Effect of salinity on food vacuole formation in *T. thermophila*

Tanner Carnegie, Katie Donohoe, Mirkka Puente, Sumeet Saini

Abstract

Tetrahymena thermophila are ciliated protozoans typically found in freshwater ecosystems. Their primary method for food consumption is through phagocytosis which results in the formation of food vacuoles. The objective of this study was to determine if an increase in salinity affects the formation of food vacuoles in wildtype T. *thermophila* and low vac. T. *thermophila* mutants. Wildtype and mutant cultures were introduced to three treatment groups of varying sodium chloride concentrations (0mM, 2mM and 50mM). The formation of food vacuoles was followed in ten minute intervals for a total of 60 minutes. Every ten minutes, a sample of the treatment group was removed and fixed. Food vacuoles of five, randomly selected cells were counted using a compound microscope at 400X magnification. A mixed-effects two-way ANOVA test was performed and significance was found between food vacuole formation and the genotype of the organism (p-value=0.0003). As such, it was concluded that there is a difference between wildtype and mutant T. *thermophila* in food vacuole formation. No significance was found between salinity level and food vacuole formation. Additionally, no interaction was found between genotype and salinity that affected food vacuole formation. These results show that T. *thermophila* have the ability to withstand some changes to salinity levels in their environment. This may be important as climate and anthropogenic changes continue to affect freshwater ecosystems by increasing the salinity levels. Understanding the full extent of this ability may help us to predict the degree to which T. *thermophila* can continue to thrive alongside these changes to enhance freshwater ecosystems and support salmon.

Introduction:

The importance of salmon in Canada can be seen in the role they play ecologically, economically, and culturally. Ecologically, we know that salmon are keystone species and play critical roles in their aquatic ecosystems, mainly by returning limiting nutrients and energy they gain from the ocean back to freshwater ecosystems (Gende et al., 2002; Hyatt and Godbout, 2000) and feeding more than 50 freshwater species (Willson and Halupka, 1995). This is especially important in Canada because many Northern freshwater and terrestrial ecosystems are nutrient limited, and adding said nutrients can increase productivity overall (Hilderbrand et al., 2004). Economically, salmon approximately amass a total of 550 million dollars per year in recreational fishing and about 300 million per year in commercial fishing in Canada (Gislason,

2017). Culturally, we also respect that salmon are traditionally intertwined with First Nations everyday life, whether that be as a food source, source of income, or whether that is their historical use of salmon in legends, arts and ceremonies, and the place it holds in First Nations cultural identity (Alfred, 2010).

Tetrahymena thermophila are a small ovoid or pear shaped protozoan ~ 30-60 μm x 50-100 μm in size and are uniformly covered in cilia. *T. thermophila* are typically found in freshwater environments (Matthews, 2004). *T. thermophila* form food vacuoles at the base of their buccal cavity upon ingestion of their food source via phagocytosis, or liquids via pinocytosis - this classifies them as filter feeders. Phagocytosis and pinocytosis are therefore imperative for their survival and longevity, as it is their primary route of nutrient and liquid consumption (Pinheiro et al., 2006). It is also important to consider the difference in phenotype between the low-vac *T. thermophila* mutant (TtVPS13AKOA4PA [low vac]) and the wild type *T. thermophila*. Which is essentially a lower amount of phagocytosis and less digestion of phagosome contents in the mutant (Samaranayke et al., 2011).

Putting all this together, we can look at the vital relationships that exist between *Tetrahymena thermophila* and salmon that enhance freshwater aquatic ecosystem. *T. thermophila* are a huge part of salmon food webs and chains since they are a food source for zooplankton, which in turn feed juvenile salmon (Eggers, 1978). Moreover, they make up biofilms alongside bacteria and other protozoa. These biofilms act as sponges and take up limiting nutrients derived from salmon such as nitrogen so that they are not lost to the surroundings (Freeman and Lock, 1995). Furthermore, *T. thermophila* also work to deactivate bacteriophage viruses that reduce bacterial population sizes (Pinheiro et al., 2006). Knowing that bacteria are a huge part of biofilms and that biofilms are really essential for ecosystem nutrient storage, we can really see the importance of this last function (Freeman and Lock, 1995).

Keeping these relationships in mind, and knowing that, one, humans are globally increasing the salt concentration of freshwaters (Argüelles et al., 2018), and second, that freshwater sources and resources are particularly vulnerable to inevitable climate change in terms

of their availability (Kundzewicz et al., 2008). Then it is imperative to investigate, for the future, the effects of increasing salinity on *T. thermophila* populations and if some variable adaptability exists within the current *T. thermophila* gene pool. This will allow us to see if there will be possible consequential impact this has on salmon in aquatic ecosystems that are so very reliant on *T. thermophila* in a multitude of ways (Eggers, 1978; Pinheiro et al., 2006; Freeman and Lock, 1995).

In the Expedition (2018) a similar study to ours exists by Ayre, Ng, Suh. In this study they tested the effect of varying salinity on the rate of food vacuole formation in *T. thermophila*, this served as the inspiration for our study and we looked to build upon it. They found that the number of food vacuoles produced by *T. thermophila* reduced with increasing salinity. They reasoned this as a result of the organism's decreased reproduction in high salinity conditions, which led to lower cell activity, and thus lower food vacuole formation in the cell. We noticed that nearly no food vacuoles formed at their 200mM NaCl concentration so we then made the decision to choose a lower concentration of 50mM as the paper had recommended to use for future studies.

We also decided to use *T. thermophila* low-vacuole mutant. This was a result of a peculiar observation from a paper by E.Orias and L.Rasmussen. It was found that a stressed no vacuole mutant of Tetrahymena was able to grow without food vacuole formation (1976). This was due to a secondary nutrient-obtaining system, rightfully named the surface uptake system. Using this system, the *Tetrahymena* cells appear to be capable of taking up, from solution in the medium, ten amino acids, a purine, a pyrimidine, seven vitamins and an incompletely determined list of inorganic ions, including Na+ and Cl-(Orias and Rasmussen, 1976). We wanted to know whether our low-vac mutant is also able to also use this surface uptake system and consequently be able to react and act differently in salinity than its wild type counterpart. This combined with the very limited research on the effects of salinity on rate of food vacuole formation in *T. thermophila* allowed us to arrive at our final proposal for our study and develop its hypotheses.

We decided to analyze the effect of varying NaCl Concentrations on the rate of food vacuole formation in both *T. thermophila* wild type and *T. thermophila* low-vac and established a set of three null hypotheses:

Null Hypothesis 1 (H_0): Food vacuole formation by *T. thermophila* will not be affected by changes in salinity.
Alternative Hypothesis 1 (H_a): Food vacuole formation by *T. thermophila* will be affected by changes in salinity.
Prediction 1: We expect that as salinity increases, food vacuole formation will decrease in *T. thermophila.* Past research has shown that food vacuole formation decreases under higher salinity conditions (Ayre et al, 2018).

Null Hypothesis 2 (H_0): There will be no difference between *T. thermophila* wild type and *T. thermophila* low-vac in food vacuole formation.
Alternative Hypothesis 2 (H_a): There will be a difference between *T. thermophila* wild type and *T. thermophila* low-vac in food vacuole formation.
Prediction 2: We expect *T. thermophila* wild type to have an overall higher food vacuole formation when compared to *T. thermophila* low-vac. We know that this is a characteristic of our mutant (Samaranayke et.al, 2011).

Null Hypothesis 3 (H_0): There will be no interaction between genotype and salinity on food vacuole formation in *T. thermophila.*
Alternative Hypothesis 3 (H_a): There will be an interaction between genotype and salinity on food vacuole formation in *T. thermophila.*
Prediction 3: We expect an interaction between genotype and salinity with respect to food vacuole formation. In higher salinity, we expect the mutant *T. thermophila* food vacuole formation to be even lower than typically expected (Samaranayke et.al, 2011) in comparison to the wild type *T. thermophila. T. thermophila* no-vac mutants do not produce food vacuoles and

under this stress, resort to a surface uptake system (Orias and Rasmussen, 1976). We believed that high salt conditions would induce a similar level of stress in our low-vac *T. thermophila* mutant. This would then cause a decrease in the rate of food vacuole formation to a degree that would mirror the no-vac *T. thermophila* mutant in the Orias and Rasmussen study.

Materials and Methods

Sample size:

The rate of food vacuole formation in the wildtype and mutant T. *thermophila* was measured at three sodium chloride concentrations (0mM, 2mM, and 50mM) at ten minute intervals as recommended by the Biology 342 lab technicians. Each concentration had three replicates and as such the sample size was three. The rate of food vacuole formation for the treatment group at each time point was the average of the three replicates.

The methods used throughout the experiment were inspired from a previous experiment published in the Expedition 2018 (Ayre et al., 2018).

Sample Preparation:

T. *thermophila* were cultured in SSP media supplemented with 2% proteose peptone, 0.1% yeast extract, 0.2% glucose and 33 μM $FeCl_3$ at pH 8. To make the dilutions, 0mM NaCl standard media with black dye and 100 mM NaCl standard media with black dye was provided in erlenmeyer flasks by the Biology 342 lab team.

To begin, all equipment was labelled based on organism, treatment group and replicate number (Figure 1). To grow the food vacuoles, 6mL test tubes with caps were used. For each organism type, nine test tubes were used.

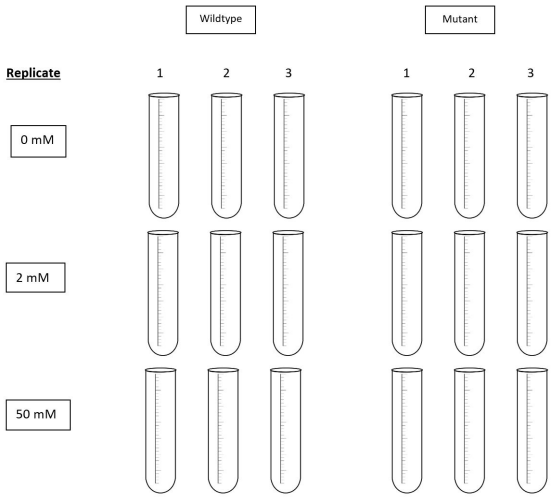

Figure 1. Set up for the test tubes used to grow the wildtype and mutant T. *thermophila*. Note each column is for a replicate and each row for a salinity.

Eppendorf tubes were used to look at the formation of the food vacuoles at each time point. As such, they were labelled and organized based on time, organism type, replicate and salinity (Figure 2). A total of 126 eppendorf tubes were used for the experiment. Once labelled, ten microliters of glyceraldehyde fixative was pipetted into each eppendorf tube.

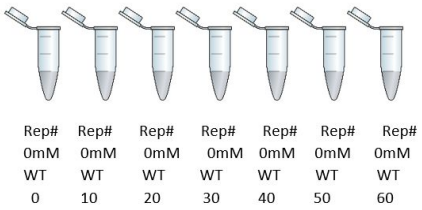

Figure 2. Set up for the eppendorf tubes for one replicate of the wild type T. *thermophila* at 0mM salinity.

To generate the different concentrations, the equation $C_1V_1=C_2V_2$ was used. The final volume for each test tube was 6 mL. For 0mM NaCl, the corresponding test tubes were composed of 5 mL of standard media with black dye and 1 mL of original stock. For 2mM NaCl, the corresponding test tubes were composed of 4.88 mL of the standard media with black dye,

0.12 mL of NaCl media with black dye and 1 mL of original stock. For 50mM NaCl, the corresponding test tubes were composed of 5 mL of NaCl media with black dye and 1 mL of original stock (Figure 3). All original solutions provided by the lab team were mixed by swirling the flask 3 to 5 times prior to pipetting into the respective test tubes. Once all cell volumes were added, a timer was set for ten minutes to allow for the cells to acclimate to the treatments.

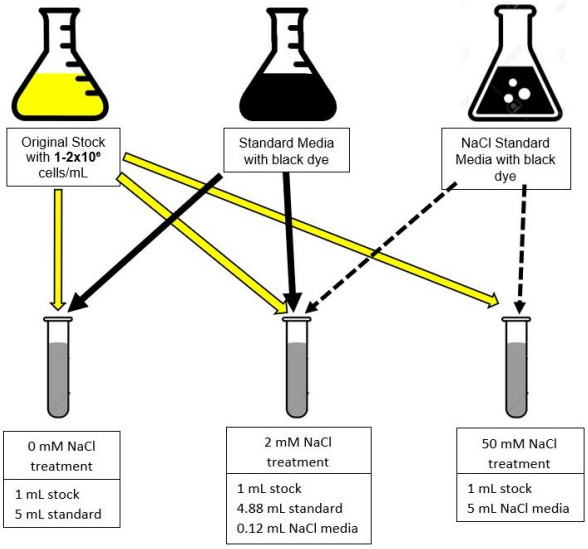

Figure 3: Composition of solutions for each treatment group.

Counting the Number of Food Vacuoles

The formation of food vacuoles were collected for a total of 60 minutes at a constant temperature of 20.2 degrees celsius. To measure the rate at which the food vacuoles formed, 100 microliters of each treatment group was pipetted into the appropriately labelled eppendorf tube every ten minutes. The sample was removed in the same order that cells were added when the treatment groups were made. Once ten minutes was finished, 100 microliters of live cells was pipetted from the test tubes. As recommended by the lab techs, the sample was drawn near the top of the solution to ensure that live cells were collected. The sample was then added to the appropriately labelled eppendorf tube depending on the treatment group, replicate number and time point (Figure 4). Upon adding to the eppendorf tube, the sample and the fixative in the

eppendorf tube were mixed. Note that during this process, the timer was not stopped. The process was repeated for 60 minutes.

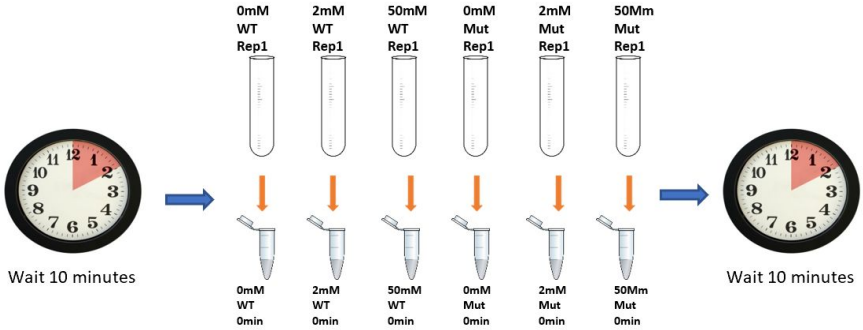

Figure 4. Steps for determining the number of food vacuoles formed at each time point. After ten minutes pass to allow food vacuoles to form, 100 microliters of sample is drawn from the test tube and placed in the correct eppendorf tube. This is repeated every ten minutes for sixty minutes. Note that figure 4 shows only one replicate. All replicates were performed at the same time.

To count the food vacuoles at each time point, ten microliters of cell solution was pipetted from the bottom of the eppendorf tube onto JohnsBrand microscope slides. GlobeScientific Inc. coverslips were placed on top of the sample. The slides were then viewed using an Axiostar compound light microscope and viewed under a lens of 40x with a total magnification of 400x. Five cells with food vacuoles were chosen at random. A cell was determined to have food vacuoles if solid black bodies were present within the cell. The average number of food vacuoles per cell was found by summing the number of food vacuoles found in the five cells and dividing by five. Figure 5 shows an example of the food vacuoles that form and the variation in their definition.

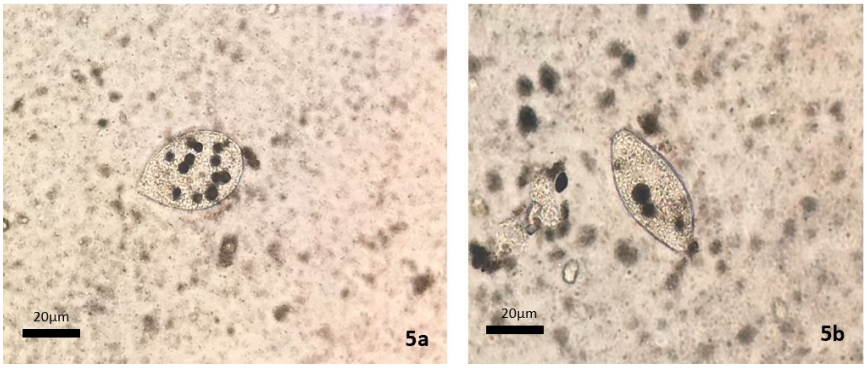

Figure 5: Examples of the food vacuoles that researchers identified and used for counting. Figure 5a shows more defined food vacuoles while figure 5b is an example of less defined food vacuoles.

Data Analysis

The number of food vacuoles per five cells was recorded every 10 minutes for each treatment and replicate. After the ratio of food vacuoles/cells was calculated, this ratio was plotted against each 10 minute interval time on Excel, resulting in a total of 6 graphs (Appendix 1). A linear equation was obtained from each group treatment and their slope was recorded to get the rate of food vacuole formation (in units of food vacuole number/(cell x time)). The rate of food vacuole formation was used as input data for the mixed-effects two-way ANOVA test. Overall, there were 3 replicates for each salinity treatment, giving a sample size n = 3.

To analyze our data, we decided to run a mixed-effects two-way ANOVA test because there was a missing value at the 50mM NaCl treatment group of the second replicate for the low food vacuole mutant. The mixed-effects two-way ANOVA test accounts for missing values by calculating a restricted maximum likelihood function for the data. Our independent variables were genotype (wild type and low food vacuole mutant) and salinity concentration (0mM, 2mM and 50mM), and our dependent variable was the rate of food vacuole formation.
The mixed-effects two-way ANOVA test was run on GraphPad Prism to determine whether or not there were significant relationships between genotype (low vacuole and wild type), salinity concentration and the rate of food vacuoles formed.

Results

The mixed-effects two-way ANOVA test resulted in three F critical values and their respective p-values. Overall, the sample size was n = 3.

Our first null hypothesis is that food vacuole formation by *T. thermophila* will not be affected by changes in salinity. For this, we got an F critical value of F(2, 6) = 0.999 and a p-value of 0.422. Therefore, we failed to reject the first null hypothesis.

Our second null hypothesis is that there will be no difference between *T. thermophila* wild type and *T. thermophila* low-vac in food vacuole formation. For this, we got an F critical value of F(1, 6) = 59.14 and a p-value of 0.0003. Therefore, we can reject our second null hypothesis.

Our third null hypothesis is that there will be no interaction between genotype and salinity on food vacuole formation in T. *thermophila.* An F critical value of F(2, 6) = 0.147 and p-value = 0.867 were obtained. Therefore, we failed to reject the third null hypothesis.

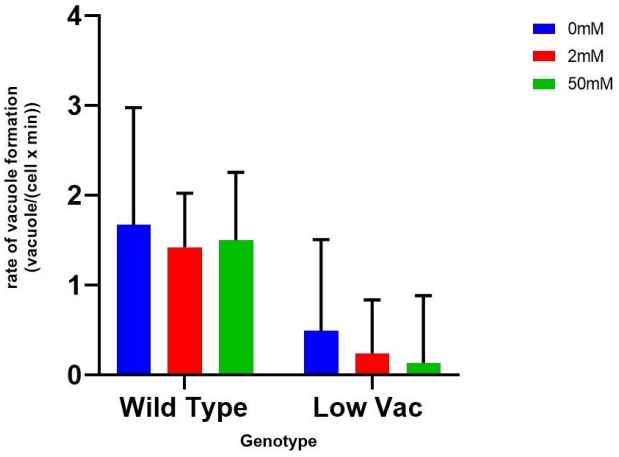

Figure 6. Means ~~(M)~~ for the rate of food vacuole formation in *T. thermophila:* wild type n=3, and low vacuole mutant n=3. The different colors represent the three salinity treatments (varies according to legend), and the error bars represent the 95% Confidence Interval (CI) of the means. F (1, 6) = 59.14 with P-value = 0.003, F (2, 6) = 0.99 with P-value = 0.422, and F (2, 6) = 0.15 with P-value = 0.867.

Figure 6 shows the mean of the rate of food vacuole formation for 0mM NaCl, 2mM NaCl and 50mM NaCl treatments in wild type *T. thermophila* to be M = 1.67 with 95% CI 1.67 ± 0.594, M = 1.42 with 95% CI 1.42 ± 0.274 and M = 1.50 with 95% CI 1.50 ± 0.34 respectively. Also, the figure shows the mean of the rate of food vacuole formation for 0mM NaCl, 2mM NaCl and 50mM NaCl treatments in low vacuole mutant *T. thermophila* to be M = 0.49 with 95% CI 0.49 ± 0.463, M = 0.24 with 95% CI 0.24 ± 0.272 and M = 0.13 with 95% CI 0.13 ± 0.301 respectively.

Discussion:

T.thermophila wild type has higher food vacuole formation than T.thermophila low-vac

We predicted that *T.thermophila* wild type would have a higher food vacuole formation when compared to *T.thermophila* low-vac. Our results confirm our prediction and we are able to reject our second null hypothesis, *T. thermophila* wild type do in fact have a higher food vacuole formation when compared to *T. thermophila* low-vac.

T. thermophila low-vac (TtVPS13AKOA4PA) are a mutant variant of *T. thermophila* with a low rate of phagocytosis and low digestion of phagosome contents (Samaranayke et.al, 2011). In our study, we observed that *T. thermophila* do indeed have lower phagocytosis compared to wild type *T. thermophila*. We do not have any contradictions to our literature analysis. Our study supports the previous research on food vacuole formation in *T. thermophila* low-vac by Samaranayke et al. (2011).

No interaction between T. thermophila genotype and salinity

We predicted for an interaction to exist between genotype and salinity with respect to food vacuole formation, in such a way that results in a lower food vacuole formation in *T. thermophila* wild type. However, our results do not agree with this idea because we failed to reject our third null hypothesis that there will be no interaction between genotype and salinity on food vacuole formation in *T. thermophila.*

T. thermophila are shown to have the ability to utilize a surface uptake system under the stress of no food vacuole formation, such as in a no-vacuole *T. thermophila* mutant (Orias and Rasmussen, 1976). This observation led us to our prediction that there would be an interaction between genotype and salinity. We believed the higher salt conditions would further decrease food vacuole formation in the already suppressed *T. thermophila* low-vac mutant when compared to wild type. The hope was that the *T. thermophila* low-vac would be induced into a stressed-state similar to the no-vac *T. thermophila* mutant in the Orias and Rasmussen paper due to high salt conditions (1976). From here it would begin to use the surface uptake system in lieu of food vacuoles, with perhaps even the possibility of beneficially utilising the additional NaCl as a source of inorganic ions (Orias and Rasmussen, 1976). However, our results disprove our theory that the mutant *T. thermophila* would behave differently than the wild type *T. thermophila* in terms of food vacuole formation in higher salinity conditions.

Salinity has no effect on T. thermophila food vacuole formation

We have intentionally saved the first hypothesis for last. We had predicted that as salinity increases, food vacuole formation would decrease in *T. thermophila.* Our results do not agree with our prediction. We were unable to reject the null hypothesis that food vacuole formation by *T. thermophila* will not be affected by salinity.

T. thermophila are typically found in freshwater environments where salinity is essentially zero (Matthews, 2004). Past research has shown that food vacuole formation decreases under higher salinity conditions (Ayre et al., 2018). Therefore, our results are peculiar because they do not agree with these sentiments. Rather, they may point to the idea that food vacuole formation in low salt environments (2mM and 50mM) is sustained and may occur to a similar degree as food vacuole formation in an environment with no salinity (Figure 6). The Ayre et al. study used a high salinity concentration of 200mM that seemed to cause Tetrahymena cell death and an impairment in cell reproduction in just 40 minutes of exposure. This consequently led to nearly no food vacuoles being formed (2018). We instead used a lower salinity level of 50mM that didn't cause this cell death or impairment and tested for 60 minutes total. In contrast, our results hint at this persistent ability in *T. thermophila* to form food vacuoles even under some low salt "stress" (Figure 6). This is definitely interesting and almost paradoxical considering *T. thermophila* are typically found in freshwater (Matthews, 2004).

Phagocytosis is imperative for *T. thermophila* survival and longevity, as it is their primary route of nutrient and liquid consumption (Pinheiro et al., 2006). Therefore our results tell us that *T. thermophila* are generally able to survive and tolerate being in low salt concentration environments since they can continue phagocytosis (Figure 6). This idea seems to be supported by the literature as well where adding low concentrations (1.71 mM-4.28mM) of reagent grade NaCl stimulated bacterivory via phagocytosis by *T. thermophila,* although the mechanism behind this survival is unknown (St. Denis et al., 2009).

We know anthropogenic effects and climate change are causing global freshwater bodies to become more saline (Argüelles et al., 2018; Kundzewicz et al., 2008). What our results then show is an adaptability that exists within the current *T. thermophila* gene pool to survive and continue phagocytosis normally in the presence of low concentrations of salt (2mM and 50mM). This tells us that there is a certain magnitude of buffer that *T. thermophila* have against changes in salinity in their global freshwater ecosystems. We also know that salmon and *T. thermophila* have vital roles in freshwater ecosystems that help to form the ecosystem's structure necessary

for its success as a whole, as well as the success of salmon specifically (Eggers, 1978; Pinheiro et al., 2006; Freeman and Lock, 1995). Knowing that *T. thermophila* have this buffer against salinity, we can be certain that even with some increasing global salinity of freshwater bodies, these relationships will remain intact. This is important because as we know the health of salmon is essential economically, ecologically and culturally in Canada (Gende et al 2002; Wyatt and Godbout, 2000; Willson and Halupka, 1995; Gislason, 2017; Alfred, 2010).

Limitations and Error

We counted food vacuoles that were well-defined and fully formed as well as food vacuoles that seemed to be in the midst of forming or partially formed. This could have led to an overestimation of food vacuoles in our experiment. However, we kept consistent with how and what we considered to be food vacuoles for our data. This overestimation does not play a huge part as an error that skews our results.

Part of our procedure was also a source of error. It is necessary to add the *T. thermophila* cell stock to our test tubes at the same time to mark the start of the experiment. However, we can only physically add cell stock to each test tube individually, one at a time, because we are doing it manually. So, we had different start times for the experiment for each test tube. This error was minimized by always extracting from each test tube at the 10 minute marks in the same one-by-one order we put the cells in to start the experiment.

When counting cells with clustered food vacuoles it may be difficult to determine the exact number of food vacuoles due to their overlap at different depths. This can be countered by adjusting the focus/depth. But, when the depth is changed, it is easy to miss vacuoles that are only one particular depth. This can be considered a limitation of our compound axiostar microscope because we see the images in something akin to a 2D-plane, rather than a full 3D image of the organism. A 3D image would provide a full picture of all the food vacuoles in the cell without having to worry about depth adjustments.

Another limitation of our study could be a small sample size, in the interest of time and completing our study we were only able to use three replicates per *T. thermophila* wild type and *T. thermophila* low-vac. Having more replicates would decrease the type II error present in our study as well as increasing the power of our test.

Additionally, we found difficulty in finding *T. thermophila* that had visible food vacuoles filled with dye. We noticed a large number of cells we came across under the microscope appeared to be completely devoid of any dyed food vacuoles. *T. thermophila* have a specialized region at the posterior end of their cells, known as the cytoproct, which mature phagosomes fuse with in order to expel their residual contents (Samaranayke et al., 2011). This could be a reason why no dye was observed in the food vacuoles of some of the cells we came across. This difficulty led us to using only 5 pseudoreplicates to come up with the averages shown in Appendix 3. Using a larger sample of cells to calculate the average number of food vacuoles is more likely to produce an accurate and representative mean of food vacuoles formation.

Future Studies

In the future, it would be wise to test beyond 50 mM NaCl to observe the degree that *T. thermophila* are able to "buffer" salinity concentrations where they continue to form food vacuoles, use phagocytosis for nutrient uptake and ultimately survive (Pinheiro et. al, 2006). Knowing the full extent of this buffer would be the first step in understanding the mechanisms behind this ability. Moreover, it will allow us to quantify the increase in global salinity of freshwater bodies that *T. thermophila* can cope with. This is important because it allows us to see to what degree of increase in global salinity of freshwater T. thermophila is able to withstand so that it may continue to support the freshwater ecosystems around them and salmon specifically. (Eggers, 1978; Pinheiro et al., 2006; Freeman and Lock, 1995). Ultimately, this is crucial because salmon play important economical, ecological and cultural roles in Canada (Gende et al, 2002; Wyatt and Godbout, 2000; Willson and Halupka, 1995; Gislason, 2017; Alfred, 2010).

Conclusion:

In conclusion, we had p-values of 0.422, 0.0003, and 0.867 for our first, second and third null hypotheses respectively. So, we cannot reject our first null hypotheses that food vacuole formation was not affected by changes in salinity. We cannot reject our third null hypothesis that there is no interaction between salinity and genotype on food vacuole formation. We were however, as predicted, able to reject our second null hypothesis that there is no difference between wild type and mutant *T. thermophila* in food vacuole formation. Most interestingly, food vacuole formation was not significantly affected by changes in salinity. This ultimately indicates that *T. thermophila* has a certain buffer and tolerance to some changes in salinity in the typical freshwaters they reside in. However, the full magnitude of this buffer as well as its mechanism are unknown.

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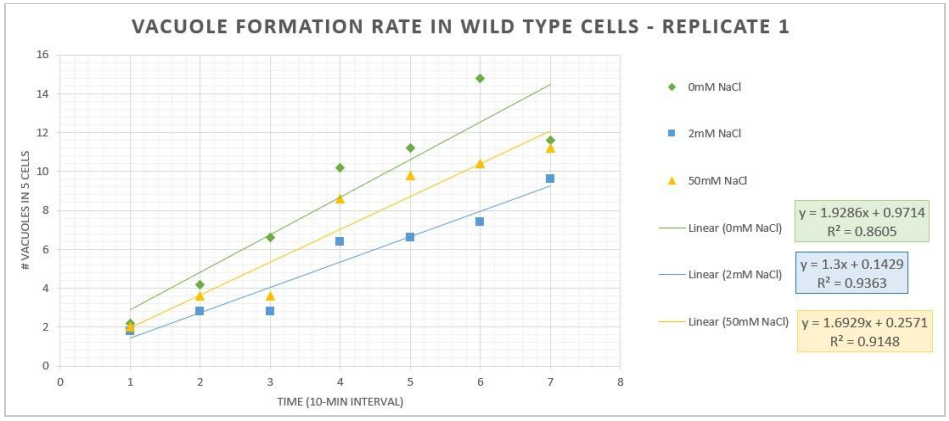
Appendix

A.



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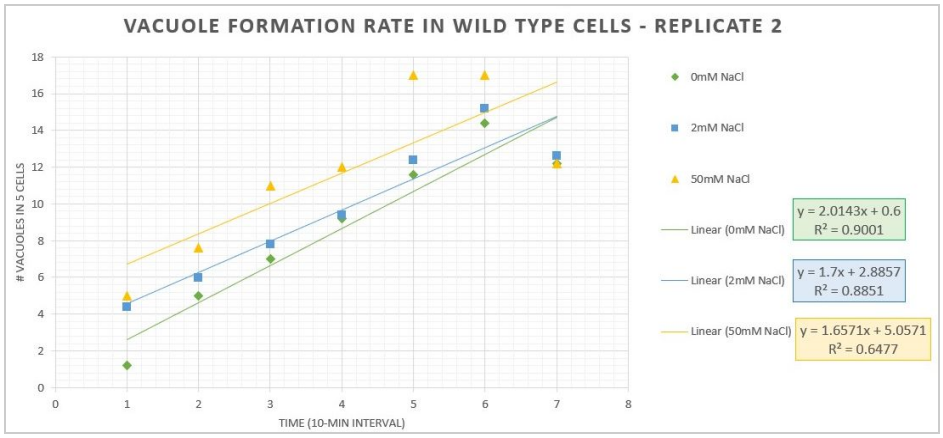

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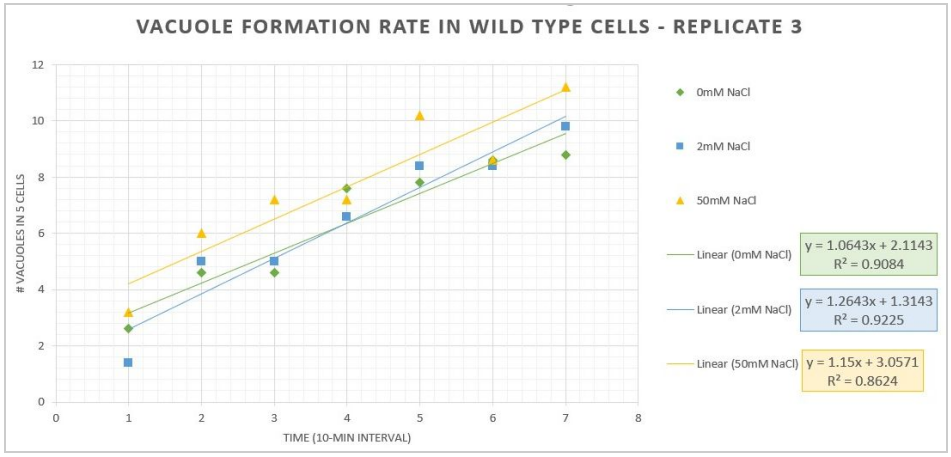

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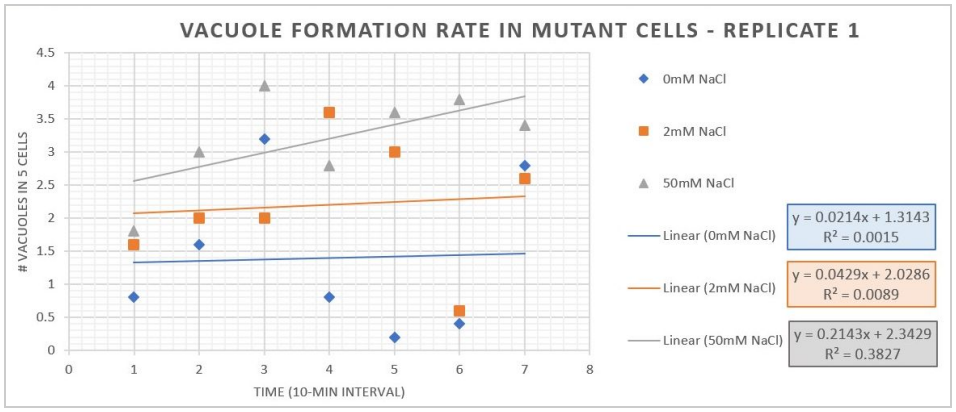

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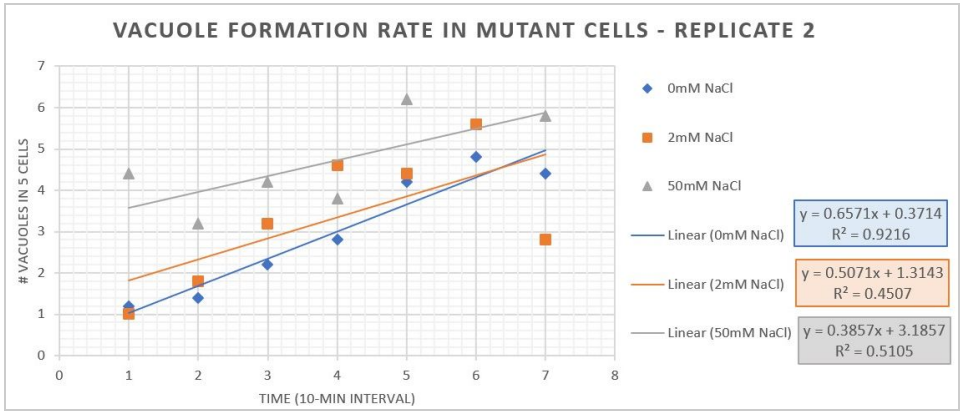

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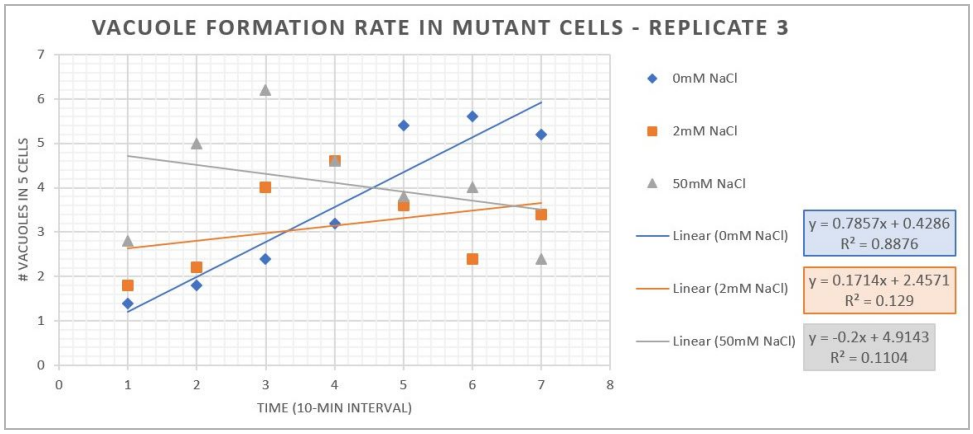

Appendix 1. Rate of food vacuole formation in *T. thermophila* at different salinity concentrations (0mM NaCl, 2mM NaCl, 50mM NaCl). Scattered points represent the number of food vacuoles per 5 cells at each timepoint (10 min interval). The slope of the trendline give the rate of food vacuole formation (food vacuole/(cell x time)). R^2 is the variance of the rate in units of food vacuole²/(cell x time)². **A.** Replicate 1 for wild type. **B.** Replicate 2 for wild type. **C.** Replicate 1 for wild type. **D.** Replicate 1 for low vacuole mutant. **E.** Replicate 2 for low vacuole mutant. **F.** Replicate 3 for low vacuole mutant.

ANOVA Table	SD	Variance	DF	F(DFn, DFd)	P Value
Genotype			1	F(1, 6)=59.14	P= 0.0003
NaCl Concentration (salinity)			2	F(2, 6)= 0.9999	P=0.4219
NaCl concentration X Genotype	0.07923	0.006277	2	F(2, 6)= 0.1466	P= 0.8666
Residual	0.3429	0.1176			

Appendix 2. Mixed-effects two-way ANOVA table. Notice that significance was only found between the genotype and rate of food vacuole formation.

Wildtype			
Replicate 1			
Time (Min)	0mM NaCl	2mM NaCl	50 mM NaCl
0	2.2	1.8	2
10	4.2	2.8	3.6
20	6.6	2.8	3.6
30	10.2	6.4	8.6
40	11.2	6.6	9.8
50	14.8	7.4	10.4
60	11.6	9.6	11.2
Replicate 2			
Time (Min)	0mM NaCl	2mM NaCl	50 mM NaCl
0	1.2	4.4	5
10	5	6	7.6
20	7	7.8	11
30	9.2	9.4	12
40	11.6	12.4	17
50	14.4	15.2	17
60	12.2	12.6	12.2
Replicate 3			

Time (Min)	0mM NaCl	2mM NaCl	50 mM NaCl
0	2.6	1.4	3.2
10	4.6	5	6
20	2.6	5	7.2
30	7.6	6.6	7.2
40	7.8	8.4	10.2
50	8.6	8.4	8.6
60	8.8	9.8	11.2
Average of Replicates			
Time (Min)	0mM NaCl	2mM NaCl	50 mM NaCl
0	2	2.53	3.4
10	4.6	4.6	5.73
20	6.07	5.2	7.27
30	9	7.47	9.27
40	10.2	9.13	12.33
50	12.6	10.33	12
60	10.87	10.67	11.53

Mutant			
Replicate 1			
Time (Min)	0mM NaCl	2mM NaCl	50 mM NaCl
0	0.8	1.6	1.8
10	1.6	2	3
20	3.2	2	4
30	0.8	3.6	2.8
40	0.2	3	3.6
50	0.4	0.6	3.8
60	2.8	2.6	3.4
Replicate 2			

Time (Min)	0mM NaCl	2mM NaCl	50 mM NaCl
0	1.2	1	4.4
10	1.4	1.8	3.2
20	2.2	3.2	4.2
30	2.8	4.6	4.8
40	4.2	4.4	6.2
50	4.8	5.6	DNE
60	4.4	2.8	5.8
Replicate 3			
Time (Min)	0mM NaCl	2mM NaCl	50 mM NaCl
0	1.4	1.8	2.8
10	1.8	2.2	5
20	2.4	4	6.2
30	3.2	4.6	4.6
40	5.4	3.6	3.8
50	5.6	2.4	4
60	5.2	3.4	2.4
Average of Replicates			
Time (Min)	0mM NaCl	2mM NaCl	50 mM NaCl
0	1.13	1.47	3
10	1.6	2	3.73
20	2.6	3.07	4.8
30	2.27	4.27	3.73
40	3.27	3.67	4.53
50	3.6	2.87	3.9
60	4.13	2.93	3.87

Appendix 3. Average number of food vacuoles found in cells at ten minute intervals for each treatment group. The values reported are the average of five pseudoreplicates at each time point. The average of the three replicates is provided for both the wildtype and mutant T. *thermophila.*