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

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Abstract

Phagocytosis and the formation of food vacuoles are essential processes involved in the growth and development of *Tetrahymena thermophila* populations. Temperature has an effect on both membrane fluidity and the rate of protein synthesis, which ultimately influence the efficiency of phagocytosis. We developed an experiment based on the nature of phagocytosis to investigate the effect of temperature on the rate of food vacuole formation in *T. thermophila.* In order to investigate this, samples of *T. thermophila* were placed in different temperature conditions and after two and five-hour acclimation periods were permitted to feed on yeast for one hour. The average number of food vacuoles formed was then counted in order to determine the rate of formation at each temperature. Our results suggest that the rate of food vacuole formation increases with both temperature and acclimation time. The rate of food vacuole formation after two hours of acclimation averaged between 5.6 and 11.5 vacuoles per hour as compared to 10.3 and 13.1 vacuoles per hour after five hours. Overall, it appears that both increasing temperature and environmental acclimation time contributes to an increase in the rate of food vacuole formation. The influence of temperature was not the same at the two acclimation-time conditions. Future research is required to confirm the effects observed as well as to provide appreciable insight into the mechanisms involved in these processes in *T. thermophila.*

Introduction

Tetrahymena thermophila belong to a group of unicellular ciliates, often measuring between 30 to 50 μm, which largely occupy freshwater environments (Collins & Gorovsky 2005). As in many unicellular organisms, *T. thermophila* have a specialized organelle on the surface of their plasma membrane utilized for the capture of particulate food matter and digestion (Orias & Rasmussen 1976). This is known as the oral apparatus, which is responsible for the ingestion of food particles greater than 0.5 μm in diameter. This process induces the formation of membranous phagosomes, also known as food vacuoles, for the digestion of such nutrients as illustrated in Figure 1 (Samaranayake, Cowan & Klobutcher 2011). *Tetrahymena* species have often been shown to demonstrate a non-specific feeding behaviour, as they not only phagocytize bacteria and yeast but will also ingest substances such as latex beads and India ink. This is a characteristic often leveraged in the laboratory to conduct

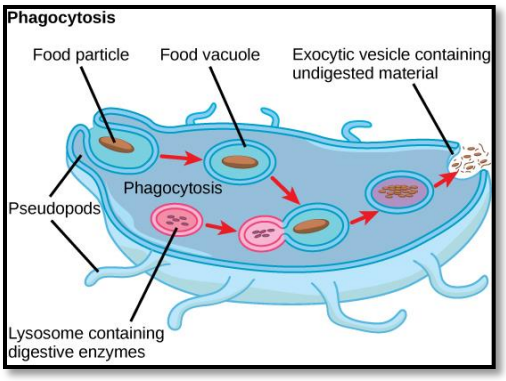

Figure 1. An illustration of the phagocytic process, including food vacuole formation, in *Tetrahymena* species (Taken from Mendel University in Brno).

studies on these organisms (Jacobs *et al.* 2006). Phagosome formation in these organisms is conditionally essential in environments that contain a great proportion of larger nutrient molecules. This is due to the fact that the uptake of nutrients such as amino acids, carbohydrates and nucleosides may be accomplished by pinocytosis across the plasma membrane when phagosome formation is inhibited (Orias & Rasmussen 1976; Rasmussen 1976). Despite this, it is thought that food vacuole formation is essential to the growth of the organism (Rasmussen 1973). Not only are these phagosomes responsible for obtaining adequate nutrients for efficient population growth, but this method of nutrient uptake has also been shown to increase the rate of population growth (Rasmussen 1973).

External environmental factors such as temperature, acidity and ion concentrations have been shown to greatly influence the process of food vacuole formation (Rasmussen 1973). As a result, we were interested in determining the particular effect that temperature would have on the formation of these phagocytic vesicles and whether or not the time spent at these temperatures would have an influence, as it is known that time has an effect on population size (Rasmussen 1973). The relationship between temperature and population growth is ultimately dependent on the efficiency of food vacuole formation in nutrient rich environments,

Ho_1: Temperature has no effect on the rate of food vacuole formation in *Tetrahymena thermophila.*
Ha_1: Temperature has an effect on the rate of food vacuole formation in *Tetrahymena thermophila*.

Ho_2: Time of incubation at a particular temperature has no effect on the rate of food vacuole formation in *Tetrahymena thermophila*.
Ha_2: Time of incubation at a particular temperature has an effect on the rate of food vacuole formation in *Tetrahymena thermophila*.

Ho_3: The effect of temperature on the rate of food vacuole formation in *Tetrahymena thermophila* is the same after two and five hours of temperature acclimation.
Ha_3: The effect of temperature on the rate of food vacuole formation in *Tetrahymena thermophila* is not the same after two and five hours of temperature acclimation.

Our model for the experiment, as depicted in Figure 2, is predicated on the assumption that the rate of food vacuole formation is an indication of the efficiency of phagocytosis in *T. thermophila*. Higher temperatures and greater intervals of time will create the conditions necessary for *T. thermophila* to efficiently carry out phagocytosis which will lead to an increased rate of food vacuole formation. In addition, varying temperatures influence characteristics of the plasma membrane, such as membrane fluidity and alter the abundance of relevant proteins to carry out phagocytosis. More specifically, increasing temperature (25°C-37°C) will contribute to increasingly greater rates of protein synthesis leading to increased rates of phagocytosis (Farewell & Neidhardt 1998). In combination, these changes are responsible for altering the dynamics of food vacuole formation (Farewell & Neidhardt 1998).

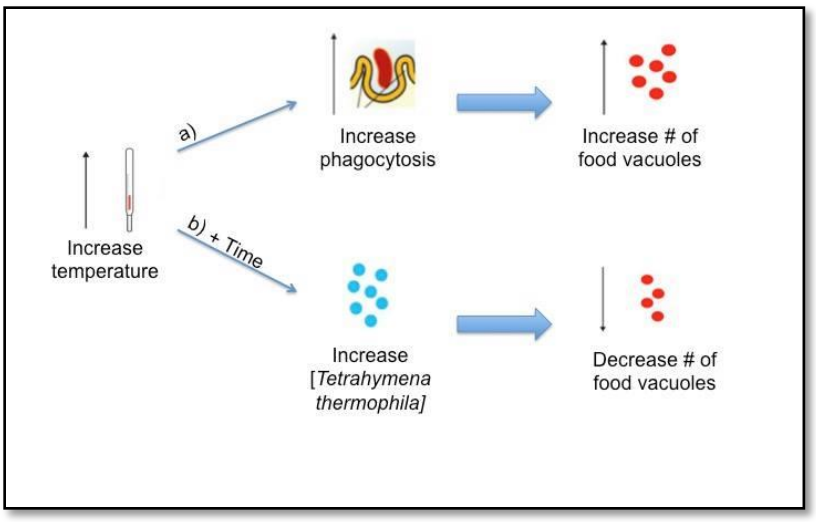

Figure 2. Model of the influence of temperature on the cellular mechanisms of *Tetrahymena thermophila* and the addition of time on the increase in concentration (population growth).

We predict that both temperature and acclimation time at a temperature will have an effect on the rate of food vacuole formation in *T. thermophila*. Specifically, we predict that the rate of food vacuole formation will increase with an increase in temperature, as previous research has indicated that exposure to higher temperature environments will lead to increased population growth, which is tightly linked to the efficiency of phagocytosis (Figure 2a) (Orias & Rasmussen 1976; Farewell & Neihardt 1998). In addition, we predict that time at the incubation temperature will have an effect on the rate of food vacuole formation in response to the doubling time of *T. thermophila*, which is between two and two and a half hours depending on environmental temperature (Orias & Rasmussen 1976). As a result, a greater concentration of cells will be present after five hours compared to the two-hour condition, which will result in greater competition for nutrients, as an assumption is being made that there is a limiting amount of yeast (Orias & Rasmussen 1976). In this case, we predict that the formation of food vacuoles will be reduced with an increased length of time spent at each temperature due to this increased competition for food particles (Figure 2b). Lastly, we predict that although the rate will differ with time, the temperature effect on food vacuole formation will be the same at each

time condition relative to one another, simply due to the influence of temperature as previously mentioned (Figure 2). The lack of scientific information regarding the function and behaviour of proteins involved in food vacuole formation in *Tetrahymena* species underscores the importance of this experiment and therefore these results have the potential to improve our understanding of the behaviour of phagocytic proteins in response to temperature and time (Jacobs *et al*. 2006).

Methods

A stock sample of *T. thermophila* was first obtained and thoroughly mixed in order to ensure an equal distribution of *T. thermophila* cells in solution to create samples of equal concentration. Sterile technique was then used to distribute the stock solution into 13 equivalent aliquots of 2 mL, which were placed in 6-mL test tubes. One sample was used to obtain baseline or control data at room temperature, while the others were allocated to each of three temperature conditions, 25°C, 30°C and 35°C, with four test tubes being placed in a water bath corresponding to each temperature (following step 2 in Figure 3) at a time denoted as zero. Simultaneously, test tubes containing Congo-red stained yeast were also placed in each of the three water baths to ensure that there was no difference in temperature between the *T. thermophila* and yeast solutions.

Room Temperature

A control treatment was conducted at room temperature (23°C), in order to assess the effect of increasing environmental temperature on food vacuole formation, with one of the 13 aliquots of stock solution. From the 2 mL sample, 500 μL were pipetted into four micro-centrifuge (mcf) tubes to create four replicates at this temperature. Following this, 500 μL of room temperature Congo-red stained yeast were added to each of the mcf tubes and gently mixed to ensure an equal distribution of yeast. With the addition of the yeast solution to the first replicate, a timer was started and yeast was

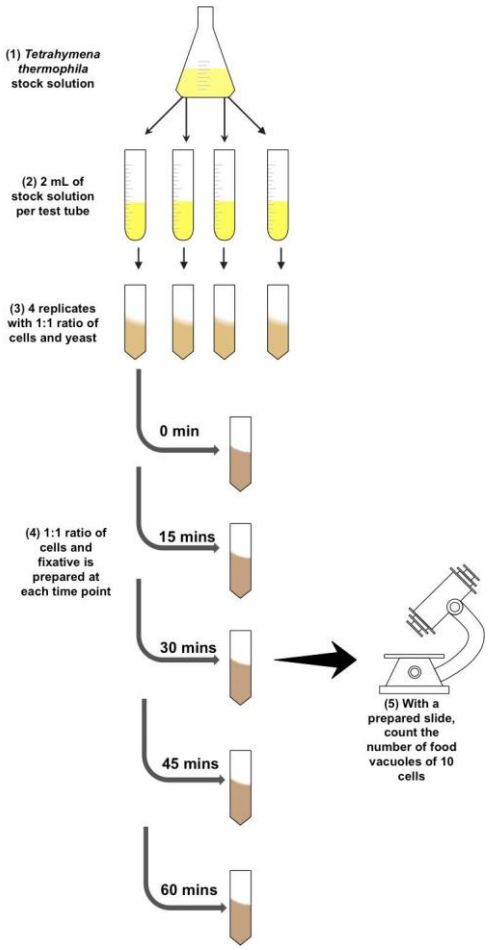

Figure 3. Illustration of methods used for each temperature treatment (25°C, 30°C, 35°C)

subsequently added to the remaining replicates every minute until each contained a sample of yeast. At 15-minute intervals, over the course of one hour, 100 µL samples were pipetted from each of the replicates, as outlined in Figure 3. Each replicate was sampled at a one-minute delay, in order to ensure that sampling consistently followed 15-minute intervals, and placed in a counting tube. In order to fix the solution, 10 µL of glutaraldehyde fixative were then added to each sample and these were stored in order to be counted at a later time.

Two and Five Hour Treatments

A similar procedure was conducted both two and five hours after the test tubes were placed in their respective water baths in order to ensure appropriate time intervals between samples, Congo-red stained yeast were again added in one minute intervals beginning with the 30°C water bath followed by 25°C and 35°C, respectively. The procedure followed was identical to that of the room temperature treatment in both the two and five-hour treatments, however the Congo-red stained yeast added were temperature appropriate in these treatments.

Counting—Concentration and Food Vacuoles

The concentration of *T. thermophila* in each sample was determined using a haemocytometer at time zero for each replicate at each temperature and time, totaling 27 samples—three at room temperature and four at each additional time and temperature. The number of food vacuoles present in 10 random cells in each sample was then determined by observing a sample of 20 μL of *T. thermophila* (with Congo-red stained yeast), at 400X magnification using an Axiostar compound microscope. Due to time constraints, the number of cells identified for counting food vacuoles was reduced to five in the latter portion of the experiment. The consistency of counting by each of the group members was established; we excluded any cells that did not possess clearly red-stained vacuoles.

Statistics

The number of food vacuoles in each replicate was averaged for each time interval, including 0, 15, 30, 45 and 60 minutes in Congo-red stained yeast, at each temperature and acclimation time. The data from 30-60 minutes were used in order to establish the rate of food vacuole formation per minute. A linear trend line was fit to the scatter plot, and the y-intercept was set to zero based on the assumption that no food vacuoles would be formed at time zero. The slope of each linear trend line thereby represented the rate of formation and was found for each of the replicates. The rates were then averaged and converted to a single rate per hour for each temperature and time condition. In order to test our hypotheses, a two-way ANOVA was then conducted with temperature and acclimation time as factors.

Results

The *T. thermophila* cells observed throughout the experiment varied greatly within each temperature treatment and after each acclimation time. As seen in Figure 4, a number of *T. thermophila* cells, identified in all treatment conditions, were in the process of dividing and did not contain stained food vacuoles. The morphology of non-dividing cells was variable, in both form and size as illustrated in Figure 5, however most of these cells contained stained food vacuoles. Lastly, it was noted that a number of cells appeared to possess food vacuoles that were not stained, as well as a mixture of stained and unstained food vacuoles, which were difficult to differentiate at times.

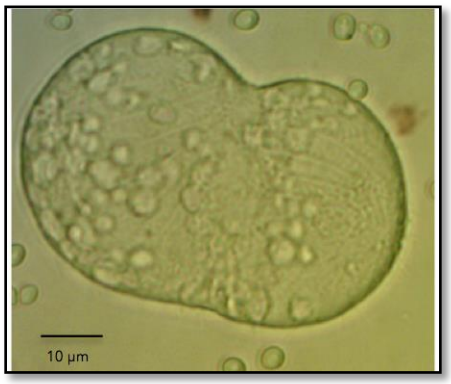

Figure 4. Dividing *T. thermophila* as viewed under 400x magnification after a two-hour acclimation period at 25°C.

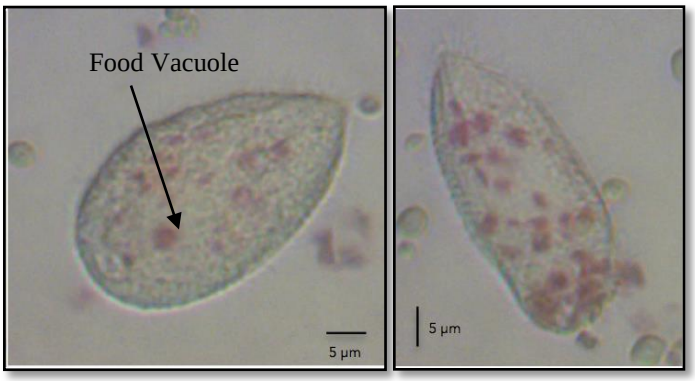

Figure 5. *T. thermophila* as viewed under 400x magnification after a five-hour acclimation period at 35°C, illustrating differential cell shape and the formation of food vacuoles.

Following a two-hour acclimation period at each treatment temperature, an increasing trend in the rate of food vacuole formation was observed with an increase in temperature, as seen in Figure 6. The average rate of formation per hour at this time was found to be 5.6 ± 0.9 (25°C), 8.5± 1.0 (30°C) and 11.5 ± 1.2 (35°C). These rates significantly differ with temperature, with an absence of overlap between confidence intervals at each temperature treatment and a p-value of $p=3.89x10^{-6}$.

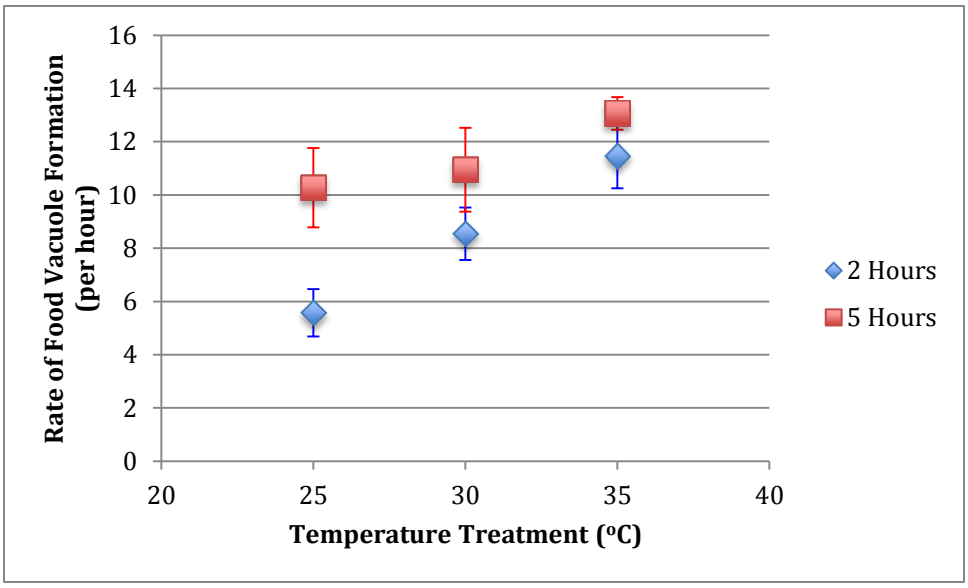

Figure 6. Mean number of food vacuoles formed per hour by *Tetrahymena thermophila* in response to increasing temperatures, following a two or five-hour acclimation period. Error bars represent 95% confidence intervals and n=4 for all treatments. Temperature $p=3.89x10^{-6}$; time at temperature $p=6.30x10^{-6}$; temperature and time at temperature p=0.02.

A similar trend was seen after a five-hour acclimation period, with an average rate per hour of 10.3± 1.5 (25°C), 10.9±1.6 (30°C) and 13.1± 0.6 (35°C) (Figure 6). The difference after five hours is also statistically significant with an absence of overlap between the 25°C and 35°C treatments and a p-value of $p=6.30x10^{-6}$. In addition, it is likely the statistically significant difference occurs with the sample at 25°C because the 95% confidence intervals do not overlap.

Discussion

Due to the influence of temperature on the phagocytic efficiency of the membrane of *T. thermophila* which is known to further impact growth rate (Figure 2) we hypothesized that temperature and time at that temperature would have an effect on the rate of food vacuole formation in response to the availability of Congo-red stained yeast. Based on our results, we reject the null hypothesis (H_o) for hypothesis one with a p-value of $p=3.89x10^{-6}$, therefore providing support for our alternative hypothesis (H_a), which states that temperature has an effect on the rate of food vacuole

formation in *T. thermophila.* This supports our prediction that an increase in environmental temperature would cause an increase in the rate of food vacuole formation, as outlined by our biological model in Figure 2a. A similar trend was found in a study by Suhr-Jessen and Orias (1979) that also investigated the effect of temperature on the rate of food vacuole formation in *T. thermophila*. Researchers found that the rate of formation at 22°C averaged 0.4 vacuoles per minute (24 vacuoles/hour) up to 1.1 vacuoles per minute (66 vacuoles/hour) at 40°C (Suhr-Jessen & Orias 1979). This suggests that food vacuole formation does in fact increase in response to an increase in temperature although the rate at which these formed in this instance is notably greater than was found in our study. Likewise, a study conducted by Rasmussen (1976) discovered the species *Tetrahymena pyriformis GL* formed food vacuoles at a rate of one per three minutes (20 vacuoles/hour) at 28°C which is a greater rate than we found.

Secondly, based on our results, we reject H_o for hypothesis two with a p-value of $p=6.30x10^{-6}$, therefore providing support for H_a, which states that time at the acclimation temperature has an effect on the rate of food vacuole formation in *T. thermophila* (Figure 2b). It appears however, that although this provides support for the influence of time at the incubation temperature on food vacuole formation, this does not provide support for our prediction. As seen in Figure 2b, we predicted that increased concentration due to natural reproduction rates would influence the ratio between *Tetrahymena* and the nutrient yeast (Orias & Rasmussen 1976). This would then result in a reduced ability for these cells to form food vacuoles in response to a greater amount of competition among the *Tetrahymena* for nutrients. Our results did not support this prediction. Although the concentration of *T. thermophila* was found to be greater in the five-hour condition (8594 cells/mL at 25°C) compared to the two-hour condition (4609 cells/mL at 25°C), the number of food vacuoles was also seen to increase. One explanation for this may involve the initial concentration ratios between *T. thermophila*

and the yeast cells present in each sample. If the yeast concentrations were not limiting as predicted, the increase in concentration of *T. thermophila* cells may not have resulted in competition for nutrients.

Lastly, we reject H_o for hypothesis three with a p-value of 0.02, therefore providing support for H_a, which states that the effect of temperature on the rate of food vacuole formation in *T. thermophila* is not the same after two and five hours of temperature acclimation. This does not support our prediction that temperature would influence food vacuole formation independent of time. These findings may have been largely influenced by the difference in concentrations between the samples both at each temperature and each time condition. For instance, temperature is known to positively influence doubling time, which is further compounded by the increase in growth rate caused by food vacuole formation itself. This may have led to a reduction in the rate differences between time of acclimatization at the higher temperatures (Orias & Rasmussen 1976; Rasmussen 1973). This may further be explained by the optimum temperature for growth rate which was described by Suhr-Jessen and Orais (1979) to be between 32°C and 38°C, which may account for the absence of significant differences between rates of formation in the 35°C condition as seen with overlapping confidence intervals in Figure 6. In order to reduce the possibility of the effect of increased concentration, it may be beneficial in future experiments to determine the concentration of *T. thermophila* cells in each replicate, following the two-hour acclimation period, to ensure that a uniform concentration is present in all three temperature treatments.

There are a variety of additional factors that may have influenced the results of this study largely relating to the methodology as well as the slight ambiguity in the visual observation of the cells. A large degree of variation existed in the number of food vacuoles in *T. thermophila* cells. In some instances, minimal food vacuole formation was observed in select cells while others exhibited a

more substantial number. There are a variety of explanations that may account for this, including the possibility that cells may have died shortly after the addition of yeast cells or that cells were in the process of preparation for division, as food vacuoles cannot be formed twenty minutes prior to cell division (Rasmussen 1976). In addition, this may be due to the improper addition of the Congo-red stained yeast cells as prior to introduction, the yeast sample was mixed with a pipet rather than a vortex mixer, which would have more thoroughly mixed the sample. This may have caused the addition of unstained yeast cells resulting in the possibility of food vacuoles being formed that were not visible under the microscope. In some instances, these unstained food vacuoles were apparent, however due to the difficulty of ensuring accuracy in counting such faint food vacuoles, only red-stained vacuoles were documented. In order to ensure consistency in our counting in these cases, cells that did not contain food vacuoles were excluded in the calculations of the mean. As a result, this may have had an influence on the accuracy of our results by incorrectly underestimating the number of vacuoles present in these samples. Furthermore, food vacuoles have been described to decrease in size after formation, which may also have attributed to the difficulty in identification and may have resulted in a reduced number of food vacuoles documented in some or all of the *T. thermophila* cells (Rasmussen 1976). In the future, one possible method for increasing the contrast of stained food vacuoles to allow for more accurate identification may be the use of an alternative ingesting medium. As previously mentioned, *Tetrahymena* are indiscriminate in their feeding behaviours and therefore allows for the use of substrate such as India Ink, as utilized by Suhr-Jessen and Orias (1979) which may be more easily visible than the red colour of stained yeast when viewed under a light microscope (Jacobs *et al* 2006).

Moreover, when determining the rate of food vacuole formation at each temperature, a variety of replicates did not produce an observable linear trend in the number of food vacuoles formed over

time. In order to determine a rate, a linear trend line was forced through the points of each replicate. This was not an accurate representation of the rate in all cases, which may have attributed to the large amount of variation in our results.

Lastly, due to time constraints, a smaller number of cells was assessed for food vacuole number. If time had permitted, the inclusion of a greater number of cells to determine the average rate may have been beneficial and may have reduced the degree of variation observed between individual *T. thermophila* cells.

Conclusion

Overall, the results of this study provide support for an effect of both temperature and time on the rate of formation of food vacuoles in *T. thermophila*, as supported by the rejection of H_{o1} and H_{o2}. In particular, higher rates of formation were observed in response to increasing temperature and acclimation time. However, temperature was not observed to influence the rates equally in each time condition, as supported by the rejection of H_{o3}. Further research is required for the confirmation of these results and would provide insight into the mechanisms involved in these processes.

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