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 test whether *Atelopus* are better at sensing high frequency acoustic sound compared to other eared and earless species in the Bufonidae family, determine whether middle ear variation within *Atelopus* affects hearing sensitivity, and test potential hearing mechanisms in *Atelopus*. We determine 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 to 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 not likely 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.

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 Sienknecht, 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). Here we investigate the high frequency hearing sensitivity and potential hearing mechanisms in harlequin frogs, genus *Atelopus*, which lack a complete tympanic middle ear (Pereyra, Womack 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, Womack 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 *Hyliola 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 to 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 both experimentally verified and has the potential to affect high frequency hearing. The lung 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 (*A. 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, Womack 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 test 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 assess whether *Atelopus* hearing differs from hearing in other bufonids by comparing the hearing of each *Atelopus* species to previously reported hearing sensitivities of other eared and earless bufonids (Womack et al., 2017). We further assess hearing differences within *Atelopus* associated with presence of a partial middle ear by comparing hearing among two partially eared and one earless *Atelopus* species. Last, we aim 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, *Rhinella tacana* n = 2) from field sites in Ecuador and Peru (Table 1), while additional eight *Atelopus* sp. 2 individuals were bred in captivity at Centro Jambatu in Ecuador. We measured the weight of each individual animal to the nearest 0.01 g (individual weights available in Supplemental Information 1) using a digital pocket scale (EHA701, Camry Industries Company Ltd., Guangdong, China) and measured the snout-vent length (SVL) of each animal to the nearest 0.1 mm (Table 1; individual SVLs available in Supplemental Information 1) using a dial caliper (31-415-3, Swiss Precision Instruments Inc., Garden Grove, CA, U.S.A). The Institutional Animal Care and Use Committee at Colorado State University approved all experiments (IACUC Protocol #12-3484A), and the Ministerio del Ambiente in Ecuador and the Servicio Nacional Forestal y de Fauna Silvestre in Peru approved collection, breeding, research, and export permits (Table 1).

Auditory Brainstem Recordings (ABRs) to test *Atelopus* hearing

We tested the hearing abilities of *Atelopus elegans* (n = 6), *Atelopus* sp. 1 (n = 9), and *Atelopus* sp. 2 (n = 4) in Ecuador and Peru using the same auditory brainstem recording methods detailed in Womack et al. (2016). Briefly, we paralyzed the bufonids with 0.05% succinylcholine chloride (Sigma-Aldrich, St. Louis, MO, U.S.A.) at a dosage of 7.5 µl/gram and then lightly anesthetized animals with a small topical application of 5% benzocaine at the sites of electrode placement. The topical application of 5% benzocaine should have brief, localized effects and wear off shortly after electrode placement. Most animals remained paralyzed throughout the 1-3 hours of testing and only received one dose of succinylcholine chloride, however, individuals that showed slight movement (active breathing) during the testing were given subsequent half-doses. We subdermally placed differential electrodes over the midbrain and VIIIth (auditory) nerve and placed a third ground within the arm contralateral to the VIIIth nerve (Supplemental Figure 2.1) to measure electrical signal generated by the VIIIth nerve. We linked the three electrodes to a pre-amplifier (RA4PA, Tucker-Davis Technologies, Alachua, FL, U.S.A.) 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, U.S.A.). We placed bufonids on a wet paper towel and positioned them perpendicular to and 46 cm away from a suspended speaker. We calibrated speaker output with a ½ inch free field microphone (46AE, G.R.A.S. Sound and Vibration A/S, Skovlytoften, Denmark).

We calibrated the experimental setup using customized software (QuickABR_burst) that controlled stimulus presentation and data acquisition using the mobile processor (RM2 Info). We played 25 ms pure tones, ranging in frequency from 200–4000 Hz (Table 2) at 5 dB increments. We averaged response signals over 400 tone bursts and measured the response to a transient generated from a half cycle 4000 Hz sinusoid at 105 dB SPL between every two frequencies to ensure that the auditory responsiveness remained stable throughout the testing session. If transient response dropped below 25% of the original signal, we omitted all subsequent measurements of that individual from analyses. We visually determined thresholds for each frequency, using the minimum stimulus decibel level that evoked a response signal amplitude of 0.002 mV (roughly twice the noise level) or greater from the auditory nerve.

Vaseline manipulations to test hearing pathways in *Atelopus*

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 Hz, 400 Hz, 1750 Hz, 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 hours 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 (*Rhaebo haematiticus, Rhinella alata, R. horribilis,* and *R. spinulosa*) with a thick layer of Vaseline to test how this affected hearing of eared 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 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 two representative individuals from a similarly sized bufonid with a complete tympanic middle ear, *R. tacana.* We euthanized two individuals of each species (total n = 8) with 20% topical benzocaine. We then decapitated the specimens, preserved the heads in 4% paraformaldehyde (diluted in Phosphate Buffered Saline from 16% paraformaldehyde solution; Electron Microscopy Sciences, Hatfield, PA, U.S.A.) for 24 hours, performed three 15-minute 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 one week at room temperature. We then put the tissue through a graded ethanol series from 30% to 100%, and embedded them in hydroxypropyl methacrylate (HPMA) plastic (Electron Microscopy Sciences, Hatfield, PA, U.S.A.). 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, U.S.A.). We then stained the tissue with Eosin and Toluidine Blue (Fisher Scientific, Pittsburgh, PA, U.S.A.) and photographed every third section with a dissection scope (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 by 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 (Figure 1), we smoothed our reconstructions within IMOD 3D.

Statistical Analysis

We visualized audiograms representing hearing sensitivity of species by graphing the thresholds from the auditory brainstem recordings using the sme (Smoothing-splines Mixed-effects models) package (Berk, 2013) in R (R Core Team, 2015). We used sme continuous graphs because comparing many species' audiograms was difficult with other graphic representations due to 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 posthoc analyses using the package lsmeans (Lenth, 2016) and lmerTest (Kuznetsova et al., 2015) in R (R Core Team, 2015). 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 (as in Boatright-Horowitz and Simmons, 1995; Miranda and Wilczynski, 2009; Shen et al., 2011), we combined data from both sexes due to limited sample size.

First, we tested hearing differences among *Atelopus* species and other eared and earless 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 random effects. We ran posthoc contrasts to compare hearing of each *Atelopus* species to both the average hearing sensitivity of other earless bufonids and the average hearing sensitivity of other eared bufonids. The least squares means gave us an estimate of the mean hearing threshold for each *Atelopus* species, all other earless bufonids, and all eared bufonids at each frequency. We compared differences in those hearing thresholds between groups and adjusted p-values using Sidak's method.

Next, we tested whether *Atelopus* with a partial middle ear were more sensitive than earless *Atelopus* species by comparing hearing among our *Atelopus* species. Using the mixed model above, we estimated the least squares means hearing thresholds for each of the three *Atelopus* species. We then ran pairwise comparisons of those least squares means hearing thresholds for each *Atelopus* species at each frequency and adjusted p-values using Tukey's method.

Last, we determined the frequencies at which individuals with and without Vaseline treatments differed in sensitivity, using only hearing data from individuals that were tested both with and without the Vaseline treatment. We ran one model that had hearing threshold as the response variable, head Vaseline treatment (yes or no), frequency, species, and their three-way interaction term as the fixed effects, and individual as a random variable. We then ran a second model that had hearing threshold as the response variable, body lung wall Vaseline treatment (yes or no) frequency, species, and their three-way interaction term as the fixed effects, and individual as a random variable. For each model, we then calculated within-species differences in least squares means of individuals' hearing thresholds with and without Vaseline treatment at all frequencies. The least squares means gave us an estimate of the mean hearing threshold for each species with and without the Vaseline treatment and compared within-species differences in those hearing thresholds. All posthoc comparisons were adjusted using Tukey's method for multiple comparisons.

RESULTS

Description of *Atelopus* middle ear structures

We found no evidence of tympanic middle ear structures in *A. elegans*, while *Atelopus* sp. 1 and *Atelopus* sp. 2 both had all middle ear structures present (columella, Eustachian tube, middle ear cavity, tympanic annulus) except the tympanic membrane. However, both *Atelopus* sp. 1 and *Atelopus* sp. 2 had an incomplete tympanic annulus in which only the ventral half is present (Figure 1). Additionally, both *Atelopus* sp. 1 and *Atelopus* sp. 2 had an extended 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* (Figure 1).

Atelopus hearing compared to other bufonids

When comparing bufonid hearing thresholds among frequencies, hearing thresholds differed among species and those threshold differences varied by frequency ($F_{179,1119.09} = 9.41$, $p < 0.001$; Figure 2). At high frequencies, all three *Atelopus* species were more sensitive than earless non-*Atelopus* bufonids (2000–4000 Hz; Figure 2A,

Table 2), and two *Atelopus* species were more sensitive than eared bufonids at select high frequencies (3000 and 4000 Hz; Figure 2B, Table 2). Meanwhile, at low-mid range frequencies, *Atelopus* were generally less sensitive than eared bufonids (Figure 2B, Table 2). All three *Atelopus* species were less sensitive than eared species from 900–1500 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 *Atelopus elegans* was more sensitive than the two partially eared *Atelopus* species (Figure 2; Table 3). The earless *A. elegans* was more sensitive than the partially eared *Atelopus* sp. 2 at a range of frequencies (200 Hz, 300 Hz, 500 Hz–900 Hz, and 4000 Hz) and was more sensitive than the partially eared *Atelopus* sp. 1 at 1300 Hz (Figure 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 ($F_{41,390.79} = 2.39$, $p < 0.001$; Figure 3A). Covering the otic epidermis of *Atelopus* sp. 2 did not decrease hearing sensitivity at any frequency (Figure 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 (Figure 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 ($F_{1,65} = 1.15$, $p = 0.288$; Figure 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 *Atelopus* have better hearing than other earless bufonids at high frequencies, however *Atelopus* still have reduced sensitivity at mid-range frequencies in comparison to eared bufonids. We found no consistent differences in hearing between our partially eared and earless *Atelopus* species, indicating the partial middle ear found in a small clade of *Atelopus* does not provide an advantage for airborne sound sensitivity. We also found no evidence that the otic epidermis or 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 other bufonids

All *Atelopus* species were 10–31 dB more sensitive to high frequency airborne sound than earless bufonids. Even more interesting, this high frequency hearing sensitivity matches the dominant call frequency a two partially eared species in this study (*Atelopus* sp. 2, df = 2250 Hz – Supplemental Information 3), as well as other *Atelopus* species (Cocroft et al., 1990). Thus, despite all species lacking a tympanum and most species completely lacking a middle ear, *Atelopus* have maintained hearing sensitivity to high frequency conspecifics 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 sensitivity 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 frequencies 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*, mid-range frequency hearing is consistently less sensitive in *Atelopus* species compared to eared bufonids. Specializations for high frequency hearing sensitivity in the extratympanic pathways or inner ear may be ineffective at sensing mid-range frequencies. Alternatively, *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). Thus, 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 are larger than those found by Jaslow and Lombard (1996), who found only a 5 dB hearing difference above 1000 Hz between the earless *Atelopus chiriquiensis* and the eared *Hyliola 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 find they have low sensitivity at mid-range frequencies, which could due to lack of a tympanic middle ear or 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 does 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 likely functioning in both earless and partially eared species.

The lack of difference in hearing sensitivity we find between our earless and partially eared *Atelopus* species conflicts with the 8–13 dB hearing advantage from 2000–2500 Hz found by Lindquist et al. (1998). Given that Lindquist et al. (1998) only compared one species with a partial middle ear (*A. flavescens*) to two earless species (*Atelopus* sp. (Nusagandi), and *A. lozanoi*) and we only compared one earless species to two species with a partial middle ear, conflict could be attributed to taxon sampling. Within our study, even partially eared species differ 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 surround 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 be 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 is not enhanced in our partially eared *Atelopus* species (Womack 2017, 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 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 frequency have 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 does not likely 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* are needed to fully understand those mechanisms within anurans more generally and their influence on middle ear evolution.

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COMPETING INTERESTS

We declare we have no competing 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. L.A.C. contributed to animal rearing, data collection, and drafting and revising the article.

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DATA AVAILABILITY

The data for this study is available in Supplementary Information 1.

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Tables

Table 1 – Snout-vent length (SVL), collection country, sites, and permit numbers for animals in the study. Asterisks in the SVL column indicate species that have missing data on a subset of individuals.

Species	SVL (mm)	Country	Region	Permit #
Atelopus elegans (Boulenger, 1882)	38.2*	Ecuador	Ecuador, Provincia Esmeraldas, Río Durango, Durango	001-13 IC-FAU-DNB/MA
Atelopus sp. 1 (*spumarius* complex)	24.4—25*	Ecuador	Ecuador, Provincia Pastaza, Reserva Otoyacu, Río Pucayacu	001-13 IC-FAU-DNB/MA
Atelopus sp. 2 (*spumarius* complex)	26.6—35.7	Ecuador	Ecuador, Provincia Morona Santiago, San Carlos de Limón (Nueva Principal)	001-13 IC-FAU-DNB/MA
Rhaebo haematiticus (Cope, 1862)	73.1—75.3	Ecuador	Reserva Otokiki, Río Baltazar, Esmeraldas Province	001-13 IC-FAU-DNB/MA
Rhinella alata (Thominot, 1884)	38.5—41.1	Ecuador	Playón de San Francisco (La Ceiba), Esmeraldas Province	001-13 IC-FAU-DNB/MA
Rhinella horribilis (Linnaeus, 1758)	77.1—107.0	Ecuador	Unión del Toachi (Chorrera del Diablo), Cotopaxi Province; and in San Francisco (La Ceiba), Esmeraldas Province	001-13 IC-FAU-DNB/MA
Rhinella spinulosa (Wiegmann, 1834)	68.1—77.6	Peru	K'iripampa Acopia in Acomayo, Departamento de Cusco	0071-2014-MINAGRI-DGFFS/DGEFFS
Rhinella tacana (Padial et al. 2006)	28.4—30.4	Peru	Quincemil, Departamento de Cusco	Permit: 0071–2014–MINAGRI–DGFFS/DGEFFS

Table 2 – Estimated least squares means differences between hearing sensitivity thresholds of *Atelopus* species and other eared and earless bufonids. Estimated least squares means differences and standard error rounded to the nearest dB are given for each frequency with significant differences between groups bolded and indicated with asterisks (p<.05=*, p<.01=**, p<.001=***). Negative estimated difference indicates that the *Atelopus* species had a lower threshold (were more sensitive) at that frequency than our eared or earless bufonids, while a positive estimated difference indicates that the *Atelopus* species had a higher hearing threshold (were less sensitive) at that frequency than other eared or earless bufonids.

Frequency (Hz)	**Airborne sound sensitivity differences between *Atelopus* and other eared bufonids dB (±SE)**			**Airborne sound sensitivity differences between *Atelopus* and other earless bufonids dB (±SE)**		
	Atelopus elegans	***Atelopus* sp. 1**	***Atelopus* sp. 2**	***A. elegans***	***Atelopus* sp. 1**	***Atelopus* sp. 2**
200 Hz	**-11 (±4)***	-8 (±4)	**11 (±3)****	**-13 (±4)****	-10 (±4)	**9 (±3)***
300 Hz	0 (±4)	-1 (±4)	**10 (±3)****	-1 (±4)	-1 (±4)	**9 (±3)***
400 Hz	8 (±4)	9 (±4)	**16 (±3)*****	0 (±4)	1 (±5)	**9 (±3)***
500 Hz	4 (±4)	10 (±4)	**15 (±3)*****	-3 (±4)	4 (±5)	**9 (±3)***
700 Hz	4 (±4)	**14 (±4)****	**21 (±3)*****	-4 (±4)	6 (±5)	**13 (±3)*****
900 Hz	**9 (±4)***	**16 (±4)****	**22 (±3)*****	-3 (±4)	3 (±5)	**10 (±3)***
1100 Hz	**9 (±4)***	**15 (±4)****	**17 (±4)*****	-7 (±4)	-1 (±5)	1 (±4)
1300 Hz	**10 (±4)***	**23 (±4)*****	**15 (±3)*****	-9 (±4)	3 (±5)	-4 (±4)
1500 Hz	**12 (±4)****	**19 (±4)*****	**12 (±3)*****	-8 (±4)	-1 (±5)	-8 (±3)
1750 Hz	8 (±4)	**16 (±4)*****	5 (±3)	**-11 (±4)***	-3 (±5)	**-15 (±4)*****
2000 Hz	**10 (±4)***	7 (±4)	**12 (±3)****	**-13 (±4)****	**-15 (±5)****	**-11 (±3)****
2250 Hz	**10 (±4)***	2 (±4)	**10 (±3)****	**-15 (±4)*****	**-23 (±5)*****	**-15 (±3)*****
2500 Hz	**10 (±4)***	2 (±4)	4 (±3)	**-15 (±4)*****	**-22 (±5)*****	**-20 (±3)*****
3000 Hz	-6 (±4)	**-18 (±4)*****	-4 (±3)	**-22 (±4)*****	**-34 (±5)*****	**-21 (±3)*****
3500 Hz	NA	NA	NA	**-22 (±4)*****	**-30 (±5)*****	**-16 (±4)*****
4000 Hz	**-14 (±4)*****	**-23 (±5)*****	-2 (±4)	**-22 (±4)*****	**-31 (±5)*****	**-10 (±4)***

Table 3 – Estimated least squares means differences between hearing sensitivity thresholds of eared and earless *Atelopus* species. Estimated least squares means differences and standard error rounded to the nearest dB are given for each frequency with significant differences between groups bolded and indicated with asterisks (p<.05=*, p<.01=**, p<.001=***). Negative estimated difference indicates that the partially eared *Atelopus* species had a lower threshold (were more sensitive) at that frequency than our earless *Atelopus* species, while a positive estimated difference indicates that the partially eared *Atelopus* species had a higher hearing threshold (were less sensitive) at that frequency than earless *Atelopus* species.

Frequency (Hz)	Airborne sound sensitivity differences between earless *A. elegans* and two partially eared *Atelopus* species dB (±SE)		Airborne sound sensitivity differences between the partially eared *Atelopus* species, *Atelopus* sp. 1 and *Atelopus* sp. 2 dB (±SE)
	***Atelopus* sp. 1**	***Atelopus* sp. 2**	
200 Hz	3 (±5)	**22 (±4)***	**-19 (±5)***
300 Hz	0 (±5)	**10 (±4)***	-10 (±5)
400 Hz	1 (±5)	9 (±4)	-8 (±5)
500 Hz	6 (±5)	**11 (±4)***	-5 (±5)
700 Hz	10 (±5)	**18 (±4)***	-8 (±5)
900 Hz	6 (±5)	**13 (±4)**	-6 (±5)
1100 Hz	6 (±5)	8 (±5)	-2 (±5)
1300 Hz	**13 (±5)***	5 (±5)	8 (±5)
1500 Hz	6 (±5)	0 (±5)	6 (±5)
1750 Hz	8 (±5)	-3 (±4)	12 (±5)
2000 Hz	-3 (±5)	2 (±4)	-4 (±5)
2250 Hz	-8 (±5)	0 (±4)	-8 (±5)
2500 Hz	-8 (±5)	-5 (±4)	-2 (±5)
3000 Hz	**-12 (±5)***	1 (±4)	**-13 (±5)***
3500 Hz	-7 (±5)	6 (±5)	**-13 (±5)***
4000 Hz	-9 (±5)	**13 (±4)**	**-21 (±5)***

Table 4 – Estimated least squares means differences between hearing sensitivity thresholds of individuals with and without vaseline treatments. Estimated least squares means differences and standard error rounded to the nearest dB are given for each frequency with significant differences between Vaseline treatments bolded and indicated with asterisks (p<.05=*, p<.01=**, p<.001=***). A negative estimated difference indicates that the Vaseline treatment increased the hearing threshold (made the anuran less sensitive) at that frequency while a positive estimated difference indicates that the Vaseline treatment decreased the hearing threshold (made the anuran more sensitive) at that frequency.

	Hearing differences with or without Vaseline dB (±SE)						
	body lung wall	**tympanic area**	**tympanic membrane**				
Frequency (Hz)	***Atelopus* sp. 2**	***Atelopus* sp. 2**	***R. alata***	***R. haematiticus***	***R. horribilis***	***R. spinulosa***
300 Hz	-5 (±4)	-1 (±4)	1 (±4)	1 (±4)	1 (±4)	-6 (±4)
500 Hz	7 (±4)	-5 (±5)	-1 (±4)	-4 (±4)	-6 (±4)	-3 (±4)
700 Hz	5 (±4)	0 (±5)	**-9 (±4)***	-4 (±4)	**-14 (±4)****	0 (±4)
900 Hz	3 (±4)	-3 (±5)	**-9 (±4)***	1 (±4)	**-18 (±4)*****	1 (±4)
1100 Hz	4 (±5)	-2 (±5)	**-9 (±4)***	-4 (±4)	**-20 (±4)*****	-4 (±4)
1300 Hz	-1 (±4)	2 (±5)	**-11 (±4)***	**-14 (±4)*****	**-25 (±4)*****	**-9 (±4)***
1500 Hz	-4 (±4)	2 (±5)	-7 (±4)	**-16 (±4)*****	**-29 (±4)*****	**-16 (±4)*****
2000 Hz	-3(±4)	1 (±5)	**-16(±4)*****	**-24(±4)*****	**-28 (±4)*****	**-18 (±4)*****
2500 Hz	-2 (±4)	-4 (±5)	**-15 (±4)*****	**-26 (±4)*****	**-24 (±4)*****	**-16 (±4)*****
3000 Hz	2 (±4)	2 (±5)	**-11 (±4)***	**-22 (±4)*****	**-13 (±5)***	**-19 (±7)****
3500 Hz	2 (±4)	-3 (±5)	**-13 (±5)***	**-17 (±4)*****	NA	NA
4000 Hz	0 (±4)	3 (±5)	-10 (±7)	**-10 (±4)***	-7 (±7)	NA

Figures

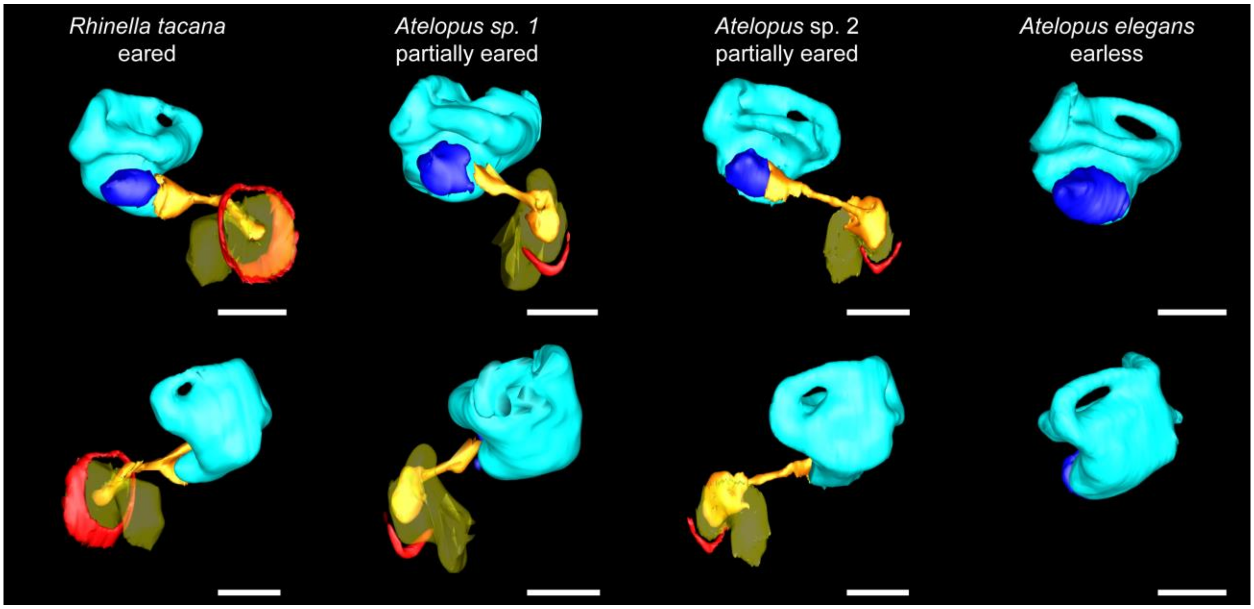

Figure 1 – 3D reconstructions from histology sections showing variation in middle ear structures among *Atelopus* species and an eared bufonid, *Rhinella tacana*. *A. elegans* has no middle ear structures, while *Atelopus* sp. 1 and *Atelopus* sp. 2 both have all middle ear structures present (columella, 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, esutachian tube + middle ear cavity – yellow, tympanic annulus – red. SVL of *R. tacana* = 28.4 mm, *Atelopus* sp. 1 = 25.0 mm, *Atelopus* sp. 2 = 32.0 mm, *A. elegans* = 26.0 mm.

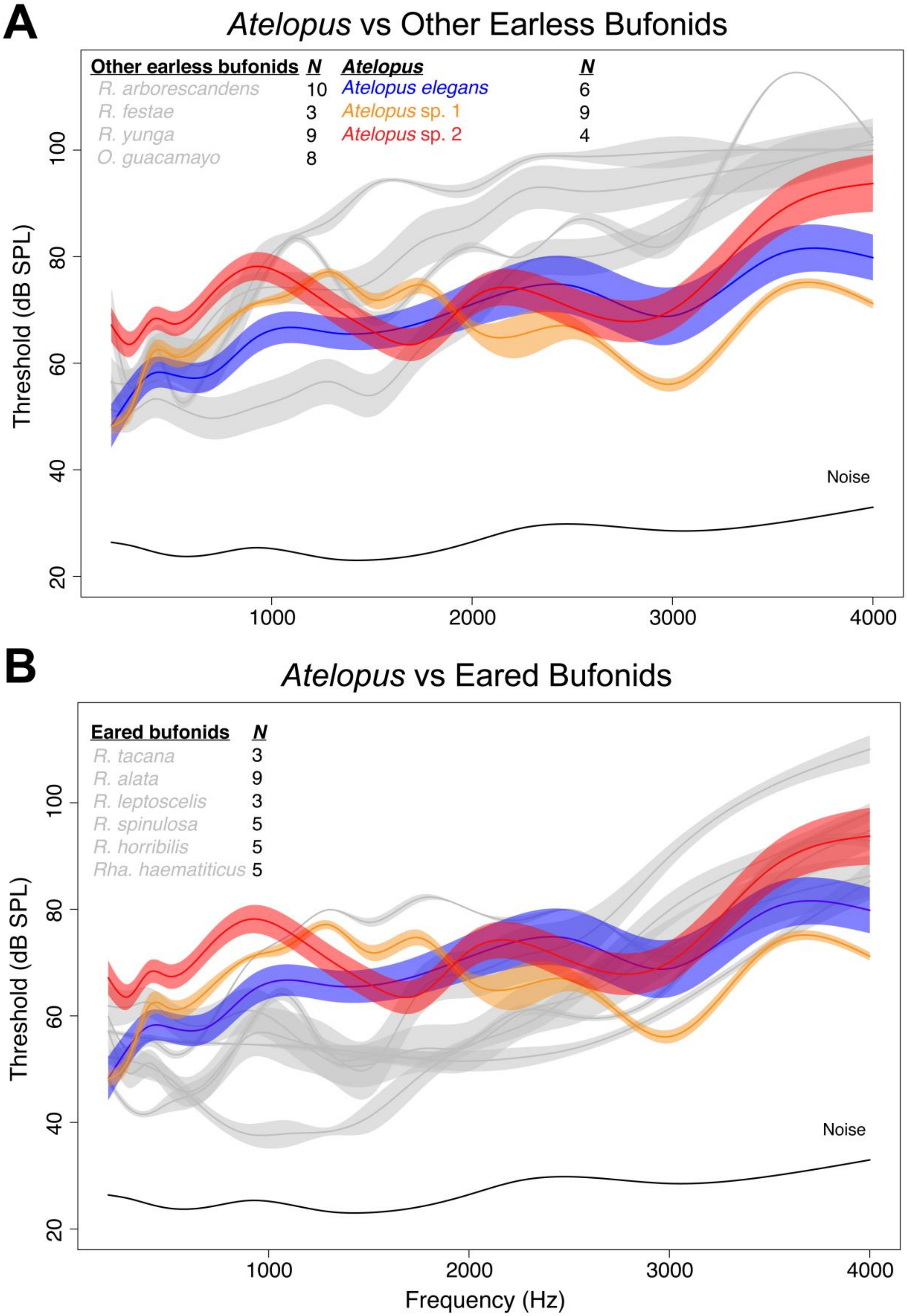
A
Atelopus vs Other Earless Bufonids
Other earless bufonids N
R. arborescandens 10
R. festae 3
R. yunga 9
O. guacamayo 8
Atelopus N
Atelopus elegans 6
Atelopus sp. 1 9
Atelopus sp. 2 4
Noise
Threshold (dB SPL)
20
40
60
80
100
1000
2000
3000
4000
B
Atelopus vs Eared Bufonids
Eared bufonids N
R. tacana 3
R. alata 9
R. leptoscelis 3
R. spinulosa 5
R. horribilis 5
Rha. haematiticus 5
Noise
Threshold (dB SPL)
20
40
60
80
100
1000
2000
3000
4000
Frequency (Hz)

Figure 2 – Hearing differences between *Atelopus* (color) and other eared and earless bufonids (grey). A – Audiograms of the three *Atelopus* species (colors) and earless non-*Atelopus* bufonid species (grey). **B –** Audiograms of the three *Atelopus* species (colors) and eared non-*Atelopus* bufonid species (grey). Separate represent different species. Within-chamber noise level shown in black. Lower x axis thresholds equate to higher hearing sensitivity. All grey species' data are from Womack et al. (2017). Genus names are abbreviated *A* = *Atelopus, R* = *Rhinella*, *Rha* = *Rhaebo*, and *O* = *Osornophryne*.

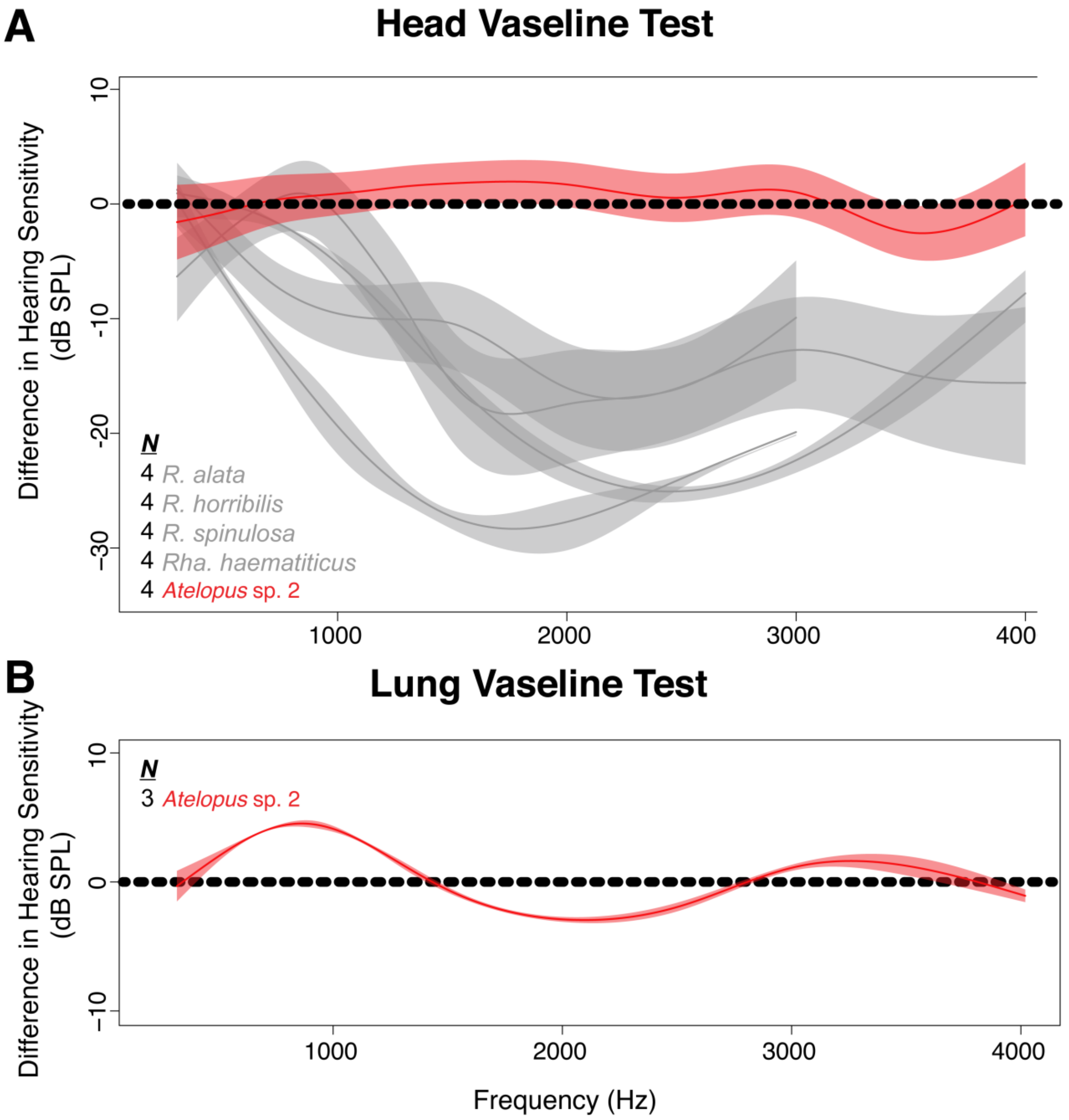

Figure 3 – Vaseline treatment effects on hearing of *Atelopus* sp. 2 (red) and other eared bufonids (grey). A – Difference in hearing between individuals before and after application of Vaseline to the cover the tympanic membrane in eared species and the otic epidermis in *Atelopus* sp. 2. **B –** Difference in hearing between individuals before and after application of Vaseline to the cover the body wall overlying the lungs. Negative values indicate a decrease in hearing sensitivity following Vaseline manipulation. Genus names are abbreviated *A* = *Atelopus, R* = *Rhinella*, and *Rha* = *Rhaebo.*

Supplemental Information 1 – Data.

Click here to Download Supplemental Data

Supplemental Information 2 – Electrode placement for Auditory Brainstem Recordings (ABRs).

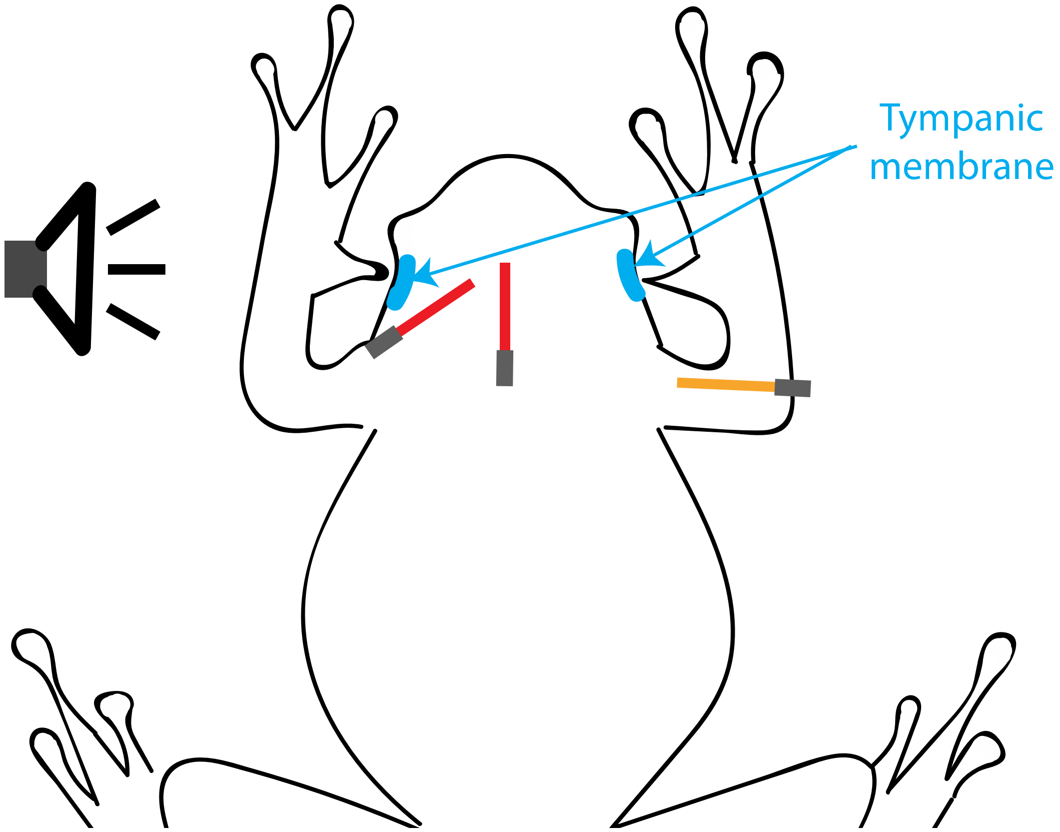

Figure S2.1 – A diagram of the electrode (red) placement for ABRs. We subdermally placed differential electrodes (red) over the midbrain and VIIIth (auditory) nerve and placed a third ground electrode (orange) within the arm contralateral to the VIIIth nerve being measured. Tympanic membranes are shown in blue.

Supplemental Information 3 – *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 6th 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 ºC.

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.

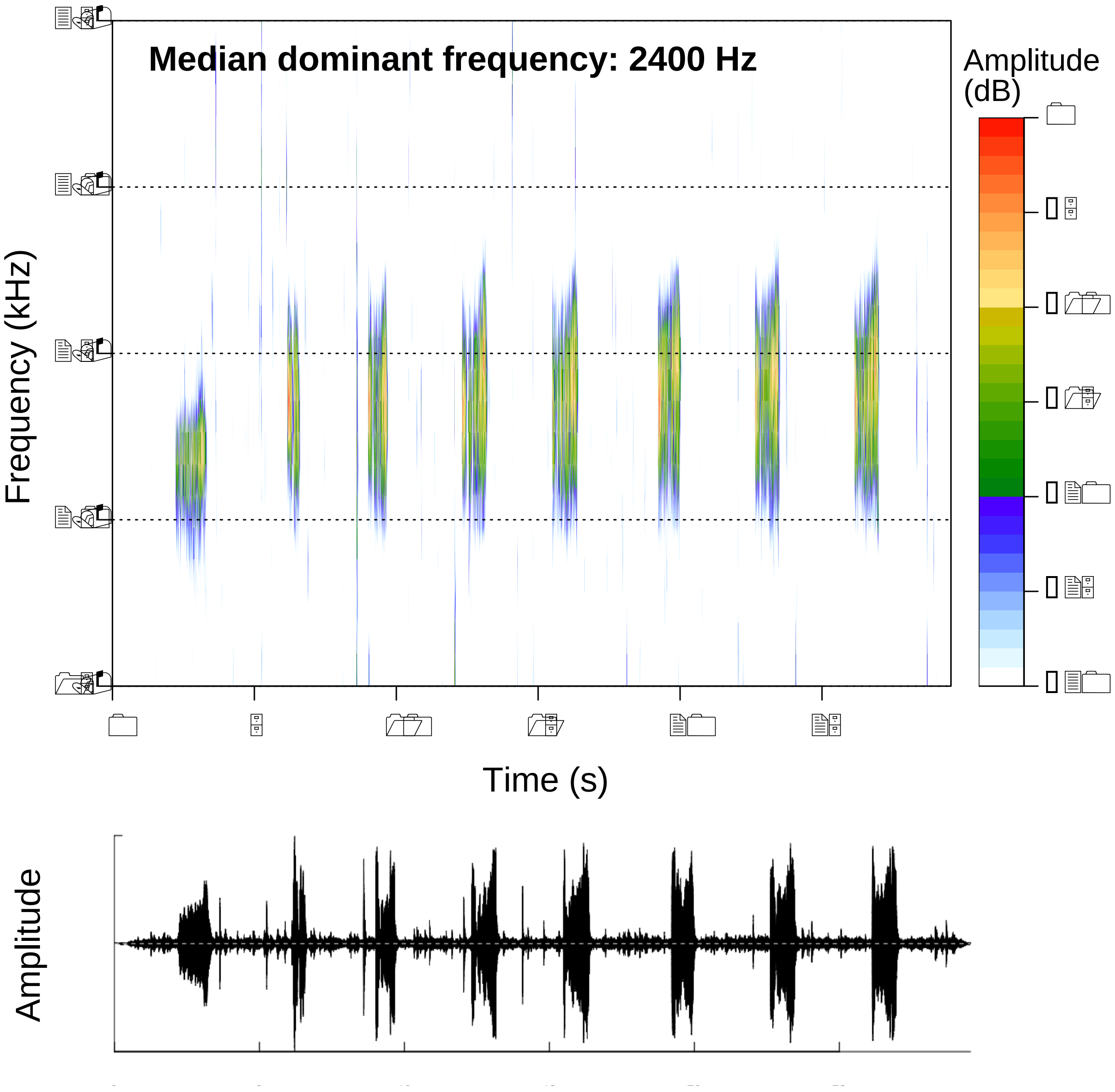

Figure S3.1 – A spectrogram (top) and oscillogram (bottom) of the advertisement call of *Atelopus* sp. 2 with median dominant frequency given. For spectrogram - window length = 512, window = "hanning", overlap = 0.

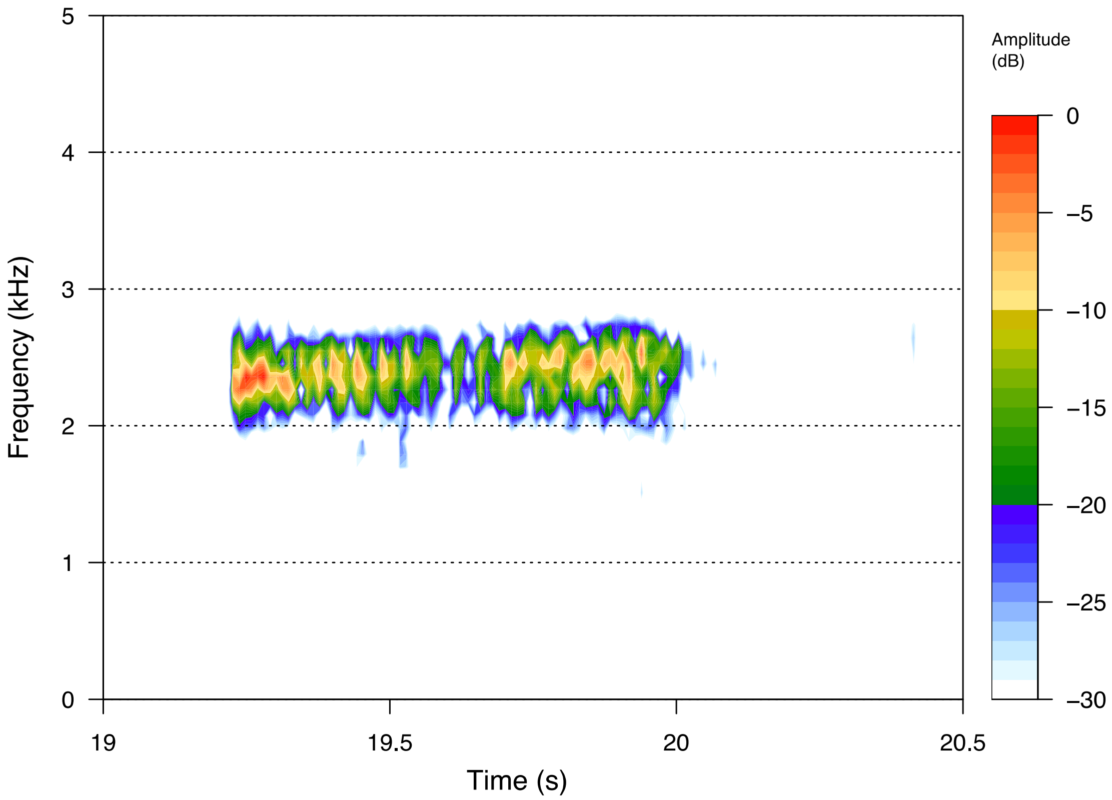

Figure S3.2– A spectrogram of one note within the advertisement call of *Atelopus* sp. 2 shown in Fig 1.1. For spectrogram - window length = 512, window = "hanning", overlap = 0.

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