

In vitro* effects of suspended sediments on fertilization success on the sand dollar *Dendraster excentricus

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ABSTRACT

The life histories of many marine invertebrates are complex and multifaceted, making them susceptible to even minor anthropogenic effects. One area of growing interest is the impact of increasing levels of suspended sediment resulting from human activities. In this study, we used an experimental laboratory approach to investigate the impact of sediment load on fertilization success in *Dendraster excentricus* (Eschscholtz 1831) at a variety of sperm concentrations. Although the results of this study did not yield a statistically significant difference among sediment treatments, our findings lay the foundation for future investigations and offer valuable insights for designing fertilization experiments involving suspended sediment as a crucial factor.

Key words: Suspended sediment, fertilization, echinoderm reproduction, anthropogenic impacts, *Dendraster excentricus*, sperm

INTRODUCTION

Anthropogenic activities are increasingly disruptive to ecological processes, including reproduction, at a variety of ecological levels. The alteration of marine turbidity levels through direct and indirect human-driven activities exemplifies the necessity for caution when considering potentially harmful activities which threaten marine environments (Airoldi, 2003a; Halpern et al., 2007; van Dover, 2011). Direct sediment alteration, such as dredging, deep-sea and terrestrial mining, industrial and domestic runoff, and agricultural practices is often conspicuous and easily identifiable (Airoldi, 2003a; van Maren et al., 2015). However, changes in sedimentation, particularly along marine coastlines, are frequently an indirect consequence of human activities that result in increased rates of soil erosion, modification of sea floor topography, or alteration of ecological dynamics at the species level (Airoldi, 2003b).

While natural phenomena also contribute to changes in turbidity levels, the alterations of sediment loads in novel habitats, due to anthropogenic activities, hold ecological significance (Airoldi, 2003a; van Dover, 2011). Increased sediment input was identified by (Halpern et al., 2007) as a significant anthropogenic threat to marine ecosystems. Moreover, attention to the alteration of breeding habitat, and the downstream effects of such changes is critically important for understanding the overall impact of human activities on the ecology of marine benthic invertebrates (Thorson, 1950, 1966).

Many marine invertebrate life histories are complicated and multidimensional, especially in their reproductive strategies and relative success (Thorson, 1950). External fertilization has a low relative success rate, despite its prevalence throughout the genera, but especially in the echinodermata (Thorson, 1950). Furthermore, some studies aimed at understanding this life history stage in the context of human impacts have already demonstrated suspended sediment significantly

hinders coral larval development (Gilmour, 1999), echinoid and molluscan larval survival and settlement (Phillips & Shima 2006) and kelp spore settlement (Deiman 2012), among others (Miller et al. 2014).

Despite the apparent wealth of studies investigating sediment impacts on invertebrate larvae (Gilmour, 1999; Miller et al., 2014; Phillips & Shima, 2006), we found relatively few studies that directly investigated sediment impacts on free spawning invertebrate gametes and their fertilization success (Gilmour, 1999; Miller et al., 2014). However, to our knowledge, no studies have demonstrated the impacts of suspended sediment on fertilization at varying sperm concentrations, and thus a key relationship has yet to be described explicitly.

We used an experimental laboratory approach to explore the impact of sediment load on fertilization success in *Dendraster excentricus* (Eschscholtz 1831) at different sperm concentrations. We chose to utilize *D. excentricus* as a model system for this experiment due to its availability, gamete size, and ease of spawning.

MATERIALS & METHODS

Sediment acquisition and preparation

The sediment we used was clay collected from False Bay on San Juan Island, Washington on 15 June 2023 and sieved through 250 μm , 150 μm , and 63 μm sieves. The finest sediment (<63 μm) was left to sit overnight to settle out and excess water was removed. We then baked this sediment in a convection oven overnight at 150°F until dry so we could achieve a dry-weight concentration. The sediment concentrations we chose were 420 mg L^{-1} for the high concentration and 170 mg L^{-1} for the low concentration (Deiman et al., 2012). We made 0.5 L stock solutions of each in separate flasks using 0.22 μm filtered sea water (FSW) and dry sediment measured using

an analytical scale. The sediment solution was imaged under a compound microscope at 20X magnification and the particles were measured along the longest axis using ImageJ. The majority of particles were about 50 μm or less and a few were as large as 100 μm .

D. excentricus spawning

D. excentricus individuals were collected from East Sound on Orcas Island by Jason Hodin in late April 2023. On 22 June 2023 we spawned sand dollars by injecting 3-6 mL 0.55M KCl with a syringe into the oral opening (von Dassow, 2003) until we had three males and three females. The syringe was positioned at an angle and injected into the soft tissue in 3-4 locations. We gently shook the sand dollars until gametes were visible on the aboral side. Females were placed aboral side down on a beaker full of sea water to collect eggs. Males were placed in a dry dish aboral side up to collect the sperm dry. Sperm was collected with a Pasteur pipet and transferred to an Eppendorf tube.

Sperm concentration

Sperm from three males was collected the day before the experiment, during a trial run, and stored at 4°C in separate Eppendorf tubes. Sperm concentration was enumerated by three replicated hemocytometer counts. FSW was used to dilute the stock sperm solution such that the final concentrations in the experimental wells are 10^5 , 10^4 , and 10^3 cells mL^{-1} . We made dilutions for each treatment so that 21.1 μL of each appropriate dilution would be added to each corresponding well.

Egg concentration

Eggs from three females were spawned into a beaker of FSW and transferred to a falcon tube with 14 mL of FSW. Eggs were not rinsed as to preserve the jelly coat integrity, but were presumed clean after two FSW dips prior to their trial. The tube was gently inverted to homogenize the mixture. We found the concentration of this mixture by pipetting 20 μL onto a microscope slide and counting the number of eggs. With this concentration, we calculated the volume needed for each well to have a concentration of 0.05 eggs μL^{-1} (Podolsky, 2002).

Experimental methods

Experiments were conducted in 12-well plates with five replicates on 22 June 2023 (Figure 1). We calculated the volumes of sperm, eggs, and sediment needed for a total volume of 3 mL per well. We first added the sediment solution to all wells, then added the egg solution. Using a timer, we added sperm to all the wells and recorded the amount of time it took. When adding sperm to the wells, we pipeted up and down once in each well. Using the time it took to pipet sperm into all wells, we added 1 mL 0.55 M KCl at the same rate to halt fertilization while allowing development to continue. We used a stopwatch to ensure 0.55 M KCl was added to the appropriate well after 15 minutes. After two hours had passed, we used a dissecting microscope to count the first 100 eggs and recorded how many were fertilized by observation of cellular division including 2-cell through blastula, similar to methods utilized by (Gilmour 1999).

Statistics

Data was collected as percent fertilization and was subsequently transformed using arcsin square root, the standard transformation for percent fertilization data (Quinn & Keough, 2002). Levene's test was run on transformed data and the obtained p-value was not significant ($p=.14$),

indicating that our data met the assumption for homogeneity of variance. Next, we plotted a normal Q-Q graph of the data alongside a Shapiro-Wilks test for normality. Results of the normality testing revealed that our data were not normally distributed, an outlier was identified and removed. Despite the lack of normality in our data, we proceeded to run the 2-Way ANOVA using R version 4.0.2 because 2-way ANOVA is a robust statistical test that can overcome issues of normality when variance in homogeneity and equal sample size assumptions are met (Quinn & Keough, 2002; R Core Team (2021)).

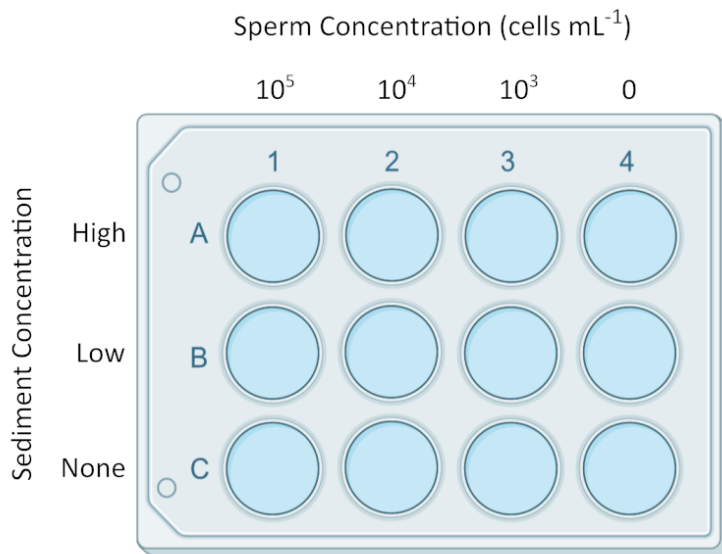


Figure 1. Schematic of experimental set up. We used a 12-well plate (n=5). Each row had a different sediment concentration, high (420 mg L⁻¹), low (170 mg L⁻¹), and none. Each column had a different sperm concentration (cells mL⁻¹), 10⁵, 10⁴, 10³, and 0. Sediment solution was added to all wells followed by eggs and then sperm. Wells were left to fertilize for 15 minutes before adding 1 mL 0.55M KCl to stop fertilization. After two hours, the first 100 eggs were counted and marked as fertilized or unfertilized.

RESULTS

Fertilization success at varying sperm concentrations

Sperm concentration had a significant impact on the percentage of eggs fertilized ($p < .001$). The high sperm concentration treatments (10^5 cells ml^{-1}) had an average of 93.2% fertilization success. The medium sperm concentration treatments (10^4 cells ml^{-1}) had an average of 2.4% fertilization success. The low sperm concentration treatments (10^3 cells ml^{-1}) had an average of 0.27% fertilization success (Figure 2).

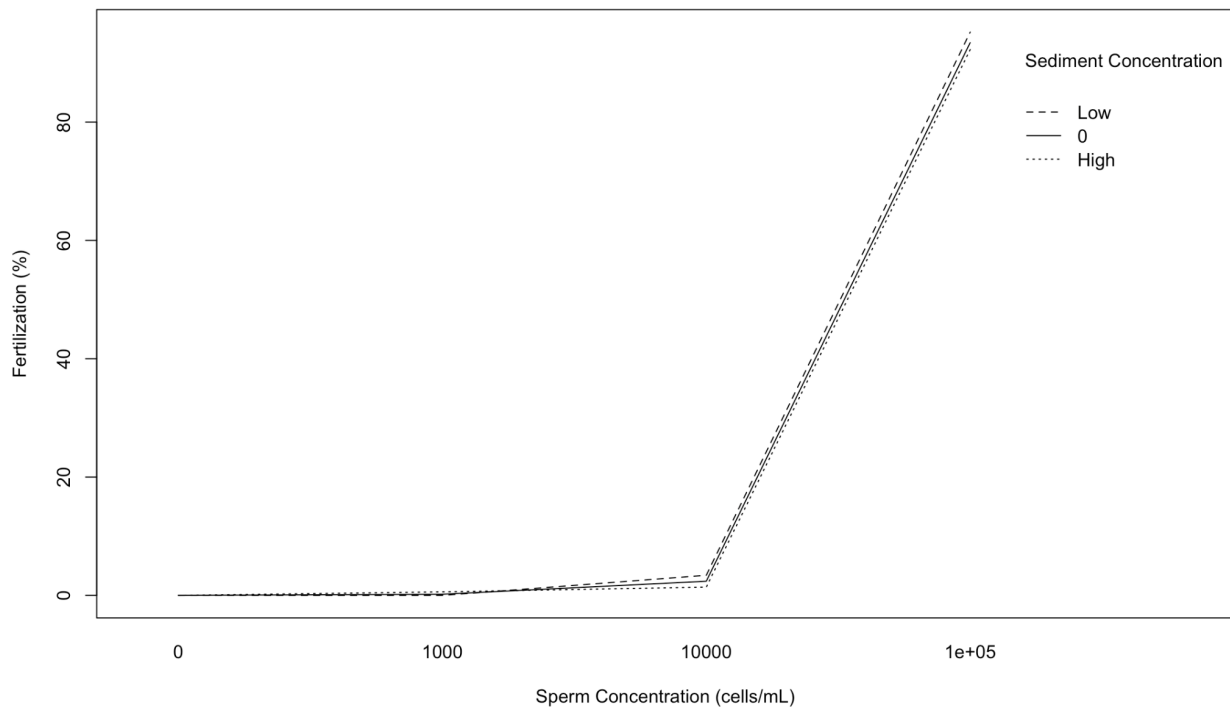


Figure 2. Average percent fertilization curve for different sediment concentrations. Lines represent the average fertilization percent for different sediment treatments. At high concentrations of sperm,

the average high sediment (dashed line) is lower than the average percent fertilization obtained in low and no sediment treatments, though not significant ($p=.36$).

Impact of sediment on fertilization

We found that relative sediment concentration alone does not have a significant impact on percent of eggs fertilized in vitro ($p \text{ adj}=.053$). The high sperm concentration ($10^5 \text{ cells mL}^{-1}$) was the most successful for fertilization and was not significantly different between sediment treatments (Figure 3). Additionally, we observed that eggs in the sediment treatments tended to have sediment attached to their jelly coat (Figure 4). Eggs covered in sediment appeared to sink faster than those without sediment and the sediment covered eggs congregated in the middle of the well, however due to time constraint no further testing was done on this observation.

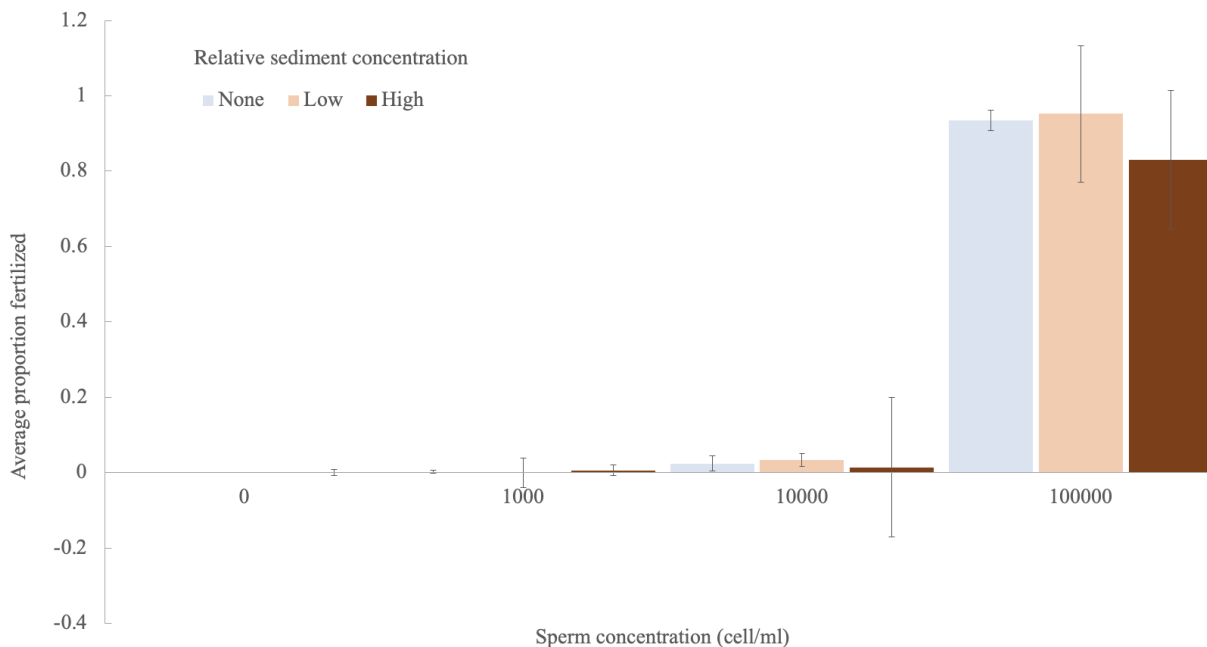


Figure 3. Average proportion fertilization of *D. excentricus* across different sediment concentrations and sperm concentrations. Error bars represent 95% confidence intervals. Percent

fertilization was not significantly different between sediment treatments, but was significantly different between the high sperm treatments (10^5 cells ml^{-1}), and medium (10^4 cells ml^{-1}) and low (10^3 cells ml^{-1}) sperm treatments. High sperm concentrations achieved the highest rates of fertilization, which dropped dramatically when concentration was decreased by an order of magnitude to medium concentration.

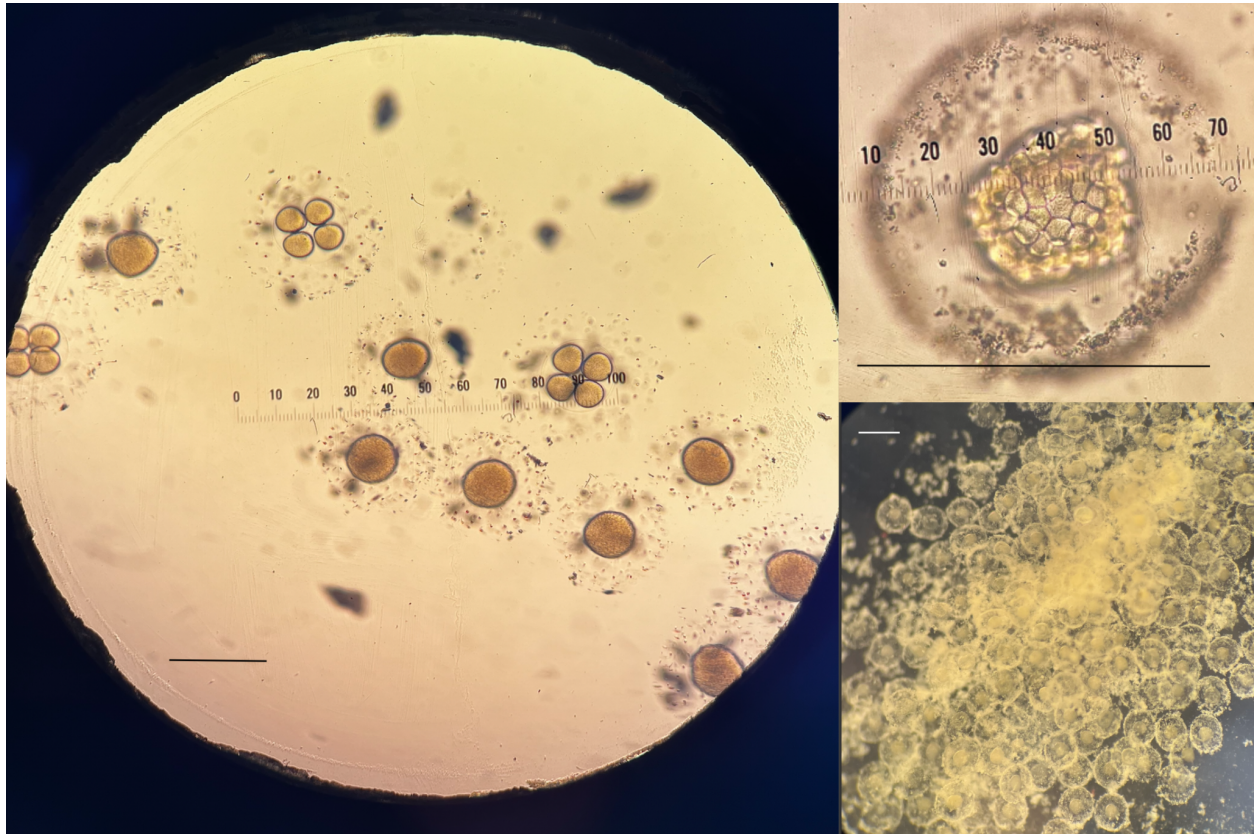


Figure 4. Photographs of sediment interaction with egg jelly coats. Sediment tended to stick to the outside (left, top right) and caused eggs to stick together (bottom right). Photos were obtained on Iphone 13 mini through dissecting (bottom right) and compound scope (left). Scale bars are $\sim 150\mu\text{m}$.

DISCUSSION

Fertilization success at varying sperm concentrations

Sperm concentration had a significant influence on fertilization success ($p < .001$). Percent fertilization in our high-concentration treatments (10^5 cells mL^{-1}) did not differ significantly between treatments. We observed an average fertilization percent of 93%, which is in line with the fertilization curve created by Levitan et al., 1991 for the echinoderm *Strongylocentrotus franciscanus*.

Impact of sediment concentration on fertilization

Our results suggest that fine sediment at 420 mg/L concentration or less does not have a significant impact on *in vitro* fertilization success in *Dendraster excentricus* ($p \text{ adj} = .053$) and no significant interaction between sperm and sediment was detected in this experiment ($p = .67$). We chose a conservative high and low sediment concentration based on concentrations reported in other literary studies (Deiman et al., 2012; Miller et al., 2014; Phillips & Shima, 2006), however, these suspended sediment loads are likely much more dilute than those observed during a direct sedimentation event such as dredging, wastewater dumping, or deep-sea mining (van Maren et al., 2015). Therefore, we may have simply chosen suspended settlement levels that were too low to show an effect on fertilization success at this scale.

It is possible that our results would differ given more replicates and an increased number of sperm concentrations. However, it is perhaps more likely that the nature of our experimental design unrealistically removed the potential for more complex interactions between gametes and sediment faced *in situ*. The added complexity of moving water, a variety of sediment sizes, and the potential for vertical movement within the water column may alter the impact of suspended

sediment on fertilization success. This idea is supported by the findings of Gilmour (1999) and Miller et al. (2014.), who found that sediments inhibited fertilization in the scleractinian coral *Acropora digitifera* and sea urchin *Evechinus chloroticus* respectively. Furthermore, these studies employed methodologies which allowed more freedom for variable interactions, which may account for our differing results.

Both Gillmore (1999) and Miller et al. (2014) implemented some form of water agitation (i.e., stirring or aeration) which may suggest that sediment impacts on fertilization are likely multivariate and not solely reliant on any one factor, such as concentration of suspended sediment, water flow and current, and overall variability of particle size. It seems likely that the results obtained from multidimensional experimental setups may more accurately portray *in situ* challenges faced by spawning invertebrates who face an abnormal sediment load.

In addition, sediment processing varied between our study and the aforementioned papers. Neither Gilmour (1999) nor Miller et al. (2014) mentioned any preparation of their sediments prior to experimentation, thus particle size between all experiments is likely variable. We observed that eggs whose jelly coats were covered in sediment appeared to sink faster than those without sediment and the sediment covered eggs congregated in the middle of the well. This finding may suggest that suspended particle size in conjunction with concentration of sediment may have a measurable impact on fertilization kinetics echinoderm fertilization.

Future studies should focus on the interaction between egg jelly coats and suspended sediment in echinoid fertilization, especially because jelly coats act to increase target size for spermatozoa and are thus directly influential on fertilization success in free-spawning echinoderms (Deaker et al., 2019).

Statistical results and analyses

Our initial observation of the data revealed an abnormal outlier which skewed subsequent statistical tests. This data point did not reflect results obtained for the other four replicates of that treatment (high sediment, high sperm concentration), and was removed.

The most likely explanation for the observed outlier is due to pipetting error. This datum showed a fertilization of 46%, while the other replicates were 98%, 88%, 94%, and 89%. We hypothesize that the viscous sperm may not have been fully evacuated from the pipet tip, and thus a lower concentration of sperm was present within that replicate; accounting for the decrease in fertilization success.

Alternatively, it is also possible that this data point could represent random chance because we subsampled our counts. However, this is unlikely because all replicates housed roughly 150 eggs, and wells with high sperm concentration were easily recognized as having a high fertilization success. It is possible, though unlikely, that roughly 50% of the eggs or sperm introduced were already duds and thus could not fertilize successfully even at the proper concentration. However, this is extremely unlikely because both male and female gametes were pooled from three adults, and eggs were visually checked periodically throughout the experimental process for viability, and unexpectedly low fertilization was not present within other high sperm concentration treatments.

Limitations

There are several limitations for this study. We were only able to obtain 5 replicates of our trials and much of our time was spent troubleshooting and designing a novel experimental strategy for an inquiry not investigated previously in the literature, to our knowledge. Furthermore, due to the compact nature of our experiment, it is possible that we did not encounter existing literature

that may be relevant to our experiment. Future work should address the limitations from this study including well randomization, increased replication, testing alternative experimental setups to decrease edge effects experienced within well plates, and testing a larger range of sperm concentrations to determine the concentration at which sperm may be necessary to overcome sediment impacts as suggested by Figure 2. Unfortunately, due to the time limitation in this study, we were not able to redesign an experiment that eliminated all such variables, but they should be noted. Our results are only indicative of sperms' ability to navigate a small, undisturbed environment with a relatively small suspended sediment load. Eggs without sediment seemed to stick to the walls of the well which may indicate that the well plate chosen for this experiment may have had a larger edge effect than originally anticipated. This may have impacted the distribution of eggs throughout the well unequally, thus introducing an additional variable to our experiment.

Conclusion and future directions

The introduction of increased suspended sediment loads to various habitats has been shown to be detrimental to reproductive success at the level of larval development (Gilmour, 1999; Phillips & Shima, 2006) and, in some cases, fertilization (Gilmour, 1999; Miller et al., 2014). Therefore continued attention to delineating the effect of sediment on early life history stages is important for a variety of benthic species who are most likely to be impacted by an abnormal increase in sediment.

While the difference in percent fertilization between sediment and control treatments in this experiment is not statistically significant, there are many other indicators that this is an avenue of research worth pursuing. Furthermore, if our results are accurate, and there is no significant interaction between sperm and suspended particles directly, it is still possible that interactions

between suspended sediment and other environmental factors *in situ* are more important than the sediment alone when influencing fertilization success in spawning benthic invertebrates (Pennington, 1985; Thorson, 1966; Vogel et al., 1982). Consideration to this possible relationship is warranted as sedimentation events are not only disruptive to normal ecological processes as they are occurring, but the impacts of such events are often long-lasting (Airoldi, 2003a; Halpern et al., 2007; van Dover, 2011).

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