Final Report for Graduate Fellowship funded by the University of California Marine Council Coastal Environmental Quality Initiative (CEQI)

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CEQI proposal objective:

The objective of this CEQI proposal was to develop a novel technique to sort marine larvae more accurately and rapidly than with traditional methods. The proposal aim was to develop this method for a number species with ecological or economical importance, and to monitor larval spillover from marine reserves. During the funding period, I worked with a post-doc to develop the larval sorting method and to apply it to two mussel groups (manuscript submitted to *Marine Ecology Progress Series*, November 2009), and we are continuing to develop the method for lobster and urchin species (see Table 1). I have since changed my research objective that utilizes this method from monitoring spillover from marine reserves to assessing whether limitation of larval dispersal is responsible for establishing species range edges in order to predict species abilities to migrate to track changes in climate. My newly developed research topic will allow me to assess, for targeted species, the likelihood that species will adjust to climate change or be at risk of extinction due to contracting habitat space. The technique developed with the support of CEQI funding can still be used by state agencies, such as the California Department of Fish and Game, to monitor the movement of marine larvae for any purpose (e.g.s, monitoring spillover from marine reserves, identifying "hot-spots" of larval recruitment, assessing the variability of larval movement or relative abundance of species' larvae). Thus, the results of this work still fulfill the 2008 CEQI funding call for research in area **(B)** ***Fisheries and marine ecosystems,*** Section **(4)** ***new tools and techniques to measure dispersal patterns and assess fisheries stocks.*** And, as monitoring marine larvae can provide insight to how marine larvae respond to changing environmental conditions, it has important implications for how marine populations will respond to global climate change, and is relevant to priority research area **(D)** ***Climate change impacts***. This proposal was funded through CEQI with the support of funds from the **California Ocean Protection Council** (OPC). This proposal supports the OPC goal to **monitor marine systems** (Section B, Objective 2, Ocean Protection Council 2006) and could be applied to the OPC goal to **monitor marine reserves** (Section E, Objective 1, Ocean Protection Council 2006). The ability to monitor marine larvae will support the protection and restoration of California's ocean resources in the face of increasing fishing pressures and global climate change.

Table 1. Species targeted in this study

Common name	Target species	Significance	Probe status	Existing data
Bay Mussel	*Mytilus trossolus/ galloprovincialis*	Fishery[1] & Fishery[1]/invasive[2]	Complete	R A L
California Mussel	*Mytilus californianus*	Ecological dominant[3]	Redesign	R A L
Horse Mussel	*Musculista senhousia*	Morphologically similar to target species	Complete	
Calif Spiny Lobster	*Panulirus interruptus*	Fishery[1]	Testing	A
Red and Purple Urchin	*Strongylocentrotus* spp	Fishery[1] & Ecological dominant	Designing	R L

R = Recruitment, A = Adult abundance, L = Lab experiments; [1]California Department of Fish and Game, fisheries landings 2006; [2]McDonald & Koehn (1988); [3]Harrold & Reed (1985)

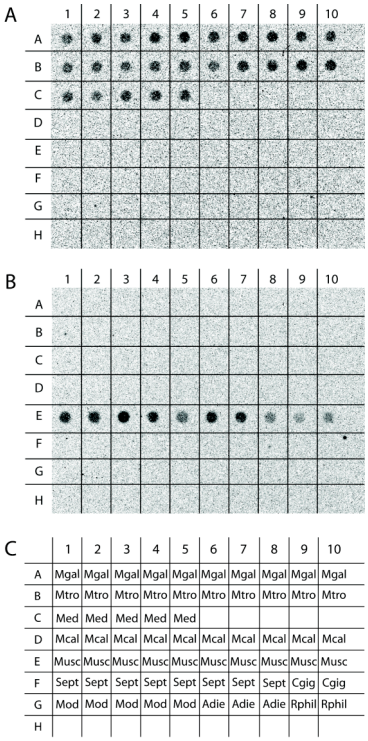

Figure 1 Dot blot probe specificity tests. A) Dot blot with *M. edulis/ galloprovincialis/ trossulus* probe. B) Dot blot with *M. senhousia* probe. C) Layout of dot blots: Mgal= *Mytilus galloprovincialis*, Mtro=*Mytilus trossulus*, Med=*Mytilus edulis*, Mcal=*Mytilus californianus*, Musc=*Musculista senhousia*, Sept=*Septifer bifurcatus*, Rphil=*Ruditapes philippanarum*, Mod=*Modiolus sacculifer*, Adie=*A. diegensis*, Cgig=*C. gigas*.

Results of work completed during the CEQI funding period:

We successfully developed the proposed technique to accurately identify and rapidly count marine larvae. Larvae are identified by fluorescently-labeled, species-specific molecular probes, which are bound to target larvae within a whole plankton sample using *in situ* hybridization (FISH). The labeled larvae are then counted from the entire plankton sample by a cell sorter (CS), which classifies each larva by fluorescence and size. We have rigorously tested our ability to sort two groups of mussels (*Musculista senhousia* and *Mytilus galloprovincialis/edulis/trossulus*), and are developing probes for additional species. Briefly, developing this method required that we (1) design molecular probes that were specific to target species or groups, (2) test probe specificity, (3) modify published *in situ* hybridization protocols to work on our samples of cultured marine larvae, and (4) test our ability to sort probed larvae from a plankton sample.

Probe design. We designed molecular probes for two bivalve species/groups: *Musculista senhousia* and *Mytilus galloprovincialis/edulis/trossulus.* Probes were designed targeting the 18S ribosomal RNA small subunit. We aligned 18S sequences from our own sequencing efforts or from sequences published in Genbank for six *Mytilus californianus*, one *M. coruscus*, five *M. edulis*, two *M. galloprovincialis*, and four *M. trossulus*. We designed a probe targeting the *M. edulis*/*galloprovincialis*/*trossulus* species complex (EGT probe), by eye from these aligned sequences, by

identifying an 18 bp region with a 3-4 bp difference between the target and non-target sequences. The specificity of these probes was tested against a wider selection of molluscs using arb (v. 07.12.07org). We used the same criteria to design a probe targeting *Musculista senhousia* (MUSC probe) the Silva comprehensive RNA ribosomal database (http://www.arb-silva.de/) search tool to download 18S mollusc sequences that had sequence, alignment and pintail qualities >75%. and searching this database using arb's Probe Design function.

Using the same methodology, we have developed or are developing probes for additional species, including the California Spiny Lobster, *Panulirus interruptus*, Red (*Strongylocentrotus franciscanus*) and Purple (*S. purpuratus*) urchins (see Table 1).

Probe specificity. The specificity of the EGT and MUSC probes was tested using dot blot hybridization. Probes were tested against ten different species (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*) and showed that the EGT and MUSC probes were specific to their target taxa (see Figure 1).

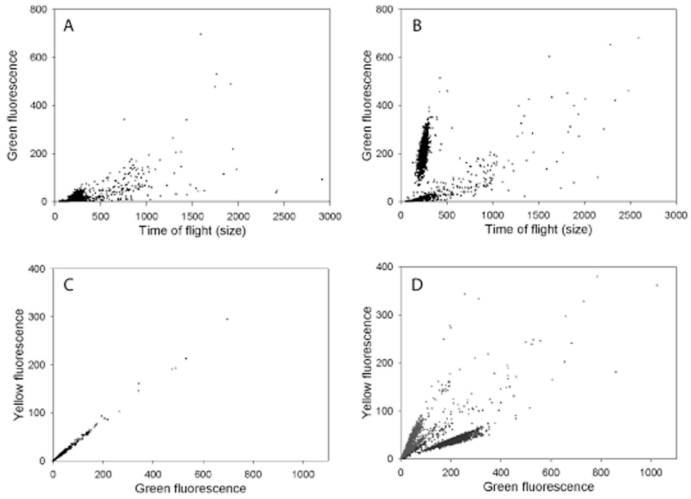

Figure 2. Cell sorter results. A & B: size vs. green fluorescence for A) control sample of mixed mussel larvae and plankton and B) mussel larvae and plankton after FISH with Alexa 488 mussel probe. C & D: Green vs. yellow fluorescence for C) control sample of mixed mussel larvae and plankton and D) mussel larvae and plankton after FISH with Alexa 488 mussel probe (black) and after FISH with TET mussel probe (gray). Note that 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.

Fluorescence in situ *hybridization.* Fluorescence *in situ* hybridization with the EGT probe was performed on five sets of samples with corresponding controls: (1) hatchery-raised mussels, *Mytilus galloprovincialis*, (2) hatchery-raised oysters, *Crassostrea gigas*, (3) plankton, (4) mussels and oysters (mussel/oyster) and (5) mussels and plankton (mussel/plankton). Probes were ordered from MWG Operon (Huntsville, Alabama) with Alexa 488 (green) or TET (yellow) fluorescent dyes attached to the 5' end. Samples were added to 2mL microcentrifuge tubes, centrifuged, and preservative solution was removed. Samples were permeabilized following Pradillon et al. (2007). Samples were probed using a FISH protocol adapted from Goffredi et al. (2006) with changes made to improve signal and reduce non-specific binding. Samples were stored in the final wash buffer in the dark until sorting. The EGT probe hybridized to the target

species *M. galloprovincialis*, but did not hybridize non-specifically to the plankton sample or to *C. gigas*,.

Sorting probed larvae from plankton and other samples. Larvae were sorted using a COPAS Plus cell sorter (Union Biometrica, Holliston, MA) with a 1x1mm square flow cell and 488nm laser. The cell sorter records time of flight past the laser (a measure of size), and fluorescence in the red (575-595 nm), yellow (532.5-557.5 nm) and green (498.5-521.5 nm) parts of the spectrum for each particle. All FISH samples described above underwent cell sorting. For the mussel/oyster sample, some of the mussel larvae were sorted and 1 larva was placed into each well of 96-well plates. The number of larvae per well was verified using a dissecting microscope. Larval DNA was extracted using Gloor et al.'s (1993) simple DNA extraction protocol and three μL were then used directly in a PCR reaction with *Mytilus*-specific, adhesive protein gene primers (Inoue et al. 1995) to verify that the sorted larvae were *M. galloprovincialis.*

As expected, plankton samples had a wide range of particle sizes and red, yellow and green autofluorescence (Fig. 1A). Normally, such autofluorescence could compromise the effectiveness of labeling techniques by yielding false positives. However, natural autofluorescence 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. When fluorescence data for any two colors is plotted, the particles in an unprocessed plankton sample fall tightly on a line (Fig. 1C). Probing with a fluorescent dye increased the fluorescence of the target larvae only in the dye's part of the spectrum (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 or oyster larvae (Fig. 1D). Of 112 larvae that FISH-CS identified as *M. galloprovincialis* and sorted from a mixed oyster (*C. gigas*) and *M. galloprovincialis* sample into 96-well plates, all were confirmed to be *M. galloprovincialis* by PCR assay with the *Mytilus*-specific ME15/16 adhesive protein primers of (Inoue et al. 1995).

Generality of method. We expect this method to work well for most marine invertebrate species. Once probes for additional species are developed and adequately tested, FISH-CS can be carried out on any number of species.

Relevance to state management objectives:

The research supported by this funding represents a significant methodological contribution that can aid both academic and state agency research in the abundance and movement of marine larvae. I will use this technique to understand the risk of extinction due to climate change faced my a group of marine animals, which is relevant to the California Department of Fish and Game's (CA DFG) commitment to manage harvested species and to protect biodiversity in general in the context of a changing climate, either by adjusting harvest levels or by protecting habitat of species threatened by climate change (Department of Fish and Game, 2008b). State agencies or academic research

scientists may apply the technique developed by this project to monitor the movement of marine larvae to obtain a better understanding of population dynamics, a stated goal of the CA DFG (2005), and a general goal of the OPC to monitor marine systems (Section B, Objective 2, Ocean Protection Council 2006). This method can also be used to monitor the movement of larvae out of marine reserves ("larval spillover"), fulfilling the goals of the OPC and the Marine Live Protection Act (MLPA) to monitor the efficacy of marine reserves and to design reserves and to create future reserves and networks based on the best science available (Section E, Objective 1, Ocean Protection Council 2006; Section 6.0, Department of Fish and Game, 2008a). In summary, both my dissertation research and the potential applications for the developed methodology support state ocean management goals.

References

Department of Fish and Game, 2005. Fish and Game Strategic Plan.

Department of Fish and Game, January 2008. California Marine Life Protection Act: Master Plan for Marine Protected Areas.

Ocean Protection Council, 2006. A vision for our ocean and Coast: 5-year Strategic Plan.
Department of Fish and Game, June 2008. White paper: Climate Change-related Research Considerations.

Harrold, C & DC Reed, 1985. Food availability, sea-urchin grazing and kelp forest community structure, Ecology 66: 1160

McDonald JH & RK Koehn, 1988. The mussels *Mytilus galloprovincialis* and *Mytilus trossulus* on the Pacific coast of North America, Marine Biology 99: 111-118.