

# Effect of mesograzers and nutrient levels on induction of defenses in several Brazilian macroalgae

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**ABSTRACT:** Herbivory can greatly modify benthic community structure by affecting the distribution of algal species. To deter herbivores, algae have developed several mechanisms, including the induction of chemical and morphological defenses, which may be influenced by nutrient availability. We tested 4 red (*Chondrophycus flagellifera*, *Cryptonemia seminervis*, *Osmundaria obtusiloba*, *Pterocladia capillacea*), 4 brown (*Dictyota menstrualis*, *Lobophora variegata*, *Sargassum vulgare*, *Styopodium zonale*), and 1 green (*Codium decortatum*) algae for inducible defenses following exposure to direct consumption by an amphipod community dominated by *Elasmopus brasiliensis*. In addition, the effects of water-borne cues from nearby grazed conspecifics and non-grazing consumers on the induction of defenses were examined in *C. decortatum* under natural and enhanced (200% natural) nutrient levels. Induction of defense was assessed in choice-feeding assays, using live algae or artificial food containing non-polar extracts of amphipod-exposed (treated) and non-exposed (control) algae. Palatability levels, estimated as the relative difference in wet mass due to consumption in feeding assays between grazer-exposed and control plants, declined significantly in 3 species after the acclimatization period. Tissue from the directly consumed red alga *P. capillacea* (live alga) was significantly less palatable than tissue from the control plants. Likewise, a significant effect was observed in the brown alga *L. variegata*. Similar, although not statistically significant, trends were observed in 6 other species. For the green alga *C. decortatum*, nutrient enrichment did not affect induction of defenses by herbivores, yet unfertilized plants were more palatable than fertilized conspecifics.

**KEY WORDS:** Plant–animal interaction · Tropical seaweeds · Mesograzer · Nutrient enrichment · Feeding preference

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## INTRODUCTION

Herbivory strongly impacts the distribution of algae and thus benthic community structure (Hay 1997, Duffy & Hay 2000) in both temperate and tropical marine environments (Hay & Fenical 1988). On coral reefs for example, herbivores can remove almost 100% of macroalgal production (Hay & Steinberg 1992, Paul

et al. 2001). To persist against natural enemies, algae must escape, tolerate or deter them (Hay & Fenical 1988, Duffy & Hay 1991).

Besides nutritional and associational defenses, chemical and morphological defenses are the most common mechanisms used by macroalgae to deter herbivores, and make algae active participants in grazer–alga interactions (Cronin 2001). Yet it has been

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suggested that defenses are costly and can only be achieved by diverting materials and energy from other life processes such as growth or reproduction (Cronin 2001). For such a mechanism to evolve, the benefits of defenses should increase fitness more than the costs reduce fitness.

Constitutive defenses require expenditure of resources even when consumers are absent. In contrast, inducible defenses allow defense-associated costs, if any, to be deferred until consumers have been detected, at which time the costs will be offset by the benefits of protection (Cronin & Hay 1996c). Besides this presumed advantage of increased resource availability for other plant processes when the defense is not induced, induction also creates variability in food quality and therefore hinders herbivores in their adaptation to host plants (Agrawal & Karban 1999). Furthermore, plants that can induce defenses are less exposed to the potential autotoxic effects of storing deterrent secondary metabolites when they are not required (Agrawal & Karban 1999). Inducible defenses should be favored when defense chemicals are costly to produce, the presence of the consumers is variable and unpredictable over temporal and spatial scales, and when individual plants are longer lived than their consumers (Pavia & Toth 2000). Furthermore, reliable cues (visual/physical contact, water-borne signals) like herbivory must be present in the environment (Toth & Pavia 2000, Sotka et al. 2002).

There is considerably less evidence of herbivore-induced defensive responses in marine macroalgae (e.g. Paul & Van Alstyne 1992, Yates & Peckol 1993, Cronin & Hay 1996c, Hammerstrom et al. 1998, Pavia & Brock 2000) compared to terrestrial plants. This inconsistency could be explained by the lack of an efficient transport system in non-vascular algae to translocate the induction stimulus throughout the algae, or simply be due to the comparatively small amount of research effort in marine systems (Cronin & Hay 1996c).

Because mesograzers are ubiquitous herbivores with rapid population growth in nearly all vegetated habitats, Duffy & Hay (2000) argued that they could have important impacts on plant assemblages, despite their low mobility and biomass consumption compared to large herbivores such as fishes. Mesograzers consume small amounts of algae over extended periods, which might allow the algae to respond to their attack (Cronin & Hay 1996c).

Most chemical deterrents produced by algae are lipid-soluble secondary metabolites (Hay & Fenical 1988, Hay & Steinberg 1992). In addition to functioning as herbivore deterrents, algal secondary metabolites may also serve other roles such as allelochemicals, antifoulants (da Gama et al. 2003), antibiotics (Cronin 2001), or even perform multiple functions (Schmitt et al.

1995, Pereira et al. 2003). Secondary metabolite levels in marine macroalgae can be influenced by or correlated with a number of environmental factors including light intensity, desiccation, salinity and nutrient levels (e.g. Ilvessalo & Tuomi 1989, Renaud et al. 1990, Yates & Peckol 1993, Cronin & Hay 1996ab, Peckol et al. 1996, Pavia et al. 1997, Pavia & Brock 2000). These factors can exert stress on plant fitness. The 'environmental stress theory' suggests that an organism in a stressed state will be less able to acquire resources and will allocate these reduced resources to maintenance rather than defense production, assuming that the provision of defenses represents a metabolic cost (Cronin 2001).

Nutrient availability (especially of N and P) tends to be a limiting factor for plant growth in marine systems (e.g. Larned 1998). Current allocation models, like the 'growth-differentiation balance hypothesis' or the 'carbon-nutrient balance hypothesis', consider resources responsible for allocation of defenses and predict phenotypic variation in the secondary defense metabolism of plants (Lerdau et al. 1997). They predict a negative correlation between growth and concentrations of C-based secondary compounds like phlorotannins or terpenes (Haukioja et al. 1998) because of a trade-off between primary and secondary metabolic pathways (Herms & Mattson 1992, Muzika 1993), which compete for limited resources within a plant (Glynn et al. 2003).

For this investigation, 9 different tropical algae were tested for induction of defense following direct grazing by amphipods. Furthermore, the effect of nutrients and different consumer activities on inducible defenses in a green alga was examined. This study attempted to determine whether the palatability levels of algae (i.e. the relative level of algae consumption by herbivores between treated and control algae) were affected by (1) different consumer activities or (2) interactive effects of consumers and nutrients, and (3) if found, which changes in palatability were caused by non-polar chemical defenses?

## MATERIALS AND METHODS

**Sampling sites and organisms.** Inducible defenses of 9 different species of algae (Table 1) were tested in 2 bio-assay induction experiments in the laboratory of the Department of Marine Biology of the Universidade Federal Fluminense (UFF) in Niterói (Rio de Janeiro state, Brazil), between January and May 2003.

Algae were collected by hand while SCUBA- or free-diving from the littoral zone of Rasa Beach and Forno Beach of the city Armação de Búzios (22° 45' to 22° 49' S, 41° 52' to 41° 57' W), and from Itaipu Beach in Niterói (23° 54' S, 43° 02' W); both places are located on the SE coast of Brazil.

Table 1. Algae tested and characteristics of sampling sites. All sites are rocky shores at Búzios (Rio de Janeiro state, Brazil), except Itaipu, which is situated at Niterói (Rio de Janeiro state, Brazil)

Alga	Location	Depth (m)	Zone
<b>Chlorophyta</b>			
<i>Codium decorticatum</i> (Woodw.) M. Howe	Itaipu	2	Subtidal
<b>Rhodophyta</b>			
<i>Chondrophyucus flagellifera</i> (J. Agardh) Nam	Rasa	1–2	Intertidal/subtidal
<i>Cryptonemia seminervis</i> (C. Agardh) J. Agardh	Rasa	1–2	Intertidal/subtidal
<i>Osmundaria obtusiloba</i> (C. Agardh) R. E. Norris	Rasa	1–2	Intertidal/subtidal
<i>Pterocladia capillacea</i> (S. G. Gmel.) Santel. & Hommers.	Forno	0.5	Intertidal
<b>Phaeophyta</b>			
<i>Dictyota menstrualis</i> (Hoyt) Schnetter, Hörnig, & Weber-Peukert	Rasa	1–2	Intertidal/subtidal
<i>Lobophora variegata</i> (J. V. Lamour.) Womersley ex E. C. Oliveira	Forno	3	Subtidal
<i>Sargassum vulgare</i> C. Agardh	Rasa	1–2	Intertidal/subtidal
<i>Styopodium zonale</i> (Lamour.) Papenf.	Forno	3	subtidal

In preliminary studies with 4 species of herbivores, the amphipod community dominated by *Elasmopus brasiliensis* was identified as the most suitable grazers for the experiments, primarily because they feed on all algae pieces with similar, continuous consumption rates. The number of amphipods per aquarium (30) was also determined in preliminary assays, so that amphipods could cause relatively small but detectable biomass loss. Amphipods were collected from Boa Viagem Beach (Niterói), where they occur in association with the common green alga *Ulva fasciata* and the bryozoans *Bugula neritina* and *Zoobotryon verticillatum*. They were immediately transferred to the laboratory and maintained in plastic trays with *U. fasciata* as a food source and shelter until needed.

**Experimental set-up.** In 2 closed water systems (ca. 9000 l), seawater was continuously aerated and filtered through a system comprised of sand, plankton mesh, activated carbon, and a protein skimmer. The water temperature was  $18.5 \pm 1^\circ\text{C}$ , pH was  $8.5 \pm 0.5$  (pH-MV-TEMP-analyzer), and salinity was  $36 \pm 1$  (Salinometer 5-10, No. 147, precision type Shibuya Optica).

Seawater was distributed by tubes (effective diameter = 0.4 cm) to the experimental units (EU), and water flux was regulated by flow controllers, exchanging the total water volume in EUs twice per day. The EUs comprised rectangular transparent plastic aquaria ( $12 \times 18 \times 11$  cm, volume = 2 l) for treatments, and round transparent plastic aquaria (diameter = 14, volume = 500 ml) for controls, each with an in- and out-flow; treatment and control aquaria were always run as pairs. Preliminary studies had shown that the size and shape of aquaria did not affect plant growth as long as the water-flow rates were identical. In each rectangular aquarium, a transparent perforated 1 mm PVC plate was inserted to create 2 compartments (Fig. 1). All EU pairs were randomly arranged below large

reservoirs that comprised part of both circulating water systems. Algae pieces were exposed to a 9:15 h light:dark cycle using daylight fluorescent lamps (40 W, Agrolux). The lamps irradiated the target algae at a mean quantum flux density (Li-185 B, Li-Cor) of  $26 \pm 18.1 \mu\text{mol m}^{-2} \text{s}^{-1}$  ( $n = 93$ ). Seawater was recycled during the acclimatization and recovery phases (without consumers, see next subsection), but was used in a flow-through mode during the treatment phase (with consumers) to avoid possible contamination by water-borne chemical cues between EUs that could influence defense induction (Toth & Pavia 2000).

**Induction experiments.** After collecting multiple individuals as a random sample from natural popula-

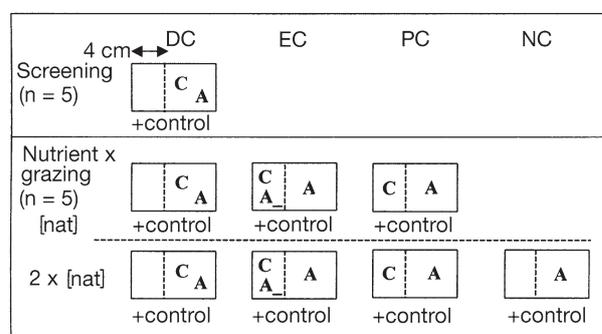


Fig. 1. Experimental units (EU) used in screening experiment and nutrient  $\times$  grazing experiment. Treatment levels ( $n = 5$ ) were DC (direct consumption), EC (nearly grazed conspecifics), PC (non-grazing consumers) and NC (no consumption; control + fertilizer). C: consumer (amphipods); A: test algae pieces; A\_: alga pieces from same species; [nat]: natural nutrient concentration;  $2 \times$  [nat]: 2-fold natural nutrient concentration; vertical dashed line inside individual aquaria indicates transparent perforated PVC-plate; n: number of algae pieces per EU; controls were always at natural nutrient concentrations and not exposed to consumers

tions of each of the 9 algae species, and freeing them of epiphytes, 50 equally sized apical pieces of each species were cut and pooled. We randomly allocated 5 pieces from each species pool, with an average wet mass of  $0.1005 \pm 0.0385$  g (*Cryptonemia seminervis*),  $0.1083 \pm 0.0289$  g (*Dictyota menstrualis*),  $0.1303 \pm 0.0605$  g (*Lobophora variegata*),  $0.2284 \pm 0.1019$  g (*Pterocladia capillacea*),  $0.3502 \pm 0.1433$  g (*Osmundaria obtusiloba*),  $0.4046 \pm 0.1424$  g (*Stypopodium zonale*),  $0.5835 \pm 0.1539$  g (*Sargassum vulgare*),  $1.7474 \pm 0.7394$  g (*Chondrophyucus flagellifera*), and  $1.7677 \pm 0.5773$  g (*Codium decorticatum*) to 5 EU pairs ( $n = 5$ ) for induction experiments. In addition, 10 pieces were deep-frozen immediately after collection and another 10 pieces were distributed pairwise into 5 rectangular EUs for comparison between natural defense level and level of defense after 14 d.

The experiments consisted of 3 phases: acclimatization phase, treatment phase (defense induction by amphipod consumption), and recovery phase. During the acclimatization phase (14 d), pieces of algae were cultivated in the absence of amphipods to allow subsidence of unknown defense levels and acclimatization to experimental conditions. This phase allowed comparable initial conditions of pieces from different individual algae which might have had different grazing histories in the field prior to the induction treatment. During the treatment phase (7 d), 30 amphipods ( $0.0441 \pm 0.0151$  g blotted wet weight) were added to each rectangular EU as defense-inducers. During the recovery phase (14 d), the algae were allowed to recover in the absence of consumers in order to assess the relaxation of defense or retarded defense responses. The duration of the treatment phase was determined according to mean induction times given in the literature (e.g. Hammerstrom et al. 1998, Toth & Pavia 2000).

We performed 2 experiments. In Expt 1, the screening experiment, 9 algae species (Table 1) were tested for induction of defense by direct amphipod grazing ( $n = 5$ ; Fig. 1). In Expt 2, the green alga *Codium decorticatum* was chosen to study the effects of herbivory and nutrients on the induction of defense in a full-cross  $3 \times 2$  factorial experiment (nutrient  $\times$  grazing experiment). This alga tended to display induced defenses after direct consumption and grew well under laboratory conditions during the screening experiment. We applied 3 consumption levels ( $n = 5$ ): (1) DC, direct consumption by amphipods; (2) EC, exposure to grazed algae (pieces of test algae were placed in the downstream compartment of a treatment EU receiving water from a compartment containing pieces of grazed algae); (3) PC, presence of consumers (test algae pieces were placed in the downstream compartment of a treatment EU receiving water from a compartment

containing amphipods only). Nutrient treatments were conducted with: (1) a natural nutrient concentration in the seawater (nitrate concentration  $18.1 \pm 0.2$   $\mu$ M); and (2) a 2-fold natural nutrient concentration (100% nitrate increase). In all, 6 treatment combinations were performed plus 1 treatment with the nutrient factor only (i.e. no consumption, NC in Fig. 1). The controls for each EU of the 7 treatments received natural seawater and were never exposed to consumers. Nutrient enrichment was achieved by addition of a nutrient stock solution, derived from slow-release NPK fertilizer beads (Planctacote pluss 4M, Aglukon). Besides nitrate, this fertilizer also provided additional potassium, phosphate, magnesium and other trace elements to the algae. At the beginning of the treatment phase, nutrients were added to the respective EUs and left in the seawater during the recovery phase.

**Choice-feeding assays.** Each phase (acclimatization, treatment, and recovery) was immediately followed by choice-feeding assays using entire pieces of live algae and artificial food containing non-polar extracts of the relevant alga. Differences in palatability between treated and control pieces of live algae can result from changes in chemical, morphological or nutritional algal traits (Hay et al. 1998) and do not allow the type of defense to be distinguished. Thus, incorporation of the algal extract into agar-based food was used to test for non-polar chemical deterrence by the algae. Only assays with artificial food containing non-polar extracts were performed in the nutrient  $\times$  grazing experiment.

Feeding assays after the acclimatization phase compared the initial defense level of the alga (non-acclimatized; assumed to depend on an unknown *in situ* level of grazing prior to the experiments) with the level of defense after 14 d without any herbivory (acclimatized). Prior to these feeding assays, acclimated seaweed pieces were frozen for 2 d to achieve the same state as the already frozen, non-acclimated pieces at the beginning of the feeding experiment. In feeding assays after the treatment and recovery phases, feeding preference between control (unexposed to grazers) and treated (grazer-exposed and herbivore-damaged during the treatment phase) pieces was examined, using not just fresh growth but the entire algae piece from the respective phase. Therefore, if induction of anti-herbivore defenses occurred mostly in new growth, we may not have detected this effect, because of dilution through inclusion of older tissues. All choice-feeding assays were performed in round aquaria (diameter = 14 cm, volume = 500 ml), using 30 amphipods ( $0.0441 \pm 0.0151$  g blotted wet weight) per aquarium. Amphipods were not used in more than 1 assay and dead individuals were replaced. The assays lasted until either ca. 50% of 1 piece/pellet was eaten or 3 d elapsed, whichever occurred first. A control for autogenic chan-

ges in live algae pieces was incorporated (pair of control and previously treated piece) to estimate change in biomass independent of consumption (Peterson & Renaud 1989). Seawater from assay containers was exchanged daily during the assays.

Consumption rates ( $\text{mg } 3 \text{ d}^{-1}$ ) were calculated to assess relative differences in palatability between treated and control algae, using the difference in wet weight (resolution 0.1 mg) of live algae pieces before and after feeding-preference assays. Consumption rates were corrected for autogenic changes according to the formula of Cronin & Hay (1996a). To minimize errors in measurement due to absorbed water, pieces were dried by blotting with absorbent paper prior to weighing. Consumption of diet containing non-polar extracts was calculated by counting the mesh squares without food.

**Preparation of artificial food containing non-polar extracts.** Artificial food was prepared according to Hay et al. (1994). We focused exclusively on the deterrent effects of lipid-soluble compounds extracted by dichloromethane (DCM) (Cetrulo & Hay 2000). DCM extracts mainly lipid-soluble, relatively non-polar secondary metabolites, which are generally responsible for chemical defenses in algae (see Paul et al. 2001 and Steinberg et al. 2001 for reviews). Algae pieces for making food pellets were dried at room temperature (1 to 2 d), then weighed and cut into small pieces prior to extraction (3 times) in 3.0 ml of DCM with the help of ultrasound (1 min). The lipophilic extract was incorporated into a powder of the chemically undefended food alga *Ulva fasciata* that was equivalent to the dry weight of the original biomass of the target alga piece. The *U. fasciata* powder was then added to 6.0 ml distilled water and mixed with a previously heated solution of 10.0 ml distilled water and 0.4 g agar, after cooling to ca. 60°C. The mixture was then poured into a mold of PVC window screen (10 × 10 squares, 1 square = 1 mm<sup>2</sup>), which provided both support for the agar pellet and an easy method of evaluating consumption by counting the squares consumed. Thin-layer chromatography (TLC) analysis of heated and non-heated extracts of all algae was performed to ensure that secondary metabolites in the extracts were not broken down by thermal degradation when added to the hot agar (Hay et al. 1987, Cetrulo & Hay 2000).

**Statistical analyses.** Effect-ratios are the preferred response-variable for the analysis of consumption rates because, as a relative measurement, effect-ratios will adjust consumer-related differences in consumption rates due to (e.g.) size or starvation level of different amphipods. However, for live algae, we could not calculate effect-ratios because some algae pieces grew faster than the amphipods grazed them, resulting in negative consumption rates. For this reason we ana-

lyzed the consumption data of live algae and artificial food in the screening experiment by different tests.

Consumption rates from live alga choice-feeding assays in the screening experiment were analyzed by a paired *t*-test for dependent samples (non-acclimatized versus acclimatized pieces; treated versus control pieces). We assessed whether artificial food from control algae was preferred over artificial food from treated algae, i.e. if an induction of alga defenses had occurred. This was done by testing whether an effect ratio of 1 (the null hypothesis) was higher and thus outside the 95% *t*-distribution ( $df = 4$ ) of effect-ratios from our experiment (Underwood 1997, p. 62).

Prior to the analysis, homogeneity of variance was tested by Cochran's *C*-test. In the case of heterogeneous data, a log-transformation was applied and the data were rechecked for homogeneity. Heterogeneous data giving significant results with the paired *t*-test cannot be interpreted without error (Underwood 1997) and were thus analyzed by a Wilcoxon signed-ranks test.

In the nutrient × grazing experiment (interaction between defenses and nutrients) a paired *t*-test was conducted after the acclimatization phase (non-acclimatized versus acclimatized pieces). The homogeneity of the data was also tested by Cochran's *C*-test and followed the same steps as the screening experiment. Effect-ratios (number of squares consumed of artificial food containing non-polar extracts from the treated piece divided by the consumption of artificial food from the control piece) after the treatment and recovery phases were compared by 2-way ANOVA to analyze whether induction varied among herbivore exposure (fixed, 3 levels), among nutrient treatments (fixed, 2 levels) and whether nutrients and grazing type interacted to affect the preference for artificial food containing non-polar extracts from *Codium decorticans*. In the advent of a significant result from the 2-way ANOVA, Tukey's post-hoc test was run to determine the position of the significant difference.

To assess whether the experiments had inadequate power to detect differences in consumption rates between control and treated plants, we used the software program 'Gpower V 2.0' ([www.psych.uni-duesseldorf.de/aap/projects/gpower](http://www.psych.uni-duesseldorf.de/aap/projects/gpower)), to estimate the percent probability of detecting differences (Erdfelder et al. 1996).

## RESULTS

### Screening experiment (Expt 1)

Differences in the palatability of live algae and the diet containing non-polar extracts in response to herbivory were recorded. Acclimatized live pieces (left

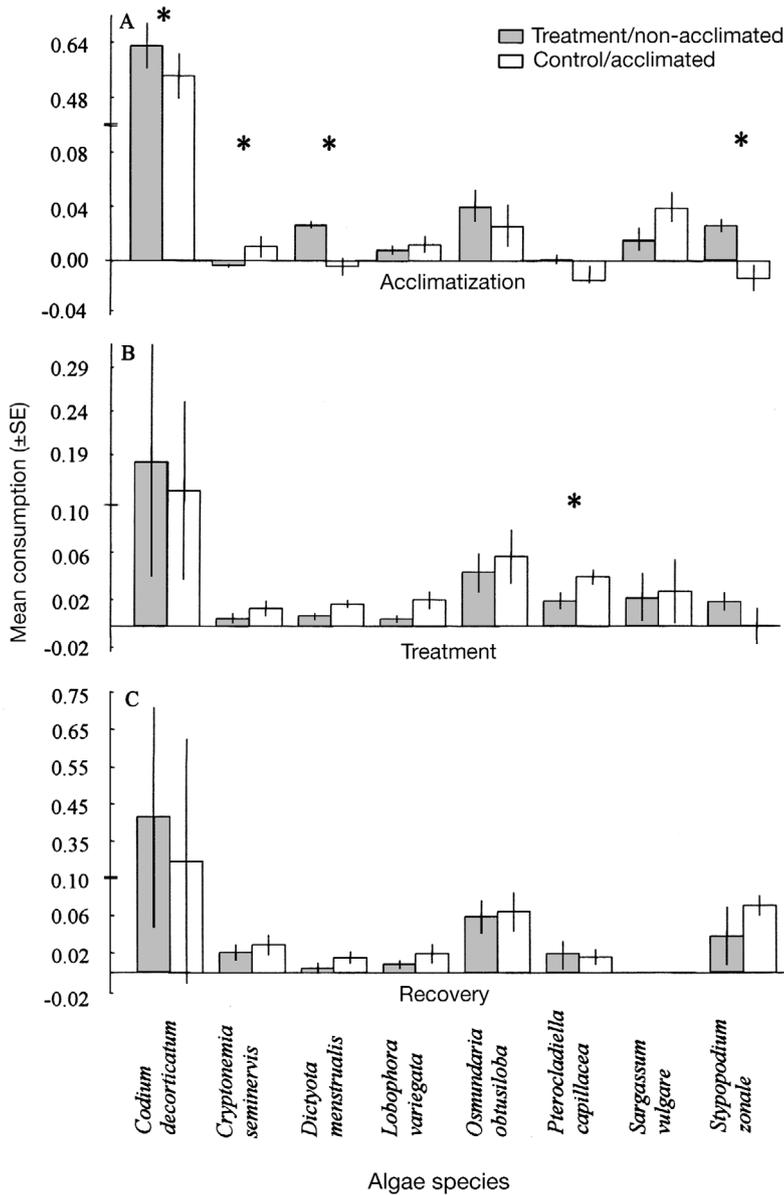


Fig. 2. Screening experiment (Expt 1). Mean ( $\pm$ SE) grazing (g blotted wet mass) of *Elasmopus brasiliensis*-dominated amphipod community on live algae. Algae incubated with 30 grazer individuals in 3 d choice-feeding assays after (A) acclimatization, (B) treatment and (C) recovery phases, to assess relative differences in palatability between amphipod-exposed and non-exposed control algae. \*Significant result of paired *t*-test comparing distribution of differences between control and treated algal pieces against null-hypothesis of no difference, note discontinuity in ordinates

ungrazed for 14 d following collection from the field) were consumed 32% more than non-acclimatized pieces for the red alga *Cryptonemia seminervis* (Wilcoxon signed-ranks test,  $Z_4 = -2.02$ ,  $p = 0.043$ ), (Fig. 2A, Table 2). In contrast, non-acclimatized live pieces were preferred by 15 and 49% over acclimatized live pieces of the brown algae *Dictyota men-*

*strualis* (paired *t*-test,  $t_4 = 3.36$ ,  $p = 0.028$ ) and *Stypopodium zonale* (paired *t*-test,  $t_4 = 3.73$ ,  $p = 0.020$ ), respectively, and by 14% for the green alga *Codium decorticatum* (paired *t*-test,  $t_4 = 3.26$ ,  $p = 0.031$ ); (Fig. 2A, Table 2). The probability of finding significant differences was <80% for all species, except for *S. zonale* (82%), *C. decorticatum* (85%), and *D. menstrualis* (86%). After the treatment phase (exposure to direct grazing by amphipods), the mean consumption of live control pieces of *Pterocladia capillacea* was 50% higher than that for previously consumed pieces (paired *t*-test,  $t_4 = -5.40$ ,  $p = 0.006$ ); (Fig. 2B, Table 2). Likewise, the palatability of ungrazed live algae pieces was higher than that of previously grazed pieces in 3 brown algae and 2 red algae, whereas the reverse pattern was found for 1 brown and 1 green alga (Fig. 2B). Besides *P. capillacea* (99% probability), feeding assays with other algae had a probability <60% of finding significant differences. At the end of the recovery phase (14 d left ungrazed following the treatment phase), no significant results were detected in any of the feeding-preference experiments (Fig. 2C, Table 2). However, 3 brown algae and 2 red algae exhibited a non-significant trend for higher palatability levels of unconsumed compared to previously consumed live algae pieces. The opposite trend was observed for 1 species each of green, brown, and red alga (Fig. 2C). Test power was consistently <50% probability at the end of the recovery phase. *Sargassum vulgare* could not be used for feeding assays after the recovery phase due to an infection by fungi. Likewise, feeding assays with live *Chondrophyucus flagellifera* pieces were not performed because this species lost too many branches (probably as a result of herbivore activities) and thus could not provide reliable consumption data.

After the acclimatization phase, mean consumption of artificial food containing non-polar extracts was not significantly different between acclimatized and non-acclimatized plants for any algae species (Fig. 3A, Table 2). Furthermore, there was no consistent trend. While acclimatized plants were preferred over non-acclimatized plants in 3 red and 2 brown algae, the opposite trend was displayed for 1 species each of green, red, and brown alga. Finally, identical consumption rates were detected between acclimatized and non-acclimatized

pieces of the brown alga *Dictyota menstrualis* (Fig. 3A, Table 3). The probability of detecting significant differences in feeding-preference assays after the acclimatization phase was >82% for all species except *Sargassum vulgare* (74%) and *Chondrophyucus flagellifera* (28%). After the treatment phase, mean consumption rate of artificial food containing non-polar extracts of grazed and ungrazed plants was not significantly different for all 9 species (Fig. 3B, Table 2). As a trend, artificial food containing extracts of ungrazed *C. flagellifera* was more consumed than that from grazed conspecifics of this red alga (upper limit of 95% *t*-distribution = 1.094). Similarly, in 2 red, 1 green, and 3 brown algae, the palatability of ungrazed plants was higher than that of grazed conspecifics. The reverse pattern was observed for 1 brown and 1 red alga (Fig. 3B, Table 3). The probability of detecting significant differences was >95% for feeding preference assays of all species, except *Pterocladia capillacea* (25%), *Lobophora variegata* (28%), and *Styopodium zonale* (53%). After the recovery phase, a significant difference was found between consumption rates of artificial food containing extracts of the brown alga *L. variegata* (Fig. 3C, Table 2). Artificial food containing non-polar extracts from ungrazed plants of *L. variegata* was preferred by 40% over artificial food from grazed conspecifics (upper limit of 95% *t*-distribution = 0.923). In addition, 2 red and 2 brown algae showed a non-significant trend for higher palatability of ungrazed

Table 2. Screening experiment (Expt 1). Statistical results of feeding assays with live algae (LA) and artificial food pellet containing non-polar algal extracts for acclimatization, treatment, and recovery phases of induction experiment. Data (n = 5) analyzed with paired *t*-test or Wilcoxon signed-ranks test (\*); n = 4 in acclimatization phase of *Dictyota menstrualis*. ns: non-significant; nc: no contribution (*Chondrophyucus flagellifera* lost branches and *Sargassum vulgare* was infected by fungi)

Alga	Acclimatization		Treatment		Recovery	
	t	p	t	p	t	p
<i>Chondrophyucus flagellifera</i>						
LA	nc	nc	nc	nc	nc	nc
Pellet	-0.69	ns	-3.32	ns	-0.53	ns
<i>Codium decortatum</i>						
LA	3.26	<0.05	0.25	ns	0.72	ns
Pellet	0.99	ns	-2.49	ns	3.95	ns
<i>Cryptonemia seminervis</i>						
LA	-2.02*	<0.05	-1.16	ns	-0.48	ns
Pellet	0.74	ns	-1.22	ns	0.02	ns
<i>Dictyota menstrualis</i>						
LA	3.36	<0.05	-2.28	ns	-1.97	ns
Pellet	0.08	ns	1.68	ns	-2.52	ns
<i>Lobophora variegata</i>						
LA	-0.51	ns	-2.25	ns	-0.99	ns
Pellet	3.32	ns	-0.13	ns	-3.51	<0.05
<i>Osmundaria obtusiloba</i>						
LA	0.57	ns	-0.41	ns	-0.18	ns
Pellet	-0.96	ns	-0.55	ns	0.70	ns
<i>Pterocladia capillacea</i>						
LA	2.08	ns	-5.40	<0.05	0.23	ns
Pellet	-2.68	ns	0.17	ns	-0.24	ns
<i>Sargassum vulgare</i>						
LA	-1.42	ns	-0.21	ns	nc	nc
Pellet	-0.82	ns	-2.58	ns	0.78	ns
<i>Styopodium zonale</i>						
LA	3.73	<0.05	1.03	ns	-1.36	ns
Pellet	-1.38	ns	-0.07	ns	-1.13	ns

compared to grazed plants. The opposite pattern was displayed by 1 brown, 1 red and 1 green alga. Finally, amphipod consumption rates showed no trend for the red algae *Cryptonemia seminervis* (Fig. 3C, Table 3). The probability of detecting a significant difference in

Table 3. Screening experiment (Expt 1). Mean ( $\pm$ SE) amphipod consumption rates (g 3 d<sup>-1</sup>) of artificial food containing non-polar extracts for each alga during 3 d choice-feeding assays (see 'Materials and methods' for details) after each phase of induction experiment. n = 5 (\*n = 4), non-a: non-acclimatized; a: acclimatized; t: grazed; c: ungrazed. See Table 2 for full species names

Alga	Acclimatization		Treatment		Recovery	
	non-a	a	t	c	t	c
<i>C. flagellifera</i>	45.6 ( $\pm$ 11.7)	58.2 ( $\pm$ 14.9)	70.8 ( $\pm$ 12.3)	78.8 ( $\pm$ 10.6)	48.6 ( $\pm$ 3.5)	52.8 ( $\pm$ 7.0)
<i>C. decortatum</i>	45.6 ( $\pm$ 7.0)	37.2 ( $\pm$ 4.9)	48.4 ( $\pm$ 6.0)	62.6 ( $\pm$ 4.6)	68.4 ( $\pm$ 5.7)	51.8 ( $\pm$ 5.1)
<i>C. seminervis</i>	54.2 ( $\pm$ 12.5)	42.2 ( $\pm$ 4.3)	69.6 ( $\pm$ 4.8)	80.4 ( $\pm$ 5.9)	55.0 ( $\pm$ 6.5)	54.8 ( $\pm$ 9.6)
<i>D. menstrualis</i>	46.5 ( $\pm$ 4.4)*	46.0 ( $\pm$ 3.6)*	70.2 ( $\pm$ 9.4)	56.6 ( $\pm$ 15.2)	37.0 ( $\pm$ 5.7)	50.8 ( $\pm$ 1.9)
<i>L. variegata</i>	53.8 ( $\pm$ 3.1)	32.6 ( $\pm$ 4.9)	49.6 ( $\pm$ 12.1)	53.0 ( $\pm$ 17.9)	47.4 ( $\pm$ 9.6)	66.4 ( $\pm$ 8.1)
<i>O. obtusiloba</i>	40.4 ( $\pm$ 6.0)	47.8 ( $\pm$ 4.1)	54.2 ( $\pm$ 7.6)	61.8 ( $\pm$ 7.2)	48.0 ( $\pm$ 5.9)	37.6 ( $\pm$ 9.6)
<i>P. capillacea</i>	41.4 ( $\pm$ 4.8)	51.0 ( $\pm$ 3.1)	56.0 ( $\pm$ 16.7)	52.2 ( $\pm$ 15.1)	53.4 ( $\pm$ 8.9)	55.6 ( $\pm$ 7.5)
<i>S. vulgare</i>	38.4 ( $\pm$ 8.3)	49.8 ( $\pm$ 8.8)	46.0 ( $\pm$ 5.7)	60.2 ( $\pm$ 4.1)	61.6 ( $\pm$ 10.1)	48.0 ( $\pm$ 10.5)
<i>S. zonale</i>	33.4 ( $\pm$ 6.4)	47.2 ( $\pm$ 5.6)	44.6 ( $\pm$ 4.9)	45.6 ( $\pm$ 10.7)	46.2 ( $\pm$ 8.0)	60.4 ( $\pm$ 9.9)

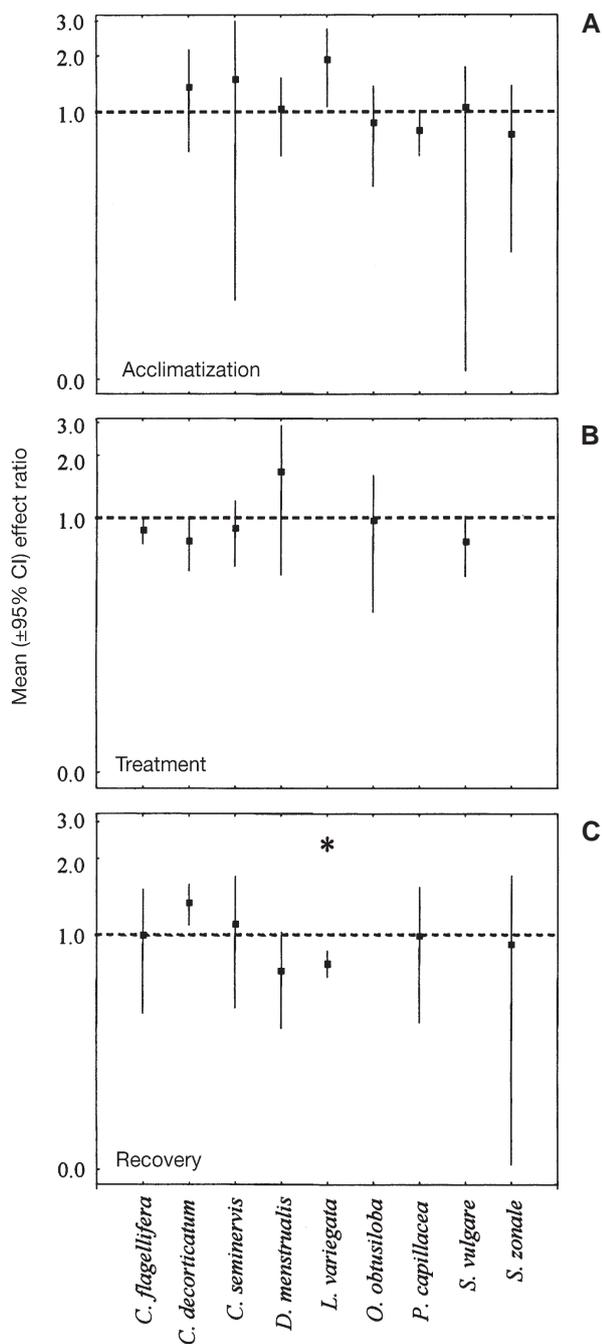


Fig. 3. Screening experiment (Expt 1). Effect-ratios (amphipod consumption rate of treated plants/consumption rate on control plants) derived from choice-feeding assays with artificial food containing non-polar extracts for each species of alga and phase of induction experiment. Horizontal dashed lines indicate effect-ratio of 1 predicted by null hypothesis. Significant defense responses (\*) recorded where the mean effect-ratio <1 and 95% confidence intervals (CI) do not cross dashed line. Note logarithmic scaling of ordinates to maximize optical resolution of effect-ratios around the null hypothesis. Effect-ratios of algae with CI limit of <0 never showed significant defense response and were omitted to maintain logarithmic scaling. Conditions of choice-feeding assays and full specific names as in Fig. 2

feeding preference assays after the recovery phase was >81% for all species except *Osmundaria obtusiloba* (25%) and *S. vulgare* (35%).

**Interaction between defenses and nutrients (Expt 2)**

Amphipods significantly preferred artificial food containing non-polar extracts from acclimatized *Codium decorticutum* pieces over artificial food from non-acclimatized pieces (Table 4). Mean consumption of acclimatized artificial food increased by 91% (paired *t*-test,  $t_4 = -7.25$ ,  $p = 0.002$ ). After the treatment and recovery phases, differences in consumption activities, nutrients, or a combination of both did not affect algal palatability relative to controls (Fig. 4, Table 5).

A 2-way ANOVA showed no effect of consumption, nutrients or both factors together after the treatment phase (Table 4). At the end of the recovery phase, the effects of the direct consumption treatment were significantly greater than those of the other consumer treatments (Fig. 4, Table 5), indicating a relatively stronger increase in palatability of algae pieces from the direct grazing treatment compared to controls than for indirect and consumer-presence treatments. During the recovery phase, the presence of nutrients resulted in decreased palatability of *Codium decorticutum* by an average of 18% (2-way ANOVA,  $F = 6.90$ ,  $p = 0.015$ ), compared to unfertilized treatments. A significant consumption × nutrient interaction indicated that consumer effects depended on nutrient levels (2-way ANOVA,  $F = 3.93$ ,  $p = 0.033$ ). During the nutrient enrichment treatment, growth of *C. decorticutum* decreased by 27% (*t*-test,  $t_9 = 2.461$ ,  $p = 0.024$ ) compared to unfertilized controls.

Table 4. Main and interactive effects between grazing and nutrient treatments. Results from choice-feeding assays (Expt 2) of artificial food containing non-polar extracts of *Codium decorticutum* after acclimatization, treatment, and recovery phases ( $n = 5$ ). *t*: test statistic (paired *t*-test); *F*: test statistic (2-way ANOVA); C: consumption; N: nutrients; C × N: interactive effects of C and N; ns: non-significant

	<i>t</i>	<i>p</i>
<b>Acclimatization phase</b>		
Effect	-6.68	<0.05
<b>Treatment phase</b>		
C	0.54	ns
N	1.86	ns
C × N	0.11	ns
<b>Recovery phase</b>		
C	2.25	ns
N	6.90	<0.05
C × N	3.93	<0.05

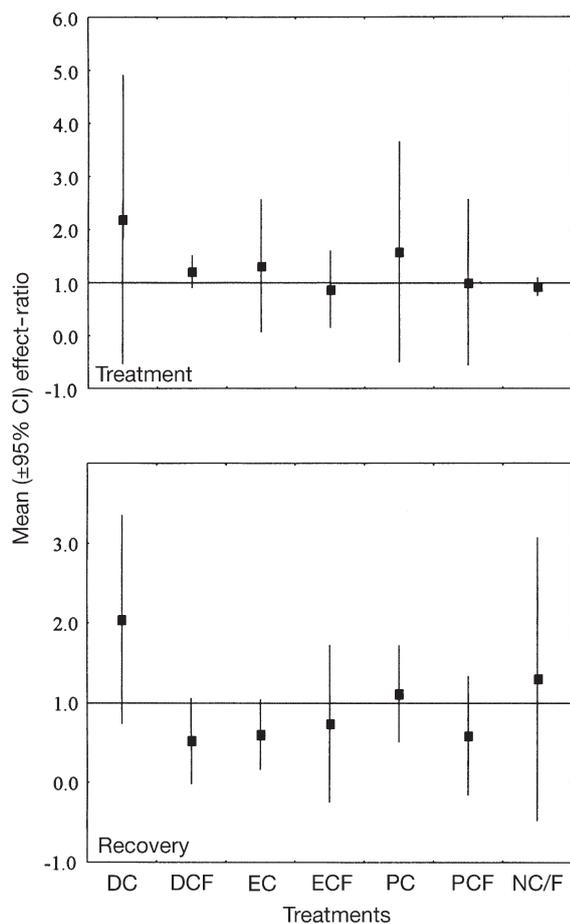


Fig. 4. Nutrient  $\times$  grazing experiment (Expt 2). Effect-ratio (amphipod consumption rate of treated artificial food/consumption rate of control artificial food) for *Codium decortiatum* after treatment and recovery phase. Horizontal continuous line indicates the effect-ratio of null hypothesis. Significant defense responses recorded where mean effect-ratio  $< 1$  and 95% confidence intervals (CI) do not cross continuous line. DC: direct consumption; DCF: DC + fertilizer; EC: nearby grazed conspecifics; ECF: EC + fertilizer; PC: non-grazing consumers; PCF: PC + fertilizer; NC/F: control + fertilizer. Conditions of choice-feeding assays as in Fig. 2

Table 5. Nutrient  $\times$  grazing experiment (Expt 2). Mean ( $\pm$ SE) amphipod consumption rates ( $1 \text{ mm}^2 \text{ squares } 3 \text{ d}^{-1}$ ) of artificial food containing non-polar extracts of green alga *Codium decortiatum* for all treatments including 'nutrients' and 'consumers' as main effects during feeding assays after treatment and recovery phases of induction experiment. n = sample size

Experiment	n	Treatment	Recovery
Direct consumption	5	51.0 ( $\pm 14.3$ )	51.8 ( $\pm 14.7$ )
Direct consumption + fertilizer	5	72.6 ( $\pm 7.5$ )	23.4 ( $\pm 5.8$ )
Nearby grazed conspecifics	5	10.0 ( $\pm 2.3$ )	43.0 ( $\pm 16.5$ )
Nearby grazed conspecifics + fertilizer	5	55.2 ( $\pm 14.4$ )	41.8 ( $\pm 12.2$ )
Non-grazing consumers	5	46.0 ( $\pm 16.2$ )	59.6 ( $\pm 7.3$ )
Non-grazing consumers + fertilizer	5	32.6 ( $\pm 9.7$ )	34.6 ( $\pm 15.1$ )
Control	35	50.8 ( $\pm 5.4$ )	59.3 ( $\pm 4.6$ )
Control + fertilizer	5	82.4 ( $\pm 8.1$ )	43.8 ( $\pm 8.0$ )

## DISCUSSION

In marine systems, inducible defenses are only known to exist in a few species of macroalgae. This study is the first to describe grazing-induced defenses among Brazilian tropical algae. Out of 4 red algae, 1 (*Pterocladia capillacea*) exhibited inducible defenses following direct consumption by amphipods. Surprisingly, only 1 of 4 brown algae studied (*Lobophora variegata*) displayed induced (delayed) defenses: the green alga *Codium decortiatum* did not respond significantly to direct grazing or water-borne signals of nearby grazers and grazed conspecifics by any detectable change in palatability, whereas nutrient enrichment seemed to trigger a change in palatability after a lag phase of 3 wk.

### Screening experiment (Expt 1)

Most differences in palatability were found after the acclimatization phase between acclimatized and non-acclimatized live algae, probably indicating that transfer to laboratory conditions requires some acclimatization time for the macroalgal species. Live algae either increased or decreased in palatability, probably due to algae-specific responses to an altered light or hydrodynamic regime under laboratory conditions, or the absence of consumers. Indeed, it is not clear whether altered abiotic conditions or changes in the defense status, or both, were responsible for the observed changes in algal palatability after this phase. However, we demonstrated the need for such acclimatization phase to achieve comparable initial conditions for detecting grazer-mediated rather than laboratory-mediated changes in algal palatability. However, changes in palatability of live algae were not confirmed with artificial food containing non-polar extracts, suggesting that either the nutritional value

(e.g. proteins, sugars) of the algae had changed under the cultivation conditions or lipid-soluble compounds extracted by DCM were not responsible for affecting amphipod preference.

After the treatment phase, 8 of 9 consumer-exposed algae were less palatable than untreated conspecifics. Pavia & Toth (2000) reported that, under natural conditions, a response to grazing by mesoherbivores within 2 to 4 wk could be considered a short-term response, since this type of small, relatively immobile mesograzers can feed on the same alga for long periods and would thus experience any changes

they induce. We have demonstrated that grazing by amphipod mesograzers can trigger resistance in the red alga *Pterocladia capillacea* within 1 wk under laboratory conditions. Live pieces of *P. capillacea* following amphipod grazing were less preferred than control pieces, but this was not the case for artificial food containing non-polar algae extracts. The higher palatability of live control over treated plants was not likely to have been simply due to grazers having eaten all the more palatable tissues in control plants, because amphipod consumption never exceeded 15% of algal wet mass during the induction experiment, suggesting that there was still enough palatable tissue available for amphipods in treated algae pieces during the feeding assay. Therefore, this result indicates the presence of a deterrent polar substance, which could not be extracted by DCM. Furthermore, almost significant results with artificial food containing non-polar extracts of the red algae *Chondrophyucus flagellifera* suggest the presence of chemical defenses. Many species of tropical red algae, including members of the genus *Chondrophyucus*, are known to produce a diverse array of halogenated secondary metabolites (Paul et al. 1987, Faulkner 2001, Garson 2001), and some have been shown to act constitutively as feeding deterrents in addition to other ecological roles (Hay et al. 1987, da Gama et al. 2003, Pereira et al. 2003); yet this study is the first to report on the ability of red algae to induce defenses. Our data do not, however, rigorously exclude the possibility that induction of defense in *P. capillacea* could be attributed to a morphological trait. Evidence for a plasticity of morphological traits in *P. capillacea* comes from the study by Scrosati (2002), who observed stress-mediated development of branches in this species. Similarly, Van Alstyne (1989) detected grazer-induced growth of adventitious branches in the brown alga *Fucus distichus* that were less palatable to snails than normal meristematic tissue of apices. The results of both studies suggest that additional thallus growth could be one indicator of induced morphological defense responses. However, this response was not observed in our experiments, increasing the probability that induced defenses in *P. capillacea* were due to a chemical mechanism.

Among the Phaeophyta screened, *Lobophora variegata* showed an induction of anti-herbivore defenses, a result that has not previously been published. In contrast to the experiments of Cronin & Hay (1996c), *Dictyota menstrualis* did not exhibit inducible defenses after exposure to amphipods. It has been shown that Brazilian *D. menstrualis* deters feeding by the amphipod *Parhyale hawaiiensis*, suggesting that this alga is constitutively defended (Pereira et al. 2000a,b). Pereira et al. (2000a,b) reported that its defensive response was quite strong ( $14 \pm 9$  amphipods died per day) dur-

ing our feeding assays. This confirms the high mortality rates of grazers which might explain the high rate of mortality and foulness on this alga (e.g. high mortality of bryozoan larvae exposed to *D. menstrualis* extracts occurred in the study of Schmitt et al. 1995). The difference in the ability of grazers to induce defenses in *D. menstrualis* between the present study and that of Cronin & Hay (1996c) indicates that induced defenses may be a grazer-specific response of algae, as has previously been assumed (Hay et al. 1987, 1988, Paul et al. 1987, Pereira et al. 2000a, Amsler 2001, Van Alstyne et al. 2001). Thus, our results may not be useful for predicting how the algae tested will respond to grazing by other herbivores of the study system or of other systems. Although *Stypopodium zonale* did not display defense induction by amphipod grazing, we did observe a strong repulsive effect, since  $27 \pm 3$  amphipods died within a 48 h treatment period during feeding assays. This corroborates the finding of Soares et al. (2003) that *S. zonale* possesses constitutive chemical defenses. Soares et al. (2003) found that natural concentrations of *S. zonale* secondary metabolites at Forno Beach deterred feeding by the sea urchin *Lytechinus variegatus* and the crab *Pachygrapsus transversus*.

The palatability levels of treated and control plants of *Pterocladia capillacea* were no longer significantly different, 2 wk after consumption ceased, indicating an as yet unreported regulatory capacity of algae to mobilize defenses when consumed and to reduce defenses when grazing pressure ceases. Furthermore, we observed retarded induction of chemical defense in artificial food made from non-polar extracts, but not in live pieces of *Lobophora variegata*. This suggests that repulsive effects of the non-polar extracts were overridden by counteracting effects of non-extracted chemicals, making (e.g.) live plants more nutritive.

### Interaction between defenses and nutrients (Expt 2)

The effects of nutrient availability and consumption on defense induction were examined in the green alga *Codium decortatum* because of its tendency to show induced chemical defenses in the screening experiment.

In contrast to the screening experiment (i.e. Expt 1), in Expt 2 the diet containing non-polar extracts from acclimatized pieces was more palatable than that from non-acclimatized pieces. The *Codium decortatum* pieces used in Expt 2 were collected from the same location as those for the screening experiment, but at different times. Thus, contrasting patterns in the palatability of *C. decortatum* after acclimatization suggest a change in its chemistry within a time span of 7 wk. Likewise, Van Alstyne et al. (2001) reported

strong changes in the chemical composition of macroalgae, over short time spans ranging from days to months, due to dynamics in herbivore populations and environmental factors. As in Expt 1, an acclimatization phase seemed to be necessary in Expt 2 to allow acclimatization of *C. decorticans* to laboratory conditions.

Neither direct consumption nor water-borne cues of consuming and non-consuming amphipods induced resistance in *Codium decorticans*. Defense induction in a green alga, triggered by the reception of water-borne cues from nearby grazed conspecifics, was only detected in *Ulva reticulata* (J. Ceh pers. comm.). Furthermore, the brown algae *Ascophyllum nodosum* (Toth & Pavia 2000), *Fucus serratus* (Rohde 2003) and *Glossophora kunthii* (E. Macaya pers. comm.) decreased in palatability after receiving water-borne signals from algae and/or herbivores. In natural habitats, *C. decorticans* individuals do not grow in dense stands (Teixeira et al. 1984, K. Weidner pers. obs.), reducing the chances of receiving water-borne cues from neighboring grazed plants. Thus, the life-history traits of *C. decorticans* make water-borne cues less likely to be an advantageous selective trait for this alga.

Unexpectedly, direct consumption by amphipods showed a trend to enhance the palatability of *Codium decorticans* (Table 5). This contrasts with the induced defense theory, but corroborates the results of the screening experiment and of a parallel study on snail-exposed *Codium platylobium* from South Africa (E. Díaz pers. comm.). Consumer-mediated damage of algal tissues may have induced the production of compounds that stimulated feeding (Cetrulo & Hay 2000), or may have facilitated access of amphipods beyond the physical defensive barriers of the alga. Likewise, consumers could have adapted to or possibly even have been stimulated by plant defense compounds, as has been described for other mesograzers (e.g. Hay et al. 1987).

Enhanced nutrient concentrations did not affect the palatability levels of *Codium decorticans*, nor was there any interactive effect of nutrient addition with grazing on defense induction. The reduced growth of fertilized plants compared to unfertilized plants could suggest that *C. decorticans* fragments were stressed by higher nutrient levels. Most studies have found that nutrient enrichment results in increased growth rates (e.g. Creed et al. 1997) or has no effect (e.g. Erftemeijer et al. 1994), and yet Schaffelke & Klumpp (1998) reported reduced growth rates in *Sargassum baccularia* due to fertilizer treatment above a growth-stimulating concentration range. Likewise, Van Alstyne & Pelletreau (2000) reported a negative effect of phosphorus and iron enrichment on the growth of *Fucus gardneri* embryos. The fertilizer beads that we used for

enrichment provided a high proportion of ammonium (7.7% out of 14% total N) that could have exceeded 50  $\mu\text{M}$ , above which level toxic effects on algae have been observed (Waite & Mitchell 1972), and this may have reduced growth. Moreover, ambient levels of nitrate may have been very high (18.1  $\mu\text{M}$ ), and thus probably well in excess of algal requirements. In regard to our observation that palatability was not affected by nutrient addition, the 'environmental stress theory' may be applicable, whereby under stress, all resources will be allocated to maintenance rather than defense.

Allocation models, such as the 'growth-differentiation balance hypothesis' or the 'carbon-nitrogen balance hypothesis', assume a negative correlation between the concentration of C-based secondary compounds (e.g. terpenes) and plant growth rates because both processes compete for limited resources within a plant (Haukioja et al. 1998, Glynn et al. 2003). Therefore, increased growth rates and a concurrent increase in the palatability of fertilized *Codium decorticans* pieces to amphipods should have occurred due to decreased defenses. However, this pattern was not found, rather, the opposite was observed after 3 wk fertilization: the fertilizer in combination with grazing seemed to improve the alga's defense capacity. This may have been due to the chemical composition of green algae. Most deterrent compounds isolated from green algae are terpenes (e.g. Paul & Van Alstyne 1988, Cronin 2001), but evidence from many studies shows that the 'growth-differentiation balance hypothesis' and the 'carbon-nitrogen balance hypothesis' predict the responses of phlorotannins (Ilvessalo & Tuomi 1989, Peckol & Yates 1997) but not of terpenes (Cronin & Hay 1996c, Puglisi & Paul 1997). This discrepancy due to nutrient availability could be explained by the 'biosynthesis-pathway hypothesis', assuming that trade-offs with growth are more likely for phenolic than for terpene biosynthesis (Muzika 1993, Haukioja et al. 1998), since there is direct competition between protein and polyphenol biosynthesis for an equal precursor whereas precursors for terpene biosynthesis are more generalized and use many pathways (Muzika 1993). However, interpretation of our data is not possible with current hypotheses.

It seems either that *Codium decorticans* does not possess deterrent secondary metabolites against *Elasmopus brasiliensis* or that laboratory conditions and nutrient enrichment stress the alga to such an extent that it is unable to respond with defenses. Our results support the suggestion of Peckol et al. (1996) that production of defenses is likely to be controlled by a complex interaction of environmental, developmental and defense-related factors.

A growing number of studies conclude that defense induction among macroalgae is not a simple, straightforward process, but rather a result of complex interac-

tions of a number of biotic and extrinsic abiotic factors (Pavia & Brock 2000, Van Alstyne & Pelletreau 2000). By manipulating these factors under controlled conditions, and screening defense induction among several species of algae, we can expect to cast some light upon the mechanisms by which macroalgae regulate herbivore consumption and thus control an important part of the food web in marine systems.

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