

**Hearing evolution across the air-water interface: lessons from comparative audiometry in turtles and salamanders**

by

Jeffrey Nathan Zeyl

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Approved by

Carol Johnston, Chair, Professor of Fisheries, Aquaculture, and Aquatic Sciences  
Craig Guyer, Emeritus Professor of Biological Sciences  
Mary Mendonça, Professor of Biological Sciences  
Daphne Soares, Assistant Professor of Biological Sciences  
Haruka Wada, Assistant Professor of Biological Sciences

## Abstract

The physical differences between water and air present unique challenges to organisms living in both environments, including challenges to the sense organs. In the auditory system, acoustic impedance mismatch is a challenge to airborne sound detection, but not aquatic sound detection. Evolutionary transitions of vertebrates across aquatic and terrestrial environments are often associated with changes in sensory systems. Comparative studies of hearing in amphibious taxa that have diversified across aquatic and terrestrial environments could illuminate the payoffs and constraints affecting hearing in aquatic-terrestrial transitions. Using evoked potentials, I collected aquatic and aerial auditory sensitivities from two amphibious tetrapod orders, Testudines and Caudata, to test the hypothesis that terrestrial clades have evolved heightened aerial sensitivity relative to aquatic ancestors. I also tested whether two aspects of extra-tympanic hearing in salamanders confer advantages to aerial auditory sensitivity: (1) metamorphosis and (2) body wall vibrations over the lungs.

In Testudines, I found a positive association between terrestrial specialization and aerial sensitivity, although the fossorial *Gopherus polyphemus* showed reduced high frequency sensitivity. A broader survey of audiograms in the literature supports this positive association. Aquatic sensitivity of terrestrial *Terrapene carolina* was comparable to that of aquatic species, indicating that augmented aerial sensitivity is not necessarily associated with marked aquatic hearing loss. In Caudata, I failed to find a comparable

positive association: aerial sensitivity of the terrestrial *Plethodon glutinosus* did not exceed that of the more sensitive aquatic species, and metamorphosis did not increase aerial sensitivity in *Ambystoma talpoideum* or *Notophthalmus viridescens*. The relationship between aerial and aquatic sensitivity varied at different frequencies and for different species. In particular, relative to the smaller species tested, the large aquatic *Amphiuma means* exhibited better aquatic-aerial carryover of auditory sensitivity at low frequencies and poorer carryover at high frequencies. Experimental blocking of the body wall over the lungs via submersion under a water surface did not change auditory thresholds in *A. talpoideum* or *N. viridescens*, failing to support a lung-based aerial auditory pathway. This dissertation develops our understanding of vertebrate hearing across aquatic-terrestrial transitions.

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## List of Abbreviations

dB	Decibel
AEP	Auditory evoked potential
RMS	Root-mean-square
SVL	Snout-vent length

## **Chapter 1 - General Introduction**

### **Abstract**

Sensory information allows animals to respond adaptively to environmental changes, but the costs, benefits, and constraints associated with sensory information vary for different organisms occupying different environments. Evolutionary transitions between aquatic and terrestrial habitats have occurred multiple times in the history of vertebrates and are generally associated with modifications to sensory structures. The different physical attributes of water and air modify structure-function relationships in sensory systems, so when organisms with senses adapted to one medium transition to the other medium, they may experience performance losses and new constraints on sensory function. Aquatic-terrestrial transitions could also affect sensory evolution as a result of changes in the sources of sensory information and modified ability to effectively use that information. The differences in acoustic impedance between water and air impose different design specifications on auditory function. Most significantly, the need for impedance matching structures constrains the ability to detect airborne sound but not aquatic sound.

Turtles and salamanders both exhibit phylogenetic diversity in ecological specialization to aquatic and terrestrial habitats, and in both groups, the most highly terrestrial members are found in more recent clades. Therefore, comparative patterns of

auditory structure and function in these groups can provide insights into the factors that may promote and constrain terrestrial hearing evolution of organisms originating in an aquatic environment. Despite the potential significance of aquatic-terrestrial transitions in altering the course of auditory evolution, the diversity of auditory sensitivity in both of these taxa has not been explicitly examined in relation to aquatic-terrestrial ecology, and relatively little is known about their auditory performance underwater. Chapters 2 and 3 test the hypotheses that terrestrial members in each taxon have evolved heightened aerial sensitivity relative to aquatic ancestors. This introduction reviews potential costs, benefits, and constraints on using auditory information across the air-water interface in turtles and salamanders.

The auditory performance of salamanders has implications for understanding terrestrial hearing evolution of early tetrapods in terms of the potential for aerial auditory function without a tympanic middle ear. Salamanders exhibit diversity in extra-tympanic auditory mechanisms implicated in aerial hearing performance (i.e., lungs, inner ear structures, body size) and they develop an auditory structure across metamorphosis (the opercularis complex). Chapters 3 and 4 explore extra-tympanic hearing mechanisms in salamanders. Chapter 3 evaluates the diversity of amphibious auditory performance in salamanders in relation to current hypotheses of extra-tympanic function and tests the hypothesis that metamorphosis improves aerial sensitivity. Chapter 4 tests the hypothesis that vibrations over the body wall in lunged salamanders confer aerial auditory sensitivity.

## **Adaptive use of sensory information**

### *Benefits, costs, and constraints in using sensory information*

Adaptive responses to changing environmental conditions depend on detecting and using information collected from the environment (Dall et al. 2005). By sampling information from the environment, animals can reduce uncertainty about potential alternative environmental conditions, allowing them to distinguish between resources, neutral features, and threats in the environment (e.g. presence and absence of food resources, mates, predators). If used properly, such information can provide an adaptive advantage.

The environmental information accessible to an organism is limited by the performance of the senses. Increased information can permit organisms to perform complex sensory tasks that support flexible behavioral responses to environmental changes (Nilssen 2013). Conversely, sensory biases can constrain behavior and adaptation by contributing to a failure to detect resources or threats in the environment (Jordan and Ryan 2015).

Although sensory information has a clear potential benefit to an organism's performance, evolution toward increasing sensory complexity is constrained by costs involved in the production and maintenance of sensory traits. Processing more sensory information requires greater investment in sensory neurons, which have high metabolic demands (Laughlin 2001; Niven and Laughlin 2008). With a limited energy budget, organisms face a trade-off in allocating resources to the development and maintenance of sensory structures versus to other vital physiological functions. In addition, trade-offs in allocation exist among different sensory modalities.

The direction of adaptive sensory evolution is determined by payoffs associated with using sensory information and constraints on the ability of an organism to detect and use sensory information. Both the utility and the amount of sensory information are determined by combined effects of intrinsic and extrinsic factors. If sensory traits have a neutral or negative payoff in relation to whole organism fitness, they may evolve via drift or selection.

#### *Useful extrinsic information sources*

Sensory information broadcast from predators, food sources, and mates has high potential adaptive utility, since these messages have a direct relationship to agents causing variation in survival and reproduction. Sensory adaptations in relation to these information categories are exemplified by specializations such as ultrasonic hearing in insects in relation to bat predation (Hoy 1992), the directional hearing capabilities of owls in relation to nocturnal prey capture (Konishi 1973), and ‘matched filters’ in the auditory systems of anurans in relation to mate localization (e.g. Moreno-Gomez et al. 2015).

In other species, sensory systems of many organisms are not as strikingly specialized for singular tasks. Generalized sensory performance could reflect the need to optimize sensory performance across a range of situations, lower needs on information for sensory tasks, or intrinsic constraints on the ability to evolve specializations (discussed below). In addition to such ‘direct’ information sources, other environmental information sources could have adaptive utility. For example, soundscapes associated with certain habitat types could be used to aid orientation for migration and habitat selection (e.g. Montgomery et al. 2006; Diego-Rasilla and Luengo 2007).

### *Intrinsic constraints on the detection and use of sensory information*

Sensory evolution is limited by phylogenetic constraints on sensory traits as well as constraints on other traits that are integral for using sensory information to improve whole organism performance, such as in the central nervous system and in motor effectors. Sensory innovations, such as tympanic middle ears or image-forming eyes, are present in some taxa but not others, generating major phylogenetic differences in the types of sensory tasks that can be performed (Nilssen 2013). Similarly, there are large phylogenetic differences in neurological and effector traits. For example, the neocortex of mammals could be associated with less constraint on neural processing relative to caecilians and salamanders. In caecilian and salamanders, large genome sizes and cell sizes, which result in a simplified brain morphology (i.e., poorly differentiated nuclei), could constrain the complexity of neural processing (Roth and Walkowiak 2015).

### *Environmental constraints on the amount of information*

In addition to qualities of an information stimulus at its source and the sensory performance in the receiver, the amount of information received is determined by environmental properties controlling stimulus transmission and masking. If environmental constraints render certain types of information inaccessible, it negates any potential performance benefit that could be gained from using that information. When animals invade new environments that restrict accessibility to certain types of sensory information, optimality models of adaptive evolution would predict regression of sensory structures. Examples of sensory regression in relation to environmental shifts restricting

access to sensory information include: loss of tympanic middle ears in anurans living near waterfalls and in those that are fossorial (Jaslow et al. 1988), loss of electrosensory systems across metamorphosis in anuran amphibians (Fritzsich 1989), and regressive evolution of eyes in subterranean animals (Jeffrey 2008).

### **Auditory evolution in aquatic-terrestrial transitions**

#### *Sensory constraints in aquatic versus terrestrial environments*

The physical differences of air and water present different environmental constraints on the types of sensory information accessible and the structures needed to access that information (Thewissen and Numella 2008). For example, the low electric conductivity and density of air make the electroreceptive and mechanosensory lateral line non-functional on land. In visual, olfactory and auditory systems, the physical differences modify structure-function relationships. The different refractive indices of water (1.3) and air (1.0) affect structure-function relationships in vision, since in air, refraction occurs at both the cornea and the lens, while underwater it occurs only at the lens (Kroger and Katzner 2008). Olfactory function is impacted by differences in chemical transmission between water and air. Diffusion rates are lower in water compared to air, and volatility is an additional restriction on airborne chemicals, which would favor different types of olfactory receptors in aquatic and terrestrial environments (Eisthen and Schwenk 2008). In the auditory system, acoustic impedance presents an impedance-matching problem in air, but not underwater (discussed below).

#### *Sensory evolution in aquatic-terrestrial transitions*

The different design specifications imposed by the physical properties of water and air on sensory function may be a significant impetus for sensory change in evolutionary transitions across aquatic and terrestrial environments. Such transitions have occurred repeatedly in the history of vertebrates, with associated changes in sensory structures (Thewissen and Nummela 2008). An evolutionary shift to a new medium can result in a potential shift or reduction in the amount of information available to an organism and can expose new phenotypic constraints on sensory functions that were not present in the previous medium. If selection pressures favor increased auditory sensitivity in both environments, adaptive sensory evolution would involve solving the different design specifications of both media.

In addition to biophysical challenges to sensory performance, aquatic-terrestrial habitat shifts could modify the course of sensory evolution due to changes in the sources of information relevant for adaptive behavior and intrinsic constraints that modify the ability to use that information. Animals entering new ecological communities are exposed to new potential agents of selection (e.g. new food resources and predators) carrying new information signatures, which are embedded within new sensory landscapes (e.g. soundscape, lighting conditions, olfactory milieu). Even if organisms can successfully detect the new, potentially useful information sources in the new environment, the ability to use that information could be impaired by the modified performance of other traits that are impacted by the physical differences between water and air. For example, feeding and locomotion have different biomechanical requirements on land - the benefit of a prey-capture behavior could be contingent on the performance of these traits in addition to sensory performance. More generally, the modified performance of many other aspects of

biological function resulting from a shift in medium (e.g. gas exchange, water balance challenges) could alter the relative strength of the correlation between sensory performance and lifetime reproductive success.

While all evolutionary lineages making aquatic-terrestrial transitions face the same biophysical challenges of water and air on sensory function, the constraints and payoffs on sensory function across aquatic-terrestrial transitions will vary among taxa, which could result in different evolutionary trajectories. With selection pressures on sensory performance in both media, appropriate phenotypic variation, and sufficient time, amphibious organisms may evolve solutions to problems of optimizing sensory function in two media. In other cases, significant constraints and poor payoffs on sensory function in the new medium could result in the regression of sensory structures. For example, relative to marine ancestors, terrestrial crabs show a reduction of olfactory pathways (Krieger et al. 2015). Similarly, penguins exhibit a reduction in olfaction receptor genes relative to other waterbirds, but possess adaptations for aquatic vision (Lu et al. 2016). The penguin example also highlights that information needs in a new medium could be met through one sensory modality over others.

#### *Medium acoustic impedance sets middle ear design specifications*

Since water has a much higher characteristic acoustic impedance than air (approximately 3,750 times greater), maximizing sound detection in each medium relies on different types of middle ear structures. Acoustic impedance is a measure of the ratio of pressure and particle velocity; a high acoustic impedance indicates that greater pressure force is needed to produce a given vibration velocity.

On land, the large acoustic impedance difference between air and animal tissue results in a significant degree of airborne sound reflection at this interface, constraining transmission of airborne sound to the ear. For airborne sound to produce oscillations in inner ear fluid, an augmented application of pressure is required. This is the ‘impedance matching’ problem of detecting airborne sound. The tympanic middle ears of tetrapods and the tympanal organs of insects solve this problem, and have evolved independently multiple times in each group (Stumpner and von Helversen 2001; Clack 2002).

The impedance-matching function of tympanic middle ears is achieved through area and lever ratios (Mason 2016a). The area ratio effect refers to the increase in force per unit area entering the inner ear as a consequence of the transfer of force from the large surface area of the tympanic membrane to the smaller stapes footplate at the oval window of the otic capsule. Pressure can also be augmented by lever mechanisms between auditory ossicles vibrating around a fulcrum point. With the second lever arm (ossicle) shorter than the first, a small force producing motion over a larger distance on the longer lever can be transformed to a larger force over a smaller distance on the shorter lever arm. The lever increases the pressure force but also decreases the vibrational velocity entering the ear. In addition to such ‘ideal transformer’ models of middle ear function, the frequency-dependent responsiveness of auditory structures depends on the mass and compliance of the vibrating structures (explored further in relation to extra-tympanic function in **Chapter 4**) (Mason 2016a).

Since the acoustic impedances of animal tissue and water are relatively close, impedance mismatch is not a significant constraint on sound detection underwater. The release from impedance matching in aquatic environments is reflected by a general

pattern of lowered area ratios in the tympanic middle ears of secondarily aquatic tetrapods (Hetherington 2008). Increasing aquatic auditory sensitivity involves amplifying particle motions. In fishes, particle motion can be detected directly by inertial motion of otolith stones, which lag behind the body of the fish (and hair cells) in phase and amplitude (Popper and Fay 1993). Many secondarily aquatic tetrapods have enlarged auditory ossicles, which provide a similar inertial motion to transmit vibrations into the ear ('inertial bone conduction') (Hetherington 2008). In the odontocete middle ear, lever mechanisms in the tympanoperiotic complex increase vibration velocities at the oval window (Nummela et al. 1999). In *Xenopus*, an aquatic frog, there are lower lever ratios in the middle ears relative to bullfrogs, which result in greater volume velocity entering the ear (Mason et al. 2009).

Adaptations for aquatic hearing in vertebrates often involve detection of vibrations produced by internal gas cavities. Since air is more compressible than water, sound reaching submerged air cavities causes the cavity to oscillate, producing local vibrations that exceed those present in the original sound wave (Alexander 1966). In fishes, these vibrations can be generated from the swim bladder or other gas cavities associated with the ear (Popper and Fay 1993). These vibrations have been attributed to the lungs in lungfishes and salamanders (Christensen et al. 2015a, b). In aquatic tetrapods with tympanic middle ears (i.e., aquatic frogs and turtles), the resonance of the air-filled middle ear cavity can drive the motion of the tympanic disc (Lombard et al. 1981; Christensen and Elepfandt 1995; Christensen et al. 2012).

Since acoustic impedance imposes different design specifications on auditory function in water and air, amphibious organisms encounter a situation where the effects

of structures on auditory function are not identical underwater versus on land, and vice versa. In addition, evolutionary lineages that have transitioned between aquatic and terrestrial environments have faced these challenges, carrying with them hearing adaptations to an ancestral environment with different design specifications on auditory function. Aquatic lineages moving to land are faced with the impedance-matching problem of airborne sound detection. Similarly, for terrestrial animals moving into aquatic habitats, although not encountering an impedance mismatch constraint, would not be equipped with other traits that would augment aquatic auditory function (e.g., hypertrophied ossicles, lever mechanisms to increase vibrational velocity, resonating bubbles).

#### *Hearing evolution in aquatic-terrestrial transitions*

Evolutionary transitions between aquatic and terrestrial environments have occurred repeatedly in the evolutionary history of vertebrates, and have often been accompanied by changes in the auditory system. Between the original aquatic-to-land transition of early tetrapods in the late Devonian, there was a period of approximately 100 million years before the appearance of tympanic middle ears in the mid-late Permian and Triassic (Müller and Tsuji 2007). Tympanic middle ears likely evolved independently in all the major tetrapod lineages (Clack 1997; Clack 2002). In addition, many terrestrial lineages have secondarily returned to aquatic environments and have evolved adaptations to enhance aquatic sound detection (Hetherington 2008). Over a period of 4-7 million years, the ears of early cetaceans underwent major changes that promoted aquatic function rather than terrestrial function, after passing through

amphibious stages with auditory structures that are presumed to have had compromised performance in both water and air (Nummela et al. 2004).

The rate and direction of evolutionary responses of hearing to aquatic-terrestrial transitions show taxonomic differences. The constraints and payoffs on auditory function are expected to vary among taxa due to different ear structures, which determine between-media carryover and the ability to solve new biophysical hearing challenges in a new medium. Auditory performance in amphibious organisms can reveal the challenges and potential solutions to hearing in two media under different phylogenetic constraints (Ashley-Ross et al. 2013). Pinnipeds are an extant amphibious taxon exemplifying that aquatic and aerial hearing can be optimized without severe constraints on hearing in both media, if there is selection (Reichmuth et al. 2013). However, in other lineages, constraints on auditory phenotypes could generate compromised auditory function in both media, as suggested for the first amphibious species preceding the evolution of early cetaceans (Nummela et al. 2004).

Amphibious clades that exhibit diversity along the aquatic-terrestrial spectrum are particularly informative for understanding hearing evolution across aquatic-terrestrial environments. Members of an amphibious clade that have become completely terrestrial or aquatic provide natural evolutionary experiments. In those members, all sensory evolution would take place under the specific design specifications of that habitat, along with release from the design constraints of the other medium. An examination of the diversity of amphibious auditory function in such groups could illuminate the conditions that promote and constrain hearing evolution across the air-water interface.

## II

### **Turtles and salamanders as models for hearing evolution in aquatic-terrestrial transitions**

Turtles and salamanders are both appropriate model organisms for examining the role of aquatic-terrestrial transitions in patterns of hearing. Both groups include members that are largely aquatic (i.e., sea turtles and paedomorphic salamanders), members that are amphibious to varying degrees, and members that are completely terrestrial (i.e., tortoises and direct-developing plethodontids). Since aquatic living is a significant part of the evolutionary history of both taxa, much of their auditory evolution would have occurred under aquatic design specifications. In both groups, the completely terrestrial members are found in more recent families (i.e., Testudinidae in Testudines and Plethodontidae in Urodela). Both turtles and salamanders are instructive models for understanding the factors that constrain and promote aerial hearing evolution following aquatic ancestry.

#### *Evolutionary histories in relation to aquatic-terrestrial ecology*

Though some of the earliest turtles were terrestrial, aquatic living is an ancestral condition in crown turtles (Joyce and Gauthier 2004) (Fig 1). Turtles appear in the fossil record in the upper Triassic, and Cryptodira families diversified in the Cretaceous and Paleocene (Shaffer 2009). The greatest extent of terrestrial invasion in Testudines is represented in the superfamily Testudinoidea (Emydidae, Geomydidae, Platysternidae, and Testudinidae), which appeared in the late Cretaceous (Joyce and Gauthier 2004; Shaffer 2009). Tortoises, the only strictly terrestrial family, appear in the Paleocene, 65-

55 Mya (Holroyd and Parham 2003; Shaffer 2009). Within emydids, one of the more terrestrial genera, *Terrapene*, is at least 11-12 million years old (Spinks and Shaffer 2009).

The oldest salamander fossils are found from the middle Jurassic, diversifying largely throughout the Jurassic and Cretaceous (Larson et al. 2006; Vieites et al. 2009). The ancestral life cycle in salamanders (and Lissamphibia more broadly) is biphasic, with aquatic larvae and metamorphosis into terrestrial adults. This developmental pattern varies phylogenetically and is correlated with aquatic-terrestrial specialization (i.e., paedomorphosis found in aquatic taxa and accelerated development characteristic of terrestrial taxa). Fully aquatic families are found across the phylogeny (Proteidae, Amphiumidae, Sirenidae, Cryptobranchidae) (Fig 2). In addition, many salamanders show facultative paedomorphosis, in which adults can complete the life cycle as paedomorphic adults if aquatic conditions are favorable.

Most fully terrestrial salamanders are found in Plethodontidae (lungless salamanders), which is comprised mostly of terrestrial members (Plethodontini and Bolitoglossini, parts of Desmognathinae). Terrestrial plethodontids have lost the free living larval stage completely, developing from eggs directly to terrestrial forms. Plethodontidae is a recent group within Caudata. A current molecular clock places the divergence of terrestrial groups such as *Plethodon* (the terrestrial plethodontid examined in this dissertation) to at least 40 Mya (Shen et al. 2016).

*Rationale, hypotheses and predictions*

Transitions across the air-water interface are expected to impact auditory evolution, and variation in aquatic-terrestrial specialization is a central feature of diversity in both turtles and salamanders. However, in both taxa, the patterns of auditory function in relation to aquatic-terrestrial ecology have not been explicitly examined. Such an analysis has been hampered by the fact that most hearing data have been collected only in air. In both taxa, comparative aerial studies under constant experimental conditions have only been conducted by Wever (1978; 1985), but generally in only a few individuals per species. Aquatic auditory function in both groups is only beginning to be investigated, although since the start of this dissertation additional studies on this topic have been published (Christensen-Dalsgaard et al. 2012; Christensen et al. 2015b). The absence of comparative data on auditory function in relation to ecological differentiation on the aquatic-terrestrial axis was a major impetus for this dissertation.

**Chapter 2 and 3 used the comparative method to test the hypothesis that terrestrial clades have evolved increased aerial sensitivity relative to aquatic ancestors.** An evaluation of this hypothesis requires data on aquatic performance and the between-media carryover in aquatic species. Those data provide a reference for the extent of aerial sensitivity in ‘prototypical’ turtle and salamander ears that are under little or no selection pressure on aerial auditory sensitivity. I compared aerial sensitivities of terrestrial and aquatic species, predicting that increased terrestrial ecology would be associated with increased sensitivity.

*AEP method*

Auditory performance was assessed using auditory evoked potentials (AEPs), a non-invasive electrophysiological method. These recordings were used to generate threshold sensitivities across a range of tone frequencies (audiograms). Auditory evoked potentials are ‘far-field’ electroencephalogram (EEG) potentials collected from a subdermal recording electrode placed above the brain. In this method, short tone pips (10 ms) are presented to the test animal a few hundred times. The EEG recordings associated with a short recording window during and after each tone repetition are averaged together. This signal averaging method removes random neurological noise from the recording, producing a voltage waveform reflecting synchronous neural activity that is time-locked to the auditory stimulus.

#### *Test species*

The Testudines compared included a highly aquatic kinosternid group (*Sternotherus odoratus* and *Sternotherus minor*), an aquatic emydid (*Trachemys scripta*), a largely terrestrial emydid (*Terrapene carolina*), and a completely terrestrial and fossorial tortoise (*Gopherus polyphemus*). In addition, Appendix 1 shows 1-2 individuals of *Kinosternon subrubrum* (Kinosternidae), which shows some terrestrial habits, particularly overwintering in terrestrial hibernacula, and *Apalone spinifera* (Trionychidae), a highly aquatic turtle.

The salamanders compared included a completely terrestrial plethodontid (*Plethodon glutinosus*), plethodontids with higher aquatic affinity (*Eurycea* spp.), an ambystomatid (*Ambystoma talpoideum*), a salamandrid (*Notophthalmus viridescens*), and one obligate aquatic amphiumid (*Amphiuma means*). Both *Ambystoma talpoideum* and

*Notophthalmus viridescens* possess a biphasic life cycle and are capable of facultative paedomorphosis.

The species selected are found within different families in their respective orders. This diverse choice of species has the disadvantage of not controlling for phylogeny. However, the deficiencies in the available data on amphibious hearing in these groups warrant a preliminary assessment into whether substantial associations exist between auditory function and an environmental factor expected to potential exert a large effect on sensory evolution (the air-water interface). In addition, a broad survey across the phylogeny allowed for the selection of species exhibiting extremes of aquatic-terrestrial specialization.

### **Potential payoffs and constraints on aerial hearing sensitivity**

#### *Auditory-mediated behaviors*

Evolving increased auditory function on land presumes a functional relationship between auditory performance and whole organism performance. The acoustic ecology of both taxa is cryptic in that neither exhibit conspicuous, highly specialized acoustically-guided behaviors on land. Nonetheless, auditory function could be a useful supplement to other primary sensory modalities such as vision and olfaction. Turtles and salamanders share some aspects of the types of relevant auditory tasks. On land, neither group exhibits long distance acoustic communication or prey capture that is primarily acoustically mediated. Anti-predator and orientation behaviors are probably relevant categories of acoustically-mediated behavior under selection in terrestrial environments.

Both taxa are generally considered non-soniferous or minimally soniferous, although there is a small literature on sound production in both (Maslin 1950; Ferrara et al. 2014). Acoustic communication has been recognized in aquatic turtles and in some tortoises in the context of courtship and copulation (Galeotti et al. 2005; Ferrara et al. 2014). In terrestrial salamanders, cases of sound production are often within distress contexts and are soft, high pitch sounds (Brodie 1978). These observations, combined with the absence of a tympanic middle ear constraining high frequency sensitivity, would suggest a stronger function of sound production in anti-predator contexts rather than conspecific communication. Intraspecific acoustic communication has been proposed in a few aquatic salamanders (e.g., Gehlbach and Walker 1970; Crovo et al. 2016). Given reports of high frequency aquatic hearing (Bulog and Schlegal 2000), the potential for acoustic communication in aquatic salamanders could be greater than in terrestrial salamanders. In general, reproductive communication in salamanders relies heavily on chemical communication.

Acoustically mediated prey capture has not been documented as a specialized sensory task in terrestrial turtles or salamanders. In the two terrestrial Testudines species examined in this dissertation, *Gopherus polyphemus* is herbivorous, and *Terrapene carolina* is omnivorous, with worms, insects and carrion comprising the animal portions of the diet (Guyer et al. 2016). Terrestrial salamanders are generally predaceous, including items such as insects, annelids, molluscs, isopods, small vertebrates, and prey capture relies more heavily on vision and olfaction than auditory function (Wake and Deban 2000). Specialized auditory-mediated prey capture is plausible in an aquatic environment but has not been documented.

Generalized predator avoidance and orientation are both plausible adaptive functions of hearing in turtles and salamanders. Turtles exhibit freeze responses and head withdrawals in response to sounds (Lenhardt 1982; Bowles et al. 1999). Increasing either aerial or seismic vibrational sensitivity would allow for the detection of predators at greater distances, improving the effectiveness of either a freeze response or an active avoidance response.

Auditory cues could aid orientation during migration events, which are important aspects of the life cycle in many turtles and salamanders. In turtles, auditory cues could facilitate orientation in relation to aquatic habitats (e.g. Tuttle and Carroll 2005), or be used as navigation landmarks. Salamanders possess a suite of sensory adaptations (chemical, visual and magnetic orientation) for orientation in migrations to aquatic breeding habitat (Sinsch 1991). The use of auditory cues from anuran calls could aid in such navigation, as has been suggested for the newts *Triturus marmoratus*, *Lissotriton helveticus*, and *Lissotriton vulgaris* (Diego-Rasilla and Luengo 2004; Diego-Rasilla and Luengo 2007; Pupin et al. 2007).

Fossoriality is one aspect of terrestrial ecology that could impact evolutionary patterns of aerial sensitivity in turtles and salamanders, since fossorial lifestyles could favor adaptations promoting seismic sensitivity rather than airborne sensitivity. Salamanders are indeed quite sensitive to vibrations (Ross and Smith 1982; Christensen et al. 2015b), and fossorial *Gopherus* tortoises have large saccule otoconial masses, which are presumed to enhance seismic sensitivity (Bramble 1982). Airborne sensitivity could still be relevant to both fossorial and non-fossorial terrestrial turtles and salamanders.

### *Peripheral auditory structures*

The presence of a tympanic middle ear in turtles (Fig. 3) and its absence in salamanders result in different constraints on and potential solutions to terrestrial hearing. The impedance matching performance of the turtle tympanic ear could be achieved by relatively minor structural changes (e.g. thinning the tympanic disc and augmenting the area ratio). In contrast, salamanders lack the basic prerequisites for impedance matching – a thin vibrating membrane over a compliant air space and a mechanism for transforming membrane vibrations into a greater force at the inner ear. However, salamanders could use alternative, extra-tympanic mechanisms to augment aerial sensitivity (discussed below). As would be expected from the auditory structures of turtles and salamanders, the best aerial hearing sensitivities of both taxa generally occurs below 1 kHz (Wever 1978; Wever 1985).

The available studies indicate that both groups are well equipped for aquatic hearing (Christensen-Dalsgaard et al. 2012; Christensen et al. 2015b). In turtles, resonance of the middle ear air cavity drives tympanic disc motion underwater (Christensen et al. 2012). The aquatic audiograms collected for turtles to date found best sensitivities below 1 kHz (**Chapter 2**). Salamanders can also make use of air cavity resonance in underwater hearing. Vibrations from the lungs improve auditory sensitivity above 120 Hz in *Ambystoma* spp. (Christensen et al. 2015b). Some observations indicate high frequency hearing extending several kHz (Bulog and Schlegel 2000, **Chapter 3**).

### III

#### **Extra-tympanic aerial hearing mechanisms in salamanders**

Early terrestrial tetrapods lacked tympanic middle ears, which would have significantly restricted their ability to detect airborne sound. Examining structure-function relationships in contemporary tetrapods lacking tympanic middle ears may broaden our view of early terrestrial tetrapod hearing evolution by revealing the constraints and potential solutions to terrestrial hearing without a tympanic middle ear. Salamanders are one such contemporary group. The amphibious life cycle of salamanders is also a useful model for the between-media carryover of auditory function experienced by early amphibious tetrapods. Early amphibious tetrapods could have used air cavity resonance to detect aquatic sounds via either the lungs (Christensen et al. 2015a) or air-filled gill pouches (Clack et al. 2003). Similarly, salamanders make use of lung-or mouth-cavity-based resonance underwater (Christensen et al. 2015b), but are restricted on land by a lack of tympanic middle ears. Therefore, in both groups, aquatic sensitivity and bandwidth could be greater underwater and more restricted on land.

Airborne sound transmission through thick, unspecialized tissues is typically restricted to low frequencies (e.g., Hetherington and Lindquist 1999; Christensen et al. 2015a,b; but see Boistel et al. 2013). However, there is a continuum of aerial sensitivity in non-tympanic taxa, which must be due, in part, to differences in specific impedances of peripheral auditory structures. Auditory traits that would improve aerial hearing of non-tympanic tetrapods include: opening the otic capsule, thinning of tissue layers over the ears, and a switch from otolithic receptors (as found in basal vertebrates) to papillae without overlying otoliths (Christensen-Dalsgaard and Manley 2013). Together, these

changes would decrease the resistance of sound transmission to the ear. Auditory receptors without an overlying otolith would remove hair cells from the inertia involved in attachment to an otolith or otoconial mass, allowing them to respond to a higher frequency range.

Salamanders possess all of these advantages for extra-tympanic aerial hearing. In addition to saccular maculae with overlying otoconial masses, the inner ears of salamanders possess the amphibian and basilar papillae, which lack overlying otoconial masses. The basilar papilla is lost in some derived clades (Lombard 1977). While salamanders don't have a tympanum on the oval window, the presence of an open oval window (with pressure release through the perilymphatic foramen) would offer a lower resistance pathway. Small body size would also be associated with thinner tissue layers overlying the ear, promoting greater sound transmission (Hetherington 1992) and potentially permitting lung-based resonance pathways (discussed below). Salamanders also share two traits with anurans that have been implicated in extra-tympanic function: lungs and the opercularis complex. Lung-based resonance can improve sound transmission through peripheral body structures in small animals, and the opercularis could provide a specialized pathway to the oval window (discussed below).

Salamanders show diversity in these structures expected to impact aerial auditory sensitivity (Kingsbury and Reed 1909; Monath 1965; Lombard 1977), but little work has been done to correlate this structural diversity with measures of auditory function.

**Chapter 3 discusses associations between structural diversity (body size, opercularis complex, lungs, presence of the basilar papilla) and auditory performance in the tested salamanders. Chapter 3 and 4 also specifically investigates two aspects of**

**extra-tympanic aerial auditory function in salamanders: metamorphosis (associated with development of the opercularis system) and lung-based hearing.**

*Metamorphic development and the opercularis complex*

Amphibians with a biphasic life cycle face the potential challenges of amphibious hearing across development. These species provide an opportunity to examine whether adaptive responses to amphibious hearing challenges involve changes to development. Structural changes could be expected to appear across metamorphosis if there is an advantage to terrestrial auditory performance beyond that conferred by aquatic auditory phenotypes.

The developmental changes to the salamander ear across metamorphosis are less pronounced compared to anurans. Anuran auditory development across and post-metamorphosis includes growth of the tympanic middle ear and reorganization of auditory nuclei (Simmons and Horowitz 2006). Although salamanders don't possess tympanic middle ears, the available data have not indicated a marked increase in complexity of auditory nuclei across metamorphosis (Fritsch et al. 1988). There are a few developmental changes in inner ear structures across metamorphosis (Lombard 1977; **Chapter 3**).

Both anurans and salamanders develop an auditory structure called the opercularis complex (Fig. 4), which is generally considered to play a role in terrestrial auditory function, either in the detection of substrate vibrations or low frequency airborne sound (Monath 1965; reviewed by Mason 2007). Aquatic larvae possess a columella (stapes), which projects forward to the palatoquadrate or squamosal. The opercularis complex

consists of a second, mobile, often cartilaginous element in the oval window (the operculum), which has a muscular attachment to the pectoral girdle. In most salamanders, this muscle is the *m. levator scapulae*, but in plethodontids it is the *m. cucullaris* (Hetherington et al. 1986). The opercularis complex is also, in general, associated with terrestrial ecology across the caudate phylogeny, being absent in aquatic taxa (Kingsbury and Reed 1909; Monath 1965) (Fig. 4).

The association between the opercularis system and terrestriality across both ontogeny and phylogeny suggests a potential adaptive function in terrestrial hearing. However, its contribution to airborne or substrate-borne sound detection in both anurans and urodeles remains controversial; there are *a priori* biomechanical considerations that question the function of the opercularis system as an effective structure that would optimize substrate detection and/or airborne sound detection (review by Mason 2007, discussed in **Chapter 3**). Alternative hypotheses for its association with terrestrial environments are: (1) a correlated response to direct selection on metamorphosis as a whole, or (2) function in buffering fluid motion through the ear during terrestrial locomotion. **Chapter 3 tested the hypothesis that metamorphosis enhances aerial sensitivity in salamanders by comparing aerial sensitivity of pre- and post-metamorphic individuals of *A. talpoideum* and *N. viridescens*.**

### *Lung-based hearing*

Extra-tympanic pathways play a significant role in the anuran terrestrial hearing. In some species, extra-tympanic pathways have been found to be as effective as tympanic pathways below 1 kHz (Lombard and Straughan 1974; Wilczyński et al. 1987). At least

eleven anuran families possess members that have lost functional tympanic middle ears, and many of these species still communicate acoustically (Jaslow et al. 1988). A few of such ‘earless’ anurans can still hear up to several kHz (Lindquist et al. 1988; Boistel et al. 2013).

Lung-based vibrations promote aerial auditory function in anurans lacking tympanic middle ears (Lindquist et al. 1998; Hetherington and Lindquist 1999) and are hypothesized to contribute to aerial extra-tympanic function in other small tetrapods (Hetherington 2001). In small animals, where the mass loading around the lungs is not excessively restrictive, lungs provide an air space that can increase the compliance of peripheral structures, lowering the impedance of the surrounding tissues at low frequencies. The significance of lungs to low frequency sound transmission is analogous to the function of enlarged middle ear cavity volumes of desert rodents (Mason 2016a, b). Lung-based vibrations could be co-opted into extra-tympanic hearing pathways if the associated vibrations can be transmitted to and detected in the ear. **Chapter 4 tested the hypothesis that body wall vibrations contribute to aerial sound sensitivity in salamanders using *A. talpoideum* and *N. viridescens* as models.**

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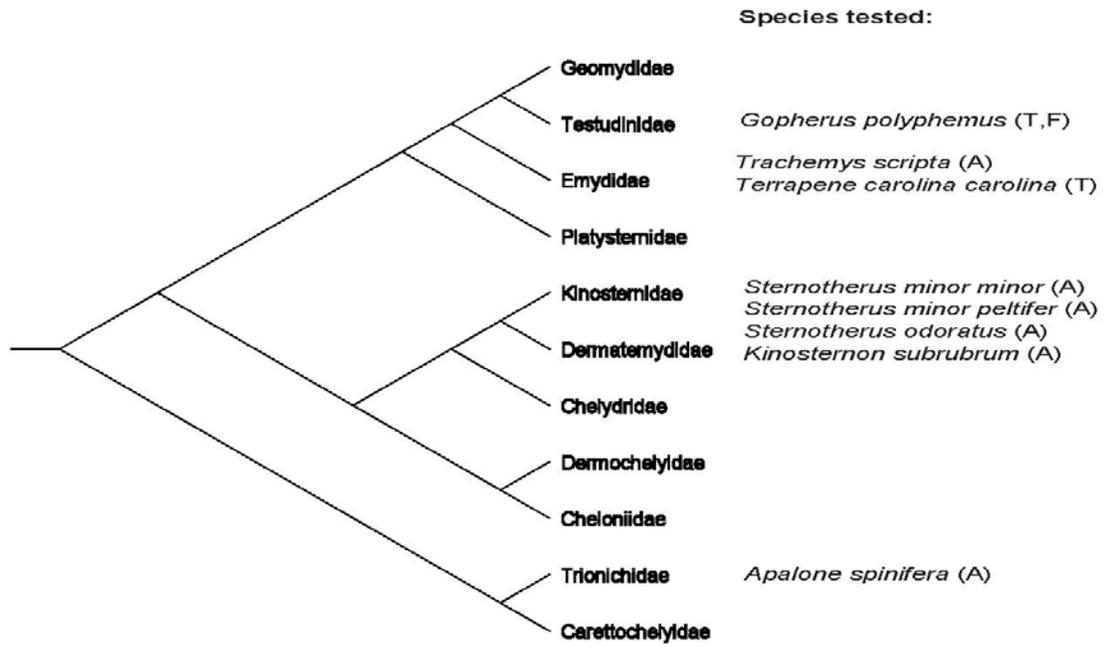
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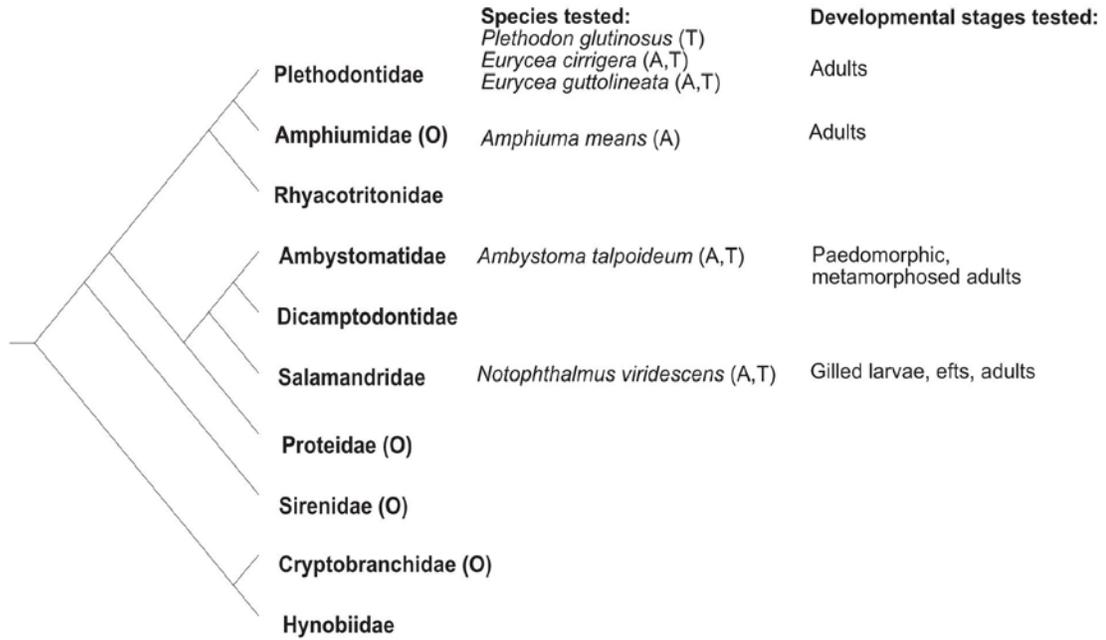
## Figures

**Fig. 1** Cladogram of Cryptodira (a suborder of Testudines) families after Shaffer (2009).

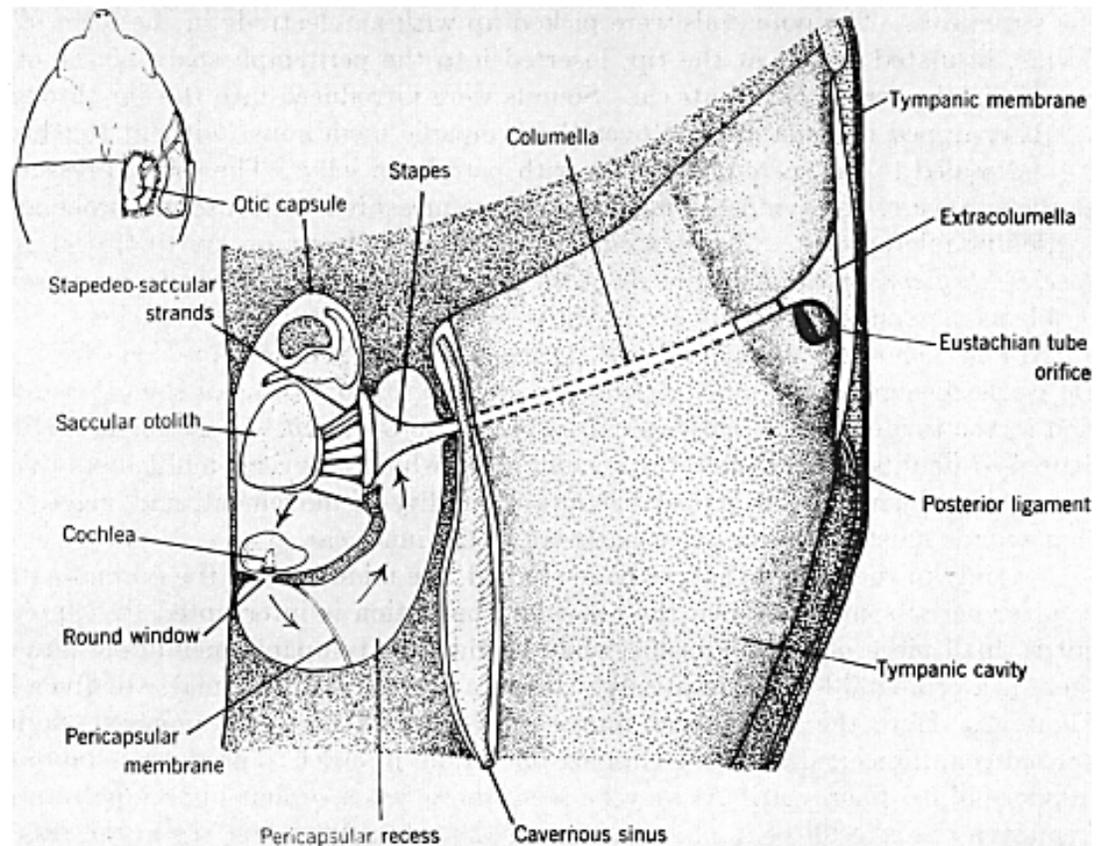
Species tested in this dissertation are listed beside the appropriate family with the dominant ecological niche in brackets (A = aquatic, T = terrestrial, F = fossorial).



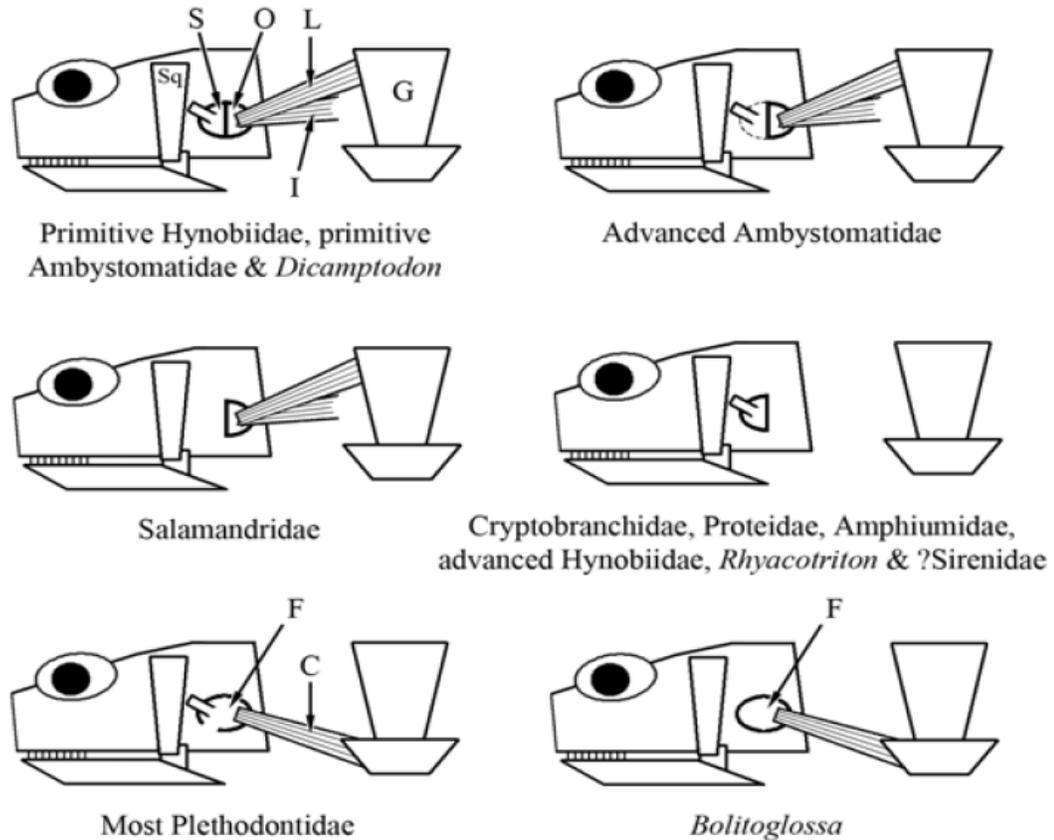
**Fig. 2** Cladogram of Urodela families after Larson et al. (2006) and the species tested in this dissertation (A = aquatic, T = terrestrial, O = obligate aquatic families).



**Fig. 3** Diagram of the turtle ear by Wever and Vernon (1956) with permission from the National Academy of Sciences. Rather than having a true tympanic membrane, turtles possess a cartilaginous tympanic disc (extracolumella). Another unique feature of the turtle ear is pressure release from the inner ear via the pericapsular recess rather than a round window opening into the middle ear cavity.



**Fig. 4.** Diagrams of diversity of the opercularis complex in salamander families from Mason (2007) with permission from Springer. The lack of the opercularis complex in the middle right panel also reflect the condition in aquatic larvae (Sq = squamosal, S = stapes/columella, O = operculum, G = pectoral girdle, L = *m. levator scapulae*, C = *m. cucullaris* F = fenestral plate).



## **Chapter 2 - Amphibious auditory evoked potentials in four North American testudine genera spanning the aquatic-terrestrial spectrum**

### **Abstract**

Animals exhibit unique hearing adaptations in relation to the habitat media in which they reside. This study was a comparative analysis of auditory specialization in relation to habitat medium in Testudines, a taxon that includes both highly aquatic and fully terrestrial members. Evoked potential audiograms were collected in four species groups representing diversity along the aquatic-terrestrial spectrum: terrestrial and fossorial *Gopherus polyphemus*, terrestrial *Terrapene carolina carolina*, and aquatic *Trachemys scripta* and *Sternotherus* (*S. odoratus* and *S. minor*). Additionally, underwater sensitivity was tested in *T. c. carolina*, *T. scripta*, and *Sternotherus* with tympana submerged just below the water surface. In aerial audiograms, *T. c. carolina* were most sensitive, with thresholds 18 dB lower than *Sternotherus*. At 100-300 Hz, thresholds in *T. c. carolina*, *G. polyphemus*, and *T. scripta* were similar to each other. At 400-800 Hz, *G. polyphemus* thresholds were elevated to 11 dB above *T. c. carolina*. The underwater audiograms of *T. c. carolina*, *T. scripta*, and *Sternotherus* were similar. The results suggest aerial hearing adaptations in emydids and high frequency hearing loss associated with seismic vibration detection in *G. polyphemus*. The underwater audiogram of *T. c. carolina* could reflect retention of ancestral aquatic auditory function.

## Introduction

Sensory traits are selected to function in relation to physical properties of the environment, which determine the types and qualities of stimuli available for perception (Endler 1992). Environmental medium is one such property affecting the function of multiple sensory systems, including the auditory system - vertebrates have evolved unique auditory adaptations for detecting sound depending on whether it is airborne, waterborne, or substrate-borne (Hetherington 2008; Christensen-Dalsgaard and Manley 2014). While airborne detection involves ‘impedance matching’ between the low acoustic impedance of air and the high impedance of the inner ear fluid, animals in contact with aquatic and solid substrate media encounter less impedance mismatch. Additionally, the large acoustic near fields in water and solid substrate allow for bone conduction to be a significant auditory pathway (Thewissen and Nummilla 2008).

When amphibious and subterranean animals shift contact with different habitat media, changes in auditory activation can occur (Higgs et al. 2002; Reichmuth et al. 2013). Such changes depend on auditory anatomy, which in turn depends both on phylogenetic history and recent adaptation. Transitions between aquatic and terrestrial media have occurred repeatedly in tetrapod lineages and have been accompanied by adaptations to detect sound in the new medium (Thewissen and Nummilla 2008).

Testudines is a useful group for studying both medium-dependent auditory activation and evolutionary responses to medium transitions since the group varies along the aquatic-terrestrial spectrum, and modern terrestrial Testudines - many of which are found in the Testudinoidea clade - are derived from aquatic ancestors (Joyce and

Gauthier 2004; Shaffer 2009). However, the diversity of Testudines auditory sensitivity in relation to the aquatic-terrestrial spectrum has not been thoroughly examined. Wever (1978) conducted a large comparative analysis of Testudines hearing using inner ear microphonic potentials, but these data consisted of only a few specimens per species, preventing quantitative analysis of species differences.

In all Testudines studied to date, best aerial auditory sensitivities have been found below 1 kHz, usually in the 200-600 Hz range (Wever 1978; Willis et al. 2013). Few studies have examined underwater and amphibious hearing capabilities of turtles, but these have also found best sensitivities below 1 kHz (Christensen-Dalsgaard et al. 2012; Lester 2012; Martin et al. 2012; Piniak 2012). In air, turtle ears conduct sound vibrations directly from the tympanum to the columella (stapes) and inner ear (Wever 1978). Underwater, resonance of the air-filled middle ear cavity drives tympanic disc motion (Christensen-Dalsgaard et al. 2012), similar to the middle ear cavity resonance that has been described for aquatic hearing of anurans (Hetherington and Lombard 1982; Christensen-Dalsgaard and Elepfandt 1995; Christensen-Dalsgaard and Manley 2014). These different sound conduction pathways in air and water suggest that hearing sensitivity could be optimized by different mechanisms in aquatic versus terrestrial species, which may affect between-media performance. However, since middle ear cavity volume is conserved across the Testudines phylogeny, terrestrial species could retain ancestral aquatic hearing performance as a consequence of middle ear cavity resonance (Willis et al. 2013).

The objective of this study was to examine medium-dependent auditory adaptation in Testudines through a comparative analysis of auditory evoked potential

(AEP) audiograms in four species groups representing different positions along the aquatic-terrestrial spectrum: *Terrapene carolina carolina*, *Gopherus polyphemus*, *Trachemys scripta*, and *Sternotherus* spp. (*Sternotherus odoratus* and *Sternotherus minor*). The eastern box turtle, *T. c. carolina* (Emydidae), is primarily terrestrial, although it can occasionally be found in aquatic habitats in hot, dry weather (Mount 1996). The gopher tortoise, *G. polyphemus* (Testudinidae), is terrestrial and also fossorial (Ernst and Barbour 1972). While both *T. scripta* (Emydidae) and *Sternotherus* (Kinosternidae) are aquatic, the basking behaviour of *T. scripta* is more strongly developed than in *Sternotherus*; thus, the former typically spends more time out of water (Ernst and Barbour 1972). According to the medium-dependant auditory adaptation hypothesis, I predicted that *T. c. carolina* and *G. polyphemus* would show the greatest aerial sensitivity, reflecting their use of a terrestrial environment, compared to the aquatic turtles *Trachemys scripta* and *Sternotherus*. Underwater, *T. scripta* and *Sternotherus* spp. were expected to be more sensitive than *T. c. carolina*.

## **Materials and methods**

### *Animal collection and husbandry*

All turtles were wild captured from ponds, rivers, roadsides, backyards, and fields in Alabama, USA by trapping and active capture. The total numbers used for testing were as follows: 11 *T. c. carolina* (mass: 83-430 g; minimum straight line carapace length: 8-13 cm), 7 *T. scripta* (mass: 210-1810 g; minimum straight line carapace length: 11-21 cm), 9 *Sternotherus* spp. [5 *S. odoratus*, 4 *S. minor*, including 2 *S. minor minor* and 2 *S.*

*minor peltifer*; mass: 70-130 g; minimum straight line carapace length: 7.0-9.3 cm], and 7 *G. polyphemus* (mass: 1.7- 2.85 kg; minimum straight line carapace length: 19-28 cm). For the duration of the study, *T. c. carolina*, *T. scripta*, and *Sternotherus* spp. were housed in an indoor vivarium, and *G. polyphemus* were housed in outdoor enclosures at the Auburn University campus. The subspecies identity of *T. scripta* (*T. s. scripta* or *T. scripta elegans*) was not determined, though one of the younger males was identifiable as *T. scripta scripta*. *Trachemys scripta* were collected in Lee Co., AL, where these two subspecies interbreed (Mount 1996). Procedures in this study were approved by Auburn University IACUC regulations (PRN# 2013-2250 and 2013-2226; collecting permits # 6736 and 6740).

#### *Audiometry*

Chemical restraint was necessary to record AEPs. In *T. c. carolina*, *T. scripta*, and *Sternotherus*, this was administered by intraperitoneal injections of 40-80 mg/kg ketamine and 20-30 mg/kg xylazine. If subjects remained active after the initial dose (40 mg/kg ketamine, 20 mg/kg xylazine), additional doses (50 % of the initial dose) were given. *Gopherus polyphemus* were tested while recovering from an ibutton implant surgery that was performed for an unrelated project. Therefore, the chemical restraint of *Gopherus polyphemus* followed the protocol of that project, which included ketamine (8 mg/kg), dexmedetomidine (75mc/kg), and morphine (1 mg/kg).

All sound tests were conducted within a 0.7 × 1.06 × 1.65 m sound-reducing booth (Tremetrics AR9S audiometric booth). Airborne stimuli were sent through an SL1 amplifier (Applied Research and Technology) to a cone speaker (Dynex, model: DX-

SP211) that rested on one end of an aquatic testing tub (Fig. 1). Turtles were placed prostrate on a table platform that was attached to the walls of the chamber and to a single wooden leg (Fig. 1). Turtles were oriented towards the speaker with heads at a distance of 14 cm from the speaker's edge. The aquatic testing tub propping the aerial speaker was not in direct contact with the table or the walls of the chamber in order to minimize potential vibrational stimulation.

The aquatic testing tub was a large piece of PVC pipe (length: 73 cm, inner radius: 15 cm) that was capped at both ends with large PVC caps and had a 15.6 x 53.5 cm hole cut in the top. Underwater sounds were sent through a UW-30 underwater speaker (UW-30, University Sound, Oklahoma City, OK) that was suspended into the testing tub from a large stand resting on the floor of the chamber (behind the aerial speaker in Fig. 1). Turtles were secured with elastic bands to an angled wooden platform, which was suspended into the water from a stand that was magnetically fixed to the table platform. This resulted in the turtles being angled at 45° relative to the surface such that nostrils were above the water and tympana were submerged at a horizontal distance of 8 cm from the underwater speaker. All except 2 of the *Sternotherus* spp. were tested at a depth of 10 cm rather than at the surface. The thresholds of these 2 were not significantly different from the other *Sternotherus* ( $F_{1,6} = 2.56$ ,  $P = 0.16$ ). Air temperature in the testing chamber ranged from 20-23°C. Water temperature was not measured directly, but water was added to the tank at least the day before testing to allow for equilibration to room temperature.

Calibrations were conducted before each trial to equalize sound frequencies to the same sound level at the head location. Underwater sound levels were determined using a

hydrophone (High Tech Inc., sensitivity: -164 dB re 1 V/ $\mu$ Pa) fed into an oscilloscope (GW GOS-6xxG dual). The peak voltage of a calibration tone was measured from the oscilloscope, converted to an approximate root mean square value by multiplying by 0.707, and divided by the hydrophone sensitivity to convert to absolute SPL in dB re 1  $\mu$ Pa. Aerial sound levels were calibrated using a Pyle sound-level meter (C-type frequency weighting, model PSPL01, sensitivity range: 40-130 dB, frequency range: 31.5Hz – 8 kHz, 40-130 dB level range, accuracy:  $\pm$  3.5 db @ 1 kHz, 94 dB). Calibrations produced normalization files that were used to modify output at each frequency to an equivalent sound level.

Signals were generated in SigGen software (Tucker Davis Technologies). Tone pips were presented at 100, 200, 300, 400, 500, 600, 700, and 800 Hz and were 10 ms in duration (5 ms rise time and 2 ms cos gating window). Evoked potential waveforms were verified to be biological and not artifact by recording responses at all frequencies from a deceased turtle at 100 dB re 20  $\mu$ Pa in the aerial condition and 120 dB re 1  $\mu$ Pa in the aquatic condition. In those recordings, sound artifacts were either canceled completely or were produced only within the first 10 ms of the stimulus period, unlike the delayed AEP responses.

The recording electrode was placed subdermally above the vertex (top of the skull), the reference electrode was placed posterior to the tympanum, and a ground electrode was inserted into one of the forelimbs (after Christensen-Dalsgaard et al. 2012). Electrodes were shielded with nail polish (except at the tip) to provide insulation (27 gauge, Rochester Electro-Medical, Inc., Tampa, FL). Electrodes were fed into a Medusa RP2.1 pre-amplifier connected to a RA16 base station processor, which was connected to

a computer running the BioSig software program (Tucker Davis Technologies). The evoked potential traces were 100 ms long, representing averaged EEGs from 250 tone repetitions (tones were repeated at a rate of  $8 \text{ s}^{-1}$ ). To cancel stimulus artifact from the recording traces, all stimuli were presented in opposite polarities and averaged together in BioSig. During data acquisition, waveforms were notch filtered at 60 Hz and bandpass filtered between 10 and 3,000 Hz. Thresholds were assessed visually as the lowest detectable sound level at which any of the peaks within the response could be clearly distinguished from the background noise.

### *Statistical analysis*

Species threshold differences were tested using one-way repeated measures ANOVAs including species group as a between subjects factors and frequencies at 100-800 Hz as a repeated measures factor. *Sternotherus* spp. were pooled together to increase sample size, since there were no differences in threshold between *S. odoratus* and *S. minor* (aquatic:  $F_{1,6} = 0.91$ ,  $P = 0.38$ ; aerial:  $F_{1,7} = 0.19$ ,  $P = 0.67$ ). Species group differences were examined with Tukey HSD post hoc tests. Straight carapace length, body mass, and sex were included as covariates but were non-significant so excluded from the final models. Greenhouse-Geisser adjusted statistics were used in instances where sphericity was violated. A species group  $\times$  frequency interaction was included, and since it was significant, the frequency-dependent responses were explored by grouping thresholds into a low frequency group (100-300 Hz) and a high-frequency group (400-800 Hz), with alpha levels adjusted by the Bonferroni correction. All statistics were conducted in SPSS statistical software.

## Results

Representative AEP waveforms for *T. c. carolina* in the aerial and aquatic conditions are shown in Fig. 2. Responses of *T. c. carolina* at low frequencies exhibited frequency following responses with frequency doubling (e.g. 200 Hz in Fig. 2). Responses at 800 Hz also exhibited multiple peaks, but they did not correspond to a doubling of the tone frequency. Latencies occurred at 5-10 ms and decreased with increasing sound level. There were no salient differences in the waveform shape between the aerial and aquatic conditions.

Aerial thresholds were frequency dependent, with lowest sensitivities found for *T. c. carolina* at 45 dB re 20  $\mu$ Pa dB at 400 Hz (Fig. 3a). There was a significant species group  $\times$  frequency interaction ( $F_{11.9,115} = 3.07$ ,  $P = 0.001$ ) that could be removed if analysed without *G. polyphemus*. At 100-300 Hz, *Sternotherus* thresholds were on average 12.1 dB higher than *G. polyphemus* (95 % CL: 1.8-22.4,  $P = 0.018$ ), 13.1 dB higher than *T. scripta* (95 % CL: 2.6-23.4,  $P = 0.01$ ), and 17.6 dB higher than *T. c. carolina* (95 % CL: 8.4-26.8,  $P < 0.001$ ). In this frequency range, *G. polyphemus*, *T. c. carolina*, and *T. scripta* were not different from each other ( $P > 0.05$ ).

Above 400 Hz, sensitivity declined with increasing frequency for all species. The lowest thresholds in this range occurred in *T. c. carolina*, with mean thresholds rising from 45 dB at 400 Hz to 83 dB at 800 Hz. *Sternotherus* thresholds were on average 18.3 dB higher than *T. c. carolina* (95% CL: 9.4-27.2,  $P < 0.001$ ), 14.8 dB higher than *T.*

*scripta* (95% CL: 4.9-24.8;  $P = 0.002$ ), but not different from *G. polyphemus* ( $P = 0.24$ ). *G. polyphemus* was 11.3 dB higher than *T. c. carolina* (95% CL: 2.0-20.5,  $P = 0.012$ ), but not different from *T. scripta* ( $P = 0.18$ ). However, there was a species group  $\times$  frequency interaction at 400-800 Hz ( $F_{8,5,116} = 3.35$ ,  $P = 0.002$ ), and the difference between *T. scripta* and *G. polyphemus* varied from 5.7 dB at 400 Hz to 13 dB at 700 Hz and 2.1 dB at 800 Hz. *T. c. carolina* and *T. scripta* thresholds were similar in this range ( $P = 0.75$ ).

Underwater audiograms of *T. c. carolina*, *T. scripta*, and *Sternotherus* were similar in shape to aerial audiograms (Fig. 3b), with the lowest thresholds at 100-400 Hz. Collapsed across species, average thresholds were 93 dB at 100 Hz and 128 dB at 800 Hz. No species group differences in underwater thresholds were detected ( $F_{2,20} = 0.43$ ,  $P = 0.66$ ). Particle acceleration values at the location of the turtle heads during testing are reported in Table 1 to facilitate comparisons with other studies conducted in different sound fields.

## Discussion

### *Aerial sensitivity*

The lowest aerial auditory thresholds were found in *T. c. carolina* and *T. scripta*, and the highest were found in *Sternotherus*. These results support the hypothesis of adaptations for airborne sound detection in Emydidae at the level of comparison to *Sternotherus*; however, the similar sensitivity between *T. scripta* and *T. c. carolina* indicates that the more terrestrial lifestyle of the latter is not accompanied by a significant increase in auditory sensitivity. When compared with other aerial AEP audiograms,

there is some association evident between terrestrial living and aerial sensitivity across the phylogeny (Fig 3c).

*Gopherus polyphemus* exhibited similar thresholds to *T. scripta* and *T. c. carolina* at 100-300 Hz, but thresholds were elevated at 400-800 Hz. When this result is considered with additional anatomical and evoked potential data for the desert tortoise, *Gopherus agassizii*, it suggests a substrate vibration adaptation in *G. polyphemus* that is accompanied by high frequency hearing loss. The lowest auditory thresholds for *G. polyphemus* were approximately 30 dB higher than those of its congener, the desert tortoise, *G. agassizii* (Bowles et al. 1999) (Fig 3c). *G. polyphemus* possess enlarged otic regions containing very large otoliths compared to *G. agassizii*, and the tympanic disc: footplate area ratios are lower in the former (3:1 vs. 28:1) (Bramble 1982). These anatomical characteristics are expected to facilitate low frequency sound and vibration detection, but interfere with high frequency airborne sound detection. *G. polyphemus* use the head as a brace during digging, which would provide direct contact with the substrate and facilitate vibration detection (Bramble 1982; Mason and Narins 2001). *Gopherus flavomarginatus*, a close relative of *G. polyphemus*, also possesses a large otolith that is intermediate in size between *G. polyphemus* and *G. agassizii* (Bramble 1982; Bramble and Hutchison 2014). High frequency hearing loss is a phenomenon also observed in fossorial mammals, suggesting similar adaptation to living in a subterranean environment (Mason 2013).

This study did not investigate anatomical mechanisms for variation in aerial auditory sensitivity, but mass, size and geometry of tympanic middle ear elements would be expected to affect sensitivity. The area ratios of Testudines are small relative to

tympanic ears of other tetrapods, but the available data indicate that variation in area ratios within the order corresponds with aerial sensitivity: 26:1 in *Gopherus agassizi* (Bramble 1982), 8.5:1 in *T. scripta* (Wever 1978), and 3:1 in sea turtles (estimated from an examination of 5 *Caretta caretta* and 1 *Lepidochelys kempii*) (Lenhardt et al. 1985). In addition to area ratio, thick tympana would facilitate low frequency sound detection but limit high frequency response in air (Christensen-Dalsgaard and Manley 2014). Therefore, the degree of differentiation of the skin on the tympanum and its attachment to the tympanic disc could affect aerial sensitivities. Lowered auditory nerve thresholds were found after removing the skin overlying the tympanic disc from the ‘common land tortoise’ (Adrien et al. 1938), although Christensen-Dalsgaard et al. (2012) found no difference in aerial evoked potential thresholds when removing this skin in *T. scripta*. The skin of *T. c. carolina* over the tympanum region is more clearly delineated than the relatively undifferentiated skin of *G. polyphemus* (personal observation), suggesting that this is a variable trait that could correlate with aerial sensitivity.

#### *Aquatic sensitivity and amphibious comparisons*

When comparing amphibious auditory sensitivities within and between taxa, it is necessary to properly characterize the sound field in each medium and to consider whether the animal is responsive to pressure, particle motion, or some combination of each. Particle motion varies for a given sound pressure in water versus air due to differences in characteristic acoustic impedance in each medium, as well as in relation to proximity to the sound source with respect to the ‘local flow’ in the acoustic near field (Bass and Clark 2003). In addition, the magnitude of particle motion relative to sound

pressure is unpredictable in small tanks due to boundary reflections and is particularly high at the surface and at tank boundaries due to pressure release (Parvulescu 1967, Popper and Fay 2011).

For an aquatic ear that is strongly sensitive to pressure across the hearing range, particle acceleration and intensity audiograms are not appropriate for examining biological differences because similar pressure thresholds may correspond to variable particle motion values that reflect tank acoustics and not the animal's auditory response to pressure (Kastak and Schusterman 1998). Conversely, if the ear responds primarily to particle motion, pressure audiogram comparisons between studies are not appropriate, since impedances (pressure/velocity) vary between tank setups (Popper and Fay 2011). In small tanks, the appropriate measurement can be complicated if the impedance of the sound field is very low, such that if the particle motions exceed the displacements produced by an animal's pressure-displacement transducer mechanism, the ear can be stimulated directly via particle motion rather than the pressure component (e.g. Christensen-Dalsgaard et al. 2011; Christensen et al. 2015).

The available data indicate that the testudine ear is sensitive to pressure in air and underwater (Wever 1978, Christensen-Dalsgaard et al. 2012). For *T. scripta elegans*, underwater sound produced tympanic vibrations within the audiogram hearing range that were 30-40 dB higher than particle motions of the surrounding water (Christensen-Dalsgaard et al. 2012). However, due to the small tank used in the current study, it is possible that high particle motions activated the ear via bone conduction. The low thresholds at 100-200 Hz could potentially indicate bone conduction responses, since whole body vibrograms for *Trachemys scripta elegans* exhibited lower best frequencies

(150-200 Hz) than underwater speaker audiograms (400-600 Hz) (Christensen-Dalsgaard et al. 2012). Alternatively, the audiogram shape could simply reflect a lack of frequency precision due to the short stimulus used (e.g. the 10 ms tone is only 1 cycle of the 100 Hz stimulus). Nonetheless, pressure is likely the appropriate measure for aquatic comparisons among Testudines (Fig. 3d), since current data indicate that the ear is activated by pressure to a large degree (Christensen-Dalsgaard et al. 2012).

The greater sensitivity of *T. c. carolina* and *T. scripta* to airborne sound was not associated with reduced sensitivity underwater compared to *Sternotherus*, yet the aerial thresholds of *Sternotherus* were elevated compared to the former two species. This indicates that the higher aerial sensitivity in the emydids does not directly trade off with aquatic sensitivity, and that aerial sensitivity may be reduced in some aquatic species. Thus, moving between aquatic and terrestrial habitat can result in different auditory sensitivity shifts for different species.

The comparable aquatic thresholds of *T. c. carolina* to *T. scripta* and *Sternotherus* could reflect retention of ancestral middle ear cavity anatomy that facilitates underwater hearing. This interpretation is supported by examination of the comparative morphology of the skull combined with the middle ear resonance model of Testudines underwater hearing (Christensen-Dalsgaard et al. 2012; Willis et al. 2013). According to the resonance model, pressure waves produce oscillations of the air in the middle ear cavity, which stimulates motion of the tympanic disc and columella (Christensen-Dalsgaard et al. 2012). Across the Testudines phylogeny, including both aquatic and terrestrial members, the ratio of the middle ear cavity volume to the total head volume is constant (Willis et al. 2013). Therefore, if middle ear cavity volume is a primary determinant of overall

sensitivity, the similar volumes in terrestrial species could explain conserved aquatic sensitivity in species such as *T. c. carolina*.

#### *Ecological significance of hearing in Testudines*

This study illustrates the potential for medium-dependent adaptation of the auditory system in Testudines. The role of hearing in turtle behavioral ecology has been poorly investigated (Young 2014), yet hearing could be important for a number of behavioral tasks. In general, the auditory sense may augment the performance of other sensory systems to improve perceptual accuracy and response times (Stevens 2013). More specific auditory tasks could include soundscape analysis and using acoustic landmarks for spatial navigation. *T. carolina* and *T. scripta* could be trained to navigate a Y maze using 500 Hz tones (Lenhardt 1981), indicating a spatial sound perception ability. Additionally, airborne sound and vibrational stimuli can elicit avoidance behavioral patterns, such as freezing and head withdrawal responses (Lenhardt 1982; Bowles et al. 1999). Finally, acoustic communication could be another potential selection pressure on hearing in some species, which has been documented in both aquatic and terrestrial species across the order (Galeotti et al. 2005; Giles et al. 2009; Ferrara et al. 2014a, b).

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## Tables

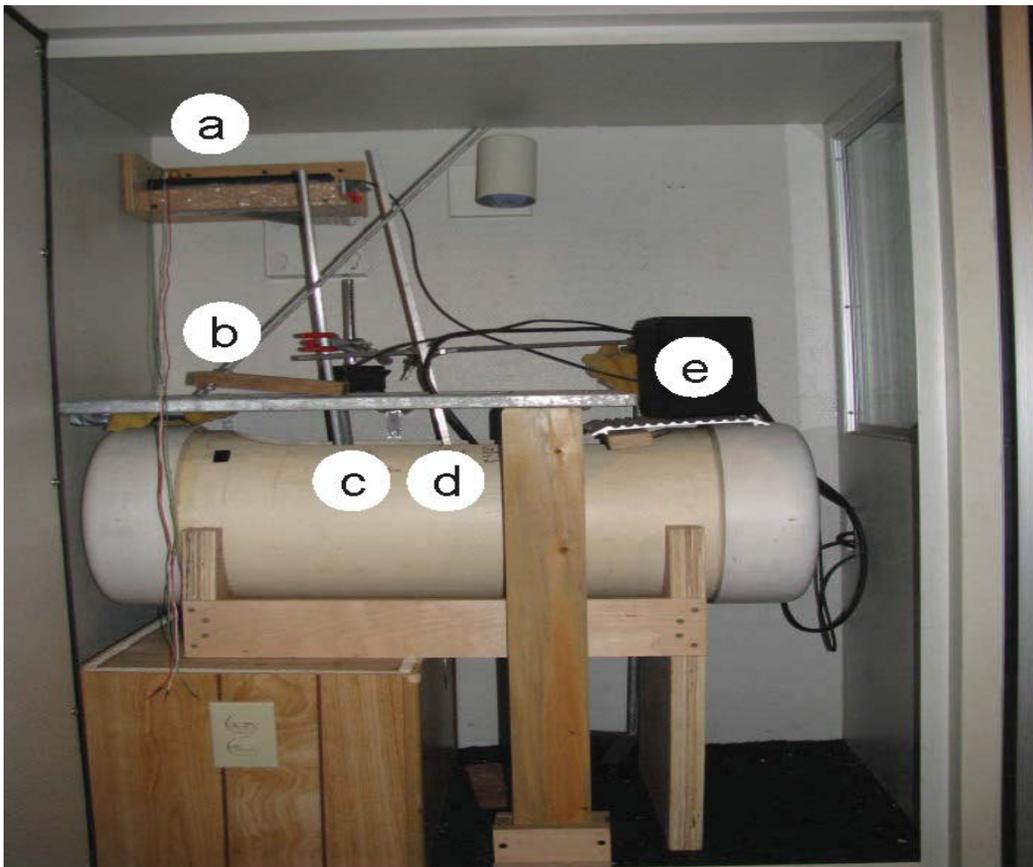
**Table 1** Particle acceleration ( $\text{m/s}^2$ ) in three orthogonal axes for a 126 dB re 1  $\mu\text{Pa}$  tone at the water surface and 10 cm deep.

Frequency (Hz)	Vertical	Horizontal (perpendicular to speaker)	Horizontal (parallel to speaker)	Combined
100 (surface)	0.0996	0.0032	0.0833	0.1298
200 (surface)	0.0641	0.0016	0.0587	0.0869
300 (surface)	0.0701	0.0031	0.0519	0.0873
400 (surface)	0.0795	0.0028	0.0737	0.1085
500 (surface)	0.0818	0.0044	0.0762	0.1119
600 (surface)	0.0761	0.0002	0.0752	0.1070
700 (surface)	0.0745	0.0101	0.0719	0.1040
800 (surface)	0.0837	0.0056	0.0776	0.1143
100 (10 cm)	0.0099	0.0576	0.0068	0.0589
200 (10 cm)	0.0122	0.0516	0.0054	0.0533
300 (10 cm)	0.0249	0.0525	0.0105	0.0590
400 (10 cm)	0.0141	0.0774	0.0137	0.0799
500 (10 cm)	0.0124	0.0768	0.0180	0.0798
600 (10 cm)	0.0210	0.0866	0.0232	0.0921
700 (10 cm)	0.0213	0.0784	0.0312	0.0870
800 (10 cm)	0.0201	0.0940	0.0344	0.1021

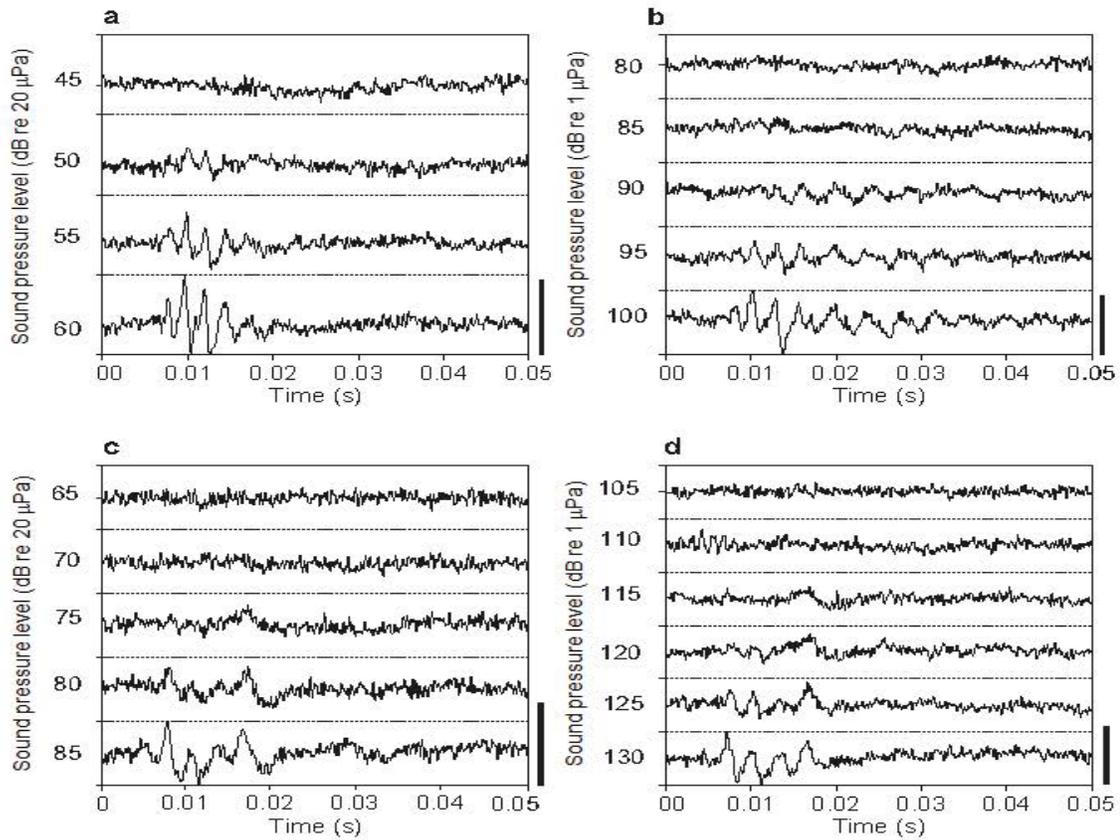
Particle acceleration was measured using the RMS pressure difference between two hydrophones divided by the distance between the hydrophones and the density of freshwater ( $997.1 \text{ kg/m}^3$ ). A significant vertical particle motion component was evident at the surface. The combined column was calculated by squaring the value for each axis, summing these values, and taking the square root.

## Figures

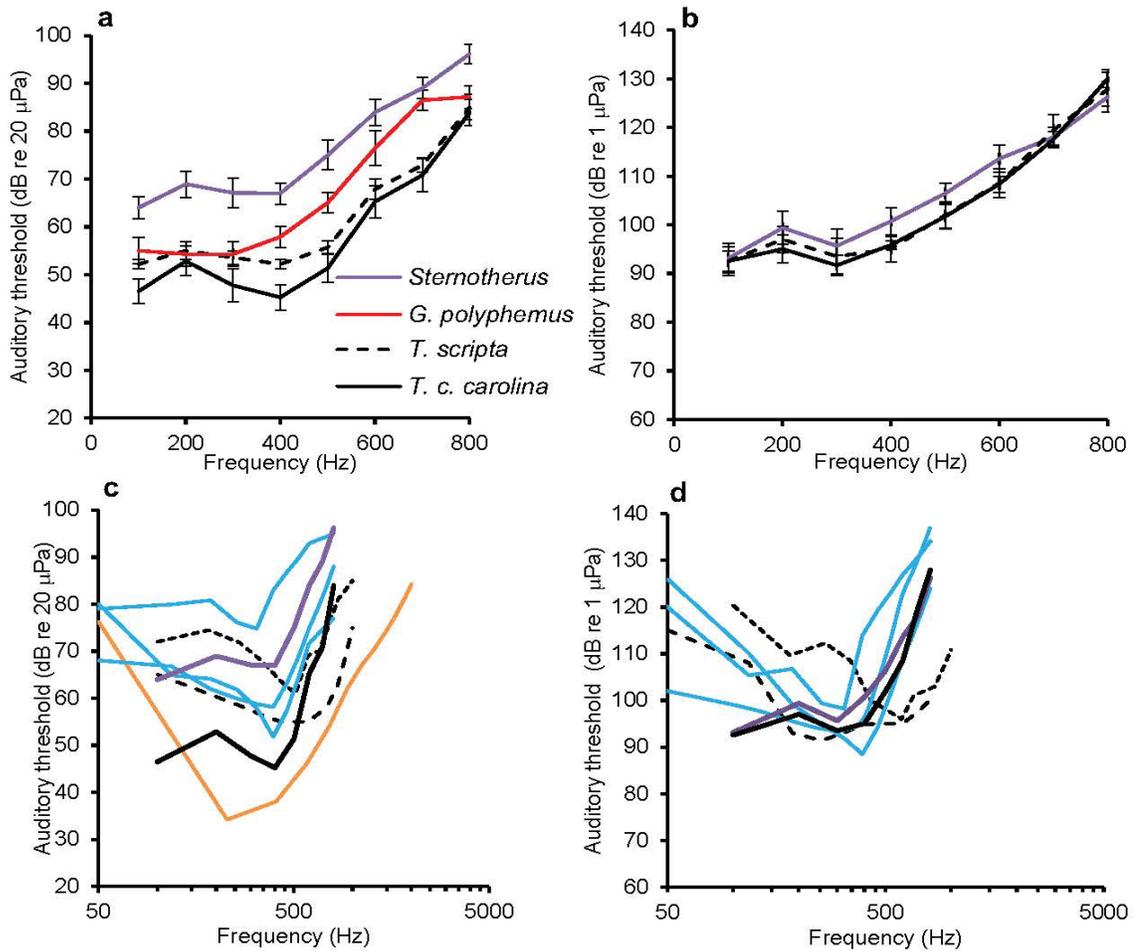
**Fig. 1** The experimental chamber and equipment used to collect auditory evoked potentials (AEP). **a** Preamplifier, **b** angled platform for aquatic testing, **c** position of the stand suspending the angled platform into the aquatic testing tub, **d** position of the stand suspending the underwater speaker into the aquatic testing tub, **e** aerial speaker. For scale, the distance from the floor of the chamber to the *top surface* of the table is 80 cm



**Fig. 2** Example AEP traces for *T. c. carolina* generated in response to 10 ms tone pips at 200 Hz (**a, b**) and 800 Hz (**c, d**). Responses on the left were collected in response to the aerial speaker stimulus, and responses on the right were collected just below the water surface in response to the underwater speaker stimulus. *Scale bars* indicate 1  $\mu$ V



**Fig. 3 a, b** Aerial and underwater audiograms for each species group (mean  $\pm$  SEM). **c, d** The range of mean AEP thresholds in the current study (*Terrapene c. carolina* in black and *Sternotherus* in purple) overlaid on mean AEP audiograms collected for other species of Testudines. Species are colored by aquatic-terrestrial lifestyle: orange terrestrial *Gopherus agassizii* (Bowles et al. 1999), blue sea turtles *Chelonia mydas*, *Dermochelys coriacea* (the highest thresholds in **c**), *Eretmochelys imbricata* (Piniak 2012), short black dashed semi-aquatic *Trachemys scripta elegans* (Christensen-Dalsgaard et al. 2013), and long black dash = *Malaclemys terrapin* (Lester 2012). The mean values for comparative audiograms in **c** and **d** were either provided in the study results or digitized from figures using Plot Digitizer software (version 2.6.3)



### **Chapter 3 - Comparative and developmental patterns of amphibious auditory function in salamanders**

#### **Abstract**

Early amphibious tetrapods may have used sound-induced lung vibrations to detect aquatic sound pressure, but their lack of tympanic middle ears would have restricted aerial sensitivity. Salamanders share these characteristics and therefore could be models for the carryover of auditory function across an aquatic-terrestrial boundary without tympanic middle ears. We measured amphibious auditory evoked potential audiograms in five phylogenetically and ecologically distinct salamanders (*Amphiuma means*, *Notophthalmus viridescens*, *Ambystoma talpoideum*, *Eurycea* spp., and *Plethodon glutinosus*) and tested whether metamorphosis to a terrestrial adult were linked to aerial sensitivity. Threshold differences between media varied between species. *A. means*' relative aerial sensitivity was greatest at 100 Hz and decreased with increasing frequency. In contrast, all other salamanders retained greater sensitivity up to 500 Hz, and in *A. talpoideum* and *Eurycea* was higher at 500 Hz than at 100 Hz. Aerial thresholds of terrestrial *P. glutinosus* above 200 Hz were similar to *A. talpoideum* and *Eurycea*, but lower than *N. viridescens* and *A. means*. Metamorphosis did not affect aerial sensitivity in *N. viridescens* or *A. talpoideum*. These results fail to support a hypothesis of terrestrial

hearing specialization across ontogeny or phylogeny. Furthermore, aerial performance is correlated with aquatic performance to different degrees, depending on species.

## Introduction

The first tetrapods appeared in the late Devonian, but tympanic ears, a major innovation for aerial hearing, did not evolve until the late Permian and Triassic periods (Clack 2002; Müller and Tsuji 2007). Without a tympanic ear to couple pressure fluctuations from airborne sound into inner ear fluid displacements, the aerial auditory sensitivity of early terrestrial tetrapods would have been restricted by sound transmission through unspecialized, non-tympanic pathways (Christensen-Dalsgaard and Carr 2008). Non-tympanic hearing typically limits sensitivity to high intensities and low frequencies (Hetherington and Lindquist 1999; Christensen et al. 2015a,b; but see Boistel et al. 2013).

Early amphibious tetrapods may have possessed the capacity to detect aquatic sounds via pressure-induced vibrations from the lungs or the air-filled spiracular chamber (Clack et al. 2003; Christensen et al. 2015a,b). Such adaptations could have extended the aquatic sensitivity range to higher frequencies, which could have pre-adapted the auditory system for high frequency aerial sensitivity (Fritsch 1992; Christensen et al. 2015a,b). However, the extent to which aquatic function translates into aerial function is expected to vary among species depending on the effectiveness of acoustic transmission between air and non-tympanic auditory structures. Studies in amphibians have indicated that non-tympanic aerial hearing is promoted by peripheral factors such as vibrations of the body wall overlying the lungs (Hetherington and Lindquist 1999), the opercularis complex (Lombard and Straughan 1974), and small body size (Hetherington 1992a,b). In the inner ear, high aerial frequency detection would be promoted by a switch from otolithic macula to papillae lacking overlying otoconial masses (i.e., amphibian and basilar papillae) (Christensen-Dalsgaard and Manley 2013). In addition, the resistance to sound

transmission through the inner ear would be lowered by opening the otic capsule with oval and round windows (Fritsch 1992; Christensen-Dalsgaard and Manley 2013).

Salamanders are useful models for non-tympanic auditory function (Hetherington 2001; Christensen et al. 2015b). Furthermore, they could be models for the carryover of auditory function across an aquatic-terrestrial boundary where there is potential to detect aquatic pressure, but no tympanic ear. The few studies that have examined aquatic auditory function of salamanders have each highlighted a role for sound pressure detection via the lung or mouth cavity (Hetherington and Lombard 1983; Bulog and Schlegel 2000; Christensen et al. 2015b). Despite lacking tympanic ears, salamanders possess peripheral characteristics that could promote non-tympanic aerial sensitivity: body walls that vibrate in response to airborne sound in lunged species (Hetherington 2001), small body size, and the opercularis complex (Mason 2007). In the inner ear, they possess both otoconial receptors (saccular maculae) and receptors lacking overlying otoconial masses (amphibian papillae and basilar papillae), with a loss of basilar papilla in several derived taxa (Lombard 1977). They possess an oval window holding a columella and/or operculum, with sound energy release via the perilymphatic foramen opening into the cranial cavity (Smith 1968; Wever 1978). These inner ear and peripheral auditory structures are diverse among salamanders, but the functional correlates of this diversity are not well understood.

Salamanders show aquatic-terrestrial specialization across both ontogeny and phylogeny, providing two levels at which medium-specific auditory adaptation can be analysed. In addition, salamanders (and frogs) possess an auditory structure, the opercularis complex, which is associated with terrestriality across both ontogeny and

phylogeny. Aquatic larvae and obligate aquatic taxa (Amphiumidae, Proteidea, Sirenidae, Cryptobranchidae) possess only a columella (stapes) in the oval window, which has an anterior ligamentous connection to either the squamosal, palatoquadrate, or ceratohyal bones (Kingsbury and Reed 1909; Monath 1965; reviewed by Mason 2007). During metamorphosis, an additional mobile element appears in the oval window (the operculum) along with a muscular attachment to the pectoral girdle (reviews by Mason 2007 and Capshaw and Soares 2016). The associations between the opercularis complex and terrestriality have led to the assumption that this structure functions in terrestrial hearing, but conclusive demonstration of this role in salamanders is still lacking (first proposed by Kingsbury and Reed 1909; reviews by Mason 2007 and Capshaw and Soares 2016). Additionally, anatomical changes to the salamander ear across development have been described (Table 1), but their effects on function have not been investigated (for a review in anurans, see Simmons and Horowitz 2007).

The purpose of this study was to provide a developmental and comparative analysis of amphibious hearing in salamanders to understand the factors that promote and constrain non-tympanic auditory function across an aquatic-terrestrial boundary. We collected auditory evoked potentials (AEPs) in response to aquatic-borne and airborne sounds in ecologically and anatomically distinct salamanders: *Amphiuma means*, *Notophthalmus viridescens*, *Ambystoma talpoideum*, *Eurycea* spp., and *Plethodon glutinosus* (Fig. 1). These species vary with respect to multiple auditory structures. *A. means* lacks an opercularis system altogether, while it is present in adult ambystomatids and salamandrids, and plethodontids (*P. glutinosus* and *Eurycea* spp.) have a unique cucullaris-fenestral complex resembling an opercularis system. *A. means* and *A.*

*talpoideum* possess a basilar papilla, whereas it is absent in *Notophthalmus* and plethodontids (Lombard 1977). Plethodontids have several additional auditory characters that could affect auditory function: lack of lungs, a longer, winding periotic canal (compared to straighter connections in other families), and a vertically-oriented amphibian papilla (Lombard 1977). In addition to these comparative analyses, we tested whether metamorphosis improves aerial sensitivity in *N. viridescens* and *A. talpoideum*, two species with a biphasic life cycle. We discuss the audiogram variation in the context of current theories of non-tympanic hearing in salamanders.

## **Materials and methods**

### *Animal collection*

Salamanders were wild-captured in the states of Alabama and Florida, USA. *Ambystoma talpoideum*, *Amphiuma means*, and both larval and eft stage *N. viridescens* were captured in and around ponds in Conecuh National Forest, Covington Co, AL. Adult *N. viridescens* (non-gilled) were captured from Blue Hole Spring, Wacissa River, Jefferson Co, FL. *Plethodon glutinosus* were captured in Macon Co, AL and in Bankhead National Forest, Winston Co, AL, and *Eurycea guttolineata* and *Eurycea cirrigera* were captured at Hatchett Creek in Clay Co, AL. The mass and snout-vent length of each species are shown in Table 2, grouped by developmental stage.

*A. talpoideum* and *N. viridescens* exhibit life cycles characterized by gilled aquatic larvae, metamorphosis to a terrestrial stage, and aquatic reproduction. Both species are also capable of facultative paedomorphosis. *Notophthalmus viridescens*

exhibits a unique terrestrial juvenile ‘red eft’ stage that is distinct from the non-gilled, fully aquatic adult phenotype and characterized by gill resorption, red coloration, and granular skin. Reproduction in both species is aquatic.

Gilled aquatic larvae of *A. talpoideum* and *N. viridescens* were collected in July of 2014 and 2015, several months after the breeding season of both species (winter and early spring). One aquatic juvenile *N. viridescens* was gilled when captured, but resorbed its gills in the laboratory, and was analysed as a larvae. In both species, a subset of captured larvae transformed spontaneously in the laboratory. The metamorphosed group of *A. talpoideum* included individuals captured as metamorphosed adults (n = 4) and individuals that metamorphosed in the laboratory (n = 5). The *A. talpoideum* paedomorphic group included large, late stage larvae and adults that did not transform in the laboratory. Two of the eft stage *N. viridescens* were captured in the field and four metamorphosed in the laboratory from gilled larvae. In both species, testing on individuals that metamorphosed in-lab was conducted after gills had fully resorbed and individuals exhibited terrestrial behavior (after at least three weeks in *Ambystoma* and after 7-20 days in *N. viridescens*). The procedures in this study were approved by Auburn University IACUC.

### *Audiometry*

Salamanders were immobilized for AEP tests by immersion in a neutral buffered bath of 0.05-0.08 g/100 mL MS-222 (Tricane methanesulfonate). Readiness for testing was assessed by loss of the righting response, which typically occurred after 20-30 min. Salamanders occasionally initiated movement during experiments and required re-bathing

before continued testing. Salamanders were periodically sprayed with water during aerial experiments to keep them hydrated. Tests were conducted throughout the year (aerial temperature range: 19-22 °C, aquatic temperature range: 17.5-21°C). The water temperature in the *A. means* experiments was a slightly colder temperature (14-16°C), presumably due to the closer contact of this tub with the ground floor of the laboratory basement. *Plethodon glutinosus* and eft *N. viridescens* were only presented with aerial sound due to their terrestrial lifestyle.

Audiometry tests were conducted within a sound booth (0.7 x 1.06 x 1.54 m, Tremetrics AR9S). The hardware equipment used for the collection of both aerial and aquatic audiograms are shown in Appendix 2. The aquatic testing chamber was a large PVC pipe with a hole cut in the top (length: 73 cm, internal diameter: 15 cm). During testing, salamanders were held within a clay saddle that rested on a horizontal stand. The horizontal stand was suspended into the aquatic testing container from a base that was magnetically attached to an overhanging metal platform. Salamanders were secured in place on the clay saddle by crossing pairs of pushpins into the clay on either side of the body. The overhanging metal platform was attached to the walls of the sound-reducing chamber with shelf brackets and propped in one corner with a leg contacting the floor. During testing, the heads of the salamanders were positioned 10 cm below the water surface and 8 cm horizontally from the underwater speaker (UW-30, University sound, Oklahoma City, OK). Salamanders were brought to the surface periodically to allow aerial respiration. The large size of *A. means* required the use of a larger aquatic testing tub, which is shown in Appendix 3 and described in detailed in Crovo et al. (2016).

For aerial tests, salamanders were placed prostrate on the metal platform and the speaker (Dynex, model: DX-SP211) was placed onto a platform that rested on one side of the cylindrical aquatic testing container. Therefore, there was no direct coupling between the speaker and the metal platform except via the walls of the sound chamber.

Salamanders were oriented towards the speaker with heads at a distance of 14 cm. In the aerial treatments for *A. means*, the hole in the metal platform was covered with two stiff boards to accommodate this species' large size: a hard plastic board (19.5 x 27 cm, 7 mm thick) closest to the speaker and holding the bulk of the body and a wood composite board (21.5 x 27.5 cm, 3 mm thick) holding a portion of the posterior length of the body. *A. means* was wrapped in a moist cloth towel along its body length during testing.

Six of the 15 adult newts tested in the aerial condition were tested under slightly different conditions in conjunction with use in a separate experiment. Rather than resting directly on the platform, these individuals rested on a layer of acoustic foam submerged into a water-filled container, which rested on another layer of acoustical foam (Appendix 4). Although the thresholds of these did not differ significantly from those collected from the newts placed directly on the platform ( $F_{1,13} = 1.55$ ,  $P = 0.23$ ), they showed a pattern of lower thresholds at 100-200 Hz (Appendix 4). These individuals were removed from aerial analyses, but since these individuals also made up a majority of the aquatic audiograms tested, they were retained in between-media analyses.

#### *Sound pressure calibration and particle acceleration calculation*

Sound pressure level was calibrated across frequencies before each trial by recording the level of a 6 s tone at the location that was occupied by the salamander's

head during testing. The measured output levels were used to create a normalization file that equalized sound level across stimulus frequencies to a common root-mean-square (RMS) sound level. Aerial sound was measured using a sound level meter (Pyle Audio, model PSPL01, sensitivity range: 40-130 dB, accuracy  $\pm 3.5$  dB at 1 kHz, 94 dB, C type frequency weighting). Aquatic sound was measured using a hydrophone (High Tech Inc., sensitivity: -164 dB re 1 V/ $\mu$ Pa) connected to an oscilloscope (GW GOS-6xxG).

Aquatic particle acceleration was calculated in both testing containers using the measured pressure gradient between two hydrophones and the following equation:  $a = -(p_1 - p_2)/d\rho$ , where  $p_1 - p_2$  is the instantaneous pressure difference,  $d$  is the distance between hydrophones (3.5 cm), and  $\rho$  is the density of freshwater ( $997.1 \text{ kg/m}^3$ ) (Mann 2006). The hydrophones were mounted on a pole and rotated in three orthogonal axes around the centroid location (position of the salamander's head), which was calibrated to a sound pressure level of  $126 \text{ dB}_{\text{rms}}$  re  $1 \mu\text{Pa}$ . Both channels were recorded into Raven Pro software (<http://www.birds.cornell.edu/raven>). The two .wav files were subtracted in Matlab (MathWorks, Inc.) and exported back to Raven Pro, where the root-mean-square (RMS) amplitude was measured and converted to Pa using the known sound level calibrated to the oscilloscope. In both aquatic test containers, measurements were taken three times and averaged to ensure repeatability.

For statistical analyses, acceleration values from all three measured axes were combined into one measure using:  $x = \sqrt{(a^2 + b^2 + c^2)}$ , where  $a$ ,  $b$ , and  $c$  are the orthogonal axes. The combined particle motion levels in the two testing containers were similar, with differences less than 5 dB across all frequencies (Appendix 5). In both containers, the axis of highest particle motion was horizontal and perpendicular to the face of the

suspended speakers, corresponding to the anterior-posterior axis of the salamanders (Appendix 5).

Aerial auditory thresholds were also expressed relative to the magnitude of vertical substrate vibrations on the table platform at the location of the salamander head. Measurements were taken with a geophone (Geospace Technologies. Model: GS-14-L3; sensitivity: 11.4 V/m/s) with the speaker playing tones at 100-2,000 Hz, calibrated to 90 dB re 20  $\mu$ Pa (Appendix 5). The geophone output was passed through an oscilloscope into a laptop computer where root-mean-square amplitude was measured in Raven Pro software, calibrated to the oscilloscope. Geophone velocity was converted to acceleration ( $a = 2\pi f * v_{\text{rms}}$ , where  $f$  is the dominant frequency of the tone measured in Raven Pro). This measurement was repeated on three separate occasions and averaged.

#### *Tone presentation and record acquisition*

Tones were presented at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 1.0, 1.5, 2.0, 2.5, 4.0, 6.0, and 8.0 kHz. Tones were 10 ms in duration with 2 ms Hanning windows and 5 ms rise times generated using SigGen software (Tucker Davis Technologies (TDT), FL, USA). The waveforms and frequency spectra of individual tone pips, recorded at the location of the salamander's head, are shown in Appendices 6 and 7. Tones were lowered in 5 dB steps until none of the peaks in the AEP responses could be distinguished from the baseline trace noise. Auditory threshold was designated as the lowest sound level at which any peaks in the evoked response could be distinguished from the baseline.

A single recording electrode was placed sub-dermally at the vertex of the skull and a reference electrode was placed posterior to the jaw. A ground electrode was placed

in the tail. Electrodes led to a Medusa RP2.1 pre-amplifier, which led to an RA16 processor, which led to a computer running BioSig acquisition software (all from TDT, FL, USA). Two traces were collected for each frequency-sound level combination, one of which was collected from the stimulus in an inverted phase. The purpose of the inverted stimulus was to cancel stimulus artifacts when the two traces were averaged together. The resulting averaged waveform represented the result of 500 tone repetitions (250 for each phase of the trace). Tones were presented at a rate of 8 Hz and the waveform acquisition sampling rate was 22.41 kHz. The bandpass acquisition filter in BioSig was set to 0.3-3 kHz with a notch filter at 60 Hz.

### *Statistical analyses*

Statistical analyses for aquatic audiograms, aerial audiograms, and between-media differences were conducted separately. The aquatic-aerial difference was computed by subtracting aerial pressure thresholds from aquatic pressure thresholds. In addition, to account for the effect of the unique impedance of the *Amphiuma means* testing container, threshold differences were also computed relative to the difference between aquatic particle acceleration and aerial vertical substrate acceleration. Before subtraction of pressure thresholds, 26 dB was added to the aerial thresholds to convert them from dB re 20  $\mu$ Pa to a common reference pressure of 1  $\mu$ Pa.

Species and developmental stage effects within *N. viridescens* and *A. talpoideum* were tested using repeated measures general linear models that included frequency as a within-subjects factor and either species or developmental stage as a between-subjects factor. Mass and SVL were tested as covariates in comparative analyses, but removed

since they were non-significant in each instance. Species differences in aerial thresholds were tested using Tukey HSD post-hoc tests at 100 Hz, 400 Hz, and 600 Hz. The effect of frequency on the aquatic-aerial pressure threshold difference was examined in each species using paired t-tests comparing 100 and 500 Hz. Species differences in between-media threshold differences were examined with Tukey HSD post hoc tests at 100 and 500 Hz.

Comparative analyses were restricted to 100-600 Hz to maximize the repeated measures sample size, since the numbers of salamanders responding decreased with increasing frequency, particularly for *N. viridescens*. Similarly, comparisons within *A. talpoideum* were restricted to 100-800 Hz for the aerial condition and 100-600 Hz for the aquatic condition. In *N. viridescens*, analyses were conducted at 100-600 Hz for aerial thresholds and 100-400 Hz for aquatic thresholds. Comparative aerial tests included only metamorphosed (M) *A. talpoideum*, and comparative aquatic tests included only paedomorphic (P) *A. talpoideum*. Both morphs of *A. talpoideum* were analysed in the comparative aquatic-aerial variable, and only adult *N. viridescens* were used in comparative tests. *Eurycea* were excluded from statistical analyses due to small sample size (n = 3).

## Results

### *Waveform morphology*

Frequency following responses were evident up to 200-300 Hz (Fig 2). Above these frequencies, waveforms could be characterized by a single positive peak and single

negative peak. Latencies decreased with increasing sound level, as is typically observed in AEP waveforms. The first negative peaks occurred at 6-12 ms. The numbers of salamanders responding declined above 1 kHz for both aquatic and aerial stimuli.

### *Aquatic audiograms*

In general, aquatic thresholds were lowest at 100 Hz and increased steadily with increasing frequency up to 1-2.5 kHz. Sensitivity levels varied between species, but the overall profile shapes of the aquatic audiograms were similar (species:  $F_{2,18} = 8.49$ ,  $P = 0.003$ ; species  $\times$  frequency:  $P > 0.05$ ). The general pattern can be illustrated with *A. talpoideum* (P), which showed the most sensitive audiogram. Thresholds in *A. talpoideum* increased from 101 dB re 1  $\mu$ Pa at 100 Hz to 108 dB at 500 Hz (-50 to -40 dB re 1  $\text{m/s}^2$ ). By 1 kHz, thresholds reached 120 dB (-29 dB re 1  $\text{m/s}^2$ ). Two individuals responded up to 2.5 kHz and one individual detected the 4 kHz tone at 150 dB. On average, the *A. talpoideum* pressure audiogram (dB re 1  $\mu$ Pa) was 10 dB lower than *A. means* ( $P = 0.053$ ) and 16 dB lower than *N. viridescens* ( $P = 0.002$ ) (Fig. 3a). When particle acceleration was examined, *A. talpoideum* and *N. viridescens* thresholds remained statistically different, but not *A. means* and *A. talpoideum* (Fig. 3b).

### *Aerial audiograms*

Aerial audiogram profiles were more diverse compared to aquatic audiograms. Species differences were strongly frequency-dependent and most distinct at 100 Hz and at 400-600 Hz (species  $\times$  frequency:  $F_{8.8, 79.1} = 12.5$ ,  $P < 0.01$ ; species:  $F_{3,27} = 8.6$ ,  $P < 0.01$ ) (Fig 4a). At 100 Hz, the lowest thresholds were found in *A. means* (60.7 dB) and *A.*

*talpoideum* (65.4 dB), which were 14.2 and 9.6 dB lower, respectively, than the highest thresholds, found in *N. viridescens* (*A. means*:  $P = 0.008$ ; *A. talpoideum*:  $P = 0.038$ ). The threshold difference between *A. means* and *N. viridescens* diminished with increasing frequency; both species reached thresholds of approximately 80 dB at 400 Hz and 95 dB by 600 Hz.

There were two distinct classes of responses at 400-600 Hz. As a group, the thresholds of *A. talpoideum*, *Eurycea* spp., and *P. glutinosus* were lower than both *N. viridescens* and *A. means*. Thresholds of *A. talpoideum* and *P. glutinosus* at 400 Hz (63-64 dB) were 15-17 dB lower than those of *N. viridescens* and *A. means* (all  $P < 0.05$ , Tukey HSD). At 600 Hz, *A. talpoideum* thresholds were 20 dB lower than *A. means* and 13.5 dB lower than *N. viridescens* (both  $P < 0.01$ ); *P. glutinosus* thresholds were 30 dB lower than *A. means* and 24 dB lower than *N. viridescens* (both  $P < 0.01$ ). The aerial audiogram of *Eurycea* spp. was similar to *A. talpoideum* and *P. glutinosus*. At 400-500 Hz, *Eurycea* spp. showed the lowest thresholds of all the species tested (55 dB).

When thresholds are expressed relative to the level of table substrate vibrations, the overall profiles are similar (Fig. 4b). However, unlike the audiograms expressed relative to pressure, there is no upward notch at 200 Hz. This corresponds to a higher table particle acceleration measured at 300 Hz relative to 100-200 Hz (Appendix 5).

#### *Comparisons between media*

Comparing using pressure thresholds, the aerial audiogram of *A. means* was much steeper with increasing frequency compared to its aquatic audiogram. Between 100 and 500 Hz, *A. means*' thresholds were elevated from 60.7 dB to 90 dB in air, but only from

112 dB to 117 dB underwater. In contrast, between 100-500 Hz, the aerial audiogram of *A. talpoideum* was flatter (65.5 to 67 dB) compared to gradually elevated thresholds underwater (101 to 108 and 103 to 116 for P and M, respectively). Thresholds of *N. viridescens* between 100 to 500 Hz increased by a similar amount (11dB) in both media (air: 75 to 86 dB; water: 114 to 125 dB). The aerial audiogram of *Eurycea* was U-shaped, compared to a flatter aquatic audiogram. In air, thresholds between 100 and 500 Hz were lowered from 68 to 55 dB, but similar underwater (108 to 110 dB).

When aerial pressure thresholds were subtracted directly from aquatic pressure thresholds for each species, the relative aerial sensitivity of *A. means* was dissimilar from the other species tested. *A. means*' aerial thresholds were 25.4 dB lower than aquatic thresholds at 100 Hz but 1.8 dB lower at 500 Hz ( $P < 0.01$ , paired t-test) (Fig 5a). In contrast, the relative aerial sensitivity of *A. talpoideum* was augmented from 100 to 500 Hz, changing 11.2 to 23.4 dB (M) and 8.4 to 14 dB (P) (M:  $P = 0.019$ ; P:  $P = 0.04$ , paired t-tests) (Fig 5b, c). Similarly, the relative aerial sensitivity of *Eurycea* spp. increased between 100 and 500 Hz (14 to 29 dB) (Fig 5d). From 100 to 500 Hz, aerial thresholds of adult *N. viridescens* were uniformly lower than aquatic thresholds (average across frequencies = 16.3 dB) and 1.8 dB lower at 600 Hz (Fig 5e). In larvae *N. viridescens*, the relative aerial sensitivity increased between 100 and 500 Hz (11.5 to 20.7 dB).

The difference in between-media carryover between *A. means* and the smaller species was evident whether analyzed using pressure or acceleration. Expressed relative to pressure, post-hoc tests revealed differences between *A. means* and *A. talpoideum* at 100 Hz and 500 Hz. At 100 Hz, the relative aerial sensitivity of *A. means* was 14.2-16.9 dB higher than *A. talpoideum* (M-P; M:  $P = 0.03$ , Tukey HSD; P:  $P = 0.0007$ , Tukey

HSD) (Fig. 6a). At 500 Hz, the pattern was reversed, with relative aerial sensitivity of *A. talpoideum* (M) 21.6 dB higher than *A. means* ( $P = 0.006$ ). Expressed relative to particle acceleration, the difference between *A. means* and *A. talpoideum* (P) at 100 Hz remained ( $P = 0.026$ ). At 500 Hz, *A. means* differed from both metamorphosed *A. talpoideum* ( $P = 0.001$ ) and also *N. viridescens* ( $P = 0.035$ ) (Fig. 6b).

#### *Developmental comparisons in N. viridescens and A. talpoideum*

Neither *N. viridescens* nor *A. talpoideum* exhibited marked changes in auditory function across metamorphosis. The aerial sensitivities of larval *N. viridescens* were overall not significantly different from aquatic adults or efts ( $F_{2,19}=0.15$ ,  $P = 0.87$ ) (Fig. 7a). Larval and adult *N. viridescens* were equally sensitive to aquatic sound ( $F_{1,15} = 0.07$ ,  $P = 0.80$ ) (Fig. 7b). Aerial sensitivities of metamorphosed adult *A. talpoideum* were similar to paedomorphic individuals ( $F_{1,16}=0.02$ ,  $P = 0.89$ ) (Fig. 8a), but aquatic thresholds were overall 7 dB above paedomorphic individuals ( $F_{1,12} = 6.49$ ,  $P = 0.026$ ) (Fig. 8b). The interaction term was non-significant ( $F_{5,60} = 2.14$ ,  $P = 0.07$ ), but the threshold difference between the groups ranged from 2.7 dB at 100 Hz up to 10 dB at 600 Hz.

## **Discussion**

### *Methodological considerations*

Both aquatic and aerial testing conditions were characterized by high particle motion conditions, which are not ideal for testing pressure-based hearing mechanisms.

Such conditions could potentially mask auditory variation between species that, if tested under lower particle motion conditions, could be attributable to pressure-to-particle motion transformers (e.g., compare Christensen-Dalsgaard et al. 2010 and Christensen et al. 2015a). Audiogram profiles collected from a speaker stimulus can vary between studies, depending on the impedance conditions and the extent to which the animal detects pressure at different frequencies (Popper and Fay 2011).

Our experimental design did not allow us to distinguish between the contributions of pressure and particle motion to the auditory response. A recent study examined the issue of the relevant stimuli in *Ambystoma*, finding aquatic pressure sensitivity above 120 Hz (Christensen et al. 2015). In our study, pressure sensitivity could have contributed to the aquatic auditory responses, and presumably to varying extent for different species. In addition, we did not control air volumes in the oral tract, which could have produced variation in air-cavity based pressure sensitivity.

Sound-induced vibrations could be sufficient to explain aerial sensitivity in salamanders (Christensen et al. 2015). Christensen et al. (2015) recently found that sound-induced head vibrations at auditory threshold from an aerial speaker are comparable to vibration thresholds measured directly. Supporting this view, we have found that thresholds expressed relative to sound pressure can become elevated when the aerial speaker and salamander are oriented to minimize sound vibrations (i.e., suspending the speaker from the ceiling and reducing coupling between the salamander and the platform substrate) (unpublished data). Therefore, the aerial thresholds expressed relative to sound pressure in our study are likely much lower than would be expected if salamanders were tested at a distance of several meters from the speaker, where

vibrations would be much lower. In addition, while we did measure the levels of vertical table substrate vibrations, these are likely an underestimate of the total sound vibration received by the salamanders. For example, in a similar experimental setup, sounds from a speaker induced head vibrations in snakes that exceeded sound-induced table substrate vibrations (Christensen et al. 2012).

Given these limitations, our between-media comparisons (whether expressed relative to pressure or acceleration) are not meaningful measures of absolute sensitivity differences of the salamander ear to aquatic versus aerial sounds. Rather, their utility is in providing a more precise comparison of species differences across frequencies under the two experimental conditions. Despite these limitations, our comparisons of species differences have internal validity within this study because all groups were presented with a similar sound field. One exception is the different aquatic impedance conditions of *A. means*, which was taken into account by analysing results in terms of acceleration in addition to pressure.

### *Aquatic audiograms*

To our knowledge, this study is the first broad comparative analysis of aquatic audiograms in salamanders. The low thresholds at low frequencies parallels the low frequency sensitivity previously described for aerial audiograms (Wever 1985). In addition, detection of sounds up to 4 kHz in *A. talpoideum* (although at very high sound levels) suggests that high frequency hearing may be a broader characteristic of aquatic salamanders, which has also been described for *Proteus* cave salamanders (Bulog and Schlegel 2000). Our study's pattern of increasing thresholds with increasing frequency is

distinct from the U-shaped audiograms of *Proteus* with best sensitivities in the 1-10 kHz range (Bulog and Schlegel 2000). Among the few species tested in our study, there was no simple relationship between aquatic specialization and aquatic auditory sensitivity - the two taxa more strongly tied to aquatic habitat (*A. means* and *Eurycea* spp.) were not more sensitive, as a group, than the species showing life histories with higher levels of terrestrial activity (*N. viridescens* and *A. talpoideum*).

#### *Metamorphosis did not improve aerial sensitivity*

The similar aerial audiograms of paedomorphic and metamorphic stages of *N. viridescens* and *A. talpoideum* challenge the hypothesis that metamorphosis improves aerial auditory sensitivity in salamanders. Similarly, no effect of metamorphosis was found when comparing the aerial sensitivity of larval *Ambystoma mexicanum*, adult *A. mexicanum*, and adult, metamorphosed *A. tigrinum* (Christensen et al. 2015b). These observations of conserved auditory function across development are paralleled by the retention of the lateral line and the lack of development of additional auditory nuclei across metamorphosis in the majority of salamanders that have been examined (Fritsch et al. 1988). The developmental changes to salamander auditory systems across development (Table 1) may be functionally redundant. This developmental trajectory contrasts the more salient anatomical and functional changes to the auditory system found in anurans, where additional auditory nuclei develop along with the tympanic middle ear and aerial sensitivity increases (Simmons and Horowitz 2007).

A study by Ross and Smith (1980) showed lower vibrational thresholds in adult *N. viridescens* relative to juveniles (efts) below 150 Hz, as measured by inner ear

microphonics in response to horizontal vibrations. The same study found that vibrational sensitivity, with sensitivity peaks near 100 Hz, could be attributed to responses from the saccule otoconial mass (Ross and Smith 1980). We noted a lowering of aerial thresholds at 100 Hz in a subset of adults *N. viridescens*, but it is likely that this effect was due to the unique positioning of these individuals rather than a continued growth of the saccule otoconial mass improving the low frequency response (Appendix 4). In either case, assuming that the opercularis system is fully developed in efts, this difference would not be attributable to the opercularis system.

The auditory function of the opercularis complex in salamanders remains to be demonstrated definitively (reviewed by Mason 2007 and Capshaw and Soares 2016). Our results challenge its presumed significance for terrestrial auditory function, which is supported by additional observations by others. Hetherington (1989) found that cutting the opercularis muscle of ambystomatids resulted in only a 4 dB threshold elevation, while Wever (1985) found that tensing the opercularis muscle in *Ambystoma* reduced microphonic responses. Cutting the opercularis muscle in juvenile *N. viridescens* and *Plethodon cinereus* had no effect on horizontal whole body vibration sensitivity and only a minor threshold elevation in adult *N. viridescens* (Ross and Smith 1980).

The expectation that soft muscle tissue can effectively transmit vibrations is not intuitive, but would be bolstered if it is tonic muscle (Hetherington et al. 1986). However, the tonic nature of the opercularis muscle in salamanders is questionable, since in *Ambystoma* the opercularis muscle consists of just 9% tonic fibers spanning 4% of the cross sectional area of the muscle (Hetherington and Tugaoen 1990). This contrasts with the 57% found in *Lithobates catesbeianus* (Hetherington and Tugaoen 1990). While the

opercularis complex has a clear low frequency auditory function in some anurans (Lombard and Straughan 1974), this role does not appear to be broadly applicable to salamanders.

Despite evidence against a role for the opercularis in terrestrial auditory function, a non-functional explanation based on constraint is not satisfying either, since there are at least two additional independent evolutionary events that link the opercularis system to terrestriality. First, the unique opercularis muscle in plethodontids, the *m. cucullaris* rather than the usual *m. levator scapulae* suggests an independent origin of the opercularis complex in this group (Hetherington et al. 1986). Second, a terrestrial-specific function for the opercularis complex is supported by the absence of the opercularis in aquatic *Xenopus* frogs (Mason et al. 2009), which otherwise exhibit advanced auditory features developing across metamorphosis (i.e., development of columella and a cartilaginous tympanic disk as well as additional auditory nuclei).

An alternative hypothesis regarding the function of the opercularis system may be in buffering pressure changes caused by large fluid motions through the ear associated with terrestrial locomotion or ventilation (Mason 2007). Fluid motions through the ear could be large during terrestrial locomotion because the ears are coupled to the cranial cavity via the perilymphatic foramen (Smith 1968; Wever 1978). Relative motion between the skull and body produces fluid motion in the ear (Smith 1968).

*Aerial sensitivity of terrestrial P. glutinosus is comparable to sensitive aquatic species*

*P. glutinosus*, the only strictly terrestrial species examined, was more sensitive to aerial sound than *N. viridescens* and *A. means* at 400-600 Hz, but comparable in

sensitivity to two other aquatic salamanders (paedomorphic *A. talpoideum* and *Eurycea*). At least in this species, a terrestrial niche is not associated with aerial function that is significantly augmented or reduced compared to the more sensitive aquatic salamanders. Terrestrial *Plethodon* fossils have been found in the lower Miocene (Tihan and Wake 1981) and a recent molecular phylogeny places *Plethodon* diversification at ~40 Ma (Shen et al. 2016).

The few comparative anatomical and neurophysiological studies on this topic also fail to provide support for specialized aerial function in terrestrial salamanders. First, *Bolitoglossa*, another direct-developing plethodontid, shows no increase in the complexity of auditory nuclei (Roth et al. 1993), though there is a loss of the lateral line and its associated nuclei (Fritzschn 1989). The only comparative study of aerial function in salamanders, to our knowledge, is by Wever (1985), which did not reveal a marked difference in aerial sensitivity between terrestrial *Aneides* and *Plethodon* spp. and more aquatic species. However, the ability of that study to detect differences is limited by the small number of individuals tested per species and biases in the microphonic potential method related to hair cell orientations (Manley 1990, p. 5-6).

#### *Role of lungs in amphibious hearing*

Body wall vibrations over the lungs are hypothesized to contribute to the aerial auditory pathway in salamanders, as has been demonstrated for anurans (Hetherington and Lindquist 1999; Hetherington 2001). The low thresholds of the lungless plethodontids in the 300-600 Hz range show that lungs are not prerequisites for aerial sensitivity. Although this result does not disprove such a pathway in lunged species

(experimental manipulations would be needed), it suggests that lungs and their associated body wall vibrations may not be critical for aerial auditory function in salamanders. In addition, although the body walls over the lungs of *Notophthalmus* and *Ambystoma* were shown to vibrate in response to aerial sounds, the peak frequencies of vibration (~ 1 kHz) are much higher than the best sensitivity range of the audiograms collected in this study (< 500 Hz) (Hetherington 2001).

Lung vibrations can facilitate aquatic sound pressure detection in salamanders (Christensen et al. 2015b). *Eurycea*'s aquatic audiograms were similar to lunged species, suggesting that lacking a lung does not constrain aquatic sensitivity. However, this interpretation must be considered in light of the high particle motion conditions in our experimental chambers. Under lower particle motion conditions, the sensitivity of *Eurycea* and other aquatic lungless salamanders could be restricted compared to salamanders that can detect pressure via the lung (e.g., *Ambystoma*, Christensen et al. 2015b). Nonetheless, the aquatic auditory sensitivity of *Eurycea* (comparable to *A. talpoideum* and *A. means*, and more sensitive than *N. viridescens*) suggests that auditory function via direct vibration detection (bone conduction) could be useful to lungless aquatic salamanders.

#### *Diverse between-media carryover*

Compared to its aquatic audiogram, *A. means* aerial sensitivity was retained at low frequencies, but restricted at high frequencies. In contrast, the smaller salamanders retained a degree of aerial sensitivity over a wider bandwidth, and in *A. talpoideum* and *Eurycea* spp., the relative aerial sensitivity at 400-500 Hz was greater than at 100-200

Hz. These results indicate that the carryover of auditory function between aquatic and aerial media can vary markedly between species.

We hypothesize that the poor carryover of high frequencies in *A. means* may be due in part to the large body size associated with thicker tissue layers and skull, which would block sound transmission to the ear. Reduced sensitivity with increasing body size is expected for non-tympanic aerial hearing (Hetherington 1992 a,b) and would be particularly evident at higher frequencies, which are more impeded by mass. Although mass and SVL were not statistically significant covariates in our analysis, the average mass of *A. means* was 40 times larger than the next largest species (*A. talpoideum*). The significance of small size permitting high frequency aerial sensitivity is also suggested by the high sensitivity of small *Eurycea* at 400-500 Hz. Underwater, body size would be a less consequential constraint on sensitivity due to the similar acoustic impedances of animal tissue and water. The low frequency sensitivity of *A. means* provides a further indication that the opercularis system is not needed to enhance low frequency aerial sensitivity.

#### *Hypotheses about the regressive evolution of the basilar papilla in caudates and caecilians*

Under a temnospondyl monophyly hypothesis of Lissamphibia origins, salamanders could have had ancestors with tympanic middle ears (Anderson et al. 2016). Both caecilians and urodeles show a pattern of loss of the basilar papilla in more derived taxa (Lombard 1977; Fritzschn and Wake 1988), which could be an indication that the function of the basilar papilla is dependent on a tympanic middle ear (Smotherman and Narins

2004). Since high frequency aerial sound detection is dependent on possession of a tympanic middle ear, this dependence could be explained if the basilar papillae of salamanders and caecilians are tuned to higher frequencies, similar to the condition in anurans. An additional prediction of this hypothesis would be that loss of the basilar papilla is associated with terrestrial niches, since high frequency aquatic sound detection would not necessarily require a tympanic middle ear.

The few data available on this topic do not support an exclusive association between possession of the basilar papilla and high frequency aquatic hearing, nor between the loss of the basilar papilla and terrestrial niches. Among the species tested in our study, *Ambystoma* was most responsive to high frequencies and has a well-developed basilar papilla. However, *Amphiuma means* also possesses a basilar papilla, but did not respond above 1.5 kHz. *Eurycea* lacks a basilar papilla, yet responded to aquatic sounds up to 2 kHz. In addition, the olm (*Proteus* spp.) has a high frequency aquatic audiogram (Bulog and Schlegel 2000), but no basilar papilla has been described for this genera (Bulog 1989) or in the other proteid genus, *Necturus* (Lombard 1977). The absence of the basilar papilla in the strictly aquatic *Siren* and *Necturus* suggests that loss of the basilar papilla is not strictly associated with terrestriality (Lombard 1977).

#### *Implications for early tetrapod hearing*

Like salamanders, early amphibious tetrapods could have detected aquatic pressure via lungs and lacked tympanic ears. The salamander ear has also been compared to microsaur (Lepospondyli) due to its short, blunted stapes and an 'accessory otic element' similar to the operculum (Clack and Allin 2004). However, salamanders possess

many derived features that would be advantageous for extending the upper limits of aerial hearing without a tympanic ear (Anderson et al. 2016; Christensen et al. 2016). In particular, aquatic-aerial carryover and aerial non-tympanic function would be impacted by small body size, lightweight columella (stapes) and/or operculum, and the possession of inner auditory receptors without an otolithic covering.

The absence of these factors in early tetrapods would have restricted high frequency aerial sensitivity and promoted low frequency sensitivity, potentially similar to the condition described for *A. means*. Lungfish, another living model for early vertebrate hearing, lack some of the derived auditory structures possessed by salamanders and have higher aerial thresholds and a more restricted aerial bandwidth than salamanders (Christensen et al. 2015a,b). Many early tetrapods were much larger than the salamanders tested in this study and possessed very large stapes (Clack 2012). The presence of non-otolithic receptors in early tetrapods is unknown, since they do not fossilize well (Clack and Allin 2004). Examining extant taxa, lungfish possess only otoconial receptors and a closed otic capsule (Platt et al. 2004), while the coelacanth *Latimeria* has a non-otolithic receptor in the ear resembling a basilar papilla (Fritzsche 1987; Fritzsche 2003; Bernstein 2003).

Our study shows that even within a non-tympanic taxon with members sharing many auditory traits, the between-media carryover of auditory function may be quite variable. The upper frequency limit of aerial sensitivity in small salamanders, as exemplified by *A. talpoideum* and *Eurycea* spp., is likely much higher than many early tetrapods. These salamanders are a useful model for the traits that promote high

frequency carryover (several hundred Hz) across an aquatic-terrestrial boundary without a tympanic ear.

### *Conclusions*

Our study suggests that metamorphic changes do not play a major role in modifying aerial sensitivity of salamanders. The moderate sensitivity of *P. glutinosus* fails to provide evidence for the evolution of heightened aerial sensitivity in terrestrial salamanders. Aerial sensitivity is significantly correlated to aquatic sensitivity, but the degree of the carryover across media may be quite variable for different species. Our study agrees with the concept of salamander terrestrial hearing being ‘better than a fish on land’ (Christensen et al. 2015b), but also highlights considerable variation in aerial performance across the phylogeny.

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## Tables

**Table 1** Major modified and conserved traits in the salamander auditory system and lateral line across metamorphosis

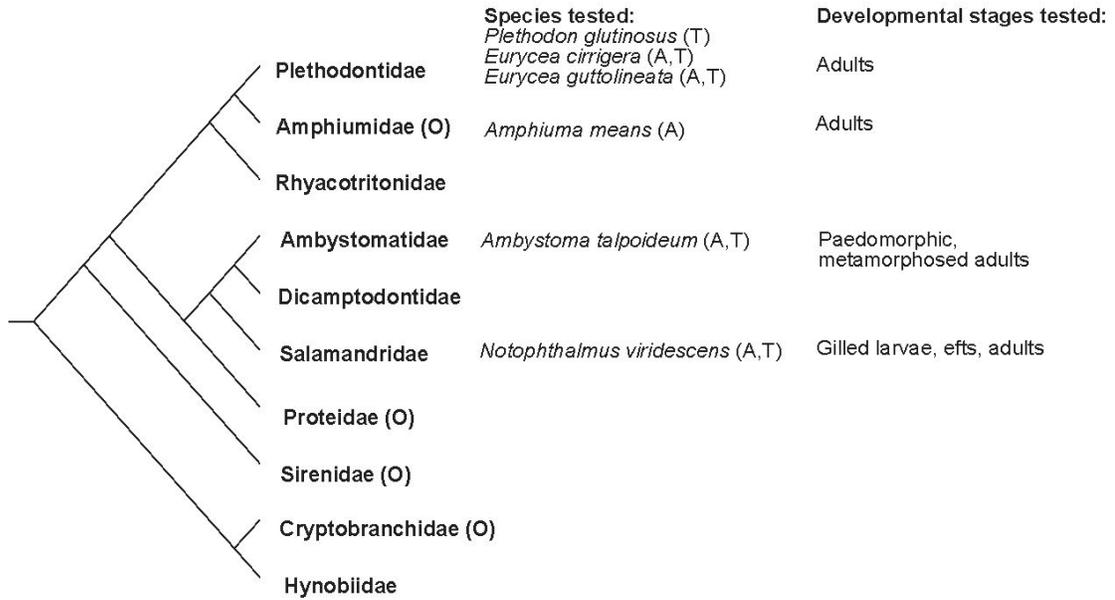
Octavolateralis trait	References
<i>Modified</i>	
○ Development of opercularis system	Kingsbury and Reed 1909; Monath 1965
○ Fusion of columella with skull (some ambystomatids, salamandrids)	Monath 1965; Christensen et al. 2015b
○ Spatial relationships in inner ear: <ul style="list-style-type: none"> <li>▪ Location of junction between periotic canal and periotic cistern changes (ambystomatids, dicamptodontids)</li> <li>▪ Orientation of amphibian papilla switches from vertical to horizontal position</li> <li>▪ Periotic cistern protrudes toward oval window (<i>Notophthalmus</i>)</li> </ul>	Lombard 1977
○ Loss of lateral line neuromasts ( <i>Chioglossa</i> , <i>Salamandra salamandra</i> )	Fritzsich 1988
<i>Conserved</i>	
○ Lateral line retained	Fritzsich 1988
○ Conserved structure of octavolateralis nuclei in brainstem	Fritzsich et al. 1988

**Table 2** Body mass and SVL of species tested

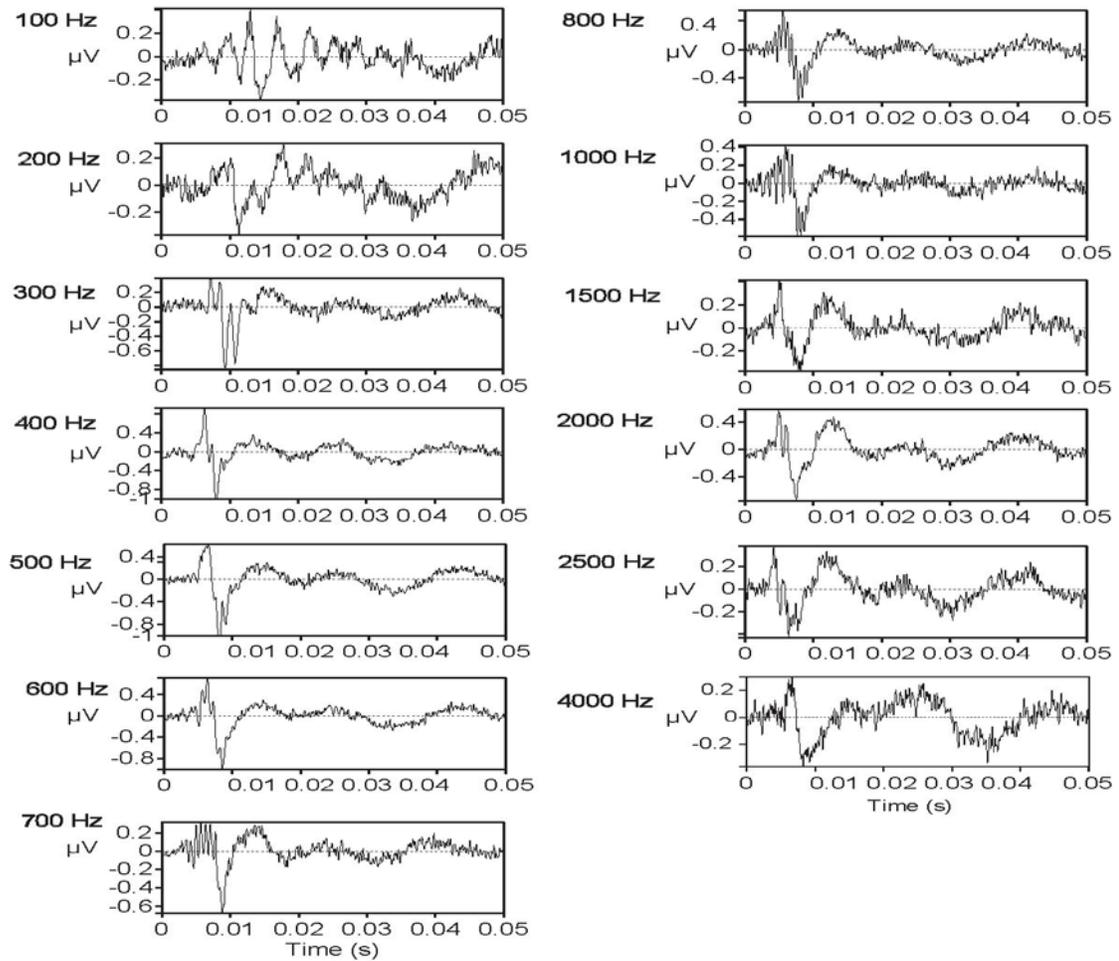
Scientific name	Developmental stage	Mass (g) (mean±SD)	SVL (mm) (mean±SD)
<i>Notophthalmus viridescens</i>	Larva	0.4 ± 0.2	20.5 ± 4.6
	Terrestrial juvenile (eft)	0.5 ± 0.4	20 ± 3.2
	Adult	4.3 ± 1.3	50 ± 3.8
<i>Ambystoma talpoideum</i>	Late stage larva and paedomorphic adult	3.7 ± 2.0	43.2 ± 5.8
	Metamorphosed adult	5.7 ± 2.1	50.7 ± 5.5
<i>Amphiuma means</i>	Adult	230 ± 116	460 ± 80
<i>Plethodon glutinosus</i>	Adult	4.3 ± 2.1	59 ± 14
<i>Eurycea cirrigera</i>	Adult	1.1, 1.1 (n = 2)	40, 42
<i>Eurycea guttolineata</i>	Adult	3.1 (n = 1)	N/A

## Figures

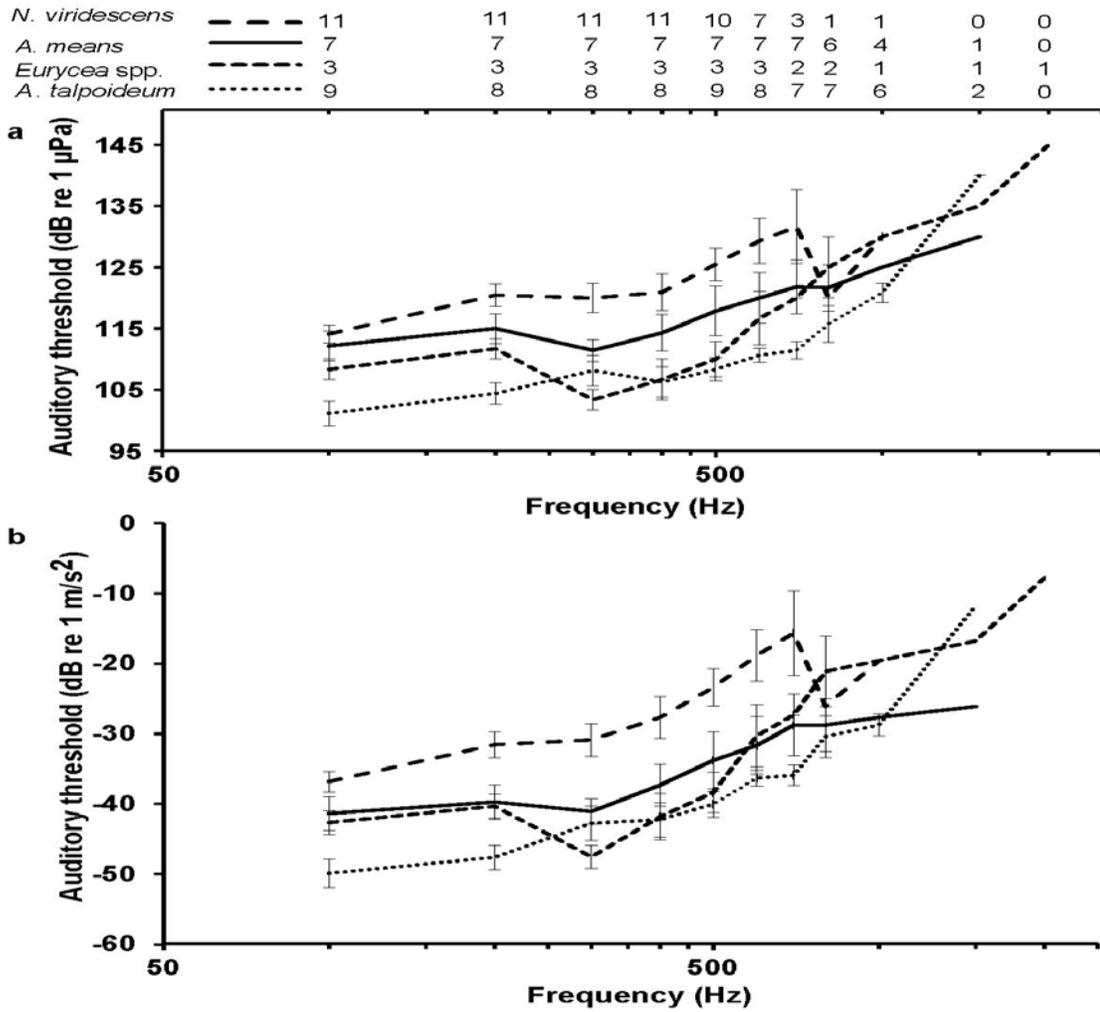
**Fig. 1** Family level salamander cladogram after Larson et al. (2006) indicating the species tested, their developmental stages, and ecological grades on the aquatic-terrestrial spectrum. O = obligate aquatic, paedomorphic families, A = aquatic, T = terrestrial



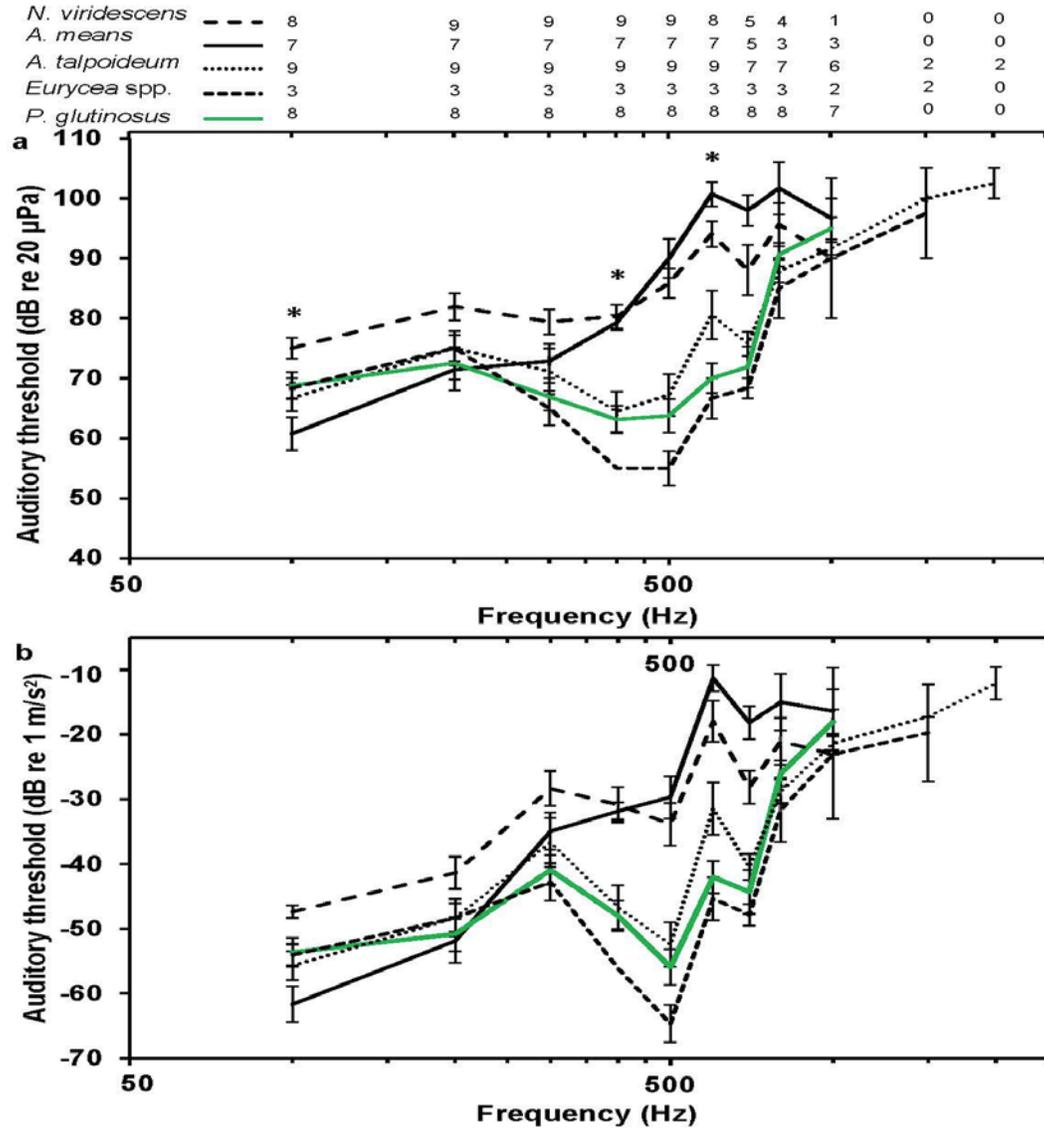
**Fig. 2** Example aquatic AEP waveforms at 100-4,000 Hz at 10 dB above auditory threshold collected from a metamorphosed *A. talpoideum*. The response at 4,000 Hz was at threshold rather than 10 dB above due to the high sound levels producing sound artifacts in the trace



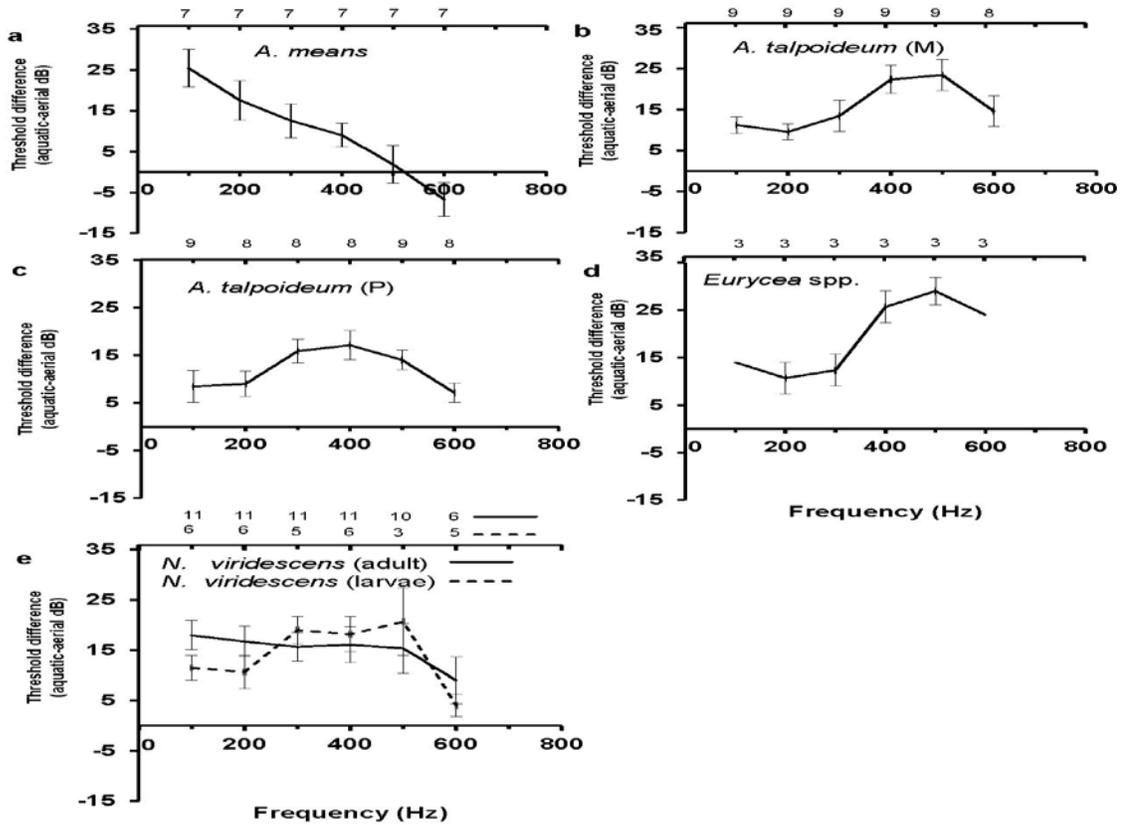
**Fig. 3** Underwater audiograms expressed relative to aquatic pressure (**a**) and particle motion (**b**) (mean  $\pm$  SEM). The number of individuals tested for each species across frequencies are indicated above the graph in (**a**)



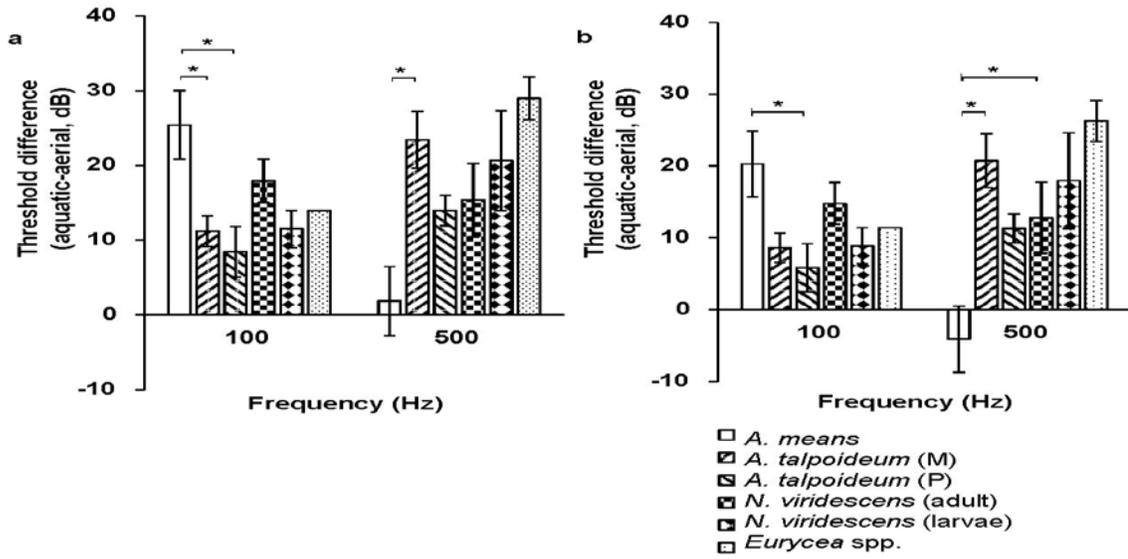
**Fig. 4** Aerial audiograms expressed relative to pressure (a) and vertical table vibrations (b) (mean  $\pm$  SEM). The number of individuals tested for each species across frequencies are indicated above the graph in (a)



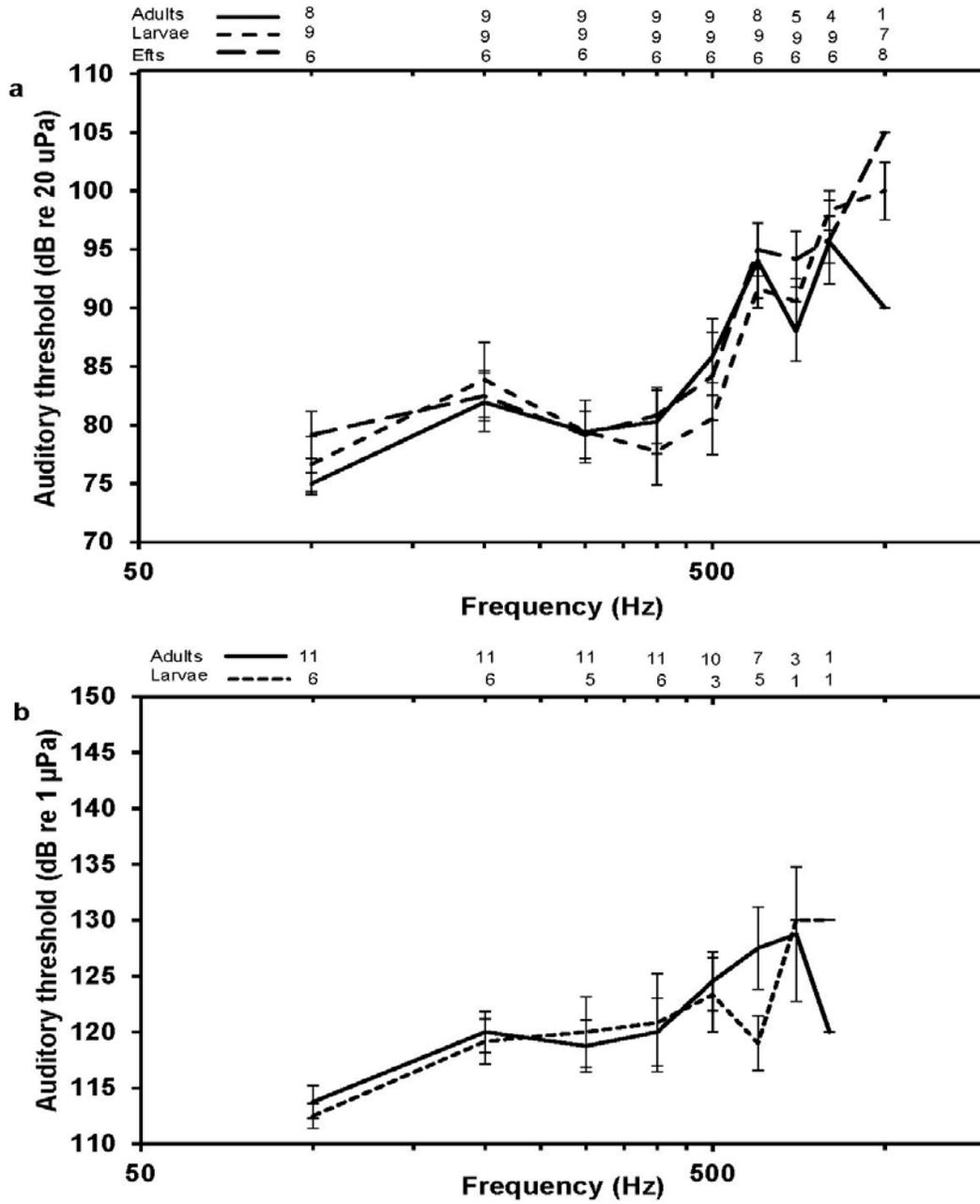
**Fig. 5** The difference between aquatic and aerial pressure thresholds in each species (a-e) (mean  $\pm$  SEM). The number of individuals tested for each species across frequencies are indicated above the graphs. ‘P’ and ‘M’ indicate paedomorphic and metamorphosed *Ambystoma talpoideum*, respectively



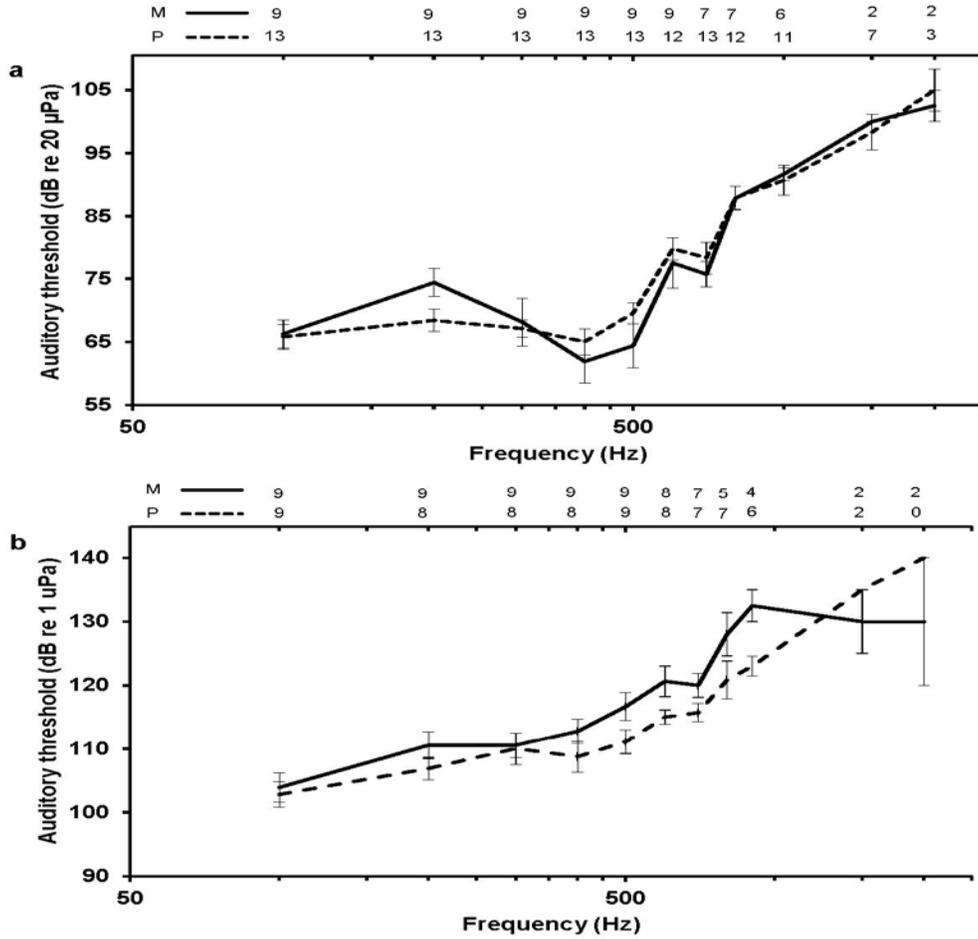
**Fig. 6** The difference between aquatic and aerial thresholds at 100 Hz and 500 Hz in each species, expressed relative to (a) pressure and (b) particle acceleration (mean  $\pm$  SEM)



**Fig. 7** Auditory thresholds expressed relative to pressure for adult, larval, and eft stage *N. viridescens* (mean  $\pm$  SEM) in response to tones from the aerial speaker (a) and under water speaker (b). The number of individuals tested for each species across frequencies are indicated above the graphs



**Fig. 8** Auditory thresholds expressed relative to pressure for paedomorphic (P) and metamorphosed (M) *A. talpoideum* (mean  $\pm$  SEM) in response to tones from the aerial speaker (a) and under water speaker (b). The number of individuals tested for each species across frequencies are indicated above the graphs



## **Chapter 4 - Do body wall vibrations facilitate aerial hearing in lunged salamanders?**

### **Abstract**

Lung-based aerial hearing pathways have been demonstrated in anurans that lack tympanic middle ears. These pathways could function more broadly in small, terrestrial tetrapods. In salamanders, the body walls overlying the lungs vibrate in response to airborne sound, indicating a potential for lung-based inputs to contribute to aerial auditory sensitivity. The current study tested the hypothesis that aerial hearing in lunged salamanders is aided by sound-induced vibrations of the body walls over the lungs. Using evoked potentials, I compared auditory sensitivities of two lunged species, *Notophthalmus viridescens* and *Ambystoma talpoideum*, before and after immersing the body walls and pectoral girdle in a foam material submerged below a water surface. I predicted that the resistance provided by the water on the body wall, by obstructing body wall vibrations communicated to the ear, would elevate auditory thresholds. In both species, sensitivities remained unchanged following this treatment. The results fail to support the hypothesis of a substantial lung-based aerial auditory pathway through body wall vibrations in salamanders. While lungs promote low frequency vibration of peripheral structures in small tetrapods, the extent to which these vibrations are coupled to and detected in the ear varies across taxa.

## Introduction

Tympanic middle ears are significant innovations in terrestrial hearing that are thought to have evolved independently in each major tetrapod lineage (Clack 1997; Christensen-Dalsgaard and Carr 2008). These structures greatly enhance the ability to detect airborne sound by facilitating sound transmission across the acoustic impedance mismatch between air and inner ear fluid. Some tetrapods lack tympanic middle ears and exhibit reduced aerial sensitivity, usually restricting sound detection to low frequencies (Hetherington and Lindquist 1999; Christensen et al. 2015; but see Boistel et al. 2013). These taxa provide an opportunity to understand the proximate factors that limit aerial auditory function in the absence of a tympanic middle ear.

Extra-tympanic inputs are involved in the aerial hearing of anurans (Hetherington 1992; Mason 2007). Lung-based resonance is one aspect of extra-tympanic function in anurans promoting aerial hearing in both ‘eared’ (possessing a functional connection between tympana and stapes) and ‘earless’ species (lacking a functional tympanum-stapes connection) (Ehret et al. 1994; Lindquist et al. 1998; Hetherington and Lindquist 1999). In ‘eared’ species, lung-based inputs can be detected on the vibrational responses of the eardrum, indicating a functional relationship between lung resonance and sound transmission through the tympanic middle ear (Narins et al. 1988; Jorgensen 1991; Ehret et al. 1994).

In ‘earless’ species, lung-based inputs can increase sensitivity by up to 20-25 dB (Hetherington and Lindquist 1999). Some ‘earless’ species can use lung-based and/or mouth cavity-based inputs to augment auditory sensitivity at high frequencies, up to 3-5 kHz (Lindquist et al. 1998; Boistel et al. 2013). The pathways connecting lung-based

vibrations to the ear in ‘earless’ species are not well understood. Hypothesized routes for sound flow include vibrations moving along spinal cord, through cranial fluid, and entering the ear through the endolymphatic and perilymphatic foramen, vibrations entering through a thinned tissue layer at the roof of the pharynx, and vibrations entering through the opercularis system (Hetherington and Lindquist 1999; Boistel et al. 2013).

The contribution of lungs to extra-tympanic auditory pathways can be understood by modeling the frequency-dependent impedances of the peripheral auditory system. Sound transmission through an object (or auditory system) is maximized at its resonant frequency ( $\omega_{\text{res}}$ ), which is determined by mass ( $m$ ) and compliance ( $c$ , the inverse of stiffness):  $\omega_{\text{res}} = \sqrt{1/(c \times m)}$  (Mason 2016a). The resonant frequency is lowered by increasing either mass or compliance. In addition, for a simple resonator, sound transmission is improved primarily by increasing compliance below the resonant frequency and by reducing mass above the resonant frequency (Mason 2016a). The frequency-dependent response of a tympanic middle ear can be modelled by adding together the mass and compliance contributions from the tympanic membrane, auditory ossicles, and middle ear cavity (Mason 2016a). Air in the middle ear cavity contributes a compliance term that varies in relation to cavity volume. For example, the enlargement of the middle ear cavity in desert rodents is considered an adaptation to improve low frequency hearing (Mason 2016a, b).

By increasing the compliance of peripheral auditory structures, the air spaces of lungs and mouth cavities would improve low frequency sound transmission, analogous to the function of enlarged middle ear cavities. However, the unspecialized tissues surrounding the lungs would generally exert a much higher mass loading than a thin

tympanic membrane, which would limit the high frequency response to lower frequencies. At a certain point, tissue thickness would impede vibratory motion altogether. Therefore, increasing body size is expected to be associated with restricted ability to use lung-based extra-tympanic pathways (Hetherington 1992). The magnitude and frequency of peak vibrations on the body wall over the lungs are correlated with body size in anurans (Hetherington 1992), and the ‘earless’ anurans capable of detecting the highest frequencies (up to 3-5 kHz) are small (< 2 grams, Lindquist et al. 1998; Boistel et al. 2013).

Vibrations on the body wall over lungs have also been measured in salamanders and lizards, suggesting that lung-based auditory pathways could apply more generally to small terrestrial tetrapods (Hetherington 2001). In salamanders, tones played from an aerial speaker elicited vibration velocities at the anterolateral body walls (i.e., over the lungs region) in the eastern newt (*Notophthalmus viridescens*) and the spotted salamander (*Ambystoma maculatum*) (Hetherington 2001). These vibrations begin to rise at roughly 500 Hz and peak at 1-2 kHz, exceeding head vibrations by up to 25 dB (Hetherington 2001). In contrast, the body wall vibrations of plethodontids, a salamander family lacking lungs, remained low and similar in magnitude to head vibrations (Hetherington 2001). Although lung-based pathways currently are thought to play a role in the extra-tympanic auditory function in salamanders (reviews by Mason 2007 and Capshaw and Soares 2016), there have not been any follow-up studies testing whether these vibrations are effectively coupled to the ear.

I tested the hypothesis that vibrations of the body wall over the lungs facilitate aerial hearing in lunged salamanders. Previous studies in anurans have found that

applying silicone grease to the external body walls interfered with lung-based pathways (Jorgensen 1991; Hetherington and Lindquist 1999). In the ‘earless’ toad, *Bombina orientalis*, this treatment reduced auditory sensitivity (measured in the midbrain) by 20-25 dB (Hetherington and Lindquist 1999). In multiple ‘eared’ frogs (*Hyla versicolor*, *Hyla gratiosa*, *Rana temporaria*, and *Eleutherodactylus coqui*) this treatment reduced displacement amplitudes measured on the eardrum (Jorgensen 1991; Jorgensen et al. 1991). Using a similar approach, I obstructed body wall vibrations in two lunged salamanders, *Notophthalmus viridescens* (Salamandridae) and *Ambystoma talpoideum* (Ambystomatidae), by submerging the body wall and pectoral girdle underwater. I predicted that this treatment would elevate auditory thresholds measured with evoked potentials (AEPs).

## Materials and methods

### *Animals*

The study involved seven metamorphosed, adult *A. talpoideum* (mass:  $6.9 \pm 1.0$  g (SD); SVL:  $5.8 \pm 0.9$  cm) and seven non-gilled, adult *N. viridescens* (mass:  $4.1 \pm 1.0$  g; SVL:  $5.0 \pm 0.5$  cm). *A. talpoideum* were captured by seining and dip-netting in Conecuh National Forest in Covington Co., Alabama, USA. *N. viridescens* were captured from Tuskegee National Forest, AL, and Blue Hole Springs, Wacissa River, FL, USA. Auburn University IACUC approved the experiments (PRN 2013-2369).

### *Anesthesia for audiometry*

Salamanders were immersed in a buffered solution of 0.05-0.12 g/100 mL MS-222 prior to AEP testing, which produced a light level of anesthesia (an MS-222 bath was also used by Hetherington (2001) to measure body wall vibrations). Readiness for testing was determined by absence of a righting response, which typically took 20-30 min. As the trial progressed, heavy breathing or body movement would sometimes occur, which required re-bathing in MS-222. Since re-application of MS-222 occurred multiple times over the course of trials (within and between each treatment conditions), I re-tested thresholds in a subset of 6 salamanders at 200, 400, 600 and 700 Hz on a second occasion.

The extent of lung ventilation was not controlled over the course of the test, which could potentially introduce a source of variability. However, two observations suggest that varied extent of lung inflation did not produce significant changes in the measured auditory responses. First, I observed that, at the start of recording trials, the gular region was typically not conspicuously pumping, but as the trial progressed subtle gular pumping could be observed. The observable gular pumping did not coincide with a marked change in AEP amplitudes (examined on three occasions in *A. talpoideum*). Second, pressing the body wall over the lungs at the beginning of a trial did not have a marked effect on amplitude (examined in two *A. talpoideum*).

#### *Salamander-speaker arrangements*

Hearing tests, conducted within an audiometric booth (0.7 × 1.06 × 1.54 m, Tremetrics AR9S), involved two treatment conditions conducted in sequence on each individual. Separate containers and speaker arrangements were used for *A. talpoideum*

and *N. viridescens* (rationale described below). Each container was filled with water. Next, a layer of egg crate acoustical foam (3.5 cm depth), which was cut to the area of the container, was submerged and pressed underwater into the container such that air bubbles in the foam were expelled. The flat, back side of the foam was kept level with the water surface. In the first condition, subjects rested on top of the wetted acoustical foam with body walls unobstructed to the sound field ('on foam' condition). In the second condition, the body (pectoral girdle plus the posterior body) was submerged into a slit that had been cut through the foam, leaving only the head above the water surface, tilted slightly upward ('in foam' condition).

*A. talpoideum* were placed on the foam at the top of an aquarium ( $27.7 \times 22.7 \times 26$  cm) with the anterior-posterior body axis positioned parallel to the speaker face, 16 cm away. The top of the speaker (Dynex, model: DX-SP211) was suspended 17 cm from the chamber ceiling with craft pipe cleaner (Fig 1a). Underwater, the foam layer was propped to the surface with (bottom-to-top): an overturned plastic jar, two flat bricks, and a wood composite board that was nearly equal to the foam/tank area. The tank was placed on a table platform that was attached to the audiometric booth walls. A series of layered boards was placed on the table, under the tank (top-to-bottom): two layers of Styrofoam ( $28.5 \times 34.3$  cm, total thickness: 3.6 cm), a wood board (area  $41.5 \times 61$  cm, thickness: 1.8 cm), and a glass pane (area:  $32 \times 60.5$  cm, thickness: 6 mm).

The speaker arrangement and container used for testing *A. talpoideum* was not useful for testing *N. viridescens*, since using that setup for *N. viridescens* would have resulted in very high thresholds, particularly in the frequency range of interest for examining lung-based resonance effects (> 500 Hz). Overall, the *N. viridescens*

audiogram is less sensitive than *A. talpoideum* (Chapter 3), and pilot tests of two *N. viridescens* under the setup used to test *A. talpoideum* revealed very high thresholds (95 dB by 500 Hz). Therefore, *N. viridescens* were tested with a different speaker-subject arrangement, which produced lower thresholds that were similar to previous tests directly on the table (Chapter 3). This arrangement involved a smaller plastic container (5 × 10 × 15 cm) that rested on a layer of acoustical foam on the table platform. The speaker was at the same vertical level as *N. viridescens*, resting on another platform that was separate from the table platform (Fig 1b). *N. viridescens* were positioned on the foam with the anterior-posterior axis perpendicular to the speaker face, 14 cm away.

To further investigate the effects of speaker-subject arrangement on thresholds, the thresholds collected from these two speaker arrangements were compared to thresholds collected from individuals tested directly on the table. In *A. talpoideum*, the same seven individuals were tested again directly on the table platform. In *N. viridescens*, thresholds from ten different individuals tested directly on the table platform were compared to the original seven. For these additional tests taken with the salamanders directly on the table ('table-direct'), the position of these salamanders and the speaker was identical to that described above for *N. viridescens*, except that animals were placed directly on the table platform rather than resting on foam.

#### *Stimuli and record acquisition*

Tones were played from a speaker (Dynex, model: DX-SP211) at 100, 200, 300, 400, 500, 600, 700, 800 and 1,000 Hz at a rate of 8 Hz (duration: 10 ms, filter: 2 ms Hanning, rise time: 5 ms). Sounds were presented in 5 dB steps at initially supra-

threshold levels and lowered until the response dropped below the noise floor. Before each trial, sound pressure levels of tone stimuli were equalized across frequencies by taking measurements with a Pyle sound level meter (Pyle Audio, model PSPL01, sensitivity range: 40-130 dB, accuracy  $\pm 3.5$  dB at 1 kHz, 94 dB, C type frequency weighting) to create a normalization file that adjusted sound levels as appropriate for each test frequency.

The electrode set included a recording electrode placed subdermally above the vertex, a reference electrode placed posterior to the jaw, and a ground electrode placed in the tail. Electrodes connected to a Medusa pre-amplifier, which connected to a RA 16 processor, which connected to the computer running BioSig acquisition software (all from Tucker Davis Technologies, FL, USA). At each stimulus-frequency combination, two sets of traces were collected, each representing the averaged EEG response collected from 250 tone repetitions. Since each trace was collected in response to one of two opposing stimulus phases, when these two traces were averaged together it cancelled out stimulus artifact. Trace acquisition was bandpass filtered at 0.3-3 kHz and notch filtered at 60 Hz, and the sampling rate was 22.41 kHz. Auditory threshold was determined by visual inspection of the trace, defined as the lowest sound level where one or more peaks in the response were distinct from the baseline. If the peaks in the response at thresholds were uncertain, additional traces were taken above and below threshold to verify repeatability.

### *Statistical analysis*

Statistical tests of *A. talpoideum* involved two-way repeated measures ANOVAs including frequency and treatment ('on foam', 'in foam', 'table-direct') as within-subject factors. The comparison between 'on foam' and 'table-direct' thresholds in *N. viridescens* included speaker-subject position as a between-subjects factor. Statistics were conducted at 100-700 Hz and 100-500 Hz for *A. talpoideum* and *N. viridescens*, respectively. Above these frequency ranges, not all individuals responded. In *A. talpoideum*, one individual exhibited thresholds above 100 dB at 600 Hz, and two individuals exhibited thresholds above 100 dB at 700 Hz ('on foam'). Threshold in these individuals was designated as 100 dB to maximize the sample size for repeated measures tests. Statistics were conducted in SPSS.

## Results

*A. talpoideum* thresholds on the foam ranged from 82 at 100 Hz to 98 dB at 800 Hz, with a slight dip down to 79 dB at 400 Hz. Thresholds measured from the unobstructed and obstructed body walls conditions did not differ significantly ( $F_{1,6} = 4.8$ ,  $P = 0.07$ ; Fig. 2a). However, thresholds collected from the suspended speaker with salamanders on the foam were higher than those collected with salamanders placed directly on the table (unobstructed) ( $F_{1,6} = 23.1$ ,  $P = 0.003$ ). The largest threshold differences between the two arrangements occurred at 100 and 500 Hz (14 and 16 dB lower, respectively) and the smallest difference occurred at 700 Hz (4 dB, Fig 2b). Repeatability tests revealed mean threshold differences that overlapped zero at 200, 400, 600, and 700 Hz (Fig. 2b).

*N. viridescens* thresholds on the foam ranged from 67.5 dB at 100 Hz to 96 dB by 600 Hz. The aquatic body wall obstruction did not shift auditory thresholds (foam:  $F_{1,6} = 2.3$ ,  $P = 0.18$ ; Fig 2c), nor did placement directly on the table ( $F_{1,15} = 1.8$ ;  $P = 0.20$ ).

## Discussion

### *Lung-based hearing hypothesis not supported*

Auditory thresholds were unchanged following obstruction of the body walls, which fails to support the hypothesis that vibrations are coupled from the body walls over the lungs to the ear. Additional observations corroborate the view that body wall vibrations over the lung do not play a substantial role in the aerial hearing of salamanders. First, lungless plethodontids can exhibit comparable or higher aerial sensitivity relative to lunged species (Chapter 3). Second, the regions of best sensitivities in the *N. viridescens* and *A. talpoideum* audiograms (100- 400 Hz) are mismatched to the frequency range of peak body wall vibrations previously measured for *N. viridescens* and *A. maculatum* (Fig. 2d; Hetherington 2001).

Third, sound-induced head vibrations may be sufficient to explain aerial sensitivity (Christensen et al. 2015). A recent study found that at auditory threshold sound levels measured in relation to an aerial speaker stimulus, salamander head vibrations (approximated using head vibrations measured from pythons in a similar setup) are comparable to vibration thresholds measured directly with a shaker table (Christensen et al. 2015). In the present study, the threshold differences between the two speaker-subject arrangements in *A. talpoideum* ('on foam' versus 'table-direct') highlight

the need to measure salamander aerial hearing in terms of acceleration in addition to sound pressure (i.e., the higher thresholds in the ‘on foam’ arrangement are presumed to be due to lower vibration levels at the subject location in that setup).

Two objections could be offered concerning the ability of the current study to detect lung-based effects. First, blocking the body walls might not affect contributions of air in the lungs and mouth cavity to sound transmission through pathways that do not involve body wall vibrations. Second, although repeatability tests indicated a mean difference of approximately 0 between tests on different days, the variation indicates a limited ability to detect minor threshold differences between the treatments.

#### *Lung-based contributions to aerial hearing vary among small tetrapods*

Lungs contribute to a resonance response that can be measured from the overlying body walls in anurans, salamanders and lizards (Hetherington 2001), but the extent to which these vibrations affect auditory sensitivity varies across taxa. In lizards, the body walls over the lungs show a resonant response, but these vibrations have not been found to strongly couple to the ear, as indicated by lack of a vibrational peak measured on the tympanum corresponding to lung-resonance (Hetherington 2001; Christensen-Dalsgaard and Manley 2005). This contrasts the stronger lung-based inputs on the ear in ‘eared’ anurans (Narins et al. 1988; Jorgensen 1991; Jorgensen et al. 1991).

Salamanders share several auditory characteristics with ‘earless’ anurans, but unlike ‘earless’ anurans, they do not appear to rely on lung-based vibrations for aerial auditory function. In addition, the extent to which lung-based and mouth cavity-based resonance contributes to auditory sensitivity varies among ‘earless’ anurans. Both

salamanders and the ‘earless’ anuran, *B. orientalis* exhibit a high frequency peak in body wall vibrations (1 kHz or higher) that is mismatched with the regions of best sensitivity in the audiograms (100-300 Hz) (Hetherington and Lindquist 1999). This indicates that both taxa do not appear to make use of this high frequency vibration peak from the body wall. However, obstructing the body wall in *B. orientalis* elevated thresholds uniformly across frequencies (rather than peaking at 1 kHz), which could be linked to the overall greater vibrations measured from both the head and body walls of *B. orientalis* below 1 kHz (Hetherington and Lindquist 1999; Hetherington 2001).

In contrast to the mismatch found in *B. orientalis*, there is a tighter correlation between peripheral peak vibrations and auditory sensitivity in ‘earless’ *Atelopus* spp. and *Sechellophryne gardineri*. In *Atelopus* spp., the frequencies of displacement peaks measured on the body wall over the lungs correspond to midbrain audiogram sensitivities, with sensitivity peaks up to 2.5 kHz (Lindquist et al. 1998). In ‘earless’ *Sechellophryne gardineri*, both the resonance in the mouth cavities and the dominant vocalization pitch occur at approximately 5 kHz (Boistel et al. 2011; Boistel et al. 2013).

### *Conclusion*

The present study failed to support the hypothesis that body wall vibrations overlying the lungs are coupled to the ear to promote aerial sensitivity in salamanders. Although lungs can promote vibrational responses in the peripheral structures of small tetrapods, responsivity of peripheral structures does not necessarily imply characteristics of the audiogram (Ruggero and Temchin 2002). The extent to which lung-based vibrations are coupled to the ear varies within anurans, and substantial contributions from

the lungs to the ear have not yet been demonstrated in non-anuran tetrapods. This study highlights functional differences in the role of lung-based vibrations in aerial hearing of small tetrapods.

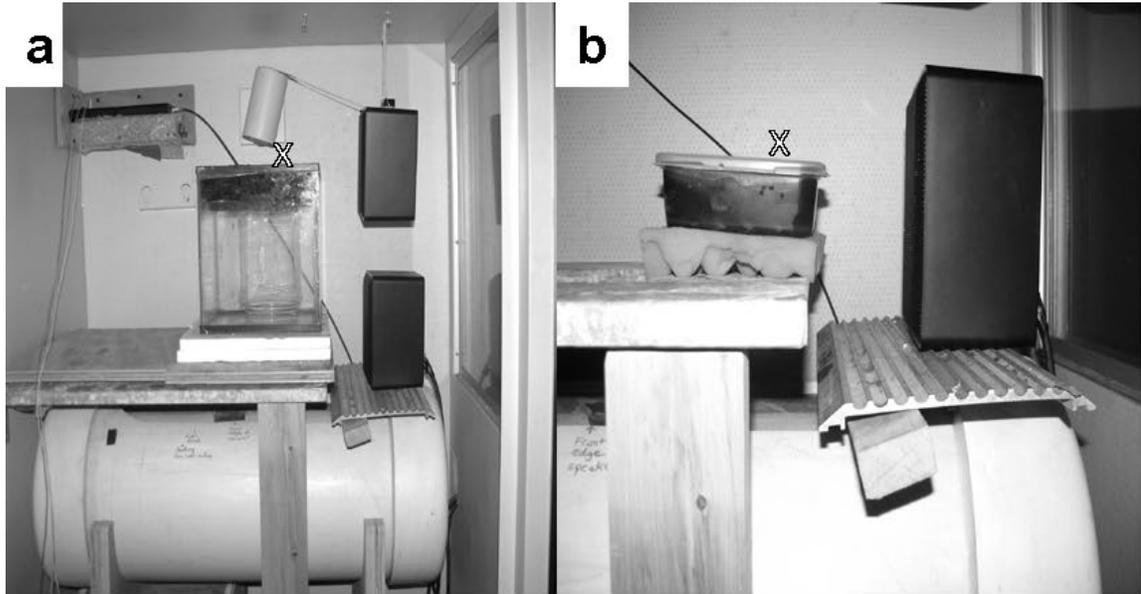
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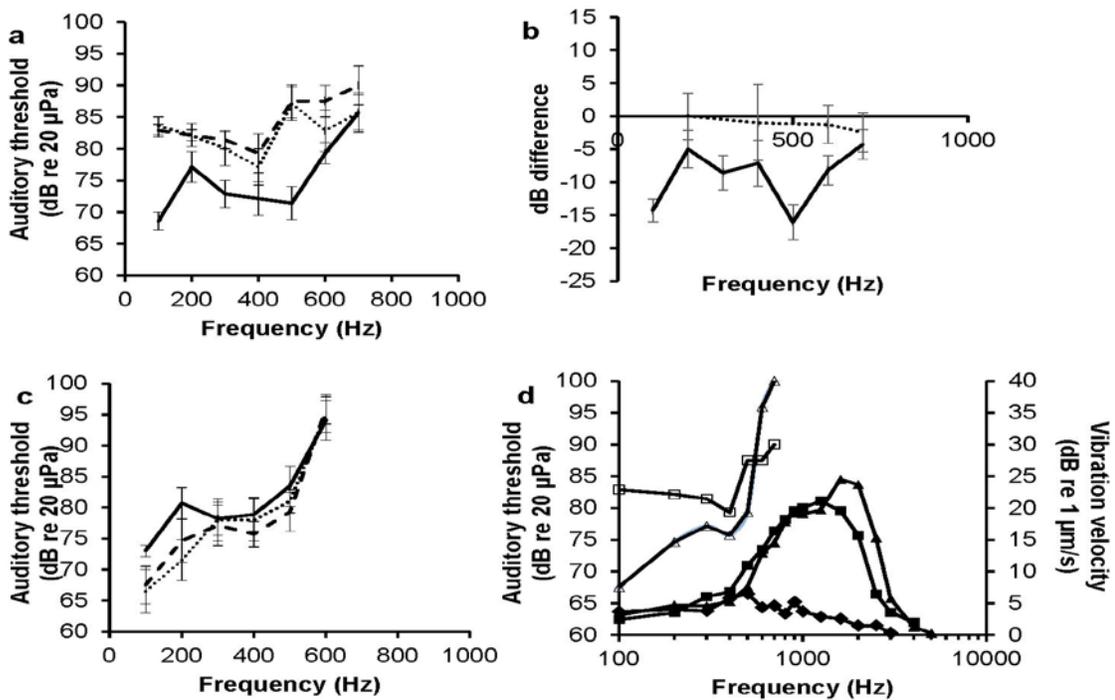
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## Figures

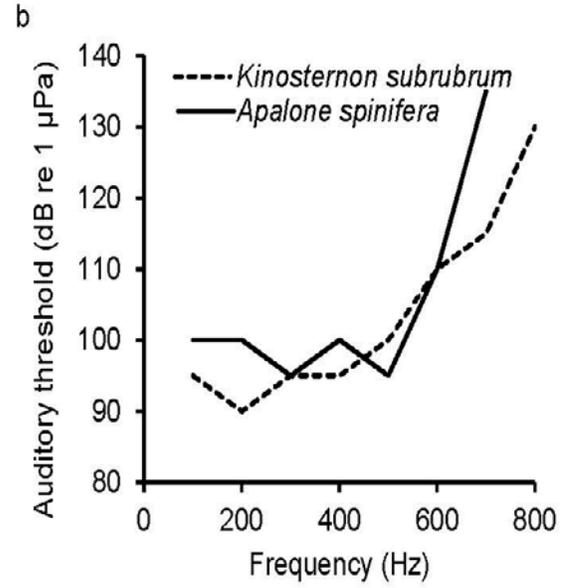
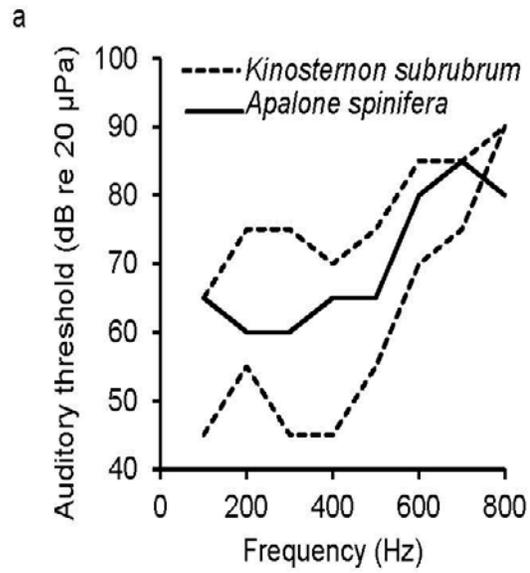
**Fig. 1** AEP testing chamber showing salamander positions in relation to the speaker for tests with *A. talpoideum* (a) and *N. viridescens* (b). The distance between the speaker and the head of the salamander (indicated with an 'x') in (a) is 16 and in (b) is 14 cm



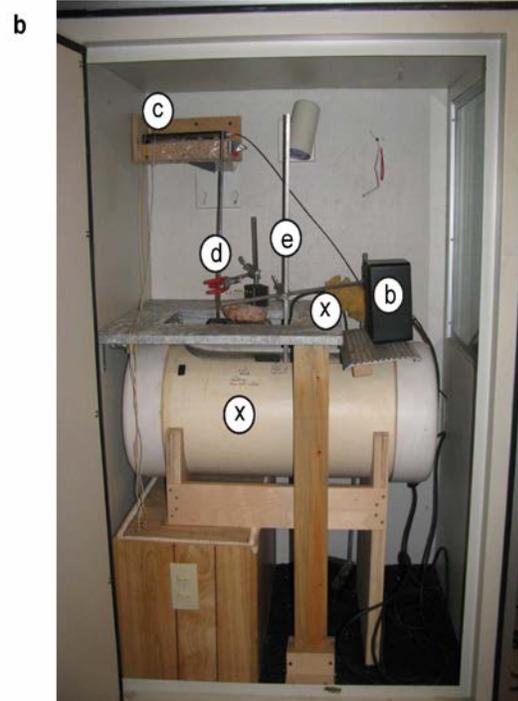
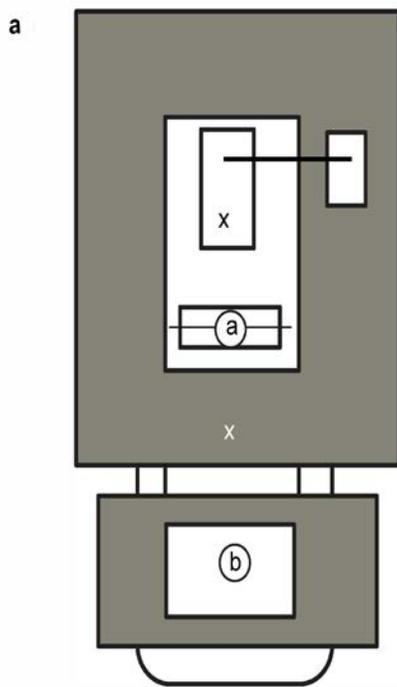
**Fig. 2** (a) *A. talpoideum* audiograms collected in the unobstructed body wall condition (dashed line), aquatic submersion condition (dotted line), and unobstructed condition placed directly on the table platform (solid line) (mean  $\pm$  SEM in all figures). (b) Difference between *A. talpoideum* thresholds collected directly on the table and on the foam (solid line) and the difference between repeated tests of individuals at 200 (n = 6), 400 (n = 5), 600 (n = 4), and 700 Hz (n = 4) (dotted line) in *A. talpoideum*. (c) *N. viridescens* audiograms on the foam (dashed line), in the foam (dotted line), and directly on the table platform (solid line). (d) ‘On foam’ audiograms of *N. viridescens* (open triangles) and *A. talpoideum* (open squares) overlaid on mean vibration velocities measured over the anterolateral body wall by Hetherington (2001) for *N. viridescens* (triangles) and *A. maculatum* (squares, single individual), and lungless *Desmognathus fuscus* (diamonds).



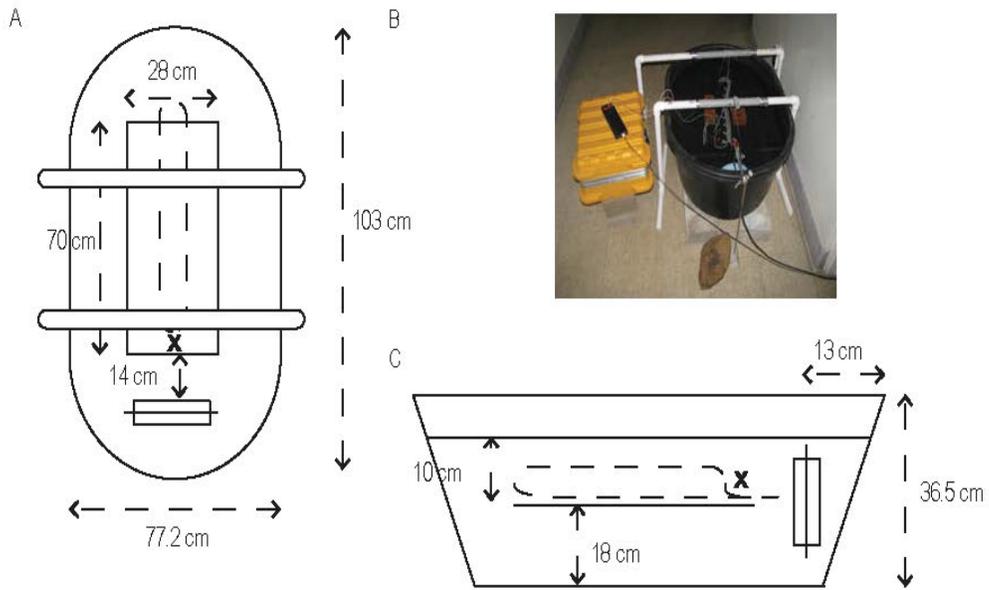
**Appendix 1** Additional audiograms from individuals of *Kinosternon subrubrum* and *Apalone spinifera*



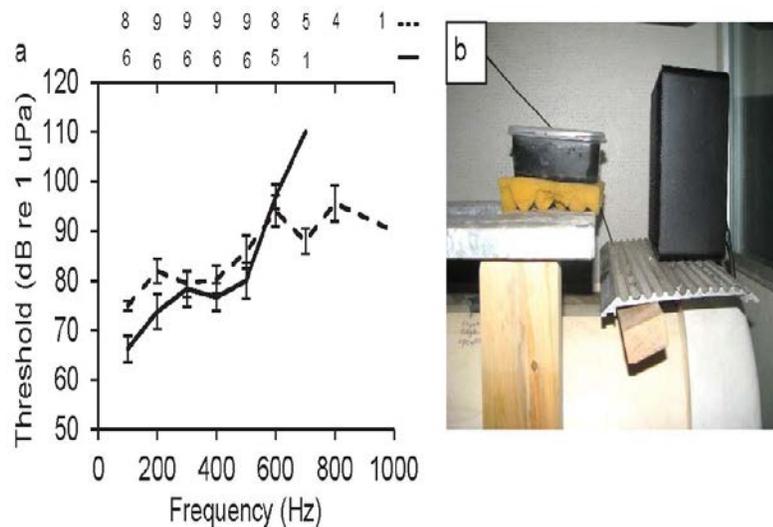
**Appendix 2** Top view (a) and side view (b) of the experimental chamber and equipment used to collect salamander auditory evoked potentials. 'X's mark the locations of the salamander head in aquatic and aerial treatments. For scale in b, the distance from the floor of the chamber to the top surface of the table is 80 cm. *a* underwater speaker, *b* aerial speaker, *c* preamplifier, *d* stand for aquatic subject platform, *e* stand for aquatic speaker



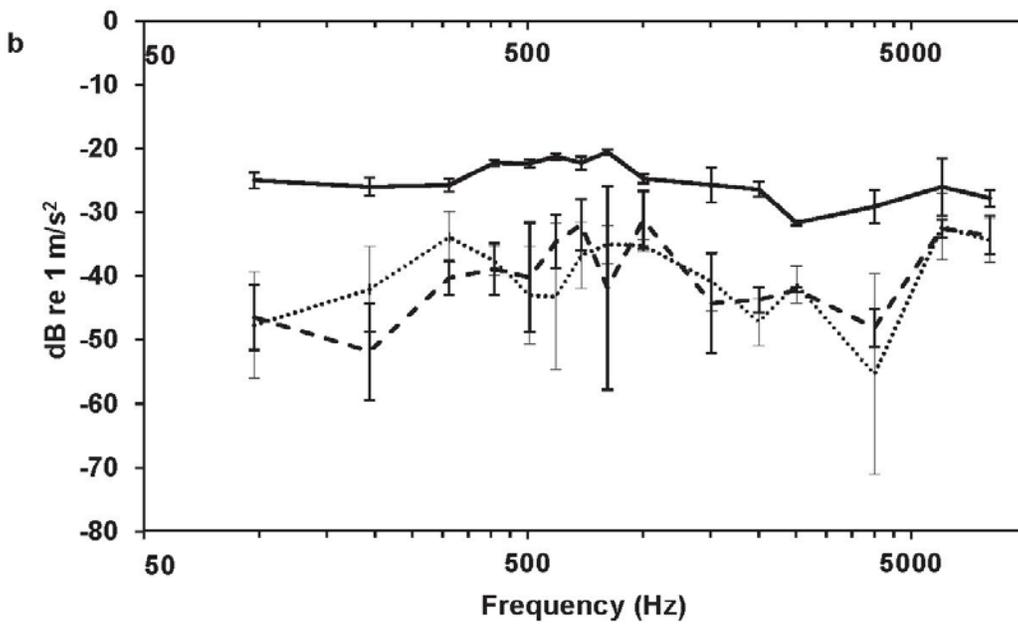
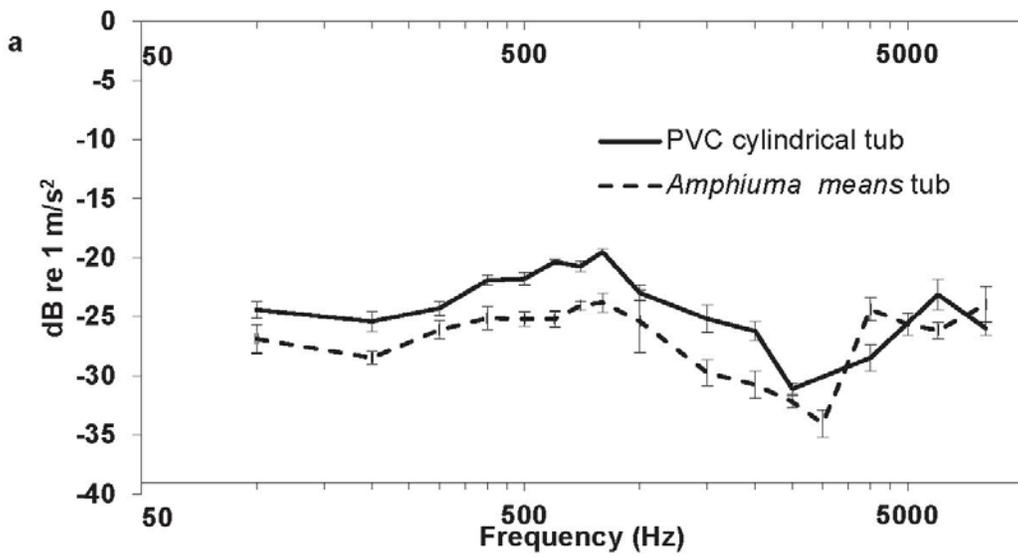
**Appendix 3** Top view (a), photograph (b), and side view (c) of the experimental tub used for audiometry tests of *Amphiuma means*. The “X” marks the location of the subject’s head.

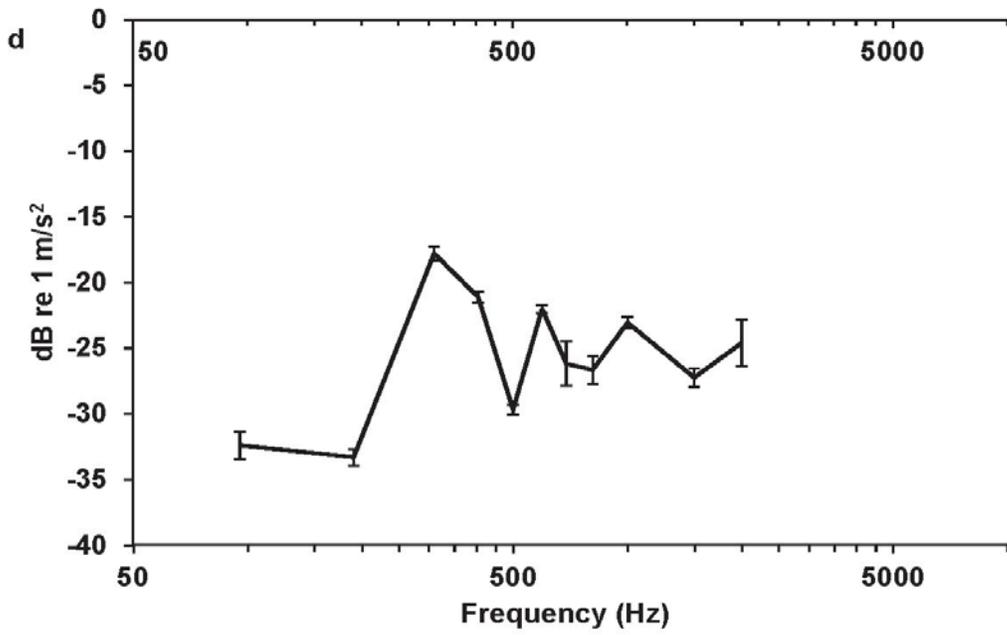
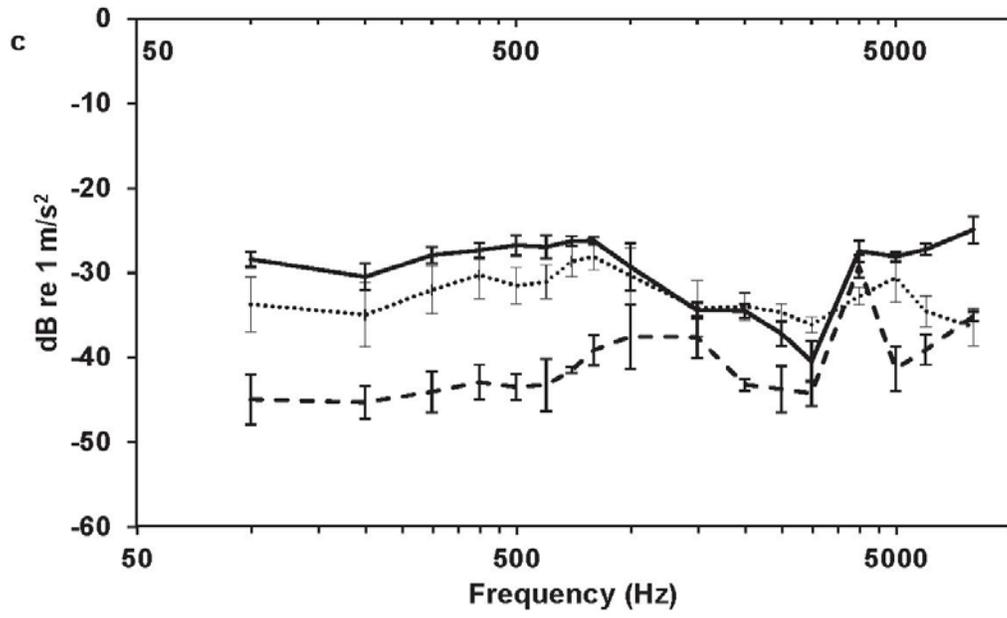


**Appendix 4** A subset of adult *N. viridescens* were tested on two layers of foam rather than directly on the table platform. The audiogram from this subset showed a trend for lower thresholds at 100-200 Hz (~8 dB lower; solid line = on the foam; dashed line = on the table platform) (**a**). In this configuration, a plastic container was filled with both water and a soaked layer of acoustical foam, which together rested on a second layer of acoustical foam on the table platform (**b**). This setup was used in conjunction with a separate experiment that involved submerging the body wall below the water surface to test contributions of body wall vibrations overlying the lungs to aerial hearing. Since this container configuration could have resulted in a larger vibrational stimulus at lower frequencies, individuals from this subset are removed from aerial analyses. However, since they make up a majority of the individuals tested in the aquatic condition, they remain in between-media analyses.

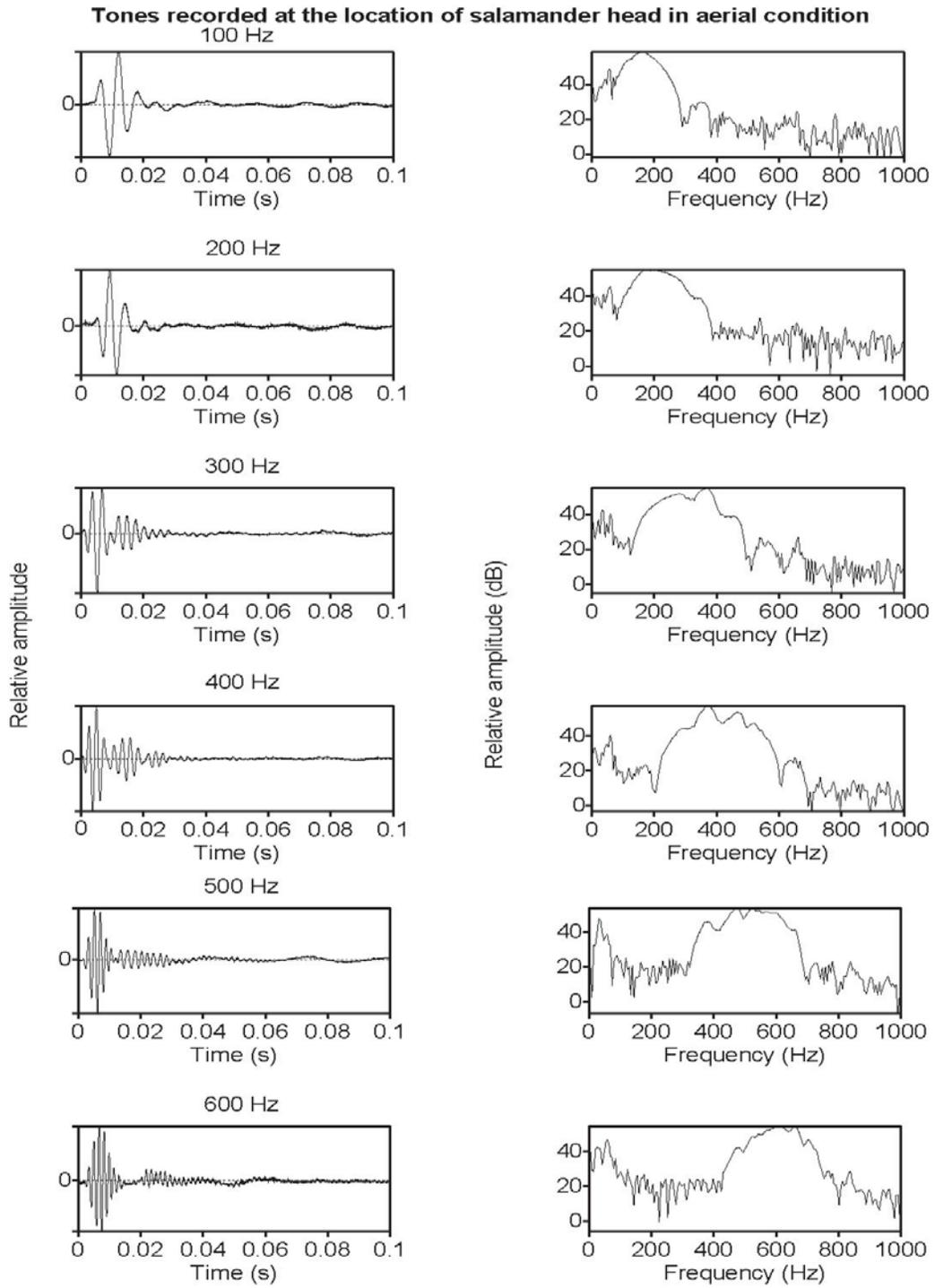


**Appendix 5 (a)** Particle acceleration (combined across three orthogonal axes) measured across test frequencies for tones calibrated to 126 dB rms re 1  $\mu$ Pa in the cylindrical chamber used for testing *N. viridescens*, *A. talpoideum*, and *Eurycea* spp. (solid line) and in the larger aquatic testing tub used for testing *A. means* (dashed line). **(b)** Particle acceleration in each axis dimension in the cylindrical chamber (vertical = dotted line, horizontal perpendicular to the speaker face = solid line, horizontal parallel to the speaker face = dashed line). **(c)** Particle acceleration in each axis dimension in the larger aquatic tub (formatting identical to **(b)**). **(d)** Vertical acceleration on the testing platform calculated from geophone velocity measurements in response to tones from the aerial speaker calibrated to 90 dB re 20  $\mu$ Pa. In all figure panels, values represent the mean  $\pm$  s.e.m. of three repeated occasions of calibration and measurement.

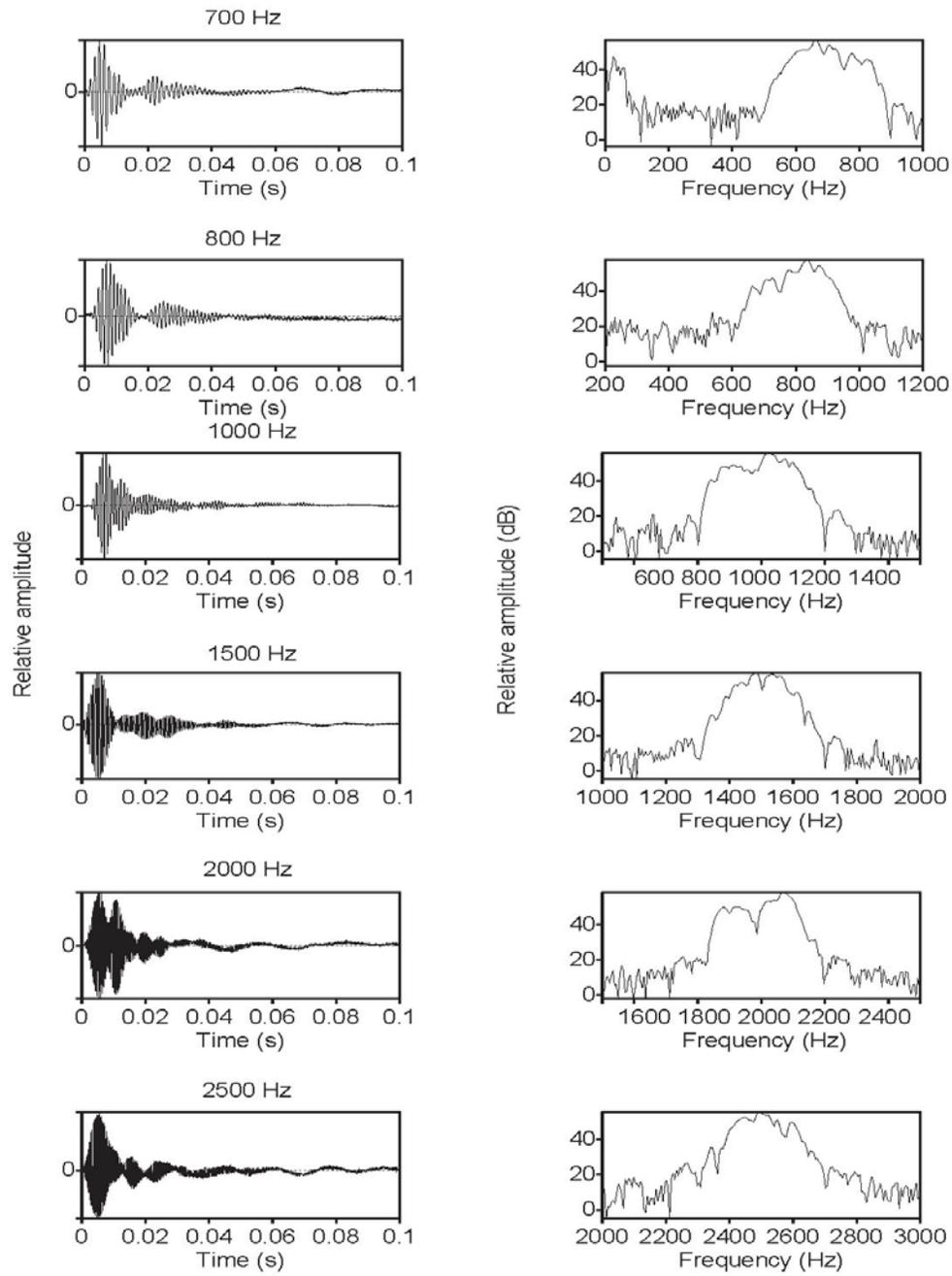




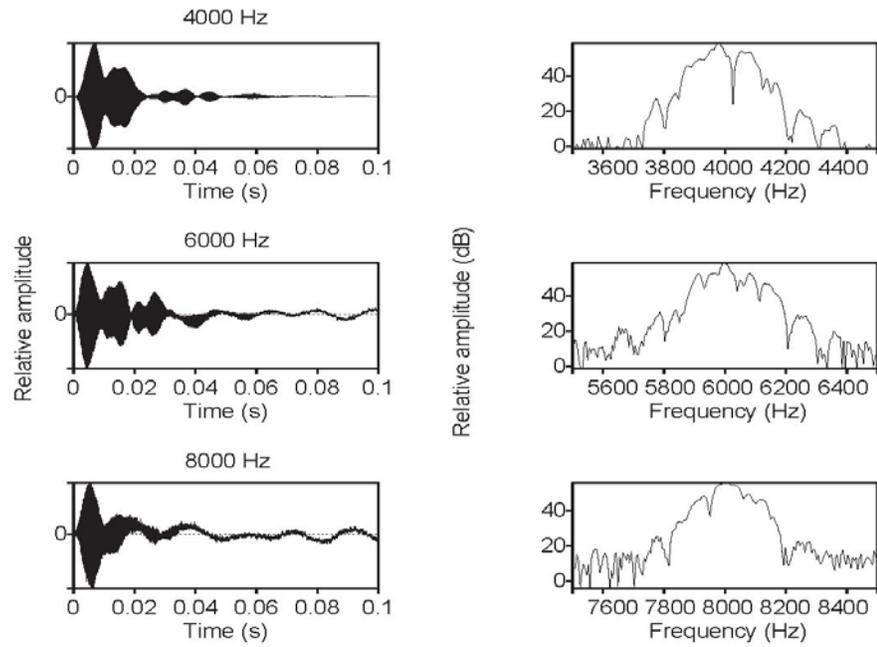
**Appendix 6** Waveforms and FFTs of 10 ms tone pip stimuli recorded at the location of the head in the aerial treatment



**Tones recorded at the location of salamander head in aerial condition (continued)**

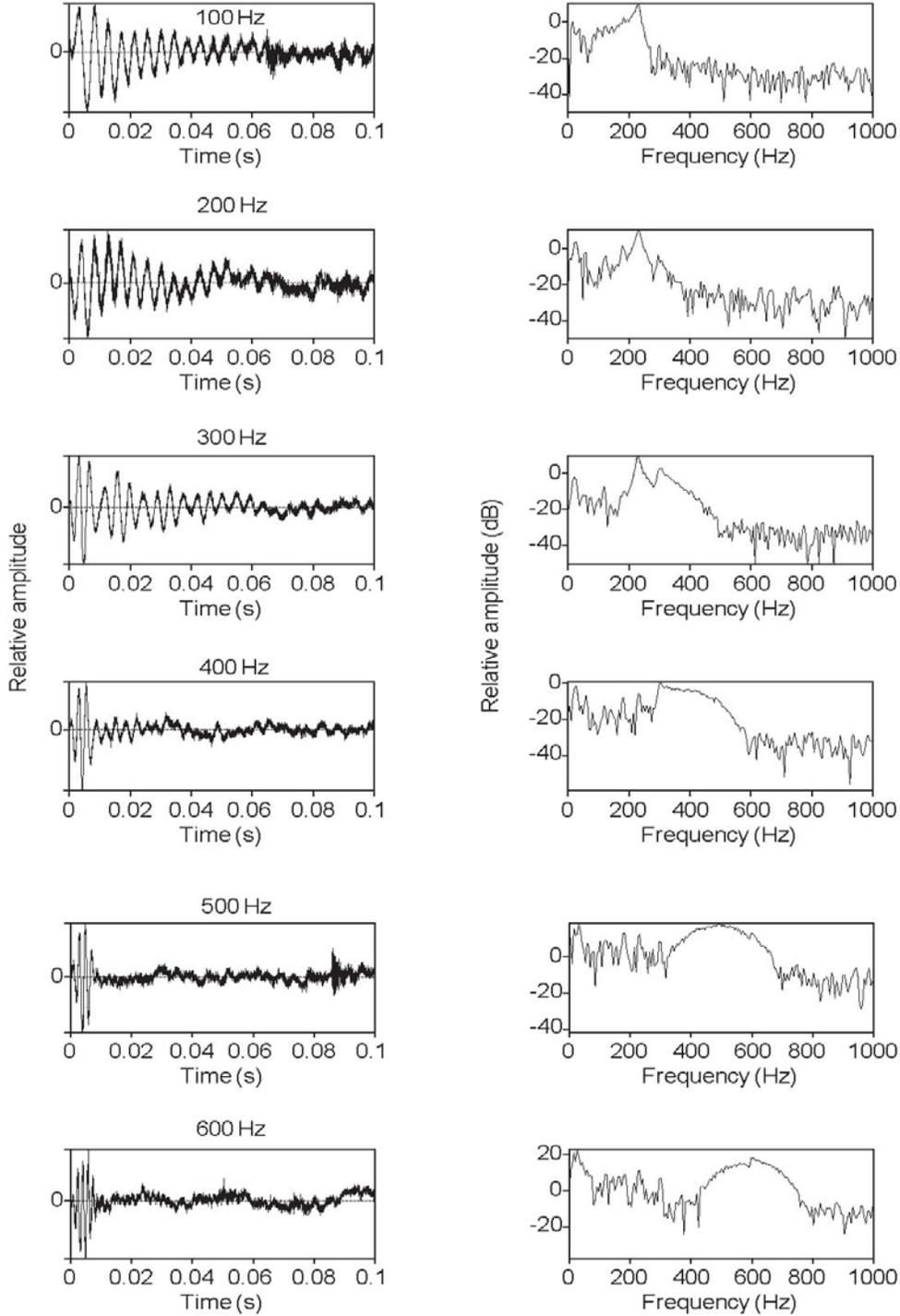


**Tones recorded at the location of salamander head in aerial condition (continued)**

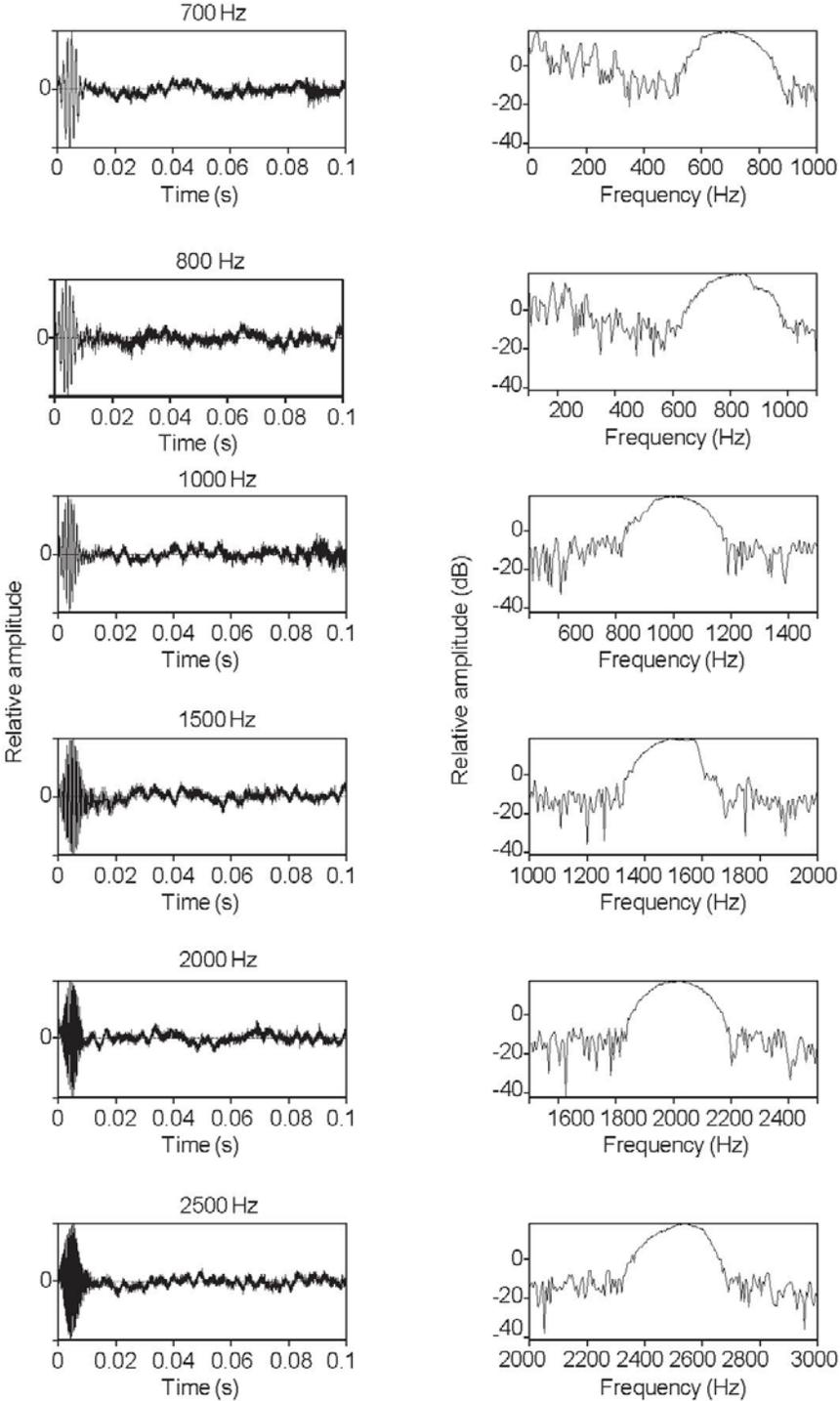


**Appendix 7** Waveforms and FFTs of 10 ms tone pip stimuli recorded at the location of the head in the aquatic treatment

**Tones recorded at the location of salamander head in aquatic condition (*A. means tub*)**



Tones recorded at the location of salamander head in aquatic condition (*A. means tub*) (continued)



Tones recorded at the location of salamander head in aquatic condition (*A. means tub*) (continued)

