



Hydrogen peroxide and other components in the ink of sea hares are chemical defenses against predatory spiny lobsters acting through non-antennular chemoreceptors

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ABSTRACT

When attacked by a predator, sea hares may release two chemically defensive secretions, ink and opaline. These secretions are mixed in the mantle cavity and then released towards the attacker. In this study of the sea hare *Aplysia californica*, we present data showing that both secretions and hydrogen peroxide (H₂O₂), a product of the reaction between escapin, an L-amino acid oxidase in ink, and lysine in opaline, are able to elicit aversive behaviors from spiny lobsters, *Panulirus interruptus*. The behaviors include mouthpart rubbing and tail flipping (for opaline and H₂O₂), deterring feeding (for ink and H₂O₂), or completely inhibiting feeding (for opaline). The aversive behavior is not elicited through long distance chemoreceptors located in the antennules, as shown by ablation experiments, nor is it due to nonspecific stimulation, as shown by the failure of KCl and highly concentrated positive stimuli to elicit it. A concentration–response curve shows that the ED₅₀ for H₂O₂ is 13.7 mM, an amount well below what is attainable *in situ*. The effect on feeding is demonstrated by the fact that lobsters do not eat shrimp laced with full strength opaline and spend a significantly greater amount of time handling shrimp laced with ink, H₂O₂, or opaline than handling control shrimp. Taken together, these data suggest that opaline, ink, and their mixture contain compounds, one of which is H₂O₂, that can alter a predator's behavior to allow the sea hare to escape attack.

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1. Introduction

Marine organisms are a rich source of secondary metabolites that function as chemical defenses against predators, and there is a very good correlation between the presence of chemical defenses and a lack of other defense mechanisms (e.g., strong shells, speed) (Pawlik, 1993). Chemical defenses are widespread in dinoflagellates (Shimizu, 1993), algae (reviewed in Paul et al., 2007), and a wide array of invertebrates such as sponges, cnidarians, mollusks, and ascidians (Pawlik, 1993). While these compounds make the bearer unpalatable and/or toxic to most predators, some predators have evolved the ability to tolerate them (Glendinning, 2007) and even take advantage of these compounds for their own defense. This is the case for sea slugs, which obtain many of their defensive compounds from red algae (MacColl et al., 1990; Coelho et al., 1998; Prince et al., 1998; Ginsburg and Paul, 2001). In fact, it has been hypothesized that shell loss was only possible after acquiring chemical defenses (Faulkner and Ghiselin, 1983).

Sea hares have a variety of defenses against predators, including passive and active chemical defenses (Johnson and Willows, 1999).

Passive chemical defenses make animals unpalatable and are especially effective when present on their skin or in mucus, as this is the first point of contact with predators (Rogers et al., 2000; Johnson et al., 2006). Passive defensive compounds include algal secondary metabolites that the sea hares derive from their diet (Kinnel et al., 1979; Rogers et al., 2000; Ginsburg and Paul, 2001). Active defenses are secreted by animals when disturbed or attacked (Carefoot et al., 1999; Johnson et al., 2006; Derby, 2007). Active defenses of sea hares include the secretions from two glands that discharge their contents in the mantle cavity, where they are mixed before being pumped towards the attacker via the siphon (Walters and Erickson, 1986; Melo et al., 1998; Carefoot et al., 1999; Kicklighter et al., 2005; Johnson et al., 2006; Derby, 2007). These secretions are a milky and sticky opaline and a purple-colored ink (Johnson et al., 2006; Derby, 2007). This secretion increases the mollusk's probability of survival when attacked by sea anemones (Nolen et al., 1995) or California spiny lobsters, *Panulirus interruptus* (Kicklighter et al., 2005).

Both ink and opaline are highly complex, which gives rise to the idea that they are protective by multiple mechanisms acting simultaneously, such as phagomimicry (i.e., stimulating the predator's feeding pathway at an inappropriate time), sensory disruption through overloading, and feeding deterrence (Kicklighter et al., 2005). The complexity of the system is further enhanced by the fact that some defensive chemicals are only produced when opaline and

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ink are mixed. For example, ink contains an L-amino acid oxidase (LAAO), called escapin in *Aplysia californica* (Yang et al., 2005; Johnson et al., 2006), dactylomelin-P in *A. dactylovela* (Melo et al., 2000; Johnson et al., 2006), and *Aplysia punctata* ink toxin (APIT) in *A. punctata* (Butzke et al., 2005). Escapin's major substrate, L-lysine, is present only in opaline. Thus, when ink and opaline are co-released and mixed, new products are formed not in either secretion alone. Escapin oxidizes lysine, producing its α -keto acid, H_2O_2 , and ammonium, and the α -keto acid spontaneously forms an equilibrium mixture of linear and cyclic molecular species (Derby, 2007; Kamio et al., unpubl. data). Then, H_2O_2 reacts non-catalytically with members of the equilibrium mixture to produce the carboxylic acid of L-lysine and the oxidative product of a cyclic member. For this reason, the α -keto acid and the other members of the equilibrium mixture are called 'escapin intermediate products' and the fully oxidized forms are called 'escapin end products'. Thus, the mixing in the mantle cavity of escapin from ink and lysine from opaline results in a cascade of chemical reactions and alterations that produces several compounds, but whether these chemicals are used as antipredatory defenses is almost completely unexplored (Derby, 2007).

The main objectives of this paper were three. First, we extended the findings of Kicklighter et al. (2005), which have shown that opaline and ink+opaline protect sea hares from lobster attacks. To do this, we performed two types of behavioral assays that complement ('feeding' assays) and extend ('squirting' assays) the previous work. Second, we began elucidating the antipredatory effect of the ink-opaline interaction by analyzing the effects of one of its products, H_2O_2 . Finally, we performed ablation experiments to identify the chemoreceptors involved in the aversive behavior as a first step in their physiological characterization.

2. Material and Methods

2.1. Animals

California spiny lobsters (*Panulirus interruptus*) were collected in the field and shipped overnight to GSU. They were acclimated and tested in individual 40-liter aquaria (50×25×30 cm) containing artificial sea water (ASW, Instant Ocean, Aquarium Systems, Mentor, OH).

2.2. Ink, opaline, and other stimuli

Aplysia californica individuals were chilled, anaesthetized by injection of 60 mL of 0.37 M $MgCl_2$, and dissected to remove opaline and ink glands, which were stored at $-80^\circ C$ until used. Ink was squeezed out of the glands and then centrifuged at $3000\times g$ for 30 min to remove any particles; the resultant supernatant is called 'ink'. Opaline glands were freeze-dried, crushed with a mortar and pestle, and extracted with methanol. The methanol was removed in a rotary evaporator and the resulting material is referred to as 'opaline'. To calculate opaline's concentration, we assumed that the wet glands had the same density as sea water and used this to calculate the volume. 'Shrimp juice' was prepared by immersing one whole frozen/thawed shrimp in 50 mL of ASW for ~1 hr. Potassium chloride and hydrogen peroxide were purchased from Sigma (St. Louis, MO) and TetraMarin (TET), a commercially available fish food, from Tetra Werke, Germany. All solutions were prepared in ASW.

2.3. 'Squirting' behavioral assay

Each assay started by introducing 0.4 mL of shrimp juice (the "search stimulus") in a lobster's aquarium directed away from the animal. This had the effect of eliciting food search behavior, thus making the otherwise difficultly accessible oral region receptors available for stimulation. Once the lobster was actively searching, 1 mL of test stimulus was applied using a handheld pipette and the

behavioral responses (as defined below) were recorded. Preliminary experiments showed that stimuli were more effective when directed at the animal's oral region, and thus this is where we aimed our stimuli. Each lobster was tested with up to 10 stimuli per day, with inter-trial intervals of 10–15 min. If an animal did not respond to the search stimulus or its behavior could not be reliably scored, those data were not used. (However, the first criterion was not used in experiments involving ablations, see Results.)

We used two behaviors as dependent measures in our study. One is tail flipping (TF), which is a very conspicuous and well-characterized defensive behavior (Newland et al., 1992; Nauen and Shadwick, 2001). The second is mouthpart rubbing (MR), a series of movements of the 3rd maxillipeds that consists of rubbing the dorsal aspect of one maxilliped against the ventral part of the other and/or spreading them far apart and/or a series of small, short movements in which the maxillipeds' dactyls are rubbed against each other. None of these movements resemble the stereotyped 'auto grooming' performed after grooming the antennules or when excited by odors (Barbato and Daniel, 1997; Ristvey and Rebach, 1999; Schmidt and Derby, 2005), because there was no dorsoventral rubbing of the maxillipeds' grooming pads. Since 100% of the lobsters that performed TF also performed MR both before and after TF, and since MR was elicited only by stimuli capable of eliciting TF, MR was considered aversive. Thus, we scored each trial as either no response (NR), mouthpart rubbing only (MRo), both mouthpart rubbing and tail flipping (MR+TF), or 'aversion' (MRo or MR+TF). We also assigned a numerical Response Value (RV) to each response category, in order of increasing intensity of aversion: 0 for no change, 1 for MRo, and 2 for MR+TF.

All behavioral observations were scored blind. To achieve this in experiments in which ink was presented, we used ASW containing blue food coloring, which had no behavioral effect (data not shown).

2.4. Effect of ablation of antennular receptors

Two types of ablations of chemoreceptors on the antennules were performed. One was exposure of the antennular flagella to deionized water, which eliminated chemoreceptor function for at least 24 hr and retained antennular integrity and much mechanosensory function (Derby and Atema, 1982; Steullet et al., 2001). Animals so treated were taken from their aquaria, secured and kept moist to avoid desiccation, and their antennular flagella were immersed in deionized water (experimental group) or ASW (sham group) for 30 min. Responses were examined to H_2O_2 both immediately before and 16 hr after treatment. To confirm that the ablation treatment was successful, a food finding assay was performed, in which a small piece of shrimp was introduced in each lobster's aquarium and we recorded the latency of the animals to detect it and to find it. The rationale behind this experiment is that food searching is mediated by antennular chemoreceptors (Derby and Atema, 1982), so that a successful ablation should result in longer times in the experimental group when compared with the sham one.

The second type of ablation consisted of the removal of antennular flagella by amputating them at their base. This treatment had the advantage of being long-lasting. The baseline responses of 14 lobsters to H_2O_2 were determined as explained above, their antennules were then ablated, and their responses to H_2O_2 were determined again 1, 7, and 18 days post ablation.

2.5. 'Feeding' behavioral assay

To evaluate the effect of test compounds on ingestion of food by spiny lobsters, we used pieces of shrimp soaked with a stimulus. Shrimp were cut into small pieces and soaked overnight in deionized water (~300 g of shrimp in 2 L of water) to dilute the chemical attractants and thus allow expression of possible feeding deterrence by additives to the shrimp. The shrimp pieces were then freeze-dried

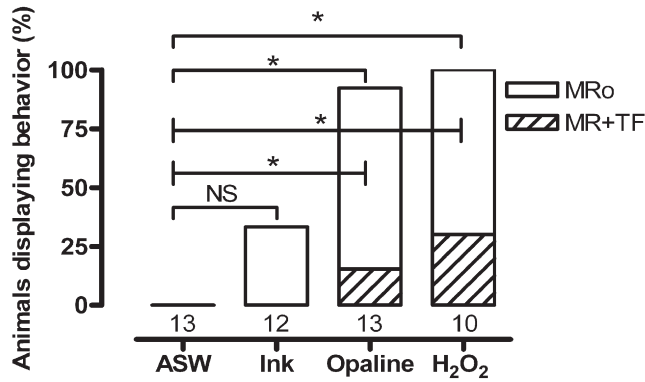


Fig. 1. Effect of opaline, ink, and H₂O₂ on the behavior of spiny lobsters. Responses are the percentages of animals performing only mouthpart rubbing (MRo) (open bars) or both mouthpart rubbing and tail flipping (MR+TF) (hatched bars) when stimulated with the different stimuli after being excited by shrimp juice. Both H₂O₂ and opaline cause a significantly greater number of lobsters to perform MRo than artificial sea water (ASW), while ink, although effective, is not significantly so. Both H₂O₂ and opaline also cause MR+TF, but not in a significant proportion. When both behaviors are taken together (=Aversion, given by the height of the stacked bars), both opaline and H₂O₂ differ significantly from ASW. Statistical differences were evaluated with a one-tailed McNemar's test in all cases. Asterisks indicate $p < 0.05$ when the corresponding section of the bar or the stacked bar are compared to the ASW control. N.S.: Non significant. Numbers below each bar represent the number of animals tested.

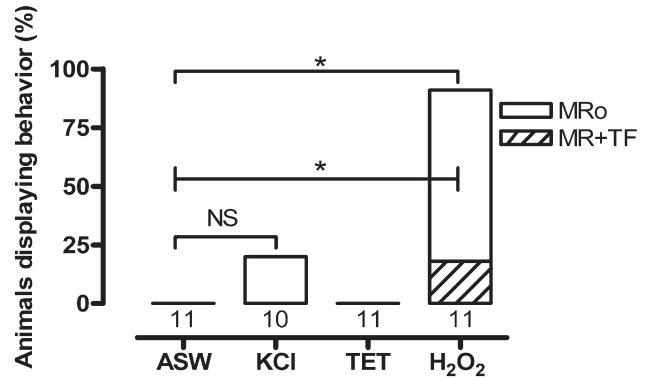


Fig. 2. Non-specific stimuli do not evoke aversive responses in spiny lobsters. Neither 145 mM KCl nor 1 mg/ml TET elicited significant MRo (open bars), MR+TF (hatched bars), or Aversion (sum of animals producing MRo and those producing MR+TF). Statistical comparisons are the test stimuli vs. ASW, using one-tailed McNemar's tests. Symbols are as in Fig. 1.

of animals (Fig. 2). Thus, the mere excitation of a high number of cells is not sufficient to evoke aversion in spiny lobsters.

3.3. Concentration-response relationship

We determined the concentration-response function for H₂O₂ (Fig. 3A and B). The number of animals exhibiting tail flip and aversion

and stored at -20°C until used. For behavioral testing, a shrimp piece was soaked in 500 μL of a test stimulus and immediately offered to a lobster at the end of a long pair of tongs. All shrimp pieces for each lobster were matched by dry weight.

2.6. Statistics

We compared groups either with nonparametric or parametric tests depending on the structure of the data. We used one-tailed tests in all cases because we had hypotheses about the outcome of each experiment regarding the direction of change in the experimental group(s). The concentration response curves were calculated using Probit regression.

3. Results

3.1. Sea hare secretions and their components elicit aversive behavior

Lobsters were made to search for food with shrimp juice and then stimulated with full strength ink, opaline, or 145 mM H₂O₂ (the maximum theoretical concentration from the escapin reaction *in situ*), and their responses were recorded. All three test stimuli caused lobsters to rub their mouthparts, although in the case of ink the effect was not statistically significant (Fig. 1). In addition to rubbing their mouthparts, some animals tail flipped when stimulated with opaline or H₂O₂, although the percentage of them performing this behavior was not significantly different from the control group. When aversion (*i.e.*, grouping together the animals that performed either MR alone or MR+TF) was considered, again both H₂O₂ and opaline produced results different from the ASW control.

3.2. Response specificity

We investigated if the aversion elicited by opaline or H₂O₂ was due to nonspecific activation of receptors located in the stimulated area. This was done by repeating the above experiment but using as test stimuli 145 mM KCl and 1 mg/ml TET. These stimuli should result in a non-specific discharge of chemoreceptors, KCl because it depolarizes neurons and TET because it is a mixture of chemicals that stimulate many chemoreceptor cells (Schmiedel-Jakob et al., 1990; Bobkov and Ache, 2007a,b). Only H₂O₂ evoked a response in a significant number

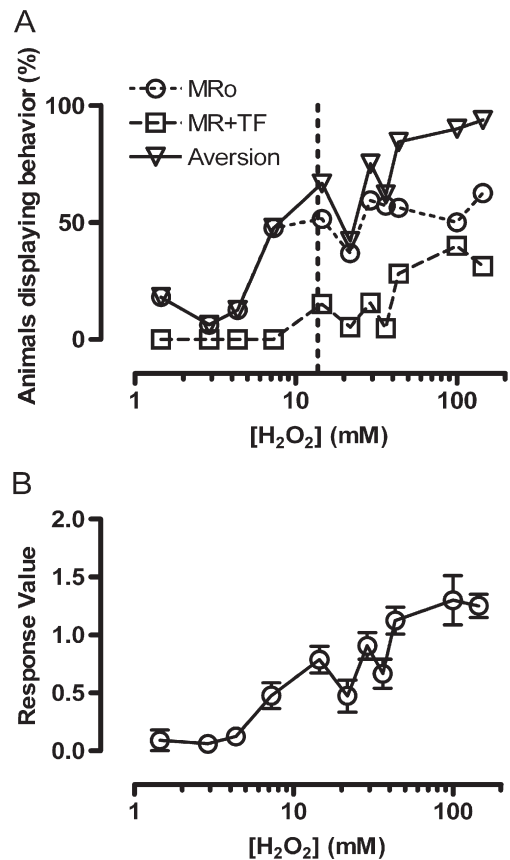
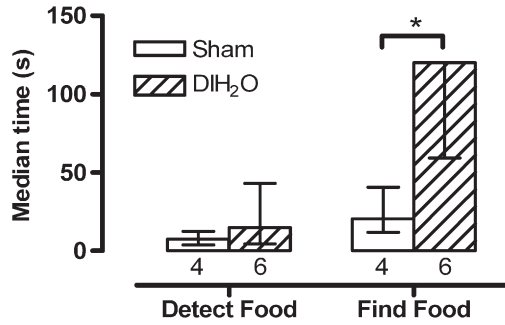


Fig. 3. Concentration-response curves for the aversive effect of H₂O₂ when aimed at the oral region of intact spiny lobsters. A: Percentage of animals within each response category as a function of H₂O₂ concentration. At low concentrations, Aversion (triangles) = MRo (circles) (*i.e.*, there is no TF [squares]); at higher concentrations, TF occurred. The vertical dotted line corresponds to the ED₅₀ for Aversion according to a probit regression: 13.7 mM. N=10–32 animals. B: The same data but showing the Response Value (RV, see Methods). Values are mean \pm S.E.M. The increase in RV with H₂O₂ concentration reflects the emergence of TF at higher H₂O₂ concentrations.

A: Food searching



B: Aversive response

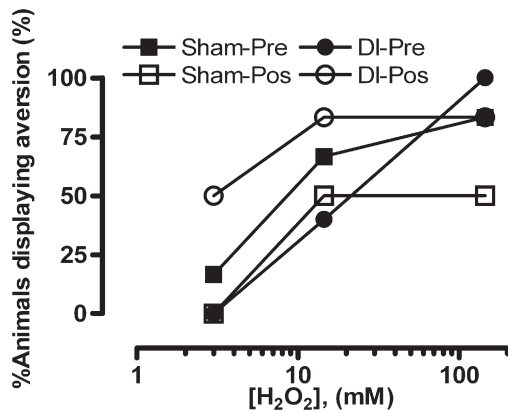


Fig. 4. Effect of antennular chemosensory ablation on search behavior and responses of spiny lobsters to H₂O₂ and food. A: Time needed to detect the introduction of food (left) and to find it (right) for animals with deionized-water ablated antennular chemoreceptors (DIH₂O, hatched bars) or sham-treated animals (Sham, open bars). DIH₂O treatment abolishes food search in experimental animals (*: p=0.0095, one-tailed Mann-Whitney U test). Numbers under bars are the number of animals used in the test. B: Response of animals in A to H₂O₂ before and after antennular treatment. Percentage of animals exhibiting aversion as a function of H₂O₂ concentration. These data show that antennular chemoreceptors are not necessary for the secretion's aversive effects.

increased with H₂O₂ concentration. A probit regression performed on the aversion (MRo or MR+TF) data showed that the median ED₅₀ for H₂O₂ was 13.7 mM, with the 95% confidence interval limits being 9.1 and 20 mM (Fig. 3A). This is also evidenced in Fig. 3B, where the data are plotted as the mean Response Value (see Methods for description of this dependent measure), which also increases with H₂O₂ concentration. Thus, at concentrations well below the maximum theoretically attainable in the secretion, H₂O₂ is capable of eliciting an aversion response in a significant number of animals. It must also be kept in mind that these concentrations are those in the stimulating pipette, and that the concentrations at the chemoreceptors responsible for the behavior may be lower. In addition, it must be kept in mind that escapin is capable of generating low millimolar quantities of H₂O₂ within a few seconds (G. Gadda, S. Collins, K.-C. Ko, C. Derby, unpublished data).

3.4. Location of chemoreceptors for aversive chemicals

Inactivation of antennular chemoreceptors by treatment with deionized water significantly reduced the animals' ability to find a piece of shrimp (Fig. 4A) but did not alter responses to several concentrations of H₂O₂ (Fig. 4B). The responses of both ablation and control groups of lobsters to the introduction of the shrimp piece were similar at first but differed markedly with time. Initially, both groups of lobsters increased the beating of their fan organs (exopodites of

the maxillipeds) and seemed to become alert. After several seconds, sham animals started to move around the aquarium, probing the substrate with their pereiopods until contacting the shrimp, which they proceeded to eat. In contrast, most animals from the experimental group (4 of 6) did not search for food and, consequently, reached the time limit of 120 sec without finding the shrimp. This is reflected in the fact that the groups did not differ in the median times to detect the presence of the shrimp (sham: 7.5 s, experimental: 15 s, p=0.1762, one-tailed Mann-Whitney U test) but the experimental group took significantly longer to find it (sham: 20.5 s, experimental: 120 s, p=0.0095, one-tailed Mann-Whitney U test). In addition, as expected, the search time of the experimental group was reduced to control levels after a further 24 hr (data not shown). In contrast, both groups of animals responded to H₂O₂ stimulation with aversive behaviors (Fig. 4B). These data indicate that antennular chemoreceptors play no role in the aversive effect of H₂O₂. Although no statistical tests were performed due to the low sample size (4–6 animals per concentration), the data suggest that abolishing antennular chemoreception has no effect on the aversive response evoked by H₂O₂. It should be pointed out that the ablation of the chemoreceptors is expected to result in a lack of searching behavior, and thus no response to the searching stimulus in the experimental group. However, we were surprised to find that even the lobsters in the sham group were unresponsive (although they did search when an actual piece of shrimp was introduced in their aquarium). In addition, both groups of lobsters were generally unresponsive to other stimuli (visual and mechanical) that generally are able to elicit responses. We considered this unresponsiveness to be the result of nonspecific effect of the immobilization and thus used an alternative ablation method, in which the lobsters had their antennular receptors ablated surgically, which has the advantage that it is long lasting and allows testing of animals several days after treatment, when their overall responsiveness has returned to control levels. This ablation did not abolish the response to H₂O₂ (Fig. 5), which is in agreement with the results of deionized water ablation. This result supports the idea that antennular receptors are not necessary for this response. It is interesting to note that in both experiments the ablations tended to increase the proportion of animals displaying aversion at low H₂O₂ concentrations.

3.5. Effects of ink and opaline on food ingestion

To examine if secretions from sea hares affect ingestion of food by spiny lobsters, we added 500 µL of test stimuli at different dilutions to pieces of freeze-dried shrimp, examined if the animals consumed or rejected them, and recorded the time it took for the pieces to be

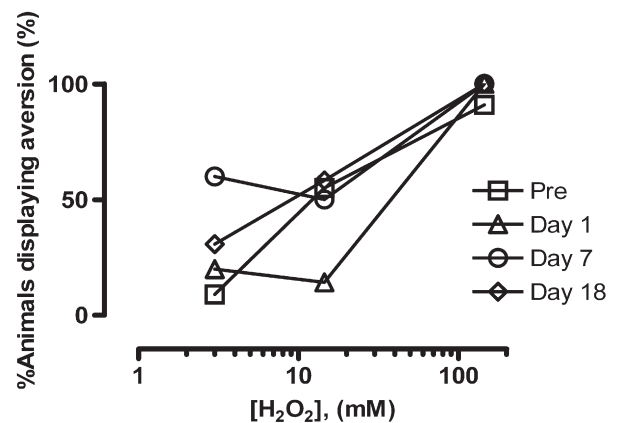


Fig. 5. Effect of surgical removal of antennular flagella on responses of spiny lobsters to H₂O₂. This treatment did not affect responses at 1, 7, or 18 days after surgery. Axes are as in Fig. 4B. N=5–12 animals per group.

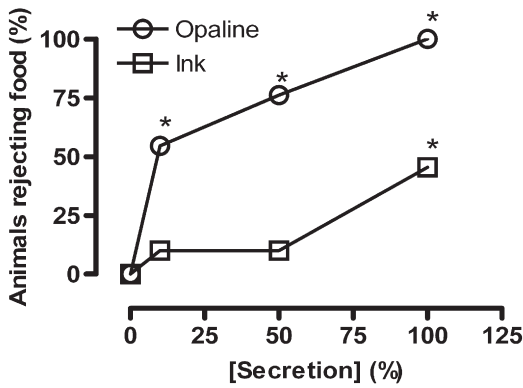


Fig. 6. Concentration-response function of the effect of opaline (circles) and ink (squares) on the percentage of spiny lobsters feeding on secretion-laced shrimp. A higher proportion of animals rejected the laced shrimp as the amount of secretion increased. The asterisks indicate a significant difference when compared with the corresponding 0% (ASW) control ($p < 0.05$ according to one-tailed McNemar's tests, $N = 9$ –12 lobsters per pair). Opaline is more effective than ink at preventing ingestion, and the effect follows a concentration-response curve with an ED_{50} of 11.4% (probit analysis); for ink, only the highest concentration (100%) is significantly different than the control.

ingested or rejected. Both secretions were deterrent in a concentration-dependent manner (Fig. 6). However, there was an important difference between them. Opaline eventually caused 100% of animals to reject the shrimp ($ED_{50} = 11.4\%$, 95% CI: 5.2–21.4%, Probit), whereas ink caused only 42% of animals to reject shrimp and the Probit regression was not significant. In addition, the proportion of lobsters rejecting the shrimp was significantly greater for opaline than for the ASW control at all three concentrations (10, 50, and 100%, $p < 0.05$, one-tailed McNemar's test, $N = 9$ –12 per pair). In the case of ink, only at 100% was it able to cause a significant proportion of the lobsters to reject the shrimp ($p < 0.05$, one-tailed McNemar's test, $N = 12$). The time that lobsters spent manipulating shrimp increased with concentration for ink but not opaline (Fig. 7). In effect, handling varied linearly with the logarithm of concentration for ink ($\text{Time} = 69.1 \log([\text{Ink}]) - 25$, $r^2 = 0.47$, $p < 0.0001$) but not for opaline ($r^2 = 0.051$, N.S.). The difference between the two secretions was further evidenced by a two-way ANOVA, which showed significant effects for secretion type ($F = 27.95$, $p < 0.0001$), secretion concentration ($F = 22.66$, $p < 0.0001$), and the interaction term ($F = 6.057$, $p = 0.0009$). Bonferroni post hoc tests showed that ink handling times were significantly greater for the two higher concentrations (50 and 100%) and that all three ink concentrations but only the highest opaline concentration caused

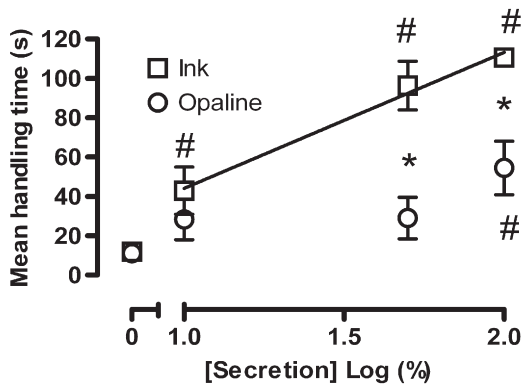


Fig. 7. Concentration-response function of the effect of opaline (circles) and ink (squares) on handling times of spiny lobsters feeding on secretion-laced shrimp. Handling time increased with concentration for ink ($y = 69.1x - 25$, $r^2 = 0.47$, $p < 0.0001$) but not opaline ($r^2 = 0.051$, N.S.). *: Significant differences between the two secretions ($p < 0.05$, Bonferroni). #: Significant difference from the corresponding ASW control (0%, $p < 0.05$ Bonferroni).

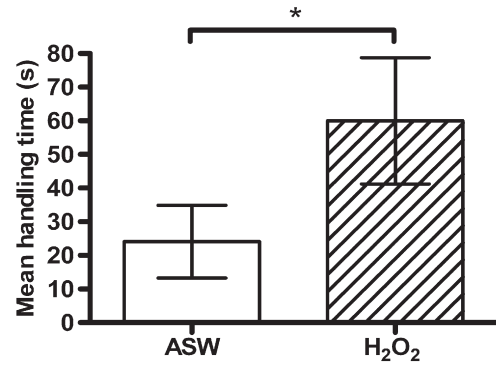


Fig. 8. Effect of H_2O_2 on the handling time of a piece of shrimp. Values are mean \pm S.E.M. Handling time was significantly longer for shrimp containing H_2O_2 than ASW ($t = 3.76$, $p = 0.001$, one-tailed paired t-test, $N = 11$).

animals to handle the laced food significantly more than the control food.

H_2O_2 was a less effective deterrent of ingestion. All animals tested ate shrimp laced with full-strength (145 mM) H_2O_2 (data not shown). Once again, however, lobsters spent significantly more time manipulating H_2O_2 -laced shrimp compared with control shrimp (Fig. 8; 24.1 vs. 60.0 s, $p < 0.001$, one-tailed paired t-test). Thus, although H_2O_2 is aversive to spiny lobsters, it is not by itself an inhibitor of ingestion.

4. Discussion

Our study aimed to understand how the defensive secretions of the sea slug *Aplysia californica* affect its sympatric predator, the California spiny lobster *Panulirus interruptus*. We focused on both ink and opaline to extend previous results from our laboratory (Kicklighter et al., 2005; Shabani et al., 2007). In particular, we wanted to compare the effects of ink and opaline presented with an appetitive stimulus (freeze dried shrimp) and also investigate the effect of ink alone, especially in light of previous findings that show that when added to a neutral carrier it elicits phagomimicry (Kicklighter et al., 2005). We also studied the effect of one of the products of the interaction between ink and opaline, H_2O_2 , which is formed from the reaction of the enzyme escapin, present in ink, with its co-secreted substrate lysine, present in opaline. Additionally, since we previously showed that both ink and opaline, when acting through the antennular chemoreceptors, are attractive to lobsters (Shabani et al., 2007), we wished to investigate the consequences of antennular chemoreceptor ablations on the aversive behavior.

The two behavioral assays used were designed to mimic events that occur when a sea hare is attacked by a lobster. The squirting assay, in which stimuli were released near the oral region of a lobster excited by food odor, represents what happens when a sea hare releases ink and opaline when attacked. The feeding assay mimics the situation in which the predator has bitten the sea hare and thus its mouthparts receive stimuli from both the food (appetitive) and the secretions (appetitive and aversive). In this latter case, the stimuli remain in contact with the chemoreceptors for a prolonged time, as is the case of the sticky opaline secretion.

Our results indicate that the effects of both secretions are complex and that their interaction gives rise to compounds that alter the predators' behavior. For example, opaline elicited aversive behavior (Fig. 1) and inhibited feeding altogether (Fig. 6), which means that in our paradigms it has a purely negative effect on the lobsters despite its high amino acid content (Kicklighter et al., 2005; Derby, 2007; Derby et al., 2007). Ink did not elicit aversive behavior in a significant number of lobsters (Fig. 1) and was only marginally effective in inhibiting feeding (Fig. 6). Finally, H_2O_2 , while very efficient at eliciting aversion (Figs. 1 and 3), was unable to inhibit feeding, and 100% of the lobsters tested consumed the H_2O_2 -laced shrimp (data not shown). It is interesting to note that H_2O_2 was the most effective in eliciting aversion, and this

may be because it lacks the stimulating compounds known to be present in ink and opaline (Kicklighter et al., 2005; Derby et al., 2007).

Ink, opaline, and H₂O₂ modified the ingestion of shrimp in another fashion, i.e., by modifying handling time. When lobsters were offered a piece of freeze-dried shrimp soaked in any of the three test solutions, they manipulated it for much longer than the control ones (Figs. 7 and 8). Once again, however, ink and opaline differed because ink was much more effective than opaline, producing this response at all tested concentrations (# symbols in Fig. 7) and with a greater magnitude than opaline (* symbols in Fig. 7).

We asked whether the aversive response was specific to these chemical defenses or simply the result of over-stimulation, an issue when we take into account that both secretions are complex and concentrated (MacColl et al., 1990; Prince et al., 1998; Russo et al., 2003; Kicklighter et al., 2005; Kicklighter and Derby, 2006; Derby, 2007; Derby et al., 2007). Our results show that this is not the case: neither KCl nor TET elicited aversion in a significant proportion of animals, whereas H₂O₂, the positive control, did so (Figs. 2 and 3). This indicates that the broad and general activity of chemoreceptors, such as is expected when stimulating with the two former solutions, is not sufficient to elicit aversion. Fig. 3A shows that the amount of H₂O₂ necessary to evoke a response in 50% of the animals is well below the maximum possible *in situ*. Furthermore, it shows how as stimulus concentration increases, the animals progress from only rubbing their mouthparts to also tail flipping. This allowed us to assign a value to each behavior, and the result of this analysis confirms that there is a gradient of aversiveness with increasing H₂O₂ concentration (Fig. 3B).

To address the question of the location of receptors for these chemical defenses, we performed two ablation experiments summarized in Figs. 4 and 5. In both cases, we targeted both antennular flagella to disrupt long distance chemoreception. However the methods and results of the two experiments differed. In the first case, we used deionized water, which has the advantage of leaving mechanoreception largely intact while impairing chemoreception for up to 24 hr (Derby and Atema, 1982). The treatment had no effect on the responses to H₂O₂ (Fig. 4B), despite confirming antennular malfunction by showing that ablated lobsters did not search for or find a piece of shrimp introduced into their aquaria (Fig. 4A), a behavior known to be mediated by antennular chemoreceptors (lobsters: Derby and Atema, 1982; Devine and Atema, 1982; Moore et al., 1991; Steullet et al., 2001; crayfish: Kraus-Epley and Moore, 2002; crabs: Keller et al., 2003). One possible issue with this experiment is that it requires immobilization and extensive handling of the animals on one day and a behavioral evaluation on the next. Thus, the profound effects of immobilization and handling *per se*, which were also seen in the sham animals and consisted in a very low level of responsiveness, were a possible confound. To eliminate this possibility, we performed the second type of ablation: amputation of both flagella, which can be done rapidly and its effects last at least until the animal molts. The results of this experiment confirm that the antennular receptors are not required to mediate the aversive behavior (Fig. 5). In addition, no changes were observed over time.

Since both opaline and ink elicit food searching behavior (Shabani et al., 2007) but opaline causes aversion, we surmised that the long- and short- range chemoreceptor pathways could be conflicting. Indeed, although the stimuli were directed at the anterior region of the animals, close to the mouth, we cannot be certain that the antennules were not stimulated, especially because decapod crustaceans direct odor-laden water currents at them (Breithaupt, 2001; Denissenko et al., 2007). For this reason, the experiments discussed above were designed as limited concentration-response ones, under the hypothesis that animals lacking antennular chemoreceptors would be more sensitive to H₂O₂. As can be seen, however, this is not the case.

The overall picture that emerges from these and previous studies (Kicklighter et al., 2005; Shabani et al., 2007) is one in which both secretions, acting through their long-distance (antennular) chemore-

ceptors, elicit food searching behavior but then, at close range, are mainly aversive or deterrent. However, it is important to point out that at close range, long-distance chemoreceptors are still active, which may result in an ambiguous signal. This may help explain why ink stimulates feeding while opaline and the ink-opaline mixture deter it (Kicklighter et al., 2005; Shabani et al., 2007). In addition, the effects of the secretions may depend on the context of presentation. Thus, ink presented in a 'neutral' carrier such as carboxymethylcellulose may cause phagomimicry to divert the lobster from the sea hare (Kicklighter et al., 2005), but when mixed with food (represented *in situ* by the sea hare), ink inhibits feeding (this paper).

It is also interesting to speculate on the effect of the ink-opaline mixture, especially since ink and opaline are usually released together (Johnson et al., 2006). One of the products of this mixture, and the one we investigated in this paper, is H₂O₂. H₂O₂ is produced by escapin's catalysis of lysine, the former in ink and the latter in opaline (Yang et al., 2005; Johnson et al., 2006; Derby, 2007). This so-called 'activated' chemical defense, in which autotoxicity is avoided by storing harmless compounds and only synthesizing harmful or irritating ones when needed (Puyana et al., 2003; Johnson et al., 2006), is known to occur in organisms as diverse as terrestrial plants (Vetter, 2000), freshwater diatoms (Pohnert, 2000), and sponges (Ebel et al., 1997). H₂O₂ was as effective as opaline in eliciting aversion (Fig. 1), and it is significantly aversive even when delivered at concentrations well below the maximum theoretically attainable (Fig. 3): the ED₅₀ for H₂O₂ is 13.7 mM, and the concentration of lysine in opaline might be as high as 145 mM. In addition, it should be stressed that since at present we do not know the exact location of the chemoreceptors mediating this behavior, the actual concentration at the site of chemoreceptors may be even lower. These facts, taken together with the ability of escapin to generate low millimolar concentrations of H₂O₂ in a few seconds (G. Gadda, S. Collins, K.-C. Ko, and C. Derby, unpublished data) make it likely that H₂O₂ plays a role in defending sea hares from lobsters. Although the mechanism of action of H₂O₂ is not clear, it is interesting to note that H₂O₂ is an irritant in other systems such as mouse respiratory system (Fraise, 1999; Gagnaire et al., 2002) and an antibiotic (Imlay and Linn, 1986; Gagnaire et al., 2002). In addition, H₂O₂ can directly activate transient receptor potential (TRP) cation channels of several subfamilies (LTRPC2: Hara et al., 2002; Wehage et al., 2002; TRPM2: Zhang et al., 2007). TRP channels are involved in chemosensory transduction: TRPC2 in olfactory transduction in the vomeronasal organ (Leypold et al., 2002; Stowers et al., 2002; Desai and Clapham, 2005; Zufall et al., 2005), and TRPM5 in vertebrate taste (Liu and Liman, 2003). In spiny lobsters, a growing body of evidence indicates that a TRP-like channel plays a key role in olfactory transduction (Bobkov and Ache, 2005; Bobkov and Ache, 2007a,b).

In addition, and since the effects of opaline and H₂O₂ do not seem to be non-specific, we speculate that there may be specialized chemoreceptors or chemical nociceptors analogous to those in *Drosophila* larvae (Hwang et al., 2007) that also use a TRP channel in the transduction pathway (Tracey et al., 2003). We are currently carrying out physiological experiments in an attempt to identify these receptors.

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