ELSEVIER

Journal of Experimental Marine Biology and Ecology 334 (2006) 256–268

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

www.elsevier.com/locate/jembe

Multiple components in ink of the sea hare *Aplysia californica* are aversive to the sea anemone *Anthopleura sola*

Cynthia E. Kicklighter *, Charles D. Derby

Department of Biology, Center for Behavioral Neuroscience, and Brains and Behavior Program, Georgia State University, Atlanta, GA 30302, United States

Received 5 December 2005; received in revised form 8 February 2006; accepted 8 February 2006

Abstract

Sea hares of the genus *Aplysia* rely on an array of behavioral and chemical defenses, including the release of ink and opaline, to protect themselves from predation. While many studies have demonstrated that ink and opaline are repellent to predators, very little is known about which components of these secretions are active against predators. Ink was previously shown to facilitate the escape of *Aplysia* from predatory anemones (*Anthopleura*) by eliciting tentacle retraction and/or shriveling, and gastrovascular eversion, but the metabolites mediating this interaction were not identified. We investigated the metabolites in *Aplysia californica* secretions that were aversive to the anemone *Anthopleura sola*, as demonstrated by tentacle shriveling and/or retraction. We found that ink elicited tentacle shriveling and/or retraction, while opaline elicited a feeding response. The active components in ink do not appear to be diet-dependent, as ink was aversive regardless of diet (natural seaweed diet vs. *Gracilaria ferox*). Furthermore, metabolites extracted from *G. ferox* were not aversive, suggesting that the aversive components are produced by the sea hares. We then examined escapin, a protein in ink with antimicrobial properties. Escapin quickly forms reaction products when mixed with the amino acids L-lysine and L-arginine, which would occur when ink and opaline are released into the sea hare mantle cavity. Neither escapin alone nor escapin mixed with its amino acid substrate L-lysine elicited aversive behaviors either immediately before or 2 min before applying to the tentacles. In addition, escapin mixed with opaline and applied to tentacles after 2 min did not elicit a significant aversive response. Using bioassay-guided fractionation, we attempted to isolate the components in *A. californica* ink that are aversive to *A. sola*. We determined that multiple components in ink, including both lipophilic and hydrophilic constituents, elicited aversive responses. We hypothesize that these components may facilitate *A. californica*'s escape from *A. sola* by eliciting tentacle shriveling and/or retraction, which lead to anemones dropping ensnared sea hares.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Chemical defense; Opaline; Predator–prey

1. Introduction

Unlike many molluscs, sea hares of the genus *Aplysia* lack a shell for protection from attacks by predators. Instead, they rely on a variety of other anti-predation strategies, such as cryptic coloration (Thompson, 1960), distasteful skin or body wall (Ambrose and Givens, 1979; Kinnel et al., 1979), and the secretions ink and

* Corresponding author. Georgia State University, Department of Biology, P.O. Box 4010, Atlanta, GA 30302-4010, USA. Tel.: +1 404 463 9995; fax: +1 404 651 2509.

E-mail address: ckicklighter@gsu.edu (C.E. Kicklighter).

0022-0981/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jembe.2006.02.002

opaline (Johnson and Willows, 1999). When a sea hare is disturbed or attacked by a predator, it may release ink and opaline independently from two glands (Tritt and Byrne, 1980; Walters and Erickson, 1986; Prince et al., 1998), mixed in the mantle cavity and directed toward the site of attack (Walters and Erickson, 1986; Walters et al., 1993; Johnson and Willows, 1999).

Several studies have demonstrated that ink and opaline can act as anti-feedants to predators such as birds, fishes, crustaceans and sea anemones (DiMatteo, 1981, 1982; Walters et al., 1993; Pennings, 1994; Nolen et al., 1995; Kicklighter et al., 2005), suggesting that ink and opaline may serve as chemical defenses facilitating the survival of *Aplysia* when attacked by predators. Thus far, only two direct tests of the survival value of ink and opaline have been performed. Nolen et al. (1995) demonstrated that ink facilitated the escape of *Aplysia californica* from the sea anemone *Anthopleura xanthogrammica* (based on the radial pattern on the oral disk of their *A. xanthogrammica*, the species was most likely misidentified and was probably *Anthopleura sola* (Pearse and Francis, 2000)), eliciting gastrovascular eversions and tentacle shriveling and retraction. Using the California spiny lobster (*Panulirus interruptus*) as a predator, Kicklighter et al. (2005) found that ink and opaline allowed *A. californica* to survive predatory attacks via multiple mechanisms including phagomimicry, sensory disruption and deterrence.

Despite the number of studies documenting that *Aplysia* ink and opaline secretions repel predators or arrest predation, only Kicklighter et al. (2005) have identified the specific active components of these secretions. In their study, free amino acids (such as taurine) in ink and opaline were identified that stimulated appetitive behavior in lobsters, causing them to attend to a false food stimulus—the secretions.

From an analytical standpoint, much is known regarding the composition of ink and opaline. Several studies have shown that ink contains pigments from the sea hare's red algal diet, along with proteins and low molecular mass components (e.g., Christomanos, 1955; Nishibori, 1960; Winkler, 1969; Troxler et al., 1981; MacColl et al., 1990; Prince et al., 1998; Yang et al., 2005). Opaline contains considerable proteinaceous material and some brominated metabolites (reviewed in Johnson and Willows, 1999). In spite of the considerable chemical and biochemical efforts to elucidate the constituents of ink and opaline, whether these components influence predator behavior is not known. Identification of chemical defensive molecules is important to help elucidate the mechanisms by which they operate against predators, leading to a better understanding of the processes governing predator–prey interactions.

One of the known components in *A. californica* ink is an L-amino acid oxidase–escapin (Yang et al., 2005). Escapin catalyzes the oxidation of L-amino acids, especially L-lysine and L-arginine, forming ammonia, hydrogen peroxide and α-keto-acids and carboxylic acids of lysine and arginine (Wellner and Meister, 1961; Lukasheva and Berezov, 2002; Yang et al., 2005). Together, these products are bacteriostatic and bactericidal (Yang et al., 2005). Opaline is the major source of substrate for escapin (Kicklighter et al., 2005; Johnson et al., 2006): opaline has 65 mM lysine and <1 mM arginine; lysine and arginine are <1 mM in ink. Thus, escapin and large doses of its substrate are mixed when sea hares release ink and opaline when attacked by predators. Escapin's natural function is not likely to be an antimicrobial agent, since escapin is not present in any other *A. californica* tissues or secretions aside from ink (Johnson et al., 2006), and the ink–opaline mixture is usually released away from the sea hare and does not normally coat the animal, as would be expected of an antimicrobial agent. Thus, we hypothesize that escapin functions as an anti-predatory defense. Escapin has been shown to lyse tentacle cells and zooxanthellae of *A. sola*, but only under unnatural conditions—at very high concentrations (higher than natural concentration) and over extremely long exposure periods (several hours) (Johnson, 2002). However, the effects of escapin or any of its orthologues (see reviews in Yang et al., 2005; Butzke et al., 2005) on the behavioral responses of potential predators have thus far not been reported in other sea hares.

Here, we investigate the components in *A. californica* secretions that may be responsible for deterring *A. sola* predation on sea hares. We chose this predator because *A. californica* secretions have been demonstrated to deter *Anthopleura* predation (Nolen et al., 1995) and *Anthopleura* has been shown to consume *A. californica* in the field (Winkler and Tilton, 1962). Using behavioral assays with the anemone *A. sola*, we demonstrate that ink, but not opaline, is aversive and that escapin is not responsible for ink's major aversive effects. Utilizing bioassay guided fractionation, we attempted to identify the aversive components in ink, and show that multiple components in ink cause anemone tentacles to shrivel and retract.

2. Materials and methods

2.1. Animals and animal care

Anemones (*A. sola* Pearse and Francis, 2000) were collected off the coast of California by Aquatic Research Consultants (San Pedro, CA). Adult sea hares

(*A. californica* Cooper, 1863) (200–300 g) used for collection of ink and opaline secretions were collected off the coast of California by Marinus Scientific (Garden Grove, CA, USA). We also obtained late juvenile *A. californica* (~90 g) from the NIH National Resource for *Aplysia* (Key Biscayne, FL) to investigate the effects of ink from mariculture-raised *Aplysia* on anemones. All animals were maintained in our laboratory in aquaria with recirculating, filtered and aerated artificial sea water (Instant OceanTM, Aquarium Systems, Mentor, OH) at 20 °C. *A. californica* was fed a diet of *Gracilaria ferox* J. Agardh (obtained from the NIH National Resource for *Aplysia* Facility), while *A. sola* was fed pieces of shrimp. Sea hares were usually dissected and their ink and opaline secretions collected within 1 week after receipt from suppliers.

2.2. Collection of secretions from Aplysia

To collect ink and opaline secretions, sea hares were chilled for 4 h at 4 °C. Animals were then anesthetized by injection of 120–180 ml isotonic $MgCl_2$ and glands were removed by dissection. Ink was collected by gently squeezing ink glands with a spatula and opaline was collected by spinning opaline glands at 30,000×*g* to separate the fluid from the gland tissue. Both secretions were frozen at −20 °C until used. The protein escapin was purified from ink via gel filtration and ion exchange chromatography, as described by Yang et al. (2005).

2.3. Anemone bioassays

2.3.1. Ink and opaline

To investigate the effects of *A. californica* ink and opaline on anemones (and all other stimuli—see below), we used a total of 15 anemones, all of which were used in multiple experiments. For each experiment, anemones were removed from their holding aquaria and placed individually in containers (14 cm×21.5 cm) filled with 1.5 l of seawater. Anemones were allowed to acclimate for several minutes until normal behavior was displayed, as demonstrated by the opening of their oral disk and extension of their tentacles. Only anemones that exhibited this behavior within 20 min were used in the bioassay. For all bioassays, 50 μl of seawater was applied slowly (over 2–3 s) to one tentacle of each anemone with a micropipette. Anemones whose tentacles responded by shriveling or retracting within 5 s were excluded from the bioassay. Tentacle retraction was defined as a shortening of at least one half of the tentacle length into the oral disk, while tentacle shriveling occurred when tentacles decreased in circumference and became wrinkled. Usually, both of these behaviors were usually observed in a given tentacle; however, sometimes only one type of behavior was observed. When this occurred, it was usually retraction (~95% of these cases). We used these behaviors as a measure of anemone aversion because Nolen et al. (1995) observed tentacle retraction and shriveling in *A. sola* when exposed to ink released by ensnared *A. californica*; these behaviors often caused the anemones to drop the sea hare, allowing it to escape. In our assay, anemones that did not exhibit shriveling or retraction of their tentacles in response to seawater were then randomly tested with 50 μl of either full strength ink or opaline (or other stimuli described below), applied as described above. Because the color and consistency of ink and opaline are very different from each other, we attempted to make imitations so that the experimenter would not know the identity of the stimuli being tested; however, this was not successful. Thus, this assay could not be conducted blind, but we attempted to apply all stimuli to the anemone tentacles in the same manner. Stimuli in all other assays were tested blind unless indicated. Responses of anemones to ink and opaline (and all other stimuli described below) versus the seawater control were analyzed with a one-tailed McNemar test ($\alpha=0.05$), since we expected an aversive response larger than the seawater control.

2.3.2. Escapin

Because ink caused aversive responses in anemones, we bioassayed the protein escapin, which is present in ink but not opaline (Yang et al., 2005; Johnson et al., 2006). First, we tested 50 μl of seawater, as a negative control. If an anemone did not respond to seawater, then 50 μl of escapin, L-lysine (which is a substrate for escapin and is present in high concentrations in the secretions (Yang et al., 2005)), escapin+L-lysine and ink (as a positive control) were tested in random order. Because escapin tends to float when applied to anemone tentacles, we solubilized it in 0.004 g/ml carboxymethylcellulose (sodium salt, high viscosity, Sigma-Aldrich, Inc., St. Louis, MO) in seawater to increase the density of the stimulus. Thus, to control for effects of the carboxymethylcellulose, all stimuli (except for ink) were solubilized in this carboxymethylcellulose solution. By solubilizing concentrated samples of escapin and L-lysine in carboxymethylcellulose, we made solutions of each at the natural concentrations at which they occur in ink and opaline, respectively (L-lysine occurs at a much lower concentration in ink than opaline

(Kicklighter et al., 2005; Johnson et al., 2006)). Thus, escapin was tested at 0.028 mg/ml (Johnson et al., 2006) and L-lysine at 65 mM (Kicklighter et al., 2005). The mixture of escapin and L-lysine was tested on anemones by pipetting 25 μl of each of these two solutions at the same time so that they would mix as they were released onto the tentacles. This release was accomplished by attaching two 1-ml syringes side-by-side such that their plungers were depressed simultaneously. We performed the assay in this way to simulate as naturally as possible the release of these stimuli. Upon attack by predators, sea hares typically co-release ink and opaline into their mantle cavity and then expel this mixture from their siphon (Walters and Erickson, 1986; Johnson et al., 2006). By this simulated natural release, escapin has several seconds to oxidize its amino acid substrates and produce its reaction products.

To address the following possibilities: (1) that a longer reaction time was necessary for escapin to react with its substrate to generate concentrations of products sufficient to elicit behavioral effects, or (2) that other components in opaline (including arginine or other unidentified molecules) react with escapin to form aversive products, we mixed 25 μl each of escapin (solubilized in carboxymethylcellulose solution) and opaline in a 1.5-ml microcentrifuge tube and waited 2 min before applying to sea anemone tentacles. This is sufficient time for escapin to completely oxidize its amino acid substrates (Yang et al., 2005). Along with escapin + opaline, the other stimuli included escapin (mixed with carboxymethylcellulose), opaline and ink (as a positive control), which were tested in random order.

2.3.3. Ink from different seaweed diets and seaweed extract

Because sea hares of the genus *Aplysia* are known to sequester compounds from their seaweed diet, we investigated whether the aversive components in ink may be diet-dependent. Thus, we also tested ink collected from *A. californica* raised solely on a diet of *G. ferox*, which does not occur off the coast of California (Setchell and Gardner, 1967; Smith, 1969), and thus would not contribute to the diet of *Aplysia* raised in the mariculture facility. To determine whether aversive components are present in seaweed (which may be sequestered by *Aplysia*), the metabolites in *G. ferox* were extracted by placing the seaweed in a volume of 100% methanol equal to approximately two times the volume of seaweed. The seaweed was then cut up into small pieces with scissors and the solvent was filtered to remove particulates. The seaweed was then extracted with 100% ethyl acetate and then 100% water. The filtered solvents from each of these three extracts were combined and placed on a rotary evaporator to remove the solvents, leaving only the chemical extract from the seaweed. This extract was solubilized in an appropriate volume of carboxymethylcellulose solution so that it was tested at natural concentration. Because the seaweed extract and ink (positive control) are of different color and because creating an adequate seaweed extract mimic was not successful, the experimenter was not blind to the stimuli being tested.

2.4. Ink bioassay-guided fractionation

To track down the molecule(s) in ink responsible for aversive responses of sea anemones, we utilized bioassay-guided fractionation, as detailed in Fig. 1. First, ink was partitioned via a Kupchan et al. (1975) scheme. This scheme yielded five partitions, soluble in hexanes, chloroform, ethyl acetate, butanol or water (Fig. 1A). Each fraction was solubilized in a volume of carboxymethylcellulose solution equivalent to the volume of ink extracted (thus, each fraction was tested at the natural concentration at which it occurs in ink) and tested in the bioassay (as described above). In these and subsequent ink fractionation assays, all anemones were tested with a carboxymethylcellulose seawater negative control and ink positive control. Most fractions were tested on all anemones; however, some anemones were not tested because they balled up on their own or were accidentally touched by the experimenter. More lipid-soluble partitions (hexanes, chloroform and ethyl acetate) were solubilized in a 20-ml vial in a 3:1 solution of hexanes, chloroform or ethyl acetate and carboxymethylcellulose solution. The solutions were rotary evaporated to remove the organic solvent, leaving the remaining extract solubilized in the carboxymethylcellulose solution. An appropriate volume of carboxymethylcellulose solution was added to the extract solution so that it was at a natural concentration. The extracts were then tested on anemones. The chloroform, ethyl acetate, butanol and water fractions each elicited a significantly higher frequency of tentacle shriveling and/or retraction than the seawater control. Thus, we attempted to isolate the aversive component(s) in each of these three fractions.

To purify the chloroform fraction, we passed it through silica gel (Davisil, 60 Å, 200–425 mesh, Fisher Scientific, Pittsburgh, PA); fractions of 5 ml volume were collected and combined according to thin-layer chromatography (TLC) characteristics, producing five fractions for bioassay (Fig. 1B). None of the fractions

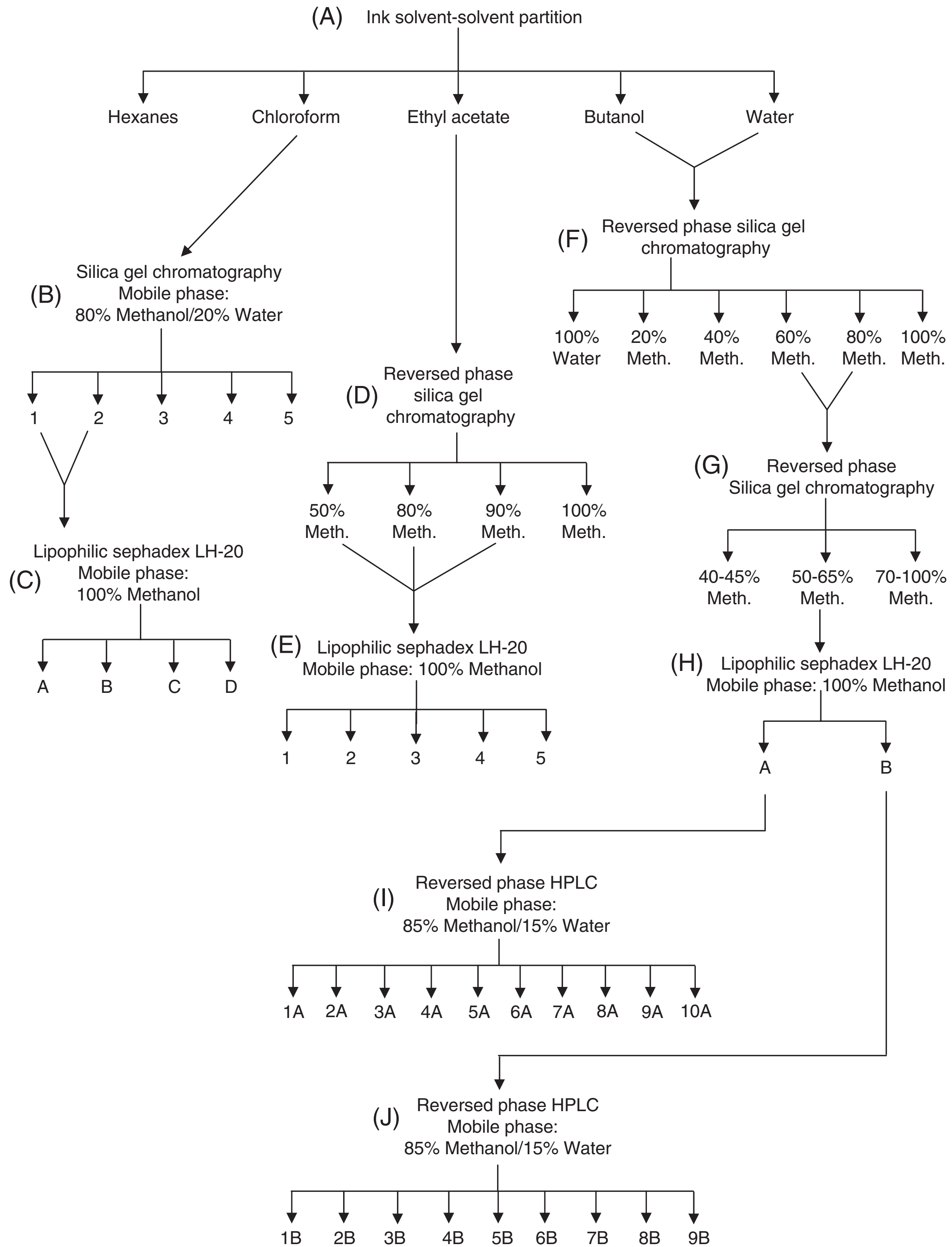

Fig. 1. Flow chart depicting the bioassay-guided fractionation of ink. (A) Solvent–solvent partition, (B) silica chromatography separation of chloroform fraction, (C) lipophilic sephadex separation of aversive fraction from B, (D) reversed phase silica separation of ethyl acetate fraction, (E) lipophilic sephadex separation of aversive fraction from D, (F) reversed phase silica separation of water+butanol fractions, (G) reversed phase silica separation of aversive fractions from F, (H) lipophilic sephadex separation of aversive fraction from G, (I) reversed phase HPLC separation of aversive fraction A from H and (J) reversed phase HPLC separation of aversive fraction B from H.

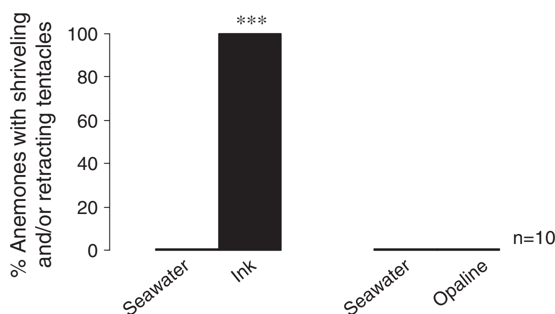

Fig. 2. Frequency of anemones showing tentacle shriveling and/or retraction in response to seawater, ink (full strength) and opaline (full strength). n = no. of anemones tested. Analysis by a one-tailed McNemar test: *** = significantly different from seawater at $p \leq 0.001$.

was aversive when tested alone; however, when fractions one and two were combined, they elicited a frequency of tentacle retraction and/or shriveling that was significantly higher than the carboxymethylcellulose control. The active components were then further purified via lipophilic sephadex LH-20 (Amersham Biosciences, Pittsburgh, PA). This produced four fractions, one of which was aversive (Fig. 1C). Further purification was not possible due to loss of aversive activity.

The ethyl acetate fraction was further purified by passing it through a reversed phase silica column, flushing with increasing concentrations of methanol (Fig. 1D). The 50%–90% methanol fractions were aversive; these were combined and purified via lipophilic sephadex (Fig. 1E). This yielded five fractions, none of which was aversive. However, when fractions one and two were combined, this combination was aversive to anemones. Further purification was unsuccessful due to loss of aversive activity.

The butanol and water fractions had similar TLC characteristics, so they were combined and further purified via reversed phase silica chromatography (Fig. 1F). Both the 60% and 80% methanol fractions were aversive to anemones; they were combined and purified by passing through another reversed phase silica column (Fig. 1G). The fractions were combined into three groups: 40–45% methanol (fraction 1), 50–65% methanol (fraction 2) and 70–100% methanol (fraction 3). Only fraction two elicited an aversive response by anemones. This fraction was purified by lipophilic sephadex, which yielded two fractions, A and B, both of which elicited an aversive response by anemones (Fig. 1H). Fraction A was further purified via high pressure liquid chromatography (HPLC) (Beckman 125S solvent module coupled to a Beckman 168 photodiode array detector, using a Phenomenex Luna reversed phase C18 silica 5 μ column [250 mm × 10 mm]). This yielded 10 fractions, two of which were aversive (Fig. 1I).

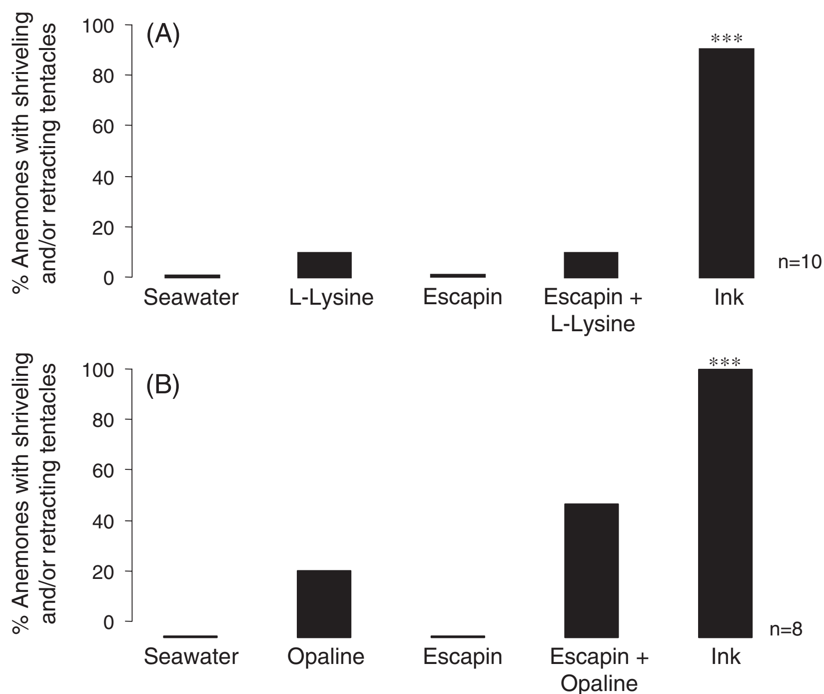

Fig. 3. Frequency of anemones showing tentacle shriveling and/or retraction in response to (A) seawater, L-lysine, escapin, escapin + L-lysine and ink, and (B) seawater, opaline, escapin, escapin + opaline and ink. Symbols and analysis are as in Fig. 1.

Fraction B was also purified in the same manner, yielding nine fractions, two of which were aversive (Fig. 1J). Further analyses of these fractions were not possible due to loss of aversive activity.

3. Results

3.1. Ink, opaline and escapin

Ink, but not opaline, elicited retraction and/or shriveling of *A. sola* tentacles. All tested animals showed aversive responses to ink, while none showed aversive responses to opaline (Fig. 2).

The primary aversive effects of ink are not due to the antimicrobial ink protein escapin. Neither escapin nor the products formed by escapin's reaction with lysine when the two were mixed as they were applied to the tentacles, elicited aversive effects at a frequency different from the seawater control (Fig. 3A). In addition, when escapin and opaline were mixed for 2 min before release onto the anemone's tentacles, thus giving time for escapin to complete the oxidation of its amino acid substrates, this mixture did not produce a significant frequency of retraction and/or shriveling (Fig. 3B).

3.2. Diet dependence

The aversive components in ink did not appear to be dependent upon a specific red algal diet. Ink from sea hares raised in the laboratory solely on *G. ferox* and ink from sea hares collected in the field (and eating a natural diet composed of multiple seaweed species), both elicited significant shriveling and/or retraction of anemone

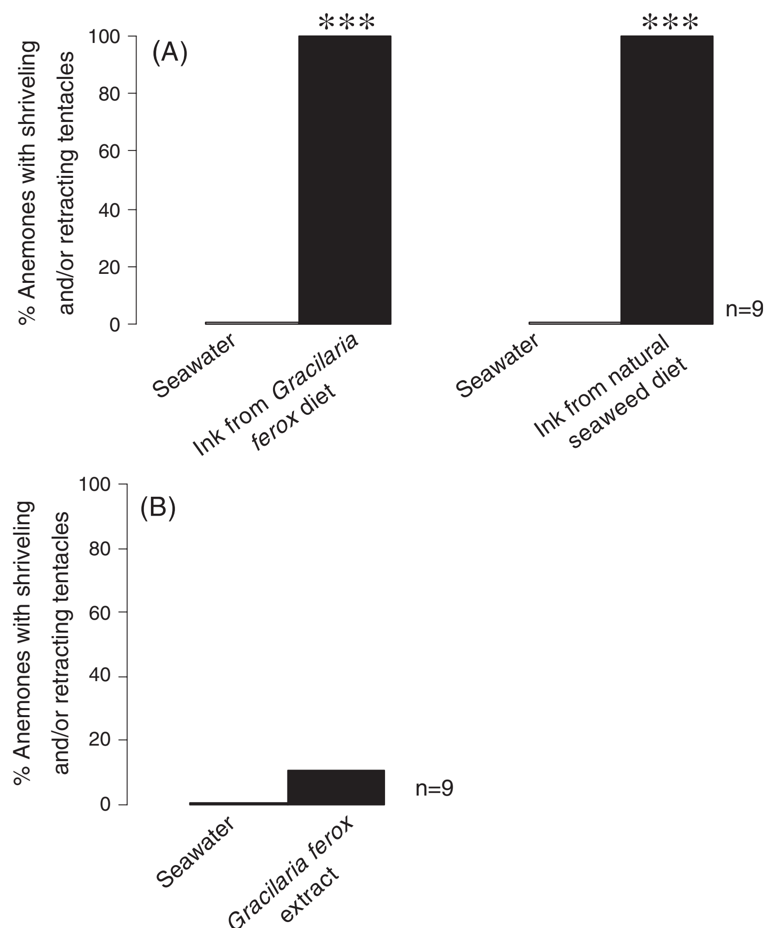

Fig. 4. Frequency of anemones showing tentacle shriveling and/or retraction in response to (A) seawater, ink from sea hares raised in the laboratory on a diet of *Gracilaria ferox*, and ink from sea hares collected from the field and thus feeding on a natural seaweed diet; and (B) seawater and an extract of the red alga *G. ferox*. Inks were tested at full strength. Symbols and analysis are as in Fig. 1.

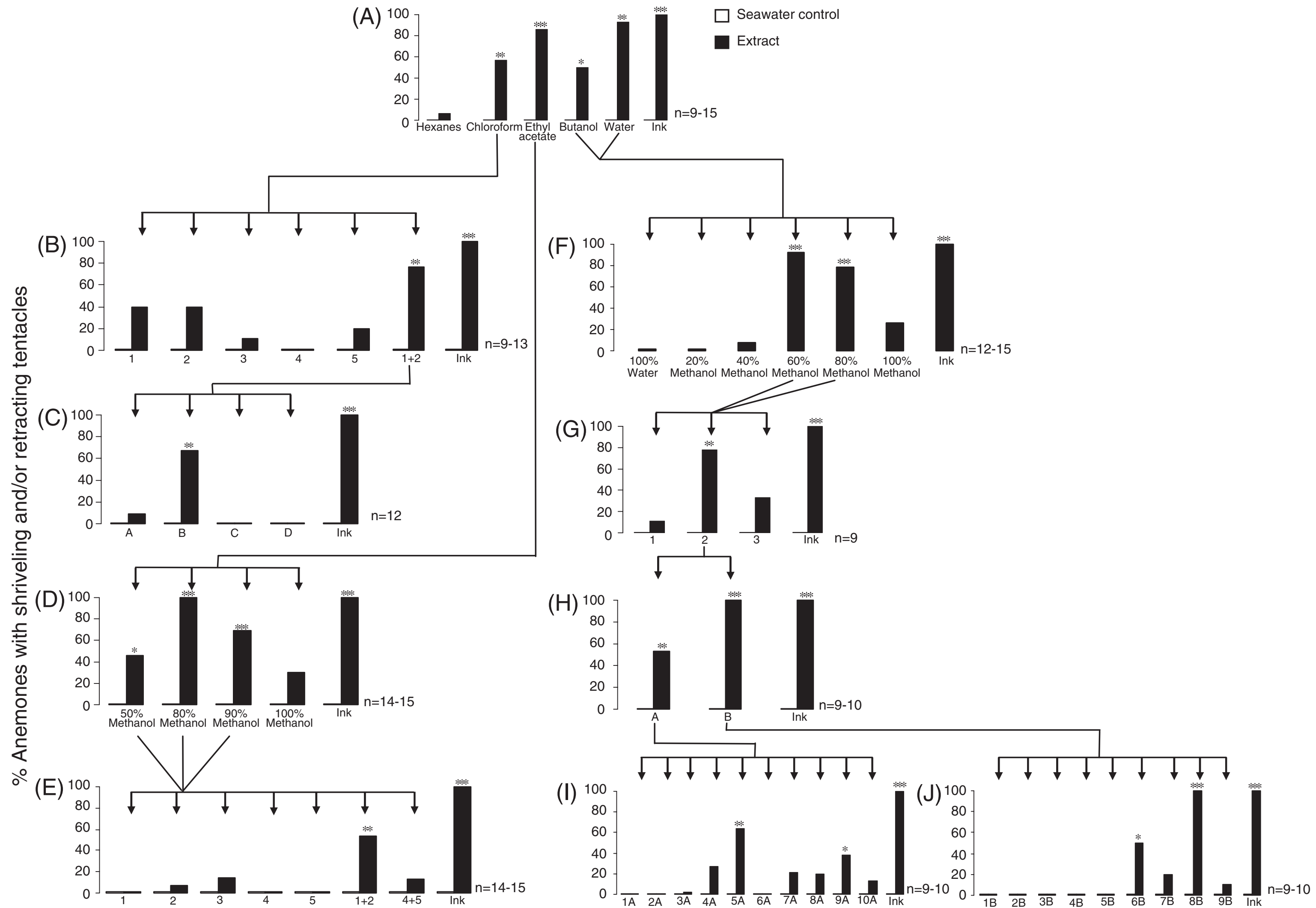

Fig. 5. Bioassay-guided fractionation of aversive components in sea hare ink based on shriveling and/or retraction of anemone tentacles. Frequency of anemones showing tentacle shriveling and/or retraction to: (A) solvent–solvent partition; (B) normal phase separation of the chloroform fraction in (A); (C) lipophilic sephadex separation of fractions 1+2 from the separation in (B); (D) reversed phase separation of the ethyl acetate fraction in (A); (E) lipophilic sephadex separation of the combination of the 50%, 80% and 90% methanol fractions from (D); (F) reversed phase separation of the water+butanol fractions from (A); (G) reversed phase separation of the combination of the 60% and 80% methanol fractions from (F); (H) lipophilic sephadex separation of fraction 2 in (G); (I) reversed phase HPLC separation of fraction A from (H); and (J) reversed phase HPLC separation of fraction B in (H). Unfilled bars represent seawater controls; black bars represent ink extracts. n = no. of anemones tested. Analysis by one-tailed McNemar test: $* = p \leq 0.05$, $** = p \leq 0.01$, $*** = p \leq 0.001$.

tentacles (Fig. 4A). In addition, the aversive components in ink did not appear to be diet-derived, as the metabolites extracted from *G. ferox* were not aversive to anemones (Fig. 4B).

3.3. Bioassay-guided fractionation of ink

Ink contains multiple components that elicited tentacle shriveling and/or retraction in *A. sola*. Solvent–solvent partition of ink yielded both lipophilic and hydrophilic aversive fractions, as metabolites soluble in either chloroform, ethyl acetate, butanol or water were aversive (Fig. 5A). Further purification of the chloroform fraction by normal phase chromatography yielded five fractions, but only the combination of fractions one and two elicited an aversive response (Fig. 5B). Further purification of fractions one and two yielded one aversive fraction (B) (Fig. 5C). Unfortunately, further examination of this fraction was unsuccessful due to loss of aversive activity.

Purification of the aversive ethyl acetate fraction yielded three fractions (50%, 80% and 90% methanol) that were aversive in the anemone bioassay (Fig. 5D). These fractions were combined and further purified by lipophilic sephadex, yielding five fractions, none of which were aversive. When fractions one and two were combined, however, they elicited a high frequency of tentacle shriveling and/or retraction (Fig. 5E). Further purification of the combination of fractions one and two was unsuccessful, due to loss of aversive activity of subsequent fractions.

The aversive water and butanol fractions were further purified, yielding six fractions, two of which (60% and 80% methanol) were aversive (Fig. 5F). These fractions shared similar characteristics, so they were combined. This extract was further purified, yielding three fractions, one of which (2) was aversive (Fig. 5G). Separation of this fraction yielded three fractions, two of which (A and B) were aversive (Fig. 5H). Fraction A was further purified to yield 10 fractions, two of which (5A and 9A) were active in the bioassay (Fig. 5I). Fraction B (Fig. 5H) was also further purified to yield nine fractions, two of which (6B and 8B) were aversive (Fig. 5J). Based on ultraviolet (UV) absorbance characteristics and HPLC retention time, fractions 5A and 6B and fractions 9A and 8B are likely the same molecule. Thus, a total of two molecules appear to be present in the initial water-soluble ink fraction that elicited tentacle shriveling and/or retraction. Unfortunately, the elucidation of molecular structure for both active molecules was unsuccessful, likely due to decomposition of the samples.

4. Discussion

4.1. Ink but not opaline is aversive to anemones

A. californica ink is aversive to the sea anemone *A. sola*, eliciting tentacle shriveling and/or retraction (Fig. 2). Opaline is not aversive to anemone tentacles (Fig. 2). To the contrary, opaline often stimulated a feeding response in anemones by eliciting tentacle movement toward the mouth and opening of the mouth, both feeding responses, as described in Lindstedt (1971). Given these observations and the fact that 87.5% of green-seaweed fed sea hares (that could not release ink, but could release opaline) in the Nolen et al. (1995) study were consumed, we conclude that ink but not opaline plays a role in defending *A. californica* against this anemone. These results are consistent with the findings of Nolen et al. (1995).

4.2. Escapin does not play a significant role in ink's aversiveness against anemones

Ink's major aversive effects are not caused by escapin, which is a major protein in *A. californica* ink (Yang et al., 2005). Neither escapin alone nor the mixture of escapin and its substrate L-lysine elicited tentacle shriveling and/or retraction different from the seawater control (Fig. 3A). We also tested escapin mixed with opaline, which was allowed to react for 2 min before applying to anemone tentacles, allowing sufficient time for escapin to completely oxidize its amino acid substrates (Yang et al., 2005). This mixture produced some tentacle retraction and/or shriveling, and there was a trend toward a significantly higher response than seawater ($p = 0.0625$), but a much lower frequency than produced by ink (Fig. 3B). Further support for escapin not being the primary aversive component comes from the fact that the contents of purple vesicles from the ink gland (which do not contain escapin: Johnson et al., 2006) elicit tentacle retraction and shriveling equivalent to the level generated by ink released from the sea hare (C. Kicklighter, pers. obs.).

Thus, escapin appears to play a minor, if any, role in the chemical defense of the sea hare *A. californica* against one of its predators, the sea anemone *A. sola*. Escapin may play a role in chemical defense against other predators, a possibility currently under investigation.

4.3. Ink's efficacy against anemones is not diet-dependent

Many studies have demonstrated that *A. californica* cannot produce ink unless it consumes a red algal diet

(Nolen et al., 1995; Prince et al., 1998), and red algal components, such as pigments and small molecules, are known to be sequestered into ink, skin, digestive gland and body wall tissues (Stallard and Faulkner, 1974a; MacColl et al., 1990; Pennings and Paul, 1993; Prince et al., 1998; Ginsburg and Paul, 2001). In this study, ink from sea hares fed either a natural diet (thought to be primarily composed of a variety of red seaweeds: reviewed in Carefoot, 1987) or an unnatural diet of the red alga *G. ferox* elicited similar frequencies of tentacle retraction and/or shriveling (Fig. 4). Thus, it appears that aversive components in ink are produced *de novo* or the sequestered components are common to many red algae. The extract from *G. ferox* did not elicit tentacle retraction and/or shriveling when tested at the natural concentration in this alga. However, it is possible that *A. californica* concentrates sequestered components (as dietary-derived metabolites can be highly concentrated in *Aplysia* (Rogers et al., 2000)) and that a higher concentration of *G. ferox* metabolites would have been aversive. It seems likely that the aversive components in ink are produced by *A. californica* or that sequestered algal components are modified by *A. californica* to metabolites aversive to anemones. Modification of sequestered compounds has been demonstrated in several opisthobranch molluscs, including *A. californica* (e.g., Stallard and Faulkner, 1974b; Paul and Van Alstyne, 1988; Paul and Pennings, 1991; Pennings and Paul, 1993), and modified compounds have been shown to deter predator feeding in at least one species—*Elysia halimedae* (Paul and Van Alstyne, 1988). No study has detected algal metabolites in *Aplysia* ink (reviewed in Johnson and Willows, 1999; Rogers et al., 2000), which supports the idea that the aversive ink components are produced de novo or are modified sequestered compounds.

4.4. Ink contains many components that are aversive to anemones

While several studies have demonstrated that ink can deter predator feeding, no study has identified specific components in *Aplysia* ink that mediate this behavior. When we attempted to isolate and identify the components in ink responsible for the aversive response by the sea anemone *A. sola*, we determined that ink fractions both lipophilic and hydrophilic in polarity were aversive to anemones (Fig. 5A), suggesting that multiple components in ink were aversive. When we attempted to purify the chloroform and ethyl acetate fractions, we were unsuccessful at isolating pure compounds due to loss of activity (Fig. 5C,E). Based on their thin layer chromatography characteristics, the ethyl acetate and chloroform fractions had some spots in common, with others unique to only the ethyl acetate or chloroform fractions. However, we could not determine whether the aversive component(s) was one of the spots the two fractions had in common or whether the aversive component(s) in the two fractions were different. Thus, we do not know if more than one lipophilic aversive metabolite exists.

We were able to isolate pure, or nearly pure, molecules from the water fraction before losing aversive activity (Fig. 5I, J). Because we believe that fractions 5A and 6B and fractions 9A and 8B contained the same molecules (based on UV absorbance characteristics and HPLC retention time), there appear to be two water-soluble aversive components. Thus, ink appears to have a minimum of three molecules that are aversive to *A. sola*. Whether these components are sufficient to facilitate the escape of *Aplysia* from anemones is unknown, as these molecules were too unstable to purify and thus could not be assayed for their efficacy. Ink is a complex mixture of proteins, pigments and other small molecules, and it may contain antioxidants that prevent the oxidation of aversive compounds. Antioxidants have been found in marine organisms, such as sponges, seaweeds, cyanobacteria and squid ink (Lucero et al., 1994; Takamatsu et al., 2003). Thus, it is possible that our purification procedures may have separated the aversive molecules from these antioxidants or other stabilizing compounds, thus leading to the instability issues that we repeatedly encountered. This occurrence may explain the problems encountered by Pennings et al. (1999) when examining the palatability of ink extract from the sea hare *Dolabella auricularia*.

Even though many components of *Aplysia* ink have been identified, predicting from current literature the identities of those that are aversive against sea anemones is difficult. Most of the identified ink constituents are likely more stable than the labile molecules that we attempted to track; if not, elucidation of their structures would have been more difficult or impossible. In addition, few polar compounds (aside from free amino acids) have been identified from ink (reviewed in Faulkner, 1992; Yamada and Kigoshi, 1997; Appleton et al., 2001). Our active polar compounds are probably not free amino acids because if they were, they would probably elute in a highly polar fraction (i.e., 100% water) and none of the fractions active against the anemone were highly polar (i.e., the 100% water fraction in Fig. 4F was not active).

Several studies investigating chemical defenses in marine organisms, such as soft corals, seaweeds,

sponges and annelids, have identified multiple compounds that deter predators; however, these compounds are usually closely related in structure and polarity (e.g., Pawlik and Fenical, 1989; Hay et al., 1987; Kicklighter et al., 2003). Multiple components were also identified in *A. californica* ink that are aversive to *A. sola*, but they are unlikely to be closely related, as some components are lipophilic and others are hydrophilic. Comparatively few studies have detected multiple defensive compounds in a single species that are very different in polarity and thus also in structural relatedness. Examples include a seaweed, a hemichordate and an annelid (Deal et al., 2003; Kicklighter et al., 2003; Kicklighter and Hay, submitted for publication). A diversity of defensive compounds may be required to deter some consumers or may be advantageous in predator-rich environments, where consumer diversity and attack strategies are both high (Lubchenco and Gaines, 1981; Hay, 1984). The use of multiple chemical deterrents may be more common than has been reported; however, some components may be so unstable that purification and elucidation is too difficult (as is the case with ink deterrents in our study and with some annelid defenses (Kicklighter et al., 2003; Kicklighter and Hay, submitted for publication)). Alternatively, compounds may be so unstable that they may go undetected altogether. Re-examination of previous studies utilizing alternative purification or bioassay strategies may reveal additional defensive components.

4.5. Sea hare defensive secretions affect a diversity of predators

Several investigations have examined the effects of ink and opaline from various sea hare species on a variety of predators, such as birds, fishes and crustaceans. In most cases, predators found these secretions unpalatable. Ink or organic extracts of ink from *Aplysia dactylomela*, *Aplysia kurodai*, *Aplysia brasiliana*, *Stylocheilus longicauda* and *D. auricularia* were all unpalatable to at least one predator (Ambrose and Givens, 1979; DiMatteo, 1981; Paul and Pennings, 1991; Pennings, 1994). In some cases, secretions from a given sea hare species affected different predators in similar ways. For example, fish tissue soaked in ink was unpalatable to laughing gulls (DiMatteo, 1981) and was avoided by the crabs *Callinectes sapidus*, *Panopeus herbstii* and *Portunus spinimanus* (DiMatteo, 1982). In addition, Carefoot et al. (1999) hypothesized that *A. dactylomela* ink functioned as a sensory irritant to predators, eliciting increased grooming behavior and decreased feeding in the crab *Mithrax sculptus*. In several cases, however, sea hare secretions affected different predators differently. Ink from *D. auricularia* was palatable to crabs (*Leptodius* sp.), but the organic extract of ink was rejected by reef fishes (Pennings, 1994). In addition, opaline from *A. juliana* was rejected by the crabs *Leptodius* sp. and *Hemigrapsus sanguineus* but was consumed by reef fishes (Pennings, 1994). In the present study, the aversive response produced by sea anemones following exposure to *A. californica* ink, and not opaline, is in contrast to the situation with predatory spiny lobsters, *P. interruptus* (Kicklighter et al., 2005). In this case, free amino acids in both ink and opaline stimulate feeding appetitive behavior of spiny lobsters by acting as phagomimics, thus causing the lobsters to attend to the secretions, rather than *A. californica*. Further, opaline has an unpalatable compound that inhibits spiny lobster ingestion, whereas opaline appears to contain no compounds that are aversive to anemones and may even contain some feeding excitants. Given that predators vary in many ways, including their sensory systems, it is not surprising that different predators react differently to sea hare defensive secretions. Thus, the use of multiple, complex secretions may be advantageous to prey that encounter multiple predators.

5. Conclusions

The ink gland secretion of the sea hare *A. californica*, which is released when sea hares are attacked by predators, is aversive to the sea anemone *A. sola*, eliciting tentacle retraction and/or shriveling. The opaline gland secretion of *A. californica*, however, is not aversive to these anemones. Escapin, a major protein in ink with antimicrobial activity, does not appear to function as a defense against this anemone predator. Instead, an array of molecules with different chemical properties, likely produced de novo, are aversive to anemones. Tentacle retraction and shriveling by anemones probably facilitates the escape of sea hares, as this behavior leads to sea hares being dropped (Nolen et al., 1995). Interestingly, the manner in which *A. californica* secretions affect *A. sola* is in opposition to their effects on spiny lobsters, which are attracted to ink and are deterred by opaline. Thus, a better understanding of the molecules mediating predator–prey interactions will yield a clearer picture of the complexity by which prey defenses may operate against different predators.

Acknowledgments

We thank Cara DiNardo, Zahra Gheewala, Emily Johnson, P.M. Johnson, Michiya Kamio, Ko-Chun Ko,

Shkelzen Shabani, P.C. Tai, Brian Vu and Hsiuchin Yang for assistance with bioassays, collection of secretions, purification of escapin and discussions. Comments by Steve Pennings and an anonymous reviewer improved the manuscript. Supported by NSF IBN-0324435 and the Georgia Research Alliance. **[PH]**

References

Ambrose, H.W, Givens, R.P., 1979. Distastefulness as a defense mechanism in *Aplysia brasiliana* (Mollusca: Gastropoda). Mar. Behav. Physiol. 6, 57–64.

Appleton, D.R., Babcock, R.C., Copp, B.R., 2001. Novel tryptophan-derived dipeptides and bioactive metabolites from the sea hare *Aplysia dactylomela*. Tetrahedron 57, 10181–10189.

Butzke, D., Hurwitz, R., Thiede, B., Goedert, S., Rudel, T., 2005. Cloning and biochemical characterization of APIT, a new L-amino acid oxidase from *Aplysia punctata*. Toxicon 46, 479–489.

Carefoot, T.H., 1987. *Aplysia*: its biology and ecology. J. Exp. Biol. 24, 167–284.

Carefoot, T.H., Pennings, S.C., Danko, J.P., 1999. A test of novel function(s) for the ink of sea hares. J. Exp. Mar. Biol. Ecol. 234, 185–197.

Christomanos, A., 1955. Nature of the pigment of *A. depilans*. Nature 175, 310.

Deal, M.S., Hay, M.E., Wilson, D., Fenical, W., 2003. Glactolipids rather than phlorotannins as herbivore deterrents in the brown seaweed *Fucus vesiculosis*. Oecologia 136, 107–114.

DiMatteo, T., 1981. The inking behavior of *Aplysia dactylomela* (Gastropoda: Opisthobranchia): evidence for distastefulness. Mar. Behav. Physiol. 7, 285–290.

DiMatteo, T., 1982. The ink of *Aplysia dactylomela* (Rang, 1828) (Gastropoda: Opisthobranchia) and its role as a defensive mechanism. J. Exp. Mar. Biol. Ecol. 57, 169–180.

Faulkner, D.J., 1992. Chemical defenses of marine molluscs. In: Paul, V.J. (Ed.), Ecological Roles of Marine Natural Products. Comstock Publishing Associates, Ithaca, pp. 119–163.

Ginsburg, D.W., Paul, V.J., 2001. Chemical defenses in the sea hare *Aplysia parvula*: importance of diet and sequestration of algal secondary metabolites. Mar. Ecol. Prog. Ser. 215, 261–274.

Hay, M.E., 1984. Predictable spatial escapes from herbivory: how do these affect the evolution of herbivore resistance in tropical marine communities? Oecologia 64, 396–407.

Hay, M.E., Duffy, J.E., Pfister, C.A., Fenical, W., 1987. Chemical defense against different marine herbivores: are amphipods insect equivalents? Ecology 68, 1567–1580.

Johnson, P.M., 2002. Multi-component chemical defense in sea hares (Gastropoda: Opisthobranchia): antipredator compounds act as both honest and deceptive signals to multiple predator species. Ph.D. Dissertation, University of Washington.

Johnson, P.M., Willows, A.O.D., 1999. Defense in sea hares (Gastropoda, Opisthobranchia, Anaspidea): multiple layers of protection from egg to adult. Mar. Freshw. Behav. Physiol. 32, 147–180.

Johnson, P.M., Kicklighter, C.E., Schmidt, M., Kamio, M., Yang, H., Elkin, D., Michel, W.C., Tai, P.C., Derby, C.D., 2006. Packaging of chemicals in the defensive secretory glands of the sea hare *Aplysia californica*. J. Exp. Biol. 209, 78–88.

Kicklighter, C.E., Hay, M.E., (submitted for publication). Avoiding versus deterring consumers: interactions among defensive and escape strategies in sabellid worms. Oecologia.

Kicklighter, C.E., Kubanek, J., Barsby, T., Hay, M.E., 2003. Palatability and defense of some tropical infaunal worms: alkylpyrrole sulfamates as deterrents to fish feeding. Mar. Ecol. Prog. Ser. 263, 299–306.

Kicklighter, C.E., Shabani, S., Johnson, P.M., Derby, C.D., 2005. Sea hares use novel antipredatory chemical defenses. Curr. Biol. 15, 549–554.

Kinnel, R.B., Dieter, R.K., Meinwald, J., Van Engen, D., Clardy, J., Eisner, T., Stallard, M.O., Fenical, W., 1979. Brasilenyne and *cis*-dihydrorhodophytin: antifeedant medium-ring haloether from a sea hare (*Aplysia brasiliana*). Proc. Natl. Acad. Sci. U.S.A. 76, 3576–3579.

Kupchan, S.M., Britton, R.W., Lacadie, J.A., Ziegler, M.F., Sigel, C.W., 1975. The isolation and structural elucidation of bruceantin and bruceantinol. J. Org. Chem. 40, 648–654.

Lindstedt, K.J., 1971. Biphasic feeding response in a sea anemone: control by asparagine and glutathione. Science 173, 333–334.

Lubchenco, J., Gaines, S.D., 1981. A unified approach to marine plant–herbivore interactions: I. Populations and communities. Annu. Rev. Ecolog. Syst. 12, 405–437.

Lucero, M.T., Farrington, H., Gilly, W.F., 1994. Quantification of L-dopa and dopamine in squid ink—implications for chemoreception. Biol. Bull. 187, 55–63.

Lukasheva, E.V., Berezov, T.T., 2002. L-Lysine α-oxidase: physicochemical and biological properties. Biochemistry (Mosc.) 67, 1152–1158.

MacColl, R., Galivan, J., Berns, D.S., Nimec, Z., Guard-Friar, D., Wagoner, D., 1990. The chromophore and polypeptide composition of *Aplysia* ink. Biol. Bull. 179, 326–331.

Nishibori, K., 1960. Studies on the pigments of marine animals: IX. Pigments of the sea-slug *Aplysia kurodai*. Publ. Seto Mar. Biol. Lab. 8, 327–335.

Nolen, T.G., Johnson, P.M., Kicklighter, C.E., Capo, T., 1995. Ink secretion by the marine snail *Aplysia californica* enhances its ability to escape from a natural predator. J. Comp. Physiol., A Sens. Neural Behav. Physiol. 176, 239–254.

Paul, V.J., Pennings, S.C., 1991. Diet-derived chemical defenses in the sea hare *Stylocheilus longicauda* (Quoy et Gaimard 1824). J. Exp. Mar. Biol. Ecol. 151, 227–243.

Paul, V.J., Van Alstyne, K.L., 1988. Use of ingested algal diterpenoids by *Elysia halimedae* Macnae (Opisthobranchia: Ascoglossa) as anti-predator defenses. J. Exp. Mar. Biol. Ecol. 119, 15–29.

Pawlik, J.R., Fenical, W., 1989. A re-evaluation of the ichthyodeterrent role of prostaglandins in the Caribbean gorgonian coral *Plexaura homomalla*. Mar. Ecol. Prog. Ser. 52, 95–98.

Pearse, V., Francis, L., 2000. *Anthopleura sola*, a new species, solitary sibling species to the aggregating sea anemone, *A. elegantissima* (Cnidaria: Anthozoa: Actiniaria: Actiniidae). Proc. Biol. Soc. Wash. 113, 596–608.

Pennings, S.C., 1994. Interspecific variation in chemical defense in the sea hare (Opisthobranchia: Anaspidea). J. Exp. Mar. Biol. Ecol. 180, 203–219.

Pennings, S.C., Paul, V.J., 1993. Sequestration of dietary secondary metabolites by three species of sea hares: location, specificity and dynamics. Mar. Biol. 117, 535–546.

Pennings, S.C., Paul, V.J., Dunbar, D.C., Hamann, M.T., Lumbang, W.A., Novack, B., Jacobs, R.S., 1999. Unpalatable compounds in the amrine gastropod *Dolabella auricularia*: distribution and effect of diet. J. Chem. Ecol. 25, 735–755.

Prince, J., Nolen, T.G., Coelho, L., 1998. Defensive ink pigment processing and secretion in *Aplysia californica*: concentration and storage of phycoerythrobilin in the ink gland. J. Exp. Biol. 201, 1595–1613.

Rogers, C.N., De Nys, R., Charlton, T.S., Steinberg, P.D., 2000. Dynamics of algal secondary metabolites in two species of sea hare. J. Chem. Ecol. 26, 721–743.

Setchell, W.A., Gardner, N.L., 1967. The Marine Algae of the Pacific Coast of North America. Wheldon and Wesley, Ltd., New York. 898 pp.

Smith, G.M., 1969. Marine Algae of the Monterey Peninsula California, 2nd ed. Stanford University Press, Stanford, CA. 752 pp.

Stallard, M.O., Faulkner, D.J., 1974a. Chemical constituents of the digestive gland of the sea hare *Aplysia californica*: I. Importance of diet. Comp. Biochem. Physiol. 49B, 25–35.

Stallard, M.O., Faulkner, D.J., 1974b. Chemical constituents of the digestive gland of the sea hare *Aplysia californica*: II. Chemical transformations. Comp. Biochem. Physiol. 49B, 37–41.

Takamatsu, S., Hodges, T.W., Rajbhandari, I., Gerwick, W.H., Hamann, M.T., Nagle, D.G., 2003. Marine natural products as novel antioxidant prototypes. J. Nat. Prod. 66, 605–608.

Thompson, T.E., 1960. Defensive adaptations in opisthobranchs. J. Mar. Biol. Assoc. U.K. 39, 123–134.

Tritt, S.H., Byrne, J.H., 1980. Motor controls of opaline secretion in *Aplysia californica*. J. Neurophysiol. 43, 581–594.

Troxler, R.R., Offner, G.D., Capo, T.R., 1981. Structural studies on aplysioviolin. Biol. Bull. 161, 339.

Walters, E.T., Erickson, M.T., 1986. Directional control and the functional organization of defensive responses in *Aplysia*. J. Comp. Physiol., A Sens. Neural Behav. Physiol. 159, 339–351.

Walters, E.T., Illich, P.A., Hickie, C., 1993. Inking and siphon response plasticity in *Aplysia*. Anti-predator and alarm signal functions. Soc. Neurosci. Abstr. 9, 578.

Wellner, D., Meister, A., 1961. Studies on the mechanism of action of L-amino acid oxidase. J. Biol. Chem. 236, 2357–2364.

Winkler, L.R., 1969. Distribution of organic bromine compounds in *Aplysia californica* Cooper, 1863. Veliger 2, 268–271.

Winkler, L.R., Tilton, B.E., 1962. Predation on the California sea hare, *Aplysia californica* Cooper, by the solitary great green sea anemone, *Anthopleura xanthogrammica* (Brandt), and the effect of sea hare toxin and acetylcholine on anemone muscle. Pac. Sci. 16, 286–290.

Yamada, K., Kigoshi, H., 1997. Bioactive compounds from the sea hares of two genera: *Aplysia* and *Dolabella*. Chem. Soc. Jpn. 70, 1479–1489.

Yang, H., Johnson, P.M., Ko, K.C., Kamio, M., Germann, M.W., Derby, C.D., Tai, P.C., 2005. Cloning, characterization and expression of escapin, a broadly antimicrobial FAD-containing L-amino acid oxidase from ink of the sea hare *Aplysia californica*. J. Exp. Biol. 208, 3609–3622.