EVALUATING REPRODUCTIVE DIFFERENCES AND HYBRIDIZATION BETWEEN ZEBRA AND QUAGGA MUSSELS

by

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ABSTRACT

Zebra mussels are a notorious invasive species that have caused significant ecological and economic impact. However, there is another species, the quagga mussels, that is as equally detrimental and even displaces zebra mussels in many locations. Because the two species have overlapping habitats, similar appearances and reproductive behaviors, hybridization between the two is a possibility. It is important to know whether hybridization will occur between the two species since they could possibly produce hybrids that might be stronger and more resistant offspring, causing even further detrimental effects to our water systems.

In this experiment, we evaluate the reproductive differences between zebra mussels (*Dreissena polymorpha*) and quagga mussels(*Dreissena bugensis*) and observe if hybridization between the two species is possible. The basic morphologies of the different gametes are similar in many aspects, while having few differences. After spawning both species, we performed fertilization experiments using intraspecific (same species) and hybrid crosses looking at fertilization and early development. Despite differences in gamete morphology, chemotaxis was observed in both trials; both eggs successfully attracted the sperm of the other species. When observing the fixed samples, sperm binding was confirmed at five minutes after fertilization. Other areas we looked at are sperm entry and egg activation.

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Introduction

The well-known zebra mussels, *Dreissena polymorpha*, and the very similar quagga mussels, *Dreissena bugensis*, are both freshwater bivalve species that have caused significant ecological and economical disruptions in North America. The zebra mussel initially arrived in North America in the Great Lakes region and has been rapidly spreading throughout the country ever since 1988(Hebert et al. 1989). However its close relative, the quagga mussels followed with its arrival in North America, also in the Great Lakes region, in 1991(May and Marsden 1992) and has since caused the same impacts as the zebra mussels, but is becoming even more numerous than zebra mussels in some parts of their distribution(Stoeckmann 2003). Predictions show that the spread of zebra mussel or quagga mussel will increase with globalization and a rise in trade (Karatayev et al., 2015).

Both zebra mussels and quagga mussels are successful invasive species because both reproduce rapidly by broadcast spawning and adhere strongly to rock surfaces and each other, making it difficult to get rid of them. Broadcast spawning allows the larva to be dispersed far and contributes to the rapid spread and infestation of the mussels. Their infestation leads to blockage of plumbing systems and water sources, while also fouling boat hulls, navigational aids such as buoys, and negatively impacting recreational locations (Claudi and Mackie, 1993). Their initial infestation around the time period of 1989 to 1991 costed hundreds of thousands of dollars in order to clear them out and control future invasions(LePage 1993), and the cost to control them has risen ever since.

The zebra and quagga mussels share many similarities in physical appearances, living environments, and reproduction. Both are small, about 1-3 centimeters as adults, and have a striped patterning on their shells. Although it is difficult to tell them apart, the zebra mussels are flat on their ventral side, allowing them to sit flat and are more triangular in shape. Quagga mussels are rounder in shape and will not sit flat. Frequently, regions initially colonized by zebra mussels subsequently become infested with quagga mussels (Ram et al. 2008). Both zebra and quagga mussels are also very similar in their reproductive characteristics, making cross fertilization a reasonable hypothesis. Both use broadcast spawning to reproduce, meaning that both release their gametes into the surrounding water environment to be fertilized and develop into larva. Both species' sperm and egg share similar appearances and structure, although there are slight differences (Walker et al. 1996, Misamore et al., 2015).

Despite their very similar natures, genetic analysis has proven that there are significant genetic differences that make them separate species. Possible hybridization of the two species could cause further ecological and economic impacts by producing a more resistant offspring. Previous studies have conflicting stances on whether hybridization between the two species is possible. In studies where naturally occurring hybrids were detected, there were limited numbers of samples (Voroshilova et al., 2010). In other papers where hybridization was not found, the conditions in those experiments may have not been optimal for producing viable hybrid offspring(Spidle et al., 1995). In this paper, we will address the question of whether gametes from zebra and quagga mussels have the ability to fertilize – the first essential step in the hybridization process.

This project's goals are to examine the possibility of hybridization between zebra and quagga mussels through inducing release of gametes and insemination in controlled

environments. Observations of the sample will be made to see whether chemotaxis occurs between the crosses, and if so, if the sperm successfully binds to the other species' eggs. We will also monitor sperm entry, egg activation through the presence of a polar body, cleavage, and subsequent larva.

Materials and Methods

Collection of mussels

Zebra mussels were collected from Lake Bridgeport, Texas. Species identification was confirmed by shell morphology. There are no reported quagga mussels in Lake Bridgeport or Texas in general. Quagga mussels were collected with the assistance of the National Park Service from Lake Mead. Species identification was confirmed by shell morphology and there are no reported cases of zebra mussels in Lake Mead or surrounding states. Collected mussels were transported to TCU and maintained in separate 40-gallon holding aquaria at 10°C in dechlorinated tap water to prevent spawning. The mussels were fed a diet of algae weekly (Shellfish 1800, Reed Mariculture).

Spawning

To spawn the mussels, 24 zebra mussels and 24 quagga mussels were chosen at random, separated, washed off with deionized water to get rid of debris, and put into individual 120-mL specimen cups full of fresh chilled pond water. The mussels were allowed to warm to room temperature overnight. This isolation also ensures no accidental cross contamination from any unexpected gametes that may have been released in the holding tanks and transferred within the mantle cavity of the isolated mussel. Twenty four hours is sufficient to confirm no extraneous viable gametes were present prior to spawning. Spawning was then induced by exposing the

mussels to serotonin (Ram et al. 1993) following Misamore et al (1996). Following the 24 hour isolation, a 1-mM solution of serotonin was made by weighing 0.45mg of 5-hydroxytryptamine(serotonin) on an analytical balance and dissolving it in 100 mL of artificial pondwater (Dietz et al., 1994) . Each mussel was then transferred from their individual cups to individual test tubes filled with the serotonin solution. This solution will induce the release of their gametes. A few hours after they were put into the serotonin solution, an observation was made whether they released their gametes or not. If any gametes were released, they were differentiated between eggs and sperm using a stereo microscope. The females of both species that released their eggs were placed into a 50-ml crystallizing dish with pondwater to avoid clumping of spawned eggs.

Insemination

After spawning the mussels, the mussels' eggs were inseminated intraspecifically and interspecifically. To inseminate the eggs, 5mL of pondwater was added into labeled 10-mL beakers, and 1mL of eggs are added using a wide bore pipette. Then, 300 μl of sperm was added to each beaker using a micropipette and the timer was started immediately. The following four crosses were performed (ZM-zebra mussel; QM-quagga mussel): ZM eggs x ZM sperm, QM eggs x QM sperm, ZM eggs x QM sperm, and QM eggs x ZM sperm.

Fixation

Before the insemination, 2-mL microcentrifuge tubes with screw caps were set for sample collection. Each tube contained 1mL of 3.2% paraformaldehyde in mussel buffer (Misamore et al., 2000). Subsamples of the insemination beakers were collected at 5 minutes and 20 minutes post-insemination and fixed 1:1 in the 2-ml sample tubes containing fixative.

Sperm Chemotaxis

Zebra and quagga mussels eggs are surrounded by a layer term egg jelly which aids in the guiding of sperm to the egg surface. Misamore et al. (2015) observed that both species produce egg jelly, but the carbohydrate composition of the jelly layers between the two species differs. Egg jelly in other invertebrate species has been shown to be species specific (Miller et al. 1985). To confirm that sperm chemoattraction occurs, shortly after insemination, an unfixed subsample of the fertilized eggs was removed and visual observation to confirm sperm chemotaxis was performed. Chemotaxis was defined as a concentration of sperm in the jelly layer surround the egg. All four experimental crosses were examined.

Sperm Binding

Intraspecific and interspecific crosses were examined to determine if sperm binding occurred. Inseminated eggs from the 5 minute post-insemination time point were removed using a wide bore disposable pipette and placed onto a glass slide then a cover slip supported by four Vaseline posts to prevent damage of eggs. Fixed samples were examined under a Zeiss Axioscope with DIC optics. To determine the extent of sperm binding, the number of bound sperm at an equatorial focus was observed (Misamore et al. 2000). Using an equatorial focus ensures the observed sperm were clearly bound to the egg surface and not resting on the egg as seen in tangential planes of focus. A total of thirty eggs for each cross were to be examined.

Sperm Entry into the egg

To determine if sperm entry into the egg cytoplasm occurred, the 20 minute post-insemination time points were to be examined. The fixed, inseminated eggs from the various timepoints were to be stained with the DNA-specific fluorochrome Hoechst 33342 at 1mg/ml concentration for 10 minutes followed by washes in mussel buffer. The stained samples were to

be examined using a Axiovert fluorescent microscope. Using the DNA dye, the sperm nucleus at 20 minutes is clearly visible in the egg cytoplasm as it begins to decondense in preparation for fusing with the egg surface. Percentage of eggs that exhibited sperm entry were to be determined for each of the four experimental crosses.

Egg Activation

Zebra mussel and quagga mussel eggs are arrested at metaphase I arrest prior to fertilization. Upon fertilization, eggs resume meiosis and produce polar bodies. Polar body formation can be utilized as an indicator of egg activation. The 20 minute post-insemination time point eggs from the four crosses were to be examined for the presence of polar body formation. Thirty eggs from each cross were to be counted and percentage of eggs with polar bodies determined. Three replicates of each cross were collected.

DNA Extraction

Extraction of DNA was performed on a random set of eight zebra and eight quagga mussels. Each individual was dissected under a stereo microscope, and a mass of each mussel's foot was placed in protease K and 400 µl lysis buffer. Then the sample was vortexed well and incubated at 60°C overnight. The next day, the vortex samples were allowed to return to room temperature. Then, 0.5 volume of 7.5M ammonium acetate was added to each sample and the mixture was vortexed vigorously for about 10 seconds. The samples were then put in a freezer for at least 10 minutes, then centrifuged at full speed for 10 minutes in order to pellet the proteins. The supernatant was poured into a clean tube, and 0.7 volumes of isopropanol was mixed in by inverting the tubes back and forth for about 15 seconds. Next, the mix was spun at full speed for 15 minutes in order to pellet the DNA. The isopropanol was poured off and inverted on paper towels. The pellet was then washed off with 400 µl of 70% ethanol by shaking

the tubes, spinning them for 5 minutes, and pouring off alcohol and inverting onto clean paper towels. The pellets were dried in a vacufuge at 60°C for 10 minutes. Then, 100 µl of 10mM Tris at pH 8.5 was added and put in a fridge overnight. The next day, the tubes were vortexed for 5 minutes to resuspend the DNA and then spun down the contents for 30 seconds. The DNA was completely extracted, but analysis was not completed due to quarantine requirement resulting from the COVID-19 pandemic.

Results

Sperm Chemotaxis

Visual observations of inseminated crosses were done to look for sperm chemotaxis. Clustering of sperm in the jelly around the egg was observed in all four crosses (Fig. 1). Sperm were observed to be concentrated immediately after insemination around the eggs in the egg jelly layer. This chemoattraction was observed for both intraspecific and interspecific crosses.

Sperm binding

Sperm binding was also observed under light microscopes in the samples fixed at 5 minutes. A total of 40 crosses was performed. Sperm binding was observed between eggs and sperm in all four crosses (Fig. 2). A minimum of 4 trials of each of the fours crosses was collected. Because female zebra mussels were having difficulty spawning, only 4 trials were performed for crosses involving zebra mussels eggs (4 trials of ZM eggs x ZM sperm, 4 trials of ZM eggs x QM sperm). A total of 16 trials were performed for each of the other experimental crosses (16 trials of QM eggs x QM sperm, 16 trials of QM eggs x ZM sperm). The mean number of equatorial bound sperm needed to be determined for each of the samples, but it was unable to be conducted due to quarantine requirement from the COVID 19 pandemic.

Sperm entry

As with sperm binding, a total of 4 trials involving zebra mussels eggs (ZM female x ZM male; ZM female x QM male) and 16 crosses involving QM females (QM eggs x QM sperm, QM eggs x ZM sperm) were performed. Samples were collected at twenty minute samples post-insemination and fixed for later examination of sperm entry. Analysis of the samples was unable to be conducted due to quarantine requirement from the COVID 19 pandemic. Existing samples can be labeled with DNA-specific fluorochromes and mean numbers of eggs with sperm entry can be determined. Future analysis of my 20 minute post-insemination samples will determine if sperm entry occurred and if the rates between interspecific and intraspecific crosses were similar.

Egg Activation

Based on live observations during sample collection, polar body formation was observed in all four crosses (Fig. 3). This indicates that interspecific sperm are able to induce egg activation in other species. Quantitative verification of percentages of egg activation need to be performed on my 20 minute post-insemination time points. This was not possible in the time frame of this study due to quarantine requirement from the COVID 19 pandemic.

DNA Analysis

Steps were taken to analyze the DNA of zebra mussels and quagga mussels and compare them to the DNA of the hybridizations, using primers discussed in Feldheim et al. 2011. The DNA was successfully extracted from the parents, but analysis of the extracted DNA was not possible due to the quarantine requirement from the COVID 19 pandemic. Future analysis of DNA of the inseminated crosses will determine whether the egg was truly activated by the other species' sperm.

Discussion

Only within the last few years has our understanding of the differences between zebra and quagga mussel reproduction begun to grow. Differences in the morphology of sperm have been known for many years (Walker et al 1996). Zebra mussel sperm are linear while quagga mussel sperm have a slightly curved sperm head. This is a simple method to easily distinguish between zebra and quagga mussel males. The egg morphology is more difficult to distinguish, but there are some differences. Quagga mussel eggs seem to have a slightly thicker jelly layer, and the carbohydrate composition based on lectin labeling differs between the two species (Misamore et al. 2015).

The first interaction between eggs and sperm of broadcast spawning species is the chemoattraction and interaction of the sperm and the egg jelly. Chemoattraction was observed in both intraspecific crosses of zebra and quagga mussels. However, chemoattraction was also observed in both of the interspecific crosses. Miller et al. (1994) reported that isolated egg jelly compounds from zebra mussel sperm were able to attract and alter the movement of quagga mussel sperm. The reciprocal cross also yielded chemoattraction. My findings are consistent with their work and here I document direct chemoattraction between eggs and sperm of the interspecific crosses. This cross-species chemoattraction is interesting and significant since many broadcast spawning species such as sea urchins show species-specific sperm chemoattraction. They utilize this species specificity to prevent hybridization. Here, as with Miller et al. (1994), we show that there is no species specificity to sperm chemoattraction. Future studies to further address this issue could observe natural chemoattraction between zebra and quagga mussels that are not induced to spawn or fertilize.

Sperm binding to the egg was also observed in both interspecific crosses. This documents for the first time that sperm binding is possible between these two species. It is interesting to note that both species of sperm are able to bind to the opposite species of egg. Further quantitative analysis of my 5 minute postsemination binding samples will help determine if the rate of sperm-egg binding between interspecific crosses occurs at a similar rate to the intraspecific crosses. Additional studies on sperm-egg binding could analyze the specific components of each species' eggs and sperm that allow binding and explain why interspecific crosses are able to bind if the components are not the same.

As with chemoattraction, sperm-egg binding is frequently species specific in broadcast spawning animals (Hirohashi, et al 2008). This is one of the main ways in which these species prevent hybridization or more importantly, ensures successful fertilization with their own species. While broadcast spawning is common in many marine invertebrates including many bivalve species (Hirohashi, et al 2008), zebra and quagga mussels are the only freshwater broadcast spawning bivalves and few other freshwater invertebrates utilize this form of external fertilization. The tight control of species-specific binding seen in marine species may be a byproduct of the numerous species releasing gametes into the water. Zebra and quagga mussel gametes do not experience that high competition and so may not need as tightly controlled system of species specific sperm-egg binding.

Along with sperm binding, sperm were also able to activate the egg and induce polar body formation in interspecific eggs. Further quantitative analysis of my samples will help determine if the ability to induce egg activation is similar between intraspecific and interspecific crosses.

Ultimately, sperm do enter into the egg in interspecific crosses. However, zygote cleavage and subsequent larval develop are the next steps that need to be addressed. Analysis of my 20 minute post-insemination samples will answer the first of these stages. Investigation of subsequent embryonic development and ultimately the production of a hybrid offspring is needed. Development of genetic tools to verify hybridization will be helpful. It will be especially interesting to see if the DNA primers used to identify adults zebra and quagga mussels will be equally useful for identifying gametes of these two species (which have half the number of chromosomes as their parents). Although hybridization might be successful in the laboratory, that does not mandate that natural hybridization can occur. Previous studies have examined naturally occurring hybridization in the Great Lakes and stated it is not likely(Spidle et al., 1995), but other regions may have better conditions for the zebra and quagga mussels to hybridize naturally. Other studies have discovered natural hybridization through genetic methods (Voroshilova et al., 2010). However, future studies must investigate whether viable larva can be produced through natural hybridization and how rapid these hybrids would be able to spread. Control of these invasive species is needed since they can cause ecological and economic disruptions that are detrimental.

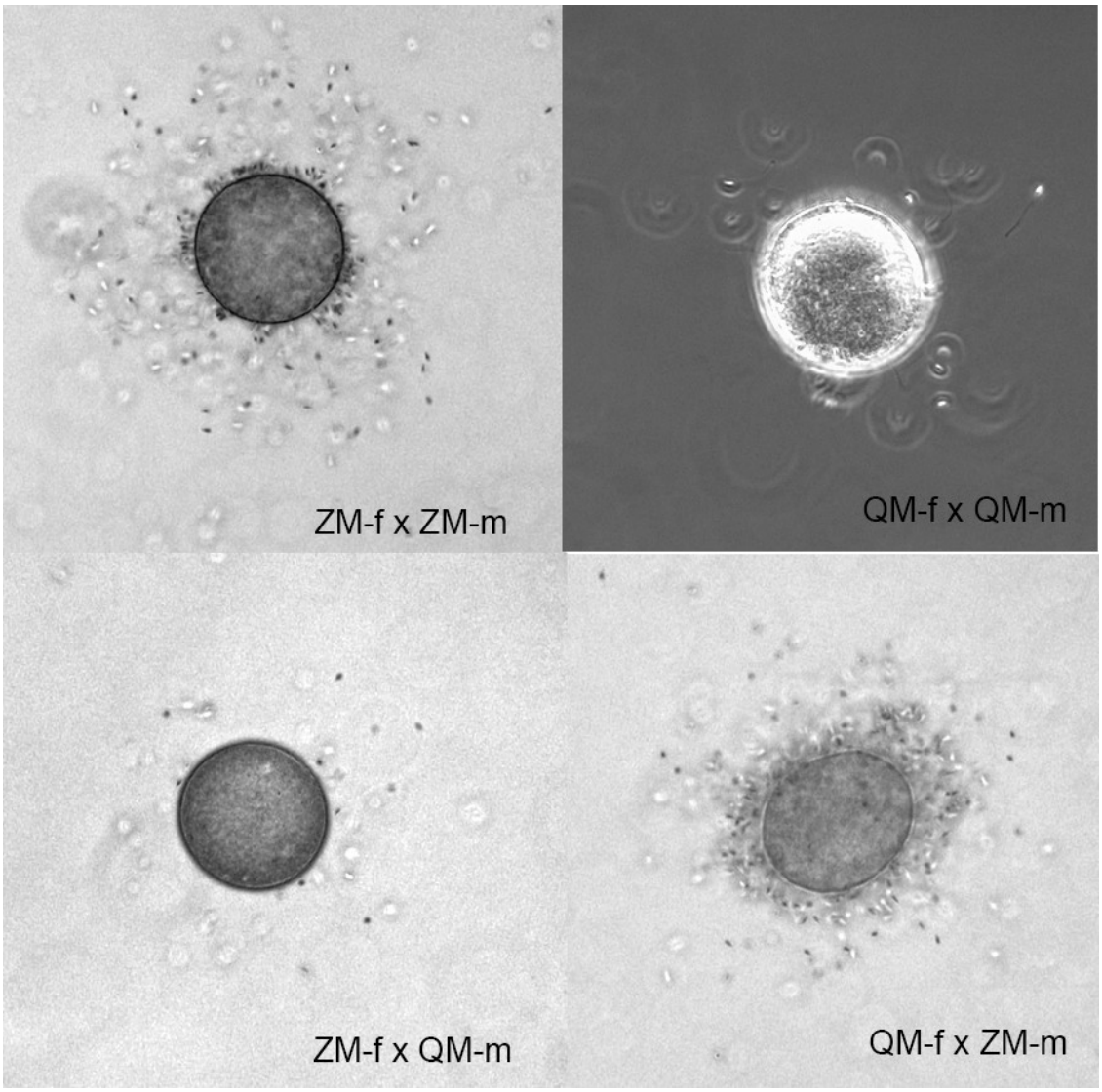

Figure 1. Light micrograph images from live samples that were inseminated. The sperm is clustering around the eggs in all crosses (f-female, m-male). The sperm were attracted to the egg by the egg jelly layer components in both intraspecific and interspecific crosses.

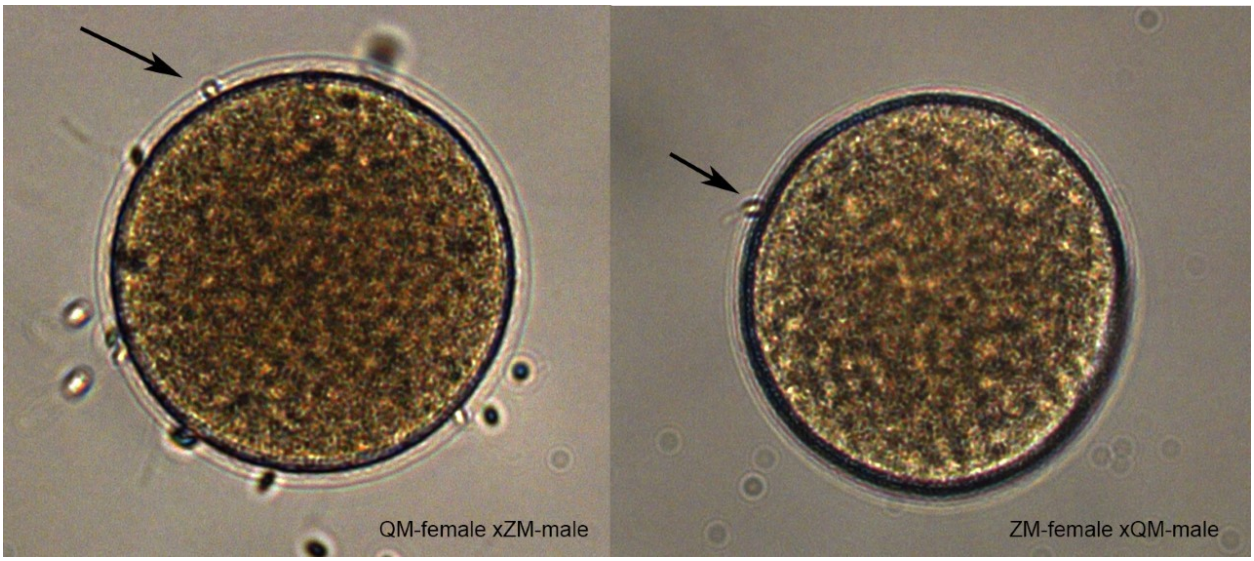

Figure 2. Light micrograph images from interspecific cross samples fixed at 5 minutes post-insemination. The sperm is bound to the egg of the other species.

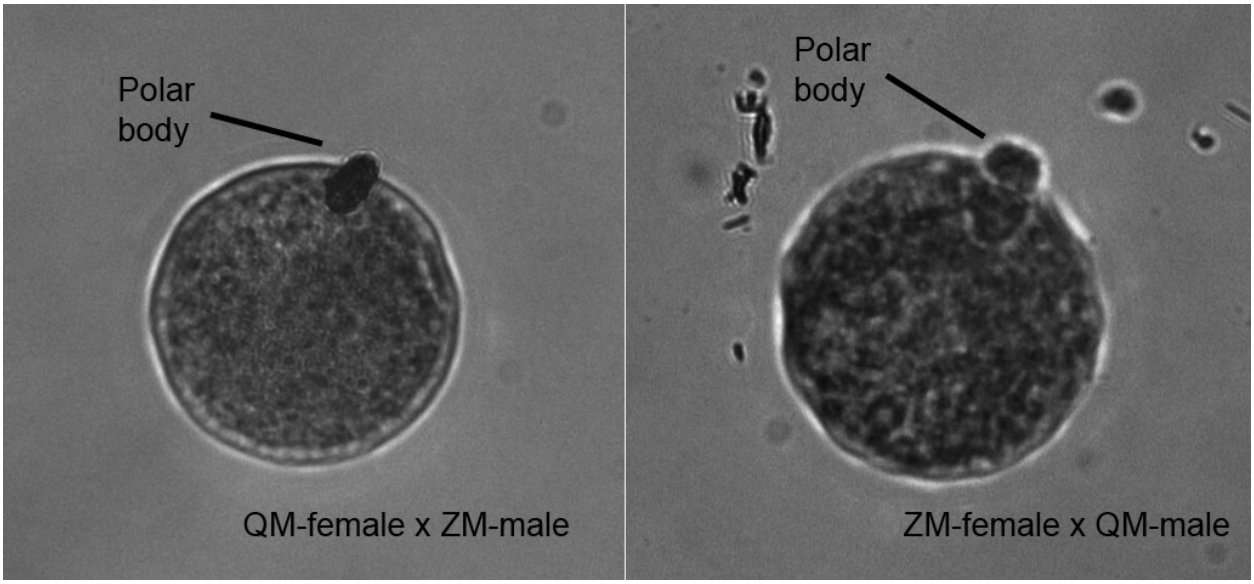

Figure 3. Captured images from interspecific cross samples fixed at 20 minutes post-insemination. The eggs were activated from the other species' sperm and polar body formation is occurring.

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