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RESEARCH ARTICLE

Prolonged middle ear development in *Rhinella horribilis*

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Abstract

Despite the benefit of the tympanic middle ear to airborne hearing sensitivity, anurans range in how soon they develop functional middle ears after transitioning to life on land. Previous evidence suggested that bufonids had particularly slow middle ear developmental rates, but precise timelines have not yet been published for this family. Here, we provide the first age-verified middle ear development timeline for a true toad species (family Bufonidae). We find that although middle ear development begins during metamorphosis in *Rhinella horribilis*, the middle ear remains incomplete 15 weeks after the transition from aquatic tadpole to land-dwelling toadlet. Using this new middle ear timeline, we discuss commonalities and differences in middle ear development among bufonids, as well as among Anura.

KEYWORDS

bufonidae, heterochrony, tympanic middle ear

1 | INTRODUCTION

In most anurans, as in other tetrapods, the tympanic middle ear functions in airborne hearing. The exceptions to this statement include a small percentage of anuran species, often termed "earless" species, which never develop a tympanic middle ear (Pereyra et al., 2016). Yet, unlike most tetrapods that complete development of the tympanic middle ear during embryonic stages, many anurans do not fully develop tympanic middle ears until well after metamorphosis when they begin living autonomously on land (Hetherington, 1987; Horowitz, Chapman, Kaya, & Simmons, 2001; Sedra & Michael, 1959; Smirnov, 1991; Vorobyeva & Smirnov, 1987; Womack, Christensen-Dalsgaard, & Hoke, 2016). Anuran inner ears are fully formed and functional during larval stages when animals hear underwater and thus would not benefit from middle ears (Bever, Jean, Fekete, 2003; Boatright-Horowitz & Simmons, 1997). After metamorphosis, however, the tympanic middle ear can take up to 1 year to complete (Sedra & Michael, 1959), and juveniles with an incomplete tympanic middle ear are 14–26 dB less sensitive to airborne sound from 900 to 2,500 Hz when compared to individuals with a fully developed tympanic middle ear (Womack et al., 2016). Unfortunately, developmental timelines of individual middle ear structures only exist for a handful of species, leaving middle ear development for most anuran species incomplete (Sedra & Michael, 1959; Hetherington, 1987; Vorobyeva and Smirnov, 1987; Smirnov, 1991) or more often, completely absent.

Tympanic middle ear development can be lengthy and difficult to track because the components of the anuran middle ear do not develop synchronously (Hetherington, 1987; Sedra & Michael, 1959). The anuran tympanic middle ear consists of a tympanic membrane surrounded by a cartilaginous ring (the tympanic annulus) that is connected to the inner ear via the columella (=stapes). The columella consists of three parts: (a) the columellar footplate, which sits within the oval window of the inner ear and is usually part cartilage (=*pars interna*) and part bone (=*pars media*); (b) the columellar shaft (=*pars* *media*), a mostly bony structure that extends from the footplate toward the tympanic membrane; and (c) the extracolumella (=*pars externa*), a cartilaginous connection between the columellar shaft and the tympanic membrane. The right and left middle ears are coupled by the middle ear cavities, which connect to the buccal cavity via the Eustachian tubes. In addition to the tympanic middle ear pathway, most anurans (with the exception of the family Pipidae; reviewed in Mason, 2007) have an operculum, a cartilaginous disc that sits next to the columellar footplate in the oval window and is proposed to help anurans sense low-frequency airborne sound and ground vibrations (Hetherington, 1985, 1988; Lombard & Straughan, 1974). The operculum forms earlier in development than the tympanic middle ear, but later than the inner ear (Fabrezi & Goldberg, 2009; Hetherington, 1987; Horowitz et al., 2001). Ontogeny of the tympanic middle ear generally follows a proximal to distal progression in anurans with the columellar footplate visible first (Sedra & Michael, 1959; Hetherington, 1987; Vorobyeva & Smirnov, 1987; Smirnov, 1991), but staging of development of the middle ear is unresolved for most anuran families.

Within Bufonidae, only limited snapshots of middle ear developmental morphology have been described from tadpole to postmetamorphic stages (Sedra & Michael, 1959; Hetherington, 1987; Vorobyeva & Smirnov, 1987, Womack et al., 2016). These intermittent data indicate that juvenile bufonids lack a complete tympanic middle ear for several months (Hetherington, 1987; Sedra & Michael, 1959; Vorobyeva & Smirnov, 1987), resulting in decreased hearing sensitivity throughout this time (Womack et al., 2016). However, existing ontogenetic timelines of bufonid middle ear development are based on body size, not age (Hetherington, 1987; Smirnov, 1991; Vorobyeva & Smirnov, 1987), leaving the duration of incomplete ear morphology and associated hearing impairment uncertain.

Here, we provide the first age-known timeline of middle ear development in a bufonid, *R.horribilis*. We document the development and growth of the tympanic middle ear components in *R. horribilis*, from tadpole stages to 15 weeks after metamorphosis.

2 | MATERIALS AND METHODS

2.1 | Specimen collection & housing

R. horribilis (Wiegmann, 1833) tadpoles of two clutches (*n* = 34; Table 1) were collected from Unión del Toachi (Chorrera del Diablo), Cotopaxi Province, Ecuador. The Institutional Animal Care and Use Committee at Colorado State University approved all experiments (IACUC Protocol #12-3484A), and the Ministerio del Ambiente in Ecuador approved collection, research, and export permits (Permit: 001–13 IC–FAU–DNB/MA). Voucher specimens (CJ 1268) were deposited at the Museum of Centro Jambatu of Amphibian Research and Conservation.

Tadpoles were reared in the lab at temperatures between 22 and 25 °C, five tadpoles per 1-L plastic containers. We fed tadpoles *Taraxacum officinale* (cooked in boiling water for about 5 min) every other day. We filtered all water to prevent the entry of chlorine, arsenic, bacteria, and other harmful agents. Once individuals grew all four limbs, we moved them to transitional containers of the same size that contained both aquatic and terrestrial features. Then, we moved metamorphs to small plastic containers of 250 mL with only terrestrial features once they routinely stayed outside of the water, maintaining two individuals per container. Juveniles and subadults were reared in Penplax containers with automatic spraying. Once in terrestrial housing, we fed individuals crickets (*Gryllus sp*., *assimilis* complex) and fruit flies (*Drosophila* spp.) three times a week and dusted the insects once a week with calcium and minerals supplement (Calcium Plus, Repashy Ventures, Inc., CA).

2.2 | Specimen fixation

Tadpoles and metamorphs were staged according to Gosner (1960) and fixed at ages ranging from tadpole Stage 17 to 15 weeks postmetamorphosis (Table 1). Due to time constraints, we were not able to raise the clutches past 15 weeks. We measured the head width and snout-vent length of postmetamorphic animals using dial calipers (31-415-3, Swiss Precision Instruments Inc., Garden Grove, CA) before fixation. We then euthanized the animals by applying 20% topical benzocaine to the ventral surface. We preserved all specimens in 4% paraformaldehyde made from 16% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA) diluted with phosphate-buffered saline (PBS). We fixed specimens in 4% paraformaldehyde for 24 hr and then performed three 15-min rinses in PBS before storing the specimens in 70% ethanol.

2.3 | Histology and 3D-reconstructions

We decalcified the specimens with 10% ethylenediaminetetraacetic acid (pH 7.4) for 1 week at room temperature. We then put the specimens through a series of ethanol washes with increasing concentration from 30 to 100% before embedding in blocks of hydroxypropyl methacrylate plastic (Electron Microscopy Sciences, Hatfield,

TABLE 1 Specimen age and size information of *R. horribilis*

Age	*n*	Snout-vent length (mm)	Head width (mm)
Stage 17	2	NA	NA
Stage 24	3	NA	NA
Stage 30	3	NA	6.5[a]
Stage 36	1	NA	7.8
Stage 41	1	13.0	8.2
Stage 44	4	8.3–12.4	3.5–5.7
1 week PM	4	8.5–12.6	3.3–4.8
2 weeks PM	1	15.0	5.05
3 weeks PM	2	14.0–16.0	5.35–5.6
4 weeks PM	2	8.8–9.3	3.5–3.8
5 weeks PM	2	14.1–15.0	5.0–5.05
10 weeks PM	2	15.6–17.5	6–6.4
12 weeks PM	1	12.9–17.8	6.8[a]
13 weeks PM	2	11.9–19	4.9–7.4
15 weeks PM	3	15.9–18.2	5.7–7.9

[a] *Note*. missing values. PM = postmetamorphosis.

PA). We drilled 1 mm holes into the surrounding plastic for future use as markers for photo alignment, sectioned the specimens at 5 μm using a microtome (RM1265, Leica, Wetzlar, Germany), and mounted every other section onto Autofrost Adhesion Microscope Slides (Cancer Diagnostics, Inc, Durham, NC). We then stained the sections with 1% Eosin and 1% Toluidine Blue. Once dry, we photographed every third section, resulting in 30 μm between photographed sections. We then examined all photos to determine presence/absence of each ear structure at all stages (data available in Supporting Information). We also noted so-called columella precursor cells that began to congregate in the area where the columella would originate. For a subset of individuals, we aligned the photos and traced the ear structures to create 3D-models of the developing ear using IMOD (Kremer, Mastronarde, & McIntosh, 1996).

3 | RESULTS

Figure 1 shows an overview of *R. horribilis* middle ear development. Overall, tympanic middle ear structures in *R. horribilis* appeared in a proximal to distal pattern, with the columellar footplate appearing first and the tympanic membrane last (Figure 1). In *R. horribilis*, the otic capsule was visible at tadpole Stage 24 (Figure 2a), but the operculum did not appear until Stage 30 (Figure 2b). At Stage 41 a dense clustering of cells was present in the anticipated position of the columellar footplate in the oval window (Figure 2c). At metamorphosis (Stage 44), the columellar footplate was the first visible structure of the tympanic middle ear (Figures 2d and 3). At 1 week postmetamorphosis the columella had extended distally to form the columellar shaft (Figures 2e and 3). The tympanic annulus was first seen in the larger of the 1-week postmetamorphosis specimens (Figure 2f). At 2 weeks postmetamorphosis, the middle ear cavity and Eustachian tube started to develop as an invagination of the buccal cavity (Figure 2g), which will first form the Eustachian tube and then continue to develop into the middle ear cavity. At two weeks postmetamorphosis the extracolumella also began to form (Figure 2h). From 3 to 12 weeks postmetamorphosis, the columella continued to thicken, the tympanic annulus grew dorsally but did not become a complete ring, and the middle ear cavity expanded (Figure 3). At 13 to 15 weeks postmetamorphosis, the tympanic annulus was sickle-shaped (Figure 3) and the middle ear cavity was not complete (Figure 2i). The tympanic membrane was present in only the largest individual (age = 13 weeks, SVL = 19 mm; Figure 4). In individuals where the tympanic membrane was not present, the tympanic region was covered with cranial epidermis that was thicker than the tympanic membrane.

4 | DISCUSSION

Our developmental timeline of middle ear structures in *R. horribilis* reveals that although development of the middle ear begins around metamorphosis, it is not complete months after metamorphosis. We selected rearing conditions to match temperatures at which this species occurs in the wild, but suggest that greater temperature variation and differences in food availability could alter developmental timelines in the wild. Below, we compare middle ear development in *R. horribilis* with that of other bufonids and anurans.

Similar to other anurans, development of the tympanic middle ear in *R. horribilis* begins with the formation of the columellar footplate (Cannatella, 1999; Hetherington, 1987; Horowitz et al., 2001; Sedra & Michael, 1959; Vorobyeva & Smirnov, 1987). Additionally, the order of middle ear structure formation in *R. horribilis* (columella –> Eustachian tube –> middle ear cavity –> tympanic annulus –> tympanic membrane) matches other anurans (Cannatella, 1999; Hetherington, 1987; Trueb & Hanken, 1992; Vorobyeva & Smirnov, 1987). Thus, the sequence of tympanic middle ear development is broadly conserved across anurans (but see Fabrezi & Goldberg, 2009 for an exception within Hylidae).

Although, the sequence of middle ear structures remained consistent among our samples, the age at which certain middle ear structures formed varied in a few cases. Body size may explain this individual variation; however, we find mixed results for greater

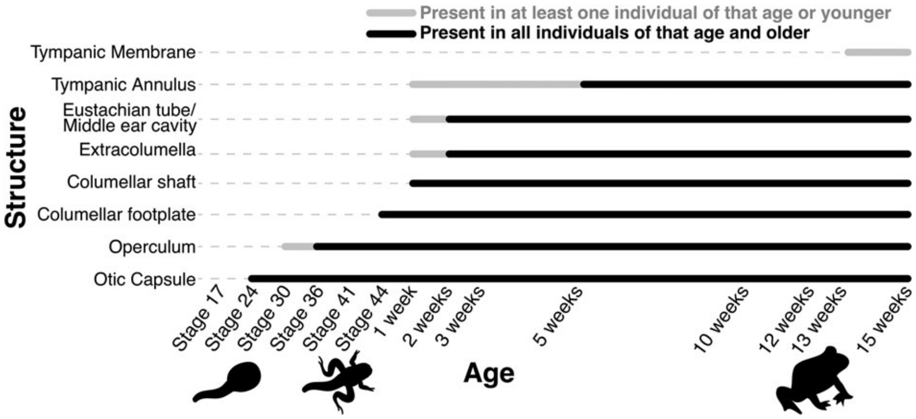

FIGURE 1 Ontogenetic timeline of *R. horribilis* ear structure appearance. Bars indicate the ages at which ear structures are present. Grey bars indicate when a structure first appears in at least one individual. black bars indicate when a structure is present in all individuals of that age as well as all individuals of older ages

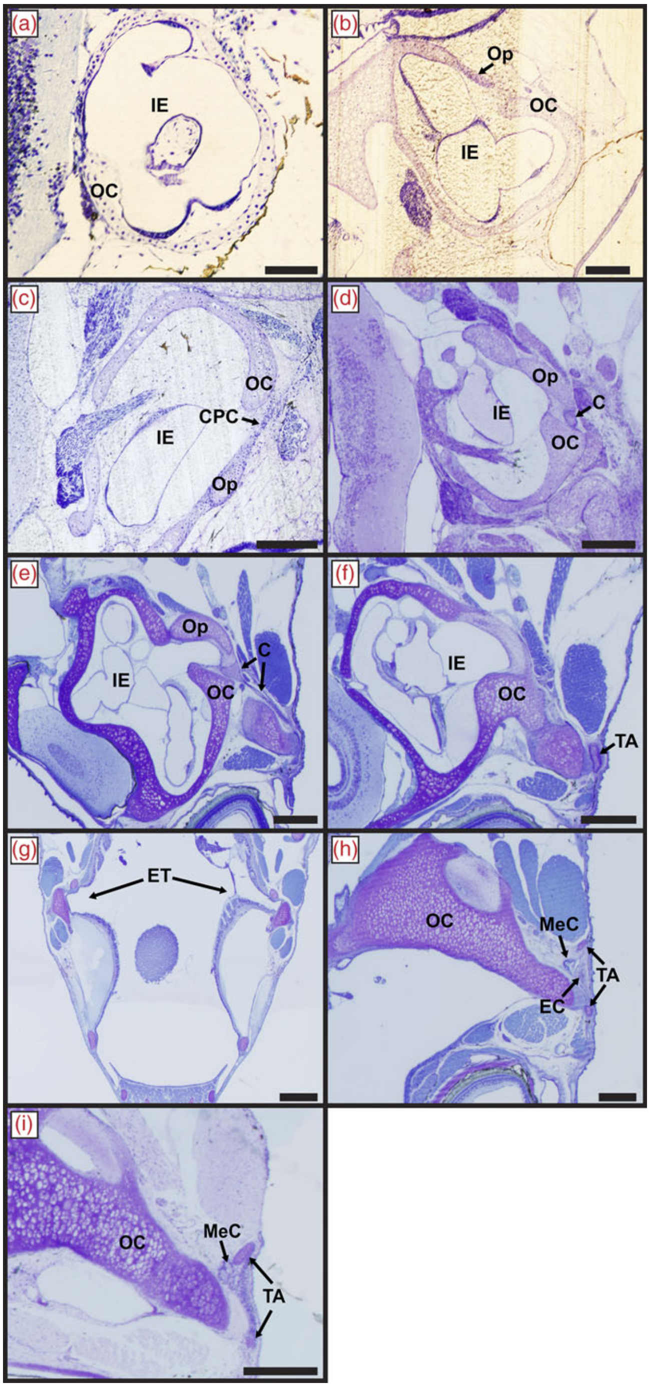

FIGURE 2 Photographs of *R. horribilis* ear development from tadpole in stage 24 (Gosner, 1960) to 15 weeks postmetamorphosis: (a) stage 24, (b) stage 30, (c) stage 41, (d) stage 44 metamorph, (e, f) 1 week postmetamorphosis, (g, h) 2 weeks postmetamorphosis (i) 15 weeks postmetamorphosis. C = columella; CPC = columella precursor cells; Ec = extracolumella; ET = Eustachian tube; IE = inner ear cavity; MeC = middle ear cavity; OC = otic capsule; Op = operculum; TA = tympanic annulus. All photographs are of transverse sections on the left ear with the exception of panels F and G, which are rotated sagittally. Top to bottom of each panel = caudal to rostral. slices vary in their dorsal to ventral position to best display the labeled characters. Scale bars = 1 mm

juvenile body size resulting in earlier formation of ear structures. The extracolumella is first visible in the smallest 1 week postmetamorphosis toadlet and likewise, the middle ear cavity is absent from the largest 1 week postmetamorphosis toadlet, providing evidence that size has no clear role at this stage of middle ear development. However, the tympanic annulus is absent from two juveniles that are older in age but smaller in body size than the 3 week postmetamorphic toadlets where the tympanic annulus is first observed. Likewise, the tympanic membrane is only seen in the largest juvenile in this study, despite being 3 weeks younger in age than the oldest individuals in this study. Thus, at later stages of middle ear development juvenile body size may play a role in the timing of individual middle ear structure formation, however, a study with larger sample sizes is needed to address this possibility.

Furthermore, our timeline of middle ear development in *R. horribilis* is consistent with previous studies that found incomplete middle ears in juvenile bufonids of comparable sizes. From this study, we know the middle ear of *R. horribilis* is not complete at 15 weeks (SVL ≤ 18.2 mm), and previous studies indicate that the columellar shaft is incompletely ossified and the tympanic annulus remains sickle-shaped at even larger body sizes (SVL = 33.6 mm, Womack et al., 2016). Other bufonid species also have incompletely ossified columellar shafts and sickle-shaped tympanic annuli at comparable juvenile sizes (*Sclerophrys* [=*Bufo*] *regularis* SVL = 22 mm—Sedra & Michael, 1959; *Anaxyrus boreas* SVL = 29 mm—Gaudin, 1978; *Bufo bufo* SVL = 18.5, *Strauchbufo* [= *Bufo*] *raddei* SVL = 20 mm, *Bufotes viridis* SVL = 19 mm—Vorobyeva & Smirnov, 1987; *Rhaebo haematiticus* SVL = 21.1 mm, *Rhinella leptoscelis* SVL = 27.2, *Rhinella spinulosa* SVL = 30.1 mm—Womack et al., 2016). Several bufonid species complete middle ear development at slightly larger body sizes (*Sclerophrys* (= *Bufo*) *regularis* SVL = 35 mm—Sedra & Michael, 1959; *Rhinella alata* SVL = 34.6, *Rhinella leptoscelis* SVL = 42.4—Womack et al., 2016). Yet, there is at least one bufonid species that has a fully developed middle ear at much smaller juvenile sizes (*Duttaphrynus* (= *Bufo*) *melanostictus*, SVL = 17 mm—Vorobyeva & Smirnov, 1987), while the columella of another species remains incompletely ossified at larger juvenile body sizes (*Bufo bufo* SVL = 40–45 mm—Vorobyeva & Smirnov, 1987). Although detailed timelines for middle ear development are unknown for these other bufonids, the high number of bufonids with incomplete ears at relatively large juvenile sizes indicate that middle ear development within Bufonidae is often delayed, but to a variable degree among species.

Variable middle ear development rate among bufonid species could be due to variability in adult body size or age at maturation. For example, larger, late-maturing bufonids may develop more slowly in general or bufonids may not rely on their middle ear until sexual maturity. Either of these hypotheses may explain *R. horribilis'* prolonged middle ear development, as *R. horribilis* is a large species (60–220 mm at maturity) and does not sexually mature until 1–2.5 years postmetamorphosis (Oliveria et al., 2017). However, when we compare the development of the middle ear among bufonids in relation to their smallest recorded adult size, we find little pattern. For example, *Duttaphrynus* (= *Bufo*) *melanostictus* has a complete middle ear at SVL = 17 mm, just 11% of adult body size (Vorobyeva & Smirnov, 1987; Oliveira et al., 2017), whereas *Rhinella leptoscelis* completes middle ear development at 53% of the smallest adult size (Padial, Chaparro, Koehler, & De La Riva, 2009; Womack

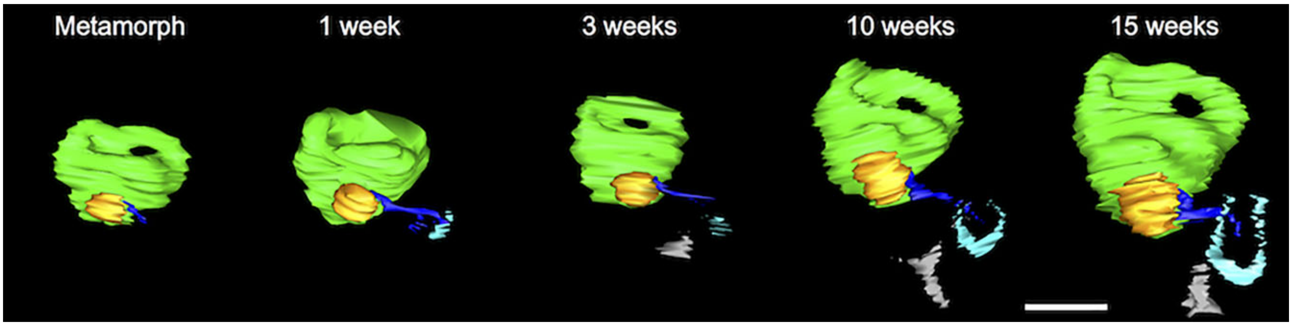

FIGURE 3 3D reconstructions of ear structures of *R. horribilis*, from metamorphosis to 15 weeks postmetamorphosis. Inner ear (green), operculum (orange), middle ear cavity and Eustachian tube (gray), columella (blue), tympanic annulus (turquoise). All models are to scale, scale bar = 1 mm

et al., 2016). Furthermore, other bufonid species have incomplete middle ears from 19% up to 52% of their adult body size (*Bufo bufo*—Vorobyeva & Smirnov, 1987; Oliveira et al., 2017 up to *Anaxyrus boreas*—Gaudin, 1978; Oliveira et al., 2017). Size or age at maturity could be predictors of middle ear development rate; however, precise age and size at maturity data are lacking for most anuran species. Given current evidence, adult body size seems to be a poor predictor of middle ear development rate among bufonids.

Our data support the conclusion that bufonids have prolonged development of the tympanic middle ear compared to nonbufonids (Sedra & Michael, 1959; Hetherington, 1987; Vorobyeva & Smirnov, 1987; Smirnov, 1991). The columellar footplate of *R. horribilis* appears at metamorphosis and is the first tympanic middle ear structure to form, which is similar to other anurans (Hetherington, 1987; Vorobyeva & Smirnov, 1987; Trueb & Hanken, 1992; Cannatella, 1999). Despite this similar start to middle ear development, other anurans complete development of the tympanic middle ear much earlier than *R. horribilis* (*Pseudacris* (= *Hyla*) *crucifer* ≤ 60 days postmetamorphosis, *Lithobates* (= *Rana*) *pipiens* = before metamorphosis completes—Hetherington, 1987; 1992; *Pyxicephalus adspersus* = before metamorphosis completes—Haas, 1999; *Pseudis platensis* = before metamorphosis completes—Fabrezi & Goldberg, 2009). Comparative work within Bufonidae and among anuran families would further inform potential drivers of variation in middle ear development among anurans.

5 | CONCLUDING REMARKS

R. horribilis follows the same proximal to distal developmental sequence of the tympanic middle ear found in other anurans, but its middle ear development is protracted, a feature that may be common within the family Bufonidae.

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AUTHOR CONTRIBUTIONS

MC Womack contributed to the conception of the study, data collection, data analysis, and writing. JL Stynoski and MK Voyles contributed to data collection, data analysis, and writing. KL Hoke contributed to the conception of the study and writing. LA Coloma contributed to specimen and data collection. All authors contributed to manuscript editing and gave final approval for publication.

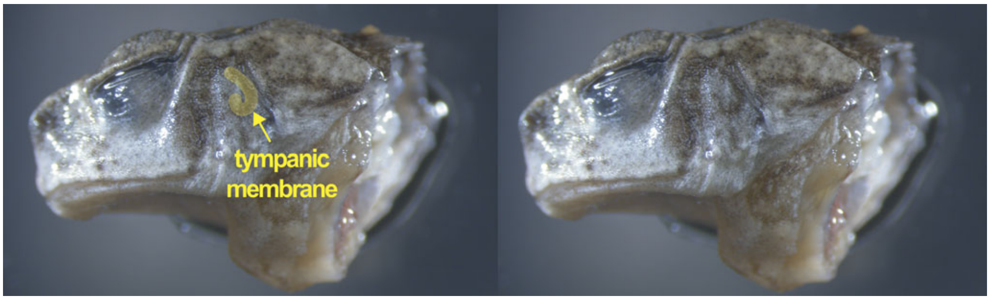

FIGURE 4 Photograph of a developing tympanic membrane at 13 weeks postmetamorphosis on a *R. horribilis* specimen. Two identical photographs (one labeled, one unlabeled) showing the external sagittal view of the developing tympanic membrane

CONFLICT OF INTEREST

We have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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