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RESEARCH ARTICLE

# Better late than never: effective air-borne hearing of toads delayed by late maturation of the tympanic middle ear structures

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## ABSTRACT

Most vertebrates have evolved a tympanic middle ear that enables effective hearing of airborne sound on land. Although inner ears develop during the tadpole stages of toads, tympanic middle ear structures are not complete until months after metamorphosis, potentially limiting the sensitivity of post-metamorphic juveniles to sounds in their environment. We tested the hearing of five species of toads to determine how delayed ear development impairs airborne auditory sensitivity. We performed auditory brainstem recordings to test the hearing of the toads and used micro-computed tomography and histology to relate the development of ear structures to hearing ability. We found a large (14–27 dB) increase in hearing sensitivity from 900 to 2500 Hz over the course of ear development. Thickening of the tympanic annulus cartilage and full ossification of the middle ear bone are associated with increased hearing ability in the final stages of ear maturation. Thus, juvenile toads are at a hearing disadvantage, at least in the high-frequency range, throughout much of their development, because late-forming ear elements are critical to middle ear function at these frequencies. We discuss the potential fitness consequences of late hearing development, although research directly addressing selective pressures on hearing sensitivity across ontogeny is lacking. Given that most vertebrate sensory systems function very early in life, toad tympanic hearing may be a sensory development anomaly.

**KEY WORDS: Acoustic communication, Sensory development, Bufonidae, Histology, Micro-CT, Auditory brainstem recordings**

## INTRODUCTION

Most vertebrate sensory systems function before or soon after an animal begins living autonomously (Ganchrow and Ganchrow, 1987; Noakes and Godin, 1988; Apfelbach et al., 1991; Fagiolini et al., 1994; Easter and Nicola, 1997; Northcutt, 2004). Yet, in anurans (frogs and toads), the tympanic hearing system is often not fully formed until days, weeks, months or even a year after metamorphosing from a free-living, aquatic larval stage to a land-based, autonomous froglet (Sedra and Michael, 1959; Hetherington, 1987). Thus, the anuran hearing system is one of few sensory systems with major structural development after the animal is fully independent. Previous work has shown that tadpoles sense underwater sound well (Simmons, 2015), but that sensitivity to airborne sound increases after metamorphosis (Shofner and Feng, 1981; Boatright-Horowitz and Simmons, 1995). Behavioral consequences of this late development are possible. Species-specific calls are known to function in mate attraction, conspecific localization, territoriality and defense in adults (Narins et al., 2006) but the fitness consequences of hearing conspecific calls or other environmental cues remains unstudied in juveniles, despite juvenile frogs showing behavioral responses to calls well before sexual maturity (Baugh and Ryan, 2010). Hearing is hypothesized to play a role in predator detection as well (Wever, 1985), though the focus of hearing research in anurans has been on its value for social communication (Wever, 1985; Narins et al., 2006, Wells and Schwartz, 2007). This late development of the ear likely has a large impact on hearing ability throughout post-metamorphic ontogeny, but little is known about how tympanic ear function changes as individual ear structures mature and grow in size from metamorphosis to adult.

The inner ear structures, which contain the sensory hair cells that transmit sound information to the brain, are fully formed and functional during the larval stages of anuran development (Simmons and Alexander, 2014; Simmons, 2015). The medulla and midbrain both respond to underwater sound in tadpoles that lack functional tympanic ears (Simmons, 2015). In addition, the operculum, a movable cartilaginous structure found solely in amphibians in the oval window, is developed by metamorphosis (Sedra and Michael, 1959; Hetherington, 1987). The operculum is thought to be involved in low-frequency sound perception (below 1 kHz) in conjunction with the opercularis muscle, which attaches the suprascapular cartilage to the operculum (Lombard and Straughan, 1974; Mason, 2006). In general, middle ear structures develop in a medial-to-lateral progression during and after metamorphosis (see Fig. 1); however, the relative timing of development of the other tympanic ear structures varies across species (Sedra and Michael, 1959; Hetherington, 1987; Vorobyeva and Smirnov, 1987). The anuran middle ear column, termed the columella, consists of three portions, the pars interna (also called the columellar footplate), the pars media and the pars externa (also called the extracolumella). The more lateral middle ear structures include the tympanic membrane and a surrounding cartilaginous ring, termed the tympanic annulus. In species that require months post-metamorphosis to complete development of the tympanic middle ear structures (species of the genus *Hyla* and *Bufo*), the order of ear structure formation is quite clear. Tympanic ear development begins with the formation of cartilaginous structures comprising the columella. First, the pars interna appears attached to the oval window by the climax of metamorphosis (Hetherington, 1987). The pars interna then grows outward, forming the pars media and eventually extends to become or join the pars externa (Hetherington, 1987). The tympanic annulus and tympanum begin to develop as the columella grows (Sedra and Michael, 1959; Hetherington, 1987). The middle ear cavity and Eustachian tube develop at the same time as the tympanic annulus and tympanum, reaching adult-like proportions before the pars media ossifies (*Hyla crucifer*;

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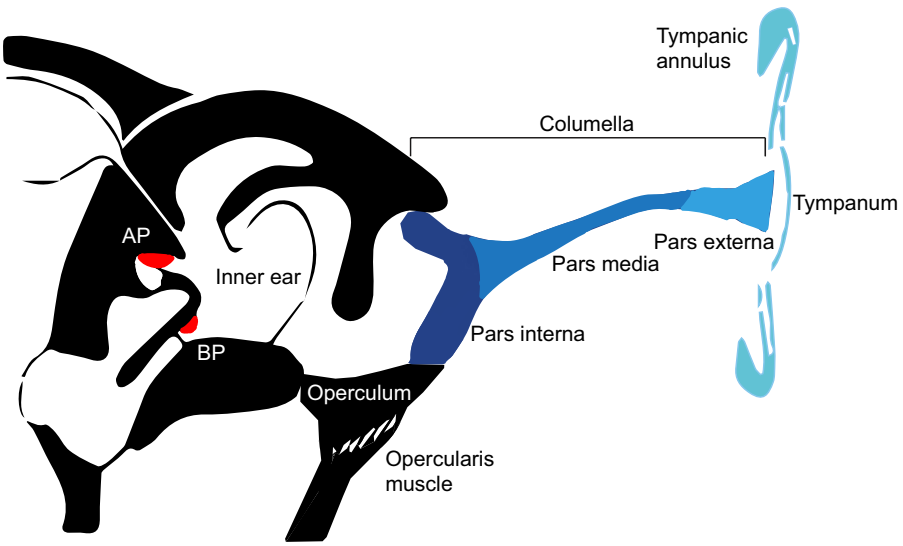


**Fig. 1. Developmental progression of tympanic middle ear structures in anurans.** Horizontal section of adult *Rana pipiens*, modified from Wever (1985) to depict the medial-to-lateral development of middle ear structures after metamorphosis. Black structures indicate muscles and bones that surround the inner ear cavity and form prior to metamorphosis. The amphibian papilla (AP) and basilar papilla (BP), depicted in red, house the sensory hair cells. The saccule is not visible in this section plane. Middle ear structures are color coded according to the relative time at which the structure initially appears in previous studies of anuran development (Sedra and Michael, 1959; Hetherington, 1987; Vorobyeva and Smirnov, 1987), as absolute timing differs in each lineage. Middle ear structures that form early are colored in darker shades of blue, with later forming middle ear structures depicted in lighter shades of blue.

Hetherington, 1987). When the middle ear is fully mature, the pars media and a portion of the pars interna are ossified, while the pars externa remains cartilaginous (Hetherington, 1987; Vorobyeva and Smirnov, 1987).

After all ear structures have formed, sound is transmitted through the tympanic hearing system in anurans similarly to the transmission of sound through comparable tympanic hearing systems across other eared tetrapods (reviewed in Christensen-Dalsgaard and Manley, 2013; Mason et al., 2015). The tympanum vibrates in response to airborne sound and transmits these vibrations to the columella. The columella footplate is in direct contact with the inner ear fluids and transmits the vibrations to the fluid, which ultimately moves hair cells, resulting in action potentials that signal to the brain (reviewed in Lewis and Narins, 1999). However, unlike the single auditory end organ found in most tetrapods, three end organs respond to sound in anurans: the sacculus, which senses very low frequencies (ca. 20–120 Hz), the amphibian papilla, which senses low–mid frequencies (ca. 120–1250 Hz), and the basilar papilla, which senses higher frequencies (ca. 1250–4000 Hz; Smotherman and Narins, 2000; Christensen-Dalsgaard and Narins, 1993).

In addition to tympanic hearing, anurans also sense airborne auditory stimuli through extratympanic hearing pathways (Wilczynski et al., 1987), which are not dependent on tympanic middle ear structures. The main extratympanic hearing pathways proposed for post-metamorphic anurans are the opercularis system and the lungs. The opercularis pathway transmits acoustic vibration to the inner ear via the operculum (Lombard and Straughan, 1974). In the lung pathway, the body wall overlying the lungs vibrates in response to sound and transmits those vibrations to the inner ear even in the absence of tympanic middle ears (Lindquist et al., 1998; Hetherington and Lindquist, 1999; Hetherington, 2001). These extratympanic hearing pathways could buffer immature anurans from potential sensory costs of late-developing ear structures.

To better understand the functional consequences of delayed middle ear development in anurans, we tested the hearing of five toad species, with individuals ranging from small juveniles at various stages of ear development to adults with completely developed ear structures. We predicted that, as in *Rana catesbeiana* (Shofner and Feng, 1981; Boatright-Horowitz and Simmons, 1995), post-metamorphic development of the middle ear structures would lead to increased sensitivity to sounds as animals grow. We further investigated which changes in tympanic middle ear structures contribute to hearing sensitivity throughout post-metamorphic development. Toad species develop their tympanic hearing system slowly in comparison to other anurans (Sedra and Michael, 1959; Hetherington, 1987), offering a unique opportunity to identify which maturational steps confer major impacts on tympanic hearing.

## MATERIALS AND METHODS

### Animal collection

Individuals of five species (*Rhinella alata* *N*=9, *Rhinella leptoscelis* *N*=8, *Rhinella marina* *N*=10, *Rhinella spinulosa* *N*=11 and *Rhaebo haematiticus* *N*=12) were collected from field sites in Ecuador and Peru (Table 1). The Institutional Animal Care and Use Committee at Colorado State University approved all experiments (IACUC protocol no. 12-3484A), and the Ministerio del Ambiente in Ecuador and the Servicio Nacional Forestal y de Fauna Silvestre (SERFOR) in Peru approved collection, research and export permits (Table 1). We identified the sex in only a subset of individuals in our study; thus, we did not examine patterns of sex differences among species, although

**Table 1. Collection country, sites and permit numbers for animals in the study**

| Species | Country | Specific region | Permit no. |
|---|---|---|---|
| *Rhaebo haematiticus* (Cope 1862) | Ecuador | Mindo Forest Reserve and Rio Guajalito Reserve in Pastaza | 001-13 IC-FAU-DNB/MA |
| *Rhinella alata* (Thominot 1884) | Ecuador | Rio Rutuno, Pastaza | 001-13 IC-FAU-DNB/MA |
| *Rhinella leptoscelis* (Boulenger 1812) | Peru | Yanachaga buffer zone of Chemillén National Park in the basins of San Alberto (CDS/CNEH-Perú) Llamaquizú, San Luis and San Daniel | 0071-2015-SERFOR-DGFFS/DGEFFS |
| *Rhinella marina* (Linnaeus 1758) | Ecuador | Canelos, Pastaza and in Mindo Forest Reserve and Maquipucuna Biological Reserve in Pastaza | 001-13 IC-FAU-DNB/MA |
| *Rhinella spinulosa* Wiegmann 1834 | Peru | K'iripampa Acopia in Acomayo, Cusco | 0071-2014-MINAGRI-DGFFS/DGEFFS |

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males and females may differ in hearing (as in Boatright-Horowitz and Simmons, 1995; Miranda and Wilczynski, 2009; Shen et al., 2011). We did not find bimodal distributions of ear structures that indicated extreme sexual dimorphism in ears is likely in this lineage.

### Auditory brainstem recordings to test hearing throughout ear development

We characterized the hearing abilities of each species using auditory brainstem recordings (ABRs) to measure the sensitivity of animals to tones across the range of audible frequencies. All ABRs were performed in Ecuador or Peru. We performed recordings in a portable wooden chamber (76 cm length×38 cm width×46 cm height) with all internal surfaces covered with acoustic foam lining. We lightly anesthetized the toads with a small topical application of 5% benzocaine and then paralyzed them with 0.05% succinylcholine chloride (Sigma-Aldrich, St Louis, MO, USA) at a dosage of 7.5 µl $g^{-1}$. We placed three 28-gauge stainless steel electrodes (Model F-E2, GRASS Technologies, Warwick, RI, USA) subdermally to measure electrical signal generated by the auditory nerve (the VIIIth nerve). Differential electrodes were placed over the VIIIth nerve and over the midbrain, and a third ground electrode was placed within the arm contralateral to the VIIIth nerve being measured. We linked the three electrodes to a pre-amplifier (RA4PA, Tucker-Davis Technologies, Alachua, FL, USA) connected to a mobile processor (RM2, Tucker-Davis Technologies) that relayed output and input signals from and to a laptop computer (Mini 210-2180, Hewlett Packard, Palo Alto, CA, USA). We played pure tones from a 3 in speaker (FF85K, Fostex, Tokyo, Japan) suspended within the audio chamber to minimize ground vibrations from the speaker reaching the animal. We amplified the acoustic stimuli with a digital AC/DC amplifier (DTA-1 Class T, Dayton Audio, Springboro, OH, USA) and calibrated with a ½ in free-field microphone (46AE, G.R.A.S. Sound and Vibration A/S, Skovlytoften, Denmark). We calibrated the free-field microphone by using a pistonphone (Type 42AA, G.R.A.S. Sound and Vibration A/S) to produce a 94 dB root mean square (RMS) re. 20 µPa tone at 1 kHz. The toads lay perpendicular to and ∼46 cm away from the speaker on a wet paper towel. To shield electrical noise, we placed a wire mesh cage over the animal during recordings that was grounded by a connection to the pre-amplifier.

We calibrated the experimental setup using customized software (QuickABR_burst) that controlled stimulus presentation and data acquisition using the RM2 processor. We played 25 ms pure tones, ranging in frequency from 200 to 4000 Hz at 5 dB increments with 40 ms intervals. We tested frequencies in order from low to high with amplitudes presented sequentially from high to low, unless additional high amplitude tones were necessary because of a lack of response at the starting amplitude. Response signals were averaged over 400 tone bursts. Between every two frequencies tested we measured the response to a transient generated from a half cycle 4 kHz sinusoid at 105 dB to ensure that the auditory responsiveness remained stable throughout the testing session. If the transient response dropped below 25% of the original signal, we omitted all subsequent measurements from analyses. We visually determined thresholds for each frequency by finding the minimum stimulus decibel level that evoked a response signal amplitude of 0.002 mV (two times the average noise level) or greater from the auditory nerve.

### Specimen fixation

After each ABR, we measured the snout–vent length (SVL) and tympanum diameter of each animal to the nearest 0.1 mm using a dial caliper (31-415-3, Swiss Precision Instruments Inc., Garden Grove, CA, USA). We then killed the animals with 20% topical benzocaine before decapitation. We preserved the head of each specimen in 4% paraformaldehyde (diluted in phosphate-buffered saline from 16% paraformaldehyde solution; Electron Microscopy Sciences, Hatfield, PA, USA) for 24 h and performed three 15 min rinses in phosphate buffered saline before storing cranial tissue in 70% ethanol.

### Micro-computed tomography scanning to compare ossification of bony ear structures

We used micro-computed tomography (micro-CT) to analyze the level of ossification of middle ear bones in 3–4 individuals per species spanning a range of body sizes. The Micro-CT Core Laboratory at the University of Texas Health Science Center scanned and reconstructed all samples. We secured toad skulls to the specimen stage using Parafilm (American National Can, Greenwich, CT, USA) and scanned the skulls in air in a high-resolution desktop micro-CT system (Skyscan 1173, Bruker Skyscan, Aartselaar, Belgium). Scan settings were: 60 kV, 133 µA beam intensity, a 0.7 deg, 4 frame averaging, and 1000 ms exposure time at each step. We used a 1.0 mm aluminium filter during scans (Kovács et al., 2009). We set pixel size to 10 µm with the exception of six very large specimens (two *R. marina*, two *R. haematiticus* and two *R. spinulosa*), which were scanned at 30 µm. We reconstructed images using NRecon (Bruker SkyScan) with a Feldkamp cone-beam algorithm (Feldkamp et al., 1984). We also used a polynomial correction to reduce beam-hardening effects during reconstructions (Kovács et al., 2009; Zou et al., 2011). We imported the bmp files from the reconstructions into Fiji (Schindelin et al., 2012) at a resolution of 60 µm and created 3D surface models for each skull at a consistent surface threshold of 50 using the 3D viewer plugin (Schmid et al., 2010). We calculated the length of visible, ossified columella using the measure tool in Meshlab (Visual Computing Lab ISTI-CNR 2015, http://meshlab.sourceforge.net/).

### Histology and 3D reconstruction to compare development of all ear structures

We decalcified two specimens per species (total $N$=10) in 10% EDTA (pH 7.4) for up to 1 week at room temperature and then put specimens through a graded ethanol series (30%, 50%, 70%, 90%, 95%, 100%, 100%), and embedded them in hydroxypropyl methacrylate (HPMA) plastic (Electron Microscopy Sciences, Hatfield, PA, USA). We drilled holes of 1 mm diameter into the plastic around each specimen before sectioning the tissue at 5 µm thickness using a microtome (RM1265, Leica, Wetzlar, Germany) and mounted every other section onto Fisher Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). We then stained the tissue with Eosin and Toluidine Blue (Fisher Scientific) and took photographs of every third section for a final resolution of 30 µm between imaged sections. We aligned the photographed sections using the drilled holes and then 3D modeled and measured ear structures within IMOD 3D (Kremer et al., 1996).

### Statistical analyses

To determine whether smaller, less developed individuals had hearing thresholds that were distinct from those of larger, adult individuals, we performed cluster analyses. We used Ward Hierarchical Clustering analysis in R to examine which individuals fell into each of the two designated clusters based on hearing thresholds. The number of clusters (two) was designated *a priori* based on a distinct split in hearing ability observed in the audiograms. We visualized audiograms representing sensitivity of animals in each hearing cluster within each species by graphing the thresholds from



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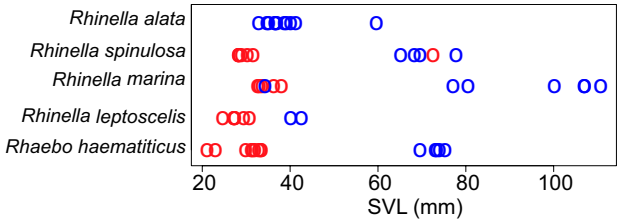


**Fig. 2. How species and size predict which individuals are assigned to the less (red) or more (blue) sensitive hearing clusters.** Each circle represents an individual used in the hearing cluster analysis, with species on the *y*-axis and body size (snout–vent length, SVL) on the *x*-axis.

the ABRs using the sme (smoothing-splines mixed-effects models) package (version 0.8, http://CRAN.R-project.org/package=sme) in R (http://www.R-project.org/).

To determine the frequencies at which our two hearing clusters differed in sensitivity, we used mixed models produced in the package lme4 (Bates et al., 2015) in R. We ran a model that had hearing threshold as the response variable, a hearing cluster by frequency interaction term as the fixed effect, and species and individual as random variables. We then calculated differences in least squares means of hearing thresholds between hearing clusters at all frequencies using the package lmerTest (R package version 2.0-29, http://CRAN.R-project.org/package=lmerTest). The least squares means gave us an estimate of the mean hearing threshold differences between hearing clusters at all frequencies.

## RESULTS

### ABRs

A hierarchical cluster analysis found that hearing thresholds distinctly changed across size/development within four out of our five species. In four of the five species (*R. haematiticus*, *R. leptoscelis*, *R. marina* and *R. spinulosa*), small individuals (<40 mm SVL) were included in the less sensitive cluster (higher hearing thresholds) and larger individuals (>40 mm SVL) were included in the more sensitive cluster (lower hearing thresholds), with two exceptions: one 34.3 mm *R. marina* clustered with the more sensitive hearing cluster, and one 72.4 mm *R. spinulosa* clustered with the less sensitive hearing cluster (Fig. 2). All individuals of *R. alata* clustered within the more sensitive hearing cluster, regardless of body size.

Hearing clusters differed in sensitivity at only a subset of frequencies. The less sensitive hearing cluster had 14–26 dB higher thresholds of acoustic responses than more sensitive conspecifics from 900 Hz to 2.5 kHz (Fig. 3A, Table 2). Fig. 3B shows within-species hearing differences between the less and more sensitive hearing clusters for *R. haematiticus*, *R. leptoscelis*, *R. marina* and *R. spinulosa*.

**Table 2. Estimated least squares means differences in hearing thresholds between the more and less sensitive hearing clusters**

| Frequency (Hz) | Hearing threshold differences (dB) |
|---|---|
| 200 | −1±3 |
| 300 | 3±3 |
| 400 | −2±3 |
| 500 | 1±3 |
| 700 | −4±3 |
| 900 | **−14±3*** ** |
| 1100 | **−22±3*** ** |
| 1300 | **−27±3*** ** |
| 1500 | **−26±3*** ** |
| 1750 | **−20±3*** ** |
| 2000 | **−15±3*** ** |
| 2250 | **−16±3*** ** |
| 2500 | **−14±3*** ** |
| 3000 | −4±3 |
| 35,000 | −2±3 |
| 4000 | 1±3 |

Estimated differences ±s.e.m. rounded to the nearest dB are given for each frequency, with significant differences between hearing stages in bold (***$P$<0.001). A negative estimated difference indicates that the more sensitive hearing cluster had a lower hearing threshold (better hearing ability) at that frequency; a positive estimated difference indicates the more sensitive hearing stage had a higher hearing threshold (worse hearing ability) at that frequency.

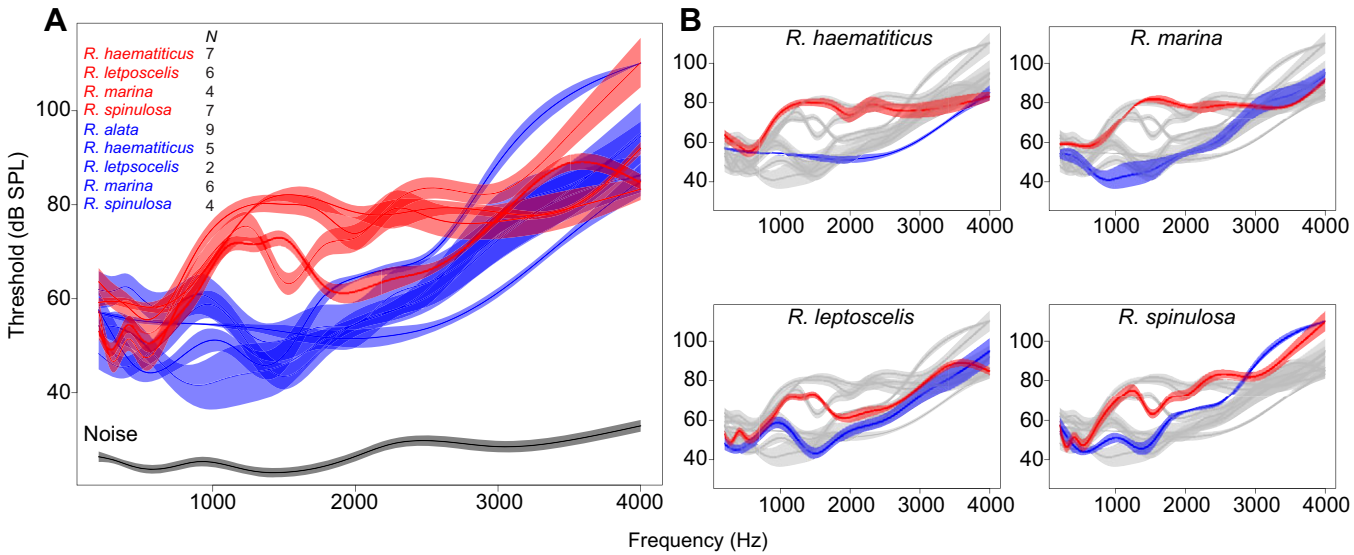


**Fig. 3. Hearing differences between the more and less sensitive hearing stages.** (A) Audiograms of less sensitive (red) and more sensitive (blue) hearing clusters. Separate lines within each stage represent different species. Within-chamber noise level is shown in black. (B) Audiograms of less sensitive (red) and more sensitive (blue) hearing stages within *R. haematiticus*, *R. leptoscelis*, *R. marina* and *R. spinulosa*. Each panel displays a species' audiogram separated by hearing stage. The separate hearing stage audiograms of the other three species are displayed in gray in each panel.

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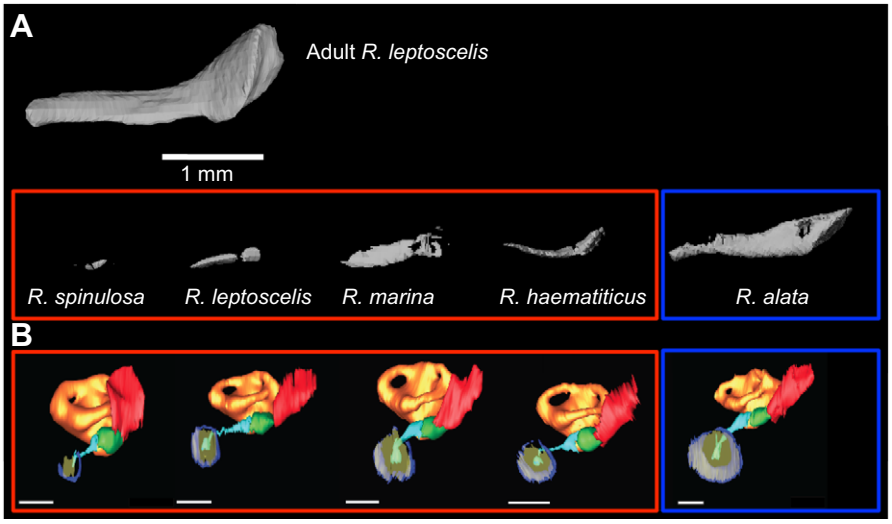


**Fig. 4. Micro-computed tomography (CT) and histology 3D reconstructions showing variation in development of tympanic middle ear structures across species and hearing cluster.** Hearing cluster assignment for each individual is indicated by the boxes (red for the less sensitive hearing cluster and blue for the more sensitive hearing cluster). (A) Micro-CT of one adult *R. leptoscelis* columella (SVL=42.4 mm) and an exemplar columella from similarly sized individuals of each species. All columellae are scaled to the 1 mm scale bar shown. Despite being similar in size (*R. spinulosa* SVL=28.7 mm, *R. leptoscelis* SVL=30.6 mm, *R. marina* SVL=32.7 mm, *R. haematiticus* SVL=29.9 mm, *R. alata* SVL=32.7 mm), different species show extreme variation in the amount of columellar ossification. (B) 3D reconstructions of ear structures showing variation in completion of the tympanic annuli (dark blue) and tympanic membranes (yellow) in similarly sized individuals of different species. All specimens examined had complete columellae (light blue), opercula (green), inner ears (orange) and opercularis muscles (red). Individuals are different between A and B, but species align vertically.

### Differences in tympanic middle ear structure development across species and size

To characterize the development of the ear structures, we used micro-CT scanning and histology. Because of limited sample size, we present qualitative observations of species differences in the relative rate of middle ear formation. Micro-CT scans revealed high variation in the ossification of the columellae (Fig. 4A). Histological analysis showed that despite incomplete columellar ossification, all columellae had completely formed from the footplate attachment at the inner ear to the extracolumella attachment at the tympanic membrane. Likewise, the inner ear and operculum were structurally complete in all specimens examined from the less sensitive hearing cluster ($N$=10, Fig. 4B). However, the tympanic annulus showed incomplete development in all examined *R. spinulosa*, *R. marina* and *R. haematiticus* juvenile specimens; the dorsal end of the tympanic annulus remained unconnected in these individuals (Fig. 4B). Species differed in the relative rate of middle ear structure development. For example, juvenile *R. leptoscelis* have minimally ossified columellae, but complete middle ear structures. In contrast, juvenile *R. haematiticus* and *R. marina* have more

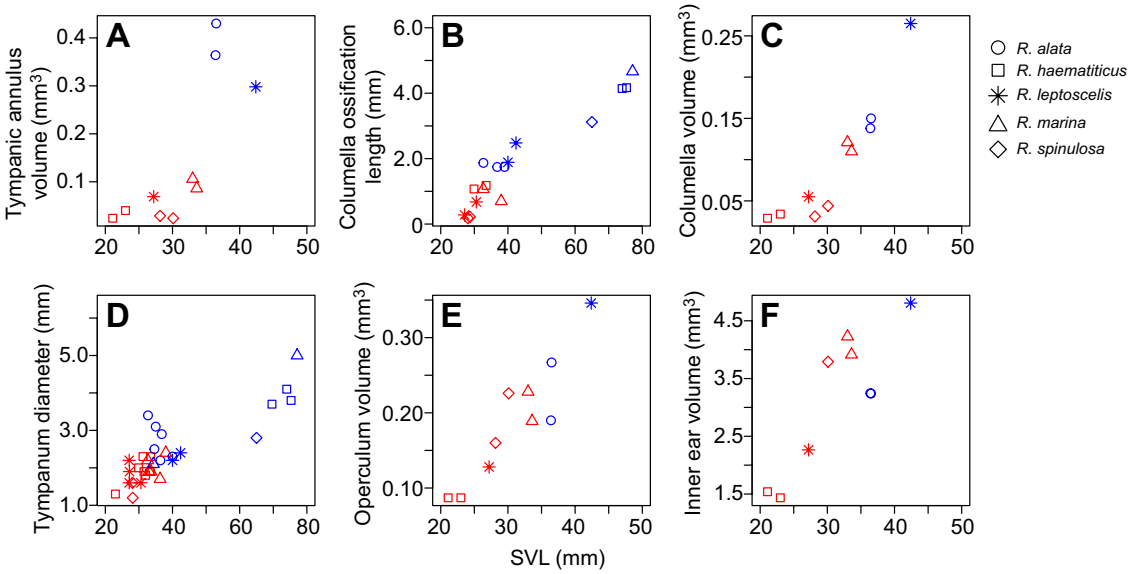


**Fig. 5. Differences in the size of hearing structures between animals in the more and less sensitive hearing clusters.** Species are indicated by symbol type and hearing cluster is indicated by color (red for the less sensitive hearing cluster and blue for the more sensitive hearing cluster). (A) Volume of an individual's tympanic annulus plotted against their SVL. (B) Length of the ossified portion of an individual's columella plotted against their SVL. (C) Volume of an individual's columella (ossified and cartilaginous portions) plotted against their SVL. (D) Diameter of an individual's tympanic membrane plotted against their SVL. (E) Volume of an individual's operculum plotted against their SVL. (F) Volume of an individual's inner ear plotted against their SVL.



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ossified columellae than juvenile *R. leptoscelis*, but incomplete tympanic annuli (Fig. 4B).

### Ear structure differences between hearing clusters

We quantified the size of middle ear structures as well as the length of ossified columellae and found three structural aspects of the ear that matched auditory threshold differences. Individuals in the more sensitive hearing cluster had thicker tympanic annuli, more ossified columellae and larger columellar volumes than less sensitive individuals (Fig. 5A–C). Individuals from the two hearing clusters overlapped in tympanic membrane diameter, operculum volume and inner ear volume (Fig. 5D–F).

## DISCUSSION

Our results support our hypothesis that protracted development of the ear confers a large hearing impairment late into juvenile development. We found a dramatic increase in hearing ability late in toad development. This increase in hearing ability was associated with two very late changes in tympanic middle ear structures: thickening of the tympanic annulus and ossification of the columella. Our results show that in toads with late-developing middle ear structures, the tympanic hearing system does not efficiently transfer sound until it has completely formed and ossified all relevant structures.

Our results are largely consistent with previous work showing that post-metamorphic development of the tympanic ear enhances sensitivity to sound, although the pattern of frequency responsiveness differs. Shofner and Feng (1981) and Boatright-Horowitz and Simmons (1995) detailed increases in overall hearing sensitivity as post-metamorphic *R. catesbeiana* (bullfrog) froglets increased in age and size. They also described a shift in the best-sensed frequencies from higher frequencies in metamorphs (~2500 Hz) to lower frequencies in adults (~1500–1700 Hz) (Shofner and Feng, 1981; Boatright-Horowitz and Simmons, 1995), which does not match our results. Our juvenile toadlets were most sensitive to low frequencies (<900 Hz). This low-frequency sensitivity remained relatively constant from juvenile to adult stages in our recordings as adults became more sensitive to frequencies between 900 and 2500 Hz. These different patterns in toads and bullfrogs likely reflect lineage differences in the developmental rate of ear structures as well as differences in adult tympanic ear function.

The novelty of our study was the ability to tease apart the formation and development of individual tympanic middle ear structures in five separate species ranging in body size, allowing us to draw strong conclusions about which ear development milestones most affect hearing ability. Previous studies on changes in post-metamorphic hearing in *Rana* did not characterize changes in the development of the tympanic hearing structures except for tympanic membrane size (Shofner and Feng, 1981; Boatright-Horowitz and Simmons, 1995). Compared with *Rana*, the toad species we examined have protracted development of the tympanic auditory system (Hetherington, 1987), which allowed us to disentangle the effects of individual structures. Individuals with thickened tympanic annuli and highly ossified columellae have much better hearing abilities than juveniles at earlier stages of tympanic ear development. Thus, we conclude that the thickening of the tympanic annulus and ossification of the columella are critical for tympanic ear functionality.

Although hearing above 1 kHz was greatly affected by development of the middle ear structures, low-frequency (<900 Hz) hearing does not rely on the tympanic hearing pathway. Previous studies have found that frequencies below 1 kHz are mainly sensed via extratympanic pathways (Wilczynski et al., 1987; Lombard and Straughan, 1974; Fox, 1995; reviewed in Christensen-Dalsgaard, 2005), and our results support this conclusion, given the lack of hearing differences among individuals with complete and incomplete tympanic hearing pathways. The opercularis pathway and the body wall overlying the lungs have both been shown to transmit sound frequencies below 1 kHz to the inner ear (Lindquist et al., 1998; Hetherington and Lindquist, 1999; Hetherington, 2001). Both the opercularis complex and the lungs and Eustachian tube were formed in all of our specimens and could be important for this similarity in low-frequency hearing between our juvenile and adult animals.

The late development of mid–high hearing sensitivity for acoustic communication may have fitness consequences, although natural history information is lacking for estimation of the costs and benefits of hearing conspecific calls, predator cues or prey sounds. All four species that displayed immature middle ear structures had poor hearing sensitivity in the 0.9–2.5 kHz range, which contains the dominant frequencies of many toad species' calls (Castellano et al., 2000; B. G. Pauly, Phylogenetic systematics, historical biogeography, and the evolution of vocalizations in nearctic toads (*Bufo*), PhD thesis, University of Texas at Austin, 2008; Guerra et al., 2011). Unfortunately, the importance of acoustic sensitivity for social communication in juvenile anurans remains unstudied, and we completely lack information about how auditory sensitivity impacts predator avoidance and prey capture, limiting our understanding of the performance and fitness consequences of late-developing hearing sensitivity.

This study provides an example of an unusually late-forming sensory system. The completion of the middle ear structures in bufonids drastically improves airborne hearing capabilities at frequencies that are vital to conspecific communication in many toad species. The ability of the tympanic hearing system to function in toads depends on columellar ossification as well as tympanic annulus volume, two very late-forming and understudied structures in the tympanic hearing system of anurans. We posit that relaxed selection on hearing sensitivity until sexual maturity may allow for the late development of these hearing structures in toads; however, more natural history studies are needed to determine the fitness consequences to juvenile anurans of reduced sensitivity above 1 kHz due to impaired detection and localization of prey, predators and conspecifics.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

M.C.W. and K.L.H contributed to all aspects of the project including experimental design, data collection, data analysis, and drafting and revising the article. J.C.-D. contributed to experimental design, data collection, drafting and revising the article.

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