# Like Mother Like Daughter?: Development Stages Over Time in the Hybrid Larvae of *Dendraster excentricus* and *Strongylocentrotus purpuratus* as Compared to Developmental Milestones of Both Parents

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## Abstract

Hybridization in individuals can happen across varying levels of taxonomic relation; within species (Byrne, 1994),(Coertze, 1990), genus (Coertze, 1990), family (Kang *et al.*, 2011), and orders (Brookbank, 1970) in plants and animals (Arnold, 1997). Although the more distinctly separate a relationship is between individuals in a hybridization event, the less biological fitness the resulting hybrid will have (Barreto, 2013). Through the methodologies of rearing the hybrid larvae of the cross *Dendraster excentricus* female and *Strongylocentrotus purpuratus* male, observations of developmental progress across stages of both echinoids are made, and have been documented by previous studies (Brookbank, 1970), (Moore, 1957). This study found no clear relationship between developmental stages reached in experimental hybrid larvae as compared to expected development stages in either parental donors. By asking if development in the cross of *D. excentricus* and *S. purpuratus* is parentally influenced, this study aims to make contributions to the lesser known *in vitro* fertilization and larval rearing processes of sand dollar and urchin hybrids.

## Introduction

Hybridization in individuals found in nature and fertilized *in vitro* experiments have been a source of controversy in the scientific community in many ways, from defining what makes an individual a hybrid (Allendorf *et al.*, 2001), to making policies and management decisions involving them (Allendorf *et al.*, 2001). Here we define hybridization as the interbreeding of individuals from genetically distinct populations, regardless of sympatry (Arnold, 1997). In nature a hybridization event can occur when gametes from two individuals of genetically distinct populations meet and successfully fertilize. Arnold (1997) discusses the importance of successful natural hybridization events to evolution and focuses only on *in vivo* hybridizations. *In vitro* work by Byrne (1994) in cross-fertilization experiments of three sympatrically occurring species of the asteroid genus *Patiriella* supports evidence that lack of hybridization in sympatric species in nature may be due to gametic incompatibility and characteristics of an individual's development and lifestyle, as well as reproductive isolation between species that have evolved despite gametic compatibility. By comparing theories from Arnold (1997) and Byrne (1994) we can make informed interpretations of data from this *in vitro* experiment as compared to naturally occurring hybridizations.

Across varying levels of taxonomic relation within a class, hybridization in individuals can happen between distinct species, genera, families, and orders. The more phylogenetically distant a relationship becomes between individuals in a hybridization event, the less biological fitness the resulting hybrid will have (Barreto, 2013). Johansen-Morris (2006) has described hybrids between two ecotypes of the selfing annual *Avena barbata* as significantly less fit on average than the midparent. As more genomes are sequenced, scientists are beginning to discover how common hybridization is in the wild (Mallet, 2007) and what we can learn about an individual's molecular biology and morphology.

In an attempt to create hybrids across orders of echinoderms, Brookbank (1970) makes contributions to the developmental history of hybrid embryos and describes the rate of development in hybrid embryos as strictly maternal with evidence from time of cleavages, hatching, and formation of primary mesenchyme in hybrid larvae of sand dollars and urchins. Flickinger (1957) tries to determine if the activity of the enzyme alkaline phosphatase in the hybridization of *Dendraster excentricus* female and *Strongylocentrotus purpuratus* male, and the reciprocal cross is characteristic of the maternal or paternal species. Moore (1957) describes the cross of *Strongylocentrotus purpuratus* female and *Dendraster excentricus* male as having biparental inheritance, and the reciprocal cross as having maternally determinate development.

Here we use a hybrid cross to investigate how development of hybrid echinoids differs from that of either parental species. Using *in vitro* hybrid crosses of *Dendraster excentricus* female and *Strongylocentrotus purpuratus* male, we qualify when crucial developmental stages differ in the hybrid group from the parental offspring. A study by Flickinger (1957) shows that maternally determined hybrid crosses develop similarly to control larvae. Therefore, we hypothesize that our experiments will yield similar results. By asking how hybrid larvae in this class of echinoid develop as compared to the control of each parent, we aim to see if development is determined by parental influence.

## Materials and Methods

*Strongylocentrotus purpuratus* were collected in March 2019 at Slip Point, Clallam Bay, WA, USA, by hand at low tide, and transported to Friday Harbor Labs (Friday Harbor, WA, USA) where they were held in subtidal cages and fed fronds of *Nereocystis luetkeana* until spawning. To induce spawning each individual was injected with 1 ML of 0.5 M KCL through the peritoneal membrane between the mouth and the test. After injection, individuals were shaken by hand for about five seconds and placed aboral side up on a table to observe spawning. Females were placed oral side over a 150 ML beaker of filtered sea water (FSW), in a sea table with running seawater of ~11-12 degrees C to keep eggs cool. Sperm was collected directly from the male gonopore under a dissecting scope using a pasteur pipette pre-moistened with FSW. Sperm was transferred to an Eppendorf tube kept cool by being dipped in a beaker of FSW between the additions of sperm, and was placed in the refrigerator upon completion of collection.

*Dendraster excentricus* were collected in July 2018 at low tide by hand on Crescent Beach (Orcas Island, WA, USA) and held in flow thru seawater on sand at the Friday Harbor Labs. To induce spawning individuals injected with 0.6-0.7 ML KCL into their mouth and subsequently shaken for 5 seconds. All sand dollars used were between 5.5 and 7.5 cm in diameter. Gametes of spawned individuals were collected similarly to that of *S. purpuratus*.

Fertilization of both controls and hybrid treatments were completed within six hours of spawning using fresh gametes to avoid any bias that may arise from age. Sperm and eggs of all specimens used were observed for motility and quality respectively prior to fertilization. Two separate spawning and fertilization events occurred over two days, with the first round including the *D. excentricus* controls and a hybrid treatment, and the second round including the *S. purpuratus* controls and a second hybrid treatment. Eggs from two females were fertilized with sperm from one male in every treatment to increase genetic diversity among replicates. All containers with embryos were left in the sea table after fertilization and between observations.

Sperm dilutions were made by adding a small amount of sperm from the tip of a pasteur pipette to ten ML of FSW in a 15 ML tube. Water was decanted off eggs until they remained undisturbed in 100 ML, and were stirred well in the beaker using a three ML plastic pipette with the tip cut off to make a wide bore. Ten drops of diluted sperm (one drop = approximately 0.1

ML) were added to the eggs, and were observed for fertilization success after ten minutes. 50 ML of FSW was then added to the beakers.

Hybrid fertilizations were conducted similarly to that of the controls, with differences in sperm concentration. In both treatments, a three ML wide bore pipette was used to transfer one ML of stirred *D. excentricus* eggs to each well in a six-welled well-plate already containing six ML of FSW in each well. Ten drops of *S. purpuratus* sperm dilution was then added to each well. Differences in fertilization protocol occurred in the second round of fertilizations where sperm concentration was changed to 300% by using a total of 30 drops.

Observations for all treatments were made at hours that coincide with documented developmental stages in both parents. Hybrid larvae were selected from each well in both well-plates and then transferred to finger bowls with 60 ML of FSW to allow room for growth. ~50% water from the surface of all hybrid treatment wells was removed and replaced with fresh FSW under a dissecting scope to decrease loss of larvae. Water in the control beakers was replaced by reverse filtration with an 80 micron mesh, using a 1.5 oz turkey baster to remove ~90% of the water, and replacing with FSW. The mesh was rinsed with cold FSW over the beaker to reduce loss of larvae during water change.

Representative photos were taken when a stage of interest occurred during development. Images were captured using BTV video capture software (Bird, B. 2000. *BTV*. London, UK: Ben Software Ltd.) and a SONY 3CCD ExwaveHAD microscope camera. Polarizing light was used to show the skeletal rod within larvae by placing a linear polarization sheet over the light source of the microscope. The experiment was terminated after the surviving hybrid treatments reached 96 hours after fertilization and all control treatments reached 98-122 hours. Feeding of larvae was not necessary due to the termination period.

## Results

Both experimental hybrid treatments reached a stage of development between mid and late gastrula between 48 and 64 hours (Figure 2 A&B). The hybrids fertilized in the first round of fertilizations all died after four days and had remained in the late gastrula stage for ~64 hours, while the hybrids fertilized from the second round of fertilizations had remained in the late gastrula stage for ~32 hours with many surviving until termination of the experiment. Growth rate was not measured, as such no statistical analysis is provided.

*S. purpuratus* controls from the second fertilization reached the early pluteus stage at ~88 hours after fertilization and remained in that stage until termination at 98 hours. There was a positive trend in development of control groups with individuals displaying cleavages at two, three, four, and six hours (Figure 2 C-F). *D. excentricus* controls from the first fertilization reached the four armed pluteus stage between 48-70 hours, as expected (Strathman, 1987), and remained in that stage until termination at hour 122. This also shows a great positive trend of development as a control.

Hybrid larvae in treatments from the first fertilization were the least conclusive as they died after reaching the late gastrula stage at ~26 hours and had no clear relationship between developmental stages reached in either parent larvae. Hybrid larvae in treatments from the second fertilization reached the late gastrula stage between 44-64 hours and showed abnormalities within the rudiment during invagination. Abnormal development observed in the hybrid treatment from the second fertilization is compared to a properly developed rudiment after invagination in a *S. purpuratus* control larva (Figure 3). Without significant findings of any relationship between developmental stages reached in experimental hybrid larvae as compared to expected development stages in either controls, no conclusion can be made about developmental pattern in these hybrid larvae.

## Discussion

*In vitro* embryo culturing methodology has been successful in crosses of *D. excentricus* and *S. purpuratus* hybrid larvae as previously demonstrated by Brookbank (1970), Flickinger (1957), and Moore (1957). These methodologies are important to the success of experimental hybrid larvae for the analysis of parental inheritance and morphology. The results of this experiment fail to show a correlation between maternally determinate development and expected times for crucial developmental stages in *D. excentricus.*

Animal hybrids with distinct separation of taxonomic lineage tend to be of low fitness and will likely be sterile when they come to fruition, or will have developmental processes halted in early larval stages (Strathman, 1987). These experimental hybrids will likely not develop past mid to late gastrula stage because of a block in the early larval stage, as multiple hybrid crosses have expressed primarily maternal gene activity and blocks in early larval stages (Strathman, 1987). The results of my experiment support the theory that long distance hybrids within a class of echinoderms do not develop far (Arnold, 1997). As the data collected from this experiment were qualitative, no statistical analysis could be done and more studies are needed to provide significant data for answering questions about parental influence in development and rates of development in echinoid hybrid larvae.

For future research on hybrid larvae, it would be useful to consider the possibilities of bias from genetically defective individuals and deficiency in availability of gametes when using live animals. Within this experiment the possibility of the results being biased by a unique male can not be overlooked as the methodology for mixed parent fertilization of both controls and hybrid treatments only involved sperm from one male.

Observations of the time a hybrid reaches a developmental stage marked in both parents are important to determine the influence of parental development. A study showing DNA synthesis of this hybrid provides material for studies of RNA, DNA, and proteins during early development in the reciprocal hybrid of this experiment; *S. purpuratus female* and *D. excentricus* male. (Brookbank, 1970) Further research should be conducted to find significance differences between molecular biology to morphology in hybrid development. A single anomalous larvae in the early pluteus stage of development was observed in the Hybrid Replicate 1 treatment (Figure 1). This shows potential human error in the methodology of storing treatments. While this individual larvae is treated as an anomaly in this experiment, and more studies should be done to determine if larvae in this hybrid fertilization can develop to an early pluteus stage. Methodology that would obtain quantitative data to determine the significance of events like this. One such as calculating the density of all embryo treatments should be incorporated into a replication of this experiment.

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## Figures

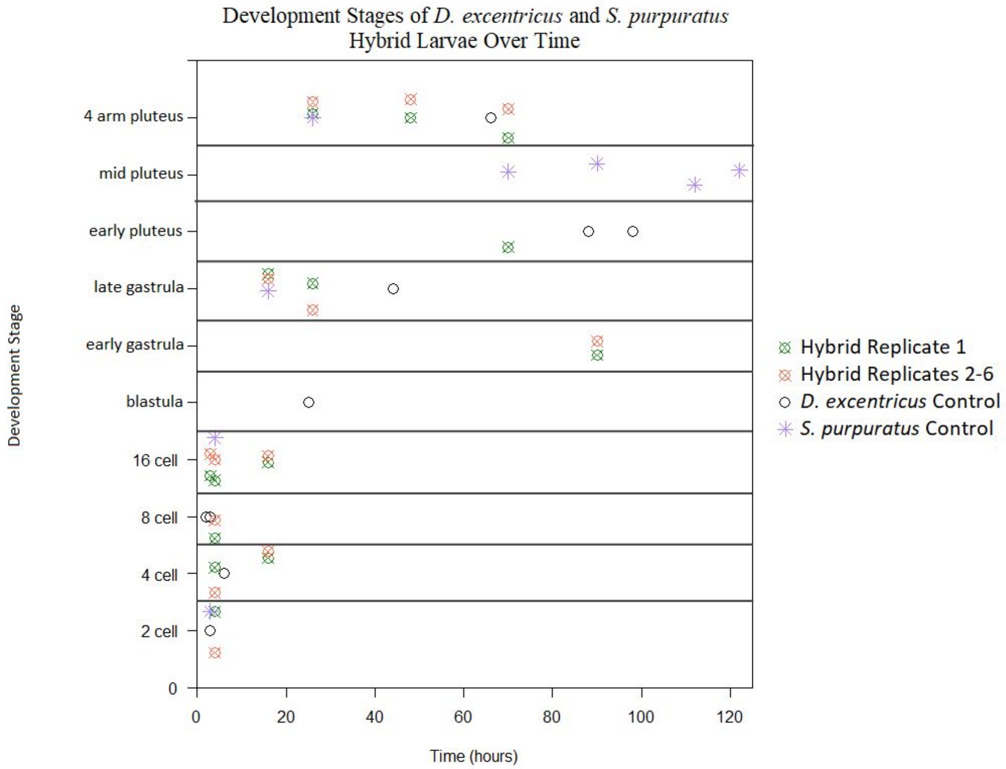

**Fig. 1** Developmental stages reached in the hybrid larvae of *D. excentricus* and *S. purpuratus* and their parent controls shown over a period of 96-122 hours. A positive trend is shown between the developmental stages reached in both experimental controls as compared to expected development stages documented in their corresponding species. No clear relationship is observed between developmental stages reached in experimental hybrid larvae as compared to expected development stages in either parental donors.

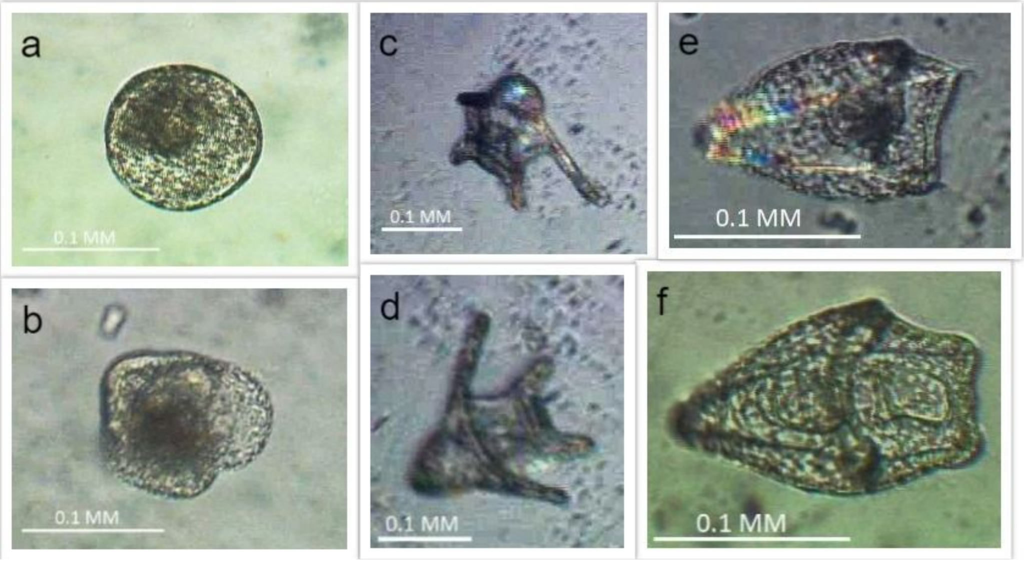

**Fig. 2** Developmental stages finally observed in *D. excentricus* and *S. purpuratus* hybrids and parental control larvae shown prior to termination of experiment. Photographs represent crucial developmental stages in hybrid and control larvae during various times within early larval development. All panels are live larvae. **(a)** Aboral view of a hybrid larvae observed 96 hours after fertilization. (100x) **(b)** Larva from the second hybrid fertilization treatment seen between mid-late gastrula with abnormal rudiment development. Development was presumably halted during invagination. Observed 96 hours after fertilization. (100x) **(c)** Aboral view of skeletal rod along postoral arms in *D. excentricus* control larvae in the four armed pluteus stage. Observed under polarized light 122 hours after fertilization. (40x) **(d)** Dorsal view of *D. excentricus* control larva in the four armed pluteus stage observed 122 hours after fertilization. (40x) **(e)** Abanal view of S. purpuratus control in the early pluteus stage with emphasis of skeletal rods in anterolateral and postoral arms before full extension. Observed under polarized light 98 hours after fertilization. (100x) **(f)** Dorsal view of S. purpuratus in the early pluteus stage before anterolateral and postoral arms fully extend. Observed 98 hours after fertilization. (100x)

**Fig. 3** A comparison of abnormal development in hybrid treatment larva to normal development of that in the *S. purpuratus* control treatment in the late gastrula stage. **(a)** Larva from the second hybrid fertilization treatment seen between mid-late gastrula with abnormal rudiment development. Development was presumably halted during invagination. Observed 96 hours after fertilization. (100x) **(b)** An dorsal view of an *S. purpuratus* control larva in the late gastrula stage properly developed. Observed 66 hours after fertilization. (40x)