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Assessing stimulus and subject influences on auditory evoked potentials and their relation to peripheral physiology in green treefrogs (*Hyla cinerea*)

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Abstract

Anurans (frogs and toads) are important models for comparative studies of communication, auditory physiology, and neuroethology, but to date, most of our knowledge comes from in-depth studies of a relatively small number of model species. Using the well-studied green treefrog (*Hyla cinerea*), this study sought to develop and evaluate the use of auditory evoked potentials (AEPs) as a minimally invasive tool for investigating auditory sensitivity in a larger diversity of anuran species. The goals of the study were to assess the effects of frequency, signal level, sex, and body size on auditory brainstem response (ABR) amplitudes and latencies, characterize gross ABR morphology, and generate an audiogram that could be compared to several previously published audiograms for green treefrogs. Increasing signal level resulted in larger ABR amplitudes and shorter latencies, and these effects were frequency dependent. There was little evidence for an effect of sex or size on ABRs. Analyses consistently distinguished between responses to stimuli in the frequency ranges of the three previously-described populations of afferents that innervate the two auditory end organs in anurans. The overall shape of the audiogram shared prominent features with previously published audiograms. This study highlights the utility of AEPs as a valuable tool for the study of anuran auditory sensitivity.

Keywords

Audiogram; Auditory brainstem response; Auditory evoked potentials; Communication; Green treefrog; Hearing

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1. Introduction

Anuran amphibians (frogs and toads) are important model organisms for comparative studies of hearing and sound communication, auditory neurophysiology, and neuroethology (reviewed in Fay and Simmons, 1999; Gerhardt and Huber, 2002; Kelley, 2004; Narins et al., 2007; Wilczynski and Ryan, 2010). However, much of what we know about the anatomy and physiology of the anuran auditory system comes from intensive study of a relatively small number of model species, such as northern leopard frogs (*Rana pipiens*), North American bullfrogs (*Rana catesbeiana*), European grass frogs (*Rana temporaria*), African clawed frogs (*Xenopus laevis*), and green treefrogs (*Hyla cinerea*). Among the issues that currently limit neurophysiological investigations to a small number of model species are the expertise and equipment required to perform survival surgeries and single-or multi-unit recordings, as well as the general invasiveness of such procedures. Here, we report results from a minimally invasive study that investigated the anuran auditory system by recording auditory evoked potentials (AEPs) through the intact skull using subcutaneous scalp electrodes. While not a substitute for more invasive neurophysiological studies, AEPs can provide useful neurophysiological measures of auditory function for use in comparative studies (e.g. Brittan-Powell et al., 2010a; Henry and Lucas, 2008; Ladich and Fay, 2013).

The auditory brainstem response (ABR) is one type of AEP that has been widely used to study auditory sensitivity in a diversity of vertebrate animals, including humans (Hall, 2007), other mammals (McFadden et al., 1999; Ramsier and Dominy, 2010; Song et al., 2006; Supin et al., 1993), birds (Brittan-Powell and Dooling, 2004; Brittan-Powell et al., 2005, 2002; Gall et al., 2011; Henry and Lucas, 2009, 2008; Lohr et al., 2013), reptiles (Brittan-Powell et al., 2010a; Higgs et al., 2002), and fish (Kenyon et al., 1998; Ladich and Fay, 2013; Wysocki and Ladich, 2003, 2001). Though a handful of previous studies have measured brainstem potentials in anurans using methods that require surgery (Bibikov and Elepfandt, 2005; Carey and Zelick, 1993; Corwin et al., 1982; Hillery, 1984; Katbamna et al., 2006b; Seaman, 1991; Yu et al., 2006), recordings of ABRs in anurans using subcutaneous electrodes have been limited to just three previous studies (Katbamna et al., 2006a; Schrode et al., in press; Zhang et al., 2012).

Greater use of ABRs to investigate the anuran auditory system would potentially confer several benefits. First, using subcutaneous electrodes to collect ABR data can be relatively fast, allowing for large sample sizes to be tested during relatively short breeding seasons (Schrode et al., 2014). Second, recording ABRs does not require surgery. Thus, animals have lower risk of infection and procedural complications, would not need to remain in long-term captivity, and would have no need for euthanasia after reaching experimental end points. Third, the same individual could be tested repeatedly over long periods, allowing for longitudinal studies into the effects of seasonality (e.g. Zhang et al., 2012), hormone levels, development, and age on auditory sensitivity. Finally, entirely portable yet low-cost and high quality ABR recording and analysis systems are technologically feasible (e.g. Valderrama et al., 2013), meaning that anuran neurophysiology could be studied outside of the laboratory environment using this method. Together, these potential benefits would

provide a means to conduct comparative research on the auditory systems of a greater diversity of anuran species.

Our broad aim in this study was to establish the use of ABR recordings for studying anuran auditory sensitivity. To this end, we chose an anuran species, the green treefrog (*Hyla cinerea*), with an exceptionally well-described auditory system and acoustic communication system. Over the last four decades, studies of hearing and sound communication in this species have investigated electrophysiological responses (Ehret and Capranica, 1980; Ehret et al., 1983; Feng and Capranica, 1978; Klump et al., 2004; Lombard and Straughan, 1974; Miranda and Wilczynski, 2009a, 2009b; Mudry and Capranica, 1987a), anatomy (Allison and Wilczynski, 1991; Almli and Wilczynski, 2009; O'Bryant and Wilczynski, 2010), endocrinology (Burmeister et al., 2001; Burmeister and Wilczynski, 2005, 2001; O'Bryant and Wilczynski, 2010), and sound-mediated behaviors (Ehret and Gerhardt, 1980; Feng et al., 1976; Gerhardt and Höbel, 2005; Gerhardt, 1987, 1981, 1978a, 1978b; Gerhardt et al., 1990; Höbel and Gerhardt, 2003; Megela-Simmons et al., 1985; Moss and Simmons, 1986; O'Bryant and Wilczynski, 2010; Rheinlaender et al., 1979; Simmons et al., 1993; Vélez and Bee, 2013; Vélez et al., 2012). Focusing our study of the anuran ABR on such a well-described species had the significant advantage of allowing us to directly compare features of the ABR to known features of auditory processing and acoustic signaling in the same species. Such an approach provides a critically important foundation for interpreting future comparative studies using noninvasive ABRs in other frog species with auditory systems that are less well-described. As with other frogs (Capranica and Moffat, 1983; Gerhardt and Huber, 2002; Narins et al., 2007), the green treefrog auditory system appears well adapted for detecting and processing conspecific advertisement calls, which consist of a single, short note (120–160 ms) repeated one to two times per second (Gerhardt, 2001). Each call has a bimodal frequency spectrum with a narrow, low frequency peak near 0.9 kHz and a broader, high-frequency peak in the range of about 2.7–3.0 kHz. Each peak is primarily encoded by a separate sensory papilla in the inner ear. The low frequency peak falls in the range of the amphibian papilla (AP) and the high frequency peak is in the range of the basilar papilla (BP) (Capranica and Moffat, 1983; Ehret and Capranica, 1980). As in other anurans (Capranica, 1976; Feng et al., 1975), the tonotopically-organized AP of green treefrogs can be further subdivided into two populations that have distinct ranges of sensitivity. In the low-frequency population, primary afferents have characteristic frequencies (CFs) lower than about 0.5 kHz; afferents in the mid-frequency population have CFs that range from about 0.5 to 1.2 kHz (Capranica and Moffat, 1983; Capranica, 1976; Ehret and Capranica, 1980). The BP acts as a single acoustic filter, with broadly tuned afferents having similar CFs near 3.2 kHz.

The specific objectives of this study were as follows. First, we sought to assess the effects of frequency, signal level, sex, and body size on ABR waveform amplitudes and latencies and characterize gross ABR morphology for comparison with ABR studies in other animals. Second, we sought to determine the extent to which ABR morphology and dependence on frequency and signal level correspond to the expected tuning of the green treefrog's peripheral auditory system based on results from previous invasive studies (Capranica and Moffat, 1983; Ehret and Capranica, 1980; Lombard and Straughan, 1974). Finally, we

sought to generate an ABR audiogram for comparison with previous behavioral (Megela-Simmons et al., 1985; Weiss and Strother, 1965) and neurophysiological (Lombard and Straughan, 1974; Miranda and Wilczynski, 2009b; Penna et al., 1992) audiograms from green treefrogs as well as ABR audiograms from Cope's gray treefrog (*Hyla chrysoscelis*) (Schrode et al., 2014), the African clawed frog (*Xenopus laevis*) (Katbamna et al., 2006a), and the Emei music frog (*Babina daunchina*) (Zhang et al., 2012).

2. Materials and methods

2.1. Subjects

Our subjects were 21 male (mean $\pm$ SD mass = 7.8 ± 1.6 g) and 24 female (7.6 ± 1.7 g) green treefrogs collected in amplexus at the East Texas Conservation Center (formerly John D. Parker East Texas State Fish Hatchery) near Jasper, Texas, U.S.A. and transported within 24–48 hours of collection to our laboratory in St. Paul, Minnesota, U.S.A. Frogs were housed on a 12-hour photoperiod in aquaria with damp moss and conditioned tap water and fed a diet of vitamin-dusted crickets. ABR recordings were made between July 10 and August 10, 2011, which is during the species' breeding season. All procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee (#1103A97192).

To record ABRs, subjects were immobilized with an intramuscular injection of d-tubocurarine chloride (6.5 – 8.0 μ g/g body mass). We allowed animals to regulate their own lung volume as the immobilizing agent took effect over several minutes. Visual inspection of immobilized animals prior to recordings revealed what appeared to us to be normal lung inflation based on lateral extension of the body walls. To facilitate cutaneous respiration, we draped frogs with a single layer of damp surgical gauze. We applied a local anesthetic (2.5% lidocaine HCl) to the scalp just prior to electrode placement. Following all procedures, we allowed the frog to recover in a dish of shallow water and returned it to its home aquarium once full mobility was regained. Experiments typically lasted 2–3 hours and full recovery was usually reached within 4–6 hours of administration of the immobilizing agent. One subject regained mobility prior to presentation of all stimuli; therefore we discarded these interrupted recordings and restarted and completed the experiment with this subject 3 days later.

2.2. Recording the ABR

All ABRs were recorded in a MAC-3 semi-anechoic sound chamber (W $\times$ D $\times$ H: 81.3 cm $\times$ 61 cm $\times$ 61 cm; Industrial Acoustics Company, Bronx, NY, U.S.A.) sitting on a vibration isolation table (TMC 68–500, Technical Manufacturing Corporation, Peabody, MA, U.S.A.). The temperature inside the chamber was equivalent to the ambient room temperature (~ 19 °C) and varied less than 1 °C across recordings of different subjects. This is a typical temperature at which these frogs breed. The consistency in temperature prevented any of the temperature-induced effects on auditory thresholds known to exist in anurans (Carey and Zelick, 1993; Hubl and Schneider, 1979; Mohneke and Schneider, 1979; Mudry and Capranica, 1987b; Walkowiak, 1980). Each subject was placed in the middle of the chamber on a 2-cm high, acoustically transparent platform with a natural posture, such

that its limbs were tucked next to its body, its mouth was closed, and its head slightly elevated. The subject directly faced a speaker (Orb Mod 1, New York, NY, U.S.A.) and was positioned so that the caudal edges of both tympanic membranes were 30 cm from the front of the speaker. Three platinum alloy subcutaneous needle electrodes (1–3 k Ω , Grass F-E2, West Warwick, RI, U.S.A.) were inserted just under the skin between the eyes (non-inverting) and next to each tympanum (inverting and ground) (Fig. 1a). The electrode leads were twisted together to reduce electrical noise and connected to a Tucker Davis Technology (TDT, Gainesville, FL, U.S.A.) RA4LI low impedance headstage and TDT RA4PA preamplifier (20 $\times$ gain, 25 kHz sampling rate). The signal was then delivered via fiber optic cable to a TDT RZ5 digital processor and stored on a computer for offline analysis. Recordings were notch filtered at 60 Hz and band-pass filtered between 30 Hz and 3.0 kHz.

Acoustic stimuli were created using TDT's SigGenRP software and presented using TDT's BioSigRP software. Signals were output via a TDT RP2.1 processor (50 kHz sampling rate, 16 bit resolution), attenuated by a TDT PA5 programmable attenuator, amplified by a Crown XLS 202 amplifier (Crown Audio, Inc., Elkhart, IN, U.S.A.), and broadcast through the speaker. The stimuli comprised short trains of either rectangular broadband clicks (0.1-ms duration, 25-ms click period) or tones (5-ms duration, 1-ms $\cos^2$ rise/fall, 25-ms tone period). Within a tone train, the frequency of the tone was held constant. Across different tone trains, we tested 23 different frequencies: 0.3 to 1.2 kHz (in 0.15-kHz steps), 1.5 kHz, 1.8 kHz, 2.1 to 3.75 kHz (in 0.15-kHz steps), 4.05 kHz and 5.1 kHz. To ensure the quality of the stimulus presentation through our setup, we digitally recorded all stimuli and measured the percent harmonic distortion (%HD) for tones and the tail-to-signal ratio (TSR) for all stimuli. Example stimulus recordings are depicted in the Supplement. Across stimuli, the median %HD was 0.9 % (interquartile range: 0 to 2.4 %). The median TSR was -16.2 dB (interquartile range: -19.4 to -9.8 dB); that is, echoes were about 10 to 20 dB lower in amplitude than the preceding signal. Given these measurements, we were confident that the neural recordings reflected activity in response to the intended stimuli.

Each recording session began and ended by presenting a train of five equal-amplitude clicks at a suprathreshold sound pressure level (80 dB SPL re 20 μ Pa, fast root-mean-square [RMS], C-weighted) followed by a 100-ms silent interval (Fig. 1b). We used recordings of the response to the five clicks to verify the presence and assess the magnitude of an ABR. Robust click-evoked responses were present at the beginning and end of each recording session and changed little over the course of the session (see Fig. 1 legend).

During a recording session, we presented stimulus trains in which we increased the signal level of successive clicks or tones; henceforth, all signal levels are given in dB SPL. Successive sounds in a train increased in 5-dB increments between 40 and 80 dB (Fig. 1b). We presented trains at a rate of 4 trains/s and alternated polarity (for clicks) or phase (for tones) between consecutive sounds in a stimulus train and between consecutive trains to cancel the microphonic. Each stimulus train was presented in two separate replicates of 400 repetitions (800 repetitions total). We collected one replicate for the click train, followed by the two replicates of all tone trains (with frequencies in a different randomized order for each subject within a replicate) and finished with the second replicate of the click train.

Stimuli were calibrated using a Larson Davis model 831 sound level meter (Larson Davis, Depew, NY, U.S.A.) by placing its ½-inch free field microphone (model 377B02) 30 cm from the speaker at the approximate location of the frog's tympana. We note that at this distance there is the potential for responses to tones with a frequency < about 1.1 kHz to be influenced by near-field particle motion. Levels of the sounds in all stimulus trains were calculated relative to the RMS level of a 1-s tone calibrated to 80 dB. For calibrating clicks, we used a tone frequency of 1.05 kHz; for calibrating tones, we used 1-s tones of equivalent frequency for each tone frequency tested.

2.3. Describing the ABR

Evoked responses were typically composed of a series of 2–3 peaks and troughs occurring between 2 and 12 ms after the stimulus reached the tympana. We used the following analyses to describe the ABR and how its features varied with sound frequency (for tones) and signal level (for clicks and tones), as well as any variation in the ABR due to subject sex and body size.

2.3.1. ABR amplitudes and latencies—An experienced observer measured the amplitude and latency of the first two consistently identifiable peaks of the ABR waveform, as illustrated in Fig. 1c, using a custom-written MATLAB program (v2010b, Mathworks, Natick, MA). Waveforms for a given train were aligned vertically (as in Fig. 2) and the observer visually identified peaks and the following troughs using an adjustable, sliding cursor. The program then recorded the peak or trough as the data point with the maximum or minimum amplitude, respectively, within ± 5 samples (± 0.20 ms) of the cursor's position. The recorded peak or trough was displayed and the observer could discard the point and choose again if necessary. Note that for convenience, we refer to the first and second peaks measured in this way as P1 and P2, respectively, without implying knowledge of the generator or generators of each peak within the nervous system. We return to this issue in the Discussion. P1 was defined as the first positive deflection of the ABR. Following P1, there were sometimes small deflections that varied greatly between the responses of individuals, and often between responses to different signal levels. We defined P2 as the first large, highly consistent deflection observed after P1. Since the shape, amplitude, and latency of P2 differed only slightly between responses, visually comparing evoked responses across signal levels allowed for P2 to be consistently identified. As illustrated in Fig. 1c, amplitudes for the two peaks were defined as the voltage difference (in μV) between the peak (P1 or P2) and the subsequent corresponding trough (T1 or T2). Latencies for each peak were defined as the time (in ms) from stimulus onset to the respective peak after subtracting the time required for the stimulus to reach the tympana (Fig. 1c). We calculated 0.88 ms as the time for sound to travel the 30 cm to the tympana given the average temperature of 19° in the acoustic chamber. Amplitude and latency values were averaged across both replicates of a stimulus for subsequent statistical analysis.

We separately analyzed ABR amplitudes and latencies using factorial analyses of covariance (ANCOVA) to assess the influences of frequency on tone-evoked responses, and the effects of signal level, subject sex, and subject body size on both click-evoked and tone-evoked responses. Before inclusion as a covariate, mass was zero centered by subtracting the mean

mass of all individuals from each individual's mass (Delaney and Maxwell, 1981). The inclusion of mass as a covariate in these analyses tested whether body size influenced either the amplitude and latency of the ABR (covariate main effect) or changes in the ABR across levels of the other factors in the models (covariate interactions). Because ABR amplitude and latency measurements required a visible response, subthreshold signal levels necessarily resulted in missing values. We minimized the impact of missing data in our analyses of ABR amplitudes and latencies in the following way. First, we limited our dataset to those frequencies (for tones) and signal levels (for clicks and tones) at which an ABR was present for the majority of individuals. By limiting our dataset to tone frequencies between 0.45 kHz and 3.6 kHz (inclusive) and signal levels between 60 dB and 80 dB (inclusive), we reduced the amount of missing data from 54% to 16% for clicks and from 50% to 9% for tones. Second, after log-transforming the data to achieve normality, we used multiple imputation to estimate the remaining missing data points (Rubin, 1976; Schafer, 1999). We derived 20 imputed datasets for each of our reduced datasets of amplitude and latency measures. The typical number of imputations recommended is usually less than five (Rubin, 1976; Schafer, 1999); however, there is evidence that increasing the number of imputations beyond five affords greater power to the analysis (Graham et al., 2007). Each imputed dataset for ABR amplitude and latency was analyzed in a separate ANCOVA. Click-evoked responses were analyzed using a 5 signal level (within subjects) $\times$ 2 sex (between subject) ANCOVA. Tone-evoked responses were analyzed using a 19 frequency (within subjects) $\times$ 5 signal level (within subjects) $\times$ 2 sex (between subjects) ANCOVA. For these analyses of imputed datasets, we report the means and ranges for F statistics, P values, and effect sizes (partial η^2). In these and other statistical analyses, which were performed using SPSS v20.0.0 (Armonk, NY, USA), we used a significance criterion of $\alpha = 0.05$ and we report adjusted P values for omnibus tests of within-subjects effects based on the Greenhouse and Geisser (1959) correction. All data are reported as the mean $\pm$ SD unless otherwise noted.

2.3.2. ABR gross morphology—We used a cross-correlation analysis to compare ABR morphology across stimulus types and tone frequency. For this analysis, recordings were examined over a 12-ms window that encompassed the entirety of the evoked response. Each response from each individual was aligned in time to the first peak of the ABR (P1) and then windowed between 2 ms preceding and 10 ms following the peak. These windowed responses were subsequently aligned in voltage such that the average amplitude of the first 1 ms (baseline) was 0 μ V for all responses. We next averaged the windowed ABR waveform across the two replicates of each stimulus for an individual and then across all individuals to obtain the population mean (and SD) ABR waveform in response to each stimulus. We then separately cross-correlated the mean click-evoked ABR and mean tone-evoked ABRs at frequencies of 0.9, 1.5, 1.8, and 3.0 kHz with the mean tone-evoked ABR at each of the 23 frequencies we tested. These four tone frequencies (0.9, 1.5, 1.8, and 3.0 kHz) were chosen because they were either similar to frequencies contained in male advertisement calls (0.9 and 3.0 kHz) or intermediate between these frequencies. We computed the maximum cross correlation within a maximum time lag of 1 ms. These cross-correlation analyses were repeated separately for signal levels of 60 to 80 dB. An additional cross correlation analysis examined ABR morphology at an approximately 10 dB sensation level (SL). For this analysis, the population mean ABR for each stimulus was generated by averaging the

responses evoked by the stimulus broadcast at a signal level that was 10 dB above each subject's threshold for that stimulus (see section 2.3.3). These cross-correlation analyses were intended to uncover broad patterns of similarities and differences in the gross morphology of the ABR waveform across stimuli. Because there was only one average waveform per stimulus, and because these analyses were not designed to test any particular *a priori* hypotheses, we have not conducted statistical analyses for any specific comparisons.

2.3.3. ABR thresholds—We used two methods to estimate the threshold signal level required to elicit an ABR. In one commonly used method (e.g. Cone-Wesson et al., 1997; Gall et al., 2011; Gorga et al., 1988; Lohr et al., 2013), we determined thresholds visually. We displayed the waveforms of responses to a given stimulus train on a computer monitor and ordered them from high to low signal level, as illustrated in Fig. 2. Two experienced observers independently estimated thresholds (Fig. 2) as the arithmetic mean between the lowest signal at which a response was present and the highest signal level at which there was no response (Brittan-Powell et al., 2010a, 2010b, 2005, 2002; Higgs et al., 2002; Lohr et al., 2013). Visual threshold detections were made without regard to identifying individual peaks, but rather were estimated based on the gross visual morphology of each response. Thresholds were scored blind with respect to the identity and sex of the frog and the type of stimulus (click or tone). We determined thresholds separately for the two replicates of each stimulus train, and then averaged across replicates. Thresholds were relatively consistent between replicates (mean difference = 0.8 ± 4.08 dB, median = 0.8 dB, mode = 0 dB). We averaged thresholds across observers for further statistical analysis. Differences in threshold estimates between the two observers were very small (across all estimates: mean = 0.05 ± 2.45 dB, median = 0 dB, mode = 0 dB, intraclass correlation = 0.83).

We used ANCOVAs (with mean-centered mass as the covariate) to evaluate the effects of stimulus frequency (for tones) and subject sex and size on visually detected ABR thresholds. Estimates of ABR thresholds for all stimuli were available for all 45 frogs, so it was not necessary to reduce the dataset and use multiple imputations to analyze this response variable. Residuals were also normally distributed for all but two tone frequencies (Shapiro-Wilk tests: *p*-values > 0.05), so no transformations were performed. Thresholds for click-evoked responses were analyzed using a univariate ANCOVA with sex as the single between subjects factor. Thresholds for responses to tones were analyzed using a 23 frequency (within subjects) $\times$ 2 sex (between subjects) ANCOVA. As a second, observer-free estimate of ABR thresholds, we used a custom-written MATLAB script to compute thresholds by comparing the RMS amplitude of evoked responses to the RMS amplitude of the biological signal in the absence of an acoustic stimulus. For each subject, the RMS amplitude of the response evoked by each of the nine stimuli in a train was computed over a 10-ms time window between 2 ms and 12 ms following stimulus arrival at the ear and plotted as a function of signal level. We then used MATLAB's *fminsearch* function to minimize the sum-of-squares of a sigmoid curve fit to the nine computed RMS values. We determined the threshold as the minimum signal level at which predicted RMS values along the fitted sigmoid curve first exceeded one of three fixed threshold criteria (see Fig. 5a in section 3.4 for an example). These criteria were equal to the mean plus 0.5, 1.0 or 2.0 standard deviations of the RMS amplitude of the biological signal computed over six 10-ms

time windows when no acoustic signal was presented. We excluded the automated threshold estimates for five individuals at the frequencies of 0.3 and 5.1 kHz from analysis, because threshold estimates were outside the range of stimulus levels presented. We provide these data for comparison with visually detected thresholds.

3. Results

3.1. ABR amplitudes

3.1.1. Click-evoked responses—In analyses of click-evoked ABRs, there was a significant effect of signal level on both P1 and P2 amplitudes (Table 1; Fig 2). Between 60 dB and 80 dB, P1 amplitudes increased monotonically from 0.33 ± 0.28 μ V to 0.85 ± 0.56 μ V; mean P2 amplitude increased monotonically from 0.48 ± 0.42 μ V to 1.20 ± 0.63 μ V over this same range of signal levels. No other effects in the ANCOVA models for the amplitudes of click-evoked ABRs were significant (Table 1).

3.1.2. Tone-evoked responses—In our analyses of tone-evoked responses, the main effects of frequency and signal level and the frequency $\times$ signal level interaction were significant for both P1 amplitudes (Table 1; Fig. 3a–c) and P2 amplitudes (Table 1; Fig. 3d–f). There were no significant effects of subject sex or body size on the amplitude of tone-evoked ABRs (Table 1). As illustrated in Fig. 3c,f, sex differences in P1 and P2 amplitudes were quite small compared with the variability observed within each sex.

We consider first the relatively straightforward effects of variation in signal level. Both P1 and P2 amplitudes increased monotonically with increasing signal level (Fig. 3b,e; see also Fig. 2). These effects of signal level were frequency-dependent and somewhat more pronounced for P1 than P2 (Table 1; cf. Fig. 3b and Fig. 3e). P1 amplitudes evoked by frequencies of 1.2 kHz and lower (e.g., 0.9 kHz; Fig. 3b), as well as frequencies of 2.1 kHz and higher (e.g., 3.0 kHz; Fig. 3b), tended to increase linearly with signal level, though the former did so with a steeper slope. At intermediate frequencies (i.e., 1.5 kHz and 1.8 kHz; open symbols in Fig. 3b), P1 amplitudes appeared to increase with signal level at two different rates. For example, between 60 dB and 65 dB for 1.5 kHz, and between 60 dB and 70 dB for 1.8 kHz, P1 amplitudes initially increased at rates similar to those observed for 3.0 kHz (Fig. 3b). At higher signal levels, however, the rate of level-dependent change in P1 amplitudes evoked by tones of 1.5 kHz and 1.8 kHz increased to rates similar to or higher than those seen at 0.9 kHz (Fig. 3b). In contrast to P1 amplitudes, P2 amplitudes at a given signal level, as well as the rate of increase in P2 amplitude across signal level, were more similar across frequencies (cf. Fig. 3b,e).

Compared to the effects of signal level, the effects of variation in frequency on ABR amplitudes were more complex. Most notably, P1 and P2 amplitudes did not change monotonically with variation in frequency. This trend is most clearly evident in the contour plots of Fig. 3a,d (showing data for all signal levels) and in Fig. 3c,f (showing data for just the 80-dB signal level). For P1, amplitude was larger at lower frequencies than at higher frequencies (e.g., 1.2 kHz versus 2.1 kHz; Fig. 3a,c). Within the lower frequency range, P1 amplitudes in response to the 0.45 kHz tone were higher than the amplitudes of responses to the adjacent frequencies of 0.3 and 0.6 kHz. P1 amplitudes at intermediate frequencies

(1.2 to 2.1 kHz) were sometimes considerably larger than amplitudes at immediately adjacent frequencies (Fig. 3c). For example, P1 amplitudes at 1.5 kHz were larger than those at 1.8 kHz and much more similar to amplitudes at lower frequencies such as 0.9 kHz than to those at frequencies > 2.1 kHz (Fig. 3b). For P2, on the other hand, there was a tendency for amplitudes to be larger at frequencies near those present in male advertisement calls (0.9 and 3.0 kHz) compared with other frequencies (Fig. 3d,f). As a result, the contour plot of P2 amplitude revealed a pattern similar to that of the ABR audiogram (see below; cf. Fig. 3d and 5b,c). There was also a trend for P2 amplitudes in response to the 0.45 kHz tone to be higher than the amplitudes of responses to the adjacent frequencies of 0.3 and 0.6 kHz, similar to the trend in P1 amplitudes.

3.2. ABR latencies

3.2.1. Click-evoked responses—The latency to P1 in click-evoked responses depended on signal level and sex (Table 2). The average latency to P1 decreased monotonically as signal level increased, ranging from 3.46 ± 1.31 ms at 60 dB to 2.77 ± 0.29 ms at 80 dB. Females had P1 latencies that were, on average, 0.23 ± 0.13 ms shorter than those observed in males. While statistically significant, the effect size associated with this sex difference was considerably smaller than that associated with the effects of signal level (Table 2). As with P1 latencies, there was a significant effect of signal level on P2 latencies of click-evoked responses (Table 2). P2 latencies also decreased monotonically with increasing signal level, ranging from 6.15 ± 1.17 ms at 60 dB to 5.26 ± 0.47 ms at 80 dB. Similar to P1 latencies, female P2 latencies were 0.22 ± 0.21 ms shorter than those of males, but the effect of sex was not significant (Table 2). There were no significant effects of body size on click-evoked ABRs for P1 or P2.

3.2.2. Tone-evoked responses—For tone-evoked responses, the ANCOVAs for both P1 and P2 latencies revealed significant effects of frequency, signal level, and their two-way interaction (frequency $\times$ signal level), although the effect sizes associated with the main effects were much larger than those for the interactions (Table 2). There was also a significant main effect of subject sex for P1, but not P2 (Table 2). There were no significant effects of body size on the P1 and P2 latencies of tone-evoked ABRs (Table 2). P1 typically occurred with latencies between 3.5 and 6.0 ms after the stimulus reached the tympana (Fig. 3g–i). P2 typically occurred a few milliseconds later, between 7.0 and 11.0 ms after the stimulus arrived at the tympana (Fig. 3j–l).

At a given frequency, both P1 latencies (Fig. 3h) and P2 latencies (Fig. 3k) decreased monotonically with increases in signal level. Latencies for both peaks also tended to decrease as a function of increasing frequency. However, these frequency-dependent changes in latency were non-monotonic, and some of the discontinuous changes in latency with frequency were similar to those reported above for changes in amplitude with frequency. For example, latencies in response to the 0.45 kHz tone were lower than latencies in response to immediately adjacent frequencies. Latencies were generally longer at lower frequencies compared with higher frequencies (e.g., 1.2 kHz versus 2.1 kHz; Fig. 3g, i, j, l). There was a sharp decrease in latencies across the intermediate frequency range between 1.2 and 2.1 kHz. We would additionally note that, across all frequencies, both P1 and P2

latencies decreased at a rate of about 4% ($4.1 \pm 1.3\%$) for every 5 dB increase in signal level. Since both P1 and P2 latencies increased with the same *percentage* change per 5 dB, the peak-to-peak latency (i.e., P2 latency minus P1 latency) decreased slightly as signal level increased, with averages of 3.86 ± 0.24 ms at 60 dB and 3.27 ± 0.22 ms at 80 dB.

The mean P1 latency of females was 0.24 ± 0.20 ms shorter than that of males when averaged across all frequencies and signal levels (Fig. 3i). This average sex difference in P1 latencies was similar in magnitude to that measured for the P1 latencies of click-evoked responses (0.23 ± 0.13 ms, as reported above). Similar to P1 latencies, P2 latencies for females were 0.23 ± 0.69 ms shorter than male responses averaged across all frequencies and signal levels (Fig. 3l).

3.3. ABR gross morphology

Our cross-correlation analyses, which measured similarity in overall waveform shape, revealed several interesting patterns of similarities and differences in the ABRs evoked by clicks and tones of various frequencies. Figure 4 depicts the average ABR for the five stimuli used as references in the cross correlation analysis (clicks and tones at frequencies of 0.9, 1.5, 1.8, and 3.0 kHz) and the resulting correlation coefficients. For clarity, coefficients are shown for only three of the five signal levels analyzed (60, 70, and 80 dB), but trends were similar for the other two signal levels. Click-evoked waveforms (Fig. 4a) were most similar (i.e., had the highest cross-correlation coefficients) to responses evoked by tones with frequencies in the range of 0.45–1.2 kHz (Fig. 4f). In response to tones, ABR waveforms had consistently different morphologies at relatively low (e.g., 0.9 kHz) and relatively high (e.g., 3.0 kHz) frequencies. This difference in shape can be most easily seen by contrasting Fig. 4b and 4g with Fig. 4e and 4j, respectively. Moreover, these frequency-dependent shapes were generally more similar in responses to tones at adjacent frequencies than to tones at more remote frequencies.

The correlation analyses revealed non-monotonic changes across the same frequency ranges observed for ABR amplitudes and latencies (Fig. 4f–j). Consider, for example, responses to the 0.9 kHz tone, which were most similar (e.g., *r*-values > 0.80) to those evoked by other low-frequency tones below about 1.2 kHz (Fig. 4g). The similarity between responses to the 0.9 kHz tone and those evoked by tones with frequencies higher than about 1.2 kHz declined sharply (e.g., to *r*-values between 0.4 and 0.65, depending on signal level; Fig. 4g).

Likewise, responses to the 3.0 kHz tone were most similar to responses to tones at other high frequencies (e.g., 2.1 kHz) and this similarity declined sharply at lower frequencies (e.g., 2.1 kHz; Fig. 4j).

Correlations computed based on responses at 10 dB SL varied across frequency in ways similar to those computed for absolute signal levels (Fig. 4f–j). This result is important, because it confirms that the frequency-dependent patterns of differences observed in both waveforms (Fig. 4b–e) and correlation coefficients (Fig. 4f–j) is not simply a result of differences in auditory sensitivity at different frequencies. In general, differences in signal level had relatively small effects on the magnitudes of correlations across frequency (Fig. 4f–j). The largest effects of signal level were observed when the intermediate frequencies of 1.5 and 1.8 kHz served as the reference frequencies for comparison (Fig. 4h–i).

3.4. ABR thresholds

Sigmoid curves for the automated method of threshold determination generally fit the RMS amplitude data well (Fig. 5a; mean $R^2 = 0.88 \pm 0.14$). Threshold estimates typically increased between 5 and 10 dB from the 0.5 to the 2.0 standard deviations criteria. For responses to clicks, the visually detected threshold of 58.7 dB was most similar to the automatically detected threshold of 59.4 dB obtained using the 1.0 standard deviation criterion. Visually detected thresholds generated an audiogram that most closely matched the automated audiogram generated using the 0.5 standard deviations criterion, with a mean difference of 1.5 ± 1.3 dB between them (Fig. 5b). The one frequency where thresholds deviated substantially between these two methods was 1.5 kHz, where the automated threshold for 0.5 standard deviations was 6 dB lower than the visually detected threshold. We could determine no reason for the large difference at this single frequency. For brevity and consistency with previous studies in frogs (Katbamna et al., 2006a; Schrode et al., 2014; Zhang et al., 2012) and other animals (e.g. Brittan-Powell et al., 2010a; Lohr et al., 2013; Wysocki and Ladich, 2005b), we focused our statistical analysis on the audiogram determined from visually detected thresholds. The thresholds of click-evoked responses were significantly affected by sex ($F_{1,43}=6.2$, $P=0.017$, partial $\eta^2=0.13$), but not the covariate of subject body size ($F_{1,42}=1.5$, $P=0.231$, partial $\eta^2=0.03$). The average click threshold for females (58.4 ± 3.14 dB) was 2.8 dB lower than the average threshold for males (61.2 ± 4.5 dB). Frequency ($F_{22,924}=244.2$, $P<0.001$, partial $\eta^2=0.85$), sex ($F_{1,42}=12.4$, $P=0.001$, partial $\eta^2=0.23$), and the frequency $\times$ sex interaction ($F_{22,924}=5.1$, $P<0.001$, partial $\eta^2=0.11$) significantly affected thresholds in response to tones. The covariate of subject body mass ($F_{1,42}=0.2$, $P=0.638$, partial $\eta^2=0.01$) and the mass $\times$ frequency interaction ($F_{22,924}=1.0$, $P=0.431$, partial $\eta^2=0.02$) were not significant for tones.

The effects of frequency on ABR thresholds were evident in the shape of the audiogram (Fig. 5c), which revealed two regions of heightened auditory sensitivity. One region extended from 0.9 to 1.2 kHz and a second, broader region extended from 2.4 to 3.15 kHz. Average ABR thresholds typically ranged between 50 dB and 55 dB within these two frequency ranges, with thresholds being 2–3 dB higher in the second region. At frequencies between these two regions, thresholds were closer to 60 dB. ABR thresholds below 0.9 kHz changed at a rate of -9.1 dB/octave, while those above 3.0 kHz changed at a rate of 20.0 dB/octave. Thresholds for females were slightly lower than those for males (Fig. 5c).

The two regions of enhanced sensitivity just described occurred at frequencies on opposite sides of the sharp differences in ABR amplitudes, latencies, and cross-correlations that occurred between 1.2 kHz and 2.1 kHz (Figs. 3, 4). We would additionally note that sensitivity to tones as a function of frequency in the range 0.3 to 0.9 kHz was also non-monotonic (Fig. 5b), mirroring the trends described for amplitudes, latencies, and cross-correlations in this same frequency range.

4. Discussion

4.1. Effects of frequency, signal level, sex, and size

Frequency strongly influenced the amplitudes and latencies of P1 and P2 in responses to tones. Amplitudes of P1 were generally smaller in response to frequencies > 1.5 kHz than to frequencies < 1.5 kHz, consistent with ABR amplitudes in gray treefrogs (Schrode et al., 2014). Amplitudes of P2 were largest for frequencies emphasized in male advertisement calls (0.9 and 3.0 kHz). When considering amplitudes of either peak as a function of frequency, we observed non-monotonicity in the frequency ranges of 0.3 to 0.9 kHz and 1.2 to 2.1 kHz. Latencies to both peaks generally decreased as a function of frequency, which was also similar to results from gray treefrogs (Schrode et al., 2014), as well as recordings from auditory nerve fibers in other species (Feng, 1982; Hillery and Narins, 1984; Stiebler and Narins, 1990; Zakon and Capranica, 1981). However, the frequency-dependent decrease in latency was non-monotonic across the same frequency ranges in which we observed non-monotonic trends in amplitudes.

Increasing signal level resulted in larger P1 and P2 amplitudes and shorter P1 and P2 latencies in responses to both clicks and tones, as has been reported in previous ABR studies (Brittan-Powell et al., 2005, 2002; Kenyon et al., 1998; Nachtigall et al., 2007; Zhang et al., 2012). As signal level increased, there was also a decrease in the interval between P1 and P2 latencies. This is in contrast to previous reports of ABRs in humans (Starr and Anchor, 1975; but see Coats, 1978; Stockard et al., 1979), budgerigars (Brittan-Powell et al., 2002), gerbils (Burkard and Voigt, 1989), and cats (Huang and Buchwald, 1978), in which inter-peak intervals were constant across signal levels. There were significant effects of the frequency $\times$ level interaction on the amplitudes and latencies of P1 and P2 in tone-evoked responses, although the effects sizes associated with the interaction were consistently smaller than those associated with the main effects of frequency and signal level. The effect of the interaction was most evident in its influence on amplitudes of P1: there was considerable frequency-dependent variation in the slopes associated with the functions relating response amplitude to signal level.

We saw little evidence for an effect of subject sex on amplitudes or latencies of either peak in responses to either clicks or tones. While subject sex did have a significant effect on P1 latencies for both stimulus types, effect sizes suggest that this factor was less important than frequency (for tones) and level in determining latencies. This small effect of subject sex is consistent with previous studies of ABRs in nonhuman animals, including frogs (Caras et al., 2010; Munro et al., 1997; Schrode et al., 2014; Zhou et al., 2006). Similarly, subject size had little effect on either of the peaks in click or tone-evoked responses.

4.2. Gross morphology

Click and tone-evoked ABRs consisted of a series of positive and negative deflections, consistent with the shape of ABRs recorded from a variety of animals (e.g. Brittan-Powell et al., 2010a, 2005; Gall et al., 2011; Higgs et al., 2002; Kenyon et al., 1998; McFadden et al., 1999; Ramsier and Dominy, 2010; Supin et al., 1993), as well as the waveforms of invasive

brainstem evoked potentials from other frog species (Carey and Zelick, 1993; Corwin et al., 1982; Katbamna et al., 2006b; Seaman, 1991).

Cross-correlation analyses revealed interesting patterns in the waveforms of responses. Click-evoked ABR waveforms were most similar to those evoked by low and mid-frequencies (0.45–1.2 kHz), in contrast to results from gray treefrogs (Schrode et al., 2014) and humans (Hall, 2007), which have found click-evoked responses to be most similar to responses evoked by tones of higher frequencies (e.g. >1 kHz). Tone-evoked waveforms tended to be most similar to waveforms evoked by other tones of nearby frequencies, which mirrors results from gray treefrogs (Schrode et al., 2014). Trends in correlation coefficients for tone-evoked responses were non-monotonic in the same frequency ranges in which we observed non-monotonic trends in amplitudes and latencies. Importantly, cross-correlation analyses of waveforms evoked at a common sensation level (10 dB SL) confirmed that the observed effects of frequency did not result from variable sensitivity to different frequencies.

4.3. Relation of the ABR to peripheral physiology

We suggest that the frequency ranges in which we noted non-monotonicity delineate responses to frequencies encoded by the three distinct populations of afferents that innervate the separate sensory papillae in the anuran inner ear, the AP and BP (Capranica and Moffat, 1983; Capranica, 1976; Ehret and Capranica, 1980; Feng et al., 1975). The AP gives rise to two of these populations, which are sensitive to low frequencies (< 0.5 kHz) and middle frequencies (~0.5 – 1.2 kHz). The third population of afferents originates from the BP, and these fibers have higher characteristic frequencies near 3.2 kHz.

The frequency range between 1.2 and 2.1 kHz was characterized by particularly large P1 amplitudes, especially at signal levels > 70 dB. Although there is little evidence that either the AP or BP has populations specifically tuned to frequencies between 1.2 and 2.1 kHz (Ehret and Capranica, 1980), behavioral work with the eastern gray treefrog (*Hyla versicolor*) indicates that at high signal levels, there is overlap in the frequency ranges to which the AP and the BP are sensitive (Gerhardt, 2005). We suggest that the observed patterns in amplitudes within this frequency range result from simultaneous excitation of the two auditory end organs by suprathreshold tones. While we cannot say with absolute certainty that the evoked potentials we recorded were generated at early stages of the auditory system, we believe this to be the most likely case. One of the most common methods of determining likely generators of ABR peaks lies in comparison of ABR latency data to latencies previously reported from studies using invasive techniques. P1 is generally considered to derive from the VIIIth nerve (Achor and Starr, 1980; Buchwald and Huang, 1975; Seaman, 1991). Consistent with this idea, invasive recordings from the VIIIth nerve of a variety of anuran species have shown latencies to range from 2 to 10 ms (Capranica, 1976; Feng, 1982; Frishkopf and Goldstein Jr., 1963; Hillery and Narins, 1987; Stiebler and Narins, 1990), which encompasses our latency measurements of P1 (2.8 to 3.5 ms for clicks and 3.5 to 6.0 ms for tones). Predicting the P2 generator is more difficult. P2 latencies ranged between 5 and 11 ms, which is consistent with both some of the longer latencies reported for the auditory nerve, as well as the 4–15 ms range of latencies reported from

single cell recordings in the first nucleus of the anuran central auditory system, the dorsal medullary nucleus (Fuzessery and Feng, 1983; Hall and Feng, 1990; van Stokkum, 1987; Yang and Feng, 2007). The overlap in latencies for auditory nerve fibers and units in the dorsal medullary nucleus thus makes the origin of P2 in green treefrogs ambiguous at present. However, the likelihood of P2 being generated at a higher level of the ascending auditory system is unlikely. In single-unit recordings from the superior olivary nucleus, latencies range from 10–50 ms (Condon et al., 1991; Fuzessery and Feng, 1983), which is rather longer than the latencies we determined for P2.

4.4. Thresholds

ABR threshold estimates revealed two regions of heightened sensitivity near frequencies that correspond to the frequencies emphasized in the advertisement calls of green treefrogs (Gerhardt, 2001). If, as in many frogs, the auditory papillae of the inner ear are tuned to spectral peaks in the call (Gerhardt and Schwartz, 2001), we would expect that these are the frequencies to which the mid-frequency region of the AP and the BP, respectively, are most sensitive. In the audiogram between 0.3 and 0.9 kHz, the decrease in thresholds was not monotonic, which may reflect responses of afferents from the region of the AP sensitive to low frequencies (Capranica and Moffat, 1983).

We detected no effect of subject size on thresholds, consistent with invasive recordings from the VIIIth nerve of frogs, which have not previously demonstrated an effect of subject size on average thresholds of fibers (Elliott et al., 2007; Frishkopf et al., 1968; Zakon and Wilczynski, 1988). Females generally had lower thresholds than males, an effect that was more pronounced for low and mid-frequencies. However, effect sizes indicated that the effect of sex plays a relatively minor role in determining thresholds relative to the effect of frequency. For many frog species, the frequency to which the BP is most sensitive is lower in females than males (Narins and Capranica, 1976; Wilczynski et al., 1992; Zakon and Wilczynski, 1988; but see Elliott et al., 2007). However, consistent with previous studies in green treefrogs (Miranda and Wilczynski, 2009b; Penna et al., 1992), we did not see any evidence for a difference in the frequencies to which males and females were most sensitive.

In Fig. 6, we compare our ABR audiogram to several published green treefrog audiograms generated based on behavioral responses (Fig. 6a; Megela-Simmons et al., 1985; Weiss and Strother, 1965) and neurophysiological responses (Fig. 6b; Lombard and Straughan, 1974; Miranda and Wilczynski, 2009b; Penna et al., 1992). All of the audiograms showed at least two regions of increased sensitivity, with at least one occurring below about 1.5 kHz and another occurring above about 2.0 kHz. However, the absolute thresholds, the exact frequencies of best sensitivity, and the bandwidth of the sensitive regions vary widely between audiograms. Our ABR thresholds were 10–20 dB higher than those reported for one of the behavioral studies (Fig. 6a). Compared with invasive neurophysiological studies, ABR thresholds ranged from 5 – 25 dB higher (Fig. 6b), which is not unusual when comparing ABR thresholds to those determined through behavioral tests or invasive neurophysiological studies (e.g. Brittan-Powell et al., 2010a, 2002; Gorga et al., 1988; Ngan and May, 2001; but see Henry and Lucas, 2008, 2009). This difference in thresholds is

typically attributed to the fact that the ABR occurs at the onset of sound and is not influenced by integration of sound over time (Gorga et al., 1984; Szymanski et al., 1999).

One feature that varied considerably between audiograms was the relative difference in threshold between the two regions of peak sensitivity. This variation is particularly extreme between behavioral audiograms (Fig. 6a), where the thresholds for the lower frequency (< 1.5 kHz) region are 20 dB *more* sensitive than those of the higher frequency (> 2.0 kHz) for reflex modification (Megela-Simmons et al., 1985), compared to 10 dB *less* sensitive for the galvanic skin response (Weiss and Strother, 1965). Our ABR audiogram had the smallest relative difference (2.5 – 3 dB) between thresholds at the two regions of peak sensitivity (Fig. 6). ABR thresholds tended to diverge more from midbrain thresholds at relatively lower frequencies than higher frequencies (< 1.5 kHz vs. > 1.5 kHz), suggesting that the small relative difference noted above may be due to overestimation of thresholds at low frequencies. It is well-established that methods for recording ABRs tend to overestimate thresholds at low frequencies; however, we chose to use these stimuli for consistency with previous studies using ABRs (e.g. Brittan-Powell et al., 2010a; Schrode et al., 2014; Zhang et al., 2012) and because increasing rise/fall time and tone duration is known to alter ABRs (see review in Hall, 2007).

ABR recordings represent a potentially important, but currently under-utilized, tool in comparative studies of audition in anurans. To illustrate the utility of ABRs for comparative work, we plot our audiogram along with ABR audiograms from three other species of frogs in Fig. 7. The audiograms for green treefrogs and Cope's gray treefrogs (*H. chrysoscelis*; Schrode et al., 2014) both have a distinctive "W" shape with peaks in sensitivity at two different frequencies. In both species, the peaks in sensitivity occur at the frequencies present in conspecific advertisement calls, and probably also represent the peak sensitivities of the AP and BP. Audiograms for African clawed frogs (*Xenopus laevis*; Katbamna et al., 2006a) and Emei music frogs (*Babina daunchina*; Zhang et al., 2012) both have a more U-like shape. That of the music frog is broad enough to encompass the frequencies containing most of the harmonic energy in male advertisement calls (Chen et al., 2011). The audiogram for *Xenopus*, on the other hand, shows peak sensitivity near 1.0 kHz and no particular sensitivity to the frequencies emphasized in the clicks they use for sexual communication (1.6 and 2.3 kHz; Picker, 1980; Vigny, 1979; Wetzel and Kelley, 1983). There is one caveat to consider in interpreting the ABR audiogram for African clawed frogs, which was generated from responses to airborne sound (Katbamna et al., 2006a). Because these frogs are aquatic, it may be more relevant to consider responses to broadcasts or simulation of waterborne sound, such as those used by to generate behavioral and midbrain neurophysiological audiograms (e.g. Elepfandt et al., 2000; Elliott et al., 2007). Audiograms generated using these methods have distinct peaks in sensitivity at frequencies of 0.6 kHz and in the range of 1.6 to 2.0 kHz, the latter of which does overlap the dominant frequencies in *Xenopus* advertisement calls.

5. Conclusions

Although rarely used in studies of anurans, the ABR is a potentially valuable method for comparative exploration of anuran auditory physiology. This study aimed to evaluate the

ABR as a tool for studying the anuran auditory system by comparing the ABR in green treefrogs with that of other animals, as well as evaluating the consistency of ABR measurements with characteristics of the green treefrog auditory system. The similarities of the green treefrog ABR with other ABR studies and the physiology of the green treefrog auditory system validate the use of ABR for further study of anuran auditory sensitivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ABR	auditory brainstem response
AEP	auditory evoked potential
AP	amphibian papilla
ANCOVA	analysis of covariance
BP	basilar papilla
RMS	root mean square
SPL	sound pressure level

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Figure 1. ABR methodology

(a) Placements of the three scalp electrodes are indicated on an outline of a green treefrog head. (b) Shown are stimuli that were presented to each subject. The top trace in (b) shows the train of equal-amplitude (80 dB) clicks presented at the beginning and end of each experiment. The middle and bottom traces in (b) show click and tone trains, respectively, in which signal level increased from 40 to 80 dB in 5-dB steps. To obtain each replicate, stimuli were repeated 400 times and click polarity and tone phase alternated between presentations. (c) P1 and P2 amplitudes were measured as the voltage from the top of the

respective peak (P1 or P2) to the bottom of the subsequent trough (T1 or T2, respectively). P1 and P2 latencies were measured as the time from arrival of the stimulus at the tympana (vertical arrow) to the top of the respective peak. Click-evoked P1 and P2 amplitudes were similar when measured at the beginning and ending, respectively, of a recording session in response to a click presented at 80 dB SPL (P1: $0.69 \pm 0.41 \mu\text{V}$ and $0.77 \pm 0.44 \mu\text{V}$; P2: $1.06 \pm 0.46 \mu\text{V}$ and $0.96 \pm 0.44 \mu\text{V}$).

Figure 2. Typical evoked responses

Shown are the first 15 ms of ABRs for a typical frog to clicks and to tones presented at four different frequencies. Vertical arrows indicate the time the sound reached the frog's tympana and horizontal arrows indicate visually detected thresholds.

Figure 3. ABR amplitudes and latencies of tone-evoked responses

Plotted are (a–c) P1 amplitudes, (d–f) P2 amplitudes, (g–i) P1 latencies, and (j–l) P2 latencies. Contour plots in (a,d,f,j) are plotted as a function of frequency and signal level. In (b,e,h,k) ABR amplitudes and latencies are plotted as a function of signal level for the four representative tone frequencies shown in Fig. 2. Points for the tone frequency of 1.8 kHz are offset in (k) to reveal underlying points. (c,f,i,l) ABR amplitudes and latencies for 80 dB, plotted as a function of frequency and separated for the two sexes. Error bars in the legends

are standard errors averaged across signal levels from 60 to 80 dB and both sexes (b,e,h,k) or all frequencies (c,f,i,l).

Figure 4. ABR gross morphology

(a–e) ABRs were aligned to P1 ($t=0$ ms) and averaged across replicates and individuals. Shown are responses to stimuli at 80 dB. The shaded region indicates ± 1 SD, across replicates and individuals. (f–j) The average responses in (a–e) were used as reference stimuli and cross-correlated with the average response to every tone frequency tested. This analysis was repeated for signal levels between 60 and 80 dB (data for 65 and 75 dB not shown) and at a 10-dB sensation level (SL).

Figure 5. ABR thresholds

(a) Automated threshold detection was accomplished by comparing the RMS amplitude of an evoked response to that of a fixed threshold criterion. Depicted here is an example of threshold detection for the responses to tones of 0.9 kHz in Fig. 2. Filled circles indicate the RMS of responses to each signal level, and the solid line is the best-fit sigmoid curve. The lines indicate calculation of the estimated threshold (vertical) from the predicted amplitude (horizontal), based on the given criterion. The criteria used were 0.5, 1.0, or 2.0 standard deviations (dashed lines) above the mean measured RMS of the neural signal in the absence

of a stimulus (indicated by the solid line labeled 0.0 SD). (b) Shown are the mean audiograms for both the visual detection and automated methods of threshold determination. We excluded the automated threshold estimates for five individuals at the frequencies of 0.3 and 5.1 kHz, because threshold estimates were outside the range of stimulus levels presented. (c) Visually detected thresholds are plotted separately for males and females. Error bars in legends for (b) and (c) show the standard error averaged across all frequencies.

Figure 6. Audiogram comparisons

(a) The ABR audiogram constructed from visually detected thresholds is compared to previously published audiograms constructed from behavioral responses in green treefrogs based on reflex modification (Megela-Simmons et al. 1985) or galvanic skin responses (Weiss and Strother 1965). (b) The ABR audiogram is compared to audiograms constructed using multi-unit recordings from the midbrain (torus semicircularis) of green treefrogs (TS1: Miranda and Wilczynski, 2009b; TS2: Penna et al., 1992; TS3: Lombard and Straughan, 1974).

Figure 7. Anuran ABR audiograms

ABR audiograms for three other species of frog are compared to our ABR audiogram for green treefrogs. The audiogram for gray treefrogs was reported by Schrode et al. (2014); the audiogram for Emei music frogs was reported by Zhang et al. (2012); and the *Xenopus* audiogram was reported by Katbamna et al. (2006a).

Results from analyses of covariance (ANCOVA) of ABR amplitudes for P1 and P2 in responses to clicks and tones. The mean and range of F statistics, P values, and effect sizes are shown for the 20 imputed data sets. Significant results (mean $P < 0.05$) are highlighted in **bold**.

Table 1

Stimulus (Peak)	Effect	df	F	P	Partial η^2
Clicks (P1)	Level	4, 168	78.0 (43.8–143.3)	< 0.001 (all < 0.001)	0.63 (0.51–0.77)
	Sex	1, 42	1.2 (0.0–3.0)	0.389 (0.088–0.865)	0.03 (0.00–0.07)
	Sex $\times$ Level	4, 168	1.8 (0.1–5.7)	0.364 (0.009–0.870)	0.04 (0.00–0.12)
	Mass	1, 42	1.1 (0.0–2.4)	0.376 (0.128–0.966)	0.02 (0.00–0.05)
	Mass $\times$ Level	4, 168	1.6 (0.1–5.7)	0.347 (0.004–0.880)	0.04 (0.00–0.12)
Clicks (P2)	Level	4, 168	100.1 (48.9–188.2)	< 0.001 (all < 0.001)	0.69 (0.54–0.82)
	Sex	1, 42	0.3 (0.0–0.6)	0.650 (0.426–0.973)	0.01 (0.00–0.02)
	Sex $\times$ Level	4, 168	2.6 (0.6–9.5)	0.181 (0.001–0.464)	0.06 (0.01–0.18)
	Mass	1, 42	0.6 (0.0–2.4)	0.547 (0.130–0.973)	0.01 (0.00–0.05)
	Mass $\times$ Level	4, 168	1.2 (0.0–4.4)	0.432 (0.035–0.907)	0.03 (0.00–0.10)
Tones (P1)	Frequency	18, 756	124.0 (117.0–130.8)	< 0.001 (all < 0.001)	0.75 (0.74–0.76)
	Level	4, 168	285.2 (238.3–333.9)	< 0.001 (all < 0.001)	0.87 (0.85–0.89)
	Frequency $\times$ Level	72, 3024	8.4 (7.4–9.7)	< 0.001 (all < 0.001)	0.17 (0.15–0.19)
	Sex	1, 42	0.9 (0.7–1.1)	0.348 (0.292–0.419)	0.02 (0.02–0.03)
	Sex $\times$ Frequency	18, 756	0.6 (0.3–0.9)	0.642 (0.463–0.810)	0.01 (0.01–0.02)
	Sex $\times$ Level	4, 168	1.1 (0.4–2.1)	0.361 (0.142–0.633)	0.03 (0.01–0.05)
	Sex $\times$ Frequency $\times$ Level	72, 3024	1.0 (0.8–1.5)	0.521 (0.072–0.744)	0.02 (0.02–0.04)
	Mass	1, 42	0.0 (0.0–0.1)	0.900 (0.749–0.999)	0.00 (0.00–0.00)
	Mass $\times$ Frequency	18, 756	1.0 (0.5–1.4)	0.421 (0.239–0.655)	0.02 (0.01–0.03)
	Mass $\times$ Level	4, 168	0.3 (0.1–0.9)	0.706 (0.415–0.851)	0.01 (0.00–0.02)
Tones (P2)	Mass $\times$ Frequency $\times$ Level	73, 3024	1.1 (0.7–1.6)	0.363 (0.047–0.839)	0.03 (0.02–0.04)
	Frequency	18, 756	11.5 (9.8–12.7)	< 0.001 (all < 0.001)	0.22 (0.19–0.23)
	Level	4, 168	236.2 (195.4–283.7)	< 0.001 (all < 0.001)	0.85 (0.82–0.87)
	Frequency $\times$ Level	72, 3024	4.4 (3.8–5.0)	< 0.001 (all < 0.001)	0.10 (0.08–0.11)
	Sex	1, 42	1.2 (1.0–1.3)	0.288 (0.263–0.331)	0.03 (0.02–0.03)
	Sex $\times$ Frequency	18, 756	1.4 (0.8–1.9)	0.229 (0.080–0.566)	0.03 (0.02–0.04)
	Sex $\times$ Level	4, 168	2.8 (1.9–3.8)	0.079 (0.027–0.155)	0.06 (0.04–0.08)

Stimulus (Peak)	Effect	df	F	P	Partial η^2
	Sex $\times$ Frequency $\times$ Level	72, 3024	1.2 (0.9–1.6)	0.294 (0.077–0.535)	0.03 (0.02–0.04)
	Mass	1, 42	1.8 (1.3–2.1)	0.189 (0.157–0.254)	0.04 (0.03–0.05)
	Mass $\times$ Frequency	18, 756	1.6 (1.2–2.0)	0.164 (0.056–0.295)	0.04 (0.03–0.05)
	Mass $\times$ Level	4, 168	0.2 (0.0–0.6)	0.867 (0.561–0.969)	0.00 (0.00–0.00)
	Mass $\times$ Frequency $\times$ Level	73, 3024	1.1 (0.8–1.5)	0.352 (0.111–0.675)	0.03 (0.02–0.03)

Results from analyses of covariance (ANCOVA) of ABR latencies for P1 and P2 in responses to clicks and tones. The mean and range of F statistics, P values, and effect sizes are shown for the 20 imputed data sets. Significant results (mean $P < 0.05$) are highlighted in **bold**.

Table 2

Stimulus (Peak)	Effect	df	F	P	Partial η^2
Clicks (P1)	Level	4, 168	47.8 (12.4–70.1)	< 0.001 (all < 0.001)	0.52 (0.23–0.63)
	Sex	1, 42	5.5 (2.6–7.8)	0.031 (0.008–0.109)	0.12 (0.06–0.16)
	Sex $\times$ Level	4, 168	1.6 (0.1–5.2)	0.384 (0.004–0.894)	0.04 (0.00–0.11)
	Mass	1, 42	1.8 (0.3–5.2)	0.237 (0.028–0.604)	0.04 (0.01–0.11)
	Mass $\times$ Level	4, 168	1.5 (0.3–5.9)	0.362 (0.010–0.748)	0.03 (0.01–0.12)
Clicks (P2)	Level	4, 168	85.8 (14.7–122.1)	< 0.001 (all < 0.001)	0.65 (0.26–0.74)
	Sex	1, 42	1.1 (0.0–2.5)	0.357 (0.121–0.960)	0.03 (0.00–0.06)
	Sex $\times$ Level	4, 168	1.8 (0.3–8.7)	0.324 (0.001–0.709)	0.04 (0.01–0.17)
	Mass	1, 42	0.3 (0.0–2.1)	0.698 (0.154–0.971)	0.01 (0.00–0.05)
	Mass $\times$ Level	4, 168	3.2 (0.3–8.2)	0.177 (0.001–0.692)	0.07 (0.01–0.16)
Tones (P1)	Frequency	18, 756	123.6 (113.4–134.8)	< 0.001 (all < 0.001)	0.75 (0.73–0.76)
	Level	4, 168	574.4 (435.4–704.3)	< 0.001 (all < 0.001)	0.93 (0.91–0.94)
	Frequency $\times$ Level	72, 3024	2.6 (1.9–4.0)	0.005 (all < 0.026)	0.06 (0.04–0.09)
	Sex	1, 42	12.8 (11.7–14.9)	0.001 (all < 0.002)	0.23 (0.22–0.26)
	Sex $\times$ Frequency	18, 756	1.4 (0.9–2.3)	0.245 (0.030–0.548)	0.03 (0.02–0.05)
	Sex $\times$ Level	4, 168	3.7 (1.1–7.3)	0.065 (0.001–0.343)	0.08 (0.03–0.15)
	Sex $\times$ Frequency $\times$ Level	72, 3024	1.1 (0.7–1.6)	0.392 (0.060–0.832)	0.03 (0.02–0.04)
	Mass	1, 42	0.6 (0.2–0.9)	0.458 (0.350–0.627)	0.01 (0.01–0.02)
	Mass $\times$ Frequency	18, 756	1.1 (0.6–1.5)	0.409 (0.157–0.792)	0.03 (0.01–0.04)
	Mass $\times$ Level	4, 168	1.5 (0.4–5.8)	0.335 (0.003–0.698)	0.03 (0.01–0.12)
Tones (P2)	Mass $\times$ Frequency $\times$ Level	73, 3024	1.1 (0.7–1.6)	0.382 (0.076–0.788)	0.03 (0.02–0.04)
	Frequency	18, 756	115.2 (101.8–127.1)	< 0.001 (all < 0.001)	0.73 (0.71–0.75)
	Level	4, 168	589.5 (436.9–761.4)	< 0.001 (all < 0.001)	0.93 (0.91–0.95)
	Frequency $\times$ Level	72, 3024	3.5 (2.6–4.5)	< 0.001 (all < 0.001)	0.08 (0.06–0.10)
	Sex	1, 42	2.6 (2.3–3.3)	0.115 (0.077–0.141)	0.06 (0.05–0.07)
	Sex $\times$ Frequency	18, 756	1.4 (0.7–2.0)	0.253 (0.052–0.640)	0.03 (0.02–0.05)
	Sex $\times$ Level	4, 168	3.3 (1.4–6.3)	0.082 (0.004–0.247)	0.07 (0.03–0.13)

Stimulus (Peak)	Effect	df	F	P	Partial η^2
	Sex $\times$ Frequency $\times$ Level	72, 3024	1.1 (0.6–1.5)	0.417 (0.105–0.828)	0.02 (0.01–0.03)
	Mass	1, 42	0.3 (0.2–0.4)	0.605 (0.542–0.695)	0.01 (0.00–0.01)
	Mass $\times$ Frequency	18, 756	0.9 (0.5–1.3)	0.511 (0.259–0.855)	0.02 (0.01–0.03)
	Mass $\times$ Level	4, 168	0.9 (0.1–3.2)	0.471 (0.001–0.800)	0.02 (0.00–0.07)
	Mass $\times$ Frequency $\times$ Level	73, 3024	1.3 (0.8–2.3)	0.254 (0.005–0.636)	0.03 (0.02–0.05)