

Loudness-dependent behavioral responses and habituation to sound by the longfin squid (*Doryteuthis pealeii*)

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Abstract Sound is an abundant cue in the marine environment, yet we know little regarding the frequency range and levels which induce behavioral responses in ecologically key marine invertebrates. Here we address the range of sounds that elicit unconditioned behavioral responses in squid *Doryteuthis pealeii*, the types of responses generated, and how responses change over multiple sound exposures. A variety of response types were evoked, from inking and jetting to body pattern changes and fin movements. Squid responded to sounds from 80 to 1000 Hz, with response rates diminishing at the higher and lower ends of this frequency range. Animals responded to the lowest sound levels in the 200–400 Hz range. Inking, an escape response, was confined to the lower frequencies and highest sound levels; jetting was more widespread. Response latencies were variable but typically occurred after 0.36 s (mean) for jetting and 0.14 s for body pattern changes; pattern changes occurred significantly faster. These results demonstrate that squid can exhibit a range of behavioral responses to sound include fleeing, deimatic and protean behaviors, all of which are associated with predator evasion. Response types were frequency and sound level dependent, reflecting a relative loudness concept to sound perception in squid.

Keywords Noise · Bioacoustics · Soundscape · Auditory scene · Invertebrate

Introduction

Squid are an abundant and ecologically vital group of marine invertebrates. Occupying a central trophic position, squid are often a key food-web link between top predators (seabirds, cetaceans, sharks, and fishes) and smaller, pelagic and mesopelagic fish and invertebrate prey (Overholtz et al. 2000; Ruiz-Cooley et al. 2004; Boyle and Rodhouse 2005). Because they are such essential taxa, addressing their sensory ecology is important to understand community relationships and environmental interactions within that ecosystem. Studies of their sensory systems have largely focused on their visual and camouflage abilities (Hanlon and Messenger 1996). Yet it is becoming increasingly apparent that squid, and other marine invertebrates, detect and respond to underwater sounds (Mooney et al. 2010; Vermeij et al. 2010; Stanley et al. 2012; Samson et al. 2014). However, the ranges and sound levels to which squid and many other marine invertebrates respond are typically unknown.

Sound is both an abundant and ecologically relevant source of information in aquatic environments; it provides an important stimulus for many vertebrates, enabling behaviors such as navigation, predator detection, and reproduction (Norris 1966; Myrberg 1981, 2001; Au and Hastings 2009). There is growing evidence that marine invertebrates may detect and respond to sound; this includes larval phonotaxis, settling in the presence of reef sounds, and physiological responses to tones (Stanley et al. 2009; Mooney et al. 2010; Lillis et al. 2013). Utilization of sound plays a key role in the behavioral ecology of vertebrates,

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and these initial data suggest a similar parallel at least for some invertebrates; therefore, there is a need to identify which sounds generate behavioral responses and the types of responses elicited for many taxa.

Historically, there has been a debate about cephalopod hearing and sound use (Moynihan 1985; Hanlon and Budelmann 1987). While early anecdotal evidence suggested that squid may respond behaviorally to sound (Dijkstra 1963; Maniwa 1976), stunning or predator avoidance responses to odontocete echolocation clicks have been hypothesized, debated and not-verified (Norris and Møhl 1983; Wilson et al. 2007). More recent work has largely focused on anatomical and physiological investigations. Squid have a lateral-line analog (Budelmann and Bleckmann 1988) that is used in predator evasion (York and Bartol 2014), and perhaps has some role in sound detection (Higgs and Radford 2016). The squid statocyst, a paired, accelerometer-like organ analogous to the fish otolith has a clear role in squid hearing (Budelmann 1990, 1992). Like many aquatic animals without compressible air cavities, squid appear only sensitive to the vibratory nature of acoustic particle motion (Packard et al. 1990; Mooney et al. 2010). Neurophysiological measurements suggest cephalopod sound sensitivities below 500 Hz (Kaifu et al. 2008; Mooney et al. 2010). Comparatively, cuttlefish behaviorally respond to sounds below 1000 Hz (although maximal sensitivities were near 150 Hz) (Samson et al. 2014). Yet corresponding behavioral data are lacking for squid and almost all other representatives of cephalopods. While the electrophysiological auditory evoked potential (AEP) data (Kaifu et al. 2008; Mooney et al. 2010) represent important results in a long debate about the auditory abilities of cephalopods (Moynihan 1985; Hanlon and Budelmann 1987), they only provide an estimate of sound levels and acoustic frequency range where behavioral responses may occur. Physiological data cannot address which behaviors are induced or influenced by sound. Unconditioned behavioral responses would be an important step in evaluating squid sound detection because such tests refer to stimulus perception (Fay 1988; Yost 1994) and can establish awareness and avoidance of sound stimuli. Addressing the gradients of behavioral responses present in cephalopods (e.g., inking, jetting and body pattern change, reaction times) and other behavioral response metrics could help evaluate more subtle perception of noise such as relative loudness (Wensveen et al. 2014). This includes using equal-latency contours, which illustrate equivalent responses at different frequencies or how response type varies based upon not only sound level but also perceived loudness, to address how different sounds such as tones of different frequencies and amplitudes, predator signals, and ship noise may be perceived and equated by the animal. Using such metrics, certain sounds may be emphasized or de-emphasized

when evaluating noise exposure criteria. Finally, addressing acoustic ecology is particularly important for squid given their global fisheries relevance (Rodhouse 2001; Hunsicker et al. 2010), numerical abundance (O'Dor et al. 2010) and aforementioned key ecological position of the taxon.

The need to understand squid acoustic ecology has been heightened by suggestions that this trophically central taxon may be impacted by increasing underwater anthropogenic noise. An initial behavioral study indicated that squid and cuttlefish may change swimming depths when exposed to distant air-gun sounds (Fewtrell and McCauley 2012). Anatomical studies of “stranded” *Architeuthis dux* revealed that statocyst hair cells may be damaged after exposures to intense sounds (André et al. 2011). Such work predicts that certain acoustic conditions could cause squid auditory damage leading to death of the exposed animals (Sole et al. 2012). If true for squid, such impacts could have ecosystem-wide repercussions.

This work seeks to address the paucity of information on squid sound sensitivity by examining how the longfin squid, *Doryteuthis* (formerly *Loligo*) *pealeii* behaviorally responds to sound. Two types of experiments were conducted. The first set of tests were used to quantify the frequency range and sound levels that generate squid behavioral responses, as well as the types of behavioral responses elicited. A second set of experiments examined whether squid behavioral response types changed over multiple acoustic exposures. The response types identified (inking, jetting and body pattern changes) have been well studied in other contexts (Hanlon and Messenger 1996; Staudinger et al. 2011) and provided a unique way to evaluate sound use by this taxon. The experiments herein aimed to fundamentally quantify the range of acoustically mediated behavioral responses in squid. In doing so, this work more broadly reflects the sounds that may be biologically relevant to many marine invertebrates.

Methods

Overview

Experiments were conducted during the summer of 2012 at the Environmental Systems Laboratory, Woods Hole Oceanographic Institution (WHOI), Woods Hole MA, USA. Adult squid (mean mantle lengths 13.4 ± 1.9 cm) were locally collected via trawl from the nearby Vineyard Sound waters, which ensured a ready supply of experimental subjects in good physical condition. Between tests, animals were maintained in two 1.2 m diameter holding tanks filled with local, flow-through, ambient temperature seawater, where they were fed daily. Two general experiments were conducted to determine: (1) the frequency range and

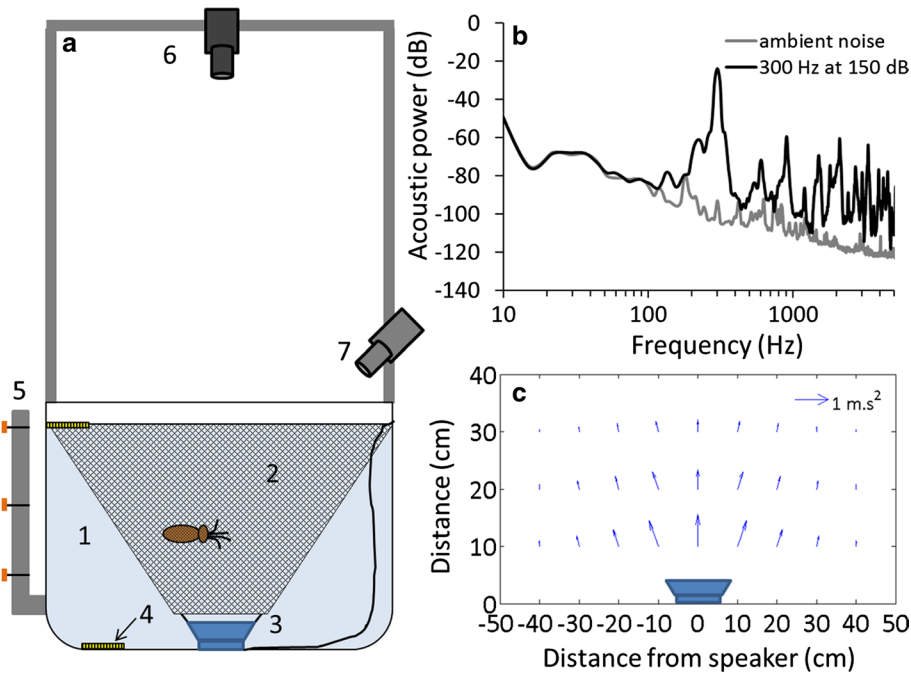


Fig. 1 **a** Schematic of the experimental set-up, side view. *1* Tank, *2* net, *3* speaker, *4* calibration ruler, *5* outflow pipe, *6* HD video camera, *7* high-speed video camera. **b** Acoustic power spectral density (dB re: $1 \mu\text{Pa}^2 \text{Hz}^{-1}$) of the 300 Hz tone (black line) at a calibrated sound level of 150 dB (received level), as recorded by calibrated hydrophone placed 10 cm above the speaker. Grey line: The spectral density of the ambient noise recorded at using the same hydrophone at the same position. **c** Vector field of the particle acceleration at

150 Hz for a calibrated sound level of 165 dB. The speaker is represented in blue, at the (0, 0) position in the tank. The vertical axes of the graph correspond to the sides of the tank and reflect distance from the tank bottom. This figure illustrates the importance of taking the distance of an animal to the speaker into account, since the sound field is very variable depending on the location in the tank. Vectors are to scale; the 1 m s^{-2} scale is noted on the figure

sound levels which generated behavioral responses and (2) the habituation occurrence and rate to repeated pure tones, following an experimental design similar to that of: (Samson et al. 2014). Tests were conducted on individual, free-swimming animals. These animals were presented a sound (a 3 s tone) and subsequent behaviors were recorded using a high-definition (HD) video and high-speed camera. Responses were scored afterwards based upon type (i.e., inking, jetting, “startle”, body pattern change, fin movement, no response) and those responses were plotted relative to stimulus type (see Supplementary Table 1 for details). Calibrations of sound pressure and particle acceleration were conducted at the beginning and end of the experiments.

Frequency and sound level tests

Behavioral response trials were conducted in a white, circular, fiberglass tank (inner diameter: 1.08 m, depth: 0.60 m), which received a continuous, low-flow of filtered sea water at ambient temperature. Animals were free-swimming in the center of a 1.08 m diameter tank. Animals were deterred from the tank wall and bottom using a stationary,

acoustically transparent, black plastic net (2 cm mesh size) hung in a conical shape from the tank rim to the speaker at the apex (see Fig. 1a). With this set-up, the animals were encouraged to swim toward the center of the tank, but their location varied at the time of the test tone. A UW30 underwater speaker (Lubell Labs Inc., Columbus, OH, USA) sat on two discs of vibration-isolating closed cell neoprene (12.7 mm each). The tank was isolated from potential vibrations through the ground by resting on two sheets of open-cell neoprene (12.7 mm each) atop a wooden platform. Care was taken to ensure animals were in the water column and not touching the sides or netting when test tones were played.

Experimental tones were generated with a custom program implemented with National Instruments LabView software (Austin, TX, USA) and a National Instruments 6062E data acquisition card, run on a laptop computer. This program allowed control of the frequency, intensity and duration of the sound pulses. Sound levels were controlled using a PYLE Chopper Series PLA2210 amplifier (Brooklyn, NY, USA) and a Hewlett-Packard 350D (Palo Alto, CA, USA) attenuator, and then played using the speaker. A Tektronix TPS 2014 oscilloscope (Beaverton,

Table 1 Matrix of initial experimental paradigm show the range of sound levels and frequencies presented to the squid

Frequency (Hz)	No sound	80	100	150	200	250	300	400	500	700	1000
165	X	–	X	X	X	–	–	–	–	–	–
160	X	X	X	X	X	X	X	–	–	–	–
155	X	X	X	X	X	X	X	X	–	–	–
150	X	X	X	X	X	X	X	X	X	X	X
140	X	X	X	X	X	X	X	X	X	X	X
130	X	X	X	X	X	X	X	X	X	X	X
120	X	X	X	X	X	X	X	X	X	X	X
110	X	X	X	X	X	X	X	X	X	X	X

OR, USA) was used to visualize the sound pulses and the signal received by the hydrophone during calibration. All tests were video recorded using a Sony HDR-XR550 HD camera (Tokyo, Japan) placed above the tank and recording at 60 fps. In order to measure response latency, a Casio EX-F1 camera (Tokyo, Japan) recording at 600 fps was fixed at an angle above one side of the tank. An LED was connected to the sound output of the computer and put in the field of view of the camera (but not visible to the squid) in order to visually record when sound signals were introduced into the tank (Fig. 1a).

Stimuli consisted of ten different test tone frequencies (80, 100, 150, 200, 250, 300, 400, 500, 700 and 1000 Hz), each 3 s in duration, plus a silent control. The experiment was initially framed in sound pressure \times frequency matrix with the range and levels of responses devised based upon physiological data (Mooney et al. 2010) (Table 1). Output levels were 110, 120, 130, 140, 150, 155, 160 and 165 dB re. 1 μ Pa rms sound pressure level (SPL) calibrated 20 cm away from the speaker. At the highest sound levels, some frequencies were distorted due to characteristics of the speaker and those sounds were not used for the experiments leaving a total of 66 combinations of sound levels and frequencies, plus the no-sound controls. Because the animals settled or swam at different distances from the speaker, the received total acceleration and sound pressure levels (SPLs) differed from the ‘source’ levels at 20 cm (noted above). Thus, the actual received levels ranged from 7.6×10^{-5} to 14.5 m s^{-2} (85–187 dB re. 1 μ Pa rms) (considering all frequencies).

A total of 101 animals were used for this experiment. At the start of each experimental day, ten individuals were randomly selected from the holding tank and kept in a separate net within that tank until used in the day’s experiments. The same individuals were typically used several days in a row. Unfortunately, it was not possible to mark individuals or separately house animals in the large tanks needed for squid husbandry (Hanlon et al. 1983; Hanlon and Messenger 1998). While this would have facilitated tracking individuals over time, keeping the individual squid separated or

in small tanks for more than a couple of hours induced high levels of stress and increased animal mortality rate. Hence, for the first experiment (frequency range and sound levels), we randomized tone presentation order and presented those tones 15–25 min apart (timing was also randomized). This specifically reduced any long-term learning effect (response rates were consistent throughout the experiment) and allowed us to quantify exposures as independent. Animals were fed daily but tended to expire within several days as is typical for the species’ breeding and semelparous life cycle (Boyle and Rodhouse 2005; Jacobson (NOAA) 2005). At the start of a trial, an animal was moved from the holding tank to the test tank where it was allowed \sim 2 min to acclimate before the tone (or silence) was presented. The behavioral responses for each squid were categorized during a timeframe which included the 3 s tone and 1.5 s immediately afterward using six response types: no response, body pattern change, fin movements, startle, jetting and inking with some gradations noted; see Supplementary Table 1 and (Samson et al. 2014). Notably, ‘inking’ only occurred with jetting, and was referred to as inking; but jetting could occur separately as was thus referred to as ‘jetting’. Body pattern changes were divided up into ‘large’ and ‘small’ where ‘large’ body pattern change included pattern change covering at least half the body area, as well as dark flashing, bleaching/paling, and stereotypical patterning such as deimatic responses, dark fin lines, eye rings or eye spots. Small body pattern changes included less than half the body area. This scoring system was based on observations of the animals before the experiments and well-established squid responses in the context of predators and human-elicited stress (Hanlon and Messenger 1996; Staudinger et al. 2011). Each day, four sound stimuli were randomly chosen from the tone matrix and those four sounds were then presented in a random order to each of the ten squid. After sound presentation, the tested animal was returned to the main part of the housing tank. The next day, four new tones were chosen and randomly presented, and the procedure was repeated until all sounds and controls in the matrix were presented. As squid deceased, they were replaced

by newly collected animals. If animals were not exhibiting normal coloration and swimming patterns (Hanlon and Messenger 1998) they were no longer used in the experiments. This included termination of the trial if the animal degraded during a trial. The order of presentations was randomized for each animal; all animals received four sounds per day. This procedure helped to prevent individual squid from potentially receiving the same sound twice. To ensure there was no order effect, response rates were compared across the experiment. Response latencies were calculated from stimulus onset to the response onset using the high-speed video recording for 46 trials where the animal was clearly visible in the limited field of view and the response was identifiable.

Habituation to repeated sounds

Specific habituation tests took place over five consecutive dates after the overall frequency-sound level tests were conducted using fifteen animals. These animals were not used previously, having been freshly acquired from the fishing boat within 0–2 days of their study sessions. Animals were chosen randomly each day and exposed to a 3-s tone, presented every minute for 30 min (i.e., 30 trials/session). The exposure sound was randomly chosen from six possible frequency-SPL combinations; frequencies were 100, 200, and 300 Hz, and SPLs were 160 and 140 dB. Habituation (or sensitization) was evaluated as the response rate overall and within each response type across the 30-trial session. Animals were presented only one frequency, but both sound levels, with sessions separated by 1 day. Responses were recorded and observed post hoc using the same prior video setup, and were then compared within an individual's session and individuals were pooled for frequency and sound level comparisons. These sounds were chosen because they spanned the most sensitive area of squid hearing and the levels induced behavioral responses in cuttlefish (Mooney et al. 2010; Samson et al. 2014). As for previous behavioral trials, exposure levels were corrected for the distance of the animal to the speaker. Standard regression analyses were used to estimate the relationship between trial number and rate of occurrence of the different response types.

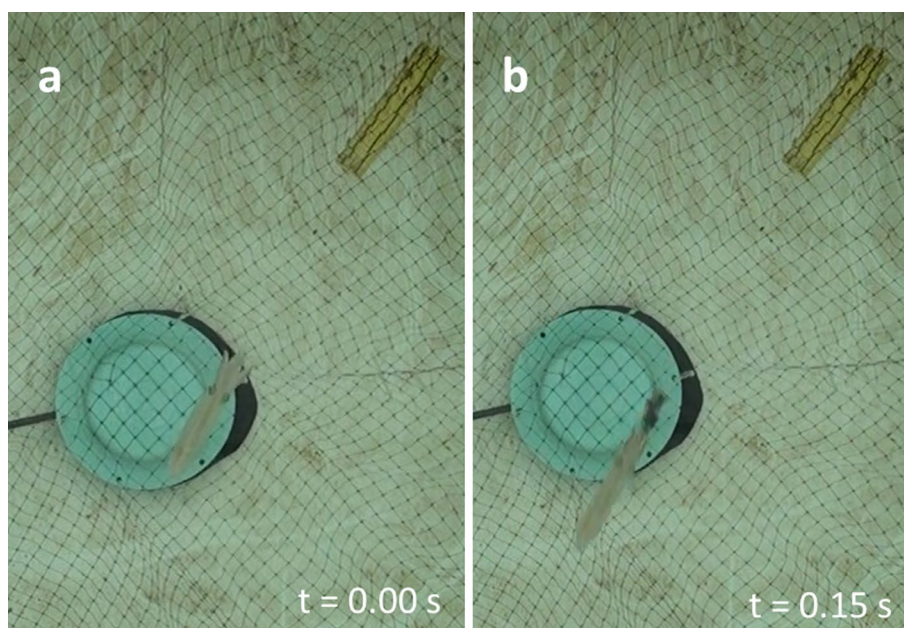
Sound calibrations

While cephalopods detect acoustic particle motion, sound pressure and particle motion are closely related and both were calibrated across the diameter and depth of the tank in 10 cm increments using each experimental test tone (Fig. 1b, c). Calibration measurements were made at the beginning and end of the experiment. Sound pressure was measured using a calibrated Reson TC 4014 hydrophone (Slangerup, Denmark) and particle acceleration values

were obtained by measuring the pressure gradient over two closely spaced sound receivers (Gade 1982; Mooney et al. 2010). For basic sound pressure measurements (dB re 1 μPa rms), the hydrophone was suspended 10 cm from the center of the speaker and moved incrementally up and to the side. The peak-to-peak amplitude of the signals was measured on the oscilloscope, and converted from voltages to SPL using a custom MatLab script. The tones were concurrently recorded using an Olympus LS-10 PCM recorder (Olympus America Inc., Center valley, PA, USA). For the particle acceleration, two custom hydrophones (-180 dB re 1 $\text{V}/\mu\text{Pa}$), vertically spaced 5 cm apart, were fixed in a location 10 cm directly above the speaker. As a stimulus was played, pressure measures at both hydrophones were concurrently measured (sampling rate: 120 kHz) and digitally stored for later analyses. The hydrophone setup was moved along the diameter and depth of the tank in 10 cm increments as described for the calibration of the sound pressure level. This two-hydrophone setup was repeated for each x, y, z direction so that particle motion could be calculated for all three dimensions. The z-plane was always the dominant axes but because animals receive sound from all three directions concurrently, the magnitude of the acceleration was computed and used for the data analysis and figures. Within the acoustic near field of the speaker, the squid was expected to act as a rigid body with respect to particle acceleration values at each location (Denton and Gray 1982; Coombs et al. 1992).

From these measurements, the actual received sound pressure levels and particle acceleration values could be calculated as functions of the distance from the animal to the speaker. Two 15 cm rulers were fixed in the tank during all trials: one was placed at water's surface and the other on the bottom of the tank (51 cm from the water surface). A custom-made MatLab tracking program was used to get the coordinates of the rulers, speaker, and squid from the video frames preceding the sound onset. The ratio of the lengths of both rulers, as observed vertically by the camera, was calculated using their respective pixel lengths in each video. The actual size of each animal (mantle length in mm) was measured and its actual depth could therefore be computed using the sizes of the rulers and the animal's mantle length observed in the videos. From the size of the animal, the expected pixel length was calculated at the water's surface and compared to its observed pixel length in each video. The ratio of observed animal length to expected animal length at the surface, compared to the ratio of the rulers' lengths, allowed us to calculate the vertical distance between the animal and the speaker. At the time of stimulus presentation, animals were all horizontal, or near horizontal, in the typical swimming position. Horizontal distance from the speaker to the center of the animal's head (measured as a point halfway between the eyes) was also

Fig. 2 Types of behavioral responses to sound. These frames are extracted from one test and illustrate how different behavioral responses can be combined. **a** Squid at rest in the experimental tank before the sound stimulus. The arms are splayed outward and the animal's color and pattern is generally matching the tank background. **b** Jetting, inking, and slight fin movement



determined. Total distance from the speaker to the center of the animal's head was computed using the horizontal and vertical distances. This total distance was then used to calculate the received sound pressure level and particle acceleration at the animal's head (where the statocysts are located) for each sound test. Analyses were conducted in Excel and MatLab.

Results

Frequency and sound level responses

Sounds generated clear behavioral responses, ranging from inking and jetting to small body pattern changes and fin movements (Fig. 2). Responses occurred at all frequencies tested but response types and occurrence rates were both frequency and sound level dependent (Figs. 3, 4). Thus, mean particle acceleration levels that elicited behavioral responses were not constant over frequencies tested; in particular, jetting and body pattern change responses varied in the levels that induced responses when compared across frequency.

Inking (which always occurred with a jet) only occurred at highest sound levels and lowest frequencies (at 6.75 m s^{-2} mean particle acceleration value, Figs. 3, 5). Lowest sound levels which induced inking occurred at 150 Hz (2.17 m s^{-2}). Jetting alone occurred more often and across a broader range of frequencies and levels although responses were still concentrated at the lower frequencies and higher sound levels (mean responses were found

at 2.55 m s^{-2}). Startle responses were not observed very often and were concentrated at the lower frequencies; mean response values were similar to jetting (2.50 m s^{-2}).

More moderate responses were categorized as large and small body pattern change and/or fin movement. Small body pattern change responses were generally exhibited at sound levels about an order of magnitude below inking (0.84 m s^{-2} , Fig. 3). These patterning responses were observed across the range tested, although fewer responses were noted at the higher frequencies (Fig. 3). The less intense patterning responses were seen at acceleration levels down to 0.001 m s^{-2} (400 Hz). Larger body pattern change and fin movements were noted at a mean level of 1.94 m s^{-2} . Multiple behaviors often occurred concurrently. For example, a 100 Hz tone at higher sound levels might induce inking, jetting and body pattern change. Finally, in many cases at all sound levels and frequencies, animals did not exhibit observable responses to sound stimuli. However, this 'no response' occurred predominantly at the lower sound levels, with a mean 'no response' at 0.62 m s^{-2} . Occurrence rates of responses were frequency and sound level dependent (Fig. 5). No responses occurred most often (Fig. 5).

Most responses occurred between latencies of 0.1–0.3 s although the fastest responses were 0.008 s for jetting and 0.01 s for body pattern change (Fig. 6). Maximum durations were greater than 1.0 s (1.41 s—jetting; 1.06 s body pattern change), such long-latency responses (greater than 1 s) occurred only once for each behavior. Thus responses were typically much more rapid. Mean latencies were significantly shorter for body pattern change ($0.14 \text{ s} \pm 0.20 \text{ SD}$) compared to jetting ($0.36 \text{ s} \pm 0.41$)

Fig. 3 Received particle accelerations and the behavioral responses they elicited. Only the highest scoring behaviors for each sound test are represented here (i.e., not all occurrences of each response types are shown). Large body pattern/fin movement: large body pattern change and/or fast fin movements. Small body pattern/fin movement: small body pattern change and/or slow fin movements. The horizontal dashed lines represent the mean particle acceleration level for that response

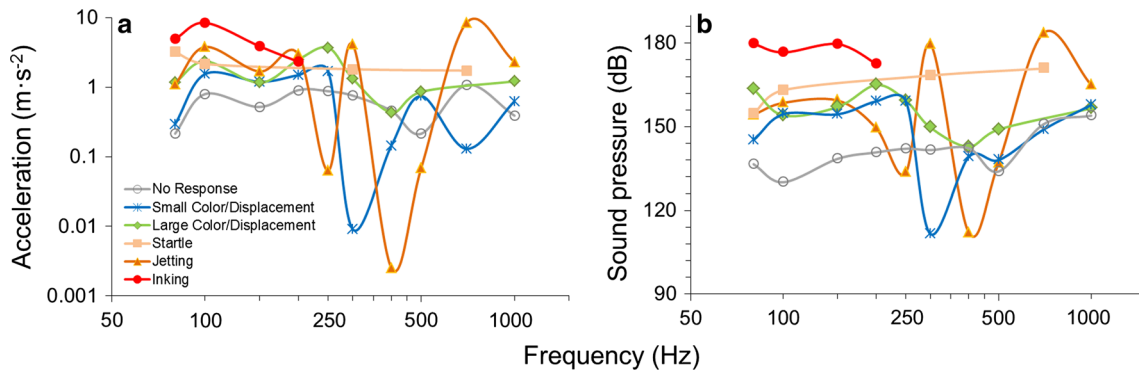
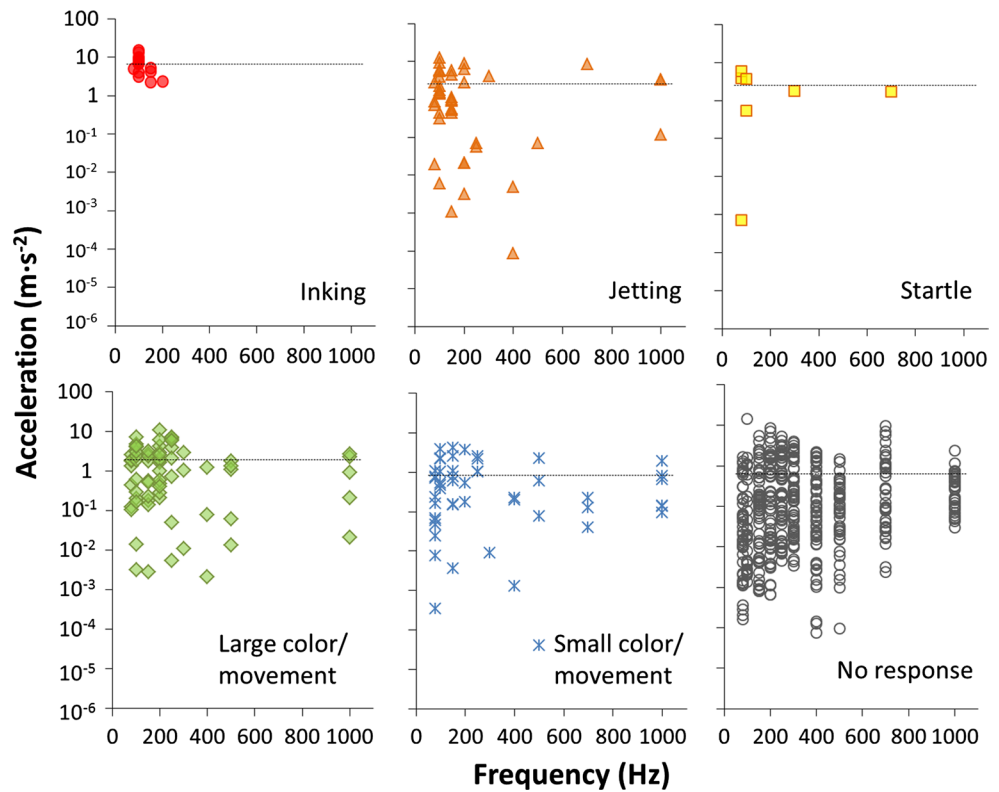


Fig. 4 Mean acceleration (a) and sound pressure (b) that elicited each behavioral response with respect to sound frequency. Response types are color-coded. Only the highest scoring behaviors for each sound test are represented here

regardless of whether these maximum latencies were considered outliers or not (two-tailed *t* test, $p < 0.05$; see Supplementary Table 2 for descriptive statistics). Latencies did not show a significant dependence on frequency (one-way ANOVA $p > 0.05$; see Supplementary Table 3 for ANOVA tables). Nor was there a relationship (linear or logarithmic) between latency of pattern change and acceleration sound level ($r^2 = 0.016$; $Y = -0.5025 \times X + 0.1588$; $p > 0.05$). However, latency of jetting responses were weakly, positively related to particle acceleration sound levels (Fig. 6; $r^2 = 0.567$; $Y = 28.006 \times X^{0.9697}$; $p < 0.01$; see Supplementary Table 4).

Habituation to repeated sounds

Animals habituated to repeated acoustic stimuli, as was reflected by the decrease of the number of animals observed responding across successive repeated tone trials (Fig. 7). This decrease was relatively rapid and logarithmic in nature for both jetting ($y = -0.398 \times \ln(x) + 1.1626$; $r^2 = 0.4235$) and body pattern change ($y = -1.119 \times \ln(x) + 3.6747$; $r^2 = 0.4965$). Habituation was also notable in the response type, which generally changed from escape responses (inking and jetting) to body pattern change. Jetting and inking responses were often no longer exhibited after a short

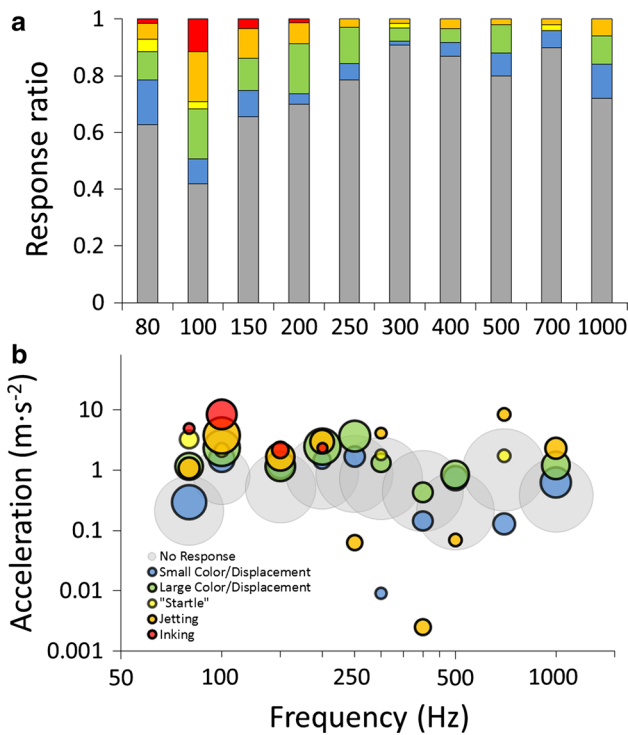


Fig. 5 **a** Relative response occurrence rate for each frequency tested. **b** Response rate (with respect to *circle area*) relative to the sound levels and frequencies presented. Behaviors are reflected by the *colors* in the inset of 'b'

number of trials (1–3). Body pattern change response rates also decreased rapidly for initial trials. However, for some animals, these reactions reoccurred in later trials. Notably, habituation tests also showed individual variations in response occurrences where some animals reflected

differences in both initial response intensities and rate of decrease. Additionally, some animals demonstrated intermittent response occurrences over the session (Fig. 7b), whereas other animals did not show sound-associated response after the initial trials (Fig. 7c).

Animals were allowed to swim freely in the tank during the sessions. During the higher source level session, animals tended to position themselves close to the surface after several repeated exposures and subsequently received lower sound levels as trials increased. For example, acceleration values were significantly higher for first trial compared to the fifth, fifteenth and thirtieth trials ($F_{3,48} = 3.67$; $p = 0.018$; one-way ANOVA). There was no significant difference during the lower source level sessions.

Discussion

The goal of this work was to define the sound levels and frequency range to which an ecologically key marine invertebrate responds and, respectively, quantify the types of responses to varying stimuli. The results reveal that squid exhibit clear acoustically mediated behavioral responses; and when those responses occur they are behaviors associated with escape and predation avoidance, particularly fleeing (jetting) but also protean responses of inking and body pattern change. Protean responses may serve to startle or confuse a predator with erratic, unpredictable escape sequences (Humphries and Driver 1970; Hanlon and Messenger 1998; Staudinger et al. 2011). Deimatic patterning changes may serve to bluff the predator (through impressions of size or behavior) or signal a warning of danger to conspecifics (Edmunds 1974; Hanlon and Messenger 1998).

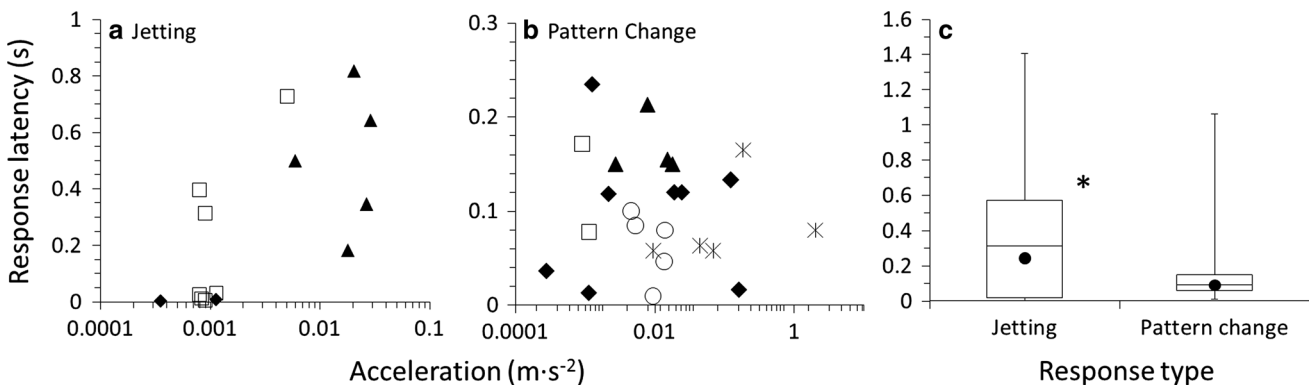


Fig. 6 Response latencies for **a** jetting and **b** large pattern change. *Shapes* reflect different frequencies (*black diamonds* 80 Hz, *black triangles* 100 Hz, *open squares* 150 Hz, *star* 200 Hz, *open circles* 250 Hz). The maximum outlier values (jetting = 1.41 s; pattern change = 1.06 s) were not plotted to better reflect the spread of most

data. **c** Box plots (median ± 25/75 quartiles; mean = dot; whiskers show data range) of all latency data for jetting and pattern change responses (including outliers). Response latency differed significantly for these two categories (two-tailed *t* test, $p < 0.05$). Note the *y*-axes scales differ

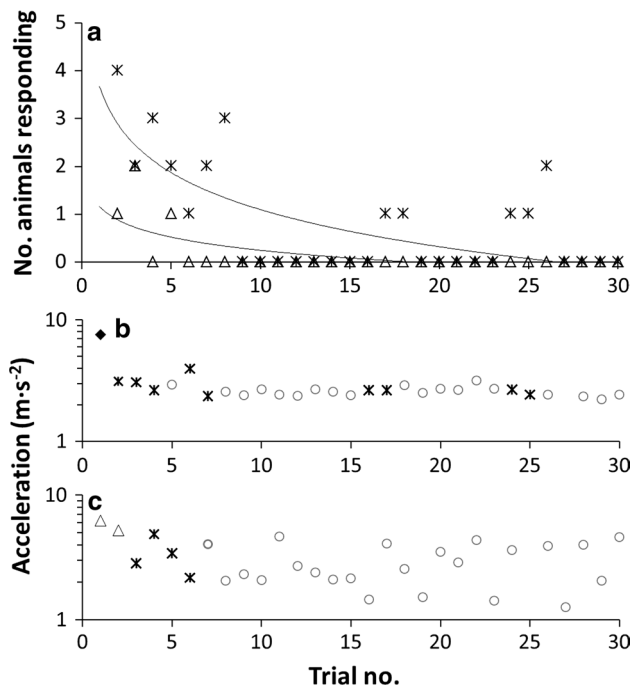


Fig. 7 **a** Habituation to a repeated sound stimulus. Data were collected using a 200 Hz tone at 160 dB (calibrated sound pressure), which was presented every minute for 30 consecutive trials. *Diamond* inking, *triangles* jetting (*bottom line*), *stars* color change (*top line*). The observations of both sound-induced jetting and color change decreased logarithmically. **b**, **c** Succession of behavioral responses of two individual squid using the 160 dB 200 Hz tone. No response for a given trial is indicated by the *open circles*, which also reflect the received level for that trial. This received level varied as the animal moved throughout the tank during the session

Reponses based on sound type and loudness contours

The frequency range and sound level data may also be used to evaluate the potential soundscape and auditory scene utilized by squid, as well as provide an initial assessment of how these animals may be influenced by anthropogenic noise. When compared to prior physiological and classically conditioned experiments (Packard et al. 1990; Mooney et al. 2010), the unconditioned behavioral responses measured here actually broaden our understanding of the sound levels and frequencies to which squid respond, noting that responses (although few) occurred up to 1000 Hz. This frequency range includes that of many of the known fish and invertebrate sounds (Fish and Mowbray 1970; Henninger and Watson 2005; Radford et al. 2008; Tricas and Boyle 2014), reflecting that squid may be able to sense and use these sounds.

At the lower frequencies (below 250 Hz), the mean response levels determined here (for all response types) were more than an order of magnitude higher than physiological thresholds measured for the same species (Mooney et al. 2010). This suggests that while inking, jetting and

pattern changes are used to evaluate responses to perceived threats, they may not be indicative of (and in fact would overestimate) hearing sensitivities and auditory sensation levels, at least at these frequencies. Thresholds lower than unconditioned response levels may be expected. Yet, at higher frequencies (300–400 Hz), auditory thresholds (Mooney et al. 2010) were similar to large pattern change and displacement response means, and were actually occasionally greater than smaller pattern and jetting mean values. At first glance these results suggest the physiological ‘thresholds’ at higher frequencies are above true detection thresholds (likely caused by differences in tanks and experimental setup). The behavioral levels may also provide insight into how squid may use sound. All responses (inking, jetting, pattern change) are clustered around similar sound levels, well above thresholds indicating that loud sounds (such as imminent predators) are required to induce these behaviors. At higher frequencies, response types are more divergent and occur at relatively low sound levels, suggesting that sound may have a different function at these frequencies, perhaps orientation, soundscape assessment or other auditory scene analyses.

One can use the general association of sound levels with response types to predict the conditions which may induce certain behaviors. The identified behaviors have a long history of association with their ecological interaction and degree of threat (predator evasion, agonistic displays, etc.) (Hanlon and Messenger 1996; Staudinger et al. 2011). Thus, it may be possible to leverage the understanding of these responses to infer the potential adverseness of these anthropogenic stimuli. Similar behavioral responses across the sound types might be a means to address relative loudness contours for squid (Fletcher and Munson 1933). For mammals and birds, equal loudness contours provide a relationship between the sound pressure level and perceived loudness across frequencies (Suzuki and Takeshima 2004). Similar contours have been proposed for cuttlefish (Samson et al. 2014), but for cuttlefish and squid, the relationship is with acceleration levels of a pure tone that have the same apparent loudness at various frequencies. These estimated loudness contours may be used as a first step to infer potential noise influences for a range of low frequency sounds.

Similar to the cuttlefish (Samson et al. 2014), levels of mean behavioral response could be separated relative to response type. Thus it was possible to discriminate the sound levels and frequencies which induced escape responses such as inking and jetting, and those which induced the milder body pattern change or subtle movements of body parts (like fins or arms). Generally, inking and jetting were confined to higher sound levels ($>1 \text{ m s}^{-2}$) and lower frequencies (200 Hz and below), although jetting showed more occurrences and variation in the frequencies and sound levels that induced response, especially above

200 Hz. Both response types are typically used for predator evasion. Their limited proportions or general absence at lower sound levels ($<1 \text{ m s}^{-2}$) suggests that sound must be of relatively high received intensity to induce these escape responses. High-level stimuli would likely be indicative of unexpected, camouflaged predators such as flounder (Staudinger et al. 2011), where the squid rapidly flee and potentially ink to avoid capture, supporting that hearing may be used to occasionally enact these behaviors. It is also possible that squid “save” the higher energetic response (inking/jetting) for when they feel a threat is eminent. An additional (visual) threat may have helped induce escape responses at lower sound levels.

Response latencies and species comparisons

Response latencies were, on average, faster for body pattern changes which perhaps reflects the relative efficiency of this neural circuitry and concomitant muscular responses (Nixon and Young 2003). However, rapid jetting responses were occasionally induced, reflecting perhaps a response mediated in part by the squid giant axon (Otis and Gilly 1990). Acoustically mediated responses suggest that squid may utilize hearing (i.e., detection of acceleration) to detect and avoid potential predator threats, which is a key adaptation in perceiving the auditory scene (Fay 2009). Particle acceleration events could arise from the head-wake of large predators such as some fishes and marine mammals (Niesterek and Hanke 2013) and may be particularly vital to detect when squid are rapidly approached by ambush predators (Staudinger et al. 2011). Detecting the head-wake of a predator via acoustic and water-motion cues would be quite important when vision is not helpful including in the aphotic zone, at night and in murky waters.

Squid did not show a decrease in response latency as sound levels increased, a phenomena which has been shown in some mammalian taxa including humans and dolphins (Green et al. 1957; Johnson 1968). In these animals, acoustic signal detection is dependent upon the overall energy in the signal, thus response detection can be improved by either an increase in signal intensity or duration (Yost 1994). Conversely, as sound levels decrease, response latencies increase. The lack of a relationship between response latency to acceleration level suggests perhaps the squid statocyst does not act as an energy detector as does the ear in mammals. Or perhaps sound levels were above the threshold for which responses are latency dependent. Additionally, the experiments were specifically designed to incorporate multiple frequencies and these differences in hearing across frequencies may have introduced variation that obscured potential trends. Response latency did vary based upon response type, reflecting that body patterning change occurs faster than jetting. Notably, body

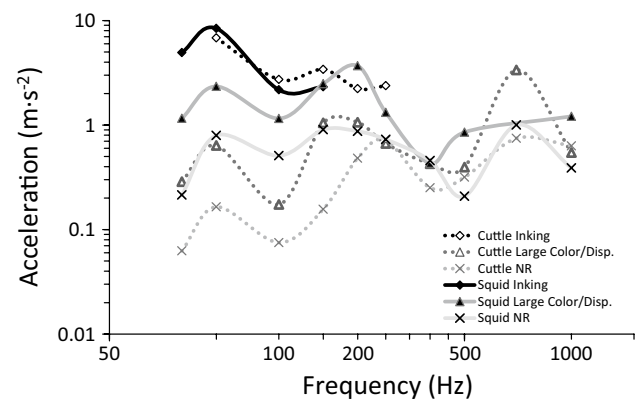


Fig. 8 Comparison of squid and cuttlefish behavioral response data (modified from Samson et al. 2014)

pattern changes also occur at lower sound levels, reflecting that in multiple ways, the initial response to a predator or other acceleratory stimuli may be body pattern changes.

The response levels were compared to those of cuttlefish with some similarities (Fig. 8; Samson et al. 2014). The inking responses observed here were comparable in sound levels to those observed previously for cuttlefish, although squid responses occurred at slightly lower frequencies. This similarity suggests that animals have similar behavioral means for escape responses. Yet, squid showed higher mean response levels for large body pattern change and ‘no response’ conditions. This may mean that squid do not respond to lower level acoustic stimuli which are potentially not life-threatening; or they may simply be less sensitive to the lower level sounds. Alternatively, the common cuttlefish may have a higher skin chromatophore density (Hanlon and Messenger 1988; Mäthger and Hanlon 2007) making responses easier to observe and thus lowering our detection threshold for this taxon. Life history might also influence these differences. For example, the three-dimensional lifestyle of pelagic squid may result in some atrophy of balance-rated sensory organs [as seen in some aquatic mammals (Ketten 1994)]. Additionally, the long-fin squid is a schooling species often found in the water column (in contrast to the epi-benthic common cuttlefish) (Hanlon and Messenger 1998). The higher ‘no response’ level of these squid might reflect that they are undisturbed by abrupt, ambush type sound, until those sounds reach a level that counteracts the protection provided by a school. Similarly, as a schooling animal, visual displays could actually serve to help a predator single you out from the school, and would thus be counterproductive to predator avoidance. Perhaps responses are also dependent upon sensory input from their neighbors in the school. Seeing conspecifics jet away or change body pattern/posture may influence response levels. Thus, future work should address

multi-modal (visual plus sound) mediated escape behaviors and responses of squid schools.

Habituation experiments

Unlike cuttlefish, squid exhibited relatively few startle responses. In the habituation tests of most squid, escape responses were not apparent after a few trials. In the cuttlefish there was often a startle response even after 45 trials (Samson et al. 2014). It is uncertain why these squid and cuttlefish may differ, but the results show that squid can essentially habituate to repeated sound stimuli. Perhaps squid are overall less ‘sound-sensitive’ compared to cuttlefish; unfortunately there are few data on comparative statocyst hair cell anatomy or physiology to address relative sensitivities. Similar to above, differences might also be due to variations in species life history or visual patterning systems. However, some squid did demonstrate occasional responses after multiple trials suggesting that at least some animals were still vigilant and continuously monitoring the auditory scene.

The habituation experiments also seemed to reveal some directional movement away from the speaker. In nearly all cases, animals moved to a location of lower sound level after the first acoustic trial and most animals moved to a quieter area after five trials. Animals were swimming freely and often had the chance to move toward the center of the speaker’s beam pattern and toward the surface, away from the speaker. But typically this movement was both higher in the water column and laterally outside the center of the speaker’s beam. This movement to lower sound level areas suggests both the ability to determine sound source directionality and an aversion to the higher sound levels.

Conclusions

These data provide the first assessment of the frequency range and sound levels to which squid behaviorally respond. Further, the responses are unconditioned behaviors. The results indicate that a variety of biologically relevant responses may be elicited by acoustic stimuli, supporting the idea that cephalopods may use sound cues to evaluate their environment. While responses could be generally characterized as predator avoidance behaviors, the demonstration of biologically relevant response implies that squid may use sound for other behaviors such as navigation or orientation. As an ecologically vital taxon, unconditioned acoustic behaviors in squid highlight the growing understanding of how important sound is to the sensory ecology of marine invertebrates and the communities they support. Generally, animals were responsive to low frequencies below 1000 Hz, and were most sensitive to sounds below 300 Hz. This low frequency sensitivity

overlaps with the predominant frequencies in ocean noise; both natural wind and wave noise, as well as anthropogenic sounds such as air guns, construction and commercial shipping occur at these lower frequency levels (Urlick 1983). As these frequencies travel efficiently in the ocean, this overlap raises concern that this noise is increasingly pervasive (Hatch et al. 2008) and cephalopods might be impacted. While there has been some suggestion that close exposures to impulse sounds could cause anatomical damage (André et al. 2011), lower level effects such as masking or behavioral responses are perhaps more likely. These results suggest that a range of response could be elicited, from jetting, to moving away from an undesired noisy area, or simple habituation to the noise. Yet, these impacts are not fully resolved and population level responses are certainly unclear. In demonstrating the overall range of responses that sounds may induce in squid, these results greatly support the need for a better understanding of noise impacts on these ecologically key taxa.

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