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

CYNTHIA E. KICKLIGHTER*†, MARKUS GERMANN‡, MICHIYA KAMIO† & CHARLES D. DERBY†
*Department of Biology, Goucher College
†Department of Biology, Georgia State University
‡Department of Chemistry, Georgia State University

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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.

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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

Correspondence: C. E. Kicklighter, Department of Biology, Goucher College, 1021 Dulaney Valley Road, Baltimore, MD 21204, U.S.A. (email: cynthia.kicklighter@goucher.edu). M. Kamio and C. D. Derby are at the Department of Biology, Brains & Behavior Program, and Center for Behavioral Neuroscience, Georgia State University, P.O. Box 4010, Atlanta, GA 30302-4010, U.S.A. M. W. Germann is at the Department of Chemistry, Brains & Behavior Program, and Center for Behavioral Neuroscience, Georgia State University, P.O. Box 4098, Atlanta, GA 30302-4098, U.S.A.

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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

by injection of 120–180 ml of isotonic $MgCl_2$, and glands were removed by dissection. Ink was collected by gently squeezing glands with a spatula, and opaline was collected by centrifuging opaline glands at $30\,000 \times g$ to separate the fluid from the gland tissue. Both secretions were frozen at −20°C until used. To collect haemolymph, two sea hares were anaesthetized and 500 µl of fluid were drawn out of the dorsal area of each animal with a syringe. These two haemolymph samples were then combined for use in assays. Mucus was collected from 10 sea hares by removing the animals from water and irritating the skin with a single-edge razor blade. The mucus produced as a result of the irritation was then scraped off the skin, stored in a centrifuge tube, and frozen at −20°C until used. We also investigated the effects of other stimuli on alarm behaviour of juvenile *A. californica*. Because the California spiny lobster *Panulirus interruptus* will prey on *A. californica* (Kicklighter et al. 2005) and the odour released from live predators elicits alarm responses in other gastropods (e.g. Chivers & Smith 1998), we tested water from a 400-litre aquarium containing more than 10 individual *P. interruptus*. Finally, we tested odour from food of sea hares by taking a sample of water from a 76-litre aquarium containing the red alga *G. ferox*. Lobster and food odour samples were tested fresh (i.e. not frozen), and all sea hares were tested the same day with aliquots of the same odour samples.

Bioassay of Alarm Responses

To examine alarm behaviour of juvenile *A. californica* in response to different stimuli, we used methods similar to Nolen et al. (1995). Each animal was tested individually in a glass arena (39 × 26 × 5.5 cm) filled with sea water to a depth of about 2.5 cm. For each trial, an animal was gently placed in the centre of the arena and allowed to acclimate for 1–2 min. After this acclimation period, as the animal locomoted forward, it was presented with 75 µl of stimulus, delivered with a 200-µl micropipettor. The stimulus was delivered about 1 cm in front of the animal at a 45° angle. After presentation of the stimulus, the sea hare was videotaped from overhead for 2 min. Animals were scored for moving away from the stimulus and galloping (saltatory crawling, in any direction). Each stimulus was tested on each animal, with at least 1 h between presentations. Sea water was used as a negative control in each set of bioassays. In all bioassays of *Aplysia* responses to stimuli other than ink, ink from *A. californica* was included as a positive control stimulus. The order of presentation of the stimuli to each animal was randomized, and the experimenter was unaware of the identity of the stimuli, with the exception of ink and opaline, as we were unsuccessful at creating coloured sea water to mimic these secretions. After each trial, the arena was emptied, rinsed with fresh water, and dried with a paper towel.

Ink Bioassay-guided Fractionation

To identify the components in ink responsible for generating the alarm response, we used various chromatography methods to separate the molecules in ink based on their polarity or size. First, the components in ink were separated based on their polarity via a modified Kupchan partition scheme (Kupchan et al. 1975). This scheme yielded five partitions that differed in solubility; these were soluble in hexanes, chloroform, ethyl acetate, butanol, or water. Each of these fractions was solubilized in a volume of sea water equivalent to the volume of ink extracted, thus reconstituting it at its natural concentration in ink, and then tested as a stimulus in the alarm cue bioassay as described above. For more lipid-soluble partitions (hexanes, chloroform, and ethyl acetate), these were solubilized in a 20-ml vial in a 3:1 solution of hexanes, chloroform, or ethyl acetate and sea water. The vial was then placed on a rotary evaporator to remove the organic solvent, leaving the remaining extract solubilized in sea water. Each animal was tested with the five partitions, as well as ink as a positive control and sea water as a negative control. To control for the presence of small amounts of organic solvents remaining in the hexanes, chloroform, ethyl acetate, and butanol partitions, the sea water controls were mixed with the appropriate solvent, which was then removed with a rotary evaporator as described above or a Speedvac evaporator.

Only the water-soluble partition elicited a frequency of alarm responses greater than sea water. Thus, the molecules in this fraction were further separated based on their polarity. Because the active components were highly polar, we used C18 reversed phase chromatography (1 g C18 disposable extraction column, Mallinckrodt Baker, Inc., Phillipsburg, NJ, U.S.A.). We passed the following series of solvents through the column: 100% water, 70:30 water:methanol, 30:70 water:methanol, and 100% methanol. All fractions were bioassayed separately for alarm response activity. We observed no differences in sea hare alarm behaviours in response to sea water that had been mixed with methanol and then removed via evaporation versus sea water that had not been previously mixed with methanol (data not shown). Thus, we omitted this procedure in this and subsequent bioassays. Only the 100% water fraction produced a frequency of alarm response different from sea water. This fraction was further purified by separating the molecules according to size by passing it through lipophilic Sephadex LH-20 (GE Healthcare, Little Chalfont, Buckinghamshire, England) with 50:50 methanol:water as solvent. This produced three fractions based on thin layer chromatography (TLC) characteristics, one of which produced an alarm response. The components of this fraction were then further separated by polarity via preparative thin layer C18 reversed phase chromatography (Partisil KC18 silica gel 6 nm, 200 µm thick, Whatman International Ltd., Maidstone, England). Based on colour and ultraviolet (UV) absorbance, three bands were collected separately in 50% methanol. Each band was dried and tested separately in the bioassay. Only the top band elicited an alarm response. Thus, this fraction was further purified by polarity via high pressure liquid chromatography (HPLC: Beckman 125S solvent module coupled to a Beckman Gold 168 photodiode array detector, using a Phenomenex Luna reversed phase C18 silica 5 µm column, 250 × 10 mm) with a mobile phase of 5:95 methanol:water at 0.8 ml/min. This produced six

peaks. Three peaks (2C.2, 2C.4, and 2C.6) elicited an alarm response. These peaks were further purified separately via another round of HPLC using a C30 reversed phase column (Develosil RP Aqueous reversed phase C30 silica 5 μm column, 250 × 4.6 mm, Nomura Chemical Co. Ltd, Seto, Japan) with a mobile phase of 100% water and a flow rate of 0.8 ml/min. We used this column because it produces good separation of highly polar molecules with a 100% water mobile phase.

Structure Confirmation

Molecular weight of peaks 2C.4 and 2C.6 were analysed via ESI (+) mass spectrometry (Waters 2695 HPLC coupled to a Micromass Q-Tof micro mass spectrometer). However, we could not reliably analyse peak 2C.2 by mass spectrometry. Retention time and ultraviolet spectrum of each peak were analysed via HPLC under the same conditions as during their purification.

All three peaks were characterized by ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy (Bruker Avance 500 NMR spectrometer equipped with a triple resonance cryoprobe) in D_2O.

Alarm Cue Quantification

To determine the natural concentrations of uracil, uridine, and cytidine in *A. californica* ink, we pooled the ink from eight adult sea hares. This was necessary because the concentration of these chemicals in ink from one individual was below the detection limit of the Beckman photodiode array (<0.01 μg). Ink glands were dissected as described above and stored at −80°C until used. Glands were thawed and squeezed to collect ink. Three 1-ml replicate aliquots of ink were collected and separately passed through a C18 reversed phase column; we omitted the Kupchan partition scheme to eliminate the possibility that the solvents create extraction artefacts. The C18 column was flushed with 250 ml of water, and the eluate from each ink sample was collected separately and dried in a Speedvac concentrator. The three dried water fractions were solubilized in 100 μl of water and analysed by HPLC (at 260 nm) using a Develosil RPAqueous reversed phase C30 silica 5 μm column (250 × 4.6 mm) with a mobile phase of 100% water and a flow rate of 0.8 ml/min. The area under the peaks for uracil, uridine, and cytidine was calculated for each injection ($N = 3$).

To determine the concentrations of the alarm cue molecules, standard curves were created by analysing the peak area of 100-μl injections of six concentrations of uracil and cytidine (0.01, 0.1, 0.6, 1, 5, and 10 μg) and eight concentrations of uridine (the above standards plus 25 and 50 μg). The linear regressions for the standard curves for uracil, uridine, and cytidine had R^2 values of 0.976, 0.998, and 0.979, respectively.

Purified Alarm Cue Bioassays

After quantifying the alarm cues in ink, we tested them in the bioassay at their measured concentrations and also at dilutions of 1:25, 1:100, and 1:500 using purchased chemicals from Sigma−Aldrich (St. Louis, MO, U.S.A.).

To examine the necessity and sufficiency of these alarm cues to elicit an alarm response in juvenile *A. californica*, we created an ink-lacking alarm cue and, as a control, ink with alarm cues removed and then added back. This was done by recombining all ink fractions resulting from a purification process as described above for quantifying the alarm cues in ink. For the reversed phase C18 separation, after the column was flushed with 100% water, it was flushed with 100% methanol and the eluate was collected. The water eluate collected from the C18 separation of ink was then passed through a C30 column via HPLC, and the three alarm cues in ink were collected and all other compounds that eluted from the column were combined. All collected fractions (100% methanol C18 column flush and the nonalarm cue portions eluting from the HPLC) were combined. This extract was divided into two aliquots, one had no alarm cues added ('ink − cues'), and the other had half of the total collected alarm cues added to it ('ink − cues + cues'). These stimuli were tested in alarm cue bioassays as described above.

Statistical Analysis

Responses of sea hares to ink, opaline, and all other stimuli were compared to the response to a paired sea water control using a one-tailed McNemar test ($P < 0.05$). Responses to stimuli were considered significant if they were statistically greater than the response to the paired sea water response.

RESULTS

Ink and Opaline Contain Alarm Cues

Both ink and opaline elicited a higher frequency of sea hare alarm responses (70−100% and 83%, respectively) than did the control stimulus, sea water (Fig. 1). The response of each animal was either, and usually both, of the alarm behaviours investigated, moving away and galloping. (This was also true for the identified ink alarm cues, as described below.) Sea hare haemolymph, sea hare mucus, odour from the predatory lobster *P. interruptus*, or odour from their food *G. ferox* did not produce significant alarm responses from sea hares (Fig. 1).

Alarm Cues in Ink

When ink was partitioned among hexanes, chloroform, ethyl acetate, butanol, and water, only the water-soluble fraction produced significant alarm responses from sea hares (Fig. 2a). Further separation via reversed phase chromatography produced four fractions, only one of which (100% water) produced significant alarm responses (Fig. 2b). This fraction was further purified via gel filtration chromatography, producing three fractions, one of which (Fraction 2) elicited a significant alarm response (Fig. 2c). This fraction was further purified via TLC, producing three

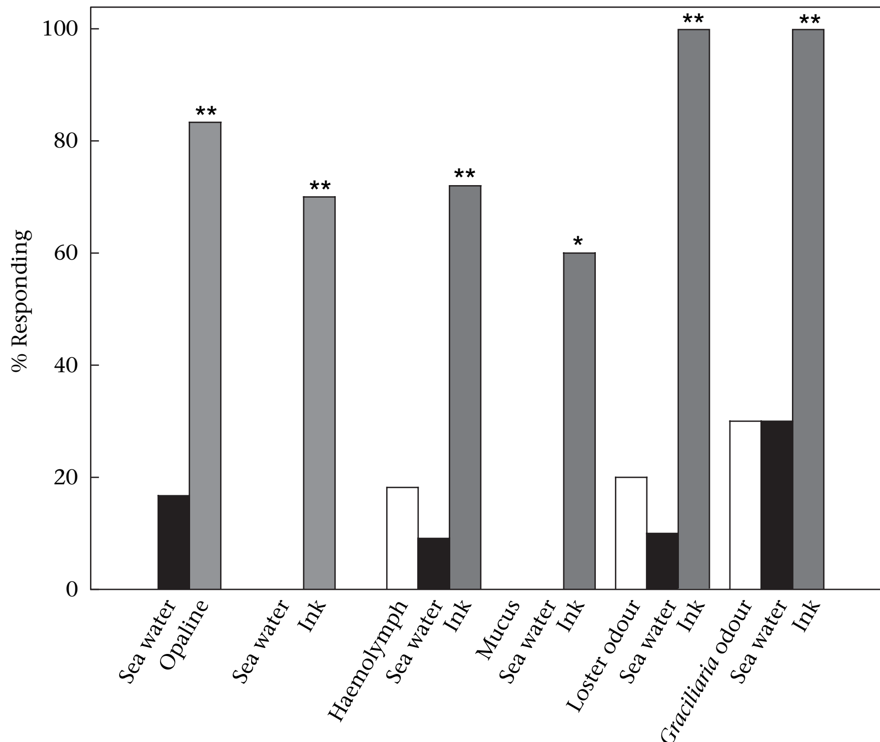

Figure 1. Frequency of sea hares (*Aplysia californica*) moving away or galloping when presented with sea hare ink or opaline (grey bars), sea hare haemolymph, sea hare mucus, lobster (*Panulirus interruptus*) odour, or seaweed (*Gracilaria*) odour (clear bars), or sea water as a negative control (black bars). In assays testing haemolymph, mucus, lobster odour, or *Gracilaria* odour, ink serves as a positive control (grey bars). $N = 10$ or 11. Analysis by a one-tailed McNemar test (*$P < 0.05$; **$P < 0.01$).

fractions, one of which (Fraction 2C) elicited significant alarm responses (Fig. 2d). Finally, this fraction was purified via reversed phase HPLC, producing six fractions, three of which produced significant alarm responses (2C.2, 2C.4, and 2C.6; Fig. 2e).

The ^{1}H NMR spectra were very similar to nucleic acid constituents. Considering these possibilities, the mass of peak 2C.4 (243.0946) matched cytidine, and the mass of peak 2C.6 (244.1965) corresponded to uridine. NMR and UV spectra and retention times on reversed phase HPLC of cytidine and uridine standards matched peaks 2C.4 and 2C.6, respectively, confirming their identity. The NMR spectrum of a uracil standard corresponded to peak 2C.2. In addition, the HPLC retention time and UV absorbance spectrum of this peak matched a uracil standard. Thus, although we could not determine the mass of peak 2C.2, this peak had all the characteristics of uracil, based on COSY, HMQC, HMBC, and NOESY.

Quantification of uracil, cytidine, and uridine in the ink yielded median values (and ranges) of 3.6 (2.8−4.8), 8.3 (8.1−9.5), and 17 (16−19) μg/ml, respectively. Uracil, cytidine, and uridine at these natural concentrations each produced a higher frequency of alarm behaviours than the sea water control (Fig. 3).

The three identified ink alarm cues were necessary and sufficient to elicit the alarm response. A mixture of uracil, uridine, and cytidine (i.e. 'three cues mixture') produced a similar frequency of responses as natural ink, when tested either at the concentration that the components occur in full-strength ink or at dilutions of 1:25, 1:100, and 1:500 (Figs 4 and 5). In addition, the three alarm cues in ink appear to be necessary and sufficient to evoke a significant alarm response from ink. Animals did not show a significant alarm response when exposed to ink with the three alarm cues removed ('ink − cues'), showing their necessity (Fig. 5). When the cues were added back to the ink − cues ('ink − cues + cues'), animals showed a frequency of alarm behaviours greater than sea water and similar to the cues ('three cues mixtures') and natural ink, showing their sufficiency (Fig. 5). Although 'ink − cues' did not elicit a statistically significant alarm response, it did elicit a modest response: 50% of the animals responded to it. We believe that this response is not because of a single additional bioactive molecule. This is indicated by testing separately the hydrophobic and hydrophilic components of 'ink − cues', in which we found that 20% and 30% of animals showed an alarm response to these stimuli, respectively (data not shown). Thus, the modest response to 'ink − cues' appears to be due to an additive effect of hydrophilic and hydrophobic components, none of which is potent individually. In addition, some of the nonactive fractions tested via the bioassay-guided fractionation of ink did show modest activity (e.g. Fig. 2b 30% methanol fraction and Fig. 2e fractions 2C.1 and 2C.5), but we believe this resulted from imperfect chemical separations rather than the existence of additional alarm cue molecules. It is possible that the alarm cues in the 100% water fraction were not completely washed out of the reversed phase column and some may have eluted in the 30% methanol fraction (Fig. 2b). In addition, the HPLC fractions were collected manually over

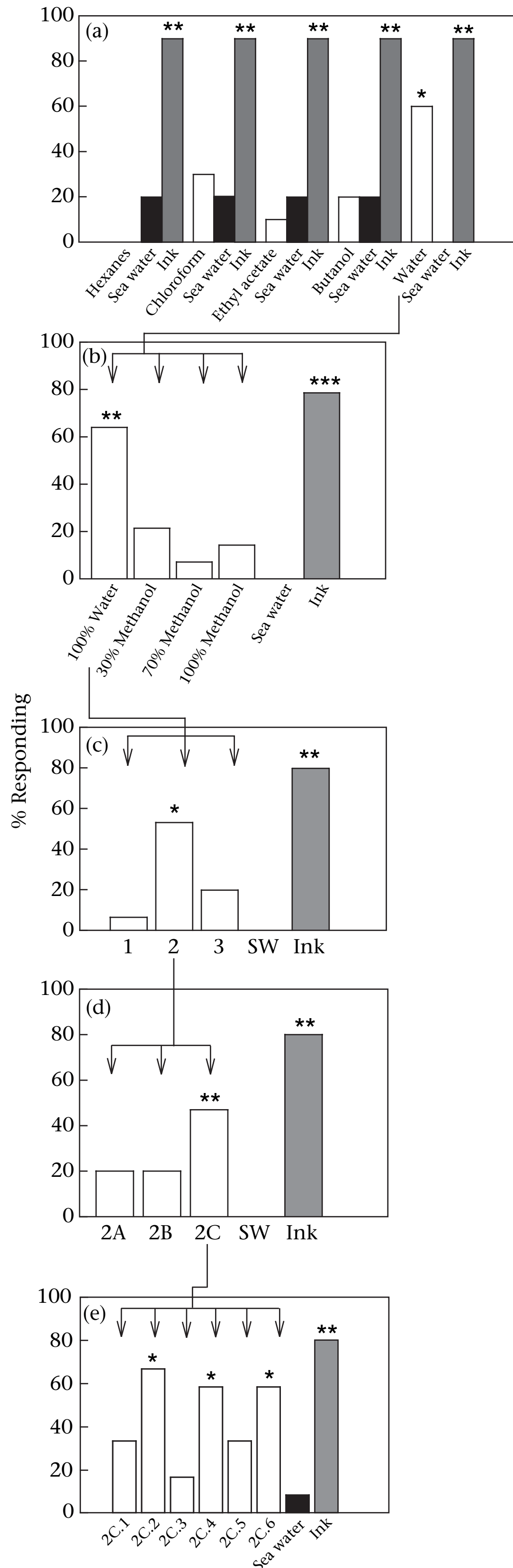

Figure 2. Isolation of the alarm cues in ink via bioassay-guided fraction. Frequency of response (moving away or galloping) by *A. californica* to chemical extracts produced by (a) solvent–solvent partition, (b) reversed phase chromatography of the water-soluble fraction from (a), (c) gel filtration of the 100% water fraction from (b), (d) reversed phase thin layer chromatography of fraction 2 from (c), and (e) high pressure liquid chromatography (HPLC) of fraction 2C from (d). Black bars represent sea water (negative control), clear bars represent ink extracts, and grey bars represent ink (positive control). $N = 10–15$. Analysis by a one-tailed McNemar test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

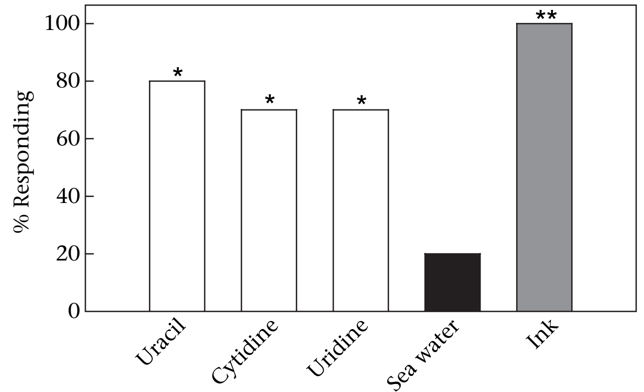

Figure 3. Frequency of *A. californica* moving away or galloping when presented with the three ink alarm cues at their natural concentrations in ink. Black bars represent sea water (negative control), clear bars represent alarm cues, and grey bars represent ink (positive control). $N = 10$, symbols and analysis as in Fig. 2.

several injections, allowing for accidental mixing during fraction collection. Thus, Fig. 2e fractions 2C.1 and 2C.5 probably contained some alarm cues. Thus, we believe that there are only three alarm cues in ink, uracil, uridine, and cytidine.

Species Specificity of the Alarm Response

The alarm response by juvenile sea hare is elicited by the defensive secretions of other inking molluscs. Ink and opaline from two other species of sea hares, *A. dactylomela* and *A. juliana*, also elicited significant alarm responses from *A. californica* juveniles (Fig. 6a). Ink from two cephalopod species, the squid *L. brevis* and the octopus *Octopus vulgaris*, also elicited significant alarm responses by *A. californica* (Fig. 6b).

The ink from both cephalopods contains uracil and uridine but not cytidine. The uracil and uridine concentrations in *O. vulgaris* ink were 63 and 41 μg/ml, respectively ($N = 2$ replicate injections from the ink from one individual). The concentrations of uracil and uridine in *L. brevis* ink were 0.021 and 0.014 μg/mg wet mass, respectively ($N = 2$ replicate injections from ink pooled from 10 individuals). These concentrations were calculated per wet mass because ink obtained from *L. brevis* ink sacs is a paste rather than a liquid.

DISCUSSION

Identity of the Alarm Cues in Ink

Numerous studies show that a variety of species avoid predators by responding to alarm cues released by injured

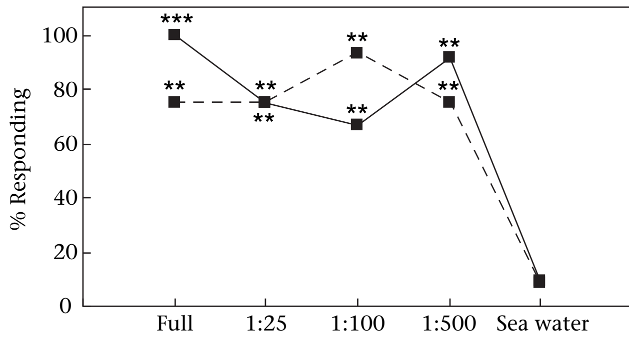

Figure 4. Frequency of moving away or galloping by *A. californica* when presented with ink or a mixture of the three ink alarm cues ('three cues mixture') at natural concentrations and dilutions of 1:25, 1:100, and 1:500. Solid line represents ink, dashed line is the alarm cue mixture. $N = 12$, symbols and analysis as in Fig. 2.

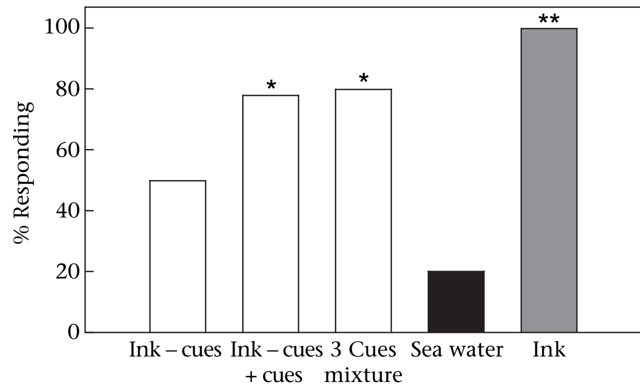

Figure 5. Frequency of moving away or galloping by *A. californica* to ink with the alarm cues removed ('ink − cues'), ink with the alarm cues added back ('ink − cues + cues'), and the three alarm cues ('three cues mixture'). Black bar represents sea water (negative control), clear bars represent ink extracts, and grey bar, ink (positive control). $N = 9$ or 10, symbols and analysis as in Fig. 2.

or disturbed conspecifics. However, very few of the molecules mediating these behaviours have been identified. Previous studies showed that the predator-evoked secretion of the sea hare *A. californica* has a defensive function not only by acting on predators but also by acting as an alarm cue for conspecifics (Walters et al. 1993; Nolen et al. 1995; Johnson & Willows 1999; Kicklighter et al. 2005; Kicklighter & Derby 2006). This defensive secretion is the product of two glands that typically release their contents simultaneously, the ink gland and the opaline gland. Which of these glandular secretions contains the alarm cues was not previously determined. In the present study, we show that both ink and opaline of *A. californica* elicit the alarm behaviours moving away and galloping in juvenile conspecifics (Fig. 1). Furthermore, we identify the base uracil and the nucleosides uridine and cytidine as the mediators of the alarm response to sea hare ink. Individually and at their natural concentrations in ink, the three molecules elicit a similar frequency of alarm response as natural ink (Fig. 3).

Uracil, cytidine, and uridine are necessary and sufficient to evoke a significant frequency of alarm behaviours relative to ink. This necessity is indicated by the finding that the frequency of alarm responses produced by ink lacking the cues was not significantly different from the frequency of responses produced by sea water (Fig. 5). The sufficiency of cues is indicated by two results. First, a mixture of the three compounds, at their natural concentration or when diluted as much as 1:500, elicited a response that was significantly greater than the response to sea water and similar to the response to ink (Figs 4 and 5). Second, when the three alarm cues were added back to the ink lacking cues, the frequency of response was significantly higher than sea water and just as high as ink (Fig. 5). Interestingly, each of the three alarm cues individually elicited a similar frequency of alarm responses as the 'three cues mixture' or ink (Figs 3–5). While uracil, uridine, and cytidine were sufficient to produce a frequency of alarm responses statistically as great as ink, the frequency of response was slightly lower than the natural secretion. It is not entirely clear why this was the case. We do not believe that it was due to the absence of other alarm cue molecules, because the 'ink − cues' and 'ink − cues + cues' stimuli elicited nearly equivalent levels of responses. It is possible, however, that active components could have decomposed when creating the 'ink − cues' stimulus. Another explanation may be pH. The pH of ink is around 4.9 (Shabani & Derby 2005, unpublished data) and the pH of the fractions and alarm cues was not adjusted to match natural ink.

In addition to moving away and galloping behaviours, we also observed head retraction in some animals tested with ink (~28% of individuals) and 'ink − cues + cues' (14% of individuals, data not shown) but not to the other stimuli. Thus, although infrequent, whole ink appears to evoke an additional alarm response that does not occur in response to opaline or the other alarm cues. Because head retraction was elicited with the 'ink − cues + cues' stimulus but not with 'ink − cues', this suggests that there is not a molecule that elicits head retraction that we did not isolate, but that the three identified ink alarm cues may interact with other components in ink to produce head retraction. In addition, the frequency of head retraction, moving away, and galloping that was elicited by natural ink versus the mixture of the three cues was not statistically different at any of the dilutions tested (McNemar's test, data not shown; Fig. 4). This further supports the conclusions that uracil, cytidine, and uridine mimic the alarm behaviours elicited by natural ink, and that we isolated and identified all alarm cues.

Why do sea hares have multiple alarm cues rather than just one? A possible function is that it compensates for significant variation in the abundance in ink of any one of these cues. This possibility remains untested, but such variation in the concentration of signal molecules could result from variability in the diet or physiological condition of sea hares.

Why was the response to a mixture of the three alarm cues for ink not greater than the response to any single component? This may result from the high similarity in the structure of these molecules, such that the sea hare's chemoreceptors do not differentiate among them. Regardless of the diversity and specificity of receptors, if these cues were present at a concentration at or above the response saturation for the

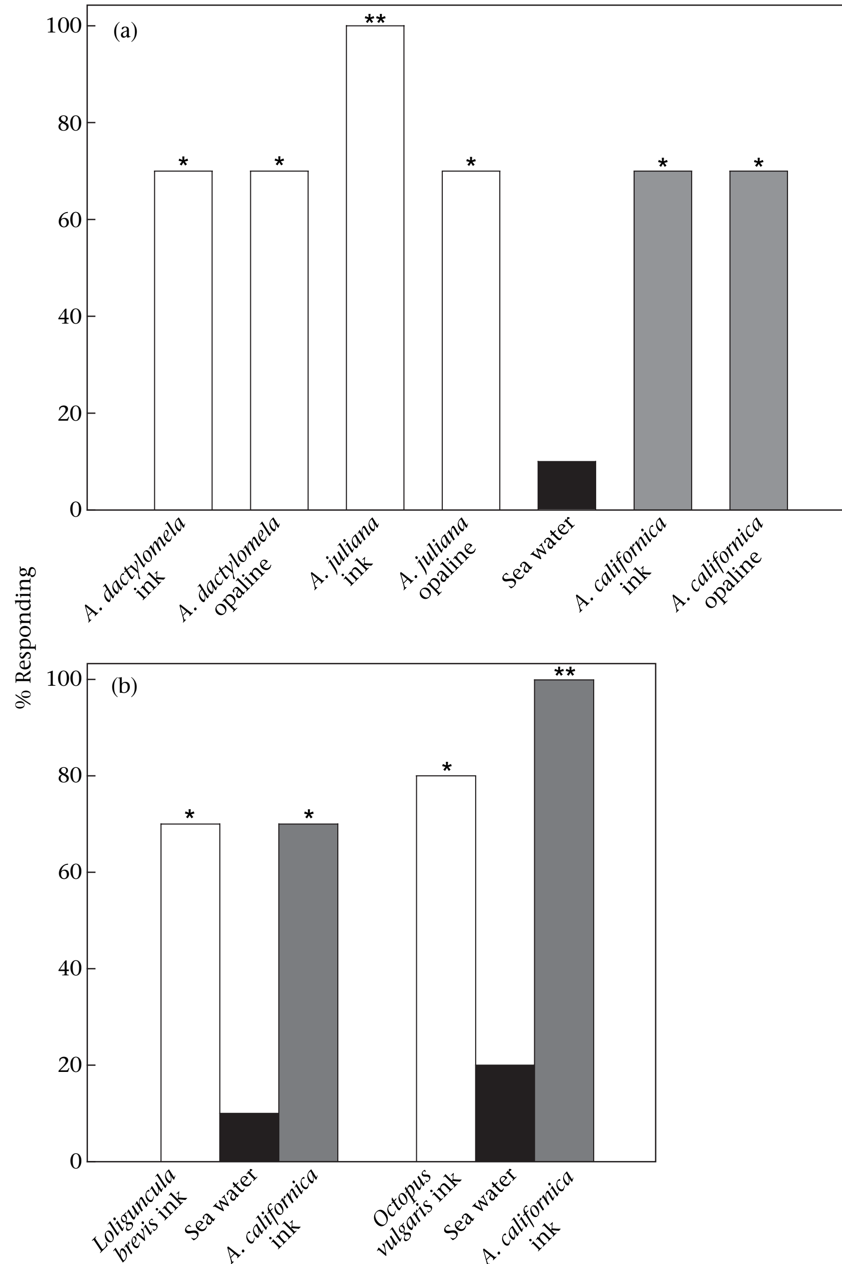

Figure 6. Frequency of moving away or galloping by *A. californica* to (a) ink and opaline from the sea hares *A. dactylomela* and *A. juliana* and (b) ink from the squid *Lolliguncula brevis* and the octopus *Octopus vulgaris*. Black bars represent sea water (negative control), clear bars represent secretions, and grey bars ink or opaline from *A. californica* (positive controls). $N = 10$, symbols and analysis as in Fig. 2.

chemoreceptor cells, then the mixture of alarm cue molecules would not produce a greater response than the individual cues from these cells or the animal. But because the greatest dilutions of ink and its components that we tested (1:500) still produced saturating responses, we are unable to comment further on receptor specificity and sensitivity.

The Nature of Alarm Cues

Antipredator behaviours are often elicited by predator odour (i.e. predator avoidance cues) or by odour from conspecifics injured by predators (i.e. alarm cues) as shown for platyhelminths, molluscs, insects, echinoderms, and fish (e.g. Snyder & Snyder 1970; Atema & Stenzler 1977; Scrimgeour et al. 1994; Chivers & Smith 1998; Rahman et al. 2000; Wisenden & Millard 2001). In our study, however, the alarm response of *A. californica* only occurred when sea hares were presented with ink or opaline but not with the odour of a sympatric predator (the spiny lobster *P. interruptus*) or other odours of conspecifics (haemolymph and mucus) that would presumably be released when sea hares are injured and/or consumed by attacking predators. This suggests that either these fluids do not

contain the isolated alarm cues or they occur at concentrations too low to evoke behavioural response. Some species show an alarm response only when odours from their conspecifics and predators are presented together and not individually (Kats & Dill 1998; Griffiths & Richardson 2006). We did not examine this in *A. californica*, so it is possible that this combination could evoke alarm behaviours.

Many if not most of the molecules that function as chemical signals, such as prey defences, pheromones, alarm cues, and settlement cues, appear to be unique to the organisms that produce them (Paul 1992; Hay 1996; McClintock & Baker 2001). This is also true for the only other identified mollusc alarm cues (Sleeper et al. 1980; Cimino et al. 1991). Thus, it seems surprising that the alarm cues in ink, uracil, cytidine, and uridine, are molecules that are present in all organisms. However, using general metabolic compounds as chemical signals is not unheard of, as indicated by the following examples. Ammonium, released by disturbed red-legged frog tadpoles, *Rana aurora*, elicits antipredator behaviour in conspecifics (Kiesecker et al. 1999). Eggs from the red abalone *Haliotis rufescens* release the amino acid L-tryptophan, to which sperm are attracted (Riffell et al. 2002). Spawning females of the marine polychaete worm *Nereis succinea* release cysteine–glutathione disulfide, which is a common tripeptide that causes males to release sperm (Zeeck et al. 1998a). The amino acid L-kynurenine is a sex pheromone released in the urine of female masu salmon, *Oncorhynchus masou* (Yambe et al. 2006). The amino acids cysteine, serine, alanine, glycine, and lysine may elicit sperm release in male rose bitterling fish, *Rhodeus ocellatus ocellatus* (Kawabata 1993). The purine nucleosides inosine and guanosine, which are present in the predatory sea star *Solaster dawsoni*, may generate an alarm response in the sea star *Asterina pectinifera* (Ukai et al. 2002). The sperm-release pheromone of the marine polychaete *Platynereis dumerilii* is uric acid, which is an end product of purine catabolism (Zeeck et al. 1998b). Finally, hypoxanthine-3-*N*-oxide, a purine nucleoside derivative, may function as an alarm cue for ostariophysan fish (Pfeiffer et al. 1985; Brown et al. 2000, 2003). Although purine nucleosides or their catabolic products are used as chemical signals in many biological systems, to our knowledge, our study is the first to show that pyridines or their nucleosides function as chemical signals between individuals. From the above examples, it is evident that the use of primary metabolites to convey information may not be that uncommon, especially for aquatic species for which polar cues, which diffuse readily in water, are advantageous. Given the primary metabolic functions of these cues, they probably did not evolve for the benefit of the sender. Rather, receivers probably evolved the ability to detect and respond to these signals, which may be released by senders together with gametes or waste products.

Species Specificity

Closely related species that respond to each other's alarm cues may share the same or very similar cue molecules (Chivers & Smith 1998). Phylogenetically distant but sympatric species may show cross responses, and this has been hypothesized to result from learning or other experiences (Chivers & Smith 1998). However, this hypothesis has not been tested because so few alarm cues have been identified. *Aplysia californica* juveniles respond to both ink and opaline from the closely related sea hare species *A. dactylomela* and *A. juliana* (Fig. 6). In addition, *A. californica* also responds to ink from phylogenetically distant species, the cephalopods *L. brevis* and *Octopus vulgaris* (Fig. 6). In this case, sea hares respond to inks from distantly related species because they contain the alarm cues uracil and uridine (but not cytidine) at concentrations higher than those found in sea hare ink. Compared to sea hare ink, uracil is six and 17 times higher and uridine is nine and 2.4 times higher in concentration in the inks of *L. brevis* and *O. vulgaris*, respectively. Sea hares and cephalopods may both possess uracil and uridine because ink release in sea hares and cephalopods may have originally evolved as a way to discharge waste. The presence of the base uracil, the nucleosides uridine and cytidine, amino acids, ammonium, and urea in inks of sea hares and cephalopods supports this hypothesis (Derby et al. 2007). The reason for cross species responses between distantly related species can only be determined by knowing the identity of the alarm cues. Thus, more work in this area is needed. Investigations may show other phylogenetically conserved alarm signals.

Cephalopods respond to ink from conspecifics with defensive or other behaviours. For example, the cuttlefish *Sepia officinalis* increases its ventilation rate following presentation of conspecific ink (Boal & Golden 1999). The squid *Loligo opalescens* increases jet escape responses when exposed to conspecific ink or L-dopa, which is present in cephalopod ink (Jimbow et al. 1992; Lucero et al. 1994). This suggests that squid ink functions as a conspecific alarm cue (Gilly & Lucero 1992). Thus, uracil and uridine may function as alarm cues for squid and octopus, and perhaps other more phylogenetically related species, an area we are currently exploring.

Sea Hare Chemical Signalling

Aplysia relies extensively on chemical signals and these three conspecific alarm cues in ink are but one example. Opaline also contains three conspecific alarm cues, but these are different molecules than those in ink (C. E. Kicklighter et al., unpublished data). Sea hares also use sex pheromones: four proteins, attractin, enticin, temptin, and seductin, make up a pheromone bouquet produced in the albumen gland and placed in egg cordons, which stimulates mate attraction (reviewed in: Cummins et al. 2006). In addition, a chemical cue released from burrowed individuals of *Aplysia brasiliana* is hypothesized to elicit burrowing in swimming conspecifics (Aspey & Blankenship 1976). *Aplysia* also uses chemical signals in interspecific interactions to arrest or deter attack by predators. Signals present in ink, opaline, and skin deter some predators, and bioactive molecules include small polar and nonpolar molecules (Ambrose & Givens 1979; Johnson & Willows 1999; Kicklighter & Derby 2006). The polar

molecules aversive to anemones are probably different from the alarm cues in ink and opaline, because the deterrent compounds are not as polar as the highly polar alarm cues (Kicklighter & Derby 2006). Other chemicals in ink and opaline, such as amino acids, ammonia, and urea, attract predators and thereby divert their attention away from sea hares, or disrupt the predator's chemosensory system (Kicklighter et al. 2005). Thus, different molecules in ink play different functional roles, serving as attractants or sensory disruptors to spiny lobsters, deterrents to anemones, and as alarm cues for conspecifics.

Broader Impacts of Alarm Cues

Sea hares can consume up to 35% of their body weight in seaweeds each day (Carefoot 1987). As a result, areas where predation is prevalent may experience changes in seaweed abundance and/or species diversity due to emigration of *Aplysia* in response to alarm cues released by attacked conspecifics. Indirect modification of sea hare behaviour by predators (trait-mediated indirect interactions) thus not only has effects at the level of the individual but may also influence the structure of invertebrate and algal communities, as shown in a several studies (reviewed in: Dill et al. 2003; Werner & Peacor 2003). For example, studies investigating indirect interactions in marine algal communities have shown that the presence of predators influences grazing intensity of a diversity of herbivores, such as echinoderms, gastropods, and arthropods, not by reducing the density of herbivores but by altering herbivore behaviour. Decreased feeding by sea urchins, snails, and crabs, due to emigration and other antipredator behaviours, has been shown in field and mesocosm studies to alter the abundance of seaweeds on which these herbivores feed (Trussell et al. 2004; Byrnes et al. 2006; Hereu 2006). Thus, responses to alarm cues may have broader impacts than simply avoiding predation, and studies investigating the mechanisms mediating predator–prey interactions may increase our understanding of community function.

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