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Settlement Of Oyster (Crassostrea-Virginica) Larvae - Effects Of Water-Flow And A Water-Soluble Chemical Cue

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Settlement of oyster (*Crassostrea virginica*) larvae: Effects of water flow and a water-soluble chemical cue

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Abstract

Although previous evidence indicates that larvae of benthic marine invertebrates can respond to waterborne cues in still water, the importance of waterborne cues in mediating natural settlement out of flowing water has been questioned. Here, we summarize the results of flume experiments demonstrating enhanced settlement of oyster larvae in small target wells (circles of 7-cm diam) with the release of a waterborne settlement cue compared to identical substrates without the cue. In concurrent still-water experiments, more oyster larvae settled in solutions of waterborne cue than in seawater controls. Velocity and electrochemical measurements of a conservative tracer verified that at low flow velocities (2 and 6 cm s^{-1}) with U_* values <0.25 cm s^{-1}, the waterborne cue was present above the targeted substrate to a height of ≤ 4 mm. Rapid vertical swimming or sinking in response to the waterborne cue can concentrate larvae in near-bottom waters and enhance larval settlement. Our investigation provides the first experimental evidence to demonstrate that dissolved chemical cues can mediate settlement by larvae under hydrodynamic conditions approaching those of natural benthic habitats.

For sessile marine invertebrates, larval settlement behavior and choice of final substratum for attachment is critical for the success of the adult organism. Many larvae appear to discriminate among settlement sites; larvae not only consistently choose between substrates in the laboratory but larval settlers cluster on preferred sites in the field (Crisp 1974; Keough and Downes 1982; LeTourneux and Bourget 1988). Chemical properties of marine benthic environments are known to provide cues for larvae actively colonizing settlement sites (Johnson and Strathmann 1989; Jensen and Morse 1990). The induction of larval settlement is most commonly thought to be mediated by substances adsorbed to the benthic substratum as overall organic enrichment (Butman et al. 1988)—a result of sediment geochemistry (Cuomo 1985)—or else sequestered in plants or bacteria encrusting surfaces (Morse et al. 1988; Scheltema 1974; Kirchman et al. 1982; Weiner et al. 1985). Presumably, the larvae cannot detect these substances until they contact the bottom (Butman and Grassle 1992). By contrast, the role of waterborne compounds in evoking larval settlement responses is only poorly understood (*but see* Hadfield and Scheurer 1985; Pawlik 1992).

Little concrete support exists at present for active habitat selection by larvae under natural flow conditions in response to chemical cues

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perceived in the water column (Butman 1986). Settlement induction by waterborne substances would be especially difficult for weakly swimming larvae. For these larvae, horizontal swimming speeds are typically much less than horizontal flow speeds in benthic boundary layers, even for low energy environments at distances less than one larval body length away from the bed (Butman 1986). Active habitat selection, while possible for weakly swimming larvae, has been proposed to occur only after the larvae come into contact with the bottom via transport by water flow (Butman and Grassle 1992). Experimental evidence for settlement induction in weakly swimming larvae by a waterborne substance while larvae are still in the water column would thus be important.

Although the importance of waterborne cues in evoking settlement out of flowing water has been questioned, evidence has accumulated from laboratory experiments in still water that suggests induction of oyster larval settlement by waterborne chemical cues. Since Cole and Knight-Jones (1939) first described gregarious settlement and metamorphosis in oysters, there has been debate about the existence and source of settlement-inducing compounds. One group of researchers has investigated compounds released by juvenile and adult oysters (Bayne 1969; Keck et al. 1971; Hidu et al. 1978), whereas another group has found that biofilms on oyster shell surfaces (Bonar et al. 1986; Fitt et al. 1989, 1990; Weiner et al. 1989) are the sources of inducer molecules. It has been proposed that films of the bacterium *Alteromonas colwelliana* produce L-dihydroxyphenylalanine (L-DOPA), a melanin precursor which stimulates oyster larval settlement (Bonar et al. 1985). Coon et al. (1985) discovered that L-DOPA induces settlement behavior of oyster larvae, supporting this hypothesis. Ammonia gas has also been suggested to induce settlement of oyster larvae (Bonar et al. 1990; Fitt and Coon 1992).

The effects of substances released by adult oysters and by biofilms were never separately assayed in a single study until recently. Tamburri et al. (1992) quantified larval responses to waterborne substances released by each source in still water. They found that oyster larvae respond similarly to waterborne compounds released from adult conspecifics and from biofilms on living oysters and on shells of dead oysters. In response to waterborne substances, larvae rapidly moved downward in the water column; their horizontal swimming speed slowed while their rate of turning increased, which focused activity near (<3 body lengths away from) the bottom; and they contacted the bottom and attached with the foot, indicating settlement.

These laboratory studies have all been performed in still water. In natural habitats, the importance of chemical factors in inducing settlement must depend on water flow and benthic boundary-layer characteristics associated with such flows (Butman 1986). Larvae in nature make settlement choices in complex hydrodynamic environments where chemical cues may be mixed or diluted. Studies of recruitment of marine larvae have clearly shown that the hydrodynamic regime is an important controlling factor in both soft-sediment (Eckman 1983; Palmer and Gust 1985; Emerson and Grant 1991) and hard-substrate environments (Pawlik et al. 1991; Walters 1992; Mullineaux and Garland 1993). Recent studies suggest that invertebrate larvae similar in size and with swimming abilities similar to oyster larvae are passively advected to settlement sites prior to any reception of chemical cues (Butman 1986; Pawlik et al. 1991; Mullineaux and Butman 1991). A recent model by Gross et al. (1992) supports the view that hydrodynamics will control the number of larvae encountering a substrate and indirectly control larval settlement as long as larvae have a high probability of accepting the substrate once they come into contact with it.

However, larvae may contact a waterborne chemical cue before encountering a surface-bound cue. Oyster larvae have been shown to have a dramatic response to waterborne chemical settlement inducers in still water (Tamburri et al. 1992). In moving water, a chemical cue emanating from the substratum can be mixed some distance away from the bed. The timely response of larvae to some waterborne chemical cue can bring them into contact with a preferred settlement site but only if the response time is short in relation to the duration of water flow over the settlement site. Ultimately, in addition to the presence or absence of any specific chemical cue, water flow will be important to settlement success.

We present results of experiments conducted in flowing seawater that compared settlement of larvae onto substrates with and with-

out the addition of a waterborne chemical settlement cue. We used a water-soluble cue known to induce settlement behavior in still water (Tamburri et al. 1992; Zimmer-Faust and Tamburri 1994) and tested the settlement response of oyster larvae in two flows: 2 and 6 cm s^{-1} (mean flow measured at 10 cm off the bottom). Simultaneously, settlement was assessed in still-water trials. In addition, we collected paired velocity and chemical concentration data to explore the dilution and advection of the chemical cue in the two flows. Oyster larval settlement in each flow and in still water is significantly elevated by the presence of the waterborne chemical cue.

Methods

Experiments in still water—Still-water experiments were performed at the University of South Carolina on 18 September and 23 October 1992, the same days on which flowing-water experiments were performed at the Virginia Institute of Marine Science (VIMS). Larvae from the same spawn at the VIMS hatchery in Gloucester Point were used in both still-water and flowing-water experiments. Charcoal-filtered seawater from the VIMS flume (salinity, 29‰) used for flowing-water experiments (described below) was frozen and delivered by overnight courier to South Carolina, where it was thawed and used to prepare test solutions. Test solutions were also prepared with artificial seawater (Instant Ocean) at the same salinity.

Test solutions consisted of either artificial seawater (ASW) or VIMS flume water (VFW) as controls and concentrations of 10^{-6} M, 10^{-8} M, and 10^{-10} M glycyl-glycyl-L-arginine (hereafter, GGR) to give a dose-response curve. Solutions were prepared from water in which oysters had been held for 2 h (oyster metabolite solution or OMS; *see* Tamburri et al. 1992; Zimmer-Faust and Tamburri 1994). Samples of metabolite solutions before and after fractionation to 500–1,000 molecular weight were also included in our experiments to assess differences between crude inducer (raw OMS), semipurified inducer (OMS after fractionation), and the peptide GGR. ASW and VFW were used in duplicate controls to ensure that the VFW was free of any organic compounds that might affect larval settlement. Multiple trials (3–5) were conducted each day for each concentration of solution.

Competent larvae (at least 16-d old and with a distinctive eyespot; henceforth called "eyed larvae") were shipped on ice via overnight courier from the VIMS hatchery at Gloucester Point and kept 1–3 d at 0.5–1.0 larvae ml^{-1} in a 1 : 1 mixture of oceanic and artificial seawater (30‰) in a 25°C incubator (cf. Tamburri et al. 1992). Larvae were fed marine diatoms (*Isochrysis galbana* and *Pavlova lutheri*) daily at concentrations of 2.5×10^4 cells ml^{-1}. Larvae were placed in ASW medium 2–3 h before testing with either a chemical test solution or a seawater control. During this time, larvae were not fed because diatom exudates influence larval swimming speed and turning (Tamburri and Zimmer-Faust unpubl. data).

For each test, 60 larvae were transferred in 5 ml of ASW solution to a Plexiglas chamber (3 × 3 × 4 cm) holding 25 ml of either test solution of seawater control. After 30 s of gentle stirring, the chamber was placed in a darkened holder, and larval swimming behavior was videotaped for 3 min. The chamber was illuminated with an infrared light source (>820 nm) oriented 90° to the axis of the video field. Swimming behavior of the larvae was filmed with a SONY IR-sensitive CCD camera (model HVM-200) equipped with a Tamron 180-mm lens. Swimming in the horizontal plane (to a depth elevated <1 mm above the bottom) was recorded from below. Both swimming and settlement were quantified with a computer-video motion analyzer (Motion Analysis Corp., model VP 110) interfaced to a Sun SPARC work station.

Settlement was recorded on the bottom surface of the Plexiglas settling chamber in a 6.75 × 6.70-mm video field. The total area of the quadrat was 0.452 cm^2, slightly less than 5% of the total surface area available for settlement in the microcosm. Settlers were counted as the larvae which, at the end of the 3-min period, were in plantigrade attachment to the bottom with the foot.

Experiments in flow—Flowing-water experiments were performed at the VIMS in Wachapreague in a recirculating raceway flume 483 cm long and 50 cm wide. Raw seawater was pumped through 2-μm filters into a tail tank to fill the flume initially. Subsequently, water was pumped through charcoal filters from the tail tank to an elevated head tank (total flume capacity, 3,516 liters), and water was allowed to recirculate in the flume for 24 h. Flow in

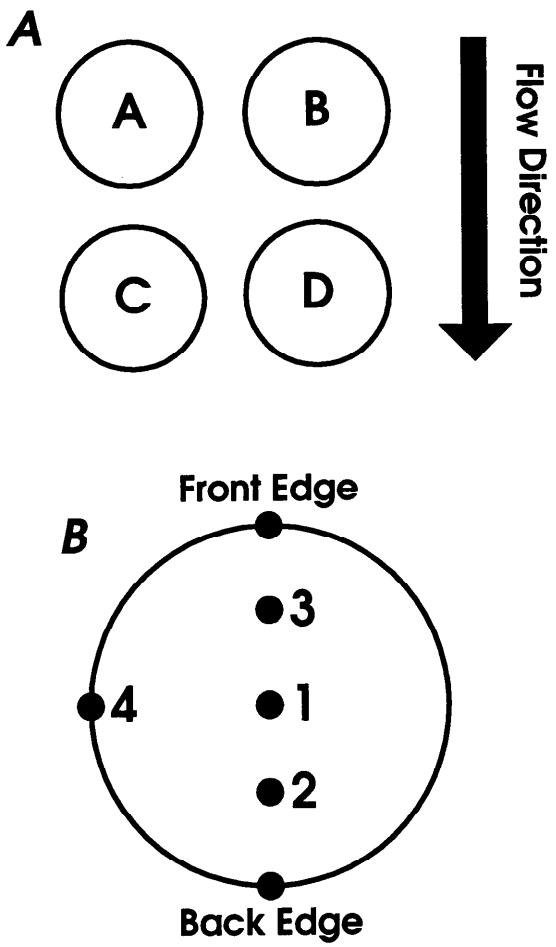

Fig. 1. A. Diagram for well locations in the flume bottom, as seen from above. In the Latin square design, peptide would be added to wells A and D, while clean seawater would be added to B and C. During the subsequent experimental run, wells B and C would receive peptide. B. Diagram showing locations of flow and chemical measurements. 1—Center of the well; 2—location of flow and chemical profiles in upstream wells; 3—location of flow and chemical profiles in downstream wells; 4—location of chemical measurement in upstream control well.

the flume was controlled by a valve at the head tank and an adjustable weir gate at the tail end of the flume. An array of soda straws provided flow straighteners at the head end.

One section of the flume bottom (a 45- × 45-cm Plexiglas plate which inserts 3.5 m downstream of the flow straighteners) was removed and modified for the purposes of this study. Four circular wells were milled out of the plate, each 6.98 cm in diameter and 1.27 cm deep. Two upstream wells were situated

Table 1. Experimental conditions for flume runs.

Larval batch age (d)	Exp. No.	1992	Estimated No. of larvae added (±SD)	Speed (cm s^{-1})
16	1	18 Sep	7,020(2,729)	2
16	2	18 Sep	8,820(1,951)	2
16	3	18 Sep	5,400(1,909)	2
23	4	13 Oct	9,450(4,497)	2
23	5	13 Oct	12,303(4,785)	6
23	6	13 Oct	14,580(7,254)	2
23	7	13 Oct	26,850(4,708)	6
24	8	14 Oct	18,900(4,604)	2
24	9	14 Oct	14,850(5,146)	6
24	10	14 Oct	25,000(3,633)	2
24	11	14 Oct	17,100(6,157)	6
24	12	14 Oct	9,450(3,974)	2
24	13	14 Oct	21,150(4,363)	6
22	14	23 Oct	20,160(4,659)	6
22	15	23 Oct	22,950(5,051)	6
22	16	23 Oct	23,400(4,800)	6

6.25 cm apart, and two identical wells were positioned 8.89 cm downstream from these (Fig. 1A). A single hole was drilled in the center bottom of each well, and hypodermic needles inserted through the holes from beneath allowed addition of seawater or seawater + peptide into each well. Wells were lined with tightly fitting Nalgene cups cut to the level of the well depth and drilled with a center hole aligned with the hole in the well below. These cups were filled with crushed oyster shell which had been rinsed and air-dried. Filled cups were inserted into the wells, leaving the shell surface as level as possible with the bottom of the flume (individual shell fragments extended up to 1.35 mm above the flat Plexiglas plate).

A solution of the waterborne chemical cue (GGR) was made in seawater at a concentration of 10^{-7} M. Concentrations of this peptide between 10^{-7} M and 10^{-8} M have been shown to maximize the induction of settlement behavior in *Crassostrea virginica* larvae in still-water trials (Zimmer-Faust and Tamburri 1994). Test solutions were added through hypodermic needles inserted into the hole in each well base. The point of entry of the needle was below the upper surface of the crushed shell, and dye studies showed that the shell acted as a diffuser, allowing solutions to slowly seep up through the shell. A peristaltic pump provided a delivery rate of 2 ml s^{-1} into the shell. Dye studies showed that this provided a "cloud" of solution over and just downstream of the

well. We used a Latin square design, such that one upstream well received seawater alone (the control well), and the other received seawater + GGR (the peptide well). Downstream of the control well, seawater + GGR was pumped in, while downstream of the peptide well, clean seawater was added (Fig. 1A). The position of the needles was switched between succeeding experimental runs so that the specific wells receiving peptide alternated between runs, always keeping to the Latin square design.

Eyed larvae (15–22 days old) were obtained from the VIMS hatchery and refrigerated at 10°C until experiments were run, usually within 24 h and never more than 48 h after delivery of the larvae (Table 1, col. 1). Cold storage of up to 98 h has been shown to be effective in maintaining other species of oyster larvae with no decrease in setting success (Holiday et al. 1991). About 1 h before each run, a subbatch of larvae was brought up to flume temperature (20°C) over the course of 60–90 min and observed repeatedly under a compound microscope (at 10×) during that time period to determine presence of eyespot, larval activity, and developmental stage (*see below*). Each subbatch contained just the larvae needed for one experimental run, so that larvae were not at room temperature for >90 min before an experimental run. Standard criteria were used to determine whether larvae were to be used in flowing-water experiments. All larvae had to be eyed, and at least 50% of the observed larvae had to be swimming or crawling on the bottom of the observation chamber. Estimates of larval abundance were made by averaging counts of 6 1-ml aliquots removed with an Eppendorf pipette from a suspension of larvae in 1 liter of flume water.

Larvae were added to the flume 120 cm upstream of the well array with a larval adder. This is a Plexiglas box (11 × 30 × 20 cm) with sliding sides designed by the flume group of Woods Hole Oceanographic Institution (described more fully by Palmer 1992). The larval adder was situated in the flume channel with the sliding sides normal to the flow. Larvae were added to the larval adder box with the sides down and observed until they could be seen swimming. The sides of the box were then raised to permit flow through the box, and most larvae were swept out of the box. When the sides of the box are raised, only the chamber base (0.96 cm thick and beveled at edges to 1-mm thickness) interferes with the bottom flow. The larval adder was left in place with the sides up throughout the experimental run.

Each experimental run lasted 15 min, and eight replicate runs were made at each flow speed (2 and 6 cm s^{-1}). We began by performing three experimental runs at the lowest flow speed in one day (runs 1–3, Table 1) but later modified this procedure. Because larval settlement response varies with age of the larvae (Fitt and Coon 1992; Butman and Grassle 1992), flow treatments were altered after every run between runs 4 and 13 (Table 1). In this way, we hoped to avoid confusion of a "flow" effect with any larval "aging" effect. After an experimental run, cups were carefully covered with lids and removed from the wells. Contents of the cups were washed into sample jars and preserved with 10% buffered Formalin dyed with Rose Bengal. The flume was not drained entirely between flume runs, but the larval adder was removed and rinsed, and the flume sides and bottom were cleaned to remove any remaining larvae. The amount of GGR added to the flume during an experimental run was ~2 liters of 10^{-7} M solution. Previous assays (unpubl. data) showed that the charcoal filters were effective in removing peptide from the seawater in the flume, so seawater was recirculated through the charcoal filters rather than draining and refilling the flume after each experimental run.

Samples were elutriated at least 3 times into a 65-μm-mesh sieve and examined under 12× magnification through a dissecting microscope. Stained larvae were easily identified, counted, and removed from the sample. Additional elutriation of each sample was continued until no more larvae were found in two successive countings.

Detailed flow measurements above the wells were not possible during experimental runs due to the possibility of interfering with larvae and with the flow field close to the bottom. During experimental runs, flow velocity was measured 1 m downstream of the well array with a Marsh-McBirney flowmeter (model 201M) 3 cm above the flume bottom. After all experiments were run, flow profiles were taken over shell-filled wells. Flume settings were the same as those used in the experiments, and chemical solutions (*see below*) were added through the wells at the same rate as in the experiments. Profiles were done with a TSI conical hot-film ane-

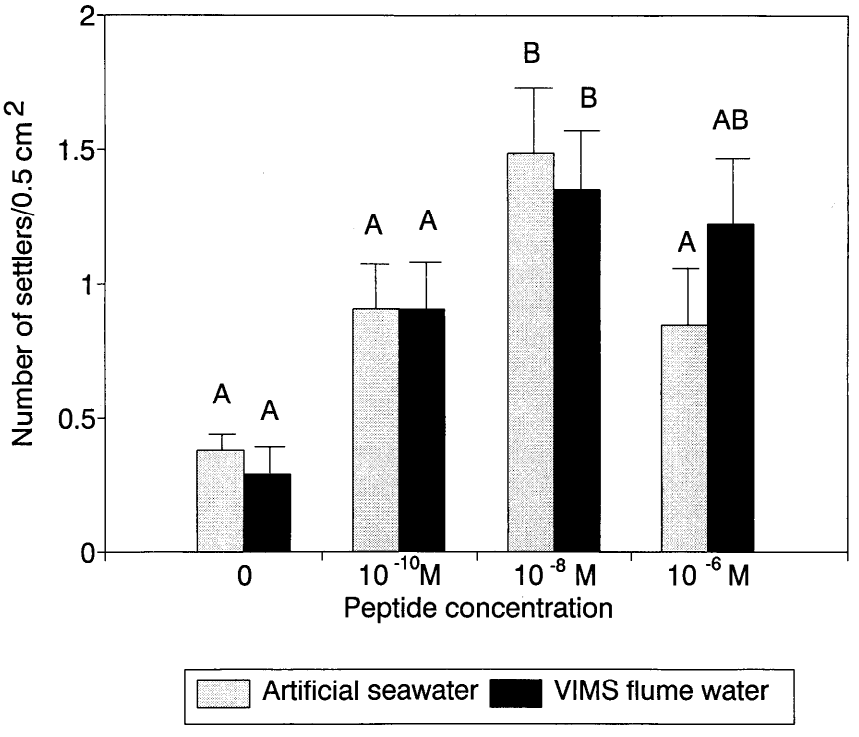

Fig. 2. Number of larvae attached per 0.5 cm^2 of bottom surface after 3-min trials at various GGR concentrations in still-water assays. Shaded bars represent solutions made with artificial seawater (ASW); black bars represent solutions made with VIMS flume water (VFW). Significant differences ($P < 0.05$) between treatments (using a Student-Newman-Keuls multiple range test with an experimentwide error rate of $\alpha = 0.05$) are indicated by different letters above the bars.

mometry system (with probe No. 1231W) measuring velocities at elevations of 20.05, 10.05, 7.05, 4.05, and 2.05 mm above the shell. A sixth velocity measurement was taken as close to the shell surface as the probe could be positioned (0.85 mm in 6 cm s^{-1}; 1.32 mm in 2 cm s^{-1}).

Electrochemical determinations were made and paired with the velocity measurements to detect chemical concentrations above the wells. For these measurements, we used dopamine as a tracer chemical. Dopamine is a water-soluble chemical not naturally present in the marine environment; thus, measurements will not be contaminated by natural sources. The molecular weight of dopamine (189) is similar to GGR (279), and the diffusional properties of dopamine in solution should be essentially identical to those of the GGR added during the settlement experiments. A solution of 200 μM dopamine in seawater (with ascorbic acid as an antioxidant and 0.01 g $liter^{-1}$ fluorescein dye as a visual tracer) was added to one upstream well (the dopamine well) to simulate addition of the water-soluble peptide. The other upstream well received clean seawater (the control well). As in the settlement experiments, the downstream wells received seawater and dopamine solution in the opposite pattern. Dopamine levels were detected with a single fiber graphite-epoxy capillary electrode attached to the probe of the TSI system and measured with IVEC-5 (In Vivo Electrochemistry Computer System, Medical Systems Corp.; Moore et al. 1992). Recordings of chemical concentrations were made at 10 Hz. Electrodes were calibrated in the flume by using an injection delivery method for solutions of dopamine assayed to generate a standard concentration curve. Calibrations exhibited excellent linearity over a concentration range of 0.5–80 μM (correlation coefficients were >0.998).

Paired electrochemistry and velocity profiles were measured in one upstream well and the well just downstream. In the upstream well,

the complete profile was taken 1.75 cm (¼ of the well diameter) downstream from the center of the well (Fig. 1B) to detect influence of the peristaltic pump on the flow regime and chemical concentrations near the input point (dye visualization studies showed that the solution added from the hypodermic needle was concentrated downstream of the center of the well). In the downstream well, the profile was taken 1.75 cm upstream of the center of the well (Fig. 1B), a region upstream of the addition of solution from the hypodermic needle.

Velocity was sampled at 10 Hz and averaged over 5 min. Average velocity was regressed on ln height above the bed to calculate shear velocity (U_*) according to the "law of the wall" (Nowell et al. 1981; Jonsson et al. 1992). In addition, to detect any cross-stream chemical movement we took chemical measurements at 2.05-mm height (very close to the bed but not within the shell substrate) in the upstream well receiving clean seawater only. These measurements were taken at the inside edge of the upstream control well, closest to the point of addition of dopamine from the adjoining well (Fig. 1B). Measurements were also taken at 2.05-mm height over the downstream dopamine well but 1.75 cm upstream of the addition point (Fig. 1B). Measurements at this point would show any upstream creep of the peptide from the addition point within the well or spreading out of the chemical signal from the upstream dopamine well on the opposite side of the flume.

Results

Larval settlement in still water—In the still-water observation chamber, the addition of peptide had a significant effect on larval settlement (Fig. 2 and Table 2). Settlement was enhanced and showed peak enhancement at a concentration of 10^{-8} M (Fig. 2). Of the larvae settling in peptide solutions, 58% remained attached to the bottom of the test chamber for the entire 3-min period and others swam back up into the overlying water and resettled. In both ASW and VFW controls, only 32% remained on the bottom until the end of the 3-min period. Addition of peptide led to enhancement of settlement in solutions made with ASW as well as those made with flume seawater, and no differences were seen between the water types (Table 2). This finding indicates that the charcoal filters effectively removed any organic materials in the VIMS seawater which might have induced or inhibited settlement. Settlement response in the peptide solution at 10^{-8} M (1.48 ± 0.24 settlers per 0.5 cm^2) was equivalent to that in the 500–1,000-Da fraction of oyster metabolite solution (1.53 ± 0.28 larvae per 0.5 cm^2) and to that in the crude metabolite solution tested without fractionation (1.61 ± 0.30 larvae per 0.5 cm^2). Analysis by HPLC indicated the presence of arginine-containing peptides at 6×10^{-9} M in the active (500–1,000 Da) metabolite fraction, implying that the synthetic peptide, GGR, used in the flume experiments imitates or closely replicates the structure and function of the natural inducer.

Table 2. Results of a 2-way ANOVA testing effects of peptide concentration and water type on the number of larvae settling in still-water experiments. Asterisks: ***—significantly different at $P < 0.001$.

Source	SS	df	MS	*F*-ratio
Peptide concn	14.842	3	4.947	11.496***
Water type	0.076	1	0.076	0.177
Pep × water	0.841	3	0.280	0.652
Error	40.023	93	0.430	

Larval settlement in flowing water—We tested for the following differences in the data: the effect of peptide on the number of larvae settled in upstream and downstream wells and a potential interaction between peptide and flow speed in the upstream well data. Electrochemistry revealed contamination of treatments in the downstream wells (*see below*), so the flow effect analysis was restricted to upstream wells.

A trend is visible that indicates a positive correlation between the number settled in upstream control wells and the number settled in upstream peptide wells (Fig. 3A,B). Some experimental runs (e.g. Exp. 2, 3, 6; Fig. 3A) had relatively high settlement in both peptide and control wells, whereas other experimental runs (e.g. Exp. 1, 4; Fig. 3A) had low overall settlement in both treatments. This correlation between the number settled in control wells and peptide wells is less evident in the downstream wells (Fig. 3C,D). We tested the significance of the correlation by regression of the number of larvae collected in peptide wells on the number collected in control treatments over all experiments for a particular flow speed. Significant correlation was found in both flow speeds in the upstream well data ($R^2 = 0.67$

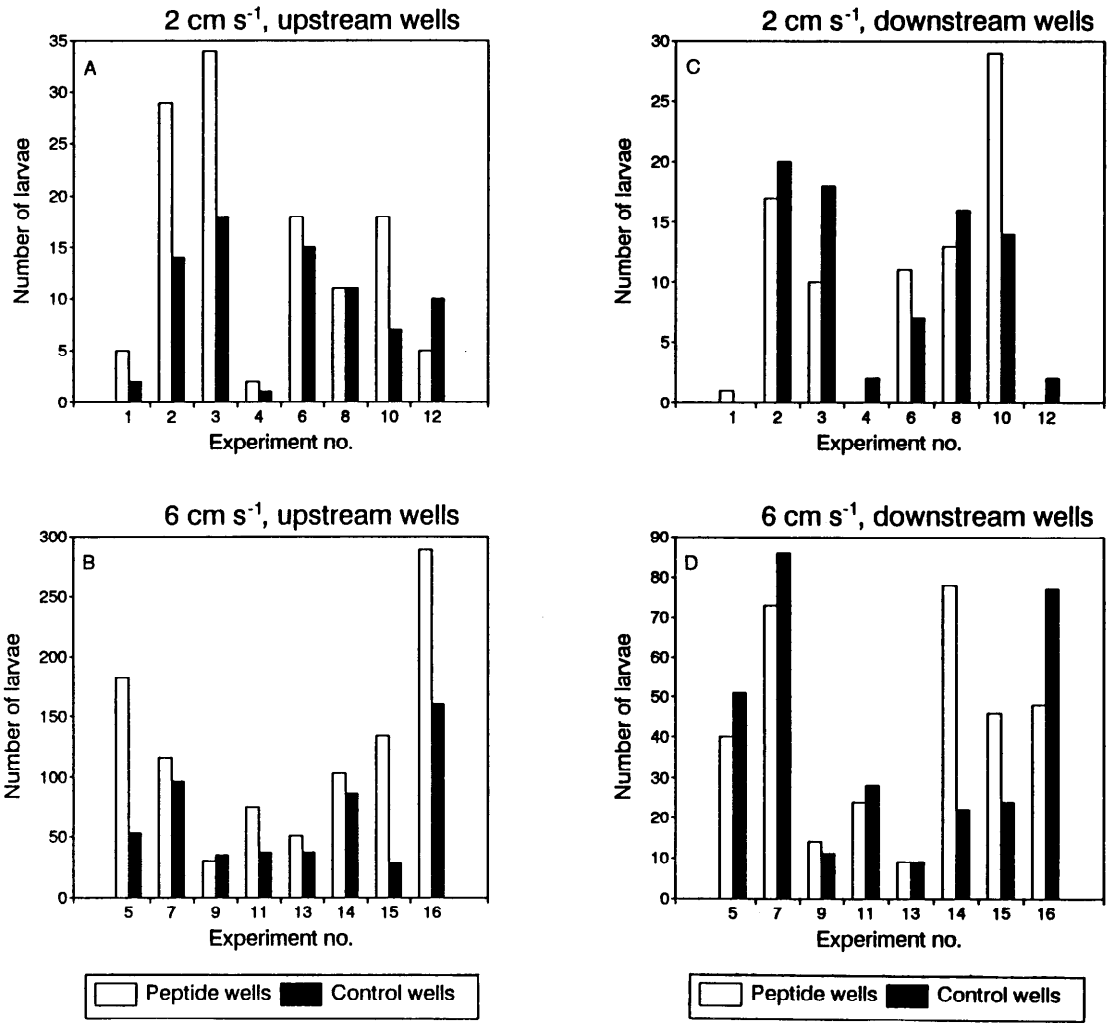

Fig. 3. Number of larvae collected from wells in different flow conditions.

for 2 cm s^{-1}, $P < 0.05$; $R^2 = 0.69$ for 6 cm s^{-1}, $P < 0.05$; Fig. 4A,B). Analysis for downstream wells revealed similar significant correlation at the slowest flow, but at the higher flow speed, the number of larvae in peptide wells and control wells was not significantly related ($R^2 = 0.53$ for 2 cm s^{-1}, $P < 0.05$; $R^2 = 0.32$ for 6 cm s^{-1}, $P > 0.05$). Thus, in the upstream wells for both flows, peptide and control wells covaried in the numbers of larvae collected.

Because of this covariation, treatments are not independent in our experiments. Analysis by traditional ANOVA (Butman et al. 1988; Butman and Grassle 1992; Grassle et al. 1992*a,b*) is therefore inappropriate. We chose to test for the effect of peptide separately for each flow speed by testing the significance of the deviation of the regression slope, regressing the number of larvae collected from peptide wells on the number collected from control seawater wells from a slope of 1.00 (i.e. no effect of peptide) with a one-tailed t-test. Upstream wells in both flows had slopes significantly different from 1 (at 2 cm s^{-1}, $t = 3.58$, $P < 0.01$; at 6 cm s^{-1}, $t = 2.44$, $P < 0.025$; Fig. 4A,B), indicating that the addition of peptide had a positive effect on the number of larvae collected. Data from downstream wells in both flows had slopes no different from 1 in a t-test (at 2 cm s^{-1}, $t = 0.76$, $P > 0.20$; at 6 cm s^{-1}, $t = 1.14$, $P > 0.10$; Fig. 4C,D),

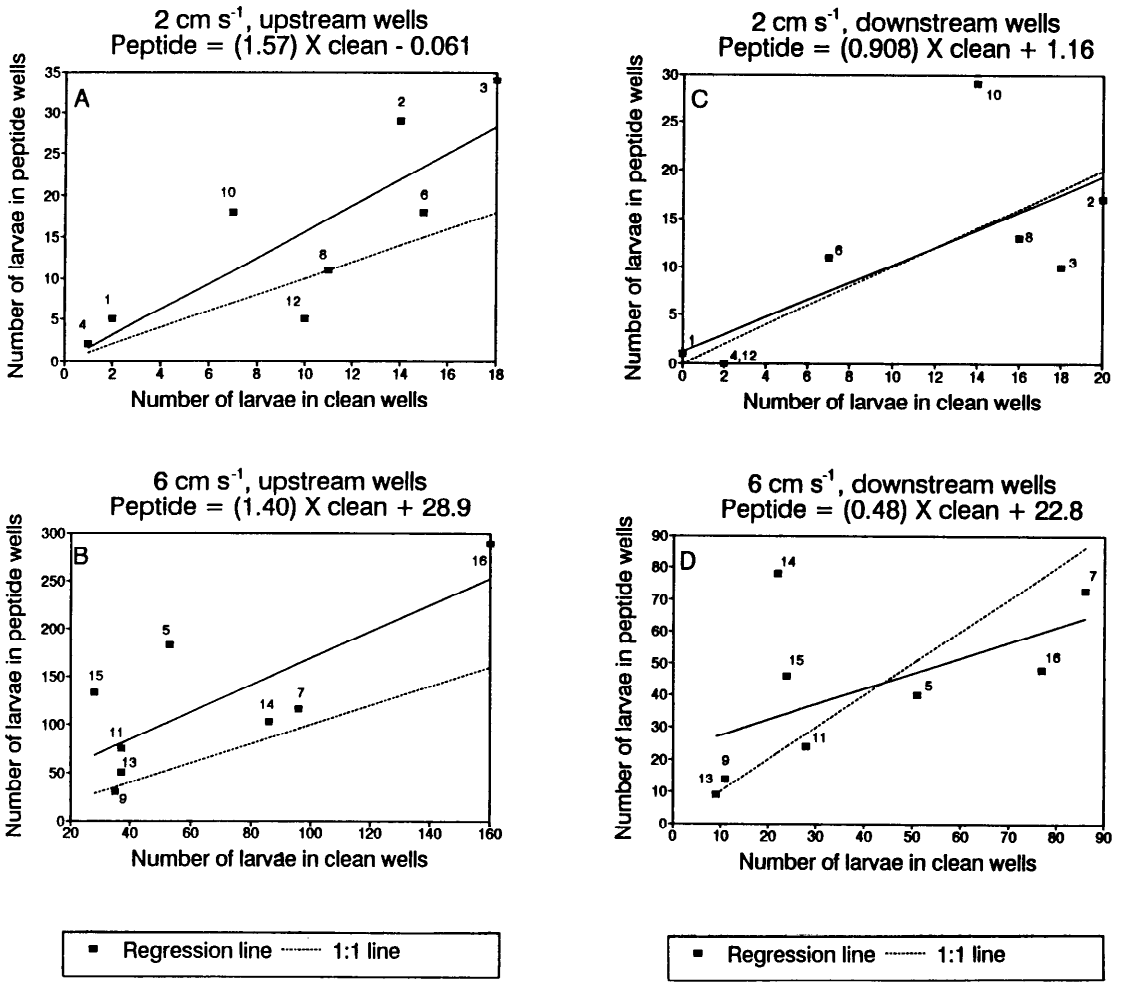

Fig. 4. Regression (solid lines) of the number of larvae collected in peptide wells on the number collected in control seawater wells. Dashed lines indicate a predicted 1 :1 ratio if larvae were settling equally in peptide and control wells. Numbers near the data points refer to experimental runs.

indicating that in downstream wells, the addition of peptide made no difference in the number of larvae collected. In summary, for both flows, the upstream wells showed greater numbers of larvae in the peptide wells than in the control seawater wells. No difference was seen due to addition of peptide in the downstream wells in either flow. Results of the electrochemistry data (*see below*) convinced us that the treatments (peptide vs. control) had been compromised in the downstream wells, so subsequent analysis was performed only on the upstream well data.

We used ANCOVA (the number of peptide well larvae covarying with the number of control well larvae) on the upstream well data to determine any interaction between flow and peptide effects which would show up as differences between the regression lines for 2 and 6 cm s^{-1} (Fig. 4A,B). There was no interaction of peptide and flow, as evidenced by the similarity of the regression lines between 2 and 6 cm s^{-1} ($P = 0.949$, Table 3A). The slopes of the lines also did not vary between flow treatments ($P = 0.325$ by ANCOVA, Table 3B), indicating that the effect of GGR was the same in both flow speeds.

The batch of larvae used in experiments aged over the course of several runs, potentially affecting settlement success. Each batch of larvae was refrigerated until 45–60 min before the start of an experimental run, and each sub-

Table 3. ANCOVA tables.

A. Test for homogeneity of slope: Model statement is

Numcue = constant + numno + speed + numno × speed.

Numcue is the number of larvae collected from the upstream wells with peptide added, constant the regression constant, numno the number of larvae collected from upstream wells without addition of peptide, speed the flow speed, and numno × speed the interaction term. Nonsignificant interaction term indicates slopes are homogeneous.

Source	SS	df	MS	*F*-ratio
Numno	2,255.975	1	2,255.975	1.378
Speed	912.302	1	912.302	0.557
Numno × speed	6.999	1	6.999	0.004
Error	19,648.559	12	1,637.38	

B. Test for ANCOVA: Model statement is

Numcue = constant + numno + speed.

Nonsignificant speed effect indicates that regression lines for both speeds are equal. Asterisks: ***—$P < 0.001$.

Source	SS	df	MS	*F*-ratio
Numno	29,023.817	1	29,023.817	19.196***
Speed	1,580.010	1	1,580.010	1.045
Error	19,655.558	13	1,511.966	

batch of larvae was brought up to flume temperature at different times in an attempt to minimize any age effect. The data show some indication of an increase in settlement in the 2 cm s^{-1} flows over the course of the day in runs 1–3 and 4–6 but not in runs 8–12 (Fig. 3A). Likewise, some evidence is shown of an increase in settlement at 6 cm s^{-1} between runs 16 and 18, but no indication of a trend is evident in runs 5–13 (Fig. 3B).

Flow and chemical profiles—Flow profiles taken above the wells generally showed the expected logarithmic relationship, but the R^2 values (0.913 upstream and 0.776 downstream at 2 cm s^{-1}; 0.831 upstream and 0.970 downstream at 6 cm s^{-1}) were lower than those needed to ensure an estimate of U_* within 5% (Nowell 1983). With this caveat in mind, calculation of U_* from the slope of the regression line gave values of 0.11 and 0.12 cm s^{-1} in the 2 cm s^{-1} flow (upstream and downstream wells, respectively) and 0.23 and 0.17 cm s^{-1} in the 6 cm s^{-1} flow.

Chemical measurements (and visual tracking of the fluorescein dye trail) indicated that little dopamine was mixed above 4 mm off the bed in the upstream dopamine well (Fig. 5A–D). No chemical was detected in the upstream control well in either flow (Fig. 6B,E). Dopamine was present at 2.05-mm height in the downstream control well due to spreading and dilution of the signal from the upstream dopamine well. The concentration of dopamine was less than that in the upstream dopamine well, but dopamine was present in the downstream control well in both flow speeds (cf. Fig. 6A and C, D and F).

Discussion

Our results show that oyster larvae do settle in response to a waterborne chemical cue in both still and flowing water. Although settlement induction by water-soluble compounds has previously been demonstrated from still-water studies (Bayne 1969; Veitch and Hidu 1971; Keck et al. 1971; Tamburri et al. 1992), our results are the first to demonstrate such a response in flows. These results are particularly noteworthy since the effect of chemical cues in flows has been questioned due to the weak swimming ability of many larvae relative to the horizontal flows which are present close to the bottom in most coastal marine habitats (Butman 1986).

The results of our experiments are striking because larval oysters are thought to be weak swimmers, with reported swim speeds of only 1.0 mm s^{-1} in the horizontal plane (Tamburri et al. 1992). Still, on contacting waterborne settlement inducer, oyster larvae respond by rapidly swimming or sinking downward at substantially higher speeds (Tamburri et al. 1992; D. S. Wethey and C. J. Finelli unpubl. data). The speeds attained by oyster larvae in the vertical dimension (up to 3.13 mm s^{-1}, Tamburri and Zimmer-Faust unpubl. data)

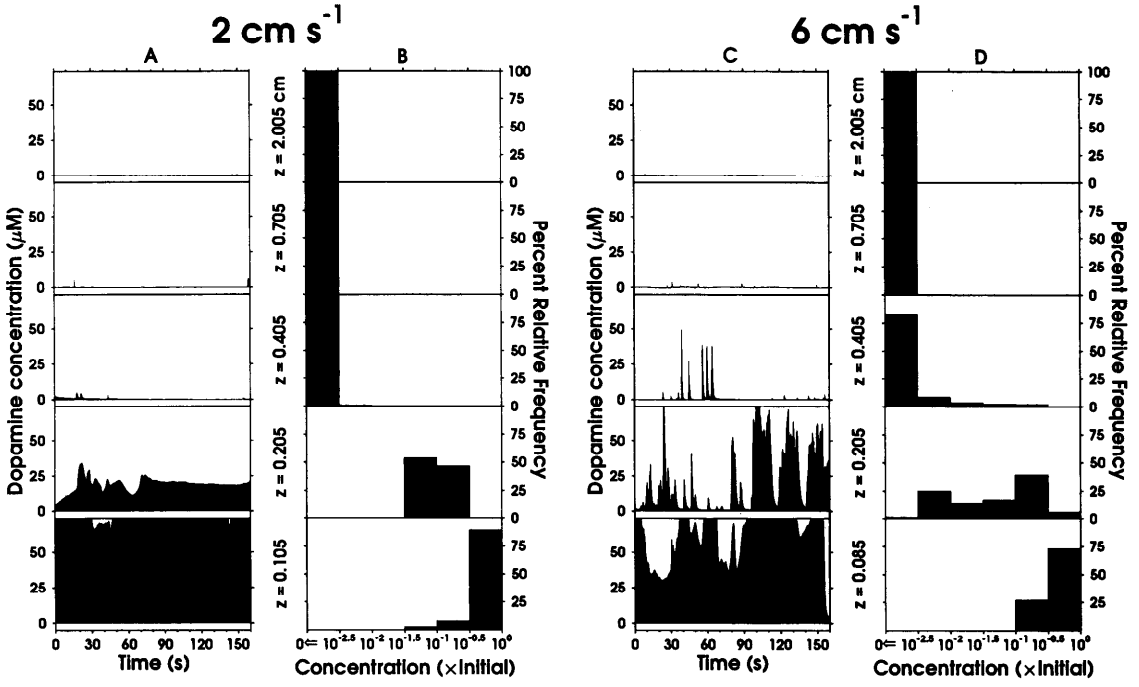

Fig. 5. Electrochemistry data collected over a well in which dopamine was introduced as a tracer for peptide. A. Time-series of 160 s of chemical data recorded at five different heights (z) above the substratum in 2 cm s^{-1} flow. B. Same data presented as a percentage of time a certain concentration was reached. C, D. As panels A and B, but in 6 cm s^{-1} flow.

could be effective in enhancing their delivery to a benthic surface, particularly since flow speeds in the vertical dimension may be very low. Further, the behavioral response of oyster larvae to the waterborne inducer takes place in the absence of any vertical chemical gradient (Tamburri et al. 1992). This finding eliminates a substantial theoretical argument against settlement induction by waterborne substances: that stable chemical gradients cannot be maintained above a substratum in turbulent benthic boundary layers. We found that larvae settle in response to any low concentration of GGR, not that they move toward a higher concentration in a chemical gradient.

Our data show a significant increase in larval settlement in a single-pass experiment over very small (6.98-cm diam) wells in response to trace amounts (10^{-7}–10^{-8} M) of chemical inducer. In addition, our chemical measurements indicate that the peptide was variable over time above the peptide wells. At 2.05 mm above the well, peptide reached high concentrations at some times, although concentrations were not consistently high (Fig. 6). Thus, the larvae had to react quickly during their one pass over the shell. A crude calculation provides some insight. If larvae are 2 mm above the bed, the horizontal velocity they experience (according to our velocity profiles) is 3.68 cm s^{-1}. Traveling downward at 3.13 mm s^{-1}, they will be carried 2.35 cm before reaching the bottom. Over our wells, larvae would need to react in <3 s to settle in the well where peptide was added. In nature, metabolites (including GGR) released by adult oysters will be dispersed over large areas of a natural reef rather than in small, isolated patches such as ours, and larvae may pass over "cued" areas for much longer periods of time.

The small size of our target wells may restrict the effects of the peptide to relatively low flow velocities in the flume, whereas the same constraints may not operate in the field. Over an oyster reef, with a much larger target area, larvae would have a longer time to sense and react

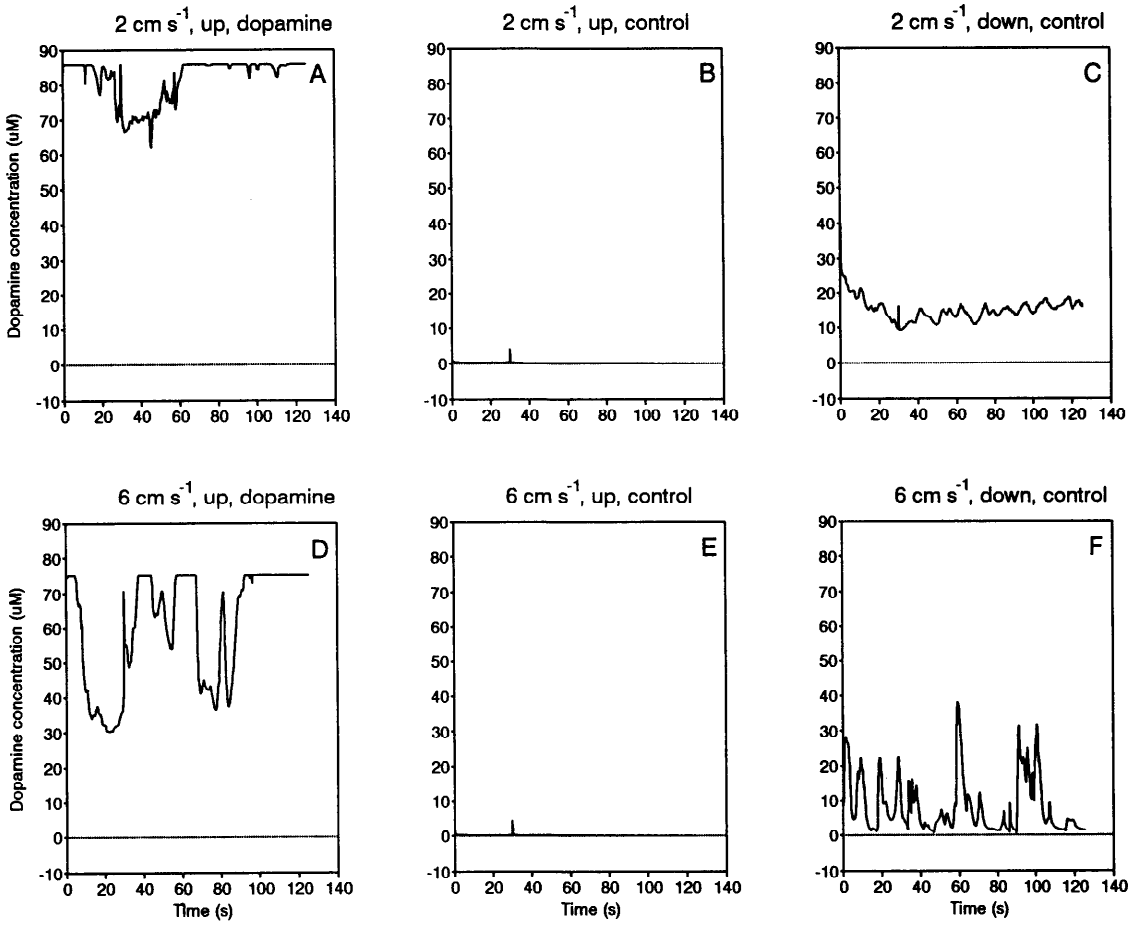

Fig. 6. Time-series of electrochemistry data from different wells and flow conditions: A, D—2 and 6 cm s^{-1}, above upstream dopamine well; B, E—2 and 6 cm s^{-1}, above upstream control seawater well; C, F—2 and 6 cm s^{-1} above downstream control seawater well.

to a waterborne cue and may be able to react to the cue at higher flow velocities. Thus, oysters may be able to use a waterborne cue more successfully than other invertebrates which recruit to smaller patches or are less gregarious.

Our electrochemistry measurements are meant to provide an analog of the behavior of the peptide during settlement experiments. We assume that the movement of GGR during the settlement experiments was not affected by the swimming of larvae through the chemical plume. Larvae in the benthic boundary layer swim in a viscous environment, and any swimming motion is unlikely to alter the plume of chemical.

Examination of the flow-electrochemistry data reveals that in upstream wells, larvae clearly would have encountered peptide over one well and clean seawater over the other. However, peptide would have been present in both downstream wells. Over the downstream control well, concentrations of dopamine were diluted up to 10-fold from the source upstream (Fig. 6). Similar dilution of peptide in our flow experiments would have resulted in concentrations of 10^{-8} M over the downstream control well. This concentration maximized settlement response in still-water assays. Thus, in the two downstream wells, larvae may have been induced to settle in both wells. Settlement could have been in response either to peptide added directly at the downstream peptide well or to peptide advected from the peptide well upstream of the downstream control well.

The concentration of dopamine above the wells was variable in time even close to the shell where the dopamine was added (Fig. 6A,D). Dopamine reached 4.05 mm above the shell for brief periods at the higher flow speed (Fig. 5C,D). Since oyster larvae have been shown to respond rapidly after contacting GGR (Tamburri et al. 1992; D. S. Wethey and C. J. Finelli unpubl. data), these brief periods may have been enough to cause larvae to rapidly approach the bottom and detect the more continual presence of peptide.

Our settlement results correspond with these electrochemistry findings. In the downstream wells, we detected no enhancement of settlement due to presence of peptide (Fig. 4C,D), not because the peptide was not inducing settlement in the test well but because the control well had been contaminated with peptide advected from an upstream source.

The effect of the peptide was the same in both flows, increasing the numbers settled in upstream wells with peptide added. The results are unequivocal ($F = 0.004$, $P = 0.949$ from ANCOVA, Table 3A) and striking; they imply that, in the two low flows used in these experiments, larvae responded in the same way, regardless of flow velocity. The behavior of the plume of dopamine was likewise similar in the two flows, with little dopamine advected above 4.05 mm from the bed and a high degree of variability shown in time, even very close to the bed (Fig. 5A,C).

The flows used in this study were below mean flows in many shallow marine areas, yet are not unrealistic flows for larvae to encounter when settling from the water column above oyster reefs and low-energy estuarine environments. Field data from Chesapeake Bay indicate that flow velocities measured 2–10 cm above oyster shell rarely exceed 7 cm s^{-1} (M. Palmer and D. Breitburg unpubl. data). In natural flow environments, very slow flow velocities will occur, whether the flow is current dominated, tidally dominated, or wind-wave dominated. Gross et al. (1992) argued that only during these times of relatively reduced flows are larvae close enough to the bed to settle out. At faster flows, turbulence suspends most larvae away from the bed.

Although we saw no interactive effect of peptide and flow, it is possible that a threshold velocity (or threshold shear stress, i.e. Gross et al. 1992) exists, above which larvae would be unable to react to chemical settlement cues. Jonsson et al. (1992) found that at flow speeds >15 cm s^{-1}, bivalve larvae (*Cerastoderma edule*) were suspended out of the near-bottom (<1 mm) layer and could not settle (*but see* Pawlik et al. 1991; Pawlik and Butman 1993; Mullineaux and Garland 1993). Flow velocities this high would rarely be seen near the bottom over oyster reefs. The U_* values calculated from our velocity profiles are in very good agreement with those obtained by Jonsson et al. (1992) for similar flows (0.19 ± 0.01 cm s^{-1} in 2 cm s^{-1} flow and 0.22 ± 0.018 cm s^{-1} in 5 cm s^{-1} flow). At these U_* values, they found that larvae were trapped in the near-bottom flow and were always within 1 mm of the bottom. Visual observation of larvae moving downstream in our experiments indicated that larvae were always close (<2 mm) to the bed, even when moving downstream in the water column. If the hydrodynamic regime traps larvae close to the bed (Jonsson et al. 1992), larvae will not need to settle very far to come into contact with the bed. Thus, even a small change in vertical movement caused by a waterborne chemical cue could be important in mediating settlement at these velocities.

Because the peptide is water-soluble, it is likely that it can be advected a considerable distance up into the water column if there is sufficient turbulence. Given that larvae respond to such low concentrations (maximum response at 10^{-8} M) of GGR, GGR might play an important early role in causing larvae to alter their swimming behavior and to approach the bottom of the water column. Thus, even if flows do not trap larvae close to the bottom, larvae may become concentrated there through active behavior. Once in this layer of water close to the bottom, other cues which may be bound to the bottom surface can be received.

This scenario contrasts somewhat with hypotheses put forward for polychaetes (Pawlik et al. 1991), barnacles, and bryozoans (Walters 1992) in which flow alone influences delivery of larvae to an area and then small-scale adjustments are made and metamorphosis occurs. It is, however, in partial agreement with Butman and Grassle (1992), who theorized that *Capitella* sp. 1 larvae may actively swim or

sink down to near-bottom waters but need contact with the sediment to accept or reject it. Grassle et al. (1992*b*) proposed a similar model whereby the bivalve *Mulinia lateralis* has a restricted ability to swim, either laterally or vertically, and is thus dependent on flow for transport to a suitable habitat. Although Pawlik and Butman (1993) related a behavioral change to flow velocity, our proposed mechanism provides a chemical cue that triggers the active vertical swimming of oyster larvae in order to reach the sediment. Oyster larvae need not be delivered completely as passive particles to the bottom and then respond to a surface-bound cue; they can respond to a waterborne cue while still some distance away from the bed if vertical velocities in the flow are low.

We have shown that oyster larvae actively approach the bottom when they contact the dissolved chemical cue in still-water trials and that their settlement out of flowing water is enhanced by the addition of GGR. However, the exact role of flow velocity (including turbulence), larval behavior, and spatial distribution of the chemical has yet to be completely determined. Because of their ability to influence their position within the water column in response to a waterborne chemical cue, oyster larvae provide an ideal model system for looking at the effects of chemistry, behavior, and hydrodynamics on recruitment to low-energy benthic environments.

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