Brain, Behavior and Evolution

Original Paper

Brain Behav Evol
DOI: 10.1159/000522549

Received: September 7, 2021
Accepted: January 21, 2022
Published online: February 11, 2022

Peripheral Auditory System Divergence Does Not Explain Species Differences in Call Preference

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Keywords
Auditory brainstem responses · Amphibian · Hearing · Mate choice · Acoustic communication

Abstract
Receiver sensory systems have long been cited as an important source of variation in mate preferences that could lead to signal diversification and behavioral isolation between lineages, with a general assumption that animals prefer the most conspicuous signals. The matched filter hypothesis posits that tuning of the frog peripheral auditory system matches dominant frequencies in advertisement calls used to attract mates. However, little work has characterized species with frequency modulation in their calls. In this study, we extend prior work characterizing the lack of correlated evolution between auditory tuning and spectral properties of male calls in *Engystomops* (=*Physalaemus*) frogs. We analyze auditory sensitivity of three cryptic species that differ consistently in female mate preferences for calls of different frequencies. The audiograms of these species differ, but the frequency at which the frog is maximally sensitive is not the most relevant difference in tuning of the auditory periphery. Rather, we identify species differences in overall sensitivity within specific frequency ranges, and we model the effects of these sensitivity differences on neural responses to natural calls. We find a general mismatch between auditory brainstem responses and behavioral preferences of these taxa and rule out the matched filter hypothesis as explaining species differences in male calls and mate preferences in this group.

Introduction

Hypotheses for signal diversification, behavioral isolation, and speciation by sexual selection place a central importance on receiver sensory systems [reviewed in Ryan, 2021]. For example, sensory drive hypotheses are founded on the premise that distinct environmental conditions select for divergent sensory systems in different lineages; signal divergence follows based on sexual selection for conspicuous signals in that environment [Endler and Basolo, 1998; Fuller et al., 2005]. Much evidence that sensory processing alters mate preferences and behavioral isolation comes from communication systems that rely on colorful visual displays. Shifts in photoreceptor sensi-

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tivity alter color discrimination and contrast in predictable ways, and these visual system differences may affect behavioral preferences both for food and mates [Cummings, 2007]. Color may be unusual, however, as visual systems compare responses in a small number of local channels, with the number of possible channels set by the number of photoreceptor classes, and color processing circuits typically subserve multiple functions in addition to mate choice. The auditory system, with its numerous peripheral channels and extensive parallel processing, may play quite different roles in shaping acoustic communication.

Capranica and colleagues proposed the idea of matched spectral filters in the peripheral auditory system of frogs, in which species-typical vocalizations contain much energy in the frequencies to which the auditory system is most sensitive, and may help filter background sounds not related to the call [Capranica, 1965; Capranica and Moffatt, 1983]. Such associations between peripheral auditory system tuning and call characteristics could reflect sexual selection on the vocalizations to best stimulate receiver sensory systems or selection on sensory systems to reduce masking by noise and thus minimize search time; both scenarios imply females mate with the most conspicuous callers. The original hypothesis applied to frog species in which females choose calls that simultaneously stimulate the two auditory end organs of frogs, the amphibian papilla (AP; sensitive to lower frequencies) and basilar papilla (BP; sensitive to higher frequencies) [Capranica and Moffatt, 1983]. Hair cells in the amphibian papilla are arranged in a spatial array in which close neighbors vibrate maximally at similar frequencies. The basilar papilla hair cells tend to have broad frequency tuning that is similar in all BP hair cells in the animal [Narins and Capranica, 1980; Zakon and Wilczynski, 1998]. Evidence for the matched filter hypothesis within anurans is widespread [reviewed in Gerhardt and Schwartz, 2001; Gerhardt and Huber, 2002; Simmons, 2013], although exceptions highlight cases in which evolution of central auditory processing is necessary to explain patterns of detection and discrimination of conspecific vocalizations [e.g., Wilczynski et al., 2001; Goutte et al., 2017]. The extent to which the matched filter hypothesis extends to species in which vocalizations excite only one auditory end organ or in which temporal patterning of sound is behaviorally relevant is largely unknown [Simmons, 2013].

One of the few examples in which auditory tuning has been compared in closely related species with critical roles for temporally patterned signal variation is the *Engystomops* (=*Physalaemus*) *pustulosus* species group. Mating calls and preferences have been extensively characterized in *E. pustulosus*, the túngara frog. Túngara frog males make compound calls with two parts: the lower frequency whine that consists of a downward frequency sweep that excites the AP, and the optional chuck, with much of its energy within the higher frequency range of the BP [Rand and Ryan, 1981]. Túngara frog females prefer whines with chucks over simple whines in phonotaxis assays [Ryan et al., 1982], and the reduction in frequency during the whine is essential, as a high-low tone pair induces positive phonotaxis but not other temporal configurations of the same tones [Wilczynski et al., 1995]. Most other species in the *E. pustulosus* species group only produce the lower frequency whine. Species in this group have quite consistent BP tuning, and the small differences in the best excitatory frequency of the amphibian papilla do not match dominant frequencies in the whine [Wilczynski et al., 2001]. The lack of correlated evolution between species-typical calls and auditory sensitivity suggests that the matched filter hypothesis might not apply in this clade, and that species divergence in calls is not explained by female preferences for the calls most conspicuous to their auditory periphery. Wilczynski et al. [2001] propose that the downward frequency sweep of the whine covers enough of the AP frequency range that the matched filter hypothesis might not be relevant in this clade. Since the time of this work, additional genetic and behavioral work has highlighted the prominent role of frequency in distinguishing species in one clade of this species group, prompting us to revisit divergence in the auditory periphery of this lineage.

To evaluate whether evolution of the auditory periphery and resulting differences in signal conspicuousness could contribute to variation in mate preferences, we examined peripheral auditory system divergence in '*Engystomops petersi*', a cryptic species complex [Funk et al., 2012] in which males differ in the spectral characteristics of their vocalizations as well as the degree of call complexity [Boul and Ryan, 2004]. We focus on three proposed species of the *E. petersi* complex that diverged an estimated 6–16 million years ago [Funk et al., 2012] in which both male and female reproductive behaviors have been characterized. We refer to the species by the preliminary names referenced in Trillo et al. [2017], as formal species descriptions and names have not yet been published. Figure 1 shows the naming scheme and inferred relationships among these species from Funk et al. [2012]. Only males vocalize in this species complex, and they produce a simple call that contains a prefix and a whine (Fig. 1).

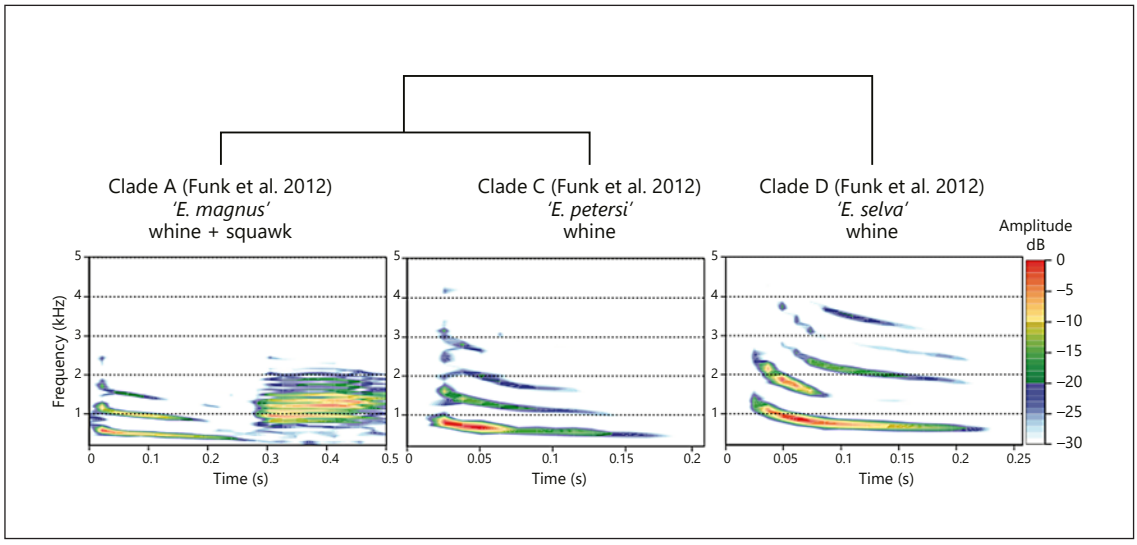

Fig. 1. Advertisement calls by male *Engystomops petersi*, *E. 'magnus'*, and *E. 'selva'* include a downward frequency sweep termed a whine, with species differences in the dominant frequencies. Males from *E. 'magnus'* facultatively add a broadband squawk after the whine to create a compound call. Species referred to using provisional names introduced by Trillo et al. [2017].

Two species tend to have lower frequency simple calls, *E. 'magnus'* males (clade A in Funk et al. [2012], exemplified by the Yasuní population in early behavioral work) and *E. petersi* males (clade C, represented by the Puyo population in behavior studies), whereas *E. 'selva'* males (clade D, named for the La Selva population in behavior work) have higher frequency calls [Boul and Ryan, 2004; Guerra and Ron, 2008; Funk et al., 2009, 2012]. In addition to these striking spectral differences of the simple calls, *E. 'magnus'* males facultatively append a syllable after the whine to produce a complex call (Fig. 1) [Boul and Ryan, 2004]. This optional syllable is similar to the chuck in *E. pustulosus*, but the acoustic properties of this syllable prompted the term "squawk" [Boul et al., 2007]. The species also differ in the call preferences of females based on two-choice phonotaxis assays in exemplar populations. Females from *E. petersi* and *E. 'magnus'* both prefer the lower frequency calls of their own males compared to the *E. 'selva'* male calls (>93% choices in *E. petersi* or *E. 'magnus'* vs. *E. 'selva'* tests) [Boul et al., 2007; Guerra and Ron, 2008], and *E. petersi* females do not discriminate between the two low frequency calls (47% choices for *E. petersi* vs. *E. 'magnus'* calls) [Guerra and Ron, 2008]. In contrast, *E. 'selva'* females prefer the higher frequency calls of their own males compared to the *E. 'magnus'* simple calls (89% choices in *E. 'magnus'* vs. *E. 'selva'* tests) [Boul et al., 2007]. Moreover, only *E. 'magnus'* females significantly prefer complex calls over simple calls (86% choosing complex calls) [Boul et al., 2007], although results from *E. petersi* females are equivocal [Guerra and Ron, 2008]. All three species overlap in distribution and can mate simultaneously at the same mating ponds, which leads to occasional amplexus between species [Trillo et al., 2017]. Thus, these cryptic species differ in various male call features and female preferences that nominate a possible role in speciation for sexual selection based on acoustic communication.

We build on prior work characterizing auditory divergence in this clade by using masked auditory brainstem responses (mABRs) to estimate species-typical audiograms for the *E. petersi*, *E. 'magnus'*, and *E. 'selva'* species of the *E. petersi* complex. Wilczynski et al. [2001] used midbrain auditory responses to report the best excitatory frequency of the AP and BP, and their sampling at Jatun Sacha [M. Ryan, pers. comm.] suggests their individuals represented the *E. petersi* lineage (clade C) [Funk et al., 2012]. Rather than focusing on best excitatory frequency measures, we compare the shape of the audiogram as a whole and overall sensitivity differences. We calculated conspicuousness of a diverse set of conspecific and heterospecific signals based on these audiograms and asked if species-typical behavioral preferences matched the calculated signal conspicuousness. We also measured the dimensions of numerous ear structures to assess the likelihood that changes in skull shape or body size altered hearing sensitivity in each lineage. We found that hearing

sensitivity and inferred signal conspicuousness are decoupled from static anatomical dimensions, and we conclude that species-typical mate preferences are not explained by divergence in tuning of the auditory periphery.

Methods

Animals

We collected adult male and female *Engystomops* frogs near Yasuní Research Station (Napo province) and La Selva Lodge (Sucumbios province) between September 2008 and January 2012 with research and collection permits from Ministerio de Ambiente del Ecuador (0032-DPO-MA, 008-09IC-FAU-DNB/MA, 001-10 IC-FAU-DNB/MA, 0036-FAU-MAE-DPO-PNY). Animals were maintained in captivity at the field stations until the experiment. All animal protocols were approved by Institutional Animal Care and Use Committee of Colorado State University (#09-1397A). Because three cryptic species of *Engystomops* frogs inhabit the area near the Yasuní station, we determined species identification after the experiments using partial sequences of tRNA-Val and 16S RNA that proved diagnostic of species [Funk et al., 2012]. We amplified the mitochondrial DNA fragment by PCR using forward primer GGCAAGTCGTAACATGGTAAG [Darst and Cannatella, 2004] and reverse primer ATGTTTTTG-GTAAACAGGCG [Goebel et al., 1999]. We aligned the resulting sequences using Geneious Pro 5.4.6 [Drummond et al., 2010] and estimated phylogenies using maximum likelihood in GARLI 2.0 [Zwickl, 2006], including all sequences from Funk et al. [2012]. Our new sequences clustered within clades A, C, and D from Funk et al. [2012] and thus were assigned to *E. 'magnus'* (n = 8 females, 8 males), *E. petersi* (n = 5 females, 7 males), and *E. 'selva'*, respectively (n = 5 females, 7 males).

Acoustic Stimuli

We recorded acoustic responses to tones using mABRs (see details below) as well as responses to pulses present in natural recordings. ABR signals depend on the coordinated responses of many neurons, and thus signals to probe responses should be brief and fit within a 40-ms stimulus window. We constructed stimulus files consisting of individual pulses that are repeated in natural *E. 'magnus'* squawks (recordings from Boul et al. [2007]). Each pulse of the squawk is 10–15 ms long. We chose three squawks with diverse spectrotemporal parameters to probe auditory responses. From these recordings we constructed three single-pulse stimuli by cutting the normalized wave file recordings using the software CoolEdit 2000 (Syntrillium Software Corp, Phoenix, AZ, USA).

Auditory Brainstem Recordings

We determined audiograms for each frog using a custom-built software program to run mABR. Briefly, mABR is a derived-response method developed by Brandt et al. [2018]. Here, sensitivity to tones is measured by the efficiency of tonal masking of the response to a transient stimulus. The tonal masking was measured by presenting at intervals of 40 ms a transient (a half-cycle 4 kHz sinusoid of duration 125 μs) with a flat spectrum within the frequencies of interest, alternating two seconds of the transients alone with two seconds of the transient mixed with a tonal sinusoid. The derived response, found by subtracting the response to the transient from the response to the masked transient, is a measure of sensitivity to the tone, as only tones that effectively excite the peripheral auditory system produce masking. This mABR method better evaluates tonal sensitivity, especially at low frequencies, compared to standard ABRs [Brandt et al., 2018]. In our experiments we first measured the response to the unmasked transient at different levels to find the near-saturation level, which we used for the derived measurements. Then, the derived response was measured at tonal masker frequencies ranging from 300 to 4,500 Hz. Figure 2 shows an example of the response to the transient, to the masked transient, and the resulting derived response. This response was measured at different levels of the tonal masker. The size of the transient response was monitored during the experiments to check the state of the animal and the quality of the recording. The stimulation was controlled by the same hardware and software as used for data acquisition (Tucker-Davis RM2 and the program QuickABR10).

We lightly anesthetized the animals first using brief immersion in 0.5% benzocaine until movement stopped, then immobilized the animals. Experiments run in 2010 relied upon continued small applications of benzocaine to immobilize animals. As animals varied greatly in their sensitivity to benzocaine, immobilizing animals using benzocaine carried a risk of fatal overdose. Starting in 2011, we injected succinylcholine chloride (15 μg/g body weight) intramuscularly after the initial benzocaine application to maintain paralysis, and used the initial benzocaine application to prevent pain from the injection and manipulations involved in preparing the animal for recordings. Including a factor for 2010 in statistical models found no differences in sensitivity based on method of immobilization.

To prepare the animals for recording differential electrical signaling across the brainstem, we positioned two metal needle electrodes (Grass Technologies, West Warwick, RI, USA) subdermally, one laterally close to the ear and one over the brainstem midline. We placed a third electrode into the arm to act as the ground. We placed the frog beside a custom-built acoustic coupler that funneled sound to the ear from a headphone (Beyer Audiometric 48.0A) using a brass housing. After sealing the coupler around the eardrum using Vaseline, we placed a moist paper towel over the animal, and enclosed the frog in a dark, quiet box. We calibrated the amplitude of sound reaching the ear using a microphone (½″ microphone calibrated with a Type 4228 pistonphone, Brüel & Kjær, Virum, Denmark).

We recorded evoked potentials in the differential electrodes attached to an RA4L1 headstage and RA4PA preamplifier (Tucker Davis Technologies, Alachua, FL, USA), and digitized the recordings with an RM2 processor (Tucker Davis Technologies). The click amplitude was set individually for each recording session as the amplitude that produced a signal just below the maximal response (typically 95–105 dB SPL). We systematically varied the amplitude and frequency of the masking tone to determine hearing thresholds at 24 frequencies between 300 and 4,500 Hz, averaging 400 traces at each amplitude-frequency combination. We recorded responses to each tone frequency at three or more amplitudes below 95 dB SPL. Traces were updated each minute to allow real-time monitoring of the stability of auditory recordings, a measure we used to assess animal health throughout the experiment. In the rare instances in which responses to the clicks decreased steadily (e.g., overdose of anesthesia), we halted recordings and revived the frog

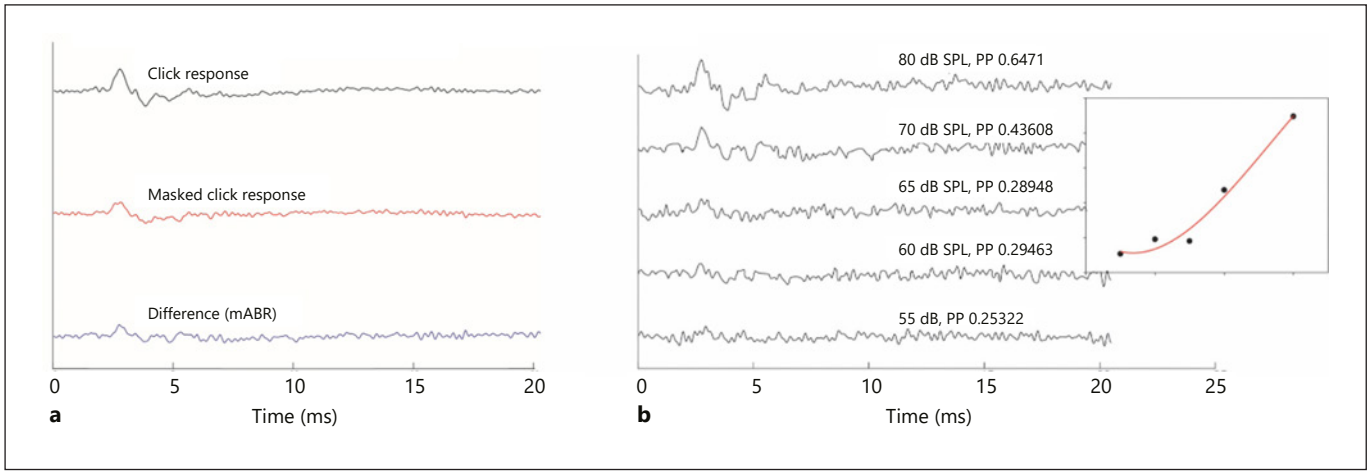

Fig. 2. Masked ABRs enable consistent measurements of sensitivity to acoustic stimuli. **a** Audible tones mask the amplitude of responses of the auditory nerve to clicks. Traces indicate responses to a click masked with 600 Hz tone broadcast at 70 dB SPL amplitude. Comparing averaged responses to masked and unmasked clicks produces a reliable difference signal if the tone is audible. **b** The threshold for acoustic sensitivity is the lowest amplitude at which playing a tone produces a difference signal. Traces indicate responses of one animal to a 600 Hz masking tone.

in clean, cool water. For a subset of animals, we complemented the masked ABR procedures by measuring ABR responses to either brief tone pulses (for comparison of masked and typical ABR procedures) or to responses to squawk pulses (for validation of estimated sensitivity to natural calls based on audiograms). Recording durations were typically three hours, after which animals were rinsed in clean water and allowed to recover. All animals recovered from the experiment once we used succinylcholine chloride as a paralytic rather than continuous benzocaine application.

Audiogram Analysis and Reliability

We estimated the threshold of response for each animal at each frequency using visual inspection of the click response, masked response, and the difference between the two traces (Fig. 2). We used several methods to validate our estimates of the sensitivity of animals at each frequency. We retested nine animals on two separate recording days when the first recording yielded incomplete data, either due to the animal's response to immobilizing drugs or other technical difficulties, and retested those animals at several frequencies for which data previously were obtained. Of 119 measurements that were repeated on two days, the median difference between the threshold estimates was 3 dB. We selected the recording session with the most complete audiogram to represent each individual's acoustic sensitivity for subsequent statistical analyses, combining data from partial audiograms assayed on different days when needed to complete the datasets. We estimated the stability of the unmasked click amplitudes over the course of the recording sessions by calculating the median coefficient of variation in click amplitudes within each recording session as 0.157. To compare our masked ABR thresholds to standard ABR methods, we also estimated thresholds to brief tone pulses in some animals with largely concordant results (not shown). Lauridsen et al. [2021] provides a comprehensive comparison of these ABR approaches.

Estimated Response to Natural Calls Based on Audiograms

To estimate the response to natural calls we used a sample of calls of the three species recorded in La Selva Lodge and Yasuní National Park. Recordings included original calls from Boul et al. [2007] and frogs recorded in a captive colony at Yasuní Research Station later identified genetically (as reported in Trillo et al. [2017]). Calls were recorded using a Sennheiser SE66 microphone [Boul et al., 2007] or an Olympus LS-10 digital recorder. To estimate amplitudes of natural calls, we recorded frog calls on an Olympus LS-10 digital recorder at known distances and gain settings. The gain settings of the recorder were subsequently calibrated by recording sound signals calibrated against a Brüel & Kjær ½″ microphone in an anechoic chamber to convert the recording levels to Pa. The anechoic room (Department of Biology, University of Southern Denmark) was custom-made with 30 cm rockwool wedges tested to be anechoic above 200 Hz. Call amplitude was estimated to be 80 dB SPL at the source.

The response to calls was estimated by filtering the calls by the audiogram (Fig. 3). A Matlab script constructed amplitude spectra of the calls and normalized the calls to the same peak amplitude. The script then binned the spectra based on the tone frequencies used to construct the audiogram, subtracted the spectral levels by the true audiogram thresholds (assumed to be 20 dB lower than the ABR thresholds, see Lauridsen et al. [2021]), and summed the linear difference across each bin. The summed difference in each bin (i.e., above-threshold stimulation) was converted to dB, reflecting that rate-level curves of auditory fibers usually show a linear dB-spike rate relationship [Zakon and Wilczynski, 1988; Christensen-Dalsgaard et al., 1998]. Finally, dB values were multiplied by 20, to approximate the maximal slope of frog auditory nerve rate-level functions [Christensen-Dalsgaard et al., 1998]. The resulting number is an estimate of neural excitation produced by each call and will be referred to as audibility. We used a threshold value of 500, although results were highly correlated for thresholds of 0 and 100.

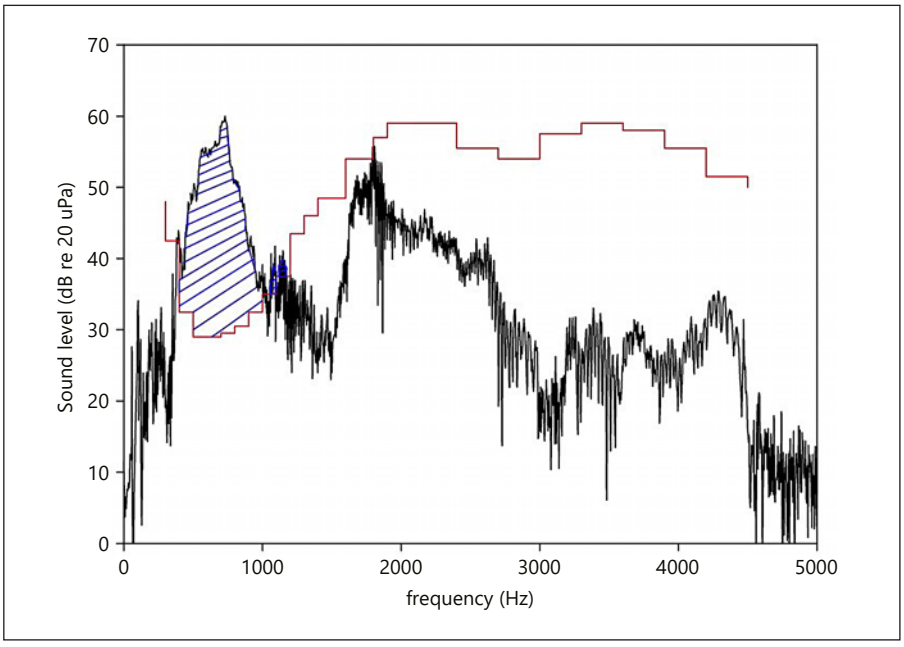

Fig. 3. Estimation methods for audibility of natural calls based on individual audiogram thresholds. Red line indicates the estimated behavioral audiogram in one *E. 'selva'* male calculated by lowering ABR relative thresholds by 20 dB (see Methods) and black trace is the sound spectrum of a recorded *E. petersi* call, normalized and played at a peak amplitude of 60 dB SPL. The area marked with diagonal lines represents the sound energy above threshold.

Ear Structural Analysis

We euthanized female specimens (6 *E. petersi*, 4 *E. 'magnus'*, and 5 *E. 'selva'*) with a topical application of 20% benzocaine. We then decapitated the animals immediately postmortem and fixed the heads in 4% paraformaldehyde for 24 h at 4°C, rinsed them three times for 15 min in phosphate buffered saline, and then stored them in 75% ethanol at room temperature. We decalcified the heads in Cal-Ex solution (Fisher Scientific, CS510-1D), dehydrated the tissue in a graded ethanol series (50%, 70%, 90%, 95%, 100%, 100%), cleared the tissue in xylene, and embedded the tissue in paraffin wax.

We sectioned the heads at 10 µm thickness using a microtome (RM1265, Leica, Wetzlar, Germany) and then mounted each section onto VWR Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA). We stained each section with Hematoxylin and Eosin Y (Fisher Scientific) and took pictures of every third section (30 µm between photos; Fig. 4). Using ImageJ [Schneider et al., 2012] we traced the inner ear, middle ear cavity, and columella (including the extracolumella) on every sixth section (60 µm between measured sections) to make area measurements and calculated volumes for each structure using intersection differences. We also measured tympanum diameter and head width with ImageJ and performed AP and BP hair cell counts on every third section. We measured all ear structures and performed hair cell counts on both the left and right ears of each female.

Statistical Analysis

We ran linear mixed models in R using the lme4 package [Bates et al., 2015] with fixed and random effects for each data type detailed below. For all models we reported type III analysis of variance table with Satterthwaite's method using the package lmerTest [Kuznetsova et al., 2017]. For significant main effects ($p < 0.05$) we reported eta-squared estimates of effect sizes using the effectsize package [Ben-Shachar et al,. 2020]. For models with a significant species effect, we reported the pairwise comparisons among species using Tukey's method for *p*-value correction in the package lsmeans [Lenth, 2016].

To evaluate audiogram differences, we predicted our outcome variable, the threshold estimates at each frequency, based on fixed factors frequency (categorical variable), sex, and species and random factors testing year and frog to account for repeated measures within individuals. We included all two- and three-way interaction terms between the fixed factors in our model. We also added as a covariate the average amplitude of the click measured for each individual at each frequency, as higher amplitude clicks should allow greater signal to noise and thus lower threshold estimates. We used post hoc pairwise tests to compare species' responses at each frequency. Species and sexes did not differ consistently in click amplitudes measured over the course of the experiment (species $F_{2,38} = 2.14$, $p = 0.13$; sex $F_{2,38} = 3.10$, $p = 0.09$; species × sex $F_{2,38} = 2.00$, $p = 0.15$). Although not statistically significant, *E. 'selva'* females had the highest click amplitudes, followed by *E. 'magnus'* males, *E. 'magnus'* females, *E. petersi* females, *E. 'selva'* males, and *E. petersi* males. A model lacking the click amplitude covariate gave nearly identical results and had larger BIC values, so we present here models with the covariate.

To test differences in estimated audibility of natural whines, we used the amplitude at which each call was above threshold as the dependent variable. Models included fixed effects of each animal's sex and species as well as the species of the animal that produced the whine, plus all two-way and three-way interactions. Random effects included the specific whine exemplar and individual frog ID. We used post hoc *t* tests to compare species' sensitivities to each type of whine for each sex. We similarly tested sex and species differences in audibility of the squawk, using multiple squawk exemplars. Fixed effects in these models were the sex and species of the animal and their interaction, with random effects including the specific squawk exemplar and individual frog ID.

To assess middle and inner ear morphological differences among the three species, we used each morphological variable (e.g., tympanum diameter) as the dependent variable, species as a fixed-effect factor, and individual and side (left or right) as random effects. Next, we ran a second set of models to determine whether head size solely

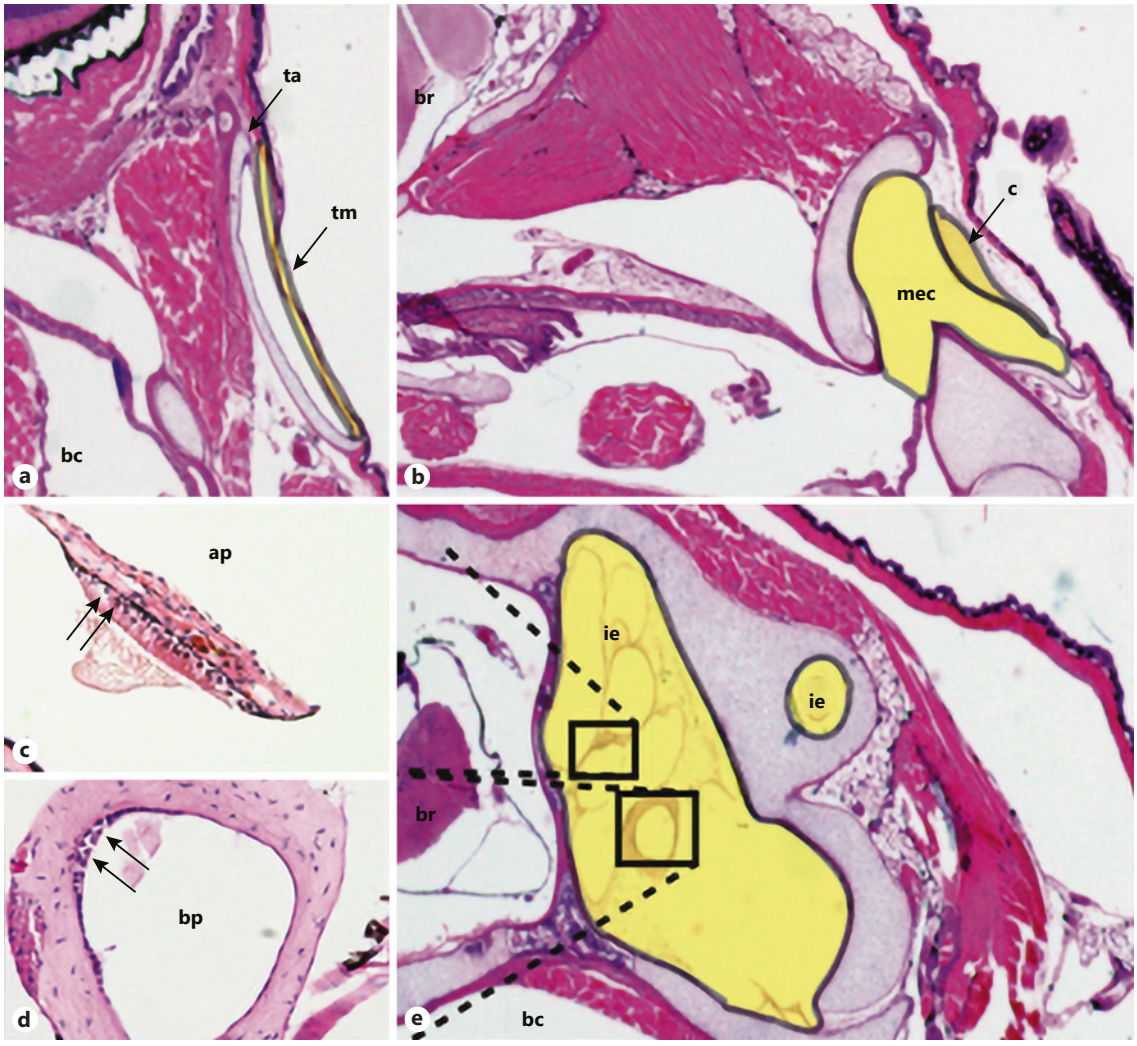

Fig. 4. Micrographs showing the middle and inner ear features we measured and compared among species. All panels are frontal sections stained with Hematoxylin and Eosin Y. **a** Tympanic annulus (ta) volume and tympanic membrane (tm) diameter. Tympanic annulus was not measured. **b** Middle ear cavity (mec) volume and columella (c) volume. **c** Amphibian papilla (ap) hair cell (black arrows) number. **d** Basilar papilla (bp) hair cell (black arrows) number. **e** Inner ear (ie) volume with boxed outlines of the amphibian and basilar papilla. The brain (br) and buccal cavity (bc) are labeled for orientation but were not measured.

explained any differences in ear morphology seen among species. We did this by running the same mixed models a second time with the addition of head width as a covariate in each model.

Results

Species Differed in Hearing Sensitivity

Audiograms demonstrated tuning curves typical of anurans, with model-estimated thresholds for each sex and species at each frequency depicted in Figure 5. Each audiogram has a lower frequency peak in sensitivity reflecting amphibian papilla responses (below 1,600) and a higher frequency response reflecting basilar papilla excitation (Fig. 5). The best-excitatory frequency (BEF) of the amphibian papilla was 500 Hz for males of all species and for *E. 'magnus'* females, whereas the BEF of *E. 'selva'* and *E. petersi* females was 600 Hz. The BEF of the basilar papilla in *E. petersi* females and *E. petersi* males was 3,000 Hz. All other groups had shallow average tuning curves, which complicated the ability to report a reliable BEF. The frequencies with the lowest model-estimated thresholds in the BP range were: 3,600 in *E. 'selva'* males, 4,200 in *E. 'magnus'* males, 3,000 in *E. 'magnus'* females, and 4,500 in *E. 'selva'* females.

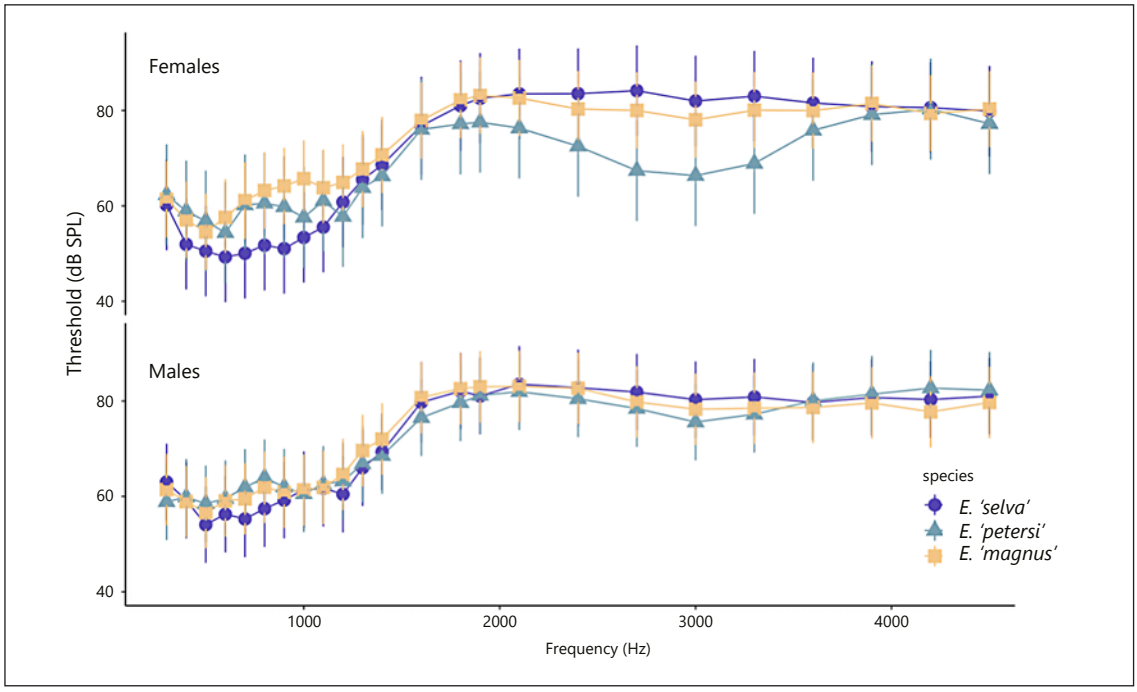

Fig. 5. Audiograms show hearing sensitivity of males and females of each species, with curves displaying model-estimated average sensitivity at each frequency. Color and symbol indicate species of animal for which auditory sensitivity is depicted, with purple illustrating *E. 'selva'* animals, blue indicating *E. petersi* frogs, and yellow indicating *E. 'magnus'* animals. Female results are plotted in the top graph, and male results are plotted in the lower graph. *E. 'selva'* females have higher sensitivity than other species between 600 and 1,100 Hz, whereas *E. petersi* females are more sensitive than other species between 2,400 and 3,300 Hz. Error bars indicate 95% confidence intervals.

Table 1. Main effects of species, sex, and frequency on hearing thresholds controlling for variation in click amplitude with degrees of freedom estimated using Satterthwaite's method and effect size estimated as eta-squared

Effect	*F* value	*p* value	Effect size (90% confidence interval)
Species	$F_{2,34.66} = 1.93$	0.16	
Frequency	$F_{23,868.09} = 185.28$	**<2e–16**	0.83 (0.82–0.84)
Sex	$F_{1,34.95} = 3.19$	0.083	
Click amplitude	$F_{1,542.1} = 5.73$	**0.017**	0.010 (0.001–0.29)
Species:frequency	$F_{46,868.13} = 4.27$	**<2e–16**	0.18 (0.11–0.19)
Species:sex	$F_{2,34.62} = 1.25$	0.3	
Frequency:sex	$F_{23,867.79} = 0.56$	0.96	
Species:frequency:sex	$F_{46,867.83} = 1.56$	**0.01**	0.076 (0.007–0.058)

The sexes differed in the frequency-specific patterns of species differences in hearing sensitivity ($F_{46,868} = 1.56$, $p = 0.011$; Fig. 5; Table 1). Post hoc analyses confirmed species differences in sensitivity to tones in females, with *E. 'selva'* frogs most sensitive between 600 and 1,100 Hz and *E. petersi* frogs more sensitive between 2,400 and 3,300 Hz (Fig. 5; Table 2). In contrast, males of the three species did not differ in sensitivity to tones at any frequency (Fig. 5; Table 2).

We estimated the communication consequences of these species differences in peripheral sensitivity. First we validated model-estimated thresholds by comparing measured responses to short portions of three squawks for seven individuals. Measured thresholds for each squawk were correlated with thresholds estimated based on the audiogram (Fig. 6a). The sexes differed in the call-type specific patterns of species differences of the model-predicted audibility of whine exemplars ($F_{4,1258} = 3.73$,

Table 2. Post hoc pairwise contrasts to test for species differences at each frequency

Frequency	Contrast	Female *p* value	Male *p* value
300	selva-petersi	0.8496	0.3818
	selva-magnus	0.9359	0.8598
	petersi-magnus	0.9638	0.6790
400	selva-petersi	0.1804	0.9776
	selva-magnus	0.2940	0.9945
	petersi-magnus	0.8717	0.9488
500	selva-petersi	0.2461	0.3407
	selva-magnus	0.4683	0.6847
	petersi-magnus	0.8113	0.8121
600	selva-petersi	0.3995	0.5507
	selva-magnus	**0.0423**	0.6304
	petersi-magnus	0.6569	0.9870
700	selva-petersi	**0.0283**	0.0878
	selva-magnus	**0.0040**	0.3507
	petersi-magnus	0.9658	0.7074
800	selva-petersi	0.0662	0.0959
	selva-magnus	**0.0026**	0.3044
	petersi-magnus	0.7482	0.7872
900	selva-petersi	0.0669	0.6570
	selva-magnus	**0.0004**	0.8521
	petersi-magnus	0.4626	0.9315
1,000	selva-petersi	0.5328	0.9610
	selva-magnus	**0.0011**	0.9999
	petersi-magnus	0.0710	0.9625
1,100	selva-petersi	0.3406	0.9621
	selva-magnus	**0.0438**	0.9981
	petersi-magnus	0.7360	0.9766
1,200	selva-petersi	0.7217	0.6563
	selva-magnus	0.4528	0.3632
	petersi-magnus	0.1274	0.8895
1,300	selva-petersi	0.9024	0.9651
	selva-magnus	0.7987	0.4655
	petersi-magnus	0.5400	0.6286
1,400	selva-petersi	0.8244	0.9656
	selva-magnus	0.8141	0.6534
	petersi-magnus	0.4510	0.4922
1,600	selva-petersi	0.9815	0.5794
	selva-magnus	0.9473	0.9454
	petersi-magnus	0.8652	0.3527
1,800	selva-petersi	0.5925	0.7159
	selva-magnus	0.9350	0.9841
	petersi-magnus	0.3571	0.5978
1,900	selva-petersi	0.4090	0.9989
	selva-magnus	0.9849	0.7996
	petersi-magnus	0.2813	0.8268
2,100	selva-petersi	0.1645	0.8708
	selva-magnus	0.9660	0.9874
	petersi-magnus	0.2009	0.9301
2,400	selva-petersi	**0.0148**	0.7395
	selva-magnus	0.6157	0.9992
	petersi-magnus	0.0869	0.7509
2,700	selva-petersi	**0.0001**	0.5133
	selva-magnus	0.4491	0.7733
	petersi-magnus	**0.0019**	0.8935
3,000	selva-petersi	**0.0003**	0.2919
	selva-magnus	0.4874	0.7864
	petersi-magnus	**0.0044**	0.6506
3,300	selva-petersi	**0.0011**	0.4781
	selva-magnus	0.6681	0.7185
	petersi-magnus	**0.0071**	0.9077
3,600	selva-petersi	0.3129	0.9928
	selva-magnus	0.8864	0.9377
	petersi-magnus	0.4993	0.8909
3,900	selva-petersi	0.9025	0.9727
	selva-magnus	0.9816	0.9277
	petersi-magnus	0.8014	0.8208
4,200	selva-petersi	0.9964	0.7238
	selva-magnus	0.9350	0.6717
	petersi-magnus	0.9689	0.2306
4,500	selva-petersi	0.7859	0.9155
	selva-magnus	0.9905	0.8996
	petersi-magnus	0.6832	0.6722

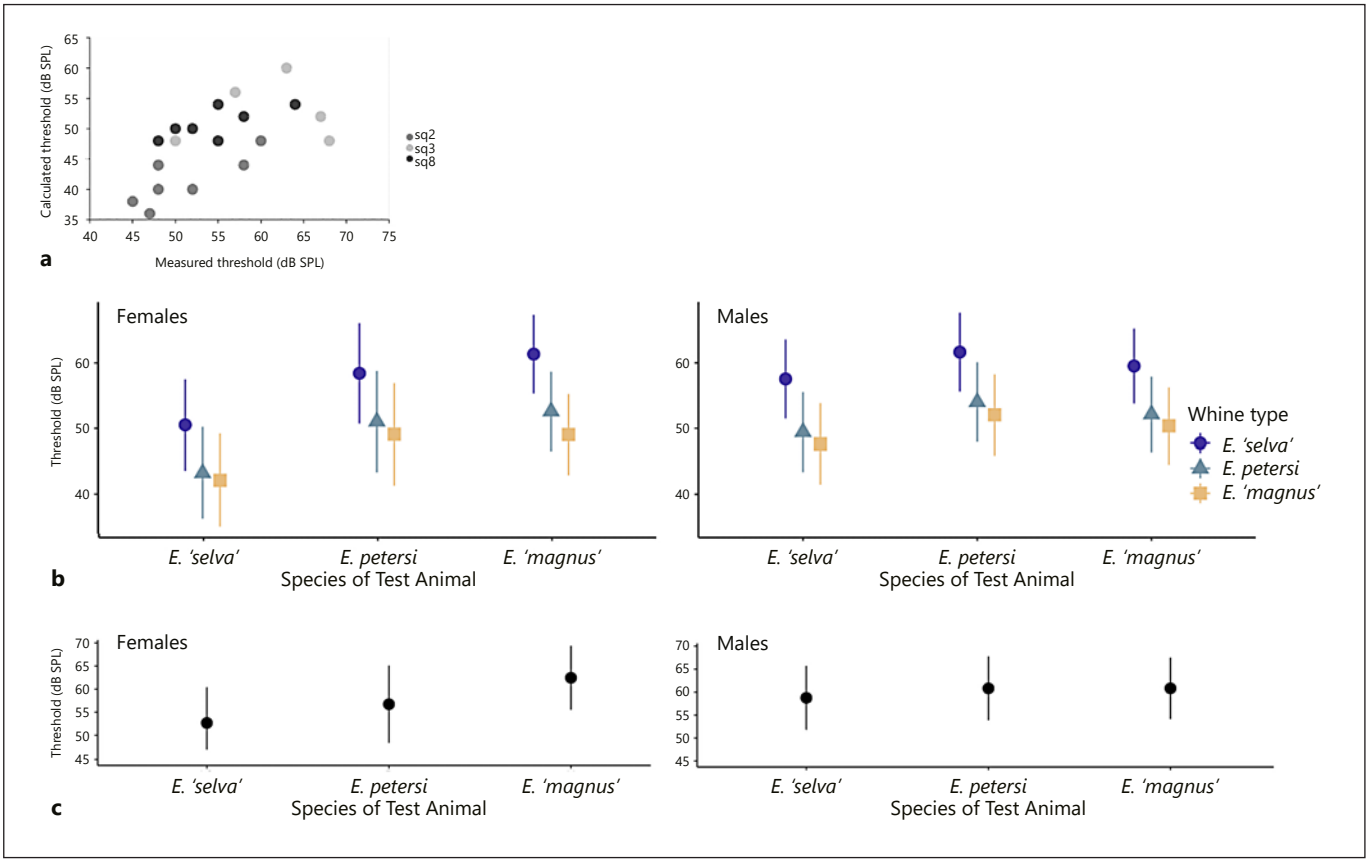

Fig. 6. Species differences in audiograms predict that *E. 'selva'* females have enhanced sensitivity to all vocalizations. **a** Calculated sensitivity of ears to squawks are largely consistent with measured thresholds of responses to individual pulses from three squawks. Each point represents one individual's measured and calculated responses to a given squawk, with grayscale indicating which squawk exemplar is plotted. **b** All species have a similar relative sensitivity to whines of each species, with greater sensitivity to *E. 'magnus'* and *E. petersi* calls than to *E. 'selva'* calls. *E. 'selva'* females are more sensitive than *E. 'magnus'* or *E. petersi* females, or males of any species. **c** All species have a similar sensitivity to squawks. Bars in **b** and **c** represent model-estimated marginal means of thresholds for response to each call type in each species, with error bars showing 95% confidence intervals.

Table 3. Main effects of species, sex, and call type (species of call exemplar) on model-predicted sensitivity to whines, with degrees of freedom estimated using Satterthwaite's method

Effect	*F* value	*p* value	Effect size (90% confidence interval)
Species	$F_{2,37.83} = 8.71$	**0.00077**	0.32 (0.11–0.48)
Sex	$F_{1,37.83} = 5.52$	**0.024**	0.13 (0.10–0.30)
Calltype	$F_{2,34.67} = 12.95$	**6.3e–5**	0.43 (0.21–0.58)
Species:sex	$F_{2,37.83} = 3.56$	**0.038**	0.16 (0.005–0.32)
Species:calltype	$F_{4,1258} = 1.57$	0.18	
Sex:calltype	$F_{2,1258} = 0.75$	0.47	
Species:sex:calltype	$F_{4,1258} = 3.73$	**0.005**	0.012 (0.002–0.02)

$p = 0.005$; Fig. 6b; Table 3). Post hoc analyses indicated that individuals of all species were more sensitive to the calls of *E. 'magnus'* and *E. petersi* males than *E. 'selva'* males. Models predicted *E. 'selva'* females to be more sensitive than *E. 'magnus'* or *E. petersi* females to all call types (Fig. 6b; Table 4), whereas males of the three species did not differ in sensitivity (Fig. 6b; Table 4). Species differed in the model-predicted audibility of squawk exemplars

Table 4. Post hoc pairwise contrasts to test for differences in audibility of each type of whine in each species

Call type	Contrast	Female *p* value	Male *p* value
E. 'selva'	selva-petersi	0.1226	0.1929
	selva-magnus	**0.0006**	0.6652
	petersi-magnus	0.2349	0.6063
E. petersi	selva-petersi	0.1376	0.1444
	selva-magnus	**0.0007**	0.4571
	petersi-magnus	0.2298	0.7166
E. 'magnus'	selva-petersi	0.1541	0.1446
	selva-magnus	**0.0008**	0.4297
	petersi-magnus	0.2262	0.7457

Call type refers to species of origin of each recorded whine, whereas the species in the contrast column refers to the species of the subject for which audiograms were measured.

Table 5. Main effects of species and sex on model-predicted sensitivity to squawks with degrees of freedom estimated using Satterthwaite's method

Effect	*F* value	*p* value	Effect size (90% confidence interval)	Pairwise contrasts
Species	$F_{2,37.407} = 4.77$	**0.014**	0.20 (0.027–0.37)	selva-petersi: $p = 0.3778$ **selva-magnus: p = 0.0174** petersi-magnus: $p = 0.3863$
Sex	$F_{2,37.407} = 3.05$	0.089		
Species:sex	$F_{2,37.407} = 2.22$	0.12		

($F_{2,37.4} = 4.77$, $p = 0.014$; Fig. 6c; Table 5), with *E. 'selva'* frogs more sensitive than *E. 'magnus'* frogs ($t_{43.3} = 1.346$, $p = 0.017$).

Ear Anatomy Does Not Explain Species Differences in Hearing

If morphological differences are responsible for the hearing differences among species, we would expect *E. 'selva'* to be distinct in aspects of morphology that affect low-frequency hearing (below 1 kHz) and *E. petersi* to be distinct in aspects of morphology that affect high-frequency hearing. However, we only find morphological differences between *E. 'magnus'* and the other two species (Fig. 7; Table 6). Without controlling for head size, we find that *E. 'magnus'* has a larger tympanic membrane, inner ear volume, middle ear cavity volume, and columella volume than the other two species. When we include head width in our model we find that the differences in tympanic membrane, inner ear volume, middle ear cavity volume, and columella volume are all best explained by the larger size of *E. 'magnus'*. Furthermore, when accounting for head width, the only morphological differences between species is in AP hair cell counts, with *E. 'magnus'* having more hair cells than the other two species.

Discussion

Peripheral auditory tuning varies among frogs in the *E. petersi* species complex, yet this divergence in auditory sensitivity does not match the species differences in preferences for conspecific whines or complex calls. *E. 'selva'* females prefer the higher frequency of *E. 'selva'* male calls, yet estimating the audibility of *E. 'selva'* females to calls does not predict greater sensitivity to their conspecific calls compared to heterospecific calls. Peripheral auditory system divergence does not explain species differences in preferences for complex calls, nor does it match the morphological divergence in ear structures. The patterns of auditory divergence suggest sensory drive or Fisher-Lande processes are unlikely drivers of signal diversification, and results are consistent with two prior hypotheses of correlated laryngeal evolution and reinforcement.

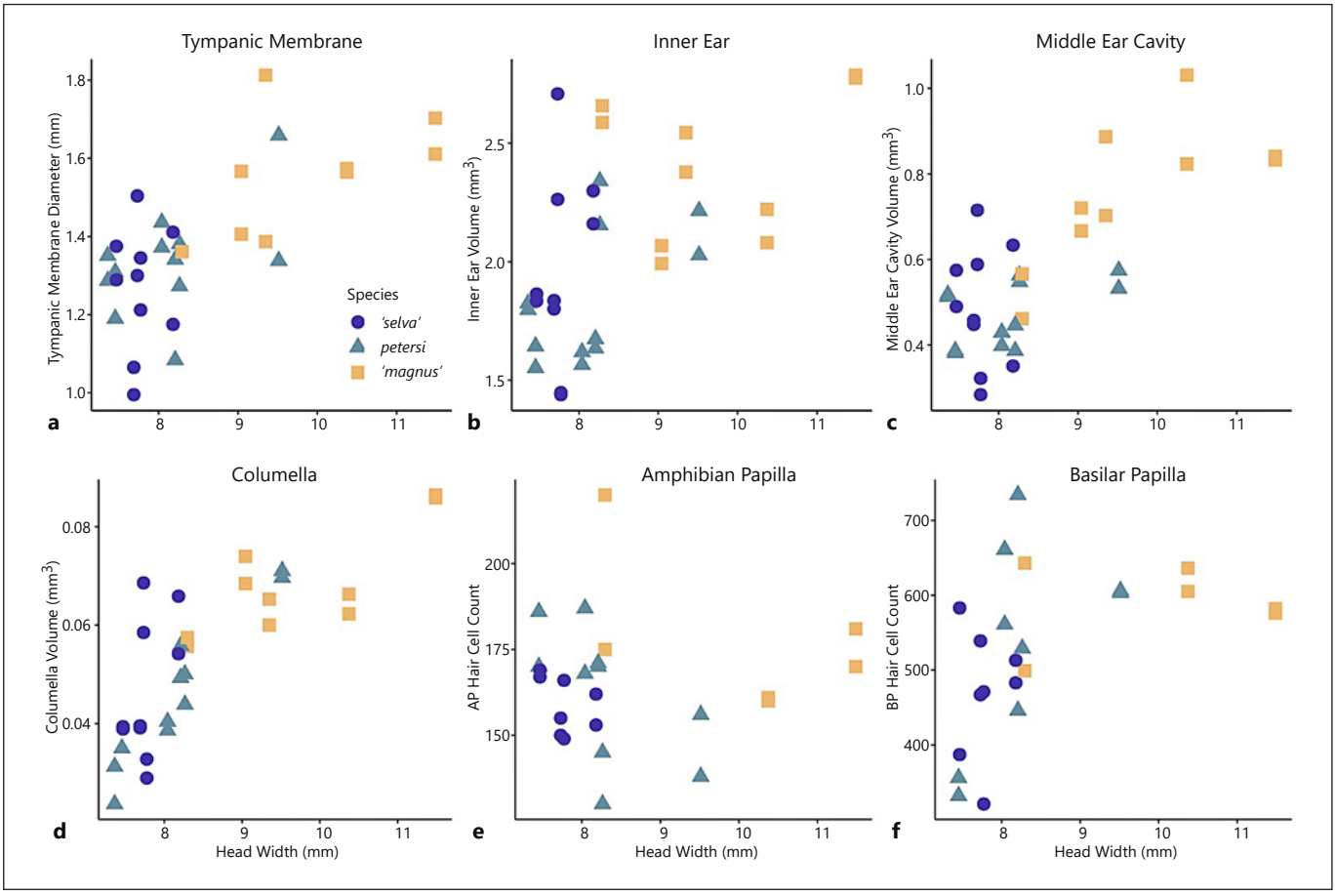

Fig. 7. Scatterplots showing the relationship between head width and the size of various middle ear features. Points represent individuals distinguished by species using color and shape: *E. 'selva'* (blue circles), *E. petersi* (teal triangles), *E. 'magnus'* (gold squares).

Species Differences in Sensitivity rather than Tuning

We find striking differences among females of the three species in sensitivity over a broad range of frequencies. *E. 'selva'* females are more sensitive than *E. 'magnus'* or *E. petersi* females over much of the amphibian papilla range (600–1,100 Hz), and *E. petersi* females are more sensitive over much of the basilar papilla range (2,400–3,300 Hz). Our evidence that tuning has diverged among species is much less clear, as *E. 'magnus'* females and all males are most sensitive to 500 Hz tones, whereas *E. 'selva'* and *E. petersi* females are more sensitive to 600 Hz tones. These differences are slight, however, and no shift is evident in the best excitatory frequency in the basilar papilla range (approximately 3,000 Hz). Several caveats limit our ability to compare auditory sensitivity using ABRs. We note that the broad tuning in the basilar papilla range essentially prevents a meaningful comparison of best excitatory frequencies among the species in this study. Frog species vary in basilar papilla tuning width [Zakon and Wilczynski, 1998], and we could not identify reliable best-excitatory frequencies for species or many individuals within the species given their equivalent sensitivity across a wide frequency range (not shown). Moreover, differences in species in transmission of electrical signals through the skull or in overall signal amplitude (e.g., number of neurons) complicate ABR threshold comparisons. We argue that such differences are unlikely to account for the differences in sensitivity we demonstrated here in these closely related, similarly shaped species. Click amplitudes did not vary consistently depending on sex and species, suggesting that overall ABR thresholds are not responsible for species differences in sensitivity.

Table 6. Main effects of species on ear morphology with and without controlling for variation in head width

Dependent variable	*F* value, *p* value, effect size with 90% confidence interval		Pairwise species comparisons
	Head width	Species	
Tympanum diameter	$F(1,29) = 5.986$ **$p = 0.021$** effect size = 0.17 (0.02, 0.37)	$F(2,29) = 1.092$ $p = 0.349$	
Columella volume	$F(1,16) = 16.998$ **$p < 0.001$** effect size = 0.51 (0.21, 0.70)	$F(2,16) = 0.6404$ $p = 0.540$	
Middle ear volume	$F(2,16) = 7.433$ **$p = 0.015$** effect size = 0.32 (0.04, 0.56)	$F(2,16) = 2.3286$ $p = 0.130$	
Inner ear volume	$F(1,16) = 1.861$ $p = 0.191$	$F(2,16) = 1.5861$ $p = 0.235$	
Amphibian papilla hair cell number	$F(1,12) = 7.432$ **$p = 0.019$** effect size = 0.39 (0.05, 0.64)	$F(2,12) = 6.6487$ **$p = 0.012$** effect size = 0.53 (0.12, 0.72)	selva-petersi: $p = 0.508$ **selva-magnus: p = 0.011** **petersi-magnus: p = 0.017**
Basilar papilla hair cell number	$F(1,12) = 1.885$ $p = 0.195$	$F(2,12) = 0.550$ $p = 0.591$	
Tympanum diameter	NA (not in model)	$F(2,15) = 10.088$ **$p = 0.002$** effect size = 0.57 (0.23, 0.73)	selva-petersi: $p = 0.534$ **selva-magnus: p = 0.0016** **petersi-magnus: p = 0.0094**
Columella volume	NA (not in model)	$F(2,16) = 5.504$ **$p = 0.015$** effect size = 0.41 (0.07, 0.62)	selva-petersi: $p = 0.986$ **selva-magnus: p = 0.036** **petersi-magnus: p = 0.021**
Middle ear volume	NA (not in model)	$F(2,16) = 10.67$ **$p = 0.001$** effect size = 0.57 (0.24, 0.73)	selva-petersi: $p = 0.978$ **selva-magnus: p = 0.0040** **petersi-magnus: p = 0.0019**
Inner ear volume	NA (not in model)	$F(2,16) = 5.258$ **$p = 0.018$** effect size = 0.40 (0.06, 0.61)	selva-petersi: $p = 0.766$ selva-magnus: $p = 0.080$ **petersi-magnus: p = 0.017**
Amphibian papilla hair cell number	NA (not in model)	$F(2,12) = 1.7956$ $p = 0.209$	
Basilar papilla hair cell number	NA (not in model)	$F(2,12) = 2.4587$ $p = 0.127$	

Decoupling Peripheral Auditory System Evolution, Signal Conspicuousness, and Mate Preferences

Simmons [2013] calls for extending the concept of Capranica's matched filter hypothesis to consider frog calls that excite only one of the two auditory end organs and taxa for which variation in temporal patterns of calls is critical for mate choice. Earlier work in the *E. pustulosus* species group did not find consistent variation across species in whine frequency and AP best excitatory frequency, and our results show a similar lack of simple concordance between species-typical mate preferences for the conspecific whine and species differences in peripheral sensitivity to acoustic signals. *E. 'selva'* females prefer higher frequency conspecific whines [Boul et al., 2007], yet the frequency at which the amphibian papilla of *E. 'selva'* females is maximally sensitive (600 Hz; Fig. 3) is the same as the best excitatory frequency of *E. petersi* females. Moreover, the model-estimated hearing thresholds for whines of each species do not predict that *E. 'selva'* females are most sensitive to calls of *E. 'selva'* males (Fig. 4). This suggests that simple estimates of conspicuousness based on tuning of the auditory periphery do not predict mate preferences in *Engystomops* frogs. All of these frogs share this downward frequency sweep in the whine that spans much of the amphibian papilla range of maximal sensitivity, hence the matched filter hypothesis

might simply not be as relevant as for species without frequency modulation, i.e., consistent changes in frequency over time.

Similarly, the species differences in sensitivity to the squawk do not predict preferences for the squawk. *E. 'magnus'* females prefer the complex whine-squawk call over the simple conspecific whine, whereas *E. 'selva'* females do not distinguish between simple and complex calls [Boul et al., 2007]. Yet *E. 'selva'* females have lower estimated thresholds, equivalent to higher sensitivity, for the squawk than do *E. 'magnus'* frogs. These species differences in sensitivities to the squawk are not striking, yet the opposite direction of effect rules out peripheral auditory tuning explaining species differences in preferences for complex calls. The acoustic structure of squawks, with their broadband energy covering an intermediate frequency range, perhaps does not lend itself to a simple matched-filter explanation. Moreover, the squawks are much less detectable than the whines for the peripheral auditory system, as models estimate approximately 10 dB higher thresholds for squawks compared to whines. We thus suggest squawk preference may not depend on higher conspicuousness of the complex calls relative to simple calls.

Despite this lack of concordance, our inferences do not preclude the possibility that signal conspicuousness impacts mate preferences in natural environments. First, our measure of thresholds is most relevant for long-distance detectability of signals, for example when females locate choruses. Mate choice likely occurs at higher signal amplitudes, as the level of *E. 'magnus'* male calls is approximately 80 dB SPL at the source (not shown). Published evidence suggests that peripheral sensitivity in frogs may remain linear over a large range of amplitudes [Mason and Narins, 2002; Penna et al., 2009], so the rank order in sensitivity may apply at closer range. Second, we estimated the detectability of signals without modeling cross-frequency interference or background noise. These cryptic species overlap in ranges, mating at times in the same pond on the same night and occasionally hybridizing [Trillo et al., 2017], so we know some individuals from each species are subject to similar background noise in their current distribution. Neural responses to complex acoustic signals in complex acoustic backgrounds as well as transmission through the environment will modify the actual conspicuousness of calls in natural settings. Further, attentional biases may also moderate conspicuousness; intrinsic biases in which spectrotemporal combinations most grab the attention of frogs may be unrelated to peripheral sensitivity. Measures of peripheral sensitivity would ideally be coupled with psychophysical measures that capture perceptual biases across various acoustic environments. In sum, our model of the relative detectability of signals gives an estimate of the auditory selectivity based on peripheral stimulation as a baseline expectation from which to compare deviations in perceptual or behavioral measures. Mismatches as we demonstrate here for the modeled auditory responses to the conspecific whine or the squawk should spark further studies characterizing how auditory and attentional processes reconfigure the inherent peripheral biases to alter signal preferences, leading to insight into the evolutionary processes underlying signal divergence and behavioral isolation.

Implications for Signal Diversification and Behavioral Isolation in E. petersi Complex

Our findings narrow the likely evolutionary scenarios for signal diversification and behavioral isolation among the cryptic species in the *E. petersi* complex. Several evolutionary scenarios for reproductive isolation and speciation in the *E. petersi* clade posit correlations between morphological traits and female mate preferences. For example, female mate preferences could have diverged as a byproduct of natural selection on body size or head shape rather than selection on auditory responses per se, or male calls and female preferences could co-evolve if both are linked to head size in a consistent fashion. We measured the ear dimensions we deemed likely to evolve as a correlated response to selection on skulls or overall body size, and found a general lack of concordance between species differences in anatomical measurements and in peripheral auditory tuning. The decoupling of head size measurements, hearing sensitivity, and mate preferences limits the scenarios by which sensory drive or Fisher-Lande processes might have created behavioral isolation.

Early studies of signal diversification in the *E. petersi* complex [Boul et al., 2007] proposed that sexual selection was driving speciation in this lineage (but see Ron [2008] for alternative proposal). Boul et al. [2007] proposed that female receivers in this group had a preexisting bias for complex calls (whine-squawks), and that genetic differences that altered male larynges to produce complex calls in *E. 'magnus'* was advantageous due to sexual selection and spread through the lineage; this divergence in larynges then lowered the dominant frequency of simple whines as well, and female preferences for simple calls diverged as a consequence (e.g., to reduce search costs of females). Because this scenario does not posit genetic cor-

relations between mate preferences based on whine frequency and for whine-squawks over whines, the lack of concordance between the divergence in hearing sensitivities and behavioral preferences does not conflict with this evolutionary scenario.

Reinforcement scenarios that posit selection against hybridization as a primary driver of divergence in the communication system remain strong candidate mechanisms for reproductive isolation in this lineage. Guerra and Ron [2008] highlighted evidence that reinforcement led to increased whine frequency and preferences for higher frequency whines in *E. 'selva'*, in contrast to the evolutionary scenario proposed by Boul et al. [2007]. We have subsequently discovered extensive hybrid inviability in some interspecific crosses [Trillo et al., 2017]. Reinforcement makes no assumptions about how preference divergence arises; genetic changes are favored if they prevent hybridization via any changes in hearing, auditory processing, or sensory-motor transformation, as long as costs are not too high. Although we have not identified the neural substrate mediating species-typical preferences, the lack of concordance between hearing sensitivity and preferences are consistent with reinforcement as a driver of behavioral isolation.

Conclusions

We find evidence of species divergence in peripheral sensitivity within the *E. petersi* cryptic species complex, yet this divergence does not match divergence in mate preferences or morphology. The major auditory changes reflect differences in relative sensitivity rather than shifts in frequency tuning. Analyzing complete audiograms rather than comparing simpler measures of the frequency of maximal sensitivity in the amphibian and basilar papilla gave a more complete understanding of the evolution of the auditory periphery in this species complex. We introduced and validated a new approach for estimating the peripheral auditory responses to mating signals, and this approach allowed us to demonstrate that divergence in mating preferences in the *E. petersi* species complex is not a simple consequence of species differences in call conspicuousness. These results further call into question how relevant the matched filter hypothesis is for frogs producing calls in which frequency modulation and temporal patterning is a key element of mating calls and preferences, and highlights the key role of central processing in preference evolution.

Acknowledgements

We acknowledge Santiago Ron for logistical support to make working at these field sites possible, including help with permits, tissue export, and DNA sequencing and analysis. We acknowledge Andrea Narvaez for frog care, administrative support, call recordings from *E. petersi* males, and species identification. We acknowledge Chris Funk for call recordings from *E. 'magnus'* and *E. 'selva'* males. We also thank three reviewers for constructive input that improved our manuscript.

Statement of Ethics

All animal protocols were approved by Institutional Animal Care and Use Committee of Colorado State University (#09-1397A).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

We thank National Science Foundation IOS-0940466 to K.L.H. for funding.

Author Contributions

K.L.H. and J.C.-D. recorded auditory sensitivity of frogs. M.C.W. prepared histological sections and conducted the imaging and analysis. All authors contributed to experimental design, analysis, writing, and editing.

Data Availability Statement

All data presented in the analysis will be available in Dryad upon publication.

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