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STEPS TOWARDS SUCCESSFUL CRYOPRESERVATION OF FISH GAMETES: IMPROVING LABORATORY PROTOCOLS IN THE ZEBRAFISH MODEL TO INCREASE GAMETE VIABILITY AND EFFICIENCY

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MASTER'S THESIS

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Acknowledgments

I dedicate this work to those who have helped me reach this point: first and foremost, to Dr. Charles Herr, for his insight, guidance, and support throughout this project; to the members of my committee, Dr. Daberkow and Dr. Raymond for investing time and energy for the ultimate betterment of this project; to my husband, Matt, for his love, unending support, proof-reading capabilities, and willingness to listen to me babble about fish eggs and sperm for hours; to Mike Baker for teaching me to weld thermocouples and mentoring me in cryobiology; to Dr. Barrie Robison for his expertise on zebrafish gamete collection; and to Josh and Henry, for investing themselves wholeheartedly in this project and for hours of cheerful labor freezing fish semen.

Furthermore:

To all those who have aided me along this journey, in capacities large or small, those named and unnamed, whom I love and hold most dear:

"I can no other answer make than thanks,
And thanks, and ever thanks."
Shakespeare: *Twelfth Night*, III.iii

Abstract

Cryopresererv ation is a powerful tool for the long term storage of genetic material from endangered species. While mammalian gametes have long been freezable, eggs and embryos from fish have yet to be successfully frozen. Furthermore, protocols for freezing fish sperm have low success rates. In an effort to overcome the limitations in gamete manipulation currently impeding successful fish gamete cryopreservation, I outline two experiment using zebrafish (*Danio rerio*) as a model for freshwater fish species. First, I attempted to extend working time in both eggs and sperm by incubating the gametes in rainbow trout oviductal fluid (RTOF), RTOF + HEPES, or RTOF under a blood-gas atmosphere. Incubation in RTOF under a blood gas atmosphere significantly extended viability in both gametes to 24 hours. Second, I attempted to minimize the number of sperm necessary to fertilize a single egg by reducing the volume in which the eggs were fertilized and by comparing the traditional fertilization system to a novel inverted drop technique. There were no differences between fertilization rates with sperm concentrations ranging from 1575 to 4 sperm/μL . Furthermore, the inverted drop technique improved overall fertilization. Both increased gamete manipulation time and reduced sperm waste have the potential to improve cryopreservation success in fish gametes.

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Introduction

Worldwide, aquatic animals are threatened with extinction due to overfishing, habitat loss, introduction of invasive species, and climate change. Even among fish species of commercial importance, populations have decreased between 60% and 85% since record keeping began (Reynolds *et al.* 2005). Worse yet, many of the remnant populations have severely bottlenecked genetics. North Sea cod live in schools of several million fish, yet these schools have a functional breeding population, a measure of genetic diversity, of 121 individuals (Hutchinson *et al.* 2003). This loss is further compounded by a lack of knowledge regarding many aquatic organisms' life history patterns, habitats, and population sizes. If this trend towards increased homozygosity compounded by decreased population size continues on, many fish species will inevitably go extinct.

Cryopreservation, or the subzero storage of biological material, is a powerful tool for endangered species preservation through the storage of eggs, sperm and embryos. Since the first mouse embryo was frozen in 1971 (Whittingham 1971), embryos and eggs have been frozen from mammalian ungulates (Wilmut & Rowson 1973), carnivores (Dresser *et al.* 1988), primates (Trounson & Mohr 1983), and marsupials (Breed *et al.* 1994). Likewise, sperm has been stored from a variety of species, mammalian or otherwise. Unfortunately eggs and embryos from birds, fish, and reptiles have yet to be successfully frozen. Portions of zebrafish embryos have been frozen and thawed successfully, but any tissue recovered can only be used to create chimeras, a process which requires tremendous amounts of skill and two generations of controlled breeding before genetics

can be properly recovered (Higaki *et al.* 2010). If any cryopreservation protocol is to be implemented on a large scale, it must be straight forward and easily reproducible.

There are several barriers to the successful cryopreservation of intact fish eggs or embryos. When eggs are first laid or stripped from a fish the eggs are permeable to ice reducing cryoprotectants, due to both an open channel in the shell-like chorion through which the egg will ultimately be fertilized and to an unpolymerized chorion. However, once fertilization or activation occurs, the channel closes and the chorion hardens (Zhang & Rawson 2007). At this point the eggs or embryos are impermeable to cryoprotectants and will significantly increase in size due to a brief influx of water (Wallace & Selman 1981). In zebrafish, this influx makes up 75% of an embryo's volume. Once an egg is laid, it must be fertilized within a tight window or it will spontaneously activate, or undergo its fertilization reaction. The end result of this process is a loss of fertilizability. Because of this tight time window, most research has focused on freezing embryos, which are easier to harvest and lack the time sensitivity of eggs.

However, the trade-off is clear. First, the increased volume of the embryo means that the surface area to volume ratio has decreased. By reducing the available surface area per unit volume, the embryos become far more difficult to rapidly cool. Second, it is difficult to expose the impermeable embryos to the same concentrations of cryoprotectants as an egg due to the polymerized chorion, which blocks water gain and ion loss in freshwater fish. As a result, during freezing ice crystals can form rapidly and without perturbation, expanding and potentially rupturing both the embryonic cell membranes and the chorion. The potential for successful cryopreservation of female fish genetic material may increase if

eggs could be held for longer periods of time without activating or degrading, thereby solving the issues of time, permeability, and size simultaneously.

Once eggs have been successfully frozen, they must be thawed and fertilized. Current fertilization protocols rely heavily on high numbers of both eggs and sperm. Cryopreservation protocols, conversely, cannot rely on these methods and often result in greatly diminished sperm quality and reduced viable sperm quantity. Protocols using cryopreserved gametes must be optimized both for high fertilization success of eggs and for minimized waste, particularly with regards to sperm. New *in vitro* protocols need to be developed to achieve both of these goals

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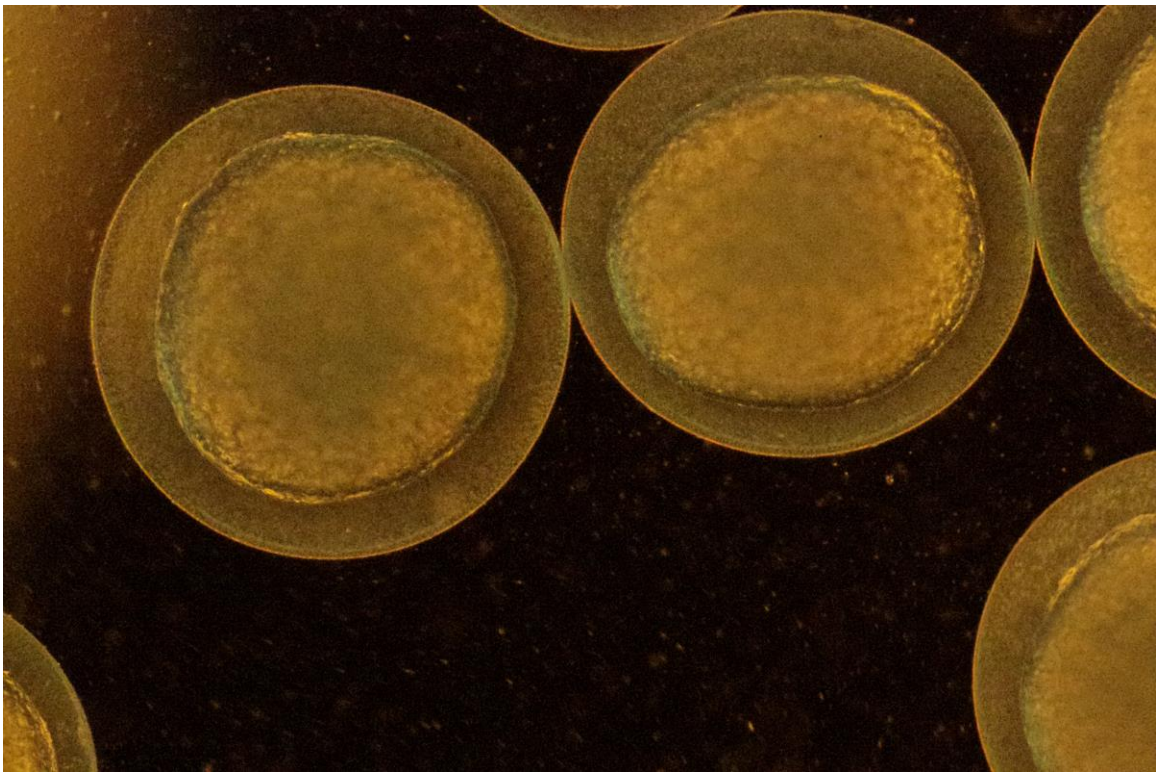
Experiment 1

Increased Holding Time in Zebrafish Eggs and Sperm



Experiment 1: Increased holding time for zebrafish eggs and sperm

The zebrafish (*Danio rerio*) is a common organism used in genetic and developmental research (Driever *et al.* 1994). Much of our understanding regarding fish development has been explored using these animals, but little work can be done on the gametes prior to fertilization. Particularly, zebrafish eggs are notoriously time sensitive when compared to eggs of other fish species. Eggs remain viable for perhaps ten minutes in water and an hour in a physiological saline (Sakia *et al.* 1997), after which they undergo rapid and irreversible changes normally associated with fertilization. During this process, known as activation, several important changes occur. First, the micropyle, a tunnel through the shell-like chorion which sperm use to gain access to the egg during fertilization, is plugged by the fertilization cone. As a result, the eggs are no longer accessible to sperm and therefore no longer fertilizable without intracytoplasmic sperm injection (Poleo *et al.* 2001). Second, the proteins which make up the shell-like chorion polymerize, creating a barrier around the egg. During polymerization, the space between the egg and the chorion fills with water and swells, displacing the chorion. Once the chorion has completely hardened the egg becomes impermeable to ion loss or gain (Loeffler 1971). This egg activation process results from egg-sperm interactions in most teleost species and is the principle block to polyspermy or multiple fertilization events (Ginsburg 1972).

Zebrafish eggs do not trigger activation due to egg-sperm interaction, but rather due to exposure to the fertilization fluid, more generally fresh water (Lee *et al.* 1999). The effect of physiological saline on egg holding hints that a major component of this activation process may be osmotically regulated. However, isotonic solutions alone are not enough to

prevent activation. Eggs still age in saline solution and either activate or break down (Sakai *et al.* 1997). Addition of serine protease inhibitors like leupeptin significantly increase storage time over physiological salines alone, indicating that the actual oviductal fluid may contain both osmotic components and small peptides or proteins critical to maintaining egg viability before mating (Minin & Ozerova 2008). This may be the method by which eggs are maintained *in vivo.* Additionally, powerful serine protease inhibitors have been found in rainbow trout semen and may be present in the seminal fluid of other fish (Ciereszko *et al.* 2005). It is possible that the act of laying eggs washes away a soluble or lower affinity inhibitor and dramatically lowers osmolites around the eggs. The male ejaculate may resupply these factors during the mating process, maximizing sperm success and overall fertilization by halting spontaneous activation.

This harmony between semen constituents and egg viability may provide a window into a potential means of holding both eggs and sperm for extended periods of time. Oviductal fluid contain components necessary for nourishing and maintaining hundreds or thousands of eggs through oogenesis. Current physiological salines like Hank's Balanced Salt Solution (HBSS) address only the osmotic component and often do not provide carbon sources for energy, as well as protein, fats, or hormones that an egg might need (Bahadabi *et al.* 2011). Simply supplementing HBSS with the protein Bovine Serum Albumin substantially increases holding time for unfertilized zebrafish eggs (Sakia *et al.* 1997).

In an ideal system, manually expressed eggs could be stored in the same oviductal fluid which has nourished them to that point, but the overall volume of oviductal fluid obtained from a single zebrafish is quite minimal and squeezing zebrafish for any usable quantity would prove fruitless. Rainbow trout (*Oncorhynchus mykiss*) have the potential to

serve as a donor for surrogate oviductal fluid. Rainbow trout are commonly farmed fish, and oviductal fluid is already collect at many of these farms for ELISA testing. Oviductal fluid can easily be removed from manually stripped clutches with a colander and a bucket, and subsequently filtered, sterilized, and frozen for laboratory use in significant quantities. Coho salmon oviductal fluid has been used in previous androgenic studies in zebrafish, but the limits with regards to storage have not been tested (Corley-Smith *et al.*, 1996).

Oviductal fluid, like many biological fluids, buffers its pH using a bicarbonate system. The CO_2 produced by cellular metabolism is combined with water and converted into the diprotic carbonic acid (H_2CO_3) which is in turn converted into bicarbonate (HCO_3^-) and H^+. CO_2, H_2CO_3, and HCO_3^- work in equilibrium to maintain systemic pH. Because atmospheric CO_2 and blood CO_2 levels differ, biological fluids will not have the same pH *in vitro* as *in vivo* unless the *in vitro* atmosphere is supplemented with physiological levels of CO_2. Improper pH may in turn have a profound effect on protein conformations and enzyme activity, as well egg metabolism and overall cell culture health.

Physiological salines are also used to hold zebrafish spermatazoa. While the overall mechanism of zebrafish sperm motility is poorly understood, previous research has linked swimming to both basic pH and low osmoloarity (Jing *et al.* 2009). HBSS provides a suitable environment for short term storage, but sperm viability drops off precipitously after 4 hours. Oviductal fluid has been shown previously to improve holding times with stickleback sperm due to its physiological osmolarity as well as potential undiscovered constituents (Elofsson *et al.* 2003). The effects of oviductal fluid properly buffered in a bicarbonate atmosphere have yet to be investigated in zebrafish.

It is important to note that if oviductal fluid proves to be a better holding medium than current physiological salines, it cannot replace sterile media. The oviductal fluid must be collected from farmed fish who are subject to a myriad of freshwater diseases. The very protocol that makes collecting large quantities of oviductal fluid possible is ELISA screening of small oviductal aliquots for freshwater diseases (Smails & Munro 2007). Filteration can reduce most bacterial and eukaryotic biological contamination, but it cannot eliminate viruses. Even in a best case scenario, oviductal fluid can only set a benchmark for gamete holding.

I hypothesized that the use of rainbow trout oviductal fluid will dramatically increase holding times in both eggs and sperm. I further hypothesized that pH and atmospheric composition is critical to extended holding times in gametes, and that a traditional blood/gas atmosphere would further increase holding times of both eggs and sperm over oviductal fluid at atmospheric pH levels or oviductal fluid brought to the equivalent pH using acid buffer instead of the bicarbonate system.

Materials and Methods

1.1 Collection and preparation of rainbow trout oviductal fluid

Rainbow trout oviductal fluid (RTOF) was collected from the Spokane Hatchery (Spokane, WA). Trout were spawned into a colander with a fine mesh cover, and the oviductal fluid was drained into a bucket. The RTOF was immediately treated with 150 mg Penicillin, 100 mg Streptomycin, 50 mg Ampicillin, and 50 mg Kanamycin per Liter and held on ice. RTOF was filtered 5 times through filters with successively smaller pore sizes,

and ultimately filtered through 1 μm pores to remove any non-viral biological contamination, and finally stored at -20°C. Repeated filtration clarifies the RTOF (Figure 1), and reduced turbidity has been correlated with increased survivorship in studies using RTOF to hold eggs from goldfish (*Carassius auratus*) for 30 minutes (Depince *et al.* 2011).

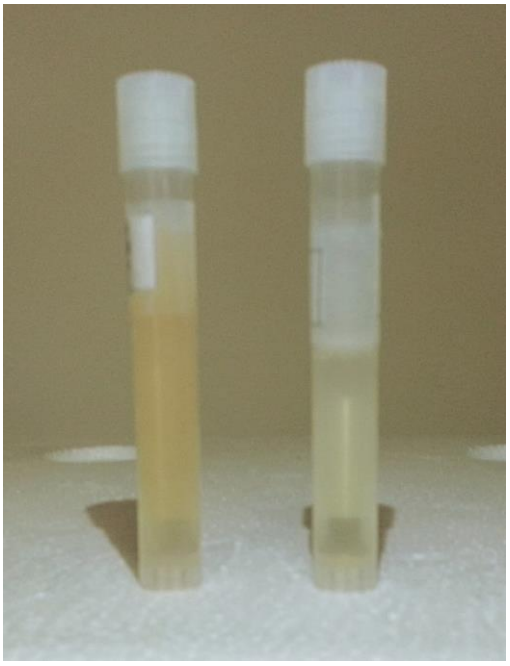


Figure 1*: Fresh, unfiltered RTOF (left) and filtered RTOF (right). Unfiltered RTOF is pungent smelling, orange in color, and highly turbid. Filtered RTOF is a clear or near clear fluid and odorless.*

RTOF pH was assessed at both room atmosphere (pH 8.0) and under blood/gas atmosphere of 7% CO_2 and 7% O_2 with balanced N_2 (pH 7.2). To mimic blood/gas atmospheric pH under ambient atmospheric conditions, RTOF was mixed with 5 mmol/mL HEPES (Sigma, H-3375), yielding a pH of 7.2.

1.2 Zebrafish Care

Zebrafish (EKK strain) were purchased from Ekkwill Waterlife Resources (Ruskin, FL). Fish were maintained at 28 ºC with a 14L:10D light cycle as outlined in Harvey and Chamberlain (1982). All animal care and experimentation was evaluated and approved by the Eastern Washington University Institutional Animal Care and Use Committee .

1.3 Gamete collection

Zebrafish were anesthetized in an 18 ppm Tricaine (MS-222) solution. Males were carefully dried and positioned ventral side upward in a slit sponge. The urogenital region was blotted a second time and semen was expressed via gentle pressure below the pelvic fin. Semen was pipetted carefully to avoid fecal contamination (Nüsslein-Volhard & Dahm 2002) (Figure 2). For the holding experiment, 10 µL semen was collected and pooled, undiluted. Sperm fertilization stock, for fertilizing eggs during the egg holding experiment, was prepared fresh before each fertilization by diluting approximately 5 µL fresh semen into 100 µL ice cold HBSS.

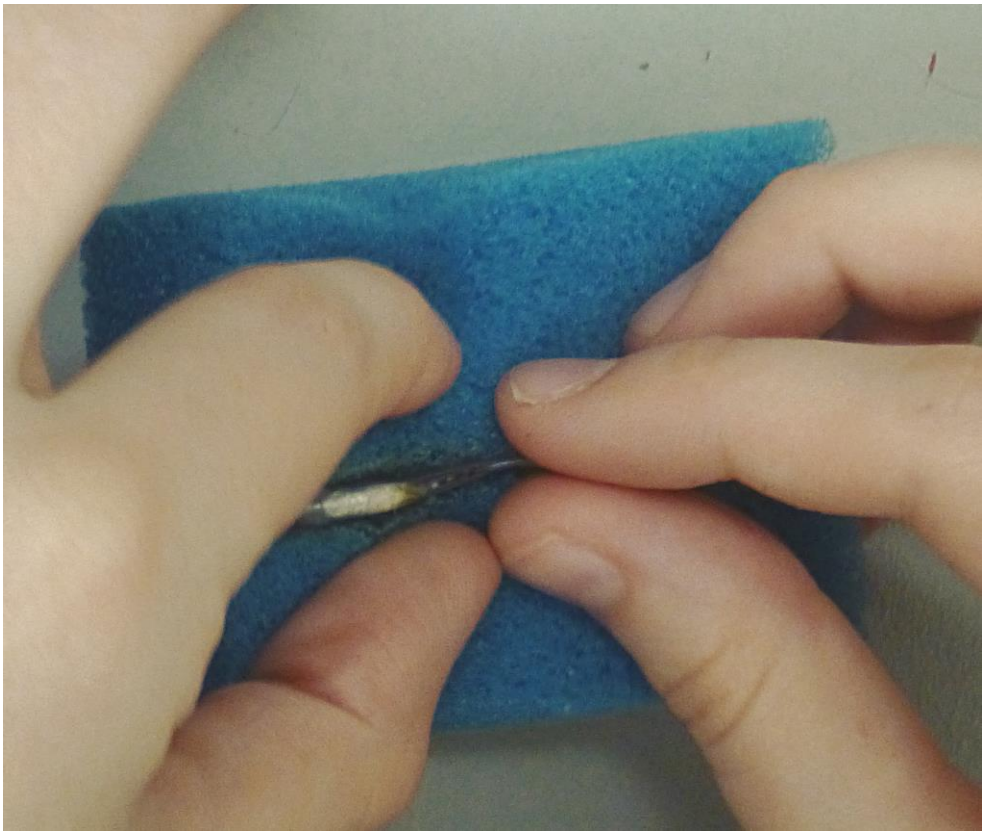


Figure 2*: A male zebrafish wedged in a slit sponge. Pressure is applied directly below the pelvic fin with the left hand.*

To collect eggs, anesthetized females were blotted and placed in a sterile petri dish. Eggs were expressed using gentle abdominal pressure sweeping from the pectoral fin towards the anal fin (Nüsslein-Volhard & Dahm 2002) (Figure 3). Clutches were divided into 4 groups and gently moved into respective treatments using a Pasteur pipette. Previous research has indicated that eggs are vulnerable to mechanical damage prior to fertilization (Hagedorn & Carter 2011), so manipulation was minimized where ever possible.

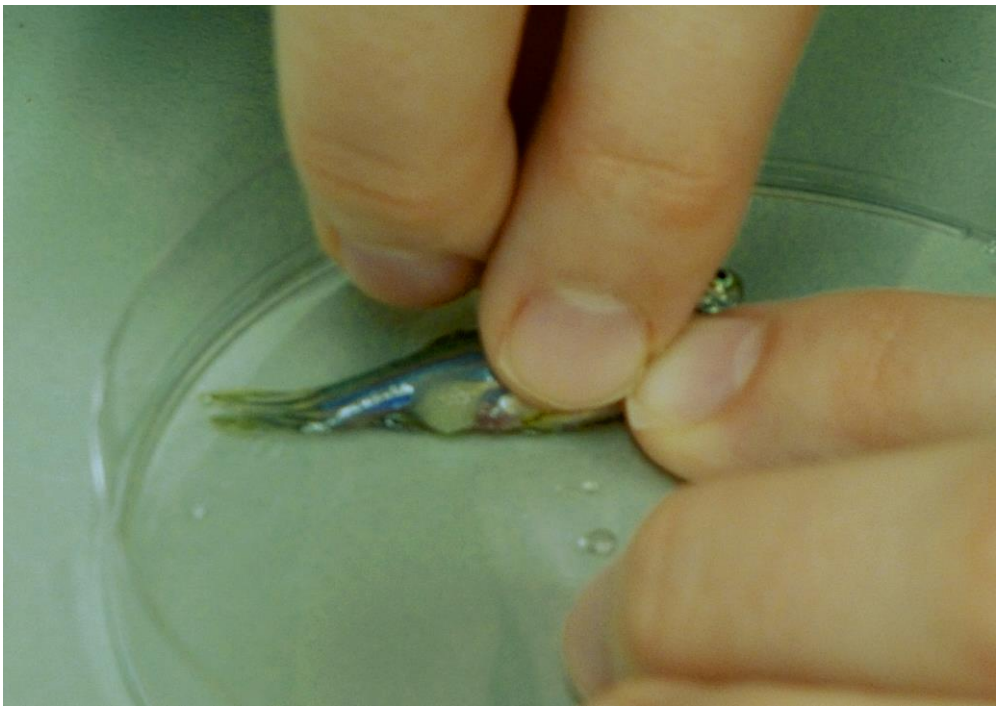


Figure 3*: Expression of eggs from a female zebrafish. Only very gentle pressure is necessary to strip eggs from a gravid female.*

1.4 Sperm holding Experiment

To evaluate different RTOF-based medias' effectiveness as sperm holding solutions, 2 µL of pooled semen was pipetted into microcentrifuge tubes containing holding solutions consisting of 100 µL of either RTOF, RTOF buffered with 5 mmol/mL HEPES, or RTOF equilibrated to a blood/gas atmosphere (7% CO_2 and 7% O_2, balanced N_2). Samples were be held at ~4 ºC and assessed at 4, 8, and 24 hours for motility. Motility at 0 hours was assessed by diluting 1 µL pooled semen in parts into 3 separate 10 µL diH_2O drops and counting motile and nonmotile sperm. Motility at 4, 8, and 24 hours was assessed by diluting 1 µL sperm containing holding solution into 10 µL diH_2O a total of 3 times and counting motile and nonmotile sperm. Observations of sperm quality were recorded during each time point.

1.5 Egg holding Experiment

Individual egg clutches were divided into 4 groups and pipetted into 1 mL RTOF, RTOF buffered with 5 mmol/mL HEPES, or RTOF equilibrated to blood/gas atmosphere (7% CO_2 and 7% O_2, balanced N_2). The 4th group, consisting of approximately 40 eggs from the clutch, was fertilized with 10 µL of sperm fertilization stock for the 0 hour measurement representative of fresh eggs unincubated in RTOF. Eggs were held at ~4 ºC and viability was assessed at 0, 4, 8, and 24 hours post incubation.

To assess viability, 40 eggs per treatment per timepoint were pipetted into fresh IVF dishes, fertilized with 10 µL freshly collected sperm fertilization stock, activated with 200 µL system water and incubated for 5 min. The IVF dish were subsequently flooded with system water and incubated at 28 ºC. Eggs were assessed for proportion fertilized after 4 hours of incubation. Successful fertilization was identified as the sphere stage of development: a spherical shaped embryo and yolk mass with a flat boundary delineating developing embryo from the and the yolk (Kimmel *et al.* 1995). At all timepoints and for all treatments, observations were made with regards to egg morphology and yolk breakdown. Any eggs which showed signs of yolk break down or chorion failure were not moved for fertilization.

1.6 Statistics

Egg and sperm viability was assessed using a binomial z-statistic, with a Holm-Bonferroni correction for familywise error. In z-statistics, a p-value $\geq$ 0.95 generally indicates significant differences between treatments. Eggs were counted as fertilized or unfertilized. Any eggs which dechorionated were removed from the experiment, as it is

impossible to assess if dechorionation happened prior to, during, or after fertilization. Sperm were counted as motile or nonmotile.

Results

Sperm Holding Experiment

Initial pooled sperm motility was 82.9%. Over the 24 experimental period, motility in all samples declined when compared to fresh motility ($p \geq 0.999$). At 4 hours, sperm motility in the RTOF + HEPES treatment (75.7%) was the first to decline compared to fresh sperm ($p = 0.997$), but these sperm were still statistically indistinguishable from those in the RTOF (motility = 77.5%, $p = 0.734$) or RTOF + blood/gas atmosphere (motility = 76.6%, $p = 0.682$). At 8 hours, however, motility in the RTOF treatment (50.9%) had declined significantly when compared to RTOF + HEPES (motility = 62.9%, $p \geq 0.999$) and RTOF + blood/gas atmosphere (motility = 62.6%, $p \geq 0.999$) at the same time point. The RTOF + HEPES and RTOF under blood/gas atmosphere were statistically indistinguishable ($p = 0.842$). By 24 hours, sperm incubated in RTOF had lost almost all motility (2.8%). RTOF + HEPES (28.4%) had significantly declined compared to RTOF + blood/gas atmosphere (motility = 56.9%, $p \geq 0.999$) (Figure 4).

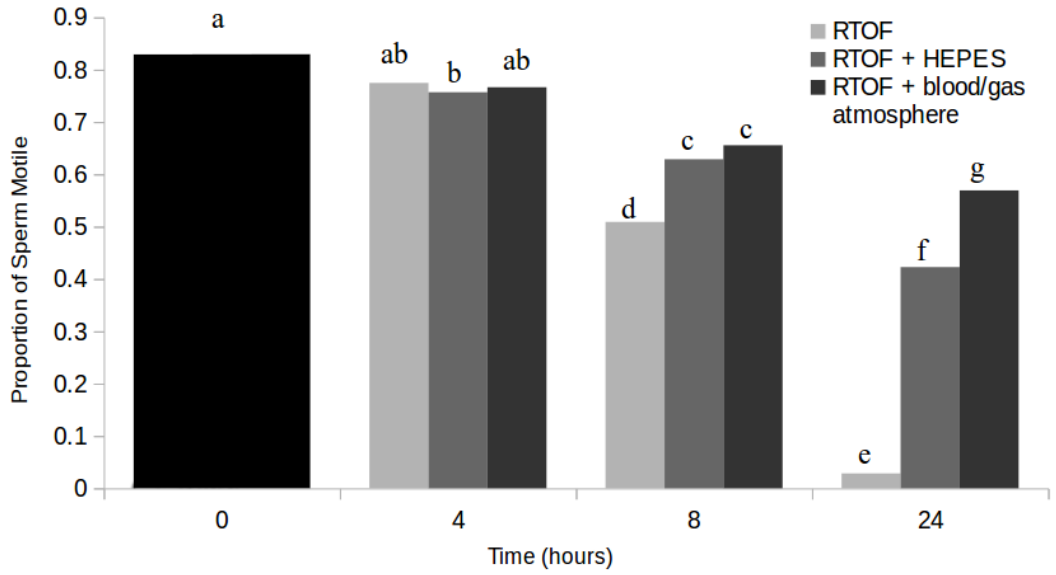


Figure 4*: Proportion of sperm motile at 0, 4, 8, and 24 hours. The black bar at 0 hr indicates initial, motility taken from the fresh, unincubated sperm sample. Letters indicate significant differences first between fresh sperm motility and motility in a sample at an time point, and then between samples within a time point.*

Observed sperm motility also varied from across treatments over time. At 4 hours, All samples had motility, speed, and direction no different from that of fresh sperm. At 8 hours, sperm in both the RTOF and the RTOF + HEPES treatment had lost speed compared to fresh sperm or sperm in the RTOF + blood/gas atmosphere treatment. Sperm in the RTOF + blood/gas treatment still swam in a manner equivalent to fresh sperm, though fewer total sperm were swimming.

At 24 hours, the few remaining motile sperm in the RTOF treatment were moving swimming more slowly yet. In the RTOF + HEPES, sperm had again lost speed compared to both fresh sperm and the 8 hour RTOF + HEPES sperm, but were still stronger, faster swimmers than those in the 24 hour RTOF treatment. Both the RTOF and RTOF + HEPES

treatments had large aggregates of dead cells which had formed in the microcentrifuge tubes. The sperm in the RTOF + blood/gas atmosphere treatment still swam in a manner equivalent to fresh sperm, and no aggregate had formed in the microcentrifuge tube.

Egg Holding Experiment

Initial viability in the egg holding experiment, assessed as the proportion of eggs fertilized, was 91.8%. At 4 hours, egg viability in the RTOF (92.3%, $p = 0.532$), the RTOF + HEPES (95.2%, $p = 0.742$), and the RTOF under blood/gas atmosphere treatments (97.3%, $p = 0.860$) were indistinguishable from 0 hour eggs. At 8 hours, egg viability in the RTOF treatment had significantly declined compared to 0 hour egg viability (72.5%, $p = 0.987$). RTOF + HEPES egg viability had improved compared to 0 hour egg viability (100%, $p = 0.964$). Egg viability in the RTOF under blood/gas atmosphere treatment remained indistinguishable from 0 hour egg viability (94.0%, $p = 0.655$).

By 24 hours, egg viability in the RTOF had further declined to 22.7% ($p \geq 0.999$). The RTOF + HEPES treatment egg viability declined compared to 0 hour egg viability (26.1%, $p \geq 0.999$). Egg viability in the RTOF under blood/gas atmosphere remained statistically indistinguishable from 0 hour egg viability (90.3%, $p = 0.722$) (Figure 5).

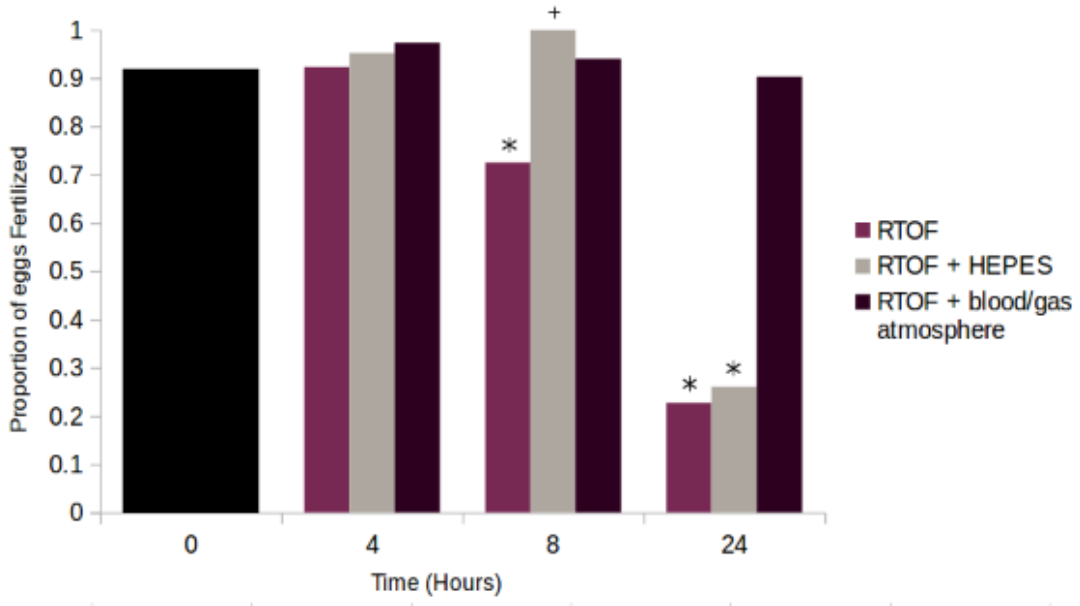


Figure 5*: A comparison of the proportion of eggs fertilized at 0, 4, 8, and 24 hours post-incubation. The black bar at 0 hours represents freshly stripped, unincubated eggs. Letters are used to group eggs by significance. Treatments labeled a are no different from hour 0 eggs. The label b indicates statistically significant negative differences from hour 0 eggs. Label c indicates statistically significant positive differences from hour 0 eggs.*

Eggs underwent profound changes in morphology and health over the course of the experiment. In the RTOF treatment, eggs appeared fresh at 4 hours, but by 8 hours the yolk material began to collapse and had an appearance similar to that of an anucleated red blood cell. By 24 hours, 10% of the eggs in the RTOF treatment had failed entirely, with both complete or near complete yolk degradation and chorion failures resulting in yolk and egg material intermingling with the external oviductal fluid. In the RTOF + HEPES treatment, eggs appeared fresh at 4 and 8 hours post incubation. At 24 hours, however, the eggs were no longer spherical, the chorion had become noticeably flaccid, and the yolk material was beginning to break down.

In the RTOF under blood/gas atmosphere treatment, the chorion had separated from the yolk material resulting in what appeared to be a perivitelline space, one of the hallmarks of activation. Eggs, however, were still no different from fresh eggs with regards to fertilization, indicating that spontaneous activation had not occurred. This separation persisted throughout the experiment's duration.

Oviductal fluid properties also varied over the course of the experiment. Both the RTOF and the RTOF + HEPES treatment oviductal fluid began to develop long, filamentous crystals at 8 hours post-incubation. The RTOF under blood/gas atmosphere did not develop similar crystals at any point during the experiment.

Discussion

In both sperm and eggs, oviductal fluid under physiological atmospheric conditions dramatically improved viable gamete holding times. Because the RTOF + HEPES treatment preserved both motility in sperm and fertilizability in eggs compared to RTOF alone, it can be inferred that physiological pH plays at least a partial role in gamete health. However, the success of RTOF under blood gas atmosphere at 24 hours in both egg and sperm incubation clearly demonstrates that pH is not the only factor necessary for maintaining gamete viability.

Several factors may be at play. First, atmospheric O_2 levels are approximately three times that of physiological O_2 levels. Reduced O_2 has several implications. Lower overall O_2 levels decrease the overall amount of oxidative phosphorylation in a cell. Reactive oxygen species production has been found at all stages of the electron transport chain

(St-Pierre *et al.* 2002, Liu *et al.* 2002). High levels of oxidative phosphorylation by necessity result in the increased and inevitable production of radical oxygen species, which can propagate and ultimately cause oxidative stress throughout fragile cells. In eggs, this process can occur in the lipid-rich yolk, where lipid peroxidation feedback loops may be the underlying cause of yolk breakdown (Halliwell & Chirico 1993). Sperm have no or minimal gene expression, and do not have the capacity to produce normal cellular responses necessary for repairing damages caused by oxidative stress (Steger *et al.* 1998). Non-physiological levels of oxygen may simply not be tolerated by zebrafish gametes as a consequence of structure and function.

The second property of the blood gas atmosphere is increased CO_2. As previously explained, CO_2 can have a profound effect of media pH due to the formation of the diprotic carbonic acid, which loses or gains protons to maintain equilibrium based on atmospheric CO_2 levels. The addition of HEPES to RTOF was meant to mimic media pH under physiological atmospheric condition without altering atmospheric composition. However, HEPES can only control media pH. The bicarbonate buffer system is unique in that it can help regulate both extracellular, or media based, pH as well as intracellular pH (Thomas 1976). CO_2 can be transported into cells to help regulate and buffer intracellular pH, and similarly bicarbonate HCO_3^- can be transported out of cells to help move ions and create membrane potential (Itel *et al.* 2012). This increased buffering capacity alone may explain sustained viability in zebrafish eggs. Sperm motility in freshwater fish is linked to membrane potential, and in trout sperm membrane potential has been found to be more important to motility than intracellular ATP concentrations (Cosson *et al.* 1991). Preserved motility quality in the RTOF under blood/gas atmosphere may be directly related to proper

intracellular buffering due to media gas composition resulting in proper membrane potential. The separation of the chorion in eggs in the RTOF under blood/gas atmosphere treatment may also be related to CO_2 and ion exchange, but any proposed mechanism would be highly speculative.

The use of physiological atmospheric conditions when incubating fish gametes is superior in all cases. Damage due to yolk breakdown is minimized in eggs, spontaneous activation is abolished in eggs, and overall motility as well as motility quality is preserved in sperm. While treatments were indistinguishable from one another at 4 hours in both the egg and the sperm experiment, the metrics used here were highly specific. Sperm incubated in RTOF alone may have lost other qualities not observed here, such as the all-important ability to fertilize an egg. Eggs were only assessed at the 4 hour post fertilization, and differences in embryo development or post-hatch fitness may have emerged with proper experimental methodologies. However, the difference in overall gamete health by these two metrics, fertilization in eggs and motility in sperm, indicates that a physiological atmosphere is the least likely to cause undue harm to these fragile cells. The improvement in the 8 hour egg RTOF + HEPES treatment also indicates that post-stripping incubation in a media with the appropriately pH may have a beneficial effect. This may be a result of either proper pH helping repair damages caused by the stripping process or improving egg maturation, ultimately resulting in more viable eggs. This improvement was not seen in the RTOF under blood/gas atmosphere egg treatment at any time point, but this may be due to lower the oxygen concentrations depressing the eggs' metabolism.

In addition to the implications for gamete storage and manipulation, the extension of holding times from 4 hours to 24 hours in eggs and sperm alike may allow for long distance

transport of zebrafish gametes and may have applications in other freshwater fish. This may open up zebrafish research to smaller labs which cannot afford to maintain a colony. Furthermore, it presents an opportunity for bringing zebrafish development into the high school classroom.

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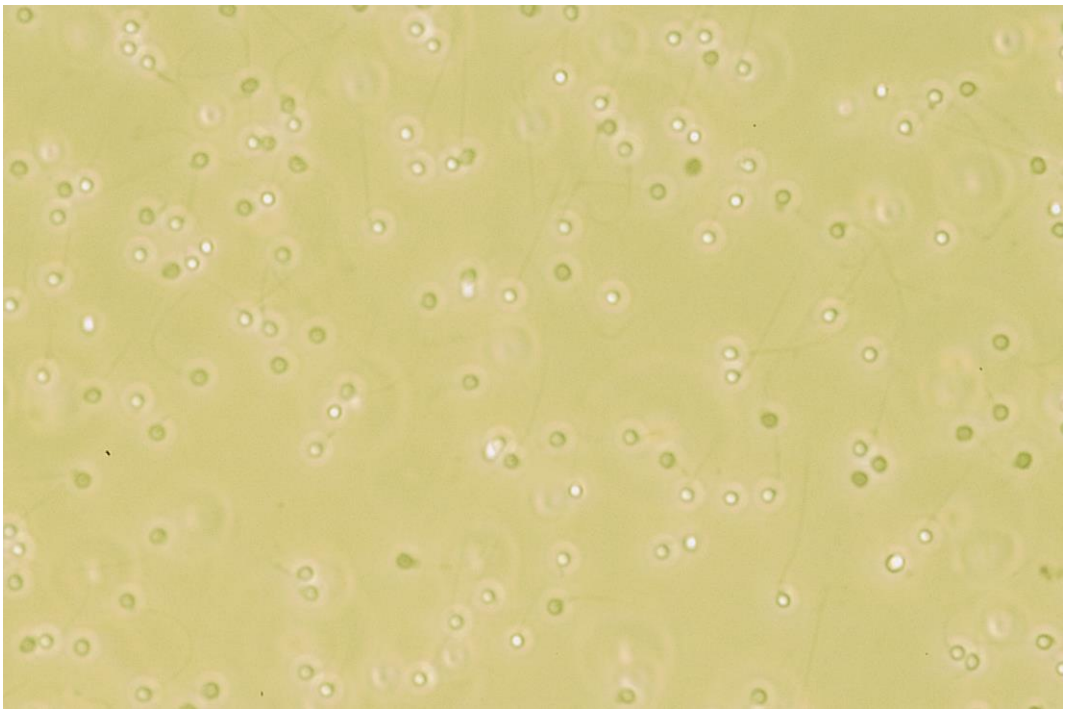
Experiment 2

Minimizing the Number of Sperm necessary to Fertilize an Egg



Introduction

Oviparous fish, like many organisms that rely on external fertilization, use high numbers of gametes to maximize reproductive success. Large clutches of eggs are fertilized by ejaculants containing millions of sperm in a large body of water. Laboratory and industrial procedures have not diverged far from the model nature provides. The widely accepted zebrafish protocol for fertilization involves semen pooled from 5 to 10 males (Westerfield 2007). The average male zebrafish produces 0.8 µL of semen (Harvey, Kelley, & Ashwood-Smith 1982) with a density of 9.96×10^7 sperm/ µL (Liu *et al.* 2010), but these concentrations can vary wildly from male to male (Hagedorn & Carter 2011). In contrast the average female zebrafish lays between 100 and 1000 eggs (Culp, Nüsslein-Volhard, & Hopkins 1991). While the pooled male semen may be used to fertilize eggs from multiple females, the sperm to egg ratio always remains astronomically high. Outside laboratory settings, the US Department of Fish and Wildlife has conducted research on the best way to mix milt, oftentimes from multiple males, and eggs from one female in large buckets (Sharbles 1998). These high volume methods are used because of their excellent success rate, but as a result, numerous sperm expire without fertilizing an egg.

Previous research in a variety of fish has shown that even in these volume-based experiments sperm concentrations are unnecessarily high. Work in catfish has shown that the optimal sperm to egg ratio is roughly 10,000-40,000 sperm per egg, and that higher ratios may be inhibitory (Rurangwa *et al.* 1998). Laboratory *in vitro* fertilization in zebrafish can be brought down to 1000 sperm/µL without affecting fertilization rates (Hagedorn & Carter 2011), but these protocols have not been widely implemented. Current

fertilization protocols require orders of magnitude more sperm than are necessary to achieve maximal fertilization success.

Spermatozoa are, most simply, packages of DNA. If we wish to implement long term preservation of richness in all species, threatened or otherwise, the easiest means of doing so is sperm cyropreservation. In 1982, Harvey *et al.* first successfully cryopreserved zebrafish sperm, and sperm from many other teleost species has been successfully frozen. Given this technology, a liquid nitrogen tank can be thought of as a frozen population containing many thousands of individual males. Aliquots of semen can be stored nearly indefinitely, and if a population of fish bottlenecks due to catastrophe, habitat loss, or disease, the old genetic richness can easily be restored by breeding available females with that frozen population. However, species preservation is incompatible with current fertilization protocols, an entire ejaculate is either used or frozen and the totality of sperm from a single male might be used before its offspring's fitness can be assessed. An ill-timed introduction of individuals susceptible to a disease or unfit for climate conditions might mean valuable genetic richness is lost forever.

In addition to waste prevention and storage optimization, sperm minimization protocols also provide an assay for overall sperm fitness. Our current metrics for cryopreserved sperm viability after thawing are motility and live/dead cell staining. However, previous research in honey bee sperm cryopreservation has shown that frozen sperm that is indistinguishable from fresh sperm in terms of motility and live/dead cell staining does not produce the same results as fresh semen when injected into virgin queens. The queens can produce diploid workers only intermittently (Hopkins *et al.* 2012). Ideally,

the best cryopreservation technique for a sperm should ultimately be measured by reproductive success, not by appearance alone.

In this experiment, I reduce the number of sperm necessary to fertilize an egg by isolating eggs in microdrops during fertilization and compare a reduced version of traditional *in vitro* fertilization to a novel inverted drop protocol. I hypothesized that by decreasing the volume of water involved through the use of microdrops and by increasing the likelihood of egg-sperm associating using the inverted drop the number of sperm per egg necessary to achieve standard fertilization rates would be substantially reduced.

Materials and Methods

2.1 Fish Care

Zebrafish (EKK strain) were purchased from Ekkwill Waterlife Resources (Ruskin, FL). Fish were maintained at 28 ºC with a 14L:10D light cycle as outlined in (Harvey and Chamberlain 1982). All animal care and experimentation was evaluated and approved by the Eastern Washington University Institutional Animal Care and Use Committee.

2.2 Gamete collection

Fish were anesthetized in 18 ppm tricaine (MS-222). Males were dried, wedged ventral side up in a slit sponge, and blotted along the urogenital region. Semen was expressed by gentle pressure above the pelvic fins (Nüsslein-Volhard & Dahm 2002). Stock solutions 0, representative of current zebrafish fertilization protocols and used here as the first semen dilution, consisted of 10 µL of semen pooled in 90 µL ice-cold Hanks Balanced Salt Solution (HBSS).

Female fish were dried and squeezed for eggs in a petri dish via gentle pressure sweeping along the abdomen from the pectoral fin towards the urogenital region (Nüsslein-Volhard & Dahm 2002). Eggs were moved to ice-cold HBSS using a Pasteur pipette and held for no more than 5 minutes prior to fertilization.

2.3 Sperm Assessment and Dilution

Sperm was serially diluted. A total of 11 sperm concentrations were made by pipetting 50 μL of the initial stock (dilution 0) into 50 μL fresh HBSS. From the newly made solution, dilution 1, 50 μL was then pipetted into 50 μL of fresh HBSS to make dilution 2, with the process subsequently repeated a total of 10 times. Sperm concentrations were therefore calculated using the expression $x_0/2^n$, where x_0 is the sperm concentration dilution 0 and n is the dilution factor. Dilution factors were assigned values from 0-10, where 0 is the stock initially collected and therefore the least dilute, and 10 is the final and most dilute solution. All dilutions were held on ice. Serial dilution was chosen over stepwise reduction because the exponential curve results in few dilutions at known successful concentrations and more resolution at lower concentrations due to fewer sperm separating each subsequent dilution (Figure 6).

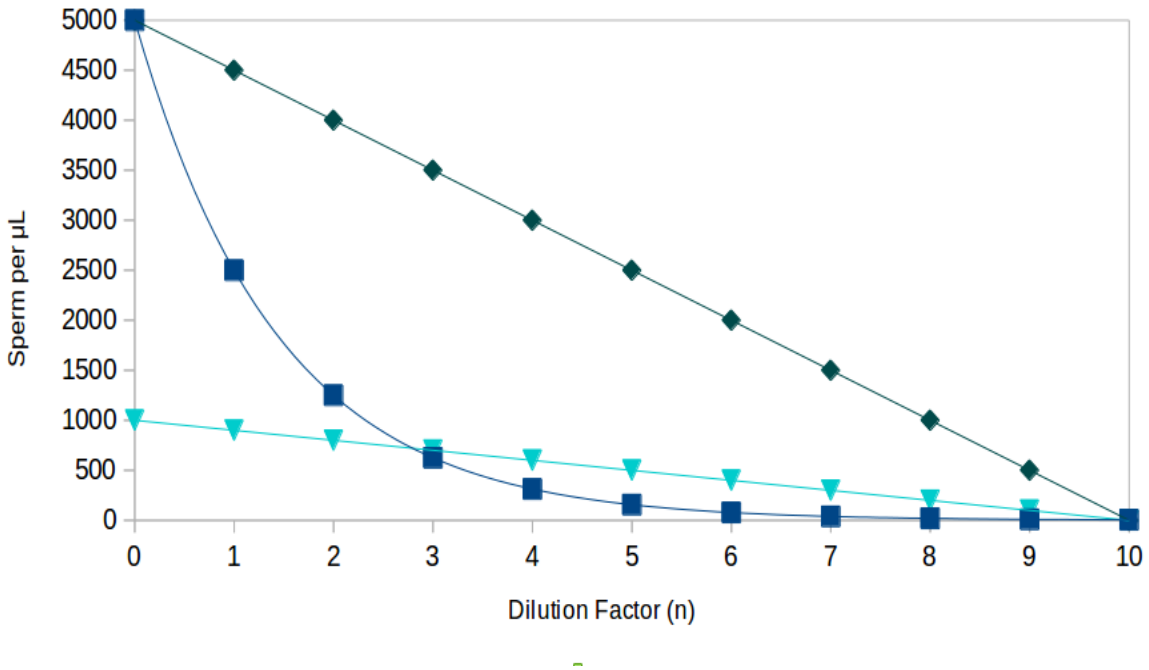


Figure 6*: A comparison of different dilution methods and the resulting sperm concentrations. Serial dilution, indicated by the blue squares, results in an exponential curve. Here, the minimal sperm/µL concentrations previously identified in other research are represented by dilutions 2 and 3. All dilutions after 3 have increased resolution due to fewer sperm/µL separating each subsequent dilution. A linear dilution beginning at the same sperm concentration, represented by the dark blue diamonds, does not achieve the previous literature concentrations until the 8th dilution, and the 10th dilution has no sperm present. Even beginning with at the previous literature best and a much lower initial concentration, the light blue triangles, resolution does not increase as sperm concentration decreases, and the 10th serial dilution once again contains no sperm. In the representative serial dilution, the 10th dilution contains 4 sperm/ µL*

Motility was estimated by mixing 2 µL of dilution 0 with 10 µL diH_2O. Because the 0th dilution contains sperm at extremely high densities, all sperm counts were taken at 1/1000th the initial concentration. To accurately count sperm, 10 µL of dilute sperm was mixed with 1 µL Bisbenzimide H33342 Fluorochrome solution (0.1 mg H33342 dissolved in 1 mL 10% DMSO), incubated for 10 min, and observed and counted using fluorescent microscopy (excitation 346 nm, emission 460 nm). H33342 is a quantum dye, and

dramatically increases its fluorescence when bound to nucleic acid (Figure 7). Sperm counts were averaged from 6, 10 µL aliquots to yield sperm/µL in dilution 0.

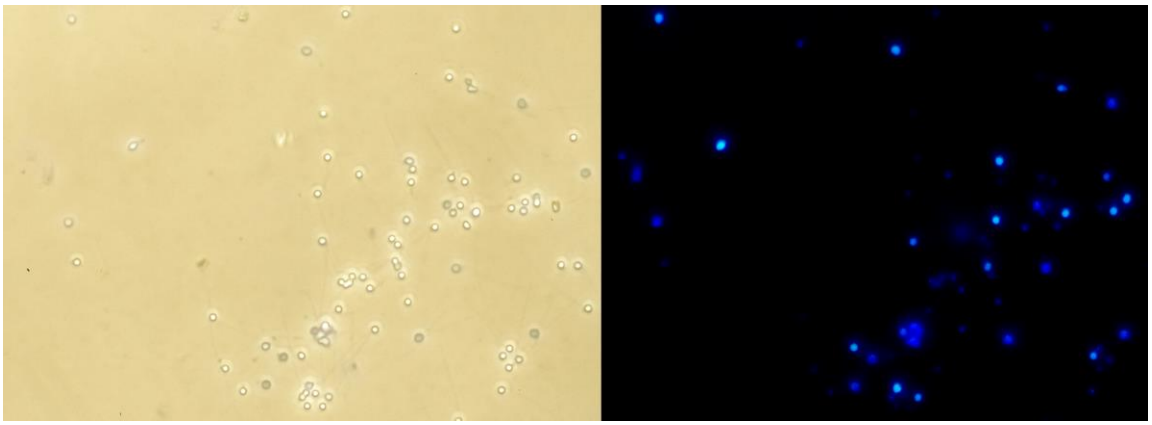


Figure 7: *A comparison of sperm stained with H33342 using traditional microscopy (left) and fluorescent microscopy (right) at 400x magnification. The visual field is identical in both photos, however, the ability to see individual sperm and to distinguish sperm from surface imperfections on the slide or from crystals in the media is dramatically improved with fluorescence.*

2.4 Preliminary Fertilization Experiment

Because most zebrafish *in vitro* fertilization techniques are performed in a petri dish, I compared fertilization in drops on both the traditional upward facing dish and a novel inverted dish, or hanging drop, technique. The upwards facing dishes serve as a reduction of the traditional laboratory technique, where the microdrop confines the sperm and eggs tightly, increasing the likelihood of association and fertilization. The inverted dishes further minimize the area over which the egg and sperm can interact by using gravity to draw both gametes to the center of the drop, rather than the base of the dish.

Both systems are prone to rapid dehydration, which is lethal to developing embryos and unfertilized eggs alike. To overcome this in the upward facing treatment, eggs were

fertilized in drops in the outer ring of an IVF dish with the central well, generally used for cell culturing, acting as a water reservoir (Figure 8).

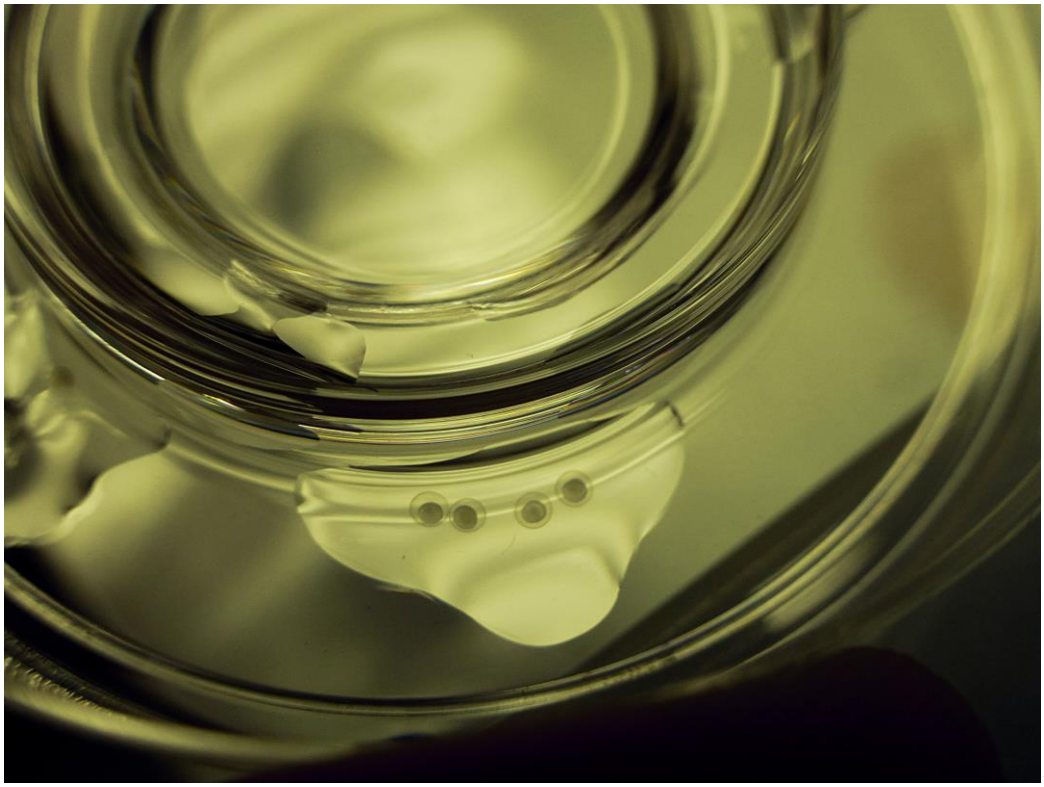


Figure 8*: Eggs fertilized and developing in the traditional fertilization system. The central well of the IVF dish has been filled with system water.*

In the inverted dish treatment, eggs were fertilized in drops on sterile petri dish lids. The dishes were then flipped and carefully placed on a tray with a shallow pool of water. Tray water must be shallow enough not to come in contact with the hanging microdrops (Figure 9).

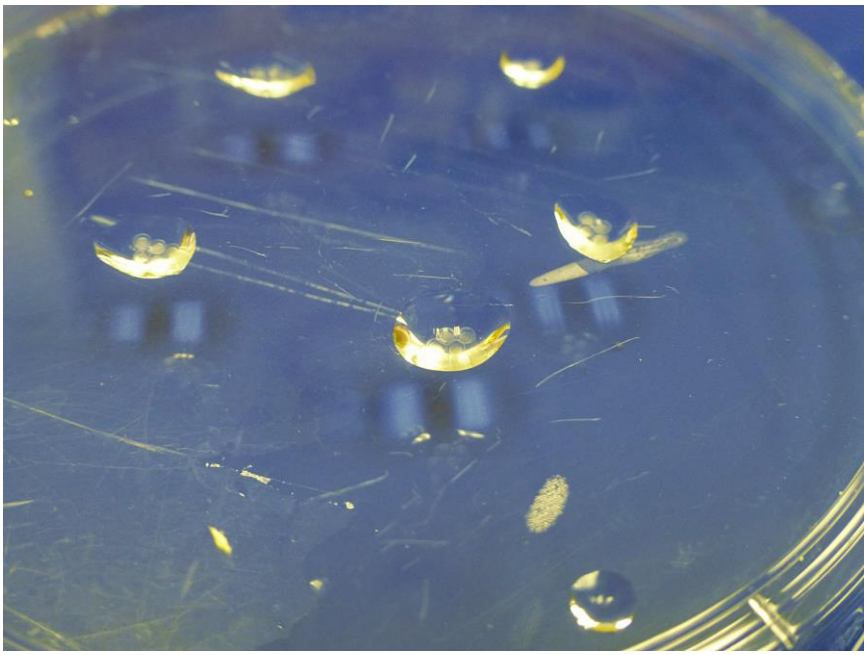


Figure 9*: Eggs fertilized and developing in the hanging drop protocol. The tray has been flooded with system water.*

Each treatment consisted of 5 microdrops, with 3-5 eggs pipetted into each microdrop, per fertilization system, per dilution. Excess HBSS was removed to reduce the final drop volume. In order to fertilize the eggs, 1 µL of sperm solution of the appropriate dilution was added to the drop, followed by approximately 20 µL of system water to activate the sperm and eggs. Inverted dishes were flipped upside-down immediately following activation with system water. IVF dishes were set aside with the lids secure. Fertilization success was assessed after 4 hours of incubation. Successful fertilizations was identified as a spherical shape with a flat boundary between the developing embryo and the yolk (Kimmel *et al.* 1995).

2.5 Statistics

Fertilization success of each serial dilution treatment was assessed with a binomial

z-statistic with pairwise comparisons between all groups, with a Holm-Bonferroni correction for familywise error. Generally, a p-value $\geq$ 0.95 indicates significant differences between treatments. Eggs were counted as fertilized or unfertilized. Any eggs which dechorionated were removed from the experiment as the moment of dechorionation was impossible to determine in relationship to the fertilization procedures.

2.6 Refined Fertilization Experiment

Because each sperm collection produces a unique set of dilutions it is difficult to combine multiple data sets from multiple fertilization tests. Additionally, the 5 minute egg incubation time limited the number of clutches which could be collected, and clutches vary in quality (Figure 10) Combining clutches from only 2 or 3 females may confound results if eggs from each clutch are not evenly distributed, as fertilization rates can vary from female to female. Furthermore, the number of treatments also makes increasing samples sizes difficult. In order to detect fine statistical differences between both fertilization system orientation and sperm concentrations, the number of overall treatments needed to be reduced.

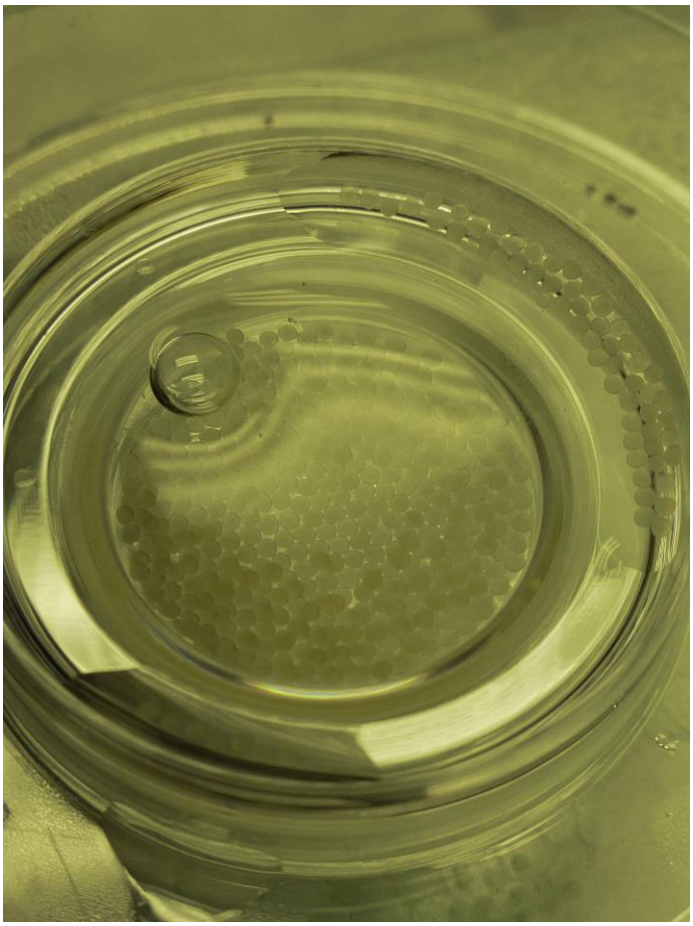


Figure 10*: Clutches pooled from 2 females. The darker eggs in the lower portion of the photograph are from one female, while the lighter eggs filling the rest of the IVF dish belong to the second female. Note the difference in opacity as well as the number of eggs. Darker eggs tend to be more prone to rapid breakdown and more sensitive to environmental perturbations.*

To refine the system 3 dilutions were proposed: the undiluted stock or dilution 0, the dilution closest to the lowest concentration of sperm found to still be indistinguishable from undiluted semen or roughly 1000 sperm/μL (Hagedorn & Carter 2011), and the dilution with the lowest concentration of sperm shown to be no different statistically from dilution 0 in the preliminary fertilization experiment. Because dilution 0 and the dilution containing 1000 sperm/μL were within 2 dilutions, only dilution 0 would be used and a

third dilution would be selected based on the findings of the preliminary fertilization experiment. A minimum of 40 eggs were used per fertilization protocol per dilution.

Results

2.1 Preliminary Fertilization Experiment

The serial dilution protocol yielded concentrations of sperm ranging from 3643 sperm/μL to 4 sperm/μL. Initial motility was estimated at 75%, yielding motile sperm/μL ranging from 2915 sperm/μL to 3 sperm/μL (Table 1).

Table 1*: A summary of the total sperm/μL and motile sperm/μL in each of the 11 serial dilutions, as well as the number of intact eggs per dilution per fertilization system. Sperm counts have been rounded up to the nearest whole number.*

Dilution Factor	Total sperm/μL	Motile sperm/μL	Eggs: Traditional	Eggs: Hanging Drop
0	3643	2915	15	10
1	1822	1457	9	12
2	911	728	8	14
3	456	365	2	14
4	228	183	18	15
5	114	92	13	9
6	57	46	21	11
7	29	23	14	14
8	15	12	23	15
9	8	6	13	8
10	4	3	8	13

No differences in fertilization rates were found between sperm concentrations in either the traditional system or the hanging drop protocol (Table 2). When comparing the two protocols within each dilution, the fertilization rate in the hanging drop protocol exceeded that of the traditional protocol only within dilution 3 (Table 3, Figure 11).

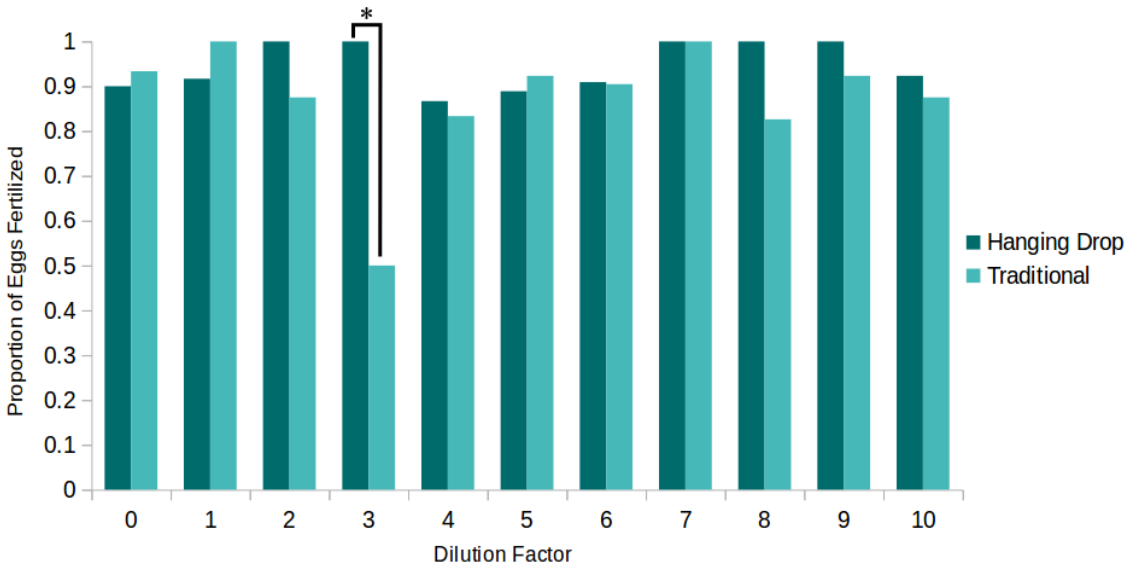


***Figure 11**: Proportion of eggs fertilized in 11 different sperm dilutions under 2 different fertilization conditions. Significant differences are indicated by an asterisk.*

***Table 2**: P-values for z-statistic comparisons between the proportion of eggs fertilized in dilution 0 and in a given dilution over 2 fertilization systems. Significant differences are marked with an asterisk.*

Dilution Factor	Traditional Fertilization System	Hanging Drop Fertilization System
1	0.8413	0.5517
2	0.5714	0.8461
3	0.9222	0.8461
4	0.7054	0.5987
5	0.5823	0.7790
6	0.5160	0.5279
7	0.9099	0.8849
8	0.7324	0.8944
9	0.5832	0.8121
10	0.5714	0.5753

***Table 3**: A comparison of the proportion of eggs fertilized between fertilization systems within a dilution. Significant differences between fertilization systems are marked with an asterisk.*

	Proportion of Eggs Fertilized		
Dilution Factor	Traditional Fertilization System	Hanging Drop Fertilization System	Fertilization System Comparison (p-value)
0	0.933	0.900	0.918
1	1	0.917	0.810
2	0.875	1	0.912
3	0.500	1	0.997*
4	0.833	0.867	0.603
5	0.923	0.888	0.606
6	0.905	0.909	0.512
7	1	1	0.500
8	0.826	1	0.955
9	0.923	1	0.788
10	0.875	0.923	0.641

2.2 Refined Fertilization Experiment

Because no statistical differences were found between dilution 0 and dilution 10 in either fertilization system, a 1:1 ratio of egg to sperm was used as the smallest allowable dilution during the refined fertilization protocol. Dilution 0 closely approximated the sperm concentrations found to be the most successful in previous experiments (Hagedorn & Carter 2011). Because dilution 0 filled both roles, the proposed third dilution was selected was chosen to match dilution 5 from the previous experiment. This selection was made because the previous dilution 5 represented an order of magnitude difference between the sperm concentrations in previous literature, and because of its "halfway" position on the exponential curve. Given these criteria, the dilutions selected for the Refined Fertilization Experiment were 0, with 1575 motile sperm/μL, dilution 4 with 99

motile sperm/µL, and dilution 9 with 4 motile sperm/µL (Table 4) Initial motility was estimated at 75%.

***Table 4**: The three chosen sperm dilutions for the refined fertilization experiment, the respective total sperm/µL and motile sperm/µL in each dilution, and the number of intact eggs used per dilution per fertilization system. Sperm counts have been rounded up to the nearest whole number.*

Dilution Factor	Total sperm/µL	Motile sperm/µL	Eggs: Traditional	Eggs: Hanging Drop
0	2100	1575	34	34
4	132	99	36	34
9	5	4	34	34

No differences in fertilization rates were found between sperm concentrations in either the traditional or hanging drop fertilization protocol (Table 5). When comparing the two fertilization protocols within each sperm dilution, the hanging drop protocol had a higher fertilization rate under all sperm dilutions and a statistically significant difference within the dilution 4 (Table 6, Figure 12).

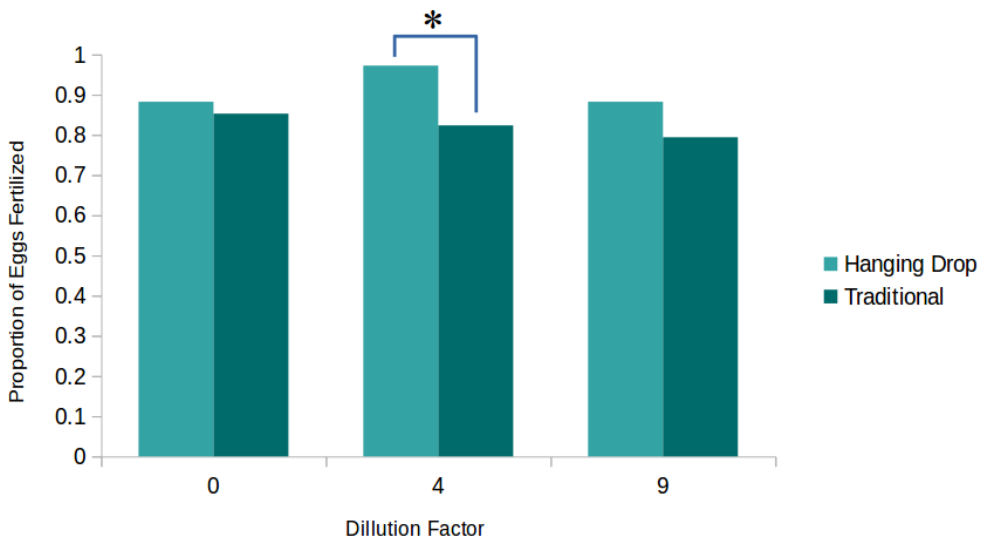


***Figure 12**: The proportion of eggs fertilized over 3 different sperm dilutions under 2 different fertilization conditions. Significant differences are indicated by an asterisk.*

***Table 5**: P-values for z-statistic comparisons between the proportion of eggs fertilized in dilution 0 and in a given dilution over 2 fertilization systems. Significant differences are marked with an asterisk.*

Dilution Factor	Traditional Fertilization System	Hanging Drop Fertilization System
4	0.6255	0.9265
9	0.7375	0.5000

***Table 6**: A comparison of the proportion of eggs fertilized between fertilization systems within a dilution. Significant differences between fertilization systems are marked with an asterisk*

	Proportion of Eggs Fertilized		
Dilution Factor	Traditional Fertilization System	Hanging Drop Fertilization System	Fertilization Systems Comparison (p-value)
0	0.882	0.853	0.637
4	0.972	0.823	0.985*
9	0.882	0.794	0.836

Discussion

In the preliminary fertilization experiment, sample sizes were low as a result of not wanting to mix clutches from only two or three females and risk unequal distribution of eggs of differing quality. As a result, dechorionation, or failure of the egg shell at some point before, during, or after fertilization, had a profound effect on the ability to detect difference between treatments. Many treatments were reduced to fewer than 10 eggs as a consequence of chorion failure. The sole statistical difference within the preliminary fertilization experiment resulted from dechorionation of all but 2 eggs in the traditional fertilization system, dilution 3. The resulting 50% viability is therefore an artifact of experimental manipulations and not an accurate representation of fertilization rates with 365 sperm/μL, nor of the traditional fertilization system. Because of this, the significant

difference between the two fertilization systems in dilution 3 should be treated as a consequence of egg manipulation and chorion integrity, and not related to the original hypothesis.

The refined fertilization experiment was originally developed to overcome these statistical shortcomings in the initial experimental design and help detect finer differences between treatments. However, no differences were found between sperm concentrations in either fertilization system. In all cases, the hanging drop protocol resulted in more fertilized eggs, though this difference was only statistically significant in dilution 4. This indicates that hanging drop is the superior protocol for maximizing fertilization. However, high fertilization rates can be achieved without the hanging drop protocol, as the traditional protocol yielded fertilization rates equivalent to average *in vitro* fertilization rates in zebrafish.

In this experiment, I have demonstrated equivalent fertilization rates between *in vitro* use of several thousand sperm per egg and one sperm per egg. This experiment was designed as a way of exploring the minimum number of sperm necessary to achieve fertilization under low water volume conditions, but the one to one sperm to egg ratio ultimately indicates something more profound. This constitutes the first demonstrations of long distance chemotaxis, both in zebrafish sperm and in other teleost species. Short distance chemotaxis near and within the micropylar canal has been demonstrated in a Pacific herring (Morissawa 2008), and generally involves distances of a few micrometers. Modelling work in sea urchins has led to the theory that fish sperm swim in close contact with the chorion to maximize the likelihood of accessing the micropyle (Cosson *et al.* 2003) This theory is further supported by the channeled surfaces of numerous fish species

(Amanze & Iyengar 1990, Chen *et al.* 2007). However, the results from this experiment indicate that an additional mechanism may exist within zebrafish gametes, allowing for individual sperm to locate micropyles on an egg in spite of both the enormous difference in size and tremendous distances that sperm must navigate quickly to reach the egg before motility expires.

The ability to reliably fertilize an single egg with a single sperm using the novel hanging drop protocol clearly demonstrates the excessive nature of standard *in vitro* fertilization protocols. The protocol outlined here may allow for cryopreservation storage of smaller volumes of semen from more individuals, as well as provide a metric by which to judge semen quality. Furthermore this protocol provides opportunities for exploring chemotaxis in zebrafish, as well as for using sperm as a vector and introducing DNA markers at the 1 cell stage of development.

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Conclusion

The overarching purpose of the experiments laid out in this document is to create a foundation for improving gamete manipulation and gamete efficiency with the ultimate goal of fish egg cryopreservation and improved cryopreservation of sperm. The increased holding time in zebrafish eggs provides a new protocol for manipulating these gametes, and more specifically for introducing and removing cryoprotectants without causing undue harm. This finding alone may be the critical step necessary for zebrafish eggs to move to the list of successfully frozen gametes. Improved holding time in sperm has broad implications, both for studying sperm motility and for short term storage of sperm, as well as for extended incubation in lower, safer concentrations of cryoprotectants. The sperm minimization experiment was designed as a protocol for fertilizing large numbers of eggs with small numbers of sperm, both to reduce cryopreserved sample volumes and to provide an assay by which to judge cryopreservation protocols. The ultimate finding of a one to one sperm to egg ratio both fulfills this purpose and subsequently raises a thousand questions with regards to the relationship between eggs and sperm. Sperm minimization may become a powerful tool for identifying the physical and chemical mechanisms by which a sperm finds and fertilizes an egg. While the methods behind cryopreservation itself are not directly addressed herein, the foundation for better gamete manipulation protocols will hopefully yield healthier cryopreserved gametes and ultimately healthier offspring, both in zebrafish and in other fish species.

Vita

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Place of Birth: Puyallup, Washington

Education—**Bachelor of Science in Biology Hillsdale College**—May 2011
—*Magna cum laude*, Minors in Biochemistry and Music
—Thesis "The Effects of Exogenous Taurine on Embryonic Chick Brain Viability"
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—**Courses Taken**: Embryology, Molecular Biology, Microbiology, Virology, Biostatistics, Biochemistry and Advanced Biochemistry, General and Organic Chemistry, Genetic, Population Genetics, Bioinformatics

—**Grants, Honors, and Awards**
Eastern Washington University
—Biology Graduate Fellowship ('12-present, EWU)
Hillsdale College
—2010 Outstanding Junior Biology Major Award
—2010 LAUREATES (Laboratory for Advanced Undergraduate Research Education Adapted for Talented and Extraordinary Students) Grant
—2010-2011 John A. Catenhusen Scholarship in Biology
—Honoraries: BBB Biology
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Posters and Presentations
Chastain MC. (2013) Fish Egg Cryopreservation. **Poster** at EWU Creative Works Symposium, Cheney, WA.

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Employment History
—**Teacher's Assistant** Eastern Washington University (2012-present)
Animal Capstone BIOL 490, Microbial Capstone BIOL 490, and Biological

Investigations BIOL 270—Supervised group projects in courses largely focused on novel experimental design through proper scientific methodology, writing, literature research, and laboratory technique.

—**Research Assistant** Population Genetics Laboratory, Hillsdale College, Hillsdale, MI. (2010-2011)
PCR, DNA extraction, and electrophoresis

—**Biology Departmental Tutor**, Hillsdale College, Hillsdale, MI (2010-2011 school year).
Tutored students of all levels in biology

—**Veterinary Assistant**, Puyallup Valley Veterinary Clinic, Puyallup, WA (Summers 2006-2011).
Worked in a small animal practice restraining pets, preparing vaccinations and prescriptions, drawing blood, and meeting with clients.

Other Experience

—Supervised projects involving: zebrafish, rats, mice, stickleback, rainbow trout, bull frog, crayfish, hornworm, *D. melanogaster* and *C. elegans*, as well as bacterial and eukaryotic cell culture

—Hillsdale College (2011): Lab Teaching Assistant for Biostatistics, Biology Tutor through Beta Beta Beta, Elementary Chinese Tutor

Skills

—Lab Skills—PCR, agarose electrophoresis and PAGE, casp-3 analysis, bacterial transformation, enzyme purification, kinetics assays, derivative synthesis, cryobiological techniques, ELISA, bioinformatics

—Instrumentation—Spectrophotometer, Gene Analyzer, Thermocycler, Microtiter plate reader, Electrophoresis equipment, NMR, Labview thermal software

—Proficient in Windows, Mac, and Linux