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RESEARCH ARTICLE

Bone conduction pathways confer directional cues to salamanders

G. Capshaw[1,*], J. Christensen-Dalsgaard[2], D. Soares[3] and C. E. Carr[1]

ABSTRACT

Sound and vibration are generated by mechanical disturbances within the environment, and the ability to detect and localize these acoustic cues is generally important for survival, as suggested by the early emergence of inherently directional otolithic ears in vertebrate evolutionary history. However, fossil evidence indicates that the water-adapted ear of early terrestrial tetrapods lacked specialized peripheral structures to transduce sound pressure (e.g. tympana). Therefore, early terrestrial hearing should have required nontympanic (or extratympanic) mechanisms for sound detection and localization. Here, we used atympanate salamanders to investigate the efficacy of extratympanic pathways to support directional hearing in air. We assessed peripheral encoding of directional acoustic information using directionally masked auditory brainstem response recordings. We used laser Doppler vibrometry to measure the velocity of sound pressure-induced head vibrations as a key extratympanic mechanism for aerial sound reception in atympanate species. We found that sound generates head vibrations that vary with the angle of the incident sound. This extratympanic pathway for hearing supports a figure-eight pattern of directional auditory sensitivity to airborne sound in the absence of a pressure-transducing tympanic ear.

KEY WORDS: Amphibian, Hearing, Extratympanic, Sound localization

INTRODUCTION

Sound is generated by mechanical disturbances, often in the vicinity of the receiver, and thus the ability to localize sound sources generally confers a clear fitness advantage, for example to facilitate navigation, detection of prey and avoidance of potential predators. The emergence of inherently directional otolithic sense organs in aquatic gnathostomes during early vertebrate evolutionary history indicates that peripheral encoding of directional cues from acoustic energy may be an ancestral feature of the tetrapod ear. However, little is known about the evolution of directional hearing during the water-to-land transition. In this study, we show that the atympanic salamander ear is directional, even without impedance-matching tympanic middle ears, which are generally considered essential for sound source localization in terrestrial tympanate vertebrates.

The water-to-land transition imposed a novel challenge for early tetrapods: their tissue was now of greater density than the

[1]Department of Biology, University of Maryland, College Park, MD 20742, USA. [2]Department of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark. [3]Department of Biological Sciences, New Jersey Institute of Technology, Newark, NJ 07102, USA.

*Author for correspondence (gcapshaw@umd.edu)

G.C., 0000-0002-5688-2538; J.C., 0000-0002-6075-3819; D.S., 0000-0002-8161-0841; C.E.C., 0000-0001-5698-6014

surrounding media (air) and therefore served as a reflective barrier preventing acoustic energy from reaching the sensory hair cells of the inner ear. The tympanic middle ear evolved separately in each major tetrapod lineage, at least five times during vertebrate evolutionary history as an adaptation to compensate for the air–skin impedance mismatch (Christensen-Dalsgaard and Carr, 2008; Clack, 1997, 2002; Kitazawa et al., 2015). However, fossil evidence demonstrates a 100 million year delay between the development of structural adaptations to support terrestrial living during the Carboniferous period and the emergence of sound-receptive tympanic ears in the Triassic. This indicates that the ancestral tetrapod ear was largely unspecialized for hearing on land (Clack, 1997, 2002). Despite this, the first terrestrial tetrapods may have had rudimentary aerial hearing capabilities similar to that observed in modern lungfish, with sensitivity to high amplitude, low frequency airborne sound pressure (Christensen-Dalsgaard et al., 2011; Christensen et al., 2015a; Clack, 2015). Recent studies in extant atympanate species indicate that aerial hearing in the absence of an impedance-matching tympanic middle ear may occur through the detection of sound-induced head vibrations (in lungfish: Christensen et al., 2015a; in snakes: Christensen et al., 2012; and in salamanders: Capshaw et al., 2020; Christensen et al., 2015b). The broad phylogenetic distribution of this extratympanic pathway for hearing indicates that it may represent a generalized terrestrial hearing mechanism for species lacking adaptations for the detection of airborne sound.

In many tetrapod lineages, including amphibians, squamates and archosaurs, the tympanic ear was formed at the spiracular openings, which connected the two tympana via the pharynx. This is an important feature, because the resulting acoustical coupling of the tympana produces inherently directional ears (Bee and Christensen-Dalsgaard, 2016; Christensen-Dalsgaard and Manley, 2005, 2008). Within the coupled ears of these taxa, sound interacts with the internal and external surfaces of the tympanic membranes to generate location-dependent responses (reviewed in Christensen-Dalsgaard, 2010). Greater directional cues are extracted from sounds for which the wavelength exceeds the size of the animal's head, hence the usual binaural cues – interaural time and level differences – are negligible. In anuran amphibians, the efficacy of these sound localization mechanisms is strongly frequency dependent. However, the anuran tympanic membrane is largely unresponsive to low frequencies, and directional responses of auditory nerve fibers to low frequency sound are therefore believed to originate through extratympanic pathways, i.e. bone conduction (Feng, 1980; Jørgensen and Christensen-Dalsgaard, 1997a,b; Wilczynski et al., 1987). The extent to which extratympanic pathways can confer directionality to an atympanate vertebrate in a terrestrial environment has never been tested. Although studies of directional hearing in frogs infer the ability of extratympanic pathways to transmit location cues to the ear, it is necessary to verify that extratympanic pathways alone are sufficient to enable terrestrial

auditory directionality in the absence of specializations for detecting aerial sound pressure.

In the present study, we explore the directionality of the atympanic salamander ear to evaluate the ability of extratympanic sound detection pathways to support directional hearing in air. Although salamanders, being atympanate, had been believed to be largely insensitive to acoustic cues, behavioral studies have reported the ability of terrestrial salamanders to use acoustic cues for navigation (Diego-Rasilla and Luengo, 2004, 2007; Pupin et al., 2007). Additionally, our previous study found that airborne sound waves are capable of generating translational vibrations in the salamander skull that are detectable to the auditory end organs of the inner ear, conferring pressure sensitivity to these atympanate animals (Capshaw et al., 2020).

Here, we tested the hypothesis that airborne sound interacts with the salamander head in a directional manner to generate directional cues at the peripheral level of the auditory system even in the absence of a terrestrially adapted tympanic ear. We used auditory brainstem response (ABR) recordings to investigate peripheral encoding of directional information in the eighth cranial nerve in response to free-field airborne sound pressure stimuli. Because extratympanic hearing in salamanders is mediated by the detection of sound-induced head vibrations (Capshaw et al., 2020), we further used laser Doppler vibrometry to assess directionality of the translational movement of the animal owing to sound pressure stimuli. We combined the results of the ABRs and laser vibrometry experimentation to provide insight into the ability of an atympanic ear that is unspecialized for terrestrial sound detection to extract directional cues from free-field airborne sound pressure.

MATERIALS AND METHODS
Directional auditory brainstem response recording
We investigated the directional sensitivity of the atympanic salamander ear using a directionally masked ABR recording technique. Our study incorporated six lungless species from the family Plethodontidae [*Desmognathus fuscus* (*n*=12), *Eurycea cirrigera* (*n*=11), *Eurycea lucifuga* (*n*=13), *Gyrinophilus porphyriticus* (*n*=10), *Plethodon cinereus* (*n*=15) and *Plethodon glutinosus* (*n*=14)]. We incorporated these six ecologically diverse plethodontid species because previous study demonstrated habitat-related differences in the salamander low frequency response that may influence directional sensitivity (Capshaw et al., 2020). Because air-filled body cavities such as the lungs may influence auditory directionality in amphibians (Bee and Christensen-Dalsgaard, 2016), we also included two salamander species with lungs [*Ambystoma opacum* (*n*=9), *Ambystoma tigrinum* (*n*=8)] to provide a natural comparison to the directional response measured in lungless plethodontids. Field-collected salamander species were collected with permission from the Tennessee Wildlife Resources Agency, permit numbers 1386, 1388, 1605 and 3838.

We anesthetized salamanders using 20% w/v benzocaine applied to the ventral body surface and placed three Teflon-coated silver wire electrodes subcutaneously at the left ear (recording electrode), on the midline of the head dorsal to the brainstem (inverting electrode), and in the tail (ground). We optimized electrode placement for each individual to maximize the signal to noise ratio for evoked potential recording. ABR measurements were performed in a custom-built anechoic chamber lined with 500 mm acoustic foam wedges and shielded by a Faraday cage to reduce electrical and acoustic noise. All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee of the University of Maryland, protocol numbers R-16-59 and R-SEP-19-41.

Masked ABR (mABR) recordings were performed using a broadband click stimulus in the presence and absence of an ongoing 200 Hz masking tone (Fig. 1) with click and masker emitted from separate loudspeakers to allow assessment of directional hearing. The mABR recording paradigm allows the use of a long duration pure tone stimulus as a simultaneous masker to ensure frequency specificity even at low frequencies (Brandt et al., 2018). The click stimulus was generated by a small speaker (ORB Audio, flat frequency response from 0.15 to 18 kHz) at a distance of 0.5 m from the left ear of the salamander. The click was one half-cycle of a 2000 Hz sinusoid with sufficient power across a frequency range of 10 to 1000 Hz and was presented at the lowest level necessary to evoke a neural response at 90% of the maximum amplitude from each individual. The evoked response was evaluated based on the presence of two to three peaks in the waveform occurring approximately 5 ms after stimulus onset that were discriminable from noise, verified by visual inspection of the ABR waveforms. The recorded response latencies, when corrected for the time required for sound to travel to the salamander, approximated the 2 ms responses from auditory nerve recordings in the grass frog (Jørgensen and Christensen-Dalsgaard, 1997b) and from ABR recordings in Cope's grey treefrog (Schrode et al., 2015), indicating that the first wave of the salamander ABR may originate in the eighth cranial nerve. The masker tone was broadcast by a low frequency loudspeaker (Electro-Voice TL606A, flat frequency response from 50 to 400 Hz) placed 1.5 m from the salamander. We used a 200 Hz tone to ensure that the frequency of the masking tone was within range of peak sound pressure sensitivity for salamanders (Capshaw et al., 2020). Equipment was calibrated, stimuli were generated and data were recorded using Tucker-Davis Technologies system 2 hardware and custom software (QuickABR, Odense, Denmark) following methods described in Capshaw et al. (2020).

The anesthetized salamander was placed on an acoustic foam block on a rotating table within an anechoic chamber. The position of the click speaker was held constant 0.5 m from the salamander and oriented to present the click stimulus to the left ear, ipsilateral to the recording electrode (−90 deg). The animal and click speaker were rotated relative to the tone speaker to present the masker tone from eight sound incidence angles separated by 45 deg intervals (Fig. 2A). We increased the amplitude of the simultaneous tone until masking was observed as a reduction in the amplitude of the click response waveform (Fig. 1C). Masked and unmasked click responses were averaged over 800 presentations, and every second stimulus presentation was phase inverted to reduce artifacts. Auditory sensitivity to the 200 Hz tone was determined as its efficiency in attenuating the neural response to the click stimulus (e.g. its masking efficiency). Detection thresholds represent the lowest level of the masker tone that reduced the amplitude of the ipsilateral click response in the masked ABR relative to the unmasked ABR (Fig. 1C). Thresholds were verified by visual inspection of evoked potential waveforms using MATLAB vR2016b (MathWorks). We randomized the order of presentation for masker tone locations and used a coding scheme to ensure that detection threshold analyses could be performed blind to the location of the sound source for each trial. Although automated algorithms are useful to ensure objective and consistent threshold detection, several studies have indicated that visual inspection and automated algorithms perform equally well with no significant variation between thresholds determined using either method (Brandt et al., 2018; Lauridsen et al., 2021; Mooney et al., 2010; Schrode et al., 2015).
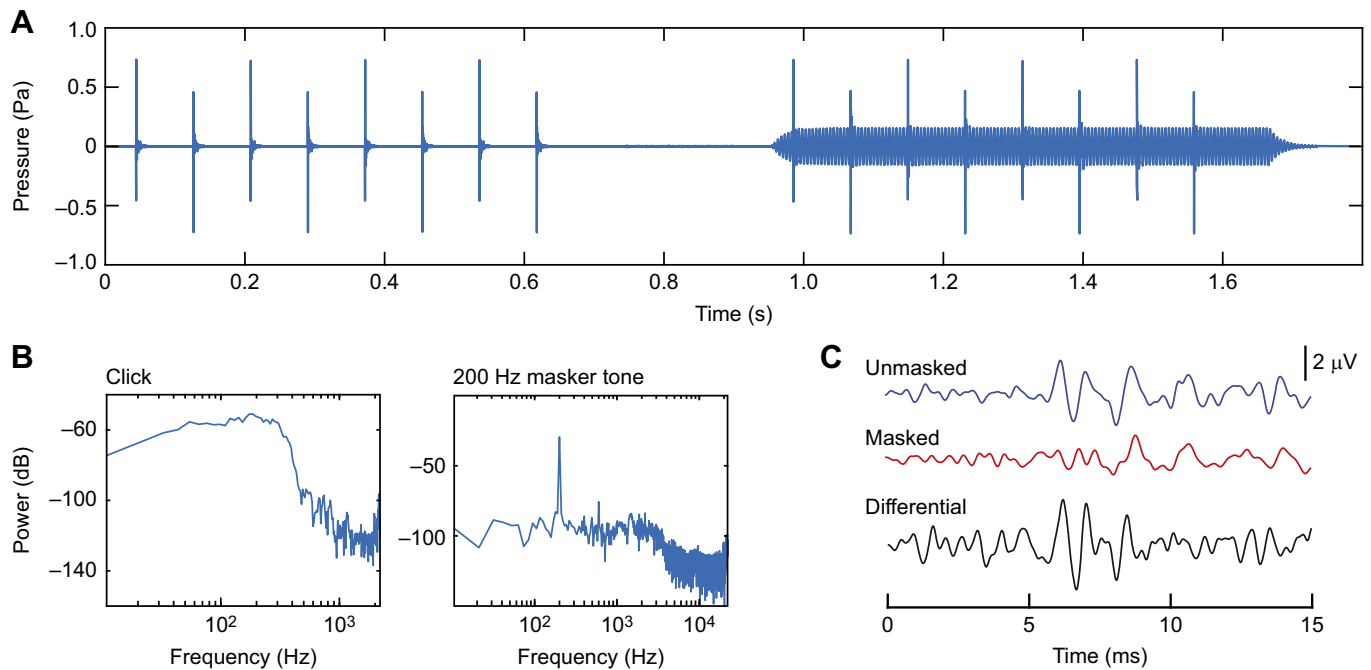
Fig. 1. Sound pressure stimuli and the auditory brainstem response (ABR) recorded in a salamander. (A) A single stimulation cycle for the masked ABR (mABR) is composed of a train of unmasked clicks followed by a series of clicks overlaid with a simultaneous 200 Hz pure tone masker. (B) Power spectra for the broadband click stimulus (left) and 200 Hz tone (right) used in mABR recordings. (C) Sensitivity to the masker tone (masking efficiency) is calculated as the difference between the unmasked and masked ABRs. Waveforms are scaled to a stimulus onset at 0 ms. A and B are reproduced from Capshaw et al. (2020, fig. 1).

Directional sensitivity was determined by comparing sensitivity thresholds among different locations for the masker tone relative to the ipsilateral response (Fig. 2B). We normalized mABR thresholds by computing the difference between thresholds recorded at different masker tone locations and the ipsilateral response. From these normalized thresholds, we created polar plots of directional sensitivity to the 200 Hz tone. We evaluated the significance of threshold differences using a linear mixed model incorporating species and masker tone position as fixed effects and individual as a random effect using the R package nlme v3.1-131 (https://CRAN.R-project.org/package=nlme). We used type III tests of fixed effects to evaluate significant differences among factors and followed these

analyses with *post hoc* pairwise testing with Bonferroni adjustments for multiple comparisons using the R package lsmeans v2.26-3 (https://CRAN.R-project.org/package=lsmeans). We assessed the influence of the air-filled lungs on the auditory directional response using linear mixed models incorporating masker tone position and presence/absence of lungs as fixed factors, and species and individual as nested random effects.

Laser vibrometry
We used laser Doppler vibrometry to measure the vibrations induced in the lateral surface of the salamander head overlying the left ear by free-field sound pressure stimuli. Vibration data were
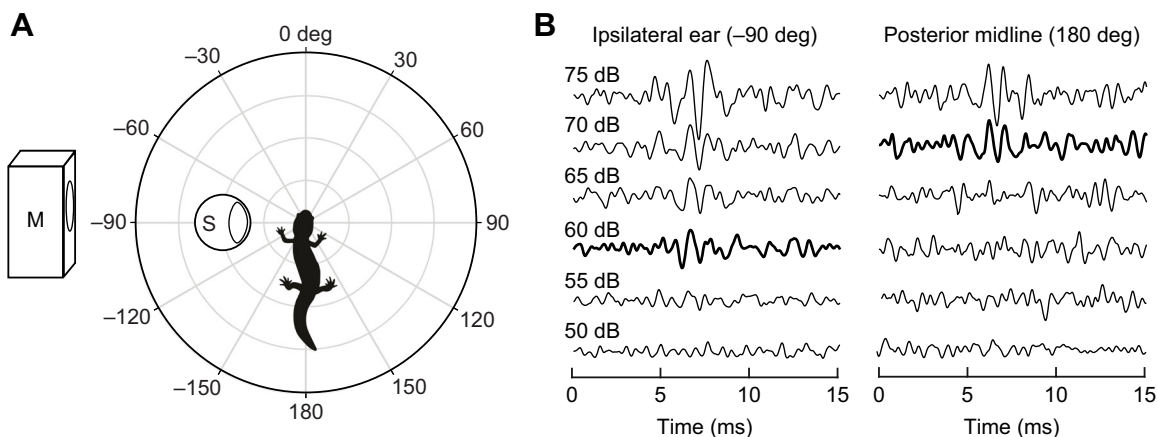
Fig. 2. Directional masked auditory brainstem response recording. (A) Schematic of the directional mABR set-up (not to scale). The click stimulus was presented by a speaker (S) 0.5 m from the left (recording) ear. Directional sensitivity was measured by rotating the heading direction of the salamander with respect to the masking tone speaker (M) located 1.5 m from the salamander. (B) Differential ABR waveforms representing the difference between the unmasked and masked click response recorded from an individual, scaled to a stimulus onset at 0 ms. Detection thresholds (bold lines) were determined as the lowest level of the tone (in dB re. 20 μPa) that attenuated the click response.

collected from *A. opacum* (*n*=4), *A. tigrinum* (*n*=4), *D. fuscus* (*n*=3), *E. cirrigera* (*n*=4), *E. lucifuga* (*n*=4), *G. porphyriticus* (*n*=4), *P. cinereus* (*n*=5) and *P. glutinosus* (*n*=3). Salamanders were placed on a heavy steel platform in an IAC Acoustics anechoic chamber covered with 500 mm foam rubber acoustic wedges to reduce reverberations. The salamander was positioned with the head facing 0 deg, the ipsilateral (recording) ear at −90 deg and the contralateral (right) ear at +90 deg (Fig. 2A). Sound pressure stimuli consisted of 100 ms frequency sweeps (0.1–10 kHz at 81 dB SPL) emitted by 12 JBL 1G loudspeakers located 1 m from the salamander and separated by 30 deg intervals. We measured vibration velocities using an OFV-5000 vibrometer with an OFV-505 sensor (Polytec, Waldbronn, Germany) at a sensitivity of 1 V mm^{-1} s^{-1}. We used a probe microphone (BK 4182) to measure sound pressure levels at the location of the salamander head.

Stimuli were generated and data were recorded using Tucker-Davis Technologies system 2 hardware and custom software (DragonQuest, Odense, Denmark). Sound and vibration recordings were collected using a 22.64 kHz sampling rate and averaged over 10 presentations. We extracted vibration velocities measured in response to a 200–400 Hz subset of the sweep to enable more direct comparison with the physiological measure of auditory directionality obtained via mABR. We calculated transfer functions as the ratio between the laser spectra measured by the vibrometer and the sound spectra recorded by the probe microphone. Vibration velocity transfer functions (in dB re. 1 mm s^{-1} Pa^{-1}) were plotted as a function of the incident angle of the sound pressure stimulus to generate polar plots of sound-induced motion of the salamander head.

RESULTS

### Directionality of the neurophysiological response

Directional mABR recordings revealed directional sensitivity to a 200 Hz sound pressure stimulus among eight atympanate salamander species. Fig. 3 shows mean detection thresholds for the masker tone recorded from each species across eight different tone locations. Auditory sensitivity to the 200 Hz tone demonstrates species-specific variation consistent with patterns observed in our previous study (Capshaw et al., 2020), in which facultative cave species *Eurycea lucifuga* and *Gyrinophilus porphyriticus* had lower detection thresholds for the masker tone relative to all other species. However, we observed no significant interspecific differences in directional sensitivity to the 200 Hz tone ($F_{7,84}$=0.59, P=0.76). Further, the presence of air-filled lungs did not significantly affect

directional hearing in salamanders (lung: $F_{1,6}$=1.27, P=0.30; lung×tone position: $F_{7,630}$=0.72, P=0.66).

A comparison of normalized detection thresholds across different masker tone positions demonstrates consistent directionality with a characteristic figure-eight pattern across all species (Fig. 4A). We observed a significant main effect of the masker tone position on its masking efficiency, measured as mABR threshold difference relative to the ipsilateral response ($F_{7,588}$=4.46, P=0.0001). The masking efficiency of the 200 Hz tone was greatest when presented from the same direction as the click stimulus (−90 deg), and decreased as the direction of the masking tone input rotated relative to the recording ear. Contralateral thresholds (+90 deg) were slightly elevated relative to the ipsilateral response; however, pairwise comparisons of the data showed that these did not deviate significantly from ipsilateral thresholds (P=0.94). The highest sensitivity thresholds for the 200 Hz masker tone were obtained at the rostral (0 deg) and caudal (180 deg) ends, averaging 5–6 dB greater than the ipsilateral response; these threshold differences were significant across all species (Bonferroni-adjusted pairwise comparisons of least square means: $P<0.001$ when comparing each masker position to the ipsilateral response).

Directionality of sound-induced head vibrations

We evaluated variation in the amplitude of vibration velocity transfer functions (in dB re. 1 mm s^{-1} Pa^{-1}) owing to the incident angle of the sound pressure stimulus at the lateral head surface overlying the left ear. The angle of sound incidence had a significant effect on amplitude of vibration transfer functions recorded at the ipsilateral ear for all species ($F_{11,385}$=2.31, $P<0.001$). Vibration velocity transfer functions were greatest in response to sound sources oriented toward the ipsilateral ear (Fig. 4B). Contralateral stimulation resulted in a reduction in transfer function amplitude averaging approximately 3 dB (range: 1–6 dB differences across species) compared with the ipsilateral response. Stimulation at the rostral and caudal ends of the animal resulted in significantly reduced transfer function amplitudes relative to ipsilateral stimulation (Bonferroni-adjusted pairwise comparisons: $P<0.0001$ for rostral and caudal stimulation). We observed no significant interspecific differences in sound-induced vibrations ($F_{7,35}$=1.96, P=0.10), and no significant effects of the air-filled lungs on measured head vibrations (lungs: $F_{1,6}$=0.05, P=0.83; lungs×tone position: $F_{11,451}$=1.35, P=0.20). Comparison of normalized sound pressure sensitivity thresholds and sound-induced head vibrations revealed a similar pattern in which detection thresholds and
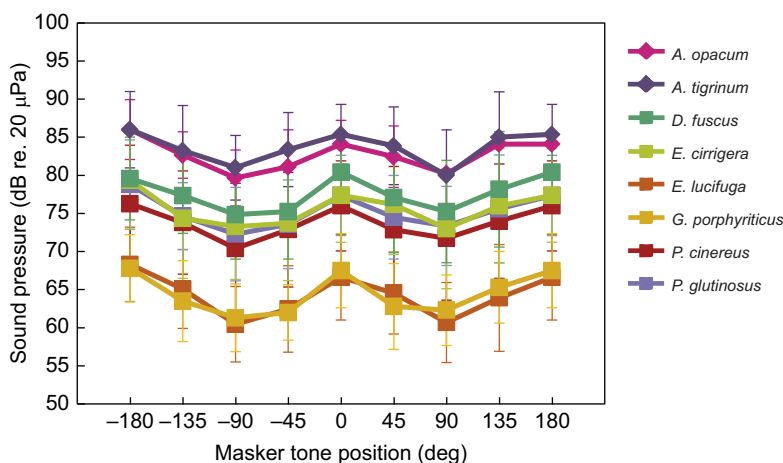
Fig. 3. Sound pressure thresholds as a function of masker tone position in eight salamander species: *Ambystoma opacum*, *A. tigrinum*, *Desmognathus fuscus*, *Eurycea cirrigera*, *E. lucifuga*, *Gyrinophilus porphyriticus*, *Plethodon cinereus* and *P. glutinosus*. Detection thresholds (means ±s.e.m.) for the 200 Hz masker tone were measured as the tone rotated relative to the ipsilateral (−90 deg) click stimulus.
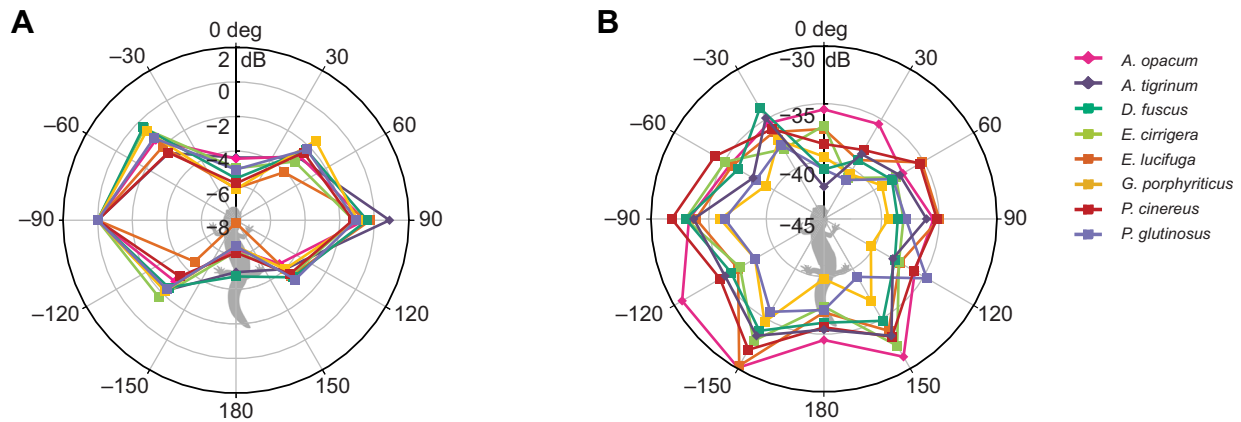
Fig. 4. Peripheral directionality of the auditory response in eight salamander species. (A) Directional auditory sensitivity represented by mean masking efficiency of the 200 Hz tone (in dB re. 20 μPa) broadcast from different locations around the salamander, normalized to the ipsilateral (−90 deg) response. (B) Directional characteristics of sound-induced head vibrations. Values represent the mean vibration velocity transfer function (in dB re. 1 mm s⁻¹ Pa⁻¹) in response to a free-field 200–400 Hz sweep broadcast from different locations around the salamander. Laser vibrometer data were collected from the lateral head surface overlying the left ear.

vibration velocity transfer functions varied with the angle of the incident sound to generate auditory directionality characterized by a rostral and caudal null. We note, however, that the figure-eight directionality was more prominent in the measures of auditory sensitivity (Fig. 4A).

DISCUSSION

We have shown that the atympanic ear of salamanders is directional, and that this directionality is most likely generated by sound-induced vibrations of the head. To assess the directional sensitivity of the eighth cranial nerve, we used mABR recordings in response to free-field sound pressure emitted from eight locations around the animal. In salamanders, mABR detection thresholds show a figure-eight pattern of directional sensitivity in the eighth cranial nerve, reflecting greatest sensitivity to sound pressure along the mediolateral axis of the body and reduced sensitivity along the rostrocaudal axis. Salamanders had the lowest detection thresholds in response to ipsilateral stimulation, although this response did not differ significantly from thresholds obtained in response to contralateral stimulation.

We observed no significant interspecific differences in the patterns of directional sensitivity among the eight species incorporated in our study, and therefore found no evidence for variation in auditory directionality among diverse salamander species. Although head sizes differ among the species tested, this variation is small relative to the wavelength of the sound waves corresponding to peak auditory sensitivity (e.g. less than 250 Hz) in salamanders. It follows that bone conduction hearing, mediated by the detection of sound-induced head vibrations, may generate a consistent pattern of directionality among species, regardless of species-specific variation in size or auditory detection thresholds. Additionally, there were no significant differences in the directional response of species with or without lungs, indicating that the presence of air-filled body cavities such as the lungs does not contribute to auditory directionality in atympanate salamanders.
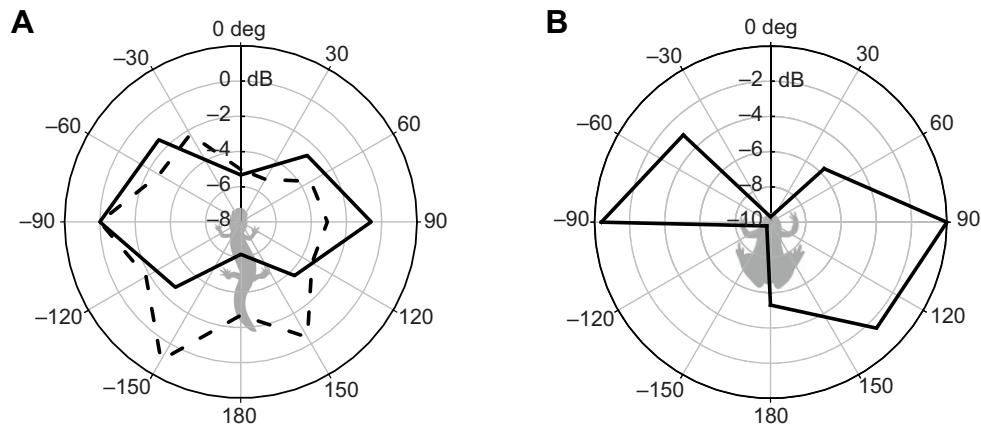
In atympanate species, auditory sensitivity to airborne sound is most likely achieved through the detection of sound-induced vibrations in the head (Capshaw et al., 2020; Christensen et al., 2012, 2015b). This extratympanic mechanism for aerial sound detection occurs when a sound pressure wave generates translational movement in the animal that is of sufficient amplitude to stimulate

the auditory end organs in the inner ear. We used laser vibrometry to assess the directionality of the sound pressure-generated vibrations in the salamander head in response to sounds broadcast from 12 locations around the animal. We observed a figure-eight-like pattern of directionality encoded in the bone-conducted vibration velocities in all species similar to that obtained from mABR evoked activity in the eighth cranial nerve. However, the figure-eight pattern of sound-induced head vibrations was not symmetrical across the midline: sounds broadcast ipsilateral to the recording ear elicited the highest vibration velocities relative to all other sound source directions, including the contralateral direction. Comparison of the amplitude of sound-induced head vibrations measured by laser vibrometry and directional sensitivity measured by mABR recording reveals a notably sharper figure-eight pattern encoded by the auditory nerve (Fig. 5A). This suggests that peripheral processing of sound at the level of the inner ear may further refine the representation of sound source location to the atympanic auditory system, beyond the directional cues provided by sound source location-dependent variation in head vibrations.

Common patterns of low-frequency directionality in eared and earless amphibians

In anurans with a functional tympanic ear, the directionality of the ear is frequency dependent. For high frequency sounds, auditory directionality shows an ovoidal pattern with the greatest activity elicited in single unit auditory fibers in response to ipsilateral sound sources (Feng, 1980; Feng and Shofner, 1981; Jørgensen and Christensen-Dalsgaard, 1997a; Wang et al., 1996). This ovoidal pattern of directional sensitivity matches the ovoidal directionality of the eardrum and is indicative of the anuran tympanic middle ear acting as a combined pressure and pressure difference receiver for frequencies greater than 500 Hz.

At low frequencies, the directionality of the frog ear transitions to a figure-eight pattern comparable to the pattern we observed in the atympanic salamander ear (Fig. 5) (Feng, 1980; Feng and Shofner, 1981; Jørgensen and Christensen-Dalsgaard, 1997a; Wang et al., 1996). This transition is also seen in the directionality of individual fibers to low and high frequency stimulation (see Jørgensen and Christensen-Dalsgaard, 1997a, their fig. 7), indicating that the origin of the directional response is peripheral to the inner ear sensory cells. Similar to the salamanders in the present study, single

Fig. 5. Comparison of the low frequency directional response in atympanate and tympanate amphibians. (A) Salamander auditory sensitivity thresholds (solid line) and head vibration transfer functions (dashed line) measured in response to free-field sound pressure stimuli. Values represent grand mean data across all species, normalized to the ipsilateral (−90 deg) response; thresholds are in dB relative to the ipsilateral response. (B) Directional response of a single auditory fiber in *Rana temporaria* recorded from the right ear (+90 deg) in response to low frequency (300 Hz) tone bursts. Values are equivalent dB, relative to the ipsilateral (+90 deg) response. Equivalent dB are calculated by referring spike rates elicited by directional stimulation to the rate-intensity function measured at +90 deg sound incidence. Redrawn from Jørgensen and Christensen-Dalsgaard (1997a, fig. 10).

unit auditory fibers in *Rana pipiens* are maximally responsive to low frequency sound sources presented from the lateral fields and minimally responsive to sounds emitted along the body midline (Feng, 1980; Wang et al., 1996). In *Rana temporaria*, the strongest directionality of auditory nerve fibers occurs at low frequencies (200–400 Hz) and exhibits a figure-eight pattern with a slightly higher response to ipsilateral stimulation (Fig. 5B) (Jørgensen and Christensen-Dalsgaard, 1997a). The recordings also showed a 180 deg phase difference in neural responses to ipsilateral and contralateral stimulus directions (Jørgensen and Christensen-Dalsgaard, 1997b). The authors suggested that this directionality was consistent with a model of the inner ear as a fluid-filled tube where sound-induced motion parallel to the tube orientation would result in maximal relative fluid motion. Thus, the source of low frequency directionality in anurans may be sound-induced translation of the head, sharpened by the orientation of the pressure-release windows of the inner ear. In the present study, we observed very similar patterns of directional auditory sensitivity in atympanate salamanders and conclude that there may be a common extratympanic pathway that mediates the low frequency directional response in the unspecialized ears of atympanate species as well as in the terrestrially adapted tympanic ears of auditory specialists.

Mechanisms for extratympanic auditory directionality in salamanders

The lung–ear pathway has previously been implicated for improving the directional response of the tympanic middle ear in amphibians (Ehret et al., 1990, 1994; Hetherington, 2001; Jørgensen et al., 1991; Narins et al., 1988). Here, we show that salamanders with and without lungs demonstrate comparable directional responses to low frequency sound pressure, indicating that the air-filled lungs do not contribute to peripheral auditory directionality in atympanate species.

In salamanders, as in other amphibians, low frequency directional responses may instead rely on extratympanic bone conduction pathways for sound. Sound-induced translation of the animal may create fluid movement within the inner ear that is directional depending on the location of pressure-relief windows of the inner ear, as discussed above (Jørgensen and Christensen-Dalsgaard,

1997a). Inner ear fluids may therefore show maximal displacement in response to sound stimulation oriented along the axis of the pressure-relief windows. For both anurans and salamanders, pressure relief primarily occurs along the mediolateral (interaural) axis. In anurans, pressure dissipates through the round window, a ventrolateral fenestration of the otic capsule, whereas in salamanders, the absence of a round window results in the majority of pressure relief occurring at the perilymphatic foramen, a fenestration that opens medially to the cranial cavity (Smith, 1968; Wever, 1978). These otic fenestrations provide compliant windows for fluid displacement within the inner ear that can support a figure-eight pattern of maximal displacement along the interaural axis. Additionally, peripheral directionality relies on differential hair cell polarity to mediate the neural response to fluid inertia within the inner ear. Hair cells must be oriented in a direction that enables maximal responsiveness along the axis of particle displacement within the ear. Bidirectional orientation of the amphibian papillar hair cells of both caecilians and salamanders support directional sensitivity with maximal responsiveness to axial motion of the tectorial membrane (Lewis, 1981; Lewis and Narins, 1999).

Atympanic sound localization and directional ambiguity

The problem with the figure-eight sensitivity of the salamander ear is that it is ambiguous with insufficient cues to distinguish ipsilateral from contralateral directions. This 180 degree ambiguity problem has been discussed extensively in the fish literature, and the general idea is that fish may require a secondary input to allow them to disambiguate ipsilateral and contralateral sound sources via the phase relationship between particle motion and sound pressure (for recent reviews, see Hawkins and Popper, 2018; Sisneros and Rogers, 2016). Access to phase comparison between the directly received acoustic stimulus and that re-radiated from a second source may also provide the basis for eliminating the ambiguity resulting from the figure-eight pattern of directional detection observed here in salamanders.

Potential second sources for phase comparisons in salamanders include the perilymphatic connections coupling the inner ears (Wever, 1978). The salamander otic capsule lacks a round window and, unlike other species that use a reentrant fluid circuit to provide pressure relief in the absence of a round window, fluid displacement

in the salamander ear passes through the cranial cavity via the perilymphatic foramina to the oval window of the opposite ear (Smith, 1968; Wever, 1978). Wever (1978) noted that direct vibratory stimulation of one ear evoked cochlear potentials in both ears, with greater ipsilateral responses. Hearing in salamanders is therefore binaural, and binaural phase differences may contribute to sound localization. Other secondary input sources may include the air-filled lungs and the mouth cavity. These could potentially act as a monopole sensor that, together with input from dipole sensors such as the otolithic saccular maculae, could resolve the 180 degree ambiguity in a manner similar to the swim bladder in auditory specialist fish. Alternatively, the operculum – via its attachment to the scapula – may provide a secondary vibration input to the inner ear.

Although the neural substrates underlying the directional processing of acoustic information in salamanders are unknown, comparisons with bony fish and frogs are instructive. In these animals, intrinsically directional inputs from the eighth cranial nerve terminate in the medulla, and projections between the first order brainstem auditory nuclei may serve to sharpen the figure-eight directional response. In fish, auditory directionality is generally encoded by fibers that innervate saccular hair cells with similar best axes, and exhibit figure-eight directionality (Edds-Walton et al., 1999; Fay and Edds-Walton, 1997). Binaural projections between the descending octaval nuclei, the first site of central processing in the auditory medulla of the fish, refine the directional response (Edds-Walton, 1998; Edds-Walton and Fay, 1998, 2009), and in toadfish, recordings from the midbrain torus semicircularis reveal further sharpening (Edds-Walton and Fay, 2003). In frogs, contralateral inhibition between the two dorsal medullary nuclei converts the figure-eight low frequency directional response in the auditory nerve fibers into a more ovoidal (i.e. disambiguated) pattern biased towards ipsilateral sounds (Christensen-Dalsgaard and Kanneworff, 2005). The anuran torus semicircularis retains this directional information, with the best responses elicited by lower frequencies presented along the interaural axis (Pettigrew et al., 1981). Salamanders share similar ascending projections to frogs. Their torus semicircularis receives contralateral auditory projections from the intermediate nucleus of the octaval column, and auditory units in the torus are more sensitive to contralateral acoustic stimulation (Manteuffel and Naujoks-Manteuffel, 1990). Thus, it seems probable that directionality of the atympanic salamander ear is supported by binaural projections in the auditory brainstem. Species comparisons indicate that binaural connections mediating sound localization are likely ancestral to tetrapods (Carr and Christensen-Dalsgaard, 2016; Walton et al., 2017).

In summary, salamander responses to low frequency sound are directional, and should permit sound source localization to facilitate navigation. Directionality in these atympanate species likely originates from differential amplitudes of sound-induced vibrations of the head that vary with incident angle of the sound source. This extratympanic mechanism for the reception of airborne sound is capable of generating fluid inertial movement within the otic capsule of the atympanic ear, where maximal displacement occurs at the locations of the pressure-relief windows along the interaural axis. Bone conduction of sound therefore supports a figure-eight pattern of extratympanic sensitivity to sound pressure, which has been observed in tympanate and atympanate amphibian species in response to low frequency acoustic stimulation, and may reflect the capacity of the ancestral atympanic tetrapod ear to detect directional cues from aerial sound in a terrestrial environment. More

research is needed to investigate how directional information is processed (and disambiguated) by the central nervous system. Also, these studies would be greatly informed by further investigations of sound localization behavior in atympanate species.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: G.C., J.C.-D., D.S., C.E.C.; Methodology: G.C., J.C.-D., C.E.C.; Software: J.C.-D., C.E.C.; Formal analysis: G.C., J.C.-D.; Investigation: G.C., J.C.-D., C.E.C.; Resources: J.C.-D., D.S., C.E.C.; Data curation: G.C.; Writing - original draft: G.C., C.E.C.; Writing - review & editing: G.C., J.C.-D., D.S., C.E.C.; Visualization: G.C., J.C.-D.; Supervision: D.S., C.E.C.; Project administration: C.E.C.; Funding acquisition: G.C., J.C.-D., C.E.C.

References

Bee, M. A. and Christensen-Dalsgaard, J. (2016). Sound source localization and segregation with internally coupled ears: the treefrog model. *Biol. Cybern.* **110**, 271-290. doi:10.1007/s00422-016-0695-5

Brandt, C., Brande-Lavridsen, N. and Christensen-Dalsgaard, J. (2018). The masked ABR (mABR): a new measurement method for the auditory brainstem response. *JARO J. Assoc. Res. Otolaryngol.* **19**, 753-761. doi:10.1007/s10162-018-00696-x

Capshaw, G., Soares, D., Christensen-Dalsgaard, J. and Carr, C. E. (2020). Seismic sensitivity and bone conduction mechanisms enable extratympanic hearing in salamanders. *J. Exp. Biol.* **223**, jeb236489. doi:10.1242/jeb.236489

Carr, C. E. and Christensen-Dalsgaard, J. (2016). Evolutionary trends in directional hearing. *Curr. Opin. Neurobiol.* **40**, 111-117. doi:10.1016/j.conb.2016.07.001

Christensen, C. B., Christensen-Dalsgaard, J., Brandt, C. and Madsen, P. T. (2012). Hearing with an atympanic ear: good vibration and poor sound-pressure detection in the royal python, *Python regius*. *J. Exp. Biol.* **215**, 331-342. doi:10.1242/jeb.062539

Christensen, C. B., Christensen-Dalsgaard, J. and Madsen, P. T. (2015a). Hearing of the African lungfish (*Protopterus annectens*) suggests underwater pressure detection and rudimentary aerial hearing in early tetrapods. *J. Exp. Biol.* **218**, 381-387. doi:10.1242/jeb.116012

Christensen, C. B., Lauridsen, H., Christensen-Dalsgaard, J., Pedersen, M. and Madsen, P. T. (2015b). Better than fish on land? Hearing across metamorphosis in salamanders. *Proc. R. Soc. B Biol. Sci.* **282**, 20141943. doi:10.1098/rspb.2014.1943

Christensen-Dalsgaard, J. (2010). Vertebrate pressure-gradient receivers. *Hear. Res.* **273**, 37-45. doi:10.1016/j.heares.2010.08.007

Christensen-Dalsgaard, J. and Carr, C. E. (2008). Evolution of a sensory novelty: tympanic ears and the associated neural processing. *Brain Res. Bull.* **75**, 365-370. doi:10.1016/j.brainresbull.2007.10.044

Christensen-Dalsgaard, J. and Kanneworff, M. (2005). Binaural interaction in the frog dorsal medullary nucleus. *Brain Res. Bull.* **66**, 522-525. doi:10.1016/j.brainresbull.2005.03.005

Christensen-Dalsgaard, J. and Manley, G. A. (2005). Directionality of the lizard ear. *J. Exp. Biol.* **208**, 1209-1217. doi:10.1242/jeb.01511

Christensen-Dalsgaard, J. and Manley, G. A. (2008). Acoustical coupling of lizard eardrums. *JARO* **9**, 407-416. doi:10.1007/s10162-008-0130-2

Christensen-Dalsgaard, J., Brandt, C., Wilson, M., Wahlberg, M. and Madsen, P. T. (2011). Hearing in the African lungfish (*Protopterus annectens*): pre-adaptation to pressure hearing in tetrapods? *Biol. Lett.* **7**, 139-141. doi:10.1098/rsbl.2010.0636

Clack, J. A. (1997). The evolution of tetrapod ears and the fossil record. *Brain. Behav. Evol.* **50**, 198-212. doi:10.1159/000113334

Clack, J. A. (2002). Patterns and processes in the early evolution of the tetrapod ear. *J. Neurobiol.* **53**, 251-264. doi:10.1002/neu.10129

Clack, J. A. (2015). The origin of terrestrial hearing. *Nature* **519**, 168-169. doi:10. 1038/519168a

Diego-Rasilla, F. J. and Luengo, R. M. (2004). Heterospecific call recognition and phonotaxis in the orientation behavior of the marbled newt, *Triturus marmoratus*. *Behav. Ecol. Sociobiol.* **55**, 556-560. doi:10.1007/s00265-003-0740-y

Diego-Rasilla, F. J. and Luengo, R. M. (2007). Acoustic orientation in the palmate newt, *Lissotriton helveticus*. *Behav. Ecol. Sociobiol.* **61**, 1329-1335. doi:10.1007/ s00265-007-0363-9

Edds-Walton, P. L. (1998). Anatomical evidence for binaural processing in the descending octaval nucleus of the toadfish (*Opsanus tau*). *Hear. Res.* **123**, 41-54. doi:10.1016/S0378-5955(98)00097-5

Edds-Walton, P. L. and Fay, R. R. (1998). Directional auditory responses in the descending octaval nucleus of the toadfish (*Opsanus tau*). *Biol. Bull.* **195**, 191-192. doi:10.2307/1542832

Edds-Walton, P. L. and Fay, R. R. (2003). Directional selectivity and frequency tuning of midbrain cells in the oyster toadfish, *Opsanus tau*. *J. Comp. Physiol. A* **189**, 527-543. doi:10.1007/s00359-003-0428-9

Edds-Walton, P. L. and Fay, R. R. (2009). Physiological evidence for binaural directional computations in the brainstem of the oyster toadfish, *Opsanus tau* (L.). *J. Exp. Biol.* **212**, 1483-1493. doi:10.1242/jeb.026898

Edds-Walton, P. L., Fay, R. R. and Highstein, S. M. (1999). Dendritic arbors and central projections of physiologically characterized auditory fibers from the saccule of the toadfish, *Opsanus tau*. *J. Comp. Neurol.* **411**, 212-238. doi:10. 1002/(SICI)1096-9861(19990823)411:2<212::AID-CNE4>3.0.CO;2-X

Ehret, G., Tautz, J., Schmitz, B. and Narins, P. M. (1990). Hearing through the lungs: lung-eardrum transmission of sound in the frog *Eleutherodactylus coqui*. *Naturwissenschaften* **77**, 192-194. doi:10.1007/BF01131168

Ehret, G., Keilwerth, E. and Kamada, T. (1994). The lung-eardrum pathway in three treefrog and four dendrobatid frog species: some properties of sound transmission. *J. Exp. Biol.* **195**, 329-343. doi:10.1242/jeb.195.1.329

Fay, R. R. and Edds-Walton, P. L. (1997). Directional response properties of saccular afferents of the toadfish, Opsanus tau. *Hear. Res.* **111**, 1-21. doi:10. 1016/S0378-5955(97)00083-X

Feng, A. S. (1980). Directional characteristics of the acoustic receiver of the leopard frog (*Rana pipiens*): a study of eighth nerve auditory responses. *J. Acoust. Soc. Am.* **68**, 1107-1114. doi:10.1121/1.384981

Feng, A. S. and Shofner, W. P. (1981). Peripheral basis of sound localization in anurans. Acoustic properties of the frog's ear. *Hear. Res.* **5**, 201-216. doi:10.1016/ 0378-5955(81)90046-0

Hawkins, A. D. and Popper, A. N. (2018). Directional hearing and sound source localization by fishes. *J. Acoust. Soc. Am.* **144**, 3329-3350. doi:10.1121/1. 5082306

Hetherington, T. (2001). Laser vibrometric studies of sound-induced motion of the body walls and lungs of salamanders and lizards: implications for lung-based hearing. *J. Comp. Physiol. A* **187**, 499-507. doi:10.1007/s003590100220

Jørgensen, M. B., Schmitz, B. and Christensen-Dalsgaard, J. (1991). Biophysics of directional hearing in the frog *Eleutherodactylus coqui*. *J. Comp. Physiol. A* **168**, 223-232. doi:10.1007/BF00218414

Jørgensen, M. B. and Christensen-Dalsgaard, J. (1997a). Directionality of auditory nerve fiber responses to pure tone stimuli in the grassfrog, *Rana temporaria*. I. Spike rate responses. *J. Comp. Physiol. A* **180**, 503-511.

Jørgensen, M. B. and Christensen-Dalsgaard, J. (1997b). Directionality of auditory nerve fiber responses to pure tone stimuli in the grassfrog, *Rana temporaria*. II. Spike timing. *J. Comp. Physiol. A* **180**, 503-511. doi:10.1007/ s003590050067

Kitazawa, T., Takechi, M., Hirasawa, T., Adachi, N., Narboux-Nême, N., Kume, H., Maeda, K., Hirai, T., Miyagawa-Tomita, S., Kurihara, Y. et al. (2015). Developmental genetic bases behind the independent origin of the tympanic membrane in mammals and diapsids. *Nat. Commun.* **6**, 1-5. doi:10.1038/ ncomms7853

Lauridsen, T. B., Brandt, C. and Christensen-Dalsgaard, J. (2021). Three auditory brainstem response (ABR) methods tested and compared in two anuran species. *J. Exp. Biol.* **224**, jeb237313. doi:10.1242/jeb.237313

Lewis, E. R. (1981). Evolution of inner-ear auditory apparatus in the frog. *Brain Res.* **219**, 149-155. doi:10.1016/0006-8993(81)90274-2

Lewis, E. R. and Narins, P. M. (1999). The acoustic periphery of amphibians: anatomy and physiology. In *Comparative Hearing: Fish and Amphibians* (ed. R. R. Fay and A. N. Popper), pp. 101-154. New York: Springer.

Manteuffel, G. and Naujoks-Manteuffel, C. (1990). Anatomical connections and electrophysiological properties of toral and dorsal tegmental neurons in the terrestrial urodele *Salamandra salamandra*. *J. Hirnforsch.* **1**, 65-76.

Mooney, T. A., Hanlon, R. T., Christensen-Dalsgaard, J., Madsen, P. T., Ketten, D. R. and Nachtigall, P. E. (2010). Sound detection by the longfin squid (*Loligo pealeii*) studied with auditory evoked potentials: sensitivity to low-frequency particle motion and not pressure. *J. Exp. Biol.* **213**, 3748-3759. doi:10.1242/jeb.048348

Narins, P. M., Ehret, G. and Tautz, J. (1988). Accessory pathway for sound transfer in a neotropical frog. *Proc. Natl. Acad. Sci. USA* **85**, 1508-1512. doi:10.1073/pnas. 85.5.1508

Pettigrew, A. G., Anson, M. and Chung, S.-H. (1981). Hearing in the frog: a neurophysiological study of the auditory response in the midbrain. *Proc. R. Soc. B Biol. Sci.* **212**, 433-457. doi:10.1098/rspb.1981.0047

Pupin, F., Sacchi, R., Gentilli, A., Galeotti, P. and Fasola, M. (2007). Discrimination of toad calls by smooth newts: support for the heterospecific attraction hypothesis. *Anim. Behav.* **74**, 1683-1690. doi:10.1016/j.anbehav.2007. 03.020

Schrode, K. M., Buerkle, N. P., Brittan-Powell, E. F. and Bee, M. A. (2015). Auditory brainstem responses in Cope's gray treefrog (*Hyla chrysoscelis*): effects of frequency, level, sex and size. *J. Comp. Physiol. A* **19**, 161-169.

Sisneros, J. A. and Rogers, P. H. (2016). Directional hearing and sound source localization in fishes. In *Fish Hearing and Bioacoustics* (ed. J. A. Sisneros), pp. 121-155. Springer International Publishing.

Smith, J. J. B. (1968). Hearing in terrestrial urodeles: a vibration-sensitive mechanism in the ear. *J. Exp. Biol.* **48**, 191-205. doi:10.1242/jeb.48.1.191

Walton, P. L., Christensen-Dalsgaard, J. and Carr, C. E. (2017). Evolution of sound source localization circuits in the nonmammalian vertebrate brainstem. *Brain Behav. Evol.* **90**, 131-153. doi:10.1159/000476028

Wang, J., Ludwig, T. A. and Narins, P. M. (1996). Spatial and spectral dependence of the auditory periphery in the northern leopard frog. *J. Comp. Physiol. A* **178**, 159-172. doi:10.1007/BF00188159

Wever, E. G. (1978). Sound transmission in the salamander ear. *Proc. Natl. Acad. Sci. USA* **75**, 529-530. doi:10.1073/pnas.75.1.529

Wilczynski, W., Resler, C. and Capranica, R. R. (1987). Tympanic and extratympanic sound transmission in the leopard frog. *J. Comp. Physiol. A* **161**, 659-669. doi:10.1007/BF00605007