

# Correspondence between evoked vocal responses and auditory thresholds in *Pleurodema thaul* (Amphibia; Leptodactylidae)

Mario Penna · Nelson Velásquez · Rigoberto Solís

**Abstract** Thresholds for evoked vocal responses and thresholds of multiunit midbrain auditory responses to pure tones and synthetic calls were investigated in males of *Pleurodema thaul*, as behavioral thresholds well above auditory sensitivity have been reported for other anurans. Thresholds for evoked vocal responses to synthetic advertisement calls played back at increasing intensity averaged 43 dB RMS SPL (range 31–52 dB RMS SPL), measured at the subjects' position. Number of pulses increased with stimulus intensities, reaching a plateau at about 18–39 dB above threshold and decreased at higher intensities. Latency to call followed inverse trends relative to number of pulses. Neural audiograms yielded an average best threshold in the high frequency range of 46.6 dB RMS SPL (range 41–51 dB RMS SPL) and a center frequency of 1.9 kHz (range 1.7–2.6 kHz). Auditory thresholds for a synthetic call having a carrier frequency of 2.1 kHz averaged 44 dB RMS SPL (range 39–47 dB RMS SPL). The similarity between thresholds for advertisement calling and auditory thresholds for the advertisement call indicates that male *P. thaul* use the full extent of their auditory sensitivity in acoustic interactions, likely an evolutionary adaptation allowing chorusing activity in low-density aggregations.

**Keywords** Acoustic communication · Audiograms · Advertisement calls · Anurans · Thresholds

## Abbreviations

BT	Best threshold
CD	Compact disc
CF	Center frequency
EVR	Evoked vocal response
RMS	Root mean square
SPL	Sound pressure level

## Introduction

Acoustic signals regulate anuran social behavior. Male frogs and toads in chorusing aggregations produce advertisement calls that are effective female attractants (reviewed in Gerhardt and Bee 2007). Vocalization exchanges among males also serve to defend calling sites. The intensity of the neighbors' signals as perceived by a resident male frog determines the characteristics of its vocal response; intensities above a certain level produce either aggressive calls or advertisement calls having a modified structure. Males typically space over distances at which the intensities of their neighbor's calls are below thresholds for eliciting aggressive vocalizations (reviewed in Wells and Schwartz 2007).

Thresholds for evoking advertisement calls are difficult to determine relative to thresholds for aggressive responses, because in the absence of any playback stimulus, frogs generally have a basal calling activity and usually engage in acoustic interactions with neighbors. This is particularly critical in tropical latitudes, where up to 20 species typically participate in a chorus (e.g. García-Rutledge and Narins 2001).

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Thresholds for evoked vocal responses (EVRs) have been measured for just a few anuran species. Field measurements have been conducted for the Caribbean treefrog *Eleutherodactylus coqui* (Narins and Capranica 1978; Narins 1982), the North American spring peeper *Pseudacris crucifer* (Gerhardt et al. 1989), and the frog of the temperate South American forest *Eupsophus emiliopugini* (Penna et al. 2005). Measurements of this behavioral response in laboratory conditions have been conducted for the North American bullfrog *Rana catesbeiana* (Megela-Simmons 1984). These studies have yielded values from about 55 to 75 dB SPL, well above the auditory thresholds of these species, which are typically within the 30–50 dB SPL range, as determined by means of multi- or single-unit recordings in the midbrain or auditory nerve (Shofner and Feng 1981; Zelick and Narins 1985; Narins 1982; Schwartz and Gerhardt 1998). Auditory thresholds of *E. emiliopugini* have not been measured.

The disparity between neurophysiological and EVR thresholds contrasts with the correspondence between thresholds for phonotactic responses reported for female hylid frogs, around 40 dB SPL (Gerhardt and Klump 1988; Beckers and Schul 2004) and their auditory thresholds (Lombard and Straughan 1974).

*Pleurodema thaul* is a leptodactylid frog distributed mainly in the mediterranean region of central Chile, where it breeds from mid winter to late spring (July–December). Males of this species call from the water surface in slow-flowing streams or pools, perched on emergent vegetation, with their vocal sacs inflated. The advertisement call consists of a long sequence of pulses containing amplitude modulations, lasting about 4 s (Penna and Veloso 1990; Solís 1994). This species does not produce a distinct aggressive call as other frogs, however, when spaced at short distances, neighbors modify the temporal structure of their calls and instead of producing uniform pulses, alternate pulses having dissimilar durations (Solís 1994). In stable chorusing aggregations, males are spaced at minimum distances of about 2 m and the intensity of the neighbor's calls at the position of focal males is about 81 dB peak SPL (Penna and Solís 1998).

We combined behavioral and electrophysiological experimentation to establish the correspondence between the thresholds for EVRs of males of *P. thaul* and the auditory sensitivity of this frog, taking advantage of the wide spacing between males in stable chorusing aggregations which facilitate measurements of behavioral thresholds in the field. The measurements conducted in the current study contribute to assess the relevance of anuran auditory sensitivity for acoustically mediated social interactions occurring in nature.

## Materials and methods

### Evoked vocal responses

#### Study site

The study was conducted during 18–21 November 2005 and 17–19 November 2006 in Los Maitenes (34° 40' S, 71° 27' W, 450 m above sea level) in central Chile. The study site during 2005 was a shallow slow-flowing stream about 3 m wide, where males of *P. thaul* called from the water surface, perched on emergent vegetation on the shores. The study site during 2006 was a stream of similar characteristics, but having a width of about 1 m and at a distance of 3 km from the 2005 study site. Nine and four experimental subjects were tested in 2005 and 2006, respectively.

#### Stimuli synthesis

Synthetic imitations of the advertisement call of *P. thaul* were generated with the Soundmaker 1.0.4 software. Because the temporal structure of this signal is dependent on water temperature (Solís 1994; Labra et al. 2008), stimuli were designed after the regression functions of temporal parameters on water temperature obtained for the calls of 45 individuals of this species recorded in previous years in the region where the study was conducted (Solís 1994). The calls were recorded with a directional microphone (AKG CK8 or AKG CK9) positioned 0.5–1 m in front of a calling frog. The microphone was connected to a cassette tape recorder (Sony TC-D5M) and the signals recorded onto chromium dioxide tape (TDK SA-90). Ten calls were analyzed for each individual with the Signalyze 3.12 software. Individual averages of call duration, number of pulses per call, pulse duration and pulse rate were obtained. Stimuli for 15, 17 and 19°C were synthesized with the following acoustic parameters 47, 54 and 61 pulses per call; pulse duration: 52, 48 and 44 ms and pulse rate: 9.8, 10.4 and 11.4 pulses/s, respectively. The envelope of the intra-pulse amplitude modulations of natural calls recorded at different temperatures was analyzed and the rise and fall envelopes were found to adjust to logarithmic and exponential functions, respectively. The synthetic stimuli were modeled accordingly, with rise and fall envelopes corresponding to the functions:  $78.80 \ln x + 3.12$  and  $1724.26 e^{-0.619x}$ ,  $74.51 \ln x + 7.36$  and  $798.92 e^{-0.489x}$ ,  $78.10 \ln x + 26.12$  and  $566.21 e^{-0.566x}$  for the stimuli of 15, 17 and 19°C, respectively. Stimuli were presented with inter-call intervals doubling the call duration. The carrier frequency for the three stimuli was 2,100 Hz, which is about the dominant frequency for the advertisement call of *P. thaul* in the region where the study was conducted (Solís 1994). The stimuli

were recorded onto a compact disc (CD) to stimulate the experimental subjects in the field. The temporal and spectral structure of these stimuli are shown elsewhere (Solís et al. submitted).

### *Experimental protocol*

The stimuli were played back with a portable CD player (Sony Walkman D-E 356 CK) connected to a custom-made impedance matching stage, an attenuator (Hewlett-Packard 350 D), a 35 W amplifier (Alpine 3540) and a 10 cm-diameter loudspeaker, mounted in a custom-made wooden cabinet (9 × 11 × 16 cm). The output of the CD player was also connected to the right channel of a cassette tape recorder (Sony TC-D5M) to record the stimuli. The EVRs of the experimental subjects were recorded on the left channel of this recorder. The frequency response of the loudspeaker was tested with pure tones of frequencies 100–5,000 Hz in 100-Hz steps in a semi-anechoic chamber, with the same set-up used for broadcasting stimuli in the field. The loudspeaker was positioned at a height of 1.5 m and at 1 m from the sound level meter microphone (Brüel and Kjær 2238), the output of which was connected to a digital tape recorder (Sony TC D10 PROII). The amplitude of the tones recorded was measured with the Signalyze 3.12 software. The frequency response of the loudspeaker was flat within ±6 dB across the 200–5,000 Hz range and within ±3 dB across the 1,000–3,000 Hz range, where most of the energy of the synthetic advertisement calls concentrates.

During the experiments at the study site, the loudspeaker was mounted on a plastic foam placed on the water surface, close to the stream border, at about 1 m of the experimental subject in the direction (right or left) of the nearest calling neighbor. The EVRs were recorded with a directional microphone (Sennheiser ME 66) positioned at 20–40 cm of the experimental subject, pointing away from the loudspeaker in order to maximize the amplitude of the EVR and minimize the broadcast stimulus in the recordings. The microphone was plugged to the left channel of the Sony TC-D5M recorder. The experiments were conducted between 2100 and 0300 h.

Before beginning playback, the intensity of the stimulus was adjusted with the attenuator to reach 70 dB SPL (fast RMS time weighting and linear frequency weighting) at the position of the experimental subject. This RMS level corresponded to 81 dB peak SPL. This is about the average intensity of the calls of the nearest neighbors to which males of this species are exposed in breeding aggregations (Penna and Solís 1998). The adjustment was done with the microphone of the sound level meter (Brüel and Kjær 2238) protected with a windscreen, positioned 5–10 cm above the experimental subject, in a vertical position, pointing downwards, minimizing the disturbance caused to the animal.

The temperature of the water near the position of the subject was measured with a digital thermometer (Digi-Sense 8528-20) and the stimulus corresponding to the closest temperature was chosen for subsequent broadcast. The stimulus was delivered briefly during a few seconds to minimize effects on the subsequent vocal activity of the subject. After this initial adjustment, any calling neighbors were silenced by gently tapping the vegetation and water around their calling sites with a twig, or if necessary, removing the frog.

The eventual spontaneous calling activity of the subjects was recorded during a period of at least 90 s (see “Results”), after which three repetitions of the stimulus were presented through the loudspeaker at 70 dB SPL. These stimulus repetitions allowed testing for the responsiveness of the subject in the experimental settings. Next, to determine EVR threshold, the SPL of the stimulus was lowered to levels between 28 and 40 dB SPL, depending on the individual tested and the stimulus was subsequently presented at increasing intensities in steps of 3–12 dB, until reaching the initial level of 70 dB SPL. Stimulation near threshold intensities was usually repeated after presentations at a higher level to check for consistency of the responses. After the second presentation of three repetitions of the stimulus at 70 dB SPL, the intensity of the stimulus was further increased in steps of 3–6 dB until reaching the maximum levels that our system could broadcast without distortion (94–100 dB SPL) or until reaching a stimulus intensity at which the frog ceased responding. To check for changes in responsiveness throughout the test session, the experiment ended with a third presentation of the stimulus at 70 dB SPL. The different SPLs at which the stimulus was presented during the experiment were calculated from the attenuation settings used. Three successive stimulus repetitions were broadcast at every intensity level.

The experimental subjects were captured and released after concluding the experiments during 2005 and 2006, to avoid repetitive recording of the same individuals. Air and water temperatures were measured again at the end of the experiment with the digital thermometer to check for changes during the recording. Water and air temperature during recordings were between 15.5–18.4 and 10.4–17.6°C. Water temperature did not differ between 2005 and 2006 (Mann–Whitney  $U$  test,  $U = 18$ ;  $P = 1.0$ ), but air temperature was significantly lower during 2005 than during 2006 (Mann–Whitney  $U$  test,  $U = 4.5$ ;  $P = 0.0372$ ).

The size and weight of 11 individuals captured after completing the experiment were between 31–35 mm and 2.2–3.3 g.

### *Analysis of responses*

Two measures were used to assess the EVR of a frog, the total number of pulses and the latency to respond, measured

from the stimulus onset to the beginning of the first pulse produced by the subject. These measures were chosen because they could be readily determined in oscillograms of the long advertisement calls of this frog. The determination of other variables, like pulse duration is an exceedingly time demanding task, in absence of automated techniques for measuring time intervals. A synthetic call was considered to evoke a response if the subject produced a call during the presentation of the stimulus and during the interval preceding the onset of the following stimulus repetition. Averages for the two EVR measures were calculated for three successive stimulus repetitions presented at the same SPL. Because all individuals were silent during the period of recording of basal vocal activity, the threshold for EVR was considered to correspond to the SPL at which the subject emitted at least one call pulse during the presentation of any of the three successive stimulus repetitions. Because presentations of three-stimulus repetitions at levels around threshold were usually broadcast 2–4 times in checking for consistency of responses (see above), averages for both EVR measures were calculated from the responses to all sets of three-stimulus repetitions broadcast at a given SPL.

Recordings of frog vocalizations and stimuli were digitized with a Macintosh G4 computer, at a sampling rate of 44,000 Hz using Peak 5.12 software. Onset and offset times of evoked calls and stimuli and number of pulses of the evoked calls were measured in sound files using Signalyze 3.12 software. The dominant frequency of evoked calls was also measured from power spectra (0–5,500 Hz, frequency resolution 20 Hz) of calls evoked by the stimulus presentations at 70 dB SPL.

Air and water temperature, size and weights of the experimental subjects and EVR measures were compared between the 2 years with the Mann–Whitney  $U$  test ( $P < 0.05$ ) to determine if the data from the 2 years could be combined. The Spearman correlation ( $P < 0.05$ ) was used to explore dependence of measures of EVRs on water temperature, relationships between stimulus intensity and EVR measures, and between EVR thresholds and distance to nearest neighbors. Comparisons among EVR measures evoked by stimuli at the initial intensity repeated at different times during the experiments were compared with Friedman ANOVA ( $P < 0.05$ ) and a posteriori differences between thresholds were analyzed with a one-tailed Wilcoxon signed-ranks test using the correction:  $P = 0.05/\text{number of comparisons}$ . To compare graphically the EVRs of different individuals to a series of stimuli, the number of pulses and latency were normalized to the maximum response for each individual. Namely, the value of an EVR measure for a given subject in response to a particular stimulus intensity was divided by the maximum value of that EVR measure produced by the frog to any one stimulus and multiplied by 100.

## Multiunit auditory responses

### *Subjects and surgical procedures*

The experiments were performed with 12 adult males of *P. thaul* (SVL  $31.7 \pm 2.7$  mm; weight  $2.7 \pm 0.8$  g). The frogs were captured in the locality of Quilimarí ( $32^\circ 06' S$ ,  $71^\circ 29' W$ , 10 m above sea level) in central Chile on 15 November 2005, the time at which the breeding season fades away in this region. The animals were kept in terraria containing vegetation from the site of capture and fed with tenebrionid larvae once per week. The terraria were maintained in a temperature-regulated room at  $15^\circ C$  under an inverted 12:12 light/dark cycle for 6–12 months, until they were used in the experiments.

The subjects were anesthetized by immersion in a 0.4% aqueous solution of MS-222 (Sigma; pH: 3.5). Under anesthesia, the skin on the dorsal surface of the head was incised and a hole was drilled on the underlying skull. Dura and pia membranes covering the brain were dissected away to expose the surface of the optic tectum. After surgery, animals were allowed to recover from anesthesia for 12–24 h. Then, the subjects were immobilized with an injection of *d*-tubocurarine chloride (9  $\mu\text{g/g}$  body weight), placed on a Peltier plate (Cambion) and covered with light moist gauze to facilitate cutaneous respiration. The recordings were conducted in a sound-attenuated booth having walls and ceiling covered with 10 cm foam wedges. Immobilization was maintained throughout the recording session with periodic injections of *d*-tubocurarine chloride. A DC current of about 1 A was supplied to the Peltier plate in order to keep the frog's body at  $15^\circ C$ , a temperature at which these frogs normally breed and call in their natural settings. Frog temperature was monitored with a miniature thermometer probe (Digisense 8528-20) inserted in the cloaca.

### Stimulus generation and recording procedures

Pure tones and noise were generated with dedicated hardware (System II, Tucker-Davis Technologies) and the Sig Gen software (Tucker-Davis Technologies) on a Pentium III computer. Two kinds of stimuli were presented in the experiments: (a) pure tones having a duration of 250 ms and rise/fall times of 50 ms, set at frequencies between 100 and 5,000 Hz and (b) synthetic calls composed of 5 pulses having the same temporal characteristics as the stimuli used in the behavioral study. A stimulus having similar characteristics was employed in a former study of neuronal responses in this species (Penna et al. 1997). Synthetic calls with three carrier frequencies (1,900; 2,100 and 2,300 Hz) were used. These frequencies encompassed the range of variation of the dominant frequency of the advertisement call in the natural population where the behavioral study

was conducted (Solís et al. submitted). Stimuli used in the electrophysiological study are shown in Fig. 1.

The amplitude of the stimuli were controlled with a programmable attenuator (PA4, Tucker-Davis Technologies), amplified (NAD C 320 BEE) and broadcast via a two-way loudspeaker (Dynaudio BM 6, frequency response 38–20,000 Hz) positioned 1 m in front of the experimental subject. Stimuli were presented at a rate of 0.5 stimulus/s.

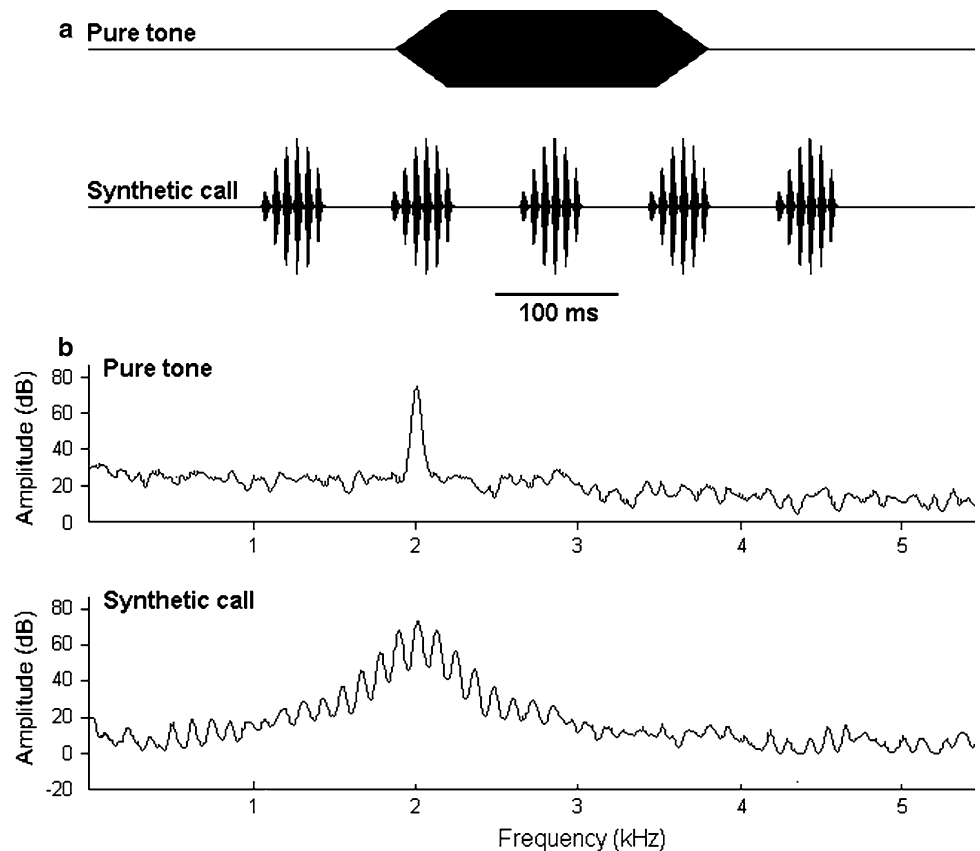
Prior to the experiment, the intensity of pure tones of frequencies 100–5,000 Hz and of the synthetic calls were measured with the microphone of a sound level meter (Brüel and Kjaer 2238) positioned 2–3 mm above the subject's head. The absolute SPLs of both types of stimuli delivered at a constant electrical peak amplitude were measured and the SPLs of the auditory thresholds were determined during the subsequent experimental session using different attenuator settings. The frequency response of the system was within  $\pm 8$  dB in the 100–5,000 Hz range and  $\pm 6$  dB in the 300–5,000 Hz range.

Multiunit responses from the torus semicircularis (TS) were recorded with custom-made gross glass-insulated tungsten electrodes (75  $\mu$ m diameter). The electrode was attached to a micromanipulator and driven with a hydraulic microdrive (Narishige MO-8), positioned on the dorsal surface of the optic tectum and advanced into the brain. The large size of the electrode relative to the optic lobe facili-

tated a regular positioning of the electrode on the brain surface at about the intersection of the limit between the mid and caudal third and the limit between medial and lateral half of the hemisphere. This placement consistently yielded the largest auditory responses; recordings for all subjects were made at this single location. The neural responses were passed through a pre-amplifier (Dagan 2400) and filtered between 10 and 3,000 Hz for multiunit recordings. Neural responses were monitored by means of an oscilloscope and a loudspeaker, and thresholds determined by audiovisual criteria. A search stimulus consisting of a white noise burst having the same temporal structure as the pure tones was presented at an intensity of about 80 dB RMS SPL while the electrode was lowered in the brain to determine the depth at which the strongest auditory response was evoked. This methodology has been used in previous studies and provides reliable measurements of auditory thresholds in different anurans (Penna et al. 1990, 1992).

The lowest threshold observed in the high frequency region of enhanced sensitivity observed in individual audiograms was termed the best threshold (BT). A center frequency (CF) for the high frequency region was calculated from a weighted average of thresholds for frequencies between 1,000 and 5,000 Hz that were within 6 dB above the BT. To calculate the CF of an individual audiogram, each of the frequencies considered was multiplied by the

**Fig. 1** Oscillograms (a) and power spectra (b) of digital files containing stimuli used in the recording of multiunit auditory responses in *P. thaul*. Stimuli shown correspond to a 2.1-kHz gated pure tone and a synthetic call having a carrier frequency of 2.1 kHz (for details of stimuli structure see “Materials and methods”)





sound pressure ( $\text{N/m}^2$ ) corresponding to the difference between the SPL at the BT and the SPL at that frequency, the products summed and divided by the sum of SPLs. This procedure was adopted instead of assigning the CF to the single frequency having the lowest threshold, because it gives a better representation of the tuning of auditory sensitivity in the high frequency region.

To obtain average thresholds of multiunit responses at each of the frequencies tested and threshold for the synthetic calls, threshold values of individuals in dB SPL were converted to  $\text{N/m}^2$  and the resulting stadigraph reconverted to dB SPL. The dependence of multiunit audiograms' CF on body size and weight was explored with Spearman correlation ( $P < 0.05$ ). Thresholds for multiunit responses among the three synthetic calls of different CF were compared with Friedman ANOVA ( $P < 0.05$ ) and a posteriori differences between thresholds were analyzed with a one-tailed Wilcoxon signed-ranks test using the correction:  $P = 0.05/\text{number of comparisons}$ . Comparisons between thresholds to these stimuli and thresholds for EVR were done with the Mann–Whitney  $U$  test ( $P < 0.05$ ).

## Results

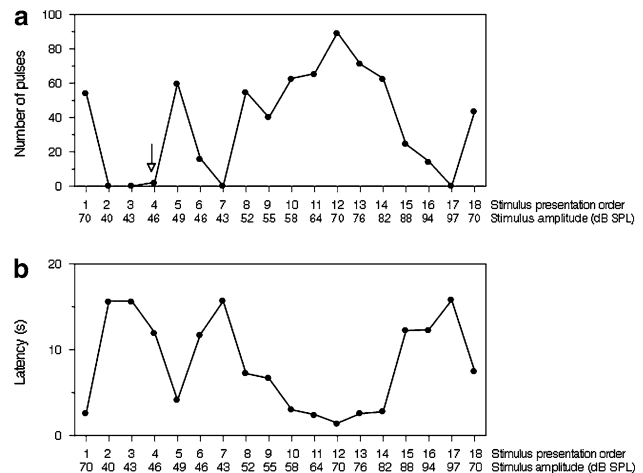
### Evoked vocal responses

The subjects' basal calling activity was monitored over an average period of 141 s (range 90–257 s). Thirteen frogs did not call during this period and were thereafter subjected to playback experiments to determine their thresholds for EVR. One individual recorded in 2006 called persistently during the period of basal recording and was discarded from the study. The 13 experimental subjects were spaced at a wide range of distances from their nearest neighbors. Eight subjects were spaced at measurable distances (average 3.56 m; range 0.5–12 m) and five subjects were at distances above 20 m from their nearest neighbors.

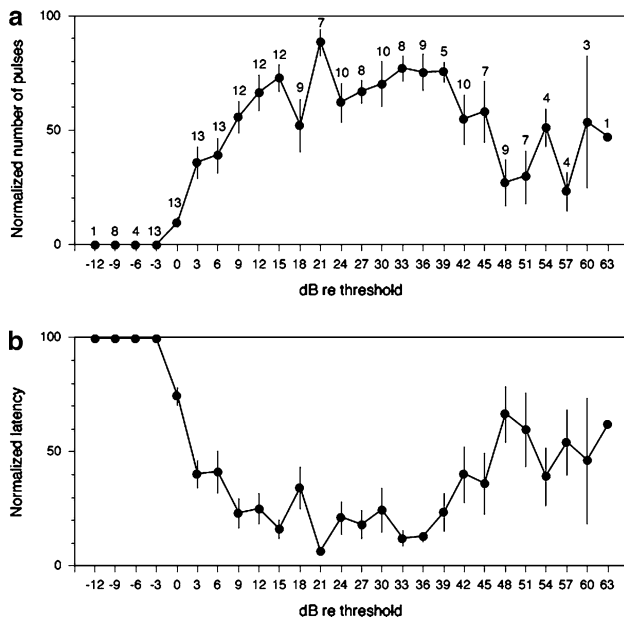
After the basal recording period, males of *P. thaul* responded readily when presented with the stimulus at 70 dB SPL, producing calls having an average dominant frequency of 2,192 Hz (range 2,007–2,340 Hz), an average of 41.1 pulses (range 17.7–63.3 pulses) and an average latency of 1.916 s (range 0.723–3.410 s). The subjects tested during 2005 and 2006 did not differ in the dominant frequency (Mann–Whitney  $U$  test,  $U = 10$ ;  $P = 0.2170$ ) and in the number of pulses (Mann–Whitney  $U$  test,  $U = 8$ ;  $P = 0.1228$ ) given in response to the presentation of the stimulus at this initial intensity but the latency to respond was significantly longer in 2006 (Mann–Whitney  $U$  test,  $U = 4$ ;  $P = 0.0307$ ). The dominant frequency of the calls was not dependent on subjects' size or weight (Spearman correlation:  $R_s = -0.1883$ ,  $P = 0.5791$ ;  $R_s = -0.1418$ ,  $P = 0.6773$ , respectively).

When stimulated with synthetic calls at low SPLs (28–40 dB SPL, depending on the subject), frogs typically remained silent. Thresholds for EVR averaged 42.9 dB SPL (range 31–52 dB SPL). The number of pulses evoked at threshold level averaged 6.36 pulses (range 0.5–15.0 pulses) and the latency averaged 11.731 s (range 7.313–15.158 s). The number of pulses and latency of the EVRs produced in response to threshold stimuli were not related to absolute threshold levels (Spearman correlation:  $R_s = 0.0910$ ,  $P = 0.7652$  and  $R_s = 0.1176$ ,  $P = 0.0702$ , respectively). Thresholds were not related either to distance from nearest neighbor (Spearman correlation:  $R_s = -0.0696$ ,  $P = 0.8957$ ) or to call dominant frequency of the experimental subjects (Spearman correlation:  $R_s = -0.2294$ ,  $P = 0.4508$ ).

The EVRs given by a frog in response to a sequence of three-stimulus repetitions in the order of presentation of different intensities are shown in Fig. 2. This individual responded to stimuli of increasing intensities with number of pulses and latencies that were clearly higher and shorter relative to those at lower intensities, respectively. The number of pulses increased and latency decreased with stimulus intensity up to 15 dB above threshold, as shown in Fig. 3 for average normalized number of pulses and latencies for the experimental subjects relative to response threshold. Stimulus level from threshold to 15 dB above threshold and the average number of pulses and latency were significantly



**Fig. 2** Number of pulses (a) and latencies (b) to playback of repetitions of a synthetic call presented at different SPLs to a male of *P. thaul*, measured at the recipient's position. After an initial presentation at 70 dB SPL, the synthetic call was presented at increasing intensities, repeating values near threshold, to check for consistency of the responses. After reaching the maximum intensity, the experiment ended with a presentation of the synthetic call at the initial amplitude of 70 dB SPL. Each symbol represents the average of responses to three consecutive stimulus repetitions at the same level (See "Materials and methods"). The arrow indicates threshold for evoked vocal response of this subject



**Fig. 3** Normalized number of pulses (a) and latencies (b) to synthetic calls presented at different levels relative to threshold for evoked vocal responses in the 13 experimental subjects. *Filled circles* and *bars* represent averages and ranges, respectively. Numbers above bars indicate number of frogs tested at a particular stimulus level

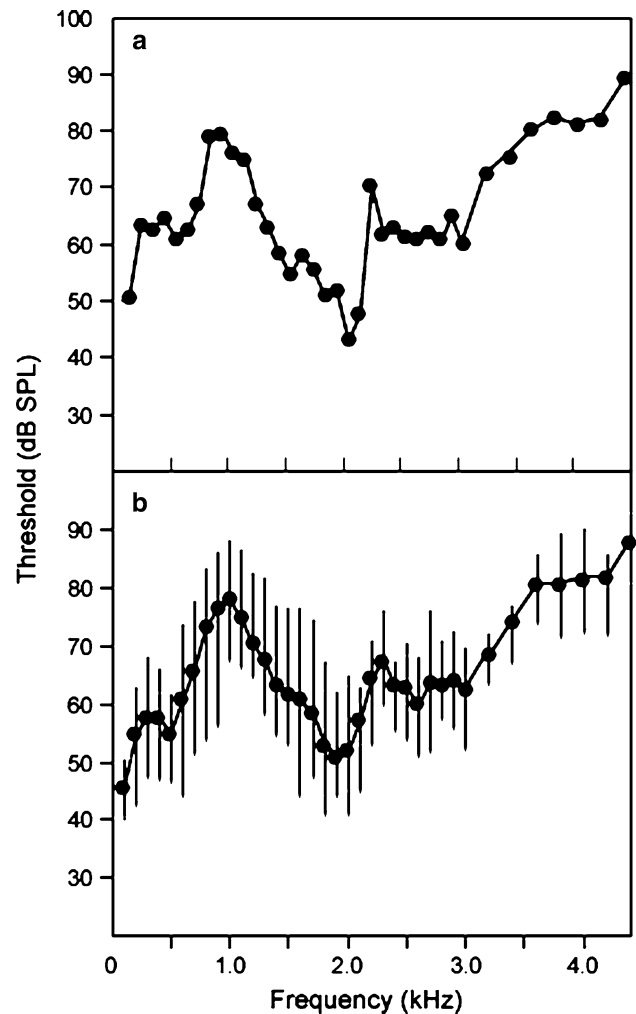
correlated (Spearman correlation:  $R_s = 1.0$ ,  $P < 0.0001$  and  $R_s = -0.8857$ ,  $P = 0.0188$ , respectively). For stimuli broadcast at intensities between 18 and 39 dB above threshold, the number of pulses and latency apparently stabilized and the average number of pulses evoked by the stimulus at these intensities was 45.9 pulses (range 31.2–56.7 pulses) and the latency was 3.161 s (range 1.050–6.393 s).

Twelve frogs were tested for EVRs to stimuli at SPLs above 70 dB SPL, until reaching the maximum intensity level that our system could broadcast (94–100 dB SPL) or until reaching an intensity level at which the frog ceased responding. This procedure attempted to explore the extent to which males of *P. thaul* persist interacting vocally. At such stimulus amplitudes, eight frogs ceased responding (average 92.5 dB SPL; range 79–100 dB SPL) and four frogs persisted responding up to 94 or 100 dB SPL. The absolute amplitudes at which the eight frogs ceased calling corresponded to an average of 50.2 dB re-threshold (range 42–60 dB re-threshold; see Fig. 3). After the presentation of the stimulus at the highest level, the subjects were presented with the stimulus at 70 dB SPL. In response to this presentation, 11 out of 12 frogs gave EVRs and one frog remained silent. In Fig. 2, responses of a frog to the three presentations of the stimulus at 70 dB SPL at the beginning of the experiment, after stimulus presentations at intensities below this level and after stimulus presentations at maximum intensity levels are shown. Number of pulses in response to stimuli was similar (Friedman ANOVA:

$\chi^2 = 3.5$ ,  $N = 12$ ,  $df = 2$ ,  $P = 0.1737$ ), but latency differed significantly among the three presentations (Friedman ANOVA:  $\chi^2 = 6.5$ ,  $N = 12$ ,  $df = 2$ ,  $P = 0.0387$ ). Latency after stimulus presentations at maximum intensity levels was longer than at the beginning of the experiment (Wilcoxon signed-ranks test,  $Z = 2.4318$ ,  $P = 0.0150$ ).

### Multiunit auditory responses

Audiograms showed regions of enhanced sensitivity at a low-frequency range (0.1–0.9 kHz) and a high-frequency range (1.0–5.0 kHz). An individual audiogram and an average audiogram for the 12 experimental subjects illustrate such pattern in Fig. 4. Both graphs also show that in the high frequency region, thresholds for 1.9 and 2.1 kHz are lower than those at 2.3 kHz. The BTs in the low- and high-frequency ranges averaged 45.1 dB SPL (range 40–51 dB SPL) and 46.6 dB SPL (range 41–51 dB SPL), respectively,

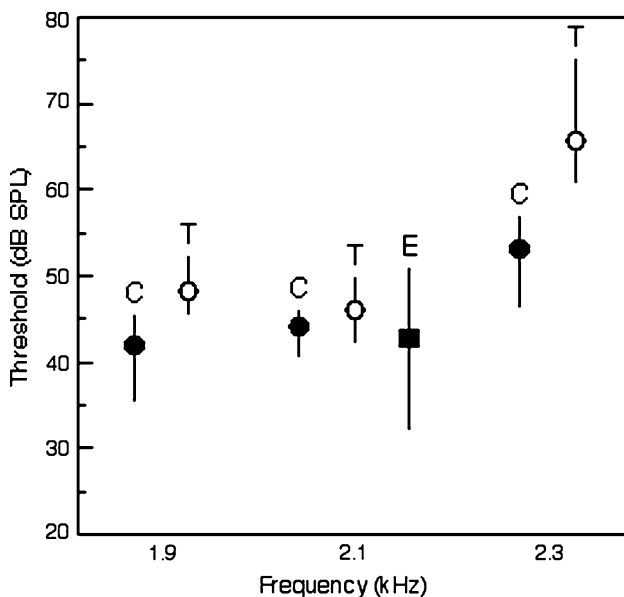


**Fig. 4** Multiunit audiogram of an individual (a) and average multiunit audiograms for 12 males of *P. thaul* (b). *Filled circles* and *bars* in (b) represent averages and ranges, respectively

and the CF calculated for the high frequency range averaged 1.9 kHz (range 1.7–2.6 kHz). The CF was not dependent on subjects' body size or weight (Spearman correlation:  $R_s = 0.4335$ ,  $P = 0.1591$ ;  $R_s = 0.5008$ ,  $P = 0.0971$ , respectively).

Thresholds of multiunit auditory responses to the synthetic calls differed significantly among the three stimuli (Friedman ANOVA:  $\chi^2 = 16.54$ ,  $N = 11$ ,  $df = 2$ ,  $P = 0.0002$ ). The response thresholds to the 1.9, 2.1 and 2.3 kHz stimuli averaged 42.0 dB SPL (range 34–46 dB SPL), 44.1 dB RMS SPL (range 39–47 dB SPL) and 53.0 dB SPL (range 45–58 dB SPL), respectively. Thresholds for the 1.9 kHz stimulus were significantly lower than thresholds for the 2.1 and 2.3 kHz stimuli (Wilcoxon signed-ranks test,  $Z = 2.4895$ ,  $P = 0.0127$  and  $Z = 2.9340$ ,  $P = 0.0033$ , respectively) and thresholds for the 2.1 kHz stimulus were significantly lower than thresholds for the 2.3 kHz stimulus (Wilcoxon signed-ranks test,  $Z = 2.8451$ ,  $P = 0.0044$ ; Fig. 5). Thresholds for the 1.9, 2.1 and 2.3 kHz stimuli were significantly lower than thresholds for tones of the corresponding frequencies (Wilcoxon signed-ranks test,  $Z = 2.9340$ ,  $P = 0.0033$  for the three comparisons).

Comparisons between the electrophysiological and behavioral experiments showed that auditory thresholds for the 1.9 and 2.1 kHz synthetic calls did not differ significantly



**Fig. 5** Thresholds of multiunit auditory responses of 12 males of *P. thaul* for synthetic calls of three carrier frequencies. Filled circles and bars represent averages and ranges, respectively. Thresholds for pure tones of the same frequencies as the synthetic calls are also represented by open circles and the corresponding bars. Filled square and the corresponding bar represent average and range of thresholds of evoked vocal responses to a 2.1-kHz synthetic call measured for 13 males of *P. thaul* in the field experiments. Abbreviations: C synthetic call; T pure tone; E evoked vocal response

cantly from thresholds for EVRs measured in the field experiments (Mann–Whitney  $U$  test,  $U = 69$ ;  $P = 0.8848$  and  $U = 51$ ;  $P = 0.2349$ , respectively). However, thresholds for the 2.3 kHz synthetic call were significantly higher than thresholds for EVRs (Mann–Whitney  $U$  test,  $U = 7$ ;  $P = 0.0001$ ).

## Discussion

### EVR thresholds

Thresholds for evoked advertisement calling in *P. thaul*, averaging 43 dB SPL and ranging from 31 to 52 dB SPL are low relative to those reported for other frogs. In field studies, EVR thresholds of 55–75 dB SPL have been measured for the Caribbean treefrog *E. coqui* (Narins and Capranica 1978; Narins 1982) and an average of 63 dB SPL for *P. crucifer* (Gerhardt et al. 1989). In a laboratory study with the North American bullfrog, *R. catesbeiana*, Megela-Simmons (1984) obtained thresholds of 60 dB SPL for this behavioral response. In the frog from the temperate austral forest, *E. emiliopugini*, thresholds for EVR are about 60 dB peak SPL (Penna et al. 2005), corresponding to about 50 dB RMS SPL (M. Penna, unpublished data). The values obtained for *E. emiliopugini* are significantly higher than those obtained in the present study (Mann–Whitney  $U$  test,  $U = 2.514$ ;  $P = 0.0108$ ).

The relatively high EVR thresholds measured in former studies for other anurans could be related to different factors, among which the noise levels of natural chorusing aggregations is likely to play a main role. *E. coqui* calls in a noisy environment in the forests of Puerto Rico, which could impair the detection of the conspecific signals (Narins and Zelick 1988). Also the relatively high density of aggregations of *P. crucifer* where behavioral measurements were conducted could have determined the high thresholds reported by Gerhardt et al. (1989). The thresholds for EVR for *R. catesbeiana* were measured in the laboratory (Megela-Simmons 1984), but responsiveness in controlled settings may differ from behavior in natural conditions. Males of this species have been shown to engage in vocal interactions with neighbors spaced up to 60 m in the field, thus responding to low intensity signals (Boatright-Horowitz et al. 2000; see below). The higher thresholds of *E. emiliopugini* for EVR relative to *P. thaul* are likely related to the low temperatures at which this frog calls in the temperate forests of southern Chile (6.5–10.9°C; Penna et al. 2005); auditory thresholds are inversely related to this environmental variable (e.g. Narins 2001).

Thresholds for advertisement calling could be subject to changes, depending on the social circumstances to which an individual is exposed, as has been shown to occur for



thresholds for aggressive calling, which increase after exposure to stimuli of high intensity (Rose and Brenowitz 1997; Marshall et al. 2003). In chorusing aggregations, males of *P. thaul* are typically spaced at distances above 2 m, however vocal interactions at distances shorter than 0.5 m also occur, during which males modify their vocal patterns, alternating pulses of irregular duration and occasionally engaging in physical struggles (Solís 1994). Conditions of very low density also occur throughout the extended breeding period of this frog, lasting from July to December in the region where the behavioral study was conducted. Isolated males, having no neighbors within a radius of 20 m, called along the stream shores at nights when this study was conducted. Frogs active in such quiet sound environment are likely to respond to low-level signals more readily than subjects calling in dense aggregations, where sensory adaptation, accommodation (sensu Rose and Brenowitz 1997) or habituation (sensu Owen and Perril 1998; Bee 2003) processes may operate. However, in our study, thresholds for EVR, spanning from 31 to 52 dB SPL were not related to nearest neighbors' distance. This lack of relationship does not support an effect of previous levels of exposure to conspecific signals on EVR thresholds.

#### Auditory correlates

Auditory thresholds measured with multi-unit electrophysiological recordings are likely to give a reliable account of hearing thresholds of this species. A study with single-unit recordings in the torus semicircularis of *P. thaul* reported thresholds spanning from 34–52 dB SPL for high frequency neurons (Penna et al. 1997), which encompass closely the 39–47 dB SPL range of responses measured in the current study to the 2.1 kHz stimulus.

It is unlikely that differences among the experimental subjects in thresholds for EVRs in the field, spanning from 31 to 52 dB SPL result from elevated auditory thresholds caused by previous exposure to different neighbor call levels. Electrophysiological recordings of auditory nerve fibers in the Puerto Rican treefrog *E. coqui* have shown that prolonged exposure to high intensity tones produce temporary elevations of thresholds which recover rapidly, in about 5 min following the cessation of overstimulation (Zelick and Narins 1985; Penna and Narins 1989). This interval is well below a minimum time of half an hour elapsed between targeting an experimental subject in the field and setting up the conditions for stimulation in absence of interference from any neighbors.

The different sensitivity of frogs for EVR could be related to hormonal factors. Testosterone plasma levels are higher in males of *P. thaul* calling spaced at distances below 7 m from their neighbors, as compared to males

spaced at distances beyond 20 m (Solís 1994). Furthermore, vocal responsiveness is directly related to plasma testosterone levels in a natural population of the Chilean leptodactylid *Batrachyla taeniata* (Solís and Penna 1997). A causal relationship between both variables has been indicated by the study of Brzoska and Obert (1980) showing that exposure to advertisement calls promotes gonadal activation in male grassfrogs. Another possible endocrine influence is the peptide arginine-vasotocin, which has been shown to activate vocal responsiveness of cricket frogs to sound stimuli in natural settings (Chu et al. 1998). The action of androgens and arginine-vasotocin in green treefrogs is apparently exerted on vocal motor pathways, without affecting auditory sensitivity (Penna et al. 1992).

The auditory thresholds of our experimental subjects, maintained in captivity for 6–12 months before recording, would not be expected to differ from those of freshly captured individuals. Auditory thresholds in anurans have shown stability across seasons and over months of captivity in controlled conditions (Brzoska et al. 1977; Penna et al. 1992; Goense and Feng 2005).

The responsiveness of males of *P. thaul* to conspecific signals at threshold levels reveal that these behavioral responses are potentially as sensitive as female phonotactical responses (Gerhardt and Klump 1988; Beckers and Schul 2004), implying that patterns of auditory sensitivity are particularly relevant for selectivity to spectral components of sounds. This is a functional alternative to nonlinearities of the auditory system affecting female phonotactic responses at higher sound levels (Schwartz and Gerhardt 1998; Gerhardt and Schwartz 2001).

The prompt and sensitive male EVRs reported in the current study could be supported by anatomical studies on anuran audio–motor interfacing showing various connections between auditory and vocal motor centers, including projections from the laminar nucleus of the torus semicircularis to the dorsal tegmental area (reviewed in Walkowiak 2007).

#### Environmental correlates

In stable chorusing aggregations of *P. thaul*, the distance between nearest neighbors is on average about 3.3 m and the intensity of the calls of the nearest neighbors at the position of a focal male is on average 81 dB peak SPL (Penna and Solís 1998), which corresponds to about 70 dB RMS SPL (see “Material and methods”). The environments where *P. thaul* communicate are shallow streams or pools across which advertisement calls propagate effectively, approximating the inverse square law (i.e. a 6-dB decrease in call SPL per doubling the distance from the sound source; Penna and Solís 1998). Assuming a 70 dB SPL at 3.3 m, the average threshold for EVR of 43 dB SPL would

be reached at 74 m, allowing long-range communication in this species.

The relatively low thresholds measured for the EVRs of *P. thaul* could also be related to the temporal characteristics of the advertisement call of this species, its long duration rendering the signal more detectable at low levels. The persistence of the signal, the favorable characteristics of the environment for signal propagation and the ability to respond to calls perceived at auditory threshold levels suggest that maximization of the communication range has been a leading selective pressure in the evolution of sound communication in *P. thaul*. The North American bullfrog, *R. catesbeiana* could also be assimilated to this condition, since diadic interactions between males spaced up to 60 m apart occur in pools where this species breeds (Boatright-Horowitz et al. 2000). The intensity of the call at this distance, calculated from the value of 80 dB SPL measured at 1 m of calling individuals by Megela-Simmons (1984) and following the inverse square law, is 44 dB SPL. Measurements of thresholds for EVRs in natural conditions are likely to yield lower values relative to the 60-dB SPL thresholds measured for this frog by Megela-Simmons (1984) in laboratory settings.

Males of *P. thaul* decreased their EVRs when presented with stimuli at high levels. Similar decrements in vocal activity have been reported for other anuran species (e.g. Harrison and Littlejohn 1985) and could represent an adaptation to avoid extremely demanding acoustic competition. Eight of the experimental subjects ceased giving EVRs at an average stimulus amplitude of 92.5 dB SPL. Following the inverse square law and considering an amplitude of 70 dB SPL at 3.3 m, such amplitude would be reached at 0.25 m from the sound source, indicating that males are able to persist interacting with nearest neighbors spaced at such short distance.

Interesting contrasts with frogs communicating over water surface are provided by anurans that breed in biotopes much less favorable for sound propagation, such as bogs and ground, where calls suffer considerable excess attenuation (Penna and Solís 1998; Penna et al. 2006). Combined measurements of thresholds for EVRs and auditory thresholds would contribute to determine the extent to which auditory sensitivity fosters the persistence of chorusing activity under such environmental constraints.

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