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Ink secretion by the marine snail *Aplysia californica* enhances its ability to escape from a natural predator

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Abstract 1. *Aplysia californica* incorporates toxins and pigments from its red seaweed diet into its body and ink, purportedly as a defense against predation. We tested ink's potential defensive function by assessing the survival of green seaweed-fed (red algal toxin deprived) snails in encounters with a natural predator, the sea anemone *Anthopleura xanthogrammica*.

2. Red seaweed-fed *Aplysia* secreted copious amounts of ink when ensnared in anemone tentacles. A similar amount of ink applied to "inkless" (green-fed) snails as they were engulfed by an anemone enhanced their survival [71% survived (ink) vs 7% (seawater control)]. Ink caused anemones to reject whitefish (a familiar food) [50% rejected (ink) vs 10% (seawater control)], triggering gastrovascular eversions, which ejected ink as well as prey from their digestive cavities. Snails with only a *passive* chemical defense (algal toxins, no ink) escaped less often than snails with only an *active* chemical defense (ink, no red algal toxins) (20% survived vs 71%) and about as often as "red algal toxin deprived" snails (20% vs 12%). Snails avoided ink by chemical orientation, thus avoiding potential sites of ongoing predation.

3. The survival value of ink and the snail's aversion to it supports ink's proposed anti-predator function.

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Introduction

The marine mollusc *Aplysia californica* is well known to neurophysiologists because its relatively simple, accessible nervous system has made it a popular organism in which to study the cellular and molecular mechanisms of learning (e.g., Kandel 1979; Klein and Kandel 1980; Walters et al. 1981; Carew et al. 1983; Hawkins et al. 1983; Carew et al. 1984; Carew and Sahley 1986; Byrne 1987; Carew et al. 1990). Despite a large literature detailing its neurophysiology, we know surprisingly little regarding its behavioral ecology and neuroethology (Leonard and Lukowiak 1986; Carefoot 1987; Kandel 1979). For example, although long assumed to be an anti-predator defense (Eales 1960), the survival value of *Aplysia's* most conspicuous behavior, the secretion of a purple ink, has not been rigorously tested. The absence of a clearly demonstrated survival value for ink has made its potential defensive function controversial (Leonard and Lukowiak 1986).

Marine snails of the genus *Aplysia* obtain a variety of secondary plant toxins as well as ink pigments exclusively from a red seaweed diet (Winkler 1961; Darling and Cosgrove 1966; Irie et al. 1969; Chapman and Fox 1969; Winkler 1969; Watson 1973; Watson and Rayner 1973; Stallard and Faulkner 1974a, b; Blankenship et al. 1975; Kinnel et al. 1979; MacColl et al. 1990). The toxins, incorporated into the animal's skin and digestive gland (Winkler 1969; Watson 1973; Stallard and Faulkner 1974a, b; Kinnel et al. 1979), probably constitute a particularly effective *passive* chemical defense against predators such as fish and birds, by making the animal distasteful (Pennings 1990a; Kinnel et al. 1979; Ambrose et al. 1979). While this passive defense may

account for a paucity of known predators, the secretion of its purplish ink in response to predation could function as an *active* chemical defense by serving as a chemical deterrent or anti-feedant (DiMatteo 1981, 1982a, b). Ink could also act as an aposematic signal of the animal's toxicity, further reducing the incidence of predation (Ambrose et al. 1979). Previous studies regarding the functional significance of ink did not control for the contribution of diet to defense; these studies confounded the possible *active* defensive function of ink with the *passive* defensive role of secondary plant toxins, thus making it difficult to determine the true survival value of either.

We have assessed the potential defensive role of ink by manipulating the animal's diet to obtain "red algal toxin deprived" snails (Chapman and Fox 1969; Stallard and Faulkner 1974a) and then directly determining ink's survival value during encounters with a natural predator, the solitary sea anemone *Anthopleura xanthogrammica*. This sessile predator captures and ingests juvenile *Aplysia californica* in tide pools off California, where they may co-occur (Eales 1960; Winkler and Tilton 1962; Kupfermann and Carew 1974). Numerous observations suggest that cnidarian tentacles (probably the nematocysts) elicit defensive behavior in *Aplysia*, including ink and opaline secretion (Winkler and Tilton 1962; Kinnel et al. 1979; DiMatteo 1982b; Tobach et al. 1989; Johnson et al. 1993; Johnson 1994). These observations suggest that sea anemone predation may represent a selective pressure on *Aplysia* – especially the more vulnerable juveniles and small adults. Our laboratory experiments show that ink secretion provides the snail with a substantial survival advantage and therefore strongly supports a role for ink as an anti-predator defense.

Preliminary results of some of this work have been reported in abstract form (Johnson and Nolen 1991; Kicklighter et al. 1992; Johnson et al. 1993; Kicklighter et al. 1993).

Materials and methods

Animals

Laboratory-cultured *Aplysia californica* were raised from eggs at the University of Miami *Aplysia* Mariculture Facility, Virginia Key, FL and fed a diet of the red seaweed *Gracilaria* sp. or the green seaweed *Ulva* sp. *A. californica* has been reported feeding on both of these seaweeds in the field (see Carefoot 1987). Our experience is that they thrive on both under the controlled rearing conditions at the *Aplysia* Mariculture Facility. *Ulva* was obtained from Harbor Branch Oceanographic Institute, Ft. Pierce, FL; *Gracilaria* was grown at the UM *Aplysia* Mariculture Facility. We transferred snails from the Mariculture Facility to the Department of Biology at least one week prior to experiments and held them in 30 gal (113.6 l) or 50 gal (189.3 l) seawater aquaria at 18–22°C under a 16:8 h L:D photoperiod. They were fed red or green seaweed every other day.

Anemones (*Anthopleura xanthogrammica*) were obtained from Marinus Inc., Long Beach, CA, held in 50 gal (189.3 l) seawater aquaria at 18–20°C under a 16:8 h L:D photoperiod, and fed fresh-frozen whitefish cubes once a week. These animals have remained healthy and active under these conditions for almost two years. Thirty-five anemones were used in this study. Some were used in several experiments, but none was used twice in the same experiment, and none was used in successive experiments within four weeks. None was exposed to *Apylsia* ink or red seaweed-fed snails (see below) twice within six months, long enough, we believe to minimize possible food aversion conditioning effects.

Red algal toxin deprived snails

Late juvenile Stage 12 snails (Kriegstein 1977; Nolen and Carew 1988; Rankin and Carew 1988) deprived of ink and red algal toxins were raised from late Stage 11 individuals on a diet of *Ulva*, from an initial size of 0.5 cm to a final mean size of 2 cm (in eight to twelve weeks). After starting their green seaweed diet, these animals were de-inked every other day (for approximately 10 days) by hand manipulation of their mantle (ink) gland, until no further ink could be expressed (see Chapman and Fox 1969). Green seaweeds such as *Ulva* do not provide the snail with distasteful secondary plant toxins (Winkler 1961, 1969; Watson and Rayner 1973; Stallard and Faulkner 1974a; Fenical 1975) nor with ink pigments (Chapman and Fox 1969). We will refer to these red algal toxin deprived snails as "green-fed". To ensure consistent handling of experimental animals, the green-fed snails were "de-inked" just prior to experimentation following the procedure described below (*De-inking*). None of these animals released any pigmented ink (purple, white or other) from their mantle glands after two weeks on the green seaweed diet and none released ink following contact with anemones.

Collection of ink

Several experiments required testing an animal's response to fresh ink. In the morning, about six hours before the experiments were to be performed, we collected fresh ink from adult *Aplysia* (raised on red seaweed and de-inked no more than 5 days previously) by holding the animal above a dry 500 ml beaker and manipulating the ink gland with a bare hand. Six to eight snails provided ink, which was pooled so that within a particular day's study, all animals treated with ink received the same ink sample and concentration (most experiments were performed over a three day period). Since *Aplysia* may secrete ink and opaline simultaneously, we took care to minimize contamination of our ink sample by passing it through a coarse (1 mm pore size) Nylon mesh screen before alliquoting it into opaque 10 ml syringes and chilling it (18–20°C) until use later the same day. We found that the sticky opaline secretion adhered to the Nylon mesh while ink seeped through. Ink collected in this manner is much less viscous than ink contaminated with opaline – its viscosity was indistinguishable from that of seawater. However, we cannot rule out the possibility that small amounts of opaline, as well as mucus – were present in our ink samples.

De-inking

Some experiments employed de-inked snails. Each day for one week prior to testing, we de-inked animals either by handling them in bare hands (Kicklighter et al. 1992) or by wrapping their foot and parapodia in a coarse paper towel (Johnson et al. 1993). All animals ($N > 60$) we have treated this way were incapable of subsequently releasing ink even upon direct massaging of the ink gland (see Chapman and Fox 1969). It takes two to three days to replenish an empty gland (Chapman and Fox 1969; Johnson et al. 1993); to ensure that these snails had no releasable ink on the day of the experiment, we did not feed them during the 24 h preceding testing.

The de-inking treatment also largely voided these snails of their opaline.

Experimental design and statistical procedures

We employed blind procedures to minimize the introduction of experimenter bias. For the experiments reported in Figs. 3 to 7, we assigned animals by a random procedure to either the experimental or control group. The individual performing the experiment was unaware of the identity of each animal's group. Before analyzing experimental effects, we ran tests of skewness and/or homogeneity of variances to determine whether parametric statistical tests were valid (Sokal and Rohlf 1981). Where they were not, we employed appropriate non-parametric statistical tests (e.g., Mann Whitney U tests) instead (Krauth 1988). We used paired comparisons, contingency tests, and Fisher's exact test of independence of a 2×2 table, to test experimental hypotheses. Pilot studies were used to estimate typical variances to establish optimal sample sizes (Sokal and Rohlf 1981). Our experience with behavioral, physiological and anatomical experiments in *Aplysia* has shown that sample sizes from 12 and 20 are adequate and that often, only non-parametric analyses are valid. Unless otherwise indicated, all significance levels reported are two-tailed.

The experimental blind

Numerous pilot studies explored the possibility of developing a "faux ink" – a food dye, or vital dye, or a mixture of chemicals – to use as a control in double blind experiments. All substances we tested caused some kind of an olfactory orienting response in the snail. We therefore chose to use seawater as a mechanical control and an opaque syringe or pipette tip in order to maintain the blind up to the instant of stimulus delivery. Blind pilot studies showed that the experimenter could not reliably determine whether ink or seawater had been delivered from the pipetter or syringe.

Arena experiments

We performed two sets of experiments in different seawater arenas. In both experiments, the arena temperatures were kept within 3 °C of the animal's home tank (20 °C).

Simulated tide pool

The interactions between snails and their potential predator were investigated in a large arena simulating a tide pool situation. Twenty-two late juvenile stage and young adult *A. californica* (2 to 6 cm, median: 4 cm) were placed in a 1.25 m × 1.25 m × 10 cm seawater arena with twenty *A. xanthogrammica* (tentacle array diameter: 6 to 10 cm, measured tentacle tip-to-tentacle tip across the oral surface), and their interactions videotaped for 2 h. These snails, raised on red seaweed, appeared to have normal, full ink glands. The anemones were not fed for four days prior to experimentation. This experiment started at 16:00 h. After the initial two hour observation period, we left the animals to interact in the arena overnight and then noted the incidence of further predation the next morning.

Chemical orientation to ink

In this experiment, we assessed the potential warning that ink could provide a young animal by asking how it responded to another snail's ink. We tested 34 red-fed Stage 12 juveniles (1.0–2.5 cm) in a 30 cm × 24 cm × 1.5 cm seawater arena for their response to a 100 µl drop of fresh ink or a 100 µl drop of seawater (control) squirted onto the head as they locomoted forward. Each animal was placed in the arena 2 min before the start of the experiment to acclimate. During this time, the animals usually locomoted around in the middle of the arena. An Eppendorf® micropipetter was used to deliver the simulus: The experimenter was careful to position the pipette tip ~ 1 cm from the animal's head at a 45° angle (or less) as the stimulus was delivered. To ensure consistency of the mechanical stimulation for both ink and seawater, we delivered each at a constant rate of 100 µl over one second. Any difference in viscosity between ink and seawater (see above) was not noticeable by the experimenter delivering the stimulus, probably due to the action of the automatic pipetter. Little disturbance occurred as the pipette tip was removed from the water and the ink drop remained relatively intact until the snail locomoted away. All experiments were performed between 10:00 and 17:00 h.

We tested each animal twice, once with ink or seawater and then approximately 2.5 h later with the alternate stimulus. The animals were not fed between trials. We randomized the order of stimulus presentation for each animal to minimize the effects of prior experience in the apparatus. After each trial we emptied the arena and scrubbed it down to prevent any effects of residual ink on subsequent trials. A blind experimental procedure, in which the ink (or seawater control) was delivered from the opaque tip of the micropipetter, effectively prevented the experimenter from knowing the treatment before its delivery. In addition, for the second test of the day, a second investigator re-coded the identification numbers used to follow animals through the two experimental trials, thereby effectively maintaining the blind for the individual performing the tests. We videotaped the animal's behavioral responses to each treatment from two angles: 1) a close-up of the head and rhinophores, and 2) an overhead view of the entire arena. Videotapes were scored by an individual unaware of the purpose of the experiment, or the hypothesis being tested. The incidences of head retraction and of head waving (Leonard and Lukowiak 1986) upon contact with the ink (or control), as well as the direction the animal locomoted within four minutes of contacting the ink (or control) was recorded. Since there was no statistically significant effect of the order of stimulus presentation, we pooled the data for both ink trials and for both seawater trials.

Prey survivability experiments

We performed two experiments to assess the survival of various types of chemically protected or red algal toxin deprived snails in forced interactions with an anemone. In each of these experiments, the survival of a snail was determined by asking whether it escaped ingestion after being placed directly on the anemone's tentacles. A similar experiment was performed to determine the palatability of the anemone's laboratory food, whitefish, when treated with ink. We withheld food from the anemones for 4 days prior to these experiments. Individual anemones (2.5 to 8.5 cm tentacle array diameter) were placed in their own 2.5 gal (9.46 l) aquarium equipped with an aerator and allowed to acclimate for 8–9 h before testing. All these experiments were performed between 18:00 and 20:00 h.

Green-fed vs red-fed snails

We placed either a de-inked (see above), red-fed snail or a "red algal toxin deprived" green-fed snail (1.5 to 3.0 cm in length) on the tentacles of the anemone and videotaped the pair of animals for 30 min, noting the snail's survival, and the anemone's incidence of gastrovascular eversion (= cycles of regurgitation, see Results and Figs. 1 and 2B). We also calculated the anemone's handling time for

the prey, as the elapsed time (in minutes) between the snail's ensnarement by a tentacle to its escape, or its ingestion (i.e., its disappearance with the anemone's gastrovascular cavity).

Green-fed snails + ink

In this experiment, the defensive role of ink was investigated using the experimental paradigm described above. We placed a green-fed snail (1.5 to 3.0 cm in length) on the tentacles of the anemone and then dispersed either 6 ml of freshly collected ink or a like amount of seawater (control) onto the snail (and the anemone's tentacles) within 1 min (range: 15 to 60 s) of the snail's contact with the anemone. Thus, ink was applied as the anemone pulled the ensnared snail toward its mouth. A blind experimental procedure, in which an opaque 10 ml syringe (*without* hypodermic needle) delivered the ink (or control), effectively prevented the experimenter from knowing the treatment before its delivery. To ensure consistency of the mechanical stimulus for both ink and seawater, we delivered the stimulus at a constant rate of 6 ml in 5 s ($\pm$ 0.5 s). Any difference in viscosity between ink and seawater (see above) was not noticeable by the experimenter delivering the stimulus, probably due to the action of the large bore syringe. We videotaped the pair of animals for 30 min and noted the snail's survival, the anemone's incidence of gastrovascular eversion, and the anemone's handling time for the snail, as described previously.

Fish cubes + ink

In this experiment, the role of ink as an anti-feedant was investigated using the above paradigm. We placed a 1 cm cube of whitefish (mean $\pm$ SEM weight: 1.4 $\pm$ 0.006 g), typical of those fed the anemones in the laboratory, on the anemone's tentacles and then dispensed either 6 ml of freshly collected ink or a like amount of seawater (control) onto the fish, tentacles and oral disk within 1 minute (range: 15 to 60 s) of the anemone contacting the fish – just as the anemone pulled the food toward its mouth. A blind experimental procedure, in which an opaque 10 ml syringe (*without* hypodermic needle) delivered the ink (or control), effectively prevented the experimenter from knowing the treatment before its delivery. To ensure consistency of the mechanical stimulus for both ink and seawater, we delivered the stimulus at a constant rate of 6 ml in 5 s ($\pm$ 0.5 s). Any difference in viscosity between ink and seawater (see above) was not noticeable by the experimenter delivering the stimulus, probably due to the action of the large bore syringe. We videotaped the animal for 30 min and noted the fate of the fish and the reaction of the anemone to *ink + fish* or to *seawater + fish*.

Results

Response of *Aplysia* to anemone predation in a simulated tide pool

Our initial observational study was designed primarily to confirm that *Aplysia* will secrete ink in response to anemone predation. As shown in Fig. 1, when a snail encounters an anemone and contacts a tentacle, it may release some ink as it begins to turn away. At this time the anemone's tentacles quickly reach over and extend down toward the snail. If the snail is still within reach, as many as eight tentacles may grab the snail and lift it off the substratum. It is at this point that an anemone might fail to capture the prey: Some of the snails escaped due to the poor grip the anemone initially secured. Anemones often simply mishandled the snails and dropped them before pulling them over the oral disk toward their mouth. If the anemone retains its hold, it will engulf the snail in a myriad of tentacles and pull it toward the mouth. We often observed at this point in the interaction, that the snail released a large amount of ink, coating both itself and the anemone's tentacles and oral disk. When the snail released ink in this manner, the anemone's tentacles quickly retracted, releasing their grip on the snail and shriveling up to about 1/10 their normal size (Fig. 2B). The snail often fell onto the anemone's oral disk and spent some time locomoting around the oral disk before getting past the tentacles and onto the substratum. Within a few minutes of being covered in ink, the anemone would start food rejection behavior – gastrovascular eversions that

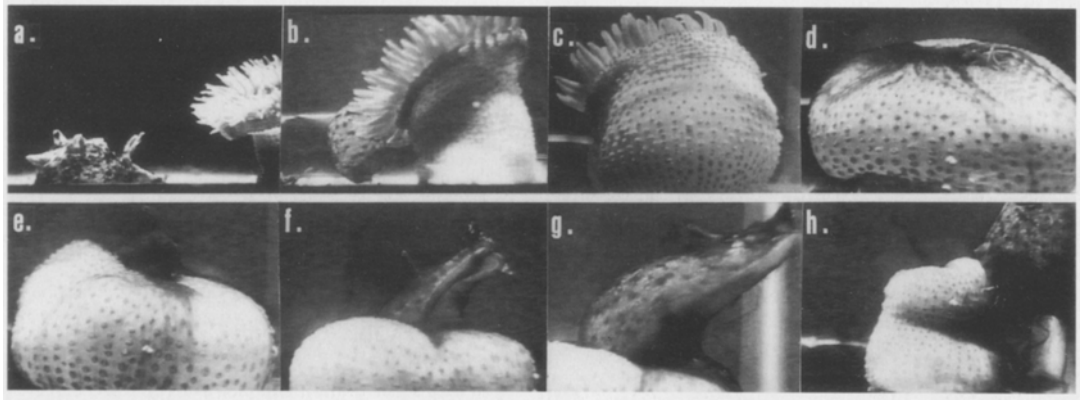

Fig. 1a–h Sea anemones are effective predators of juvenile and small adult *Aplysia* in a simulated tide pool. **a** Snail encounters an anemone; **b** is snared and releases a small amount of ink (@ time = 21 s after ensnarement); **c** is engulfed (@ 46 s); **d** inks copiously (@ 1:41 min); and **e–h** is then regurgitated relatively unharmed (@ 3:45, 5:24, 6:22 and 7:29 min respectively). A cycle of gastrovascular eversions started at **d** and continued as the snail escaped. Note the ink being expelled from the anemone's gastrovascular cavity in **h**

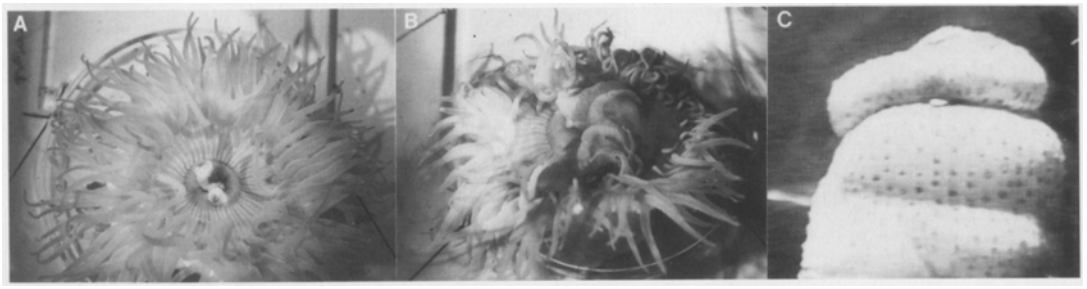

Fig. 2A–C Ink induced tentacle retraction in an anemone. **A** Normal posture of receptive anemone. **B** Approximately 2 min following an encounter with a snail and its ink, the anemone has retracted many of its tentacles and started a cycle of gastrovascular eversion. **C** Another anemone in the balled up state after undergoing gastrovascular eversions (a subsequent frame from the sequence of Fig. 1h)

consist of peristaltic contractions of the column and eversion of the digestive cavity (Fig. 1g, h; Fig. 2C). The anemone usually produced several cycles of eversions, often remaining in a "balled-up" defensive posture (tentacles pulled into the oral disk and the top most part of the column contracted over the disk) at the end of a cycle (Fig. 2C). Gastrovascular eversions always ejected ink from the anemone's gastrovascular cavity (Fig. 1f). In some cases, gastrovascular eversions ejected the snail from the anemone's oral disk or from its digestive cavity (Fig. 1e).

Within their first 2 h in the simulated tide pool arena, 68% (15 of 22) of the snails contacted an anemone; and while 93% of these became ensnared in tentacles and were picked up, only 43% (the smallest 6 in the arena, at 2 cm long) ultimately were ingested. Of the snails interacting with anemones, 60% released ink within one minute of contact (none released ink unless they contacted an anemone; $P = 0.02012$, Fisher's exact test; incidence of inking following contact different than expected by chance). More than half the snails that escaped, inked copiously after becoming ensnared (Fig. 1d). Some snails encountered several anemones, releasing ink and escaping each time. Toward the end of the two hour observation period, these snails, especially the smaller ones (2–3 cm) were releasing little ink in further encounters with predators.

We followed up on these animals the next morning (18 h after the start of the experiment) to see how many snails were able to avoid capture and to assess the longer term effects of ink on anemones that had interacted with snails. Several of the anemones that had been exposed to enough ink to cover their tentacles, remained balled up after their initial bout of gastrovascular eversions. Six of the anemones that had eaten a snail the day before had ejected the undigested remains of the snail's digestive gland. Also, at this time, we observed that two more of the smallest snails (about 2 cm) had been caught and eaten.[1] Nevertheless, despite the predatory ability of the anemones, 14 of 22 snails survived the night in this "tide pool".

While these observations show that *Aplysia* will release ink on contact with this predator, because the snail could employ several defenses simultaneously (ink, opaline, distasteful chemicals in the skin), it was impossible to assess independently the significance of each defense. For these reasons, we designed a controlled, direct assessment of the survival value of the behavioral phenotypes: *inking* versus *non-inking* in interactions with sea anemones.

Survival of red algal toxin deprived snails

Since *Aplysia californica* obtain certain toxins and ink pigments exclusively from red seaweeds, green seaweed-fed animals are relatively free of toxins and pigments (Chapman and Fox 1969; Stallard and Faulkner 1974a, b; Winkler 1969). Our green-fed animals had been deprived of red seaweed toxins and pigments for eight to twelve weeks and had been completely de-inked prior to experimentation (see Methods). We found that they had lost their releasable stores of ink, leaving only residual amounts in depleted ink vesicles in their glands [see Chapman and Fox (1969) for a discussion of this method of de-inking]. In addition, they had lost the pungent halogen chemical smell and taste that characterizes those fed a red seaweed diet. Unlike red-fed snails, we found the green-fed snails were palatable (although quite tough).

In survivability experiments (see Methods) anemones readily ate green-fed snails placed on their tentacles: anemones engulfed and ingested 87.5% (21 of 24) of the green-fed snails in about 4 minutes ($P < 0.001$, sign test, H_0: snail not palatable, expect 0% to be eaten). These snails must have been palatable because only four of the twenty-four anemones (16.7%) attempted to expel the snail from their digestive cavity by gastrovascular eversion within 2 h of ingestion[2]

[1] It may be important that these small snails were most likely to have spent their (relatively small) ink stores the day before

[2] Two hours is sufficient time for an anemone to digest a snail of this size

($P < 0.001$, sign test, H_0: snail not palatable, expect 100% gastrovascular eversions). A few of these snails (3/24) released opaline – but none released ink – in response to anemone tentacles. It is possible that the snails released opaline after they were engulfed and ingested and therefore out of view. As best we could determine, none of those that escaped, secreted opaline. Since so few escaped, this experiment suggests that opaline – even if it is secreted commonly by our green-fed snails – is not a confound for our subsequent experiments comparing the relative survival of snails treated with exogenously applied ink or the seawater control (see below).

Survival value of ink

Since green-fed snails were readily eaten and only infrequently escaped from anemones, we determined their survival when freshly collected ink was applied to the anemone as it engulfed the snail in its tentacles. Ink, in amounts comparable to those released by snails of the size we used, significantly increased the survival rate of the snail: 71.1% ($N = 14$) of the snails survived their encounter with the anemone when ink was applied, compared to only 7.1% ($N = 14$) when seawater was applied as a control ($P = 0.000717$, Fisher's exact test) – a relative survival value of 10 to 1 (Fig. 3A). There was no correlation between a snail's survival and its absolute size [mean ± SEM length: 2.2 ± 0.1 cm (Eaten) vs 2.2 ± 0.2 cm (Escaped), $t_{23} = 0.2$, $P > 0.8$] or its size relative to the anemone [mean ± SEM snail length/tentacle array diameter: 0.5 ± 0.03 (Eaten) vs 0.4 ± 0.04 (Escaped), $t_{23} = 0.7$, $P > 0.49$]. Likewise, there was no correlation between a snail's experimental group and its absolute, or relative size [2.2 ± 0.1 cm (Ink) vs 2.2 ± 0.2 cm (Seawater), $t_{23} = 0.3$, $P > 0.75$; and 0.4 ± 0.03 (Ink) vs 0.5 ± 0.04 (Seawater), $t_{23} = 1.6$, $P > 0.13$].

An examination of the anemone's handling times (see Methods) suggested that ink halts or retards the anemone's rapid consumatory response. We compared handling times for anemones that ultimately ingested their snails with those that failed to ingest their prey, regardless of whether ink was applied (Fig. 3B), and found that when an anemone ate a snail, it did so in about 4 min (median handling time: 4.1 min). However, when the anemone rejected or otherwise failed to eat the snail, the time the anemone spent handling the snail (time it could otherwise have spent attempting to capture new prey) was about 13 min (median handling time: 13.2 min; $P < 0.0216$, Mann Whitney U test, H_0: Eaten ≤ Escaped). When we examined handling times with respect to ink or the seawater treatment, we found that the effect of ink application was to extend the anemone's handling time for the prey (Fig. 3B) [median handling time: 8.0 min (ink) vs 4.5 min (seawater); $P < 0.0283$, Mann Whitney U test, H_0: Ink ≤ Seawater]. Ink appears to increase handling time because it causes the predator to drop the prey onto the oral disk; the anemone ceases consumatory behavior while the snail makes its escape.

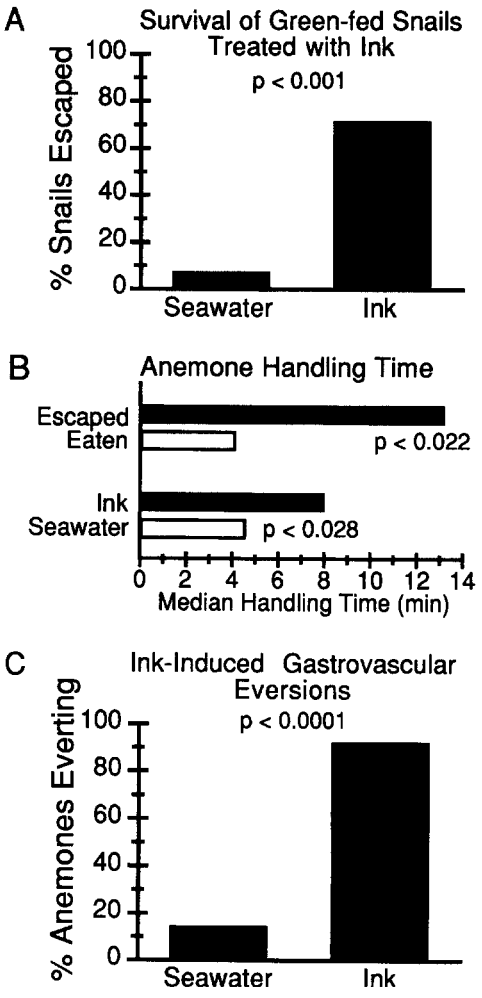

Fig. 3A–C Two groups of 14 green-fed *A. californica* were placed on the tentacles of an anemone, *A. xanthogrammica*, and then treated with either ink or seawater (control). **A** Direct test of the survival value of inking. While green-fed *A. californica* are readily eaten, application of exogenous ink to the tentacles of an anemone as it pulled a snail to its mouth resulted in a high incidence of the snail's escape: Ink and seawater groups were statistically significantly different. The result of a Fisher's exact test for ink vs seawater is shown. **B** Comparison of the anemone's handling time (median min) for a snail, with respect to the prey's fate (*top bars*) or the anemone's treatment group (*bottom bars*). Snails that ultimately escaped from the anemone (*top*) spent significantly more time on the oral disk, resulting in a longer handling time. The application of ink (*bottom*) significantly extended the anemone's handling time for the snail. Comparisons between Escaped ($N = 17$) vs Eaten ($N = 11$) and between Ink ($N = 14$) vs Seawater ($N = 14$) were made using Mann Whitney U tests [$U(17, 11) = 50.5$, $P < 0.0216$; one-tailed: escape times > ingestion times; $U(14, 14) = 56.5$, $P < 0.0283$; one-tailed: ink times > seawater times]. **C** The application of ink also induced a significantly higher incidence of gastrovascular (GV) eversions, which expelled ink and sometimes the snail (see Fig. 1e–h) from the digestive cavity. The result of a Fisher's exact test for ink vs seawater is shown

As we observed in our initial tide pool experiments, ink caused local recoiling of tentacles (Fig. 2B),

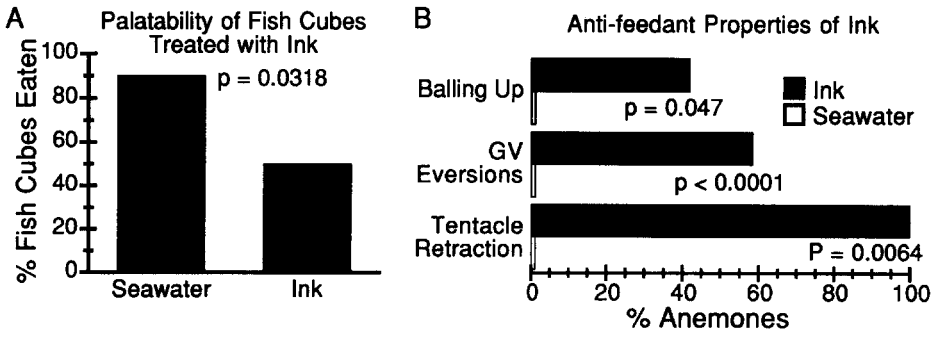

Fig. 4A, B Ink elicits defensive and anti-feeding behavior in anemones. Ink or seawater (control) was applied to a cube of whitefish (normally palatable) as the anemone engulfed it and pulled it toward its mouth. **A** The presence of ink ($N = 24$) resulted in a significant level of rejection when compared to the seawater control ($N = 9$). The percentage of fish cubes engulfed and ingested within 30 min is shown. The result of a Fisher's exact test for ink vs seawater is shown. **B** Defensive (*balling up*) and anti-feedant behaviors (*gastrovascular (GV) eversions* and *tentacle retraction*) observed in response to ink ($N = 24$) or seawater ($N = 9$) application. The percentage of anemones exhibiting these three behaviors is plotted. The results of Fisher's exact tests for ink vs seawater are shown

followed by their extension over the substratum. Ink application also increased the incidence of gastrovascular eversions by the anemone ($P = 0.0000688$, ink vs seawater; Fisher's exact test) (Fig. 3C). We often observed the snail making its escape before the anemone actively ejected it, although sometimes the snail – along with ink – was expelled from the oral disk or digestive cavity (see Fig. 1e–h).

The anti-feedant properties of ink

We next used the survivability paradigm (see Methods) to compare the effect of ink *vs* seawater (control) on the anemone as it engulfed a familiar food, a cube of whitefish. As expected from our observational study, we found that ink induced a high incidence of tentacle retraction compared to controls (Fig. 4B), thus forcing the anemone to drop and reject otherwise palatable food (Fig. 4A). Ink also induced a relatively high incidence of gastrovascular eversions and defensive balling up behavior (Fig. 4B). These results confirm ink's detrimental effect on the consumatory behavior of the anemone.

Survival value of the passive chemical defense

We next assessed the relative survival value of the snail's *passive* chemical defense against anemones. We compared the survival of green-fed snails (without red algal chemical defenses) and de-inked, red-fed snails (with only the passive algal defense) using the survivability paradigm (see Methods). As shown in Fig. 5A, de-inked, red-fed snails were just as likely to be eaten as green-fed snails. In addition, for anemones that ate the snail there was no statistically significant difference in their handling times for red-fed vs green-fed snails (Fig. 5B), suggesting that toxins in the skin of red-fed snails did not retard the consumatory behavior of the anemone. The incidence of gastrovascular eversions following ingestion of red-fed snails was not statistically significantly different from that for green-fed snails, and both rates were low (Fig. 5C). By comparing the survival of green-fed snails treated with ink (Fig. 3A) and the survival of de-inked, red-fed snails (Fig. 5A), we found that snails with only the benefit of an "active" defense (green-fed + ink) had a significant survival advantage over those with only a passive defense (red-fed, de-inked): 71% ($N = 14$) vs 20% ($N = 19$), respectively, a survival advantage of 3.55 to 1 ($P = 0.02534$, Fisher's exact test, see Fig. 6A). Likewise, by comparing the anti-feedant properties of these two defensive phenotypes (percentage of anemones induced to produce gastrovascular eversions following contact with the snail), we found snails with the active defense (green-fed + ink) to be significantly more aversive than snails with the passive defense (red-fed, de-inked) ($P = 0.000956$, Fisher's exact test; see Fig. 6B).

Orienting responses of juveniles to ink

We found that when a juvenile encountered ink as it locomoted forward, it produced a rapid head retraction (Fig. 7A) followed by head waving (characteristic olfactory orientation behavior), at which point the animals usually turned and rapidly locomoted away from the ink (Fig. 7B). The statistically significant differences in response to ink and seawater (see Fig. 7A and B), suggested that each animal responded to the chemicals inherent in the ink and not simply to the mechanical disturbance of stimulus delivery.

When we performed a similar experiment on a stationary animal and completely covered its head in ink, the animal retracted its head and waved it back and forth (6/6, ink *vs* 0/6, seawater: $P = 0.00216$, Fisher's exact test), but it did not start locomoting [nor turn

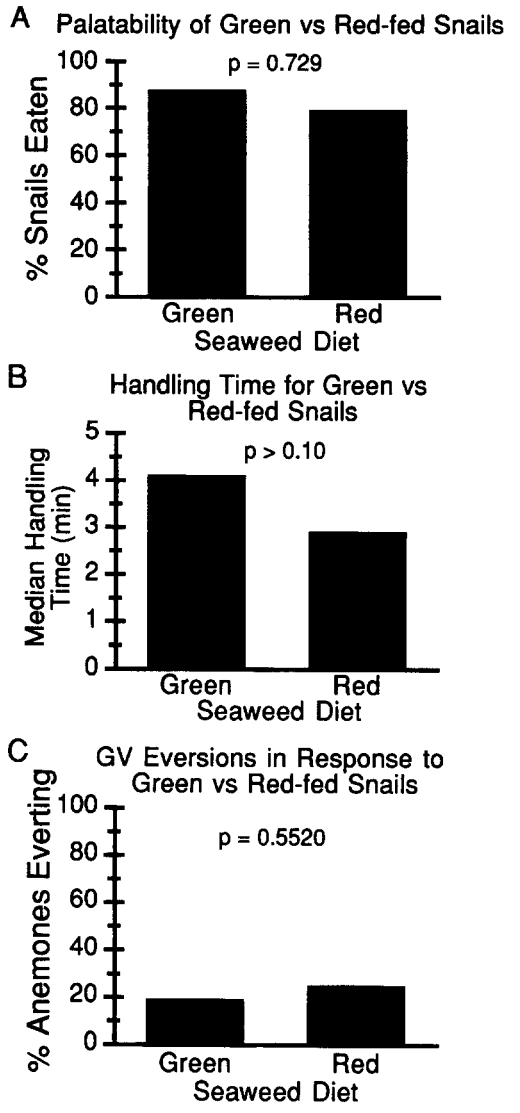

Fig. 5A–C Anemones do not respond significantly differently to de-inked red-fed or green-fed snails. Animals were placed on the tentacles of an anemone and observed for 30 min. **A** De-inked red-fed snails are just as palatable as green-fed snails. The percentage of snails engulfed and ingested within 30 min is shown. (Fisher's exact test comparing green-fed ($N = 24$) and red-fed ($N = 19$) snails was not significant, as shown). **B** For snails they ultimately ingested, the anemone's handling time for green-fed and de-inked red-fed snails was not significantly different (Mann Whitney U test: $U(20, 15) = 103$, $P > 0.10$). The median handling time spent by an anemone to ingest or reject a snail is shown. **C** For anemones that ultimately ingested their snail, the incidence of gastrovascular (GV) eversions was not statistically significantly different for green-fed vs de-inked, red-fed snails. The outcome of a Fisher's exact test comparing green-fed ($N = 21$) vs red-fed ($N = 15$) snails is shown. A similar analysis comparing the incidence of GV eversions by all anemones also revealed no significant differences for green-fed ($N = 24$) and de-inked, red-fed snails ($N = 19$) (Fisher's exact test for ink vs seawater: $P = 0.4315$). The percentage of anemones exhibiting GV eversions within 30 min of engulfing and ingesting a snail is shown

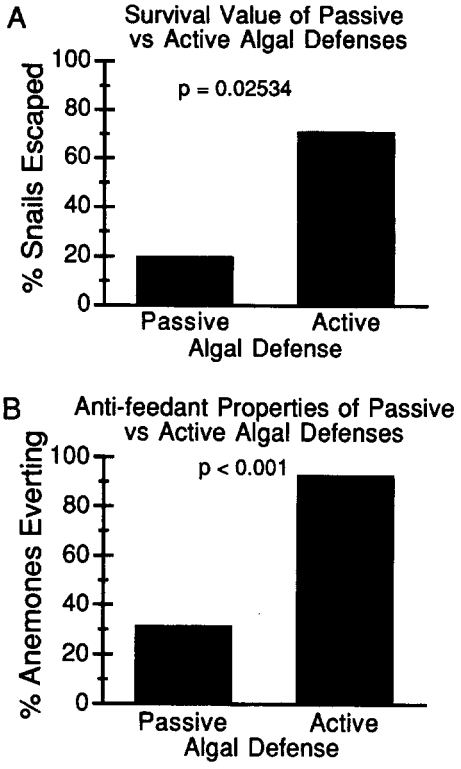

Fig. 6A, B A summary comparison of the survival values of passive vs active red algal defenses. **A** The survival of snails with only a passive red algal defense (red-fed, de-inked, $N = 19$) and snails with only an "active" red algal defense (green-fed + exogenous ink, $N = 14$) are compared. The result of a Fisher's exact test was significant, as shown. **B** The anti-feedant properties of these two defensive phenotypes are compared, as the percentage of anemones induced to produce gastrovascular eversions within 30 min of contact with the snail's defense (either the skin of the snail with the passive defense, or the ink of the snail with the "active" defense). The result of a Fisher's exact test was significant, as shown

away from the ink: 5/6 stayed put in response to ink *vs* 0/6 for seawater (these snails commenced locomoting straight ahead): $P = 0.0152$, Fisher's exact test]. In this situation, the ink adhered to the animal's head and rhinophores, so that the head's side to side motions did not move it out of the ink. We believe that in this experiment the animal could not assess the ink concentration gradient. These results suggest that the animal will stay put when it cannot locate the source of ink.

Discussion

Ink as an active chemical defense

This study represents the first direct experimental assessment (Dawkins 1986) of the survival value of ink secretion for *Aplysia* as an active anti-predator defense. Several other indirect lines of evidence also support this defensive role for ink. First, ink secretion involves an elaborate neural trigger mechanism (Carew and Kandel 1977a, b, c; Byrne et al. 1979; Shapiro et al. 1979; Byrne 1980a, b, c) that commands specific actions of the siphon and mantle organs to direct ink toward the site of stimulation (Walters and Erickson 1986). This ability

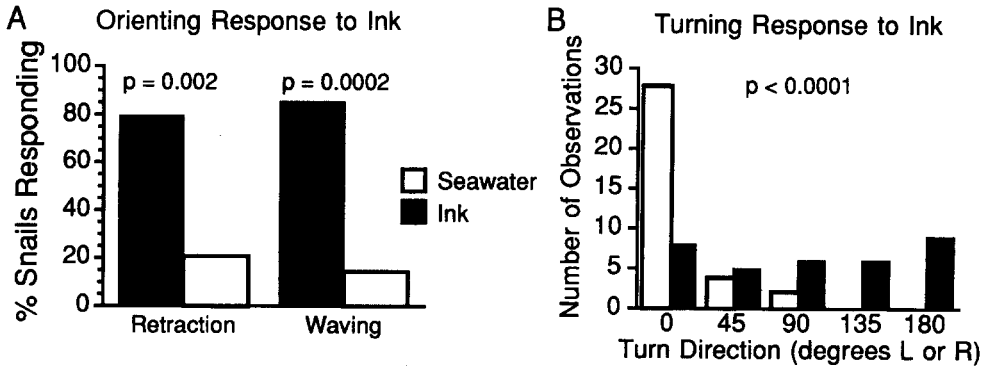

Fig. 7A, B Ink elicits orienting and negative chemotaxis reactions in Stage 12 juveniles ($N = 34$). **A** The percentage of juvenile snails responding to a 100 μl bolus of fresh ink or seawater (control) is shown for head retraction (a startle response to a novel stimulus) and head waving (chemical orientation). Ink induced significantly higher incidences of these behaviors than did the seawater control. (The results of contingency tests, with continuity corrections, are: head retraction, $\chi^2 = 9.6$, $P = 0.002$; head waving, $\chi^2 = 14.2$, $P = 0.0002$.) **B** Once oriented to the bolus of ink, juveniles usually turned and rapidly locomoted away from the ink drop. The number of observations for each general turn direction (0°, 45°, 90°, 135°, 180° Left or Right) are shown (see Methods) in response to ink and seawater (control). For statistical purposes, the turn categories were collapsed to 0°, 45 + 90° and 135° + 180°. (Results of contingency test: $\chi^2 = 27.6$, $P = 0.0001$.)

to direct ink appears important to the snail since inking takes precedence over other potentially conflicting behaviors. For example, exogenous ink application inhibits the siphon withdrawal reflex (Stopfer et al. 1993); and noxious stimuli, which elicit inking, also specifically inhibit the siphon withdrawal reflex (Marcus et al. 1988) and act to transform the siphon's response for use in defensive ink secretion (Illich et al. 1994). Since the siphon directs ink toward the site of stimulation, inhibition of the siphon withdrawal reflex in the context of predation (presence of ink, noxious stimulation, etc.) may function to prevent interference with the siphon's crucial role as an effector of an anti-predator behavior (Illich et al. 1993).

Previous studies of the defensive function of ink have employed straightforward observations of the snail's interactions with a potential predator (Tobach et al. 1965; Kupfermann and Carew 1974; Ambrose et al. 1979; DiMatteo 1982b; Tobach et al. 1989). These types of studies are useful in determining how a defense might be employed and the appropriate behavioral context for its expression. For example, we observed that following capture, a snail often released a copious amount of ink when positioned over the anemone's oral disk. However, observational studies are not adequate to test adaptive or functional hypotheses (Alcock 1993). One problem with these "natural experiments" is our inability to control important variables, especially the expression of the purported defense. Consider the following: If a particular defense is *not* employed in an encounter with a predator, we cannot infer anything useful about its effectiveness or about its survival value. We cannot know why an animal did not employ the defense nor whether it might have relied on another, less obvious one. Finally, we cannot compare predator-prey encounters such as these and be confident that any difference in the prey's survival was due solely to the use or non-use of the defense.

We chose to avoid the uncertainty and variation inherent in simple observational studies by directly assessing the relative survivability (Dawkins 1986) of individuals with and without the purported defensive adaptation: ink secretion [see Tinbergen (1967) and Roeder (1967) for examples of other direct assessments of purported defensive adaptations]. Moreover, we determined the survival value for ink secretion at the critical time when the anemone has positioned the snail over its mouth (Fig. 1) because this is when the largest amounts of ink are released by the snail. Our survivability tests demonstrate that ink causes the anemone's tentacles to retract, forcing it to drop the snail (Fig. 3). Ink also causes the anemone to undergo gastrovascular eversions (Figs. 3C and 4B), which can force the snail out of the anemone's mouth (Fig. 1e–h) or push it off the oral disk to safety. While ink's anti-feedant properties are crucial for its survival value for the prey, the snail's active participation is important as well – the snail often walked off the oral disk before the anemone actively ejected it.

One common, valid criticism of ink as an active chemical defense against predators such as fish, for example, has been that it would quickly dissipate in the subtidal disturbances characteristic of *Aplysia's* habitat (as noted by Kupfermann and Carew 1974). However, in encounters with anemones, unlike those with fish, ink appears to coat the snail just as the predator engulfs it and attempts to ingest it. In this environment, protected from currents within the anemone's tentacles, ink persists until the anemone releases the snail and it begins its escape (e.g., see Fig. 1e–h).

Active vs passive defenses

Ink may be especially useful in deterring predation by sea anemones because of the ease with which this

predator can selectively regurgitate toxic or inedible parts of their prey (Hyman 1940). Indeed, Winkler (1961) reported that anemones digest the least toxic, outer tissues of the snail and then selectively regurgitate the most toxic part, the digestive gland [which may contain 58x the level of toxins found in other parts of the body (Winkler 1969)], an observation we have confirmed in our initial arena experiments.

Besides its secondary plant toxins, *Aplysia* may *synthesize* other potentially defensive compounds. For example, opaline, a white, sticky and pungent-smelling material, is secreted from a specialized gland on the floor of the mantle in response to disturbance (Carefoot 1987; Kandel 1979). While we cannot exclude the possibility that opaline, as well as mucus, plays some role in the effects of mantle secretions on anemones, our experimental design minimized the impact this might have on our interpretations regarding the survival value of ink: De-inking the snails the day prior to experimentation (see Methods) caused them to release their opaline, as well as ink. This probably accounts for the low incidence of opaline secretion during the snail's initial encounter with an anemone – a situation in which animals with full ink glands (e.g. those used in the simulated tide pool experiments) release opaline as well as ink. Moreover, while opaline secretion might be obscured by our application of ink, few of the control animals (e.g. Fig. 3A) released opaline in the 4 min between their initial contact with the anemone and ingestion; we would expect that animals in the experimental (ink) group would be similarly unlikely to secrete opaline. Finally, it is possible the snail secreted opaline after becoming obscured from view within the anemone's tentacles or gastrovascular cavity. However, since few of the green-fed control snails ever escaped, opaline cannot account for the effect of exogenous ink as determined by comparing the survival of snails in the experimental (ink) and control (seawater) groups (Fig. 3A). However, this does not mean that opaline does not function as an anti-predator defense – direct tests of its survival value should be performed to verify a potential defensive function. It would be interesting to directly test the potential synergistic function of opaline and ink, since they often are secreted together.

Whatever other defensive chemicals *Aplysia* possesses, green seaweed-fed snails lack the toxic terpenoid and halogenated organics obtained from red seaweeds. The anemone's ability to deal with *Aplysia's* passive chemical defense was apparent in our survivability experiments. Any secondary algal toxins that these snails normally may harbor appear to be of little significance for the anemone: red-fed snails were eaten as readily as green-fed snails (Fig. 5). Since ink's antifeedant properties provide a degree of survivability above that provided by its passive chemical defense alone (Fig. 6), we believe ink may provide the snail with its most effective defense against anemone predation.

Acquisition of defensive secondary plant chemicals

Ink secretion is a predominant component of the elaborate arsenal of chemical defenses characteristic of this genus (Carefoot 1987). Thirty-five of the thirty-seven species of *Aplysia* are known to possess a mantle (ink) gland; thirty of these secrete purple ink (Table 1) (Carefoot 1987) whose principle component, phycoerythrobilin, is derived from the breakdown of phycobiliproteins found in red seaweeds (MacColl et al. 1990). While the diets of most species have not been carefully studied (Carefoot 1987), since most *Aplysia* forage on macroalgae, the source of these ink pigments is likely red seaweed (MacColl et al. 1990; Carefoot 1987). Generally, only a diet of red seaweed provides the snail with the benefit of a dual chemical defense; green and brown seaweeds do not have phycobilisome accessory photosynthetic pigments used to produce ink, and green seaweeds do not contain the halogenated anti-herbivory toxins typical of many species of red algae (Winkler 1961; Chapman and Fox 1969; Winkler 1969; Watson 1973; Watson and Rayner 1973; Stallard and Faulkner 1974a, b; Blankenship et al. 1975; Kinnel et al. 1979; MacColl et al. 1990). Some brown seaweeds possess certain toxins and some species of *Aplysia* may sequester these materials as well (see Table 1).

Acquisition of red seaweed-derived chemicals appears to be of utmost importance to juvenile *Aplysia*. Veliger larvae can delay metamorphosis indefinitely [up to 311 days post competence (Kempf 1981)] unless provided a preferred (usually red) seaweed on which to settle (Strenth and Blankenship 1978; Switzer-Dunlap 1978). The trigger for metamorphosis is likely the water soluble phycoerythrobilin that leaches out of red macroalgae (Mianmanus 1988). While the halogenated plant compounds are toxic to the veliger (in higher concentrations) (Mianmanus 1988) post-metamorphic juveniles nevertheless choose to remain on red seaweed, which is the only natural source for their dual chemical defense.

Testing adaptive hypotheses

Ideally, one should test adaptive (functional) hypotheses such as the above by demonstrating that the trait increases the individual's inclusive fitness, and by showing an evolutionary history of selection for the purported function (West-Eberhard 1992; Burian 1992; e.g., see Tinbergen 1967). For potential anti-predator adaptations, a first step would be to show an enhanced rate of survival in encounters with a natural predator – as we have reported here for inking in *Aplysia californica*.

Field studies with natural populations of anemones could add significantly to our understanding of ink's potential anti-predator function. It is possible that

Table 1 The thirty-seven species of *Aplysia*: The extent of current knowledge about each species' mantle (ink) gland secretion, diet, secondary plant toxins and ability to swim is tabulated. The species are organized by subgenus with *Aplysia* (*Neaplysia*) *californica*, the subject of this study, located at the top [data from Eales (1960), Carefoot (1987) and Bebbington (1977)]. *yes*, presence confirmed in several studies; *no*, absence confirmed in several studies; ?, not determined, or no observations; *maybe*, unconfirmed, anecdotal report

Subgenus	Species	Red[b] Seaweed Diet?	Mantle[b] Gland Secretion	Halogenated Organics?	Swims?
Neaplysia	*californica*	yes	purple	yes	no
Pruvotaplysia	*parvula*	yes	purple	?	no
Pruvotaplysia	*punctata*	yes	purple	?	no
Varria	*brasiliana*	yes	purple	yes	yes
Varria	*fasciata*	yes	purple	yes	yes
Varria	*dactylomela*	yes	purple	yes	no
Varria	*kurodai*	yes	purple	yes	no
Varria	*cervina*	yes	purple	?	no
Varria	*keraundreni*	yes	purple	?	no
Varria	*extraordinaria*	?	purple	?	yes
Varria	*morio*	?	purple	?	yes
Varria	*pulmonica*	?	purple	?	yes
Varria	*maculata*	?	purple	?	maybe
Varria	*winneba*	?	purple	?	maybe
Varria	*cornigera*	?	purple	?	no
Varria	*denisoni*	?	purple	?	no
Varria	*gigantea*	?	purple	?	no
Varria	*gracilis*	?	purple	?	no
Varria	*inca*	?	purple	?	no
Varria	*oculifera*	?	purple	?	no
Varria	*reticulata*	?	purple	?	no
Varria	*robertsi*	?	purple	?	no
Varria	*sagamiani*	?	purple	?	no
Varria	*sowerbyi*	?	purple	?	no
Varria	*sydneyensis*	?	purple	?	no
Aplysia	*depilans*	no	white[a]	yes[c]	yes
Aplysia	*juliana*	no	white	maybe	no
Aplysia	*vaccaria*	no	white[a]	maybe	no
Aplysia	*cedrosensis*	?	white	?	no
Aplysia	*nigra*	?	white or black	?	no
Aplysia	*dura*	?	?	?	no
Phyciphila	*euchlora*	?	purple	?	no
?	*tanzanesis*	?	purple	?	yes
?	*geographica*	?	purple	?	no
?	*reticulopoda*	?	purple	?	no
?	*spuria*	?	purple	?	no
?	*rehderi*	?	?	?	no

[a] It is unclear whether these species may also secrete a purple ink (Christomanos 1955; Carefoot 1987). The white secretion is known to be distinct from opaline.
[b] Purple ink is believed to be obtained from a diet of red macroalgae. However, most species' diets have not been studied (Carefoot 1987). Blue green algae contain some of the pigments found in red algae (Gantt et al. 1979; Cohen-Bazire and Bryant 1982); but they do not contain the toxic secondary compounds and normally are not a component of the diet of *Aplysia*.
[c] Several unusual terpenes derived from the brown seaweed *Dictyota* are sequestered by this species (Minale and Raffaele 1976; Danse et al. 1977; Finer et al. 1979)

we would have obtained different results had we performed initial experiments in the field with wild anemones and snails. The difficulties associated with carrying out experiments in the field would have limited the tests we could have performed; and without understanding how and when ink might function as an anti-predator defense, we might have focused on less fruitful hypotheses. Now that we have delineated several important characteristics of the effects of ink on anemones, it will be possible to extend this investigation to comparative studies in the field. However, future field work should control for the possibility that anemones (or other predators) have become averse to *Aplysia* due to prior experience with this prey's chemical defenses.

While *Aplysia* are not a major part of the anemone's diet[3], since these snails can range over a considerable

[3] While a particular prey species may be inconsequential to a particular predator's feeding ecology, the outcome of predation is always of significance to the prey individual. Any trait reducing predation should be favored by natural selection.

distance up and down the sub- and intertidal zones during their lifetime (Carefoot 1987), it is possible that many species of *Aplysia* are at risk of anemone predation sometime in their life history (Winkler and Tilton 1962; Kupfermann and Carew 1974). Even if snails encounter anemones only rarely in their excursions up and down the sub- and intertidal zones, ink could provide small adults – and juveniles in particular – with a significant survival advantage, thus enhancing the inking individual's fitness.[4]

Given the widespread occurrence of the ink gland (Table 1) in this genus (Carefoot 1987), it is likely that ink secretion is a phylogenetically old trait. While one of the gland's functions may be defensive, it probably evolved as part of the physiological requirements of bile pigment metabolism when this gastropod's ancestors first started to exploit red seaweeds and make use of their anti-herbivory toxins. Thus, natural selection may have co-opted the gland's modern defensive function from an original excretory function. A reasonable evolutionary sequence would include: 1) The ancestor's exploitation of chemically protected red seaweed, 2) sequestering of anti-herbivory chemicals, 3) selection for efficient extraction, storage, packaging and excretion of bile pigments, 4) selection on the survival advantage of ink released in response to predation, and 5) selection for efficient predator-triggered ink release mechanisms. Comparative studies of other species of *Aplysia* and related genera may help to support this evolutionary scenario.

Ink as an adaptation

Ink's potential anti-predator function continues to be controversial (Leonard and Lukowiak 1986). Several investigators have proposed numerous alternative functions for ink, which fall into three broad categories: 1) active chemical defenses, 2) chemical signaling, and 3) waste excretion. Our results shed light on several of these hypotheses, so we will briefly consider the evidence for several of the most prominent ones.

Active chemical defense

1. *Anti-feedant. Aplysia* secretes ink in response to other predators as well as anemones [e.g., lobsters and crabs (Walters et al. 1993)]. Ink's anti-feeding effects in anemones (food rejection, Figs. 3 and 4), are similar to those produced in other potential predators [e.g., crabs and birds (DiMatteo 1981, 1982a; Walters et al. 1993)]. Our survivability tests show that in encounters with anemones, ink provides the snail a high level of survivability – above that provided by its passive chemical defense alone (which, for anemone predators, seems to provide no demonstrable benefit, Figs. 5 and 6). However, since direct tests of the survival value of ink have only been performed on anemones, it is unclear whether ink's survival value is unique to this one type of predator.

Indeed, ink is not an effective anti-feedant for all predators. A conspicuous predator of *Aplysia californica* is *Aglaja inermis* (navanax) itself a gastropod (Leonard and Lukowiak 1986; Pennings 1990a, b; Paine 1963). This specialized predator may be well suited to effectively deal with *Aplysia's* chemical defenses. In most all cases, when navanax strikes at relatively small *Aplysia californica*, this predator prevails, regardless of whether the snail releases ink – [though, curiously, ink is seldom released in these situations (Leonard and Lukowiak 1986)]. Likewise, lobsters preying on *A. brasiliana* do not appear to be deterred by the prey's ink (Walters et al. 1993) and find both green-fed and red-fed snails palatable (Pennings 1990a). The failure to provide a defense against a particular predator (especially a specialized one such as navanax), however, does not mitigate ink's defensive role against anemones or other predators.

2. *Camouflage: A visual or chemical smoke screen. Aplysia's* ink commonly is compared to squid and octopus ink, which seems to function in these species as a "smoke screen" (Eales 1960) by providing the animal some cover (visual or olfactory) as it makes a retreat (MacGinitie and MacGinitie 1968). However, unlike squid and octopus, most species of *Aplysia* move too slowly to take any advantage of ink as a screen. Moreover, in small, natural tide pools (Kupfermann and Carew 1974), or even in an enclosed aquarium (Nolen, pers. obsv.), ink starts to dissipate before the animal has moved substantially.

3. *Startle.* While we can dismiss ink's potential smoke screen function for most species, for the seven (to nine) species that swim, it is possible that ink could startle a predator, much as the eye spots on an underwing moth startle bird predators (Sargent 1976), giving the prey some chance to swim away as the predator hesitates.

Chemical signaling

Several chemical signaling hypotheses for ink have been presented [see Leonard and Lukowiak (1986) and Carefoot (1987) for recent reviews], these include ink acting as: 1) an aposematic signal of the animal's distastefulness, or 2) an alarm signal, or 3) a pheromone. Another, more parsimonious explanation is that ink may function simply as a cue of nearby predation danger, rather than as an evolved "signal" in an adaptive communication system (Johnson and Nolen 1991).

[4] Even traits with small (or rarely employed) selective advantages can be favored (Huxley 1942).

1. *Aposematism.* The diets of most species of *Aplysia* are not well described (Table 1), so it is not yet possible to determine whether all can sequester red algal toxins. Five of the six species known to sequester halogenated secondary plant compounds (e.g., various terpenes), are known to eat red seaweeds and secrete purple ink. Although the composition and content of toxins in red seaweeds varies across species (Fenical 1975), and perhaps within species as well, the correlation between the possession of ink and secondary plant toxins (Table 1) is likely to be high enough that ink could function as a useful aposematic signal of the snail's potential toxicity or distastefulness, as first suggested by Ambrose et al. (1979). Some support for this hypothesis comes from Ambrose et al.'s (1979) study showing that laughing gulls find *Aplysia* distasteful. DiMatteo (1981, 1982a) later found that ink acted as an anti-feedant for sea gulls, as well as crabs. However, ink's aposematic function is unclear for crabs who find fresh *Aplysia* flesh palatable (DiMatteo 1982a). Our study indicates that aposematism probably is not a factor for anemones since they are not deterred by the snail's passive chemical defense (Fig. 5), as are birds (Ambrose et al. 1979), some fish (e.g., wrasses, Pennings 1990a) and sharks (Kinnel et al. 1979).

2. *Alarm signal.* If ink acts as an alarm signal (Sherman 1985), then the response of the receiver must benefit the sender's inclusive fitness [see Grier and Burk's (1992) for one of many discussions of communication, pp. 502–504]. We have seen no evidence in *Aplysia californica* of a benefit for the "sender" of such an alarm signal – the animals seem to move too slowly to affect a confusion effect, especially for active predators such as fish or even starfish. Interestingly, seven species of *Aplysia* are active swimmers (see Table 1), but it is unknown if ink could trigger a mass "flight" in a group of these animals, thereby confusing a predator. If swimming is a primitive trait (and this is not yet clear, although swimming is found in three subgenera; see Table 1), it is possible that an alarm function could explain the initial selective advantage of defensive ink secretion in this entire genus.

Ink secretion could be a form of kin selected alarm signaling. For example, it could be an act of genetic altruism [an indirect fitness enhancing alarm signal (Hamilton 1964; Sherman 1985)] (Stopfer et al. 1993), but only if close relatives live in proximity and only if individuals direct their ink selectively toward non-descendent kin. In addition, an individual's fitness could benefit if ink were directed toward descendent kin [this would fall under the category of parental care, rather than genetic altruism and would be due to direct fitness enhancing selection (Brown 1987)]. However, *Aplysia's* extended pelagic larval phase (Kempf 1981; Strenth and Blankenship 1978; Switzer-Dunlap 1978) and its widespread larval dispersal make it unlikely that siblings or offspring settle in the same local patch (Carefoot 1987), thus reducing the potential for indirect or direct selection of inking as an altruistic act of alarm signaling.

3. *Pheromone.* Various authors have shown some response of *Aplysia* to their own or another's ink and have suggested that ink in some species acts as a pheromone (Tobach et al. 1965; Fiorito and Gherardi 1990; Stopfer et al. 1993). As argued above, if ink acts as an *intra*species chemical signal (pheromone), its reception must benefit the inclusive fitness of the sender (and some would argue the receiver as well). We have yet to observe, or see documentation of, any instance where ink induces any response by the intended receiver that results in any short or long term benefits for the sender.

4. *Adventitious cue of danger.* If ink serves a defensive, rather than an alarm function, then a neighboring snail's ink could act as an indicator of the proximity of a predator. This could allow other snails in the vicinity to avoid the source of the ink and, presumably, the predator (Johnson and Nolen 1991). In this situation, ink reception benefits the "receiver" and not the "sender". Therefore, ink would not function as a "signal" in an adaptive chemical communication system, but rather as an adventitious cue of nearby predation danger. According to this hypothesis, if the snail can determine the location of the source of ink, it should avoid it (Johnson and Nolen 1991). Furthermore, if the animal cannot determine the location of the ink, e.g., when ink covers its head and rhinophores or its whole body, the safest strategy would be to stay put, where there are no predators (for the moment). Juveniles [Fig. 7; and Johnson and Nolen 1991] as well as adults (Stopfer et al. 1993; Walters et al. 1993) appear to do just this. This strategy would be especially advantageous against a sessile predator such as an anemone.

Bile excretion

Another, non-functional hypothesis is that the ink gland (and thus ink secretion) has no defensive role and is merely a consequence of bile metabolism (Chapman and Fox 1969; Tobach et al. 1989). Thus, ink simply would be a waste product excreted from an excretory gland. This hypothesis seems unlikely for a variety of reasons previously stated. In particular, the probability of ink secretion is not a function of the amount of "waste product" stored in the gland, as this hypothesis would suggest. The inking neural circuit within the CNS (Carew and Kandel 1977a, b, c; Byrne et al. 1979; Shapiro et al. 1979; Byrne 1980a, b, c) triggers ink secretion in response to external stimuli to the head, tail, foot, parapodia or body wall (e.g., contact with anemone tentacles) rather than simply as a function of the amount of ink stored in the gland. We have found (Johnson et al. 1993) that the glands of snails kept undisturbed in the laboratory for up to three months

are full of old, oxidized ink, which is evident in many large, red-brown ink vesicles. Snails with freshly sequestered ink contain a lower density of moderately sized, dark purple vesicles. The animals with full glands did not release ink spontaneously (or when their glands were ostensiby full). Yet both groups readily released ink in response to anemone tentacles.

Neuroethology

Our identification of at least one class of predator for which inking is a defense requires a re-evaluation of our understanding of the functional organization of the inking neural network. Ink secretion is thought to be an all-or-none, high threshold, fixed act (e.g., Carew and Kandel 1977a, b, c; Byrne 1980b). This belief is based on laboratory studies using unnatural, noxious stimuli and, usually, animals fed a diet of dried seaweed. However, in our observations of interactions between *Aplysia* and anemone predators we have seen that, depending on the behavioral context, ink release is *not* necessarily all-or-none: The snail may release variable amounts of ink and multiple inking episodes are possible (Kicklighter et al. 1992; Johnson et al. 1993). One explanation for differences between our experiments and previous ones could be the animals' diets. Perhaps it is more difficult to extract and process ink pigments from dried red seaweed than from fresh red seaweed. In the earlier studies, this difference could have led the animals to release all of their (small stores of) ink, but at a high threshold (Leonard and Lukowiak 1986). That ink release is not necessarily all-or-none is further supported by a recent study of Illich et al. (1994) in which a noxious stimulus, which elicited ink release, acted to the reduce the threshold for subsequent ink release.

Finally, since it can take a snail up to three days to replenish its ink gland (Johnson et al. 1993; Chapman and Fox 1969), any neuroethological studies of the mechanism of inking must consider the animal's diet and its recent experience with predators. Our study suggests that in a natural predator-prey context, inking should be a graded response, reflecting its potential adaptive role in a diversity of predator-prey situations in which the amount of ink released would be correlated with the degree of predation risk. Future studies will address the potential plasticity in *Aplysia's* food preferences and inking behavior, which are likely to depend on the state of the animal's ink gland, the nature of its diet and its recent experience with predators.

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