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The purple pigment aplysioviolin in sea hare ink deters predatory blue crabs through their chemical senses

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Sea hares release an ink secretion composed of purple ink and white opaline as a potential chemical defence against predators. The aim of our study was to identify deterrent molecules in the ink of *Aplysia californica* against an allopatric generalist crustacean predator, the blue crab *Callinectes sapidus*, and to define the mechanisms of action of the deterrents against crabs. We used two behavioural assays, a squirting assay and an ingestion assay, to show that ink is highly effective and that opaline is moderately effective in suppressing feeding of crabs. Results with reversibly blinded crabs demonstrate that the deterrence is mediated through the crabs' chemical senses. We used bioassay-guided fractionation to identify the purple molecules aplysioviolin and phycoerythrobilin as a major and minor deterrent, respectively, in ink against crabs. These molecules derive from a light-harvesting protein in the photosynthetic system of dietary algae. This is the first demonstration of an animal converting a photosynthetic pigment into a chemical deterrent. Mixing opaline and ink enzymatically produces hydrogen peroxide, which also functions as a chemical deterrent against crabs. Our results and those of other studies show that sea hares use a diversity of molecules in their skin, mucus and ink secretion to chemically defend themselves against their potential predators. Aplysioviolin, phycoerythrobilin and hydrogen peroxide also exist in ink secretion of *Aplysia dactylomela*, a sea hare sympatric to blue crabs, and thus we posit that these molecules are potentially effective in ecologically relevant predator–prey interactions and need to be scrutinized in more ecologically relevant experiments.

Many species with low mobility, from plants to animals, defend themselves from attack by consumers using constitutive chemical defences that cover their body surface or are embedded in their tissues (McClintock & Baker 2001; Clark et al. 2005; Paul & Ritson-Williams 2008; Wink 2008). In addition, many organisms also respond to predatory attacks using activated chemical defences in their tissues (Hadacek 2002; Wittstock & Gershenzon 2002; Van Alstyne & Houser 2003; Thoms & Schupp 2008; Walling 2009) or by releasing chemicals to the external environment around them to deter predators (Eisner & Aneshansley 1999; Wood 1999; Shiomi et al. 2001; Williams & Gong 2007). Many of these released molecules probably affect the chemosensory systems of consumers, as has been experimentally demonstrated in some cases (Kicklighter et al. 2005; Nusnbaum & Derby 2010). Some of these molecules are pigmented (i.e. absorb light in the visible range of predators) and thus might act as defences through the visual modality of predators. Such 'ink' can protect an animal by acting as a smoke screen behind which it can hide from a predator, as a decoy that attracts the attention of the predator, or as a stimulus that startles the predator (Derby 2007; Wood et al. 2008). Thus, pigmented molecules have the potential to affect both the visual and chemical senses of the releaser's enemies. Animals can sequester chemical deterrents from their food and modify them (Garson 2001; Hadacek 2002; Clark et al. 2005), or synthesize them de novo (Garson 2001; Cimino & Ghiselin 2009).

Sea hares (Opisthobranchia: Anaspidea) live in marine benthic communities where they have relatively specialized diets, concentrating on red and green algae (Carefoot 1987). They are mobile but slow and have a soft body without a shell for protection. Consequently, they chemically defend themselves against a range of predators by sequestering secondary metabolites from their dietary red algae and mobilizing them into their skin and digestive glands (Paul & Pennings 1991; Ginsburg & Paul 2001; Kamiya et al. 2006). In addition to these constitutive chemical defences in their skin and tissues, sea hares also have a behaviourally activated chemical defence, ink secretion, which is released when they are attacked by predators (Nolen et al. 1995; Johnson & Willows 1999; Pennings et al. 1999). Ink secretion is composed of two co-released glandular products: ink, which is a dense, dark purple product released from the ink gland, and opaline, which is a translucent,

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whitish product released from the opaline gland. Ink secretion of the sea hare *Aplysia dactylomela* suppresses feeding of laughing gulls and blue crabs (DiMatteo 1981, 1982), ink secretion from *Dolabella auricularia* is unpalatable to reef fishes (Pennings et al. 1999), and ink secretion from *Aplysia californica* is a deterrent of sea anemones, spiny lobsters and a variety of fishes (DiMatteo 1981; Nolen et al. 1995; Kicklighter et al. 2005; Kicklighter & Derby 2006; Aggio & Derby 2008; Sheybani et al. 2009; Nusnbaum & Derby 2010, in press). However, in only a few cases have the molecules responsible for the chemical deterrence of ink been identified (Kicklighter et al. 2005; Aggio & Derby 2008). One case is the amino acid components of ink and opaline, which function as a deterrent against spiny lobsters. Ink and opaline contain millimolar concentrations of amino acids, which by themselves are highly attractive to many predators including crustaceans and fishes (Derby & Sorensen 2008). Because of its stimulatory amino acids, ink secretion itself can sometimes overcome unpalatable molecules in ink and stimulate appetitive feeding responses of predatory spiny lobsters, a process called phagomimicry (Kicklighter et al. 2005). Another case is molecules generated by escapin. Escapin is an L-amino acid in ink (Yang et al. 2005). When ink and opaline are co-secreted, escapin oxidizes L-lysine, which is present in high millimolar amounts in opaline (Johnson et al. 2006). This reaction produces three molecules: hydrogen peroxide, ammonia and the alpha-keto acid of lysine. The alpha-keto acid exists as an equilibrium mixture of imine, enamine and other forms in an aqueous solution (called 'escapin intermediate products'), and these compounds react with hydrogen peroxide to produce another set of compounds (called 'escapin end products') (Kamio et al. 2009). Products of the escapin pathway are deterrent to spiny lobsters (Aggio & Derby 2008) and wrasses (Nusnbaum & Derby, in press). Despite these examples, and despite abundant knowledge that ink secretion is unpalatable to many animals, no deterrent molecules in ink secretion itself have been identified through bioassay-guided fractionation.

The overall goal of our study was to find chemosensory deterrent molecules in ink or opaline using the sea hare *Aplysia californica* and the predatory blue crab *Callinectes sapidus* as animal models. Towards this goal, we had several aims in our experiments. The first aim was to determine whether sea hares use ink and opaline as a phagomimetic defence or as a deterrent defence, and to assess the relative importance of ink and opaline. The second aim was to identify the deterrent molecules in the ink. The third aim was to identify which of the predators' sensory modalities is affected by these molecules. We also tested the deterrence of the enzymatic reaction products that are produced by co-secretion of ink and opaline.

We used blue crabs in our study because they are generalist feeders (Hines 2007) whose feeding behaviour is easy to study in the laboratory and which use chemoreception in many aspects of their behaviour including feeding (Pearson & Olla 1977; Rittschof 1992; Weissburg & Zimmer-Faust 1994). Sea hares typically release ink when vigorously attacked by predatory blue crabs presented with small juvenile (ca. 1 g) or adult (200–300 g) sea hares (M. Kamio, unpublished observations). Sea hares can release ink before or after being bitten or pinched by crabs, and sea hares can control the direction of inking towards the attacking predator (Walters & Erickson 1986). During encounters in which ink is released, ink can stimulate different sensory organs on the predator's body. For crustaceans such as blue crabs, these sensors can include the first and second antennae, the mouthparts, or the legs, which are major chemosensory organs that control different aspects of feeding behaviour (Derby et al. 2001). Those sensors might be stimulated in the presence or absence of body fluids released from damaged sea hares, defensive ink secretions, or when the predator has the sea hare in its mouth. The inking often stops blue crabs' attacking behaviour and gives sea hares a chance to escape. We used ink from the sea hare *Aplysia californica*, which is allopatric to blue crabs, rather than from the blue crab's sympatric sea hare, *Aplysia dactylomela*, because *A. californica* is better studied and readily available.

METHODS

Animals

All animals used in our study were collected from the field. Mature male blue crabs, *Callinectes sapidus*, with carapace length of 10–12 cm were obtained from Gulf Specimen Marine Lab (Panacea, FL, U.S.A.) or commercial fisherman in St Augustine, Florida, maintained in our laboratory in aquaria with recirculating, filtered and aerated artificial sea water (Instant Ocean, Aquarium Systems, Mentor, OH, U.S.A.) at 20 °C, and fed squid and shrimp. Adult (200–300 g) sea hares, *Aplysia californica*, were collected by Marinus Scientific (Garden Grove, CA, U.S.A.), and adults of *Aplysia dactylomela* were obtained from the Keys Marine Laboratory (Layton/Long Key, FL). Sea hares were used immediately upon their arrival in the laboratory. Animal care and experiments were performed within university regulations and national guidelines. Only animals that ate pieces of food or responded appetitively to food-related chemical stimuli were used in our experiments.

Chemical Stimuli and Reagents

To collect ink and opaline, we dissected ink and opaline glands from anaesthetized sea hares immediately upon their arrival in our laboratory. Ink and opaline glands were frozen at −80 °C until used. Ink was collected by gently squeezing dissected ink glands in a petri dish with the blunt end of a scalpel handle. Opaline was collected by centrifuging opaline glands at 30 000 × g for 1 h at 4 °C. Shrimp juice was prepared by homogenizing 1 g of fresh shrimp abdominal muscle in 50 ml of sea water. Sea water was either artificial sea water (Instant Ocean®) or filtered sea water (pumped and filtered from sea water near the Whitney Laboratory, University of Florida). Freeze-dried shrimp was made from frozen shrimp using a lyophilizer. Pieces of freeze-dried shrimp of ca. 5 × 5 × 10 mm (90–130 mg dry mass) were used in feeding experiments. Bilirubin (to determine structure–activity relationships), sodium alginate and calcium chloride (to prepare food pellets) were purchased from Sigma–Aldrich (St Louis, MO, U.S.A.). The intermediate products of escapin's oxidation of L-lysine (escapin intermediate products, or EIP) were prepared by incubating L-lysine monohydrochloride, escapin (purified from ink) and catalase in double distilled water as described in Kamio et al. (2009).

Experimental Approaches

We tested substances for their effects on blue crabs in two general categories of assays: an assay that measures the chemical's ability to stimulate feeding behaviour and thus serve in defence as a phagomimic; and assays that measure the chemical's ability to inhibit appetitive responses to food odours and ingestion of food. Then, we used bioassay-guided fraction, based on an ingestion–inhibition assay developed from our earlier observations of the behavioural responses of crabs, to identify components in ink responsible for its deterrent effects. We also used the ingestion–inhibition assay to test the activity of escapin products. The effect of the deterrents on the chemical sense of blue crabs was confirmed using eye caps to reversibly deprive animals of visual cues.