CHEMICAL DEFENSES IN THE SEA HARE *APLYSIA PARVULA* AND ITS HOST ALGA *PORTIERIA HORNEMANNII* AND THEIR EFFECTS ON PREDATION

BY

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Title: Chemical defenses in the sea hare *Aplysia parvula* and its host alga *Portieria hornemannii* and their effects on predation.

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Marine algae produce a variety of secondary metabolites that function as herbivore deterrents. Algal metabolites, however, often fail to deter damage by some herbivores such as mesograzers that both live and feed on their host alga. In addition, the degree to which intraspecific chemical variation in an alga affects a mesograzer's feeding behavior and its ability to deter predators is poorly understood. The red alga *Portieria hornemannii* contains the secondary metabolites apakaochtodene A and B, which have been shown to vary from site-to-site on Guam and act as significant deterrents to fish feeding. On Guam, the sea hare *Aplysia parvula* preferentially consumed and demonstrated the greatest growth on their algal host *P. hornemannii.* However, such a relationship did not come without a cost to sea hares grazing on this alga. High concentrations of *P. hornemannii* crude extract and the pure compounds apakaochtodene A and B acted as feeding deterrents to *A. parvula.* Despite site-to-site differences in the levels of total apakaochtodenes A and B, *A. parvula* showed no significant preference for *P. hornemannii* from any one location. However, differences in the intraspecific chemical and nutrient content of *P. hornemannii* affected the feeding behavior of *A. parvula* and its ability to choose between specific parts of algal thalli. There was no evidence of the induction of apakaochtodenes in *P. hornemannii* via

grazing by *A. parvula*. Lastly, *A. parvula* acquired apakaochtodenes, and whole animals as well as body parts were unpalatable to reef fishes. While these defenses were not effective against all predators, this observation is consistent with the hypothesis that diet-derived algal metabolites in sea hares play a role in deterring predation.

TO THE OFFICE OF GRADUATE SCHOOL AND RESEARCH

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INTRODUCTION

Marine algae produce a variety of secondary metabolites that function as herbivore deterrents (Hay and Fenical 1988, Paul 1992, Hay 1996). Under natural conditions, these compounds provide a selective advantage to the algae (Hay 1992, 1996, Paul 1992). Algal secondary metabolites may also have other roles as defenses against pathogens and fouling organisms, thus increasing the adaptive value of these metabolites (Paul 1992, Schmitt et al. 1995). Despite the clear importance of these latter roles, ecological studies that have examined algal secondary metabolites have most commonly focused on these metabolites as defenses against herbivores and predators (Hay and Steinberg 1992, Paul 1992, Hay 1996).

Algal metabolites often fail to deter damage by some herbivores, and may stimulate feeding by small, relatively sedentary herbivores such as amphipods, crabs, polychaetes, and some gastropods. These herbivores, collectively termed mesograzers, both live on and feed on their host alga (Hay et al. 1989, 1990a, b, Brawley 1992, Hay 1992, Trowbridge 1992). It has been hypothesized that the preference of some mesograzers for chemically-defended algae has been driven primarily by the advantages of decreased predation that mesograzers might experience while living and feeding on a host plant that is deterrent to fish predators (Hay et al. 1987, 1989, 1990a, b, Hay 1992, 1996).

The degree to which intraspecific chemical variation in an alga affects a mesograzer's feeding behavior and its ability to deter predators is poorly understood. While several studies have examined quantitative variation in algal secondary chemistry

(Carlton et al. 1989, Meyer and Paul 1992, 1995, de Nys et al. 1996, 1998, Puglisi and Paul 1997, Matlock et al. in press), few authors have examined the effects of within-algal variation on the feeding preferences of mesoherbivores (Cronin and Hay 1996a, Poore 1994, VanAlstyne 1989). Such studies are necessary not only to understand the factors affecting the production of chemical defenses, but also to provide insight into the ecological consequences of such variation (Hay 1996, Becerro et al. 1998).

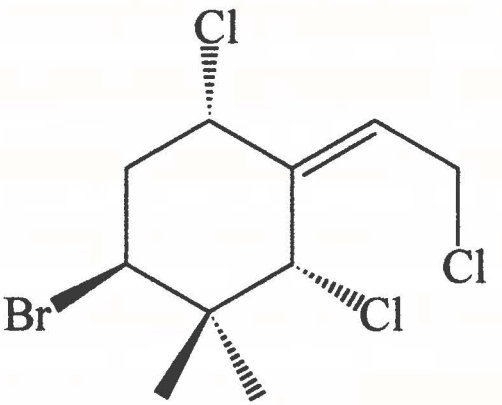
Factors affecting intraspecific variation in seaweed metabolites may include the induction (i.e., the increase in production or concentration of a metabolite) of chemical defenses in response to herbivory, examples of which are well known in terrestrial plants (Karban and Baldwin 1997). Herbivore induced responses in marine algae are known for only two genera, *Fucus* (Van Alstyne 1988, Yates and Peckol 1993) and *Dictyota* (Cronin and Hay 1996b), in which mesograzers (a snail and an amphipod, respectively) were implicated. Van Alstyne (1988) hypothesized that the induction of chemical defenses in seaweeds may cause levels of algal secondary metabolites to vary spatially and temporally. Conversely, intraspecific chemical variation may influence herbivory, which may result in variation in benthic algal assemblages (Hay 1996). Such variation in algal resources may have considerable effects on the susceptibility of mesograzers, which are relatively immobile, to potential predators such as reef fishes (Van Alstyne 1988, Hay 1996).

Few studies have examined how intraspecific variation in secondary metabolites of marine algae affects feeding by mesoherbivores which, in turn, may influence the vulnerability of these herbivores to predation (Poore 1994, Cronin and Hay 1996a, b, de

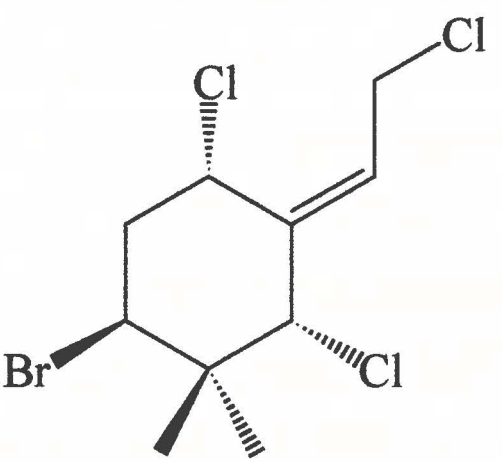
Nys et al. 1996). These types of interactions can be described by examining the chemistry as well as the palatability of organisms within an alga-mesograzer-predator, or tri-trophic, system. I chose to study the red alga *Portieria hornemannii* (Lyngbye) Silva (Gigartinales: Rhizophyllidaceae), the sea hare *Aplysia parvula* Mörch (Opisthobranchia: Anaspidea) and generalist, reef fish predators.

The red alga *Portieria hornemannii* was selected for this study because it exhibits notable site-to-site variation in secondary metabolite production (Paul et al. 1987, Fuller et al. 1992, 1994, Puglisi and Paul 1997, Matlock et al. in press) and is host to the sea hare *Aplysia parvula* (Carefoot 1987). On Guam, the major secondary metabolite of *P. hornemannii* is apakaochtodene B (Figure 1) which is an effective feeding deterrent against herbivores (Paul et al. 1987, 1990, 1992, Meyer et al. 1994). Several other halogenated monoterpenes including apakaochtodene A (Figure 1) are minor metabolites (Paul et al. 1987, Puglisi and Paul 1997, Gunatilaka et al. submitted).

The sea hare *Aplysia parvula* is an oligophagous herbivore that feeds primarily on red algae (Carefoot 1987, Gosliner 1987, Rogers et al. 1995) and is able to sequester algal metabolites from its diet which have been hypothesized to function in defense against predators (Faulkner 1992, de Nys 1995). Switzer-Dunlap and Hadfield (1981) reported that, in laboratory cultures, larvae of *A. parvula* preferentially metamorphose in the presence of *Portieria hornemannii*. On Guam, *A. parvula* preferentially graze and are primarily found to live on *P. hornemannii* from which animals sequester the algal metabolites apakaochtodene A and B (this study).



apakaochtodene A



apakaochtodene B

Figure 1. Chemical structures of apakaochtodene A and B.

To investigate how seaweed chemical defenses mediate ecological interactions among the sea hare *Aplysia parvula*, its algal host *Portieria hornemannii* and predatory reef fishes, I asked the following questions: (1) Do *A. parvula* prefer to consume and demonstrate the greatest growth on *P. hornemannii* compared to other algae? (2) Do site-to-site differences in the concentrations of apakaochtodenes found in *P. hornemannii* affect the feeding behavior of *A. parvula*? (3) Is there within-alga chemical variation in the production of apakaochtodenes within the tips and bases of *P. hornemannii* thalli and what is the affect of this variation on the feeding behavior of *A. parvula*? (4) Does grazing by *A. parvula* induce the production of apakaochtodenes in *P. hornemannii* thalli? (5) Are *A. parvula* (whole animals and body parts) deterrent to reef fish predators? (6) Lastly, do the varying concentrations of apakaochtodenes found in *P. hornemannii* from different sites, which are sequestered by *A. parvula*, differentially affect predation on sea hares by reef fishes?

MATERIALS AND METHODS

Collection sites and sampling methods

Portieria hornemannii and *Aplysia parvula* were collected from five sites on Guam (Figure 2): four sites on the leeward side, Double Reef (13°36′N; 144°48′E), Gun Beach (13°31′N; 144°48′E), Apaca Point (13°24′N; 144°40′E), and Anae Island (13°23′N; 144°38′E) and one site on the windward side of the island, Pago Bay (13°25′N; 144°48′E) (see Puglisi and Paul 1997 for a full description). Double Reef and Gun Beach are located at the northern end of Guam. Sea hares and algae were collected from Double Reef's northeastern reef and from the northern end of Gun Beach. Pago Bay, Apaca Point and Anae Island are located are located further south on Guam. Samples from Pago Bay were collected behind the University of Guam Marine Laboratory and collections from Apaca Point were made from the southern end of the point. Specimens collected from Anae Island, located ≈450 m offshore, were collected from the fringing reef on its eastern side.

Portieria hornemannii grows as tufts comprised of small individual thalli 3 to 9 cm tall with primary branches 0.5 to 1 mm in diameter, attached to rocks or dead corals in subtidal areas with heavy current (Trono 1969, 1997, Calumpong and Meñez 1997, Puglisi and Paul 1997). Tufts occur 0.5 to 3 m apart and were collected haphazardly from the reef.

Aplysia parvula is a small (0.4 to 3.5 cm), oligophagous herbivore that feeds primarily on red algae (Carefoot 1987, Gosliner 1987, Rogers et al. 1995, de Nys et al.

Figure 2. Map indicating location of study sites and collection areas.

1996). These animals are camouflaged with coloration similar to their host algae (Rogers et al. 1995). Due to their small size and camouflage, collection of animals in the field was impractical. Therefore, specimens of *A. parvula* were obtained by collecting whole tufts of *Portieria hornemannii*. Bulk collections of algae, from their respective sites, were brought back to the laboratory and placed in flow-through aquaria. Sea hares were gleaned from *P. hornemannii* as they were needed for each experiment.

Chemical extraction

Organic compounds were extracted from individual *Portieria hornemannii* thalli. Fresh or frozen whole individual thalli were rinsed in freshwater and cleaned of epiphytes, spun in a plastic salad spinner 20 revolutions and blotted dry. After recording fresh weights (to the nearest 10^{-4} g), thalli were ground with a Virtis high speed homogenizer in a 1 : 1 (vol : vol) solvent mixture of dichloromethane (DCM)-methanol (MeOH). The solvent and solid material were transferred to a flask and the volume brought to 50 ml. After 8 h, the extract was decanted through weighed filter paper (Whatmann GF/A Glass Microfibre filter), sealed and stored in a freezer at -20 °C. The solvents left in the flask were resuspended in 50 ml of fresh solvent and extracted an additional 8 h. A total of three successive extractions were performed on each sample, after which all remaining solids were dried on the filters and weighed. The combined extract from each thallus was concentrated by rotary evaporation and weighed. These crude extracts were then dissolved in hexanes, filtered through glass wool, dried, weighed, and stored at -20 °C prior to quantitative chemical analysis of the

apakaochtodene compounds. The extract yields of individual thalli were calculated using the dry weights from filtered, crude extracts.

Monoterpenes were also extracted from *Aplysia parvula.* Individual sea hares were frozen, dissected and their digestive gland and mantle removed. Because of the small size of *A. parvula*, it was necessary to combine replicate digestive gland and mantle parts to obtain sufficient material for chemical analysis. Pooled samples (digestive gland and mantle parts, respectively) were freeze dried, chemically extracted using acetone and prepared for analysis of apakaochtodenes as described above.

Chemical analysis

Dried extracts for gas chromatography-mass spectrometry (gc-ms) were redissolved in hexanes with naphthalene as an internal standard at a concentration of 100 $\mu g\ ml^{-1}$. Samples were diluted to a concentration of 1 ml of hexanes (with the internal standard naphthalene) per 1 mg crude extract (1 $mg\ ml^{-1}$). Gas chromatography was conducted using a Hewlett-Packard 5980 Series II Plus gas chromatograph with a cross-linked 5% methyl silicone column (HP-5, 30 m x 0.25 mm). Injections were made in the splitless mode with an inlet pressure of 13.9 kPa at 70 ° C. The injection port was held at 250 ° C with a 70- 290 ° C temperature ramp at 10 °C per minute. The carrier gas was helium at a flow rate of 0.5 $ml\ min^{-1}$.

Mass spectrometry was conducted with an HP 5972 Mass Selective Detector (MSD). Ions characteristic of the internal standard naphthalene and apakaochtodenes A and B were monitored in the selected ion monitoring (SIM) mode and were

quantitatively analyzed using purified standards. Quantification was performed with a multiple point (6 dilutions) external standard method using the HP ChemStation (1993) software. The peak areas of apakaochtodenes A, B and the internal standard naphthalene were measured and their ratio (compound/internal standard) calculated and converted to concentration by reference to standard curves specific to each pure compound. For both apakaochtodenes A and B, the amount of pure compound was converted to percent yield based on total dry mass of the individual thalli.

Performance and dietary breadth of *Aplysia parvula*

Seaweed preference assays

Experiments examining the dietary preference of *Aplysia parvula* were conducted using choice assays in laboratory aquaria (Paul and Pennings 1991). *Aplysia parvula* used in each set of choice assays were collected from either Apaca Point or Gun Beach. Animals (n = 8 - 15) were housed individually in separate 1- L flow-through aquaria (diameter = 16 cm, maximum depth = 8 cm). Each sea hare was offered a choice between two species of algae (*Portieria hornemannii* versus another species [see below]) that were weighted with clothespins and placed at opposite ends of the aquarium. Individual sea hares were placed in the middle of each aquarium at the beginning of the experiment and their presence or absence on either of the two algae was recorded hourly for 4 - 6 h. Following the methods of Paul and Pennings (1991), individual animals were dropped from the experiment if they were not recorded on algae at least 3 times. Sea hares were recorded as preferring the alga upon which they were observed the most (ties

were dropped). Individual sea hares were used only once during the experiment. Five preference tests were conducted: *P. hornemannii* vs. *Acanthopora spicifera; P. hornemannii* vs. *Asparagopsis taxiformis*; *P. hornemannii* vs. *Dictyota cervicornis*; *P. hornemannii* vs. *Galaxaura marginata*; *P. hornemannii* vs. *Gracilaria tsudae*; and *P. hornemannii* vs. *Laurencia papillosa.* Preference results were analyzed using a two-tailed binomial test (Sokal and Rohlf 1995).

Sea hare growth and consumption

Experiments examining the growth of *Aplysia parvula* were conducted in the laboratory by feeding sea hares 1 of 5 algal diets: *Acanthophora spicifera*; *Dictyota cervicornis; Gracilaria tsudae*; *Laurencia papillosa*; or *Portieria hornemannii* (see Paul and Pennings 1991). Sea hares were blotted dry and weighed (to the nearest 10^{-4} g). Each algal species was provided *ad libitum* to individual sea hares (n = 10) kept in separate 250 ml flow-through aquaria (diameter = 7 cm, maximum depth = 9 cm). Animals that died or crawled out of their aquaria were dropped from the experiment. After six days, each animal was weighed again and its relative change in mass was calculated using the following equation $[(T_f - T_o)/T_o]$: where T_o and T_f are the weight of sea hares before and after the assay. Growth data were checked for normality and for homoscedasticity using Bartlett's test of equal variances. Data were log transformed as needed and compared by ANCOVA with the initial weights of *A. parvula* held as the covariate. Tukey's Honestly Significant Difference (HSD) pairwise comparison of means test was used to determine which means were significantly different from one

another. When the data did not meet the assumptions of ANOVA (Sokal and Rohlf 1995), even after transformation, a Kruskal-Wallis test was conducted. Differences between means were determined using a comparison of mean ranks test. Statistical analyses were performed using *Statistix* (1996).

During the growth experiments described above, measurements of the consumption of algae by *Aplysia parvula* were also conducted (see Paul and Pennings 1991). Sea hares were fed 1 of 5 algal diets as described in the growth experiment above. Each species of alga was divided into treatments and no-herbivore controls, blotted dry and weighed on an electronic balance (to the nearest 10^{-4} g). No-herbivore controls were used to control for changes in mass in the absence of herbivores. After six days, each alga was reweighed and the amount of algal tissue consumed by sea hares was calculated with the following equation $[(T_o \times C_f/C_o) - T_f]$: where T_o and T_f are the weight of the algal pieces exposed to herbivory before and after the assay, and C_o and C_f are the weight of the controls for autogenic changes before and after the assay (Cronin and Hay 1996b). Data were analyzed the same way as in the previous experiment except consumption data were compared by one-way ANOVA.

Whole seaweed samples of *Acanthophora spicifera, Dictyota cervicornis, Gracilaria tsudae, Laurencia papillosa, Portieria hornemannii* were analyzed for their nutritional content. Portions of each algal thallus (n = 3) were rinsed in seawater to remove extraneous material and blotted dry. Samples were oven dried at 62 °C for 48 h, stored in tightly capped vials, and analyzed for total Kjeldahl nitrogen (TKN) (Lachat

Instruments 1992) and organic carbon (Nelson and Sommers 1975) using a Lachat QuickChem Automated Ion analyzer.

Nutritional data were calculated as the percent yield of nitrogen and carbon from total dry mass of the seaweed. Data were checked for normality and for homoscedasticity using Barlett's test of equal variances. TKN present in different species of seaweed were arcsin-squareroot transformed. Organic Carbon and TKN data were analyzed and compared by one-way ANOVA. Tukey's HSD test was used to determine which data were significantly different from one another. Statistical analyses were performed using *Statistix* (1996).

Chemical bioassays

Bioassays examining the preference of *Aplysia parvula* for crude extract and pure apakaochtodenes from *Portieria hornemannii* were conducted in the laboratory in 1- L flow through aquaria. Thalli of the palatable red seaweed *Acanthophora spicifera* (≈ 5 - 7 mg wet mass) were trimmed with a razor blade to a length of 5 cm and were coated with a solution of either crude *P. hornemannii* extract or pure apakaochtodene A or B dissolved in hexanes (Hay et al. 1998). These treatment thalli, coated with either crude *P. hornemannii* extract or pure apakaochtodenes were tested over a range of concentrations that approximates their natural wet mass concentrations. Controls consisted of *A. spicifera* thalli that were coated with hexanes only. Since metabolites extracted from *P. hornemannii* are lipid-soluble, they adhere to the surface of the *A. spicifera* thalli after the hexanes evaporate. Similar studies with lipid soluble

metabolites from other seaweeds found that compounds were not lost after 24 h underwater (Hay et al. 1998). Preference bioassays were run as described above (Seaweed preference assays) over an interval of 24 - 48 h. Preference results were recorded by measuring the amount of *A. spicifera* eaten (cm) by each sea hare and were analyzed using a paired *t*-test (Sokal and Rohlf 1995).

Site-to-site chemical variation

Experiments testing the preference of individual *Aplysia parvula* for *Portieria hornemannii* collected from different locations were conducted in laboratory aquaria. *Portieria hornemannii* was collected from different sites around Guam, and each collection was housed in a separate tank. Individual sea hares (n = 10 - 15), collected at Gun Beach, were placed in 1- L flow-through aquaria and offered a choice between individual *P. hornemannii* thalli from two different sites. Sea hare choice assays were run as described above (Seaweed preference assays) and were scored hourly for 5 - 7 h. Three choice tests were conducted (different collections of *P. hornemannii* are listed by site): Apaca Point vs. Double Reef; Pago Bay vs. Apaca Point; and Pago Bay vs. Double Reef. Assays testing algae from Apaca Point vs. Pago Bay were conducted on two separate days using a different set of sea hares on each day. The assay results from these two days were pooled. Preference results were analyzed using a two-tailed binomial test. Additionally, individual *P. hornemannii* were separately collected from Apaca Point (n = 7), Double Reef (n = 6) and Pago Bay (n = 7), and chemically analyzed by gc-ms for their concentrations of apakaochtodenes A and B. Site-to-site chemical data were checked for

normality and homoscedasticity using Barlett's test of equal variances and were compared by one-way ANOVA. Tukey's HSD test was used to determine which data were significantly different from one another. Statistical analyses were performed using *Statistix* (1996).

Within-alga chemical variation

Experiments testing whether individual *Aplysia parvula* prefer to graze the tips versus the bases of individual *Portieria hornemannii* thalli were conducted in laboratory aquaria. *Aplysia parvula* used in each set of preference tests were collected from either Double Reef or Gun Beach. Individual animals (n = 15 - 19), housed in separate 1- L flow-through aquaria, were offered a choice between the tips or bases from an individual *P. hornemannii* thallus. Whole thalli were divided into thirds (tips, middles and bases) laterally, and the tips and bases placed in separate, paired clothespins as described above (Seaweed Preference Assay). Middles were not used in this experiment. Three preference assays were conducted with seaweed collected from Apaca Point, Pago Bay and Double Reef, respectively. Choice assays were scored hourly for 6 - 7 h. The assay with Pago Bay algae was conducted on two separate days using a different set of sea hares on each day. The assay results from each of these two days were pooled. Preference results were analyzed statistically using a two-tailed binomial test (Sokal and Rohlf 1995). The tips and bases of whole thalli (n = 15) from Anae Island were also chemically analyzed for their apakaochtodene B content by gc-ms. However, unlike the chemical extraction protocol previously outlined, thallus parts were freeze dried and

extracted in DCM. Thallus parts were compared using a paired *t*-test (Sokal and Rohlf 1995).

Individual algal parts of *Portieria hornemannii* (tips, middles, bases) were analyzed for their nutritional content. Each algal part (n = 3) was rinsed in seawater to remove extraneous material and blotted dry. Algal tissues were prepared and analyzed for TKN and organic carbon as previously described. Nutritional data were calculated as the percent yield of nitrogen and carbon from total dry mass of the seaweed. Data were checked for normality and homoscedasticity using Barlett's test of equal variances. Total Kjeldahl nitrogen and organic carbon in algal parts were compared using a two-way mixed-model ANOVA without replication, with thallus part (fixed factor) and individual ålga (random factor) as the two factors (Sokal and Rohlf 1995). Tukey's test for nonadditivity indicated no significant interactions for neither analysis; thus, the two-way ANOVA without replication was valid. Tukey's HSD test was used to determine which data were significantly different from one another (Sokal and Rohlf 1995). Statistical analyses were performed using *Statistix* (1996).

Induction of chemical defenses in *Portieria hornemannii*

A paired assay examining the induction of apakaochtodenes A and B in *Portieria hornemannii* via herbivory by *Aplysia parvula* was conducted in laboratory aquaria. On two separate occasions, individual *P. hornemannii* thalli were collected from Double Reef, brought back to the laboratory, cleaned of epiphytic material and placed in separate 1- L flow-through aquaria. Each thallus (n = 12) was monitored for 7 days to insure that

A. parvula was not present on the algae before beginning the experiment. Each thallus (n = 12) was then split into 2 halves, and subsampled for chemical analysis. Each half was designated either a treatment or no-herbivore control and placed in an individual 1- L flow-through aquarium. Three sea hares were placed on each treatment thallus and allowed to graze for 48 h. Controls were not grazed. After 4 days, a final subsample was taken from each thallus half for chemical analysis. The remaining portions of no-herbivore control and treatment thalli were offered to individual *A. parvula* in paired choice assays. Preference tests were run for both experiments as described above (Seaweed Preference Assay) and were scored hourly for 4 - 6 h. Preference results were compared using a two-tailed binomial test (Sokal and Rohlf 1995). A laboratory error prevented algae collected in the first experiment from being chemically analyzed. Initial and final subsamples from the second experiment were extracted and chemically analyzed by gc-ms. Chemical analyses of initial and final controls and treatments were compared using a paired *t*-test (Sokal and Rohlf 1995).

The preference of *Aplysia parvula* for individual *Portieria hornemannii* in which tips were removed or left intact was determined using a paired preference assay in laboratory aquaria. Individual *P. hornemannii* thalli were collected from Anae Island, brought back to the laboratory, cleaned of extraneous material and placed in separate 1-L flow-through aquaria. Each thallus (n = 20) was split into 2 halves and designated either a treatment or control. The tips of treatment thalli were cut with scissors whereas the tips of control thalli were left intact. After 4 d, treatment and control thalli were offered to *A. parvula* (n = 20) in paired choice assays which were run as described above.

Preference results were compared using a two-tailed binomial test (Sokal and Rohlf 1995).

Palatability of *Aplysia parvula* to predators

Experiments examining the palatability of *Aplysia parvula* to reef fish predators were conducted at two sites on Guam; Gun Beach and Western Shoals (Figure 2) (Paul and Pennings 1991). At both sites, frozen, whole *A. parvula* collected from Anae Island (n = 10) and Double Reef (n = 10) were tossed in random order into the water column and the fate of each animal (rejected or eaten) was recorded. At Western Shoals, live sea hares from Anae Island (n = 10) were also tested. Chunks of squid (n = 10) served as controls. A *G*-test of independence was used to statistically analyze the results via a 3 × 2 contingency table for Gun Beach and a 4 × 2 contingency table for Western Shoals. (Sokal and Rohlf 1995).

Individual *Aplysia parvula* from Apaca Point (n = 10) and Double Reef (n = 10) were frozen, dissected, and their body parts (digestive gland and mantle) offered to predatory fishes at Gun Beach. Digestive gland (n = 10) and mantle parts (n = 10) were cut into equal-sized pieces and tossed in random order into the water column. Squid chunks (n = 10) served as controls. Assays were analyzed using a 3-way log-linear model (Sokal and Rohlf 1995), classifying responses based on site of collection (Apaca Point or Double Reef), type of body part (digestive gland or mantle), or number of body parts eaten or rejected. Statistical analyses were performed using *SYSTAT* (Wilkinson et al. 1992). Fishes preference for digestive gland and mantle from Apaca Point and Double

Reef, respectively, was analyzed using Fisher's Exact Test (Sokal and Rohlf 1995). Additionally, sea hares from Cocos Lagoon (n = 10) (Figure 2) were frozen, dissected and their digestive gland and mantle parts chemically analyzed for their concentrations of apakaochtodenes A and B by gc-ms.

RESULTS

Performance and dietary breadth of *Aplysia parvula*

Seaweed preference assays

Individual *Aplysia parvula* preferred *Portieria hornemannii* compared to all other seaweed diets which included the red algae, *Acanthophora spicifera*, *Asparagopsis taxiformis*, *Galaxaura marginata*, *Gracilaria tsudae*, *Laurencia papillosa*, and the brown alga, *Dictyota cervicornis* (two-tailed binomial, $p < 0.05$) (Table 1). Of all choice tests performed, only one individual *A. parvula* preferred an alga other than *P. hornemannii*, and chose *G. tsudae*.

Sea hare growth and consumption

Three growth and consumption experiments were conducted using *Aplysia parvula*. The first assay was performed as described earlier, except that fewer sea hares ($n = 5$) were placed on individual algal diets. Also, the brown alga *Dictyota cervicornis* was not used as a treatment diet. There was no significant difference in the relative change in mass of sea hares on any of the no-choice seaweed diets (ANCOVA, $F_{3, 13} = 2.85$, $p = 0.078$) (Figure 3a). There was, however, a significant difference in the mean amount of algae consumed by individual sea hares (ANOVA, $F_{3, 14} = 4.65$, $p = 0.018$) (Figure 3b). The amount of *Acanthophora spicifera*, *Gracilaria tsudae* and *Portieria hornemannii* consumed by sea hares was significantly greater than the amount of

Table 1. Feeding preference assays testing the choice of *Aplysia parvula* for different species of algae. P values were determined with a binomial test (two-tailed).

Alga	Sample Size	Number of Preferring	P value
Portieria hornemannii	10	7	0.015
Acanthophora spicifera		0	
P. hornemannii	12	10	0.002
Asparagopsis taxiformis		0	
P. hornemannii	12	8	0.007
Dictyota cervicornis		0	
P. hornemannii	11	10	0.002
Galaxaura marginata		0	
P. hornemannii	10	9	0.021
Gracilaria tsudae		1	
P. hornemannii	8	8	0.007
Laurencia papillosa		0	

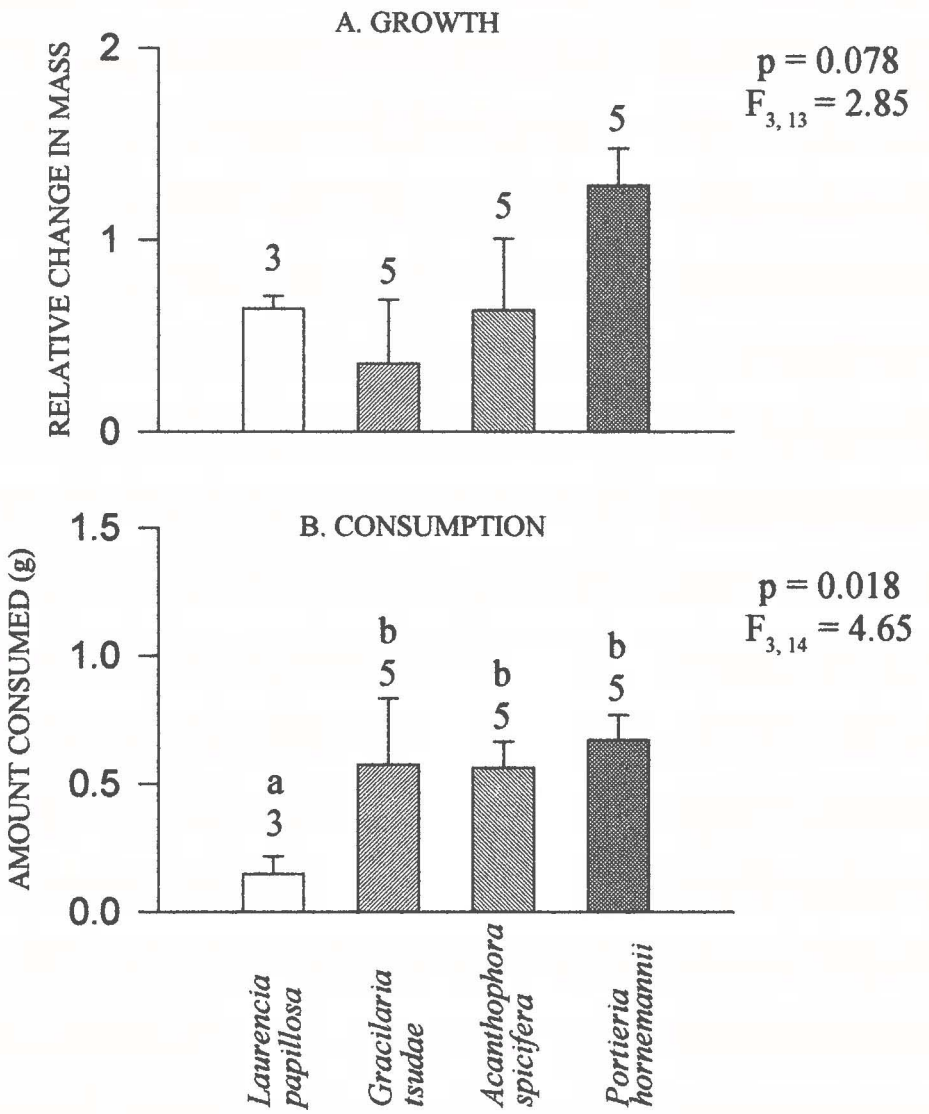


Figure 3. Growth (A) and consumption (B) of *Aplysia parvula* on 4 different algal diets. Growth data were compared by ANCOVA with the initial weights of *A. parvula* held as the covariate. Consumption data were log transformed and analyzed by one-way ANOVA. Histogram bars represent the mean relative change in mass and amount of algae consumed by individual sea hares (+ 1 SE), as calculated by the equations $[(T_f - T_o)/T_o]$ and $[(T_o C_f/C_o) - T_f]$, respectively, where T_o and T_f are the weight of treatments and C_o and C_f are the weight of controls for autogenic changes before and after each assay. Identical letters above bars indicate means that are not significantly different (Tukey's HSD, $p>0.05$). Numbers above bars represent the sample sizes of individual treatments. Untransformed means are presented for clarity.

Laurencia papillosa consumed (Figure 3b). Because of the small sample size used in the first assay, the growth and consumption experiment was repeated.

In the second assay, a post-hoc analysis showed that the initial weights of sea hares used for the different seaweed diets were significantly different (ANOVA, $F_{4, 43} = 3.75$, $p = 0.01$). Specifically, the mean initial weights of sea hares placed on the *Portieria hornemannii* diet were smaller compared to animals placed on either the *Acanthophora spicifera*, *Dictyota cervicornis*, *Gracilaria tsudae*, or *Laurencia papillosa* diets (Tukey's HSD, $p < 0.05$). This skewness in the *P. hornemannii* treatment diet could bias any conclusions made from these data. Thus, the growth and consumption assay was repeated for a third time. The mean initial weights of sea hares placed on either the *A. spicifera*, *D. cervicornis*, *G. tsudae*, *L. papillosa*, or *P. hornemannii* treatment diets in the third experiment did not differ significantly (ANOVA, $F_{4, 42} = 0.36$, $p = 0.834$).

In both the second and third experiments, the relative change in mass of *Aplysia parvula* on the 5 seaweed diets was significantly different (Kruskal-Wallis, $df = 4$, $\chi^2 = 31.94$, $p < 0.001$, and ANCOVA, $F_{4, 41} = 12.07$, $p < 0.001$) (Figures 4a and 5a). Individual sea hares grew best on *Portieria hornemannii* compared to all other seaweed diets. The amount of algae consumed by *Aplysia parvula* was also significantly different among treatments (ANOVA, $F_{4, 43} = 4.40$, $p = 0.005$, and Kruskal-Wallis, $df = 4$, $\chi^2 = 17.30$, $p < 0.001$) (Figures 4b and 5b). In the second experiment, the amount of *Acanthophora spicifera*, *Laurencia papillosa* and *Portieria hornemannii* consumed by sea hares was significantly greater than *Dictyota cervicornis* or *Gracilaria tsudae* (Figure 4b), whereas, in the third experiment, the amount of *A. spicifera*, *G. tsudae* and *P.*

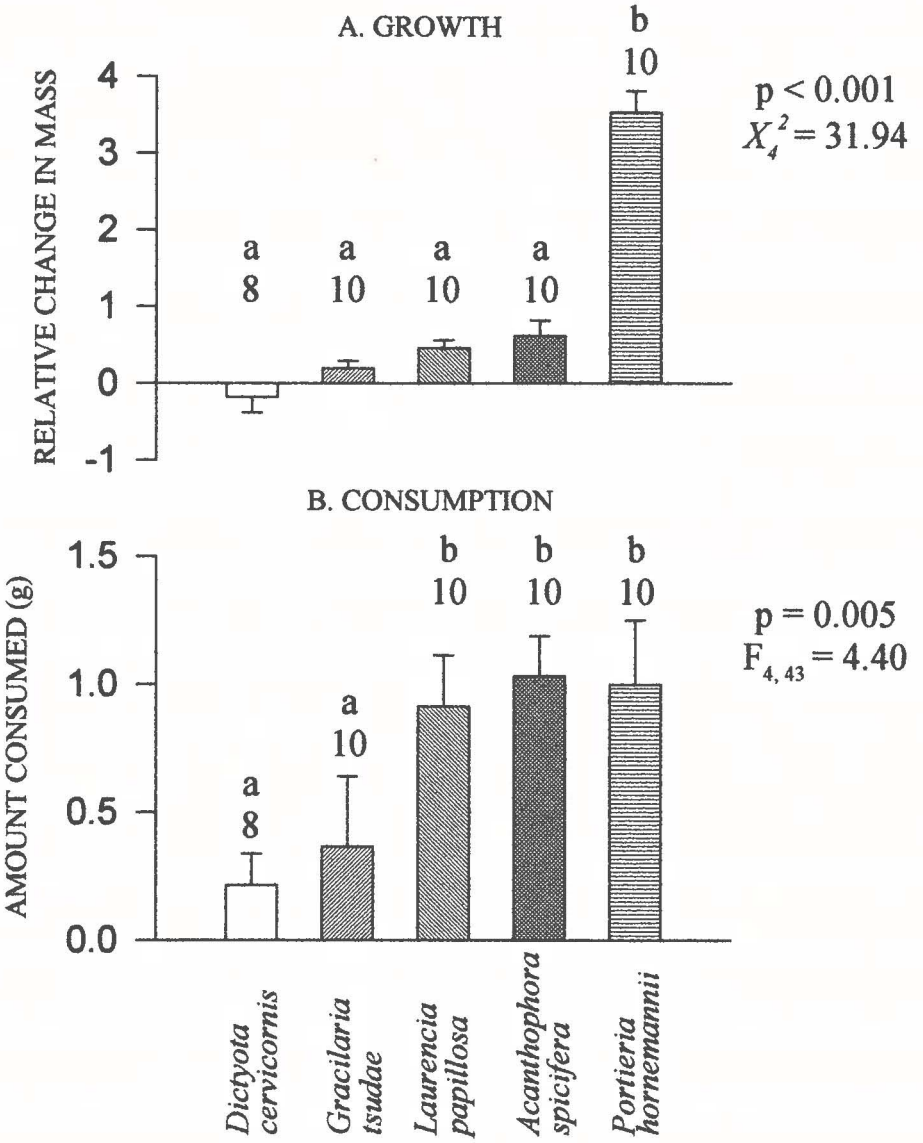


Figure 4. Growth (A) and consumption (B) of *Aplysia parvula* on 5 different algal diets. Growth data were compared using a Kruskal-Wallis test. Consumption data were analyzed by one-way ANOVA. Histogram bars represent the mean relative change in mass and amount of algae consumed by individual sea hares (+ 1 SE), as calculated by the equations $[(T_f - T_o)/T_o]$ and $[(T_o C_f/C_o) - T_f]$, respectively, where T_o and T_f are the weight of treatments and C_o and C_f are the weight of controls for autogenic changes before and after each assay. Identical letters above bars indicate means that are not significantly different [comparsion of mean ranks (A) and Tukey's HSD (B), $p>0.05$]. Numbers above bars represent the sample sizes of individual treatments.

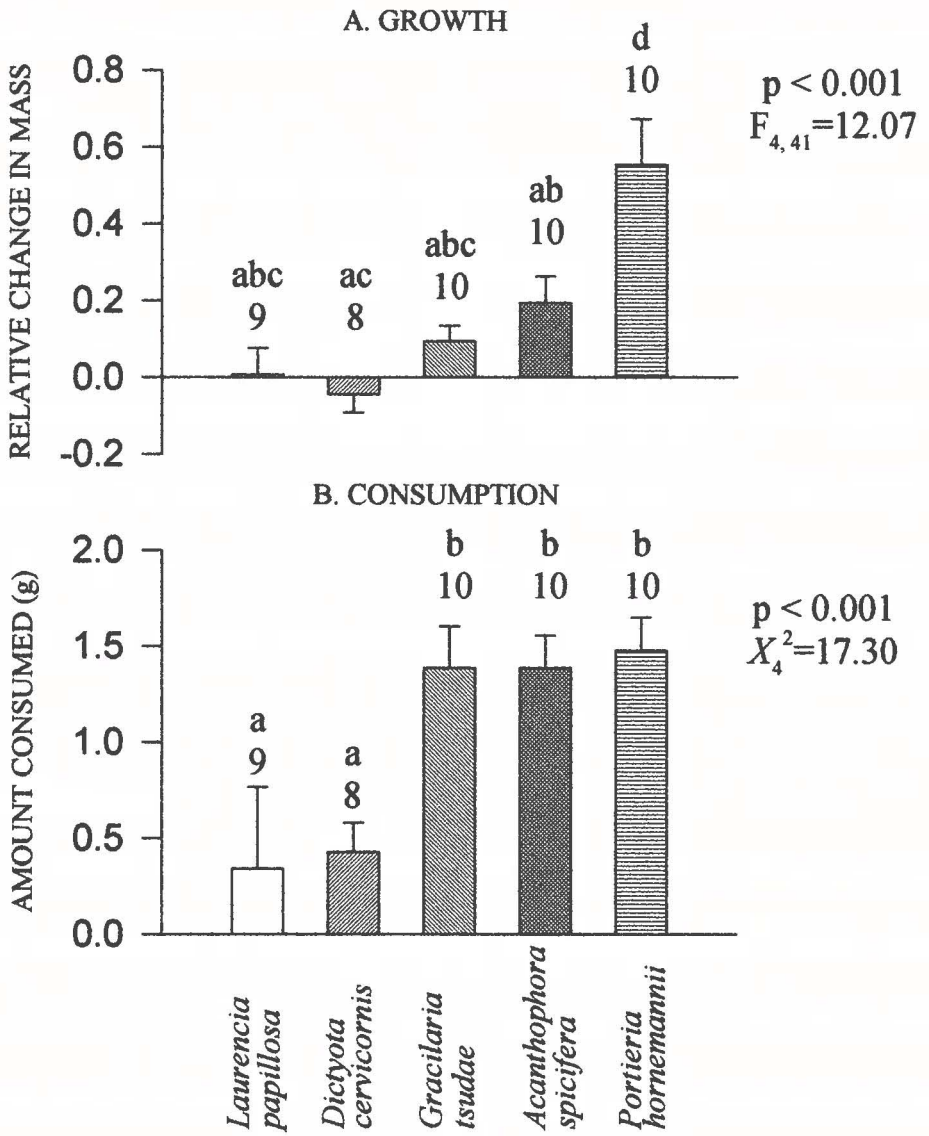


Figure 5. Growth (A) and consumption (B) of *Aplysia parvula* on 5 different algal diets. Growth data were log transformed and compared by ANCOVA with the initial weights of *A. parvula* held as the covariate. Consumption data were analyzed using a Kruskal-Wallis test. Histogram bars represent the mean relative change in mass and the amount of algae consumed by individual sea hares (+ 1 SE), as calculated by the equations $[(T_f - T_o)/T_o]$ and $[(T_oC_f/C_o) - T_o]$, respectively, where T_o and T_f are the weight of treatments and C_o and C_f are the weight of controls for autogenic changes before and after each assay. Identical letters above bars indicate means that are not significantly different [Tukey's HSD (A) and comparison of mean ranks (B), $p>0.05$]. Numbers above bars represent the sample sizes of individual treatments. Untransformed means are presented for clarity.

hornemannii consumed was significantly greater than *D. cervicornis* or *L. papillosa* (Figure 5b).

There were significant differences observed in the mean amounts of organic carbon and total Kjeldahl nitrogen (TKN) within the 5 species of seaweed analyzed (ANOVA, $F_{4, 10} = 5.90$, $p = 0.01$, and $F_{4, 10} = 11.67$, $p = 0.001$) (Figures 6a and 6b). Despite significant differences between algal treatments, high levels of organic carbon were present in the tissues of all algal species tested (Figure 6a). Levels of TKN present in different algal treatments were more distinct with levels highest in *A. spicifera*, *G. tsudae* and *P. hornemannii* and lowest in *D. cervicornis* and *L. papillosa* (Figure 6b).

Chemical bioassays

Crude *Portieria hornemannii* extract and pure apakaochtodenes were tested at varying concentrations to determine levels of deterrence (Figures 7 and 8). Mean natural wet mass concentrations of *P. hornemannii* crude extract are between 0.5%- 4%. Likewise, average natural wet mass concentrations of pure apakaochtodenes are approximately 10% of crude extract (0.01%- 0.6% per wet mass). While crude *P. hornemannii* extract did not significantly deter feeding by *Aplysia parvula* at 0.1%, 0.5%, 1%, and 2%, it did deter feeding at 4% and 6% (Figure 7a). In a direct comparison, sea hares were indifferent to natural concentrations of crude *P. hornemannii* extract (0.9% per wet mass) from Anae Island and Gun Beach (Figure 7b). Both apakaochtodenes A and B, at all concentrations tested, deterred feeding by sea hares (Figures 8a and 8b).

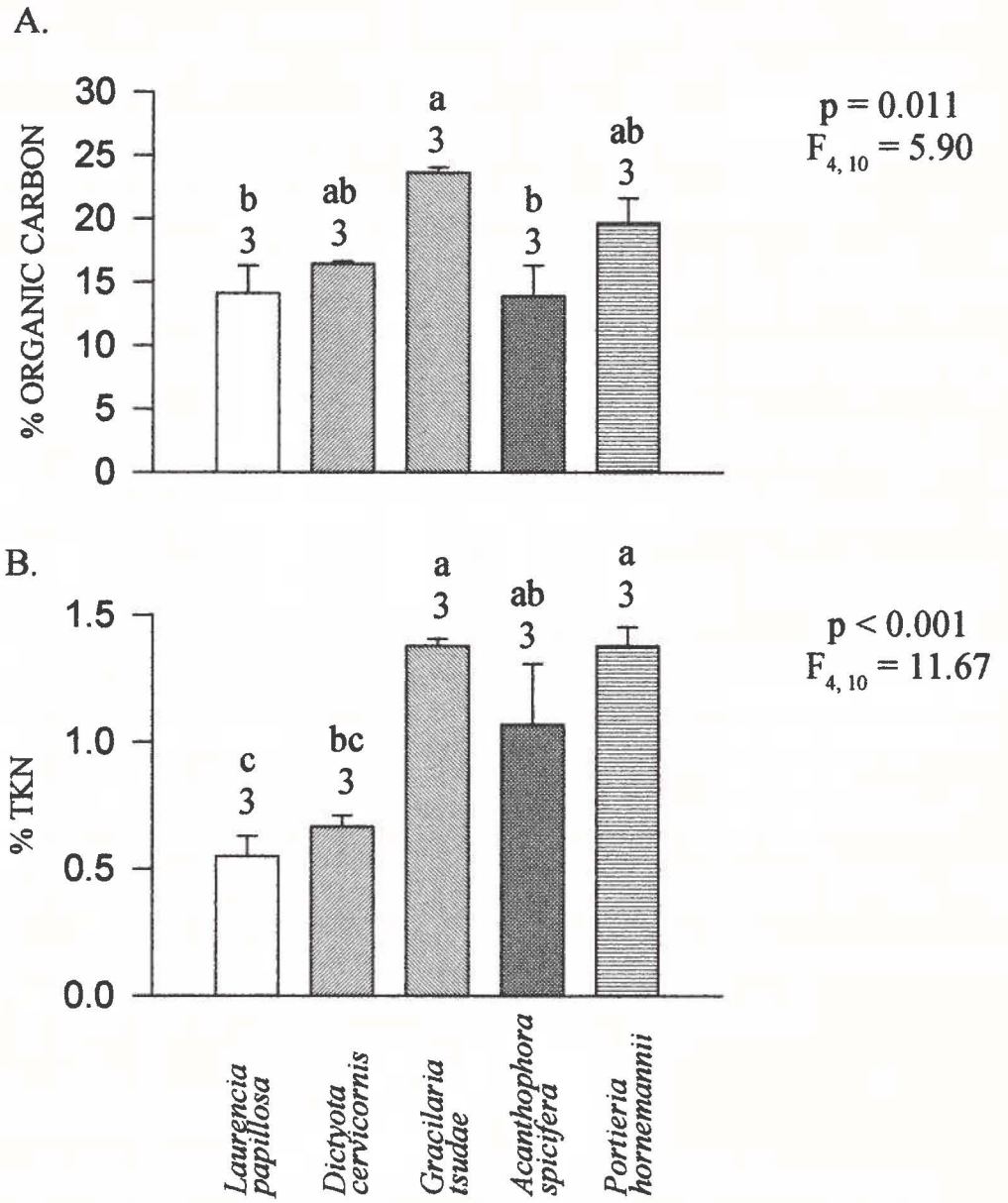


Figure 6. Nutritional content, organic carbon (A) and TKN (B), of 5 different algal diets. Organic carbon and TKN data were each separately analyzed by one-way ANOVA. TKN data were arcsin-squareroot transformed. Histogram bars represent the mean percent organic carbon and TKN (wt. N or C/algal dry mass) within individual algae (+1 SE). Identical letters above bars indicate means that are not significantly different (Tukey's HSD, $p>0.05$). Numbers above bars indicate the size of each algal treatment. Untransformed means are presented for clarity.

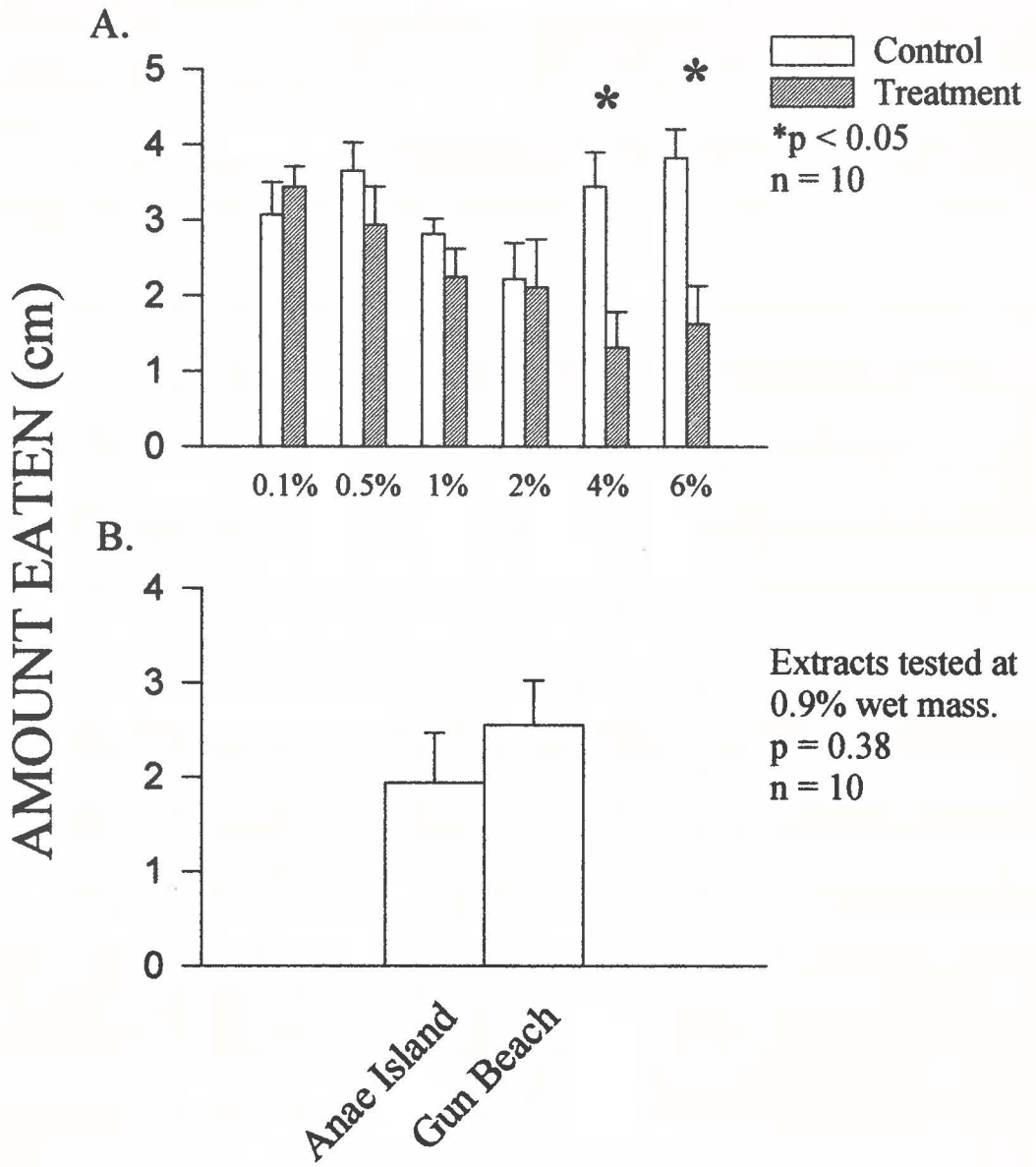


Figure 7. Chemical bioassays with crude extracts from *Portieria hornemannii*. Crude extracts were tested at wet mass concentrations that span the natural range of concentrations found in the alga (A) and in a direct comparison between *P. hornemannii* from two different locations (B). Histogram bars represent mean amount eaten + 1 SE. Data were analyzed using paired *t*-tests. N = 10 paired replicates for each concentration.

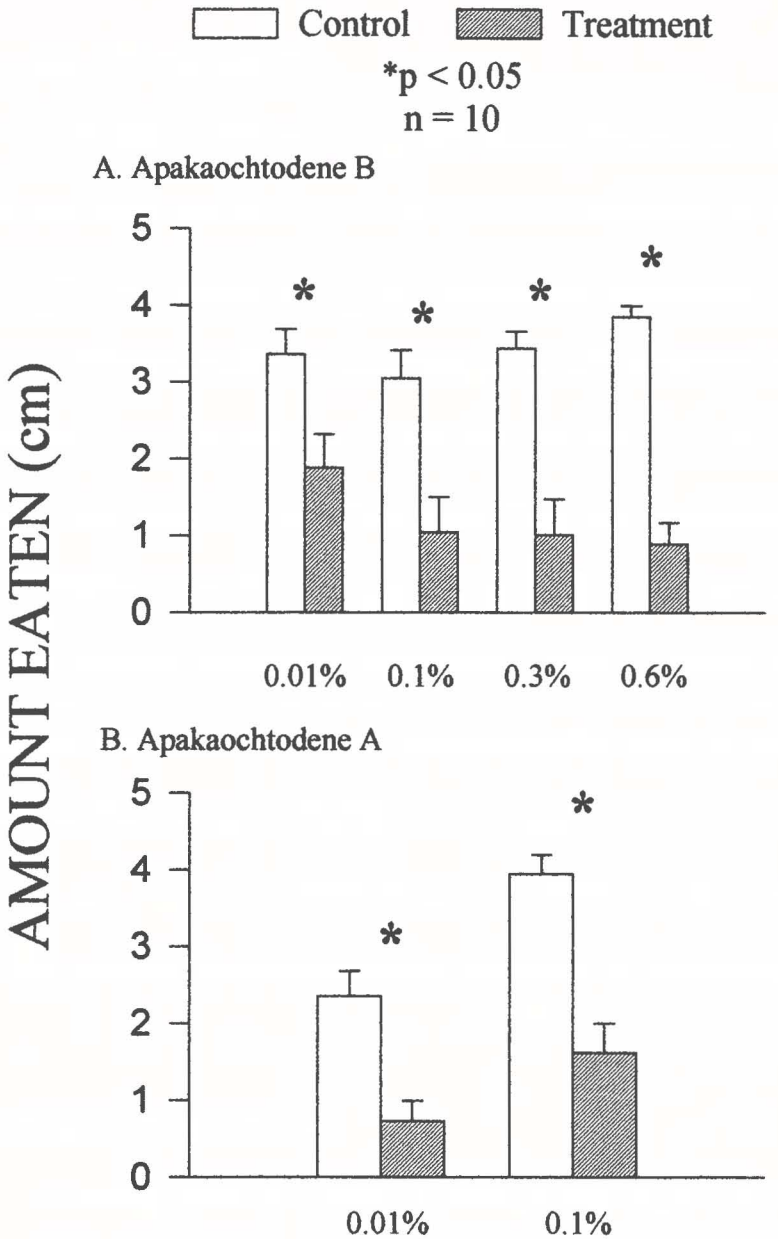


Figure 8. Chemical bioassays with pure apakaochtodenes from *Portieria hornemannii.* Pure compounds (A and B) were tested at wet mass concentrations that span a natural range of concentrations found in the alga. Histogram bars represent mean amount eaten + 1 SE. Data were analyzed using paired *t*-tests. N = 10 paired replicates for each concentration

Site-to-site chemical variation

In feeding preference assays, individual *Aplysia parvula* did not show a significant preference for *Portieria hornemannii* collected from either Apaca Point, Double Reef or Pago Bay (two-tailed binomial, $p > 0.05$) (Table 2). Chemical analysis by gc-ms indicated significant differences in the total amount of apakaochtodenes A and B between *P. hornemannii* from Apaca Point, Double Reef and Pago Bay (ANOVA, $F_{2, 15} = 5.22$) (Figure 9). Algae from Apaca Point had significantly higher levels of apakaochtodenes A and B than Pago Bay, but neither algae from Apaca Point or Pago Bay were different from Double Reef (Tukey's HSD, $p < 0.05$). However, when tested separately, there was no significant difference in the levels of apakaochtodene A or B between individual algae from different sites (ANOVA, $F_{1, 10} = 4.14$, $p = 0.069$, and ANOVA, $F_{2, 15} = 2.94$, $p = 0.084$) (Figure 9). Levels of apakaochtodene A in algae from Pago Bay were less than detection limits and could not be quantified in this experiment.

Within-alga chemical variation

In feeding preference assays, individual *Aplysia parvula* preferred the tips compared to bases of *Portieria hornemannii* thalli collected from Double Reef and Pago Bay (two-tailed binomial test, $p < 0.05$) (Table 3). Sea hares showed no preference for tips or bases from Apaca Point although a similar trend for preferences of tips was observed (two-tailed binomial test, $p > 0.05$) (Table 3). Additionally, chemical analysis of individual *P. hornemannii* from Anae Island indicated significantly higher levels of apakaochtodene B in thalli tips compared to bases (Figure 10). Levels of

Table 2. Feeding preference assays testing the choice of *Aplysia parvula* for *Portieria hornemannii* collected from different sites. P values were determined with a binomial test (two-tailed).

Site	Sample Size	Number of Preferring	P value
Apaca Point	13	10	0.092
Double Reef		3	
Pago Bay	10	6	0.507
Double Reef		3	
Apaca Point	15	8	0.387
Pago Bay		4	

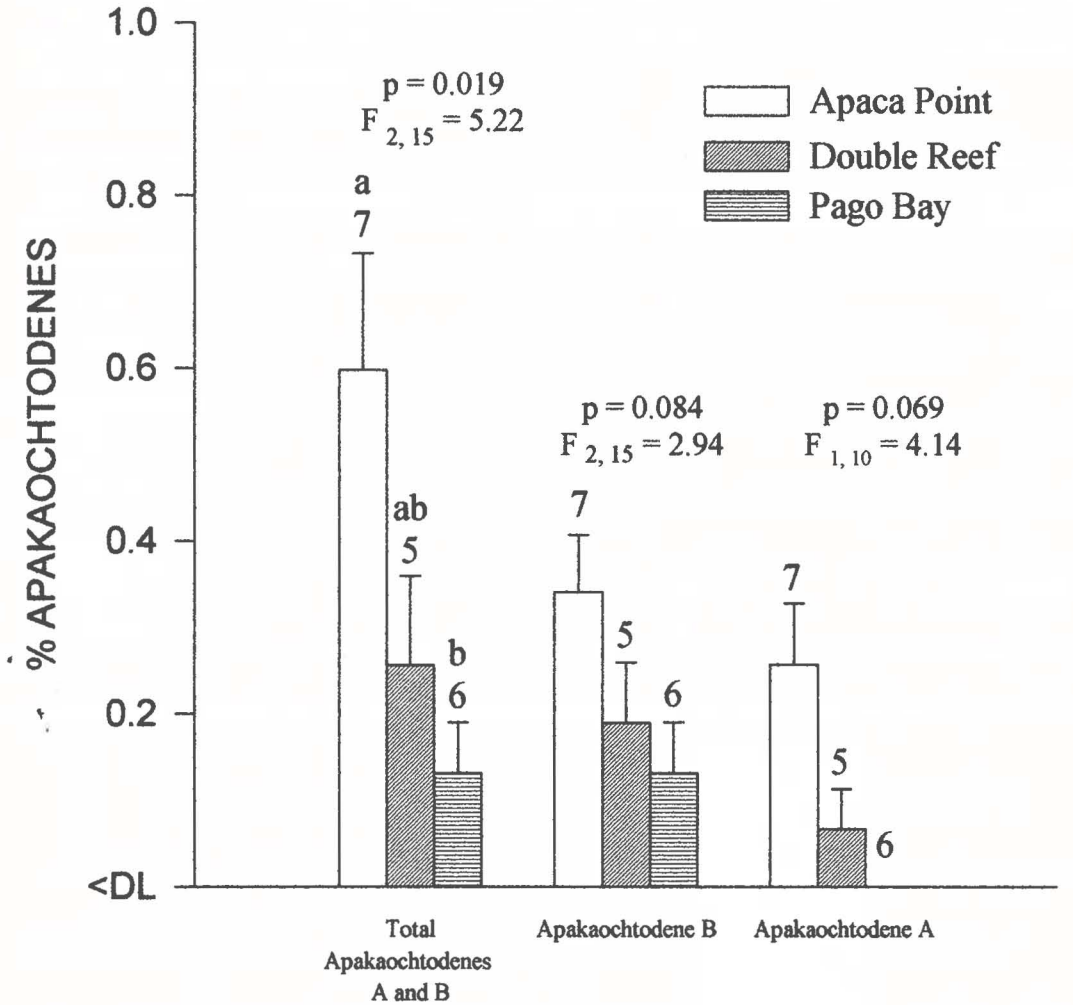


Figure 9. Site-to-site chemical variation of apakaochtodenes in *Portieria hornemannii*. The amount of pure compound was converted to percent yield based on total dry mass of the individual thalli. Data are means + 1 SE. <DL: less than detection limits. Numbers above bars represent sample sizes of individual treatments. Identical letters above bars indicate means that are not significantly different. Mean concentrations of total apakaochtodenes A and B, apakaochtodene A and apakaochtodene B were separately analyzed by one-way ANOVA with Tukey's HSD, $p<0.05$.

Table 3. Feeding preference assays testing the choice of *Aplysia parvula* for the tips or bases from an individual *Portieria hornemannii* thallus. P values were determined with a binomial test (two-tailed).

Site	Sample Size	Number of Preferring	P value
Apaca Point	15		
Tips		11	0.056
Bases		3	
Double Reef	15		
Tips		10	0.012
Bases		1	
Pago Bay	19		
Tips		10	0.019
Bases		2	

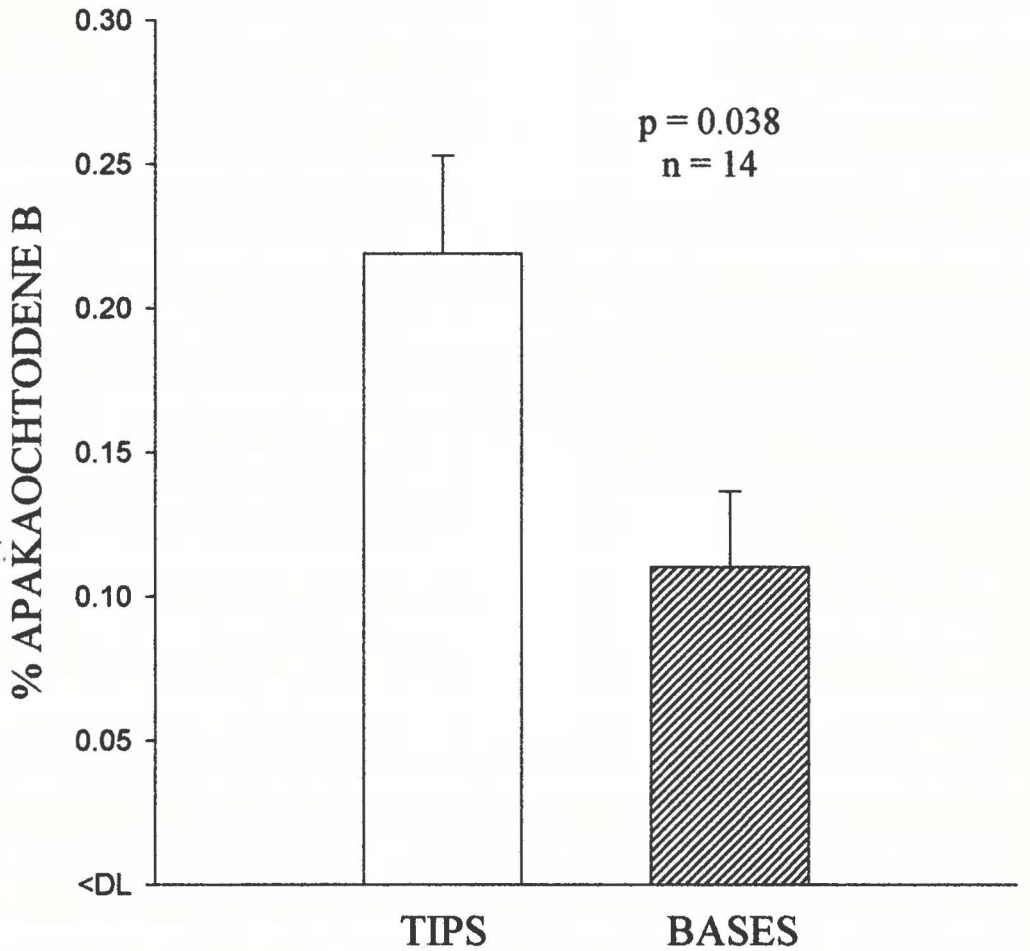


Figure 10. Concentrations of apakaochtodene B in the tips and bases of *Portieria hornemannii* thalli from Anae Island. The amount of pure compound was converted to percent yield based on total dry mass of the individual thalli. Data are means + 1 SE. <DL: less than detection limits. Levels of apakaochtodene A could not be quantified in this experiment. Data were analyzed using a paired *t*-test ($p<0.05$). N = 14 replicates for each treatment group.

apakaochtodene A were less than detection limits and could not be quantified in this experiment. These tips and bases were extracted in DCM, which may have resulted in lower extraction efficiency.

Nutritional analysis of individual *Portieria hornemannii* thalli showed that the amount of organic carbon present between the tips, middles and bases did not significantly differ (two-way ANOVA, $F_{2,8} = 5.97$, $p = 0.063$) (Figure 11a). In contrast, levels of TKN within thalli were dissimilar. Tips had significantly higher levels than bases, but neither tips nor bases were different from middles (two-way ANOVA, $F_{3,8} = 9.26$, $p = 0.032$) (Figure 11b).

Induction of chemical defenses in *Portieria hornemannii*

The apakaochtodene B content of *Portieria hornemannii* thalli did not significantly increase as the result of grazing by *Aplysia parvula* (paired *t*-test, $p > 0.05$) (Figure 12). There were no significant differences in the concentrations of apakaochtodene B between initial or final controls and treatments. Levels of apakaochtodene A were not detected in *P. hornemannii* thalli in this experiment. Additionally, in the first feeding preference experiment, *A. parvula* preferred final control versus final treatment (grazed) *P. hornemannii* thalli (two-tailed binomial, $p < 0.05$) (Table 4). Although a similar trend was observed in the second feeding preference experiment, sea hares did not significantly prefer final control compared with grazed thalli (two-tailed binomial, $p > 0.05$) (Table 4). Subsequent choice assays demonstrated

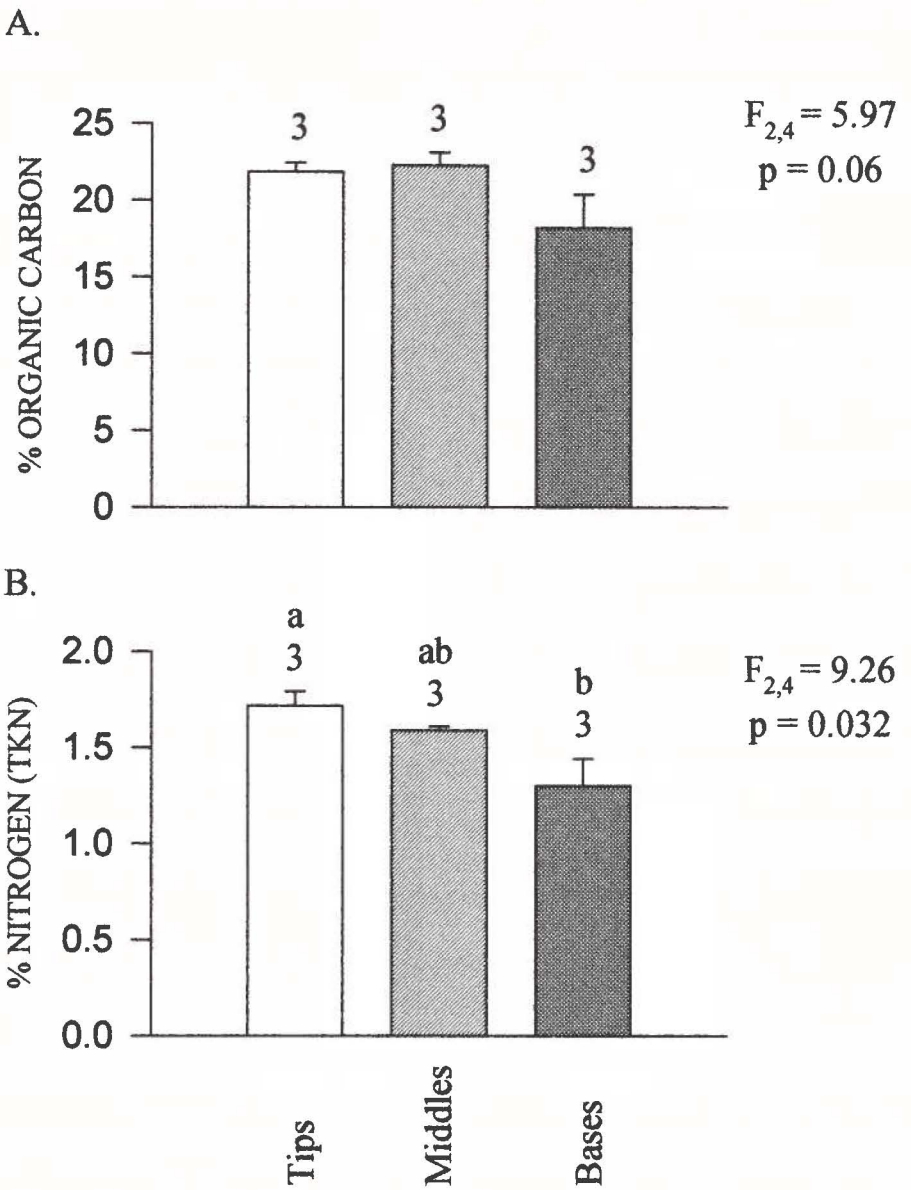


Figure 11. Nutritional content, organic carbon (A) and TKN (B), of different parts of individual *Portieria hornemannii* thalli. Organic carbon and TKN data were analyzed by two-way ANOVA. Histogram bars represent mean percent organic carbon and TKN (+ 1 SE). Identical letters above bars indicate means that are not significantly different (Tukey's HSD, $p>0.05$). Numbers above bars indicate sample sizes of individual treatments.

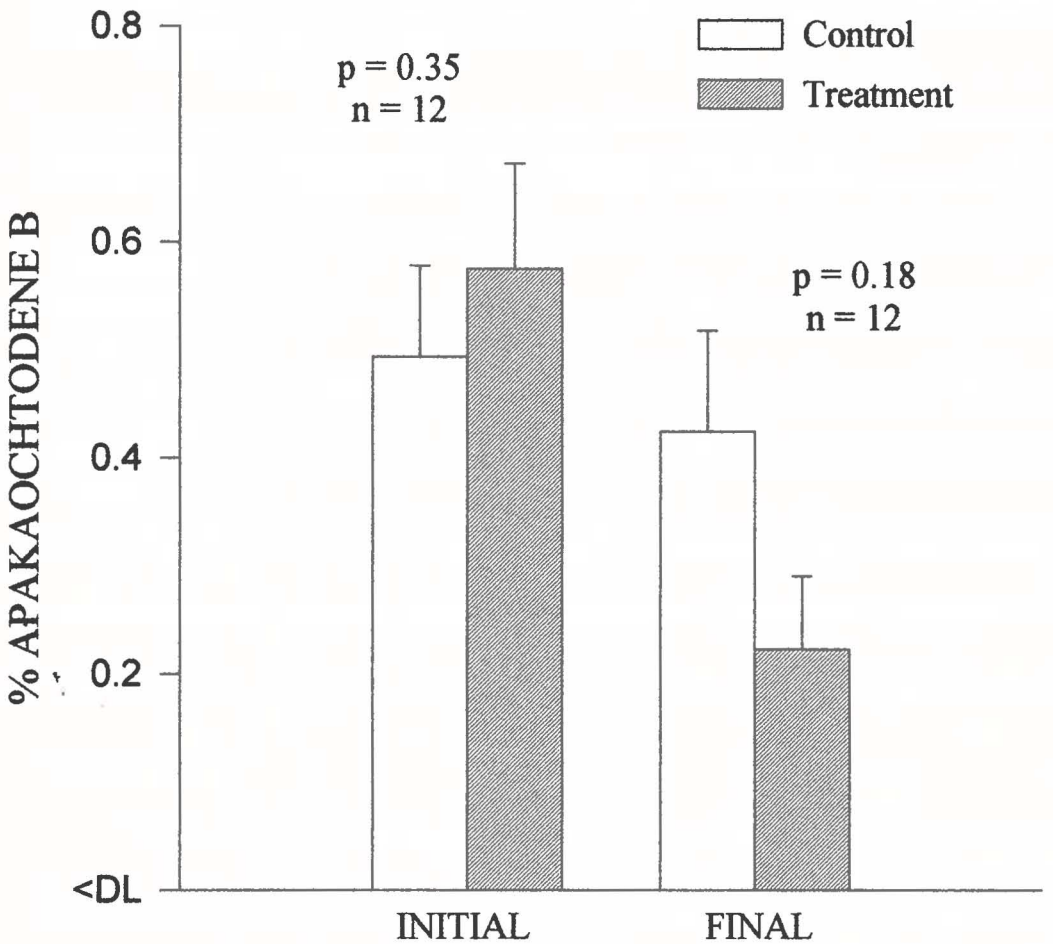


Figure 12. Assay testing the induction of apakaochtodene B in *Portieria hornemannii* via grazing by *Aplysia parvula.* The amount of pure compound was converted to percent based on total dry mass of the individual thalli. Data are means + 1 SE. <DL: less than detection limits. Apakaochtodene A was not present in *P. hornemannii* thalli in this experiment. There was no significant difference between initial or final controls and treatments,(paired *t*-test, $p>0.05$). N=12 replicates for each treatment group.

Table 4. Induction experiment feeding preference assays testing the choice of *Aplysia parvula* for undamaged or grazed *Portieria hornemannii* thalli. P values were determined with a binomial test (two-tailed).

Treatment	Sample Size	Number of Preferring	P value
Experiment 1	10		
Controls		9	0.021
Grazed		1	
Experiment 2	11		
Controls		9	0.064
Grazed		2	

that *A. parvula* significantly preferred thalli with the tips left intact compared to thalli in which the tips were removed (two-tailed binomial, $p < 0.05$) (Table 5).

Palatability of *Aplysia parvula* to predators

In field assays at Gun Beach and Western Shoals, neither sea hares from Anae Island nor Double Reef were palatable to reef fishes relative to squid pieces (3×2 contingency table, G-test, $G = 31.60$, $df = 2$, $p < 0.001$, and 4×2 contingency table, G-test, $G = 9.21$, $df = 3$, $p < 0.01$, respectively) (Figure 13). At Gun Beach, *Thalassoma lutescens*, *Halichores hortulanus*, *Balistapus undulatus*, and a serranid wrasse, preferentially ate sea hares. In contrast, *Scarus* sp. and *Naso vlamingii* ate sea hares at Western Shoals. For nearly all cases, sea hares were eaten only after being mouthed by numerous fishes.

Field assays at Gun Beach also indicated that digestive gland and mantle parts from *Aplysia parvula* were not palatable to reef fishes (Figure 14). Reef fish readily consumed squid parts. However, so as not to confound the specific question of the palatability of *A. parvula* body parts from different sites, squid data were dropped from statistical analyses. Since a log-linear model including all possible two-way interactions did not significantly fit the observed preference data ($df = 1$, $G = 4.54$, $p = 0.033$), a three-way interaction could not be used to account for this variation. By inspection, fishes ate similar amounts of digestive gland parts from either Apaca Point (1 eaten: 9 rejected) or Double Reef (1: 8), whereas, the number of mantle parts eaten from Apaca Point (6: 4) and Double Reef (0: 10) were different. However, fishes showed no

Table 5. Feeding preference assays testing the choice of *Aplysia parvula* for individual *Portieria hornemannii* thalli in which tips were either removed or left intact. P values were determined with a binomial test (two-tailed).

Treatment	Sample Size	Number of Preferring	P value
Anae Island	16		
Tips		14	0.004
Without Tips		2	

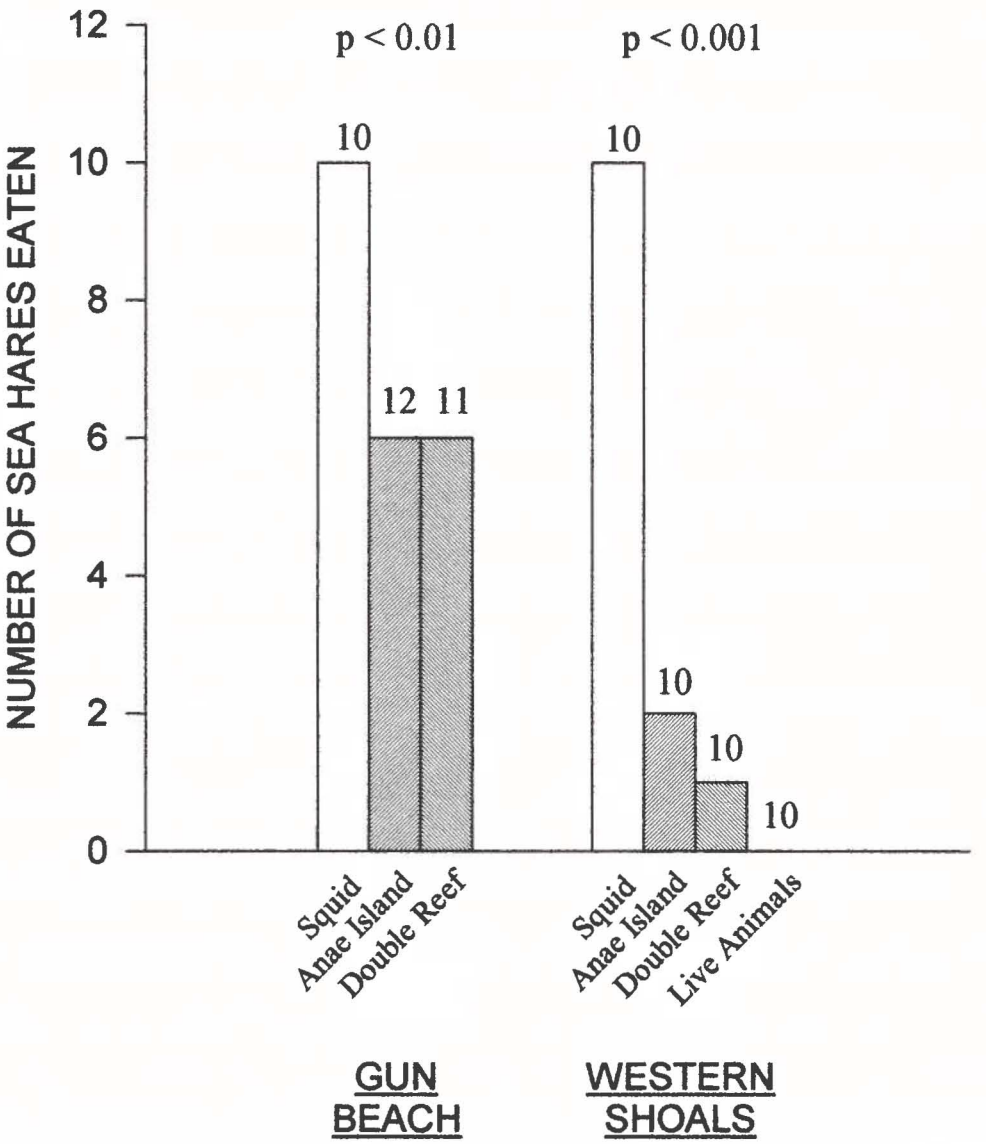


Figure 13. Reef fish assay testing the palatability of whole *Aplysia parvula* at Gun Beach and Western Shoals. In both assays, sea hares collected at Anae Island and Double Reef were offered to reef fishes. At Western Shoals, live animals from Anae Island were also tested. Squid chunks served as controls. Histogram bars represent the number of sea hares eaten. Assays were analyzed using a *G*-test of independence.

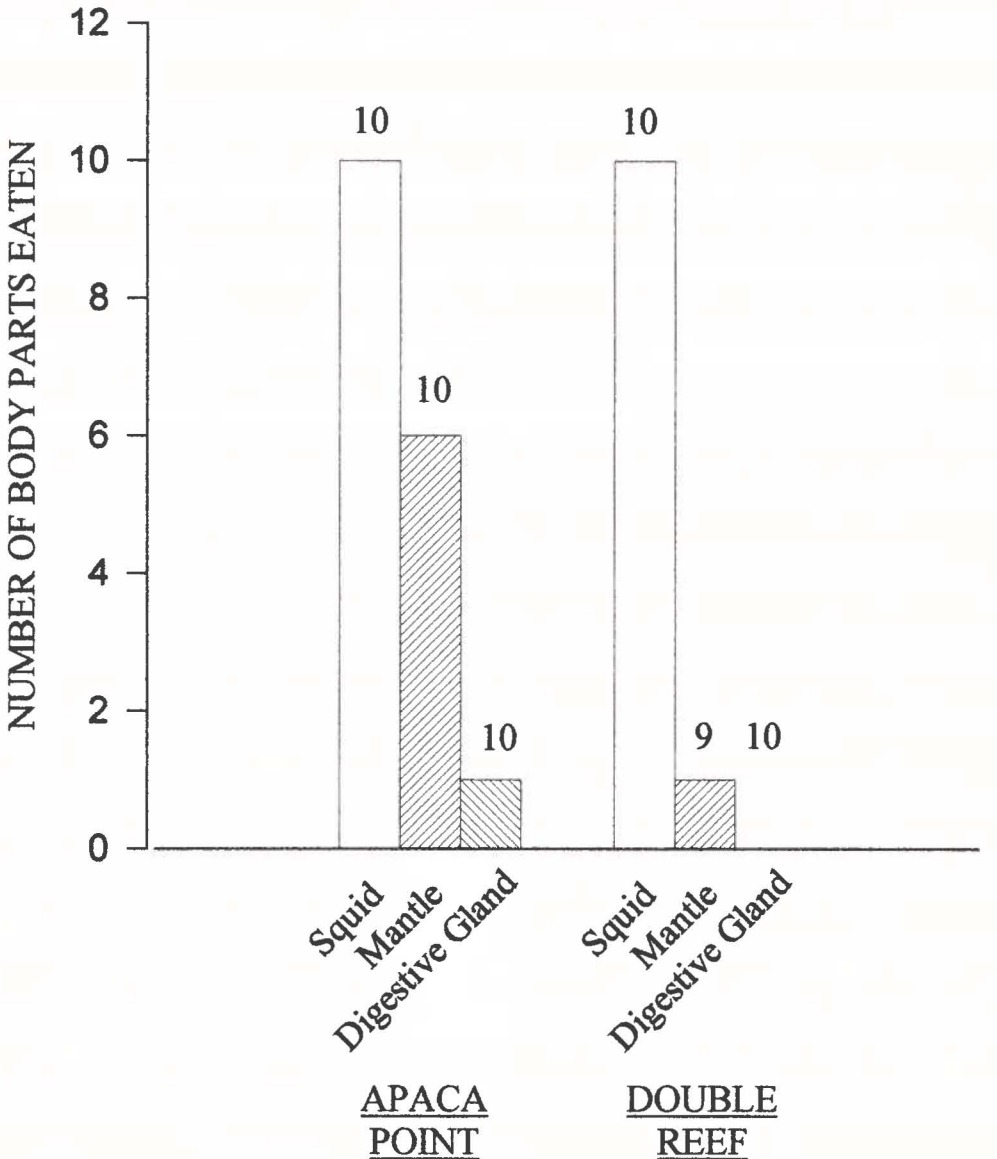


Figure 14. Reef fish assay testing the palatability of sea hare digestive gland and mantle parts at Gun Beach. In both assays, body parts from Apaca Point and Double Reef were offered to reef fishes. Squid chunks served as controls. Histogram bars represent the number of body parts eaten. Numbers above bars represent individual sample sizes. Assays were analyzed using a 3-way log-linear model classifying responses based on site of collection, type of body part, and whether body parts were eaten or rejected. This model, which included all possible two-way interactions, did not significantly fit the observered preference data (df=1, G=4.54, p=0.033). The number of squid chunks eaten was not included in the model.

significant preference for digestive gland or mantle from either Apaca Point or Double Reef (Fisher's Exact, $p = 0.057$, and $p = 1.00$, respectively) (Figure 14). Sea hare body parts were consumed by *Abudefduf saxatilis*, *Thalassoma lutescens* and *Arothron manilensis*. Lastly, chemical analysis by gc-ms demonstrated that concentrations of apakaochtodenes A and B were solely present in the digestive gland of *A. parvula* (Figure 15). Apakaochtodenes were not detected in mantle parts.

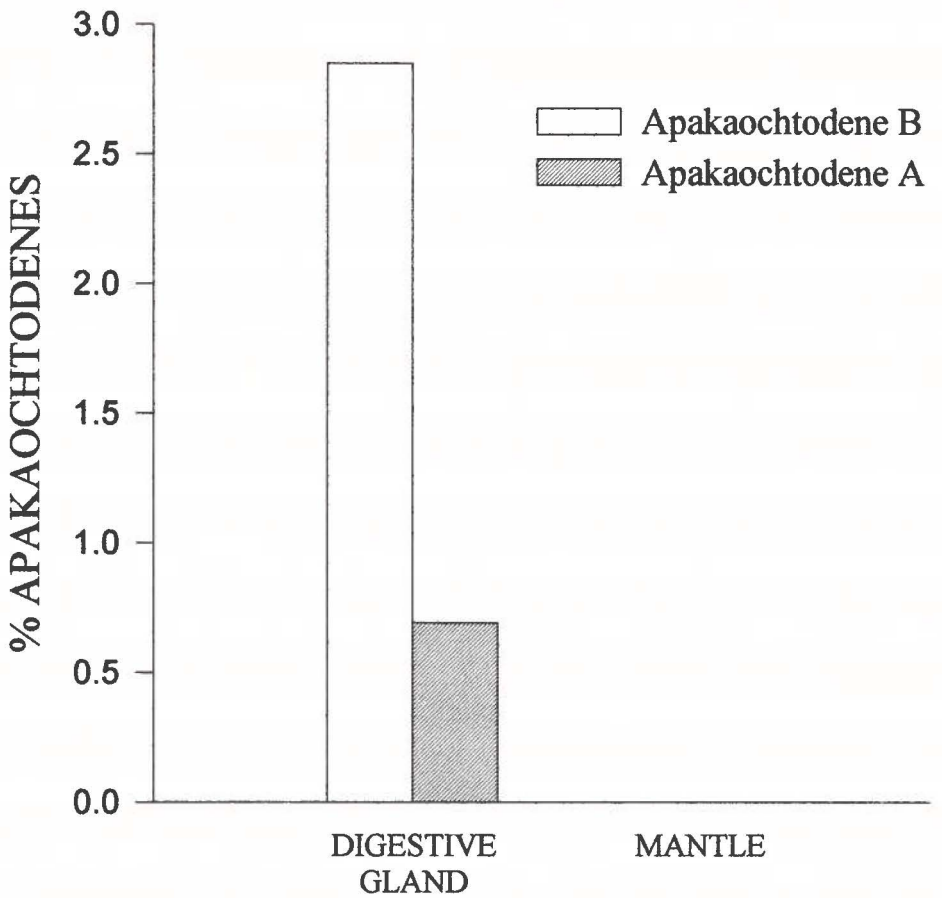


Figure 15. Concentrations of apakaochtodenes in digestive gland and mantle of *Aplysia parvula* from Cocos Lagoon. Digestive gland and mantle parts were pooled (n = 10, respectively) to obtain sufficient material for chemical analysis. The amounts of apakaochtodenes A and B were converted to percent yield based on total dry mass of sea hares. Histogram bars represent the mean amount of apakaochtodenes present in sea hare body parts. Data were compared by visual inspection.

DISCUSSION

Dietary specialization by *Aplysia parvula*

On Guam, *Aplysia parvula* preferred *Portieria hornemannii* compared to all other seaweed diets (Table 1). While sea hares consumed algae that were equally nutritious (as measured by algal organic carbon and TKN), the relative growth of animals was greater when they consumed *P. hornemannii* (Figures 3 to 6). Similarly, in other areas of the Pacific, *A. parvula* consumes a variety of red algae that are rich in secondary metabolites (Carefoot 1987, Rogers et al. 1995), many of which contain halogenated secondary metabolites (Hay and Fenical 1988). For example, in New South Wales, Australia, Rogers et al. (1995) reported that *A. parvula* preferentially consumes the nutritionally and chemically rich red algae *Laurencia obtusa* and *Delisea pulchra*, which are the animal's primary algal hosts. Surprisingly, however, sea hares do not grow when fed *D. pulchra*, from which animals sequester secondary metabolites as protection against predators, but grow best on *L. obtusa*. Rogers et al. (1995) suggested further that, at the level of interspecific comparisons, the dietary preferences of sea hares and the nutritional content of seaweeds which they consume are unrelated (Carefoot 1987, Duffy and Hay 1991). Indeed, feeding specialization by *A. parvula* on chemically defended algae may reflect their need for algal hosts to serve as refuges against predation rather than some unique dietary requirement (but see Futuyma and Moreno 1988, Rogers et al. 1995).

Aplysia parvula may become less susceptible to predation by selectively living and feeding on *Portieria hornemannii*. The major secondary metabolite produced by this alga is apakaochtodene B which is an effective feeding deterrent to herbivorous fishes (Paul et al. 1987, 1990, 1992, Meyer et al. 1994). Therefore, *P. hornemannii* may offer *A. parvula* "enemy-free space" (Jeffries and Lawton 1984); that is, sea hares may escape accidental predation by herbivores or omnivores by inhabiting an alga that large consumers avoid (Rogers et al. 1995). These results are consistent with other studies of alga-mesograzer-predator interactions and agree with the hypothesis that mesoherbivores can minimize predation by specializing on chemically defended seaweeds that are unpalatable, and thus approached less by fish predators (Hay et al. 1987, 1989, 1990a, b, Hay and Fenical 1988, Duffy and Hay 1991, 1994, Hay and Steinberg 1992, de Nys et al. 1996).

Dietary specialization by *Aplysia parvula* may also be influenced by the types and concentrations of algal secondary metabolites found in its host algae. As previously mentioned, the work of Rogers et al. (1995) with *A. parvula* in Australia suggested that sequestration of secondary metabolites from the red alga *Delisea pulchra* may restrict the ability of sea hares to grow. On Guam, *A. parvula* encountered individual *Portieria hornemannii* thalli that contain crude extract concentrations ranging from 0.5% to 4% of algal wet mass. At low concentrations, crude extract had little effect on sea hare grazing, but it acted as an effective feeding deterrent at higher concentrations (Figure 7a). Crude extract was a deterrent at 4%, a concentration found to be present in ≤2% of individual *P. hornemannii* collected from several different locations on Guam (Matlock et al.

unpublished data). These findings are consistent with previous studies reporting that it is not uncommon for mesograzers, who by definition both live on and feed on their host alga, to be indifferent to low concentrations of seaweed chemical defenses, yet they are deterred at higher concentrations of these same metabolites (Hay et al. 1989, 1990, Pennings and Paul 1993, Steinberg 1995, Hay 1996, Nagle et al. 1998).

In contrast, the pure compounds apakaochtodene A and B that are produced by *Portieria hornemannii*, were strongly deterrent to *Aplysia parvula* at all concentrations tested (Figure 8); a surprise, considering that sea hares were indifferent to equivalent proportions of crude *P. hornemannii* extract. The underlying causes of this disparity between the ability of crude extract and apakaochtodenes to deter feeding by sea hares are not known. It is possible that compounds such as triglycerides or sterols within the crude extract of *P. hornemannii* mask the potency of apakaochtodenes A and B. At present, however, the compound (or compounds) that may be responsible for such a masking effect have not been identified.

Intraspecific chemical variation and its affects on *Aplysia parvula*

Site-to-site variation

Despite site-to-site differences in the levels of total apakaochtodenes A and B, *Aplysia parvula* showed no significant preference for *Portieria hornemannii* from any one location (Figure 9, Table 2). These results are consistent with previous studies that have demonstrated significant variation in the production of apakaochtodenes by *P. hornemannii* among different locations on Guam (Puglisi and Paul 1997, Matlock et al.

in press). Individual concentrations of apakaochtodenes A and B, however, were not significantly different between sites in this study which could be due in part to the limited replication of this assay (Figure 9). My data suggest that at the site level, the feeding behavior of *A. parvula* was not influenced by the composition or concentration of apakaochtodenes produced by *P. hornemannii*, and are consistent with findings by Steinberg (1995) in which he showed that feeding by the herbivorous sea urchin *Holopneustes purpurescens* is not affected by variation in levels of phlorotannins in its host kelp *Ecklonia radiata.*

Portieria hornemannii has been shown to exhibit significant variation in apakaochtodene content among thalli within the same site (Matlock et al. in press). It is possible that such differences in the chemical makeup of *P. hornemannii* may present a problem to *Aplysia parvula* in that its algal host may vary in its acceptability as a resource both temporally and spatially (Nagle et al. 1998, Matlock et al. in press). Site-to-site chemical variability of *P. hornemannii* may be an evolutionary response to grazing by generalist feeders such as herbivorous reef fishes (Nagle et al. 1998). Differential grazing by large consumers between sites has been suggested to play a strong influence in selecting for algae to evolve higher levels of chemical defenses (Hay 1996). However, variability in algal chemistry may also be a evolutionary response to specialist-feeders such as *A. parvula* (Hay 1996, Becerro et al.1998, Nagle et al. 1998).

Within-alga variation

Few studies have examined the ecological consequences of within-thallus chemical and nutritional variation on feeding by mesograzers, and most research to date has focused on brown algal species. For example, in the alga *Fucus distichus,* high concentrations of phlorotannins in older algal tissues are less preferred by two species of herbivorous snails than young apices (Van Alstyne 1989). The apical tips of the alga *Zonaria angustata,* which contain lower densities of phlorotannin containing-physodes and are less tough than other tissues, are more susceptible to grazing by the amphipods *Tethygeneia* sp. and *Hyale rubra* (Poore 1994). In addition, the amphipod *Ampithoe longimana* and the sea urchin *Arbacia punctuata* prefer to consume the apical meristems of the alga *Dictyota ciliolata* which contains lower levels of diterpenoid metabolites than older, less palatable algal tissues (Cronin and Hay 1996a). No other previous studies on the intraspecific variation of algal secondary metabolites have experimentally demonstrated how chemical defenses affect the ability of mesoherbivores to choose between specific parts of algal thalli (but see de Nys et al. 1996). Thus, *Portieria hornemannii* is the first red alga for which such an interaction has been described.

In this study, *Aplysia parvula* preferred the tips compared to the bases of *Portieria hornemannii* thalli (Table 4). More than 2 times the levels of apakaochtodene B were observed in the tips of *P. hornemannii* compared to bases (Figure 10). Algal chemistry alone, however, cannot explain these results since *A. parvula* was not attracted to these monoterpenes. *Aplysia parvula* may prefer the tips of thalli compared to bases for their higher nutritional content. Cronin and Hay (1996a) suggest that soluble protein

can act as a feeding stimulant to herbivores. Analysis of tips revealed that levels of TKN were significantly higher than levels in the bases (Figure 11). The preference for algal tips by mesograzers may be due to variation in the nutritional qualities of different parts of their host algae (Rogers et al. 1998). Thus, the variation in nutritional quality in *P. hornemannii* thalli helps to explain the observed feeding preferences of *A. parvula*.

While *Aplysia parvula* clearly prefer *Portieria hornemannii* tips relative to bases, it is not known whether this selectivity of algal tissues may play a role in deterring predation. Predation by large, generalist predators such as reef fishes may restrict the movement of some mesograzers such as *A. parvula* to the cryptic, basal sections of their host algae (Hay et al.1989, Rogers et al. 1998). Individual sea hares may face a tradeoff in their choice between the nutritious tips of *P. hornemannii* as food versus their use of algal bases as a refuge from predators. Duffy and Paul (1992) have demonstrated that, among different reef organisms, prey nutritional quality can be of equal or greater importance than secondary metabolites in determining the dietary preferences of their consumers. Therefore, as suggested by Rogers et al. (1998), *A. parvula* may be limited to diel patterns of vertical movement on their host algae; sea hares may hide in algal bases during the daytime hours, coming out only at night when they are less likely to be consumed by visual predators such as reef fish.

Inducible chemical defenses in *Portieria hornemannii*

Grazing by *Aplysia parvula* did not positively induce the production of apakaochtodene B in *Portieria hornemannii* (Figure 12). The apakaochtodene B content

of final controls was higher than that found in final treatments; a surprise considering that *A. parvula* preferred control thalli that had not been previously damaged (Table 4). It is possible that the feeding preferences of sea hares may have been influenced by intraspecific changes in the nutritional quality of thalli (Table 5). As previously discussed, *A. parvula* preferentially fed on *P. hornemannii* tips which contained notably higher levels of TKN and monoterpenes compared to bases. Thus, the disparate levels of apakaochtodene B that were found among final controls and treatments may be best explained by the assumption that sea hares selectively remove the highly nutritious tips of *P. hornemannii*, leaving portions that are on average lower in secondary chemistry.

Numerous investigations using natural herbivores (Steinberg 1995) and artificial clipping (Paul 1992, Pfister 1992, Paul and Van Alstyne 1992, Steinberg 1994) to damage seaweeds have failed to show evidence of induction of chemical defenses in marine algae. Several authors argue that mechanical damage to algae does not effectively mimic actual herbivores and suggest that studies should use herbivores rather than artificial clipping whenever possible (Steinberg 1994, Cronin and Hay 1996b, Hay 1996). Specifically, Hay (1996) has suggested that mesograzers, because of their small size and limited mobility, are the most likely cue for the induction of chemical defenses in their benthic hosts. My results, however, do not support this claim. It is possible that the number of *Aplysia parvula* (n = 3) that I used to damage individual algae in the laboratory did not effectively simulate natural densities of sea hares in the field. Perhaps, not only mesograzers themselves, but the number of herbivores grazing on their host prey may play a factor in eliciting an induction response. The natural densities of *A. parvula*

on *Portieria hornemannii* were not addressed in this study, but should be considered as a possible factor in the induction of apakaochtodenes in *P. hornemannii* on Guam.

Predation on *Aplysia parvula*

Similar to other sea hares (Faulkner 1992, Pennings 1994), *Aplysia parvula* sequesters diet-derived chemical defenses from its host algae (Rogers et al. 1995, de Nys et al 1996), which on Guam was *Portieria hornemannii*. My field experiments to test the affects of site-to-site chemical variation in *P. hornemannii* on the susceptibility of *A. parvula* to predation by reef fishes indicated that sea hares, regardless of where they were collected, were unpalatable to reef fish (Figure 13). Thus, despite differences in the composition and concentrations of apakaochtodenes in *P. hornemannii* between sites, *A. parvula* which fed on *P. hornemannii* from Apaca Point were no more vulnerable to predation than when they had fed on *P. hornemannii* from Double Reef. My data on the fish species that consumed whole *A. parvula* (*Thalassoma lutescens*, *Halichores hortulanus*, *Balistapus undulatus*, *Naso vlamingii*, and *Scarus* sp.) are similar to those that are reported (*T. hardwickii* and other wrasses) to eat the sea hare *Stylocheilus longicauda* (Paul and Pennings 1991). The susceptibility of *A. parvula* to predators was slightly higher at Gun Beach than at Western Shoals. Generally, there were more parrotfish and damsels (*Scarus* spp. and *Amblyglyphidodon* spp., respectively), and fewer wrasses (*Thalassoma* spp.) present at Western Shoals compared with Gun Beach. This is, perhaps, of no surprise since Western Shoals is a popular tourist site where divers hand feed fish several times a day. As suggested by several authors (Pennings and Paul

1991, Avila and Paul 1997), diet-derived secondary metabolites in sea hares may deter some fish predators, but are ineffective against others such as tolerant (e.g., wrasses) or opportunistic predators (e.g., damselfish).

The body parts (digestive gland and mantle) of *Aplysia parvula* were also unpalatable to reef fish (Figure 13 and 14). However, while reef fishes showed no preference for individual body parts from Double Reef, fishes preferred mantle over digestive gland parts from Apaca Point (Figure 14). Concentrations of apakaochtodenes in *A. parvula* varied among different body parts with algal compounds present only in the digestive gland and not in the mantle (Figure 15). These data are consistent with previous findings of quantitative (de Nys et al. 1996, Pennings and Paul 1993) and qualitative variation (Winkler 1969) of algal secondary metabolites in sea hares. However, since sea hares sequester algal metabolites in their digestive gland (where they are not optimally located for defense) rather than mantle tissues, the anti-predatory role of these compounds have been disputed (Pennings and Paul 1993, Pennings 1994, de Nys et al. 1996). However, several studies have demonstrated that sea hare mantle is unpalatable to predators (Ambrose et al. 1979, Pennings 1990, 1994). It is possible that sea hares themselves may produce an unpalatable compound which is of a greater defensive value than diet-derived metabolites (Pennings 1994, Pennings and Paul 1993). While this latter argument cannot be ignored, my data indicate that *A. parvula* body parts are unpalatable to most reef fish and are consistent with the hypothesis that algal secondary chemistry acquired by sea hares function as predator deterrents (Paul and Pennings 1991, Faulkner 1992, de Nys 1996).

Conclusions

On Guam, *Aplysia parvula* preferentially consumed and grew best on their algal host *Portieria hornemannii*. However, such a relationship did not come without a cost to sea hares grazing on this alga. High concentrations of *P. hornemannii* crude extract and the pure compounds apakaochtodene A and B acted as feeding deterrents to *A. parvula*. Additionally, differences in the intraspecific chemical and nutrient content of *P. hornemannii* affected the feeding behavior of *A. parvula* and its ability to choose between specific parts of algal thalli. *Portieria hornemannii* is the first red alga for which such an alga-mesograzer interaction has been described. There was no evidence of the induction of apakaochtodenes in *P. hornemannii* via grazing by *A. parvula*. While chemical induction may not occur in *P. hornemannii*, it is possible that the number of sea hares used to graze thalli tissue in this study were not sufficient to evoke an induction response. Lastly, *A. parvula* sequestered apakaochtodenes and whole animals as well as body parts were deterrent to reef fishes. While these defenses were not effective against all predators, this observation is consistent with the view that diet-derived algal metabolites in sea hares play a role in deterring predation (Paul and Pennings 1991, Faulkner 1992, de Nys 1995).

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