Vol. 72: 117–123, 1991 | MARINE ECOLOGY PROGRESS SERIES
Mar. Ecol. Prog. Ser. | Published May 23

Settlement behaviour of ascidian larvae: preliminary evidence for inhibition by sponge allelochemicals

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ABSTRACT: Recruitment in many marine invertebrates is inherently variable; the behaviour of larvae at settlement may be an important contributor to this variation. Yet there are few in situ observations of settlement behaviour of marine invertebrate larvae. Direct observations in the field showed that 63 % (n = 89) in larvae of the colonial ascidian *Podoclavella moluccensis* Sluiter settled on their first contact with the substratum, while a small percentage made 10 or more contacts prior to settlement. Two species of subtidal sponge, *Mycale* sp. and *Crella incrustans*, were consistently avoided by these settling larvae. *Mycale* sp. was often contacted repeatedly, but invariably rejected. In contrast, even contacts with *C. incrustans* were rare, relative to the space that this sponge occupied. We investigated the role of sponge allelochemicals, at natural concentrations, in inhibiting settlement of these larvae. Settlement of larvae onto substrata impregnated with an ether-soluble extract of *Mycale* sp. was significantly inhibited, relative to controls. Several fractions of this extract were equally inhibitory to settling larvae. Butanol and ether extracts of *C. incrustans* were also inhibitory and the repellent activity was in the more polar lipophilic fractions. The least polar fraction was attractive to larvae. Extracts appeared to be non-toxic to the larvae and we failed to detect any antibacterial activity. We conclude that settlement of these ascidian larvae may be inhibited by natural products released from or at the surface of these sponges.

INTRODUCTION

Variation in the initial settlement of marine invertebrates may have a significant influence on distribution and abundance of adults, and also on community structure (Connell 1985, Gaines & Roughgarden 1985, Davis 1988). The behaviour of larvae can, in part, determine these patterns of recruitment. For example, some larvae seek the protection of pits and crevices (Connell 1961, Keough & Downes 1982) while others avoid settling on or near organisms that may lower their survival (Grosberg 1981, Davis 1987). The cues by which larvae distinguish between substrata are poorly understood and are likely to be complex (Burke 1983).

Despite the perceived importance of settlement to community structure and dynamics, direct observations of larval settlement in the field are few. Davis (1987) observed the initial response of settling larvae of the colonial ascidian *Podoclavella moluccensis* Sluiter (Clavelinidae: Aplousobranchia) on their first encounter with 5 types of substratum: bare space (wood) and 4 species of sponge. These observations indicated that larvae were highly selective and showed a clear preference for bare space, rarely settling on the unfouled matrix of sponges. One sponge, *Mycale* sp., was consistently rejected. Others, including *Crella incrustans* [previously called *Tedania* sp. A in Davis (1987)], were selected less often than expected from their relative abundances.

In this paper we present additional field observations on settlement behaviour of *Podoclavella moluccensis* larvae. We then test the hypothesis that allelochemicals contribute to this behaviour by examining the responses of larvae to crude and fractionated extracts of 2 species of sponge that were consistently avoided.

MATERIALS AND METHODS

Field observations and settlement trials were conducted beneath Edithburgh pier, South Australia. This

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0171-8630/91/0072/0117/$ 03.00

site and its fauna are described in Kay & Keough (1981), Kay & Butler (1983) and Davis (1987).

Field observations of ascidian settlement. *Podoclavella molluccensis* larvae were released from adults for ca 6 wk of each year, beginning in late spring. Traps were fitted over reproductive colonies and the larvae released were followed directly using SCUBA (Davis 1987, Davis & Butler 1989). Individual larvae were followed until they either settled or were lost. The substrata touched by each larva and the number of contacts were recorded. The time a larva had spent in a trap did not appear to alter its behaviour (Davis & Butler 1989). The area of substrata available for contact was estimated with the haphazard placement of 13 × 17.5 cm quadrats (227.5 cm^2) onto the pier pilings. The substratum that dominated each of the 150 quadrats was recorded.

The frequency of observed contacts was then compared to that expected, based on the area of each substratum available for settlement. Low expected frequencies were apparent with some of the substrata. Sokal & Rohlf (1981) maintain that it is appropriate to use a *G*-test to compare observed and expected frequencies if there are 5 or more groups with expected frequencies of 3 or more. Some substrata were pooled to meet this condition.

Preparation of extracts. We investigated extracts of 2 sponges, *Mycale* sp. (SP20 code of voucher specimens held at the University of Adelaide, South Australia, and deposited in the South Australian Museum) and *Crella incrustans* (Northern Territory Museum numbers Z3581, Z3582, Z3583).

Mycale sp. was collected from beneath the Edithburgh pier in May 1988 and stored frozen at −14 °C until extraction commenced in October 1988. The frozen sponge was extracted by blending it with acetone (4 × 1 l) in a Waring Blender. All solvents were redistilled. The acetone-sponge mixture was filtered through diatomaceous earth (Celite) after each extraction. The acetone was removed under vacuum to provide a thick aqueous mixture. The volume was made up to 600 ml with water and the mixture extracted with diethyl ether (5 × 500 ml). The ether extract was dried with anhydrous $MgSO_4$ and the ether removed under vacuum to give the crude lipophilic extract (5.4 g; 2.6 %, based on the weight of the dried sponge residue remaining).

A portion of the extract (1.2 g) was chromatographed on Sephadex LH-20 (Pharmacia) using methanol: chloroform 1:1 as the eluent, to give 4 successive fractions: 2-99A (80 mg), 2-99B (220 mg), 2-99C (670 mg), 2-99D (5 mg).

Crella incrustans was collected under the Edithburgh pier in October 1989 and kept frozen until November 1989, when extraction commenced. The sponge was extracted as above using acetone (4 × 5 l) and the aqueous residue extracted with ether (4 × 1 l) followed by *n*-butanol (3 × 800 ml) to give, after drying and removal of solvents, crude extracts 2-181A (16 g) and 2-181B (3.5 g) respectively. The yields based on the weight of dried sponge residue were 3 and 0.6 % respectively.

A portion of 2-181A (ether extract, 6.1 g) was submitted to rapid vacuum chromatography on silica-gel (Coll & Bowden 1986) to give 7 fractions [2-183A (0.54 g) to 2-183G]. 2-183A was the least polar fraction and was eluted with mixtures ranging from 0 to 40 % dichloromethane and light petroleum.

A second portion of 2-181A (1.7 g) was chromatographed on Sephadex LH-20 using chloroform:light petroleum:ethanol (10:10:1) as the eluent. Seven fractions were collected: 2-181J, K, L (680 mg); M (300 mg); N (43 mg); P (51 mg); Q (41 mg). 2-181Q was the most polar fraction.

To estimate a 'natural' concentration of crude extract for use in tests, we assumed that all of the active compound(s) is concentrated at the surface and made an estimate of the surface area of the extracted sponge. This was more straightforward for *Mycale* sp. than for *Crella incrustans* because the former has a thin encrusting growth form, whereas *C. incrustans* is more mound-form and lobed.

In the case of *Crella incrustans*, each chromatographic fraction was tested at a concentration corresponding to its percentage recovery from the column. Thus any active compounds in the chromatographic fractions of *C. incrustans* would be present in the tests at less than or equal to the concentration present in the original crude ether extract (2-181A). Concentrations (in μg cm^{-2}) used in each test were as follows. *Mycale* sp.: (1) Total ether extract, 1500; (2) 2-99A, 350; (3) 2-99B, 970; (4) 2-99C, 1460. *Crella incrustans:* (5) 2-181A, 500; (6) 2-181B, 123; (7) 2-181L, 200; (8) 2-181M, 87; (9) 2-181P, 14; (10) 2-181Q, 12; (11) 2-183A, 44; (12) Cholesterol, 500.

The *Mycale* sp. extracts were tested in the field on 3 to 5 November 1988 and the *Crella incrustans* extracts on 9 to 14 November 1989. Extracts (5) and (6) were also tested at 0.1 × the above concentrations, and the cholesterol (Extract 12) at 0.1 and 0.01 dilutions. Cholesterol (Aldrich) was used as supplied. The reason for including cholesterol was that, in the 1988 trials with *Mycale* sp. extract, all 3 fractions were repellent (see 'Results'). A possible reason for repulsion was that we were observing a non-specific response to the physical nature of the ether extract rather than one mediated by the unique chemical structure of an active compound(s). Cholesterol is a significant component of many sponges (Goad 1979) and was chosen because it seemed that it would ade-

quately mimic the physical characteristics of the ether-soluble extract of *Mycale* sp.

Responses of larvae to sponge extracts. The responses of larvae to extracts were tested by giving a single larva a binary choice: filter paper soaked in extract or an equal area of paper soaked in solvent only. This was done beneath the Edithburgh pier where larvae were obtained by fixing larval traps over adult colonies (Davis 1987), by gently shaking reproductive colonies to dislodge brooded larvae or by catching newly released larvae in mid-water with a large syringe (20 ml, with the nozzle enlarged). Each trial was done in a 70 ml screw-top plastic container with 1 larva per container. In the laboratory, discs of filter paper (Whatman # 1) of the same diameter (45 mm) as the container lids were folded to mark the mid-line, and then extract was carefully pipetted onto one half first, followed by control solvent onto the other half. This order had the effect, if there was an error at the boundary, of spreading solvent onto the extract side rather than extract onto the solvent side. This was merely a precautionary technique; in fact the pipetting could be done quite accurately. The disc was allowed to dry and then placed into the lid, which was screwed on. Containers were transported to the pier thus, containing dry treated papers in air. Underwater, the container was opened to allow seawater to enter, then a single larva was released into it, and the lid replaced so that the paper was gripped under the lid even when the container was upright. Containers were placed lid-upwards in a weighted rack on the seafloor, direction of the control and extract sides of the papers being varied haphazardly. Larvae invariably swam upwards in these containers; thus, although about one third of them settled on the plastic sides of the container, most settled on the paper. Many larvae settled within seconds; a minority swam for an hour or more and finally settled. Racks were left on the seafloor for times varying from 1.5 to 3 h and then scored. Only larvae settling on paper were scored, and the model for analysis was that if the extract had no influence, half would be expected to settle on the extract side and half on the control side of the paper; this was tested using log-likelihood ratio (G) tests for goodness-of-fit (Sokal & Rohlf 1981).

Antibacterial activity. Ether extracts of both sponge species were tested for their ability to inhibit growth of bacteria. In the laboratory a standard disc bioassay (White et al. 1986) was used to test the following bacteria. Gram-positive: *Bacillus subtilus* and *Staphylococcus epidermis*; Gram-negative: *Escherichia coli*, *Pseudomonas aeruginosa* and *Serratia morcescens*.

RESULTS

In situ observations of settlement

Most larvae (63 %, n = 89) settled on their first encounter with a substratum, but 6.7 % made 10 or more contacts prior to settlement (Fig. 1).

The number of initial contacts differed significantly from that expected (Fig. 2). The data-set was not homogeneous ($G_6 = 28.25$, $p < 0.001$) and a simultaneous test procedure (Sokal & Rohlf 1981) identified homogeneous groups (groups joined by lines were not significantly different at the 5 % level of significance), as shown in Table 1.

Crella incrustans and *Tedania* sp. B received less than one seventh of the contacts expected, fewer contacts than for any other category of substratum. Furthermore, when repeated contacts (i.e. total contacts) are considered, *C. incrustans* and *Tedania* sp. B received < 1 % of all contacts despite accounting for almost 14 % of the available area.

Responses of larvae to extracts

***Mycale* sp.** Trials were run over 3 d. Data pooled across these 3 d revealed that the crude extract and each of the fractions were avoided by settling larvae, relative to solvent-treated filter paper, and that they did

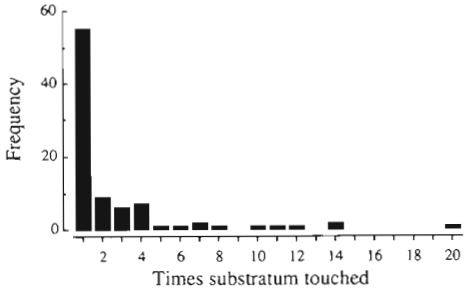

Fig. 1. *Podoclavella moluccensis*. Number of contacts made by larvae (n = 89) with substrata, prior to settlement. All observations made using SCUBA beneath Edithburgh pier, South Australia

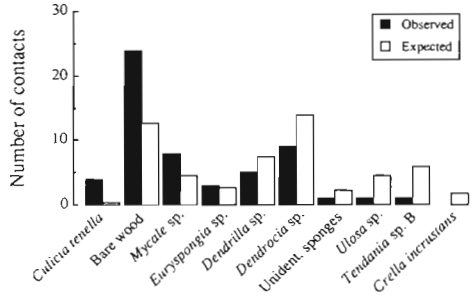

Fig. 2. Observed and expected number of initial contacts by *Podoclavella moluccensis* larvae on 12 substrata beneath Edithburgh pier, South Australia

Table 1. *Podoclavella moluccensis*. Settlement of larvae on various substrata. Homogeneous groups of substrata were identified using a simultaneous test procedure (Sokal & Rohlf 1981). Groups joined by lines were not significantly different at the 5 % significance level

Bare wood	*Mycale* sp.	*Euryspongia* sp., *Culicia tenella* and unidentified sponges	*Dendrilla* sp.	*Dendrocia* sp.	*Ulosa* sp.	*Tedania* sp. B and *Crella incrustans*

not differ significantly from each other (Table 2). To check for heterogeneity in larval behaviour between days their settlement on crude ether extract was examined each day (Table 3). Significant heterogeneity between days and an analysis of subtables showed that relatively more larvae settled on the crude extract on Day 2. Fractions 2-99A and 299-C were tested on Day 2; the proportion settling on them is not significantly higher than for other extracts and so whatever the 'day effect' was, it does not appear to have affected our conclusion. Davis (1989) has suggested that daily release of *Podoclavella moluccensis* larvae is correlated with water temperature. We did find differences between days in the number of larvae obtainable for tests, but there was no evidence that this influenced the results of the trials.

Table 2. Settlement of *Podoclavella moluccensis* larvae on filter papers treated with ether extract of *Mycale* sp. and 3 fractions, vs papers treated with solvent only. For heterogeneity $G_3 = 1609$, non-significant; for fit of totals to 1:1 ratio $G_1 = 44.27$, $p < 0.001$

Extract	Settled on control	Settled on extract
Crude (ether)	38	11
2-99A	16	4
2-99B	18	2
2-99C	16	4
Total	88	21

Table 3. Settlement of *Podoclavella moluccensis* larvae on filter papers treated with ether extract of *Mycale* sp. vs papers treated with solvent only (control), on 3 consecutive days, November 1988. G_2 for heterogeneity between rows = 9.42, $p < 0.01$

Day	Settled on control	Settled on extract
1	14	3
2	12	8
3	12	0

Crella incrustans. In the 1989 trials on extracts of *Crella incrustans*, up to 5 different extracts were tested simultaneously on a given day, and certain extracts (or cholesterol) were run on successive days to check for heterogeneity in larval responses between days. There was never significant heterogeneity between runs on a given extract, and so the pooled results for each extract are given in Table 4.

Extracts 2-181A and B were significantly avoided, and did not differ significantly from one another (Table 4; G_1 for heterogeneity = 1.07, non-significant). Results from the trials with cholesterol were compared with these extracts and did not differ significantly from them (G_2 for heterogeneity = 1.24, non-significant). When diluted to 1:10 or further, the extracts and cholesterol no longer showed detectable deterrence by this test (Table 4).

The set of fractions from the ether extract, 2-180Q, P, M, L and 2-183A, were heterogeneous (Table 5; $G_4 = 17.5$, $p < 0.005$) but the Sephadex fractions, 2-181Q, P, M and L, were homogeneous ($G_3 = 5.07$, non-significant) and their pooled totals (94 control; 57 extract) were significantly different from a 1:1 ratio ($G_1 = 9.16$, $p < 0.005$). The entire table was heterogeneous because 2-183A was significantly preferred over the control.

In summary, the butanol extract and the more polar fractions of the ether extract were repellent, and did not differ significantly from one another; the most non-polar fraction of the ether extract was attractive to settling larvae and was significantly different from all the rest.

Antibacterial activity. Non antibacterial activity was observed for either extracts of either sponge species; zones of inhibition were not apparent.

DISCUSSION

Field observations of settlement

A high proportion of larvae settled on their first encounter with a substratum, even though highly preferred substrata (such as bare wood; Fig. 2) accounted

Table 4. Settlement of *Podoclavella moluccensis* larvae on filter papers treated with crude extracts of *Crella incrustans* or cholesterol at 0.1 or 0.01 dilutions vs papers treated with solvent only (control), in November 1989. NS: not significant

Extract	Settled on control	Settled on extract	G_1 for fit to 1:1	Significance
Ether extract 2-181A	26	11	6.26	$p < 0.025$
0.1 × 2-181A	6	11	1.49	NS
Butanol extract 2-181B	22	5	11.56	$p < 0.001$
0.1 × 2-181B	7	4	0.83	NS
Cholesterol	12	3	5.78	$p < 0.025$
0.1 × cholesterol	7	3	1.65	NS
0.01 × cholesterol	7	5	0.34	NS

Table 5. Settlement of *Podoclavella moluccensis* larvae on filter papers treated with 4 Sephadex fractions (2-181Q, P, M and L) and the least polar vacuum chromatography fraction (2-183A) of ether extract 2-181A of *Crella incrustans*. NS: not significant; G_4 for heterogeneity between rows = 17.5, $p < 0.005$

Extract	Settled on control	Settled on extract	G_1 for fit to 1:1	Significance
2-181Q (most polar)	16	3	9.77	$p < 0.005$
2-181P	43	29	2.74	NS
1-181M	15	10	1.01	NS
1-181L	20	15	0.72	NS
2-183A (least polar)	5	17	6.92	$p < 0.01$

for less than 15% of the area available for settlement. Larvae of a tropical ascidian also made a high proportion of initial contacts with highly preferred substrata (D. Stoner pers. comm.).

It is noteworthy that *Mycale* sp. was contacted by larvae more frequently than expected (Fig. 2). Yet this sponge was not a preferred site for actual settlement (Davis 1987). These data indicate that deterrence occurs on contact, not at a distance. In contrast, contact with *Crella incrustans* was so rare that it is worth considering the hypothesis that it releases waterborne chemicals that act prior to contact. The release of such substances has been demonstrated in laboratory trials and near in situ conditions, although their effects in the field remain unclear (Young & Chia 1981, Thomson 1985, Walker et al. 1985).

Responses of larvae to extracts

Ether-extractable compounds inhibited larval settlement and may therefore account for the observed rejection of the sponge *Mycale* sp. Many epibenthic taxa, especially colonial species, possess secondary metabolites which presumably function to deter epibionts, endobionts and predators (Dyrynda 1986, Davis et al. 1989, Wahl 1989).

Over the past 5 yr, sponges of the genus *Mycale* have been reported as possessing a number of secondary metabolites. Most are polar lipophilic compounds with a variety of chemical structures: nucleosides (Kato et al. 1985), macrolides (Fusetani et al. 1989), terpenes (Capon & MacLeod 1985, 1987, Corriero et al. 1989) and compounds of amino acid/acetogenin origin (Perry et al. 1988, 1990, Fusetani et al. 1989). They have been shown to have antifungal, antiviral, antitumor and antimicrobial activity (Capon & MacLeod 1985, Perry et al. 1988, 1990, Fusetani et al. 1989). In addition, some inhibit cell division of fertilized starfish eggs (Kato et al. 1985, Fusetani et al. 1989).

In preliminary trials with *Mycale* sp., we found some indication that the antifouling activity lay amongst the polar lipophilic compounds but this has not been easy to confirm. Results of the trials with cholesterol indicate that a range of steroid compounds may also influence settlement preferences. This may explain the fact that we observed activity in all fractions of the *Mycale* sp. extract (Table 2). The lipophilic metabolites from this species of *Mycale* are mainly facts, lipids and steroids (van Altena unpubl.). This suggests that, in this type of study, care should be taken when ascribing a specific biological activity to a particular fraction.

We found no convincing evidence that allelochemicals from *Crella incrustans* act at a distance. During our

trials larvae often settled on the plastic or on control paper in very close proximity to the filter paper coated with extract. It was also observed (Davis & Butler unpubl.) that larvae of *Podoclavella moluscensis* settled (on wood) very close to or touching colonies of *C. incrustans* and survived for at least a month. Nevertheless it is possible that water-borne components of the extract of *C. incrustans* may act to delay the metamorphosis of larvae, as Young & Chia (1981) demonstrated for a didemnid ascidian.

It does appear, however, that some component of the extracts deters settlement on contact and that, in the case of *Crella incrustans*, the activity resides amongst the polar lipophilic components. The most non-polar fraction appeared to be significantly attractive, and there was a suggestion that the strongest repellant activity was at the polar end of the remainder. In contrast to *Mycale* sp., very few if any secondary metabolites have been reported from the genus *Crella*. This work is continuing and it should be possible to use the bioassay to isolate a pure active compound (or compounds) from *C. incrustans*.

It is interesting that the non-polar fraction of *Crella incrustans* was apparently attractive. This material is essentially similar to the major components present in *Ulosa* sp. (van Altena unpubl.), a sponge which appears to have less inhibitory effect on the larvae (Davis 1987). This type of 'essential oil' appears to be present in many sponges, especially the highly coloured ones. It is plausible that these attractive substances are present as a result of basic biochemical processes and that selection from fouling has led to the evolution of other, secondary compounds that mask their attractive effect and deter larval settlement. This could explain the presence of both attractive and deterrent compounds in the same species.

The toxicity of secondary metabolites is a potentially potent selective force in moulding the settlement preferences of larvae, and in the Porifera the degree of microfouling has been negatively correlated with the toxicity of metabolites (McCaffrey & Endean 1985, Thompson et al. 1985). There was, however, no evidence that the inhibition of settlement on *Mycale* sp. or *Crella incrustans* was mediated by toxicity. Deaths of ascidian larvae in the test containers were few and unrelated to the extract under test.

In determining the appropriate concentrations of extracts we assumed that metabolites were concentrated at the surface of both sponge species. Yet 1:10 dilutions of the inhibitory extracts did not prove effective against settling larvae. If our assumption is not justified then these data would suggest that secondary metabolites may not be the only inhibitors of settlement in these sponges. The settlement of larvae on the surface of an organism may be inhibited physically or chemically – by various skeletal structures, mucus, production of secondary substances, or by a combination of these defences (Davis et al. 1989, Wahl 1989). In addition, invertebrate larvae frequently show preferences for surfaces possessing a microbial film (Davis et al. 1989). We failed to detect any antibacterial activity in ether extracts of either sponge species and hence found no evidence to suggest the inhibition of film formation.

The role of steroids (noting the effect of cholesterol in our trials) is unclear. Many sponges either produce steriods or aquire them from symbiotic micro-organisms (Goad 1979). These steroids could have many functions (e.g. structural), although a defensive function is possible. The effective concentrations of cholesterol (in comparison with the effective concentrations of ether extract) suggest that the concentrations, at least in *Mycale* sp., are high enough to have an inhibitory effect. The validity of this suggestion remains to be tested.

Acknowledgements. For assistance in the field we thank A. T. D. Bennett, J. M. Davis, I. Magraith, G. A. Powell and M. Robinson. The antibacterial assays were done by D. Tscharke and V Vingelis. We are grateful to J. Hooper for the identification of sponge specimens. D. J. Ayre and R. J. Whelan made helpful comments on the manuscript. A.R.D. was financially supported by the Ecological Society of Australia, the Victorian Institute of Marine Science and a University of Adelaide Postgraduate Research Award, A.J.B. and I.V.A. by an Adelaide University Research Grant. This a joint contribution from the Coobowie Marine Research Station and the Ecology and Genetics Group (contribution number 80) at the University of Wollongong.

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This article was presented by A. J. Underwood, Sydney, Australia

Manuscript first received: December 12, 1990
Revised version accepted: March 5, 1991