PUBLISHED

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Originally published at: http://dx.doi.org/10.1242/jeb.198184

http://hdl.handle.net/2440/121134

SHORT COMMUNICATION

Underwater hearing in sea snakes (Hydrophiinae): first evidence of auditory evoked potential thresholds

Lucille Chapuis[1,2,*], Caroline C. Kerr[2], Shaun P. Collin[2,3], Nathan S. Hart[4] and Kate L. Sanders[5]

ABSTRACT

The viviparous sea snakes (Hydrophiinae) are a secondarily aquatic radiation of more than 60 species that possess many phenotypic adaptations to marine life. However, virtually nothing is known of the role and sensitivity of hearing in sea snakes. This study investigated the hearing sensitivity of the fully marine sea snake *Hydrophis stokesii* by measuring auditory evoked potential (AEP) audiograms for two individuals. AEPs were recorded from 40 Hz (the lowest frequency tested) up to 600 Hz, with a peak in sensitivity identified at 60 Hz (163.5 dB re. 1 µPa or 123 dB re. 1 µm s^{-2}). Our data suggest that sea snakes are sensitive to low-frequency sounds but have relatively low sensitivity compared with bony fishes and marine turtles. Additional studies are required to understand the role of sound in sea snake life history and further assess these species' vulnerability to anthropogenic noise.

INTRODUCTION

Sea snakes (Elapidae; Hydrophiinae) are a highly diverse and critical component of reef and coastal ecosystems in tropical and subtropical areas of the Indian and Pacific Oceans (Rasmussen et al., 2011; Sanders et al., 2013). Some sea snake populations have experienced alarming declines in abundance over decadal time scales, highlighting the need to better understand the basic biology and conservation status of these unique marine reptiles (Udyawer et al., 2018). Particularly concerning is the drastic loss of sea snake species diversity and abundance that was reported at Ashmore Reef in the Timor Sea between 1994 and 2004 (Guinea, 2007; Lukoschek et al., 2013), which lies within a protected marine reserve. Concomitantly, the North West Shelf of Western Australia, including many reef ecosystems within the Timor Sea, has been experiencing heightened activity in petroleum exploration. Within these regions, potential oil and gas reserves are surveyed using seismic techniques, which employ arrays of airguns that produce high-intensity impulsive acoustic signals of predominantly low frequency (20–500 Hz) that may travel for many kilometres from the source (Ainslie and de Jong, 2016). The impacts of these seismic surveys on marine fauna can range from displacement from feeding or breeding areas to auditory damage, tissue trauma and mortality (Kunc et al., 2016). Behavioural responses, such as changes in vocalisation and avoidance, have also been observed in sound-sensitive marine mammals at ranges of tens or hundreds of kilometres (Gordon et al., 2003). Similarly, bony fishes can react with changes in behaviour and startle responses and, in some species, inner ear damage, a reduction in hearing thresholds and physiological signs of stress have also been demonstrated (Carroll et al., 2017). With invertebrates, airguns have elicited death of zooplankton (McCauley et al., 2017a), while rock lobsters showed damage to their primary mechanosensory organs (statocysts) (Day et al., 2016). However, some of these findings are contradictory (Carroll et al., 2017), suggesting differential responses depending on the taxon and the sound source.

Only a few studies have investigated the effects of seismic surveys on marine reptiles (Nelms et al., 2016). Caged loggerhead (*Caretta caretta*) and green (*Chelonia mydas*) turtles responded to increasing airgun intensity levels by increasing their swimming speed and displaying erratic behaviour (McCauley et al., 2000). Similarly, loggerhead turtles dived to lower depths in response to airguns during seismic surveys (DeRuiter and Larbi Doukara, 2012). To the best of our knowledge, the effects of anthropogenic noise on sea snakes have not previously been studied, and no information exists on the hearing abilities of these species. It is impossible therefore to predict and evaluate the effect of anthropogenic noise, including seismic airguns, on sea snakes without a deeper understanding of the frequency range and sensitivity of these marine reptiles.

Snakes lack both an external ear and a tympanic middle ear and thus are thought to have a reduced sensitivity to airborne sound compared with other tetrapods (Hartline and Campbell, 1969; Wever, 1978). Christensen et al. (2011) showed that the terrestrial royal python (*Python regius*) responded to low-frequency (80–160 Hz) vibration of the substrate, rather than airborne pressure variations, and suggested that all snakes may have lost pressure-transduced hearing and instead use vibration sensitivity for communication and detection of predators and prey (Hetherington, 2008). There is also the possibility that sensory systems in addition to their inner ear contribute to the detection of waterborne vibrations in sea snakes. The cephalic scales of sea snakes are covered in numerous mechanoreceptors known as scale sensillae (Crowe-Riddell et al., 2016; Crowe-Riddell et al., 2019a). Although the sensitivity of these scale mechanoreceptors is not yet known, they may effectively detect low-frequency (<150 Hz) underwater hydrodynamic stimuli produced by swimming fishes, predators or prey items, and thus enhance the auditory sensitivity of sea snakes (Westhoff et al., 2005).

In this study, we investigated the hearing abilities of Stokes's sea snake *Hydrophis stokesii* (previously known as *Astrotia stokesii*) using auditory evoked potentials (AEPs). *Hydrophis stokesii* is a

[1]Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter EX4 4QD, UK. [2]Oceans Graduate School and the UWA Oceans Institute, The University of Western Australia, Perth, WA 6009, Australia. [3]School of Life Sciences, La Trobe University, Bundoora, VIC 3086, Australia. [4]Department of Biological Sciences, Macquarie University, North Ryde, NSW 2109, Australia. [5]School of Biological Sciences, University of Adelaide, Adelaide, SA 5005, Australia.

*Author for correspondence (l.chapuis@exeter.ac.uk)

L.C., 0000-0003-3001-983X; S.P.C., 0000-0001-6236-0771; N.S.H., 0000-0002-7289-9399; K.L.S., 0000-0002-9581-268X

fully aquatic sea snake that inhabits varied shallow marine habitats from the Arabian Gulf to Australia, reaches almost 2 m in total length, and is a specialist predator of spiny benthic fishes, including toadfish (*Halophryne* sp.) (McCosker, 1975; Sherratt et al., 2018).

MATERIALS AND METHODS

Animal ethics statement

This study was carried out with the approval of The University of Western Australia Animal Ethics Committee (Application RA/3/100/1369) and in strict accordance with the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013).

Animal acquisition, maintenance and anaesthesia

Two wild individuals of *Hydrophis stokesii* (Gray 1846) were caught offshore from Broome, Australia, by dip netting from a boat. The sea snakes were transported to The University of Western Australia, where they were held in separate 1500 l recirculating seawater aquaria (at 27.5–28°C, pH ~8.0, salinity 30,000 ppm) for a maximum of 12 weeks.

Snakes were fasted for a minimum of 48 h prior to each procedure. Animals were netted from their holding tank, weighed and measured and transferred into a clear Perspex tube sealed at one end to restrain the head of the snake. The snake was then anaesthetised with an intramuscular (i.m.) injection of Alfaxan (Jurox Pty, Rutherford, NSW, Australia; 2.5 mg kg^{-1} alfaxalone). The level of anaesthesia was monitored until the animal reached an anaesthetic stage suitable for minor surgery (Sladky and Mans, 2012). To prevent hypoxia due to apnoea, the animal was intubated with an endotracheal tube and artificial respiration was performed manually at regular intervals during the whole procedure. Because of the fragile nature of the lung and air sacs, extreme care was taken to prevent over-inflation during assisted ventilation. Once the required stage of anaesthesia was reached, the subject was transferred to the test chamber and secured in a sling 200 mm below the water surface. Maintenance injections of Alfaxan were performed if required (maintenance dose 1.0–1.5 mg kg^{-1} i.m.). An observer monitored the depth of anaesthesia at 5 min intervals between recordings by evaluating muscle tone and reflexes and using a Doppler ultrasonic monitor to assess heart rate. The level of anaesthesia and oxygen supply were adjusted and administered accordingly. Recovering animals were manually ventilated with 100% oxygen until breathing spontaneously and were returned to their holding tank only when full muscle control was regained.

Recording of the AEP

Hearing responses were determined for each individual by measuring AEPs in response to tonal stimuli at a known sound pressure level (SPL) and particle acceleration level (PAL). AEPs were recorded in a test chamber which consisted of an upright Schedule 60 steel pipe, 1 m high and 0.37 m internal diameter (Fig. 1). The steel pipe was designed to create a standing wave, as previously described in Hetherington and Lombard (1983, 1982) and Christensen et al. (2015). The pipe was sealed at one end with a steel plate that formed the base of the chamber. The chamber was positioned on a platform that rested on rubber car tyres to reduce the transmission of structural vibration from the floor of the building. The test chamber was filled with the same seawater as the holding tank to a height of 800 mm. Water temperature was held at ~28°C throughout the recordings. The test chamber was mounted inside a plywood enclosure that was lined with foil to minimise electromagnetic interference and with damping material to minimise airborne noise. An underwater speaker (Diluvio, Clark Synthesis; frequency response 20 Hz to 17 kHz) was placed at the bottom of the pipe on rubber mounts. A PVC pipe frame (cradle) was placed inside the chamber to support the snake but was isolated from the chamber with patches of rubber to reduce the transmission of vibrations (Fig. 1). The body of the snake was coiled to fit in the pipe frame and secured in place with Velcro straps. The head was strapped in place in the cradle, flat just below the surface and perpendicular to the pipe. Stainless steel needle electrodes (25 mm×29 gauge) were used to record the AEP signals. A recording electrode was inserted subdermally into the dorsal surface of the animal, directly over the brainstem and the ears. A reference electrode was inserted into the dorsal musculature. A ground electrode was placed directly into the water near the sea snake (Fig. 1).

Sound stimuli were produced and AEP waveforms recorded using a National Instruments (NI, Austin, TX, USA; USB 6353 X-Series) data acquisition system controlled by custom-written software (scripts written in Microsoft Visual Studio 2015 by N.S.H. using NI Measurement Studio DaqMX libraries). Sound stimuli were created by generating a voltage output from the USB 6353, which was subsequently passed through a Pi-pad passive attenuator to control acoustic stimulus intensity in steps of 1 dB. The resultant signal was amplified using a power amplifier (Response Precision AA0452) connected to a Diluvio underwater sound transducer (Fig. 1). Evoked biopotentials were amplified (10,000 times) and bandpass filtered (10 Hz to 3 kHz) with an AC-coupled differential amplifier (DAM50; World Precision Instruments, Sarasota, FL, USA). The system was implemented on a HP ProBook 6570b laptop computer.

Test sound stimuli were chosen following preliminary experiments (AEPs previously measured on two other individuals of the same species to test the setup and refine the anaesthetic regime) which showed that AEPs were recorded only with stimuli below 1000 Hz, i.e. sinusoidal tone bursts at fundamental frequencies of 40, 60, 80, 100, 200, 300, 400, 500, 600, 800 and 1000 Hz. All bursts were windowed in the time domain using a 2 ms Hanning window to reduce spectral leakage and provide a ramped onset and decay (Kenyon et al., 1998). The number of cycles in a tone burst was adjusted according to frequency in order to obtain the best compromise between stimulus rapidity and frequency bandwidth (Silman and Silverman, 1991). Three cycles were used for 40–200 Hz tone bursts, and five cycles for 500–1000 Hz (Fig. S1). Tones were presented in one phase, then in the other phase, and this stimulus pair was repeated for averaging. The 40 and 60 Hz tones were presented within a 200 ms recording epoch and were averaged over 300 repetitions (of the stimulus pair), while the other frequencies (100–1000 Hz) were presented within a 100 ms recording epoch and were averaged over 500 repetitions. The number of repetitions was adapted for best compromise in time efficiency and the ability to extract the signal from the noise floor. Mean AEPs corresponding to each phase of the tone were subtracted from one another to remove electrical or inductive electrical artefacts caused by the speaker. Experiments were also conducted with two deceased sea snakes to confirm that identified AEP responses were not stimulus artefacts.

A descending threshold search protocol started at an intensity level that was expected to elicit a detectable response based on preliminary studies (data not shown). If there was no response at that intensity level, the protocol was set to increase the intensity. After response detection, the sound level was decreased by 1 dB until a
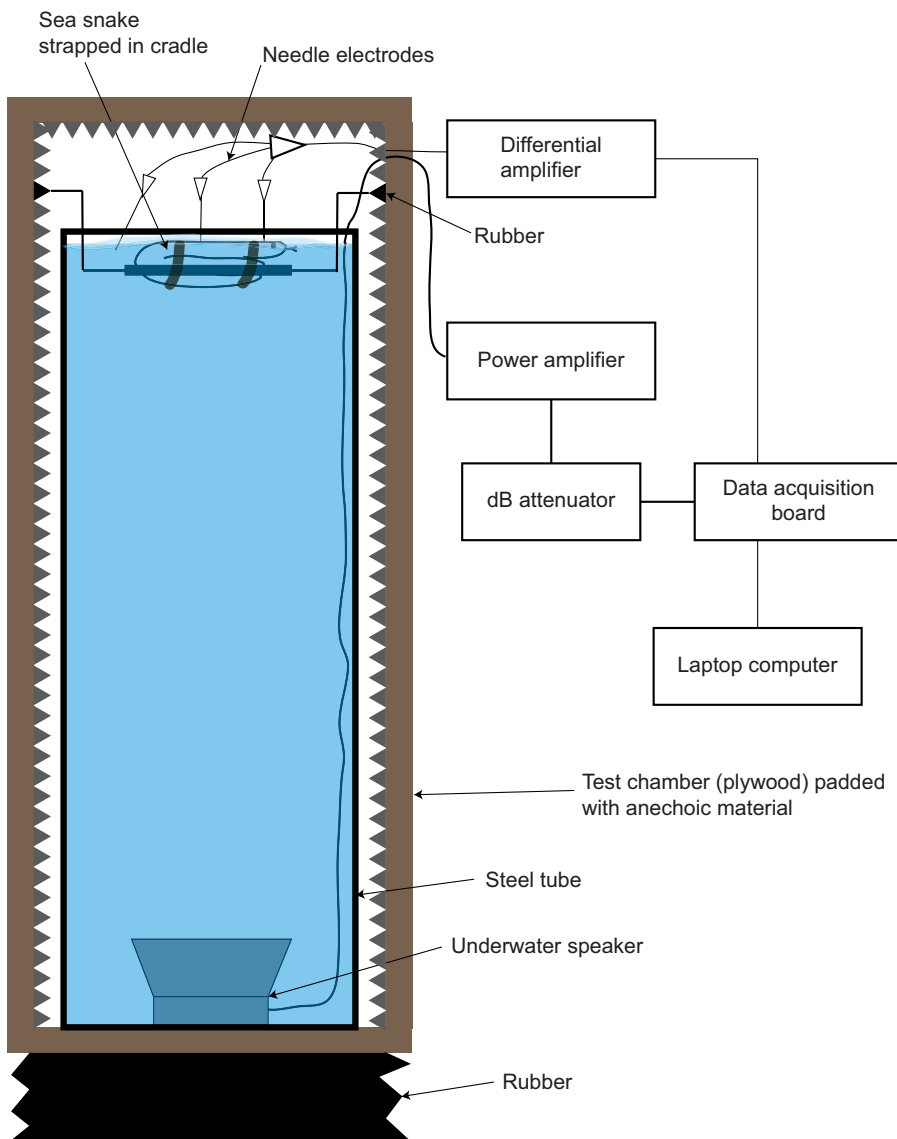
Fig. 1. Auditory evoked potential (AEP) test chamber and setup. The wooden chamber enclosed a 1 m high steel pipe, with an underwater speaker placed at the bottom and was padded with anechoic material. Two electrodes (recording and reference) were sub-dermally inserted into a restrained sea snake, and a ground wire was placed loose in the water. The whole structure was positioned on a rubber base to isolate the tube from any floor vibrations.

response was no longer identified. Auditory thresholds were defined visually as the minimum sound level that elicited a detectable AEP response above the background level of brain activity. Although objective methods to detect thresholds are sometimes preferred (Dobie and Wilson, 1989, 2005), visual detection has been shown to produce comparable results to the use of statistical approaches (Brittan-Powell et al., 2002; Higgs et al., 2004; Mann et al., 2001). One individual was tested twice, with 1 week recovery between trials. In that case, the threshold values were averaged for the two trials. The other individual was tested only once.

Sound calibration
Both particle acceleration and sound pressure were calibrated in the test chamber in the absence of a sea snake. Particle acceleration was estimated by measuring the pressure gradient over two closely spaced sound receivers (Gade, 1982). Two hydrophones (HTI 90U, High Tech, Inc., Long Beach, MS, USA), with responses that were considered linear from 2 Hz to 20 kHz, were vertically spaced 11 cm apart, and fixed at the location of the sea snake head. System gain was estimated with a white noise calibrator of known level (McCauley et al., 2017b).

Acoustic stimuli from all frequencies and intensity levels used for the study were measured with the hydrophone array subsequently oriented in all three orthogonal directions. Consistent with previous studies on aquatic vertebrate hearing (Casper and Mann, 2006; Horodysky et al., 2008; Wysocki et al., 2009), the x-axis was considered to be anterior–posterior along the animal's body, the y-axis was considered to be lateral (right–left) relative to the animal, and the z-axis was considered to be vertical (i.e. up–down) relative to the animal. The root mean square (rms) amplitude of the largest single cycle (360 deg) in the hydrophone recording was used as the calibrated SPL in dB re. 1 µPa. Background noise was also measured and its particle acceleration, although variable, was consistently below 1×10^{-6} m s^{-2} (SPL rms <100 dB re. 1 µPa) (Fig. S2).

RESULTS AND DISCUSSION
Hydrophis stokesii responded to frequencies from 40 Hz (lowest frequency tested because of the limits of the underwater transducer) up to 600 Hz, with a peak in sensitivity at 60 Hz (163.5 dB re. 1 µPa or 123 dB re. 1 µm s^{-2}) and another peak between 300 and 500 Hz (individual 1: 300 Hz, 169.1 dB re. 1 µPa or 128.6 dB re. 1 µm s^{-2}, individual 2: 500 Hz, 162.1 dB re. 1 µPa or 122.2 dB re. 1 µm s^{-2})
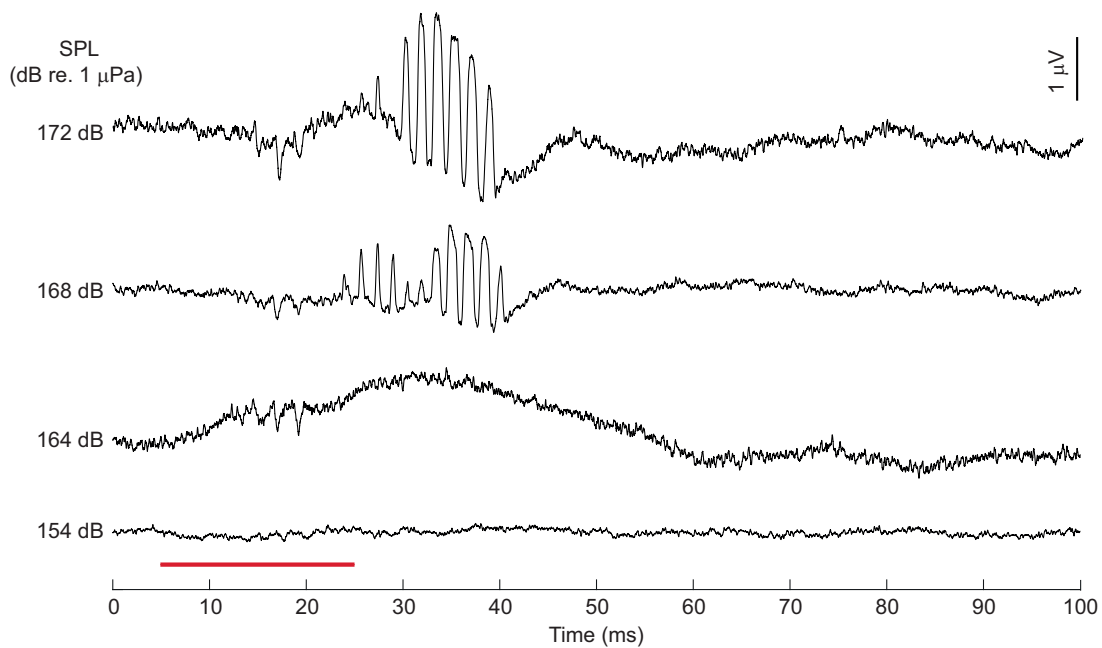
Fig. 2. AEP waveforms of one individual *Hydrophis stokesii* responding to 300 Hz tone burst stimuli. Stimuli were given at a sound pressure level (SPL) of 154–172 dB re. 1 µPa. The red horizontal bar above the time axis indicates a stimulus duration of 30 ms (starting after a 5 ms pre-stimulus interval). Response amplitude decreased as stimulus intensity decreased.

(Figs 2 and 3). The evoked potential to sinusoidal tone bursts consisted of an asymmetric wave and the latency of stimulus onset to response depended upon the frequency and intensity of the stimuli (Fig. 2). Evoked potentials were assumed to be from biological events in response to the sound, rather than electrical or mechanical artifacts, because no evoked potentials could be recorded from deceased snakes or without the sound source playing underwater, and the responses appeared with a notable latency of stimulus onset. In Fig. 2, the response stimulus is clearly present from 25 ms. The first apparent signal (15–20 ms) may be an artefact created by the underwater speaker or a biological response. The two different signals may be caused by a response from two different sensory pathways, i.e. the scale mechanoreceptors distributed over the head (Crowe-Riddell et al., 2016, 2019a) and the inner ears. Thresholds were successfully determined for both SPL and PAL based on one full protocol completed for one snake and two full protocols repeated after a 1 week interval for the other individual (Fig. 3).

Overall, our findings suggest that *H. stokesii* (and probably other sea snakes) present a relatively low hearing sensitivity compared with other marine vertebrates, in terms of both sound pressure and particle acceleration. In general, bony fishes detect sound pressure at around 80 dB re. 1 µPa and respond to frequencies up to 3 kHz (Ladich and Fay, 2013). Sea turtles can only detect frequencies from 50 to 800 Hz, with best sensitivity at 100 Hz, but possess a higher sensitivity (98 dB re. 1 µPa at 100 Hz) than *H. stokesii* or other snakes previously tested (Martin et al., 2012). Wever (1978) found measured cochlear potentials in response to airborne sounds in the sea snake *Hydrophis* (previously *Pelamis*) *platurus* and measured responses over a range from 30 to 5000 Hz, with the highest sensitivity below 100 Hz, but poor sensitivity overall compared with other snakes. The terrestrial snake *P. regius* showed highest sensitivity to sound stimuli from 80 to 160 Hz at 78 dB re. 20 µPa (Christensen et al., 2011), which represents a comparatively higher sensitivity than found here in *H. stokesii*, even if considering the difference in reference (air versus water).

These results appear to confirm that snakes, including sea snakes, can hear but may have a limited sensitivity to sound pressure compared with previously studied marine vertebrates (Christensen et al., 2011). The PAL recorded at best threshold sensitivity (123 dB re. 1 µm s^{-2} at 60 Hz) was also higher than in bony fishes, which typically show threshold levels between 30 and 70 dB re. 1 µm s^{-2} (Ladich and Fay, 2013). However, this relatively low sensitivity would still allow sea snakes to detect high-amplitude sounds (e.g. air gun blasts) and vibrations in the substrate, in addition to hydrodynamic stimuli (Westhoff et al., 2005).

The range in sensitivity of *H. stokesii* overlaps with most sources of anthropogenic noise, including shipping (<1000 Hz), pile driving (400 Hz) and seismic airgun arrays (20–500 Hz) (Ainslie and de Jong, 2016; Hildebrand, 2009). The sound level of one airgun can peak at 230 dB re. 1 µPa rms at 1 m from the source, and therefore could affect sea snakes located in close proximity to regions being surveyed by seismic activity. Ultimately, the area over which a seismic survey may affect these animals will depend on many factors, including the extent of sound propagation underwater, which is relative to depth, properties of the local waters (e.g. pH, temperature) and the geochemical characteristics of the substrate.

Because it lacks a tympanic middle ear, the terrestrial snake auditory system is thought to respond mostly to low-frequency sound and vibrations from the substrate (Hartline and Campbell, 1969; Wever, 1978). Vibrations are conducted through the snake's jawbone, relaying a signal to the brain via the inner ear (Christensen et al., 2011; Wever, 1978). Underwater, vibrations at a similar magnitude from a moving object in the water column, or directly transmitted through the substrate, could elicit a 'hearing' response from the inner ear. It is also possible that the first peak in sensitivity found in *H. stokesii* (at 60 Hz, Fig. 3) was driven by the scale mechanoreceptors distributed over the head rather than the inner ear, which could be driving the second peak found at higher frequencies (300–500 Hz) with higher inter-individual variability. In this case,

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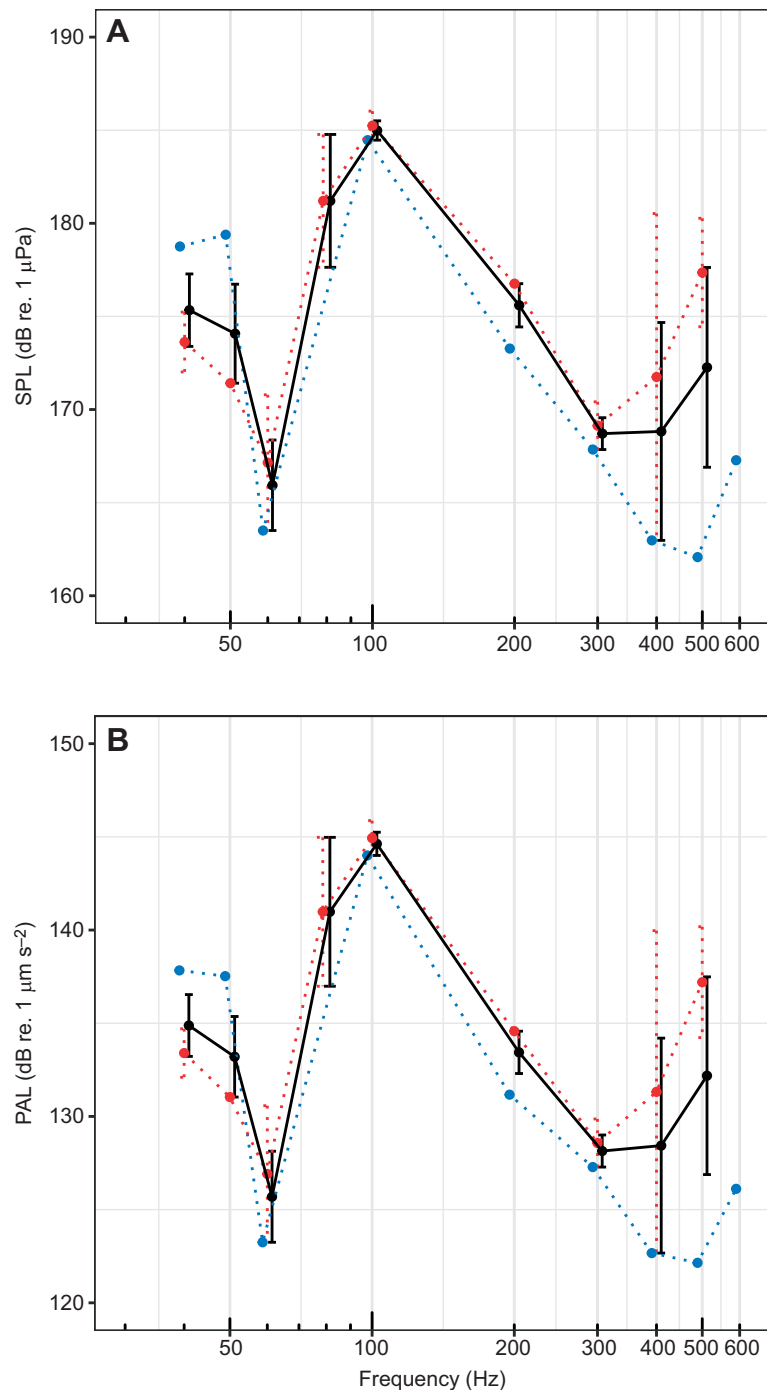
Fig. 3. Sound pressure level (SPL) and particle acceleration level (PAL) audiograms for two individuals of *Hydrophis stokesii*. (A) SPL and (B) PAL over a frequency range from 40 to 600 Hz. The black line shows the mean (±s.e.m.) threshold. The red dotted line shows the average of the results of two trials on the same individual. Only one trial was performed on the other individual (blue dotted line).

both the ear and the scale organs may play an integrative role in detecting 'acoustic' stimuli.

Our experimental setup did not allow us to separate the components of sound (pressure and particle acceleration) when testing for hearing capability. It is possible that the recorded evoked potentials were elicited from pressure alone, particle acceleration alone, or a combination of pressure and particle acceleration. Sea snakes possess a right lung that extends almost the entire length of their body (Graham et al., 1975), allowing them to remain submerged for long periods, and some species perform deep (>200 m) dives (Crowe-Riddell et al., 2019b). The sea snake lung could also act as a pressure detector underwater, similar to the swim bladder of bony fishes. The fact that we intubated and supplied oxygen to the lungs in

between AEP recordings may have affected the animal's detection of pressure waves. However, a similar process would be expected to occur in natural conditions because the lung is compressed during dives and prolonged submersion (Graham et al., 1975).

In this study, we showed that the sea snake *H. stokesii* can detect underwater sounds of low frequency (<600 Hz) with a relatively low sensitivity compared with other aquatic vertebrates. To further assess the acoustic ecology of sea snakes, more studies are required to discriminate whether sea snakes are primarily responsive to sound pressure or particle motion. As sea snakes are ecologically very diverse, it will be critical to explore the auditory sensitivity of other sea snake species, and to examine the hearing abilities of sea snakes at different life stages. To assess the potential influence of

anthropogenic noise on sea snake persistence, future work should focus on assessing the individual and population-level responses of these animals to noise, and the effect of noise on critical behaviours (mating, feeding and antipredator behaviour), physiology (auditory threshold shift) and anatomy (scale mechanoreceptors, inner ear and lung damage).

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: L.C., K.L.S.; Methodology: L.C., C.C.K., N.S.H., K.L.S.; Software: L.C., N.S.H.; Validation: L.C.; Formal analysis: L.C.; Investigation: L.C., C.C.K., K.L.S.; Resources: S.P.C., N.S.H., K.L.S.; Data curation: L.C.; Writing - original draft: L.C.; Writing - review & editing: L.C., C.C.K., S.P.C., K.L.S., N.S.H.; Visualization: L.C.; Supervision: S.P.C., K.L.S.; Project administration: L.C., C.C.K., S.P.C., K.L.S.; Funding acquisition: K.L.S.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.198184.supplemental

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