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Ecological relevance of a conspecific, waterborne settlement cue in *Balanus amphitrite* (Cirripedia)

Peter D. Elbourne, Anthony S. Clare *

School of Marine Science and Technology, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK

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ABSTRACT

Planktonic marine invertebrate larvae are now considered to exhibit varying degrees of control over their transition back to benthic habitats through behavioural and ontogenetic adaptations. Gregarious settlement in barnacles is attributed to the settlement-inducing protein complex (SIPC), the cypris larva temporary adhesive and a waterborne cue; the latter obtained by conditioning seawater with adult *Balanus amphitrite* (=*Amphibalanus amphitrite*). By responding to a waterborne settlement cue, a swimming larva may elect to settle without contacting a surface. The evidence for such a role is, however, limited. This theme is examined here by evaluating the behavioural response of cyprids to the cue through various laboratory techniques – settlement assays, motion tracking and enumeration of antennule movements – and linking the cue to recruitment of larvae in the field. The cue is detected in solution, remains active upon dilution, induces a similar response in young and aged larvae, and only a brief (3–15 min) exposure to conditioned seawater is required to stimulate settlement. Seawater collected *in situ*, close to piling fouled with *B. amphitrite* at Duke University Marine Laboratory, North Carolina, induced settlement over samples collected at a distance from the piling. The evidence derived from experiments on laboratory-conditioned and field-collected seawater is consistent with an important role for the waterborne cue in the settlement of barnacle cypris larvae.

1. Introduction

Settlement by the planktonic larvae of marine invertebrates was once considered to be a passive process, primarily due to the limited capacity for significant movement in flow (e.g. Abelson and Denny, 1997). Observations of delayed metamorphosis and habitat selection revealed, however, that larvae are not inert particles and are able to exert some control over their dispersal (Crisp, 1974). Indeed, larval behaviour is now an integral component of settlement models (e.g. Jonsson et al., 2004, Koehl et al., 2007). Many marine invertebrate larvae exhibit a gregarious response to the presence of their own, or closely-related, species (Crisp, 1974). Gregarious behaviour is an important regulator of larval settlement (Burke, 1986) and benthic community structure (Hills and Thomason, 1996). Barnacles develop through planktonic nauplii that metamorphose into lecithotrophic cyprids. This settlement stage possesses sensory structures (Walker et al., 1987) that enable the larva to select suitable sites for permanent fixation during surface exploration (Lagersson and Høeg, 2002).

Gregarious behaviour in barnacle cyprids is mediated by a suite of chemical cues originating from both larvae and adults (reviewed by Clare and Matsumura, 2000). The earliest studies demonstrated settlement induction by "arthropodin" – now termed the settlement-inducing protein complex (SIPC; Matsumura et al., 1998a,b; Dreanno et al., 2006a) – through a tactile chemical sense (Crisp and Meadows, 1963; Dreanno et al., 2006b). The temporary adhesive, deposited as 'footprints' during searching behaviour (Walker and Yule, 1984), induces settlement in other cyprids (Yule and Walker, 1985; Clare et al., 1994). Matsumura et al. (1998b) and Dreanno et al. (2006c) showed that the adhesive either contains, or is equivalent to the SIPC. A third settlement inducer is a waterborne cue that is detectable by bioassay in seawater conditioned with adult barnacles (Elbourne et al., 2008). The cue is reported to be a small peptide with a basic carboxy terminus and a neutral or basic amino terminus, with its settlement-inducing activity mimicked by several di- and tripeptides with these structural features, e.g. glycyl-glycyl-arginine (GGR) (Tegtmeyer and Rittschof, 1989; Browne et al., 1998; Browne and Zimmer, 2001). However, Clare and Yamazaki (2000) were unable to confirm the activity of GGR. Recently Endo et al. (2009) isolated a settlement-inducing protein from *Balanus amphitrite* that is unrelated to the SIPC. This ~32-kDa protein rapidly induced searching behaviour by cyprids and it was proposed that the protein acts as a waterborne settlement pheromone. The authors further noted that evidence of the release of this protein by adult barnacles needs to be obtained to substantiate this hypothesis. Based on the molecular mass of this protein, it is clearly not synonymous with low a molecular weight barnacle waterborne cue (Clare and Matsumura 2000), which is the subject of this study.

* Corresponding author. Tel.: +44 0191 2225918.
E-mail address: a.s.clare@ncl.ac.uk (A.S. Clare).

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Despite earlier observations that waterborne cues induce larval settlement (e.g. Scheltema, 1961), Crisp (1974) argued that larvae could not respond to cues in the water column through chemotactic behaviour. It was reasoned that marine invertebrate larvae could neither detect a chemical gradient across their small body nor respond to concentration changes during movement, which is considered implausible due to the slow swimming speeds in the majority of larvae. Furthermore, a turbulent flow regime would rapidly dilute a waterborne cue at a short distance from its origin. More recent studies have provided evidence to suggest that waterborne cues may play an important role in larval settlement. Waterborne cues have been shown to remain active upon serial dilution (Boettcher and Targett, 1998; Koehl and Hadfield, 2004; Santagata, 2004) and chemicals released from flat and rough surfaces form fine filaments within neutral water (Hadfield and Koehl, 2004; Koehl et al., 2007). Indeed, larvae have been shown to exhibit a binary (on/off) response to waterborne cues (Turner et al., 1994; Hadfield and Koehl, 2004), which is typically manifested by increasing downward movement. This behaviour enhances contact rate (Eckman et al., 1994) and may be achieved through passive sinking (Zhao and Qian, 2002; Hadfield and Koehl, 2004) or active swimming in both still water (Tamburri et al., 1992; Santagata, 2004) and flow (Turner et al., 1994; Tamburri et al., 1996). Finelli and Wethey (2003) observed a rapid acceleration by *Crassostrea virginica* larvae to approximately 0.8 cm s^{-1}, which allows a larva to contact a surface in free-stream velocities of up to 50 cm s^{-1}. The rapid swimming speeds attained by barnacle cyprids (e.g. Walker, 2004) implies they can attach in conditions of high flow and, potentially, successfully respond to a waterborne cue.

Many cues implicated in larval settlement have yet to be fully characterised. Some possess attributes that raise questions about whether they could be effective inducers of settlement in the marine environment (Steinberg et al., 2002). Cues have been found to be comparatively insoluble in seawater (Kato et al., 1975), derived from a species that is not associated with the target habitat (Yvin et al., 1985), or have inconsistent effects in settlement assays (Jaccarini et al., 1983). Lumichrome was isolated from both conditioned seawater and extracts of adult and larval ascidians and induced settlement at low concentrations (Tsukamoto et al., 1999), which makes it perhaps the first ecologically relevant waterborne cue of known origin to be fully characterised. Nevertheless, as this compound is available commercially, it is surprising that it has not been tested to confirm the cue's identity (Hadfield and Paul, 2001). Only one waterborne cue has been fully characterised, quantified *in situ* and related to larval recruitment in the natural habitat. Swanson et al. (2004) detected a rapid behavioural change in competent sea urchin, *Holopneustes purpuracens*, larvae in response to histamine isolated from the red alga *Delisea pulchra*. Further research quantified the concentration of histamine in active samples collected adjacent to algae in the field, which corresponded to concentrations active in laboratory assays (Swanson et al., 2006).

This study examines whether the waterborne cue derived from adult barnacles is an ecologically relevant inducer of cyprid settlement, in that the characteristics of the response to the cue are consistent with behaviours that would enhance settlement under natural conditions in the field. The response of *Balanus amphitrite* Darwin (=*Amphibalanus amphitrite*) cyprids to the waterborne cue is evaluated through settlement assays that follow the solitary-larva design advocated by Elbourne et al. (2008) and enumeration of antennule movements of tethered larvae to determine whether the cue is detected in solution. Lateral flicking of the fourth segment occurs during surface exploration to facilitate the movement of stimuli over chemoreceptive setae (Lagersson and Høeg, 2002). Behavioural changes of larger *Semibalanus balanoides* (L.) cyprids are recorded using motion tracking adapted from the methodology developed by Marechal et al. (2004). In addition, pulse-exposure experiments are used to determine how long *B. amphitrite* cyprids must be in contact with the cue for settlement to be induced. Finally, the settlement-inducing activity of seawater collected adjacent to wild *B. amphitrite* is considered in relation to the results of assays of field-collected water performed by Rittschof (1985).

2. Materials and methods

2.1. Collection and maintenance of barnacles

Oyster shell, bearing *B. amphitrite*, was delivered to Newcastle from Duke University Marine Laboratory (DUML), Pivers Island, Beaufort, North Carolina, and maintained in daily exchanges of aerated 10 μm-filtered natural seawater (FSW) warmed to room temperature (23 ± 2 °C). Barnacles were brushed clean every day and sustained on a diet of *Artemia* sp. nauplii (Artemia International LLC) with supplementary feeds of laboratory-cultured algae — mostly the diatom *Skeletonema costatum*. A second population of *B. amphitrite* was cultured on slides using laboratory-reared cyprids (see 'Larval culture, collection and ageing') obtained from another brood-stock delivered from DUML. After maintaining the barnacles on algae for one month, the population was transferred to artificial seawater (ASW) – mixed to 32 PSU using Tropic-Marin® salt and reverse-osmosis water (ROW) – in a recirculating aquarium system maintained at 25 °C. Barnacles were fed the same diet as populations in the static-renewal system, except that flow was removed for 1–2 h to allow time for feeding. Limpet shells, fouled with *Semibalanus balanoides*, were collected at Seaton Sluice, Northumberland, shucked and cleaned thoroughly. The population was subsequently maintained in a second recirculating aquarium system with FSW kept at 17 °C and fed in the same way as *B. amphitrite*.

2.2. Preparation of barnacle-conditioned seawater

The specific details of each of the four batches of barnacle-conditioned seawater used in this study are summarised in Table 1, including the filtration date that they will be referred to hereafter. Three batches were prepared by placing scrubbed *B. amphitrite* in various containers with enough aerated ASW to cover all barnacles. After 18 h at 25 °C, the water was vacuum-filtered through a 0.45 μm membrane, divided into centrifuge tubes and stored at −20 °C until use. The fourth batch was obtained by conditioning FSW at 17 °C with *S. balanoides*, with filtration and storage as for the batches of *B. amphitrite*-conditioned seawater. An equivalent volume of ASW was submitted to identical treatments as each batch of conditioned seawater to serve as a control. Preliminary experiments demonstrated no relationship between the numbers of barnacles used for conditioning and settlement-inducing activity.

Table 1
Details of four batches of barnacle-conditioned seawater prepared using adult *Balanus amphitrite* and *Semibalanus balanoides* examined in this study.

Date of filtration	Species (origin)	Number of barnacles	Container type	Seawater volume (salinity)
04/05/05	*S. balanoides* (seaton sluice)	~500	Glass dish	500 ml (34 PSU)
12/05/05	*B. amphitrite* (laboratory-reared)	125	Glass dish	1500 ml (35 PSU)
21/07/05	*B. amphitrite* (laboratory-reared)	~475	Plastic box	5000 ml (35 PSU)
15/06/06	*B. amphitrite* (North Carolina)	44	Glass dish	2000 ml (32 PSU)