What to expect when you're expecting (larvae): The effects of four algal diets on the development of *Pisaster ochraceus* larvae

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Abstract

Echinoderm larvae, particularly those of class Asteroidea, are well-studied in a variety of fields ranging from marine ecology to developmental biology. Larvae are useful study organisms in that they are generally easily accessible and can be raised in large numbers at minimal cost. A proper diet is essential for raising larvae to metamorphosis and through the juvenile stage. This study investigates the effects of four algal diets on *Pisaster ochraceus* larvae in their bipinnaria stage: *Rhodomonas sp.*, *Dunaliella tertiolecta*, *Isochrysis sp.*, and a combination of the three. Larval size was quantified sixteen days post-fertilization by measuring the maximum larval length, maximum larval width, maximum gut width, and right enterocoel length. Only the maximum larval width proved to be significantly different due to the diet treatments, with the *D.tertiolecta* treatment growing the widest. One replicate of the combined diet treatment yielded larvae with a fused enterocoel, indicating an advanced stage of development, likely due to a decreased larval density as a result of high mortality. Although largely inconclusive, this study offers further insight into the effects of different algal diets on developing echinoderm larvae.

Introduction

The study of larvae, marine or otherwise, offers a unique opportunity to examine a variety of developmental processes. Echinoderm larvae, in particular, make it possible to observe the emergence of juvenile structures (such as podia and ossicles), a change in symmetry from bilateral to pentameral, and the habitat shift from the water column to the seafloor. They can also be reared to metamorphosis at relatively low cost in large numbers. For these reasons, echinoderm larvae make good study organisms in a wide variety of subjects including

developmental biology, evolutionary biology, biomechanics, physiology, and the marine sciences (Hodin et al. 2019).

Echinodermata is an extraordinarily diverse phylum, with Asteroidea being one of the most well-studied classes. Even within this class, post-embryonic development proceeds in different ways: via benthic broods underneath the mother (e.g., *Leptasterias*), a non-feeding planktonic stage (e.g., *Porania*), or a feeding planktonic stage (e.g., *Pisaster*) (Strathmann 1975). The planktonic larvae generally pursue a pelagic lifestyle before undergoing metamorphosis and settling on a substrate to begin the benthic juvenile phase (Heyland et al. 2005). This study focuses on *Pisaster ochraceus* larvae beginning immediately after fertilization. These larvae are feeding, or planktotrophic, and first develop through a bipinnaria stage. This stage is characterized by a bilaterally symmetrical larva with ciliated swimming and feeding bands, an ovoid body, and a complete gut (reviewed in McEdward and Miner, 2001). Successfully rearing these planktotrophic larvae requires a live algal diet that is typically administered soon after changing the water in their culture vessel.

Common laboratory algal diets for *P. ochraceus* larvae usually include three species: *Rhodomonas sp.*, *Dunaliella tertiolecta*, and *Isochrysis sp*. (Hodin et al. 2019). These three algal species have proven to be sufficient for raising echinoderm larvae to competency either as single or mixed diets (George 1999, Schiopu et al. 2006, George and Walker 2007). In his observations of the appearance of algal cells in echinoderm larvae guts Strathman (1971) observed *Dunaliella* being rapidly digested in the stomach of *Pisaster* larvae, while *Isochrysis* cells did not disintegrate quickly. Wray et al. (2004) recommended a 1:1 mix of *Rhodomonas sp.* and *D. tertiolecta*, and noted that *Isochrysis sp.* should be utilized in a mixed diet only. Schiopu et al.

(2006) found that a mixed diet of *Isochrysis sp.* and *D. tertiolecta* yielded the highest rate of larval survival, as well as the quickest development to metamorphosis. The second best results came from a diet of only *Dunaliella sp.*. George et al. (2008) stated that all three common algae species have successfully raised echinoderm larvae to metamorphosis as a single diet, despite having different nutritional capabilities. In this study, *P. ochraceus* larvae were reared from fertilization to the bipinnaria stage via four different algal diets: three single-alga diets of the most common species described above, and one diet that combined the three.

The objective of this study was to further investigate whether *P. ochraceus* larvae will undergo the fastest development towards metamorphosis on a mixed algal diet consisting of three common species of algae (*Rhodomonas sp.*, *D.tertiolecta*, and *Isochrysis sp.*) compared to respective single-algal diets via measuring both larval structures and the elongating coelomic structure, which develops into the juvenile rudiment in more advanced larvae.

Materials and Methods

Fertilization

Three *Pisaster ochraceus* adults were collected from the University of Washington's Friday Harbor Laboratories at low tide: one male and one female from 48°32'45.9"N 123°00'27.3"W and one female from 48°32'44.2"N 123°00'45.8"W. It was not necessary to inject the male with a spawning inducer as it began releasing sperm soon after collection, which was gathered and immediately transferred to the refrigerator in an embryo safe vial. To obtain eggs, 1 mL of 1-methyladenine for every 100 mL of sea star volume was injected with a 16 gauge needle inside two opposite arms of the two female sea stars. *P. ochraceus* exhibits a lifting behavior

when the gonads contract, after which each female was placed ventral side up into a beaker to collect eggs. Fertilization was achieved on May 13, 2019 by combining 250 μL of eggs and a pipette with a minimal amount of sperm (to prevent multiple fertilization events on one egg). Approximately five days post fertilization (dpf), the embryos had developed complete guts and were separated into the experimental treatments.

Experimental Setup: Water Changes and Food Preparation

Twelve half-gallon, embryo-safe jars were obtained and washed thoroughly with cold tap water and reverse-osmosis micron filtered (RO) water. A 0.45 micron filter was used to purify seawater (~28-30 ppt) for the experiments. All jars were placed in a sea table with continuously flowing water of about 10.5°C (Fig 1). Paddles were placed in the jars and attached to a motor-driven stirring mechanism which allowed for continuous mixing of the larvae and their food (See Strathmann 1987, Hodin et al. 2019). Approximately 1,000 larvae were added to each jar, for a density of 1 larva per 1.5 mL. Water changes were made every other day by concentrating the bipinnariae into about 100 mL of water via reverse-filtration through a 70-micron Nitex mesh (see Figure 1A, Hodin et al. 2019). The larvae were then gently poured into a small bowl while each jar was thoroughly rinsed with cold tap water, followed by RO water, and scrubbed every other water change. The 0.45-micron filter was used to add 1400 mL of FSW back into each jar, after which the larvae and paddle were returned to the jar and placed back on the stirring mechanism in a random position to minimize any effect of position in the sea table (e.g. ambient light levels) on development rate.

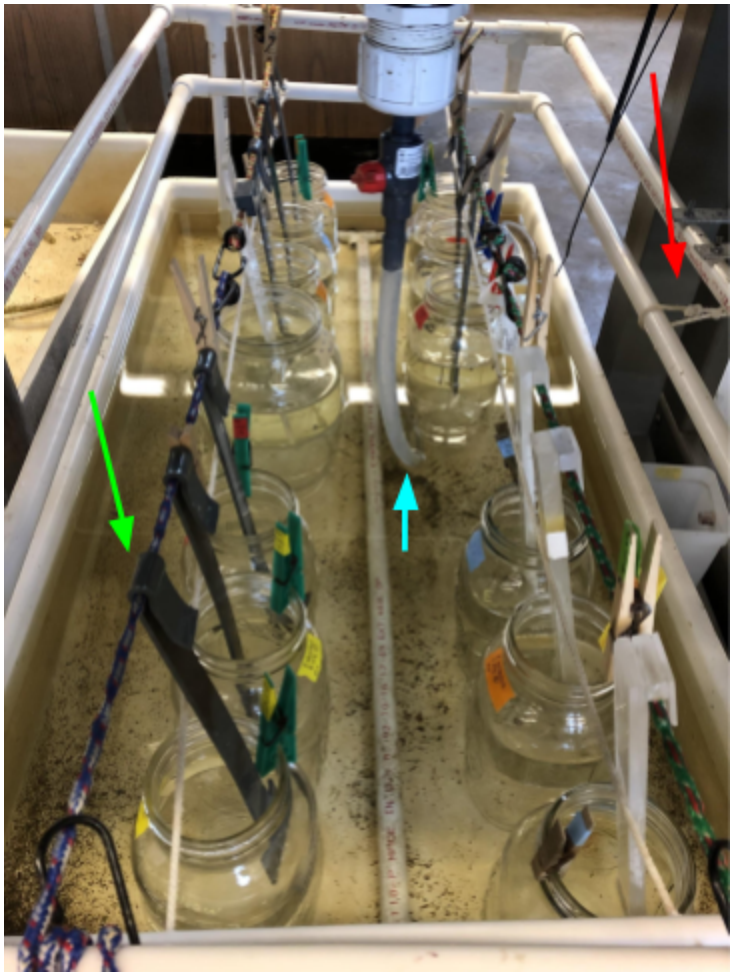

Figure 1. Experimental Setup showing the seawater hose (blue arrow), paddle connected to the stirring mechanism (green arrow), and where the motor connects to the stirring mechanism (red arrow). Motor itself is offscreen.

Algal cultures of *Rhodomonas sp.*, *Dunaliella tertiolecta*, and *Isochrysis sp.* were grown in vitamin-enriched F/2 medium. Four experimental groups, each with three replicates, were fed one of four food treatments: 2500 cells/mL of *Rhodomonas sp.* (R treatment), 3000 cells/mL of *D. tertiolecta*, *sp.* (D treatment), 20,000 cells/mL of *Isochrysis sp.* (I treatment), and a combined treatment of 933.3 cells/mL *Rhodomonas sp.*, (2,333.3) cells/mL of *D. tertiolecta*, and 26,666.7 cells/mL of *Isochrysis sp.* The combined treatment concentrations were obtained from Pia et al. (2012). Each culture was counted on a hemocytometer and concentrated using an International

clinical centrifuge, model CL (International Equipment Co., Needham Heights, Mass.), for 15 minutes at medium speed. The culture medium was decanted off the resulting pellet, which was resuspended with FSW and distributed to the corresponding treatments. For details, see Strathmann (2014).

Data Collection and Statistical Analysis

Larval measurements were collected sixteen dpf on May 29, 2019. Ten larvae per jar were randomly selected by pipetting them onto slides with a raised coverglass (via molding clay). The microscope was slightly out of focus in an attempt to eliminate any selection bias. The following larval structures were measured at 40X magnification via an Olympus compound microscope (Model BH-2, Scientific Instrument Co.): maximum larval length, maximum larval width, maximum stomach width, and length of the right enterocoel (Fig 2). Each of the four metrics was averaged and plotted to obtain one value per metric per treatment for visualization purposes.

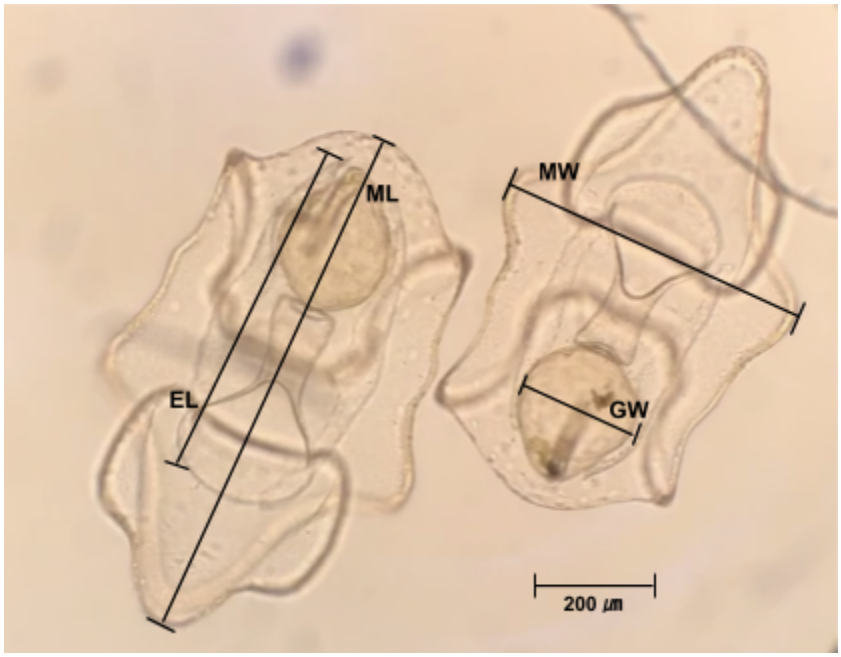

Figure 2. Four metrics were measured to investigate the effects of different algal diets on development: maximum larval length (ML), right enterocoel length (EL), maximum larval width (MW), and maximum gut width (GW).

The larval size metrics for each jar (n=10) were averaged to obtain a single value for each metric, per jar. The Analysis ToolPak, installed on Microsoft Excel (v. 16.25), was used to conduct four single-factor ANOVA tests (significance of $p<0.05$) to determine if the food treatments significantly affected larval development.

Results

There was a significant effect of algal diet on average larval maximum width at the $p<0.05$ level [$F(3, 8) = 3.977$, $p=0.052$] (Table 1). Algal diet did not have a significant effect on average larval maximum length [$F(3,8) = 2.298$, $p=0.154$], average maximum gut width [$F(3,8) = 1.132$, $p=0.393$], and average right enterocoel length [$F(3,8) = 2.617$, $p=0.123$].

Table 1. ANOVA Single-Factor Alpha .05 results for each metric.

Measurement	p value	Significant?
Avg Max Length	0.154	No
Avg Max Width	0.052	Yes
Avg Max Gut Width	0.393	No
Avg R Enterocoel Length	0.123	No

The average maximum larval lengths and widths were similar in that the larvae fed only *Isochrysis sp.* had the smallest measurements, and the *Dunaliella tertiolecta* treatment had the largest measurements. Interestingly, the *Rhodomonas sp.* and combined treatments were similar (Fig 3A&B). The combined and *D. tertiolecta* treatments had the largest average gut width, although the values only ranged from 190-218.5 μm (Fig 3C). The combined treatment resulted in individuals with the largest average right enterocoel length, whereas the *Isochrysis sp.* treatment had the smallest values (Fig 3D). The third replicate of the combined diet treatment had a significantly lower larval density than the others, which was noticed when selecting the larvae for measurement. This replicate was the only one in which merged enterocoels were observed.

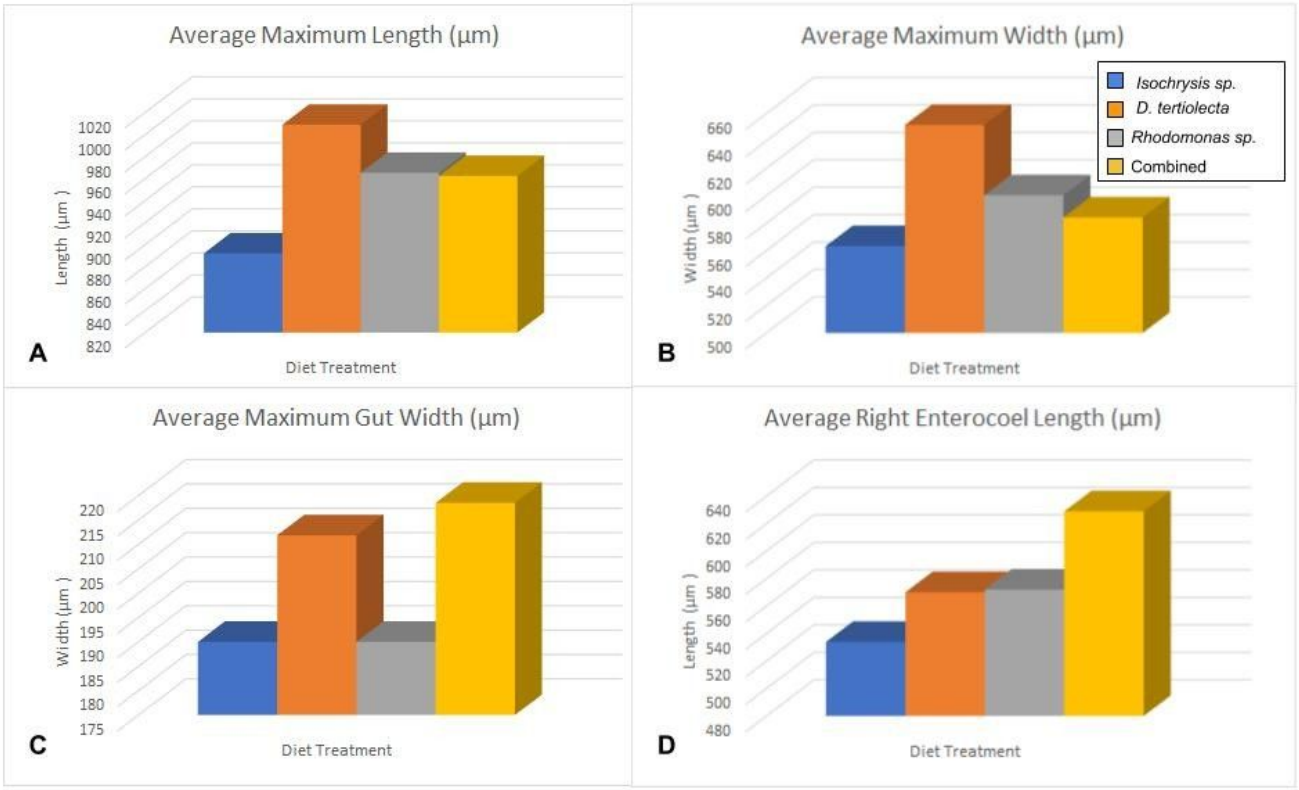

Figure 3. Average larval measurements in micrometers (µm) for each diet treatment.

Discussion

The short nature of this study resulted in only early-stage bipinnaria larvae being observed. As such, the results from this study should only be used to infer the effects of separate algal diets on this stage, and not later developmental stages, considering such conclusions would be speculative. It is not surprising that there was no significant difference between three of the four metrics, given that development was only allowed to take place for sixteen days (Table 1). The *Dunaliella tertiolecta* treatment had a significantly larger average maximum larval width than the other three treatments (Table 1, Fig 3B), but it is difficult to say exactly what mechanism is responsible for this difference. Some larvae exhibit larger feeding structures when

food resources are scant, while juvenile structures (such as the coelom) develop at a faster rate when food is widely available (Bertram and Strathmann 1998). Strathmann (1971) stated that a larva's energy would likely go into developing feeding structures that could accommodate a high particle intake rate. This could indicate that the *D. tertiolecta* treatment developed a greater average maximum larval width because the concentration of food was too low. Bertram and Strathmann (1998) also stated that large amounts of quality food would lead to the accelerated development of juvenile structures in an effort to approach competency faster. Three larvae in the third replicate of the combined diet treatment exhibited joined enterocoels, indicating a stage of development later than all other larvae observed; however, this cannot be attributed to the experimental treatments since the enterocoel length measurement was not significantly different with the four diets. Additionally, that particular jar had a noticeably lower larval density, so is it possible that the larvae in that jar were further developed for that reason.

Several avenues could be taken to improve future variations of this study. When counting the algae cells using the hemocytometer, a more consistent count could be obtained by killing the cells using a drop of Lugol's fixative, as proposed by Strathmann (2014). Ideally, this experiment would run until the larvae transitioned through the brachiolaria stage and became competent to undergo metamorphosis and settle. This study focused only on the bipinnaria stage due to time constraints. This experiment could be repeated with a lower larval density, which may further quicken development due to less resource competition. Bertram and Strathmann (1998) only used 200 larvae per 1.5L compared to the 1,000 used in this study. If the experiment could go longer, with a lower density, conducting multiple days of measurements would remove larvae periodically and take away some of the stress that comes as the larvae grow and develop

faster rates of food intake. Future experiments could include a treatment consisting only of unfiltered seawater, or of plankton gathered from the field, to further investigate larval dietary needs.

Investigating the effects of various diets on *Pisaster ochraceus* larval development is helpful not only because of the active role the adults play in shaping the intertidal, but because of their potential as easily attainable test organisms for a variety of subjects. Rearing these larvae to metamorphosis has been made possible by the work of pioneers like Strathmann, George, and Hodin. Determining which algal diet allows the most rapid, successful larval development would perhaps encourage others to perform experiments on these widely accessible organisms. Studying the developmental stages of this echinoderm can likely provide valuable insight in fields with ranges of applicability far outside marine ecology.

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assisted me with the experimental setup and along with Ally Arnold, helped me keep a cool head during the entire experimental process. Ashley Cullen, Justin Harshman, Ryu Kawada, and Sanna Titus were an incredible help during water changes; they rinsed materials in between jars and monitored the reverse-filtration mechanisms so I could thoroughly inspect each treatment. Jason Hodin and Karly Cohen provided incredible guidance both on this project and its write-up, but also over the course of the entire quarter. Karly, in particular, was essential in the data analysis phase and helped me decide what statistical method would be best. The other two teaching assistants associated with the Zoobot program, Dr. Christopher Wells and Mo Turner, also provided immense help in the form of guidance on statistical analyses, moral support, and how to construct scientific papers. The entire Zoobot 2019 program has my thanks for being a supportive, inclusive, and motivating group to work with. Thank you for an incredible quarter!

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