

Inactivity of glycyl-glycyl-arginine and two putative (QSAR) peptide analogues of barnacle waterborne settlement pheromone

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The effect of two putative tripeptide analogues (isoleucine-isoleucine-arginine [IIR] and valine-leucine-arginine [VLR]) and a reported analogue, glycyl-glycyl-arginine (GGR), of the waterborne cue to settlement of *Balanus amphitrite amphitrite* Darwin cypris larvae has been investigated. Settlement in the presence of these tripeptides was not significantly different from that in filtered seawater. Because crude waterborne cue and settlement-inducing protein complex both significantly evoked settlement, the cyprids used in this study were competent to respond. The overall results, therefore, demonstrate that IIR, VLR and GGR are not analogues of barnacle waterborne settlement pheromone.

The chemical basis of gregarious settlement behaviour in barnacles has long been attributed to the detection of an adult glycoprotein by a tactile chemical sense (Crisp & Meadows, 1962; Gabbott & Larman, 1987; Clare & Matsumura, 2000). More recently, evidence has been presented to support the existence of an additional waterborne cue to barnacle, *Balanus amphitrite*, cypris settlement (Rittschof, 1985). Barnacle waterborne settlement pheromone (BWSP) (see Clare & Matsumura, 2000), is a 3–5 kDa peptide with a basic carboxy terminus and a neutral, or basic, amino terminus. Tegtmeier & Rittschof (1989) found that certain di- and tripeptides with arginine or lysine at the carboxy terminus could mimic the activity of the pheromone. Remarkably, one of these peptide mimics, glycyl-glycyl-arginine (GGR), is also a potent mimic of the waterborne cue (a <500 Da peptide) to oyster, *Crassostrea virginica*, settlement (Zimmer-Faust & Tamburri, 1994). Browne et al. (1998) modelled quantitative structure–activity relationships (QSARs) for the oyster and barnacle cues and predicted the activity of a number of untested tripeptides.

The aim of the present study was to examine the settlement-inducing activity of two of these tripeptides (isoleucine-isoleucine-arginine [IIR] and valine-leucine-arginine [VLR]), relative to GGR, crude BWSP, and crude settlement-inducing protein complex (SIPC) (Matsumura et al., 1998) on cypris larvae of *Balanus amphitrite*. Cyprids were reared at the Marine Biological Association from adult broodstock (Clare, 1996). The adults were collected from Beaufort, North Carolina (American broodstock) and Lake Hamana, Shizuoka Prefecture (Japanese broodstock) and arrived in Plymouth on 17 November 1998 and 9 December 1998 respectively. Once filtered from batch culture, cyprids were held at 6°C prior to use in settlement assays. The latter were carried out in 24-well plates (Iwaki, Japan), with 2 ml solution and ten cyprids per well, at 28°C, in the dark. Five replicates of each treatment and 0.45 µm filtered seawater control were tested and the assays were carried out with cyprids raised from both 'Japanese' and 'American' broodstock.

To obtain crude BWSP, approximately 500 adult *B. amphitrite*, attached to bamboo ('Japanese' broodstock), were held without food in 1-l of 0.45 µm filtered aerated seawater in a 26×14×9.5 cm (l:w:h) plastic sandwich box (Stewart Plastics) at 25°C for 24 h. The seawater was then filtered, consecutively, through Whatman no. 1 filter paper and 0.45 µm Millipore filters to obtain crude BWSP. The latter, which was prepared on

15 December 1998, was assayed for activity and then stored at –20°C. Crude SIPC was extracted as required from Japanese broodstock specimens according to Matsumura et al. (1998). Assays of BWSP were done without dilution; 2 ml of test solution corresponding to ~1 barnacle equivalent. Settlement-inducing protein complex was added in solution at a concentration of 10 µg ml⁻¹ protein, which for an adult of ~1 g wet weight, approximates to 0.0025 barnacle equivalents in 2 ml of test solution.

Glycyl-glycyl-arginine, as the acetate salt, was obtained from Sigma Chemical Company (code G 887), while IIR and VLR were synthesized by Alta Bioscience (University of Birmingham). The former was assayed in the concentration range 10⁻⁶ to 10⁻¹⁰ M, overlapping the threshold concentrations reported by Tegtmeier & Rittschof (1989). All solutions were freshly prepared in 0.45 µm-filtered seawater prior to the assay. Concentrations quoted in the present study were not adjusted to account for the 70% peptide concentration of the acetate salt. In the absence of corresponding information in Tegtmeier & Rittschof (1989), direct comparison between the two studies is problematic. Isoleucine-isoleucine-arginine and VLR were assayed at and below the predicted (QSAR) threshold concentration (Browne et al., 1998). The protein concentration of crude SIPC was determined by a microplate version of the Bradford (1976) assay with BSA as standard. The peptide concentration of BWSP was assayed using 2,4,6-trinitrobenzene sulphonic acid (Habeeb, 1966) with GGR as standard.

Figure 1A,B shows the results of settlement assays using day 1 cyprids from 'Japanese' (scored after 19 h) and 'American' (scored after 16 h) broodstock respectively. Although settlement values were higher at subsequent time intervals (24–48 h), a similar pattern of settlement in the respective treatments and controls was observed. Settlement in the seawater control in both assays was low, thereby facilitating the detection of settlement-inducing effects. Settlement was induced significantly by both crude SIPC and BWSP compared to the filtered seawater control (16-h assay: $P < 0.01$ [SIPC and BWSP]; 19-h assay: $P < 0.05$ [SIPC]; $P < 0.01$ [BWSP]; Dunnett's test). No significance is attached at this stage to the increased settlement in BWSP compared to SIPC, since it is difficult to compare the respective concentrations. Moreover, SIPC is maximally effective when bound to a surface, not when in solution (Crisp & Meadows, 1962; Matsumura et al., 1998). None of the tripeptides

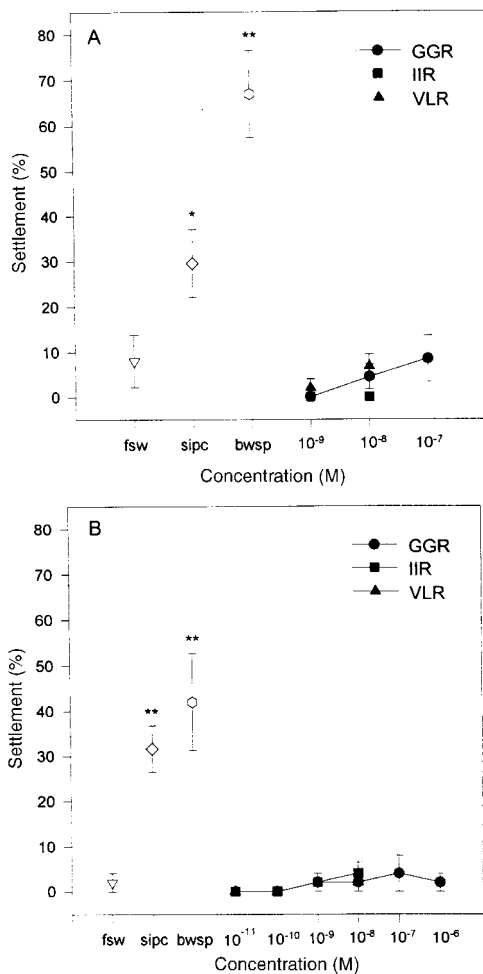


Figure 1. Effect of crude pheromones and tripeptides on settlement (\pm SE) of day 1 *Balanus amphitrite* cyprids. (A) From Japanese broodstock after 19-h exposure and (B) from American broodstock after 16-h exposure. *, $P < 0.05$; **, $P < 0.01$. FSW, filtered seawater; SIPC, crude settlement-inducing protein complex; BWSP, crude barnacle waterborne settlement pheromone.

induced settlement compared to the seawater control ($P > 0.05$) at any of the concentrations tested. A repeat of the GGR (10^{-6} – 10^{-12} M) assay with day 0 cyprids (the age used by Tegtmeier & Rittschof, 1989) also failed to induce settlement ($P > 0.05$).

The peptide concentration of crude BWSP was at the lower limit of detection of the standard curve ($\sim 1.75 \mu\text{g ml}^{-1}$, taking account of GGR as $\sim 70\%$ peptide). Assuming that BWSP is a peptide of less than 500 Da, i.e. of no more than three amino acid residues, its concentration would be $\sim 3.5 \times 10^{-6}$ M. Crude BWSP is likely to contain a mixture of peptides and amino acids. The concentration of active pheromone present will therefore be lower than the aforementioned concentration and probably within, or below, the concentration range at which the tripeptides were tested. Since the cyprids used in this study were clearly competent to settle in response to stimulatory cues, it is concluded that GGR, IIR and VLR do not mimic BWSP at the concentrations tested. The possibility that one or more of these tripeptides are components of BWSP cannot be ruled out, however, since the latter could well comprise a bouquet of peptides.

By analogy with the role of waterborne cues in oyster settlement (Zimmer-Faust & Tamburri, 1994), our working hypothesis is that BWSP alters the behaviour of cypris larvae such that they are more likely to contact a surface. Induction of settlement

in the present study may therefore be indirect and modulated by increased deposition of cypris temporary adhesive—itsself a settlement pheromone (Yule & Walker, 1985; Clare et al., 1994) during searching behaviour. This hypothesis might help to explain the previous report of settlement induction by GGR (Tegtmeier & Rittschof, 1989), since cyprids were used at densities ranging from 30 to 150 per 5 ml. In this range, density effects on settlement have been noted, due presumably to the cypris temporary adhesive's secondary role as a settlement pheromone (Clare et al., 1994; Yule & Walker, 1985).

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REFERENCES

- Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248–254.
- Browne, K.A., Tamburri, M.N. & Zimmer-Faust, R.K., 1998. Modelling quantitative structure–activity relationships between animal behaviour and environmental signal molecules. *Journal of Experimental Biology*, **201**, 245–258.
- Clare, A.S., 1996. Signal transduction in barnacle settlement: calcium re-visited. *Biofouling*, **10**, 141–150.
- Clare, A.S., Freet, R.K. & McClary, M.J., 1994. On the antennular secretion of the cyprid of *Balanus amphitrite*, and its role as a settlement pheromone. *Journal of the Marine Biological Association of the United Kingdom*, **74**, 243–250.
- Clare, A.S. & Matsumura, K., 2000. Nature and perception of barnacle settlement pheromones. *Biofouling*, **15**, 57–71.
- Crisp, D.J. & Meadows, P.S., 1962. The chemical basis of gregariousness in cirripedes. *Proceedings of the Royal Society B*, **156**, 500–520.
- Gabbott, P.A. & Larman, V.N., 1987. The chemical basis of gregariousness in cirripedes: a review (1953–1984). In *Barnacle biology* (ed. A.J. Southward), pp. 377–388. Rotterdam: A.A. Balkema.
- Habeeb, A.F., 1966. Determination of free amino acid groups in proteins by trinitrobenzene sulfonic acid. *Analytical Biochemistry*, **14**, 328–336.
- Matsumura, K., Nagano, M. & Fusetani, N., 1998. Purification of a settlement-inducing protein complex (SIPC) of the barnacle, *Balanus amphitrite*. *Journal of Experimental Zoology*, **281**, 12–20.
- Rittschof, D., 1985. Oyster drills and the frontiers of chemical ecology: unsettling ideas. *American Malacology Bulletin*, **1**, 111–116. [Special edition.]
- Tegtmeier, K. & Rittschof, D., 1989. Synthetic peptide analogs to barnacle settlement pheromone. *Peptides*, **9**, 1403–1406.
- Yule, A.B. & Walker, G., 1985. Settlement of *Balanus balanoides*: the effect of cyprid antennular secretion. *Journal of the Marine Biological Association of the United Kingdom*, **65**, 707–712.
- Zimmer-Faust, R.K. & Tamburri, M.N., 1994. Chemical identity and ecological implications of a waterborne, larval settlement cue. *Limnology and Oceanography*, **39**, 1075–1087.

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