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Acoustically evoked potentials in two cephalopods inferred using the auditory brainstem response (ABR) approach

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ABSTRACT

It is still a matter of debate whether cephalopods can detect sound frequencies above 400 Hz. So far there is no proof for the detection of underwater sound above 400 Hz via a physiological approach. The controversy of whether cephalopods have a sound detection ability above 400 Hz was tested using the auditory brainstem response (ABR) approach, which has been successfully applied in fish, crustaceans, amphibians, reptiles and birds. Using ABR we found that auditory evoked potentials can be obtained in the frequency range 400 to 1500 Hz (*Sepiotheutis lessoniana*) and 400 to 1000 Hz (*Octopus vulgaris*), respectively. The thresholds of *S. lessoniana* were generally lower than those of *O. vulgaris*.

1. Introduction

It has been speculated for more than a century, whether cephalopods can hear. Baglioni (1910) observed that blind *Octopus vulgaris* responded to water movements and low frequency vibrations by behavioural changes. Fifty years later, Wells and Wells (1956) reported that blind octopus could locate the direction of a sound source which was produced by tapping on the tank. *Sepia officinalis* responded to a stimulus of 180 Hz by changing its colour (Dijkgraaf, 1961) and Maniwa (1976) convincingly demonstrated that the squid *Todarodes pacificus* could be attracted by a pure tone sound of 600 Hz which was emitted from commercial squid fishing boats. An electrophysiological approach was used by Budelmann and Bleckmann (1988) to demonstrate the detection of water vibrations ranging from 3.5 Hz to 200 Hz by the epidermal head lines of juvenile specimens of the cuttlefish *S. officinalis*, but response to higher frequencies (indicating possible underwater audition), were not observed.

Sound perception among cephalopods has been a controversial issue since the early 20th century, due ostensibly to debate regarding the definition of hearing in an aquatic environment. As most cephalopods lack gas filled chambers, such as a swim bladder and, thus, most likely cannot detect the pressure wave component of sound. However, like fish, cephalopods (Young, 1989) and shrimp (Lovell et al., 2005) have statocysts (otoliths) that in principle can be used to detect whole body motions such as those caused by the displacement component of a sound wave. Young (1960) pointed out that the statocyst might serve as a detector for vibrations, or sound, in a similar way as the vertebrate vestibular system. The cephalopod statocyst with its macula–statolith system shows many comparative features similar to the fish inner ear with the macula–otolith complex. It is well accepted that fish (Webster et al., 1992; Kenyon et al., 1998; Yan, 1998; Fay and Popper, 1999; Yan and Curtsinger, 2000; Simpson et al., 2005) and shrimp (Lovell et al., 2005) have the ability to detect acoustic underwater stimuli of a wide frequency range using either their inner ear (in fish) or statocyst (in shrimp). In these examinations the auditory brainstem response (ABR), an electrophysiological far-field recording method that was originally used in clinical evaluation of the patients' hearing ability (Hall, 1992), had been applied. The ABR technique has never been used on cephalopod species, as these animals have no real brainstem. However, they show the presence of afferents in the statocyst and existence of neural pathway terminating in the brain, indicating that the physiology of cephalopods is suitable for the recording of acoustically evoked potentials (AEPs) with the use of ABR (Williamson and Budelmann, 1985; Hanlon and Messenger, 1996). In a study on European prawn *Palaemon serratus*, the ABR technique had clearly demonstrated hearing ability via the statocysts ranging from 100 Hz to 3000 Hz by this invertebrate (Lovell et al., 2005). The results of this study prompted us to formulate a hypothesis that cephalopod may also detect sound stimuli with frequencies higher than 400 Hz.

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The goal of our study was to investigate whether AEPs can be recorded from cephalopods and whether cephalopods can hear sound frequencies above 400 Hz. To investigate the role of the statocyst in the generation of the AEPs, we also chemically ablated the statocyst function to offer the proof that hearing ability is coded by the statocyst.

2. Materials and methods

2.1. The oval squid Sepioteuthis lessoniana Lesson, 1830

Forty five specimens of mixed sex and ranging in length from 8 cm to 20 cm mantle length (mantle length is from the mantle tip to the end of the gladius superior to the head) were obtained from a local dealer. For transportation back to the laboratory 5 animals were transferred into a 20 l plastic sac and slightly anaesthetized with 0.2–0.5% $MgCl_2$ which is a widely used non-toxic anaesthetic for cephalopods (Messenger et al., 1985). Air pumps with bubble stones were installed to guarantee a proper oxygen supply and the water was kept at a temperature of 18–20 °C using ice packs inside the cooler box. As soon as they were transported to the laboratory, the animals were held in groups of 10–20 individuals per tank. The flow-through tanks had a volume of 8000 l, and the seawater flow rate was approximately 2 l/min. The seawater temperature was kept at a 28–30 °C, the salinity varied between 30 and 32 ppt. The animals were provided with 13 h of natural daylight and 1 h of fluorescent light tubes after dark. Each animal was fed daily with 10–20 palaemonid shrimp (approx. 5 cm body length) which were caught in the estuary waters near the laboratory.

2.2. The octopus Octopus vulgaris Cuvier, 1797

Ten specimens of mixed sex ranging in weight from 250–500 g were obtained from a local dealer and transported to the laboratory in 20 l plastic tanks with oxygen supply from an air pump. The animals were held in 250 l seawater flow-through tanks with one individual per tank. The flow rate was approximately 1 l/min. and the seawater was kept at a temperature of 28–30 °C. Salinity ranged from 32 to 35 ppt. Each animal was fed with 1–2 living mangrove crabs (2–3 cm carapace length) per day.

2.3. ABR methodology

For ABR measurement each test subject was anaesthetized with 1% $MgCl_2$ in a rectangular plastic tank with air supply, until their mantle movements slowed down and showed no reaction to the touch with a glass rod. Additionally the ambient water was cooled down with the placement of sealed ice packs inside the holding tank to approximately 18 °C (but not below 15 °C). Afterwards the animals were immobilized by an injection of a neuromuscular junction blocker, gallamine triethiodide (Flaxedil; Sigma-Aldrich G-8134, St. Louis, MO., USA) in a 15 mg/10 mL dilution. Flaxedil is a non-depolarising muscle relaxant. It acts by binding with the cholinergic receptor sites in muscle and competitively blocking the transmitter action of acetylcholine (Raghavendra, 2002). Flaxedil has been proven not to influence excitatory postsynaptic potentials (EPSP) in marine mollusk (Panchin et al., 1995), therefore, it is a good choice of anesthetic for use in this experiment. The injected volume was 200 µl/kg in *O. vulgaris* and 120 µl/kg for *S. lessoniana*. In both species Flaxedil was injected into the arm base. When the mantle movements almost stopped the anaesthetized animals were transferred to a holder inside a rectangular plastic tub. The holder consisted of an acrylic glass board with wholes where the animal was placed on. Additionally a soft rubber tube was used to fix the animals head slightly onto the board by firmly laying the rubber tube around the neck of the squid, the ends of the tube were placed into the holes of the board. For the octopus the rubber band was placed around the narrow connection between arms and head. Except for the fact that the animal was laying on a substrate, slightly fixed, the body could move freely, ensuring the aeration of gills by slow pumping movements. Additionally, the gills were irrigated with fresh seawater by two soft rubber tubes inserted into the mantle cavity, without destroying the cartilage lock in *S. lessoniana*. The flow rate that provided the gills with seawater was 1.5 l/min. The plastic tub was filled with seawater and equipped with an overflow drain tubing connected to a canister under the vibration-free table. The animal was positioned so that the nape of the head and dorsal parts of the mantle margin were 1–2 mm above the water surface. The whole setup was placed on a vibration isolation table (Kinetic Systems model 1201), which was enclosed in a walk-in sound-proof room (1.8 m × 1.6 m × 2.7 m). The inside of the room was covered with a fine mesh metal net, i.e., Faraday cage, to filter out noise from electric sources during recording. A rectangular piece of wet Kimwipe tissue paper was placed on the tentacles and parts of the head, to prevent them from drying out during recording.

The acoustically evoked potentials were recorded using two subcutaneous silver electrodes. The recording electrode was placed on the head between the eyes on the "donut-shaped" brain. The reference electrode was positioned on the dorso-anterior margin of the mantle. Both electrodes were pressed firmly against the skin of the test subject. The electrodes consisted of a teflon-insulated silver wire (0.25 mm in diameter) with a ca. 1 mm exposed tip. Wires were fixed with epoxy, and covered by a plastic pipette housing and clamped into micromanipulators. Shielded electrode cables (60 cm in length) were plugged into the differential inputs of an AC amplifier (Grass P-15, 40 dB gain, high-pass at 30 Hz, low-pass at 3000 Hz). The ground terminal of the preamplifier was connected via a wire to the water in the tub. To measure the sound level that was transmitted through the water to the test subjects a hydrophone (Celesco LC-10) was placed inside the tub at the same depth adjacent to the animals head. The hydrophone was connected to a Grass P-15 amplifier. Speakers were mounted 1 m above the testing tub to place the animal outside the near field in order to minimize unnecessary disturbance caused by wave actions (400 Hz; wavelength < 1 m) (Kenyon et al., 1998). For frequencies less than 3000 Hz, a 30 cm "woofer" speaker (Pioneer; frequency response 19 Hz–5 kHz) was used. Output terminals of the preamplifiers and speakers were hooked to shielded leads that routed through a junction box on the wall of the sound-proof chamber.

2.4. The ABR recording apparatus and stimulus presentation

Sound stimuli and ABR waveform recording were accomplished with a Tucker–Davis Technologies (Gainesville, FL, USA) modular rack mount system controlled by an optical cable-linked Dell OptiPlex 1.2 GHz PC containing a TDT AP 2 Digital Signal Process board and running TDT "Bio-Sig" software. Sound stimuli waveforms were generated using TDT "Sig-Gen" software, and fed through a DA1 digital-analog converter, a PA4 programmable attenuator, and a power amplifier (QSC Audio products, Model USA 370) which fed the speaker. The stimuli had a duration of 20 ms. Two thousand ABR traces of opposing polarities were recorded and averaged to avoid any stimulus artefacts. For each frequency and sound level tested, two traces were recorded and overlaid to examine conformities. The lowest sound level, where a repeatable ABR trace could be obtained, by overlaying replicate traces, was considered as the threshold. The visual method for threshold determination is the traditional method in ABR audiometry (Jacobson, 1985; Hall, 1992). Such a visual inspection of threshold determination had been proven to be effective and reliable in comparing with Spearman Rank Order correlation coefficient between two replicates (Yan, 1998).

2.5. Ablation of the statocyst during measurement of acoustically evoked potentials

For ablation of the statocyst, one specimen of the oval squid of medium size (13 cm mantle length) was chosen, and anaesthetized as described above. The test subject was placed with its ventral side up in order to get to access to the statocysts, on the holder inside the tub with fresh seawater supply. The recording electrode was placed 3 mm anterior of the statocyst and the reference electrode was positioned on the ventral anterior margin of the mantle. After recording of several electrophysiological responses to 1000 Hz stimuli (at sound levels ranging from 149.4 dB to 139.4 dB; re 1 μPa), two micro glass needles clamped into micromanipulators, were inserted into the left statocyst chamber. One glass needle was connected to a syringe via a silicone tube. For chemical ablation purpose the endolymph was sucked out of the statocyst, and with a new syringe, a volume of 1–2 μl neomycin (concentration: 0.8 mM, solvent: molluscan physiological saline) (Sanchis and Mascitti, 1970) was delivered into the cavity. Neomycin is a known ototoxicant which blocks calcium channels of auditory sensory hair cells (Yan et al., 1991; Harris et al., 2003). Therefore, the chemical ablation experiment was used to investigate the role of statocyst in audition. Afterwards ABR recordings as described above were made in several time points. The differences observed in ABR recordings before and after chemical ablation were compared, offering proof that the statocyst is involved in sound perception.

3. Results

3.1. Electrophysiological responses to auditory stimuli

Auditory evoked potentials were recorded from both, *S. lessoniana* and *O. vulgaris*. To prove that the recorded evoked potentials from live animals were genuine and not artefact, we also recorded from one dead individual from each species and only system random noises and no response to any acoustic stimuli given (data not shown) could be obtained. This standardized false check procedure (see Kenyon et al., 1998) validated our recordings. The trace of acoustically evoked potentials from living specimens was a downward peak signal that varies in shape and amplitude. Measured signals had amplitudes ranging from 60 nV to 300 nV. In general, recorded potentials from the common octopus had smaller amplitude (60 nV–120 nV) than those of oval squid (100 nV–300 nV). Additionally a typical frequency dependent shift of traces was observed (Fig. 1). During the 20 ms stimuli the recorded potential appears earlier with increasing frequency resulting in a latency time difference of 2.5 ms between 400 Hz and 1200 Hz.

3.2. ABR threshold determination

Thresholds of five individuals of each species were determined visually from the sequentially arranged waveform for each frequency tested, according to method by Kenyon et al. (1998). Fig. 2a shows the evoked waveforms recorded from one *S. lessoniana* in response to a 1000 Hz sound with sound levels ranging from 149.4 dB to 129.4 dB (re 1 μPa at 1 m), attenuated in 5-dB steps. The threshold was reached when two replicate waveforms showed opposite polarities or no common peak could be detected (Fig. 2a at 129.4 dB).

3.3. Audiograms of S. lessoniana and O. vulgaris

The audiograms obtained for the two species based on the sequential ABR threshold determinations show a hearing range of 400 Hz to 1000 Hz for *O. vulgaris* and 400 Hz to 1500 Hz for *S. lessoniana* (Fig. 3). For 400-, 500-, 700 and 800 Hz no significant difference of thresholds between squid and octopus was observed (t-test $p>0.05$), whereas for 600 Hz and above 800 Hz the thresholds of *S. lessoniana* were significantly lower than those of *O. vulgaris* (t-test $p<0.05$). In addition, *O. vulgaris* failed to respond to acoustical stimuli higher than 1000 Hz and therefore the upper reach of hearing frequency for *O. vulgaris* was at 1000 Hz with a sound pressure level of about 150 dB. On the other hand, the hearing frequency of *S. lessoniana* extended beyond 1000 Hz and reached up to 1500 Hz with a threshold of 140 dB. We presented a frequency range of 400 Hz to 4000 Hz to the test subjects but they failed to respond to frequencies higher than those mentioned above. On a comparative basis, *S. lessoniana* is characterized by a wider hearing frequency range in combination with lower sound thresholds than of *O. vulgaris* (Fig. 3).

4. Effects of chemical ablation of the statocyst on acoustically evoked potentials

The ablation of statocyst function was achieved by injection of neomycin (at a concentration of 0.8 mM) into *S. lessoniana*. Prior to the injection, normal evoked brainwaves were observed and recorded (Traces 1 and 2 in Fig. 4). From 0 to 3 min after the injection, AEP signals started showing signs of subtle changes (Traces 3 and 4 in Fig. 4). Three to 6 min after the injection, the first peak as seen previously had disappeared whereas the second upward peak showed altered course and amplitude (Traces 5 and 6 in Fig. 4). These newly formed upward signals become 2–4 small upward peaks until 15 min after the injection (Traces 7 and 8 in Fig. 4). Between 21 and 24 min after the injection, inconsistent wave shapes of the two replicate tracks were observed (Traces 9 and 10 in Fig. 4). Then, no peak could be observed on the recorded evoked brainwaves between 25 and 28 min (Traces 11 and 12 in Fig. 4), probably an indication that sensory hair cells lost their function to respond to acoustical stimuli. After measurement, it was confirmed that the subject was still alive and even at the final time point (almost 3 h after the injection) of terminating the experiment, the

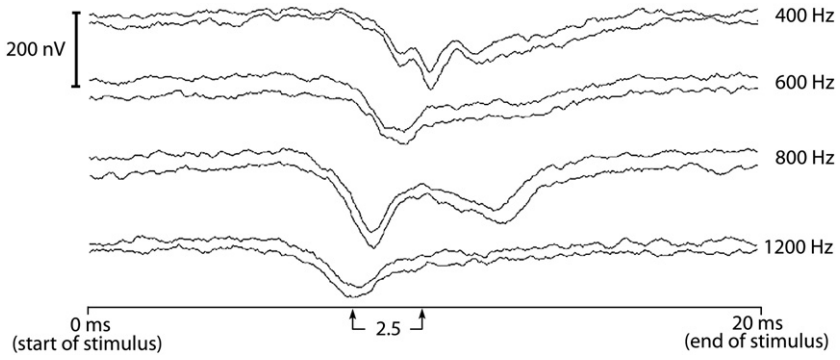

Fig. 1. ABR of *Sepioteuthis lessoniana* to the sound frequencies indicated on the right (sound pressure level of 149.9 dB). Note that peak latencies decreased by about 2.5 ms when the stimulus frequency was increased from 400 to 1200 Hz.

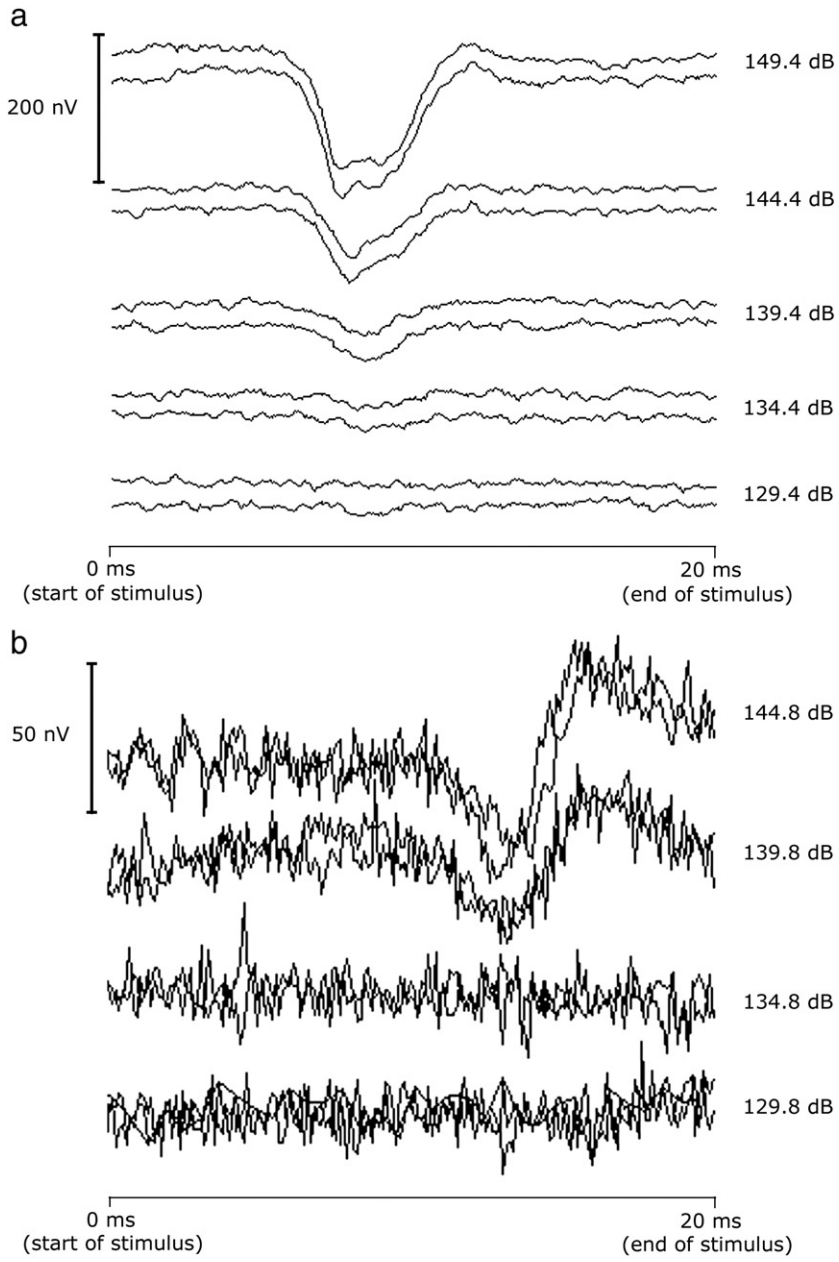

Fig. 2. (a) ABR of *Sepioteuthis lessoniana* (stimulus frequency 1000 Hz, stimulus amplitude 129.4 to 149.4 dB). Replication of two traces in each sound pressure level is used as criteria for threshold determination. The amplitude of the signal ranges from 200 nV (149.4 dB) to approximately 50 nV (134.4 dB). At a sound level of 129.4 dB there is no conformity between two traces. In this case the threshold would be 134.4 dB. The data for this figure were smoothed by calculating in each case the mean of six neighbouring points. (b) ABR of *Octopus vulgaris* (stimulus frequency 1000 Hz, stimulus amplitude 139.8 to 144.8 dB). The course of the waves are not as smooth as it is in the plot of Fig. 3(a) because of the weaker, received potentials (max. 70 nV) in relation to recorded noise. But a threshold can also be determined for 139 dB. The data for this figure are smoothed by averaging of every two points.

subject was still alive with mantle movement as well as pumping of branchial hearts.

5. Discussion

This work has clearly demonstrated that at least two cephalopod species, *S. lessoniana* and *O. vulgaris* are able to detect sounds ranging from 400 Hz to 1500 Hz and from 400 Hz to 1000 Hz, respectively.

Earlier studies demonstrated that cephalopods respond to local (near field) water movements up to 200 Hz by using their epidermal sensory receptors (Budelmann and Bleckmann, 1988). However, there was no proof for the detection of sounds above 400 Hz except an observation made by Maniwa (1976) who noted that squids could be attracted by 600 Hz sounds. Additionally Komak et al. (2005) offered another behavioural evidence for detection of local sinusoidal water movements up to 600 Hz in the cuttlefish *S. officinalis*. In the present study, lowest thresholds for both species were around 600 Hz. Essentially, the electrophysiological findings from the present study corroborate with behavioural observations made by Maniwa (1976). Combining both electrophysiological and behavioural data, it is confirmed that cephalopods can detect under water sounds with frequencies higher than 400 Hz.

The question regarding a specialized organ that is responsible for the detection of underwater pressure waves or particle motion above 400 Hz has been debated by earlier work. Several receptors can be considered as possible candidates for squids´ and octopus´ response to sound stimuli, such as epidermal head and arm lines and the statocyst (Budelmann and Bleckmann, 1988; Bleckmann et al., 1991). We

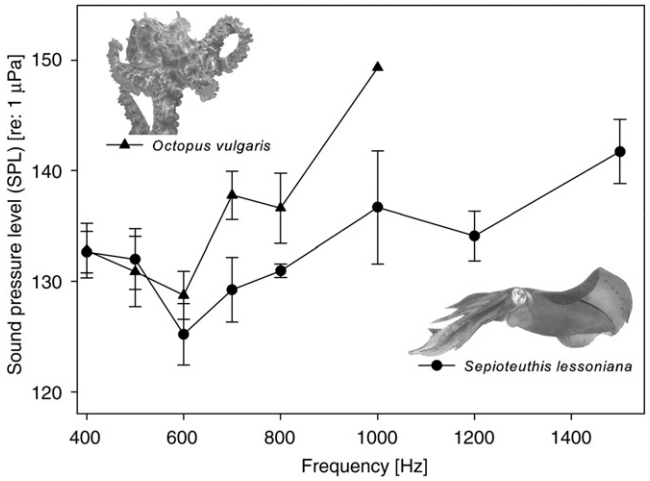

Fig. 3. Auditory thresholds of *Octopus vulgaris* and *Sepioteuthis lessoniana* presented in the form of an audiogram. In both cases data were based on five animals. For each animal and frequency, respectively, five threshold values were averaged.

consider the most likely receptor system for frequencies >400 Hz to be the statocyst. These equilibrium receptors consist of a statolith–macula complex inside an endolymphe filled cavity (Young, 1989). With respect to the mechanism of sound detection, the statolith–macula complex includes all structural components for detection of the displacement component of a sound wave. Since cephalopods are acoustically buoyant they move with the sound waves and, thus, the higher inertial mass of the statolith causes relative motion between the macula and statolith. Chemical ablation of the sensory hair cells confirms that the AEP response to sound stimulation is mediated by the statocyst organ.

All members of aminoglycoside antibiotics (neomycin is one of them) are known to cause the death of sensory hair cells in the hearing organ (Yan et al., 1991; Lombarte et al., 1993), as well as neuromasts of the lateral line system (Song et al., 1995). Neomycin induced abnormal auditory evoked potentials were very striking and intriguing. Three to 15 min after neomycin injection, the amplitudes of upward peaks increased when comparing with the amplitudes of potentials prior to the injection. This might indicate that more hair cells were abnormally activated and therefore stronger electric signals were generated. We cannot be sure whether the injection of neomycin in this study caused death of hair cells or physical changes of statocyst parameters (pressure or ionic composition) led to hair cell malfunction, but at least their proper function was disturbed. The cession of evoked potential signals to the sound stimulus after 25 min and onward might be due to the ototoxic effect of neomycin on the physiological functions of sensory hair cells inside the statocyst. Due to the fact that we only injected neomycin into one statocyst chamber a complete cession of evoked potentioal was unexpected. Thus, it might be possible that the animal was brain dead at the final time point of 25 min, although mantle and branchial hearts were still moving. Nevertheless, the observation before the complete cession of signals supports the possibility that the statocyst contribute to the auditory ability of cephalopods. Further investigations are needed in order to clearly identify the responsible organ.

The cephalopod statocyst shows many comparable features to the fish inner ear such as the macula and otolith inside an endolymph filled cavity, but apart from these similarities there is a major difference: gas filled structures. Gas filled structures that are connected directly or indirectly to the inner ear of fish function as amplifiers to pick up the pressure component of sound and pass it onto the inner ear. Deflation of the swim bladder of goldfish (Yan and Curtsinger, 2000) and removal of gas from otic gasbladder in mormyrid fish (Yan and Curtsinger, 2000) all caused a decrease in the hearing ability. As cephalopods do not have any gas filled chambers, except of *Sepia* spp. and *Nautilus* spp., there is no possibility for amplification of sound pressure waves. On a relative scale, the hearing ability of *O. vulgaris* and *S. lessoniana* is comparable to those of fish without a mechanically coupled gasbladder to the inner ear and is also comparable to hearing ability of prawns, which also have no gas filled structure for the amplification of pressure waves (Lovell et al., 2005). Another striking similarity between vertebrate inferred potentials and those of the two examined cephalopod species is a frequency dependent change in response latency. This is typical for ABR recordings (Hall, 1992; Kenyon et al., 1998). It is concluded that the frequencies detected in both *O. vulgaris* and *S. lessoniana* were in

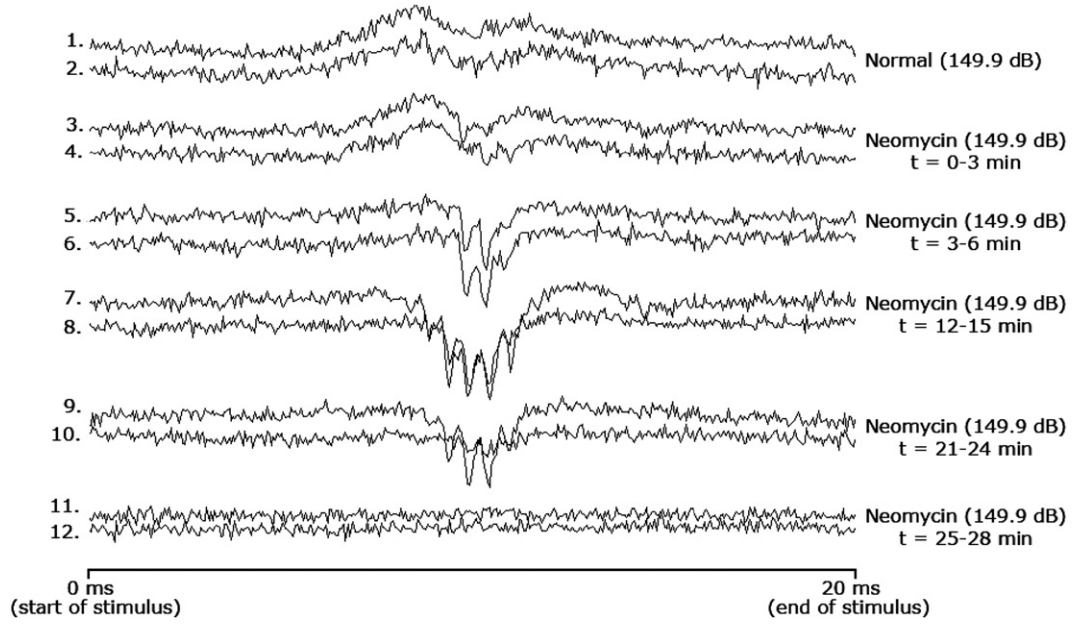

Fig. 4. Traces of acoustically evoked potentials before, during and after injection of neomycin (0.8 mM) into the left statocyst chamber of one individual of *Sepioteuthis lessoniana*. After 20–25 min no potential was recorded. The sound frequency tested was 1000 Hz. The animal continued showing pumping of the branchial hearts and slow mantle movements for more than 3 h until it died.

the range of other animals that lack gas filled chambers and, thus, these cephalopods are probably only sensitive to the motion of water particle displaced by sound with frequencies up to 1000 Hz–1500 Hz.

The differences in audiograms of the oval squid and the common octopus clearly indicated that varying hearing abilities exist among cephalopods. The difference in hearing ability of *S. lessoniana* compared to *O. vulgaris* could perhaps be explained by long term selection pressure exerted on them by the ecological niches they occupied. The oval squid is a pelagic and group forming organism with very limited hiding possibilities, while on the other hand the common octopus is a demersal organism that resides in rocky habitats and has very strong camouflage ability. In light of this difference in habitat usage the auditory sense would play a role of higher importance for the squid in order to escape from predators before they are visually detected. The ecological importance as well as biological significance for cephalopods to hear sound frequencies in the range as presented in this study needs to be investigated. Sounds in the 400 Hz to 2000 Hz range are either produced by anthropogenic, abiotic or animal sources in the marine environment. Odontocete cetaceans are common predators feeding on cephalopods. These animals can produce sounds in the range of 1–20 kHz with sound pressure levels as high as 168 dB re 1 μPa at 1 m (Miller, 2006). Especially dolphins that prey on cephalopods can emit sounds in the frequency range of 1–2 kHz with high sound pressure levels (Schultz et al., 1995; Monteiro-Filho and Monteiro, 2001). In respect to the results of this work it can be established, that at least *O. vulgaris* and *S. lessoniana* are able to detect one of their main predators by audition.

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These experiments comply with the “Principles of animal care”, publication No. 86-23, revised 1985 of the National Institute of Health, and the animal use protocols were approved by the Academia Sinica Institutional Animal Care and Use Committee (No. RFiZOOYH2007012).

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