

Spatial hearing in Cope's gray treefrog: II. Frequency-dependent directionality in the amplitude and phase of tympanum vibrations

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Abstract Anuran ears function as pressure difference receivers, and the amplitude and phase of tympanum vibrations are inherently directional, varying with sound incident angle. We quantified the nature of this directionality for Cope's gray treefrog, *Hyla chrysoscelis*. We presented subjects with pure tones, advertisement calls, and frequency-modulated sweeps to examine the influence of frequency, signal level, lung inflation, and sex on ear directionality. Interaural differences in the amplitude of tympanum vibrations were 1–4 dB greater than sound pressure differences adjacent to the two tympana, while interaural differences in the phase of tympanum vibration were similar to or smaller than those in sound phase. Directionality in the amplitude and phase of tympanum vibration were highly dependent on sound frequency, and directionality in amplitude varied slightly with signal level. Directionality in the amplitude and phase of tone- and call-evoked responses did not differ between sexes. Lung inflation strongly affected tympanum directionality over a narrow frequency range that, in females, included call frequencies. This study provides a foundation for further work on the biomechanics and neural mechanisms of spatial hearing in *H. chrysoscelis*, and

lends valuable perspective to behavioral studies on the use of spatial information by this species and other frogs.

Keywords *Hyla chrysoscelis* · Eardrum vibrations · Auditory periphery · Hearing · Lung inflation

Abbreviations

IAD	Interaural amplitude difference
ILD	Interaural level difference
IPD	Interaural phase difference
ITD	Interaural time difference
IVAD	Interaural vibration amplitude difference
IVPD	Interaural vibration phase difference
IVTD	Interaural vibration timing difference
TVA	Tympanum vibration amplitude
TVP	Tympanum vibration phase

Introduction

Spatial hearing is critically important in the reproductive biology of anuran amphibians (Gerhardt and Huber 2002; Gerhardt and Bee 2007). Female frogs, which commonly exhibit robust phonotaxis toward calling males (e.g., Gerhardt 1995; Baugh and Ryan 2010; Christie et al. 2010), must often locate potential mates in structurally and acoustically complex environments, such as a breeding chorus in a densely vegetated wetland or forest. Likewise, male frogs, which often defend discreet calling sites or territories using both vocalizations and physical aggression (e.g., Wells 1978; Robertson 1986; Wagner 1989), must be able to locate reproductive rivals that begin calling in close proximity (Gerhardt and Rheinlaender 1980; Wilczynski and Brenowitz 1988; Ursprung et al. 2009). In some species, the sounds of the chorus itself may function as an acoustic

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beacon that frogs use to localize breeding aggregations (e.g., Gerhardt and Klump 1988; Bee 2007a). Importantly, both sexes can solve these tasks of sound source localization using only the acoustic cues provided by vocalizations. Spatial hearing is also important in other perceptual and cognitive tasks not directly related to sound source localization. Three such likely tasks involve auditory grouping (Schwartz and Gerhardt 1995; Farris et al. 2002, 2005; Bee and Riemersma 2008; Bee 2010; Farris and Ryan 2011), spatial release from masking (Schwartz and Gerhardt 1989; Bee 2007b, 2008; Nityananda and Bee 2012; Ward et al. 2013a), and cross-modal sensory integration (Narins et al. 2005; Taylor et al. 2011).

The directional sensitivity of the anuran ear is believed to derive largely from mechanical coupling of the left and right tympanic membranes via the Eustachian tubes and mouth cavity (reviewed in Christensen-Dalsgaard 2005, 2011). In addition, sound energy can be transmitted to the internal surfaces of the tympanic membranes via alternate pathways, such as through the body wall and lungs (Ehret et al. 1990; Jørgensen et al. 1991). The interactions between sounds reaching the external and internal surfaces of the tympanic membranes give rise to amplitude and phase differences that impart inherent directionality to the frog's ears, which function in part as pressure difference receivers (reviewed in Christensen-Dalsgaard 2005, 2011). This directionality provides the peripheral input for spatial hearing in frogs. The directionality of frogs' ears in response to free-field sound presentations from different incident angles has been studied most extensively using laser Doppler vibrometry (Vlaming et al. 1984; Michelsen et al. 1986; Jørgensen 1991; Jørgensen and Gerhardt 1991; Jørgensen et al. 1991; Ho and Narins 2006) and recordings of auditory nerve fibers (Feng 1980; Schmitz et al. 1992; White et al. 1992; Wang et al. 1996; Wang and Narins 1996; Jørgensen and Christensen-Dalsgaard 1997a; Klump et al. 2004), with limited effort also directed toward modeling (Palmer and Pinder 1984; Aertsen et al. 1986). Together, these previous studies of ear directionality in frogs provide a solid foundation for understanding spatial hearing in these animals. However, much of this work has used species (e.g., *Rana pipiens* and *R. temporaria*) for which parallel data on behavioral performance in a diversity of spatial hearing related tasks are lacking.

This study investigated ear directionality in Cope's gray treefrog, *Hyla chrysoscelis*. Recent behavioral studies of this species have focused on the performance of several different perceptual tasks dependent on spatial hearing, such as sound source localization (Caldwell and Bee 2014), spatial release from masking in signal recognition and discrimination tasks (Bee 2007b, 2008; Nityananda and Bee 2012; Ward et al. 2013a), and auditory grouping and stream segregation based on spatial coherence (Bee and Riemersma

2008; Bee 2010). Ear directionality has been previously examined for only a single *H. chrysoscelis* individual (Jørgensen 1991; Jørgensen and Gerhardt 1991), precluding any description of the range of typical responses or of inter-individual variation. Furthermore, data for this individual were not shown separately from those recorded from two individuals of its sister species, the eastern gray treefrog, *H. versicolor*.

Here, we report results from a study that used laser Doppler vibrometry to measure the tympanum response to free-field sounds. Our study had four main goals. The first was to describe the directionality of the tympanum of *H. chrysoscelis* in response to sounds presented from different azimuthal angles. We did this by examining absolute measures of tympanum vibration amplitude (TVA) and tympanum vibration phase (TVP), as well as relative measures of interaural vibration amplitude differences (IVADs) and interaural vibration phase differences (IVPDs). Second, we investigated the extent to which these measures of ear directionality varied with frequency and signal level. Third, we characterized how air contained within the lungs shaped patterns of directionality. Finally, we assessed the extent to which size differences and size-independent sex differences affected ear directionality.

Materials and methods

Subjects

The subjects in this study were 16 males and 16 females of Cope's gray treefrog, *H. chrysoscelis*. Animals were collected in May and June, 2012, as amplexed pairs from wetlands at three field sites in eastern Minnesota, USA (Carver Park Reserve, Carver county; Crow-Hassan Park Reserve, Hennepin county; and Lake Maria State Park, Wright county). The female in each pair was used as a subject in various behavioral experiments prior to measurements of tympanum vibrations. Cope's gray treefrogs in Minnesota are from the western mtDNA lineage (Ptacek et al. 1994). Most frogs (22 of 32) were tested in a bioacoustics laboratory on the St. Paul campus of the University of Minnesota. The remaining frogs were tested in a bioacoustics laboratory at Syddansk Universitet, in Odense, Denmark. Prior to testing, all frogs were chemically immobilized with intramuscular injections of d-tubocurarine chloride (1 µg/g). We allowed the frogs to regulate their own lung volume as the immobilizing agent took effect. After full immobilization was achieved, lung inflation (based on body wall extension) resembled that observed for unmanipulated frogs sitting in a natural posture. Unless we purposefully manipulated it, lung inflation remained steady throughout a recording session, as assessed for a subset of 12 subjects by

measuring their maximum lateral body width with calipers at the start and end of a test session (Wilcoxon signed-rank test: $Z = -1.178$, $P = 0.239$, $N = 12$).

Tone presentations

Testing procedure

To determine how the vibrational response of the tympanum varies with sound presentation angle, frequency, and signal level, we presented six male and six female *H. chrysoscelis* with free-field broadcasts of a series of pure tones. Recordings were made in a custom-built, semi-anechoic sound chamber (3 m \times 2.5 m \times 2 m, L \times W \times H, inside dimensions; Industrial Acoustics Company, Bronx, NY) at the University of Minnesota. The sound chamber had a carpeted, vibration isolation floor. The walls and ceiling of the chamber were lined with Sonex acoustic foam panels (Model VLW-60; Pinta Acoustic, Inc. Minneapolis, MN) to reduce reverberation. During the experiment, we placed a subject on a 30 cm pedestal made from acoustically transparent wire mesh (0.9-mm diameter wire, 10.0-mm grid spacing). We rested the very tip of the mandible on a raised arch of thin wire, such that the frog sat in a typical posture with head raised and in line with its body, and jaw parallel to the ground. The pedestal was suspended 90 cm above the chamber floor by a horizontal, 70-cm long, rigid metal beam (unistrut) attached to its base. The beam itself was mounted to a vibration isolation table (Technical Manufacturing Corporation, Peabody, MA) on one side of the chamber. Both the beam and the vibration isolation table were covered with acoustic foam.

Stimuli were broadcast and responses recorded using custom software (Stimprog v.5.42, written by NL) in MATLAB (v.2011a, MathWorks, Natick, MA) via an external digital and analog interface device (NI USB 6259, National Instruments, Austin, TX). Signal levels were controlled using a programmable attenuator (PA5, Tucker-Davis Technologies, Alachua, FL). The signal was amplified (Sonamp 1230, Sonance, San Clemente, CA) and presented through a single speaker (Mod1, Orb Audio, New York, NY) 50 cm away from the approximate center of a subject's head measured along the interaural axis. The speaker was attached to a rotating arm made of 2.1-cm diameter metal tubing filled with and covered with acoustic foam and suspended from the ceiling of the chamber. This apparatus allowed us to position the speaker at any azimuthal angle relative to the frog's snout.

Just prior to commencing tympanum measurements, we placed a microscopic retroreflective glass bead (3M, St. Paul, MN) at the center of the left tympanum of each frog to enhance the reflectance of this membrane. We also swabbed the opening to each Eustachian tube using

a lint-free wipe to clear away any mucus. All measurements from an individual were taken during a single session with the animal in the same body position. At the start of measurements, we focused a laser vibrometer (PDV-100, Polytech, Irvine, CA) on the bead at the center of the tympanum. The laser was mounted to the vibration isolation table 80 cm from the test subject, and positioned at 75° relative to the frog's snout. This angle was chosen so that the speaker never obstructed the laser beam. We also positioned the tip of a probe microphone (40SC, G.R.A.S., Holte, Denmark) approximately 2 mm to the rear of same tympanum. The analog microphone signal was amplified (MP1, Sound Devices, Reedsburg, WI) to match the voltage range output by the vibrometer prior to arriving at the digital and analog interface. The analog vibrometer signal was fed directly into the interface. We refer to the side of the animal from which measurements were taken (left) as the ipsilateral side, and sound presentation angles on the ipsilateral side of the animal will be positive. Negative sound presentation angles will indicate that the speaker was on the contralateral (right) side of the animal.

We presented each frog with 180 tone stimuli corresponding to factorial combinations of 12 azimuthal sound presentation angles (0°, $\pm 30^\circ$, $\pm 60^\circ$, $\pm 90^\circ$, $\pm 120^\circ$, $\pm 150^\circ$, and 180°), 5 tone frequencies (600, 1,250, 1,625, 2,500, and 3,200 Hz), and 3 signal levels (76, 85, and 94 dB SPL, re 20 μ Pa, C-weighted at 50 cm). Angles of 0° and 180° correspond, respectively, to the rostral and caudal ends of the midline. All frequencies fell within the audible range of *H. chrysoscelis* (Hillery 1984; Schrode et al. 2014), and frequencies of 1,250 and 2,500 Hz correspond to the two peaks present in the bimodal spectrum of male advertisement calls in our study population (Schrode et al. 2012). Signal levels between 76 and 94 dB are within the range of call amplitudes likely experienced in breeding choruses (Gerhardt 1975). Tones were 200 ms in duration, had \cos^2 onset and offsets of 10 ms, and were repeated five times with a period of 300 ms at each combination of sound presentation angle, frequency, and signal level before the next combination was tested. Signal levels were calibrated for each subject prior to commencing measurements using a Brüel and Kjær Type 2250 sound level meter (Norcross, GA) with its microphone (Type 4189) suspended by an extension cable from the ceiling of the sound chamber and positioned in the same location where the center of a frog's head was positioned during recordings of tympanum responses.

Analysis

We analyzed digital recordings (48 kHz, 16-bit) of tympanum responses by the laser and sounds by the probe microphone. Two absolute measures of the ipsilateral

tympa-num's response were analyzed using MATLAB: TVA, (in dB re 1 mm/s/Pa) and TVP, (in degrees). We determined TVA as the RMS velocity of ipsilateral tympanum movements measured over the duration of each tone. We compared TVAs to the RMS sound pressure level (in dB SPL) measured with the probe microphone immediately adjacent to the ipsilateral tympanum. The response of the laser vibrometer was flat ($\pm 1\%$) across the range of frequencies examined in our study. Measurements of recordings made with the probe microphone were corrected for the frequency response of that sensor. We determined TVP as the shift in phase that occurred between tympanum vibrations at a particular sound presentation angle and a reference angle of 0° (frontal direction). TVP was measured using custom MATLAB code modified from the FitSignal script (v.120825, Markus Nentwig). TVP was compared with the corresponding phase shifts in probe microphone recordings made for the same sound presentation angle and the 0° reference angle. Negative values of TVP and phase shifts in sound recordings indicate a delay in temporal response relative to presentation at 0° , and positive shifts indicate that oscillations preceded those measured at 0° . All measurements were averaged across the five presentations of each stimulus. All experimental measurements were corrected for small inter-angle variation in signal level ($< \pm 1.4$ dB at 600 Hz, $< \pm 0.6$ dB across all other frequencies) and shifts in stimulus timing as sound arrived at the pedestal (within ± 40 μ s at 600 Hz and ± 15 μ s across all other frequencies) using measurements taken with the calibration microphone when no frog was present.

TVA and TVP were our primary measures of ear directionality, and its potential dependence on frequency, signal level, sex and body size. Analysis of covariance (ANCOVA) was used to compare variation in TVAs and TVPs across treatments. For TVAs, we constructed a 12 sound presentation angle (within subjects) \times 5 frequency (within subjects) \times 3 signal level (within subjects) \times 2 sex (between subjects) ANCOVA with mean-centered body size (snout-to-vent length in mm) as the covariate. For TVPs, we constructed a similar ANCOVA model that did not include measurements at sound presentation angles of 0° , because these were used as references for calculating relative phase shifts at the 11 other angles. Female *H. chrysoscelis* are generally larger than males, and this was the case for the individuals used in tone and call presentations (males: 35.43 ± 4.60 mm, females: 40.0 ± 1.66 , mean \pm SD.; Mann–Whitney: $U = -3.125$, $P = 0.001$, $N_m = 11$, $N_f = 11$). To assess whether TVAs and TVPs measured from male and female frogs differed without controlling for any effects of body size, we also constructed additional versions of both statistical models that did not include subject body size as a covariate (ANOVA: 12 or 11 sound presentation angle \times 5 frequency \times 3 signal

level \times 2 sex). The data for TVAs and TVPs fit the assumptions of parametric statistical tests. For each model, we report Greenhouse and Geisser (1959) corrected P values for within-subjects effects with more than a single numerator degree of freedom. We report partial η^2 , which ranges in value from 0 to 1, as a measure of effect size for all main effects and interactions in the ANCOVA and ANOVA models. We used a significance criterion of $\alpha = 0.05$ for all analyses.

We made the following predictions. First, if tympanum responses are directional, we predicted a significant main effect of sound presentation angle. Second, to the extent that directionality in tympanum responses is frequency dependent, we further predicted significant interactions between sound presentation angle and stimulus frequency. Third, if the directionality of the tympanum response depends on sound amplitude, we expected a significant interaction between sound presentation angle and signal level. Fourth, body size may affect directionality in TVA or TVP, or the frequency dependence of this directionality. If this was the case, we expected a significant interaction between sound presentation angle and body size, or a three-way interaction between sound presentation angle, frequency, and body size. And finally, if the sex of a frog, independent of body size, affects directionality in TVA or TVP, or the frequency dependence of this directionality, we expected a significant interaction between sound presentation angle and sex, or a three-way interaction between sound presentation angle, frequency, and sex in the full ANCOVA models that included body size as a covariate.

Assuming bilateral symmetry between the ears, as shown for northern leopard frogs by Ho and Narins (2006), we estimated responses of the contralateral tympanum based on those measured from the ipsilateral side. These estimates provided a means to assess the ipsilateral tympanum's amplitude and phase responses relative to those of the contralateral ear. Such interaural comparisons are relevant because frog must use similar comparisons to extract spatial information about a sound source. We computed the IVAD by subtracting the TVA estimated at each angle on the contralateral side of the subject from the TVA recorded at the corresponding ipsilateral angle (e.g. TVA at 30° minus TVA at -30°). A measure of the IVPD was computed similarly based on TVPs at corresponding angles on the contralateral and ipsilateral sides of the animal. To quantify the contribution of the pressure difference mechanism to tympanum directionality, we assessed IVAD and IVAP relative to the differences in sound measured at the external surfaces of the tympana. We computed these interaural amplitude differences (IADs) and interaural phase differences (IPDs) as the difference in sound pressure level and phase, respectively, between paired sound presentation angles of opposite sign as measured with the probe microphone adjacent to

the ipsilateral tympanum. Note that IADs are equivalent to interaural level differences (ILDs). Where useful to compare absolute, in addition to frequency dependent, measures of the timing of tympanum vibrations, we also report IVPDs and IPDs as interaural vibration timing differences (IVTDs) and interaural time differences (ITDs), respectively.

Call presentations

To better characterize ear directionality in response to behaviorally relevant stimuli, we broadcast synthetic advertisement calls to 10 *H. chrysoscelis* (5 males, 5 females). Synthetic calls were created in MATLAB to have spectral and temporal properties near the average values of calls recorded at our field sites in Minnesota. The call contained 30 pulses, each shaped with a species-typical amplitude envelope and having harmonically related spectral peaks at frequencies (and relative amplitudes) of 1,250 (−9 dB) and 2,500 Hz (0 dB) (see Ward et al. 2013b, for additional details of call synthesis). For each subject, calls were broadcast at signal levels of 76, 85, and 94 dB in separate trials. The methods used for presenting calls and recording tympanum responses were similar to those for tone presentations with two exceptions. First, calls were played at four additional azimuthal angles (16 total angles: 0°, ±30°, ±45°, ±60°, ±90°, ±120°, ±135°, ±150°, and 180°). Second, recordings were made of the right tympanum; thus, ipsilateral (positive) sound presentation angles occurred on the subject's right side. As with our statistical analyses of tones, we used separate ANCOVA and ANOVA models to compare TVA and TVP in response to calls. TVP of the lower (1,250 Hz) and upper (2,500 Hz) spectral peaks in the call were measured separately. To obtain a measure of frequency-dependent directionality similar to that used for tone-evoked responses, we included spectral peak (1,250 and 2,500 Hz) as a within-subjects factor in our analysis of call-evoked responses.

Frequency sweep presentations

We examined TVA in response to frequency-modulated (FM) sweeps in 12 animals (7 males, 5 females). Recordings were made in an anechoic room (4 m × 4 m × 3 m, L × W × H) at Syddansk Universitet. The ceiling, walls, and most of the floor were covered with 55-cm rockwool wedges. The room has been determined to be anechoic to below 200 Hz. During measurements, the immobilized frog was positioned on a thin wire mesh platform suspended 1.2 m above the floor from a thin metal pole. An additional thin wire supported the frog's mandible such that the animal's head and body were in a naturalistic position during measurements. The platform on which the frog was positioned was at the center of a 12-speaker array (JBL 1G),

with each speaker separated from the platform by 1 m and from adjacent speakers by 30°. The frog's snout was aimed toward the speaker designated as having a position of 0°. Positive and negative angles correspond to broadcasts from speakers that were ipsilateral and contralateral, respectively, to the recorded tympanum. Stimulation and data recording were controlled by Tucker-Davis (TDT) System II hardware and customized software. The acoustic signal consisted of a 175-ms FM tone that swept linearly upward in frequency between 200 and 7,500 Hz. FM sweeps were amplified (Cambridge Audio Azur 740A) and broadcast at signal levels between 80 and 90 dB SPL. Prior to tympanum measurements, the transfer functions of all 12 speakers were determined for the FM sweep using a 0.5 in. microphone (Brüel and Kjær Type 4190) placed at the center of the set-up at the approximate position of a frog's head during measurements. For subsequent tympanum measurements, the signal broadcast from each speaker was deconvoluted by dividing the spectrum of the sweep by the transfer function of the speaker. This procedure resulted in signals that varied ±0.5 dB at the position of the frog's head across all 12 speakers. We made tympanum measurements using a Polyttec laser (Waldbronn, Germany, OFV-505) with its beam focused on the center of the right tympanum. Measurements were made of responses to 16–20 repetitions of the FM sweep from each speaker position and were averaged online. All repetitions were completed at one speaker position before moving to the next, adjacent speaker position. During tympanum measurements, sound at the eardrum was also measured using a probe microphone (Brüel and Kjær Type 4182) placed 5 mm from the eardrum.

For a small subset of subjects ($N_{\text{males}} = 3$, $N_{\text{females}} = 3$), we investigated the influence of lung inflation on the frequency response of the tympanum. For these animals, we made initial tympanum measurements from all speaker angles with the animal's lungs in a natural state of inflation (as described above). We then manually deflated the lungs by pressing gently on the animal's lateral body wall to express air through the glottis. Then the frog was repositioned, the laser was refocused on the tympanum, and a second complete set of measurements from all speaker angles was acquired. Finally, we manually re-inflated the lungs by blowing air through the glottis using a narrow, blunt glass tube until the extension of the body wall resembled the state of extension prior to lung deflation. Then a third, and final, complete set of tympanum measurements was made in response to sounds from all angles. To elucidate the relationship between the natural frequencies of lung vibration and frequencies of tympanum vibrations resulting from the lung input, we recorded vibration of the lateral body wall directly covering the ipsilateral lung during a sweep broadcast at a sound presentation angle of 90° in one subject with naturally inflated lungs. Aside from the

location where vibrations were measured, methods for this quantification of body wall response were identical to those employed to measure the response of the tympanum.

Results

Responses to tones

The ear of *H. chrysoscelis* exhibited frequency-dependent directionality in both the amplitude and phase of the tympanum response to tones presented from different azimuthal angles. Figure 1 depicts TVA (Fig. 1a) and TVP (Fig. 1d) measured with the laser in response to tones, as well as the sound pressure levels (Fig. 1b) and phases (Fig. 1e) of the tones as measured near the external surface of the tympanum with a probe microphone. Also shown in Fig. 1 are the magnitudes of difference between TVA and sound pressure measurements taken adjacent to the tympanum (Fig. 1c), and between TVP and sound phase adjacent to the tympanum (Fig. 1f). Figure 2 depicts the relative differences of each of these same measures between the two ears in the form of polar plots of IVAD (Fig. 2a) and IVPD (Fig. 2c) based on laser measurements, and IAD (Fig. 2b) and IPD (Fig. 2d) based on probe microphone recordings. Figure 2 also shows IVPD and IVP results re-plotted as IVTDs and ITDs (Fig. 2e, f). In both figures, data are shown as a function of sound presentation angle for different frequencies after averaging over signal level.

Directionality of tympanum vibration amplitude

Sound presentation angle had a significant effect on TVA (Table 1). Relative to presentations from a frontal direction (0°), TVAs were higher in response to tones from ipsilateral (positive) sound presentation angles and lower in response to contralateral (negative) angles (Fig. 1a). As the sound source moved from a frontal position along the ipsilateral side, TVAs initially increased, then decreased as the sound source move further toward a rearward direction (180°). The opposite trend was observed for sound presentations from the contralateral side (i.e., between 0° and -150° ; Fig. 1a). Probe microphone measurements of sound pressure level at the tympanum showed very little variation across sound presentation angles at lower frequencies (mean maximal directional variation between any two angles was <1 dB for responses to 600, 1,250, and 1,625 Hz tones; Fig. 1b). Somewhat larger variation across angles was observed at higher frequencies (mean maximal directional variation was 1.7 dB at 2,500 Hz, and 2.7 dB at 3,200 Hz), likely due to diffraction caused by the frog's head. For each frequency tested, maximum directionality, defined as the largest difference in TVA (or TVP, below)

between any two angles, was typically 2–3 dB greater than the maximum directional variation in sound pressure level recorded by the probe microphone (cf. Fig. 1a–c). Averaged across frequencies, the largest IVAD, 3.31 ± 0.24 dB (mean \pm s.e.m, here and elsewhere; Fig. 2a), occurred at a sound presentation angle of 60° . This was 2.61 dB greater than the mean IAD at the same angle (0.70 ± 0.07 dB, Fig. 2b).

TVA also depended on a significant interaction between sound presentation angle and sound frequency, indicating that ear directionality was frequency dependent (Table 1). The maximum directionality occurred at 2,500 Hz (Fig. 1a), which corresponds to the most intense peak in the bimodal spectrum of the advertisement call. At this frequency, the maximum directionality was 4.87 ± 0.66 dB and occurred between responses at -90° and 30° . Aside from frequency-dependent directionality, the ear also showed higher overall sensitivity to some tone frequencies compared to others. For example, mean TVAs spanned a nearly 30 dB range across frequencies in response to tones presented at 94 dB (600 Hz: -29.5 ± 0.4 dB; 1,250 Hz: -9.7 ± 0.6 dB; 1,625 Hz: -3.7 ± 0.6 dB; 2,500 Hz: 1.0 ± 0.6 dB; 3,200 Hz: -0.1 ± 0.3 dB). Consequently, the main effect of sound frequency had a large influence on TVA (Table 1). The ear was particularly insensitive to tones at 600 Hz.

Signal level also had a significant effect on TVA (Table 1). Mean TVAs, averaged across tone frequency and sound presentation angle, for signal levels of 76, 85, and 94 dB were -24.4 ± 0.4 , -16.8 ± 0.4 , and -8.4 ± 0.5 dB, respectively. Given the 9-dB step size between signal levels, the corresponding increases in TVA of 7.6 dB (76–85 dB) and 8.4 dB (85–94 dB) indicate that the tympanum's response increased nearly linearly over the range of signal levels used in this study. However, signal level also entered into significant two-way and three-way interactions with sound presentation angle and frequency. These significant interactions indicate that the frequency-dependent directionality of TVA depended on absolute signal level. This dependence was largely driven by the tympanum's response to sounds at the two lowest frequencies, 600 and 1,250 Hz. At these frequencies directionality in TVA decreased as signal level decreased (Fig. 3). At higher frequencies, the tympanum response was remarkably linear with respect to signal level.

In the ANCOVA model for TVA, the main effects of sex and body size were not significant, nor were there any significant interactions between subject sex or body size and sound presentation angle, frequency, or signal level (Table 1). A reduced ANOVA model that did not include body size as a covariate also showed no influence of subject sex on TVA. Tone-evoked responses, therefore, provided no evidence for an effect of body size or a size-independent effect of sex on ear directionality. We would note,

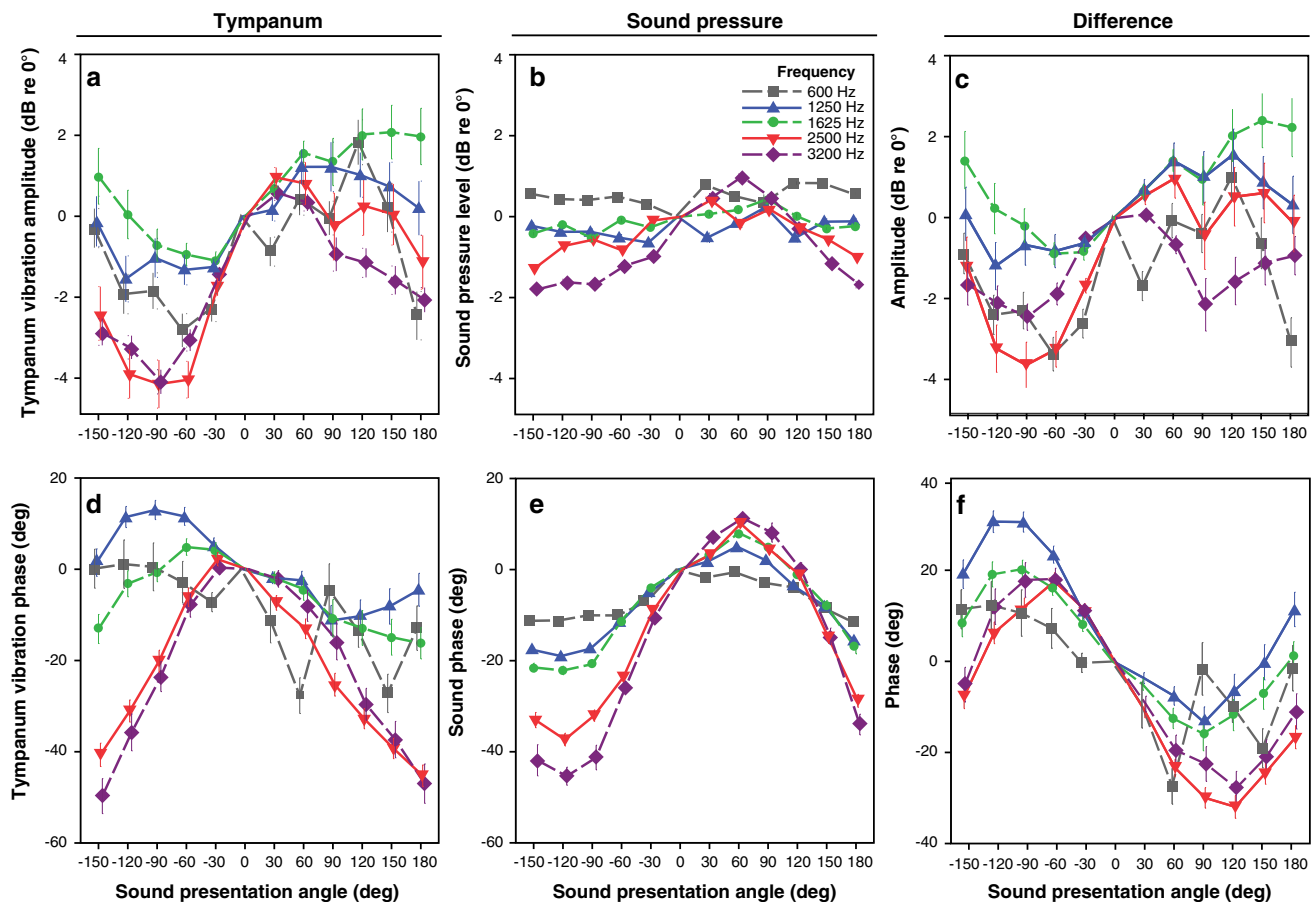


Fig. 1 Directionality in TVA, TVP, and the amplitude and phase of sound pressure adjacent to the tympanum during tone presentations. **a** Mean TVAs from 12 *H. chrysoscelis* in response to tones presented from 12 azimuthal angles and 5 frequencies. For playbacks at each tone frequency, results across all signal levels have been standardized to velocity measured at 0° (frontal direction). Ipsilateral directions are positive, contralateral directions are negative. **b** Amplitude of sound recorded adjacent to the tympanum. **c** Mean differences

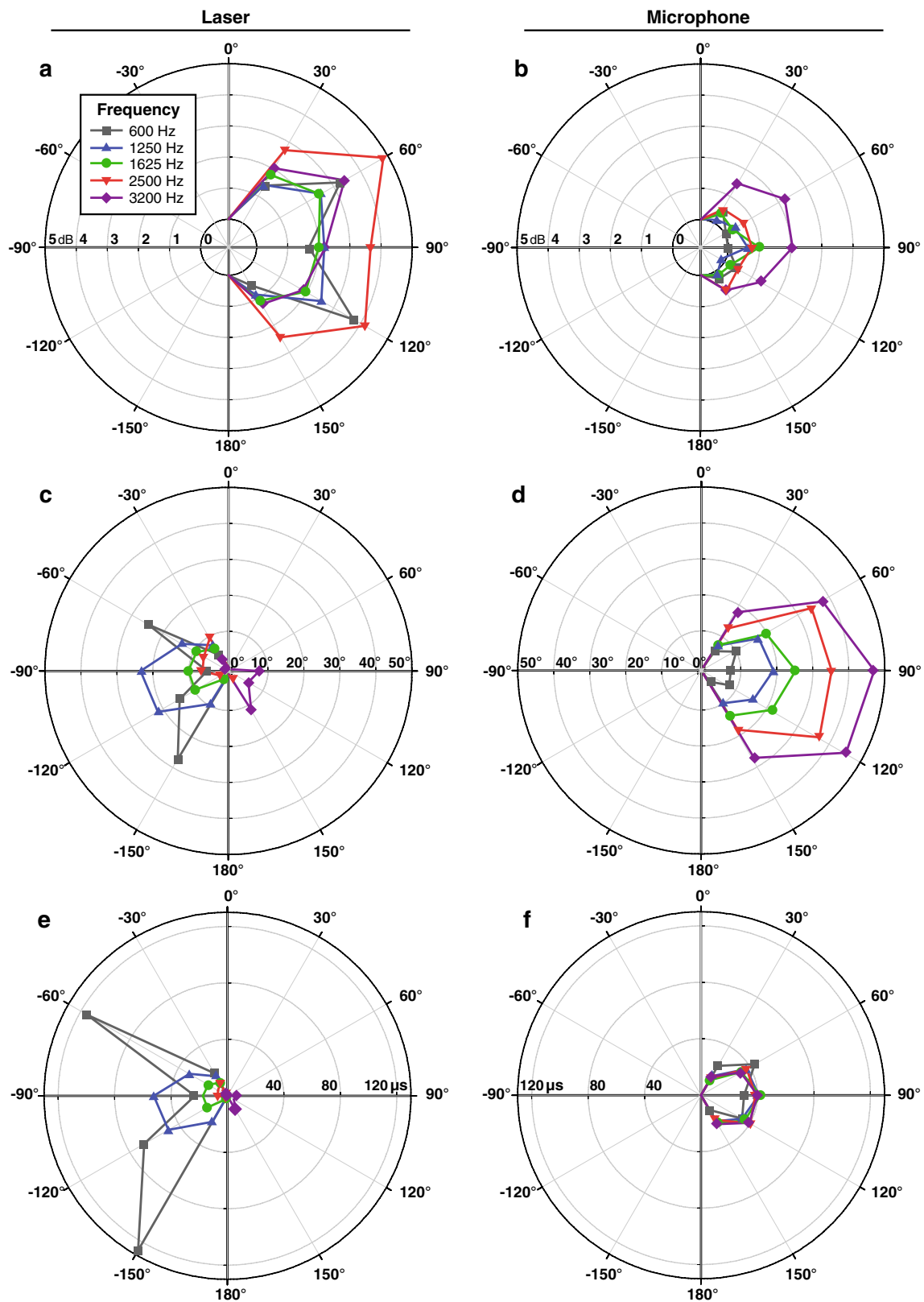
between TVA and sound amplitude measured at the tympanum, calculated from data presented in **a** and **b**. **d** TVPs in response to tones. Negative phase shifts indicate lags relative to response at 0°. **e** Phase of sound pressure adjacent to the tympanum. **f** Mean differences between TVP sound phase measured at the tympanum, calculated from data in **d** and **e**. Error bars represent s.e.m. In some cases, errors are obstructed by data markers

however, that in the ANCOVA model, the non-significant frequency \times sex interaction was associated with a moderate effect size (partial $\eta^2 = 0.279$, $P = 0.055$; Table 1). Relative to males, females had larger average TVAs at frequencies of 600 Hz (+2.3 dB), 1,250 Hz (+5.1 dB), and 1,625 Hz (+9.2 dB), but slightly smaller TVAs at frequencies of 2,500 Hz (−1.1 dB) and 3,200 Hz (−0.3 dB).

Directionality of tympanum vibration phase

The main effects of sound presentation angle and frequency, and their two-way interaction, were significant in the ANCOVA models for TVP (Table 2). When sounds were shifted from a frontal position (0°) to one more ipsilateral, TVP generally became increasingly negative, indicating a phase lag in the ipsilateral direction relative to responses at 0° (Fig. 1d). This lag was most pronounced

for relatively higher sound frequencies. A different pattern in TVP emerged when the sound source was moved from a frontal position to one contralateral relative to the measured tympanum. At relatively high sound frequencies (2,500 and 3,200 Hz), TVP became more negative as the absolute sound presentation angle increased, though the peak TVP at these frequencies occurred at a contralateral angle of −30° (Fig. 1d). At 1,250 Hz (the lower spectral peak in advertisement calls), TVP continued to increase at larger contralateral angles, with a peak occurring at −90° (Fig. 1d). The TVPs for 1,625 Hz on the contralateral side were intermediate between those measured for 1,250 and 2,500 Hz. There was little consistent variation in TVP across sound presentation angles at a frequency of 600 Hz (Fig. 1d). Overall, the maximum directionality in TVP (50.1°) occurred at 3,200 Hz between −30° and −150°. Similar maximum directionality was observed at 2,500 Hz



(47.5° between -30° and 180°), which is the higher spectral peak in advertisement calls. Neither the main effect of signal level nor any of its interactions were significant in

the ANCOVA model for TVP, and hence, we found no evidence to suggest signal level affected the directionality of TVP. As was the case for TVA, sex and body size also did

◀ **Fig. 2** Interaural comparisons of the amplitude (IVAD) and phase (IVPD) of tympanum vibrations and sound pressure adjacent to the tympanum. **a** Mean IVADs in response to tones presented to 12 *H. chrysoscelis* at 12 azimuthal angles and 5 frequencies. The *black circle* towards the center of the plot indicates no difference between response of the ipsilateral and contralateral ears, and the outermost circle indicates 5 dB of difference. Positive sound presentation angles occur on the side of the frog ipsilateral to the focal tympanum. Plot values extending radially at *ipsilateral angles* indicate higher amplitude response in the ipsilateral versus the contralateral tympanum at ipsilateral sound presentation angles. **b** IADs during tone presentations. **c** IVPDs during tone presentations. Plot values extending radially in the ipsilateral direction indicate a phase lead at the ipsilateral ear, *Values extending in the contralateral direction* indicate a phase lag at the ipsilateral ear. **d** IPDs during tone presentations. **e** IVTDs during tone presentations. **f** ITDs during tone presentations. The function of the two ears is assumed to be symmetrical in the calculation of interaural differences

not affect the directionality of TVP in the ANCOVA model (Table 2), nor was there a significant effect of subject sex in an ANOVA model that excluded the body size covariate.

Importantly, directionality in TVP was relatively small (e.g., $<50^\circ$ for call frequencies, Fig. 1d) and on the same order of magnitude as the phase differences in acoustic recordings made adjacent to the tympanum (e.g., $<60^\circ$, Fig. 1e). As a result, IVPDs were not notably larger in magnitude than IPDs. In fact, IVPDs were smaller than IPDs at many combinations of sound presentation angle and

frequency (Fig. 2c, d). When IVPDs and IPDs were converted to *interaural vibration timing differences* (IVTDs) and *interaural time differences* (ITDs), respectively, it became more apparent that the timing of tympanum vibrations varied strongly with frequency, while the timing of sound pressure oscillations external to the tympanum was consistent across frequencies (Fig. 2e, f). Maximum IVTD's varied between 127 μ s for 600 Hz tones presented at 150° , and 11 μ s for 2,500 Hz tones presented at 30° . Maximum ITDs, by contrast, were approximately 40 μ s across all tone frequencies tested.

In contrast to TVP, sound recordings of all tones above 600 Hz in frequency revealed an initial phase lead adjacent to the ipsilateral tympanum as the sound source moved from a frontal position toward the ipsilateral side (Fig. 1e). The maximum phase lead in sound pressure occurred at 60° for all frequencies except 600 Hz, where sound phases lagged behind those measured at 0° for all angles. Across all frequencies, a lag in sound phase occurred when the sound source was moved from 0° in the contralateral direction, with a maximum lag at a sound presentation angle of -120° . The pattern of directionality in sound phase was, therefore, distinct from that measured for TVP, with these two measures having phase shifts of opposite sign for most ipsilateral angles (cf. Fig. 1d, e). Thus, although maximum directionality in sound phase was similar in magnitude to

Table 1 Results of ANCOVA examining factors influencing tympanum vibration amplitude (TVA) during tone presentations

Factor	<i>df</i>	<i>F</i>	<i>P</i>	Partial η^2
Sound presentation angle	11, 88	6.24	0.020	0.438
Signal level	2, 16	3,406.12	<0.001	0.998
Frequency	4, 32	123.33	<0.001	0.939
Sex	1, 8	0.47	0.511	0.056
Body size	1, 8	0.01	0.914	0.002
Sound presentation angle \times signal level	22, 176	3.89	0.007	0.327
Sound presentation angle \times frequency	44, 352	3.69	0.017	0.316
Sound presentation angle \times sex	11, 88	0.55	0.540	0.064
Sound presentation angle \times body size	11, 88	0.50	0.565	0.058
Signal level \times frequency	8, 64	160.01	<0.001	0.952
Signal level \times sex	2, 16	1.06	0.338	0.117
Signal level \times body size	1, 8	0.005	0.959	<0.001
Frequency \times sex	4, 32	3.10	0.055	0.279
Frequency \times body size	4, 32	0.49	0.671	0.057
Sound presentation angle \times signal level \times frequency	88, 704	3.88	0.004	0.327
Sound presentation angle \times signal level \times sex	22, 176	1.06	0.394	0.117
Sound presentation angle \times signal level \times body size	22, 176	1.19	0.332	0.130
Sound presentation angle \times frequency \times sex	44, 352	0.97	0.435	0.108
Sound presentation angle \times frequency \times body size	44, 352	1.04	0.399	0.115
Signal level \times frequency \times sex	8, 64	1.32	0.293	0.142
Signal level \times frequency \times body size	8, 64	0.37	0.654	0.044
Sound presentation angle \times signal level \times frequency \times sex	88, 1	1.35	0.257	0.145
Sound presentation angle \times signal level \times frequency \times body size	88, 1	1.24	0.306	0.134

Bold values indicate statistically significant results

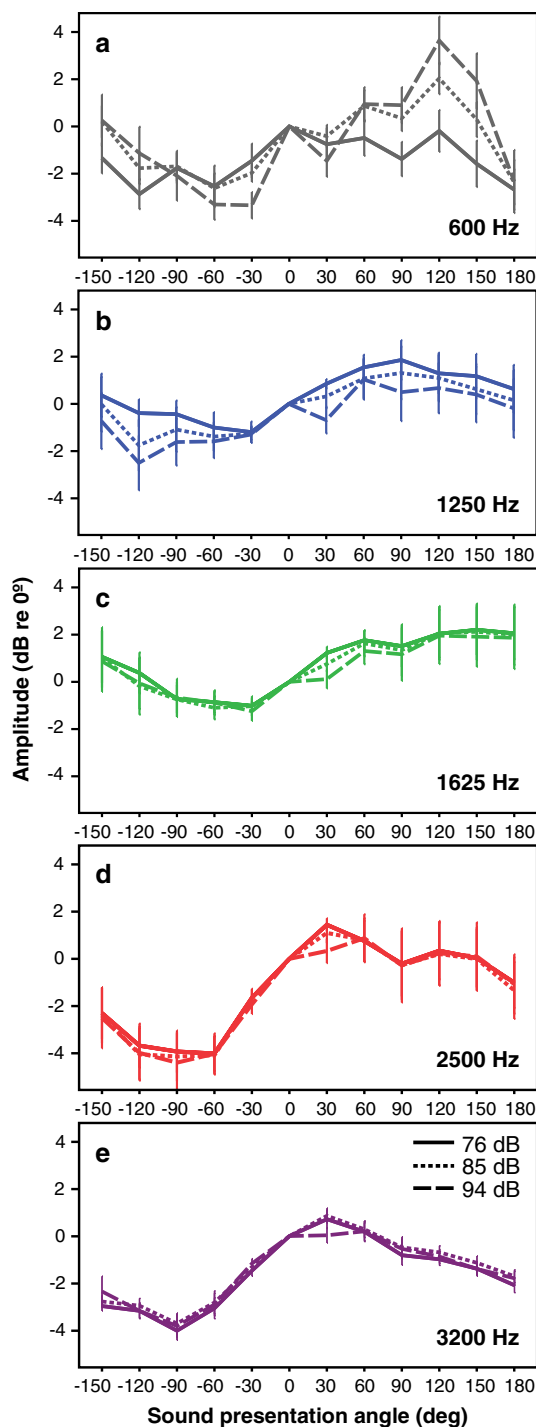


Fig. 3 Signal level and frequency dependence of directionality in TVA. Shown are mean and s.e.m for TVAs from 12 *H. chrysoscelis* recorded in response to tones presented from 12 azimuthal angles and 3 signal levels across 5 frequencies: **a** 600 Hz, **b** 1,250 Hz, **c** 1,625 Hz, **d** 2,500 Hz, and **e** 3,200 Hz. All responses are standardized to the velocity of tympanum vibration at a sound presentation angle of 0°

that observed for TVP, directionality in TVP was not simply due to pressure variation external to the ipsilateral tympanum. This can be seen clearly in Fig. 1f, after accounting

for variation in sound pressure, the transfer function of tympanum vibration still showed strong directionality.

Responses to calls

Figure 4 depicts TVA (Fig. 4a) and TVP (Fig. 4b), averaged across signal levels, in response to calls broadcast from 16 sound presentation angles. Also shown are the corresponding measures of calls made with the probe microphone near the tympanum. There were significant effects of sound presentation angle in the ANCOVA models, confirming that TVA and TVP were both highly directional in response to calls (Tables 3, 4). Directionality in both TVA and TVP was very similar to that evoked by pure tones (cf. Figs. 1, 4). Maximum directionality in TVA occurred between -90° and 135° , and was 3.88 ± 0.19 dB. Maximum IVAD's (3.55 ± 0.18 dB; Fig. 4c) occurred at 90° , and were approximately 3 dB greater than the corresponding IADs. Angular variation in TVP at both the lower (1,250 Hz) and upper (2,500 Hz) spectral peaks of the call was nearly identical to that observed in response to the tones of the same frequency (cf. Figs. 1c, 4b). The interaction between sound presentation angle and spectral peak was significant in the ANCOVA model for TVP (Table 4), indicating that directionality in the phase of the tympanum response varied between the two spectral peaks. A similar sound presentation angle \times frequency interaction was observed in responses to tones (Table 2). Furthermore, similar to the differences observed between IVPDs (Fig. 2c) and IPDs (Fig. 2d) in responses to tones, IVPDs in response to calls were comparable to IPDs measured for calls at the lower spectral peak (IVPDs $<27^\circ$ and IPDs $<21^\circ$, corresponding to IVTDs <60 μ s and ITDs <47 μ s; Fig. 4d), whereas IVPDs were considerably smaller than IPDs measured for the upper spectral peak (IVPDs $<10^\circ$ and IPDs $<60^\circ$, corresponding to IVTDs <11 μ s and ITDs <44 μ s; Fig. 4d).

As was the case for tone presentations, the main effect of signal level was significant in the ANCOVA model for TVA, but not in that for TVP (Tables 3, 4), indicating that changes in the signal level of calls affected the amplitude but not the phase of tympanum vibrations. Importantly, signal level had no effect on directionality in TVA or TVP, and the interaction between signal level and sound presentation angle was not significant in either model. Neither the effects of body size or sex nor any of their interactions were significant in the ANCOVA model for TVP in response to calls (Table 4), nor was the effect of sex significant in the reduced ANOVA model. There was, however, a significant interaction between the effects of sound presentation angle, frequency and body size on TVP, indicating that body size did affect frequency-dependent directionality in tympanum phase during call presentations. There was no effect of sex on TVP in either the ANCOVA or the ANOVA model.

Table 2 Results of ANCOVA examining factors influencing tympanum vibration phase (TVP) during tone presentations

Factor	<i>df</i>	<i>F</i>	<i>P</i>	Partial η^2
Sound presentation angle	10, 80	10.89	<0.001	0.609
Signal level	2, 16	1.23	0.322	0.149
Frequency	4, 32	4.41	0.031	0.387
Sex	1, 8	0.49	0.508	0.065
Body size	1, 8	0.86	0.383	0.110
Sound presentation angle \times signal level	20, 160	1.25	0.317	0.152
Sound presentation angle \times frequency	40, 320	4.37	0.012	0.385
Sound presentation angle \times sex	10, 80	0.58	0.619	0.077
Sound presentation angle \times body size	10, 80	0.42	0.724	0.056
Signal level \times frequency	8, 64	0.40	0.704	0.054
Signal level \times sex	2, 16	0.44	0.644	0.060
Signal level \times body size	2, 16	0.69	0.515	0.089
Frequency \times sex	4, 32	0.69	0.525	0.089
Frequency \times body size	4, 32	0.60	0.568	0.079
Sound presentation angle \times signal level \times frequency	80, 640	1.92	0.159	0.215
Sound presentation angle \times signal level \times sex	20, 160	0.63	0.596	0.082
Sound presentation angle \times signal level \times body size	20, 160	0.49	0.683	0.065
Sound presentation angle \times frequency \times sex	40, 320	1.22	0.327	0.148
Sound presentation angle \times frequency \times body size	40, 320	0.68	0.538	0.089
Signal level \times frequency \times sex	8, 64	0.89	0.472	0.112
Signal level \times frequency \times body size	8, 64	0.89	0.470	0.113
Sound presentation angle \times signal level \times frequency \times sex	80, 640	0.72	0.550	0.093
Sound presentation angle \times signal level \times frequency \times body size	80, 640	0.63	0.599	0.083

Bold values indicate statistically significant results

Response to FM sweeps

The frequency response of the tympanum in subjects with naturally inflated lungs was bimodal, with a narrow low-frequency peak (center frequency ranging between 1.3 and 2.1 kHz), a narrow trough of reduced sensitivity at intermediate frequencies (10–20 dB lower amplitude as compared to the low-frequency peak, center frequency ranging between 1.7 and 2.3 kHz), and a broad peak of sensitivity over a large range of higher frequencies (Figs. 6, 7). The lower peak and trough were usually located between the two spectral peaks of the advertisement call (1,250 and 2,500 Hz), although the trough partially overlapped with the higher call peak in the females tested (Fig. 5). In fact, the locations of both the low- and the high-frequency peaks in tympanum response were shifted towards higher frequencies in females (Lower peak: Mann–Whitney, $U = -2.66$, $P = 0.008$, $N_m = 5$, $N_f = 5$, median_m = 1.3 kHz, median_f = 2.0 kHz; Higher peak: $U = -2.47$, $P = 0.016$, $N_m = 5$, $N_f = 5$, median_m = 2.5 kHz, median_f = 2.8 kHz; Fig. 8). This difference between the frequency response of male and female subjects does not appear to be due to the size difference between the sexes normally observed in this species, as there was no significant difference between the size of males and females used for FM sweeps ($U = 0.32$, $P = 0.841$, $N_m = 5$, $N_f = 5$, median_m = 4.1 g, median_f = 3.6 g; Fig. 8).

As with responses to tones and calls, the ear of *H. chrysoscelis* was directional with respect to the angle at which FM sweeps were presented (Fig. 5). At the frequencies examined in tone presentations, we found a similar level of directionality in TVA in response to FM sweeps as in responses to tones. For example, maximum directionality at the lower spectral peak of the call, 1,250 Hz, was on the order of 3 dB, and maximum directionality at the higher spectral peak, 2,500 Hz, was between 5 and 10 dB for most subjects, with ipsilateral sound presentation angles producing higher mean TVAs at both frequencies. We observed substantially greater maximum directionality in TVA (e.g., 10–15 dB, Fig. 5) at frequencies in the trough of reduced tympanum sensitivity than was seen in response to any frequency we presented during tone trials.

The effects of lung inflation on tympanum vibration

Experimental alteration of lung air volume had pronounced effects on the overall frequency response and directionality in TVA. As illustrated in Figs. 6, 7, the low-frequency peak and the trough were absent in tympanum recordings when the lungs were deflated, and returned when the lungs were re-inflated. Furthermore, the depth of the trough varied strongly with sound presentation angle, and this directionality was eliminated in frogs with deflated lungs (Fig. 6).

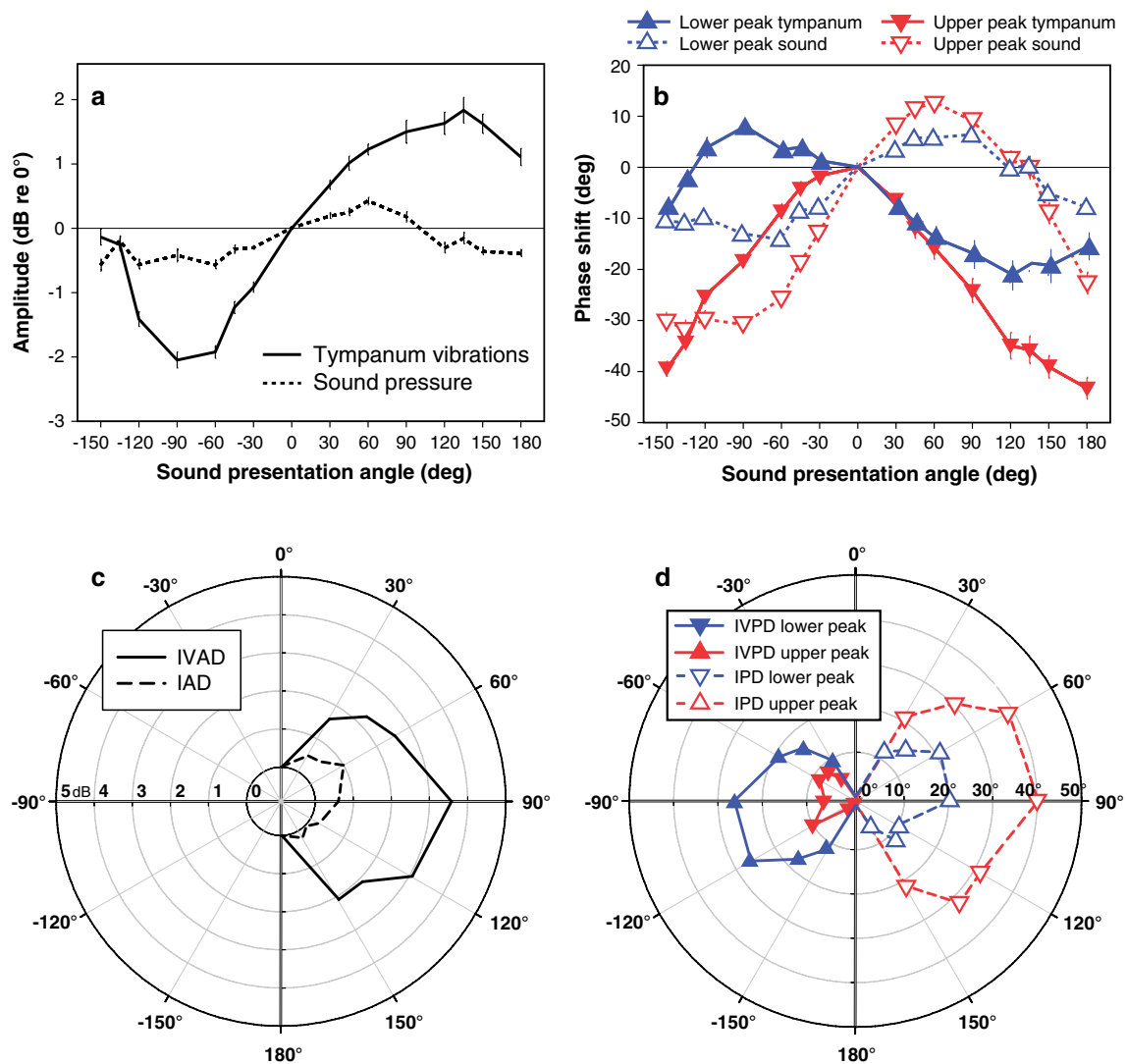


Fig. 4 Directionality in amplitude and phase in response to calls. **a** Mean \pm s.e.m. amplitude of tympanum vibrations (TVA, solid line) and sound pressure adjacent to the tympanum (dashed line) in 10 *H. chrysoscelis* in response to calls from 16 azimuthal angles. Results across all signal levels have been standardized to velocity measured at 0°. **b** Mean phase shift for tympanum vibrations (TVP, solid line) and sound pressure adjacent to the tympanum (dashed line) relative to phase when sound presentation angle was 0°. Shown are shifts

for the lower (1,250 Hz) and upper (2,500 Hz) call peaks. Negative phase shifts indicate that measurements at the contralateral ear lagged behind those measured at 0°. **c** IVADs (solid line) and IADs (dashed line). Plot values extending towards positive sound presentation angles indicate that amplitude was greater on the ipsilateral side of the frog. **d** IVPDs (solid line) and IPDs (dashed line) for the lower and upper call peaks. Plot values extending towards ipsilateral angles indicate a lead in phase relative to 0°

The frequency response curve generated from direct measurement of body wall vibrations in one subject showed a single peak at approximately 1,400 Hz, which corresponded well with the lower spectral peak from the tympanum response of same subject with inflated lungs (Fig. 7).

Discussion

The four main goals of this study of *H. chrysoscelis* were to describe the directionality of the tympanum response,

to investigate the extent to which directionality varied with frequency and signal level, to characterize how lung volume influenced patterns of directionality, and to assess the influence of size and sex differences on directionality. The main results in relation to these four goals can be summarized as follows. First, both the amplitude and phase of tympanum vibrations, TVA and TVP, respectively, varied significantly with azimuthal sound presentation angle. This resulted in clear ovoid directional patterns in estimates of interaural differences in tympanum amplitude (IVAD), and these differences were consistently larger than the corresponding

Table 3 Results of ANCOVA examining factors influencing tympanum vibration amplitude (TVA) during call presentations

Factor	<i>df</i>	<i>F</i>	<i>P</i>	Partial η^2
Sound presentation angle	15, 75	44.48	<0.001	0.899
Signal level	2, 10	8,843.05	<0.001	0.999
Sex	1, 5	1.57	0.266	0.238
Body size	1, 5	4.66	0.083	0.482
Sound presentation angle \times signal level	30, 150	1.30	0.315	0.206
Sound presentation angle \times sex	15, 75	1.30	0.313	0.207
Sound presentation angle \times body size	15, 75	0.73	0.514	0.127
Signal level \times sex	2, 10	0.01	0.922	0.002
Signal level \times body size	2, 10	1.21	0.323	0.194
Sound presentation angle \times signal level \times sex	30, 150	1.30	0.402	0.206
Sound presentation angle \times signal level \times body size	30, 150	1.02	0.726	0.170

Bold values indicate statistically significant results

Table 4 Results of ANCOVA examining factors influencing tympanum vibration phase (TVP) during call presentations

Factor	<i>df</i>	<i>F</i>	<i>P</i>	Partial η^2
Sound presentation angle	14, 84	16.20	0.001	0.730
Signal level	2, 12	0.10	0.794	0.192
Spectral peak	1, 6	45.60	<0.001	0.884
Sex	1, 6	0.19	0.896	0.003
Body size	1, 6	0.05	0.834	0.008
Sound presentation angle \times signal level	28, 168	0.92	0.393	0.133
Sound presentation angle \times spectral peak	14, 28	36.78	<0.001	0.860
Sound presentation angle \times sex	14, 84	0.05	0.930	0.008
Sound presentation angle \times body size	14, 84	0.25	0.743	0.040
Signal level \times spectral peak	2, 12	0.72	0.453	0.108
Signal level \times sex	2, 12	0.15	0.743	0.024
Signal level \times body size	2, 12	0.04	0.880	0.006
Spectral peak \times sex	1, 6	0.13	0.735	0.020
Spectral peak \times body size	1, 6	0.39	0.554	0.061
Sound presentation angle \times signal level \times spectral peak	28, 168	0.89	0.390	0.129
Sound presentation angle \times signal level \times sex	28, 168	0.11	0.810	0.018
Sound presentation angle \times signal level \times body size	28, 168	0.50	0.546	0.077
Sound presentation angle \times spectral peak \times sex	14, 84	1.87	0.153	0.237
Sound presentation angle \times spectral peak \times body size	14, 84	3.22	0.033	0.350
Signal level \times spectral peak \times sex	2, 12	0.78	0.434	0.115
Signal level \times spectral peak \times body size	2, 12	0.02	0.928	0.004
Sound presentation angle \times signal level \times spectral peak \times sex	28, 168	0.89	0.720	0.129
Sound presentation angle \times signal level \times spectral peak \times body size	28, 168	0.26	0.649	0.042

Bold values indicate statistically significant results

differences in sound pressure level external to the two ears (IAD). Typical differences between IVADs and IADs ranged between 1 and 4 dB in responses to tones and calls. By contrast, the directional patterns observed for interaural differences in the phase response of the tympanum (IVPD) were small, and similar in magnitude to phase differences in sound pressure measured at the two ears (IVP). When these phase differences were considered in terms of timing differences, ITDs at the external surfaces of the tympanum were small (≤ 40 μ s) and IVTDs were also small (≤ 60 μ s) for most frequencies and for calls. Second,

directionality in TVA and TVP varied with frequency and, to a lesser extent, with signal level. While there was appreciable directionality at the frequencies emphasized in calls, especially at 2,500 Hz, and in response to calls themselves, FM sweeps revealed that the greatest TVA directionality occurred at frequencies in the trough of reduced tympanum response, which for all males and one female were found between the spectral peaks of the advertisement call. Third, lung volume strongly influenced the tympanum's sensitivity to relatively low frequencies as well as directionality in TVA, especially for frequencies in the range of the trough

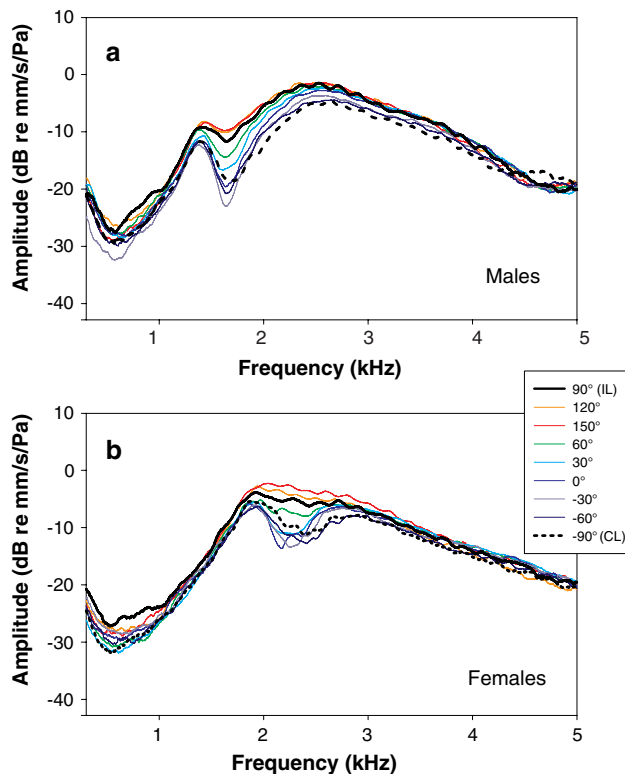


Fig. 5 Directionality in TVA for male and female *H. chrysoscelis*. Mean TVA spectra (peak standardized) of **a** males ($n = 5$) and **b** females ($n = 5$) in response to FM sweeps presented at 12 azimuthal angles

of low sensitivity. Finally, while there was no evidence to indicate that TVA or TVP varied strongly with body size or depended on sex differences in responses to tones and calls, TVAs recorded during FM sweeps revealed consistent differences between the tympanum responses of males and females at frequencies not included in our tone presentations or emphasized in calls. These differences did not appear to be due to size variation between the males and females presented with FM sweeps.

Directionality of the tympanum response

Tympanum vibration amplitude

The pattern of directional sensitivity in TVA across angles in *H. chrysoscelis* was similar to that described previously for its sister species, *H. versicolor* (Jørgensen 1991; Jørgensen and Gerhardt 1991). Likewise, the magnitudes of IVADs for tones at frequencies emphasized in advertisement calls were similar between the two species (<5 dB for *H. chrysoscelis* versus <6 dB for *H. versicolor*). The directionality observed in TVA was consistently larger than that observed in sound pressure measurements made adjacent to the tympanum.

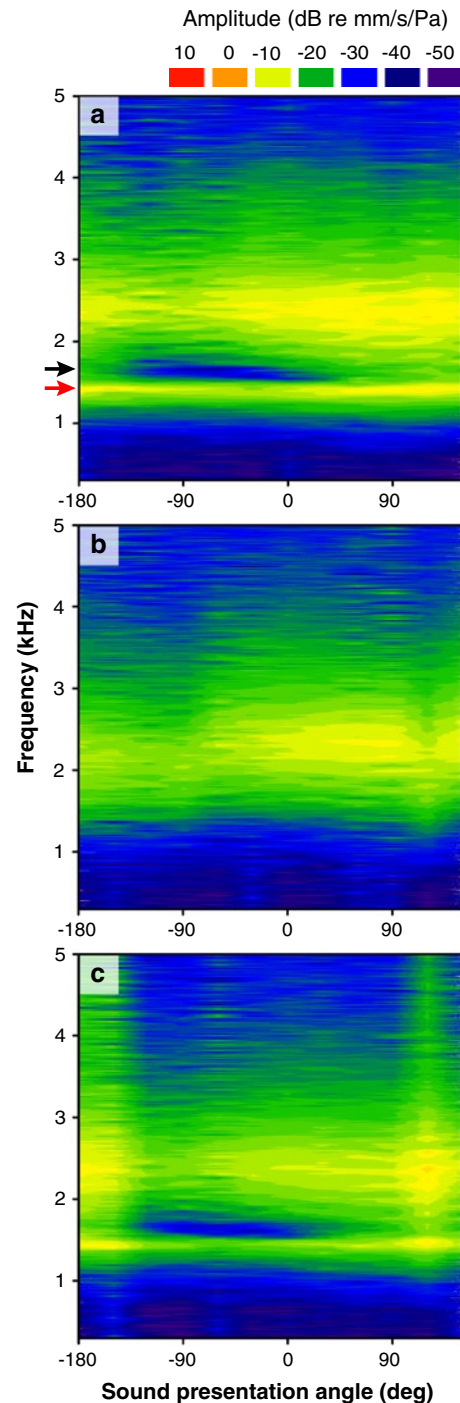


Fig. 6 The effects of lung inflation on directionality in TVA. Tympanum response to FM sweeps presented from 12 azimuthal angles in a typical male *H. chrysoscelis* with **a** normally inflated lungs, **b** deflated lungs, and **c** lungs that were refilled with air. Note that the spectral peak at 1,450 Hz (“peak”, red arrow) and strong directionality between 1,600 and 1,900 Hz (“trough”, black arrow) disappears when the lungs are deflated

Recordings from the torus semicircularis of green treefrogs, *H. cinerea*, suggest this species is capable of detecting IADs as small as 1 or 2 dB (Feng and Capranica

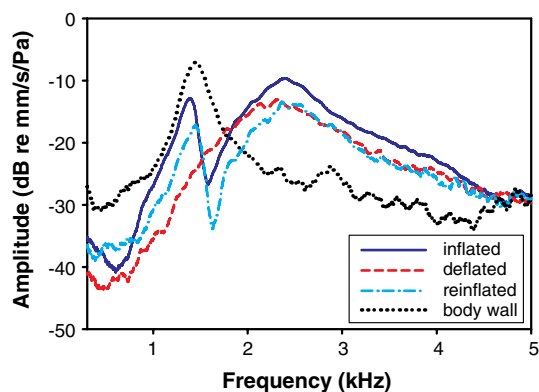


Fig. 7 Lung effects and directionality in *H. chrysoscelis*. Shown is the TVA spectrum of a typical male *H. chrysoscelis* presented with FM sweeps from a sound presentation angle of 90°. Plots indicate spectra measured when lungs of this subject were either in a natural state of inflation, deflated, or re-inflated. Also shown is the spectrum of body wall vibrations recorded in response to the same frequency sweeps

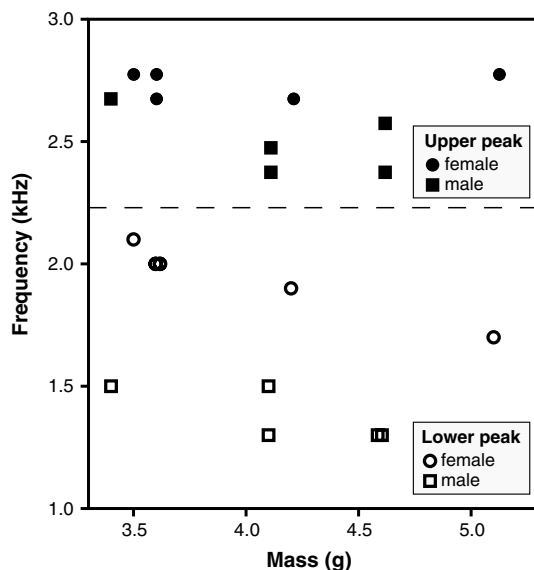


Fig. 8 Frequencies of the lower and upper peaks in the tympanum response for male and female *H. chrysoscelis*. Shown are lower (open symbols) and upper (closed symbols) peaks for the five males (squares) and five females (circles) presented with FM sweeps, along with the mass of these individuals. Overlapping data markers have been offset

1978). Were *H. chrysoscelis* similarly sensitive, it would, presumably, be able to derive some spatial information from IADs, which we found to reach a maximum of approximately 1 dB for advertisement calls (Fig. 4c). In a companion study (Caldwell and Bee 2014), we show that females are able to localize male calls with a mean orientation error of 23.5° across forward angles of sound incidence ($\pm 45^\circ$ relative to the snout). If we assume this value

of mean orientation error estimates the precision with which the animals can localize a call in azimuth, then we can use the maximum rate of angular change in IAD and IVAD between any two forward angles to conservatively estimate the minimum IADs that can influence localization behavior. Based on results shown in Fig. 4c, we can compute the maximum rate of angular change for IAD as 0.0163 dB/°, and that for IVAD as 0.0531 dB/°. In both cases, these values occurred between 0° and 30° (Fig. 4c). If *H. chrysoscelis* uses IADs alone to localize sounds, then they would need to assess IADs of about 0.4 dB, or IVADs of about 1.25 dB, to achieve orientation with a precision of 23.5°. In another study, it was demonstrated that the attractiveness of *H. chrysoscelis* calls is considerably reduced when speakers separately broadcasting each of the call's two spectral peaks are spatially separated by just 7.5° (Bee 2010). Were this spatial sensitivity based on IADs alone, our results suggest that this would require an ability to assess IADs as small as 0.1 dB, or IVADs as small as 0.4 dB. Such an acute sensitivity to changes in IADs would be remarkable, and it seems likely that these frogs attend to additional cues to refine their estimates of sound source location. The most obvious candidate for such a cue would be IPDs. However, our results also indicate that IPDs and IVPDs are quite small in *H. chrysoscelis*.

Tympanum vibration phase

In addition to directionality in TVA, several anuran species also show strong, consistent patterns of directionality in the phase of tympanum vibrations or in neural response to IPDs (Schmitz et al. 1992; Jørgensen and Christensen-Dalsgaard 1997a; Klump et al. 2004; Ho and Narins 2006). It is therefore likely that IPDs play an important role in the spatial hearing of some anurans. Our results show that for *H. chrysoscelis*, sound presentation angles on the ipsilateral side of the frog were associated with a lag in the phase of tympanum vibrations relative to the corresponding contralateral angles (Figs. 2c, 4d). This finding is consistent with measures of phase directionality of the tympanum and auditory nerve responses in *R. pipiens* (Schmitz et al. 1992; Ho and Narins 2006) and auditory nerve responses in *R. temporaria* (Jørgensen and Christensen-Dalsgaard 1997a). Our results differ somewhat from those reported for *H. cinerea*, in which ipsilateral sound presentation angles resulted in phase advances in auditory nerve responses relative to presentation at 0° (Klump et al. 2004).

In contrast to our results for TVA and IVAD, the maximum directionality observed in TVP was similar in magnitude to that observed in sound pressure recordings made at the tympanic membrane. In addition, our estimates of IVPD were generally smaller and less consistent across direction and sound frequency compared with IPD (Fig. 2c,

d). Although the phase response of the tympanum of *H. chrysoscelis* is directional, the magnitude of this directionality is quite small when phase is considered in terms of IVTDs and ITDs. At tone frequencies corresponding to the two spectral peaks in calls, 1,250 and 2,500 Hz, *H. chrysoscelis* had maximum mean IVPDs, averaged across signal levels and subjects, of approximately 23.5° and 9.6°, respectively (Fig. 2c). These IVPDs correspond to IVTDs of approximately 52.2 and 10.7 μ s, respectively (Fig. 2e). We measured similarly small maximum IVPDs during presentation of advertisement calls (Fig. 4d, lower peak: 26.4°, corresponding to an IVTD of 58.7 μ s; upper peak: 9.8°, corresponding to 10.9 μ s). Recordings from cells in the superior olivary nucleus and torus semicircularis of green treefrogs, *H. cinerea*, suggest that frogs may be capable of detecting ITDs, and likely therefore IVTDs, in the range of 100 μ s (Feng and Capranica 1978). It remains to be seen whether frogs are capable of detecting IVTDs on the scale of those we measured in *H. chrysoscelis*. It should be noted, however, that changes in IVTDs potentially result in much larger shifts in the relative timing of auditory nerve responses from the left and right ears, which in turn could provide a basis for using IVTDs in binaural comparisons at higher levels of the central auditory system. For example, in *R. pipiens*, changes in the preferred firing phase of auditory nerve fibers in response to changes in sound azimuth are several times greater than corresponding changes in IVPDs (Schmitz et al. 1992; Ho and Narins 2006). Intensity dependent shifts in firing latency may be partially responsible for this pattern of auditory nerve response. In the more closely related *H. cinerea*, fibers in the auditory nerve show timing shifts that are an order of magnitude greater than ITDs (Klump et al. 2004). With no published IVTD data for the tympanic response of *H. cinerea*, however, it is unclear whether the discrepancy between ITDs in sound at the two ears and timing differences measured from auditory nerve fibers are introduced by the mechanics of the pressure difference ear, or whether they are caused by some other mechanism.

While the inherent directionality in both the amplitude and phase of tympanum vibrations is well documented in anurans, and it is apparent that directional information in both amplitude and phase is processed by the auditory system, it is not clear how frogs actually use these two sources of information in perceptual tasks. For example, there have been no behavioral studies investigating the relative importance of IVAD and IVPD information in the assessment of spatial information in anurans. Such research would be invaluable to our understanding of how frogs extract and process spatial information from sounds in their environment. However, given the complexity and potential variability in how sound energy reaches the anuran inner ear (Mason 2007), it may prove difficult to design behavioral

tests that distinguish between the use of amplitude and phase as separate sources of directional information.

Frequency dependence of tympanum directionality

We found that directionality in both the amplitude and phase of tympanum vibrations was strongly frequency dependent. Frequency-dependent directionality in the tympanum's response to sound is a common pattern across anurans (Jørgensen and Gerhardt 1991; Jørgensen and Christensen-Dalsgaard 1997b), but does not necessarily occur over behaviorally relevant frequency ranges in all species (Michelsen et al. 1986). While the pattern of amplitude directionality was fairly consistent across frequencies—ovoid with greater amplitudes at ipsilateral sound presentation angles, and only the magnitude of this directionality varying between frequencies (Fig. 1a)—this was not the case for directionality in phase. The pattern of directionality in phase varied strongly between frequencies, even differing in the sign of the phase shift for some angles between frequencies (Fig. 1d).

Our results do not indicate that the mechanical response of the ear of *H. chrysoscelis* is specially tuned to localize the sound frequencies emphasized in conspecific advertisement calls. While the largest IVADs in response to pure tones did occur at a frequency corresponding to the higher spectral peak in the call, 2,500 Hz, IVADs were notably smaller at the frequency of the lower spectral peak, 1,250 Hz (Fig. 2a). Furthermore, IVPDs were not particularly large at either spectral peak (Figs. 2c, 4d). Examining responses to FM sweeps, it is evident that the frequency range of maximal amplitude directionality does not necessarily include the frequencies of either spectral peak in the call (e.g. for males). This result is consistent with past work showing that maximal directionality is not found at call frequencies for other frogs (Jørgensen 1991; Jørgensen et al. 1991; Jørgensen and Christensen-Dalsgaard 1997a), including *H. versicolor*, the sister species of *H. chrysoscelis* (Jørgensen and Gerhardt 1991). In the few anurans for which it has been measured, the frequency of maximal directionality appears to be influenced by sound-induced vibrations in the lungs, and these 'lung effects' can occur at frequencies between the two frequency peaks of the advertisement call (Jørgensen 1991; see below for additional discussion of lung inputs).

Little is known about how frequency-dependent directionality in the ear might affect the use of spatial information among anurans. However, one study has investigated the role of sound frequency in spatial release from masking in *H. chrysoscelis*. Nityananda and Bee (2012) measured signal recognition thresholds in response to calls presented from speakers that were either colocated with a source of masking noise or separated from it by 90° in azimuth.

Females experienced masking release in the separated condition regardless of whether the signal had the usual, bimodal frequency spectrum, with peaks at 1,300 and 2,600 Hz, or a unimodal spectrum with just one of the two peaks. Interestingly, when stimuli contained only the lower spectral peak, females experienced about 2 dB more masking release as compared to when only the higher spectral peak was broadcast. This result is not necessarily what we would expect based on tone-evoked tympanum responses if *H. chrysoscelis* uses primarily amplitude directionality to extract spatial information from calls. Tones presented at the frequency of the lower spectral peak of the call (1,250 Hz) produced 1–3 dB less directionality over a 90° separation than did signals at the higher call peak (2,500 Hz; Fig. 1a). In fact, the results of Nityananda and Bee (2012) are more consistent with the use of tympanum phase to extract spatial information. In response to tones, TVPs at the lower spectral peak of the call were more directional than those at the higher peak (Figs. 2c, 4d).

Level dependence of directionality

In responses to tones and calls, the overall pattern and magnitude of directionality in TVA and TVP was generally consistent across signal levels. We found no evidence that signal level affected directionality of TVP. However, directionality in TVA did vary with signal level during tone, but not call, presentations (Table 1). This dependence of directionality in TVA on signal level was driven, in part, by decreases in directionality at the lowest two tone frequencies, 600 and 1,250 Hz, at the lower signal levels (76 and 85 dB, Fig. 3). It is not clear what led to this level dependence in directionality, but it is possible that this result stems from limitations in our recording equipment. Of the tone frequencies tested, the tympanum was least sensitive to 600 and 1,250 Hz, and it is possible that the tympanum response to these low frequencies was somewhat obscured by the noise floor of our recordings at the 76 dB signal level. Previous investigations in other anurans have found that the magnitude of the tympanum response is linear across the amplitudes tested (Pinder and Palmer 1983; Vlaming et al. 1984; Jørgensen et al. 1991; Ho and Narins 2006; Penna et al. 2009). Our results are generally consistent with these previous findings.

Lung inputs

The importance of a sound pathway through the body wall and lungs in anuran hearing has been appreciated for some time (Wilczynski et al. 1987; Narins et al. 1988; Ehret et al. 1990; Jørgensen 1991; Jørgensen et al. 1991; Ehret et al. 1994; Lindquist et al. 1998). In the present study, manipulation of lung air volume strongly affected the

frequency response and directionality of tympanum vibrations (Figs. 5, 6). The tympana of subjects with naturally inflated lungs had bimodal frequency responses, with a narrow low-frequency peak, a narrow trough of lower sensitivity at intermediate frequencies, and another peak at higher frequencies. When the lungs were deflated, the low-frequency peak and the trough in the frequency response of the tympanum disappeared, indicating that these features are the result of lung input. The strong congruence between the single peak in the frequency response of the body wall and the lower peak in the response of the tympanum is further evidence that input from the lungs plays a major role in shaping TVA over the frequency range affected by lung inflation. In response to FM sweeps, maximum directionality for all subjects occurred within the frequency range corresponding to the trough produced by lung effects. Although several studies have documented that sound-induced vibrations in the lungs affect tympanum vibrations in other species (Wilczynski et al. 1987; Narins et al. 1988; Ehret et al. 1990; Ehret et al. 1994; Lindquist et al. 1998), few have examined the role of the lung input in tympanum directionality. Two such studies, focusing on *H. gratiola* and *Eleutherodactylus coqui*, found results similar to ours, with maximum directionality in TVA occurring at a trough in the frequency response of the tympanum formed by lung input (Jørgensen 1991; Jørgensen et al. 1991). In our FM sweep presentations, although the trough was usually centered between the two spectral peaks of the call, it did overlap with the upper spectral peak in some individuals (Fig. 5). Further investigation is needed to quantify the effect that lung input has on the ear's directional response to conspecific communication signals.

Sex and size dependence of directionality

Both male (Gerhardt and Rheinlaender 1980; Shen et al. 2008; Ursprung et al. 2009) and female (Rheinlaender et al. 1979; Gerhardt and Rheinlaender 1982; Caldwell and Bee 2014) frogs exhibit accurate phonotaxis toward calling males, but because their behavioral roles differ, males and females may assess conspecific calls differently. The potential for sex differences in spatial hearing among anurans has received very little research attention thus far. The present study identified a clear pattern of sex differences among *H. chrysoscelis* in the frequency responses of male and female tympana presented with FM sweeps (Fig. 5). Both the lung effects (i.e., the low-frequency peak in sensitivity and the trough of lower sensitivity) and the high-frequency peak in sensitivity of the tympanum's frequency response were shifted towards higher frequencies in females, as compared to males. Moreover, as mentioned above, for females presented with FM sweeps, the increased directionality found at frequencies within the trough of reduced tympanum

sensitivity partially overlapped with the 2,500 Hz spectral peak in the call (Fig. 5). As we found no difference between the mass of males and females presented with FM sweeps, it is unlikely that differences in the frequency responses of their tympana can be attributed to size variation between the sexes (Fig. 8). It is, however, not clear how relevant these sex differences are to the behavior of this species. During tone and call presentations, which both included focused examinations of the frequencies most likely to be behaviorally relevant (1,250 and 2,500 Hz), we found no strong evidence to suggest that there was either a size-independent or a size-dependent sex difference in TVA or TVP, or in how these two measures varied with sound presentation angle. In fact, after controlling for sex differences in body size, the main effects of sex and the sound presentation angle \times sex interaction in our analyses of tone-evoked responses were associated with some of the smallest effect sizes in our study (partial $\eta^2 < 0.08$; Tables 1, 2). Though still not significant, the effect sizes associated with these two effects were somewhat larger in analyses of call-evoked TVAs ($0.20 < \eta^2 < 0.24$, Table 3). These larger effect sizes may reflect an influence of the partial overlap observed in females presented with FM sweeps between the upper spectral peak of the call and the range of frequencies in the trough of reduced sensitivity, but increased directionality, in the tympanum response. We found no evidence that body size affected directionality in either TVA or TVP during tone presentations. Our analysis of directionality in TVP during call presentations, however, revealed an interaction of moderate effect size between sound presentation angle, spectral peak, and body size (Table 4), indicating body size may play a role in the frequency-dependent directionality in phase in response to calls.

Treefrogs as models for integrating physiological and behavioral studies of spatial hearing

A thorough understanding of directionality in the auditory periphery is key to the study of spatial hearing in any vertebrate animal. While considerable progress has been made in describing the directionality of anuran ears, relatively few attempts have been made to integrate biomechanical and neurophysiological data with behavioral studies of spatial hearing in the same species. One reason for this is that behavioral experiments are not tractable in all frog species. Consequently, there is a mismatch between the species most often used to study the biomechanics and neurophysiology of hearing and those used to study acoustically mediated behaviors. For example, for the two best-studied anuran species in terms of the biomechanics and neurophysiology of spatial hearing—*Rana pipiens* (Feng 1980; Feng and Shofner 1981; Schmitz et al. 1992; White et al. 1992; Wang et al. 1996; Wang and Narins 1996; Ho

and Narins 2006) and *R. temporaria* (Vlaming et al. 1984; Aertsen et al. 1986; Melssen and Epping 1992; Jørgensen and Christensen-Dalsgaard 1997a, b)—no studies exist on their use of acoustic spatial information in perception, decision making, or in the performance of behaviorally relevant tasks (e.g. phonotaxis). In contrast to these species, both physiological and behavioral aspects of spatial hearing have been studied previously in a few treefrog species in the genus *Hyla*. These species include the green treefrog, *H. cinerea* (Feng et al. 1976; Feng and Capranica 1978; Rheinlaender et al. 1979, 1981; Gerhardt and Rheinlaender 1982; Michelsen et al. 1986; Schwartz and Gerhardt 1989; Klump et al. 2004), the barking treefrog, *H. gratiiosa* (Feng et al. 1976; Klump and Gerhardt 1989; Jørgensen 1991), and the eastern gray treefrogs, *H. versicolor* (Jørgensen 1991; Jørgensen and Gerhardt 1991; Schwartz and Gerhardt 1995).

Most recently, Cope's gray treefrog, *H. chrysoscelis* has been used to investigate spatial hearing in a variety of different contexts. The present study of this species complements a companion paper investigating open and closed loop localization acuity in azimuth, including quantification of the effects of broadband noise on localization and phonotaxis behaviors (Caldwell and Bee 2014). Several studies have now demonstrated spatial release from masking in Cope's gray treefrogs, and have shown that it reduces response latencies and increases response probabilities in phonotaxis tests (Bee 2007b; Ward et al. 2013a), reduces signal recognition thresholds (Nityananda and Bee 2012), and improves temporal pattern discrimination in the context of species recognition (Bee 2008; Ward et al. 2013a). Studies of auditory grouping and auditory stream segregation in this species indicate spatial cues might be relatively more important for perceptually binding simultaneous signal components, such as the harmonics in the bimodal frequency spectrum of calls, than for binding sequentially produced signal components, such as pulses (Bee and Riemersma 2008; Bee 2010). While the present study represents an important first step, there is a clear need for additional investigations of the biomechanics and neurophysiology of spatial hearing in Cope's gray treefrogs. At present, data similar to those available, for instance, for *R. pipiens* and *R. temporaria*, simply do not exist for *H. chrysoscelis*. By quantifying the frequency-dependent directionality of the amplitude and phase responses of the tympanum, we have provided a starting point for further mechanistic studies of spatial hearing in this species.

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