Analysis of Various Cryptobiotic States Exhibited by Phylum Tardigrada

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Abstract

Phylum Tardigrada is a fascinating group of microorganisms known for their unique physical appearance and ability to survive through some of Earth's most extreme conditions. Tardigrades do so by entering a "hibernation state" known as cryptobiosis. There are several different cryptobiotic states that differ slightly in their mechanism of survival dependent on the extreme condition that forced the organism into "hibernation." Through a series of experiments, this thesis aims to answer the question of whether there is a substantial difference in the amount of time that it takes the organisms to exit the different cryptobiotic states when the extreme stressor is removed. Additionally, a second set of experiments tested the hypothesis that when the organisms are starved and lacking glucose, they will be less effective at entering cryptobiosis. Through substantial data collection, it was shown that significant differences do exist in exit times between cryptobiotic states and that lack of glucose does correlate with an inability to hibernate, dependent on the extreme condition. There is still further work that could be done using these procedures and data to continue to expand our knowledge of Phylum Tardigrada.

Keywords: tardigrade, cryptobiosis, anhydrobiosis, cryobiosis, osmobiosis , trehalose

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Chapter 1: Introduction

When considering their unique appearance and amazing ability to survive extreme conditions, it comes as no surprise that tardigrades have long been the subject of scientific research. Tardigrades, first discovered in 1773 by J.A.E Goeze, a German pastor, were then given the name Tardigrada, meaning "slow stepper," later by Lazzaro Spallanzani, an Italian biologist (Bordenstein, 2017). Since their initial discovery, researchers have gained extensive knowledge about their classification, anatomy, and distribution. Researchers now place the over 1,000 species of tardigrades into three classes: Heterotardigrada, Eutardigrada, and Mesotardigrada (Wright, 2014). The organisms used for these experiments were of Order Apochela within Class Eutardigrada. Anatomically, all tardigrades have very similar external anatomy with only a few slight variations between classes and orders. Animal Diversity Web gives this description of the external anatomy of Phylum Tardigrada :

> "Tardigrades are small (average 0.1 to 0.5 mm long), bilaterally symmetrical animals, with four pairs of lobopodious legs terminating in adhesive pads, discs, or claws. All tardigrades have intrinsic musculature and some species have telescopic legs. Their bodies are covered by a thin cuticle, which is uncalcified, may be divided into dorsal and lateral plates, and is often ornamented" (Wright, 2014).

This cuticle, which provides the organism with protection and durability, is one factor that allows tardigrades to live in diverse and inhospitable habitats. Tardigrades can truly live in any environment on Earth provided some moisture is available to them. According to Smithsonian magazine, such inhospitable environments can include cold as low as -

328°F (-200°C) or highs as great as 300°F (148.9°C). Tardigrades “can also survive radiation, boiling liquids, massive amounts of pressure of up to six times the pressure of the deepest part of the ocean and even the vacuum of space without any protection” (Bradford, 2017). Their ability to live through these extreme conditions is entirely dependent on cryptobiosis. Without having cryptobiotic ability, tardigrades would not inhabit the vast environments they do.

It is important to note that tardigrades are not considered true extremophiles because, while they can survive these extreme environmental conditions, they are not adapted to permanently live in them (Bordenstein, 2017). In fact, cryptobiosis brings the animal nearer to death than it does life. In the cryptobiotic state, metabolic activities come to a standstill, with most organisms actually dying due to the cessation of metabolism (Bordenstein, 2017). As mentioned earlier, tardigrades can enter cryptobiosis in response to many different environmental extremes. From this, researchers have found that there are different cryptobiotic states with differing mechanisms for survival dependent on the environmental stressor to which they are exposed. In the experimental work for this thesis, four different cryptobiotic states were induced and observed:

- Anhydrobiosis - induced by desiccating the organisms (lack of water)
- Cryobiosis - induced by exposing the organisms to extreme cold
- Extreme heat cryptobiosis (no given name) - induced by exposing the organisms to extreme heat
- Osmobiosis - induced by drastically changing the solute concentration of the organism's environment

Although extensive work has been done with these four cryptobiotic states (specifically anhydrobiosis, which is the most widely studied), no research could be found on how long it takes the organisms to "exit" the differing cryptobiotic states once the environmental stressor is removed.

This thesis seeks to answer that question. It was hypothesized that due to the differing mechanisms that allow for the different cryptobiotic states, there would be a discernible difference between the exit times of the different states. The research did show this to a certain extent (see chapter 3). Additionally, a brief experiment was done to test if the exit times of osmobiosis differed depending on the change in salinity to which the tardigrade was exposed (see chapter 4). Lastly, experiments were done testing the role of glucose stores and food availability on the organisms' ability to enter cryptobiosis. Anhydrobiosis, the most extensively studied of the cryptobiotic states, is known to rely on trehalose, a glucose disaccharide, for tun formation. Knowing this, the experiment sought to test the hypothesis that under starvation conditions, with no glucose stored in the body, the tardigrade would not be able to synthesize the trehalose needed to enter the cryptobiotic state. This experiment was done not only with anhydrobiosis, but with the cryobiotic and extreme heat cryptobiotic states as well. As with the first experiment, the hypothesis was partially supported (see chapter 5).

Chapter 2: Procedures and Methods

Tardigrade Care and Visualization

All experimental work for this thesis was done using tardigrades ordered from Carolina Biological Supply Company. In total, six vials of tardigrades were purchased and used. Upon arrival, the samples were aerated and then placed into six small glass beakers. Each 50mL beaker contained one vial of tardigrades, 25 mL of spring water, and 5mL of algae food source (Carolina Biological Supply). Throughout the duration of these experiments, which took approximately two months, the beakers were regularly exposed to sunlight, and additional spring water and food source were added as needed. When removed from the beakers for observation before and after enduring extreme conditions, the tardigrades were placed in a depression slide, and a compound light microscope was used for visualization. Tardigrades can be seen under a dissecting scope, but not to the magnification and focus level needed to differentiate between a "normal" tardigrade, and an individual in the tun state. For this reason, all observations of the organisms were done using a compound light microscope on scanning power.

Establishing Extreme Conditions

In total, the tardigrades were exposed to four extreme conditions: heat, cold, desiccation, and salinity change. These conditions were established and tested independently of one another to individually test each of the four induced cryptobiotic states. The extreme heat condition was established by simply heating a beaker of water on a hot plate to temperatures ranging from 70°C-75°C. The temperature was maintained within this range during the time of exposure. To expose the tardigrades, a small sample was removed from the beakers and observed under the microscope to both quantify the

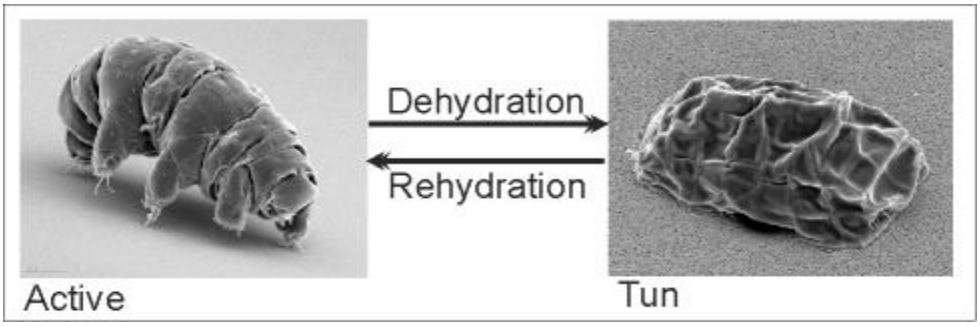
number of tardigrades present and ensure that all were alive. The tardigrades were then placed in a glass test tube with a small amount of spring water. The test tube was placed in the 70°C-75°C water for 20 minutes. After that time period, the tardigrades were removed from the tube and observed for their exit time from cryptobiosis (discussed in further detail later). The extreme cold condition was established similarly. A large beaker was filled with a salt and ice water mixture that maintained a temperature of -1°C. Tardigrades removed from the beaker, observed, and quantified were then placed in glass test tubes that were placed in the ice water for a duration of 30 minutes (ten minutes longer than the extreme heat conditions). After 30 minutes, the tardigrades were removed from the tube and observed for their exit time from cryobiosis, the cryptobiotic state in response to extreme cold. The dessication state was established as a sample of tardigrades was simply removed from the beakers, quantified, observed and placed in watch glasses with just 3 mL of spring water in them. The watch glasses were then left to evaporate for a duration of three days, desiccating tardigrades accustomed to living in the aqueous environment. After the three days, the tardigrades were rehydrated and observed as performed after the other extreme conditions. Finally, the tardigrades' response to extreme salinity was studied by exposing them to two differing salt water concentrations: 1.7M and 2.1M. Following the procedures of other researchers who have worked with Phylum Tardigrada osmobiosis, the tardigrades were gradually exposed to the salinity change. As others have done, the changes in salinity were performed gradually over several hours by periodically increasing the salinity of the solution by adding water with a pre-determined salinity (Halberg, 2009). Once the desired salinity was established, the specimen were left in the salt water for fifteen minutes and then removed and placed back

in spring water for observation, as was done with the other experiments. The final extreme condition to which the tardigrades were exposed was starvation. The specimens were removed from the beakers containing spring water and food source and placed in beakers containing only spring water with no food source. The organisms were left in this "starvation extreme" for four days before undergoing the same extreme experimentation as above, excluding the cryobiosis experiment which was only done with "fed" tardigrades.

Distinguishing and Quantifying Mortality Rates and Exit Times

After the tardigrades were removed from the extreme condition and put back into conditions for sustainable life, they were immediately observed. This observation was two-fold: 1. determining the mortality rate of the sample and 2. recording the time taken for each living tardigrade to "exit" the cryptobiotic state. Both tardigrades that are dead and those in the cryptobiotic state sink to the bottom of the beaker, petri dish, or compression slide. A difference can be seen however, in that tardigrades in cryptobiosis exhibit a protective shell covering and curl into a ball, as seen in Figure 1 (Raz, 2005).

Figure 1



Source: Media and Society Journal (Chelguin, 2015)

Once it was determined which individuals were dead and which were in cryptobiosis, the tuns were watched for any sign of movement. Similar to another group of researchers performing similar research, the standard was accepted that specimens that retained their locomotory function after being exposed to the extreme condition were considered alive (Bordenstein, 2017). Thus, upon the first sign of movement, the animal was officially known to be alive and the exit time was recorded. This exit time data was then compiled and underwent statistical analysis, as seen in the coming chapters.

Chapter 3: Analysis of Cryptobiotic State Exit Times Under Normal Conditions

Practicing the procedures as previously outlined, data was gathered for the four different cryptobiotic states examined in these experiments. The data focused on two specific statistics: mortality rate and exit time of the cryptobiotic state, both of which were compared across the different states for substantial differences.

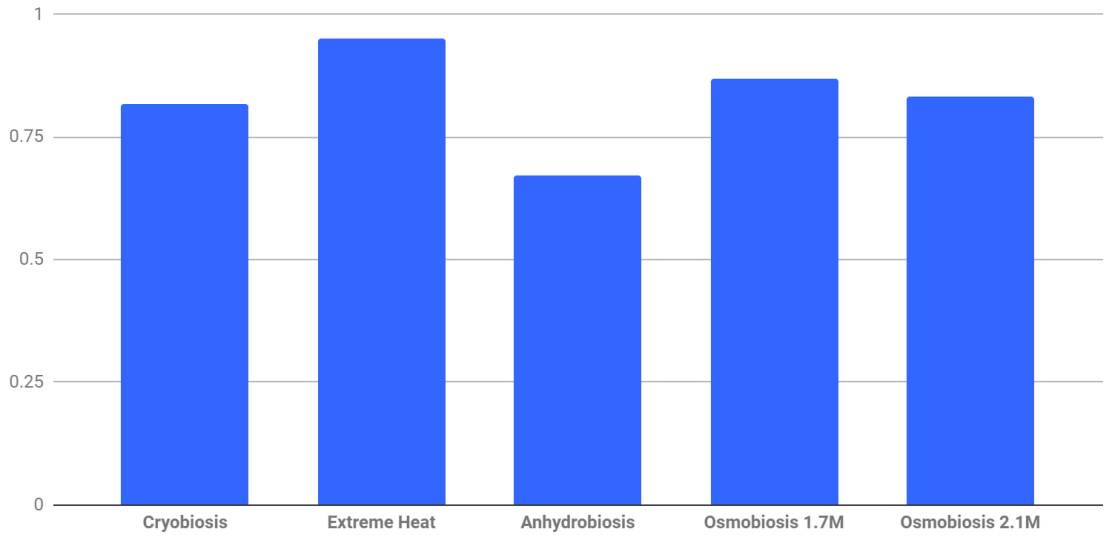
Mortality Rate Comparison

The data collected for the mortality rates of the different cryptobiotic states can be seen here:

Figure 2 (Lost refers to tardigrade not being found after being exposed to the extreme)

State	Original #	Survivors	Deaths	Lost	% Survivorship
Cryobiosis	55	45	7	3	81.8%
Extreme Heat	40	38	1	1	95%
Anhydrobiosis	61	41	17	3	67.2%
Osmobiosis 1.7M	38	33	5	0	86.8%
Osmobiosis 2.1M	65	54	7	4	83.1%

Figure 3 (P-Value = 0.6309399157)



It can be clearly seen that there is a difference in the mortality rates of the different states after being exposed to different extreme conditions. Figure 3 shows, however, that these differences in mortality rate are not statistically significant. With a P-value of 0.62, it is likely that the difference in mortality rates occurred because of random chance and not because of a significant difference in the average mortality of the different cryptobiotic states. With that said, further experimentation and larger sample sizes could possibly yield a significant difference between the different states. Thus, an analysis of why the different mortality rates may vary is included in this chapter.

The extreme heat cryptobiotic state exhibits the highest survivorship rate (and thus lowest mortality). This low mortality rate was expected as a similar study performed in 2001 which examined exposure to extreme temperatures on the eutardigrada *Richtersius (Adorybiotus) coronifer* found that 80% of tardigrades survived when exposed to temperatures of approximately 70°C, similar to the temperatures to which the tardigrades in these experiments were exposed (Worland, 2009). Unfortunately, the exact

mechanisms behind how tardigrades survive extreme heat are not yet known. Two important facts to note are that tardigrades more effectively survive extreme heat when dessicated (in the anhydrobiotic state) and that the high-temperature tolerance of different species differ greatly (Worland, 2009). This does not reveal any clear information about the mechanisms of the cryptobiotic state induced under extreme heat, but it does indicate that this state may be in some way similar or connected to the anhydrobiotic state, possibly by the sugar trehalose. This may also indicate that this state is anatomically based which is why species differing anatomically exhibit different heat tolerances.

Anhydrobiosis, on the other hand, exhibited the lowest survivorship rate in these experiments. These were not the expected results and could be due to some procedural error that led to this high mortality rate. Above all other cryptobiotic states, tardigrades are known for their ability to survive dessication, making anhydrobiosis the most widely studied cryptobiotic state. Researchers know not only the mechanisms behind this cryptobiotic state (discussed in chapter 5), but also that desiccation typically yields a very low mortality rate. One study by John H. Crowe, from the University of California Davis, showed that if the tardigrades are exposed to moist air before being dessicated, as were the conditions used in this experiment, nearly all of them should survive (Higgins, 1975). Countless accounts similar to this can be found of researchers examining anhydrobiosis. Thus, it can be assumed that an experimental error of some sort likely led to the low survivorship seen in this experiment, or that different tardigrade species were utilized.

Finally, cryobiosis and osmobiosis both produced a survivorship rate of approximately 80%. No previous studies on typical mortality rates for these states could be found for comparison. Thus, it is believed that these are accurate measures, although

comparison to other previous studies would be useful. The mechanisms behind these two cryptobiotic states will be discussed in the coming pages, with an extensive look at the osmobiotic state in chapter four.

Exit Times Comparison

Along with examining mortality rates, a more extensive analysis was performed on the data collected of the exit times for the differing states. The following information if given for reference while reading this section:

1.) The exit time data collected for each individual tardigrade in the different states
2.) A table of statistical analysis data derived from the exit times (Figure 4)
3.) A One-Way ANOVA test done with the exit time data (Figure 5)
4.) A connecting letters report of the data (Figure 6)
5.) Histograms showing the exit time data of each individual state (Figure 7)

Cryobiosis

6, 6, 9, 12, 13, 14, 18, 18, 19, 19, 19, 19, 20, 20, 20, 20, 20, 20, 21, 21, 21, 21, 21, 22, 22, 23, 23, 23, 24, 25, 26, 26, 27, 33, 34

Extreme Heat (no given name)

4, 7, 9, 12, 12, 13, 13, 14, 14, 14, 14, 15, 15, 15, 15, 15, 16, 16, 17, 18, 18, 18, 18, 19, 19, 20, 21, 22, 36, 42

Anhydrobiosis

4, 7, 13, 23, 27, 28, 31, 34, 36, 39, 40, 41, 41, 42, 43, 43, 44, 45, 45, 46, 46, 47, 47, 48, 48, 49, 49, 49, 50, 51, 52, 52, 57

Osmobiosis

See Chapter 4

Figure 4

State	Cryobiosis	Extreme Heat	Anhydrobiosis	Osmobiosis 1.7M	Osmobiosis 2.1M
% Survivorship	81.8%	95%	67.2%	86.8%	83.1%
Mean	20.14	16.7	41.03	15.96	30.05
Minimum	6	4	7	5	14
Quartile 1	19	14	37.5	12.5	26
Median	20	15	44	15	30
Quartile 3	23	18	48.5	18.5	33
Maximum	34	42	57	34	46
Standard Deviation	6.04	7.24	11.11	5.63	7.13

Figure 5 [F(4,157) = 49.1214, p <.0001]

****Confidence intervals found using Agresti-Coull Method **

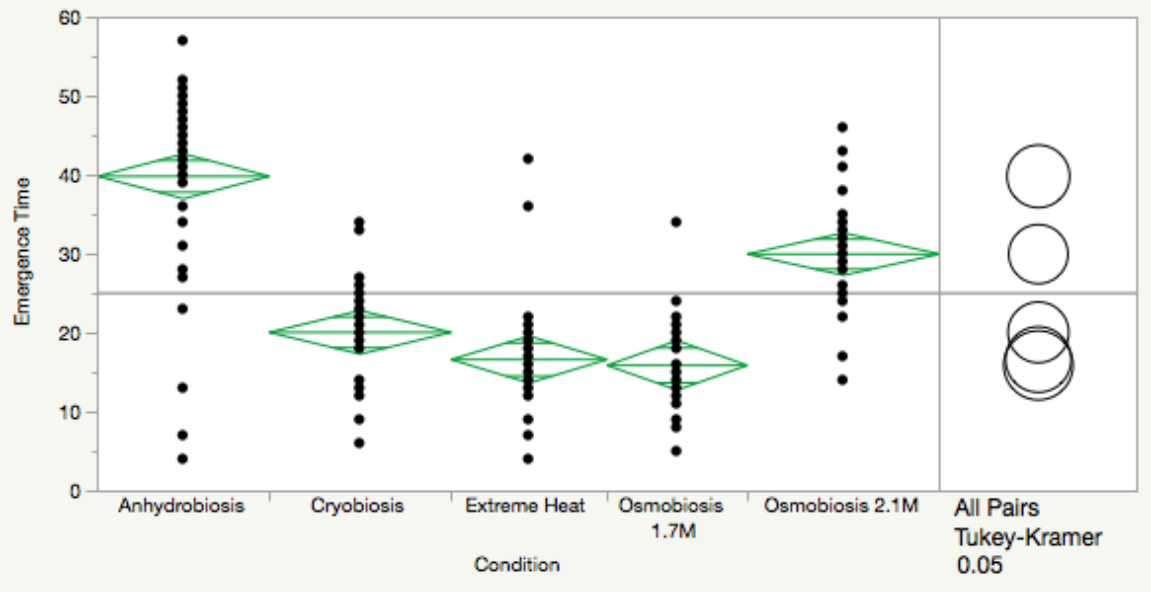
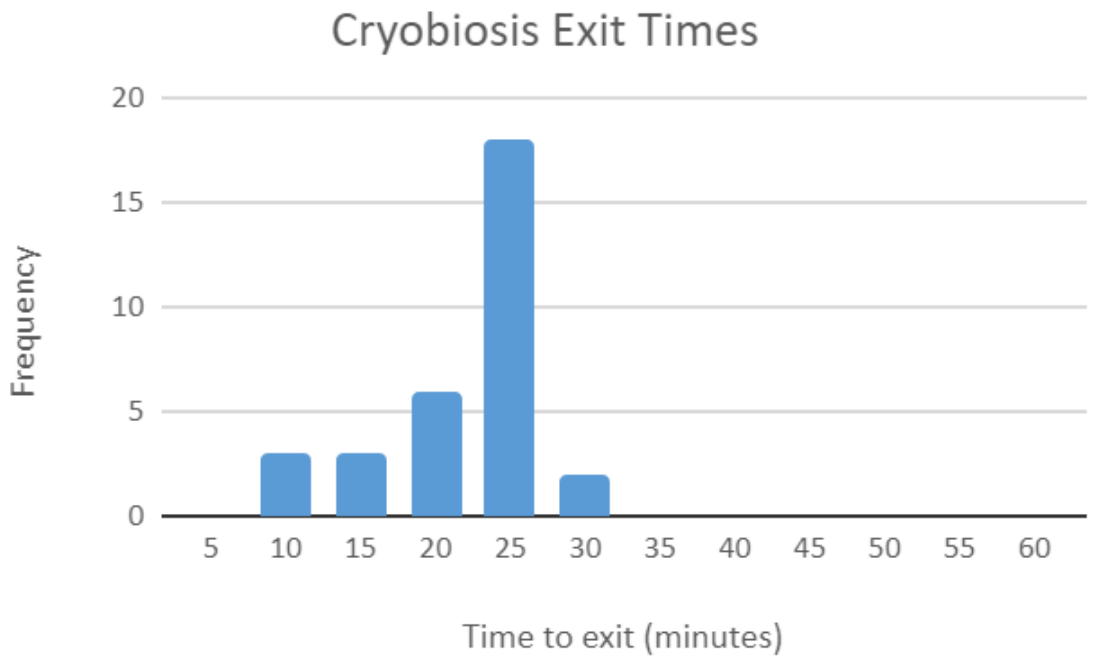
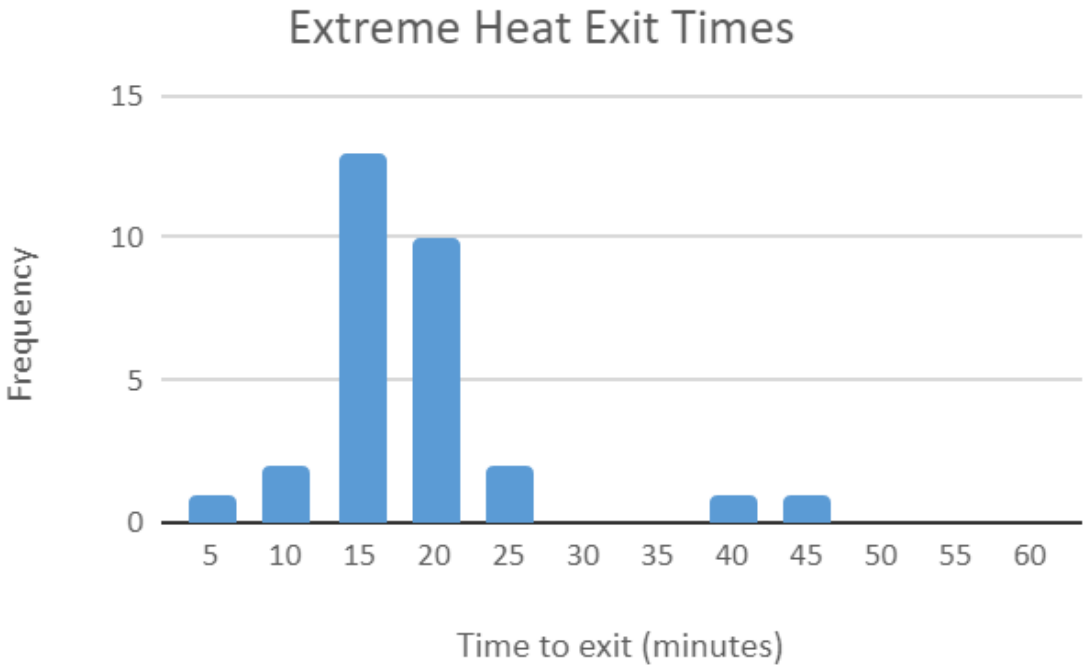


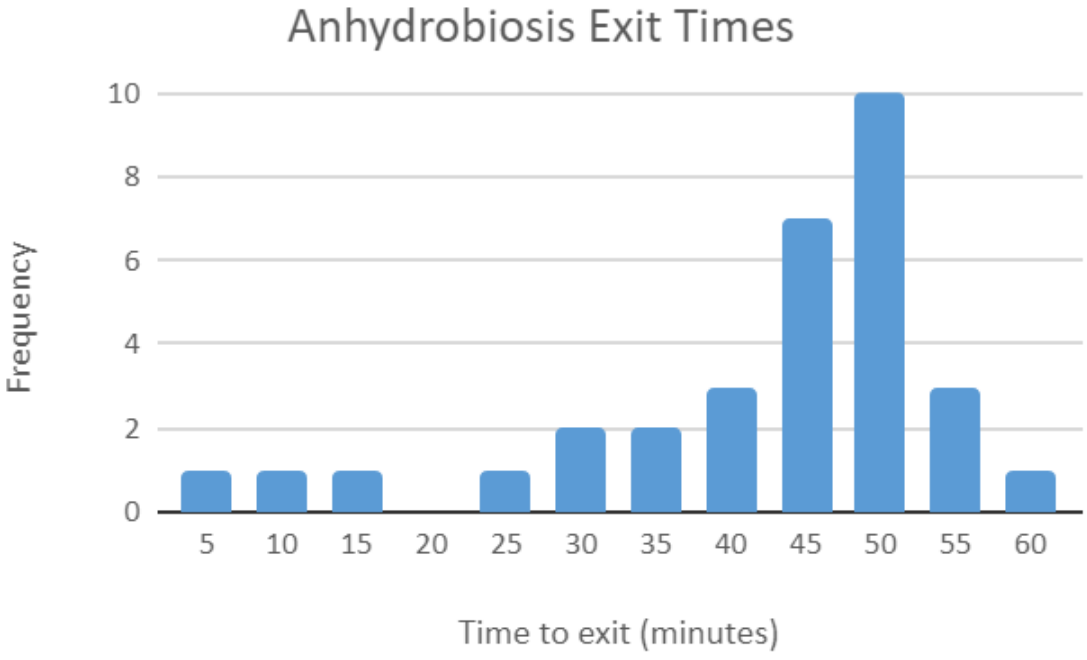
Figure 6

Level				Mean
Anhydrobiosis	A			39.909091
Osmobiosis 2.1M		B		30.054054
Cryobiosis			C	20.142857
Extreme Heat			C	16.700000
Osmobiosis 1.7M			C	15.962963

Figure 7




Extreme Heat Exit Times
Frequency
15
10
5
0
5
10
15
20
25
30
35
40
45
50
55
60
Time to exit (minutes)


Anhydrobiosis Exit Times
Frequency
10
8
6
4
2
0
5
10
15
20
25
30
35
40
45
50
55
60
Time to exit (minutes)

Unlike the mortality rates for anhydrobiosis and extreme heat conditions, where previous experimental work provided a basis for comparison, these exit time data are similar to the osmobiosis and cryobiosis mortality rates in that there is no previous data available. Accordingly, the rest of this section will be a comparison and explanation of the statistics shown, as well as providing possible hypotheses underlying the variable exit times.

The most obvious statistic from the data is the vast difference in average exit time for the different states. Most notably, anhydrobiosis takes much longer to exit on average than the other cryptobiotic states. This could be due to the anhydrobiotic mechanism outlined in chapter 5, but it is more likely that this is due to the longer duration the tardigrades were desiccated in comparison to the other extreme conditions. As stated in the procedures section, the tardigrades were dessicated for three days, while the other extreme conditions were only inflicted for a matter of hours or less than one hour. It was not evident until after completion of the experiments that the amount of time spent in the extreme condition affects the "difficulty" for the tardigrade to exit cryptobiosis. As explained by a team who studied tardigrades in many different extreme environments, "recent investigations indicate that the longer the time spent in anhydrobiosis, the more damage is inflicted to DNA, which would explain the prolonged recovery time" (Mobjerg, 2011). Thus, prolonged exit time may not be due to the mechanism of the anhydrobiotic state, but rather the length of time the tardigrades were exposed to dessication.

It is also notable that the anhydrobiosis exit times exhibit a left skew of the histogram. This reflects that some tardigrades in the sample exited much earlier than the others did. It is likely that these tardigrades may not have been as dessicated as the others

and were still clinging to some water molecules, allowing them to exit their anhydrobiotic state earlier.

It is also evident that cryobiosis and the extreme heat cryptobiotic states displayed the lowest average exit times. The reason for this is believed to be similar to the reasoning for why the anhydrobiotic state gave a longer exit time than the others. The organisms were only placed in the extreme heat for 20 minutes and the extreme cold for 30 minutes, a substantial difference from the three days they were dessicated. Further support for this hypothesis comes from an Italian research team who found that the mortality rate and amount of damage done to the organism are directly related to not only the environmental stressor the organism is exposed to, but also the time spent in the cryptobiotic state (Guidetti, 2011). While the abiotic and biotic conditions present throughout experimentation were well controlled and as uniform as possible in the different conditions, the time spent in each extreme condition should have been more controlled and uniform. While this theory requires repeat experiments, it is hypothesized that the differing exit times result not from the mechanism of the different cryptobiotic states, but rather from the varying times that these samples were exposed to each extreme condition.

A final point that should be made about the histograms in Figure 7 is the absence of histograms for the osmobiotic state. These are provided in the following chapter which focuses specifically on osmobiosis and a comparison of these two different salinities.

Chapter 4: Analysis of Osmobiosis Exit Times Under Different Salinity Conditions

While the other cryptobiotic states were observed after being exposed to one uniform extreme condition, osmobiosis was exposed to two salinities differing in concentration. The two salinities were decided after initially testing five different salinity concentrations. The tardigrades were exposed to salinities of .85M, 1.3M, 1.7M, 2.1M, and 2.6M. The .85M and 1.3M concentrations were not an extreme enough change to induce cryptobiosis while the 2.6M was too extreme of a change that the tardigrades were not able to enter the cryptobiotic state resulting in their death. Thus, while the other states had only one set of data to analyze, the osmobiotic state produced two sets of data for the remaining two salinities: 1.7M and 2.1M. The purpose of this experiment was to determine if there is a substantial difference in mortality rate or exit time for the tardigrades dependent on the salinity to which they are exposed.

Mortality Rate Comparison

The data were compared to findings from an experiment performed in 1950. In this experiment, the tardigrades were placed in various salt solutions. One observation found that the time it takes to enter the tun conformation is dependent on the concentration of the solution (Higgins, 1975). This supports the data found in this study and will be discussed in the final section of this chapter. Secondly, it was found that "while most of the animals they observed could survive one day's exposure to seawater, none survived exposure to a 1200 mosm solution of NaCl (osmotically equivalent to sea water) for the same period" (Higgins, 1975). This both supports and differs with the data collected in this thesis. While the tardigrades studied did exhibit a fairly uniform mortality rate across the different salinities, as was found in the previous study, the high

survivorship rate was not expected. Considering that none of the organisms in the previous study survived the NaCl exposure, it is interesting that in the present study tardigrades yielded such a low mortality rate.

Unfortunately, so little is known about the osmobiotic state that extrapolating this small sample of results into a hypothesis of some sort would be futile in the absence of more data. In fact, the osmobiotic state is so understudied that some researchers argue "there is no evidence for the existence of osmobiosis" at all (Guidetti, 2011). It is for this reason that a study from 1950 is being used as the model for studying this cryptobiotic state. A more recent study would be preferred for the purpose of comparison, but none could be found. In conclusion, no clear hypothesis can be made from this study's data other than the simple observation that osmobiosis does in fact seem to exist and it is possible that some species of tardigrades are able to exist in an osmobiotic state in NaCl solutions while others cannot.

Exit Times Comparison

While the majority of the 1950s study did not provide much support for the data from this experiment, one important aspect of the prior experiment did support the findings. Unlike in this experiment, which observed organisms' exit times, the prior study observed the time taken for the tardigrades to enter the tun formation and begin living in the osmobiotic state. As stated previously, the researchers found that the organisms entered the tun at a rate determined by the concentration of the solution (Higgins, 1975). It is no surprise, therefore, that a correlation may be seen between the exit time of the tardigrades and the salinity in which they are placed. Provided below for reference are the histograms generated for these exit times of the two different salinities (figure 8). There

does appear to be a correlation between the salinity to which the tardigrade is exposed and the time taken to exit osmobiosis once placed back in a “normal” concentration.

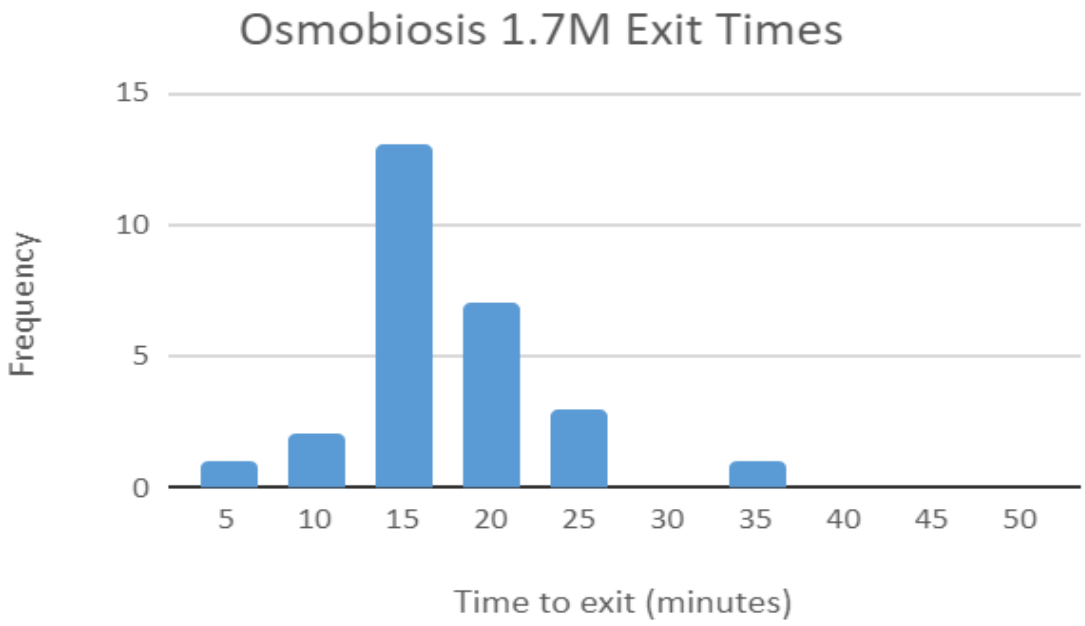
Osmobiosis (1.7 M)

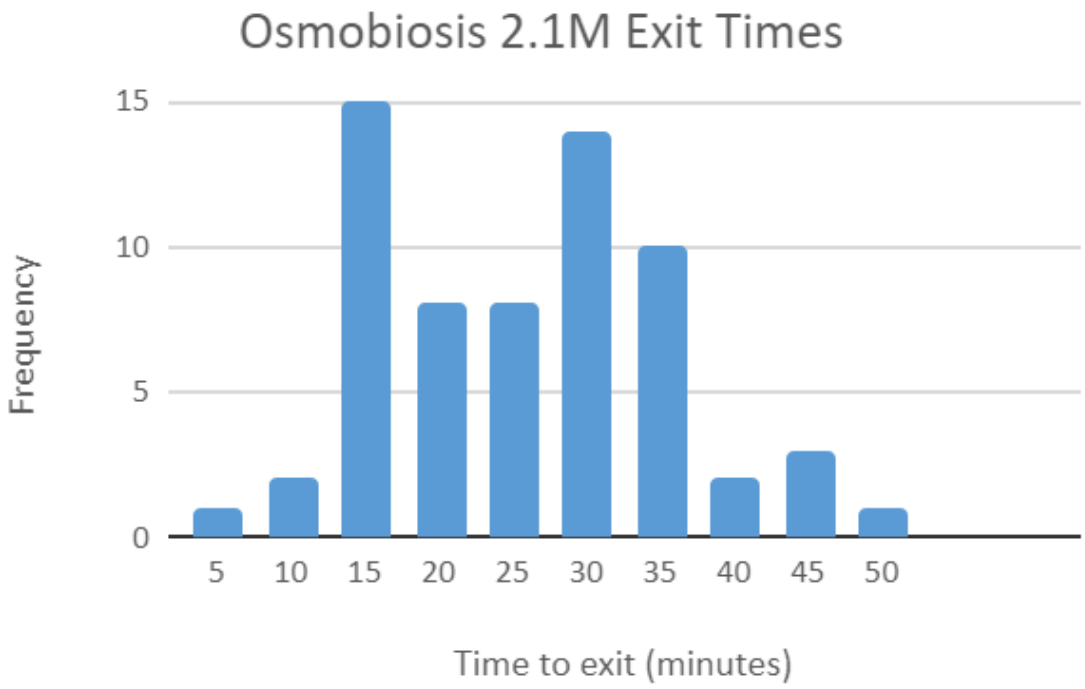
5, 8, 9, 11, 12, 12, 13, 14, 14, 14, 14, 15, 15, 15, 15, 15, 16, 18, 18, 18, 19, 20, 20, 21, 22, 24, 34,

Osmobiosis (2.1 M)

14, 14, 17, 22, 24, 24, 25, 25, 26, 28, 28, 28, 28, 28, 29, 29, 30, 30, 30, 30, 30, 30, 31, 31, 32, 32, 32, 33, 34, 34, 35, 38, 38, 41, 43, 43, 46

Figure 8


Osmobiosis 1.7M Exit Times
Frequency
15
10
5
0
5
10
15
20
25
30
35
40
45
50
Time to exit (minutes)


Osmobiosis 2.1M Exit Times
Frequency
15
10
5
0
5
10
15
20
25
30
35
40
45
50
Time to exit (minutes)

Chapter 5: Analysis of Cryptobiotic State Exit Times Under Starvation Conditions

The final experiment done was intended to function as a test of trehalose's role in three cryptobiotic states: extreme heat, cryobiosis, and anhydrobiosis. Trehalose is a simple glucose disaccharide made of two alpha glucose molecules joined by an alpha, alpha-1,1-glycosidic linkage (Cotton, 2012). Trehalose is often used as an energy storage molecule in insects and has attracted attention recently as being an effective food additive and stabiliser to preserve cells, tissues, and organs, an idea which will be expanded on in the final chapter (Cotton, 2012). In this experiment, however, trehalose was studied for its ability to preserve membrane integrity and protein structure in the organisms during extreme conditions (Hengherr, 2008). Of the three cryptobiotic states examined in this experiment, trehalose is only known to be an active agent working in anhydrobiosis.

> "Two models for the mechanism of the protective role of trehalose have been proposed that are not mutually exclusive: The water replacement hypothesis states that trehalose forms hydrogen bonds with macromolecules and cellular structures in place of water during dehydration and thus preserves native structures. In addition, the vitrification hypothesis proposes the formation of amorphous sugar glasses during desiccation, which protects proteins and membranes" (Hengherr, 2008).

Knowing that trehalose has an important function in the anhydrobiotic state, this study sought to provide further support for this idea and to explore the question of whether trehalose is important for the other cryptobiotic states as well. It is known that tardigrades are typically better able to survive exposure to extreme heat when dessicated than when they are fully hydrated (Worland, 2009). Thus, the hypothesis can be made that the

presence of trehalose does in some way aid in surviving extreme temperatures and may somehow aid in surviving other extremes.

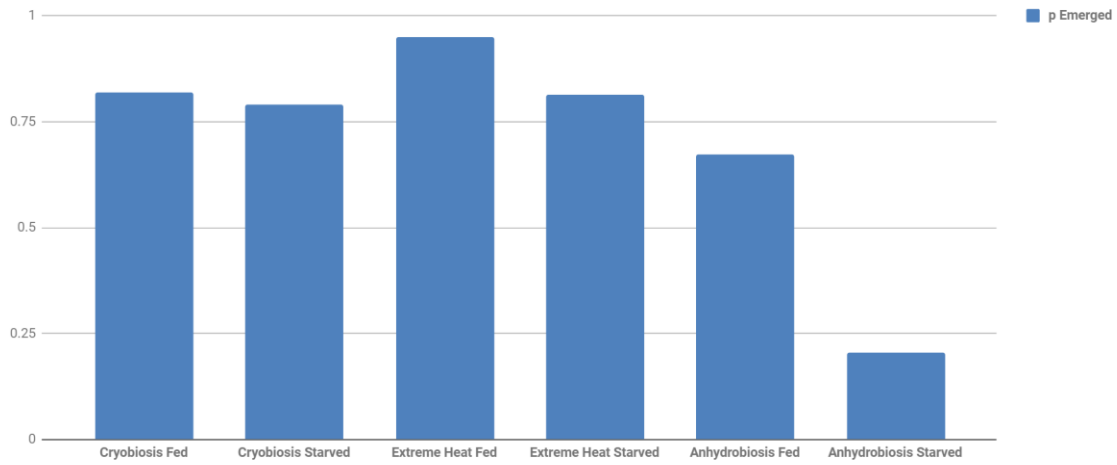
Considering that trehalose is a glucose disaccharide, it can be hypothesized that the tardigrade requires some glucose source to synthesize the trehalose sugar. By removing the tardigrades from their food source for four days, their nutrient (glucose) source would have been either completely exhausted or at least greatly depleted. In theory, this could make it difficult or even impossible to synthesize trehalose to enter cryptobiosis. Future work could include a trehalase assay in order to truly quantify the trehalose concentration of the organism (discussed in chapter 6).

The following pages provide the data gathered for each of the cryptobiotic states both in their starved and normal conditions. First, Figures 9 and 10 provide a comparison of mortality rates of the different cryptobiotic states under normal and starved conditions. Figure 11, provides the statistical analysis of the exit time data for the cryptobiotic states under normal and starved conditions. Figures 12 and 13 give a graphical representation and table information of the average values (and confidence intervals) of each of the cryptobiotic states' exit time. Finally, Figure 14 is composed of several histograms that act as a visual comparison of the cryptobiotic state exit times under normal and starved conditions. At the conclusion of this chapter, are short potential explanations of trehalose's role for each of the three cryptobiotic states.

Figure 9

Cryptobiotic State	Original #	Survivors	Deaths	Lost	% Survivorship
Cryobiosis	55	45	7	3	81.8%
Starved Cryobiosis	43	34	7	2	79.1%
Extreme Heat	40	38	1	1	95%
Starved Extreme Heat	48	39	8	1	81.3%
Anhydrobiosis	61	41	17	3	67.2%
Starved Anhydrobiosis	39	8	31	0	20.5%

Figure 10 (P-Value = 0.001779936787)



Exit Times (Minutes): See chapter 3 for normal condition times

Starved Cryobiosis

14, 14, 15, 15, 16, 16, 18, 18, 18, 18, 19, 19, 19, 19, 20, 20, 20, 20, 20, 21, 22, 22, 23, 23, 25, 25, 28, 36

Starved Extreme Heat (no given name)

6, 12,14, 15, 15, 17, 17, 18, 18, 18, 18, 19, 19, 20, 20, 20, 20, 20, 21, 22, 22, 22, 23, 23, 24, 24, 26, 26, 28

Starved Anhydrobiosis

29, 32, 35, 38, 38, 44, 45, 47

Figure 11

Cryptobiotic State	Cryobiosis	Starved Cryobiosis	Extreme Heat	Starved Extreme Heat	Anhydrobiosis	Starved Anhydrobiosis
% Survivorship	81.8%	79.1%	95%	81.3%	67.2%	20.5%
Mean	20.14	20.11	16.7	19.55	41.03	38.5
Minimum	6	14	4	6	7	29
Quartile 1	19	18	14	17.5	37.5	33.5
Median	20	19.5	15	20	44	38
Quartile 3	23	22	18	22.5	48.5	44.5
Maximum	34	36	42	28	57	47
Standard Deviation	6.04	4.60	7.24	4.53	11.11	6.44

Figure 12

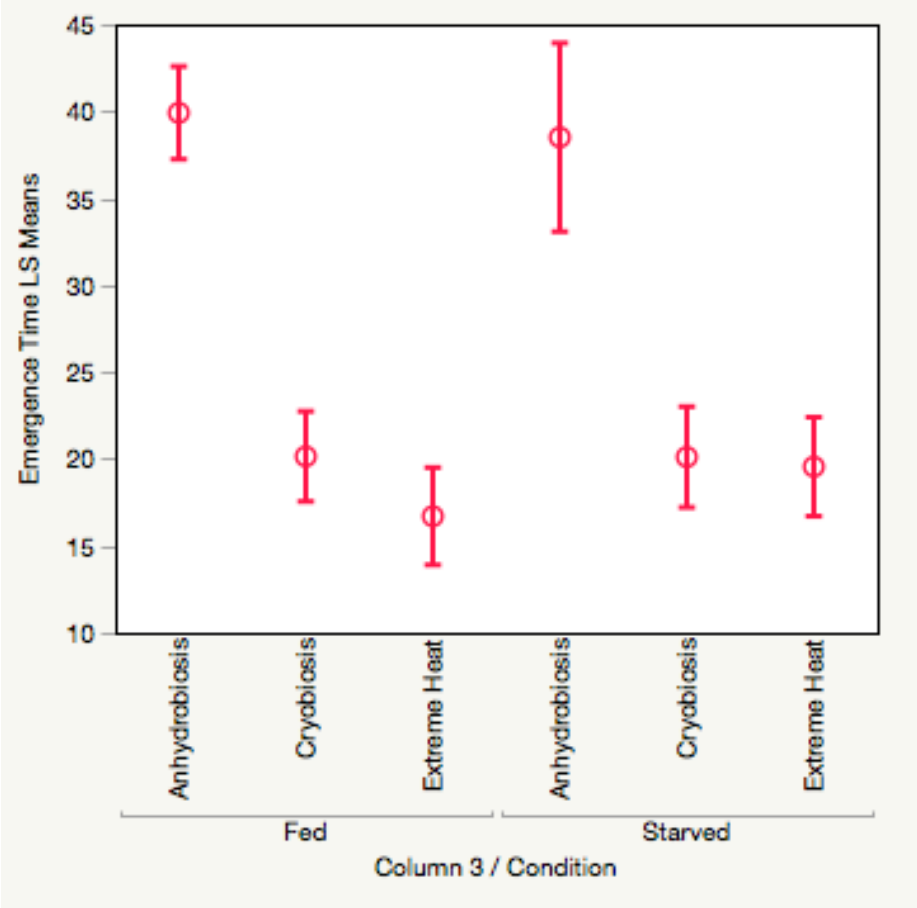
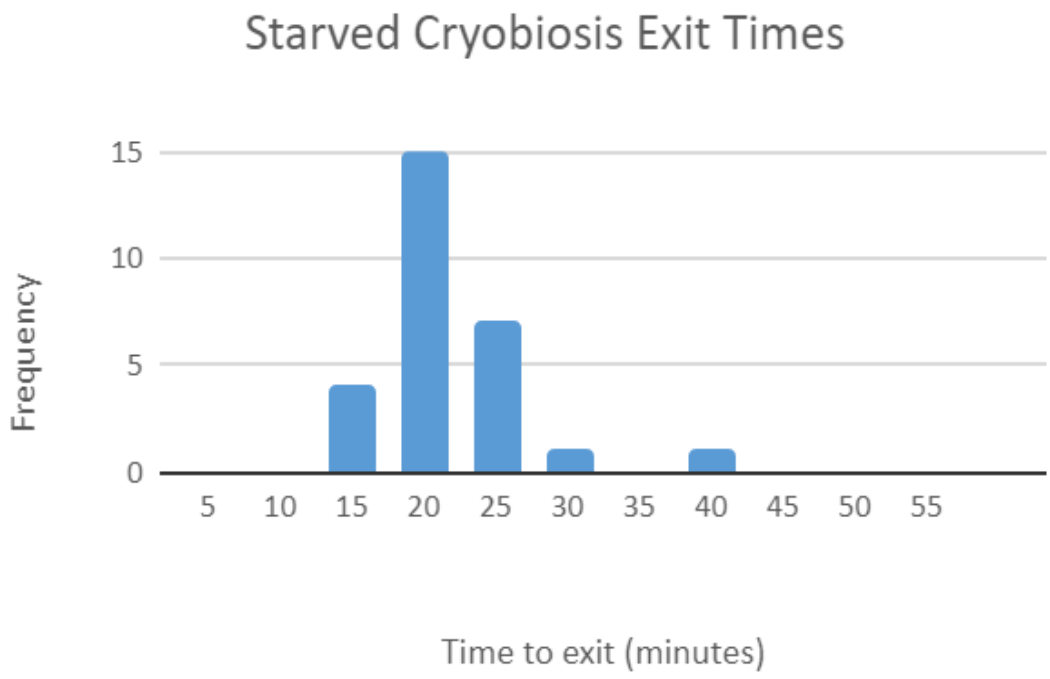


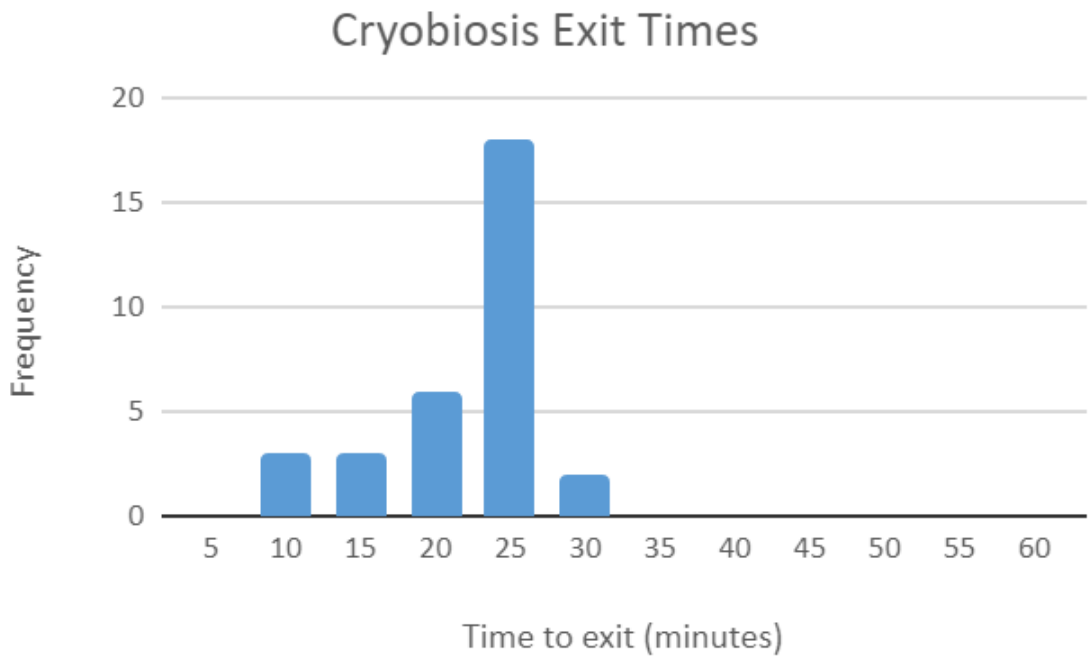
Figure 13

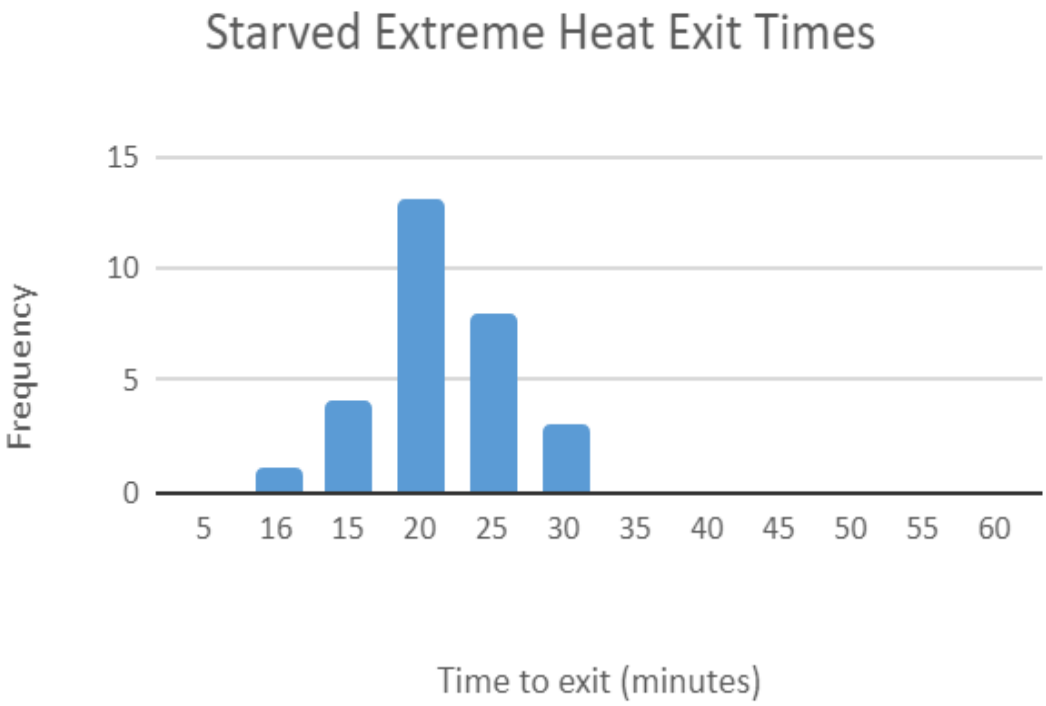
Level	Least Sq Mean	Std Error
Fed,Anhydrobiosis	39.909091	1.3521994
Fed,Cryobiosis	20.142857	1.3129968
Fed,Extreme Heat	16.700000	1.4181987
Starved,Anhydrobiosis	38.500000	2.7463299
Starved,Cryobiosis	20.107143	1.4679751
Starved,Extreme Heat	19.551724	1.4424431

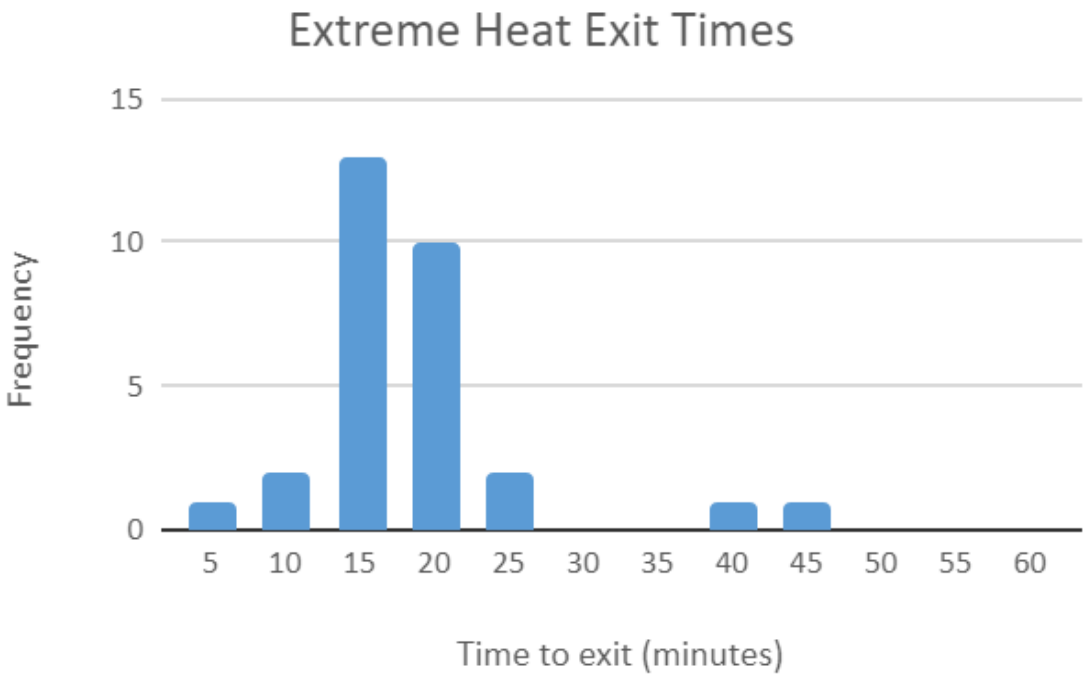
Level	Least Sq Mean	Std Error	Mean
Fed	25.583983	0.7862493	25.7449
Starved	26.052956	1.1439659	22.1231

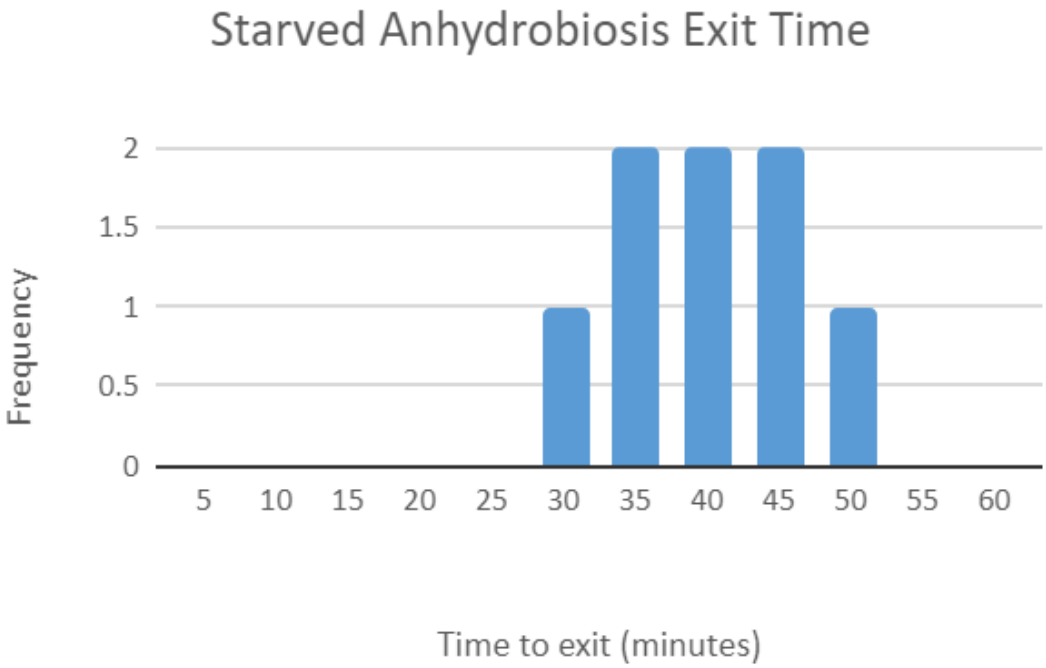
Figure 14

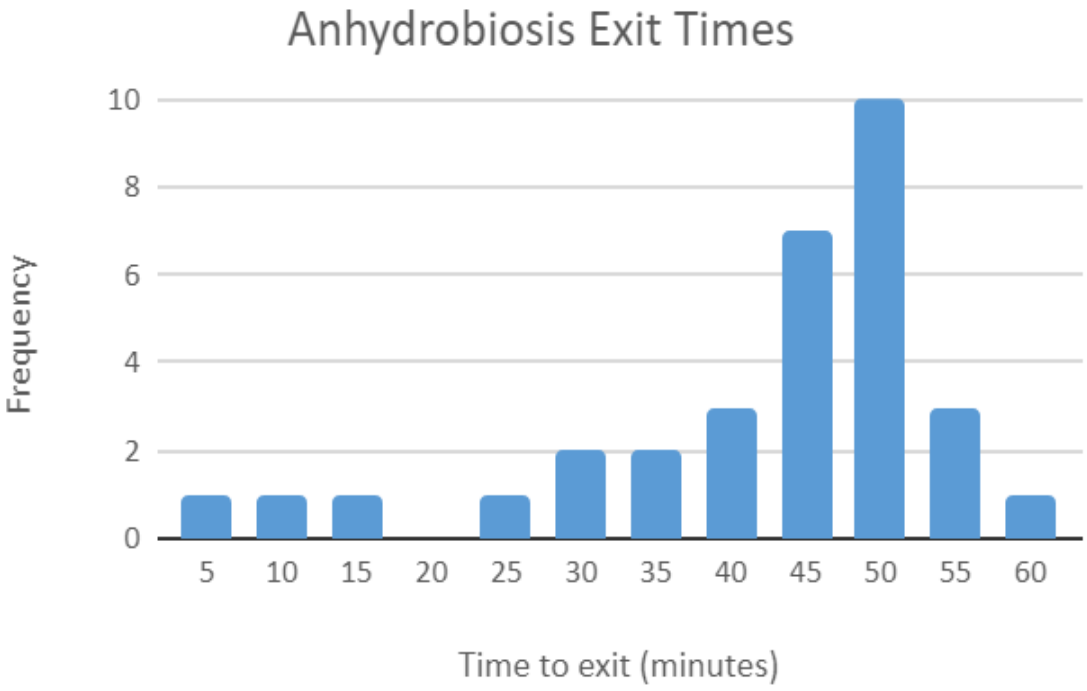

Starved Cryobiosis Exit Times
Frequency
15
10
5
0
5 10 15 20 25 30 35 40 45 50 55
Time to exit (minutes)


Cryobiosis Exit Times
Frequency
20
15
10
5
0
5 10 15 20 25 30 35 40 45 50 55 60
Time to exit (minutes)


Starved Extreme Heat Exit Times
Frequency
15
10
5
0
5
16
15
20
25
30
35
40
45
50
55
60
Time to exit (minutes)


Extreme Heat Exit Times
Frequency
15
10
5
0
5
10
15
20
25
30
35
40
45
50
55
60
Time to exit (minutes)


Starved Anhydrobiosis Exit Time
Frequency
2
1.5
1
0.5
0
5 10 15 20 25 30 35 40 45 50 55 60
Time to exit (minutes)


Anhydrobiosis Exit Times
Frequency
10
8
6
4
2
0
5 10 15 20 25 30 35 40 45 50 55 60
Time to exit (minutes)

Extreme Heat

As mentioned, it is not known if trehalose is an essential part of the extreme heat cryptobiotic state mechanism. The data yielded from this experiment suggest that it is not. This is an interesting conclusion given the fact that tardigrades survive extreme heat more successfully in their dessicated state than in their hydrated state. It can be seen in the data that neither the exit times nor the mortality rates have any significant changes in starved or normal conditions. This data supports the conclusion that trehalose is not an essential part of the extreme heat cryptobiosis mechanism.

Cryobiosis

Cryobiosis is the most understudied of all the cryptobiotic states. Thus, it is not known if trehalose is a part of the cryobiosis mechanism or not. Unlike with the extreme heat state, in which it is known that the organism is more likely to survive if it is dessicated (utilizing trehalose), there are no data relating to the connection between cryobiosis and anhydrobiosis in tardigrades. While extreme heat yielded a small difference in mortality rates between the two conditions, cryobiosis gave nearly identical values for both mortality rate and exit time. This supports the conclusion that trehalose is likely not an active part of the cryobiosis mechanism.

Anhydrobiosis

The findings in this study of the anhydrobiotic state strongly supported previous reports. Much research has already been done to support the important role of trehalose in the dessicated state of tardigrades, and this study further supports the importance of this role. Unlike the other states, which yielded similar exit times and mortality rates both while fed and starved, organisms in the anhydrobiotic state were less likely to survive

without available glucose stores. In fact, a substantial difference was seen, with the survivorship rate dropping from 67.2% when fed to 20.5% when starved. On the other hand, the exit times for the two groups were very similar. Thus, lacking glucose seems to effect the tardigrades' ability to create trehalose and enter the anhydrobiotic state, but does not seem to affect the mechanism underlying this state among survivors.

Chapter 6: Implications and Possible Continuing Work

Additional work could include further statistical analysis of the data collected in these experiments. While quite a bit of statistical analysis was done with this data, as can be seen throughout this paper, it is likely much more could be done. All contributors to this thesis were biology based in their interests. Thus, a math or statistics professional's view of the data in this thesis would be extremely interesting and helpful.

Other additional work that could be done is a trehalose assay similar to the test done by a team at the University of Utah School of Medicine. The team gives a detailed outline of their process of trehalose quantification in the article cited in the references section of this thesis. An overview of their trehalose quantification assay is as follows:

> "Trehalose is quantified by an assay that is based on the protocol for measuring glucose levels ... The difference is that trehalase is added to the extract to digest the trehalose into free glucose, which can then be quantified by standard assays and compared to the background level of glucose present in the original sample" (Tennessen, 2014).

This assay would be helpful in exploring the mechanism responsible for the findings. One possibility for the increase in death rate during starvation conditions of anhydrobiosis is an inability of the tardigrades to make trehalose. If this is the case, a trehalose assay should reveal that the tardigrades who survived desiccation when fed would have a much higher trehalose concentration than the starved tardigrades who could not survive the dessication. Similarly, another trehalose assay could be done comparing the tardigrades in anhydrobiosis with those in cryobiosis or the extreme heat cryptobiotic state. It can be hypothesized that, because the starved tardigrades put under extreme cold and heat

conditions did not exhibit as great a drop in survival rate as the dessicated samples did, trehalose is not an active part of cryptobiosis in response to extreme heat and extreme cold. Unfortunately, this trehalose assay could not be performed due to time constraints.

While the human implications of these microscopic creatures and experiments done with them may not be obvious, these organisms do hold great scientific research importance. Aside from further understanding tardigrades' ability to survive such extreme conditions and the sense of accomplishment that comes from understanding such a complex process, is how these findings can be applied to human medicine and other fields of science. In his book, S*uspended Animation: The Research Possibility That May Allow Man to Conquer the Limiting Chains of Time*, famed chemist Robert Prehoda explains that "much of the future progress in cryobiology will depend on success in unravelling the secrets of how some of the simpler forms of life can survive a cessation in their metabolism for long periods of time" (1969). Cryobiology, the study of the effects of low temperature on living organisms, is just one extreme that can be further studied with the knowledge obtained about tardigrades (and other organisms capable of cryptobiosis) under adverse conditions. This knowledge can then be applied to other research fields, such as organ donations, food preservation in harsh climates, and perhaps even extending the human lifespan. If it can be understood how small-scale organisms can achieve cryptobiosis, humans may be able to replicate this on a larger scale. This could be used to save countless lives every year by preserving organs longer for the donation process, getting crops through bad seasons, and much more. As Prehoda states, these advancements are only possible if "man learns to duplicate through science what nature has achieved through evolution" (1969).

This thesis presents a more complete understanding of three ideas that could be central to future work with cryptobiosis in human medicine or other fields:

1.) The mechanisms between the different cryptobiotic states differ.

    Understanding this is essential to how these different cryptobiotic states will be approached in future research. The great drop in survivorship with a lack of glucose only for the anhydrobiotic state suggests that trehalose is an essential sugar only for the anhydrobiotic state and could be supported by replicating this experiment and performing a trehalose assay with the control and starved organisms. Additionally, the substantial difference in exit times between the different cryptobiotic states under "normal" conditions indicates that the mechanisms of these different states may be different, although they do not provide any evidence or explanation as to how they differ.

2.) The cryptobiotic states differ substantially in their exit times.

    Knowledge of the different exit times for each cryptobiotic state, specifically their relation to one another, could be valuable if cryptobiosis were to be utilized on a larger scale. For example, if an organ is put in a cryptobiotic state in order to preserve it for transplant, the surgeon may have an idea of how long it will take for that organ to be "ready" for transplant, depending on what cryptobiotic state is utilized.

3.) The salinity change is correlated with the exit time of the osmobiotic state.

    Similarly, if the osmobiotic state specifically is being utilized to preserve an organ (or other organism), then knowing what salinity is needed to

achieve osmobiosis with a given organism without killing it is essential. Additionally, precisely controlling the salinity appears to provide greater control over the exit time of the cryptobiotic state. This is another facet of this experiment that would be more effective and give more conclusive results to make this hypothesis if the experiment were replicated on a larger scale with more variable salinities.

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