The auditory brainstem response in two lizard species

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Although lizards have highly sensitive ears, it is difficult to condition them to sound, making standard psychophysical assays of hearing sensitivity impractical. This paper describes non-invasive measurements of the auditory brainstem response (ABR) in both Tokay geckos (Gekko gecko; nocturnal animals, known for their loud vocalizations) and the green anole (Anolis carolinensis, diurnal, non-vocal animals). Hearing sensitivity was measured in 5 geckos and 7 anoles. The lizards were sedated with isoflurane, and ABRs were measured at levels of 1 and 3% isoflurane. The typical ABR waveform in response to click stimulation showed one prominent and several smaller peaks occurring within 10 ms of the stimulus onset. ABRs to brief tone bursts revealed that geckos and anoles were most sensitive between 1.6–2 kHz and had similar hearing sensitivity up to about 5 kHz (thresholds typically 20–50 dB SPL). Above 5 kHz, however, anoles were more than 20 dB more sensitive than geckos and showed a wider range of sensitivity (1–7 kHz). Generally, thresholds from ABR audiograms were comparable to those of small birds. Best hearing sensitivity, however, extended over a larger frequency range in lizards than in most bird species.

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I. INTRODUCTION

The tympanic ear probably originated independently in all major tetrapod groups (Clack, 1997). Lizards therefore represent an independent experiment in tympanic hearing and are highly interesting in comparative studies of ear evolution in tetrapods. Most lizards have delicate eardrums and sensitive ears (Manley, 1990), but the lack of behavioral assays of auditory sensitivity has made it difficult to compare lizard hearing to other vertebrate groups. Relatively non-invasive methods, such as cochlear microphonics (reviewed in Wever, 1978) and measurements of compound action potentials recorded from the round window (Werner et al., 2008) have been used successfully, but the thresholds and general frequency response of the ear are dependent on the method used and the surgical preparation needed (Manley, 1990; Werner et al., 2001). There is therefore a need for standardized, non-invasive measurements of auditory sensitivity.

The anatomy of the basilar papilla shows considerable variation (see reviews in Manley, 2000, 2002, 2004). The species chosen for this study, anoles and geckos, were selected to reflect this variation. Anoles belong to the Iguanidae and typically have short basilar papillae (range 300–500 μm) while the basilar papilla is both highly specialized and extended in Gekkonids (range 1800–2100 μm) (Manley, 2002, Manley et al., 1999). Furthermore, lizard papillae are divided into at least two distinct areas, one where most hair cells respond best to frequencies below 1 kHz and a second area (or two in some species) that responds to higher frequencies (see Fig. 4.3 in Manley, 2000). This high frequency region is particularly well developed in gekkonids (Manley et al., 1999; Manley, 2002).

Despite the diversity in their papillae, all lizard species share a similar hearing frequency range of about 0.1–5 kHz (see Wever, 1978 and reviews in Manley, 2000; 2004). In all lizards investigated thus far, the auditory nerve fibers have V-shaped tuning curves with lowest thresholds at 5 dB SPL and show phase locking to low-frequency stimuli (below approximately 1 kHz; Manley, 1981; Manley, 2000). The responses of most auditory nerve fibers are primary-like and characterized by robust onsets (for review, see Manley, 2000). Auditory nerve fibers project to the auditory brainstem (Szpir et al., 1995, for review see Grothe et al., 2005); further neural processing along the auditory pathway has not yet been investigated in detail (Manley, 1981).

The auditory brainstem response (ABR) is as an effective tool to study hearing sensitivity as well as the function-
ility of the auditory system, and is similar across most vertebrate classes (e.g., Corwin et al., 1982; Walsh et al., 1992). Most importantly, the ABR audiogram provides a good estimate of the shape of the behavioral audiogram (Borg and Engström, 1983; Brittan-Powell et al., 2002; Stapells and Oates, 1997; Wenstrup, 1984). For most animals tested, the ABR is unaffected by sleep and/or sedation (Hall, 2007; Picton and Hillyard, 1974; but see Santarelli et al., 2003). It is manifested as a series of peaks occurring within the first 10 ms following stimulation and represents the progressive propagation of neural activity through the ascending auditory pathway.

This study compared hearing sensitivity of a vocal lizard (Tokay gecko, *Gekko gecko*) to a non-vocal lizard (anole, *Anolis carolinensis*). Tokay geckos are nocturnal Southeast Asian animals. Gekkonoids are noted among lizards, both for their ability to vocalize and for the complexity of their vocal apparatus (Blair, 1968; Bogert, 1960; Moore et al., 1991). Their vocalizations vary from quiet, insect-like chirrups to loud clucks, barks, and whistles (Marcellini, 1977; Seufer, 1991; Tang et al., 2001) and are distinguishable from those of most other reptiles in that the sound emitted often has tonal and harmonic qualities (Brown, 1985; Steck, 1908; Werner et al., 1978). In contrast, the green anole is a diurnal, non-vocal animal found in the southeastern United States. We have used the ABR to measure hearing sensitivity since it is a simple, non-invasive measurement that allows comparisons of sensitivity and frequency response not only within the group of lizards but also with other vertebrate groups. To our knowledge, this is the first such study in lizards.

II. METHODS

A. Animal preparation

Geckos were placed in a walk-in sound-attenuating chamber (IAC) for all measurements. Closed, custom-made sound systems were placed at the entrance of both ear canals, containing commercial miniature earphones and miniature microphones (Knowles EM 3068). After the sound systems were sealed into the ear canal using Gold Velvet II ear impression material (Earmold and Research Laboratories, Wichita, KS), the sound systems were calibrated individually at the start of each experiment, using built-in miniature microphones (Knowles EM3068, Itasca, IL).

B. Auditory brainstem response (ABR)

Hearing sensitivity was measured in 5 geckos and 7 anoles using ABRs. Anesthesia was induced in intubated animals by isoflurane inhalation via a chamber. Once the lizards were anesthetized, a constant gas flow of carbogen mixed with isoflurane at 1 ml/min was connected via a loose fitting tube into the trachea (Bennett, 1998). Animals were sedated with isoflurane in concentrations of 1% and 3%. At levels below 1%, animals could be kept sedated but occasionally took deep breaths and would respond to toe-pinch. When changes to isoflurane levels were made, at least 30–60 min were allowed before the next data collection began. Body temperature was maintained at 27 °C by a heating blanket wrapped around the animal. To measure ABRs, platinum electrodes were inserted just under the skin at the vertex, behind the stimulated ear and grounded at the other side of the head. Responses to brief tone bursts, emitted through the coupler sealed over the eardrum, were evoked at frequencies between 0.2–10 kHz with intensity levels of 5 to 90 dB SPL.

The stimulus presentation, ABR acquisition, equipment control, and data management are similar to those used in previous bird and alligator studies (Brittan-Powell et al., 2002; Brittan-Powell et al., 2005; Higgs et al., 2002). Briefly, the system was coordinated using a Tucker-Davis Technologies (TDT; Gainesville, FL) System 3 modular rack-mount system USB linked to a Dell laptop running TDT ‘BIOSIG’ and ‘SIGGEN’ software. Sound stimuli were generated using SIGGEN and fed through a RP2.1. The output of the RP2.1 was connected to a programmable attenuator (PA5), which directly drove the speaker. Recording electrodes were connected to the low-impedance Medusa Digital Biological Amplifier System (RA4L Headstage and RA16PA PreAmp), which added an additional 10–20× gain, and then to the RA16BA (Medusa Base station) via fiber optic cables. All biological signals were notch filtered at 60 Hz during data collection. After data collection, the signals were band-pass filtered below 30 Hz and above 3000 Hz using BIOSIG.

As in previous studies, ABR thresholds were measured in response to multiple-intensity stimulus trains consisting of 9 single frequency tone-bursts of increasing intensity and presented at a rate of 2/s. Tone burst stimuli were 10 ms in duration (1 ms cos² rise/fall) with 20 ms ISI. Each ABR represented the average of 200 alternating phase stimulus presentations, sampled at 25 kHz for 285 ms following onset of the stimulus. Each frequency-intensity combination was replicated. Latency to wave 1 was calculated as the time (ms) to the first positive peak while the amplitude was measured from baseline-to-peak for wave 1 [see inset Fig. 2(B)]. We defined ABR threshold as the intensity 2.5 dB (one-half step in intensity) below the lowest stimulus level at which a response could be visually detected on the trace between 1 and 6 ms, regardless of whether the peak in the waveform was positive or negative (Brittan-Powell and Dooling, 2004; Brittan-Powell et al., 2005).

The authors recognize that the brief stimuli and short rise/fall times of the tone bursts used in this study were not ideal for accurately determining thresholds for low frequency sounds. However, increased rise/fall times and longer stimulus durations affect the brainstem response morphology. In humans, ABR peak latency was directly and positively correlated with stimulus rise time (e.g., Hecox et al., 1976; Kodera et al., 1977; Kodera et al., 1979) although Beattie and Torre (1997) found that increasing rise/fall time from 1 to 4 ms at 500 Hz had no effect on ABR threshold. Other studies showed that increasing the duration of the tone resulted in changes in waveform morphology, with decreased amplitudes and offset responses in stimuli as short as 10 ms (e.g., Kodera et al., 1977, see review in Hall, 2007). Given these constraints, we chose a similar methodology (i.e., 5 or 10 ms tone trains with 1 ms rise/fall times) in order to compare ABR thresholds for lizards with ABR thresholds from small birds and alligators (collected by the first author).
III. RESULTS

The typical ABR showed two to three prominent peaks occurring within 4–6 ms of the stimulus onset (Fig. 1). Peak 1 of the ABR is the far-field representation of the negatively-oriented peak of the compound action potential (CAP N1) of the auditory nerve, likely originating from along the tonotopic gradient within the basilar papilla (Jewett, 1970; Köppl and Gleich, 2007). Both geckos (n=3) and anoles (n=4) showed a similar sequence of peaks in response to a click, but geckos (median: 27.5 dB pSPL) exhibited lower click thresholds than anoles (median 42.5 dB pSPL).

As with all animals tested to date, ABR latency decreased [Fig. 2(A)] and ABR amplitude increased [Fig. 2(B)] with increasing sound pressure (SPL) in both geckos and anoles. There were no species differences in the latency functions [F(1,33)=1.6, p>0.05]. There were noticeable species differences in the amplitude functions [species; F(1,33)=16.35, p<0.001]. In anoles, click-evoked ABRs gradually increased in amplitude with increasing SPL, while geckos showed large amplitudes and steeper slopes in response to clicks. The slopes of the amplitude functions did not differ [species x intensity F(6,33)=2.18, p>0.05], but geckos showed significantly larger amplitude responses in response to suprathreshold levels [55 dB and higher; F(2,33)=13.75, p<0.0001] compared to anoles at the same SPL. When intensity decreased below 55 dB pSPL, closer to threshold, there were no differences in amplitude between the two species [F(5,33)=0.71, p>0.05].

Exemplar tone evoked waveforms are shown for a single gecko and anole (Fig. 3). Gecko ABRs showed better synchrony, with more defined peaks, than anoles. Occasionally, a small, low amplitude shoulder was evident on the rising phase of wave 1; we assume this is a summating potential. Some of the responses to low frequency (e.g., 0.4 kHz) stimulation revealed well-defined stimulus-locked peaks for many of the geckos tested (seen at 400 Hz in gecko in Fig. 3 but not in anole). This frequency following response (FFR) was evoked at high intensity levels and masked later response peaks at these levels. When intensity levels decreased, however, the amplitude of the FFR decreased rapidly. Also, since we used alternating phases during data acquisition, note that the averaged FFR was about twice the frequency of the stimulation. When tone stimulation increased in frequency, sharper, more synchronized responses were evoked, especially in the gecko. Note also that the gecko response at 2 kHz was larger and better defined with respect to the other frequency responses shown. Similarly, enhanced responses were apparent in the anole ABR traces but to a lesser degree.

Both species had U-shaped ABR-derived audiograms and thresholds that were similar below 5 kHz [Fig. 4(A)]. However, there were significant species [F(1,105)=75.05 p<0.0001] and species by frequency [F(13,105)=11.91 p<0.0001] interaction differences. Although geckos appeared to have lower ABR thresholds than anoles, these were not significant below 3 kHz (with the exception of 1 kHz) [post hoc contrasts from species by frequency interaction showed no significant differences at frequencies from 0.2–4 kHz, F(8,105)=1.88, p>0.05]. Anoles were, however, about 20 dB more sensitive than geckos above 5 kHz [post hoc contrasts from species by frequency interaction showed significant differences from 5–10 kHz, F(6,105)=29.23, p<0.0001]. The
gecko showed well defined peaks in sensitivity around 0.6 and 1.6 kHz (~30 dB SPL) but a steeper high frequency cutoff; whereas, the anole ABR-derived audiogram showed extended sensitivity between 1–7 kHz.

The effects of varying isoflurane levels are shown in Figs. 4B and 4C. Changing the level from 3% (producing full anesthesia) to 0.5%–1% (animal is lightly anesthetized or sedated), produced no significant changes in sensitivity [sedation level by frequency by individual F(32,84)=0.047 p >0.05].

ABR-derived audiograms for budgerigars, screech owls, alligators and lizards show striking similarities but also differences (Fig. 5). At low frequencies, both lizards and alligators appeared to show increased sensitivity at and below 1 kHz when compared to the budgerigar and the screech owl tested using the same stimuli, although sounds were delivered in free field for the birds. The differences between birds and alligators were significant at 1 kHz [F(4,43)=7.73, p <0.0001]. Post-hoc Turkey’s HSD tests showed that birds had significantly higher ABR thresholds at 1 kHz than anoles or geckos at the 0.05 level of significance. All other comparisons were not significant. The opposite held for the mid-frequency range (between 2–4 kHz), where geckos, anoles and birds had similar thresholds, with alligators showing a steep increase in ABR thresholds (these were derived in air, not water). There were significant differences in thresholds at 2 kHz [F(4,43)=6.47, p <0.0001] and 4 kHz [F(4,43)=91.78, p <0.0001]. While post-hoc Tukey’s HSD showed that the alligators had significantly higher thresholds than the lizards and the birds at 2 kHz and 4 kHz, budgerigars and geckos had similar thresholds, as did anoles and screech owls, at 4 kHz. As frequency increased above 4 kHz, gecko and budgerigar thresholds showed similar trajectories (steeper increases in threshold). ABR thresholds for anoles were more similar to, but not as low as, the screech owl, an

![FIG. 3. Representative ABR waveforms for a single gecko and anole, evoked by tones of 0.4, 0.8, 2, 4 and 5 kHz. Different scales for the highest stimulus intensities are shown to the left of each averaged waveform. Arrows indicate estimated threshold. (A) Response waveforms for 0.4 kHz at 70 dB reveal well-defined frequency following response that masked later response peaks. Note that the use of alternating phases during data acquisition produces an averaged FFR of about 2F. (B) Response waveforms in the anole were smaller than those measured in the larger gecko. Note the large and sensitive response to increasing stimulus frequency.]
auditory specialist. In both the anoles and screech owls, ABR thresholds began to increase dramatically around 7 kHz. At 8 kHz, thresholds were significantly different \( F_{4,24} = 44.58, p < 0.0001 \), with post-hoc Turkey’s HSD tests showing significantly lower thresholds in anoles compared to all other species.

IV. DISCUSSION
A. Lizard hearing

The ABR proved a robust and useful method to evaluate lizard hearing. We found no evidence that level of anesthesia affected ABR thresholds in either species (but see Dodd and Capranica, 1992; Santarelli et al., 2003; Stronks et al., 2010). Overall, signals were relatively large in the two lizards, with geckos showing better neural synchrony (sharper peaks in waveform) than anoles. The increased synchrony may be due to geckos having three times as many auditory nerve fibers as the anole (Manley, 2000; Miller, 1985). Interestingly, the increased synchrony and larger amplitudes at higher sound intensities did not translate to lower thresholds. As intensity neared threshold levels, amplitude was similar in both species. The audiograms of the two lizard species were similar in frequency range and show similar peak sensitivity at 30 dB SPL. However, anoles were sensitive to a larger range of frequencies thresholds 30–35 dB SPL from 1–7 kHz and had significantly lower thresholds than geckos between 5–10 kHz. The increased high-frequency sensitivity in anoles was consistent with experiments showing high frequency spontaneous otoacoustic emissions among anolis lizards (7.7 kHz, Manley and Gallo, 1997) and laser vibrometry measurements showing extended high-frequency sensitivity of the Anolis eardrum (Christensen-Dalsgaard and Manley, 2008). Unlike the anole, geckos showed distinct peaks in sensitivity, one of which corresponds to the fundamental frequency of their advertisement calls. The rattle (mononote) and the ge ko (binote) call both show fundamental frequencies around 500 Hz (Tang et al., 2001). Geckos also showed increased sensitivity around 1.6 kHz which may correspond to the second frequency peak at 1.3 kHz (Brillet and Paillette, 1991). Lastly, low frequencies evoked FFR in high intensity signals, especially in geckos. These responses were twice the stimulation frequency, which is typical in studies when opposite phases are added together (see Aiken and Picton, 2008; Sohmer et al., 1977).

We occasionally saw summating potentials that are gen-
generated by summed inner-ear receptor potentials. Such summating potentials have been reported in round window recordings in several species of lizards (Johnstone and Johnstone, 1969) and should be small in far field ABR recordings. Although small, we consider summating potentials here because they could contribute differentially to the ABR and result in differences in thresholds in lizards with different papilla organization. We think this unlikely because the summating potentials, when observed, were much smaller than wave 1 of the ABR. Furthermore, we defined ABR threshold as the intensity 2.5 dB (one-half step in intensity) below the lowest stimulus level at which a response could be visually detected on the trace between 1 and 6 ms, regardless of waveform (Brittan-Powell and Dooling, 2004; Brittan-Powell et al., 2005). Thus, although it is possible that the waveforms contain far field components of the summating potential, given our definition of threshold, it is unlikely that their small contribution affected the threshold estimations.

The comparable low best frequency ABR thresholds in lizards, alligators and birds may reflect the similar organization of their auditory systems (Carr, 1992; Carr and Code, 2000; Gleich and Manley, 2000; Manley, 1970). Birds, alligators and lizards have unidirectional (hair bundles oriented in the same direction) populations of hair cells covered by a tectorial membrane and similar auditory nerve projections (Gleich and Manley, 2000; Leake, 1974). Manley has proposed that the primitive lepidosaur papilla supported unidirectional hair cells covered with a tectorial membrane. Such a papilla would only have responded to low sound frequencies (below about 1 kHz). The evolution of additional unique populations of high frequency hair cells in most lizards may have increased lizard high frequency sensitivity (Köppl and Manley, 1990; Manley, 1990; Miller, 1980).

**B. Comparisons with earlier Tokay data**

Comparison of previous data on audiograms in the Tokay gecko obtained from single unit nerve recordings (Eatock et al., 1981; Manley, 1972; Manley et al., 1999; Sams-Dodd and Capranica, 1996), cochlear microphonics (CM) (Werner and Wever, 1972), and our ABRs, reveal differences in audiogram shape (Fig. 6). The data fall into two groups: one which shows a distinct low best frequency peak and another which is more bimodal. The first group (gray lines) includes CM studies (Werner and Wever, 1972) and a single-unit study (Sams-Dodd and Capranica, 1996) and shows best sensitivity around 4–500 Hz and a relatively monotonic decline of sensitivity from 600 Hz to 8 kHz. The second group (black lines) includes audiograms based on the lowest thresholds of cochlear nucleus neurons (Manley, 1972), primary nerve fibers (Eatock et al., 1981; Manley et al., 1999) and our ABR data, and shows bimodal audiograms with best sensitivities at 400 Hz and 1–2 kHz. Each method comes with its own set of limitations. CM measurements may be biased toward low-frequency responses, where the synchronization is greatest. Furthermore, Manley (1990) has pointed out that CMs from the bidirectional higher best frequency population may largely cancel each other out because there are equally large populations with both orientations (Manley, 1990; Köppl and Authier, 1995).

Experimental data will further be influenced by the surgical method used to access the auditory nerve, and both the ventral surgical approach used by Sams-Dodd and Capranica (1996) and the approaches used to record CM and compound action potentials (CAP) from the round window are considerably more invasive than the present method. CAP and laser interferometry measurements carried out in the same species of geckos (Eublepharis macularius and Oedura marmorata) showed that the shape of the CAP audiograms (very similar to the CM audiogram in the Tokay) was not predicted well by the tympanic membrane response. For example, the peak response of the adult E. macularius velocity function was approximately 2.2 kHz while the most sensitive CAP threshold was at 0.7 kHz (Werner et al., 2002). It seems reasonable, as suggested by Werner et al. (2001), that these differences are caused by the ventral surgical approach; surgical fenestration of the ventral throat wall may cause artificial enhancement of sensitivity at low frequencies and erratic responses at high frequencies. The role of the intact middle ear cavity in high frequency hearing is also supported by recent laser vibrometry studies and mathematical modeling of the gecko middle ear that show a pronounced resonance of the middle ear cavities (Christensen-Dalsgaard and Manley, 2008).

The bimodal audiogram is most consistent with laser vibrometry studies of the Tokay gecko since the ABR peak sensitivity at 2 kHz also reflects the maximal sensitivity of the eardrum with laser measurements (Christensen-Dalsgaard and Manley, 2005; Werner et al., 2002). At low frequencies, the slope of the present ABR audiogram is slightly shallower than for the previous physiological measurements (see Fig. 6). Below 400 Hz, however, the very
brief tone pulse (10 ms) is more broad-band than the nominal frequency which may lead to frequency splatter and recruitment of additional auditory responses from adjacent regions of the papilla.

The 20 dB general difference in sensitivity between the ABR audiogram and the single-unit audiograms is easier to explain. As previously stated, ABR studies have shown that frequency-dependent ABRs are good predictors of audiogram shape but not of absolute auditory sensitivity. ABR thresholds are generally about 10–30 dB greater than behavioral thresholds (e.g., Brittan-Powell et al., 2002; Gorga et al., 1988; but see Bullock et al., 1968; Popov and Supin, 1990). The synchrony required for an ABR response may be well above the threshold for behavioral sensation (Hecox et al., 1976; Szymanski et al., 1999). In the barn owl, where there is a large nerve data set, Köppl and Gleich (2007) compared measures of behavioral sensitivity with CAP, CM, and single units and found very similar shapes of the CM and CAP audiograms and relative thresholds of single auditory-nerve fibers, although the CAP and CM thresholds were much larger than the single unit or behavioral measures.

It should be noted that behavioral thresholds have not been measured in most lizards (or in other reptiles except birds), and although non-avian reptiles produce a variety of sounds ranging from the rattling of snake tails to the bellows of crocodilians (see review Gans and Maderson, 1973), in no case has communication by sound been well documented except in the gekkonid lizards. In the Tokay gecko, the dominant frequencies of the call match the region of best sensitivity in the ABR. Peak energy of vocalizations and maximum sensitivity of the audiogram usually match in birds and frogs (Ryan and Wilczynski, 1988, Dooling et al., 2000), and the same relationship may hold for gekkonid lizards. However, the present results show that a non-vocal lizard (anole) has a more extended high frequency range and is as sensitive as or more sensitive than the vocal gecko. This would suggest that sound communication is not the prime function of the exquisite auditory sensitivity in lizards.

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