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Author(s): Peter J. Schupp and Valerie J. Paul

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CALCIUM CARBONATE AND SECONDARY METABOLITES IN TROPICAL SEAWEEDS: VARIABLE EFFECTS ON HERBIVOROUS FISHES¹

PETER J. SCHUPP AND VALERIE J. PAUL²

University of Guam Marine Laboratory, UOG Station, Mangilao, Guam 96923 USA

Abstract. On coral reefs where herbivorous fishes are both numerous and diverse, many seaweeds combine mineral and chemical defenses. We hypothesized that combined defenses affect a greater number of herbivore species than either single defense, and in this study we experimentally tested this hypothesis. We first incorporated calcium carbonate (aragonite form) alone at a range of concentrations into an artificial diet composed of freeze-dried samples of the noncalcified, green alga *Enteromorpha clathrata*, carrageenan, corn-starch, and water. In field experiments where diets were made available to a natural fish community, aragonite had variable effects: in some trials aragonite deterred feeding, in others it had no significant effect, and in one trial it even stimulated feeding. Parrotfishes (Scaridae) and the surgeonfish *Ctenochaetus striatus* (Acanthuridae) showed a significant preference for food containing aragonite in field observations, while the surgeonfish *Naso vlamingii* and three damselfishes (Pomacentridae) were observed to feed preferentially on food without added aragonite. In the laboratory, the surgeonfishes *Acanthurus nigrofasciatus*, *A. triostegus*, *A. xanthopterus*, and *Naso unicornis* and the rabbitfish *Siganus spinus* (Siganidae) significantly avoided food with added aragonite. However, the parrotfishes *Scarus sordidus* and *S. schlegelii* consumed significantly more food containing aragonite than plain food. *Ctenochaetus striatus* avoided aragonite only at the highest aragonite concentration and showed no preference at lower aragonite concentrations.

Artificial diets containing natural concentrations of aragonite (80% dry mass), diterpenoid secondary metabolites from *Halimeda macroloba* (1% dry mass), and a combination of both defenses (80% aragonite and 1% aragonite-free dry mass of diterpenes) were tested in the laboratory against four fish species. *Scarus sordidus* and *Ctenochaetus striatus* avoided secondary metabolites but not aragonite, and *Naso lituratus* and *Siganus spinus* were deterred by aragonite but not by secondary metabolites. All fishes were deterred by one defense or the other, and all fishes but *Scarus sordidus* avoided food containing the combined defenses. Combinations of aragonite and secondary metabolites increased the number of fish species that were effectively deterred from grazing relative to either single defense.

Key words: aragonite; calcified seaweed; calcium carbonate; chemical defense; herbivorous fish; mineral defense; western Pacific.

INTRODUCTION

Calcified algae, including corallines and articulated calcareous algae, are among the most conspicuous algae in coral reef habitats characterized by intense herbivory (reviewed in Littler and Littler 1984, Hay 1991). Several correlative studies have shown that herbivorous fishes and other generalist herbivores have a low preference for heavily calcified algae (Tsuda and Bryan 1973, Bryan 1975, Littler et al. 1983, Hay 1984, Lewis 1985, Paul and Hay 1986, Paul and Van Alstyne 1988a, Wylie and Paul 1988, Pennings and Paul 1992). However, because many species of calcified algae are also chemically defended (Hay 1984, Paul and Fenical 1984, Paul and Hay 1986, Paul and Van Alstyne 1988b), these correlative studies do not unambiguously dem-

onstrate a defensive role for calcium carbonate. In fact, the role of calcium carbonate as a general defense mechanism has been disputed by Padilla (1985, 1989), who found that algal mechanical resistance to grazing by limpets was not greater in calcified algae compared to noncalcified algae. However, Padilla's results may not be applicable to all herbivores, because limpets feed by rasping with a radula whereas many other herbivores feed by biting tissue from plants. Heavily calcified algae are tougher (based on penetrometer readings) than many noncalcified algae (Pennings and Paul 1992), and some herbivore types may be deterred by the mechanical properties of calcified seaweeds.

Moreover, the issue of whether calcification deters feeding by increasing mechanical resistance may be moot in some instances. Pennings and Paul (1992) found that powdered calcium carbonate (both calcite and aragonite) incorporated into artificial diets deterred feeding by the sea hare *Dolabella auricularia* even though it did not increase toughness. Calcium carbonate may

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² Send reprint requests to this author.

affect herbivores by lowering nutritional value of the food (Duffy and Paul 1992) or by having negative physiological effects on some herbivores (Smith and Paulson 1975, Hutchings 1986).

Many of the studies of seaweed defenses to feeding by herbivorous fishes have been conducted in the field, but this approach may obscure important differences among fish species in their responses to algal defenses. Herbivorous fishes are known to respond differently to algal secondary metabolites (Hay 1991, Paul 1992, Paul et al., *in press*) and also to have different diets (Horn 1989). For example, lightly calcified algae and those with tough, leathery thalli are generally eaten by scarids (parrotfishes), but often avoided by acanthurids (surgeonfishes), which instead feed on noncalcareous red algal species (Lewis 1985). Generally, scarids ingest filamentous algae and inorganic sediment, while many acanthurids consume filamentous and foliose algae (Hiatt and Strasburg 1960, Jones 1968, Horn 1989). These dietary differences are mainly related to the differences in (1) dentition: parrotfishes have large mouths with fused teeth, while surgeonfishes have small mouths with close-set lanceolate or incisiform teeth, and (2) digestive physiology: parrotfish grind their food in a pharyngeal mill; some surgeonfishes and mullets (mugilids) grind their food in a thick-walled, gizzard-like stomach; and some surgeonfishes, damselfishes (pomacentrids), and probably rabbitfishes (siganids) process their food with moderately to highly acidic stomach fluids (Lobel 1981, Horn 1989, 1992, Choat 1991). These morphological and physiological differences suggest that different fishes might respond differently to algal calcium carbonate as a defense mechanism.

To test the putative defensive role of aragonite in marine algae, we conducted field experiments with natural populations of herbivorous fishes and laboratory experiments with eight fish species (two scarids, one siganid, five acanthurids). These experiments confirmed the hypothesis that the effect of aragonite on fish feeding depends on the species involved. We also compared the effectiveness of aragonite and secondary metabolites alone and in combination as defenses against four fish species. Two fish species that were deterred by secondary metabolites were not deterred by aragonite, and two species that were deterred by aragonite were not deterred by secondary metabolites. These results emphasize the importance of multiple lines of algal defenses against herbivores.

METHODS

Study sites

Field assays were conducted at a patch reef (named Val's Reef) in Cocos Lagoon near Cocos Island and at Western Shoals in Apra Harbor on Guam. Val's Reef is a small (35 × 65 m) reef of mostly dead coral rubble and some scleractinian coral heads. The coral rubble is covered with an algal turf, with *Halimeda* spp. and

Dictyota spp. as the dominant macroalgae. Western Shoals is a larger patch reef inhabited by a variety of corals (dominated by *Porites* spp.). The most abundant macroalgae here are *Padina tenuis* and *Halimeda* spp. Fishes observed feeding during assays at Val's Reef included *Scarus sordidus*, *Scarus schlegeli*, *Abudefduf sexfasciatus*, *Amblyglyphidodon curacao*, *Ctenochaetus striatus*, and *Epibulus insidiator*. At Western Shoals the same fish species were observed feeding, as well as *Abudefduf vaigiensis* and *Naso vlamingii*.

Food preparation

Calcium carbonate occurs primarily in two forms in algae, either calcite or aragonite (Borowitzka 1977). Aragonite is the more common polymorph in marine foliose algae and was therefore used in the experiments of this study. Its effect on feeding was assessed by incorporating aragonite into an artificial food mixture. *Halimeda macroloba* was collected and soaked for 1 d in a 1:1 mixture of commercial bleach (5.25% sodium hypochlorite) and water to remove organic components. It was then rinsed in running seawater for at least 2 d to remove bleach residues, since the dilute hypochlorite solution would be readily soluble in seawater. Aragonite crystals in *Halimeda* plants form a fused, unoriented network of primary and secondary crystals that adhere even after the plant dies (Multer 1988). The remaining aragonite skeleton was dried at 40°C and then ground through a 0.6-mm mesh sieve to mechanically break up the segments. To determine that large amounts of organic material did not remain undigested in the *Halimeda* skeletons, we ashed some of the aragonite as well as commercial calcite (Sigma Chemical Company) for 12 h at 450°C in a muffle furnace. Commercial calcite lost 0.1–0.2% ($N = 2$) and our prepared aragonite lost 2.5–2.7% ($N = 2$) of its starting mass. Therefore, the organic components in the aragonite are low and would not affect the aragonite levels in the diets.

The food mixture consisted of 5.0 g freeze-dried *Enteromorpha clathrata* (ground to a powder), 6.0 g carrageenan (Type I, Sigma Chemical Company), and 3.0 g commercial cornstarch per 200 mL water. *Enteromorpha* grows in nutrient-enriched shoreline habitats on Guam. Nitrate levels are high due to percolating groundwaters, and C:N ratios of *Enteromorpha* are 2–3 times lower than other Guam seaweeds (FitzGerald 1978, Wylie and Paul, *in press*). For this reason, we added cornstarch as a carbohydrate to our mixture to add to the gelling capacity of the mixture and to increase the percent carbon in the diet. We also aimed to keep the food value of our mixture relatively low because the dry mass of *Halimeda* and other calcified algae is only 15–30% organic material. Duffy and Paul (1992) have shown that the effects of secondary metabolites may vary in high quality (protein rich) vs. low quality foods and that metabolites deter fish feeding more effectively in low quality foods. The carrageenan,

cornstarch, and water were heated in a microwave oven for 90 s. Then *Enteromorpha* and aragonite were added and heated for 30 s, and for some experiments the extracts or solvents were stirred in. The mixture was poured into 1-cm³ molds to cool. A rubber O-ring embedded in each cube was used to attach it to a yellow polypropylene rope (by a safety pin) for field assays.

Different amounts of aragonite were added to correspond to different levels of calcification in algae. For example, a diet containing 70% aragonite means that 70% of the total food dry mass was aragonite (aragonite mass/[algal + aragonite + carageenan + cornstarch mass] = 0.70). Because of the addition of aragonite, the calcified diet weighed more per unit volume than the plain diet. The volume of the various diets did not detectably differ even when aragonite was added (235–240 mL total volume for diets containing 0–70% aragonite); therefore, approximately the same amount of *Enteromorpha* per unit volume was available to herbivores in plain and aragonite diets.

Feeding preferences of fish assemblages

Different percentages of ground aragonite were added to the artificial diet (20, 30, 40, 50, 60, and 70% aragonite by dry mass at Val's reef and 10, 20, 30, 40, 50, and 70% aragonite at Western Shoals) to assess the deterrent effect of aragonite against natural assemblages of herbivorous fishes. These aragonite concentrations cover a range from lightly to heavily calcified algae (*Padina tenuis* is $\approx 48\%$ aragonite and *Halimeda macroloba* as a heavily calcified alga contains $\approx 80\%$ aragonite) (Pennings and Paul 1992). Four plain *Enteromorpha* food cubes without aragonite or four *Enteromorpha* cubes with aragonite were attached to a rope. The ropes were placed on the reef in pairs of one treated (aragonite) and one control (plain) rope and were attached to coral heads in 3–5 m of water. Several pairs of each aragonite concentration were placed on the reef at the same time. The pairs were removed when approximately half of the cubes were eaten (normally after 10–15 min). Twenty replicate pairs were tested for each concentration of aragonite. Duration of the entire assay (time to test 20 pairs of ropes) was usually <1 h. Dozens of individual fishes would feed from the ropes during the assays; however, because these field assays were of short duration, it was common to have different species or densities of fishes present for different assays.

Results were analyzed in two ways. Some assays were scored as the number of cubes completely eaten and the results were analyzed with a Wilcoxon signed-rank test for paired comparisons (two-tailed). In later experiments the cubes were weighed before and after the feeding assay and the changes in mass of plain and calcified food were compared with a paired (by site and time) *t* test (two-tailed). In one field experiment, ropes with treated and control food cubes were placed in a mesh bag in the water as no-herbivore controls to check

for autogenic changes in mass due to handling and potential dissolution. The results for these no-herbivore controls are reported but were not used in the analyses.

To test for a difference in the deterrent effect of calcite vs. aragonite, we conducted three assays at Val's Reef, comparing diets with (1) *Enteromorpha* cubes containing 60% aragonite against plain *Enteromorpha* cubes, (2) *Enteromorpha* cubes containing 60% calcite against plain *Enteromorpha* cubes, and (3) *Enteromorpha* cubes containing 60% aragonite against *Enteromorpha* cubes containing 60% calcite. We used commercial reagent-grade calcite powder from Sigma Chemical Company (Number C-6033).

Field experiments: effect of aragonite on individual fish species

To determine the food preferences of different fish species, we observed fish in field feeding experiments similar to those described above, at Val's Reef (Cocos Lagoon), Western Shoals, and Fingers Reef (in Apra Harbor near Western Shoals) in Guam. Ropes with 10 cubes of 80% aragonite or plain *Enteromorpha* food were set on the reef in pairs. Ten pairs of ropes were set at the beginning, and pairs were replaced when half of the cubes from one of the ropes in a pair was eaten. We counted the number of bites taken by individual fish from the plain or aragonite food cubes. Observations were stopped once an individual took a total of 20 bites or stopped feeding (fish which did not take a minimum of 10 bites were not used in the data analysis). Individual fish of each species (distinguished by size, coloration, and scars) were used as replicates. For each fish species, results were analyzed by determining the proportion of total bites taken by each individual fish from calcified food cubes. A null model of no preference (e.g., proportion of bites from calcified food = 0.5) was tested by comparing the observed proportion with 0.5 using a one sample *t* test.

Laboratory experiments: effect of aragonite on individual fish species

To further assess the effect of aragonite on feeding by some of the major herbivorous fish species, we conducted feeding assays in 12 46-L plastic tanks with running seawater. We selected species of herbivorous fish that were common around Guam and could be readily caught and maintained in laboratory aquaria. We also tried to select species representing several different families of herbivorous fishes. Parrotfishes (*Scarus sordidus*, *Scarus schlegeli*), surgeonfishes (*Ctenochaetus striatus*, *Acanthurus triostegus*, *Acanthurus nigrofusus*, *Acanthurus xanthopterus*, *Naso unicornis*), and the rabbitfish *Siganus spinus* were caught with barrier and scoop nets and held in the tanks in species groups of two (*C. striatus*, *N. unicornis*, *S. spinus*) or three individuals (*A. triostegus*, *A. nigrofusus*, *A. xanthopterus*, *S. sordidus*, and *S. schlegeli*). The fish

were given daily aragonite and plain *Enteromorpha* artificial food until they started eating, and then the feeding experiments were started. At least six replicate groups were used for each species. Different fish species and different aragonite levels were assigned randomly to the experimental tanks, and experiments with different fish species and different aragonite levels were run simultaneously. Taxonomic identifications were verified by scale and fin-ray counts for 3–6 individuals of each fish species, following keys in Myers (1991). A subsample of each fish species was measured to the nearest 0.5 cm: *Scarus sordidus* (total length [$\bar{X} \pm 1$ SD] = 8.9 ± 1.9 cm, $N = 17$), *S. schlegeli* (8.0 ± 2.1 cm, $N = 2$), *Ctenochaetus striatus* (14.6 ± 2.7 cm, $N = 5$), *Acanthurus nigrofusus* (12.0 ± 1.0 cm, $N = 3$), *A. triostegus* (10.4 ± 2.0 cm, $N = 12$), *A. xanthopterus* (9.6 ± 2.8 cm, $N = 11$), *Naso unicornis* (14.1 ± 1.8 cm, $N = 11$), *Siganus spinus* (12.2 ± 2.2 cm, $N = 9$).

The fish in each tank were offered a choice between a plain *Enteromorpha* food cube ($3.5 \times 1.5 \times 1$ cm high) and an *Enteromorpha* cube containing aragonite at 30, 50, or 70% dry mass. Each group of fish was tested once at each aragonite level, then released. Additional plain and aragonite food cubes were placed in a small plastic beaker with a mesh opening on one side inside the experimental tank to serve as a no-herbivore control for the same length of time as the feeding assays. This controlled for possible changes in mass of the food due to autogenic changes such as water absorption or handling (Peterson and Renaud 1989). All food cubes were weighed both before and after each feeding trial. Food cubes were checked regularly for mass loss. Trials took between 4 and 36 h. Replicates in which fish did not eat more than one-third of the total food or in which fish ate all of one food before the experiment was terminated were excluded from the data analysis. The difference in mass change between plain and aragonite food was compared between fish (experimental) and no-fish (control) cubes, yielding two sets of differences (experimentals and no-herbivore controls), which were analyzed with a paired *t* test (Peterson and Renaud 1989). Fish and no-fish cubes were paired because they were in the same aquarium and immersed for the same period of time. Two-tailed *P* values are reported.

Multiple defenses as feeding deterrents

To compare the effects of aragonite and *Halimeda* secondary metabolites as feeding deterrents individually and in combination, we conducted additional experiments with four species of herbivorous fishes (*Scarus sordidus*, *Ctenochaetus striatus*, *Naso lituratus*, *Siganus spinus*), using at least seven replicates of each treatment for each species. New fish (or groups of fish) were used for each replicate, and fish were tested once at each of the three food treatments and then released. Three different choice experiments were conducted: food containing 80% aragonite vs. plain *Enteromorpha*

food, food containing *Halimeda macroloba* diterpenes at 1% dry mass vs. food with added solvent (dichloromethane), and a combination of both aragonite and secondary metabolites (80% aragonite and diterpenes at 1% of the aragonite-free dry mass of the food) vs. *Enteromorpha* food containing only solvent. These levels were selected because they represent natural concentrations of aragonite and secondary metabolites in *Halimeda macroloba* (Paul and Fenical 1984, Paul and Van Alstyne 1988a, b). For food containing both defenses, the same mass of the diterpene mixture was added that was added to the food containing only secondary metabolites. This kept the concentration of *Halimeda* diterpenes constant at 1% of aragonite-free dry mass, and approximately constant per volume of food; however, the concentration of secondary metabolites relative to dry mass of food was only 0.2% in the calcified food because it was diluted by the added aragonite. Even though this dry mass concentration is relatively low for *H. macroloba*, we felt it was important not to use a fivefold increase in the amount of diterpenes in the food testing combined defenses compared with the food testing *Halimeda* diterpenes alone.

Halimeda macroloba secondary metabolites were obtained by extracting the alga in a 1:1 volume: volume mixture of dichloromethane and methanol, and the major secondary metabolites, halimeditetraacetate and halimedatriol, were purified by vacuum-flash silica gel column chromatography with ethyl acetate/hexane mixtures (Paul and Fenical 1984). The 20, 25, and 35% ethyl acetate/hexane fractions contained the major secondary metabolites found in *H. macroloba*, mainly halimeditetraacetate and minimal amounts of halimedatriol (Paul and Fenical 1984, Paul and Van Alstyne 1988a, b). These fractions were combined to obtain the purified diterpenes used in the experiments. Because *H. macroloba* contains halimeditetraacetate as $\approx 80\%$ of the organic extract (Paul and Fenical 1984), the major diterpene is easy to isolate from this alga. Thin layer chromatography (TLC) and proton nuclear magnetic resonance (NMR) spectroscopy were used to confirm that the purified fraction consisted of these two deterrent secondary metabolites (Paul and Van Alstyne 1988a, b). Trace amounts of chlorophyll pigment were also detected in the mixture by TLC. The presence of the two diterpenes in the food after the experiments were finished was confirmed by extracting 10 food cubes after the feeding trials and examining the extract by TLC. In all cases, the two metabolites were present, although we could not quantify the amounts remaining by TLC.

Caged plain and treated *Enteromorpha* food cubes in each tank served as no-herbivore controls during each trial. Plain and treated *Enteromorpha* food cubes were weighed both before and after each trial. Replicates in which fish did not eat more than one-third of the total food or in which fish ate all of one food before the experiment was terminated were excluded from the

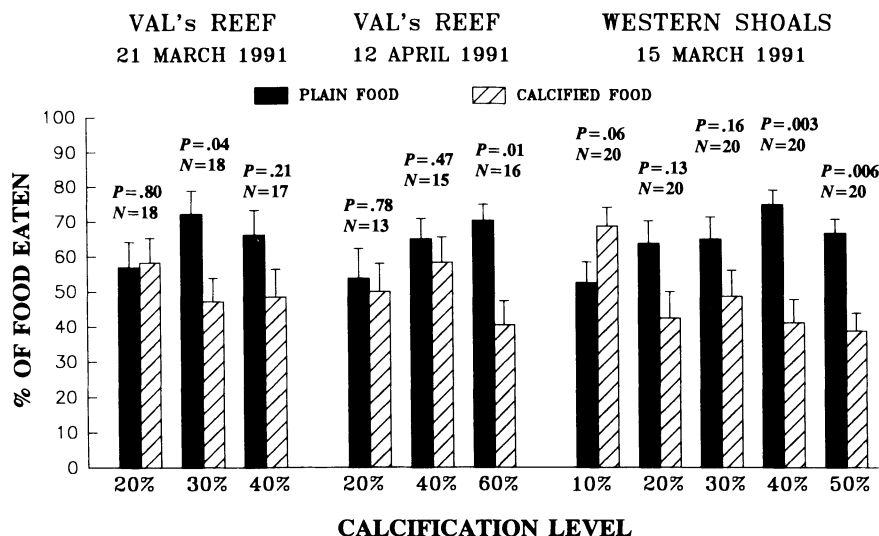


FIG. 1. Results of feeding experiments testing different concentrations of aragonite in the *Enteromorpha* diet. The histogram bars represent mean percent of number of food cubes completely eaten, with narrow vertical bars representing 1 SE. N = number of pairs of ropes (with experimental food cubes attached to them) used in the assays. Data were analyzed with a Wilcoxon signed-ranks test for paired comparisons (two-tailed).

data analysis. The difference in mass change between plain and treated food cubes was compared for fish and no-fish (controls) with a paired t test (two-tailed), as previously described.

We also compared fish responses to the three food treatments (aragonite, chemistry, and combined defenses) with a two-way ANOVA without replication, with treatment and individual fish as main effects. To compare the responses of the four fish species to the different treatments, we further analyzed the data with

a two-way ANOVA, with species and treatment as main effects and individual fish as replicates. The variable used for these analyses was the difference in mass change (in grams) between plain and treated food cubes for fish minus the difference for similar cubes protected from fish (to correct for autogenic changes in mass). This variable was used because all experiments were conducted as paired assays and all replicate fish were offered both plain and treated food cubes simultaneously.

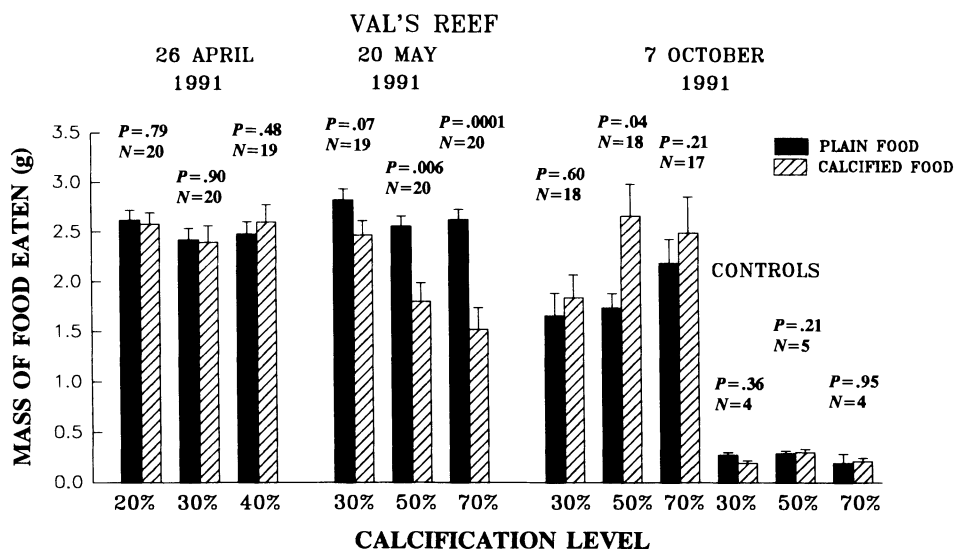


FIG. 2. Results of feeding trials testing different concentrations of aragonite on different dates. The histogram bars represent means, with narrow vertical bars representing one standard error. N = number of pairs of ropes used in the assays. Data were analyzed with a paired t test (two-tailed). Controls (no fish) were put in a mesh bag in the water for the duration of the trial.

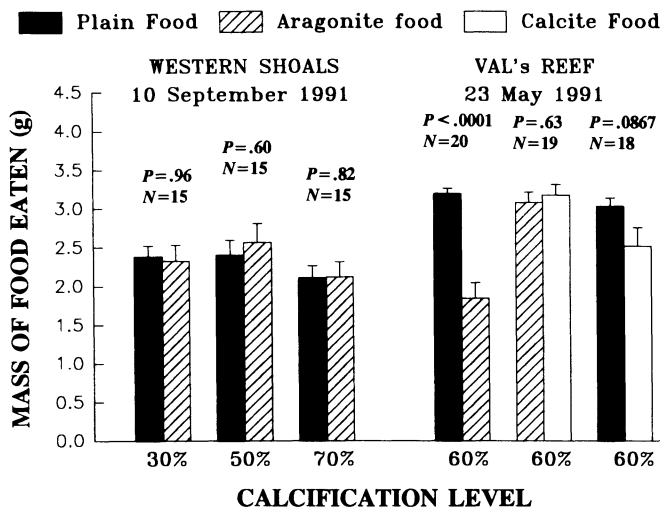


FIG. 3. Results of feeding trials testing different concentrations of aragonite and aragonite vs. calcite. The histogram bars represent means, with narrow vertical bars representing 1 SE. N = number of pairs of ropes used in the assays. Data were analyzed with a paired t test (two-tailed).

RESULTS

Aragonite sometimes deterred feeding by herbivorous fishes in the field, but the results varied among different dates and different reefs. Aragonite deterred feeding by fish at concentrations as low as 30% dry mass with the scored ropes on which we counted the number of cubes completely eaten (Fig. 1). However, for the weighed food cubes (cubes were weighed before and after each feeding trial), feeding was reduced only at the 50% and 70% aragonite concentrations on 20 May 1991 at Val's Reef ($P = .006$ and $P = .0001$, Fig. 2), and feeding was not affected at Western Shoals (Fig. 3). In one experiment, the 50% aragonite concentration even showed an attractant effect at Val's Reef on 7 October 1991 ($P = .04$). The no-herbivore controls showed no significant differences in mass loss between aragonite and plain food cubes and only small mass changes relative to food cubes eaten by fishes (Fig. 2). Fish did not differentiate between the two crystal forms of calcium carbonate, aragonite and calcite (Fig. 3). Aragonite was significantly deterrent at 60% compared with plain food ($P < .001$, Fig. 3) and calcite showed a trend toward deterrence ($P = .087$, Fig. 3). In the direct comparison between the two forms of calcium carbonate, there was no significant difference in herbivore preference between aragonite and calcite (Fig. 3).

Different fish species showed significant preferences for either plain or calcified food during the feeding observations at Val's Reef, Western Shoals, and Fingers Reef (Fig. 4). The parrotfishes *Scarus sordidus* and *S. schlegelii* and the surgeonfish *Ctenochaetus striatus* preferred food containing aragonite ($P < .0001$ for both species of scarids and $P = .016$ for *C. striatus*). The

FEEDING OBSERVATIONS

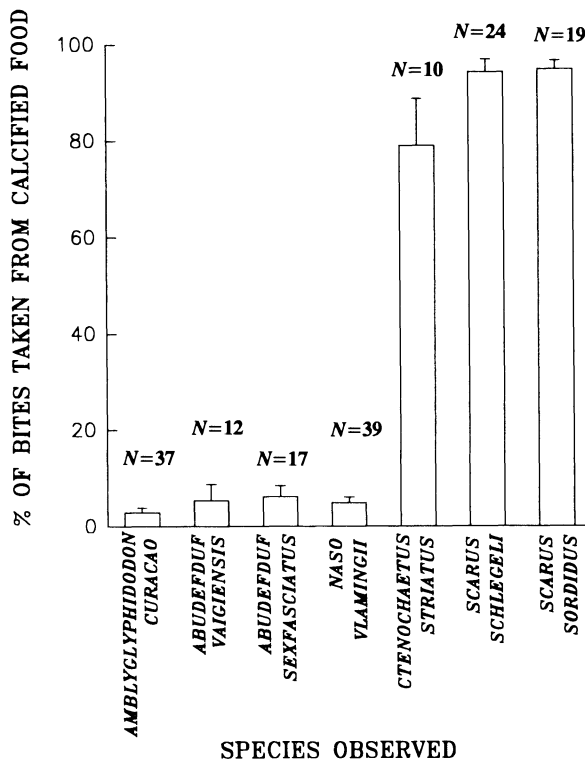


FIG. 4. Feeding observations of the deterrent effect of aragonite on individual fish species from Val's Reef, Western Shoals, and Fingers Reef. Histogram bars show mean percent of bites taken from calcified food, and narrow vertical bars 1 SE. N = number of different individual fish observed. A null model of no preference (e.g., proportion of bites from calcified food = 0.5) was tested by comparing the observed proportion with 0.5 using a t test.

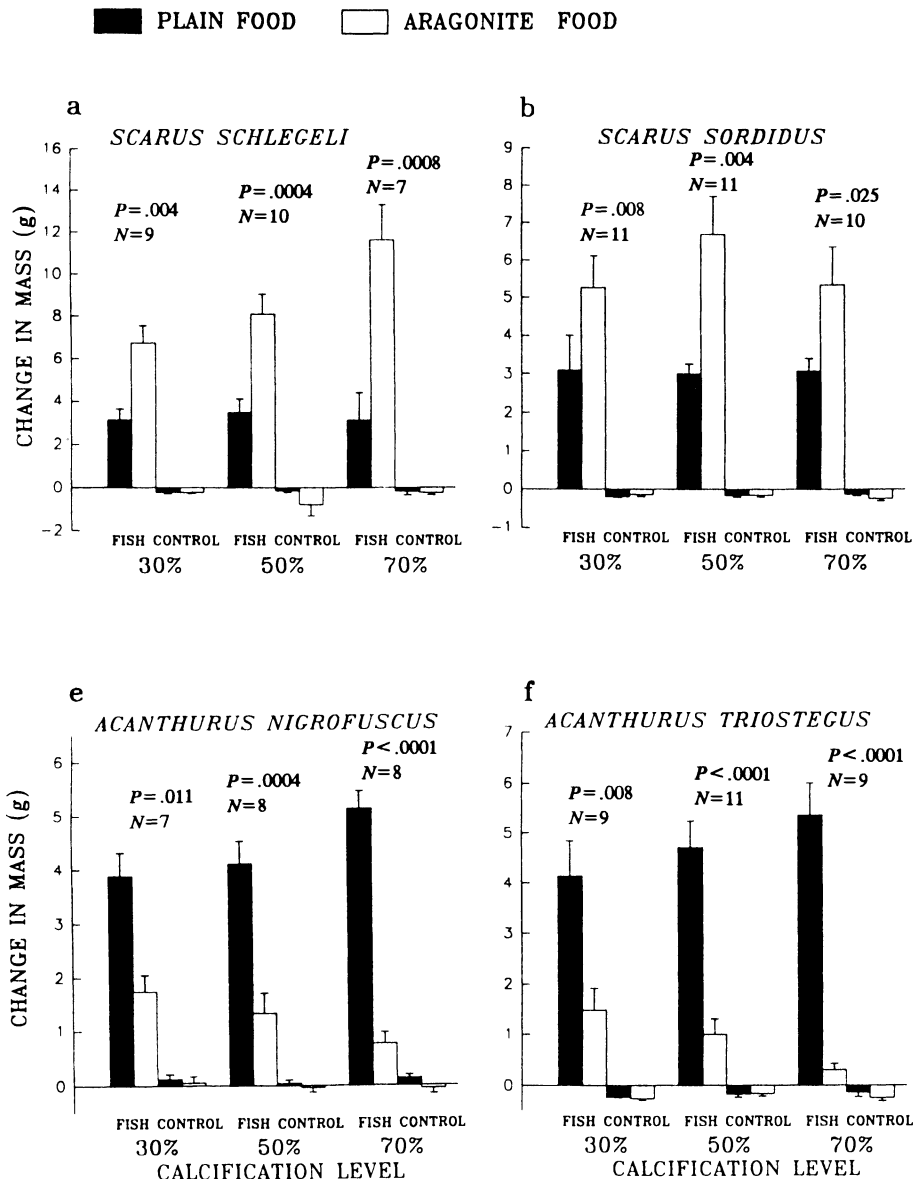


FIG. 5. Results of laboratory feeding trials testing three different aragonite concentrations on eight fish species. Histogram bars represent means, with narrow vertical bars representing 1 SE. N = number of replicate groups of fish. Data were analyzed with a paired t test (two-tailed).

surgeonfish *Naso vlamingii* and the damselfishes *Amblyglyphidodon curacao*, *Abudefduf sexfasciatus*, and *Abudefduf vaigiensis* preferred the noncalcified food ($P < .0001$ for each).

In most cases, the preferences of the fishes observed in the laboratory experiments corresponded to the preferences of fish species in the field. The eight fish species tested differed considerably in their responses to aragonite in their diet (Fig. 5a–h). The scarids preferred aragonite over plain food at all concentrations (Fig. 5a, b). Most acanthurids and *Siganus spinus* significantly avoided calcified food at all concentrations (P values ranging from $P = .01$ to $P < .0001$, except *A. xan-*

thopterus with $P = .132$ at 30% aragonite, Fig. 5d–h). To ensure that the nonsignificant P value for the *A. xanthopterus* feeding experiment at 30% calcium carbonate was not due only to the low power of $N = 6$ replicates, we randomly reduced the replicates to $N = 6$ for both the 50 and 70% aragonite levels as well. Both the 50 and 70% aragonite levels were still shown to have strongly deterred feeding by *A. xanthopterus* when only six replicates were used in the analyses ($t = 8.11$, $P = .0005$ for 50% aragonite; $t = 5.50$, $P = .003$ for 70% aragonite). *Ctenochaetus striatus* was the exception within the surgeonfishes tested, because feeding was reduced only by the highest aragonite level in one

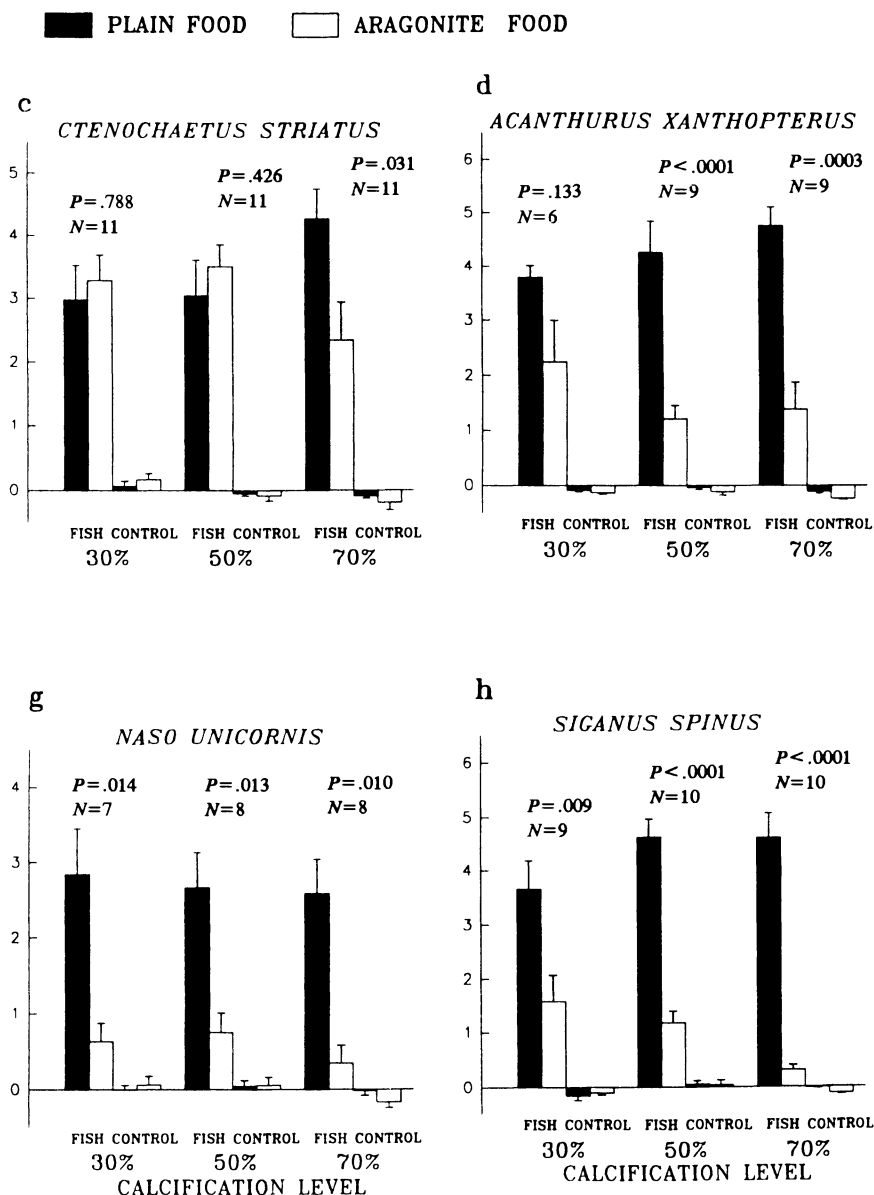


FIG. 5. Continued.

experiment (Fig. 5c), but not in another (Fig. 6b), and these fish seemed to prefer calcified food in the field observations (Fig. 4).

To see if the scarids actually exhibit a preference for calcified food, we considered two possible null models. First, if we assume that fish feed randomly by mass, they should consume the same mass of plain and calcified food, regardless of the content of aragonite. (Percent by mass of diet made up of calcified food would be 50%.) Second, if we assume that fish feed randomly by volume, they would consume a greater mass of calcified food than plain food, since the aragonite food weighs more at the same volume than the plain food.

Also, the ratio of aragonite to plain food eaten by fish would increase with increasing aragonite content, because the difference in density between plain and aragonite cubes increases with increasing aragonite concentration. To calculate the null model, if 50% of the volume of food eaten is aragonite food, then percent by mass of diet made up of calcified food = (mass of calcified diet) ÷ [(mass of calcified diet) + (mass of plain diet)] since the volume of the different diets is the same. These values equate to 50.7% by mass for 30% aragonite food, 51.6% by mass for 50% aragonite food, and 53.5% by mass for 70% aragonite food if equal volumes are consumed. One-sample *t* tests were

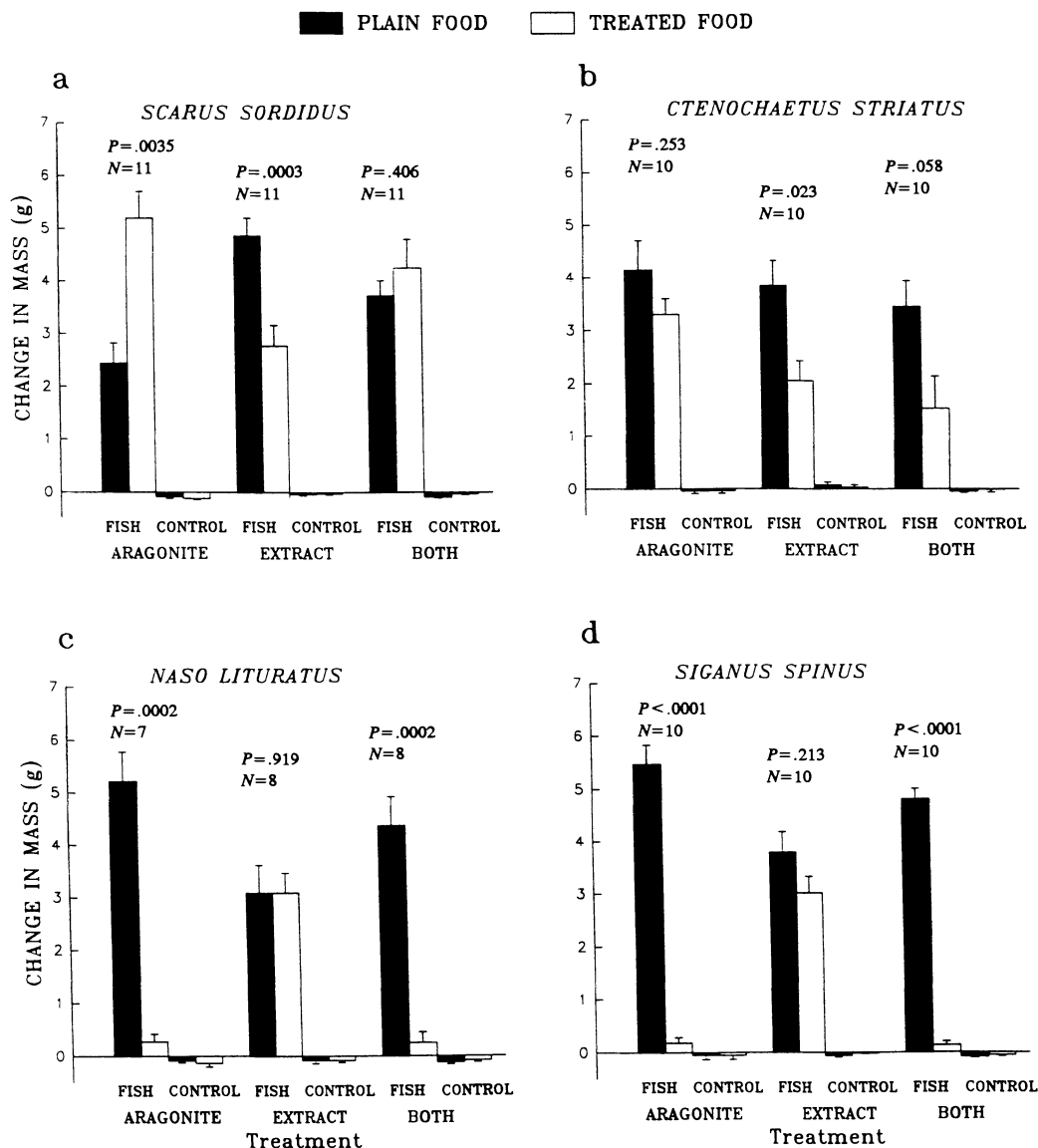


FIG. 6. Results of laboratory feeding trials testing 80% aragonite (by dry mass) against plain food, *Halimeda macroloba* secondary metabolites (1% dry mass) against plain food, and a combination of both (same amount of aragonite and secondary metabolites as in single defense experiments) against plain food. Histogram bars show means and narrow vertical bars 1 SE. N = number of replicate groups of fish. Data were analyzed with a paired t test (two-tailed).

used to compare percent calcified food eaten minus these values with 0 to determine significance levels at the different aragonite concentrations.

The scarids had a significant preference for aragonite food at all concentrations by mass (Fig. 5a, b) and by volume (P values ranging from .049 for *Scarus sordidus* eating 70% aragonite food to <.0001 for *S. sordidus* eating 50% aragonite food). For the surgeonfishes and rabbitfish, all species significantly avoided aragonite food at all concentrations when compared by mass (Fig. 5c–h) and by volume except for *Acanthurus xanopterus*, which did not significantly avoid 30% aragonite food by mass (Fig. 5d) or volume ($N = 6$, $t = 2.12$, P

= .09, one sample t test) and *Ctenochaetus striatus*, which did not significantly avoid 30 or 50% aragonite food by mass (Fig. 5c) or volume, but significantly avoided 70% aragonite food by mass (Fig. 5c) and volume ($N = 11$, $t = 3.15$, $P = .01$).

In the laboratory experiments testing multiple defenses, all four fish species tested were deterred by either aragonite or secondary metabolites (Fig. 6a–d). The parrotfish *Scarus sordidus* showed a significant preference for the food containing 80% aragonite, but was deterred by *Halimeda macroloba* secondary metabolites (Fig. 6a). A combination of both defenses did not have a significant effect on feeding. Similarly, ara-

TABLE 1. Analysis of variance tables for comparisons of fish responses to the three food treatments (aragonite, chemistry, and combined defenses). (A–D) are results for analyses of individual fish species separately (two-way ANOVA without replication, treatment and individual fish as main effects). (E) reports results of an analysis of all fish species combined (two-way ANOVA, species and treatment as main effects).

	Source	df	ss	F	P
A) <i>Scarus sordidus</i>	Treatment	2	129.58	24.58	<.0001
	Individual	10	52.14	1.98	.093
	Error	20	52.73		
B) <i>Ctenochaetus striatus</i>	Treatment	2	7.15	0.80	.464
	Individual	9	8.15	1.83	.132
	Error	18	4.45		
C) <i>Naso lituratus</i>	Treatment	2	105.27	18.92	.0001
	Individual	7	23.28	1.19	.370
	Error	13	36.18		
D) <i>Siganus spinus</i>	Treatment	2	122.10	35.60	<.0001
	Individual	9	13.87	0.90	.546
	Error	18	30.87		
E) All species	Treatment	2	44.72	6.42	.0025
	Species	3	308.51	29.52	<.0001
	Treatment × species	6	374.48	17.92	<.0001
	Error	104	362.30		

gonite did not deter feeding by the surgeonfish *Ctenochaetus striatus*, but secondary metabolites did significantly reduce feeding, and combined defenses were marginally deterrent ($P = .058$, Fig. 6b). The surgeonfish *Naso lituratus* and the rabbitfish *Siganus spinus* were both deterred by aragonite but not by secondary metabolites alone (Fig. 6c, d). A combination of defenses also deterred feeding by these fishes, but the effect of combined defenses was no greater than for aragonite alone (Fig. 6c, d).

Comparisons by two-way ANOVA confirmed that *Scarus sordidus*, *Naso lituratus*, and *Siganus spinus* (Table 1A, C–D) but not *Ctenochaetus striatus* (Table 1B) responded differently to the different food treatments; however, the individual fish effect was never significant (Table 1A–D) and therefore was dropped for the following analysis. The comparison of the responses of the four different species to the different treatments (Table 1E) showed that the effects of treatment and species were both highly significant. The ANOVA term of primary interest was the treatment × species term, which was also highly significant, confirming that the pattern of responses to the three food treatments differed among the fish species.

DISCUSSION

Calcified algae consistently rank low in preference by herbivorous fishes (reviewed by Horn 1989, Hay 1991). However, many calcified algae are also chemically defended by secondary metabolites (Hay 1984, Paul and Fenical 1984, Paul and Hay 1986, Paul and Van Alstyne 1988b), and very few studies have experimentally addressed the link between calcium carbonate alone and susceptibility to herbivores (Pennings and Paul 1992). Our field experiments supported the

hypothesis that some fish avoid aragonite; however, the results varied among different dates and reefs. This variation probably is explained by field observations and laboratory experiments that showed that the effect of aragonite depended on the fish species. Similarly, the effect of secondary metabolites from the green alga *Halimeda* also depended on the species of fish involved. Fish that were not deterred from feeding by one defense were deterred by the other. However, in no case did the combination of defenses work better than a single defense toward a susceptible herbivore. In fact, for *Scarus sordidus* the addition of aragonite to the secondary metabolites appeared to cancel out the deterrent effect of the diterpenes (Fig. 6a). Nonetheless, a combination of aragonite and secondary metabolites may be more consistently effective in reducing feeding by a diverse herbivore assemblage than either of the defenses alone.

The effect of aragonite on feeding by fishes

Our experiments allowed us to test the effects of calcium carbonate on fish feeding without affecting the toughness of the foods. We tested aragonite in powdered form, and toughness of the aragonite food did not differ from toughness of the controls. Essentially, we studied calcium carbonate as a chemical defense, not a structural defense, and we term it a mineral defense to differentiate it from chemical defenses based on organic molecules.

Aragonite more commonly deterred than stimulated feeding by different herbivorous fish species, but the results varied among different reefs and between different dates on the same reef. This variability was probably caused by differences in the species of fish that ate during the short duration of the experiments. Although

dozens of individual fish ate from the ropes during the experiments, the presence of a few large individuals that fed selectively could affect the results. For example, the attractant effect of aragonite at Val's Reef on 7 October 1991 (Fig. 2) was apparently due to the presence of a few *Scarus sordidus* that we observed feeding selectively on the calcified food cubes that day. Generally, aragonite did not have an overall deterrent effect when many parrotfish were feeding. The variability in the field experiments is more understandable if we examine the results of feeding observations carried out at these reefs (Fig. 4). The fish fell into two distinct groups, with parrotfishes and the surgeonfish *Ctenochaetus striatus* showing a significant preference for the calcified food, and three damselfishes and the surgeonfish *Naso vlamingii* significantly avoiding the calcified food (Fig. 4).

Differential results such as these were also obtained in laboratory experiments. The parrotfishes *Scarus sordidus* and *S. schlegeli* were not deterred by aragonite and clearly preferred calcareous food. Surgeonfishes (with the exception of *Ctenochaetus striatus*) and the rabbitfish strongly avoided aragonite, especially at concentrations $\geq 50\%$. The deterrent effect for some of these fishes increased with increasing aragonite level. *Ctenochaetus striatus* only avoided aragonite at the highest concentration, at least in some cases (Fig. 5c vs. Fig. 6b).

The preference of parrotfishes for calcified food and the deterrent effect of aragonite on feeding by many surgeonfishes and the rabbitfish are consistent with what we know about the biology of these fishes. The fishes we studied fall into three groups based on digestive mechanisms: (1) trituration in pharyngeal jaws (parrotfishes); (2) acid lysis in a thin-walled stomach (some surgeonfishes, damselfishes, and probably rabbitfishes); and (3) trituration in a gizzard-like stomach (some surgeonfishes and mullets) (Horn 1992).

Parrotfish have fused teeth and a robust pharyngeal mill, which enables them to take bites out of tough or heavily calcified algae and to grind them up using their pharyngeal mill, making them more digestible (Smith and Heemstra 1986, Bellwood and Choat 1990, Myers 1991). Parrotfish are therefore capable of consuming calcified foods and commonly feed on calcified algae or consume calcium carbonate incidentally as they scrape algae off hard surfaces (Randall 1967, Steneck and Adey 1976, Lobel and Ogden 1981, Lewis 1985, Wolf 1985, Lewis et al. 1987, Horn 1989, 1992). Parrotfish lack a stomach, have a more or less neutral intestinal pH (pH 6.4–8.6), and do not hydrolyze their food through acid lysis as do many surgeonfishes (Lobel 1981, Horn 1989, 1992). This possibly avoids the release of large amounts of CO_2 within the digestive tract that would otherwise occur when ingested calcium carbonate encountered acidic stomach fluids. Since we were able to remove the effects of toughness in our experiments, it appears that parrotfish like the taste or

texture of aragonite or have a physiological need for calcium carbonate in their diets (Smith and Paulson 1975).

In contrast, surgeonfishes and rabbitfishes have small mouths and small close-set lanceolate or incisiform teeth, which are more effective at browsing on filamentous and foliose algae (Hiatt and Strasburg 1960, Choat 1991, Horn 1992). A preference for noncalcified algae in these species is supported by several feeding observations and transplant experiments (Tsuda and Bryan 1973, Bryan 1975, Lewis 1985, Wolf 1985, Fishelson et al. 1987, Paul et al. 1990). Furthermore, some surgeonfishes have thin-walled stomachs and an acidic gut pH (pH 2.9–5.7, Horn 1989, 1992). They use the often highly acidic stomach fluids to lyse the algal cell walls. Lobel (1981) suggested that fish with an acidic stomach avoid calcium carbonate in their diet because the buffering capacity of calcium carbonate would increase the pH and prevent lysis of algal cell walls. Rabbitfishes, closely related to surgeonfishes, also have thin-walled, presumably acidic stomachs (Horn 1989, 1992). Since our calcified foods were not physically tougher than the controls, these fishes do not appear to be avoiding calcified foods simply because of structural or morphological defenses. It appears that these fishes do not like the taste or texture of calcium carbonate, or may have adverse physiological reactions to a calcified diet.

Ctenochaetus striatus and *Acanthurus xanthopterus* fall within the group of surgeonfishes that use a gizzard-like stomach to grind their food. They consume calcareous sediment while feeding on algal and detrital films on rocks and in sand patches (Hiatt and Strasburg 1960, Horn 1989). *Ctenochaetus striatus* has slender moveable teeth, which are not designed to bite off algal pieces, but are used to feed in a scraping and sucking manner (Randall 1955). The stomachs of *Ctenochaetus striatus* may contain up to 75% inorganic sediment (Nelson and Wilkins 1988). *Acanthurus xanthopterus* also has large amounts of inorganic sediment in its gut (reviewed in Jones 1968). *Acanthurus xanthopterus* and *Ctenochaetus striatus* have a neutral gut pH like the parrotfish and process their food by grinding it up in their thick-walled, gizzard-like stomachs (Horn 1989, 1992). This could explain why these two surgeonfishes were not deterred at the lower calcification levels, since they both normally consume calcium carbonate during grazing. *A. xanthopterus* avoided aragonite more than *C. striatus*, especially at higher concentrations, so even fishes within the group with gizzard-like stomachs and neutral gut pH can be deterred by high concentrations of calcium carbonate.

The effect of multiple defenses against feeding by fish

Algal secondary metabolites are well known to deter feeding by herbivorous fishes (reviewed by Hay and Fenical 1988, Hay 1991, Paul 1992). Of the four species

we tested, *Scarus sordidus* and *Ctenochaetus striatus* were deterred by *Halimeda* diterpenes but not by aragonite, whereas *Naso lituratus* and *Siganus spinus* were deterred by aragonite but not by diterpenes. It is interesting that none of the four species avoided both secondary metabolites of *Halimeda* and aragonite when each putative defense was tested singly.

These results probably explain why many algae combine mineral and chemical defenses, since a combination of calcium carbonate and secondary metabolites increases the number of fish species that are deterred. However, our results suggest that for some fish species, such as *Scarus sordidus*, combinations of calcium carbonate and secondary metabolites are less effective as a defense than secondary metabolites alone because these fish prefer to eat calcified foods. This observation may be an artifact of our experimental design because we decreased the overall concentration of diterpenes (based on percent dry mass of the food, not based on percent volume or percent aragonite-free dry mass of the food) in the combined aragonite and diterpene food to keep the amount of compounds we added to each diet the same. The deterrent effect of the diterpenes seemed to be diluted by the addition of the highly preferred aragonite. *Halimeda macroloba* actually contains higher levels of diterpenes than we tested here (1% total dry mass including CaCO_3 mass, Paul and Fenical 1984), although other *Halimeda* species produce lower levels of diterpenes closer to the levels we tested (Paul and Fenical 1984).

Nevertheless, the preference parrotfishes show for calcified foods suggests that calcified algae may require high levels of secondary metabolites or very toxic and deterrent secondary metabolites to defend against grazing in areas where parrotfishes are abundant. *Halimeda* chemistry may actually reflect this pattern of defense, since diterpenes are sometimes produced in high concentrations especially in new growth and young plants (Paul and Fenical 1984, Hay et al. 1988, Paul and Van Alstyne 1988b), and a potent feeding deterrent, halimediatriol, is actively produced in damaged tissues in *Halimeda* (Paul and Van Alstyne 1992) and is also found in high concentrations in new growth (Hay et al. 1988, Paul and Van Alstyne 1988b). We might also expect to find chemical defenses in many other calcified seaweeds found in areas subject to high herbivory by parrotfishes.

Calcium carbonate does not occur in high concentrations in terrestrial plants. Probably the most comparable mineral-based defense is silica, which occurs in many grasses. Silica appears to be an inducible defense against herbivores (McNaughton and Tarrants 1983) and concentrations are higher in more heavily grazed grasslands and early in the growing season (McNaughton et al. 1985, but see review by Vicari and Bazely 1993). Silica effectively deters some mammalian grazers (Gali-Muhtasib et al. 1992). Grasses also produce a variety of secondary metabolites (Vicari and

Bazely 1993). Grassland ecosystems would seem to be another good place to look for combined chemical and mineral defenses; however, little evidence suggests that silica and secondary metabolites co-occur in grasses (Bernays and Barbehenn 1987, Vicari and Bazely 1993), even though Serengeti grasslands may support a high diversity of grazing mammals and some of Earth's largest herds (McNaughton et al. 1985). Not all seaweeds use chemical and mineral defenses, but in coral reef habitats where fish grazing is intense many macroalgae contain calcium carbonate and secondary metabolites (Hay 1984, Paul and Hay 1986, Paul and Van Alstyne 1988b).

Even on coral reefs, however, many seaweeds grow fast, do not produce any defenses, and tolerate herbivory, and others produce secondary metabolites but do not calcify (Hay 1991). It is likely that this variability reflects different benefits and costs of defenses. For example, calcified seaweeds are slow growing, low primary producers (reviewed in Littler and Littler 1984), suggesting a cost to calcification. However, the calcareous, chemically defended seaweeds and coralline algae seem to predominate in many high-herbivory habitats. It is not clear whether the apparent absence of combined chemical and mineral defenses in terrestrial grasses illustrates a contrast in plant defensive strategies between high herbivory habitats in terrestrial and marine ecosystems or reflects a lack of research on multiple defenses in grasses and how these might interact to affect potential herbivores.

A combination of aragonite and *Halimeda* diterpenes reduced feeding by all fish species except *Scarus sordidus*, and *S. sordidus* was significantly deterred by natural concentrations of diterpenes. This suggests that the utilization of two very different defenses, secondary metabolites and calcium carbonate, allows tropical algae to defend themselves better against a wider range of herbivores than would the use of only one or the other single defense.

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