Title

A Novel Approach to Evaluating Patterns of Vertical Distribution in Cryptic Larvae: Application of Fluorescence in situ Hybridization (FISH) and a Large-Particle Cell Sorter

Author

Levin, Lisa A.

Publication Date

2011-01-15

California Sea Grant Sea Grant
Final Project Progress Report

01/15/2011
Preparer Information
Paola López-Duarte
plopezduarte@ucsd.edu
858-534-3579

R/ENV-149PD
01/01/2009–12/31/2010

A novel approach to evaluating patterns of vertical distribution in cryptic larvae: application of fluorescence in situ hybridization (FISH) and a large-particle cell sorter

Lisa A. Levin
Scripps Institution of Oceanography
Integrative Oceanography Department
llevin@ucsd.edu
858-534-3579

Project Hypotheses
We hypothesized that differences in the distribution of adult mussels are the result of differences in the selection pressures on their larvae, such that the vertical distribution of larvae from the inner bay species (*M. senhousia*) facilitates bay retention, while that of the open-coast species (*M. californianus*) enhances alongshore dispersal.

Project Goals and Objectives
The preliminary research to be conducted was designed to address the following questions: (1) Can new imaging and particle analysis techniques (fluorescence in situ hybridization and a large-particle cell sorter - FISH-LPCS) be used to successfully isolate and distinguish among three mytilid species in field-collected plankton samples? (2) Do larvae from the three mytilid species display different vertical distribution patterns that may influence their horizontal transport? (3) Does vertical distribution change with tide, light or ontogenetic stage?

Briefly describe project methodology
(1) LAB-SPAWNED LARVAE. Adult *M. californianus*, *M. galloprovincialis*, and *M. senhousia* were collected and brought back to the lab where spawning was induced. After quantifying larval densities, test mixtures of known concentrations of mussel larvae were created and used to test the accuracy of the large-particle cell sorter to quantify samples containing mixed species. During phase I, the reproductive status of the mytilids was assessed to ensure future field sampling was scheduled during known reproductively active periods (Please see “Project Modifications” section).
(2) FIELD LARVAL DISTRIBUTIONS. After several failed attempts to schedule our field sampling due to weather (Please see “Project Modifications” section), sampling was conducted at the Scripps Marine Facility (MARFAC), located near the mouth of San Diego Bay

(32.706221 N, 117.235881 W) where *M. californianus*, *M. galloprovincialis*, *M. senhousia* adults and larvae can co-occur. Constant volumes (6000 L per sample) of seawater were pumped using a stainless steel pump, powered by an electric generator and fitted with a vortex impeller to minimize damage to larvae. The pump intake was adjusted to obtain samples at near surface (0-1.5 m depth), and (~1 mab). The tidal range at this site was between 5.5-7.5 meters. Sampling was conducted every 2 h for 24 h (May 24-25). This sampling resolution was set to permit comparison of larval distributions during different phases of the tide (high, falling, low and rising tide). Seawater was pumped through a 75 micrometer mesh to concentrate samples and retained material and fixed in a modified salt ethanol solution (70% ethanol with salt, EDTA and Tris-HCl added) and transported to the FISHsort Lab at the University of California, Santa Barbara, for tagging and sorting.
(3) FISH-LPCS AND LARVAL SORTING. Samples gathered from phases (1) and (2) were labeled with species-specific probes for each of the three target species using a whole-larva fluorescent in situ hybridization protocol. Three different-colored fluorescent labels were used for each of the species-specific probes, permitting samples to be probed for all three species simultaneously. After probe-binding was completed, samples were sorted and counted with a large-format cell sorter (COPAS Plus model manufactured by Union Biometrica). The number of larvae of each color (i.e., species) were counted, and larvae were sorted into four groups, the three target species and a fourth group of all other particles in the sample. The accuracy of the technique was determined by manually counting the samples using fluorescence microscopy (to confirm counting accuracy and ensure that sorted samples only include the target mussel species) for the known samples from phase (1). Once the accuracy of the method was verified, plankton samples containing field-caught larvae (phase 2) were labeled and sorted. For a subset of the field samples, accuracy of identification was verified genetically.

Describe progress and accomplishments toward meeting goals and objectives

(1) LAB-SPAWNED LARVAE: Adult mytilids were spawned and test mixtures were sent to the FISHsort Lab, where fluorescent probes were designed for all three species and the accuracy of the large-particle cell sorter was tested. To increase the probability that we would in fact collect mussel larvae in the field, we regularly collected mussels for spawning throughout the year (~3 weeks) and scheduled the field sampling during May, when all three species spawned in the lab.
(2) FIELD LARVAL DISTRIBUTION: A 24-hr. field sampling was conducted in May 24-25, 2010. Samples were fixed and transported to the FISHsort Lab.
(3) FISH-LPCS AND LARVAL SORTING: All sorting and analysis was recently completed and the findings are presented below.

Project modifications

We encountered delays in field applications of the research due to lack of spawning by mussels and to the need for further genetic verification of specimens following initial analysis. A preliminary sampling effort on the open coast did not yield larvae and thus the

decision was made to move sampling into San Diego Bay to maximize the effectiveness of the vertical distribution test.

Project outcomes
See attached document "RENV-149PD Project Outcomes"

Impacts of project
New image and particle analysis techniques employed for this project can be used to successfully isolate and distinguish among similar planktontic larval species - in this case the three mytilid species. A more thorough description of the accuracy of this technique is included in Henzler et al 2010.

Benefits, commercialization and application of project results
The FISHsort Lab at UCSB is currently collaborating with several groups that are seeking to make plankton identification more effective and efficient. This work has contributed to improving and validating the application of this technique to marine larvae by providing both laboratory and fieldtests with closely related mussel species. This should facilitate dispersal and connectivity research that is often stymied by our inability to distinguish similar larvae of related species in plankton samples.

Economic benefits generated by discovery
NA

Issue-based forecast capabilities
Connectivity information is highly desired for design and management of marine protected areas. It is also important to effective management of natural marine resources, invasive species and aquaculture species. This tools developed in this project will advance connectivity science which becomes increasingly important in the face of changing biogeographic and environmental regimes.

Tools, technologies and information services developed
Project provided testing and application of Coupled fluorescence in situ hybridization and cell sorting (FISH-CS)(Henzler et al. 2010)

Publications
Technical reports
NA

Conference papers, proceedings, symposia
Hoaglund, EA, CM Henzler and SD Gaines (2010) Species Migration, Dispersal Limitation and Technological Innovation: The application of a novel technique to identify and count marine larvae to assess dispersal at species range boundaries. Ocean Science Meeting, Portland, Oregon. [Poster]
Hoaglund, EA, CM Henzler, GE Hofmann and SD Gaines (2009) Species Migration, Dispersal Limitation and Technological Innovation: The application of a novel technique to identify and count marine larvae to assess dispersal at species range boundaries. 90th Meeting of the Western Society of Naturalists, Monterey, California. [Poster]

Peer-reviewed journal articles or book chapters
Henzler CM, Hoaglund EA, and Gaines SD (2010) FISH-CS - a rapid method for counting and sorting species of marine zooplankton. Mar Ecol Prog Ser 410:1-11.

Please list any workshops/presentations given
P. Lopez-Duarte presented at Cabrillo National Monument, as part of the San Diego Science Festival in March 2009. The presentation, entitled "Mussel Madness! Connectivity of Mytilid Mussel Populations in Southern California", targeted a wide (nonscientific) audience including children and adults (approximately 30 attendees).

Students
Elizabeth A. Hoaglund
University of California Santa Barbara
Department of Ecology, Evolution and Marine Biology
Degree program enrolled in: Ph.D.
Theses/dissertation title: The influence of oceanography on marine species' distribution
Supported by Sea Grant funds? [] yes [x] no

How many students/volunteers were involved in the project? 0

Cooperating organizations
Academic Institutions
University of California, Santa Barbara (Christine Henzler [Postdoctoral Researcher] and Elizabeth Hoaglund [Graduate Student])

International implications
NA

Awards
NA

Keywords
mussel larvae, larval dispersal, larval vertical distribution, *Mytilus californianus*, *Mytilus galloprovincialis*, *Musculista senhousia*, population connectivity

Project Outcomes (RENV-149PD)

Species-specific Probe Development

During phase I of this project, probes were successfully designed and tested for the target bivalve taxa (*Mytilus galloprovincialis*, *Mytilus californianus*, and *Musculista senhousia*) and the fluorescence *in situ* hybridization large particle cell sorting (FISH-LPCS) technique was verified. A method to circumvent the problem of plankton autofluorescence, which has stymied other attempts to develop FISH for identifying marine larvae (discussed in Pradillon et al. 2007) was also developed. A manuscript detailing the method and probe development has been published [Henzler CM, Hoaglund EA, and Gaines SD (2010) FISH-CS - a rapid method for counting and sorting species of marine zooplankton. Mar Ecol Prog Ser 410:1-11].

We developed and tested short (18bp), oligonucleotide probes to target 18S rRNA of *Mytilus edulis/galloprovincialis/trossulus* (EGT), *Musculista senhousia* (MUSC) and *Mytilus californianus* (MCAL) (Table 1). All three probes were tested by dot blot hybridization against 18S DNA of 65 individual bivalves representing ten different species found in southern California waters: 10 *Mytilus galloprovincialis,* 10 *Mytilus trossulus*, 5 *Mytilus edulis*, 10 *Mytilus californianus*, 10 *Musculista senhousia*, 8 *Septifer bifurcatus*, 5 *Modiolus sacculifer*, 3 *Adula diegensis*, 2 *Ruditapes philippanarum* and 2 *Crassostrea gigas.* Results of the dot blot hybridizations demonstrate that the EGT and MUSC probes are specific to their target taxa. The *M. californianus* probe was not specific; in addition to *M. californianus*, it also bound to *S. bifurcatus* and *A. diegensis* DNA. Further work is underway to develop a species-specific *M. californianus* probe, but in the interim, a PCR-RFLP assay to differentiate *M. californianus*, *S. bifurcatus* and *A. diegensis* larvae has been designed and tested.

Table 1. Probes designed for this study. T_m = oligonucleotide melting temperature.

Probe Name	Probe sequence (5'-3')	Target taxa	T_m
EGT	AGGTCAGGAGCAGGCAGT	*Mytilus edulis* *Mytilus galloprovincialis* *Mytilus trossulus*	62.2°C
MUSC	GTAAACCGACGGTGTCGG	*Musculista senhousia*	62.2°C
CAL	AGGACGAGCAGTAACCGA	*Mytilus californianus* (probe also binds to *Septifer bifurcatus* & *Adula diegensis*)	59.9°C

Fluorescence *in situ* hybridization (FISH) was tested with the EGT probe, but not the MUSC or CAL probes, using cultured larvae; pure cultures of *Musculista senhousia* and *Mytilus californianus* were not available during the testing phase. Fluorescence *in situ* hybridization with the EGT probe, labeled with either Alexa 488 (green) or TET (yellow) fluorescent dyes, was performed on five sets of samples with corresponding controls: (1) hatcheryraised mussels, *Mytilus galloprovincialis*, (2) hatchery-raised oysters, *Crassostrea gigas*, (3) plankton, (4) mussels and oysters (mussel/oyster) and (5) mussels and plankton (mussel/plankton). FISH successfully labeled only *M. galloprovincialis* larvae in both *M. galloprovincialis/Crassostrea gigas* and *M. galloprovincialis*/plankton samples. Images of samples hybridized with Alexa 488-labeled probe and corresponding control samples are shown in Figure 2. After FISH, all samples

underwent cell sorting using a COPAS Plus cell sorter (Union Biometrica, Holliston, MA). As expected, plankton samples had a wide range of particle sizes and red, yellow and green autofluorescence (Fig. 3A). When the sample of mixed hatchery-raised mussel larvae and plankton sample was probed with the Alexa 488-labeled (green) probe, the autofluorescence of some of the plankton sample was as bright as the probed larvae (Fig. 3B).

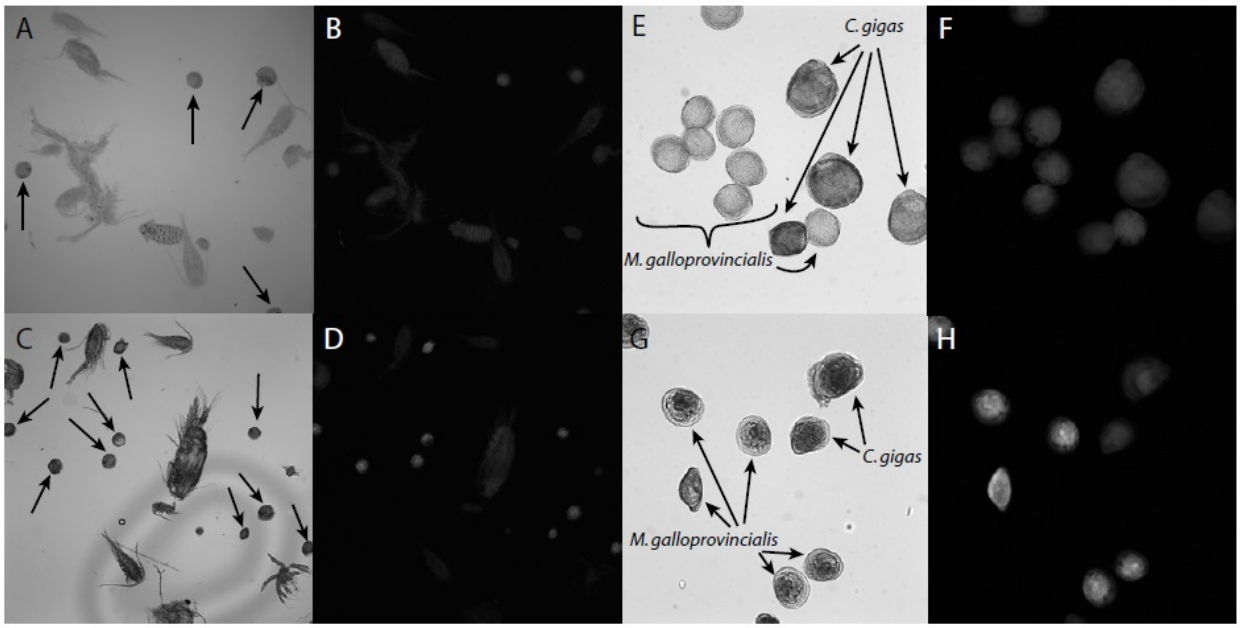

Figure 2. A-D: *Mytilus galloprovincialis* larvae & plankton sample, E-H: *Mytilus galloprovincialis* & *Crassostrea gigas* larvae. A) White light and B) fluorescein filter of control treatment showing autofluorescence; C) white light and D) fluorescent image under fluorescein filter after FISH with Alexa 488 Megt probe. E) White light and F) fluorescent image under fluorescein filter of control treatment; G) white light and H) fluorescent image after FISH with Alexa 488 Megt probe. Arrows in A & C denote the four and ten *M. galloprovincialis* larvae, respectively, in each image. A-D are at 100X magnification, and E-H are at 200X magnification.

Normally, such autofluorescence could compromise the effectiveness of labeling techniques by yielding false positives. However, natural autofluorescence of preserved samples showed a convenient pattern that can be used to resolve its confounding effects. The ratio of autofluorescence in any two of the three colors is remarkably constant among particles in a plankton sample and among plankton samples, regardless of size or type of particle (larvae, detritus, etc.). When fluorescence data for any two colors is plotted, the particles in an unprocessed plankton sample fall tightly on a line (Fig. 3C). Probing with a fluorescent dye increased the fluorescence of the target larvae only in the dye's part of the spectrum (e.g. green for Alexa 488, yellow for TET) while not correspondingly increasing target larvae fluorescence in other parts of the spectrum. This changed the ratio of fluorescence brightness between colors in target larvae, but not non-target taxa. Both Alexa 488 (a green dye) and TET (a yellow dye) allowed target mussel larvae to be easily differentiated from the plankton sample (Fig. 3D) and even oyster larvae of the same size and shape.

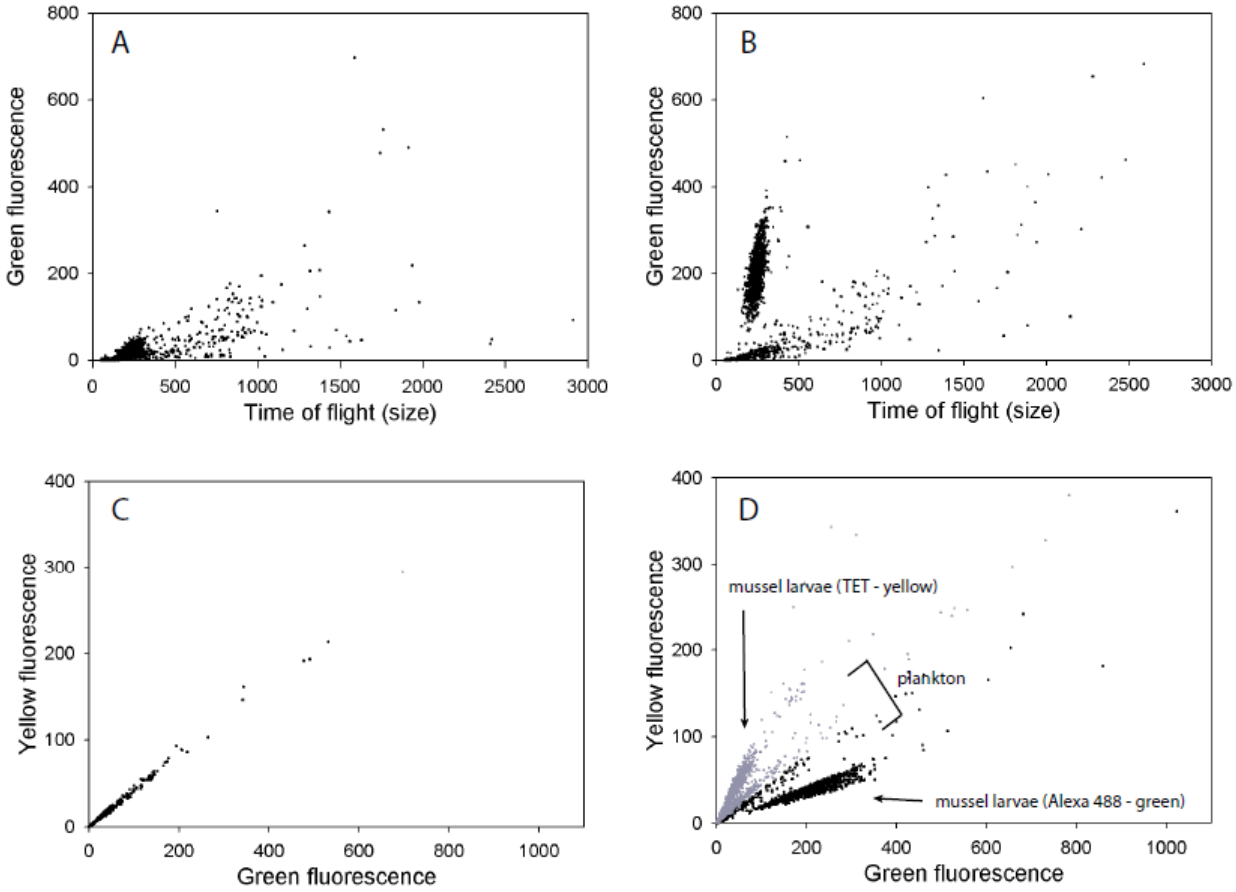

Figure 3. Cell sorter results. A & B: size vs. green fluorescence for A) control sample of mixed mussel larvae and plankton and B) mussel larvae and plankton after FISH with Alexa 488 mussel probe. C & D: Green vs. yellow fluorescence for C) control sample of mixed mussel larvae and plankton and D) mussel larvae and plankton after FISH with Alexa 488 mussel probe (black) and after FISH with TET mussel probe (gray). Note that in D, while plankton from both samples stay in the same position as the control sample in C, mussel larvae hybridized with the Alexa 488 probe have more green fluorescence and mussel larvae hybridized with TET have more yellow fluorescence than the plankton in the samples.

DNA extraction & PCR amplification of sorted mussel larvae

To test the accuracy of the FISH-CS method, we used the cell sorter to sort some of the mussel larvae from the mussel/oyster sample, 1 larva per well, into two 96-well plates. Larvae were extracted using a simple DNA extraction protocol, and then used directly in a PCR reaction with *Mytilus* adhesive protein gene primers (Inoue et al. 1995) to verify that the sorted larvae were *Mytilus galloprovincialis*. Of 112 larvae that FISH-CS identified as *Mytilus galloprovincialis* and sorted from a mixed oyster (*Crassostrea gigas*) and *M. galloprovincialis* sample into 96-well plates, all were confirmed to be *M. galloprovincialis* by PCR assay with the *Mytilus* adhesive protein primers of (Inoue et al. 1995).

Identification of Field-Collected Larvae

Over the 24hr sampling (05/24/10 at 1730 – 05/25/10 at 1530) inside the mouth of San Diego Bay, we collected a total of 200 mytilid larvae. Seventy eight percent of these larvae were identified as *M. galloprovincialis* (n=156). These results were expected because the dominant mussel species in rocky habitats in this area is *M. galloprovicalis* and their main spawning season (Sprin) coincides with the time of our study. Only 10% of the larvae were identified as *M. senhousia*. We were expecting to obtain higher numbers of *M. senhousia* because the adults are known to form high density mats in the sediments throughout the bay. However, the number of adults in San Diego Bay, as well the adjacent Mission Bay, have fluctuated dramatically in the past and our own collections between 2008 and 2009 suggest the numbers are considerable less than in previous years. Finally, only 12% of the larvae were identified as *M. californianus*. This species occurs mainly in the open coast are their larvae aren't expected to occur in high numbers within the bay. In addition, their main spawning season is thought to be later in the Fall.
The molecular verification process (PCRs and RFPL) for the larval IDs is accurate, but not as efficient as we had predicted. Therefore, only a subsample of the larvae in our samples was verified using molecular methods. Thus far, all the larvae that were identified as *M. galloprovincialis* by the cell sorter have been confirmed to be *M galloprovincialis*. Completion of the verification process is underway.

Larval Abundance and Vertical Distribution

Day/Night Cycle: Our results suggest that there was no difference in larval abundance between the day and night samples. While 46% of total mytilid larvae were collected in the day, 54% were collected during night hours. Differences in abundance are more evident when each species is considered individually. Most *M. musculista* (70%) and *M. californianus* (62%) were collected during the day. In contrast, most M. galloprovincialis (60%) were collected at night.

Temperature and Salinity: Mean temperature throughout the water column was 16.5±1.14ºC and ranged from 14.8ºC to 18.3ºC. Surface temperatures were 17.5±0.5ºC and bottom temperatures 15.5±0.6ºC. We were unable to discern any abundance or vertical distribution patterns based on temperature fluctuations. Salinity measurements were very consistent at 33.5±0.16.

Surface vs. Bottom: Overall, 62% of the larvae were collected at 1 meter above bottom. While 67% of all *M. californuanus* and *M. gallporvincialis* were collected at the bottom, most of *M. senhousia* (80%) were collected at the surface. Unfortunately, we do not have larval size data (see *Larval Size* section below) and therefore cannot speculate as to the changes in vertical distribution that may occur at different larval ages/sizes.

Tides: The frequency distributions of larvae of all three species throughout the 24 hr sample period were examined with respect to predicted tides at the MARFAC facility (Tides and Currents Software). At the surface, most larvae were present during the times of high tide (2 high tides) or immediately following (Figure 4a), at the time of falling tide (ebb tides). Larvae that maintain their position at the surface during outgoing tides would be transported towards the mouth of the bay and out into coastal waters. At the bottom, the only spike in larvae was observed was observed during the early morning, falling tide (Figure 4b).

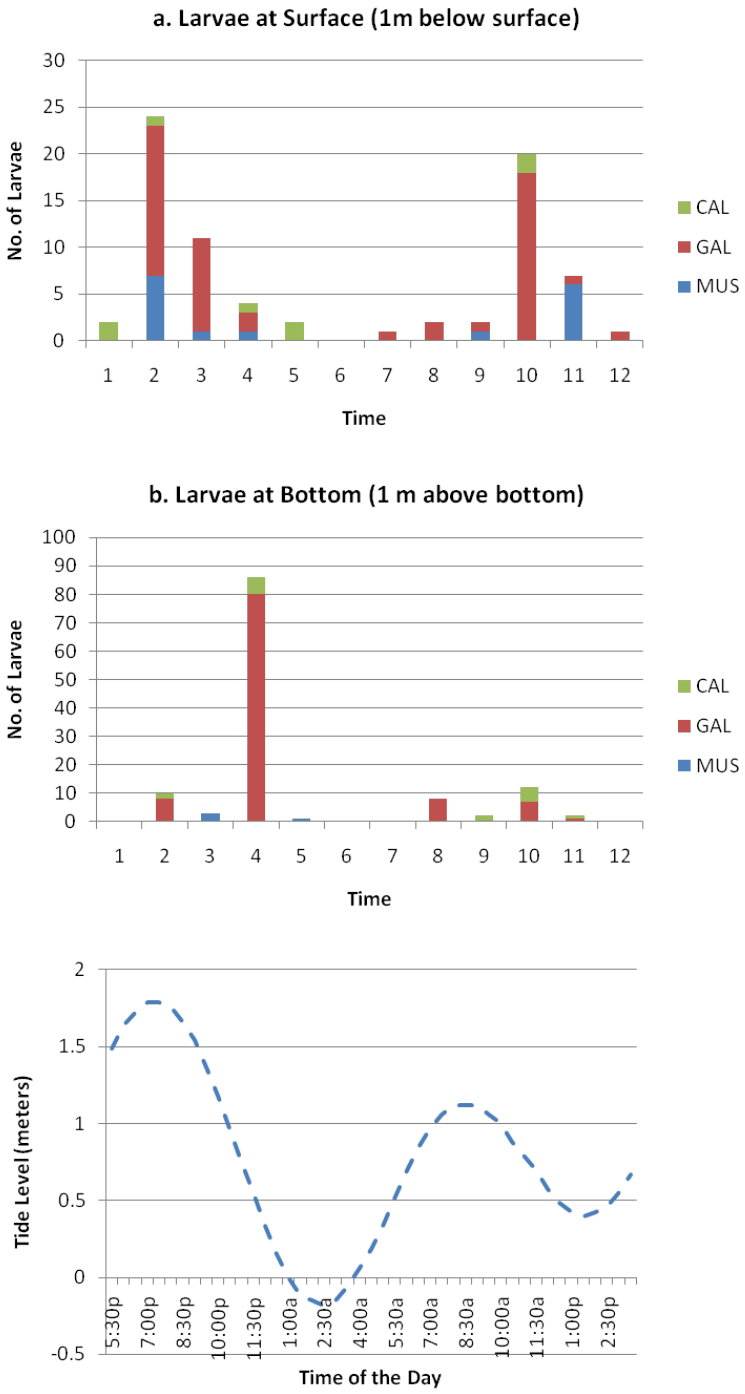

Figure 4. Frequency distribution of larval abundance over a 24 hr cycle in San Diego Bay. Frequency bars are divided into the number of larvae collected for each of three mytilid species. (CAL in green, GAL in red, MUS in blue). (a) Larvae collected 1 m below the surface, (b) and 1 m above bottom. Bottom panel represents predicted tidal level (2 m range).

Larval Size

One of our original goals was to determine if vertical distribution changed with ontogeny among the targeted species. We predicted that to favor dispersal smaller (younger) larvae would be found near the surface, while larger (older) larvae seeking settlement habitats would be found near the bottom. The large-particle cell sorter measures particle size. However, when the particles being sorted are in the order of hundreds to thousands, as was in this case, even a very small false positive rate results in tens to hundreds of particles being sorted that are not the bivalves of interest. After the samples goes through the cell sorter, the particles are sorted into a small dish, at which point there is no way of pairing a particle (which includes non-bivalves) with its corresponding size.

To obtain an idea of the size range, a subsample of the processed mussel larvae were manually measured and averaged 226.6±57.7 □m ($\overline{X}$ ± SD). Mussel larvae identified as EGT (*M. galloprovincialis*) or CAL (*M. californianus*) averaged 224±49.6 □m (n=10) and 212.5±57.2 □m (n=10), respectively. The largest mussel larvae measured were both identified as MUSC (n=2, 375 □m and 312.5 □m). While we were not able to test the hypothesis that vertical distribution changes with ontogeny, our results indicate that larvae found at the surface and near the bottom are similar in size (239.6±61.4 □m and 222.2±57.6 □m, respectively).

Conclusions

Our results thus far suggest that:

- New image and particle analysis techniques employed for this project can be used to successfully isolate and distinguish among the three mytilid species targeted in field-collected plankton samples. A more thorough description of the accuracy of this technique is included in Henzler et al 2010. The FISHsort Lab at UCSB is currently collaborating with several groups that are seeking to make plankton identification more effective and efficient. Our work has contributed to improving and validating this technique.
- Larvae of the three mytilid species targeted in this study do not appear to display different vertical distribution patterns that could result in different dispersal patterns (i.e., net horizontal movement). This suggests that there are other mechanisms that result in the adult distributions that we see in southern California among *M. californianus* (coastal areas), *M. galloprovincialis* (front bay), and *M. senhousia* (back-bay). However, further evidence in the form of (1) additional sampling replicates and (2) higher sample sizes are needed to strengthen our conclusions.

Improvements to the Protocol

Sub sampling. To improve the efficiency of larval identification process using a particle cell sorter, we suggest future users consider sub sampling. There was a wide variation in the biomass contained within our samples, which translated into a wide variation in sorting time. It is important to note that this factor did not affect accuracy, but it is important to consider because

of the additional time required to sort denser samples. Most of our samples contained tens of thousands of particles. However, some were closer to one million particles. While the smaller samples were processed in an hour, the largest samples required three to four hours to complete analysis, significantly hindering our ability to complete the analyses in sooner.

Literature Cited

Henzler CM, Hoaglund EA, and Gaines SD (2010) FISH-CS - a rapid method for counting and sorting species of marine zooplankton. Mar Ecol Prog Ser 410:1-11

Inoue K, Waite JH, Matsuoka M, Odo S, Harayama S (1995) Interspecific variations in adhesive protein sequences of Mytilus edulis, M-galloprovincialis, and M-trossulus. Biological Bulletin 189:370-375.

Pradillon F, Schmidt A, Peplies J, Dubilier N (2007) Species identification of marine invertebrate early stages by whole-larvae in situ hybridisation of 18S ribosomal RNA. Marine Ecology-Progress Series 333:103-116.