STUDIES ON THE CHEMICAL ECOLOGY AND REPRODUCTIVE BIOLOGY OF SARCOPHYTON TROCHELIOPHORUM VON MARENZELLER (COELENTERATA: ALCYONACEA) ON GUAM, MARIANA ISLANDS

By

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A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER of SCIENCE

in

BIOLOGY

UNIVERSITY OF GUAM

August 25, 2000

AN ABSTRACT OF THE THESIS OF John A. Starmer for the Master of Science in Biology presented on August 25, 2000.

Title: Studies on the chemical ecology and reproductive biology of *Sarcophyton trocheliophorum* Von Marenzeller (Coelenterata: Alcyonacea) on Guam, Mariana Islands.

Approved: _____
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Sarcophyton trocheliophorum colonies collected at Piti Bay, Guam were found to contain organic chemicals and calcium carbonate spicules that deterred predation by sympatric reef fishes. Laboratory feeding assays showed the deterrent effect to be caused in part by the compound, isosarcophytoxide B. An additional, multicompound fraction was also found to be antifeedant. The effectiveness of either sclerites or extracts was found to vary among different fish species tested. The soft coral specialist *Chaetodon melannotus* was attracted to extract which deterred most other species tested. Concentrations of crude organic extract and sclerites have an inverse relationship within *Sarcophyton* colonies. Organic extract levels are highest in the polyp-bearing capitulum and decrease towards the colony's base. Protein concentrations were highest in the polyp anthocodia and were similar throughout the rest of the animal. Egg and larval development of *S. trocheliophorum* was similar to other alcyonarian species. These soft corals were found to produce eggs with a two-year cycle and to spawn synchronously during several nights each year. Low levels of isosarcophytoxide B occur in the spawned eggs and larvae of *S. trocheliophorum*. The eggs of *S. trocheliophorum* were deterrent to *Canthigaster solandri* in laboratory trials.

TO THE OFFICE OF GRADUATE SCHOOL AND RESEARCH

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Acknowledgments

The assistance of Cyrena Aguon, from George Washington High School, and Jason Biggs, Lisa Kirkendale, Jesse Manglona, Melany Puglisi, Marc Slattery from the University of Guam Marine Laboratory, was helpful in the field and laboratory. Bruce Bowden, James Cook University, provided reference NMR spectra of *Sarcophyton* compounds. Lolita Penland provided much-appreciated editorial, graphical, and motivational assistance. Supported in part by National Institutes of Health Grant GM38624.

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Introduction

Soft corals often rival or surpass the abundance of other sessile organisms on Indo-Pacific coral reefs (Dinesen 1983, Bakus *et al.* 1989, Dai 1991). The presence of these soft-bodied, sessile animals in a predator-rich community would suggest that soft corals should be at least moderately preyed upon (Grigg *et al.* 1984). However, with the exception of certain specialized predators, soft corals are relatively free from predation (Coll *et al.* 1983, Coll and Sammarco 1986, Bakus *et al.* 1989, Wylie and Paul 1989, Aliño, et *al.* 1992, Griffith 1994, McDonald and Nybakken 1999). There is ample evidence that the reasons for this resistance to predation and the resulting abundance of soft corals on reefs are their chemical and structural defenses (terpenes and calcareous spicules) (La Barre *et al.* 1986, Sammarco *et al.* 1987, Harvell *et al.* 1988, Bakus *et al.* 1989, Wylie and Paul 1989, Van Alstyne *et al.* 1992, 1994).

Predators, including specialists, may have their prey choices affected by variation in concentrations and types of chemical defenses or structural defenses in potential prey species (La Barre 1986, Aliño, et *al.* 1988, Wylie and Paul 1989, Becerro *et al.* 1998). The functions of octocoral sclerites (calcareous spicules) as structural as well as defensive elements can impose limitations on their effectiveness as defenses (West 1998). The ability to compensate with defensive chemistry could be advantageous to those colonies whose structural defenses are environmentally limited. Additionally, as no single defense is likely to deter all predators; combining structural and chemical defenses may allow soft corals to overcome the limitations of using a single type of defense (Van Alstyne and Paul 1992, Schupp and Paul 1994, Van Alstyne *et al.* 1994).

Those species that are known to feed on soft corals have been shown to be more resistant to soft coral defenses, but can still be affected (Wylie and Paul 1989). *Chaetodon unimaculatus* was undeterred by natural concentrations of *Sinularia*

extracts, but a 2-3x increase in extract concentrations had a significant deterrent (Wylie and Paul 1989). West (1998) showed that *Cyphoma gibbosum* showed a preference for artificial food containing short rather than long sclerites.

The effects of different levels of extracts and sclerites on the predation rates of reef fishes have also been investigated (Harvell *et. al* 1988, Van Alstyne and Paul 1992, Van Alstyne *et. al* 1994). Van Alstyne *et al.* (1994) showed that concentrations of sclerites and organic extracts varied between bases and tips in *Sinularia* species. The levels of sclerites in the bases were more deterrent than the lower and nondeterrent levels found in the tips, and high levels of organic extract were more deterrent than the lower levels in the bases (Van Alstyne *et. al* 1994).

In this study, I chose to use *Sarcophyton trocheliophorum* Von Marenzeller, 1886, a widely distributed soft coral on Pacific reefs (Verseveldt 1977 and 1978, Dai 1991), to investigate the effects of chemical and structural defenses on predation by ecologically relevant fishes. On Guam, *S. trocheliophorum* is common on forereef slopes and in reef flat, lagoon and harbor environments (Benayahu, 1997). On the reef flat and shallow fore reef of Piti Bay it is a common to dominant animal (pers. obs.). These factors made the bay a reasonable location to make observations on natural history, collect animals for extraction and to run feeding assays. Assays were run, both in the field at Piti Bay and in the laboratory, to test the following hypotheses:

1. Levels of secondary metabolites and organic extracts vary inversely with the concentration of sclerites among parts of S. *trocheliophorum colonies.*
2. Secondary metabolites and sclerites in *S. trocheliophorum reduce* predation on adult soft corals.
3. Generalist (polyphagous) predators are more likely to be deterred by these secondary metabolites than soft coral-specialist (oligophagous, monophagous) predators.

4. Structural defenses (sclerites) should show less effectiveness as a defense against preadapted predators.

Laboratory assays allow a greater control over which species are tested and allow for the control of variables in a manner, which is not possible in the field. However, the behavior of a fish in the laboratory may also be influenced by the conditions imposed in the laboratory. Although they are still artificial constructs, field assays allow for a better understanding of the reaction of fishes in their natural environment.

While investigating the above hypotheses, I was additionally able to make some observations on the natural history of *S. trocheliophorum.* These included records of spawning patterns, egg development, and larval ecology.

Materials and Methods

Secondary Metabolite Isolation

Portions of three haphazardly chosen *S. trocheliophorum* colonies with a contracted stalk diameter of approximately 10 cm were collected from the Piti Bay reef flat on March 20, 1996. The colonies were kept in seawater in plastic bags and returned to the lab in a cooler. These colonies were the source of all extracts and sclerites used in subsequent feeding assays.

The colonies were exhaustively extracted (c.a. 5 times) following methods of Van Alstyne and Paul (1992). The corals were extracted with a 1:1 dichloromethane (DCM): methanol (MeOH) solution until freshly added solvent was not discolored after 24 hours. The organic layer was separated from the aqueous layer with a separatory funnel and dried by rotary evaporation with a Buchi R114 or RE111 Rotavapor. This material, hereafter referred to as crude extract was used for all ecological feeding assays in this study. The crude extract was further separated by silica gel flash column chromatography (Fig. 1). Feeding assays conducted in the laboratory with *Canthigaster solandri* (Richardson, 1844) (see below for methods) were used to determine which fractions contained antifeedant compounds. The process was repeated until nearly pure antifeedant compounds were identified.

In one case, the deterrent fraction was further separated with a Waters 501 high performance liquid chromatograph (HPLC) equipped with a Waters R401 differential refractometer to isolate a pure antifeedant compound. The fraction was first dissolved in the HPLC solvent system (30% ethyl acetate (EtOAc): hexanes) and filtered through glass wool to remove insoluble materials. The solvent was then evaporated using a Savant SC110 Speed Vac, and sufficient solvent to just dissolve the fraction was added. Approximately 50mg of dissolved material per run was loaded on the HPLC

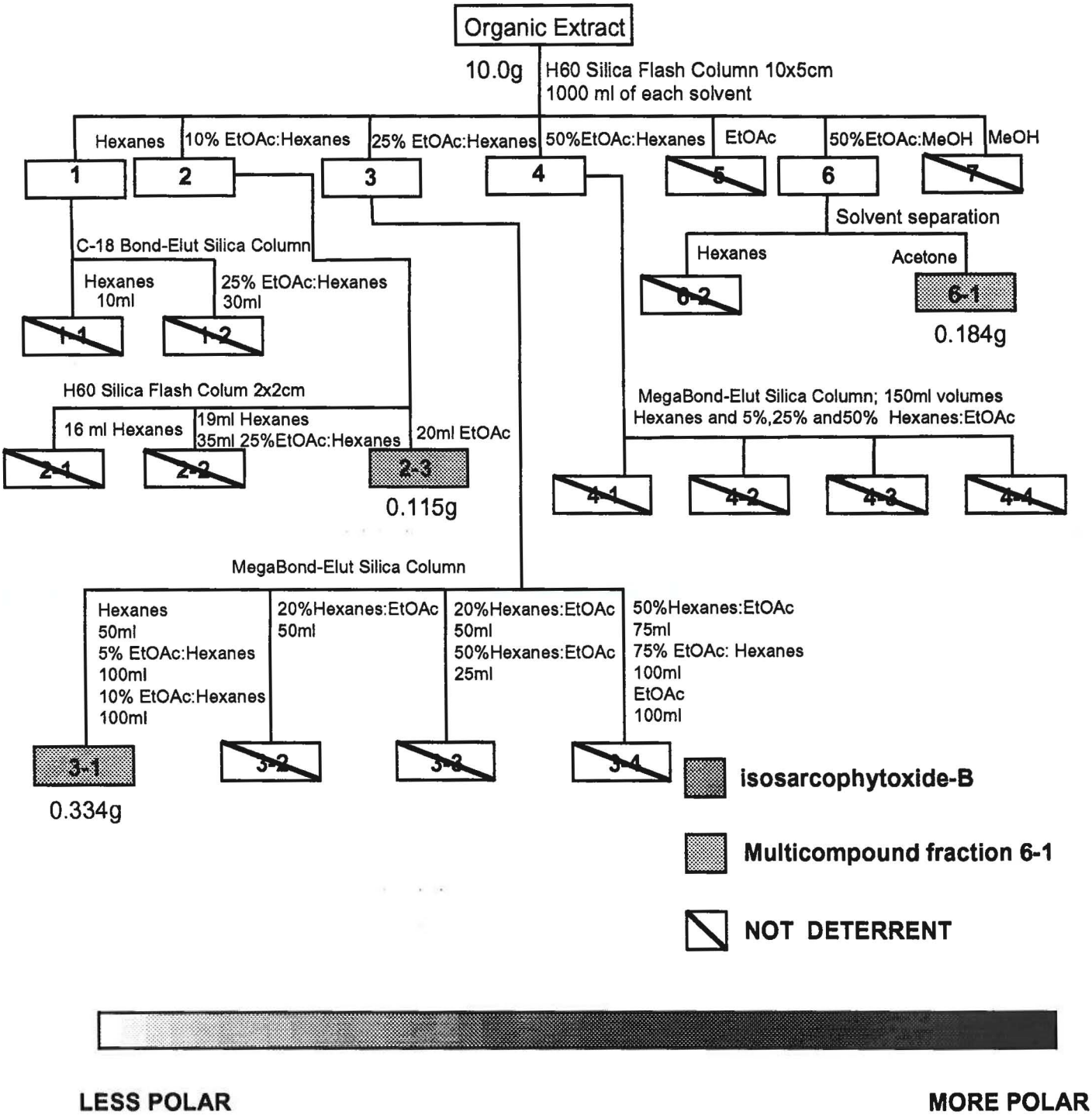

Figure 1. Secondary metabolite isolation scheme for *Sarcophyton trocheliophorum.*

and run through an Alltech Econosil 10u (250mm x 10mm) silica column at a rate of 3ml/min using the 30% EtOAc: hexanes solvent system. The compound isolated was examined by proton nuclear magnetic resonance (NMR) spectroscopy to determine its identity and purity. The compound was identified by comparison of its NMR spectrum to those of known soft coral compounds.

Feeding Assays

Chemical Defenses

The antifeedant effect of the crude extract was tested on the fishes *Canthigaster solandri* (Richardson, 1844), *Dascyllus aruanus* (Linnaeus, 1758), *Chaetodon melannotus* Bloch, 1801, and *Chlorurus sordidus* (Forsskål, 1775) in the laboratory using the methods developed by Hay *et al.* (1994). The crude extract, which was derived from the whole colony extraction procedure, was used for all assays. When testing for the effectiveness of chemical feeding deterrence, the extract was used at the average concentration for the whole colony, base or capitulum was used as indicated. Assays were all evaluated for significant differences at the $p \leq 0.05$ level.

The fishes used in the assays were selected to provide a range of ecologically relevant predators on which to test the effectiveness of *S. trocheliophorum*'s defenses. *Canthigaster solandri* is an omnivorous pufferfish common on most reef flats on Guam (Myers 1983). *Dascyllus aruanus* is an omnivorous damselfish generally associated with colonies of branching hard corals such as *Acropora* spp. (Sano *et al.* 1984, Myers 1999). The butterflyfish *C. melannotus* is reported to restrict its feeding primarily to scleractinian and octocoral polyps (Harmelin-Vivien and Bouchon-Navaro 1981, Sano *et al.* 1984, Myers 1999). The parrotfish *Chlorurus sordidus* was included to provide comparison with a fish that typically includes a large amount of calcium carbonate in its diet (Sano *et al.* 1984, Schupp and Paul 1994).

The artificial food mix consisted of 1.5g ground catfish food pellets (Kruse's Perfection brand, El Monte, California) sifted through a 300um screen, 20ml water, and 0.62g carrageenan (Type 1, Sigma Chemical Company). The food was prepared by mixing the carrageenan with water and then heating the mixture in a microwave oven for approximately one minute. The test extract or compound was then added at a natural concentration (extract/dry weight) and mixed with the prepared food at high speed with a Virtis homogenizer. The control food lacked the extract, but was treated identically. The catfish pellets contain ~36% protein and the finally assay mix contained approximately 25% protein, which is equivalent to average protein concentrations in *S. trocheliophorum*.

As most extracts were oils at room temperature, or melted readily when heated, solvents were generally not used. Those compounds or extract fractions, which were not prone to melting, were mixed with a small amount of ethyl acetate as a solvent, added to the catfish food, and dried by rotary evaporation. The control food was treated identically in this case, with the same amount of solvent added.

The mixed, liquid food was then poured onto a 2mm high Formica mold placed over plastic window screen. The two resulting 2.5x 25cm strips of food (control and test) formed by the mold were leveled using a microtome blade. The screen was then cut into 1cm wide strips. The resulting strips contained a 1 x 2.5cm area of control and test mix each covering 120 screen squares. The strips were presented to individual fish maintained in 4-liter, flow-through system aquaria. The assays were run until over half of either the control or test food was consumed or until 24 hours had passed. The results were scored by counting the number of squares of screen exposed by the consumption of food mix. The tests were evaluated by paired t-tests (two- tailed) for each species of fish. Crude extracts, flash-column fractions, and HPLC fractions were tested in the same manner. Those fractions, which showed antifeedant activity, were further separated until pure, antifeedant compounds were isolated.

Sclerites

Sclerites were prepared following the methods outlined in Van Alstyne and Paul (1992). Extracted tissues from the capitulum and base of the stalk of colonies collected for the initial chemical extraction were oven dried, weighed and dissolved in 5.25% sodium hypochlorite (bleach) to remove all organic material. The sclerites were then washed in tap water five times and once in acetone, dried, and weighed. The sclerites from the base were eventually combined for feeding assays and the capitulum sclerites were kept separate from the base sclerites. Assays testing the effects of sclerite feeding deterrence from the capitulum or base use the sclerites isolated from the same section.

Assay sclerite concentrations were based on the average values determined from the soft corals extracted for the crude extract (35%, capitulum) ; 75%, base). The sclerites were added to the food mix in a ratio of sclerites/whole tissue dry mass. They were mixed into the test food mix in the same manner as for the chemical feeding assays. The antifeedant effectiveness of the sclerites was compared in assays using a ground catfish food control as previously described. These assays were run in the laboratory with the assays described in the previous section.

Combinations of Sclerites and Extracts

Two sets of three-way assays were used to compare the deterrent effects of capitulum sclerites and extracts and base sclerites and extracts to each other in addition to the standard control food. These assays used average sclerite and extract concentrations determined by the results of the samples collected for internal variation. Internal and external section values were combined to give the following averages: capitulum sclerites, 44%; capitulum extract, 18%; base sclerites, 77%; base extract, 5%. These assays used the same extract and sclerites used for the other assays.

One three-way assay was run in the field at Piti Bay. A food mix identical to that used in the laboratory assays was poured into a steel mold to produce 1cm^3 food cubes. The cubes were allowed to cool, removed from the mold, and placed in numbered plastic ziploc bags. In the field an assistant haphazardly tossed one of two test food cubes or a control food cube into the water column. Cubes were scored as being eaten, partially eaten or rejected. An assistant then indicated which bag the scored food had come from. The assay results were analyzed with a G test. Those assays with significant differences were then subdivided to compare treatments.

The three-way laboratory assays with *Canthigaster solandri* were similar to those used in the previous tests; however, in these assays screen strips with three rather than two artificial food areas were used. Each test strip had one section of control food and two test sections. The results were analyzed using Wilcoxon's signed rank test for paired comparisons. Post hoc comparisons were conducted with the Wilcoxon test with alpha adjusted to p=0.0167 (Bonferroni correction) because three multiple comparisons were tested.

Eggs

Mature *S. trocheliophorum eggs* were removed from a frozen colony collected during a spawning event in 1998. The eggs were presented to the sharpnose pufferfish *Canthigaster solandri*. *Pocillopora damicornis* (L., 1758) larvae harvested from colonies maintained in the laboratory served as controls. This coral produces larvae similar in size to the eggs *of S. trocheliophorum, and* the larvae have proven palatable to *C. solandri* in the laboratory (pers. obs.). Eight fish were haphazardly offered a *P. damicornis* larva or *S. trocheliophorum* egg followed with the other species' egg or larva. Eggs and larvae were not reused. Each fish was used only once. A Fisher's exact test was used to determine significance.

Variation

Intracolony Protein, Chemical, and Sclerite Variation

Ten *Sarcophyton trocheliophorum* colonies from the Piti Bay reef flat were collected on November 17, 1998 to investigate the variation in secondary chemistry, protein and sclerites within colonies. Colonies with a disc diameter of approximately 15cm were removed intact with their bases still attached to the substrate on which they were growing. The colonies were placed in seawater-filled 1-gallon plastic bags. Seawater was also collected in 0.5 gallon plastic bags approximately 30cm above three *Sarcophyton* colonies. Each of the samples was collected approximately five meters from the closest previous sample. The seawater and *Sarcophyton* samples were placed in a cooler and transported to the laboratory in an insulated cooler.

In the laboratory, each colony was placed upside-down in a glass beaker for three hours to collect the mucus produced by the colony. The resulting exudate was then frozen and freeze-dried along with 50ml of the seawater samples. The freeze-dried material was kept at -20°C and was extracted three times with 25ml DCM (1h: 1h: 12h). The seawater and mucus extracts were dried with a rotary evaporator, transferred to preweighed vials using the extraction solvent and dried using a Savant Speedvac. The extracts were then weighed and diluted with hexanes to 1mg/ml in preparation for GC/MS analysis.

The colonies were then subsampled for protein and chemical analysis (fig. 2). Polyp anthocodia, the portion of the polyp, which can be extended above the surface of the colony, were collected haphazardly from the surface of a colony and placed into, preweighed vials. One hundred polyp anthocodia (hereafter referred to as anthocodia) were collected for chemical analysis and 25 anthocodia were collected for protein analysis from each colony. In each colony, all other samples for chemical and protein analysis were taken directly adjacent to each other. Roughly 10mm^2 and 1mm thick

sections of surface tissue were cut from the capitulum with a scalpel. All polyp anthocodia and as much coenenchyme as possible was removed from the sections and the remaining tissue was placed into preweighed vials. Approximately 0.75cm^3 sections of the underlying capitulum coenenchyme were sampled after being cleaned of anthocodia. This process was repeated with the stalk directly above the biofouled basal portion.

All samples for secondary metabolite analysis were then freeze dried and extracted three times with 2ml 1:1 DCM: MeOH (1h: 1h: 12h) at -20°C. The extracts were dried in preweighed vials with a Speed Vac, weighed, and then dissolved in 1mg/ml naphthalene dissolved in hexanes to produce a final extract concentration of 1 mg/ml. A series of standards consisting of isosarcophytoxide B dissolved in 1mg/ml naphthalene: hexanes were prepared. The standards were serially diluted with the naphthalene: hexanes solution to produce concentrations of isosarcophytoxide B at 1, 0.5, 0.25, 0.125, and 0.625 mg/ml.

The extracts were analyzed with a Hewlett Packard 5890 Series 2 gas chromatograph equipped with a Hewlett Packard 5972 mass spectrometer (GCMS) to locate and quantify the concentrations of isosarcophytoxide B. The GCMS run was started with an oven temperature of 250ºC and increased to 300ºC in 3.5 minutes. Helium was used as a carrier gas at 8psi with a flow rate of 1.0 ml/minute. Samples were left at room temperature during the run. Sample ratios were converted into percent isosarcophytoxide B using the regression equation for the standard curve.

A Biorad Bradford assay kit was used for protein analysis. Samples were digested in 1N NaOH for 24 hrs. The kit's lyophilized bovine serum albumin standard was prepared with NaOH to produce a 1N NaOH solution. Diluting the stock standard with 1N NaOH produced a series of standards. Samples and standards were incubated with the kit's dye reagent for 20 minutes. Absorbance was read at 595um on a Spectronic 21D spectrophotometer. The spectrophotometer was blanked with distilled

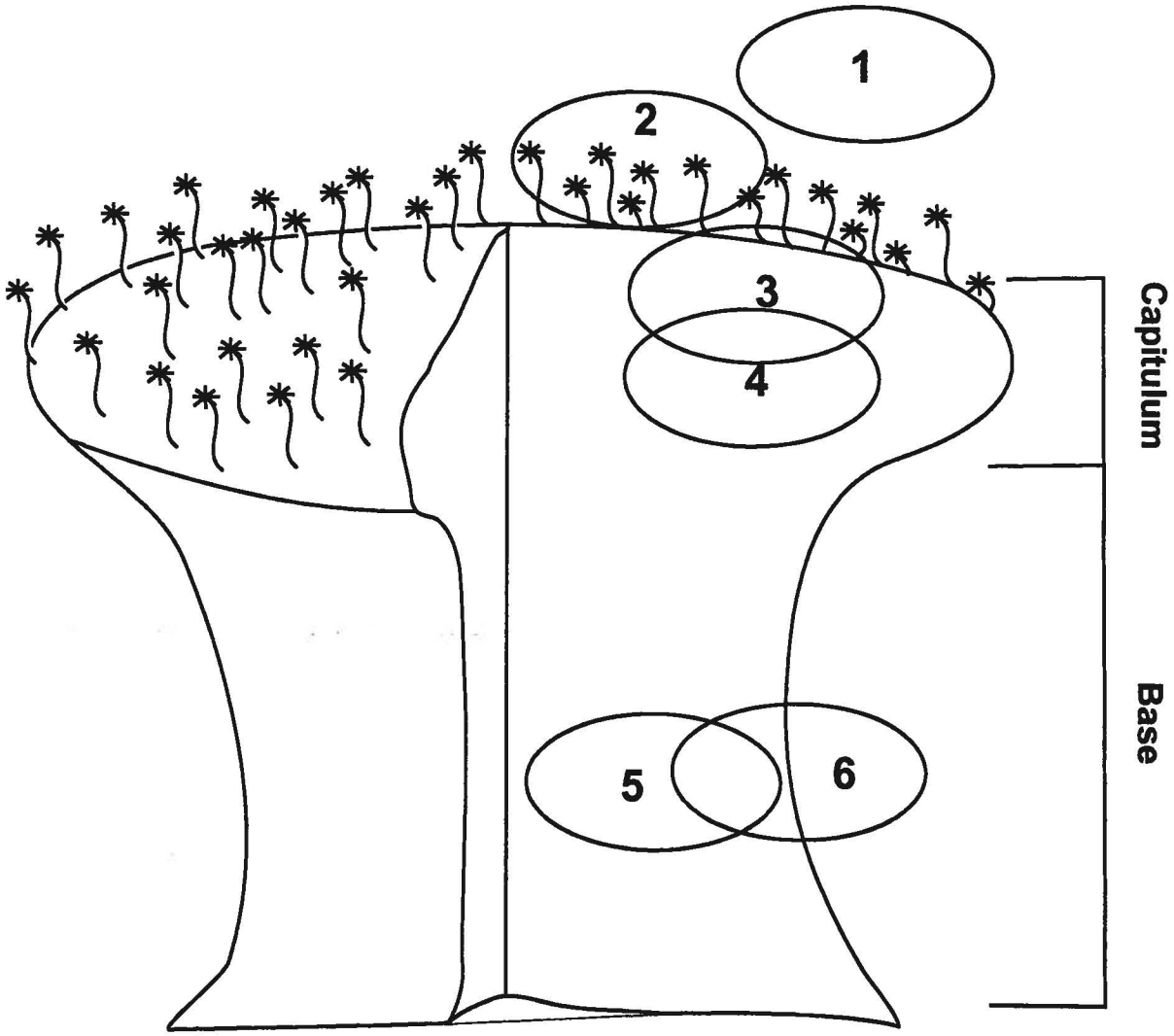

Figure 2. Regions in a *Sarcophtyton trocheliophorum* colony sampled for intracolony variation: 1- mucus, 2-polyp anthocodia, 3- capitulum surface, 4- capitulum interior, 5- basal interior, and 6- basal surface.

water. Percent protein was determined by converting absorbance readings using the regression equation from the standard curve.

Egg, Larval, and Primary Polyp Chemical Variation

During the July 1995 *Sarcophyton* spawn, eggs from several actively spawning female colonies were collected. The colonies selected were separated by at least 5m to reduce the likelihood of sampling the same clone. The colonies were enclosed in clear 10-gallon polyethylene garbage bags that were secured with elastic bands fastened around the base of each colony. The bags were collected after approximately one hour and sealed. Sperm collected incidentally with the eggs was sufficient to provide high (~80% fertilization (pers. obs.) The collected broods of eggs were then placed in coolers and transported to the University of Guam Marine Laboratory. Seven broods were kept separate in plastic containers (50x35x25cm). The number of eggs in each brood was not quantified, but numbered in the thousands. Each brood was sampled once a day for three days during the initial development from egg to larva. Approximately 1 ml of eggs or larvae was collected for each sample. As development presumably slowed after the arrival at the larval stage, a fourth sample was not taken until a week later. By this time, most larvae had died and surviving broods had to be pooled for sampling. The remaining larvae were placed together in a new container. After another week, approximately 30 metamorphosed primary polyp stage corals from the pooled batch were sampled. Samples were then frozen, freeze-dried and analyzed by GCMS using the procedures previously described.

Eggs size

Five haphazardly selected adult colonies were sampled per month from March to September of 1996. This period included the expected spawning dates of 9-11 days after the full moon in June and July and was intended to allow for possible variation in

spawning date. A 1cm wide section was cut from the center to the edge and down to the base of each colony and brought back to the lab in seawater. The sex of each colony was noted in the field. If eggs were noticeable in the sampled colony in the field, two additional sections were cut from the colony at angles of approximately 120º from the initial section around the vertical colony axis. The strips from each colony were placed together in a water-filled ziploc bag. The samples were then placed in a cooler and brought back to the laboratory.

The eggs from these strips were removed from the adults in the laboratory. All the eggs were removed from five, haphazardly selected, exposed, intact polyps from each strip using fine forceps under a dissecting microscope. Egg diameters were measured using a calibrated ocular micrometer under a compound microscope. The number of eggs per polyp were counted and averaged for each coral. The color of maturing eggs in their second year of development was noted as well. Immature eggs in their first year of development were counted when seen but not included in total egg counts.

Results

Secondary metabolite isolation

Bioassay guided fractionation of the crude organic extract using *Canthigaster solandri* identified five factions as initially deterrent. Two of these lost their antifeedant effect after further fractionation (Fractions 1,4, Fig. 1). Of the remaining three deterrent fractions ($p<0.05$), two proved to be composed primarily of isosarcophytoxide B (Fig. 1). Another fraction (fraction 6-1) was deterrent ($p<0.05$), but pure deterrent compounds were not isolated from this fraction. The complex mixture of compounds in this fraction did not contain isosarcophytoxide B.

Feeding Assays

Chemical Defenses

Laboratory assays using the average whole colony concentration of extract (9%) showed variable results, deterring feeding by *C. sordidus* ($p<0.001$) and *C. solandri* ($p<0.001$), stimulating *C. melannotus* ($p<0.05$), and not affecting *D. aruanus* (Fig. 3).

Sclerites

In *Sarcophyton trocheliophorum,* sclerites are present in lower concentrations in the capitulum (35% of dry mass) relative to the base of the stalk (75% of dry mass), which is almost a solid mass of sclerites. The average natural concentration of base sclerites deterred feeding by *C. solandri* in the laboratory ($p<0.05$) but not C. sordidus; C. melannotus was not significantly deterred, but had a tendency to avoid test food ($p=0.073$)(Fig. 4A). Sclerites isolated from the capitulum tested at the natural average concentration (35% of dry mass) did not deter any of the species tested (Fig. 4B). A comparison of basal versus capitulum sclerites at natural average concentrations

showed the basal sclerites to be more deterrent for all species tested ($p<0.05$, Fig. 4C). In this comparative assay, base sclerites were incorporated into the artificial food mix at 75% of dry weight and capitulum sclerites were incorporated at 35% of dry weight.

Combinations of Sclerites and Extracts

Two sets of three-way comparisons, one conducted in the field at Piti Bay and one run in the laboratory, tested the antifeedant effects of capitulum extracts (tested at 5% of dry weight), base extracts (tested at 76% of dry weight), capitulum sclerites (tested at 44% of dry weight), and base sclerites (tested at 77% of dry weight).

In three way comparisons testing sclerites and extracts at natural average concentrations, results were consistent among four trials. Capitulum extract was significantly more deterrent than capitulum sclerites ($p<0.001$,Fig. 5A) or control food ($p<0.001$, Fig. 5A, 5D). Base sclerites were more deterrent than either base extract ($p<0.005$, Fig. 5B).or control food ($p<0.001$, Fig. 5B). Base sclerites were also more deterrent than either capitulum sclerites (Fig. 5C, $p<0.005$) or control food ($p<0.001$, Fig. 5C). Capitulum extract was more deterrent than or base extract ($p<0.005$, Fig. 5D) or control food ($p<0.001$, Fig. 5D).

In the laboratory, three-way assays using *Canthigaster solandri* found that capitulum and base extracts were more deterrent than control food ($p<0.005$, Fig. 6A) and capitulum extract was more deterrent than base extract ($p<0.05$, Fig. 6A). No difference was observed between capitulum sclerites, base sclerites, or control food (Fig 6B). Extract of the capitulum was significantly more deterrent than capitulum sclerites or control food ($p<0.005$, Fig. 6C). In the same assay there was no difference between capitulum sclerites and control food (Fig. 6C). The fishes consumed more base extract mix and base sclerite mix than control food ($p<0.005$, Fig. 6D). The same assay showed base extracts to be more deterrent than base sclerites ($p=0.025$, Fig. 6D).

Eggs

Of eight *Canthigaster solandri* offered *Pocillopora damicornis* larvae or *Sarcophyton trocheliophorum* eggs, seven consumed the larvae while all but two rejected the eggs. The results of a Fisher's exact test showed that the eggs were significantly less palatable ($p=0.04$) than *P. damicornis* larvae.

Variation

Intracolony Protein, Extract, and Sclerite Variation

Sclerites and crude organic extracts showed an inverse relationship from top to bottom of *Sarcophyton* colonies. Organic extracts averaged 80% of dry mass in the polyp anthocodia while sclerites comprised an average of 20% of the dry mass (Fig. 7). Sclerites accounted for an average of 75% of the dry mass in the base, and extract was less than 10% (Fig. 7). While protein levels were relatively high in the polyp anthocodia compared to the rest of the colony, levels in upper or lower parts of the colonies were fairly consistent at around 15%-20% of colony dry mass (Fig. 7). As the organic extract includes lipids and proteins as well as secondary metabolites, the totaled values of protein, sclerites and extract exceed 100%.

The trend in isosarcophytoxide B levels mirrored those of the organic extract. In the anthocodia, a maximum of 18% isosarcophytoxide B was found. The compound averaged only 3% of dry mass as most samples did not contain detectable levels of the compound (Table1). Half of the sampled colonies had isosarcophytoxide B in the capitulum with an overall average concentration of 2% (Table1). The capitulum interior and base exterior contained less than 1% of the compound and the interior of the base averaged 0.1 % (Table1). In two samples of *Sarcophyton* mucus, isosarcophytoxide B was detected but not quantified (Table1). The compound was not detected in three seawater controls (Table 1).

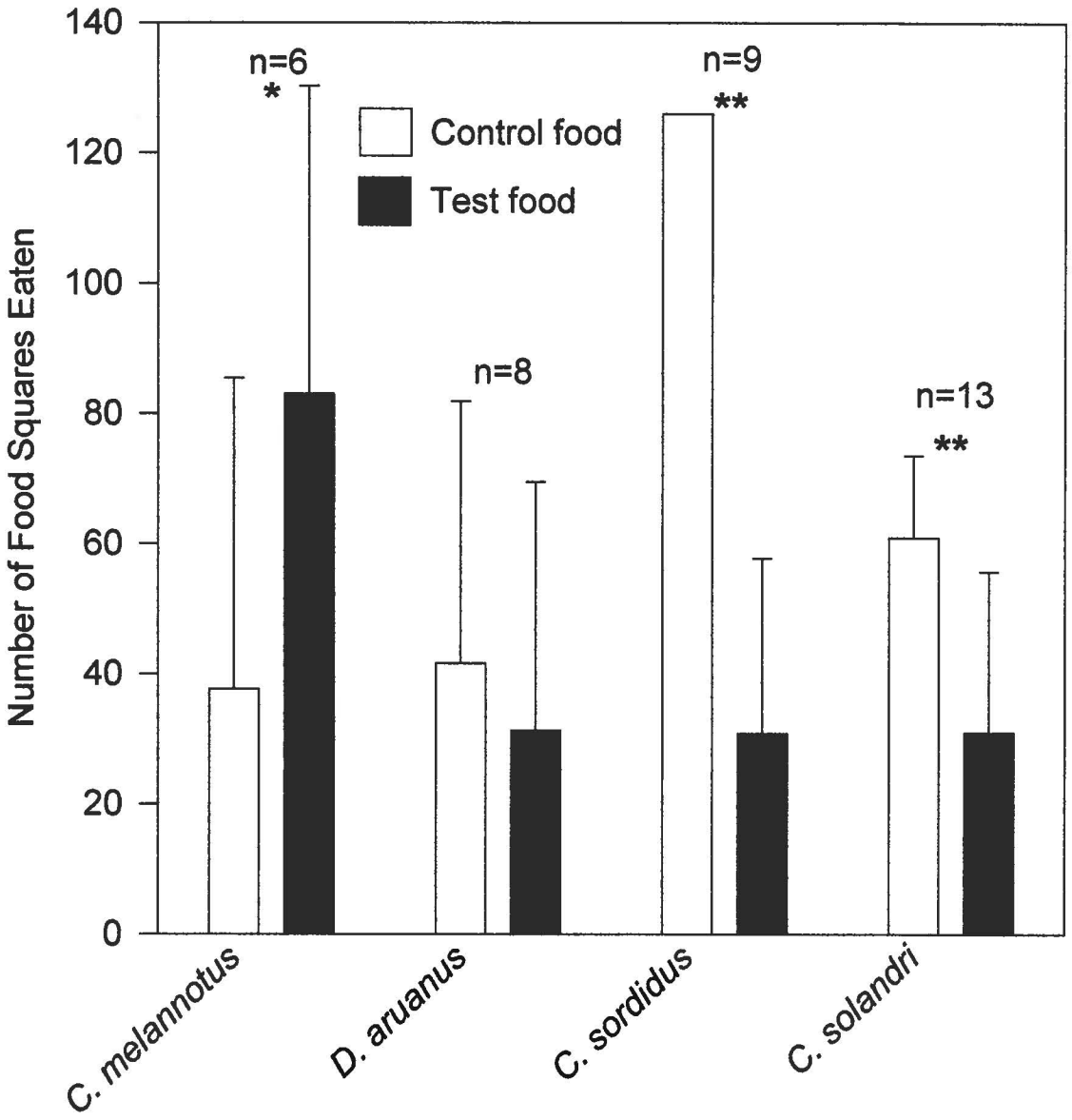

Figure 3. Laboratory feeding assays testing organic extract of *Sarcophyton trocheliophorum* against different species of consumers. Extract was tested at a 9% concentration. Analyses were made with paired t-tests (*$p<0.05$, **$p<0.001$). Error bars represent one standard deviation.

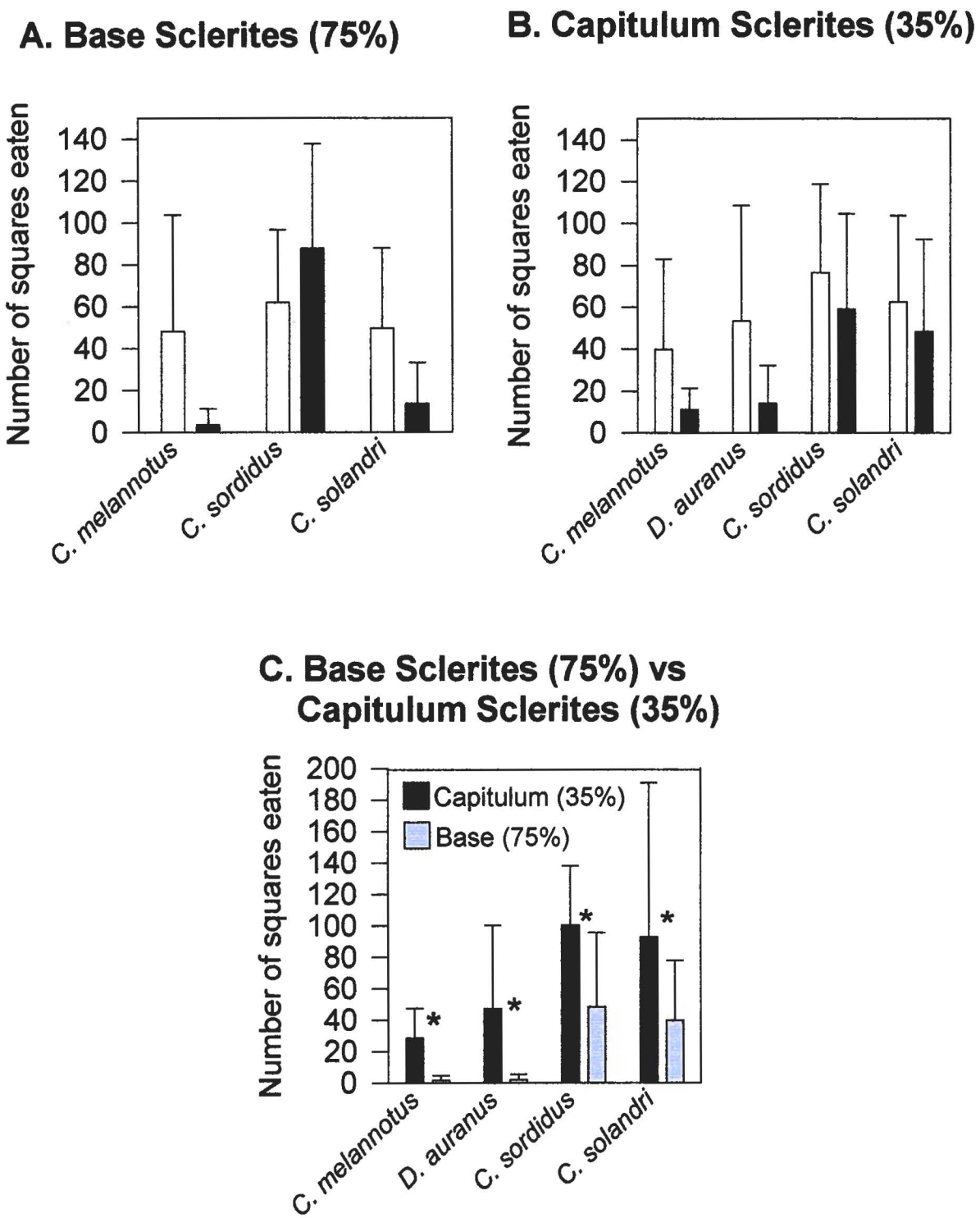

Figure 4. Sclerite feeding assays conducted in laboratory aquaria. Error bars represent one standard deviation. White bars represent control food and black or gray bars indicate test food. Significant differences determined by paired t-tests are indicated by * ($p<0.05$).

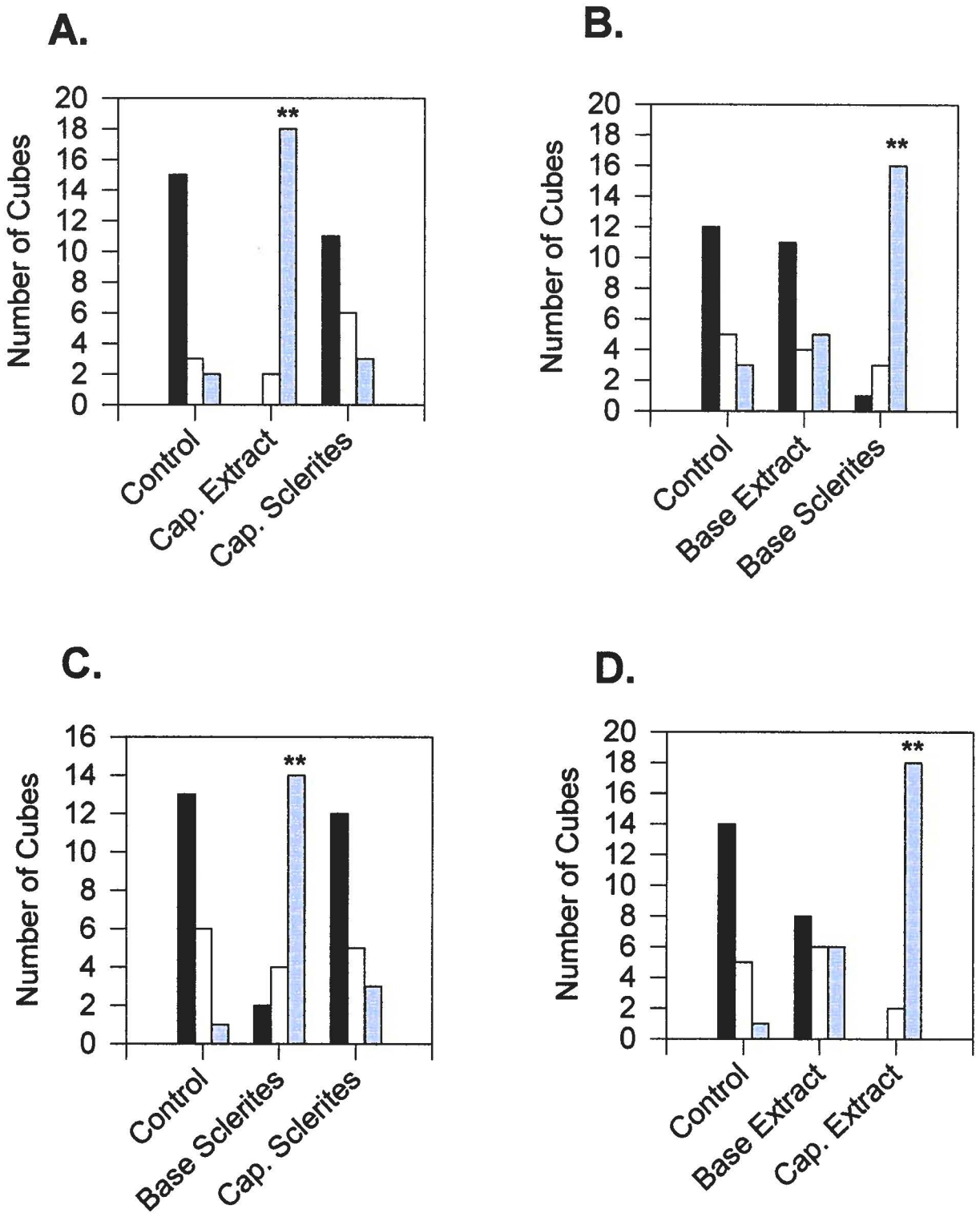

Figure 5. Piti Bay field assays. Grey bars represent rejected cubes, white bars represent partially eaten cubes, and black bars represent completely eaten cubes. Treatments signifcantly different from controls indicated by ** ($p<0.001$) as determined by G test analysis.

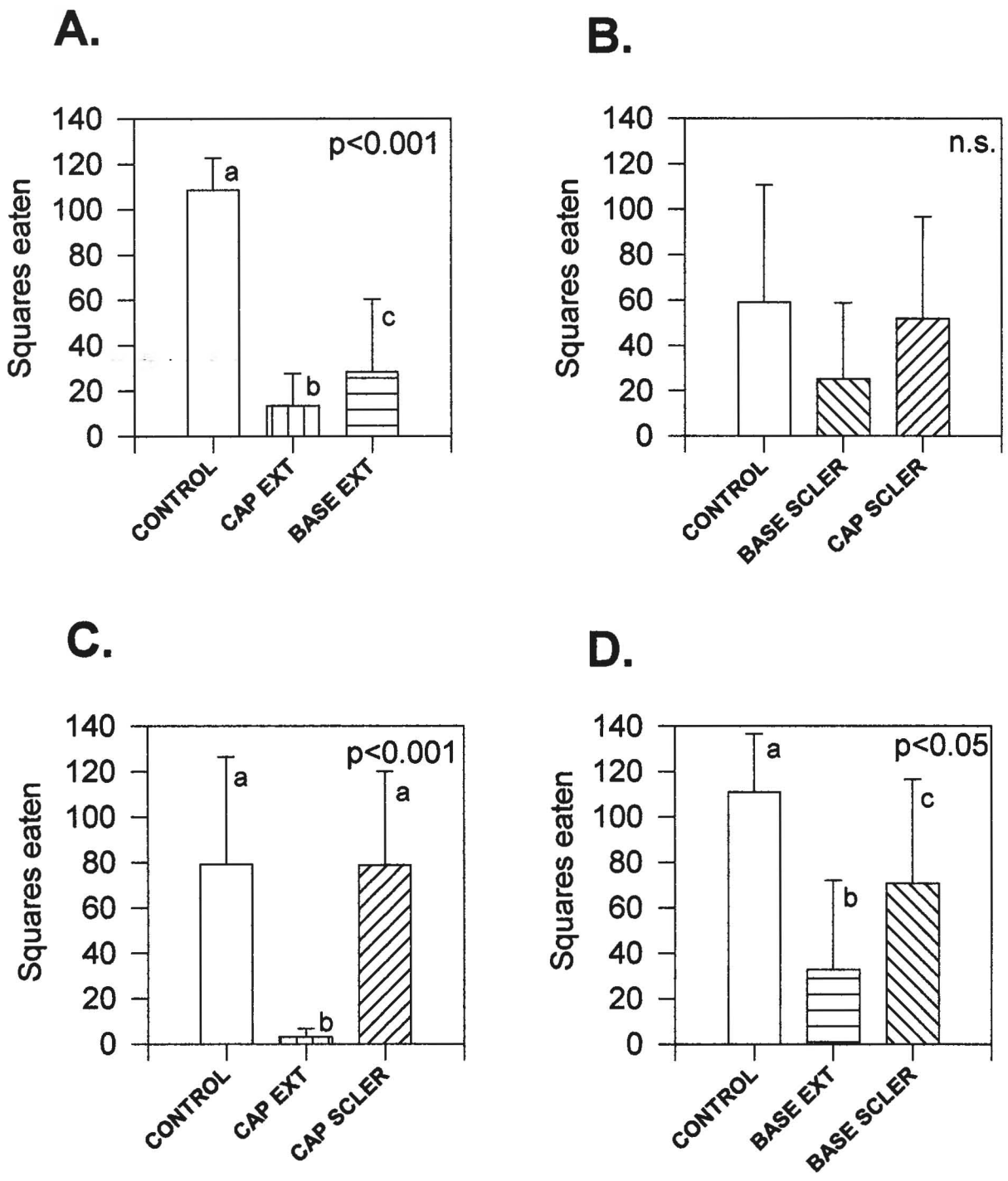

Figure 6. *Canthigaster solandri* feeding assays comparing preferences for capitulum and base sclerites and extracts. Significance values based on Friedman's tests. Post hoc paired comparisons were conducted with the Wilcoxon test for paired comparisons. Error bars represent on standard deviation.

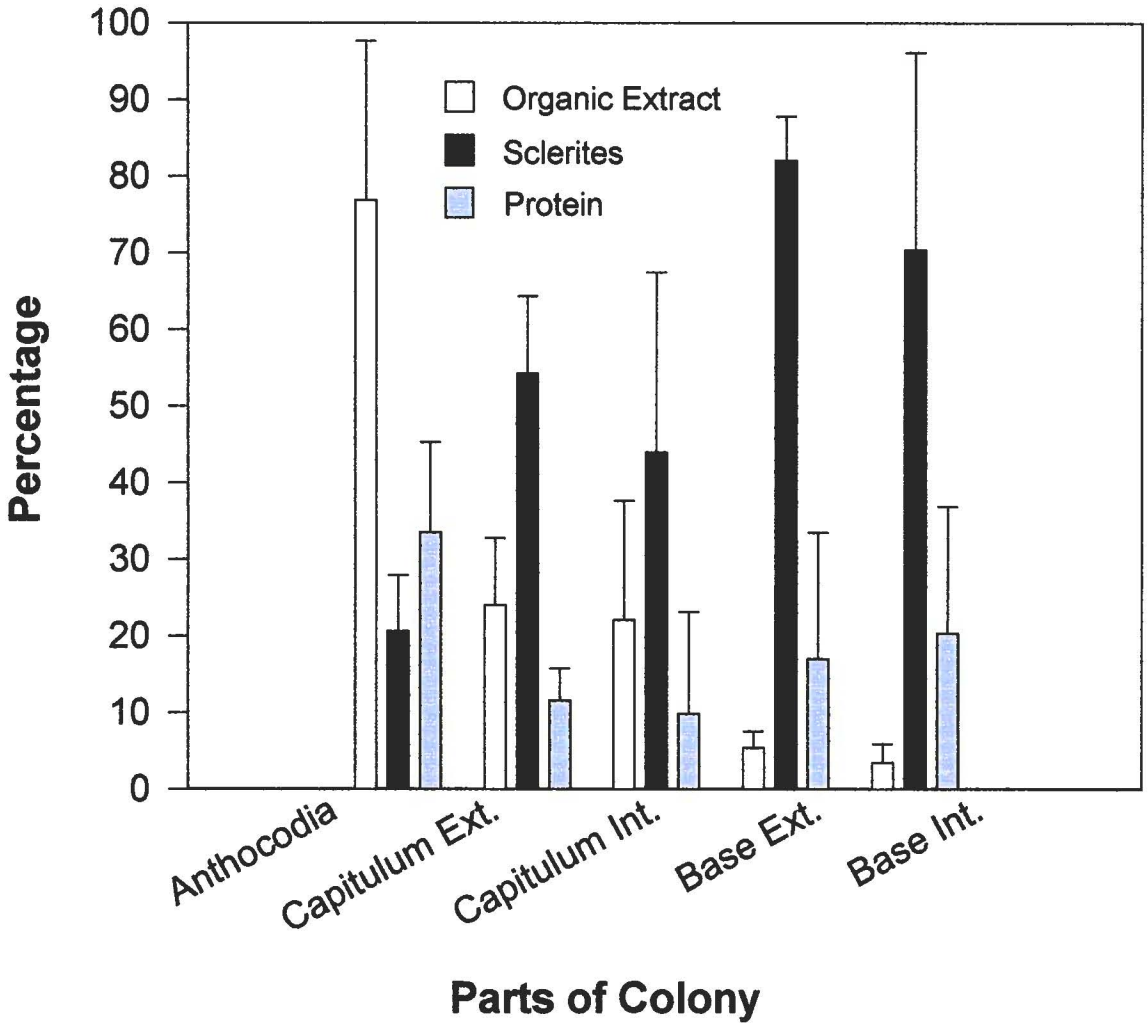

Figure 7. Relative concentrations of sclerites,organic extract, and protein in the freeze dried tissue of *Sarcophyton trocheliophorum*. See figure 1 for location of sampling sections. (Int.= interior, Ext.= exterior). Error bars represent one standard deviation.

Egg, Larval, and Primary Polyp Chemical Variation

After the July, 1995 spawning, several broods from seven female colonies were sampled for chemical analysis. After initially sampling the developing eggs and larvae daily, high larval mortality made it necessary to pool subsequent samples. Isosarcophytoxide B was detected in three of seven broods analyzed (Table 2). In broods one, four, and five, the compound disappeared during development and a pooled larval sample and pooled primary polyps did not contain detectable amounts of isosarcophytoxide B (Table 2).

Eggs

Mean egg diameter doubled from March to July 1996 and then stayed at 0.6-0.65mm from July to September (Fig. 8). Full spawning did not occur as expected during June or July, possibly due to abnormally high water temperatures resulting from that year's ENSO event. Eggs were concentrated in the capitulum and were generally found within one centimeter of the surface.

Eggs of different colors were often interspersed within single polyps with no systematic arrangement within polyps or within colonies. Sampling sections could contain polyps with all pink, all white, mixed or no eggs. Eggs generally deepen in color from a white to salmon pink color as they mature. The average monthly percentage of white eggs varied between 24% and 38 % from April to September after an initial high of 70% in March. Despite the variety of colors within polyps, the color of spawned eggs was generally uniform within a single colony. There were noticeable differences in the intensity of pink coloration among colonies.

Immature white eggs were first noted in May and ranged in size from 0.09 to 0.25 mm. The average size of these eggs gradually increased from 0.17 to 0.20 mm from May to September (Fig. 7). These eggs differed from the more mature eggs in being translucent rather than opaque, and their color remained consistent.

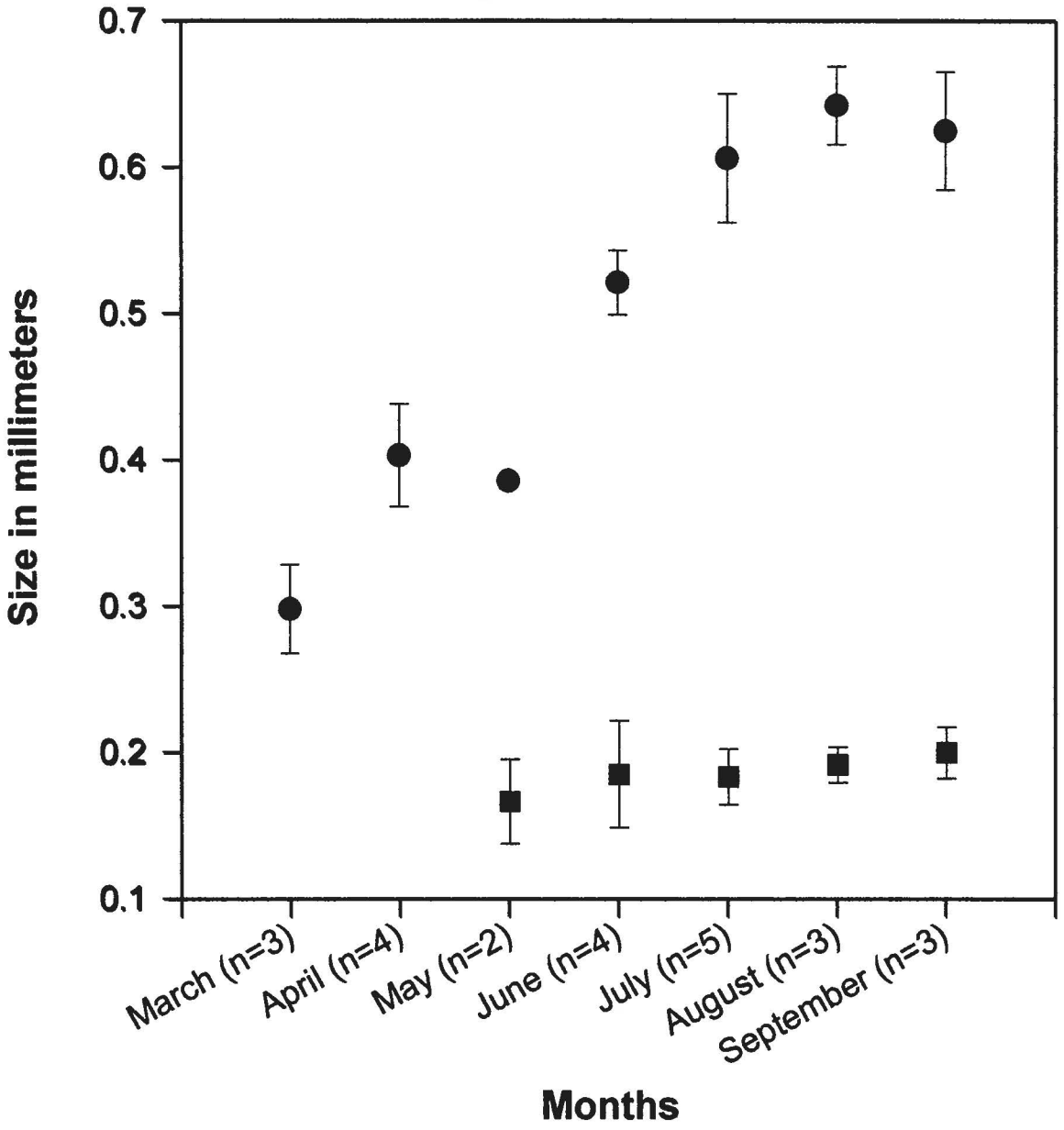

Figure 8. Mean colony egg size for summer 1996. Maturing eggs represented by circles, immature eggs by squares. n= umber of female colonies sampled for each month. Error bars represent one standard deviation.

Spawning

Spawning was observed during three summers (Table 3). The events occurred nine to twelve days after the full moon in June and July. Spawning started around sunset and was coincident with a falling high tidal cycle. During the June spawn of 1996 only females were observed spawning. However, eggs collected on June 12 developed into larvae, which suggests that at least a few males spawned.

Spawning generally began shortly prior to sunset. In the short time before darkness, several fish species were noted consuming eggs. *Amblyglyphidodon curacao*, *Chaetodon unimaculatus* Bloch, 1787, *Chaetodon lineolatus* Cuvier, 1831, and *Chaetodon melannotus* could all be found in mixed groups above spawning female colonies.

Laboratory Observations

In the laboratory, eggs were positively buoyant and covered the surface of static water containers in which they were kept. By the second day after spawning the eggs were irregular in shape and sank into the water column. Larvae developed by the third day after spawning and actively moved through the water column. Sizes varied from 1000x500um to 1600x550um. Actual dimensions varied as larvae elongated and contracted. Larvae began to settle within 7 days of being spawned, but some were kept alive in small containers for one month.

Table 1. Percent dry weight of isosarcophytoxide B in the sampled parts of ten *Sarcophyton trocheliophorum* colonies and three seawater controls.

Colony	Anthocodia	Capitulum Exterior	Capitulum Interior	Base Exterior	Base Interior	Mucus	Sea Water
1	8.13	1.04	1.65	0.21	0.19	0	0
2	0	3.73	0	0.99	0.24	present	0
3	1.34	6.93	0	1.47	0.49	present	0
4	0	6.52	0.95	1.28	No data	0	
5	18.14	3.29	0	0	0	0	
6	0	No data	0	0	0	0	
7	0	0	0	0	0	0	
8	0	0	0	0	0	0	
9	0	0	0	0	0	0	
10	0	0	0	0	0	0	
Average	2.76	2.15	0.26	0.40	0.09	N/A	0
Std. Dev.	5.97	2.79	0.57	0.60	0.17	N/A	0

Table 2. Presence or absence of isosarcophytoxide B in eggs, larvae and juveniles of *Sarcophyton trocheliophorum* spawned on 22 July, 1995. Isosarcophytoxide B is listed as percent dry weight. Dashes indicate no data. Broods were collected from separate individual female colonies except for number 8, which contains offspring of several colonies.

Brood Number	22-Jul-95 eggs	23-Jul-95 embryos	24-Jul-95 larvae	29-Jul-95 larvae	4-Aug-95 polyps
1	5.6	0	-	-	-
2	-	-	0.06	-	-
3	0	0	0	-	-
4	0.28	0	-	-	-
5	0.02	0	-	-	-
6	0	0	-	-	-
7	0	0	-	-	-
8	-	-	-	0	0

Table 3. Spawning dates at Piti Bay, Guam.

Year	Month	Days after full moon	Spawning activity
1995	June	not known	Spawn noted (VP pers. comm.)
	July	9	Strong (7:30PM)
		10	Strong (6:45PM)
		11	None (no eggs in colonies)
1996	June	9	Single female (8:30 PM)
		10	Weak (Few females)
		11	Weak
		12	Weak
	July	9	Weak
		10	Weak (Few colonies; 8:30 PM)
1998	July	8	Weak (Single male; 7:30 PM)
		9	Moderate
		10	Moderate
		11	No spawning (11:00 PM)

Discussion

The chemical and structural defenses found in *Sarcophyton trocheliophorum* show variation in their effectiveness to deter predation by sympatric reef fishes on Guam. Neither type of defense alone was completely effective at deterring all fishes tested in feeding assays. In combination, however, these defenses were able to deter all the fishes tested. Indeed, the results of this study add to the growing evidence for the presence of chemical and structural defense in exposed, sessile invertebrates on coral reefs (Stoeker 1980, Pawlik *et al.* 1987, 1995, Van Alstyne *et al.* 1994, McClintock *et al.* 1997).

The third initial hypothesis, that generalist (polyphagous) predators are more likely to be deterred by these secondary metabolites than soft coral-specialist (oligophagous, monophagous) predators was strongly supported. Whole colony organic extracts proved to be deterrent to generalist fishes tested in the field and to *Chlorurus sordidus* and *Canthigaster solandri* when tested in the laboratory. Van Alsytne *et al.* (1994) had similarly found the extracts of *Sinularia* species to deter feeding by natural assemblages of reef fishes on Guam.

Contrary to the above results, feeding assays with *Chaetodon melannotus*, a soft coral predator showed average colony extract level to be a feeding stimulant rather than a deterrent. As *C. melannotus* is a soft coral predator, this is possibly to be expected. However, these findings are in contrast to those of Wylie and Paul (1989) who found another soft coral predator, *Chaetodon unimaculatus*, to be unaffected by natural average extract concentrations in *Sinularia* species. In their case, extracts were deterrent at 2-4x levels; however, an attractant effect was not noted (Wylie and Paul 1989).

Interestingly, *Dascyllus aruanus* was not deterred by average, whole colony extract levels either. This fish is generally considered a planktivore which may

occasionally benthic prey (Myers, 1999) and would not be expected to be a preadapted soft coral predator. However, this result may not be completely without precedent as *D. aruanus* and other pomacentrids and have been observed consuming soft coral eggs during mass spawning events (Slattery *et al.* 1999). Tursch (1982) also found another species of damselfish, *Abudefduf leucogaster*, was more resistant to *Litophyton* extracts in toxicity tests than other sympatric species. While this study isolated the major deterrent compound isosarcophytoxide B from *S. trocheliophorum* extract, at least one other deterrent compound exists in the species. The mixture of compounds in fraction 6-1 is likely to contain at least one deterrent compound. Additional deterrent compounds may also exist in *S. trocheliophorum.* The loss of deterrent effects in fraction 1 and 4 (Fig. 1) suggests that some compounds may be deterrent in combination and lose their effectiveness once purified.

Feeding assays comparing base and capitulum sclerites showed results consistent with those of Van Alstyne *et al.* (1992) for *Sinularia* species. Capitulum ("tip") concentrations were less deterrent than base concentrations. While this may be explained by the difference in concentration of the sclerites, the differing morphology of the sclerites from the two regions may also have an effect. Capitulum sclerites are smaller and have less pronounced projections than base sclerites (pers. obs.). These projections may make the food containing base sclerites harder to tear or chew as the projections interlock with each other. It is unlikely that the sclerites are injurious to predators due to their relatively small size.

The results of the feeding assays with *Chlorurus sordidus* complement those of Schupp and Paul (1994) in which *Chlorurus [Scarus] sordidus* was attracted to food with added aragonite and deterred by secondary metabolites from *Halimeda macroloba.* Similarly, C. *sordidus* was not deterred by capitulum sclerites or base sclerites from the soft coral (Fig. 4 A, B) and *Sarcophyton* extract was deterrent (Fig. 3).

When testing the base sclerites, the parrotfishes actually consumed more of the test food than the control, however the difference was not statistically significant.

Sclerite and extract concentration in *S. trocheliophorum* support the initial hypothesis of this study that levels of secondary metabolites and organic extracts vary inversely with the concentration of sclerites among parts of the colonies. These results generally match previous findings in *Sinularia* species (Wylie and Paul 1989, Van Alstyne *et al.* 1992, 1994)(Figure 7). However, the finer scale of sampling within Sarcophyton colonies showed an additional gradient in isosarcophytoxide B which was higher in the surface tissue compared to the coenenchyme (Table 1). As a result of both chemical gradients, the more exposed portions of the soft coral have the highest levels of chemical defense.

Secondary metabolites and sclerites in *S. trocheliophorum do* reduce predation on adult soft corals as initially hypothesized. As previously noted, specialized predators that have presumably evolved to cope with specific types of defenses may still be affected by another type of defense. While I did not investigate the possibility of synergistic effects between chemistry and sclerites in this species of soft coral, the sclerites present in the capitulum may still offer some measure of added protection despite not having shown specific antifeedant activity.

Variation in isosarcophytoxide B levels is somewhat perplexing. Those colonies that contained the compound showed levels consistent with a defensive chemical, with high levels in exposed portions, anthocodia and capitulum surface and very low levels in the interior of the base (Table1). However, half of the colonies did not have detectable levels of the compound (Table 1). One possible explanation could be that isosarcophytoxide B is an inducible defensive compound and those colonies which contained the compound were producing it in response to predation.

As some of the fishes tested were either undeterred or even attracted by either chemistry or sclerites of *S. trocheliophorum* alone, combining defenses is likely to

reduce the animal's overall attractiveness to any single predator species. Schupp and Paul (1994) noted the feeding stimulant effects of aragonite on *Scarus [Chlorurus] sordidus* was eliminated by the addition of naturally occurring levels of diterpenoid chemistry from the alga *Halimeda macroloba*. A similar combination of defenses may improve the overall effectiveness of resistance to predation by *S. trocheliophorum*.

Chaetodon melannotus on Guam have been observed to feed on *S. trocheliophorum* by removing a few polyp anthocodia and then moving to another colony (pers. obs.). While capitulum sclerites were not deterrent in laboratory assays, they may reduce the level of feeding preference in *C. melannotus* enough to shift the fishes foraging to the abundant anthocodia rather than the main part of the colony. Aliño *et. al* (1992) noted that the *C. melannotus* in Australia did take bites from the coenenchyme of the soft corals they tested, though *Sarcophyton* was not included in their study. Aliño *et. al* (1992) only observed *C. melannotus* feeding from the surface of the stalk in the field and hypothesized mucus feeding as an explanation. Certainly further study will need to be undertaken before any generalizations can be made about the feeding preferences of this species.

A number of examples of deterrent invertebrate larvae have been documented (Young and Bingham 1987, Harvell *et al.* 1996, Lindquist and Hay 1996, McClintock and Baker 1997). In this study, *Sarcophyton trocheliophorum* eggs proved to be unpalatable to *Canthigaster solandri* in a laboratory assay in comparison to *Pocillopora damicornis* larvae. Slattery *et al.* (1999) had previously found that *Sinularia polydactyla* eggs were deterrent to *C. solandri.* However, *S. polydactyla* larvae contain different deterrent terpenes, 11B -acetoxypukalide and pukalide.

Despite being deterrent in the above laboratory assay, large quantities of eggs were observed being consumed by chaetodontids and pomacentrids during a spawning event in 1995 (pers. obs.). Predation on octocoral eggs during mass spawning events has been previously noted on the Great Barrier Reef and on Guam (Aliño and Coll

1989, Coll *et al.* 1990, Slattery *et al.* 1999). In these cases terpenes in the eggs do not appear to prevent predation on the eggs. However, the fishes feeding on the eggs seem to be restricted to Chaetodontids and Pomacentrids, which have a greater tolerance for soft coral secondary metabolites. The deterrent effect of isosarcophytoxide B may work on more selective predators.

The location of the developing eggs near the surface of the colonies may suggest another function for isosarcophytoxide B. Terpenes in the eggs may augment the colony's defense while they are present to counteract the presumed increase in nutritive value from the eggs while they are in the adult colony.

Isosarcophytoxide B may also have a relationship to the actual spawning event. Pass *et al.* (1989) have previously found that compound (-) sarcophytoxide caused strong contractions in *Xenia elongata* polyps and proposed that a threshold level of the metabolite may be necessary to induce spawning in those colonies producing the metabolite.

The mode of reproduction and development in *S. trocheliophorum* matches fairly closely with information reported for *Sarcophyton glaucum* (Benayahu and Loya 1986) and for *Lobophytum crassum* (Uehara *et al.* 1987), and the general information in Aliño and Coll (1989). Eggs develop over a two-year period and immature eggs can be found with mature eggs for several months prior to spawning (Fig. 8, Benayahu and Loya 1986, and Uehara *et al.* 1987). Mature and immature eggs were found intermixed in S. trocheliophorum. This is in contrast to the arrangement in *Lobophytum*, where mature eggs are found closer to the surface than immature eggs (Uehara *et al.* 1987). The colonies are single sexed and males and females spawn together shortly after dark. Egg sizes closely match these related species, around 600um (Benayahu and Loya 1986, Aliño and Coll, 1989). Larvae of *S. trocheliophorum* were larger than those reported for other species but this could simply be a species-specific difference. Larvae actively moved through the water column and could contract and elongate. A

qualitative assessment of larval settlement preference suggests that *S. trocheliophorum* larvae prefer to settle under available substrate (pers. obs.) as noted by Aliño and Coll (1989) for *Lobophytum* and *Sarcophyton* spp.

This study is the first to show differing responses to soft coral defense by several species of ecologically relevant reef fishes. While showing the general efficacy of these defenses against sympatric reef fishes, the results highlighted the ability of specialized predators to overcome these defenses. The investigation of fine scale variation in both chemical and structural defense showed an increased level of chemical defense in external versus internal tissues. This study also identified isosarcophytoxide B to be the major antifeedant compound in *S. trocheliophorum* adults, eggs and larvae.

The differences in this study from prior investigations of soft coral reproduction and chemical ecology highlight the lack of knowledge about many of the basic aspects of soft coral biology. As many of the studies cited as references were completed in Australia or the Red Sea, some of the differences found may relate to environmental or genetic differences between these regions and Guam. However, such differences suggest interesting areas for further study. The possibility of synergistic effects between sclerites and metabolites or different metabolites at different live history stages would be a productive area of investigation. The role of isosarcophytoxide B in the adult colonies and the reasons for the large variation in amount and presence or absence of the compound in different colonies is still in need of an explanation.

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