In Vitro* Kelp Crab (*Pugettia producta)* Embryo in Various Salinities with Comparisons to Embryos Raised *In Vivo

Allison Arnold

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Friday Harbor Laboratories, University of Washington, Friday Harbor, WA 98250

Contact Information:

Allison Arnold
Biology Department
University of Washington
Seattle, WA 98105
allyjean@uw.edu

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Allison Arnold
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University of Washington Friday Harbor Labs

Abstract

Developing embryos of near-shore marine organisms are subject to freshwater influx, which exposes them to low salinity conditions. This experiment tracked embryo development in kelp crabs (*Pugettia producta*) exposed to three salinity treatments (32, 23, and 9 p.p.t.). Data from this experiment indicate that the rate of embryo development is not affected by salinity within the range of 9 – 32 p.p.t. Survival, determined by presence of heart beats, was highest in 32 p.p.t. No embryos survived after 24 hours in the 9 p.p.t. treatment. In addition to salinity testing, rates of embryo development were compared between the embryos cultured in dishes (*in vitro*) and those allowed to develop in the abdomens of live adult females (*in vivo*). The results indicate that embryos develop slightly faster when raised *in vivo* rather than *in vitro*, but that embryos can be reared successfully *in vitro* without the use of antibiotics.

Introduction

Kelp crabs (*Pugettia producta*) have several qualities which make them convenient for the study of metamorphosis. Their reproductive cycle is not seasonally limited, meaning that gravid females with embryos at different stages can be found year-round (Strathmann, 1987). In addition, thousands of eggs are brooded on the pleopods of female crabs and develop synchronously (Strathmann, 1987). Therefore, each developing brood of eggs provides a large sample size of embryos that can begin an experiment in the same stage of development. In addition to their convenient reproductive patterns, they are abundant and relatively easy to capture from dock pillars along the West Coast of North America. Their diet is mostly brown algae including *Nereocystis luetkeana* and *Fucus distichus*, which are abundant and easy to collect in the Salish Sea.

Shallow, near-shore waters are susceptible to salinity fluctuations caused by fresh water influx from nearby streams. Horizontal fronts of variable salinity, temperature, and density, which create convergent circulation, are common in estuaries and nearby areas (Epifanio & Cohen, 2016). Since *P. producta* feed on photosynthetic brown algae, their habitat falls within the euphotic zone, where salinity fluctuations are common. It is therefore plausible that kelp crab embryos may be adapted to low salinity environments. If this is true, then one would predict that embryos exposed to low salinity will be able to survive and develop normally. This study analyzed survival and development rates of *P. producta* embryos exposed to salinities of 32, 23, and 9 p.p.t. to test the hypothesis that their embryos are well-adapted for low salinity conditions.

Many salinity experiments have been conducted on crab zoea (Tilburg et. al, 2009), but relatively few have been conducted on crab embryos. A recent study on the Blue Swimming Crab (*Portunus pelagicus*) tested the effects of various salinities on embryo development and hatching rate, allowing embryos to be raised *in vivo* (Ikhwanuddin et. al, 2016). Research has been done on the *in vitro* effects of salinity on early embryonic development of *Chasmagnathus granulata* and *Cyrtograpsus angulatus* (Bas & Spivak, 2000). Embryo development and reproductive cycles of *Pugettia producta* have been observed (Boolootian et. al, 1959), but I am unaware of any descriptions of this species' embryo development in varied salinity conditions.

Effective methods for rearing kelp crab embryos are useful for anyone interested in their embryology. An additional component of this study focused on development differences between embryos raised in dishes and embryos raised *in vivo,* on the mother's pleopods. Similar to this study, comparisons between *in vivo* and *in vitro* embryo development in various salinities were conducted on *Palaemonetes argetninus* (Giovagnoli et. al, 2014). Most literature on Brachyuran embryology deals with large-scale larval rearing of crabs rather than small-scale laboratory work, and with commercially important species such as *Rhithropanopeus harrisii* (Costlow et. al, 1966) and *Portunus pelagicus* (Ikhwanuddin et. al, 2016). *P. producta* have unique reproductive cycles and diets compared to other Brachyuran crabs, so their embryos may require different conditions than those of their relatives. Some risks associated with small-scale *in vitro* culturing of embryos include susceptibility to infection, exposure to chemicals, and temperature shock. Extra care must be taken when working with embryos to maintain their viability. Therefore, careful methodology on this topic is important. Documentation of the differences between embryos developing within the mother's abdomen and those raised in dishes can inform scientists whether or not *in vitro* experiments can accurately represent embryo development in the wild.

Methods

Six gravid kelp crabs (*Pugettia producta*) were collected in Friday Harbor, WA (USA) in May (**Table 1**). Most were found on the pillars under the Friday Harbor Labs dock. They were kept in a sea table (~11-12 degrees Celsius) with flow-through sea water and fed *Nereocystis luetkeana, Fucus distichus,* and *Alaria marginata*. Their sea table was cleaned weekly. Each female was tagged with a different colored zip tie for identification. The lengths and widths of each female's carapace were recorded, as well as the date and location of collection (see Table 1).

Table 1: Gravid female Kelp Crabs used for experiment. Embryos for *in vitro* salinity experiments were taken from Crabs 1 and 4 only. * Crab 2 was released on 05/22/19, because she was infested with worms.

Crab ID #	Date Collected	Location Collected	Carapace Width (cm)	Carapace Length (cm)	Initial Embryo Stage
1	05/11/2019	Friday Harbor Labs (FHL) dock pillars	6.5	6.3	5
2*	05/16/2019	Brown Island	8.1	7.5	2
3	05/16/2019	FHL dock pillars	5.5	6.2	2
4	05/10/2019	Brown Island	7	6	4
5	Unknown	Unknown; found in a sea table at FHL	7.5	6.8	4
6	05/21/2019	FHL dock pillars	6.5	6	7

On May 21, 2019, stages of embryos in all six gravid females were noted. Embryo removal from gravid females required one researcher to hold the crab while another gently pulled open the abdomen, picked up approximately ten embryos with forceps, and transferred them to a wet mount. Embryo stages described in a previous study on *P. producta* reproductive cycles

(Boolootian et. al, 1959) were assessed at 40x magnification (**Table 2**). Photos of the observed embryo stages are shown in **Figure 1.**

Table 2: Brachyuran crab embryo stages taken directly from Boolootian et. al (1959). The second column lists the panel in Figure 1 that corresponds to the listed stage.

Stage	Image (Figure 1)	Description
1	A	No segmentation observable. Circle completely crosshatched.
2	B	Cleavage has taken place. Circle completely crosshatched.
3	C	A yolk-free (transparent) part becomes apparent. This stage coincides with the appearance of endoderm cells and the beginning of invagination. Circle ¼ clear.
4	D	A more distinct division into a yolk-free and yolk-containing part becomes clearly visible. Circle 1/3 clear.
5	E	Eye pigment of the embryo becomes clearly visible. Circle ¼ clear.
6	F	Pigment bands of the embryo become visible. Circle ½ clear.
7	G	Larvae become strongly segmented but still contain much yolk. Circle 2/3 clear.
8	H	The yolk is reduced to 2 small separate patches. Circle ¾ clear.
9	I	Zoea larvae become recognizable. Clear circle.
10	n/a	Swimming larvae appear. Clear circle.

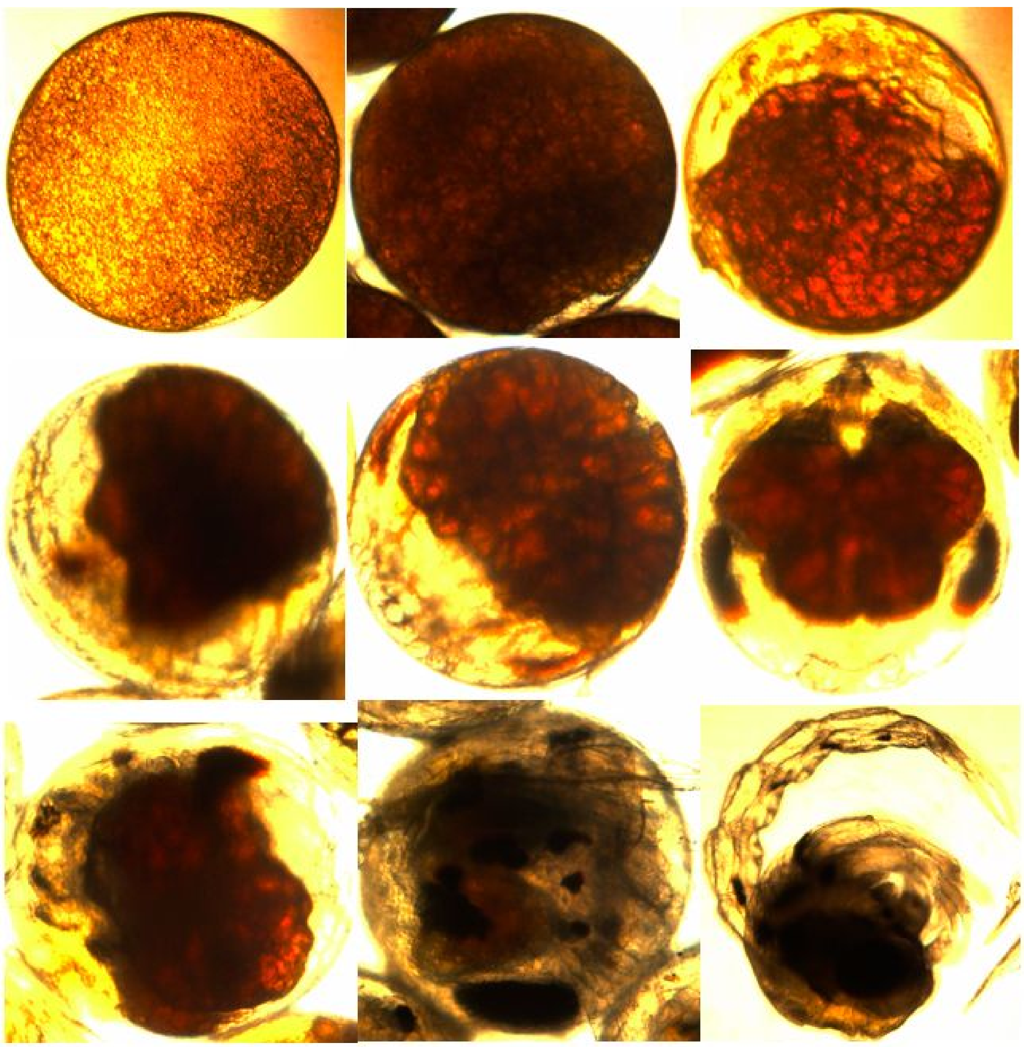
Also on May 21, 500-800 embryos were removed from Crabs 1 and 4, which were selected because they were at similar stages, to be used for *in vitro* salinity testing (see Table 1). Prior to the procedure, the adult crabs were kept on ice to numb any pain and slow their movements. Three salinity treatments were prepared in 1000 mL Erlenmeyer flasks using approximately 5 µm-filtered natural sea water, sterilized by boiling (henceforth SFSW). Treatment 1 was undiluted SFSW (32 p.p.t.). Treatment 2 was SFSW diluted with reverse-osmosis filtered fresh water to a final salinity of 23 p.p.t. Treatment 3 was SFSW likewise diluted to 9 p.p.t.. An estimated 200-300 embryos were pipetted into finger bowls containing 70 mL of each salinity treatment. Two replicates of each treatment were made for each experimental crab for a total of 12 bowls. Finger bowls were arranged in a shallow sea table with flowing water to maintain them at the same temperature as their mothers (~11-12 degrees Celsius). SFSW at the three salinities was maintained in the sea table and covered for future use in water changes.

In all replicates and treatments, the stages, sizes, survival, and worm infestation levels of ten randomly chosen embryos were assessed on a wet mount at 40x magnification every other day, starting on May 22, the day after initiation of treatments. Water changes of the salinity treatments were done by reverse filtration every other day using an 80 µm mesh filter. Size was recorded as the diameter of the embryos. Survival was assessed in embryos at Stage 4 or later by noting the presence or absence of heart beats. Infestation was noted by counting the number of worms, which appeared as gray lumps on the outside of embryos when they were not moving.

In addition, ten randomly chosen embryos from each of the five adult crabs were extracted and assessed every other day in the same manner, starting on May 23, two days after the initiation of the experiment. This allowed for comparisons between rates of development *in vivo* and *in vitro*, and also for comparisons of embryo progression among all five females.

ANOVA tests were conducted in R to compare the rates of embryo development in the three salinity treatments for each crab (R Core Team, 2014). A Tukey HSD test was used to compare embryo development amongst the five crabs (R Core Team; Mendiburu, 2019).

Figure 1: The stages of *Pugettia producta* embryos, based on the 10 stages described by Boolootian et. al (1959) (**Table 2**). All photos were taken at 400x magnification. A) Stage 1, diameter = 472 µm. B) Stage 2, diameter = 472 µm. C) Stage 3, diameter = 472 µm. D) Stage 4, diameter = 472 µm. E) Stage 5, diameter = 472 µm. F) Stage 6, diameter = 586 µm. G) Stage 7, diameter = 611 µm. H) Stage 8, diameter = 625 µm. I) Stage 9, diameter = 625 µm.



Results

Salinity and Embryo Development

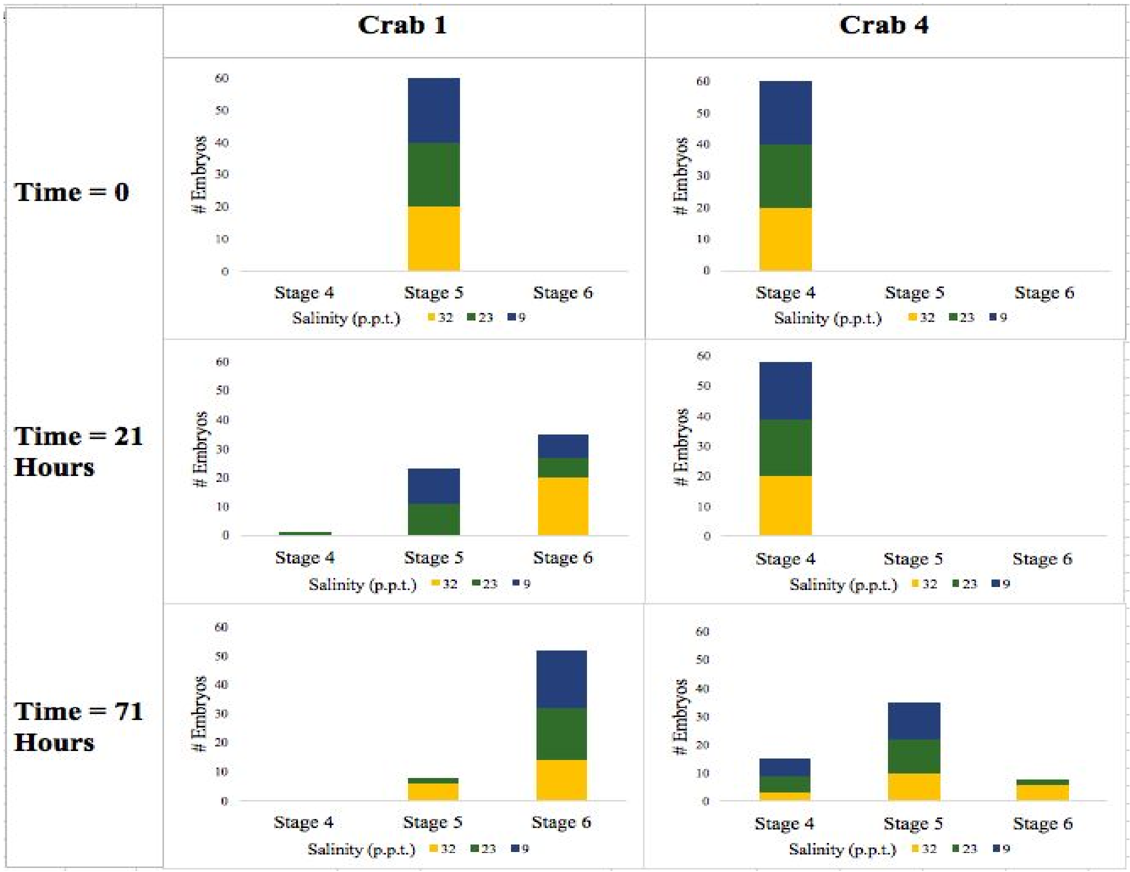
The combined data from both experimental crabs shows that Treatment 1 (32 p.p.t.) had the highest percentage of embryo survival; heart beats were seen in at least one of ten embryos observed in every replicate, although heart beats were only visible in Stage 4 or higher. Since heart beats could only be seen in certain embryo orientations, any signs of heart beats in the batch, or the ten embryos observed from each replicate, were considered a positive indication that the other embryos in the batch were still alive as well. No embryos had heart beats in any of the observed batches from Treatment 3 (9 p.p.t.), even after only 24 hours. Deflated embryos, cloudy orange yolks, and burst eggs were commonly seen in Treatment 3, but were rare or nonexistent in other treatments. Of Treatment 2 (23 p.p.t.) embryos, 75% of observed batches had at least one heart beat after 21 hours, and 25% of batches had at least one heart beat after 71 hours. (**Table 3**)

Differences in the rates of embryo development in the three salinity treatments were not statistically significant in either Crab 1 or Crab 4 (ANOVA $p = 0.372$, $p = 0.94$ respectively). After 71 hours, the following percentages of embryos from Crab 1 had progressed from Stage 5 to Stage 6: 70% in 32 p.p.t., 90% in 23 p.p.t., and 100% in 9 p.p.t. No embryos from Crab 1 progressed by more than one stage. In Crab 4 however, 32 p.p.t. yielded the highest total rate of development. Over 71 hours, progressions from Stage 4 to Stage 5 were as follows: 50% in 32 p.p.t., 60% in 23 p.p.t., and 65% in 9 p.p.t.; progressions from Stage 4 to Stage 6 were 30% in 32 p.p.t., 10% in 23 p.p.t., and 0% in 9 p.p.t. (**Figure 2**) Because random sampling was used at each observation time, some embryos were seen in lower stages at 71 hours.

Table 3 shows the survival percentages of embryos raised *in vitro* in various salinities, determined by presence of heart beats.

Treatment	**Salinity (p.p.t.)**	**% Alive (Time = 0)**	**% Alive (23 hours)**	**% Alive (71 hours)**
1	32	100	100	100
2	23	100	75	25
3	9	100	0	0

Figure 2: Embryo development from each salinity treatment over a 71-hour period. There was no significant difference between the rates of embryo development and salinity treatment.

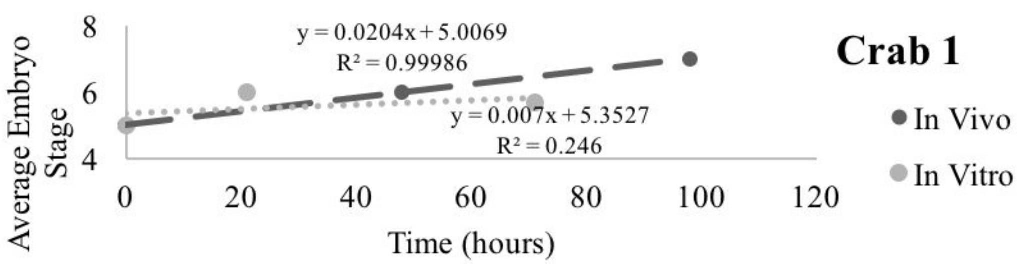


In Vitro vs. *In Vivo* Embryo Development

For this comparison, development of *in vitro* embryos from Crab 1 and Crab 4 in Treatment 1 (32 p.p.t.) was compared to that of *in vivo* embryos of the respective mother. (**Figure 3**) *In vitro* and *in vivo* embryos for this analysis were all at 32 p.p.t. salinity.

Assessments of the embryo stages in all five adult females over time indicate that embryos in the first stages of development have lower rates of development than those in mid to late stages. (**Figure 4**) The differences in rates of embryo development between crabs were statistically significant according to a Tukey HSD test ($p < 2e\text{-}16$). Embryos in Stages 1-6 were red-orange in color, but they were light brown in stages 7 and higher. Worms were seen in Crabs 1, 3, and 4.

Figure 3: A comparison of embryo development between embryos raised *in vivo* and *in vitro*. (Salinity = 32 p.p.t.)



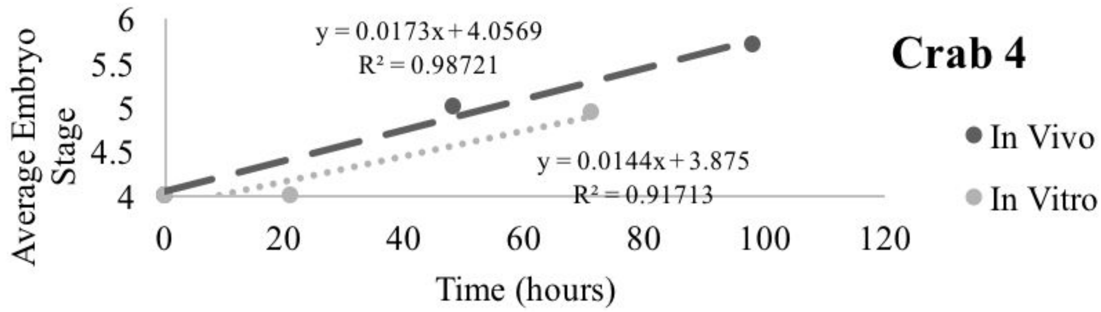
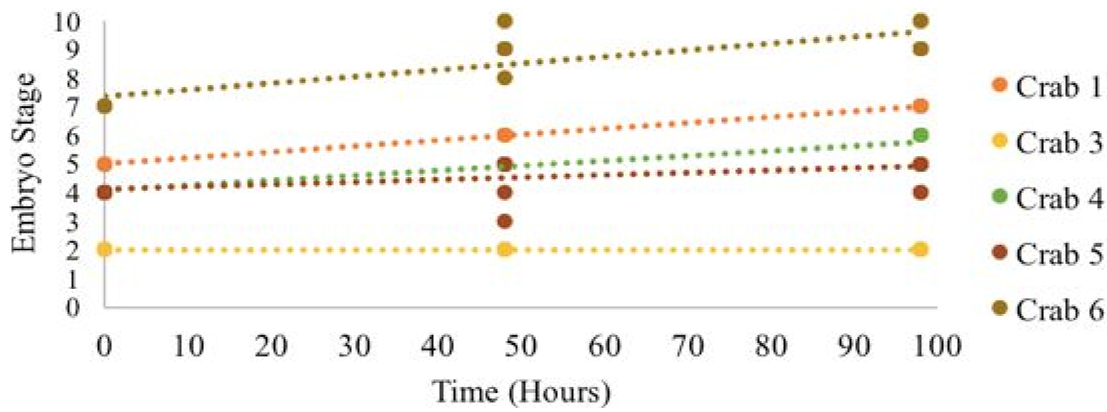


Figure 4: The development of embryos raised *in vivo* in five adult females.



Discussion

The results suggest that low salinity does not impact rate of embryo development, but it does negatively affect embryo survival. Studies of the estuarine crab *Rhithropanopeus harrisii* show no detrimental effects on larval development at salinities between 3 p.p.t and 25 p.p.t. (Costlow et. al, 1966; Rosenberg & Costlow Jr., 1979), providing supporting evidence for the hypothesis that zoea can be adapted for low salinity conditions. However, embryos may have different salinity responses than zoea. This experiment shows that embryos in 9 p.p.t. do not survive. If zoea are more tolerant of low salinity than embryos are, then gravid females with late-stage embryos would perhaps be found in lower salinity waters than those with early-stage embryos. One study has shown that female fiddler crabs, *Uca terpsichores,* release larvae in response to various spatial and temporal cues (Christy, 2011). *P. producta*, also in the Brachyuran family, may show increased embryo development rates in 23 p.p.t. salinity at later stages because decreased salinity is a cue for zoea release. Although the collection location of each adult female collected for this experiment was recorded, one crab's location was unknown, and the salinity of the water where they were collected was not sampled. More research should be done to find out if gravid kelp crabs migrate to lower salinity waters near the end of their reproductive cycle.

The data collected from the five gravid females suggest that rates of development may vary between embryo stages, with higher rates in mid to late stages. For a more accurate representation of *P. producta* embryo development, more research should be done to document average development time from stage to stage. The analysis presented in this report may be slightly skewed if some embryo stages take longer to develop than others; however, the two groups of experimental embryos used in the salinity experiment were in Stages 4 and 5 at the start, which provided relatively comparable replicates.

This experiment looked at the effects of salinity as a lone factor. However, salinity may have a more dramatic effect on embryo development when combined with other abiotic stressors including temperature and ambient oxygen. One study found that the detrimental effects of low salinity (5 p.p.t.) on American horseshoe crab embryos were less dramatic at lower temperatures (Vasquez et. al, 2015). If this is true, then rising global temperatures may interfere with crab embryo survival at lower salinities, which could potentially lead to a decline in crab populations.

Worms were seen in three of five crabs, likely of *Carcinonemertes epialti* (Nemertea: Hoplonemertea), which were observed in a past study of *P. producta* embryos (Boolootian et. al, 1959). It is unlikely that the extraction procedure was the cause of infestation. However, I did not start noting the presence of worms until midway through the experiment, when I first noticed a worm moving. Until that point, I did not know that the unmoving gray lumps on the outside of the embryos were worms. If this experiment were repeated, it would be useful to track the presence of worms several times from the day the crabs were collected to see if captivity introduces them to worms. Additionally, it may be better to keep each adult female in a separate tank to avoid transfer of worms between individuals. For further research, the effects of worms on embryo viability should be tested.

For future research on crab embryos, it is useful to know that embryo development rates are slightly higher *in vivo* than *in vitro.* The mother provides protection and circulation, which may increase embryo fitness. Nevertheless, the *in vitro* embryos in 32 p.p.t. salinity survived during the course of the experiment, indicating that the culturing techniques used were sufficient. Some literature on the subject recommends using antibiotics when culturing embryos *in vitro* (Caceci et. al, 1966), but this experiment's success indicates that antibiotics are unnecessary if care is taken to change the water frequently and sterile sea water is used.

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