Where should I settle?

An investigation into the larval settlement patterns of *Mesocentrotus franciscanus*

Leland Wood

Research in Marine Biology: Metamorphosis in the Ocean and Across Kingdoms (FHL 470)

Spring 2019

Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250

Contact Information:

Leland Wood

ljwood@uw.edu

Keywords: *Mesocentrotus franciscanus,* Settlement, Cues, Larvae, Coralline algae, Biofilm

Abstract

The red sea urchin, *Mesocentrotus franciscanus,* is an ecologically and economically important species. Although there has been extensive work on the life of the adults, little work has been done on the larval stage of its life history, namely the settlement cues of competent larvae. This study tested 6 treatments, with 24 hour exposure, to test which factor induced the most efficient larval settlement. At the conclusion of this study, *Calliarthron tuberculosum,* showed significantly higher settlement results for larvae, with an average of 12 ± 0.632 out of 15. Biofilms from purple urchins and urchin free environments also seemed to have an effect on settlement, but this was not as significant of a difference as the coralline algae. Red urchin biofilm, kelp detritus, and sea water did not have a significant effect on the settlement of the larvae.

Introduction

Red sea urchins are ecologically important due to their grazing capabilities and subsequent impact on the food web (Rodgers-Bennett 1995). In the absence of predators, urchins are known to make urchin barrens, which decimate kelp forests that would otherwise normally support a productive ecosystem (Filbee-Dexter and Scheibling 2014). In addition to their large ecological impact, sea urchins are also farmed commercially for consumption (Solan 1986). With ecological and economical importance, it is critical to understand the drivers behind the shift between urchins' planktonic, larval stage and their benthic, adult stage. This metamorphic shift has been well studied and described as a vital turning point in the life history of many organisms (Hodin et al. 2019).

Although urchins are found in virtually every benthic oceanic habitat, a given urchin species occupies very specific sub-habitats. How is it their larvae, which spend the entire phase of their life cycle floating in the water and riding currents, decided where and when to settle? Environmental cues are vital to the larvae, as they are signals on when to settle and continue in their life cycle.

Species-specific settlement cues are seen in organisms across multiple phyla. Past studies show, for example, unique cues for corals (Morse et al. 1988), chitons (Barnes and Gonor 1973), limpets (Steneck 1982), tubeworms (de Silva 1962), sea stars (Henderson and Lucas 1971), and urchins (Rowley 1989). Although each species has its own cue, there are several that appear frequently for more than one organism. Coralline algae, crustose or upright, has been shown to induce settlement in a variety of organisms (e.g., Daume et al. 1999; Morse et al. 1988; Johnson 1991). Biofilms, the collection of microbes on a surface, have been proven to be effective settlement cues for marine invertebrates (see Hadfield 2011 for review). Although there is a breadth of understanding on the settlement of larvae, there are still organisms to which the cues are unknown. In the case of the red urchin, *Mesocentrotus franciscanus,* little research has been done on specific cues. This study's aim is to narrow down possible cues for the settlement of *M. franciscanus*, by investigating the responses of urchin larvae to coralline algae, multiple biofilms, and kelp.

Methods

Culturing larvae

On 4/15/2019, two males and two females were spawned and eggs were fertilized and cultured according to standard methods (Strathmann 1987; Hodin et al. 2019). Adults spawned were collected by SCUBA divers on various dates throughout 2017-2018. The adults were kept in large aquaria in Friday Harbor Laboratories or in subtidal cages off the Friday Harbor Laboratories floating dock, and periodically fed drift kelp, mostly *Nereocystis luetkeana*. Larvae were placed in a 3-liter glass jar with 10.5 °C, ~5μm bag filtered sea water (referred to as CFSW) at an initial density of approximately 1 larva per 2 mL water. The jars with larvae were labelled and submerged into a sea table with flowing water, as to keep the jars at constant temperature. A plexiglass paddle was placed into each jar and attached to a stirring apparatus to keep water moving inside the jar (Strathmann 1987). Every two days, the water was changed and the larvae were fed *Rhodomonas sp.* and *Dunaliella sp.* at a concentration of 2500 and 3000 cells/mL respectively (Hodin 2019). After about 20 days, the larvae were split into two jars,

to reduce the density of larvae to an approximate concentration of 1 larva per 5 mL to allow for optimal growth.

Determining Competency

Before settlement treatments can start, the competency of the larvae must be tested. It has been shown that an excess of KCl in seawater can induce settlement to test competency (e.g, Carpizo-Ituarte et al. 2002; Hodin et al. 2019). Larvae will undergo settlement when they are competent or close to being so. The exposure of KCl induces settlement in competent larvae, thus a baseline for the competence of a stock can be determined. For *Mesocentrotus franciscanus*, competency was reached in just over 30 days. To check if the larvae were competent, 10 larvae were placed in an 8 mL of 100 mM KCl. After 1-hour exposure, the larvae were counted and then transferred to clean sea water before being released.Collection and preparation of settlement substrates

In total, 5 exposure treatments and 1 control were tested. Plastic well plates were used in which the tissue culture treated surface had been thoroughly washed off and thus removed . 36 hours prior to the settlement experiments, three well plates were put into different enclosures to begin building a biofilm.

One plate was placed in the touch tank in the basement of Fernald Labs. This tank was home to several adult *M. franciscanus* and this plate became the “Red Urchin Biofilm.” This tank also held many other organisms including cucumbers, anemones, worms, and gunnels. A second plate was placed into a tank that held several adult *Strongylocentrotus purpuratus* as well as several adult *Strongylocentrotus droebachiensis* as well as other invertebrates, including mussels, anemones, sea stars, sand dollars, scallops, and snails. This was called the "Purple Urchin" biofilm treatment. A third plate was placed into a tank that held several crabs and snails, but no urchins. This was to grow an “Urchin absent” biofilm. However, at some unknown time, four *S. purpuratus* were placed in this tank, despite its designation as “urchin-free.” It is speculated that said urchins were placed in the last 4-8 hours of the biofilm growth so the impact on the biofilm is unknown.

Calliarthron tuberculosum was collected from Cattle Point, San Juan Island (48.450229, -122.962236) during low tide on April 23. This alga was broken into 2.5 cm segments one segment was placed into each well. A section of *Nereocystis luetkeana* stipe and fronds was broken up in a blender to create kelp detritus. The detritus was filtered at 75 μm, left to break down for 6 days and finally frozen for storage (Hodin, Jason. Personal communication. 2019*)*. Kelp detritus was placed in wells at a concentration of 6000 cells/mL. Finally, the control plate of filtered sea water was prepared.

Settlement trials

The larval culture jars were reverse filtered into volume of 500 mL to make selection easier. Using a dissecting scope, larvae were individually selected into the appropriate well. For each of the 6 treatments (including the control), 6 replicates of 15 larvae each were set up. Each well plate had one exposure type as explained above and contained 10 mL of CFSW in addition to the potential settlement inducers in the well, and were prepared before the larval selection began.

Larvae were selected only if they were not already settled, not deformed, and did not appear malnourished. Start time order for replicates of each treatment were randomized. This was done to reduce bias and increase randomness of when the trials began. After the larvae were placed into the well plate, a second count was done to ensure 15 larvae were in each well. During the process of selecting larvae, intervals of about 3 minutes were utilized, to allow adequate time for counting. During counting, if the count did not use the full time of that interval, a short break was taken. When scoring each larva after the exposure, it takes longer to score than it does to select larvae out, therefore planning the trials in intervals is necessary so that 24 hours is achieved with all larvae and there is not a time difference for trials. For example, if the selection took 1 minute each, but the counts afterwards took 3 minutes, the last count would have 1.2 hours longer exposure than the first. After selection and the exposure began, the well

plates with larvae were kept in a small chiller maintained at 10 °C, with limited ambient light exposure over the duration of the exposure.

Observing Settlement

After exposures of 3 hours and 24 hours, larvae were counted into 4 categories according to specific characteristics: settled, attached, swimming or on bottom. Settled larvae are those who have begun metamorphosis. They have visible tube feet and their outer skin has started retracting down the skeletal rods in the larval arm (Heyland and Hodin 2014). Settled larvae range from ones that have started to retract the skin from their spines, to those who have fully undergone metamorphosis to become young juveniles (Figure 1.a and Figure 1.b, respectively). Attached larvae are those that have an advanced rudiment and their tube feet attached, but the skin over their spines has not retracted yet (Figure 1.c). The attached larvae may or may not still have their cilia beating, but are stuck to the bottom. Swimming larvae have not attached their tube feet, nor in any way have they started the process of metamorphosis, and are actively swimming throughout the well (Figure 1.d). Larvae on bottom have the same appearance as swimming larvae (Figure 1.d), the only difference being not swimming throughout the water column, but they are on the bottom, alive, and not doing anything else that could be scored.

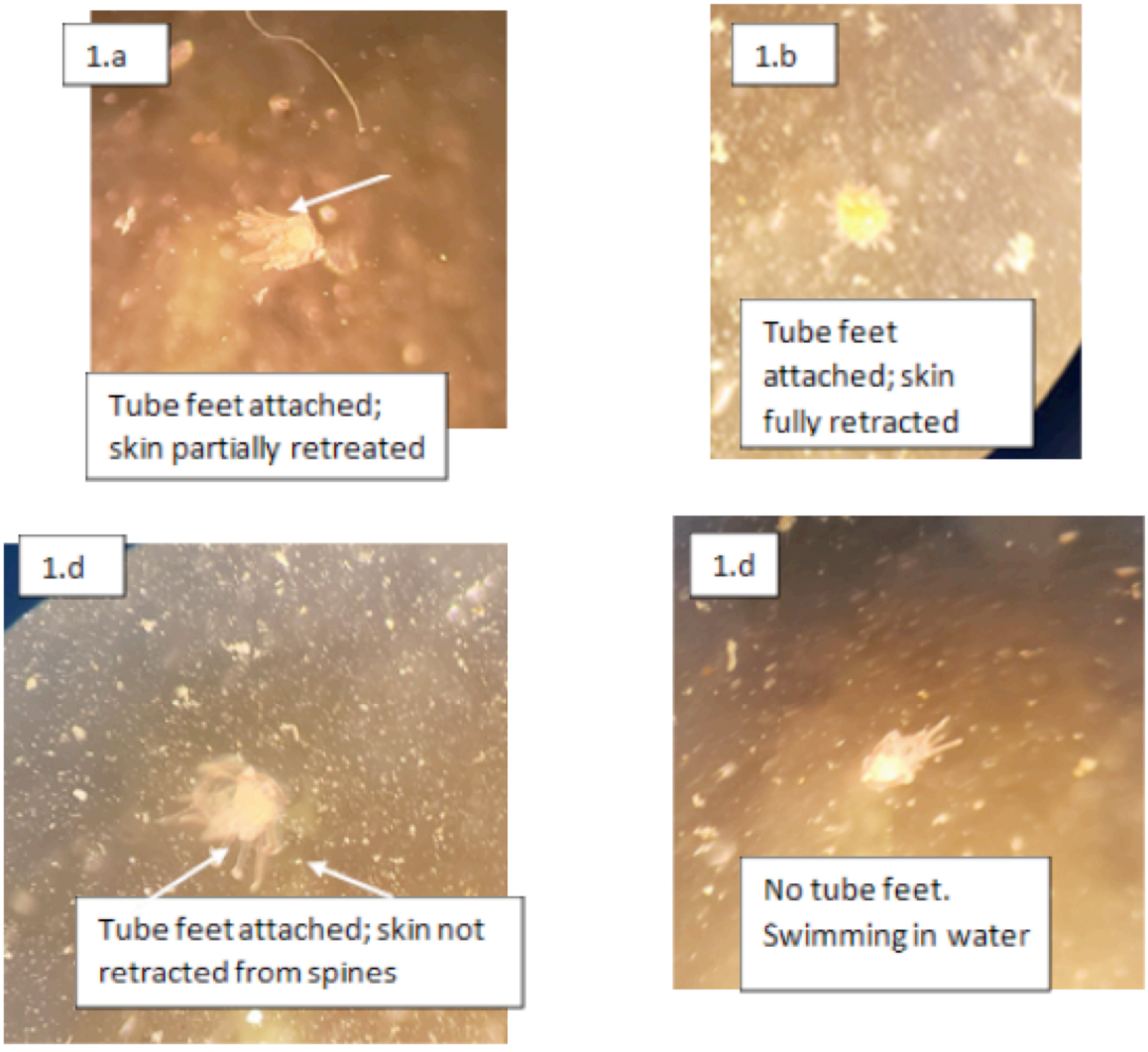

Figure 1: 1.a and 1.b show two settled larvae. 1.c shows attached larva. 1.d shows swimming or on bottom larva.

Statistical analysis was performed with R (v. 3.5.1) using an ANOVA test and Tukey's HSD test, from "agricolae" package.

Results

At the conclusion of the experiment, both the 3 hour and 24 hour exposures had significantly different results (ANOVA, P=4.65e-10 and P=2.31e-13 respectively). For this ANOVA test, the counts of settlement and attachment were combined and compared to the larvae that were still swimming or on bottom. This is because although the process of the tube feet invagination is a reversible step (Lebesgue et al. 2016), it is the beginning of the settlement process. With the study conducted, it is not possible to tell if the larvae would detach and become swimming larvae again. Since we cannot know the fate of the larvae, it was assumed they were on their way to fully settling, and they were combined with the settled group for statistical analysis. The post hoc Tukey HSD test showed the average settlement for the exposure with coralline algae at 3 hrs was significantly different than any of the other groups, with a mean of 5.33 ± 0.067 larvae out of 15 settled or attached. For this study, and from henceforth, all settlement values and means are out of 15 since each trial had 15 larvae exactly. The test at 3 hrs showed no significantly different averages in red biofilm, purple biofilm, urchin absent biofilm, kelp detritus, and sea water. After 24 hours of exposure there were differences between the groups. Coralline algae exposure was the most significantly different with an average of 12 ± 0.632 at 24 hours. The Tukey showed pairs of remaining treatments had no significant difference between the each other, but having significance from the rest (Figure 2). Figure 3 shows the count of larvae at each stage. Totals are the same since each treatment had 6 replicates with 15 larvae each, however the percentages in each stage is variable.

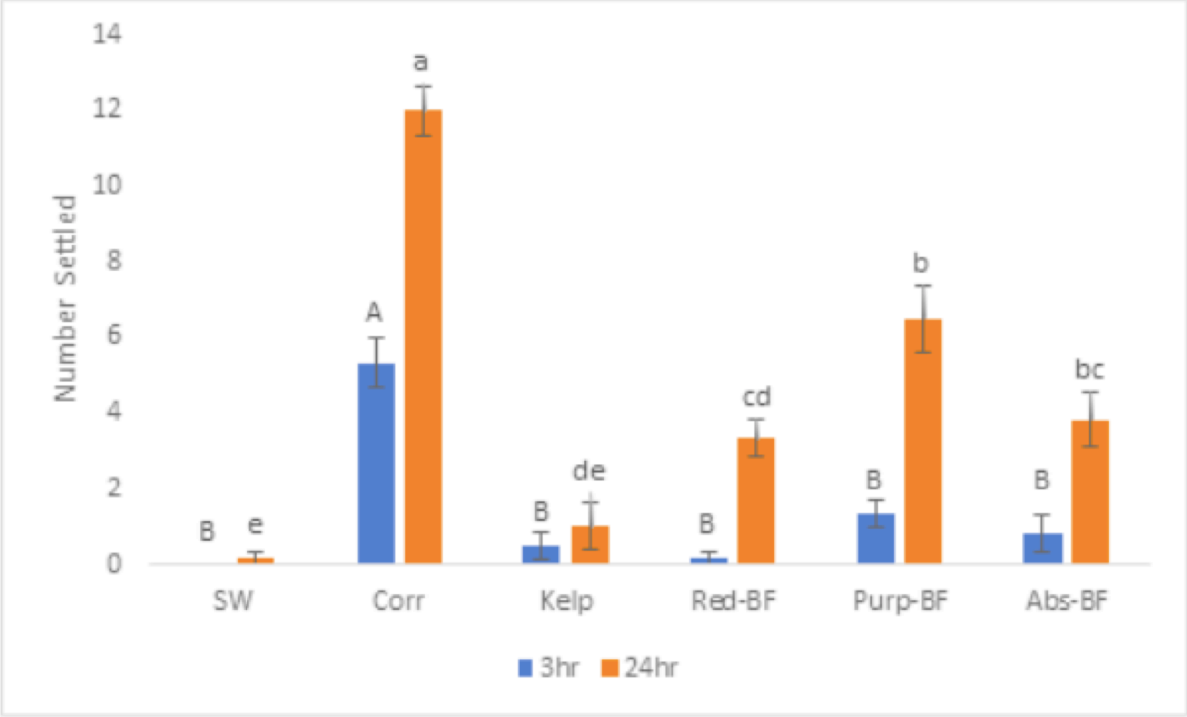

Figure 2: This graph shows the average settlement for the different treatments. Error bars are standard error. Letters above each bar show the grouping as a product of the Tukey test with uppercase referring for the 3 hour groups and lowercase referring to the 24 hour groups.

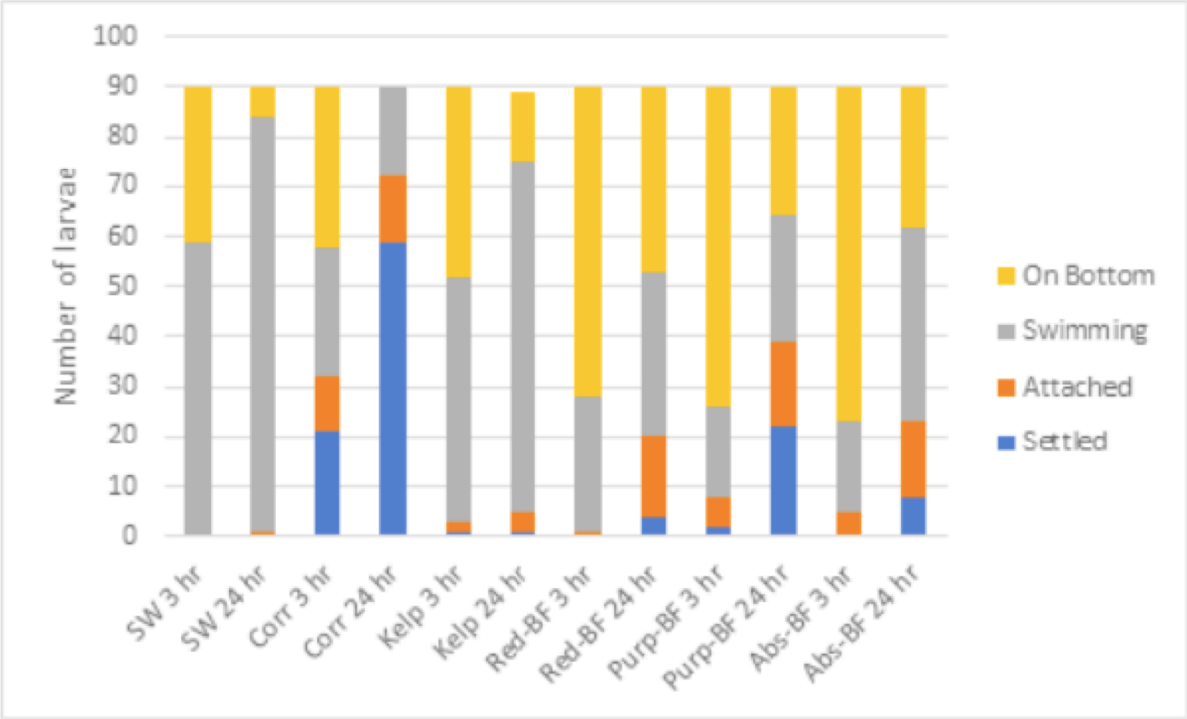

Figure 3: This graph shows the total number of larvae in each stage for each category

Discussion

These results show with confidence, *M. franciscanus* larvae preferentially settle on coralline algae among the various substrates tested. As shown above, the exposure of coralline algae was a strong environmental cue for the larvae to settle. This gives clues to possible co-evolution, as the algae and urchins are commonly abundant in the same areas (Rogers-Bennett and Bennett 1995). As the urchins graze the algae of the rocks, encrusting and upright algae have more space to inhabit (Rogers-Bennett and Bennett 1995), which possibly provides more space for the larvae to settle. This also raises concern with the adverse effects the coralline algae are facing with climate change (Kuffner et al. 2008). As the oceans become more acidic, the carbonate fixing algae are beginning to decrease in abundance, which has negative implications for not only red urchins, but any species that settle on the algae (Kuffner et al. 2008). Although the algae had a strong correlation to the settlement of *M. franciscanus,* their intertwined fates may be in peril with the encroaching climate change.

There seemed to be another influence of biofilms on the settlement. It is interesting that, although the biofilm did seem to have some influence on the settlement of the larvae, it was not the biofilm collected from the same species of urchins. The biofilm that was collected from adult *M. franciscanus* did prove to be an effective cue as the settlement was not significant when compared to the other two biofilms. The purple urchin and urchin absent biofilms had higher settlement averages, at 6.8 ± 0.88 and 3.833 ± 0.703 respectively. This raises the question that there may have been something other than the target species affecting the biofilm.

In the tank with the “Absent” and “Purple” urchins, there were more invertebrates, which may have contributed to the settlement rates observed. For example, in the urchin “Absent” biofilm tank, there was a number of small crabs. Using a different species of crab, a study done by Clemente (2013) showed crabs are a predator of the juvenile urchin. Future studies could

investigate the effects of presence of predators on the settlement of the larvae and the growth of the juveniles.

Another possible discrepancy is the age of the biofilms used in this study were fairly young, at 36 hours, compared to other studies, which used biofilms as old as 21 days (Wieczorek and Todd 1998). The maturity and outright variability in microbiomes can be inconsistent and are not always uniform across a sample (Wieczorek and Todd 1998). Although the results of biofilms inducing settlement is supported by these studies, Cameron and Schroeter (1980), show red and purple urchins actually do show settlement preferences with adults in the wild, which contradicts the results of this study. Therefore, it would be valuable to revisit the biofilm exposures by creating a biofilm with a single, isolated species and with more mature biofilm to further investigate influences of adults and that of other invertebrates.

Exposure of kelp detritus was not shown to be an effective inducer of settlement as its average settlement of 1 ± 0.632 was not significantly different from the control, according to the Tukey test (Figure 2). The average of the number swimming went up from 8.1 ± 0.341 to 11.16 ± 0.632 between the 3 hour and 24 hour exposure. This suggests the larvae did not find the cues sufficient for settlement and returned to the water column in order to find new cues. A recent study done shows how kelp detritus is an effective food for larvae and produces optimal growth to competency (Feehan et al. 2018). Although this kelp detritus, which is produced in the wild through thallus erosion, is a beneficial source of food, it is not shown to be an effective settlement cue in this study. A possible reason for this is that once the larvae are fertilized, they are able to feed on the detritus in the water column while they grow and they do not receive this as a cue to settle since they feed on it though their larval stages. When they reach competency while feeding on detritus, they receive a new cue to induce settlement. However, more research would be needed to prove this concept.

Acknowledgements

I especially thank Jason Hodin for the personal assistance with protocol development, paper revisions, and answering all my questions along the way. Your teachings have been eye opening and invaluable to the success of this paper. To Mo Turner for assistance with data analysis and presentation; this would not be possible without you. To Karly Kohen, with paper revisions, encouraging feedback, and helpful words. To Fleur Anteau, Morgan Thompson, Helen Kesting, and Katie Gonzalez, for late night and early morning motivation to write this paper. To Allison Jean Arnold, Katie Gonzalez, Katie Hearther, Fleur Anteau, Haleh Mawson, and the other members of the ZooBot program: your feedback and revisions on my paper were integral to the success of this paper and the quality of this paper would not be reached without you.

References

Barnes, J. R., and J. J. Gonor. "The larval settling response of the lined chiton Tonicella lineata." *Marine Biology* 20.3 (1973): 259-264.

Cameron, R. A., and S. C. Schroeter. "Sea urchin recruitment: effect of substrate selection on juvenile distribution." *Mar. Ecol. Prog. Ser* 2.3 (1980): 243-247.

Carpizo-Ituarte, E., A. Salas-Garza, and G. Pares-Sierra. "Induction of metamorphosis with KCl in three species of sea urchins and its implications in the production of juveniles." *Ciencias Marinas* 28.2 (2002): 157-166.

Clemente, S., et al. "Predators of juvenile sea urchins and the effect of habitat refuges." *Marine Biology* 160.3 (2013): 579-590.

Daume, Sabine, Sascha Brand-Gardner, and Wm J. Woelkerling. "Settlement of abalone larvae (Haliotis laevigata Donovan) in response to non-geniculate coralline red algae (Corallinales, Rhodophyta)." *Journal of Experimental Marine Biology and Ecology* 234.1 (1999): 125-143.

De Silva, P. H. D. H. "Experiments on choice of substrata by Spirorbis larvae (Serpulidae)." *Journal of Experimental Biology*39.3 (1962): 483-490.

Feehan, Colette J., et al. "Kelp detritus provides high-quality food for sea urchin larvae." *Limnology and Oceanography*63.S1 (2018): S299-S306.

Filbee-Dexter K, Scheibling RE (2014) Sea urchin barrens as alternative stable states of collapsed kelp ecosystems. Mar Ecol Prog Ser 495:1-25. https://doi.org/10.3354/meps10573

Hadfield, Michael G. "Biofilms and marine invertebrate larvae: what bacteria produce that larvae use to choose settlement sites." *Annual review of marine science* 3 (2011): 453-470.

Henderson, J. A., and J. S. Lucas. "Larval development and metamorphosis of Acanthaster planci (Asteroidea)." *Nature*232.5313 (1971): 655.

Heyland, Andreas, and Jason Hodin. "A detailed staging scheme for late larval development in Strongylocentrotus purpuratus focused on readily-visible juvenile structures within the rudiment." *BMC developmental biology* 14.1 (2014): 22.

Hodin, Jason, et al. "Culturing echinoderm larvae through metamorphosis." *Methods in cell biology* 150 (2019): 125-169.

Johnson, Craig R., et al. "Settlement of crown-of-thorns starfish: role of bacteria on surfaces of coralline algae and a hypothesis for deepwater recruitment." *Marine ecology Progress series* (1991): 143-162.

Kuffner, Ilsa B., et al. "Decreased abundance of crustose coralline algae due to ocean acidification." *Nature Geoscience*1.2 (2008): 114.

Lebesgue, Nicolas, et al. "Deciphering the molecular mechanisms underlying sea urchin reversible adhesion: A quantitative proteomics approach." *Journal of proteomics* 138 (2016): 61-71.

Morse, Daniel E., et al. "Control of larval metamorphosis and recruitment in sympatric agariciid corals." *Journal of Experimental Marine Biology and Ecology* 116.3 (1988): 193-217.

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Rogers-Bennett, L. , Bennett, W. A., Fastenau, H. C. and Dewees, C. M. (1995), Spatial Variation in Red Sea Urchin Reproduction and Morphology: Implications for Harvest Refugia. Ecological Applications, 5: 1171-1180. doi:10.2307/2269364

Rowley, R.J. Mar. Biol. (1989) 100: 485. https://doi.org/10.1007/BF00394825

Steneck, Robert S. "A limpet-coralline alga association: adaptations and defenses between a selective herbivore and its prey." *Ecology* 63.2 (1982): 507-522.

Strathmann MF. 1987. Reproduction and development of marine invertebrates of the northern Pacific coast. Seattle: University of Washington Press.