RESEARCH ARTICLE

Hearing with an atympanic ear: good vibration and poor sound-pressure detection in the royal python, *Python regius*

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SUMMARY

Snakes lack both an outer ear and a tympanic middle ear, which in most tetrapods provide impedance matching between the air and inner ear fluids and hence improve pressure hearing in air. Snakes would therefore be expected to have very poor pressure hearing and generally be insensitive to airborne sound, whereas the connection of the middle ear bone to the jaw bones in snakes should confer acute sensitivity to substrate vibrations. Some studies have nevertheless claimed that snakes are quite sensitive to both vibration and sound pressure. Here we test the two hypotheses that: (1) snakes are sensitive to sound pressure and (2) snakes are sensitive to vibrations, but cannot hear the sound pressure *per se*. Vibration and sound-pressure sensitivities were quantified by measuring brainstem evoked potentials in 11 royal pythons, *Python regius*. Vibrograms and audiograms showed greatest sensitivity at low frequencies of 80–160 Hz, with sensitivities of $-54\,dB\,re.\,1\,m\,s^{-2}$ and $78\,dB\,re.\,20\,\mu Pa$, respectively. To investigate whether pythons detect sound pressure or sound-induced head vibrations, we measured the sound-induced head vibrations in three dimensions when snakes were exposed to sound pressure at threshold levels. In general, head vibrations induced by threshold-level sound pressure were equal to or greater than those induced by threshold-level vibrations, and therefore sound-pressure sensitivity can be explained by sound-induced head vibration. From this we conclude that pythons, and possibly all snakes, lost effective pressure hearing with the complete reduction of a functional outer and middle ear, but have an acute vibration sensitivity that may be used for communication and detection of predators and prey.

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Key words: vibration detection, hearing, evoked potentials, snakes, royal python, *Python regius*.

INTRODUCTION

Hearing provides vital information about the surrounding environment in most vertebrates, and so sound detection and auditory scene analysis are utilized by animals in many different ways to navigate, avoid predators, find prey and communicate (Webster et al., 1992). In water, where the impedance of animals is close to the impedance of the surrounding medium, most animals detect particle motion using an otolith-based ear functioning as an accelerometer (Sand and Karlsen, 2000), where the differential acceleration of tissue and otoliths leads to hair-cell deflection. In air, most of the sound energy is reflected off of an animal because of the large difference in impedance between air and tissue. The evolution of the tympanum and middle-ear ossicles have played a crucial role in solving this problem of impedance mismatch in the evolutionary transition from water to land. The tympanic middle ear evolved independently at least five times, in all the major tetrapod lineages (Clack, 1997), and enables detection of sound pressure by transforming sound energy in air to fluid motion in the inner ear.

Some tetrapods have secondarily lost their tympanum and middle ear, including the snakes, which have a reduced tympanic cavity and no tympanum or Eustachian tube. Nevertheless, snakes have maintained a single middle-ear ossicle, the columella auris (Wever, 1978), whose proximal end rests in the vestibular window while the distal end is connected to the quadrate. Thus, instead of being connected to the tympanum as in most other tetrapods, the columella is connected to the jaw suspension (Fig. 1, also see the interactive three-dimensional model presented in supplementary material Fig. S1). This marked difference in morphology compared with other tetrapod groups is believed to be a result of a secondary reduction (Manley, 2010) in a fossorial (Walls, 1940) or aquatic ancestor (Caldwell and Lee, 1997; Tchernov et al., 2000), attributable to the evolution of the ophidian feeding mechanism where the jaws are used to manipulate prey (Berman and Regal, 1967), while maintaining the capability to detect vibrations (Thewissen and Nummela, 2008).

In other reptiles, the tympanum and columella auris function, as in most other tetrapods, as an impedance-matching device to facilitate the transduction of pressure waves in air to movement of fluid in the inner ear (Saunders and Johnstone, 1972). The lack of an impedance-matching middle ear therefore implies that snakes should have poor pressure sensitivity and hence poor aerial hearing. Instead, the connection of the columella and the quadrate has been interpreted as a specialization for detecting substrate vibrations (Tumarkin, 1948; Bellairs and Underwood, 1951), consistent with a fossorial lifestyle, where vibration detection is likely to be advantageous compared with pressure hearing (Christensen-Dalsgaard and Carr, 2008).

Despite these morphological predictions, previous studies on the auditory system of snakes have reached very different conclusions
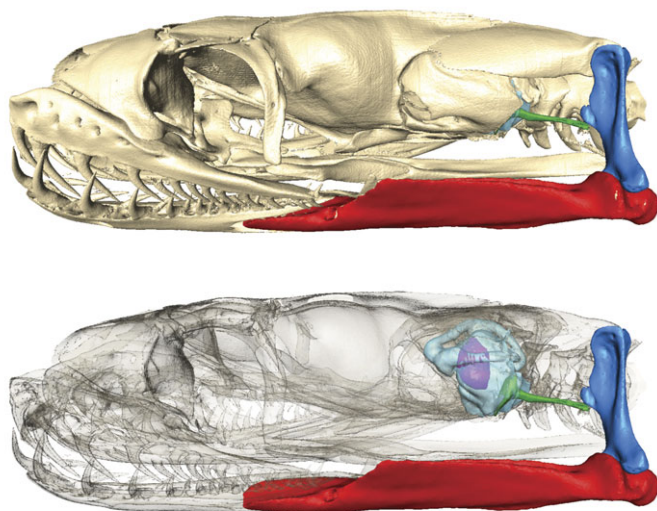
Fig. 1. Micro-CT scan of a royal python, *Python regius*, head. Upper: solid skull; lower: transparent skull. Red: mandible; dark blue: quadrate; green: columella auris; purple: saccule; light blue: inner ear space. See supplemental material Fig. S1 for a three-dimensional model of the snake skull and bones.

regarding the ability of snakes to hear airborne sound [for an overview, see Young (Young, 2003)]. Manning (Manning, 1923) reported that rattlesnakes of the species *Crotalus adamanteus*, *C. horridus* and *C. atrox* were practically unresponsive to sound. Manning did, however, find that one specimen of *C. atrox* responded to low-frequency sound and that placing the speaker in contact with the substrate doubled the frequency range where a response could be recorded.

On the contrary, several later studies suggested that snakes are seemingly quite sensitive to airborne sound. Wever and Vernon reported that several species of the family Colubridae responded to airborne sound with best sensitivity of 30–50 dB re. 20 µPa at approximately 200 Hz (Wever and Vernon, 1960), which is comparable to the hearing thresholds of other squamates with a tympanic ear (e.g. Brittan-Powell et al., 2010; Werner et al., 1998). Hartline found that species of the families Crotalidae, Colubridae and Boidae responded both to airborne sound and to vibrations of the substrate, with best sensitivities of 53 dB re. 20 µPa and −62 dB re. 1 m s^{-2} at 250 and 200 Hz, respectively (Hartline, 1971). Both studies dismissed the common view at the time that sound is only detected by air-pressure-induced substrate vibrations. Hartline also concluded that the head vibrations caused directly by the impinging sound were not intense enough to be detected by the snakes (Hartline, 1971), implying that the snakes used in his study indeed had actual pressure hearing.

Recently, behavioral studies have been made using both sound pressure (Young and Aguiar, 2002; Young and Harris, 2006) and vibration stimulation of snakes (Young and Morain, 2002). Young and Aguiar found a significant decrease in body movement and tongue flicking, and a significant increase in head jerks and tail rattling when presenting sounds of 150–450 Hz in the range of 65–75 dB re. 20 µPa to rattlesnakes *C. atrox* (Young and Aguiar, 2002). Further, Young and Morain reported that olfactory-denervated and temporarily blinded Saharan sand vipers (*Cerastes cerastes*) were able to capture freely mobile prey utilizing vibrational clues, although strike distance, strike angles and strike accuracy were significantly reduced (Young and Morain, 2002).

Thus, there is a conflict between the morphology of the atympanous auditory system of snakes – which implies a lack of pressure hearing – and studies reporting that snakes indeed seem quite sensitive to sound pressure.

In an attempt to resolve these conflicting reports, we here test the two hypotheses that: (1) snakes are sensitive to sound pressure and (2) snakes are sensitive to vibrations, but cannot hear the sound pressure *per se*, and instead detect sound *via* sound-induced head vibrations. We determined the sound and vibration sensitivity of the royal python, *Python regius*, using evoked potentials, while quantifying the head and substrate vibrations induced by sound and vibration. We find that royal pythons have very high vibration sensitivity, but very poor sound-pressure sensitivity. Further, the sound-pressure sensitivity can be explained by the sound-induced head vibrations. We therefore conclude that the ancestors of royal pythons lost the ability to hear sound pressure *per se* with the loss of the tympanic middle ear, but have developed an acute vibration sensitivity for detection of predators and prey, and possibly for communication.

MATERIALS AND METHODS
Masked evoked potentials

When exposed to sound, the inner-ear hair cells that are sensitive to the frequency band around the stimulus will increase the discharge level of their associated afferent neurons. Following this increase in discharge, the auditory neurons are refractory and respond to a lesser degree or not at all to a subsequent stimulation. This phenomenon is known as forward masking, and can be used to determine thresholds of pure tones in evoked potential studies (Berlin et al., 1991; Christensen-Dalsgaard et al., 2011; Manley and Kraus, 2010).

A click is characterized by a short duration and a broad frequency band. Stimulation with a broadband click will excite all the hair cells covering the bandwidth of the click. Presentation of a detectable tone, with a frequency contained within the frequency band of the click, prior to the click, results in a reduction of the overall evoked potential response to the click because of the masking effects of the tone. Thus, by comparing the auditory brainstem response to masked and unmasked click stimulations, we used forward masking to determine the thresholds of vibrational and acoustic sinusiodals. Investigation of the click response enabled the use of longer tones, which further allowed us to find thresholds at lower frequencies than is possible with normal evoked potential approaches.

Experimental setup and calibration

The study was conducted using 11 specimens of *Python regius* (Shaw 1802) with a mass of 165–299 g and a snout–tail length of 63–78 cm. The snakes were obtained commercially and housed in individual terraria under a 12 h:12 h light:dark cycle in a room heated to 28°C. They were provided with water *ad libitum* and maintained on a diet of mice. All recordings were made at temperatures of 23–27°C, slightly below the normal temperature of the animals. This may have led to an increased sensitivity at the lower frequencies and a decrease in sensitivity at the higher frequencies, leading to a downshift in the best frequency of the snakes (Werner, 1972). Prior to measurements, the snakes were lightly anesthetized by subcutaneous injection of Hypnorm (fentanyl citrate 0.315 mg ml^{-1} and fluanisone 10 mg ml^{-1}) and ketamine (100 mg ml^{-1}) at a dosage of 0.50 ml kg^{-1} Hypnorm and 0.75 ml kg^{-1} ketamine. The anesthesia was judged as light because slightly reduced dosages did not abolish movement of the snakes. The snakes usually recovered after 4 h.

The experiments were licensed by the Danish Animal Experimentation Board.

A schematic of the recording setup is shown in Fig. 2. All recordings were conducted in a Cremer principle anechoic room ($5.75 \times 4.5 \times 3.65$ m length×width×height) to minimize acoustic noise and reflections. Further, the shaker and a swing, holding the body of the snake, were placed on consecutive layers of mineral wool and flagstone to minimize vibrational noise coupling from the floor, and in a wire-mesh Faraday cage to minimize electrical noise. Electrodes (disposable subdermal needle electrode, 27 gauge, 12 mm, Rochester Electro-Medical Inc., Lutz, FL, USA) used to measure the evoked potentials were connected to an RA4PA four-channel Medusa Preamplifier [Tucker-Davis Technologies (TDT), Gainesville, FL, USA] and a TDT RM2 Mobile Processor sampling with a rate of 24,414 Hz and controlled by a laptop computer. The stimulator (speaker or shaker) was controlled by the laptop *via* the RM2 and an Azur 740A power amplifier (Cambridge Audio, London, UK).

The speaker, an 8 inch V8 installation speaker (Tannoy Ltd, Coatbridge, UK), placed 80 cm above the head of the snakes, was suspended by strings from the ceiling to minimize coupling of vibrational noise from the speaker to the setup during sound experiments. The sound field was calibrated using a G.R.A.S. ½ inch Microphone Type 40AF (G.R.A.S., Holte, Denmark), oriented perpendicularly to the speaker and connected to the RM2 through a G.R.A.S. ½ inch Preamplifier Type 26AM and a G.R.A.S. Power Module Type 12AD. The microphone was calibrated using a Brüel & Kjær Acoustical Calibrator Type 4231 (Brüel & Kjær, Nærum, Denmark) with an output of 94 dB SPL ($dB_{r.m.s.}$ re. 20 μPa) at 1000 Hz.

The shaker, a Brüel & Kjær Vibration Exciter Type 4809, was placed directly under the snake's head, so that the lower jaws of the snake rested on it. It was calibrated using a Brüel & Kjær Accelerometer Type 4381 connected to the RM2 through a Brüel & Kjær Conditioning Amplifier Type 2692-A-0S4. The accelerometer was calibrated using a Brüel & Kjær Calibration Exciter Type 4294 with an output of 10 m s^{-2} at 159.15 Hz.

The experimental equipment was calibrated and controlled by routines written in MATLAB 2007b (MathWorks, Natick, MA, USA) and RPvdsEx v72 (TDT). Data were analysed using MATLAB 2007b. The Lilliefors test was used to test for homoscedasticity of data.

Fig. 1 was made using a micro-computer tomography (micro-CT) data set acquired by scanning a 5 cm long python head with a μCT 40 scanner (SCANCO Medical AG, Brüttisellen, Switzerland). Segmentation and modelling were accomplished in Amira 5.3.3 (Visage Imaging, Berlin, Germany). This data set was also used to make the three-dimensional model, which can be seen in supplementary material Fig. S1.

Recording of evoked potentials

Evoked potentials were recorded as a response to both vibration and sound pressure stimuli by inserting three needle electrodes subcutaneously. Two measuring electrodes were inserted on top of the snake's head, one dorsal to the brainstem and one dorsal to the VIIIth cranial nerve. The electrode placement was optimized in two steps: (1) we located the VIIIth cranial nerve and brainstem by dissecting three snakes, and (2) by subsequent fine-scale changes in electrode placement we found a configuration that rendered the highest evoked potentials for a one-cycle 300 Hz standard click of -5 dB re. 1 m s^{-2} peak–peak (pp). By placing the measuring electrodes close to the VIIIth cranial nerve and the brainstem, it was possible
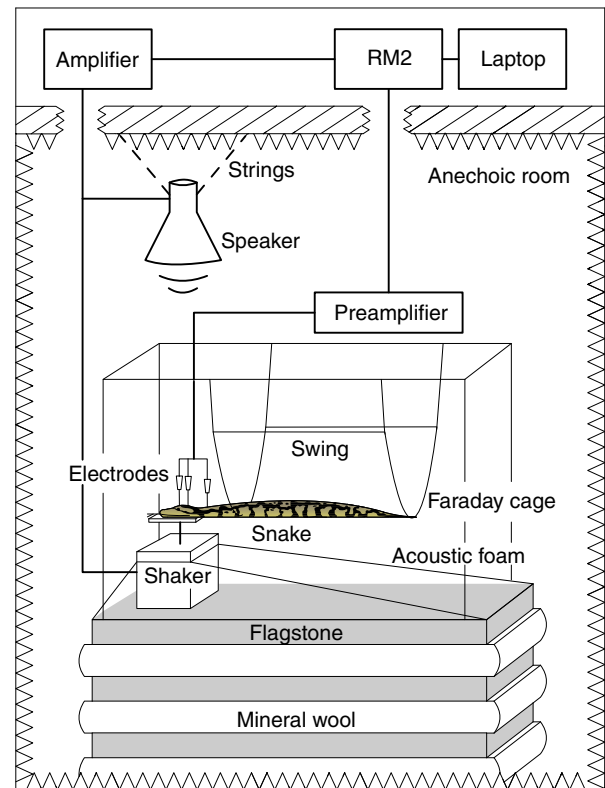

Fig. 2. Experimental setup. The setup was placed in an anechoic room to minimize acoustic noise and reflections. The speaker was suspended by strings from the ceiling to minimize the direct coupling of vibrational noise. The swing and the shaker were placed on consecutive layers of mineral wool and flagstone to minimize the vibrational noise from the floor, and within a Faraday cage to minimize electrical noise.

to record the neural response in both the auditory nerve and brainstem. The reference electrode was inserted into the neck of the snake well away from the VIIIth cranial nerve and brainstem. The neural responses to stimulation were recorded as the voltage difference between the two measuring electrodes relative to the reference electrode. Subtracting the two measuring potentials reduced the electrical noise and potentials made by muscle contractions. The signal-to-noise ratio was further improved by filtration of the recordings with a second-order 200 Hz high-pass and a fourth-order 2000 Hz low-pass Butterworth filter and by averaging over 320 consecutive measurements aligned in time by the click onsets.

The stimuli used in the masking experiment were designed as alternations between a click train added to a masking pure tone and the click train alone (Figs 3, 4). To suppress stimulation artifacts, every second stimulus train was phase inverted.

The click train (Fig. 3A, Fig. 4A) was composed of four clicks consisting of a single cycle at 300 Hz and an inter-click interval of 45 ms. We used a single cycle at 300 Hz as this produced a broadband signal giving the largest neural click response for both sound pressure and vibration across a frequency range of 40 to 1000 Hz. For every snake, the click level giving 50% of the maximum evoked potential amplitude was determined and used for subsequent recordings. This level was chosen as a compromise between avoiding backward masking of the masking tone by the click and not recording a click evoked potential with sufficient signal-to-noise ratio.
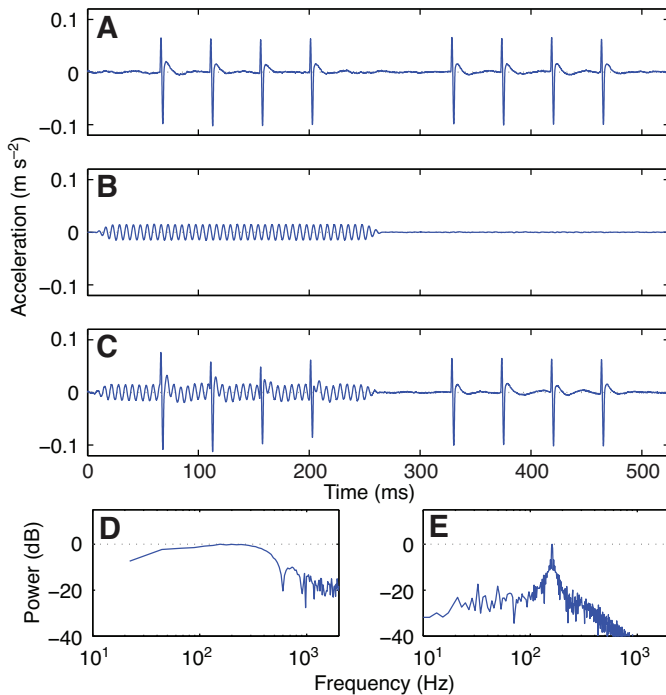
Fig. 3. Vibration stimuli composed of a 300 Hz one-cycle click of −16 dB re. 1 m s⁻² [peak–peak (pp)] and a 160 Hz masker tone of −40 dB re. 1 m s⁻² (r.m.s.). (A) Recorded waveform of the click trace of one stimulation cycle. (B) Recorded waveform of the pure tone trace of one stimulation cycle. (C) Recorded waveform of the combined click and tone traces. (D) Power spectrum of the click. (E) Power spectrum of the masker.

Fig. 4. Sound stimuli composed of a 300 Hz one-cycle click of 103 dB re. 20 µPa (pp) and a 160 Hz masker tone of 80 dB re. 20 µPa (r.m.s.). (A) Recorded waveform of the click trace of one stimulation cycle. (B) Recorded waveform of the pure tone trace of one stimulation cycle. (C) Recorded waveform of the combined click and tone traces. (D) Power spectrum of the click. (E) Power spectrum of the masker.

The pure tone was windowed with a Tukey window to avoid speaker transients and to provide a ramped rise and fall of the tone. The alpha value of the Tukey window was dependent on tonal frequency, so that both rise and fall of the window covered three cycles (Fig. 3B, Fig. 4B). The stimulus rise was followed by a 45 ms delay before the first click and lasted until 45 ms after the last click (Fig. 3C, Fig. 4C), giving a length of the tone stimulus at full intensity of 225 ms. We used pure tones of 40–1000 Hz for testing vibration detection, whereas the speaker could not be calibrated adequately at frequencies below 80 Hz. Therefore, sound-pressure experiments were conducted at frequencies from 80 to 1000 Hz, covering the frequency range tested in previous studies (Wever and Vernon, 1960; Hartline, 1971).

As can be seen in Fig. 3D and Fig. 4D, the click design resulted in a decrease in power above 600 Hz for both vibration and sound clicks compared with the lower frequencies. This decrease in power at frequencies above 600 Hz means that the click could be masked by a less intense pure tone at these frequencies as compared with a flat click spectrum. Therefore, thresholds at these frequencies found in the present study may be overestimations and slightly lower than threshold found using a click with a flat spectrum.

Different objective methods of threshold determination were tried without luck, and thresholds for sound pressure and vibration were therefore determined by visual inspection of the evoked potentials to different stimulus intensities following Brittan-Powell et al. (Brittan-Powell et al., 2010) and Manley and Kraus (Manley and Kraus, 2010). This approach may lead to higher thresholds compared with those found by regression (Manley and
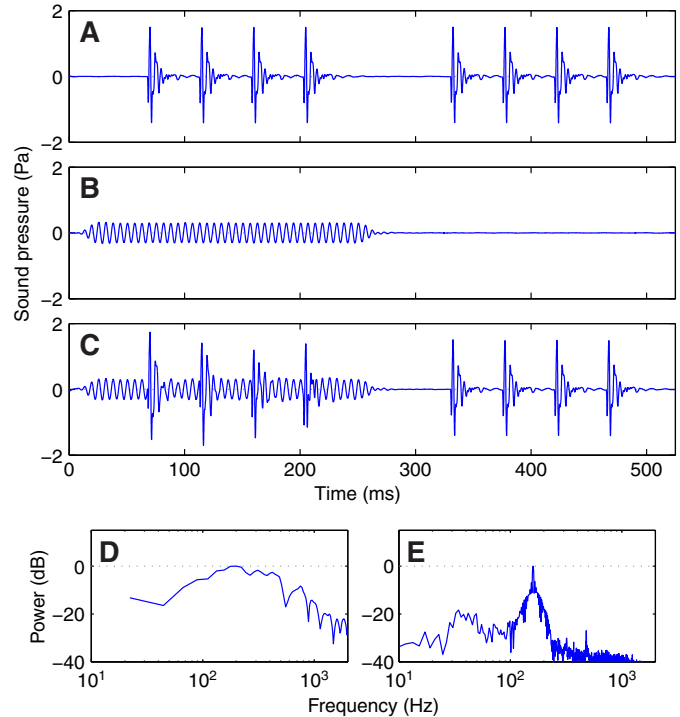
Kraus, 2010). However, as thresholds of both stimuli suffer from such a bias, the error is likely small in a comparative study of thresholds for multiple stimulus types. A threshold was defined as the lowest tone level that had a masking effect on the click response (Fig. 5), i.e. where the first, second and third peaks of the masked and unmasked click responses differed more than what could be attributed to noise. Initially we tested the masking effect of the pure tone by recording the evoked potential of the click while attenuating the pure tone level in 6 dB steps until no masking was found. Thereafter we tested the masking effect 3 dB above the level which had no masking effect on the click, giving a threshold accuracy of 3 dB. To ensure that vibration thresholds were not overestimated, shaker vibrations were also measured along the x- and y-axes (the horizontal plane), and the overall vibrations were calculated and plotted in the vibrogram.

Sound-induced vibrations of shaker and head

The evoked potentials from sound exposure could, in addition to direct auditory stimulation, be elicited by sound-induced substrate or head vibrations, or by a direct coupling of vibrations from the speaker to the setup. To quantify the sound-induced substrate vibrations, we measured vibrations of the shaker platform when stimulating with sound at threshold intensities previously found by evoked potentials. We measured the vibrations in all three dimensions with the Type 4381 accelerometer and were thereby able to quantify the overall substrate vibrations induced by the sound stimulation during sound experiments.

To investigate the effect of sound-induced head vibrations, a small one-dimensional Brüel & Kjær Miniature Accelerometer Type 4517-
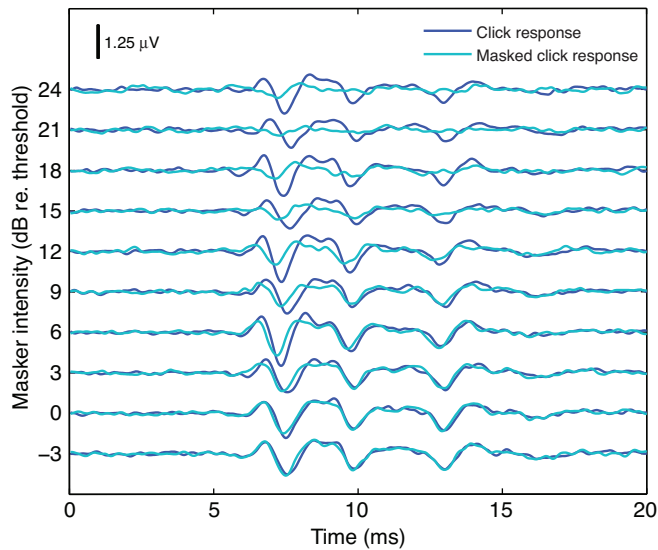
Fig. 5. The masked click response decreases compared with the unmasked click response when increasing the intensity of the masker. The threshold was determined as the lowest level having a masking effect.

Fig. 6. Click stimulus and auditory brainstem response to click stimulation. (A) Vibration click stimulus, one-cycle 300 Hz click at −16 dB re. 1 m s^{-2} (pp) band-pass filtered with a 200–1000 Hz second-order Butterworth filter. (B) Click response to click shown in A, high-pass filtered at 200 Hz with a second-order Butterworth filter and low-pass filtered with a fourth-order Butterworth filter with a cutoff frequency of 2000 Hz. (C) Sound-pressure click stimulus, one-cycle 300 Hz click at 103 dB re. 20 µPa (pp), band-pass filtered with a 200–1000 Hz second-order Butterworth filter. (D) Click response to click shown in C, high-pass filtered at 200 Hz with a second-order Butterworth filter and low-pass filtered with a fourth-order Butterworth filter with a cutoff frequency of 2000 Hz.

C with a mass of 0.6 g was glued onto the head of the snakes. In this way the head vibrations were measured in all three dimensions at threshold intensities for both sound and vibration stimuli, one dimension at a time, and the overall head vibration was calculated as the vector norm of these vibrations. This was done to ensure that no vibration vector was overlooked, as both vibration and sound stimuli induced multidirectional head vibrations. The accelerometer was amplified by the Brüel & Kjær Conditioning Amplifier Type 2692-A-0S4 and sampled using the TDT RM2 Mobile Processor. By measuring the head vibrations in response to vibration stimuli of −30 dB re. 1 m s^{-2} with and without accelerometer using a laser vibrometer (OFV-505 sensor and OFV-5000 vibrometer, Polytec, Waldbronn, Germany), the added mass of the miniature accelerometer was shown to have a mean effect of 0.6 dB on the head vibrations across all frequencies and in all three dimensions. Because the self-noise of the miniature accelerometer exceeded the derived detection thresholds for some frequencies, the head vibrations were calculated by linear regression of head vibrations measured at higher intensities. The head vibrations followed the stimuli intensities of both shaker and speaker linearly. To ensure that the regressions were not noise biased, only data points 3 dB above the self-noise of the accelerometer were used. The laser vibrometer could not be used to measure head vibrations because it was impossible to avoid sound-induced vibrations of the laser itself in the present setup.

Measurements of sound and vibration noise

Both sound and vibration background noise levels were measured to ensure that background noise did not mask the experimental stimuli. To quantify the background noise, we made 240 recordings with a time window of 800 ms corresponding to the time span of three normal measurements. Each recording was windowed by a Tukey window (α=0.1) and filtered by a third-order Butterworth filter with cutoff 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 r.m.s. noise level for the filtered 800 ms recording.
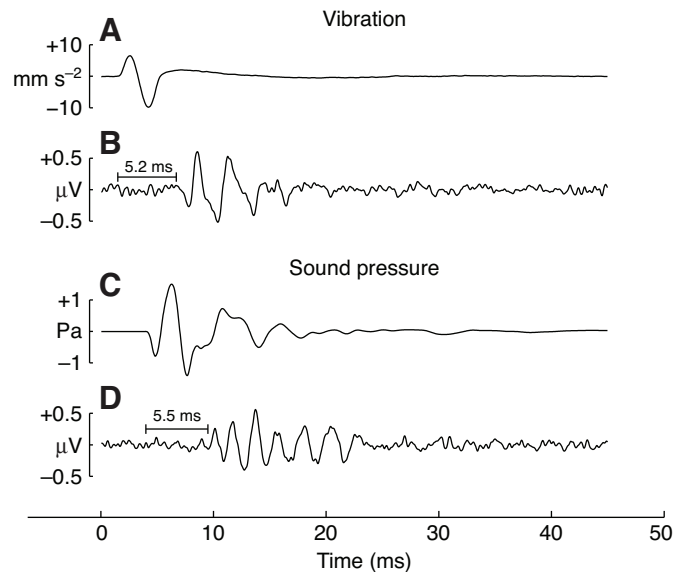
The noise levels shown in the vibrogram (Fig. 7) and the audiogram (Fig. 8) are the mean noise levels of 240 recordings each. Vibration noise levels were calculated as the vector norms of recordings in the x-, y- and z-axes. Further, the noise levels were checked after each calibration by making 10 recordings of 800 ms and using the same filtration as above. Because the critical bands in lizards are equal to or smaller than one octave (Sams-Dodd and Capranica, 1996), this procedure generates an upper band estimate of the ambient noise masking, if the critical bands in snakes are comparable to those in lizards.

RESULTS
Evoked potentials

We measured the evoked potentials of 11 royal pythons, *P. regius*, to determine thresholds for vibration and sound. The evoked potential to broadband click stimulation consisted of an asymmetric wave with multiple peaks for both vibration and sound stimuli (Fig. 6). The latency from stimuli onset to response was approximately 5 ms for both vibration and sound recordings.

Thresholds for both vibration and sound were determined for 10 snakes as the lowest tone level having a masking effect on the auditory brainstem response to the broadband click. Thresholds for both vibration and sound pressure were obtained as a function of frequency for one snake at a time, without changing the placement of the recording electrodes between recordings, allowing for comparison of head vibrations at thresholds. For one snake we were only able to measure the vibration thresholds, and hence a mean vibrogram of 11 snakes and a mean audiogram of 10 snakes could be established. After determining thresholds for both vibration and sound stimuli, overall shaker and head
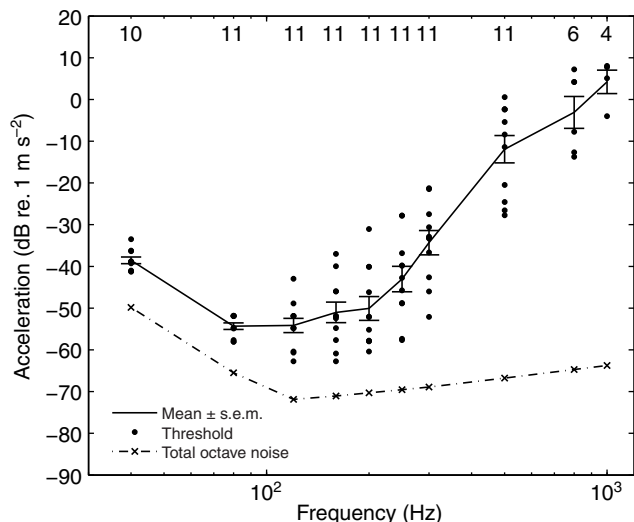
Fig. 7. Vibrogram of *Python regius*. Individual thresholds (filled circles); total octave noise (crosses). *N*-values are shown above each set of data points.
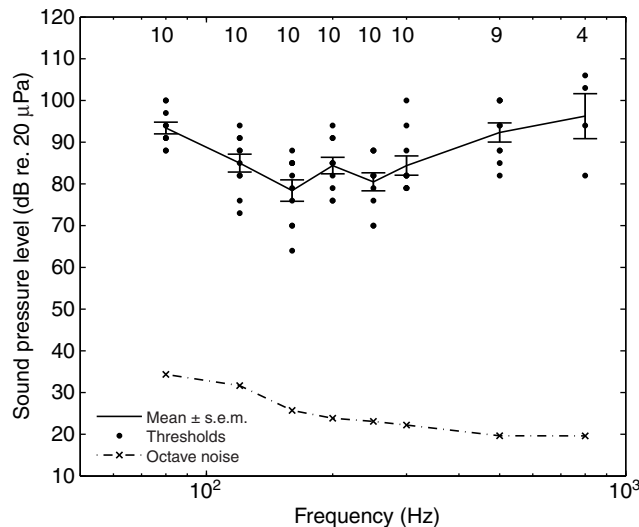

Fig. 8. Audiogram of *Python regius*. Individual thresholds (filled circles); one-octave noise (crosses). *N*-values are shown above each set of data points.

vibrations at threshold intensities were found for both sound and vibration stimuli.

Vibrogram

Individual vibration thresholds of the 11 snakes are shown in Fig. 7. The detection thresholds at higher frequencies were higher for some of the snakes than the maximum output levels of the shaker system ($7\,dB\,re.\,1\,m\,s^{-2}$ at 800 and 1000 Hz). We were therefore not able to determine thresholds for all snakes at these frequencies. Although the thresholds at low frequencies were almost equal, they were more scattered at the higher frequencies. As can be seen in Fig. 7, the mean vibration sensitivity increased from 40 to 80 Hz, changed little from 80 to 200 Hz and then declined rapidly at frequencies above 200 Hz. Snakes had the highest vibration sensitivity at frequencies of 80 and 120 Hz, being -54 ± 0.8 and $-54\pm1.8\,dB\,re.\,1\,m\,s^{-2}$ (means ± s.e.m.), respectively. All thresholds found were at least 8 dB above the overall octave vibration noise level (Fig. 7).

Audiogram

The individual sound pressure thresholds of the 10 snakes and the resulting mean audiogram are shown in Fig. 8. For frequencies below 500 Hz, the masking thresholds for all 10 snakes could be established. At 500 and 800 Hz, masking threshold could be established for nine and four snakes, respectively. At 1000 Hz, no masking effect was seen for any of the snakes when using tones of up to $110\,dB\,re.\,20\,\mu Pa$.

Snakes had a best frequency of 160 Hz with a mean (±s.e.m.) sensitivity of $78\pm2.6\,dB\,re.\,20\,\mu Pa$. After having increased 15 dB from 80 to 160 Hz, little change was seen in the sensitivity between 160 and 300 Hz, after which it was reduced by 14 dB at 500 Hz and 18 dB at 800 Hz, relative to the best sensitivity (Fig. 8). All thresholds found were at least 39 dB above the octave noise levels of the tested frequencies (Fig. 8).

Sound-induced shaker vibrations

To investigate whether the sound stimuli induced substrate vibrations large enough to elicit the evoked potentials recorded during sound experiments, we recorded the vibrations of the shaker platform in

all three dimensions at sound pressures corresponding to the threshold levels from each of the snakes. The total shaker vibrations were computed as the vector norm of the three axes and plotted in Fig. 9A, together with the overall shaker vibrations at vibration threshold levels. Sound induced shaker vibrations at all frequencies, though vibrations were most pronounced at 80, 300 and 800 Hz. Because the vibration sensitivity of the snakes was reduced at 300 and 800 Hz, the shaker vibration induced by the sound stimuli was only significantly higher than the vibration thresholds of the snakes at 80 Hz (paired *t*-test, $t=3.221$, $P=0.0105$; Fig. 9B). At 120 Hz, the sound-induced shaker vibrations were below, but not significantly smaller than, the vibration threshold at that frequency. In the frequency range between 160 and 500 Hz, shaker vibrations induced by sound were significantly below the vibration thresholds, and thereby not large enough to elicit the recorded evoked potentials. At 800 Hz, the shaker vibrations were below the vibration threshold, but could not be evaluated because of the small sample size at this frequency.

Head vibrations at thresholds

The sound-induced head vibrations were investigated by measuring the head vibrations at threshold levels of both sound pressure and vibration. Because sound and vibration stimuli both induce head vibration magnitudes along the *x*- and *y*-axes that are comparable to the head vibrations along the *z*-axis (Fig. 10), we measured head vibrations in all three dimensions and calculated the overall head vibrations as the vector norm.

Total head vibrations induced by threshold level sound pressures and vibrations are plotted in Fig. 11A. At 80–250 Hz, the head vibrations caused by threshold level sound pressure were 3–14 dB greater than head vibrations caused by threshold-level vibration stimuli. At 300–800 Hz, head vibrations caused by threshold-level sound pressure were 2–15 dB below those caused by vibrations.

To provide a better picture of the differences in head vibrations induced by the two stimuli types, we plotted head vibrations as relative head vibrations, found by subtracting the individual head vibrations induced by threshold-level vibration stimuli from the head
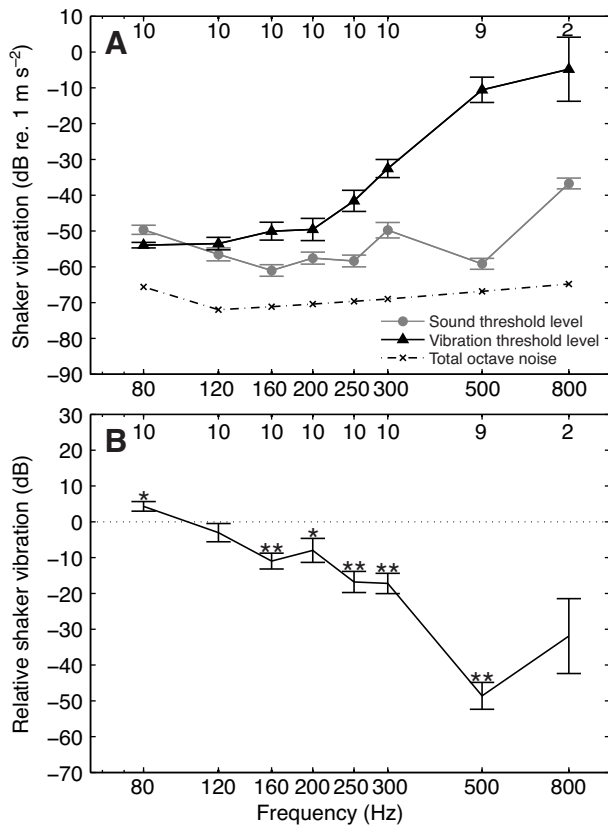
Fig. 9. Shaker vibrations. (A) Vibrations of the shaker platform induced by sound stimulation at threshold levels (filled circles; ±s.e.m.). Also shown are vibration thresholds (filled triangles; ±s.e.m.) and octave noise summed over all three dimensions (crosses). (B) Induced vibrations at sound thresholds relative to vibration thresholds (±s.e.m.). Asterisks indicate statistical significance (paired *t*-test, *P<0.05; **P<0.01). N-values are shown above each data point.
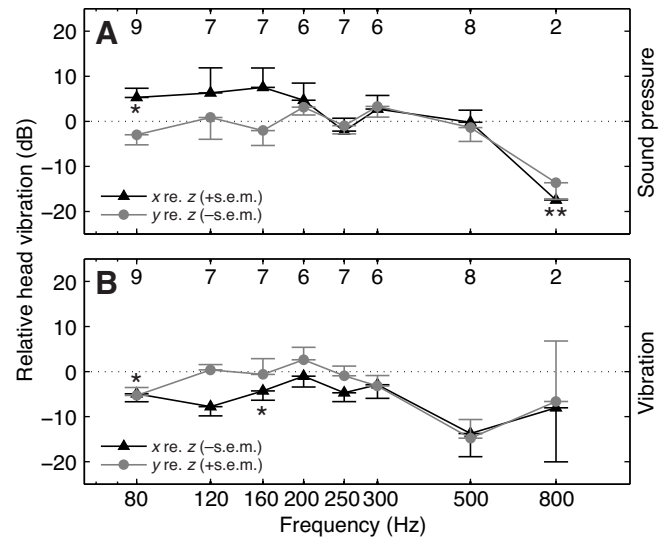

Fig. 10. Head vibrations of *x*- and *y*-axes relative to head vibrations of the *z*-axis. When exposed to sound pressure (A) and vibration (B), snake heads vibrated in all three dimensions. N-values are shown above each data point.

decreased with increasing frequency, with a maximum of –27 dB re. 1 mm s^{-1} Pa^{-1} at 80 Hz, falling to –54 dB re. 1 mm s^{-1} Pa^{-1} at 800 Hz.

DISCUSSION
In this study, we used evoked potentials to estimate the vibration and sound-pressure sensitivity of royal pythons, *P. regius*. We also quantified the substrate and head vibrations induced by sound pressure to experimentally test whether snakes have sound-pressure hearing *per se*, or whether they instead detect the sound-induced head vibrations when exposed to sound. The main result is that the pythons are very sensitive to low-frequency vibrations (best sensitivity=–54 dB re. 1 m s^{-2} at 80–120 Hz) and that the sensitivity to airborne sound is generated by sound-induced head vibrations.

The evoked potential approach to hearing and vibration studies is much faster than psychophysical experiments (which are especially challenging for reptiles), and less invasive than single- or multi-unit recordings with high-impedance electrodes. Using the method of measuring the differences between masked and unmasked responses is not without technical difficulties, as the signal-to-noise ratio and thus the thresholds found by this method are to some extent dependent on electrode placement. We addressed this problem by optimizing the electrode placement according to prior dissections, and by configuring the electrodes for every snake so as to maximize the amplitudes of the click-evoked potentials. Thresholds found by evoked potentials are generally approximately 10–30 dB higher than thresholds found by single-cell recordings or behavioral studies (Brittan-Powell et al., 2010), and hence thresholds found in the present study likely underestimate the true detection thresholds for both vibration and pressure. Although the method of evoked potentials may give higher thresholds than those determined by behavioral detection methods, the method is well suited for comparative studies of sensitivity to different stimuli. As long as electrode placements are not changed between the evoked potential measurements for the two stimuli types, the offset from absolute thresholds will be

vibrations induced by threshold-level sound pressures (Fig. 11B–E). The differences in head vibration were significant at frequencies of 80, 120, 250 and 500 Hz (Fig. 11B). At 80, 120 and 250 Hz, the relative head vibrations induced at thresholds were significantly above zero, which corresponds to head vibrations induced by threshold-level sound stimuli being higher than those induced by threshold-level vibration stimuli (*t*-test, 80 Hz: *t*=10.33, P<0.0001; 120 Hz: *t*=2.825, P=0.03; 250 Hz: *t*=2.569, P=0.04). At 500 Hz, however, the head vibrations induced by threshold-level sound stimuli were significantly lower than those induced by threshold-level vibration stimuli (*t*-test, *t*=–3.898, P=0.0059).

The head vibrations at sound pressure thresholds plotted relative to head vibrations at vibration thresholds of the three individual axes can be seen in Fig. 11C–E. Relative head vibrations along the *x*-axis (the medial axis) were significantly greater than zero at 80, 160 and 250 Hz (*t*-test, 80 Hz: *t*=7.166, P=0.0001; 160 Hz: *t*=2.481, P=0.05; 250 Hz: *t*=2.848, P=0.03). Along the *y*-axis (the lateral axis), the relative head vibrations were significantly greater than zero at 80 Hz (*t*-test, *t*=3.323, P=0.0105). Along the *z*-axis (the dorsoventral axis), relative head vibrations were significantly less than zero when exposed to a pure tone of 500 Hz (*t*-test, *t*=–3.510, P=0.0099).

To assess the amount of head vibration induced by sound pressure, we calculated the transfer functions between sound and head vibrations for the snakes (Fig. 12). Vibration amplitudes
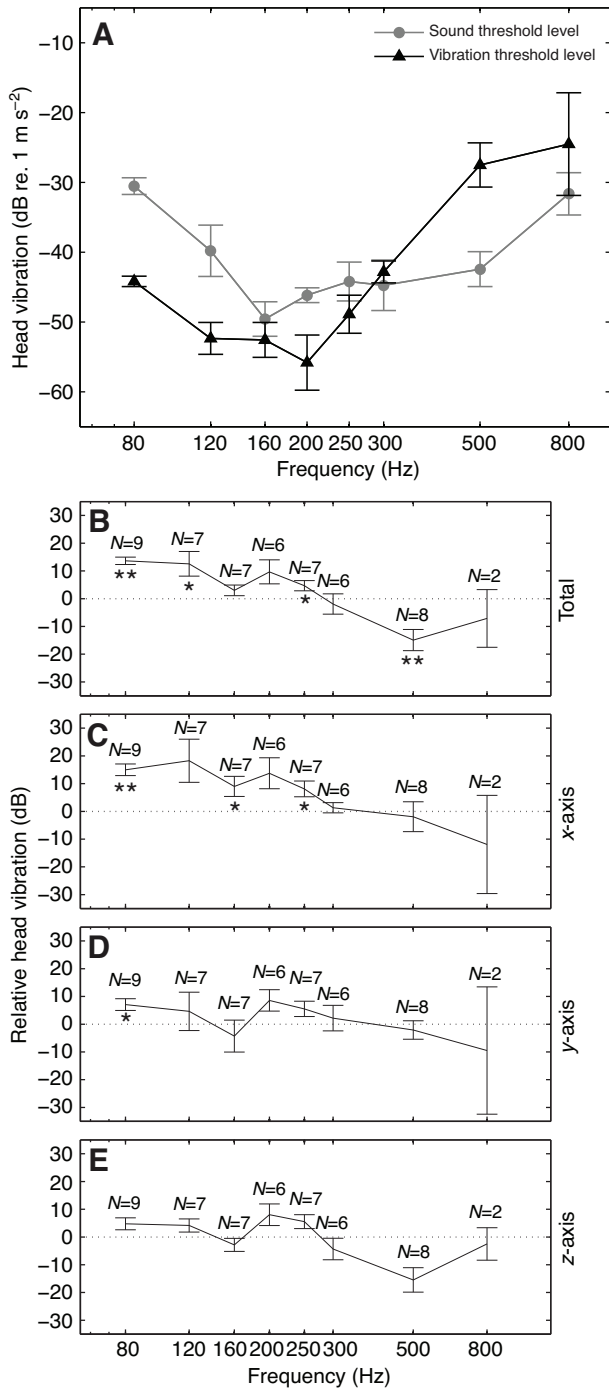
Fig. 11. Overall head vibrations, and head vibration in all three dimensions individually. (A) Overall head vibrations at sound-pressure thresholds (filled circles) and vibration thresholds (filled triangles). (B) Overall head vibrations at sound-pressure thresholds relative to head vibrations at vibration thresholds. (C–E) Head vibrations at sound-pressure thresholds relative to head vibrations at vibration threshold along the *x*-, *y*- and *z*-axes. Asterisks indicate statistical significance (*t*-test, *P<0.05; **P<0.01). Data are means ± s.e.m.; *N*-values are shown above each data point.

constant assuming that it is the same neurons or higher nuclei that respond to both types of stimuli. This assumption is probably reasonable in snakes, as the response to sound pressure can be masked by vibrating the head of the snake (Hartline, 1971).
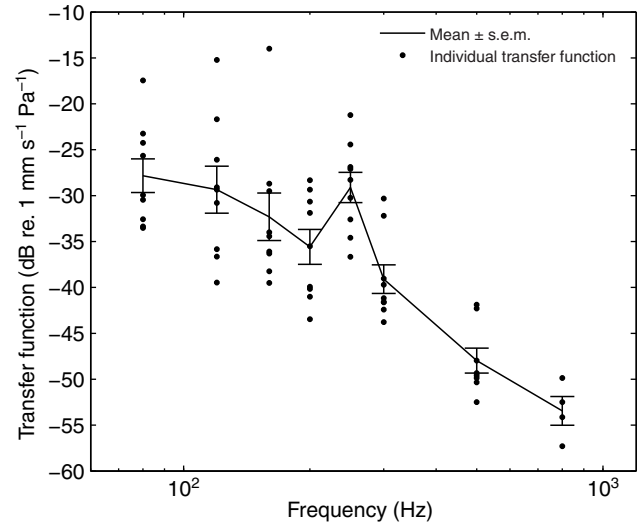

Fig. 12. Transfer functions between sound and velocity of the snake head.

When determining sensitivity of an animal to a stimulus, it is crucial to quantify the ambient noise to ensure that masking is not affecting the derived thresholds and thereby flattening the sensitivity curve. We quantified ambient noise levels in one-octave bands around all tested frequencies for both sound and vibration and measured the ambient vibration noise levels in all three dimensions. Because all thresholds were 39 and 8 dB above octave noise levels for the audiograms and vibrograms, respectively, sensitivity curves in the present study should not be limited by ambient noise for either sound or vibration. However, we cannot preclude that vibration thresholds at 40 Hz were affected by background noise, as the decrease in sensitivity seemed to follow the increase in noise level at this frequency (Fig. 7).

Hearing and vibration sensitivity

To test the hypotheses that (1) snakes are sensitive to sound pressure and (2) snakes are sensitive to vibrations but cannot hear the sound pressure *per se*, we examined the vibration and sound-pressure sensitivities and could thereby derive a vibrogram and an audiogram. Hartline reported sensitivity of two systems: the auditory system, referring to the inner ear and the VIIIth cranial nerve, and the somatic system, referring to skin receptors (Hartline, 1971). We believe that the sensitivity curves shown in the present study are of the auditory system only, as the signal disappeared with little change in position of the electrode with respect to the VIIIth cranial nerve. Further, snakes used in the present study were juveniles and so best frequencies for adult snakes could be expected to be lower, as the resonance frequency of the snake head is expected to decrease with increasing size of the snakes.

We showed that royal pythons are very sensitive to vibrations at frequencies below 1 kHz. The pythons have a U-shaped sensitivity curve with best sensitivity of -54 dB re. 1 m s^{-2} at frequencies of 80 and 120 Hz (Fig. 7). This is comparable to the sensitivity of rattlesnakes found by Hartline (Hartline, 1971), with best sensitivity of approximately -62 dB re. 1 m s^{-2} at 200 Hz (Fig. 13).

Two of the most prominent substrate vibrations are Lowe and Rayleigh waves, which contain most energy below 1 kHz with a peak at 340–370 Hz when propagated in sand (Aicher and Tautz, 1990). The frequency range and sensitivity of the snakes seem well adapted to detect these types of substrate vibrations. The
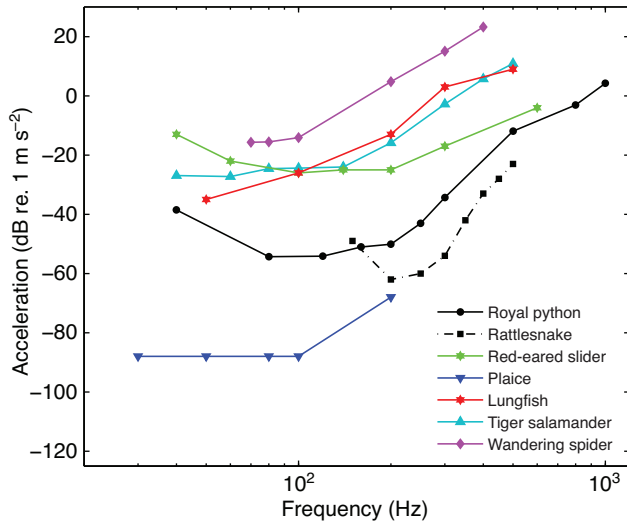
Fig. 13. Vibration sensitivity of royal python (*Python regius*, present study), rattlesnake [*Crotalus viridis* (Hartline, 1971)], red-eared slider [*Trachemys scripta elegans* (J.C.-D., C. E. Carr, P.T.M., C.B., K. Willis, C.B.C., D. Ketten, P. Edds-Walton and R. R. Fay, in preparation)], plaice [*Pleuronectes platessa* (Chapman and Sand, 1974)], lungfish [*Protopterus annectens* (Christensen-Dalsgaard et al., 2011)], tiger salamander [*Notophthalmus viridescens* (Ross and Smith, 1979)] and wandering spider [*Cupiennius salei* (Barth, 1986)]. Some of the differences in sensitivity may be caused by the different methods used.
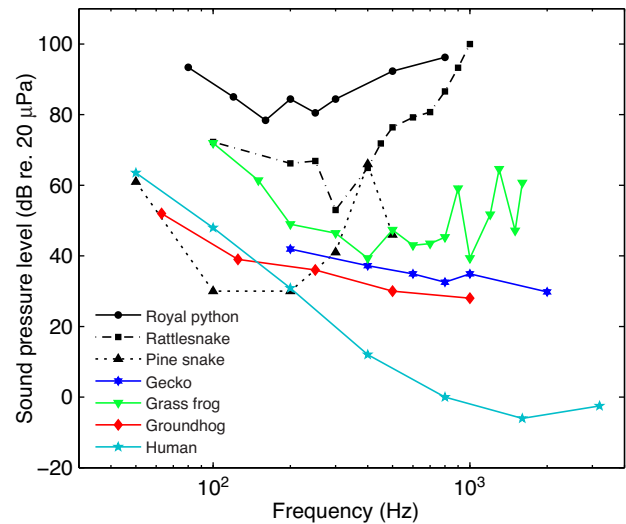

Fig. 14. Audiograms from the present study (*Python regius*) and previous studies on snakes [*Crotalus viridis* (Hartline, 1971) and *Pituophis melanoleucus* (Wever and Vernon, 1960)] and other animal groups. Reptiles: gecko [*Gekko gecko* (Brittan-Powell et al., 2010)]; amphibians: grass frog [*Rana temporaria* (Christensen-Dalsgaard et al., 1998)]; and mammals: groundhog [*Marmota monax* (Heffner et al., 2001)] and humans (Von Békésy, 1960). Some of the differences in sensitivity may be caused by the different methods used.

pronounced vibration sensitivity could enable snakes to use vibrations as a source of information regarding potential prey, predators and conspecifics, and for communication, as reported for many other animals (e.g. Aicher and Tautz, 1990; Caldwell et al., 2010; Hill, 2009). In one of the few studies on snake vibration detection, Young and colleagues (Young et al., 2000) reported a maximum detection distance to mice of 128 cm, from which Young concluded 'that vibration detection may only be critical to predation under special circumstances' [p. 311 of Young (Young, 2003)]. However, vibration detection and its use remain to a large degree an unexplored branch in the field of snake sensory physiology. In comparison to the vibration thresholds found for snakes, Christensen-Dalsgaard and Narins reported vibration thresholds of single fibers in the amphibian papilla in the range of –80 to –40 dB re. 1 m s^{-2} in a study of the northern grass frog (*Rana pipiens pipiens*) and the white-lipped frog (*Leptodactylus albilabris*) (Christensen-Dalsgaard and Narins, 1993). So, taking the possible 10–30 dB offset of thresholds found by evoked potential into account, snakes may be as sensitive to vibration as frogs. Furthermore, it has been hypothesized that snakes are able to detect the direction of vibration signals by differential vibration of the two sides of the lower jaw (Friedel et al., 2008).

The evoked potentials show that the royal python has poor sound-pressure sensitivity and can only detect high-intensity sound below 1 kHz. Pythons have a rather flat audiogram with a best sensitivity of 78 dB re. 20 µPa at 160 Hz, which is consistent with the expectations from the morphology of the ophidian auditory system predicting poor pressure sensitivity because of the lack of an impedance-matching tympanic ear. We thus confirm that snakes can detect aerial sound, but because the sound-pressure sensitivity of the royal python is poor and limited to low frequencies, we reject the first hypothesis, that pythons are

specifically sensitive to sound pressure. Comparison of the snake audiograms of Hartline (Hartline, 1971) and Wever and Vernon (Wever and Vernon, 1960) with our data reveals large discrepancies in the auditory sensitivity (Fig. 14). The audiogram found by Hartline (Hartline, 1971) is 10–30 dB below the thresholds found in the present study, although the shape is comparable. This difference may be due to interspecific differences in hearing sensitivity, but it may also (perhaps more likely) reflect that thresholds found by evoked potentials are usually approximately 10–30 dB higher than thresholds found by single-cell recordings or behavioral studies (Brittan-Powell et al., 2010). Thus, the hearing sensitivity found in the present study might be comparable to that found by Hartline (Hartline, 1971), which still is quite insensitive. The thresholds found by Wever and Vernon (Wever and Vernon, 1960), however, were up to 60 dB lower than those from the present study at low frequencies, and indicated that snakes have good sound-pressure sensitivity. In fact, Wever and Vernon (Wever and Vernon, 1960) found sensitivities of pine snakes (*Pituophis melanoleucus*) comparable to those of geckos [*Gekko gecko* (Brittan-Powell et al., 2010)], groundhogs [*Marmota monax* (Heffner et al., 2001)] and humans (Von Békésy, 1960) at low frequencies indicating that despite of their lack of a tympanic ear, pine snakes can seemingly detect sound pressure very well at low frequencies (Fig. 14).

This large discrepancy between our data for pythons and those of the pine snakes raises the question of whether the loss of a functional tympanic ear in snakes in fact has any significance for sound pressure hearing. The different thresholds for snakes could reflect interspecific differences, but given that all snakes share the complete lack of a tympanic ear, such a consistent absence of impedance matching structures is hard to reconcile with a species-specific 60 dB difference in auditory sensitivity. That, in turn, raises the possibility that at least part of the difference is due to the different

methodologies between researchers and not to actual differences in the hearing capabilities of different snake species. It is likely that Wever and Vernon (Wever and Vernon, 1960) may have underestimated the vibrations of their setup during sound experiments, which in turn would lead to a significant overestimation of hearing thresholds.

Sound-induced vibrations of the substrate and the head

Because sound stimuli induce vibrations when impinging on an object, it is not a trivial task to examine sound-pressure sensitivity in animals that are very sensitive to vibrations. To test the second hypothesis that snakes are sensitive to vibrations but cannot hear the sound pressure *per se*, we quantified the sound-induced substrate and head vibrations to assess whether these were intense enough to be detected by the snakes. Such a test is crucial to avoid reporting sensitivity to an inadequate stimulus, i.e. reporting vibration sensitivity as sound-pressure sensitivity. To ensure that vibrations were not underestimated, we measured both substrate and head vibrations induced by sound pressure and vibration in all three dimensions, so the reported total vibrations are the vector norm of the three dimensions. A popular view has been that snakes detect aerial sound by detecting sound-pressure-induced substrate vibrations, which are transmitted to the inner ear *via* the jawbones. Hartline (Hartline, 1971) and Wever and Vernon (Wever and Vernon, 1960) tested this hypothesis and reported that substrate vibrations were not intense enough to explain the recorded response to sound pressure as vibration detection. They did not, however, go into detail on how they measured the substrate vibrations or whether they measured the vibrations in all three dimensions; therefore, they may have underestimated the true vibration magnitude.

Sound caused low-level substrate vibrations at all frequencies (Fig. 9A), but only the sound-induced substrate vibrations at 80 Hz were significantly above the vibration thresholds of the snakes in the present study. Furthermore, the substrate vibrations induced by sound pressure levels at threshold were not significantly lower than the vibration threshold at 120 Hz (Fig. 9B). This indicates that the evoked potentials recorded at 80 and 120 Hz may be elicited by the sound-induced substrate vibrations of the experimental setup.

In addition to substrate vibrations, sound exposure may also cause vibration of the snake body itself. Quantification of head vibrations show that the head vibrations induced by threshold-level sound pressures are within the vibration sensitivity range of the snakes at all frequencies except 500 Hz (Fig. 11). This is the outcome both when considering the overall head vibrations (Fig. 11B) and when considering vibration in the vertical axis alone (Fig. 11E). These findings conflict with those of Hartline, who found that the sound-induced head vibrations were not sufficient to elicit the neural response seen at sound-pressure thresholds (Hartline, 1971). Hartline (Hartline, 1971) did, however, only examine the vertical axis and, therefore, may have overlooked significant head vibrations in the two other axes, which may also have contained considerable vibration magnitudes (Fig. 10A). We therefore suggest that at least part of the discrepancy between our and previous measured hearing thresholds for snakes relates to the degree to which the substrate and the head may have been vibrated when exposed to sound. It is therefore plausible that the good hearing sensitivities of the pine snakes in the study of Wever and Vernon (Wever and Vernon, 1960) may in fact stem from resonances in the experimental setup, and that the snakes may have been responding to vibration rather than sound pressure.

Either way, in the present study, the high sound-pressure hearing thresholds can be explained by sound-induced head vibrations alone, leading us to conclude that pythons do not hear sound pressure *per se*, but instead use their vibration sensitivity to detect airborne sound *via* sound-induced head vibrations. Our findings explains the early report of Manning (Manning, 1923), who found that coupling of vibrations from the speaker to the setup increased the response frequency range of the snakes, and is also consistent with the predictions from the morphology of the atympanate snake heads. Wever and Vernon suggested that the quadrate bone acts as a receiving surface for aerial sounds, and communicates its movements to the cochlear duct through the columella and the fluid of the inner ear (Wever and Vernon, 1960). Hartline showed that there are only minor differences in the frequency response for head vibrations and columella vibrations, also indicating that the columella plays an active role in transmission of vibration to the cochlea (Hartline, 1971). In light of the present results, it therefore seems that the end organs of the snake cochlear duct, the papilla and the lagenar macula (Wever, 1978), are sensitive to vibrations rather than to sound pressure because of the lack of a functional tympanum and an impedance-matching middle ear.

Our results suggest that the evolutionary history of snakes, with the loss of the outer ear and the tympanum, has rendered them unable to detect sound pressure *per se*. This reinforces the importance of the tympanic middle ear in pressure hearing, and is consistent with the evolution of a tympanum in all the tetrapod lineages. It also implies that the early tetrapods, which were atympanate, were quite insensitive to sound pressure (Christensen-Dalsgaard et al., 2011). According to several hypotheses, the ancestors of snakes lost the tympanum as a secondary reduction because of a fossorial or aquatic lifestyle (Manley, 2010; Walls, 1940; Caldwell and Lee, 1997; Tchernov et al., 2000), where it would be less costly for snakes to dispense with a tympanic ear because of the reduced importance of impedance matching. However, it is important to note that a modified middle ear can still be useful in aquatic animals. Underwater hearing in the aquatic clawed frog and the red-eared slider turtle is greatly facilitated by a modified tympanic ear (Christensen-Dalsgaard and Elepfandt, 1995; J.C.-D., C. E. Carr, P.T.M., C.B., K. Willis, C.B.C., D. Ketten, P. Edds-Walton and R. R. Fay, in preparation). Therefore, a reduction of the tympanic middle ear because of a fossorial lifestyle, with development of a feeding mechanism that often involves dislocation of the lower jaw (Berman and Regal, 1967) and an alternative stimulation route *via* the quadrate to the inner ear, seems most convincing.

Role of the tympanum

To evaluate the costs of dispensing with a tympanic ear, the auditory sensitivities found in the present study were plotted along with audiograms found in former studies of snakes and other animal groups (Fig. 14). The royal python is approximately 40 dB less sensitive to sound pressure than geckos [*G. gecko* (Brittan-Powell et al., 2010)], groundhogs [*M. monax* (Heffner et al., 2001)] and humans (Von Békésy, 1960), which all possess a tympanic middle ear.

The discrepancy between hearing sensitivities in the present study and those previously reported from other animal groups indicates that the lack of a tympanic ear comes at the cost of a reduction in hearing sensitivity by some 40 dB or more, as would be expected from the impedance mismatch between the air and tissue or inner-ear fluids. To assess the ability of the snakes to transform sound pressure into tissue motion, compared with animals with a

tympanum, we computed the transfer function between sound and the vibration of the snake's head (Fig. 12). For all frequencies, the transfer function was below $-27\,dB$ re. $1\,mm\,s^{-1}\,Pa^{-1}$, and at $160\,Hz$ it was $-32\,dB$ re. $1\,mm\,s^{-1}\,Pa^{-1}$. In comparison, Christensen-Dalsgaard and Manley reported a transfer function of $2\,dB$ re. $1\,mm\,s^{-1}\,Pa^{-1}$ between sound and the tympanum at the best frequency of $1.8\,kHz$ in the gecko (Christensen-Dalsgaard and Manley, 2005). Hence, sound induces $34\,dB$ less vibration to the snake's head than to the tympanic membrane of the Tokay gecko at the best frequency, consistent with the measured $40\,dB$ reduction in hearing sensitivity. However, it should be remembered that the transfer function in the gecko tympanic ear is much smaller at the low frequencies relevant for snakes. The laser vibrometry measurements reported by Christensen-Dalsgaard and Manley (Christensen-Dalsgaard and Manley, 2005; Christensen-Dalsgaard and Manley, 2008) are very noisy at low frequencies, but it seems safe to assume a reduction by at least $20\,dB$ at low frequencies compared with the peak frequency. Therefore, at very low frequencies, the tympanic ear in those animals is not very effective (Christensen-Dalsgaard and Carr, 2008), and that in itself could lead to changes of the tympanic ear in animals specializing in low-frequency hearing.

Wever and Vernon found that removal of the quadrate reduced the hearing sensitivity by 10 to $18\,dB$, supporting a role for the quadrate in detection of aerial sound (Wever and Vernon, 1960). This was interpreted by the authors as if the quadrate serves the function of the tympanic membrane of other reptiles, birds and mammals. We pose the hypothesis that the quadrate does not function like the tympanum, i.e. detecting differences in sound pressure, but rather that snakes detect aerial sounds when the quadrate, being in contact with the substrate *via* the lower jaw, is accelerated differentially with respect to the rest of the head, leading to movements of the columella and hence to motion in the perilymphatic fluid.

Conclusions

We conclude that pythons, and possibly all snakes, can hear, but that they lost effective pressure hearing with the loss of a functional outer and middle ear. Instead, snakes have maintained or developed vibration sensitivity as good as that found in any terrestrial vertebrate, enabling them to maintain insensitive sound detection *via* sound-induced head vibrations. The loss of effective pressure hearing is reinforced by the observation that the same sensory cells detect both substrate vibrations and aerial sounds (Hartline, 1971).

The high thresholds to sound pressure measured in the present study suggest that sound-pressure hearing may be of little biological relevance to pythons in the detection of prey or predators, or in interspecific communication. Usually, the frequency of maximum sensitivity of the audiogram matches the peak energy of vocalizations, but in snakes the maximum sensitivity is at much lower frequencies than their sound production (Young, 2003). The peak energy of snake sounds seems to match the sensitivity of lizards (Brittan-Powell et al., 2010), perhaps as a relic from the nearest common ancestor of these two groups. Therefore, snake vocalizations are likely directed at mammalian or bird predators and not towards other snakes. This further implies that snakes, with the loss of the tympanic middle ear, may also have lost the ability to communicate with conspecifics by means of sound pressure. Their high vibration sensitivity, however, enables them to sense vibrations at very low levels, which likely serves a function in communication and for detecting predators and prey.

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