Acta Spartae Vol. 1 No. 1 2015
Pages 7–15

Experimental Hybridization Between Two Species of Subtropical Sea Urchins from Tampa Bay, *Arbacia punctulata* and *Lytechinus variegatus*: An Analysis of Skeletal Morphology Using the Scanning Electron Microscope

Erin M. Meadows and Stanley A. Rice[1]

Department of Biology, University of Tampa, Tampa, FL 33606, [1]Faculty Advisor

ABSTRACT

Two genera of subtropical sea urchins, *Arbacia punctulata* and *Lytechinus variegatus* are common in the Tampa Bay area. *A. punctulata* is typically found in deeper coastal waters in the Gulf of Mexico, while *L. variegatus* typically inhabits the inshore sea grass beds of Tampa Bay. *A. punctulata* has recently expanded its range to include the shallow coastal waters near the mouth of Tampa Bay and now overlaps the range of *L. variegatus*. The two species broadcast spawn during overlapping seasons and hybrid embryos, from *L. variegatus* male with *A. punctulata* female and *A. punctulata* male with *L. variegatus* female, have been generated and raised in the laboratory up to the pluteus larval stage. In order to determine if the two genera are hybridizing in the field, specimens from Tampa Bay have been collected, acclimated to lab conditions, spawned, and the skeletal morphometrics of the larvae from four crosses, including two within species crosses used as controls and the two reciprocal hybrid crosses, have been examined at both the light and electron microscopy level. Mean percent fertilization was highest, 87%, in the *L. variegatus* control cross, 45% in the *A. punctulata* control cross, 37% in the *L. variegatus* female $\times$ *A. punctulata* male hybrid cross, and below 5% in the *A. punctulata* female $\times$ *L. variegatus* male hybrid cross. The prism stage of each control cross had minor differences so the focus of this study was on the four arm pluteus stage skeletal features at the Scanning Electron Microscopy (SEM) level. The pluteus larvae from each control cross for *A. punctulata* and *L. variegatus* had distinctive skeletal features. The skeletal features of the pluteus larvae from the hybrid cross were very similar to those of the maternal parent, but some variations were observed. In the *A. punctulata* control cross, the Anterolateral Rods (AR) of the larvae initially grew at day four and then shrank at day six while the Post-oral Rods (PR) continued to grow. Total Rod Length of *A. punctulata* control was generally always smaller than *L. variegatus* control. Also, the total rod length of *L. variegatus* female $\times$ *A. punctulata* male hybrids was about the same as *L. variegatus* control and sometimes larger. Growth patterns of all treatments, *L. variegatus* and *A. punctulata* controls as well as *L. variegatus* female $\times$ *A. punctulata* male hybrids, did not follow a clear trend as expected; as time proceeded total rod length shrunk, grew, and shrunk again. When live larval and skeletal measurements (SEM) were compared, skeletal measurements seemed to be more accurate.

1 INTRODUCTION

Sea urchins belong to the Phylum Echinodermata and are benthic marine invertebrates that feed on algae and various other forms of marine plant life. More is known about the embryonic development of the sea urchin than most any other marine invertebrate because they are easy to collect and shed millions of gametes at one time (Hinegardner, 1969). There has been extensive research done on culturing both *Arbacia punctulata* and *Lytechinus variegatus* larvae to complete metamorphosis into adult urchins in laboratory conditions (Cameron et al., 1989; George et al., 2004). It was found in a classic study on the culture of *A. punctulata* that a special diet, such as the diatom *Nitzchia closterium*, is needed to raise *Arbacia sp.* and hybrid larvae past the pluteus stage (Harvey, 1949). The techniques for raising several species of sea urchins, including *A. punctulata* and *L. variegatus*, to complete metamorphosis under laboratory conditions were reported by Hinegardner (1969). It was found that the larvae of different species of sea urchins have different food preferences. The complete development, from zygote to complete metamorphosis, of *L. variegatus* in synthetic seawater was described by Mazur & Miller (1971). The specific anatomical features that occur during metamorphosis of both *A. punctulata* and *L. variegatus* were described by Cameron & Hinegardner (1974). It was suggested that two stimuli are needed to initiate metamorphosis: a non-particulate organic chemical cue of bacterial origin and a bacterial film substratum. It was also observed that *A. punctulata* metamorphosis could be induced by an electrical stimulation.

Most sea urchins release their gametes into the water column, a phenomenon called broadcast spawning, after which prezygotic mechanisms, such as geographical isolation or gamete incompatibility, can play a role in inhibiting the successful union of the sperm and egg of different species (Cameron & Hinegardner, 1974; Rahman et al., 2001). Once fertilization occurs, there are postzygotic mechanisms that can also inhibit full maturation of hybrid embryos (Rahman et al., 2001). In spite of naturally occurring mechanisms that prevent hybridization, laboratory studies have shown that even distantly related taxa can be crossed under certain conditions. For example, Brookbank (1970) used laboratory-produced hybrids between *Strongylocentrotus purpuratus* (a sea urchin) and *Dendraster excentricus* (a sand dollar) to study DNA synthesis during larval development. In hybrids of these same two species, Moore (1957) described developmental rates and larval morphology in hybrid and control crosses. *Lytechinus variegatus* from the Atlantic Coast was shown to be cross-fertile with Pacific Coast sea urchins including *L. pictus*, *Strongylocentrotus*

purpuratus, and *S. franciscanus* in spite of estimated maximum divergence times of 30–40 million years (Minor et al., 1991).

In more closely related species, viable larvae have been produced under laboratory conditions even in cases where no hybrid individuals have been observed in the field. Rahman et al. (2001) crossed two sympatric species of the sea urchin, *Echinometra*, in the laboratory and raised the larvae to maturity. Hybrid individuals displayed combinations of maternal and paternal characteristics with some intermediate characters. Extensive field surveys failed to identify any individuals of hybrid origin suggesting that hybridization between these two species does not occur in nature. In the genus *Lytechinus*, two species from California (*L. pictus* and *L. anamesus*), that occupy different microhabitats were crossed in the laboratory by (Cameron, 1994) and hybrids raised to metamorphosis. It was not evident if these species produce hybrids in the ocean.

The specific mechanisms that inhibit hybrid fertilization when gametes of different species come into contact involve the acrosome reaction in the sperm and sperm-egg binding following contact of the sperm and egg plasma membranes (Hirohashi et al., 2008). Even if these mechanisms fail to prevent hybrid fertilization, genetic incompatibility usually arrests development of the hybrid embryos. The acrosome reaction in *Lytechinus* is non-specific and occurs spontaneously in both within and between species crosses (Minor et al., 1991). An acrosomal protein in sea urchin sperm, called bindin, facilitates the species-specific binding of sperm and egg. Analysis of bindin gene and amino acid sequences between species revealed that these proteins have various combinations of species-specific and conserved regions (Minor et al., 1991). In addition, evolution of gamete recognition proteins has been shown to be very rapid under certain natural conditions and may be related to population density and sperm competition (Levitan & Ferrel, 2006). Gamete recognition proteins tend to play a larger role in preventing hybridization between sympatric species since these proteins are under more intense evolutionary pressure (Minor et al., 1991). In four allopatric species of *Arbacia*, Metz et al. (1998) found little variation in the bindin sequences and showed that the Atlantic and Gulf of California species (the two most divergent species) were cross-fertile suggesting that there was little differentiation in gamete recognition systems. The evolution of bindin proteins in the genus *Lytechinus* was reported by Zigler & Lessios (2004). In a review of prezygotic and postzygotic mechanisms inhibiting the hybridization of different species of sea urchin, Lessios (2007) suggested that there is no single barrier that isolates species completely, but that reproductive isolation in sea urchins is from a combination of various factors.

In Tampa Bay, the distribution patterns and spawning seasons of *A. punctulata* and *L. variegatus* overlap in the spring and early summer suggesting that hybrids could potentially occur in Tampa Bay and the Gulf of Mexico (Sharp & Gray, 1962). Laboratory studies indicate that hybridization is possible in reciprocal crosses but hybrid larvae have not been documented in nature.

The present study attempted to establish the baseline morphology of *L. variegatus* and *A. punctulata* prism and pluteus larvae and to compare these to the morphology of laboratory-produced hybrid embryos using the scanning electron microscope. If distinctive morphological features can be identified in the hybrid larvae, then it may be possible to sample plankton during the overlapping spawning seasons and identify hybrid larvae in the field.

2 METHODS

Sea Urchin Collection

Sea urchins were collected from the main shipping channel off North Beach, Fort DeSoto Park near the mouth of Tampa Bay ($27°37.049'$N, $82°41.437'$W). An otter trawl was deployed in 7–9 m of water and a total of 150 *L. variegatus* and 105 *A. punctulata* were collected during two trips on 12 December 2012 and 11 March 2013. Water temperature at the time of collection was $21\,°\mathrm{C}$. Urchins were kept in aerated coolers until arrival at the University of Tampa Marine Science Field Station and then acclimated to laboratory conditions in 400 gal seawater tanks. Sea urchins were fed raw spinach.

Seawater

Seawater was collected at the same time as urchins from Egmont Channel, and filtered using P5 Filter paper (Fisherbrand) and transferred to a carboy. Salinity was 32 parts per thousand (‰) and an airstone was kept in the carboy for aeration. Seawater was used for gamete collection, fertilization experiments, and for larval cultures.

Collection of Gametes

Urchins were injected with $6\,\mathrm{mL}$ of $0.53\,\mathrm{M}$ KCL ($39.22\,\mathrm{g}$ l-1) through the peristomial membrane to induce gamete shedding. Each injected urchin was then placed aboral side up in a plastic petri dish and gametes, coming out of the gonopores, examined to determine sex; milky white gametes were sperm and orange, yellow or red (dependent on species) gametes were eggs.

Lytechinus variegatus males were placed aboral side down in a clean petri dish slightly slanted, to reduce mixing with coelomic fluid. *Arbacia punctulata* males were placed aboral side up and gametes collected with a glass pipette. Undiluted sperm, referred to as "dry sperm", was collected in a clean $15\,\mathrm{mL}$ test tube.

Both *L. variegatus* and *A. punctulata* females were placed aboral side down in a $250\,\mathrm{mL}$ beaker filled with filtered seawater. Eggs fell to the bottom of the beaker and were collected with a clean plastic pipette into a $50\,\mathrm{mL}$ centrifuge tube with filtered seawater. Eggs were allowed to settle and washed with filtered seawater 2–3 times to remove any residual coelomic fluid, then resuspended in filtered seawater.

Sperm Dilutions

One to two drops of dry sperm were added to 10 ml of filtered seawater, and sperm were counted using a Makler Chamber, specifically made for counting sperm. Initial sperm concentrations were adjusted to roughly $10 \times 10^6\,\mathrm{cells/mL}$. A fresh dilution was used when generating crosses to reduce the effects of sperm age on fertilization success.

Generation of Crosses

Eggs collected during each injection were distributed equally among four $300\,\mathrm{mL}$ beakers filled 1/3 full with filtered seawater. One plastic pipette full of the sperm dilution was added to each beaker. Beakers were then set on an orbital shaker (GeneMate, BioExpress) for about an hour and then percent fertilization for each cross

was determined by removing a sample of 100 embryos/eggs and identifying them as fertilized or unfertilized.

Four sets of crosses were generated including 13 replicates of *L. variegatus* control, 11 replicates of *A. punctulata* control, four replicates of *L. variegatus* female × *A. punctulata* male hybrids, and four replicates of *A. punctulata* female × *L. variegatus* male hybrids. Not all crosses could be generated during each attempt due to lack of female and/or male gametes of a particular species. Measurement and skeletal morphometric data, comparing hybrids to controls, were only collected when both the hybrid and control crosses were successful using the same batch of gametes. Data are not presented for larval skeleton size in the *L. variegatus* male × *A. punctulata* female hybrid cross due to small sample size.

Larval Cultures

Each 300 mL glass container with live larvae was screened every day using a 35 µm mesh screen and larvae were rinsed back into container using a squirt bottle filled with filtered seawater. Larvae were examined under a dissecting microscope for developmental stage, density, and overall health, and observations were recorded. The larvae in each container were resuspended in 100 mL of fresh filtered seawater and placed back on an orbital shaker. Once larvae reached the pluteus stage, food was added. Live cultures of *Isochrysis glabra* and *Dunaliella salina* were maintained for a food source, and Kent Marine MicroVert (Invertebrate food for fine filter feeders) was also used for a food source. Live algae was spun down in a clinical centrifuge (Centra CL2) and resuspended in filtered seawater before adding to larval cultures. If the density of a container was too high (dead larvae on the bottom) it was split into multiple containers to maintain an optimal density (high survival rate). Once density of larvae in a particular container reached a count lower than 3, it was terminated.

Larval Skeletal Preps

Serial larval skeletal preps of the prism and pluteus larval stages from each cross were made in increments of every other day and replicated to document developmental rate and for skeletal morphology analyses. Larvae were collected using a glass pipette, after the larvae had been concentrated and layered onto a 1 µm Nucleopore filter on top of filter paper in a plastic petri dish. Larvae were allowed to dry for one day to prevent them from falling off the filter when bleached and rinsed. Nucleopore filters containing the larvae were then transferred to a separate petri dish for bleaching. A 50% solution of commercial bleach was applied to the Nucleopore filters using a glass pipette until only the larval skeletons remained. Nucleopore filters were then transferred to a new petri dish with filter paper, rinsed with deionized water, and placed in a 60 °C oven for at least one day to dry. Once dry, Nucleopore filters were placed on an aluminum stub and coated with gold and palladium using a Cressington 108 Auto Sputter Coater.

Examination of Morphometric Features

A JEOL 6010LA scanning electron microscope was used to examine the skeletal features of each cross. Terminology for prism stage spicules following Okazaki (1975), was used to refer to structures examined. Terminology for later stage skeletal structures, follows McEdwards & Herrera (1999).

Measurements of Skeletal Structures

Measurements of the three primary prism stage spicules were taken with at least 25 larvae measured per replicate cross. Each spicule of the four-arm pluteus, as described by McEdwards & Herrera (1999), of each individual larva was measured including Body Rods (BR), Transverse Rod (TR), Postoral Rods (PR), Anterolateral Rods (AR), and Recurrent Rods (RR). Total Rod Length in microns was calculated for BR + PR + AR. Measurements were made using the InTouch JEOL software calibrated with a size standard (X-Checker Calibration Standard, Electron Microscopy Sciences). Live larval measurements were done on an Olympus BH-2 compound microscope using an ocular micrometer calibrated with a stage micrometer. The length of each long arm (BR + PR) was measured from the apex of the larva to the end of the long arm (PR) while the short arms (AR) were measured from the point where the arm emerged from the central body to the end of the arm. For live larval measurements, the long arm length corresponds to the SEM measurements, BR+PR, and the short arm length corresponds to the SEM measurement, AR. The total rod length from SEM measurements and live larval measurements were compared using a t-test. Data were analyzed in Microsoft Excel.

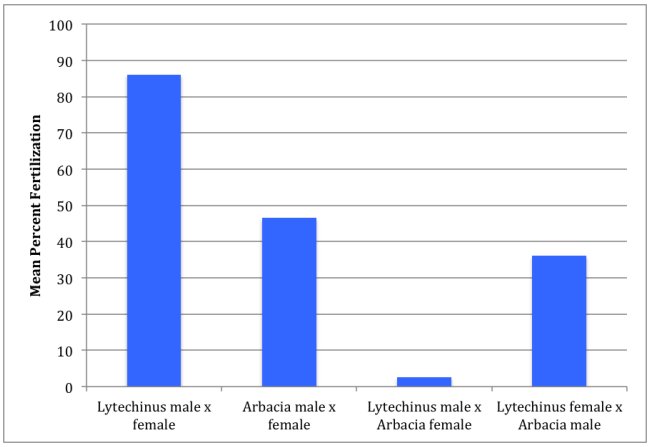


Fig. 1: Percent Fertilization in control and hybrid crosses. Values are the mean percent fertilization of each replicate ($n = 100$).

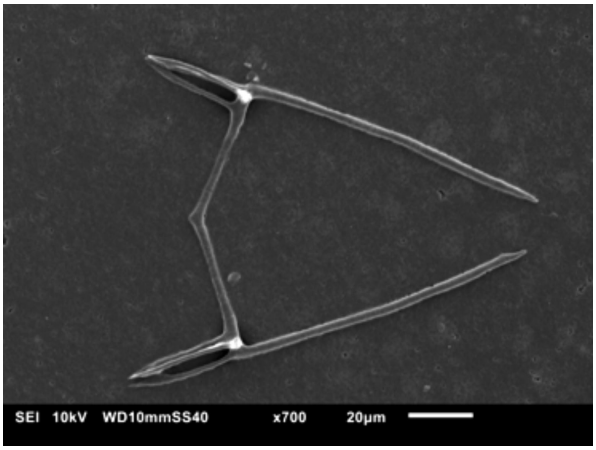
3 RESULTS

Percent Fertilization

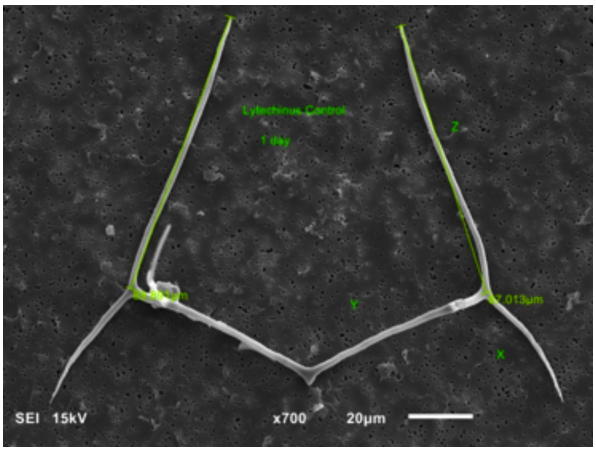
Percent fertilization was highest, 87%, in the *L. variegatus* control cross, 45% in the *A. punctulata* control cross, 37% in the *L. variegatus* female × *A. punctulata* male hybrid cross, and below 5% in the *A. punctulata* female × *L. variegatus* male hybrid cross (Figure 1).

Skeletal Morphometrics

Terminology for skeletal rods described by McEdwards & Herrera (1999) was used for the purposes of describing skeletal morphometrics. At the prism stage, only the Apex (A), Body Rods (BR), Transverse Rods (TR), and the beginning of the Post-oral Rods (PR) are present (Figure 2). The prism stage of each control cross, *A. punctulata* and *L. variegatus*, had minor differences.



(a)



(b)

Fig. 2: SEM Micrographs. Prism stage skeletal morphology. (a), *A. punctulata* prism stage electron micrograph, (b), *L. variegatus* prism stage electron micrograph, in (b), an example of the measurements taken of each skeletal element.

In both control crosses A was fused, BR were smooth, and TR was fused. The only difference was observed in the PR where *A. punctulata* had three separate spicules and only one spicule was observed in *L. variegatus*. (Figure 2).

At the base of the four-arm pluteus in Figure 3, the Apex (A) is purple. Extending from the Apex are the two Body Rods (BR) in purple. The Postoral Rods, in blue, are an extension of the BR. The Transverse Rods (TR), in green, connects the two BR and PR. At the base of the PR the Anterolateral Rods (AR), in red, begin to form. After formation of the AR, the Recurrent Rods (RR), in yellow, begin to form at the base of the AR (Figure 3).

The four-arm plutei from each control cross, *A. punctulata* and *L. variegatus*, had very distinctive skeletal features. The most prominent differences were seen in the A, TR, and PR. The Apex of the *L. variegatus* control cross was not fused and consisted of very spine-like spicules whereas the Apex of *A. punctulata* was fused and consisted of flat spines (Figure 4). The BR of *L. variegatus* consisted of short spike-like spines while the BR of *A. punctulata* were smooth

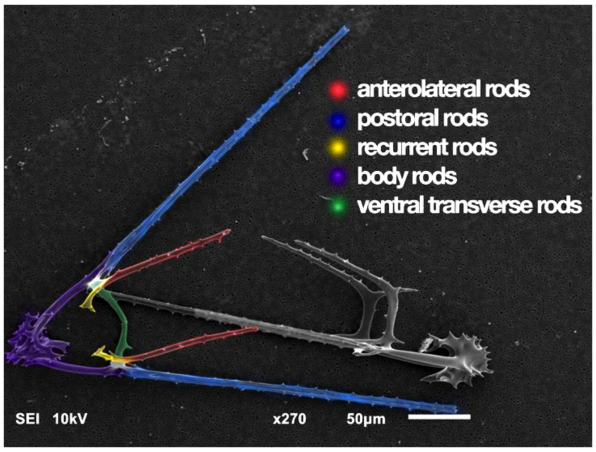
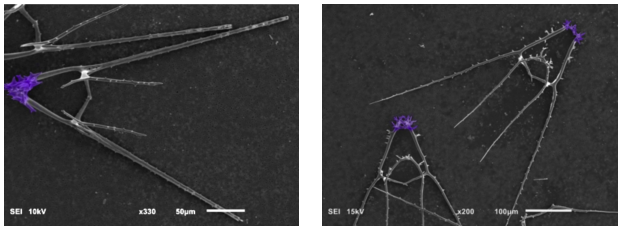
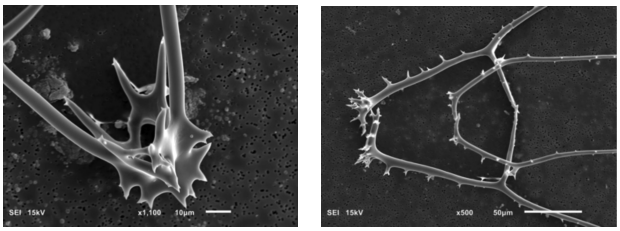


Fig. 3: SEM Micrograph. Skeletal terminology of the four arm pluteus larvae after McEdwards & Herrera (1999). Front view (colored) and side view of a 6-day *A. punctulata.* Apex (A) and Body Rods (BR) in purple, Postoral Rods (PR) in blue, Transverse Rods (TR) in green, Anterolateral Rods (AR) in red, and Recurrent Rods (RR) in yellow.



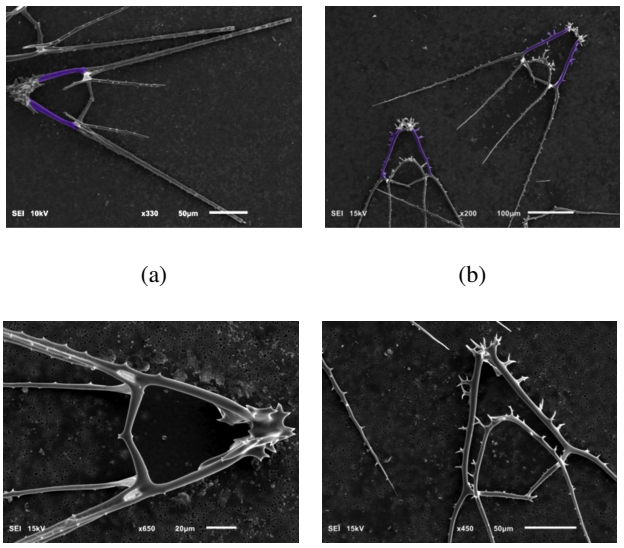
(a) (b)



(c) (d)

Fig. 4: SEM Micrographs. Comparison of the Apex, in purple, of (a), *A. punctulata* and (b), *L. variegatus*. (c), The Apex of *A. punctulata* is fused with flat spines. (d), The Apex of *L. variegatus* is not fused with several short spike-like spines.

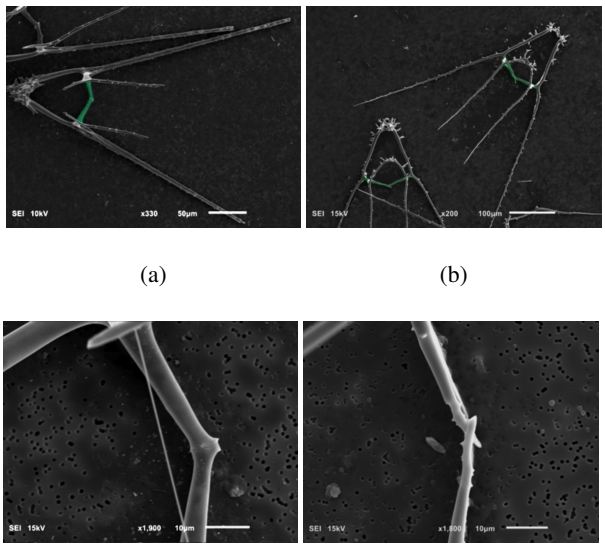
(Figure 5). The PR of *L. variegatus* consisted of one spicule with several long spike-like spines, but the PR of *A. punctulata* were made up of three fused spicules resulting in a fenestrated arm (Figure 6). The TR of *L. variegatus* was not fused and had several short spike-like spicules, while the TR of *A. punctulata* was fused and smooth (Figure 7). There were some minor differences in the



(a) (b)

(c) (d)

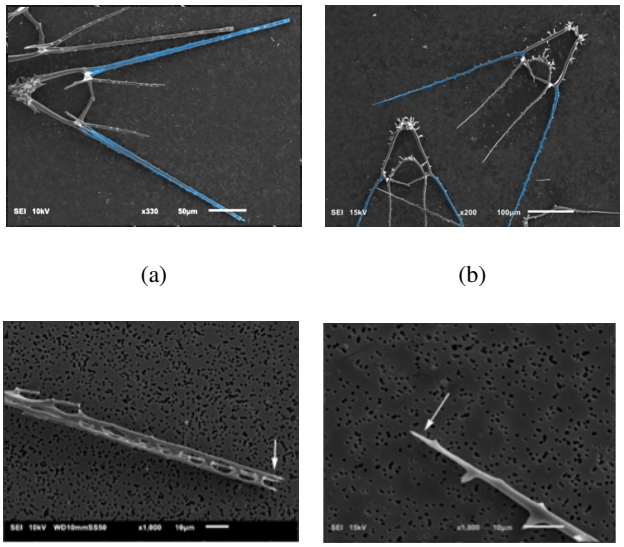
Fig. 5: SEM Micrographs. Comparison of the Body Rods, in purple, of (a), *A. punctulata* and (b), *L. variegatus*. (c), The Body Rods of *A. punctulata* are relatively smooth. (d), The Body Rods of *L. variegatus* have lateral spike-like spines.



(a) (b)

(c) (d)

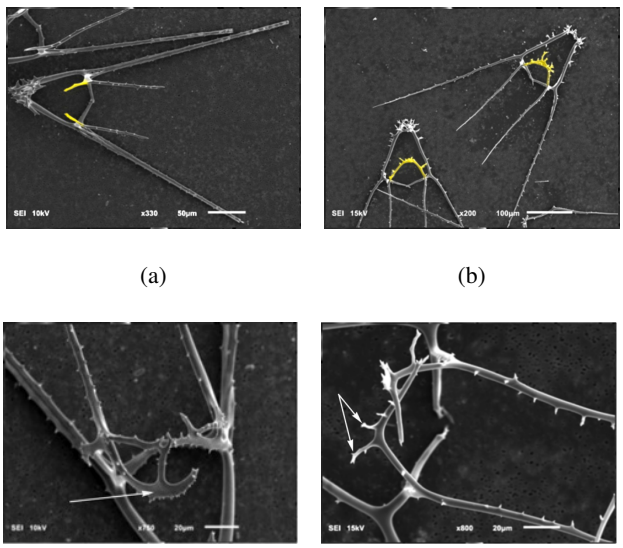
Fig. 7: SEM Micrographs. Comparison of the Transverse Rods (TR), in green, of (a): *A. punctulata* and (b): *L. variegatus*. (c): The TR of *A. punctulata* are fused and smooth. (d): The TR of *L. variegatus* are not fused and have several short spines.



(a) (b)

(c) (d)

Fig. 6: SEM Micrographs. Comparison of the Postoral Rods (PR), in blue, of (a), *A. punctulata* and (b), *L. variegatus*. (c), The PR of *A. punctulata* are fenestrated consisting of three fused spicules, note the three spicules at the tip of the PR (arrow). (d), The PR of *L. variegatus* is a single spicule with longer spike-like spines (arrow).

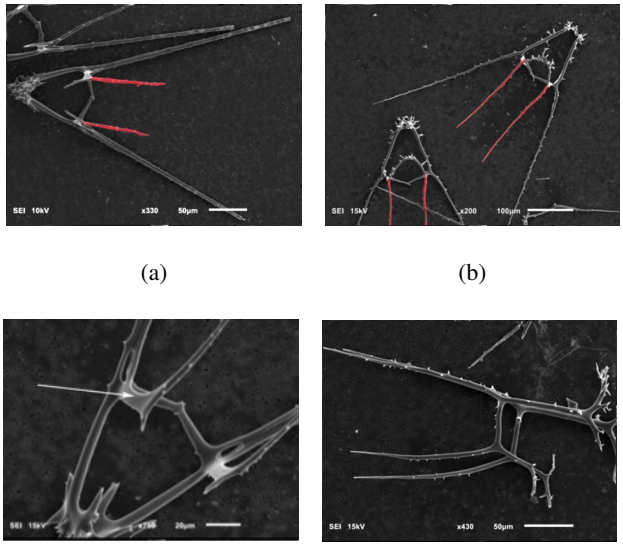


(a) (b)

(c) (d)

Fig. 8: SEM Micrographs. Comparison of the Recurrent Rods (RR), in yellow, of (a): *A. punctulata* and (b): *L. variegatus*. (c): The RR of *A. punctulata* curve inward at a circular angle and have several short spines (arrow) (d): The RR of *L. variegatus* curve inward at more of a right angle and have several longer spike-like spines (arrows).

BR, and RR of each control cross, *L. variegatus* and *A. punctulata*. The RR of *L. variegatus* curved inward at a sharp angle and consisted of long spike-like spines, but the RR of *A. punctulata* curved inward at a smooth circular angle and had several short bristle like spines (Figure 8). The AR of each control cross,



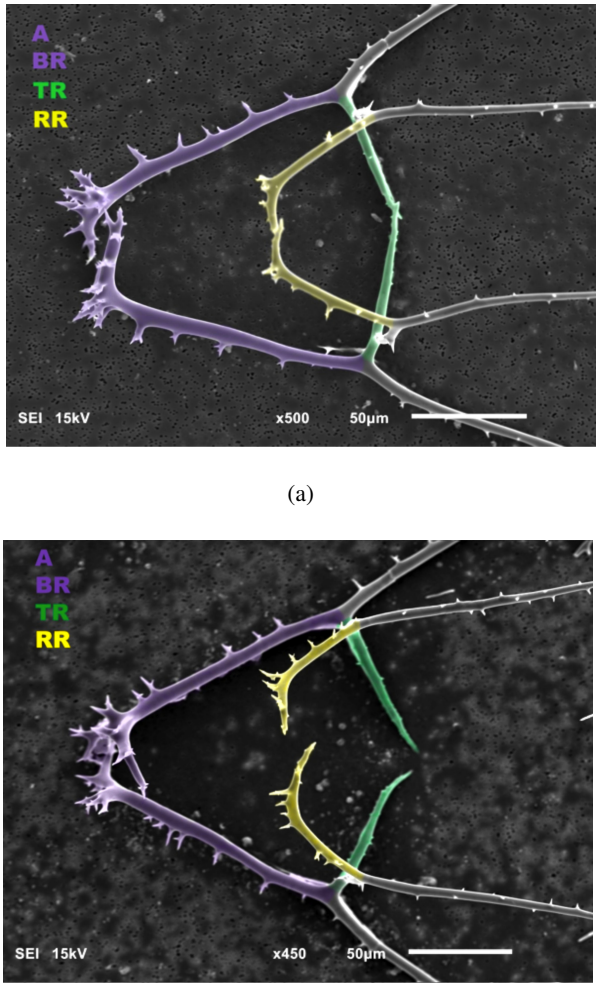
(a) (b)

(c) (d)

Fig. 9: SEM Micrographs. Comparison of the Anterolateral Rods (AR), in red, of (a): *A. punctulata* and (b): *L. variegatus*. (c): The base of the AR in *A. punctulata* is flattened (arrow), unlike the rest of the rod which is circular. (d): The base of the AR in *L. variegatus* is circular and is similar to the rest of the rod.

L. variegatus and *A. punctulata*, had no differences, they were both one spicule consisting of several short spines (Figure 9).

The hybrid cross, *L. variegatus* female × *A. punctulata* male, four-arm pluteus larvae were almost identical to the control, *L. variegatus* × *L. variegatus*, with a few minor differences observed in some larvae. The A, in purple, of the hybrid and the control crosses was not fused and had several spike-like spines (Figure 10). The BR, in purple, of the hybrid and control crosses had several spike-like spines (Figure 10). The TR, in green, of the hybrid and control crosses was not fused and had several short spines (Figure 10). Some minor differences were observed in the PR, AR, and RR. The RR, in yellow, of the hybrids appeared to have longer spines than the control crosses (Figure 10). At the base of the AR, in red, in the hybrid four arm pluteus, there was a series of spikes, whereas the base of the AR in the control was smooth (Figure 11). The PR, in blue, in the hybrid larvae had a tip with two spicules that fused further up the PR instead of just a single spicule observed in the control (Figure 12).



(a)

(b)

Fig. 10: SEM Micrographs. Comparison of (a): *L. variegatus* control larvae and (b): *L. variegatus* female × *A. punctulata* male hybrid larvae. Both the Apex (A), in purple, of the control (a) and hybrid (b) are not fused and have several spike-like spines. The Body Rods (BR), purple, of each larva have several spike-like spines. The Transverse Rods (TR), in green, are not fused and have several short spines. The Recurrent Rods (RR), in yellow, curve in at almost a right angle, however, the spines on the RR of the hybrid (b) appear longer than the control cross (a).

Preliminary Measurement Data

In a replicate of the *A. punctulata* control cross, the Anterolateral Rods (AR) initially grew until day four and then shrank at day six while the Post-oral Rods (PR) continued to grow (Figure 13). Total Rod Length, (BR + AR + PR), of the *A. punctulata* control was generally smaller than the *L. variegatus* control (Figure 14). Also, the total rod length of *L. variegatus* female × *A. punctulata* male hybrid was slightly larger than *L. variegatus* control (Figure 14). When live larval size and prepared skeletal spicule size (SEM) were compared, the skeletal spicule measurements produced consistently smaller total sizes. (Figures 15, 16 & 17).

4 DISCUSSION

Percent Fertilization

The mean percent fertilization was highest in the *L. variegatus* control crosses (87%), lower in the *A. punctulata* control crosses (45%), still lower in the *L. variegatus* female × *A. punctulata* male hybrid crosses (37%), and lowest in the *A. punctulata* female × *L. variegatus* male hybrid crosses (5%). Percent fertilization in the *L. variegatus* control cross followed trends seen in previous experiments, however *A. punctulata* had a lower than predicted percent fertilization.

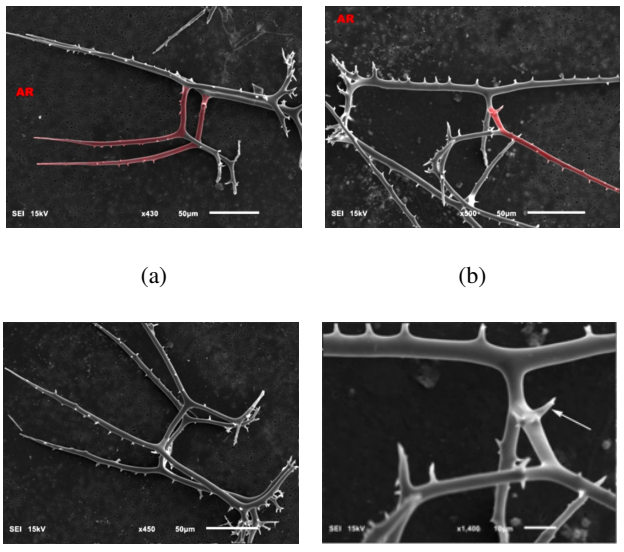


Fig. 11: SEM Micrographs. Comparison of the Anterolateral Rods (AR), in red of (a): *L. variegatus* control cross and (b): *L. variegatus* female × *A. punctulata* male. (c): The base of the AR in the control larvae appears to have a normal spine pattern, whereas, (d): The base of the AR in the hybrid larvae has a series a longer spike-like spines (arrow).

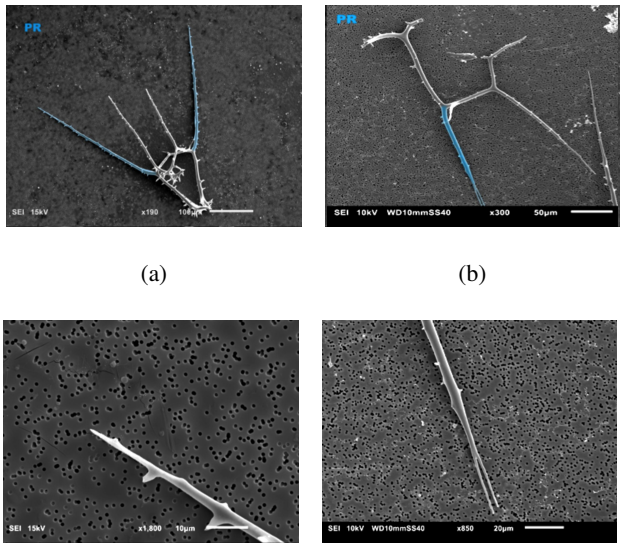


Fig. 12: SEM Micrographs. Comparison of the Postoral Rods (PR), in blue, of (a): *L. variegatus* control cross and (b): *L. variegatus* female × *A. punctulata* male. (c): The tip of the PR in the control larvae shows the rod consists of only one spicule. (d). The tip of the PR in the hybrid cross splits into two spicules, suggesting that the rod is made up of two or more spicules.

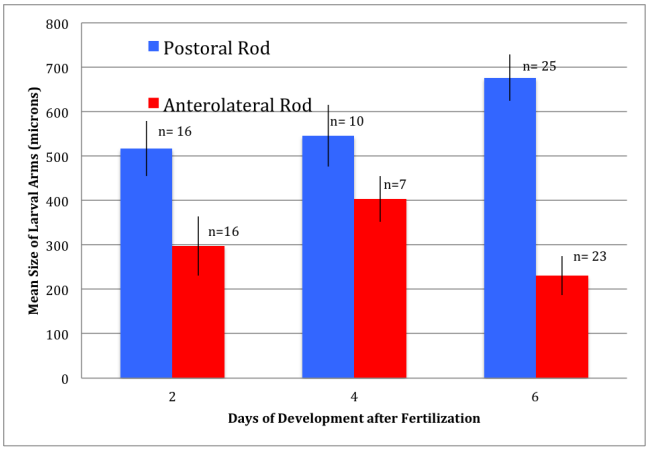


Fig. 13: A comparison of the Postoral Rod (PR), in blue, versus Anterolateral Rod (AR), in red, growth in *A. punctulata*. It appears that the PR continue to grow in length while the AR begin to grow then shorten. Bars indicate one standard deviation.

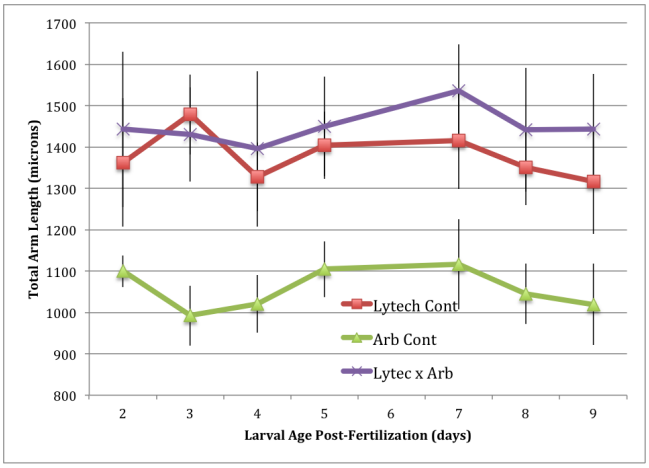


Fig. 14: Total Arm Length, the sum of long arms (BR+PR) plus short arms (AR) for live larvae for each treatment: *L. variegatus* control, *A. punctulata* control, and *L. variegatus* female × *A. punctulata* male, over a span of nine days. Values represented are means of at least 25 larvae measured for each day. Bars represent one standard deviation.

fertilization in the *Arbacia* control was likely due to the time of adult collection (March) at the beginning of their normal spawning season. The low mean percent fertilization in the hybrid crosses was not unexpected due to the considerable phylogenetic distance between these genera (Minor et al., 1991). The percent fertilization in reciprocal hybrid crosses using more closely related species of sea urchins has been reported to be higher (Rahman et al., 2004). Asymmetry in hybrid crosses between different species of sea urchins has been reported previously (Rahman et al., 2001).

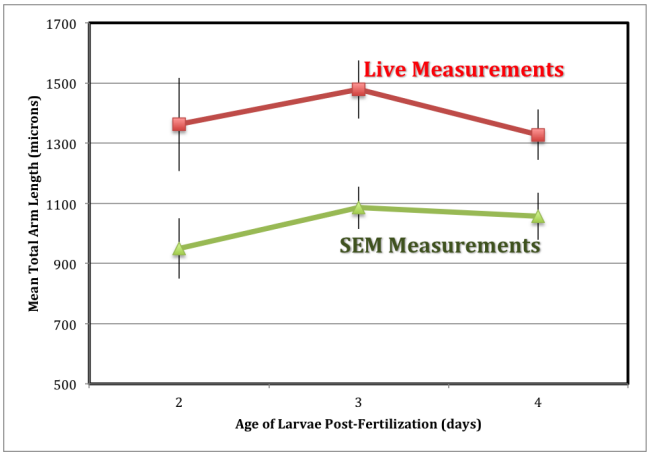


Fig. 15: Live measurements (sum of arm lengths) compared to SEM measurements (sum of corresponding spicule lengths) for *L. variegatus* control. Number measured at each interval was 25 or more. Bars represent one standard deviation.

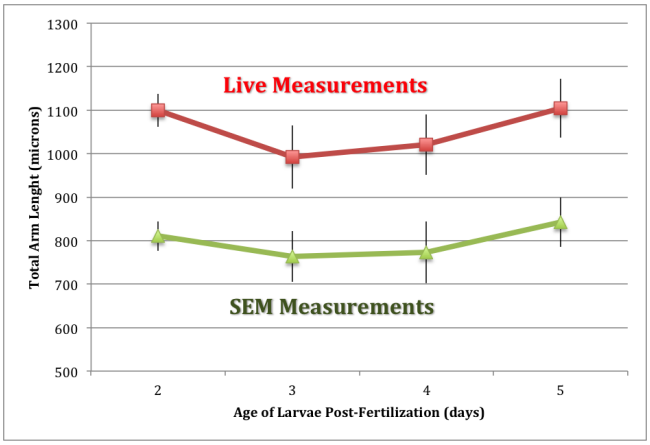


Fig. 16: Live measurements (sum of arm lengths) compared to SEM measurements (sum of corresponding spicule lengths) for *A. punctulata* control. Number measured at each interval was 25 or more. Bars represent one standard deviation.

Skeletal Morphometrics

The prism stages of each control cross were very similar except for the early postoral rods which were composed of a single spicule in *L. variegatus* and three converging spicules in *A. punctulata*. (Figure 2).

The four-arm plutei from each control cross had very distinctive skeletal features. The most prominent differences were seen in the Apex (A), the Body Rods (BR), the Transverse Rods (TR), and the Postoral Rods (PR) (Figures 4, 6 & 7). The fused tips of the BR in *A. punctulata* formed a complex, spiny apex and were very different from the unfused, overlapping tips of the BR in *L. variegatus*. The PR in *A. punctulata* were fenestrated, being composed of three fused skeletal spicules and very different from the single spicule PR in *L. variegatus*. There were some minor differences in the Anterolateral Rods (AR), and Recurrent Rods (RR) of the control larvae (Figures 5, 8 & 9). These differences serve as a good marker for use in comparison with hybrid skeletons of these two species.

The hybrid four-arm pluteus larvae from *L. variegatus* female × *A. punctulata* male were almost identical to the *L. variegatus* control larvae with a few minor differences observed in the PR, AR, and RR (Figures 10, 11, 12). The differences described were only observed in a few larvae; more hybrid larvae need to be generated for analysis to try to quantify these differences. Also, a method for quantifying spine type needs to be investigated for a better determination of differences in the control versus the hybrid crosses. If differences in the hybrid larvae are identified, then these markers can be used to determine if hybridization is occurring in the field by taking plankton tows in the area of distribution overlap during overlapping spawning season and analyzing the pluteus larvae found. The similarity of hybrid larvae to the maternal parent suggests that hybrid larvae may be parthenogenic, in which the sperm activates the fertilization process but does not contribute to the larval genome (Moore, 1957). Chromosome counts in hybrid and control larvae were not successful. Methods involving stained sperm and fluorescent microscopy are being tested for use in determining whether the zygote genome has incorporated the male genome. The skeletal features of the four-arm pluteus larvae, *A. punctulata* female × *L. variegatus* male, still need to be examined. Most larvae of this cross did not survive to the desired four-arm pluteus stage.

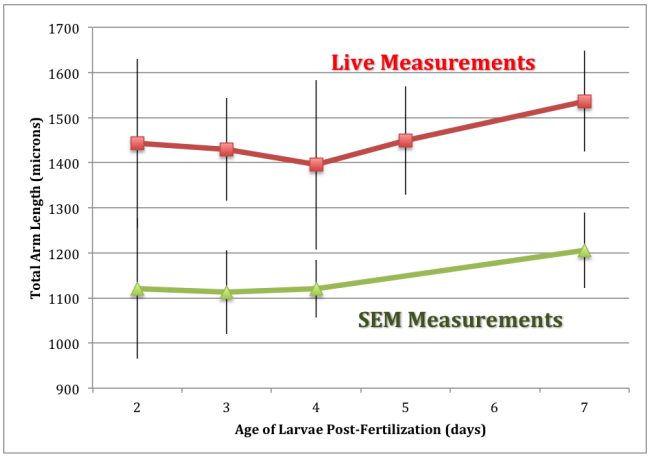


Fig. 17: Live measurements (sum of arm lengths) compared to SEM measurements (sum of corresponding spicule lengths) for *L. variegatus* female × *A. punctulata* male hybrid cross. Number measured at each interval was 25 or more. Bars represent one standard deviation.

Preliminary Measurement data

Measurement data indicated that for all larvae produced, the Postoral Rods (PR) (Figure 12) shrunk as larval growth proceeded and Anterolateral Rods (AR) continued to grow. *A. punctulata* control larvae were generally smaller than *L. variegatus* control larvae and *L. variegatus* female × *A. punctulata* male hybrids.

Growth of larvae in all treatments, *L. variegatus* control, *A. punctulata* control, and *L. variegatus* female × *A. punctulata* male hybrid, did not continue to increase over time as expected but rather displayed an unusual pattern of increases and decreases in size. This unpredicted pattern is likely not due to sample size since 30 larvae were typically analyzed at each time interval for each treatment. Factors such as ambient temperature in the lab and food availability may have contributed to the variations in growth data. Temperature has been known to have an effect on growth, in which cold temperatures slow growth rates and higher, tolerable, temperatures increase growth rates (McEdwards, 1984, 1986a,b). In laboratory experiments conducted with *L. variegatus* by (McEdwards & Herrera, 1999), larvae continued to increase in size until near metamorphosis. The differences in measurement size data between live larvae and prepared skeletons was likely due to the larval tissue that surrounds the skeletal rods in the live larvae. This would explain the larger sizes measured in live larvae compared to skeletal spicule preparations where all soft tissue was removed.

This work was made possible by a Delo Research Grant from the University of Tampa to one of us (SAR) and a Biology Summer Research Fellowship Grant to one of us (EEM). This research was part of a BIO 450 Research Capstone experience for EEM with SAR serving as advisor. Individuals who helped with this project include Matt Lyon, Lyndie Jordan, Rhonda Johnson and Jennifer Messink. The manuscript was improved through comments from an anonymous reviewer.

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