

Trait independence primes convergent trait loss

Molly C. Womack,^{1,2,3} Tyler S. Fiero,¹ and Kim L. Hoke¹

¹Department of Biology, Colorado State University, Fort Collins, Colorado 80523

²Department of Vertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, District of Columbia 20560

³E-mail: mollywo@berkeley.edu

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The repeated, independent evolution of traits (convergent evolution) is often attributed to shared environmental selection pressures. However, developmental dependencies among traits can limit the phenotypic variation available to selection and bias evolutionary outcomes. Here, we determine how changes in developmentally correlated traits may impact convergent loss of the tympanic middle ear, a highly labile trait within toads that currently lack adaptive explanation. The middle ear's lability could reflect evolutionary trade-offs with other skull features under selection, or the middle ear may evolve independently of the rest of the skull, allowing it to be modified by active or passive processes without pleiotropic trade-offs with other skull features. We compare the skulls of 55 species (39 eared, 16 earless) within the family Bufonidae, spanning six hypothesized independent middle ear transitions. We test whether shared or lineage-specific changes in skull shape distinguish earless species from eared species and whether earless skulls lack other late-forming skull bones. We find no evidence for pleiotropic trade-offs between the middle ear and other skull structures. Instead, middle ear loss in anurans may provide a rare example of developmental independence contributing to evolutionary lability of a sensory system.

KEY WORDS: Bufonidae, developmental bias, pleiotropy, tympanic middle ear, Williston's law.

The repeated, independent evolution of traits (convergent evolution) is often attributed to shared environmental selection pressures (Rüber and Adams 2001; Harmon et al. 2005; Rosenblum 2005; Edwards et al. 2012; McCurry et al. 2017). However, developmental dependencies among traits can contribute to convergent evolution by limiting the phenotypic variation available to selection (Arthur 2002; Beldade et al. 2002; Kavanagh et al. 2013). Thus, developmental factors can bias evolutionary lability and may help explain convergent traits that lack adaptive explanation. For example, convergent eye loss in cavefish, once ascribed to a combination of relaxed selection pressures and high energetic cost, is now attributed to selection for enhanced taste buds that are genetically linked to eye reduction (Yamamoto et al. 2009). Thus, trait lability can result from genetic and/or developmental links among traits (trait integration) that cause traits to change in concert (Wright 1964, 1980; Mayr 1976; Kavanagh et al. 2013). On the other hand, traits capable of evolving independently of

other traits are freed from pleiotropic trade-offs and may be more flexible (Beldade et al. 2002; Esteve-Altava et al. 2014). Here, we investigate whether convergent loss of the tympanic middle ear in anurans, which currently lacks adaptive explanation, is associated with changes in other skull features or whether the middle ear shows evolutionary independence with respect to developmentally linked skull features.

Most tetrapods have a tympanic middle ear, which functions to transmit airborne sound from the environment to the inner ear sensory hair cells (reviewed in Manley 2010). The anuran middle ear functions similarly to those found in other tetrapods and consists of numerous structures: a tympanic membrane surrounded by a cartilaginous tympanic annulus, a mostly bony middle ear column, and a middle ear cavity connected to the buccal cavity via the Eustachian tube (Lewis and Narins 1999). In species that have lost their tympanic middle ears, a condition termed earlessness, the inner ear remains but all middle ear structures are missing

(Wever and Capranica 1985; Jaslow et al. 1988), eliminating this mode of transferring sound from the environment to the inner ear. Middle ear loss, which is associated with reduced high-frequency hearing sensitivity (Womack et al. 2017), is especially perplexing in anurans because frogs and toads are well known for their use of acoustic communication in finding mates, territoriality, and defense (reviewed in Wells and Schwartz 2007). Yet, at least 38 independent losses of middle ear structures have occurred across anurans (Pereyra, Womack et al. 2016) without evidence of shared environmental selection pressures across ear transitions (Jaslow et al. 1988). It could be that the middle ear is genetically and/or developmentally integrated with other parts of the skull, such that its loss is linked to changes in other skull features with fitness impacts that might explain ear loss. Alternatively, the middle ear may be genetically and developmentally independent from the rest of the skull, allowing for its repeated loss by selection or drift processes. These hypotheses are explained in turn in the following paragraphs.

Developmental integration with other skull features could lead to middle ear lability when skull evolution occurs. In anurans, middle ear structures arise from three separate neural crest streams (Cerny et al. 2004; Gross and Hanken 2008; Chapman 2011), linking development of the ear to the formation of most other skull structures. Furthermore, the genes patterning middle ear structures also pattern many other skull features in vertebrates (Chapman 2011). These genetic and developmental links to the rest of the skull make the middle ear a likely candidate for pleiotropy (Paaby and Rockman 2013). An example of pleiotropy driving sensory loss is found in beetles of the genus *Onthophagus*. *Onthophagus* eyes and horns are genetically linked, and eye reduction allows for larger sexually selected horns (Nijhout and Emlen 1998). Like *Onthophagus* eyes, middle ear transitions could result from advantageous changes in genetically or developmentally linked skull structures that could offset any negative consequences of reduced hearing. However, genetically correlated structures do not always evolve together, and within anurans, little work has tested whether the middle ear is lost in concert with other skull changes that may have adaptive value.

On the other hand, traits that can evolve independently may be more easily modified by selection or vulnerable to loss via nonadaptive processes because of the lack of negative pleiotropic consequences. Evidence for evolutionary loss of developmentally independent traits is found throughout the evolution of the tetrapod skull, which has consistently lost bones over evolutionary time (Williston's law; Gregory 1935). Bones lost from the tetrapod skull are usually less developmentally and functionally dependent on other skull bones (Esteve-Altava et al. 2014). However, bones lost from the tetrapod skull typically lack specialized functions (Esteve-Altava et al. 2014), making the loss of the middle ear bone in anurans an unusual case. Development data indicates the

middle ear may be weakly linked to the rest of skull development in the anuran skull. The middle ear is known to vary in developmental timing (Gaudin 1978; Hanken and Hall 1984; Wiens 1989; Kerney et al. 2007), indicating developmental lability. Moreover, the middle ear develops quite late compared to other skull features (Sedra and Michael 1959; Gaudin 1978; Hanken and Hall 1984; Hetherington 1987; Wiens 1989; Kerney et al. 2007; Weisbecker and Mitgutsch 2010), which could allow loss via a change in late development that avoids affecting earlier developing traits. Lack of developmental links to other skull features, in concert with either direct selection pressures or accumulated nearly neutral mutations via genetic drift, could explain the evolutionary lability of middle ears.

To address whether middle ear lability is associated with pleiotropic changes in developmentally linked skull features or whether the middle ear is evolving largely independently of the rest of the skull, we compared skull morphology of 55 species (39 eared, 16 earless) within the family Bufonidae. The family Bufonidae has the largest number of ear loss events, and also has potential regains of the ear structures (Pereyra, Womack et al. 2016), making it ideal for comparing closely related eared and earless species. Our sampling captured six hypothesized independent middle ear transitions (Pereyra, Womack et al. 2016), allowing us to distinguish changes in the skull associated with earlessness from changes due to shared phylogenetic history. Specifically, we tested (1) whether earless toad skulls shared changes in skull shape, (2) whether earless toad skulls show different evolutionary rates compared to eared toad skulls, and (3) whether earless toad species lack other skull bones. Evidence supporting any these hypotheses could illuminate potential pleiotropic trade-offs of earlessness in bufonids. In contrast, a lack of support for these hypotheses would demonstrate that the middle ear is evolving independently of the rest of the skull, allowing it to be lost by active or passive processes without large pleiotropic consequence to other skull features. Testing these hypotheses will illuminate how developmental processes could bias middle ear evolvability in anurans.

Materials and Methods

SPECIMEN COLLECTION AND FIXATION

We examined 102 total specimens from 55 species (39 eared, 16 earless) in this study. These species were chosen to capture closely related eared and earless species across many independent ear loss events within the family Bufonidae (Pereyra, Womack et al. 2016). Our chosen earless species were previously shown to lack all middle ear structures and our eared species were previously shown to have all middle ear structures, with the exception of our eared *Atelopus* species that lack a tympanic membrane but have all other middle ear structures. For most species, two

specimens were available, however, a few species were represented by only one specimen and two species had three specimens. We obtained 49 species (89 specimens) from the Smithsonian National Museum of Natural History (Supporting Information 1). We collected an additional six species (13 specimens) from field sites in Ecuador and Peru (Supporting Information 2). The Institutional Animal Care and Use Committee approved all collections methods (IACUC Protocol #12-3484A) and the Ministerio del Ambiente in Ecuador and the Servicio Nacional Forestal y de Fauna Silvestre in Peru approved collection, research, and export permits. Field-caught animals were euthanized with 20% topical benzocaine and then decapitated. We preserved the head of each specimen in 4% paraformaldehyde diluted in phosphate-buffered saline from 16% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA) for 24 hours and performed three 15-minute rinses in phosphate-buffered saline before storing cranial tissue in 70% ethanol.

MicroCT SCANNING

We used X-ray micro-computed tomography (microCT) to analyze differences in skull shape and presence/absence of specific bones. The microCT Core Laboratory at the UT Health Science Center scanned and reconstructed all samples. We secured toad skulls to the specimen stage using Parafilm (American National Can, Greenwich, CT) and scanned the skulls in air in a high-resolution desktop microCT system (Skyscan 1173, Bruker Skyscan, Aartselaar, Belgium). Scan settings were: 60 kV, 133 μ A beam intensity, a 0.7°, four frame averaging, and a 1000 millisecond exposure time at each step. We used a 1 mm aluminum filter during scans (Kovács et al. 2009). We set the pixel size to 10 μ m for most skulls under 25 mm head width and 30 μ m for most skulls with a head width greater than 25 mm. We reconstructed the images with NRecon (Bruker SkyScan, Aartselaar, Belgium) with a Feldkamp cone-beam algorithm (Feldkamp et al. 1984). We used a polynomial correction to reduce beam-hardening effects during reconstructions (Kovács et al. 2009; Zou et al. 2011). We imported the bmp files from the reconstructions into Fiji (Schindelin et al. 2012) at a resolution of 60 μ m and created a 3D surface model for each skull using the 3D viewer plugin (Schmid et al. 2010).

LANDMARK PLACEMENT

For the geometric morphometrics analysis of overall skull shape, we created 3D surface models of the skulls within the 3D viewer plugin (Schmid et al. 2010) of Fiji (Schindelin et al. 2012) and selected thresholds manually for each skull to best depict all skull features. We then imported these 3D surface models into Landmark (Wiley et al. 2005) and placed 57 3D landmarks on specific cranial elements that were reliably found in each skull, defined

the borders of many bones, and characterized overall skull shape (Fig. 1).

SKULL SHAPE ANALYSES

We initially aligned all of the skulls and landmark data generated from placing the 57 landmarks on each of our skulls in the program MorphoJ (Klingenberg 2011). We imported our landmark data into MorphoJ and performed a Procrustes fit to the landmark data, which superimposes the landmarks from each skull onto one another so that they can be appropriately compared. We then averaged the skull size (centroid size) and shape data from the 2–3 specimens for each species to use in downstream comparative analyses, which were all performed within the package *geomorph* (Adams and Otarola-Castillo 2013) in R (R Core Team 2015).

Next, we visualized differences between eared and earless skulls by performing a Principal Component Analysis on size-corrected shape data. First, we ran a Procrustes ANOVA to correct our Procrustes-aligned data for size while accounting for phylogenetic relationships. We used the Procrustes data from MorphoJ as the dependent variable, log skull centroid size as the independent variable, and the most recent phylogenetic tree of amphibians (Pyron 2014), which we trimmed to our species using the package *phytools* (Revell 2012) in R (R Core Team 2015). We then performed a Principal Components Analysis on the residuals from this size corrected Procrustes ANOVA. We plotted the results of the Principal Component Analysis in phylomorphospace, which allowed us to visualize skull shape differences between and among eared and earless species that were not due to differences in skull size while examining phylogenetic relationships. Furthermore, we determined the amount of nonindependence in skull shape due to phylogenetic relatedness by estimating phylogenetic signal in skull shape after correcting for size using the multivariate version of the K-statistic (Kmult: Adams 2014).

To determine if eared and earless species differed consistently in skull shape, we used a phylogenetic analysis of variance for shape data (Procrustes ANCOVA) in the package *geomorph* (Adams and Otarola-Castillo 2013) within R (R Core Team 2015). We used the Procrustes data from MorphoJ as the dependent variable, log skull centroid size as an independent variable, and ear presence/absence as a second independent variable. The same phylogeny of amphibians used above (Pyron 2014) was used in this analysis. Using this model, we were able to test for differences in skull shape between eared and earless species while controlling for both the effects of phylogeny and size in a single model.

Finally, we determined whether the skulls of earless species were changing shape at a faster evolutionary rate than eared species using the *compare.evol.rates* function within the package *geomorph* (Adams and Otarola-Castillo 2013) within R (R Core Team 2015). Our first shape analysis would only detect differences between eared and earless species that were shared among

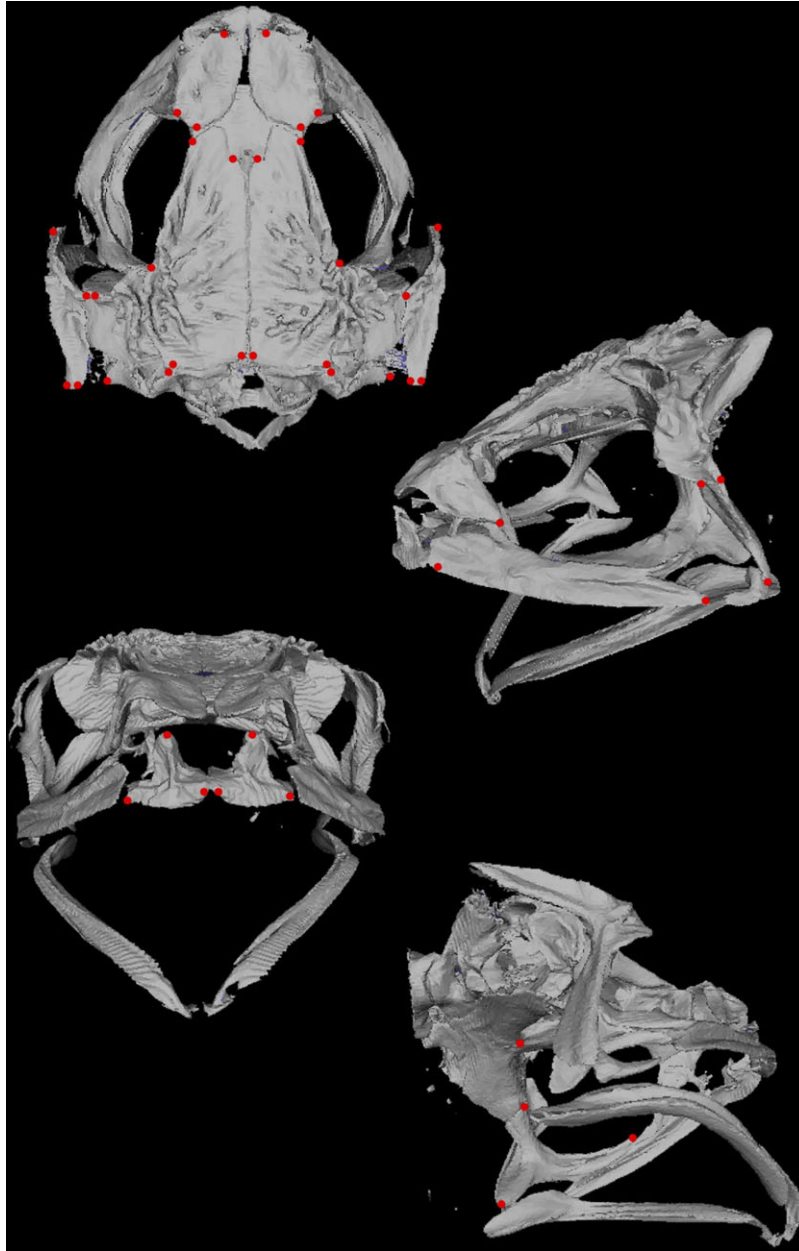


Figure 1. Landmark placement for size and shape analyses. The placement of all 57 landmarks is indicated with red dots on the skull.

earless species, but this second analysis only requires that earless skulls change shape more than eared species and does not require those shape changes to be shared among earless species. Thus, this evolutionary rate analysis could elucidate whether earless skulls were changing in lineage-specific ways compared to eared skulls because the shape changes do not have to be similar among earless species. We phylogenetically transformed our size-corrected shape data from our Procrustes ANOVA and then calculated the net rate of shape evolution for eared and earless species in the multidimensional space. We first calculated the evolutionary rate ratio for eared and earless species. We then tested for differences in evolutionary rate between eared and earless species by performing

a phylogenetically controlled simulation of the data under Brownian Motion (a stochastic model where the expected variance under Brownian motion increases linearly through time) using a common evolutionary rate pattern for all species and then determined whether the observed evolutionary rate ratio significantly differed from the 10,000 iterations of simulated evolutionary rate ratios.

BONE PRESENCE/ABSENCE SURVEY

To determine whether eared and earless species were missing any other bones consistently, we examined the 3D surface models in Meshlab (Cignoni et al. 2008). We examined all skulls for the loss

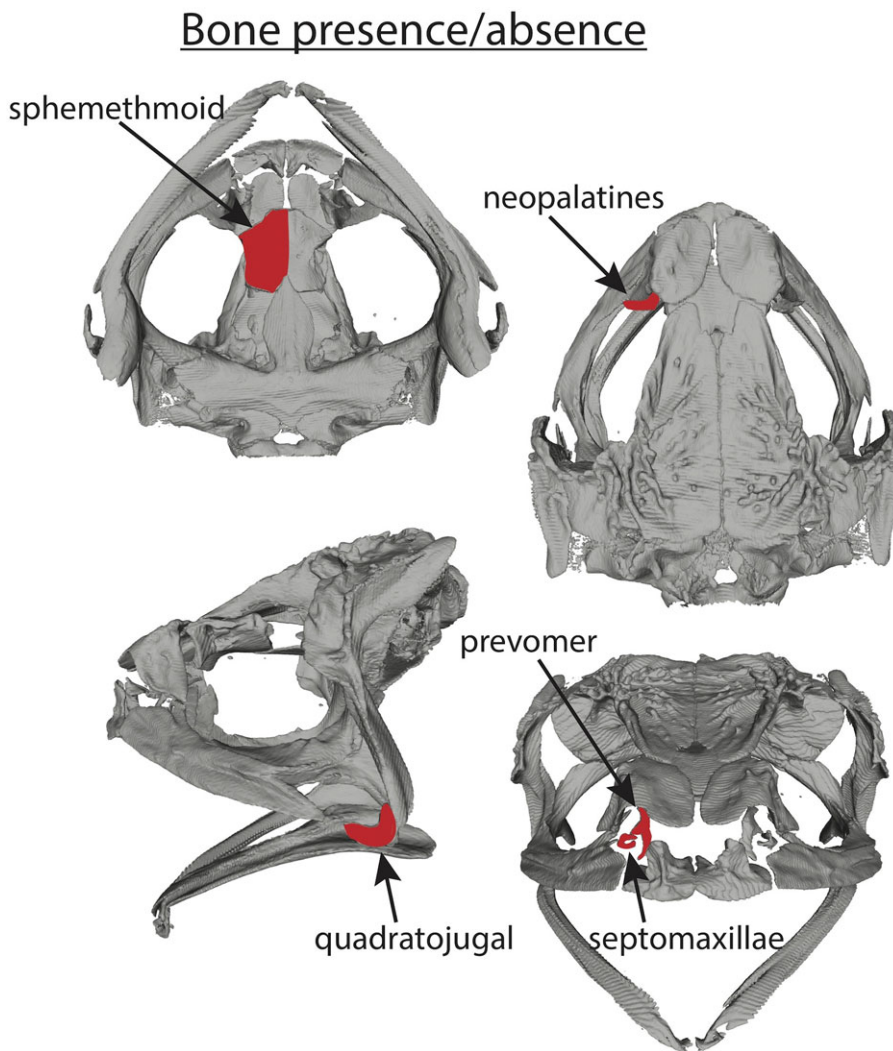


Figure 2. Bones surveyed for presence/absence. The five late-forming bones scored in our bone presence/absence analysis are highlighted in red and labeled.

of the neopalatines, quadratojugal, prevomers, septomaxillae, and sphenethmoid (Fig. 2) because presence/absence of these bones varies among anuran species (Yeh 2002) and they form around the same developmental period as the middle ear (Gaudin 1978; Weisbecker and Mitgutsch 2010).

RESULTS

COMPARISON OF SKULL SHAPE AND EVOLUTIONARY RATE BETWEEN EARED AND EARLESS SPECIES

We first confirmed ear status for all our species. Each eared species had a visible middle ear bone (Fig. 3), and each earless species completely lacked a middle ear bone. When we compared skull shape between our eared and earless species, we found that skull shape varied across the phylogeny (Fig. 4), and we detected phylogenetic signal for skull shape after controlling for

skull size ($K = 0.533$, $P = 0.001$). Eared and earless skulls did not differ in shape when accounting for both phylogeny and size ($F_{1,52} = 1.276$, $P = 0.368$) shown by the overlap in skull shapes of eared and earless species after correcting for size (Fig. 4). Furthermore, eared and earless skulls did not differ in evolutionary rate of skull shape (rate ratio (eared/earless) = 1.617; $P = 0.99$), indicated by the lack of significantly longer phylogenetic branches for earless lineages (Fig. 4).

LOSS OF LATE FORMING SKULL BONES IN EARLESS SPECIES

Late forming bones (neopalatines, quadratojugal, prevomers, septomaxillae, and sphenethmoid) were not consistently missing in eared or earless species. In addition to the middle ear, *Nectophryne batesii* was missing neopalatines and was the only earless species in which all examined specimens (a single specimen,

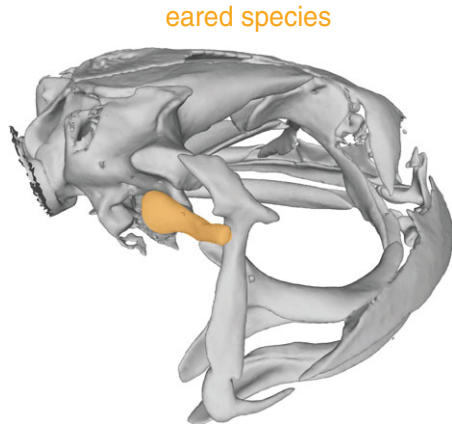


Figure 3. An example 3D mesh of an eared skull. The middle ear bone is highlighted in orange.

SVL = 21.3 mm) were consistently missing a skull structure other than the middle ear bone. A single *Dendrophryniscus brevipollicatus* specimen (SVL = 16.9 mm) lacked palatines however, a second, slightly larger specimen (SVL = 20.5 mm) had visible palatines. Three other earless species, *Werneria mertensiana*, *Melanophryniscus stelzneri*, and *Dendrophryniscus brevipollicatus*, had very small quadratojugals, however the bone was still present (full structures displayed in Fig. 2). No eared species

had reduced or missing skull bones (full dataset Supporting Information 3).

Discussion

Here, we provide evidence that the middle ear is able to evolve independently of other skull bones, which may contribute to middle ear evolvability and prime bufonids for convergent middle ear loss. No differences in skull shape or skull shape evolutionary rates distinguish earless from eared skulls, and we found no evidence of general bone loss in earless species. Overall, eared, and earless skulls were remarkably similar, indicating that middle ears are evolving independently in the bufonid skull, without large pleiotropic links to other cranial features. Below we discuss this independence as a contributor to trait evolvability and heterochrony as a potential mechanism for convergent middle ear loss.

MIDDLE EAR INDEPENDENCE AS A CONTRIBUTOR TO TRAIT LABILITY

Here, we show no changes in skull shape, evolutionary rate of skull shape, or skull bone loss associated with middle ear loss, supporting that the middle ear evolved independently of the rest of the anuran skull. Thus, middle ear loss in anurans may reflect

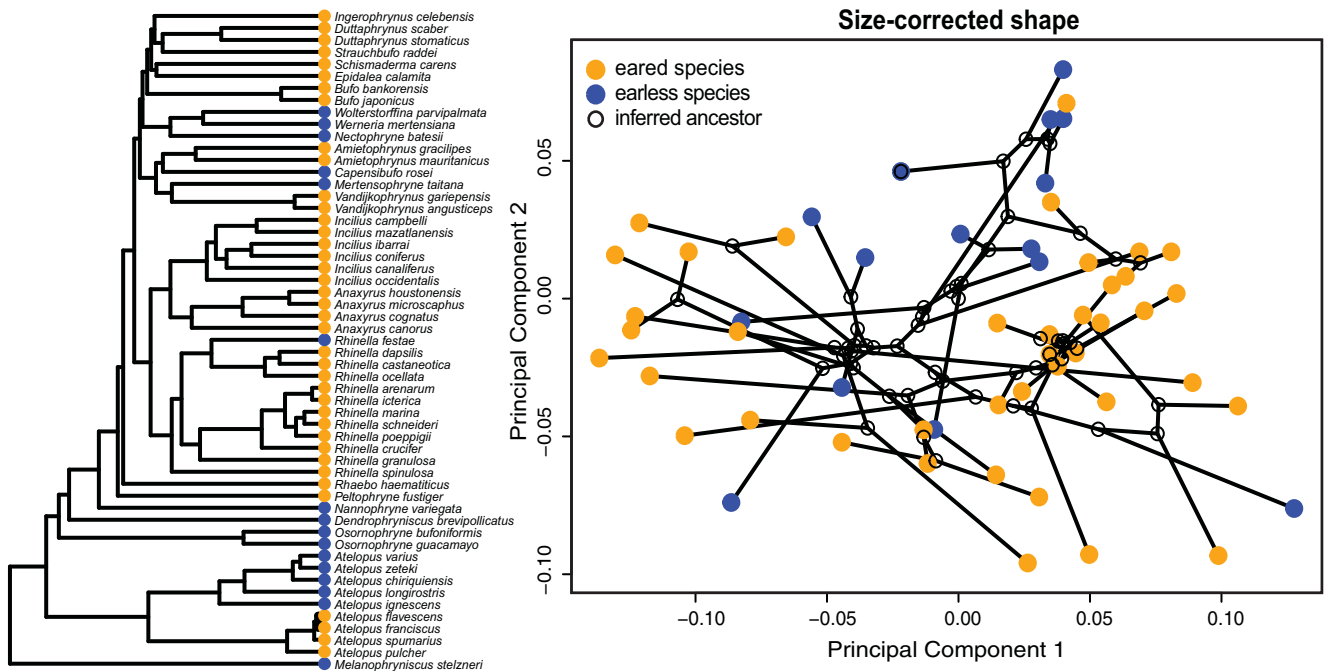


Figure 4. Skull shape differences between eared (orange) and earless (blue) species after adjusting for overall skull size. Left—Published phylogeny (Pyron 2014) trimmed to show only the relationships of the species in this study. Right—We plotted the first two principal components of variation in our size-corrected shape data. Each point represents the average skull shape after controlling for overall skull size for one species. Phylogenetic relationships and estimated ancestor relationships are indicated with black lines. Ancestral shape estimates are indicated at each node with the black, unfilled circle.

different underlying processes from other convergent sensory losses or reductions, such as eye reduction in beetles and eye loss in cavefish, in which pleiotropic trade-offs contribute to their repeated loss or reduction (Nijhout and Emlen 1998; Yamamoto et al. 2009). Soft tissue or noncranial traits not examined in this study may display pleiotropic trade-offs with anuran middle ear loss, however no morphological traits robustly correlated with earlessness have been identified to date, and no qualitative differences in muscles surrounding the ear have been found between closely related eared and earless species (Womack et al., unpubl. data). We therefore lack evidence that shape differences between eared and earless skulls confer fitness advantages that could offset potential fitness costs of reduced hearing sensitivity associated with earlessness (Womack et al. 2017).

Anuran middle ear loss may present a unique opportunity to study how developmental independence in concert with yet unknown selection pressures or accumulation of nearly neutral mutations via genetic drift can result in a surprising convergent phenotype. Other selection pressures to consider include indirect selection for faster developmental rate (see discussion below in heterochrony section), reduced energetic costs, or other shared ecological factors. It remains to be investigated whether earless species save energy from lack of middle ear growth or reduced neural firing due to diminished high frequency hearing sensitivity. And although alternative mating strategies such as visual signaling and explosive breeding may relax selection on high frequency hearing in earless species, we lack sufficient information about mating strategies of earless species for rigorous comparative studies, and many earless species produce high frequency advertisement calls (Loftus-Hills 1973; Lindquist et al. 1998; Boistel et al. 2011). The ultimate explanation of middle ear loss remains elusive; however, the independent evolution of the middle ear adds a new piece to this evolutionary puzzle.

THE POTENTIAL ROLE OF HETEROCHRONY IN CONVERGENT MIDDLE EAR LOSS

The combination of middle ear independence from the rest of the anuran skull (evidenced here) and its late formation (Hetherington 1987; Kerney et al. 2007) makes heterochrony (changes in development rate or timing) a likely contributor to the middle ear loss in anurans. Specifically, if middle ear development was delayed beyond all other skull structures (heterochrony via postdisplacement; Gould 1977; McNamara 1986), the middle ear could be lost without large effects on other skull features. Bufonids have a strikingly high number of convergent middle ear losses compared to other anuran families (Pereyra, Womack et al. 2016) and also show late completion of the tympanic middle ear compared to other anurans (Gaudin 1978; Hetherington et al. 1987; Smirnov 1991; Womack et al. 2016), indicating postdisplacement may be a mechanism of middle ear loss. In fact, Smirnov (1991)

proposed postdisplacement as a mechanism for incomplete differentiation of the tympanum in *Bufo bufo* (Smirnov 1991). Thus, heterochrony (as postdisplacement), which could result from selection for faster developmental rate, genetic drift, or genome size changes, is a viable mechanism for the evolution of earlessness. Comparing the development timing and rate of cranial structure formation among eared and earless species will provide critical insight into whether heterochrony contributes to middle ear loss and can explain how the middle ear is lost while other structures are retained.

CONCLUDING REMARKS

In sum, our analyses find little evidence for shifts in other skull features associated with lack of a middle ear in bufonids suggesting that middle ear structures evolve relatively independently from the rest of the skull. This puts the anuran middle ear within a considerable list of bones that have been lost from the tetrapod skull over time (Gregory 1935; Esteve-Altava et al. 2014). Like these other lost bones, the anuran middle ear is less developmentally and functionally dependent on other skull bones (Esteve-Altava et al. 2014). However, anuran middle ear loss is distinct from these other bone losses due to its specialized function in hearing (reviewed in Manley 2010) and the reduced auditory sensitivity of earless bufonids (Womack et al. 2017). We propose that the pervasiveness of ear transitions in anurans may be due to heterochrony via postdisplacement or other developmental mechanisms that enable mutations to alter middle ear development without detrimental effects on other traits. Why this seemingly important sensory system is commonly lost in anurans remains a perplexing; however, the apparent independence of middle ear development could be a key contributor to middle ear lability in anurans.

AUTHOR CONTRIBUTIONS

M.C. Womack contributed to the conception of the study, data collection, data analysis, and writing. T.S.F. contributed to data collection and writing. K.L. Hoke contributed to the conception of the study, data analysis, and writing.

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CONFLICT OF INTERESTS

The authors have declared no conflict of interests.

DATA ARCHIVING

The doi for our data is <https://doi.org/10.5061/dryad.6tn2n>.

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