

Escape by Inking and Secreting: Marine Molluscs Avoid Predators Through a Rich Array of Chemicals and Mechanisms

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Abstract. Inking by marine molluscs such as sea hares, cuttlefish, squid, and octopuses is a striking behavior that is ideal for neuroecological explorations. While inking is generally thought to be used in active defense against predators, experimental evidence for this view is either scant or lacks mechanistic explanations. Does ink act through the visual or chemical modality? If inking is a chemical defense, how does it function and how does it affect the chemosensory systems of predators? Does it facilitate escape not only by acting directly on predators but also by being an alarm signal for conspecifics? This review examines these issues, within a broader context of passive and active chemical defensive secretions. It focuses on recent work on mechanisms of defense by inking in sea hares (*Aplysia*) and extends what we have learned about sea hares to other molluscs including the cephalopods.

Neuroecology of Chemical Defenses: Mechanisms From a Comparative Perspective

There are numerous examples of how inks and other chemical secretions are used for protection from predation. Identification of bioactive compounds from defended organisms is often a research focus. Here, I explore chemical defenses in model systems, including identifying chemicals and their cellular and molecular mechanisms of defense and elucidating their comparative, evolutionary, and ecological relationships.

Investigations of mechanisms of chemical protection include many interesting examples. Nonetheless, how chem-

ical defenses act through the nervous system, particularly the sensory systems, is relatively poorly understood. Defensive secretions are usually complex mixtures of chemicals that are often assumed, but infrequently shown, to be distasteful, harmful, toxic, or some combination of these. The best examples of sensory mechanisms of chemical defenses in natural contexts are for herbivores in plant-insect interactions. The vast majority of such examples are of plants having compounds that are distasteful or toxic to herbivores, and the identity and characteristics of the sensors of these compounds are known for many insect species (*e.g.*, Blaney *et al.*, 1986; Stowe *et al.*, 1995; Schar *et al.*, 2001; Glendinning *et al.*, 2002; Bernays and Singer, 2005; Glendinning, 2007; Conner *et al.*, 2007). In the marine environment, many chemical defensive compounds are identified (*e.g.*, Hay, 1996), but for almost all of them the sensory mechanisms of action remain unexplored.

A chemically defended animal not only avoids being eaten by using its chemical arsenal, but it also avoids predators by detecting chemicals released by attacked conspecifics and subsequently producing evasive behaviors. These conspecific chemicals, called alarm signals, are known from many species (Blum, 1996; Wisenden, 2000; Wyatt, 2003). (Note that here and throughout the text, I use “signal” in the sense of Bradbury and Vehrencamp (1998), defined as communication with benefits to the transmitting organism.) An interesting but often neglected question is, what is the relationship between conspecific alarm signals and heterospecific antipredator compounds released in the same secretion? Are the sensory pathways used in detecting conspecifics alarm molecules the same as or different from those that detect heterospecific defensive compounds?

An understanding of basic principles and thematic variations in mechanisms of chemical defense requires a com-

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Abbreviations: LAAO, L-amino acid oxidase; MAA, mycosporine-like amino acid.

parative and evolutionary perspective. For this, one needs to consider a diversity of animals varying in phylogenetic and phenotypic relatedness, even if a few are the focus of in-depth studies.

This paper explores the neuroecology of chemical defenses, from molecules to mechanisms and with a comparative perspective, focusing on marine molluscs and their inking behavior.

Chemical Defenses of Molluscs

Molluscs are a diverse lot of organisms and include snails, cephalopods, and bivalves. Many molluscs are enjoyed as food not only by humans but also by other species from diverse taxa including but not limited to fish, crustaceans, sea stars, and sea anemones. For their part of the evolutionary arms race, molluscs have adopted an impressive array of defensive strategies. Some molluscs are protected by shells, but many, including opisthobranch gastropods and cephalopods, are not. Chemical defenses are used extensively by both shelled and shell-less molluscs, and consequently molluscs are a valuable resource for examining the neuroecology of chemical defenses. Inks are just one class of these defenses. The following section examines the nature of these chemical defenses, first focusing on passive defenses for background and context, then exploring inking in greater detail.

Chemical defenses of gastropods

The gastropods are relatively well studied in terms of the chemical defenses in their mucus, skin, and digestive glands. For example, skin and mucus of snails can deter predatory attacks because they contain distasteful and deterrent compounds, some of which have been identified. Mucus can have a mechanical protective effect. It can also carry chemical cues, thereby enhancing the persistence of the chemicals and reducing their dispersal in water (Denny, 1989; Kicklighter *et al.*, 2005). Mucus can also contain chemical deterrents, as shown for shelled gastropods and nudibranchs (Johannes, 1963; Branch, 1981; Reel and Fuhrman, 1981; Rice, 1985; Denny, 1989; Avila *et al.*, 1991; Ehara *et al.*, 2002). The skin of marine gastropods has deterrent chemicals (Stallard and Faulkner, 1974; Kinnel *et al.*, 1979; Faulkner and Ghiselin, 1983; Paul and Van Alstyne, 1988; Faulkner, 1992; Pennings and Paul, 1993; Yamazaki, 1993; Pennings, 1994; de Nys *et al.*, 1996; Yamada and Kigoshi, 1997; Pennings *et al.*, 1999; Gallimore and Scheuer, 2000; Ginsburg and Paul, 2001; Barsby *et al.*, 2002; Cimino and Gavagnin, 2006). Many of these deterrents are diet-derived and not synthesized *de novo*, and many are terpenoids, especially sesquiterpenoids and diterpenoids (Avila, 1995; Cimino *et al.*, 1999; Kamiya *et al.*, 2006). Rarely have these compounds been tested experimentally for their biological effects; however, some of these

compounds or the tissues containing them have been demonstrated to have toxic effects (Thompson, 1960; Marin *et al.*, 1999). Other passive defenses in the skin do not depend on particular diets but rather are synthesized *de novo* (Pennings, 1994; Barsby *et al.*, 2002), which is probably a more derived evolutionary condition (Cimino and Ghiselin, 1999; Wägele and Lussmann-Kolb, 2005).

Skin glands that secrete acids, such as sulphuric acid, in response to disturbance are known from many marine gastropods (Thompson, 1983, 1986, 1988; Gillette *et al.*, 1991; Wägele and Lussmann-Kolb, 2005; Shabani *et al.*, 2007). Acid secretions may have direct deterrent effects on predators, may be part of a positive feedback loop that reinforces the aversive behavior of snails themselves, or may enhance other chemical defenses (Thompson, 1988; Gillette *et al.*, 1991; Shabani *et al.*, 2007).

Passive chemical defenses are not limited to mucus and skin. Some gastropods have deterrent compounds in their egg masses and capsules to prevent them from being ingested by other animals (Pennings, 1994; Benkendorff *et al.*, 2001). These compounds might also have an antimicrobial function. For example, L-amino acid oxidases are present in the albumen gland and egg mass and have antimicrobial activity (Kamiya *et al.*, 1986, 2006; Yamazaki *et al.*, 1989a, b, c; Jimbo *et al.*, 2003; Iijima *et al.*, 2003b; Cummins *et al.*, 2004).

In addition to these chemical defenses, sea hares have active chemical defensive behavior—releasing ink when attacked by a predator. Inking is the focus of this review. Ink is usually a mixture of two glandular secretions that are simultaneously released into the mantle cavity and then expelled through a siphon toward the site of predatory attack (Walters and Erickson, 1986). The ink gland of most species secretes a deep purple ink. The purple color of the secretion (aplysiocyanin) is derived from pigments (phycocyanin) found in the sea hare diet of red algae (Rüdiger, 1967; Chapman and Fox, 1969; MacColl *et al.*, 1990). Without this diet, purple ink cannot be produced (Nolen *et al.*, 1995; Nolen and Johnson, 2001). The second secretion is released from the opaline gland, and it is a clear-to-whitish liquid that polymerizes and becomes highly viscous upon contact with water. Production of opaline is not diet-dependent, and opaline appears to be made *de novo* (Johnson and Willows, 1999; Kicklighter *et al.*, 2005). Both glands are under separate neural control in different ganglia and can release their secretions independently or together (Carew and Kandel, 1977; Tritt and Byrne, 1980). Healthy, undisturbed animals do not release either secretion without provocation.

Sea hare ink might have visual or chemical defensive effects. In analogy with the inking behavior of cephalopods, ink might act as a visual mimic, distracter, or smoke screen against attacking predators, giving sea hares an advantage in escape as the would-be predator visually focuses on the ink

(Caldwell, 2005; Derby *et al.*, 2007). Certainly the dark color of ink in some ways visually mimics sea hare coloration, and this is consistent with a visual function, as has been speculated but never experimentally tested (reviewed in Carefoot, 1987, and Johnson and Willows, 1999). However, unlike cephalopods, sea hares move rather sluggishly, so a purely visual decoy or smoke screen is likely to have at best short-term effects on survival. Thus, it is not surprising that evidence has accumulated that sea hare ink works through the chemical medium. Several studies have demonstrated that the ink secretions of *Aplysia* contain chemicals that inhibit the feeding of potential predators such as birds, fishes, crustaceans, and sea anemones (reviewed in Carefoot, 1987, and Johnson and Willows, 1999). But so far, the survival value of ink secretions has been directly tested on only two species: the sea anemone *Anthopleura sola* and the spiny lobster *Panulirus interruptus* (Nolen *et al.*, 1995; Kicklighter *et al.*, 2005; Kicklighter and Derby, 2006). Much is known about the composition of ink secretions (Johnson and Willows, 1999; Yang *et al.*, 2005; Kamiya *et al.*, 2006; Kicklighter and Derby, 2006; K.-C. Ko *et al.*, unpubl. data). Some compounds in the ink or opaline of opisthobranchs are derived directly from a diet of red algae (Quinoa *et al.*, 1989; Rogers *et al.*, 2000; Bezerra *et al.*, 2004; Capper *et al.*, 2005). Others are produced *de novo* (Prince *et al.*, 1998; Bezerra *et al.*, 2004; Johnson *et al.*, 2006). Notably, in spite of the many chemical studies of ink and opaline, very few bioactive components of these secretions have been identified.

In addition to chemical defenses that act on predators, gastropods—like many other animals—release chemicals when attacked by predators that serve as alarm signals to nearby conspecifics to elicit predator avoidance behaviors (Snyder and Snyder, 1971; Atema and Stenzler, 1977; Wyatt, 2003; Jacobsen and Stabell, 2004). The chemicals can be released from the flesh of injured conspecifics or from the body fluid (*e.g.*, urine) of threatened or disturbed conspecifics. The responses include burrowing and escape locomotion. The defensive ink secretions of *Aplysia fasciata* and *A. californica* produce such alarm signals that evoke escape behaviors (Fiorito and Gherardi, 1990; Nolen *et al.*, 1995).

Sea hares: models of the neuroecology of chemical defenses

Sea hares are nearly shell-less opisthobranch gastropod molluscs. They are known throughout the world's temperate and tropical oceans, where they face a variety of predatory threats (Sarver, 1978; Pennings, 1990; Paul and Pennings, 1991; Johnson and Willows, 1999; Ginsburg and Paul, 2001). Sea hares have a number of antipredatory defenses, among them cryptic coloration and form, large size as adults, mucus, and chemical defenses. The chemical de-

fenses of sea hares have been studied in the field and the laboratory by natural products chemists and chemical ecologists (*e.g.*, Kinnel *et al.*, 1979; Yamazaki, 1993; Pennings, 1994; de Nys *et al.*, 1996; Carefoot *et al.*, 1999; Gallimore and Scheuer, 2000; Ginsburg and Paul, 2001). Other advantages of studying sea hare neuroecology are that a diversity of species are available; they are often intertidal or subtidal and therefore readily accessible for collection or study in the field; and one species, *Aplysia californica*, is commercially raised and can be obtained year round at any developmental stage.

Chemical defenses against predatory spiny lobsters: a panoply of mechanisms. When attacked by spiny lobsters (*Panulirus interruptus*), sea hares (*Aplysia californica*) often release defensive secretions from ink and opaline glands. The ink-opaline mixture facilitates the escape of sea hares in complex ways. Some bioactive molecules are present in ink alone and some in opaline alone, and others are generated only when ink and opaline are co-secreted and mixed in the mantle cavity. These compounds include feeding stimulants, feeding deterrents, and aversive compounds. For example, the secretion contains some compounds that stimulate appetitive and ingestive feeding behavior; these are amino acids, present in either ink or opaline, some at extremely high concentrations—*e.g.*, taurine, a major feeding stimulant, is present at $>200 \text{ mmol l}^{-1}$ in opaline (Kicklighter *et al.*, 2005; Derby *et al.*, 2007). Other compounds inhibit ingestive behavior (Kicklighter *et al.*, 2005). Still other compounds are aversive and inhibit appetitive feeding behavior without affecting ingestive behavior. An example is the products of the oxidation of L-lysine (present in high doses in opaline) by escapin (an L-amino acid oxidase that is present only in ink), which are produced only when ink and opaline are co-secreted and mixed in the mantle cavity just prior to release (Kicklighter *et al.*, 2005; Johnson *et al.*, 2006; Kamio *et al.*, 2007). Not surprisingly, the ink-opaline mixture can produce a variety of responses in spiny lobsters (Kicklighter *et al.*, 2005; Kamio *et al.*, 2007). Some spiny lobsters treat the secretion as food, reacting with both appetitive and ingestive behavior such as digging with the legs into the substrate covered by the secretion or moving the first two pairs of legs to the mouth. This effect, which is termed phagomimicry, is a deception that stimulates the spiny lobster's chemosensory neural pathway and leads it to attend to a false food stimulus (Johnson, 2002). The highly viscous nature of opaline may create a tactile sensation of food, contributing to the mimicry. Other spiny lobsters avoid the ink-opaline secretion, produce escape responses, show grooming behavior, or exhibit a combination of these responses. These latter effects might be produced by sensory disruption, in which the sticky and potent secretions cause high-amplitude, long-lasting chemo-mechanosensory stimulation; or they might be produced by aversive or

deterrent compounds that oppose the appetitive effects of the phagomimics. In a sense, a reptile dropping its tail (Dial and Fitzpatrick, 1984) or an octopus dropping an arm (Norman, 2000) when attacked by predators, with the detached appendage distracting or attracting predators, is an example of phagomimicry. But these are visual rather than chemical mimics, and losing a piece of the body is much more energetically costly than a chemical mimic such as ink. There are no other demonstrations of chemical phagomimics, though anecdotal reports suggest some candidates (Maschwitz *et al.*, 1981; Hölldobler *et al.*, 1982; LaMunyan and Adams, 1987). Phagomimicry may be related to other forms of chemical mimics that involve false scents of food to attract mates, prey, and pollinators (Stowe, 1988).

Acids enhance the phagomimetic defense. Ink and opaline are highly acidic: at full strength, their pH values are 4.9 and 5.8, respectively. Does acidity influence the efficacy of these chemical defenses? To answer this question, we used spiny lobsters to examine the behavioral and electrophysiological responses of antennular and mouthpart chemosensory neurons to phagomimetic chemical defenses—ink and opaline, as well as seawater control—at the pH levels of 4.9, 6.3, and 7.7 (Shabani *et al.*, 2007). The response intensity of chemosensory neurons to phagomimetic chemical defenses and the appetitive behavioral responses controlled by them—attraction, grabbing, and ingestion—are greater at lower pH. Additionally, low-pH seawater by itself elicits responses from antennular and mouthpart chemosensory neurons and appetitive behavioral responses from spiny lobsters. The results indicate that the phagomimetic defense of sea hares against spiny lobsters is significantly enhanced at the naturally low pH of the defensive secretions.

Another predator; different mechanisms. Against the predatory sea anemone *Anthopleura sola*, sea hares use ink, but not opaline, as a chemical defense. Ink acts only as a feeding deterrent through aversion: it causes tentacle retraction or shriveling and gastrovascular eversion. Ink contains many constituents of aversive compounds (Kicklighter and Derby, 2006).

These studies on lobsters and sea anemones point out a more general property of chemical defenses—chemically defended species contain an array of bioactive molecules. Sometimes defensive compounds are closely related to one another (*e.g.*, Hay *et al.*, 1987; Pawlik and Fenical, 1989; Kicklighter *et al.*, 2003). In other cases, such as in sea hares, the multiple defensive compounds in a single species have low structural relatedness (Kicklighter *et al.*, 2003; Kicklighter and Hay, 2007). Having a medley of defensive compounds may be useful in deterring a variety of predatory species in predator-rich environments (Hay, 1984).

More predators. Antipredatory effects of ink and opaline include feeding deterrents to crabs, fish, and other species, though most of the bioactive molecules have not been identified (Ambrose and Givens, 1979; DiMatteo, 1981; Kamio *et al.*, 2007). Future work will undoubtedly reveal a greater diversity and richness of mechanisms.

L-Amino acid oxidases in marine gastropods: a comparative case study

L-Amino acid oxidases (LAAOs) are enzymes that oxidatively deaminate L-amino acids, producing a mixture of compounds that include hydrogen peroxide, ammonium ions, α -keto acids, and carboxylic acids. LAAOs are found widely in nature, and because their reaction products often damage tissues or cells, they often have offensive or defensive functions, as described later in this section. A variety of LAAOs are known from marine gastropods. Because of their ubiquity in a range of opisthobranch species and organs, and because LAAOs are well-studied molecules, they make an interesting case study.

Diversity of LAAOs. In the opisthobranchs, LAAOs occur with both phylogenetic and organ-level differences (Fig. 1). They are found in the ink gland and its purple ink, in egg masses, and in the albumen gland, which packages the egg masses. Other organs, including opaline gland, skin and body wall, gill, and internal organs, of *Aplysia* spp. and *Dolabella* have been examined for LAAOs, but these organs typically lack them (Butzke *et al.*, 2005; Johnson *et al.*, 2006). Exceptions are the skin, body wall, and coelomic fluid of *Dolabella auricularia*, which contain smaller polypeptides with LAAO activity (Kisugi *et al.*, 1989a; Iijima *et al.*, 2003a). A likely homolog is present in *Bursatella leachii*, though its partial characterization leaves some doubt that this protein is an LAAO (Rajaganapathi *et al.*, 2002). Each species of sea hare typically expresses several LAAOs, with each type of protein having an organ-specific expression pattern, as described below and in Figure 1. A given organ of a given species can express more than one type of LAAO (*A. punctata*: Petzelt *et al.*, 2002; Butzke *et al.*, 2004, 2005; *Dolabella*: Iijima *et al.*, 2003a, b). The functions of this variety of LAAOs have largely been ignored, since most characterizations have been driven by biomedical or drug-discovery interests rather than by chemical ecology. Orthologs and paralogs of *A. californica*'s escapin abound (Fig. 1). The molecular structures of a subset of these LAAOs have been characterized, allowing a phylogenetic comparison of their sequences (Fig. 2).

Biochemistry of LAAOs and their products. Comparison of the biochemical and enzymatic properties of escapin, *Aplysia punctata* ink toxin (APIT), and other LAAOs is instructive in understanding their relationships and functions. All

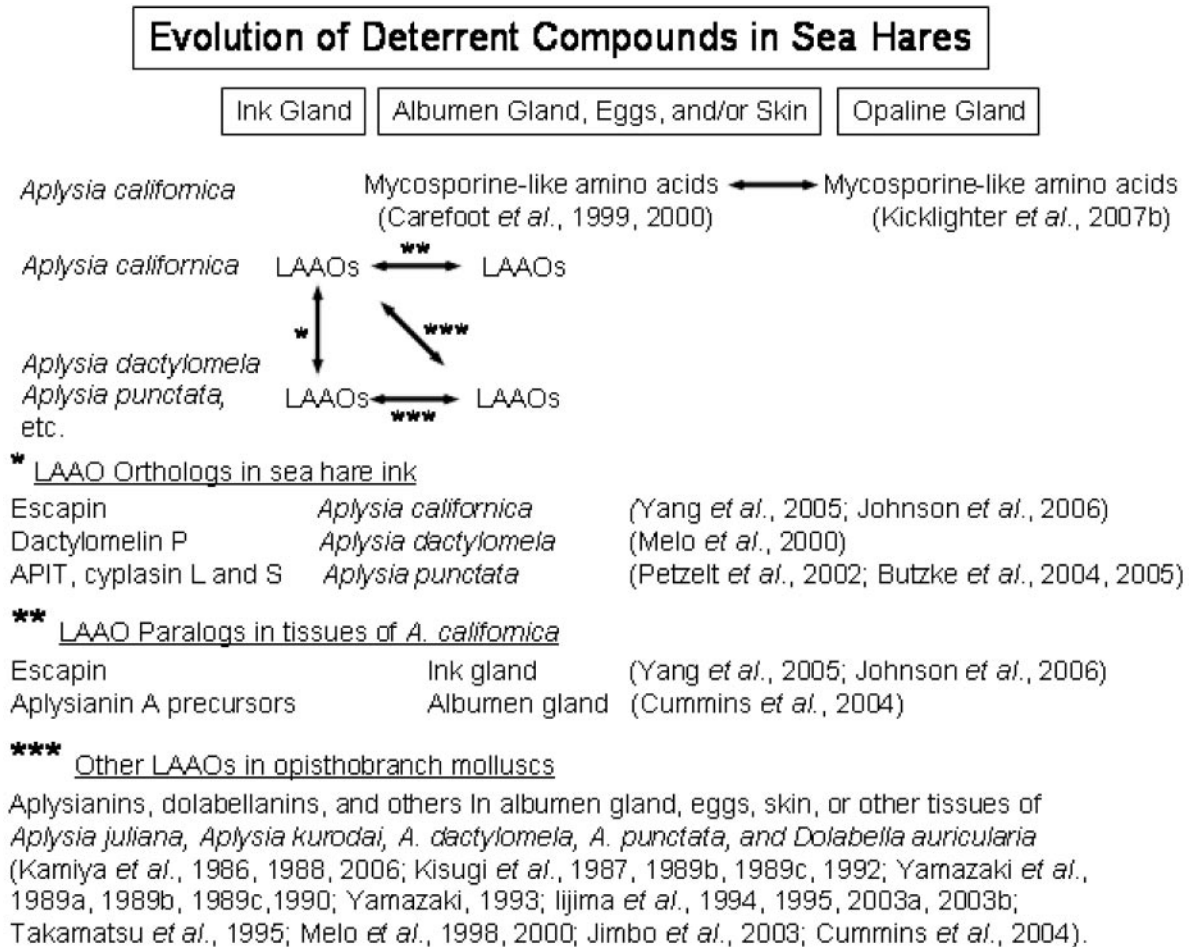


Figure 1. Deterrent compounds in sea hare ink and their evolution. See text for explanation.

contain flavins as co-factors, which are necessary for their activity. These LAAOs are minimally glycosylated. Many studies suggest that glycosylation is not important for their activity (Melo *et al.*, 2000; Yang *et al.*, 2005; Butzke *et al.*, 2005), though others suggest otherwise (Obara *et al.*, 1992; Takamatsu *et al.*, 1995; Ehara *et al.*, 2002; Petzelt *et al.*, 2002). Opisthobranch LAAOs can occur as monomers (Yamazaki, 1993; Butzke *et al.*, 2004, 2005; Yang *et al.*, 2005), though others are polymers (Yamazaki, 1993) as is the LAAO of pit vipers (Torii *et al.*, 2000).

These LAAOs have different substrate specificities. Some prefer basic amino acids such as L-lysine and L-arginine (Jimbo *et al.*, 2003; Butzke *et al.*, 2004, 2005; Yang *et al.*, 2005). Others have broader specificity, utilizing L-leucine, L-methionine, and L-tyrosine (Ponnudurai *et al.*, 1994; Torii *et al.*, 2000; Macheroux *et al.*, 2001; Du and Clemetson, 2002; Ehara *et al.*, 2002; Lu *et al.*, 2002). Since L-lysine is much more abundant than L-arginine in the ink-opaline secretion of sea hares, it is the dominant natural substrate (Kicklighter *et al.*, 2005; Derby *et al.*, 2007). Kinetic studies of LAAOs indicate that they function ex-

remely quickly upon release. For example, incubation of escapin and lysine at natural concentrations produces millimolar concentrations of hydrogen peroxide, ammonia, α -keto acids, carboxylic acids, and other reaction products within seconds (Yang *et al.*, 2005; K.-C. Ko *et al.*, unpubl. data).

Sea hare LAAO activity produces a mixture of ingredients that changes quickly over time. The identities of these products have often been assumed from studies of other LAAOs, but one case—escapin and L-lysine—has been analyzed in detail (K.-C. Ko *et al.*, unpubl. data). Figure 3 provides a summary. The first step in the reaction is catalytic and produces the α -keto acid of lysine, plus hydrogen peroxide and ammonium. The α -keto acid spontaneously cyclizes to form enamine/imine tautomers; but at the naturally acidic pH of ink, the α -keto acid dominates. The second step in the reaction is noncatalytic and involves reactions of hydrogen peroxide with the α -keto acid and enamine/imine tautomers, which produces, eventually, the carboxylic acid of L-lysine and the oxidative product of the enamine/imine tautomers. It is essential to note that the

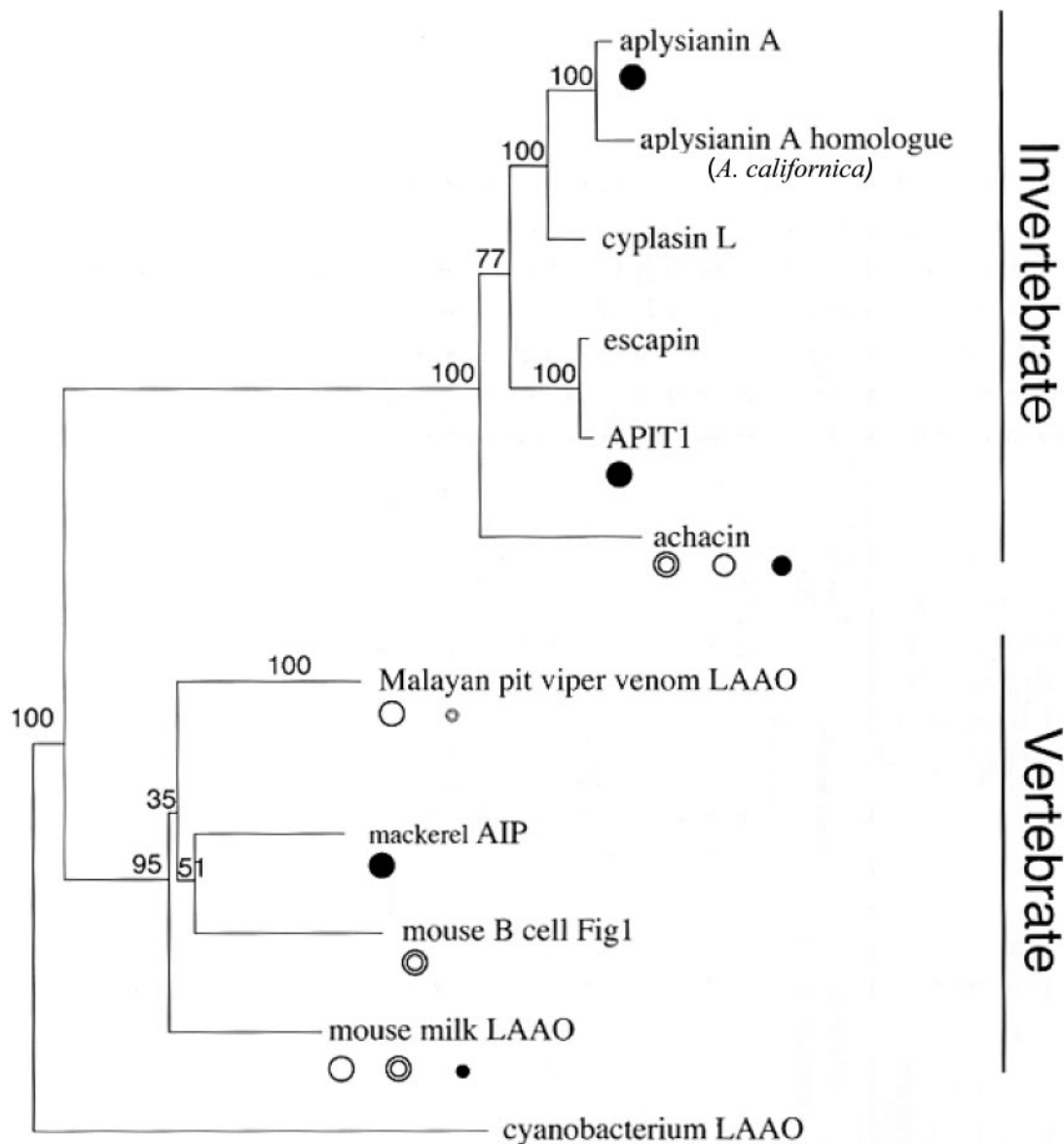


Figure 2. A phylogenetic tree and substrate specificity of L-amino acid oxidases from diverse sources based on gene sequence. See Kamiya *et al.* (2006) for a description of the methods for producing this phylogeny. Substrate specificity is indicated by the symbols, where closed circles represent basic amino acids, open circles represent aromatic amino acids, and double circles represent aliphatic amino acids, and size of circles represents relative activity. Aplysianin A (accession # Q17043, *Aplysia kurodai*: Jimbo *et al.*, 2003); aplysianin A homologue (AAN78211, *Aplysia californica*: Cummins *et al.*, 2004); cyplasin L (CAC1936, *Aplysia punctata*: Petzelt *et al.*, 2002); escapin (AAT12273, *Aplysia californica*: Yang *et al.*, 2005); APIT1 (AAR14185, *Aplysia punctata*: Butzke *et al.*, 2004, 2005); achacin (CAA45871, *Achatina fulica*: Obara *et al.*, 1992; Ehara *et al.*, 2002); pit viper venom (AR20248, *Calloselasma rhodostoma*: Ponnudurai *et al.*, 1994); AIP (CAC00499, *Scomber japonicus*: Jung *et al.*, 2000); mouse B cell Fig 1 (AAO65453, *Mus musculus*: Mason *et al.*, 2004); mouse milk LAAO (NP598653, *Mus musculus*: Sun *et al.*, 2002). From figure 10.4 of Kamiya *et al.* (2006), and used with permission from Springer-Verlag.

summary scheme of Figure 3 does not include compounds produced by the reaction of reactive oxygen species with compounds described above, some of which are strongly bioactive (K.-C. Ko *et al.*, unpubl. data).

Function of LAAOs. LAAOs are used by a number of non-molluscan organisms as a defense against microbes or other potential attackers. Apoptosis-inducing protein, an LAAO in fish, fights infections by larval nematodes (Jung *et*

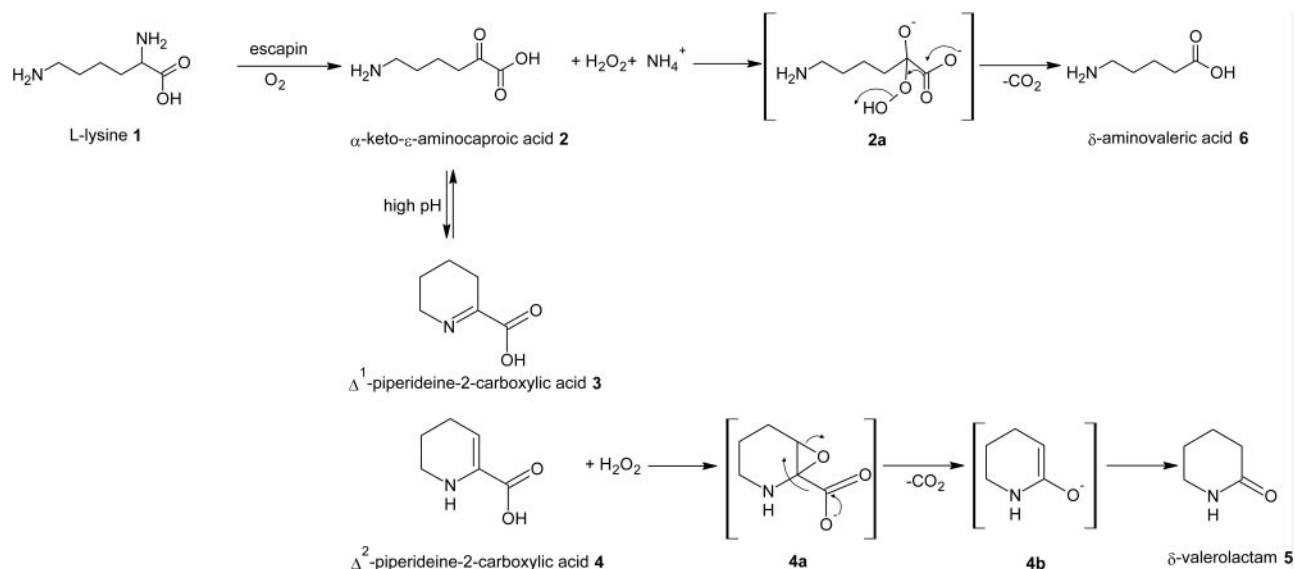


Figure 3. The chemistry of the escapin/ L-lysine pathway of *Aplysia californica*. See text for explanation. [Reprinted, with kind permission of Springer Science and Business Media, from Figure 10.4 of H. Kamiya, R. Sakai, and M. Jimbo. 2006. Bioactive molecules from sea hares. Pp. 215–239 in *Molluscs: From Chemoeological Study to Biotechnological Application*. G. Cimino and M. Gavagnin, eds. Progress in Molecular and Subcellular Biology, Vol. 43/Marine Molecular Biotechnology, Springer-Verlag, Berlin. Copyright Springer Berlin Heidelberg 2006.]

al., 2000). The red seaweed *Chondrus crispus* uses an LAO as a defense against infections by the green algal endophyte *Acrochaete operculata* (Weinberger *et al.*, 2005; Weinberger, 2007). This LAO uses L-asparagine released from the endophyte following induction by the red alga as the major substrate, in an interesting signaling exchange between the red alga and its endophyte. The bioactive defensive compound in this example is probably H₂O₂. In another example, the marine bacterium *Marinomonas mediterranea* produces marinocine, an LAO with L-lysine as its major substrate. Marinocine has antimicrobial effects due to H₂O₂ production (Lucas-Elío *et al.*, 2006). Marinocine is probably used by this bacterium as it competes against other microbes. The fungus *Trichoderma* has a lysine LAO, probably with defensive functions (Kusakabe *et al.*, 1980; Lukasheva and Berezov, 2002). In viper snakes, LAOs are a major contributor to the toxicity of the venom (Torii *et al.*, 2000; Du and Clemetson, 2002; Kanzawa *et al.*, 2004). These examples all support the idea that LAOs are broadly used for defensive functions by diverse organisms.

Unfortunately, the function of LAOs in molluscs is almost completely unexplored in either laboratory or field studies. Characterization of the biological activity of molluscan LAOs is largely limited to their antimicrobial or antiviral activity in the context of drug discovery. From a chemical ecological point of view, an antimicrobial function makes sense in some contexts, but antipredatory functions make more sense in most functional contexts. For example, it is adaptive to have antimicrobial agents in egg masses or

in the albumen glands that package them (Fig. 1). Molluscs lay eggs in strings on the substrate, so having antimicrobial agents would prevent fouling, an obvious advantage. This is a strategy used by many other invertebrates and involves a range of compounds (Barbieri *et al.*, 1997; Benkendorff *et al.*, 2001). Having antipredatory compounds in the egg masses is also adaptive, preventing ovovores from ingesting the eggs. It is more difficult to explain a function for antibacterial compounds in ink or opaline. An argument can be made that antimicrobial compounds in ink or opaline could serve as a salve if they coated the sea hare's skin during an attack and subsequently prevented infections resulting from any injury to the skin. However, a more efficient way to fight skin infections after an attack would be to have the antimicrobial compounds in the skin itself or in skin secretory glands. In fact, there are examples of this. The skin and body wall of the sea hare *Dolabella auricularia* contain an antimicrobial peptide (Iijima *et al.*, 2003a, b). To date, no LAO has been reported in the skin or body wall of *Aplysia* spp.

Our initial investigations of the antipredatory effects of the products of the escapin/L-lysine pathway show that specific products suppress the foraging behavior of spiny lobsters, and other products affect ingestion by blue crabs (Kamio *et al.*, 2007). Escapin products seem not to deter feeding by one species of sea anemone or four species of fish, suggesting species specificity in their effects (Kamio *et al.*, 2007). This is the first demonstration of the antipredatory effects of sea hare LAOs and shows that they can

affect the behavior of potential predators through the attackers' chemosensory systems.

Sea hares: sluggish bombardier beetles? Sea hare LAOs demonstrate how defensive compounds can be packaged to prevent autotoxicity (Johnson *et al.*, 2006). Escapin is present only in the ink gland—actually only in a subset of vesicles in that gland. Escapin's substrate, L-lysine, is present in high concentrations only in the opaline gland. Thus, the enzyme and its substrate are mixed only when ink and opaline are co-released upon predatory attack. The mixing takes place in the mantle cavity, where the bioactive molecules are generated before and while the defensive secretion is pumped out the siphon toward the attacker. This mechanism of generating deterrent compounds only when needed, by mixing, at the time of predatory attack, two inactive chemicals that react to form active deterrents, is reminiscent of bombardier beetles and other arthropods, only much slower (Aneshansley and Eisner, 1969; Eisner *et al.*, 2005).

Learning to avoid predators through chemical defense. Some predators learn to avoid sea slugs on the basis of odor. Fish are one example, and they learn in different ways to avoid the chemically defended nudibranch *Doriopsilla pharpa* (Long and Hay, 2006). One fish, the mummichog *Fundulus heteroclitus*, learns to specifically avoid sea hares via a sesquiterpene, polygodial. In the study by Long and Hay (2006), mummichogs initially ate food containing this compound and learned to avoid food containing it, but they continued to eat foods initially associated with this compound (specific learned aversion). Another fish, the striped blenny *Chasmodes bosquianus*, also initially ate food containing polygodial and learned quickly to avoid food containing it; however, this species avoided food that was initially associated with polygodial but no longer contained it (generalized learned aversion). Importantly, this study shows that the type of learning affects food selection, including how predators respond to new foods, and that these shifts in food preferences subsequently affect the functional organization of communities.

Chemical defenses of cephalopods

Cephalopods are famous for their defenses, from their fast jetting escape movements; to changes in coloration that can be cryptic, disruptive, or startling; to arm autotomy, toxic venom, and (by no means least) inking (Hanlon and Messenger, 1996; Norman, 2000). Cephalopod inks are chemical secretions produced by and released from the ink sac, which is not a homolog of the ink glands of gastropods but rather a modified hypobranchial gland (Roseghini *et al.*, 1996; Lindberg and Ponder, 2001).

Ink secretions are of two types: clouds and pseudo-

morphs. Pseudomorphs are well-defined objects composed of ink and mucus. They keep their form and physical integrity for some time after release by the cephalopod, and they can be almost as large as the animal releasing them (Hanlon and Messenger, 1996; Caldwell, 2005; Bush and Robison, 2007). Although ink secretions are a well-known feature of cephalopods, their function is not well understood. They are generally thought to function in defense as a visual stimulus. This might be as a smoke screen behind which the cephalopod can escape unseen—especially true for clouds; or it might be as a distracting decoy that attracts the attention of the predator while the cephalopod escapes—especially true for pseudomorphs (Lucero *et al.*, 1994; Hanlon and Messenger, 1996; Norman, 2000; Caldwell, 2005). An example of the use of the secretion as a decoy is the “Blanch-Ink-Jet manoeuvre,” in which a squid changes its color (from dark to light, or light to dark) and at the same time moves quickly away and releases ink, thus giving a would-be predator the illusion that the cloud of ink is the squid while the ‘invisible’ squid disappears (Hanlon and Messenger, 1996).

Beyond its function in the visual realm, cephalopod ink has been hypothesized to function through the chemical realm as well. The idea is that ink contains chemicals that disrupt a predator's chemical senses (MacGinitie and MacGinitie, 1968; Fox, 1974; Kittredge *et al.*, 1974; Prota *et al.*, 1981; Moynihan and Rodaniche, 1982). Theoretically, the effect could be chemical inactivation of the predator's sensory systems, or chemical deterrence through noxious chemicals (Kittredge *et al.*, 1974). Despite its appeal, this idea lacks experimental evidence to support it, except for anecdotes such as observations that octopuses squirt ink at crabs or snails approaching their brooding eggs (Eibl-Eibesfeldt and Scheer, 1962). As described below, cephalopod ink has been examined within the context of drug discovery, which has led to demonstrations that ink is toxic to microbes, retroviruses, and cell lines. But few studies have tested the compounds in ink in natural situations as antipredatory chemicals. (Note the parallels with sea hare ink in this respect.) I offer the following evidence and arguments as to why cephalopod ink functions as a chemical defense.

One unpublished report tested the hypothesis that octopus ink is aversive to predatory moray eels (Grüniger, 1997). This study did not identify aversive qualities to octopus ink; in fact, it reported the intriguing result that octopus ink was actually attractive to moray eels, evoking searching and attack behaviors similar to those of liquefied food, supporting previously published anecdotal observations (Fox, 1974). The results of this study suggest phagomimicry as a mechanism.

If cephalopods were using phagomimicry against predatory fish, one would expect ink to contain phagostimulants for fish. Fortunately, fish and their chemosensory systems

have been intensely studied, including identifying many of the more potent components of their food and the receptor systems that detect these chemicals and activate feeding behaviors. These stimulants include amino acids, amines, polyamines, and nucleotides (Carr *et al.*, 1996; Rolen *et al.*, 2003). Thus, if cephalopod ink operated through phagomimicry, one might expect considerable levels of some of these phagostimulants. As an initial experimental approach to the question of whether cephalopod ink contains phagostimulants, we have chemically analyzed the amino acid content of the inks of six cephalopod species, including squid, cuttlefish, and octopus (Derby *et al.*, 2007). All of these inks have millimolar concentrations of amino acids, and the amino acids in highest concentration—taurine, aspartic acid, glutamic acid, alanine, and lysine—have specific receptor systems in crustaceans and fish, which are major predators on these molluscs. This chemical analysis supports the idea that inking cephalopods have the potential to use phagomimicry and sensory disruption as chemical defenses.

Squid ink, like sea hare ink, contains alarm signals—chemicals that elicit escape responses in conspecifics (Gilly and Lucero, 1992). The alarm signals in squid ink include dopa and dopamine (Gilly and Lucero, 1992; Lucero *et al.*, 1994). The synthesis of these signals in ink is reasonably well elucidated (Fiore *et al.*, 2004). Dopamine is produced in specific compartments of mature ink gland cells, distinct from the production of melanin. Other evidence points to detection of other conspecific signals by cuttlefish and squid, but neither the molecular nature of these signals nor details of the nature of the signaling system are known (Boal and Golden, 1999; Buresch *et al.*, 2003, 2004).

Another major component in cephalopod ink is melanin. Although its synthetic pathway and release mechanism are well described (*e.g.*, Prota *et al.*, 1981; Palumbo *et al.*, 1997), its function is far from clear. Melanin, which provides the dark pigment of ink, may be involved in the visual smoke screen effect of ink. Fiore *et al.* (2004) demonstrate that dopamine in secreted ink is adsorbed onto melanin granules, and from this observation speculate that the melanin granules in secreted ink may serve as a carrier of dopamine, possibly preventing dilution after ejection and/or adding the delivery of dopamine to chemosensors on conspecifics or other species. Melanin is an antioxidant/reductive agent, removing H_2O_2 in living systems. Thus, another of its possible functions is to stabilize L-dopa and dopamine by preventing their oxidation, although ink may contain other antioxidants (Lucero *et al.*, 1994).

Ink from cephalopods can be a cytotoxin, though the toxic components are often not identified. For example, ink from cuttlefish and squid has antimicrobial activity (Mochizuki, 1979; Sheu and Chou, 1990) and inhibits reverse transcriptase (Rajaganapathi *et al.*, 2000). Tyrosinase, a compound in ink, appears to have toxic properties. The

ability of cephalopod ink to kill tumor cells may be due to tyrosinase activity, possibly through immunostimulation of macrophages (Takaya *et al.*, 1994; Naraoka *et al.*, 2000, 2003). Tyrosinase from the cuttlefish *Sepia officinalis* is toxic to several cell lines, probably through apoptosis (Russo *et al.*, 2003). This effect requires tyrosinase's catalytic activity but does not require added tyrosine as substrate. Catalase also does not affect tyrosinase's cytotoxicity, which supports the notion that tyrosinase acts on specific molecules on target cells (Russo *et al.*, 2003). For example, tyrosinase can catalyze the conversion of tyrosine residues in proteins to dopaquinone and eventually to protein-bound dopa (Ito *et al.*, 1984); the latter can cause oxidative damage to DNA, lipids, and proteins (Pattison *et al.*, 2002).

In summary, cephalopod ink has been shown to be toxic to some cells, but it is virtually untested against predators in controlled experiments. Chemical analysis of cephalopod ink shows that the enzymatic metabolism of tyrosine leads to production of many compounds, some of which function as alarm signals for conspecifics (dopa, dopamine), but most of which are untested in any assays (dopaquinone, dopachrome, 5,6-dihydroxyindole, melanin). The enzymes themselves (tyrosine hydroxylase, dopa decarboxylase, tyrosinase, peroxidase, dopachrome-rearranging enzyme) could theoretically have direct biological effects, but evidence for this exists for only one (tyrosinase). The presence of millimolar concentrations of compounds that stimulate the chemical senses of their predators (Derby *et al.*, 2007) and the demonstration that octopus ink activates food-related behaviors in predatory moray eels (Grüniger, 1997) raise the possibility that cephalopod ink might have antipredatory effects through phagomimicry, sensory disruption, or both.

Intraspecific Chemical Communication of Predatory Threats Through Alarm Signals

Chemicals released when marine gastropods are attacked by predators can sometimes elicit escape behaviors from neighboring conspecifics. Such alarm signals may come from passively released fluids of damaged flesh or from actively released secretions (Snyder and Snyder, 1971; Atema and Stenzler, 1977; Jacobsen and Stabell, 2004). Alarm signals are present in the ink secretions of at least two species of sea hares, in which they elicit escape responses such as head withdrawal, moving away from the stimulus, and escape locomotion (Fiorito and Gherardi, 1990; Nolen *et al.*, 1995). Alarm signals of *Aplysia californica* are present in both ink and opaline, and they evoke rapid turning and escape locomotion. The response is especially prevalent in juvenile sea hares. Ink contains three compounds that account for most of its alarm activity—the nucleosides uridine and cytidine, and the base uracil (Klicklighter *et al.*, 2007a). Opaline contains three alarm signaling molecules that constitute its bioactivity—all are mycospo-

rine-like amino acids (MAAs), two of which have never been described before (Kicklighter *et al.*, 2007b) (Fig. 1). Opaline contains other MAAs, but they appear not to function as alarm signals (Kicklighter *et al.*, 2007b, and unpubl. data). MAAs are thought to be diet-derived, UV-absorbing molecules that act as sunscreens in some tissues, including in *Aplysia* spp. (Carefoot *et al.*, 2000; Shick and Dunlap, 2002; Bandaranayake, 1998). The discovery that MAAs can be chemical signals raises an entirely new direction for exploring the potential functions and evolution of MAAs (Fig. 1).

The alarm response by *A. californica* is not species-specific, as *A. californica* also avoids ink from the octopus *Octopus bimaculoides* and the squid *Loligunculus brevis* (Kicklighter and Derby, 2006). Chemical analysis of these inks demonstrates that they contain uridine and uracil at levels that induce escape responses in sea hares. Dopamine and dopa in the ink of squids are alarm signals in squids (Gilly and Lucero, 1992; Lucero *et al.*, 1994). Together, these results suggest that this alarm signaling occurs in many ink-producing molluscs. This raises interesting issues related to the comparative biology and evolution of alarm signaling in molluscs. For example, did these molluscs independently evolve the ability to make use of common chemical cues, or do they have specialized signals?

Evolution of Molluscan Chemical Defenses

L-Amino acid oxidases in the ink gland

Escapin and its orthologs in the ink gland of sea hares may be derived from paralogs in other tissues with an original antimicrobial or antiparasitic function. Which appeared first—those in the ink gland or those in the albumen gland—is not known, but I speculate that LAAOs first appeared in the albumen glands. Sequence data, though limited, supports this idea (Fig. 2). LAAOs in the albumen gland have antimicrobial functions, which is adaptive given that the eggs are laid onto substrates and take weeks to develop, during which time antimicrobial activity would limit attack by microbes. LAAOs in albumen glands and egg capsules have not been tested for antipredatory functions. However, given their similarity to escapin and the antipredatory function of some of escapin's products, it is reasonable to speculate that the LAAOs in albumen glands and egg capsules confer antipredatory properties on the egg capsules. This would certainly be an immense functional advantage given that the eggs are vulnerable to a vast array of consumers (*e.g.*, Benkendorff *et al.*, 2001). Such an evolutionary scenario would explain why LAAOs in ink glands have an antimicrobial function, which is functionally questionable, in addition to their antipredatory function, which seems so reasonable (Wyatt, 2003).

Other defensive chemicals in ink and opaline

The original function of the ink and opaline glands may have been digestion or excretion, which might explain the presence of some diet-derived compounds in them. Such function may in some cases persist: digestive glands accumulate secondary compounds from consumed algae, as do opaline and ink glands, and the LAAOs may function in detoxifying these wastes and toxins. But a derived function in defense is likely. Modification of sequestered compounds has been demonstrated in several opisthobranch molluscs, including *Aplysia californica* (*e.g.*, Stallard and Faulkner, 1974; Paul and Van Alstyne, 1988; Paul and Pennings, 1991; Pennings and Paul, 1993), and modified compounds can deter predator feeding (*Elysia halimeda*: Paul and Van Alstyne, 1988). Additionally, some defensive compounds—most notably LAAOs—are specifically synthesized and packaged in these glands (Johnson *et al.*, 2006). Finally, ink and opaline are released only when animals are attacked. Ink and opaline glands are under neural control, and the necessary stimulus is a high-threshold disturbance, which typically occurs only when an animal is attacked by a predator. If the function of these glands were excretion, one might expect them to release their contents in a manner uncoupled from attack by predators. Together, these observations argue that these glands currently function in chemical defense, although this function may be derived from an original function in waste removal or as a visual smoke-screen.

Alarm signals

How might alarm signaling have evolved? What would be the selective advantage to inking animals of producing signals that warn conspecifics of danger? One candidate is kin selection (Wyatt, 2003). If neighboring animals tend to be genetically related, then the alarm signal would enhance the signaler's inclusive fitness by increasing the survival of close relatives even if the behavior put the signaler at risk. This explanation is not likely for *Aplysia*. *Aplysia* neighbors are unlikely to be highly related genetically because *Aplysia* has a long post-hatching, planktonic veliger larval stage, which would lead to dispersal of sea hares away from relatives. The veliger larval stage of *A. californica* lasts a minimum of 34 days in laboratory cultures (Kriegstein *et al.*, 1974), and other *Aplysia* spp. have similar or longer planktonic phases (*e.g.*, Kempf, 1981; Plaut *et al.*, 1995). A more likely scenario for the evolution of alarm signaling using ink and opaline is that these molecules had some other function, as cues, and that these molecules were then co-opted for use in signaling to conspecifics. The sex pheromones of goldfish may have originated as hormones with a reproductive function, but because they leaked out into the water, they served a predictive function for detecting reproductively receptive animals, and the chemicals became co-

opted as pheromones (Sorensen and Stacy, 1999). Another example is chemicals having a defensive function, then gaining a conspecific-signaling function (Wyatt, 2003). Some ant species and other arthropods use the same chemicals for defense (detering enemies) and for alarm (alerting nestmates to join the attack) (Blum, 1981). Other examples are alarm pheromones originating as unpalatable or toxic compounds, which function against predators, parasites, or microbes (Wyatt, 2003; Zimmer and Ferrer, 2007). These include bufotoxins and larval skin extracts with antipredatory functions, which evoke alarm responses in toad tadpoles. Other examples are alarm pheromones that are released by injured fish but that may have had an original function in controlling skin diseases (Wyatt, 2003). In the case of *Aplysia*, there is no evidence yet that the alarm signals in ink—cytosine, uridine, uracil—had or have a defensive function. Yet it remains a possibility, especially in the phagomimicry defense, since nucleotides and nucleosides are known as chemical stimulants for fish and crustaceans, though not necessarily these particular nucleosides (Carr *et al.*, 1996). The alarm signals in opaline—mycosporine-like amino acids (Kicklighter *et al.*, 2007b)—are probably derived from the algae upon which they feed and may have originally functioned as sunscreens in algae and even in their own tissues and egg masses (Carefoot *et al.*, 1999, 2000).

Principles Derived from Studies of Sea Hare Chemical Defenses

Several principles are emerging from studies of chemical defenses of sea hares. (1) Chemical defenses are typically multicomponent mixtures of diverse chemicals. (2) Defensive compounds can be either produced *de novo* or sequestered from chemicals, in particular diet-derived algal food. (3) These chemicals act through several mechanisms even against a single predatory species. (4) One chemical can mediate different mechanisms. (5) A given mechanism acts through different compounds for different predators. (6) Similar chemicals and mechanisms may occur across closely related species (*e.g.*, the orthologs escapin in *Aplysia californica* and APIT in *A. punctata*) but also across distantly related species (escapin and achacin in the land pulmonate snail *Achatina fulica*). (7) A species may use paralogues for different functions: in *A. californica*, escapin in ink for defense in predatory attacks, and aplysianin in eggs for protecting its eggs from consumption.

Why Should an Animal Have So Many Chemical Defenses?

Why does a sea hare, or any animal for that matter, have so many chemical defenses? The answers come from multiple perspectives, including evolutionary, ecological, and biochemical. One explanation is evolutionary—that an an-

imal uses different levels of defenses that are produced under different degrees of risk and have different degrees of cost. Passive chemical defenses, such as those produced chronically in the skin and mucus in the absence of predatory attacks, have a different cost/risk benefit than active chemical defenses such as ink, which are released only after a predator attacks and thus might be costly to produce. A second reason for the diversity of chemical defenses is ecological–dietary differences. For those defensive chemicals that are diet-derived, the diversity could come from what is available. Additionally, to compensate for some foods not being constantly available, having a variety of sources and thus chemicals would be adaptive. A third reason for the diversity of defensive chemicals is that sea hares and other animals require protection from many and varied species of predators. Having a diversity of chemical defenses, some more effective against certain predators, would be beneficial. A fourth reason is that even against a single species, having multiple parallel or sequential levels of action is an advantage. This principle is evident from studies of toxins in cone snails (Olivera *et al.*, 2002). For defenses that act on a predator's chemical senses, some chemicals might work on olfactory pathways and others through gustatory pathways, each affecting the behavior of the animal in different ways. A fifth reason is biochemical. As is evident from the LAAO pathway, the chemistry of the reaction of a single enzyme with a single substrate can be complex and can produce a diverse array of chemicals, each of which can act in different ways on different species of would-be predators.

Future Experiments in Neuroecology of Mollusc Inking

One future direction might be to determine the ubiquity of phagomimicry and sensory disruption as mechanisms of chemical defense. Do sea hares use these defenses against fish and other predatory crustaceans besides spiny lobsters? Species to be tested should include potential predators, including those that are electrophysiological models of chemoreception so that mechanistic studies can be pursued.

Field studies of the defensive functions of inking behavior are needed. One approach is testing animals that have been experimentally manipulated to alter their ink content. Two types of manipulation might be used. One is to experimentally deplete animals of their ink, opaline, or both, as has been done in laboratory antipredation studies (Kicklighter *et al.*, 2005), but in this case to expose them to predators in the field under natural conditions. A second type of manipulation is to deprive animals of specific compounds by diet manipulation or double-stranded RNA inhibition, which has worked in gastropods (Korneev *et al.*, 2002; Park *et al.*, 2006). There is also a need for field tests

of alarm functions of ink and opaline, to determine how these operate in natural contexts.

Can sea hares tailor their defenses to likely predators? Theoretically, this could be accomplished by selective production of chemicals, selective feeding on certain algae, or even selective release of ink or opaline. The latter may be possible, since the ink and opaline glands are under independent neural control.

Another important area for future studies is the consequences of defenses, avoidance, and alarm signals for processes operating at the population or community ecological levels. Do these defenses affect the abundances and distributions of organisms, as has been shown in other systems (e.g., Duffy and Hay, 2001; Cruz-Rivera and Villareal, 2006; Paul *et al.*, 2007; Zimmer and Ferrer, 2007)? Is there evidence for consumer resistance, trophic transfer, or other effects of these compounds on abundances and distributions of organisms?

Finally, expanding neuroecological studies to other inking molluscs should be of immense value.

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