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Responses of the sea catfish *Ariopsis felis* to chemical defenses from the sea hare *Aplysia californica*

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ABSTRACT

Ink secretion of sea hares (*Aplysia* spp.), which is a mixture of co-released ink from the ink gland and opaline from the opaline gland, protects sea hares from predatory invertebrates through diverse mechanisms. These include both aversive or deterrent compounds and also high concentrations of amino acids that stimulate the predators' chemical senses and divert the attack through phagomimicry or sensory disruption. The aim of the present study was to examine if sea hares also defend themselves from predatory vertebrates by interacting with their chemical senses. We used sea catfish, *Ariopsis felis*, in behavioral and electrophysiological experiments. Behavioral tests on sea catfish show that ink is aversive: when ink is added to palatable food items (noodles with food flavoring), the noodles are no longer eaten, and when ink is added to noodles without food flavoring, the noodles are avoided more than unflavored noodles. Behavioral tests also show that opaline and the amino acid components of either opaline or ink are appetitive. Electrophysiological recordings of chemosensory neuronal activity in the olfactory epithelium and maxillary barbels show that the olfactory and gustatory systems of sea catfish are highly stimulated by ink and opaline, and that the amino acid components of ink and opaline significantly contribute to these responses. Compounds generated by the activity of escapin, an L-amino acid oxidase in the secretion, are moderately stimulatory to both olfactory and gustatory systems. Taken together, our results support the idea that sea hares are chemically defended from predatory sea catfish largely through unpalatable chemical deterrents in ink, but possibly also through amino acids stimulating olfactory and gustatory systems and thus functioning through phagomimicry or sensory disruption.

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1. Introduction

Predatory fish have many sensory systems, including vision, mechanoreception, chemoreception, and electroreception (Hara and Zielinski, 2007), that they might use in selecting prey. The importance of each depends on many factors such as the species, availability of light, and turbidity of the water.

Chemoreception is probably used by all fish species in some aspect of feeding (Sorensen and Caprio, 1998; Valentinčič, 2005; Hara and Zielinski, 2007; Derby and Sorensen, 2008). Olfaction is used by many fish to locate food from a distance, though some fish, such as catfish, can also use an external taste system to perform this task. Gustation controls the ingestion of food, even for fish that use other senses to detect and approach the food. Some of the chemicals influencing how fish locate potential food, evaluate its palatability, and decide whether or not to swallow are known. The chemicals in potential prey that stimulate feeding tend to be small, nitrogen containing molecules, especially amino acids, nucleosides and nucleotides, amines, and polyamines (Hara, 1994; Carr et al., 1996; Rolen et al., 2003; Valentinčič, 2005). Mixtures of such molecules are highly stimulatory to the olfactory and gustatory receptor cells of many species of fishes, and are also attractive and palatable to these animals. The neural mechanisms controlling the sensory detection and processing of these chemicals are well described (Caprio et al., 1993; Sorensen and Caprio, 1998; Caprio and Derby, 2008). Many animals also contain chemicals that decrease palatability as a defense from predators. These include many examples of chemical defenses against predatory fish (Duffy and Paul, 1992; Paul, 1992; Pawlik, 1993; Hay et al., 1998; McClintock and Baker, 2001; Kubanek et al., 2002; Lindquist, 2002; Long and Hay, 2006; Paul et al., 2006). However, the neural basis for their chemical deterrency is almost completely unexplored. For a notable exception, see Cohen et al. (2008).

One group of animals that are well-defended chemically is the sea hares, which are soft bodied marine gastropod molluscs (Johnson and Willows, 1999). Their chemical defenses include compounds in their skin and tissues that are distasteful and in some cases toxic, and an ink secretion – a colored, sticky secretion released when sea hares are

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vigorously attacked by predators. Ink secretion is a mixture of two co-released glandular products: opaline from the opaline gland, and ink from the ink gland. Inking has been shown to protect sea hares against attacks of two species of predators – sea anemones and spiny lobsters (Nolen et al., 1995; Kicklighter et al., 2005).

The mechanisms whereby inking protects sea hares are beginning to be understood, and can be classified into three categories: distastefulness, phagomimicry, and sensory disruption. The first category is the best known, with several examples of ink and/or opaline inhibiting ingestive behavior of predators, although the molecular identities of the bioactive substances are rarely identified (Ambrose and Givens, 1979; DiMatteo, 1981; Kicklighter et al., 2005; Kicklighter and Derby, 2006; Kamio et al., 2007). Phagomimicry and sensory disruption occur because ink and opaline contain amino acids and other compounds at extraordinarily high concentrations and mixed in a sticky secretion. For example, taurine is present at >200 mM in opaline (Kicklighter et al., 2005; Derby et al., 2007) (also see Table 1). These chemicals stimulate the chemosensory systems of crustacean predators, such as spiny lobsters, affecting their behavior in any of a number of ways (Kamio et al., 2007). Spiny lobsters sometimes treat the secretion as food, reacting with both appetitive and ingestive behavior, a behavior called phagomimicry (Kicklighter et al., 2005; Shabani et al., 2007). The combination of stickiness and high concentrations of stimulants in the secretion may produce an unusually long, sustained, and extremely high magnitude chemosensory stimulation, possibly causing adaptation and sensory disruption, and leading to reduced predatory attacks (Kicklighter et al., 2005). These three mechanisms are not necessarily mutually exclusive, and they can mediate defense even against a single predatory species (Derby, 2007).

A major component in ink that is a prime candidate for playing a defensive role, but with little evidence so far, is escapin. Escapin is an L-amino acid oxidase present in ink of *Aplysia californica*, and it reacts with its substrate L-lysine, present in opaline, to produce to complex mixture of compounds, as is summarized in Fig. 1 (Yang et al., 2005; Johnson et al., 2006; Derby, 2007; Kamio et al., in press). Escapin also oxidizes L-arginine and produces a similar set of compounds; however, since L-arginine is 300 times less concentrated than L-lysine in the secretion, these compounds are in much lower concentration than their lysine analogues. Orthologs of escapin exist in all other sea hares examined, including *Aplysia dactylomela* (Melo et al., 2000; Kamiya et al., 2006; Derby, 2007). Some of the molecules in this complex mixture are known to have bacteriostatic and bactericidal effects (Ko et al., 2008), but their effects on predators are almost completely unexamined (Kicklighter and Derby, 2006; Kamio et al., 2007).

Table 1
Composition of artificial ink and artificial opaline

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

These mixtures are based on concentrations of amino acid and ammonia in natural ink and opaline from wild *Aplysia californica* reported in Kicklighter et al. (2005) and Derby et al. (2007).

The exceedingly high concentrations of amino acids in sea hare ink and the high sensitivity of fish predators to amino acids raise the distinct possibility that sea hares may use amino acids as a phagomimetic and/or sensory disruptive defense against predatory fish. We tested this phagomimicry hypothesis and also the hypothesis that ink defends sea hares through distasteful chemicals, using the sea catfish *Ariopsis felis*. Sea catfish are opportunistic and generalist feeders and are sympatric with sea hares *Aplysia dactylomela* and *Aplysia brasiliana* in subtidal waters of coastal Florida and the Gulf of Mexico, and thus sea catfish are potential predators of sea hares (Eales, 1960; Marcus, 1972; Muncy and Wingo, 1983; Carefoot, 1987). The sea catfish has previously been developed as a model for mechanisms of neural processing in olfaction and gustation (Silver et al., 1976; Caprio, 1980; Michel and Caprio, 1991; Michel et al., 1993; Kiyohara and Caprio, 1996; Kohbara and Caprio, 1996; Smith, 2000). The olfactory and gustatory systems of sea catfish are highly sensitive to amino acids, with L-cysteine and L-methionine being the more stimulatory olfactory stimuli (physiological threshold of ~10 nM) (Caprio, 1980) and L-alanine, glycine, and D-alanine being the more stimulatory taste stimuli (physiological thresholds of 1-10 nM) (Michel and Caprio, 1991; Michel et al., 1993).

In this study, we use sea catfish as a model organism to study mechanisms whereby sea hare defensive secretions potentially defend against predatory fish.

2. Materials and Methods

2.1. Stimuli

2.1.1. Collection of sea hare secretions

Natural ink (NI) and natural opaline (NO) were collected from dissected ink and opaline glands of adult sea hares (*Aplysia californica*) purchased from Marinus Inc., according to the procedures of Yang et al. (2005).

2.1.2. Escapin and its reaction products

Escapin was purified 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 natural ink+natural opaline, EIP+H_2O_2, or EEP+ H_2O_2 was tested, the two components of each were mixed 15 sec or 5 min before presentation. This is because there are chemical reactions of ingredients in the two components, including but not limited to the cascade of time-dependent reactions beginning with escapin's oxidative deamination of L-lysine and L-arginine as described in the Introduction and reviewed in Derby (2007). Comparing responses over time allowed us to examine the importance of these time-dependent compositional changes.

2.1.3. Artificial ink and opaline mixtures

Artificial ink (AI) and artificial opaline (AO) were formulated from amino acid and ammonium profiles of natural ink and opaline of *A. californica*, based on the results of Kicklighter et al. (2005) and Johnson et al. (2006). The component amino acids (Sigma-Aldrich) were brought up at natural concentration in artificial sea water to create AI and AO, with the formulation shown in Table 1. The amino acid compositions for *A. californica*, *A. dactylomela*, and other sea

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