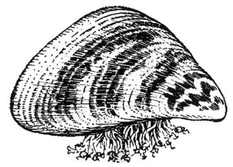
Investigating the role of proteases in fertilization in the invasive zebra mussels (*Dreissena polymorpha*).



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Abstract

Zebra mussels (*Dreissena polymorpha)* are an invasive bivalve of significant ecological and economic importance due to their widespread invasion and disruption of aquatic ecosystems and commercial infrastructure. Their ability to spread from the northern Great Lakes to the southern areas of the United States is due in large by their reproductive strategy. Zebra mussels release eggs and sperm into the water column where fertilization and subsequent larval development occurs. Two key steps in the fertilization process are the ability of sperm to bind and penetrate the egg surface and the ability of the egg to prevent more than one sperm from entering the egg (polyspermy). In many other species, proteases play a key role in these processes; however, there is there is variability between aquatic species, such that elucidating specific mechanisms is unique to individual organisms. Here, I investigate the potential role of proteases in sperm binding and entry. To discern these mechanisms in zebra mussels, I exposed fertilization processes to small-molecule inhibitors. Based on the observations of the phenotypic changes upon exposure, implications can be made to specific molecules or groups of molecules involved in *Dreissena polymorpha* sperm-egg interactions. These implications point to the further investigation and development of small-molecule inhibitors of *Dreissena polymorpha* fertilization.

Introduction

Invasive species.

- Invasive freshwater bivalve originating from Eurasia
- Transported to the Great Lakes in the 1980s through ship ballast water
- Spread extensively across the continent, reaching as far south as Texas.
- Distinct "zebra" stripe pattern
- Filter feeders and consume the algae, outcompeting many native species
- Attach to native clams and kill them
- Have few predators within the United States
- Cause significant ecological damage and ecosystem modification
- Couse significant economic damage to industrial infrastruce by clogging pipes and recreational impact by attaching to boats

Reproduction

- Broadcast spawners which release their gametes into the water column
- External fertilization and planktonic larval development
- Model organism for studying fertilization

Steps in Fertilization

- Sperm attacted to egg via jelly layer surrounding egg
- Sperm binds to extracellular coat surrounding egg
- Sperm acrosome reaction releases enzymes
- Sperm acrosome filament binds to egg surface
- Sperm nucleus enters egg cytoplasm
- Egg releases cortidcal granuales releasing enzymes
- Non-fertilizing sperm detach from egg

Importance of Proteases in Fertilization in other animals

- Sperm acrosome contains proteases
  - Proteases released during acrosome reaction
  - Needed to degrading coat surrounding egg allowing sperm to reach egg plasma membrane

Egg cortical granules contain proteases

- Granules underlie egg plasma membrane
- Granules exocytose at fertilization
- Released proteases clip sperm binding sites on egg surface
- Prevents polyspermy (multiple sperm fertilizing one egg

Presences and role of proteases in zebra mussels is unknown

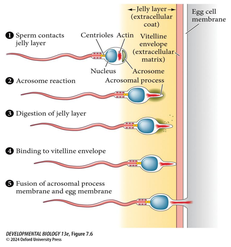
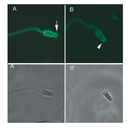
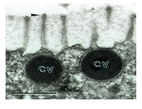


Figure above shows process of sperm contact to egg entry.





Top figure shows unreacted sperm (top) and acrosome reacted sperm (bottom).

Bottom figure show cortical granule vesicles at egg membrane surface prior to release.

Experimental Design

Maintaining Zebra Mussels

- Kept in insulated cooler at 49-50 F
- Mussels were fed 15mL algae solution 1-2 times weekly

Spawning Mussel Gametes

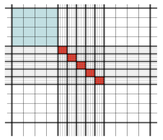
- Sets of 24 mussels were placed individually into spawning cups containing chilled dechlorinated water
- Mussels were then kept in spawning cups for 12-24 hours to allow them to acclimate to room temperature
- Spawning solution was prepared consisting of 30.8 mg 5-hydroxytryptamine (serotonin) per 80 mL 'pond water'
- Mussels were then rinsed with deionized water and placed into test tubes containing spawning solution
- After 15 minutes, males spawned sperm and were removed from their tubes and placed into separate spawning dishes to prevent re-siphoning of sperm
- After 45-60 minutes, females spawned eggs and were removed from their tubes and placed into separate spawning dishes that were filled with 'pond water' to continue spawning

Spawning male indicated by opaque solution (below).

Serotonin causes mussels to open shell to release gametes. Females moved into larger dish to observe egg release (below).

Sperm Count via Hemocytometer

- To measure sperm concentration, 100 µL of sperm was added to 100 µL para fixative and 800 µL 'pond water'
- This solution was then injected onto a hemocytometer (diagram right) where 5 cells were counted and averaged
- This average was input into the formula: # cells x 5 x 5 x 10^4, to find the concentration of the sperm sample
- This concentration was then input into the formula: sperm sample concentration / desired concentration x 5 mL, to find the quantity of sperm sample needed for the trial



Hemocytometer illustrated above.

Protease Solution Preparation and Storage

- Solutions were stored as:
  - SBTI: Stock solution made of 10 mg SBTI dissolved in 1 mL 'pond water' was stored between 2-8 C
    - 500 µL of stock solution was dissolved in the 5 mL fertilization trial to reach a working concentration of 1 mg/mL
  - E64: Stock solution made of 1 mg E64 dissolved in 2.8 mL deionized water was stored between 2-8 C
    - 50 µL of stock solution was dissolved in the 5 mL fertilization trial to reach a working concentration of 10 µM
  - Chymostatin: Stock solution made of 1 mg chymostatin dissolved in 165 µL DMSO to reach a 10 mM concentration
    - 50 µL of stock solution was dissolved in the 5 mL fertilization trial to reach a working concentration of 100 µM
  - PMSF: Stock solution made of 15 mg PMSF dissolved in 10 mL ethanol
    - 38.7 µL of stock solution was dissolved in the 5 mL fertilization trial to reach a working concentration of 1 mM
  - Pepstatin A: Stock solution made of 1 mg pepstatin A dissolved in 1 mL DMSO
    - 3.35 µL of stock solution was dissolved in the 5 mL fertilization trial to reach a working concentration of 1 µM
  - Aprotinin: Stock solution made of 10 mg of aprotinin dissolved in 1 mL water
    - 10 µL of stock solution dissolved in 100 µL water to reach a working concentration of 2 µg/mL in the 5 mL fertilization trial

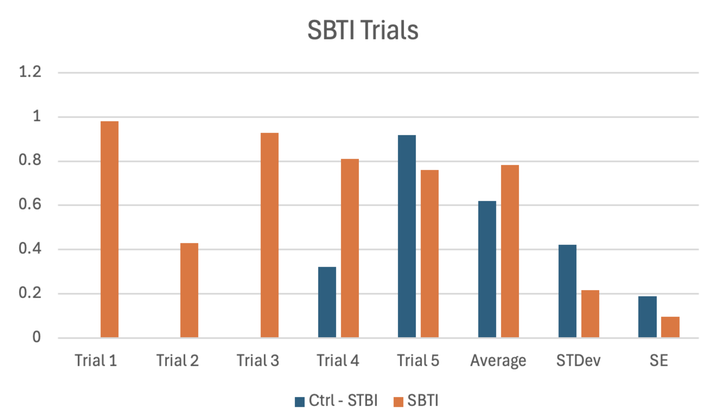
Fertilization Trial

- The calculated mL of sperm from a single male was mixed with 4 mL of eggs from a single female
- At 1 minute post-insemination, the protease solution was added
- To preserve the stages of fertilization, 500 µL of the fertilization sample was added to 500 µL para fixative at 5 minutes, 20 minutes, and 30 minutes post-insemination
- For the control trials, the same process was repeated using the same spawning male and female, but rather than treatment with a protease solution, samples were treated with the same quantity of 'pond water' or protease solution medium instead

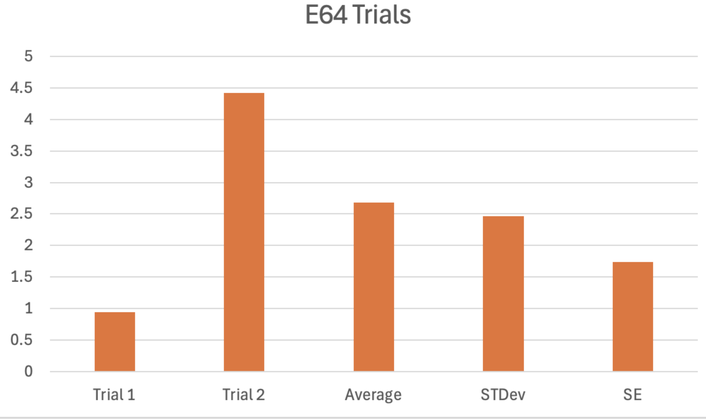
Sperm Binding Assay at 5 Minutes Post-Insemination

- The 5 minute timepoint of fertilization samples were viewed using light microscopy
- Equatorial sperm were counted and recorded

Preliminary Results



- 5 trials were performed with SBTI treatment
  - Trials 4 and 5 contained controls
  - Controls for trials 1, 2, and 3, were not viable
- At the time of testing, our samples of zebra mussels were out of their breeding season and had likely passed maturity
- Preliminary results do not appear to suggest polyspermy induced via SBTI treatment
  - However, we suggest complete replication of testing with viable controls before reaching conclusions



- Several trials were performed with E64 treatment
  - Of these, 2 trials contained gametes
  - No control trials were viable
- At the time of testing, our samples of zebra mussels were out of their breeding season and had likely passed maturity
- Preliminary results of E64 trial 2 appear to suggest polyspermy induction
- However, without controls, no conclusions can be reached
- We suggest complete replication of testing with viable controls and new samples of mussels before reaching conclusions
- Based on the initial results, E64 would be of our greatest interest in replication of trials
- At this time we plan to continue testing of further proteases including: chymostatin. PMSF, Pepstatin A, and Aprotinin
- We additionally plan to rerun trials for SBTI and E64

Literature Cited

- Togo, T., & Morisawa, M. (1997). Aminopeptidase-like protease released from oocytes affects oocyte surfaces and suppresses the acrosome reaction in establishment of polyspermy block in oocytes of the mussel Mytilus edulis. *Developmental Biology*, *182*(2), 219–227. https://doi.org/10.1006/dbio.1996.8483
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- Nathan Cress Final Thesis

Future Studies

- While in the early stages of testing, we encountered difficulties with spawning both male and female gametes
- In response to this, several trials were run with each of the collected zebra mussel samples we had maintained to determine the viability of our samples
  - The results of this testing suggested that all of our samples were no longer spawning gametes and had likely passed maturity
  - Additionally, at the time of testing, our zebra mussels were out of their regular spawning season
  - This halted fertilization trials until new samples of zebra mussels could be obtained
- We began to search local Texas aquatic ecosystems for the presence of known populations of zebra mussels, finding that the populations had collapsed
- As such, we were unable to continue further testing

- Our initial design planned to test several protease inhibitors including PMSF, E64, Pepstatin A, SBTI, Aprotinin, and Chymostatin
  - We chose these inhibitors based on previous studies by Togo and Morisawa, 1997 in which aminopeptidase inhibitors were found to inhibit polyspermy block in oocytes of *Mytilus edulis* mussels
  - Based on the several protease inhibitors tested by them, we chose the five most successful which included PMSF, E64, Pepstatin A, SBTI, Aprotinin, and Chymostatin
- Future testing for the ability of these protease inhibitors to inhibit polyspermy block in oocytes of *Dreissena polymorpha* may provide critical information into elucidating the polyspermy blocking mechanism of *Dreissena polymorpha*
- Due to our lack of controls and several nonviable trials, future studies may look to replicate the trials performed here, as well as expanding upon them in trial number and size
- Additionally, a broader testing of protease inhibitor classes beyond the five we have suggested may provide further details into the mechanism of polyspermy blocking in zebra mussels

Acknowledgements

Zebra mussels were sourced from