Interspecific differences in fouling of two congeneric ascidians (*Eudistoma olivaceum* and *E. capsulatum*): is surface acidity an effective defense?

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Abstract

Ascidian specimens were collected from the Indian River Lagoon, Florida, USA, in November 1987. *Eudistoma olivaceum* (Van Name) was fouled by only two species of epifaunal invertebrates and the percent cover of epibionts was negligible. Colonies of *E. capsulatum* (Van Name) were fouled by up to 17 species of epifaunal invertebrates, epibionts covered an average of 12.5% of the surface area of these colonies. Extracts of *E. olivaceum* exhibited potent cytotoxic, antimicrobial and antiviral activity in laboratory bioassays, while the activity of extracts of *E. capsulatum* was insignificant. The toxicity of extracts of each ascidian was negatively related to the percent cover of fouling organisms. Surface acidity of each ascidian was positively related to the percent cover of epibionts: *E. olivaceum* was nearly neutral (pH = 6), while *E. capsulatum* was highly acidic (pH = 1 to 2). Larval settlement and growth of ancestrulae of the cheilostome bryozoan *Bugula neritina* (Linnaeus) were inhibited at very low concentrations of crude extract of *E. olivaceum*. Crude extracts of *E. capsulatum* inhibited settlement and growth of *B. neritina* only at concentrations of approximately an order of magnitude greater than those of *E. olivaceum*. Differences in the degree to which these congeneric ascidians are fouled is related to differences in the chemical defenses they possess. Acidity is not an effective inhibitor of settlement by epifaunal invertebrates.

Introduction

Space is often a limiting resource in epibenthic communities (Jackson and Buss 1975). If free space is not available, the overgrowth of neighbouring organisms or the settlement of larvae onto the surface of organisms may be the only means of procuring space. Yet epibiosis is rarely observed in these communities, despite indications that all but the most delicate of surfaces may be suitable for settlement (Ware 1984). The disadvantages inherent in possessing epibionts have most probably acted as strong selective pressures, producing a range of antifouling defenses.

Mechanisms believed to inhibit the settlement of epibionts include the production of mucus, sloughing of the epithelium, skeletal reinforcement and the production of secondary metabolites (Bakus et al. 1986, Dyrynda 1986). These antifouling mechanisms may, in addition to reducing or halting epibiosis, have important implications for community structure and dynamics. Habitat selection by settling larvae can be an important determinant of subsequent survivorship (Buss 1979, Davis 1987) and evidence indicates that settlement is an important determinant of adult distribution and abundance (Connell 1985, Davis 1988).

In ascidians, elevated levels of vanadium and surface acidity have been proposed as mechanisms by which the settlement of epibionts may be inhibited (Stoecker 1978, 1980). Members of the family Polycitoridae are the only ascidians which possess both of these potential inhibitory mechanisms (Stoecker 1980). Yet, contrary to predictions that polycitorids should be free of epibionts, field observations in Florida revealed that a member of this family, *Eudistoma capsulatum*, was heavily fouled. The closely related *E. olivaceum* was, on the other hand, relatively free of epibionts. Members of the genus *Eudistoma* are colonial ascidians and the heavy fouling of *E. capsulatum* is contrary to Jackson's (1977) prediction that colonial organisms should be relatively free of epibionts and Stoecker's (1980) affirmation of his prediction for ascidians.

The purpose of this study was to determine which species fouled these ascidians in the field and determine the relative contributions of overgrowth and settlement to epibiosis, and by means of laboratory settlement assays to evaluate the defences employed by each species.

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Materials and methods

Levels of fouling

Twenty three colonies, or portions of colonies, of *Eudistoma olivaceum* and *E. capsulatum* were collected from the shallow subtidal zone near the Fort Pierce Inlet of Indian River Lagoon, Florida, USA, in November 1987. The projected surface area of each colony and the identity, number and, where appropriate, the projected surface area of epizooites was determined in the laboratory. Surface-area measurements were made by projecting samples onto a video screen and digitizing the image (Houston Hipad).

Colonization stemming from settlement was distinguished from overgrowth. Only clonal organisms were capable of overgrowth; those that were clearly connected to the border of the "host" colony, or within 2 cm of the border of that colony were recorded as overgrowth. Colonization by solitary organisms and arborescent colonial species was recorded as settlement, as were any colonial species that did not have at least part of the colony within 2 cm of the border of the host.

Preparation of extracts

The projected surface areas of five colonies of each species of ascidian were estimated as described above. The upper half of colonies of *Eudistoma olivaceum* were separated from their bases, while colonies of *E. capsulatum* were left intact. Following the removal of epibionts from these colonies, crude-solvent extracts were prepared. Each colony or portion of a colony was macerated with methanol in a Waring blender, steeped for 1 h, filtered, and concentrated to dryness by distillation under reduced pressure. The crude-solvent extracts were then partitioned into ethyl acetate and aqueous fractions to remove salts and evaporated again to dryness under vacuum. This procedure yielded ten extracts for *E. olivaceum* and five for *E. capsulatum*. The ethyl acetate fraction of the extract was weighed and divided by the original area of each colony, yielding an extract concentration per cm^{-2} for each species. Extract yields from the upper and lower halves of *E. olivaceum* were pooled for this calculation. The mean extract concentration per cm^{-2} was 4.85 mg (SE = 0.94) for *E. olivaceum* and 6.84 mg (SE = 0.56) for *E. capsulatum*. These values were taken to represent a concentration of 100% and all dilutions used them as reference values.

Activity of extracts

Each of the replicate extracts were screened in bioassays for cytotoxic (P388), antimicrobial and antiviral (HSV-1) activity. In vitro cytotoxicity assays were carried out against P388 murine lukemia tumor cells (obtained from Dr. J. Mayo, National Cancer Institute, Bethseda, Maryland, USA) using the microculture tetrazolium assay (Alley et al. 1988). Antimicrobial assays were carried out against *Candida albicans* (ATCC Strain 44506), *Aspergillus nidulans* (ATCC Strain 36321), *Bacillus subtilis* (ATCC Strain 6633), *Pseudomonas aeruginosa* (ATCC Strain 27853), and *Escherichia coli* (HBOI/BMR Strain) using the disk-diffusion assay (White et al. 1986). Assays against KOS Strain *Herpes simplex* Virus Type 1 were carried out using the antiviral disc assay (Russell 1962).

pH determinations

We tested the acidity of the ascidian tunic by placing small pieces of epibiont-free ascidian onto pH paper (ColorpHast™ Indicator strips, EM Science, Cherry Hill, New Jersey, USA) and applying gentle pressure until body fluids were extruded. Strips of pH paper were also laid gently across the surface of intact *Eudistoma capsulatum*.

Settlement bioassays

A settlement bioassay was developed with the larvae of the arborescent cheilostome bryozoan *Bugula neritina*. Larvae of this species were induced to settle on fragments of polystyrene petri dishes by the procedure of D. Rittschof (personal communication). Reproductive colonies of *B. neritina* were collected from the Indian River Lagoon; larvae were obtained after exposure to bright light. Batches of larvae (20 to 80) were pipetted into 5 ml glass stender dishes containing 3 ml of the same seawater in which the adult colonies had been steeped. After the addition of larvae, the quantity of seawater was made upto 3.5 ml in all dishes.

Squares of polystyrene (1.5 × 1.5 cm) were cut from petri dishes (Labtek) and carefully coated with extracts of either *Eudistoma olivaceum* or *E. capsulatum*. At all dilutions, 150 µl of methanol was added to the treatment and control squares of polystyrene; controls only received solvent. The solvent was allowed to evaporate prior to beginning trials. Treated and control polystyrene squares were added to dishes, paired with respect to the number of larvae they contained. Dishes were transferred to a 24 °C constant-temperature cabinet and kept in absolute darkness. The number of larvae which settled on the polystyrene was determined 24 h later.

On the completion of trials, the heights of five ancestrulae (the elongated processes into which metamorphosing larvae develop) were measured per dish with an ocular graticule at 250×. Since most larvae settled on the edge of the polystyrene and grew on the same plane as the water surface, these measurements could be made without removing settlers from the dishes.

The effects of extracts on the growth of settled bryozoans was also determined. Squares of polystyrene measuring 1 × 1 cm were coated with ethyl acetate extracts of either *Eudistoma olivaceum* or *E. capsulatum*. Control squares only received methanol. The solvent was allowed to evaporate and the squares were pushed to the bottom of 5 ml glass

stender dishes containing 3 ml filtered seawater. Ancestrulae of *Bugula neritina*, which had been allowed to settle on 1.5 × 1.5 cm polystyrene squares during the previous 24 h, were then added to the dishes. The polystyrene with settlers "floated" just below the water surface and did not come into direct contact with the extract-coated squares.

Experimental dishes were incubated at 24 °C for 48 h and the heights of ancestrulae from five randomly selected individuals were measured at 24 and 48 h (equivalent to 48 and 72 h after the larvae had been first left to settle). The percentage of ancestrulae with their lophophores extended was also recorded. Pilot trials revealed that settled individuals could be maintained for 96 h without any obvious detrimental effects.

Results

Levels of fouling

The levels of fouling were markedly disparate. Only two invertebrate species were recorded on *Eudistoma olivaceum*, while 17, mostly ascidians, encrusted *E. capsulatum* (Table 1).

Colonization events from settlement averaged 0.47 cm^{-2} on *Eudistoma olivaceum* (SE = 0.43, $n = 23$) and 0.87 cm^{-2} (SE = 0.44, $n = 23$) on *E. capsulatum* and were significantly different at the 1% level (Mann-Whitney U-test = 636). On *E. olivaceum*, the arborescent bryozoan *Bugula neritina* accounted for almost 95% of settlement, with an unidentified barnacle responsible for the remaining 5%. The barnacle *Balanus eburneus* accounted for almost 75% of all settlement events recorded on *E. capsulatum*, ascidians around 16%, bryozoans 10%, and sponges < 1%.

Eudistoma olivaceum was not overgrown, while an average of 12.5% (SE = 4.1%, $n = 23$) of each *E. capsulatum* colony was overgrown by encrusting organisms. One colony had more than 60% of its surface encrusted by epibionts. Ascidians were responsible for almost 84% of this overgrowth, with bryozoans accounting for 11% and sponges for 5%.

Activity of extracts

Extracts of *Eudistoma olivaceum* showed potent cytotoxic, antimicrobial and antiviral activity, while extracts of *E. capsulatum* showed no significant biological activity (Table 2). Antimicrobial activity of *E. olivaceum* was restricted to two of the five microorganisms tested – *Bacillus subtilis* and *Escherichia coli*. Only extracts from the upper lobes of *Eudistoma olivaceum* colonies inhibited the growth of *E. coli*. No other differences in biological activity were detected between the tops and bases of *E. olivaceum* colonies (Table 2).

All significant biological activity was restricted to the ethyl acetate partitions of the extracts, although all aqueous partitions were tested.

Table 1. Epibionts fouling ascidians *Eudistoma olivaceum* and *E. capsulatum*

Taxon	Epibionts on:	
	E. olivaceum	*E. capsulatum*
Ascidiacea	none	*Aplidium antillense* *Botryllus planus* *Didemnum* sp. (white) *Didemnum* sp. (off-white) *Diplosoma glandulosum* *Perophora viridis* *Symplegma rubra* *Symplegma viride* *Didemnum psammathoides* encrusting colonial ascidian
Bryozoa	*Bugula neritina*	*Bugula neritina* *Bugula stolonifera* *Zoobotryon verticillatum*
Crustacea	*Balanus* sp.	*Balanus eburneus*
Cnidaria	none	*Aiptasia* sp. unidentified anemone
Porifera	none	encrusting orange demosponge

Table 2. *Eudistoma olivaceum* and *E. capsulatum*. Activity exhibited by ethyl acetate and aqueous partitions of ascidian extracts in bioassays. Cytotoxicity assay yields concentration ($\mu g\ ml^{-1}$) at which 50% inhibition of growth occurs (IC_{50}); an IC_{50} concentration of more than 20 $\mu g\ ml^{-1}$ is considered to indicate no significant activity. Antimicrobial assay yields mean size of the zone of inhibition in mm at a concentration of 200 μg/6.35 mm disk; the standard error of the mean is in parentheses. Antiviral assay gives degree of inhibition of viral plaque formation (+ + +: complete inhibition; −: no inhibition), with concentration (μg/5.6 mm disk) in parentheses. All tests were done with five replicate extracts

Assay	*E. olivaceum*		*E. capsulatum*
	upper lobes	colony base	
Ethyl acetate partition			
Cytotoxicity assay (P-388)	< 0.175	< 0.175	> 20
Antimicrobial assay			
Bacillus subtilis	12.8 (1.5)	11.4 (1.12)	0 (−)
Escherichia coli	4.6 (2.8)	0 (−)	0 (−)
Candida albicans	0 (−)	0 (−)	0 (−)
Aspergillus nidulans	0 (−)	0 (−)	0 (−)
Pseudomonas aeruginosa	0 (−)	0 (−)	0 (−)
Antiviral assay (HSV-1)	+ + + (2)	+ + + (2)	− (200)
Aqueous partition			
Cytotoxicity assay (P-388)	4.46 1.25	14.0 3.1	> 20 −

pH determinations

Fluids extruded from the tunic of *Eudistoma olivaceum* were near neutral (pH = 6, $n = 6$), while those of *E. capsulatum* were highly acidic (pH = 1 to 2, $n = 6$). pH paper that was

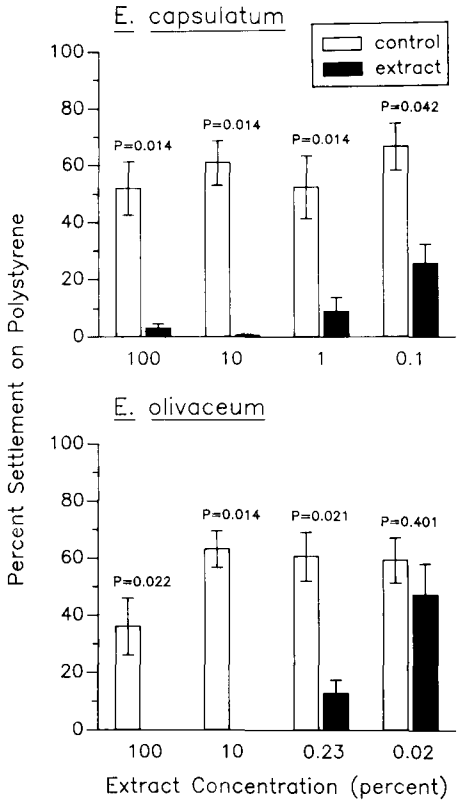

Fig. 1. *Bugula neritina.* Settlement responses of bryozoan larvae to different concentrations of *Eudistoma olivaceum* or *E. capsulatum* extracts. Bars are means from eight trials, and error bars represent standard errors. *P* values above each pair of bars were calculated from a Student's *t*-test of arcsine-transformed data or a Wilcoxon paired-rank test if data were not normally distributed

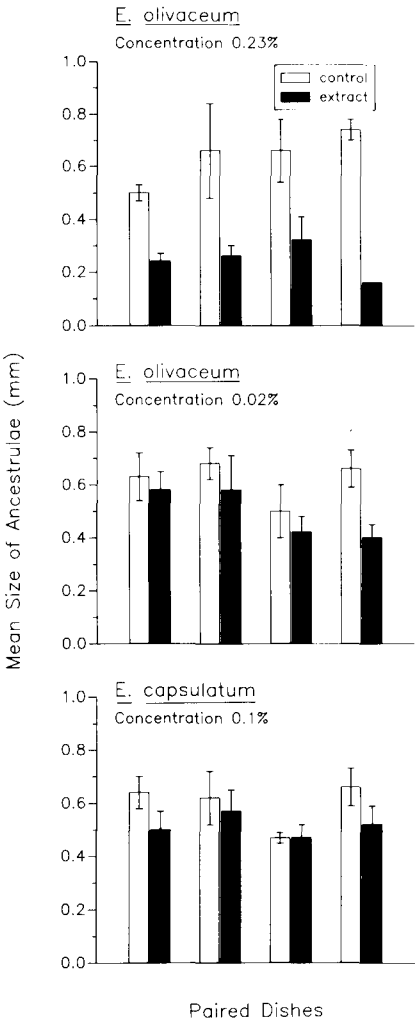

Fig. 2. *Bugula neritina.* Mean height of bryozoan ancestrulae 24 h after settling on polystyrene squares coated with *Eudistoma olivaceum* or *E. capsulatum* extracts. Paired bars represent means of five height measurements on ancestrulae in paired treatment and control dishes. Error bars are standard deviations

lightly pressed against the surface of fouled and unfouled portions of intact colonies of *E. capsulatum* showed a pH of 2.

Settlement bioassays

The settlement of *Bugula neritina* onto polystyrene coated with ascidian extract was a function of extract concentration. Settlement occurred at all concentrations of extract of *Eudistoma capsulatum*, but only at the two lowest concentrations (0.23 and 0.02%) in *E. olivaceum* (Fig. 1). At the lowest concentration tested (0.02%), *E. olivaceum* extract did not significantly inhibit settlement of larvae of *B. neritina* relative to controls. *E. capsulatum* extract at a concentration of 0.1% inhibited *B. neritina* settlement, but the level of significance was very close to 5%.

Methanol did not affect the settlement of larvae of *Bugula neritina* (Student's $t=0.57$, $P=0.58$). On average, 48.7% of larvae settled within 24 h on polystyrene coated with methanol (SE = 5.5, $n=12$) and 52.7% settled on polystyrene in the absence of the solvent (SE = 4.9, $n=12$).

No evidence suggests that the numbers of larvae of *Bugula neritina* in each dish influenced the subsequent per-

Table 3. *Bugula neritina.* Nested analysis of variance table for heights of bryozoan ancestrulae 24 h after their introduction into dishes containing extract of *Eudistoma olivaceum* or *E. capsulatum.* Factors were treatment (Treat) with two levels (ascidian extract and solvent control) and a nested dish-factor (Dish). Cochran's test was used to ensure that variances were homogeneous. Levels of significance = ** $P<0.01$; *** $P<0.001$. MS: mean square

Taxon (extract conc)	Source of variation	MS (×1 000)	DF	*F*	*P*
E. olivaceum (0.23%)	Treat	1 460	1,6	34.9	**
	Dish	42	6,28	5.4	***
	Error	8			
E. olivaceum (0.02%)	Treat	148	1,6	3.6	NS
	Dish	41	6,32	6.3	**
	Error	6			
E. capsulatum (0.1%)	Treat	58	1,6	2.9	NS
	Dish	20	6,28	4.2	**
	Error	5			

Table 4. *Bugula neritina.* Nested analysis of variance table for heights of bryozoan ancestrulae 48 and 72 h after their introduction into dishes containing extract of *Eudistoma olivaceum* or *E. capsulatum*. Bryozoan larvae did not come into direct contact with ascidian extract. Factors were treatment (Treat) with two levels (ascidian extract and solvent control) and a nested dish-factor (Dish). Cochran's test was used to ensure that variances were homogeneous; two data sets required transformation to meet this assumption. Levels of significance = * $P<0.05$; ** $P<0.01$. SV: source of variation; MS: mean square

Taxon (extract conc)	Time since settlement									
	48 h					72 h				
	SV	MS (×1 000)	DF	*F*	*P*	SV	MS (×1 000)	DF	*F*	*P*
E. olivaceum (0.23%)	Treat	198	1,4	11.0	*	Treat	140	1,4	27.9	**
	Dish	18	4,24	2.8	*	Dish	5	4,24	3.0	*
	Error	6				Error	2			
E. olivaceum (0.02%)	Treat	61	1,6	1.8	NS	Treat	198	1,6	14.2	**
	Dish	33	6,32	1.9	NS	Dish	14	6,30	2.5	*
	Error	17				Error	5			
E. capsulatum (10%)	Treat	0.2	1,6	0.05	NS	Treat	51	1,6	1.9	NS
	Dish	3.3	6,33	0.46	NS	Dish	27	6,32	3.1	*
	Error	7.3				Error	9			

centage of settlers. The percent settlement was not related to the number of larvae per control dish ($r^2=0.004$, $P>0.4$, DF = 132).

Extract concentration influenced the height of ancestrulae of *Bugula neritina*. Twenty-four hours exposure to extract of *Eudistoma olivaceum* at a concentration of 0.23% significantly slowed the growth of ancestrulae relative to controls ($P<0.01$, Fig. 2, Table 3). At the lower concentration of 0.02%, ancestrulae were still shorter relative to controls, but not significantly so. No significant reduction in the height of ancestrulae was seen with extract of *E. capsulatum* at a concentration of 0.1%. Insufficient larvae settled at higher concentrations to warrant measurements of the height of ancestrula. Significant differences were detected between dishes (the nested factor) in all analyses, rendering pooling across dishes inappropriate.

Measurements of ancestrulae that had settled previously onto plates and were not in direct contact with extracts of the ascidians provided the best estimates of the effect of ascidian extracts on growth; these growth estimates were independent of the time at which larvae had settled. Extract of *Eudistoma olivaceum* at a concentration of 0.23% significantly inhibited the growth of ancestrulae relative to controls after periods of 48 and 72 h. At the lower concentration (0.02%) no significant differences were detected after 48 h, and at 72 h ancestrulae exposed to extracts were significantly taller than controls ($P<0.01$, Fig. 3, Table 4). Extracts of *E. capsulatum* showed much lower activity. The height of ancestrulae exposed to an extract concentration of 10% were not significantly different from controls.

The concentration of *Eudistoma olivaceum* extract influenced the number of lophophores extended by *Bugula neritina* 48 and 72 h after settlement (Fig. 4). A concentration of 0.23% completely halted the extension of lophophores. In contrast, a concentration of 0.02% produced no difference

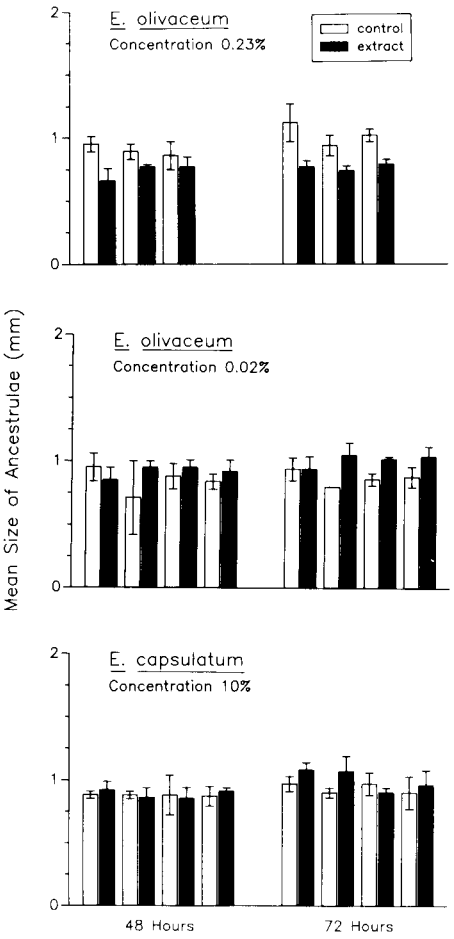

Fig. 3. *Bugula neritina.* Mean height of bryozoan ancestrulae after 48 and 72 h in glass dishes with *Eudistoma olivaceum* or *E. capsulatum* extracts. Settlers were not in direct contact with extract. Paired bars represents means of five height measurements on ancestrulae in paired control and treatment dishes. Error bars are standard deviations

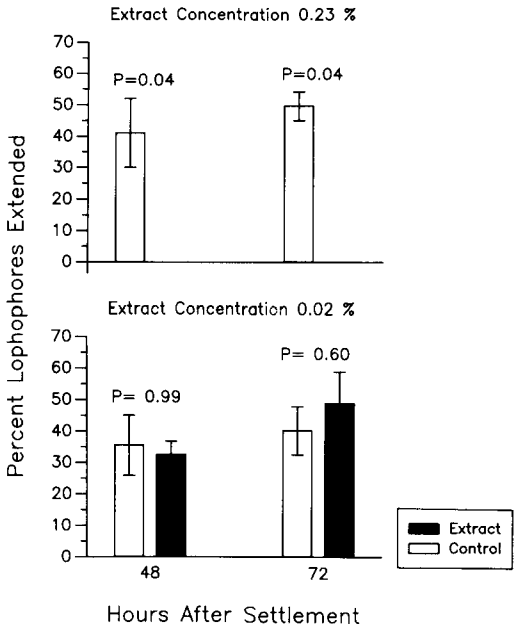

Fig. 4. *Bugula neritina*. Mean percentage of lophophores extended by metamorphosed bryozoans in the presence of *Eudistoma olivaceum* extract. (Lophophores were not extended at extract concentration of 0.23%.) Bars are means of six trials and error bars are standard errors. *P* values above each pair of bars were calculated from a Student's *t*-test of arcsine-transformed data or a Wilcoxon paired-rank test if data were not normally distributed

in the percentage of lophophores extended by the treatment and control groups.

Discussion

Fouling in the two ascidians was negatively correlated with the toxicity of their extracts. Extracts of *Eudistoma olivaceum* showed cytotoxic, antimicrobial, and antiviral activity, and this species was only lightly fouled. All fouling on *E. olivaceum* was the result of settlement: no overgrowth was observed. On the other hand, extracts of *E. capsulatum* showed no significant activity in any of the bioassays. Overgrowth and the settlement of epibionts were responsible for the fouling observed. A negative relationship between fouling and toxicity occurs in temperate and tropical sponges (McCaffrey and Endean 1985, Thompson et al. 1985).

Settlement bioassays with the bryozoan *Bugula neritina* were consistent with the findings of the bioassays. *Eudistoma olivaceum* extract inhibited larval settlement and subsequent growth at concentrations of approximately an order of magnitude lower than *E. capsulatum* extract. Although concentrations of extract were calculated on the basis of the surface area of the colonies, differential settlement and growth of *B. neritina* at different concentrations of ascidian extracts were real and not an artifact of colony thickness, as colonies of both species were roughly the same thickness.

Inhibition of growth of *Bugula neritina* by *Eudistoma olivaceum* extract was concentration-dependent. Inhibition of growth occurred in the absence of direct contact between ancestrulae and the *E. olivaceum* extract, indicating that growth inhibition may occur in response to water-diffusible compounds. Slow rates of growth by ancestrulae are related to interference with lophophore extension and feeding.

Eudistoma capsulatum was fouled despite the low pH of the surface of this species. None of 13 Bermudian species with a surface pH ≤ 2 had epibionts (Stoecker 1980). A pH of 2 rapidly killed hydroid and ascidian larvae in laboratory trials (Stoecker 1978). The discrepancy between her laboratory findings and the data we assembled from field samples probably arose because seawater rapidly neutralizes acid (Parry 1984). In the field, acidity is not an effective defense against fouling organisms.

Vanadium is an inhibitor of larval settlement (Stoecker 1978), at least for larvae of a hydroid in laboratory settlement trials. Inhibition of larval settlement occurred only at concentrations >250 ppm (Stoecker 1978; her Table V). Vanadium determinations for *Eudistoma olivaceum* average less than 95 ppm (SD = 19) (Stoecker 1980). If these vanadium levels occur in *E. olivaceum* in South Florida, the inhibition of larval settlement observed in this species would not be due to vanadium.

Colonies of *Eudistoma olivaceum* could inhibit the settlement of epibionts by modifying their cues for settlement. Submerged surfaces usually develop a microbial or primary film prior to the settlement of macro-organisms. The antimicrobial activity of *E. olivaceum* may be sufficient to inhibit or slow the formation of a primary film and thereby inhibit subsequent macrofouling. The initial presence of a microbial film prior to macrofouling cannot be taken as evidence that such films facilitate or are a necessary prerequisite to the subsequent fouling of macro-organisms (Crisp 1984). Bacterial films do not always facilitate larval settlement (Maki et al. 1988).

Inhibition of larval settlement on *Eudistoma olivaceum* probably results from the presence of eudistomins (Davis and Wright 1988), alkaloids which are responsible for the potent biological activity of *E. olivaceum* in bioassays. Rinehart et al. (1984) noted that "Of the 650 marine species assayed during the 1978 Alpha Helix Caribbean Experiment . . . *Eudistoma olivaceum* was the most active against *Herpes simplex* virus, type 1 (HSV type 1)". Twenty eudistomins have been isolated from this ascidian species (Kinzer and Cardellina 1987, Rinehart et al. 1987).

Our findings are consistent with the production of chemical antifouling agents by the ascidian species that was relatively free of epibionts (*Eudistoma olivaceum*). Acidity is not an effective inhibitor of larval settlement or overgrowth.

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