ORIGINAL PAPER



Biophysical heterogeneity in the tympanic membrane of the Asian water monitor lizard, *Varanus salvator*

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Received: 13 September 2017 / Revised: 16 January 2018 / Accepted: 20 January 2018 / Published online: 6 February 2018 © Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

The tympanic membrane of the Asian water monitor lizard (*Varanus salvator*) is functionally divided into dorsal, more pliant, and ventral, stiffer regions. The ventral portion is smaller and vibrates significantly (up to 12×) more to the same stimuli. The pattern of tympanic membrane response is frequency-dependent with a peak response centered around 2.5 kHz, and a lower response at 0.5 kHz. This pattern of tympanic membrane frequency response coincides with the high- and low-frequency ranges of the varanid auditory response. The functional relationship between the tympanic heterogeneity and the internally coupled ears of the varanid auditory system is uncertain, as is the exact role of the tympanum in modulating pharyngeal pressure. The middle ear dynamics of *Varanus salvator*, and likely those of other squamates, are more complex than previously reported.

Keywords Audition · Squamata · Hearing · Vibration

Introduction

The water monitor lizard, *Varanus salvator*, like most squamate reptiles, has internally coupled ears (or ICE). In animals with ICE, the contralateral tympanic membranes are physically linked by large patent conduit(s) through the head, this conduit being formed by the pharynx, Eustachian tube, and possibly other cavities in the skull (e.g., Christensen-Dalsgaard and Manley 2008; Bierman et al. 2014). The vibrational displacements of the linked tympanic membranes provide an ICE animal with neural cues (termed the internal level difference and internal time difference) that have different amplitudes, frequencies, and directional relationships than those available to non-ICE animals (Christensen-Dalsgaard and Manley 2005; Vedurmudi et al. 2016a, b).

The ICE auditory system is possible because displacement of one tympanic membrane creates pressure waves which propagate through the patent conduit, involving the Eustachian tubes and the pharynx, to reach the other tympanic membrane (Young 2016). But what about the pressure waves generated within the pharynx? ICE animals, like other vertebrates, exhibit a wide range of behaviors and/or functions that involve changes in the pressure within the pharyngeal space, including, but not limited to yawning (Gallup 2011), vocalizing (Dudley and Rand 1991), drinking (Bels et al. 1994), and pharyngeal pumping (Brainerd 1999). There is experimental evidence that at least some of these pharyngeal motions alter the vibrational response of the tympanic membrane to airborne pressure waves (Han and Young 2016).

Though our previous study (Han and Young 2016) provided experimental evidence that the middle ear of *Varanus* was more dynamic than previously described, that study simplified the auditory system by treating the tympanic membrane as a homogenous structure with uniform responses. Similarly, the previous models of ICE in squamates have modeled the tympanum as a plate and assumed that the tympanum responds equally to vibrations everywhere on its surface (Vossen et al. 2010; Vedurmudi et al. 2016a, b). The purpose of the present study is to explore these assumptions about the squamate tympanum by testing for differences in composition, vibrational response, and biomechanics, between different regions of the tympanum of *V. salvator*.

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Materials and methods

Live animals

Two hatchling (total lengths of 34 & 36 cm), and six subadult (total lengths of 85–148 cm) *Varanus salvator* (Laurenti 1768) were obtained commercially. The lizards were housed in individual $(60 \times 120 \times 35 \text{ h})$ terraria within a special reptile holding facility with a 12:12 light cycle, water ad libitum, and a temperature range of 28 to 32 °C. The animals were maintained on a diet of previously frozen rodents (Colorado Rocky Mountain Rodents, Paonia, CO, USA).

Compliance with ethical standards

The authors declare that they have no potential conflicts of interest arising from this research. The housing, care, and use of the live *Varanus salvator* followed all applicable national guidelines. All procedures performed were in accordance with the ethical standards of the institution at which the studies were conducted and were approved by the Institutional Animal Care and Use Committee of A.T. Still University (Protocol #177, approved 10/17/2013, and #186 approved 10/15/2014).

Auditory brainstem responses (ABR)

To ensure that the results of this study had relevance to behavioral ecology, it was essential that the trial tones used fell within the auditory response range of the species. Previous studies of *Varanus* have used two very different techniques—cochlear microphonics (Wever 1978) and single unit recordings (Manley 1977)—to demonstrate the frequency range and sensitivity level of the "low" and "high" frequency ranges of auditory response. Since this study is looking at dynamic distribution of acoustic energy between these two auditory regions, an additional technique (ABR) was used to corroborate the earlier studies.

Three of the sub-adult lizards were anesthetized with isoflurane, intubated, then the free end of the tracheal tube was placed in an open chamber containing cotton wadding wetted with isoflurane. This was adequate to maintain anesthetic depth for the duration of the ABR trials without requiring active airflow (which can interfere with the ABR). Small (~1 mm) bilateral incisions were made in the margins between two cephalic scales over the inner ear, and custom fabricated silver chloride electrodes were inserted through the incisions to serve as recording electrodes. A reference electrode (RCS, World Precision Instruments, Sarasota, FL, USA) was placed on the dorsal surface of the snout and covered in electrolyte gel (Signagel, Parker laboratories, Inc., Fairfield, NJ, USA). These electrodes were connected to an ABR system (SmartEP, Intelligent Hearing Systems, Miami, FL, USA). Signal output from the ABR system was routed to a 1208-8 SPS BWX speaker (Peavey Electronics Corp., Meridian, MS, USA) suspended within a custom fabricated acoustic analysis chamber. Stimuli were presented as 25 ms pure tones, presented at a rate of 21 Hz for 1024 repetitions. The resulting neural response traces were judged (by eye, Fig. 1) to determine the minimum pressure level that evoked a response in the auditory pathway.

The individual auditory response curves from the ABR trials on the three specimens exhibited less than 5 dB variation; the three curves were averaged and offset by 20 dB to account for the differential sensitivity of the ABR technique (Gorga et al. 1988). The data from Wever (1978) were converted to SPL with a 20 μ Pa reference level; peak sensitivity values from the results graph in Manley (1977) were used to create low-frequency and high-frequency response curves.

Laser Doppler vibrometry

Individual specimens were anesthetized with isoflurane, then placed on a narrow (head width) padded elevated platform atop a vibration isolation table (Micro-G; Backer-Loring Corp.; Peabody, MA) located within a custom-built acoustic isolation chamber. Two different tympanic response experiments were performed; an initial (hereafter referred to as the narrow-band) and a later (hereafter referred to as the broad-band). For the narrow-band experiment, pure tones (of 200, 550, and 1500 Hz) were generated using Tone Generator software (NCH Software, Greenwood Village, CO) and presented to the animal (at a duration of 0.1 s) using



Fig. 1 Raw ABR data tracings showing the sub-threshold (above) and supra-threshold (below) responses recorded from the auditory system of *Varanus salvator*. The two traces shown were responses to a 500 Hz stimulus, but for the lower trace, the sound pressure was 30 dB (SPL) greater (50 dB compared to 20 dB)

a 1208-8-SPS BWX speaker (Peavey Electronics Corp., Meridian, MS, USA) positioned perpendicular to the long axis of the head, at the same horizontal level as the tympanic membrane, and at a distance of 95 cm. The pressure level of the trial stimuli were held constant at 70 dB (SPL); the pressure level was verified using a digital sound meter (WS1361, Wensn, ShenZhen City, China) and a calibrated USB microphone (UMIK-1, MiniDSP, Hong Kong, China,). Each trial frequency was presented 10 times with a minimum of 0.5 s between stimuli; the presentation sequence of the three frequencies was randomized.

A laser Doppler vibrometer (OFV 534 and OFV5000; Polytec Inc., Irvine, CA, USA) was used to quantify the velocity of the tympanic membrane. The output from the laser Doppler system (tympanic velocity) was recorded (at 10 kHz) using a MiDAS (Xcitex Inc., Woburn, MA, USA) data acquisition system; synchronized output from the tone generator was also recorded. In *Varanus*, the tympanic membrane is ovoid with the longer axis being dorsal–ventral and the extracolumella being asymmetrically attached to the inner surface of the caudodorsal portion. Tympanic velocity was recorded from 10 locations on the tympanic membranes of two sub-adult monitors, and from five locations on the (smaller) tympanic membranes of the two hatchling specimens (Fig. 2a, b).

For the broad-band experiment, Tone Generator software was used to present 0.1 s stimuli with frequencies of 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, and 8.0 kHz. The tones were presented to the animal using a 1208-8-SPS BWX speaker positioned perpendicular to the long axis of the head, at the same horizontal level as the tympanic membrane, and at a distance of 95 cm. The pressure level of each stimulus was individually calibrated to 70 dB (SPL). Each stimulus was presented 10 times with a minimum 0.5 s interval between successive stimuli; the presentation sequence of the different stimuli was randomized.



Fig. 2 Schematic of the tympanic membrane of *Varanus slavator* indicating the sites where the velocity responses were recorded; the arrows indicate the anatomical position for the three tympanic membranes (Ca caudal, Cr cranial). **a** The 10 points used for the narrow-band trials on the larger specimens, **b** the 5 points used during the narrow-band trials on the hatchlings; **c** the 3 points used on all specimens during the broad-band trials

The broad-band trials were performed on two sub-adult and one hatchling specimen of *Varanus salvator*. Tympanic velocity was recorded, using the laser Doppler vibrometer, from three locations on the tympanic membranes (Fig. 2c).

The signals from the laser Doppler system closely tracked the supplied stimuli (Fig. 3), with a roughly 3.5 ms delay due to the distance to the speaker and the inertia of the tympanic membrane. We compared tympanic velocities (using Tukey's W procedure) within and between the 10 trials of each stimulus; in each case, there were no statistical differences within any given stimulus train. The trains were then re-sampled and the tympanic velocities compared using a two-way ANOVA to test for differential responses between the recording sites on the tympanic membranes, as well as between the stimuli frequencies. Significant results from the ANOVA were further analyzed using Tukey's HSD post hoc tests (both performed using IBM SPSS Statistics v. 20.0.0).

Tympanic tensiometry

Immediately following the laser Doppler vibrometry, while still under anesthesia, the specimens were euthanized. The right tympanic membrane was excised with care being taken to avoid any tears or cuts in the membrane itself. The extracolumella was severed, so that the distal end of the extracolumella, which is integrated into the tympanic membrane (Han and Young 2016), was left intact and attached to the tympanic membrane. The excised tympanic membrane was positioned (medial side up) on a glass cover slip that was coated with reptilian Ringer's solution (formula taken from Barfuss and Dantzler 1976) to minimize both dessication of the tympanic membrane and friction drag on the lateral surface of the tympanic membrane.



Fig. 3 Raw data traces from the laser Doppler vibrometer showing the 2.5 kHz stimulus (upper trace) and the tympanic velocity response (lower trace). The traces on the right have a finer temporal resolution and demonstrate the delay between stimulus presentation and tympanic response

One side of the excised tympanic membrane was clamped to a mechanical infusion pump (model 901, Harvard Apparatus, Holliston, MA) which enabled a constant rate of strain to be applied to the tympanic membrane. The opposing edge of the tympanic membrane was clamped to a force transducer (FT03, GRASS Instruments, Pleasanton, CA) connected to a strain gage amplifier (P122 GRASS Instruments, Pleasanton, CA, USA) the output of which was sampled (at 10 kHz) using the MiDAS (Xcitex Inc., Woburn, MA, USA) data acquisition software. The opposing clamps were placed on the anterior and posterior surfaces of the tympanic membrane, both above and below the level of the extracolumella (hereafter referred to as the dorsal and ventral portions of the tympanic membrane).

A digital photograph was taken of the clamps in place immediately prior to each trial; the distance between the clamps was quantified using ImageJ (NIH). The withdraw rate of the infusion pump was constant (the exact value and repeatability of the pump was verified using high-speed digital videography), which enabled the calculation of stress/ strain curves (Fig. 4). Standard linear regression curve fitting was used to identify the initial displacement (the slope of which is herein termed the initial Young's modulus), the linear portion of the deformation curve (the slope of which is herein termed the tangent Young's modulus), and the deflection point between these two regions of the curve (Fig. 4). To test for differences between the dorsal and ventral portions of the tympanic membranes, these three aspects of the



Fig. 4 Raw data tracing from the tympanic tensiometry experiments. The experimental values (the stress/strain curve) are indicated by the black line. The three quantified features for each stress/strain curve were the slope of the initial displacement (indicated by the blue dashed line), the slope of the linear portion of the displacement (indicated by the blue solid line), and the strain value corresponding to the deflection point after the initial linear displacement (red vertical line)

tympanic stress/strain curves were compared using a oneway ANOVA and Tukey's HSD post hoc test.

Morphology

The left tympanic membrane, and the adjacent skeletal and muscular tissue were excised from each specimen, and preserved in chilled neutral-buffered formalin (NBF) for 72 h. The excised blocks were decalcified in RDO Rapid Decalcifier (Apex Engineering Products, Aurora, IL, USA) for 24 h prior to dehydration and paraffin embedding. Transverse, frontal, and parasagittal sections were cut at 10 µm; mounted sections were stained with either hematoxylin and eosin, Verhoff's elastin stain, or Masson's trichrome stain (following Luna 1968). Microscopic anatomy was documented using a DM 4000B microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA). The dorsal and ventral portions of three tympanic membranes were excised from the extracolumella, photographed, then weighed on an analytical balance (100A, Denver Instruments, Bohemia, NY, USA). The surface area of the excised tympanic portions was determined by importing the digital photographs into ImageJ (NIH).

Results

Morphology

The extracolumella attaches to the inner surface of the tympanic membrane; the cranial tip of this attachment occurs near the midpoint of the tympanic membrane (Han and Young 2016; Fig. 5a). In conscious and anesthetized specimens, the central contact point (between the pars inferior of the extracolumella and the inner surface of the tympanic membrane) is the most lateral portion of the tympanic membrane (Fig. 5a). Once the central segment (supporting the extracolumellar attachment) was excised, the dorsal portion of the membrane had a significantly (t=4.21, p=0.0449)smaller surface area (mean 19.9 mm², s.d. = 5.39) than the ventral portion (mean 36.08 mm², s.d. = 3.88). The dorsal and ventral portions of the tympanic membrane had similar (t=0.91, p=0.28) densities (0.0038 and 0.0046 g/mm³, respectively). Tympanic thickness ranged from 16.7 µm in the smallest hatchling to 49.2 µm in the largest specimen; no significant difference was found between the thickness of the dorsal and ventral portions of the tympanic membrane.

The tympanic membrane of *Varanus salvator* is not supported by a fibrocartilage ring, annular ligament, or anulus tympanicus (Decraemer and Funnell 2008). The peripheral edge of the tympanic membrane is approximately 4 times thicker than the remainder; the membrane tapers down quickly (Fig. 5b, c) and other than the local thickening associated with the attachment of the extracolumella and



Fig. 5 Morphology of the tympanic membrane of *Varanus salvator*. **a** Photograph of the tympanic membrane of a live specimen. The pars inferior of the extracolumella is located near the center of the tympanic membrane and causes the membrane to bulge outward slightly; note the appearance in the tympanic membrane of radiating lines away from this central bulge. **b** Dorsocaudal margin of the tympanic membrane showing the epithelial transitions on the two surfaces of the membrane and the rapid decrease in the size of the lamina propria. **c** Caudal margin of the tympanic membrane; the elastin-rich post-tympanic band courses along the posterior margin of the mem-

the tympanus muscle (Han and Young 2016), no distinct variation in membrane thickness was noted histologically. The deep (medial) surface of the thicker peripheral edge of the tympanic membrane exhibits a transition from a mucoid (and in patches ciliated) epithelium to a thin non-keratinized epithelium (Fig. 5b, c). The superficial (lateral) surface of the tympanic membrane is covered by a relatively thin keratinized epithelium which is continuous with the adjacent scalation (Fig. 5b, c). In V. salvator, the caudal attachment of the tympanic membrane involves the post-tympanic band, a dorsal-ventral thickening of the epimysium and connective tissue surrounding the depressor mandibulae. Han and Young (2016) recently reported that this post-tympanic band includes elastic connective tissue; the elastin extends into the tympanic membrane itself (Fig. 5c) and the amount of elastin is greater in the ventral portion of the post-tympanic band.

The internal structure, or lamina propria, of the varanid tympanic membrane consists of multiple layers of collagenous fibers, with distinct changes in fiber orientation

brane and elastin fibers (stained black in this Verhoff's preparation) integrate into the lamina propria. **d** Frontal section through the middle of the tympanic membrane near the attachment of the extracolumella; this region of the membrane is thicker than most, and clearly demonstrates the presence of layers of collagen fibers at different orientations. **e** parasagittal section through the ventral portion of the tympanic membrane showing the integrated layers of collagen fibers within the lamina propria. *e* extracolumella; *l* lamina propria; *m* middle ear cavity; *p* post-tympanic band

between adjacent layers (Fig. 5d, e). Parasagittal sections through the membrane (Fig. 5e) suggest interwoven layers of collagen fibers, rather than discrete radial and circular fiber layers. The membrane is thickest, and most organized, in the central portion immediately adjacent to the attachments of the extracolumella (Fig. 5c, d); for the remainder of the membrane, the collagen is sparse enough that it is difficult to discern the fiber orientations histologically in transverse sections (Fig. 5b).

ABR frequency response range

The ABR results indicate peak sensitivity from 500 to 800 Hz. The drop-off in sensitivity with higher frequency flattens out somewhat between 1.5 and 3.5 kHz, indicative of the high-frequency response range. The difference in relative sensitivity between the low-frequency and high-frequency hearing ranges is approximately 40 dB (Fig. 6).



Fig. 6 The auditory range of *V. salvator*. Blue lines—data taken for *Varanus* from Wever (1978); Red lines—data taken from Manley (1977); Black lines—results of the ABR analysis. There is general agreement for a peak sensitivity range centered around 700 Hz, and a high-frequency range around 2 kHz which is approximately 40 dB less sensitive. There is a steep decrease in auditory sensitivity on either side of this auditory range



Fig. 7 Biomechanics of the tympanic membrane of *Varanus salvator*. The presence of the extracolumella is used to divide the membrane into dorsal (orange lines) and ventral (blue lines) portions; the stress/ strain curves were determined separately for each portion. The individual trials are indicated by the dashed lines, while the mean values for both halves are given by the heavier solid line. The strain level at deflection and the tangent Young's modulus were significantly different in the two halves of the tympanic membrane

Tympanic biomechanics

The resistance to the initial extension of the dorsal and ventral portions of the tympanic membrane (the initial Young's modulus and identified as the linear portion of the stress/ strain curve that begins at the origin, Fig. 7) was larger in the ventral portion (B=0.539, s.e. = 0.09) than in the dorsal portion (B=0.313, s.e. = 0.07); however, this difference was not significant (one-way ANOVA, 1 df, F=3.192, p=0.108). In both portions of the membrane, the stress response increased relative to the applied strain, such that the linear relationship that characterized the initial Young's modulus no longer held. This deflection point occurred at a strain value of 0.57% (s.e. = 0.03) for the ventral portion of the membrane and at 0.82% (s.e. = 0.07) for the dorsal portion. The difference between these two deflection points was significant (one-way ANOVA, 1 df, F=13.132, p=0.006). Beyond this initial point of deflection, the stress/ strain curves all demonstrated a second linear phase, the tangent Young's modulus. The slope of the tangent Young's modulus for the dorsal portion of the membrane (B=2.162, s.e. = 0.46) was significantly (one-way ANOVA, 1 df, F=5.29, p=0.015) lower than the slope in the ventral portion (B=3.574, s.e. = 0.41).

Laser Doppler vibrometry

The narrow-band experiments were performed on two hatchlings and two sub-adult specimens of Varanus salvator; due to their marked differences in tympanic surface area, different recording sites were used on the sub-adult and hatchlings. The narrow-band experiments on the hatchlings involved five recording sites on the tympanic membrane (Fig. 2). There was a consistent pattern with the ventral portion of the tympanic membrane vibrating more to the stimulus than the dorsal or middle portions (Fig. 8). In both specimens, there were significant (F = 476.61 and 506.82, both with df = 4, for p < 0.001) differences between the five recording sites. Post hoc tests (Tukey's HSD) in both specimens revealed the same pattern; the two recording sites from the ventral portion of the tympanic membrane vibrated significantly (p < 0.001) more than the other sites (Fig. 8). There was also a differential response to the three trial tones presented (F = 417.04 and 113.31, both with 2 df, for p < 0.001); in both specimens, the 200 Hz stimulus produced less tympanic response than either the 550 or 1500 Hz stimuli, the responses to which were not significantly different (Fig. 8).

The larger tympanic surface area of the sub-adults enabled replicable recordings from more sites, but the same pattern of results was found. In both specimens, the ventral, and particularly the cranioventral, portion of the tympanic membrane vibrated more in response to the trial stimuli (Fig. 9). Both specimens had significant differences (F = 2046.99 and 1536.5, 9 *df*, p < 0.001) between the 10 recording sites. Tukey's HSD post hoc analyses revealed that the sites on the ventral portion of the tympanic membrane had significantly (p < 0.001) greater vibrational response to the trial stimuli than did the sites on the dorsal portion (Fig. 9). There was a differential response to the three trial stimuli (F = 2585.97 and 4727.88, 9 *df*, p < 0.001); with less response to the 200 Hz tone than to either the 550 or 1500 Hz tones, the latter two yielded similar levels of response (Fig. 9).



Fig. 8 Tympanic velocity recorded from 5 locations on the tympanic membrane of *Varanus salvator* during the presentation of three trial stimuli; note that all of the vertical scales are the same. Colored

dashed lines are from the individual specimens, the solid black line is the mean; the mean line is plotted on the left graph

The broad-band experiments were performed on three sub-adult specimens which exhibited very similar responses. In all three specimens, there was significant differences in tympanic response between the three recording sites (*F* values of 1288.24, 1726.67, and 1031.2, df = 2, all *p* values < 0.001). Post hoc tests demonstrated that the

responses recorded from the ventral portion of the tympanic membrane (Fig. 10) were significantly larger than those recorded from the dorsal portion or the extracolumella. There was a significant differential response to the 15 trial stimuli presented (F values of 385.45, 989.54, and 360.28, df = 14, all p values < 0.001). Peak responses



Fig.9 Tympanic velocity recorded from 10 locations on the tympanic membrane of *Varanus salvator* during the presentation of three trial stimuli; note that all of the vertical scales are the same. Colored

dashed lines are from the individual specimens, the solid black line is the mean; the mean line is plotted on the left graph

were found around 2.5 kHz, with a second (lower) peak at 500 Hz. The vibrational responses to the 500 Hz and 2.5 kHz tones were not significantly different at the dorsal recording point, though they were at the extracolumellar and ventral points (Fig. 10).

Discussion

In the narrow-band trials the peak tympanic velocities recorded from the ventral portion of the tympanic



Fig. 10 Tympanic velocity recorded from 3 locations on the tympanic membrane of *Varanus salvator* during the presentation of 15 trial stimuli; note that all of the vertical scales are the same. Colored dashed lines are from the individual specimens, the solid black line is the mean

membrane were significantly (3-4x) greater than those recorded from the dorsal portion (Figs. 8, 9). During the broad-band trials the peak velocities recorded during the 2.5 kHz stimulation of the ventral portion of the tympanic membrane were significantly (13x) greater than those recorded in the dorsal portion (Fig. 10). In all of the specimens, and over both laser Doppler trials, the ventral portion of the tympanum exhibited a greater response than the dorsal portion.

In terrestrial or amphibious vertebrates, the auditory system takes one of four general configurations: (1) a disc or plate-like receptive surface with coupling between the two middle ear cavities (e.g., *Xenopus*; Mason et al. 2009); (2) a disc or plate-like receptive surface with no coupling between the two middle ear cavities (e.g., aquatic turtles; Christensen-Dalsgaard et al. 2012); (3) a drum or membrane-like receptive surface with coupling between the two middle ear cavities (e.g., *Varanus*); or (4) a drum or membrane-like receptive surface with no coupling between the middle ear cavities (e.g., mammals). Regional differences in tympanic responsiveness are well-known in mammals,

where the tympanic membrane is frequently divided into a more-responsive "pars tensa" and a less-responsive "pars flaccida" (e.g., Lim 1995). Little is known about the pattern of tympanic displacements in the other vertebrate groups. Manley (1972) mapped the tympanic response of the Tokay gecko (Gekko gecko) to stimuli ranging from 0.25 to 10 kHz; though the results were not analyzed statistically, he found "some asymmetry" as well as a frequency-dependent pattern to the displacements. Muyshondt et al. (2016) measured the tympanic displacements of the duck (Anas) and found a similar asymmetric pattern. The gecko and duck have a similar tympanic and middle ear configurations to that of Varanus, but not enough comparative work has been done to generalize the patterns of tympanic displacement in anything but the mammalian group. The pars flaccida of the mammalian tympanum has been proposed to function in middle ear volume/pressure regulation (e.g., Stenfors et al. 1979; Dirckxa et al. 1998); it remains to be determined how pressure/volume regulation is accomplished in the other "types" of vertebrate ears.

The pattern of differential vibrational response in the tympanic membrane of *Varanus* was significantly frequencydependent. The narrow-band trials resulted in significantly greater tympanic velocities at 550 and 1500 Hz, than at 200 Hz (Fig. 8, 9). The broad-band trials also recovered the response at 500 Hz, but demonstrated that the response seen in the earlier trials at 1500 Hz is the lower side of a high-frequency response region which peaks at 2.5 kHz (Fig. 10). The broad-band trials revealed little tympanic response to either 100 Hz stimuli, or stimuli > 7 kHz; in these frequency regimes, the different portions of the tympanic membrane responded similarly.

There was good agreement between the results of the ABR experiments, and the previous studies of varanid audition (Fig. 6). The portions of the varanid audiogram typically ascribed as the low-frequency and high-frequency hearing range correspond to the two frequencies of greatest tympanic response (0.5 and 2.5 kHz). The frequency ranges under which differential tympanic responses were documented (0.1-7.0 kHz) span the range of varanid auditory sensitivity. If the mean tympanic responses obtained during the broad-band experiment (Fig. 10) are overlapped with the auditory sensitivity curve obtained from the ABR data (Fig. 11), it suggests that the ventral portion of the tympanum has the best fit to the low-frequency portion of the audiogram. None of the high-frequency tympanic responses are reflected in the audiogram where the high-frequency portion of the hearing range is little more than a subtle decrease in the fall-off of auditory sensitivity, rather than a second "peak." The roughly 40 dB difference between the low- and high-frequency sensitivities is more congruent with the differential velocities recorded from the ventral portion of the tympanum (Fig. 11).



Fig. 11 Mean ABR audiogram (solid line, inverted from Fig. 6) right *Y*-axis, with the mean tympanic velocity responses (left *Y*-axis) from the ventral (short dash), extracolumellar (medium dash) and dorsal (long dash) portions of the tympanic membrane

Classical membrane theory (e.g., Fletcher 1992) and more recent theoretical models of the reptilian tympanum (Vedurmudi et al. 2016b) predict that with increasing frequency, the tympanum will switch vibratory modes, and that this switch will be marked by a more asymmetric response. It is likely that this transition between the first and second mode of the varanid tympanum is reflected in the differential responses at 0.5 and 2.5 kHz (Fig. 10).

Despite the general agreement it affords, the application of classic membrane theory to the varanid tympanum is problematic. The applications of membrane theory to the tympanum presented by Fletcher (1992) and (Vedurmudi et al. 2016b) both assume a homogeneous tympanum that is rigidly clamped around its perimeter. The greater vibrational response in the ventral portion of the varanid tympanum may be due, in part, to the intrinsic structure of the membrane itself. When compared to the upper portion, the lower portion of the tympanic membrane deforms earlier and deforms to a greater degree for a given force (stress) level (Fig. 7). The lower portion of the tympanic membrane is stiffer, and the upper portion is more compliant. This is surprising given that no significant difference in tympanic thickness was found between the two regions; at the level of light microscopy, there was no evident anatomical difference between the two portions of the tympanic membrane. A more detailed 3-D reconstruction of the collagen in the tympanic membrane may reveal differences in fiber orientation and layering that were not evident in this study (more complex fiber architectures have been described from mammalian tympana; Knutsson et al. 2007). Studies of the tympanic membranes of mammals have found differences in collagen fiber composition, with resulting differences in biomechanical properties (Jackson et al. 2008; O'Connor et al. 2008). There was no evidence of smooth muscle within the tympanic membrane of *Varanus salvator* (though this has been reported in mammals; Henson and Henson 2000).

In *Varanus*, as in most lizards, the dorsal margin of the tympanic membrane is attached to the squamosal and quadrate bones, the cranial margin to the quadrate, the ventral margin to the retroarticular process of the mandible, and the caudal margin to the depressor mandibulae and the post-tympanic band (Han and Young 2016). In *Varanus*, where there is no annulus tympanicus or fibrocartilage anchor for the periphery of the tympanum, it is not clear how rigid, or uniform, these tympanic attachments are. This is particularly true along the caudal margin where elastin in the post-tympanic band not only contacts the tympanum, but integrates into the ventral portion of the membrane.

The formalizations of the internally coupled ears of lizards, like Varanus salvator, have treated the surface area of the tympanic membrane as the responsive area (Vossen et al. 2010; Vedurmudi et al. 2016a, b). Wever (1978; and in Wever and Werner 1970) extrapolated from studies of the mammalian system (including Wever and Lawrence 1954) to posit that in reptiles only the central part of the tympanic membrane was free to move and so the effective area of the tympanic membrane should be taken as 66% of the total surface area. Manley (1972) directly challenged this assumption by taking precise measurements of tympanic displacement at different locations over varying frequencies; Manley's clear results allowed him to dismiss Wever's proposed effective area as a "gross over-simplification." The current study supports Manley's view; at 2.5 kHz a region in the ventral portion of the tympanic membrane exhibits a vibrational response that is more than 12x that of the rest of the membrane (Fig. 10). At this frequency, the effective area would be far closer to 30% than the 66% proposed by Wever.

As part of their foundational work on the mechanics of lizard hearing, Wever and Werner (1970) and Werner and Wever (1972) explored the functional basis of impedance matching and amplification in the middle ear. They argued that the principle determinant of impedance would be the hydraulic ratio of tympanic surface area to stapedial footplate surface area. Without disputing the basic mechanics described by Wever and Werner (1970) and Werner and Wever (1972), the tympanic vibrational responses in *Varanus salvator* means that the impedance of the middle ear would follow a frequency-dependent pattern with a marked decrease at higher frequencies.

An earlier study of this species (Han and Young 2016) described a skeletal muscle that inserts onto the distal extracolumella and integrates into the tympanic membrane; contraction of this muscle alters both the pattern of tympanic vibrational response and the amplitude of that response (Han and Young 2016). Much of the length of the interaural canal of *Varanus salvator* is made up of the pharynx. *V. salvator* is capable of actively modulating the air pressure within the pharynx, and thus the pressure acting on the medial surface of the tympanic membrane (Owerkowicz et al. 1999). Han and Young (2016) showed that the amplitude and pattern of the tympanic membrane's vibrational responses could be significantly altered by changing the air pressure within the interaural canal. Given the intrinsic biomechanical differences between the dorsal and ventral portions of the tympanic membrane (Fig. 7), it is not clear if the entire membrane would exhibit the same shifts in vibrational response due to pharyngeal pressure changes or tensile forces exerted by the tympanus muscle.

In mammals the pars flaccida of the tympanic membrane has been interpreted as functioning to accommodate changes in middle ear pressure (e.g., Hellström and Stenfors 1983; Sadé 1997). The findings of the present study, in conjunction with the earlier work on dynamic control of tympanic tension in *Varanus* (Han and Young 2016), suggest that *Varanus* has evolved a pressure-regulation system that is analogous, but not homologous, to the mammalian system. In the varanid system, the majority of the tympanic membrane, but particularly the dorsal portion, may function more in pressure regulation, while the ventral portion is the effective center for vibrational response.

Acknowledgements The authors wish to thank P. Kondrashov and the administration of ATSU, as well as C. Montgomery and the Department of Biology of Truman State University for their support of this research.

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