WARNING COLORATION ASSOCIATED WITH NEMATOCYST-BASED DEFENCES IN AEOLIDIOIDEAN NUDIBRANCHS

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(Received 22 January 2004; accepted 18 August 2006)

INTRODUCTION

Opisthobranch gastropods display a high diversity of morphological variation related to their evolutionary reduction, and loss of the shell. Many of the shell-less opisthobranchs possess dorsal processes or cerata while others, like some chromodorids, are flattened and do not bear cerata. With the evolution of chemical defence mechanisms, opisthobranchs have not only altered their morphological appearance from shelled gastropods but have changed their use of colour as well. Most shell-less marine molluscs have chemical secretions or nematocyst-based defences that deter predators from eating them (Faulkner, 1992; Gavagnin et al., 1994), and they advertise themselves to predators with bright colours. Aeolidioidean nudibranchs are known to sequester nematocysts from cnidarian prey for defensive purposes (Edmunds, 1966). Nematocysts are ingested with other prey tissues and pass through the nudibranch’s digestive system to the tips of the cerata, the dorsal extensions of the digestive gland. The nematocysts are stored in specialized structures, known as cnidosacs, at the tips of the cerata (Greenwood & Mariscal, 1984). When the aeolidioideans are disturbed, the nematocysts are squeezed out from the cnidosacs. Aeolidioideans exhibit striking variation in colour, from dull, cryptic, patterns in some species to conspicuous colours (red, pink, yellow, blue) in others. The cerata are often brightly coloured in a manner that contrasts with the background colour of the mantle. Putative predator-induced body or mantle damage is rarely seen in aeolidioideans. Several authors have argued that aeolidioideans use dorsal protuberances as defensive lures. Predators may be attracted to the most unpleasantly flavoured part of the body, which they can bite and be repelled, without serious damage to the aeolidioidean’s body (Thompson, 1960; Edmunds, 1966). When attacked, an aeolidioidean usually holds the cerata erect and may wave them towards the enemy. Predators are intercepted by the cerata, and a direct attack towards the vital head and visceral mass of the aeolidioid is avoided.

Warning colours are colours and patterns of prey that are adaptive because they signal to predators a potential cost of making an attack. This definition implies that the prey can have a punitive effect that causes negative reinforcement; that predators learn the warning colour pattern, and in doing so may cause death or injury to the prey, and that a warning colour pattern is a better signal than a nonwarning or cryptic pattern (Mallet & Singer, 1987). Some Mediterranean aeolidioidean nudibranchs may exhibit warning colours, while others have shapes that closely match those of their food. The cerata of Cratena peregrina (Gmelin, 1791) have an iridescent blue and orange coloration with white areas on the upper parts that contrast with the white body. In addition, two conspicuous orange spots can easily be seen between the bases of the oral tentacles. This brilliant colour pattern suggests that C. peregrina is aposematic. In contrast, the aeolidioidean Spurilla neapolitana exhibits a near-perfect crypsis on its prey. It feeds on anemones of the genus Anemonia and retains symbiotic zooxanthellae and nematocysts from its prey. The cerata resemble the morphology and colour (due to symbiotic zooxanthellae) of anemone tentacles (Marin & Ros, 1991). Thus, studies focused on aeolidioidean nudibranchs with nematocyst-based defences located in dorsal protuberances could offer insights into how colour and body morphology are related to the evolution of opisthobranchs.

In the present study, we examined the role of warning colour, cerata and nematocyst-based defences in the aeolidioidean C. peregrina. The predator–prey relationship is one of the stronger factors that contribute to the evolution of behaviour. We analysed the learning interaction between aeolids and...
predatory fish using both live aeolids and artificial models to address the following questions: (1) Do retained nematocysts reduce palatability of this aeolid? (2) Do the aeolids use mantle appendages as a defensive lure? (3) Does the presence of cerata reduce damage to the aeolid’s body during a predatory attack? (4) Do fish learn to avoid the warning coloration pattern of this mollusc? (5) Does the risk of predation depend on the population density of the aeolid?

**MATERIAL AND METHODS**

*Field assays with live Cratena peregrina*  
Field studies with living *Cratena peregrina* were conducted in May 2003 in three localities in Murcia, southeastern Spain. The first experimental site, Aguilica Cape, was selected because *C. peregrina* was abundant in the benthic communities (density 1 individual/m²), indicating that fish would encounter this species. *Cratena peregrina* was less abundant in the second experimental area (density 0.01 individual/m²), Hornillo Bay, but we observed the presence of other aeolid species including *Dondice banyulensis* and *Plabinella affinis*. In the third experimental area, Gachero Cove, *C. peregrina* was very scarce (density less than 0.0001 individual/m²).

The experiment was carried out with live *C. peregrina* of two different colour patterns, normally coloured and blue. The blue colour was obtained by dyeing *C. peregrina* with Janus Green. The aeolids were put in a glass beaker with 20 ml seawater and 0.05 g Janus Green stain (Sigma) until they had changed to a dark blue colour. Observation under a stereo microscope showed that the change of colour was not due to coloration of the skin but of the mucous secretion, and after 60 min they lost the vital stain. To check whether the stain had some effect on the defensive capacity of the aeolids, they were tested again once the animal had lost the blue colour. To transfer the aeolids, we used single piece moulded polyethylene pipettes of 7 ml capacity with the tip cut. The blue-dyed and non-dyed aeolids were transported underwater in polyethylene jars (250 ml). The scuba divers waited for 10 min, until fish were habituated to their presence, then the aeolids were dropped into the water column using pipettes about 0.5 m above the rocky substrate. The diver who had dropped the aeolid slowly retreated 20 m, while a second diver located 5 m from the experimental site registered the number of attacks until the molluscs finally landed on the substrate.

*Feeding assays with artificial aeolids*  
Artificial models: The following experiments with artificial aeolids were employed to test the effects of colour pattern and nematocysts on fish learning. The assays were performed using carrageenan pellets incorporating pieces of the mantle of the common cuttlefish (*Sepia officinalis*) as the feeding attractant. We used the common cuttlefish because this mollusc conferred a white colour on the food mixture. These artificial models were made by stirring 1 g of carrageenan and 5 g of trituated cuttlefish mantle into 20 ml of distilled water. This mixture was heated in a boiling water bath until all the carrageenan had dissolved. The base of the artificial models was made by pouring the cuttlefish–carrageenan mixture into a mould of polyvinyl chloride (PVC) plastic. The mould had two parallel 1.5 × 12 cm openings cut in it with a depth of 0.5 cm. A razor blade was used to smooth the food into the mould and ensure a uniform thickness of 0.5 cm. When the carrageenan had cooled in the mould, it was cut into 1.5 × 4 cm rectangles. The dorsal protuberances of the artificial aeolids were made with the same food as the base, but cut into 0.5 × 0.5 × 4 cm square rods, which were glued with a hot drop of cuttlefish–carrageenan mixture; each model received six square rods. The different colours tested with the models were obtained with liquid food dyes added to the cuttlefish–carrageenan mixture when this was heated (Fig. 1).

*Laboratory assays with fish:* We performed feeding assays to investigate the effect of nematocyst-based defences and conspicuous colour patterns on feeding by Mediterranean fish. In the laboratory, we offered artificial aeolids to the wrasse *Thalassoma pavo* in separate aquaria. Fish were collected from a natural population in Gachero Cove, where *C. peregrina* was very scarce. We selected this fish species because it was the main predator of live aeolids and of artificial models in the field experiments. The fishes were individually housed in aquaria at a room temperature of 20°C. Training and testing took place in rectangular aquaria that were 40 × 60 cm and 35 cm deep. The walls were covered with brown plastic, apart from the front walls that were used for observation. The aquaria were fit with 40-W fluorescent lamps with a 12:12 h light:dark regime. Before the experiments, we trained the fish to forage on the cuttlefish–carrageenan mixture. Each feeding assay used different fish in order to avoid learned behaviours from previous experiments. After the experiments, we returned the wrasses to their original locality.

To test whether fish can learn to avoid aeolids because they recognize their shape, we conducted a learning experiment with F and S models, which have the same brown colour and amount of food, but different morphology. To determine if fish can learn to avoid the colour pattern of *C. peregrina*, we trained fish with artificial models of different colour patterns but the same shape (R and S models). A similar design was employed in both experiments. We performed three successive tests with the same fish in order to study the effects of the coloration or morphology of models on fish behaviour. On the first day of each experiment (session 0), palatable models of both types were offered to the wrasses. The different colour patterns or
morphologies of the artificial aeolids might be avoided due to innate responses, or previous experiences of the experimental wrasses. Fishes that refused to eat any palatable model were excluded from the further experiments. The training period began the following day and consisted of three sessions on consecutive days (sessions 1, 2 and 3). The duration of each training session was 15 min. The S and R models were treated with nematocysts as negative stimulus during training sessions. We obtained nematocysts by grinding Eudendrium in a mortar and pestle, and passing the liquid through a 1 mm mesh screen. Controls, F and B models, were not bathed with hydroid sauce. In each training session, we randomly offered palatable (group 1: F; group 2: B) and distasteful artificial models (group 1: S; group 2: R). The number of attacks on each model was recorded during training sessions. On the third day, we tested if the wrasses had learned to avoid the distasteful models. The post-training tests (session 4) were carried out under the same conditions as the pre-training test (session 0). In session 4, S and R models were not treated with nematocysts.

Field assays with artificial models: Artificial aeolids were also tested in the field using the same cuttlefish–carrageenan mixture to determine how avoidance learning of aposomatic aeolid nudibranchs occurs in natural marine habitats. Fish predators may avoid nasty prey that they have earlier seen or caught. In assays with live aeolids we did not know whether the fish had previous experience of C. peregrina. Thus, assays with models allow us to simulate the conditional learning process of a species with warning colours such as C. peregrina in marine ecosystems where the aeolids live surrounded by hundreds of potential predators. Field assays were carried out in Fraile Island, a protected natural area frequently visited by scuba divers where the fish are habituated to their presence. For field assays, each artificial aeolid was attached to plaques of metal mesh (10 × 10 cm). Plaques with nematocyst-treated and control artificial aeolids were scattered in the rocks of Fraile Island at 10–15 m depth. After an hour, the plaques were retrieved and the percentage of food eaten per artificial aeolid was scored. The experimental design was similar to that described for laboratory assays, but all the experiments were carried out on the same day in each locality. Prior to training, we offered wild fish blue control models (B) and orange and white models (R) without nematocysts. The training period consisted of four consecutive sessions of 30 min. In each session, blue models and red–white models without nematocysts were randomly distributed in the same area. The scuba divers at 10–15 m from the experimental models recorded the number of attacks on both models.

RESULTS

Field studies with live Cratena peregrina

Assays to determine the effect of density of natural populations, and of colour of Cratena peregrina, indicated that the number of attacks by fish was independent of the density of the prey, but not of their colour pattern (Fig. 2). A two-factor ANOVA on square-root transformed data showed that there were significantly more attacks on blue than on control aeolids (two-way ANOVA, $F = 122.91, P < 0.0001$), whereas site had no significant effect (two-way ANOVA, $F = 1.13, P > 0.05$). However, the interaction between colour pattern and site was significant (two-way ANOVA, $F = 5.36, P < 0.01$) due to the different fish behaviour in the locality with a medium density of aeolids. Even though the aeolids that were dropped in the water column were immediately attacked by fish, every non-dyed C. peregrina survived without visible damage. The molluscs were snapped up and spat out several times before reaching the substrate. On the other hand, fish attacks caused 15–20% mortality on blue-dyed C. peregrina. The mortality of blue aeolids did not differ between the three experimental localities (Kruskal–Wallis, $H = 5.02, df = 2, P > 0.5$). In order to assess how the Janus Green stain might be affecting the defensive capacity of C. peregrina, they were tested again when they had lost the blue colour. No significant differences were found in the number of attacks between previously dyed aeolids and control C. peregrina (paired $t$-test, $P > 0.05$).

Laboratory assays with artificial aeolids

Morphology: The fish attacked palatable aeolid-like models (S models) significantly more than palatable flat (F models) in the pre-training test (Fig. 3; paired $t$-test, $P = 0.001$; Fig. 4). In training sessions, there were significantly fewer attacks on flat F models than S models in sessions 1 and 2 (paired $t$-test, $P < 0.05$), but no significant difference in session 3 (paired $t$-test, $P > 0.05$). The number of attacks on F models did not differ significantly during the four sessions (one-way ANOVA, $P > 0.05$). However, there was a significant effect of session number on attacks on S models (ANOVA, $P < 0.05$). This effect was not significant in the pre-training test (session 0; paired $t$-test, $P > 0.05$).
difference in the number of attacks was due to session 4 (post hoc Tukey test, \( P < 0.05 \)). The post-training test trial showed differences in the number of attacks on the S models (paired \( t \)-test, \( P < 0.05 \)) but not on the F models (paired \( t \)-test, \( P > 0.05 \)). These data show that wrasses began to learn the shape of the unpalatable S model in the last training session (Fig. 4).

Warning colour: There was no significant difference between the number of attacks by wrasse on R and B models in the pre-training session (paired \( t \)-test, \( P > 0.05 \)). In training sessions, treated R models were significantly less attacked than control B models in sessions 1, 2 and 3 (paired \( t \)-test, \( P < 0.05 \)). The number of attacks during the training trials showed a significant and progressive decline on the attack rate on unpleasant, conspicuous models (repeated-measures ANOVA of the sessions 1, 2 and 3, \( P < 0.05 \)). In session 4, no treated R model was attacked by fish (Fig. 7). The fishes learned to avoid aposematic R models after the first training session (post hoc Tukey test, \( P < 0.05 \)). The controls, B models, were attacked with the same intensity in all training sessions (repeated-measures ANOVA, \( P > 0.5 \)). Post-training test showed that fish avoided palatable R models (paired \( t \)-test, \( P < 0.01 \)) (Fig. 8). These results suggest that the fishes have learned the colour pattern of our R model in only four training sessions.

Field assays with artificial aeolids

The pre-training test with palatable R and B models showed that there was no significant difference between the number of attacks by fish in the field on either model (paired \( t \)-test, \( P > 0.05 \)). In training sessions, treated R models were significantly less attacked than control B models in sessions 1, 2 and 3 (paired \( t \)-test, \( P < 0.05 \)). The number of attacks during the training trials showed a significant difference between the number of attacks by fish on either model (paired \( t \)-test, \( P > 0.05 \)). In session 4, no treated R model was attacked by fish (Fig. 7). The fishes learned to avoid aposematic R models after the first training session (post hoc Tukey test, \( P < 0.05 \)). The controls, B models, were attacked with the same intensity in all training sessions (repeated-measures ANOVA, \( P > 0.5 \)). Post-training test showed that fish avoided palatable R models (paired \( t \)-test, \( P < 0.01 \)) (Fig. 8). These results suggest that the fishes have learned the colour pattern of our R model in only four training sessions.
of the attack, and vigilance results in early predator detection. Shoaling by fish can lead to predator confusion or to dilution in groups, which may decrease the threat of other fish predators. Effective because potential fish predators frequently live in the water column. Dropping the aeolids into the water column. On several occasions even where the density of aeolids was high.

**DISCUSSION**

Faulkner (1992) noted that the hypothesis that nudibranchs store chemicals from their diet for defensive purpose seems reasonable, but needs thorough testing before it can be accepted. First, it must be shown that the chemicals can deter potential predators. Second, an attack by a predator must elicit a behavioral response that results in the predator being repelled by a chemical secretion. Edmunds (1991) gave four criteria to demonstrate that a nudibranch is aposematic: (1) it is sufficiently noxious that some predators will not eat it; (2) it is conspicuously coloured, or advertises itself by means of some other signals; (3) some predators avoid attacking it because of its signals; (4) these conspicuous signals provide better protection to the individual or to its genes than would other signals. We have obtained experimental evidence that *Cratena peregrina* is an aposematic nudibranch. The field and laboratory assays have shown that living aeolids and artificial models with nematocyst-based defences were unpalatable to potential fish predators, *C. peregrina* is conspicuously coloured in benthic habitats where it feeds on hydroids. The wrasse *Thalassoma pavo* quickly learned to avoid artificial models coupled with nematocysts, while continuing to attack control models. *T. pavo* learned to avoid artificial aeolids with colour pattern similar to *C. peregrina* during avoidance training sessions in aquaria. During the conditioning process, the wrasses explored the artificial aeolids very intensively. The process of conditioning was gradual in subsequent trials, until the wrasses had learned the task. Then, in the final trial, wrasses did not bite palatable warning-coloured models. Field assays with artificial aeolids showed results identical to laboratory assays. However, the field assays with live *C. peregrina* indicated that avoidance learning was not perfect, fish attacked on several occasions even where the density of aeolids was high. It seems that unusual stimuli induce exploration in fish, e.g., dropping the aeolids into the water column.

The defensive mechanism found in *C. peregrina* is particularly effective because potential fish predators frequently live in groups, which may decrease the threat of other fish predators. Shoaling by fish can lead to predator confusion or to dilution of the attack, and vigilance results in early predator detection (Csányi & Doka, 1993). When first encountering live *C. peregrina* or artificial models, individual fishes leave the shoal and approach close to the potential prey. The field assays suggest that *C. peregrina* can live in low density because the negative signal transmission of these ‘inspector’ fish inhibited attack from the shoal. The fish acquired a conditioned avoidance reaction against aposematic artificial models and live *C. peregrina* without a direct contact with the prey, through observation of the consumption by other members of the shoal. The aposematic coloration of aeolids may work as well for solitary species able to survive predator attacks, as for gregarious species. The evolution of unpalatability and aposematic coloration has long been a subject of debate. An important question has been whether these traits evolved in solitary or in aggregated prey (Guilford, 1990). Gregariousness is more common in aposematic than in palatable and cryptic species (Gagliardo & Guilford, 1993). Gagliardo & Guilford (1993) indicated that it was not the appearance of an aggregation that was the important stimulus for the enhanced aversion learning when the prey was grouped, but the opportunity for the predator to see the prey’s coloration when, or immediately after, noticing the prey’s distastefulness. Fisher (1930) suggested that unpalatability may have evolved in kin-grouped prey, and kin selection has been applied to the evolution of aposematic coloration as well. The existence of many solitary opisthobranch species suggests that aposematism can evolve through individual selection, especially considering that most opisthobranchs have pelagic larvae, and therefore probably seldom settle in kin groups (Faulkner & Ghiselin, 1983; Tullibot, 1994); planktonic larval dispersal excludes kin grouping in most marine organisms (Rosenberg, 1989). Some experiments with wild birds (great tit, *Parus major*) indicated that there is no evidence that aggregation facilitated the evolution of warning coloration (Tullberg, Leimar & Gamberale-Stille, 2000). Our experiments show that the number of attacks on live *C. peregrina* was independent of its density in natural habitats. The avoidance mechanism worked as well at Aguilica Cape (high density) as in Hornillo Bay (medium density), and Gachero Cove (low density). Aggregation might not, therefore, be a prerequisite for the evolution of aposematism in aeolids. Our results suggest that nematocyst-defence combined with expendable, unpalatable papillae have led to the evolution of aposematic coloration.

Muellerian mimicry is common among Mediterranean nudibranchs (Ros, 1976, 1977). Our results suggest that selection could favour mimicry of aeolidioidean species with the same ceratal pattern. Mimicry among small Mediterranean aeolidioids may be explained by warning colour of cerata and not by their body colour pattern. A similar colour pattern of cerata in the Mediterranean aeolidioids can be found in *Flabellina babai*, *Coryphella lineata*, *Pseustegites gabinieri*, *Cathana caerulea*, *Berghia verrucinornis*, *Facelina botomiosmis*, *F. corona* and *Dondice banyulensis*.

**ACKNOWLEDGEMENTS**

The authors thank Dr M. Edmunds for his valuable comments on the manuscript.

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