DOES A SHELL MATTER FOR DEFENCE? CHEMICAL DETERRENCE IN TWO CEPHALASPIDEAN GASTROPODS WITH CALCIFIED SHELLS

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ABSTRACT

Opisthobranch molluscs show an evolutionary trend to reduce, internalize and lose the shell. Many of them base their defensive strategies on natural deterrent products and current evolutionary theory suggests that the acquisition of chemical defences preceded shell reduction and loss, which has characterized the evolution of this group. Here we show that basal, shelled opisthobranch molluscs are defended against sympatric predators even if their protective shell is removed. The cephalaspideans *Bulla striata* and *Haminoea orbignyana*, both with distinct shell calcification, significantly deterred feeding by sympatric crab and fish predators, both in laboratory and field assays. However, our results argue against a progressive increment of chemical defences associated with shell reduction, because the cephalaspidean with the more fully calcified shell, *Bulla striata*, was also the more deterrent. These findings suggest that effective chemical defences might have evolved independently from shell loss, at least in basal opisthobranchs such as cephalaspideans.

INTRODUCTION

Many slow-moving and sessile organisms lack structural defences, live in habitats with a large number of predators and yet are rarely preyed upon (Thompson, 1960; Faulkner & Ghiselin, 1983; Rudman, 1991; Griffith, 1994). In recent decades, the defensive role that secondary chemistry could play in deterring predators has become widely accepted, and the evidence for the effectiveness of chemically mediated defences is increasing (Leimu & Koricheva, 2006; Paul, Puglisi & Ritson-Williams, 2006). Chemical defences are so effective at deterring predators that it has been suggested they evolved as a response against predation, driving evolution in groups as diverse as butterflies (Berenbaum, 1983; Feeny, 1991), birds (Martin, 1995) and molluscs (Cimino & Ghiselin, 1998).

With over 93,000 living species and a great variety of forms and lifestyles, molluscs have acquired defensive strategies based on structural and chemical defences (Brusca & Brusca, 2003). Gastropods and bivalves rely heavily on structural defences provided by the growth form and thickness of their shells, and the degree of protection can vary as a function of predation (Vermeij, 1978, 1982; Appleton & Palmer, 1988; Trussell, 1996; West & Cohen, 1996; Leonard *et al*., 1999). Nonetheless, among gastropods, many opisthobranchs have abandoned the protection of the shell, which is small, internal or absent in this group, and have shifted from structural to other defensive strategies such as autotomy, mimicry, crypsis, cleptodefence and the retention or production of defensive chemicals (reviews by Ros, 1977; Cimino & Sodano, 1993; Avila, 1995). Due to these latter defences, opisthobranch molluscs are regarded as good models to understand chemically mediated predator–prey interactions and their role in marine ecosystems (Avila, 1995; Paul & Puglisi, 2004).

It has been suggested that chemical defences are in fact the driving force behind opisthobranch evolution (Faulkner & Ghiselin, 1983; Cimino & Ghiselin, 1998, 1999; Cimino, Fontana & Gavagnin, 1999). Some opisthobranch lineages that originated as shelled animals presumably without chemical defences, have since evolved into forms with no structural defences but with chemical ones that are sequestered from the diet or biosynthesized *de novo* (Faulkner & Ghiselin, 1983; Cimino & Ghiselin, 1999; Fontana *et al*., 2004). The evolution of chemical defences is supposed to be preadaptive, since they need to be functional in the mollusc before shell loss (Faulkner & Ghiselin 1983; Wägele & Klussmann-Kolb, 2005). Despite the evolutionary implications, data on chemical defences of shelled opisthobranchs are limited to a small number of species.

Living cephalaspidean molluscs have the most ancestral traits within opisthobranchs and are considered the basal opisthobranch group (see Mikkelsen, 1996 and references therein; Rudman & Willan, 1998). Cephalaspideans include species with robust external shells into which the animals completely retract when under attack (e.g. *Acteon*, *Bulla*; Rudman & Willan, 1998), species with small and fragile external shells that fail to provide protection to the whole animal (e.g. *Haminoea*; Rudman & Willan, 1998; Malaquias & Cervera, 2006), species with small internal shells (e.g. *Sagaminopteron*; Carlson & Hoff, 1973, 1974) and species with no shells at all (e.g. some *Siphopteron*; Gosliner, 1989).

In this study, we tested whether the shelled cephalaspideans *Bulla striata* Bruguière, 1792 and *Haminoea orbignyana* (Férussac, 1822) are chemically defended against sympatric predators. *Haminoea orbignyana* has a thin, fragile and translucent shell with a globular shape, and the animal, measuring 1–2 cm in length, cannot retract totally inside it (Rudman & Willan, 1998). In Portugal, *H. orbignyana* is diurnally active and usually found in populations with a high mean annual density. This cephalaspidean feeds upon the epiphytes that grow on the leaves of the seagrass *Zostera noltii* or on the green algae *Ulva* (Malaquias *et al*., 2004; personal observations). On the other hand, *B. striata*, 3–4 cm in length, has a robustly calcified

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shell, with an aperture at least as long as the shell, into which the animal can retract completely. On Portuguese coasts, *B. striata* is usually found in the same places as *H. orbignyana*, but is always burrowed in the sediment during daytime. The diet of *B. striata* is mainly composed of green algae, seagrasses, diatoms and cyanobacteria (Malaquias *et al.*, 2009).

Because of the differences in structural defences between these two species, and in agreement with the predictions from evolutionary theory, we expected *Haminoea orbignyana* to be chemically defended and more deterrent than *Bulla striata*, which does not need a chemical defence owing to the presence of a full protective shell. Our results failed to agree fully with the prediction and in fact showed that each species is chemically defended against predators, regardless of their differences in structural defences.

MATERIAL AND METHODS

Cephalaspidean collection and extraction

Bulla striata and *Haminoea orbignyana* were collected by hand intertidally at localities along the Portuguese coast, from December 2004 to May 2005. *Bulla striata* was collected at Ria Formosa (37°00′N, 07°88′) and Ria de Alvor (37°07′N, 08°35′W); *H. orbignyana* was collected at Ria de Alvor and Ria de Aveiro (40°42′N, 08°40′W).

Immediately after collection, we transferred the animals to 32-l aquaria. They were weighed and frozen at −24°C. For chemical extraction of *B. striata*, frozen animals were macerated in acetone (1 ml of solvent/g of fresh weight) for 10 min in an ultrasound bath. The acetone solution was filtered, and the extraction was repeated twice with the same amount of solvent. The solvent was evaporated (at 25°C) under vacuum to yield a residual crude extract free of solvent and water. We obtained 3.82 g of total extract and 202.5 g of dry residue from 290.5 g (fresh weight) of *B. striata* (49 specimens).

For *H. orbignyana* we used the same extraction procedure to obtain the original acetone solution. Due to the very high percentage of water present in this solution, only the acetone was totally evaporated. The resulting aqueous solution was extracted with diethyl ether (3×450 ml) to give an aqueous extract (396 ml) and an organic extract (4 g). Both extracts and a dry residue of 76.9 g were obtained from 688.2 g of fresh weight of *H. orbignyana* (540 specimens).

Extracts were analysed by thin-layer chromatography (TLC) to confirm the presence of deterrent metabolites found in previous studies on Mediterranean populations of these species (Cimino, Sodano & Spinella, 1987; Cutignano *et al.*, 2003; Spinella *et al.*, 1993).

We ran a series of experiments to determine whether: (1) *B. striata* and *H. orbignyana* deterred sympatric predators; (2) deterrence is functional despite the absence of shell; (3) deterrence is chemically mediated.

Laboratory assay – experiment 1

We first tested in the laboratory whether in the absence of shells, soft tissues of *B. striata* and *H. orbignyana* deterred feeding by the generalist predator *Carcinus maenas*. This crab is easily maintained under laboratory conditions, and is a common inhabitant of shallow-water habitats (Crothers, 1968; Pawlik, Albizati & Faulkner, 1986) including those where *B. striata* and *H. orbignyana* occur. We collected crab specimens from the same locations where we collected the molluscs and, once in the laboratory, we placed individual crabs in 32-l aquaria with running sea water. We trained crabs to feed on pieces of bivalve mantle tissue for a number of days prior to running the experiments. Crabs were starved for 24 h before performing the experiments. We offered each of 12 individual crabs either soft tissues of *B. striata* or *H. orbignyana* or a piece of mantle tissue of equivalent size of the bivalve *Donax trunculus* as a control. Ten minutes later we recorded whether the food was eaten or rejected (when the crab ignored or failed to eat it). We randomly offered treatment or control food first, and then repeated the experiment with the remaining food item. When treatment food, i.e. a cephalaspidean species, was offered and rejected in the second trial, we offered an additional control food as a third trial to differentiate true food rejection from satiation. Crabs that failed to eat this third control item were considered satiated, and were not used in the analysis. Since we offered multiple food items to the same individual crabs, i.e. we tested the same individuals with both control and treatment foods, the McNemar test for significance of changes (Sokal & Rohlf, 1995) was used to test for significant differences between control and treatments.

Intertidal field assay – experiment 2

We also tested whether *B. striata* and *H. orbignyana* deter sympatric predators in the field by using modified methods based on Hay (1984), Van Alstyne *et al.* (1992) and Gimenez-Casalduearo, Thacker & Paul (1999). These have been successfully used to evaluate molluscan deterrent activities (Becerro *et al.*, 2001). Briefly, we used safety pins to attach unshelled specimens of *B. striata* (treatment), specimens of unshelled *H. orbignyana* (treatment) or pieces of equivalent sizes of mantle tissues of the bivalve *Donax trunculus* (control) to individual 25-cm long ropes. We tied three ropes to an iron bar that was buried in the muddy bottom. Each rope had 12 safety pins and a specimen of one of the treatments or the control was attached to each of them. At the opposite end of each rope, a small buoy kept the ropes vertical in the same area where each cephalaspidean species was collected. During low tide, we placed the device on one of our collecting sites and removed it at the following low tide (*c.* 12 h) when we counted the number of control and treatment foods eaten. Differences in consumption of treatment *vs* control food pieces were evaluated with the Pearson χ^2 test.

SCUBA field assay – experiment 3

We also ran field experiments to determine whether *B. striata* and *H. orbignyana* are chemically defended against a natural assemblage of fish predators. Methods were similar to those of Pawlik *et al.* (1986), Pawlik & Fenical (1989) and Becerro *et al.* (2003). We added extracts from *B. striata* and *H. orbignyana* to an artificial diet consisting of 1.4 g agar (Iberagar), 2.8 g powdered fish food (Hagen, Laguna Goldfish & Koi), 1.3 g paraffin and 27.5 ml distilled water. We added the necessary amount of extracts or fractions relative to the total weight of the artificial food pellet to mimic the natural concentrations (fresh weight) found in each species. These were added by mixing with the cooling food. The amount of total extract (dissolved in water:acetone 1:1) or fractions (the organic fraction was dissolved in diethyl ether) varied according to the percent yield (per weight) of each particular extract or fraction. For *H. orbignyana* the aqueous and organic fractions were tested both together and separately. Control and treated food were identical except that we added solvent alone to the control food instead of solvent and extract in the treated food. The mixture was then poured into PVC molds from which we cut cubes of side 0.5 cm with a scalpel. We ran feeding assays at Sesimbra, Portugal (38°26′N, 09°06′W) at a depth of 4–8 m. At this location the major generalist fish predators that participated in the feeding assay were *Coris julis*, *Centrolabrus* *exoletus* and occasionally *Parablennius pilicornis*. Control and treated cubes were offered one at a time and we randomly changed the order of the food offered within replicates to prevent predators from learning any sequence that might affect the outcome. This field assay was performed for *c.* 2 h. We used a total of 15 replicates and tested for differences in consumption between food types with a Pearson χ^2 test (Sokal & Rohlf, 1995).

RESULTS

From the crude extracts of each cephalaspidean collected off Portugal, we isolated the major metabolites using the procedure previously described for the same species from the Mediterranean Sea (Cimino *et al*., 1987; Spinella *et al*. 1993; Cutignano *et al*., 2003). From three specimens of *B. striata* we isolated the polypropionates aglajne-1 (9 mg) and aglajne-3 (24 mg), and from 200 specimens of *Haminoea orbignyana* the 3-alkylpiridine alkaloids haminol-1 (1 mg) and haminol-2 (2 mg). All metabolites were identified by comparison of their NMR data with those previously reported in the literature. These compounds have already been described from populations of the same species inhabiting the Mediterranean Sea (Cimino *et al*., 1987; Spinella *et al*., 1993; Cutignano *et al*., 2003). Their presence in the organic extracts used in laboratory and field assays was confirmed by TLC.

In the absence of shells, each cephalaspidean species was eaten significantly less by the generalist crab *Carcinus maenas* than the control food (experiment 1 – McNemar test, *B. striata*: $P < 0.001$, *H. orbignyana*: $P = 0.046$; Fig. 1).

Bulla striata was eaten significantly less by predators in the field than either control food or *H. orbignyana* (Pearson χ^2 test, $P < 0.001$; Fig. 2).

The crude extracts of *B. striata* and *H. orbignyana* (experiment 3) significantly deterred consumption by fish predators in the field (Pearson χ^2 test, $P < 0.001$ and $P = 0.023$, respectively; Fig. 3). Note that no *B. striata* extracts were consumed. We found no differences in consumption between food treated with the organic fraction, aqueous fraction or total extract of *H. orbignyana* combined (Pearson χ^2 test, $P = 0.301$). The crude extract of *B. striata* was significantly more deterrent than the total crude extract of *H. orbignyana* (Pearson χ^2 test, $P < 0.001$).

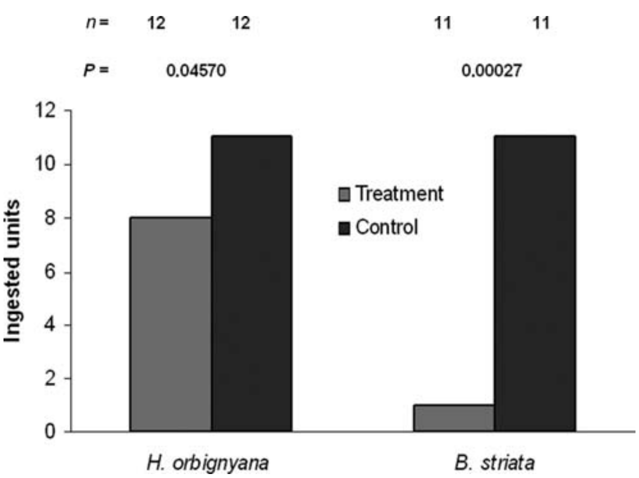

Figure 1. Laboratory assays with fresh material. Consumption by *Carcinus maenas* of paired *Haminoea orbignyana* or *Bulla striata* and control (*Donax trunculus*). n = number of pairs used in statistical analysis. Probability (P) calculated using the McNemar test.

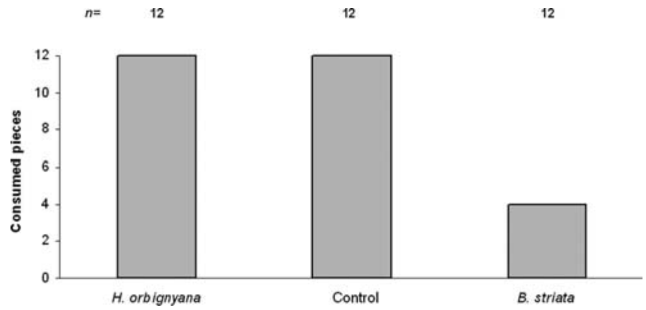

Figure 2. Intertidal field assay. Consumption, by unknown predators, of treatment food pieces (*Bulla striata* or *Haminoea orbignyana*) and control food pieces (*Donax trunculus*). n = number of food pieces (treatment and control) left in the field between two high tide periods and used for statistical analysis.

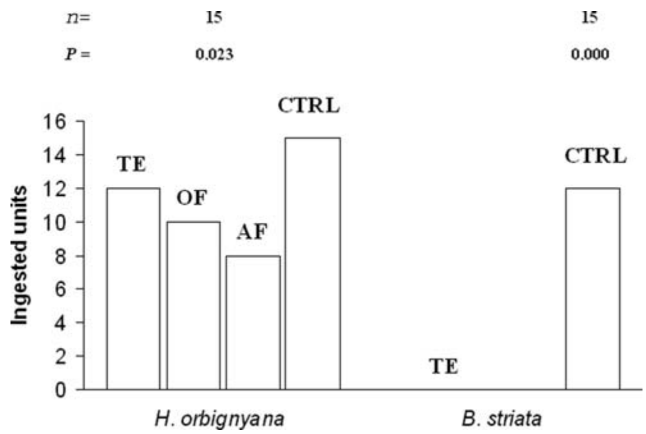

Figure 3. SCUBA field assay. Consumption, by some fishes, of treated pellets (artificial food with chemical extracts from each species studied) and control pellets (artificial food with no extracts). Probability (P) calculated using Pearson χ^2 test. Abbreviations: TE, total extract; OF, organic fraction; AF, aqueous fraction; CTRL, control; n, number of food pellets (treatment and control) offered.

DISCUSSION

Among opisthobranchs, progressive shell reduction and acquisition of chemical defences have occurred independently in many groups and are considered the driving force in their evolution (Faulkner & Ghiselin, 1983). This scenario requires the acquisition of chemical defences prior to shell loss (Edmunds, 1987), with the overlooked implication that basal, shelled opisthobranchs might also be chemically defended. We proposed the hypothesis that *Haminoea orbignyana* would counteract its weak structural defences and ready availability to predators with strong chemical deterrents, whereas *Bulla striata* would rely on structural and behavioural characteristics to avoid predators. However, we found that although *H. orbignyana* is chemically defended, *B. striata* is significantly more so. The three experiments performed, using different putative predators that could respond differently to the same defences, all support this conclusion. The results partially agree with previous studies on the main metabolites from these cephalaspideans; whereas aglajne-1 and aglajne-3 (present in *B. striata*) were deterrent to fish, haminol-1 and haminol-2 (present in *H. orbignyana*) act as alarm pheromones (Marín *et al*., 1999).

Bulla striata has few known predators (Paine, 1963; Burn, 1966, Cimino *et al*., 1987; Villani, 1991; Spinella *et al*., 1993), which is consistent with its structural, chemical and behavioural characteristics. However, *H. orbignyana* occurs in large numbers on the surface of the sandy or muddy bottoms that it inhabits, where it is readily available to predators, it lacks structural defences, and its chemical defences only minimally and inconsistently deterred consumption. With these biological traits, the lack of known predators of this species is surprising (Paine, 1963; Rudman, 1972; Boulch-Bleas, 1983; Villani, 1991). Without a strong investment in structural and chemical defences, *H. orbignyana* has a highly synchronous reproductive period and life cycle, which might be an alternative survival strategy, in agreement with the predator satiation hypothesis (Mills, 1982; but see Magro *et al.*, 2002). This is consistent with the large densities found in populations of this species (Malaquias & Sprung, 2005), although it fails to explain the lack of natural predators.

Beyond the biological and ecological consequences of the defensive strategies of these species, our results also raise some broader evolutionary questions. We demonstrate that chemical defences might have evolved independently from shell loss, at least in basal opisthobranchs such as cephalaspids. Thus, a co-evolutionary process linking structural and chemical defences may not be as coordinated as previously thought. Although opisthobranchs still provide most of the known examples of molluscan chemical defence mediated by secondary metabolites, there is growing evidence for this in other shelled molluscs, including patellogastropods (Pawlik *et al.*, 1986) and bivalves (Eufemia *et al.*, 2002; Kicklighter, Fisher & Hay, 2004). Moreover, chemical defence is also present in other marine benthic invertebrates with calcified shells such as brachiopods (McClintock *et al.*, 1993; Mahon *et al.*, 2003).

The evolutionary trend to reduce, internalize and lose the shell in opisthobranchs is uncommon among other molluscs (Gosliner, 1994). To counteract the lack of structural defences, opisthobranchs must incorporate new defensive strategies prior to shell loss (Faulkner & Ghiselin, 1983) and this shift must provide selective advantages. There are multiple examples of progressive loss of the shell in the evolution of opisthobranchs and the question remains whether or not reduced structural protection is coupled with an increase in alternative defence mechanisms, including chemical defences.

Our results could argue against increased chemical defence being associated with shell reduction as we found that the cephalaspidean with the fully calcified shell, *B. striata*, was also the more chemically deterrent. However, the information available is limited, preventing a robust test of the hypothesis. Present knowledge supports a shift from structural to chemical defence, but it is unclear whether such a shift is cause or consequence of opisthobranch evolution. The testing of falsifiable hypotheses such as that in our study will advance knowledge of the biology of opisthobranch species and will contribute to understanding the mechanisms behind their evolution.

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