Low environmental pH inhibits phagosome formation and motility of *Tetrahymena pyriformis*

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SUMMARY

*Tetrahymena pyriformis*, single-celled protozoans, populate ponds, lakes, and streams. As 2.57 million tons of carbon dioxide enter the atmosphere every second, Earth’s bodies of water acidify rapidly, creating harmful habitats for organisms such as ciliates at the bottom of the food chain, like *T. pyriformis*. Investigating the ability of *T. pyriformis* to feed in acidic pHs presents a deeper understanding of the short-term ramifications of carbon dioxide emissions on freshwater ecological communities. In this experiment, we varied pH from 4.5 to 7.0 by diluting carbonated water. To observe *T. pyriformis* food vesicle formation, we counted the number of phagosomes located in iodine-fixed *T. pyriformis* after a 10-minute feeding period. We hypothesized that increased suspension acidity would reduce *T. pyriformis’* consumption of food vesicles due to inhibited motility functions and phagosome formation during phagocytosis. Our data suggests *T. pyriformis* best generate phagosomes within a pH range of 6.0 to 7.0. Data displayed a low average vesicle count of 0.60 ± 0.16 at a pH of 4.5 and a high average vesicle count of 3.73 ± 0.18 at a pH of 7.0. At a pH of 6.0, average vesicle count plateaued as its rate of increase slowed. We posit that as pH levels decrease, *T. pyriformis* lose feeding competence due to three probable mechanisms: increased membrane density, weakened myosin necessary for vesicle transport, and inhibition of ciliary movement — all components necessary to initiate and complete phagocytosis.

INTRODUCTION

Since the late 19th century, our industries, modes of transportation, agricultural operations, and residential needs have required the burning of fossil fuels, a process which releases harmful greenhouse gases into the atmosphere (1, 2). As global emissions have steadily increased due to population growth and its consequent demand, Earth’s bodies of water have absorbed unexpected amounts of carbon dioxide, resulting in their acidification through the formation and subsequent dissociation of carbonic acid (3, 4). Even subtle changes in environmental conditions, such as water acidity, can disturb the balance of ecosystems (4). Investigating the potential consequences of acidification on aquatic organisms gives insight on the ecological changes human fossil fuel emissions can cause. Our study of *T. pyriformis* initiates this investigation, starting at the bottom of the food chain.

*T. pyriformis* are ciliated eukaryotes that inhabit fresh bodies of water, feeding on bacteria through the process of phagocytosis (5, 6). These unicellular paramecia function as model organisms due to their distinct, universal cellular functions such as their food vesicle maturation process (5). Using their cilia (Figure 1A), *T. pyriformis* pull bacterial particles into the base of their complex oral groove where they organize their food into fatty membrane vesicles, or phagosomes, using four ciliated membranelles (7). The phagosomes then enter the complex network of membrane trafficking pathways before exiting the organism through egestion (6). Membrane trafficking depends heavily on phospholipids residing in the cytoplasmic leaflet, called phosphoinositides, for vesicle recognition and transport (8, 9). In addition, vesicle transport from the oral apparatus requires the use of myosin motor proteins, composed of fibrous yet low environmental pH inhibits phagosome formation and motility of *Tetrahymena pyriformis*
Figure 2: Overview of the stepwise experimental procedure for preparing the samples and the observing the fixed T. pyriformis. Step 1.) shows the configuration of the T. pyriformis solution, which includes the setup of the multi-well dish, as well as the preparation of the various carbonated water solutions used to alter the environmental acidity. Step 2.) incorporates and describes both the feeding and the fixing processes of the organism. Each of the 8 100 mL carbonated water solutions were separate treatments that varied in pH measurement. Lastly, Step 3.) depicts the sampling and data collection procedures.

dynamic actin molecules crucial for motility (9). All of the molecules work in concert to complete maturation; therefore, if any step is interrupted along the way because of extreme habitat conditions, phagocytosis may not be possible.

The goal of this experiment was to investigate the effect of varying environmental pH on T. pyriformis’ ability to produce phagosomes containing 2% India Ink, an experimental proxy for food. Through simulating the acidification of freshwater environments due to excess CO₂ in the atmosphere, we aimed to discover optimal feeding pH for T. pyriformis. We hypothesized that as acidity increased, T. pyriformis would consume less, producing fewer food vesicles, which would implicate their inability to successfully complete phagocytosis. We propose that as environmental pH drops below the neutral freshwater surface conditions of a pH ranging from 7.5–8.5 (10), increased amounts of hydrogen ions (lower pH/acidic conditions) might negatively alter the molecular composition of cilia and membranes, inhibiting T. pyriformis’ motor functions and vesicle formation processes. Our data uncovers that T. pyriformis consume the most food vesicles within an optimal pH range of 6.0–7.0. At any point below a pH of 6.0, T. pyriformis lose feeding competence, consuming fewer food vesicles. These results display the danger freshwater acidification poses to even small ciliated organisms, providing reasonable evidence to further research acidification’s impact on other aquatic organisms and to uncover the urgency of limiting our fossil fuel emissions.

RESULTS

To determine whether our hypothesis that increased acidity would inhibit phagocytosis and prevent T. pyriformis from generating a baseline amount of food vesicles, we prepared seven different treatment groups of the varying acidities: 4.5, 5.0, 5.5, 6.0, 6.2, 6.5, and 7.0, which acted as a negative control, replicating a standard freshwater habitat pH. We created separate treatments by diluting carbonated water, which had a pH of 4.5, with differing amounts of distilled water. Carbonated water mimicked the conditions of a freshwater habitat after absorbing carbon dioxide, and each treatment simulated a more severely acidified environment. After quantifying each treatment’s pH using paper pH strips, we mixed T. pyriformis and India Ink, a dark dye and proxy for food, into a treatment solution (Figure 2). After a 10 minute feeding period, we fixed the T. pyriformis with iodine and then, under 400X magnification, counted the number of India Ink food vesicles used to alter the environmental acidity. Step 2.) incorporates and describes both the feeding and the fixing processes of the organism. Each of the 8 100 mL carbonated water solutions were separate treatments that varied in pH measurement. Lastly, Step 3.) depicts the sampling and data collection procedures.

As pH increased, T. pyriformis formed more India Ink vesicles, consuming the largest mean number of food vesicles of 3.73 ± 0.18 (± standard error) at a pH of 7.0 (negative control), and the smallest mean number of food vesicles of 0.60 ± 0.16 at a pH of 4.5 (Figure 3). At a pH of 7.0 with no food available and no carbonation added (second negative control), no T. pyriformis held any food vesicles within their bodies. We observed no qualitative changes in morphology T. pyriformis across different pH suspensions (Figure 1B).

A one-way analysis of variance (ANOVA) performed on our dataset yielded significant variation among our tested conditions (F(7, 112) = 58.41, p = 1.74 × 10⁻³⁴). A post hoc Tukey’s test revealed that mean food vesicle count increased with significance (p < 0.001) from 0.60 ± 0.16 to 2.07 ± 0.21 while suspension pH increased from 4.5 to 5.0; then, from a treatment pH of 5.0 to 5.5, mean vesicle count significantly increased (p < 0.05) again from 2.07 ± 0.21 to 2.87 ± 0.26 (Table 1). No statistical significance was identified between
all sequential treatment pairings with pHs of 5.5 and higher (Table 1). However, a comparison between pH treatments of 5.5 and 7.0 with mean vesicle counts of 2.87 ± 0.26 and 3.73 ± 0.18, respectively, also yielded statistical difference ($p < 0.025$, Table 1). The data set fits the trend of a quadratic polynomial curve with an r2 value of 0.955. Additionally, p-values calculated using two-tailed T-tests between different sets of pseudo-trials within each run were all greater than 0.05, showing statistical similarity, with the exception of two different trials from testing in a pH of 6.0 ($p < 0.0004$).

**DISCUSSION**

Our data supports the hypothesis that a lower environmental pH decreases the amount of visible food vesicles within *T. pyriformis* after a 10-minute feeding period. However, the data collected also revealed a new element to this trend: as pH increased from acidic to neutral, food vesicle count increased at a decreasing rate. Mean food vesicle count plateaued at pHs higher than 5.5 (Figure 3), when data points no longer were statistically different from one another ($p > 0.05$, Table 1). But mean vesicle counts between pH treatments of 5.5 and 7.0 remained statistically different ($p < 0.025$), demonstrating that the optimal feeding environment of *T. pyriformis* lies within a pH range of 6.0 to 7.0. The most probable explanation for this phenomenon is acidity's direct effect on *T. pyriformis’* process of phagocytosis. As the hydrogen ion concentration of their suspension increases, *T. pyriformis’* cell membrane thickness and structure can change rapidly and drastically. Studies investigating other membranes such as phosphatidylcholine bilayers, which occur naturally in eggs, reveal that acidic environments cause decreased head repulsions in phospholipids around a pH of 5.5, increasing interfacial tension and density of the bilayer within nanoseconds (11, 12). Bilayers containing phospholipids with amphiphilic heads occurring in eukaryotic cells, such as *T. pyriformis*, may react similarly to protonation caused by high hydrogen ion concentrations. Increased membrane density could directly inhibit the function of phosphoinositides, which would prevent phagosome transport and organization. Phosphoinositides are essential elements in endosome dynamics, as well as various cell signaling pathways, necessary for regulating the movement of food vesicles throughout phagocytosis (9). Without functional phosphoinositides, food vesicles may not successfully enter membrane trafficking pathways or even reach their respective destinations after phagocytosis, potentially explaining why fewer vesicles were observed in lower pH environments. Though we saw no visible alterations in food vesicle morphology, even subtle changes in membrane density may have hindered phosphoinositide function. Finally, phagosomes rely on actin-based myosin motors to move from the oral apparatus into the cell's interior; yet, acidic environments with a pH of lower than 6.5 can weaken myosin's average force by 20% (13). Even if weakened myosin can still transport phagosomes, they must do so at a slower rate, a discovery which may explain the drastic drop in vesicle count seen in pHs lower than 6.5 (Figure 3).

**Figure 3:** The mean value of India Ink vesicles counted within iodine-fixed *T. pyriformis* in variable pH environments. Means of food vesicles result from 15 replicates ($n = 15$) collected over 3 trials. Vesicles were counted (at 400X magnification) after *T. pyriformis* were fed India Ink for 10 minutes in various acidic concentrations of carbonated water ($4.5 \leq \text{pH} \leq 7.0$). Mean vesicle counts in treatments with a pH of 5.5 and below were statistically significant ($p \leq 0.05$), while data collected in a pH of 6.0 and higher was statistically similar ($p \geq 0.05$). When no carbonation and no India Ink were added to the environment, there were no vesicles observed in any *T. pyriformis* (not shown above). Error bars denote the calculated standard deviation (SD) of each run.

**Table 1:** Results of a post-hoc Tukey's Test indicating statistical significance between treatments groups. Calculated Tukey HSD p-values are listed for treatment group comparisons labeled in the leftmost column. Green coloration denotes statistical significance ($p \leq 0.05$) while red coloration signals statistical similarity ($p \geq 0.05$).
the decimal of the pH. Instead, using an electronic pH meter may have increased precision because of its ability to detect
minute changes in acidity.

In the future, we could initiate feeding periods after T. pyriformis have acclimated to their respective pH suspensions
for longer periods of time, to investigate whether T. pyriformis
can adapt to more acidic environments, better simulating the
long-term process of fresh-water acidification. Additionally,
studying phagocytosis or other bodily processes in different
organisms may further solidify our claims and reveal more
consequences of freshwater acidification. We could also
collect samples from freshwater ponds, lakes, or streams to
study the changing pH levels of our local habitats and how
the T. pyriformis that reside in them are coping with their
environment’s varying conditions.

METHODS

Variables and Control Groups

By carbonating water, acidic freshwater environments were
replicated by lowering water pH through the reactions: H$_2$O +
CO$_2$ → H$_2$CO$_3$ → H$^+$ + HCO$_3^-$ Adding different concentrations
of carbonated water to the T. pyriformis suspension varied
environmental acidity and consequently lowered the overall
pH to 4.5, 5.0, 5.5, 6.0, 6.2, and 6.5 respectively. For these six
conditions, three trials each consisting of five pseudo-trials
were conducted to determine the amount of India Ink vesicles
within random T. pyriformis in solution. In addition to the six pH
solutions listed above, two other control groups were included
in the experiment. The first negative control was the original
T. pyriformis solution with food added but no pH change, and
the second negative control group was the same T. pyriformis
solution with no pH change or food. The first control group
served as a baseline for the other tests, since the natural pH
that T. pyriformis exist in is an average freshwater pH of 7.0
(10). The second control group was necessary to confirm that
without food, the T. pyriformis would hold no food vesicles.

Configuration of T. pyriformis Solutions

T. pyriformis solution (50 μL) was added into 8 separate
wells of a 12-well dish. The carbonic acid concentrations,
or proportionate mixtures of CO$_2$ and distilled H$_2$O, were
combined in a separate multi-well dish. The carbonic acid
was prepared by placing a bottle of distilled water in a
SodaStream® and pressing the carbonation button for 10
seconds, as suggested by the instructions. The pH of the
purely carbonated water was around 4.5; this was established
as the minimum bounds of the IV. To achieve the other less
carbonated solutions, the original carbonated water solution
was diluted with distilled water to raise solution pH. Preliminary
testing demonstrated that adding 20 μL increments of distilled
water to 100 μL of carbonated water would increase the pH by
roughly 0.5 per addition (Table 2).

Feeding the T. pyriformis

After the concentrations were made and the pHs were
quantified prior to each trial using pH strips (Table 2), the
acidic dilutions and food proxy were simultaneously added
to the T. pyriformis solutions. First, 100 μL of the 4.5 pH
solution and 50 μL of 2% India Ink were added to 50 μL of
T. pyriformis in the first dish well, for a 1:1 ratio of 2% India
Ink to T. pyriformis. These values, along with the amount of
carbonated solution added, were controlled every single trial

### Table 2: Ratios of distilled water to carbonated water to obtain 6 distinct carbonated water solution pHs. Micropipettes were used to control amounts of each component added into the mixture. Accuracy values were measured using paper pH strips.

<table>
<thead>
<tr>
<th>Distilled H$_2$O (μL)</th>
<th>Carbonated H$_2$O (μL)</th>
<th>Resulting pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>≈ 4.5</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td>≈ 5.0</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td>≈ 5.5</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td>≈ 6.0</td>
</tr>
<tr>
<td>80</td>
<td>100</td>
<td>≈ 6.2</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>≈ 7.0</td>
</tr>
</tbody>
</table>
and run to prevent any other variables from influencing our results. Exactly 30 seconds later, a sufficient window of time to refill micropipettes, 100 μL of the 5.0 pH solution and 50 μL of 2% India Ink were added to the 50 μL of *T. pyriformis* in the second well of the dish. This process was continued for the rest of the remaining pH solutions at 30 second intervals (Figure 3). In the penultimate well of the dish, 50 μL of 2% India Ink was mixed with 100 μL of distilled water and combined with the 50 μL of *T. pyriformis* solution. In the last well of the dish, the 50 μL of *T. pyriformis* solution was unaltered; it was expected that this *T. pyriformis* solution would confirm that without food, *T. pyriformis* held no food vesicles (Figure 4). They were given exactly 10 minutes to feed.

**Preparation and Administration of Fixing Solution**

During the 10-minute waiting period, the dilute iodine solution used to fix the *T. pyriformis* was prepared. This dilution was generated by mixing 200 μL of 50% iodine with 500 μL of distilled water to create 700 μL of diluted 20% iodine. The 20% solution was used because the 50% iodine made the *T. pyriformis* too dark to inspect under the microscope, an observation confirmed by preliminary testing. After the 10-minute feeding, 50 μL of the 20% iodine solution was added to the first well containing the 4.5 pH solution to fix the *T. pyriformis*, allowing them to be easily observed under a microscope. Next, after 30 seconds, 50 μL of the iodine dilution was added to the second well containing the 5.0 pH solution. This same fixing procedure was repeated for all solutions in the other wells of the dishes again at 30 second intervals, including both control groups (Figure 4). After this process was completed, 50 μL of solution from each run was added to a slide and labeled. (Figure 2).

**Data Collection**

These slides were then inspected under a microscope at 400X magnification, and the number of large, dark food vesicles (Figure 1B) within five random *T. pyriformis* for each of the eight slides was counted and recorded. Occasionally, microscope focus was adjusted when looking at a single *T. pyriformis* to observe all of its bodily contents, as vesicles can lay on different focus planes within their bodies. After collecting the vesicle data for five random *T. pyriformis*, the first trial was concluded. The second and third trials, each with five pseudo-trials, were commenced in the same manner as the first. Once all data was collected, mean, SD, and standard error calculations were completed. Additionally, an ANOVA and post hoc Tukey’s Test were performed in Excel to assess statistical variance among our dataset. In Figure 2, SD calculations were used to produce error bars, and Excel was employed to produce a best fit polynomial trend to the data.

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**REFERENCES**


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