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.

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; Hanlon and Messenger, 2018). In behavioral chemosensory 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; Stauinger 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; Mobjley 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
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 pealei 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...
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 μL 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. bimacu- loides 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 administration and arm response for most (9 of 15) octopus experiments. Video resolution was 640 x 480, 30 frames s⁻¹, and video was synchronized to the electrophysiological recording by using the multimedia feature in Spike2 software (CED). 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 x 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 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
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...
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 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.
showed a strong positive correlation (Kendall's \( \tau \) also showed a significant positive relationship with the number of events (Kendall's \( \tau = 0.5364, P < 0.001 \)).

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 \( \tau = 0.5436, P < 0.0001 \)). (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 µL) 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. In general, trials with little to no movement had little to no events recorded on the nerve.

**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**


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