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

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21 **Abstract**

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

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38

39 **Introduction**

40 Squid are an abundant and ecologically vital group of marine invertebrates. Occupying a  
41 central trophic position, squid are often a key food-web link between top predators (seabirds,  
42 cetaceans, sharks, and fishes) and smaller, pelagic and mesopelagic fish and invertebrate prey  
43 (Overholtz et al. 2000; Ruiz-Cooley et al. 2004; Boyle and Rodhouse 2005). Because they are  
44 such essential taxa, addressing their sensory ecology is important to understanding community  
45 relationships and environmental interactions within that ecosystem. Studies of their sensory  
46 systems have largely focused on their visual and camouflage abilities (Hanlon and Messenger  
47 1996). Yet it is becoming increasingly apparent that squid, and other marine invertebrates, detect  
48 and respond to underwater sounds (Mooney et al. 2010; Vermeij et al. 2010; Stanley et al. 2012;  
49 Samson et al. 2014). However, the ranges and sound levels to which squid and many other  
50 marine invertebrates respond are typically unknown.

51 Sound is both an abundant and ecologically relevant source of information in aquatic  
52 environments; it provides an important stimulus for many vertebrates, enabling behaviors such as  
53 navigation, predator detection, and reproduction (Norris 1966; Myrberg 1981; Myrberg 2001; Au  
54 and Hastings 2009). There is growing evidence that marine invertebrates may detect and respond  
55 to sound; this includes larval phonotaxis, settling in the presence of reef sounds, and  
56 physiological responses to tones (Stanley et al. 2009; Mooney et al. 2010; Lillis et al. 2013).  
57 Utilization of sound plays a key role in the behavioral ecology of vertebrates, and these initial  
58 data suggest a similar parallel at least for some invertebrates; therefore there is a need to identify  
59 which sounds generate behavioral responses and the types of responses elicited for many taxa.

60 Historically, there has been a debate about cephalopod hearing and sound use (Moynihan  
61 1985; Hanlon and Budelmann 1987). While early anecdotal evidence suggested that squid may

62 respond behaviorally to sound (Dijkgraaf 1963; Maniwa 1976), stunning or predator avoidance  
63 responses to odontocete echolocation clicks have been hypothesized, debated and not-verified  
64 (Norris and Møhl 1983; Wilson et al. 2007). More recent work has largely focused on anatomical  
65 and physiological investigations. Squid have a lateral-line analog (Budelmann and Bleckmann  
66 1988) that is used in predator evasion (York and Bartol 2014), and perhaps has some role in  
67 sound detection (Higgs and Radford 2016). The squid statocyst, a paired, accelerometer-like  
68 organ analogous to the fish otolith has a clear role in squid hearing (Budelmann 1990;  
69 Budelmann 1992). Like many aquatic animals without compressible air cavities, squid appear  
70 only sensitive to the vibratory nature of acoustic particle motion (Packard et al. 1990; Mooney et  
71 al. 2010). Neurophysiological measurements suggest cephalopod sound sensitivities below 500  
72 Hz (Kaifu et al. 2008; Mooney et al. 2010). Comparatively, cuttlefish behaviorally respond to  
73 sounds below 1000 Hz (although maximal sensitivities were near 150 Hz) (Samson et al. 2014).  
74 Yet corresponding behavioral data are lacking for squid and almost all other representatives of  
75 cephalopods. While the electrophysiological auditory evoked potential (AEP) data (Kaifu et al.  
76 2008; Mooney et al. 2010) represent important results in a long debate about the auditory  
77 abilities of cephalopods (Moynihan 1985; Hanlon and Budelmann 1987), they only provide an  
78 estimate of sound levels and acoustic frequency range where behavioral responses may occur.  
79 Physiological data cannot address which behaviors are induced or influenced by sound.  
80 Unconditioned behavioral responses would be an important step in evaluating squid sound  
81 detection because such tests refer to stimulus perception (Fay 1988; Yost 1994) and can establish  
82 awareness and avoidance of sound stimuli. Addressing the gradients of behavioral responses  
83 present in cephalopods (e.g., inking, jetting and body pattern change, reaction times) and other  
84 behavioral response metrics could help evaluate more subtle perception of noise such as relative

85 loudness (Wensveen et al. 2014). This includes using equal-latency contours, which illustrate  
86 equivalent responses at different frequencies or how response-type varies based upon not only  
87 sound level but perceived loudness, to address how different sounds such as tones of different  
88 frequencies and amplitudes, predator signals, and ship noise may be perceived and equated by  
89 the animal. Using such metrics, certain sounds may be emphasized or de-emphasized when  
90 evaluating noise exposure criteria. Finally, addressing acoustic ecology is particularly important  
91 for squid given their global fisheries relevance (Rodhouse 2001; Hunsicker et al. 2010),  
92 numerical abundance (O'Dor et al. 2010) and aforementioned key ecological position of the  
93 taxon.

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

102         This work seeks to address the paucity of information on squid sound sensitivity by  
103 examining how the longfin squid, *Doryteuthis* (formerly *Loligo*) *pealeii* behaviorally responds to  
104 sound. Two types of experiments were conducted. The first set of tests were used to quantify the  
105 frequency range and sound levels that generate squid behavioral responses, as well as the types  
106 of behavioral responses elicited. A second set of experiments examined whether squid behavioral  
107 response types changed over multiple acoustic exposures. The response types identified (inking,

108 jetting and body pattern changes) have been well-studied in other contexts (Hanlon and  
109 Messenger 1996; Staudinger et al. 2011) and provided a unique way to evaluate sound use by  
110 this taxon. The experiments herein aimed to fundamentally quantify the range of acoustically  
111 mediated behavioral responses in squid. In doing so, this work more broadly reflects the sounds  
112 that may be biologically relevant to many marine invertebrates.

113

## 114 **Methods**

### 115 *Overview*

116 Experiments were conducted during the summer of 2012 at the Environmental Systems  
117 Laboratory (ESL), Woods Hole Oceanographic Institution (WHOI), Woods Hole MA, USA.  
118 Adult squid (mean mantle lengths  $13.4 \pm 1.9$  cm) were locally collected via trawl from the nearby  
119 Vineyard Sound waters, which ensured a ready-supply of experimental subjects in good physical  
120 condition. Between tests, animals were maintained in two 1.2 m diameter holding tanks filled  
121 with local, flow-through, ambient temperature seawater, where they were fed daily. Two general  
122 experiments were conducted to determine: (1) the frequency range and sound levels which  
123 generated behavioral responses and (2) the habituation occurrence and rate to repeated pure  
124 tones, following an experimental design similar to that of: (Samson et al. 2014). Tests were  
125 conducted on individual, free-swimming animals. These animals were presented a sound (a 3 s  
126 tone) and subsequent behaviors were recorded using a high-definition (HD) video and high speed  
127 camera. Responses were scored afterwards based upon type (i.e., inking, jetting, “startle,” body  
128 pattern change, fin movement, no response) and those responses were plotted relative to stimulus  
129 type. Calibrations of sound pressure and particle acceleration were conducted at the beginning  
130 and end of the experiments.

131

132 *Frequency and sound level tests*

133 Behavioral response trials were conducted in a white, circular, fiberglass tank (inner  
134 diameter: 1.08 m, depth: 0.60 m), which received a continuous, low-flow of filtered sea water at  
135 ambient temperature. Animals were free-swimming in the center of a 1.08 m diameter tank.  
136 Animals were deterred from the tank wall and bottom using a stationary, acoustically  
137 transparent, black plastic net (2 cm mesh size) hung in a conical shape from the tank rim to the  
138 speaker at the apex (see Fig 1a). With this set-up, the animals were encouraged to swim toward  
139 the center of the tank, but their location varied at the time of the test tone. A UW30 underwater  
140 speaker (Lubell Labs Inc., Columbus, OH, USA) sat on two discs of vibration-isolating closed  
141 cell neoprene (12.7 mm each). The tank was isolated from potential vibrations through the  
142 ground by resting on two sheets of open-cell neoprene (12.7 mm each) atop a wooden platform.  
143 Care was taken to ensure animals were in the water column and not touching the sides or netting  
144 when test tones were played.

145 Experimental tones were generated with a custom program implemented with National  
146 Instruments LabView software (Austin, TX, USA) and a National Instruments 6062E data  
147 acquisition card, run on a laptop computer. This program allowed control of the frequency,  
148 intensity and duration of the sound pulses. Sound levels were controlled using a PYLE Chopper  
149 Series PLA2210 amplifier (Brooklyn, NY, USA) and a Hewlett-Packard 350D (Palo Alto, CA,  
150 USA) attenuator, and then played using the speaker. A Tektronix TPS 2014 oscilloscope  
151 (Beaverton, OR, USA) was used to visualize the sound pulses and the signal received by the  
152 hydrophone during calibration. All tests were video recorded using a Sony HDR-XR550 HD  
153 camera (Tokyo, Japan) placed above the tank and recording at 60 fps. In order to measure

154 response latency, a Casio EX-F1 camera (Tokyo, Japan) recording at 600 fps was fixed at an  
155 angle above one side of the tank. An LED was connected to the sound output of the computer  
156 and put in the field of view of the camera (but not visible to the squid) in order to visually record  
157 when sound signals were introduced into the tank (Fig. 1a).

158         Stimuli consisted of ten different test tone frequencies (80, 100, 150, 200, 250, 300, 400,  
159 500, 700 and 1000 Hz), each 3 s in duration, plus a silent control. The experiment was initially  
160 framed in sound pressure x frequency matrix with the range and levels of responses devised  
161 based upon physiological data (Mooney et al. 2010) (Table 1). Output levels were 110, 120, 130,  
162 140, 150, 155, 160 and 165 dB re. 1  $\mu$ Pa rms sound pressure level (SPL) calibrated 20 cm away  
163 from the speaker. At the highest sound levels, some frequencies were distorted due to  
164 characteristics of the speaker and those sounds were not used for the experiments leaving a total  
165 of 66 combinations of sound levels and frequencies, plus the no-sound controls. Because the  
166 animals settled or swam at different distances from the speaker, the received total acceleration  
167 and sound pressure levels (SPLs) differed from the ‘source’ levels at 20 cm (noted above). Thus,  
168 the actual received levels ranged from  $7.6 \times 10^{-5}$  to  $14.5 \text{ m}\cdot\text{s}^{-2}$  (85 to 187 dB re. 1  $\mu$ Pa rms)  
169 (considering all frequencies).

170         A total of 101 animals were used for this experiment. At the start of each experimental  
171 day, 10 individuals were randomly selected from the holding tank and kept in a separate net  
172 within that tank until used in the day’s experiments. The same individuals were typically used  
173 several days in a row. Unfortunately, it was not possible to mark individuals or separately house  
174 animals in the large tanks needed for squid husbandry (Hanlon et al. 1983; Hanlon and  
175 Messenger 1998). This would have facilitated tracking individuals over time keeping the squid  
176 separated or in small tanks for more than a couple of hours induced high levels of stress and



177 increased animal mortality rate. Hence, for the first experiment (frequency range and sound  
178 levels), we randomized tone presentation order and presented those tones 15-25 min apart  
179 (timing was also randomized). This specifically reduced any long term learning effect (response  
180 rates were consistent throughout the experiment) and allowed us to quantify exposures as  
181 independent. Animals were fed daily but tended to expire within several days as is typical for the  
182 species' breeding and semelparous life cycle (Boyle and Rodhouse 2005; Jacobson (NOAA)  
183 2005). At the start of a trial, an animal was moved from the holding tank to the test tank where it  
184 was allowed ~2 min to acclimate before the tone (or silence) was presented. The behavioral  
185 responses for each squid were categorized during a timeframe which included the 3 s tone and  
186 1.5 s immediately afterward using six response types: no response, body pattern change, fin  
187 movements, startle, jetting and inking with some gradations noted; see Supplementary Table 1  
188 and (Samson et al. 2014). Notably, 'inking' only occurred with jetting, and was referred to as  
189 inking; but jetting could occur separately as was thus referred to as 'jetting'. Body-pattern  
190 changes were divided up into 'large' and 'small' where 'large' body pattern change included  
191 pattern change covering at least half the body area, as well as dark flashing, bleaching/paling,  
192 and stereotypical patterning such as deimatic responses, dark fin lines, eye rings or eye spots.  
193 Small body pattern changes included less than half the body area. This scoring system was based  
194 on observations of the animals before the experiments and well-established squid responses in  
195 the context of predators and human-elicited stress (Hanlon and Messenger 1996; Staudinger et al.  
196 2011). Each day, four sound stimuli were randomly chosen from the tone matrix and those four  
197 sounds were then presented in a random order to each of the ten squid. After sound presentation,  
198 the tested animal was returned to the main part of the housing tank. The next day, four new tones  
199 were chosen and randomly presented, and the procedure was repeated until all sounds and

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

209

#### 210 *Habituation to repeated sounds*

211       Specific habituation tests took place over five consecutive dates after the overall  
212 frequency-sound level tests were conducted using fifteen animals. These animals were not used  
213 previously, having been freshly acquired from the fishing boat within 0-2 days of their study  
214 sessions. Animals were chosen randomly each day and exposed to a 3-s tone, presented every  
215 minute for 30 min (i.e., thirty trials/session). The exposure sound was randomly chosen from six  
216 possible frequency-SPL combinations; frequencies were 100, 200, and 300 Hz, and SPLs were  
217 160 dB and 140 dB. Habituation (or sensitization) was evaluated as the response rate overall and  
218 within each response type across the 30-trial session. Animals were presented only one  
219 frequency, but both sound levels, with sessions separated by 1 day. Responses were recorded and  
220 observed post-hoc using the same prior video setup, and were then compared within an  
221 individual's session and individuals were pooled for frequency and sound level comparisons.  
222 These sounds were chosen because they spanned the most sensitive area of squid hearing and the

223 levels induced behavioral responses in cuttlefish (Mooney et al. 2010; Samson et al. 2014). As  
224 for previous behavioral trials, exposure levels were corrected for the distance of the animal to the  
225 speaker. Standard regression analyses were used to estimate the relationship between trial  
226 number and rate of occurrence of the different response types.

227

### 228 *Sound calibrations*

229 While cephalopods detect acoustic particle motion, sound pressure and particle motion  
230 are closely related and both were calibrated across the diameter and depth of the tank in 10 cm  
231 increments using each experimental test tone (Fig. 1b,c). Calibration measurements were made at  
232 the beginning and end of the experiment. Sound pressure was measured using a calibrated Reson  
233 TC 4014 hydrophone (Slangerup, Denmark) and particle acceleration values were obtained by  
234 measuring the pressure gradient over two closely spaced sound receivers (Gade 1982; Mooney et  
235 al. 2010). For basic sound pressure measurements (dB re 1  $\mu$ Pa rms), the hydrophone was  
236 suspended 10 cm from the center of the speaker and moved incrementally up and to the side. The  
237 peak-to-peak amplitude of the signals was measured on the oscilloscope, and converted from  
238 voltages to SPL using a custom MatLab script. The tones were concurrently recorded using an  
239 Olympus LS-10 PCM recorder (Olympus America Inc., Center valley, PA, USA). For the  
240 particle acceleration, two custom hydrophones (-180 dB re 1 V/ $\mu$ Pa), vertically spaced 5 cm  
241 apart, were fixed in a location 10 cm directly above the speaker. As a stimulus was played,  
242 pressure measures at both hydrophones were concurrently measured (sampling rate: 120 kHz)  
243 and digitally stored for later analyses. The hydrophone setup was moved along the diameter and  
244 depth of the tank in 10 cm increments as described for the calibration of the sound pressure level.  
245 This two-hydrophone setup was repeated for each x, y, z direction so that particle motion could

246 be calculated for all three dimensions. The z-plane was always the dominant axes but because  
247 animals receive sound from all 3 directions concurrently, the magnitude of the acceleration was  
248 computed and used for the data analysis and figures. Within the acoustic near-field of the  
249 speaker, the squid was expected to act as a rigid body with respect to particle acceleration values  
250 at each location (Denton and Gray, 1982; Coombs et al., 1992).

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

269 the animal's head (where the statocysts are located) for each sound test. Analyses were  
270 conducted in Excel and MatLab.

271

## 272 **Results**

### 273 *Frequency and sound level responses*

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

280 Inking (which always occurred with a jet) only occurred at highest sound levels and  
281 lowest frequencies (at  $6.75 \text{ m}\cdot\text{s}^{-2}$  mean particle acceleration value, Figs. 3, 5). Lowest sound  
282 levels which induced inking occurred at 150 Hz ( $2.17 \text{ m}\cdot\text{s}^{-2}$ ). Jetting alone occurred more often  
283 and across a broader range of frequencies and levels although responses were still concentrated  
284 at the lower frequencies and higher sound levels (mean responses were found at  $2.55 \text{ m}\cdot\text{s}^{-2}$ ).  
285 Startle responses were not observed very often and were concentrated at the lower frequencies;  
286 mean response values were similar to jetting ( $2.50 \text{ m}\cdot\text{s}^{-2}$ ).

287 More moderate responses were categorized as large and small body pattern change and/or  
288 fin movement. Small body pattern change responses were generally exhibited at sound levels  
289 about an order of magnitude below inking ( $0.84 \text{ m}\cdot\text{s}^{-2}$ , Fig. 3). These patterning responses were  
290 observed across the range tested, although fewer responses were noted at the higher frequencies  
291 (Fig. 3). The less intense patterning responses were seen at acceleration levels down to 0.001

292  $\text{m}\cdot\text{s}^{-2}$  (400 Hz). Larger body pattern change and fin movements were noted at a mean level of  
293  $1.94 \text{ m}\cdot\text{s}^{-2}$ . Multiple behaviors often occurred concurrently. For example, a 100 Hz tone at higher  
294 sound levels might induce inking, jetting and body pattern change. Finally, in many cases at all  
295 sound levels and frequencies, animals did not exhibit observable responses to sound stimuli.  
296 However, this ‘No response’ occurred predominantly at the lower sound levels, with a mean ‘No  
297 response’ at  $0.62 \text{ m}\cdot\text{s}^{-2}$ . Occurrence rates of responses were frequency and sound level dependent  
298 (Fig. 5). No responses occurred most often (Fig. 5).

299         Most responses occurred between latencies of 0.1-0.3 s although the fastest responses  
300 were 0.008 s for jetting and 0.01 s for body pattern change (Fig. 6). Maximum durations were  
301 greater than 1.0 s (1.41 s – jetting; 1.06 s body pattern change), such long-latency responses  
302 (greater than 1 s) occurred only once for each behavior. Thus responses were typically much  
303 more rapid. Mean latencies were significantly shorter for body pattern change ( $0.14 \text{ s} \pm 0.20 \text{ s.d.}$ )  
304 compared to jetting ( $0.36 \text{ s} \pm 0.41$ ) regardless of whether these maximum latencies were  
305 considered outliers or not (two-tailed t-test,  $p < 0.05$ ; see Supplementary Table 2 for descriptive  
306 statistics). Latencies did not show a significant dependence on frequency (one-way ANOVA  
307  $p > 0.05$ ; see Supplementary Table 3 for ANOVA tables). Nor was there a relationship between  
308 latency of pattern change and sound level ( $r^2 = 0.016$ ;  $Y = -0.5025 * X + 0.1588$ ;  $p > 0.05$ ).  
309 However, latency or jetting response did seem weakly related to particle acceleration sound level  
310 (Fig 6;  $r^2 = 0.567$ ;  $Y = 28.006 * X^{0.9697}$ ;  $p < 0.01$ ; see Supplementary Table 4).

311

### 312 ***Habituation to repeated sounds***

313         Animals habituated to repeated acoustic stimuli, as was reflected by the decrease of the  
314 number of animals observed responding across successive repeated tone trials (Fig. 7). This

315 decrease was relatively rapid and logarithmic in nature for both jetting ( $y = -0.398 \cdot \ln(x) +$   
316  $1.1626$ ;  $r^2 = 0.4235$ ) and body pattern change ( $y = -1.119 \cdot \ln(x) + 3.6747$ ;  $r^2 = 0.4965$ ).  
317 Habituation was also notable in the response type, which generally changed from escape  
318 responses (inking and jetting) to body pattern change. Jetting and inking responses were often no  
319 longer exhibited after a short number of trials (1-3). Body pattern change response rates also  
320 decreased rapidly for initial trials. However, for some animals, these reactions reoccurred in later  
321 trials. Notably, habituation tests also showed individual variations in response occurrences  
322 where some animals reflected differences in both initial response intensities and rate of decrease.  
323 Additionally, some animals demonstrated intermittent response occurrences over the session  
324 (Fig. 7b), whereas other animals did not show sound associated response after the initial trials  
325 (Fig. 7c).

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

332

### 333 **Discussion**

334         The goal of this work was to define the sound levels and frequency range to which an  
335 ecologically key marine invertebrate responds and respectively quantify the types of responses to  
336 varying stimuli. The results reveal that squid exhibit clear acoustically mediated behavioral  
337 responses; and when those responses occur they are behaviors associated with escape and

338 predation avoidance , particularly fleeing (jetting) but also protean responses of inking and body  
339 pattern change. Protean responses may serve to startle or confuse a predator with erratic,  
340 unpredictable escape sequences (Humphries and Driver 1970; Hanlon and Messenger 1998;  
341 Staudinger et al. 2011). Deimatic patterning changes may serve to bluff the predator (through  
342 impressions of size or behavior) or signal a warning of danger to conspecifics (Edmunds 1974;  
343 Hanlon and Messenger 1998).

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

353         At the lower frequencies (below 250 Hz), the mean response levels determined here (for  
354 all response types) were more than an order of magnitude higher than physiological thresholds  
355 measured for the same species (Mooney et al. 2010). This suggests that while inking, jetting and  
356 pattern changes are used to evaluate responses to perceived threats, they may not be indicative of  
357 (and in fact would overestimate) hearing sensitivities and auditory sensation levels, at least at  
358 these frequencies. Thresholds lower than unconditioned response levels may be expected. Yet, at  
359 higher frequencies (300-400 Hz), auditory thresholds (Mooney et al. 2010) were similar to large  
360 pattern change and displacement response means, and were actually occasionally greater than



361 smaller pattern and jetting mean values. At first glance these results suggest the physiological  
362 ‘thresholds’ at higher frequencies are above true detection thresholds (likely caused by  
363 differences in tanks and experimental setup). The behavioral levels may also provide insight into  
364 how squid may use sound. All responses (inking, jetting, pattern change) are clustered around  
365 similar sound levels, well above thresholds indicating that loud sounds (such as imminent  
366 predators) are required to induce these behaviors. At higher frequencies, response types are more  
367 divergent and occur at relatively low sound levels, suggesting that sound may have a different  
368 function at these frequencies, perhaps orientation, soundscape assessment or other auditory scene  
369 analyses.

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

383            Similar to the cuttlefish (Samson et al. 2014), levels of mean behavioral response could  
384 be separated relative to response type. Thus it was possible to discriminate the sound levels and  
385 frequencies which induced escape responses such as inking and jetting, and those which induced  
386 the milder body pattern change or subtle movements of body parts (like fins or arms). Generally,  
387 inking and jetting were confined to higher sound levels ( $> 1 \text{ m}\cdot\text{s}^2$ ) and lower frequencies (200  
388 Hz and below), although jetting showed more occurrences and variation in the frequencies and  
389 sound levels that induced response, especially above 200 Hz. Both response types are typically  
390 used for predator evasion. Their limited proportions or general absence at lower sound levels ( $<$   
391  $1 \text{ m}\cdot\text{s}^2$ ) suggests that sound must be of relatively high received intensity to induce these escape  
392 responses. High-level stimuli would likely be indicative of unexpected, camouflaged predators  
393 such as flounder (Staudinger et al. 2011), where the squid rapidly flee and potentially ink to  
394 avoid capture, supporting that hearing may be used to occasionally enact these behaviors. It is  
395 also possible that squid “save” the higher energetic response (inking/jetting) for when they feel a  
396 threat is eminent. An additional (visual) threat may have helped induce escape responses at lower  
397 sound levels.

398            Response latencies were, on average, faster for body pattern changes which perhaps  
399 reflects the relative efficiency of this neural circuitry and concomitant muscular responses  
400 (Nixon and Young 2003). However, rapid jetting responses were occasionally induced, reflecting  
401 perhaps a response mediated in part by the squid giant axon (Otis and Gilly 1990). Acoustically  
402 mediated responses suggest that squid may utilize hearing (i.e., detection of acceleration) to  
403 detect and avoid potential predator threats, which is a key adaptation in perceiving the auditory  
404 scene (Fay 2009). Particle acceleration events could arise from the head-wake of large predators  
405 such as some fishes and marine mammals (Niesterok and Hanke 2013) and may be particularly

406 vital to detect when squid are rapidly approached by ambush predators (Staudinger et al. 2011).  
407 Detecting the head-wake of a predator via acoustic and water-motion cues would be quite  
408 important when vision is not helpful including in the aphotic zone, at night and in murky waters.

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

423 The response levels were compared to those of cuttlefish with some similarities (Fig 8;  
424 Samson et al. 2014). The inking responses observed here were comparable in sound levels to  
425 those observed previously for cuttlefish, although squid responses occurred at slightly lower  
426 frequencies. This similarity suggests that animals have similar behavioral means for escape  
427 responses. Yet, squid showed higher mean response levels for large body pattern change and 'No  
428 response' conditions. This may mean that squid do not respond to lower level acoustic stimuli

429 which are potentially not life-threatening; or they may simply be less sensitive to the lower level  
430 sounds. Alternatively, the common cuttlefish may have a higher skin chromatophore density  
431 (Hanlon and Messenger 1988; Mäthger and Hanlon 2007) making responses easier to observe  
432 and thus lowering our detection threshold for this taxon. Life history might also influence these  
433 differences. For example, the 3-dimensional lifestyle of pelagic squid may result in some atrophy  
434 of balance-rated sensory organs (as seen in some aquatic mammals (Ketten 1994)). Additionally,  
435 the longfin squid is a schooling species often found in the water column (in contrast to the epi-  
436 benthic common cuttlefish) (Hanlon and Messenger 1998). The higher ‘no-response’ level of  
437 these squid might reflect that they are undisturbed by abrupt, ambush type sound, until those  
438 sounds reach a level that counteracts the protection provided by a school. Similarly, as a  
439 schooling animal, visual displays could actually serve to help a predator single you out from the  
440 school, and would thus be counterproductive to predator avoidance. Perhaps responses are also  
441 dependent upon sensory input from their neighbors in the school. Seeing conspecifics jet away or  
442 change body pattern/posture may influence response levels. Thus, future work should address  
443 multi-modal (visual plus sound) mediated escape behaviors and responses of squid schools.

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

452 occasional responses after multiple trials suggesting that at least some animals were still vigilant  
453 and continuously monitoring the auditory scene.

454         The habituation experiments also seemed to reveal some directional movement away  
455 from the speaker. In nearly all cases, animals moved to a location of lower sound level after the  
456 first acoustic trial and most animals moved to a quieter area after 5 trials. Animals were  
457 swimming freely and often had the chance to move toward the center of the speaker's beam  
458 pattern and toward the surface, away from the speaker. But typically this movement was both  
459 higher in the water column and laterally outside the center of the speaker's beam. This  
460 movement to lower sound level areas suggests both the ability to determine sound source  
461 directionality and an aversion to the higher sound levels.

462         These data provide the first assessment of the frequency range and sound levels to which  
463 squid behaviorally respond. Further, the responses are unconditioned behaviors. The results  
464 indicate that a variety of biologically relevant responses may be elicited by acoustic stimuli,  
465 supporting the idea that cephalopods may use sound cues to evaluate their environment. While  
466 responses could be generally characterized as predator-avoidance behaviors, the demonstration  
467 of biologically relevant response implies that squid may use sound for other behaviors such as  
468 navigation or orientation. As an ecologically vital taxon, unconditioned acoustic behaviors in  
469 squid highlight the growing understanding of how important sound is to the sensory ecology of  
470 marine invertebrates and the communities they support. Generally animals were responsive to  
471 low frequencies below 1000 Hz, and were most sensitive to sounds below 300 Hz. This low  
472 frequency sensitivity overlaps with the predominant frequencies in ocean noise; both natural  
473 wind and wave noise, as well as anthropogenic sounds such as air guns, construction and  
474 commercial shipping occur at these lower frequency levels (Urick 1983). As these frequencies

475 travel efficiently in the ocean, this overlap raises concern that this noise is increasingly pervasive  
476 (Hatch et al. 2008) and cephalopods might be impacted. While there has been some suggestion  
477 that close exposures to impulse sounds could cause anatomical damage (André et al. 2011),  
478 lower level effects such as masking or behavioral responses are perhaps more likely. These  
479 results suggest that a range of response could be elicited, from jetting, to moving away from an  
480 undesired noisy area, or simple habituation to the noise. Yet, these impacts are not fully resolved  
481 and population level responses are certainly unclear. In demonstrating the overall range of  
482 responses that sounds may induce in squid, these results greatly support the need for a better  
483 understanding of noise impacts on these ecologically key taxa.

484

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494

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626  
627



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

631

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

632

633

634

635

636 Fig 1. a: Schematic of the experimental set-up, side view. 1: tank, 2: net, 3: speaker, 4:  
637 calibration ruler, 5: outflow pipe, 6: HD video camera. 7: High-speed video camera. B: Power  
638 spectrum from a 300 Hz sound at a calibrated sound level of 150 dB (received level), as recorded  
639 by a calibrated hydrophone placed 12.5 cm above the speaker. b: power spectrum from the  
640 ambient noise recorded by the same hydrophone at the same position. c: Vector field of the  
641 particle acceleration at 150 Hz for a calibrated sound level of 165 dB. The speaker is represented  
642 in blue, at the (0, 0) position in the tank. This figure illustrates the importance of taking the  
643 distance of an animal to the speaker into account, since the sound field is very variable  
644 depending on the location in the tank. Vectors are to scale; the  $1 \text{ m}\cdot\text{s}^{-2}$  scale is noted on the  
645 figure.

646

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

652

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

659

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

663

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

667

668 Fig 6. Response latencies for a: Jetting and b: large pattern change. Shapes reflect different  
669 frequencies (black diamonds: 80 Hz, black triangles: 100 Hz, open squares: 150 Hz, star: 200  
670 Hz, open circles: 250 Hz). The maximum outlier values (jetting = 1.41 s; pattern change = 1.06  
671 s) were not plotted to better reflect the spread of most data. (c) Box plots (median  $\pm$ 25/75  
672 quartiles; mean = dot; whiskers show data range) of all latency data for jetting and pattern  
673 change responses (including outliers). Response latency differed significantly for these two  
674 categories (two-tailed t-test,  $p < 0.05$ ). Note the y-axes scales differ.

675

676 Fig 7. a: Habituation to a repeated sound stimulus. Data were collected using a 200 Hz tone at  
677 160 dB (calibrated sound pressure), which was presented every minute for 30 consecutive trials.  
678 Diamond: inking, triangles: jetting, stars: color change. The observations of both sound-induced  
679 jetting and color change decreased logarithmically. b,c: Succession of behavioral responses of  
680 two individual squid using the 160 dB 200 Hz tone. No response for a given trial is indicated by



681 the open circles, which also reflect the received level for that trial. This received level varied as  
682 the animal moved throughout the tank during the session.

683

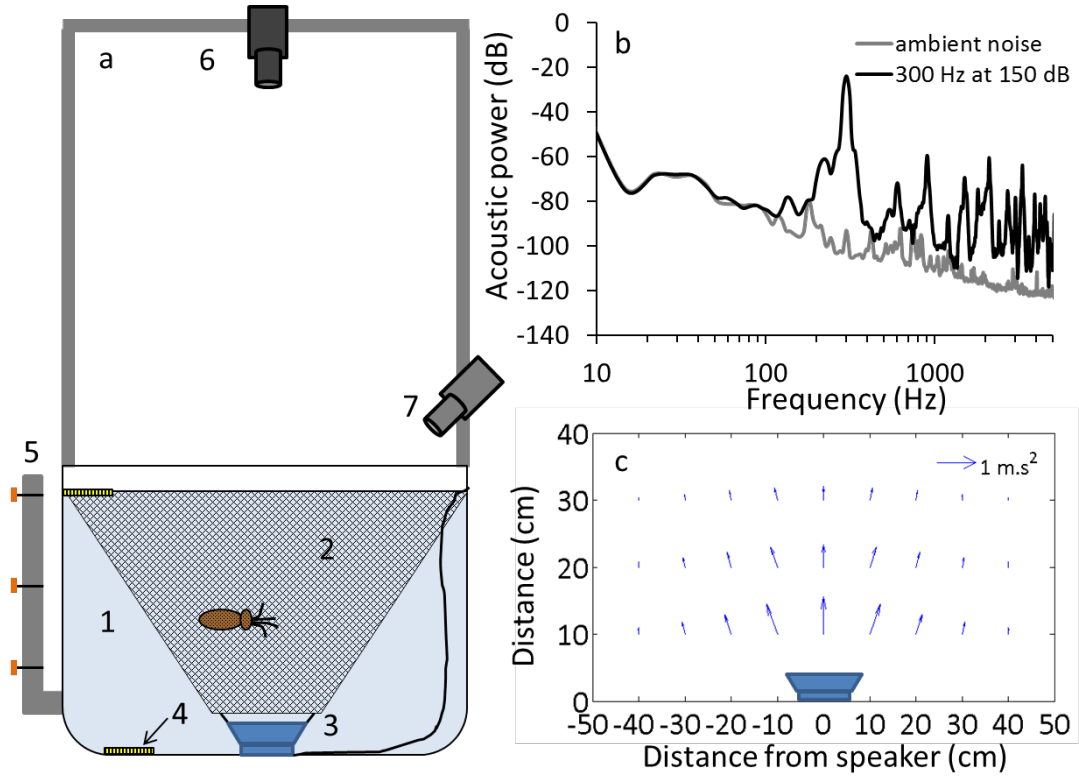
684 Fig 8. Comparison of squid and cuttlefish behavioral response data.

685

1 Figure 1.

2

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4

5

Figure 2.

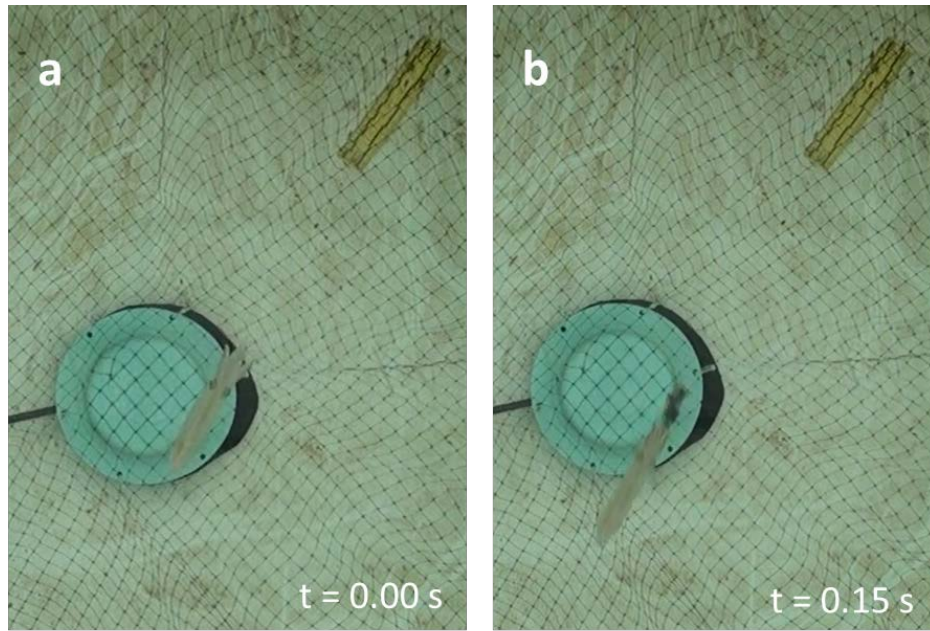


Figure 3.

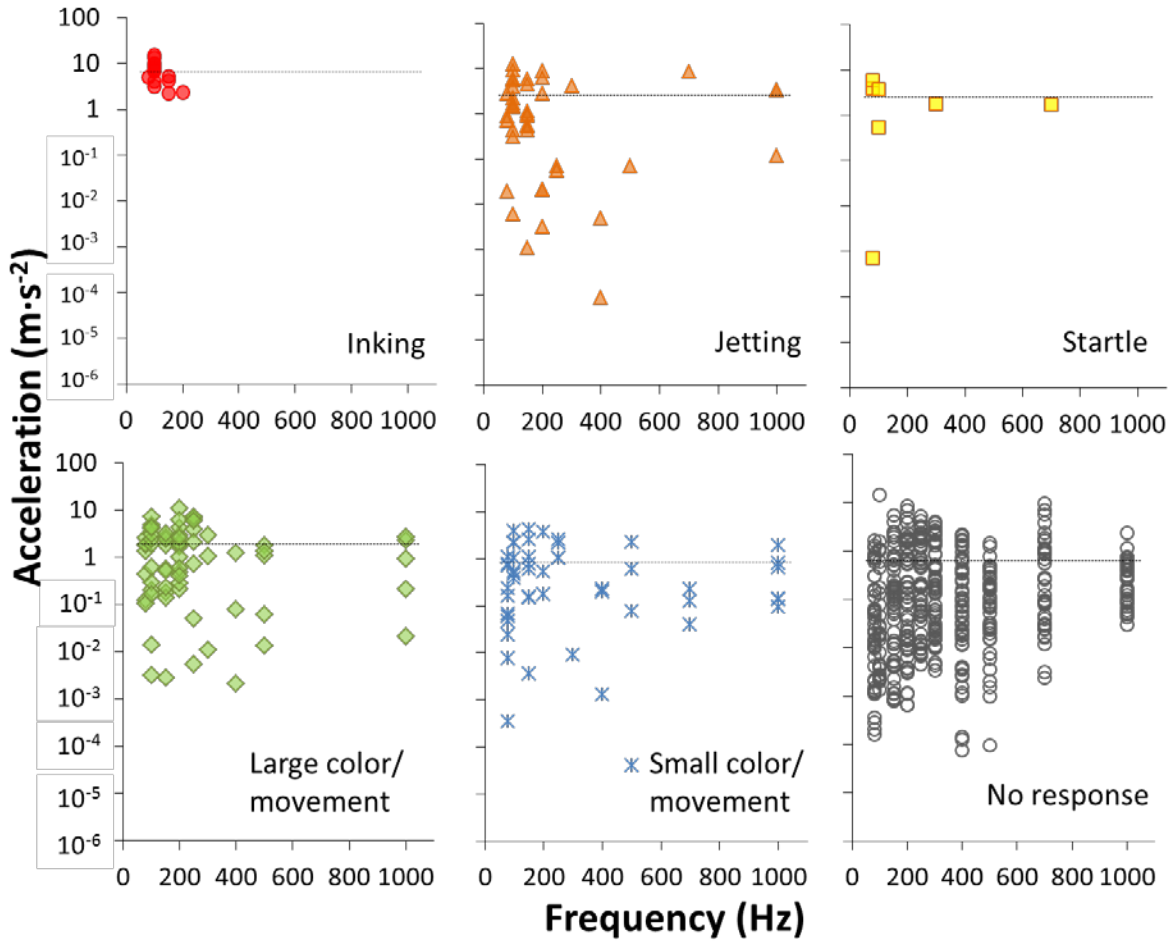


Figure 4.

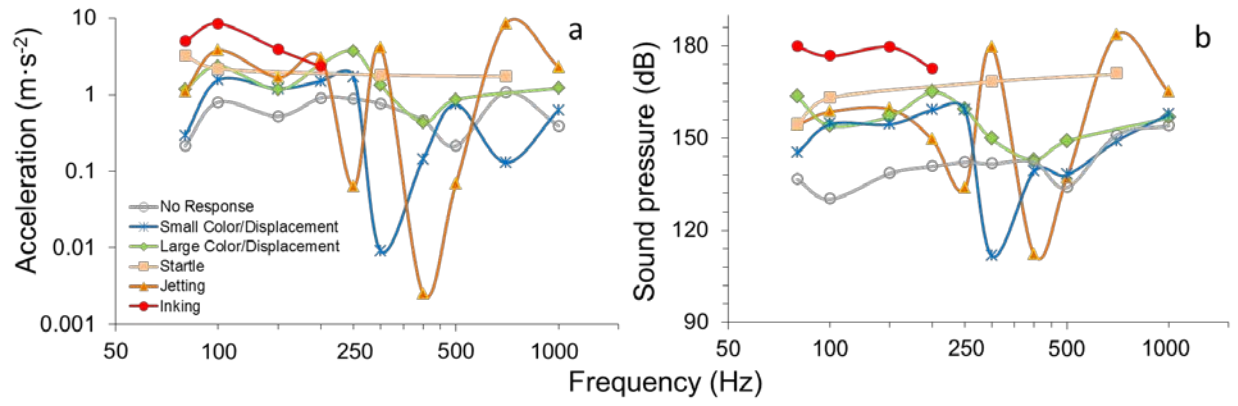


Figure 5.

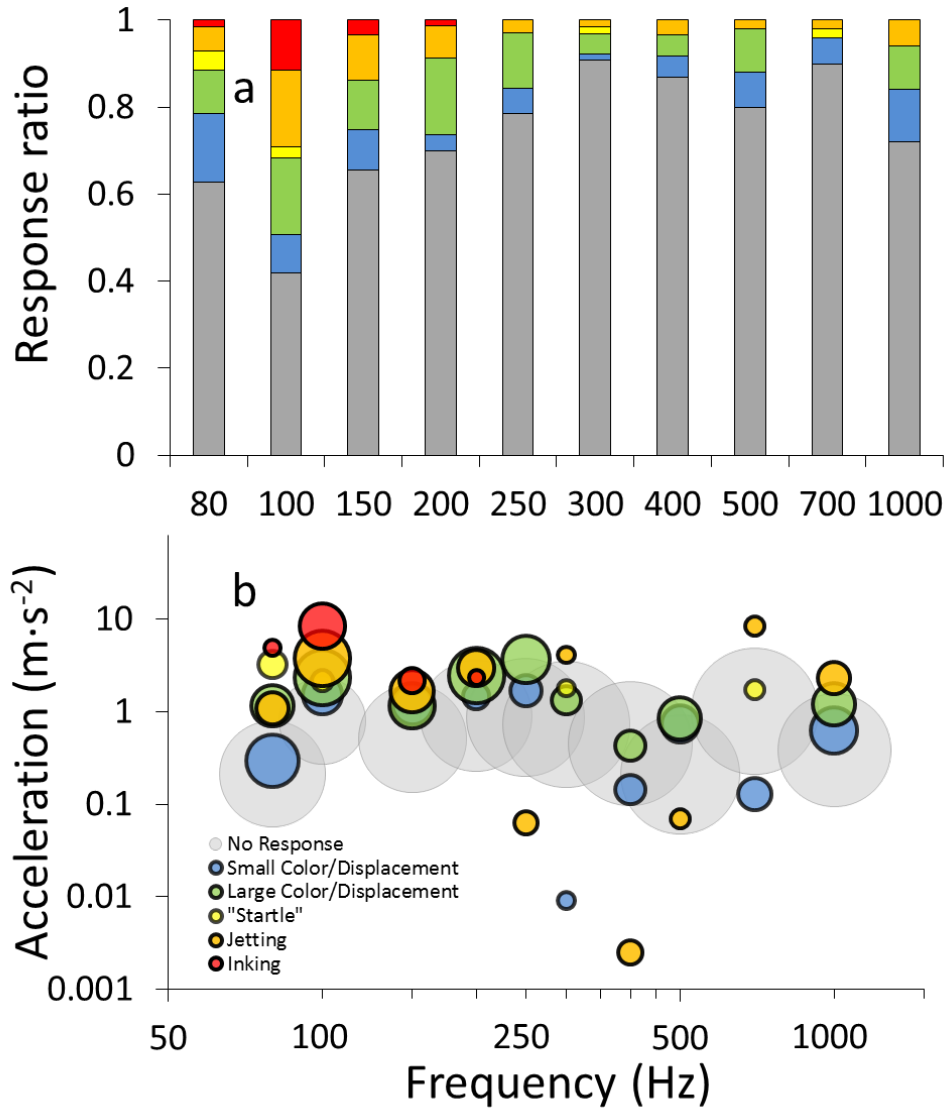


Figure 6.

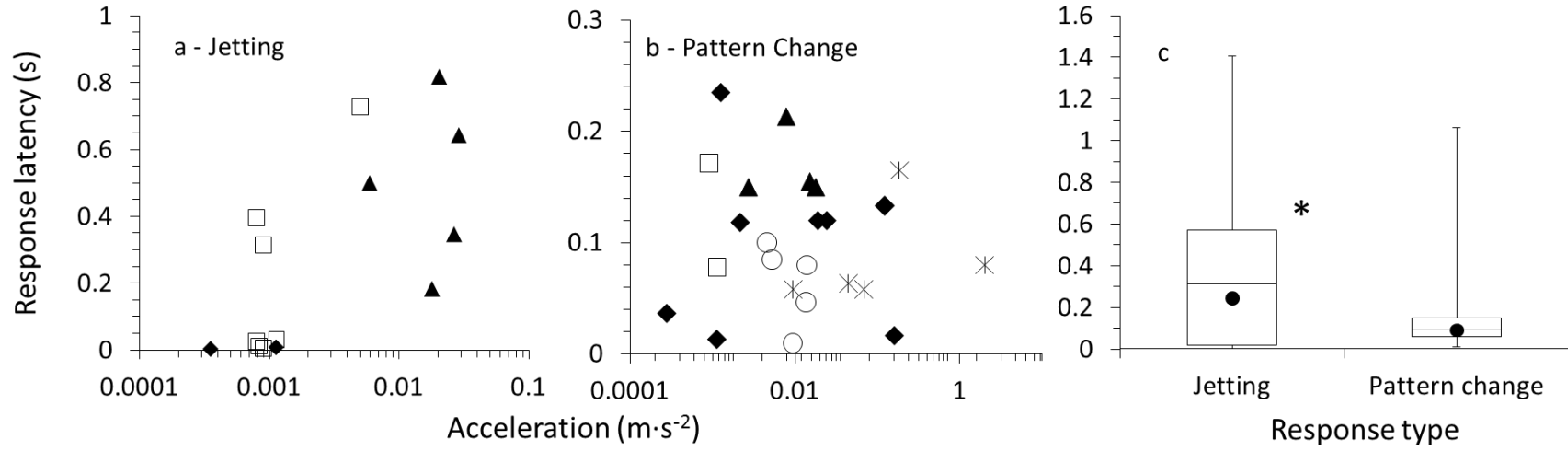


Figure 7.

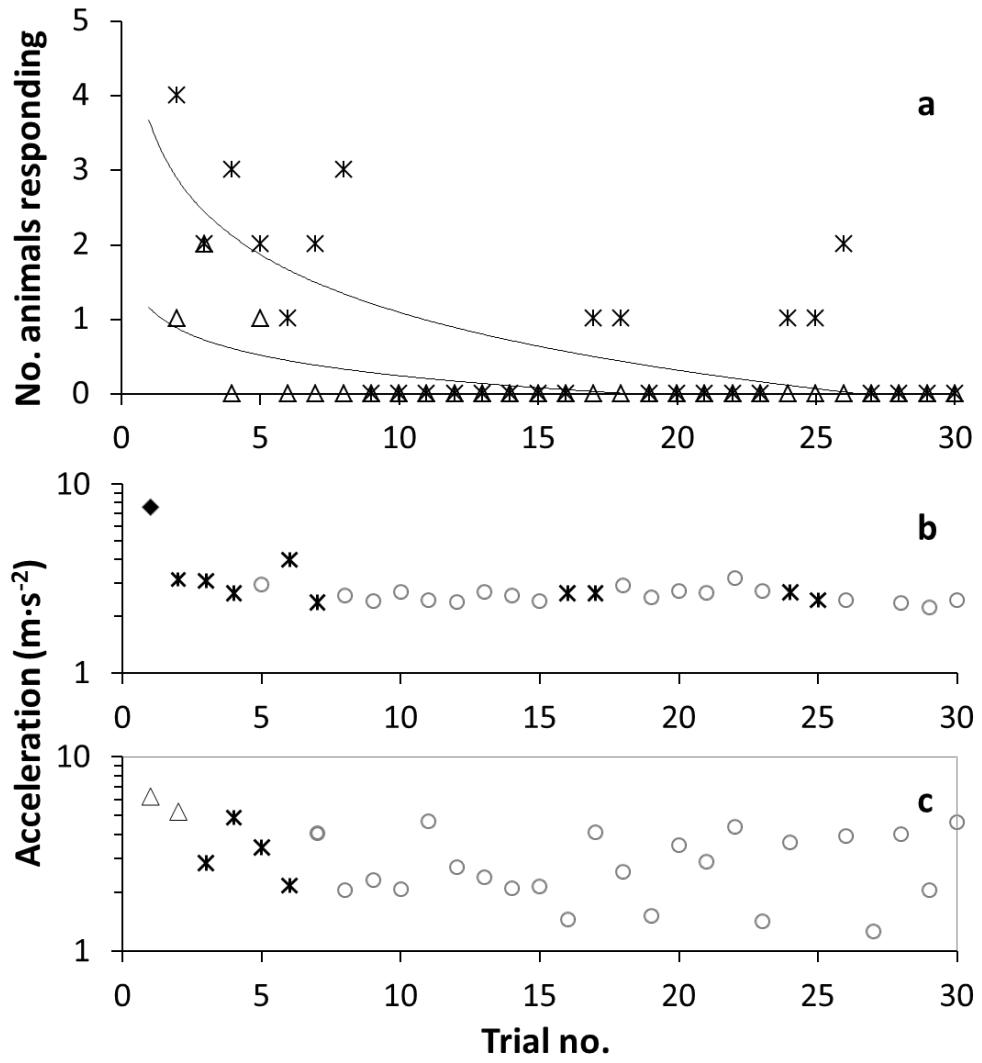
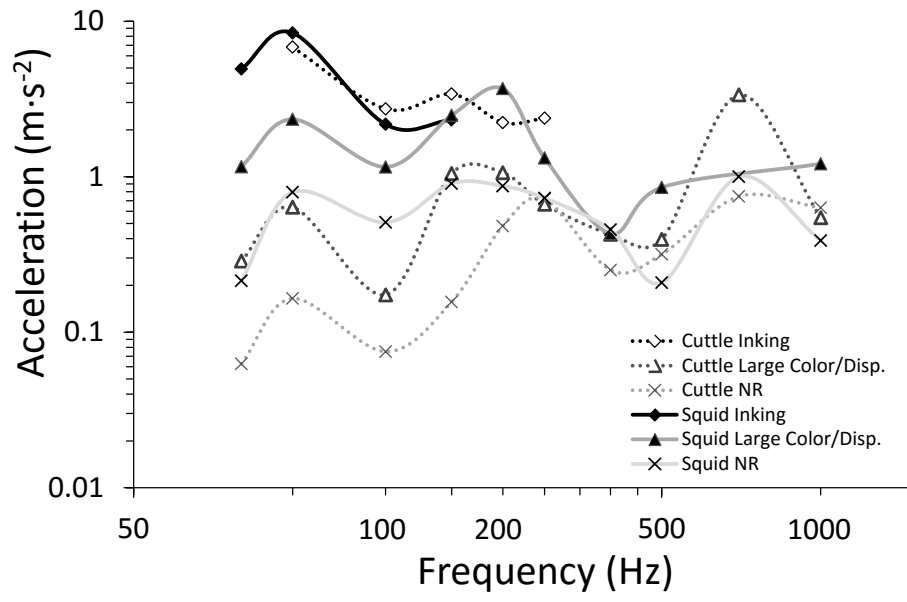




Figure 8.



## TABLES

**Supplementary Table 1.** Overview of the types of responses and their intensities used to score the behavioral responses of *Doryteuthis pealeii* to sound stimuli

<b>Response type</b>	<b>Intensity</b>	<b>Description</b>
No response	-	No change in behavior observed, no acceleration or deceleration in fin movement, no body pattern change or flickering of chromatophores, no displacement.
Body pattern change	Small	Body pattern change covering less than half the body area.
	Big and Deimatic	Body pattern change covering at least half the body area, includes dark flashing, bleaching, deimatic, etc. Body pattern including some or all of the following: flattened body shape, paling of the skin, paired dark mantle spots, dark fin line, dark eye rings, pupil dilation.
Fin movements	Slow	Slow fin undulations resulting in slow displacements (undulation rate estimated to be less than 1 Hz).
	Fast	Intense fin undulations resulting in rapid, marked displacements (undulation rate estimated to be more than 1 Hz).
Startle	Small	Small contraction of the mantle and/or arms, often followed by slow fin movements with or without displacement.
	Big	Big, marked contraction of the mantle and arms, usually followed by big displacements and/or jetting.
	“Stereotyped”	Arm twitch, sometimes with a small mantle contraction. The arms go back to their initial position immediately after the response. In some cases, the arms only twitch at the tips and a contraction of the pupils is observed. No displacement.
Jetting	Small	Small jet(s), distance covered is less than two body

		lengths, speed is relatively slow. The number of jets was also recorded.
	Big	Big jet(s), distance covered is at least two body lengths, displacement is fast. The number of jets was also recorded.
Inking	-	Expulsion of ink. The number of inking events was also recorded.
Other	Elongating	Body is stretched along the longitudinal axis, especially the arms are stretched.

### Supplementary Table 2.

t-test and Descriptive statistics		
	Jetting	Pattern change
Minimum	0.008	0.010
Q1	0.019	0.060
Median	0.315	0.093
Q3	0.572	0.150
Maximum	1.407	1.063
Mean	0.362	0.140
Variance	0.164	0.041
s.d.	0.405	0.201
n	15	25
<i>p</i>	<0.05	
t Stat	1.976	
df	18	

### Supplementary Table 3a and 3b.

3a. One-way ANOVA

Pattern change latency vs. Acoustic frequency

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
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Between Groups	0.07361	4	0.0184	0.4285	0.78639	2.8401
Within Groups	0.9019	21	0.04295			
Total	0.97551	25				

3b. One-way ANOVA

Jetting latency vs. Acoustic frequency

<i>Source of Variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P-value</i>	<i>F crit</i>
Between Groups	0.89875	2	0.44937	3.86767	0.05054	3.88529
Within Groups	1.39425	12	0.11619			
Total	2.293	14				

**Supplementary Table 4.**

Linear relationship between latencies (for jetting and pattern change) vs. acceleration

Regression	Jetting	Pattern change
$r^2$	0.443	0.016
$p$	0.007	0.555

Logarithmic relationship between latencies (for jetting and pattern change) vs. Acceleration

Regression	Jetting	Pattern change
$r^2$	0.567	0.016
Equation	$Y = 28.006 * X^{0.9697}$	$Y = -0.5025 * X + 0.1588$
$p$	0.007	0.555