Animal Behaviour 79 (2010) 1067–1076

Contents lists available at ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Ink secretion protects sea hares by acting on the olfactory and nonolfactory chemical senses of a predatory fish

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ARTICLE INFO

Article history:
Received 24 August 2009
Initial acceptance 8 October 2009
Final acceptance 31 December 2009
Available online 12 March 2010
MS. number: A09-00561R

Keywords:
chemical defence
chemoreception
deterrent
opisthobranch
wrasse

Ink secretion of sea hares, *Aplysia californica*, is a mixture of ink from the ink gland and opaline from the opaline gland. Its defensive mechanisms against predatory spiny lobsters include deterrent compounds that are unpalatable and amino acids that stimulate appetitive responses (phagomimicry) or interfere with chemoreception (sensory disruption) by predators. The current study aimed to identify mechanisms whereby sea hares use ink secretion to defend against a fish predator, in this case the bluehead wrasse, *Thalassoma bifasciatum*. We show that inking by live sea hares decreased the probability that wrasses strike sea hares. Ink protected sea hares by affecting two phases of feeding. First, an ink cloud between a wrasse and food decreased the probability that the wrasse captured the food. Second, if the wrasse captured food treated with ink, then that food was less likely to be accepted. In neither assay did opaline have a significant effect. Inactivating the olfactory sense of fish through nares occlusion eliminated the deterrent effect of ink on food capture but not the effect of ink on food acceptance, thus showing that olfaction mediates responses to deterrents during the capture phase of feeding and that nonolfactory chemical senses mediate responses to deterrents during the acceptance phase. These nonolfactory chemical senses may be intraoral senses, as fish did not reject pellets until after they were captured. Ink did not protect through phagomimicry, since neither ink nor opaline was accepted, despite the fact that mixtures containing the amino acid components of ink and opaline were accepted.

Animals use a wide variety of defences against predators, including speed, stealth, crypsis, size, physical defences and chemicals (Pawlik 1993; McClintock & Baker 2001; Hay 2009). Opisthobranch molluscs, which include sea hares, are soft bodied and slow moving, and thus would be highly vulnerable to predators if not for the possession of a variety of defences. These include cryptic coloration and behaviour, large size, ability to produce copious mucus, and, most notably, chemical defences (Carefoot 1987; Johnson & Willows 1999; Wägele & Klussmann-Kolb 2005). Chemical defences of sea hares include passive ones, which are constitutively present, and active chemical defences, which are released only when the animal is attacked by a predator (Nolen et al. 1995; Johnson & Willows 1999). One active chemical defence is inking, which is the release of a purple, sticky secretion. The ink secretion of sea hares is the product of two glands that co-release their contents: the ink gland, which releases a purple fluid; and the opaline gland, which releases a white, highly viscous substance. These secretions are mixed in the sea hare's mantle cavity and squirted out of the body through the muscular pumping of the mantle.

Sea hares use ink to defend themselves from a diversity of predators using a variety of mechanisms. Mechanisms of chemical defence by ink of *Aplysia californica* have been described for two potential predators, a Pacific sea anemone, *Anthopleura sola*, and the California spiny lobster, *Panulirus interruptus*. Ink reduces predation by *P. interruptus* through a variety of mechanisms including unpalatability, sensory disruption and phagomimicry (Kicklighter et al. 2005; Shabani et al. 2007; Aggio & Derby 2008). Against sea anemones, ink is an unpalatable deterrent that causes tentacular withdrawal (Nolen et al. 1995; Kicklighter & Derby 2006). Injection of ink from *Aplysia dactylomela* into pieces of fish fillet resulted in rejection by laughing gulls, *Larus atricilla* (DiMatteo 1981). Studies on a number of sea hare species indicate that diets consisting of chemically depauperate plants alter the ink secretion and reduce its efficacy as a feeding deterrent, indicating that some chemical defences are diet derived (Pennings & Paul 1993; Nolen et al. 1995; Prince et al. 1998; Ginsburg & Paul 2001; Pennings et al. 2001). Thus, ink has the potential to chemically defend sea hares from predatory invertebrates, fish, birds and perhaps even marine or terrestrial vertebrates.

To expand our understanding of sensory mechanisms of chemical defence by sea hare ink, we examined a fish predator's

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0003-3472/$38.00
doi:10.1016/j.anbehav.2010.01.022

response to various components of chemical defences used by sea hares. Fish occupy the niche of top predators in most marine systems and represent a potentially strong selective pressure for the slow-moving, soft-bodied sea hares. There is little evidence of fish predation on sea hares in the wild, probably because of a combination of defences, including chemical defences such as ink release during an attack (Carefoot 1987; Johnson & Willows 1999). Fish are good model systems to study mechanisms of chemical senses, as their chemosensory systems are well characterized and they can be effectively studied both behaviourally and electrophysiologically (Nikonov & Caprio 2001; Rolen et al. 2003; Sato & Sorensen 2003; Caprio & Derby 2008; Cohen et al. 2008; Sheybani et al. 2009). The process of predatory attack, in general, and by fish specifically, involves two phases: approach and capture of food, when the prey is taken into the mouth, and the acceptance phase, when the prey is swallowed and consumed (Endler 1986; Ritson-Williams & Paul 2007). The approach and capture of prey by fish can be controlled by many senses. Of the chemical senses, the olfactory system is often involved in this phase, but other extraoral chemical senses, such as external gustatory systems, can also control this behaviour in some fish (reviewed in Caprio & Derby 2008). The acceptance and consumption of food is controlled by intraoral gustation (Valentinčič & Caprio 1994; Kasumyan & Døving 2003; Caprio & Derby 2008). Chemical defences might function at either or both of these phases and be effective in protecting potential prey species (Ritson-Williams & Paul 2007). Deciphering the phases in predation in which chemical defences function will allow further identification of the chemosensory modalities involved and therefore further elucidation of the functional mechanisms of the defences.

We chose to use the bluehead wrasse, *Thalassoma bifasciatum*, in our study because it is a good laboratory model as well as a potential predator of the sympatric sea hare *A. dactylomela*. Bluehead wrasses are found in the waters around Florida and the Caribbean islands, often associated with reefs but also found in inshore, nonreef areas and sea grass beds (Feddern 1965; Clifton & Motta 1998). *Aplysia dactylomela* occupies a similar ecological niche as *A. californica*. Like *A. californica*, *A. dactylomela* releases purple ink and white opaline, and its ink and opaline contain many of the same or similar diet-derived and metabolized defensive compounds, including ammonia, amino acids, L-amino acid oxidases (dactylomelin P in *A. dactylomela* and escapin in *A. californica*), and the pigments aplysioviolin and phycoerythrobilin, which are or generate aversive compounds (Melo et al. 1998; Kicklighter et al. 2005; Derby et al. 2007; Kamio et al. 2007; Kamio et al. 2009; M. Kamio, T. V. Grimes, M. H. Hutchins, R. van Dam & C. D. Derby, unpublished data). The advantages of using the bluehead wrasses for aquarium bioassays have been detailed previously (Pawlik 1987). It is a common fish species for testing antipredatory chemical defences, since it is easy to maintain and train to feed on artificial diets (Lindquist & Hay 1996; Kubanek et al. 2000; Odate & Pawlik 2006). In other studies, we found that ink of *A. californica* is an effective deterrent against five other fish species, including wrasses sympatric with *A. californica*, señorita wrasses, *Oxyjulis californica*, as well as pinfish, *Lagodon rhomboides*, mummichogs, *Fundulus heteroclitus*, and bonnethead sharks, *Sphyrna tiburo*. All of these fish responded to presentation of *A. californica* secretions in the same way as *T. bifasciatum* and sea catfish, *Ariopsis felis* (Sheybani et al. 2009; M. Nusnbaum & C. D. Derby, unpublished data).

To test the protective capabilities of the ink secretion, we presented either normal or de-inked *A. californica* to bluehead wrasses and observed whether inking affected predatory attacks. To test whether ink acts extraorally as a chemical defence to prevent fish from taking sea hares into their mouths, we presented food to bluehead wrasses in a cloud of ink and examined whether that condition reduced food capture. To test for phagomimicry, we added a mixture of amino acids to an alginate pellet at concentrations identical to those in natural ink and opaline to determine whether this increased acceptance of pellets. To test for unpalatability, we added ink and/or opaline to shrimp-flavoured alginate pellets and examined whether addition of these substances affected bluehead wrasses' acceptance of pellets. We inferred palatability, or lack thereof, from the results of the pellet assays. To examine whether olfaction contributes to the effect of ink on fish, we performed nares occlusions and tested anosmic fish in cloud assays as well as pellet assays.

METHODS

Animals

Juvenile yellow phase bluehead wrasses, 5–10 cm long, were wild caught in south Florida and maintained at Georgia State University in individual 40-litre glass aquaria ($50 \times 25 \times 30$ cm) containing 28 ppt sea water (Instant Ocean, Aquarium Systems, Mentor, OH, U.S.A.) that was filtered and aerated (Whisper Filters: Tetra, Blacksburg, VA, U.S.A.) and maintained at 21 °C. Fish were fed frozen shrimp and brine shrimp ad libitum twice daily. Fish were kept on a 14:10 h light:dark cycle and maintained in the same aquaria in which they were tested. Small ($\sim$1 g) specimens of *A. californica* were obtained from the National Institutes of Health National Resource for *Aplysia* (Miami, FL, U.S.A.) and kept in separate 40-litre glass aquaria before being used in the feeding assay. Sea hares were raised on an exclusive diet of laboratory-grown *Gracilaria ferox* prior to being shipped to our laboratory and were not fed during the 1-week period following their arrival at our laboratory prior to experimentation. Wrasses were kept in captivity for no longer than 3 months during behaviour assays and were euthanized at the end of the study.

Collection of Sea Hare Secretions

Ink and opaline were collected from adult sea hares caught in waters off the coast of California by Marinus, Inc. (Garden Grove, CA, U.S.A.) immediately after their arrival in our laboratory. The diet of these wild-caught individuals was not known, but the presence of purple ink indicated that their diet included red algae. Secretions were collected from dissected ink and opaline glands. Ink glands were gently squeezed to release ink. Opaline glands were centrifuged at $30\,000 \times g$ for 1 h at 4 °C to separate opaline secretion from gland tissue. Secretions collected from individual animals were pooled to reduce effects of individual variability in contents of glands. Secretions were frozen at −80 °C until needed.

Feeding Assay Using Live Sea Hares

Small specimens of *A. californica*, $\sim$1 g and 2.5 cm in length, were presented to bluehead wrasses to examine effects of inking on attacks by predatory fish. The fish were deprived of food for 1 week to ensure that they would readily attack the unfamiliar prey item. Twenty-nine individual fish were each tested with a single sea hare that was either completely intact (i.e. with ink) or de-inked. Each fish was tested once to avoid biasing the data as a result of predator experience. Fifteen sea hares were de-inked by repeatedly applying high concentrations of sea salt to the holding water, which induced head retraction and ink release. These sea hares were rinsed in sea water and allowed to rest for 5 min between salt applications and allowed at least 1 h to rest before being used in feeding assays. If a de-inked sea hare did not return to normal mobility and