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## Localized induction of a generalized response against multiple biotic agents in Caribbean sea fans

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**Abstract** Although inducible defenses are an important component of the defense systems of a range of modular organisms, little is known about inducible defenses of octocorals. A localized inducible response against multiple enemies was detected in the Caribbean sea fans *Gorgonia ventalina* L. and *G. flabellum* L. Field surveys showed that localized purpling, resulting from an increase in the proportion of purple sclerites, was associated with overgrowth by *Millepora alcicornis* and macroalgae, and infection by the fungus *Aspergillus sydowii*. To confirm inducibility and to assess specificity of the response, a range of treatments—abrasion, cable tie, *M. alcicornis*, sea fan tissue infected with *A. sydowii* (diseased allografts), and healthy allografts—was applied to healthy sea fans. After 10 days, all biotic treatments induced a localized physical response consisting of changes in concentration, type, and color of sclerites; however, antifungal activity of non-polar chemical extracts was unaffected. The purpling response appears to be specific to biotic agents and reduces tissue damage in subsequent interactions.

**Keywords** Multiple enemies · Pathogen · Specificity of defense · Inducible resistance · Coral reefs

### Introduction

Pathogens, parasites, and herbivores have promoted the coevolution of a diverse set of host defenses (O'Brien and Evermann 1988; Linhart 1991), and, indeed, having multiple enemies may be a common feature of most organisms (e.g., Thompson 1998). Because different enemies can place conflicting demands on host defenses, how an organism should invest in defenses to maximize fitness is likely to be complicated (Linhart 1991; Sih et al. 1998; Thompson 1998; Agrawal and Karban 1999; Tollrian and Harvell 1999). For instance, the freshwater snail *Lymnaea stagnalis* expels blood when avoiding a crayfish predator; however, the blood loss reduces the proportion of hemocytes capable of phagocytosis, thus increasing susceptibility to pathogens (Rigby and Jokela 2000). In some cases, induction of defenses following an attack by one enemy can increase resistance to subsequent attacks by another. In the dock-beetle-fungus (*Rumex-Gastrophysa-Uromyces*) interaction, beetle herbivory on dock induces local and systemic antifungal resistance. However, fungal infection reduces the nutritive value of dock which in turn affects growth rate, survivorship, and fecundity of the beetles (Hatcher et al. 1994a, 1994b). Although these studies illustrate the importance of multiple enemies in shaping the evolution of host defenses, studies examining the effectiveness of inducible host defenses against multiple enemies, under simultaneous or sequential attacks, are rare.

Defending against multiple enemies is often a necessity for tropical corals, which live in an environment of high predation, competition, and an abundance of potentially pathogenic microorganisms (Vermeij 1978; Jackson and Hughes 1985; Chornesky 1989; Hixon 1997; Furhman 1999). Corals, as sessile invertebrates, cannot evade biotic interactions behaviorally and have an arsenal of chemical and physical defenses. Gorgonian octocorals, in particular, produce chemicals with antimicrobial, antifouling, predator deterrent, and allelopathic properties (Bakus 1981; Targett et al. 1983;

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Gerhart 1984; Standing et al. 1984; Pawlik et al. 1987; Harvell et al. 1988; Kim 1994; Jensen et al. 1996; Slattery 1999; Kim et al. 2000a, 2000b). Structural responses of gorgonians include induced sweeper tentacles used in competitive interactions (Sebens and Miles 1988) and sclerites that deter fish and predatory gastropods (Gerhart et al. 1988; Harvell et al. 1988; Van Alstyne and Paul 1992; Nowlis 1994; West 1997, 1998). Plasticity in these defenses has been shown for sweeper tentacles, which are induced by contact with corals, zoanthids, ascidians, and algae (Sebens and Miles 1988), and sclerite morphology in *Briarium asbestinum* and *Plexaura dichotoma*, which is induced by physical damage, wave exposure, and corallivorous gastropods (Nowlis 1994; West 1997, 1998). Although these studies highlight specific responses to specific interactions, little is known about the defensive strategy against multiple enemies.

The Caribbean sea fans, *Gorgonia ventalina* and *G. flabellum*, are subject to a range of predators and pathogens against which a variety of defenses have been documented. For instance, both sea fan sclerites and chemical extracts are known to deter feeding by fish and the predatory gastropod *Cyphoma gibbosum* (Van Alstyne and Paul 1992; Cronin et al. 1995; Slattery 1999). Sea fans form tumors consisting of gorgonin (a protein that makes up the axial skeleton) which encapsulate an *Ostreobium*-like alga (Morse et al. 1977, 1981). Chemical extracts of *G. ventalina* inhibit the pathogen *Aspergillus sydowii* and are more inhibitory adjacent to lesions, which suggests the inducibility of this chemical response (Kim et al. 2000a). In addition, aspergillosis in sea fans (the disease caused by *Aspergillus sydowii*) is characterized by purpling of tissue, gall formation, and tissue necrosis (Nagelkerken et al. 1996, 1997; Smith et al. 1998). These purpled areas result from an increase in the abundance of purple sclerites (Smith et al. 1998), are often associated with tissue necrosis, and are devoid of polyps (Kim and Harvell 2002). Thus, the purpling is associated with a suite of responses to an invading agent. In this study, we assessed the specificity of the purpling response to a series of biotic and abiotic agents, and determined the degree of localization, timing, and consequences of the purpling response in defense against subsequent attacks.

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## Methods

The field work was carried out on shallow water (< 2 m), near-shore patch reefs in San Salvador, Bahamas, and Akumal, Mexico.

### Purpling survey (Bahamas)

To assess prevalence of the purpling and to identify correlates and potential causative agents, visual surveys were carried out at three reefs in San Salvador (June 1999). At Lindsay Reef and Rocky Point, all sea fans

(*Gorgonia ventalina* and *G. flabellum*) encountered 1 m on either side of two 50-m transects were counted. The transects were haphazardly selected within sea fan beds. At Dump Reef, where most of the sea fans were located on the edge of a system of shallow patch reefs (< 1 m depth), the first 200 sea fans encountered during a swim along the edge were examined. Species identity, presence of purpling, and direct contact of suspected purpling agent were noted for each sea fan. The relationships among species, site, biotic interactors, and purpling were analyzed using a log-linear model (Sokal and Rohlf 1981).

### Short-term multiple inducer experiment (Bahamas)

The short-term response of sea fans to a range of potential purpling inducers was assessed in 15 *G. ventalina* of uniform size (approximately 50 cm tall and 30 cm wide) that were disease-free and located within a 100 m<sup>2</sup> area of Dump Reef (San Salvador, June 1999). Each sea fan received three grafts: 1. a diseased (i.e., aspergillosis) allograft (2×2 cm); 2. a healthy allograft (2×2 cm); and 3. a *Millepora alcicornis* (Cnidaria, Hydrozoa) xenograft (2×1 cm). Aspergillosis is a known inducer of purpling (Nagelkerken et al. 1996) and can be transmitted via grafts (Smith et al. 1998). A healthy allograft was used as a control for any grafting effect. *M. alcicornis* was chosen because it overgrows sea fans (Wahle 1980) and is associated with purpling (personal observation). All treatments were located 5 cm from the colony edge, equidistant from one another, and fastened using cable ties. This experiment began with 12 sea fans (0 d); three additional sea fans were added at 2 d. The experiment was terminated at 10 d (therefore the three fans added at 2 d were stopped 8 d after setup), at which time the inducer and surrounding host tissue were excised and stored at -20 °C for sclerite analysis and antifungal assays. An area of healthy tissue ~10 cm away from the graft also was collected from each fan as a control. The pieces were taken at an equal distance from the edge as there is a natural gradient of antifungal activity, with the edge regions being most active (Kim et al. 2000a).

### Long-term multiple inducer experiment (Bahamas)

Long-term responses to inducer grafts were examined over a 7-month period at Rice Bay (San Salvador, June 1998). In this experiment, both sea fan species—*Gorgonia ventalina* ( $n = 11$ ) and *G. flabellum* ( $n = 5$ )—were treated with four inducers: *M. alcicornis*, diseased allograft, cable tie, and tissue removal using a dive knife (i.e., abiotic abrasion). The two latter treatments were used to examine sea fan responses to non-biotic agents. The colonies were approximately 50 cm tall and 30 cm wide. All inducers were located 5 cm from the colony edge, equidistant from each other, and in random order. *M. alcicornis* and diseased allografts were secured onto

the sea fan with cable ties. Tissue removal was carried out by scraping off the tissue from a  $\sim 1 \text{ cm}^2$  area using a dive knife until the underlying axial skeleton was visible. The amount of purpling, tissue necrosis, and overgrowth were noted on days 2, 10, and 17, and finally after 7 months (January 1999). All sea fans were collected and the areas around the treatments excised for sclerite analysis in the same manner as described in the short-term experiment.

### Sclerite analysis

Sclerite analyses for the short-term experiment were carried out using samples adjacent to the graft (near) and 10 mm away (far). Antifungal assays were performed using a square area ( $9 \text{ cm}^2$ ) of the host tissue surrounding the area where the graft was placed. Fragments of sea fan tissue were removed using a cork borer (7.5 mm in diameter), dried overnight at  $60^\circ \text{C}$ , weighed, and then placed in  $\sim 2.6\%$  sodium hypochlorite solution (half dilution of commercial bleach) to dissolve the tissue. The remaining sclerites and axis were rinsed three times in de-ionized water and once in ethanol, dried overnight at  $60^\circ \text{C}$ , and re-weighed to determine the contribution of sclerites and axis to whole sample mass. Percentage of dry weight was used instead of absolute dry weight in order to standardize each sample. A subsample of the sclerites was mounted onto glass slides and examined with a compound microscope fitted with a CCD camera (Cohu, San Diego, CA) attached to a computer running NIH Image (v. 1.62, U.S. National Institutes of Health). For each slide, the first 50 axial sclerites completely within the field of view were categorized according to sclerite type (scaphoid or spindle) and color (purple or clear), and measured for maximum length and width (see Bayer 1961).

All statistical analyses were performed in DataDesk (v. 6.01, Data Description, Ithaca, NY). Both short- and long-term inducer experiments were analyzed with separate nested MANOVA. All variables in both experiments met assumptions of normality (Kolmogorov Normality test:  $P > 0.1$  except for % purple sclerites in the long-term experiment where  $p = 0.053$ ) and homogeneity of variances (Levene test:  $p > 0.2$  in all cases). Individual sea fans were nested within species and inducer location was nested within colony; colony was treated as a random factor. Where there were significant differences, univariate nested ANOVAs and Scheffé post hoc tests were subsequently applied. Multivariate analysis was performed first because of increased power when variables are correlated. In addition, it reduces Type I errors in subsequent univariate tests when the null hypothesis is rejected (Rencher 1995; Velleman 1997). Multiple measures of sclerite size were tried but none were significant and therefore this variable was removed from the model. Potential outliers were detected by examining residual plots of all variables and scatter plots of the principle components. One outlier

was removed from the analysis because it was two standard deviations away from the mean. There was no obvious biological reason to explain the outlier, nor did it alter the results qualitatively. For the short-term experiment, correlations between dependent variables were explored using the Pearson product-moment correlation.

### Antifungal assay

Antifungal activity of sea fan extracts from the short-term experiment was measured using a fungal growth assay (Alker et al. 2001; Dube et al. 2002). Briefly, equal-sized gorgonian samples ( $\sim 9 \text{ cm}^2$ ) surrounding the three treatments and a control area (10 cm away) were individually extracted twice overnight at  $-20^\circ \text{C}$  in dichloromethane. The extracts were dried under continuous flow of  $\text{N}_2$ , weighed, and re-suspended in acetone to a stock solution of 20 mg/ml. To perform the assay, 75  $\mu\text{l}$  of these extracts were spread evenly on PYG agar plates: 0.1% peptone, 0.1% yeast extract, 0.3% glucose, 3% Instant Ocean (Aquarium Systems, Mentor, Ohio, USA), and 0.005% tetracycline (Calbiochem, California, USA). Each extract was tested on three replicate plates. Nine control plates were also included: three plates with 75  $\mu\text{l}$  hygromycin B (a known antifungal agent, Calbiochem, California, USA) in acetone at 20 mg/ml; three plates with 75  $\mu\text{l}$  acetone; and three plates to which nothing was added. After the extracts were added, the plates were placed in a laminar flow hood for 40 min to evaporate the acetone. Each plate was then inoculated with 2  $\mu\text{l}$  of stock spore solution (see Alker et al. 2001) of *Aspergillus sydowii* (San Salvador strain) at  $2.25 \times 10^6$  spores/ml. The plates were sealed to minimize changes in humidity and incubated at  $25^\circ \text{C}$ . Using the digital video-microscopy set-up described above, average diameter (from two perpendicular measurements) of circular colonies (mm/d) were recorded at 4, 5, and 6 days after initial inoculation. Fungal growth rate was measured as the average daily change in fungal colony diameter; thus, highly active extracts are indicated by slow fungal growth rates. Inducibility of the antifungal activity was tested by comparing antifungal activities of sea fan extracts from inducer-treated control areas using a nested ANOVA (treatments within colony).

### Timing and consequences of purpling (Mexico)

To quantify the timing of the purpling response and to determine if purpling confers a benefit to the sea fan in subsequent interactions, a time series experiment using *M. alaicornis* as an inducer was performed at Half Moon Bay in Akumal, Mexico, in May 2000. In this experiment, one fragment of *M. alaicornis* was attached to a "pre-purpled" area (cause of purpling was undetermined) and another fragment to a non-purpled area on

the same sea fan ~10 cm away. As added controls, completely healthy sea fans were also treated with two *M. alvicornis* fragments attached within 10 cm of each other. Only *M. alvicornis* was used in this experiment because of its ability to induce a detectable response in sea fans (see below) and because inoculum size of *Aspergillus sydowii* in diseased allografts could not be adequately controlled in this series of paired treatments. Tissue samples were taken from sea fans before *M. alvicornis* fragments were attached to establish baseline conditions ( $n = 10$  pre-purpled, 10 healthy). An additional set of 20 pre-purpled and 20 healthy colonies were treated with *M. alvicornis* fragments; tissue samples were taken from random subsets of these sea fans at 4 days (10 pre-purpled, 9 healthy) and at 8 days (10 pre-purpled, 6 healthy). At the end of the experiment, lesions surrounding the *M. alvicornis* attachment were measured using calipers, and tissue samples were collected for sclerites analysis (see below). For each tissue sample, three sclerite preparations were examined, noting only the proportion of purple sclerites. The mean percent of purple sclerites of the three subsamples was analyzed in a two-way ANOVA (Kolmogorov normality test,  $p = 0.167$ ; Levene homogeneity of variances,  $p = 0.249$ ). The lesion data were analyzed using a three-way ANOVA, having met the assumptions of normality (Kolmogorov Normality test,  $p = 0.320$ ) and homogeneity of variances (Levene's test,  $p = 0.229$ ).

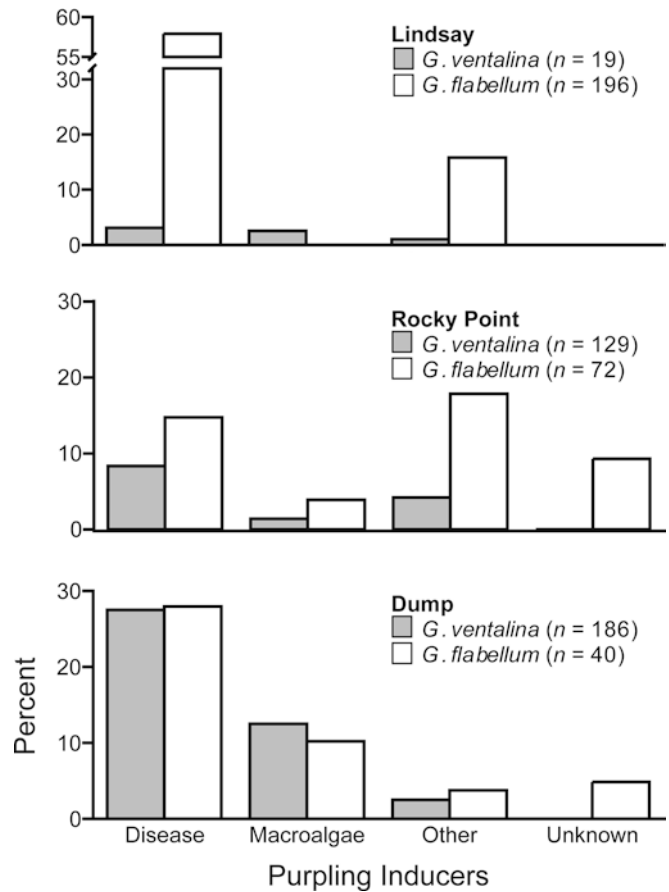
## Results

### Surveys

Purpling was associated with aspergillosis, macroalgae, abrasion against other corals and the hydrozoan *Millepora alvicornis*, and scars from predation by *Cyphoma gibbosum* (Fig. 1). While not observed in the Bahamas, sea fan purpling in the Florida Keys was also associated with the growth of hydroids and tubeworms on the colony (personal observation). Purpling was more prevalent in *Gorgonia ventalina* than in *G. flabellum* (55 vs. 21%; log-linear test:  $\chi^2 = 77.1$ ,  $df = 1$ ,  $p < 0.001$ ) and most frequently associated with aspergillosis. Frequency of purpling varied across sites, with the most purpled sea fans occurring at Dump Reef (43% of all *Gorgonia* spp. colonies; log-linear test:  $\chi^2 = 48.4$ ,  $df = 2$ ,  $p < 0.0001$ ). In addition, there were second-order interactions: site  $\times$  species ( $\chi^2 = 10.9$ ,  $df = 2$ ,  $p = 0.004$ ), site  $\times$  inducer ( $\chi^2 = 35.5$ ,  $df = 6$ ,  $p = 0.004$ ), and species  $\times$  inducer ( $\chi^2 = 10.6$ ,  $df = 3$ ,  $p = 0.014$ ). No third-order interactions were detected ( $\chi^2 = 7.26$ ,  $df = 6$ ,  $p > 0.100$ ).

### Short-term, multiple-inducer experiment

Starting date (i.e., 0 or 2 d) was not significant in any of the measured variables (split-plot ANOVA,  $p > 0.05$  in



**Fig. 1** Frequency of purpling in *Gorgonia ventalina* and *G. flabellum*. Data are from surveys of three reefs (Lindsay, Rocky Point, and Dump) in San Salvador, Bahamas, June 1999. Possible purpling inducers are categorized as: disease, macroalgae, other (*Cyphoma* feeding scars and abrasion with other corals), and unknown (no inducer apparent)

all cases) and thus was not considered in subsequent analyses.

*M. alvicornis*, healthy allografts, and diseased allografts all caused tissue necrosis and changes in sclerites in sea fans. While in most cases the tissue necrosis was limited to the immediate vicinity of the grafts, in two sea fans there were large ( $> 25 \text{ cm}^2$ ) areas of tissue necrosis, stemming from the diseased allografts. When plated onto sterile medium, small fragments of the necrotic tissue gave rise to fungal colonies identified as *Aspergillus sydowii* based on colony and spore morphology.

Sclerite analysis of the short-term experiment revealed localized responses to all three treatments when compared to untreated area of the sea fan (MANOVA, Table 1). The response consisted of increases in both axial weight and relative proportions of purple and spindle sclerites (Fig. 2). These variables differed between treatment locations within a colony indicating a very localized and multivariate response (Table 1); however, only the sclerite variables (i.e., % spindle and % purple) were correlated with each other (Table 2). In contrast, there was no significant difference in antifungal

**Table 1** MANOVA and ANOVA results for the short-term inducer experiment. Response variables are: % spindle and % purple, given as percentages of spindle and purple sclerites relative the total number of sclerites counted. Sclerite and axis weight are the percentage of the total dry weight of whole tissue sample

A. MANOVA

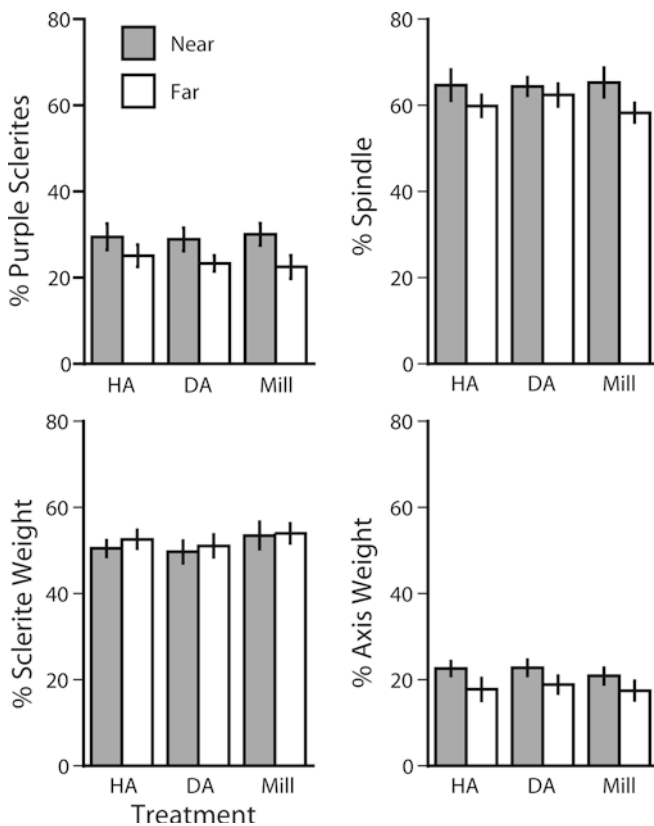
Source	Wilks $\lambda$	Apprx. F	df	$p$
Sea fans	0.001	5.32	70	$\leq 0.001$
Inducer	0.780	0.583	10	0.819
Fan x inducer	0.007	1.51	130	0.012
Location	0.039	1.42	75	0.048

B. Univariate ANOVA

Source	% Spindles	% Purple	Sclerite wt.	Axis wt.
Sea fans	$\leq 0.001$	$\leq 0.001$	$\leq 0.001$	$\leq 0.001$
Inducer	0.790	0.860	0.440	0.730
Fan x inducer	0.010	0.080	0.600	0.160
Location	$\leq 0.001$	0.010	0.130	0.040

C. Scheffe's post-hoc tests for location (near-far comparisons)

Variable	Difference	SE	$p$
% Spindle	4.73	1.87	0.016
% Purple	-5.78	1.64	0.001
Axis wt.	-4.45	1.41	0.003



**Fig. 2** Multivariate response of sea fans to short-term inducer experiment. Patterns of induction of structural (sclerites and axis) changes resulting from attachment of all three inducer agents onto each sea fan ( $n = 15$ ) after 10 days are shown. Data are for tissue samples taken adjacent to the inducer (near) and 10 mm away (far). Error bars indicate standard error of the mean. HA healthy allograft; DA disease allograft; Mill *Millepora alcicornis*

activity of extracts from treatment and control areas (nested ANOVA for treatments,  $p = 0.686$ ).

Long-term, multiple-inducer experiment

The non-biotic abrasion, caused by the use of a dive knife to scrape off sea fan tissue, completely healed over and was undetectable within 2 days so that the exact location of the initial treatment was difficult to discern. Thus, this component of the experiment was excluded in further analyses. Similarly, sea fans grew completely over the cable tie without any purpling; however, because the location of the cable tie treatment was obvious, it was included in the sclerite analysis.

Diseased allografts initially caused tissue necrosis in the sea fan and by 17 days, 94% ( $n = 16$ ) of the sea fans had purpled around the treatment. After 7 months, sea fans treated with diseased allografts had completely healed and on 81% of the sea fans, the host appeared to have grown confluent with the allograft; however, we did not perform histological examinations to fully characterize this interaction. Overall, purpling was noted in 44% of the cases. No disease symptoms were present at this time and consequently we could not discern whether the initial response to the diseased allograft was due to the pathogen or the foreign sea fan tissue.

The outcome of the *M. alcicornis*-sea fan interaction varied among replicates and over time. After 17 days, 31% of sea fans had necrotic regions associated with the *M. alcicornis* graft, 25% were partially overgrown by *M. alcicornis*, and 13% were overgrowing *M. alcicornis*. In the remaining 31% of the sea fans, no visible changes were seen on either the host or the graft. Purpling of the sea fan tissue accompanied 50% of all these treatments but was not associated with the status of the interaction ( $\chi^2 = 5.82$ ,  $df = 3$ ,  $p > 0.100$ ). After 7 months, the interaction between *G. ventalina* and *M. alcicornis* reversed: there was a decrease in the number of cases in which *M. alcicornis* was overgrowing the sea fan (31 to 25%) with a concomitant increase in the number of sea fans overgrowing *M. alcicornis* (13 to 44%). The number of "stand-offs" remained unchanged at 31%. Thus, there appeared to be no correlation between status of the interaction at 17 days and at 7 months ( $\chi^2 = 2.22$ ,  $df = 4$ ,  $p > 0.100$ ). At 7 months, areas surrounding *M. alcicornis* were purple in all sea fans examined.

There was no significant difference between the responses of *Gorgonia ventalina* and *G. flabellum* to the

**Table 2** Pearson product-moment correlation between response variables ( $n = 15$ ) in the short-term experiment. Asterisk indicates  $p < 0.05$

	% Spindle	% Purple	Sclerite wt.
% Purple	0.562*		
Sclerite wt.	-0.329	-0.167	
Axis wt.	0.464	0.290	-0.446

inducers (ANOVA, Table 3A). In general, sea fans responded differently to *M. alcicornis* than to the abiotic control (cable tie) and the diseased allograft (MANOVA,  $F = 3.3$ ,  $p = 0.003$ ; Fig. 3). *M. alcicornis* attachment resulted in an increase in the relative proportion of both purple sclerites and spindles in the sea fan (ANOVA, Table 3B). The increase in purpling was highly localized; tissue samples 10 mm away from the graft were significantly less purple.

#### Timing and consequences of purpling

The purpling response following *M. alcicornis* attachment was visible by 4 days but detectable statistically only after 8 days (two-way ANOVA:  $F = 8.81$ ,  $df = 2$ ,  $p = 0.001$ ; Fig. 4). The increase in purple sclerites was linear over time, leading to an approximately 2-fold increase in the proportion of purple sclerites over 8 days. This increase was independent of the status of the tissue (i.e., pre-purpled vs. healthy areas, ANOVA,  $F = 13.1$ ,  $p = 0.001$ ). The rate of increase in purple sclerites was also independent of initial sea fan condition (i.e., diseased or healthy; fan status  $\times$  time,  $F = 0.733$ ,  $p = 0.495$ ).

To determine if purpling affects the outcome of subsequent biotic interactions, *M. alcicornis* was placed directly onto a previously purpled area and a nearby healthy area of the same sea fan (Fig. 5). In all cases, *M. alcicornis* caused necrosis and lesions on the sea fan. However, by 8 days, lesions on healthy areas were larger than lesions on purpled areas of the same sea fan (three-way ANOVA,  $F = 9.21$ ,  $df = 1$ ,  $p = 0.014$ ). Lesion size on healthy areas did not differ, regardless of whether they were adjacent to a previously purpled area or on a sea fan colony devoid of any areas of purpling ( $F = 2.88$ ,  $df = 1$ ,  $p > 0.100$ ).

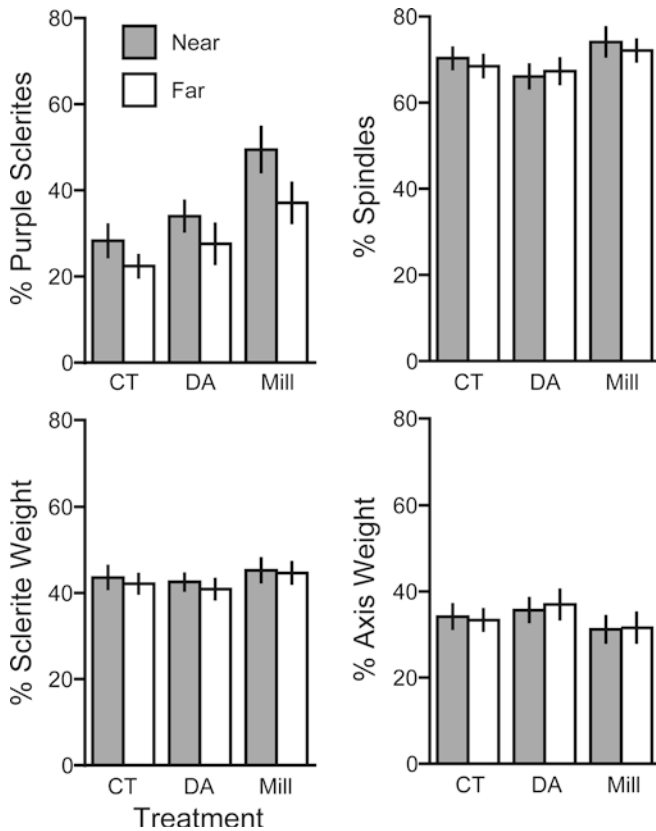
## Discussion

Purpling in sea fan corals, which results from an increase in the relative concentration of purple sclerites embedded in the tissue, appears to be part of a general and inducible response to biotic agents. We found that *Millepora alcicornis* and sea fan allografts induced purpling whereas abrasion and attachment of a cable tie did not. In addition, this response was rapid (Fig. 4) and highly localized to the area of contact. Indeed, purpling was significantly reduced only 10 mm away from the contact area (Fig. 2). Field surveys also showed that sea fan purpling was associated with aspergillosis and contact with macroalgae and other benthic species (Fig. 1). The results of this study are consistent with observations of purpling in the gorgonian *Plexaura kuna* in reaction to allografts (Lasker and Coffroth 1985) and *Plexaura flexuosa* in response to abrasion by adjacent corals and to feeding by the polychaete *Hermodice carunculata* (personal observation). Our findings of rapid induction, specificity of response, and, most importantly, increased resistance of purpled areas to subsequent interactions point to purpling as an inducible defense against biotic agents.

An important parameter in evolutionary models of inducible responses is the time course of the response. Padilla and Adolph (1996) suggested that long lag times in response might be a constraint against production of inducible defenses. However, the sea fan response is relatively quick. Changes in sclerite color were noticeable as early as 4 days, and significant increases in purpling were detected by 8 days. This rate is comparable to the induction of spines and stolons in a marine bryozoan (Harvell 1999) and is faster than the 2 weeks required for production of sweeper tentacles in corals

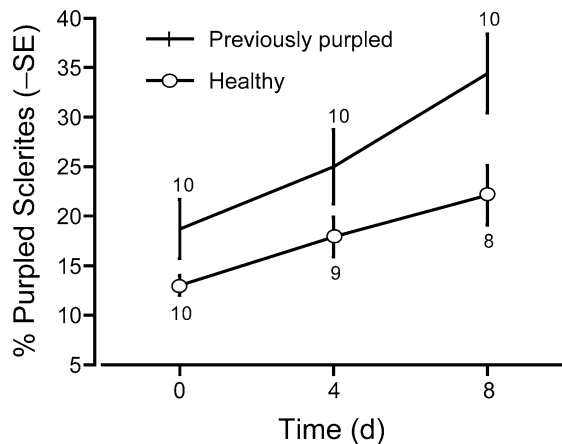
**Table 3** MANOVA and ANOVA results for the long-term inducer experiment. Percents spindle and purple are proportions of spindle and purple sclerites relative to the total number of sclerites counted. Sclerite and axis weight are the percentage of the total dry sample weight. *Mill* *Millepora alcicornis*, *DA* diseased allograft, *CT* cable tie

A. MANOVA						
Source		Wilks $\lambda$	Aprpx. F	df	P	
Species		0.758	0.638	5	0.676	
Fan		0.0002	10.0	70	$\leq 0.001$	
Inducer		0.351	3.30	10	0.003	
Fan $\times$ inducer		0.005	2.06	140	$\leq 0.001$	
Location		0.038	1.76	80	0.002	
B. Univariate ANOVA						
Source	df	% Spindle	% Purple	Sclerite wt.	Axis wt.	
Species	1	0.190	0.450	0.940	0.750	
Fan	14	$\leq 0.0001$	$\leq 0.0001$	$\leq 0.0001$	$\leq 0.0001$	
Inducer	2	0.020	$\leq 0.0001$	0.310	0.350	
Fan $\times$ inducer	28	0.330	0.010	0.010	$\leq 0.0001$	
Location	16	0.760	$\leq 0.0001$	0.090	0.090	
C. Scheffé's post-hoc tests						
Response	Factor	Comparison	Diff.	SE	P	
% Purple	Location	Near-far	15.7	2.50	$\leq 0.001$	
		Inducer	Mill-CT	17.1	4.45	0.003
		DA-CT	4.73	4.45	0.574	
		DA-Mill	-12.4	4.45	0.033	
		Mill-CT	-3.93	2.31	0.253	
% Spindle	Inducer	DA-CT	2.40	2.31	0.589	
		DA-Mill	6.33	2.31	0.037	



**Fig. 3** Multivariate response of sea fans to long-term (7 months) inducer experiment ( $n = 16$ ). Data are for tissue samples taken adjacent to the inducer (*near*) and 10 mm away (*far*). Error bars indicate standard error of the mean. CT cable tie; DA disease allograft; Mill *Millepora alcicornis*

(Chornesky 1983; Sebens and Miles 1988; Lang and Chornesky 1990) and the approximately 30 days required for the development of bent morphology in barnacles exposed to a predatory snail (Lively 1999). Once induced, the purpling may be maintained for ex-

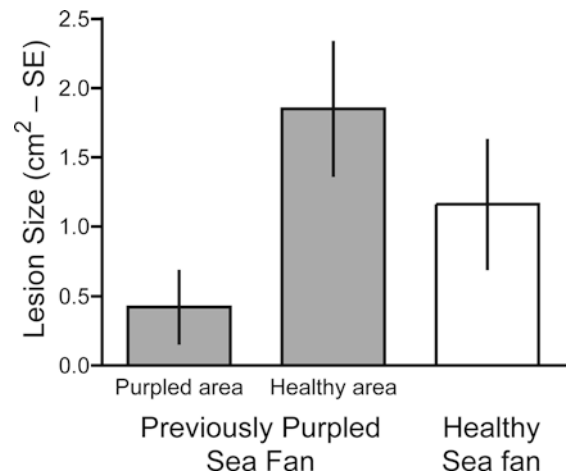


**Fig. 4** Changes in proportion of purple sclerites over time in response to *Millepora alcicornis* for healthy and previously purpled sea fan tissue. Error bars indicate standard error of the mean and numbers indicate sample sizes

tended periods if the inducer remains in contact. For instance, the purpling induced by *Millepora alcicornis* remained unchanged after 7 months, whereas purpling caused by diseased allografts was either reduced or completely undetectable.

*Millepora alcicornis* caused smaller lesions when grafted onto pre-purpled areas than onto healthy areas of the same sea fan (Fig. 5), highlighting a potential function of the purpling response. This response in sea fans appears analogous to the hypersensitive response of plants, which involves structural changes (e.g., lignification and the formation of a cork layer) and biochemical changes, when challenged by a pathogen. In both cases, infected cells quickly die (i.e., loss or removal of polyps in corals), but the concomitant production of defenses limits the spread of the pathogen (Kuc 1995; Kim et al. 2000a). Indeed, a longitudinal study where we photo-monitored individual colonies revealed that most small purpled areas on sea fans (90%,  $n = 332$ ) either did not change in size or healed over a 3-year period (Alker et al. in preparation). The cost of this response, however, is that the purpled areas are devoid of polyps (Kim and Harvell 2002) and thus are unable to contribute to colony feeding and reproduction.

The lack of purpling in response to tissue removal or cable tie attachment suggests that physical damage alone is not the cue for mounting the response. This apparent specificity of the purpling response to biotic agents suggests the presence of a recognition system in sea fans. Recognition systems in gorgonians previously have been demonstrated by antagonistic reactions against foreign tissue in grafting studies (Theodor 1976a; 1976b; Lasker and Coffroth 1985; Leddy and Green 1991; Salter-Cid and Bigger 1991). However, the similarity in response to all biotic challenges also suggests that the recognition system in sea fans is relatively simple and does not dis-



**Fig. 5** Consequences of purpling in sea fans. Size of lesions resulting from *Millepora alcicornis* attached to previously purpled and healthy (i.e., non-purpled) area of the same sea fan. *M. alcicornis* was also grafted onto healthy sea fans (i.e., free of purpling) for comparison. Error bars indicate standard error of the mean

tinguish among different biotic agents. A high level of recognition specificity is hypothesized to be costly compared to a more generalized one (Frost 1999) and thus, a generalized system is favored if it confers intermediate fitness across all possible inducers (Moran 1992). Many of the induced defensive structures in corals are general responses (Leddy and Green 1991). For example, the sweeper tentacles in the gorgonian *Erythropodium caribaeorum* are induced by ascidians, zoanthids, algae, and many scleractinian corals (Sebens and Miles 1988). The gorgonian *Swiftia exserta* exhibits specific reactivity with a memory component to allografts (Salter-Cid and Bigger 1991), but it is unknown how universal this trait is among gorgonians or cnidarians in general.

What is unclear from our study is whether the purpling itself is the defense or a marker associated with the defense. Given that purple and clear sclerites are similar in size and that purpled areas were not chemically better defended, we suggest that the purpling is a by-product of underlying structural and chemical changes associated with mounting a response against invading biotic agents.

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