Swimming by microscopic organisms in ambient water flow

M. A. R. Koehl · Matthew A. Reidenbach

Abstract When microscopic organisms swim in their natural habitats, they are simultaneously transported by ambient currents, waves, and turbulence. Therefore, to understand how swimming affects the movement of very small creatures through the environment, we need to study their behavior in realistic water flow conditions. The purpose of the work described here was to develop a series of integrated field and laboratory measurements at a variety of scales that enable us to record high-resolution videos of the behavior of microscopic organisms exposed to realistic spatio-temporal patterns of (1) water velocities and (2) distributions of chemical cues that affect their behavior. We have been developing these approaches while studying the swimming behavior in flowing water of the microscopic larvae of various bottom-dwelling marine animals. In shallow marine habitats, the oscillatory water motion associated with waves can make dramatic differences to water flow on the scales that affect trajectories of microscopic larvae.

1 Introduction

Studies of swimming organisms are usually done in still water or in a flume in which unidirectional flow is adjusted so that an animal swimming steadily upstream maintains its position in the working section of the tank. In contrast, organisms swimming in nature can be buffeted by unsteady ambient water motions. The smaller or more weakly swimming the organism, the greater the effect of environmental water motion on its trajectory. When microscopic organisms swim in their natural habitats, they are simultaneously transported by ambient currents, waves, and turbulence. Therefore, to understand how swimming affects the movement of very small creatures through the environment, we need to study their behavior in realistic water flow conditions. However, measuring the swimming behavior of an individual microscopic organism (e.g., instantaneous velocity of the organism, beating of cilia or appendages) requires high-magnification imaging of that organism.

The purpose of the work described here was to develop a series of integrated field and laboratory measurements at a variety of scales that enable us to record high-resolution videos of the behavior of microscopic organisms exposed to realistic spatio-temporal patterns of water velocities and distributions of water-borne chemical cues that affect their behavior. We used microscopic larvae of marine animals to study the effects of ambient water flow on trajectories of very small swimming organisms.

1.1 Swimming by microscopic planktonic larvae of benthic marine animals

Many bottom-dwelling marine animals disperse to new sites by producing microscopic planktonic larvae that are dispersed by ocean currents. Where those larvae settle back down onto the substratum can affect not only the population dynamics of those species, but also the structure of the benthic communities into which they recruit (reviewed by Roughgarden et al. 1991; Ólafsson et al. 1994; Rothlisberg and Church 1994; Palmer et al. 1996). When competent larvae (larvae old enough to be capable of undergoing

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metamorphosis into the bottom-dwelling form) move from the water column to the substratum, they pass through the benthic boundary layer. A number of studies conducted in flumes with unidirectional currents have shown that turbulent flow in the benthic boundary layer affects the delivery of larvae to the substratum (reviewed in Nowell and Jumars 1984; Butman 1987; Eckman et al. 1990; Abelson and Denny 1997; Crimaldi et al. 2002; Koehl and Hadfield 2004; Koehl 2007). Many shallow coastal sites where larvae recruit into benthic communities are exposed to waves, yet our knowledge of how ambient water motion affects rates of larval settlement has been based on studies in steady currents. Boundary layers are thinner in waves and shear stresses along the bottom are higher than in unidirectional flow at the same free-stream velocity (Charters et al. 1973; Nowell and Jumars 1984). However, net horizontal transport across a habitat in back-and-forth, wave-dominated flow is slow, even though instantaneous velocities in waves can be high (e.g., Koehl and Powell 1994).

Experiments done in still water have shown that dissolved chemical cues can induce the larvae of many types of benthic marine animals to undergo metamorphosis into the bottom-dwelling form (reviewed by Hadfield and Paul 2001). These chemical cues are released by organisms living on the substratum, such as adults of the same species as the larvae, their prey, or bacterial biofilms. A few behavioral studies of swimming competent larvae have shown that these chemical cues can also induce downward motion in still water (Hadfield and Koehl 2004) and in a unidirectional ambient water current (Tamburri et al. 1996). However, the low magnification of the video records of larval trajectories in those studies did not reveal whether such downward motion was due to active swimming or passive sinking, nor did those experiments reveal the spatial distributions in the water of the chemical cues inducing the changes in larval trajectories.

We used larvae of the nudibranch *Phestilla sibogae* and of the tube worm, *Hydroides elegans*, both of which swim with cilia, to investigate the effects of ambient water flow on the motion through the environment of swimming microscopic organisms.

2 Materials and methods

To investigate how swimming by microscopic organisms interacts with ambient water motion to determine the movement of the organisms in their natural habitats, we studied how the larvae of benthic marine animals move from the water column to the substratum to settle into suitable habitats. We have focused on the larvae of the sea slug, *Phestilla sibogae*, which settle onto the substratum in response to a water-borne species-specific metabolite of their prey, *Porites compressa*, the abundant coral that forms reefs in shallow habitats in Hawaii (Hadfield 1977; Hadfield and Koehl 2004; Koehl and Hadfield 2004). To study the interaction of swimming larvae with ambient water flow, we measured water velocities and mass transport on a variety of scales to determine realistic conditions under which to measure the swimming responses of individual larvae.

2.1 Field measurements of water flow

Our first step in determining how ambient water flow affects the motions of swimming larvae was to measure water velocities in the field in the habitats in which the larvae make their way from the water column to the substratum. For the larvae of *Phestilla sibogae*, we measured water velocities above coral reefs in Kaneohe Bay on the island of Oahu, HI (N21°27′, W157°47′).

As is typical of Hawaiian reefs, the dominant coral species at our study sites was *Porites compressa*. We measured water velocities above *P. compressa* throughout the tidal cycle, when water depth above the reef ranged between 0.10 and 0.80 m. On the days when we measured water velocities, water temperatures were 23–26°C, mean wind speeds were 6.6–6.8 m s^{-1}, and maximum wind speeds were 9.2–10.8 m s^{-1} (weather station in Kaneohe Bay of the Hawaiian Institute of Marine Biology, University of Hawaii).

Water velocities were measured using a Sontek SP-AV10M01 Acoustic Doppler Velocimeter (ADV) with a measurement volume of $\sim$0.25 cm^3 and a sampling rate of 25 Hz. A cable from the ADV was run to a boat anchored nearby, where the data were recorded on a laptop computer. Water velocities were recorded for periods of 3 min at heights of 4 and 8 cm above the surface of the reef. The distance of the sampling volume above the reef was measured both by the ADV and by ruler (see Finelli et al. 1999), and these distances agreed in all cases. The ADV was held in position by a rigid scaffolding placed not to interfere with the flow being recorded.

Our field measurements revealed that the shallow coastal habitats in which the larvae of *P. sibogae* must make their way from the water column to a coral reef are characterized by turbulent, wave-driven flow. Some examples of our field water velocity data are shown in Figs. 1 and 2. How are microscopic larvae transported in such flow? How does such water flow disperse the dissolved chemical cues released by the coral *P. compressa* that induce the larvae to settle onto the substratum? We addressed these questions in a series of laboratory flume experiments.

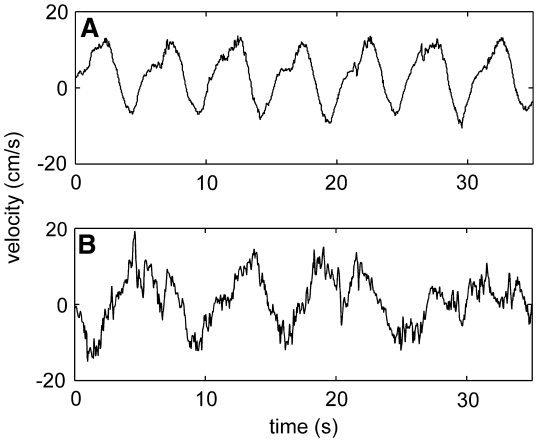

Fig. 1 Comparison of horizontal velocities measured using LDA at a height of 2 cm above the top of the *P. compressa* "reef" in the laboratory wave-current flume (*top graph*), and measured using ADV at a height of 2 cm above the top of a living *P. compressa* reef in Kanehoe Bay, HI (*lower graph*). In both cases the mean water depth above the top of the reef was ∼25 cm. The flume is diagrammed in Fig. 3

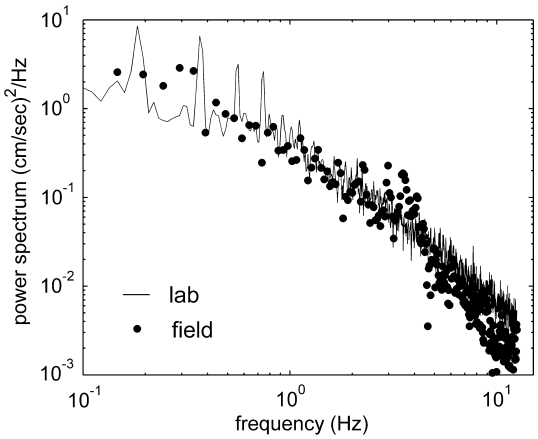

Fig. 2 Comparison of the power spectra for vertical velocity fluctuations measured using LDA at a height of 2 cm above the top of the *P. compressa* "reef" in the laboratory wave-current flume (*black line*), and measured using ADV at a height of 2 cm above the top of a living *P. compressa* reef in Kanehoe Bay, HI (*black circles*). These are data from the same flow records plotted in Fig. 1. In both cases the mean water depth above the top of the reef was ∼25 cm

2.2 Wave-current flume

Our field measurements of water velocities above coral reefs were used to design the water flow in a wave-current flume (12.5-m long by 1.2-m wide) that could produce both a mean current and surface waves (Fig. 3). A steady re-circulating water current was produced by a centrifugal pump commanded by a digital frequency controller. Waves could be generated simultaneously by a paddle-type wavemaker that was driven by a servo motor and linear actuator (Pidgeon 1999). The wavemaker paddle produced waves at a single frequency (which could be adjusted) that propagated in the direction of water motion. A sloping, broad-crested weir at the downstream end of the tank minimized reflection of wave energy, since the flow over the weir was supercritical.

A section of coral "reef" was constructed in the wave-current flume from skeletons of the branching, reef-forming coral *Porites compressa* (provided by M. Hadfield, State of Hawaii collecting permit #1999–2005, after they had been used in other experiments). Left-over coral skeletons, rather than living corals, were used both to minimize the impact of this study on wild populations of *P. compressa*, and to avoid the difficulty of maintaining healthy corals in the laboratory. Baird and Atkinson (1997) found little difference between the overall drag, Re_*, roughness length scale, and mass transfer coefficients of living *P. compressa* and of their skeletons, thus our use of coral skeletons to study water flow over reefs is justified. The skeletons of coral heads (typically about 15 cm tall) were packed together tightly, as they are in the field, such that the constructed reef completely covered the floor of the test section of the flume (1.8-m long and 1.2-m wide). The coral skeletons used in the flume had branch widths and inter-branch spacings of approximately 1 cm (Reidenbach et al. 2006). The total water depth in the flume for all experiments was 40 cm, with the depth of water above the canopy being on average 25 cm.

2.3 Measurements of water velocities in the flume using Laser Doppler Anemometry

A Dantec two-component Laser Doppler Anemometer (LDA), operated in forward scattering mode, was used to measure streamwise, u, and vertical, w, velocities. The

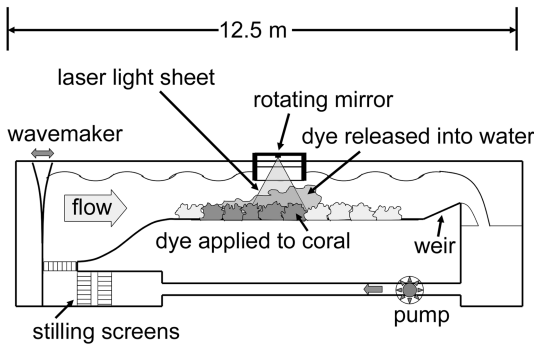

Fig. 3 Diagram of the wave-current flume (details given in the text). Planar-laser induced fluorescence was used to image the flux of Rhodamine 6G dye from the surfaces of the coral under a combined wave-current flow. Examples of velocity measurements and a turbulence spectrum used in this flume are shown in Figs. 1 and 2, respectively

LDA optics were coupled with a laser (Coherent Innova 90), operated at a wavelength of 514.5 nm. The measurement volume was 0.1 mm in the vertical and streamwise directions, and 1 mm in the cross-channel direction. Velocity measurements in the horizontal and vertical directions were made by detecting the Doppler frequency shift of laser light scattered by small particles moving with the fluid. The LDA system was positioned by a 3-axis motorized rail-bearing traverse with positioning in the vertical direction to a precision of 50 μm. Velocities were acquired above the top of the coral canopy (z = 0 defined at the tip of the uppermost branch of the coral) at heights z = 0.2, 0.4, 0.7, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, 20.0, 30.0, 40.0, 50.0, 75.0, and 100.0 mm. Velocity measurements at each height above the canopy were collected at 50 Hz for 30 min. The free-surface displacements of the air–water interface in the flume directly over the LDA measurement volume was simultaneously measured using a capacitance wave height gauge (Richard Brancker Research Ltd., Model WG-30).

2.4 Comparison of water flow in the field and in the flume

Although water flow over coral reefs in Kaneohe Bay was more variable than in the laboratory wave-current flume, the small-scale features of the flow that should affect the movement of larvae and of dissolved cues above a reef were reproduced quite well in the flume. The peak velocities and periods of waves in the flume (Fig. 1a) fell within the ranges of those measured in the field (Fig. 1b), although the waves were more regular in the flume. While large-scale, low-frequency flow features in the field could not be replicated in the flume, the turbulence structure of field and flume flow were very similar (Fig. 2).

2.5 Effects of waves on fine-scale flow

Our LDA measurements of velocity profiles along coral surfaces in the flume revealed that the superposition of waves onto a unidirectional current can have dramatic consequences to water motion at the fine spatial scales encountered by larvae swimming in the water near a coral reef. Examples of such LDA measurements are shown for the case of a unidirectional current (Fig. 4a) and a wave-dominated current (Fig. 4b). The root-mean-squared (rms) velocity for a unidirectional current over the coral increased logarithmically away from the canopy, as expected for a turbulent boundary layer (i.e., Gross and Nowell 1983). In contrast, the profile of the rms velocities for the wave-dominated case revealed drastically different flow structure. A very steep gradient in rms velocities, as well as in peak velocities, occurred very near the surface of the coral. Due to the oscillatory nature of the flow, which is caused by strong pressure gradients that oscillate with the period of the wave forcing (Sleath 1987), water parcels must be quickly diverted around the coral structure, causing accelerations in the flow and ultimately inducing much higher velocities immediately adjacent to the coral roughness elements (Lowe et al. 2005). While flow diversion around the coral also occurred in unidirectional flows, the flow directly adjacent to the coral was much slower than in waves because a thicker boundary layer developed due to the lack of an oscillating pressure in the flow field.

The higher velocities near the coral due to wave action had the effect of increasing both the fluid shear stress imposed on the coral and the turbulent motions near the coral surfaces. Peak Reynolds stresses measured near the surfaces of the coral under the oscillatory flow were

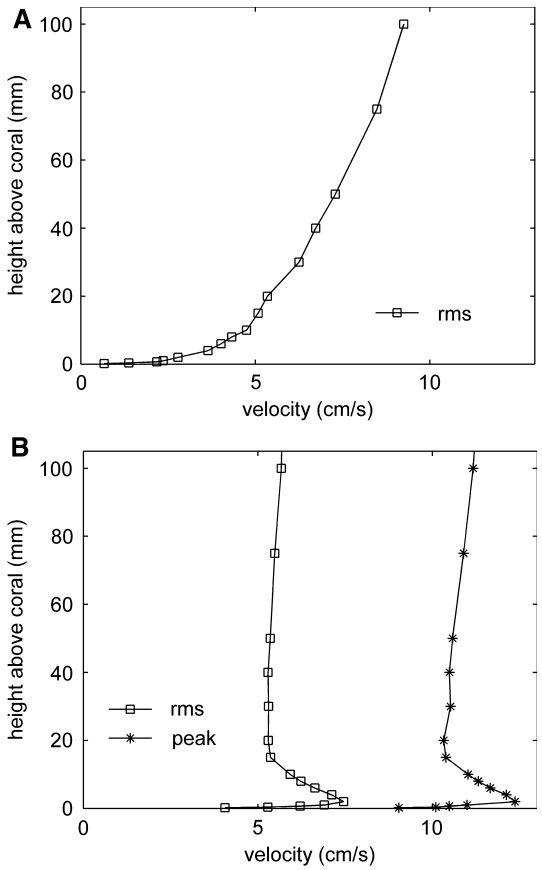

Fig. 4 Root-mean-squared (rms) water velocity profiles, calculated from LDA measurements made above a coral "reef" in a wave-current flume for a unidirectional current with a free-stream velocity of 9.7 cm/s (**a**), and for a wave-dominated flow with a background mean current (**b**). In this example, the wave period was 3s, and the peak freestream horizontal velocity in the downstream direction attained during each wave was 11.3 cm/s, while the peak horizontal velocity near the corals was 12.4 cm/s

$\langle u'w' \rangle_{max}/U^2_{rms}$ = 0.04 ± 0.005 while that for the unidirectional flow were 0.008 ± 0.001, indicating a five-fold increase in turbulence due to waves. While these flow dynamics may be small in scale relative to the depth of the water column, they can have a dramatic effect on a microscopic larva attempting to settle onto the reef.

2.6 Planar laser-induced fluorescence (PLIF) measurements of chemical cues leaching from corals

To investigate how realistic wave-driven water flow affects the dispersal of the dissolved chemical cues that affect larval behavior, we measured the fine-scale instantaneous distributions of chemicals released from a reef in the wave-current flume. Rhodamine 6G fluorescent dye was used as an analogue for dissolved substances, such as larval settlement inducer, released by the corals. This is justified because the Schmidt numbers (Sc = {kinematic viscosity of the fluid}/{molecular diffusivity of the dissolved substance}) for chemicals dissolved in water are quite high. The Sc of Rhodamine 6G in water is 1,250 (Barret 1989). Although we do not yet know the Sc's of settlement inducer released by corals, even if it were an order of magnitude different from that of the dye, the fine-scale patterns of concentration distribution of the two would be quite similar because molecular diffusivity is so low relative to water's kinematic viscosity.

To mimic the release of dissolved inducer from the corals, we painted coral skeletons with a mixture of equal volumes of a solution of Rhodamine 6G dye (500 ppm in fresh water) and gelatin powder (Difco Laboratories Bacto-Gelatin). A layer 1-mm thick of this dye-gelatin solution was painted onto the surfaces of the coral skeletons. A strip along the midline of the coral "reef" 1.2-m long (parallel to the flow direction) and 0.20-m wide was painted in this manner and allowed to set in air for 30 min. These coated corals were then placed into the water in the flume. As the gelatin slowly dissolved, the dye was released into the water to simulate dissolved substances leaching from living corals. For each experiment, coated corals could be exposed to water flow in the flume for approximately 10 min before uneven wear of the dye coating along the surfaces of the corals occurred.

Planar laser-induced fluorescence (PLIF) was used to determine the fine-scale spatial and temporal structure in the water above the reef of the concentrations of dye released from the corals. By illuminating a thin slice of the water column with a sheet of laser light (1-mm thick) that caused the rhodamine dye to fluoresce, we could measure concentrations of this analogue for inducer on a spatial scale relevant to the ambits of microscopic swimming larvae (Fig. 5). Our PLIF system consisted of a laser, imaging optics to expand and focus the laser light, a scanning mirror to produce a sheet of light, and a digital CCD camera to record the dye fluorescence in the flowing water. Laser light was emitted by an Argon-Ion laser (Coherent Innova 90) at an output of 1 watt. The laser beam was first expanded using a 3× laser expander (Melles Griot) to minimize transmission losses and then focused using a 2 m focusing lens. A light sheet was created using a moving-magnet optical scanning mirror (Cambridge Technology model 6800HP). As the dye passed through the laser light sheet, the fluoresced dye was imaged with a CCD camera (Silicon Mountain Design with 1024 by 1024 pixels and 12 bit resolution) fitted with a Micro-Nikkor 55 mm flat-field lens to reduce curvature effects at the image edges. A filter on the receiving optics, with a center frequency of 555 nm and bandwidth of 30 nm, was used to remove laser and ambient light wavelengths, leaving only emitted light from the fluorescing dye. Pixel brightness was proportional to dye concentration (calibration procedures described in Crimaldi and Koseff 2001). Raw images were processed to remove biases in the data, including varying pixel dark response, varying pixel response to fluorescence intensity, slow background changes in pH and temperature, lens and optics aberrations, and laser attenuation due to the background dye concentrations, as described in Crimaldi and Koseff (2001).

Each image (of an area in the flume 21 × 21 cm), was exposed by a single laser scan with a total integration time

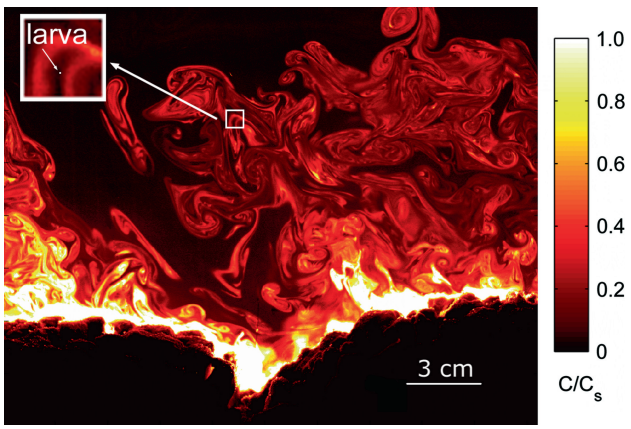

Fig. 5 Planar laser-induced fluorescence (PLIF) image of chemical flux from the surfaces of two *P. compressa* coral heads (which appear *black* at the *bottom* of the image) in the "reef" on the floor of the wave-current flume. The inset image is a ×5 magnification of the portion of the scalar field indicated by the *white box*. The *white dot* in the inset indicates the size of a larva relative to the dye filaments. The color scale bar indicates dye concentration, which is normalized by C_s, the concentration along the surface of the coral. Mean flow was from right to left with a mean current of 7.8 cm/s and a superimposed wave with a period of 5 s and orbital wave velocity amplitude of ±11 cm/s

of 30 ms. The advection of dye during this integration time was much smaller than the typical pixel dimension of 200 μm, thus ensuring accurate mapping of the scalar structure onto the pixel. Images were collected at a rate of 10 Hz. Typically, 100–500 sequential images were taken during each experiment sequence.

By using PLIF to examine the instantaneous spatial distribution of dissolved inducer in the water moving above a reef on the fine spatial scale relevant to a microscopic larva, we learned that filaments of inducer swirl around in inducer-free water. Therefore, we reasoned that as larvae swim or sink through the water, they move into and out of inducer filaments (see insert in Fig. 5), rather than encountering a continuous diffuse concentration gradient as has been assumed in models of larval settlement in response to aromas from the substratum (e.g., Eckman et al. 1994). How do brief encounters with settlement inducer affect the swimming of larvae of *P. sibogae*?

2.7 Rapid reactions of larvae to encounters with filaments of inducer

The larvae of *Phestilla sibogae* swim by beating cilia along the edges of a two-lobed swimming organ, the "velum" (Fig. 6a). To determine how larval swimming is affected by brief encounters with filaments of dissolved inducer, we had to record the action of the cilia and the velum of individual larvae as the animals moved into and out of inducer filaments, hence the animals had to be viewed using a microscope. Because the field of view of a microscope is small, we could not follow the velar actions of freely swimming larvae. Instead, we used videomicrography to record the actions of the velar lobes of individual larvae tethered in a small flume (mini-flume) that moved water past each larva at the velocity of water motion relative to an untethered swimming larva, $\sim$0.2 cm s^{-1} (Hadfield and Koehl 2004).

The Plexiglas "mini-flume" had a working section that was small enough (3-cm wide $\times$ 3-cm deep $\times$ 14.5-cm long) to permit a larva to be viewed using a microscope so that ciliary beating and the position of the velum could be discerned. We videotaped individual larvae (60 frames s^{-1}) using a SPI Minicam mounted on the ocular of a Wild stereomicroscope. A steady flow rate of filtered sea water through the flume was maintained by a constant-head tank, arrays of screens were used to create a flat velocity profile across the middle of the working section where the larva was positioned, and velocity was adjusted by raising or lowering the constant-head tank with a lab jack. The mini-flume was designed to be a flow-through system so that background levels of dye and chemical cues would not build up over the course of an experiment. Water velocity past a larva in the mini-flume was measured by videotaping the movement of small neutrally buoyant particles carried in the moving water. The microscope was focused on the mid-line of the flume (where the larva was positioned) and only particles in sharp focus were digitized and used to calculate water velocities (using SCION Image software) to avoid errors due to parallax.

An individual larva was tethered by using Vaseline® to stick its hydrophobic shell to the tip of a fine stainless steel insect pin (0.24-mm diameter) and was held in a fixed position in the mini-flume in the field of view of the microscope. The pin and larva were gently lifted from the larval culture dish and positioned with a micromanipulator in the mini-flume so that the larva could "swim" into the flow (i.e., the water flow relative to the tethered larva was the same as the water flow relative to a freely swimming larva) (Fig. 6a).

Tethered larvae were exposed to filaments of test solutions (filtered sea water, or various concentrations of chemical inducer released by the coral, *P. compressa*) labeled with 0.05 or 0.1% fluorescein that were carried past them in the flowing water (Fig. 6b) (Hadfield and Koehl 2004). These filament encounters were designed to mimic the exposure to inducer filaments that a freely swimming larva would encounter in the turbulent flow above a coral reef (Fig. 5). We estimated the time course of encounters with odor filaments by a larva above a coral reef using a computer simulation of larval motions (due to swimming, ambient waves, and turbulence) through the changing concentration fields recorded in PLIF videos (e.g., Fig. 5) (Koehl et al. 2007). Swimming velocities measured for untethered larvae in still water in aquaria (Hadfield and Koehl 2004) were used in these simulations. Our calculations indicated that a larva moving through the water passes into and out of filaments of inducer, and that larvae close to the reef encounter filaments more often than do larvae higher above the reef (Koehl et al. 2007). We simulated swimming through an inducer filament in the mini-flume by gently releasing (using a syringe pump, Sage Instruments model No. 351) a narrow filament of test solution into the water through a syringe needle (bore diameter of 0.5 mm) positioned by a micromanipulator perpendicular to the flow upstream from the larva. The needle was higher in the water column than the larva, so the larva did not experience the wake of the syringe. The filament of test solution was carried downstream across the tethered larva by the water flowing in the mini-flume, as though the larva were swimming through the filament. Video records of these fluorescein-labeled filaments showed that before they reached a larva, the momentum induced by the filament-releasing system was damped out.

We videotaped the instantaneous responses of larvae, including cessation and resumption of beating of velar

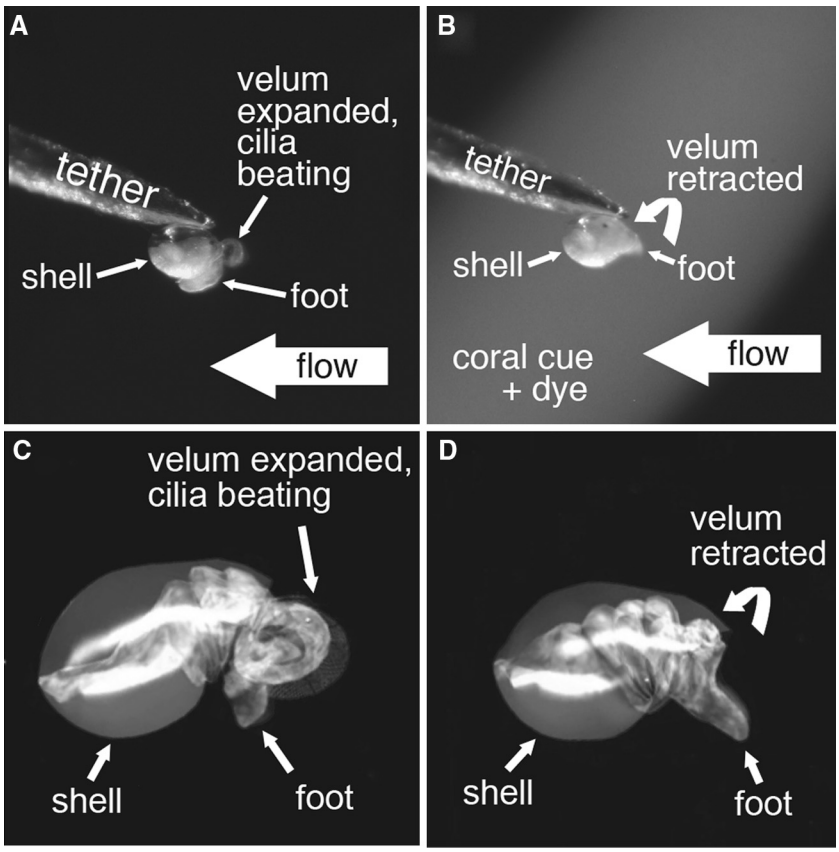

Fig. 6 Side views of a larva of *Phestilla sibogae*. **a**, **b** are frames of a video taken of a larva tethered in a small flume in which water was moved past the larvae at the same speed and opposite direction as the swimming velocity of the larva (video made using a SPI Minicam mounted on the ocular of a Wild dissecting microscope). The tether was a fine insect pin (diameter 0.24 mm). **c**, **d** are diagrams of larvae in the same postures as shown in **a**, **b**, respectively. When the tethered larva was "swimming" in filtered seawater, its velum was extended and its velar cilia were beating (video frame in **a** and diagram in **c**). When a tethered larva encountered a filament of inducer from *P. compressa* (labeled with fluorescien dye), it stopped beating its cilia and retracted its velum into the shell, but left its foot protruding out of the shell (video frame in **b** and diagram in **d**)

cilia, and partial or complete retraction or re-extension of the velum or foot. Using frame-by-frame analyses of these video records, we measured (to the nearest 0.017 s) the time lag between the onset or cessation of these behaviors and the time when the edge of a filament encountered or left the chemoreceptive organ of a larva (Hadfield et al. 2000). The mini-flume permitted us to expose larvae to different temporal patterns of inducer encounters and different concentrations of the chemical cue. We found that swimming competent larvae of *P. sibogae* did not respond to fluorescein dye alone, but that they stopped beating their cilia and retracted their velum into the shell when they encountered inducer above threshold concentration, and they re-expanded the velum and resumed ciliary beating upon exiting an inducer filament (Hadfield and Koehl 2004). If they had not been tethered, the larvae would have sunk through the water when the velum was retracted.

Our high-magnification records of what larvae did during brief encounters with inducer filaments revealed that they kept the foot protruded out of the shell when the velum was retracted. This observation explains why larvae moving downwards in coral inducer in still water traveled more slowly than both swimming larvae and than sinking dead, fully retracted larvae (Hadfield and Koehl 2004): the high drag on the foot of a larva sinking in response to inducer slows down its rate of descent. The foot of a competent *P. sibogae* larva is sticky, thus a larva with its foot extended from the shell can adhere to a surface on which it lands (Hadfield and Koehl 2004; Koehl and Hadfield 2004). Past analyses of larval settlement onto surfaces have often relied on sinking velocities measured for dead or anesthetized larvae (e.g., Butman et al. 1988), but our *Phestilla* results illustrate the importance of knowing the postures and behaviors of larvae before assuming that living larvae move like passive, dead ones.

How do the instantaneous behavioral responses of larvae of *P. sibogae* to brief encounters with filaments of coral inducer affect their motion relative to a coral reef in nature? To address that question, we must put microscopic larvae (whose responses to inducer were measured in the mini-flume, and whose swimming and sinking velocities were measured in still water in aquaria, Hadfield and Koehl 2004) back into the wave-driven ambient flow over a coral reef.

2.8 Simultaneous measurements of water velocities (particle image velocimetry, PIV) and of scalar concentrations (PLIF)

Microscopic organisms such as larvae are carried along by the water in which they are swimming, Because they are so small, such organisms are transported across the habitat by

net current flow, and are carried around locally in turbulent eddies. Those turbulent eddies also carry the filaments of chemical cues to which the larvae react (Fig. 5). The only way that a microscopic organism can move *relative to the water and chemical cues* around it is to swim or sink. In contrast, the organism moves *relative to the substratum* both by swimming or sinking and by being carried by the ambient water flow. Therefore, to determine the temporal pattern of inducer encounters by a larva or the trajectory of a microscopic animal relative to the substratum, we must simultaneously measure both the fine-scale velocity vector field of the flow and the fine-scale patterns of concentration of dissolved inducer in the water.

Instantaneous velocity vector fields of turbulent flowing water can be measured using particle image velocimetry (PIV; technique described in detail in e.g., Cowen and Monismith 1997). A method which combines measurements of planar laser-induced fluorescence and particle image velocimetry can be used to simultaneously measure inducer concentration and velocity structure in two dimensions. For our PLIF imaging, we used a laser with an output wavelength of light at 488 nm to excite fluorescein dye (mean excitation at 490 nm) leaching from coral skeletons, as described above for rhodamine. A sheet of laser light (produced with a scanning moving-magnet mirror, as described above) illuminated a thin slice through the water column in the flume, and the fluorescein dye in that slice emitted light at a mean wavelength of 520 nm. We recorded the dye motions in the flume using a digital camera (Redlake motionscope with 480 × 420 pixel resolution) fitted with an optical bandpass filter of 520 ± 10 nm (Andover Corp.) so that only emitted light from the fluorescent dye was imaged. This laser sheet was scanned to illuminate the imaging field every 0.02 s, with a wait period of 0.02 s between each scan. A second laser with an output wavelength of light at 532 nm was simultaneously used for our PIV imaging. This second laser illuminated silver-coated glass spheres (11 μm in diameter, Potter Industries) carried in the water moving in the flume, but did not excite the fluorescein dye. The second laser was also pulsed at 0.02 s intervals, but only during the time periods when the PLIF laser was not being scanned. The pulsed laser passed through a 30° cylindrical lens to create a light sheet that was aligned along the same two-dimensional plane as the PLIF laser. Particle motions illuminated by the PIV laser were recorded using a second Redlake motionscope digital camera (Fig. 7a). Both cameras were aligned so that they imaged the same field. Images of particle trajectories were analyzed with PIV software (MatPIV 1.6.1) to calculate a velocity vector in every subwindow (16 by 16 pixels), and these vector fields were superimposed on the simultaneously recorded scalar concentration field (Fig. 7b). Such data permits us to assess the instantaneous water flow and inducer concentration encountered by a microscopic larva at any defined position in the portion of the water column that we have imaged.

2.9 Putting data from different scales together to determine larval trajectories through the environment

A computer simulation of the transport of larvae relative to the substratum can be conducted using data measured at the

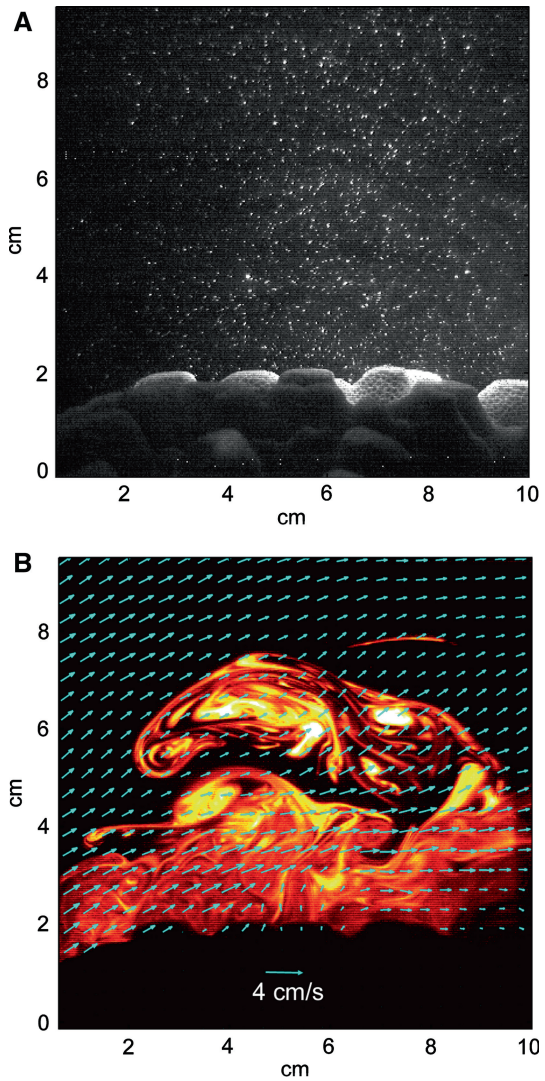

Fig. 7 Example of simultaneous PIV and PLIF measurements over *P. compressa* coral in wave-dominated flow in a flume. The flow was oscillatory with a mean freestream velocity (from left to right in the image) of U = 10 cm/s. Images shown here were collected when the oscillatory flow was beginning to reverse the current from right to left. **a** Frame of a PIV video. **b** PLIF image of dye concentrations recorded 0.02 s after the image shown in **a**. The velocity vectors of water motion that occurred during this interval are shown in *blue*

different spatial scales described above. Larvae can be simulated by behavioral algorithms (based on microscope observations on the scale of micrometers of larvae in the mini-flume) and can be assigned swimming and sinking velocities (measured on the scale of millimeters to centimeters s for freely swimming larvae in aquaria). These simulated larvae can be placed randomly in the PLIF/PIV data (scale of 0.1 mm's to cm's) recorded in a wave-flume, in which water flow and coral reef structure measured in the field on the scale of centimeters to meters was mimicked. A larva sinks or swims, depending on the concentration of inducer in the pixel in which it is located in the PLIF video frame. The vector sum of the swimming (or sinking) velocity of a microscopic larva and of the water velocity in the PIV subwindow in which the larva is located predicts where that animal will be in the next frame of the PLIF video of the scalar (i.e., inducer) field. By repeating such calculations for successive frames of the PLIF/PIV videos, the trajectories of larvae swimming and sinking in the turbulent, wave-driven, inducer-laden water above a coral reef can be determined.

2.10 Larval swimming near surfaces in water currents and waves

When swimming animals encounter surfaces, their behavior can be altered. For example, in still water, the swimming behavior of microscopic larvae of the marine tube worm, *Hydroides elegans*, changes if they touch a surface covered with biofilm (The mechanisms responsible for these behavioral changes are not yet understood.). Surfaces in marine habitats are first colonized by biofilms of bacteria and other microorganisms; then larvae of larger animals recruit onto the biofilmed substratum (e.g., reviewed by Koehl 2007). Videos of larvae of *H. elegans* in dishes of still water showed that they continued to swim when over clean glass surfaces, but spent more time crawling on the substratum than swimming above biofilmed surfaces (J. Zardus, M. Hadfield, T. Cooper, M. A. R. Koehl, unpublished data). Contact with biofilms in still water also induces the larvae of *H. elegans* to undergo metamorphosis (Unabia and Hadfield 1999). If these larvae are being carried past the substratum by ambient water flow, how do contacts with biofilmed surfaces affect their trajectories?

Hydroides elegans are abundant members of the "fouling communities" that grow on man-made structures, such as ships and docks, in warm-water harbors worldwide. To study the trajectories of the microscopic larvae of *H. elegans* in realistic flow conditions, we first measured water flow across surfaces in Pearl Harbor, HI, where *H. elegans* are abundant. Our measurements of water velocity profiles (using a small electromagnetic flow meter, Marsh-McBirney Model 523) adjacent to dock surfaces, showed that the flow oscillated due to wind chop superimposed on slow ambient currents (Fig. 8a).

We videotaped the behavior of competent larvae of *H. elegans* near different substrata in the "mini-flume" described above. An array of screens upstream from the working section was used to produce a velocity profile similar to that measured in Pearl Harbor, and a plunger upstream of the screens was used to superimpose velocity oscillations on the net downstream water motion in the flume to simulate the wind chop measured in the field (Fig. 8b). Water velocity profiles were measured as a function of time by digitizing (Matplotlib 0.83) videos (60 fps) of the paths of neutrally buoyant marker particles carried in the water.

Competent larvae of *H. elegans* were placed in the upstream reservoir of the flume and were carried by the moving water through the working section of the flume. By working in the mini-flume, we were able to make high-magnification videos in which individual larvae could be followed (Fig. 9). The water was not recirculated through the flume so that we only recorded the first encounters of larvae with a test substratum. We videotaped the larvae (60 fps) and digitized their trajectories using Matplotlib 0.83.

3 Results and discussion

3.1 Putting data from different scales together to determine larval trajectories through the environment

Our computer simulation of the transport of larvae *Phestilla sibogae* relative to the substratum was an

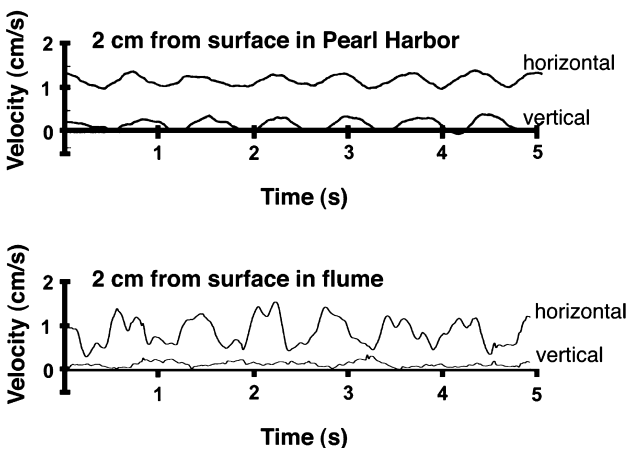

Fig. 8 Water velocities measured as a function of time 2 cm above surfaces covered with the tube worm *Hydroides elegans* in Pearl Harbor, HI (**a**), and in the lab in the working section of the "mini-flume" in which larval behaviors can be recorded

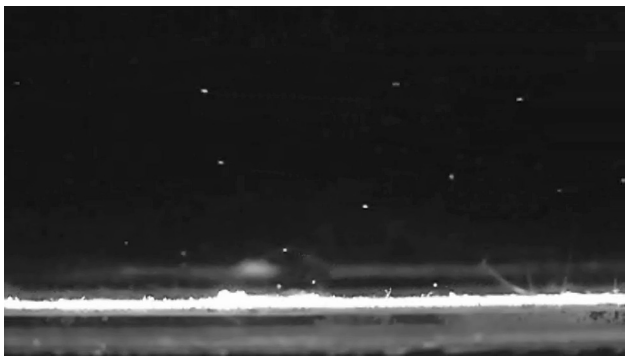

Fig. 9 Frame of a video of competent larvae of the tube worm *Hydroides elegans* swimming in wave-driven flow near a surface covered with a biofilm in a small wave-current flume. The larvae, which look like white dots, are about 100-μm long. The magnification was chosen to achieve the largest field of view in which the larvae were still big enough to be clearly visible

individual-based model that coupled behavioral algorithms [measured for microscopic larvae exposed to realistic patterns of encounters with coral odor (Hadfield and Koehl 2004)] with fine-scale patterns of time-varying instantaneous flow velocities and odor concentrations [measured above a coral reef in a flume (Reidenbach et al. 2007) exposed to wavy, turbulent water movement like that measured in the field (Koehl and Hadfield 2004)]. Our calculations revealed that the simple behavior of sinking during brief encounters with odor filaments can enhance the rate of larval settlement onto a reef by about 20% (Koehl et al. 2007). Thus, we learned that the behavioral responses of slowly moving microscopic larvae to chemical cues can affect their trajectories in the environment, even in turbulent, wave-driven ambient water flow.

Is it worth the effort to measure fine-scale time-varying velocity and odor distributions under field flow conditions? Past analyses of larval settlement in response to odors assumed a constant, diffuse concentration gradient of dissolved chemical cues from the benthos (e.g, Crisp 1974; Eckman et al. 1994). When we used the diffuse time-averaged odor concentration gradient measured above our reef in the flume to calculate larval transport rates, we overestimated larval transport by about 15% compared with rates we calculated using the time-varying instantaneous filamentous pattern (Koehl et al. 2007). Many studies of larval settlement assumed that larvae are transported in the benthic boundary layer like passive, negatively buoyant particles (e.g., Hannan 1984; Butman 1987). When we ran our model with the assumption that larvae sink continuously like passive particles, we over-estimated the rate of transport of larvae to the substratum compared with rates we calculated using the behavior measured for larvae of *Phestilla sibogae* that sink only while in coral odor above a threshold concentration (Koehl et al. 2007). Thus, our calculations indicate that ignoring time-varying, fine-scale flow and odor distributions or instantaneous larval responses can lead to overestimates of the rates of transport of larvae to the substratum.

3.2 Larval swimming near surfaces in water currents and waves

H. elegans larvae in still water swim along helical paths (Fig. 10a). Such helical swimming has been shown to enable organisms in still water to navigate relative to odors, light, and gravity (e.g., reviewed in McHenry and Strother 2003). However, we found that when *H. elegans* were swimming in the miniflume in water flow like that encountered near surfaces in harbors, they were carried distances of a cm or more (i.e., ≥100 body lengths) relative to the substratum for each turn of the helix (Fig. 10b, c) (M. A. R. Koehl, D. Sischo, T. Cooper, T. Hata, and M. Hadfield, unpublished data). Furthermore, when the oscillations due to wind chop were added to the slow currents typical of harbors, larval trajectories were more varied and showed more vertical motion (Fig. 10c) than in flow without the wind chop (Fig. 10b). The importance of helical swimming in navigation by larvae carried by turbulent or wave-driven ambient water motion has not yet been determined.

Fig. 10 Digitized trajectories of competent larvae of *H. elegans*. Each colored line shows the path of an individual larva. Larvae in still water swam along helical paths (*left panel*), but were carried downstream in a unidirectional water current (*middle panel*), or were carried in a slow current plus small waves (typical of wind chop in harbors) (*right panel*). The larvae had more variable trajectories with more vertical motion when waves were superimposed on the current

By producing small-scale flow in the miniflume that was similar to water motion measured in the field, we were also able to record the behavior of individual larvae of *H. elegans* as they encountered the substratum under realistic flow conditions (Koehl, Sischo, Cooper, Hata, and Hadfield, unpublished data). We learned that when competent larvae of *H. elegans* contacted the flume floor, they appeared to "hop" along the bottom, touching down two–three times per centimeter that they moved downstream. The touchdown durations of larvae that contacted biofilmed surfaces were longer than for larvae that contacted clean glass, the duration of a larva's contact with the substratum was shorter in flowing water than in still water, and the variation in touchdown time was greater in waves than in unidirectional flow. Our results indicate flowing water and contact with biofilmed surfaces have dramatic consequences for the trajectories of larvae of *H. elegans.*

3.3 Ambient water flow affects the motion of swimming microscopic organisms

Studies of swimming are often done in still water or in unidirectional currents in flumes. However, when microscopic organisms swim in their natural habitats, they simultaneously are being transported by ambient currents, waves, and turbulence. Therefore, to understand how swimming affects the movement of very small creatures through the environment, we need to study their behavior in realistic water flow conditions. However, quantifying the swimming behavior (e.g., ciliary beating, body trajectory) of an individual microscopic organism requires high-magnification imaging of that organism over time, which is very difficult in their natural habitats or in a large flume. In this paper, we have described a series of integrated field and laboratory measurements at a variety of scales that have enabled us to record high-resolution videos of the behavior of microscopic marine larvae exposed to realistic spatio-temporal patterns of water velocities and of chemical cues that affect their behavior.

We found that the oscillatory water motion associated with waves can make dramatic differences to water flow on the scale of microscopic larvae (Reidenbach et al. 2007). Although shallow marine habitats are often exposed to waves or wind chop, most flume studies of the effects of water flow on larval settlement onto the substratum have been done in unidirectional flow (reviewed in Abelson and Denny 1997; Koehl 2007). Fortunately, the tools are now available to produce in the laboratory more realistic water flow, based on field measurements on the spatio-temporal sales relevant to larval swimming.

Not only do ambient currents affect the trajectories of microscopic organisms by carrying them across the habitat, but water movement also can stimulate them to change their swimming behavior. For example, some larvae curtail their swimming in rapid water currents (e.g., lobsters, Rooney and Cobb 1991; polychaetes, Pawlik and Butman 1993). Similarly, snail larvae were induced to sink when high turbulence dissipation rates were produced in a tank by oscillating screens (Fuchs et al. 2004). Some types of larvae orient their locomotion relative to the direction of ambient water flow or shear (e.g., barnacles, Crisp 1955; bivalves, Jonsson et al. 1991; bryozoans, Abelson 1997). Larvae that have landed on a surface can stay or they can reject the surface and resume swimming (reviewed by Krug 2006); the larvae of some species of barnacles resume swimming after landing more often in rapidly moving water than they do in slow flow (Mullineaux and Butman 1991; Jonsson et al. 2004; Larsson and Jonsson 2006). As these studies illustrate, laboratory swimming studies conducted in still water may not reveal ecologically relevant behaviors for those animals that alter their locomotion in response to water movement in the environment.

3.4 Swimming by microscopic organisms can affect their transport by ambient water flow

Whether marine larvae are simply transported like passive particles by moving water or actively seek suitable habitats has long been debated (e.g., reviewed by Butman 1987; Woodin 1991; Jumars 1993; Koehl 2007). Evidence has been accumulating that, although microscopic organisms such as larvae are small and swim slowly relative to ocean currents, their locomotory behavior can affect where they are transported by ambient water flow.

As described above, we found that the behavior of sea slug larvae that are being carried in realistic wave-driven water flow near surfaces can affect their transport to the surfaces (Koehl et al. 2007). Similarly, the swimming of bivalve larvae in unidirectional flow in flumes affects their transport to the bottom. (Tamburri et al. 1996; Finelli and Wethey 2003). In addition, studies of ascidian larvae, which are large enough to be seen and tracked by investigators in the field under natural flow conditions, have shown that larval swimming responses to light and gravity affect their transport across the habitat and their settlement onto the substratum (reviewed in Young 1990; Worcester 1994; Koehl et al. 1997).

Not only can the behavior of larvae affect their transport across the benthic boundary layer, but their locomotion also can affect their horizontal transport over long distances. Large-scale water movements in the ocean carry marine larvae between sites and from offshore waters to the coast (e.g., reviewed by Roughgarden et al. 1991; Rothlisberg et al. 1995; Shanks 1985). The vertical position

of larvae in the water column can determine where they are carried, since water at different depths can move in different directions. Thus, the vertical swimming or sinking behavior of larvae can affect their horizontal transport despite how slowly they locomote relative to ambient water currents (e.g., Cronin and Forward 1986; Shanks 1985; Epifanio et al. 1989; Bingham and Young 1991; Rothlisberg et al. 1995; Tankersley and Forward 1994; Tankersley et al. 1995; Queiroga and Blanton 2005). Many lab studies have documented the behavioral responses of larvae to environmental cues (e.g., light, gravity, pressure, salinity), as well as the endogenous rhythms of such behaviors and the ontogenetic changes in swimming, and have related these responses to where larvae are carried by currents (e.g., Forward and Cronin 1980; Forward et al. 1995; Tankersley and Forward 1994; Tankersley et al. 1995; and others reviewed by Chia et al. 1984; Young and Chia 1987; Young 1995). However, some larvae swim too weakly for such mechanisms to be important to their dispersal (e.g., Stancyk and Feller 1986), while others are such strong swimmers that they can actively move horizontally in spite of ambient currents (e.g., crabs, Luckenbach and Orth 1992; lobsters, Katz et al. 1994).

4 Conclusions

Studies of swimming are often done in still water or in unidirectional currents in flumes. However, the motion of microscopic organisms swimming in their natural habitats cannot be understood without considering how ambient water flow affects their trajectories and the transport of chemical cues that induce behavioral responses. In shallow marine habitats, the oscillatory water motion associated with waves can make dramatic differences to water flow on the scale of microscopic organisms such as larvae.

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