THE EFFECTS OF SPERM CONCENTRATION ON POLYSPERMY IN QUAGGA MUSSELS

by

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THE EFFECTS OF SPERM CONCENTRATION ON POLYSPERMY IN QUAGGA MUSSELS

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INTRODUCTION

The quagga mussel (*Dreissena bugensis*) is a bivalve of the same family as the infamous zebra mussel (*Dreissena polymorpha*). They are both aquatic pests that have had enormous success in their spread, both across Europe and North America (Ram and Palazzolo, 2008). These mussels originated from the Paratethys region of Southwestern Asia, where *D. bugensis* and *D. polymorpha* species first diverged about 13 million years ago (Ram and Palazzolo, 2008). It is believed that the spread of the dreissenid mussels across Europe was due to canal systems implemented during the Industrial Revolution, namely along the Danube River (Ram and Palazzolo, 2008).

The quagga mussel was first observed in Lake Ontario and the Erie Canal in the year 1991, three years after the zebra mussel in Lake St. Clair (Ram and Palazzolo, 2008). Quagga mussels spread more slowly than zebra mussels, but are sometimes found in greater amounts (Ram and Palazzolo, 2008). This difference is due to a characteristic called respiration rate (Ram and Palazzolo, 2008). Quagga mussels have a lower respiration rate than zebra mussels, and because of this, it is believed that they can assign more energy to reproduction, allowing for them to takeover lakes previously populated by zebra mussels (Ram and Palazzolo, 2008).

Interestingly, the quagga mussel has had much more success crossing the Continental Divide, whereas the zebra mussel has remained, in the large part, eat of the Mississippi River (Ram and Palazzolo, 2008) (Fig. 1). Quagga mussels are found in multiple parts of the Colorado River system, in locations throughout California, Arizona and Nevada (Ram and Palazzolo, 2008). Recently, the zebra mussel has reached a few lakes in northern Texas, namely Lake Texoma (Fig. 1).

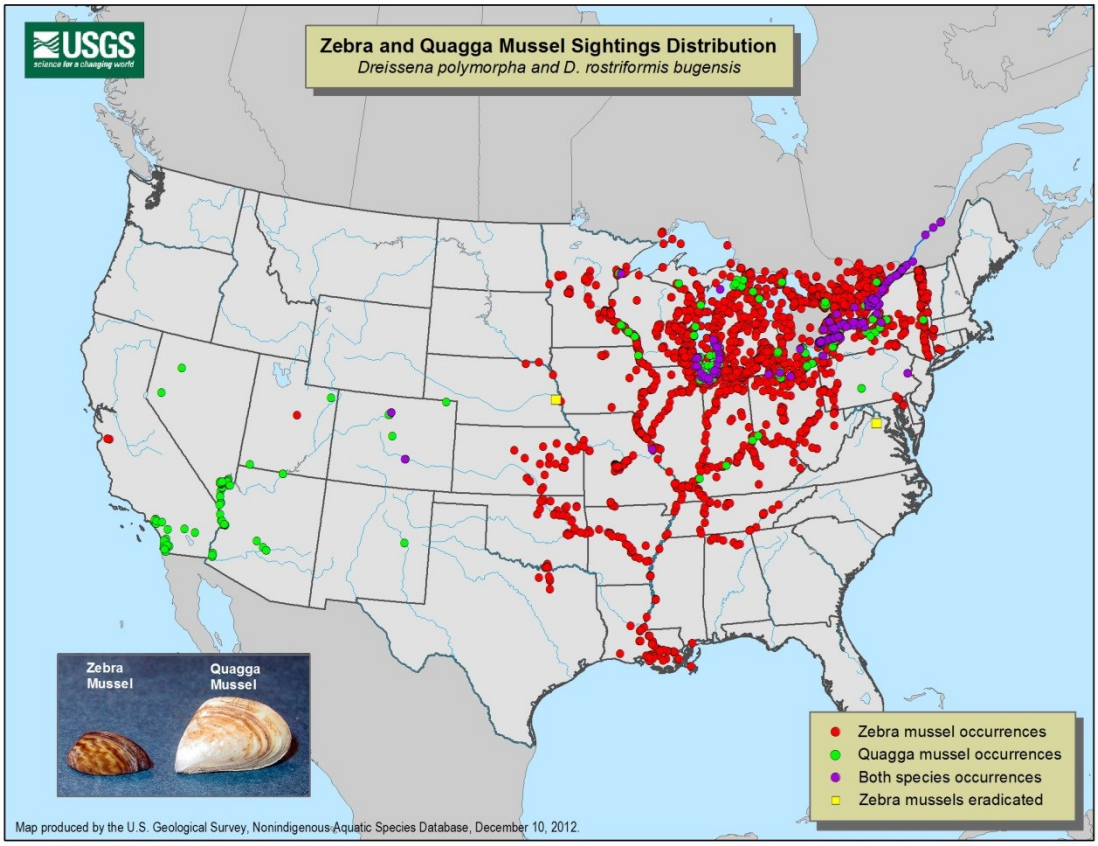


Fig. 1. The most recent locations of zebra and quagga mussels in the United States. Inset shows the difference in size and color between the two mussels (Fuller, 2012).

Quagga mussels spread by two main methods: transportation of adults primarily via boats and movement downstream by veligers (Ram and Palazzolo, 2008). While the spread of adults can only be prevented by mindful boat owners, the transportation of larvae via water presents a unique problem, especially because they can live up to 5 weeks before settling down to mature (Ram and Palazzolo, 2008). Originally, there was thought to be a limit on the southward spread of these animals due to their origins in cold water. However, these mussels have consistently moved beyond what was previously thought possible (McMahon, 1996).

These mussels are considered aquatic pests for a couple of reasons. One is that they simply overtake the ecosystem of the lakes they invade, covering every surface with their colonies (Ram and Palazzolo, 2008). Secondly and more importantly, in lakes and rivers where raw water is used as a coolant, they are able to enter in as veligers and settle down in the plumbing (Fig. 2). They can attach and grow in these pipes in very high densities, posing a significant threat to water companies and power plants alike (Ram and Palazzolo, 2008). With ample access to algae in the coolant water, there are few limitations to growth, leading to high reproductive success and large colonies (Ram and Palazzolo, 2008). Left undisturbed, these large populations of mussels can lead to very high resistance or complete blockage of coolant pipes, causing machinery to overheat and fail (Ram and Palazzolo, 2008). Therefore, in order to protect the important function of water treatment and power plants, an effective method must be found to combat the spread of quagga and zebra mussels.

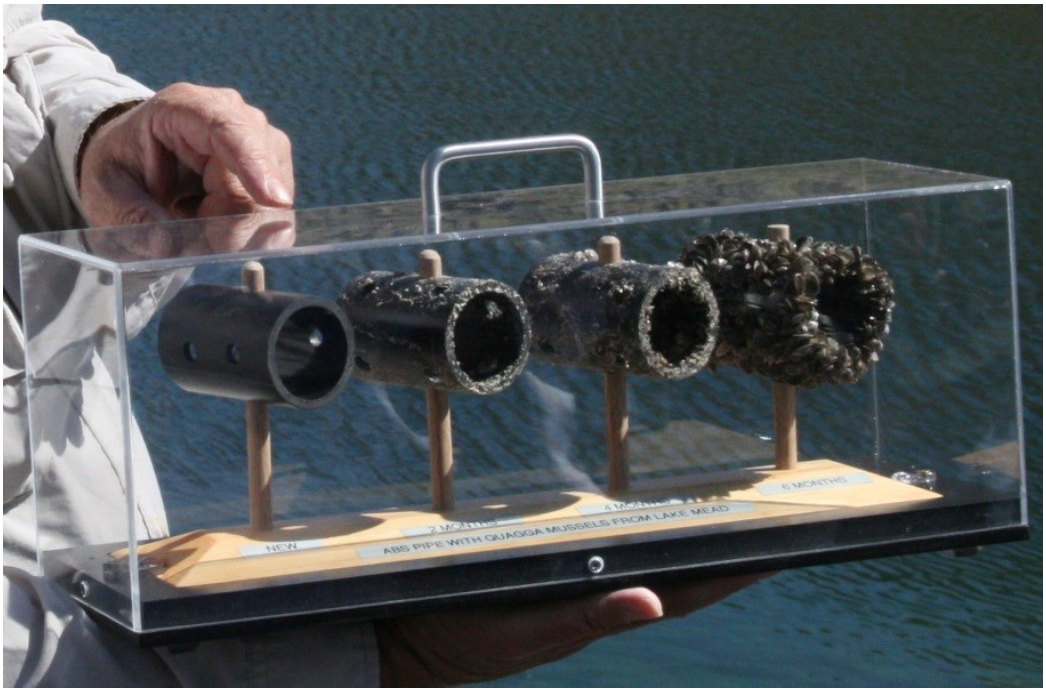


Fig. 2. The progression of a qugga mussel invasion of a pipe in Lake Mead, NV (Wilson, 2013).

Quagga mussels are broadcast spawners, a common reproduction strategy for many marine invertebrates, but rare in freshwater (Fallis, Stein, Lynn, Misamore, 2010). Broadcast spawning involves males and females releasing their sperm and eggs into the water column where fertilization and development of the veligers occurs (Fallis *et al*., 2010). Also, in broadcast spawning the proximity and concentration of sperm are paramount, so low concentrations of sperm restrict reproduction, but excessive concentrations can prevent reproduction also. In the latter case, polyspermy is a major factor is restricting successful fertilizations.

The definition of polyspermy is when more than one spermatozoon penetrates an egg (Dufresne-Dubé, Dubé, Guerrier, and Couillard, 1983). This condition is lethal in most species due to abnormal chromosome number leading to a gene dosage imbalance and abhorrent cell division due to multiple cleavage planes. There are many different methods that various animals employ in an attempt to combat polyspermy. The most well-known block to polyspermy for invertebrates is the two-step block in sea urchins. They first undergo a fast, partial block (2-3 seconds), which is an electrical change on the egg surface diminishing the receptivity of the egg to sperm (Dufresne-Dubé *et al.*, 1983). Secondly, a slow block to polyspermy occurs around 1-2 minutes after initial sperm binding when, the egg releases cortical granules that alter the egg surface, preventing subsequent sperm binding (Dufresne-Dubé *et al*., 1983; Gould and Stephano, 2003). The electrical block in sea urchins is believed to result from an action taken on the sperm (Alliegro and Wright, 1983). It is believed that the same type of action to prevent polyspermy can be observed in the oyster, *Crassostrea virginica* (Alliegro and Wright, 1983).

Another method employed by invertebrates to prevent polyspermy was studied by McAnlis, Lynn and Misamore (2010), as well as many others. This block has to do with binding of sperm to the egg surface and subsequent detachment of non-fertilizing sperm following successful fertilization. McAnlis *et al.* (2010) found that a carbohydrate-containing basal ring in the sperm acrosome was present at the site of sperm-egg binding. Non-fertilizing sperm were detached from the surface of the egg separating this sperm basal ring from the egg surface (Fallis *et al*., 2010). The detachment of a basal ring is known to be a part of a polyspermy block in zebra mussels, and is a possible element of the quagga mussel block (McAnlis *et al*., 2010; Fallis *et al*., 2010). Similar mechanisms for cleavage of sperm binding proteins to prevent polyspermy are found in mammals (Burkart, Xiong, Baibakov, Jiménez-Movilla, and Dean, 2012)

While there are many known methods of blocking polyspermy in invertebrates, even mostly in the zebra mussel, the method for the quagga mussel is essentially a mystery. In this experiment, an attempt was made to discover at what levels of sperm concentration quagga mussels can successfully resist polyspermy in an attempt to better understand the animal. Also, in order to further analyze the details of the quagga mussel's physiology and anatomy, it is necessary to know the most efficient way to breed them.

METHODS

Collection and Maintaining

The animals were obtained by the Nation Park Service from Lake Mead in Nevada. Once the animals arrived at TCU, they were placed immediately into a pond water tank. The animals were kept at 9-9.5° C in the tank until use. The animals were

fed once per week and had their water changed once per week to assure their healthy condition.

Spawning

Spawning followed protocols described by McAnlis *et al.* (2010). Animals were moved from the chilled holding tank and individually isolated in specimen cups containing approximately 50 mL of chilled pond water. The animals were allowed to gradually warm to room temperature overnight and remained in the cups for 1-2 days. After 1-2 days of isolation, each animal was rinsed twice with deionized water, transferred to 25-mL test tubes, and covered with a 0.1 mM serotonin solution. After 20 minutes in serotonin, the animals were removed from the serotonin solution, rinsed twice, and placed back in the test tubes that contained pond water. Finally, the animals were allowed to release their gametes. Males spawned within 30 minutes and females spawned within 90 minutes. When females began spawning, they were transferred to crystallizing dishes to continue spawning into a larger volume of pond water to lessen damage to the eggs.

Gamete Counting

Once the males had spawned, they were removed from their test tube to get a final sperm concentration within the tube. The sperm concentration was calculated as follows: first, an aliquot from the test tube was diluted to a 5:1 ratio. This was accomplished by combining 200 μL of the sperm in pond water, 200 μL of paraformaldehyde, and 600 μL of deionized water. Then, a hemocytometer was then used to count the sperm, with a minimum of 80 sperm counted to assure the valid concentrations. Three counts of the

same animal's sperm were done and the mean of these samples was used to determine the final sperm concentration in each test tube.

Fertilization

Sperm used for insemination was diluted to the desired concentrations prior to fertilizations. The fertilizations were done in a volume of 3 mL of water, so the dilutions were calculated to that volume. For example, if the counted sperm concentration was 6.32×10^6 cells/mL, then 483.9 µL of sperm was added to 2.5161 mL of DI water with the eggs to attain a total of 3 mL. Once a female was identified (which typically occurred around 1 hour after the serotonin treatment), she was moved to a 70 x 50 mm crystalizing dish. The eggs were later obtained by pipetting the eggs from dish with the animal into a 10 mL beaker. During this egg transfer, care was taken while collecting the eggs with as little water as possible, ensuring that the eggs became more concentrated, which allows for satisfactory results.

Sample Collection and Fixation

At time points of 3 minutes and 20 minutes post-insemination, a subsample of eggs was removed from the fertilization dishes and fixed with 2% paraformaldehyde in mussel buffer (McAnlis *et al*., 2010). This was done at a 1:1 ratio, using 500 µL of fixative and 500 µL of the spawned eggs. The samples were stored at 4° C until examination.

Counting Bound Sperm

In order to count the bound sperm per egg, the samples were wet mounted onto a glass slide between four silicone grease posts. The coverslip that was positioned on four silicon grease posts so that the eggs would not be crushed. Once counting began, a

systemic scanning method was used to ensure that no egg was counted twice. Also, two important criteria were used to determine whether or not a sperm was actually bound to an egg, or just in close proximity. These criteria were: (1) the sperm must be positioned perpendicularly to the surface of the egg (± 10°, in order to account for the bent nature of the quagga mussels' sperm), and (2) the sperm must be on the equatorial axis of the egg (this prevents counting of nearby sperm above and below the egg). In addition, if an egg had six or more bound sperm according to the chosen criteria, the first six sperm were documented.

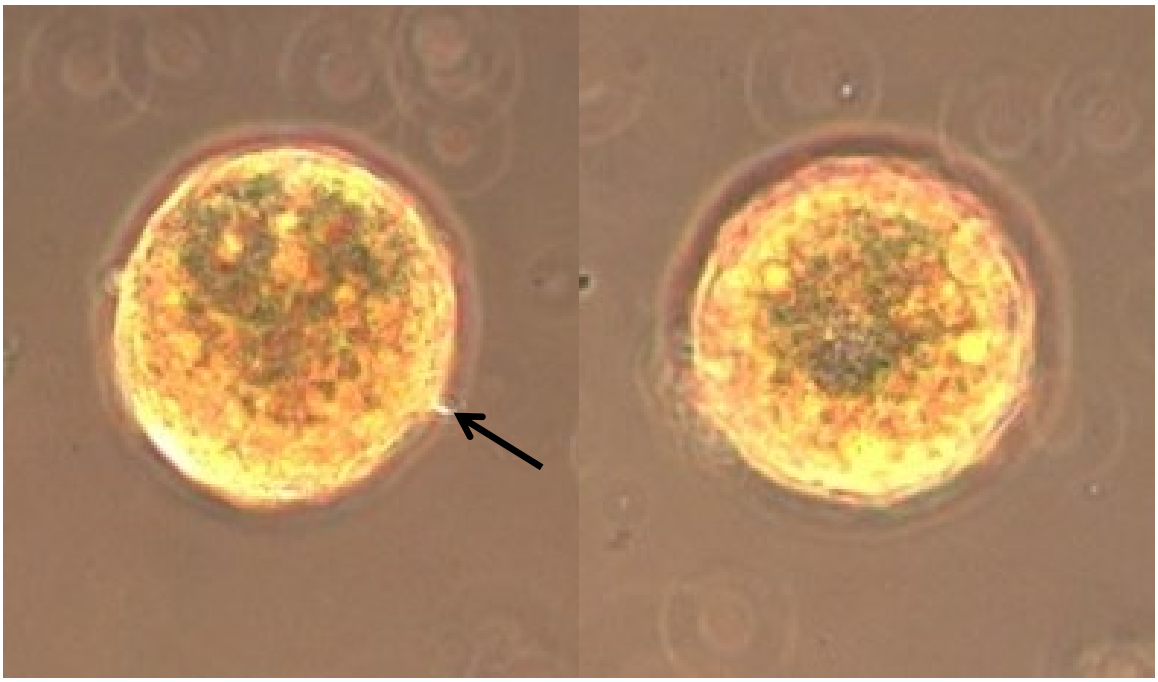


Fig. 3. Phase micrograph of an egg with one sperm bound (left, arrow) and an egg with no sperm attached (right).

Staining and Counting Incorporated Sperm

To determine the number of sperm nuclei that entered into the egg cytoplasm (sperm incorporation), fertilized eggs were stained with the DNA-binding fluorochrome. Slowfade Gold antifade reagent with DAPI (4', 6-diamidino-2-phenylindole) from Invitrogen was used (Fig. 4). The same method of mounting for the slide was used, but

before the coverslip was added, 2-3 drops of the DAPI stain was added to the eggs. Next, the stain was allowed to penetrate the sample for 15-20 min before viewing the eggs under fluorescent light. The same scanning method from counting the bound sperm was applied here. The criteria to determine whether or not a sperm had begun to fertilize an egg were standard. If a pronucleus and the female's chromosomes were distinguishable, then the number of sperm pronuclei was documented.

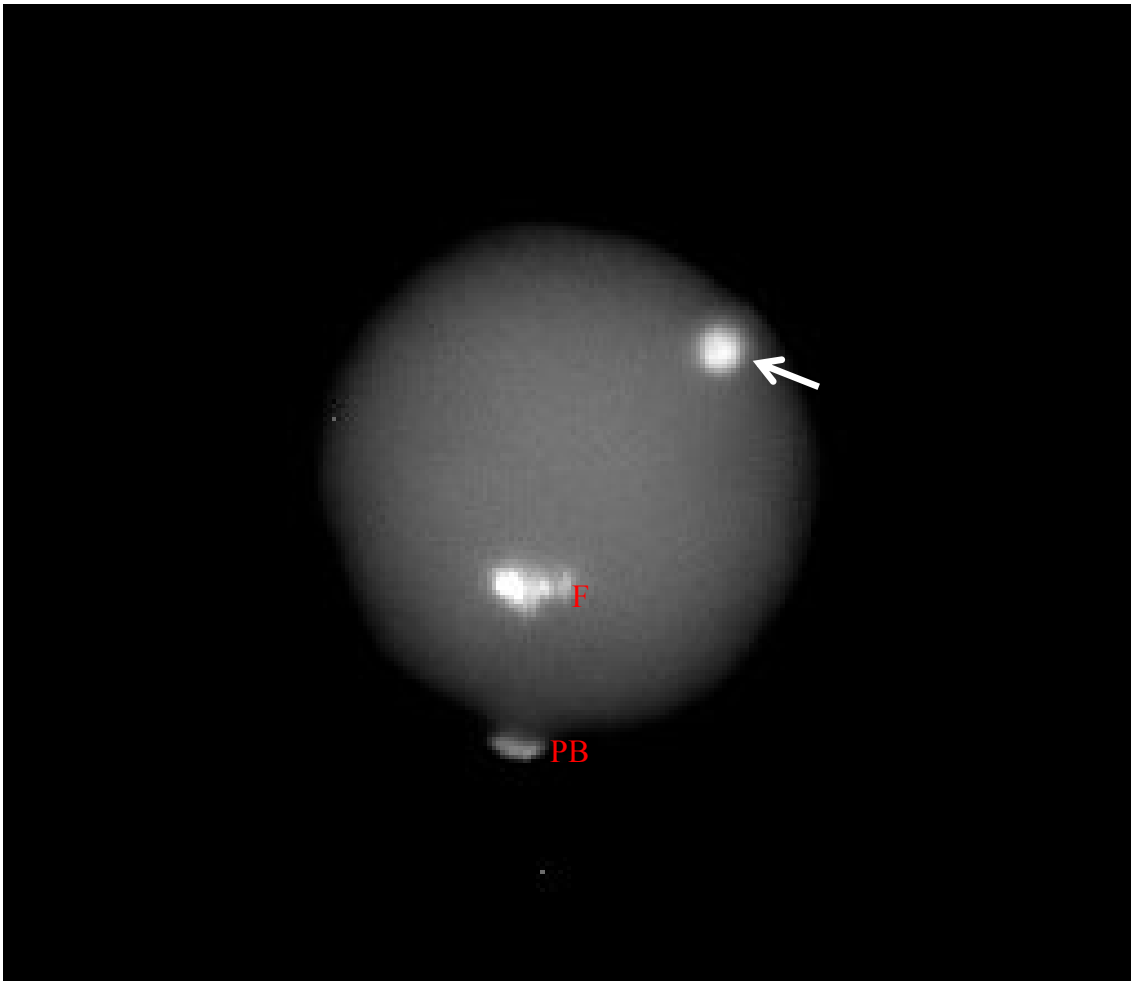
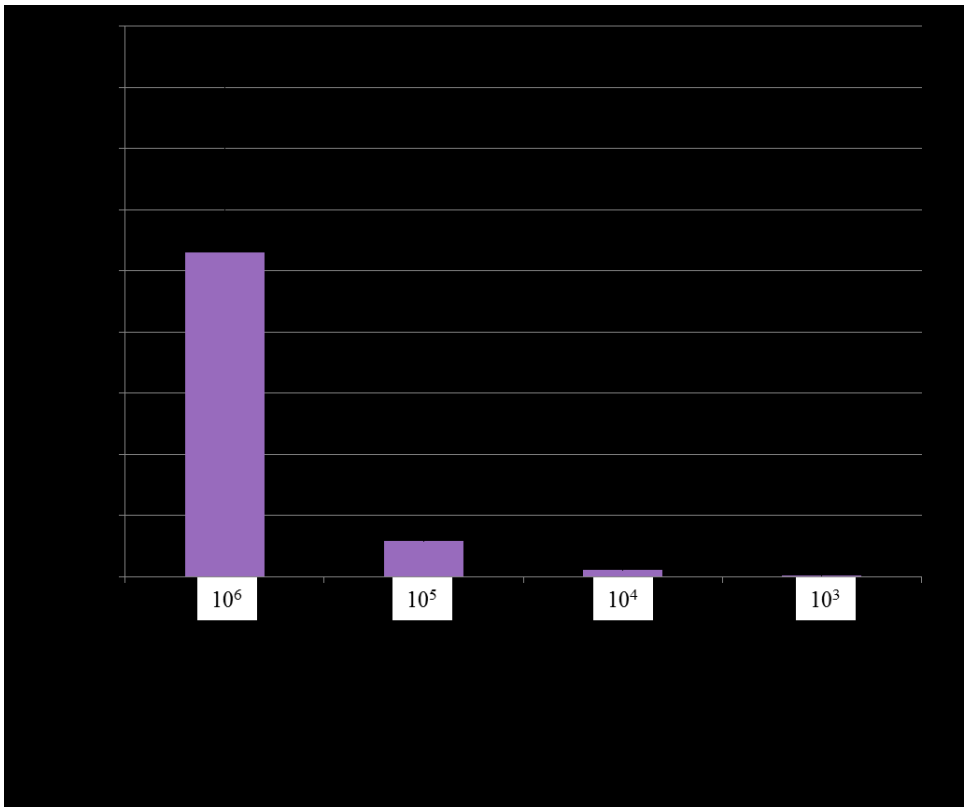


Fig. 4. Fluorescent micrograph of an egg with one sperm decondensing nucleius (arrow) that has been incorporated into the egg cytoplasm. F – indicates egg DNA and PB – indicated polar body. Eggs labeled with the DNA-specific fluorochrome DAPI.

RESULTS

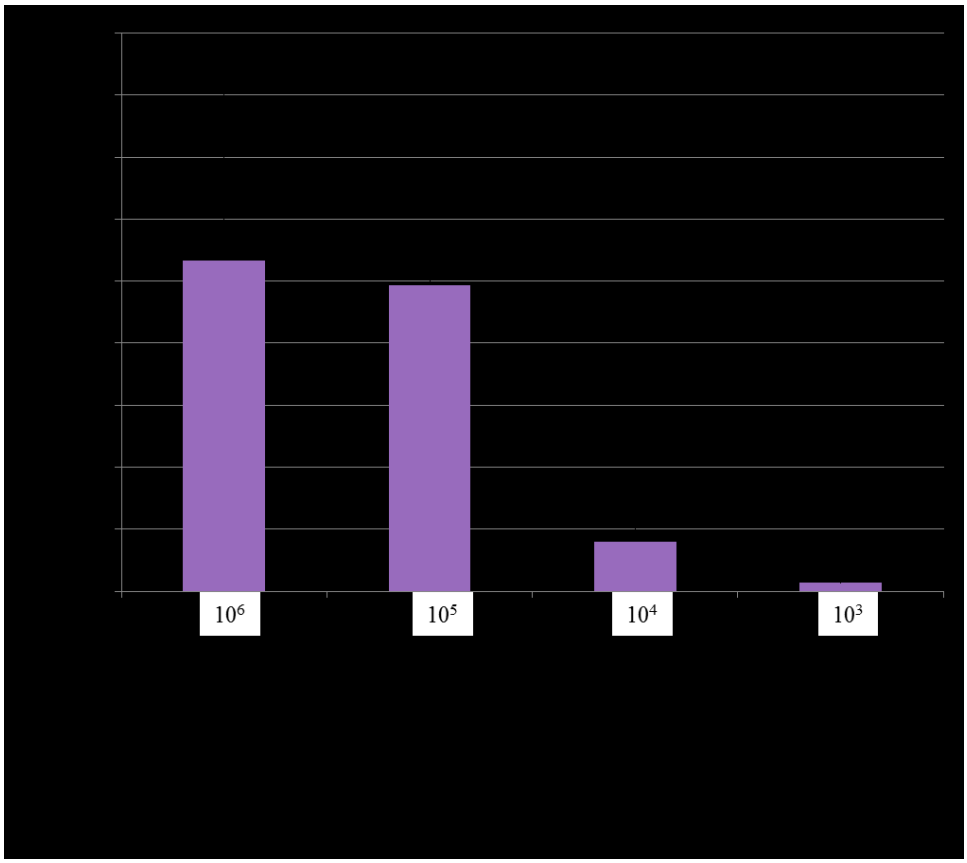
Sperm Binding

The results appeared to be quite interesting. It was found that bound sperm increased significantly as sperm concentration increased (Fig. 5). At 10^6 sperm/mL, a high level of polyspermic binding was observed (2.65 sperm/egg, Fig. 5). There were some polyspermic bindings that occurred at 10^5 sperm/mL; however, there were also many eggs without any sperm associated. At 10^4 and 10^3, there were no polyspermic bindings.



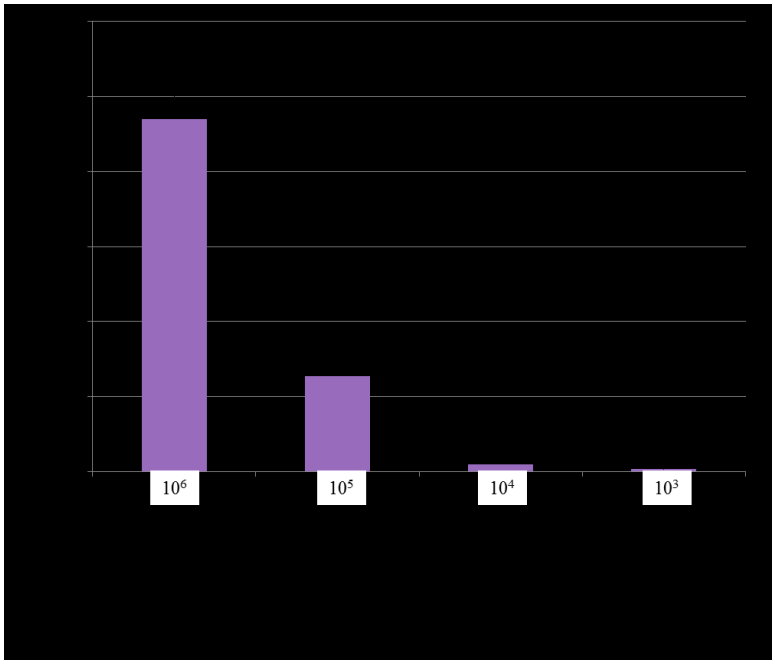
When calculating the percentage of eggs with only a single sperm bound (monospermic binding) (Fig. 6), it was found that at 10^6 sperm/mL there were many monospermic bindings, as well as polyspermic bindings (Fig. 5). Interestingly, at 10^5 sperm/mL, no statistical difference in number of monospermic bindings relative to 10^6

sperm/mL was observed (~25%, Fig. 6). However, there were significantly fewer monospermic bindings at 10^4 and 10^3 sperm/mL relative to the higher concentrations (Fig. 6).

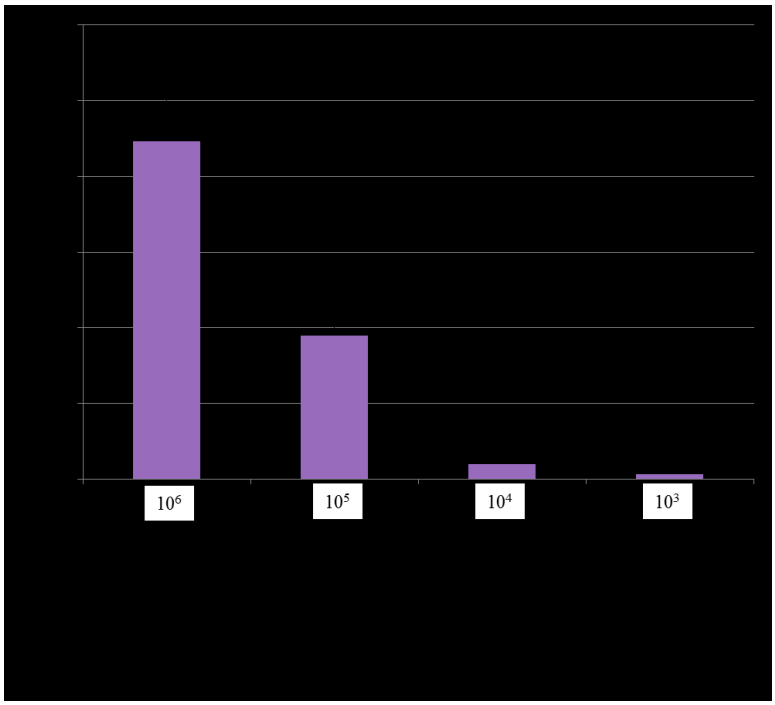


Sperm Incorporations

When looking for sperm incorporation inside the egg following sperm binding at the 20 min time point, a statistically significant difference between treatments was found (Fig. 7). Specifically, a statistically much higher average of incorporated sperm at the concentration of 10^6 sperm/mL relative to the lower three concentrations was observed (Fig. 7). At 10^5 sperm/mL, all but one egg with an incorporated sperm was monospermic. The lower concentrations only produced monospermy.



When percent monospermy for each concentration of sperm was examined, it was found that at 10^6 sperm/mL, monospermy was the most abundant (Fig. 8). At 10^5 sperm/mL, percent monospermy falls from 44.67% to 18.98%, and at the lower two concentrations, monospermy is nearly absent.



DISCUSSION

There has been some research with quagga mussels in the past, mainly revolving around the mechanism of their polyspermy block (McAnlis *et al.*, 2010). However, the experiment described here was the first account that aimed to analyze quagga mussel eggs' reaction to exposure to high levels of sperm. This experiment tested the strength of the quagga mussels' block to polyspermy by attempting to simply overwhelm the block. It was found that the quagga mussel's ability to block polyspermy does indeed depend on the sperm concentration.

Quagga mussels and zebra mussels alike are susceptible to polyspermy is the presence of large concentrations of sperm (Misamore *et al.*, 1996). It is also known that both of these species have a slow block to polyspermy at around 15 post-insemination, which is the detachment of non-fertilizing sperm (McAnlis *et al*., 2010). One of the goals for this project was to determine if the quagga mussel exhibits any difference in susceptibility to polyspermy relative to zebra mussels. However, the data only suggested that quagga mussels, similar to zebra mussels, lack a fully effective block to polyspermy in the presence of high levels of sperm.

When exposed to low concentrations of sperm ($>10^4$ sperm/mL), quagga mussels showed an effective block to polyspermic binding (Fig. 5) and sperm incorporation (Fig. 7). From these sets of data, it could be suggested that either quagga mussel's block to polyspermy is essentially 100% effective or that the sperm were so dilute that polyspermy was statistically improbable, or potentially both scenario occurred at the same time. In any case, only 1 egg out of 300 eggs counted at the lower two concentrations exhibited polyspermy.

However, at 10^5 sperm/mL, and especially at 10^6 sperm/mL, quagga eggs exhibited little ability to resist the binding of multiple sperm. Also, at the 20-minute time point, many polyspermic incorporations were observed. It is suggested that at these concentrations of sperm, the quagga mussel's block to polyspermy can be overwhelmed by sheer number of sperm. Interestingly, the highest percentage of polyspermic binding and incorporations was observed at 10^6 sperm/mL. Due to these findings, it is believed that while many eggs incorporated multiple sperm, that the eggs possess a method to eliminate secondary sperm invasions. At the 10^5 sperm/mL concentration, fewer eggs bound multiple sperm, but a much lower percentage of monospermic incorporations were observed. This is probably due to many eggs binding zero sperm, which obviously leads to an unfertilized egg. Because both polyspermy and non-fertilized eggs both result in an unsuccessful fertilization, it was found that losses due to polyspermy must be overlooked to achieve a maximum amount of monospermic incorporations.

In the environment, quagga mussels reproduce extremely successfully, and the current understanding of their reproductive cycle is too limited for a successful attempt at eradicating them. A species' specific block to polyspermy is an essential part of that reproductive cycle. The experiment presented here aimed at understanding more about this somewhat mysterious invertebrate, and as such, was quite beneficial.

In addition, the results offer a large step in the direction of mass culture for larvae. This is important because the larvae are an obvious target for eradication in the wild. In order for methods to be developed to achieve this eradication, a large, controlled sample size of larvae must be cultured to work with. Because these larvae are in the water column they can be collected from plankton tow in the wild. However, many other

planktonic species including algae and invertebrates such as rotifers are much more abundant than the mussel larvae. It is both costly and inefficient to obtain and isolate large numbers of mussel larvae from plankton tows. In addition to the logistical issues with plankton tows, the isolated larvae would be at varying stages of development. There is no way of knowing how long they have been in the water column. Due to all of these deterrents from harvesting wild larvae, culturing them is the only feasible option.

Based on the presented results, a concentration of 10^6sperm/mL would most likely give rise to the most successful larvae from a given sample of eggs. Further tests need to be done to confirm this hypothesis, but based on the available data, 10^6 sperm/mL produces the most monospermic incorporations indicating that it will also lead to the most larvae.

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ABSTRACT

The quagga mussel, cousin of the well-known zebra mussel, is an invasive Eurasian bivalve that has invaded the lake of the United States. Quagga mussels have been extremely successful, and one reason for that is their strong ability to reproduce. Here, an attempt to analyze the quagga mussels' ability to resist polyspermy (multiple sperm fertilizing one egg) when exposed to very high concentrations of sperm was undertaken. Individual animals were separated 24-48 hours before spawning in order to ensure no previous mating had occurred and to help stimulate spawning. Spawning was then fully induced using serotonin and isolated samples were collected after 20 min. The eggs were then fertilized with four different concentrations of sperm (10^6, 10^5, 10^4, and 10^3 cells/mL). At time points 3' and 20', samples were fixed to identify sperm bound and sperm entered, respectively. It was found that 10^6 cells/mL, the eggs lacked the ability to consistently resist polyspermic bindings, but by far, it also had the highest percentage of monospermic incorporations.