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Smelly feet are not always a bad thing: the relationship between cyprid footprint protein and the barnacle settlement pheromone

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A critical phase in the life cycle of sessile benthic marine invertebrates is locating a suitable substratum for settlement. For barnacles, it is the lecithotrophic cypris larva that makes this plankto–benthic transition. In exploring possible substrata for settlement, the cyprid leaves behind 'footprints' of a proteinaceous secretion that reportedly functions as a temporary adhesive, and also acts as a secondary cue in larval–larval interactions at settlement. Here, we show that two polyclonal antibodies raised against peptides localized at the N- and C-terminal regions of the adult settlement cue—the settlement-inducing protein complex (SIPC)—could both detect 'temporary adhesive' indicating that the SIPC is either a component of this secretion or that they are the same protein.

Keywords: arthropod; barnacle; *Balanus amphitrite*; cyprid; larval settlement; settlement-inducing protein complex

1. INTRODUCTION

Many benthic marine invertebrates select specific settlement sites using cues of both biotic and abiotic origin (e.g. Pawlik 1992). The settlement stage of barnacles is the highly specialized lecithotrophic cypris larva (Walker 1987). For gregarious species, and especially those that are obligate cross-fertilizers, settlement in isolation may compromise future reproductive fitness. Even though barnacles are capable of lateral movement postmetamorphisis (Crisp 1960), they are essentially sessile in habit and potential mates need to be within a penis length to achieve internal cross-fertilization. Barnacles have thus evolved mechanisms to locate conspecifics at settlement (e.g. Crisp 1985) that necessitate adhesion to overcome the hydrodynamic forces that would otherwise dislodge them from the substratum. A proteinaceous secretion, released onto the surface of the paired attachment discs of the ambulatory antennules (Walker 1987) is thought to confer the ability to adhere temporarily while 'walking' over the substratum. This temporary adhesive is left behind as 'footprints' that can be detected by staining with the protein dye reagent Coomassie Brilliant Blue (CBB; Walker & Yule 1984; Clare *et al.* 1994), particularly on a high protein-binding surface such as nitrocellulose membrane (Matsumura *et al.* 1998*b*).

In addition to serving as a putative temporary adhesive, the secretion acts as a pheromone by inducing conspecific settlement (Yule & Walker 1985; Clare *et al.* 1994). Consequently, surfaces that are explored by cyprids become progressively more attractive; gregarious settlement may thus occur in the absence of conspecific adults (Yule & Walker 1985; Clare *et al.* 1994). Walker & Yule (1984) highlighted the similarities between the temporary adhesive and another settlement pheromone, the adult settlement-inducing protein complex (SIPC; Matsumura *et al.* 1998*a*) and postulated their relationship; first, because they are proteinaceous; and second, because of their connection with the cuticle. SIPC has recently been shown to be a cuticular protein (Dreanno *et al.* submitted), possibly an exocrine secretion, while the temporary adhesive is thought to be secreted by modified tegumentary glands; unicellular glands located in the second article of the antennules (Nott & Foster 1969). The hypothesis of relatedness gained support from our previous finding that a polyclonal antibody raised against the 76 kDa subunit of SIPC could immunolabel footprints deposited on nitrocellulose membrane and 'adhesive' on the surface of the attachment discs (Matsumura *et al.* 1998*b*). We now provide further evidence consistent with that hypothesis by using polyclonal antibodies, SIPC-N and SIPC-C, raised against separate peptides located in the N- and C-terminal regions of the SIPC, respectively (Dreanno *et al.* submitted).

2. MATERIAL AND METHODS

(a) *Larval culture*

Adult broodstock, *Balanus amphitrite* were collected from Lake Hamana (Japan) and Beaufort, NC (USA). Cyprids were raised in the laboratory from naupliar releases by established methodology (e.g. Vogan *et al.* 2003) and maintained in the dark for 3 d, at 6 °C, prior to use.

(b) *Antibody synthesis*

Polyclonal antibodies SIPC-N and SIPC-C were raised in rabbit (Eurogentec) to two peptides (coupled to BSA), H_2N–C+STHKKYESHVKTEF–$CONH_2$ (amino acid positions 424–437) and H_2N–C+PEERNIQEYELTPAA–COOH (amino acid positions 1533–1546), located near the N- and C-terminal regions, respectively, of the SIPC protein. Each antibody was purified by affinity chromatography by coupling the antigenic peptide to a thiopropyl-Sepharose matrix. The specificity of each antibody will be reported elsewhere (Dreanno *et al.* submitted).

(c) *Whole-mount immunohistochemistry*

Cyprids were first anaesthetized in a menthol bath and then fixed in 4% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) at 4 °C for 1 h, followed by cold (−20 °C) methanol for 15 min. The cyprids were then washed three times with PBT (PBS, 0.5% Tween 20) and then blocked with a solution of PBT containing 5% goat serum for 2 h at room temperature. After incubation with SIPC-N and SIPC-C, each at a 1 : 50 dilution in the blocking buffer at 4 °C overnight, the cyprids were washed with PBT and incubated with the secondary antibody (1 : 500 dilution, alkaline phosphatase conjugated anti rabbit IgG goat antibody, Jackson ImmunoResearch) for 1 h at room temperature. Following several washes with PBT, the signal was developed with the detection buffer (100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 1 mM levamisol, 100 mM Tris–HCl, pH 9.5) containing 337.5 μg ml^{-1} nitroblue tetrazolium (NBT, Roche) and 175 μg ml^{-1} 5-bromo-4-chloro-3-indolyl

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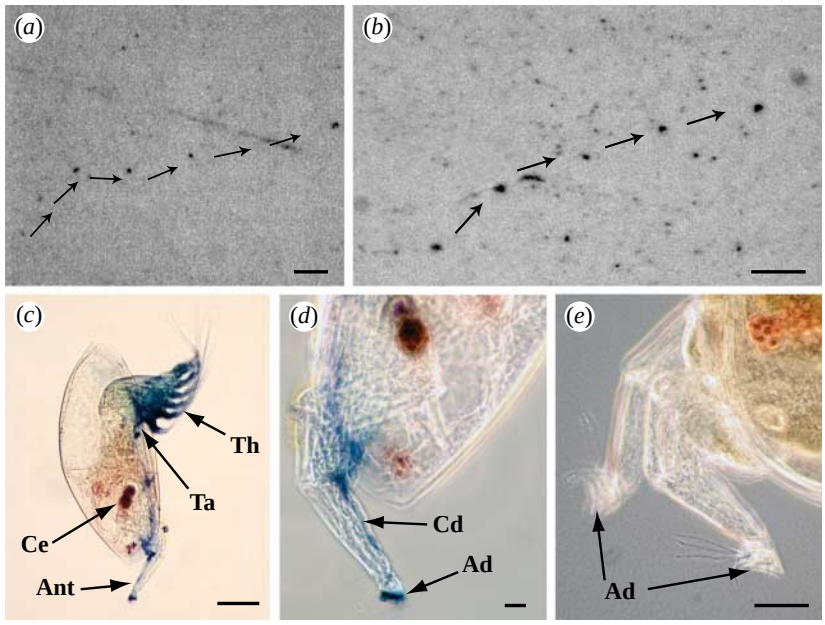

Figure 1. Cyprid footprint tracks on nitrocellulose membranes and localization of the SIPC in the cyprid antennule. (*a*) A track of cyprid footprints detected by the polyclonal antibody SIPC-N. Scale bar, 200 µm. (*b*) As in (*a*) detected by the polyclonal antibody SIPC-C. Scale bar, 200 µm. (*c*) Localization of the expressed SIPC in the cyprid. Scale bar, 100 µm. (*d*) Localization of the expressed SIPC in the cyprid antennules. Scale bar, 50 µm. (*e*) Negative control. Abbreviations: Ad, attachment disc; Ant, antennule; Cd, cement duct; Ce, compound eye; Ta, thorax; Th, thoracopods.

phosphate (BCIP, Roche). The reaction was stopped by washing the larvae with the detection buffer. Cyprids were mounted with a PBS : glycerol (1 : 9) solution on glass slides.

(**d**) ***Detection of cyprid footprints***

Cyprid footprints were visualized using a modified version of the CBB (R250, Sigma) staining and immunoblotting methodology described by Matsumura *et al.* (1998*b*). Briefly, 100 cyprids were added to 100 ml of sterile artificial seawater (Tropic Marin) in a polypropylene container (Hipack, Japan) that had an untreated nitrocellulose membrane (Schleicher & Schuell) attached to the base by carbon adhesive tape (Agar Scientific). After dark incubation at 25 °C for 16 h the membrane was removed, rinsed three times with PBS and the footprints visualized by staining with 0.2% CBB for 15 min followed by microwave destaining for 2 min in 50% methanol and 1% acetic acid.

Immunostaining of the footprints was carried out with the anti-76 kDa polyclonal antibody developed by Matsumura *et al.* (1998*b*) and the two polyclonal antibodies described above. The membranes were blocked with PBT containing 5% skimmed milk for 1 h at room temperature and then incubated with the primary antibody (anti-SIPC) in PBT with 5% skimmed milk, at 4 °C overnight. After several washes with PBS, the membranes were incubated with the secondary antibody (1 : 5000 dilution, PA-conjugated anti rabbit IgG goat antibody, Jackson ImmunoResearch) and the footprints visualized with BCIP/NBT (Sigmafast, Sigma) and observed under a binocular microscope.

The controls for the immunoblotting and immunohistochemistry, which showed no staining, substituted non-immune serum for the primary antibody.

3. RESULTS

The footprints stained by CBB (not shown) were roughly oval in shape and measured 34.8±6.5 µm, comparable to previous reports (Clare *et al.* 1994; Matsumura *et al.* 1998*b*). Footprints of similar morphology to those stained with CBB were revealed by immunostaining with all three antibodies. The anti 76 kDa subunit antibody has been used for this purpose before (Matsumura *et al.* 1998*b*). The two new antibodies, SIPC-C and SIPC-N, gave results that were indistinguishable from the positive control (figure 1*a*,*b*). Tracks characteristic of the wide cyprid search behaviour (Crisp 1976; Lagersson & Høeg 2002) were revealed with a typical pace distance of 361.4±52.5 µm; again very close to the value reported by Clare *et al.* (1994). No footprints were observed for the non-immune serum control (not shown).

The results of cyprid whole-mount immunohistochemistry with both the SIPC-C and SIPC-N antibodies were also indistinguishable. Figure 1*c* shows that the most intense staining is associated with the appendages—the antennules and the thoracopods. Figure 1*d* highlights the strong staining of the attachment discs of the third segment of the antennules with the anti-SIPC-C antibody. It is not clear at this level of resolution whether staining corresponds to adhesive secretion, cuticular villi or both. The cuticle lining the cement duct (Nott & Foster 1969) also reacted with the antibodies. Figure 1*e* is the control.

4. DISCUSSION

Our results support our earlier suggestion that *B. amphitrite* SIPC and the cyprid 'temporary adhesive' are related immunologically (Matsumura *et al.* 1998*b*) by providing strong evidence that the SIPC is either a component of, or is equivalent to the footprint temporary adhesive.

The SIPC is a cuticular glycoprotein contact pheromone that functions to induce gregarious settlement of conspecific cyprids (Crisp & Meadows 1963; Clare & Matsumura 2000). This glycoprotein is expressed in all cuticle (Dreanno *et al.* submitted), though why it is expressed so strongly by the thoracopods is not clear. In the cyprid, all three antibodies resulted in prominent staining of the surface of the attachment discs, which may in part reflect the high surface area presented by the numerous

cuticular villi that cover their surface (Nott 1969). However, as the temporary adhesive is secreted onto the disc's surface (Nott & Foster 1969; Yule & Walker 1985), immunostaining may correspond to a reaction with this secretion. This explanation seems reasonable since areas of nitrocellulose membrane explored by cyprids show numerous footprints when immunostained; these are the antennular secretions left behind during surface exploration (Walker & Yule 1984), which are considered to derive from a group of unicellular glands—modified tegumental glands—in the second antennular segment (Nott 1969; Walker & Yule 1984). These glands release their content onto the disc surface via two concentric rings of pores (Nott & Foster 1969).

Whether the antennular disc secretion is an adhesive is open to conjecture. A difficulty with the temporary adhesion hypothesis is that *B. amphitrite* footprints are rarely observed on surfaces that are readily explored, e.g. glass. This could be explained either by inappropriate staining protocols or the favourable protein-binding characteristics of nitrocellulose membrane, which may retain the protein better during staining and destaining. However, a problem with that explanation is that *Semibalanus balanoides* footprints retain their ability to induce settlement of conspecific cyprids for over three weeks of exposure to running natural seawater (Yule & Walker 1985). An alternative untested explanation is that the nanotextured surface of the attachment disc may itself provide adhesion based on van der Waals forces in a mechanism akin to that of the gecko foot (e.g. Autumn & Peattie 2002). The secretion might under these circumstances serve to increase adhesive forces, or conversely it may serve as a release agent. The plethora of footprints on nitrocellulose membrane, an unfavourable settlement surface, may represent an artefact of adhesive failure between the disc and the secretion, or of cohesive failure of the secretion, a consequence of the strong protein-binding characteristics of the membrane.

It is now of particular interest to determine whether the SIPC is produced by tegumental glands, i.e. whether it is an exocrine secretion, because the unicellular glands that produce the temporary adhesive are hypothesized to be a modified type of tegumental gland (Walker & Yule 1984). A functional link has already been established as both the SIPC and the temporary adhesive promote settlement of conspecific cyprids (Yule & Walker, 1985, Clare *et al.* 1994). Here, by using two different antibodies—SIPC-C and SIPC-N—directed at peptides that span a major portion of the SIPC molecule, we have explicitly established that the secretion released onto the antennular disc and deposited as footprints during surface exploration, contains the SIPC (or at least a major portion of the molecule). This lends support to the possibility that the temporary adhesive and the SIPC may be synonymous as first suggested by Walker & Yule (1984). An advanced analytical characterization (e.g. by mass spectrometry) of isolated footprint protein is required to test the latter hypothesis.

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