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Dietary preferences of the opisthobranch mollusc *Stylocheilus longicauda* for secondary metabolites produced by the tropical cyanobacterium *Lyngbya majuscula*

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Abstract Pure compounds isolated from the cyanobacterium *Lyngbya majuscula* Gomont were evaluated in an artificial diet for their influence on the feeding preferences of the sea hare *Stylocheilus longicauda* (Quoy and Gaimard, 1824), which lives in and feeds on this filamentous cyanobacterium (blue-green alga). Microcolin B, ypaoamide, malyngolide and other natural products acted as feeding deterrents at natural concentrations. At lower concentrations, sea hares were indifferent to ypaoamide and malyngolide in their diets. In contrast, barbamide stimulated sea hare feeding at the concentrations normally found in *L. majuscula*. Malyngamides and majusculamides, the most common natural products found in samples of *L. majuscula* from Guam, increased sea hare feeding at low concentrations and inhibited feeding at the higher concentrations that occurred in some collections of *L. majuscula*. Dietary selection of cyanobacteria by *S. longicauda* may be regulated by the concentration of specific chemical cues produced by *L. majuscula*.

Introduction

The feeding specificity of herbivores may be regulated by several factors, including the availability of "enemy-free space", resource-partitioning, mating behavior, and host-plant chemistry (Fox and Morrow 1981; Bernays and Graham 1988; Schultz 1988; Thompson 1988; Bernays and Cornelius 1989; Hay 1992). The relative importance of these factors is a topic of considerable debate. Because specialist herbivores are relatively rare in marine systems, at least compared with insects in terrestrial systems, the importance of different factors that select for host-plant specialization may be easier to determine in marine communities (Hay 1991, 1992). Like many specialist terrestrial herbivores (Rank 1992; Rowell-Rahier and Pasteels 1992), marine specialist herbivores are often unaffected by, or even stimulated to feed by, host natural products (Hay et al. 1989, 1990).

The sea hare *Stylocheilus longicauda* (Quoy and Gaimard, 1824) (Opisthobranchia: Anaspidea) specializes on the benthic, filamentous cyanobacterium *Lyngbya majuscula* Gomont (Oscillatoriaceae) [= *Microcoleus lyngbyaceus* Kützing] (Switzer-Dunlap and Hadfield 1979; Paul and Pennings 1991), and sequesters natural products, which can function in defense against predators, from this cyanobacterium (Paul and Pennings 1991; Pennings and Paul 1993a; Pennings et al. 1996). Switzer-Dunlap and Hadfield (1977) reported that larval *S. longicauda* metamorphosed on *L. majuscula* and several species of red algae, but that only *L. majuscula* supported rapid growth of juveniles. Large numbers of *S. longicauda* can be found on *L. majuscula* in the field (Switzer-Dunlap and Hadfield 1979; Paul and Pennings 1991; Nagle and Paul personal observation), *S. longicauda* prefers *L. majuscula* over other algae in laboratory choice-tests, and of six algae tested, only *L. majuscula* supported rapid growth in laboratory assays (Paul and Pennings 1991). Thus, considerable evidence suggests that this sea hare is a specialist on *L. majuscula*.

Like many cyanobacteria, *Lyngbya majuscula* is a rich source of biologically active natural products including both deterrent and toxic compounds (Moore 1981, 1982; Orjala and Gerwick 1996). The *L. majuscula* metabolites Malyngamides A and B (Paul and Pennings 1991; Pennings et al. 1996; Thacker et al. 1997), Majusculamides

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A and B (Pennings et al. 1996), and malyngolide (Thacker et al. 1997) have been demonstrated to act as feeding deterrents to reef fishes. Aplysiatoxins, produced by *L. majuscula*, were among the first natural products to be identified from Hawaiian specimens of *Stylocheilus longicauda* (Kato and Scheuer 1974, 1975; Mynderse et al. 1977). The cyanobacterial lipopeptides Malyngamides A and B (Paul and Pennings 1991) and Majusculamides A and B (Pennings et al. 1996) have since been isolated from extracts of *S. longicauda*. Initial studies have suggested that cyanobacterial chemistry does not determine diet selection by *S. longicauda*, because Malyngamides A and B deterred feeding by *S. longicauda* as effectively as a variety of other secondary metabolites from macroalgae and sponges (Pennings and Paul 1993b). However, these experiments tested *L. majuscula* compounds only at relatively high concentrations (2% of dry mass). Thus, the underlying mechanisms responsible for the cyanobacterial diet specialization of *S. longicauda* have remained unclear.

During numerous collections of *Lyngbya majuscula* from Guam and Micronesia, we observed that *Stylocheilus longicauda* was only found on some chemotypes of this cyanobacterium. While a structurally diverse array of natural products can be found in *L. majuscula*, on Guam, *S. longicauda* is most commonly observed on *L. majuscula* containing Malyngamides A and B and Majusculamides A and B (Paul and Pennings 1991; Nagle and Paul personal observation). In this study, we evaluated pure compounds isolated from numerous collections of *L. majuscula* for their ability to influence *S. longicauda* feeding-preferences. We hypothesized that dietary selection of cyanobacteria by *S. longicauda* is regulated by the concentrations and types of specific chemical cues produced by *L. majuscula*.

Materials and methods

Sea hares

Specimens of *Stylocheilus longicauda* (Quoy and Gaimard, 1824) were collected from the walls of tanks at the University of Guam Marine Laboratory; juveniles periodically recruit into these tanks, which are supplied with continuously-flowing unfiltered sea water. Benthic specimens were also collected from *Lyngbya majuscula* (Gomont) in the field and added to our holding tanks. Even without an available source of *L. majuscula*, the sea hares eventually grew to a size of 2 to 5 cm (reproductive adults, suitable for assay) while feeding on film-forming cyanobacteria which grow on the walls of these outdoor tanks. During the course of this study, the sea hares were maintained in several 20-liter tanks, and were rotated throughout the assays. Most experiments were conducted when large numbers of sea hares were available, and individuals were not re-used. Occasionally, when numbers of sea hares were low, an individual was used for more than one feeding assay; however, the same sea hare was never used more than once in the same assay.

Evaluation of *Lyngbya majuscula* metabolites

Metabolites were isolated from numerous collections of *Lyngbya majuscula* made throughout Micronesia, Okinawa, and Curaçao (Caribbean). Chemically diverse natural products with a wide variety of biological activities were selected for the study (Fig. 1). The antibiotic lipid, malyngolide (Cardellina et al. 1979), has recently been demonstrated to act as a feeding deterrent to reef fishes (Thacker et al. 1997). Debromoaplysiatoxin is a cytotoxic (P-388 lymphocytic leukemia, Mynderse et al. 1977) chemical irritant (Kato and Scheuer 1974, 1975). Peptide- and lipopeptide-derived metabolites were also evaluated. Malyngamides and majusculamides, the most commonly encountered metabolites in *L. majuscula* from Guam, deter feeding by herbivorous and omnivorous fishes (Paul and Pennings 1991; Pennings et al. 1996). Barbamide, an unusual polychlorinated metabolite isolated from a Curaçao collection of *L. majuscula*, has been shown to be potently lethal to the snail *Biomphalaria glabrata* (Orjala and Gerwick 1996). Curacin A is a new antimitotic agent, with potent toxicity to brine shrimp (*Artemia* sp.) (Gerwick et al. 1994). Microcolins A and B are cytotoxic and immunosuppresive at n*M* concentrations (Koehn et al. 1992). Ypaoamide is a recently identified new lipopeptide which acts as an effective feeding deterrent to several species of herbivorous reef fishes (*Scarus schlegeli*, *Siganus argenteus and S. spinus*) and the sea urchin *Echinometra mathaei* (Nagle et al. 1996).

Isolation of natural products

Samples of *Lyngbya majuscula* from Guam and Micronesia were either extracted immediately after collection or stored at −20 °C until extracted with 1:1 (v/v) dichloromethane:methanol (4×). Solvents were removed from combined crude extracts by rotary evaporator. Crude extracts were fractionated by silica gel vacuum-flash column-chromatography (a series of hexanes:ethyl acetate:methanol fractions of increasing polarity). Sephadex LH-20 size exclusion columns (1:1 dichloromethane:methanol) were used to remove impurities in an intermediate purification step. Final purifications were typically accomplished by normal-phase (silica gel) high-performance liquid chromatography (HPLC) of column fractions with mixtures of hexanes:ethyl acetate. Metabolites were identified spectroscopically by comparison of proton or carbon nuclear magnetic resonance (NMR) spectra with those of published standards. Several pure compounds extracted and isolated as described in the original literature were supplied by Dr. W.H. Gerwick (Oregon State University): barbamide (Orjala and Gerwick 1996), Curacin A (Gerwick et al. 1994), Malyngamide I (Todd and Gerwick 1995), and Microcolins A and B (Koehn et al. 1992). Compound concentrations were based upon direct measurements of compound yield as a percentage of cyanobacterial dry mass, and were determined in our laboratory and W.H . Gerwick's laboratory. Most of the yields are also published in the original chemical literature.

Feeding assays

To measure preferences between different foods, sea hares were offered artificial diets molded onto fiberglass window-screening, in a method similar to that of Hay et al. (1994) and Lumbang and Paul (1996). The artificial food consisted of 0.9 g finely powdered freeze-dried *Enteromorpha clathrata* (Roth), 1.7 g commercial catfish food (Kruse's Perfection Brand, O.H . Kruse Grain and Milling, El Monte, California), 0.6 g agar, and 25 ml water. This food was developed based on previous research with *Stylocheilus longicauda* in our laboratory. Pennings and Paul (1993b) found that *S. longicauda* lost mass on diets that contained less than 10% protein by dry mass, and they developed a high-protein diet (29% protein per dry mass) containing the protein-rich cyanobacteria *Spirulina* spp. for their feeding assays. This diet supported growth of the sea hares. For this study, we decreased the protein content of the diet to 19% protein per dry mass and substituted the catfish pellets (36% protein) for *Spirulina* spp. (46% protein). This modified diet was readily consumed by the sea hares and also sustained their growth. While this artificial diet is higher in protein than a typical algal diet, some species of cyanobacteria have a high protein content (40 to 60% of dry mass) (Becker 1994: p. 178). *Lyngbya*

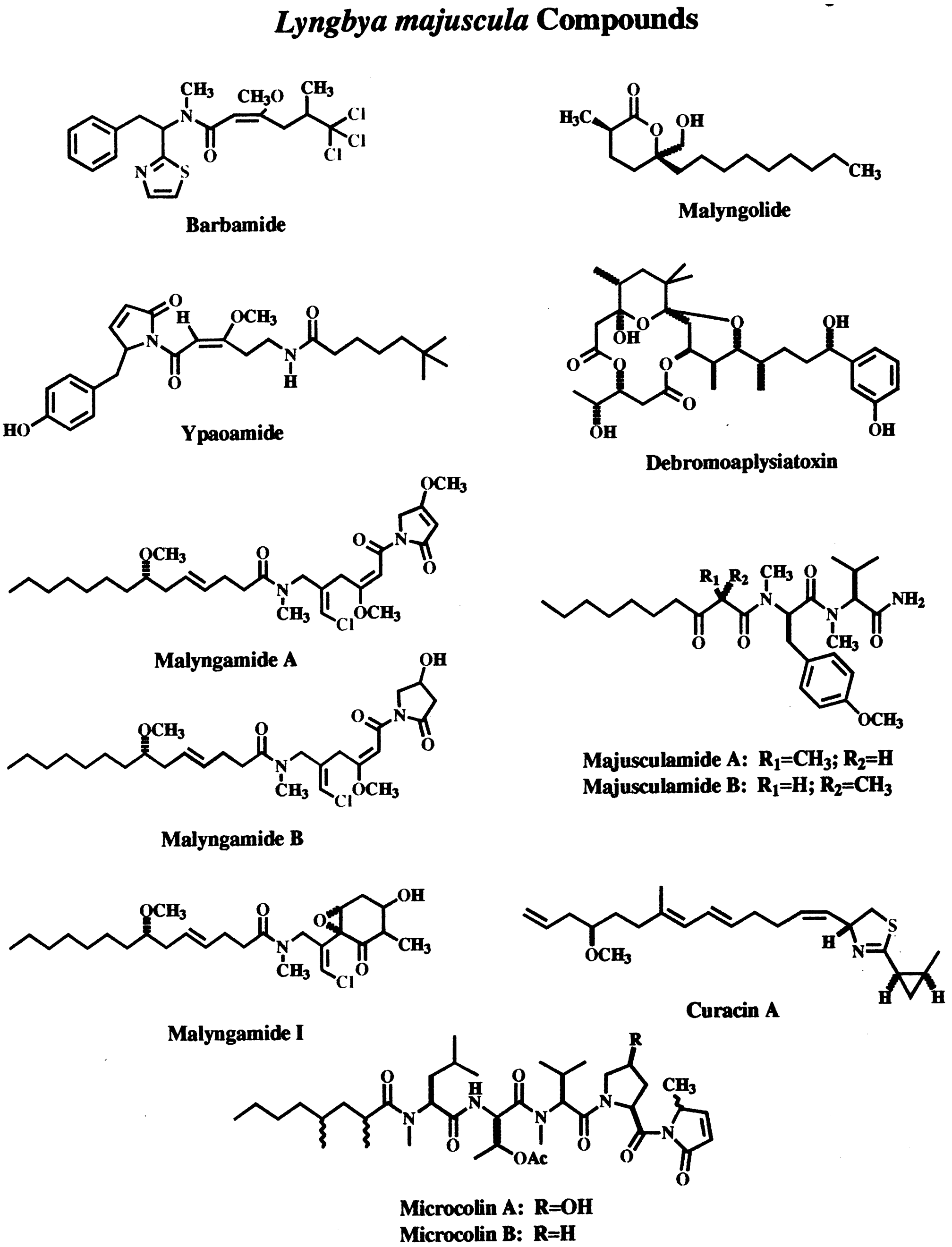


Fig. 1 *Lyngbya majuscula*. Structures of secondary metabolites studied

majuscula is no exception; possessing amongst the lowest C:N ratios of 92 marine plants surveyed (Atkinson and Smith 1983), *L. majuscula* is a good source of nitrogen (2.9 and 4.8% N, ≃ 18 and 30% protein based on the standard conversion factor of 6.25 × nitrogen wt = protein wt, for two collections from Enewetak and Hawaii, respectively), and is a much better source than other reef macroalgae (including other cyanobacteria such as *Schizothrix* spp.) (Atkinson and Smith 1983).

Pure compounds were added to the artificial food as a percentage of dry mass that reflected natural concentrations in *Lyngbya majuscula*. These concentrations were based on direct measurements of compound yield as a percentage of cyanobacterial dry mass; most of these yields are published in the original chemical literature. To incorporate these compounds into the artificial foods, the compound was dissolved in either dichloromethane or diethyl ether and added to the *Enteromorpha clathrata* powder and catfish food. Solvents were removed by rotary evaporation. Solvent was also added to and subsequently removed from all controls. Foods were prepared by bringing the agar and water to the boil in a microwave and then mixing in the *E. clathrata* and catfish food (with or without test compound) to the agar and water mixture while it was cooling, thus reducing the possibility of thermal decomposition. The mixture was then poured into a mold backed with fiberglass window-screening. The mold had two parallel 2.6 × 25 cm openings cut in it with a depth of 2 mm (see Fig. 1 in Hay et al. 1994 for diagram). One opening of the mold was filled with control food and the other with treated food containing *L. majuscula* compounds. The food assumed the shape of the mold and became attached to the screening. The molds were removed, and the screening was cut so that a length of screening contained both control and treated foods, each on a screen strip 1 cm wide with 17 × 7 or 17 × 8 square openings in the screen. The window-screen provided an easily readable grid to measure food consumption by number of squares eaten.

Individual sea hares, each in a plastic container with running sea water, were offered a choice between two food types (control and treatment). When at least one-half of one side of a food strip was eaten, the strip was scored and the differences between numbers of squares consumed for control and treated foods analyzed with a paired *t*-test. Differences were tested for normality by a Wilk–Shapiro/Rankit plot test (Statistix 4.0). In one case where the data were not normally distributed, a Wilcoxon signed-ranks test was performed. After the assays, the treated food strips were extracted with dichloromethane and examined by thin-layer chromatography to ensure that the cyanobacterial compounds had not decomposed and were still present in the extracts of treated food strips. No decomposition was noted.

Results

Stylocheilus longicauda showed a variety of feeding responses to the different *Lyngbya majuscula* metabolites. Some *L. majuscula* metabolites acted as feeding stimulants to *S. longicauda* at or even above natural concentrations (Fig. 2). Barbamide showed a pronounced feeding attractant activity when tested at the low concentration found in *L. majuscula* (0.007% or 70 ppm, $P < 0.0001$). Malyngamide I may somewhat stimulate *S. longicauda* feeding at 0.23%; however, its effect was not statistically significant ($P = 0.088$).

Many metabolites from *Lyngbya majuscula* were either inactive or acted as feeding deterrents to *Stylocheilus longicauda* at natural concentrations (Fig. 3). Microcolin B effectively deterred *S. longicauda* feeding at a concentration of 0.022% ($P = 0.0028$), while the more major epimer microcolin A showed no significant effect even at twice the concentration of Microcolin B. The feeding deterrent effects of neither Curacin A nor debromoaplysiatoxin were statistically significant when tested at their respective natural concentrations. Ypaoamide and malyngolide were evaluated at two different concentrations (Fig. 4). Ypaoamide had no significant effect at 0.23%; however, it acted as a powerful deterrent to feeding at 0.5%. The sea hares consumed about five times more control diet than ypaoamide treatment at 0.5% of dry mass ($P = 0.0046$). Similarly, malyngolide had no effect on feeding at 0.2%, but was strongly deterrent at the 0.4% concentration found in *L. majuscula* on Guam. Sea hares consumed almost none of the treated food at this higher concentration.

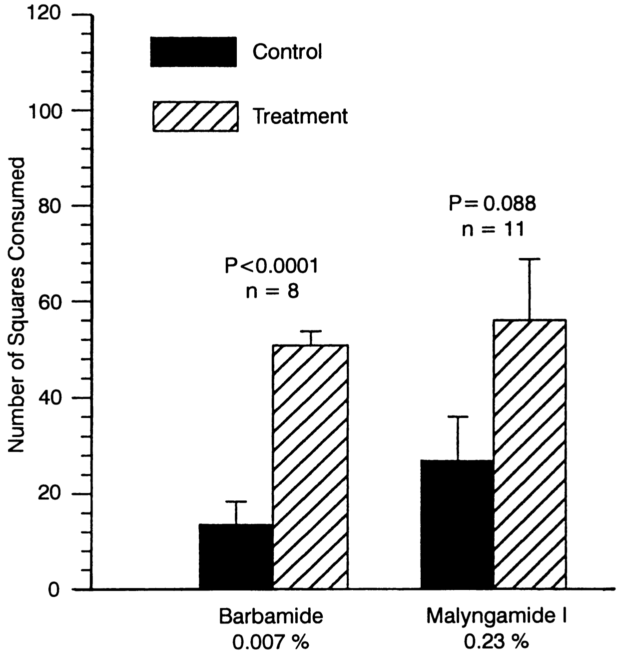


Fig. 2 *Stylocheilus longicauda*. Consumption of control foods and foods treated with compounds from *Lyngbya majuscula* that stimulate feeding at natural concentrations. Histogram bars show mean number of food squares consumed +1 SE. Control foods contained solvents only, treatment foods contained the compounds at concentrations indicated. Number of replicate pairs (*n*) and *P*-values are indicated for each paired *t*-test

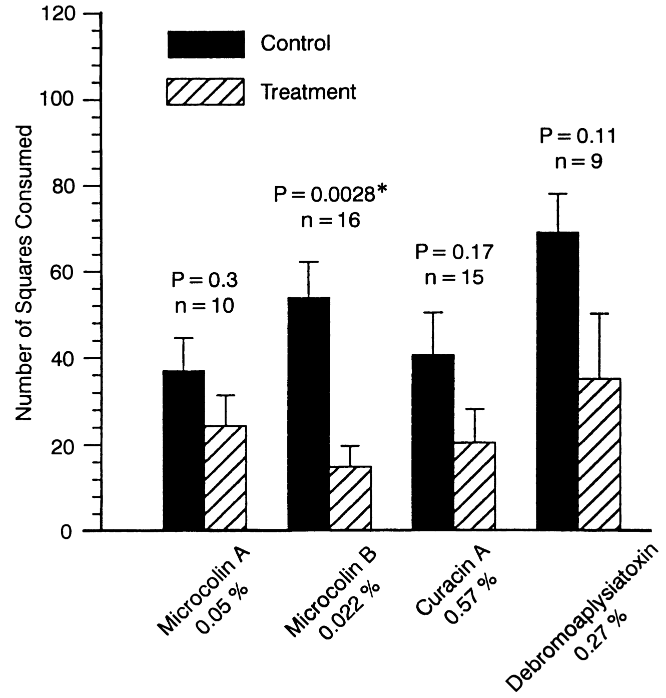


Fig. 3 *Stylocheilus longicauda*. Consumption of control foods and foods treated with compounds from *Lyngbya majuscula* that deter feeding (* significant effect) or have no significant effect at natural concentrations. Means and standard errors are plotted for each paired *t*-test as for Fig. 2

The malyngamide and majusculamide content of *Lyngbya majuscula* collections are highly variable. Malyngamide A and B concentrations ranged from non-detectable levels to ~2.0% of dry extracted mass in samples obtained throughout Micronesia and elsewhere (Pennings et al. 1996; Nagle and Paul personal observation). We therefore tested these compounds at a variety of concentrations in order to observe their effects over a concentration range (Fig. 5). Malyngamides and majusculamides acted as feeding attractants at the low end of their concentration range, but acted as powerful deterrents at higher concentrations.

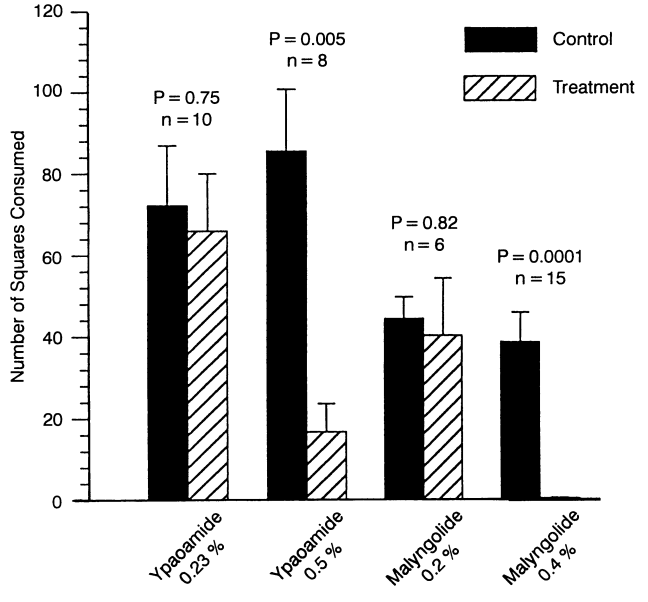


Fig. 4 *Stylocheilus longicauda*. Consumption of control foods and foods treated with compounds from *Lyngbya majuscula* that either deter feeding at natural concentrations or have no effect on feeding at slightly below natural concentrations. Means and standard errors are plotted for each paired *t*-test as for Fig. 2

When tested at lower concentrations (0.5 and 2.0% for Malyngamide A; 0.5% and 1.5% for Malyngamide B) *Stylocheilus longicauda* consumed from 2 to 6 times as much treated food as the control diet. However, when tested at slightly higher concentrations (4.0% Malyngamide A; 2.0% Malyngamide B) the sea hares were strongly deterred from feeding. In fact, the sea hares nearly stopped eating treated material, consuming from 17 (Malyngamide B) to 28 (Malyngamide A) times more control food than treated diet at the high end-concentrations.

Majusculamides A and B (combined) produced a "mixed" response feeding-activity similar to those observed for Malyngamides A and B (Fig. 4). The majusculamides strongly stimulated *Stylocheilus longicauda* feeding at 0.2%. Like the malyngamides, the majusculamides effectively deterred sea hare feeding at higher concentrations. The sea hares consumed about seven times more 0.2% treated food than control food ($P < 0.0001$). This ratio was completely reversed when the majusculamide concentration was raised to 1.0% ($P < 0.0001$).

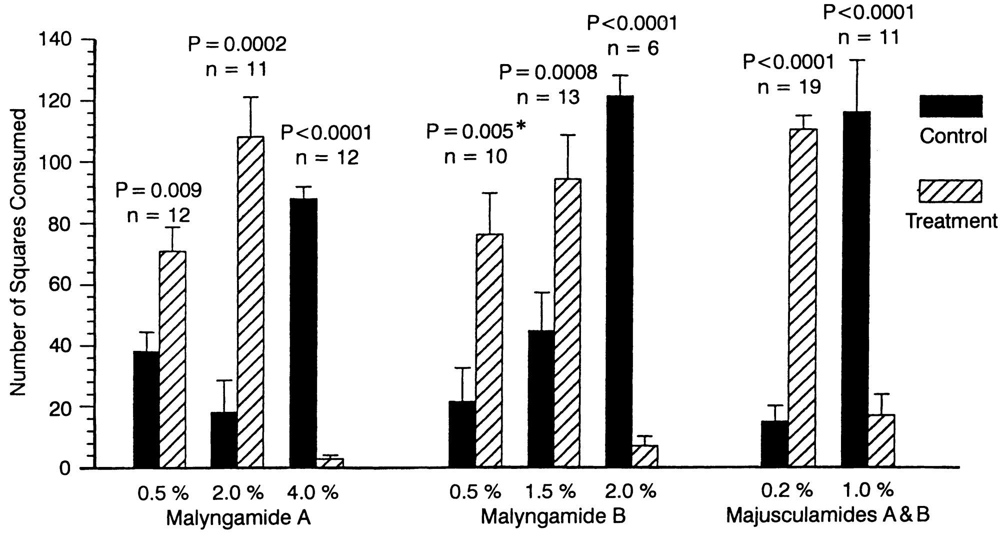
Discussion

Cyanobacterial metabolites which act as potent feeding-deterrents to herbivorous reef fishes (Paul and Pennings 1991; Pennings et al. 1996; Thacker et al. 1997) were tested for their ability to affect food preference of the specialist *Stylocheilus longicauda*. Barbamide acted to effectively stimulate feeding by *S. longicauda* at the low concentration of 0.007% (70 ppm), and is thus one of the most potent metabolites we have encountered. This is especially remarkable since barbamide is toxic to the snail *Biomphalaria glabrata* (LD_{100} = 100 ppm, in water, Orjala and Gerwick 1996).

Neither debromoaplysiatoxin nor Curacin A were statistically significant as sea hare feeding-deterrents at natural concentrations (Fig. 3); a surprise, considering the potent biological activities associated with these metabolites. Debromoaplysiatoxin is strongly cytotoxic (Mynderse et al. 1977), and Curacin A is a potent brine-shrimp toxin (*Artemia salina* LC_{50} = 0.0025 ppm in water: Gerwick et al. 1994; Orjala and Gerwick 1996). Toxicity and deterrency are often not correlated (Hay et al. 1987; Bernays 1991; Schulte and Bakus 1992; Pawlik et al. 1995), and *Stylocheilus longicauda* does not appear to select nor sequester diet-derived compounds based on their relative toxicities (Pennings et al. 1996).

We have never observed *Stylocheilus longicauda* feeding on the bloom-forming *Lyngbya* sp., which grows in mixed assemblages with other cyanobacteria.

Fig. 5 *Stylocheilus longicauda*. Consumption of control foods and foods treated with compounds from *Lyngbya majuscula* that either stimulate or deter feeding in concentration-dependent manner over range of natural concentrations. Means and standard errors are plotted for each paired *t*-test (* analyzed by Wilcoxon signed-ranks test for paired comparisons) as for Fig. 2



Ypaoamide, a broadly-acting herbivore feeding-deterrent, has recently been isolated from one such bloom on Guam (Nagle et al. 1996). Ypaoamide had little effect on sea hare feeding at low concentrations, but acted as a powerful feeding deterrent at only slightly higher concentrations (Fig. 4). Ypaoamide is inactive when tested at 0.23%, a concentration slightly below that found in the assemblage with the other cyanobacterium *Schizothrix calcicola*. Ypaoamide is an effective antifeedant at 0.5%, a concentration only slightly higher than that found in this assemblage (<0.4%) yet probably less than that occurring in the *L. majuscula* strands themselves, which seem to be the source of this compound (Nagle et al. 1996).

Malyngamides A and B and Majusculamides A and B are produced by numerous populations of *Lyngbya majuscula* found throughout Micronesia (Paul and Pennings 1991; Pennings et al. 1996; Nagle and Paul work in progress). The concentrations of these metabolites may range from only a trace to ~2% of *L. majuscula* dry weight (Nagle and Paul personal observation). These metabolites stimulated sea hare feeding at low concentrations and inhibited feeding at the high end of physiologically relevant concentrations (Fig. 5), and they showed this type of mixed activity within an extremely narrow concentration range. Similar effects have been observed in grasshopper feeding-assays, where compounds that deterred feeding at the higher end of natural concentrations acted as significant phagostimulants at lower concentrations (Bernays 1991).

Our results are consistent with the findings of Pennings and Paul (1993b) that relatively high concentrations of Malyngamides A and B deter feeding by *Stylocheilus longicauda*; however, we have now shown that lower concentrations of these same compounds and other *Lyngbya majuscula* metabolites can stimulate feeding. It is unclear whether this indicates adaptations to particular *L. majuscula* compounds or simply a generic tolerance to some secondary metabolites at lower concentrations, since we did not test the effects of secondary metabolites from a diversity of non-*Lyngbya* sources in this study. Nonetheless, our results clearly indicate that different chemotypes of *L. majuscula*, which differ in composition or concentration of secondary metabolites, should differ dramatically in acceptability to *S. longicauda*. This is consistent with our field observations from dozens of collections. We find that *S. longicauda* usually feeds on *L. majuscula* that contains malyngamides and majusculamides (Pennings et al. 1996; Nagle and Paul personal observation).

While other specialist or relatively specialized marine herbivores have been shown to be attracted to or unaffected by algal compounds (Hay et al. 1989, 1990; Steinberg 1995), to the best of our knowledge this is the first study to examine the responses of a specialist marine herbivore to the variety of types and concentrations of compounds found in its host. The feeding behavior of many insect specialists can also be strongly affected by variation in the chemistry of host plants (Rank 1992; Stadler 1992; Adler et al. 1995). Some insect specialists are stimulated to feed by higher concentrations of secondary metabolites (Rank 1992), others can be deterred at higher concentrations but stimulated to feed at lower concentrations of secondary metabolites (Bernays 1991), and some are negatively affected by some host-plant compounds but not others (Adler et al. 1995). Generalist herbivores in both marine and terrestrial habitats have usually been found to be deterred by higher concentrations of host-plant compounds when chemical variation and its effects on herbivory have been examined (Van Alstyne 1988; Zangerl 1990; Poore 1994; Cronin and Hay 1996).

This study provides evidence that dietary selection of cyanobacteria by *Stylocheilus longicauda* may be regulated by the concentrations and types of specific chemical cues produced by *Lyngbya majuscula*. The chemical complexity and variability of *L. majuscula* may create problems for *S. longicauda* in that its host may vary tremendously in quality both temporally and spatially. This diverse arsenal of natural products may be required by *L. majuscula* in order to deal with both generalist-grazers such as reef fishes and specialist-feeders such as *S. longicauda* in tropical reef habitats.

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