Developmental Effects of Predator Cues on *Dendraster excentricus* Larvae: The Effects of *Pugettia producta* Effluent and Crustacean Dominant Plankton Effluent

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Abstract

Previous findings supporting increased cloning in *Dendraster excentricus* (*D. excentricus*) larvae as a response to predator cues, in particular fish slime. Such findings report a "visual predator hypothesis", suggesting that the larvae clone in order to become smaller and thereby avoid visual predators and possibly even non-visual predators. The experiment reported here builds upon earlier findings by studying the exposure of *D. excentricus* larvae to a kelp crab effluent (using *Pugettia producta*) and a crustacean dominant plankton effluent. Individual larvae were exposed to one of three treatments: the kelp crab effluent, plankton effluent, or filtered sea water, for approximately 66 hours. After this period, number of clones, number of larval arms, and the rudiment stage of each larvae was determined. Linear modeling showed significant results when comparing the kelp crab treatment to the control for cloning ($p=0.024$) and rudiment stage ($p= 0.032$); they also displayed significant differences for larval arm stage when comparing both the kelp crab effluent treatment ($p= <0.001$) and plankton effluent treatment ($p= <0.001$) to the control. These findings may support the visual predator theory, depending on whether *D. excentricus* larvae are able to differentiate predator cues, and, if so, to what specificity.

Introduction

Dendraster excentricus (*D. excentricus*) is an organism of ecological importance from the Hecate Straits in central British Columbia to Baja California Sur in Mexico. The species are used as a substrate for certain organisms, while their beds act as a habitat for a variety of different organisms such as many coelenterates, mollusks, crustaceans, echinoderms, and fishes (Merrill et al., 1970). Due to the ease with which they can be worked with and observed, *D. excentricus* also serves as an attractive organism for studying both fertilization and development (Ettensohn, 2017). These examples all serve to form excellent reasoning for enlarging the body of knowledge of the species.

The development of *D. excentricus* larvae, and echinoderm larvae in general, has been shown to be influenced by a multitude of different factors including temperature, pH, salinity, and many others (e.g., George and Walker, 2007; Chan et al., 2015). In 2008, a study on the effect of predator cues on cloning in *D. excentricus* larvae was conducted by Vaughn and Strathmann, 2008, results displayed instances of increased cloning when exposed to external fish mucus. Larval cloning has been seen in echinoderm larvae to be caused by various stressors and typically result in smaller size and asymmetry. Based off of these findings, it was hypothesized that reduced size, caused by cloning, may be beneficial to the larvae by causing them to be less vulnerable to visual predators and some nonvisual predators (Vaughn and Strathmann, 2008).

To attempt to further test the theory proposed by Vaughn and Strathmann, 2008, a variety of different possible predator cues should be tested with *D. excentricus* larvae and cloning response should be measured. In order to accomplish this, in the current study, *D. excentricus* larvae were placed in the effluent of two different possible predators: *P. producta* and crustacean dominant plankton. In addition to denoting instances of cloning, the number of larval arms and

rudiment stage of each larvae was also determined; such measurements can be used to allow the data to be more comprehensive and possibly explain changes in development in response to predator cues.

Methods

All materials used in spawning, fertilization, and rearing of *D. excentricus* larvae have been handled to be embryo safe.

D. excentricus Spawning and Fertilization

Spawning of the parent organisms were conducted on May 22nd, 2019 using *D. excentricus* collected by hand from Crescent Beach in East Sound, Orcas Island, WA, USA in the month of April 2018 and held in flow-through aquaria until spawning. A 25 G 1 ½ needle with syringe was filled with 0.7 mL of 0.5 M KCl, then used to inject each organism in the gonad through the mouth; one male specimen was spawned using a 0.6 mL dose of KCl before switching to a 0.7 mL dosage due to lack of response from several specimens. Each dose was given in three parts by pulling out the needle slightly and reinserting in a new position, attempting to access different gonads. The needle was rinsed with reverse osmosis water in order to avoid contamination and possibly fertilization, then filtered seawater between organisms. Organisms were slightly shaken and placed oral side down to be observed for evidence of spawning. Upon evidence of spawning, females releasing eggs were placed oral side up on the rim of a 150 mL beaker filled to the brim with filtered seawater. Once all eggs appeared to be released, the organisms were set aside. In the case of a male, a glass Pasteur pipette was used to collect the sperm straight from the gonopore, then placed immediately into a labeled Eppendorf tube (labeled M1 or M2 depending on the specimen). Out of spawning, gametes were collected

from 2 males (M1 and M2) and 2 females (F1 and F2), sperm motility and egg quality were then checked for all specimens. Beakers with eggs were placed in the sea table in FHL Lab 4 and sperm was frozen from collection until the beginning of fertilization.

To prepare for fertilization, two beakers were filled with 100 mL of filtered seawater. 50 mL of water was decanted off from the two beakers containing eggs from for a total of 100 mL of filtered seawater and eggs left in each beaker. Approximately four hours had passed from end of spawning to beginning of fertilization. A wide bore plastic transfer pipette was then used to move half the eggs from one beaker to another, splitting up the eggs from each into two beakers. Upon completion of egg preparation, sperm from each male was diluted by first filling a 15 mL Eppendorf tube with 10 mL of fresh seawater. Using a glass Pasteur pipette, a small 'notch' of sperm, equivalent to the space between the notches at the end of the pipette, was then taken from each of the samples and placed into its own 10 mL of filtered seawater. This suspension was then repeatedly pipetted up and down and thoroughly mixed. 10 drops of the sperm suspension were then added into the respective labeled beaker containing eggs after stirring those as well. Beakers were then placed in the sea tables of University of Washington's Friday Harbor Laboratories' (FHL) Lab 4 for 10 minutes in order to allow them to settle. After 10 minutes, percent fertilization was determined by examining 10 eggs from each beaker and then measuring egg diameter. In cases where percent fertilization was below 80%, a second notch of sperm was added. Eggs were then allowed to settle before sperm water was decanted off and replaced with clean, cold, Millipore filtered seawater; cultures were left on the benchtop in FHL Lab 4 at room temperature (19°C). Larvae were then poured into a large glass gallon jar with a density of 1 larva per 1 mL after hatching. On day two post-fertilization, the larvae were separated into two separate glass gallon jars in order to achieve a density of 1 larva per 2 mL.

Preparation of Treatment Effluents

In order to produce the plankton effluent, a LED light source was placed into the water next to the FHL docks. After about 30 minutes, crustacean dominant plankton was collected from around the edge of the light source. Fishes were removed from the collected waters and the remaining organism were strained through a 243 μm mesh before being placed into a tank filled with 2.5 L of fresh seawater, complete with air stone. To produce the kelp crab effluent, a male *Pugettia producta* (*P. producta*) was collected off the FHL docks (it should be noted the left pincher was missing) and placed into a bucket of fresh seawater. All organisms were left to soak for 24 hours in their respective waters before being strained through Whatman Grade GF/C filter paper (due to convenience), leaving only the effluent concentrate; the control water was also produced using fresh seawater and the same filter paper. Enough concentrate was collected to produce 480 mL of the final treatment solution, this amount was calculated using the biomass of the organisms used and the amount of water they were soaked in. This method required the kelp crab effluent concentrate to be heavily diluted using the control water. An ultimate ratio of 114.2 g biomass to 1 μL of seawater was achieved within both the kelp crab effluent and plankton effluent. Each well in 3 new and rinsed, tissue-culture treatment free, 6 well-well plates were then filled with 8 mL of treatment water, resulting in a total of 3 full plates for each treatment and 9 plates total.

Experimental Setup

On May 25th (approximately three days post fertilization), over the course of 90 minutes, select larvae from the May 22nd fertilization were placed into each well of the treatment plates for a total of 18 replicates each; larvae were selected to be free of obvious asymmetry and deformations. 2500 cells/mL of *Rhodomonas sp.* and 3000 cells/mL of *Dunaliella tertiolecta*

were also added to each well as a food source for the larvae. Larvae were left in treatment for approximately 66 hours next to a southeast facing window; temperature was set to 19°C and larvae saw approximately 16 hours of daylight and 8 hours night conditions. Larvae were then removed from treatment on May 27th, in random order, over the course of seven and a half hours. In order to collect data from each treatment, clones were first counted in every well in the plate. The larva in each well was then mounted on a slide using a raised cover glass and studied under a compound scope. Number of larval arms were then counted using polarized light created with two pieces of polarized film in/on a compound scope, in order to look for evidence of $CaCO_2$ extending into the postoral arm. Rudiment stage was determined using the rudiment staging scheme detailed by Heyland and Hodin (2014). Larvae displaying an early or late stage rudiment were recorded by subtracting or adding 0.5 to their rudiment stage value respectively. Photos were taken of average, asymmetrical, and deformed larvae from each treatment through the compound scope using an IPhone X camera.

Statistical Analysis

Collected data was then analyzed using R coding language to create three separate linear models comparing number of larval arms, rudiment stage, and number of clones between treatments. Figures were created using R coding language (R Core Team, 2013), sciplot (Morales, 2017), plyr (Wickham, 2011), and ggplot2 packages (Wickham, 2016).

Results

The linear model comparing treatment to number of larval arms showed significant results when comparing both the kelp crab effluent treatment (p= <0.001) and plankton effluent treatment (p= <0.001) to the control. Linear modeling comparing treatment to rudiment stage displayed significant differences when comparing the kelp crab effluent treatment to the control (p= 0.032); the linear model comparing treatment and number of clones showed significance in the comparison of the kelp crab effluent treatment to the control (p=0.024) as well.

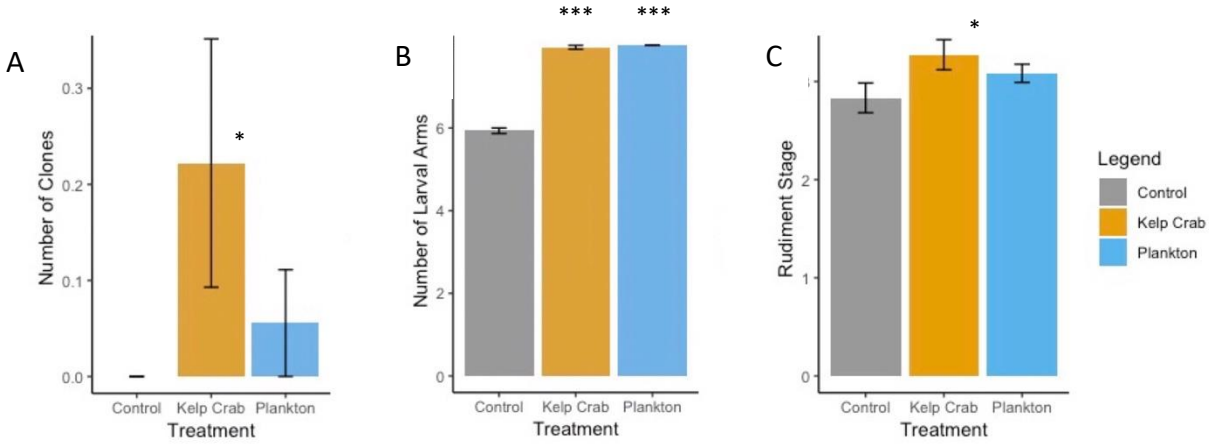

Figure 1. Graphs depicting the mean number of clones per larvae (A), number of arms (B), and rudiment stage (C) within each treatment. Asterisks denote significant differences from the control according to the linear models conducted. "" is used to denote P<0.05, "**" to denote P<0.01, and "***" to denote P<0.001.*

Larvae from the control treatment were all found to be in the 6 arm stage (presenting no $CaCO_2$ spicule extension in the postoral arms), while larvae from the two effluent treatments were all in the 8 arm stage (besides one 7 arm clone in the kelp crab effluent treatment) (Fig. 2).

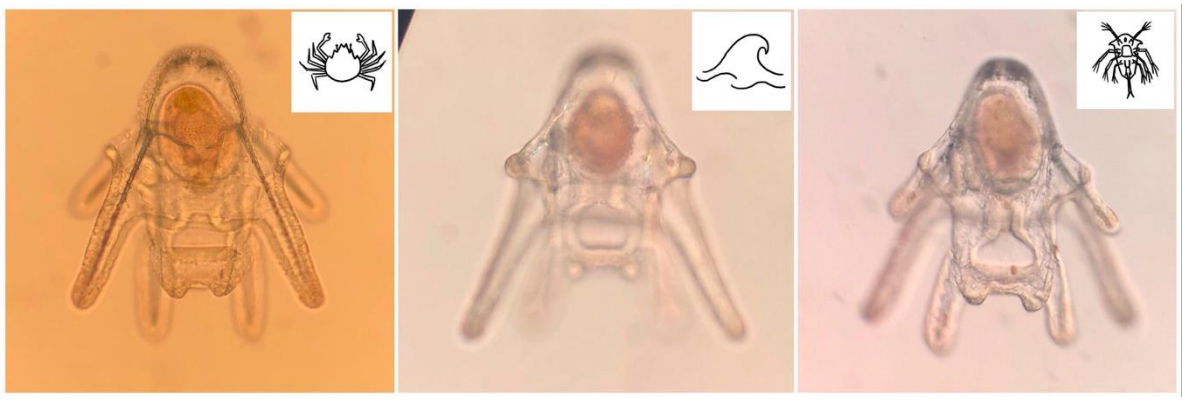

Figure 2. Appearance of average larvae. Treatment denoted by icon in upper right; crab icon represents kelp crab effluent treatment, water icon represents control, and nauplii icon represents the plankton effluent treatment.

More instances of cloning were observed in the kelp crab effluent treatment than any other group (Fig. 1A), however, one instance of cloning was seen in the plankton effluent treatment (Fig. 3).

Figure 3. Examples of cloning. Asymmetry in arms obvious in last three images. See Fig. 2 for icon information.

Asymmetry and deformities without clear evidence of cloning were more heavily observed in the plankton effluent treatment (Fig. 4).

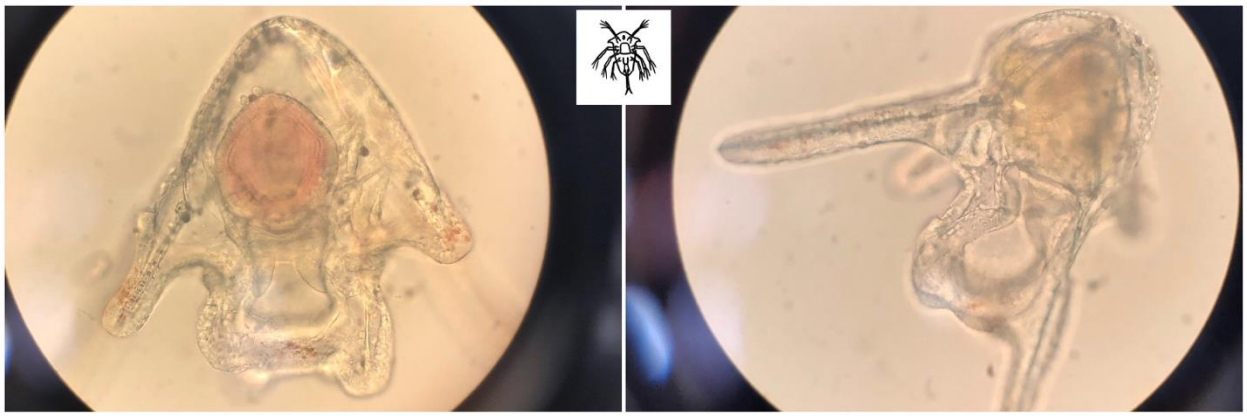

Figure 4. Examples of asymmetry and deformities in the plankton effluent treatment. See Fig. 2 for icon information.

Discussion

Although the predators of *D. excentricus* larvae are somewhat unknown, *P. producta* nauplii is a likely candidate due to their size and distribution. Due to constraints with obtaining *P. producta* larvae, an adult specimen was used, and a general crustacean dominant plankton was utilized as a second example of a predator cue. Cultures were performed at room temperature,

about 19°C, in order to obtain more advanced larvae in a shorter period. Larvae development at this temperature can also be seen in the natural habitat of *D. excentricus* in areas such as Baja California, an area where *P. producta* and planktonic crustaceans can also be found; it should be noted however that the planktonic crustaceans in that area most likely differ greatly when compared to those from the Straight de Juan de Fuca. Cultures contained larvae from a fertilization of two female specimens of *D. excentricus* and two males, helping to eliminate the possibilities that results were due to parentage; though, for larval cloning, this was disproven by Vaughn, 2009. 18 different *D. excentricus* larvae were exposed to each predator cue, resulting in increased cloning and rudiment stage in those exposed to the kelp crab effluent treatment ($p=0.0241$ and $p=0.0315$ respectively) and increased larval arm stage in both kelp crab and plankton effluent treatments ($p= <2e-16$ for both); this, in tandem with observations made by Vaughn and Strathmann, 2008, furthers evidence on predator cues effecting larval development in *D. excentricus*.

Increased larval arms and rudiment stage are indicative of increased development and therefore, being closer to settlement. By settling, larvae leave their planktonic stage and become benthic larvae. These benthic larvae experience a new trophic position and environmental factors; at this stage predators such as *P. producta* and crustacean dominant plankton may no longer be or are no longer a concern. Settlement may serve to increase fitness in *D.* excentricus when exposed to predator cues; this theory is not outlandish considering preferential settlement has been observed in *D. excentricus* larvae when exposed to various factors (Crisp, 1974; Gray, 1974; Highsmith et al., 1982; Woodin, 1991). The fact that cloning was not seen to increase within the plankton effluent treatment, but did in the kelp crab effluent treatment, is not

supported by the aforementioned theory unless the organisms present in the plankton effluent are not considered predators by the larvae.

The visual predator hypothesis discussed by Vaughn and Strathmann, 2008 is not necessarily supported by this data due to the lack of cloning occurring in the plankton effluent treatment and the nature of *P. producta* as a possible predator. The plankton used to produce the effluent, contained a large number (unquantified) of crustaceans such as copepods and general crustacean larvae; these both, and especially together, should have no trouble forming a predator cue for the larvae as their most likely predator should be zooplankton in general. If it is necessarily just a predator cue that encourages cloning, more examples of cloning should be seen in the plankton effluent treatment. However, there is also the possibility that the larvae are able to differentiate predator cues; they may see predators such as fish as a larger threat than those such as zooplankton. The presence of cloning in the kelp crab effluent treatment, however, does not necessarily support the visual predator theory either. Adult kelp crabs are herbivores and are not, themselves, actual predators of *D. excentricus* larvae; their larvae on the other hand most likely are (Hines, 1982). If *D. excentricus* larvae are unable to differentiate predator cues from adult versus larval stage of *P. producta*, the visual predator theory may still be supported. This last hypothesis is most likely, considering the increased development in larvae exposed to kelp crab effluent, as they are likely to view adult *P. producta* as a predator and see the environment as one they should readily exit.

Though no molecular analysis was completed, when considering the kelp crab effluent, urea can be presumed to dominate composition when compared to other substances due to the lack of water flow in the containment vessel during the 24-hour soaking of *P. producta*; recognizing this, there is a large possibility that the behavior of the larvae in response to this

treatment was due developmental issues in response to a stressor such as poor water quality (Weihrauch et al., 2004). It should also be noted that the *P. producta* specimen used to produce the effluent was missing one arm with chelae, meaning it may have produced different cues than an uninjured specimen. These points, however, do not disprove any possibilities of the larvae's' behavior being due to the presence of a possible predator.

Conclusion

The results described can be explained by a variety of different factors. Although the primary goal of conducting this experiment was to test the visual predator hypothesis proposed by Vaughn and Strathmann, 2008, instead more hypotheses were raised. These hypotheses build upon hose raised by Vaughn and Strathmann, 2008 and expand the list of possible research topics concerning *D. excentricus* larvae. When considered simultaneously, many of these hypotheses can be used to support the visual predator theory; however, the same hypotheses can also be used to disprove it. In order to determine if the theory proposed by Vaughn and Strathmann, 2008 is supported, it is necessary for more research to be done concerning the possibility that *D. excentricus* larvae have the capacity to differentiate predator cues. It must also be determined to what precision they may be able to differentiate such cues (such as differentiating adult versus larval *P. producta*).

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