

Seeing Double: Taking a Look at Cloning in *Dendraster excentricus*

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Abstract

Cloning is a form of asexual reproduction that occurs in *Dendraster excentricus* and results in a decrease in size and developmental stage. Previous research has shown that *D. excentricus* larvae at the 4-6 arm stage clone both in response to predator cues and to an increase in nutrients. It is not known if the 4-6 arm is the stage where larvae clone the most or if they can even clone at other developmental stages. In the current study, individual larva were given food pulses at one of two developmental stages: the 4 arm stage (4dpf) and at the 6-8 arm stage (7dpf). While it cannot be said for certain there were any cloning events, there was a large decrease in size of the larvae given the 4dpf pulse from 5dpf to 11dpf as well as some morphological oddities that could have indicated cloning; one larva appeared to be budding.

Introduction

The growth and development of echinoid larvae has been well studied. Most pluteus larvae are obligate planktotrophs, which means that they require food to grow and ultimately reach metamorphosis. There are clearly identifiable embryonic stages that start with the blastula, after that is gastrulation, then prism (when body skeletal rods appear), and lastly the pluteus stage. The pluteus starts out in the 4-arm stage then grows to the 6 and 8 arm phases soon after. One of the first pairs of arms to develop are post oral arms that are used for feeding (reviews by McEdward and Miner 2001, Hodin et al. 2019). Next, the juvenile rudiment begins to form, then the skeleton develops (Heyland and Hodin 2014). Eventually settlement occurs which is characterized by the appearance of tube feet sticking out of the body and firmly attach to the bottom (Heyland and Hodin 2004). These phases are important pieces to the life history of an echinoid and should carry out in a linear fashion, however, echinoids have a trick up their sleeves - cloning.

Cloning is a process of reproducing asexually by budding or fission (Eaves et al. 2013). Previous research has shown that *D. excentricus* use cloning as a response to predation cues from fish or to a sudden increase in food - (Vaughn 2010; McDonald and Vaughn 2010).

Cloning results in a decrease in size and possibly a reduction in developmental stage such as going from a 6-arm stage to 4-arm stage and it has been shown that cloning decreases predation rates (Vaughn 2010).

Work has also been done to describe some of the mechanisms. It has observed that origin of cloning can be the lateral body wall, arms, oral hoods, and the posterior end. The clone itself can be in a number of developmental stages from blastula to full formed larvae and the event always results in a decrease in size of the original larvae (Eaves and Palmer 2013; Jaeckle 1994).

Cloning has been regularly observed in *Dendraster excentricus*, a common species of sand dollar found in the pacific northwest that grows at such an exceptional rate they can be competent to settle as early as 10 dpf at room temp (Hodin et al. 2015) which makes them an ideal study organism for looking at morphology and growth rate. As previously mentioned, during the 4-6 arm phase *D. excentricus* clone in response to a sudden increase in food (McDonald and Vaughn 2010). However, not much work has been done on looking at the morphology in when larvae are cloned at different stages or if they are even able to clone closer to settlement. The goal of this study is to see if the increase in cloning is limited to the 4-6 arm developmental time period or can it also be applied to the 6-8 arm phase and if so what effect does this have on growth.

Methods

Adult *Dendraster excentricus* were collected of Crescent Beach (East Sound, Orcas Island, WA, USA) by hand at low tide in July of 2018 and maintained in flow thru aquaria (FHL, Friday Harbor, WA, USA) until May 16, 2019 when a handful of the largest adults were collected for spawning. Two males and two females were induced to spawn by injecting them with 0.75mL of 0.55M KCl through the mouth and into the gonads. Eggs were collected by placing spawning females aboral side first into 150mL beakers filled with filtered sea water (FSW). Sperm was collected from males by using a pipet to suck the sperm directly out of the

gonopore. The quality of the sperm and the eggs were assessed and then a sperm dilution was made by taking a notch of sperm and suspending it in 10mL of FSW ('Sea Urchins for Education' 2018). The eggs from the females were divided in half and put into new beakers with 150mL FSW to make two F1 and two F2 beakers. Ten drops of the sperm suspension were used to make four different parent crosses. Percent fertilization was over 80% in all crosses and then embryos from each cross were measured. The newly hatched larvae were poured off into new beakers, leaving behind unfertilized eggs and old sperm. Then density counts were used to assess what volume should be poured into the 3L jar so that each cross contributed 750 larvae. The larval density was one per mL 1 dpf and then was decreased to one larva per two mL 23dpf. The large culture was reverse filtered the water every other day and were fed a low food treatment of 250 cells per mL of *Dunaliella tertiolecta* and 500 cells per mL of *Isochrysis*.

On 4 dpf individual larvae that were in the 4-arm stage were placed in 6 well plates so that when a cloning event happened there would be no doubt that the larvae was a clone, and the morphology of the cloned vs the clonee could be compared as well as the growth of both. All well plates were kept on a shaker table going 75 rpm and all were on a 12 hour day night cycle controlled by a incandescent light that gave off $16.09 \mu\text{mol}^{-1} \text{ m}^{-1} \text{ s}^{-1}$. Well plates were rotated so there was not a disproportionate amount of light on any one plate.

Four 6-well plates were given a 'pulse' feeding of 10,000 cells per mL of *Isochrysis* and 5,000 cells per mL of *Dunaliella* with the intent of inducing them to clone, these plates were labeled 'A Clones'. Four more 6 well plates were given the low food treatment until 7 dpf, which were then given the 'pulse' feeding and were labeled 'B Clones'. Individual larvae were kept at a density of 1 larva per 8 mL and complete water changes were performed every day to check for clones, and then the old wells were kept overnight and rechecked in the morning to be confident no clones had been missed the first time around. Complete water changes entailed moving the clone into an entirely new well with new water and food. All larvae were measured 5 dpf and 11 dpf so comparison records could be kept in the event that they cloned. Statistical analysis was

performed using R version 3.5.2 (R Core Team, 2018) to perform ANOVA tests that looked at the relationship between the mid body length across both cloning treatments and time and post oral arm length in relation to the cloning treatments and time.

Results

Measurements were taken of each individual larvae at 5 dpf and 11 dpf of mid body length (MBL) and post oral arms (POA). Anovas were ran on all the data and a subsequent post-hoc Tukey test revealed that MBL of A clones was

significantly smaller than A and B clones at 5dpf and B clones 11dpf ($F(1,92) = 11.43$, $P = .0011$) (Figure 1).

Another ANOVA test was ran to see the relationship between the POA length in A Clones and B Clones with each other and across time. The post-hoc Tukey test showed that the POA length in A clones had a significant decrease from 5 dpf to 11dpf. B Clones on the other hand were not significantly different from one time period to another ($F(1,92) = 12.86$, $P < 0.001$) (Figure 2).

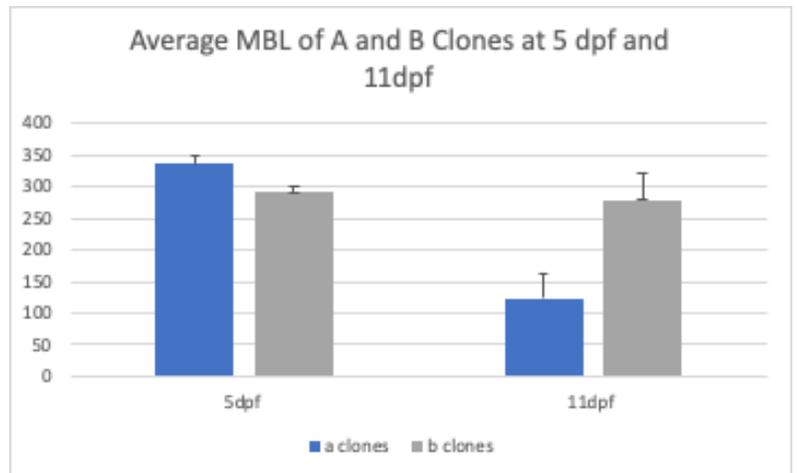


Figure 1. Average MBL of A Clones and B Clones at 5 dpf and 11 dpf. This illustrates how A Clones were significantly smaller than they were at 5 dpf and smaller than B Clones at any point in time. Error bars are standard error of the mean. Y axis in length in micrometers

While no clones were observed in individual well plates, there were two larvae (#16 and #19) from plates “A Clone 3” and “A Clone 4” respectively that had a reduction in size. When initially measured (5 dpf) larva #19 had a mid-body length of $270\mu\text{m}$, a post oral arm length of $216\mu\text{m}$ and was in the 6-arm stage. At 10 dpf this same larva was in the 4-arm stage and had a mid-body length of $243\mu\text{m}$ and a post oral arm length of $162\mu\text{m}$. At 5 dpf, larva #16 was at the four 4-arm stage, had a mid-body length of $270\mu\text{m}$ and a post oral arm length of $216\mu\text{m}$; the midbody length decreased to $216\mu\text{m}$ and the post oral arms decreased to $162\mu\text{m}$ at 10 dpf. The average midbody and post oral arm lengths for the A Clone 3 well plate was $270\mu\text{m}$ and developed rudiments and were getting ready to settle (Figure 3).

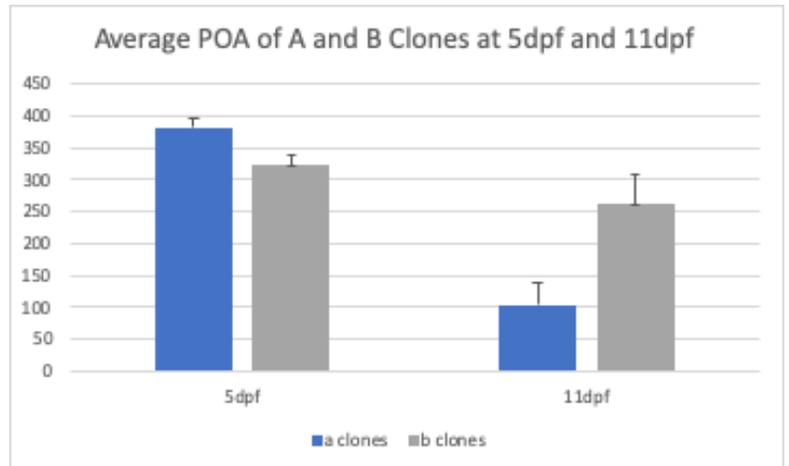


Figure 2. Average POA length for A Clones and B Clones at 5 dpf and 11 dpf. This shows that A Clones had a large drop in post oral arm length from 5 dpf to 11 dpf. There was no significant difference from B Clones 5 dpf to 11 dpf. Error bars are standard error of the mean. Y axis is length in micrometers.

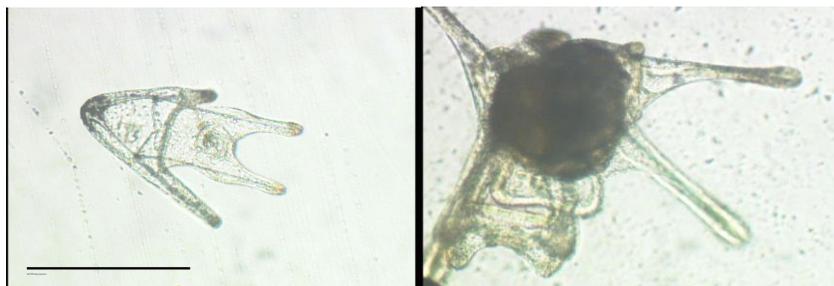


Figure 3. Larvae 11 dpf both from “A Clone 3” # 19 (left) is a possible clone and #25 (right) is representative of the rest of the well plate which have developed rudiments and are almost ready to settle. Scale bar is 250 micrometers and applies to all images.

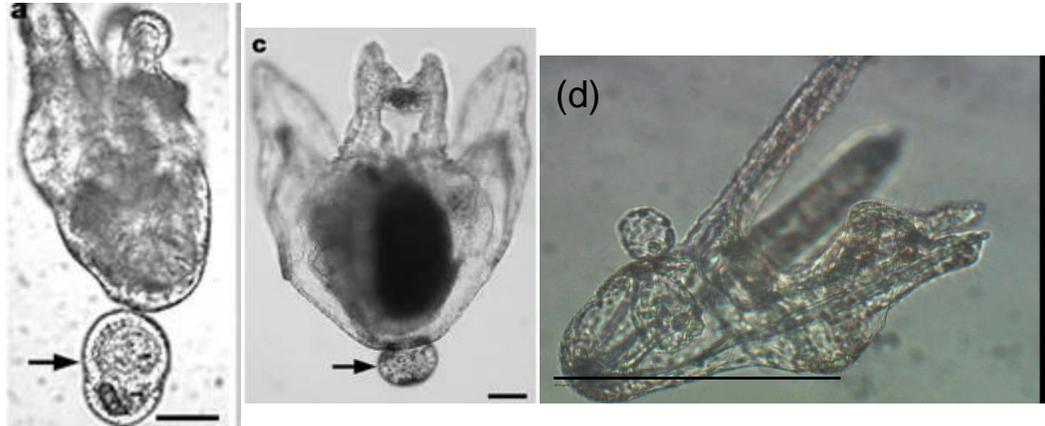


Figure 4. Images from Eaves et al. 2003 of a (a) Sea cucumber (*Parastichopus californicus*) undergoing budding (left) (c) as well as a Sea urchin (*Strongylocentrotus purpuratus*) that is undergoing budding (middle). Scale bars are 50 micrometers. (d) To the right is larvae #16 which may be undergoing budding. Scale bar is 150 micrometers.

Number 16 also exhibited a bizarre morphology that resembled that of budding (Figure 2d) which can be compared to two cases of budding in the literature (Figure 2 a. & b.) The first two images show asexual budding in the sea cucumber (*Parastichopus californicus*) and Sea urchin (*Strongylocentrotus purpuratus*). In this image the budding is occurring at the posterior end (Eaves and Palmer 2003).

Probable clones were found in the large culture jars (Figure 3.) It cannot be said for sure if these are clones since the only way to determine if there is cloning in a large culture is to do density analysis which was not performed. However, it can be said that these are most likely clones as their morphology is asymmetric, and extraordinary small compared to the rest of the cohort, and in many cases didn't even have distinguishable arms (Vaughn 2018)

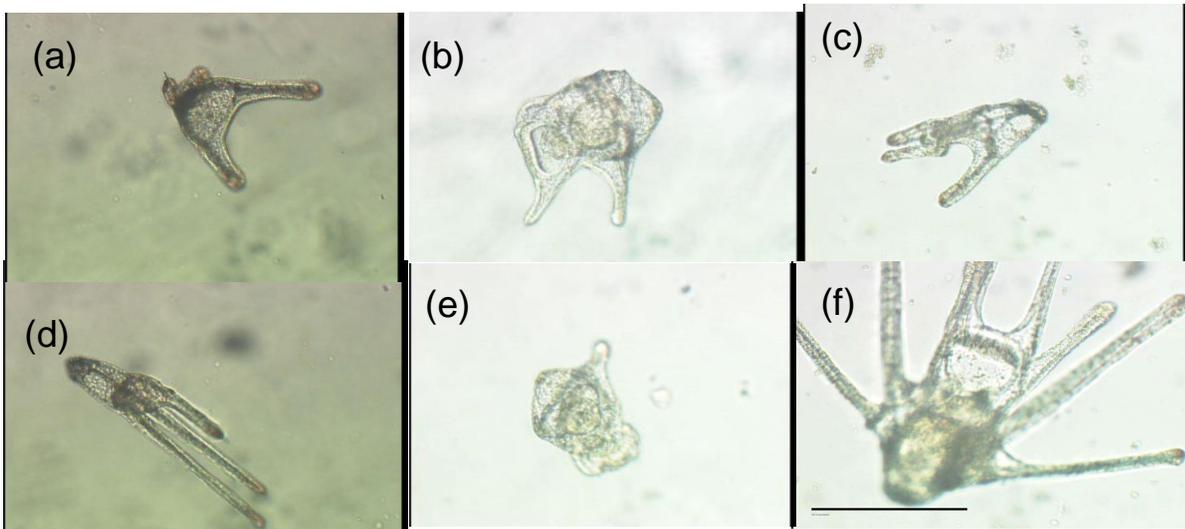


Figure 5. Larvae 8dpf from large culture. (a)-(e) probable clones (e) representative of what an 8 dpf larvae should look like. Scale bar 250 micrometers and applies to all images

Another interesting observation was the large mortality of larvae across all plates. On a couple of occasions, the body was wrapped in debris, however the majority of missing larvae did just that- they went missing. After transferring larvae in to the new well plates the entire plate was re checked to make sure none of the larvae were lost when transferred from old well plates into new ones. On May 23rd an entire well plate lost its larvae and by the end of the experiment on May 28th 35.4% of larvae had been lost.

Discussion

It has been previously shown that a sharp increase in food between the 4-arm and 6-arm phase leads to increased cloning in individual well plates by 20% and in large cultures by 50-100% (McDonald and Vaughn 2010). This is why it is so intriguing that in my experiment there were no clones out of the 48 larvae that were given the exact same feeding protocol. The McDonald and Vaughn protocol was adapted to keep many of the factors the same as possible.

However, there were still many differences that could have contributed to the discrepancy in their percent cloned when compared to the current study. The major factor that could have made a large difference is that 5 μm bag filtered sea water (FSW) was used and in the McDonald and Vaughn protocol 0.45 μm FSW was used. This means that there were possibly more contaminants and undesired organisms that were able to get through the mesh filter and may account for the high mortality. Ciliates, part of the phylum Ciliophora are a very large group of protists characterized by having cilia. Ciliates eat an extremely wide variety of things (Pierce 1992) and it is possible that the clones, if they were small enough, were broken down on a cellular level. It is also possible that the clones died shortly after the event and subsequently broke down and became unrecognizable.

Another possible explanation for this high mortality was well plates had to be transferred from my workstation to another room containing the shaker table. Extra care was kept to walk slow and keep all of the well plates level when being moved. However, it is possible that some water with a larva in it could have accidentally been tipped too far and fallen out of the well.

The significant decrease in size of the POA and the MBL in A Clones from 5 dpf to 11 dpf (Figure 1 & 2) is interesting. As the larvae age they should be going through the developmental stages and increasing in size not decreasing. This evidence combined with photo data that shows delayed development and some indicators of clone morphology such as decrease in size and stage (Figure 3) as well as a larva that appears to be budding (Figure 5) makes an argument that these could have undergone cloning at some point in time. Observation has shown that some clones don't separate until the primary larvae has begun to metamorphose (Eaves and Palmer 2003). Larva #16 (Figure 5) had not yet begun to metamorphose as it was still quite small and still had the possible bud by the termination of the experiment. It is possible that it was just taking a while to clone and a longer amount of time for this study would have allowed for closer monitoring of this larva.

Further studies conducted should utilize 0.45- μm FSW so that there are no possible contaminants. I think it would be worthwhile to run the experiment again with more larvae from different fertilizations just to diversify and see if the same results are obtained.

Acknowledgments

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