J. Mar. Biol. Ass. U.K. (2002), **82**, 117–129
Printed in the United Kingdom

The effect of pedal mucus on barnacle cyprid settlement: a source for indirect interactions in the rocky intertidal?

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Laboratory assessment of barnacle cyprid settlement showed that it was increased by a multiple of ~6 and by a multiple of ~3 by the pedal mucus produced by *Patella vulgata* and by *Littorina littorea*, respectively. Field experiments showed that pedal mucus produced by *P. vulgata* could increase cyprid settlement by a multiple of ~4, but that there was no effect of the pedal mucus produced by *L. littorea*. Evaluation of the effect of pedal mucus coated with nitro-cellulose and various pedal extracts, on cyprid settlement, ascertained that there appeared to be no chemotactic or chemotaxic effect of pedal mucus on cyprid settlement. In contrast, the use of a physical analogue to pedal mucus, silicon grease, increased cyprid settlement by a multiple of ~ 18.

Pedal mucus produced by *P. vulgata* and by *L. littorea* increased the time spent by cyprids in surface suitability testing by a multiple of ~ 10 and ~3, respectively. Only the pedal mucus produced by *P. vulgata* had any effect on the exploratory behaviour of cyprids increasing the time spent on this behaviour by a multiple of ~3. Pedal mucus affects the settlement of cyprids through adhesive enmeshment, resulting in positive feedback to the mechanoreceptors housed in the antennules of cyprids, in what is effectively a settlement cascade. Pedal mucus produced by *P. vulgata* and *L. littorea* can affect the settlement of the majority of settling marine organisms through physical entrapment. Pedal mucus produced by *L. littorea* will have little, if any, effect on the settlement of organisms in the field whereas the pedal mucus produced by *P. vulgata* may be of major importance in determining the adult distribution patterns.

INTRODUCTION

The larvae of many sessile intertidal marine invertebrates have been shown to use both environmental and biological cues to assess the suitability of settlement sites (see Crisp, 1974, 1984; Pawlik, 1992 for reviews). For some species, these settlement cues can originate from non-trophically related organisms that occupy the same habitat as the adult form of the settling larvae (see De Silva, 1962; Hadfield & Scheuer, 1985; Mokady et al., 1992 for examples). This indirect interaction between settling organisms and a non-trophically related co-habiting species, can offer an alternative explanation to the adult distribution patterns of settling organisms that have classically been attributed to post-settlement mortality, arising from predation, competition and/or biological disturbance (Connell, 1961; Menge, 1976; Hawkins, 1981; Berlow & Navarrete, 1997; Buschbaum, 2000). The importance of the indirect interactions occurring between grazers and co-existing species, with regard to community formation and succession, has only been realized in the last two decades (Connell & Slayter, 1977; Dethier & Duggins, 1984; Dugan, 1986; Van Tamlen, 1987; Farrell, 1991; Geller, 1991; Povey & Keough, 1991; Menge, 1995). Studies have shown that pedal mucus produced by such molluscs can either stimulate or inhibit the settlement of organisms, depending upon both the species of the settling organism and the species of the mollusc (see Connor & Quinn, 1984; Connor, 1986; Raimondi, 1988; Johnson & Strathmann, 1989; Davies et al., 1992; Proud, 1994; Santelices & Bombadilla, 1996).

Three species, which lend themselves to the investigation of the possible indirect effects of pedal mucus on the settlement of organisms, are *Patella vulgata* L., *Littorina littorea* (L.) and *Semibalanus balanoides* (L.). These organisms can comprise the dominant fauna of the mid-littoral zone on most north-eastern Atlantic temperate rocky shores (Southward, 1958; Moyse & Nelson-Smith, 1963). Both *P. vulgata* and *L. littorea* are mobile grazing gastropods which use mucus exuded from their foot, in combination with muscular locomotor waves on the sole of their foot, as their means of locomotion (Fretter & Graham, 1994). In contrast, *S. balanoides* is a sessile marine invertebrate (barnacle), whose adult distribution is determined by the settlement and survival of its non-feeding larval stage, the cyprid. In choosing a suitable settlement site, the settling cyprids interact with a complex series of cues including biomolecules, hydrodynamics, surface texture, colour and elemental composition (see Barnes, 1970; Crisp, 1974, 1984; Lewis, 1978 for reviews).

Of all the cues perceived by cyprids, many authors have suggested that chemical cues can elicit the strongest settlement responses (see Crisp, 1974, 1984; Yule & Walker, 1987; Hui & Moyse, 1987 for reviews). For cyprids, these cues are not limited to water-soluble chemotaxic cues, i.e. as commonly observed for other marine settling larvae (see Hahn, 1989; Pawlik 1990, 1992 for reviews), but also include a range of surface-bound chemotactic cues, e.g. arthropodin (see Hui & Moyse, 1987; Gabbot & Larman, 1987 for reviews). For this paper, chemotaxic cues are

defined as 'water-soluble chemical cues that can elicit a settlement response in an organism, when that organism is able to detect the presence of that cue within the water column'. In contrast, chemotactic cues are defined as 'chemical cues which are irreversibly bound to a substratum that can elicit a settlement response in an organism only when that organism makes physical contact with that chemical cue'.

Differentiation between the potential chemotaxic and chemotactic effects of a substance on cyprid settlement can be made by examining the effects of various extracts of the material, both coated on surfaces and diffused within the water column (see Knight-Jones, 1953; Crisp & Meadows, 1962) and/or by coating the surface coated with the substance with a permeable membrane (e.g. nitro-cellulose; see Knight-Jones, 1953). Permeable membranes will allow water soluble compounds to diffuse into the water column but prevent the sensory apparatus of an organism from contacting it.

In addition to chemotaxic and chemotactic cues, there are two further sources of stimuli that can originate from a material, with specific reference to pedal mucus, which may affect the settlement of cyprids. These are, the physio-chemical properties of the material (see Holmes et al., 2002) and the ability of a material to mechanically enmesh settling cyprids (see Zobell, 1938, 1939). For the former effector, Holmes et al. (2002) have documented how the physio-chemical properties of the pedal mucus produced by *P. vulgata* and by *L. littorea* may effect the settlement of organisms. Assessment of the potential effect of the latter effector on cyprid settlement can be made in the following ways: (1) by using a chemically inert analogue to the material. That is, if the effects of the analogue either match or approximate the results obtained for the effects of the material, then it is likely that individually or in combination with chemical cues, physical entrapment of the cyprids in the material is responsible for any observed increase in cyprid settlement; (2) by examining the behaviour of cyprids in response to the material. That is, Visscher (1928), Crisp (1955, 1974), Crisp & Meadows (1962), Barnes (1970), Yule & Walker (1984, 1987), Rittschof et al. (1984) and Neal & Yule (1992) have all recorded various facets of cyprid behaviour in response to various surfaces. Of particular interest are the cyprid behaviours of exploration and surface suitability testing (antennule tugging). Exploratory behaviour is generally regarded as the initial ambulatory phase in cyprid behaviour, exhibited when a cyprid contacts a surface. The second behavioural phase, antennule tugging, occurs when a cyprid uses its antennules to determine exactly how suitable a surface is for settlement. If the cue is tactile/adhesive in origin then an increase in the two behaviours should be observable resulting in a settlement cascade that may be abandoned at any time in the face of unsuitable stimuli (Neal & Yule, 1992).

With regard to the potential effects of pedal mucus on cyprid settlement Raimondi (1988), Johnson & Strathmann (1989) and Proud (1994) have all found that the pedal mucus produced by various molluscs can increase the settlement of barnacle cyprids. In particular, Proud (1994) has shown that surfaces pre-conditioned, in the field, with the pedal mucus produced by *L. littorea* can, in their absence, increase cyprid settlement. Similarly Davies et al. (1992) have shown that the pedal mucus produced by *P. vulgata* can increase the settlement of macroalgal propagules and microalgae. It is possible then that, the pedal mucus produced by *P. vulgata* and/or *L. littorea* may have important consequences for the settlement and success of organisms in the rocky intertidal. The aims of this paper are as follows: (i) to determine, quantitatively, the potential effect of the pedal mucus produced by *P. vulgata* and by *L. littorea* on the settlement and settling behaviour of *S. balanoides* cyprids; (ii) to elucidate the mechanism of the effect (i.e. the source) of the pedal mucus on cyprid settlement.

MATERIALS AND METHODS

General methods

All experiments were carried out over two cyprid settlement seasons, at a different site for each season. The first set of experiments were carried out in Spring 1995 at the University Marine Biological Station, Millport, Isle of Cumbrae, Scotland, and the second set of experiments were carried out in Spring 1996 at Port Erin Marine Laboratory, Port Erin, Isle of Man. For some experiments, data were collected only during one settlement season, whilst for other experiments the experimental procedure was repeated at both sites. All laboratory experiments utilized an identical procedure, in which new borosilicate ground glass slides (Chance Propper Ltd, Warley, UK), 75×25×1 mm, were used as a uniform substratum, after they had been autoclaved for 4 h at 1.2×10^5 Pa to ensure sterility (Prescott et al., 1993). Slides were prepared in the following manner according to each experiment, as follows: (A) the glass slides were placed on to the bottom of a tank, which was filled with running filtered (45 μm) seawater (10°C), for 12 h as a control; (B) as (A), but with the exception that freshly collected *Patella vulgata*, enough to completely cover all of the exposed surface of the slides, were placed onto the submerged slides and allowed to crawl over them for 12 h; (C) as (B), but using *Littorina littorea* instead of *P. vulgata* as the producer of pedal mucus. The periwinkles were prevented from crawling off the slides and up the sides of the tank by placing a wire mesh (5×5 mm) approximately 10 mm above the height of the largest individual; (D) an extract was prepared by dissecting out the feet of 40 limpets and then liquidizing them using a food blender in 400 ml of ultra violet irradiated seawater (UVFS) (0.2 μm). The resultant solution was then centrifuged at 4500*g* for 5 min and the supernatant decanted off (hereafter referred to as the limpet standard). The glass slides were then dipped into the limpet standard and left to dry for 5–6 h at 20°C; (E) as (D), but using 100%, AristaR grade ethanol (BDH Ltd, Poole, UK) as the extracting agent. Ethanol was used as the extracting agent because of its ability to dissolve lipids and lipid complexes (Zubay, 1993); (F) as (D), but using, a periwinkle standard prepared in an identical manner to the limpet standard (hereafter referred to as the periwinkle standard) with the exception that 1000 periwinkle feet were used as the source material; (G) as (F) but using 100%, AristaR grade ethanol as the extracting agent; (H) the glass slides were lightly smeared, by hand, with silicon grease (10°C) (Aqua Seal, California, USA) to a depth of ~0.2 mm and used within 1 h of preparation.

For treatment B, the glass slides coated with the pedal mucus produced by *P. vulgata* were individually inspected, by eye, for the presence of pedal mucus and the area covered (always $\geqslant 70\%$ of the slide surface) by the pedal mucus was marked on the underside of the slide with a non-toxic marker. Areas on the control slides (i.e. treatment A) were randomly marked, in an identical manner, as a procedural control. For the slides coated with the pedal mucus produced by *L. littorea* it was assumed that the entire slide surface had been coated with pedal mucus. Enumeration of the number of cyprids adhering to the pedal mucus produced by *P. vulgata* was only made for cyprids adhering to areas previously marked as coated with pedal mucus. Any slides not obviously bearing any pedal mucus were rejected from further experimentation.

Following the application of the treatments, pertinent to the experiment, the glass slides were placed in a random order (treatment face up) on to the bottom of a Perspex (acrylic plastic) tank (50×50×30 cm, height×width× depth, respectively) that had been filled with 15 l of UVFS. Individual treatments were placed in separate tanks, with the exception of the appropriate control treatments, to minimize the possibility of cross contamination occurring, from diffuse water-soluble chemical cues, between treatments. Approximately 1000 freshly trawled *Semibalanus balanoides* cyprids were then introduced in to each tank. The cyprids were collected by hand from the shores of the Isle of Cumbrae and Isle of Man, using a 210-μm mesh size plankton net. The water in the tanks was maintained at 10°C under constant fluorescent illumination (4.73 μE m^{-2} s^{-1} at Millport and 6.95 μE m^{-2} s^{-1} at Port Erin) and stirred using a roto-mix stirrer at 10 ± 2 mm s^{-1} (mean water current ±SE), calculated from 100 randomly selected points within the experimental tanks) throughout the experimental period (12 h). Before experimentation, all slides and cyprids were maintained at 10°C to prevent thermal shock occurring. At the end of the experimental period, the glass slides were removed from the tank and washed in UVFS to displace all but the adhered cyprids. The number of cyprids attached to each slide was then counted, by eye, and noted. For any one replicate experiment, 10–15 slides per treatment were used. For experiments carried out on the Isle of Cumbrae, *P. vulgata* and *L. littorea* were collected from the mid-shore of White Bay (National Grid Reference (NGR) NS 177592), whilst for the experiments carried out on the Isle of Man, *P. vulgata* were collected from the mid-shore at St Mary's Ledges (NGR SC 213673) and *L. littorea* from Niarbyl Bay (NGR SC 214775).

All data analysis was made in accordance with the appropriate models and methods of calculation, for both the *F* statistics and pooled mean square values, as outlined in Sokal & Rohlf (2000). For all experiments, the treatment model term in the analyses is considered to be a fixed factor and all other terms random (see Bennington & Thayne, 1994 for discussion).

Experiment 1. The effects of the pedal mucus produced by P. vulgata *and by* L. littorea *on the settlement of* S. balanoides *cyprids: laboratory experiments*

All experiments were conducted at the University Marine Biological Station and repeated at Port Erin Marine Laboratory. Treatments A, B and C were applied individually, to 10–15 glass slides for each treatment, per replicate experiment and the protocol followed, as outlined. Eleven replicate experiments were performed on the Isle of Cumbrae and ten replicate experiments on the Isle of Man.

Experiment 2. The effect of P. vulgata *and* L. littorea *pedal extracts on the settlement of* S. balanoides *cyprids*

All experiments were performed at the University Marine Biological Station and at Port Erin Marine Laboratory. Treatments A, D, E, F and G were made to 15 glass slides for each treatment and the protocol followed, as outlined. For all treatments, ten replicate experiments were made.

To determine if an aqueous solution of limpet standard would affect cyprid settlement, 50 ml of the limpet standard was added to 5 l UVFS. Ten untreated glass slides were then placed on the bottom of a tank, which had been filled with UVFS containing the limpet standard and half the batch of freshly trawled cyprids. A control tank was then prepared, which contained ten slides, the other half of the cyprid trawl and 5.05 l of UVFS. Both tanks were maintained under the standard experimental conditions for 12 h. At the end of the experimental period, the slides were removed, washed in UVFS, and the number of adhered cyprids counted, by eye. The whole experiment (all treatments) was repeated a further nine times on consecutive days (i.e. ten replicate experiments in total). An identical procedure was adopted for *L. littorea* using the periwinkle standard, ten replicate experiments in total.

Experiment 3. The effect of the pedal mucus produced by P. vulgata *and by* L. littorea, *encased in a nitro-cellulose barrier, on the settlement of* S. balanoides *cyprids*

Treatments A, B and C were applied, individually, to 15 glass slides, per treatment per replicate experiment. The glass slides (i.e. pedal mucus coated and control) were then dipped into a 25% (v/v, di-ethylamine/chloroform) nitro-cellulose solution (Colloidin, BDH Ltd, Poole, UK) and left to dry for 1 h. Nitro-cellulose was selected as a thigomo-tactic barrier (see Knight-Jones, 1953) to allow diffuse settlement stimuli to leach off surfaces but prevent any direct physical contact. Each treatment was then run, following the protocol outlined, separately against a corresponding number of control slides, i.e. to minimize the potential of cross contamination occurring between treatments, under the standard experimental conditions. For all treatments, ten replicate experiments were conducted at the Port Erin Marine Laboratory.

Experiment 4. The effect of a pedal mucus analogue (silicon grease) on the settlement of S. balanoides *cyprids*

Treatments A and H were made to ten glass slides, for each treatment and replicate experiment. Ten replicate experiments were carried out, in total, at the University Marine Biology Station. Silicon grease was used as a pedal mucus analogue because it has not been shown to affect cyprid settlement chemotaxically (Walters, 1992)

https://doi.org/10.1017/S0025315402005234 Published online by Cambridge University Press

and has been used elsewhere as an analogue (Holmes et al., 2002).

Experiment 5. The effects of the pedal mucus produced by P. vulgata *and* L. littorea *on the settlement behaviour of* S. balanoides *cyprids*

Glass slides, coated with the pedal mucus produced by *P. vulgata,* and control slides were prepared in an identical manner as for treatments A, B and C. A glass slide coated with the pedal mucus produced by *P. vulgata* (i.e. treatment slide) was then placed in a clear Perspex box (105×75×75 mm), along with a second control slide. The box was filled with UVFS and 100 freshly trawled cyprids added. The box was maintained throughout the experimental period at 10°C under a diffuse illumination of 14.23 μE m^{-2} s^{-1}. The behaviour of the cyprids was recorded on videotape for 3 h using a Panasonic F10 video camera with a ∼×40 magnification macro lens connected in line with a video recorder. This whole procedure was repeated a further five times on consecutive days (i.e. six replicate experiments in total) at the University Marine Biology Station. At the end of the experimental period the slides were gently washed in UVFS and the position of any adhered cyprids noted.

Using the video recording, in combination with the data recorded for the position of any adhered cyprids at the end of the experiment, the individual time spent in exploratory behaviour and then the individual time spent in surface suitability, i.e. the behaviour following exploratory behaviour, testing by ten randomly selected cyprids, for each treatment, was noted (i.e. N=10 for each treatment in each replicate experiment). Exactly the same procedure was repeated for the pedal mucus produced by *L. littorea*, with six replicate experiments being made in total at the Port Erin Marine Laboratory.

Experiment 6. The effects of the pedal mucus produced by P. vulgata *and by* L. littorea *on the settlement of* S. balanoides *cyprids: field experiments*

All experiments were performed at the University Marine Biological Station, Millport, Isle of Cumbrae, Scotland. Polished slate coupons (30×100×10 mm) of an average surface rugosity of 0.70 ±0.08 μm, measured using a mechanical stylus (N=100) (see Holmes et al., 1997), were used as a uniform experimental substratum after they had been autoclaved for 4 h at 120°C at 1.2×10^5 Pa to ensure sterility. For experiments using the pedal mucus produced by *P. vulgata*, two treatments were prepared as follows: (A) 24 coupons were randomly selected out of a total of 48 and placed onto the bottom of a tank filled with running filtered (45 μm) seawater, (10°C, 20 cm depth) subject to a 12:12 h L:D cycle, for 24 h as a control; (B) as (A), using the remaining 24 coupons, with the exception that the upper surface of the coupons were covered in freshly collected *P. vulgata*, from the mid-shore at White Bay, such that all of the exposed surface of the coupons was covered.

Once prepared, all coupons were removed from their tanks and fixed in a random order, to a floating boom (4×0.1 m, the coupons were suspended at a depth of 0.1 m throughout the tidal cycle) attached to Keppel Pier (NGR: NS 177546) and left for 24 h. After this period, the coupons were removed from the boom, washed in UVFS and the number of cyprids adhering to their surface counted by eye. This whole experimental procedure was repeated a further six times on consecutive days, i.e. seven replicate experiments in total. Because of limited coupon availability, after each experiment the coupons were soaked in 3M NaOCl solution for 1 h and then scrubbed and washed in freshwater, to which a small amount of detergent had been added (Tween, BDH Ltd, Poole, UK), in order to neutralize any effects of surface bound arthropodin (see Knight-Jones, 1953). They were then autoclaved at 120°C, 1.2×10^5 Pa for 4 h to ensure sterility and re-used.

An identical procedure was adopted for examining the effects of the pedal mucus produced by *L. littorea*, with the exception that only 11 coupons per treatment per replicate experiment were used. There were seven replicate experiments in total. The periwinkles were prevented from crawling off the coupons and up the sides of the tank by placing a wire mesh (5×5 mm) cage 10 mm above the shell of the largest individual. Complete coverage of the coupon surface by the pedal mucus produced by *L. littorea* was ensured by placing 1000 periwinkles into the tank and confirmed by visual inspection of the coupon surfaces after treatment.

Table 1. *Two-way, three factor, mixed model nested ANOVA, for the effect of the pedal mucus produced by* Patella vulgata *and* Littorina littorea *on the settlement of* Semibalanus balanoides *cyprids: laboratory experiments.*

	Patella vulgata pedal mucus$^{\#}$				*Littorina littorea* pedal mucus$^{\#}$			
Source of variation	df	Mean square	*F*	*P*	df	Mean square	*F*	*P*
Treatments ^{f} (1)	1	22.497	1963.368	* (***)	1	463.434	1118.757	* (***)
Sites ^{r} (2)	1	4.98×10^{-4}	7.68×10^{-3}	ns	1	0.232	0.067	ns
Replicate experiments ^{r}	19	0.243	3.750	***	18	10.116	2.946	***
1×2	1	1.15×10^{-2}	0.177	ns	1	0.414	0.121	ns
Residual	573	6.48×10^{-2}			680	3.434		
Total	595				701			

Probability levels: ns, not significant; ***, ⩽0.001; *, ⩽0.05; the characters in the parentheses indicate the probability calculated from the pooled mean squares (Sokal & Rohlf, 2000). $^{\#}$, denotes data log$_{10}$ transformed; ^{r}, that the variable has been defined as a random factor; ^{f}, denotes that the variable has been defined as a fixed factor.

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RESULTS

Experiment 1. The effects of the pedal mucus produced by Patella vulgata *and by* Littorina littorea *on the settlement of* Semibalanus balanoides *cyprids: laboratory experiments*

Analysis of the $\log_{10}$ transformed data, using a two-way, three factor, mixed model nested analysis of variance (ANOVA), revealed that cyprid settlement was increased on the pedal mucus produced by *P. vulgata* by a multiple of ~6 (6.35 ±0.75) relative to the control treatment (Table 1). The pooled means ±SE of cyprids settled on the *P. vulgata* pedal mucus and control slides were 1780 ±100 and 280 ±30 cyprids m^{-2}, respectively. Cross comparison, between both the sites (i.e. Isle of Cumbrae and Isle of Man) and replicate experiments analysis terms, revealed that there were no differences in the propensity of cyprids to settle between sites but that there were differences between replicate experiments, i.e. sampling days (Table 1).

Analysis of the data, using a two-way, three factor, mixed model nested ANOVA, revealed that cyprid settlement was increased on the pedal mucus produced by *L. littorea* by a multiple of ~3 (3.35 ±0.34) relative to the control treatment (Table 1). The pooled means ±SE number of cyprids settled on the *L. littorea* treatment and control slides was 940 ±60 and 280 ±24 cyprids m^{-2}, respectively. As for the effect of the pedal mucus produced by *P. vulgata*, cross comparison between both the sites and replicate experiments analysis terms revealed that there were no differences in the propensity of cyprids to settle between sites but that there were differences between replicate experiments (Table 1).

Experiment 2. The effect of P. vulgata *and* L. littorea *pedal extracts on the settlement of* S. balanoides *cyprids*

Analysis of the $\log_{10}$ transformed data, for each experiment, using a mixed model two-way ANOVA determined that cyprid settlement was not affected by any of the *P. vulgata* pedal extracts, both fixed and diffuse (Table 2A). The mean ±SE numbers of cyprids settled were 690 ±80, 550 ±60 and 629 ±55 cyprids m^{-2} for the glass slides coated with the water and ethanol extracts and the diffuse extract experiment, respectively, and 640 ±90, 620 ±80 and 608 ±40 cyprids m^{-2} for the respective control slides. The replicate experiments model term, within two of the analyses, was statistically significant indicating that there were differences in the numbers of cyprids settling between replicate experiments (Table 2A).

Analysis of the $\log_{10}$ transformed data, for each experiment, for the pedal extracts of *L. littorea* revealed that cyprid settlement, as for *P. vulgata*, was not affected by any of the pedal extracts, fixed or diffuse (Table 2B). The mean ±SE numbers of cyprids settled was 310 ±30, 340 ±30, 327 ±47 and 340 ±50, 370 ±30, 357 ±52 cyprids m^{-2} for the water extracts, ethanol extracts, diffuse experiment and respective control treatments. The replicate experiments model term in all of the analyses were statistically significant indicating that there were differences in the numbers of cyprids settling between replicate experiments (Table 2B).

Experiment 3. The effect of the pedal mucus produced by P. vulgata *and by* L. littorea, *encased in a nitro-cellulose barrier, on the settlement of* S. balanoides *cyprids*

Individual analysis of the data using a mixed model two-way ANOVA obtained for the effect of the nitro-cellulose encased pedal mucus produced by either *P. vulgata* or by *L. littorea* (data $\log_{10}$ transformed) revealed that cyprid settlement was not affected by the pedal mucus of either species (Table 3). The mean ±SE number of cyprids settled on the control and *P. vulgata* treatment glass slides were 260 ±30 and 220 ±30 cyprids m^{-2}, respectively, and for the *L. littorea* treatment and control glass slides 192 ±20 and 170 ±20 cyprids m^{-2}, respectively. The replicate experiments model terms within both of the analyses were statistically significant indicating that there were differences in the numbers of cyprids settling between replicate experiments (Table 3).

Experiment 4. The effect of a pedal mucus analogue (silicon grease) on the settlement of S. balanoides *cyprids*

Analysis of the $\log_{10}$ transformed data using a mixed model two-way ANOVA revealed that silicon grease could increase cyprid settlement by a multiple of ~18 (17.63 1.33), with respect to the control surface (Table 3). The mean ±SE numbers of cyprids settled were 6700 ±890 and 380 ±60 cyprids m^{-2}, on the treatment and control glass slides, respectively. The replicate experiments model term within the analyses was statistically significant indicating that there were differences in the numbers of cyprids settling between replicate experiments (Table 3).

Experiment 5. The effects of the pedal mucus produced by P. vulgata *and by* L. littorea *on the settlement behaviour of* S. balanoides *cyprids*

Direct observation of the settlement behaviour of the cyprids during the experimental period revealed the following: (i) that when cyprids contacted a surface coated with pedal mucus their antennules appeared to become entrapped within the pedal mucus matrix; (ii) that following their entrapment the cyprids would vigorously kick back (tug), in a similar manner to surface suitability testing, in what appeared to be an attempt to free themselves from the pedal mucus.

Individual analysis of the $\log_{10}$ transformed data, using a mixed model two-way ANOVA, obtained for the time spent by the cyprids in both exploratory and surface suitability testing behaviour on the pedal mucus produced by *P. vulgata* revealed that for both behavioural categories, cyprids spent more time in either behavioural category on pedal mucus, than they did on the control surface (Table 4A). The mean ±SE time period spent in exploratory behaviour was 122.5 ±8.3 and 44.3 ±3.3 s for the slides coated with the pedal mucus produced by *P. vulgata* and the control slides, respectively. The mean ±SE time periods spent in surface suitability testing were 75.1 ±6.0 and 7.5 ±1.0 s for the slides coated with the pedal mucus produced by *P. vulgata* and for the control slides, respectively. Therefore, the pedal mucus produced by *P. vulgata*

https://doi.org/10.1017/S0025315402005234 Published online by Cambridge University Press

https://doi.org/10.1017/S0025315402005234 Published online by Cambridge University Press

Table 2 *Two-way mixed model ANOVA, for the effects of the pedal extracts on the settlement of* Semibalanus balanoides *cyprids.*

A. *Patella vulgata.*

	Water based extract#				Ethanol based extract#				Diffuse extract#			
Source of variation	df	Mean square	*F*	*P*	df	Mean square	*F*	*P*	df	Mean square	*F*	*P*
Treatments [f] (1)	1	4.42×10^{-2}	1.373	ns (ns)	1	2.88×10^{-5}	4.74×10^{-4}	ns (ns)	1	1.46×10^{-4}	3.19×10^{-3}	ns (ns)
Replicate experiments [r] (2)	9	0.242	3.758	***	9	0.103	1.800	ns	9	0.181	3.078	**
1×2	9	3.22×10^{-2}	0.500	ns	9	6.08×10^{-2}	1.063	ns	9	4.58×10^{-2}	0.779	ns
Residual	180	6.4×410^{-2}			180	5.72×10^{-2}			180	5.88×10^{-2}		
Total	199				199				199			

B. *Littorina littorea.*

	Water based extract#				Ethanol based extract#				Diffuse extract#			
Source of variation	df	Mean square	*F*	*P*	df	Mean square	*F*	*P*	df	Mean square	*F*	*P*
Treatments [f] (1)	1	1.42×10^{-3}	0.034	ns (ns)	1	5.86×10^{-2}	3.311	ns (ns)	1	8.65×10^{-2}	1.856	ns (ns)
Replicate experiments [r] (2)	9	7.79×10^{-2}	2.782	**	9	8.69×10^{-2}	3.195	***	9	7.79×10^{-2}	2.782	**
1×2	9	4.23×10^{-2}	1.511	ns	9	1.77×10^{-2}	0.651	ns	9	4.66×10^{-2}	1.664	ns
Residual	280	2.80×10^{-2}			280	2.72×10^{-2}			280	2.80×10^{-2}		
Total	299				299				299			

Probability levels: ns, not significant; ***, ⩽0.001; **, ⩽0.01; the characters in the parentheses, indicate the probability calculated from the pooled mean squares (Sokal & Rohlf, 2000). #, denotes data $\log_{10}$ transformed; [r], that the variable has been defined as a random factor; and [f], denotes that the variable has been defined as a fixed factor.

Table 3. *Two-way mixed model ANOVA, for the effects of the pedal mucus produced by* Patella vulgata *and by* Littorina littorea*, encased in nitro-cellulose, and silicon grease on cyprid settlement.*

Source of variation	*Patella vulgata* pedal mucus				*Littorina littorea* pedal mucus#				Silicon grease#			
	df	Mean square	F	*P*	df	Mean square	*F*	*P*	df	Mean square	*F*	*P*
Treatments [f] (1)	1	0.403	2.056	ns (ns)	1	1.52×10^{-3}	0.559	ns (ns)	1	27.561	423.364	*** (***)
Replicate experiments [r] (2)	9	1.334	3.737	***	9	7.39×10^{-2}	3.079	**	9	0.139	2.135	*
1×2	9	0.196	0.549	ns	9	2.72×10^{-3}	0.113	ns	9	4.91×10^{-2}	0.754	ns
Residual	280	0.357			280	2.40×10^{-2}			180	6.51×10^{-2}		
Total	299				299				199			

Probability levels: ns, not significant; ***, ⩽0.001; *, ⩽0.05; the characters in the parentheses, indicate the probability calculated from the pooled mean squares (Sokal & Rohlf, 2000). #, denotes data $\log_{10}$ transformed; [r], that the variable has been defined as a random factor; and [f], denotes that the variable has been defined as a fixed factor.

can increase the time spent by cyprids in exploratory behaviour by a multiple of ∼3, and the time spent by cyprids in surface suitability testing by a multiple of ∼10, with reference to the control surface. The replicate experiments model term for the analysis of the surface suitability testing behaviour was statistically significant, indicating that there were differences in the relative behaviour of the cyprids between replicate experiments.

Individual analysis of the $\log_{10}$ transformed data, using a mixed model two-way ANOVA, obtained for the time spent by the cyprids in both exploratory and surface suitability testing behaviour on the pedal mucus produced by *L. littorea*, revealed that there was no statistically significant difference in the time spent by cyprids in exploratory behaviour on either the control or treatment slides (Table 4B). The mean ±SE time periods spent in exploratory behaviour were 52 ±8 and 45 ±7 s for the slides coated with the pedal mucus produced by *L. littorea* and for the control slides, respectively. In contrast, however, comparison between the treatments for the time spent by cyprids in surface suitability testing, revealed that the time spent by the cyprids was longer on pedal mucus than it was on a control surface (Table 4B). The mean ±SE time periods spent in surface suitability testing were 30 ±2 and 10 ±1 s for the slides coated with the pedal mucus produced by *L. littorea* and for the control slides, respectively. Therefore, the pedal mucus produced by *L. littorea* can increase the time spent by cyprids in surface suitability testing by a multiple of ∼3, with reference to the control surface. As for

Table 4. *Two-way mixed model ANOVA, for the effect of the pedal mucus produced by A or B on the settlement behaviour of* Semibalanus balanoides *cyprids.*

A. *Patella vulgata.*

Source of variation	Exploratory behaviour#				Surface suitability testing#			
	df	Mean square	*F*	*P*	df	Mean square	*F*	*P*
Treatments [f] (1)	1	4.158	176.186	*** (***)	1	26.309	339.033	*** (***)
Replicate experiments [r] (2)	5	4.88×10^{-2}	1.755	ns	5	0.171	2.984	*
1×2	5	2.36×10^{-2}	0.849	ns	5	7.76×10^{-2}	1.354	ns
Residual	108	2.78×10^{-2}			108	5.73×10^{-2}		
Total	119				119			

B. *Littorina littorea.*

Source of variation	Exploratory behaviour#				Surface suitability testing#			
	df	Mean square	*F*	*P*	df	Mean square	*F*	*P*
Treatments [f] (1)	1	7.03×10^{-2}	3.056	ns (ns)	1	5.814	77.211	*** (***)
Replicate experiments [r] (2)	5	4.80×10^{-2}	1.727	ns	5	0.172	2.930	*
1×2	5	2.30×10^{-2}	0.827	ns	5	7.53×10^{-2}	1.283	ns
Residual	108	2.78×10^{-2}			108	5.87×10^{-2}		
Total	119				119			

Probability levels: ns, not significant; ***, ⩽0.001; *, ⩽0.05; the characters in the parentheses indicates, the probability calculated from the pooled mean squares (Sokal & Rohlf, 2000). #, denotes data $\log_{10}$ transformed; [r], that the variable has been defined as a random factor; and [f], that the variable has been defined as a fixed factor.

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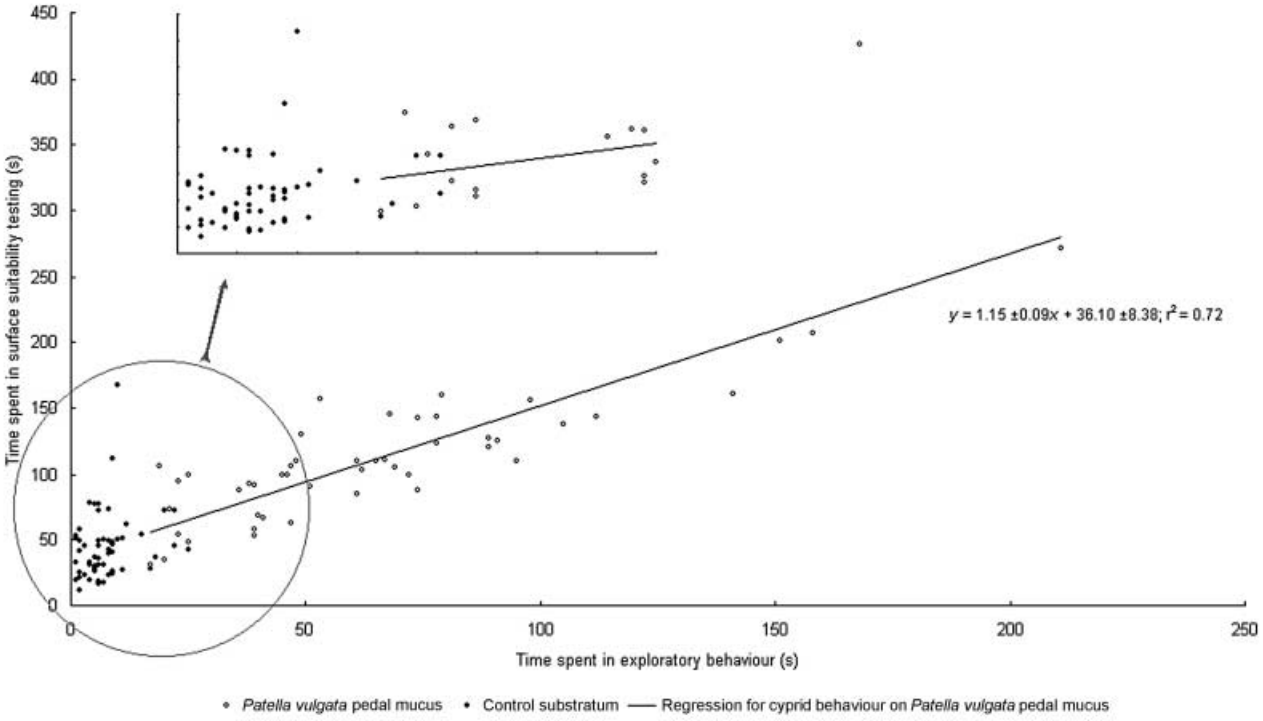

Figure 1. The relationship between cyprid exploratory behaviour and surface suitability testing on the pedal mucus produced by *Patella vulgata* and the control surface.

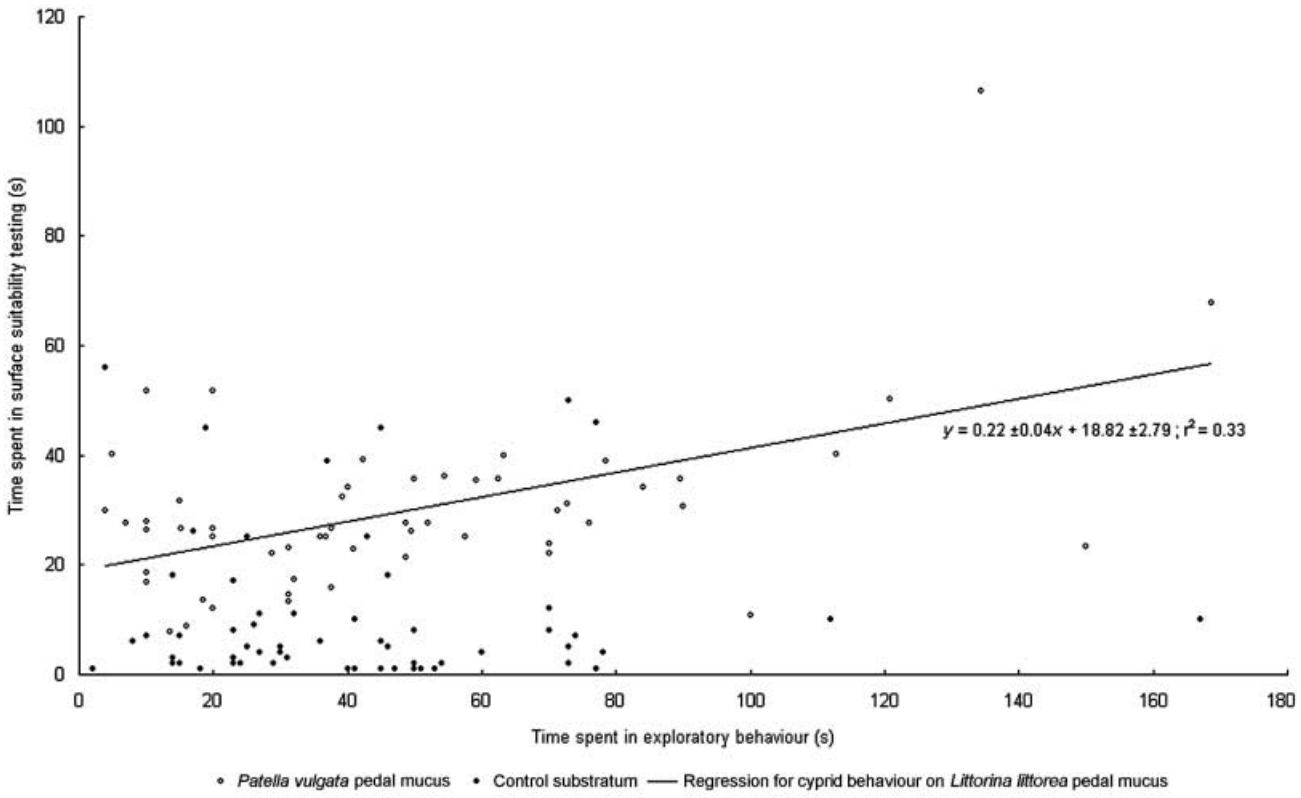

Figure 2. The relationship between cyprid exploratory behaviour and surface suitability testing on the pedal mucus produced by *Littorina littorea* and the control surface.

P. vulgata, the replicate experiments model term for the analysis of the surface suitability testing behaviour was statistically significant, indicating that there were differences in the relative behaviours of the cyprids between replicate experiments.

Linear regression of the behaviour of cyprids (i.e. exploratory behaviour, x axis, vs surface suitability testing, y axis) on the control slides, for both treatments, revealed that no linear relationship could be approximated (Figures 1 & 2). In contrast, for the glass slides coated with the pedal

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Table 5. *Two-way mixed model ANOVA, for the effect of the pedal mucus produced by* Patella vulgata *and by* Littorina littorea *on the settlement of* Semibalanus balanoides *cyprids: field experiments.*

	Patella vulgata pedal mucus#				*Littorina littorea* pedal mucus#			
Source of variation	df	Mean square	*F*	*P*	df	Mean square	*F*	*P*
Treatments [f] (1)	1	14.038	88.848	*** (***)	1	8.71×10^{-3}	0.531	ns (ns)
Replicate experiments [r] (2)	6	0.314	3.326	**	6	4.75×10^{-2}	1.915	ns
1×2	6	0.158	1.674	ns	6	1.64×10^{-2}	0.661	ns
Residual	322	9.44×10^{-2}			140	2.48×10^{-2}		
Total	335				153			

Probability levels: ns, not significant; ***, ⩽0.001; **, ⩽0.01; the characters in the parentheses, indicates the probability calculated from the pooled mean squares (Sokal & Rohlf, 2000). #, denotes data $\log_{10}$ transformed; [r], that the variable has been defined as a random factor; and [f], denotes that the variable has been defined as a fixed factor.

mucus produced by *P. vulgata* or the pedal mucus produced by *L. littorea*, significant linear relationships were derivable (r^2=0.72, P=0.001 and r^2=0.33, P=0.001 for the settlement behaviour of cyprids on the pedal mucus produced by *P. vulgata* and the pedal mucus produced by *L. littorea*, respectively) (Figures 1 & 2). Comparison between the gradients calculated for each of the pedal mucus regressions, using a *t*-test (see Zar, 1999) revealed that the slopes were different (t=14.76; $P\leqslant0.001$). For both treatments, the time spent in one behaviour by the cyprids is directly related to the time spent in the other behaviour.

Experiment 6. The effects of the pedal mucus produced by P. vulgata *and by* L. littorea *on the settlement of* S. balanoides *cyprids: field experiments*

Analysis of the $\log_{10}$ transformed data using a mixed model two-way ANOVA revealed that the pedal mucus produced by *P. vulgata* could increase the settlement of *S. balanoides* cyprids (Table 5). The overall mean ±SE number of cyprids settled for the limpet-treated and control coupons were 1500 ±100 and 400 ±100 m^{-2}, respectively. This represents an overall mean increase in cyprid settlement, on the coupons coated with the pedal mucus produced by *P. vulgata*, by a multiple of ∼4 (3.73 ±0.95), with respect to the control coupons. The replicate experiments model term, for the analysis, was statistically significant indicating that there were differences in the numbers of cyprids settling between replicate experiments (Table 5). In contrast to the results obtained above, analysis of the $\log_{10}$ transformed data for *L. littorea* determined that cyprid settlement was not affected by the pedal mucus produced by *L. littorea* (Table 5). The overall mean ±SE number of cyprids settled was 100 ±20 and 90 ±30 cyprids m^{-2} for the coupons coated with the pedal mucus produced by *L. littorea* and control coupons, respectively.

DISCUSSION

Laboratory examination of the effect of the pedal mucus produced by *Patella vulgata* and by *Littorina littorea*, on the settlement of *Semibalanus balanoides* cyprids, revealed that pedal mucus could increase settlement by a factor of ∼6 and ∼3, respectively. Correspondingly, the pedal mucus produced by *P. vulgata* was found to increase cyprid settlement by a factor of ∼3 in the field while no effect was observable for the pedal mucus produced by *L. littorea*. The failure of the pedal mucus produced by *L. littorea* to elicit a settlement response in the field is in contrast to those of Proud (1994) who found that surfaces pre-conditioned with the pedal mucus produced by *L. littorea* could increase cyprid settlement. The most likely explanation for this difference is that the pedal mucus produced by *L. littorea* was rapidly abraded from the surface of the coupons during the experiment. Davies et al. (1992) have found that the pedal mucus produced by *P. vulgata* has a longevity in the field ∼4 times that of the pedal mucus produced by *L. littorea* and that the pedal mucus produced by *L. littorea* can be rapidly abraded from a substratum when that substratum is exposed to flowing seawater. The current flowing under the pier, used for the experiments, ranged from 0–5 kn, depending upon the state of the tide and observation of the *L. littorea* treatment coupon surfaces at the end of each experiment showed little, if any, presence of pedal mucus. The reduced levels of cyprid settlement in the field, for the pedal mucus produced by *P. vulgata*, probably arises from a reduction in the amount of pedal mucus left on the surface of a coupon when that coupon is exposed in the field.

Pedal mucus can potentially affect cyprid settlement, individually or in combination, through chemotaxic cues, through physio-chemical cues, through chemotactic cues and/or by mechanically entrapping cyprids. Examination of the effect of pedal extracts and its soluble components, i.e. experiment 3, of pedal mucus on cyprid settlement provided no evidence for the presence of any chemotaxic or chemotactic cue, which is in contrast to the suggestions of Johnson & Strathmann (1989) and Proud (1994). However, for species that produce mucus that inhibits settlement it is likely that such effects are chemotaxic/tactic in origin, i.e. Johnson & Strathmann's (1989) 'the ghost of predation future' (see also Proud, 1994 for details on the effect of the pedal mucus produced by *Nucella lapillus* on cyprid settlement. Raimondi (1988), however, has shown that the pedal mucus produced by a predatory gastropod (*Acanthina angelica*) can increase cyprid (*Chthamalus anisopoma*) settlement and it is therefore possible, that the response of an organism to the pedal mucus produced by a mollusc may be determined by its perceived threat. For non-predatory or species with a reduced predatory threat the positive response of settling organisms, that coexist within the same habitat, to pedal mucus may reflect their response to a suitable settlement

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cue (see Raimondi, 1988 for discussion). Alternatively, the ability of pedal mucus to stimulate settlement and hence act as a provendering agent, thereby offsetting the energetically expensive cost of its production (see Holmes et al., 2001 for discussion), would be a sensible strategy for more generalist feeding species. For such species, it is unlikely that the pedal mucus produced by them will contain a chemotaxic cue as the presence of such a cue could allow a settling organism to evolve a negative response.

Other authors have suggested that the sources of effect of mucus matrices on the settlement of organisms are physio-chemical in origin (Zobell, 1938, 1939; Loeb et al., 1984; Maki et al., 1990, 1992; Peduzzi & Herndl, 1991; Szewzyk et al., 1991; Neal & Yule, 1994a). Holmes et al. (2002) however, have suggested that such factors are of little consideration given the adhesive potential of pedal mucus. Nott & Foster (1969) have proposed that the tactile settlement response of cyprids to a substratum is solely chemotactic in origin. In contrast, Crisp (1975) has suggested that the tactile settlement response of cyprids to a substratum arises from the strength at which the antennules of a cyprid bonds to a substratum, mediated by mechanoreceptors responding to the force (pull) required to remove the adhered antennules from that substratum (see also Crisp et al., 1984; Yule & Walker, 1987). No evidence for the presence of any chemotactic cue was found, within the pedal extracts of either *P. vulgata* or *L. littorea*, which could affect the settlement of cyprids. This suggests that the tactile settlement response of cyprids to pedal mucus is not chemotactic in origin. Observation of the settlement behaviour of the cyprids revealed that there was some form of effector cue, although not chemotactic in origin, present in the pedal mucus. That is, the cyprids, which physically contacted a surface coated with pedal mucus, increased their time spent in exploratory behaviour and surface suitability testing. Similarly, Neal & Yule (1994a,b) have found that the mucus matrices produced by microbes can increase the time spent by *Elminius modestus* cyprids in both exploratory behaviour and surface suitability testing (see also Neal & Yule, 1992). The results obtained for the effect of the pedal mucus produced by *L. littorea* should be viewed with caution because of the relatively low r^2 value obtained, i.e. the calculated regression only accounted for 33% of the total variance. In addition, it should be noted that in this study the direct effect of pedal mucus extracts on the settlement of cyprids has not been examined and therefore the presence of some chemotactic cue within the pedal mucus produced by both *P. vulgata* and by *L. littorea* cannot be discounted.

An alternative possibility, which is tactile in origin and is in line with both the hypotheses of Crisp (1975) and those of Holmes et al. (2002), is that the pedal mucus are in some way physically enmeshing the settling cyprids. Such a proposal arises from the work of Zobell (1938, 1939) who formulated two hypotheses as to the source of effect: (i) that mucus matrices could act to physically enmesh free-swimming larvae; and (ii) that by doing so the mucus matrices could modify the settlement behaviour leading to an increase in settlement. The use of a chemically inert analogue to pedal mucus appears to corroborate the first hypothesis of Zobell (1938, 1939). That is, silicon grease, which has a high adhesive potential (see Holmes et al., 2002), increased cyprid settlement by a multiple of ~18. Similar results have been recorded by Walters (1992), who used silicon vacuum grease to entrap *Balanus amphitrite* cyprids when they first contacted a substratum. She found that by preventing cyprids from re-locating, using silicon grease, cyprid attachment to a particular location could be increased by a multiple of ~10. Correspondingly, the video recordings revealed that, following the initial contact of the cyprids with a surface coated with pedal mucus, the antennules of those cyprids became enmeshed within the pedal mucus matrix. Once stuck, the cyprids then tugged vigorously away from the surface in an attempt to free themselves. Instances of organisms using self-produced mucus matrices to facilitate surface adhesion (see Percival, 1979; Boney, 1981; Fletcher & Callow, 1992; Abelson et al., 1994) and to entrap particles for consumption (see Kohn, 1983 for review) have been well-documented.

Neal & Yule (1992) have shown that there is a strong correlation between exploratory behaviour, cyprid tenacity (i.e. surface suitability testing), and the propensity of a cyprid to settle (see also Visscher, 1928; Crisp & Meadows, 1962; Crisp, 1974; Yule & Walker, 1987). Exploratory behaviour and surface suitability testing by cyprids are regarded as late stages in a behavioural settlement cascade, motivated by positive physiological and ecological feedback, which may be abandoned at any time in the face of unsuitable stimuli (Neal & Yule, 1992). Exploratory behaviour and surface suitability testing were linearly related for each of the pedal mucus. It is likely then, as already suggested, that the mechanism of increased cyprid settlement in response to pedal mucus is probably a consequence of the physical entrapment of cyprid antennules within the pedal mucus matrix, resulting in a positive feedback to the cyprid's mechanoreceptors. This is observable both as an increase in the time spent by cyprids in exploratory behaviour/surface suitability testing and ultimately in the number of cyprids settled.

If this proposal is correct, then explanation of the differences in the numbers of cyprids settling on the pedal mucus produced by *P. vulgata*, on the pedal mucus produced by *L. littorea* and on silicon grease can be made in the following way. Holmes et al. (2002) have shown that the adhesive potential of the pedal mucus produced by *P. vulgata* is ~1.5 times that of the pedal mucus produced by *L. littorea*. Therefore, if the source of effect of pedal mucus on the settlement of cyprids is through the enmeshment (entrapment) of the antennules of the cyprids in the pedal mucus, then the numbers of cyprids settling on the pedal mucus produced by *P. vulgata* should be greater than the number of cyprids settling on the pedal mucus produced by *L. littorea*, as is the case. Similarly, Holmes et al. (2002) have shown that the adhesive potential of silicon grease is far greater than the adhesive potential of the pedal mucus produced by *P. vulgata* and as such is reflected in the results recorded for its use as a physical analogue. As such, this argument may explain the differences between the positive effect recorded by Raimondi (1988) and the negative effect recorded by Johnson & Strathmann (1989) for the pedal mucus produced by predatory gastropods (*Acanthina angelica* and *Nucella lamelisoa*, respectively) on cyprid settlement. That is, the pedal mucus produced by *N. lamelisoa* may have a reduced adhesive potential when

compared to that produced by *A. angelica* and hence cyprids can respond negatively to the mucus produced by *N. lamelisoa* but are prevented from doing so by the mucus produced by *A. angelica*.

In the natural environment, the stimulatory effect of pedal mucus on cyprid settlement will depend on the density of the mucus producers present on the shore. For both species a low to medium density of the mucus producers may both increase the rate and success with which a shore is colonized. However, at high densities the effects of biological disturbance (see Connell, 1961; Hawkins, 1981, 1983; Bertness et al., 1983; Kim, 1997) will probably outweigh any positive effects of pedal mucus on settlement although settlement may still be increased because grazers may open up space, remove matter (algae) that usually prevents settlement and/or generate microfloral assemblages stimulatory to settlement (see Strathmann et al., 1981; Hartnoll & Hawkins, 1985; Berlow & Navarrete, 1997 for examples).

In the author's opinion, given both the failure of the pedal mucus produced by *L. littorea* to increase cyprid settlement in the field for the experiments reported here and the high mobility of *L. littorea*, i.e. in contrast to that of *P. vulgata* (see Fretter & Graham, 1994) the pedal mucus produced by *L. littorea* has no effect on cyprid settlement on a shore. Correspondingly, Proud (1994) found that surfaces preconditioned with the pedal mucus produced by *L. littorea*, in the presence of *L. littorea*, negatively affected cyprid settlement, i.e. settlement was prevented/reduced through biological disturbance (see also Buschbaum, 2000). Care, should be taken when extrapolating these results to the 'real' environment because of the nature of the test substrata used, i.e. they were clean. It is entirely possible that if previously filmed glass slides/coupons had been used that the settlement on the control treatment may have been much greater than that on the relevant treatment substrata. Many authors have shown that settlement can be stimulated or inhibited by the biofilms present on preconditioned slides when compared to virgin substrata (Maki et al., 1990; O'Connor & Richardson, 1998; Olivier et al., 2000). Given, however, that Proud (1994) determined that there was an effect of pedal mucus on the cyprid settlement on naturally filmed substrata, it is probable that the results recorded here are in some part in accordance with those occurring in the environment. In addition, the experiments that were carried out only examined one possible factor, i.e. pedal mucus, that may affect settlement. In the natural environment there will be many factors operating at different spatial scales (see Chabot & Bourget, 1988; Miron et al., 2000; Holm et al., 2000 for examples and Bourget, 1989 for discussion), which will operate in concert to determine whether or not an organism will settle. Hence, until further field studies are made whereby the effect of pedal mucus on settlement is evaluated on natural substrata (i.e. those with a resident community representative of the normal settlement site) under normal environmental conditions we cannot truly assess the importance or otherwise of the effect of pedal mucus when compared to other natural cues.

For *P. vulgata* the indirect interaction between *P. vulgata* and *S. balanoides* cyprids, mediated through the pedal mucus trails produced by *P. vulgata*, is an example of the potential effect of an indirect interaction on the settlement of another species. Whether this relationship is contramesalistic (−, +), commensalistic (0, +) or mutualistic (+, +) in nature, needs further research. The potential importance of this interaction in determining the shape of a community is self-apparent. If the pedal mucus produced by some grazer species, as is evidenced here, can act so effectively on a relatively large motile marine invertebrate, i.e. a cyprid, the implications of such indirect interactions should not be overlooked when considering the potential effects of grazer-produced pedal mucus trails on the settlement of smaller non-motile species.

I would like to thank Professors J. Davenport and T. Norton for the generous use of their facilities at the University Marine Biological Station and Port Erin Marine Laboratory. Special thanks to Dr Jaap van de Meer for the statistical advice and Dr Graham Walker for his invaluable help. Finally, I would like to note that the Ecology Centre at the University of Sunderland funded this research.

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Submitted 19 February 2001. Accepted 13 November 2001.