Settling Sand Dollars with Sand-Water

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Abstract

Chemical cues are commonly used to convey information to conspecifics and in marine invertebrates are often associated with larval settlement. This research set out to examine methodology surrounding adult-associated pheremonal settling cues observed in *Dendraster excentricus.* I hypothesized settlement of juvenile *D. excentricus* could be induced via exposure to water that had been saturated in sand from adult sand dollar beds then filtered of visible particulate matter. This was tested by exposing larvae to both filtered and unfiltered concentrations of water associated with sand dollar sand and comparing the settlement rates. Data were inconclusive as only 1.3% of larvae settled and no significant trends were observed. Research still has potentially broad implications in refining established methodology surrounding *D. excentricus* settlement inducers for use in laboratory based research.

Introduction

Chemical cues are commonly used to convey information to other organisms of the same, or different, species. This phenomenon is well documented in arthropods, and research indicates the chemical processes may be similar in marine invertebrates. (Burke, 1986). How specific pheromonal cues impact marines invertebrates does vary, however, in several genera they are implicated in the selective settlement of planktonic larvae that may stay in their larval forms for extended lengths of time until exposed to cues which induce settlement. (Hadfield & Paul, 2001).

Settlement in marine larvae can be associated with numerous environmental factors such as such as substrate roughness, light intensity or exposure to turbulence (Hodin et al. 2015). *Dendraster excentricus* is a well studied species whose planktonic larvae are known to exhibit gregarious settlement, meaning they preferentially settle in response to pheromonal cues from conspecific adults (Burke, 1984; Woodin, 1986). Several studies have shown that despite these

enticing environmental cues *Dendraster* larvae can spend several months in the planktonic stage (Burke, 1984). This leave individuals vulnerable to predation for an extended period of time; therefore it is important to understand exactly what cues impel *D. excentricus* larvae to settle.

Here we are interested in the effects of chemical cues on the rate of settlement in *D. excentricus*. Previous research conducted at Friday Harbor Labs (FHL) examining settlement in this species (Highsmith,1982, Hodin et al. 2018, unpublished data, Zoobots 2019) support the idea that settlement in *D. excentricus* can be induced through the use of sand containing an unknown pheromone produced by adult dollars. Some data suggest the chemical cue to be a 960-dalton peptide (Burke, 1984) capable of diffusing through materials sand particles are too large to diffuse through, while maintaining its ability to induce settlement (Highsmith, 1982). This research was intended to test the hypothesis that the settlement of juvenile *Dendraster* can be induced via exposure to water that has been saturated in sand from adult sand dollar beds then filtered of visible particulate matter.

Methods and Materials

For this study I used four adult *D. excentricus* that were collected at Crescent Beach (East Sound, Orcas Island, WA, USA) by hand at low tide in July 2018 and held in flow through aquaria located at the Friday Harbor Labs (FHL), San Juan Island, WA, USA. Spawning was induced in two male and two female by injecting 0.75mL of 0.55 M KCl into their mouth opening using 1mL syringes. Females were placed aboral side down on top of 150 mL beakers filled to the brim with filtered seawater. Eggs collected at the bottom. Males had sperm removed from their aboral surface via pipette, which were then refrigerated in 1.5mL Eppendorf tubes for preservation until fertilization. When ready to begin fertilization, sperm suspension was made by

adding one Pasteur pipette notch of sperm to 10mL of filtered sea water. Gametes were then fertilized in four beakers, each with 50% of the eggs from a single female, and adding 10 drops of sperm suspension from the two males in a M1xF1, M1xF2, M2xF1, M2xF2 pattern.

Fifteen minutes after fertilization sperm water was decanted off the fertilized eggs and replaced with filtered sea water. Eggs were then left alone to mature until they hatched. At that point hatched embryos were carefully decanted into 150mL beakers containing 20mL filtered sea water. Additional filtered seawater was added as needed to bring total volume of each beaker to 100mL. Embryo densities were then calculated by counting and averaging the number of embryos in 3-500µL samples per beaker. Volumes equating to 750 embryos per beaker were then removed and consolidated into a single 3L jar containing 2L of 19**°C** filtered sea water. Half the larvae were then split into an additional 3L jar, already containing 2L filtered sea water. Both jars were then filled to 3L, resulting in 2-3L jars containing approximately 1,500 genetically varied larvae each.

From this point, larvae were cared for under optimal conditions for maturation according to personal communications with J. Hodin and protocol established by Zoobots Research class, 2019, unpublished data. Larvae were kept in 19°C filtered sea water under low intensity natural light conditions, and were cultured on a low velocity shaker table that ensured constant gentle motion. From this stage forward, even when not specified, all sea water used was room temperature, 19**°C.** Every other day they were fed 2,500 cells/mL of cultured *Rhodymenia* sp. and 3,000 cells/mL of cultured *Dunaliella sp.*. Water was changed and jars were cleaned of debris before every feeding. The larvae remained in 2-3L jars until on day 4 they were divided into 4-3L jars, each containing approximately 750 larvae.

After developing for 11 days, larvae were tested for competency via hour long submersion in 5 mL of 40 M excess KCl millipore filtered seawater. Two days later, 300 larvae

were randomly selected for the settlement experiment, wherein they were divided into well plates at a concentration of 12 larvae per well. Each well was filled with 5mL of one of five seawater treatments. The treatments were: filtered seawater (a negative control), filtered seawater that had been exposed to sand dollar sand (SDS) from an adult bed (a positive control), and three concentrations of filtered seawater that had been exposed to SDS then filtered again with a 80μm mesh filter to remove sand particles.

Sand-water treatments were prepared by collecting 100mL of SDS from FHL flow thru tanks and dividing it equally into 4-50mL centrifuge tubes. The tubes were then filled with 25mL of filtered seawater and then shaken vigorously for 30 seconds. After 10 seconds of rest, water was decanted off the top into clean beakers. From this point, high concentration filtered sand-water was prepared by using a pipette to siphon the decanted water through an 80μm mesh filter, leaving large particle dregs behind. In the medium concentration filtered sand-water, the decanted water was mixed in a 1:1 ration with filtered seawater before being filtered the same way. Low concentration filtered sand-water was made by diluting the 1:1 ratio of sand-water to filtered seawater an additional 66%, before the filtered water was also filtered using the 80μm mesh. Unfiltered sand-water followed the same dilution procedure as low concentration filtered sand-water but was not filtered at the end.

Observations began once larvae were submerged in treatments. Wells were examined 15 minutes, 30 minutes, 2 hours, and 4 hours after initial exposure. The number of settled larvae at each time interval were counted and recorded.

Results

Competency Test

On day 11 larvae visually appeared to be in the “precompetent” stage (Hodin et al., 2015)

wherein larvae legs are relatively long and equal in length. Only 2% settled upon exposure to 40 mM excess KCl filtered seawater.

Settlement Treatments

No larvae settled within the first 30 minutes in any treatment. After two hours, one larvae had settled in single replicates of both the high and medium concentrations filtered SDS water. After four hours one additional larvae had settled in one replicate of medium concentration filtered SDS water, and one new larvae had settled in a replicate of unfiltered SDS water. Of the 300 total larvae tested, four had settled by the end of the experiment.

Treatments	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5
High Concentration Filtered SDS Water	0	0	1	0	0
Medium Concentration Filtered SDS Water	1	0	0	0	1
Low Concentration SDS Water	0	0	0	0	0
Non-Filtered SDS Water	0	0	0	1	0
Filtered Sea Water	0	0	0	0	0

Fig 1. Data table representing total number of settled larvae across replicates of all treatments. Each replicate had 12 total larvae.

Treatments	15 Min	30 Min	2 Hours	4 Hours
High Concentration Filtered SDS Water	0	0	1	1
Medium Concentration Filtered SDS Water	0	0	1	2
Low Concentration Filtered SDS Water	0	0	0	0
Non-Filtered SDS Water	0	0	0	1
Filtered Sea Water	0	0	0	0

Fig 2. Data table representing time at which larvae settled in treatment. Each column represents total number of larvae settles at indicated time point.

Discussion

In this study I set out to examine the impact of chemical cues on larval settlement under

controlled conditions. Unfortunately, my results were inconclusive and neither support nor refute my hypothesis that settlement can be induced with filtered SDS water. While there appears to be a very minute trend as larvae did in fact settle in the positive control and in the filtered SDS water treatments and none at all settled in the plain sea water, the numbers are simply too low to indicate any significant relationship. Before conducting the experiment, I had planned to graph the data comparing the average settlement numbers across all five treatments and to use an ANOVA test to measure their significance. As only 1.3% of all larvae settled, and the average across replicates were <1, both graphs and statistical analysis would have been pointless.

Evaluating my results and my processes, I find it likely that the low settlement rates in my experiment had little relation to the SDS water treatments themselves. They were more likely a result of the short length of time the larvae were given to mature, and the environmental conditions used to culture them. On day 11, when competency was tested using excess KCl millipore filtered seawater, only 2% of larvae were capable of settling. At this point testing was postponed for two days to allow larvae additional time to mature, however it is entirely possible that those two days were insufficient and the larvae were still only precompetent. This is not entirely unexpected. While Hodin et al. (2015) found that larvae fed regularly and raised in 20-22 degree water may mature as quickly as 12-14 days, according to Burke (1984), 4-6 weeks is the standard length of time lab cultured *D. excentricus* take to reach competency. While it is unclear exactly which factors lead to larvae maturing slower than Hodin et al. described in their research, it is possible my larvae required a higher concentration of food than I provided, and I suspect that at 19 degrees the water temperature was insufficient for maximum speed of development.

In addition to my data being inconclusive due to immature larvae, I did find flaws in my experimental design. The 80μm mesh I used for filtration was almost entirely ineffective at

filtering the SDS water. While large particles and unwanted organisms were blocked, the majority of sediment were fine enough to fit through the mesh, thus defeating the point of the filter. There was a slight qualitative difference in water clarity between low concentration filtered SDS water and unfiltered SDS water (both of which followed the same dilution procedure), but the high and mid concentrations of filtered SDS water had the lowest visibility of all treatments. However, this is simply a technical issue that could be easily resolved with the right materials. Had I the opportunity to repeat this experiment, I would likely give my larvae longer to mature and use a much finer mesh filter.

Despite my inconclusive results and flaws in my experimental design, there are still broad applications of my research. My underlying goal approaching this topic was inspired by the Zoobots, 2019, research class, and was focussed on improving current methodology used to study *D. excentricus* using pheromonal cues. In the protocol used by the Zoobots, sediment in the SDS water impeded visibility, contained nematodes, and provided layers of opaque sand in which the larvae were able to burrow out of sight. By designing a more efficient protocol for using the pheromonal cues in SDS, my research could be used in future research ventures to reduce the impact of human error in studying the chemical factors impacting the transition from planktonic to benthic stages *D. excentricus* larvae.

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Citations

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