

# Induction of Settlement of Larvae of the Sea Urchin *Holopneustes purpurascens* by Histamine From a Host Alga

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**Abstract.** Larvae of the Australian sea urchin *Holopneustes purpurascens* are induced to settle and metamorphose (termed settlement herein) by a water-soluble compound produced by the red alga *Delisea pulchra*, the main host plant of new recruits. The settlement cue for *H. purpurascens* had previously been identified as a floridoside-isethionic acid complex, and this paper presents new evidence correcting that finding. The actual settlement cue produced by *D. pulchra* was isolated from the polar extract by cation-exchange chromatography and identified as histamine, using one- and two-dimensional nuclear magnetic resonance spectrometry. The chemical identity of the cue was confirmed by gas chromatography–mass spectrometry (GC-MS) and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry. Synthetic histamine and histamine at 4.5  $\mu\text{M}$  isolated from *D. pulchra* both induced rapid settlement in 80%–100% of the larvae of *H. purpurascens*. Lower concentrations of histamine (0.9–2.3  $\mu\text{M}$ ) induced larval settlement, but this response varied from 0%–90%. The histamine content of two host plants of *H.*

*purpurascens*—*D. pulchra* and *Ecklonia radiata*—and of four other common species was quantified using GC-MS. *D. pulchra* had the highest histamine content, which is consistent with *H. purpurascens* recruiting to this species. Histamine was also detected in the seawater surrounding these host algae. This is the first time that a settlement cue has been quantified in the habitat of a marine organism.

## Introduction

Most marine invertebrates have complex life histories in which a dispersive larval phase alternates with benthic juvenile and adult phases. The demography of such species is highly dependent on larval recruitment to a favorable habitat (Pawlik, 1992; Underwood and Keough, 2000), and the question of how planktonic larvae locate an appropriate benthic habitat in which to settle has long been a focus for marine biologists. The current view is that hydrodynamic processes dominate at large spatial scales (meters, kilometers), with active habitat selection becoming progressively more important at smaller spatial scales (centimeters, millimeters, micrometers) (Keough and Downes, 1982; Mullineaux and Butman, 1991; Harvey and Bourget, 1997; Zimmer and Butman, 2000).

Active habitat selection requires that larvae discriminate among potential settlement sites, which is possible through the detection of habitat-specific cues. Many laboratory experiments have confirmed that larvae from a diverse range of phyla respond both behaviorally (settlement—sinking to the bottom for substrate exploration) and morphologically (metamorphosis—ontogenesis into the benthic form) to such physical factors of a habitat as light (Maida *et al.*,

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**Abbreviations:** CX, cation-exchange; F-I, floridoside-isethionic acid; GC-MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; ISTD, internal standard; MALDI-TOF MS, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry; NMR, nuclear magnetic resonance; SSW, sterile seawater.

1994), surface orientation (Raimondi and Morse, 2000), flow conditions (Mullineaux and Butman, 1991), crevices (Keough and Downes, 1982), and surface texture (Bernstson *et al.*, 2000). Larvae can also be induced to settle and metamorphose (collectively termed settlement in this paper) by surface-bound or waterborne chemical cues, which are thought to indicate a suitable habitat for the benthic stage (Hadfield and Paul, 2001). The source of such chemical cues (inducers) may be conspecifics (Burke, 1986), host organisms (Williamson *et al.*, 2000), prey (Hadfield and Scheuer, 1985), or biofilms (Wieczorek and Todd, 1998).

The chemical cues for larval settlement that have been isolated from natural sources within the habitat appear to be diverse; however, most have been only partially characterized. These include small peptides (the sand dollar *Dendraster excentricus*—Burke, 1984; the oyster *Crassostrea virginica*—Zimmer-Faust and Tamburri, 1994; the jellyfish *Cassiopea xamachana*—Fleck and Fitt, 1999), uncharacterized low-molecular-weight water-soluble compounds (the nudibranch *Phestilla sibogae*—Hadfield and Pennington, 1990; the cephalaspidean *Haminaea callidegenita*—Gibson and Chia, 1994), carbohydrates (the coral *Agaricia humilis*—Morse and Morse, 1996; the ascoglossan *Alderia modesta*—Krug and Manzi, 1999), and glycoproteins (the barnacle *Balanus amphitrite*—Clare and Matsumura, 2000).

In contrast to the numerous partially characterized inducers, there are only a few examples in which the chemical structure of a settlement cue isolated from a natural source has been determined. Delta-tocopherols from *Sargassum tortile* induced settlement of the hydroid *Coryne uchidai* (Kato *et al.*, 1975), jacarone isolated from the red alga *Delesseria sanguinea* induced settlement of the scallop *Pecten maximus* (Yvin *et al.*, 1985), narains and anthosamines A and B isolated from marine sponges induced settlement of ascidian larvae (Tsukamoto *et al.*, 1994, 1995), and lumichrome isolated from conspecifics induced settlement of larvae of the ascidian *Halocynthia roretzi* (Tsukamoto *et al.*, 1999). In most cases, the ecological relevance of these compounds *in situ* is not clear, either because the source of the settlement cue is not necessarily related to the recruitment patterns of the organism (Yvin *et al.*, 1985; Tsukamoto *et al.*, 1994, 1995), or because the availability of the cue to settling larvae has not been demonstrated (Tsukamoto *et al.*, 1999).

A naturally occurring characterized settlement cue that appears to strongly affect the demography of the sea urchin *Holopneustes purpurascens* Agassiz 1872 (Temnopluridae: Echinodermata) was recently reported by Williamson *et al.* (2000). *H. purpurascens* is an endemic Australian echinoid that lives in shallow subtidal habitats in the canopy of macroalgae, particularly *Delisea pulchra* Greville (Montagne) 1844 and *Ecklonia radiata* (C. Agardh) J. Agardh 1898 at Bare Island, Sydney (Williamson *et al.*, 2000, 2004). Although abundant on both host plants, the smaller

size classes of *H. purpurascens* were most abundant on *D. pulchra*, with the smallest size class (test diameter  $\leq 5$  mm) found only on that species. This suggested that *D. pulchra* might produce a settlement cue for larval *H. purpurascens* (Williamson *et al.*, 2000). Fresh pieces of *D. pulchra* (but not *E. radiata*) and seawater collected *in situ* near *D. pulchra* plants induced settlement in larvae of *H. purpurascens*. The water-soluble cue was subsequently isolated and characterized as a complex between the sugar floridoside and isethionic acid (F-I complex; Williamson *et al.*, 2000).

During further research on this system, we obtained inductive fractions that contained isethionic acid but not floridoside, and we were also unable to reproduce a synthetic F-I complex that induced settlement of larval *H. purpurascens*. Subsequently, we hypothesized that the F-I complex was not a natural settlement cue for this urchin. This paper identifies the true nature of this chemical cue from *D. pulchra* for settlement of *H. purpurascens* larvae, correcting the previous finding of Williamson *et al.*, (2000). In addition, we quantify the settlement cue in host and non-host algae of *H. purpurascens*—the first time that a natural settlement cue has been quantified in the habitat of a marine organism.

## Materials and Methods

### Study site

All animals and algae used in this study were collected from sublittoral habitats (1–3 m depth) at Bare Island (33° 59' 38" S, 151° 14' 00" E) at the north head of Botany Bay, Sydney, Australia. At this site individuals of *Holopneustes purpurascens* are primarily found wrapped in the laminae of the brown kelp *Ecklonia radiata* (Laminariales: Phaeophyta) or in the fronds of the red foliose alga *Delisea pulchra* (Bonnemaisoniales: Rhodophyta). A more detailed description of this habitat and the ecology of this system are found in Wright and Steinberg (2001) and Williamson *et al.* (2004).

### Preparation of the polar extract of *Delisea pulchra*

The results of Williamson *et al.* (2000) indicated that any settlement cues were contained within the polar fraction of the crude extract of *D. pulchra*. A polar extract of *D. pulchra* was thus prepared from 1.0 kg (wet weight) of algae collected from Bare Island. Epibiota were removed, the plants blotted dry, and the thallus exhaustively extracted in methanol (OmniSolv, EM Science). The methanol extract was filtered (Whatman #1), dried by rotary evaporation *in vacuo* at 40 °C, and partitioned between dichloromethane (OmniSolv) and Milli-Q water. The Milli-Q phase was filtered (Whatman #1) and dried *in vacuo* at 40 °C. The dried crude polar extract was dissolved in absolute ethanol

three times, pooling each extract, and dried *in vacuo* at 40 °C to yield the polar extract.

*Isolation of the settlement cue in Delisea pulchra by bioassay-guided fractionation*

**High-performance liquid chromatography.** The polar extract of *D. pulchra* was fractionated using reversed-phase high-performance liquid chromatography (HPLC—Adsorbosil C18 column, 5- $\mu\text{m}$  particle size, 250 mm  $\times$  4.6 mm, Waters R410 RI-detector) (100% Milli-Q water at 1 ml  $\cdot$  min<sup>-1</sup>). The polar extract was dissolved in Milli-Q water (50 mg  $\cdot$  ml<sup>-1</sup>), filtered (0.22  $\mu\text{m}$ ), and manually injected (20  $\mu\text{l}$ ). HPLC resolved two major peaks, peak 1 with a retention time (rt) of 2.7 min, and peak 2 with rt = 3.4 min (Fig. 1A). Each peak fraction was collected from multiple injections and dried by rotary evaporation *in vacuo* at 40 °C. Peak fractions were tested for bioactivity in

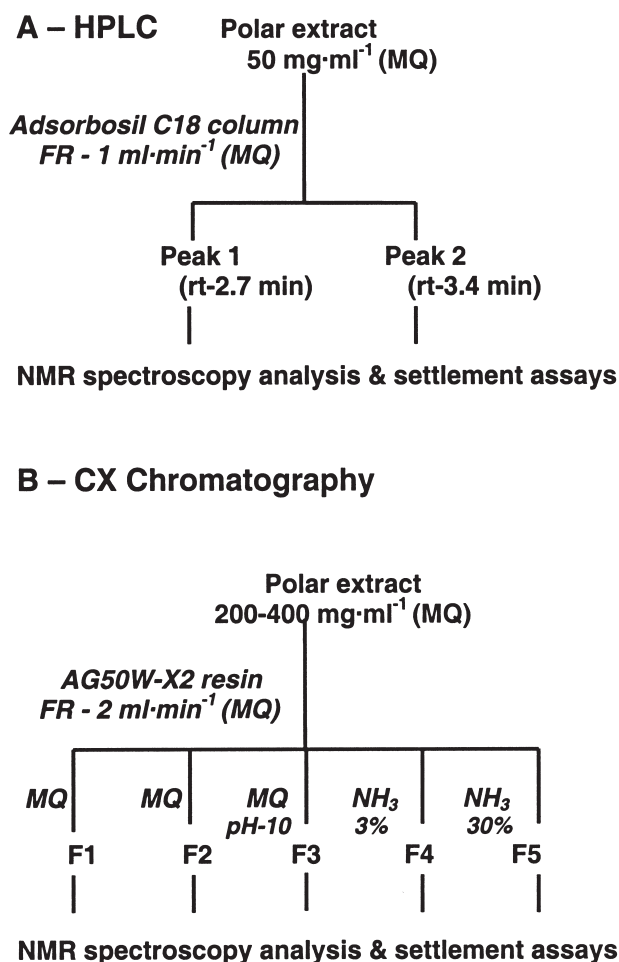
settlement assays and analyzed by <sup>1</sup>H- and <sup>13</sup>C-nuclear magnetic resonance (NMR) spectroscopy (Bruker DMX 500).

**Cation-exchange chromatography.** The settlement cue could not be isolated as a pure fraction using HPLC, so an alternative procedure, cation-exchange (CX) chromatography, was used to fractionate the polar extract of *D. pulchra*. CX resin (AG50W-X2 [H<sup>+</sup> form], BioRad) in Milli-Q water was poured into a 50-ml burette, taking care to exclude air bubbles. The resin (25-ml bed volume) was equilibrated with Milli-Q water at 2 ml  $\cdot$  min<sup>-1</sup> until the eluant was pH 5–6. The polar extract of *D. pulchra* (1–2 g) was dissolved in 5 ml of Milli-Q water, filtered (0.22  $\mu\text{m}$ ), and gently loaded onto the column. Unbound compounds were collected in 100 ml of Milli-Q water (fraction 1) and another 100 ml of Milli-Q water (fraction 2). Retained compounds were eluted using a series of basic solutions: 30 ml of dilute NH<sub>3</sub> in Milli-Q water (pH 10; fraction 3), 30 ml of 3%-NH<sub>4</sub>OH w/w (fraction 4), and 30 ml of 30%-NH<sub>4</sub>OH w/w (fraction 5, Fig. 1B). Fractions 1–5 were collected as controls, using the same method but without loading any *D. pulchra* extract on the column; none of these fractions had any subsequent activity. CX-fractions were dried in a centrifuge *in vacuo* (speed-vac SVC200, Savant), tested for bioactivity in settlement assays, and analyzed by <sup>1</sup>H-NMR spectroscopy.

*Identification of isolated settlement cue*

**Nuclear magnetic resonance spectroscopy.** Bioassay-guided fractionation of the polar extract of *D. pulchra* by cation-exchange chromatography yielded one active fraction (CX-fraction 5, F5). The inducing compound in F5 was identified by <sup>1</sup>H and <sup>13</sup>C-NMR experiments (D<sub>2</sub>O), and a high-field two-dimensional <sup>1</sup>H-<sup>15</sup>N HMBC NMR experiment (*d*<sub>4</sub> MeOH, Bruker DMX 500). To confirm the putative structure of F5 as histamine, 3 mg of F5 was dissolved in D<sub>2</sub>O and analysed by <sup>1</sup>H-NMR spectroscopy. Synthetic histamine (3 mg) was added to F5 and the sample re-analysed. The <sup>1</sup>H-NMR spectra of the unspiked F5 sample and the spiked F5 sample were then compared.

**Gas chromatography–mass spectrometry.** NMR spectroscopy analyses identified the isolated settlement cue as histamine, and this was confirmed by gas chromatography–mass spectrometry (GC-MS). Putative (naturally isolated) histamine (1 mg) and synthetic histamine (1 mg) were derivatized with heptafluorobutyric anhydride (Aldrich) and then acetic anhydride (Aldrich), using the method of Barancin *et al.* (1998). Derivatized samples were diluted 100-fold in ethyl acetate before analysis. A Zebtron ZB-5 column (15 m, 0.25  $\mu\text{m}$   $\times$  0.25 mm ID; Phenomenex) was used on a



**Figure 1.** Diagram of the bioassay-guided fractionation of the polar extract of *Delisea pulchra*, using either reversed-phase HPLC (A) or cation-exchange (CX) chromatography (B). MQ = Milli-Q water, FR = flow rate, rt = retention time, F = fraction.

Hewlett Packard (HP) 5980 series II gas chromatograph equipped with an HP5971A or HP5972 mass selective detector. Injections (2  $\mu\text{l}$ ) were in the splitless mode with an inlet pressure of 170 kPa. The injection port was held at 290 °C and the interface at 300 °C. The gas chromatograph was held at 90 °C for 2 min and ramped at 10 °C  $\cdot$  min<sup>-1</sup> to 200 °C, then at 50 °C  $\cdot$  min<sup>-1</sup> to 310 °C and held for 2 min (17.2 min per run). Helium was used as the carrier gas. The mass selective detector was operated in scan mode ( $m/z$  50–550). The average retention times of derivatized putative histamine and derivatized synthetic histamine were recorded from five injections of each sample (mean  $\pm$  SD,  $n = 5$ ). The electron impact ion-spectra of derivatized putative histamine and derivatized synthetic histamine were compared.

*Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry.* The elemental formula of putative histamine was determined by matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (Bucknall *et al.*, 2002). A Perseptive Voyager DE STR (Perseptive Biosystems, Framingham, MA) MALDI-TOF MS was operated in both positive-ion linear delayed-extraction mode and reflector delayed-extraction mode for accurate mass analysis. The test samples were prepared in acetonitrile/Milli-Q water (50:50) and contained either 100 ng  $\cdot$   $\mu\text{l}^{-1}$  of putative histamine or synthetic histamine.  $\alpha$ -Cyano-4-hydroxycinnamic acid (5 mg  $\cdot$  ml<sup>-1</sup>) prepared in acetonitrile/Milli-Q/trifluoroacetic acid (80:20:0.02) was used as the matrix. Glycine (500 ng  $\cdot$   $\mu\text{l}^{-1}$ ) and [sarcosine-<sup>15</sup>N-methyl-d<sub>3</sub>]creatinine  $\cdot$  HCl (5 ng  $\cdot$   $\mu\text{l}^{-1}$ , Cambridge Isotope Laboratories #DNLM-2171) were added as internal mass calibrants for accurate mass determinations. An accurate mass for the putative protonated histamine molecular ion  $[\text{M}+\text{H}]^+$  was determined by 10 repeat analyses of each sample. The mean molecular weight was calculated for these mass spectra and compared with both the theoretical molecular weight for histamine and the molecular weight measured for synthetic histamine using the same analytical technique. The standard deviation for these mass measurements was taken as an estimate of the mass measurement error.

#### Settlement assays

*H. purpurascens* larvae were cultured as previously described (Williamson *et al.*, 2000). Larvae reached competency (*i.e.*, become developmentally ready for settlement) within 6 days, as recognized by the presence of five well-developed tube feet. All settlement assays were done at 19 °C with a 12-h-light/12-h-dark regime, in 40-mm petri dishes and 5 ml of sterile seawater (SSW). Replicates were randomly assigned among treatments, with 10–15 replicates per treatment and one 6-day larva per replicate dish. We were unable to use multiple larvae per dish in these assays

because this species is a “dribble” spawner and generally yields low numbers of larvae (settlement is not gregarious; Williamson *et al.*, 2000). Larvae were added once all petri dishes were prepared, and percent settlement (*i.e.*, percent metamorphosed) was recorded at set time intervals.

*HPLC peak fractions.* Peak 1 and peak 2 fractions were tested against larvae to determine the presence of a settlement cue. Peak fractions were dissolved in Milli-Q water (10 mg  $\cdot$  ml<sup>-1</sup> stock solution) and aliquots of each stock solution were added to assigned petri dishes for final test concentrations of 25  $\mu\text{g} \cdot$  ml<sup>-1</sup> of peak 1 and 51  $\mu\text{g} \cdot$  ml<sup>-1</sup> of peak 2. A floridoside-isethionic complex sample (“F-I complex”) from the previous study (Williamson *et al.*, 2000) was also tested in the assay at a final concentration of 76  $\mu\text{g} \cdot$  ml<sup>-1</sup>. Pieces of fresh *D. pulchra* (~10 mg) were used as a positive control, and Milli-Q water and SSW were used as the negative controls. Percent settlement was scored after 18 h ( $n = 12$  replicates per treatment).

*Cation-exchange fractions.* Each CX-fraction (F) was tested against larvae to determine the presence of a settlement cue. F1, F2, F3, F4 and the polar extract of *D. pulchra* (used as a positive control) were dissolved in Milli-Q water at 5 mg  $\cdot$  ml<sup>-1</sup>. Aliquots of the appropriate fraction were added to the petri dish to give final test concentrations of 50  $\mu\text{g} \cdot$  ml<sup>-1</sup> for each treatment. F5 was dissolved in Milli-Q water at 100  $\mu\text{g} \cdot$  ml<sup>-1</sup>, and aliquots were added to assigned petri dishes for final test concentrations of 0.1–1.0  $\mu\text{g} \cdot$  ml<sup>-1</sup> (much lower concentrations of F5 were tested because of a low yield in F5). Initial settlement assays showed that only F5 induced settlement; therefore, CX-control-fraction 5 (CF5) was tested in future settlement assays as the procedural control. CF5 was dissolved in Milli-Q water at 100  $\mu\text{g} \cdot$  ml<sup>-1</sup> and tested at 1.0  $\mu\text{g} \cdot$  ml<sup>-1</sup>. Milli-Q water and SSW were used as the negative controls. Percent settlement was scored after 1 h ( $n = 10$  replicates per treatment).

*Natural versus synthetic histamine.* Settlement assays were used to compare the responses of larvae to (i) natural histamine isolated using CX chromatography. (ii) synthetic histamine, and (iii) synthetic histamine run through the same procedure used to isolate natural histamine. Stock solutions of 900  $\mu\text{M}$  of each histamine treatment were prepared in Milli-Q water, and aliquots of the appropriate stock solution added to assigned petri dishes for final test concentrations of 0.9–9.0  $\mu\text{M}$ . Pieces of fresh *D. pulchra* (~10 mg) and 50  $\mu\text{g} \cdot$  ml<sup>-1</sup> of the polar extract *D. pulchra* were used as the positive controls, and Milli-Q water and SSW were used as the negative controls. Percent settlement was scored after 1 h ( $n = 12$  replicates per treatment).

*Delisea pulchra* treated with antibacterial agents. Because some marine bacteria produce histamine (Fujii *et al.*, 1997), the identification of histamine as the settlement cue (see Results) raises the possibility that the bacterial biofilm on the surface of *D. pulchra* may be the source of the cue. To test this, the ability of *D. pulchra* to induce settlement after various antibacterial treatments was examined in a settlement assay. Antibacterial treatments were adapted from previous studies in which treatments were shown to be effective in reducing surface bacteria (Xue-Wu and Gordon, 1987; Aguirre-Lipperheide and Evans, 1993; Johnson and Sutton, 1994). Seven plants of *D. pulchra* were collected from Bare Island and brought back to the laboratory, where portions of each plant were allocated to each of seven treatments. There were six antibacterial treatments and a procedural control. All antibacterial treatments included a 5-min soak in a 10% betadine-SSW solution, followed by three rinses in SSW and a 24-h treatment in either (1) SSW (the “soak” treatment); (2) SSW containing 20 mg · l<sup>-1</sup> streptomycin (Aldrich), 10 mg · l<sup>-1</sup> penicillin G (Aldrich), and 10 mg · l<sup>-1</sup> kanamycin (Aldrich; “SPK” treatment); (3) SSW containing 10 mg · l<sup>-1</sup> ciprofloxacin (Bayer, “ciprofloxacin” treatment); (4) SSW after pieces of *D. pulchra* were gently wiped across an agar plate, before and after the 24-h soak, to physically remove bacteria (“wipe” treatment); and the combination treatments (5) “wipe + SPK”, and (6) “wipe + ciprofloxacin.” The procedural control was a 24-h soak in SSW without the initial betadine soak (“soak control” treatment). The next day, subsections of several *D. pulchra* plants were collected as a “fresh control” treatment and used in the settlement assay on that day. Pieces of *D. pulchra* (~10 mg) from each treatment were added to assigned sterile petri dishes, and percent settlement was scored after 20 h (*n* = 15 replicates per treatment).

#### Quantitative analysis of histamine in various algae

If histamine is a natural settlement cue for this urchin, we would expect *D. pulchra*, the primary host plant of new recruits of *H. purpurascens*, to have higher levels of histamine than other algae in the habitat. To test this, we quantified the histamine content of six species of algae from the habitat of *H. purpurascens*. The two primary host plants (*D. pulchra* and *E. radiata*) and four other prominent species of algae (*Amphiroa anceps*, *Corallina officinalis*, *Homeostichus olsenii*, and *Sargassum vestitum*) were collected from Bare Island in January 2003. Five replicates of each alga were analyzed, with each replicate consisting of three small sections taken from different parts of one thallus, which were then pooled into a single sample for analysis (2–4 g wet-weight). A polar extract of each algal sample was prepared as described above. Polar extracts were dissolved in Milli-Q water (200 μl) and acidified with 50 μl of glacial acetic acid. [ $\alpha$ ,  $\alpha$ ,  $\beta$ ,  $\beta$ -d<sub>4</sub>]Histamine · 2HCl (1 μg, Cam-

bridge Isotope Laboratories, #DLM 2911) was added to each sample as the internal standard (ISTD). Strong cation-exchange solid-phase extraction cartridges (50 mg, Alltech) were equilibrated with Milli-Q water (5 ml) at a flow rate of 1 ml · min<sup>-1</sup>, and the sample was loaded. Unbound compounds were eluted in 2 ml of Milli-Q water (fraction 1) and another 2 ml of Milli-Q water (fraction 2). All retained compounds were eluted in 1 ml of 30% NH<sub>4</sub>OH w/w (fraction 3) and dried in a speed vac. Standards that contained 1-μg ISTD and either 0.1, 0.5, 1.0, 5.0, or 10 μg of synthetic histamine were prepared. Standards and fraction 3 samples were derivatized with heptafluorobutyric anhydride and acetic anhydride using the method of 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; Smythe *et al.*, 2002) were installed on the GC-MS instrument previously described, and the same run conditions were used. The mass selective detector was operated in selected ion monitoring mode using ions characteristic of the analyte (derivatized histamine—*m/z* 94, 307, 349) and the ISTD (*m/z* 97, 311, 353). Extracted ion chromatograms were used to manually integrate the area under each ion peak (which is proportional to the amount of analyte in the sample). For each standard and sample, the areas of the analyte ions (*m/z* 94, 307, 349) were added together and the areas of the ISTD ions (*m/z* 97, 311, 353) were added together. The ratio of the combined areas of analyte:ISTD in standards was used to generate a standard curve. The histamine content of the samples was calculated by reference to the standard curve and expressed in terms of micrograms per gram (wet weight) of algal tissue (μg · g<sup>-1</sup>).

After checking that the data met the assumptions of the test, the histamine content of different algae was transformed [ln(*x* + 1)] and compared by using a one-factor analysis of variance. We excluded *A. anceps* and *C. officinalis* from the analysis because no histamine was detected in these species. Bonferroni's post hoc test was used to determine which species differed in their histamine contents (SYSTAT ver. 7.0). We were concerned that one high value for *D. pulchra* might be unduly influencing our analysis, but the outcome was unchanged when we repeated the analysis with this value omitted. Therefore, we report the results of the initial analysis.

#### Reanalysis of samples from Williamson *et al.* (2000)

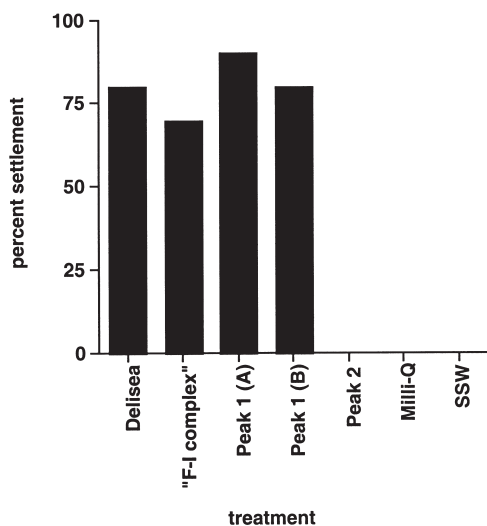
Samples remaining from the study published in Williamson *et al.* (2000) were analyzed by GC-MS for the presence of histamine. Any histamine in the old samples was isolated using cation-exchange solid phase extraction cartridges, as outlined previously for isolating algal histamine, and then derivatized with heptafluorobutyric anhydride and acetic anhydride, using the method of Barancin *et al.* (1998) for quantitative GC-MS analysis.

## Results

### Isolation of the settlement cue in *Delisea pulchra* by bioassay-guided fractionation

**HPLC fractions—NMR spectroscopy analysis and settlement assays.** The polar extract of *Delisea pulchra* was separated into two fractions using HPLC (peak 1 and peak 2, Fig. 1A). These were analyzed by NMR spectroscopy (1-min  $^1\text{H}$ - and 30-min  $^{13}\text{C}$ -NMR experiments) and tested in settlement assays. Peak 1 displayed the pattern of isethionic acid (Barrow *et al.*, 1993), as determined by  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectroscopy, as well as some additional signals that were not characteristic of floridoside (see next section). The  $^{13}\text{C}$ -NMR spectrum of peak 2 corresponded to previously published data for floridoside [ $\alpha$ -D-galactopyranosyl-(1-2)-glycerol] (Karsten *et al.*, 1993). Therefore, the isethionic acid and floridoside components of the F-I complex eluted separately, in peak 1 and peak 2, respectively. Peak 1 induced settlement of *Holopneustes purpurascens* larvae in settlement assays, but peak 2 did not (Fig. 2). Four batches of peak 1 ( $25 \mu\text{g} \cdot \text{ml}^{-1}$ ) induced 80%–100% settlement in five assays, whereas neither of two batches of peak 2 ( $51 \mu\text{g} \cdot \text{ml}^{-1}$ ) induced settlement in two assays (representative data shown in Fig. 2). These data suggested that the F-I complex is not a settlement cue for *H. purpurascens* and that peak 1 (which lacked floridoside) contained the settlement cue.

Isethionic acid and taurine were the major compounds in peak 1, as determined by  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectroscopy



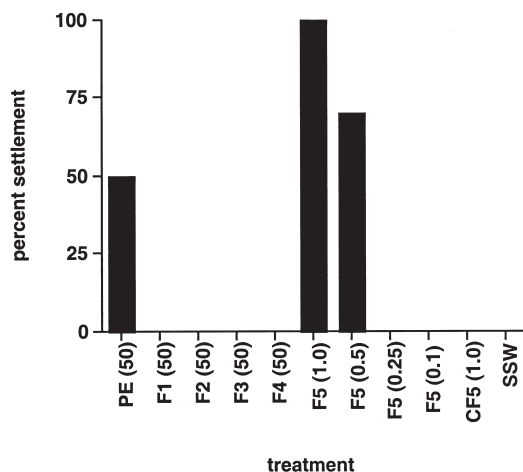
**Figure 2.** The settlement (%) of larvae of *Holopneustes purpurascens* after 18 h incubation with fresh *Delisea pulchra* (~10 mg) or HPLC peak fractions of the polar extract of the alga. Peak 1 (batch A or B) was tested at  $25 \mu\text{g} \cdot \text{ml}^{-1}$ , peak 2 was tested at  $51 \mu\text{g} \cdot \text{ml}^{-1}$ , and a floridoside-isethionic acid complex sample ("F-I complex") from Williamson *et al.* (2000) was tested at  $76 \mu\text{g} \cdot \text{ml}^{-1}$ . Milli-Q water and sterile seawater (SSW) were included as the negative controls ( $n = 10$ ).

and comparison with synthetic samples. When isethionic acid ( $1\text{--}25 \mu\text{g} \cdot \text{ml}^{-1}$ ), sodium isethionate ( $15\text{--}30 \mu\text{g} \cdot \text{ml}^{-1}$ ), and taurine ( $1\text{--}13 \mu\text{g} \cdot \text{ml}^{-1}$ ) were tested in settlement assays with *H. purpurascens* larvae, none of these compounds induced settlement (data not shown). Different combinations of these compounds were tested together (*e.g.*,  $15 \mu\text{g} \cdot \text{ml}^{-1}$  of isethionic acid and taurine) in case two cues were required for settlement of *H. purpurascens*. There was no settlement in the combination treatments (data not shown). Following these results, we hypothesized that one or more trace compounds in peak 1, not yet detected by NMR analysis, were inducing settlement. To test this, a larger amount of peak 1 was collected and a much longer (24-h)  $^{13}\text{C}$ -NMR experiment run on the sample. The  $^{13}\text{C}$ -NMR spectrum showed about 20 additional carbon signals not detected previously by NMR spectroscopy, indicating that additional compounds were present in peak 1 in trace amounts. The finding that peak 1 induced settlement of larvae of *H. purpurascens* but the identified major components (isethionic acid, taurine) in peak 1 did not implied that one of the compounds present in trace amounts was the settlement cue.

**Cation-exchange fractions—settlement assay.** The settlement cue could not be isolated as a pure fraction using HPLC, so the polar extract of *D. pulchra* was fractionated using CX chromatography (Fig. 1B). Five CX-fractions (F) were obtained and tested in settlement assays; only F5 induced settlement of larvae of *H. purpurascens* (Fig. 3). F5 at a concentration of  $1.0 \mu\text{g} \cdot \text{ml}^{-1}$  induced 100% settlement in larvae after 1 h,  $0.5 \mu\text{g} \cdot \text{ml}^{-1}$  induced 70% settlement, and  $0.1\text{--}0.25 \mu\text{g} \cdot \text{ml}^{-1}$  did not induce settlement. There was no settlement in the control fraction CF5 ( $1.0 \mu\text{g} \cdot \text{ml}^{-1}$ ) and SSW treatments (Fig. 3).

### Identification of the settlement cue for *Holopneustes purpurascens*

**Nuclear magnetic resonance spectroscopy.** The  $^1\text{H}$ -NMR ( $\text{D}_2\text{O}$ ) spectrum of F5 showed proton signals at  $\delta$  2.76 (2H, t, J 7.0 Hz, H2), 3.03 (2H, t, J 8.2 Hz, H1), 6.86 (1H, s 2H, imidazole H), and 7.57 (s, 1H, imidazole H). The  $^{13}\text{C}$ -NMR ( $\text{D}_2\text{O}$ ) and DEPT spectra of F5 showed carbon signals at  $\delta$  25.9, 39.5 ( $\text{CH}_2$ ); 116.4, 136.5 (CH) and 134.0 (quaternary C). These signals supported the assignment of F5 as histamine (2-[1*H*-imidazol-4-yl]-ethylamine, MW 111.15). The structure of F5 was further confirmed by a high-field two-dimensional  $^1\text{H}$ - $^{15}\text{N}$  HMBC NMR experiment in which the methylene triplet at 2.76 ppm showed two three-bond correlations to the ethylamine  $\text{NH}_2$  group and the imidazole nitrogen. The identity of F5 was further confirmed by a spiking experiment. All F5 signals increased in intensity and no additional signals were detected, confirming the identity of F5 as histamine.



**Figure 3.** The settlement (%) of larvae of *Holopneustes purpurascens* after 1 h incubation with the polar extract of *Delisea pulchra* (PE) and cation-exchange fractions (F) of the PE. The different test concentrations of each treatment are shown in brackets ( $\mu\text{g} \cdot \text{ml}^{-1}$ ); note the lower concentrations for F5 and the procedural control (CF5). Sterile seawater (SSW) was used as the negative control ( $n = 10$ ).

**Gas chromatography–mass spectrometry.** The identity of putative histamine (F5) isolated from *D. pulchra* was confirmed using GC-MS. The retention times (rt) of the heptafluorobutylracyl derivative of putative histamine ( $\text{rt} = 9.728 \pm 0.0045$ , mean  $\pm$  SD,  $n = 5$ ) and synthetic histamine ( $\text{rt} = 9.732 \pm 0.0045$ , mean  $\pm$  SD,  $n = 5$ ) were nearly identical, suggesting that they were the same compound. The electron-impact ion spectra of both derivatized compounds displayed the same major fragment ions ( $m/z$ —54, 69, 81, 94, 138, 169, 226, 307, 349) and overall fragmentation pattern, confirming that they were the same compound. The electron-impact ion spectra for derivatized histamine matched that reported in the literature (Barancin *et al.*, 1998).

**Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry.** The elemental formula of putative histamine isolated from *D. pulchra* was confirmed by accurate mass measurements using MALDI-TOF MS. The measured accurate mass of the putative protonated histamine molecular ion  $[\text{M}+\text{H}]^+$  was  $112.08878 \pm 0.0026$  ( $n = 10$ , mean  $\pm$  SD), and the measured mass for synthetic histamine was  $112.08853 \pm 0.0025$  ( $n = 10$ , mean  $\pm$  SD). The measured masses of the two samples were different by only 2.2 ppm. These values were different from the calculated monoisotopic mass for protonated histamine (112.08692—elemental formula  $\text{C}_5\text{H}_{10}\text{N}_3$ ) by only 15 ppm for synthetic protonated histamine and 17 ppm for putative protonated histamine. This is most likely due to measurement bias introduced by the very different chemical properties of histamine, glycine, and creatinine (the internal calibrants).

An elemental calculator was used to generate all possible

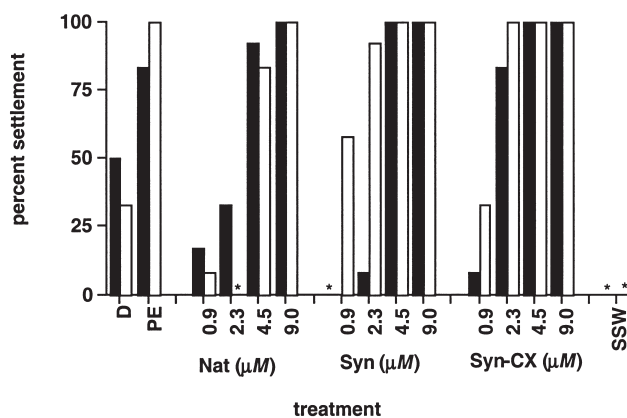
elemental formulas with a mass of approximately 112.08878. The nearest other candidate was  $\text{C}_6\text{H}_{10}\text{NO}$  at 112.07569 with a difference of 117 ppm from the measured mass of putative protonated histamine. This difference was much higher than 17 ppm (difference of measured mass for putative histamine relative to calculated mass for  $\text{C}_5\text{H}_{10}\text{N}_3$ ), confirming that the putative protonated histamine had the elemental formula of  $\text{C}_5\text{H}_{10}\text{N}_3$ .

#### *The response of Holopneustes purpurascens larvae to natural and synthetic histamine*

Natural histamine isolated from *D. pulchra* by using CX chromatography, synthetic histamine, and synthetic histamine eluted from CX resin all resulted in very similar responses in larvae when assayed concurrently (Fig. 4). More than 80% of the *H. purpurascens* larvae settled within an hour of incubation in 4.5 and 9  $\mu\text{M}$  natural and synthetic histamine. The lowest test concentration of synthetic histamine that consistently induced rapid settlement of all larvae was 4.5  $\mu\text{M}$  (in 10 separate assays). Larvae exhibited a more variable response to 0.9 and 2.3  $\mu\text{M}$  histamine, both within and across different batches (Fig. 4). Up to 80% of larvae settled in response to 0.09–0.45  $\mu\text{M}$  synthetic histamine, but only after long incubation times (up to 96 h) or as larval age increased to 13–21 days (data not shown).

#### *Response of larvae to Delisea pulchra after antibacterial treatments*

In response to *D. pulchra* that had received antibacterial treatments, larvae of *H. purpurascens* settled at levels equivalent to (or greater than) those in response to control

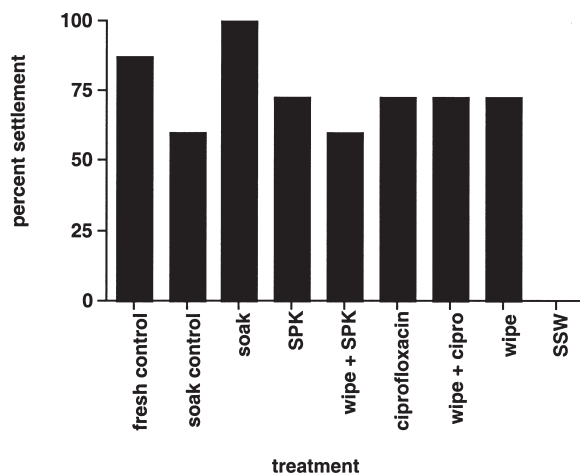


**Figure 4.** The settlement (%) of larvae of *Holopneustes purpurascens* after 1 h incubation with fresh *Delisea pulchra* ( $\sim 10$  mg), its polar extract ( $50 \mu\text{g} \cdot \text{ml}^{-1}$ ) and 0.9–9.0  $\mu\text{M}$  of natural histamine isolated from *D. pulchra* (Nat), synthetic histamine (Syn), or synthetic histamine eluted from cation-exchange resin (Syn-CX). Sterile seawater (SSW) was used as a negative control. Data from two experiments using different batches of larvae are shown (black and white bars). \* Indicates no settlement in treatment ( $n = 12$ ).

*D. pulchra* treatments (Fig. 5). SSW did not induce settlement.

#### Quantitative analysis of histamine content in algae

The histamine content of six algal species was determined by GC-MS (Table 1). *D. pulchra*, the alga on which new recruits of *H. purpurascens* are found (Williamson *et al.*, 2000), had the highest histamine content of all algae surveyed. Histamine was not detected in any samples of *Amphiroa anceps* or *Corallina officinalis*. The histamine content of *D. pulchra*, *Ecklonia radiata*, *Homeostrichus olsenii*, and *Sargassum vestitum* differed significantly from each other ( $F_{3,16} = 9.903$ ,  $P = 0.0006$ ). Pairwise comparisons showed that the histamine content of *D. pulchra* ( $11.82 \pm 6.56 \mu\text{g} \cdot \text{g}^{-1}$ ) was significantly higher than the histamine content of *E. radiata* ( $1.28 \pm 1.01 \mu\text{g} \cdot \text{g}^{-1}$ ,  $P = 0.0092$ ), *S. vestitum* ( $0.35 \pm 0.32 \mu\text{g} \cdot \text{g}^{-1}$ ,  $P = 0.0016$ ), and *H. olsenii* ( $0.25 \pm 0.09 \mu\text{g} \cdot \text{g}^{-1}$ ,  $P = 0.0015$ ). The amount of histamine in different *D. pulchra* plants was highly variable, ranging from 1.88–34.22  $\mu\text{g} \cdot \text{g}^{-1}$  wet weight of algal tissue. The variability in histamine levels of *E. radiata* was also high, with no histamine detected in two samples, yet another contained 4.73  $\mu\text{g} \cdot \text{g}^{-1}$  wet weight of algal tissue. Likewise, histamine was not detected in three



**Figure 5.** The settlement (%) of larvae of *Holopneustes purpurascens* after 20 h incubation with *Delisea pulchra* subjected to antibacterial treatments. All antibacterial treatments included a 5-min soak in a 10% betadine solution, followed by 3 rinses in sterile seawater (SSW) and a 24-h treatment in either SSW (“soak”); SSW containing streptomycin (20  $\text{mg} \cdot \text{l}^{-1}$ ), penicillin G (10  $\text{mg} \cdot \text{l}^{-1}$ ) and kanamycin (10  $\text{mg} \cdot \text{l}^{-1}$ , “SPK”); or SSW containing ciprofloxacin (10  $\text{mg} \cdot \text{l}^{-1}$ , “ciprofloxacin”). Other treatments involved wiping pieces of *D. pulchra* across an agar plate gently, to physically remove bacteria, before and after a 24-h soak in SSW (“wipe”), SSW containing SPK (“wipe + SPK”), or SSW containing ciprofloxacin (10  $\text{mg} \cdot \text{l}^{-1}$ , “wipe + cipro”). *D. pulchra* soaked in SSW for 24 h (without betadine soak) was the procedural control (“soak control”), fresh *D. pulchra* was used as a positive control (“fresh control”), and SSW was used as a negative control ( $n = 15$ ).

**Table 1**

The histamine content of six algal species was significantly different (ANOVA,  $F_{3,16} = 9.903$ ,  $P = 0.0006$ )

Species	Histamine ( $\mu\text{g} \cdot \text{g}^{-1}$ [ww]) mean $\pm$ SE, $n = 5$
<i>Delisea pulchra</i>	$11.82 \pm 6.56$
<i>Ecklonia radiata</i>	$1.28 \pm 1.01^*$
<i>Sargassum vestitum</i>	$0.35 \pm 0.32^*$
<i>Homeostrichus olsenii</i>	$0.25 \pm 0.09^*$
<i>Corallina officinalis</i>	nd
<i>Amphiroa anceps</i>	nd

\* Indicates species in which histamine content differs significantly from *D. pulchra* (pairwise comparisons,  $P < 0.0092$ ); nd, not detected; ww, wet weight.

of the *S. vestitum* samples analyzed, but another contained 1.48  $\mu\text{g} \cdot \text{g}^{-1}$  wet weight of algal tissue. The *H. olsenii* plants analyzed showed consistently low levels of histamine, ranging from 0.05–0.46  $\mu\text{g} \cdot \text{g}^{-1}$  (wet weight) of algal tissue.

#### Reanalysis of samples from Williamson *et al.* (2000)

Several samples remaining from the previous study were analyzed by GC-MS for the presence of histamine. Histamine was detected in F-I complex fractions from *D. pulchra* ( $1.5\text{--}46 \mu\text{g} \cdot \text{mg} [\text{sample}]^{-1}$ ) in a synthetic F-I complex sample ( $0.35 \mu\text{g} \cdot \text{mg} [\text{sample}]^{-1}$ ), and in a batch of floridose used to make the synthetic complexes ( $0.45 \mu\text{g} \cdot \text{mg} [\text{sample}]^{-1}$ ).

## Discussion

Habitat-specific cues play an important role in the settlement of many benthic marine invertebrates (Pawlik, 1992; Hadfield and Paul, 2001). Larvae presumably maximize their chances of post-settlement survival by responding to habitat-specific cues, as settlement in a preferred habitat should provide shelter and food to the vulnerable juvenile life-history phase (Gosselin and Qian, 1997). Chemical cues for larval settlement are derived from conspecifics (Burke, 1986), host organisms (Williamson *et al.*, 2000), prey (Hadfield and Scheuer, 1985), or biofilms (Wieczorek and Todd, 1998); they include a diverse range of compounds from small peptides (Zimmer-Faust and Tamburri, 1994) to complex macromolecules (Clare and Matsumura, 2000). The complete characterization of chemical settlement cues has, however, proved difficult because of the low endogenous or environmental levels of such compounds and the rapid dilution of water-soluble cues. Few studies have definitively characterized settlement cues (reviewed by Hadfield and Paul, 2001; Steinberg *et al.*, 2001).

Williamson *et al.* (2000) reported on one such putative

characterized cue, a metabolite complex isolated from the red algal host *Delisea pulchra* that induced settlement in larvae of the sea urchin *Holopneustes purpurascens*. At Bare Island (Sydney, Australia), *H. purpurascens* is found primarily on two algal hosts, *D. pulchra* and *Ecklonia radiata*, with the smallest size class (test diameter  $\leq 5$  mm) only found on *D. pulchra*. Larvae metamorphosed in response to pieces of *D. pulchra* and the polar extract, but not to pieces or extracts of *E. radiata* (Williamson *et al.*, 2000). A water-soluble cue was implicated when seawater collected near *D. pulchra* plants *in situ* also induced settlement of larvae. The settlement cue in *D. pulchra* was isolated and characterized as the floridoside-isethionic acid (F-I) complex (Williamson *et al.*, 2000).

New evidence presented in this paper shows that histamine, not the F-I complex, is a natural inducer of settlement in *H. purpurascens*. The settlement cue was isolated from the polar extract of *D. pulchra* by using bioassay-guided fractionation by cation-exchange chromatography. The isolated compound at  $0.5 \mu\text{g} \cdot \text{ml}^{-1}$  induced settlement in 80%–100% of larvae within an hour. The settlement cue was identified as histamine using NMR spectroscopy, and this was confirmed by GC-MS and MALDI-TOF MS. The response of larvae to synthetic histamine in settlement assays mirrored their response to natural histamine isolated from *D. pulchra*. *D. pulchra*, the primary plant on which new recruits of *H. purpurascens* are found, had the highest average histamine content ( $11.82 \pm 6.56 \mu\text{g} \cdot \text{g}^{-1}$  wet weight), approximately an order of magnitude higher than other algae surveyed. Seawater collected near *D. pulchra* plants in the study by Williamson *et al.* (2000) induced rapid settlement of larval *H. purpurascens*; however, those samples were used completely in bioassays and are therefore not available for histamine analysis. We have since detected histamine in seawater surrounding *D. pulchra* and *E. radiata* (at concentrations ranging from 20 to 70 nM), but not in samples 2 m away from the macroalgae. A comprehensive analysis of histamine levels in seawater will be reported in another manuscript. Although the histamine concentrations measured in seawater do not induce rapid settlement in larvae that have just attained competence, this concentration can induce settlement of *H. purpurascens* larvae over longer time periods and in older larvae (data not shown). In addition, the natural habitat may contain other settlement cues that if detected in conjunction with histamine, may lower the threshold concentration of histamine required for rapid induction of settlement. These findings support our proposal that histamine released from macroalgae is a natural settlement cue for *H. purpurascens*.

Reanalysis of samples from the study by Williamson *et al.* (2000) provides an explanation for the incorrect conclusion that the F-I complex is a settlement cue for larvae of *H. purpurascens*. The F-I complex was isolated from the polar extract of *D. pulchra*, using reversed-phase HPLC and

methanol as the mobile phase, and eluted as a single peak (Williamson *et al.*, 2000).  $^{13}\text{C}$ -NMR spectroscopy analysis of this peak showed only  $^{13}\text{C}$ -signals for floridoside and isethionic acid (Williamson *et al.*, 2000). However, trace amounts of histamine were also present but not detected, because their levels were below the limit of detection for  $^{13}\text{C}$ -NMR spectroscopy. Histamine elutes in the first peak from reversed-phase (C18) columns regardless of the mobile phase, so any histamine in the polar extracts of *D. pulchra* used by Williamson *et al.* (2000) would have co-eluted with the F-I complex fraction. Consequently, the “F-I complex” samples contained histamine, detected here using GC-MS, and induced settlement of *H. purpurascens* larvae. Although a synthetic F-I complex induced rapid settlement in *H. purpurascens* larvae in the previous study (Williamson *et al.*, 2000), not all batches induced settlement (R. de Nys, pers. obs.). The synthetic F-I complexes were made using natural floridoside isolated from *D. pulchra* and synthetic isethionic acid. The floridoside used to make the synthetic F-I complex was contaminated by histamine and thus induced settlement. Confirming this, histamine was detected by GC-MS in a floridoside sample (used for preparation of the synthetic complex) and a synthetic F-I complex sample prepared by Williamson *et al.* (2000). In summary, histamine was present in trace amounts in the “F-I complex” samples that induced settlement of larval *H. purpurascens* in the previous study, and histamine was the inductive compound in the “F-I complex” samples.

The finding that histamine is a natural settlement cue for *H. purpurascens* is of considerable interest in the context of linking ecological patterns with physiological mechanisms. Histamine is a biogenic amine produced by the decarboxylation of the amino acid histidine. It is one of five primary biogenic amines in invertebrates, along with serotonin, octopamine, dopamine, and tyramine (Blenau and Baumann, 2001). Biogenic amines, all decarboxylation products of amino acids, play critical roles in initiating and controlling behavior, and in the physiology of invertebrates, by acting as classical neurotransmitters, neuromodulators, and neurohormones (Katz, 1995; Beltz, 1999). For example, dopamine activates hunting behavior in an opisthobranch mollusc (Norekyan and Satterlie, 1993), and serotonin controls aggressive behavior in crustaceans (Huber *et al.*, 1997). The photoreceptors in all classes of arthropod eyes are histaminergic; that is, they synthesize histamine and use it as their neurotransmitter (Stuart, 1999). Also, histamine is thought to be an inhibitory neurotransmitter in the stomatogastric and cardiac ganglia and the sensory system of lobsters (Claiborne and Selverston, 1984; Bayer *et al.*, 1989; Hashemzadeh-Gargari and Freschi, 1992). Importantly, in the context of this study, histamine directly gates a chloride channel in the receptor cells of the olfactory pathway of lobsters (McClintock and Ache, 1989). Fast neurotransmitters directly gate ion channels, which leads to fast behav-

ioral and physiological outcomes. We have observed that the settlement response of *H. purpurascens* to histamine is rapid, with complete metamorphosis within half an hour. This fast response is consistent with the notion that the larvae of *H. purpurascens* have specific receptors that bind histamine and act directly on ion channels, leading to rapid settlement.

Neurotransmitters, or their precursors, have been suggested to mimic the function of natural settlement cues (Morse, 1985; Bonar *et al.*, 1990). The best-known example is the gamma-aminobutyric acid (GABA)-mimetic peptide (or peptides), present on the surface of crustose coralline algae, which Morse and colleagues proposed as a settlement cue for abalone (Morse *et al.*, 1979, 1984). Another example comes from oyster larvae, where L-3, 4-dihydroxyphenylalanine (L-DOPA) induced stereotypical searching behavior, while epinephrine and norepinephrine induced metamorphosis (Coon *et al.*, 1985). Endogenous levels of neurotransmitters, and their precursors, also appear to modulate the behavioral and physiological processes accompanying settlement (Coon and Bonar, 1987; Pires *et al.*, 2000). Our findings show that a naturally produced neurotransmitter is in fact a settlement cue for larvae, a phenomenon that may be widespread in the marine environment.

The finding that histamine, rather than the F-I complex, is a settlement cue for *H. purpurascens* potentially complicates the previous interpretations of the relationship between settlement cues and the demography of this sea urchin (Williamson *et al.*, 2000, 2004). Histamine, a simple breakdown product of the amino acid histidine, may be broadly distributed across the natural habitat of *H. purpurascens*; for example, in algal and animal tissue, and in bacterial communities living on their surfaces. For histamine to be an ecologically relevant settlement cue, its distribution in the natural habitat must relate to the recruitment patterns of *H. purpurascens*. This was in fact the case. The histamine content of the algae surveyed was consistent with the recruitment patterns of the organism, with much higher levels of histamine measured in *D. pulchra*, the primary plant on which we find new recruits.

*D. pulchra* had the highest average histamine content ( $11.82 \pm 6.56 \mu\text{g} \cdot \text{g}^{-1}$  wet weight), ranging from 1.88 to  $34.22 \mu\text{g} \cdot \text{g}^{-1}$  wet weight. Similarly, levels of histamine varied for *E. radiata*, with concentrations ranging from 0 to  $4.73 \mu\text{g} \cdot \text{g}^{-1}$  wet weight. Since only subsections of plants (not whole plants) were extracted, these results may reflect within-plant variation, within-species variation, or both. Future histamine analyses will extract whole plants, as well as specific regions of thalli, to directly test these possibilities. The low (or absent) levels of histamine typically measured in *E. radiata* samples may explain why pieces and extracts of *E. radiata* did not induce settlement in the study by Williamson *et al.* (2000). However, we have observed that some pieces of *E. radiata* do induce settlement of *H. pur-*

*purascens*, which is consistent with the variation we measured in levels of histamine in the alga. Given this, and the large biomass of *E. radiata* kelp beds in the natural habitat of *H. purpurascens*, *E. radiata* may contribute to environmental levels of histamine, inducing the settlement of larvae in this habitat. Histamine was not detected in the turfing coralline algae *Corallina officinalis* and *Amphiroa anceps*, although they induce settlement of larvae of *H. purpurascens* (Williamson *et al.*, 2000; R. Swanson, pers. obs.) and provide a habitat for new recruits (R. Swanson, pers. obs.). Larger samples of *A. anceps* (up to 180 g wet weight) were extracted and no histamine was detected. The coralline algae may produce a different settlement cue for *H. purpurascens*, or histamine may only be produced and released *in situ*—for example, by surface-associated bacteria.

Finally, the two possible sources of histamine in *D. pulchra* are the host alga or the surface-associated bacterial community (or both). *D. pulchra* treated with various antibacterial agents still induced high levels of settlement in *H. purpurascens*, suggesting that the host alga produces the histamine. A bacterial source of histamine is, however, possible, as a known histamine-producing bacterium, *Photobacterium phosphoreum* (Fujii *et al.*, 1997) is a constituent of the microbial community on local algal species (M. Watson, UNSW Australia; pers. comm.). If histamine-producing bacteria are colonizing algal surfaces within the habitat, then it is possible that they produce and release histamine to seawater, which could lead to the induction of settlement of *H. purpurascens*.

## Conclusion

Many larval species have the ability to respond to low-molecular-weight, water-soluble settlement cues (Hadfield and Scheuer, 1985; Zimmer-Faust and Tamburri, 1994; Boettcher and Targett, 1996; Lambert *et al.*, 1997; Fleck and Fitt, 1999). This paper has presented evidence that histamine is a natural settlement cue for the sea urchin, *H. purpurascens*, correcting the previous study of Williamson *et al.* (2000). Histamine at  $4.5 \mu\text{M}$  induces settlement (metamorphosis) in 80%–100% of *H. purpurascens* larvae within half an hour, fulfilling two essential criteria for an effective water-soluble settlement cue: (1) larvae must perceive low concentrations of inducer, and (2) larvae must respond rapidly to the inducer. *D. pulchra* had the highest histamine content of all the species surveyed, consistent with the recruitment patterns of *H. purpurascens*. In a preliminary analysis, we detected histamine in seawater near *D. pulchra* and *E. radiata* plants, but not in seawater collected 2 m away, supporting our proposal that histamine leaches from algae that produce this settlement cue. This hypothesis is consistent with the finding of Williamson *et al.* (2000) that seawater collected very near to *D. pulchra* induced settlement in larvae of *H. purpurascens*. We have shown that

histamine, an invertebrate neurotransmitter, is also a natural settlement cue for larvae of *H. purpurascens*, linking the physiology and ecology of the organism.

### Acknowledgments

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