The effect of starvation on the rate of food vacuole formation in *Tetrahymena thermophila*

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ABSTRACT: The objectives of the study were to determine if starvation has an effect on food vacuole formation in *Tetrahymena thermophila* and also to see if the effect from starvation on food vacuole formation is different in the mutant versus the wild-type. We began by placing mutant and wild-type *T. thermophila* into starvation medium (10mM Tris-HCl pH 7.5) and regular culture medium for 17 hours, and then reintroducing both into nutrient medium (SPP). India ink (1%) was then added to both the mediums to make the food vacuoles more easily visible. Glutaraldehyde (1%) was added to samples taken at the given time intervals as a fixative and to make counting food vacuoles less difficult. Food vacuoles were then counted at 5 different time intervals. The mean rate of food vacuole formation found was 0.911±0.142, 1.406±0.257, 0.760±0.195 and 2.742±0.789 in starved wild-type cells, wild-type control cells, starved mutant cells, and for mutant control cells respectively. Our results suggest the controls have an increased mean number of food vacuoles over time compared to starved cells. Further, another trend seen was that the controls generally have a higher rate of food vacuole production compared to the starved wild-type and starved mutant as indicated by the mean rate of food vacuole formation. The three factors that contributed to the results include sexual immaturity, uptake via pinocytosis and the inefficiency of phagocytosis of the mutant. In conclusion, we found that with our data we reject our H_{O1}, H_{O2} and H_{O3} and provide support for our H_{a1}, H_{a2}, H_{a3}.

INTRODUCTION

Tetrahymena thermophila is a freshwater, free-living ciliate protozoa found in various climates almost anywhere around the world (Winey *et al.* 2012). It feeds via phagocytosis and has a sexual reproduction cycle of approximately twelve hours (Rodgers and Karrer 1985). The mutant strain used in our experiment is VPS13A::NEO and is also known as TtVPS13AA4PA (Cornell University 2014). This mutation is known as a knockout mutation that affects the *T. thermophila* vacuolar protein sorting 13 protein or TtVPS13A protein and is created by the complete replacement of the VSP13A gene (Samaranayake *et al.* 2011). Knocking out the gene

prevents the TtVPS13A protein from localizing to the phagosome membrane making phagocytosis inefficient (Samaranayake *et al.* 2011). The mutant also feeds via phagocytosis (Cornell University 2014).

Our objective in this research was firstly to learn if starvation has an effect on food vacuole formation in *T. thermophila*. Our second objective was to observe if there was a difference in the effect that starvation has on food vacuole formation in the mutant vs. the wild-type or if the cell type does not matter.

T. thermophila lose food vacuoles during reproduction and must be starved in order for reproduction to occur, therefore, this experiment was important in helping us get a visual understanding of how the food vacuoles are lost and why they are lost during the reproduction cycle as well as if a mutation has any effect on the duration of the reproduction cycle (Suhr-Jessen and Orias 1979). This experiment was also important in helping us understand that the mutation has no effect on the production of food vacuoles and that the mutant has the same response to being starved as the wild type.

Our first null hypothesis (H_{01}) for this experiment was: starvation has no effect on food vacuole formation in *T. thermophila*. Whereas, our first alternate hypothesis (H_{a1}) for this experiment was: starvation has an effect on food vacuole formation in *T. thermophila*. Our second null hypothesis (H_{02}) for this experiment was: presence of the mutation has no effect on food vacuole formation of *T. thermophila*. On the contrary, our second alternate hypothesis (H_{a2}) for this experiment was: presence of the mutation has an effect on food vacuole formation of *T. thermophila.* Our third null hypothesis (H_{03}) for this experiment was: the effect of

starvation on the food vacuole formation of *T. thermophila* is the same in wild type and mutant. Our third alternate hypothesis (H_{a3}) for this experiment was: the effect of starvation on the food vacuole formation of *T. thermophila* is not the same in wild type and mutant.

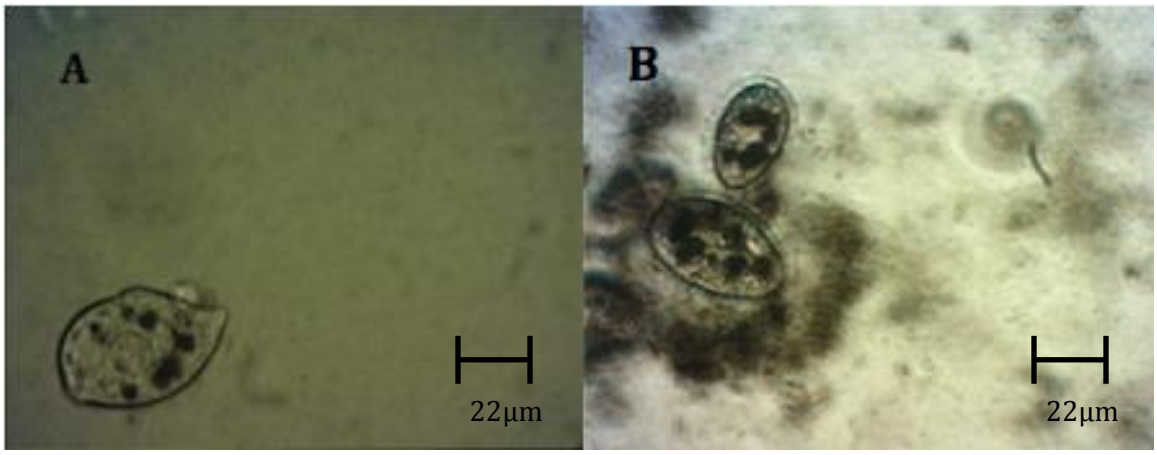

Figure 1. A) Mutant *Tetrahymena thermophila* after starvation B) Wild-type *Tetrahymena thermophila* after starvation at 60 minutes and at 400X magnification. Black dots represent food vacuoles that have absorbed India ink.

METHODS

Starvation

We began by placing both wild-type and mutant *T. thermophila* into 10mM Tris-HCl pH 7.5 approximately 17 hours before reintroducing them to their normal nutrient medium SSP. In order to do so we began by recording cell counts of both wild-type and mutant populations with a haemocytometer. Based on these cell counts we diluted (with the nutrient medium) the samples in order to have approximately the same number of cells in both the wild-type and the mutant. With four 15mL centrifuge tubes labeled as mutant (M), mutant control (Mc), wild-type (WT) and wild-type control (WTc) we prepared our samples. Once the samples

were similar in count, we placed 5mL of mutant sample into each M and Mc tubes and 5mL of wild-type sample into each WT and WTc tubes using sterile technique, then centrifuged all four tubes at maximum speed for 5 minutes.

T. thermophila will swim up in the medium after centrifugation so it was important to quickly decant, using micropipettes, as much liquid as possible from the centrifuge tubes. Next, we proceeded to add the starvation medium, with the use of plastic pipettes and pipettes, into both the wild-type and mutant tubes until the 7mL mark. For the controls, the same amount is added but of the normal nutrient medium. Once this was complete, we used new plastic pipettes, micropipettes and sterile technique to transfer the 7mL of each sample into labeled large glass tubes for storage over night. Lastly, the temperature at the bench was recorded.

Data Collection

After the starvation period we began by resuspending all tubes in a nutrient medium. Before doing so we performed cell counts again. Then, we pipetted, using sterile technique, the 7mL out of each glass tube and placed them into four new 15mL centrifuge tubes with appropriate labels and centrifuged at maximum speed for 5 minutes. Next, we decanted and resuspended all samples in the nutrient medium and India ink solution at a 1:1 ratio (India ink was already in a nutrient medium). Using the cell counts to calculate how much to dilute each sample, we performed serial dilutions to get approximately the same number of cells in each sample and prepared all samples to 9.6mL. We allowed all four samples to stay in the India ink solution for a minimum of 5 minutes before beginning data collection.

Four researchers prepared a sample of the M, Mc, WT, and WTc using sterile technique by extracting 2.3mL of sample into the labeled smaller glass tubes.

After 5 minutes we began timing the experiment. At each time interval of collection (for us it was at 5, 20, 40, 60 and 80 minutes) 100μL of each four samples was placed into a labeled microcentrifuge tube along with 10μL of 1% glutaraldehyde. Once the samples were fixed we could then prepare slides for counting. To do so, we extracted 20μL of each sample and placed it onto a welled slide (allowing approximately 30 seconds before adding the cover slide to allow cells to sink to the bottom). Using a compound microscope at 100x or 400x magnification, we located the first 10 visible cells and counted the number of food vacuoles present and averaged those 10 numbers. We also found the average size of the vacuoles in ocular meters. Once all data was recorded we performed appropriate two-way ANOVA test and 95% confidence intervals for statistical analysis.

RESULTS

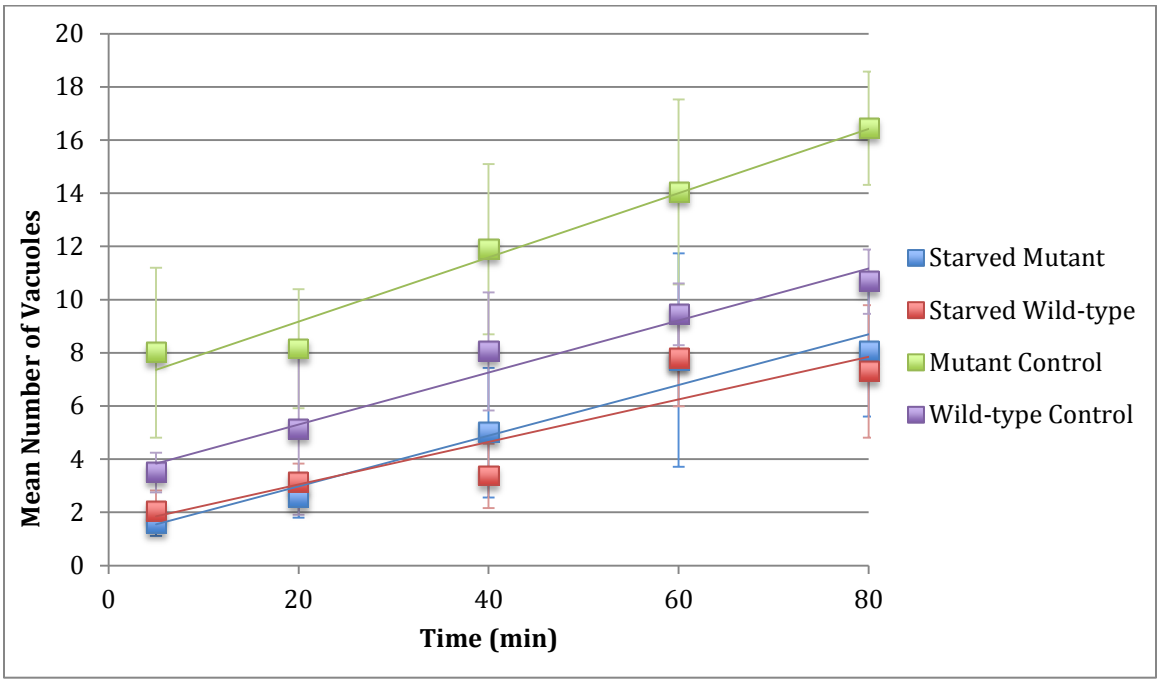

Figure 2. Mean number of food vacuoles in starved mutant, starved wild-type, mutant control, and wild-type control cells of *Tetrahymena thermophila* at 5, 20, 40, 60, and 80 minutes. Bars represent 95% confidence intervals, trend lines represent best-fit lines, n=4 for each time for each cell type.

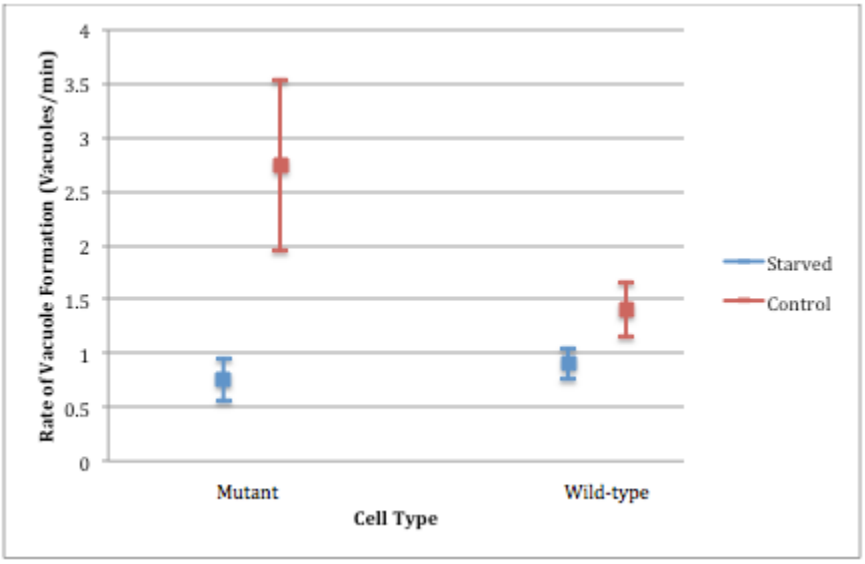

Figure 3. Rate of food vacuole formation (vacuoles/min) in starved mutant, mutant control, starved wild-type, and wild-type control cells of *Tetrahymena thermophila.* Bars represent 95% confidence intervals, n=4 for each cell type.

Figure 2 shows there is an increase in mean number of food vacuoles as time increases. A trend that is seen in Figure 2 is that the controls have an increased mean number of food vacuoles over time compared to starved cells. The starved mutant mean number of food vacuoles for 5, 20, 40, 60, and 80 minutes are 1.575±0.455, 2.550±0.759, 5.000±2.486, 7.725±4.097, and 8.050±2.501 respectively (Figure 2). The starved wild-type mean number of food vacuoles for 5, 20, 40, 60, and 80 minutes are 2.050±0.770, 3.125±0.704, 3.375±1.209, 7.925±1.802, and 7.300±2.488 respectively (Figure 2). The mutant control mean number of food vacuoles for 5, 20, 40, 60, and 80 minutes are 8.000±3.194, 8.150±2.238, 11.90±3.204, 114.05±3.479, and 16.45±2.130 respectively (Figure 2). The wild-type control mean number of food vacuoles for 5, 20, 40, 60, and 80 minutes are 3.500±0.746, 5.125±3.213, 8.050±2.221, 9.450±1.158, and 10.68±1.213 respectively (Figure 2).

Figure 3 displays the mean rate of food vacuole formation in starved mutant *T. thermophila* cells was 0.760±0.195 (vacuoles/min), and for mutant control cells, the mean was 2.742±0.789 (vacuoles/min, Figure 3). The mean rate of food vacuole formation in starved wild-type cells was 0.911±0.142 (vacuoles/min), and for wild-type control cells, the mean was 1.406±0.257 (vacuoles/min, Figure 3). There is a trend of the controls having an increased rate of food vacuole formation compared to the starved cells in Figure 3. In addition, Figure 3 shows that there is no overlapping of confidence intervals and there is a significant difference in the rate of food vacuole formation between starved wild-type and wild-type control cells and between starved mutant and mutant control cells. Figure 3 also shows no overlap of

confidence intervals and a significant difference between the mutant and wild-type controls. However, Figure 3 shows an overlap in confidence intervals and no significant difference between starved mutant and wild-type cells.

We reject our H_{O1} since we have a calculated p-value of 0.000112496, and Figure 2 also supports this. Further, we reject our H_{O2} because we have a calculated p-value of 0.019746297, and Figure 2 also supports this. We also reject our H_{O3} because the calculated p-value for this was 0.005534446.

DISCUSSION

From our results we are able to reject all three null hypotheses and provide support for the alternative hypotheses. After performing a two-way ANOVA test on the data, we obtained p-values of 0.000112, 0.019746 and 0.005534 for H_{O1}, H_{O2} and H_{O3}, respectively. All three of these calculated p-values are found to be statistically significant as they are ≤0.05 meaning there is a less than 5% chance the values are from the same population; this allows us to confidently reject H_{O1}, H_{O2} and H_{O3}. Following from the support of H_{a1}, we are able to say that starvation does indeed have an effect on food vacuole formation in *T. thermophila*. Furthermore, by rejecting our H_{o2} and H_{o3} we can firstly support that the presence of the mutation does have an effect on food vacuole formation and that secondly, the effect of starvation on food vacuole formation is not the same in the wild-type and mutant.

The effect of starvation on food vacuole formation is a decrease in number of food vacuoles formed. This is supported by the data in Figure 2 in which we can see that the starved mutant and starved wild-type both have lower mean numbers of

food vacuoles compared to the mutant control and wild-type control. Another trend that can support this effect and one that could possibly be the reason for the decrease in number of vacuoles formed can be seen in Figure 3. From Figure 3 we can see the rate of vacuole formation is significantly decreased in the starved mutant and starved wild-type compared to their respective controls. This could possibly be owing to the fact that *T. thermophila* begin the sexual reproduction cycle, also known as conjugation, when they are starved in at least one nutrient (Rodgers and Karrer 1985). After conjugation, the progeny are normally unable to mate again and are considered immature *T. thermophila* and must undergo 70-105 fissions before reaching maturity (Rodgers and Karrer 1985). During this immature, phase the cell has no food vacuoles formed which can be seen as the lag in the number of food vacuoles initially formed (Rodgers and Karrer 1985). Due to the fact the cell needs nutrients to undergo fission and successfully reach sexual maturity it may first prepare to divide before collecting nutrients. Another possibility may be that in the immature phase because they are not sexually mature the necessary components for efficient phagocytosis may also be underdeveloped.

Although our data support that the presence of the mutation has an effect on food vacuole formation, the effect is not what we expected. Our expectation was that the starved mutant and mutant control would have fewer food vacuoles at earlier times, compared to the starved wild-type and wild-type control. However, in contrast to our expectations, we observed that the mutant control had many more food vacuoles at earlier times than the wild-type control (Figure 2). Our reasoning was taken from Samaranayake and colleagues' (2010) work on our mutant strain

where it was found that phagocytosis and subsequently, food vacuole formation is partially affected in the mutants with VPS13A gene knockout. This is because the VPS13A protein, coded by the VPS13A gene, localizes to the *T. thermophila* phagosome membrane (food vacuole membrane) and is necessary for efficient phagocytosis (Samaranayake *et al*. 2010). It was found that the VPS13A protein actually remains associated with the phagosomes throughout the entirety of phagocystosis (Samaranayake *et al.* 2010). From the previous finding it is clear that this protein must play an important role in phagocytosis.

A very probable reason we see many food vacuoles in the mutant control is the fact they are taking up nutrients via pinocytosis. Pinocytosis is the receptor-mediated endocytosis of particles less than 0.5 μm in size, whereas phagocytosis is when particles larger than 0.5 μm are taken into the cell (Samaranayake *et al.* 2010). Our medium was not restricted in particle size and thus did not inhibit receptor-mediated pinocytosis by cells. Cells that have taken up small particles via pinocytosis have noticeably smaller food vacuoles when compared to particles ingested via phagocytosis (Figure 4). It would seem that the mutant had no challenges in forming food vacuoles compared to the wild-type and this can be attributed to the environment they were grown in. Samaranayake *et al.* (2010) states, that only under conditions where phagocytosis is essential, the mutant will show a delay in forming food vacuoles. Due to our conditions making phagocytosis inessential, the mutant forms food vacuoles through other endocytotic uptakes such as pinocytosis and grows normally. We can see that the food vacuoles formed by the mutants likely are through other pathways (most likely pinocytosis) as they are very

small, approximately 1.2 µm- 2.7 µm. The larger food vacuoles measured from 5.7µm- 11.8µm and are consistent with the size of phagosomes (Samaranayake *et al.* 2010).

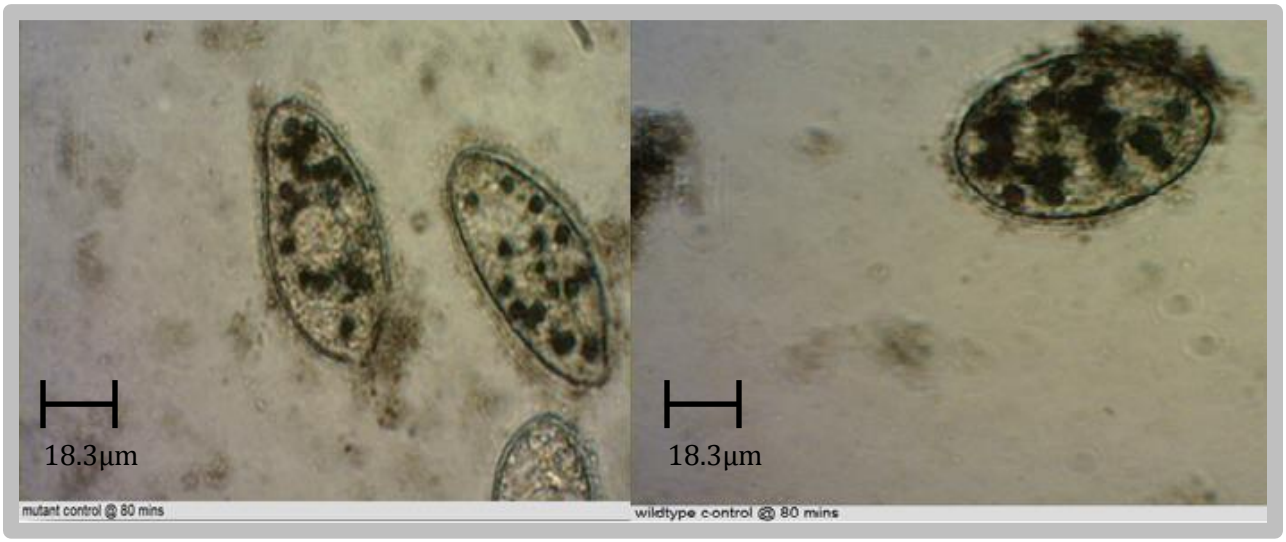

Figure 4. Mutant control (left) at 80 mins vs wild-type control (right) at 80 mins. Both at 400X magnification. The difference between the sizes of food vacuoles is noticeable when you compare the mutant control to wild-type control.

The effect of starvation on food vacuole formation is not the same in the wild-type and mutant and is supported by our data and Figure 2. From the figure, we see that the starved mutant has fewer food vacuoles compared to the mutant control; the same can be seen for the starved wild-type and wild-type control. Looking at the wild-type and mutant controls in Figure 2, we see that the mutant control has more food vacuoles at later times compared to the wild-type control; this was the expected effect. As mentioned earlier, under conditions where phagocytosis is essential, the mutants are slow growing and exhibit delayed food vacuole formation (Samaranayake *et al.* 2010). This would mean that the mutant

would have more food vacuoles at later times due to the inefficiency of phagocytosis. The wild-type undergoes a normal cycle of phagocytosis as it is not affected by the VPS13A mutation. The normal time to complete a cycle of phagocytosis is roughly 2 hours and as we near the end of the cycle, there are fewer food vacuoles as the cells begin ejecting the undigested materials (Samaranayake *et al.* 2010). As a result of the mutant's inefficient phagocytosis, their cycle becomes longer and we would then expect to see mutants at later times with more food vacuoles than wild type. While our conditions were not phagocytically restricted, we did see that there were more food vacuoles in mutants after 80 minutes than in the wild type; this can be seen in Figure 5 below.

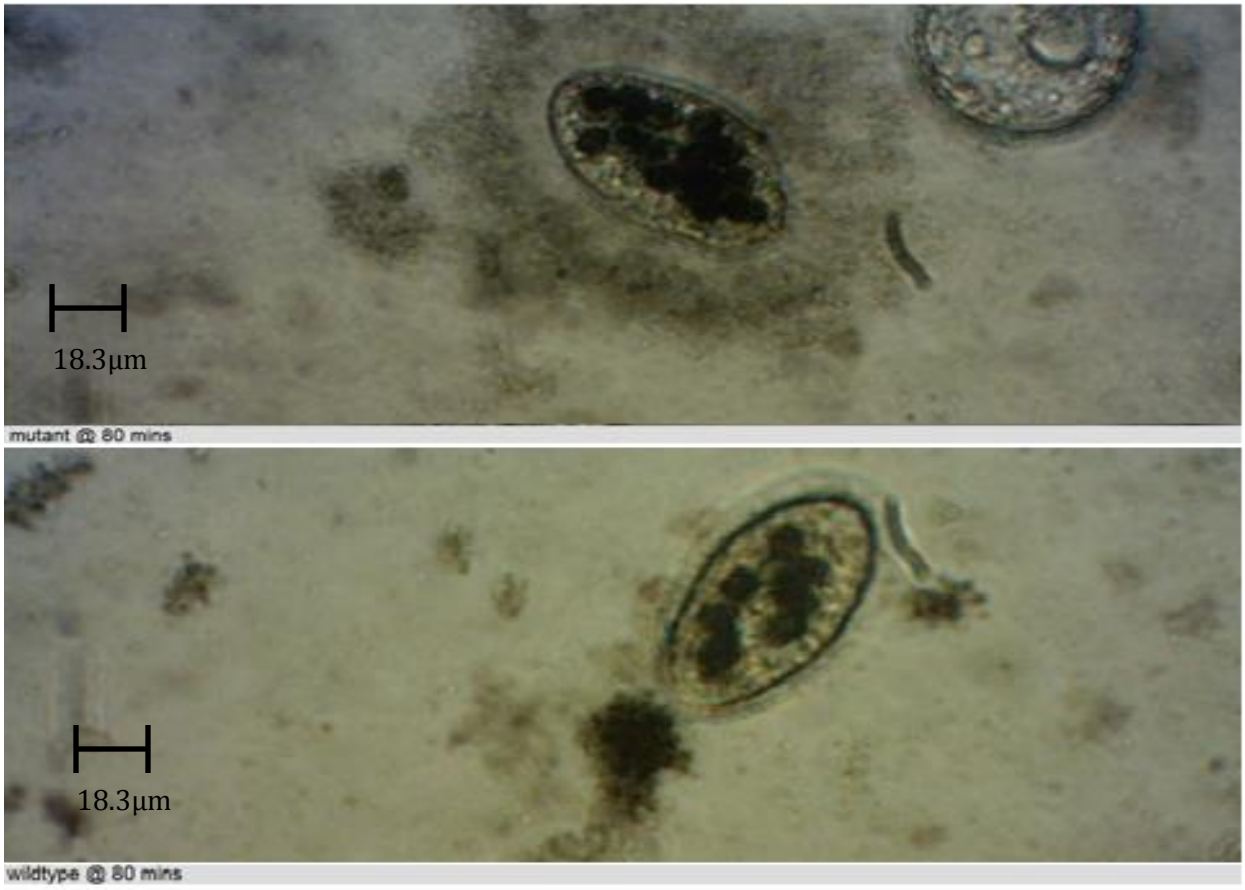

Figure 5. Starved mutant (top) vs. starved wild type (bottom) at 80 min, both at 400X magnification. The mutant has more food vacuoles present compared to the wild type. This was expected as the mutant is slower at digesting food and therefore has more phagosomes later.

Samaranayake's (2010) work is the most detailed on our mutant strain and in it she does use different methods and had a slightly different research direction; she wanted to see what the VPS13A gene was really responsible for. Some sources of error and variation may include the use of a medium where phagocytosis was not essential, as this may have given us different results altering our hypotheses. Another source of error is the improper counting of cells and which may be responsible for skewing the data as in counting immature cells, counting recently divided cells, miscounting or counting immature food vacuoles.

CONCLUSION

In summary we were able to reject our H_{O1}, H_{O2} and H_{O3} and support H_{a1}, H_{a2}, H_{a3} with our data. Firstly, we found that starvation does have an effect on food vacuole production in *T. thermophila*, as fewer food vacuoles are formed in the starved mutant and wild-type. Secondly, our data also supports that the presence of the VPS13A mutation does indeed have an effect on food vacuole formation; however, it was not the expected effect of the starved mutant and mutant control having fewer food vacuoles at earlier times. Thirdly, we are also able to support that starvation does not elicit the same response in the mutant and wild-type; with our data we can support that the mutant control has more food vacuoles at later times than the wild-type control. These findings show that under normal conditions

where phagocytosis is not essential, the mutant can form food vacuoles as efficiently as the wild-type.

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LITERATURE CITED

Rodgers, M.B., and Karrer, K.M. 1985. Adolescence in *Tetrahymena thermophila*. Proceedings of the National Academy of Sciences of the United States of America, **82**(2): 436–439.

Samaranayake, H. S., Cowan, A. E., and Klobutcher, L. A. 2011. Vacuolar Protein Sorting Protein 13A, TtVPS13A, Localizes to the *Tetrahymena thermophila* Phagosome Membrane and Is Required for Efficient Phagocytosis [online]. Eukaryotic Cell, **10**(9): 1207–1218. doi:10.1128/EC.05089-11.

Suhr-Jessen, P. B., and Orias, E. 1979. Mutants of *Tetrahymena thermophila* with temperature sensitive food vacuole formation: II. Physiological and morphological studies [online]. Experimental Cell Research, **124**: 317-327. doi: 10.1016/0014-4827(79)90207-6.

Tetrahymena Stock Center. [ ]. Cornell University [online]. Available from https://tetrahymena.vet.cornell.edu/display.php?stockid=SD01758 [accessed 24 October 2014].

Winey, M., Stemm-Wolf, A. J., Giddings Jr., T. H., and Pearson, C. G. 2012. Chapter 13 – Cytological Analysis of *Tetrahymena thermophile* [online]. Methods In Cell Biology, **109**: 357–378. doi: 10.1016/B978-0-12-385967-9.00013-X.