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# Electrophysiological and Motor Responses to Chemosensory Stimuli in Isolated Cephalopod Arms

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**Abstract.** While there is behavioral and anatomical evidence that coleoid cephalopods use their arms to "taste" substances in the environment, the neurophysiology of chemosensation has been largely unexamined. The range and sensitivity of detectable chemosensory stimuli, and the processing of chemosensory information, are unknown. To begin to address these issues, we developed a technique for recording neurophysiological responses from isolated arms, allowing us to test responses to biologically relevant stimuli. We tested arms from both a pelagic species (*Doryteuthis pealeii*) and a benthic species (*Octopus bimaculoides*) by attaching a suction electrode to the axial nerve cord to record neural activity in response to chemical stimuli. *Doryteuthis pealeii* arms showed anecdotal responses to some stimuli but generally did not tolerate the preparation; tissue was nonviable within minutes *ex vivo*. *Octopus bimaculoides* arms were used successfully, with tissue remaining healthy and responsive for several hours. Arms responded strongly to fish skin extract, glycine, methionine, and conspecific skin extract but not to cephalopod ink or seawater controls. Motor responses were also observed in response to detected stimuli. These results suggest that chemosensory receptor cells on *O. bimaculoides* arms were able to detect environmentally relevant chemicals and drive local motor responses within the arm. Further exploration of potential chemical stimuli for *O. bimaculoides* arms, as well as investigations into the neural processing within the arm, could enhance our understanding of how this species uses its arms to explore its environment. While not successful in *D. pealeii*, this technique could be attempted with other cephalopod species, as comparative questions remain of interest.

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*Abbreviations*: ASW, artificial seawater; FSW; filtered seawater; MBL, Marine Biological Laboratory.

*Online enhancement*: video.

## Introduction

Coleoid cephalopods, the soft-bodied octopods and decapods that make up the vast majority of extant cephalopods, have complex nervous systems with remarkable cognitive and sensory abilities that evolved separately from vertebrate nervous systems (Budelmann, 1995, 1996; Mather and Kuba, 2013; Hanlon and Messenger, 2018). Their distinct genetics and neural organization, therefore, make them fascinating organisms to study (Albertin *et al.*, 2015; Liscovitch-Brauer *et al.*, 2017; O'Brien *et al.*, 2018). The coleoid cephalopods that have been examined for chemosensory abilities, including various species of octopus, squid, and cuttlefish, detect chemical cues in their environment through multiple chemosensory organs, including olfactory pits located near the eye (Woodhams and Messenger, 1974; Gilly and Lucero, 1992; Budelmann, 1996; Mobley *et al.*, 2008; Polese *et al.*, 2016), chemoreceptors on the lips and mouth parts (Graziadei, 1965; Emery, 1975), and chemoreceptors on the suckers of the arms and tentacles (Graziadei, 1962, 1964b; Santi, 1975).

Chemosensation in the arms and tentacles is particularly interesting because of the organization of the axial nerve cords that run down the length of each. Unlike vertebrate peripheral nerves, which consist only of axons, the axial nerve cords that run down each arm or tentacle consist of a continuous series of ganglia (apparent from swellings along the nerve cord), with extensive local circuitry (including sucker ganglia that lie outside the nerve cord; for diagrams see Graziadei, 1962; Rowell, 1963), resembling a spinal cord more than a nerve (Graziadei, 1962; Rowell, 1963; Budelmann and Young, 1985; Mather and Kuba, 2013; Hanlon and Messenger, 2018). This underlies the relative autonomy of arm function, where local sensory information can evoke motor responses within an arm even

when disconnected from the brain or other arms (*e.g.*, Rowell, 1963; Nesher *et al.*, 2014). Afferent pathways also send sensory information to the brain, where responses can be coordinated across arms and behavioral patterns altered based on additional sensory input, such as from the visual system (Budelmann and Young, 1987; Budelmann, 1995; Hanlon and Messenger, 2018).

Morphology and function of cephalopod arms and tentacles vary considerably between species (Budelmann, 1996; Hanlon and Messenger, 2018); thus, the extent and function of chemosensation in arms and tentacles surely vary as well. Cephalopods are diverse, occupying many different ecological niches within the ocean that place different demands on their sensory systems (Budelmann, 1996; Nixon and Young, 2003; Hanlon and Messenger, 2018). Benthic octopods, such as *Octopus bimaculoides* used in this study, forage for food by reaching their arms into crevices and under rocks and using chemosensation and somatosensation to identify prey (Budelmann, 1996; Hanlon and Messenger, 2018). During foraging, cuttlefish are known to manipulate their environment with their arms, such as moving sand around or jetting water to expose prey, which requires recognition of their prey and the associated environment and which could involve chemosensation (Mather and Kuba, 2013). Pelagic squid, such as *Doryteuthis pealeii* studied here, use vision as the dominant sense for hunting; but they use their arms to grasp prey, as well as in reproductive interactions (Hanlon *et al.*, 2013; Hanlon and Messenger, 2018). Thus, the density and specificity of chemoreceptors would be expected to vary between these species. Indeed, anatomic descriptions of putative chemoreceptive cells on suckers report higher densities of such cells on the suckers of benthic octopods than pelagic squid (Graziadei, 1962, 1964a; Santi, 1975).

When vision is unavailable, octopus foraging behavior is dependent on arm chemotactile sensation, reaching into dark crevices and probing other parts of their surroundings (Budelmann, 1996; Walderon *et al.*, 2011; Mather and Kuba, 2013). In behavioral chemosensation studies, octopuses have been shown to distinguish between hydrochloric acid, quinine, and sucrose, indicating "taste-by-touch" chemosensory capabilities (Wells, 1963). Octopuses can also detect and respond to chemicals dissolved in seawater, although the anatomic location of the chemoreceptors is unclear in these studies (Chase and Wells, 1986; Walderon *et al.*, 2011). Additionally, *O. vulgaris* was shown to use chemosensory cues at its suckers to prevent self-attachment, which is essential for an octopus in order to avoid self-entanglement (Nesher *et al.*, 2014).

There is also evidence that squid have specialized chemosensory functions in their arms. A protein found on *D. pealeii* egg casings has been shown to trigger aggressive behaviors in *D. pealeii* males when they touch the eggs with their arms (Cummins *et al.*, 2011; Hanlon *et al.*, 2013). This pheromonal signal seems to be mediated by chemosensory receptors on the arms or suckers of the males, although no receptor or response pathway has been identified yet. Squid touch conspecifics with their arms in aggressive and reproductive contexts, as well as touching prey items (Budelmann, 1996; Hanlon *et al.*, 2013; Hanlon and Messenger, 2018). The possibility that arm chemoreceptors are used in such interactions has yet to be explored. Ink, which can act as a conspecific alarm cue in cephalopods (Gilly and Lucero, 1992; Wood *et al.*, 2008; Staudinger *et al.*, 2011; Derby, 2014), has been shown to trigger responses in chemosensory receptor cells in the olfactory pits of *Doryteuthis opalescens* (Lucero *et al.*, 1992); but it is not known whether it is also detected by chemoreceptors on other parts of the body.

Despite initial observations of sucker chemoreceptors decades ago (Graziadei, 1962, 1964b; Santi, 1975), our understanding of how different cephalopods use sucker chemosensation remains sparse. In contrast to research on mechanoreception and muscular control (Gutfreund *et al.*, 2006; Sumbre *et al.*, 2006; Kier, 2016), there is little known about the physiological coding and neural processing of chemical information. In this study, the physiological and behavioral responses to chemical stimuli were recorded from isolated arms from *D. pealeii* (longfin inshore squid) (formerly known as *Loligo pealeii* Lesueur, 1821) and *O. bimaculoides* (California two-spot octopus) (Pickford and McConnaughey, 1949). We tested stimuli inspired by previous studies, including amino acids, fish skin extract, *D. pealeii* egg casing extract, *O. bimaculoides* skin extract, *D. pealeii* ink, and *O. bimaculoides* ink (Chase and Wells, 1986; Lucero *et al.*, 1992; Mobley *et al.*, 2008; Wood *et al.*, 2008; Cummins *et al.*, 2011; Walderon *et al.*, 2011; Hassenklover *et al.*, 2012; Nesher *et al.*, 2014). We hoped to establish an isolated arm protocol that would enable further research, and we hypothesized that both cephalopods would sense and respond to environmentally relevant chemicals through arm chemoreceptors.

## Materials and Methods

### *Experimental animals*

Adult longfin inshore squid (*Doryteuthis (Amerigo) pealeii* (Lesueur, 1821)) were collected by trawl from the Vineyard Sound near Woods Hole, Massachusetts, by the Marine Biological Laboratory (MBL) *Gemma* vessel between June 12 and August 9, 2018. Both male and female squid were used, for a total of 20 individuals. The animals were maintained in large collection tanks with oxygenated natural seawater that varied with ambient sea temperature (18–20 °C) and pH (7.5–8.5). California two-spot octopus (*Octopus bimaculoides* Pickford & McConnaughey, 1949) were reared by the Cephalopod Breeding Initiative at the MBL. Nine juveniles aged 40–70 days (0.95–3 g), sex not determined, were used for experiments. The octopuses were individually housed in plastic tanks with plastic partitions

and continuously circulating, oxygenated filtered seawater (FSW) kept at 24 °C, 34 ppt salinity, and pH ~8.1. The octopuses were maintained in 12h : 12h dark : light cycles and were fed a diverse diet of crustaceans, fish, and snails between 10:00 and 16:00. To ensure the ethical and humane treatment of animals, all animal handling and experimental procedures were conducted at the MBL, in accordance with MBL regulations, in consultation with MBL veterinarian Lisa Abbo.

### *Physiological solutions and chemical stimuli*

For all octopus and some squid experiments, arms were bathed in, and stimuli were created with, FSW. For some squid experiments, artificial seawater (ASW) was created using Crystal Sea Marine Mix (Marine Enterprises International, Baltimore, MD) and deionized water. Stimuli applied in *D. pealeii* experiments included 1 mmol $L^{-1}$ L-methionine in ASW or FSW; 1 : 10 homogenized fish skin extract in FSW; 20 mmol $L^{-1}$ glycine in ASW or FSW; and homogenized *D. pealeii* egg casing extract in FSW. Stimuli used in *O. bimaculoides* experiments included 1 : 10 homogenized fish skin extract in FSW; 1 mmol $L^{-1}$ L-methionine in FSW; 20 mmol $L^{-1}$ glycine in FSW; *D. pealeii* ink in home tank water (natural seawater); homogenized *O. bimaculoides* skin extract in FSW; and *O. bimaculoides* ink in home tank water (FSW). Concentrations of amino acid stimuli were based on a review of related literature (Chase and Wells, 1986; Mobley *et al.*, 2008; Wood *et al.*, 2008; Hassenklover *et al.*, 2012) but increased slightly from reported values to account for the rapid (and uncontrolled) dilution of stimuli into the bath. Ink was collected by carefully pipetting freshly released ink from the home tanks of *D. pealeii* and *O. bimaculoides* when the organisms expelled ink in response to capture or handling. *Octopus bimaculoides* did not ink often, which delayed our ability to collect this stimulus; only later experiments used *O. bimaculoides* ink, and *D. pealeii* ink was used in earlier experiments. Fish skin (species unknown, obtained from chopped fish used as food for marine organisms at the MBL), *D. pealeii* egg case, and *O. bimaculoides* skin (taken from arms used in prior experiments as well as one euthanized octopus mantle, samples from multiple animals combined) were cut into small pieces and homogenized separately into FSW with a manual glass homogenizer. Because it took time to collect octopus skin, only later experiments used this stimulus. All stimuli, including ASW and FSW controls, were made in batches, aliquoted, and kept frozen (−20 °C) until use. Osmolality of stimuli was tested using a freezing point osmometer; and samples were found to be comparable to FSW, with the exception of the fish skin extract, which had a slightly lower osmolality (~850 mOsm). We diluted FSW with deionized water to create a comparable low-osmolality control, which was tested on three octopus arms and did not evoke a response (similar to FSW controls; data not shown).

### Doryteuthis pealeii *arm preparation*

To isolate arms from the squid body for experimentation, squid were euthanized by decapitation between the mantle and eyes, followed immediately by decerebration (a series of cuts through the brain between the eyes with surgical scissors). A segment of one arm was then removed; placed in oxygenated, cooled ASW or FSW for experimentation; and attached to an electrode. The entire procedure typically took three to five minutes. The head with attached arms was then transferred to home tank seawater and kept on ice, and additional arms were sometimes amputated later for additional trials.

The removed arms were pinned down onto a Sylgard-coated 50-mm petri dish filled with 12–14 mL ASW or FSW. Cold ASW or FSW was perfused over the arm at 3–4 mL $min^{-1}$. For some experiments, seawater was oxygenated by bubbling $O_2$ through an air stone in the bath reservoir; different bath temperatures were tried in different experiments, ranging from 6 °C to 22 °C. For most experiments, a plastic suction electrode, containing a silver chloride wire and filled with bath seawater, was attached to the nerve cord by drawing the cut end of the axial nerve into the pipette; a second silver chloride wire attached to the outside of the pipette served as the reference electrode. For a subset of experiments, two metal pin electrodes were pinned through the axial nerve cord about 1 cm apart.

### Octopus bimaculoides *arm preparation*

Nine octopuses were anesthetized, and arms were surgically removed for experimentation. Animals were anesthetized in 1.5% ethanol in FSW for 8–10 min (L. Abbo, Marine Biological Laboratory, pers. comm.). Depth of anesthesia was assessed by animal coloration, ventilation rate, and response to arm pinch. Once an animal was adequately anesthetized, one-half to two-thirds of the arm was removed with surgical scissors and placed in oxygenated ambient-temperature FSW. One to three arms were taken from each octopus. One octopus was euthanized after amputation by incremental increases of 2% ethanol in FSW every 2 min for 16 min total (mantle skin from this animal was used to generate stimuli; other tissue was used for separate anatomy experiments). All other anesthetized octopuses were transferred to FSW immediately after amputation for recovery. All recovered animals resumed normal ventilation rate, color, and behavior within 10 minutes and exhibited typical locomotion and feeding behavior after the experiment. Arm regeneration was observed to begin within days after the lesion.

Each arm was transferred to a Sylgard-coated 50-mm petri dish filled with 12–14 mL FSW. The exposed axial nerve cord protruded from the surrounding tissue upon amputation of each arm and was drawn into the tip of a suction electrode (as previously described for *Doryteuthis pealeii* arm preparation). Ambient-temperature (~22–23 °C) oxygenated FSW was perfused over the arm at a rate of 3–4 mL $min^{-1}$ once the electrode was successfully attached. If more than one arm was removed

from an animal, amputated arms were kept in oxygenated FSW until use. Time from amputation to first stimulus ranged from 6 min to 1 h 40 min.

*Electrophysiological recordings and stimulus delivery*

For both squid and octopus experiments, differential recordings were amplified with an A-M Systems (Sequim, WA) head stage and 1800 amplifier (gain = 1000x; filtration: low cutoff = 10 Hz, high cutoff = 1 kHz, notch filter in), digitized with a Digidata Micro 1401 (CED, Cambridge, UK), and continuously recorded at a rate of 10 kHz with Spike2 software (CED).

Each experiment began with tactile stimulation by manually pinching the distal end of the arm with plastic forceps to ensure that the experimental instrumentation worked and the arm tissue was responsive; the pinch response was used as a positive control. Chemical stimuli were administered manually by gently pipetting 5 $\mu$L of the stimulus into the bath ~3–5 mm away from the suckers (see supplemental video, available online, for an example of stimulus delivery).

For *O. bimaculoides* experiments, each trial proceeded as follows. The bath perfusion was paused, then a FSW stimulus was applied. Twenty (or more) seconds after the FSW stimulus, a randomly selected test stimulus was applied from the following set of test stimuli: fish skin extract, methionine, glycine, *D. pealeii* ink, *O. bimaculoides* skin extract, and *O. bimaculoides* ink. After 20 s of exposure, the bath perfusion was resumed at a high rate (15–20 mL $min^{-1}$) for at least 90 s to wash out the stimulus. During pilot experiments, clearing of dye "stimuli" (monitored visually) and concentrated saltwater "stimuli" (monitored with an osmometer) was achieved after 30–60 s of perfusion; we selected a 90-s-minimum wash to be conservative. This process was repeated, starting with a FSW control, until all stimuli in the set had been tested, thus completing the trial. We ran three trials on each arm, randomizing the order of the stimuli for each trial. Occasionally during an experiment, we noted an error in stimulus delivery or a spontaneous burst of activity in the nerve just before stimulus application (often associated with a spontaneous arm movement). In those cases, the single affected stimulus exposure was disregarded in further analyses. The stimulus was repeated later in the trial if time allowed. This occurred in about 3% of stimulus applications.

For experiments with *D. pealeii*, trial structure was not systematic because of the lack of a reliable preparation. Six octopus arm preparations were used for pilot experiments during which we developed our procedures; they did not follow this exact protocol and are not included in analyses.

*Electrophysiological analyses*

Nerve recordings in *O. bimaculoides* experiments measured the activity of a large population of axons in the axial nerve cord. While single units were occasionally discernible in spontaneous activity, evoked responses clearly reflected the sum of activity of a large population. To quantify the neural activity, the mean and standard deviation (SD) for each full trial were calculated; since the vast majority of the trial was made up of wash and rest periods between stimuli, the mean accurately represented the baseline, and SD approximated background noise. Thresholds at ±3 SD were then applied to the recordings. Each time the trace crossed the threshold it was counted as an event. Events were binned by seconds post-stimulus and averaged across trials of the same stimulus within each arm to examine the time course of the response. Then the total number of events in the first 10 seconds after each stimulus was analyzed statistically to compare test stimuli to the FSW control.

Stimuli that were used in all 15 experiments (FSW, pinch, amino acids, and fish skin extract) were analyzed using a repeated-measures design to account for variation in excitability or quality of recordings between arms. Specifically, a series of pairwise Wilcoxon tests (JMP Pro14, SAS Institute, Cary, NC) was conducted to analyze the number of events in the first 10 seconds, comparing each test stimulus (pinch, methionine, glycine, or fish skin extract) to FSW. A Bonferroni correction was applied to account for multiple comparisons, $\alpha = 0.0125$. In this and other analyses, non-parametric tests were selected because of relatively low sample sizes.

Cephalopod ink and skin stimuli were not tested in all 15 arms, so these data were analyzed with a Kruskal-Wallis test followed by Dunn's control test (JMP Pro14) to compare the responses to these stimuli with the responses to FSW from the same subset of experiments.

*Video recording and analyses*

A webcam was mounted adjacent to the bath used for physiological recordings and was used to record video of stimulus administration and arm response for most (9 of 15) octopus experiments. Video resolution was 640 × 480, 30 frames $s^{-1}$, and video was synchronized to the electrophysiological recording by using the multimedia feature in Spike2 software (CED). Video was used to confirm stimulus application timing when needed, as well as to observe any gross motor responses by the arm.

Motor responses were quantified during the 10 seconds immediately following stimulus administration by a practiced observer, blinded to stimulus identification. The movement was scored on a 0 to 4 scale according to the following criteria: 0, no movement; 1, a discrete twitch; 2, some region of the arm tightened or contracted, but the arm stayed in the same basic coil or orientation; 3, the whole or a large portion of the arm contracted and wriggled or changed shape or orientation; 4, the whole arm dramatically flipped, extended, or whipped about (for examples of movements scored as 0 and 3, see supplemental video, available online). This scale was meant to capture both the intensity and the complexity of the motion, considering the entire 10-second window; the maximal movement during the 10 seconds is reflected in the score. Mean movement scores for each stimulus type were calculated for each arm; and then

scores for test stimuli were compared to scores for FSW and pinch controls with a Kruskal-Wallis test, followed by a Dunn's *post hoc* control test (JMP Pro14). This non-parametric analysis was selected because of small sample sizes and non-normal distribution of movement score data.

The timing and duration of motion were recorded by noting whether the arm was moving or stationary during each of five two-second bins following stimulus application. For this quantification, any movement (small or large) resulted in a score of 1 (moving), while no visible movement resulted in a score of 0 (stationary). For each two-second time bin in which movement was assessed, the percent of trials with movement was calculated for each experiment. These data were then used to create a heat map of responses over time and stimulus type.

To assess whether arm movement in response to stimuli was correlated with the electrophysiological response to stimuli, movement scores and movement "duration" (the number of two-second bins that showed visible movement) for individual trials were compared with the number of events generated in the physiological recordings of those same trials. The Kendall rank correlation test (a non-parametric test suitable for ordinal data) was used to determine correlation coefficients (Kendall's tau) between each movement variable and the number of electrophysiological events, as well as between the two movement variables (JMP Pro14).

## Results

### Doryteuthis pealeii *experiments*

Squid arms did not remain viable over the course of the experiment, as indicated by a rapid decline in both spontaneous and evoked activity in nerve recordings across the first few minutes of the experiment (Fig. 1). The deterioration of arm physiology was further apparent in gross observations of the arms, with arm movement stopping within the first 10 minutes and the tissue becoming opaque shortly thereafter. Numerous adjustments were made to the experimental procedures, including selecting only the healthiest-looking squid, changing the bath temperature (temperatures from 6 °C to 22 °C were tried), oxygenating the bath, changing the type of electrodes (suction or pin electrodes), and changing the source of the bath water (FSW or ASW); no change produced any significant improvement in the preparation. Of the 20 squid used, only 5 experiments showed notable responses to pinch and chemical stimuli. In some of these experiments, fish skin extract or amino acids did elicit a response that appeared to be greater than the seawater control stimuli (*e.g.*, Fig. 1). Yet, even in the most responsive arms, recordings were unsuccessful after a short amount of time post-amputation (Fig. 1). Thus, no systematic analyses could be performed.

### Octopus bimaculoides *experiments*

Octopus arms were generally healthy, showing both spontaneous and evoked motor activity, as well as electrophysiological responses to various stimuli, for several hours after amputation (Fig. 2; supplemental video, available online). It was common to see arms and suckers move after stimuli were applied; sometimes they showed very small movements (twitches) and other times large movements that involved the full arm bending or extending. Suckers were also observed to extend toward stimuli on some trials.

The time course of responses varied between stimuli (Fig. 3A). Pinch stimuli evoked a strong but short-lived response, with most events occurring in the first one to three seconds. Amino acids also evoked a rapid response, with the highest average rates of events in the early time bins but a slightly longer time course, with responses consistently lasting for three to five seconds. Fish skin and octopus skin extracts evoked the

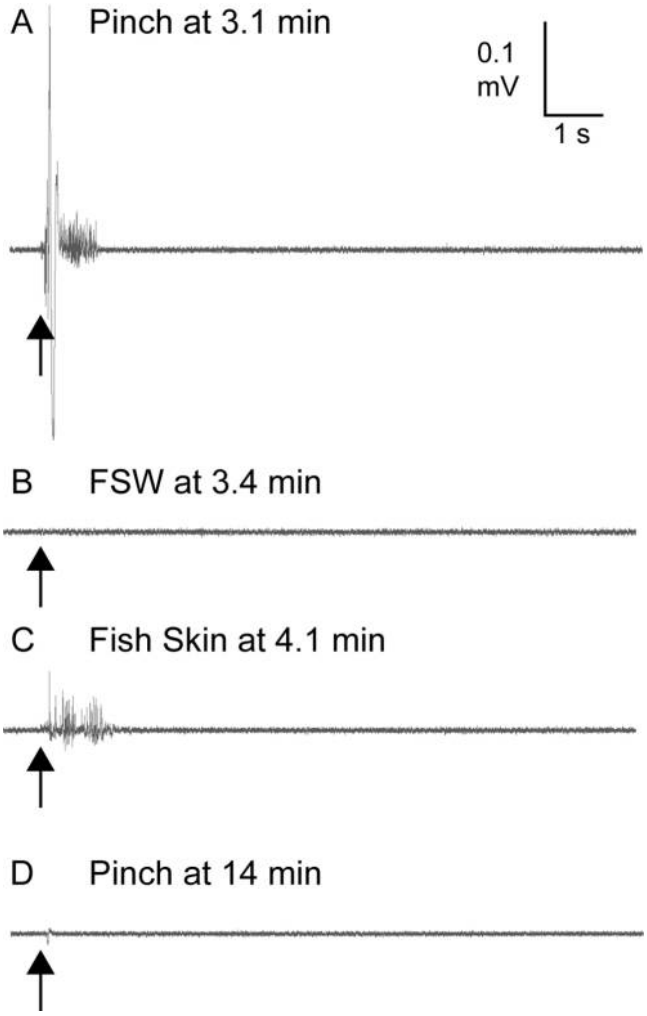


**Figure 1.** *Doryteuthis pealeii* arms did respond to stimuli in some cases, but responsiveness did not persist. Sample recordings from the axial nerve cord in one *D. pealeii* arm show activity (mV) over time (s). Arrows indicate start time of stimulus administration, 0.5 s into each trace. (A) Response to pinch stimulation at 3.1 min post-amputation. (B) No response to control (filtered seawater [FSW]) stimulus at 3.4 min post-amputation. (C) Response to fish skin at 4.1 min post-amputation. (D) Little to no response to pinch stimulation at 14 min post-amputation.

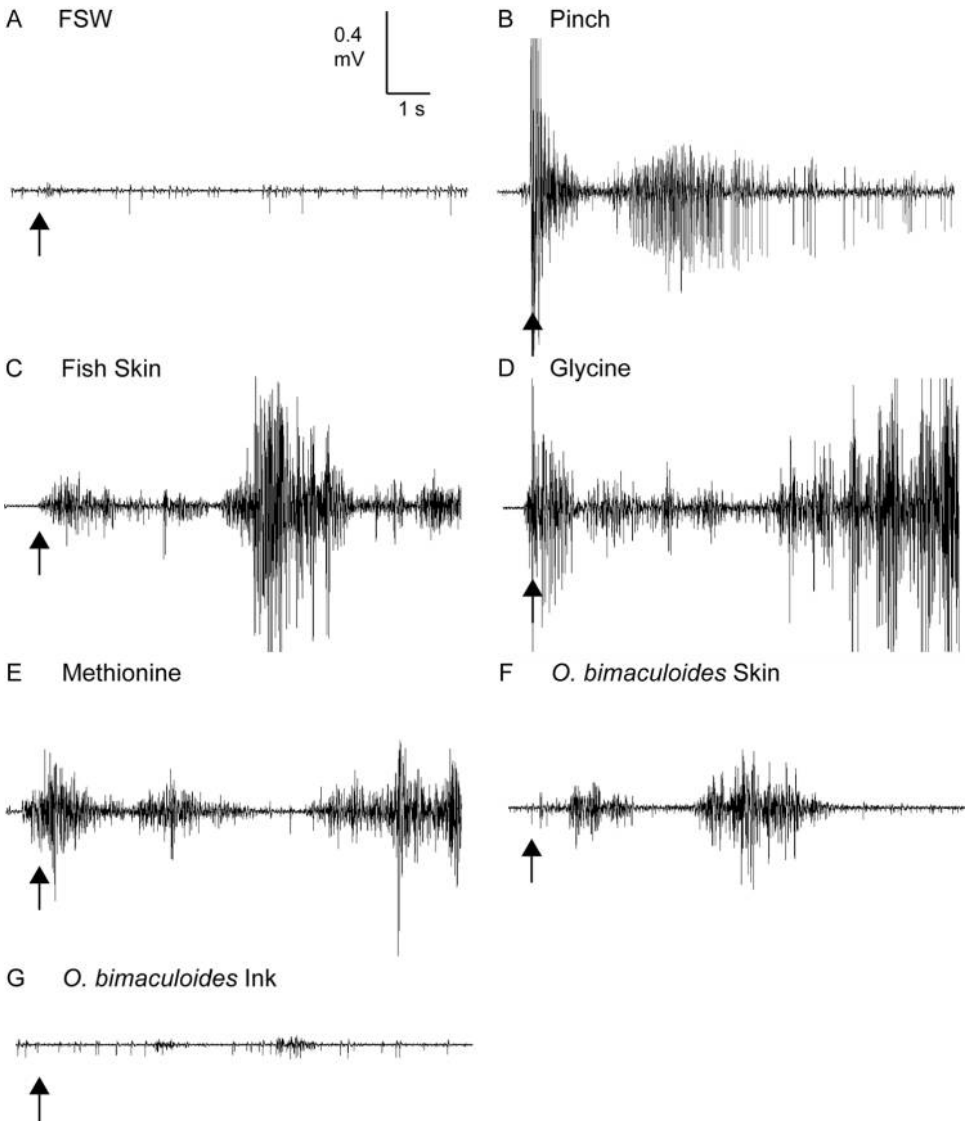


**Figure 2.** Axial nerve recordings show considerable activity in response to chemosensory stimuli and pinch in a representative *Octopus bimaculoides* arm. Electrical nerve cord activity (mV) was measured over time (s). Arrows indicate the time of stimulus administration, 0.5 s into each trace. Representative recordings are all from a single arm. Test stimuli were (A) filtered seawater (FSW), (B) pinch, (C) fish skin extract, (D) glycine, (E) methionine, (F) *O. bimaculoides* skin extract, and (G) *O. bimaculoides* ink.

most prolonged responses, typically lasting six to eight seconds. FSW and cephalopod inks evoked little to no response.

Arm movement timing was quantified in 2-s bins across the first 10 s after stimulus exposure for a subset of experiments, and we found a similar time course of response to the various stimuli (Fig. 3B). Filtered seawater and cephalopod ink evoked few movements, while pinch reliably evoked rapid movements that were not typically sustained. Fish skin extract evoked movement that was both reliable and lasting, while the amino acids produced a response that was still robust but slightly less consistent and shorter lived. The sample sizes for octopus skin and cephalopod ink stimuli were small ( just three experiments), but octopus skin data suggest a somewhat delayed or prolonged response.

Collapsing electrophysiological events across the first 10 seconds after stimulus delivery, we found that pinch, methionine, glycine, and fish skin extract produced significantly greater responses than FSW (pairwise Wilcoxon tests, $n = 15$, Bonferroni correction, $P < 0.0125$; Fig. 4A). *Octopus bimaculoides* skin extract also evoked responses that were significantly greater than FSW (Kruskal-Wallis, $P < 0.0001$, followed by Dunn *post hoc*, $n = 9$, $P < 0.05$; Fig. 4B). Both *D. pealeii* and *O. bimaculoides* ink samples were not statistically different from FSW.

Degree of movement intensity was quantified using a 0 to 4 scale (0 representing no movement and 4 representing dramatic, whip-like motion; see *Materials and Methods* for more detail). Similar patterns were seen, with average movement

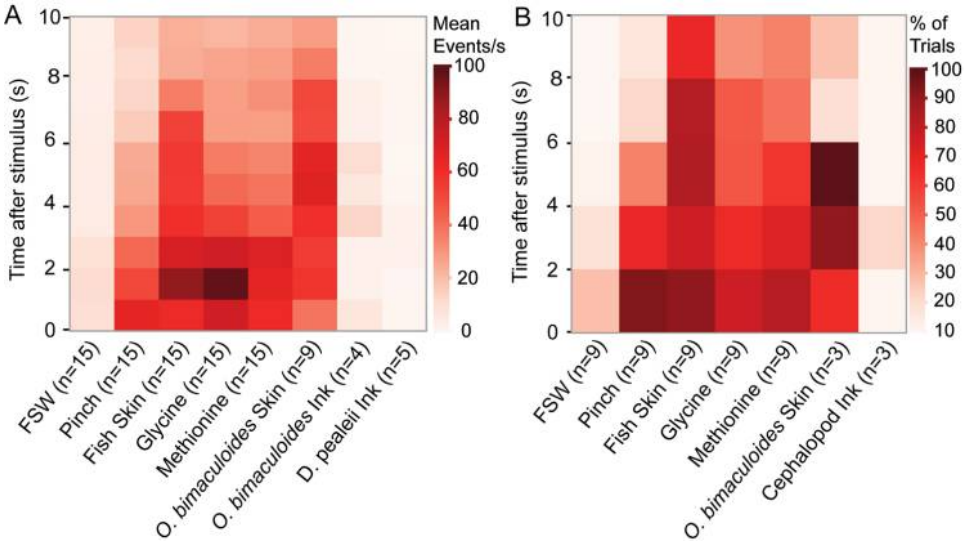


**Figure 3.** Both electrophysiological and movement responses were prolonged in *Octopus bimaculoides* for more complex stimuli. The number of arms (*n*) is listed in parentheses for each stimulus. (A) The mean number of events per second was calculated for each arm tested for the first 10 s after stimulus administration. Peak responses to pinch, glycine, and methionine occurred in the first 1–3 s, while responses to fish skin extract and *O. bimaculoides* skin extract continued for more than twice as long. Filtered seawater (FSW) and ink stimuli evoked little to no response at any time. (B) Movement was assessed with a binary metric (movement or no movement) in 2-s time bins for 10 s after stimulus administration. The percentage of trials with movement for each stimulus and time bin was calculated for each arm tested. Then the average percentage of trials with movement was plotted on the heat map. FSW and cephalopod ink evoked little movement at any time (two experiments using *Doryteuthis pealeii* ink and one experiment using *O. bimaculoides* ink were combined because of small *n* and similar results). Pinch evoked reliable movement that tended to be short-lived, while fish skin extract evoked movement that tended to last throughout the 10 s measured.

scores for fish skin extract, pinch, glycine, and methionine significantly greater than for FSW (Kruskal-Wallis, $P < 0.0001$, Dunn *post hoc*, $n = 9$, $P < 0.05$; Fig. 4C). Octopus ink generated mean movement scores between 2 and 3, while cephalopod inks produced movement scores between 0 and 1 (Fig. 4C); but with only $n = 3$ for each stimulus, they were not included in the statistical analysis.

Given the apparent relationship between electrophysiological events and arm movement, the number of events, movement score, and movement duration (quantified as the number of 2-s bins that showed movement) were compared on a trial-by-trial basis. Greater numbers of electrophysiological events were indeed correlated with greater movement score (Kendall's tau = 0.5364, $P < 0.0001$; Fig. 5A) and greater movement duration (Kendall's tau = 0.5436, $P < 0.0001$; Fig. 5B). Movement scores and duration were also positively correlated (Kendall's tau = 0.874, $P < 0.0001$; Fig. 5C). Despite the clear association between arm movement and electrophysiology, the correlation coefficients and the scatter apparent on the graphs indicate that the number of electrophysiological events could not be predicted reliably from the duration or degree of movement alone.

## Discussion

The physiological responses recorded in this study suggest that chemosensory receptor cells on isolated cephalopod arms were able to detect ecologically relevant chemicals in the water, leading to neural signals sent toward the animal's brain, as well as local motor responses, evoked without input from the brain. These conclusions are largely drawn from experiments using juvenile *Octopus bimaculoides*, although anecdotal evidence suggests that *Doryteuthis pealeii* arms may also show chemosensory responses. We also found that *O. bimaculoides* arms are well suited to an *ex vivo* electrophysiological preparation, unlike *D. pealeii* arms, which we were unable to maintain after amputation. An additional advantage of using *O. bimaculoides* is that animals are easily anesthetized and recover well after amputation. We feel it would be worthwhile to attempt this technique in other cephalopod species that have established anesthesia protocols, including other species of octopus and cuttlefish, such as *Sepia bandensis* (Lewbart and Mosley, 2012; Gleadall, 2013; Butler-Struben *et al.*, 2018). This would allow comparative studies of the use of chemosensation in arms across the coleoid cephalopods. We had hoped to compare a benthic species and a pelagic species, and we remain interested in how arm chemosensory systems may have adapted in animals that use their arms differently.

In chemosensory research, a distinction has often been made between taste (contact chemosensation, where stimuli are often solid or liquid) and olfaction (distance chemosensation, where stimuli are often volatile compounds) (Caprio, 1977). It is not yet clear how this distinction might be applied to cephalopods,

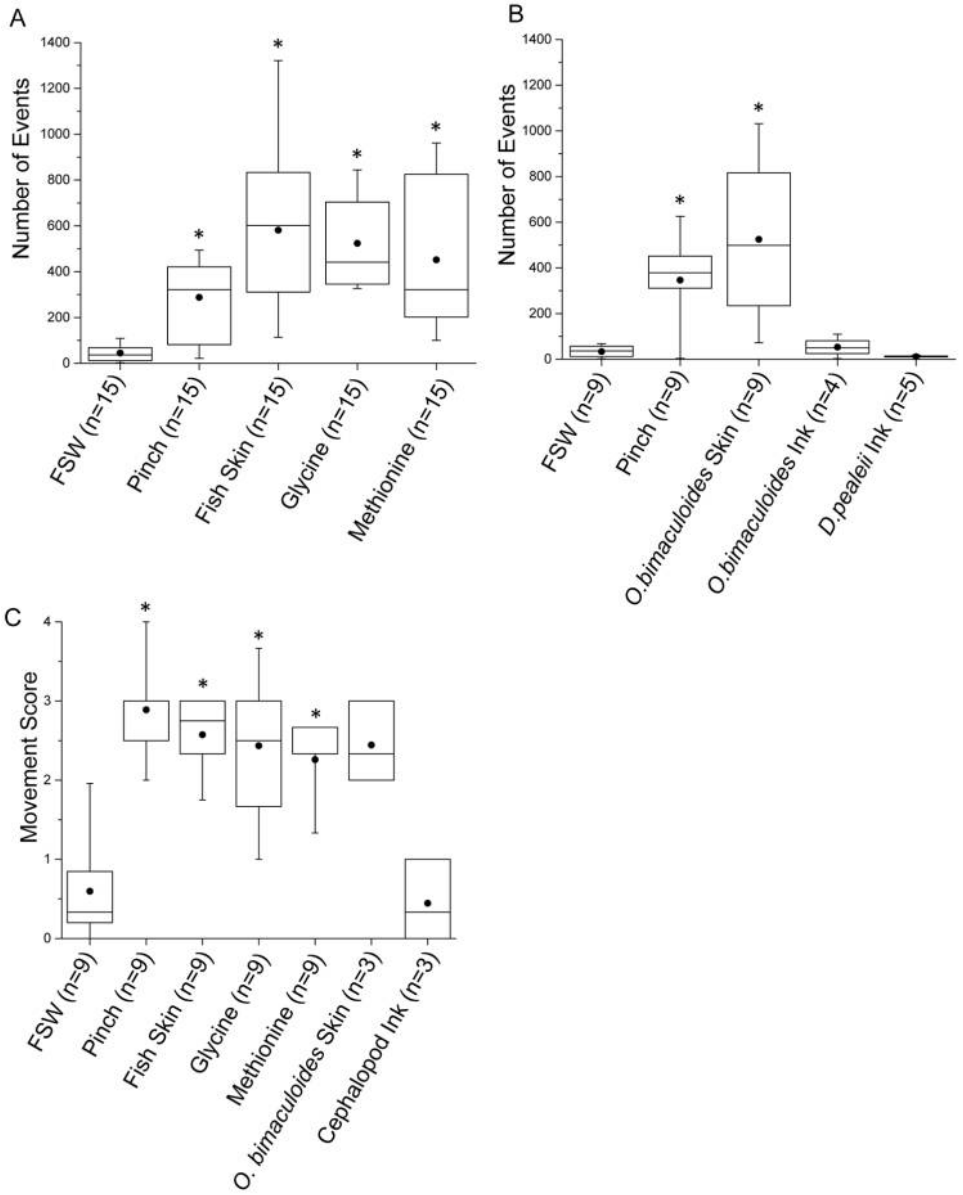


**Figure 4.** Most test stimuli evoked significant levels of neural activity and arm movement in *Octopus bimaculoides* arms. Boxplots show the median and quartiles, whiskers are tenth and ninetieth percentiles, and black dots show the mean. The number of arms examined for each stimulus is indicated in parentheses. (A) The number of events during the first 10 s after stimulus administration was significantly greater for pinch, fish skin extract, glycine, and methionine than for filtered seawater (FSW) (*$P < 0.0125$ for stimulus compared with FSW, pairwise Wilcoxon tests with Bonferroni correction). These stimuli were tested on all 15 arms. (B) The number of events during the first 10 s after stimulus administration was significantly greater for *O. bimaculoides* skin and pinch than for FSW (*$P < 0.05$, Kruskal-Wallis, Dunn *post hoc*). *Doryteuthis pealeii* ink and *O. bimaculoides* ink were not significantly different from FSW. These stimuli were tested on subsets of arms. (C) Movement was assessed on a scale of 0 (no arm movement) to 4 (large-scale movement) for each stimulus on a subset of arms. Pinch, fish skin extract, glycine, and methionine induced significantly greater movement scores than FSW (*$P < 0.05$, Kruskal-Wallis, Dunn *post hoc*). *Octopus bimaculoides* skin and cephalopod ink (two experiments using *D. pealeii* ink and one experiment using *O. bimaculoides* ink were combined here) were not included in statistical analyses because of the small sample size of each, but they are graphed here to show that data do follow the expected trend.

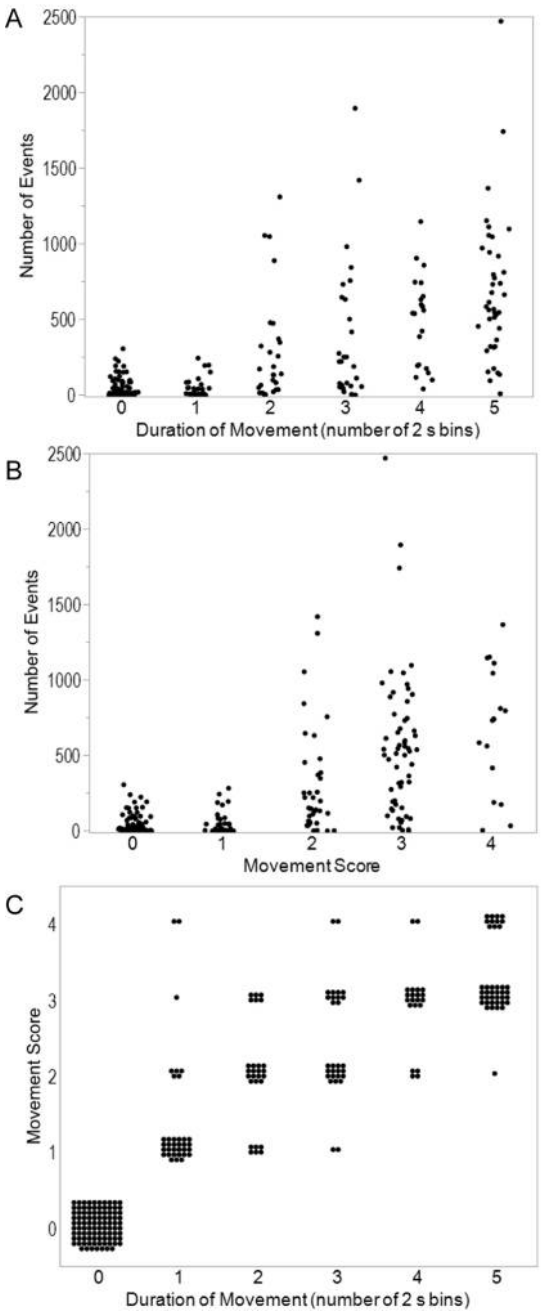


**Figure 5.** Movement variables correlate with electrophysiological results in *Octopus bimaculoides*. Duration of arm movement (defined as the number of 2-s bins that showed any type of motion), movement score, and number of electrophysiological events were compared, trial by trial, for nine *O. bimaculoides* arms; and correlations between pairs of variables were assessed. (A) Duration showed a significant, positive relationship with number of events (Kendall's tau = 0.5436, $P < 0.001$). (B) Movement score also showed a significant, positive relationship with the number of events (Kendall's tau = 0.5364, $P < 0.001$). (C) The two movement variables showed a strong positive correlation (Kendall's tau = 0.874, $P < 0.001$).

where the same receptors may serve to detect chemicals with both local and distant sources; and concentration, rather than state, may be the critical factor in detection. An alternative organizing principle might be based on the function of the body part that contains the chemoreceptors rather than the distance or state of the stimulus source. Thus, we think it will be important to consider cephalopod behavior and ecology in exploring chemosensation (Derby and Zimmer, 2012).

The response of *O. bimaculoides* arms and suckers to our amino acid and fish skin extract stimuli was consistent with previous literature describing that octopuses are able to detect waterborne chemical cues, particularly those associated with prey (Wells, 1963; Chase and Wells, 1986; Walderon *et al.*, 2011). Amino acids and compounds in the fish skin extract are likely important for prey identification (Chase and Wells, 1986; Mobley *et al.*, 2008; Hassenklover *et al.*, 2012; Mather and Kuba, 2013). A chemosensory system located in the arms allows octopuses to reach into dark areas to search, sample, and collect prey items based on stimuli encountered by an arm alone (Mather and Kuba, 2013; Hanlon and Messenger, 2018). Future studies could explore a broader range of stimuli derived from different prey sources and could test specific chemicals that may signal the presence or quality of food, such as additional amino acids, lipids, and their metabolites.

*Octopus bimaculoides* skin extract also elicited robust afferent nerve activity and motor responses. This stimulus was inspired by Nesher *et al.* (2014), who found that amputated octopus arms would not reflexively suck onto objects coated with self or conspecific skin extract (they found no difference between self and conspecific extracts); but they would readily attach to control objects or objects coated with fish skin extract. While we cannot assess any valence difference in the signals we recorded, we believe our study is consistent with Nesher *et al.* (2014), because to inhibit the sucker reflex, octopus skin must be first detected, likely resulting in afferent signal. Conspecific recognition is needed to avoid self-entanglement, as well as for social or reproductive encounters. While *O. bimaculoides* individuals are often solitary, they do fight conspecifics with arm contact (Hanlon and Messenger, 2018). Detection of conspecific chemical cues may allow for identification of sex and reproductive status as well (Walderon *et al.*, 2011). For this study we combined skin samples from multiple animals to create our extract. The isolated arm preparation could be used to test whether there were differences in response to self *versus* conspecific and to test specific compounds of interest that may underlie conspecific- and self-recognition mechanisms.

Interestingly, the arms of *O. bimaculoides* did not show any significant response to either *D. pealeii* or *O. bimaculoides* ink samples. Inking is used by cephalopods to confuse predators and provide an alarm call for conspecifics (Boal and Golden, 1999; Wood *et al.*, 2008; Staudinger *et al.*, 2011; Derby, 2014; Hanlon and Messenger, 2018). Ink may be detected through other sensory pathways, such as the olfactory pits located on the head (Gilly and Lucero, 1992; Lucero *et al.*, 1992) and the visual system (Wood *et al.*, 2008), but not by arm chemoreceptors. Evolutionary history, specialized function of the various chemosensory organs, and receptor sensitivity could

play a role in observed lack of response to ink in the arms. This should be tested in additional species that are more social and that use ink more frequently than *O. bimaculoides*, in order to determine whether this finding is typical of other cephalopods as well.

We observed responses (both electrophysiological and behavioral) to our stimuli that persisted for 10 seconds or more in some instances. During that time period, the stimulus (volume: 5 $\mu$L) rapidly diluted from its original concentration as it diffused through the bath (volume: 12–14 mL), sometimes facilitated by the movement of the arm itself. The continued response may represent persistent firing of the same receptors or recruitment of additional receptors as the stimulus spread through the bath. The more chemically complex stimuli often led to longer responses, which may also suggest an additive effect of multiple chemical stimuli. More experiments are needed to better understand the dynamics of these chemosensory responses. It would be useful to test the sensitivity of arm chemoreceptors to various stimuli by using controlled dilutions. Likewise, the time course of the responses to various stimuli, including adaptation or habituation, would be interesting to know.

Observed gross movements of the arm were correlated with afferent nerve activity, but there was significant scatter in the trial-by-trial comparison. In general, trials with little to no movement had little to no events recorded on the nerve. But trials with movement showed tremendous variation in the number of electrophysiological events. This variability likely reflects the complexity of neural processing within the arm that occurs between the point of stimulation and the two distinct end points of nerve cord recording and motor output. The signals we recorded are not simple receptor potentials or action potentials from primary sensory axons; rather, they include some or all of the following: afferent sensory information (chemosensory and somatosensory), efferent copies of motor commands, sensory and motor signals being sent to the proximal segments of the amputated arm, and local activity within the arm ganglia adjacent to the electrode. Thus, while more refined electrophysiological techniques will be needed to understand the nature of the signals being recorded and the processing of chemosensory information within the arm, these nerve recordings are well suited to initial explorations of the chemosensory stimulus space (*i.e.*, asking the arm, "Do you taste this?") in *O. bimaculoides* and, potentially, other cephalopods.

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## Ethical Care Considerations

The care and treatment of cephalopods, as invertebrates, are not covered by animal care laws in the United States. However, we worked closely with a veterinarian who has experience and expertise with cephalopods to ensure humane and ethical treatment of our animals, as well as adherence to all guidelines and practices of the MBL, where the experiments were conducted.

## Data Accessibility

All raw data (in the form of electrophysiological recordings and videos) and analyzed data (event counts and movement scores) can be made available upon request.

## Literature Cited

**Albertin, C. B., O. Simakov, T. Mitros, Z. Y. Wang, J. R. Pungor, E. Edsinger-Gonzales, S. Brenner, C. W. Ragsdale, and D. S. Rokhsar. 2015.** The octopus genome and the evolution of cephalopod neural and morphological novelties. *Nature* **524:** 220–224.

**Boal, J. G., and D. K. Golden. 1999.** Distance chemoreception in the common cuttlefish, *Sepia officinalis* (Mollusca, Cephalopoda). *J. Exp. Mar. Biol. Ecol.* **235:** 307–317.

**Budelmann, B. U. 1995.** The cephalopod nervous system: what evolution has made of the molluscan design. Pp. 116–138 in *The Nervous Systems of Invertebrates: An Evolutionary and Comparative Approach*, O. Breidbach and W. Kutsch, eds. Birkhauser, Basel.

**Budelmann, B. U. 1996.** Active marine predators: the sensory world of cephalopods. *Mar. Freshw. Behav. Physiol.* **27:** 59–75.

**Budelmann, B. U., and J. Z. Young. 1985.** Central pathways of the nerves of the arms and mantle of *Octopus*. *Philos. Trans. R. Soc. B Biol. Sci.* **310:** 109–122.

**Budelmann, B. U., and J. Z. Young. 1987.** Brain pathways of the brachial nerves of *Sepia* and *Loligo*. *Philos. Trans. R. Soc. B Biol. Sci.* **315:** 345–352.

**Butler-Struben, H. M., S. M. Brophy, N. A. Johnson, and R. J. Crook. 2018.** *In vivo* recording of neural and behavioral correlates of anesthesia induction, reversal, and euthanasia in cephalopod molluscs. *Front. Physiol.* **9:** 109.

**Caprio, J. 1977.** Electrophysiological distinctions between the taste and smell of amino acids in catfish. *Nature* **266:** 850–851.

**Chase, R., and M. J. Wells. 1986.** Chemotactic behavior in *Octopus*. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* **158:** 375–381.

**Cummins, S. F., J. G. Boal, K. C. Buresch, C. Kuanpradit, P. Sobhon, J. B. Holm, B. M. Degnan, G. T. Nagle, and R. T. Hanlon. 2011.** Extreme aggression in male squid induced by a β-MSP-like pheromone. *Curr. Biol.* **21:** 322–327.

**Derby, C. D. 2014.** Cephalopod ink: production, chemistry, functions and applications. *Mar. Drugs* **12:** 2700–2730.

**Derby, C. D., and R. K. Zimmer. 2012.** Neuroecology of predator-prey interactions. Pp. 158–171 in *Chemical Ecology in Aquatic Systems*, C. Bronmark and L. A. Hansson, eds. Oxford University Press, Oxford.

**Emery, D. G. 1975.** Ciliated sensory neurons in lip of squid *Lolliguncula brevis* Blainville. *Cell Tissue Res.* **157:** 323–329.

**Gilly, W. F., and M. T. Lucero. 1992.** Behavioral responses to chemical stimulation of the olfactory organ in the squid *Loligo opalescens*. *J. Exp. Biol.* **162:** 209–229.

**Gleadall, I. G. 2013.** The effects of prospective anaesthetic substances on cephalopods: summary of original data and a brief review of studies over the last two decades. *J. Exp. Mar. Biol. Ecol.* **447:** 23–30.

**Graziadei, P. 1962.** Receptors in suckers of *Octopus*. *Nature* **195:** 57–59.

**Graziadei, P. 1964a.** Electron microscopy of some primary receptors in the sucker of *Octopus vulgaris*. *Z. Zellforsch. Mikrosk. Anat.* **64:** 510–522.

**Graziadei, P. 1964b.** Receptors in the sucker of the cuttlefish. *Nature* **203:** 384–387.

**Graziadei, P. 1965.** Sensory receptor cells and related neurons in cephalopods. *Cold Spring Harbor Symp. Quant. Biol.* **30:** 45–57.

**Gutfreund, Y., H. Matzner, T. Flash, and B. Hochner. 2006.** Patterns of motor activity in the isolated nerve cord of the octopus arm. *Biol. Bull.* **211:** 212–222.

**Hanlon, R. T., and J. B. Messenger. 2018.** *Cephalopod Behaviour*. Cambridge University Press, Cambridge.

**Hanlon, R. T., K. Buresch, H. Moustahfid, and M. Staudinger. 2013.** *Doryteuthis pealieii*, longfin inshore squid. Pp. 205–240 in *Advances in Squid Biology, Ecology and Fisheries*, Pt. I, *Myopsid Squids*, R. Rosa, R. O'Dor, and G. Pierce, eds. Nova Biomedical, New York.

**Hassenklover, T., L. P. Pallesen, D. Schild, and I. Manzini. 2012.** Amino acid- vs. peptide-odorants: responses of individual olfactory receptor neurons in an aquatic species. *PLoS One* **7:** e53097.

**Kier, W. M. 2016.** The musculature of coleoid cephalopod arms and tentacles. *Front. Cell Dev. Biol.* **4:** 10.

**Lesueur, C. A. 1821.** Descriptions of several new species of cuttlefish. *J. Acad. Nat. Sci. Phila.* **2:** 86–101.

**Lewbart, G. A., and C. Mosley. 2012.** Clinical anesthesia and analgesia in invertebrates. *J. Exot. Pet Med.* **21:** 59–70.

**Liscovitch-Brauer, N., S. Alon, H. T. Porath, B. Elstein, R. Unger, T. Ziv, A. Admon, E. Y. Levanon, J. J. C. Rosenthal, and E. Eisenberg. 2017.** Trade-off between transcriptome plasticity and genome evolution in cephalopods. *Cell* **169:** 191-202.e111.

**Lucero, M. T., F. T. Horrigan, and W. F. Gilly. 1992.** Electrical responses to chemical stimulation of squid olfactory receptor cells. *J. Exp. Biol.* **162:** 231–249.

**Mather, J. A., and M. J. Kuba. 2013.** The cephalopod specialties: complex nervous system, learning, and cognition. *Can. J. Zool.* **91:** 431–449.

**Mobley, A. S., W. C. Michel, and M. T. Lucero. 2008.** Odorant responsiveness of squid olfactory receptor neurons. *Anat. Rec.* **291:** 763–774.

**Nesher, N., G. Levy, F. W. Grasso, and B. Hochner. 2014.** Self-recognition mechanism between skin and suckers prevents octopus arms from interfering with each other. *Curr. Biol.* **24:** 1271–1275.

**Nixon, M., and J. Z. Young. 2003.** *The Brains and Lives of Cephalopods*. Oxford University Press, Oxford.

**O'Brien, C. E., K. Roumbedakis, and I. E. Winkelmann. 2018.** The current state of cephalopod science and perspectives on the most critical challenges ahead from three early-career researchers. *Front. Physiol.* **9:** 700.

**Pickford, G. E., and B. H. McConnaughey. 1949.** The *Octopus bimaculatus* problem, a study in sibling species. *Bull. Bingham Oceanogr. Collect.* **12:** 1–66.

**Polese, G., C. Bertapelle, and A. Di Cosmo. 2016.** Olfactory organ of *Octopus vulgaris*: morphology, plasticity, turnover and sensory characterization. *Biol. Open* **5:** 611–619.

**Rowell, C. H. F. 1963.** Excitatory and inhibitory pathways in the arm of *Octopus*. *J. Exp. Biol.* **40:** 257–270.

**Santi, P. A. 1975.** The morphology of putative receptor neurons and other neural components in the suckers of the squid, *Lolliguncula brevis*. Ph.D. dissertation, Florida State University, Tallahassee.

**Staudinger, M. D., R. T. Hanlon, and F. Juanes. 2011.** Primary and secondary defences of squid to cruising and ambush fish predators: variable tactics and their survival value. *Anim. Behav.* **81:** 585–594.

**Sumbre, G., G. Fiorito, T. Flash, and B. Hochner. 2006.** Octopuses use a human-like strategy to control precise point-to-point arm movements. *Curr. Biol.* **16:** 767–772.

**Walderon, M. D., K. J. Nolt, R. E. Haas, K. N. Prosser, J. B. Holm, G. T. Nagle, and J. G. Boal. 2011.** Distance chemoreception and the detection of conspecifics in *Octopus bimaculoides*. *J. Molluscan Stud.* **77:** 309–311.

**Wells, M. J. 1963.** Taste by touch: some experiments with *Octopus*. *J. Exp. Biol.* **40:** 187–193.

**Wood, J. B., K. E. Pennoyer, and C. D. Derby. 2008.** Ink is a conspecific alarm cue in the Caribbean reef squid, *Sepioteuthis sepioidea*. *J. Exp. Mar. Biol. Ecol.* **367:** 11–16.

**Woodhams, P. L., and J. B. Messenger. 1974.** Note on ultrastructure of *Octopus* olfactory organ. *Cell Tissue Res.* **152:** 253–258.