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Sound detection by the longfin squid (*Loligo pealeii*) studied with auditory evoked potentials: sensitivity to low-frequency particle motion and not pressure

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SUMMARY

Although hearing has been described for many underwater species, there is much debate regarding if and how cephalopods detect sound. Here we quantify the acoustic sensitivity of the longfin squid (*Loligo pealeii*) using near-field acoustic and shaker-generated acceleration stimuli. Sound field pressure and particle motion components were measured from 30 to 10,000 Hz and acceleration stimuli were measured from 20 to 1000 Hz. Responses were determined using auditory evoked potentials (AEPs) with electrodes placed near the statocysts. Evoked potentials were generated by both stimuli and consisted of two wave types: (1) rapid stimulus-following waves, and (2) slower, high-amplitude waves, similar to some fish AEPs. Responses were obtained between 30 and 500 Hz with lowest thresholds between 100 and 200 Hz. At the best frequencies, AEP amplitudes were often >20 μV. Evoked potentials were extinguished at all frequencies if (1) water temperatures were less than 8°C, (2) statocysts were ablated, or (3) recording electrodes were placed in locations other than near the statocysts. Both the AEP response characteristics and the range of responses suggest that squid detect sound similarly to most fish, with the statocyst acting as an accelerometer through which squid detect the particle motion component of a sound field. The modality and frequency range indicate that squid probably detect acoustic particle motion stimuli from both predators and prey as well as low-frequency environmental sound signatures that may aid navigation.

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Key words: cephalopod, statocyst, auditory, acceleration, hearing, invertebrate.

INTRODUCTION

Sound propagates with less attenuation underwater relative to many other stimuli, and marine animals regularly utilize acoustic signals for important biological activities such as intraspecific communication (reproductive behavior), predator avoidance, habitat identification, foraging or orientation. Sound production and reception have been demonstrated for many aquatic vertebrates including teleost fish (Myrberg, 1981), elasmobranch fish (Kritzler and Wood, 1961), reptiles (Bartol et al., 1999) and marine mammals (Johnson, 1967; Norris et al., 1961).

However, relatively little is known about how most marine invertebrates may use sound (for reviews, see Budelmann, 1992a; Budelmann, 1992b). Crustaceans are perhaps the best studied marine species. Spiny lobster and snapping shrimp produce sounds (Patek, 2001; Versluis et al., 2000), and recent studies indicate that shrimp are sensitive to acoustic stimuli (Lovell et al., 2005). However, for cephalopods and particularly squid, auditory receptive capability has remained an intriguing topic that has stimulated considerable debate but for which there are few experimental data.

Anecdotal evidence initially suggested squid may be attracted to 600 Hz pure tones (Maniwa, 1976), and one relative, the cuttlefish (*Sepia officinalis*), was reported to have startle responses to 180 Hz stimuli (Dijkgraaf, 1963). It has also been hypothesized that squid may be stunned by, and therefore perhaps be sensitive to, intense ultrasonic echolocation clicks produced by foraging toothed whale predators (Norris and Møhl, 1983). These hypotheses stimulated the proposal that squid evolved to be 'deaf' to the effects of intense sound exposure (Moynihan, 1985). Further work citing morphological data (Budelmann, 1976) and behavioral observations outlined why squid are probably sensitive to sound (Hanlon and Budelmann, 1987). This last contention has been corroborated by behavioral response studies and classical behavioral conditioning experiments showing that squid, cuttlefish and octopus are sensitive to local water movement and low frequency particle motion (Komak et al., 2005; Packard et al., 1990). Additional behavioral studies argue against the Norris and Møhl hypothesis of squid sensitivity to ultrasounds by demonstrating that exposures of squid to simulated odontocete ultrasonic clicks do not elicit anti-predator responses or debilitation in squid (Wilson et al., 2007).

Conclusions that cephalopods only detect the low-frequency particle motion component of the sound field (Packard et al., 1990) have recently been contested by preliminary evoked potential measurements (Hu et al., 2009). These data suggest that squid and octopus might detect sounds up to 1600 Hz, reviving the discussion of squid acoustic detection of toothed whales. Thus, there remains a controversy between older anatomical and physiological data that clearly suggest a low-frequency accelerometer-like detector and recent suggestions that squid can hear higher frequencies and detect

the pressure component of the sound field (Hu et al., 2009). There is therefore a need for further study to resolve what squid actually detect and over what frequency range.

The probable organ for sound detection in cephalopods is the statocyst (Budelmann, 1990). The squid statocyst is relatively complex for an invertebrate, having multiple lobes arrayed in three planes populated with heavily innervated hair cells coupled to a mass (the statolith or cupula) (Budelmann, 1990; Young, 1984). There are two separate receptor systems within the statocyst, a macula that provides orientation information on the gravitational field and on linear acceleration, and a crista–cupula system that acts as an angular accelerometer (Budelmann, 1990). Consequently, the general morphology and vestibular role of the statocyst organ functions like that of the fish inner ears (de Vries, 1950; Fay and Popper, 1975). As with vertebrate otoliths (Chapman and Sand, 1974), the statocyst in squid may sense sound-induced displacement between the statolith and its hair cells (Budelmann, 1992b), and as an accelerometer may play an auditory role (Packard et al., 1990). Because a sound field consists of both particle motion and pressure components available for potential detection (Chapman and Sand, 1974; Fay and Popper, 1974), hearing can be defined as the auditory detection of either of these two sound field components (Chapman and Sand, 1974; Webster et al., 1992). Hearing may involve detection of the pressure component, as is the case for certain fish with auditory specializations that use the swimbladder as a pressure-to-particle motion transducer, or the particle motion component, as is the case for most aquatic animals. Hearing in the form of detecting the particle motion component of a sound field has been demonstrated in many marine organisms, including cartilaginous and teleost fish that do not have specialized adaptations to detect or transduce sound pressure (Kalmijn, 1988; Popper and Fay, 1997).

In classical studies of animal audition, psychophysical approaches such as behavioral responses or cardiac conditioning have often been used. Recently, evoked potential studies have been applied to investigate auditory responses in animals that do not lend themselves easily to conventional psychophysical measurements. Auditory evoked potentials (AEPs) reflect synchronous neural activity as afferent responses are conducted from the auditory end-organ to higher centers (Burkhard et al., 2007). Responses have been elicited by acoustic or acceleration stimuli as demonstrated in mammals (Jewett and Williston, 1971), fishes (Fay and Edds-Walton, 1997; Kenyon et al., 1998) and, recently, invertebrates (Lovell et al., 2005). In aquatic animals such as teleost and cartilaginous fishes, AEPs are commonly initiated at the otolithic endorgans. Responses can be elicited by sound pressure and particle motion stimuli (Casper et al., 2003; Kenyon et al., 1998). The frequency of AEP responses are similar to results from behavioral studies but often with a decreased sensitivity in the response thresholds. Electrophysiological data indicate otolith afferents generate responses when the related direction-sensitive hair cells are stimulated (Fay and Edds-Walton, 1997). In all fish tested with sinusoidal stimuli, AEP response rates are twice the stimulus frequency (e.g. Egner and Mann, 2005). This is attributed to simultaneous responses from two groups of hair cells oriented in opposite directions.

Because classical conditioning studies in squid are problematic (Gilbert et al., 1990; Packard et al., 1990), direct neurophysiological approaches such as AEPs are a viable alternative to address auditory abilities. These auditory studies also facilitate having a controlled sound field where both the particle motion and pressure components of the sound field are quantifiable (Chapman and Sand, 1974; de Vries, 1950; Sand and Karlsen, 1986). In a plane wave, particle velocity and pressure are fundamental elements of specific acoustic impedance (defined as $Z=pu$ where p is pressure and u is particle velocity); they are also affected by the characteristic impedance of the medium, the product of density and sound speed (ρc) of a propagating sound wave. An additional source of particle motion is generated by hydrodynamic flow from the motion of the sound emitter (Gade, 1982; Au and Hastings, 2009). This near-field flow effect of a sound source will attenuate rapidly with distance ($1\,\text{distance}^{-2}$) compared with the attenuation of pressure of the propagating sound wave ($1\,\text{distance}^{-1}$). Particle motion will therefore dominate close to the sound source (i.e. 'near-field'). With distance ('far-field'), the effect of excess particle motion is negligible, whereas particle velocity (u) is proportional to the sound pressure p of the propagating sound wave: $u=p/\rho c$. For large water volumes that are effectively acoustic free-field systems, the acoustic impedance can be estimated reliably, but in small water volumes, such as most experimental tanks, the acoustic impedance will be affected by factors such as source wavelength and tank dimensions (Au and Hastings, 2009). Standing waves in a small tank can generate complex pressure and particle velocity patterns compared with the free field and therefore require careful measurements of actual particle motion and pressure fields to determine what the organisms are receiving in the experimental setup.

In the present study, AEP techniques were used to test whether the statocyst is the organ used for acoustic detection and to determine the range and sensitivity of squid to pressure and particle velocity components of a sound field. We show that squid use their statocysts to detect low frequency particle motion, and we discuss the implications of how, and for what, squid use their auditory system.

MATERIALS AND METHODS

Animal preparation and experimental set-up

Evoked potential measurements of the longfin squid (*Loligo pealeii* LeSueur) were conducted from June to August 2008 at the Marine Biological Laboratory, Woods Hole, MA, USA. Squid were collected locally by a trawler from surrounding North Atlantic waters 4 days per week, which ensured a ready supply of experimental subjects in good physical condition. The animals were maintained in an oval holding tank filled with chilled seawater until used for experiments. Nineteen animals were used in these experiments; 15 for *in vivo* evoked potential measurements conducted in a saltwater tank and four for evoked potential measurements using a shaker displacement system. The mean animal wet mass and mantle length were 54.7±18.9 g and 14.8±3.1 cm, respectively.

On experimental days, one or two healthy squid were collected from the holding tank and transferred to a 10 liter plastic bin (30 cm×18 cm×12 cm) filled with seawater (14°C) from the holding tank. The bin was then immediately covered with black plastic and carried to the experimental area. Animals were sedated for each experiment in a bath of $MgCl_2$ solution ($0.15\,\text{mol}\,l^{-1}$) (Mooney et al., 2010). $MgCl_2$ sedation does not have an apparent effect on cephalopod evoked responses (Messenger et al., 1985; Mooney et al., 2010; Pruess and Budelmann, 1995).

Acoustic evoked potential measurements

Anesthetized squid were moved from the $MgCl_2$ bath to a rectangular plastic tank (82.6 cm×50.2 cm×47.3 cm) for evoked potential measurements (Fig. 1, item 1). The tank rested inside a larger plywood box lined with acoustically dampening open-cell foam (2). The foam and wood served to reduce noise and dampen vibrations from the surroundings. This box sat on four rubber gaskets that further isolated the tank from the substrate. Aerated, chilled (14°C)

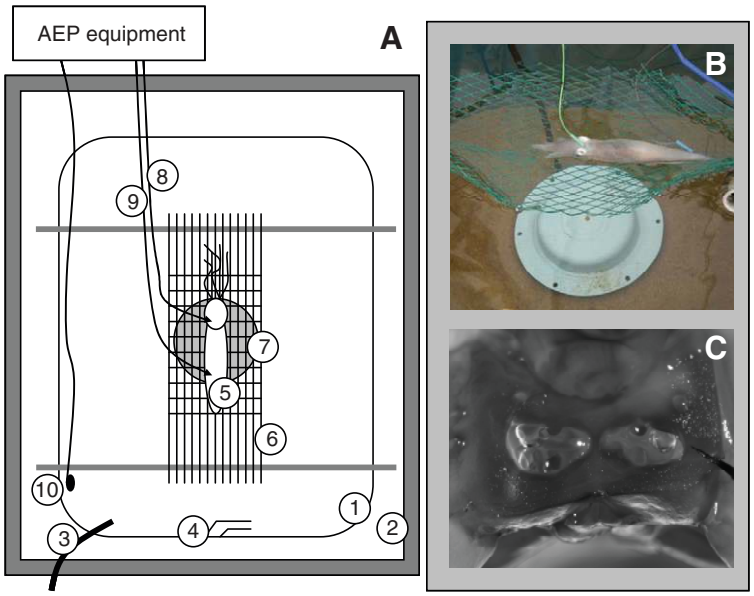

Fig. 1. (A) Schematic of experimental set-up and (B) photograph of squid during AEP measurements. 1, rectangular plastic tank; 2, larger plywood box lined with acoustically dampening open-cell foam; 3 and 4, seawater incurrent and outfall, respectively; 5, the squid; 6, acoustically transparent plastic mesh; 7, speaker; 8, 'recording' electrode; 9, reference electrode; 10, ground wire; for further details see the text. (C) Electrode in the recording location of the cartilage surrounding the squid statocyst after AEP measurements. Some of the surrounding soft-tissue has been removed for improved viewing. View is toward the anterior, dorsal is top.

seawater flowed slowly through the tank (3, incurrent; 4, outfall). The squid (5) rested near the surface (3.0 cm depth) in acoustically transparent plastic mesh (6; Fig. 1B). An underwater speaker (UW-30; Lubell Labs Inc., Columbus, OH, USA) rested on the bottom of the tank, partially buried in sand. The speaker was located directly below the squid eye region (7) and its statocyst organs (40 cm depth).

The squid was placed ventral side up (dorsal side towards the speaker) in the tank. The siphon of the anesthetized squid was surgically trimmed for improved viewing of the internal anatomy of the brain and statocyst. The 'recording' electrode (8) was inserted medially, from the squid's ventral side, posterior to the eye but on the anterior side of the statocyst and into the surrounding cartilage (Fig. 1C). This was the standard location for all recordings with the exception of one experiment described below. A reference electrode (9) was inserted into the muscle of the squid body, 5–10 cm from the head. Both electrodes were modified by coating the entire stainless steel portion, except the very tip (0.5–1 mm), with a thin layer of Por-15 (Morristown, NJ, USA), which reduced extraneous electrical noise. The connection of the stainless steel to the electrode cable was also lightly coated in epoxy resin to prevent saltwater from penetrating the connection. The electrodes and an additional ground wire (10) were connected to a battery-powered Grass CP-511 biological amplifier and filter (Astro-Med Inc., West Warwick, RI, USA) that amplified the signal 10,000 fold and, for most recordings, bandpass-filtered responses from 100 to 3000 Hz. The received signal was then connected to a BNC connector box and converted from analog to digital *via* a National Instruments 6062E data acquisition card (DAQ; Austin, TX, USA) and custom AEP program (using National Instruments LabView software) on a laptop computer. Evoked potential records using stimuli of 1000 Hz and below were sampled at 16 kHz. Stimuli above 1000 Hz (3000 and 10,000 Hz) were sampled at 64 kHz and the low pass AEP filter was set to 10,000 Hz. The high-pass filter was 30 Hz for stimuli below 100 Hz. To obtain an AEP record, 1000 sweeps (responses) were collected and averaged. Evoked potential activity was monitored using a Tektronix TDS 1002 oscilloscope (Beaverton, OR, USA).

The same laptop, custom program and data acquisition card were used to generate acoustic signals for all test measurements. Using an update rate of 256 kHz, amplitude modulated tone-pips of 50–10,000 Hz (50, 80, 100, 120, 150, 170, 200, 220, 250, 300, 400, 500, 1000, 3000 and 10,000) were presented *via* the UW-30, although frequencies below 100 Hz were somewhat difficult to generate and were not pure tones. Each signal was at least six cycles in duration, thus signal length varied relative to frequency (Table 1; see supplementary material Fig. S1). Sounds were played from the data acquisition card to a custom attenuator that could adjust sound

Table 1. Characteristics of underwater loudspeaker stimuli played to the squid in the seawater tank

Stimuli (Hz)	Duration (ms)	No. of cycles	Rec. window (ms)	Presentation rate (s^{-1})	Start SPL (dB re. 1 μPa, rms)
10,000	20	200	30	20	152
3000	20	60	30	20	145
1000	20	20	30	20	139
500	20	10	30	20	140
400	20	8.0	30	20	141
300	20	6.0	30	20	142
250	25	6.25	40	16.7	144
220	30	6.6	50	14.2	145
200	30	6.0	50	14.2	145
170	40	6.8	60	12.5	151
150	40	6.0	60	12.5	153
120	50	6.0	80	10	152
100	60	6.0	80	10	151
80	75	6.0	100	8.3	145
50	120	6.0	150	5.8	142

pressure levels in 1 dB steps, and then to an HP 465A amplifier (Palo Alto, CA, USA) which was then connected to the underwater speaker. Outgoing stimuli were monitored using an oscilloscope.

Sound presentations digitally triggered AEP recordings; thus, stimuli and evoked potential records were synchronized. Stimuli durations were variable, but never more than 200 ms (50 Hz) and were as short as 30 ms (for stimuli ≥300 Hz). Records between 50 and 200 Hz were 40–100 ms in duration. Measurements typically started at maximum sound pressure levels (SPLs) for each frequency (139–153 dB re. 1 μPa; 4.3–160.9 m s^{-2} depending on the frequency) and decreased in 5–10 dB steps depending on response amplitude.

The source of AEPs was investigated using ablation experiments. Evoked potentials (150 Hz stimuli) were recorded from six animals and the recording electrode was then removed. All surgical procedures were performed under full anesthesia. Following the method of Messenger (Messenger, 1970), the funnel was trimmed, and soft-tissue down to the statocyst cartilage was removed. Three sham operations stopped here. For three other subjects, the statocysts were exposed and opened with a pointed scalpel, the statoliths removed and the statocyst interior swabbed. Animals were then confirmed to be ventilating normally and otherwise healthy. The electrodes were reinserted, and AEP recordings were made. Statocyst ablations were confirmed by post-mortem examination of the animals using a dissecting microscope.

Shaker evoked potential measurements

To test the effects of just acceleration alone, a custom-built moving-coil shaker table system (Fay and Edds-Walton, 1997; Fay, 1984) was used to provide sinusoidal vertical stimulation similar to the tone-pips in the tank. This motion stimulus was free of pressure and interference phenomena found in the tank set-up, thus providing primarily acceleration of the animal and a very limited pressure gradient. Anesthetized animals rested at the bottom of an aluminum bowl and were kept moist by a 1 cm layer of sea water (i.e. they were not fully submerged). Sinusoidal vertical motion was produced by a Brüel and Kjær (Nærum, Denmark) 4809 shaker supporting the bowl. Acceleration was determined by a Brüel and Kjær 4500 accelerometer glued to the top of the bowl that measured dorsoventral vibrations. The accelerometer was connected to a charge amplifier (2635; Brüel and Kjær) and the signal was recorded by a digital signal processor [RM2; Tucker-Davis Technologies (TDT), Alachua, FL, USA]. The accelerometer was calibrated by an accelerometer calibrator (Brüel and Kjær 4294) producing 10 m s^{-2} at 159 Hz. Stimuli for the AEP measurements were acceleration impulses produced by one cycle of a 200-Hz-shaker-generated sinusoid (resulting in a broad-band signal) masked by shaker-generated tones (frequencies 20–1000 Hz). The impulse had a constant peak acceleration of 1 m s^{-2}, whereas the masker tones could be varied in frequency as well as amplitude. Impulses were presented at a constant rate (every 40 ms) whereas the masker was alternately on and off every 2 s. The sensitivity to the tone was measured as the difference between responses to the impulse and responses to the masked impulse for different masker levels. The rationale for this procedure was to have sufficiently long signals for a well-defined frequency at low frequencies, but still with a well-defined onset response. AEPs are generally onset responses that can be generated best by broadband stimuli. Short tone bursts may not initiate sufficient onset responses at low stimulus levels. This impulse+masker method combines generation of a reliable onset (impulse) with detection (i.e. masking) of pure tones (Brandt et al., 2008). Similar methods have been employed for human ABR experiment (Berlin et al., 1991). The evoked auditory responses to alternating impulse and impulse+masker presentations (40 ms each) were recorded by electrodes placed in the squid as described above, but the electrode signal was now amplified by another head stage and preamplifier (RA4LI, RA4PA, TDT) and recorded on the digital signal processor (RM2, TDT). Stimulation and data recording was controlled by QuickABR custom software (Brandt et al., 2008) using 400 averages per masker level.

Sound and particle calibrations

Sound pressure and particle motion in the acoustic tank were calibrated in the absence of a squid. Sound pressure measurements were made using a single receiving hydrophone (Brüel and Kjær 8103) placed at the planned location for the squid's head as well as in the surrounding waters (±5 cm). The hydrophone was connected to a Brüel and Kjær 2635 charge amplifier, and incoming sound levels were monitored on the oscilloscope. The same test stimuli presented in the tank hearing experiments were presented *via* the UW-30. The received peak-to-peak voltage (V_{p-p}) at each location was measured on the oscilloscope and converted to peak-equivalent root-mean square voltage (peRMS) by subtracting 9 dB. Because of the size of tank used in the experiment, competing reflections rendered SPLs a few dBs higher than the true rms levels found in a free field. Stimuli were also digitally recorded for reference using a custom program and the previously mentioned computers and DAQ card.

Particle accelerations values at the position of the squid's head were obtained by measuring the pressure gradient over two closely spaced sound receivers (Gade, 1982). Two Brüel and Kjær 8103 hydrophones, vertically spaced 2 cm apart, were fixed at the location of the squid's head (3 cm depth). Each hydrophone was connected to a charge amplifier (Brüel and Kjær 2635) which was connected to an analog-to-digital preamplifier (RA8GA; TDT) and a digital signal processor (RM2; TDT). Stimuli were then played and particle acceleration was computed from the pressure gradient across the two hydrophones:

$$a = -\Delta sig / (\rho\, \Delta r),$$

where Δsig is the magnitude of the difference between the waveforms of the two hydrophones (in Pa), ρ is the density of the medium, and r is the distance between the hydrophones. The particle motion was measured in three dimensions by positioning the two hydrophones along three orthogonal axes (Kalmijn, 1988; Wahlberg et al., 2008). Subsequently, particle acceleration values for the pressure-derived AEP thresholds were determined by relating the measured pressure at threshold with the corresponding particle acceleration at the head of the squid. Squid probably act as a rigid bodies in the acoustic near-field (Denton and Gray, 1982), thus measurements at the head were compared with additional measurements ±5 cm along the anterior–posterior axis to confirm the sound acceleration field. These measurements were similar (±2 dB) to those at the squid head.

The tank noise was recorded using an ITC-1032 hydrophone (–193 dB re. 1 V/μPa, ±2 dB up to 40 kHz; Santa Barbara, CA, USA) connected to the HP amplifier (+20 dB gain) and the DAQ card, which sampled at 256 kHz. The average background noise was compiled as the mean of a 1-s time window from 10 sound files. Noise levels in the tank were typically below that of the recording equipment (~70 dB re. 1 μPa^2/Hz in the frequency range from 100 Hz to 40 kHz).

Data analysis

During AEP measurements, responses were initially assessed visually from online averaging in the custom program. Evoked potentials were recorded at decreasing SPLs until responses were not detectable. Then, one to three additional measurements, at 5–15 dB below this 'threshold' were made to ensure responses were not missed. Threshold analyses were completed offline using EXCEL and custom scripts written in MatLab 7.4 (Mathworks). Thresholds were determined by two methods. First, AEP waveform data and records were visually assessed to determine presence of response events and the levels at which no response could be visually detected, a method commonly used in fish and invertebrate hearing investigations (Kenyon et al., 1998; Lovell et al., 2005). Alternatively, threshold estimates of the tank data were made by calculating the fast Fourier transform (FFT) power spectra (512 pt, Hann window) of the averaged waveforms. As with fish AEPs, the FFT spectra revealed peaks at twice the stimulus frequency at supra-threshold sound pressure levels (Egner and Mann, 2005; Maruska et al., 2007). Decreases in FFT peak amplitude corresponded with decreasing SPLs. These values at twice the stimulation rate were plotted relative to the corresponding stimulus amplitude and an approximated linear regression was calculated addressing the values. Five-to-ten records were collected per threshold (mean=6.2) and the points with the highest r^2 value were used to calculate the regression (Mooney et al., 2009; Nachtigall et al., 2007). The point at which the regression line transected the abscissa was taken as the theoretical sound level at which no AEP response would occur and constituted the animal's probable threshold at that frequency, as has been done for some vertebrate species (Nachtigall et al., 2007).

RESULTS

AEP waveform characteristics

The AEP waveforms obtained in all animals in the seawater tank followed a consistent pattern: after a temporal delay, multiple, rapid, sinusoidal waves developed, superimposed on a longer duration, initially negative wave. These waves were produced by tone-pip stimuli and occurred with tones of constant or alternating waveform polarities (Fig. 2). Responses were not found when the electrodes were placed in the water without the squid present, when the active electrode was placed in a location away from the statocyst recording site (such as an arm, near the lateral line analogue or other distant body regions), when electrodes were in the statocyst cartilage of a deceased animal, or when statocysts were ablated. Sham-surgery squid, operated on up to the point of ablation had normal AEP responses (see supplementary material Fig. S2). Shaker-generated responses were evoked by the impulse stimuli and followed the waveform of the single cycle (Fig. 3).

When stimulus intensities were high, relative to the animal's threshold, AEPs were discernable well above the noise level (Fig. 4). As stimulus levels decreased, there was a corresponding decrease in response amplitude. This was found at all frequencies tested (Fig. 5). Some response attributes varied with the stimulus. For example, the temporal latencies of AEP waves for frequencies 300 Hz and higher began approximately 7–8 ms after the onset of the tone-pip. As stimulus frequencies decreased (200–100 Hz), responses initiated closer to 10 ms. At 50 and 80 Hz, responses began 21 and 13 ms, respectively, after the stimulus. The time from stimulus onset to the minima of the first negative wave was designated as the onset latency (see supplementary material Table S1). Latencies were measured using six squid with clear AEP records at the highest respective stimulus levels. The rapid-wave durations were also dependent on the stimulus durations. Thus, the duration of rapid waves for a 300 Hz stimulus was 10 ms. For 200 Hz, this duration was 20 ms. The duration of rapid waves was near 30 ms for 80–150 Hz. And at 50 Hz, the 'rapid waves' were 40–50 ms in duration. Similarly, AEP response amplitudes depended strongly on stimulus frequency. Maximal responses were found between 100–200 Hz. Response amplitudes diminished substantially at the higher (300 Hz) and lower response limits (50–80 Hz). Of note were

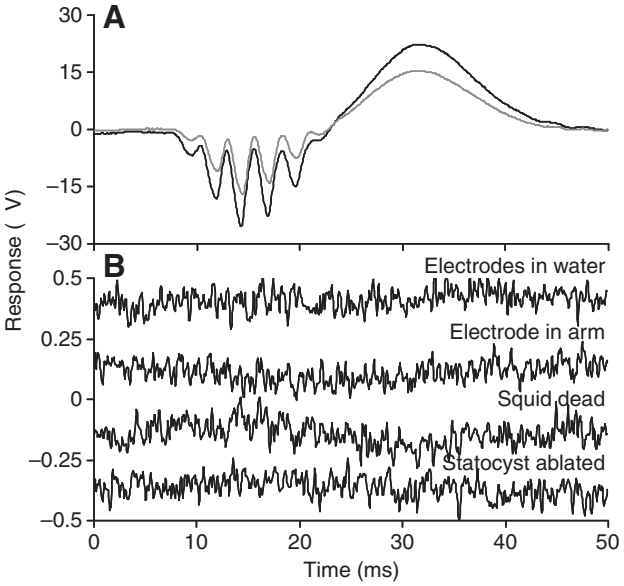

Fig. 2. Squid evoked potentials to a 200 Hz tone presented *via* an underwater speaker at 106 dB re. 1 μPa (rms) using 1000 averages. (A) AEPs from two animals (grey and black traces) in the standard recording position (in the cartilage surrounding the statocyst; Fig. 1C), obtained in response to tone bursts of alternating polarities. (B) Responses in 'control' situations (from top to bottom) when (1) electrodes were placed in the water in the absence of the animal; (2) the active electrode was moved to the arm of the animal, (3) the active electrode was in the standard recording position but animal was dead, and (4) the statocyst was ablated. Sample sizes of respective control tests were: *N*=19, 6, 8 and 3. Data were recorded using the same 200 Hz stimulus and 1000 averages.

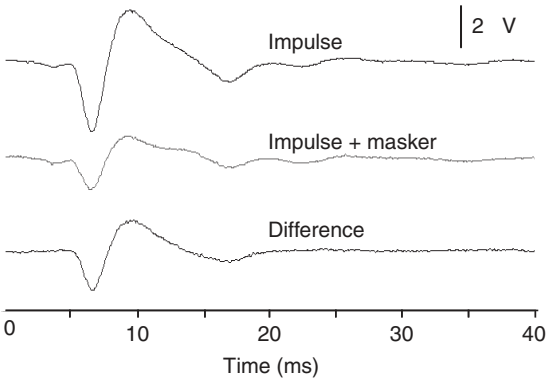

Fig. 3. Shaker-generated AEPs from one squid. Top trace: the responses to only the broadband impulse, presented at a peak acceleration of 1 m s^{-2}. Middle trace: response to the impulse plus a 50 Hz masker, level 0.1 m s^{-2}. Bottom trace: the difference between the impulse and the masking tone. Detecting the difference represents detection of the masker tone. 400 sweeps were averaged for each response. In all, four animals were tested using the shaker.

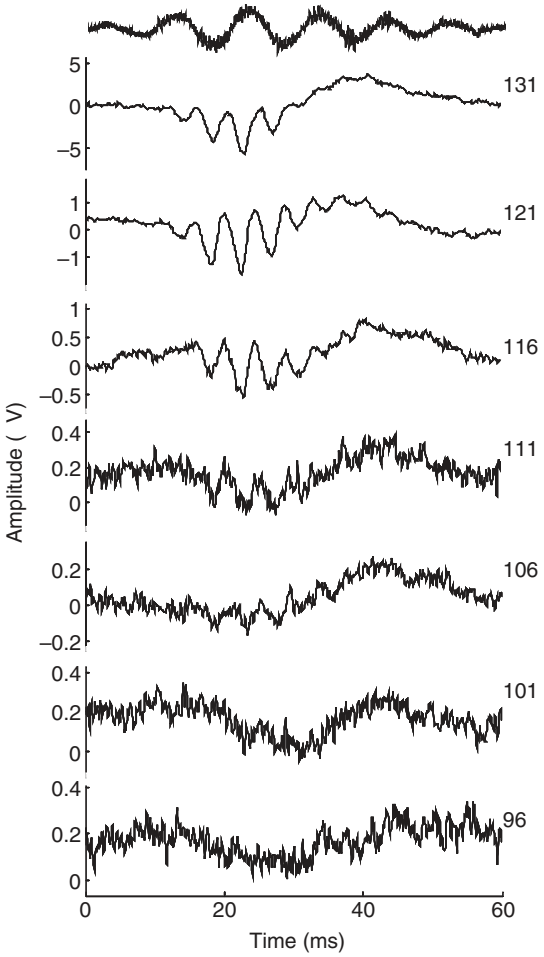

Fig. 4. Squid AEPs to visual threshold using a 100 Hz tone-pip stimulus from 131 to 96 dB re. 1 μPa (rms) in 5–10 dB steps. Responses were generated using the underwater speaker and collected using 1000 sweep averages. Note the ordinate scales are variable relative to the response amplitude. Visually determined threshold here was 101 dB. The stimulus waveform is overlaid at the top (see also supplementary material Fig. S2).

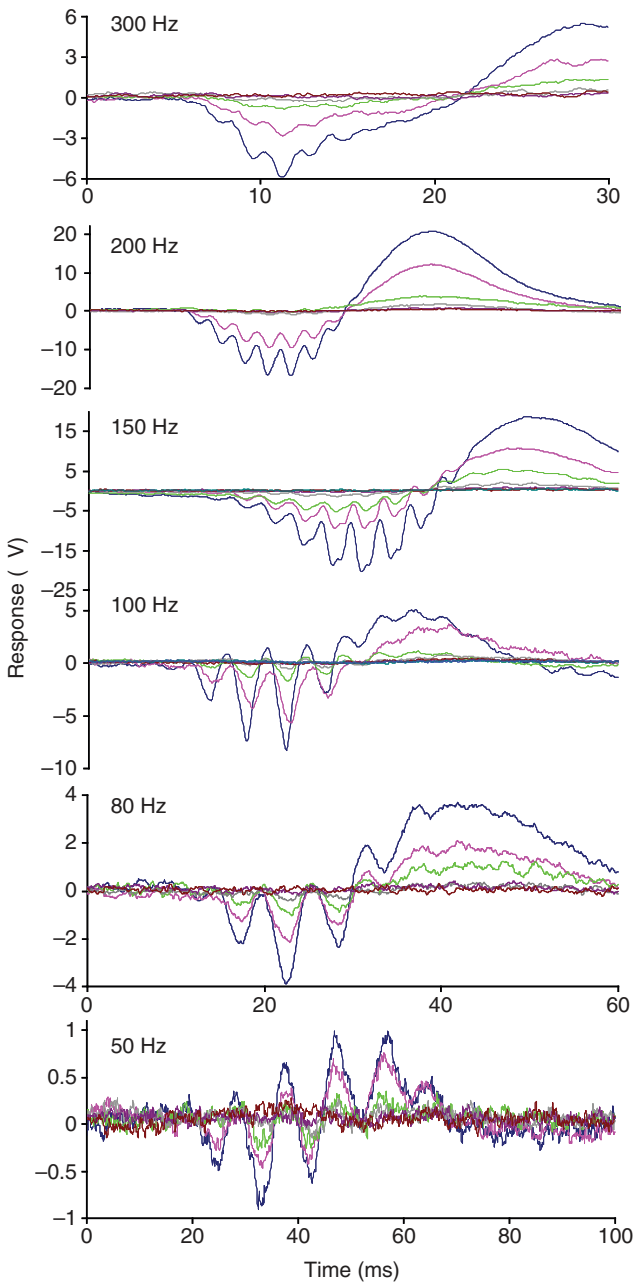

Fig. 5. Examples of speaker-generated AEP responses using frequencies from 50–300 Hz (listed on each plot). Responses to different SPLs are overlaid for each frequency. Start frequencies are listed in Table 1 and SPLs decrease in 5 dB steps according to color. Colors in order of descending SPL are: blue, pink, green, grey, magenta, brown, teal and black. Note the varying response amplitudes for each stimulus frequency. Data are from one animal.

often large amplitudes of the AEP responses, particularly at frequencies of best sensitivity. At these frequencies fast-wave p–p response amplitudes were often 15–20 μV (Fig. 2). Slow-wave amplitudes occasionally approached levels near 50 μV. At all frequencies, when sound levels were decreased to near threshold values, the fast-wave responses typically diminished earlier although slow-waves remained at lower sound levels (Fig. 4).

Evoked responses and temperature

The effect of temperature on AEPs was also investigated in two squid. Evoked potential recordings were made using 150 and 200 Hz stimuli (the frequencies of maximal responses) using the same acoustic tank and underwater speaker. Initial recordings were first made at 16 or 20°C to assess baseline AEP response levels and confirm characteristics were similar to those previously established. Water temperatures were then decreased to 7–8°C and responses were measured. Finally, as temperatures were increased to the starting level, responses were progressively measured, to monitor any changes. Responses for the first squid (Fig. 6) at the initial 16°C resembled 'normal' amplitudes and latencies. However, at the lower temperature of 7°C the initial recording showed no response. Succeeding recordings at 8°C found a small response, approximately 25% of the p–p value of 16°C measurements. The final measurements, made at 14–20°C, demonstrated response amplitudes that returned to the initial levels. A second squid had similar response variations with ambient temperature changes.

Recording location

To determine if neurons near the statocyst were the likely source of the AEP responses, we measured responses anterior and posterior

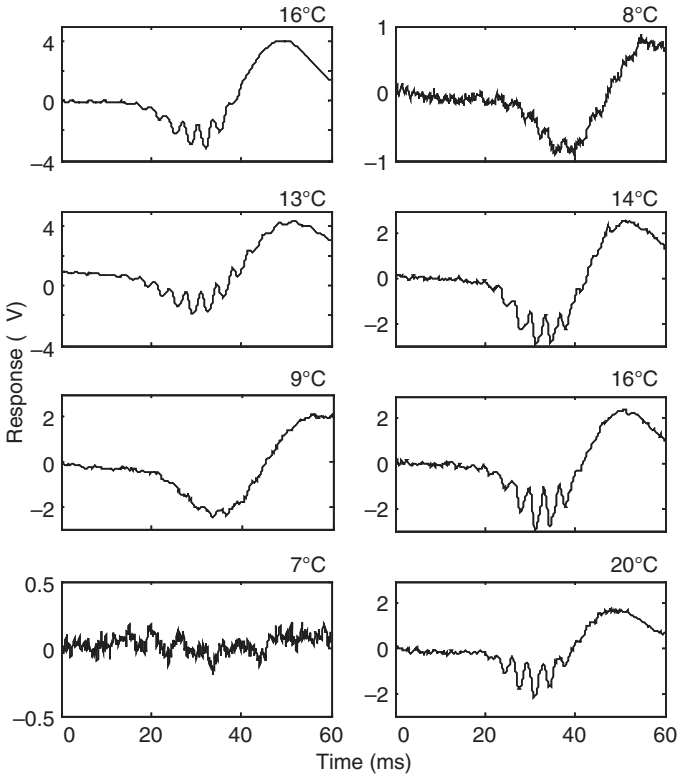

Fig. 6. AEP waveforms recorded at different ambient water temperatures (responses were generated using the underwater speaker). Recordings are shown sequentially, from 16°C to 7°C and back up to 20°C. Note the varying ordinate scales. Two squid were tested.

to the primary recording site used throughout the experiment (the cartilage on the ventral–anterior side of the statocyst but posterior to the brain). In two squid, distance moved was measured relative to the 0 mm location. This location (ventral–anterior side of the statocyst) was the primary recording site for all other AEP recordings. The position of the active electrode was then moved in 3–5 mm steps, anteriorly and posteriorly, along the animal's midline (Fig. 7). Response amplitudes (p–p) and latencies (peak) were assessed from each recording site and measured until they were no longer obtainable. Recordings were made from two squid using a 150 Hz tone-pip (seven to eight locations per squid) in the acoustic tank using the underwater speaker. Amplitudes and latencies of only the four most prominent waves were compared between recording location and these data were pooled for each squid.

Maximum response values were found both at the primary recording site, or 3 mm anterior, and near the brain (Fig. 8). All other locations yielded significantly lower AEP amplitudes (one-way ANOVA: F=25.16; P<0.001). Latencies of these fast-wave responses were similar for both squid tested, demonstrating the shortest responses were from the 'usual' location, on the anterior side of the statocyst. These durations were not significantly different from recording from locations immediately anterior or posterior. Latencies were significantly greater in recordings made more than 5 mm anterior or posterior from the statocyst recording site (one-way ANOVA: F=774.51, P<0.001). As noted earlier, latency was calculated as the time from stimulus onset to the minima of the first negative wave. No responses were detected beyond ±10 mm, thus these latencies could not be plotted or compared. Interestingly, recordings made more than 3–4 mm anterior of the standard recording station had reversed polarity relative to recordings at or posterior to the statocyst (Fig. 8C). These reversed polarity recordings were made when the recording electrode was directly ventral, or ventral and anterior, to the brain.

Threshold determinations

Sound-generated speaker thresholds were determined by both visual and FFT-based methods. Both methods gave similar results, supporting the use of either technique and demonstrating relatively sensitive AEP thresholds to the near-field sound stimuli. Similar to fish, the FFT method revealed frequency following responses and, at supra-threshold stimulus levels, a peak in the frequency response spectrum at twice the stimulus frequency (Fig. 9).

Squid were most sensitive at frequencies between 100 and 300 Hz (Fig. 10). At lower frequencies, pressure thresholds increased gradually at a rate of 4–5 dB octave^{-1}. At higher frequencies, the visually determined thresholds reflected a steep slope to cut-off (20 dB octave^{-1}). This was not evident in the FFT thresholds, although neither threshold method detected responses above 400 Hz, despite relatively high stimulus intensities (149 dB re. 1 µPa; 60.4 m s^{-2}).

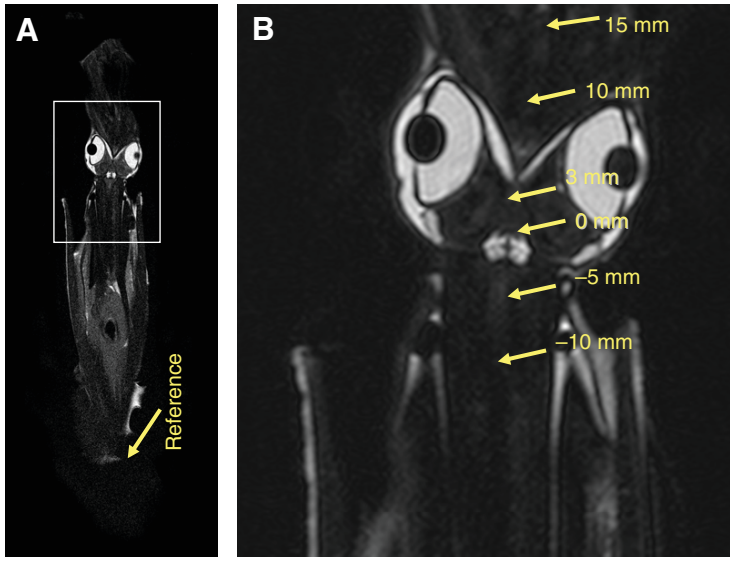

Fig. 7. (A) Magnetic resonance image (MRI) of whole squid. (B) Enlarged view of the eyes, brain (arrow at 3 mm) and statocysts (centered) in A. Arrows indicate scale and locations of recording electrode as response amplitudes and latencies are gauged. 0 mm is the recording location in the anterior portion of the statocyst cartilage, which was the primary active electrode placement during the experiment. The 0 mm location is shown *in situ* in the Fig. 1C image. The statocysts' complexity is roughly reflected in its irregular shape and visible nodes. The location of the reference electrode did not change and is shown in A.

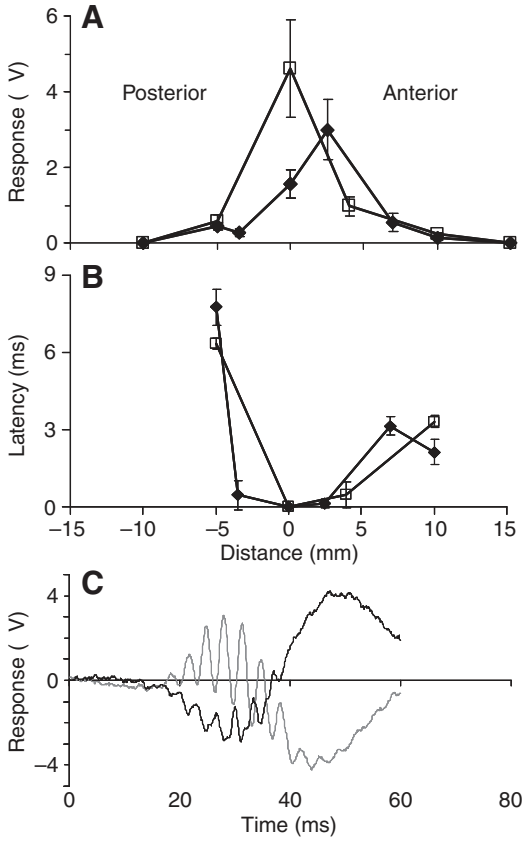

Fig. 8. (A) Fast-wave peak-to-peak response amplitudes to a 150 Hz tone-pip relative to recording location where 0 mm is the primary recording location in the medial part of statocyst cartilage. Responses were generated using the underwater speaker. Distance is relative to this portion of the statocyst. Data from both the animals tested are plotted (squares and diamonds, respectively). Data represent measurements at the four most prominent fast waves and error bars show the s.d. (B) Latency (ms) of the peak-to-peak responses where 0 ms is the standard recording location. Responses greater than 10 mm were not detectable therefore these latencies could not be plotted. (C) AEP responses recorded at the 0 mm location (black trace) and 3 mm anterior to statocyst (grey trace) ventral to the brain. These traces are from one of the squids used for A (black diamonds). All records ventral and anterior of the brain were inverted relative to the primary recording location and points posterior.

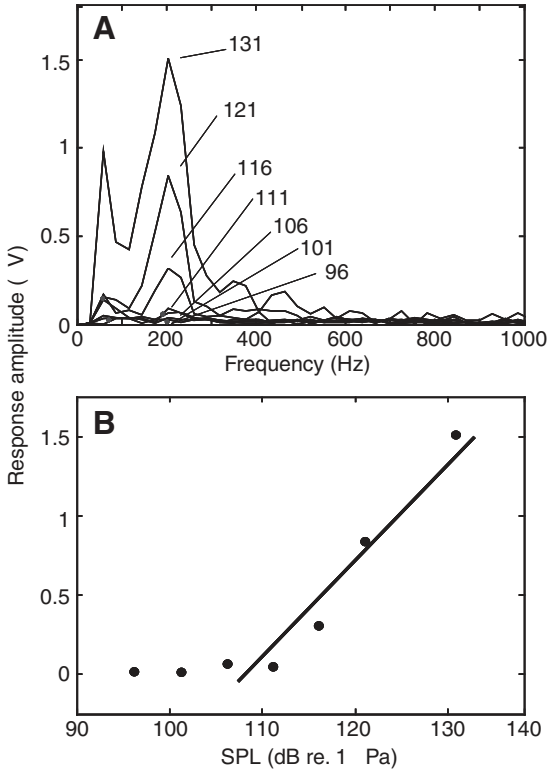

Fig. 9. (A) FFT frequency spectra of the squid AEPs shown in Fig. 4. Responses were generated from a 100 Hz tone-pip using the underwater speaker in the acoustic tank. As noted in many fish, peak responses corresponded to twice the stimulus frequency, thus 200 Hz for a 100 Hz tone. This is probably indicative of the polarization of hair cells in the statocyst responding as the squid body oscillates in two directions with the particle motion waves (see text for details). Amplitudes are listed in dB relative to the corresponding FFT spectra. (B) FFT spectra values at 200 Hz plotted relative to the corresponding stimulus amplitude. The points with the highest r^2 value were used to calculate the regression; i.e. points from 106–131 dB re. 1 μPa (peRMS; r^2=0.93). The threshold for the tone is defined as the point where the regression line crosses zero on the response scale, in this case 108 dB re. 1 μPa (rms).

Shaker-generated response curves showed similar trends, with a region of best frequencies between 100 and 300 Hz and values of –26 dB re. 1 m s^{-2}. Below this region, response thresholds slowly increased and then leveled off at –16 dB re. 1 m s^{-2}. Loss of sensitivity at higher frequencies increased more rapidly (8 dB octave^{-1}) and responses were not detected at frequencies above 500 Hz. Acceleration thresholds from the shaker were compared with values calculated from acceleration thresholds in the acoustic tank (Fig. 11). Both measurement techniques provided comparable thresholds at regions of best sensitivity although shaker thresholds were lower at the upper and lower frequency ranges. The frequency range of response was similar for both methods.

DISCUSSION

As a sound wave propagates through a medium, regions of compression and rarefaction generated by local particle motion are concomitant with pressure fluctuations. Hair cells transduce particle motion through attendant deflection of their cilia. This deflection can be increased by coupling the hair cells to higher density objects such as otoliths in some vertebrates (Chapman and Sand, 1974; de Vries, 1950) and statoliths in invertebrates (Budelmann, 1976; Budelmann, 1992b), where acceleration of the higher density objects relative to an associated hair-cell-sensory matrix generates larger differential motion and greater deflection of the hair cell cilia (de Vries, 1950). Sound pressure detection requires compressible components that can act as pressure-to-particle motion transducers, as is the case for swimbladders in fish (Fay and Popper, 1974; Sand and Karlsen, 2000). In aquatic species for which we are attempting to define hearing abilities, it is crucial to measure both particle motion and sound pressure to determine which stimulus the animals are detecting. In small tanks, the pressure and particle motion fields may be exacerbated by reflections and can be detected by careful calibration of acoustic fields.

In this experiment, we measured both sound pressure and particle motion in water at the location of the squid head and statocyst. Although AEPs were generated using sound, shaker-generated AEPs allowed us to isolate the acceleration components without sound pressure. Thus, we were able to discriminate between responses to the two acoustic components. The similar high frequency cut-offs

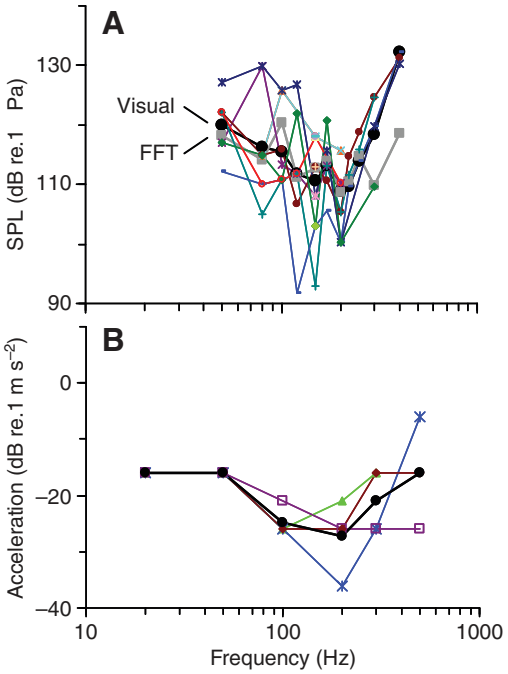

Fig. 10. (A) Thresholds of 15 squid presented in dB re. 1 μPa determined by visual inspection (full audiograms were not collected for all animals). Responses were generated using the underwater speaker. Black circles and the solid black line show the mean visually determined thresholds; grey squares and the thick grey line show the mean FFT determined thresholds. (B) Shaker-derived acceleration thresholds (dB re. 1 m s^{-2}) for four squid and mean threshold (black circles). No responses were detected above 500 Hz by either method.

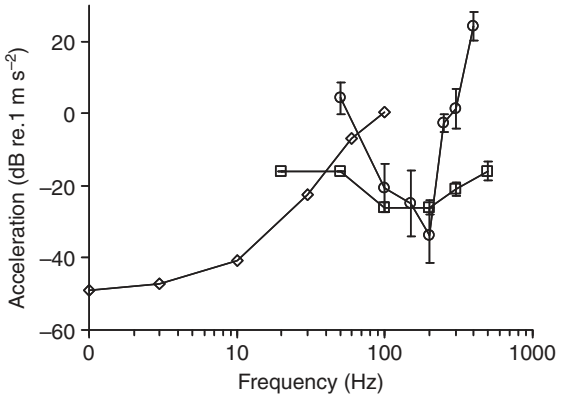

Fig. 11. Acceleration-derived (visual) AEP thresholds measured in the shaker (squares) and speaker (acoustic) tank (circles; ±s.d.) as well as conditioned behavior measured thresholds (diamonds) using a standing wave acoustic tube (Packard et al., 1990). Behavioral thresholds are from Packard et al. (Packard et al., 1990) and are the sums of squid, octopus and cuttlefish data as sample sizes were low.

and comparable acceleration thresholds for shaker vibration in air and particle motion in water lead us to conclude that it is not pressure, but rather the acceleration and particle motion components of a sound field, that a squid can detect (Fig. 11).

These results are considerably different from the finding that squid detect sound pressure and high frequencies up to 1600 Hz (Hu et al., 2009). However, the data presented here agree with established morphological and classical conditioning results (Budelmann, 1992b; Packard et al., 1990) reporting that the squid statocysts act as accelerometers. Present data also agree with the original notion that the statocyst is primarily a balance- and motion-related organ that detects movements and acceleration of the animal while swimming (for a review, see Budelmann, 1990; de Vries, 1950). Sound pressure sensitivity, as suggested by Hu et al., is not likely without a coupling to air-filled cavities, which are not consistently found in squid. Hu et al. (Hu et al., 2009) only measured the pressure component of the sound field, and it is likely that a large pressure release at the water surface, where Hu et al. held their squid during measurements, caused very large, but unquantified, particle velocities to which squid actually responded, rather than directly to the frequencies reported, which we conclude are outside their normal hearing range.

The absolute particle acceleration thresholds derived here for some frequencies compare well with the data of Packard et al., (Packard et al., 1990) using a completely different technique. This suggests that AEP techniques are promising methods for studying auditory abilities in other invertebrates as well as cephalopods. However, the two studies found differences near 100 Hz and below ~30 Hz. Increased response thresholds near the upper end of sensitivity has been shown in several other fish hearing studies using the acoustic tube method (Karlsen, 1992; Sand and Karlsen, 1986). Hearing tests that do not involve the tube method, ours included, are challenged when testing at very low frequencies. There are often additional problems with high vibration noise levels at low frequencies as well (Packard et al., 1990). Finally, recording evoked potentials at very low frequencies is often difficult because of long wavelengths and low AEP onset response. In the present study, the apparent decreased sensitivity at frequencies below 100 Hz (Fig. 11) probably stem from such AEP and noise issues.

Here we show that the statocyst and associated nerves were the probable source of the AEPs (Fig. 2; supplementary material Fig. S2). Ablation experiments show that responses were clearly not originating from the lateral line or proprioceptive neck hair cells (Pruess and Budelmann, 1995). Responses were not detected from locations on the head where the lateral-line analogue is located (however, they reached maxima near the statocyst). The AEPs were maximal in response amplitude and minimal in latency at the anterior end of the statocyst. This also suggests responses were generated near this organ. The lower amplitude responses detected away from the statocyst were probably still originating in the same place (near the statocyst) but responses attenuated as they were conducted farther through tissue to the electrode. The reversed polarity of the AEP waveforms as electrode position was moved anterior and over the brain supports the notion that recordings were made near the AEP source. Such a phenomena is seen in other taxa when electrode position is shifted relative to the axis of the evoked potential dipole (Burkhard et al., 2007; Zhang and Hood, 2004). Thus, by measuring at the statocyst, we are probably measuring from one side of this dipole source as responses are conducted toward the brain of the squid. The range of frequency response compares well to microphonic potentials recorded from squid and cuttlefish lateral lines (Budelmann and Bleckmann, 1988) implying that more than one hydroacoustic cephalopod sensory systems is adapted to low frequency detection.

Temperature effects

Decreasing temperatures diminished evoked potential amplitude (Fig. 6); raising temperature restored amplitudes and waveforms. Similar temperature-sensitive effects have been demonstrated on the

excitatory postsynaptic potential amplitude and pre-synaptic spike height of *L. pealeii* (Weight and Erulkar, 1976). However, *in vivo* tests involving the giant axon of *L. opalescens* indicated that although some physiological response characteristics may be less effective in chilled water, there are apparent compensations that maintain critical jetting pressures and escape responses (Neumeister et al., 2000).

The reduction in AEP amplitudes corresponding with decreasing water temperature was intriguing as this species is often associated with cooler water (10–15°C) (Summers, 1983). The provocative implication of the lack of AEP responses at low temperatures would seem to be that these squid do not detect sound as well in cold water. The temperature-dependent physiological effects shown here and in other studies began near 9°C but were most substantial at and below approximately 7–8°C, which is the lower thermal limit where these animals are found in nature (Hanlon and Messenger, 1996; Summers, 1983). If behavioral responses are essentially uninhibited below their thermal limit (Neumeister et al., 2000), squid may have alternative physiological response mechanisms for some stimuli. Thus, another, but possibly sub-optimal, neuronal escape system may exist for cold temperatures. Consequently, squid tend to remain in environments above a certain temperature for greater efficiency in physiological responses.

Alternatively, temperatures in these experiments may have been lowered too rapidly, causing cold stunning. Squid may be able to adapt to changes in temperature over longer (seasonal) time scales but not in tens of minutes, as administered here. However, this leaves uncertain how or if animals adapt naturally with short-term changing temperatures and depth.

Comparisons with fish hearing

Squid evoked potential generation, latency of waves and waveform characteristics appear very similar to those of some teleost fish. Like fish and elasmobranchs without auditory specializations, squid bodies have a similar density to water and are without pressure-to-particle motion transducers such as swimbladders coupled to their statocysts (Fig. 7). Given that statolithic organs act as accelerometers, the statocyst sensory epithelia will encode movements of the squid and hence also sound-induced movement of the body as it oscillates back-and-forth with the water. The fast waves are likely the result of the squid, and its hair cells, moving relative to the denser statolith. Evoked potential slow-waves may also be hair cell responses or higher-order AEPs, subsequent to the initial hair-cell deflection.

A statocyst detector system, which may primarily be for measuring acceleration and orientation, is innately equipped to also detect the particle motion component of a sound field for an animal with an impedance similar to the surrounding medium. The analogous systems of squid and some fish produce similar frequency response curves (Fay, 1988; Johnstone and Hawkins, 1978) and AEP-derived thresholds (Egner and Mann, 2005; Mann et al., 2001). This suggests that they have faced similar evolutionary pressures to orient in a gravitational field, as well as detect the linear accelerations and particle motion components of a sound field. Cephalopod thresholds from present data and Packard et al. are substantially higher than acceleration detection capabilities in many fish (e.g. Sand and Karlsen, 2000). However, at this point it is difficult to say if such differences are real or the result of the methodology. One must take into account that AEP thresholds are generally higher than thresholds measured by behavioral experiments (Karlsen, 1992), and the differences between thresholds suggest that calibrations and response measurements may not be as sensitive as the animals examined. Future experiments should involve behavioral thresholds of free swimming squid to controlled sound exposures. Also, it might be preferable (less stressful to the squid) to measure the vibration thresholds by vibrating the squid in water rather than the vibration in air used in the present study.

Squid statocyst hair cell action and responses seem similar to that of teleost fish. That is, FFT peaks to AEP responses found here were noted at twice the stimulus frequency. This is considered to be a function of hair cells that are oriented (and maximally stimulated) in-line and in opposition but parallel to the direction of the acoustic waves (Egner and Mann, 2005; Fay, 1974). One set of hair cells responds with the relative motion of the fish in the direction of the sound wave while another separate set of hair cells responds as the fish is moved in the opposite direction. Given that the statolith-statocyst functions similarly to otolithic organs, these results are not that surprising. However, beyond basic hair cells, the inner ears of squid and fish evolved convergently (Webster et al., 1992). Similarities in function and morphology reinforce the idea that both animal groups face similar aquatic challenges of orientation, predator avoidance, and prey detection and capture (O'Dor and Webber, 1986).

Biological relevance

Strong behavioral reactions to infrasound in fish, with little sign of habituation, suggest that low-frequency sound detection has been driven primarily by predator avoidance (Knudsen et al., 1992). As this is a strong evolutionary force, similar pressures may have shaped the evolution of squid audition (Hanlon and Messenger, 1996; O'Dor and Webber, 1986). Furthermore, there are field observations which suggest that squid detect and avoid swimming-generated low-frequency cues of certain fish predators (Hanlon and Budelmann, 1987). The sensitivity of the accelerometer-like auditory system will also probably allow squid to detect the water displacement generated in the head-wake produced by larger predators such as toothed whales (Wilson et al., 2007). Detection of head-wakes or similar water-motion may be limited in range but perhaps be large enough to mediate giant-axon based escape responses. However, the low-frequency auditory range (Fig. 11) and the behavioral observations of Wilson et al. (Wilson et al., 2007) do not support detection of whistles and clicks produced by echolocating odontocete predators as suggested by Hu et al. (Hu et al., 2009).

Navigation is another potential use for low-frequency hearing (Sand and Karlsen, 1986; Sand and Karlsen, 2000). Near-shore and near-surface sounds, such as waves breaking and reef-fish communication, may be useful cues for orientation. Furthermore, the large wavelengths of internal waves and their interactions with the bottom and other structures could allow pelagic animals such as squid to detect the presence of shelves or seamounts or even rafts of organisms on which they feed. This potential detection of ambient sound sources for habitat identification and general orientation has been suggested as the source of auditory capabilities in many animals (e.g. Sand and Karlsen, 1986). Sound detections of prey may also be one of the auditory functions. Such capabilities might be similar to sharks (and fish without swim-bladders), which are thought to use the low frequency movements of struggling fish as a potential cue to locate prey (Casper and Mann, 2007; Nelson and Gruber, 1963). Anthropogenic noise from shipping and airgun activities also contain a considerable amount of low frequency noise. Sensitivity to these sounds may contribute to masking of the biologically relevant stimuli or induce acoustic traumas to the receptor system, and may be worthwhile to test in squid and other marine invertebrates, as either the presence or absence of impact are important to understand.

To date, there is no indication that cephalopods themselves make sounds (Hanlon and Budelmann, 1987; Hanlon and Messenger, 1996) nor are there substantial data that support molluscan sound production. Thus, there is little support for hearing in this case to be used in intraspecific communication. However, squid jetting does generate low frequency water-flow with strong particle motion. Thus, eavesdropping of such cues may indicate to schooling squid when a nearby conspecific has jetted away, and the receiver may also jet or increase vigilance.

Detecting primarily local, near-field stimuli would limit the range of detection at higher frequencies as excess particle motion in the flow near field around a sound source attenuates relatively rapidly compared with the kinetic component of sound in an acoustic free field. Yet, at lower frequencies, which squid appear to hear, the excess particle motion signature of the flow near field extends reasonable distances (e.g. 4.8 m at 100 Hz) (Coombs et al., 1992; Wahlberg and Westerberg, 2005). Significant levels of sound-generated particle motion will also be present in the far field for high pressures, thus also available for detection. The likely directional capabilities of the squid statocyst (Budelmann, 1976; Budelmann and Williamson, 1994) may allow the squid to establish sound direction (Sand and Bleckmann, 2008). The lateral line may further allow detection of relative water motion around the squid body (Budelmann and Bleckmann, 1988).

In summary, squid use their statocysts to detect low frequency, particle motion stimuli with a frequency response similar to the accelerometer ears of most elasmobranch and teleost fishes. Evoked potential response characteristics also parallel those found in many fish species that lack auditory specializations. This acceleration-detecting auditory system overlaps with, and can probably detect, much of the low frequency natural biotic (invertebrates, fish communication, fish cues, conspecific movement) and abiotic (wind, waves) sounds in the ocean. Thus squid may use their auditory system for orientation, navigation and predator and prey detections similar to many fish, although these functions, as well as the potential for negative impacts from anthropogenic noise, remain to be addressed behaviorally.

LIST OF SYMBOLS AND ABBREVIATIONS

AEP	auditory evoked potential
c	sound speed
DAQ	data acquisition card
FFT	fast Fourier transform
p	pressure
peRMS	peak-equivalent root-mean square
p–p	peak-to-peak
SPL	sound pressure level
u	particle velocity
V_{p-p}	peak-to-peak voltage
ρ	density

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