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FEATURE ARTICLE

In situ quantification of a natural settlement cue and recruitment of the Australian sea urchin *Holopneustes purpurascens*

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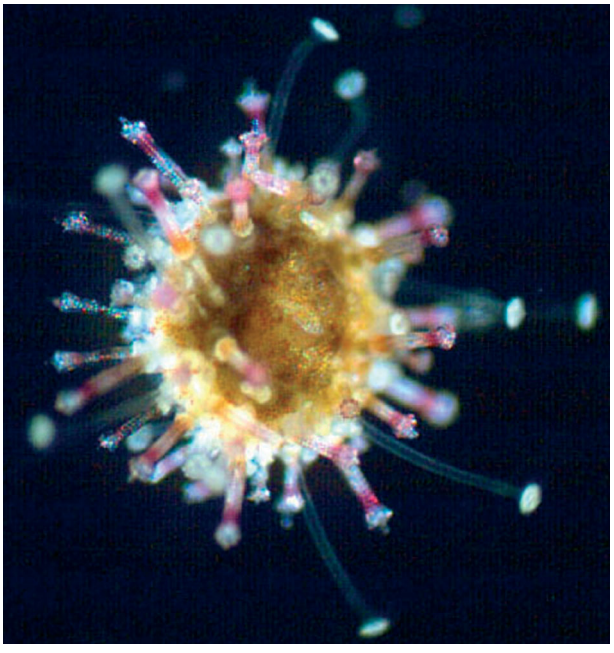
ABSTRACT: In the first study of its kind, the recruitment of the Australian sea urchin *Holopneustes purpurascens* is compared to quantitative *in situ* measurements of a naturally occurring settlement cue, histamine. More than 90% of new recruits were found on either the foliose red alga *Delisea pulchra* or on coralline turf algae while 8% of recruits were found on the brown alga *Homeostrichus olsenii*. These algae induced the settlement (and metamorphosis) of almost all larvae in laboratory assays after 24 h. No new recruits were found on *Ecklonia radiata* or *Sargassum vestitum* and unfouled *E. radiata* induced low levels of settlement in laboratory assays. Thus, the algae on which we found the most recruits in the field induced the highest rate of settlement in laboratory assays. *D. pulchra* contained far higher levels of histamine than all other algae and the coralline algae lacked measurable histamine. Seawater collected *in situ* adjacent to *D. pulchra* induced low levels of settlement of aged larvae in laboratory assays and contained the highest concentration of histamine (~5 nM). With the exception of coralline algae, variation in settlement and recruitment was consistent with the variation among species histamine contents. Antibacterial treatment of *Amphiroa anceps* greatly reduced the number of larvae settling in response to the alga. Biofilms of 2 bacterial strains isolated from the surface of coralline algae induced settlement of 20 to 43% larvae in laboratory assays after 96 h and media in which these strains were cultured contained more histamine. These initial results support a biofilm derived settlement cue for larval *H. purpurascens* from coralline algae.

KEY WORDS: Settlement cue · *In situ* quantification · Histamine · Recruitment · Sea urchin · Invertebrate larvae · *Holopneustes purpurascens* · *Delisea pulchra*



Larvae of the Australian sea urchin *Holopneustes purpurascens* settle with preference on the red alga *Delisea pulchra* and on coralline turf, and they metamorphose in response to histamine, which is produced in high quantities by *D. pulchra*. The settlement cue from coralline algae may derive from bacteria.

Photo: Rebecca Swanson

INTRODUCTION

Settlement of larvae and recruitment of new individuals into marine habitats are fundamental ecological processes for population structure and community

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dynamics in benthic ecosystems (Underwood & Keough 2000). Most marine invertebrates have complex life histories with recruitment into a population occurring through a planktonic larval phase which settles and metamorphoses into the benthic juvenile. The transition from the swimming larva to the less mobile, benthic juvenile is a crucial stage in the life history of such organisms, as survival is dependent on larvae choosing an appropriate habitat in which to settle. The question of how these planktonic larvae return to and choose an appropriate benthic habitat has been a major focus of marine ecology for over 50 yr. Over large spatial scales (kilometres, miles) prevailing hydrodynamic conditions are probably the most important factor affecting the distribution of larvae. However, when currents/eddies/tides return larvae to benthic or near shore environments, behaviour on small spatial scales (centimetres, millimetres, microns) influences whether they settle into a specific habitat (Mullineaux & Butman 1991, Harvey & Bourget 1997). Larvae of many benthic species display active habitat selection by responding to chemicals emanating from conspecifics (Burke 1984), host plants (Williamson et al. 2000), prey (Hadfield & Scheuer 1985), or surface-associated bacterial communities (biofilms)(Wieczorek & Todd 1998). Such chemicals may be surface-bound or waterborne and either trigger settlement (sinking, substrate exploration, attachment), metamorphosis (morphological transformation) of larvae, or both (in the current study the 2 processes together are termed settlement).

The importance of chemical cues in triggering the settlement of marine invertebrate larvae has been recognised for a wide range of species. A large number of chemical settlement cues have been partially characterised including many classes of compounds, from small peptides (Zimmer-Faust & Tamburri 1994, Lambert et al. 1997, Fleck & Fitt 1999) to large glycoproteins (Clare & Matsumura 2000), low molecular weight (LMW) water-soluble compounds (Hadfield & Pennington 1990, Gibson & Chia 1994) to high molecular weight surface-bound carbohydrates (Morse & Morse 1991, Krug & Manzi 1999) and proteins (Jensen & Morse 1990), and lipids (Takahashi et al. 2002). However, the exact chemical identity of settlement cues has rarely been reported, and even in these cases, the ecological relevance of the identified compounds *in situ* is often not clear. For example, jacarone (Yvin et al. 1985) and anthosamines A and B (Tsukamoto et al. 1994, 1995) are putative settlement cues that were isolated from organisms which are not associated with recruitment of the target species. Alternatively, the accessibility of the cue to settling larvae has not been demonstrated, as is the case for lumichrome, which was isolated from the tunic tissue of adult conspecifics (Tsukamoto et al. 1999). Remarkably, after 60 yr of research, there is not a single settlement cue for invertebrate larvae which has unequivocally been: (1) structurally characterised, (2) quantified *in situ*, and (3) related to the recruitment of the organism by quantifying variation in the distribution of the settlement cue in the habitat.

We recently reported that the LMW water-soluble amino-compound histamine induced settlement of the Australian sea urchin *Holopneustes purpurascens* (Temnopluridae: Echinodermata) (Swanson et al. 2004). A survey of the size distribution of *H. purpurascens* found that the smallest size class (new recruits) only occurred on the red foliose alga *Delisea pulchra* with larger individuals primarily found on the kelp *Ecklonia radiata* (Williamson et al. 2000, 2004). In settlement assays *D. pulchra*, its polar extract and seawater collected near *D. pulchra* plants *in situ* all induced settlement of *H. purpurascens* larvae, with no (or minimal) settlement observed in response to fronds or extracts of *E. radiata* (Williamson et al. 2000). These findings supported a water-soluble settlement cue for *H. purpurascens* emanating from *D. pulchra*. Histamine was isolated from *D. pulchra* using cation-exchange chromatography, and its identity confirmed by spectroscopy and mass spectrometry (Swanson et al. 2004). Histamine at low micromolar concentrations isolated from *D. pulchra* induced rapid settlement of *H. purpurascens* larvae in static water laboratory assays within a hour (Swanson et al. 2004). An initial analysis of the histamine content of *D. pulchra*, *E. radiata* and co-occurring algae indicated that the histamine content of *D. pulchra* was 10-fold greater than these other algae. Here we describe the distribution of histamine in the habitat of *H. purpurascens* and relate this to recruitment of this species, thereby for the first time relating quantitative variability of a naturally occurring settlement cue with observed variation in settlement and recruitment of the target organism.

MATERIALS AND METHODS

Study site. All urchins, algae and seawater used in this study were collected from sublittoral habitats (1 to 3 m depth) at Bare Island (33° 59′ 38″ S, 151° 14′ 00″ E) at the north head of Botany Bay, Sydney, Australia. A detailed description of this habitat is reported elsewhere (Wright & Steinberg 2001, Williamson et al. 2004).

Recruitment survey. *Holopneustes purpurascens* is an 'arboreal' sea urchin, typically living off the benthos enmeshed in the fronds/laminae of seaweeds. A range of algae were collected from Bare Island each month for 2 yr from December 2002 to November 2004 (excluding June 2003/2004 and February 2004) and searched for new recruits to determine recruitment

patterns of *H. purpurascens*. Algae sampled included the main host plants of *H. purpurascens* at Bare Island, *Ecklonia radiata* (Laminariales: Phaeophyta) and *Delisea pulchra* (Bonnemaisonales: Rhodophyta), as well as the common and co-occurring species, *Sargassum vestitum* (Fucales: Phaeophyta), *Homeostrichus olsenii* (Dictyotales: Phaeophyta), *Corallina officinalis* and *Amphiroa anceps* (Corallinales: Rhodophyta). Five plants (or parts thereof) of *E. radiata*, *D. pulchra*, *S. vestitum*, *H. olsenii* were collected, or 10×10 cm^2 (n = 5) of the turf algae *C. officinalis* and *A. anceps* (in an attempt to sample approximately equal amounts of each algal type). Algae were rinsed in fresh water and the epifauna collected. The algae were blotted dry with paper towel and the wet weight (ww) recorded. New recruits (urchin test diameter ≤5 mm) were measured using a dissecting microscope and identified by comparison to juveniles of *H. purpurascens* reared from larvae in the laboratory. The total number of new recruits found each month on each alga (pooling data from the coralline turf algae) was standardised to number found per 100 g of algae sampled (no. 100 g^{-1} ww). The variation between years in the number of recruits found on each alga in each month is presented (mean ± SE, see Fig. 1). The recruitment survey was analysed by a 1-way (alga = fixed) ANOVA (transformed data, $x + 1$) using months as replicates (n = 21). Bonferroni's post-hoc test was used to determine which treatments differed significantly at p = 0.05.

Settlement assays with algae and seawater. *Holopneustes purpurascens* larvae were cultured as previously described (Williamson et al. 2000). Larvae reached competency (developmentally ready for metamorphosis) within 6 d, recognised by the presence of 5 well-developed tube-feet. All settlement (defined as attachment and metamorphosis) assays with competent larvae were done with 6 d old larvae at 19°C with a 12 h light:12 h dark regime, in 36 mm sterile petri-dishes and 4 ml sterile seawater (SSW) under static conditions. Dishes containing 10 µM histamine or SSW were included in all assays as positive and negative controls, respectively. Replicates were randomly assigned among treatments and larvae were added once all petri-dishes were prepared.

A range of host algae were assayed against larvae of *Holopneustes purpurascens* to test for a settlement response. We tested *Delisea pulchra*, *Ecklonia radiata*, *Homeostrichus olsenii*, and *Amphiroa anceps* as these species host recruits, juveniles or adults of *H. purpurascens* (Williamson et al. 2000). Plants of *D. pulchra*, fouled and unfouled *E. radiata*, *H. olsenii*, and *A. anceps* were collected and a piece of alga, or fouling (epiphytes) from the surface of *E. radiata* fronds (approximately 20 mg ww), were placed in assigned dishes (n = 10). Five larvae were added to each dish and the percent settlement (i.e. percent metamorphosed) recorded at 1 and 24 h. The response of larvae to different algae in the settlement assay was analysed by univariate repeated measures ANOVA (proportions untransformed). We compared the effect of treatments within each level of time using planned comparisons. We first compared the effect of inductive algae (*D. pulchra*, *A. anceps* and *Homeostrichus olsenii*), as we *a priori* believed that these treatments were unlikely to be different. Treatments in which there was no significant difference were pooled and tested against the non-inductive kelp (*E. radiata*). Similarly, the effects of fouled *E. radiata* and epiphytes were compared, which did not differ significantly at p = 0.05; therefore pooled treatments were tested against unfouled *E. radiata.*

In order to test whether settlement cues for *Holopneustes purpurascens* were present in seawater (SW) as leachate from algae, we collected seawater adjacent to and at some distance from algae, to test against competent and aged larvae of *H. purpurascens* in settlement assays. Seawater was collected by sterile syringe, placing the tip within 5 mm of algal fronds and drawing 10 ml of seawater into the barrel. One sample was collected from 10 individual plants of *Delisea pulchra* (*Delisea*-SW) and *Ecklonia radiata* (*Ecklonia*-SW), or from 10 patches of *Amphiroa anceps* (*Amphiroa*-SW). Control seawater was collected at the sea surface approximately 2 m away from any macroalgae (Surface-SW). Samples were stored on ice and filtered (0.22 µm) upon return to the laboratory. Five samples of *Delisea*-SW, *Ecklonia*-SW, *Amphiroa*-SW and Surface-SW were randomly chosen and tested in settlement assays (n = 5 dishes, 4 ml) with competent larvae on the day of collection with 3 larvae added per dish. Percent settlement was scored after 24 and 48 h. Seawater samples were stored at −4°C until more larvae were available, at which time the remaining 5 samples were thawed and tested against competent larvae (as above). All seawater samples (n = 10) were later thawed and tested against aged larvae (3 wk post-fertilisation) with 10 larvae added per dish. Percent settlement was scored after 24, 44 and 72 h. The response of aged larvae to *in situ* seawater in the settlement assay was analysed by univariate repeated measures ANOVA (proportions untransformed) and by planned comparisons at 24 h. First, we compared the effect of Surface-SW and SSW as we *a priori* believed that these treatments were unlikely to be different. As there was no significant difference, these treatments were pooled (Control-SW) and tested against each seawater treatment collected *in situ* adjacent to algae (*Delisea*-SW, *Ecklonia*-SW or *Amphiroa*-SW).

Temporal analysis of the histamine content of algae. We quantified the histamine content of *Delisea pulchra*, *Ecklonia radiata*, *Homeostrichus olsenii* and *Sargas-*

sum vestitum over 4 seasons (September 2003, January 2004, April 2004 and July 2004) using a modified gas chromatography-mass spectrometric analysis which had greater sensitivity than the previous analysis (Swanson et al. 2004). The coralline turf algae were excluded from these analyses because histamine was not detected in several previous extractions and analyses (Swanson et al. 2004, unpubl. data). *D. pulchra*, *E. radiata*, *H. olsenii* and *S. vestitum* (n = 5) collected in September 2003, January 2004, April 2004 and July 2004 as part of the recruitment surveys were freeze-dried for histamine analysis. *D. pulchra* (5 plants) was also collected in successive months September, October, November 2003 and July, August, September 2004 in order to assess the short-term variation in the histamine content. A polar extract of each algal sample was prepared as previously described (Swanson et al. 2004), except that freeze-dried algae were extracted instead of wet algae, and the internal standard (ISTD) for quantitative histamine analysis was added to the methanol extract of each algal replicate before drying it. The addition of a known amount of ISTD at the beginning of sample work-up allows for most accurate quantification of the analyte (histamine). Ideally, the ISTD should be a dueterated form (or another isotopic form) of the analyte so that they behave equally to chemical treatment (i.e. extraction and derivatisation) but have slightly different masses allowing for quantification. The ISTD was [α, α, β, β-d_4]Histamine·2HCl (Cambridge Isotope Laboratories #DLM 2911) and 100, 10, 3 and 3 µg of ISTD was added to the methanol extracts of *D. pulchra*, *H. olsenii*, *E. radiata* and *S. vestitum*, respectively. Polar extracts were dissolved in Milli-Q (200 µl) and acidified with 50 µl of glacial acetic acid. Strong cation-exchange solid phase extraction cartridges (50 mg, Alltech) were equilibrated with Milli-Q (5 ml) at a flow rate of 1 ml min^{-1} and the sample loaded. Unbound compounds were eluted in 5 ml Milli-Q and discarded, then all retained compounds were eluted in 200 µl of 30% NH_4OH and dried in a Speed-Vac. Two sets of standards were prepared that contained synthetic histamine and ISTD (0.1 to 10 µg histamine and 3 µg ISTD, or 10 to 250 µg histamine and 100 µg ISTD). Standards were run through the cation-exchange cartridges as per the algal samples. Extracted standards and algal samples were derivatised with heptafluorobutyric anhydride and acetic anhydride (Barancin et al. 1998).

A DB-5MS column (15 m, 0.25 µm × 0.25 mm ID; J & W Scientific) and a packed liner (3% SP-2250, Supelco) were installed on an electron-ionisation (EI) GC-MS instrument described in Swanson et al. (2004) and the same run conditions were used for data acquisition (Swanson et al. 2004). Briefly, the Mass Selective Detector was operated in selected ion monitoring mode (derivatised histamine, mass to charge ratio [*m/z*] 307 and 349, and derivatised ISTD, *m/z* 311 and 353) and extracted ion chromatograms were used to manually integrate the area under each ion peak (which is proportional to the amount in the sample). Although the analyte and ISTD coelute in the gas chromatogram, the peaks consist of different masses for each compound and form distinct peaks in extracted ion chromatograms. The ratio of the amount of histamine:ISTD in standards was used to generate a standard curve, from which the histamine content of the samples was calculated (Swanson et al. 2004). The histamine content of the samples was expressed in terms of µg g^{-1} dry weight (dw) of algal tissue (based on ww/dw conversion factors determined for each species). The statistical analysis of temporal variation of algal histamine levels used a 2-factor (alga = fixed, month = random) ANOVA (transformed, ln[x + 1]) excluding data on *Sargassum vestitum* from the analysis (this alga was not present in the habitat in April). The short term temporal variation in histamine content of *D. pulchra* was analysed by a 1-way (month = fixed) ANOVA (transformed [ln(x + 1)]).

Variation in histamine across the thallus of *Delisea pulchra* and *Ecklonia radiata*. Variable results were often obtained in settlement assays when testing fresh pieces of *Delisea pulchra* and *Ecklonia radiata* against larvae, suggesting that there may be some within-plant variation of histamine content. To test this hypothesis, different regions of the thallus of *D. pulchra* and *E. radiata* were extracted for histamine analysis. *D. pulchra* (n = 5) were collected in September 2004 and 1 branch of each plant was sectioned into tip (upper 1 cm), mid (top half of remaining branch), and base (lower half of remaining branch). The histamine content of the tip, mid, base and whole plant (n = 5) was determined using the methods outlined above, except that 50 µg-ISTD was added to the methanol extracts of tip, mid and base sections (100 µg-ISTD for whole plant). Unfouled and fouled *E. radiata* (n = 3) were collected in September 2004 and the unfouled plants were divided into primary (1°) and secondary (2°) laminae. Fouling epiphytes were scraped from the surface of the 2° laminae of the fouled plants and blotted with tissues. The histamine content of each region was determined using the methods outlined above, except that 5, 10 and 100 µg-ISTD was added to the methanol extracts of the 1° and 2° laminae and the fouling epiphytes, respectively. The statistical analysis of within plant variation of histamine content of *D. pulchra* (untransformed) and *E. radiata* (transformed [ln(x + 1)]) both used a 1-way (algal part = fixed) ANOVA and Bonferroni's post-hoc test to determine which treatments differed significantly at p = 0.05.

Histamine concentration of seawater in the habitat. To test for the presence of histamine in seawater as

leachate from algae we collected seawater, adjacent to and at some distance from algae, for histamine analysis. Microcon-SCX adsorptive micro-concentrators (Mic-SCX, Millipore) were used to extract histamine from seawater samples. Approximately 2 ml of each seawater sample (n = 10 collected adjacent to each plant) remained after retesting in settlement assays, to which 100 ng ISTD was added. A duplicate set of standards was prepared in sterile seawater containing either 25, 10, 5, 1 or 0 ng of synthetic histamine and 500 ng ISTD. Samples and standards were acidified with 10 µl glacial acetic acid. Mic-SCX membranes were washed with methanol (500 µl) and then Milli-Q (500 µl) by centrifuging for 15 s at 7000 × *g* and discarding the filtrate. Samples (500 µl) were applied to Mic-SCX and centrifuged at 1200 × *g* for 1 min, discarding the filtrate. This binding step was repeated 4 times until the total sample/standard was applied. Mic-SCX were washed with 10 mM HCl by centrifuging at 1200 × *g* for 1 min and discarding the filtrate. A new vial was used to collect bound compounds, which were eluted with two 50 µl applications of freshly prepared desorption reagent (500 µl methanol, 400 µl Milli-Q and 100 µl NH_4OH) and centrifuged at 14 000 × *g* for 15 s. Eluted compounds from 5 samples from each plant were pooled together to form 2 samples for each plant. Pooled samples and standards were dried in a Speed-Vac in labeled culture tubes and were derivatised with heptafluorobutyric anhydride and acetic anhydride (Barancin et al. 1998).

The histamine content of seawater samples and standards was analysed by electron-capture negative-ionisation (ECNI) GC-MS, which can detect much lower concentrations of histamine (~1000-fold) than EI GC-MS. The ECNI GC-MS instrument used is described in Smythe et al. 2002). The gas chromatograph oven temperature was held at 70°C for 2 min, ramped at 30°C min^{-1} to 250°C and held for 2 min (10 min run). The Mass Selective Detector was operated in selected ion monitoring mode (derivatised histamine, *m/z* 306 and derivatised ISTD, *m/z* 310) and extracted ion chromatograms were integrated. The histamine content of the samples (ng) was calculated by reference to the standard curve and converted to ng ml^{-1} (nM). The histamine concentration of seawater was analysed by a 1-way (seawater = fixed) ANOVA (transformed [ln(x + 1)]) and Bonferroni's post-hoc test was used to determine which treatments differed significantly at p = 0.05.

Settlement assay with *Amphiroa anceps* treated with antibacterial agents. Larvae of *Holopneustes purpurascens* settle rapidly in response to fronds of *Amphiroa anceps* in laboratory assays and the majority of new recruits of *H. purpurascens* were found on this alga. However, extracts of *A. anceps* lacked detectable histamine (Swanson et al. 2004) and these extracts only induced a slow rate of settlement of larvae at high concentrations (R. L. Swanson unpubl. data). The absence of histamine, or another rapid acting settlement cue, in extracts of *A. anceps* raises the possibility that the biofilm on *A. anceps* produces and releases a settlement cue *in situ* for *H. purpurascens.* The settlement cue from coralline turfing algae may in fact be bacterial derived histamine (or another compound) that is only produced and released *in situ* and hence not detectable in extracts of the algae (Swanson et al. 2004).

To test this hypothesis, *Amphiroa anceps* was subjected to antibacterial treatments and then tested against *Holopneustes purpurascens* larvae in settlement assays, to determine whether the settlement cue(s) from *A. anceps* is produced by the alga or the biofilm. Antibacterial treatments were adapted from previous studies where treatments were shown to be effective in reducing the diversity of surface-associated bacterial communities (Aguirre-Lipperheide & Evans 1993, Johnson & Sutton 1994, Huggett et al. 2005, Huggett et al. in press). The same experiment was run previously with *Delisea pulchra* in which antibacterial-treated *D. pulchra* induced equivalent levels of settlement to control plants, implying an algal derived cue (Swanson et al. 2004). *A. anceps* (n = 4) were collected and brought back to the laboratory, where portions of each plant were allocated to each of 4 treatments. All antibacterial treatments of *A. anceps* included a 5 min soak in a 10% betadine-SSW solution, followed by 3 rinses in SSW, and a 48 h treatment in either: (1) SSW containing 20 mg l^{-1} streptomycin, 10 mg l^{-1} penicillin G and 10 mg l^{-1} kanamycin ('AB' treatment); (2) SSW after pieces of *A. anceps* were gently wiped across an agar plate, before and after the 48 h soak, to physically remove bacteria ('wipe' treatment); (3) the combination of 'AB+wipe' treatment. The procedural control was a 48 h soak in SSW ('soak' treatment). Subsections of several *A. anceps* plants were collected on the day of the assay as a positive control ('fresh' treatment) and SSW was used as a negative control. Pieces of *A. anceps* (~10 mg ww) from each treatment were added to assigned dishes with 5 competent larvae (n = 10) and percent settlement scored after 24 h. The response of larvae to treated *A. anceps* in the settlement assay was analysed by a 1-way (treated alga = fixed) ANOVA (proportions untransformed) and Bonferroni's post-hoc test was used to determine which treatments differed significantly at p = 0.05.

Bacterial biofilms from coralline algae: settlement assay and histamine analysis. Some species of marine bacteria are known to produce histamine (Fujii et al. 1997); hence we further explored the possibility that

the settlement cue from coralline turfing algae may be bacterial derived histamine. Five bacterial isolates from the surface of *Amphiroa anceps* or *Corallina officinalis* (which also induces settlement of *Holopneustes purpurascens*) were screened against larval *H. purpurascens* in settlement assays. Surface bacteria from *A. anceps* and *C. officinalis* were cultured by adding pieces of each alga (n = 3) to 1 ml SSW, vortexing and diluting to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} and 100 µl of each dilution was spread onto Marine Agar plates. Plates were observed daily for 2 wk and each bacterial morphotype was re-streaked until purity, and stored in glycerol at −70°C until sequencing/screening. Single isolates were grown overnight, spun down and extracted as reported elsewhere (Dahllöf et al. 2000). PCR of 16s rDNA was then performed using primers F27 (5′-GAGTTTGATCCTGGCTCAG-3′) and R1492 (5′-ACGGTTACCTTGTTACGACTT-3′). Purified PCR product (50 to 100 ng) was sequenced unidirectionally using BigDye™ terminator cycle sequencing reaction mix (Applied Biosystems), using the F27 and R1492 16S rDNA primers and a 530F primer (5′-GTGCCAGCMGCCGCGG-3′). Sequences were analysed on an ABI 310 DNA system at the Sydney University Prince Alfred Molecular Analysis Centre, aligned and analysed using the BLAST search algorithm (www.ncbi.nlm.nih.gov).

A total of 5 bacterial isolates from the surface of *Amphiroa anceps* (A) or *Corallina officinalis* (C) were selected based on initial sequence data (all gammaproteobacteria, Table 1), for screening against larval *Holopneustes purpurascens* in settlement assays using an adapted method (Negri et al. 2001). We selected isolate C1 because its closest match using the BLAST search algorithm (at the time) was a known histamine producer, *Photobacterium phosphoreum* (Fujii et al. 1997). Two other isolates (A1, C2) that closely matched *Photobacterium* spp. were also selected, along with 2 additional isolates (A2, A3) from *A. anceps* for comparison (Table 1). Isolates were inoculated onto plates of Marine Agar and colonies were transferred 24 h later to suspended Marine Broth culture media (20 ml, n = 5). Sterile coverslips were added to tubes containing inoculated or control Marine Broth (containing no bacteria), for the formation of biofilms, which were agitated at room temp (20°C) for 24 h. Coverslips with a visible biofilm were selected from each treatment (n = 5) and rinsed gently with SSW before the settlement assay. Coverslips (biofilms) were placed in sterile petri-dishes with 4 ml of SSW and 5 competent larvae, and percent settlement scored at 94 h.

Marine Broth cultures from each treatment were kept for histamine analysis (n = 2). Broth cultures were centrifuged at 10 000 × *g* for 20 min to separate cells (pellet) from the culture media which contains any exogenous compounds released by bacteria (supernatant). The supernatants were filtered (0.2 µm) into sterile tubes and frozen until analysis. The supernatants were transferred to large vials containing 1 µg ISTD and were extracted 3 times with equal volumes of dichloromethane (DCM). The DCM extract was left to evaporate and the vials rinsed with 5 ml ethanol which was dried in a Speed-Vac. A set of standards containing either 10, 1, 0.1 or 0 µg of histamine and 1 µg ISTD were extracted with DCM as per samples. Extracted compounds and standards were derivatised and analysed by GC-MS (ECNI) as described above for the analysis of the histamine concentration of seawater.

The response of larvae in the biofilm assay and the histamine content of bacterial supernatants were both analysed by a 1-way (bacteria = fixed) ANOVA (proportions/content untransformed) and planned comparisons. Following the ANOVA, we compared the effect of isolates which induced >5% settlement (i.e. inductive treatments), as we *a priori* believed that these treatments were unlikely to be different. There was no significant difference between the 2 treatments so they were pooled. Next, the effects of isolates which induced <5% settlement (i.e. non-inductive treatments) were compared with the control as we *a priori* believed that these treatments were unlikely to be different. Non-inductive treatments did not differ significantly from the control and were therefore also pooled. Inductive treatments (pooled) were then tested

Table 1. *Corallina officinalis* and *Amphiroa anceps*. BLAST analysis and designated accession numbers for bacterial isolates from the surface of *C. officinalis* (C) and *A. anceps* (A) which were tested in settlement assays with larval *Holopneustes purpurascens*. The nearest matching bacterial strain (with designated accession number) and percent similarity (%) from 2 search dates are shown

Isolate ID	Accession number	Nearest match (initial search Aug 2003)	%	Nearest match (recent search Apr 2005)	%
C1	DQ005883	*Photobacterium phosphoreum* AJ746360	99	*Photobacterium damselae* AY147861	95
C2	DQ005897	*Photobacterium* sp. AY781193	97	*Photobacterium eurosenbergii* AJ842344	98
A1	DQ005851	*Photobacterium* sp. AY582934	96	*Pseudoalteromonas* sp. AY626830	99
A2	DQ005882	*Thalassomonas viridans* AJ294748	99	*Thalassomonas viridans* AJ294748	99
A3	DQ005869	*Vibrio mediterranei* X74710	99	*Vibrio medeterranei* X74710	98

against non-inductive treatments (pooled). The histamine content of bacterial supernatants from the respective inductive and non-inductive treatments were analysed using the same planned comparisons outlined for the settlement assay.

Statistical treatment. All experiments were balanced designs which were analysed by factorial analysis of variance (ANOVA) models using SYSTAT® 10.0 for Windows. Data were transformed when necessary to meet the assumptions of the test and were used only when the transformation improved the data spread or the homogeneity of variance. Details of each analysis are in the relevant sections.

RESULTS

Recruitment survey

Recruitment of *Holopneustes purpurascens* at Bare Island from December 2002 to November 2004 onto the seaweeds *Delisea pulchra*, *Corallina officinalis*, *Amphiroa anceps*, and *Homeostrichus olsenii* was generally low (1 to 7 total new recruits mo^{-1}) but recruits were found in all months except for February 2003 and September 2004 (Fig. 1). The highest number of new recruits was recorded in July 2004 on coralline turf algae (7 new recruits 100 g^{-1} alga ww); however, it was more common to find 1 to 4 new recruits mo^{-1} (100 g^{-1} alga ww). No new recruits were found on *E. radiata* or *S. vestitum* in any month. New recruits with test diameters ≤2.0 mm were found in 13 of the 21 mo sampled and represented about 20% of all new recruits found. Another 20% of new recruits had test diameters between 2.1 and 3.0 mm while the remaining 60% were split equally between the 2 larger size classes of test diameters between 3.1 and 4.0 mm or 4.1 and 5.0 mm. In total, 38% of all new recruits were found on *A. anceps*, 32% on *D. pulchra*, 22% on *C. officinalis* and 8% on *H. olsenii*. New recruits of *H. purpurascens* were differentially distributed among algal species at Bare Island (1-way ANOVA, $F_{alga\ (4,100)} = 9.061$, $p < 0.001$), with more recruits found on *D. pulchra* and the coralline turf algae than on *E. radiata* or *S. vestitum* (Bonferroni's pairwise comparisons, all $p < 0.02$).

Settlement assays with algae and seawater

A range of host algae were assayed against larvae of *Holopneustes purpurascens* to test for a settlement response. The algae tested induced varying levels of settlement of larvae after 1 and 24 h with no settlement observed in SSW (Fig. 2). Fresh pieces of *Delisea pulchra* and *Amphiroa anceps* induced approximately 50% settlement of larvae after 1 h whereas *Homeostrichus olsenii* and unfouled *Ecklonia radiata* induced significantly lower responses of 27 and 4% settlement, respectively (Fig. 2). Thus, the algae on which we found the most new recruits in the field induced the highest rate of settlement of *H. purpurascens* larvae in laboratory assays. Fouled

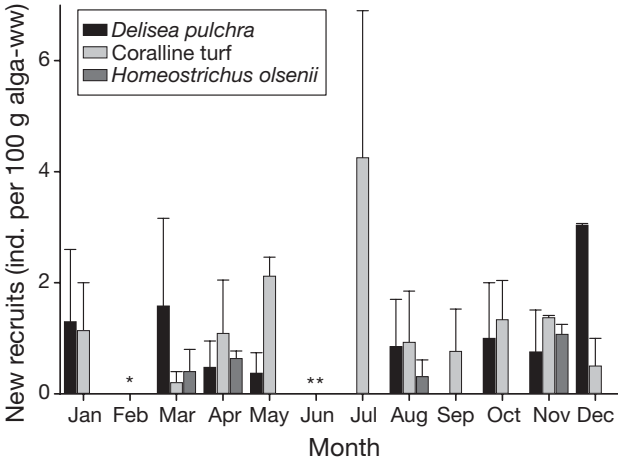

Fig. 1. *Holopneustes purpurascens*. Mean numbers (+SE) of new recruits (test diameter ≤5 mm) found each month (n = 2 yr) on *Delisea pulchra*, the coralline turf algae (*Amphiroa anceps* and *Corallina officinalis*) and *Homeostrichus olsenii* (total from n = 5 plants of each alga mo^{-1} standardised to number of recruits 100 g^{-1} ww alga sampled). New recruits were not found on *Ecklonia radiata* or *Sargassum vestitum* at any time. *not sampled in Year 2, **not sampled in Years 1 and 2

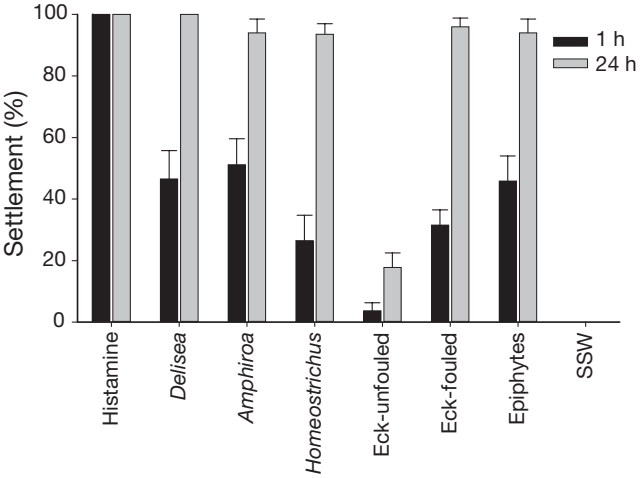

Fig. 2. *Holopneustes purpurascens*. Percent settlement of larvae (mean + SE, n = 10, 5 larvae per dish) after 1 and 24 h in response to a range of host algae: *Delisea pulchra*, *Amphiroa anceps*, *Homeostrichus olsenii*, fouled *Ecklonia radiata* (Eck-fouled), unfouled *E. radiata* (Eck-unfouled) and the fouling epiphytes removed from laminae of *E. radiata* (epiphytes). Histamine (10 µM) and SSW (sterile seawater) were included as the positive and negative controls

Table 2. *Holopneustes purpurascens.* (a) Univariate repeated measures analysis of the response of larvae to algae in settlement assays after 1 and 24 h, and (b) planned comparison p-values. *E.r.*: *Ecklonia radiata*, *D.p.: Delisea pulchra*, *A.a.*: *Amphiroa anceps*, *H.o.*: *Homeostrichus olsenii*, –: not analysed

(a) Source	df	MS	*F*	p
Between subjects				
Alga	5	1.142	32.421	<0.001
Error	54	0.035		
Within subjects				
Time	1	7.016	274	<0.001
Time × Alga	5	0.183	7.167	<0.001
Error	54	0.026		

(b) Planned comparisons (df = 1); p-values:	1 h	24 h
D.p. vs. *A.a.*	0.637	0.233
D.p. + *A.a.* vs. *H.o.*	0.011	0.420
D.p. + *A.a.* vs. unfouled *E.r.*	<0.001	–
D.p. + *A.a* . + *H.o.* vs. unfouled *E.r.*	–	<0.001
Epiphytes vs. fouled *E.r.*	0.151	0.689
Epiphytes + fouled *E.r.* vs. unfouled *E.r.*	<0.001	<0.001

E. radiata and epiphytes alone induced 32 and 49% settlement, respectively, after 1 h, significantly higher than the 4% settlement observed with unfouled *E. radiata* (Fig. 2). By 24 h all algal treatments had induced over 90% settlement of larvae except for unfouled *E. radiata*, which had induced a significantly lower response of 18% settlement (Fig. 2, Table 2). Statistical analysis found a significant interaction between alga × time, which was explored further by a series of planned comparisons of algal effects within each level of time (Table 2). There were no major qualitative differences in the statistical outcome of comparisons at 1 and 24 h, and hence we concluded that there were algal effects over and above any interaction.

We collected seawater samples *in situ*, adjacent to and at some distance from algae, to test whether the settlement cue for *Holopneustes purpurascens* leaches from these seaweeds. None of these seawater samples induced settlement in competent larvae of *H. purpurascens* (6 d post-fertilisation). However, the same seawater samples were subsequently tested with aged larvae (3 wk post-fertilisation) which are known to respond to lower concentrations of histamine (R. L. Swanson et al. unpubl. data). Aged larvae responded differentially to seawater collected *in situ*, adjacent to and at a distance from algae (Fig. 3, Table 3). Nine and 16% of aged larvae settled in response to *Delisea*-SW by 24 and 44 h, respectively (Fig. 3),

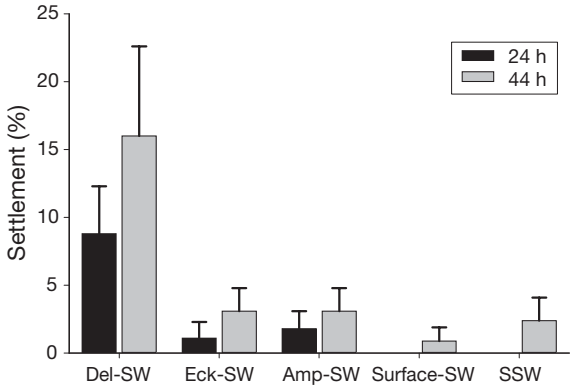

Fig. 3. *Holopneustes purpurascens*. Percent settlement (mean + SE, n = 10, 10 larvae per dish) of aged larvae (3 wk post-fertilisation) after 24 and 44 h in response to seawater collected *in situ* near *Delisea pulchra* (Del-SW), *Ecklonia radiata* (Eck-SW) and *Amphiroa anceps* (Amp-SW), or at the sea surface (Surface-SW). Histamine (10 µM: not shown, 100% settlement at 24 h) and SSW were included as the positive and negative controls

which was greater than settlement in Surface-SW and SSW (Control-SW, Table 3). Similar larval responses were observed in response to 2 other batches of *in situ* seawater samples, that is, significantly more aged larvae settled in response to *Delisea*-SW than to Control-SW (data not shown).

Temporal analysis of the histamine content of algae

Levels of histamine in *Delisea pulchra* (108 to 288 µg g^{-1} dw) were 2 to 3 orders of magnitude higher than

Table 3. *Holopneustes purpurascens.* (a) Univariate repeated measures ANOVA of the response of aged (3 wk post-fertilisation) larvae to seawater after 24 and 44 h in settlement assays. (b) Planned comparisons at 24 h. Seawater collected adjacent to algae, *Delisea pulchra* (*Delisea*-SW), *Ecklonia radiata* (*Ecklonia*-SW) and *Amphiroa anceps* (*Amphiroa*-SW), or at the sea surface (Surface-SW). SSW = sterile seawater, Surface-SW-SSW (pooled) = Control-SW

(a) Source	df	MS	*F*	p
Between subjects				
Seawater	4	0.059	5.836	<0.001
Error	45	0.010		
Within subjects				
Time	1	0.023	9.118	0.004
Time × Seawater	4	0.004	1.586	0.194
Error	45	0.003		
(b) Planned comparisons				
Surface-SW vs. SSW	1	0.000	0.000	1.000
Delisea-SW vs. Control-SW	1	0.062	21.66	<0.001
Amphiroa-SW vs. Control-SW	1	0.002	0.779	0.382
Ecklonia-SW vs. Control-SW	1	0.001	0.286	0.595

Table 4. *Delisea pulchra*, *Homeostrichus olsenii*, *Ecklonia radiata* and *Sargassum vestitum*. (a) Histamine content of 4 species of algae over time (µg g^{-1} dw, mean ± SE, n = 5 [except n = 4 for *Ecklonia radiata* in April]). (b) Histamine contents of the 4 species of algae over time were significantly different (2-factor ANOVA results shown). –: *S. vestitum* not present in the habitat in April

(a)	Sep 2003	Jan 2004	Apr 2004	Jul 2004
Delisea pulchra	288 ± 77	129 ± 92	108 ± 39	262 ± 52
Homeostrichus olsenii	2.62 ± 1.06	0.41 ± 0.14	0.64 ± 0.15	2.85 ± 0.91
Ecklonia radiata	0.27 ± 0.08	0.06 ± 0.03	0.28 ± 0.11	0.05 ± 0.01
Sargassum vestitum	0.10 ± 0.02	0.07 ± 0.01	–	1.12 ± 0.52
(b) Source	df	MS	*F*	p
Alga	2	133.9	271.2	<0.001
Month	3	1.936	6.172	0.001
Alga × Month	6	0.494	1.575	0.175
Error	48	0.313		

those of the other algae at all times (Table 4). Histamine levels in *D. pulchra* were 100 times greater than levels in *Homeostrichus olsenii* (0.41 to 2.85 µg g^{-1} dw), and approximately 1000 times greater than *Ecklonia radiata* (0.05 to 0.28 µg g^{-1} dw) and *Sargassum vestitum* (0.07 to 1.12 µg g^{-1} dw). The histamine content of *H. olsenii*, on which we found 8% of new recruits of *Holopneustes purpurascens*, was always higher than that of *E. radiata* and *S. vestitum*, on which no new recruits were found. The histamine content of *D. pulchra* was higher in July and September and this trend was explored further. *D. pulchra* plants sampled in July, August and September 2004 had the highest average histamine contents (262, 507 and 1041 µg g^{-1} dw, respectively) while those sampled in January 2003/2004 and April 2004 contained the least histamine (80 to 130 µg g^{-1} dw). The histamine content of *D. pulchra* varied considerably from month to month (1-way ANOVA, $F_{8,36}$ = 12.649, p < 0.001), as reflected by the lowest (10 µg g^{-1} dw, January 2003) (Swanson et al. 2004) and highest (1470 µg g^{-1} dw, September 2004) content recorded for individual plants.

Fig. 4. *Holopneustes purpurascens*. Percent settlement of larvae (mean + SE, n = 10, 5 larvae per dish) after 24 h in response to *Amphiroa anceps* that had been treated to reduce the surface bacterial community. The anti-bacterial treatment of *A. anceps* included 'AB' (streptomycin, penicillin G and kanamycin), 'wipe' (across agar) and 'AB + wipe' treatments. Control treatments were 'soak' (in SSW), 'fresh' *A. anceps* and SSW. Treatments that share a line induced a similar larval response (ANOVA, p < 0.001; Bonferroni pairwise comparisons, p < 0.001)

Variation in histamine across the thallus of *Delisea pulchra* and *Ecklonia radiata*

The histamine content varied significantly across the thallus of *Delisea pulchra*, with levels in the base of the plant approximately 2-fold greater than the mid section and 4-fold greater than the tips (Table 5). The primary and secondary laminae of *Ecklonia radiata* both contained very low levels of histamine. The histamine content of the fouling epiphytes (6.0 µg g^{-1} dw) was 100-fold greater than the laminae and comparable to the lowest levels recorded in *D. pulchra* (Swanson et al. 2004).

Histamine concentration of seawater in the habitat

Delisea-SW contained the highest concentration of histamine (4.20 ± 0.75 nM) of all seawater samples, with significantly lower levels in *Ecklonia*-SW (1.54 ± 0.15 nM), *Amphiroa*-SW (0.58 ± 0.16 nM) and Surface-SW (0.42 ± 0.17 nM; 1-way ANOVA, $F_{3,4}$ = 56.256, p = 0.001; Bonferroni pairwise comparisons, all p < 0.02).

Settlement assay with *Amphiroa anceps* treated with antibacterial agents

Amphiroa anceps treated with antibiotics (AB), and *A. anceps* treated with antibiotics and an agar-wipe (AB+wipe), induced minimal settlement (<5%) relative to fresh and soaked (procedural control) *A. anceps*, which induced 52 to 64% settlement of *Holopneustes purpurascens* larvae (Fig. 4, 1-way ANOVA, $F_{4,45}$ = 15.985, p < 0.001; Bonferroni pairwise comparisons, p < 0.001).

Table 5. *Delisea pulchra* and *Ecklonia radiata*. The histamine content (µg g^{-1} dw, mean ± SE) of different regions of the thallus of *D. pulchra* (n = 5) and *E. radiata* (n = 3). 1-way ANOVA results shown. *Histamine content is significantly different to base content, p = 0.003, **significantly different to base or laminae content, p < 0.001 (Bonferroni pairwise comparisons)

	Thallus	Histamine mean ± SE
Delisea pulchra	Base	2657 ± 286
$F_{2,12}$ = 18.850 p < 0.001	Mid	1231 ± 194*
	Tip	716 ± 285**
Ecklonia radiata	1°	0.059 ± 0.029
$F_{2,6}$ = 205.74 p < 0.001	2°	0.044 ± 0.006
	Epiphytes	6.01 ± 1.17**

Bacterial biofilms from coralline algae: settlement assay and histamine analysis

Holopneustes purpurascens larvae settled to C1-biofilms (43%) and A2-biofilms (20%) by 94 h (Fig. 5a). Settlement in dishes containing these inductive biofilms ranged from 0 to 100% and the settlement rate was slow. The larval settlement response to C1-biofilms and A2-biofilms was significantly higher than settlement to other bacterial biofilms and control biofilms which contained no bacteria (1-way ANOVA, $F_{5,24}$ = 1.922, p = 0.128; planned comparisons [df = 1, 24]: C1 vs. A2, p = 0.20; A3 vs. control, A3-control vs. A1, A1-A3-control vs. C2, all p > 0.8; C1-A2 vs. C2-A1-A3-control, p = 0.01). The histamine content of the supernatants from broth cultures of isolates in which biofilms were cultured correlated with the level of settlement induced by the different biofilms in the assay (Fig. 5a,b). Supernatants from the most inductive isolates in the assay (C1 and A2) contained higher concentrations of histamine (0.091 and 0.068 µg ml^{-1}, Fig. 5b). In contrast, supernatants from the non-inductive isolates including A3 (≤5% settlement) had similar concentrations of histamine (0.017 to 0.033 µg ml^{-1}) as the control supernatant (0.021 µg ml^{-1}) (1-way ANOVA, $F_{5,6}$ = 1.71, p = 0.265; planned comparisons [df = 1,6]: A3 vs. control, A3-control vs. A1, A1-A3-control vs. C2, all p > 0.65). Although low concentrations of histamine were detected in the broth media containing no bacteria (control supernatant) and non-inductive isolates, significantly higher histamine concentrations were detected in supernatants of broth media in which inductive isolates C1 and A2 were cultured (planned comparisons [df = 1,6]: C1 vs. A2, p = 0.51; C1-A2 vs. C2-A1-A3-control, p = 0.032).

DISCUSSION

Recruitment of new individuals into an appropriate habitat is fundamental for the survival of the new recruit and for the persistence of populations in benthic ecosystems (Underwood & Keough 2000). Improved knowledge of settlement cues that govern this crucial stage in the natural history of marine organisms could lead to advancements in aquaculture and antifouling technologies, and better management of ecologically important ecosystems. However, despite over 60 yr of research effort no settlement cue for a marine invertebrate has been unequivocally identified and quantified *in situ*. In the current paper we present the first investigation of the settlement and recruitment of a marine invertebrate with respect to the quantitative distribution of a chemical settlement cue in the organism's habitat. We have shown that the distribution of new recruits of *Holopneustes purpurascens* at the study site can be partially explained by the distribution of histamine in the habitat. We found recruits of *H. purpurascens* (≤5 mm) in almost every month of 2 yr of sampling and recruits less than 2 mm were found in all

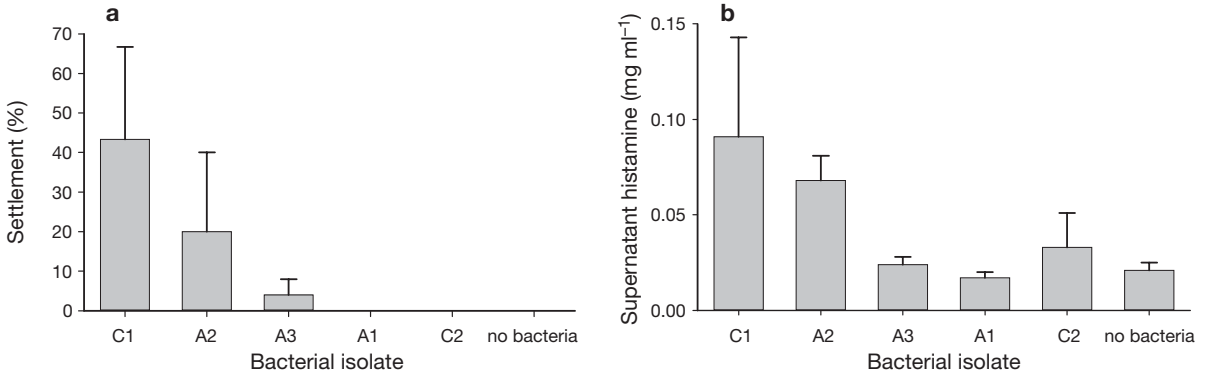

Fig. 5. *Holopneustes purpurascens*. (a) Percent settlement of larvae (mean + SE, n = 5 dishes, 5 larvae per dish) after 94 h in response to biofilms of bacterial isolates from the surface of *Amphiroa anceps* (A1, A2, A3) or *Corallina officinalis* (C1, C2; see Table 1). Biofilms containing no bacteria were used as the negative control and histamine (10 µM) was used as the positive control (not shown). (b) Histamine concentration (µg ml^{-1} ± SE) of the supernatants from the culture of the bacterial isolates (n = 2)

months except February (sampled 1 yr), June (not sampled) and August. The distribution of recruits through time suggests that recruitment of *H. purpurascens* occurs throughout the year at this site. These findings are consistent with the continuous production of the settlement cue by *D. pulchra* and the reproductive cycle reported for *H. purpurascens* (Williamson & Steinberg 2002). The actual breeding period of *H. purpurascens* is throughout spring and early summer; however, vitellogenesis appears to be continuous in this species, meaning they are capable of reproducing sporadically throughout the year, which is rare among urchins (Williamson & Steinberg 2002). This ability of *H. purpurascens* to reproduce throughout the year is reflected in our experience with larval culture. Larvae were obtained in all seasons over a 5 yr period, albeit with low yields of larvae (50 to 100) in some months (R. L. Swanson pers. obs., Williamson & Steinberg 2002).

With the exception of the coralline algae for which histamine could not be detected, variation in settlement and recruitment was consistent with the variation among species' histamine contents, supporting the link between histamine production and settlement on these algae. Of all new recruits 32% were found on *Delisea pulchra*, which contained far greater levels of histamine (up to 1000-fold) than the other algae surveyed (see Fig. 1, Table 4). The algal histamine data reported here are consistent with a previous study (Swanson et al. 2004) although differences between *D. pulchra* and *Ecklonia radiata*/*Sargassum vestitum* in this paper are 100-fold greater, due to greater accuracy and sensitivity of the analysis. As regards new recruits of *Holopneustes purpurascens*, 60% were collected from the coralline turf algae *Amphiroa anceps* (38%) and *Corallina officinalis* (22%), which are far more abundant at this site than *D. pulchra*, forming dense tufts in sub-canopies below foliose algae (including *D. pulchra*) and kelp. Another study at Bare Island found new recruits of *H. purpurascens* on *D. pulchra*, but none on coralline turf algae (Williamson et al. 2004). The coralline algae also induced rapid settlement of *H. purpurascens* larvae (Fig. 2); however, treatment with antibiotics greatly reduced the settlement in response to this alga (Fig. 4). This is in contrast to *D. pulchra*, which induced a high settlement response in *H. purpurascens* larvae even after being treated with these antibacterial agents (Swanson et al. 2004). This treatment of *A. anceps* with antibiotics reduced the diversity of the surface-associated bacterial community (biofilm) by ~75% but does not remove all bacterial strains (Huggett et al. in press). Despite the persistence of some surface bacterial strains after treatment, the antibiotics drastically reduced the capacity of *A. anceps* to induce settlement of *H. purpurascens* which supports a biofilm based settlement cue from *A. anceps* for *H. purpurascens*.

Biofilms on the surface of animate and inanimate marine surfaces produce and release settlement cues for a range of invertebrate larvae (Wieczorek & Todd 1998). Biofilms of C1 and A2, bacterial components of the natural biofilm from the surface of *Corallina officinalis* and *Amphiroa anceps*, induced 43 and 20% settlement of larval *Holopneustes purpurascens*, respectively (Fig. 5a). Settlement in dishes containing inductive biofilms ranged from 0 to 100% and the rate of settlement was slow, which was possibly due to differences in the density and condition of the biofilm on the coverslips. The rate of settlement induction was too slow (96 h) to be conclusive, given that the composition of C1- and A2-biofilms may have been altered by the introduction of bacteria along with the larvae; however, there was no settlement in the control (bacteria-free biofilm) by this time. The broth media used to culture isolates contained low concentrations of histamine. However, significantly higher concentrations of histamine were detected in supernatants of media in which the inductive isolates, C1 and A2, were cultured and biofilms formed (Fig. 5b). The higher concentrations of histamine in these supernatants suggests bacterial production and release of histamine *in vitro* by C1, as reported previously for this bacterium (Yoguchi et al. 1990). Together, these initial findings support a settlement cue of bacterial origin from coralline algae for *H. purpurascens* which may be bacterial derived histamine. Histamine was not detected in extracts of the coralline algae nor in *in situ* seawater samples collected adjacent to *A. anceps*; however the biofilm on the algal surface may only produce histamine *in situ* at certain times, releasing histamine into surrounding seawater. If the bacterial settlement cue proves to be histamine, this would be the first system described for which larval settlement of a marine invertebrate is induced by one compound that is produced by both eukaryotes and prokaryotes in the natural habitat. However, evidence for a bacterial source of histamine is at a much earlier stage than our case for algal derived histamine acting as a settlement cue for larval *H. purpurascens*.

The majority of the literature on chemical settlement cues for larvae focuses on surface-bound, non-polar chemicals that induce larval settlement, because for many years the only effective settlement cues were considered to be surface-bound (Morse 1990, Pawlik 1992). It was argued that dissolved settlement cues would be ineffective due to rapid dilution in seawater and, even if detected by larvae, the weak swimming abilities of most would prevent them from swimming towards the source of the cue, particularly in turbulent environments (Crisp 1974, Butman 1987). However, it is now clear that a number of species of marine invertebrate settle in response to dissolved chemical cues

(Hadfield & Paul 2001), in still water assays (Williamson et al. 2000, Swanson et al. 2004), laboratory flumes under realistic flow conditions (Turner et al. 1994, Tamburri et al. 1996), and in the field (Browne & Zimmer 2001).

This paper reports the first *in situ* quantitative measurements of a characterised chemical cue in seawater. Very low concentrations of dissolved histamine (~5 nM) were measured in seawater collected *in situ* adjacent to *Delisea pulchra*, with significantly lower levels detected in seawater collected adjacent to *Ecklonia radiata*, *Amphiroa anceps* or at the sea surface. For a settlement cue to be ecologically relevant it must be present in the habitat at inductive concentrations. None of the *Delisea*-SW tested here induced settlement in newly competent *Holopneustes purpurascens* larvae. However, 16% of aged larvae settled in response to *Delisea*-SW (compared to <3% settlement in SSW), indicating that a dissolved settlement cue was present *in situ* in the seawater surrounding *D. pulchra* plants (Fig. 3). In other experiments, 10 nM of histamine induced 10 to 40% settlement of aged larvae (3 to 4 wk post-fertilisation) after 24 to 72 h with less than 5% settlement in SSW at 72 h (R. L. Swanson et al. unpubl. data). Newly competent larvae do not settle in response to 10 nM histamine which suggests that aged larvae show an increased sensitivity to histamine while at the same time maintaining their selectivity for histamine as a settlement cue for at least 4 weeks post-fertilisation (R. L. Swanson et al. unpubl. data). Thus, levels of histamine measured in the habitat induce settlement of larvae, though at low rates and only for aged larvae, which may be more typical in the natural habitat than newly competent larvae.

An earlier study of this system used *Delisea*-SW which induced 100% settlement of newly competent *Holopneustes purpurascens* larvae after 4 h (Williamson et al. 2000). The histamine concentration of these samples was not measured but may have contained higher concentrations of histamine than reported here. This earlier result suggests that inductive levels of histamine do occur *in situ* in seawater surrounding *Delisea pulchra* plants in the habitat. We found considerable variation in the histamine content of *D. pulchra* plants sampled simultaneously and through time (Tables 4 & 5). Thus, higher concentrations of histamine may occur in seawater surrounding *D. pulchra* plants during times when the histamine content of the plants is high (e.g. in September 2004), or in the boundary layer at the surface of *D. pulchra* plants. It is also possible that low concentrations of histamine are more effective at inducing settlement in the natural habitat, when the cue is detected in unison with other chemical cues or physical factors such as flow.

In a previous study, *Ecklonia radiata* and *Sargassum vestitum* induced minimal settlement of newly competent (6 d old) larval *Holopneustes purpurascens* while red algae induced 20 to 100% settlement after 24 h (Williamson et al. 2004). The brown algae/kelp induced 20% of 18 d old larvae to settle (Williamson et al. 2004) suggesting that older larvae show a decreased selectivity for red algae. The increased settlement response to brown algae/kelp may be due to an increased sensitivity of larvae to the lower concentrations of histamine present in these algae (R. L. Swanson et al. unpubl. data). In this study, we tested only 6 d old larvae in the algal assay of which ~20% settled in response to unfouled *E. radiata* after 24 h (Fig. 2). The difference in settlement responses of 6 d old larvae to *E. radiata* in this study and Williamson et al. (2004) may be explained by the histamine analysis of the kelp and its epiphytes (Table 5). Unfouled *E. radiata* contained negligible amounts of histamine and induced a low settlement response. However, the epiphytic fouling community, predominantly filamentous brown and red algae (*Sphacelaria* sp., *Polysiphonia blandii* (Jennings & Steinberg 1997) contained levels of histamine that were comparable to the lowest amounts recorded in *Delisea pulchra* (Swanson et al. 2004) and consequently fouled *E. radiata* and epiphytes alone induced a high rate of settlement. Given the biomass of fouled *E. radiata* in the habitat of *H. purpurascens*, fouled kelp may leach histamine into surrounding seawater, particularly in dense beds of *E. radiata*. The seawater analysis supports this claim as higher concentrations of histamine were detected in *Ecklonia*-SW than in Surface-SW ($p = 0.038$). The subsequent absence of new recruits or small juveniles on fouled *E. radiata* may be due to the morphology of kelp: the new recruits are probably not able to attach to, or enmesh themselves in, the large and tough laminae of kelp. However, post-recruitment migration of juvenile *H. purpurascens* from *D. pulchra* and coralline algae to *E. radiata*, once the urchins are large enough to enmesh themselves in the laminae, is crucial to the fitness of this species as *H. purpurascens* of all ages perform poorly on *D. pulchra* (Williamson et al. 2004), while coralline algae only provide adequate nutrition for the growth of small (<10 mm) juvenile urchins (Meidel & Scheibling 1999, Williamson et al. 2004).

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