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Article

Increased carmine red exposure periods yields a higher number of vacuoles formed in *Tetrahymena pyriformis*

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SUMMARY

***Tetrahymena pyriformis* (*T. pyriformis*) are single celled eukaryotes that use the process of phagocytosis to ingest food by forming vacuoles. Vacuoles are compartments in the cytoplasm of a cell which store the essential nutrients for the cell. *T. pyriformis* can use phagocytosis to create vacuoles of carmine red, a dye which is made using crushed insects and is full of nutrients (1). Establishing a relationship between vacuole formation and duration of exposure to food can demonstrate how phagocytosis occurs in *T. pyriformis*. We hypothesized that if *T. pyriformis* was incubated in a carmine red solution, then more vacuoles would form over time in each cell. This was because with increasing amounts of time, *T. pyriformis* would have a longer period to create new vacuoles. Our results revealed that during the 2 and 5-minute incubation periods, the number of vacuoles increased exponentially, and vacuole formation increased linearly after 5 minutes. The correlation between elongated carmine red exposure and the number of vacuoles formed suggests that the effectiveness of phagocytosis may depend on exposure to the dye, which is used to simulate the particles that they ingest. This may shed light on how other phagocytes manage their consumption of food for energy storage and other metabolic processes.**

INTRODUCTION

The unicellular protozoan *T. pyriformis* is phagocytic. *T. pyriformis* lives in freshwater lakes, and propels itself using its cilia. This protozoan is a model organism because it has two nuclei: a micronucleus and macronucleus. It is also easy to access and multiplies in a simple culture, because it eats bacteria. *T. pyriformis* is unique in that it is a phagocyte with feeding patterns that are easily observed with a simple microscope. A phagocyte is a type of cell that has the ability to engulf foreign bodies. Phagocytic cells perform phagocytosis: the digestion process in which the cell uses its plasma membrane to engulf foreign particles on the cell surface and draw them inward (2). Furthermore, phagocytosis is an active transport mechanism, which is the movement of ions or molecules across a cell membrane into a region of higher concentration, assisted by enzymes and requiring energy (3).

When *T. pyriformis* encounters food particles, its cilia sweep the food into the cell's oral groove, and the cell forms a vacuole, an organelle in a cell's cytoplasm which stores the essential nutrients for the cell. Following the formation, hydrolysis reactions in the vacuole break down the food to allow the cell to absorb its nutrients (4). Since phagocytosis in *T. pyriformis* is such a dramatic cell behavior, it is simple to track the rate of vacuole formation under an ordinary microscope. Living in freshwater environments, *T. pyriformis* tend to eat mostly bacteria or other small cells (4).

Forming vacuoles in response to the presence of food may be an efficient strategy for *T. pyriformis* because they don't have to spend as much energy on feeding when limited food is available. The goal of this experiment was to determine the effect of a carmine red solution, a diluted dye which is made using crushed insects and is full of nutrients (1), over varied increments of time on the number of vacuoles formed in the cytoplasm of *T. pyriformis*. This experiment is vital to understanding how phagocytes conserve their energy through phagocytosis.

We hypothesized that if phagocytes create more vacuoles over time and we expose *T. pyriformis* to a carmine red solution for varied amounts of time, then we should see more vacuoles formed for every increasing incubation period. Previous research has examined nutrient uptake in *T. pyriformis* using 2% India ink (5). We used carmine red to feed *T. pyriformis* as opposed to 2% India ink because when using India ink in preliminary testing, the vacuoles were harder to see than when we used the carmine red. The process of phagocytosis is visualized by the carmine red dye, as the *T. pyriformis* ingest the dye as they would ingest the nutrients they rely on for survival. (6) We allowed the cells to incubate with carmine red for 0, 5, 15, 20 and 60 minutes (4). The results of our experiment revealed that vacuoles formed in a linear trend over time. We concluded that phagocytes conserve energy with phagocytosis because by forming these food storages, they can avoid feeding when there is limited food for them to ingest.

RESULTS

The change in the rate at which *T. pyriformis* can create vacuoles after exposure to food for different amounts of time can be generally informative about how productive phagocytosis is and how energy is distributed to create vacuoles. *T. pyriformis* were exposed to a 1:2 carmine red concentration and water solution. After 0, 5, 15, 20 and 60 minutes, the cells were fixed with iodine for viewing of vacuoles

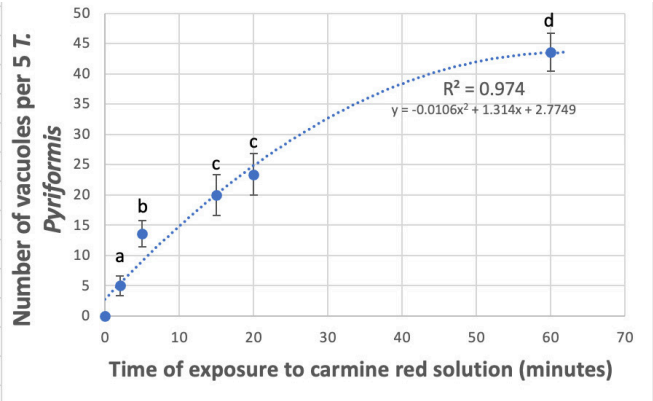

Figure 1: Number of vacuoles per 5 *T. Pyriformis*. Each data point represents the average number of vacuoles of all five trials for the given length of exposure of *T. pyriformis* to carmine red. The blue best fit line represents a polynomial relationship between the time increments and number of vacuoles. The R2 value indicates that our data has a strong correlation to the ideal regression line. The letters depict the statistical relationship between each run. The same letter indicates that those points were not statistically different. A different letter is indicative of a statistical difference (calculated with a two tailed t-test with Bonferroni Correction) between the points. Error bars represent mean ± standard deviation.

under a microscope. The vacuoles were then counted by eye using a counter.

Our results showed a linear increase in vacuole formation following the exposure of carmine red (**Figure 1**). Vacuole formation appeared to be consistent throughout the initial exposure to the dye, which indicates that *T. pyriformis* distributed their eating during the 60-minute time period. However, there was a more rapid increase in vacuole formation between the 2- and 5-minute incubation periods. There were 5.0 ± 1.6 vacuoles per *T. pyriformis* at 2 minutes, and 13.60 ± 1.927 at 5 minutes (mean ± standard deviation). The slope of these two points alone is 2.23 vacuoles per minute higher than the slope encompassing the data points from 0, 5, 15, 20 and 60 minutes (**Figure 1**). Longer exposure times increased the number of vacuoles per 5 *T. pyriformis* at a rate of 2.75 vacuoles per minute for the first 5 minutes and 0.65 vacuoles per minute for the following 45 minutes. After 5 minutes, vacuole production increased exponentially. Two tailed t-tests were performed between every assay time combination. The t-tests for the 15- and 20-minute data, the two closest data points, revealed that there was no statistical difference between the two (t-value of 1.57 at 8 degrees of freedom).

DISCUSSION

The outcome of our experiment supported our hypothesis, more vacuoles would form over longer exposure, since the average number of vacuoles in *T. pyriformis* increased throughout the 60 minutes of exposure to the carmine red solution. This increase is most likely due to the fact that with longer exposure to the dye, the cells had more time for phagocytosis and were able to create more vacuoles. There was a shorter incubation period between 2 and 5 minutes than 15 and 20, yet the data points for 2 and 5 minutes were statistically different while15 and 20 were not. This further supports how *T. pyriformis* ingest more following immediate exposure to food, then they distribute their eating afterwards in a linear fashion.

When *T. pyriformis* feed, the lysosomes in the cell break down the food particles to form a vacuole, which allows *T. pyriformis* to absorb the nutrients (4). Since we found that this process accelerates once the organisms are exposed to carmine red but declines over time, it is possible that *T. pyriformis* can only form a finite number of vacuoles at once. This process of phagocytosis could be a survival mechanism for these eukaryotes. Having the ability to conserve energy and distribute their eating might grant *T. pyriformis* enough time to digest the food they consume while utilizing the nutrients required.

Some possible sources of the variability are that the drops of *T. pyriformis*, dye, and iodine might not have been exactly the same size because the pipettes used did not deliver exact volumes. This variance in volume could have changed the ratio of dye to *T. pyriformis* between replicates and altered the rate at which *T. pyriformis* created vacuoles. In future experiments, a micropipette could be used to deliver precise microliter amounts of *T. pyriformis*, dye, and iodine rather than drops from a pipette. Additionally, we observed that the iodine didn't always completely fix *T. pyriformis* cells, giving them more time to create vacuoles when they were being prepared for the microscope. This problem could be remedied by adding more iodine. Another alternative fixation could be performed with isopropyl alcohol.

Further testing at 5-minute intervals between 20-minute and 60-minute exposures could help determine if the relationship between length of exposure to carmine red and number of vacuoles is linear or exponential between those two data points. Additional testing at 5-minute intervals after 60 minutes could also help to answer if the number of vacuoles in each *T. pyriformis* cell plateaus, or if they completely stop feeding.

Since phagocytosis is an active transport mechanism, *T. pyriformis* requires energy to digest food. In order to survive, it is necessary for these eukaryotes to move substances like ions, glucose, or amino acids against a concentration gradient, which will allow only specific materials to cross through the cell membrane. When *T. pyriformis* eat their food, they need to budget their energy in order for digestion to occur. Although carmine red does not imitate the nutrients that *T. pyriformis* feed on for metabolic processes to occur, the manner in which *T. pyriformis* ingest the carmine red does simulate how they will ingest the nutrients in their habit.

However, the carmine red solution does not give the *T. pyriformis* any energy, the process of forming vacuoles only exhausts the protozoan. This could be an explanation for the approaching plateau for vacuole formation. (**Figure 1**) The *T. pyriformis* could have prioritized dedicating their remaining

energy for other survival processes, as opposed to ingesting the carmine red. Many aquatic organisms will experience food shortages due to upwelling events and other ecological changes. It is important to know not only the optimal conditions for survival, but also how these organisms will allocate energy to survive even when faced with these conditions.

MATERIALS AND METHODS

Culturing *T. pyriformis*

In making *T. pyriformis* (Carolina, cat. no.131620) subcultures, three drops of alive *T. pyriformis* were dropped into an axenic medium (Carolina, cat. no. 132315) using a sterile pipette. The culture was grown at room temperature in a glass test tube and given three days to mature before being examined under the microscope (Swift Optical M225C cat. no. S67300). This guaranteed an equal increment of time to let *T. pyriformis* mature and culture, so that it was certain that they were all functioning the same no matter which culture of *T. pyriformis* was used. The cap of the graduated cylinder sheltering the medium was not fully closed to allow oxygen to pass through and prevent *T. pyriformis* suffocation. The organisms were cultured in a cabinet set to limit the amount of light entering and keep the temperature constant.

Exposing *T. pyriformis* to carmine red dye

Carmine red dye is very thick and condensed. This made it harder to observe *T. pyriformis* under the microscope because the dye would heavily contaminate and cloud the whole solution. The carmine red dye was therefore diluted with water in a 1:2 ratio to reduce this issue. Three drops of *T. pyriformis* were placed into five wells of a plastic tray. Two drops of the 1:2 carmine red concentration and water solution were then dropped into each well.

The dye in each well was then mixed into *T. pyriformis* thoroughly using a toothpick. A stopwatch was immediately started to keep track of exposure time. After the allotted time for exposure was completed (0, 5, 15, 20 and 60 minutes), two drops of iodine were added to each well to fix *T. pyriformis* and stop them from creating more vacuoles (**Figure 2**). One drop of *T. pyriformis* was taken from each well and dropped onto a microscope slide and then the cover slip was placed on top (**Figure 3**).

Using the 40X objective on the Swift Optical M225C microscope, the total number of visible vacuoles containing carmine red dye in five *T. pyriformis* cells was recorded for each run using a counter. Exposing *T. pyriformis* to carmine red dye was performed a total of 5 times for each increment of time: 0, 5, 15, 20 and 60 minutes (**Figure 4**). The vacuoles for each microscope slide were counted twice, one time by two different people. That number was then averaged to determine a final value.

Clean pipettes were used for each treatment to eliminate the possibility that runs would mix with each other and skew the number of vacuoles counted. The same type of slide was used for each run to eliminate any possible differences in the viewing and counting of *T. pyriformis* under the microscope.

Figure 2: Experimental Set-Up. Displayed is the plastic tray with the wells where the carmine red solution was added to *T. pyriformis*. In each well, two drops of iodine were added to the fed *T. pyriformis* after the respective amount of time for each trial. The wells were labeled above with the amount of time allotted for *T. pyriformis* to feed off the carmine red solution.

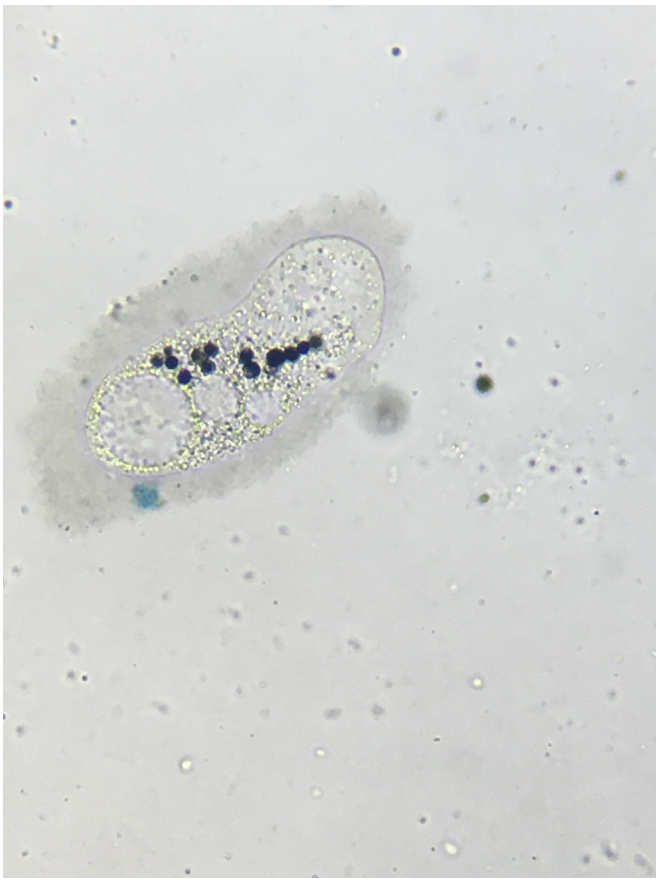

Figure 3: Fed *T. pyriformis*. Displayed is the image of the fed *T. pyriformis* under the 40x lens of a microscope. The darker filled regions are the vacuoles *T. pyriformis* formed following fifteen minutes of carmine red dye exposure.

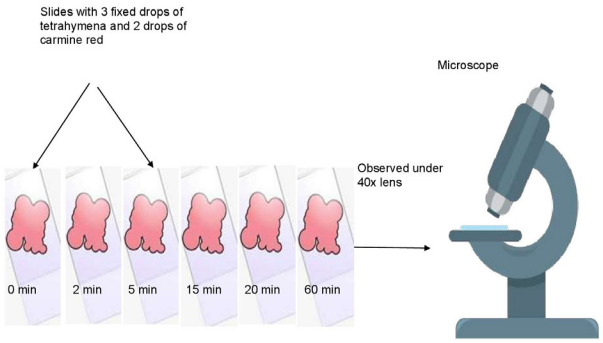

Figure 4: Microscope set-up. After the different exposure times, one drop of the fixed *T. pyriformis* and carmine red was placed onto slides for observation under the 40X lens of a microscope.

Statistical methods

A two tailed t-test via Google Sheets was chosen as a statistical testing mechanism to determine whether each combination of treatments was statistically different from the others. The two-tailed t-test was chosen because the distribution is both positive and negative - more and less vacuoles, respectively.

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