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Article

Longer Exposure to 2% India Ink Increases Average Number of Vacuoles in *Tetrahymena pyriformis*

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SUMMARY

***Tetrahymena pyriformis* are single-celled phagocytes that feed by forming food vacuoles in a process known as phagocytosis. The goal of this experiment was to examine to what extent exposure of *Tetrahymena* to India ink affects the average number of vacuoles formed. We hypothesized that the increase of feeding time would increase the amount of India ink food vacuoles per *Tetrahymena* in a linear fashion. By observing *Tetrahymena* vacuole formation in response to India ink over a 60-minute period, we found that that vacuole formation in *Tetrahymena* initially increased linearly with increased India ink exposure time. After a certain time, however, vacuole formation ceased due to either a lack of remaining ink or a deficiency in the energy or resources necessary to continue performing phagocytosis. The relationship between India ink exposure time and food vacuole formation also provides insight into correlated changes in metabolic rate in *Tetrahymena* and other organisms for which *Tetrahymena* serves as a model. The way in which *Tetrahymena* budget their energy over time between vacuole formation, metabolism, and the countless other biological processes necessary for survival, particularly when supplied a non-nutritional particle such as India ink, could also be important for further studies on various organisms' reactions to food shortages.**

INTRODUCTION

Phagocytes are cells that are capable of ingesting foreign particles via phagocytosis. Phagocytosis takes place when the cilia of a phagocyte sweep food particles into the oral groove of the cell so that the plasma membrane can surround the food particles to form a food vacuole. After the vacuole is formed in the cell, the lysosomes, digestive organelles where food is broken down using hydrolysis, fuse to the food vacuole and break down its contents, so the cell can absorb its nutrients (1).

Tetrahymena pyriformis, one type of phagocyte, are ciliated, single-celled, microscopic organisms (2). The goal of this experiment was to determine the effect of *Tetrahymena* exposure to 2% Speedball Superblack India ink on the average number of food vacuoles formed. This experiment is also significant because studies have shown that food vacuole formation rate correlates to respiration rate; specifically, a 1978 experiment conducted by L. Skriver and J. R. Nilsson found that inducing a high rate of vacuole formation in *Tetrahymena pyriformis* caused increased oxygen consumption, indicating increased respiration rate (3). Because of this correlation, the relationship that we find between time exposed to ink and food vacuole formation may also have implications on metabolic rate. This information can give us insight on how organisms budget their energy when exposed to varying amounts of ingestible particles.

While conducting our background research, we found a report for a very similar experiment. In 2016, Carpenter-Boesch et al. analyzed food vacuole formation over time in *Tetrahymena* exposed to various concentrations of Congo Red. The results of their research revealed a positive, linear relationship between time and mean number of Congo Red vacuoles (4). In our lab, we fed the *Tetrahymena* India ink rather than Congo Red or Carmine Red, primarily due to practical reasons; the red colored particles were more difficult to distinguish under the microscope because the *Tetrahymena* had been stained orange by the Iodine used to fix them, and Carmine Red is a larger molecule that would likely take longer to ingest. Nonetheless, we suspected that the linear increasing relationship observed in the Carpenter-Boesch experiment would apply to our findings as well. Additional background research revealed that the process of phagocytosis merely involves intaking food particles nearby and forming food vacuoles. This process neither accelerates nor deccelerates unless affected by another factor (1). Thus, based on the Carpenter-Boesch experimental results and our own background research on the limiting aspects of the process of phagocytosis, we hypothesized that as the time that we exposed *Tetrahymena* to India ink increased, the average number of food vacuoles would increase in a linear fashion.

RESULTS

In order to test ingestion of India ink, food vacuoles were counted over a period of 60 minutes following initial exposure to the ink. We observed a linear increase in average number of vacuoles per *Tetrahymena* during the first 20 minutes **(Figure 1)**. We measured a rate of 0.5 food vacuoles per minute with a high R^2 value of 0.987, indicating that this linear model closely fit our data. After 20 minutes, the number of food vacuoles remained relatively constant, as supported by the near-zero rate (0.005 food vacuoles per minute) of the linear best fit line for the final three data points. At 20

minutes of exposure to India ink, there were 10.00 ± 1.627 vacuoles per *Tetrahymena*; at 45 minutes, there were 10.024 ± 0.334 vacuoles per *Tetrahymena*; and at 60 minutes, there were 10.208 ± 0.514 vacuoles per *Tetrahymena* (average ± absolute deviation). The first four time points were significantly different (all *p*-values < 0.05, two-tailed t-test with Bonferroni correction), while the final three time points were not significantly different (all *p*-values > 0.05, two-tailed t-test with Bonferroni correction), as labelled in **Figure 1**.

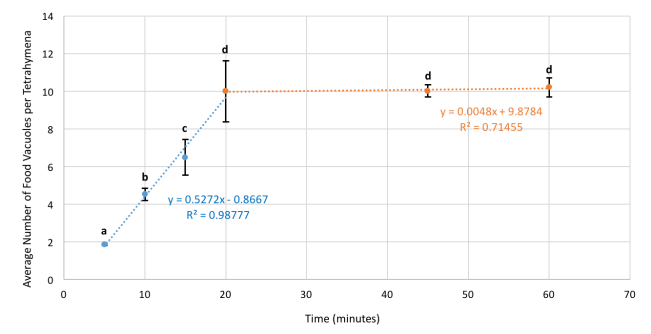

Figure 1: Effect of time exposed to 2% India ink on the average number of food vacuoles per *Tetrahymena*. The blue best fit line represents the increasing linear portion of the graph. The orange best fit line represents the linear horizontal portion of the graph. The equations of the two best fit lines were included to show the difference in slopes; the near-zero slope of the second best fit line shows that a maximum number of food vacuoles was reached after about 20 minutes. The letters represent the statistical relationship between each run. Independent, two-tailed t-tests with Bonferroni correction were calculated between every possible combination of assay time points, comparing the set of five averages (one for each trial). The same letter indicates that those time points were statistically not different and a different letter indicates that those time points were statistically different. Uncertainty bars represent absolute average deviation (AAD). Fifteen *Tetrahymena* were recorded for each trial. Five trials were conducted for each run (n=5)

DISCUSSION

Assuming there was a constant rate of phagocytosis, we originally hypothesized that increasing the duration of *Tetrahymena* exposure to India ink would increase the average number of food vacuoles per *Tetrahymena* in a linear trend, as found in the Carpenter-Boesch experiment (4). We found that the increase in the amount of time exposed to India ink did, in fact, increase the number of food vacuoles per *Tetrahymena* at a rate of 0.5 food vacuoles per minute for the first 20 minutes. This linear increase was also supported by our finding that the first four data points were statistically different (all *p*-values < 0.05, two-tailed t-test with Bonferroni correction). The increasing, linear portion of the graph may be explained by the fact that phagocytosis, the process by which *Tetrahymena* ingest food particles and form vacuoles, is a very specific process. The cilia sweep the food particle into the oral groove, the phagocyte engulfs the food particle and forms a food vacuole, and the lysosomes break down the contents of the food vacuoles using hydrolysis so that the cell can absorb the nutrients. This process does not accelerate as the *Tetrahymena* continue eating, nor does it slow down. The cilia sweep up any food particle close to them until another factor prevents them from doing so. Since only a finite number of food vacuoles can be formed at once, *Tetrahymena* eating is limited by the process of phagocytosis (1).

After 20 minutes of exposure to India ink, the average number of India ink vacuoles remained relatively constant. Here, the rate of increase in food vacuoles was only 0.005 food vacuoles per minute and there was no statistical difference between 20 minutes, 45 minutes, and 60 minutes of exposure to India ink (all *p*-values > 0.05, two-tailed t-tests with Bonferroni correction). One possible explanation for this horizontal linear portion of the graph is that once 20 minutes of exposure to India ink passed, the *Tetrahymena* may have eaten all of the ink that we had given to them. To test this explanation, additional trials could be conducted in which we exposed the *Tetrahymena* to a larger volume of ink. If the number of food vacuoles continued increasing for longer than 20 minutes before becoming constant, then the results would show that the plateau was dependent on the amount of India ink. On the other hand, if no additional food vacuoles formed after adding more ink, this would support another explanation for the plateau in food vacuole formation: the *Tetrahymena* essentially could not consume any more ink, even if additional ink was available, perhaps due to a lack of energy, resources, or space. During our experimentation, after 20 minutes, we observed that the *Tetrahymena* appeared black, as they were filled with India Ink vacuoles. This observation supports the second explanation for the plateau, because it appeared as if no more ink could be consumed.

A source of uncertainty was letting the iodine sit out on the slides. To prepare the slides for the *Tetrahymena* and ink, iodine was placed onto each slide in advance. The amount of time that the iodine was sitting on the slide may have been different for each run. Since iodine is light sensitive and prone to decomposition, the *Tetrahymena* were potentially fixed with different concentrations of iodine. Because of the strong iodine dilution, this uncertainty did not seem to affect most of the trials, as all of the *Tetrahymena* were fixed despite the slightly varied concentrations. However, for one of the 60 minute trials, one *Tetrahymena* was observed moving in the slide after being treated with iodine. If this had been the case for other trials, it would have skewed the averages upwards, because the *Tetrahymena* would have had additional time feeding. A solution for this uncertainty would be to put iodine on the slide right before putting the *Tetrahymena* and ink on the slide. This solution would require multiple people and pipettes.

Several further experiments could be conducted to both improve the precision of this experiment and examine the effects of other variables on *Tetrahymena* feeding. First, the results of this experiment showed that vacuole formation ceased after about 20 minutes of feeding time. To determine the nature of the transition into this plateau (whether there is a sharp change from linear increasing to linear horizontal or a more gradual curved decrease in the feeding rate), more

testing could be conducted at 1-minute intervals between 15 minutes and 25 minutes. To examine other factors potentially affecting *Tetrahymena* feeding and vacuole formation, an experiment could be conducted during which the concentration of India ink was varied. Additionally, another type of ink, such as carmine red, could be used instead of India ink, in order to examine whether various foods, with various molecular sizes, structures, and properties might affect vacuole formation and the time at which the number of ink vacuoles becomes constant. Finally, the same experiment could be conducted to compare feeding rates under varying conditions, for instance different temperatures or pHs.

Despite our uncertainty, the background research on the process of phagocytosis, the experimental data, and the statistical testing support the hypothesis that an increasing linear relationship exists between time exposed to 2% India ink and average number of vacuoles formed per cell in *Tetrahymena pyriformis*. After 20 minutes of feeding time, the average number of vacuoles per *Tetrahymena* remained constant at about 10 vacuoles, as shown by the near-zero rate of vacuole formation (0.005 food vacuoles per minute). Independent, two-tailed t-tests revealed no statistical difference between the 20-minute, 45-minute, and 60-minute runs (all *p*-values > 0.05, two-tailed t-test with Bonferroni correction). Overall, this experiment revealed that after the linear increasing relationship that was hypothesized, the rate of *Tetrahymena* vacuole formation decreases until the average number of ink vacuoles stops at a constant maximum, due to either a lack of remaining ink or lack of energy to create more food vacuoles. Our results could potentially relate to the metabolic rate of organisms and how these organisms budget their energy. Although we exposed the *Tetrahymena* to India ink, a substance that does not provide sugar or nutrients, the creation of food vacuoles would be the first step in the metabolic process when digesting actual nutrient-rich particles (5). In our case, the *Tetrahymena* likely had sugar stored from before the experiment that they used in respiration to generate ATP to create food vacuoles.

Because the India ink does not give the *Tetrahymena* any energy, the process of making food vacuoles only depletes energy overall. Potentially, as a result, they stop ingesting India ink and use their remaining energy for other processes in order to survive. This cessation in phagocytosis could explain the plateau in food vacuole formation on our graph. This explanation could also shed light on the mechanisms organisms use to budget their energy when they have a shortage of food in their environment. Many organisms will face shortages in food caused by environmental and ecological changes (6). 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 suboptimal conditions.

MATERIALS AND METHODS

Tetrahymena Culture

Two pipette drops of a *Tetrahymena* culture were put into a test tube of *Tetrahymena* medium from Carolina Biological Supply Company and incubated at 25 °C. After five days, Iodine-Potassium Iodide Solution (Carolina Biological Supply Company) was diluted to 50% by combining 11 drops each of iodine and water in a microtube and vortexing. The 50% dilution of iodine (Iodine-Potassium Iodide Solution) was chosen because a lower concentration might have failed to kill all of *Tetrahymena*. To fix the *Tetrahymena*, three drops of the 5-day old culture were combined with one drop of this 50% iodine dilution in another microtube and vortexed. One drop of this solution was pipetted into a hemocytometer, which was then placed under a Swift compound microscope (400x magnification). The cells were visible due to the iodine stain. 52 *Tetrahymena* were counted in the 0.9mm^3 square. This value was converted to cell concentration in units of cells/mL by the following calculations:

Conversion: $1\text{mm}^3 = 1000\mu\text{l} = 1\text{ml}$
4/3 accounts for 3:1 dilution of *Tetrahymena* to iodine

Thus, the *Tetrahymena* culture used for experimentation had an approximate concentration of approximately 77,000 cells/mL. For the second round of final testing, the *Tetrahymena* culture's cell concentration was again counted using a hemocytometer. This time, 111 cells were counted, so a 1:1 dilution of *Tetrahymena* culture to *Tetrahymena* medium was created to standardize the concentration used for both days of final testing.

Preparing Solutions

In five different microtubes, one for each trial, 12 pipette drops of the 77,000 cells/mL *Tetrahymena* culture were combined with 8 pipette drops of 2% India ink, as shown in **Figure 2**. The *Tetrahymena* to ink ratio was kept to 3:2 for all of final testing because the Carpenter-Boesch experiment indicated that variation of food concentration might affect the number of vacuoles formed (4). Immediately after the first drop of India ink was added to each microtube of *Tetrahymena*, an iPhone stopwatch was started. Microtubes were then closed, vortexed, and returned to the tray to incubate.

Assays

One drop of 50% iodine was pipetted in the center of each of the five microscope slides, corresponding to the five microtube trials **(Figure 3)**. Five sterile pipettes were used to remove one drop from each of the five microtubes at each of the predetermined time points (5 minutes, 10 minutes, 15 minutes, 20 minutes, 45 minutes, and 60 minutes). The 60-minute time point served as the control group because maximum vacuole formation was expected at 60 minutes. In order to fix the *Tetrahymena* at the correct time, these *Tetrahymena* and ink drops were immediately placed atop the 50% iodine drops on the corresponding microscope slides.

Meanwhile, the remaining *Tetrahymena* in the microtubes continued eating ink as the timer continued. With the microscope set at 400x magnification, the total number of ink vacuoles in each of the first 15 *Tetrahymena* observed were counted per trial, as shown in **Figure 4**.

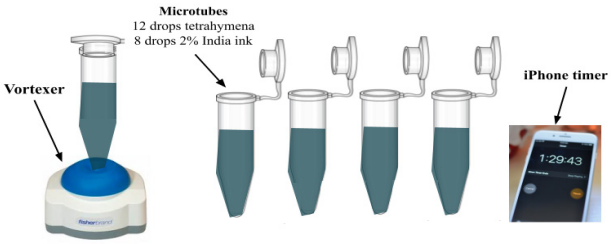

Figure 2: Microtube set-up. Five microtubes, one for each trial, were filled with 12 drops of Tetrahymena culture and 8 drops of 2% India ink, then closed and vortexed. The iPhone timer was started after the initial drop of India ink was dropped into the microtube.

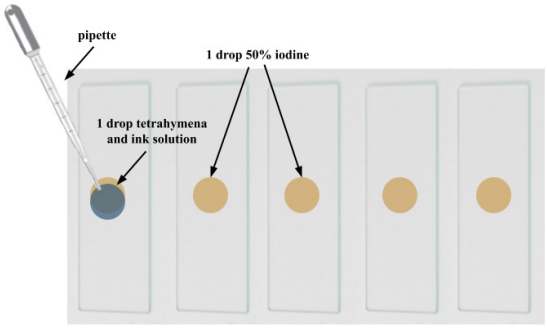

Figure 3: Microscope slide set-Up and iodine procedure. One drop of 50% iodine dilution was pipetted into the center of five microscope slides, one for each trial. At each of the different time points (5 min, 10 min, 15 min, 20 min, 45 min, 60 min), one drop of the *Tetrahymena* and ink solution was removed from each microtube and dropped atop the iodine on the corresponding slide.

Statistical Testing

Within each trial, the average number of vacuoles in the 15 counted *Tetrahymena* was calculated; then the average of these averages was calculated, and this value was displayed on the graph for each time interval. Independent, two-tailed t-tests with Bonferroni correction were calculated, using excel spreadsheets, between the set of averages for every possible combination of assay time points. Furthermore, the slopes of the two best fit lines were used to represent the rate of food vacuole formation.

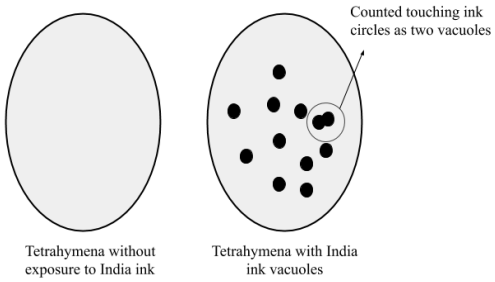

Figure 4: Example of *Tetrahymena* before and after exposure to India ink. Under microscope magnification 400x, the total number of India ink vacuoles was counted in the first 15 *Tetrahymena* observed.

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