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Less inhibited with age? Larval age modifies responses to natural settlement inhibitors

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Abstract
As larvae of marine invertebrates age, their response to settlement cues can change. This change can have significant consequences to both the ecology of these organisms, and to their response to antifouling coatings. This study examines how larval age affects the settlement response of larvae to two naturally derived settlement inhibitors, non-polar extracts from the algae *Delisea pulchra* and *Dilophus marginatus,* the former of which contains compounds that are in commercial development as antifoulants. Two species of marine invertebrates with non-feeding larvae were investigated: the bryozoans *Watersipora subtorquata* and *Bugula neritina*. Larval age strongly affected larval settlement, with older larvae settling at much higher rates than younger larvae. Despite having strong, inhibitory effects on young larvae, the non-polar extracts did not inhibit the settlement of older larvae to the same degree for both species studied. The results show that the effects of ecologically realistic settlement inhibitors are highly dependent on larval age. Given that the age of settling larvae is likely to be variable in the field, such age specific variation in settlement response of larvae may have important consequences for host-epibiont interactions in natural communities.

Keywords: *Biofouling, bryozoan, desperate larva hypothesis, natural antifoulants, settlement inhibition*

Introduction

Chemical cues mediate the colonisation of a range of marine propagules. These cues can come from a variety of sources and act in either a positive (inducing) or negative (inhibitory) manner (see review in Steinberg et al. 2002). From the perspective of host organisms, the settlement of epibionts onto their surfaces can have deleterious effects on host organisms and this has been suggested to lead to the evolution of host-derived settlement inhibitors ('natural antifoulants'). Although the actual chemical characterisation and quantification of ecologically relevant antifoulants is rare, the inhibition of settlement of fouling organisms by extracts from marine algae has been demonstrated (Steinberg et al. 2002; Steinberg & de Nys, 2002; Nylund & Pavia, 2003).

Whilst the effects of natural antifoulants on settlement can be strong, the responses of larvae to these compounds are not consistent and variation in the response of larvae exists. Several studies have found temporal variability in the response of fouling organisms to algal extracts with antifouling properties that are consistent with seasonal variation in the production of the compounds (Hellio et al. 2004; Maréchal et al. 2004). However, variability in larval settlement response to antifoulants as the larvae age may also contribute to the observed patterns, but this has received little consideration. Whilst larvae are often viewed (and modelled) as a homogenous population, variability in the response of larvae to settlement surfaces can be substantial (Raimondi & Keough, 1990; Marshall & Keough, 2003).

There are a number of sources of variation in larval settlement behaviour including size (Isomura & Nishimura, 2001; Marshall & Keough, 2003), genetics (Toonen & Pawlik, 2001a; 2001b) and most commonly studied, larval age (Knight-Jones, 1951; 1953; Gibson, 1994; Miron et al. 2000). It has long been known that, as larvae age, they become less discriminating with respect to settlement surfaces and larvae that previously required a settlement inducer will later settle in the absence of that inducer (Knight-Jones, 1951; 1953). This is typically viewed as being an energetic argument, as larvae age, they deplete their energetic reserves and become

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'desperate' to settle (Pechenik, 1999). This concept has received further support for a range of taxa by the recent work by Pechenik and others demonstrating strong post-metamorphic consequences of extending the larval period (Pechenik & Cerulli, 1991; Pechenik et al. 1998; Marshall et al. 2003b).

Given that delaying the metamorphosis of non-feeding larvae reduces their fitness, and older larvae require fewer positive settlement cues to initiate settlement, it seems likely that older larvae may also react differently to negative settlement cues. It may be that as larvae age, the inhibitory effects of negative settlement cues are diminished due to the increasing costs of further delaying metamorphosis. Older larvae of the barnacle *Balanus amphitrite* in general have higher rates of settlement (Head et al. 2004), are less discriminating between settlement surfaces (Rittschof et al. 1984) and settle more readily in the presence of a negative settlement cue (e.g. low frequency sound waves) (Branscomb & Rittschof, 1984) than younger larvae. Variability in the settlement response of *B. amphitrite* cyprids to bacterial biofilms has also been demonstrated (Maki et al. 1988; 1990).

However, this issue has rarely been considered in the context of host/epibiont interactions, in part because demonstrations of natural antifoulants in an ecologically realistic context are still rare (Steinberg et al. 2002). If naturally occurring inhibitors are less effective against older larvae, then the older larvae of any species will represent the greatest 'challenge' to the host producing the inhibitors. Pragmatically, the ecological relevance of natural settlement inhibitors (for example on macroalgae) may be misinterpreted if differently aged larvae react differently to settlement inhibitors. Rather than preventing the settlement of all larvae, natural antifouling substances may merely be discouraging the settlement of young larvae. Therefore comparisons of the effectiveness of natural antifouling substances on young *vs* old larvae of the same species are important in an ecological context as well as an applied (Branscomb & Rittschof, 1984; Rittschof et al. 1984) context.

In this study, the response of old and young larvae of two species of bryozoans to naturally occurring antifoulant substances derived from marine macroalgae is examined. Concentrations of antifouling substances that were ecologically relevant surface concentrations for each macroalgal species were used, as well as larval periods for each bryozoan that are likely to occur under field conditions.

Methods

Collection of larvae

All experiments were carried out during March 2004. To collect larvae from the bryozoans, reproductively mature colonies of *Bugula neritina* and *Watersipora subtorquata* were collected from Rose Bay, Sydney Harbour, New South Wales, Australia. The colonies were held in aquaria (as part of a recirculating system) in constant darkness for 2 d, then exposed to bright light. *B. neritina* and *W. subtorquata* colonies begin spawning, approximately 15 min and 1 h, respectively, after exposure to light. Larvae from at least 15 colonies were pooled together and collected within 20 min of release. Larvae of both species are competent to settle immediately after release (Marshall & Keough, 2003).

Manipulation of larval age

After collecting the larvae for each species, larvae were randomly allocated into either a 'delayed' or 'non-delayed' treatment. All the larvae were put into new, 90 mm diameter glass Petri dishes ($\sim$5 larvae per Petri dish) containing 10 ml of 0.2 μm filtered seawater. The dishes allocated to the delay treatment were then placed on a shaker table, the surface of which had been covered by aluminium foil. The shaker table was placed 30 cm under two fluorescent tubes (36 watts) and the table was set to rotate at 30 rpm. *W. subtorquata* larvae were delayed for 6 h and *B. neritina* larvae were delayed for 4 h. No settlement was observed for those larvae whose settlement was being delayed. Larvae allocated to the non-delayed treatment were immediately placed into the surface-treated Petri dishes (see below).

Extraction of surface chemicals

Non-polar surface chemicals were extracted from the red alga *Delisea pulchra* (Greville) and the brown alga *Dilophus marginatus* J. Agardh. *D. pulchra* produces a range of biologically active non-polar secondary metabolites (furanones; de Nys et al. 1992; 1993) which are known to reduce the attachment of bacteria (Maximilien et al. 1998) and inhibit settlement of epiphytes and invertebrate larvae (de Nys et al. 1995). *D. marginatus* produces biologically active diterpenes (Ravi & Wells, 1982) and non-polar metabolites extracted from the surface of *D. marginatus* inhibited the settlement of gametes from the green alga *Ulva lactuca* and larvae from the serpulid polychaete *Hydroides elegans* in laboratory assays (de Nys et al. unpublished data).

Plants of each species were randomly collected from sublittoral habitats (1 – 3 m water depth) at Bare Island (33° 59′ 38″ S, 151° 14′ 00″ E) at the north head of Botany Bay, Sydney, Australia. Any epibionts were removed from each alga (all the plants were relatively free of fouling organisms). The algae were then spun in a salad spinner to remove excess surface water. To create the surface treated Petri

dishes, pieces of *D. pulchra* and *D. marginatus* (approx. 130 mg and 53 mg each, respectively) equal in surface area to the surface area of the Petri dishes ($\sim 9\,cm^2$) (de Nys et al. 1998; Dworjanyn & Steinberg, 1999), were removed from each plant and vortexed in vials containing 4.5 ml of hexane for 30 s. This is sufficient time to extract non-polar surface compounds without lysing the cells on the surface of the algae (de Nys et al. 1998). Because the extraction efficiency of the procedure is not known, for *D. pulchra* dishes at twice natural concentrations were also created by vortexing 260 mg of plant $vial^{-1}$. After vortexing, the pieces of algae were removed and the vials placed in a fume hood and the hexane allowed to evaporate overnight. The following day, the residual extracts in each vial were redissolved in 0.5 ml hexane and transferred to labeled $9\,cm^2$ Petri dishes. The Petri dishes were then placed on an orbital shaker in the fume hood until the hexane had evaporated. Hexane controls were prepared as for the algal extracts except that no algae were added to the control vials. A total of three experimental runs were performed. For *W. subtorquata*, delayed and undelayed larvae were exposed to hexane controls, *D. marginatus* extract and *D. pulchra* extract. For *B. neritina*, in one run delayed and undelayed larvae were exposed to controls and to *D. marginatus* extract and in another run delayed and undelayed larvae were exposed to *D. pulchra* extract at natural and twice natural surface concentrations. For *B. neritina*, five replicates were used for each treatment combination and for *W. subtorquata* three replicates were used for each treatment combination.

Settlement assays

Sixteen larvae were placed in each Petri dish in a temperature controlled room (20°C). The surface-treated Petri dishes contained 4 ml of 0.2 μm filtered seawater and sat on a black surface to encourage settlement (Marshall & Keough, 2003). Larvae were checked for settlement 1 h after placement into the Petri dishes, and were classed as 'settled' if they had begun metamorphosis and 'not settled' if they were still free swimming. No larvae died during the course of the experiments.

Data analysis

We examined the effects of larval age and settlement surface type on the percentage settlement of larvae within each Petri dish (Petri dish was the replicate) using ANOVA. When significant interactions between settlement surface and larval age were found, simple main effects tests were used to examine the source of the interaction. Where a significant main effect of settlement surface was found, one sided Dunnett's tests were used to examine which extractions differed from the control surface.

Results

In the first experiment, the settlement of young *W. subtorquata* larvae was inhibited by *D. pulchra* and *D. marginatus* extracts (one sided Dunnett's tests: *Dilophus vs* control, $p = 0.036$, *Delisea vs* control, $p = 0.0045$). Overall, older larvae settled at higher rates on all three surfaces (see Table I, Figure 1). Similarly, for *B. neritina* larvae, *D. marginatus* extracts inhibited settlement but older larvae showed much higher settlement than younger larvae (Table I, Figure 2).

In the second experiment with *D. pulchra* and *B. neritina*, there was an interaction between settlement surface and larval age (Table II). Older larvae

Table I. ANOVA for the effects of surface type and metamorphic delay on the settlement of *W. subtorquata* and *B. neritina*. Surfaces were *D. marginatus* and *D. pulchra* extracts for *W. subtorquata* and *D. marginata* only for *B. neritina*. Significant *p* values shown in bold.

Source	df	MS	F	*p*
a) *W. subtorquata*				
Delay	1	0.173	4.8	**0.048**
Surface	2	0.201	5.6	**0.019**
Interaction	2	0.025	0.7	0.513
Residual	12	0.063		
b) *B. nertina*				
Delay	1	0.5970	6.9284	**0.0181**
Surface	1	0.5818	6.7514	**0.0194**
Interaction	1	0.0630	0.7307	0.4053
Residual	16			

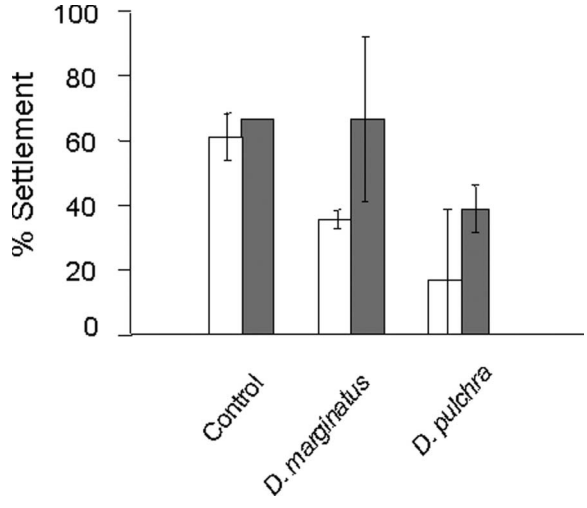

Figure 1. Effect of larval age and surface type on the mean (% ± SE) settlement of *W. subtorquata* larvae. □ = the settlement of newly released larvae; ■ = the settlement of delayed larvae.

settled more than younger larvae on both surfaces containing *D. pulchra* surface extract but there was no difference in the settlement of old and young larvae on the control surface (Table III, Figure 3). The 2× surface extract values did not unduly influence the results as omitting these values did not change the outcome of the analysis (Interaction: $F_{1,16} = 6.3$, $p = 0.02$).

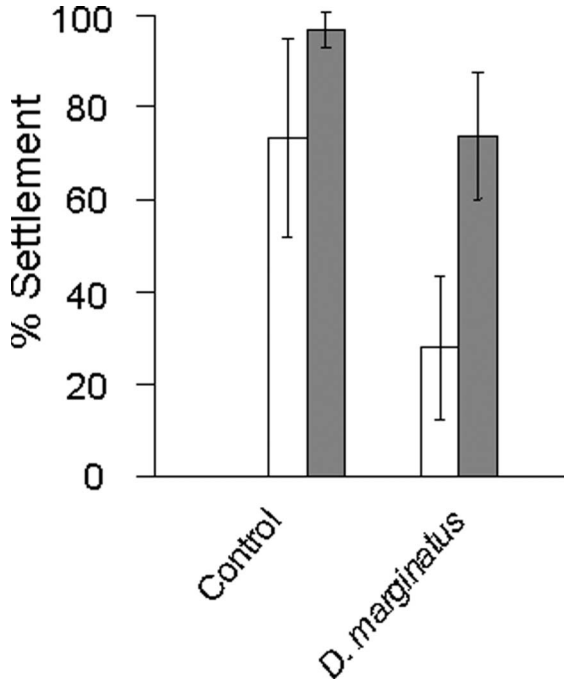

Figure 2. Effect of larval age and surface type on the mean (% ± SE) settlement of *B. neritina* larvae. □ = the settlement of newly released larvae; ■ = the settlement of delayed larvae.

Table II. Effect of surface type and larval age on settlement of *B. neritina* for control surfaces, *Delisea* extraction and double *Delsea* extraction. Significant *p* values shown in bold.

Source	Df	MS	F	*p*
Delay	1	0.592	21.3	**<0.001**
Surface	2	0.458	16.5	**<0.001**
Interaction	2	0.115	4.2	**0.028**
Residual	24	0.028		

Table III. Simple main effects test for delay *vs* not delayed *B. neritina* larvae for each settlement surface type. All f-ratios calculated using a model denominator of 0.028 with degrees of freedom of 1 and 24 for the numerator and denominator respectively. Significant *p* values shown in bold.

Surface	F	*p*
D. pulchra (1×)	7.610	**0.011**
D. pulchra (2×)	21.422	**0.000**
Control surface	0.330	0.571

Discussion

Delaying the metamorphosis of larvae generally resulted in increased settlement in the presence of a settlement inhibitor. This delay was likely to be an ecologically relevant delay period and for one species at least (*W. subtorquata*) represents a typical delay period in the field (Marshall & Keough, 2003). The delay period used here did not interfere with the normal metamorphosis of *B. neritina* and *W. subtorquata*. In other words, larvae that were delayed for the period used in this study were still capable of post-metamorphic survival and growth although growth may be lower in delayed larvae (Wendt, 1998). Larvae of each species that had experienced a metamorphic delay double that was used here can still successfully metamorphose (Marshall, unpublished data). From the results, it appears that larval settlement behaviour is very dynamic. Larvae appear to be altering their behavioural responses according to their energetic state, as young larvae that presumably have higher energetic reserves reject poor settlement surfaces but older larvae settle despite the presence of antifouling substances. Given that the metamorphic delay period did not deplete larval resources completely, it appears that the costs of settling on a poor settlement surface are outweighed by the costs of further delaying metamorphosis.

From the perspective of organisms that produce antifouling substances, the results show that older larvae were much less inhibited by antifouling

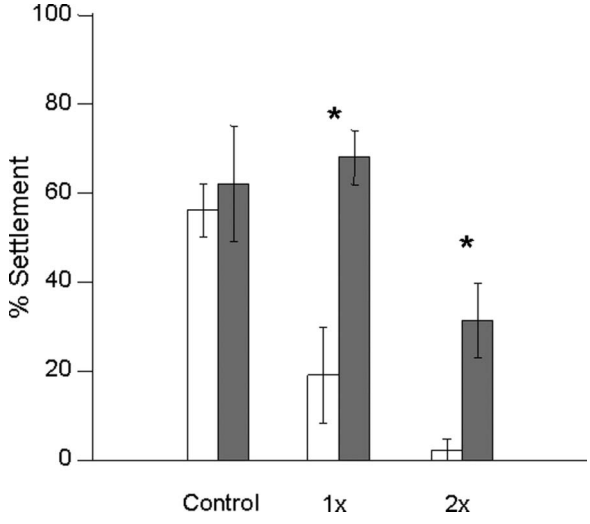

Figure 3. Effect of larval age and surface type on the mean (% ± SE) settlement of *B. neritina* larvae for three types of settlement surface: controls, non-polar extracts from the surface of *D. pulchra* and non-polar extracts from the surface of *D. pulchra* at twice the normal concentration. □ = the settlement of newly released larvae; ■ = the settlement of delayed larvae; * = significant differences found for the settlement of old and young *B. neritina* larvae using *post hoc*, simple main effects tests.

substances than younger larvae. Indeed, doubling the natural surface concentration of antifouling substances for *D. pulchra*, whilst strongly inhibiting young larvae did not have a significant inhibitory effect on old *B. neritina* larvae. At twice natural concentrations of surface extracts of *D. pulchra*, Steinberg et al. (2001) found that the settlement of *B. neritina* larvae was comparable to those for young larvae in this study, although settlement was slightly higher at natural concentrations. This suggests that rather than inhibiting the settlement of all potentially fouling propagules, *D. pulchra* and *D. marginatus* are producing enough antifouling substances to inhibit only some of the propagules. Given the reduced sensitivity of older larvae to the inhibitory substances, it may be more efficient to produce enough antifoulant to deter only a proportion of the fouling propagule population. Clearly, the fact that some larvae appear to be unaffected by the negative settlement cues changes the way the cost/benefit of producing antifouling substances by organisms in the real world (Steinberg & de Nys, 2002) should be viewed.

It is now recognised that the quality of settling larvae can vary substantially within a single population due to differences in exposure to pollutants, maternal provisioning, age and nutritional history (Pechenik et al. 1998; Phillips & Gaines, 2002; Marshall et al. 2003a; Ng & Keough, 2003). Larval condition can significantly influence the habitat choice and settlement success of larvae (Pechenik et al. 1993; Jarrett, 1997). This variability in larval quality has not been examined in detail in the context of antifouling studies, or has been recognised but excluded from experimental designs with the consequence that most previous studies have employed either young larvae or only used larvae of a single age when assessing the settlement response of larvae to antifoulants (e.g. de Nys et al. 1995; Cho et al. 2001; Nylund & Pavia, 2003; Hellio et al. 2004; Maréchal et al. 2004; Nogata et al. 2004). In one of the few studies incorporating larval variability in an antifouling context, gregarious settlement of cyprids of *Balanus amphitrite* exposed to organic settlement promoters and inhibitors significantly affected the interpretation of results (Head et al. 2003). Subsequent work has found that the gregarious settlement behaviour of the cyrids of *B. amphitrite* and *B. improvisus* (in the absence of settlement inducers or inhibitors) was affected by both the age of larvae and the duration of the experiment (Head et al. 2004). Settlement of both *B. improvisus* and *B. amphitrite* increased with experimental duration, whereas, the effect of larval age decreased settlement in *B. improvisus* and increased settlement in *B. amphitrite*. The present study has shown that larval age significantly affects the settlement response of larvae to natural antifoulants. As a consequence, any assessment of the efficacy of antifoulants (either natural or artificial) must include consideration of the potential variability in response by larvae of different ages. Indeed, testing of potential antifouling substances in an applied context should arguably be on the most difficult to deter (older, in this case) proportion of the larval population as this should lead to the most conservative estimate of the potential for a substance to inhibit settlement in the field.

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