Earless toads sense low frequencies but miss the high notes

Molly C. Womack1,1, Jakob Christensen-Dalsgaard2, Luis A. Coloma3,4, Juan C. Chaparro5,6 and Kim L. Hoke1

1Department of Biology, Colorado State University, Fort Collins, CO, USA
2Department of Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark
3Centro Jambatu de Investigación y Conservación de Anfibios, Fundación Otonga, Giovanni Farina 566 y Baltra, San Rafael, Quito, Ecuador
4Universidad Regional Amazónica Ikiam, Mayuya, Tena, Ecuador
5Museo de Biodiversidad del Peru, Cusco, Peru
6Museo de Historia Natural de la Universidad Nacional de San Antonio Abad del Cusco, Cusco, Peru

Sensory losses or reductions are frequently attributed to relaxed selection. However, anuran species have lost tympanic middle ears many times, despite anurans’ use of acoustic communication and the benefit of middle ears for hearing airborne sound. Here we determine whether pre-existing alternative sensory pathways enable anurans lacking tympanic middle ears (termed earless anurans) to hear airborne sound as well as eared species or to better sense vibrations in the environment. We used auditory brainstem recordings to compare hearing and vibrational sensitivity among 10 species (six eared, four earless) within the Neotropical true toad family (Bufonidae). We found that species lacking middle ears are less sensitive to high-frequency sounds, however, low-frequency hearing and vibrational sensitivity are equivalent between eared and earless species. Furthermore, extratympanic hearing sensitivity varies among earless species, highlighting potential species differences in extratympanic hearing mechanisms. We argue that ancestral bufonids may have sufficient extratympanic hearing and vibrational sensitivity such that earless lineages tolerated the loss of high frequency hearing sensitivity by adopting species-specific behavioural strategies to detect conspecifics, predators and prey.

1. Introduction

Evolutionary biologists typically attribute sensory loss or reduction to the colonization of an environment that relaxes selection on that sensory system [1,2] and/or to selection for another trait that inhibits development of sensory structures [3–8]. However, many species of frogs and toads have lost tympanic middle ears, a condition termed earlessness, without a consistent adaptive explanation. The tympanic middle ear functions in hearing airborne sound by transferring sound energy to fluid vibrations sensed by hair cells in the inner ear (reviewed in [8]). That similar tympanic hearing systems evolved independently in the major tetrapod lineages [8–11] signifies the importance of tympanic middle ears for hearing on land. Despite these benefits of tympanic hearing and fossil evidence that the middle ear evolved in the latest common ancestor of all extant anurans [12–16], at least 38 lineages within the order Anura secondarily lost middle ears [17]. These independently evolved (convergent) losses of middle ears are puzzling given that anurans use acoustic communication for mating, defense and territoriality (reviewed in [18]). Furthermore, earless species occupy diverse habitats, making it unlikely that earless species share common environmental selection pressures that relax selection on their tympanic middle ears [19–22]. Here we examine the hypothesis that ancestral alternative sensory pathways maintain hearing sensitivity in earless species, relaxing selection on the tympanic middle ear.
Extratympanic hearing pathways do not require a tympanic middle ear and provide some degree of hearing sensitivity in all anurans [23–30]. The extent to which extratympanic mechanisms preserve airborne hearing sensitivity in anurans lacking tympanic ears is debated. Experimentally removing the tympanum of two eared frog species, *Hyla regilla* (= *Hyla regilla*) and *Drosophytes versicolor* (= *Drosophytes versicolor*), decreased hearing sensitivity by 25 dB above 1 kHz [23]. Yet, closely related eared and earless species from the genus *Atelopus* differ in sensitivity by only 8–13 dB between 2000 and 2800 Hz [26], and similarly sized, but distantly related, *H. regilla* (eared) and *A. chiriquiensis* (earless) differ by only 5 dB in sensitivity [31]. The limited number of species tested makes it hard to generalize the extent to which ancestral extratympanic pathways transmit low and high frequency vibrations in earless anurans. Moreover, earless anurans may not all have equivalent extratympanic sensitivity. Lineage-specific hearing mechanisms may allow some species better mid-to-high frequency hearing sensitivity than others, however, few studies compare hearing among earless species [26].

In addition to preserved airborne hearing, earless anurans may have increased sensitivity to substrate vibrations through extratympanic pathways. Anurans are thought to sense substrate vibrations via the operculum, a cartilaginous disc in the oval window of the inner ear in amphibians [32–34]. Low-frequency auditory fibres are sensitive to both sound and substrate vibrations [30,53]. The middle ear and the operculum are in physical contact within the oval window of the inner ear and their movement is coupled [36]. Therefore, loss of the middle ear could allow freer movement of the operculum, increasing sensitivity to substrate vibrations in earless species. Earless species that call when very close or in direct contact with conspecifics [37,38] could communicate via substrate vibrations that travel through the ground or plants. However, no one has compared vibrational sensitivity between eared and earless species to determine whether earless species have enhanced vibrational sensitivity.

Here we determine whether alternative sensory pathways could enable conspecific communication and predator and prey detection in earless anurans by (1) uniformly preserving airborne hearing sensitivity; (2) providing lineage-specific hearing sensitivity; or (3) enhancing sensitivity to substrate vibrations. To address these hypotheses, we compared hearing and vibrational sensitivity among six eared and four earless species within the true toad family (*Bufoidae*). *Bufoidae* has the highest prevalence of ear transitions within a single family and may also have examples of ear regain, and moreover, enable comparisons of closely related species that differ in ear structures [17]. Our earless species completely lacked all middle ear structures, as verified histologically (electronic supplementary material, S1). The 10 species of bufonids used in this study encompass four independent ear loss or regain events [17] as well as a range of body sizes (figure 1). First, we compared the hearing sensitivities of eared and earless species to determine whether extratympanic hearing mechanisms buffer earless bufonids from hearing loss and hence relax selection on tympanic middle ears. Second, we considered hearing sensitivity differences among earless species to verify the existence of species-specific extratympanic hearing mechanisms. Third, we tested whether earless species have enhanced sensitivity to substrate vibrations compared with eared bufonids. Commonalities across independent transitions in ear structures inform us about sensory capabilities associated with middle ear lability in bufonids.

## 2. Material and methods

### (a) Animal collection

Ten species of bufonids (*Rhaebo haematiticus* *n* = 14, *Rhinella alata* *n* = 10, *Rhinella arborescendens* *n* = 9, *Rhinella festae* *n* = 4, *Rhinella leptoscelis* *n* = 10, *Rhinella horribilis* *n* = 10, *Rhinella spinulosa* *n* = 11, *Rhinella tacana* *n* = 5, *Rhinella yunga* *n* = 13 and *Osornophryne guacamayo* *n* = 9) were collected from field sites in Andes and Amazonian forests from Ecuador and Peru (electronic supplementary material, S2). We measured the snout-vent length (SVL) of each animal using a dial caliper (31–415–3, Swiss Precision Instruments Inc., Garden Grove, CA, USA).

### (b) Auditory brainstem recordings to test hearing and vibrational sensitivity

We tested the hearing and vibrational sensitivities of each species to measure sensitivity of animals to tones across a range of frequencies. All auditory brainstem recordings (ABRs) were performed at laboratories in Ecuador and Peru. We measured
sensitivity to airborne sound in four bufonid species as in Womack et al. [39] and used hearing sensitivity data from Womack et al. [39] for an additional six species (adult animals only, R. haematiticus, Rhinella alata, Rhinella leptoscelis, Rhinella horribilis, Rhinella spinulosa, Rhinella tacana). Briefly, we paralysed bufonids 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 and amplified (RA4PA, Tucker-Davis Technologies, Alachua, FL, USA) and measured the electrical signals generated by the auditory nerve (the VIIIth nerve). We broadcast acoustic stimuli to the bufonids using a three-inch speaker (FF85 K, Fostex, Tokyo, Japan) and a digital AC/DC amplifier (DTA-1 Class T, Dayton Audio, Springfield, OH, USA). We calibrated the amplifier using a 0.5 inch free-field microphone (46AE, G.R.A.S. Sound and Vibration A/S, Skovlytoften, Denmark) and a pistonphone (Type 42AA, G.R.A.S. Sound and Vibration A/S). We used customized software that controlled calibrated stimulus presentation and data acquisition using a mobile processor (RM2, Tucker-Davis Technologies). We presented 25-ms tone bursts at a rate of 25 Hz. Tone bursts ranged in frequency from 200 to 4000 Hz and were played in order from low to high frequency. We increased sound level in 5 dB increments and averaged response signals over 400 tone bursts. We measured background noise level within the testing chamber to verify that it was below the hearing thresholds of the animals and not interfering with the testing.

To test sensitivity to vibrational stimuli of all 10 bufonid species, we placed the bufonids on a small plastic platform connected to a mini-shaker (Type 4810, Bruel & Kjær Sound & Vibration A/S, Nærum, Denmark) within the audio chamber. We calibrated the mini-shaker with a charge accelerometer (Type 4527-C, Bruel & Kjær Sound & Vibration Measurement A/S) using customized software that controlled stimulus presentation and data acquisition using the RM2 processor. We stimulated the platform with 25-ms tone bursts, ranging in frequency from 200 to 900 Hz. Responses to each stimulus were averaged over 400 tone bursts presented at a 25 Hz rate. We measured responses to increasing amplitude tone bursts at each frequency in 5 dB increments and tested frequencies in order from low to high frequency.

During both the auditory and vibrational ABRs, we measured the response to a transient signal between every two frequencies tested. For the auditory tests, the transient was generated from a half cycle 4 kHz sinusoid at 105 dB re SPL, and for vibrational tests we used a half cycle 2 kHz sinusoid at ~30 dB re 1 m s⁻². We verified that the individual’s responsiveness to this signal remained stable throughout the testing session. If transient responses dropped below 25% of the original signal, we omitted all subsequent measurements from analyses (reducing datasets for nine of the 69 total individuals in the hearing study and six of the total 76 individuals in the vibrational study). 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.

### (c) Testing effects of middle ear presence on hearing and vibrational sensitivity

To effectively visualize hearing differences among numerous species with overlapping hearing thresholds, we graphed smoothed splines, which fit a smoothed curve to the estimated hearing thresholds, using the package smooth (40) in R [41]. To quantify the overall effect of ear presence/absence on hearing thresholds, we used linear mixed-effects models in the package lme4 [42]. We ran a model with hearing threshold as the dependent variable, ear (presence/absence), frequency (as a factor), and their interaction as fixed effects, and included species, by frequency interaction and individual as nested random effects (table 1: Model 1). We then used Type III tests of fixed effects to determine the effect of ear presence/absence on overall hearing sensitivity and on hearing sensitivity across frequency. Finally, to examine differences in hearing sensitivity between eared and earless bufonids at each frequency, we calculated least-squares means of the hearing thresholds for eared and earless species and their interaction as fixed effects, and examined patterns of sex differences among species despite the possibility that males and females may differ in hearing (as in [44–46]).

Because overall ABR signal amplitudes might influence the signal:noise ratio and hence hearing thresholds, we tested whether click response (the response to the transient signal played between every two frequencies tested) accounted for hearing sensitivity differences between eared and earless species. Therefore, we added click response level as an additional fixed effect to our hearing threshold model (table 1: Model 2). Type III fixed effects showed that the main effect of ear (presence/absence) was still significant (F(1,12) = 9.6, p = 0.009) as was the interaction between ear (presence/absence) and frequency (F(15,130) = 3.5, p < 0.001), consistent with our results from Model 1. We do not discuss this model in the main text or interpret least-squares means differences using this

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**Table 1.** Mixed models used in the likelihood-ratio testing.

| model no. | model (y ~ fixed effects + (1|random effects)) | model purpose |
|-----------|-----------------------------------------------|---------------|
| 1         | hearing thresholds ~ frequency + ear (Y/N) + frequency × ear + (1|species) + (1|species:frequency) + (1|individual) | eared versus earless airborne hearing sensitivity |
| 2         | hearing thresholds ~ frequency + ear (Y/N) + frequency × ear + click + (1|species) + (1|species:frequency) + (1|individual) | model 1 + control for click sensitivity |
| 3         | hearing thresholds ~ frequency + ear (Y/N) + frequency × ear + SVL + (1|species) + (1|species:frequency) + (1|individual) | model 1 + control for SVL |
| 4         | vibration thresholds ~ frequency + ear (Y/N) + frequency × ear + (1|species) + (1|species:frequency) + (1|individual) | eared versus earless vibration sensitivity |
| 5         | earless hearing thresholds ~ frequency × species + (1|individual) | interspecific differences in earless airborne hearing sensitivity |
| 6         | earless hearing thresholds ~ frequency × species + SVL + (1|individual) | model 5 + control for SVL |
model because click response may be confounded with overall hearing sensitivity.

Body size can also affect anuran hearing sensitivity [47,48], and the diversity of body sizes among eared and earless species in this study allowed us to verify that hearing sensitivity differences between eared and earless species were not being driven by differences in body size. We ran a model to control for the effects of body size on hearing sensitivity differences between eared and earless species by adding SVL as an additional fixed effect to our hearing threshold model (table 1: Model 3). Type III fixed effects showed that, when controlling for body size \((F_{1,41.1} = 8.5, p = 0.006)\), the main effect of ear (presence/absence) was still significant \((F_{1,122.5} = 8.0, p = 0.015)\) as was the interaction between ear (presence/absence) and frequency \((F_{15,128.4} = 3.4, p < 0.001)\), consistent with our results from Model 1. We do not interpret overall differences in hearing sensitivity between eared and earless species using this model because the species differ substantially in their ranges of body size (figure 1).

We used similar mixed-effects models to compare vibrational sensitivity between eared and earless species. We ran a model with vibrational thresholds as the dependent variable, ear (presence/absence), frequency (as a factor) and their interaction as fixed effects, including species, species by frequency interaction and individual as nested random effects (table 1: Model 4). We then calculated the effect of ear presence/absence on vibrational sensitivity thresholds using Type III tests of fixed effects. Because we found no differences between eared and earless species, we did not assess vibration sensitivity using models that controlled for body size or click response.

**Table 2.** Least-squares means differences between hearing sensitivity thresholds of eared and earless species. Least-squares means differences and standard error (s.e.) rounded to the nearest dB are given for each frequency with Tukey's honest significant differences between hearing stages italic. A negative difference indicates that the eared species had a lower threshold (were more sensitive) at that frequency while a positive difference indicates that earless species had a higher hearing threshold (were less sensitive) at that frequency.

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>Airborne sound sensitivity differences between eared and earless species, dB (±s.e.)</th>
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<tbody>
<tr>
<td>200</td>
<td>-2 (±6)</td>
</tr>
<tr>
<td>300</td>
<td>0 (±6)</td>
</tr>
<tr>
<td>400</td>
<td>-7 (±6)</td>
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<tr>
<td>500</td>
<td>-6 (±6)</td>
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<tr>
<td>700</td>
<td>-8 (±6)</td>
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<td>900</td>
<td>-12 (±6)</td>
</tr>
<tr>
<td>1100</td>
<td>-16 (±6)*</td>
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<tr>
<td>1300</td>
<td>-19 (±6)**</td>
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<tr>
<td>1500</td>
<td>-20 (±6)**</td>
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<tr>
<td>1750</td>
<td>-19 (±6)**</td>
</tr>
<tr>
<td>2000</td>
<td>-23 (±6)**</td>
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<tr>
<td>2250</td>
<td>-25 (±6)**</td>
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<tr>
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<td>-16 (±6)*</td>
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<tr>
<td>3500</td>
<td>-18 (±7)*</td>
</tr>
<tr>
<td>4000</td>
<td>-10 (±7)</td>
</tr>
</tbody>
</table>

* \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\).

3. Results

(a) **Hearing sensitivity differences between eared and earless species**

To determine whether earless species maintain sensitivity to airborne sound through extratympanic hearing pathways, we compared hearing sensitivity thresholds between eared and earless species. Overall, we found that earless species were less sensitive to airborne sound compared with eared species and that hearing differences between eared and earless species varied by frequency \((F_{15,859.6} = 12.3, p < 0.001); table 1: Model 1\). At and below 900 Hz and at 4000 Hz, eared and earless species had equivalent hearing, but between 1100 and 3500 Hz, earless species were 16–25 dB less sensitive than eared species (table 2; figure 2a).

(b) **Hearing sensitivity differences among earless species**

To determine whether earless species have species-specific extratympanic hearing sensitivity, we compared hearing among earless lineages. We found differences in hearing sensitivity among earless species that varied by frequency \((F_{15,439} = 4.9, p < 0.001); table 1: Model 5\). Most differences in hearing among earless species occurred at higher frequencies (above 1100 Hz; table 3). Figure 2b shows the variation in hearing sensitivity among earless species across the frequency range tested.

(c) **Vibrational sensitivity differences between eared and earless species**

To determine whether earless species had enhanced vibrational sensitivity, we compared sensitivity to substrate
vibrations between eared and earless species. We found no consistent differences in vibrational sensitivity ($F_{1,108.5} = 2.4, p = 0.121$; table 1: Model 4; figure 3). Furthermore, responsiveness to substrate vibrations did not vary between eared and earless species depending on frequency ($F_{5,52.5} = 1.4, p = 0.239$; table 1: Model 4; table 2).

4. Discussion

Overall, we found support that alternative sensory pathways provide sensitivity to low frequency sound in all earless species and facilitate species-specific sensitivity at high frequencies. Although earless species were generally less sensitive to sound at high frequencies (above 900 Hz), species varied in hearing sensitivity in ways that could affect conspecific communication. Additionally, earless species did not exhibit higher sensitivity to substrate vibrations than eared species. Below we discuss the implications of these results for proposed extratympanic hearing pathways and social communication in earless anurans.

(a) Extratympanic hearing may reduce stabilizing selection on middle ear structures in anurans

Earless bufonid species have a 16–25 dB hearing sensitivity reduction at high frequencies. Earless species show no reduction in hearing sensitivity at low frequencies ($\leq 900$ Hz), consistent with the idea that tympanic hearing is mainly important for anurans above 1 kHz and that extratympanic pathways play a larger role in hearing below 1 kHz [23].
Table 3. Least squares means differences among hearing sensitivity thresholds of earless species. All pairs of earless species with differences in least-squares means hearing thresholds are reported for each frequency. The species with less sensitive hearing is always to the left of the less-than symbol. Least-squares means differences and standard error (s.e.) rounded to the nearest dB are given for each pair.

<table>
<thead>
<tr>
<th>frequency (Hz)</th>
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<tbody>
<tr>
<td>200</td>
<td>Rhinella arborescandens &lt; O. guacamayo, 15 (± 4)</td>
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<tr>
<td>300</td>
<td>O. guacamayo &lt; R. yunga, 11 (± 4)</td>
</tr>
<tr>
<td>400</td>
<td>R. arborescandens &lt; R. yunga, 10 (± 4)</td>
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<tr>
<td>500</td>
<td>NA</td>
</tr>
<tr>
<td>700</td>
<td>NA</td>
</tr>
<tr>
<td>900</td>
<td>R. festae &lt; R. yunga, 21(± 5)</td>
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<tr>
<td>1100</td>
<td>NA</td>
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<tr>
<td>1300</td>
<td>O. guacamayo &lt; R. arborescandens, 15 (± 5)</td>
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<td>1500</td>
<td>O. guacamayo &lt; R. arborescandens, 20 (± 5)</td>
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<tr>
<td>1750</td>
<td>NA</td>
</tr>
<tr>
<td>2000</td>
<td>R. arborescandens &lt; R. yunga, 17 (± 4)</td>
</tr>
<tr>
<td>2250</td>
<td>O. guacamayo &lt; R. festae, 21 (± 7)</td>
</tr>
<tr>
<td>2500</td>
<td>R. arborescandens &lt; R. yunga, 13 (± 4)</td>
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<td>3500</td>
<td>NA</td>
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<td>4000</td>
<td>NA</td>
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</table>

The decreases in hearing sensitivity we describe are comparable to previous studies in anurans (discussed below) and not as dramatic as might be expected from the reduction in sensitivity in gekkonid lizards lacking middle ears (up to 62 dB) [49]. Equivalent measurements in other tetrapod groups have not been published to more broadly quantify the hearing sensitivity benefits of tympanic ear structures. As at least some vocalizations of earless bufonids have energy primarily above 900 Hz (see below), the lack of a tympanic middle ear would restrict the distance over which conspecifics acoustically communicate. Reduced hearing sensitivity might similarly limit predator or prey detection in earless lineages, although the auditory system’s contribution to these functions has not been examined in bufonids. Moreover, the moderate reduction in airborne hearing sensitivity we describe may not capture all of the sensory consequences associated with earlessness, as current models of sound localization in eared anurans require the middle ear cavity and Eustachian tubes [50]. We argue that extratympanic hearing does not completely compensate for hearing reduction associated with tympanic middle ear loss in anurans, but the moderate reduction in sensitivity compared with those in lizards prompt future study to test whether anurans have more effective extratympanic hearing pathways than other terrestrial vertebrates.

Although the magnitude of hearing loss associated with earlessness in our study (16–25 dB between 900 and 4000 Hz) is less dramatic than that measured in lizards, our results are generally consistent with previous estimates of extratympanic hearing sensitivity in other anurans. Lombard & Straughan [23] experimentally removed the tympanum in two hylid species and measured up to 25-dB reductions in sensitivity above 1 kHz [23]. Comparisons among species concluded that earless bufonids from the genus Atelopus were only 5 dB less sensitive than the eared Hylidae regilla (=Hyla regilla) [26] and 8–13 dB less sensitive than Atelopus species that have a columella but lack a tympanic membrane [31]. Although these previous estimates of hearing loss associated with earlessness within bufonids are smaller than our estimates, they are difficult to compare directly given differences in methodology and sampling design. That hylids [23] and bufonids (this study) both gain approximately 25 dB sensitivity from tympanic ears suggests that extratympanic hearing provides similar sensitivity to airborne sound in both groups. Our results thus provide no evidence that middle ears are especially labile in bufonids compared with other anurans because extratympanic hearing in this family is exceptional.

(b) Enhanced vibrational sensitivity unlikely to affect ear lability

Owing to the proximity of the operculum and columellar footplate in the oval window, loss of the middle ear could impact the free-motion of the operculum and increase sensitivity to low frequency sound sensed via vibration, either induced in the skull or in the substrate and transduced by opercular motion. Bufonids, like other anurans [30, 51–53] and salamanders [54–56], are at least as sensitive to substrate
vibrations as most other vertebrates [57–63]. Substrate vibrations may thus be an important sensory modality in all batrachians. Studies in a few species have characterized the use of substrate vibrations by adult anurans for communication and feeding (e.g. [64–66]), but more extensive analyses are necessary to draw conclusions about possible fitness consequences of vibrational sensitivity. We found that eared and earless bufonid species are equally sensitive to vibration, providing no support for the hypothesis that enhanced vibrational sensitivity compensated for the reduced airborne sensitivity in animals lacking tympanic middle ears.

(c) Diverse communication strategies may enable species to tolerate reduced hearing sensitivity

Earless bufonids may adopt different communication strategies to compensate for their reduced hearing sensitivity. Broadly speaking, species could either vocalize at frequencies to which they are most sensitive, or reserve acoustic communication for short-range interactions. Earless anurans might vocalize at lower frequencies at which their extratympanic hearing is effective, a strategy adopted by *R. yunga* (JL Stynoski, FA Trama, FL Rizo Patrón, E Tapia, KL Hoke, 2017, unpublished data). By contrast, species might communicate at short range, either employing substrate vibrations or airborne hearing. For example, *R. arborescandens* has been observed calling when close to conspecifics perched on ferns and other plants that would be excellent vibrational conduits (JC Chaparro and JL Stynoski 2016, personal communication). More natural history studies that characterize how airborne hearing and substrate vibrations function in conspecific communication, predator detection, and feeding across species. Alternative sensory pathways, acoustic environments and signalling behaviour may jointly create unique selection regimes within different clades that enabled the widespread convergent loss of what is a vital sensory structure to most terrestrial tetrapods.

5. Concluding remarks

Our results show that earless bufonids have reduced high-frequency hearing sensitivity but may rely on their sensitivity to low frequencies and/or species-specific extratympanic hearing mechanisms for conspecific communication and for predator and prey detection. These and other behavioural strategies could relax selection on tympanic middle ears within some lineages if ancestral, or compensate for ear loss if derived. To infer possible fitness consequences of reduced high-frequency hearing sensitivity in early earless ancestors, more natural history studies are needed to characterize broadly how extratympanic pathways for both airborne and substrate borne vibrations function in conspecific communication, predator detection, and feeding across species. Alternative sensory pathways, acoustic environments and signalling behaviour may jointly create unique selection regimes within different clades that enabled the widespread convergent loss of what is a vital sensory structure to most terrestrial tetrapods.

**Acknowledgements.** We thank Elicio E. Tapia (CJ), Peter Condori (MUBI) and Amanda Delgado (MHNC) who helped to obtain the wild-caught animals for this study. Special thanks to María Dolores Guarderas (CJ), Amanda Delgado and Florencia Trama (Center for Conservation and Sustainable Development, CDS, Oxapampa, Peru) for facilitating collection and transportation permits. We also thank Jennifer Stynoski for sharing her observations on conspecific communication used by earless species in this study.

**Figure 3.** Eared and earless species have equivalent vibrational sensitivity. Average eared (orange) and earless (blue) species’ vibrational thresholds are plotted as a function of frequency. The least-squares means thresholds are plotted with error bars showing 95% confidence intervals.
References


