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DEFENSIVE INKING IN *APLYSIA* SPP.: MULTIPLE EPISODES OF INK SECRETION AND THE ADAPTIVE USE OF A LIMITED CHEMICAL RESOURCE

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Summary

The seahare *Aplysia* spp. extracts many of its defensive chemicals from its red seaweed diet, including its purple ink, which is an effective deterrent against predators such as anemones and crabs. It is believed that the inking behavior is a high-threshold, all-or-none fixed act that nearly completely depletes the seahare of its ink supply. If a seahare depletes its gland of ink, it must seek out a source of red seaweed and then feed for at least 2 days to replenish its ink supply. This suggests that the animal would not be able to deploy ink more than once in rapid succession in response to successive attacks from one or more predators.

However, we found that *Aplysia* spp. can secrete ink in response to three or more successive stimulations with (i) anemone tentacles, (ii) a mechanical stimulus, consisting of grabbing and lifting the animal from the substratum, or (iii) a noxious electric shock. A spectro-photometric measure of ink secretion showed that only approximately 48 % of the gland's releasable ink reserves are deployed initially. Thus, deployment of this defensive chemical is not strictly all-or-nothing, although the trigger mechanism is. Moreover, the animal tends to secrete a relatively fixed proportion (30–50 %) of its available ink reserves even after its gland has been depleted to approximately half its initial content. Since an animal need only use a proportion of its ink reserves to deter an attacker effectively, the inking behavior is adaptive in its economical use of a limited resource.

Key words: chemical defence, anti-feedant, all-or-none fixed act, phycoerythrobilin pigment, ink, conservation of limited resources, opisthobranch mollusc, behavioural ecology, eco-physiology, *Aplysia californica*, *Aplysia brasiliana*.

Introduction

Certain behaviors tend to be especially economical in their use of energy, time or other limited resources unless there is a compelling fitness-enhancing reason to be otherwise (see Heinrich, 1979; Holmes, 1984). For example, anti-predator behaviors tend to favor short-term survival at a cost to long-term conservation or economy. However, if an anti-predator behavior uses a limited resource, we would expect economy of use to prevail as well. Previously, we showed that ink secretion by the seahare *Aplysia californica* (a large marine snail) provides the animal with an active chemical defense against predators such as sea anemones (Nolen et al., 1995), and it may do so for crabs and lobsters as well (Ambrose et al., 1979; DiMatteo, 1981; DiMatteo, 1982a; DiMatteo, 1982b; Walters et al., 1993; Carlson and Nolen, 1997). Since *Aplysia* spp. obtain their ink pigments and many other defensive chemicals almost exclusively from red seaweed (Winkler, 1961; Winkler, 1969; Irie et al., 1969; Chapman and Fox, 1969; MacColl et al., 1990), they must invest time and energy to find an appropriate food source to replenish their chemical arsenal. Depending on the predatory environment, *Aplysia* spp. should be conservative in their deployment of these valuable chemical resources, and we would expect them to use only that amount of ink necessary to deter a predator.

In simulated tide pool encounters between *Aplysia californica* and anemones, we previously observed *Aplysia californica* releasing variable amounts of ink depending on the kind of interaction it had with a predator (Nolen et al., 1995). In each case, the ink elicited a defensive response in the anemone. More importantly, we observed *Aplysia californica* deploying ink many times in succession as they met the same or a different anemone in the tide pool (Nolen et al., 1995; Johnson, 1994). Furthermore, *Aplysia californica* that had depleted their ink supplies after several encounters with predators were captured and eaten. In addition, we found that ink provided the seahare with its best defense against anemones, which are not deterred by the distasteful secondary plant toxins incorporated into the animal's skin (Nolen et al., 1995): seahares with only a passive chemical defense (distastefulness) were eaten 3.5 times more often than those with only an active chemical defense (ink). Finally, we found that the entire content of a full ink gland (T. G. Nolen and B. Carlson, unpublished observations) is more than adequate

to deter predation by an anemone (Johnson, 1994; Nolen et al., 1995).

These observations (Nolen et al., 1995) seemingly contradict the long-held belief that inking is an all-or-none fixed act. Carew and Kandel (Carew and Kandel, 1977a; Carew and Kandel, 1977b; Carew and Kandel, 1977c) used a noxious electric shock to show that a triad of electrically coupled neurons ($L14_{a–c}$) in the abdominal ganglion triggered inking in an all-or-nothing manner (see also Byrne, 1980). The behavior had a high threshold and a stimulus/response relationship that resembled the step function often attributed to a fixed action pattern (Lorenz, 1953). [Although, according to Eibl-Eibesfeldt (Eibl-Eibesfeldt, 1970, p. 43), the most important considerations are that a fixed action pattern 'is always an inborn, internally coordinated sequence, which merely requires a releasing stimulus'.] Moreover, they found that, once threshold had been reached, the animal tended to secrete almost all (approximately 86 %) of its ink stores in a single episode (Carew and Kandel, 1977a). They concluded that inking has two 'all-or-none' characteristics: (i) the behavior is triggered in an all-or-none fashion (Carew and Kandel, 1974b; Carew and Kandel, 1974c), and (ii) the amount of ink secreted once threshold is reached is all-or-none, i.e. the animal secretes almost the entire content of its ink gland (Carew and Kandel, 1977a). If the animal's gland contains more than enough ink to deter a predator, then secreting almost all of its ink is wasteful of a limited resource. Furthermore, it is counter to our recent observations of interactions between the seahare and its natural predators (see Nolen et al., 1995) as well as to a study (Illich et al, 1994) in which a noxious stimulus triggered ink release and acted to reduce the threshold for subsequent ink secretion.

This study was designed to assess the deployment of ink in two different species in response to natural stimulation as well as that elicited by noxious electric shocks commonly used in studies of learning in *Aplysia* spp. Our goals were (i) to document that *Aplysia* spp. are capable of secreting ink in several sequential episodes; (ii) to determine how quickly a seahare can replenish a completely depleted gland; (iii) to determine how much ink is left in the gland following a suprathreshold inking episode; and finally (iv) to determine how much of the gland's available ink stores are deployed against a potential predator. We found that *Aplysia* spp. may deploy ink in successive episodes (<5 min inter-trial interval) and that it does not necessarily secrete all its ink stores following stimulation by a natural predator or by an electric shock. In addition, this ability is not restricted to just one species or to a benthic or swimming lifestyle. These observations make adaptive sense because it takes days to replenish an empty gland, and the animal need not use all its stores to deter a predator (Nolen et al., 1995). In this light, the nature of the ink's deployment is probably adaptive: the wasteful use of ink is minimized (i) by a trigger mechanism that requires a specific or high-threshold releaser (Carew and Kandel, 1977b; Carew and Kandel, 1977c; Byrne, 1980) and (ii) by a gland that deploys a minimum effective amount of ink against the predator.

Materials and methods

Animals

Laboratory-cultured *Aplysia californica* Cooper 1863 and *A. brasiliana* Rang 1828 were raised from eggs at the NCRR National Resource for *Aplysia* at the University of Miami, Virginia Key, FL, USA. The animals were fed a diet of the red seaweed *Gracilaria tikvahiae* or the green seaweed *Ulva lactuca*. *Gracilaria tikvahiae* was grown at the *Aplysia* Resource Facility; *Ulva lactuca* was obtained from Harbor Branch Oceanographic Institute, Ft Pierce, FL, USA. We transferred seahares from the *Aplysia* Resource Facility to our laboratory at least 1 week before the experiments and held them in 113.6 l (30 gallon) or 189.3 l (50 gallon) recirculating seawater aquaria at 18–22 °C under a 16 h:8 h L:D photoperiod. While in holding conditions, unless indicated otherwise, the animals were fed *ad libitum* every other day. Anemones, *Anthopleura xanthogrammica*, were obtained from Marinus Inc. (Long Beach, CA, USA) and maintained in 189.3 l (50 gallon) recirculating seawater aquaria at 18 °C under a 16 h:8 h L:D photoperiod. Anemones were fed whitefish cubes twice a week.

Quantification of ink release

In several experiments in which we wanted to verify the occurrence of inking behavior (e.g. in sequential trials), we quantified the behavior by using a subjective ordinal score (Krauth, 1988): 0=no ink, 1=very little ink, 2=small amounts of ink, 3=moderate amounts of ink, 4=large amounts of ink and 5=the largest amounts of ink we observed. Ink scores of 3–5 were associated with mantle pumping and escape locomotion, together with ink secretion. Our subjective ink score was positively correlated with spectrometric measures of ink pigment concentrations (see below) in the mantle secretion produced in response to noxious (electric shock) stimulation (Spearman r=0.727, N=35, P<0.0001).

Natural stimulation with anemone tentacles

A. brasiliana (100–200 g) were anesthetized in chilled sea water (2–5 °C) for 20 min before being tethered by the parapodia with five pairs of hooks and suspended in a 36 cm×21 cm×15 cm deep chamber containing 8 l of aerated sea water at 22 °C (Fig. 1A). The parapodia of *A. brasiliana* are quite large and muscular and are specialized for extended periods of swimming (Kandel, 1979). Rarely did such animals ink in response to the tethering procedure. The lateral edge of the ink gland was glued using cyanoacrylate adhesive to a platform and reflected back so that its ventral surface could be positioned under a stereomicroscope. In this way, the secretion of ink from the gland could be observed and photographed (for details, see Prince et al., 1998).

Ink secretion from the ink gland was elicited by stimulating the seahare's tail or head in the following manner. An anemone was placed on a 3 cm×3 cm Plexiglas platform beneath the animal. Each corner of the platform was attached to a nylon line suspended from a threaded nylon bolt on the upper edge of the chamber. The nylon lines suspended the platform off the

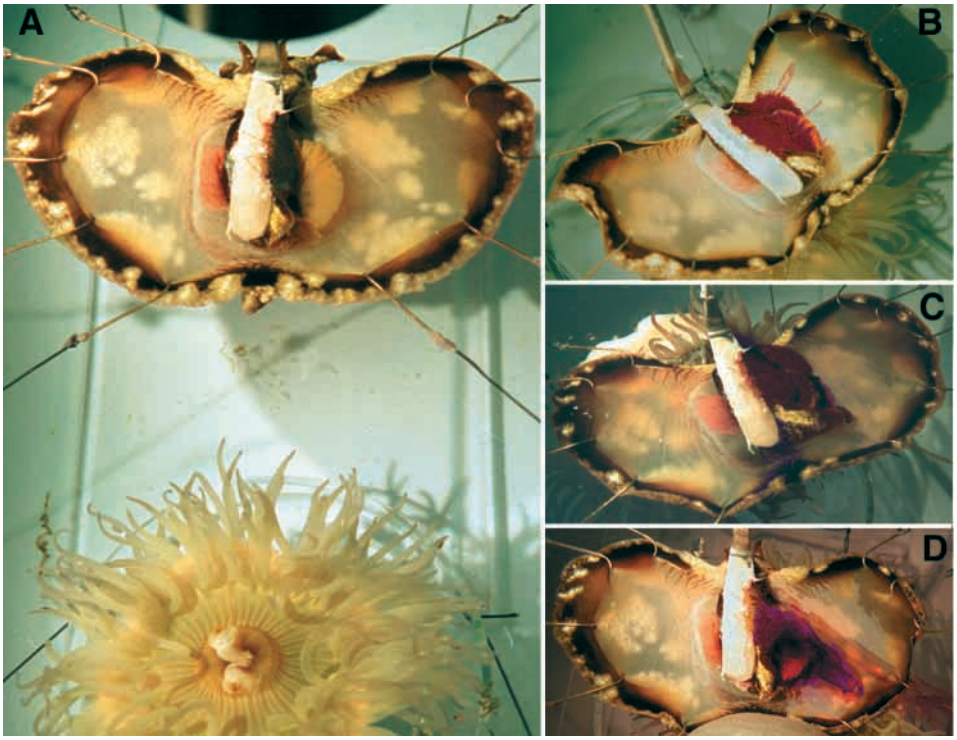

Fig. 1. Multiple episodes of ink release in response to a natural predator. (A) The swimming chamber. A seahare (*Aplysia brasiliana*) is tethered in the chamber. An anemone is visible at the bottom of the picture. (B–D) The response of the seahare to three successive stimulations from an anemone (interstimulus interval 5–10 min). (B) First trial: tail stimulation, ink score 4. (C) Second trial: head stimulation, ink score 4. (D) Third trial: tail stimulation, ink score 2. The photographs show inking behavior within 1 min of the start of ink secretion. Ink was aspirated from the chamber after each trial.

bottom of the chamber and allowed it to be raised towards the seahare above as the bolts were turned. The anemone was allowed to relax its tentacles after transfer to the chamber (usually within 20 min). The platform was then slowly raised until one or more tentacles contacted the seahare (Fig. 1B). This type of stimulation mimics the situation when swimming *A. brasiliana* land on an anemone (T. G. Nolen, unpublished observation). The responses of the seahare and anemone were recorded on video and/or 35 mm film.

Mechanical stimulation: modeling some aspects of predatory attacks

Ink secretion was also elicited using two different mechanical stimulators, a focal sucker that delivered a moderate vacuum and an array of smaller suckers.

A focal suction device (termed 'Sucker' in Fig. 4) was used to lift the seahare off the substratum as follows. A 6 l aquarium was filled with approximately 4 l of sea water from the animal's holding tank. The bottom was covered to a depth of 2 cm with medium-sized gravel. An animal (50–100 g) was placed in the tank and allowed to acclimate for 5–10 min. A 5 ml syringe with a Teflon probe tip (0.8–0.9 mm inner diameter) was held against the middle of the parapodium using a micromanipulator. A vacuum pump delivered 56 Pa of negative pressure to the parapodium. The seahare then was lifted off the bottom of the tank and lightly jostled for up to 60 s or until it inked. This action mimicked some of the prey-capture behavior of an anemone or a crab or lobster (i.e. grabbing and lifting).

A suction array consisting of either a single element (covering 12.6 mm^2) or a 3×3 array of identical suckers arranged in a square covering 113.1 mm^2 was used to lift the animal off the substratum. In each case, a sucker consisted of a 1 ml Tuberculin syringe (without plunger) connected *via* Tygon tubing to a second 1 ml syringe (with plunger), which was used to apply suction. Each of the syringes of the 3×3 array was activated simultaneously by a specially built holder, and each delivered the same amount of vacuum. Suction was applied by pulling the plungers of the remote syringes out by 0.3–0.5 ml and holding for up to 150 s. (We could not measure the vacuum delivered, but it was probably considerably smaller than that used with the focal suction device described above.) For both the single syringe and the 3×3 array, we used the same procedure as with the focal sucker (see above) except that the vacuum was increased stepwise as follows: 0.3 ml for 60 s, then 0.4 ml for 60 s and then 0.5 ml for 30 s.

Noxious stimulation by electric shock

Ink secretion was also elicited by electric shocks delivered to the tail or neck using a paired capillary stimulating electrode (a pair of silver/silver chloride wires separated by 1.5 cm and each enclosed in a 1 cm diameter×1 cm length of seawater-filled Tygon tubing) similar to that used by Marcus et al. (Marcus et al., 1988). The amount of current delivered during stimulation was monitored with a custom-built current monitor. We also monitored the electrical resistance of the circuit to ensure that a good electrical seal against the seahare's body was achieved when delivering the shock; results from experiments in which the electrical resistance dropped suddenly (indicating the loss of electrical contact between the electrode and the body wall) were discarded. Noxious stimuli consisted of a.c. shocks of 1–2 s duration (60 Hz) and 2.5–120 mA (see below). In some experiments, to control for

the possibility that mechanical stimulation elicited inking, the electrode was placed on the animal but no current was applied; the occurrence of inking behavior was monitored for 90 s (see Touch in Fig. 4). Similarly, we controlled for the possibility that handling elicited inking (Kicklighter et al., 1992) by allowing animals 30–60 min to acclimate in the chamber undisturbed before starting the experiment. In addition, in some experiments, we omitted the use of the shock electrode while maintaining all other actions necessary to carry out the stimulation and recording of behavior; this control was to ensure that other stimuli associated with the procedure were not causing ink secretion (termed None in Fig. 4).

Quantifying ink reserves in the gland following noxious stimulation

Two groups of animals (*A. californica*, 75.6±5.8 g, mean ± S.E.M., *N*=15) were tested for ink secretion in response to noxious shocks. One group had been kept on a diet of dried red seaweed (nori, obtained from a local health food store) for 1 week following de-inking (see below). The other group remained on its diet of fresh red seaweed (*Gracilaria tikvahiae*) for 1 week after de-inking. We elicited ink secretion by applying a strong shock to the neck with the paired capillary a.c. electrode (as described above) for four trials at a 10 min inter-trial interval. The current intensity was twice the threshold value (based on a 50 % population inking criterion; see Carew and Kandel, 1977a) (and see Fig. 4). This current intensity (100–120 mA for 1.5 s) was sufficient to elicit inking in approximately 83 % of the animals when applied to the tail (strong shock in Fig. 4) or 80 % when applied to the neck (see Fig. 5). Our shock stimulus was presumably noxious because it caused tissue damage in repeated presentations (depigmentation was the overt sign of this damage). In a previous study, 100 mA shocks from such an electrode were sufficient to cause sensitization in similarly sized *Aplysia californica* (Marcus et al., 1988).

The animals were held individually in beakers in 500 ml of fresh artificial sea water (Instant Ocean) at 20 °C. The seahare sat on a small platform situated above a magnetic stir bar that was used to mix the secreted ink in the sea water. An 8–10 ml sample of the mixed ink was collected after each trial, and relative ink concentration was calculated from the absorbance (B&L Spec80) of samples at two characteristic wavelengths (500 nm and 565 nm) for the main ink pigment, r-phycoerythrin (MacColl et al., 1990). To ensure that the sample's pigment concentration was below saturation for the spectrophotometer, we ran a dilution series and verified a linear relationship between dilution factor and absorbance. Ink samples were read against a seawater blank. At the end of the experiment, the animal's gland was hand-expressed, which generally depleted the gland of releasable ink (Nolen et al., 1995; see also Chapman and Fox, 1990; but see Discussion below). We ensured that all the ink secreted into the mantle cavity was expelled into the surrounding sea water by waiting until the vigorous pumping actions associated with inking emitted no more ink. Finally, we ensured that the final hand expression was effective by assaying the animals' residual ink content using the paper towel test (see below).

The relative ink concentration of a sample was determined at both wavelengths and then averaged to obtain an individual score for each animal in each of the four trials plus the hand expression. We corrected our concentration determinations for the volume of samples we took from the 500 ml (initially) of sea water in the animal's beaker. The total initial ink content of the gland was then calculated as the sum of ink released on each of the four trials plus that released in the final hand expression. Two different relative measures of ink secretion were calculated: (i) as a percentage of the gland's total initial ink content (i.e. the total ink secreted in the experiment, see Carew and Kandel, 1977a); and (ii) as a percentage of the gland's ink stores available for release in a given trial (i.e. the amount of ink remaining in the gland). The amount of ink remaining in the ink gland after a stimulus trial was calculated by subtracting the amount of ink secreted on that trial from the amount remaining before the trial. The animals were stimulated, and ink samples were collected and analyzed using a blind procedure so that the experimenter did not know the animal's group identity (i.e. diet).

Ink replenishment experiments

Adult *A. californica* (181.35±8.8 g, mean ± S.E.M., *N*=40) raised on the green seaweed *Ulva lactuca* from stage 11 (<0.5 cm; Kriegstein, 1977) were randomly assigned to one of five groups (*N*=8 each). Each animal was de-inked by handling (Nolen et al., 1995) and placed on a diet of *Gracilaria tikvahiae* (red seaweed diet) or maintained on the green seaweed diet (control). These two diet groups were housed in separate 189.3 l (50 gallon) tanks and provided with fresh seaweed *ad libitum* each day. Since the groups were housed communally for practical reasons, we do not know how much each individual ate each day, but consumption in the tanks was between 25 and 50 g animal^{-1} day^{-1}. This level of feeding was adequate to support growth over the duration of the experiment: on average, the animals were 15.95±2.61 % (mean ± S.E.M., *N*=40) heavier than at the start of the experiment (one sample *t*-test; null hypothesis, 0 % growth; t_{39}=6.1093, P<0.0001).

We subsequently tested each animal for its ability to secrete ink by wrapping it in a coarse paper towel for up to 120 s. Pilot studies indicated that a paper towel covering the foot and both parapodia is a potent stimulus, reliably eliciting inking in 100 % (*N*=40, see Fig. 4) of the animals tested (Nolen et al., 1995). This test was especially sensitive because we could directly observe the ink gland in the mantle cavity during stimulation to monitor ink secretion. One group was tested 1 day after the start of the red seaweed diet; the other groups were tested 3, 5 and 11 days after the start of the red seaweed diet. Ink secretion was scored on an ordinal scale (as described above). Previously, we had found that *Aplysia californica* is capable of extracting small amounts of red and blue pigments from the blue-green algae that were always present in our tanks (Prince et al., 1998). Since green-seaweed-fed seahares

sometimes released a small amount of ink (mean ink score 1.125±0.12), we compared the ink scores of the red-seaweed-fed seahares with those of the green-seaweed-fed control group to ensure that we were determining the time course of red-seaweed-based ink production. Therefore, a statistically significant difference in ink scores (with red-fed>green-fed) was interpreted as evidence that seahares had extracted pigments from the red seaweed and produced releaseable ink from that source. Threshold was determined as the duration of paper towel stimulation (in seconds) required to trigger ink release, as observed directly from the ink gland.

Statistical analyses

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. Kruskal–Wallis tests) instead (Krauth, 1988). Unless indicated otherwise, all significance levels reported are two-tailed, and values reported are means ± S.E.M. Statistical tests were performed with InStat 2.03 for Macintosh (GraphPad Software) or with GB-Stat PPC 6.5.2 (Dynamic Microsystems, Inc).

Results

Multiple inking episodes

We first examined the ability of a natural stimulus (anemone tentacles) and of a mechanical model of predatory attacks (the lifting sucker devices, see Materials and methods) to elicit multiple episodes of inking. We tested two different species, *A. brasiliana* and *A. californica*, in situations in which they might meet anemone predators. *A. brasiliana* is capable of swimming and can land on anemones at the end of a swimming bout and may be eaten if snagged (Tobach et al., 1980). We investigated the ability of anemone tentacles to trigger inking in *A. brasiliana* when it was suspended in the swimming chamber. *A. californica* do not swim and are likely to meet anemones or other predators such as crabs and lobsters only when walking about (see Nolen et al., 1995; Pennings, 1990; DiMatteo, 1982a; DiMatteo, 1982b). Because the anemone must grab and then pick up the snail, we could not easily stage multiple trials with walking *A. californica* using live anemones. Therefore, we chose the mechanical stimulus as an approximation of the action of the predator (i.e. grabbing and lifting). We tested *A. californica* with the mechanical (sucker) stimulators (see below) while it was walking on the substratum. We compared these more 'natural' releasing stimuli with noxious shock to the neck or tail (see below and Fig. 4).

Natural stimulation in the swimming chamber

Contact between an anemone tentacle and the tail or head of *A. brasiliana* suspended in the chamber resulted in ink secretion from individual ink storage/release vesicles throughout the ink gland, as observed under the dissection microscope (Fig. 1B–D). Swimming was also elicited (>90 % of the time) if the seahare was not already swimming. Successive stimulation with the anemone produced substantial ink release (inking scores of 2–4) in each trial (Fig. 1B–D).

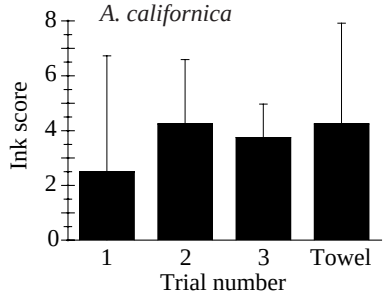

Fig. 2. Multiple episodes of ink release in response to a mechanical stimulus. Ink scores of *Aplysia californica* (means + 95 % confidence interval, *N*=7) when stimulated three times (trials 1–3) with the 'focal sucker' device (see Materials and methods) and lifted off the substratum for 60 s. After the third sucker stimulus, the animals were assessed for further inking ability with the paper towel test (see Materials and methods).

Since the nematocysts of *Anthopleura xanthogrammica* are not armed with neurotoxins (Hyman, 1967), such (noxious) chemicals cannot be necessary to release inking behavior in the seahare. Rather, the mechanical nature of the tentacles grabbing the seahare's body wall seems to be the salient feature of the releaser (see below).

Mechanical stimulators

Focal sucker. Grabbing the parapodium and lifting the seahare off the substratum (see Materials and methods; Fig. 4, Sucker) caused slowly walking or standing *A. californica* to secrete substantial amounts of ink (ink scores of 3–4) in 77 % of the animals (*N*=13). Successive stimulations (10 min inter-trial interval) with a suprathreshold grabbing/lifting stimulus triggered ink release in subsequent trials (Fig. 2). The high ink scores (approximately 4) in response to the final (paper towel) stimulus indicated that substantial amounts of ink were still present even after the third mechanical stimulation (Fig. 2).

Sucker array. When a suction device of either one or nine tuberculin syringes (see Materials and methods) was placed on the parapodium and weak suction applied as the animal was lifted off the substratum, the probability of inking increased as a function of the number of syringes (i.e. of the area of skin stimulated). Table 1 shows that 28.6 % of the animals secreted ink in response to weak suction applied to 12.6 mm^2 of skin through one syringe, whereas 64.7 % secreted ink in response to the 3×3 array distributed over 113.1 mm^2 of skin. Of those animals releasing ink, there was no difference in ink scores between the single and 3×3 array (P>0.5, Mann–Whitney U-test).

In general, the sucker stimulus alone did not trigger inking (i.e. when first applied to the seahare's parapodium). Only after the animal had been lifted did it (eventually) ink.

Table 1. *Probability of ink secretion as a function of the area stimulated by a sucker array*

Stimulus	Behavioral outcome		
	Ink	No ink	Total
Single	4 (28.6 %)	10 (71.4 %)	14
3×3 array	11 (64.7 %)	6 (35.3 %)	17

Fourteen *Aplysia californica* (weighing 50–100 g) were stimulated with the single syringe sucker and 17 were stimulated with the 3×3 sucker array (see Materials and methods).

The number (and percentage) producing ink or not producing ink are recorded.

A Fisher exact test (one-sided) showed a significant difference between stimulus types (P=0.0475).

Noxious shocks in the swimming chamber

A strong shock to the tail or head of *A. brasiliana* suspended in the chamber caused ink release from the ink gland. Multiple stimulus trials with a moderately strong shock (45 mA) resulted in substantial ink release in each successive trial (Fig. 3). (Multiple shocks were also effective releasers in walking *A. californica*; see below.)

Quantification of ink deployment

A strong shock to the tail of *A. californica* while it was walking slowly or standing produced inking in 83 % of those tested (Fig. 4). Weaker stimuli caused proportionately fewer animals to ink: a weak shock (2.5 mA) or simply touching the animal with the electrode elicited inking in less than 5 % of the animals (Fig. 4). Approximately half produced inking behavior in response to a shock of 50 mA. Inking behavior may have involved tail withdrawal, escape locomotion, mantle pumping and inking. Sometimes, all the defensive behaviors associated with inking were produced, but no measurable ink was secreted (T. G. Nolen and P. M. Johnson, unpublished observations). Our ink score only considered ink secretion. On a population level, the 50 mA stimulus represents threshold for a 50 % response criterion (see Carew and Kandel, 1977a).

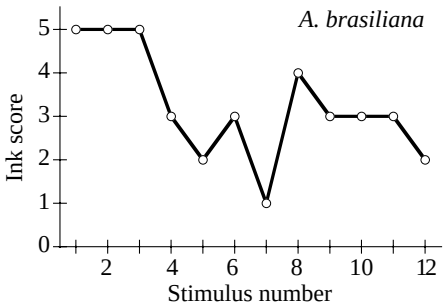

Fig. 3. Multiple episodes of ink release in response to an electric shock. An example of an individual *Aplysia brasiliana* in the swimming chamber that secreted ink in 12 successive trials (stimulated on the neck with a medium-intensity shock; 45 mA, inter-trial interval 5 min). This particular animal had an unusually good supply of ink (most were depleted in 4–6 trials).

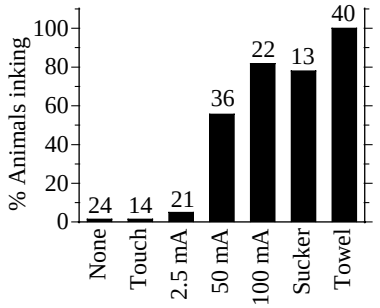

Fig. 4. Various stimuli can elicit inking behavior in *Aplysia californica*. Weak shocks (2.5 mA) did not elicit significant inking, nor did simple mechanical stimulation with the electrode (Touch). Seahares never inked spontaneously (None). Only intermediate to strong shocks (50 mA and 100 mA) and the focal (Sucker and Towel) stimuli caused a significant incidence of inking. The latter two stimuli were as effective as a strong shock. Animals were tested while they stood still or walked slowly; they were tested once with one stimulus. In total, 170 animals (mass 60–120 g) were used in this comparison. Values of N are given above the columns.

Successive noxious shocks to the neck of *A. californica* while the animal was walking or standing elicited multiple episodes of ink secretion. As shown in Fig. 5A, the first stimulus released approximately 40–60 % of the total amount of ink contained in the gland initially; successive stimulation elicited less ink, but the amount was between 10 and 20 % of the initial content of the gland (Fig. 5A). There was no significant effect of diet (e.g. fresh *versus* dried red seaweed) on the amount of ink release in successive trials (Fig. 5A) or on the total amount of ink the animals secreted during the experiment (total ink secreted, P>0.39, one-way analysis of variance, ANOVA, $F_{6,9}$=0.788), so we combined the results for the two diet groups and calculated the amount of ink remaining in the gland (relative to the initial content) after each stimulus (Fig. 5B). The first stimulus left 48 % of the initial stores in the gland (Fig. 5B). By the last trial, 20 % of the initial stores remained (Fig. 5B). Furthermore, the animals secreted approximately 30–50 % of the available ink present in their glands on a particular trial (see Materials and methods) (Fig. 5C). Thus, the amount of ink secreted was consistently less than 80–90 % of the gland's available content even in later trials (trials 2–4, Fig. 5C) when the gland was partially depleted.

We determined that hand expression was a relatively effective method of estimating ink reserves by including a paper towel test at the end of the experiment. For the whole group, the amount of secreted ink as a result of hand expression accounted for 15.7±7.3 % (N=12) of the total ink released during the experiment. Only three animals released ink in response to the paper towel (with low ink scores, between 1 and 2), indicating that hand expression of the gland after four shocks was generally effective in depleting the animal of releaseable ink. However, for the seahares that did secrete ink in response to the paper towel, the amount of ink released by

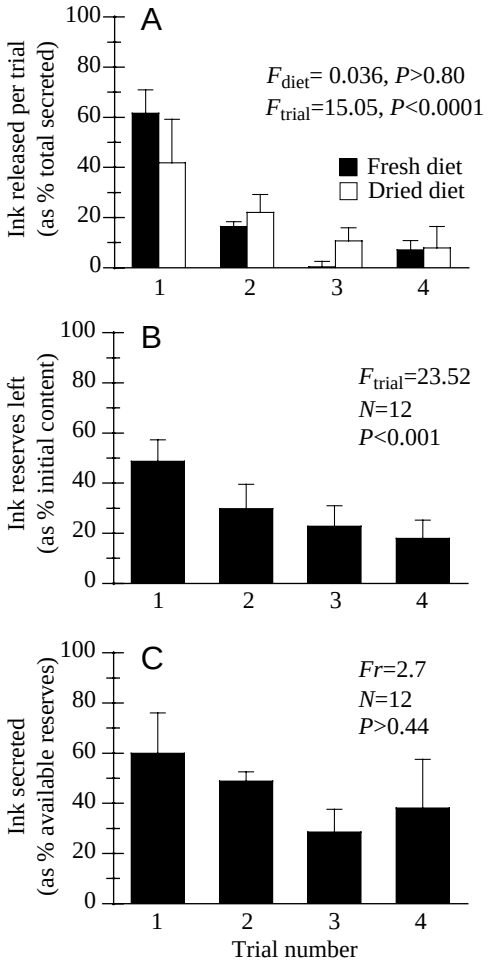

Fig. 5. Spectrophotometric quantification of ink secretion. *Aplysia californica* (46–119 g, N=15) were stimulated in four trials with a 1 s a.c. shock (120–121 mA) to the neck (inter-trial interval 10 min; see Materials and methods). The amount of ink secreted was measured spectrophotometrically. (A) The amount of ink secreted in each trial as a percentage (means + S.E.M.) of the ink gland's initial total reserves (see Materials and methods) for the two diet groups (fresh diet, N=9; dried diet, N=6). A two-factor repeated-measures ANOVA indicated that significantly less ink was secreted in later trials (F_{trial}=15.05). However, there was no effect of diet (F_{diet}=0.036) on the amount of ink secreted. Of the 15 animals tested, 12 % inked in trial 1. Those that did not ink were not included in the statistical tests (or in this figure). (B) The amount of ink remaining in the gland (as a percentage of the initial ink content) at the end of each trial (means + S.E.M.). There was a significant downward trend as a function of trial number (F_{trial}=23.53, P<0.001, N=12). (C) The percentage (means + S.E.M.) of the gland's reserves secreted as a function of trial number. A Friedman non-parametric repeated-measures ANOVA indicated no effect of trial number (Fr=2.7, P>0.44, N=12).

hand expression accounted for 45.8±18.1 % (N=3) of the ink they secreted during the experiment compared with 5.7±4.5 % (N=9) for the subset that did not respond to the paper towel. So, seahares that had residual ink in their glands after hand expression (i.e. those that subsequently inked in response to the paper towel) also contained relatively large amounts of ink before hand expression. These observations suggest that, when the gland is relatively full (e.g. after trial 1, Fig. 5), hand expression leaves some residual ink. Since our animals had been induced to release ink four times before hand expression, most (9 out of 12) had depleted glands by the time of the paper towel treatment. Thus, our estimates of total ink gland content are probably slightly low rather than too high.

Replenishment of the ink gland

Aplysia spp. process red seaweed in their digestive diverticulae, where they extract phycobilin pigments from the rhodoplasts of the red algal cells. They then incorporate the pigments, along with newly synthesized proteins, into ink storage/release vesicles in the ink gland (Coelho et al., 1998; Prince et al., 1998). This metabolically active process should require substantial time to make enough ink for use in defense. We examined the time it takes a purple-ink-depleted seahare to produce releasable ink stores once it starts feeding on red seaweed.

Animals raised from juvenile stage 11 to adulthood (>50 g) on the green alga *Ulva lactuca* do not release significant amounts of purple ink when disturbed (Nolen et al., 1995; also see Chapman and Fox, 1969; MacColl et al., 1990). When switched to a diet of red algae (*Gracilaria tikvahiae*), only 50 % of the animals released any ink (ink scores ⩾1) after 1 day on the red seaweed diet (Fig. 6A), and their mean ink scores were not significantly different from those of controls (freshly de-inked animals on a green seaweed diet; see Materials and methods). Only after the third day of the red seaweed diet were ink scores statistically significantly different from those of the green-seaweed-fed controls (Fig. 6A). The amount of ink secreted increased with the number of days the animal was fed the red seaweed diet compared with the green-seaweed-fed controls (Fig. 6A). Moreover, all animals (8/8 in each group) secreted measurable ink (ink scores ⩾1) after 3 days on the red seaweed diet (Fig. 6A).

The threshold amount of stimulation (in s) necessary to cause inking decreased with the number of days the animal had been fed the red seaweed diet (Fig. 6B). Regardless of the number of days on the red seaweed diet, there was a negative correlation between ink score and threshold (Fig. 6C). Thus, animals with low stores and those secreting small amounts (low ink scores) had relatively high thresholds. However, the unusually high threshold for an ink score of 1 (approximately 64 s; Fig. 6C) and the relatively low threshold for higher ink scores (9–18 s) suggest that, when ink stores are low (and therefore ink scores at their lowest, e.g. 1 in this experiment), thresholds are at their highest. But once a gland has even partially replenished its stores (day 5 onwards), its threshold is relatively low regardless of the amount of ink it releases (Fig. 6C).

Discussion

When a predator captures prey such as *Aplysia* spp., it first must grab the animal and then manipulate it to its mouth.

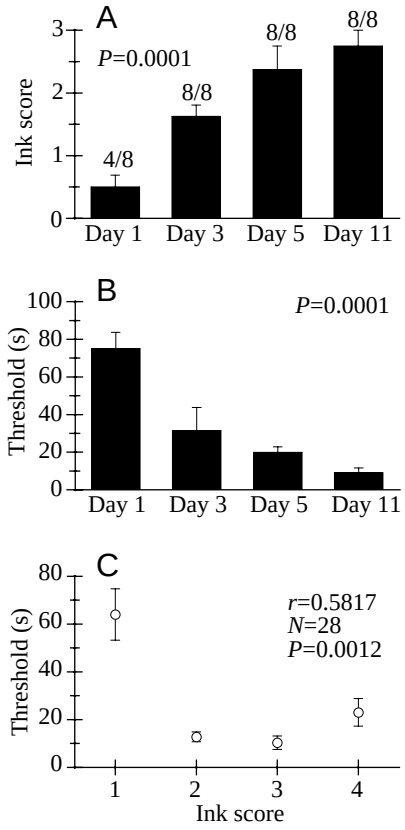

Fig. 6. The replenishment of the ink gland requires several days on a red seaweed diet. Forty *Aplysia californica* (51–297 g) raised on *Ulva lactuca* from stage 11 of juvenile development (<0.5 cm) were de-inked by hand and assigned to one of five groups (N=8 in each). Four groups were then fed on a diet of fresh *Gracilaria tikvahiae* while the fifth group remained on the diet of *Ulva lactuca* (as a control). Each group was then tested once for inking ability with the paper towel test (see Materials and methods) 1, 3, 5 or 11 days after de-inking (the control group was tested on day 0). (A) Ink scores for the four red-seaweed-fed groups. Ink scores increased as a function of the amount of time the seahare had been on the red seaweed diet (Kruskal–Wallis non-parametric ANOVA, KW=31.5, P<0.0001). Only animals feeding for three or more days on red seaweed after de-inking secreted more ink than green-seaweed-fed controls (Dunn's multiple-comparison tests for day 1, Diff=5.00, P>0.05; day 3, Diff=16.56, P<0.05; day 5, Diff=22.19, P<0.01; day 11, Diff=26.25, P<0.001; all comparisons *versus* green-seaweed-fed controls). The proportion of animals inking for each group is shown above each column, although all were included in the calculation of mean ink scores. Values are means + S.E.M. (B) Thresholds for inking (in seconds of paper towel stimulation) for each of the four red-seaweed-fed groups. Thresholds for ink release decreased as a function of time on the red seaweed diet (Kruskal–Wallis non-parametric ANOVA, KW=13.26, P<0.004). Values are means + S.E.M. (C) Inking thresholds were negatively correlated with ink scores (r is the Pearson non-parametric correlation coefficient). Values are means ± S.E.M.

Previously, we observed that a seahare's encounter with a single anemone tentacle could elicit the secretion of a small amount of ink; large ink secretions occurred whenever several tentacles grabbed the seahare and lifted it towards the predator's oral disc (Nolen at al, 1995). Similarly, when a crab or spiny lobster grabs a seahare, it surrounds the prey with its legs and pulls it to its mouth (T. G. Nolen, unpublished observation; B. Carlson and T. G. Nolen, in preparation). Crabs rarely grab the snail with their chelae; they usually employ their mouthparts and legs. Spiny lobsters have no enlarged chelae and necessarily use their mouthparts and walking legs (T. G. Nolen, personal observation). Thus, important features of the releasing stimulus for inking could include distributed tactile stimulation over the animal's body and loss of contact of the foot with the substratum. We do not know whether a high-threshold stimulus, such as an electric shock (or a pinch), or a specific configurational releasing stimulus, such as that produced by a predator grabbing the body and lifting, is more effective (Nolen et al., 1995). However, our sucker stimulators, modeling grabbing and lifting, were as effective as strong, noxious shocks (compare the 100 mA shock and Sucker in Fig. 4). In addition, we have found that ink secretion is much more likely to result from a barehanded investigator picking up the animal than from a poke, pinch or jab (with sharp forceps) or even a weak electric shock to the tail (Johnson et al., 1993). This suggests that more distributed mechanical stimuli (i.e. covering much of the animal's body, as happens when the animal is picked up by an experimenter or by a crab, lobster or anemone) are more effective releasers of inking behavior (Johnson et al., 1993; S. Robinson, personal observation). For example, our 3×3 sucker array distributed over the seahare's parapodium was more effective than a single sucker delivering the same negative pressure (Table 1; S. Robinson, unpublished data). We conclude from our behavioral studies with natural predators and with mechanical models of predator stimulation (Figs 1–3; Johnson et al., 1993; Nolen et al., 1994) that noxious stimulation *per se* (i.e. sufficient to cause tissue damage) is not necessary to elicit inking, although it may be sufficient (Fig. 4; Nolen et al., 1995).

Ink deployment

We have found that *Aplysia californica* and *A. brasiliana* do not necessarily secrete all the ink in their gland, as suggested by earlier work. This was shown in our experiments with a tethered seahare that we could stimulate repeatedly with an anemone (Fig. 1): such stimulation elicited many episodes of inking within minutes of each other. Noxious shocks to the neck were also effective in eliciting multiple inking episodes in both species (Figs 3, 5). Furthermore, our sucker stimuli were also capable of releasing multiple inking episodes (Fig. 2). These results show that, irrespective of the locomotory situation (walking, swimming) or the type of stimulus (natural anemone tentacles, mechanical models or noxious electric shocks), both these species of *Aplysia* are capable of secreting ink in several sequential episodes.

We also found that 48 % of the animal's initial ink stores remained after a first suprathreshold shock and that each successive stimulation caused the animal to secrete 30–50 % of its available stores (Fig. 5). Since it takes the animal several

days of feeding on red seaweed to substantially replenish its ink gland (Fig. 6A; Chapman and Fox, 1969), none of these results can be due to replenishment of ink stores between stimulations (i.e. within the 10 min inter-trial interval). In addition, these observations hold for two different species of *Aplysia* with different behavioral ecologies (*A. californica* is benthic, while *A. brasiliana* can swim), but with historically similar types of predation (Nolen et al., 1995).

Our results seemingly contradict the original findings of Carew and Kandel (Carew and Kandel, 1977a), who first described the 'all-or-none' characteristics of this behavior. In particular, they showed that strong electric shocks to the neck triggered inking in an all-or-none fashion. Even stimulation at twice and four times the threshold current did not elicit larger (or subsequent) ink secretions (Carew and Kandel, 1977a). Moreover, they calculated that the animal used more than 86 % of its releasable stores in response to a suprathreshold shock and therefore concluded that the amount of ink released relative to the gland's stores was 'all-or-none' (Carew and Kandel, 1977a).

We have considered several possible explanations for the discrepancy between our results and these earlier experiments. First, like ours, the calculations of Carew and Kandel (Carew and Kandel, 1977a) of the amount of ink released from the gland involved measuring the residual amount of ink expressed following hand manipulation of the gland. In our experiment, after the first suprathreshold stimulus trial, the seahares still had nearly 50 % of their gland's reserves intact (Fig. 5B). Moreover, those with substantial ink reserves at the end of four trials also had measurable ink remaining after hand expression, as shown by the subsequent paper towel stimulation (see Results). A *post-hoc* analysis of these data suggested that, when the gland is relatively full, hand expression will leave some residual ink. This was seen in the cases of the three seahares releasing ink in response to the paper towel treatment: hand expression released almost 46 % of the ink secreted in the entire experiment up to that point, indicating that the gland was relatively full even after four previous inking episodes. Carew and Kandel (1977a) used only one suprathreshold shock: if substantial ink remained after their stimulus (as it did after our first shock, see trial 1, Fig. 5B), then their hand expression probably left some ink. Therefore, it is possible that, rather than overestimating the gland's total initial (maximum) releasable content as they believed they had, they would have underestimated it. If so, their calculated amount of ink released would have been less than 86 % of the gland's initial content.

Originally, we anticipated that some of the differences between our earlier results and the original findings of Carew and Kandel (1977a) could be due to differences in diet that might have led their animals to have low ink stores and to deplete their gland on the first trial. This possibility was considered initially because, unlike our animals, which are fed a diet of fresh red seaweed, most laboratory-held animals in the past were fed dried red seaweed (or other dried seaweeds or romaine lettuce, if they were fed at all), and they could have had reduced ink pigment stores. (We find that animals do not eat dried seaweed as readily as fresh seaweed.) In addition, we knew that animals with low ink stores could have high thresholds (Fig. 6B) and might secrete a larger proportion of their (small) reserves. However, we found no statistically significant difference between our two diet groups ($P>0.80$, Fig. 5A), and there was no evidence that our dried-seaweed-fed seahares had lower ink stores (the total amount of ink released during the experiment was not significantly different for the two diet groups, Fig. 5). Undoubtedly, in some cases (e.g. trial 4, Fig. 5A), the amount of ink secreted depletes the gland in a given trial. But for relatively full glands, several episodes of inking are apparently needed to deplete the gland (e.g. see Fig. 2). Indeed, Carew and Kandel (1977a) state that the amount of ink released in response to their first suprathreshold shock obscured the animal. Such a description of inking in a volume of 400 ml suggests an ink score of at least 3 and, therefore, a relatively full gland initially.

A third potential source of disparity between our findings and those of Carew and Kandel (1977a) is the possible difference between laboratory-reared animals (ours) and the wild-caught animals of the earlier studies. For example, if wild-caught animals have experienced predation, then they may respond differently from naive laboratory-reared animals. In addition, there were differences in age/maturity/size of the experimental animals in the two studies: our animals were smaller and probably younger (smaller animals are more susceptible to predation; Pennings, 1990) and may been more conservative in their use of ink as a defense. However, wild-caught animals have been observed to ink several times in succession (T. G. Nolen, unpublished observation; Illich et al., 1994), so it is not clear whether this explanation is the most likely one for the difference between our study and that of Carew and Kandel (Carew and Kandel, 1977a). Clearly, more work needs to be done to resolve the discrepancies between these experiments. In any case, our observation that the amount of ink secreted is not necessarily all-or-none and the possibility that different rearing conditions, handling or other experience (e.g. see Illich et al., 1994) affect inking behavior is of significance to future studies.

Replenishment of the gland

Aplysia spp. obtain their ink pigments (e.g. phycoerythrobilin) from the accessory photosynthetic pigments of the red algae in their diet (MacColl et al., 1990). Previously, we confirmed the results of Chapman and Fox (Chapman and Fox, 1969) by showing that animals kept on a non-red-seaweed diet do not secrete substantial amounts of purple ink (Nolen et al., 1995). Our results reported here show that secretion of purple ink is not possible until several days after the start of a red seaweed diet. Similarly, Chapman and Fox (Chapman and Fox, 1969) found that seahares raised on red seaweed and then completely de-inked required 3 days on a red seaweed diet to replenish their ink stores sufficiently to release observable ink.

We chose the paper towel method to assess inking ability because it reliably triggers the behavior even when a very

strong shock does not (compare the 100 mA shock with the Towel results in Fig. 4). In addition, application of the paper towel allowed us to deliver a constant level of stimulation and conveniently record stimulus duration as a measure of threshold. This was important because Shapiro et al. (Shapiro et al., 1979) found that ink release was more likely the longer the duration of a noxious (shock) stimulus to the head. Our threshold measure is thus a reasonable one on the basis of our present understanding of the ink trigger mechanism (Byrne et al., 1979; Byrne, 1980). In addition, this technique allowed us to be fairly certain when even small amounts of ink were secreted because we could directly observe the ink gland in the mantle cavity by pulling back the parapodia (which stick to the paper towel). Despite the potency of the paper towel stimulus for normal animals, when ink stores were low (i.e. 1 day after the start of a red seaweed diet, Fig. 6A), the paper towel elicited inking in only 50 % of the animals (for up to 120 s of stimulation). Moreover, the amounts of ink secreted were small (ink scores of 1, which typically is a tiny dribble of ink) and the thresholds were high (an average of 75 s of stimulation was necessary to trigger inking behavior, Fig. 6B). In contrast, when ink stores were larger (i.e. after 11 days on the red seaweed diet), only 9 s of stimulation was required to trigger the secretion of a moderately large amount of ink (ink scores of 2–3; Fig. 6).

These results suggest that inking thresholds may vary as a function of recent experience (see Illich et al., 1994), diet and the state of the ink gland. They further suggest that inking can be a low- or a high-threshold behavior (see also Shapiro et al., 1979). However, Illich et al. (Illich et al., 1994) found that, with noxious stimulation (which itself elicited ink secretion), inking triggered by a shock to the tail or parapodium sensitized (measured as a decrease in the threshold current), even though the gland had been partially depleted. This is not necessarily contrary to our observations of a six- to sevenfold higher threshold for nearly depleted glands compared with less-depleted glands (e.g. day 1 *versus* day 11 animals in Fig. 6B or ink scores of 1 *versus* 2 or more in Fig. 6C). In Fig. 6C we see that, while the threshold is very high for ink scores of 1 (i.e. a nearly depleted gland), it is low and relatively constant for ink scores between 2 and 4 (which would be typical of the inking produced in our Fig. 5 or, perhaps, in Illich et al., 1994). It is possible that the reduction in threshold in the sensitization experiments of Illich et al. (Illich et al., 1994) was within the range of thresholds we found for ink release from full to moderately full glands.

Inking release mechanism

We found that *A. californica* consistently secrete approximately 30–50 % of the ink present in their gland in any particular trial (Fig. 5C). Thus, even after the gland had been substantially depleted (i.e. by trials 2–4; Fig. 5), the animals did not secrete all their remaining stores. This mode of release (30–50 %) is consistent with a trigger mechanism (Carew and Kandel, 1977b; Carew and Kandel, 1977c; Byrne et al., 1979) that activates a relatively fixed number of ink storage/release vesicles (Prince et al., 1998) irrespective of their state of fullness. As long as vesicles are randomly activated and do not release all their contents when so activated, the amount of ink secreted would be a function of a fixed proportion of the gland's vesicles. Previously, we found that individual vesicles within the gland are stochastically activated on successive trials (Prince et al., 1998). Moreover, individual ink vesicles do not release all their contents, but may be activated several times to release graded amounts of ink (Johnson et al., 1993; Prince et al., 1998). In addition, we found that the amount of ink released from individually isolated vesicles is a function of the concentration of acetylcholine, the presumed neurotransmitter (Prince et al., 1998). Such a random mode of vesicle activation can result from (i) a network of highly branched ink motoneuron terminations on a large number of vesicles, and (ii) relatively common branch point conduction failures throughout the motoneuron axonal array. The high discharge rate of the three identified ink motoneurons ($L14_{a,b,c}$; Carew and Kandel, 1977b) could result in only a small number of action potentials reaching any presynaptic terminals, thus causing weak activation of the vesicle and only partial release. Preliminary anatomical studies of the ink gland (J. Prince and T. G. Nolen, unpublished results) suggest that the ink motoneuron terminal branches are indeed quite small ($\leqslant 1\,\mu m$ in diameter), numerous and distributed throughout the gland. Branch point conduction failures (Baccus et al., 2000) are more common in such small axons and would be more common with high-discharge bursts of action potentials in the ink motoneurons (but see Cox et al., 2000). We are currently modeling such a possibility to account for the relatively constant proportion of vesicles activated during ink secretion.

Economy of use: adaptive deployment of a limited resource

Because ink provides *Aplysia* spp. with such an effective anti-predator defense (Nolen et al., 1995; Carlson and Nolen, 1997), secreting all its ink stores is not only wasteful of a limited resource but also makes the animal more susceptible in subsequent encounters with predators. This is especially true for anemones because the seahare's other (passive) chemical defense (its distasteful secondary plant toxins) is less effective against predators that can regurgitate the most toxic components of their partially digested prey (Nolen et al., 1995). Similarly, crabs and lobsters are able to deal with the seahare's passive chemical defense by rasping the body wall and lapping up the animal's hemolymph before discarding the more toxic body parts (Walters et al., 1993; T. G. Nolen, unpublished observations). Since the seahare may encounter a predator several times in rapid succession (Nolen et al., 1995), the observations that *Aplysia* spp. need not secrete all their ink stores and that they can deploy their active defense in response to several subsequent encounters with a predator make functional adaptive sense, especially because (i) it will take several days to replenish their ink supply (Fig. 6A; Chapman and Fox, 1969) and (ii) the ink gland contains more than enough ink to deter the feeding of anemones and possibly other predators (Nolen et al., 1995; T. G. Nolen and B. Carlson,

unpublished observations). Our estimate of the anti-predator effectiveness of ink suggests that a well-fed snail with a relatively full ink gland could release 4–6 defensive salvos (Carlson and Nolen, 1997; B. Carlson and T. G. Nolen, in preparation). The most efficient use of ink would be to deploy the smallest amount that effectively deterred the predator. If the first deployment of ink were not adequate, then a second or third deployment would still be possible without having wasted the whole arsenal in a first response. Such economical deployment of its valuable ink reserves should provide the seahare with an enhanced chance of surviving predation (Nolen et al., 1995).

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