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Chemical Defenses of Cryptic and Aposematic Gastropterid Molluscs Feeding on their Host Sponge *Dysidea granulosa*

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Abstract Numerous opisthobranchs are known to sequester chemical defenses from their prey and use them for their own defense. Information on feeding biology is critical for understanding the ecology and evolution of molluscs, yet information on feeding biology is still scarce for many groups. Gastropterid molluscs are often found on sponges, but there is controversy as to whether they are true sponge feeders. On Guam, we found the gastropterids *Sagaminopteron nigropunctatum* and *S. psychedelicum* on the sponge *Dysidea granulosa*. They seem to rely on contrasting defense strategies as *S. psychedelicum* has vivid colors, consistent with the warning coloration found in many chemically defended opisthobranchs, whereas *S. nigropunctatum* is highly cryptic on the sponge. *S. nigropunctatum* is avoided by the pufferfish *Canthigaster solandri* in aquarium assays. We analyzed the secondary metabolites of the two species and found that both share polybrominated diphenyl ethers (BDEs) with their host sponge *D. granulosa*. *S. psychedelicum* and *S. nigropunctatum* sequester the major BDE in the sponge and accumulate it in the mantle at approximately the same concentration as in the sponge (4.03 and 2.37%, respectively), and concentrate it in their parapodia at over twice the sponge concentration (7.97 and 10.10%, respectively). We also detected trace amounts in the mucus secretion of *S. psychedelicum*, and quantified significant amounts in the mucus (1.84%) and egg masses (2.22%) of *S. nigropunctatum*. Despite contrasting color patterns displayed by the two gastropterid species, they seem to share a similar

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chemical defense strategy, i.e., they feed on *D. granulosa* and accumulate the major BDE of the sponge in their tissues.

Keywords Brominated diphenyl ethers (BDEs) · Chemical defense · *Dysidea granulosa* · Feeding specialists · *Sagaminopteron nigropunctatum* · *Sagaminopteron psychedelicum*

Introduction

Feeding biology plays a major role in the ecology and evolution of molluscs, particularly opisthobranchs (Kohn, 1983; Willan, 1984; Avila, 1995; Johnson and Willows, 1999). Many traits that drive the evolution of opisthobranchs, such as shell reduction or loss, have resulted from coevolution with diet organisms (Faulkner and Ghiselin, 1983; Cimino and Ghiselin, 1998, 1999; Cimino et al., 1999, 2001). Without shells, many opisthobranchs rely on other defense mechanisms. Camouflage or cryptic coloration is the most common strategy, with examples in a variety of organisms including opisthobranch mollusk. Since organisms must resemble their backgrounds (Endler, 1978), appropriate coloration is background-specific. Many opisthobranch molluscs are specialist predators, and cryptic species show evidence of a close association with their diets (Rudman, 1981; Morrow et al., 1992; Becerro et al., 2001, 2003). Aposematic or warning coloration is at the opposite extreme, and evolutionary theory postulates that aposematic coloration evolves in deterrent species because it is a more effective warning signal than the alternate cryptic coloration (Wallace, 1867; Poulton, 1890). In opisthobranchs molluscs, warning coloration is based on the benefits provided by chemical defenses often sequestered from the opisthobranch food source (Faulkner and Ghiselin, 1983; Avila, 1995), and it seems associated with evolutionarily more derived, rather than more basal, taxa (Gosliner, 2001).

Despite the importance of feeding biology in the life history strategies of opisthobranchs, there is a lack of information on the feeding biology of many groups. Gastropterid molluscs are often found on sponges, which suggests they are sponge feeders. Despite the close association between gastropterids and sponges, it remains doubtful whether gastropterids actually feed on sponges since their radular teeth are similar to the closely related philinoid bubble shells, which feed on hard-shell prey (Hurst, 1965; Mikkelsen, 1996). Two sympatric gastropterid species of the genus *Sagaminopteron*, *S. nigropunctatum* and *S. psychedelicum*, show contrasting color patterns and are associated with the sponge *Dysidea granulosa* on which they are usually found. *S. nigropunctatum* displays cryptic coloration (Gosliner, 2001); it is well camouflaged with a pattern that is almost identical in color and texture to the sponge (Carlson and Hoff, 1973). In contrast, *S. psychedelicum* has a bright and remarkable color pattern that makes it obvious (Carlson and Hoff, 1974), which is consistent with an aposematic coloration (Gosliner, 2001). Both sagaminopteran species are associated with their host sponge and, in contrast to other gastropterids, neither is a spectacular swimmer, especially *S. nigropunctatum*, which seldom swims (Carlson and Hoff, 1973).

Many opisthobranchs feed on chemically defended prey (Faulkner, 1992; Avila, 1995). In a predator, the presence of secondary metabolites produced by its prey is considered evidence of a trophic relationship. Studies in chemical ecology show that

opisthobranchs sequester secondary metabolites from prey, accumulate, concentrate, and distribute them in their tissues, and use them for their own defense (Carté and Faulkner, 1983; Paul et al., 1990; Becerro et al., 2001, 2003). This strategy of sequestration, concentration, modification, and distribution of metabolites from the prey into the body of the predator is found widely in nudibranchs (Cimino and Ghiselin, 1999 and references therein), sacoglossans (Cimino and Ghiselin, 1998; Cimino et al., 1999; Becerro et al., 2001), notaspideans (Teeyapant et al., 1993; Ebel et al., 1999; Becerro et al., 2003), and sea hares (Johnson and Willows, 1999). *D. granulosa* is a chemically defended sponge that produces a number of polybrominated diphenyl ethers (Becerro and Paul, 2004). Given the close and consistent association of both gastropterid species with *D. granulosa* and the widespread occurrence of compound sequestration and biomagnification observed in specialist predators, we set forth the hypothesis that, if they are true specialist predators, both *S. nigropunctatum* and *S. psychedelicum* will share secondary chemistry with their host sponge *D. granulosa*. However, given the contrasting color patterns shown by the two species, they might differ in their defense strategies. Thus, we present two working hypotheses: (1) consistent with its warning coloration, *S. psychedelicum* will accumulate, concentrate, and distribute in its parapodia compounds sequestered from *D. granulosa*; and (2) the cryptic *S. nigropunctatum* will sequester sponge secondary metabolites in its digestive gland and will fail to concentrate and adequately distribute these metabolites in its tissues thus, relying on camouflage.

Methods and Materials

On Guam, *D. granulosa* (Bergquist 1965, n. sp., aff. *granulosa*; Kelly et al., 2003) (Family Dysideidae, Order Dictyoceratida) is a common shallow-water sponge mostly found on reef flats, where it can be abundant. *D. granulosa* is abundant up to a depth of 5–6 m in Gun Beach, a reef slope habitat with a series of 1- to 5-m-wide channels with vertical walls perpendicular to the shoreline and open to wave action and surge. On this location, *D. granulosa* has three polybrominated diphenyl ethers (BDEs) as major secondary metabolites (Becerro and Paul, 2004). On Gun Beach, the gastropterids *S. psychedelicum* and *S. nigropunctatum* are consistently found on *D. granulosa*, sometimes sharing the same specimen. Neither is highly abundant, and because of their small size (smaller than 0.5 cm in our study site), they are difficult to observe.

Chemical Analysis

We collected five specimens of *S. psychedelicum*, nine specimens of *S. nigropunctatum*, and 10 egg masses of *S. nigropunctatum*. We found no egg masses of *S. psychedelicum* at the time of collection. All samples were collected by scuba diving, and taken to the laboratory where they were frozen at −20°C and stored until analyzed and used in experiments.

Frozen gastropterids were thawed and rinsed with distilled water to remove any secretion released by the animals. Gastropterids were dissected to separate parapodia and "tail," digestive gland, and the remainder of the body. Because of the small size and in order to have enough material for chemical quantification, the same body parts from all specimens were pooled in preweighed vials and freeze-

dried. Eggs were rinsed in distilled water and freeze-dried. Samples were then extracted with 1:1 (v/v) DCM/MeOH. This extraction procedure differs slightly from that used by Becerro and Paul (2004) to extract BDEs from sponge samples, since we used DCM/MeOH instead of DCM as the extraction solvent. However, BDEs are exhaustively extracted by both solvent mixtures, and we did not find more polar secondary metabolites in the sponges or the gastropterids. The crude extract was redissolved in DCM and filtered through glass wool and a 1-cm, 60–200 mesh silica gel pipette column in preparation for gas chromatography.

We used gas chromatography/mass spectrometry (GC-MS) to quantify BDEs in the gastropterid body parts. For quantification by GC-MS, we dissolved crude extracts in DCM (2 ml/mg) containing naphthalene (50 μg/ml) as internal standard (IS). Quantification was done with a Hewlett-Packard 5890 Series II GC fitted with an HP-5 capillary column (5% phenyl methyl crosslinked silicon siloxane, 30 m long, 0.25 mm i.d., 0.25 μm film thickness) and coupled to a Hewlett-Packard 5972 MS. Gas chromatography was performed following methods of Becerro and Paul (2004). One μl of extract solution at a concentration of 0.5 mg/ml was injected. We quantified the % yield of compounds by comparing the ratio "area of compound divided by area of IS" in the samples to a calibration curve calculated with known concentrations of pure compounds. To calculate the % yield of BDEs per sample dry mass, we multiplied the yield of BDEs in the extract by the yield of crude extract in the sample × 100.

We compared the concentration of compound **2** [3,5 dibromo-2-(2′,4′-dibromophenoxy)phenol] in gastropterid body parts to the concentrations found by Becerro and Paul (2004) in the sponge ectosome and choanosme. Because we pooled the same gastropterid body parts from the same species, we have a single concentration value for each body part, i.e., we obtained an average value from our replicates but we lack variance data. To test whether our single values differ statistically from the replicated ectosome and choanosome data ($N = 10$, respectively), we used a t-test for a single specimen compared with the mean of a sample (previous arcsin transformation to meet parametric assumptions) as described by Sokal and Rohlf (1995).

Ecological Experiments

We ran two sets of experiments to investigate the relationship between the gastropterids and the sponge, and to assess whether gastropterids are defended against generalist predators. All experiments were run with *S. nigropunctatum* because we found no *S. psychedelicum* at the time of collection. We collected pieces of *D. granulosa* hosting *S. nigropunctatum* at Gun Beach. To reduce the likelihood of the animals discharging secretion, we collected the opisthobranchs by breaking off pieces of sponge rather than removing the animal from the sponge. The gastropterids and sponges were placed in large plastic bags with seawater and transported to the University of Guam Marine Laboratory in a cooler. In the laboratory, sponges were cleaned of epifauna and cut into equal sized pieces of ectosome and choanosome. Ectosome refers to the outer layer of sponge (about 2 mm in thickness), whereas choanosome refers to the gray interior. The ectosome contains abundant cyanobacteria that makes this zone a greenish-purple color. There also are bacterial and chemical differences between these two sponge parts (Becerro and Paul 2004). In the field, gastropterids are found on the surface of the sponge (ectosome), and we tested whether gastropterids show a preference for the

ectosome over the inner choanosme. Paired ectosome and choanosome sponge pieces were placed in watch glasses with single gastropterids ($N = 17$) and left until the animal chose one of the sponge pieces up to a maximum of 1 hr. We then scored the number of gastropterids present. Animals were left in the bowl for an additional hour and scored again. We used a *binomial distribution* ($P = Q = 0.5$) to test for significant preferences between ectosome and choanosome sponge pieces (two-tailed P values reported) at the two time intervals.

To test whether *S. nigropunctatum* was defended against generalist predatory fish, the same specimens used in the previous choice experiment were then offered to the sharpnose pufferfish *Canthigaster solandri* (Richardson, 1845). The pufferfish were kept in individual flowthrough aquaria (10 l) and maintained on artificial commercial catfish food pellets. We recorded whether *S. nigropunctatum* was eaten, tasted and rejected, or ignored by the fish. Immediately after this trial, we offered the same fish a piece of equally sized skinned octopus tentacle. Since we tested the same individual fish twice (first for treatment and then control tests), we used a McNemar test to check whether *S. nigropunctatum* inhibited feeding of the pufferfish compared to control food (Sokal and Rohlf, 1995).

Results

The extract of *D. granulosa* from Gun Beach has three major BDEs. The compound 3,5 dibromo-2-(2′,4′-dibromo-phenoxy)phenol (hereafter referred to compound **2**, as in Becerro and Paul 2004) is the major metabolite in the extract and corresponds to a % yield per dry mass of sponge of 2.78 ± 0.27 (mean ± SE) in the ectosome and 3.21 ± 0.67 in the choanosome (Becerro and Paul 2004). Other BDEs closely related to **2** that are found in lower concentrations in the sponge were not detected in the gastropterids (see Becerro and Paul, 2004 for a more comprehensive analysis of the chemical variation of the sponge *D. granulosa*).

Both *S. psychedelicum* and *S. nigropunctatum* share secondary chemistry with the sponge *D. granulosa*. We detected **2** in all body parts from both species, including the egg mass of *S. nigropunctatum* (Fig. 1). However, concentration of **2** between body parts and species differed quantitatively (Fig. 1, note lack of variance in body parts due to replicate pooling). Concentration of **2** sequestered in the digestive gland may depend on the amount of food in the gut and the actual concentration of compounds in the material ingested. *S. nigropunctatum* concentrates **2** in the parapodia or mantle's edge up to an average concentration of 10.10% per dry mass, which is a significant increase as compared to the concentration found in the sponge ectosome and choanosome ($P < 0.001$ and $P = 0.019$, respectively). *S. psychedelicum* also concentrates **2** in the parapodia up to an average concentration of 7.97%, which is a significant increase as compared to the ectosome ($P = 0.001$) and almost as much as the choanosome ($P = 0.055$). The body tissues of both gastropterid species and the egg masses and secretion of *S. nigropunctatum* had concentrations of **2** similar to those found in either the sponge ectosome or choanosome ($P > 0.05$ for all comparisons). Compound **2** was also present in the secretion of *S. psychedelicum* but below the quantification threshold.

In laboratory choice experiments, *S. nigropunctatum* significantly selected the outer ectosome of *D. granulosa* over the interior choanosome. At the initial choice, 12 out of 17 molluscs selected the ectosome over the choanosome (binomial test,

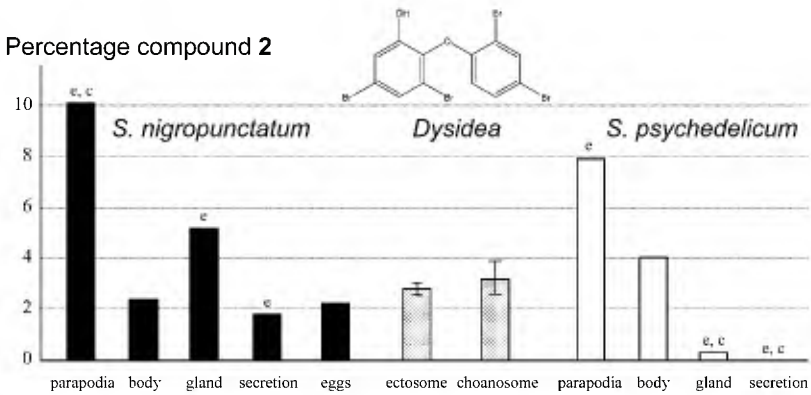

Fig. 1 Concentration (as % dry mass) of compound **2** (3,5-dibromo-2-(2′,4′-dibromo-phenoxy)phenol) found in several tissues of the gastropterid *Sagaminopteron negropunctatum* and *S. psychedelicum*. Note lack of error bars in gastropterid body parts due to replicate pooling. Mean (±1 SE) percent of compound **2** in the ectosome and choanosome ($N = 10$) of the sponge *Dysidea granulosa* is shown for comparison. Sponge data from Becerro and Paul (2004). Letters "e" or "c" above bars denote significant differences between the bar value and the ectosome or choanosome data, respectively ($P < 0.05$)

$P = 0.047$). After 1 hr, three of the five specimens that chose pieces of choanosome had moved away and were observed on the ectosome ($N = 1$) or crawling on the aquarium ($N = 2$) (binomial test, $P = 0.018$). Individuals that chose a piece of ectosome remained on it until the end of the experiment.

In feeding assay experiments, *S. nigropunctatum* deterred feeding of the pufferfish *C. solandri* (McNemar $G = 4.159$, $P < 0.05$); eight out of 10 pufferfish tasted and rejected the gastropterid in the laboratory, three of which consumed the control food. No fish fed on the gastropterid and rejected control food.

Discussion

Cephalaspideans are a polyphyletic group of opisthobranch molluscs that consume a variety of diets such as polychaetes, molluscs, urchins, or foraminifera (Mikkelsen, 1996). There is some controversy about gastropterid feeding habits and whether or not they are true sponge feeders, with indirect evidence either supporting or casting doubts on this hypothesis (see below). The hypothesis that gastropterid are sponge feeders is mostly based on the close association between gastropterids and sponges, including the close association between *S. psychedelicum* and *S. nigropunctatum* and their host sponge *D. granulosa* investigated in this study (*Dysidea* cf. *reticulata* in Carlson and Hoff, 1973, 1974). Yet, a close association may not necessarily involve a trophic relationship. Nudibranch species of the genus *Trapania* are associated with sponges, which were assumed to be their food source. However, there is some evidence that *Trapania* species seem to feed on entoprocts that grow on top of the sponges rather than feeding on the sponges themselves http://www.seaslugforum.net/display.cfm?id=3767).

Anatomical data argue against *Sagaminopteron* species as sponge feeders. Gastropterid radular teeth are similar to those of their relatives, none of which

feed on sponges but instead on hard-shell prey such as the polychaete *Pectinaria*, small clams, snails, sea urchins, and foraminiferans (Hurst, 1965; Burn and Bell, 1974a,b; Shonman and Nybakken, 1978). Accordingly, it is reasonable to hypothesize that *S. nigropunctatum* and *S. psychedelicum* feed on food sources they encounter while crawling on the sponge surface. For example, aeolid nudibranchs typically feed on cnidarians, but species of the genus *Calma* have a modified radular structure (a species-level character) to feed efficiently on fish egg masses (Calado and Urgorri, 2002). Alternatively, gastropterids could feed on sponges without evolving a modified radula. Sacoglossans are considered highly specialized herbivores that feed on green algae (Williams and Walker, 1999), but some species of the genera *Favorinus*, *Stiliger*, and *Olea* are oophagous rather than herbivorous (Thompson and Brown, 1984)—yet they show no radular adaptations that reflect their change of diet. Likewise, polyceratid nudibranchs typically feed on bryozoans, but species of the genera *Roboastra* and *Nembrotha* feed on nudibranchs and ascidians, respectively (Carté and Faulkner, 1986; Paul et al., 1990; Megina and Cervera, 2003), and show no radular modifications to do so.

Our study shows that *S. nigropunctatum*, *S. psychedelicum*, and their host sponge *D. granulosa* share secondary chemistry, which supports the hypothesis that gastropterids are sponge feeders. Pawlik et al. (1988) showed that the Spanish dancer nudibranch *Hexabranchus sanguineus* sequesters its chemical defense from its food sponge *Halichondria* sp., and distributes and concentrates the chemical defenses in the mantle, mucus secretion, and the egg masses. The gastropterids *S. psychedelicum* and *S. nigropunctatum* seem to share the same strategy, even though they are a fraction of the size. Both species sequester, distribute, and accumulate sponge BDEs in their parapodia, a strategy similar to that found not only in *H. sanguineus* but in many other opisthobranch molluscs that feed on chemically defended prey (Pawlik et al., 1988; Faulkner, 1992; Avila, 1995; Avila and Paul, 1997). Thus, compound **2** (3,5-dibromo-2-(2′,4′-dibromo-phenoxy)phenol), the major BDE in *D. granulosa*, is present in the mantle of the gastropterids at over twice the concentration found in the sponge, and additionally is present in the digestive glands. When disturbed, both gastropterids discharge a mucus secretion that include **2**, which suggests BDEs contribute to predator deterrence. Also, *S. nigropunctatum* transfers **2** to egg masses, where it could help prevent predation or fouling by bacteria, fungi, or other benthic organisms (Matsunaga et al., 1986). BDEs have a variety of biological activities (Sharma and Vig, 1972; Fu et al., 1995; Handayani et al., 1997) including defense against predators (Duffy and Paul, 1992; Pennings et al., 1994). Compound **2** deters a variety of predators including pufferfish *C. solandri*, xanthid crabs *Leptodius* spp., and the sea hare *Stylocheilus longicauda* (Duffy and Paul, 1992; Pennings and Paul, 1993; Pennings et al., 1994) even at concentrations below those found here. The pufferfish *C. solandri* tasted and rejected specimens of *S. nigropunctatum* in the laboratory suggesting that it is defended against them.

Coral reefs are among the ecosystems with the highest levels of predation (Carpenter, 1986) including generalist fish predators that use visual cues to obtain food (Lowe-McConnell, 1987). *S. nigropunctatum* is highly cryptic on *D. granulosa* since the gastropterid mimics both the color and texture of the sponge. Egg masses and some orange parts on the head shield and siphon of *S. nigropunctatum* are the only indications of its presence. *S. nigropunctatum* also mimics *D. granulosa* chemically by incorporating the sponge major metabolite in its tissues. Consequently, predators may overlook *S. nigropunctatum* in the field as a result of visual and

chemical camouflage. When cryptic coloration fails, chemical defenses may help the gastropterid overcome predators.

In contrast, *S. psychedelicum* has a conspicuous color pattern, which suggests a true aposematic coloration (Edmunds, 1991; Gosliner, 2001). Being conspicuously advertised is just one of the four criteria needed to fit the definition of aposematic coloration (Edmunds, 1991). It is also necessary to know whether or not (1) the organism deters predators, (2) predators avoid attacking a potential prey because of its colors, and (3) the aposematic color pattern is more effective than being cryptic (Edmunds, 1991). Although not tested, it is reasonable to think that *S. psychedelicum* is also chemically defended against predators, because it shares the same chemical as *S. nigropunctatum* at concentrations above those known to deter potential consumers. Despite the high numbers of generalist predators sharing habitat with the gastropterid, we have seen no sign of predation on this species in the field. Whether or not the striking color pattern of *S. psychedelicum* is more effective than the alternative cryptic coloration found in *S. nigropunctatum* is unknown, but these gastropterids may be an excellent system to understand better the role that color patterns and chemistry play in predation.

Our data also show that *S. nigropunctatum* significantly prefers the ectosome of *D. granulosa* over the interior choanosome. Some species of the genus *Dysidea* are known to contain large number of cyanobacteria. The cyanobacteria *Oscillatoria spongeliae* may represent up to 50% of the tissue volume of *D. herbacea* and is thought to be responsible for the production of BDEs in the sponge (Unson et al., 1994; Faulkner et al., 2000). *D. herbacea* and *D. granulosa* have genetically distinct populations of *O. spongeliae* (Thacker and Starnes, 2003), which are restricted to the ectosome of *D. granulosa* (Becerro and Paul, 2004). Cyanobacteria might also be responsible for the production of the BDEs in *D. granulosa* (although see Elyakov et al., 1991; Voinov et al., 1991). Whether they are behind the preference of *S. nigropunctatum* for the ectosome as described for the opisthobranch *Tylodina perversa* (Becerro et al., 2003) is unknown. The possibility that other chemical or structural differences between the ectosome and choanosome of the sponge or other microorganisms associated with the sponge may be responsible for the selection by the gastropterid cannot be completely ruled out. The use of standard methods in chemical ecology along with appropriate experimentation may provide new light on the feeding biology of opisthobranch molluscs and help us understand the role that color patterns and chemical defenses play in their biology, ecology, and evolution.

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