**Effect of Temperature on the Rate of Vacuole Formation in *Tetrahymena thermophila***

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**ABSTRACT**

*Tetrahymena thermophila* are protists that are primarily found in ponds near vegetated shores. *T. thermophila* undergo a physical transformation during feeding that involves the formation of vacuoles through phagocytosis. Since *T. thermophila* are involved in the freshwater ecosystem, and temperatures varies between location and season, it is is important to know whether temperature will affect *T. thermophila* feeding. We visualize feeding by using black watercolour that is incorporated into vacuoles while they form. We incubate *T. thermophila* in 3 different temperatures (13ºC, 30ºC, and 34ºC) to determine the rate of vacuole formation in temperatures under, in, and above their optimal range. We found that the rate of vacuole formation in not significantly different from each other in the different temperatures. This may be due to *T. thermophila* ability to correct oral abnormalities during development that may occur due to the temperature.

**INTRODUCTION**

*Tetrahymena thermophila* are heterotrophic, ciliated, unicellular protists that live in temperate freshwater environments, primarily in ponds and drainage streams (Doerder & Brunk, 2012). Like most ciliates, *T. thermophila* typically feed on microorganisms such as bacteria, algae, and yeasts, as well as organic debris. To feed, *Tetrahymena* use their cilia to sweep food into their oral groove, into their cell mouth, and finally into their "stomach", called a gullet. When the amount of food in their gullet reaches a certain size, it breaks off and forms a food vacuole (Gray et al., 2012). The food vacuole travels through the cell and fuses with a lysosome, where the food gets degraded slowly. This process is known as phagocytosis and it is essential for acquiring the nutrients necessary for growth (Gronlien et al., 2002). This process is non-specific for *T. thermophila*, so phagocytosis can be visualized by feeding the cells particles of black ink to effectively stain the vacuoles black for observation under a compound

microscope (Jacobs et al., 2006). The rate of phagocytosis in a cell can be quantified by counting the number of vacuoles in a defined time period (Bozzone, 2000).

These ciliates are key consumers of bacteria and influence the health of fish through their interactions with fish pathogens (Pinheiro & Bols, 2013). *T. thermophila* have been shown to phagocytose *Yersinia ruckeri* (Pinheiro & Bols, 2013), a pathogenic bacteria that can cause the often fatal Enteric redmouth disease in salmonids. With the earth's climate changing, it is important to study how temperature may affect the rate of *T. thermophila* vacuole formation to better predict the interactions between *Tetrahymena thermophila*, the bacteria and fish that reside in a temperate freshwater ecosystem.

*T. thermophila* reproduce asexually in nutrient-rich media and sexually by conjugation in the absence of sufficient food (Zufall, 2016). The optimal temperature for *T. thermophila* is between 27ºC and 32ºC when other factors besides growth are considered. At optimal conditions, their doubling time is about 2 hours (Cassidy-Hanley, 2012). The rapid reproduction of *T. thermophila* coupled with their non-selective feeding makes *T. thermophila* the ideal candidate to study vacuole formation on.

The objective of this experiment was to determine whether temperature has an effect on the rate of vacuole formation in *T. thermophila*. The temperatures this experiment investigated were 13ºC, 30ºC, and 34ºC. The control was 30ºC, as it is the closest to the optimal temperature for *T. thermophila*.

**Null Hypothesis ($H_0$):** Temperature has no effect on the rate of vacuole formation in *T. thermophila*.

**Alternative Hypothesis ($H_A$):** Temperature has an effect on the rate of vacuole formation in *T. thermophila*.

We predicted that the rate of vacuole formation will be highest at 30ºC because it is within the optimal temperature range for *T. thermophila*.

## METHODS

### Cell Culture

*T. thermophila* cell were cultured in SSP media supplemented with 2% proteose peptone 0.1% yeast extract 0.2% glucose 33 μM $FeCl_3$.

### Preparation

*T. thermophila* cell cultures were incubated for 24 hours at 13ºC, 30ºC, and 34ºC. To prepare cultures for each temperature condition, a 250 mL Erlenmeyer flask containing *T. thermophila* culture was swirled to evenly distribute the cells, and then 5 mL was aliquoted into three 10 mL test tubes, which were then placed in incubators set to the appropriate temperatures. After 24 hours of incubation, we added 3 mL of regular SSP media with black watercolour, which was prepared by adding 50 μL of "Lamp Black" watercolour paint from Art Advantage to 28 mL of SSP media, to the test tubes and incubated them again for 1 hour. Figure 1 graphically summarizes preparatory steps.
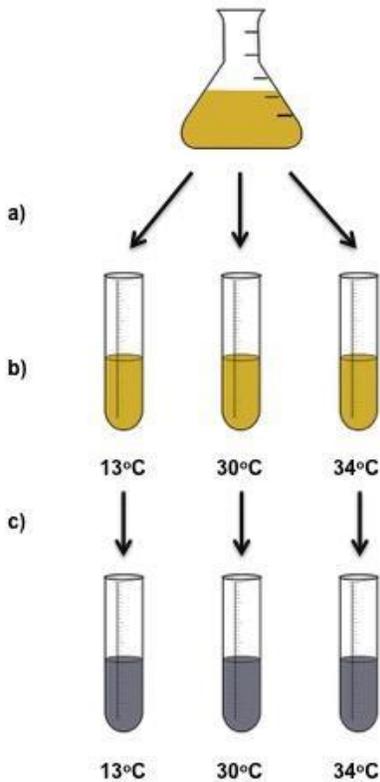
**Figure 1.** a) 5 mL of *T. thermophila* culture into three 10 mL test tubes, b) Each test tube was placed in an incubator at different temperatures (13ºC, 30ºC, and 34ºC) and allowed to incubate for 24 hours, c) 3 mL of SSP media with black watercolour was added to each test tube, and then placed back into their respective incubator for 1 hour.

**Sample Collection**

For each of the 3 temperatures, we transferred 200 µL of the incubated cells with watercolour into a 0.5 mL Eppendorf tube with 4 µL Glutaraldehyde every 10 minutes for 90 minutes, as shown in Figure 2. 2% Glutaraldehyde was added to fix cells so samples did not continue to feed after collection. Samples were taken from all 3 temperatures simultaneously.
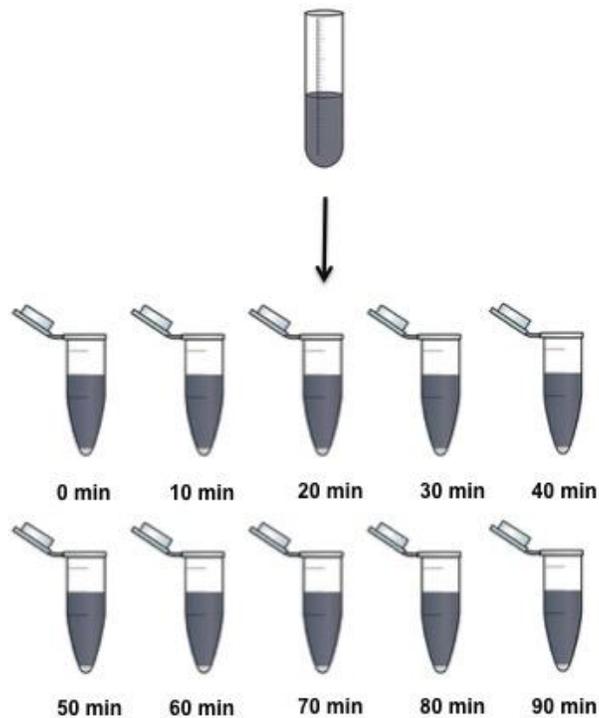
**Figure 2**. 200 µL from the culture was incubated every 10 minutes for 90 minutes. This was done for the temperatures at 13ºC, 30ºC, and 34ºC simultaneously.

**Vacuole Counting**

For each sample collected, 20 µL was transferred onto a microscope slide, and then viewed under a Zeiss Axiostar compound microscope. To obtain fixed cells, we pipetted from the bottom of the Eppendorf tube because fixed cells sink. First, 100X magnification in phase mode was used to find the cells on the slide, and then 400X brightfield was used to count the vacuoles. Five countable cells were randomly selected to count the number of vacuoles present. We randomized by first viewing the slide at the right edge, and moving left to a new field of view to find a new countable cell until we counted 5 cells. A countable cell, such as the one shown in Figure 3, was a cell that contained 1 or more stained vacuoles. A vacuole was counted when it was as dark as

the watercolour we used, and had a well-defined round shape. We adjusted the soft

focus to view different layers of the cells to ensure we counted all vacuoles within the
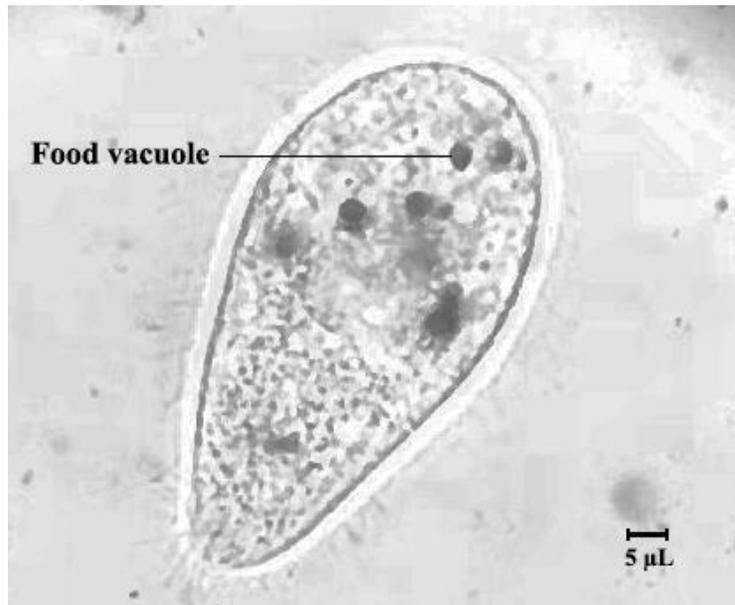
cell.



**Figure 3.** *T. thermophila* with black-stained food vacuoles, as viewed with a total
magnification of 400X. At this focus level, 4 vacuoles were counted.

**Data Analysis**

      For each temperature (13ºC, 30ºC, and 34ºC), the average number of vacuoles

per cell was determined for each time interval. After determining the average number of

vacuoles per cell, the data was plot against time on a scatterplot, and a linear trendline

was fit to find the rate of vacuole formation (Figure 4). Next, the average vacuole

formation rate for each temperature was calculated by combining the rates of 3

replicates. A one-way ANOVA was conducted using GraphPad to determine whether or

not the means of the vacuole formation rates were significantly different at different

temperatures. Figure 5 shows a graph of the mean vacuole formation rate at each temperature with error bars included so that we could compare the means +/- 95% CI.

**RESULTS**

The vacuole formation rates of replicates were found at each temperature by plotting the average number of vacuoles per cell against time and adding a linear regression line. The rate is visualized by the trendline and is expressed as the slope of the equation given in the legend (Figure 4). At the lowest temperature (13ºC), the vacuole formation rates were close to zero at 0.032, -0.008, and 0.014 vacuoles/cell/minute. At the median and highest temperature (30ºC and 34ºC), the vacuole formation rates were higher overall as shown by the increased slopes, reaching 0.071 vacuoles/cell/ minute in 30ºC and 0.064 vacuoles/cell/minute in 34ºC. The variance ranged from 0.027 to 0.194 (vacuoles/cell)$^2$/minute$^2$ in 13ºC, 0.028 to 0.339 (vacuoles/cell)$^2$/minute$^2$ in 30ºC, and 0.091 to 0.443 (vacuoles/cell)$^2$/minute$^2$ in 34ºC as indicated by the least squares value, R$^2$ (Figure 4). The variance was random in all replicates across all temperatures, as demonstrated by the varying distances of points above and below their respective trendlines.
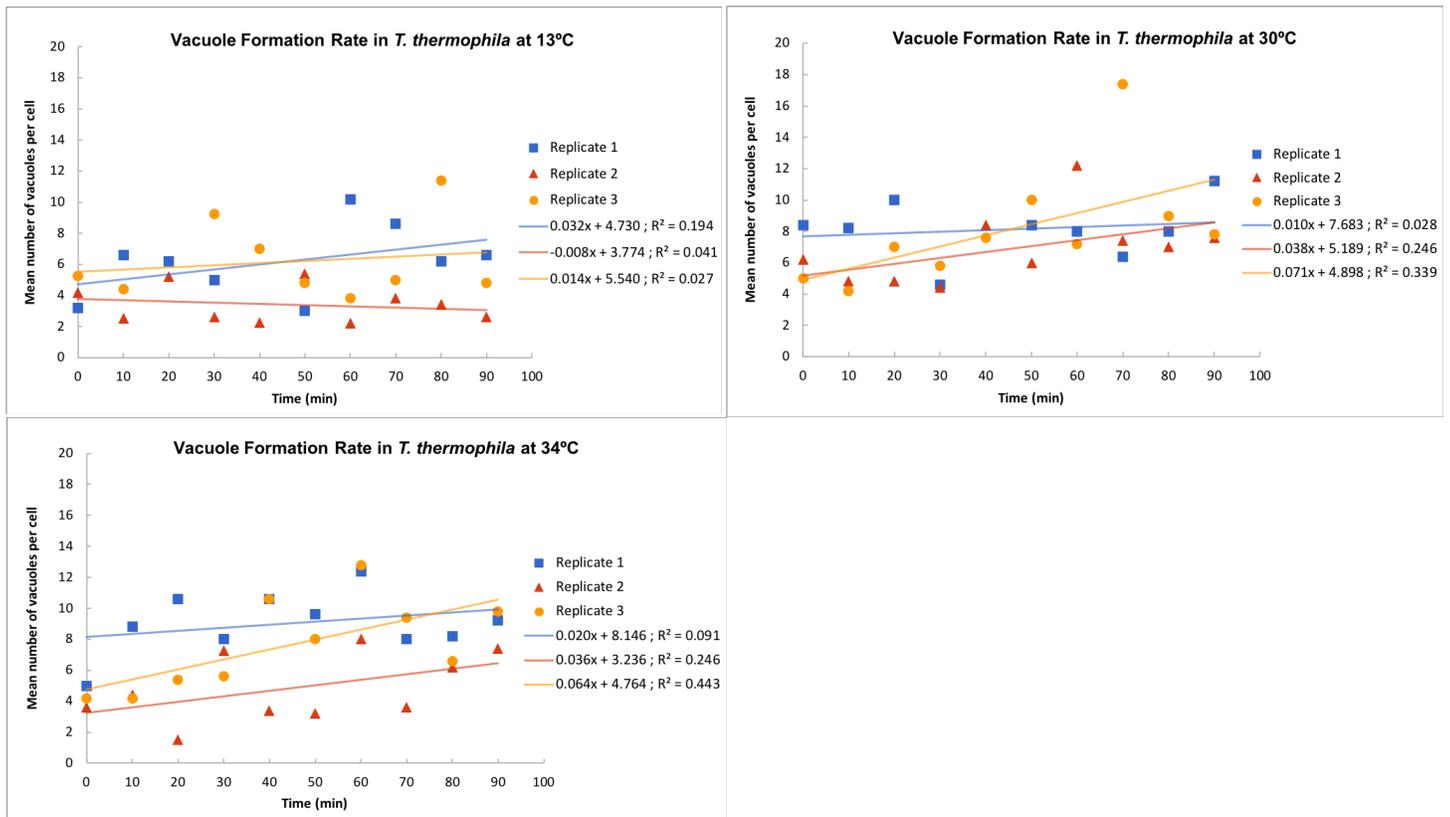
**Figure 4.** Food vacuole formation rates in *T. thermophila* at **a)** 13ºC, **b)** 30ºC, and **c)** 34ºC. Points represent the average number of vacuoles per cell (n=5) at each timepoint. The three points at each timepoint correspond to three replicates. The slope of the trendline gives the rate of vacuole formation while the R²-value gives the variance for the rate.

The vacuole formation rate was computed with 3 replicates at the three temperatures. Using 95% confidence intervals, the mean vacuole formation rate was 0.0124 ± 0.0493 vacuoles/cell/minute at 13ºC, 0.0396 ± 0.0762 vacuoles/cell/minute at 30ºC, and 0.0401 ± 0.0550 vacuoles/cell/minute at 34ºC. Figure 5 illustrates that at the median and highest temperature, vacuole formation rates were similar and were higher than the rate at the lowest temperature by nearly 400%. The intervals for the lowest and

highest temperature are similar and are smaller than the interval for the median

temperature by approximately ⅓. This indicates that there was the most variance in the

replicate measurements at 30ºC. The one-way ANOVA calculated the $p$-value to be

0.36, which shows that the mean vacuole formation rate is not significantly different

between the three temperatures for our data at the significance level α=0.05.
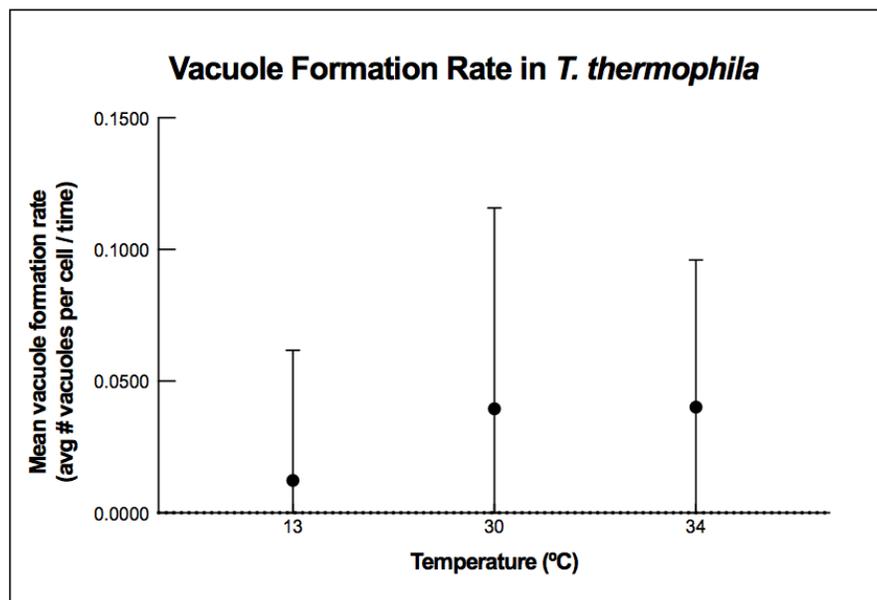


**Figure 5**. The mean vacuole formation rate in *Tetrahymena thermophila* at three different temperatures. Bars represent the mean ± 95% CI in average number of vacuoles per cell over time observed in 13ºC (n=3), 30ºC (n=3) and 34ºC (n=3). One-way ANOVA, $p$-value = 0.3595.


**DISCUSSION**

We failed to reject our null hypothesis since the vacuole formation rate at

different temperatures was not significantly different. Our findings suggest that

temperature does not have an effect on the vacuole formation rate in *T. thermophila*.

This further suggests that *T. thermophila* ingestion and digestion of food is similar across a range of temperatures.

Food vacuole formation indicates phagocytosis of nutrients that were swept into the cell mouth during feeding (Gray et al., 2012). Although the optimal temperature range for *T. thermophila* to thrive is small (27ºC-32ºC), our study shows that when temperature is not optimal, there is no impact on vacuole formation rate, which is an indicator of feeding. The rate of vacuole formation may be similar across all treatment temperatures because feeding is essential, so it would be necessary to survive, thus within their tolerance range, *T. thermophila* feed at similar rates, otherwise the cell would die.

In the *Tetrahymena* family, *T. thermophila* is the most resistant to high temperatures (Frankel & Nelson, 2001). In supraoptimal temperatures, oral abnormalities were infrequent and mild in *T. thermophila* because any abnormalities in oral primordia in the early stages of membrane formation were usually corrected during later stages of ciliary development (Frankel & Nelson, 2001). This correction, that may involve positional shift of structures or resorption of misaligned structures (Frankel & Nelson, 2001), may account for why supraoptimal temperatures do not affect vacuole formation. Furthermore, a study on mutant *T. thermophila* with defects in oral development resulted in food vacuoles being unable to form at supraoptimal temperatures after 2 days (Suhr-Jessen & Orias, 1979). Together, this suggests that oral development is integral to vacuole formation since the oral cavity is where nutrients enter the cell during ingestion, which leads to vacuole formation. It is plausible that there is no significant difference in vacuole formation between temperatures because *T.*

*thermophila* are able to correct any oral abnormalities that would affect feeding that may occur due to non-optimal temperatures.

Our results that indicate *T. thermophila* vacuole formation rate is not affected by temperature are not in agreement with previous research. In a study by Suhr-Jessen and Orias (1979), the food vacuole formation rate of *T. thermophila* was found to increase with increasing temperature. The rate increased from an average of 0.4 vacuoles/cell/minute at 22ºC to an average of 1.1 vacuoles/cell/minute at 40ºC. Studies published in different volumes of The Expedition also found an increase in vacuole formation rate with increasing temperature: Luan, Miller, Ngui, and Siddiqui (2013) saw an increase from 0.4 vacuoles/cell/minute at 12ºC to 2.8 vacuoles/cell/minute at 30ºC; Chan, Pinter, and Sohi (2017) saw an increase from 5.6 vacuoles/cell/hour at 25ºC to 11.5 vacuoles/cell/hour at 35ºC. The discrepancies in our results could be explained by differences in experimental methods, such as longer incubation time, different temperatures used, differerent sampling intervals, and different sampling duration.

We allowed for 1 hour incubation after the black watercolour was added, which provided enough time for the cells to ingest the watercolour and form vacuoles, but also enough time for metabolism of the vacuoles to start. Since the processing period for vacuoles to be egested is 45 minutes (Fok & Shockley, 1985), 1 hour was sufficient for vacuoles with watercolour to form and be digested. If vacuoles were formed at a similar rate to vacuoles being egested, the incubation time prior to collecting samples may account for the near steady vacuole formation rate across our 90 minute sampling time. Previous experiments (Luan et al., 2013; Suhr-Jessen & Orias, 1979) had shorter incubation time, 20 minutes, which is not sufficient time for both ingestion and digestion.

This difference in incubation time may be why our results differ; by sampling after a shorter incubation time, vacuoles formed during ingestion would be counted, without enough time for vacuoles to begin being egested, thus there would likely be a greater increase in vacuole count between sampling intervals compared to counting after a longer incubation period that allows for sufficient time for both ingestion and digestion, like we did in our experiment.

It is also possible that our results did not show significant differences in the vacuole formation rate between temperatures, while some literature did, because of data collection methods and their associated uncertainties, as well as variation in data analyses. Due to incubator locations, it was difficult to ensure that different treatments were incubated for equal times once we began sampling every 10 minutes. The 34ºC treatment was closest to the lab so it had an overall longer incubation time than the other treatments since we were able to return the sample to the incubator faster due to the shorter walking distance. Another source of uncertainty was the few data points for each timepoint. Vacuoles were counted in only 5 cells due to time constraints, but some samples had less than 5 cells, so only the number of cells present were counted. If we had looked at more cells or a wider range of temperatures, our results could have a more definitive trend. In data analyses, the variation from the mean number of vacuoles per cell propagated into calculating vacuole formation rates, and the variation in the best-fit line for the rates propagated into the calculation of the mean rates. The mean rates from only 3 replicates were then used to determine whether or not they were significantly different between temperatures. All of these sources of uncertainties and variation may have led us to commit a Type II error where our analyses show no

significant difference in mean vacuole formation rate at different temperatures, but they may actually be different in the true population.

Future studies should investigate the effect of incubating cells for different periods of time prior to adding watercolour to see if the time spent in the treatment temperature affects the cells, and whether in longer term than 24 hours if there is a difference in vacuole formation rates. Different temperature ranges should also be investigated to see if extreme temperatures have an effect on vacuole formation rates.

**CONCLUSION**

Our study suggests that temperature does not have an effect on *T. thermophila* food vacuole formation rate. Our data shows that vacuole formation rates were not significantly different at different temperatures, so we failed to reject our null hypothesis that temperature has no effect on the rate of vacuole formation in *Tetrahymena thermophila*.

**ACKNOWLEDGEMENTS**

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# APPENDIX

**Table 1.** Data collection and simple summary for 3 replicates at 13ºC. The number of vacuoles were counted in 5 cells every 10 minutes over a 90 minute period. Some cells had less than 5 cells so all vacuoles were counted in all cells present. The average number of vacuoles per cell at each timepoint was also computed.

Temperature: 13ºC

Replicate: 1

| | Number vacuoles in n-th cell | | | | | Avg # vacuoles / cell |
| Time | 1 | 2 | 3 | 4 | 5 | |
|---|---|---|---|---|---|---|
| 0 | 1 | 3 | 6 | 3 | 3 | 3.2 |
| 10 | 6 | 6 | 5 | 9 | 7 | 6.6 |
| 20 | 11 | 3 | 3 | 9 | 5 | 6.2 |
| 30 | 6 | 3 | 2 | 8 | 6 | 5 |
| 40 | | | | | | #DIV/0! |
| 50 | 6 | 3 | 2 | 1 | 3 | 3 |
| 60 | 12 | 9 | 9 | 20 | 1 | 10.2 |
| 70 | 1 | 2 | 15 | 15 | 10 | 8.6 |
| 80 | 10 | 7 | 2 | 4 | 8 | 6.2 |
| 90 | 20 | 3 | 6 | 1 | 3 | 6.6 |

Replicate: 2

| | Number vacuoles in n-th cell | | | | | Avg # vacuoles / cell |
| Time | 1 | 2 | 3 | 4 | 5 | |
|---|---|---|---|---|---|---|
| 0 | 8 | 6 | 3 | 3 | 1 | 4.2 |
| 10 | 1 | 4 | | | | 2.5 |
| 20 | 7 | 4 | 7 | 5 | 3 | 5.2 |
| 30 | 1 | 2 | 2 | 7 | 1 | 2.6 |
| 40 | 4 | 3 | 1 | 1 | | 2.25 |
| 50 | 4 | 6 | 6 | 8 | 3 | 5.4 |
| 60 | 1 | 1 | 2 | 6 | 1 | 2.2 |
| 70 | 7 | 1 | 1 | 4 | 6 | 3.8 |
| 80 | 1 | 6 | 7 | 1 | 2 | 3.4 |
| 90 | 2 | 2 | 2 | 3 | 4 | 2.6 |

Replicate: 3

| | Number vacuoles in n-th cell | | | | | Avg # vacuoles / cell |
| Time | 1 | 2 | 3 | 4 | 5 | |
|---|---|---|---|---|---|---|
| 0 | 2 | 6 | 3 | 10 | | 5.25 |
| 10 | 1 | 6 | 6 | 4 | 5 | 4.4 |
| 20 none | | | | | | #DIV/0! |
| 30 | 9 | 12 | 3 | 13 | | 9.25 |
| 40 | 3 | 13 | 2 | 4 | 13 | 7 |
| 50 | 6 | 3 | 2 | 8 | 5 | 4.8 |
| 60 | 1 | 12 | 1 | 3 | 2 | 3.8 |
| 70 | 8 | 14 | 1 | 1 | 1 | 5 |
| 80 | 16 | 18 | 8 | 13 | 2 | 11.4 |
| 90 | 4 | 2 | 1 | 16 | 1 | 4.8 |

**Table 2**. Data collection and simple summary for 3 replicates at 30ºC. The number of vacuoles were counted in 5 cells every 10 minutes over a 90 minute period. Some cells had less than 5 cells so all vacuoles were counted in all cells present. The average number of vacuoles per cell at each timepoint was also computed.

**Temperature: 30ºC**

**Replicate: 1**

| | Time | Number vacuoles in n-th cell | | | | | Avg # vacuoles / cell |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| | 0 | 13 | 1 | 2 | 11 | 15 | 8.4 |
| | 10 | 6 | 2 | 17 | 8 | 8 | 8.2 |
| | 20 | 6 | 13 | 14 | 14 | 3 | 10 |
| | 30 | 7 | 1 | 3 | 4 | 8 | 4.6 |
| | 40 | | | | | | #DIV/0! |
| | 50 | 15 | 8 | 6 | 4 | 9 | 8.4 |
| | 60 | 18 | 9 | 2 | 5 | 6 | 8 |
| | 70 | 7 | 10 | 6 | 3 | 6 | 6.4 |
| | 80 | 5 | 4 | 10 | 15 | 6 | 8 |
| | 90 | 6 | 17 | 5 | 9 | 19 | 11.2 |

**Replicate: 2**

| | Time | Number vacuoles in n-th cell | | | | | Avg # vacuoles / cell |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| | 0 | 5 | 9 | 6 | 10 | 1 | 6.2 |
| | 10 | 6 | 3 | 4 | 3 | 11 | 4.8 |
| | 20 | 1 | 5 | 8 | 4 | 6 | 4.8 |
| | 30 | 1 | 8 | 8 | 3 | 2 | 4.4 |
| | 40 | 10 | 2 | 11 | 8 | 11 | 8.4 |
| | 50 | 4 | 7 | 8 | 7 | 4 | 6 |
| | 60 | 12 | 12 | 20 | 13 | 4 | 12.2 |
| | 70 | 9 | 1 | 2 | 13 | 12 | 7.4 |
| | 80 | 1 | 5 | 8 | 6 | 15 | 7 |
| | 90 | 2 | 16 | 10 | 6 | 4 | 7.6 |

**Replicate: 3**

| | Time | Number vacuoles in n-th cell | | | | | Avg # vacuoles / cell |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| | 0 | 8 | 5 | 6 | 2 | 4 | 5 |
| | 10 | 7 | 2 | 3 | 6 | 3 | 4.2 |
| | 20 | 5 | 8 | 8 | 8 | 6 | 7 |
| | 30 | 5 | 7 | 5 | 6 | 6 | 5.8 |
| | 40 | 5 | 11 | 2 | 12 | 8 | 7.6 |
| | 50 | 13 | 10 | 5 | 10 | 12 | 10 |
| | 60 | 5 | 10 | 5 | 9 | 7 | 7.2 |
| | 70 | 7 | 10 | 11 | 31 | 28 | 17.4 |
| | 80 | 9 | 8 | 10 | 6 | 12 | 9 |
| | 90 | 5 | 7 | 9 | 7 | 11 | 7.8 |

**Table 3.** Data collection and simple summary for 3 replicates at 34ºC. The number of vacuoles were counted in 5 cells every 10 minutes over a 90 minute period. Some cells had less than 5 cells so all vacuoles were counted in all cells present. The average number of vacuoles per cell at each timepoint was also computed.

| Temperature: 34ºC | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Replicate: 1** | | | | | | | |
| | | Number vacuoles in n-th cell | | | | | |
| | Time | 1 | 2 | 3 | 4 | 5 | Avg # vacuoles / cell |
| | 0 | 2 | 4 | 8 | 5 | 6 | 5 |
| | 10 | 13 | 3 | 5 | 8 | 15 | 8.8 |
| | 20 | 12 | 14 | 9 | 9 | 9 | 10.6 |
| | 30 | 8 | 12 | 7 | 4 | 9 | 8 |
| | 40 | 11 | 9 | 7 | 13 | 13 | 10.6 |
| | 50 | 11 | 8 | 5 | 9 | 15 | 9.6 |
| | 60 | 24 | 9 | 1 | 19 | 9 | 12.4 |
| | 70 | 10 | 13 | 4 | 5 | 8 | 8 |
| | 80 | 5 | 2 | 11 | 11 | 12 | 8.2 |
| | 90 | 12 | 5 | 3 | 10 | 16 | 9.2 |
| **Replicate: 2** | | | | | | | |
| | | Number vacuoles in n-th cell | | | | | |
| | Time | 1 | 2 | 3 | 4 | 5 | Avg # vacuoles / cell |
| | 0 | 3 | 3 | 5 | 6 | 1 | 3.6 |
| | 10 | 4 | 5 | 6 | 1 | 6 | 4.4 |
| | 20 | 2 | 1 | | | | 1.5 |
| | 30 | 13 | 12 | 3 | 1 | | 7.25 |
| | 40 | 2 | 1 | 2 | 5 | 7 | 3.4 |
| | 50 | 2 | 4 | 6 | 1 | 3 | 3.2 |
| | 60 | 9 | 6 | 4 | 13 | 8 | 8 |
| | 70 | 1 | 4 | 8 | 3 | 2 | 3.6 |
| | 80 | 11 | 1 | 8 | 9 | 2 | 6.2 |
| | 90 | 4 | 6 | 16 | 8 | 3 | 7.4 |
| **Replicate: 3** | | | | | | | |
| | | Number vacuoles in n-th cell | | | | | |
| | Time | 1 | 2 | 3 | 4 | 5 | Avg # vacuoles / cell |
| | 0 | 2 | 1 | 9 | 4 | 5 | 4.2 |
| | 10 | 6 | 2 | 6 | 1 | 6 | 4.2 |
| | 20 | 12 | 4 | 2 | 8 | 1 | 5.4 |
| | 30 | 10 | 6 | 7 | 4 | 1 | 5.6 |
| | 40 | 22 | 4 | 8 | 5 | 14 | 10.6 |
| | 50 | 15 | 11 | 4 | 1 | 9 | 8 |
| | 60 | 2 | 11 | 21 | 17 | 13 | 12.8 |
| | 70 | 14 | 13 | 8 | 9 | 3 | 9.4 |
| | 80 | 3 | 9 | 9 | 5 | 7 | 6.6 |
| | 90 | 6 | 8 | 12 | 13 | 10 | 9.8 |