J Exp Biol Advance Online Articles. First posted online on 13 November 2014 as doi:10.1242/jeb.113365
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.113365

Graded behavioral responses and habituation to sound in the common cuttlefish, *Sepia officinalis*

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Keywords: bioacoustics, cephalopod, hearing, noise, loudness, invertebrate, ear, statocyst, lateral line

Short title: Cuttlefish behavioral responses to sound

List of symbols and abbreviations:

fps	frames per second
rms	root mean square
SPL	sound pressure level

SUMMARY

Sound is a widely available and vital cue in aquatic environments yet most bioacoustic research has focused on marine vertebrates, leaving sound detection in invertebrates poorly understood. Cephalopods are an ecologically key taxon that likely use sound and may be impacted by increasing anthropogenic ocean noise, but little is known regarding their behavioral responses or adaptations to sound stimuli. These experiments identify the acoustic range and levels that elicit a wide range of secondary defense behaviors such as inking, jetting, and rapid coloration change. Secondarily, it was found that cuttlefish habituate to certain sound stimuli. The present study examined the behavioral responses of 22 cuttlefish (*Sepia officinalis*) to pure-tone pips ranging from 80-1000 Hz with sound pressure levels of 85–188 dB re 1 μPa rms and particle accelerations of 0-17.1 $m.s^{-2}$. Cuttlefish escape responses (inking, jetting) were observed between frequencies of 80-300 Hz and at sound levels above 140 dB re 1 μPa rms and 0.01 $m.s^{-2}$ (0.74 $m.s^{-2}$ for inking responses). Body patterning changes and fin movements were observed at all frequencies and sound levels. Response intensity was dependent upon stimulus amplitude and frequency, suggesting that cuttlefish also possess loudness perception with a maximum sensitivity around 150 Hz. Cuttlefish habituated to repeated 200 Hz tone pips, at two sound intensities. Total response inhibition was not reached, however, and a basal response remained present in most animals. The graded responses provide a loudness sensitivity curve and suggest an ecological function for sound-use in cephalopods.

INTRODUCTION

Sound in aquatic environments is a widely available cue that many marine vertebrates use during vital biological activities such as foraging, predator detection, mate attraction, and habitat selection (Webster et al., 1992; Fay and Popper, 1998; Au et al., 2000). Consequently, for vertebrates, sound detection is considered a primary sensory modality and an important component of vital intraspecific interactions and a key way to detect the surrounding environment. The ability of marine invertebrates to detect and potentially use sound is far less understood (Budelmann, 1992a, b; Mooney et al., 2012). This is somewhat surprising given their relative abundance and central role in many marine ecosystems.

Yet a growing body of literature suggests that marine invertebrates respond to sound in a variety of ways. For example, coral reef invertebrates (crabs and coral larvae) may swim toward or away from reef sounds, with the actual direction being taxon specific (Vermeij et al., 2010; Simpson et al., 2011). Reef sounds from certain habitats can generate settlement behaviors and increased rates of metamorphosis (Stanley et al., 2009, 2012). Perhaps not surprisingly, variations in the frequencies and levels of these sounds can affect whether the behavior is induced (Simpson et al., 2011; Stanley et al., 2011). However, thresholds have rarely been established and we still know little regarding the frequencies to which most invertebrates respond. Furthermore, it is vital to quantify acoustic particle motion, a stimulus often overlooked. Both sound pressure and acoustic particle motion are generated by sound sources, but it is particle motion [i.e., the back-and-forth hydrodynamic flow from the motion of the sound emitter (Gade, 1982; Au and Hastings, 2009)] that is the likely stimulus for most marine animals without compressible air cavities (Mann et al., 2007; Mooney et al., 2010; Popper and Fay, 2011). Despite a burgeoning literature, there is a poor understanding of the frequencies and levels of sounds that generate functional behavioral responses in invertebrates.

Cephalopods offer a unique means to quantify the frequency range and sound levels that generate behavioral responses for several reasons. First, the potential behavioral responses of several species, such as the common cuttlefish, *Sepia officinalis* L., 1758, are both dynamic and well described (Hanlon and Messenger, 1996). Previous behavioral studies have shown that these cuttlefish exhibit a range of responses to sensory stimuli, including changes in body patterning, locomotor activity, jetting and inking events (Hanlon and Messenger, 1996). Secondly, these behavioral responses show a gradation in intensity, from primary defense responses (usually crypsis or camouflaging against the background) to secondary defenses such as deimatic behaviors used to deter the potential predator and ultimately flight responses involving jetting and inking (Hanlon and Messenger, 1998; Langridge et al., 2007; Langridge, 2009; Staudinger et al., 2011). A similar gradation in response intensity may be generated by acoustic stimuli (Fewtrell and McCauley, 2012). Finally, many cephalopods occupy central positions in food chains; thus, understanding their sensory ecology is required to accurately determine relationships between this taxon and other marine species, and could provide indications on how other invertebrates may use sound.

The statocyst is generally considered the primary sound detection organ in cephalopods (Budelmann, 1990, 1992a), although peripheral hair cells may play a role in detecting local water movements (Bleckmann et al., 1991; Coombs et al., 1992). With regard to acoustic stimuli, the statocyst likely acts as an accelerometer in response to the vibratory particle motion component of sound (Budelmann, 1990; Packard et al., 1990; Mooney et al., 2010). Besides the hair cells in the statocysts, common cuttlefish also have eight lines of epidermal hair cells running over their head and arms which are able to detect local water movements generated by a vibrating sphere (Budelmann and Bleckmann, 1988; Komak et al., 2005).

There is some anecdotal evidence suggesting that cephalopods respond to sounds such as tapping on the tank wall (Baglioni, 1910; Dijkgraaf, 1963). Other observational evidence includes cephalopods swimming away from sound-generating predators in the sea (Hanlon and Budelmann, 1987). More recently, conditioned responses were generated in common octopus (*Octopus vulgaris*), squid (*Loligo vulgaris*) and cuttlefish (*S. officinalis*) using low frequency acceleration stimuli (Packard et al., 1990). Juvenile *S. officinalis* exhibited body patterning changes and locomotor responses when exposed to water movements ranging between 0.01 and 1000 Hz (Komak et al., 2005), and octopus showed changes in respiratory rates when presented sound stimuli between 50 and 150 Hz (Kaifu et al., 2007). Furthermore, there are suggestions that anthropogenic noise may impact cephalopod behavior or anatomy (André et al., 2011; Fewtrell and McCauley, 2012). Understanding the frequency ranges and sound levels that generate behavioral responses, whether they adapt (habituate) and the types of behavioral responses elicited would help evaluate the likely influences of noise on cephalopods.

Accordingly, the aim of this study was to quantify the sounds that generate behavioral responses and identify the potential behaviors elicited. Animals were presented tones that varied in both frequency and sound level, and response types were quantified. The three main goals were to: (1) determine the frequency range and sound levels to which behavioral responses are observed, (2) describe and quantify the types of responses and their occurrence rates, and (3) investigate the potential for habituation to repeated sound stimuli. In addressing these goals, both sound pressure and particle acceleration were quantified.

RESULTS

Acoustic frequency range and sensitivity

All animals showed clear behavioral responses to acoustic stimuli (Figs. 1, 2), and the intensity of the response was associated with the amplitude and the frequency of the signal. Multiple response types were elicited (Table 1). Responses occurred at all frequencies tested; occurrence rates and response types were dependent upon both the frequency and sound level received (Fig. 1). Some individual variations in response intensities were observed but the general pattern of response intensities was conserved. Greatest intensity responses (i.e., inking; Fig. 2) were found at the highest sound levels, typically between 100 and 300 Hz. As sound levels got lower, response intensity decreased to jetting, startle, large body patterning changes and/or fast fin movements, and small body patterning changes and/or slow fin movements. The controls most often showed "no response," but small and large fin movements and/or body pattern changes were also observed. These responses were more often noted in the more active animals. More frequently, these animals would swim and change body pattern in their housing tanks (outside of the experiments) where they might interact with other animals and respond to prey presentation during feedings. Inking, jetting and startle were not observed in the controls.

The sound parameter matrix did not take into account the distance between the animal and the speaker, meaning that the sound levels in Fig. 1 are the calibrated sound levels at 20 cm from the speaker, not the sound levels actually received by the animal. This resulted in discrepancies in the observed pattern of response intensities. For example, small body pattern changes and/or slow fin movements are often placed at higher sound levels than big body pattern changes and/or fast fin movements (Fig. 1).

Corrections for the distance between the animal and the speaker were made and the behavioral responses were plotted relative to the actual received particle acceleration (Fig. 3) for each sound trial (pressure data are shown in the Supplementary Material). Only the most intense response for each trial was plotted; i.e., if the animal showed several responses during a test, only the highest scoring response was plotted (inking in the case of Fig. 2). Cuttlefish escape responses (inking, jetting, startle) were highly dependent on the sound frequency and level. Inking was only observed for sounds between 80 and 300 Hz, and above 0.73 $m.s^{-2}$ (particle acceleration) and 140 dB (sound pressure level – SPL - presented in dB re 1 μPa rms). Jetting and startle responses were observed primarily between the same frequencies, with occasional incidences at higher frequencies. These responses also occurred predominantly above 0.01 $m.s^{-2}$ (above 140 dB) with a few occurrences at lower sound levels, stretching the range of particle acceleration eliciting those responses by an order of magnitude compared to inking. No escape responses were observed below particle accelerations of $3.3x10^{-3}$ $m.s^{-2}$ or 110 dB. Less intense responses (body patterning changes and fin movements) were more widespread along both the frequency and sound intensity range and had much lower mean acceleration levels (dashed lines in Fig. 3 and SM1). The less intense responses were seen at acceleration levels down to $4x10^{-4}$ $m.s^{-2}$ and sound pressure levels as low as 85 dB. The absence of response (no response) was typically found at lower sound levels, similar to the levels eliciting body patterning changes and fin movements.

The mean sound pressure level and particle acceleration eliciting behavioral responses was not constant over the frequency range (Fig. 4). The lowest sound levels eliciting a response were found at 150 Hz, regardless of whether they were measured as particle acceleration or sound pressure. At this frequency, animals demonstrated responses to sound stimuli at a mean particle acceleration of 0.025 $m.s^{-2}$ (and mean SPL of 124 dB), and the elicited response was a small body patterning change and/or small fin movement. The absolute lowest sound levels eliciting a response at 150 Hz were $4x10^{-4}$ $m.s^{-2}$ and 85 dB; the observed behavior in these cases was a small body patterning change and/or fin movement. On the other hand, 1000 Hz and 700 Hz required relatively high sound levels to elicit responses from the animals (Figs. 1, 4).

There were no changes in response rates while the animals were subject to the conditions in the matrix (including across the eleven consecutive days or within single test days with four tests per day, at least 20 minutes between each test). For example, we were concerned that animals might have reacted less at the end of the two weeks of testing (i.e. a cuttlefish getting a 300 Hz/140 dB sound on day 3 might react more than a cuttlefish getting that same sound on day 10 because the latter had already been exposed to multiple tones for nine days). However, response types and occurrences showed no consistent pattern with respect to the order of sound presentations. This suggests that: (1) cuttlefish behaviors were not influenced by the prior exposures. Thus, it is possible the animals did not learn or otherwise anticipate the sound presentation when signals were presented in this randomized order and schedule. (2) The repeated sound presentations did not impact their hearing enough to change their responses. Consequently, individual sound presentations were considered independent trials.

Habituation to repeated sounds

While response rates did not change in the random matrix, which spread sound trials over several days, habituation to acoustic stimuli was observed when identical sounds were presented closer in time. When tones of the same source level and frequency were presented every minute for 30 min, the number of animals showing escape responses (inking and jetting) decreased logarithmically as the number of repeated stimuli increased (Fig. 5). This was true for both higher and lower sound levels although higher sound levels tended to reflect less variation in the number of animals responding and, correspondingly, higher regression-based r^2 values (Table 2). For example, inking response occurrence rates were significantly related to trial number for both the higher and lower sound levels, but higher sound levels produced a higher r^2 value (r^2 for high and low sound levels were 0.60 and 0.42, respectively; see Table 2). Jetting responses, also tied to trial number, occurred more often in the early trials, allowing for a steeper decline in response rates for both high and low sound levels. Occurrence rates decreased significantly with increased trial number (r^2 for high and low sound levels were 0.72 and 0.70, respectively). Similar trends were seen for the large body patterning changes as well, but with greater overall variation (r^2 = 0.25 at the higher sound level; r^2 = 0.49at the lower sound level). Startle responses, fin movements, and smaller body patterning changes showed reverse trends with slight increases in occurrence rates as trial number increased. This was likely because the escape responses tended to dominate at the beginning of test series (only the highest scoring behavior was taken into account for each trial); as trial number increased and habituation set in, the lower intensity responses became more prevalent.

The differences in particle acceleration shown in Fig. 6 provide an indication of the movements of the animal because the received acceleration level depended on the distance of the animal to the speaker. Cuttlefish often settled themselves near the speaker, at the bottom of the netted space, so the first trial of each test series tended to be at a relatively high recieved level (Fig. 6A, black symbols). The animal in Fig. 6A then moved higher in the water column, away from the speaker, and received a relatively lower sound level in the second trial (the particle acceleration is lower). The cuttlefish subsequently moved around in the tank and finally settled back down after 5 to 10 exposures (reflected in the more or less constant sound level from trial 10 onward). This pattern is also noticeable in the lower part of Fig. 6A and in Fig. 6B.

Total response inhibition was never reached; individuals repeatedly exhibited a "stereotyped startle" response. The order in which the sounds were presented (i.e., higher intensity sound on the first or second test day) and the age of the animals did not have an effect on the observed decrease in response type. Greater variation in the responses given by different animals was also seen in the early trials, but sound levels were also more variable as the animal tended to move around in the sound field as a result of the acoustic stimuli.

DISCUSSION

Acoustic frequency range and sensitivity

A primary aim of this research was to address the frequency range and sound levels that induce behavioral responses in a cephalopod, the common cuttlefish. This work provides the only unconditioned, sound-mediated behavioral response data set for cephalopods, and the only work that describes both the range and sensitivity of such responses for marine invertebrates. The data may be applicable for evaluating the auditory scene that some cephalopods may utilize, and help define the noise conditions that may impact these animals.

The sound levels generating behavioral responses in this study were quite low, often lower than the physiological thresholds previously measured in cephalopods. Body pattern changes and fin movements were observed at the lowest sound levels, lower 10^{-4} $m.s^{-2}$ and down to 85 dB. Neurophysiological responses in longfin squid and common octopus were generated using slightly higher amplitude signals [between 10^{-3} to 10^{-4} $m.s^{-2}$ (Kaifu et al., 2008; Mooney et al., 2010)]. The differences between physiology and behavior results could reflect that the evoked potential methods are not as sensitive as the animal's auditory system and these behavioral metrics. Or there could be taxonomic based differences as this study used cuttlefish, while Kaifu et al., (2007; 2008) and Mooney et al., (2010; 2012) used octopus and squid species. Yet, Packard et al., (1990) used classical conditioning to address *S. officinalis* sound detection and response thresholds were still two orders of magnitude higher than here. This suggests that *S. officinalis* is more sound-sensitive than previously thought. Furthermore, the unconditioned method used here provides a robust way to address the behavioral response range and apparent sensitivity for this species.

The overall frequency range and upper limit that generated responses was somewhat greater than previous acceleration-based cephalopod sound detection studies (Packard et al., 1990; Kaifu et al., 2008; Mooney et al., 2010), but results were similar to those of many fish without auditory specializations (Popper and Fay, 2011). This reinforces the notion that cephalopods, like many fish, have an accelerometer-like "auditory" system that detects the particle motion component of sound stimuli. Furthermore, cephalopod auditory scenes and sound use may be very similar to fish without specializations.

Mean response levels fluctuated with stimulus frequency. To some extent, this may be the result of greater sensitivities at lower frequencies. These variations may also be due to sound reflections and interferences linked to the size of our experimental tank. In small tanks, sound does not attenuate as in the free field, and despite the detailed calibrations conducted here it is impossible to determine the exact levels received by a moving animal for every location within the tank. However, the variations may also reflect individual differences in auditory or behavioral response thresholds. Such variation was evident from general observations of the animals and is reflected within the individual data (e.g. Fig. 6).

The occurrence of escape responses was strongly linked to the characteristics of the sound stimulus. For example, inking was only found at lower frequencies and higher sound intensities. Jetting was also only found at the higher sound levels. Yet all stimuli had relatively rapid rise times and short onsets of the stimuli (tens of ms) suggesting these were not vital to inducing the escape responses. The link to sound intensity suggests that sound level could provide some behavioral relevance to the animals, and that higher levels infer closer predators, thus inducing the escape behaviors. More basically, sound detection could be a mechanism for predator detection in these animals. This idea of predator detection is reinforced by the observation of deimatic displays to some of the acoustic stimuli. The deimatic display is usually elicited by visual stimuli, e.g. a model of a predator (King and Adamo, 2006; Cartron et al., 2013) or an actual predator (Langridge et al., 2007), with the purpose of deterring said predator. The observation of deimatic displays in the absence of a visual stimulus suggests that sound could play a role in predator detection by cuttlefish, as surmised by Hanlon and Budelmann (1987).

The behaviors exhibited were clustered relative to frequency and received levels (Fig. 3). Higher levels and lower frequencies induced escape responses (as noted above) and more moderate responses (body pattern changes and fin movements) were observed at lower sound levels and higher frequencies. This trend generally follows what we know regarding cephalopod hearing: they detect lower frequencies better, suggesting a sensation level response curve for these behaviors. The clustering also indicates a potential for the perception of loudness in the common cuttlefish (and perhaps other cephalopods); that is, the behavioral response curves (Figs. 4, 5) could be taken as preliminary loudness sensitivity measures. As in several other studies (Stebbins, 1966; Kastelein et al., 2011), these assessments would be subjective and based on certain response characterizations, and would probably not be as accurate as protocols aimed specifically at generating loudness curves (Finneran and Schlundt, 2011). Yet, *S.officinalis* appears to differentially respond to acoustic stimuli based upon relative perceived sound levels, not solely absolute values.

Overall, the dynamic range of potential responses that cuttlefish can generate in response to acoustic stimuli are relatively well characterized in regard to their behavioral and ecological relevance in other contexts (Hanlon and Messenger, 1996). These prior descriptions of behaviors and the clarity of the responses seen during this study indicate cuttlefish are a suitable subject for future bioacoustic studies.

Habituation to repeated sounds

All tested cuttlefish showed habituation to repeated stimuli. Habituation was noted by a logarithmic decrease in the occurrences of certain responses over the course of 30 exposures (30 min) of repeated 200 Hz tone stimuli. This decrease was notable in the more dramatic escape responses (inking and jetting), and for large body patterning changes; this pattern of habituation is similar to that reported in the squid *Lolliguncula brevis* (Long, et al, 1989). It was significant across both sound levels, suggesting the robustness of this form of habituation. The decrease in response intensity was more marked at lower sound intensities; this is in agreement with one of the characteristics of habituation described by Rankin et al. (2009): weaker stimuli generate more rapid and/or more pronounced habituation.

Similar to the first experiment, escape responses were initiated by relatively higher intensity stimuli (likely of greater sensation level); but in this experiment, earlier signals also showed a greater response rate. These evasion responses suggests that the cuttlefish initially reacted to the stimulus as they would react to a predator or other form of danger, and that sound detection could be a mechanism for predator detection in these animals. After several exposures and no eminent threat, the number of escape responses decreased, suggesting the cuttlefish were able to filter out the "irrelevant" acoustic stimuli, allowing for a refocusing of sensory mechanisms.

This present study is one of the few measuring habituation in cephalopods and the only one focusing on habituation to acoustic stimuli. Previous studies using visual stimuli in squid showed a sharp decrease in the number of jetting responses over the first five minutes but total inhibition of responses was not observed and the squid continued to show a ring pattern when exposed to the fish predator models (Long et al. 1989). Those results are very similar to the results obtained for acoustic habituation in cuttlefish. While both overall response intensities and the number of escape responses decreased over time, total response inhibition was not observed. Cuttlefish often ended test series with a startle or "stereotyped startle" response, which seemed to be a residual startle response and was often limited to a twitch of the median arms. The continued elicitation of the "stereotyped" response could indicate that sound is an important source of information for these animals. It may be vital for cuttlefish to keep a certain level of (neural) vigilance when it comes to gathering acoustic information from the environment and continuously processing an auditory scene.

Cephalopod acoustic ecology

Cuttlefish responded to a range of sound levels and frequencies, and response intensities depended on the sounds to which the animals were exposed. Moreover, cuttlefish showed habituation to repeated sound stimuli over time. These findings indicate that cuttlefish, and perhaps cephalopods in general, can use sound as a source of information and have the level of neural development required to process acoustic information from their environment, for example by selecting or learning which sounds can be "ignored" (i.e., habituation to sound). It remains unclear, however, what the function of sound is in the lives of cephalopods, especially in relation to their other well-developed sensory systems, particularly vision. Sound production has been proposed (Iversen and Perkins, 1963) but remains highly speculative. Defense against predators (Hanlon and Budelmann, 1987), prey detection, or navigation are possible functions of sound sensitivity because the natural marine soundscape offers a wide range of natural and animal sounds. How invertebrates, in general, use sound is not well understood.

The results herein also provide some indication of sound-induced directional responses by the cuttlefish. While the direction of displacements was not measured explicitly, the animals' locations in the tank were noted at the time of stimulus presentations. During the habituation experiments, the cuttlefish tended to start testing sessions near the speaker (i.e., a preferred location). At the start of nearly all second sound stimuli, cuttlefish were located higher in the water column and farther from speaker, suggesting an initial movement away from the sound source. This is in agreement with the earlier indications that the observed responses tended to be avoidance behaviors. From an anatomical perspective, cephalopod statocysts could support directional hearing. Hair cells of the squid and cuttlefish statocyst are polarized and directionally oriented (Budelmann, 1979). Directional response movements have already been proposed in larval invertebrates (Vermeij et al., 2010) but have yet to be shown in adults. The experiments here were not designed to test the directionality of behavioral responses and follow-up examinations would best address such a hypothesis.

Although the sound frequencies and levels used in this study could be produced by natural factors, they are also similar to many anthropogenic noises such as shipping, air guns and drilling (Urick, 1983). Cephalopods may be anatomically impacted by exposures to such sounds, and may even be stranding as a result of intense sound exposures (André et al., 2011). Yet few detailed behavioral quantifications exist. Behavioral responses may have significant impacts on cephalopod populations, even at lower sound levels or more distant exposures. Measuring the effects of noise from different sources (recordings from shipping or industrial activities, white noise, etc.) on cephalopod behavior and physiology is important to predict how increasing anthropogenic noise in the ocean will affect cephalopod populations and their distribution, key variables because of the importance of cephalopods in marine food webs. Thus, quantifying behavioral responses as well as potential habituation to anthropogenic noise in multiple species could provide a foundation to understanding how cephalopods may respond to noise exposures.

MATERIALS AND METHODS

Experimental overview

Two general experiments were addressed: (1) the frequency range and sound levels which generated behavioral response and (2) the rate of habituation to pure tones. Animals were free-swimming in the center of a 1.08 m diameter tank (Fig. 7). To test the range and levels of responses, a matrix of sound stimuli was devised based upon physiological data (Fig. 1), and ten animals were presented each sound (a 3 s tone) in a random sequence (with no animal receiving more than 4 sounds per day). Behavioral responses were recorded using HD video and scored based upon response type (i.e., inking, jetting, "startle," color change, fin movement, no response) and responses were plotted relative to stimulus condition. Habituation trials consisted of presenting ten individual animals a 3 s tone at 200 Hz every min for 30 trials. Responses were scored in similar manner and addressed relative to trial number. Calibrations of sound pressure and particle acceleration were conducted at the beginning and end of the experiments.

Animals

Experiments were conducted between January and July 2012. The twenty-two cuttlefish used for the experiments were hatched and raised at the Marine Biological Laboratory (MBL) in Woods Hole, MA, USA. Ten animals were used for the first set of experiments addressing frequency range, sensitivity and habituation (January and February); six "older" cuttlefish (approximately 1.5 year old) and four juveniles (approximately six months old). This experiment was designed around a matrix that utilized 10 animals at all exposure levels and frequencies (Fig 1). However, during the first series of experiments, one old cuttlefish and one juvenile died due to events unrelated to the tests. They were replaced by new individuals of corresponding age; all animals were included in the analyses (thus, a final n = 12). Based upon these results, tests for frequency range and sensitivity were expanded in July 2012 using ten additional cuttlefish (one year old). The older animals were accustomed to being handled for visual experiments but were naïve to acoustic tests; the juveniles had never been used for experiments before. During the testing period, the animals were kept at the Woods Hole Oceanographic Institution (WHOI) in Woods Hole, MA, USA. Animals were housed individually in partitioned, shallow tanks with a permanent flow of filtered seawater and were fed defrosted shrimp once a day.

Experimental set-up and protocol

The same basic experimental set-up was used for all tests (Fig. 7). Behavioral response trials took place in a circular fiberglass tank (inner diameter: 1.08 m, depth: 0.60 m), the inside of which was painted white. There was a continuous, low flow of filtered seawater to maintain constant water temperature (14˚C) and aerated conditions. Three valves were mounted at different heights on the outflow pipe to allow for regulation of the water level in the experimental tank and partial water changes (in the case of inking for example). An acoustically transparent black plastic net (2 cm mesh size) was strung in a conical shape from the tank rim to the speaker at the apex. This ensured that the animals swam above the speaker in the water column and prevented them from settling on the bottom of the tank or the speaker, and from touching the sidewalls of the tank. The speaker was isolated from the tank by two discs of closed-cell neoprene (12.7 mm each) to reduce the potential transmission of vibrations from the speaker to the tank. The tank itself was also isolated from the floor by elevating it on a platform and adding two sheets of open-cell neoprene (12.7 mm each) between the platform and the tank. The netting was loosely hooked to the sides of the tank and hung in a conical fashion generally encouraging the animals toward the center of the tank, but their location could vary. Because the net only hung loosely and due to the neoprene gaskets, there was little transmission of sound or vibration to the netting or tank. There was no detectable particle motion from these structures into the water column (see calibrations below). Care was taken to ensure animals were in the water column and not touching the sides or netting when we initiated the test tones.

Experimental test tones were produced using a UW30 underwater speaker (Lubell Labs Inc., Columbus, OH, USA). The speaker was connected to a Panasonic CF-52 Toughbook (Bizco Technologies, Lincoln, NE, USA) with a National Instruments 6062E data acquisition card (DAQ, Austin, TX, USA) and running a custom program using the National Instruments LabView software. This program allowed us to control the frequency and intensity of the sound and the duration of the sound pulses. A PYLE Chopper Series PLA2210 amplifier (Brooklyn, NY, USA) and a Hewlett-Packard 350D (Palo Alto, CA, USA) attenuator were used to adjust the output from the computer to the speaker. A Tektronix TPS 2014 oscilloscope (Beaverton, 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 camera (Tokyo, Japan) placed above the tank and recording at 60 fps.

Sixty-seven different tones, including a silent control, were used to determine the frequency range and sound levels that induced behavioral responses (Fig. 1). These tones lasted 3 s and differed in frequency (80, 100, 150, 200, 250, 300, 400, 500, 700 and 1000 Hz) and intensity (110, 120, 130, 140, 150, 155, 160 and 165 dB re. 1 μPA rms, as calibrated 20 cm away from the speaker). This initial matrix was based on the physiological responses to sound obtained from the longfin squid (Mooney et al., 2010). Based on the behavioral results from the first series of sound tests, an additional set of 10 sound combinations using 700 and 1000 Hz was tested on 10 new animals in July 2012. These animals were housed and tested as described above. At the highest sound levels, some frequencies were distorted due to characteristics of the speaker; those sounds were not used for the experiments (blank cells in Fig. 1). Because the animals settled or swam at different distances from the speaker, the received sound pressure levels differed from the calibrated ones. Thus, by changing the speaker output levels (in the range noted above) and the animal varying its location in the tank (swimming and thus the distance to the source), the received levels ranged from 85 to 188 dB re. 1 μPa rms (considering all frequencies). Unless stated otherwise, sound pressure levels (SPL) are presented in dB re. 1 μPa rms.

Prior to a sound test, the animal was gently moved from the housing tank to the test tank using a glass container. Before the start of the experiments, the animal was given 1-2 min to settle. All cuttlefish were tested individually and exposed to four different sounds a day (each tone lasted 3 s), but each animal was only exposed once to a specific frequency-sound level combination. The order in which the cuttlefish were tested was randomized every day, with the condition that there should be at least four trials using other individuals between two consecutive tests of one animal, leaving enough time for recovery from handling and exposure to sound. By the end of the testing period (two to three weeks), the animals had been presented each sound (66 sounds in total) and the silent control once in a randomized order.

Sound calibrations

Both sound pressure and particle motion were calibrated across the diameter and depth of the tank using the experimental test tones. Calibration measurements were made at the beginning and end of the experiment with essentially the same results. Experimental tones of all tested frequencies were recorded at each location. Sound pressure was measured using a calibrated Reson TC 4014 hydrophone (Slangerup, Denmark) and for the particle acceleration calculations described in the next paragraph. For basic SPL (dB re 1 μPa rms) the hydrophone was suspended 10 cm from the center of the speaker and then moved to the surface in 10 cm steps. This procedure was repeated along the diameter of the tank, with horizontal distance from the speaker increasing in 10 cm increments. The peak-to-peak amplitude of the signals was measured on the oscilloscope, and converted from voltages to SPL using a custom script. The tones were concurrently recorded using an Olympus LS-10 PCM pocket recorder (Olympus America Inc., Center valley, PA, USA).

Particle acceleration values were obtained by measuring the pressure gradient over two closely spaced sound receivers (Gade, 1982). Two custom hydrophones (sensitivity -180 dB re 1V/μPa), 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 was 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. A total of three depths and 11 positions along the diameter were used and the hydrophones were placed in three different orientations to record sound pressure in all three directions at each measuring point. Particle acceleration was computed from the pressure gradient across the two hydrophones:

$$a = \frac{-\Delta p}{\rho \Delta r} \qquad (1)$$

where Δp is the magnitude of the difference between the waveforms of the two, ρ is the density of the medium and r is the distance between the hydrophones (Kalmijn, 1988; Wahlberg et al., 2008). The particle motion was measured in three dimensions by positioning the two hydrophones along three orthogonal axes. The magnitude of the acceleration was computed and used for the data analysis and figures. Comparisons of particle acceleration values for the pressure-derived thresholds were determined by relating the measured pressure at the location with the corresponding particle acceleration at each corresponding location. Within the acoustic near-field of the speaker, the cuttlefish 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 the calibration results, 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: 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 cuttlefish 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. Knowing the actual size of the animal, we could compute its expected pixel length at the water's surface and compare this 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 from the animal to 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 animal's head (between the eyes) was also determined. Total distance from the speaker to the center of the animal's head (between the eyes) 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.

Sound pressure levels were calibrated at the start of the experiments in January 2012 and again later in July 2012. The calibrations were found to be similar. Accelerations were calibrated once, after all the tests were performed.

Scoring behavior

The behavioral responses for each cuttlefish at each sound combination were categorized using six types of response: no response, body pattern change, fin movements, startle, jetting and inking. Within each type of response, some gradations were defined (Table 1). This scoring system is based on observations of the animals before the experiments and on previous research on the response of cuttlefish to predators and human-elicited stress (Hanlon and Messenger, 1998; Staudinger et al., 2011).

Two behaviors described in Table 1 deserve more extensive explanations: the deimatic pattern and the startle response. The deimatic display is usually observed in experiments involving visual stimuli (Langridge, 2009; Mather, 2010; Staudinger et al., 2011); it is considered a threat (or startle) display to deter potential predators (Staudinger et al, in press) and is defined by a flattened body shape, paling of the skin, the presence of paired, dark mantle spots, a dark fin line, dark eye rings and a dilation of the pupil (Hanlon and Messenger, 1988; Hanlon and Messenger, 1998). The startle response has been described for several taxa, mostly vertebrates and insects (Hoy et al., 1989; Pilz and Schnitzler, 1996; Koch, 1999; Kastelein et al., 2008) and is provoked by an intense and unexpected stimulus, has a short delay, and involves a fast motor response including escape responses and subtler movement such as eye blinks (Hoy et al., 1989; Koch, 1999; Götz and Janik, 2011). Based on these descriptions of the startle response, we defined one of the responses in *Sepia officinalis* as a startle response. During the habituation tests (see below), we observed a decrease in startle response intensity and termed this the "stereotyped startle" response (Table 1). The notion of "stereotyped" is preferred to "reflex" in this case because of the lack of neurological investigation.

Habituation to repeated sounds

Two weeks after the initial behavioral responses tests, 10 animals were tested for potential habituation to sound stimuli. Animals were divided into two groups and exposed to repeated 200 Hz, 3 s tones, presented every minute for 30 minutes. This frequency was chosen because of the general sensitivity and diversity of responses it elicited in the first series of experiments. Responses to two sound intensities were compared using calibrated sound levels of 150 and 165 dB. Each of the two groups consisted of three old animals and two young ones. The first group started with the sound at 165 dB on the first day and received the 150 dB sound on the second day; the second group got the opposite treatment. As for previous behavioral trials, exposure levels were corrected for the distance of the animal to the speaker. Tests were performed with 30 to 45 stimuli and behaviors were recorded and scored as noted above. Standard regression analyses were used to estimate the relationship between trial number and rate of occurrence of the different response types.

ACKNOWLEDGEMENTS

We thank Kimberly Ulmer, Kendra Buresch, Liese Siemann and other members of the Hanlon Lab for providing advice on husbandry and experimental set-up. Thanks also to Vicke Starczak, Jesús Pineda and Michael Moore from WHOI for suggestions on experimental design and analyses. Scott Gallager, Houshou Jiang and Gareth Lawson lent us the facilities space. Members of Mooney's SPASE Lab assisted with the experiments at various stages, including Margot Wilsterman, Max Kaplan, Amy Streets, and Samantha Zacarias. Rick Galat, Joe, Ed, Steve Allsopp, Kristopher Newhall and Jim Dunn helped make the tank and seawater adjustments. Thanks to Sander Kranenbarg, Henk Schipper and Kees Voesenek from the Experimental Zoology Group at the Wageningen University for their help with the MatLab program. Words 6729.

COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

J.E.S. and T.A.M. designed the experiments, collected the data, and conducted the analyses. S.W.S.G. and R.T.H. assisted with the data analyses and writing the paper.

FUNDING

The work was initially posed through a MBL Grass Fellowship to TAM. Funding was provided by the Sholley Foundation (for RTH) and WHOI's Ocean Life Institute.

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TABLES

Table 1 Overview of the types of responses and their intensities used to score the behavioral responses of *Sepia officinalis* to sound stimuli

Response type	**Intensity**	**Description**
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	Body pattern change covering at least half the body area, includes dark flashing, bleaching, deimatic, etc.
	Deimatic	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.

Table 2 Logarithmic regression statistics to evaluated response occurrence rates vs. trial number

Sound level	Response type	Line equation	r2	*p*	n
	jetting	y = -2.32*ln(x) + 8.03	0.72	< 0.001	30
high	inking	y = -0.81*ln(x) + 2.42	0.6	< 0.001	30
	large color change	y = -0.78*ln(x) + 3.97	0.25	< 0.05	30
	jetting	y = -1.72*ln(x) + 6.16	0.7	< 0.001	30
low	inking	y = -0.50*ln(x) + 1.54	0.42	<0.01	30
	large color change	y = -0.99*ln(x) + 3.74	0.49	< 0.001	30

FIGURE CAPTIONS

Figure 1. Matrix of the behavioral responses of an individual cuttlefish to different sounds. The matrix reflects the stimuli presented as part of the experimental design. The responses shown are from 1.5-year old cuttlefish for the frequencies between 80 and 500 Hz, and from a different, one-year old animal for 700 and 1000 Hz. The blank cells indicate sound combinations that were not played due to technical limitations of the set-up. NR: no response. The control is not represented in the matrix. SPL: sound pressure level dB re 1 µPa.

Figure 2. Types of behavioral responses to sound. These frames are extracted from one test and illustrate how different behavioral responses can be combined. A: Cuttlefish at rest in the experimental tank before the sound stimulus. The median arms are dark and are held backward over the head. B: Jetting and inking. C: Large body pattern change (darkening) and fast fin movements resulting in a displacement of the animal after having jetted away.

Figure 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: large body pattern change and/or fast fin movements, Small body pattern/fin: small body pattern change and/or slow fin movements. The dashed lines represent the mean acceleration value for that response.

Figure 4. Mean (A: acceleration; B: sound pressure) behavioral responses. Only the highest scoring behaviors for each sound test are represented here. At lower frequencies (below 500 Hz), the escape responses (jetting, inking and large body pattern change) were elicited at higher sound levels. Above 500 Hz a relatively high sound level was needed to induce any type of response.

Figure 5. **Habituation to a repeated sound stimulus.** Data were collected using a 200 Hz tone at 165 dB (calibrated sound pressure), which was presented every minute for 30 consecutive trials. The occurrence of both response types (inking and jetting) decreased logarithmically; details in Table 2 (high sound level). N = 10 cuttlefish.

Figure 6. Succession of behavioral responses of two individual cuttlefish. A 200 Hz tone was presented every minute for 30 consecutive trials. A: old animal (1.5-year old), B: young animal (6 months old). Black symbols represent the responses to the first test series; grey symbols represent the responses to the second test series.

Figure 7. Experimental set-up. A: Schematic side view. 1: tank, 2: net, 3: speaker, 4: calibration ruler, 5: outflow pipe, 6: HD video camera. B: Detail from a video as recorded by the HD camera above the tank.

FIGURES

Figure 1

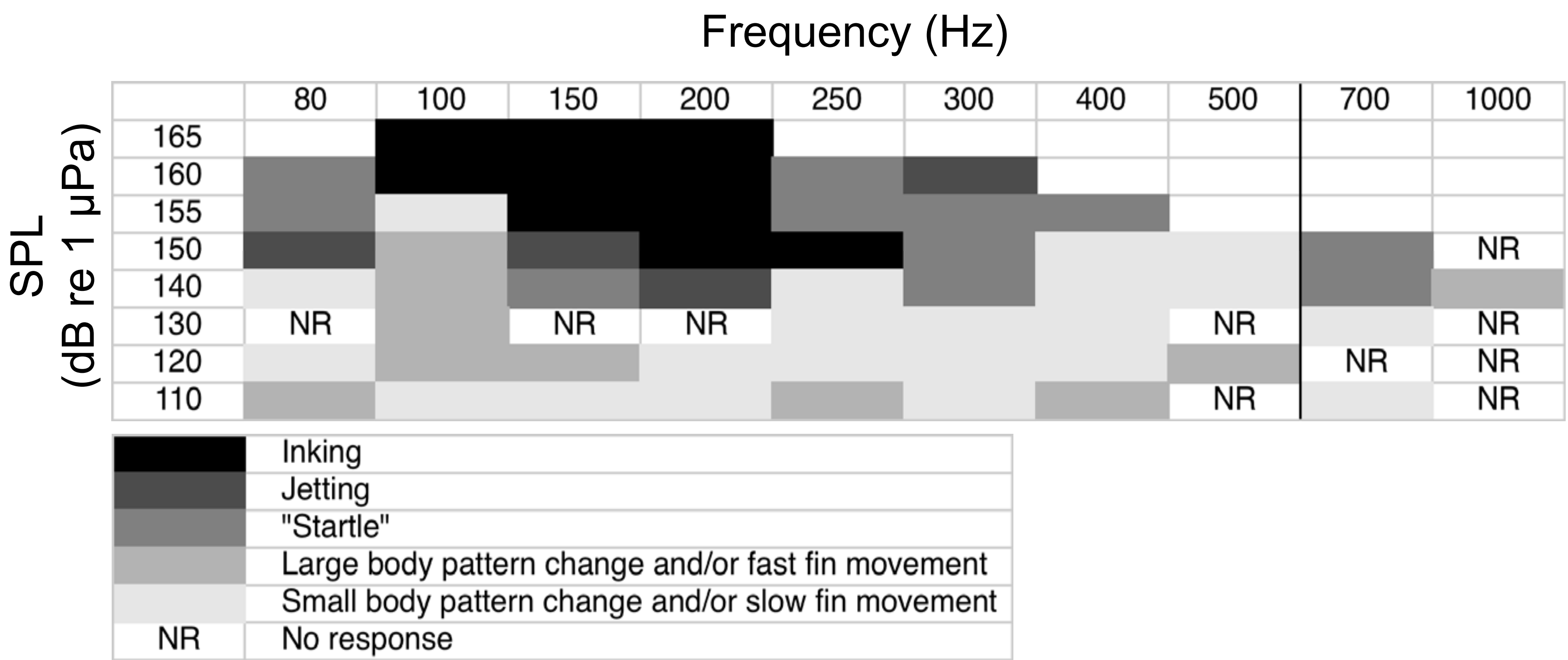

Figure 2

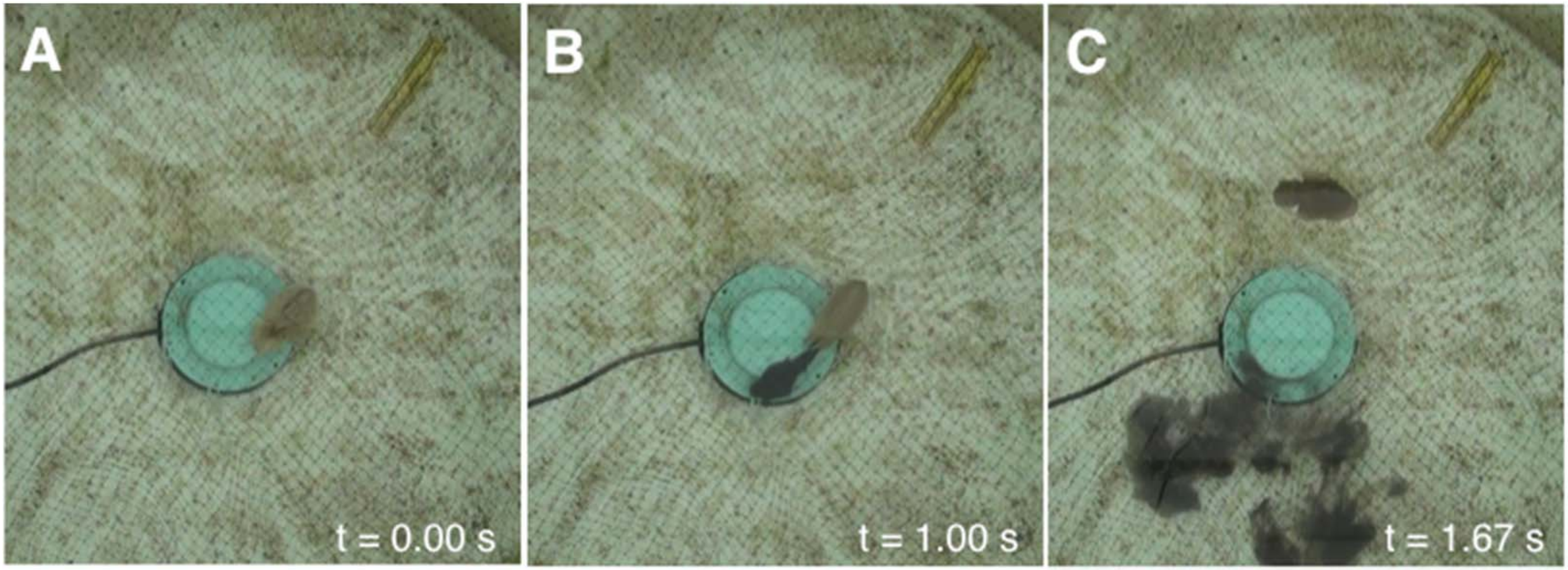

Figure 3

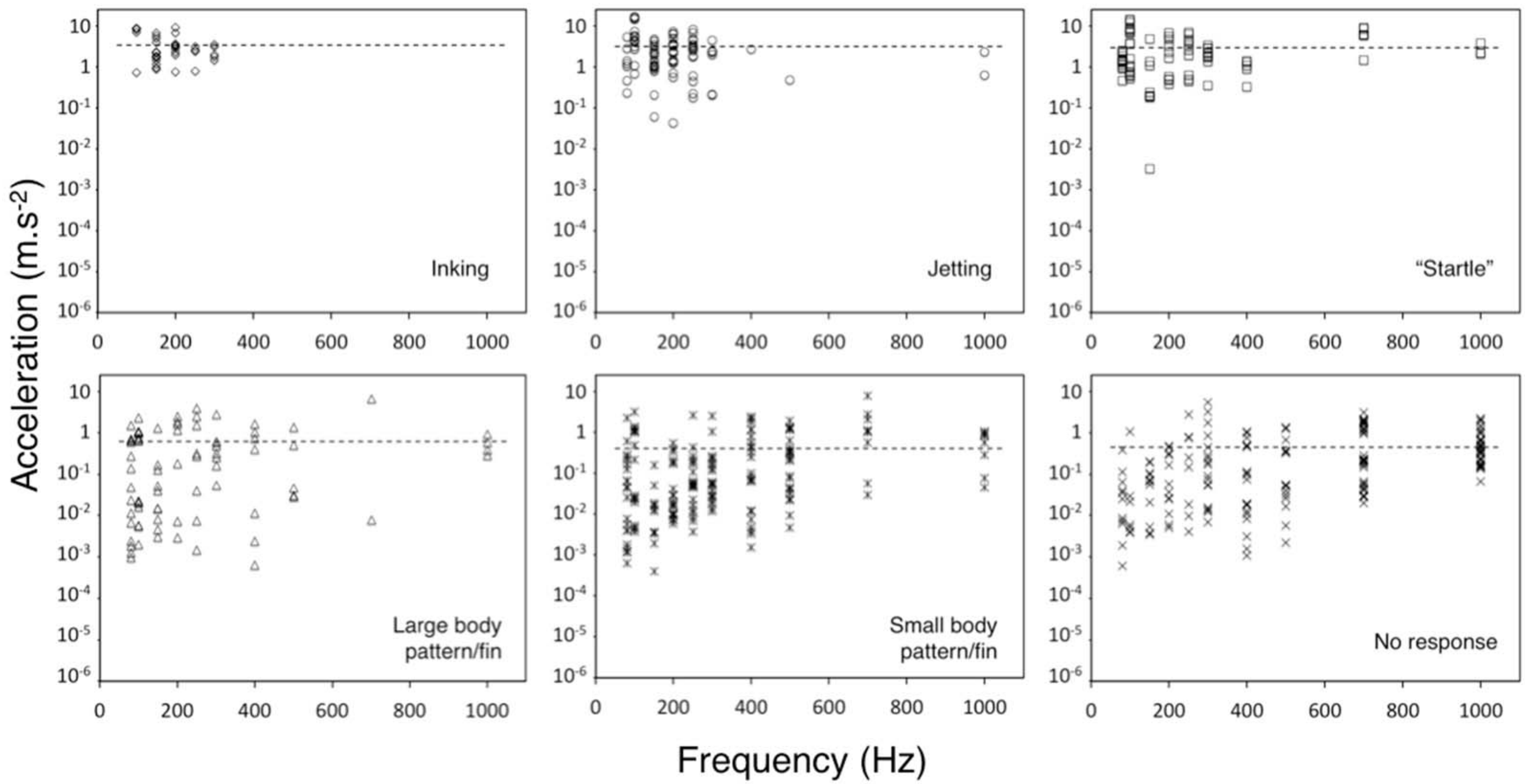
Inking
Jetting
"Startle"
Large body pattern/fin
Small body pattern/fin
No response
Acceleration (m.s-2)
Frequency (Hz)
10
1
10-1
10-2
10-3
10-4
10-5
10-6
0
200
400
600
800
1000

Figure 4

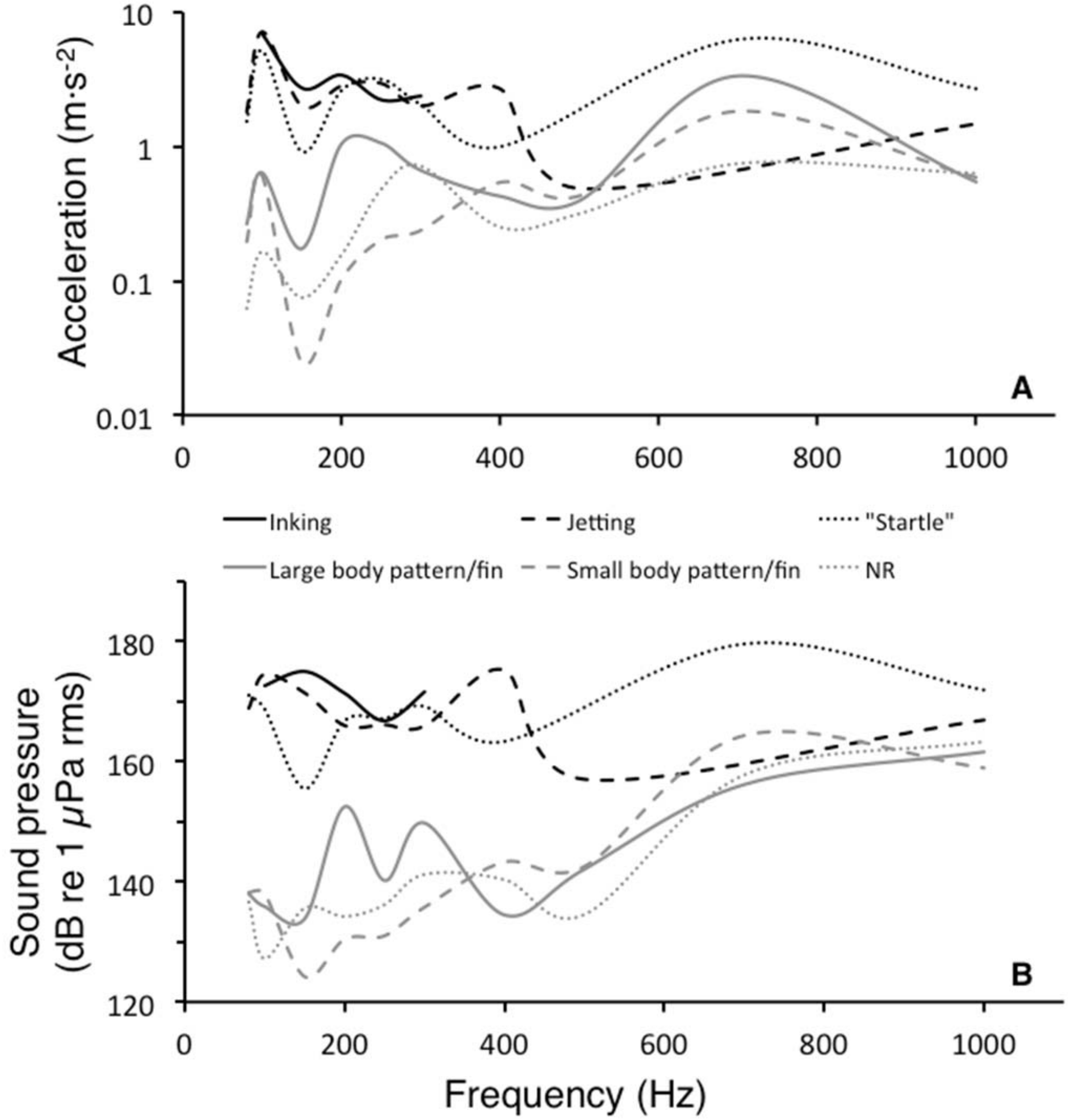
10
1
0.1
0.01
Acceleration (m·s^{-2})
A
0
200
400
600
800
1000
Inking
Jetting
"Startle"
Large body pattern/fin
Small body pattern/fin
NR
180
160
140
120
Sound pressure
(dB re 1 μPa rms)
B
0
200
400
600
800
1000
Frequency (Hz)

Figure 5

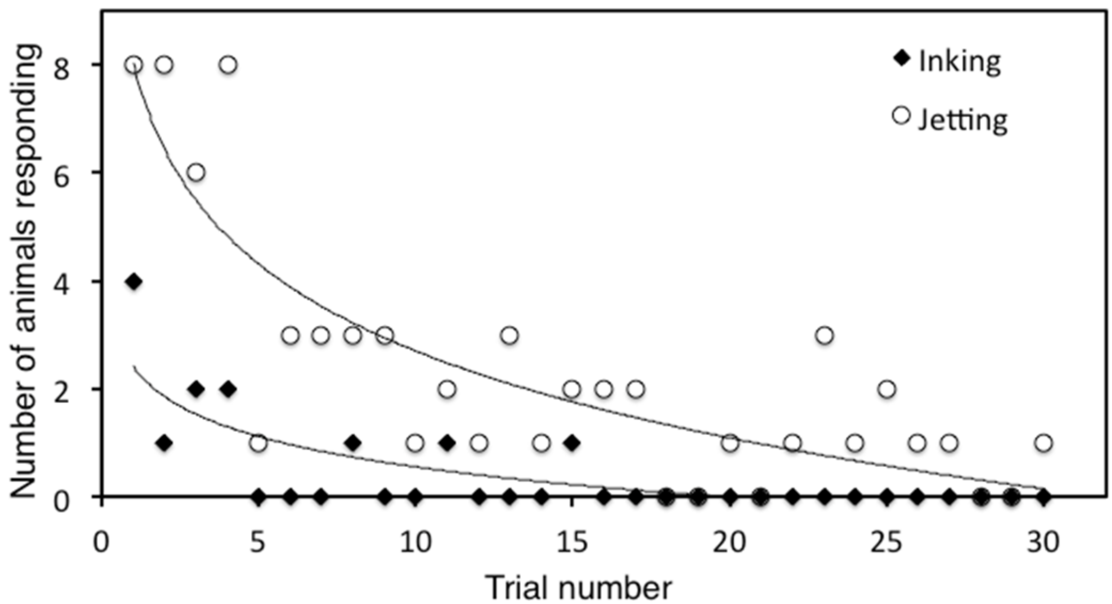
Inking
Jetting
Number of animals responding
0
2
4
6
8
0
5
10
15
20
25
30
Trial number

Figure 6

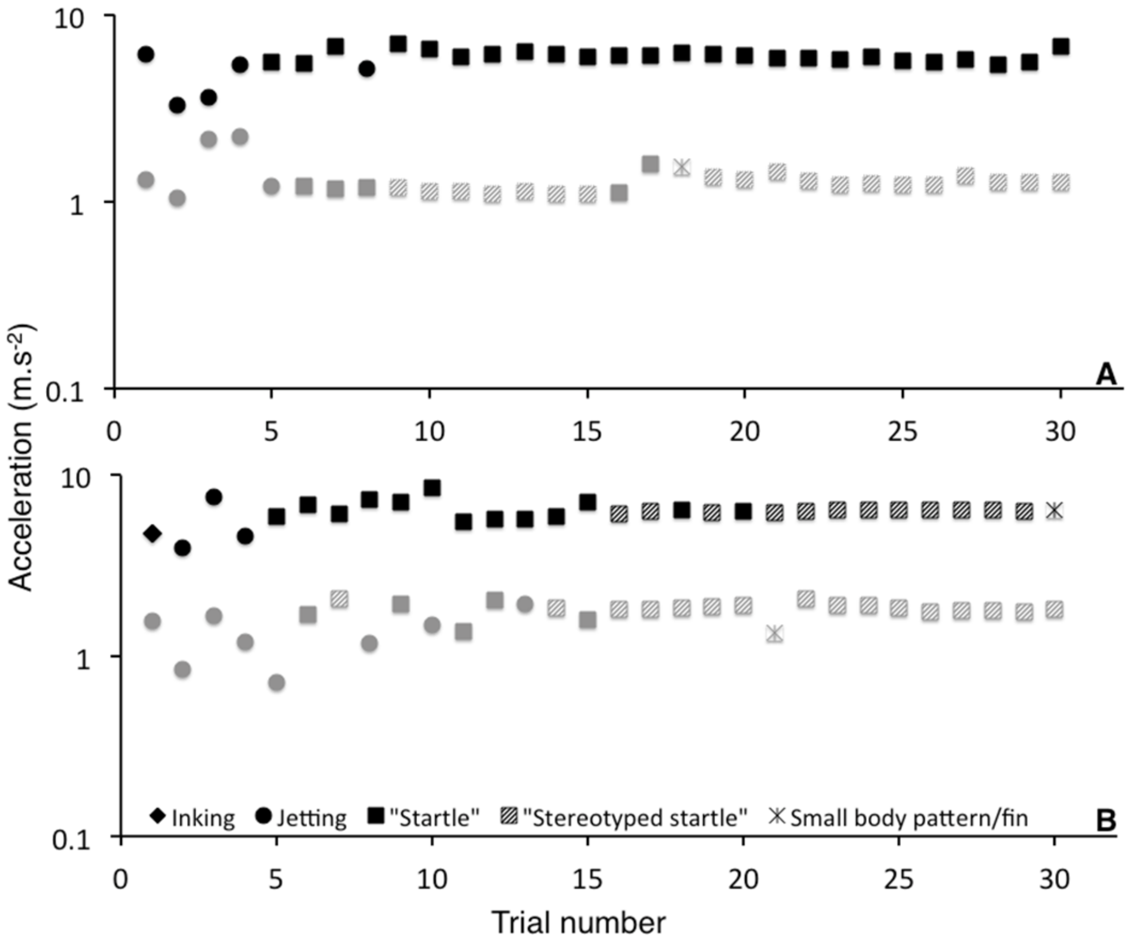
10
1
0.1
A
0
5
10
15
20
25
30
Acceleration (m.s-2)
10
1
0.1
◆ Inking ● Jetting ■ "Startle" ▨ "Stereotyped startle" ✳ Small body pattern/fin
B
0
5
10
15
20
25
30
Trial number

Figure 7