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Whispers from vestigial nubbins: Arrested development provokes trait loss in toads

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Abstract

Despite the use of acoustic communication, many species of toads (family Bufonidae) have lost parts of the tympanic middle ear, representing at least 12 independent evolutionary occurrences of trait loss. The comparative development of the tympanic middle ear in toads is poorly understood. Here, we compared middle ear development among two pairs of closely related toad species in the genera *Atelopus* and *Rhinella* that have (eared) or lack (earless) middle ear structures. We bred toads in Peru and Ecuador, preserved developmental series from tadpoles to juveniles, and examined ontogenetic timing and volume of the otic capsule, oval window, operculum, opercularis muscle, columella (stapes), and extracolumella in three-dimensional histological reconstructions. All species had similar ontogenesis of the otic capsule, oval window, operculum, and opercularis muscle. Moreover, cell clusters of primordial columella in the oval window appeared just before metamorphosis in both eared and earless lineages. However, in earless lineages, the cell clusters either remained as small nubbins or cell buds in the location of the columella footplate within the oval window or disappeared by juvenile and adult stages. Thus, columella growth began around metamorphosis in all species but was truncated and/or degenerated after metamorphosis in earless species, leaving earless adults with morphology typical of metamorphic anurans. Shifts in the timing or expression of biochemical pathways that regulate the extension or differentiation of the columella after metamorphosis may be the developmental mechanism underlying convergent trait loss among toad lineages.

KEYWORDS

3D reconstruction, comparative development, heterochrony, progenesis

1 | INTRODUCTION

The evolutionary loss or reduction of a structure often results from the early termination of its developmental trajectory (Lande, 1978). For example, the loss of skeletal appendages such as limbs, pelvic girdle, sternal girdle, or digits in caecilians, salamanders, snakes, legless lizards,

amphisbeanians, flightless birds, and cetaceans has been attributed to an arrested step in embryonic development; the relative extent of evolutionary loss is determined by the timing of developmental truncation (Bejder & Hall, 2002; Essex, 1927; Galis et al., 2018; Lande, 1978; Rahmani, 1974; Saunders, 1948; Senter & Moch, 2015; Thewissen et al., 2006). Similarly, sensory organs such as

eyes that are reduced or lost in blind cavefish and cave salamanders are initiated in the lens and/or retina but then fail to fully differentiate and later degenerate (Berti et al., 2001; Dufton et al., 2012; Eigenmann & Denny, 1900; Tovar et al., 2018). Vestigial structures can offer clues to the embryonic history of mesenchymal condensations that failed to complete growth or differentiation as in their evolutionary ancestors (Bejder & Hall, 2002; Lande, 1978; Rahmani, 1974; Senter & Moch, 2015). In some cases, primordial buds are ontogenetically transient and regress during development via apoptosis, such as the horns of *Onthophagus* beetles (Moczek et al., 2006), hind limbs of cetaceans (Andrews, 1921; Thewissen et al., 2006), appendages of limbless lizards and snakes (Lande, 1978; Rahmani, 1974), teeth of baleen whales (Ishikawa & Amasaki, 1995), and eyes of cavefish (Berti et al., 2001; Dufton et al., 2012), and subterranean salamanders (Tovar et al., 2018). The initial condensation of a structure may be required due to developmental or genetic pleiotropy (Bejder & Hall, 2002; Dufton et al., 2012; Galis et al., 2018; Pottin et al., 2011; Wilkens, 2007); for example, correct formation of the forebrain and some cranial bones in blind cavefish requires the presence of the eye rudiments in early development (Dufton et al., 2012; Pottin et al., 2011; Wilkens, 2007). Vestigial rudiments can shed light on the evolutionary history and developmental mechanisms of lost structures, but the developmental underpinnings of many cases of evolutionary loss are poorly understood (Schoch, 2014; Senter & Moch, 2015).

The loss of skull bones has occurred frequently in tetrapod evolution, and is hypothesized to have resulted from truncated development (Gregory, 1935; Maddin et al., 2010; Schoch, 2014; Smirnov, 1991). In general, developmental arrest and degeneration can result from changes in gene expression due to mutation of cell proliferation or organizational genes (Bejder & Hall, 2002; Lande, 1978; Rahmani, 1974) and/or changes in the timing of developmental events (heterochrony; Alberch et al., 1979; Dufton et al., 2012; Pottin et al., 2011). Proposed mechanisms for the truncation of cranial bone development include failure to ossify (Weisbecker & Mitgutsch, 2010), primordial fusion of mesenchymal structures (Koyabu et al., 2011; Maddin et al., 2010), and/or heterochronic processes (see Table 1 in Albrech et al., 1979) such as progenesis (early offset; Shkil & Smirnov, 2015; Smirnov, 1991), postdisplacement (late onset; Smirnov, 1991), or neoteny (slowed developmental rate; Womack et al., 2019). The developmental processes that underlie the loss of skull bones remain unclear in many cases, and maybe taxon- or structure-specific. Empirical data that would allow for a comparison of the morphogenesis of skull structures among closely related species that do and do not show cranial reductions are limited.

One skull structure, the tympanic middle ear, has been lost independently in at least 38 anuran lineages despite its importance in hearing (Pereyra et al., 2016; Womack et al., 2017). Independent losses have occurred in 10–17 lineages just within the true toads (family Bufonidae), leaving over 200 of 618 toad species “earless” (Pereyra et al., 2016). The tympanic middle ear facilitates hearing of high-frequency sound—such as most anuran calls—through the reception of airborne vibrations and transmission to sensory hair cells via fluid in the inner ear (Wever, 1985; Womack et al., 2017, 2019). This complex of structures is comprised, distal-to-medial, of the epithelial tympanic membrane, cartilaginous tympanic annulus, and the three components of the columella (or stapes) situated within the middle ear cavity: cartilaginous extracolumella (*pars externa plectri*), bony and cartilaginous columella shaft (*pars media plectri*), and cartilaginous columella footplate (*pars interna plectri*; reviewed in Pereyra et al., 2016). The columella footplate sits within the connective tissue of the oval window in the otic capsule of the inner ear, adjacent to the cartilaginous operculum, which is a key middle ear structure implicated in a second, independent pathway by which vibrations reach sensory neurons in the inner ear via the tonic opercularis muscle (Hetherington, 1987; Lombard & Straughan, 1974).

During evolutionary reduction or loss, the first structures to disappear are generally late-developing and distal rather than medial, and thus are eliminated from ancestral morphology via the progressively earlier onset of cell death and subsequent degeneration (Bejder & Hall, 2002; Eigenmann & Denny, 1900; Essex, 1927; Lande, 1978; Wilkens, 2007; Weisbecker & Mitgutsch, 2010). Within anuran lineages with tympanic middle ear loss, structures are predictably reduced in a distal to medial pattern; a lineage may lose all five components or some distal structures, but never loses medial structures while distal structures are present (see Pereyra et al., 2016). The tympanic middle ear is known to develop late in bufonid ontogeny, completing development weeks or even months after metamorphosis (Fabrezi & Goldberg, 2009; Hetherington, 1987; Smirnov, 1989; Womack, Stynoski et al., 2018). Some experimental work suggests that the development of middle ear structures is obligatorily sequential such that truncation of medial structures is always associated with the loss of distal structures (Pereyra et al., 2016): ablation of the otic capsule leads to the loss of the *pars interna* and *pars media plectra* in the mouse, chick, turtle, and salamander (Thompson et al., 2012) and ablation of the anuran tympanic annulus results in the loss of the tympanic membrane (Helff, 1928). Furthermore, ear structures that are only partially developed, such as a sickle-shaped

rather than fully circular tympanic annulus, lack the hearing functionality of complete tympanic middle ears in toads (Womack, Christensen-Dalsgaard et al., 2018). However, the ontogenesis of tympanic middle ear structures has not been described in closely related eared and earless species (Hetherington, 1987; Womack et al., 2016; Womack, Stynoski, et al., 2018), which precludes comparative analysis of developmental trajectories to elucidate mechanisms that provoke trait loss in these anuran skull bones.

In this study, we contrasted the ontogeny of tympanic middle ear structures within pairs of eared and earless toad species. Our main objective was to investigate whether the developmental processes associated with the loss of tympanic middle ear structures in the anuran skull are consistent with the developmental mechanism of arrest and degeneration of distal structures as seen in other cases of evolutionary trait loss such as appendages and eyes. In addition, we sought to investigate the proposed hypothesis that heterochronic processes such as postdisplacement are associated with loss of tympanic middle ear structures in toads (see Shkil & Smirnov, 2015; Smirnov, 1991; Womack et al., 2019) via comparisons of the developmental onset and offset of the columella, extracolumella, otic capsule, oval window, operculum, and opercularis muscle among eared (*Rhinella alata*, *Atelopus* sp. [*spumarius* complex]) and earless (*R. yunga*, *A. elegans*) species pairs.

2 | MATERIALS AND METHODS

2.1 | Animal breeding and rearing

In this study, we reared four bufonid species that vary in tympanic middle ear completeness as adults: *Atelopus* sp. (*spumarius* complex) has a complete columella, partial sickle-shaped tympanic annulus, and no tympanic membrane; *A. elegans* has no middle ear structures; *R. alata* has a complete middle ear; and *R. yunga* lacks all middle ear structures (Pereyra et al., 2016; Womack et al., 2017). The procedures employed in this study complied with guidelines for the care and use of animals and were ethically reviewed and approved by the Institutional Animal Care and Use Committee at Colorado State University (protocol 15-5715A). Samples of *Atelopus* were produced as part of a captive breeding program for these critically endangered species (IUCN, 2019).

To produce developmental series of *R. alata*, *A. elegans*, and *A.* species (*spumarius* complex, *A.* sp 2 *sensu* Womack, Christensen-Dalsgaard, et al., 2018), we collected several adults from field sites in Ecuador in 2014–2016 (see Womack, Christensen-Dalsgaard, et al., 2018:

Table 1 for collection sites) and placed them in laboratory conditions at the Centro Jambatu de Investigación y Conservación de Anfibios in Quito, Ecuador (CJ). We fed adults with crickets (*Gryllus* sp., *assimilis* complex), dusted once weekly with mineral powder (Calcium Plus, Repashy Ventures, Inc., CA, USA). Clutches were deposited in containers 15 cm deep with oxygenated and chlorine-filtered water. In the case of *R. yunga*, we collected several amplexant pairs from the field during the wet seasons of 2014–2016 and housed them overnight at the Centro de Capacitación en Conservación y Desarrollo Sostenible (CDS/CNEH-Perú) in Oxapampa, Peru. We kept fertilized eggs in 2–3 cm of natural spring water until hatching a few days later.

Upon hatching, *Atelopus* and *R. alata* tadpoles were maintained in the lab at 20–24°C and pH 8.1–8.4 in chlorine-filtered water and fed with powder of boiled *Taraxacum officinale* every other day. We maintained *R. yunga* tadpoles in 40 × 30 × 10 cm open plastic containers (~200 tadpoles per container) at ambient temperatures in natural spring water that was changed multiple times weekly, and fed them ad libitum with spirulina powder and commercial trout food pellets. With all four species, when all four limbs emerged, we moved tadpoles to containers with both aquatic and terrestrial features that allowed them to climb out of the water. When metamorphic individuals spent most of their time out of the water, we moved them to small plastic lidded containers (10 per container) with wet paper towels and fed them with springtails and fruit flies (*Drosophila* spp.) a few times per week for up to 6 months. Toads reached metamorphosis after 50–60 days or more (mean snout-vent length (SVL) of metamorphs: *A. elegans* 7.5 mm, $n = 9$; *A.* sp. (*spumarius* complex) 6.9 mm, $n = 47$; *R. alata* 8.1 mm, $n = 20$; *R. yunga* 8.9 mm, $n = 24$).

2.2 | Histological samples

We fixed and sectioned individuals from all four species at tadpole Stages 17, 24–27 (grouped hereafter as Stage 25), 30, and 44 according to Gosner (1960), as well as juveniles at 1 week after metamorphosis and adults. In some species, as available, we fixed and sectioned individuals at Stages 33, 34, 37, 41, and 42 as well as 1, 2, 3, 5, or 6 months following metamorphosis (see subscripts in Figure 2 and Table S1). Animals were euthanized with 20% topical benzocaine on the ventral surface, preserved in 4% paraformaldehyde (diluted from 16% paraformaldehyde solution [Electron Microscopy Sciences] with phosphate-buffered saline [PBS]) for 24 h, and then rinsed thoroughly in PBS before being stored in 70% ethanol. We deposited voucher specimens at the Museo

de Biodiversidad del Perú (MUBI) in Cusco, Perú (*R. yunga* $n = 53$; MUBI 15593, 15594, 15595, and 15596) and the Centro Jambatu de Investigación y Conservación de Anfibios (CJ) in Quito, Ecuador (*R. alata* $n = 225$; CJ 5548–5623, 5631–5640, 5646–565, 5659–5682, 5691–5700, 5718–5722, 5725–5734, 5740–5744, 5755–5773, 5784–5803, 5811–5819, 5832–5836, and 5849–5869; *A. elegans* $n = 69$; CJ 3914–3918, 4222, 5641–5645, 5656–5658, 5683–5690, 5745–5754, 5774–5783, 5789–5798, 5804–5808, 5820–5824, 5828–5831, and 5838–5846; *A. sp.* (*spumarius* complex) $n = 15$; CJ 5708–5712, 5735–5739, 5825–5827, and 5841–5842).

To soften bones for sectioning, we decalcified specimens with 10% ethylenediaminetetraacetic acid (pH 7.4) at 23°C for 1 week. Then, we dehydrated specimens gradually from 30% to 100% ethanol and embedded them in hydroxypropyl metacrylate plastic (Electron Microscopy Sciences). To create markers for aligning tissue sections, we drilled 1 mm diameter holes into the plastic surrounding the specimen in the block. We sectioned blocks at 5 μm with a microtome (RM1265, Leica), mounting every other section onto Autofrost adhesion microscope slides (Cancer Diagnostics, Inc.) and staining with 1% eosin and 1% toluidine blue. We photographed one section per slide, leaving 30 μm total between sections, with a microscope camera (Olympus DP71) attached to a microscope (Olympus SZX10) at $\times 0.63$ to $\times 3$ magnification depending on the size of the specimen.

Using the program 3Dimod (Kremer et al., 1996), we aligned photos and reconstructed three-dimensional (3D) models to measure the volume of the following structures on the left and right side of each animal when present (excluding adults): otic capsule, oval window, operculum, opercularis muscle, columella (*pars interna* and *pars media*), and extracolumella (*pars externa*). In addition, to account for differences in overall size, we divided all measures of volume by the distance between geometric center of the otic capsules in the 3D model of that individual, a size index that scales to the overall body and head size more consistently across the time scale of this study than measures of length (given that anuran body length increases from hatching until metamorphosis and then decreases as the tail is reabsorbed). Based on observations in 3D models and in stained histological sections, we noted whether each structure was present or not at each developmental stage. When present, we also noted whether the structure could best be categorized as a mesenchymal condensation, a condensation with differentiating cells, or a delineated structure. Because verification of tissue type via immunohistochemistry was outside of the scope of this study, we distinguished the approximate categories as described in Thorogood and Hinchliffe (1975; see Figure 1 for examples and descriptions).

3 | RESULTS

Premetamorphic development of ear structures such as the inner ear and opercularis complex is similar in all four species. The otic capsule is present as an organized cluster of differentiated cells or a clearly delineated structure by Stage 30. The oval window is present by Stage 37, the operculum by Stage 37, the columella by Stage 42, and the opercularis muscle by 1 week after metamorphosis (Figure 2).

At metamorphosis (Stages 42–45), all four species show an organized cluster of developing mesenchyme or cartilage in the position of the primordial columella footplate (*pars interna*) within the oval window, located between the operculum and the anterior border of the otic capsule (Figure 3). A vestigial “nubbin” of the columella footplate was present in all three metamorphic and six postmetamorphic *R. yunga* (top row of Figure 3), some shaped like a finger and marginally adjoined to the otic capsule at one extreme and others floating independently within the connective tissue of the oval window. A “bud” of cartilaginous cells protrudes from the anterior border of the otic capsule into the oval window in the direction of the operculum in two Stage 44 *A. elegans* (third row of Figure 3). A cartilaginous cell cluster floats within the connective tissue of the oval window in the position of the primordial columella footplate in Stage 44 *R. alata* and *A. sp.* (*spumarius* complex; Figure 3).

In all postmetamorphic *R. alata* and *A. sp.* (*spumarius* complex), the columella extends distally (*pars media*) and joins the extracolumella (*pars externa*; Figure 3). Meanwhile, neither an extension of the distal end of the columella (*pars media*) nor a primordial extracolumella are visible in 1, 2, 3, 5, or 6-month-old specimens of *R. yunga* or *A. elegans* (Figure 2). Unextended cartilaginous nubbins (*pars interna*) are present in two of three adult *R. yunga* (Figure 4a,b) and cartilaginous buds on the anterior otic capsule wall are present in two juvenile and two adult *A. elegans* (Figure 3c,d). Only adult *R. alata* and *A. sp.* (*spumarius* complex) show evidence of a tympanic annulus or tympanic membrane.

Based on measurements of 3D models, the relative volumes of ear structures at different developmental stages did not show a predictable pattern when comparing eared and earless species in each pair; within *Rhinella* or *Atelopus*, sometimes a structure was larger in the earless species during early ontogeny (e.g., *Rhinella* otic capsule) and sometimes it was larger in the eared species (e.g., *Atelopus* operculum; Figure 5). These observations and measurements were similar between left and right sides of the head in all specimens. Size-correction of volumes (based on a distance between otic

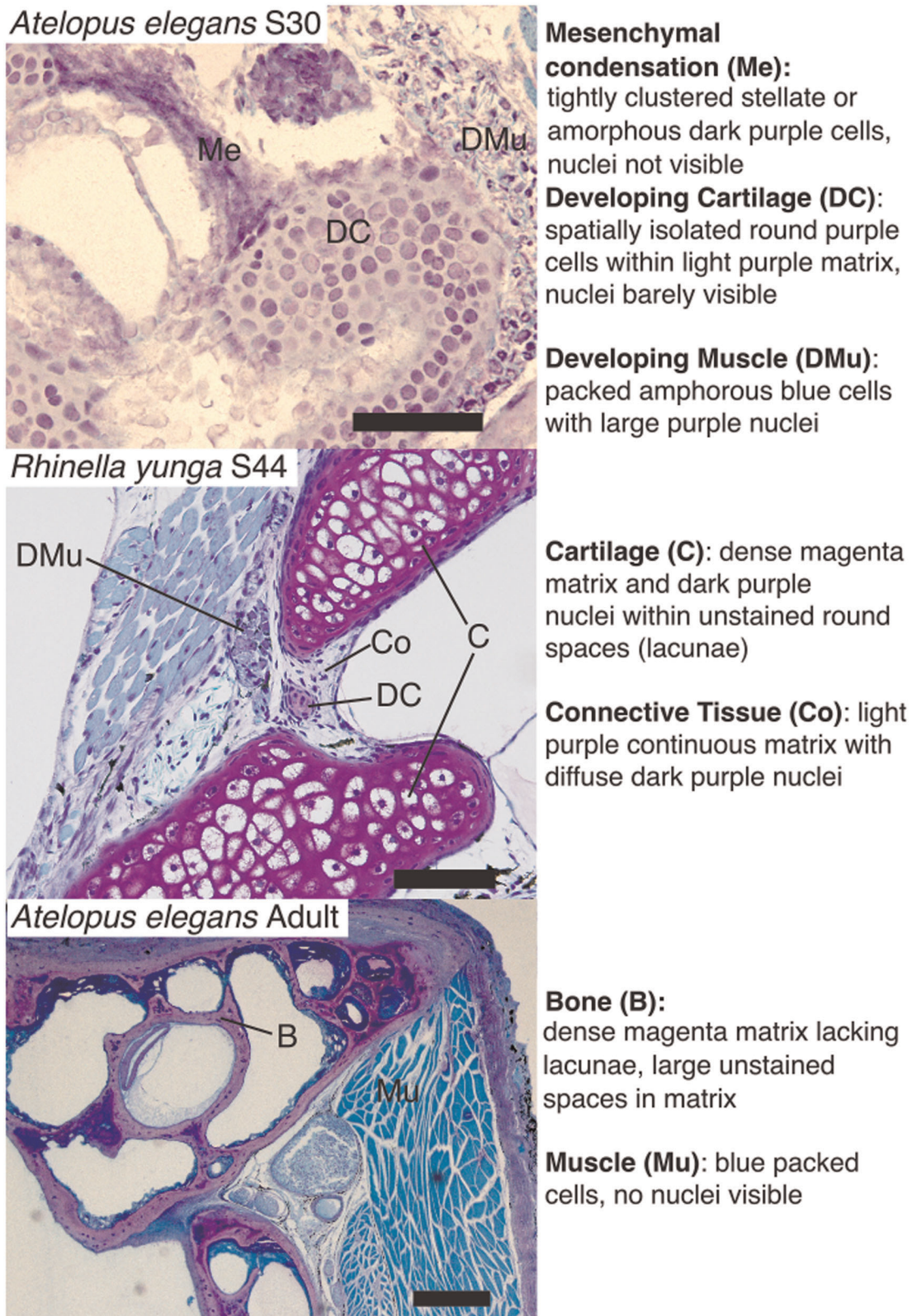


FIGURE 1 Examples of tissue types as categorized in histological sections of developmental series. Structures were classified as mesenchymal condensations (Me, undifferentiated mesenchyme), condensations with differentiating cells (DC, developing cartilage and DMu, developing muscle), or delineated structures (B, bone, C, cartilage, Co, connective tissue). S refers to tadpole stage according to Gosner (1960) and scale bars = 1 mm











							
		Stage 25–27	Stage 30–34	Stage 37	Stage 41–44	1 Week	1 Month–Adult
 <i>Rhinella yunga</i>	Otic Capsule	M ₂	D ₁ S ₁	S ₁	S ₄	S ₁	S ₈
	Oval Window		M ₂	S ₁	S ₄	S ₁	S ₈
	Operculum		M ₂	D ₁	D ₁ S ₃	S ₁	S ₈
	Opercularis Muscle				M ₄	S ₁	S ₈
	Columella		M ₁ *	M ₁ *	M ₁ D ₃ *	D ₁ *	D ₇ *
	Extracolumella						
 <i>Rhinella alata</i>	Otic Capsule	M ₂	D ₂	- ₀	S ₁	S ₁	S ₂
	Oval Window		M ₂	- ₀	S ₁	S ₁	S ₂
	Operculum		M ₂	- ₀	S ₁	S ₁	S ₂
	Opercularis Muscle				M ₁	S ₁	S ₂
	Columella				M ₁ *	D ₁	D ₁ S ₁
	Extracolumella				M ₁	M ₁	M ₁ S ₁
 <i>Atelopus elegans</i>	Otic Capsule	D ₂	S ₁	- ₀	S ₂	S ₂	S ₂
	Oval Window		M ₁	- ₀	S ₂	S ₂	S ₂
	Operculum				S ₂	S ₂	S ₂
	Opercularis Muscle				M ₂	S ₂	S ₂
	Columella				M ₂ *	D ₂ *	D ₂ *
	Extracolumella				M ₂ *	M ₂ *	
 <i>Atelopus sp.</i> (<i>spumarius</i> complex)	Otic Capsule	D ₁	S ₁	- ₀	S ₁	S ₁	S ₄
	Oval Window				S ₁	S ₁	S ₄
	Operculum				S ₁	S ₁	S ₄
	Opercularis Muscle				M ₁	S ₁	S ₄
	Columella				M ₁ *	M ₁ *	S ₄
	Extracolumella				M ₁	M ₁	M ₁ S ₃

FIGURE 2 Ontogenetic timeline of middle ear development in species pairs of eared (*Rhinella alata*, *Atelopus sp. (spumarius complex)*) and earless (*R. yunga*, *A. elegans*) toads. Structures are noted as being (M) mesenchymal condensations, (D) differentiating clusters of cartilage or muscle cells, or (S) clearly delineated structures with an overall shape similar to that seen in adults. Subscript numbers indicate the number of individuals identified in each developmental condition for each structure. If no specimens were available for a given stage (e.g. *R. alata* Stage 37), colors were carried over from the preceding category and indicated with a dash. Asterisks indicate a nubbin or bud of cells restricted to the oval window rather than a complete columella that extends distally. Stages follow Gosner (1960)

capsules) did not alter any conclusions about developmental patterns or growth rates among species pairs (see Figure S1 for graphs with adjusted volumes).

4 | DISCUSSION

In this study, we show that arrested development and subsequent degeneration of a late-developing structure is responsible for the loss of a skull bone and its associated distal elements. This study provides the first evidence of a vestigial nubbin or bud of the columella footplate within

the oval window of two species of earless toads, which establishes truncation as a proximate mechanism underlying earlessness in this clade. Initiation of the primordial columella footplate (*pars interna plectri*) of the tympanic middle ear occurred during metamorphosis in both eared and earless toad species, but was interrupted in earless species before distal extension, and sometimes was resorbed during the juvenile stage. Thus, trait loss results from arrest of the mesenchymal extension, differentiation, or ossification of the columella soon after metamorphosis, followed by the degeneration in some individuals. The developmental mechanism underlying

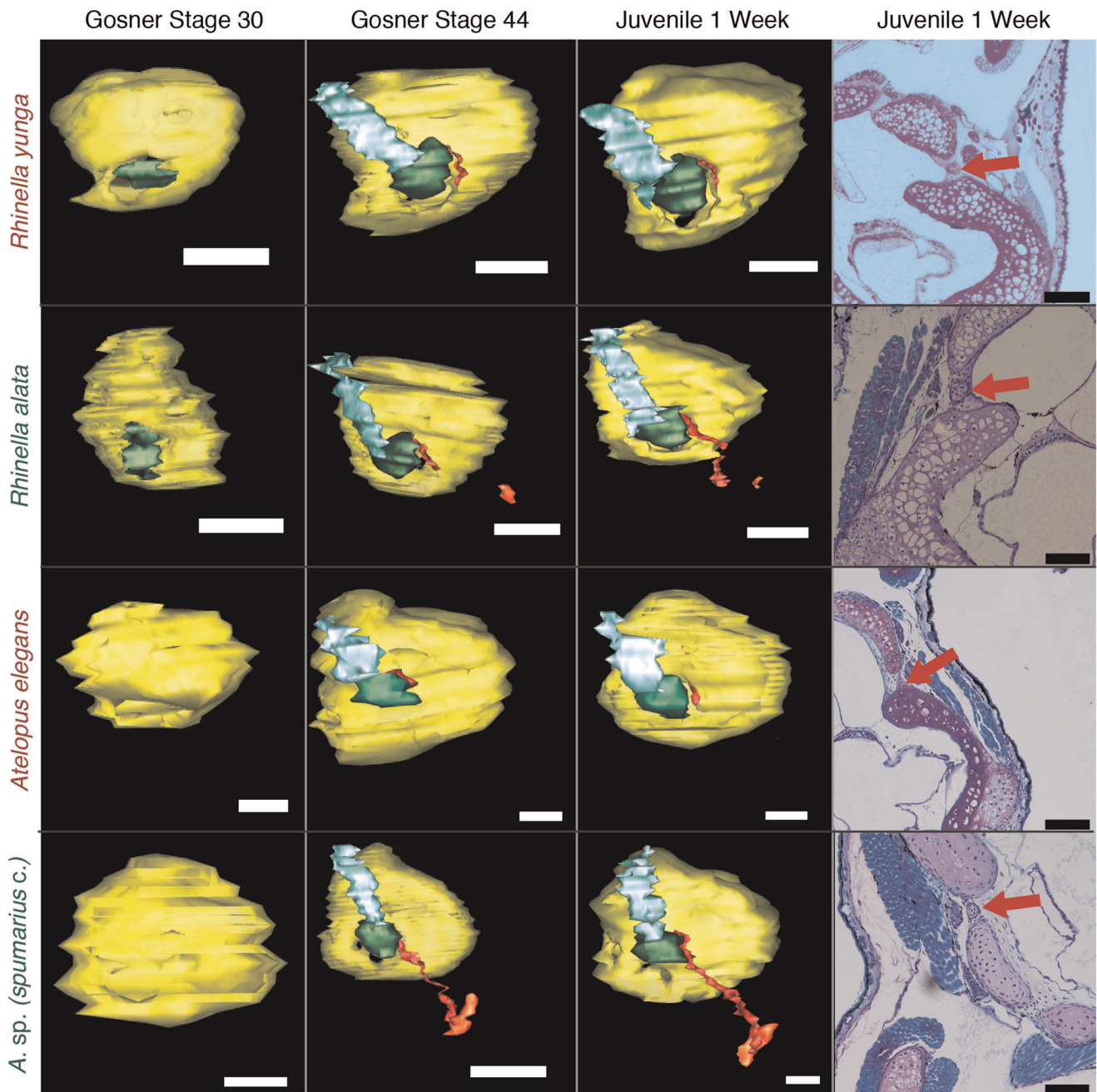


FIGURE 3 Three-dimensional reconstructions (first three columns) and histological images (fourth column) of the otic capsule (yellow), operculum (green), opercularis muscle (turquoise), columella (red), and extracolumella (orange) in developing toads of four species, two of which form complete tympanic middle ears as adults (*Rhinella alata*, *Atelopus* sp. [*spumarius* complex]) and two of which lack the tympanic annulus, tympanum, extracolumella, and parts of the columella as adults (*R. yunga* and *A. elegans*). Red arrows identify the nubbin (*R. yunga*), bud (*A. elegans*), or columella footplate (*R. alata*, *A. sp. [spumarius complex]*) in week-old specimens, each positioned within the oval window between the wedge-shaped operculum and the anterior border of the otic capsule. Scale bars = 1 mm

the loss of the tympanic middle ear is consistent with losses of other structures in vertebrates in that a mesenchymal condensation is initiated, but is prematurely arrested and regresses partially or entirely before the adult stage (Berti et al., 2001; Eigenmann & Denny, 1900; Rahmani, 1974; Senter & Moch, 2015).

We identified a vestigial cartilaginous nubbin within the oval window in six of six postmetamorphic juveniles and two of three adult *R. yunga* (sometimes fused to the ventral border of the otic capsule; Figure 3a,b), and a cartilaginous bud of cells bordering the otic capsule was present in the same location in four postmetamorphic

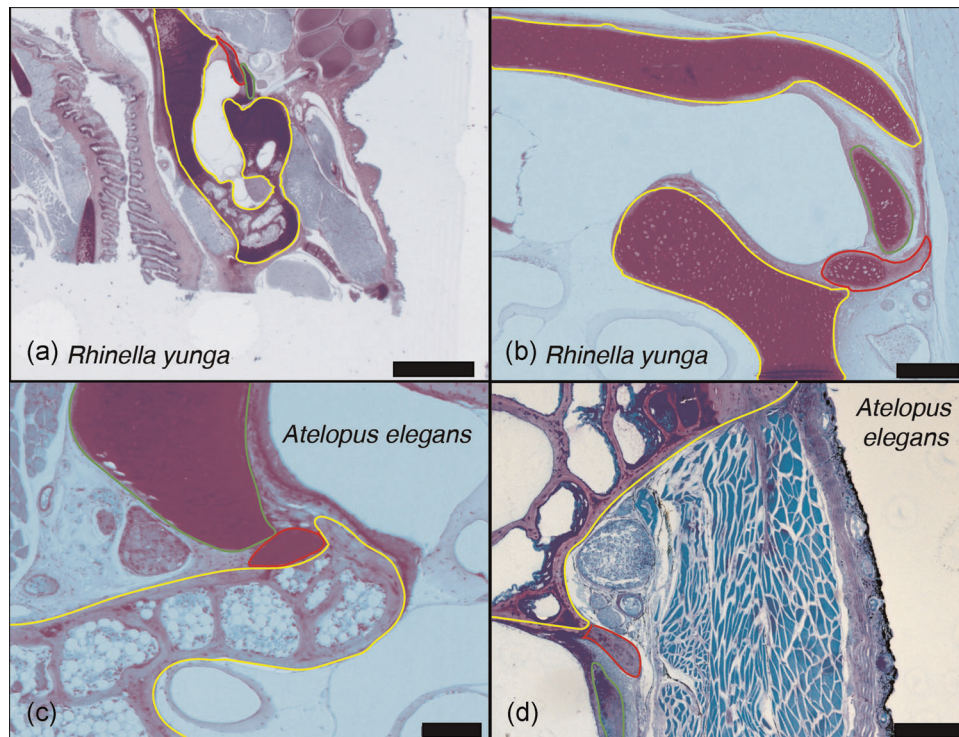


FIGURE 4 Histological frontal sections of the inner and middle ear of (a) and (b) two adult *Rhinella yunga* and (c) and (d) two adult *Atelopus elegans*, stained with eosin and toluidine blue. Vestigial nubbins or buds of the columella footplate (outlined in red) are situated between the anterior border of the otic capsule (outlined in yellow) and the operculum (outlined in green; situated posterior to other outlined structures). Whereas the columella extends to meet the extracolumella and tympanum in *R. alata* and *A. sp. (spumarius complex)*, it does not extend distally in adjacent sections in *R. yunga* or *A. elegans*. Scale bars = 1 mm

A. elegans (Figure 3c). Because the interruption of columella development was similarly timed between *Rhinella* and *Atelopus* species pairs, but differed in having either a distinct nubbin or a bud fused to the otic capsule, the truncation of middle ear development could have occurred at the cellular level in distinct ways in lineages showing independent losses, reductions, and regains within the bufonid clade. These findings suggest that the evolutionary loss of tympanic middle ears in toads involves the arrest and gradual degeneration of late-developing distal structures via failure to ossify, fusion with other elements, and some degree of apoptosis, similar to losses of sensory structures and appendages in other vertebrate clades (Essex, 1927; Galis et al., 2018; Lande, 1978; Pereyra et al., 2016; Wilkens, 2007). All juvenile *R. yunga* and all juvenile and adult *A. elegans* exhibited nubbins and buds, respectively, of the columella footplate, but one adult *R. yunga* did not exhibit a vestigial structure. Similarly, vestigial middle ear structures were not detected in juveniles or adults of numerous earless bufonid species (M. Womack, unpublished data), suggesting that many earless toads exhibit either complete degeneration of initiated columella elements

during or soon after metamorphosis or that they fail to initiate columella development entirely. Previous reports have depicted partial tympanic middle ears (Table 1), including in adult *R. paraguas* (Grant & Bolivar-G, 2014). Substantial variation in the form and persistence of vestigial nubbins and buds in premature and adult toads supports the idea that the cellular processes that provoke the sequential loss of distal structures are not consistent across earless toad lineages.

More comparative developmental studies that achieve a broader phylogenetic context and finer temporal specificity will be needed to determine the degree to which the pattern of loss across the bufonid clade is due to shifts in expression levels of cell proliferation or regulatory genes (as in lost or reduced tetrapod digits; Saxena et al., 2017), and/or to heterochrony (as in blind cavefish; Pottin et al., 2011). Progenesis (early offset), post-displacement (delayed onset) and neoteny (slowed developmental rate) are the heterochronic processes proposed to generate paedomorphic developmental traits that reverse phylogenetic trajectories (see Table 1 in Alberch et al., 1979). All three mechanisms have been speculated to underlie the loss of the tympanic middle

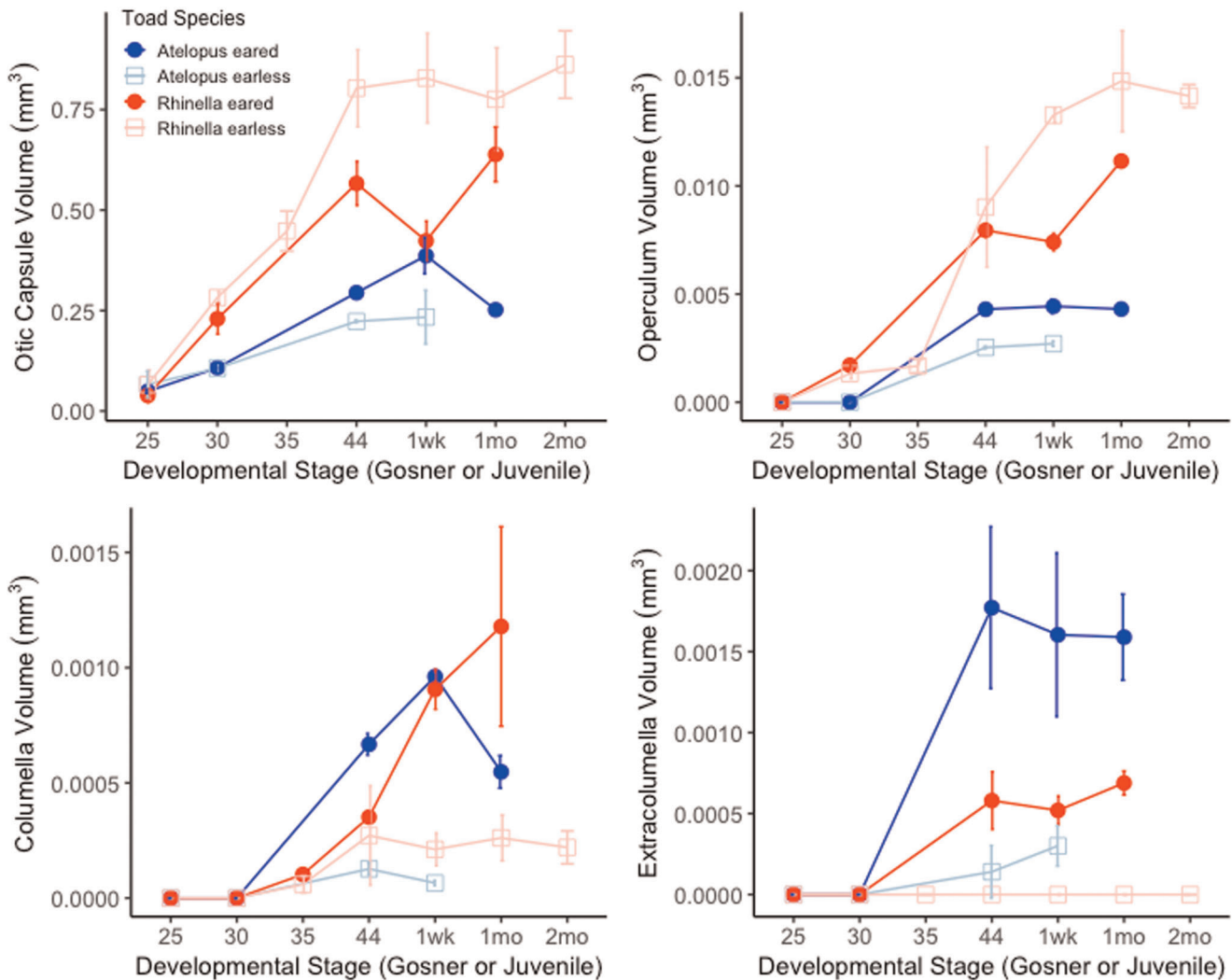


FIGURE 5 Volume across development of the otic capsule, operculum, columella, and extracolumella in species pairs of eared and earless *Atelopus* and *Rhinella* toads

ear in anurans (Shkil & Smirnov, 2015; Smirnov, 1991; Womack, Fiero, et al., 2018; Womack et al., 2019). The findings of the current study are most consistent with the hypothesis that the tympanic middle ear was lost via progenesis of the development of the columella, given that two species known to lack complete tympanic middle ears as adults initiated the development of a columella footplate in the oval window around metamorphosis, just as in fully eared species, but development was truncated (early offset) at the juvenile stage, and in some cases, subsequent degeneration occurred. In contrast to suggestions by Smirnov (1991), our evidence is not consistent with postdisplacement as a proximate mechanism for tympanic middle ear reduction, because the sequential timing of onset and growth of medial structures (otic capsule, oval window, operculum, opercularis muscle, and columella footplate) was not delayed in earless species relative to eared species (Figure 2).

Although neoteny has also been suggested as a potential mechanism for anuran ear losses (Smirnov, 1991; Womack et al., 2019), our findings do not show obvious evidence for neoteny because rates of development of ear structures do not appear to be relatively slower in earless species; as seen in Figure 5, various structures stop growing between Stage 44 and 1 week postmetamorphosis, but growth rates (slopes; see Alberch et al., 1979) before arrest are not qualitatively different between eared and earless *Rhinella* or *Atelopus* species pairs. However, given limited sampling in this exploratory study ($n = 1-3$ at most time points), we cannot rule out the possibility that deeper and finer-scale examination at critical developmental phases (e.g., between Stage 37 and 1-week postmetamorphosis), as well as comparisons of ear development relative to toad age rather than to developmental stage, could reveal reduced developmental rates (less steep slopes) of key structures

TABLE 1 Examples of an incomplete or vestigial columella (stapes) in anurans

Anuran species	Stage	Figure; Reference
<i>Rhinella horribilis</i> [= <i>marina</i>]	S37	Figure 2c; Womack, Stynoski et al. (2018)
<i>Hyla crucifer</i>	S40–42	Figures 3 and 4; Hetherington (1987)
<i>Rana catesbeiana</i>	S42–44	Figure 1d,e; Horowitz et al. (2001)
<i>Rana temporaria</i>	Metamorph	Figure 14; Villy (1890)
<i>Hyperolius horstocki</i>	Metamorph	Figure 7; du Toit and De Villiers (1932)
<i>Rana clamitans</i>	Metamorph	Figure 2; Witschi (1949)
<i>Anaxyrus</i> [= <i>Bufo</i>] <i>americanus</i>	Metamorph	Figure 7a; Hetherington (1987)
<i>Hyla crucifer</i>	Metamorph	Figure 7b; Hetherington (1987)
<i>Pseudacris triseriata</i>	Metamorph	Figure 7c; Hetherington (1987)
<i>Hyla versicolor</i>	Metamorph	Figure 7d; Hetherington (1987)
<i>Rana sylvatica</i>	Metamorph	Figure 7e; Hetherington (1987)
<i>Atelopus elegans</i>	Adult	Current study
<i>Rhinella yunga</i>	Adult	Current study
<i>Rhinella paraguas</i>	Adult	Figure 4b; Grant and Bolivar (2014)
<i>Rana catesbeiana</i>	Adult (unilateral)	Figure 4; Horowitz et al. (2005)
<i>Bombina orientalis</i>	Adult	Figures 4–27; Wever (1985)
<i>Bombina variegata</i>	Adult	Figures 4–20; Wever (1985)
<i>Bombina maxima</i>	Adult	Stadtmüller (1931)
<i>Bombina pachypus</i>	Adult	Stadtmüller (1931)
<i>Beelzebufo ampinga</i>	Adult	Figure 29; Evans et al. (2014)
<i>Nectophrynoides asperginis</i>	Adult	Figure 3e,f; Arch et al. (2011)

Note: Bufonid toads are listed in bold. S = Stage of tadpole according to Gosner (1960).

in earless species. It is also possible that both progenesis of the columella and neoteny of the tympanic middle ear act as concerted mechanisms leading to tympanic middle ear loss across the bufonid clade, which is known for having slow columella development relative to other anuran clades (Fabrezi & Goldberg, 2009; Hetherington, 1987; Smirnov, 1989; Womack, Stynoski, et al., 2018). Our results are thus consistent with shifts in gene expression, progenesis, and possibly neoteny, but not postdisplacement, as potential mechanisms for arrested development and degeneration of ear structures in toads.

During eye loss in blind cave salamanders (Eigenmann & Denny, 1900; Tovar et al., 2018), degeneration of the latest developing (and most distal) structure occurs during and shortly after metamorphosis, which concurs with the timing seen here in *Rhinella* and *Atelopus* earless species. In amphibians, developmental mechanisms underlying the loss or reduction of traits

may be inherently tied to metamorphosis (Eigenmann & Denny, 1900); it has been suggested that pre-metamorphic stages are more fixed than late labile stages, and that postmetamorphic changes in gene expression or timing serve to compartmentalize trait evolution and loss (Moran, 1994; Wilkens, 2007). Given that toad development is prolonged overall (Fabrezi & Goldberg, 2009; Hetherington, 1987; Smirnov, 1989; Womack, Stynoski, et al., 2018), postmetamorphic truncation of the columella could have resulted from desynchronization of gene regulatory networks for endochondral chondrification and ossification such as *runx2*, *col2a1*, and *sox9* (Chapman, 2011; Gómez-Picos & Eames, 2015; Kerney et al., 2018; Sienknecht, 2013), and/or release of thyroid hormone, which stimulates both metamorphosis and skeletogenesis in anurans (Bassett & Williams, 2016; Hanken et al., 1989). The reversal of postmetamorphic degeneration via resynchronization

could explain the inferred regain of tympanic middle ear structures in at least two bufonid lineages, including within *Atelopus* (Lande, 1978; Pereyra et al., 2016).

Many evolutionary losses are attributed to relaxed selection: larvae of cave salamanders have eyes but do not use them (Eigenmann & Denny, 1900), whales moved to the water before their hind limbs regressed (Bejder & Hall, 2002), and snakes and legless lizards exhibited burrowing and creeping locomotion and elongated bodies before losing digits, limbs, and pelvic and sternal girdles (Essex, 1927; Lande, 1978). Losses of the tympanic middle ear have occurred in 10–17 independent toad lineages and impair hearing sensitivity at sound frequencies within the range of earless toad calls (>1 kHz; Boistel et al., 2011; Cocroft et al., 1990; Lombard & Straughan, 1974; Womack et al., 2017; but see Womack, Christensen-Dalsgaard, et al., 2018). Such impaired sensitivity does not appear to be compensated by improved extratympanic sensation via the opercular system (Hetherington, 1987; Smirnov, 1991; Womack et al., 2017), and in the current study, the onset and growth rates of opercular structures were similar in eared and earless species pairs. We reason that the cartilaginous vestiges found in the oval window do not likely serve a useful function in hearing, because even partial loss of the most distal tympanic middle ear structure, the tympanic annulus, severely reduces hearing sensitivity in toads (Womack et al., 2016) and because vestiges of digits, eyes, and other evolutionarily reduced structures are nonfunctional (Bejder & Hall, 2002; Lande, 1978; Rahmani, 1974; Senter & Moch, 2015). That neither the opercularis system nor vestigial nubbins offer an apparent benefit to hearing suggests that tympanic ear loss is consistent with relaxed selection.

Although middle ear loss in toads shares proximate developmental mechanisms with other evolutionary trait losses, ultimate explanations for repeated loss across the bufonid clade remain unresolved. Earless toads live in a wide variety of ecological contexts and exhibit a diversity of behavioral adaptations that could either relax selection on tympanic middle ears or compensate for their loss (Jaslow et al., 1988): short-range communication (Cocroft et al., 1990; Gluesenkamp & Acosta, 2001), prolonged amplexus (32 days; Crump, 1988), explosive breeding (Jacobson & Vandenberg, 1991; Lips & Krempels, 1995), low-frequency calls (Stynoski et al., 2020), female–female competition (Stynoski et al., 2020), multimodal or visual signaling (Crump, 1988; Lindquist & Hetherington, 1998), and environmental vibrational cues (Rueda Solano & Warkentin, 2016; Womack et al., 2017). Given the diversity of selection pressures on auditory systems across the bufonid clade, evolutionary processes other than adaptive selection may contribute to the lability of ear

structures (Womack, Fiero, et al., 2018; Womack et al., 2019). Developmental bias—limitations on how mutations can alter development—can work alongside natural selection to influence trait evolution (Arthur, 2002). For example, under pleiotropic influence, pythons lost their external ears to allow prey manipulation (Christensen et al., 2012) and some crickets lost tympanic ears when wings were reduced (reviewed in Pascoal et al., 2014; Strauß & Stumpner, 2015). Future studies could explore the degree to which progenesis or neoteny (see above) as well as pleiotropy primed repeated ear loss across diverse selective contexts in the bufonid clade (Pereyra et al., 2016; Smirnov, 1991; Womack et al., 2016; Womack, Fiero, et al., 2018; Womack, Stynoski, et al. 2018).

Our findings document anatomical changes that hint at a developmental mechanism for the loss of tympanic middle ears in toads and other anurans, and highlight pathways that could underlie the early arrest of distal extension and differentiation of the columella around metamorphosis. Shifts in developmental signaling or timing may prime bufonid lineages for the loss of late-developing structures such as the *pars media*, extra-columella, and tympanum, and thus may prove to be a recurrent mechanism underlying the lability of the tympanic middle ear among anurans more broadly.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Jennifer L. Stynoski, Molly C. Womack, and Kim L. Hoke designed the study. Jennifer L. Stynoski, Molly C. Womack, Florencia A. Trama, Luis A. Coloma, and Kim

L. Hoke collected and interpreted the data. Jennifer L. Stynoski wrote the manuscript with input from Molly C. Womack, Florencia A. Trama, Luis A. Coloma, and Kim L. Hoke.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article or available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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