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Responses of the Sea Catfish, Ariopsis felis, to Chemical Defenses from the Sea Hare, Aplysia californica

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by

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Abstract:

Sea hares (*Aplysia spp*.) emit two chemical substance, ink and opaline, which protect them from predators. Extracellular, electrophysiological recordings determined whether olfactory and gustatory systems of a potential predator, the sea catfish (*Ariopsis felis*), detect these substances. Further experiments determined the behavioral effects of ink and opaline on the sea catfish. Both defensive secretions contain high concentrations of amino acids, which are stimulatory to both olfactory and gustatory systems of all predatory fishes studied. To determine the role of amino acids in the defensive secretions, mixtures of the same amino acids found in ink and opaline were tested electrophysiologically at equivalent concentrations and compared to responses to both natural and artificial ink and to opaline. Escapin, an L-amino acid oxidase which is present in ink and reacts with opaline and basic amino acids, was not a potent stimulus of either chemosensory system. Ink and opaline were both found to be potent olfactory and gustatory stimuli in the sea catfish, and the amino acids within the two substances were responsible for a significant portion of the electrophysiological responses. Behavioral experiments showed that sea catfish exhibited an aversive response to ink, but were attracted to both ink and opaline which is likely due to the amino acid content of these substances. However, when ink was mixed with a food substance, the composite became unpalatable. In summary, the electrophysiological results indicate that both taste and olfactory systems of the sea catfish detect molluscan defensive secretions, and behavioral

studies show that opaline and the amino acid fraction of molluscan ink are attractive, but that whole ink is aversive.

Introduction:

Chemoreception is defined as the ability to detect specific chemicals in either air or water. In fish, olfaction and gustation are used in the process of feeding, including finding food and determining its palatability (Sorenson and Caprio 1998). Fish generally have taste buds around the oral cavity and on the gill rakers. Sea catfish, however, possess taste buds over their entire body surface, thus gustation plays an even more important role in locating food (Michel and Caprio 1991, Caprio and Derby 2008). Through both olfactory and gustatory systems, sea catfish are able to detect free amino acids, important feeding cues (Michel and Caprio 1991), in the water.

Sea hares, squid and octopi all defend themselves from predators through inking (Derby et al. 2007). Inking is thought to act on a predator by three general mechanisms: phagomimicry, chemical deterrence, and sensory disruption. Phagomimicry is a deception that results in the predator demonstrating a feeding behavior. Phagomimicry is a result of the presence of a high concentration of feeding cues (Derby 2007). Chemical deterrence is an activity that is represented by a decrease in feeding behaviors (Kicklighter et al. 2005). Sensory disruption is caused by continued excitation of chemosensory systems due a substance either adhering to the predator or somehow resulting in long term stimulation. This continued excitation will then make the predator less sensitive to other stimuli (Kicklighter et al. 2005).

The sea hare co-releases two chemicals, ink and opaline, which contain numerous amino acids (Derby et al. 2007). The high concentration of amino acids suggests that their presence could result in a predator exhibiting phagomimetric behavior when it comes into

contact with ink or opaline. A study with spiny lobsters showed that they exhibited feeding behaviors when presented with ink, while contact with opaline elicited aversive behaviors (Kicklighter et al. 2005).

Along with the high concentration of amino acids found in ink and opaline, escapin, an L-amino acid oxidase is found in ink. Escapin was shown to inhibit the growth of bacteria. The main substrate of escapin is L-lysine, an amino acid which is found in opaline. The oxidase activity of escapin produces hydrogen peroxide, which was shown to have antimicrobial properties (Yang et al. 2005). The products from this reaction are effective in deterring lobsters and blue crab from attacking, but are not effective deterrents to sea anemone or other fish, indicating that the role of escapin is species specific (Nolen et al. 1995; Johnson et al. 2006; Derby 2007).

In the present study, sea catfish were used as a model organism to test whether olfactory and/or gustatory systems are responsible for the detection of defensive secretions of the sea hare. Sea catfish are potential predators of sea hares as they are sympatric with *Aplysia dactylomela* and *Aplysia brasiliana* (Muncy and Wingo, 1983). The sea catfish was previously developed as a model organism for studies in olfaction and gustation (Silver et al. 1976; Caprio 1980; Michel and Caprio, 1991). The electrophysiological and behavioral data in this study support the idea that sea hares defend themselves through a variety of mechanisms including phagomimicry and chemical deterrence.

Materials and Methods

Stimuli

Collection of sea hare secretions

Natural ink (NI) and natural opaline (NO) were collected by my collaborator (Matthew Nusnbaum, Georgia State University) from dissected ink and opaline glands of adult sea hares purchased from Marinus Inc., according to the procedures of Yang et al. (2005).

Escapin and its reaction products

Escapin was purified by Matthew Nusnbaum from whole ink according to Yang et al. (2005). Escapin end products of L-lysine (EEP-K) and escapin end products of L-arginine (EEP-R) were prepared by incubating escapin with 145 mM L-lysine or 350 μM L-arginine at 30°C in 50 mM potassium phosphate buffer for 48 to 72 hr. Production of escapin intermediate products of L-lysine (EIP-K) and escapin intermediate products of L-arginine (EIP-R) followed the same protocol except that 4 mg/ml of catalase (Sigma-Aldrich, C1345) was added to the solution to scavenge H_2O_2 and prevent the completion of the reaction. Escapin and catalase were removed from the solution by filtration, and the solution was dried down for storage at -20°C.

When I tested natural ink+natural opaline, EIP+H_2O_2, or EIP+ H_2O_2, the two components of each were mixed 15 sec, 5 min, or 8 min before presentation. This procedure was followed due to chemical reactions that occur with the ingredients in the two components. These reactions included, but were not limited to, the cascade of time-dependent reactions beginning with escapin's oxidative deamination of L-lysine and L-arginine as previously described (introduction) and reviewed in Derby (2007). Comparing responses over time allowed for an examination of the importance of these time-dependent compositional changes.

Artificial ink and opaline mixtures

Artificial ink and opaline were formulated from amino acid and ammonium profiles of whole secretions based on the results of Kicklighter et al. (2005) and Johnson et al. (2006). The component amino acids (Sigma-Aldrich) were prepared by my collaborator, Matt Nusnbaum at Georgia State University, at natural concentration in artificial sea water to create artificial ink (AI) and artificial opaline (AO), with the formulation shown in Table 1.

Chemical	Artificial Ink (µM)	Artificial Opaline (µM)
L-Alanine	1024	339
L-Asparagine	51	41
L-Aspartic acid	2231	2512
L-Cystine	84	16
L-Glutamic acid	1166	1616
L-Glutamine	121	9
L-Glycine	181	791
L-Histidine	255	7185
L-Isoleucine	135	96
L-Leucine	327	10
L-Lysine	0	65190
L-Methionine	122	35
L-Phenylalanine	130	648
L-Proline	131	7
L-Serine	214	68
L-Threonine	193	236
L-Tyrosine	297	14
L-Valine	301	56
Taurine	7830	231200
Ammonia	24360	6810
TOTAL (µM)	X	Y

Table 1. Composition of artificial ink and artificial opaline. These mixtures are based on the amino acid and ammonia concentrations in natural ink and opaline from wild *Aplysia californica* based on analyses reported in Kicklighter et al. (2005).

Animals and their maintenance

Sea catfish, *Ariopsis felis* (Linnaeus, 1766) (formerly *Arius felis*), were caught by hook and line in Grande Isle, Louisiana, and were transported to the Animal Care Facility in the Life Sciences Building at Louisiana State University. The catfish were maintained in a 280-L, aerated aquarium with circulating artificial seawater (Instant Ocean) (ASW), and both the salinity (25-28 ppt) and temperature (25ºC) of the ASW were adjusted to the levels where

the fish were captured. All fish were fed fresh shrimp several times a week and were used for the physiological recordings within two weeks of capture. The weight and length of the 13 fish ranged between 108-174 g and 21.6-27.0 cm, respectively.

Olfactory physiology

Animal immobilization

Sea catfish were immobilized with an intramuscular injection of Flaxedil (gallamine triethoiodide, 1.2 mg/kg body weight), wrapped in tissue paper, and positioned in a holding container. When required, additional Flaxedil was delivered to the fish administered from a 1-ml syringe attached to a hypodermic needle in the flank musculature. Aerated ASW containing a general anesthetic (0.05% ethyl-m-aminobenzoate methane sulfonic acid, MS-222) circulated through the mouth and over the gills throughout the experiment. The tissue paper in which the fish was wrapped was wetted with this gill irrigation water to keep the skin of the fish moist.

Fish preparation

A flap of tissue dorsal to the olfactory epithelium was removed to expose the olfactory mucosa, and a constant 6 ml/min flow of ASW was delivered to the exposed olfactory organ. Stimuli were introduced to the constant flow for 3 sec using a gravity-feed delivery system with a spring-loaded valve (Model 5301, Rheodyne, Cotati, CA) driven by a pneumatic actuator (Model 5300). Stimuli were tested at 2-min intervals.

Electrophysiological recordings

The underwater electro-olfactogram (EOG), a slow negative potential change, was recorded in the water above the surface of the olfactory mucosa in response to chemical stimulation. EOGs were recorded with calomel electrodes via Ringer-agar filled capillary pipettes, amplified by a d.c. amplifier (Grass P-18) and displayed on a chart recorder. The

magnitude of each EOG response was measured as the height in mm of the phasic displacement from the baseline level.

All test stimuli were first administered to determine their relative potencies. Subsequently, a cross adaptation protocol was performed to determine if natural ink and opaline contained stimulants besides amino acids. This was accomplished by replacing the constant flow of ASW with artificial ink or artificial opaline. After 5 min, stimuli were then tested. The adapting solution served as the solvent for the test stimuli and the adapting solution served as a control. To confirm the initial activity prior to adaptation, the constant flow of the adapting solution was replaced with ASW and all stimuli were tested again.

Multiunit recordings of taste neural activity

Fish preparation

Animals were immobilized as in the experiments involving olfactory recordings. A constant flow (6 ml/min) of ASW was delivered to the left maxillary barbel, which was inserted into a glass capillary tube. Taste stimuli were introduced into the glass sleeve using the same method as described for the olfactory experiments. A local anaesthetic (tetracaine) was applied around the ocular socket prior to and subsequent to deoculation.

Electrophysiological recordings

A branch of the facial nerve that innervates taste buds on the maxillary barbel was exposed, cleared of connective tissue, transected, and its peripheral cut end lifted onto a tungsten hook electrode. After confirming that the nerve was responsive to taste stimuli, the nerve and electrode were placed under halocarbon oil to prevent desiccation of the nerve. Neural activity was a.c. amplified (Grass P511; bandpass 30-3000 Hz), integrated (0.5 s time constant), and displayed on a chart recorder. Taste activity was quantified by measuring the amplitude of the integrated phasic response from baseline.

Behavioral experiments

Preparation of noodles and shrimp

'Noodles' were created to test the effect of added test stimuli on the feeding behavior of the sea catfish, especially the ingestion of the food. Shrimp purchased at a seafood market were freeze dried, then ground into a powder using a mortar and pestle. Powdered shrimp and alginate (Sigma) were combined in a 5:3 ratio by weight, and 8 gm of this mixture was added to 100 ml deionized water. This shrimp-alginate solution was drawn with a 50-µl pipette and exuded into a 0.25 M $CaCl_2$ solution, creating a solid matrix that could be cut into 3-mm long noodles. Unflavored alginate noodles were produced by following the same procedure except that shrimp was not added. Preliminary behavioral tests showed that shrimp-alginate noodles were attractive to fish, whereas unflavored alginate noodles were neutral (i.e. did not cause arousal and were not taken into the mouth). Shrimp-alginate noodles and unflavored noodles were added to different test solutions by combining liquid extracts of those test solutions with the alginate gel, to create 'test noodles'. The test solutions were natural opaline, natural ink, artificial opaline, and artificial ink. Positive controls were pieces of shrimp and shrimp-alginate pellets.

Feeding assays

Feeding assays were conducted in a 280-L aquarium in the Animal Care Facility at LSU. The same five sea catfish were tested on different days. The catfish were fed fresh gulf shrimp 48 hr prior to each experiment. Each control and test pellet was attached to a 1-g piece of clay with a pin to ensure that they sank to the bottom of the aquarium. Two such pellets of similar type were simultaneously introduced into the aquarium. The pellets were kept in the aquarium until multiple fish came into contact with them and exhibited a consistent pattern of behavior. If the test noodles were not consumed, they were removed after 2 min. Fish were given shrimp alginate pellets at the beginning and end of the

experiments to ensure that the fish exhibited feeding behavior. All behavior was recorded with a digital video camera.

The behaviors monitored included the number of fish that (a) ingested the pellets, (b) were attracted to but did not ingest the pellets, (c) exhibited no obvious response, and (d) were initially attracted to, but after coming within 5 cm of the test pellet, quickly swam and stayed away from the pellet. An attraction to the pellet was defined as a fish swimming towards the pellet and coming within 5 cm of the pellet.

Results

Behavior

Behavioral responses to sea hare secretions and their components embedded either alone or in combination with shrimp into alginate pellets (i.e., a food stimulus) are reported in Table 2. To determine if sea hare secretions are phagostimulants, natural or artificial ink or opaline was added to unflavored alginate pellets. Unflavored alginate pellets did not evoke arousal or attraction and were neither taken into the mouth nor ingested. Addition of full-strength natural ink to the pellets made the pellets aversive, such that sea catfish avoided the pellets rather than exhibiting their normal indifference to them. In contrast, the addition of natural opaline to the pellets increased the arousal of sea catfish and their attraction towards the pellets, although not causing ingestion. Addition of both natural opaline and natural ink to the unflavored alginate pellets also caused an aversive response. Artificial opaline or artificial ink also increased attraction and arousal to unflavored alginate pellets, but did not increase their ingestion.

To determine if sea hare secretions contain feeding deterrents, we added natural or artificial ink or opaline to shrimp-alginate pellets. Shrimp-alginate pellets were readily ingested by sea catfish. Addition of natural opaline, artificial opaline, or artificial ink at full strength to 100 times dilution to shrimp-alginate pellets did not affect their palatability as all

were ingested. In contrast, the addition of natural ink did affect the ingestion of shrimp-alginate pellets. The addition of full-strength natural ink caused aversion to the pellets, such that they were not ingested, whereas the addition of 10 or 100 times diluted natural ink to shrimp-alginate pellets resulted in attraction, but the fish did not ingest the pellets. Furthermore, the addition of natural ink (even at 100 times dilution) to shrimp-alginate pellets that also contained natural opaline was also avoided and was not ingested. Thus, natural ink is a phagodeterrent, making food unpalatable, whereas artificial ink and artificial opaline were neutral.

Table 2: Behavioral Responses of Sea Catfish to Alginate Pellets

Additives to Alginate Pellet			Response[3]
Shrimp?[1]	Sea Hare Secretion?[2]		
-	-		No response
-	Natural Ink		Aversion
-	Natural Opaline		Attracted but not ingested
-	Natural Opaline + Natural Ink		Aversion
-	Artificial Ink		Attracted but not ingested
-	Artificial Opaline		Attracted but not ingested
+	-		Ingested
+	Natural Ink		
		Full strength	Aversion
		10 x dilution	Attracted but not ingested
		100 x dilution	Attracted but not ingested
+	Natural Opaline		
		Full strength	Ingested
		10 x dilution	Ingested
		100 x dilution	Ingested
+	Natural Opaline + Natural Ink		
		Full strength	Aversion
		100 x dilution	Aversion
+	Artificial Ink		
		Full strength	Ingested

	10 x dilution	Ingested
+	Artificial Opaline	
	Full strength	Ingested
	10 x dilution	Ingested
	100 x dilution	Ingested

[1] + = added to the alginate pellet, - = not added to the alginate pellet
[2] Concentration is full strength, unless otherwise noted
[3] Response is based on 3 sets of 5 fish, with each stimulus being tested on 1 set, except for the positive control (shrimp alginate pellets), which was tested on all three sets of animals

Electrophysiology

Olfaction: EOG responses

The olfactory system of sea catfish is highly stimulated by sea hare secretions, and amino acids significantly contributed to this. Natural secretions or their artificial mixtures caused robust, concentration-dependent, EOG responses (Figs. 1, 2).

Natural secretions were more stimulatory than artificial mixtures, and the combination of opaline and ink was more stimulatory than either alone (Figs. 1A, 2). Artificial ink and artificial opaline were somewhat less effective than their respective natural secretions (Figs. 1A, 2). The responses to natural opaline and natural ink were partially, but not completely cross-adapted by equal concentration of the corresponding artificial mixture (Figs. 1B, 1C). Adaptation to artificial ink reduced the response to natural ink by 49-64%, and adaptation to artificial opaline reduced the response to natural opaline by 48-70%. These results showed that the amino acid fraction of opaline and ink was a highly effective olfactory stimulant, but that other components in opaline and ink were also highly stimulatory to the olfactory receptor neurons.

Our results showed that reaction products of escapin were stimulatory to olfactory receptor neurons of the sea catfish, but were significantly less so than their original amino

acid substrates (Figs. 1A, 2). One of the products of escapin's reaction, H_2O_2,was barely stimulatory. Furthermore, the addition of H_2O_2 to EIP-K or EIP-R did not increase the stimulatory effectiveness of those mixtures (Figs. 1A, 2).

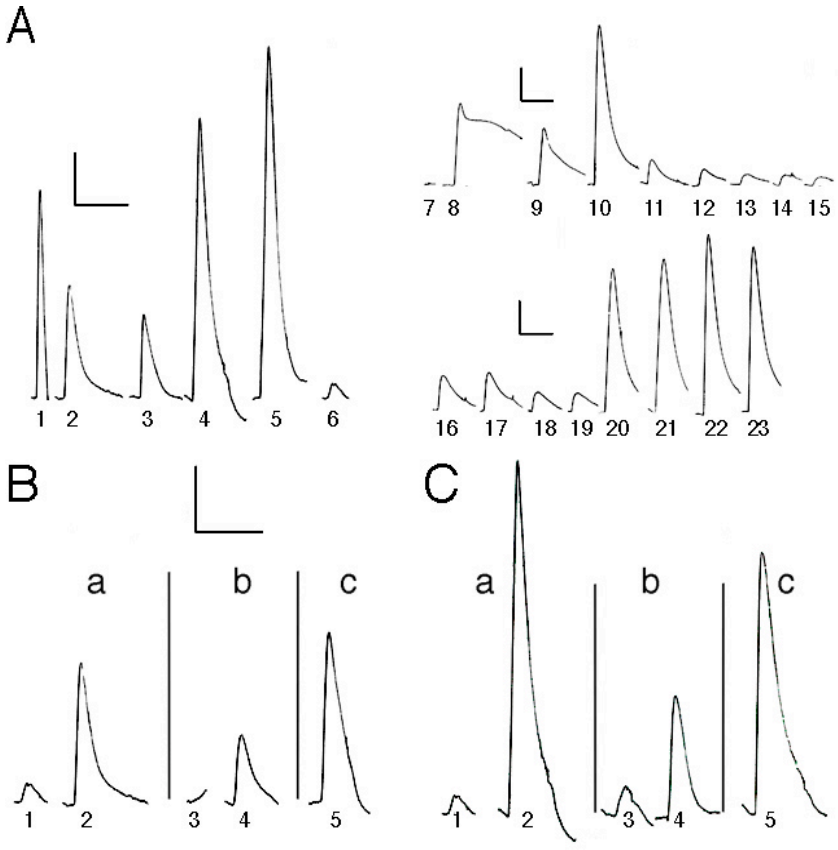

Figure 1. (A) Representative electro-olfactogram (EOG) recordings from the sea catfish to tested stimuli. (1) 10 μM L-methionine (standard), (2) natural ink (NI) diluted 100,000x, (3) artificial ink (AI) diluted 100,000x, (4) natural opaline (NO) diluted 100,000x, (5) artificial opaline (AO) diluted 10,000x, (6) artificial seawater (ASW) (control), (7) ASW (control), (8) 10 μM L-methionine (standard), (9) 100 μM L-arginine, (10) 100 μM L-lysine, (11) escapin intermediate products of lysine (EIP-K) diluted 1,000x, (12) escapin intermediate products of arginine (EIP-R) diluted 1,000x, (13) escapin end products of lysine (EEP-K) diluted 1,000x, (14) escapin end products of arginine (EEP-R) diluted 1,000x, (15) 1.45 mM H_2O_2, (16) EIP-L diluted 1,000x + 1.45 mM H_2O_2 tested 15 sec after mixing, (17) EIP-K diluted 1,000x + 1.45 mM H_2O_2 tested 5 min after mixing (18) EIP-R diluted 1,000x + 1.45 mM H_2O_2 tested 15 sec after mixing, (19) EIP-R diluted 1,000x + 1.45 mM H_2O_2 tested 5 min after mixing, (20) NI diluted 10,000x, (21) NO diluted 10,000x, (22) NI diluted 10,000x + NO diluted 10,000x 15 sec after mixing, and (23) NI diluted 10,000x + NO diluted 10,000x 5 min after mixing. B, C. EOG recordings in adaptation experiments . (B) The adapting solution was AI diluted 10,000x. (a) Responses prior to adaptation to: (1) ASW (control) and (2) NI diluted 100,000x. (b) Responses during continuous adaptation to: (3) AI diluted 10,000x (control) and (4) NI diluted 100,000x. (c) Post-adaptation responses to (5) NI diluted 100,000x. (C) The adapting solution was AO diluted 10,000x. (a) Responses prior to adaptation to: (1) ASW (control) and (2) NO diluted 100,000x. (b) Responses during continuous adaptation to: (3) AO diluted 10,000x (control) and (4) NO diluted 100,000x. (c)

Post-adaptation response to (5) NO diluted 100,000x. A1-A6, B, and C were recorded from the same fish; A7-A23 were recorded from a different fish. Time bar, 1 min; amplitude bar, 380 μV.

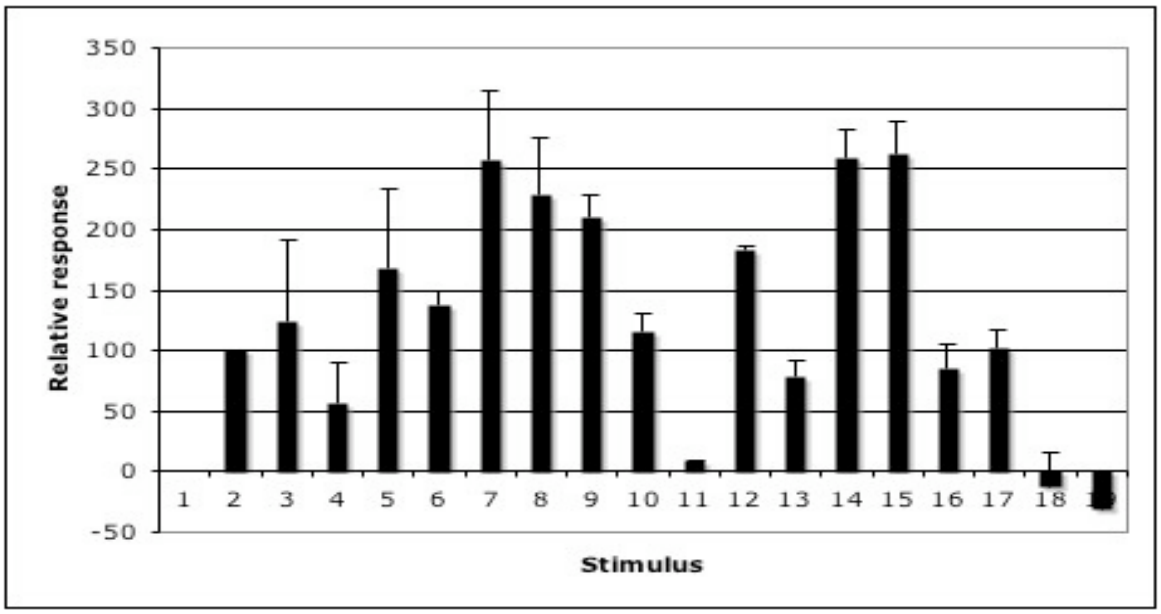

Figure 2. Mean response to (1) aSW, (2) 10 μM L-methionine, (3) NI diluted 10,000x, (4) AI diluted 10,000x, (5) NO diluted 10,000x, (6) AO diluted 10,000x, (7) NI diluted 10,000x and NO diluted 10,000x 15 seconds after mixing, (8) NI diluted 10,000x and NO diluted 10,000x 5 minutes after mixing, (9) 100 μM L-lysine, (10) 100 μM L-arginine, (11) 14.5 μM H_2O_2, (12) EIP-L diluted 100x, (13) EIP-R diluted 100x, (14) EIP-L diluted 100x and 14.5 μM H_2O_2 tested 15 seconds after mixing, (15) EIP-L diluted 100x and 14.5 μM H_2O_2 tested 5 minutes after mixing, (16) EIP-R diluted 100x and 14.5 μM H_2O_2 tested 15 seconds after mixing, (17) EIP-R diluted 100x and 14.5 μM H_2O_2 tested 5 minutes after mixing, (18) EEP-L diluted 100x, (19) EEP-R diluted 100x. See Fig. 1 legend for definitions of abbreviations. Stimuli 3,5,9,10 were each tested on 3 fish. All other stimuli were tested on 2 fish. Stimuli 1 and 2 (control and standard) were tested on 6 fish.

Taste responses

Natural and artificial ink and opaline resulted in large magnitude taste responses compared to that of the standard, 100 μM L-alanine (Figs. 3A, 4). The response to natural ink was partially, but not completely, cross-adapted with an equal concentration of the artificial ink as the response to natural ink at 10,000 dilution was reduced by 44-64% following adaptation to artificial ink at 10,000 dilution (Fig. 3B). The response to natural opaline was almost completely cross-adapted by artificial opaline as the response to natural opaline at 10,000 dilution was reduced by 92-100% following adaptation to artificial opaline

at 10,000 dilution (Fig. 3C). These results suggest that artificial opaline contains many of the gustatory stimulants in natural opaline, and natural ink contains some additional gustatory stimulants than are present in artificial ink.

The reaction of escapin with L-lysine or L-arginine did not affect the gustatory efficacy of L-lysine and L-arginine since they were weak gustatory stimulants. Also, there was no response to 1.45 mM H_2O_2. The addition of H_2O_2 to EIPs did not increase their stimulatory effectiveness (Figs. 3A, 4).

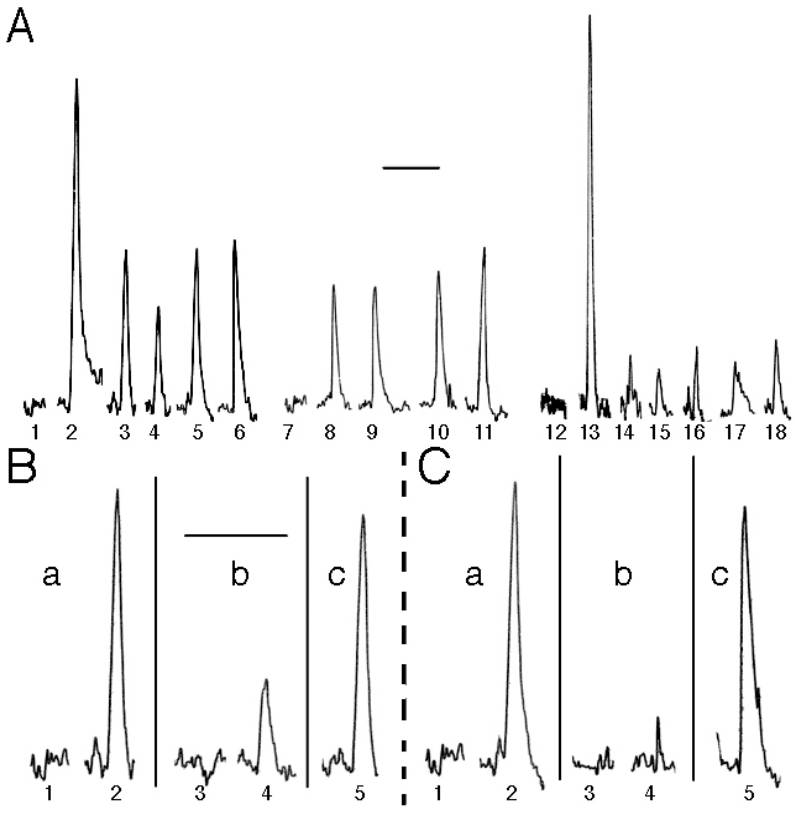

Figure 3. Typical records of integrated facial taste recordings from the sea catfish to: (A) (1) artificial seawater (aSW) (control), (2) 100 μM L-alanine (standard), (3) NI diluted 10,000x, (4) AI diluted 10,000x, (5) NO diluted 10,000x, (6) AO diluted 10,000x, (7) aSW, (8) NI diluted 10,000x, (9) NO diluted 10,000x, (10) NI diluted 10,000x and NO diluted 10,000x 15 seconds after mixing, (11) NI diluted 10,000x and NO diluted 10,000x 5 minutes after mixing, (12) aSW , (13) 100 μM L-alanine, (14) EIP-L diluted 100x, (15) EIP-R diluted 100x, (16) EEP-L diluted 100x, (17) EIP-L diluted 100x and 14.5 μM H_2O_2 tested 15 seconds after mixing, (18) EIP-R diluted 100x and 14.5 μM H_2O_2 tested 5 minutes after mixing. The following stimuli evoked no significant responses and are not shown: 100 μM L-arginine, 100 μM L-lysine, 14.5 μM H_2O_2, EEP-R diluted 100x. B,C. Integrated facial taste recordings of adaptation experiments. (B) adaptation to AI diluted 10,000x. (a) responses prior to adaptation to: (1) aSW (control) and (2) NI diluted 10,000x, (b) responses during continuous adaptation to (3) AI diluted 10,000x (control), (4) NI diluted 10,000x, and (c) post-adaptation response to (5) NI diluted 100,000x. (C) the adapting solution was AO diluted 10,000x. (a) responses prior to adaptation to: (1) aSW (control) and (2) NO diluted 10,000x, (b) responses

during continuous adaptation to: (3) AO diluted 10,000x (control) and (4) NO diluted 10,000x; (c) post-adaptation response to (5) NO diluted 10,000x. A1-A11 and B,C were recorded from the same fish; A12-A18 were recorded from a different fish. Time bar, 1 min. See Fig. 1 legend for definitions of abbreviations.

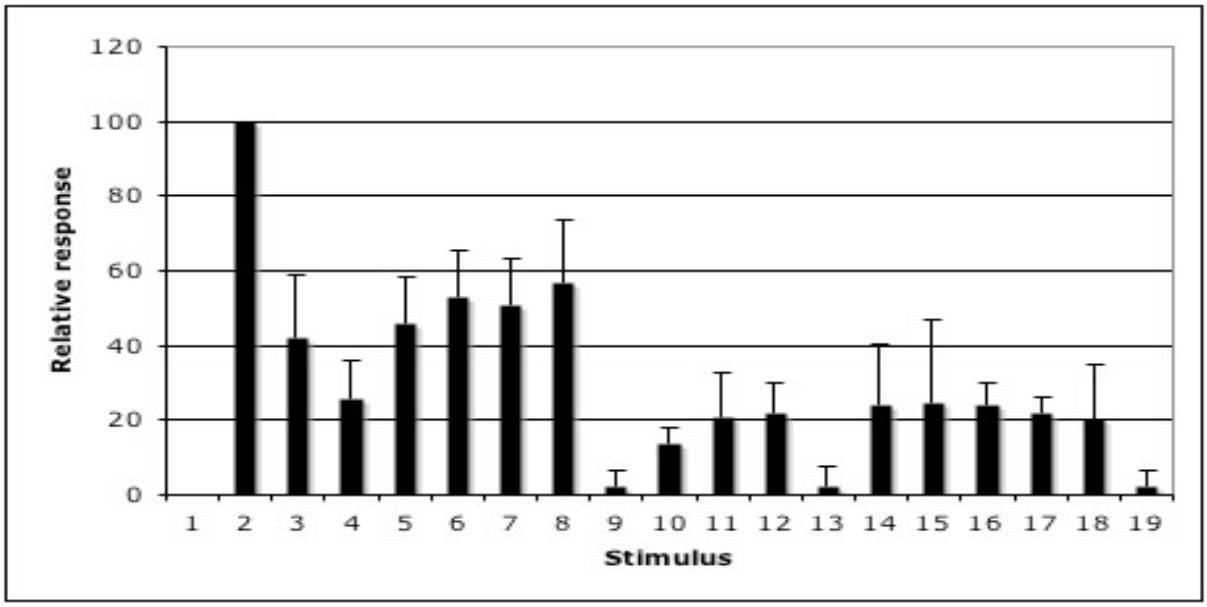

Figure 4. Mean response to (1) aSW, (2) 10 μM L-methionine, (3) NI diluted 10,000x, (4) AI diluted 10,000x, (5) NO diluted 10,000x, (6) AO diluted 10,000x, (7) NI diluted 10,000x and NO diluted 10,000x 15 seconds after mixing, (8) NI diluted 10,000x and NO diluted 10,000x 5 minutes after mixing, (9) 100 μM L-lysine, (10) 100 μM L-arginine, (11) EIP-L diluted 100x, (12) EIP-R diluted 100x, (13) 14.5 μM H_2O_2, (14) EIP-L diluted 100x and 14.5 μM H_2O_2 tested 15 seconds after mixing, (15) EIP-L diluted 100x and 14.5 μM H_2O_2 tested 5 minutes after mixing, (16) EIP-R diluted 100x and 14.5 μM H_2O_2 tested 15 seconds after mixing, (17) EIP-R diluted 100x and 14.5 μM H_2O_2 tested 5 minutes after mixing, (18) EEP-L diluted 100x, (19) EEP-R diluted 100x. See Fig. 1 legend for definitions of abbreviations. Stimuli 1-10 were tested on 3 fish each. Stimuli 11-19 were tested on 5 fish each. Stimuli 1 and 2 (control and standard) were tested on 8 fish.

Discussion

The results suggest that the sea catfish, *Ariopsis felis*, is an excellent model organism for investigating physiological mechanisms of action of chemical defenses. Using the ink of sea hares, *Aplysia californica*, as the defensive secretion, the behavioral and electrophysiological studies examined how chemical defenses affect sea catfish through interactions with their olfactory and gustatory systems.

Ink secretion, which is a mixture of two glandular components, ink and opaline (Derby 2007), is unpalatable to sea catfish (Table 2). Catfish avoided ink secretion when presented alone and rejected food when ink secretion was added to it. But ink and opaline contribute differently to the overall ink secretion. Ink was unpalatable and aversive when presented by itself and caused rejection when added to food, similar to the effects of the mixed secretion. Opaline by itself, however, increased the animals' arousal and attraction although not to the point of causing ingestion and did not reduce the palatability of food when added to it.

The compounds responsible for the unpalatability of ink secretion to sea catfish were not identified. Future behavioral work on the molecular identity of the unpalatable compounds in sea hare ink will be important for pursuing electrophysiological investigations into the sensory mechanisms of their perception and deterrence.

The appetitive effect of opaline is mediated in part by its amino acid components, which total over 300 mM in full-strength opaline (Kicklighter et al., 2005; Derby et al., 2007). An artificial mixture mimicking the amino acid composition of opaline had a similar effect on sea catfish as opaline itself increased arousal and attraction but did not cause ingestion when presented alone and did not affect the palatability of food. Ink itself also has a high concentration of amino acids, and an artificial mixture of ink, like artificial opaline, induced attraction but not ingestion. Our electrophysiological recordings demonstrated that sea hare

ink secretion is highly stimulatory to both olfactory and gustatory systems of sea catfish . The amino acids in ink and opaline are responsible for a large portion of the neural response to the natural secretions, but not all of it, as demonstrated by two results. First, the magnitude of the responses to the natural secretions was greater than that to the corresponding artificial mixture of the component amino acids. Secondly, cross-adaptation experiments showed that adaptation to the amino acid components of ink or opaline only partially reduced the responses to the corresponding natural secretion. Compounds generated by the activity of escapin, an L-amino acid oxidase in the secretion (Yang et al., 2005; Johnson et al., 2006), were moderately stimulatory to both olfactory and gustatory systems (although generally less so than their precursor) and thus also contributed to the stimulatory activity of the secretion. The large neural responses to the secretions, particularly the amino acid fraction, are supportive of the hypothesis that sensory disruption and/or phagomimicry might contribute to the antipredatory effect of the secretion.

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