Journal of Experimental Marine Biology and Ecology 388 (2010) 20–27

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Caribbean reef squid, *Sepioteuthis sepioidea*, use ink as a defense against predatory French grunts, *Haemulon flavolineatum*

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ARTICLE INFO

Article history:
Received 17 November 2009
Received in revised form 20 March 2010
Accepted 22 March 2010

Keywords:
Cephalopod
Chemical defense
Chemoreception
Ink
Sepioteuthis
Squid

ABSTRACT

Many animals release secretions in defense against predators. Some marine molluscs, including cephalopods (squid, octopus, cuttlefish) and gastropods (sea hares), release a colored ink secretion. Observational evidence supports the idea that inking is a defensive behavior that protects cephalopods from predators by forming a visual smokescreen or visual mimic (pseudomorph). Another possible function of cephalopod ink is to act against the chemical senses of predators either as a deterrent or distracting food mimic (phagomimic). Experimental tests of both hypotheses are lacking for cephalopods. In our study, we tested the hypothesis that squid use ink as a defense against attacks by predatory fish by performing three sets of experiments to examine the behavior of juvenile French grunts, *Haemulon flavolineatum*, toward ink from Caribbean reef squid, *Sepioteuthis sepioidea*. In the first set of experiments, a pseudomorph assay, in which ink was presented between a fish and a piece of food, assessed effects of ink on the approach and capture phase of a predator's attack. This showed that a pseudomorph of squid ink hindered the attack by significantly delaying food capture as well as evoking significantly more avoidance of or biting at the pseudomorph compared to a control pseudomorph of carboxymethylcellulose. A pseudomorph of carboxymethylcellulose plus food color to simulate the color of squid ink had a similar effect to the squid ink pseudomorph. In a second set of experiments, a disc assay, in which ink was added to meat-flavored paper discs, examined ink's effect on the consumption of food, simulating ink's protective effect if a squid and its ink are taken into a predator's mouth. This showed that squid ink added to meat-flavored discs significantly changed handling of the discs and increased, though non-significantly, their rejection. The same food color as used in the pseudomorph assay, when added to meat-flavored discs, significantly affected handling and rejection of the discs, showing that the food color itself, intended as a control, is unpalatable. In the third set of experiments, the disc assay was used to show that ink did not increase the acceptance of unflavored (i.e. without meat) discs, a result suggesting that ink is not a phagomimic. Our study presents the first experimental results supporting the hypothesis that inking protects squid against predatory fish, and that it acts during both the capture and consummatory phases of attacks: during the capture phase through visual and/or chemical effects against predators, and during the consummatory phase through unpalatable chemicals.

1. Introduction

The evolution of coleoid cephalopods – squid, cuttlefish, and octopus – is strongly driven by active mobile vertebrate predators such as fish, birds, and mammals (Packard, 1972; Aronson, 1991; Hanlon and Messenger, 1996; Wood et al., 2003). As an example of the high exposure of cephalopods to predators, one study of the Caribbean reef squid, *Sepioteuthis sepioidea*, reported an average of seven encounters per hour (Hanlon and Messenger, 1996). Cephalopods have several types of defenses against predators. One of these defenses is the ability to change color, shape, and texture, which can provide crypsis and/or deimatic behaviors that threaten, startle, frighten, or bluff predators (Hanlon and Messenger, 1996; Adamo et al., 2006; King and Adamo, 2006; Hanlon, 2007; Bush et al., 2009). Cephalopods also produce protean behaviors, which include unpredictable erratic escape behavior such as jetting and inking (Hanlon and Messenger, 1996).

Inking involves the ejection of a black mass of chemicals that can take different forms. It may be a diffuse plume, a gelatinous mass known as a pseudomorph, or other forms and shapes (Shimek, 1983;

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doi:10.1016/j.jembe.2010.03.010

Anderson and Mather, 1996; Hanlon and Messenger, 1996; Huffard and Caldwell, 2002; Bush and Robison, 2007). Observational evidence suggests that ink is a visual stimulus that acts as a smokescreen behind which a cephalopod hides or escapes, as a decoy that attracts the attention of the attacking predator, or as some unknown agent that startles or distracts the predator (Hanlon and Messenger, 1996; Bush and Robison, 2007). As an example, Caribbean reef squid threatened by a predator often turn dark before releasing a dark ink pseudomorph (Wood, personal observation). When the squid inks and jets, it often turns lighter and the dark pseudomorph may become the new object of attention of the predator. The fact that some deep water cephalopods produce bioluminescent ink supports the argument that ink functions through visual channels (Dilly and Herring, 1978; Young et al., 1979; Bush and Robison, 2007).

A second possible function of cephalopod ink is that it is a chemical defense against predators. Another group of molluscs, sea hares, use ink as an anti-predatory chemical defense in several different ways. First, sea hare ink acts against a predator's chemical senses, as either an aversive substance that deters the predator's attack to reduce the chance of capture, an unpalatable substance that causes the predator to reject an inking animal once it is taken into the predator's mouth, a phagomimic that distracts or attracts the predator away from the inking animal, or a sensory disruptor that interferes with the predators' sensory systems and thus affects the predator's ability to capture or consume the animal (Nolen et al., 1995; Kicklighter et al., 2005; Kicklighter and Derby, 2006; Derby, 2007; Sheybani et al., 2009; Kamio et al., in press; Nusnbaum and Derby, 2010a,b). Second, sea hare ink acts as an alarm cue for conspecifics, causing them to flee from the vicinity of the ink (Kicklighter et al., 2007).

Experimental support for squid ink acting as a conspecific alarm cue has recently been provided for Caribbean reef squid (Wood et al., 2008). This study demonstrated that squid respond to conspecific ink with deimatic, cryptic, and protean behaviors, which could enhance their escape. This alarm cue was shown to function through visual modality, and might also operate through chemosensory modalities. Earlier support for squid ink as a chemical alarm cue came from the observation that ink from the squid *Doryteuthis opalescens* evokes escape jetting behavior in restrained conspecifics (Gilly and Lucero, 1992; Lucero et al., 1994).

The possibility that cephalopod ink acts as a direct chemical deterrent against predators is supported only by fragmentary and largely anecdotal observations of unrestrained animals (Eibl-Eibesfeldt and Scheer, 1962; MacGintie and MacGintie, 1968; Moynihan and Rodaniche, 1982; Hanlon and Messenger, 1988, 1996; Hanlon et al., 1999; Caldwell, 2005). The fact that some species of squid that live in low-light conditions also release non-luminescent ink can be used as an argument that ink may act chemically (Bush and Robison, 2007). Furthermore, ink from cephalopods is toxic to some animal cell lines (Russo et al., 2003) and protective towards animal cell types (Zhong et al., 2009), raising the possibility that cephalopod ink acts at a cellular level against predators. Ink from some octopuses contains tetrodotoxin (Williams and Caldwell, 2009; Williams, 2010), although its contribution to any deterrent effects has not been experimentally demonstrated.

The possibility that squid ink acts as a phagomimic or sensory disruptor is suggested by chemical analysis of ink of several representative species of squid, octopus, and cuttlefish, which have been shown to contain total free amino acid concentrations of 0.5 to 50 mM (Derby et al., 2007; Wood et al., 2008). Given that amino acids are strong excitants of chemosensory systems and stimulators of feeding behavior of many species of fish, including predators of cephalopods (Valentinčič and Caprio, 1994; Valentinčič et al., 1999; Caprio and Derby, 2008; Derby and Sorensen, 2008), the presence of high concentrations of amino acids in cephalopod ink raises the possibility that ink functions against fish through phagomimicry and/or sensory disruption. In fact, Grüninger (1997) showed that ink from *Octopus bimaculoides* is stimulatory and attractive to moray eels, a finding consistent with the phagomimicry hypothesis.

In this paper, we experimentally examine the effects of ink from the Caribbean reef squid *S. sepioidea* on the behavior of juvenile French grunts *Haemulon flavolineatum*. We chose these fish because they are generalized carnivores that live sympatrically with and are potential predators on young *S. sepioidea*, and they behave well under laboratory conditions (Randall, 1967; Burke, 1995; McFarland and Wahl, 1996). We tested them in two assays, which analyze different phases of predatory attacks: the approach and capture of the prey, and the swallowing of that prey (Endler, 1986; Ritson-Williams and Paul, 2007; Nusnbaum and Derby, 2010b). A 'pseudomorph assay' was used to examine the approach phase of attack. In this assay, ink was presented to grunts as a pseudomorph between them and a piece of food that they were attacking, and we examined if the ink pseudomorph slowed the attack or prevented capture. A 'disc assay' examined the second phase of attack, in which ink was added to meat-flavored discs to determine if ink decreased the likelihood of grunts accepting the discs and thus decreases the palatability of food. We also examined if ink itself was palatable by determining if it induced acceptance of discs without meat flavoring. Our results provide support for ink being a deterrent against grunts, operating during both the approach and consummatory phases of their feeding.

2. Materials and methods

2.1. Animals

Caribbean reef squid, *S. sepioidea* (Blainville, 1823), and juvenile French grunts, *H. flavolineatum* (Desmarest, 1823), were collected in waters around Bermuda using seine and hand nets per Bermuda Government Permit No. SP051002. Squid weighed 43.7 ± 17.0 gm (mean ± S.D.) and had a ventral mantle length of 68 ± 14 mm (mean ± S.D.) upon capture. Squid were kept in a large aquarium and fed live locally-collected silversides, including hogmouth fry *Anchoa choerostoma*, blue fry *Jenkinsia lamprotaenia*, rush fry *Allanetta harringtonensis*, pilchard *Harengula humeralisi*, and anchovy *Sardinella anchovia*. Juvenile grunts were 2.5 to 5 cm long and were held in large aquaria until used for behavioral testing. The fish were fed pieces of squid mantle and other food. Sea hares, *Aplysia dactylomela*, were collected by hand in waters around Bermuda and fed a diet of red algae collected from the same area.

2.2. Collection of ink and preparation of stimuli

Ink was obtained from 18 individual live squid, *S. sepioidea*, with the goal of keeping it as concentrated as possible. Squid were acclimated in the large aquarium for at least one week before collection of their ink. For ink collection, an individual squid was placed in a 32 × 18 × 20 cm acrylic aquarium with sea water and induced to release ink by striking the aquarium. Ejected ink in the pseudomorph form is viscous and held together by mucous. This ink was collected using a turkey baster and placed in a finger bowl. Sea water surrounding the ink was then removed using a 10-ml syringe. This ink was tested on the day of collection as described below.

Ink secretion, which is a mixture of ink and opaline, was collected from five sea hares acclimated in aquaria for several days. Ink was collected by removing a sea hare from the water, handling it until it released ink, and collecting the ink with a pipette. Sea hare ink secretion was tested on the day of collection.

2.3. Behavioral testing arena

Plastic aquaria, 27 cm long × 19 cm wide × 17 cm deep, were placed in a large bench with flow-through sea water pumped in from local waters (19.9–21.8 °C) and in the presence of overhead artificial lighting. Each aquarium had a rock for enrichment, an inlet tube introducing sea water, and an air tube providing additional aeration and circulation. Two grunts were placed in each aquarium to simulate the natural conditions of these schooling fish. Animals were held at least 5 days before behavioral testing, during which time they were fed pieces of food while a pipette was placed in the water, which acclimated them to being hand fed. Once they did not flee upon introduction of the pipette, the experiment began. All fish were fed small pieces of squid mantle or a prepared shrimp several times a day. Fish were not fed 24 h prior to testing. Data were used only if fish ate food at the beginning and end of a behavioral test, which was the case for most fish. An individual fish was used in only one of the two assays described below.

2.4. Pseudomorph assay

This assay examined if squid ink released between grunts and food affected the time to capture the food. This assay is meant to simulate the effect of squid ink on a predator as it approaches the squid. Food was krill-flavored pellets. Pellets were made by combining 3 g of alginic acid (sodium salt) and 5 g of a blend of freeze-dried baby shrimp, mini krill, and jumbo krill that was ground into a powder and mixed in 100 ml of deionized water. This mixture was sucked into a 1-ml syringe and then ejected into a bath of $CaCl_2$. This resulted in a pellet that was subsequently cut into 1-mm long pieces.

Each trial began with the introduction of a food pellet, which was hand delivered by dropping the food at the water surface, and lasted for 120 s. As the fish noticed and began to approach the pellet, 1 ml of a chemical stimulus was gently released from a pipette between the pellet and at the attacking fish. The 1-ml volume released roughly matched the volume of ink released by *S. sepioidea* in natural situations. Three stimuli were used. One stimulus was squid ink, collected as described above. When released from the pipette, the ink formed a pseudomorph between the fish and the food. The ink was more rope-like than the typical pseudomorphs of Caribbean reef squid, being approximately 2–3 mm diameter due to extrusion from the pipette. Rope-shaped pseudomorphs are produced by some species of squid (Bush and Robison, 2007). The flow of water into the aquarium and the aeration were low, such that the ink maintained its structure for the course of the trial. The second stimulus, carboxymethylcellulose, was intended to mimic the consistency of squid ink but lack ink's chemical and visual components. It was prepared by mixing 3 g of carboxymethylcellulose (Sigma-Aldrich #C-5013) in 50 ml sea water to produce a mixture with the consistency of squid ink. The third stimulus, carboxymethylcellulose plus food color, was intended to mimic both the consistency and color of squid ink but lack ink's chemical composition. It was prepared by mixing carboxymethylcellulose at the same concentration as above, plus a mixture of three Food Colors (McCormick and Co., Inc., Sparks, MD) in the following formulation: three drops of Red, three drops of Blue, and two drops of Green, into 50 ml of sea water. The ingredients in these food colors are listed as propylene glycol, propylparaben, and FD&C colors Red 40, Red 3, Yellow 5, and Blue 1. The time from introduction of the pellet until the fish took the pellet into its mouth (i.e. capture) was measured using a hand-operated stopwatch. Each trial was performed by two experimenters, one who introduced the pellet and released the stimulus, one who timed events, and both of whom were observers. Each fish received each of the three stimuli, presented in random order, over the course of one day. At the end of each trial, a turkey baster was introduced into the aquarium and used to remove the stimulus. Data were used only if fish ate food at the beginning and end of a behavioral test. Differences in time to take food pellets into its mouth were statistically analyzed using one-tailed Wilcoxon matched-pairs signed-ranks tests ($n=26$ fish). We used this non-parametric test because the paired data did not meet the assumption of parametric statistical tests, and we used the one-tailed version because we expected that squid ink would increase the time to capture food pellets.

In addition to recording the time necessary for each fish to take each pellet into its mouth, we also recorded other behaviors produced by the fish toward the pseudomorph. These included: ‘avoid’ in which the fish did not move into the pseudomorph but rather swam around it to reach the food pellet; ‘pause’ in which the fish hesitated at the pseudomorph but swam through it to reach the pellet, ‘bite’ in which the fish snapped at the pseudomorph; and ‘no reaction’ in which the fish moved through the pseudomorph without any changes in behavior as it captured the pellet. We compared the distribution of responses to squid ink versus the other stimuli using 2×4 contingency table and χ^2 statistic ($n=26$ fish).

2.5. Disc assay

This assay examined the effect of ink on ingestion of food by a predator once that food is taken into the predator's mouth, and thus is an assessment of squid ink's effect on the palatability of food. This assay is meant to simulate the situation in which a predatory fish takes a squid into its mouth, at which time it experiences the squid's ink. In this assay, fish were presented with a paper disc (2-mm diameter one-ply absorbent paper) soaked until saturated in one of the following five solutions: 1 ml of meat (=squid mantle macerated in seawater) combined with 1 ml of seawater; 1 ml of meat combined with 1 ml of fresh squid ink; 1 ml of meat combined with 1 ml of food color (same as in the pseudomorph assay), used as a visual control; 1 ml of meat combined with 1 ml of sea hare ink, used as a positive control since sea hare ink is deterrent to many fish (Sheybani et al., 2009, Nusnbaum and Derby, 2010a); or sea water.

A trial began with the introduction of a disc into the aquarium, and lasted for 120 s. A hand-help stopwatch was used to time when the first of the two fish took a paper disc into its mouth (capture) or spit it out (reject). Each trial was performed with two experimenters, one to introduce the food, the other to time the events, and both to observe. At the end of each trial, a turkey baster was introduced into the aquarium, either to remove uneaten paper disc or as a control for those trials in which the disc was eaten. Each fish received each of the five discs, presented in random order, over the course of one day. Data were included in the analysis only for fish that ate a piece of squid mantle prior to testing and at the conclusion of the final trial, and only for trials where the fish took the disc into its mouth. We quantified behaviors using two dependent measures of palatability: the percent of fish that ate a disc, and the amount of time the disc was in the mouth or stomach of the fish (i.e. when it was not visible in the aquarium) ($n=39$ fish). The percentages were statistical analyzed using a McNemar test, and the time data were analyzed using a one-tailed Wilcoxon matched-pairs signed-ranks test because the data did not meet the assumptions of parametric statistics and because we expected that squid ink would decrease the palatability of food.

2.6. Phagomimicry assay

This assay and data analysis were the same as the disc assay as described above, except that the discs were soaked in 1 ml of either fresh squid ink combined with 1 ml of seawater, or sea water alone.

3. Results

3.1. Pseudomorph assay: effect of squid ink on approach to food by grunts

We used the pseudomorph assay to measure the amount of time it took a fish to attack a food pellet introduced into the aquarium with different stimuli released between the fish and pellet to determine if squid ink was the stimulus that most strongly delayed attacks. Once a fish initiated an attack and approached the pellet, squid ink was presented as a pseudomorph between the food and fish. Carboxymethylcellulose mixed in sea water to a consistency that simulated the physical properties of squid ink served as one control. Fish ($n = 26$) took significantly longer to take a food pellet into its mouth when squid ink was released (median = 4.10 s) versus carboxymethylcellulose (median = 2.80 s) (Fig. 1A). Thus, the presence of squid ink between a grunt and the food it attacked increased the median time for the fish to take the food into its mouth by 1.30 s compared to the control, which is a 46.4% increase in attack time. The second control was a pseudomorph of carboxymethylcellulose dyed with food color, which produced a delay (median = 4.41 s) statistically similar to that for the squid ink pseudomorph (Fig. 1A). We also tested sea water without carboxymethylcellulose or food color on 19 of the 26 fish, a control that was a diffuse, uncolored cloud compared to the pseudomorphs. The sea water cloud produced a delay similar to that of the carboxymethylcellulose pseudomorph (median = 2.72 s; 25th and 75th quartile intervals = 2.15 and 4.13 s respectively).

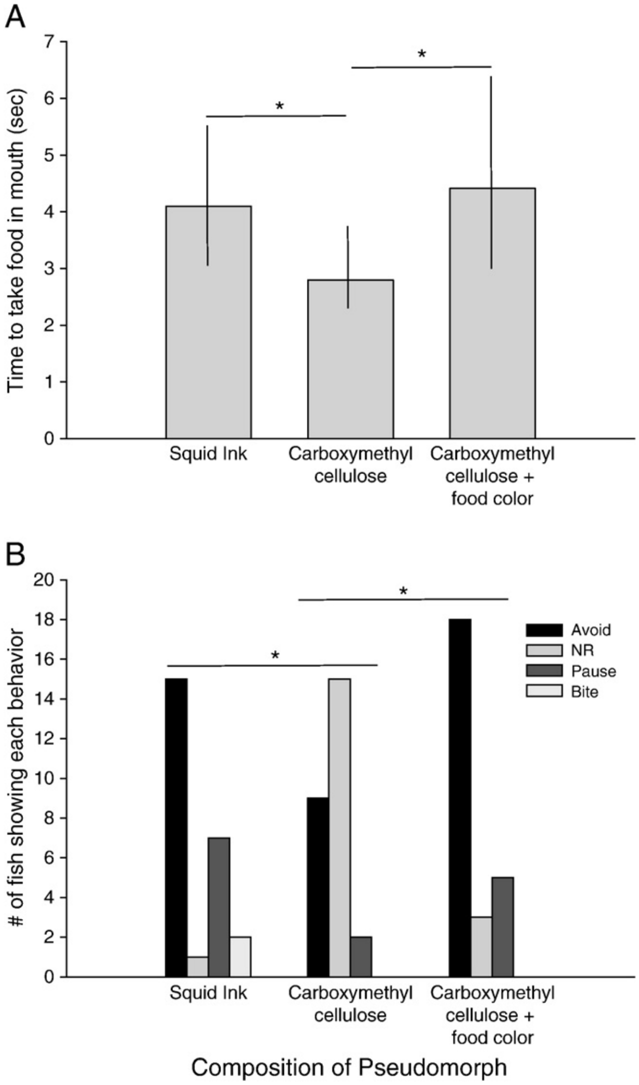

Fig. 1. Pseudomorph assay. Each grunt ($n = 26$) was presented with a food pellet, and as the fish attacked the pellet, the indicated substance was released as a pseudomorph directly between the fish and pellet. The substance was either squid ink, carboxymethylcellulose (at a consistency similar to squid ink), or carboxymethylcellulose with a food color (to simulate the consistency and color of squid ink) (see Materials and methods). A. Time to take the pellet into the mouth. Values are median ± 25th and 75th interquartile intervals for 26 fish. The time to take food into the mouth significantly differed in the presence of these three pseudomorphs (Friedman ANOVA, $\chi^2_{[0.05,\ 2]} = 16.69$, $n = 26$, $p < 0.00024$). Fish took significantly longer to take food pellets into the mouth when presented with a squid ink pseudomorph or pseudomorph of carboxymethylcellulose + food color compared to a carboxymethylcellulose pseudomorph (one-tailed Wilcoxon matched-pairs signed-ranks tests, $p < 0.001$; indicated by asterisks). Responses to the squid ink pseudomorph and pseudomorph of carboxymethylcellulose + food color were not different from each other ($p = 0.790$). B. Behavioral responses of fish to a pseudomorph during feeding attacks. Behaviors were 'avoid', 'no response' (NR), 'pause', and 'bite' at the pseudomorph (see text for definitions). The frequencies of these behaviors in response to a squid ink pseudomorph or a pseudomorph of carboxymethylcellulose + food color were significantly different from those to a carboxymethylcellulose pseudomorph (2×4 contingency tables, $p < 0.00001$; indicated by asterisks) but were not different from each other ($p = 0.452$).

To verify the accuracy of our measurements, two observers independently scored 15 attacks. Scores for the two observers were 4.27 ± 1.68 and 4.22 ± 1.68 s (mean ± S.D.), and the mean difference between the two observers per event was 0.10 ± 0.09 s (mean ± S.D). Thus, our inter-observer accuracy was within 0.10 s, which is a small fraction of value of the dependent measure values themselves. The values of the two observers were highly correlated (Pearson product moment correlation, $R^2 = 0.993$, $p < 0.0000001$).

Behavioral observations also showed a difference in effect of a carboxymethylcellulose pseudomorph and a squid ink pseudomorph on attack behavior of fish (Fig. 1B). Most fish (58%) swam through the carboxymethylcellulose pseudomorph of to reach the food without a change of behavior, and thus scored as 'no response'. Some fish showed 'avoid' (35%) or 'pause' (7%) to the carboxymethylcellulose pseudomorph. They did not 'bite' at the carboxymethylcellulose pseudomorph (0%). On the other hand, to squid ink, fish showed 'avoid' most often (58%). 'Pause' was the next most common behavior (27%). The most common response to carboxymethylcellulose, 'no response' occurred only 7% of the time to ink treatment. 'Bite', which was not observed to carboxymethylcellulose, occurred to ink 11% of the time. The frequencies of these behaviors to carboxymethylcellulose and squid ink were statistically significantly different (Fig. 1B). The behavioral responses to the colored carboxymethylcellulose pseudomorph were statistically similar to that for the squid ink pseudomorph (Fig. 1B).

3.2. Disc assay: effect of squid ink on palatability of food to grunts

We used a disc assay to examine if grunts are less likely to swallow food that contains squid ink. Paper discs soaked in different substances were presented to fish and two measures of palatability were examined.

The first measure of palatability was whether or not the disc was consumed during the 120-s assay. 61.5% of fish ate discs soaked in meat, and 46.1% ate discs soaked in meat + squid ink. The addition of squid ink to meat reduced the percentage of fish accepting discs from 61.5% to 46.1%, which is a strong trend but not statistically significant at α level of 0.05 (Fig. 2A). As a comparison to squid ink, we used sea hare ink, which is highly unpalatable to a variety of marine predators (reviewed in Derby, 2007) including fish (Sheybani et al., 2009, Nusnbaum and Derby, 2010a,b). Discs soaked in meat + sea hare ink were not eaten by any fish, thus demonstrating that a strong chemical deterrent can completely deter feeding of grunts in this assay. Grunts also did not eat the negative control: discs soaked only in sea water. Interestingly, discs soaked in meat + food color (the same as used in the pseudomorph assay) were eaten by only 23.1% of the fish, significantly less than for meat alone.

The second measure of palatability was the time that the fish held the disc in its mouth or stomach during the 120-s assay. The higher the palatability of the disc, the higher the value of this variable. The median time for discs soaked in meat was 115 s. Fish in this condition took the disc into their mouth quickly and accepted it, thus swallowing it immediately. This time is significantly longer than the median time for discs soaked in meat + squid ink, which was 9 s (Fig. 2B). Fish in this condition typically also quickly took the disc into their mouth, but then rejected it quickly. As a comparison, discs soaked in meat + sea hare ink were held in the mouth only 10 s (6 and 21 s = 25th and 75th quartiles), showing similar behavior as to the

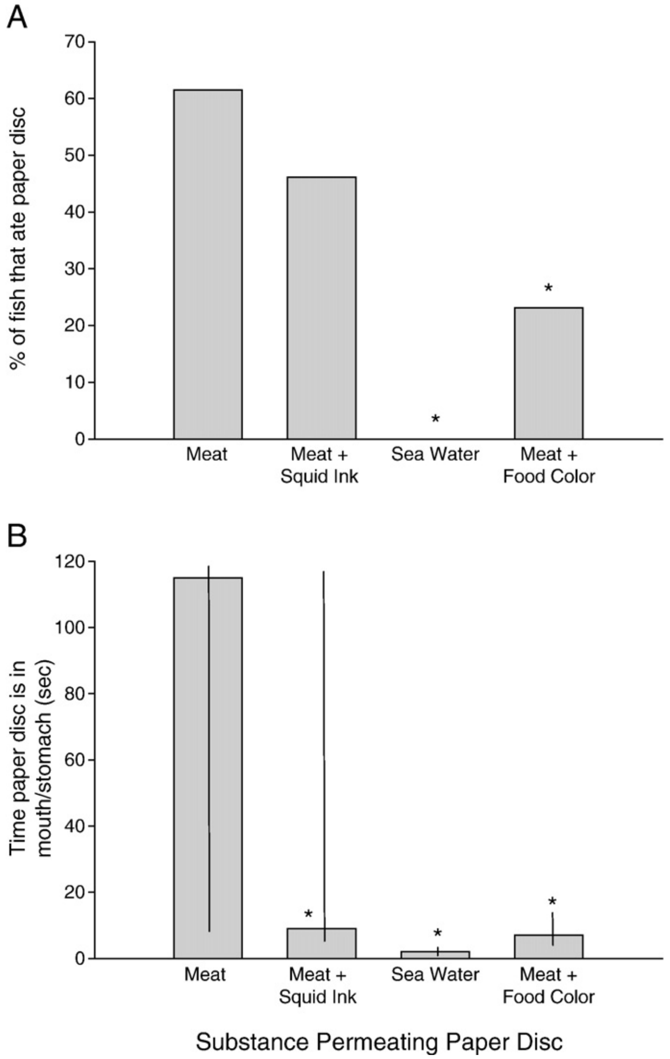

Fig. 2. Disc assay. Each grunt ($n=39$) was presented with a paper disc soaked in each of the indicated substances, one at a time, and assayed for palatability using (A) acceptance of disc (eaten or not within the 120-s assay) and (B) time that the disc was in the mouth or stomach during the 120-s assay. Substances were 1) Meat, 2) Meat + Squid ink, 3) Sea water, and 4) Meat + Food color. Values are median ± 25th and 75th interquartile intervals for 39 fish. (A) Adding squid ink to meat reduced the % fish accepting (i.e. consuming) the discs from 61.5% to 46.1%, which is a strong trend but not statistically significant (one-tailed McNemar test, $n=39$ fish, $p=0.119$). Adding food color to meat significantly reduced the acceptance of meat-flavored disc, to 23.1% acceptance (one-tailed McNemar test, $p<0.001$). Discs soaked in sea water only were not eaten. Conditions significantly different than the meat-flavored discs are indicated by an asterisk. (B) The time that the paper disc was in the mouth/stomach of the fish significantly differed for the four conditions (Friedman ANOVA, $\chi^2_{[0.05,\ 3]}=67.955$, $n=39$ fish, $p<0.00001$). Conditions significantly different than the meat-flavored discs are indicated by an asterisk. Adding squid ink to meat significantly decreased the time that fish held the disc in their mouth or swallowed it (one-tailed Wilcoxon matched-pairs signed-ranks test, $Z=1.846$, $p=0.0324$). Adding food color to meat also significantly reduced this variable ($Z=3.543$, $p=0.0002$). Discs soaked only in sea water were held in the mouth/stomach significantly less than the meat-flavored discs ($Z=5.428$, $p<0.0000001$).

disc with meat + squid ink except that the 75th quartile was much lower for discs with meat + sea hare ink, signifying that few fish held the disc in their mouth for very long or swallowed it. Discs soaked in sea water only were held in the mouth for only 2 s (Fig. 2B), signifying that they quickly rejected these discs because they did not have the palatable meat flavoring. Additionally, discs soaked in meat + food color were held in the mouth for only 7 s (4 and 15 s = 25th and 75th quartiles), signifying that the food color, meant to dye the discs without adding any flavor, was in fact unpalatable.

3.3. Phagomimicry assay: is squid ink by itself palatable to fish?

We also tested discs soaked only in squid ink, to examine if fish find squid ink palatable. Only 1 of 39 fish tested ate such discs (2.6%), and the median time in mouth or stomach was 3 s (25th and 75th quartiles = 1 and 5 s respectively). This response is not different from discs soaked in sea water (median = 2 s, 25th and 75th quartiles = 1 and 3 s respectively, as shown in Fig. 2B). The behavioral responses to these two conditions were similar: fish quickly rejected the discs after taking them into their mouth. This shows that squid ink by itself is not palatable to grunts, thus providing no evidence for phagomimicry.

4. Discussion

4.1. Ink as a defense against predatory fish

Our experimental study tested the hypothesis that squid use ink as a defense against attacks by an ecologically relevant fish predator. We examined the effects of ink from the Caribbean reef squid, *S. sepioidea*, on the behavior of juvenile French grunts, *H. flavolineatum*, in three sets of experiments. In one set, an ink pseudomorph released between the food and fish significantly changed the fish's behavioral responses, causing avoidance of or biting at the ink pseudomorph and a delay in time to capture the food. In the second set of experiments, ink added to a meat-flavored paper disc reduced its palatability as measured by handling and acceptance. In the third set, ink added to an otherwise unflavored disc did not increase the disc's palatability as measured by handling and acceptance. Although observational evidence from prior studies supports a defensive role of cephalopod ink against predators (Eibl-Eibesfeldt and Scheer, 1962; MacGintie and MacGintie, 1968; Fox, 1974; Moynihan and Rodaniche, 1982; Shimek, 1983; Hanlon and Messenger, 1988, 1996; Anderson and Mather, 1996; Grüninger, 1997; Hanlon et al., 1999; Caldwell, 2005; Bush and Robison, 2007; Wood et al., 2008), ours is the first experimental demonstration that squid ink is an effective defense against predators.

4.2. Mechanisms of deterrence by squid ink

The deterrent properties of squid ink could result from any of its sensory attributes. Ink is generally assumed to defend cephalopods by acting through the visual modality of predators. Ink could form a visual smoke screen behind which the inking cephalopod can hide or escape, as a decoy that attracts the attention of the predator, or as some unknown stimulus that startles or distracts the predator (Huffard and Caldwell, 2002; Caldwell, 2005; Adamo et al., 2006; Bush and Robison, 2007). Alternatively, ink might function through the chemical sensory modalities of predators, as a chemical defense. Ink of other marine animals such as sea hares and other gastropods are chemical defenses against a diversity of predators (Carefoot, 1987; Johnson and Willows, 1999; Wägele et al., 2006; Derby, 2007). Ink is also viscous due to mucus, which could contribute to its defensive properties as has been demonstrated for other viscous secretions (Greenwood et al., 2004; Brown and Bythell, 2005; Lim et al., 2006).

Our analysis of the sensory modalities through which squid ink affects the behavior of fish demonstrated that ink acts through the chemical senses and possibly also through vision. The disc assay demonstrated that squid ink acts through the chemical senses of fish by showing that meat-flavored discs taken into the mouth of fish were not as readily eaten if squid ink was added to them (Fig. 1). Thus, the disc assay provides clear evidence that ink is a chemical defense that can make food less palatable. In nature, ink's protective effect might occur if a fish predator took a squid into its mouth and at which time it experienced the squid's ink, leading to an unpalatable experience and subsequent rejection of the squid. The disc assay might also simulate encounters in nature when a fish bites an ink pseudomorph or ink cloud during an attack, leading to an unpalatable experience that derails the attack. It is also possible that a negative sensory experience in an encounter of a fish predator with ink could lead to a learned avoidance of squid in subsequent encounters, a hypothesis consistent with the observation by Caldwell (2005) that green sea turtles that bit an ink pseudomorph of *Octopus bocki* during an attack on the octopus did not attempt to bite a second octopus.

Our pseudomorph assay allowed us to examine ink's mechanism of action when encountered by a fish as it approaches a squid. In this assay, we used two types of control stimuli. One control, a carboxymethylcellulose pseudomorph, was designed to mimic the consistency of ink but to lack the color and chemicals of ink. A second control, carboxymethylcellulose plus food color, was designed to mimic both the consistency and color of ink. The use of both controls was designed to determine if ink operates through the visual and/or chemosensory modalities of the fish. We found that the carboxymethylcellulose pseudomorph was less effective than the squid ink pseudomorph in slowing attacks. Grunts took significantly longer to take a food pellet into its mouth when released with a squid ink pseudomorph compared to a carboxymethylcellulose pseudomorph (median of 4.10 s for squid ink versus 2.80 s for carboxymethylcellulose) (Fig. 1A). This 1.30 s difference is a 46.4% increase in attack time compared to the carboxymethylcellulose pseudomorph. The second control, the pseudomorph of carboxymethylcellulose dyed with food color, produced a delay (median = 4.41 s) statistically similar to the squid ink pseudomorph (Fig. 1A). The effect of the carboxymethylcellulose pseudomorph was similar to that of a diffuse cloud of sea water without either carboxymethylcellulose or food color, suggesting that tactile features of ink, in and of themselves, do not significantly slow attacks. The pseudomorph of carboxymethylcellulose plus food color had the same effect as the squid ink pseudomorph, with both significantly compromising appetitive feeding behaviors compared to a pseudomorph of only carboxymethylcellulose (Fig. 1). At face value, this might lead one to conclude that ink protects squid from fish through the visual modality and not through chemosensory modalities. But upon further consideration, this conclusion is suspect, because the food color used in the pseudomorph assay, when used in the disc assay, was an unpalatable chemical deterrent. When added to a meat-flavored disc, the food color significantly reduced acceptance of the disc, from 61.5% acceptance for meat-flavored discs with no additives to 23.1% acceptance for meat-flavored discs with food color. In comparison, 46.1% of the fish accepted meat-flavored discs with squid ink, 17.9% accepted meat-flavored discs with sea hare ink, and 0% accepted discs with only sea water (Fig. 2). Thus, the food color was more unpalatable than squid ink and as unpalatable as sea hare ink secretion — which is highly unpalatable to many marine predators (Kicklighter et al., 2005; Kicklighter and Derby, 2006; Derby, 2007; Aggio and Derby, 2008; Kamio et al., in press; Nusnbaum and Derby, 2010a,b). The ingredients of the McCormick Food Colors are propylparaben (a preservative), propylene glycol (a carrier), and FD&C Red 40, Red 3, Yellow 5, and Blue 1. We do not know which of these are contributing to the unpalatability of the food color in our experiments or if they also affect extra-oral function. It is interesting to note that McCormick Blue 1 (also known as Brilliant Blue) elicits escape jetting in the squid *D. opalescens*, and this is due to propylparaben (Gilly and Lucero, 1992). Given that the food color used in our experiments caused rejection of food taken into the mouth and thus is probably acting on intra-oral chemical senses, it is also quite possible that the food color acts in the pseudomorph assay extra-orally as an aversive stimulus, operating through the fish's olfactory or other extra-oral chemical senses. Thus, we cannot be certain whether the food color's effect in the pseudomorph assay was due to its visual or chemical properties. In addition, the mucus consistency of squid ink probably contributes to its effectiveness by providing the physical form of the pseudomorph. The mucus could act in concert with the chemical components in ink as a sensory disruptor by sticking to chemosensory surfaces and interfering with reception of chemical cues, as is the case for sea hare ink (Kicklighter et al., 2005; Derby, 2007). More experiments are necessary to identify the mechanisms of deterrence in the pseudomorph assay.

The functional importance of the effect of the squid ink on attacks by fish – i.e., a 1.30 s increase in time for the fish to capture the food in our assay – is revealed by considering the escape swimming speeds of squid. Speeds attained during escape swimming have been estimated for *Loligo* spp. of the size of *S. sepioidea* used in our assay to be from ~1 m/s (O'Dor, 1982, 1988) up to 10 m/s (Vogt, 1987). Using O'Dor's (1988) estimate of an average escape velocity of 1.4 m/s, an ink-induced delay of 1.30 s during a predatory attack on a 17.5-cm long squid allows the squid to move 1.82 m, or 10.4 body lengths. Thus, a 1.30-s increase in predation time gives a squid sufficient of time to accelerate away, change color, and eject more ink pseudomorphs.

We did not find support for squid ink functioning as a phagomimic. French grunts did not eat discs soaked in squid ink, and thus squid ink as a whole did not defend by evoking feeding responses and therefore distracting the fish away from the squid, as was shown for sea hare ink against predatory spiny lobsters (Kicklighter et al., 2005). Whether the amino acids or other components of squid ink have a phagomimetic or sensory disruptive effect remains to be tested.

4.3. Comparative biology of molluscan defenses

Our work, taken together with past work, shows that squid defend themselves through a variety of mechanisms. The ability of squid and other coleoid cephalopods to rapidly change their appearance by altering their color, shape, and texture is well documented. These behaviors allow them to become cryptic or to threaten, startle, frighten, or bluff predators (Hanlon and Messenger, 1996; Messenger, 2001; Mather, 2004; King and Adamo, 2006; Adamo et al., 2006). Fast escape through jetting is another effective defense. We show here that ink can slow the attack of predators. Another effect of ink is as an alarm cue, evoking escape responses from conspecifics that are not directly attacked by the predator but that are in the vicinity and detect the ink released by neighbors (Wood et al., 2008).

Our work adds support to a more general conclusion that inking molluscs use their ink secretion as a defense, both by acting directly on predators to deter attacks, and by acting as an alarm cue that neighboring conspecifics detect and respond with escape behaviors. Prior work with other inking molluscs, sea hares, shows that their secretion provides these two forms of defense (Derby, 2007; Kicklighter et al., 2007). Sea hare chemical defenses, however, appear to be much stronger than those of squid as is evident from the many passive defensive compounds identified from the body tissues of sea hares compared to squid (Kamiya et al., 2006; Lim et al., 2007). This was shown for active chemical defenses in our study in which sea hare ink was more effective than squid ink in causing French grunts to reject otherwise palatable food. The high effectiveness of sea hare ink as a conspecific alarm cues is evident from the fact that it evokes escape responses in most animals, even at a 1:500 dilution (Kicklighter et al., 2007). The greater effectiveness of sea hare ink compared to squid ink as a chemical defense may be because the sluggish sea hares have fewer options in their defensive repertoire than do squid. Sea hares are slower and cannot change their visual appearance as rapidly as squid. Chemicals also persist for some time after release, which makes them a more useful defensive option for sea hares given that their slow rate of locomotion requires more time for escape. It may be that squid living in deep and dark waters with predators that rely less on visual cues have more effective chemical defenses compared to species such as the Caribbean reef squid that live in relatively shallow and clear waters.

Our work is the first to directly test and demonstrate the deterrent properties of squid ink on predatory fish. Future work could determine the chemical basis for these deterrent effects, test whether

Caribbean reef squid use ink to deter attacks of other fish species or whether other species of squid and other cephalopods use ink to deter attacks by its predators, and compare the behavioral responses to and chemical properties of ink from cephalopods living in different environments.

Acknowledgments

We thank NSF-REU Award (OCE-0552453) of Bermuda Institute of Ocean Sciences for supporting NSF-REU interns Eva Sawyer and Kelly Pennoyer. This work was partially supported by NSF grant IBN-0614685 to Charles Derby. We also thank Kim Zeeh for logistical support. **[SS]**

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jembe.2010.03.010.

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