Characterizing the Role of Extracellular Zinc in the Slow Block to Polyspermy for the African Clawed Frog

by

Beatrice L. Milnes

Submitted to the Graduate Faculty of the

University Honors College in partial fulfillment

of the requirements for the degree of

Bachelor of Philosophy

University of Pittsburgh

2022

UNIVERSITY OF PITTSBURGH

UNIVERSITY HONORS COLLEGE

This thesis was presented

by

Beatrice L. Milnes

It was defended on

April 11, 2022

and approved by

Jeffrey D. Hildebrand Ph.D., Associate Professor, Department of Biological Sciences

Kirill Kiselyov Ph.D., Associate Professor, Department of Biological Sciences

Michael C. Puljung Ph.D., Assistant Professor, Department of Chemistry and Neuroscience Program, Trinity College

Thesis Advisor: Anne E. Carlson Ph.D., Associate Professor, Department of Biological Sciences

Characterizing the Role of Extracellular Zinc in the Slow Block to Polyspermy for the African Clawed Frog

Beatrice L. Milnes, BPhil

University of Pittsburgh, 2022

Fertilization of an egg by multiple sperm, a condition referred to as polyspermy, is lethal for developing embryos from nearly all sexually reproducing animals. In response, various mechanisms known as polyspermy blocks have evolved to prevent supernumerary fertilizations and the developmental problems resulting from fertilization of an egg by more than one sperm. One of the egg-based polyspermy blocks involves the release of cortical granules docked at the egg's plasma membrane, into the extracellular milieu, a process known as the slow block to polyspermy. During the slow block, eggs from diverse animals release zinc ions, and this extracellular zinc is hypothesized to contribute to the mechanism that keeps sperm out of fertilized eggs. This hypothesis is supported by the finding that insemination of eggs from diverse species in concentrations of zinc similar to the amount released during the slow block, prevents fertilization and the initiation of embryonic development. Using fertilization in the African clawed frog *Xenopus laevis*, I have demonstrated that treatment of both eggs and sperm with zinc prior to insemination was sufficient to stop fertilization. Using the cell-impermeant zinc chelator ZX1, I have shown that the zinc block of fertilization is reversible in both gametes, consistent with a role for extracellular zinc in preventing fertilization. Further, I demonstrated that the zinc induced infertility phenotype of gametes can extend to other transition metals. This data indicates that extracellular zinc may target both sperm and eggs, suggesting that the classic belief that the slow block to polyspermy only changes the egg may not provide a complete picture of how multiple sperm are kept out of the nascent zygote. Understanding how zinc blocks polyspermy could have implications in optimizing clinical conditions used for *in vitro* fertilizations in humans.

Table of Contents

List of Figures

Preface

Many thanks to Rachel Bainbridge, Kayla Komondor, and Dr. Anne Carlson for their wonderful mentorship and support during my pursuit of this thesis. I would also like to thank Dominique Summerville for technical assistance and Dr. Joel Rosenbaum for help troubleshooting, as well as Dr. Wase Tembo and other previous members of the lab. Many thanks to the undergraduates in the Carlson lab, Paushaly Sau, Eirlys Barbara, Sarah Khanzada, Julia Shack, and Risha Musuku for their support and assistance with experiments.

Further, I am grateful for the members of my committee, Dr. Jeffrey Hildebrand, Dr. Kirill Kiselyov, and Dr. Michael Puljung for contributing their insight and expertise during my undergraduate experience.

Funding for my project was contributed in part by the University Honors College Chancellor's Undergraduate Research Fellowship and the Department of Biological Sciences Norman H. Horowitz Fellowship.

1.0 Introduction

Fertilization is a unique cellular process that fuses two haploid gametes to create a diploid zygote. This is the first step towards successful embryonic development and ultimately the formation of a new organism. The molecular mechanisms regulating this process have long been a subject of interest within the field of developmental biology but have not been fully elucidated (Evans, 2020).

1.1 Monospermic Fertilization

There is a vital ratio required for successful embryonic development, the fertilization of a single egg by a single mature sperm. This condition, known as monospermy, is a tightly regulated process between the egg and sperm gametes. The fertilization of an egg by multiple sperm, or polyspermy, is lethal to developing embryos from nearly all sexually reproducing animals, and results in chromosomal abnormalities, developmental defects, and embryonic mortality (Hassold and Hunt, 2001). Only a handful of animals can survive polyspermic fertilization including the axolotl and the fruit fly (Iwao et al., 2020). Consequently, eggs from diverse animals have developed an arsenal of protective processes to stop additional sperm from entering an already fertilized egg (Wong and Wessel, 2006).

1.2 Polyspermy Blocks

In order to prevent supernumerary fertilizations, most animals utilize diverse molecular systems known as polyspermy blocks (Kim et al., 2011). The two best studied mechanisms are the fast and slow polyspermy blocks, named for their timing relative to fertilization. Eggs from many externally fertilizing species use the fast block to polyspermy (Wozniak and Carlson, 2020). In external fertilizers, fast block activates within seconds following fertilization. Prior to fertilization, the membrane of eggs which use the fast block generally rests at a specific membrane voltage known as the resting potential (Wozniak and Carlson, 2020). During the fast block, fertilization signals a depolarization of the egg's membrane. As a result, sperm may bind to but do not penetrate a depolarized egg. The fast block's immediate depolarization protects the nascent zygote. By contrast, the slow block takes several minutes to enact (Wozniak and Carlson, 2020). Notably, only eggs from external fertilizers use the fast block; mammalian eggs do not depolarize at fertilization, and depolarizing mammalian eggs does not stop sperm entry (Jaffe LA, 1985).

The slow block to polyspermy involves the release of the contents of cortical granules into the extracellular milieu (Wong and Wessel, 2006). The cortical granules are like synaptic vesicles and are docked at the egg membrane and their contents somehow transform the external matrix that surrounds the egg from one that allows sperm entry, to one that prevents sperm penetration. This external matrix is comprised of a network of glycoproteins, sugar-containing proteins which are highly conserved amongst diverse animals including human and abalone (Swanson and Vacquier, 2002). Despite a high degree of homology amongst the proteins that comprise this matrix, the structures are referred to by various names including the vitelline envelope in the African clawed frog *X. laevis*, chorian in teleost fish, and zona pellucida in mammals (Wong and Wessel, 2006). The slow block transforms this matrix from a structure penetrable by sperm into a

hardened, impenetrable “shell” (Fig. 1). The cortical granule contents include enzymes, such as the protease ovastacin, known to modify this glycoprotein matrix (Bianchi et al., 2014). The metal ion zinc is one of the proposed contents of the cortical granules (Wozniak et al., 2020). While the contents of these vesicles are not completely known, it is clear that their ultimate effect is to modify the external matrix of both mammalian and non-mammalian eggs in order to develop the physical barrier to sperm entry.

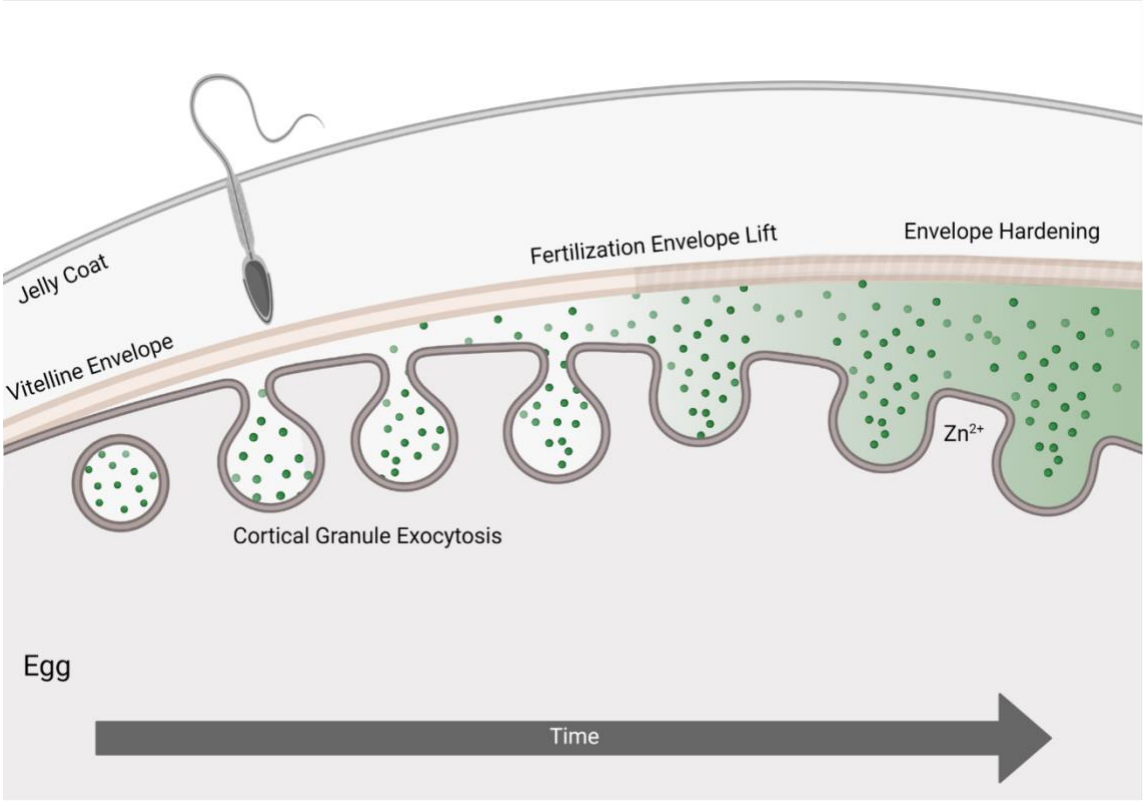

Figure 1. The slow block to polyspermy prevents additional sperm entry into already fertilized eggs from diverse animals. Illustration depicting the processes of fertilization and cortical granule release in *X. laevis* eggs. Following cortical granule exocytosis, a release of zinc and other proteins and ions cause the fertilization envelope to lift and ultimately harden. Figure created with Bio Render.

1.3 Zinc Ions in Fertilization

In comparison to other metal ions such as copper and iron, zinc ions are far more abundant in the *X. laevis* eggs (Nomiza et al., 1993). Natural waters generally contain between 5 and 10 ppb zinc where the predominant species is the +2 valency state (Stumm and Morgan, 1996). In the last decade, a zinc efflux was observed from mammalian eggs during cortical granule exocytosis (Kim et al., 2011). We now understand that eggs from diverse animals, including internal fertilizers and external fertilizers alike, release zinc during the slow block (Wozniak et al., 2020). This extracellular zinc is thought to protect the nascent zygote from multiple fertilizations as part of the slow block to polyspermy (Levendosky et al., 2016; Tokuhiro and Dean, 2018).

Zinc release during the slow block has been documented from eggs of the *X. laevis* fluorescent microscopy (Fig. 2) (Seeler et al., 2021; Wozniak et al., 2020). This can be observed using FlouZin-3 a florescent zinc indicator (Wozniak et al., 2020). Progression of zinc release around the egg circumference, in the same pattern as cortical granule release which moves around the egg starting from the point of sperm entry (Fig. 2) (Runft et al., 2002; Wozniak et al., 2020).

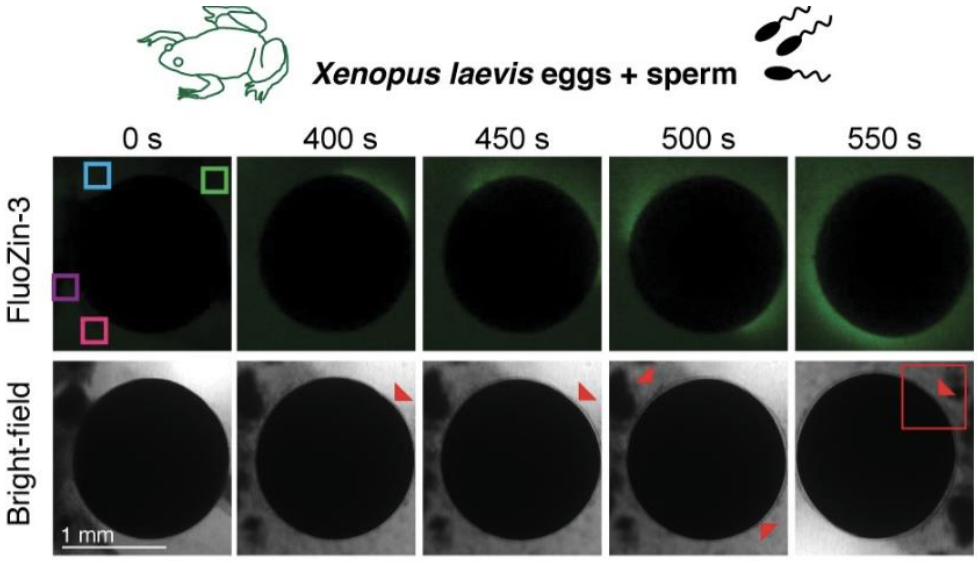

Figure 2. Representative fluorescence and bright-field images of *X. laevis* eggs releasing zinc at fertilization, indicated using the fluorescent zinc marker FlouZin-3. Time relative to sperm addition noted above corresponding images. Zinc release conincided with the lifting of the envelope (red arrows). Time is relative to sperm addition. Scale bar represents 1 mm. (Wozniak et al., 2020)

The cortical granule exocytosis can be induced by either fertilization or artificial activation, via the calcium ionophore ionomycin (Seeler et al., 2021; Wozniak et al., 2020). The multiple studies which indicate zinc release from eggs which have been fertilized or artificially activated, support the hypothesis that zinc is a content of the cortical granules since their contents are known to be released upon activation.

1.4 Zinc Release after Fertilization is Highly Conserved

Zinc release during the slow block to polyspermy has been observed from the eggs of several mammals including humans (Que et al., 2017), mice, and cows (Kim et al., 2011; Que et al., 2017; Que et al., 2019). Zinc release has also been observed from eggs from *X. laevis*, the

neotenic salamander axolotl, the teleost fish *Danio rerio* (Wozniak et al., 2020), and the fruit fly *Drosophila melanogaster* (Hu et al., 2020). Insemination of eggs from *X. laevis*, the purple sea urchin *Strongylocentrotus purpuratus*, and the hydroid *Hydractinia symbiolongicarpus* in the presence of zinc reduced the incidence of fertilization and the initiation of embryonic development (Wozniak et al., 2020). This revealed that not only is zinc release a highly conserved process through evolution, but zinc is able to inhibit fertilization in diverse animals (Fig. 3).

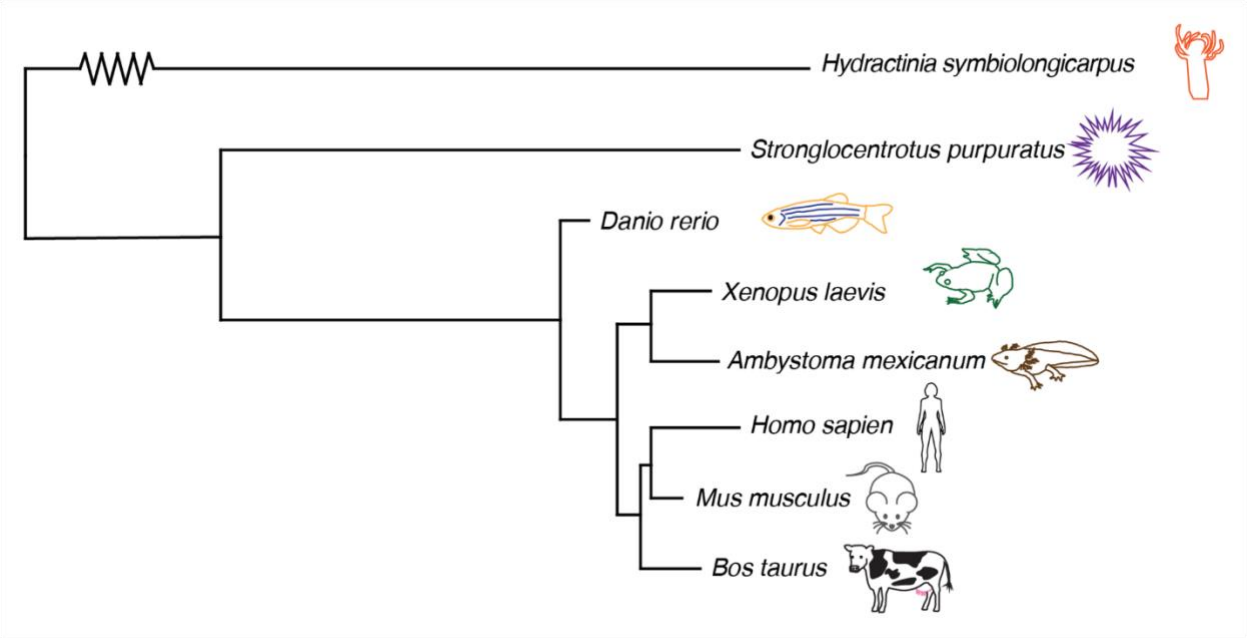

Figure 3. An evolutionary tree showing the animals whose eggs have been shown to release zinc following activation, or have fertilization inhibited in the presence of notable extracellular zinc (Bainbridge, unpubl. data).

1.5 Extracellular Zinc Decreases Incidences of Fertilization

The amount of zinc released during activation of *X. laevis* eggs, has been quantified using fluorometry with the zinc indicator FluoZin-3 (Fig. 4). Fertilization induced an average release

of 5.5 ± 2.7 x 10^{13} zinc ions per egg while 1.9 ± 1.0 x 10^{13} zinc ions are released upon fertilization or activation with ionophore ionomycin. The extracellular jelly layer surrounding *X. laevis* eggs is known to bind and buffer other metals like calcium (Wozniak et al., 2017) and similarly trap zinc near the egg to create a region of high local concentration immediately surrounding the egg (Wozniak et al., 2020). Together with the volume of the jelly surrounding *X. laevis* eggs, and number of zinc ions released at fertilization, we estimate the local concentration of zinc surrounding the egg after fertilization to be approximately 377 µM .

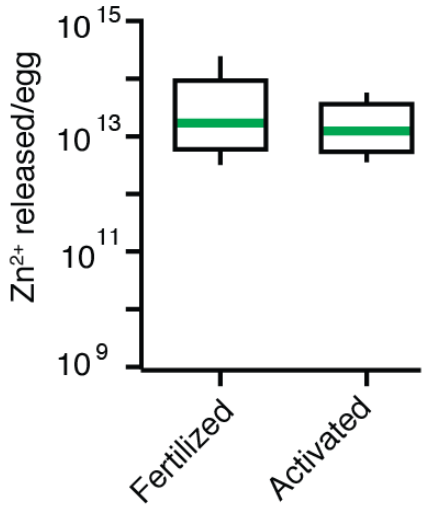

Figure 4. Box plot distribution of zinc ions released per X. laevis egg signaled by fertilization or egg activation via ionomycin treatment as detected by FluoZin-3 fluorometry (21-62 eggs, 5-9 trials) (Wozniak et al., 2020).

If extracellular zinc stops supernumerary fertilizations when released during the slow block, then its presence in the environment should prevent fertilization altogether. Indeed, previous work from the Carlson lab demonstrated that insemination of eggs from the African clawed frog *X. laevis*, the purple sea urchin *Strongylocentrotus purpuratus*, and the hydroid *Hydractinia symbiolongicarpus* in micromolar concentrations of extracellular zinc, reduced the incidences of fertilization and the initiation of embryonic development (Wozniak et al., 2020). Extracellular zinc and various other metal ions have been shown to influence fertility in similar yet distinct manners

to prevent fertilization (Seeler et al., 2021). It was previously believed that the cortical granule contents simply created a physical barrier to sperm entry around the egg (Bianchi et al., 2014). However, the molecular details of how zinc and other metal ions target gametes and contributes to the slow polyspermy block have yet to be uncovered. Here, I demonstrate that not only does extracellular zinc interfere with egg fertility thought the slow block, but also disrupts the ability of sperm to fertilize in *X. laevis*.

1.6 The African Clawed Frog, *Xenopus Laevis*

My research characterizing the inhibitory effect of zinc on fertilization uses *X. laevis* as a model system. I chose this systems because *X. laevis* eggs that have undergone successful fertilization, develop cleavage furrows within two hours of sperm application thereby facilitating the study of the initial stages of development (Fig. 5) (Wozniak et al., 2018). By assaying for the incidence of cleavage furrow development, I can identify the proportion of eggs which are undergoing embryonic development, an indicator of successful fertilization.

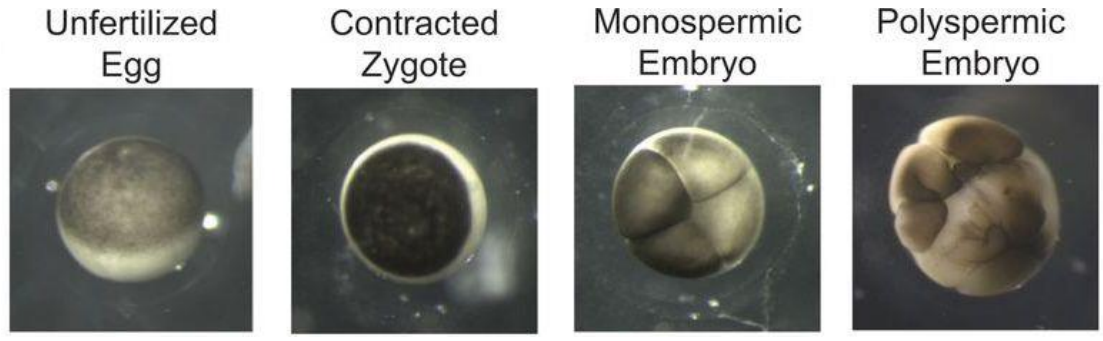

Figure 5. Images of X. laevis eggs before sperm addition (left), after fertilization with the animal pole contracted (center left), a normally developing monospermic embryo (center right), and a polyspemic embryo exhibiting disorganzed cleavage furrows (right). (Wozniak et al., 2018)

1.7 Objectives

The central hypothesis of my research is that extracellular zinc targets both *X. laevis* sperm and the egg to protect the nascent zygote from fertilization by additional sperm. Using *in vitro* fertilization, I explored the mechanism of how extracellular zinc stops fertilization by probing for effects on both egg and sperm. My primary aim was to determine if the inhibitory effect of environmental zinc on *X. laevis* fertilization is only targeting the egg or interacting with both gametes. My second aim was to determine whether zinc chelators reversed the effects of zinc on *X. laevis* eggs and sperm pre-treated with zinc. Finally, my third aim was to determine if the ability of zinc to target both sperm and eggs applied to other divalent transition metals known to inhibit fertilization. These experiments lay the foundation for further understanding the molecular mechanisms that enable the slow polyspermy block during fertilization in *X. laevis*.

1.8 Significance and Clinical Relevance

Understanding the molecular mechanisms by which polyspermy blocks stop fertilization of an egg by more than one sperm will uncover the mechanism imperative for the survival of embryos in many sexually reproducing animals, including humans. With more than 48.5 million couples struggling with infertility worldwide and a low average success rate of in vitro fertilization (IVF), it is important to continue to develop assisted reproductive technologies (CDC, 2019). It is estimated that hundreds of thousands of embryos develop polyspermy during IVF and are therefore inviable for implantation (CDC, 2019). With a more detailed understanding of the slow polyspermy block, better procedures for IVF can be developed, and fewer eggs will be wasted as

rates of polyspermy decrease. These studies could also lend predictions to how environmental contaminants such as heavy metals interfere with fertilization in vulnerable animal populations like amphibians and may be vital to conservation efforts across diverse phyla.

2.0 Zinc in the Slow Block to Polyspermy

To characterize how extracellular zinc stops fertilization, I inseminated *X. laevis* eggs in various concentrations of extracellular zinc and assayed for the appearance of cleavage furrows. Similar to previous reports, I found that insemination in increasing concentration of zinc prevented fertilization (Wozniak et al., 2020). No embryonic development was evident in eggs inseminated in extracellular zinc concentration over 300 μM Zn^{2+}, which failed to develop based upon the absence of cleavage furrows with half the eggs dividing at 25 μM (IC_{50}) (Fig. 6). This is 15x less than the estimated amount of zinc released after fertilization. At 377 μM, the estimated concentration of zinc released from eggs, no development is observed. Building on this knowledge, this thesis aims to explore the specific effects of extracellular zinc on each gamete.

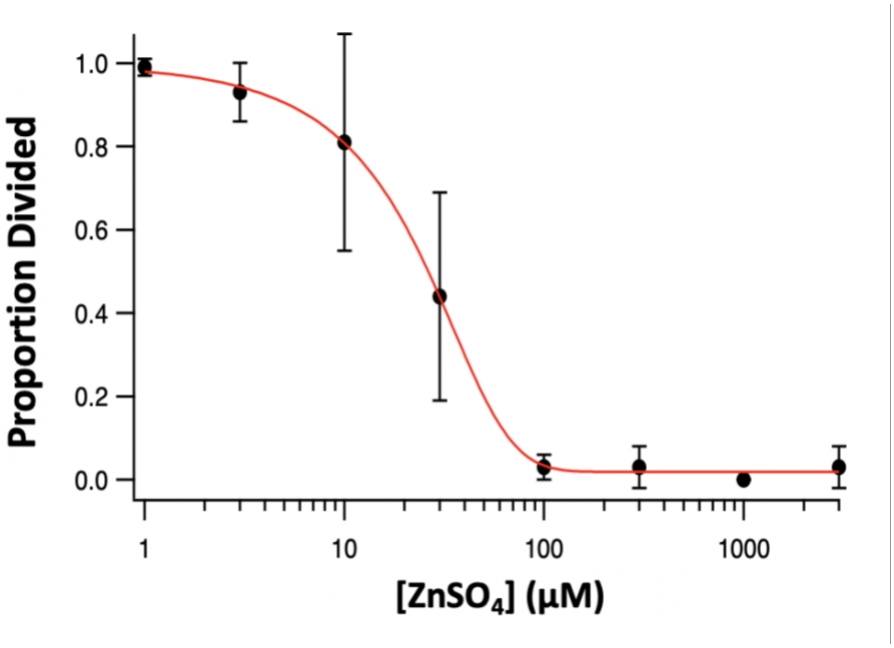

Figure 6. Extracellular zinc inhibits fertilization of *X. laevis* in a dose dependent manner. Proportion of inseminated eggs that developed cleavage furrows versus applied concentrations of ZnSO4. IC50 = 25.16 ± 10.6 μM (103-146 eggs, 5 trials).

Because the slow block to polyspermy is known to modify the egg, I speculated that the zinc may directly target the egg to stop sperm from entering an already fertilized egg. Pre-treatment of *X. laevi*s eggs with biologically relevant concentrations of zinc was sufficient to block early development (Wozniak et al., 2020), even when eggs were inseminated under normal conditions with no added zinc (Fig. 8). These data confirm the egg as a target of zinc's inhibitory function.

2.1 Zinc-induced Infertility of Eggs is Reversible

Theoretically, zinc could stop fertilization by acting on the outside of the gametes or entering the gametes to exert intracellular effects. To discriminate between these possibilities, I used zinc chelators that can or cannot pass the plasma membrane. Zinc chelators function by binding zinc with high affinity and thereby preventing zinc from interacting with other targets. The permeant zinc chelator, N,N,N',N'-tetrakis(2-pyridinmylmethyl)-1,2-ethanediamine (TPEN), binds both extracellular and intracellular zinc. The impermeable zinc chelator, 2-([Bis(2-pyridinylmethyl)amino]ethylamino) benzenesulfonic acid hydrate sodium salt (ZX1) does not pass through the membrane and should solely bind to extracellular zinc (Radford and Lippard, 2013). Thus, ZX1 treatment should chelate extracellular zinc only, but not alter zinc accumulated within the gametes (Fig. 7).

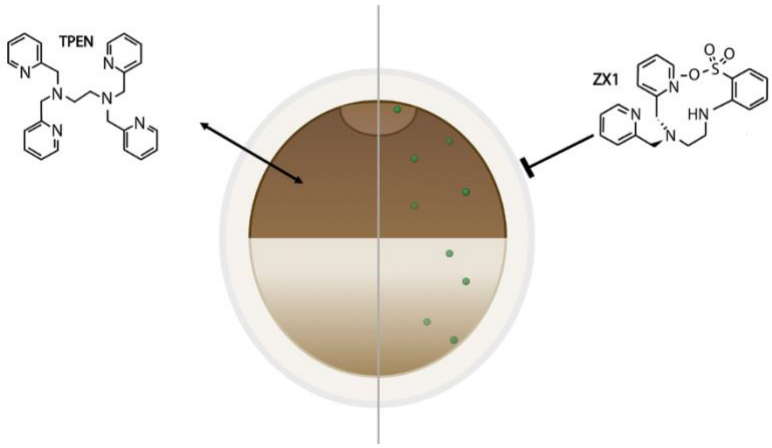

Figure 7 . Schematic of TPEN (left) and ZX1 (right) targeting of zinc ions in relationship to an *X. laevis* egg. Figure created with Bio Render.

To accomplish this, I treated eggs with 300 μM zinc for 10 minutes, washed the control solutions, and then inseminated in the presence of the membrane permeant zinc chelator TPEN or the cell impermeant chelator ZX1. *X. laevis* eggs were pre-treated with zinc in a zinc-free solution as a positive control, 300 μM and 1 mM $ZnSO_4$ as negative controls, and each of the chelators to ensure that there was no off-target effect of either compound on fertility (Fig. 8). Eggs pre-treated with 1 mM $ZnSO_4$ for 10 minutes did not develop cleavage furrows, even when inseminated in a solution with no added zinc (Fig. 8). However, if those zinc pre-treated eggs were inseminated in a solution containing 300 μM ZX1 or TPEN, cleavage furrows appeared in a proportion of eggs similar to those who had no contact with zinc (Fig. 8). The full recovery of fertility of zinc pre-treated eggs in the presence of ZX1 suggests that zinc is either acting on the extracellular structures surrounding the eggs, or that any zinc that enters the egg readily leaves the gamete upon chelation. This regime resulted in a 90% restoration of fertilization, similar to published findings (Wozniak et al., 2020).

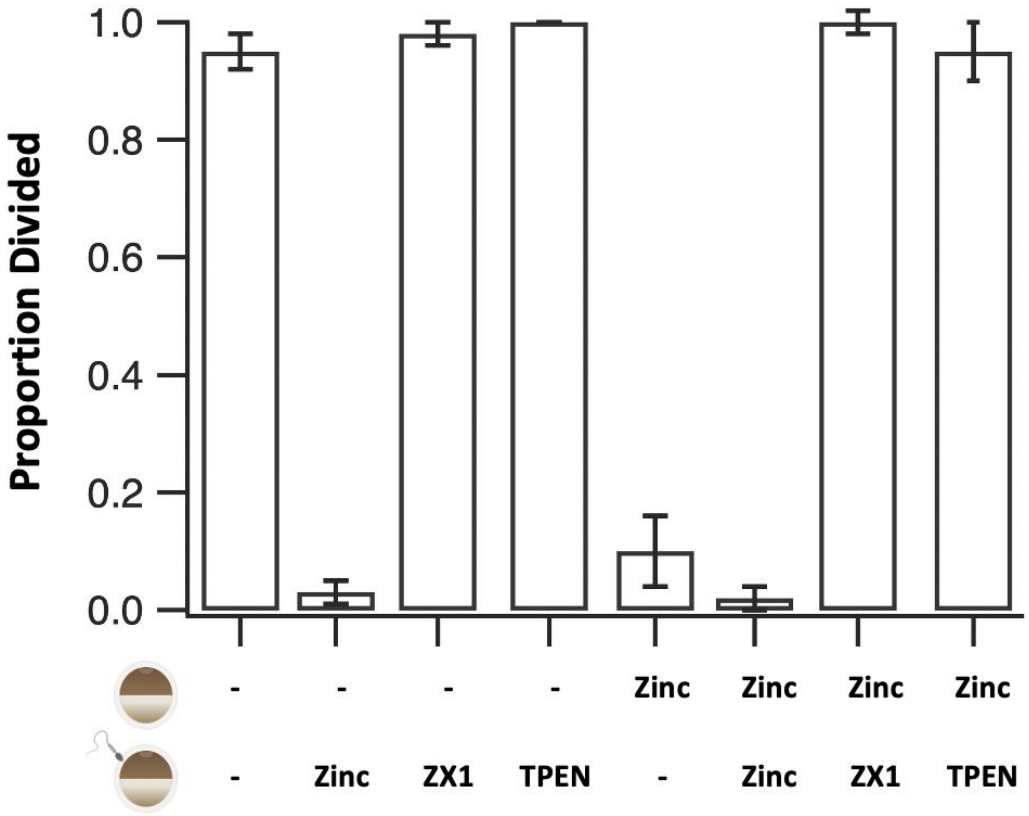

Figure 8. Zinc disruption of *X. laevis* fertilization with $ZnSO_4$ was reversed by chelation. Fertilizing or pretreating eggs in 1 mM $ZnSO_4$ reduced the proportion of eggs that developed cleavage furrows. Eggs pre-treated in a control solution and then inseminated in the presence of ZX1 or TPEN showed high proportions of development. Insemination of zinc pre-treated eggs in control solution shows a slight proportion (~15%) of eggs as unable to develop. Insemination of zinc pre-treated eggs in 300 µM $ZnSO_4$ resulted in a lack of cleavage furrow formation. Insemination of zinc pre-treated eggs 300 µM zinc-chelators ZX1 and TPEN all show notably high levels of divided eggs. (N = 34-73 eggs in 4 independent trials). Error bars represent SEM.

2.2 Zinc-induced Infertility of Sperm is Reversible

Most studies on the role of extracellular zinc on fertilization have focused on how it regulates the egg (Wozniak et al., 2020). However, it is possible that the zinc also targets sperm to

stop their entry into an already fertilized egg. To determine whether zinc alters the ability of sperm to fertilize, I pre-treated *X. laevis* sperm with various concentrations of $ZnSO_4$ for 10 min, and inseminated eggs in the absence of added zinc. Increasing concentrations of zinc stopped fertilization in a concentration responsive manner with a half-maximal concentration (IC_{50}) of 729 ± 51.9 μM (Fig. 9). No eggs developed cleavage furrows following insemination with sperm pretreated with 1 mM $ZnSO_4$. Compared to the concentration dependence of fertilizations performed with both gametes in the presence of zinc, the effect of zinc on sperm is much less potent (729 μM vs 25 μM).

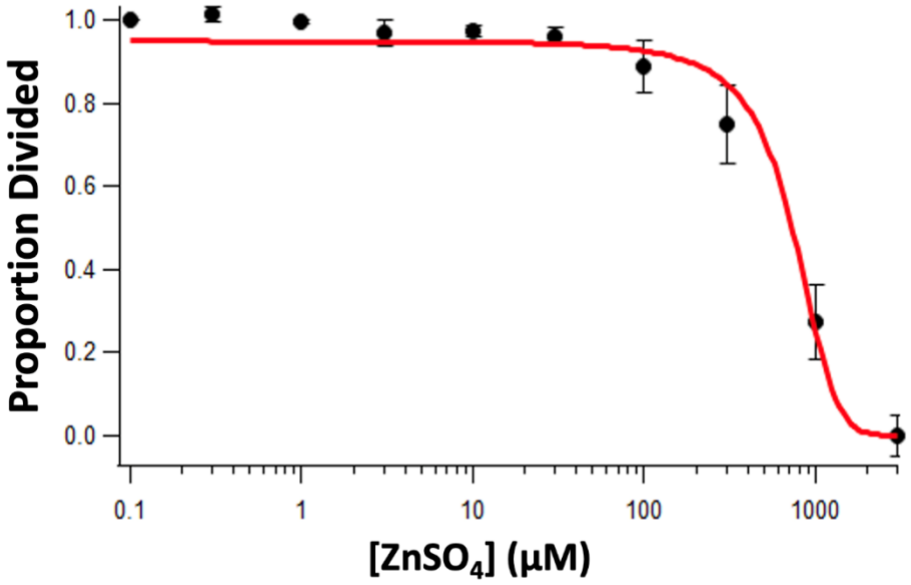

Figure 9. Pre-treating X. laevis sperm with various concentraions of zinc, stopped fertilization. A plot of the proportion of inseminated eggs that developed cleavage furrows versus the concentrations of $ZnSO_4$ used to pretreat sperm. (IC_{50} = 729.14 ± 51.9 μM, 92-354 eggs, 3-15 trials)

Having observed that zinc pretreatment is sufficient to inhibit sperm fertility, I next sought to determine whether this interaction was reversible by chelation. To do so, I again used both the cell permeant zinc chelator TPEN and the cell impermeant zinc chelator ZX1 to determine whether

sperm pre-treated with zinc could fertilize in the presence of these chelators. Sperm were pretreated with 1 mM zinc, a concentration that I have found to completely stop the ability of sperm to fertilization (Fig. 10), before insemination of eggs in the presence of 300 μM TPEN or ZX1. Indeed, I found that insemination in the presence of TPEN was sufficient to reverse the zinc inhibition of fertilization by *X. laevis* sperm (Fig. 10).

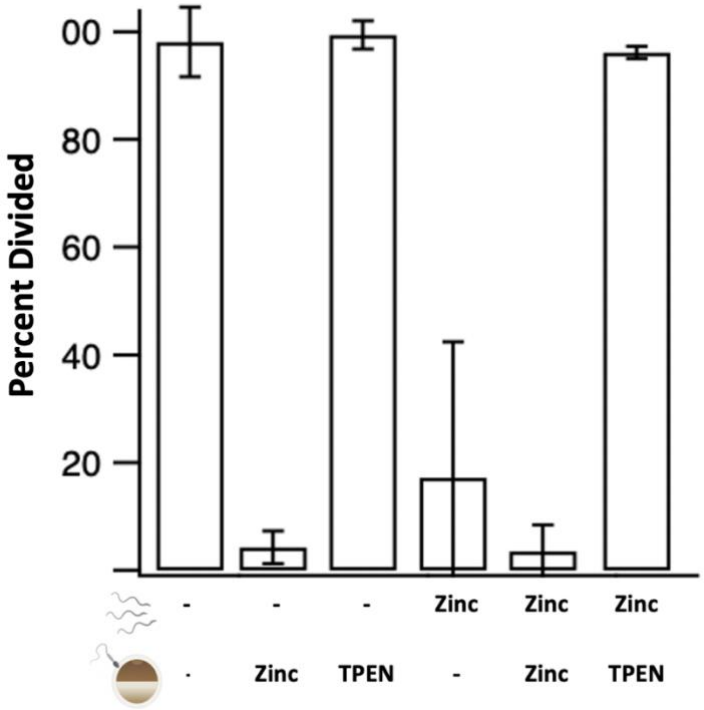

Figure 10. The cell permeant zinc chelator TPEN was sufficent to restore fertility of zinc pre-treated sperm. Pre-treatment of sperm with zinc prevented successful divisions of *X. laevis* eggs, however insemination in the presence of 300 μM TPEN restored fertilization and the appearance of cleavage furrows. (N = 44-82 eggs in 6 independent trials). Error bars represent SEM.

Eggs insemination in the presence of ZX1 developed cleavage furrows indicating that extracellular chelation was sufficient to rescue zinc inhibition of *X. laevis* sperm fertility (Fig. 11).

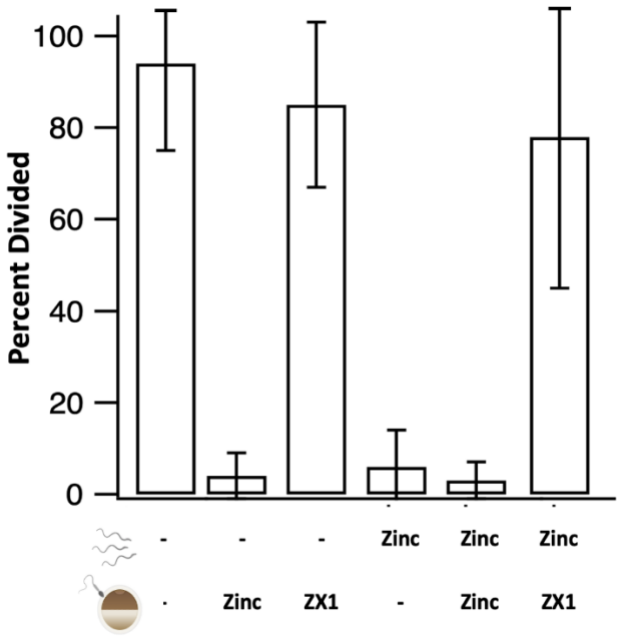

Figure 11. The cell impermeant zinc chelator ZX1 was sufficent to restore fertility of zinc pre-treated sperm. Pre-treatment of sperm with zinc prevented successful divisions of *X. laevis* eggs, however insemination in the presence of 300 μM ZX1 restored fertilization and the appearance of cleavage furrows. (N = 38-94 eggs in 5 independent trials). Error bars represent standard deviation.

Zinc pre-treatments were not washed off of the sperm, so some would have been transferred into the insemination solution. However, any labile zinc in the sperm pre-treatment was diluted away upon insemination in the chelator solution, reducing the overall zinc concentration so that no fertility effects would be expected. The zinc chelators should then be able to bind to the remaining zinc bound to the egg or extracellular vestments. It is worth noting that ZX1 was used 300 μM, which is less than the 1 mM zinc treatment, which may result in the incomplete rescue of fertilization block (Fig. 11). However, treatment with a comparable concentration of TPEN was sufficient to reverse fertilization block following zinc pre-treatment (Fig. 11). This suggests that the chelation of extracellular zinc was insufficient to fully reverse the loss of fertility and suggests that the lower concentration of ZX1 is not the reason for incomplete rescue.

2.3 Other Transition Metals Inhibit Gamete's Ability to Fertilize

Finally, I explored the ability of other divalent transition metals to target sperm to stop fertilization. Each transition metal has a different potency and targets, so comparing their ability to disrupt fertilization may provide insight into the other molecular components of the slow block. Zinc's inhibitory effect is supported by decreased incidence of fertilization in the presence of increasing concentrations of extracellular zinc, and other divalent transition metals such as manganese or copper (Seeler et al., 2021; Wozniak et al., 2020). Like zinc, extracellular copper stopped *X. laevis* gametes from fertilizing in a concentration responsive manner (Fig. 12). I found that the half-maximal concentration was found to be 24 μM, comparable to that of zinc. This not consistent with expected potency based on the Irving-Williams series which depicts the strength of complexes between divalent transition metals and proteins (Williams, 1987). The metal-protein interactions become more potent correspondingly: Co < Ni < Cu > Zn. Thus, copper is the only metal ion which would be expected to have a more potent effect on fertilization as opposed to zinc.

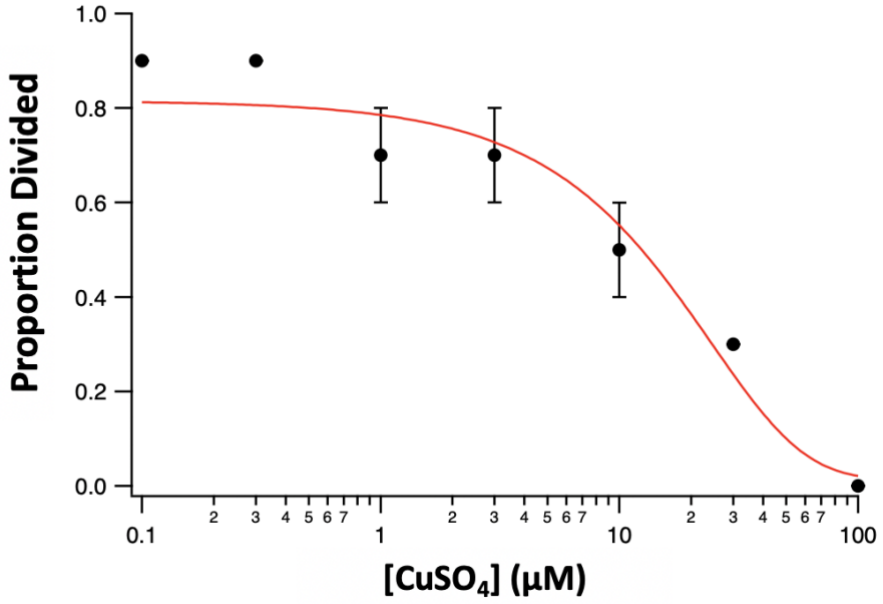

Figure 12. Insemination in the presence of various concentrations of extracellular copper, stopped the appearance of cleavage furrows. Plotting incidence of the appearance of cleavage furrows versus copper concentration. (IC50 = 24 + 3 µM, 136-156 egs, n = 4). Error bars represent standard deviation.

Copper also appears to have gamete specific effects on sperm fertility. Moreover, copper's effect on sperm was more potent, exerting similar effects as zinc at concentrations of 300 µM $CuSO_4$ in comparison to 1 mM $ZnSO_4$. This shift in the IC_{50} suggests a higher sensitivity of eggs to copper than zinc. Insemination of *X. laevis* eggs in the presence of copper showed a dramatic decrease in the incidence of cleavage furrow formation (Fig. 13). Similar to zinc, pre-treatment of *X. laevis* sperm with copper also showed a marked decrease in the rates of fertilization (Fig. 13).

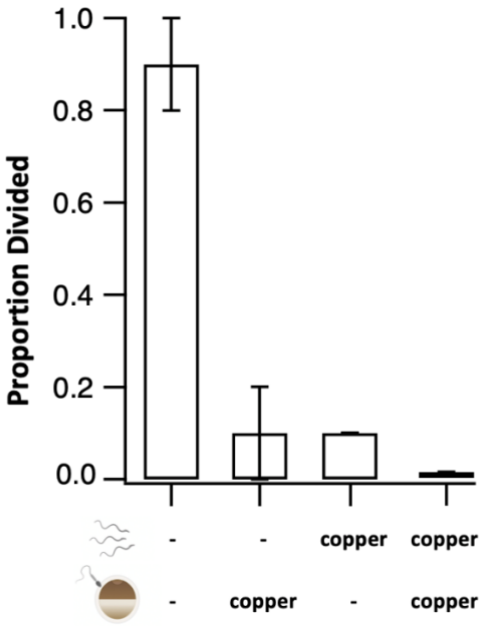

Figure 13. Insemination in the presence of or pre-treatment of *X. laevis* sperm with copper decreased rates of fertilization. Bar graph showing proportion of eggs successfully dividing in the combinations of incubation (top) and insemination (bottom) in 300 µM $CuSO_4$. Error bars are standard deviation.

2.4 Materials and Methods

2.4.1 Animals and Ethics Statement

All vertebrate animal studies conformed to the regulatory standards adopted by the Institutional Animal Care and Use Committee at the University of Pittsburgh, which approved this study under a protocol listed by the Carlson Lab (20026806). Animal care and use protocols adhered to the guidelines established by the National Institutes of Health (United States). *X. laevis* (frog) adults were obtained commercially (Xenopus1, Dexter, MI) and were housed at 18˚C with a 12/12-hour light/dark cycle.

2.4.2 Reagents and Solutions

A 0.1 M $ZnSO_4$ solution was purchased from Ricca (Arlington, TX). TPEN was purchased from Tocris (Bristol, United Kingdom), and human chorionic gonadotropin (hCG) was purchased from Henry Schien (Melville, NY). Unless noted otherwise, all remaining materials were purchased from Thermo Fisher Scientific (Waltham, MA). Modified Ringer's (MR) solution was used for *X. laevis* fertilization experiments. MR contains (in mM) 100 NaCl, 1.8 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, and 5.0 HEPES (pH 7.8) and filtered using a sterile 0.2 μm polystyrene filter (Heasman et al., 1991). Embryonic development assays were performed in 33% MR diluted in DDH_2O (MR/3). Dilutions of all metal ion and chelator solutions were performed in MR/3 for egg application or 1x MR for sperm application.

2.4.3 Collection of *X. laevis* Gametes

Using methods previously established by the Carlson lab, egg laying was stimulated in sexually mature females by injection with 1,000 IU of hCG into their dorsal lymph sac (Wozniak et al., 2020). Following injection, females were housed overnight for 12–16 hours at 16˚C. Typically, egg laying began within 2 hours of moving to room temperature. Eggs were collected on dry petri dishes and used within 10 minutes of being laid. Sperm were obtained from whole testes harvested from sexually mature *X. laevis* males (Wozniak et al., 2017). Following euthanasia by a 30-minute immersion in 3.6 g/L tricaine-S (pH 7.4), testes were dissected and cleaned by manual removal of residual fat and vasculature. Cleaned testes were stored at 4˚C in 1x MR for use on the day of dissection or in L-15 medium for use up to 1 week.

2.4.4 Fertilization and Embryonic Development Assays

For each experimental trial, development of *X. laevis* embryos was assessed from approximately 20–40 eggs in each experimental condition. In total, 20–90 μL of the sperm suspension was used to fertilize eggs depending on the volume of the dish (Wozniak et al., 2020). Approximately 90–120 minutes after insemination, the appearance of cleavage furrows was used to assess the initiation of embryonic development. Bright-field images were taken and analyzed using the ImageJ software package (Schneider et al., 2012). Each assay was repeated at least three times with gametes from different males and females and on different experiment days.

Sperm were extracted by mincing 1/10 of a testis in 100 μL of 1x MR and were used within 1 hour of collection. For experiments sequentially treating eggs with different conditions, eggs were incubated in an initial experimental solution, washed three times by moving between MR/3 1% agar petri dishes using plastic transfer pipettes, and then placed in the final treatment for experimental observation. Two types of these sequential treatment assays are reported here: transfer before insemination and transfer after insemination. When transferred between treatments before fertilization, eggs were incubated in the starting solution for 15 minutes and inseminated in the transfer solution. Sperm were incubated in the starting solution for 10 minutes at 4˚C before used to inseminate eggs in the transfer solution. When transferred after insemination, eggs and sperm were incubated together in the starting solution for 30 minutes and then transferred.

3.0 Discussion and Future Directions

For successful embryonic development, a single egg must be fertilized by a single sperm, a condition known as monospermy (Wong and Wessel, 2006). Should more than one sperm enter the egg, a condition known as polyspermy occurs, resulting in a catastrophic disorganization of developmental processes will ensue (Evans, 2020). Progression beyond this stage results in early embryonic lethality in the eggs of the vast majority of sexually reproducing animals. Therefore, the egg has an arsenal of protective processes known as polyspermy blocks, which prevent more than one sperm from fertilizing an egg. Two of the most studied blocks are named for the time that they are enacted relative to an initial fertilization event. They are referred to as the fast and slow blocks to polyspermy, as they respectively initiate just seconds and minutes after sperm fusion (Tahara et al., 1996).

A release of zinc has been observed from the cortical granules during the slow block process suggesting a role for zinc in the polyspermy block mechanism (Wong and Wessel, 2006). Insemination of eggs from many species, in physiologic concentrations of extracellular zinc, prevents fertilization and the initiation of embryonic development (Wozniak et al., 2020). Data in this thesis reveals that extracellular zinc targets both the egg and the sperm in order to prevent fertilization. This metal ion may be a key component of the slow block and involved in protecting the nascent zygote from fertilization by additional sperm. Zinc inhibition of fertilization in vertebrate and invertebrate species demonstrates that its role in the slow block to polyspermy is an ancient and highly conserved phenomenon (Wozniak et al., 2020.

I reasoned that a reversal of the inhibitory effect of zinc will be observed in the presence of both TPEN and ZX1 treated gametes, indicating that the external localization of zinc is vital to

preventing incidences of fertilization. Extracellular chelation of zinc ions was shown to rescue fertility of both gametes independently, suggesting that zinc released by the egg upon fertilization targets both egg and sperm to enact the slow block. Zinc's inhibitory effect is reversable as normal developmental patterns can be restored in the presence of a zinc chelator, suggesting a non-covalent interaction.

To further leverage the similarities and differences between other transition metals, copper was shown to inhibit sperm fertility and fertilization overall. Copper can interact with thiol groups on gamete extracellular matrix proteins. Future work will seek to better understand the interaction of zinc with each gamete's extracellular matrix by exploring the characteristics of copper inhibition of egg fertility.

Zinc is known to be a co-factor for a protease released by *X. laevis* eggs that acts on the glycoprotein matrix surrounding it (Lindsay and Hedrick, 2004). Many proteases are also known to bind zinc in active enzymatic sites, such as zinc-dependent metalloproteases. However, if zinc's primary role were to activate a protease, we would not expect its effect to be readily reversible with chelators. There are other potential non-protein binding sites for zinc including the sugars on glycoproteins themselves. Copper inhibition of fertility suggests alternative mechanisms of action from protease activation, such as strengthening the extracellular matrix components with metal ion binding.

Further work needs to be done to identify how directly these iterations of the slow block resemble one another, as there are some minute differences between species. This is key to ultimately translating this research into the modern medical field. Understanding how zinc blocks polyspermy could have far reaching implications from understanding the first moments of new life

to optimizing clinical conditions used for *in vitro* fertilizations in humans and artificial insemination in livestock animals.

3.1 Molecular Mechanisms and Timing of Zinc Inhibition

To confirm that zinc release occurs within the window of the slow block, studies were done to elucidate the impact of this zinc release on the timing of these methods. With the knowledge that inseminating in the presence of zinc inhibits fertilization, researchers transferred *X. laevis* eggs inseminated in these conditions to a zinc-free solution 30 minutes after development. These developing eggs failed to develop cleavage furrows, which indicates that there is a temporal aspect to the zinc regulation of fertilization, acting in the first half hour after fertilization (Wozniak et al., 2020). When these solutions were flipped, and eggs were inseminated in a zinc-free solution before being transferred to an inhibitory concentration of zinc, development proceeded accordingly.

This study is crucial to our understanding of the polyspermy block, as it differentiates the inhibition of fertilization by zinc, from its potential function as an inhibitor of development. The successful progression of embryonic development in *X. laevis* was assayed by the appearance of cleavage furrows, which typically occurs within two hours of sperm application. The results further reinforced the notion that zinc must be acting within this first 30-minute window to prevent fertilization. After that, the metal ion does not interfere with later stages of embryonic development (Que et al., 2019; Seeler et al., 2021; Wozniak et al., 2020).The release of zinc just minutes after fertilization thus corresponds to the function of altering the egg to prevent fertilization by more than one sperm. This process is consistently time dependent across all of these species and will

only enact within a specific window after fertilization (Que et al., 2019; Seeler et al., 2021; Wozniak et al., 2020).

To further ensure that extracellular zinc blocks fertilization, a timing assay can be conducted to examine how rapidly ZX1 application can restore embryonic development. Eggs will be incubated in the presence or absence of zinc before fertilization and transferred to ZX1. If zinc blocks fertilization, the appearance of cleave furrows should shift by the amount of time between insemination and chelator addition. This assay was conducted using the cell-permeant chelator TPEN which found that sperm were unable to penetrate eggs in the presence of zinc until application of the TPEN chelator. If solely extracellular zinc is interacting with the slow block to polyspermy, an identical result should be seen with a time course assay using ZX1. Fertilization can additionally be assayed using microscopy to determine if zinc is preventing sperm from binding to, or penetrating the egg.

Additionally, further research needs to be done regarding the potential function of intracellular zinc. While there are many studies indicating its release from the egg, it is possible that the extracellular zinc may be taken up by the egg or sperm and mediate the slow block to polyspermy from the interior of the gametes.

3.2 The Potential Zinc Targeting of Non-Mammalian Sperm

The slow block to polyspermy creates a physical barrier to sperm entry. The “shell” or envelope surrounding eggs provides an obstacle for sperm access to the egg. If the zinc acting in the slow block is located outside the egg’s envelope, it may also be interacting with nearby sperm. Unpublished data suggests that zinc targets sperm cells to inhibit fertilization in a similarly dose-

dependent manner as it does the eggs. The half-maximal concentration for zinc pre-treated sperm is far higher than that for inhibition of egg fertility. However, it remains within the range of physiological concentrations which indicates that both the egg and sperm could be exposed to these levels of zinc in natural conditions. Unlike the hardening of the egg envelope, zinc treated sperm cells exhibit no morphological changes which could contribute to the slow block. However, there are many processes which support sperm movement through the extracellular matrix, allowing for sperm fusion with the egg membrane. These mechanisms include flagellar hyperactivation and the acrosome reaction, both of which may be inhibited by zinc ions in the environment (Traboulsie et al., 2007).

Given that zinc is known to function extracellularly, it may be using egg-based methods, sperm-based methods, or both to inhibit fertilization. Extracellular zinc may prevent multiple fertilizations by targeting the sperm to halt capacitation and block the acrosome reaction. At mating, mammalian sperm are unable to fertilize an egg. Sperm undergo changes, termed capacitation, that provide them with the capacity to fertilize after entering the female reproductive tract (Jin et al., 2011). One such change is hyperactivation, a shift in flagellar beat from a linear swimming motion to an asymmetric whip-like motion (Traboulsie et al., 2007). This change is predicted to enable sperm penetration through the egg's extracellular vestments. Zinc is known to be able to block some of the ion channels which allow for hyperactivation to occur (Traboulsie et al., 2007). Thus, zinc may be targeting sperm to prevent its hyperactivation as a method of decreasing the rates of fertilization.

Another sperm-based target of extracellular zinc could be the acrosome reaction. During the acrosome reaction, hydrolytic enzymes are released from the sperm head (Jin et al., 2011). Specifically, the proteins are released from the acrosome, region on top of the sperm head. The

acrosomal membrane fuses with the cell membrane to release the contents trapped between them and expose key components of the sperm head. This process is believed to be necessary for sperm penetration through the zona pellucida that surrounds the egg (Evans, 2020). The aforementioned ability of zinc to block ion channels also comes in to play here. Voltage-gated calcium channels are necessary to signal for both hyperactivation, and the acrosome reaction (Traboulsie et al., 2007). Similarly to artificial activation in an egg, the calcium ionophore ionomycin can chemically induce the acrosome reaction (Jin et al., 2011). This suggests that zinc may prevent sperm from being able to fertilize an egg by inhibiting the activation of the acrosome reaction.

Existing evidence of high zinc concentrations and lowered sperm activity in addition to knowledge of other ion channel-based mechanisms in fertilization indicates that zinc could be targeting sperm as well as egg gametes (Wozniak and Carlson, 2020). These studies suggest that zinc could interfere with the initiation of two key process that allow the sperm to "drill" through the external matrix of the egg (Que et al., 2019). Combined with the increased strength of the membrane barrier produced during the egg's slow block, this may be sufficient to prevent supernumerary fertilizations. Conceptually, this proposed model aligns well with the current understanding of the membrane block. Increased defenses of the egg in addition to lowering the penetrative ability of sperm cells could result in the overall slow block to polyspermy. This would make it one of the few polyspermy blocks known to function by targeting both types of gametes in order to achieve its desired outcomes.

Bibliography

Bianchi, E., Doe, B., Goulding, D. and Wright, G. J. (2014). Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature* **508**, 483-487.

CDC. (2019). ART Success Rates: National Center for Chronic Disease Prevention and Health Promotion, Division of Reproductive Health.

Evans, J. P. (2020). Preventing polyspermy in mammalian eggs-Contributions of the membrane block and other mechanisms. *Molecular Reproduction and Development* **87**, 341-349.

Hassold, T. and Hunt, P. (2001). To ERR (meiotically) is human: The genesis of human aneuploidy. *Nature Reviews Genetics* **2**, 280-291.

Heasman, J., Holwill, S. and Wylie, C. C. (1991). Fertilization of cultured Xenopus oocytes and use in studies of maternally inherited molecules. *Methods Cell Biol* **36**, 213-30.

Hu, Q., Duncan, F. E., Nowakowski, A. B., Antipova, O. A., Woodruff, T. K., O'Halloran, T. V. and Wolfner, M. F. (2020). Zinc Dynamics during Drosophila Oocyte Maturation and Egg Activation. *iScience* **23**, 101275.

Iwao, Y., Kimoto, C., Fujimoto, A., Suda, A. and Hara, Y. (2020). Physiological polyspermy: Selection of a sperm nucleus for the development of diploid genomes in amphibians. *Molecular Reproduction and Development* **87**, 358-369.

Jaffe LA, G. M. (1985). Polyspermy-preventing mechanisms. In *Biology of Fertilization*, vol. 3 (ed. M. A. Metz CB). New York: Academic Press.

Jin, M., Fujiwara, E., Kakiuchi, Y., Okabe, M., Satouh, Y., Baba, S. A., Chiba, K. and Hirohashi, N. (2011). Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 4892-4896.

Kim, A. M., Bernhardt, M. L., Kong, B. Y., Ahn, R. W., Vogt, S., Woodruff, T. K. and O'Halloran, T. V. (2011). Zinc Sparks Are Triggered by Fertilization and Facilitate Cell Cycle Resumption in Mammalian Eggs. *Acs Chemical Biology* **6**, 716-723.

Levendosky, R. F., Sabantsev, A., Deindl, S. and Bowman, G. D. (2016). The Chd1 chromatin remodeler shifts hexasomes unidirectionally. *Elife* **5**.

Lindsay, L. L. and Hedrick, J. L. (2004). Proteolysis of Xenopus laevis egg envelope ZPA triggers envelope hardening. *Biochemical and Biophysical Research Communications* **324**, 648-654.

Nomiza, T., Falchuk, K. H. and Vallee, B. L. (1993). Zinc, Iron, and Copper Contents of Xenopus Zaeuis
Oocytes and Embryos. *Molecular Reproduction and Development*.

Que, E. L., Duncan, F. E., Bayer, A. R., Philips, S. J., Roth, E. W., Bleher, R., Gleber, S. C., Vogt, S., Woodruff, T. K. and O'Halloran, T. V. (2017). Zinc sparks induce physiochemical changes in the egg zona pellucida that prevent polyspermy. *Integrative Biology* **9**, 135-144.

Que, E. L., Duncan, F. E., Lee, H. C., Hornick, J. E., Vogt, S., Fissore, R. A., O'Halloran, T. V. and Woodruff, T. K. (2019). Bovine eggs release zinc in response to parthenogenetic and sperm-induced egg activation. *Theriogenology* **127**, 41-48.

Radford, R. J. and Lippard, S. J. (2013). Chelators for investigating zinc metalloneurochemistry. *Current opinion in chemical biology* **17**, 129-136.

Runft, L. L., Jaffe, L. A. and Mehlmann, L. M. (2002). Egg Activation at Fertilization: Where It All Begins. *Developmental Biology* **245**, 237-254.
Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**, 671-675.
Seeler, J. F., Sharma, A., Zaluzec, N. J., Bleher, R., Lai, B., Schultz, E. G., Hoffman, B. M., LaBonne, C., Woodruff, T. K. and O'Halloran, T. V. (2021). Metal ion fluxes controlling amphibian fertilization. *Nat Chem* **13**, 683-691.
Stumm, W. and Morgan, J. J. (1996). Aquatic chemistry. New York: John Wiley & Sons.
Swanson, W. J. and Vacquier, V. D. (2002). The rapid evolution of reproductive proteins. *Nature Reviews Genetics* **3**, 137-144.
Tahara, M., Tasaka, K., Masumoto, N., Mammoto, A., Ikebuchi, Y. and Miyake, A. (1996). Dynamics of cortical granule exocytosis at fertilization in living mouse eggs. *American Journal of Physiology-Cell Physiology* **270**, C1354-C1361.
Tokuhiro, K. and Dean, J. (2018). Glycan-Independent Gamete Recognition Triggers Egg Zinc Sparks and ZP2 Cleavage to Prevent Polyspermy. *Developmental Cell* **46**, 627-+.
Traboulsie, A., Chemin, J., Chevalier, M., Quignard, J. F., Nargeot, J. and Lory, P. (2007). Subunit-specific modulation of T-type calcium channels by zinc. *Journal of Physiology-London* **578**, 159-171.
Williams, R. J. P. (1987). The biochemistry of zinc. *Polyhedron* **6**, 61-69.
Wong, J. L. and Wessel, G. M. (2006). Defending the zygote: search for the ancestral animal block to polyspermy. *Curr Top Dev Biol* **72**, 1-151.
Wozniak, K. L., Bainbridge, R. E., Summerville, D. W., Tembo, M., Phelps, W. A., Sauer, M. L., Wisner, B. W., Czekalski, M. E., Pasumarthy, S., Hanson, M. L. et al. (2020). Zinc protection of fertilized eggs is an ancient feature of sexual reproduction in animals. *PLoS Biol* **18**, e3000811.
Wozniak, K. L. and Carlson, A. E. (2020). Ion channels and signaling pathways used in the fast polyspermy block. *Molecular Reproduction and Development* **87**, 350-357.
Wozniak, K. L., Mayfield, B. L., Duray, A. M., Tembo, M., Beleny, D. O., Napolitano, M. A., Sauer, M. L., Wisner, B. W. and Carlson, A. E. (2017). Extracellular Ca2+ Is Required for Fertilization in the African Clawed Frog, Xenopus laevis. *PLOS ONE* **12**, e0170405.
Wozniak, K. L., Phelps, W. A., Tembo, M., Lee, M. T. and Carlson, A. E. (2018). The TMEM16A channel mediates the fast polyspermy block in Xenopus laevis. *Journal of General Physiology* **150**, 1249-1259.