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Molecular identification of alarm cues in the defensive secretions of the sea hare *Aplysia californica*

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Prey species possess numerous strategies to reduce predation. One tactic is to respond with antipredator behaviours when conspecific alarm cues are detected. The sea hare *Aplysia californica* defends itself from predators in many ways, one of which is releasing ink and opaline upon attack. Previous work showed that a mixture of ink and opaline from *A. californica* causes conspecifics to respond with antipredator behaviours such as moving away and/or 'galloping'. We examined the specificity of the alarm response, including identifying the molecules mediating it. Either ink or opaline alone evokes the full alarm response, but conspecific mucus, conspecific haemolymph, odour from predatory spiny lobsters, or odour from algal food do not. Thus, the defensive secretions, ink and opaline, specifically act as alarm cues to nearby conspecifics. We isolated and identified the alarm cues in ink as the base uracil and the nucleosides uridine and cytidine. Each of these molecules individually elicits frequencies of alarm behaviours as great as ink. Ink without its alarm cue molecules does not elicit a significant frequency of alarm behaviours. Thus, these three molecules together are necessary and sufficient to produce alarm responses. *Aplysia californica* antipredator behaviours are also elicited by ink from the congener *Aplysia juliana* or *Aplysia dactylomela*. Furthermore, ink from the squid *Lolliguncula brevis* or the octopus *Octopus bimaculoides* also elicits antipredator behaviours by *A. californica*, owing to the presence of uracil and uridine. Thus, these alarm cues may be common among inking molluscs.

Keywords: alarm signal; *Aplysia californica*; chemical defence; ink; opaline; predator–prey; sea hare; trait-mediated indirect interaction

Prey species use various strategies to avoid being detected, captured, and consumed by predators. One strategy is performing antipredator behaviours in response to cues released by injured or disturbed conspecifics (alarm cues) or to cues released by predators (predator avoidance cues) (Chivers & Smith 1998; Kats & Dill 1998). Antipredator behaviours should decrease the probability that prey will be detected or captured by predators and may include decreasing activity or movement, avoiding the alarm or predator avoidance cue, seeking refuge, or increasing movement (Mathis & Smith 1993; Hagen et al. 2002; Smee & Weissburg 2006; Zimmer et al. 2006). In particular, responding to alarm cues should be advantageous because cues that are released from prey that are attacked or injured are reliable indicators of predation risk. Thus, prey that can identify and respond to these cues with appropriate behaviours decrease the likelihood of being attacked by predators.

Numerous species of fish, amphibians, molluscs, arthropods, annelids, platyhelminths, echinoderms, and other animals alter their behaviour when exposed to alarm cues (e.g. Chivers & Smith 1998; Forward & Rittschof 2000; Rosenberg & Selander 2000; Wisenden & Millard 2001; Watson et al. 2005). This strategy is particularly common

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among aquatic species, where the use of chemical cues is widespread (Grant & Mackie 1974; Zimmer & Butman 2000; McClintock & Baker 2001). However, despite the plethora of studies showing alarm responses in marine and aquatic species, very few investigations have identified molecules and showed their function as alarm cues (Chivers & Smith 1998). The few identified alarm molecules include anthopleurine (Howe & Sheikh 1975), navanones A, B, and C (Sleeper et al. 1980), haminols A and B (Cimino et al. 1991), tetrodotoxin (Zimmer et al. 2006), and possibly hypoxanthine-3-*N*-oxide (Pfeiffer et al. 1985; Brown et al. 2000, 2003). Anthopleurine elicits tentacle withdrawal and mouth closing in the anemone *Anthopleura elegantissima*. Navanones A, B, and C and haminols A and B, which are laid down in the slime trails of the opisthobranch molluscs *Navanax inermis* and *Haminoea navicula*, respectively, arrest following of slime trails by conspecifics and cause them to move away from the cues. Tetrodotoxin induces hiding behaviour in juvenile newts, *Taricha torosa*, and hypoxanthine-3-*N*-oxide elicits alarm behaviours such as changes in dorsoventral orientation, increased shoaling, and movement towards the substrate. Given the paucity of identified alarm molecules, this is an area in dire need of attention. Knowing the identities of the alarm cues that mediate predator–prey interactions is beneficial in facilitating our understanding of the neural processing of these signals by prey species. Very few studies have investigated the mechanisms at the neural level by which prey avoid or deter predation (Kicklighter et al. 2005; Zimmer et al. 2006). Thus, such studies would allow a more complete understanding of the role of chemoreception in predator–prey encounters, linking physiological, behavioural, and ecological mechanisms influencing these interactions (Zimmer et al. 2006).

Sea hares of the genus *Aplysia* use a variety of defensive strategies against would-be predators. These defences include crypsis, blending in with the seaweed on which they feed, and adding distasteful compounds in the skin either by sequestration of metabolites derived from their seaweed diet or de novo production of deterrent metabolites (Pennings 1990, 1994; Pennings & Paul 1993; Ginsburg & Paul 2001). In addition, when attacked by predators, sea hares release the defensive secretions, ink and opaline. Ink facilitates the escape of *Aplysia californica* from the anemone *Anthopleura*, and this is mediated by at least one lipophilic molecule and two hydrophilic molecules (Nolen et al. 1995; Kicklighter & Derby 2006). Both ink and opaline contain high concentrations of amino acids, which defend sea hares from predatory spiny lobsters through phagomimicry and/or sensory disruption (Kicklighter et al. 2005). Furthermore, opaline contains chemicals that are unpalatable to spiny lobsters (Kicklighter et al. 2005; C. E. Kicklighter, unpublished data). Ink and opaline also deter feeding by a variety of other predators, such as birds, fish, and crabs (Ambrose & Givens 1979; DiMatteo 1981; Paul & Pennings 1991; Pennings et al. 1999). Because ink and opaline are almost always released in response to predatory attacks and are not generally released by undisturbed animals, these secretions could indicate predator presence. Walters et al. (1993) and Nolen et al. (1995) showed that secretions from conspecifics elicit head retraction and turning and moving away in juvenile and adult *A. californica*. For these experiments, secretions were collected from live animals stimulated to release ink and opaline, and thus whether ink or opaline contains the alarm cues is unknown.

The goal of our study was to determine which sea hare secretion, ink or opaline, contains the alarm cues, and to identify the bioactive molecules. Our results show that either ink or opaline, but not other sea hare fluids and not stimuli associated with sea hare predators or food, elicits alarm behaviours in juvenile *A. californica*. Using bioassay-guided fractionation, we showed that ink contains alarm cues, identified as the nucleosides uridine and cytidine and the base uracil. These three molecules are necessary and sufficient to account for the alarm activity of ink. These intraspecific alarm molecules are different from those already identified as affecting sea hare predators, such as spiny lobsters and sea anemones (Kicklighter et al. 2005; Kicklighter & Derby 2006).

METHODS

Animal Care and Collection

Juvenile sea hares, *A. californica*, used in behavioural assays were ~1 g when obtained from the NIH/University of Miami National Resource for *Aplysia* (Key Biscayne, FL, U.S.A.). They were used in experiments until they reached about 5 g, which occurred within 2–3 weeks of receipt. For collection of secretions and fluids, we used adult (150–300 g) *A. californica* collected off the coast of California by Marinus Scientific (Garden Grove, CA, U.S.A.). All animals were 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. Sea hares were fed with the red seaweed *Gracilaria ferox* obtained from the NIH/University of Miami National Resource for *Aplysia*. Adult sea hares were dissected and their ink and opaline secretions collected, usually within 1 week after receipt. We also collected ink and opaline secretions from *Aplysia dactylomela* from waters around Bermuda and *Aplysia juliana* off the coast of Warkworth, New Zealand. We also obtained ink from the sacs of the three immature squid *Lolliguncula brevis*, collected off the coast of Texas by the National Resource Center for Cephalopods (Galveston, TX, U.S.A.) and of one adult octopus *Octopus vulgaris*, collected off the coast of St. Augustine, FL, U.S.A. *Aplysia dactylomela* and *A. juliana* were dissected immediately after field collection. *Lolliguncula brevis* and *O. vulgaris* were held in laboratory aquaria before dissection.

Sea Hare Secretions, Alarm Cues, and Other Stimuli

To investigate the effect of sea hare secretions and fluids on juvenile conspecifics, we obtained ink, opaline, haemolymph, and mucus from adult *A. californica*. Ink and opaline secretions were collected by chilling sea hares in sea water for 4 h at 4°C. Animals were then anaesthetized