

Acidity enhances the effectiveness of active chemical defensive secretions of sea hares, *Aplysia californica*, against spiny lobsters, *Panulirus interruptus*

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Abstract Sea hares such as *Aplysia californica*, gastropod molluscs lacking a protective shell, can release a purple cloud of chemicals when vigorously attacked by predators. This active chemical defense is composed of two glandular secretions, ink and opaline, both of which contain an array of compounds. This secretion defends sea hares against predators such as California spiny lobsters *Panulirus interruptus* via multiple mechanisms, one of which is phagomimicry, in which secretions containing feeding chemicals attract and distract predators toward the secretion and away from the sea hare. We show here that ink and opaline are highly acidic, both having a pH of ~5. We examined if the acidity of ink and opaline affects their phagomimetic properties. We tested behavioral and electrophysiological responses of chemoreceptor neurons in the olfactory and gustatory organs of *P. interruptus*, to ink and opaline of *A. californica* within their natural range of pH values, from ~5 to 8. Both behavioral and electrophysiological responses to ink and opaline were enhanced at low pH, and low pH alone accounted for most of this effect. Our data suggest that acidity enhances the phagomimetic chemical defense of sea hares.

Keywords Chemoreception · Ink · Opaline · pH · Phagomimicry

Introduction

Sea hares, like many other opisthobranch gastropods, compensate for the reduction or loss of shell by using an array of defenses (reviewed in Johnson and Willows 1999; Wägele and Klussmann-Kolb 2005). Included in this array are passive and active chemical defenses (Kinnel et al. 1979; Faulkner 1992; Nolen et al. 1995; Johnson and Willows 1999; Cimino and Gavagnin 2006). Passive chemical defenses are not released under nervous system control during predatory attacks, but are present as deterrent compounds in the skin, some of which are acquired through diet (Thompson 1960; Ginsburg and Paul 2001). Active chemical defenses however are released under nervous system control only during physical attacks by predators (Carew and Kandel 1977; Johnson and Willows 1999). A dramatic example of an active chemical defense is inking by sea hares (*Aplysia*), wherein a purple cloud composed of pigmented and other substances is formed. This slimy secretion is composed of two different glandular products, ink and opaline, that are typically released simultaneously (Tritt and Byrne 1980; Prince et al. 1998; Nolen and Johnson 2001). Ink is diffusible and purple, whereas opaline is whitish and highly viscous.

The secretion of ink and opaline not only generates a visual cloud that can affect visually oriented predators, but chemicals in it can interact with the chemosensory organs of predators. The mechanisms whereby the chemicals in the defensive secretion of *Aplysia* manipulate predators' chemosensory systems and provide a defense have only recently begun to be understood.

One mechanism for *Aplysia* chemical defenses is through deterrent chemicals that inhibit ingestive behaviors. Examples of this include predatory sea anemones, fish, and spiny lobsters, though the molecular identities of the

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deterrents are largely unknown (Kicklighter et al. 2005; Kicklighter and Derby 2006; but see Kamio et al. 2007).

A second mechanism for *Aplysia* chemical defenses is phagomimicry, which involves predators such as spiny lobsters being diverted toward the secretion and away from the sea hare (Kicklighter et al. 2005). This mechanism operates because ink and opaline have high quantities of amino acids and other food-associated compounds that are highly stimulatory to the chemical senses of predators such as spiny lobsters. For example, ink and opaline, as well as artificial mixtures mimicking them based on chemical analysis of the secretions, are attractive, and can evoke feeding behavior from the California spiny lobster, *Panulirus interruptus* (Kicklighter et al. 2005).

A third mechanism is sensory disruption, which is based on the fact that the secretions not only contain very high concentrations of stimulants but are also very sticky and can adhere to the predators' sensory organs. For example, the highly stimulating secretions stick to the chemosensory organs of spiny lobsters—the antennules, legs, and mouthparts (Kicklighter et al. 2005). This secretion stickiness may functionally disrupt the spiny lobsters' chemosensory organs. Spiny lobsters rely heavily on their chemosensory organs during foraging and feeding. To forage, they use their antennules for detection and orientation toward distant food odors. To feed, spiny lobsters use legs and mouthparts to taste and handle food (Garm 2004; Garm et al. 2005; Garm and Høeg 2006). Functional disruption of chemosensory activity by sea hare secretions may explain spiny lobster behaviors to sea hare secretions, such as extensive grooming of mouthparts and antennules, which allow the sea hare to escape (Kicklighter et al. 2005).

A striking feature of some chemical defenses of sea hares and other opisthobranchs is the use of acids. Acid glands of many opisthobranchs release their contents following disturbance of the skin (Thompson 1960, 1983, 1986, 1988). For example, notaspids release copious amounts of sulfuric acid from their skin when irritated, creating mucus with a pH of ~ 2 (Gillette et al. 1991). Likewise, the mucus of sea hares (*Aplysia californica*) becomes acidic (pH ~ 4.0) immediately after physical aggravation of the skin (unpublished data).

Acidity is not limited to the skin. Ink and opaline secretions also are acidic. Our preliminary studies showed that ink and opaline are highly acidic. The acidity of the ink secretions may contribute to their efficacy as chemical defenses. Therefore, we asked whether acidity of sea hare secretions enhances the phagomimetic defense by enhancing the neural and behavioral responses of predators to ink and opaline secretions. To answer this question, we measured behavioral and neural chemosensory responses of spiny lobsters to secretions of sea hares presented at a range of pH values. The spiny lobster is a good model predator

for this work, since it has been used already to demonstrate effects of ink and opaline secretions on the function of chemosensory neurons and on chemically evoked behavior (Kicklighter et al. 2005), and since the spiny lobster's chemosensory systems are well studied (e.g. Derby 2000; Derby et al. 2001). First, we measured behavioral responses that are controlled by either the antennules (attraction) or mouthparts and legs (handling and ingestion). Second, complementary to behavior we measured the electrophysiological responses of chemoreceptor neurons in the mouthparts and antennules.

Materials and methods

Animals

California spiny lobsters (*P. interruptus*) with carapace lengths of 60–90 mm were collected in the waters near San Diego, CA, USA. Sea hares (*A. californica*) were supplied by the National Institutes of Health National Resource for *Aplysia*, in Florida. Both were shipped to our laboratory and maintained in aquaria containing filtered and re-circulated artificial seawater (Instant Ocean[®], Aquarium Systems, Mentor, OH, USA) at room temperature 20–25°C and a 12:12 h light:dark cycle. Spiny lobsters for behavioral experiments were housed individually in 80-l aquaria, and spiny lobsters for electrophysiological assays were held in groups of ~ 15 in 800-l aquaria. Spiny lobsters were fed shrimp and squid three times per week. Spiny lobsters that were pre-molt or that were not attracted to shrimp or squid juice were not used in our assays.

Determination of the pH of natural ink and opaline

The pH values of ink and opaline at full strength were measured from seven individual sea hares. Ink and opaline were collected from ink and opaline glands according to Kicklighter et al. (2005) and their pH values were tested immediately after collection. pH values were measured using an Accumet AB 15 pH meter and a combination pH electrode with calomel reference (Fisher Scientific, Pittsburgh, PA, USA).

Stimuli and solutions

Ink and opaline were collected from ink and opaline glands, respectively, of 12 sea hares according to Kicklighter et al. (2005) and stored at -20°C until used. Full strength squid juice was prepared by homogenizing 20 g of squid mantle in 100 ml of artificial sea water (ASW) (Derby 1995) and then filtering through a Whatman 3 filter paper. Full strength shrimp juice was prepared as was squid juice

except using frozen shrimp (*Penaeus* sp.). Ink, opaline, and squid juice were diluted with ASW for the behavioral and electrophysiological assays, except for the loose-patch electrophysiological recordings for which dilutions were made with *Panulirus* saline (PS) (Derby 1995). The pH of all stimuli in all of our assays was adjusted using sodium hydroxide or hydrochloric acid, which affected osmolarity by less than 0.1%. The pH of solutions remained stable over the course of experiments, since they contained buffers. Sea water contained sodium bicarbonate and saline contained HEPES. We measured the pH of solutions at the beginning, middle, and end of each experiment, and they remained within ± 0.2 pH units. For the behavioral assay and electrophysiological recordings from chemoreceptor axons, we made fresh dilutions and pH adjustments every day. For the electrophysiological recordings from olfactory receptor neuronal somata, dilutions and pH adjustments were made once and solutions were then stored at -20°C . After thawing the stimuli diluted in saline, we adjusted the pH, if necessary, and checked it before, during, and after experiments. The solutions were adjusted to pH values of 7.7 (the pH of sea water), 4.9 (the pH of ink when released from a sea hare), and 6.3 (midway between the pH of sea water and ink). The pH of natural sea water (~ 8.1) is slightly higher than the pH of sea water in our aquaria (7.7). Since lobsters behaved normally in our aquaria, and we needed to test the same pH values in all experiments, we used 7.7 as the ‘natural’ pH of sea water in both behavioral and electrophysiological experiments.

Behavioral assays

In our behavioral assays, we examined whether the acidity of sea hare secretions had a significant effect on the behavioral responses of spiny lobsters. Assays were conducted during the light phase of the 12:12 L:D photoperiod. Stimuli were delivered to each spiny lobster using a hand-held 1-ml pipette, which allowed introduction of 1 ml of stimulus approximately 8 cm from the antennules of the animal. The assays had two phases: conditioning phase and testing phase.

Conditioning phase

Spiny lobsters normally respond to a food odor by moving forward and flicking their antennules in the direction of odor. Animals normally do not respond to ASW. However, at the beginning of our experiments, animals sometimes responded to introduction of the delivery pipette or delivery of ASW. Thus, we conditioned the animals to respond to delivery of food odor (shrimp juice) but not ASW by acclimating them to the delivery procedure. We conditioned them daily, beginning one week prior to the testing phase of

the experiment, by presenting to each animal 1 ml of shrimp juice and 1 ml of ASW three times per day at 1 h intervals. This procedure eventually led to animals consistently responding to shrimp odor by moving forward at least a half body length toward the point of stimulus delivery, and by not moving toward ASW.

Testing phase

Once the conditioning phase was complete, we proceeded with the testing phase. We began by determining the minimum concentration that induced at least 20% of the spiny lobsters to respond to ink, opaline, and squid juice. We tested stimuli, all at pH 7.7, ranging down to 0.0001% full-strength. We identified the minimal effective concentrations to be 0.001% full-strength ink, 0.0005% full-strength opaline, and 0.001% squid juice, which we then used to determine the effect of pH on behavioral responses of spiny lobsters.

The independent variables in our experiment were stimulus type and stimulus pH. We tested four stimulus types (ink, opaline, squid juice, ASW), each at three pH levels (7.7, 6.3, 4.9) in separate behavioral assays. In each behavioral assay, spiny lobsters were tested with a positive control (0.01% squid juice), negative control (ASW), and one stimulus type at three pH values, for a total of five trials. We waited approximately 30 min between each stimulus delivery. A response was measured through dichotomous scoring: positive response versus no response. Significance was established using a non-parametric statistical test, the McNemar pair test. A positive response was defined as forward movement of the spiny lobster toward the stimulus by at least a half body length. A no-response was defined as no forward movement within 20 s of stimulus delivery. Spiny lobsters that failed to respond to the positive control stimulus or that responded to negative control stimulus were not included in our analysis.

Electrophysiological assays

Antennules of spiny lobsters have ten types of sensilla (Cate and Derby 2001). One of these, the aesthetasc sensilla, contains only chemoreceptor neurons, whereas the other types, collectively called non-aesthetasc sensilla, contain both mechanoreceptor neurons and chemoreceptor neurons. Either aesthetascs or non-aesthetascs are sufficient for allowing spiny lobsters to detect and locate food (Stuellet et al. 2001). Unlike antennules, mouthparts of spiny lobsters mainly have bimodal sensilla composed of both mechano- and chemoreceptor neurons (Derby 1989; Corotto et al. 1992; Garm et al. 2005; Garm and Høeg 2006). Chemoreceptor neurons in the mouthparts of *Panulirus* detect the same chemicals as antennules but have broader

tuning properties (Garm et al. 2005). Thus, mouthparts complement antennules in assessing chemical compounds before items are ingested. For example, spiny lobsters are attracted to either ink or opaline secretions of *Aplysia*, but they ingest only ink because of feeding deterrent compounds in opaline (Kicklighter et al. 2005).

Two types of preparations were used to examine the effect of pH on the responses of spiny lobster chemoreceptor neurons to defensive secretions. One preparation was used to record responses from the axons of chemoreceptor neurons in the mouthparts and antennular lateral flagella. This preparation did not allow us to identify the sensillum type innervated by the recorded chemoreceptor neurons. A second preparation was used to record responses from the somata of identified receptor neurons—olfactory receptor neurons in the aesthetascs.

Single-unit extracellular electrophysiological recordings from axons

The effect of pH on response of chemoreceptor neurons (CRNs) in the antennular lateral flagellum and second maxilliped (one of the six pairs of mouthparts) was examined as follows. Axonal recordings were performed via single-unit extracellular electrophysiology (Derby 1995; Garm et al. 2005). We recorded from 14 mouthparts and 18 antennular lateral flagella, often isolating more than one neuron per preparation.

Stimuli were presented to the sensilla via an olfactometer with electronically driven valves. Responsive CRNs were identified using 1% ink or opaline, with ASW as the negative control. These stimuli were also presented periodically over the course of an experiment to ascertain that the responsiveness of the neuron did not change. Our independent variables were stimulus type (ASW and 1% of ink, opaline, and squid juice) and stimulus pH (7.7, 6.3, and 4.9). These stimuli were presented in a random order. Each stimulation was immediately followed with three rinses of ASW and a rest period of 90 s before the next stimulation.

We analyzed the activity of single CRNs using spike sorting (Spike 2, v. 5.05, CED, Cambridge, UK). In each recording, we collected data for total of 16 s, divided into three time periods: 2 s prior to chemical stimulation, 2 s during chemical stimulation, and 12 s after stimulation. Our dependent variable was spike frequency in the first 500 ms of response to a chemical stimulus. We analyzed these data for statistical differences through both parametric GLM repeated measures ANOVA and non-parametric Wilcoxon two-related-sample tests using SPSS software (SPSS 12.0). We performed the same type of analysis for all the electrophysiological data. Data were transformed to square root to meet sphericity and normal distribution criteria as needed. After we determined significance for each stimulus, we

performed three post-hoc comparisons between pH values: 7.7 vs. 6.3, 7.7 vs. 4.9, and 6.3 vs. 4.9. The appropriate significance levels ($P < 0.05$) were determined after Bonferroni corrections for either parametric or non-parametric tests. Single-unit activity from recordings of axons of antennular CRNs most likely was from non-aesthetasc sensilla rather than aesthetasc olfactory receptor neurons (Steullet et al. 2002).

Extracellular recordings from somata of olfactory receptor neurons

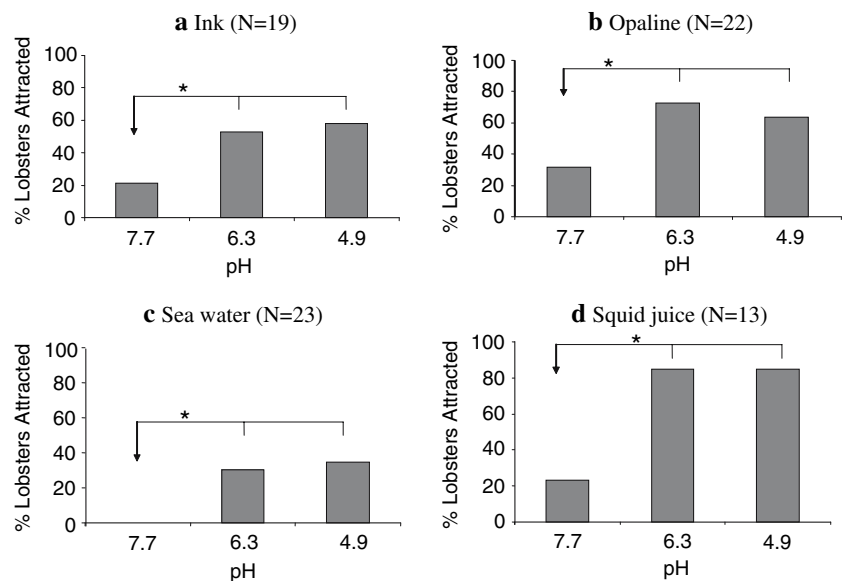
Our antennular preparation and method of recording olfactory receptor neurons (ORNs) were modified from Bobkov and Ache (2003, 2007). The middle section of the aesthetasc region of the lateral flagellum was cut from intermolt animals and bathed in a dish filled with PS. From this section, ~12 individual annuli were cut. The dorsal half of each annulus was removed to expose the ORN clusters. To remove sheath tissue around ORN clusters, annuli were placed in 1 mg trypsin/ml PS for 1 min, after which they were rinsed in PS for 20 min. The distal-most row of ORN clusters in the annulus was then removed to visualize and access the remaining ORN somata. An annulus was then transferred to a 60 × 15 mm dish and filled with PS, and the annulus was secured to the bottom using a hair that was secured by dental wax. This preparation was placed on the stage of an inverted microscope (Olympus CK2) and perfused continuously with fresh PS (0.6 ml/min). Stimuli were delivered directly to aesthetascs via electronically controlled rapid-solution changer (RSC-160, Bio-Logic, Claix, France). The stimulus was released approximately 1 mm from the aesthetascs. In each recording, we collected 2 s of data before and 13 s after a 4-s stimulus recording.

Extracellular recordings were taken from ORN somata of aesthetasc sensilla using loose-patch techniques (Bobkov and Ache 2003, 2007). Borosilicate patch pipettes (GMBH GB150-8P, Science Products, Germany) were pulled using a Narishige PP830 pipette puller (Japan) to produce electrodes with a tip diameter of ca. 1 μm. These electrodes were filled with PS and had a resistance of 2–3 MΩ in the PS bath. The electrodes usually formed seals with resistance of ~30 MΩ. The electrode tip contacted the soma of a single ORN innervating a single aesthetasc sensillum. Data acquisition was performed using an amplifier (Axopatch-1D), digitizer (Digidata-1320A), and software (pClamp 8.2) (Axon instruments, Inc.). Spike sorting was accomplished with Spike 2 v.5.05 software. The dependent variable was spike frequency, measured during the first 3 s of response to chemical stimulation. We measured the spike rate at 3 s instead of 0.5 s since the spike rate of ORN responses increased at a much slower rate than single unit recordings (see above), and thus a 3-s measurement is a

more reliable indicator of spiking rate. For example, the CRNs in single unit recordings reached their maximum spike rate during their first 0.5 s, whereas the ORNs in single soma recordings reached their maximum spike rate in 2–3 s.

This preparation was used to examine the effect of pH on the response of ORNs at various concentrations of ink. Our positive control was the fish food TetraMarine (TM) (Tetra, USA) dissolved with PS and filtered (pore diameter 0.22 μm , Fisherbrand) to 0.125 g/ml (full strength). TM was further diluted with PS to 1% when used as a positive control. ORNs that responded to 1% TM (pH 7.7) or in some cases to 0.1% ink (pH 7.7) but not PS alone (pH 7.7) were considered as odor-responsive ORNs and used in our assay. ORNs that showed intrinsic bursting activity, as reported by Bobkov and Ache (2007), were avoided since stimulation with ink or TM did not appreciably change their spiking activity. Each 15-s recording consisted of measuring spontaneous activity for 2 s before stimulus delivery, then the response to the chemical stimulus during 4 s of its delivery, followed by 9 s of post-stimulation recordings. The interval between stimulations was 90 s. Our experimental stimuli included ink at dilutions of 0.1, 0.01, and 0.001% full strength, each at three pH values—7.7, 6.3 and 4.9. Stimuli were delivered in a random order from one preparation to another. However, within a single preparation, a block of stimulations consisted of testing a single concentration of ink at all three pH values, presented in random order, followed by other blocks at different ink concentrations. Within a block, the first ink stimulus was re-tested as a positive control at the end of that block. At the end of all blocks, TM and PS were tested, followed by a measurement of spontaneous activity.

Fig. 1 The percentage of lobsters attracted to acidic stimuli was significantly higher for all stimuli. **a** 0.001% ink, **b** 0.0005% opaline, **c** sea water, and 0.001% squid juice. $N = \#$ of spiny lobsters tested. Asterisks indicate responses at pH 4.9 or 6.3 were significantly different than the response to the stimulus at pH 7.7 sea water (McNemar pair test, $\alpha = 0.05$)



Results

The defensive secretions of sea hares are acidic

Full-strength ink had a pH of 4.9 ± 0.07 (mean \pm SEM, $n = 7$), and full-strength opaline had a pH of 5.9 ± 0.08 (mean \pm SEM, $n = 7$). Both had lower pH values than sea water: the pH of sea water in our aquaria was ~ 7.7 .

Phagomimetic behavioral response of spiny lobster to ink and opaline is enhanced at acidic pH

Our previous study showed that spiny lobsters were attracted to the defensive secretions of sea hares—ink and opaline—even if they did not consume one of the secretions, a process called phagomimicry (Kicklighter et al. 2005). In those studies, both secretions were tested at their natural pH values. To determine whether the pH values of secretions increase phagomimetic attraction, we tested both secretions at near-threshold concentrations, and at pH values ranging from that of ink at full strength (pH 4.9) to that of sea water (pH 7.7). We recorded the number of lobsters that showed attractive responses to ink, opaline, squid juice (SJ, a positive control), and sea water (SW, a negative control), at pH values of 7.7, 6.3, and 4.9, and from these data we calculated the percentage of responding lobsters out of all tested lobsters. The percentage of lobsters that showed attractive responses to stimuli of low pH was significantly higher than that responding to stimuli of high pH for all four stimuli (Fig. 1a–d). For instance, the percentage of lobsters showing attractive responses to ink, opaline, and sea water of low pH was ~ 30 –40% points higher than the percentage of animals responding to the same stimuli at pH 7.7. Similarly, the percentage of lobsters showing attractive

responses to squid juice at low pH was ~60% points higher than the percentage responding to squid juice at pH 7.7.

Responses of mouthpart and antennular chemoreceptor neurons to ink and opaline are higher at acidic pH

Axonal recordings of second maxilliped chemoreceptor neurons

CRNs from mouthparts clearly fell into two groups: one group was sensitive to changes in pH alone (i.e. sea water at low pH), and another group was insensitive to pH changes. These two groups differed in another way: those sensitive to pH changes alone were also sensitive to a pH change in other stimuli, while those insensitive to pH change alone were generally insensitive to pH changes in these other stimuli. The group of pH-sensitive CRNs responded on average with higher spike frequency to sea water, ink, opaline, and squid juice at low pH than at the pH of sea water (7.7) (Fig. 2a), with responses to sea water and ink being significantly higher at pH 4.9 than at pH 7.7 (Fig. 2a). Note that CRN responses to sea water at any pH were much lower than responses to ink and opaline, and thus the increase in response at pH 6.3 and 4.9 compared to 7.7 was high on a percentage scale but not on an absolute scale. In other words, the increase in absolute responses at lower pH values was in the same range for sea water, ink, and opaline. In fact, subtracting sea water responses at each pH from the corresponding stimulus of the same pH removed the pH effect for each stimulus. Thus, pH change alone accounted for most of the enhanced chemosensory responses toward either defensive secretions or a food odor. The pH-insensitive CRNs responded with similar intensity across the pH values of each stimulus (Fig. 2b). The pH-insensitive CRNs showed little or no response to sea water at any pH, although they showed high responses to other stimuli such as ink and opaline.

Axonal recordings from antennular chemoreceptor neurons

CRNs from antennules also partitioned into two groups: one group that was sensitive and another that was insensitive to changes in pH alone. The CRNs sensitive to pH change alone were also sensitive to pH change in other stimuli, and the CRNs insensitive to pH change alone were minimally or not at all sensitive to pH change in other stimuli. The group of pH-sensitive CRNs from antennules responded on average with higher spike frequency to sea water, ink, opaline, and squid juice at low pH than at 7.7 (Fig. 3a), with significantly greater responses to pH 4.9 than 7.7 for both sea water and ink (Fig. 3a). Furthermore, sea water induced much lower responses than other stimuli, so that the change in absolute response with increasing pH

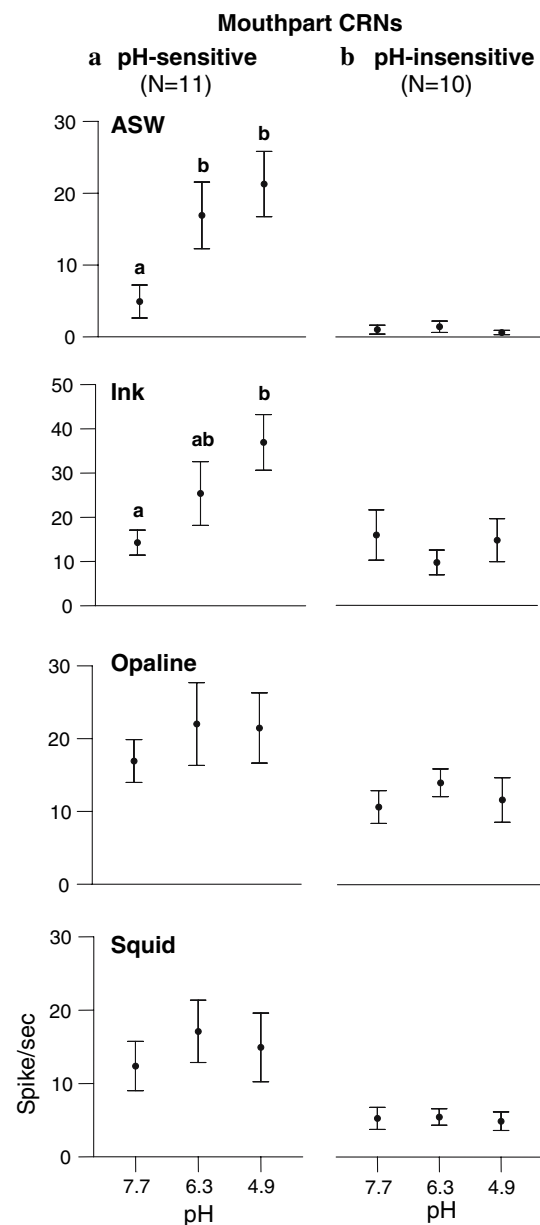


Fig. 2 In the 2nd maxillipeds, over 50% of chemoreceptor neurons were highly sensitive to pH change alone and to other stimuli (a), the rest were insensitive to pH change across stimuli (b). Activity is expressed as mean \pm SEM of the frequency of action potentials (# of spikes/s) to ASW, 1% ink, 1% opaline, and d 1% squid juice. The letters represent statistical differences between responses to the pH levels (ANOVA or Wilcoxon followed by post-hoc tests after Bonferroni corrections, $\alpha = 0.05$)

was similar for sea water, ink, and opaline. Subtracting the sea water responses from each stimulus of corresponding pH removed the pH effect for each stimulus. Thus, pH change alone accounted for the enhanced responses of antennular CRNs to either defensive secretion or food odors. The pH-insensitive CRNs from antennules, similar to those from mouthparts, responded with the same intensity to sea water across the pH values, and unlike those

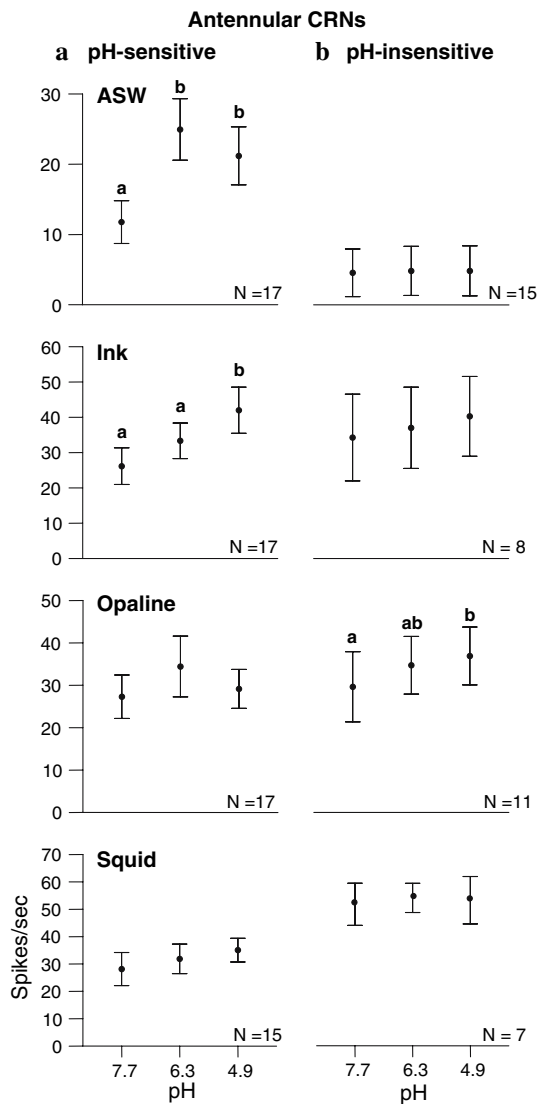


Fig. 3 In the antennules, over 50% of chemoreceptor neurons were highly sensitive to pH change alone and to other stimuli (a), while the rest were insensitive to pH change alone but sensitive to specific stimuli (b). Activity is expressed as mean \pm SEM of the frequency of action potentials (# of spikes/s) to ASW, 1% ink, 1% opaline, and 1% squid juice. The letters represent statistical differences between responses to the pH levels (ANOVA or Wilcoxon followed by post-hoc tests after Bonferroni corrections, $\alpha = 0.05$)

from mouthparts, responded greater to ink and opaline at low pH than at high pH (Fig. 3b). In fact, these CRNs responded significantly greater to opaline at pH 4.9 than at pH 7.7. Furthermore, when pH-sensitive and insensitive CRNs were grouped together, they responded to stimuli (ink, opaline, squid, and sea water) with significantly greater intensity to low pH (4.9) than high pH (7.7) ($P < 0.05$ ANOVA). However, the pH-insensitive CRNs from antennules, like those from mouthparts, showed little or no response to sea water at any pH, although they responded well to other stimuli such as ink and opaline.

Soma recordings from aesthetasc olfactory receptor neurons (ORNs)

Taken in its entirety, the population of 35 aesthetasc ORNs responded to ink in a manner dependent on both stimulus concentration and stimulus pH (Fig. 4). In statistical terms, concentration explained a significant proportion of response variance (partial $\eta^2 = 0.55$ or $F = 46.4$, $P < 0.0001$), with the highest concentration of ink (0.1%) but not the lower concentrations (0.01 and 0.001%) producing significant responses. pH also explained a significant portion of response variance (partial $\eta^2 = 0.12$ or $F = 5.1$, $P < 0.008$). This pH effect was apparent only at the highest concentration of ink, since this was the only concentration that produced significant responses from the overall ORN population. For 0.1% ink, the response to ink was significantly greater at the lowest pH (4.9) than at 6.3 or 7.7.

While the above description is for the entire population of ORNs, the response spectrum of individual members of this population of ORNs varied from each other. This can be seen from qualitative observations. Three examples of ORN responses to 0.1% ink are shown in Fig. 5a–d. The first example is an ORN that responded more to lower pH ink than to higher pH ink (Fig. 5a). Over 60% of recorded ORNs responded similar to this ORN. A second example of the diversity of response spectra of the aesthetasc ORNs, as shown in Fig. 5b, responded more to higher pH ink. A third

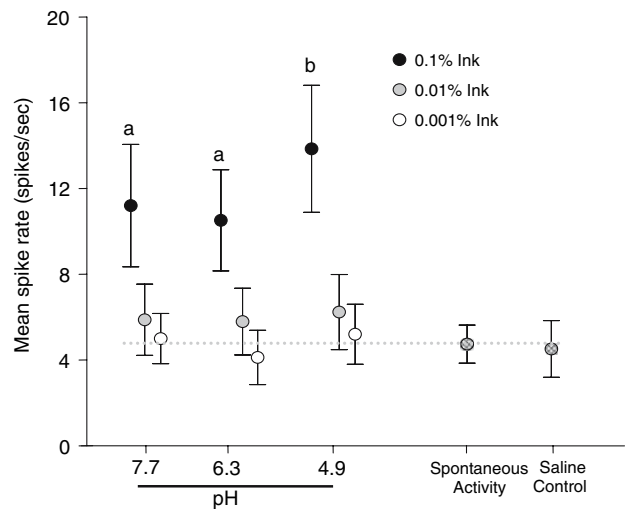
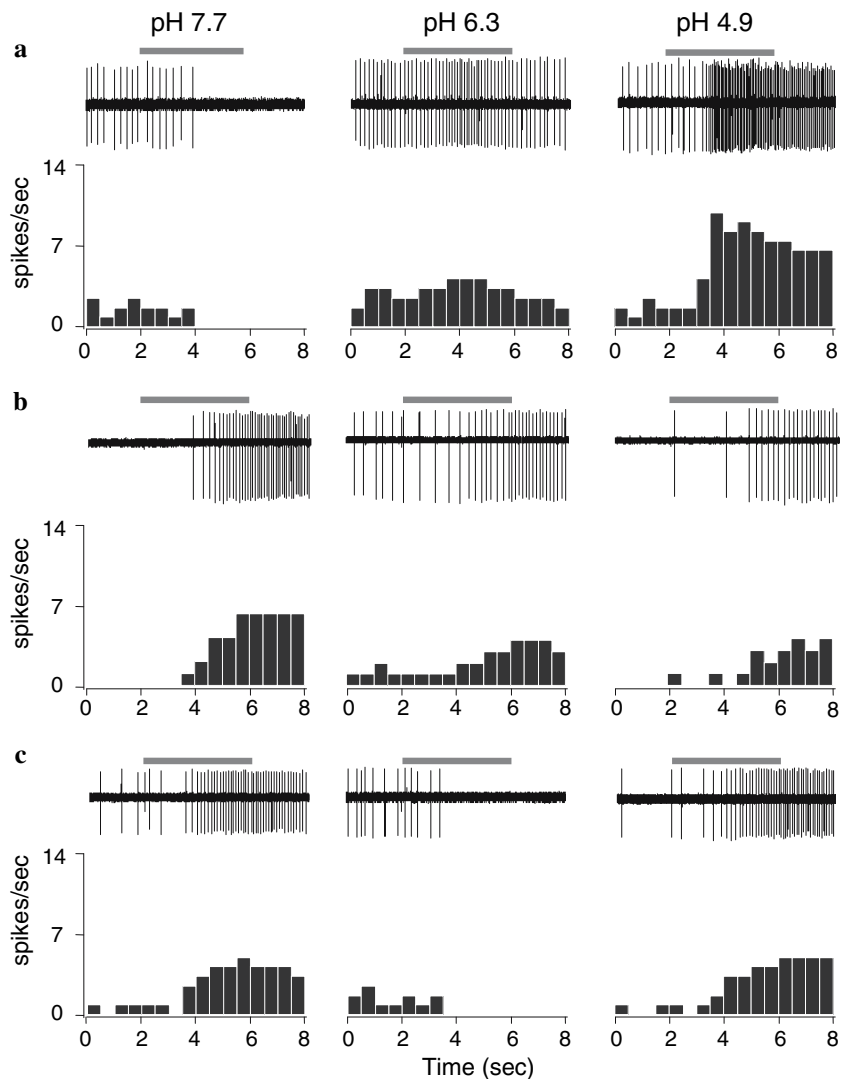


Fig. 4 The response of a population of olfactory receptor neurons ($n = 39$) to high concentrations of chemicals was enhanced at acidic pH. At high concentration of ink (0.1%), the ORN population responded with significantly higher spike frequencies at low pH (4.9) compared to 6.3 and 7.7. The circle and bars represent mean \pm SEM. A dashed line indicates mean spontaneous spiking rate. The letters represent statistical differences between responses to the pH levels at that concentration (ANOVA followed by post-hoc tests, $\alpha = 0.05$). This effect of pH was only seen at the highest concentration of ink (0.1%) and not at the lower concentrations (0.01 and 0.001%)

Fig. 5 Three examples of the diversity of responses of ORNs to stimulation with 0.1% ink at three pH values—7.7, 6.3, and 4.9 (gray line). **a** This ORN responded greatest to acidic ink. **b** This ORN responded greatest to high pH ink. **c** This ORN responded greatest to high and low pH ink but not to mid pH ink



example is an ORN that responded most to either low or high pH ink but little to pH 6.3 ink (Fig. 5c).

Discussion

Our behavioral and electrophysiological study showed that the acidity of *Aplysia's* active defensive secretions enhanced its phagomimetic properties against the predatory spiny lobster *P. interruptus*. The acidity significantly increased the behavioral attraction of spiny lobsters to the secretions, and the effect of pH alone accounted for most of this increase. These behavioral results correspond closely to changes in responses of specific chemoreceptor neurons in the spiny lobster's antennules and mouthparts. Low pH significantly and consistently increased the pH-sensitive neurons' responses to defensive secretions, and low pH alone accounted for most of the increased excitability.

Acidity enhanced CRNs responses to secretions, depending on the type of CRNs, primarily by direct activa-

tion and perhaps secondarily by interacting with secretions. In half of the recorded antennular and mouthpart CRNs, acidity directly activated them and in so doing strongly enhanced the response to the secretions. In the other half of these mouthpart CRNs, acidity did not directly activate them and it did not at all enhance the response to the secretions. Surprisingly however, the antennular pH-insensitive CRNs, though not sensitive to acid alone, showed moderately enhanced responses to acidic secretions. This suggests that for some cells, pH modulates the response to secretions. Overall for the population of antennular or mouthpart CRNs, acid strongly enhances their responses to the secretions.

Similarly, acid enhanced the responses of olfactory receptor neurons in the aesthetasc sensilla (ORNs) to ink, although these ORNs often showed complex response profiles to ink at different concentration and pH values. For example, acid increased the average response of ORNs to ink only at high ink concentration. This may be due to low, and sometimes high, concentration of ink evoking inhibitory

rather than excitatory responses at some pH values (Fig. 5a, c). Such responses were not seen in recordings from antennular and mouthpart CRNs. Because of these complexities in ORNs responses, the overall population of ORNs did not show enhanced excitatory responses to low concentrations of ink.

Acid may affect cellular responses in several ways. One possibility is that the defensive compounds in ink and protons bind to similar sites on the same receptor molecules, i.e. they are competitive agonists. For example, protons enhance nociceptor sensitivity to capsaicin molecule because they bind to and activate similar sites on capsaicin receptors (Jordt et al. 2000; Tominaga and Julius 2000). Similarly, acids interact with bitter, sweet, and amino acid tastants on similar sites of the taste receptor TRPM5 (Liu et al. 2005). A second possibility is that protons affect post-receptor components of the transduction pathway separate from that by other components of ink, i.e. a non-competitive effect. A third possibility is that protons affect the molecular structure of the defensive compounds themselves. For example, ink changes from purple to reddish when pH is increased, indicating that at least some chemical properties are changed. In addition, previous studies have suggested that protons change amino acid charge and ultimately amino acid effectiveness in inducing chemosensory responses (Tierney and Atema 1988). Because low pH sea water can stimulate both behavioral and physiological responses, we do not believe that this third possibility is responsible for our reported effects.

Our results suggest that the acidity of ink and opaline, by enhancing the response of chemosensory neurons to these secretion, may enhance the phagomimetic and/or sensory disruptive defensive properties of these secretions. The secretions are effective in diverting the attack of spiny lobsters toward the defensive secretions, causing grabbing and digging behaviors, and away from the sea hare itself and thus allowing it to escape (Kicklighter et al. 2005). We show here that the acidity of ink and opaline enhances their effectiveness. The acid's stimulation of chemosensory neurons should likewise enhance the sensory disruptive defensive properties of these secretions, although this was not studied in our behavioral tests.

The use of acids as chemical defenses is widespread among marine gastropods. Such acids, including sulfuric acid, are packaged in special glands in the skin (Thompson 1960, 1983, 1986, 1988). When attacked by predators, these glands quickly release copious amounts of mucus laden with sulfuric acid, making the skin highly acidic. For example, irritation of the skin causes skin mucus of *Pleurobranchaea californica* to reach a pH of ~2 (Gillette et al. 1991) and the mucus of *A. californica* to reach a pH of 3–4 (personal observation). How ink and opaline are made acidic—whether by sulfuric acid or other acids—is

unknown. How the acidity in these gastropod skin secretions affects chemical responses of predators is also unknown. Spiny lobsters that grab sea hares will encounter the acidic skin mucus with their legs, mouthparts, and possibly even the antennules. We hypothesize that the acidity of the skin secretions enhances their defensive properties in a manner similar to that which we showed here for ink and opaline.

In summary, our results demonstrate another weapon in the arsenal of active chemical defenses by sea hares—the acids. These acids modify the behavior of predators either by directly stimulating or by indirectly enhancing the chemosensory responses of predators such as spiny lobsters. Anecdotal reports have previously indicated that acids alone or a change in the pH of amino acid stimuli can affect the activity of crustacean chemoreceptor neurons, but without suggesting an ecological context for this effect (Case 1964; van Weel and Christofferson 1966; van Weel and Correa 1967; Johnson and Ache 1978). Therefore, our report provides the first demonstration within a neuroethological and functional context that acids enhance the chemosensory responses of crustaceans.

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