

## RESEARCH ARTICLE

# Hearing of the African lungfish (*Protopterus annectens*) suggests underwater pressure detection and rudimentary aerial hearing in early tetrapods

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**ABSTRACT**

In the transition from an aquatic to a terrestrial lifestyle, vertebrate auditory systems have undergone major changes while adapting to aerial hearing. Lungfish are the closest living relatives of tetrapods and their auditory system may therefore be a suitable model of the auditory systems of early tetrapods such as *Acanthostega*. Therefore, experimental studies on the hearing capabilities of lungfish may shed light on the possible hearing capabilities of early tetrapods and broaden our understanding of hearing across the water-to-land transition. Here, we tested the hypotheses that (i) lungfish are sensitive to underwater pressure using their lungs as pressure-to-particle motion transducers and (ii) lungfish can detect airborne sound. To do so, we used neurophysiological recordings to estimate the vibration and pressure sensitivity of African lungfish (*Protopterus annectens*) in both water and air. We show that lungfish detect underwater sound pressure via pressure-to-particle motion transduction by air volumes in their lungs. The morphology of lungfish shows no specialized connection between these air volumes and the inner ears, and so our results imply that air breathing may have enabled rudimentary pressure detection as early as the Devonian era. Additionally, we demonstrate that lungfish in spite of their atympanic middle ear can detect airborne sound through detection of sound-induced head vibrations. This strongly suggests that even vertebrates with no middle ear adaptations for aerial hearing, such as the first tetrapods, had rudimentary aerial hearing that may have led to the evolution of tympanic middle ears in recent tetrapods.

**KEY WORDS:** Auditory evoked potentials, Early tetrapods, Auditory system, Vibration detection

**INTRODUCTION**

Hearing in the first tetrapods was most likely impaired by the impedance mismatch between air and tissue in their transition from an aquatic to a terrestrial lifestyle during the early Carboniferous. This problem may not have been solved until the convergent evolution of tympanic middle ears in all the tetrapod lineages during the early Triassic (Christensen-Dalsgaard and Carr, 2008; Clack, 1997; Clack, 2011). Consequently, there may have been a period of up to 100 million years when early tetrapods were unable to hear aerial sound. Lungfish are the closest living relatives of the tetrapods

(Liang et al., 2013) and the auditory system of recent lungfish is considered to be the best available model for the auditory systems of early tetrapods such as *Acanthostega* (Clack, 2002; Clack, 2011). Experimental studies on the sensory capabilities of lungfish are therefore relevant for discussing the hearing capabilities of early tetrapods before the appearance of the tympanic middle ear and for understanding the selection pressures that drove the evolution of aerial hearing via tympanic middle ears.

Sound travels almost unhindered into the inner ears of animals underwater as the impedance of tissue is comparable to the impedance of water. Accordingly, differential inertial movements of otoliths or otoconia relative to the hair cells enable detection of particle motion in the auditory systems of fish (Popper and Fay, 2011; Sand and Karlsen, 2000). Compressible air volumes coupled to the inner ear enable pressure detection that confers auditory advantages as it may lead to increased sensitivity and a broader frequency range of the auditory system (Chapman and Sand, 1974; Fay and Popper, 1974; Popper and Fay, 2011). Transformation of pressure waves into detectable particle motion can be accomplished by gas-filled cavities like swim bladders or lungs that provide up to two orders of magnitude more particle motion than the surrounding water when ensonified by a pressure wave (Alexander, 1966). The auditory gain from pressure-to-particle motion transduction is highly dependent on the distance and mechanical coupling between the gas-filled cavities and the inner ears (Kalmijn, 1988). Hence, gas-filled bullae close to the ears, swim bladder extensions and Weberian ossicles are known to enhance the transmission of the converted particle motion to the sensory epithelia in the inner ears and thereby increase hearing sensitivity (reviewed in Braun and Grande, 2008). Nonetheless, even species with no specialized connection between the swim bladder and the inner ear, such as the European eel (*Anguilla anguilla*), have been shown to obtain an auditory gain from the pressure-to-particle motion transduction of air cavities in their swim bladder (Jerkø et al., 1989). Similarly, air cavities in the lungs of lungfish may also be hypothesized to work as pressure-to-particle motion transducers and provide lungfish with an auditory gain at higher frequencies (>100 Hz).

In contrast to vertebrates in water, tetrapods face the challenge that the impedance of tissue is much higher than the impedance of air, and most of the sound energy is therefore reflected when it impinges on animals in air. In most tetrapods, this problem is solved by the tympanic middle ear, which converts aerial sound pressure into detectable particle motion in the endolymph of the inner ears to provide stimulation of the papilla end organs. Lungfish are, however, completely unadapted to aerial hearing having no middle ears and a closed otic capsule containing otolith organs only (Platt et al., 2004). They may therefore be hypothesized to be unable to detect aerial sound pressure. However, vibration sensitivity has been

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shown to enable atympanic vertebrates such as snakes and likely also salamanders to sense airborne sound through detection of sound-induced head vibrations (Christensen et al., 2012; Christensen et al., 2015). It is therefore possible that lungfish are also able to sense airborne sound via a similar mechanism.

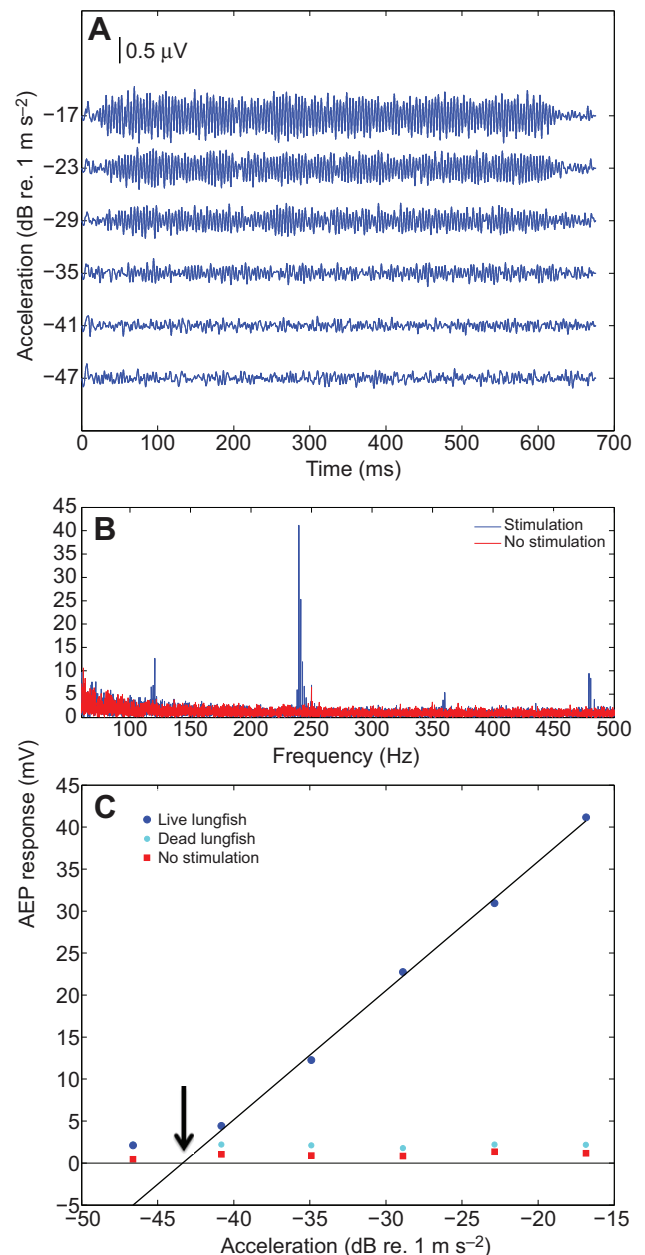
The only previous study of hearing capabilities of lungfish showed sensitivity to underwater particle motion and substrate vibrations in air, but apparently no sensitivity to either underwater or aerial sound pressure (Christensen-Dalsgaard et al., 2011). The inability to detect aerial sound pressure is consistent with the morphology of the lungfish auditory system. Still, it is surprising that lungfish cannot detect the underwater sound pressure utilizing the pressure-to-particle motion transduction by air in their lungs. The underwater experiments of the previous study (Christensen-Dalsgaard et al., 2011) were, however, probably conducted in excessive particle motion conditions in the near field of the underwater loudspeaker (Kalmijn, 1988; Parvulescu, 1964; Rogers et al., 2015). It is therefore possible that potential pressure detection was masked by excessive particle motion.

Here, we investigated the underwater hearing capabilities of lungfish in a standing wave tube where the ratio between pressure and particle motion can be manipulated. This enables determination and comparison of underwater hearing capabilities under both high particle motion and high pressure conditions. This setup allowed us to re-test the hypothesis that lungfish are sensitive to pressure by using their lungs as pressure-to-particle motion transducers. Additionally, we investigated the vibration detection and sound pressure hearing of the lungfish in air to test the hypothesis that lungfish can detect airborne sound. We show that lungfish detect underwater sound pressure in a frequency range matching the resonance frequency of the air volumes in their lungs. The morphology of lungfish shows no specialized connection between these air volumes and the inner ears and so our results imply that air breathing may have enabled pressure detection as early as in the Devonian era. Additionally, we demonstrate that lungfish in spite of their atympanic middle ear can sense airborne sound through detection of sound-induced head vibrations. This strongly suggests that even vertebrates with no middle ear adaptations to aerial hearing, such as the first tetrapods, likely had rudimentary aerial hearing that may have led to the evolution of tympanic middle ears in recent tetrapods.

## RESULTS

### Evoked potentials and threshold determination

Auditory evoked potentials (AEPs) were recorded from eight African lungfish, *Protopterus annectens* (Owen 1839), to determine AEP-derived thresholds for particle motion and pressure in water, and substrate vibrations and airborne sound in air. Pure tone stimulation resulted in an evoked potential response at twice the stimulation frequency (Fig. 1), and so sensitivity thresholds could be determined by monitoring the peak size of the second harmonic in the Fast Fourier Transform (FFT) of the evoked potential response (Egner and Mann, 2005). The evoked response was derived from the combined neural responses of 20 pure tone stimulations as measured by the FFT peak size relative to the noise floor measured in 20 periods of no stimulation. The FFT peak size at the second harmonic increased sigmoidally with stimulation, whereas no increase in FFT peak size was found for periods of no stimulation or in a dead animal used as a control (Fig. 1). The extrapolation (linear regression) of the steep part of the sigmoidal response function to the zero crossing defined the threshold (Mooney et al., 2010).



**Fig. 1. Neural response to 120 Hz pure tone stimuli of increasing intensity.** Pure tone stimulation resulted in a characteristic signal in the evoked potentials at twice the stimulation frequency that was used to determine sensitivity thresholds. (A) Average neural response underwater to 120 Hz pure tones, band pass filtered at 80 and 360 Hz by a second-order Butterworth filter. (B) Fast Fourier transform (FFT) of the neural response to 120 Hz tone of  $-17$  dB re.  $1 \text{ m s}^{-2}$  and to no stimulation. The FFT of the neural response to the tone stimulus shows a significant peak at twice the stimulation frequency, whereas the FFT of the neural response to no stimulation contains no such peak. (C) Auditory evoked potential (AEP) response as a function of acceleration stimulation. The FFT peak size at the second harmonic of the combined AEP response was found to increase with increasing stimulation intensity for tone stimulation (blue circles), but not for periods of no stimulation, in live animals. In comparison, no increase was seen in a dead lungfish used as control. Thresholds were defined as the zero-crossing of the regression line as indicated by the arrow.

### Sound detection underwater

The underwater experiments were conducted in a standing wave tube where the particle motion-to-pressure ratio of the underwater

**Table 1. Relative particle motion-to-pressure ratios of the sound field at recording depths**

	Frequency (Hz)						
	80	120	160	200	240	320	640
High particle motion depth (cm below the surface)	15	15	15	15	15	15	94
High particle motion (dB re. free field)	26	23	20	19	17	15	24
High pressure depth (cm below the surface)	85	85	85	85	85	94	56
High pressure (dB re. free field)	10	5	4	3	-2	-5	-4

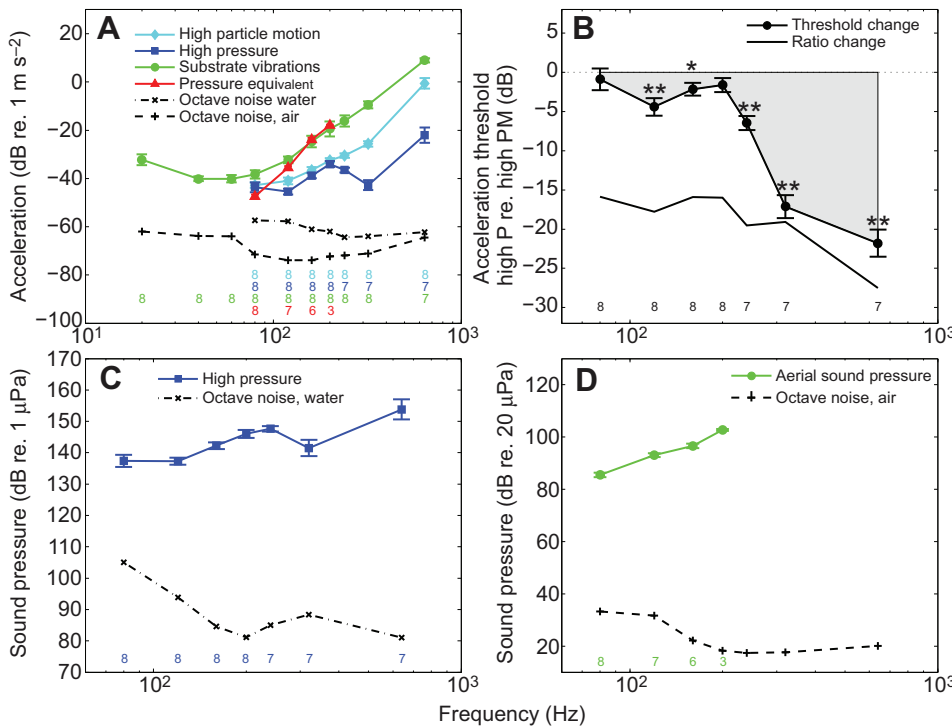
Data are recording depths and particle motion-to-pressure ratios relative to free field ratios under high particle motion conditions and high pressure conditions.

sound field varies with depth (Hetherington and Lombard, 1982; Hetherington and Lombard, 1983). Particle motion thresholds could therefore be determined at two depths with different particle motion-to-pressure ratios (Table 1; supplementary material Fig. S1). The sensitivity curve for underwater particle motion determined under high particle motion conditions showed best sensitivity at 80 Hz with a mean ( $\pm$ s.e.m.) threshold of  $-43.0 \pm 1.1$  dB re.  $1 \text{ m s}^{-2}$  (Fig. 2A). From there, sensitivities were gradually reduced with increasing frequency to  $-1.0 \pm 2.2$  dB re.  $1 \text{ m s}^{-2}$  at 640 Hz. Mean individual particle motion thresholds determined under high pressure conditions were comparable to those determined under high particle motion conditions at 80 and 200 Hz (Fig. 2A,B). At frequencies of 120–160 Hz and especially 240–640 Hz, high pressure condition thresholds were, however, significantly lower than those determined under high particle motion conditions (paired

*t*-test: 120 Hz:  $t=-3.931$ ,  $P=0.006$ ; 160 Hz:  $t=-2.658$ ,  $P=0.033$ ; 200 Hz:  $t=-7.147$ ,  $P<0.001$ ; 320 Hz:  $t=-11.704$ ,  $P<0.001$ ; 640 Hz:  $t=-12.543$ ,  $P<0.001$ ; Fig. 2A,B). The sensitivity curve determined under high pressure conditions was shallow and W-shaped with best frequencies of 120 and 320 Hz and thresholds (mean  $\pm$  s.e.m.) of  $137.3 \pm 1.1$  and  $141.5 \pm 2.6$  dB re.  $1 \mu\text{Pa}$ , respectively (Fig. 2C).

**Internal air volumes**

Computed tomography (CT) was performed to determine air volumes in lung and mouth cavities of the lungfish (Fig. 2E). No air was observed in the mouth cavities of awake animals, and only small air volumes (mean  $\pm$  s.d.,  $0.2 \pm 0.2$  ml) were occasionally found in the mouth cavity of anesthetized and handled animals. Anesthesia and handling did not affect air volumes found in the lungs (paired *t*-test:  $t=0.599$ ,  $P=0.571$ ): the lungs of awake animals contained



**Fig. 2. Hearing and vibration sensitivity of African lungfish in water and air.**

(A) Underwater hearing sensitivity of African lungfish determined under high particle motion and high pressure conditions in water, along with acceleration sensitivity in air (substrate vibrations), and sound pressure-equivalent vibration sensitivity calculated from aerial sound pressure thresholds and transfer functions. (B) Individual high pressure (P) condition acceleration sensitivity relative to high particle motion (PM) condition sensitivity (filled circles) along with the change in particle motion-to-pressure ratio. The shaded area indicates the increase in hearing sensitivity of the lungfish when increasing the sound pressure relative to the particle motion. Asterisks indicate statistical significance in paired *t*-test: \* $P<0.05$  and \*\* $P<0.01$ . (C) Underwater sound pressure sensitivity determined under high pressure conditions. (D) Sound pressure sensitivity in air. *N*-values for each dataset are indicated in the bottom of all plots. Bars indicate means  $\pm$  s.e.m. Both aerial (plus signs) and underwater (crosses) octave noise levels were significantly below all thresholds at all frequencies tested (see A, C and D). (E) Computed tomography-based illustration of lung air volumes (blue) and inner ears (red) of the lungfish.

**Table 2. Transfer functions**

	Frequency (Hz)			
	80	120	160	200
Head vibrations re. shaker vibrations (dB)	3.8±2.1	3.6±1.0	-0.6±2.9	-6.4±1.0
Head vibrations re. sound pressure (dB re. 1 m s <sup>-2</sup> Pa <sup>-1</sup> )	-35.3±0.4	-31.0±1.2	-27.2±4.8	-33.1±3.0

Data (means ± s.d.) are head vibrations induced by shaker vibrations and aerial sound pressure.

5.2±3.4 ml of air (mean ± s.d.), while lungs of anesthetized and handled animals contained 6.6±6.6 ml of air.

### Vibration detection and hearing in air

The aerial experiments were conducted in a combined acceleration and sound pressure setup that enabled determination of thresholds for both substrate vibrations and aerial sound pressure. The sensitivity curve for substrate vibrations (vibrogram) showed the same shape as the particle motion sensitivity curve determined in water under high particle motion conditions. Mean thresholds determined in air were, however, on average 11.2±3.9 dB (mean ± s.d.) above those determined in water, with best sensitivity (mean ± s.e.m.) at 40 and 60 Hz of -40.1±1.0 and -40.1±1.5 dB re. 1 m s<sup>-2</sup>, respectively (Fig. 2A).

Sound pressure thresholds could be determined at frequencies of 80–200 Hz with best sensitivity (mean ± s.e.m.) of 85.5±0.9 dB re. 20 µPa at 80 Hz (Fig. 2D). Sound-induced shaker vibrations were below vibration thresholds at all frequencies tested, but equivalent vibration thresholds calculated from sound pressure thresholds and measured transfer functions (Table 2) matched the vibration thresholds at 120–200 Hz (Fig. 2A).

## DISCUSSION

### Pressure detection enhances underwater hearing capabilities

It is not a trivial task to demonstrate pressure detection of aquatic vertebrates in laboratory setups as sound fields in small tanks are complex (Parvulescu, 1964; Rogers et al., 2015), with excessive near-field particle motion that can easily mask potential pressure detection (Kalmijn, 1988). To mitigate this problem, we used a standing wave tube setup to resolve how sound pressure affects the hearing capabilities of lungfish underwater. This is advantageous as the pressure-to-particle motion ratio varies along the length of such a tube, enabling manipulation of the relative pressure intensity by changing the measuring depth (Table 1; supplementary material Fig. S1). We found that a relative increase in the sound pressure (high pressure condition thresholds relative to high particle motion condition thresholds) significantly increased the hearing sensitivity of lungfish (shaded area in Fig. 2A,B), especially at frequencies above 200 Hz, showing that lungfish are able to detect sound pressure underwater (Fig. 2C). Experimentally determined sensitivities may be affected by the methodology used and, furthermore, the sound fields used in such studies are largely affected by the individual laboratory setup. Therefore, it can be problematic to compare hearing sensitivities between different studies (Fay, 1988; Ladich and Fay, 2013; Sisneros et al., 2015). Still, the sensitivity of lungfish appears significantly poorer than in specialized fish such as the goldfish (*Carassius auratus*) (e.g. Fay and Popper, 1974; Ladich and Fay, 2013), but comparable to that of other unspecialized fish such as the European eel (Jerkø et al., 1989) and damselfish (Egner and Mann, 2005; Ladich and Fay, 2013) when taking the difference in methodologies into account.

Our finding of pressure sensitivity is in contrast to the findings of an earlier study of hearing in lungfish (Christensen-Dalsgaard et al.,

2011) which, because of comparable acceleration thresholds in water and air, found no evidence of underwater sound pressure detection. The lungfish in Christensen-Dalsgaard et al.'s study were placed 50 cm above an underwater loudspeaker and 20 cm below the surface. When a sound source vibrates to create a propagating acoustic wave, it simultaneously creates incompressible local flow and hence the sound source near-field is characterized by excessive particle motion (Kalmijn, 1988). Further, the water surface works as a pressure release window as the air above it is compressible, and therefore the underwater sound field is characterized by low pressure and excessive particle motion close to the surface. Collectively, this suggests that the underwater experiments of the earlier study were conducted under excess particle motion conditions. Such conditions are unfavorable for testing pressure detection in aquatic vertebrates as the pressure-to-particle motion transduction of the gas-filled cavities has to exceed the near-field amplified particle motion. Potential pressure detection may therefore easily be masked by detection of the excessive particle motion.

We used CT (Fig. 2E) to measure internal air volumes that can be responsible for the required pressure-to-particle motion transduction in the lungfish. Small air bubbles were occasionally found in the mouth cavity, but with estimated resonance frequencies above 1000 Hz, these were unable to explain the pressure detection found. However, the air volumes measured in the lungs correspond to a resonance frequency of 300 Hz calculated from a model based on fish swim bladders (Alexander, 1966). This resonance frequency matches the high frequency peak in particle motion sensitivity at 320 Hz determined under high pressure conditions (Fig. 2A). This strongly suggests that pressure detection in lungfish is enabled through detection of the pressure-induced particle motion generated by the resonating air volumes in the lungs. As there is no specialized mechanical connection between the lungs and the inner ears in lungfish (Platt et al., 2004), these results suggest that air-filled cavities provide even unspecialized aquatic vertebrates with some ability to detect sound pressure underwater (Jerkø et al., 1989).

This leads us to speculate that pressure detection of aquatic vertebrates may have evolved initially as an unavoidable side-effect of the evolution of air breathing in fish driven by low oxygen levels in fresh water in the early Devonian (Clack, 2007). As air-filled cavities such as lungs and swim bladders resonate mainly above the frequencies of normal best sensitivities in fish accelerometer ears (Chapman and Sand, 1974; Jerkø et al., 1989), air gulping and subsequent development of lungs may have provided a selection pressure for evolving high frequency tuning of inner ear hair cells. This is considered to play an important role in effective aerial hearing (Manley, 2000) and, hence, the aquatic ancestors of tetrapods may have been pre-equipped for aerial hearing through a high frequency extension of their sensory epithelia, driven by pressure hearing via air breathing in water.

### Vibration detection enables aerial hearing

Our air measurements show that lungfish despite being atympanic, surprisingly, can detect high level (>85 dB SPL) airborne sound at

low frequencies (Fig. 2D). The detection of airborne sound found here is in contrast to the previous study on lungfish hearing, where the authors were unable to determine hearing thresholds to airborne sound stimuli using the method of masked AEP (Christensen-Dalsgaard et al., 2011). Masked AEP, however, depends on a good signal to noise ratio in the recordings, as it involves the subtraction of masked and unmasked responses. Here, AEP was also used to determine thresholds, but in contrast to a short click stimulus, relatively long (510 ms + rise and fall of 10 cycles) pure tone stimuli were used to evoke neural responses, thus improving the signal-to-noise level of the responses. The integration time of the lungfish otolith organs is unknown, but in goldfish tone durations greater than 400 ms apparently do not lead to improvement of pressure thresholds (Fay and Coombs, 1983). We therefore assume that the pure tone stimuli used here exceed the integration time of lungfish and thereby maximize activation of the lungfish auditory system.

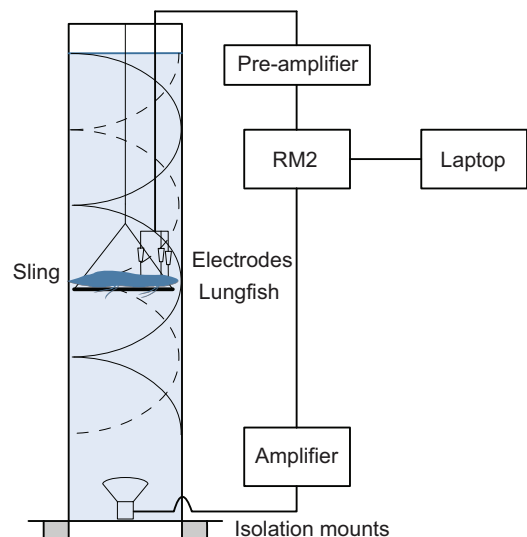
The lack of middle ear adaptations suggests that lungfish are unable to detect the aerial sound pressure per se, but instead detect the sound-induced vibrations of the head either directly or indirectly through induced substrate vibrations. We found that lungfish are very sensitive to substrate vibrations in air (Fig. 2A), confirming the frequency range and sensitivities reported earlier (Christensen-Dalsgaard et al., 2011). Substrate vibrations induced by threshold-level sound pressure were, however, below vibration thresholds of the lungfish and therefore cannot explain the detection of aerial sound pressure. Rather, equivalent head vibration thresholds calculated from aerial sound pressure thresholds (Fig. 2A) and transfer functions (Table 2) match the vibration thresholds determined in air (Fig. 2A), showing that lungfish hear in air by detecting the sound-induced head vibrations. This demonstrates that even aquatic vertebrates with no middle ear adaptations to aerial hearing, such as the early atympanic tetrapod *Acanthostega* (Clack, 1992; Clack, 1997; Clack, 1998), may have been able to detect higher levels of low frequency airborne sound. This limited sensitivity may have provided rudimentary hearing that led to the gradual evolution of low mass skin areas and bony structures, eventually forming the tympanic middle ear. Our lungfish results therefore imply a gradual change from the particle motion-sensitive ears of aquatic ancestors to the pressure hearing of most modern tetrapods where high frequency tuning in aquatic air-breathing forms, along with detection of sound-induced head vibrations in early terrestrial tetrapods, drove the evolution of aerial hearing.

## MATERIALS AND METHODS

The study was conducted using eight African lungfish (*P. annectens*) with a mass of 170–441 g and a total length of 32.3–41.0 cm obtained commercially and kept on a 12 h:12 h light:dark cycle at ~25°C. Before recordings, the fish were anesthetized by submergence in a 0.25% benzocaine (Sigma-Aldrich, St Louis, MO, USA) water solution. Benzocaine was also added to the tank water, resulting in a 0.10% solution to keep the lungfish anesthetized during the underwater recordings. During measurements in air, the lungfish were kept moist by wrapping them in wet paper towels and dripping benzocaine solution on them several times. The animals recovered from anesthesia in about 30 min when put in clean water after measurements. The experiments were licensed by the Danish Animal Experimentation Board.

### Experimental setup and calibration

The underwater neurophysiological experiments were conducted in a standing wave tube setup (Fig. 3) comparable to that previously described (Hetherington and Lombard, 1982), but here, we use a 2 m long steel tube, with an inner diameter of 30 cm and 1 cm thick walls, standing in an upright position. Electrodes (Neuroline subdermal needle electrode, 27 gauge,



**Fig. 3. Standing wave tube setup.** The underwater experiments were conducted in a standing wave tube where pressure (solid line) and particle motion (dashed line) are out of phase and the particle motion-to-pressure ratio therefore varies with depth. The setup was placed on four passive isolation mounts to minimize coupling of vibrational noise from the floor.

12×0.40 mm, Ambu®, Ballerup, Denmark) used to measure the evoked potentials were connected to an RA4PA four-channel Medusa pre-amplifier (Tucker-Davis Technologies, Gainesville, FL, USA). From here, the signal was sampled by a Tucker-Davis Technologies RM2 Mobile Processor at 24,414 Hz and sent to a laptop computer. An underwater speaker (AQ339-Underwater Speaker, Aquasonic Clark Synthesis, Littleton, CO, USA), placed on the bottom of the tube, was controlled by the laptop via the RM2 and an Azur 740A power amplifier (Cambridge Audio, London, UK).

Pressure and particle motion magnitude [ $\sqrt{x^2+y^2+z^2}$ ] profiles were measured in the tube (supplementary material Fig. S1) using two Reson hydrophones (TC 4013) connected to the laptop computer, through the RM2 and a Brüel & Kjær Conditioning Amplifier Type 2692-A-0S4 (Nærum, Denmark). The hydrophones were calibrated using a Brüel & Kjær hydrophone calibrator (Type 4223) with an output of 165.7 dB rms re. 1  $\mu$ Pa at 250 Hz. Particle motion was calculated using the instantaneous pressure gradient measured between the two hydrophones spaced 3 cm apart (Christensen-Dalsgaard et al., 1990) along all three dimensions. Measuring depths with significantly different particle motion-to-pressure ratios were then chosen (Table 1) from the pressure and particle motion profiles. At these depths, both pressure and particle motion were calibrated in the center of the tube without a fish present. Further, both pressure and magnitude of particle motion were measured  $\pm 10$  cm in the *x*- and *y*-axes and found not to vary significantly in these axes. During water measurements, the lungfish were suspended in a sling of nylon mesh on a PVC frame. The sling did not distort the sound field significantly, but air volumes (in the lung and mouth cavities of the lungfish) affected the effective sound intensity in the tube. Air-filled balloons were used to investigate this effect (supplementary material Fig. S2). Because the pressure (supplementary material Fig. S2A) and the particle motion (supplementary material Fig. S2B) components of the sound field were affected equally, no change was seen in the particle motion to pressure ratios (supplementary material Fig. S2C) away from air volumes. Close to the air volumes, particle motion was enhanced by the pressure-induced vibration of the air volumes, whereas the sound pressure was unaffected. Therefore, we assume that the particle motion-to-pressure ratio of the input sound stimuli coming from the speaker was affected in the same way as measured away from the air volumes. The calibrations made without fish present in the tube could hence be corrected before each electrophysiological trial by quantifying the effect of introducing the individual fish in the used measuring depths. The standing wave tube was mounted on four passive isolation mounts (PWA075, Thorlabs, Göteborg, Sweden) to minimize the vibrational noise coupling from the floor.

The neurophysiological experiments in air were conducted in a combined acceleration and sound pressure setup previously described (Christensen et al., 2012). To minimize the vibrational noise coupling from the floor, the shaker was put on a 60×60 cm iso-plate passive isolation system (PTT600600, Thorlabs), four passive isolation mounts (PWA075, Thorlabs) and successive layers of flagstone and styrofoam. The lungfish were placed in the setup with the head resting on a shaker platform 80 cm below a loudspeaker in order to determine acceleration and sound pressure sensitivities. The shaker (Brüel & Kjær Vibration Exciter, Type 4809) was calibrated using a Brüel & Kjær Accelerometer (Type 4381), that was in turn calibrated using a Brüel & Kjær Calibration Exciter (Type 4294) with an output of  $10 \text{ m s}^{-2}$  at 159.15 Hz. The speaker (8 in V8 installation speaker, Tannoy Ltd, Coatbridge, UK) was calibrated using a 0.5 in free-field microphone (Type 40AF, G.R.A.S., Holte, Denmark), calibrated with a Brüel & Kjær Acoustical Calibrator (Type 4231) with an output of 94 dB rms re. 20  $\mu\text{Pa}$  at 1000 Hz.

A small one-dimensional Brüel & Kjær Miniature Accelerometer Type 4517-C with a mass of 0.6 g was glued on to the head of two dead lungfish to investigate the effect of sound-induced head vibrations and determine transfer functions. The head vibrations were measured in all three dimensions in response to 1 Pa and  $1 \text{ m s}^{-2}$  sound and vibration stimuli, respectively, one dimension at a time, and the overall head vibration calculated as the vector norm of these vibrations. The accelerometer was amplified by the Brüel & Kjær Conditioning Amplifier Type 2692-A-0S4 and sampled using the Tucker-Davis Technologies RM2 Mobile Processor.

The experimental equipment was calibrated and controlled by routines written in Matlab 2007b (The MathWorks, Natick, MA, USA) and RpvdsEx v72 (Tucker-Davis Technologies) in both the underwater and aerial setup. Data collection and analyses were made using Matlab and RpvdsEx v72 (Tucker-Davis Technologies). To quantify the acoustic background noise level, we made 240 recordings with a time window of 800 ms. Each recording was windowed by a Tukey window ( $\alpha=0.1$ ) and filtered by a third-order Butterworth filter with cut-off frequencies corresponding to an octave filter band around the center frequency as defined in ANSI s1.1-1986 (www.ansi.org). The noise level for each recording was then calculated as the rms noise level for the filtered 800 ms recording. The noise levels shown in the vibrogram (Fig. 2A) and the audiograms (Fig. 2C,D) are the mean noise levels of 240 recordings each. Vibration noise levels were calculated as the vector norms of recordings in the  $x$ -,  $y$ - and  $z$ -axes. Further, the noise levels were checked after each calibration by making 10 recordings of 800 ms and using the same filtration as above.

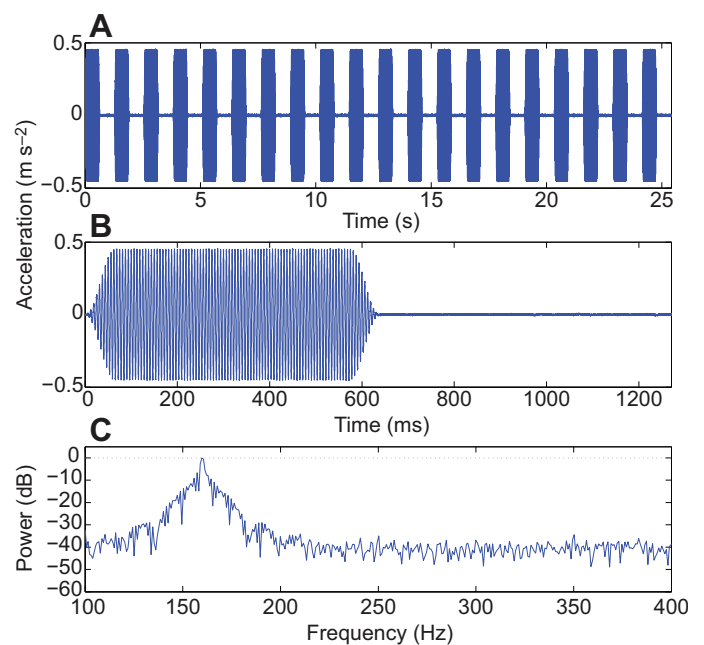
### Recording of evoked potentials

Evoked potentials (Fig. 1) were recorded from the brainstem and VIIIth cranial nerve by inserting three stainless steel needle electrodes subcutaneously. Two measuring electrodes were inserted on top of the head of the lungfish; one medial dorsal to the brainstem and one mediolateral dorsal to the inner ear and VIIIth cranial nerve, enabling recording of the neural response from both the auditory nerve and brainstem. The reference electrode was inserted into the neck of the lungfish well away from the VIIIth cranial nerve and brainstem. The neural response to stimulation was recorded as the potential difference between the brainstem and the VIIIth cranial nerve, reducing the electrical noise and potentials made by muscle contractions.

The stimulus consisted of pure tone bursts in both water and air (Fig. 4). Each trial consisted of 20 tone bursts interspaced by equal length periods of no stimulation (Fig. 4A). To avoid transients and to provide a ramped rise and fall of the tone of 10 cycles, the 510 ms pure tones were gated with frequency-dependent Tukey windows (Fig. 4B). Pure tones of 80, 120, 160, 200, 240, 320 and 640 Hz were used in underwater experiments as the sound field contained a range in pressure-to-particle motion ratio of 16 dB or more at these frequencies (Table 1). In air, pure tones of 20–1280 Hz were used for testing vibration detection. The speaker could not, however, be calibrated adequately below 80 Hz, and therefore sound pressure experiments in air were conducted at frequencies from 80 to 1280 Hz.

### Determination of internal air volumes

CT was performed using a Siemens Somatom Definition (Siemens Medical Solutions, Germany; 472×472 mm<sup>2</sup> field-of-view, 512×512 matrix, 0.6 mm



**Fig. 4. Pure tone stimuli.** Both vibration and sound stimuli were composed of 20 pure tones interspaced by periods of no stimulation. (A) Recorded waveform of an entire trial of vibration stimulus at 160 Hz and  $-16 \text{ dB re. } 1 \text{ m s}^{-2} \text{ rms}$ . (B) Recorded waveform of one stimulation cycle. (C) Power spectrum of 160 Hz tone stimulus. The power at the second harmonic (here 320 Hz) was found to be 40 dB below the power at the fundamental frequency.

slice thickness, 100 kVp tube voltage, 260 mAs tube current and acquisition time of 20 s) to measure air volumes of the lungs and pharynx of the lungfish. The animals were placed in separate water-filled plastic containers on the scanner bed and CT was performed on both awake undisturbed and anesthetized and handled animals, to evaluate any effect of anesthesia and handling on internal air volumes.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

All authors contributed to the conception and design of the study, interpretation of the findings, and drafting and revising the manuscript. C.B.C. executed the experiments and analyzed the data.

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### Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.116012/-DC1>

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