Freezing behaviour facilitates bioelectric crypsis in cuttlefish faced with predation risk

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Cephalopods, and in particular the cuttlefish Sepia officinalis, are common models for studies of camouflage and predator avoidance behaviour. Preventing detection by predators is especially important to this group of animals, most of which are soft-bodied, lack physical defences, and are subject to both visually and non-Visually mediated detection. Here, we report a novel cryptic mechanism in S. officinalis in which bioelectric cues are reduced via a behavioural freeze response to a predator stimulus. The reduction of bioelectric fields created by the freeze-simulating stimulus resulted in a possible decrease in shark predation risk by reducing detectability. The freeze response may also facilitate other non-visual cryptic mechanisms to lower predation risk from a wide range of predator types.

1. Introduction

The importance of preventing detection by predators is most obvious in visually camouflaged species, which are often matched to their background to render themselves nearly invisible to receivers [1–3]. Cephalopods have long been of interest to researchers due to their ability to modify their appearance under changing conditions [1–3]. Within this group, behavioural mechanisms that either prevent detection by predators (camouflage) or that avoid attack after detection has occurred (visual displays, fleeing and inking) have been frequently studied in the common cuttlefish Sepia officinalis [4–11].

Selective pressures for the evolution of visual camouflage have also influenced adaptive cryptic mechanisms in non-visual modalities [3], but these mechanisms remained understudied [4,7,8]. Freezing behaviours, defined as temporary cessations of body movement or ventilation, often co-occur with background matching and visual displays to evade predation by both visual and non-visual predators [8,9,12,13]. Freezing has been noted in a diversity of taxa, including cephalopods. For example, the longfin squid Loligo pealeii stops swimming and drops to the substrate as a way to reduce motion that signals its presence to nearby teleost predators [9]. Cephalopods, and in particular S. officinalis, present an ideal system for studying non-visual crypsis as they fall prey to a diverse group of predators [14–20], many of which employ acute, non-visual sensory modalities while foraging [21–24]. For instance, elasmobranch fishes consume a variety of cryptic prey, including several cephalopod species [15,16,25,26], and can locate prey using their electroreceptive system alone [21,22,27]. Therefore, visual camouflage may not afford full protection against these predators and freezing may facilitate non-visual cryptic mechanisms (e.g. bioelectric crypsis) when non-visual foragers, such as sharks, are present.

Here, we show that the cuttlefish S. officinalis employs a freeze response. Due to high risk of predation by elasmobranchs [17], we hypothesized that the freeze response in S. officinalis enables protection via bioelectric crypsis. To address this hypothesis, our goals were to (i) quantify electric potentials associated with freezing and (ii) assess shark responses to Sepia-simulating weak electric fields that simulate normal (i.e. resting) and freezing behaviour.
2. Material and methods

(a) Animal collection and maintenance
Cuttlefish *S. officinalis* (mantle length 68–105 mm) were captive-hatched by Monterey Bay Aquarium (Monterey Bay, CA, USA) or aquarium suppliers and obtained as post-hatchlings. All animals were individually housed and maintained in a closed, temperature-controlled seawater system on a 12 L: 12 D cycle.

Sharks were collected via gillnet from the Florida Keys (adult bonnethead sharks, *Sphyra tiburo*; total length 45–66 cm) and Tampa Bay (juvenile blacktip sharks, *Carcharhinus limbatus*; total length 55–57 cm) and transported to Mote Marine Laboratory in Sarasota, FL, USA where they were maintained in indoor 150 000–225 000 l tanks fitted with seawater-recirculating systems. Sharks were held in captivity for one to two months and then fasted for 24 h (blacktip sharks) or 48 h (bonnethead sharks) before experiments began.

(b) Baseline voltage and frequency measurements
The electric potential (voltage) and frequency of the bioelectric field that arises from ventilation were recorded from live *S. officinalis* (*n* = 3) using electrophysiological techniques described by Bedore & Kajura [28]. Briefly, an individual cuttlefish was placed within a 15 × 15 × 8 cm compartment inside a clear acrylic experimental tank (89 × 43 × 21 cm) filled with continuously recirculating, aerated seawater. A non-polarizable Ag–AgCl recording electrode (Warner Instruments, Hamden, CT, USA) was fitted with a seawater- and agar-filled glass capillary tube (diameter: 1.5 mm) and positioned approximately 1 mm from the animal. An identical reference electrode was positioned in the corner of the internal tank wall, mantle height was measured at three points (i.e. the stimulus was at its maximum size), defined as the looming fundamental frequency. Using a 1 cm calibration mark on the internal tank wall, mantle height was measured at three points preceding the onset of the looming stimulus, defined as the resting mantle height. The mantle height during the looming stimulus was measured when the stimulus was at its maximum size, defined as the looming mantle height. For consistency in measurements on actively ventilating animals, each mantle height measurement was recorded during an ‘inhale’ phase of the breathing cycle.

Each response was scored as no response, freeze or jet (escape). ‘Freeze’ was defined as a 2 s or longer reduction of at least three of the following criteria: more than or equal to 20% in body movements, more than or equal to 5% in mantle height, and covering of the siphons, funnel or mantle cavity (cf. [13], adapted for cuttlefish behaviour). ‘No response’ was assigned to those responses when fewer than three of the criteria were met. ‘Jet’ was defined as any sudden, erratic movement made as an attempt to flee the immediate area. In preliminary analyses, there was no evidence of within-subject habituation across multiple trials (repeated measures ANOVA, *p* = 0.83; see electronic supplementary material, figure S1), so trial number was excluded as a factor in the final analysis. The 5 min inter-stimulus interval was sufficient to allow at least 3 min of full recovery before the next stimulus was presented (electronic supplementary material, figure S2), and therefore trials could be considered as independent events for further analysis. When the frequency and amplitude of body movements returned to within 1 s.d. of the pre-stimulus level, the animal was considered ‘recovered’ after the predator presentation.

(c) Experiment 1: do cuttlefish freeze in response to a looming predator stimulus?
We used a randomized block within-subjects design to quantify cuttlefish responses to looming stimuli. A cuttlefish was placed inside the experimental tank and allowed to acclimate until it rested quietly on the bottom of the tank (approx. 30 min). Looming videos were presented to cuttlefish on an iPad (v. 2; Apple, Cupertino, CA, USA) positioned along one wall of the clear experimental tank within the cuttlefish’s field of view. Details regarding video content can be found in the electronic supplementary material. For an experimental session, a cuttlefish (*n* = 4) was shown a total of seven videos in successive random order with approximately a 5 min inter-stimulus interval. Each video either served as a control (no predator) or contained a silhouette of a looming predator (see electronic supplementary material, movie S2). Every individual was used in one experimental session for a total of seven trials (one video is one trial), and all trials in which cuttlefish remained in the field of view of the camera were used in analysis (22 total looming trials).

All trials were recorded with a digital video camera at 30 frames s⁻¹ (HDR-CX260, Sony, Tokyo, Japan) positioned next to the tank, opposite of the iPad. Videos were imported into Tracker software (v. 4.81; © Douglas Brown, Open Source Physics, Cabrillo, CA, USA). A marker was positioned on the centre of the eye facing the camera, and the position of the eye was marked in every third frame using the Autotracker function (evolution rate = 10%, automark level = 6). The x−y coordinates produced by Autotracker were exported to LabChart for fast Fourier transform (FFT) analysis using 256 points and a Hamming window. The fundamental frequency of body movement and its full-width at half maximum (FWHM) were quantified for the 10 s segment immediately preceding the onset of the looming stimulus and was defined as the resting fundamental frequency. The fundamental frequency and FWHM during each looming period were quantified for a 3 s period when the looming stimulus transitioned between approach and retreat (i.e. the stimulus was at its maximum size), defined as the looming fundamental frequency. Using a 1 cm calibration mark on the internal tank wall, mantle height was measured at three points preceding the onset of the looming stimulus, defined as the resting mantle height. The mantle height during the looming stimulus was measured when the stimulus was at its maximum size, defined as the looming mantle height. For consistency in measurements on actively ventilating animals, each mantle height measurement was recorded during an ‘inhale’ phase of the breathing cycle.

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(d) Experiment 2: does the freeze response affect bioelectric field characteristics?
To determine if the freeze response reduces the frequency or voltage components of the electric field, the electric potential was recorded from cuttlefish throughout video presentations of a looming generalized fish predator (see the electronic
supplementary material for more information). Each naive cuttlefish (n = 7) was shown a total of six videos in random order; five of these videos had a looming stimulus and served as experimental stimuli, whereas one video lacked a looming predator and served as the control. The experimental protocol was identical to experiment 1, with the addition of electrical recordings. Voltage and frequency were recorded at the opening of the mantle cavity using the electrophysiology technique described in §2b.

Each cuttlefish was used in a single experimental session, which provided a total of 35 predator response sequences for analysis (n = 5 for each of seven individuals). All trials were video recorded with overhead and horizontally positioned Sony HD 1080i Handycam video cameras at 30 fps for analysis of behavioural responses. Each response was first scored as no response, freeze or jet as described for experiment 1. All freeze responses were subjected to additional analyses as follows. Body movement (frequency and mantle height) was quantified as described for experiment 1 except that looming mantle height and frequency were measured at the onset of the freeze response. Electrical data were analysed in a similar manner using waveform dynamics recorded directly in LabChart. The electrical frequency associated with ventilation was analysed using FFT analysis with 16384 points and a Hamming electrical frequency associated with ventilation was analysed to quantify voltage, the mean of the electrical potential with waveform dynamics recorded directly in LabChart. The mean voltage at the stimulus to define the resting potential. The mean voltage at the onset of the freeze response was quantified and defined as the freeze voltage. Paired t-tests were used to compare rest and freeze or jet responses to alternating current voltages have not been well characterized, so a DC stimulus was used in all trials. When the shark began to forage at the perimeter of the electrode array, a 4 µA (freeze-simulating), 6 µA (rest-simulating) or 26 µA (jet-simulating) electric current was applied randomly to one of the four electrode pairs to create a localized electric field. Electric currents were selected based on measurements recorded by Bedore & Kajiura [28] and voltage recorded in §2b. Once a shark bit at an active dipole, the electrode pair was immediately switched off and another dipole/stimulus combination was randomly activated. Trials lasted a maximum of 1 h and each shark was tested twice. Responses were recorded with a Sony HDR-CX260 digital video camera (30 frames s$^{-1}$) mounted above the centre of the array. Videos were later analysed and responses were recorded. Each time a shark approached an active dipole, its behaviour was scored as follows: 1 = no response (shark’s head broke the plane of a 20 cm reference circle on the acrylic plate, but shark did not bite at the active dipole), 2 = orient without bite (shark’s trajectory turned greater than 20° and oriented towards the active dipole, but did not demonstrate a bite response), 3 = pause (shark slowed and directed its mouth at the active dipole, but did not bite), 4 = bite (shark bit at the dipole). Response levels 1 and 2 were considered ‘no response’, whereas levels 3–4 were considered a ‘bite response’.

(e) Experiment 3: do freezing and jetting electric stimuli affect shark responses to electric fields?

A behavioural assay was employed to determine the relative responsiveness of blacktip sharks (C. limbatus, n = 9) and bonnethead sharks (S. tiburo, n = 7) to Sepia freeze, resting and jet-simulating dipole electric fields following methods by Kajiura & Holland [22]. Briefly, four 1 cm electric dipoles, each created by a pair of underwater electrodes, were equally spaced on a 76 × 91 cm clear acrylic plate (see electronic supplementary material, movie S2). The four electrodes were connected to a current-regulated electric stimulator powered by a 9 V battery with a multimeter (27/FM, Fluke Corporation, Everett, WA, USA) connected in series to monitor the applied current. Elasmobranch responses to alternating current voltages have not been well characterized, so a DC stimulus was used in all trials. When the shark began to forage at the perimeter of the electrode array, a 4 µA (freeze-simulating), 6 µA (rest-simulating) or 26 µA (jet-simulating) electric current was applied randomly to one of the four electrode pairs to create a localized electric field. Electric currents were selected based on measurements recorded by Bedore & Kajiura [28] and voltage recorded in §2b. Once a shark bit at an active dipole, the electrode pair was immediately switched off and another dipole/stimulus combination was randomly activated. Trials lasted a maximum of 1 h and each shark was tested twice. Responses were recorded with a Sony HDR-CX260 digital video camera (30 frames s$^{-1}$) mounted above the centre of the array. Videos were later analysed and responses were recorded. Each time a shark approached an active dipole, its behaviour was scored as follows: 1 = no response (shark’s head broke the plane of a 20 cm reference circle on the acrylic plate, but shark did not bite at the active dipole), 2 = orient without bite (shark’s trajectory turned greater than 20° and oriented towards the active dipole, but did not demonstrate a bite response), 3 = pause (shark slowed and directed its mouth at the active dipole, but did not bite), 4 = bite (shark bit at the dipole). Response levels 1 and 2 were considered ‘no response’, whereas levels 3–4 were considered a ‘bite response’.
of body movements during ventilation and deimatic displays. Jetting did not occur in any trial.

(c) Experiment 2: bioelectric crypsis
Cuttlefish demonstrated both freezing and jetting (fleeing) behaviours, although jetting was only elicited in two of the predator presentations and in response to overhead looming stimuli (i.e. experimenter movement during electrode positioning). Freeze responses occurred in the remaining 32 of the 34 trials used in statistical analyses (figure 3). The freeze response significantly decreased both voltage and frequency (figure 4; paired t-tests, \( p < 0.01 \); electronic supplementary material, figure S3), and significantly decreased mantle height and the frequency of ventilation-associated body movements (figure 4; paired t-tests, \( p < 0.01 \)) as described in experiment 1 (electronic supplementary material, movie S1). The amplitude of voltage fluctuations and body movements was also reduced by 16.2 \( \pm 3.7\% \) and 42.3 \( \pm 6.2\% \), respectively (mean \( \pm \text{s.e.} \)).

(d) Shark detection of electric fields
Both shark species readily exhibited bite responses to all stimulus magnitudes (figure 5; \( n = 346 \) total bite responses; 200 from seven bonnethead sharks, \( S. \ tiburo \), and 146 from nine blacktip sharks, \( C. \ limbatus \)). Although blacktip sharks responded to rest-simulating electric fields less often than bonnethead sharks (ANCOVA, \( F = 14.5, p = 0.0005 \)), both species demonstrated fewer bites to freeze-simulating electric fields with a mean reduction of approximately 50% compared with the resting-state stimulus (ANCOVA, \( F = 23.8, p < 0.0001 \)). Jet-simulating stimuli elicited bite responses by both species in nearly all of the trials. The median detection distances were 5.4, 8.0 and 10.6 cm for freeze, rest and jet stimuli, respectively. Maximum detection distances were 15.5, 20.3 and 38.5 cm for freeze, rest and jet stimuli, respectively.

4. Discussion
Cryptic species that face an approaching predator generally have two decisions: freeze or flee [9,12,29]. Because fleeing may alert a predator that has not yet detected the cryptic prey, freezing may be considered the more favourable option [29]. Perhaps the most apparent characteristic of the freeze response is the reduction in movement that breaks the camouflage of background-matching organisms [30]. Freezing also maximizes crypsis of non-visual signals that are generated by movement [12]. For example, voles that freeze in response to calls of predatory owls reduce auditory cues produced from movement in the grasses that they inhabit [12,31]. Most studies of freezing as a mechanism of crypsis consider only its effects on motion and sound [12,13,32]. However, freezing in juvenile and embryonic elasmobranchs has been hypothesized to reduce the bioelectric field used by foraging elasmobranchs to locate and identify prey [33–35].

Electric fields in aquatic animals arise from ion exchange at mucous membranes and respiratory structures that directly contact the seawater, which contributes a baseline DC voltage. Rhythmic pumping of water over the gills temporally modulates this DC voltage, resulting in a fluctuating (AC) voltage [28]. In laboratory studies, such as those used here,
elasmobranch predators readily respond to weak dipole electric fields that simulate prey DC voltage with a feeding strike. These strikes are initiated within 50 cm of the dipole and demonstrate a sensitivity of less than 1 nV cm\(^{-1}\) [21,22]. Further, the stimuli used in prey-simulating behavioural assays are purely electric, indicating that electroreceptive systems can be used to precisely localize prey when no other cues are present [21,22].

Together with predatory teleosts and cephalopods, elasmobranchs represent a primary predator group of *S. officinalis* [15–18,20]. Most species of elasmobranchs are thought to rely more heavily on non-visual senses, such as electroreception and olfaction, to localize individual prey items and initiate a feeding strike [36,37]. We show here that *S. officinalis* uses a freeze response that reduces bioelectric signals arising at the gills (figures 3 and 4). Reductions in voltage recorded by our equipment during freeze responses probably represent a conservative estimate of electric potential reduction because the recording electrode was positioned inside the opening of the mantle cavity (electronic supplementary material, movie S1). Cuttlefish either constricted or covered their cavity openings in 91% of freeze responses (electronic supplementary material, movies S1 and S2). Skin is relatively impermeable to ion diffusion, making it electrically resistive, and therefore an insulator of bioelectric fields [28]. Voltage recordings adjacent to the mantle opening revealed that voltage was reduced by up to 89% when these openings were covered by mantle or arm tissue (figure 1). Our results suggest that in addition to the passive reduction in the electric field that occurred with reductions in ventilation, *S. officinalis* further reduced propagation of electric cues by actively insulating their ion-leaking structures in the presence of certain predators. Occlusion of the gill-associated cavities (i.e. the siphons and mantle cavity) during the freeze response occurred almost exclusively in the presence of fish stimuli (figure 2; electronic supplementary material, movie S2; binomial GLM, \(p < 0.0001\), suggesting that active insulation of these cavities is important in preventing detection by predatory fishes. Although the specific cues that elicit covering behaviour were not tested here, previous studies have described predator-specific behaviours in the longfin squid *L. pealei* [9] and *S. officinalis* [4,8]. Such cryptic behaviours have been detailed in numerous species of colour-changing and camouflaged species (see [38] for review).

We propose that *S. officinalis* occluded these orifices to maximize their crypsis to approaching predators in which it would be most beneficial. It is generally assumed that predator–prey interactions between sedentary, cryptic prey and their larger, cruising predators begin when the prey detects the predator [29]. As a shark predator closes the distance between itself and cuttlefish prey, the chance that the cuttlefish will be detected increases because stimuli are distance-dependent such that the signal strengthens with decreasing distance. In this case, all shark detections of rest stimuli occurred within a 20 cm radius of the dipole and resulted in feeding strikes in 62% of trials in which they were encountered. The freeze stimulus reduced shark detection distance by approximately 5 cm and reduced responsiveness to 30% (figure 5). Conversely, jetting (fleeing) cuttlefish stimuli were detected in 94% of trials and were detected up to 38 cm away. These results collectively suggest that fleeing from sharks is a riskier option for cuttlefish. Although inking that often occurs with jetting in cephalopods chemically deters certain predators from initiating an attack [39], the shark species in this study were chemically attracted to and readily consumed ink products from *S. officinalis* (C. Bedore 2013, personal data).

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**Figure 3.** Representative response of *S. officinalis* to a video simulation of a looming predator. Both the frequency and amplitude of body movements and bioelectric cues were reduced by the freeze response. Amplitude data are normalized to the peak point in each trace for presentation purposes. Cuttlefish illustrations depict the typical camouflage and mantle cavity opening states for each phase of the recording. Rest = quiescent, non-active animals. The gills were exposed laterally at the mantle cavity opening at the junction between the mantle and the head. Freeze = response characterized by motionlessness, flattening of the body, covering of the gills and reductions in the amplitude and frequency of electrical cues. Recovery = transition from freeze response to the resting state. Camouflage and the amplitude and frequency of body movements and electric potential returned to within 1 s.d. of the resting state at the beginning of the recovery period. Resting mantle height and baseline voltage returned to within 1 s.d. of resting at the end of the recovery period (not shown). Black/primary y-axis = body movement/motion, grey/secondary y-axis = electric potential/voltage.
Blacktip sharks were generally less responsive to electric stimuli than bonnethead sharks (figure 5). The difference in responsiveness is likely to be due to differences in foraging strategy, which may result in differences between species with regard to the role of electroreception in prey detection. Bonnethead sharks are primarily benthic foragers [20,26,37] and search for food almost exclusively along the bottom, where the experimental array was placed. Juvenile blacktip sharks, on the other hand, are benthopelagic foragers [25,37] and frequently feed at the water surface in captivity.

Although our results suggest robust responses both by _S. officinalis_ responding to predator stimuli and by shark responses to _Sepia_ -simulating electric fields, no laboratory experiment is without limitations. The baseline ventilatory parameters used to define resting values may not precisely represent that of a cuttlefish in its natural habitat; the physical constraints of the experimental tank and lack of appropriate substrate for burying unavoidably impart stress on experimental animals, which consequently may inflate ventilatory frequency and amplitude of resting animals (see electronic supplementary material, figure S1). It may seem intuitive that the exaggerated ventilation necessarily increases electric potentials. However, voltage does not arise from active ventilation itself; rather, it is a product of osmoregulation at the gill surface, and the resulting charge is then propagated away from the gills in water currents resulting from the pumping behaviour during ventilation. In previous work that quantified electric potentials of aquatic invertebrates and fishes [28,40], data were carefully inspected for interactions among voltage, frequency and stress level (indicated by ventilatory rate). Data for the current work were subjected to similar scrutiny. No correlations were present in any of the datasets (electronic supplementary material, figure S1), indicating that voltage was not affected by stress in these experiments.

We are also limited in our predictions of predation risk in natural settings due to isolation of electric signals in an otherwise complex predator–prey system. Although the combined effectiveness of visual and bioelectric crypsis for decreasing detection by shark predators was not tested here, we expect the contribution of visual cues to a foraging shark to be minimal. Together with low spatial resolving power (2–11 cycles/degree) [41], a large blind area in front of the head produced by lateral placement of the eyes [42], and lack of colour vision [41,43] visual detection of cryptic prey would be difficult for most sharks. The functional range of the electroreceptive system overlaps with that of the visual blind area that extends several centimetres in front of shark heads [42], so most sharks presumably rely on electroreceptive input at close range. Because elasmobranchs place substantial predation pressure on cephalopods, bioelectric crypsis may afford cephalopods extra protection when under threat from nearby foraging sharks. Freezing in cephalopods may also decrease longer-range chemical cues, which are used by most elasmobranch species during the initial stages of prey searching [36]. These modalities should be evaluated in future studies for their contribution to detection of cryptic prey by elasmobranchs.

**Ethics.** All work was conducted in accordance with Duke University and Mote Marine Laboratory IACUC-approved protocols (Duke A221-13-08 and A226-13-08; MML 13-05-CB1).
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