SENSITIVE HIGH-FREQUENCY HEARING IN EARLESS AND PARTIALLY EARED HARLEQUIN FROGS (ATELOPUS)

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ABSTRACT
Harlequin frogs, genus Atelopus, communicate at high frequencies despite most species lacking a complete tympanic middle ear that facilitates high-frequency hearing in most anurans and other tetrapods. Here, we tested whether Atelopus are better at sensing high-frequency acoustic sound compared with other eared and earless species in the Bufonidae family, determined whether middle ear variation within Atelopus affects hearing sensitivity and tested potential hearing mechanisms in Atelopus. We determined that at high frequencies (2000–4000 Hz), Atelopus are 10–34 dB more sensitive than other earless bufonids but are relatively insensitive to mid-range frequencies (900–1500 Hz) compared with eared bufonids. Hearing among Atelopus species is fairly consistent, evidence that the partial middle ears present in a subset of Atelopus species do not convey a substantial hearing advantage. We further demonstrate that Atelopus hearing is probably not facilitated by vibration of the skin overlying the normal tympanic membrane region or the body lung wall, leaving the extratympanic hearing pathways in Atelopus enigmatic. Together, these results show Atelopus have sensitive high-frequency hearing without the aid of a tympanic middle ear and prompt further study of extratympanic hearing mechanisms in anurans.

KEY WORDS: Extratympanic hearing, Auditory brainstem recordings, Bufonidae, Lung hearing

INTRODUCTION
Most tetrapods have tympanic middle ears, which allow them to better sense their acoustic environment on land (Christensen-Dalsgaard and Carr, 2008; Manley, 2010; Manley and Sienknect, 2013); however, a minority of tetrapods rely on alternative methods for sensing acoustic stimuli (Hartline, 1971; Christensen et al., 2012; Mason and Narins, 2002; Wever, 1975). Yet, these alternative hearing methods are only efficient at low frequencies (Hartline, 1971; Christensen et al., 2012; Mason and Narins, 2002; Wever, 1975), and tetrapods without a tympanic middle ear that both communicate and have effective hearing above 1000 Hz are uncommon (Boistel et al., 2011). In this study, we investigated the high-frequency hearing sensitivity and potential hearing mechanisms in harlequin frogs, genus Atelopus, which lack a complete tympanic middle ear (Pereyra et al., 2016) but are known to communicate at high frequencies (1750–3780 Hz; Cocroft et al., 1990; Boistel et al., 2011).

Atelopus may be unique among bufonids in their ability to hear high frequencies (above 1500 Hz) without a middle ear. Earlessness, lack of all middle ear structures, has evolved at least 38 times in anurans (Pereyra et al., 2016), and is associated with a 16–25 dB decrease in hearing sensitivity above 1000 Hz in non-Atelopus bufonids (Womack et al., 2017). Likewise, two other anuran species show a 25 dB decrease in hearing sensitivity above 1000 Hz when the tympanic membrane is removed [Hyliola regilla (=Hyla regilla) and Dryophytes versicolor (=Hyla versicolor); Lombard and Straughan, 1974]. Yet, hearing tests on a limited number of Atelopus species show sensitivity to high-frequency sound above 1000 Hz. Atelopus chiriquiensis is only 5 dB less sensitive than the eared species H. regilla (Jaslow and Lombard, 1996) and three other Atelopus species {Atelopus flavescens, Atelopus sp. (Nusagandi) and Atelopus lozanoi {Atelopus sp. (Chingaza)} have sensitive hearing well above 1000 Hz (Lindquist et al., 1998). However, Atelopus hearing has not been compared with the hearing of closely related eared and earless species and the extratympanic hearing pathways used by earless Atelopus lack experimental verification.

Researchers have proposed several anuran extratympanic hearing pathways, but only one has been experimentally verified and also has the potential to affect high-frequency hearing: the lung pathway. This pathway, which transfers airborne sound waves that vibrate the body lung wall to the inner ear (Narins et al., 1988), mediates hearing sensitivity at frequencies up to 1000 Hz (Ehret et al., 1990; Hetherington and Lindquist, 1999), and the body lung wall of three Atelopus species {Atelopus flavescens, Atelopus sp. (Nusagandi) and A. lozanoi} were shown to vibrate at even higher frequencies (~2500 Hz) that relate to their species’ dominant call frequency (Lindquist et al., 1998). Thus, the lung pathway is a strong candidate for a potential extratympanic hearing mechanism in Atelopus (Lindquist et al., 1998; Boistel et al., 2011). However, no one has experimentally tested this pathway in any species that has high-frequency hearing sensitivity but lacks a tympanic middle ear.

In addition to effective extratympanic hearing pathways, some Atelopus species have a partial middle ear that may provide a high-frequency hearing benefit. Although all Atelopus lack a complete middle ear, a small clade of Atelopus species have either retained or regained a partial middle ear that has the middle ear bone and cavity but lacks a tympanic membrane (Lindquist et al., 1998; Boistel et al., 2011; Pereyra et al., 2016). These middle ear components may function relatively normally, with the skin overlying the attachment to the middle ear bone (herein referred to as the otic epidermis) vibrating in response to airborne sound and transferring those vibrations through the middle ear bone to the inner ear. Comparison of a single partially eared Atelopus species (A. flavescens) and two earless Atelopus species {Atelopus sp. (Nusagandi) and A. lozanoi} found the partially eared species was 8–13 dB more sensitive to
airborne sound from 2000 to 2500 Hz (Lindquist et al., 1998). High-frequency hearing in partially eared *Atelopus* may be mediated by this incomplete middle ear.

Here, we tested the hearing of three *Atelopus* species [*Atelopus elegans*, *Atelopus* sp. 1 (spumarius complex) and *Atelopus* sp. 2 (spumarius complex)] to assess their hearing sensitivity and to better understand mechanisms of hearing without a complete tympanic middle ear. First, we describe the ear structures of the three *Atelopus* species. Next, we assessed whether *Atelopus* hearing differs from hearing in other bufonids by comparing the hearing of each *Atelopus* species with previously reported hearing sensitivities of other eared and earless bufonids (Womack et al., 2017). We further assessed hearing differences within *Atelopus* associated with the presence of a partial middle ear by comparing hearing among two partially eared and one earless *Atelopus* species. Last, we aimed to identify the mechanisms of high-frequency hearing in *Atelopus* by manipulating two potential hearing pathways: the otic epidermis and the body lung wall. These studies provide broad hearing comparisons within the family Bufonidae, inform hypotheses of middle ear evolution within *Atelopus*, and test potential extratympanic hearing pathways in anurans.

**MATERIALS AND METHODS**

**Animal collection**

Adult animals were collected (earless *Atelopus elegans*, *n*=6; partially eared *Atelopus* sp. 1, *n*=9; partially eared *Atelopus* sp. 2, *n*=4; eared *Rhaebo haematiticus*, *n*=4; eared *Rhinella alata*, *n*=4; eared *Rhinella horribilis*, *n*=4; eared *Rhinella spinulosa*, *n*=4; eared *Rhinella tacana*, *n*=2) from field sites in Ecuador and Peru (Table 1), while an additional eight *Atelopus* sp. 2 individuals were bred in captivity at Centro Jambatu in Ecuador. We measured the mass of each individual animal to the nearest 0.1 mm (Table 1; individual SVLs available in Dataset 1) and measured the snout–vent length (SVL) of each animal to the nearest 0.01 g (individual masses available in Dataset 1) in Ecuador Playón de San Francisco (La Ceiba), Esmeraldas Province – Cope 1862 73.1  – (Wiegmann 1833) 77.1  – (Wiegmann 1834) 68.1 – 82.4 Peru Peru – K’iripampa Acopia in Acomayo, Departamento de Cusco 001-13 IC-FAU-DNB/MA 001-13 IC-FAU-DNB/MA 001-13 IC-FAU-DNB/MA 001-13 IC-FAU-DNB/MA 001-13 IC-FAU-DNB/MA 001-13 IC-FAU-DNB/MA 001-13 IC-FAU-DNB/MA 0071-2014-MINAGRI-DGFFS/DGEFFS 0071–2014-MINAGRI–DGFFS/DGEFFS

**Auditory brainstem recordings (ABRs) to test *Atelopus* hearing**

We tested the hearing ability of *Atelopus elegans* 

**Table 1. Snout–vent length (SVL), collection country, sites and permit numbers for animals in the study**

<table>
<thead>
<tr>
<th>Species (taxonomic authority)</th>
<th>SVL (mm)</th>
<th>Country</th>
<th>Region</th>
<th>Permit no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atelopus elegans</em> (Boulenger 1882)</td>
<td>38.2*</td>
<td>Ecuador</td>
<td>Provincia Esmeraldas, Río Durango, Durango</td>
<td>001-13 IC-FAU-DNB/MA</td>
</tr>
<tr>
<td><em>Atelopus</em> sp. 1 (spumarius complex)</td>
<td>24.4–25*</td>
<td>Ecuador</td>
<td>Provincia Pastaza, Reserva Otoyacu, Río Pucayacu</td>
<td>001-13 IC-FAU-DNB/MA</td>
</tr>
<tr>
<td><em>Atelopus</em> sp. 2 (spumarius complex)</td>
<td>26.6–35.7</td>
<td>Ecuador</td>
<td>Provincia Morona Santiago, San Carlos de Limón (Nueva Principal)</td>
<td>001-13 IC-FAU-DNB/MA</td>
</tr>
<tr>
<td><em>Rhaebo haematiticus</em> Cope 1862</td>
<td>73.1–75.3</td>
<td>Ecuador</td>
<td>Reserva Otokiki, Río Bailtazar, Esmeraldas Province</td>
<td>001-13 IC-FAU-DNB/MA</td>
</tr>
<tr>
<td><em>Rhinella alata</em> (Thominton 1884)</td>
<td>38.5–41.1</td>
<td>Ecuador</td>
<td>Playón de San Francisco (La Ceiba), Esmeraldas Province</td>
<td>001-13 IC-FAU-DNB/MA</td>
</tr>
<tr>
<td><em>Rhinella horribilis</em> (Wiegmann 1833)</td>
<td>77.1–107.0</td>
<td>Ecuador</td>
<td>Unión del Toachi (Chorrera del Diablo), Cotopaxi Province; and in San Francisco (La Ceiba), Esmeraldas Province</td>
<td>001-13 IC-FAU-DNB/MA</td>
</tr>
<tr>
<td><em>Rhinella spinulosa</em> (Wiegmann 1834)</td>
<td>68.1–77.6</td>
<td>Peru</td>
<td>K’iripampa Acopia in Acomayo, Departamento de Cusco</td>
<td>0071-2014-MINAGRI-DGFFS/DGEFFS</td>
</tr>
</tbody>
</table>

Asterisks in the SVL column indicate species that have missing data for a subset of individuals.

We performed additional ABRs on a subset of *Atelopus* sp. 2 individuals to test potential hearing pathways in a partially eared *Atelopus* species. All manipulation ABRs were recorded exactly as described above with the exception of four frequency omissions (200, 400, 1750 and 2250 Hz) to reduce test length. We chose not to
randomize the order of non-treatment ABRs and Vaseline treatment ABRs because it was not possible to completely remove the Vaseline without stressing the animal between tests. Most animals remained paralyzed throughout the full 1–3 h of testing (including non-manipulation and manipulation ABRs) and only received one dose of succinylcholine chloride. We gave subsequent half-doses of succinylcholine chloride to animals that showed slight movement. However, the click response was monitored throughout both tests to ensure that overall response levels did not drop during the Vaseline ABR or change after a subsequent dose of succinylcholine chloride.

With four Atelopus sp. 2 individuals, we tested whether vibration of the otic epidermis was contributing to hearing by covering the otic epidermis on both sides of the head with a thick layer of Vaseline. If vibration of these regions is important to hearing, then the Vaseline applied to these surfaces should affect hearing by weighing the otic epidermis down and affecting its ability to vibrate in response to sound waves. For comparison, we covered the tympanic membranes of four individuals per species of other bufonids (R. haematiticus, R. alata, R. horribilis and R. spinulosa) with a thick layer of Vaseline to test how this affected hearing of earless bufonids that rely on vibration of the tympanic membrane. These additional four species were tested in either Ecuador or Peru with the same experimental set-up and protocol as for the Atelopus sp. 2 individuals and their unmanipulated ABR results were published in Womack et al. (2017).

With three other Atelopus sp. 2 individuals, we tested whether vibration of the body lung wall was involved in the hearing of Atelopus sp. 2 by wrapping the body lung wall along with the complete mid-region of the frog’s body in a thick layer of Vaseline and cheesecloth. These manipulations are similar to those performed by Hetherington and Lindquist (1999), which resulted in decreased hearing sensitivity in Bombina orientalis.

Specimen fixation, histology and 3D reconstruction

After ABRs, we fixed two representatives from the three Atelopus species (A. elegans, Atelopus sp. 1 and Atelopus sp. 2) and compared their middle ear morphology with that of two representative individuals from a similarly sized bufonid with a complete tympanic middle ear, R. tacana. We killed two individuals of each species (total n=8) with 20% topical benzocaine, then decapitated the specimens, preserved the heads in 4% paraformaldehyde (diluted in phosphate-buffered saline from 16% paraformaldehyde solution; Electron Microscopy Sciences, Hatfield, PA, USA) for 24 h, performed three 15 min rinses in phosphate-buffered saline, and finally stored the cranial tissue in 70% ethanol.

We sliced the heads in half (sagittal) to isolate a single ear of each specimen and then decalcified the tissues in 10% EDTA (pH 7.4) for up to 1 week at room temperature. We then put the tissues through a graded ethanol series from 30% to 100%, and embedded them in hydroxypropyl methacrylate (HPMA) plastic (Electron Microscopy Sciences). We drilled holes of 1 mm diameter into the plastic around each tissue, sectioned through the ear structures at 5 µm thickness with a microtome (RM1265, Leica, Wetzlar, Germany), and mounted every other section onto Autofrost Adhesion Microscope Slides (Cancer Diagnostics, Inc., Durham, NC, USA). We then stained the tissue with Eosin and Toluidine Blue (Fisher Scientific, Pittsburgh, PA, USA) and photographed every third section with a dissection microscope (Olympus SZX10) and digital camera (Olympus DP71) for a final distance of 30 µm between imaged sections. We took images with a resolution of 1360 pixels×1024 pixels. We aligned the photographed sections using the drilled holes and then 3D modeled and measured ear structures within IMOD 3D (Kremer et al., 1996). For the 3D reconstruction figures (Fig. 1), we smoothed our reconstructions within IMOD 3D.

**Fig. 1. Middle ear variation within Atelopus.** 3D reconstructions from histology sections showing variation in middle ear structures among Atelopus species and an eared bufonid, Rhinella tacana. Atelopus elegans has no middle ear structures, while Atelopus sp. 1 and Atelopus sp. 2 both have all middle ear structures present (columella, Eustachian tube, middle ear cavity, tympanic annulus) except the tympanic membrane. The partially eared species, Atelopus sp. 1 and Atelopus sp. 2, have an incomplete tympanic annulus and an extended extracolumella, when compared with R. tacana. Inner ear, light blue; operculum, blue; columella, orange; Eustachian tube+middle ear cavity, yellow; tympanic annulus, red. Snout–vent length (SVL) of R. tacana, 28.4 mm; Atelopus sp. 1, 25.0 mm; Atelopus sp. 2, 32.0 mm; A. elegans, 26.0 mm. Scale bars: 1 mm.
Statistical analysis
We visualized audiograms representing hearing sensitivity of species by graphing the thresholds from the ABRs using the sme (smoothing-splines mixed-effects models) package (http://CRAN.R-project.org/package=sme) in R (http://www.R-project.org/). We used sme continuous graphs because comparing many species’ audiograms was difficult with other graphic representations because of the large number of overlapping data points. We then tested for hearing differences between species and groups of species using a mixed model produced in the package lme4 (Bates et al., 2014) and post hoc analyses using the package lsmeans (Lenth, 2016) and lmerTest (http://CRAN.R-project.org/package=lmerTest) in R (http://www.R-project.org/). Because we wanted to test overall species differences in hearing sensitivity, we did not examine sex differences. Even though males and females may differ in hearing (see Boatright-Horowitz and Simmons, 1995; Miranda and Wilczynski, 2009; Shen et al., 2011), we combined data from the two sexes because of the limited sample size.

First, we tested hearing differences among Atelopus species and other earless and eared bufonids using the previously published hearing data for bufonid species measured with identical methods (Womack et al., 2017). We ran a model that had hearing thresholds of all species as the response variable, species, frequency (as a factor) and their interaction as fixed effects, and individual as a random effect. For each model, we then calculated within-species contrasts to compare hearing of each species to the tympanic membrane, when compared with a non-Atelopus species with a complete middle ear, R. tacana (Fig. 1).

Additionally, both Atelopus sp. 1 and Atelopus sp. 2 both had all extracolumella, the most distal portion of the columella that attaches to the tympanic membrane, when compared with a non-Atelopus bufonid with a complete middle ear, R. tacana (Fig. 1).

Atelopus hearing compared with that of other bufonids
When comparing bufonid hearing thresholds, we found that thresholds differed among species and varied by frequency...
At high frequencies, all three Atelopus species were more sensitive than earless non-Atelopus bufonids (2000–4000 Hz; Fig. 2A, Table 2), and two Atelopus species (A. elegans and Atelopus sp. 1) were more sensitive than eared bufonids at select high frequencies (3000 and 4000 Hz; Fig. 2B, Table 2). All three Atelopus species were less sensitive than eared species from 900 to 1500 Hz, and individual Atelopus species were less sensitive than eared species at a number of other frequencies below 2500 Hz (Table 2).

**Testing effects of the partial middle ear on Atelopus hearing**

Overall, the earless A. elegans was more sensitive than the two partially eared Atelopus species (Fig. 2, Table 3). The earless A. elegans was more sensitive than the partially eared Atelopus sp. 2 at a range of frequencies (200, 300, 500–900 and 4000 Hz) and was more sensitive than the partially eared Atelopus sp. 1 at 1300 Hz (Fig. 2, Table 3). Only at one frequency (3000 Hz) was a partially eared species (Atelopus sp. 1) more sensitive than the earless A. elegans.

**Testing hearing mechanisms in Atelopus**

Covering the tympanic region with Vaseline affected hearing in ways that varied by species and frequency ($F_{41,390.79}=2.39$, $P<0.001$; Fig. 3A). Covering the otic epidermis of Atelopus sp. 2 did not decrease hearing sensitivity at any frequency (Fig. 3A, Table 4). In contrast, covering the tympanic membrane of bufonid species with complete tympanic ears resulted in a 9–29 dB decrease in hearing sensitivity at frequencies ranging from 700 to 3500 Hz (Fig. 3A, Table 4).

Covering the body lung wall with Vaseline did not result in any overall difference in hearing sensitivity for the partially eared Atelopus sp. 2 ($F_{1,65}=1.15$, $P=0.288$; Fig. 3B, Table 4), nor did the effects of the Vaseline lung treatment vary by frequency ($F_{11,65}=1.43$, $P=0.183$).

**DISCUSSION**

Atelopus species are rare examples of tetrapods lacking tympanic middle ears yet sensing and communicating with high-frequency airborne sound. We showed that Atelopus have better hearing than other earless bufonids at high frequencies; however, Atelopus still have reduced sensitivity at mid-range frequencies in comparison to...
provide an advantage for airborne sound sensitivity. We also found no evidence that the otic epidermis and the body lung wall function as extratympanic pathways transmitting sound waves to the inner ears of *Atelopus* sp. 2. We discuss our hearing sensitivity data in relation to previous hearing studies on *Atelopus* and other anurans, middle ear lability in *Atelopus*, and hypotheses of extratympanic pathways in *Atelopus* and other anurans.

**Atelopus hearing in comparison to that of other bufonids**

All *Atelopus* species were 10–31 dB more sensitive to high-frequency airborne sound than other earless bufonids. Even more interesting, this high-frequency hearing sensitivity matches the dominant call frequency of a partially eared species in this study (*Atelopus* sp. 2, d.f.=2250 Hz; Fig. S2), as well as other *Atelopus* species (Cocroft et al., 1990). Thus, even though all *Atelopus* species lack a tympanum and most completely lack a middle ear, they have maintained hearing sensitivity to high-frequency conspecific calls, despite reduced sensitivity at these high frequencies in other earless bufonids (Womack et al., 2017). This study is the first to test hearing differences between earless *Atelopus* and other earless anurans, but our results agree with previous studies on hearing using other *Atelopus* species, which found *Atelopus* were sensitive to high-frequency sound (Jaslow and Lombard, 1996; Lindquist et al., 1998; Boistel et al., 2011). These results all suggest that *Atelopus* have mechanisms for hearing high-frequency sound that other earless anurans lack.

However, these extratympanic hearing mechanisms in *Atelopus* do not seem to function very well at mid-range frequencies (900–1500 Hz). Despite the high-frequency hearing capabilities of *Atelopus*, their mid-range frequency hearing is consistently less sensitive compared with that of eared bufonids. Specializations for high-frequency hearing sensitivity in the extratympanic pathways or

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**Table 3. Estimated least squares means differences between hearing sensitivity thresholds of partially eared and earless *Atelopus* species**

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>Earless <em>A. elegans</em> versus partially eared <em>Atelopus</em> (dB)</th>
<th><em>Atelopus</em> sp. 2 versus <em>Atelopus</em> sp. 1 (dB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>3±5</td>
<td>−19±5***</td>
</tr>
<tr>
<td>300</td>
<td>0±5</td>
<td>−10±5**</td>
</tr>
<tr>
<td>400</td>
<td>1±5</td>
<td>−8±5</td>
</tr>
<tr>
<td>500</td>
<td>6±5</td>
<td>−5±5</td>
</tr>
<tr>
<td>700</td>
<td>10±5</td>
<td>−8±5</td>
</tr>
<tr>
<td>900</td>
<td>6±5</td>
<td>−6±5</td>
</tr>
<tr>
<td>1100</td>
<td>6±5</td>
<td>−2±5</td>
</tr>
<tr>
<td>1300</td>
<td>13±5*</td>
<td>8±5</td>
</tr>
<tr>
<td>1500</td>
<td>6±5</td>
<td>6±5</td>
</tr>
<tr>
<td>1750</td>
<td>8±5</td>
<td>12±5</td>
</tr>
<tr>
<td>2000</td>
<td>−3±5</td>
<td>−4±5</td>
</tr>
<tr>
<td>2250</td>
<td>−8±5</td>
<td>−8±5</td>
</tr>
<tr>
<td>2500</td>
<td>−8±5</td>
<td>−2±5</td>
</tr>
<tr>
<td>3000</td>
<td>−12±5*</td>
<td>−13±5*</td>
</tr>
<tr>
<td>3500</td>
<td>−7±5</td>
<td>−13±5*</td>
</tr>
<tr>
<td>4000</td>
<td>−9±5</td>
<td>−21±5***</td>
</tr>
</tbody>
</table>

Estimated least squares means (±s.e.) rounded to the nearest dB are given for airborne sound sensitivity differences at each frequency, with significant differences between groups in bold (*P<0.05, **P<0.01, ***P<0.001). A negative estimated difference indicates that the partially eared *Atelopus* species (*Atelopus* sp. 1 and 2) had a lower threshold (were more sensitive) at that frequency than the earless *Atelopus* species (*A. elegans*), while a positive estimated difference indicates that the partially eared *Atelopus* species had a higher hearing threshold (were less sensitive) at that frequency than the earless *Atelopus* species.
inner ear may be ineffective at sensing mid-range frequencies. Alternatively, as Atelopus species often breed near streams (Savage, 1972; Cocroft et al., 1990; Hödl and Amézquita, 2001), which create high levels of ambient noise, reaching maximum levels at lower frequencies (below 900 Hz; Hödl and Amézquita, 2001; Brumm and Slabbekoorn, 2005), stream noise may relax selection for hearing sensitivity at mid-range frequencies. In general, the hearing differences between Atelopus and the eared bufonids in this study were larger than those found by Jaslow and Lombard (1996), who found only a 5 dB hearing difference above 1000 Hz between the earless A. chiriquiensis and the eared H. regilla. Given Jaslow and Lombard’s (1996) results rely on the hearing sensitivity of a single Atelopus species and single eared non-bufonid, the discrepancy between our study and theirs could largely be due to their limited sampling. Thus, although Atelopus are able to hear high frequencies better than other earless bufonids, we found they have low sensitivity at mid-range frequencies, which could be due to the lack of a tympanic middle ear or to ambient stream noise and relaxed selection.

### Hearing in earless and partially eared Atelopus and its implications for middle ear evolution within Atelopus

Despite containing almost all the functionally relevant pieces of a tympanic middle ear, the partial middle ear found in some Atelopus species does not consistently benefit hearing sensitivity. Our manipulation experiment provides evidence that the partial middle ear of Atelopus does not function similarly to the tympanic middle ear of other anurans. Putting Vaseline over the otic epidermis did not appear to decrease hearing sensitivity in Atelopus sp. 2. It is therefore unlikely that this Atelopus with a partial middle ear relies on vibration of the tympanic region for transferring sound waves to the inner ear. Thus, Atelopus are capable of hearing via some other extratympanic pathway that is probably functioning in both earless and partially eared species.

The lack of difference in hearing sensitivity between our earless and partially eared Atelopus species conflicts with the 8–13 dB hearing advantage from 2000 to 2500 Hz found by Lindquist et al. (1998). Given that Lindquist et al. (1998) only compared one species with a partial middle ear (A. flavescens) with two earless species [Atelopus sp. (Nusagandi) and A. lozanoi] and we only compared one earless species with two species with a partial middle ear, conflict could be attributed to taxon sampling. Within our study, even partially eared species differed in hearing, so selecting small numbers of species in each study could lead to ambiguities in estimating any hearing advantages of partial middle ears. Furthermore, the ring of cartilage that normally surrounds the tympanic membrane (the tympanic annulus) was incomplete in both partially eared species in this study, and an incomplete tympanic annulus is associated with non-functional tympanic middle ears in developing bufonids (Womack et al., 2016). Whether it is due to the incomplete tympanic annulus or the lack of tympanic membrane, the partial middle ear in Atelopus appears to convey little to no hearing sensitivity benefits; thus, its presence is perplexing and requires further research.

To interpret the species differences in hearing within Atelopus, we need more extensive sampling among numerous species in a phylogenetic context to infer evolutionary shifts in acoustic sensitivity, vibrational sensitivity and sound localization. Partial middle ears may enhance sensitivity to substrate-borne vibration, or the coupling of the middle ears via the middle ear cavities and Eustachian tubes may provide sound localization benefits. Although sound localization was not explored in this study, sensitivity to vibration was not enhanced in our partially eared Atelopus species (M.C.W., unpublished data). To relate those patterns to selection, we need natural history data that characterize communication strategies across the same set of species.

### Extratympanic hearing mechanisms for Atelopus

Our manipulative experiments suggest that vibration of the body lung wall is not an important extratympanic pathway in Atelopus. This is surprising given the body lung wall has been shown to vibrate in response to frequencies around 2500 Hz in other Atelopus species (Lindquist et al., 1998), and covering the body lung wall of Bombina orientalis with silicon grease resulted in a 20–25 dB decrease of hearing sensitivity at all frequencies tested in that study (100–1000 Hz; Hetherington and Lindquist, 1999). Despite being one of the most discussed and experimentally investigated extratympanic hearing pathways in anurans (Narins et al., 1988; Ehret et al., 1990, 1994; Hetherington, 1992; Hetherington and Lindquist, 1999; Mason, 2006; Boistel et al., 2013), the lung pathway does not appear to contribute to high-frequency hearing sensitivity in Atelopus via vibration of the body lung wall.

Other extratympanic pathways could contribute to the high-frequency hearing of Atelopus. Boistel et al. (2013) proposed bone
Conduction enhanced by resonance of the oral cavity to explain high-frequency communication in the earless Sechellophryne gardineri. However, the effectiveness of this pathway at high frequencies has not yet been tested experimentally. Thus, the mechanisms of high-frequency hearing in Atelopus and other anurans without a middle ear remain unverified.

Concluding remarks

Atelopus species are sensitive to high-frequency airborne sound despite lacking a tympanic middle ear. The mechanisms of Atelopus high-frequency hearing remain unclear, but vibration of the body lung wall probably does not contribute. Additionally, the partial middle ear found in some Atelopus species does not convey a strong hearing advantage, making it unlikely that direct selection pressures for increased hearing sensitivity are acting to retain or regain middle ear structures within Atelopus. Future research into extratympanic hearing mechanisms in Atelopus is needed to fully understand those mechanisms within anurans more generally and their influence on middle ear evolution.

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Competing interests

The authors declare no competing or financial interests.

Author contributions


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Supplementary information

Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.169664.supplemental

References

Fig. S1. A diagram of the electrode (red) placement for auditory brainstem recordings (ABRs). We subdermally placed differential electrodes (red) over the midbrain and VIIIth (auditory) nerve and placed a third ground electrode (orange) within the arm contralateral to the VIIIth nerve being measured. Tympanic membranes are shown in blue.
Median dominant frequency: 2400 Hz
Fig. S2. *Atelopus* sp. 2 (*spumarius* complex) call collection & analysis.

The call of *Atelopus* sp. 2 was analyzed for this study. Elicio E. Tapia recorded the call on July 6\textsuperscript{th} 2016 at 9:00 H and 10:00 H with a digital recorder (Olympus Linear PCM Recorder LS 10S) and microphone (Sennheisser). These recording was done (under lab conditions) of a male born in captivity from parents from San Carlos de Limón, Morona Santiago Province, Ecuador. The frog was inside a terrarium 40x40 cm partially opened in the upper cover with other males in the terrarium. The microphone was at about 5 cm from the frog. Atmospheric pressure was about 730.78 mb, the altitude of Centro Jambatu lab is 2700 m asl, and the frog’s temperature was about 20 °C.

The call was analyzed using the package seewave (Sueur et al., 2008) in R (R Core Team, 2015). The call was visualized with spectrograms and then trimmed to the time frame of the call. We eliminated background noise at frequencies above and below the call using a single band pass frequency filter (window length = 1024, window = "hanning", overlap = 75). From these trimmed call files, we calculated the median dominant frequency by performing an instantaneous frequency extraction by zero crossing.

(A) A spectrogram (top) and oscillogram (bottom) of the advertisement call of *Atelopus* sp. 2 with median dominant frequency given. For spectrogram - window length = 512, window = "hanning", overlap = 0.

(B) A spectrogram of one note within the advertisement call of *Atelopus* sp. 2 shown in A. For spectrogram - window length = 512, window = "hanning", overlap = 0.