USING ASCORBIC ACID AND HIGH SALINITY TO EXTEND THE VIABILITY OF *PROALES SIMILIS* (ROTIFERA) DIAPAUSING EGGS

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USING ASCORBIC ACID AND HIGH SALINITY TO EXTEND THE VIABILITY OF PROALES SIMILIS (ROTIFERA) DIAPAUSING EGGS

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Table of Contents

List of Figures

Page

Abstract

Rotifers are sensitive indicators of environmental conditions and serve as model organisms for assessing toxicity. It is understood that rotifer cysts (diapausing eggs) are convenient for toxicity assessment because they remove the need to maintain animal cultures, reduce variability in tests, and can be stored for on-demand use. Indeed, cyst-based toxicity tests for the rotifer *Proales similis* have helped to fill a need for an additional marine animal model to the rotifer *Brachionus plicatilis* in ecotoxicology. A challenge to implementing tests is the need for readily available *P. similis* diapausing eggs, which need to be reliably preserved and hatched on demand. This study explores preservation methods to extend the viability of *P. similis* eggs. We explore factors including storage temperature, salinity, and the addition of ascorbic acid to measure their effects independently and combined. We found that storing the diapausing eggs at approximately 4℃ in the presence of 20 μM ascorbic acid and a 220ppt salinity is effective for extending egg viability. With longer viability, *P. similis* diapausing eggs are more readily available and thereby valuable as a tool in toxicity assessments.

Chapter I

Introduction

Ecotoxicology addresses how toxicants can impact the biological systems in an environment, often through the and use of model organisms, to assess environmental health. Rotifers have been a useful model animal in ecotoxicology because they are sensitive indicators to changes in ecosystems (Declerck & Papakostas, 2017). Their small size, fast population growth, and production of dormant embryos called diapausing eggs make them easy to use in laboratories. It is understood that rotifer diapausing eggs (cysts) are convenient for toxicity assessment because they remove the need to maintain animal cultures, form a similar cohort of animals once hatched that reduces testing variability, and can be stored in cool, dry conditions for on-demand use (Snell & Persoone 1988; Snell & Janssen, 1995). Marine aquatic tests have relied on the rotifer *Brachionus plicatilis,* although another, a smaller species of rotifer, *Proales similis,* is more sensitive to environmental contaminants (e.g., mercury; Rebolledo et al., 2018). Testing with multiple species, however, can provide a better assessment of environmental health (Snell, 2000), particularly as *s*ensitivity differences have implications for expanding the battery of marine rotifer cysts used in ecotoxicology.

Prior studies have focused primarily on the applications of *P. similis* in aquaculture, aquatic animal farming, as a small-bodied organism useful in feeding smaller fish larvae (Wullur et al., 2009). However, recent ecotoxicological studies in my lab showed that *P. similis* perform well in and are a valuable addition to marine toxicity cyst-based tests (Snell et al., 2019). Preliminary experiments in our lab showed that *P. similis* diapausing eggs remained viable for two months before a significant decline in hatching. This two-month period of viability is very

short compared to the decades *B. plicatilis* diapausing eggs can be stored, so the goal is to extend the viability of *P. similis* diapausing eggs.

The goal of this research is to extend the viability of *P. similis* diapausing eggs. We focused efforts on finding compounds and conditions known to demonstrate preservation and assessed their effects on *P. similis* diapausing eggs. One of these compounds was a common food preservative with antioxidant properties: ascorbic acid. *P. similis* diapausing eggs are stored at high salinities, which can be an environmental stressor. Fish larvae feeding on ascorbic acid supplements have shown higher tolerance to environmental and physiological stress (Merchie et al., 1996). We want to see if ascorbic acid can similarly improve stress tolerance and extend the preservation of *P. similis* diapausing eggs.

With longer storage, *P. similis* can be more widely used in ecotoxicology tests and provide more comprehensive toxicity assessments. This has implications to improve the assessment of environmental health in aquatic ecosystems. To determine if ascorbic acid could aid in preserving *P. similis* diapausing eggs, we examined the number of animals hatching weeks after storage in a range of ascorbic acid concentrations to measure how many eggs remain viable. Additionally, we conducted experiments on the effects of storage temperature and desiccation salinity, to form the best combination of preservation factors to extend the viability for *P. similis* diapausing eggs.

Chapter II

Literature Review

Rotifers are sensitive indicators of environmental conditions, such as pollution, which makes them great model organisms for assessing toxicity. For marine waters, the rotifer *Brachionus plicatilis* has been investigated for use in cyst-based toxicity assessments (Snell & Persoone, 1988). There has been a need for additional marine animal models in ecotoxicology because using a single species is not a comprehensive assessment. A recent study developed *Proales similis* as a similar, rotifer-cyst-based, marine toxicity test (Snell et al., 2019). To be readily available for testing, *P. similis* diapausing eggs need only to be preserved.

Most research surrounding *P. similis* focuses on their use in aquaculture because their small body size and fast population growth make them advantageous for feeding small fish larvae (Wullur et al. 2009). The features that make *P. similis* useful in aquaculture also suggests that the species could also be used in ecotoxicology. *P. similis* can be used in marine ecotoxicology, specifically, because the species can tolerate a wide range of salinities (Rebolledo et al., 2018). While historically most ecotoxicological assessments are done in freshwater, the fact that marine waters make up 70% of the Earth's surface suggests that more efforts should focus on expanding marine model organisms (Raisuddin et al. 2007).

Marine ecotoxicology has thus far relied on the rotifer *B. plicatilis*. Acute toxicity tests using the species have been researched and standardized (ASTM 1991). However, Rebolledo et al. (2018) provided evidence that *P. similis* are 10-38% more sensitive to mercury than *B. plicatilis*, suggesting that *P. similis* should be an additional, useful marine bioassay. According to Snell (2000), toxicity is best assessed when measuring a toxicant's effect on endpoints, like reproduction or mortality, for different species because of the broad rotifer species sensitivity

distribution. Using *P. similis* alongside *B. plicatilis* can therefore better characterize environmental toxicity.

Cyst-based toxicity assessments are appealing because diapausing eggs can be stockpiled. Stockpiling the eggs for long-term storage avoids the need to keep an ongoing proales culture in the laboratory. It has been studied that rotifer cysts remain dormant through storage in a desiccated state and hatch when reintroduced to favorable environmental conditions (Snell and Janssen 1995; Gilbert and Schroder 2004). For *B. plicatilis,* diapausing eggs can be stored for decades while retaining viability. This allows for tests to be performed on an on-demand basis, by eliminating the need for keeping a laboratory culture. This factor is largely why the species is so useful in ecotoxicology. Snell et al. (2019) observed that *P. similis* diapausing eggs can remain viable after storage in a high salinity brine for several months, which is a short period compared to *B. plicatilis.* Shorter shelf-life could limit the use of *P. similis* as a cyst-based toxicity test, but it is yet unknown whether *P. similis* diapausing eggs can be preserved for longer without losing viability.

The current study explores preservation methods to extend the viability in *P. similis* diapausing eggs. Kranner and Birtić (2005) linked viability loss in other desiccation-tolerant organisms to the breakdown of antioxidants, so the breakdown of antioxidants may be a contributing factor to the viability loss in *P. similis* diapausing eggs. For that reason, the addition of the antioxidant ascorbic acid is one of our focuses to curb viability loss in *P. similis* diapausing eggs is. Ascorbic acid will be studied in addition to factors like temperature and salinity to determine the most effective preservation method. If we can find a successful preservation method to extend the viability of *P. similis* diapausing eggs, the species will have more value in ecotoxicology and improve the assessment of toxicants in aquatic ecosystems.

Chapter III

Methods

Rotifer Culture and Desiccation

The *Proales similis* culture was maintained in a 200 ml flask containing 15ppt artificial seawater (ASW) and 10% *Tetraselmis suecica* algae to maintain growth. The flasks were kept at 25°C under fluorescent light. More algae were added to the culture as necessary, based on the green color in the flask, to keep the culture fed.

To collect the diapausing eggs, *P. similis* was added in 0.5ml aliquots to 0.6ml microcentrifuge tubes. The tubes were left uncapped at room temperature for about one week to allow the medium to evaporate. As evaporation progressed, *P. similis* naturally deposited diapausing eggs to the bottom of the tubes. Tubes then were capped when there were no actively swimming animals observed. This point signified that the tube's environment was uninhabitable, and the diapausing eggs would remain dormant. Salinity is an important marker in the desiccation process because with little liquid left in the tube the salinity will be at a level that limits the eggs from hatching. Before storage, we used a refractometer to measure the salinity. This salinity value was approximately 100ppt and would remain so throughout storage. Elements of this desiccation process were then altered to optimize the best preservation methods for *P. similis* diapausing eggs.

Rehydrating the Tubes & Scoring

Animals were hatched out from storage to allow for scoring of viability. Thus, for experiments, we removed tubes from storage and rehydrated them by adding approximately 500

µL of DI water. By adding water, the salinity in the tube would decrease causing the environment in the tube to be favorable for hatching. To further promote hatching, so we incubated the rehydrated tubes under fluorescent light at room temperature (25°C) for 24 hours.

Weekly scoring, counting the number of live animals present, showed us whether the diapausing eggs were still viable after storage because if the eggs were not viable they would hatch. We scored the animals 24 hours after rehydration by adding the full volume of a rehydrated tube to a well on a well plate with an additional 0.5ml of DI water to fill up each well.

Storage Temperature Comparison

To examine the effects of storage temperature on diapausing egg viability, 80 tubes were prepared following the outlined desiccation process. After being capped, half of the tubes were kept in the refrigerator at approximately 4°C and the rest at room temperature (25°C). Both sets of tubes were stored in the dark. Three replicates of each treatment were rehydrated and scored following the above methodology for 5 months so we could observe the trend in hatching and determine the effect storage temperature had on extending viability.

Ascorbic Acid Concentration Comparison

To measure the preservative effects of ascorbic acid on *P. similis* diapausing eggs we tested 5 concentrations in addition to a control (0 µM ascorbic acid). An 800 µM stock solution of ascorbic acid was diluted with ASW to 5,10,20,40, and 80 µM concentrations. Each concentration was added to the 0.6ml microcentrifuge tubes followed by the 0.5ml aliquots of *P. similis*. For each concentration, 12 tubes were prepared to have 3 replicates of each treatment to test over 4 weeks. The original desiccation process was followed once the tubes were prepared

and three replicates of each concentration followed the rehydration and scoring process each week. We again were looking to see a trend in the number of animals hatching each week for a month, and we wanted to determine which concentration was most effective in extending the viability of the diapausing eggs. The concentration showing the longest viability was noted to be used in further tests using additional preservation methods.

Desiccation Salinity Comparison

We conducted a salinity comparison to determine if a higher salinity during storage could act as a preservative and extend the viability of *P. similis* diapausing eggs. In the original desiccation process, tubes were allowed to desiccate until the salinity was approximately 100ppt. We used this as the control to compare the effects of letting tubes reach a salinity of approximately 240ppt before being stored. To reach this higher endpoint salinity, tubes were left uncapped longer, allowing for more evaporation. For both salinity endpoints, 12 tubes were prepared with a 0.5ml aliquot of *P. similis* to test over 4 weeks. Three replicates of each treatment were rehydrated and scored each week. Tubes in the salinity comparison were scored again 48 hours after rehydration due to small initial hatching after a 24-hour incubation.

Combination effects

With the data collected from the comparison assays, we then tested the preservation effects of multiple factors acting together. This was done under the assumption that the treatments that worked best in the ascorbic acid and salinity assays may further extend the viability of the diapausing eggs when acting together. The desiccation process was repeated, but this time using the best concentration from the ascorbic range finding, 20µM, and the best

endpoint salinity. For this combined treatment, our endpoint salinity only reached 220ppt, but believed this would be as effective as the 240ppt endpoint. 80% of the prepared tubes were stored in the refrigerator at 4°C, and some were kept at room temperature to recreate and reconfirm our initial temperature comparison. Over 6 months, rehydration and scoring were conducted comparing again the storage temperature and seeing how long the combined treatment could keep P. *similis* diapausing eggs viable.

Statistical Analysis

Welch Two Sample t-tests were performed in R for treatments with observed extension of diapausing egg viability to determine if the difference between the mean number of animals hatching in the treatment group and control group was statistically significant. We compared calculated p-values to a 0.05 reference alpha value.

Chapter IV

Results

Storage Temperature Comparison

Proales similis eggs that were stored at different temperatures showed variation in viability each week following full desiccation (Figure 1). When stored in the refrigerator, the mean hatching per tube did not greatly decline until seven weeks after desiccation, at which point hatching remained around 20 animals per tube for the next 12 weeks. For the tubes stored at room temperature, this decline occurred by week four, with little to no animals hatching past week seven.

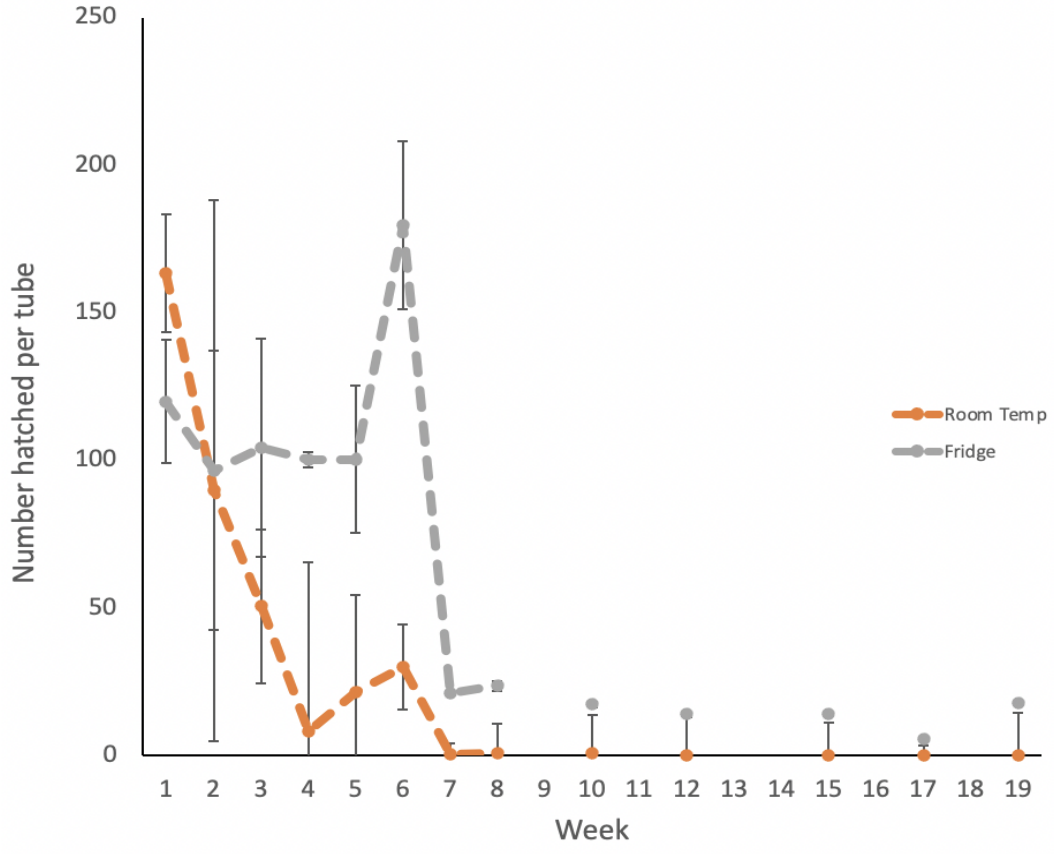

Figure 1. *P. similis* hatching viability after desiccation and storage at room temperature (25°C) compared to refrigerated (4°C). Each data point is a mean of three replicates. The vertical lines indicate standard error.

Ascorbic Acid Concentration Comparison

Repeating the desiccation process with ascorbic acid at six different concentrations showed an increase in preservation from ascorbic acid (Figure 2). Ascorbic acid at 20 µM had the greatest mean hatching out of the six tested concentrations past one week after desiccation (Figure 3). At week 3, 20 µM ascorbic acid had significantly higher hatching per tube than the control (Welch two-sample t-test, t=-3.528, df= 3.9218, p= .02507). Week four also had a greater number of hatched animals at 20 µM compared to the control, but the difference was not significant. Although hatching at concentrations greater than 20 µM also displayed higher mean hatching than the control, the 20 µM of ascorbic acid was all that was necessary to see an increase in hatching and remained more consistent.

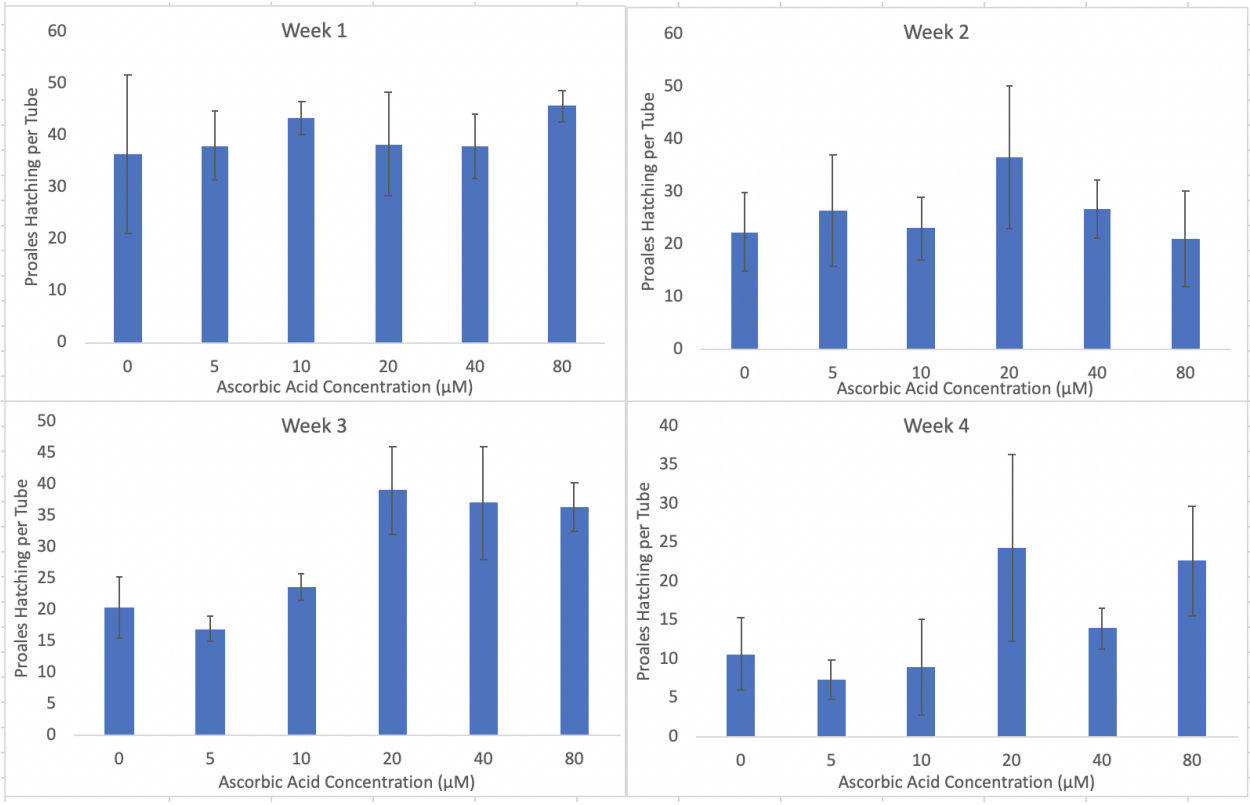

Figure 2. Comparison of *P. similis* hatching up to four weeks after desiccation in the presence of ascorbic acid. The data shows the mean of three replicates at six different concentrations of ascorbic acid (0, 5, 10, 20, 40, 80 µM). The vertical lines indicate standard error.

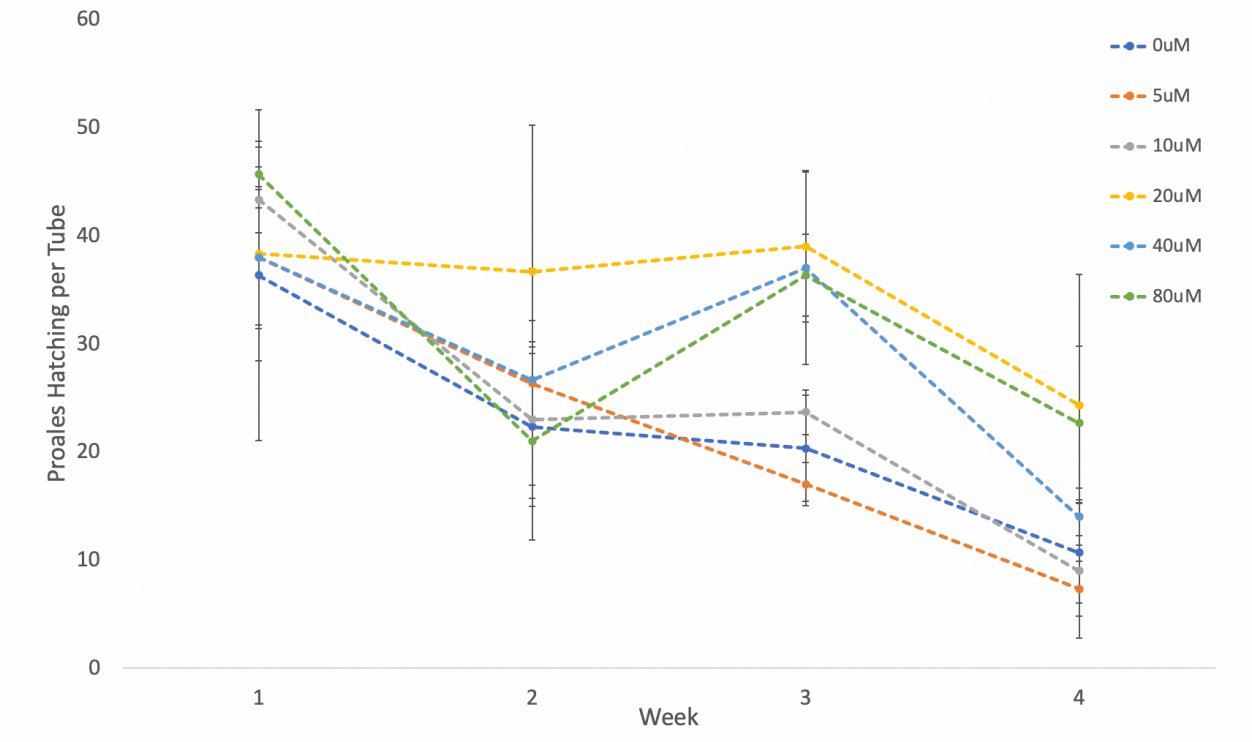

Figure 3. Hatching of *P. similis* after desiccation in the presence of ascorbic acid concentrations. Each data point is a mean of three replicates. The vertical lines indicate standard error.

Desiccation Salinity Comparison

The effects of higher salinity during storage were compared over four weeks after desiccation (Figure 4). In weeks 2 and 4, tubes that had a 240ppt salinity endpoint had significantly higher hatching per tube than the control (Welch two-sample t-test, week 2: t-value =-3.6875, df=2.6718,p=0.04192, week 4: t-value=-4.4709,df=3.9772, p=0.0112), but only 48 hours after rehydration. The hatching per tube after 48 hours after dehydration was consistently greater than 24 hours after (Figure 5).

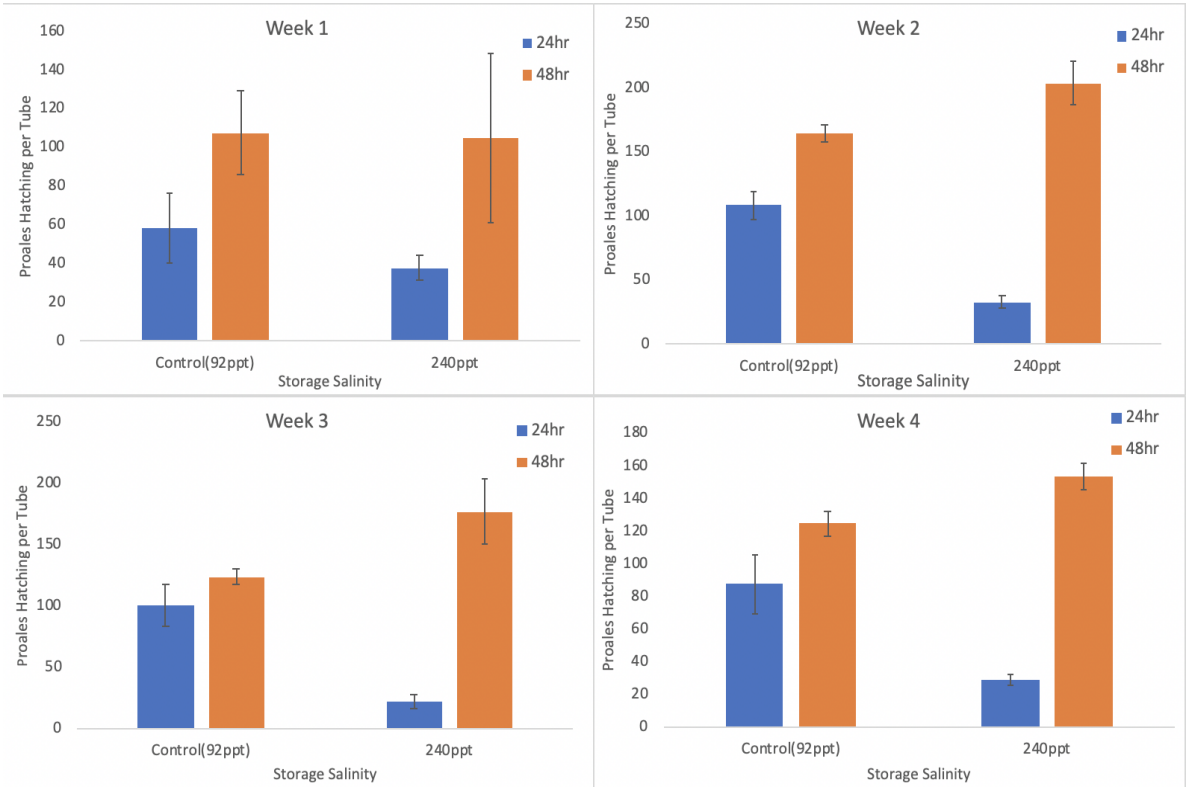

Figure 4. Comparison over four of *P. similis* hatching viability after desiccation to a control salinity(92ppt) and 240ppt. Data shows hatching twenty-four and forty-eight hours after rehydration. The salinity after rehydration was 16ppt for the control and 18ppt for the 220ppt treatment. Vertical lines indicate standard error.

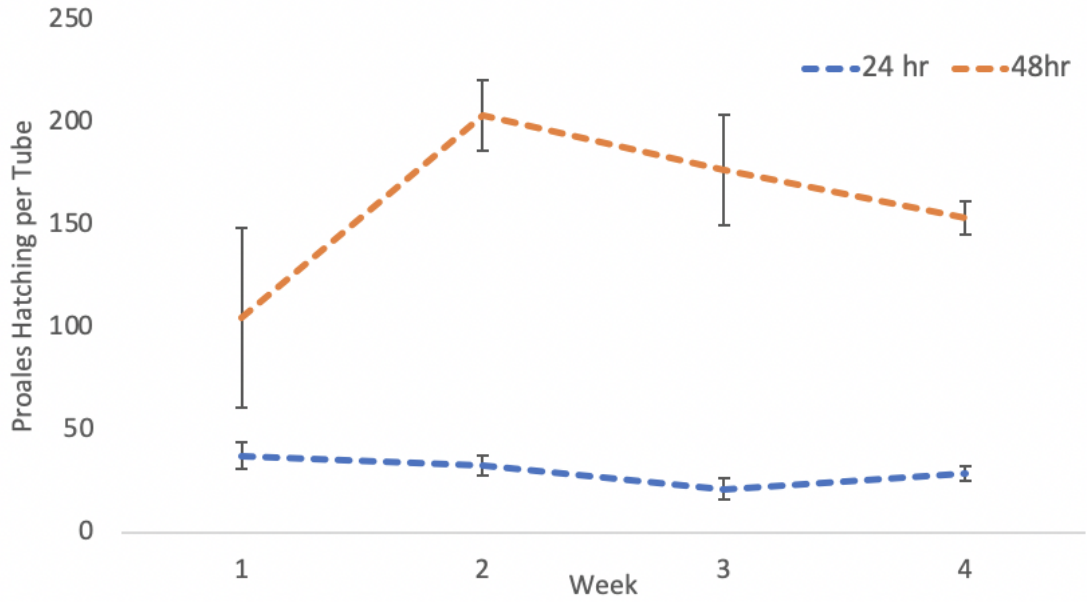

Figure 5. *P. similis* hatching viability of tubes desiccated to 240ppt twenty-four and forty-eight hours after rehydration measured over four weeks. Each data point is a mean of three replicates. Vertical lines indicate standard error.

Combination effects

The combined effects of 20 µM of ascorbic acid and a higher salinity endpoint for desiccation were compared at room temperature and refrigerator storage (4°C) until the animals hatching per tube reached 0. Room temperature tubes reached this endpoint by week 14, and refrigerator tubes remained above 0 through week 24 (Figure 6). Similar to before, the average number of animals hatching per tube was greater after week 2 for the tubes kept in the refrigerator (Figure 6). Being stored in the refrigerator with 20µM of ascorbic acid and 220ppt salinity allowed hatching to avoid the significant drop observed at week 7 for refrigerated tubes with lower salinity and no ascorbic acid (Figure 1). From the maximum number of animals hatched a value 10% of that was hit by week 10 without treatment and not until week 22 for tubes with treatment.

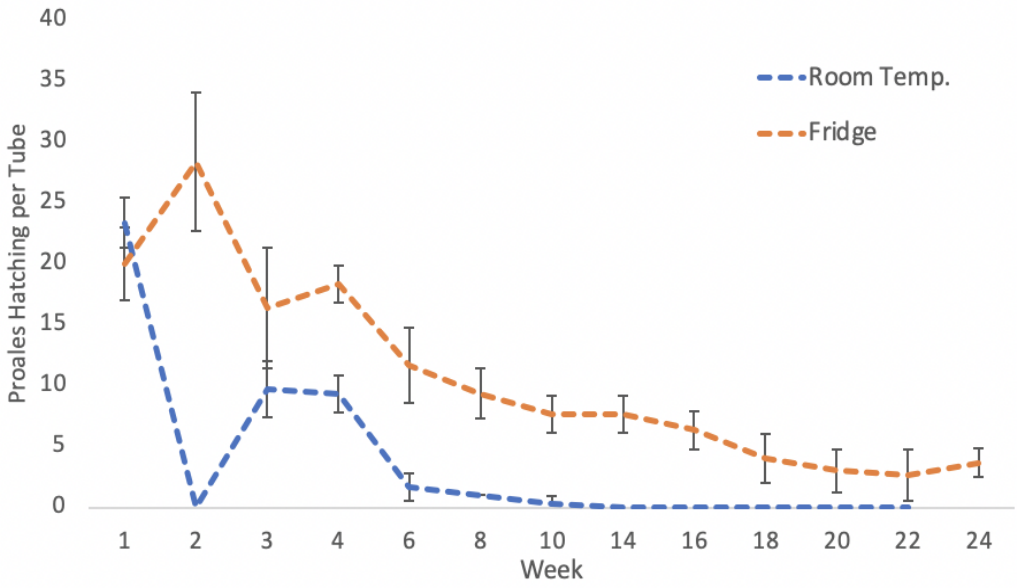

Figure 6. Hatching of *P. similis* after desiccation to 220ppt in the presence of 20 µM ascorbic acid. The data points are a mean of three replicates and show hatching forty-eight hours after rehydration. The vertical lines represent standard error**.**

Chapter V

Discussion

Our experiments tested methods to extend the viability of *Proales similis* diapausing eggs. From the temperature comparisons, we concluded that refrigerator storage keeps the diapausing eggs viable for longer than storage at room temperature. Ascorbic acid at 20 μM and an endpoint salinity 220-240 ppt showed longer viability than control conditions (0 μM ascorbic acid and 92ppt salinity). These factors all showed significantly higher hatching than the control tubes proving that they are beneficial in preservation. The collected data supports that these factors when all working together extend the viability of *P. similis* diapausing eggs from a period of 2 months to 6 months.

Antioxidants are useful for preserving viability in desiccation-tolerant organisms. (Kranner and Birtic 2005) and ascorbic acid specifically has helped organisms fight oxidative stress caused by high salinities (Shalata et al. 2001). Our findings from the ascorbic acid comparisons showed that ascorbic acid present in the tubes during storage provides a preservation effect for the diapausing eggs. Our findings in preserving rotifer diapausing eggs reinforce the findings of the previous studies showing there is a connection between viability and compounds with antioxidant properties.

Past studies have shown that storing resting eggs at lower temperatures yield higher hatching rates compared to those stored at room temperature (Hagiwara et al. 1997), and our results confirm this trend. We saw greater hatching in the tubes stored in a refrigerator both with and without the combined effects of ascorbic and higher salinity. Cool temperature along with darkness and salinity over 40% were found to prevent hatching (Minkoff et al. 1983), and for that reason, they are key factors in storing diapausing eggs. The results from our salinity

comparison affirms this as well. Since high salinity keeps cysts in their dormant state, having the *P. similis* diapausing eggs stored in 240ppt salinity likely decreased any sporadic hatching and was an effective preservation method.

The combined effects of refrigerator storage, 220ppt endpoint salinity, and 20 μM ascorbic preserved diapausing egg viability through 6 months of storage. This shows that we were successful in extending the viability of *P. similis* diapausing eggs, which were originally only viable in storage for 2 months. There are likely other preservation methods that can be explored to bring *P. similis* diapausing egg preservation closer to that of *Brachionus plicatilis*. Caprioli et al. (2004) and Gilbert (1974) studied the connection between carbohydrates, such as trehalose, that form the outer protective layer of diapausing eggs and the egg's deterioration patterns. Based on their findings, trehalose could be an additional compound to explore for extending diapausing egg viability. Additionally, research done on using disinfectants to preserve *B. plicatilis* resting eggs from harmful bacterial growth (Balompapueng et al. 1997) may be applicable in preserving *P. similis* diapausing eggs. The key to preserving *P. similis* diapausing eggs could be any combination of factors, but our research shows steps in the right direction.

Chapter VI

Conclusion

The use of rotifers as model organisms in ecotoxicology is largely due to their production of diapausing eggs that can be stored for on-demand use in cyst-based toxicity assessments. For marine ecotoxicology, these assessments previously relied on the rotifer species *Brachionus plicatilis*, whose diapausing eggs can be stored for decades at a time. Performing toxicity assessments on more than one species, however, gives a better understanding of the toxicant's environmental impact. Filling this need for an additional species, Proales *similis* is a recent addition to the model organisms used in marine ecotoxicology. *P. similis* diapausing eggs remained viable in storage for only 2 months which limits their value for on-demand cyst-based toxicity assessments. Our findings showed ascorbic acid in addition to factors like storage temperature and salinity is effective in extending the viability of *P. similis* diapausing eggs. With this newfound preservation for *P. similis* diapausing eggs, the species can be a more valuable tool for marine ecotoxicology. Its wider use will provide a more comprehensive assessment of environmental health, which will continue to be crucial for protecting our diverse, marine ecosystems.

References

Caprioli, M., A. K. Katholm, G. Melone, H. Ramløv, C. Ricci, and N. Santo. 2004. Trehalose in desiccated rotifers: a comparison between a bdelloid and a monogonont species. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology **139**:527-532.

Declerck, S. A., and S. Papakostas. 2017. Monogonont rotifers as model systems for the study of micro-evolutionary adaptation and its eco-evolutionary implications. Hydrobiologia **796**:131-144.

Gilbert, J. J. 1974. Dormancy in rotifers. Transactions of the American Microscopical Society:490-513.

Hagiwara, A., M. Balompapueng, N. Munuswamy, and K. Hirayama. 1997. Mass production and preservation of the resting eggs of the euryhaline rotifer Brachionus plicatilis and B. rotundiformis. Aquaculture **155**:223-230.

Kranner, I., and S. Birtić. 2005. A modulating role for antioxidants in desiccation tolerance. Integrative and Comparative Biology **45**:734-740.

Merchie, G., P. Lavens, P. Dhert, M. G. U. Góamez, H. Nelis, A. De Leenheer, and P. Sorgeloos. 1996. Dietary ascorbic acid requirements during the hatchery production of turbot larvae. Journal of Fish Biology **49**:573-583.

Minkoff, G., E. Lubzens, and D. Kahan. 1983. Environmental factors affecting hatching of rotifer (Brachionus plicatilis) resting eggs. Hydrobiologia **104**:61-69.

Shalata, A., and P. M. Neumann. 2001. Exogenous ascorbic acid (vitamin C) increases resistance to salt stress and reduces lipid peroxidation. Journal of Experimental Botany **52**:2207-2211.

Snell, T. 2000. The distribution of endpoint chronic values for freshwater rotifers. Pages 185-190 New Microbiotests for Routine Toxicity Screening and Biomonitoring. Springer.

Snell, T. W., and C. R. Janssen. 1995. Rotifers in ecotoxicology: a review. Hydrobiologia **313**:231-247.

Snell, T. W., R. K. Johnston, A. B. Matthews, N. Park, S. Berry, and J. Brashear. 2019. Using Proales similis (Rotifera) for toxicity assessment in marine waters. Environmental toxicology **34**:634-644.

Snell, T. W., and G. Persoone. 1989. Acute toxicity bioassays using rotifers. I. A test for brackish and marine environments with Brachionus plicatilis. Aquatic toxicology **14**:65-80.

Raisuddin, S., K. W. Kwok, K. M. Leung, D. Schlenk, and J.-S. Lee. 2007. The copepod Tigriopus: a promising marine model organism for ecotoxicology and environmental genomics. Aquatic toxicology **83**:161-173.

Rebolledo, U. A., S. Nandini, O. E. Sánchez, and S. Sarma. 2018. Combined effects of temperature and salinity on the demographic response of Proales similis (Beauchamp, 1907) and Brachionus plicatilis (Müller, 1786)(Rotifera) to mercury. Chemosphere **202**:312-321.

Wullur, S., Y. Sakakura, and A. Hagiwara. 2009. The minute monogonont rotifer Proales similis de Beauchamp: Culture and feeding to small mouth marine fish larvae. Aquaculture **293**:62-67.