The Effect of Caffeine on the Swimming Speed of *Tetrahymena thermophila*

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Abstract

Caffeine is a prevalent chemical that is often found in ecosystems. Previous research has shown that high concentration of caffeine has a negative impact on the growth and survival of protists. The objective of this experiment is to determine the effect of caffeine on the swimming speed of *T. thermophila*, a unicellular microorganism found in freshwater environments. We predict that higher caffeine concentrations will lead to decreased swimming speeds of *T. thermophila.* To test this prediction, we incubated *T. thermophila* in solutions of 0 μM, 10 μM, 100 μM, or 1000 μM of caffeine for 10 minutes and observed their movement under a dissecting microscope. Cell movement was recorded and average swimming speeds were determined. Our results show there is no significant difference in the swimming speed of *T. thermophila* between the different caffeine treatment groups, thus we fail to reject the null hypothesis that caffeine concentration has no effect on the swimming speed of *T. thermophila.* However, the cells exposed to the highest caffeine concentration had the highest swimming speeds, at 1.5 times faster than those in the control group, and *T. thermophila* exposed to the medium caffeine concentration had the lowest swimming speed. This study allows us to further understand the impact of chemicals upon the microbe community.

Introduction

Tetrahymena thermophila is a unicellular, ciliated eukaryote that has been widely used as a model organism in biological studies (Collins & Gorovsky, 2005). Found in freshwater habitats within temperate regions (Collins & Gorovsky, 2005), *T. thermophila* swims with a helical trajectory by beating their cilia (Marumo et al., 2021). Various species of *Tetrahymena* have been utilized to test the toxicity of several chemicals in aqueous solution, as they are known to show decreases in their swimming speed or vary from their usual swimming patterns when exposed to harmful chemicals in their environment (Noever et al., 1994).

Beverages containing caffeine, a chemical in the group of methylxanthines, have become widely consumed, and as a result, caffeine is entering aquatic environments through waste water (Gibson et al., 2012). Caffeine is highly soluble in water, allowing it to remain in aquatic systems for prolonged periods

of time, and it has the ability to biomagnify up the food chain (Gibson et al., 2012). Several studies have shown that caffeine has negative mutagenic effects upon various bacterial species, notably damage to DNA and decreased lifespans (Gibson et al., 2012). Additionally, the effect of caffeine upon the growth rate of *T. thermophila* has been investigated (Wolff, 1973), however there is a distinct knowledge gap, wherein the physiological impacts of caffeine upon the swimming behaviors of *T. thermophila* are not well understood.

The swimming motion of *T. thermophila* is generated through the beating of their cilia (Murofushi, 1974). The movement of cilia depends upon cyclic adenosine monophosphate (cAMP), and the rate of ciliary beating is decreased when *Tetrahymena* are exposed to compounds that increase levels of cAMP (Wolff, 1973). Caffeine prevents the action of phosphodiesterase which breaks down the bonds in cAMP. As the degradation of cAMP is decreased, its intracellular concentration increases over time (Wolff, 1973). Thus, as the presence of caffeine increases the levels of cAMP within *T. thermophila*, it can be expected that the force, rate, or coordination of the ciliary beating would decrease, thereby resulting in a measurable decrease in swimming speed (Wolff, 1973).

The objective of this study is to determine how exposure to various levels of caffeine will impact the swimming speed of *T. thermophila*. The null hypothesis is that caffeine concentration has no impact on the swimming speed of *T. thermophila*, and the alternate hypothesis is that the swimming speed of the cells will differ between the different caffeine concentration treatments. Since previous studies have shown that caffeine exposure increases cAMP levels, which in turn resulted in decreased ciliary beating and decreased swimming speeds in *T. thermophila* (Wolff, 1973), we predicted that as the caffeine concentration increases, the swimming speed of *T. thermophila* will decrease.

Methods

Supplies and Equipment

For this experiment the lab equipment needed was a dissection microscope, *T. thermophila* stock, *T. thermophila* standard growth medium, a 2000 μM caffeine stock solution, micropipettes, 25 mL flasks, 15 mL test tubes in which the *T. thermophila* and the treatment solution are mixed, watch glasses, a squirt bottle with distilled water, a laptop with the DinoCapture 2.0 software, an ocular camera, and the ImageJ program with the Manual Tracking plugin.

Experimental preparation

Prior to conducting the experimental trials, a test of the DinoCapture 2.0 camera in conjunction with the dissecting microscope was conducted to confirm connectivity of the camera to the laptop, and to ensure the *T. thermophila* cells were clearly visible and remained in the field of view for long enough to track their movement before they moved out of view (at least five seconds). Through this process it was determined that the appropriate magnification of the dissecting microscope was 4.0 times to allow for an adequately wide field of view. Further, it was found that loading 3 mL of sample onto the watch glass resulted in sufficient amount of solution to evenly cover the entirety of the watch glass with a thin layer, resulting in a cell density of approximately 50 to100 cells on the screen at any given time. A random number generator was used to determine the order in which the treatments were conducted.

Incubating *T. thermophila* in caffeine solutions and recording cell movement

The goal of this experiment was to expose *T. thermophila* to various concentrations of caffeine and record their movement for further analysis. Prior to preparing solutions, all glassware and test tubes were sterilized over a flame. From the initial caffeine stock solution of 2000 μM, a serial dilution was performed to prepare stock solutions for each treatment, with the treatments being the control of 0 μM of caffeine, a low treatment of 10 μM of caffeine, a medium treatment of 100 μM of caffeine, and a high

treatment of 1000 μM of caffeine (Figure 1). A total of 12 trials were conducted, with three biological replicates for each treatment. For each treatment, 2 mL of *T. thermophila* culture was added to 2 mL of the appropriate caffeine treatment solution, or 2 mL of standard *T. thermophila* growth medium (SSP solution) in the case of the control in a 15 mL test tube. The test tube was gently mixed on the vortex set at 4.5 speed for two seconds. Then, 3 mL of the mixture was pipetted onto a watch glass that had been labeled with four quadrants (Figure 2) The *T. thermophila* were acclimated to the solution for 10 minutes with no exposure to light from the microscope. During the acclimation period, the microscope, set at 4.0 times magnification, was focused on the cells in the randomly chosen quadrant of the watch glass. After 10 minutes had elapsed, the microscope light was turned on, and it was ensured that the microscope was correctly focused and that there was a sufficient cell density in the field of view. Then, the movement of the cells was recorded through the use of the DinoCapture 2.0 which had been placed in one of the ocular lenses of the microscope and connected to a laptop. Each trial was recorded for 60 seconds, after which the recording was stopped for 60 seconds, and then recorded again for an additional 60 seconds. From this, two 60 second recordings were obtained for each trial. After each trial was conducted, the used watch glass was thoroughly rinsed with distilled water and dried. As three trials were conducted for each treatment, this resulted in six 60 second recordings for each treatment.

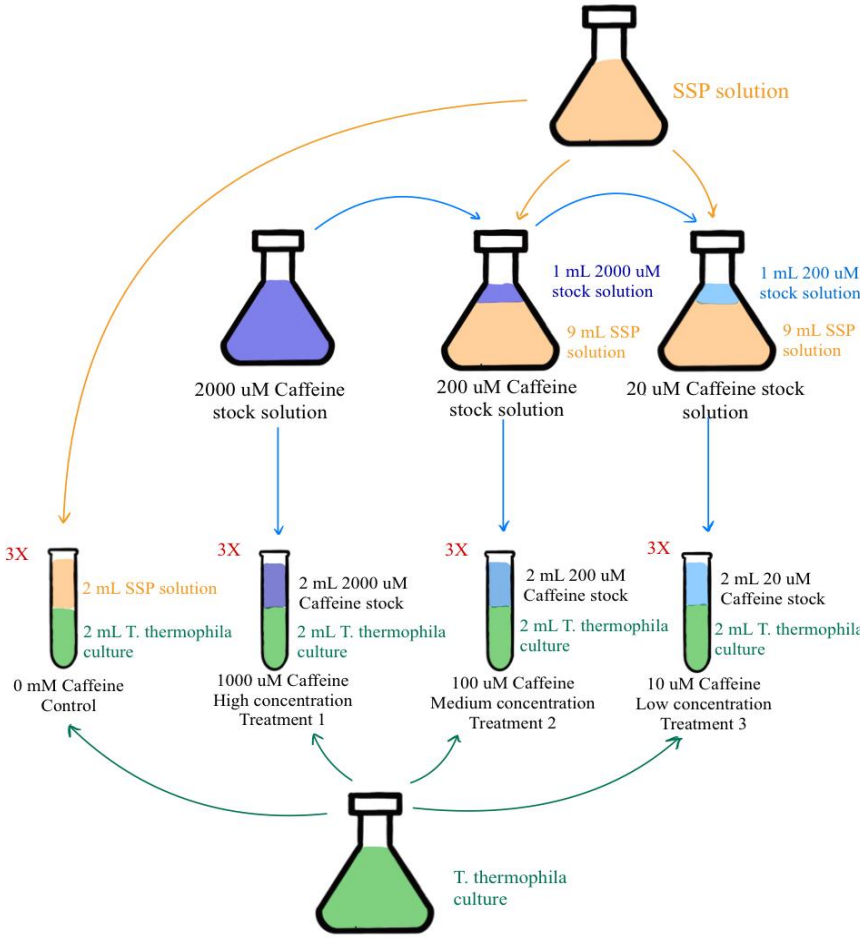

Figure 1. Diagram for experimental treatment preparation of serial dilutions.

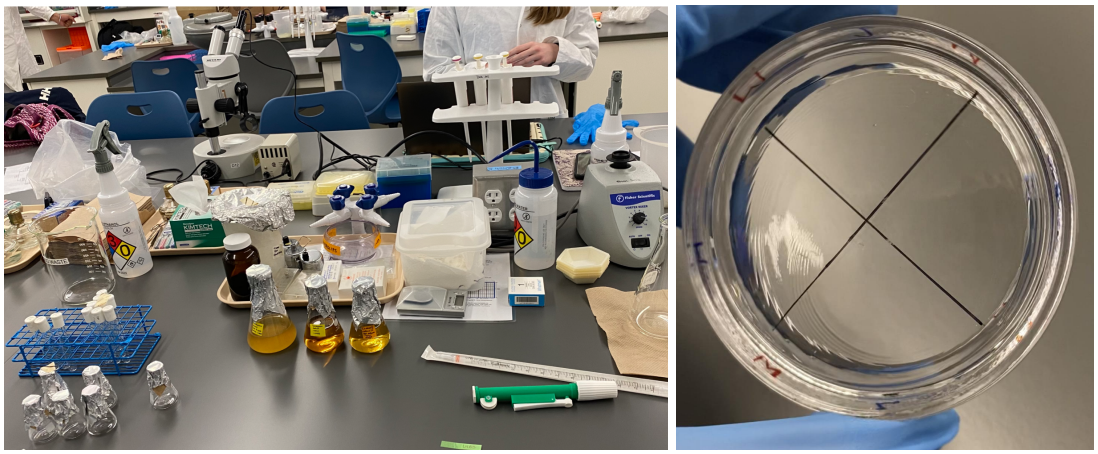

Figure 2. Experimental setup and equipment required for this experiment (Left). The watch glass with labeled quadrants (Right).

Video analysis

Video analysis was performed using the Manual Tracking plugin of ImageJ. From each video that had been recorded, three cells were selected for analysis. The first cell was chosen in the time frame of 0-20 seconds into the video, the second cell from 20-40 seconds into the video, and the third cell from 40-60 seconds into the video. To ensure cells were randomly selected, a random number generator was used to select an exact timestamp within each 20 second timeframe. Once an exact time had been selected, all cells visible on the screen at that time were numbered in ImageJ, and the random number generator was used to select a number, and the cell with the corresponding number was analyzed (Figure 3). If the randomly selected cell was moving too fast, moved out of view or out of focus, or was stationary, a different cell was randomly selected. Overall, since each of the four treatments had six recordings, and three cells were selected from each recording, this resulted in a total sample size of 72 cells that were analyzed.

To find the velocity of cells that had been randomly selected, the actual length represented by each pixel was determined. Then, the Manual Tracking function was used to track the path traveled by the chosen cell. ImageJ provided the coordinates of the cell in pixels which was used to calculate the number of pixels traveled by the cell (Figure 4). Finally, the number of pixels traveled by the cell was converted into the actual distance traveled, and the velocity of the cell was calculated using this formula:

$$\textit{Velocity of cell } (\mu m/s) = \textit{Number of pixels traveled} \times \textit{Actual distance of each pixel } (\mu m) \div \textit{Time } (s)$$

During the process of tracking the distance each cell moved, a velocity between each consecutive point tracked is found, and the overall velocity of the cell is found by taking the average of all of the velocities of the cell between two consecutive different points. Once the average velocity of the individual cells had been determined, the average velocity of the cells across each treatment was calculated.

Statistical analysis

All statistical analysis was carried out using RStudio. As a normal distribution of data is a requirement to perform the one-way ANOVA, a check to determine if the data was normally distributed in each sample was conducted. If the data was not normally distributed, a Kruskal-Wallis test would be carried out which is a non-parametric alternative to one-way ANOVA that does not assume normal distribution of data.

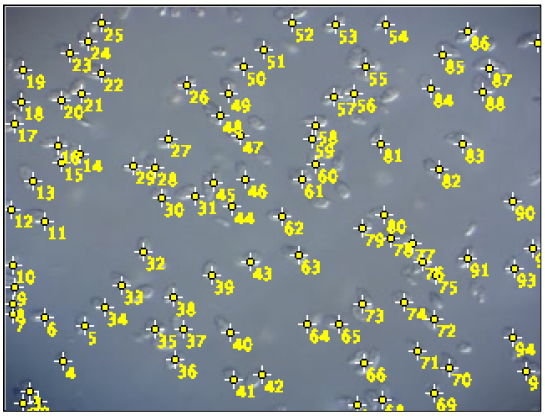

Figure 3. A screenshot of cells numbered through ImageJ using the Manual Tracking plugin option. Later an individual cell was selected through a random number generator.

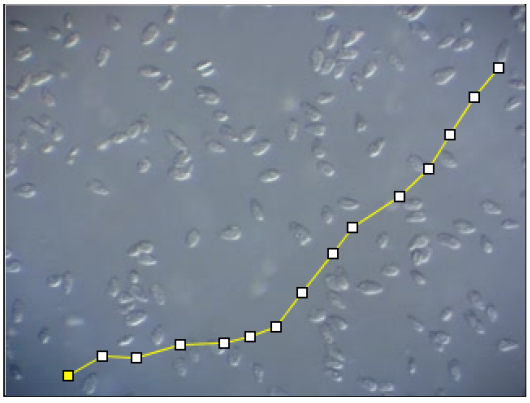

Figure 4. A traced path of *T. thermophila* in a control treatment trail. Image captured in the ImageJ program with movement tracing produced using the Manual Tracking plugin feature.

Results

From this experiment, cells in the control treatment, which were incubated with only SSP solution and 0 µM of caffeine, demonstrated an average swimming speed of 101.7475 µm/s with a confidence interval of 102.7475 ± 35.3 µm/s. The low treatment, which exposed cells to a 10 µM caffeine solution, demonstrated an average swimming speed of 112.0314 µm/s and a confidence interval of 112.0314 ± 36.6 µm/s. The medium treatment, which exposed cells to 100 µM of caffeine, demonstrated an average swimming speed of 101.7254 µm/s with a confidence interval of 101.7254 ± 34.8 µm/s. Lastly, the high treatment exposed cells to a caffeine solution of 1000 µM, and demonstrated an average swimming speed of 157.7649 µm/s with a confidence interval of 157.7649 ± 57.5 µm/s.

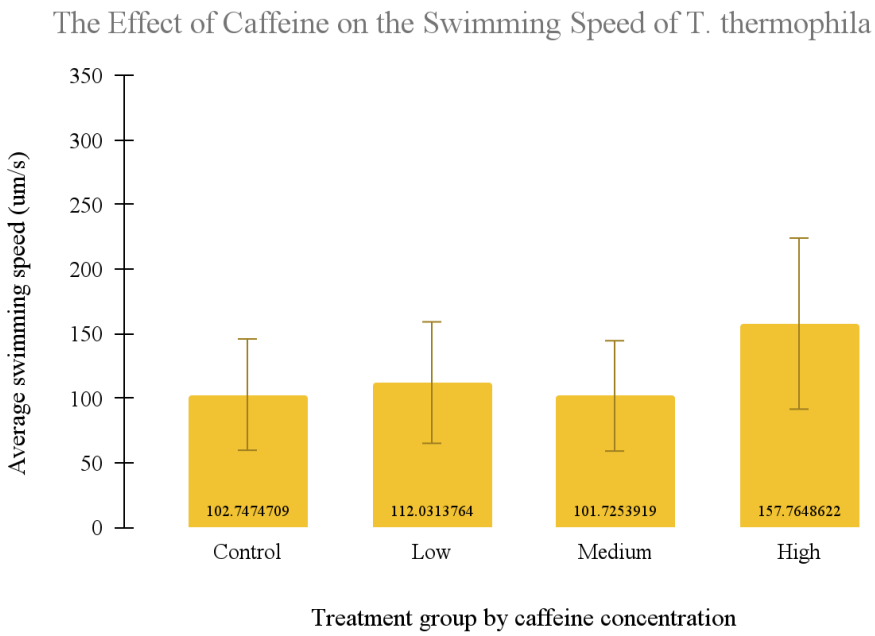

Figure 5. The average swimming speed of *T. thermophila* in caffeine treatment levels of 0 µM of caffeine, 10 µM of caffeine, 100 µM of caffeine, or 1000 µM of caffeine (control, low, medium, and high treatments respectively). The bars represent the mean swimming speed in micrometers per second of all cells exposed to the treatment, and error bars represent the 95% confidence intervals. The calculated mean swimming speed for each treatment is represented by the numbers at the bottom of the bars. 18 cells analyzed for each treatment (n=18). The Kruskal-Wallis test among the four treatments resulted in a *p*-value of 0.3699 ($p > 0.05$).

As seen in Figure 5, the highest concentration of caffeine resulted in cells with the highest average swimming speed at 157.76 μm/s, and the medium caffeine concentration had *T. thermophila* with the lowest average velocity at 101.73 μm/s. The overall trend in the data is such that as the concentration of caffeine increases from 0 μM in the control treatment to 1000 μM in the high treatment, the average swimming speed of *T. thermophila* also increases, with the exception of the decrease in average speed seen in the medium treatment. The cells in the high treatment showed the greatest increase in average swimming velocity compared to the cells in control, at 55.02 μm/s faster than the average velocity of the cells in the control treatment. The average swimming velocity of the *T. thermophila* from high concentration treatment was approximately 1.5 times faster than the control group. In contrast, the average velocities of the low and medium caffeine treatments were relatively similar to the average swimming velocity of the control. The average swimming speed of cells exposed to the low caffeine concentration was 9.28 μm/s (or 1.09 times) faster than control group, and the average swimming speed of cells in the medium caffeine concentration was 1.02 μm/s (or 0.99 times) slower than the control group.

The data did not satisfy the assumptions of normality required to conduct the one-way ANOVA test. Since the experiment contained one explanatory variable, the concentration of caffeine solution upon the difference between the swimming speed of *T. thermophila* cells, we conducted a Kruskal-Wallis test by rank through RStudio as a non-parametric alternative to the one-way ANOVA. The test returned a *p*-value of 0.3699, which is greater than the alpha of 0.05. There was no need to conduct a multiple pairwise-comparison between groups, as there was no significant difference in the average swimming speeds overall, and therefore no difference between pairs of groups.

Discussion

The purpose of this study is to determine the impact of caffeine concentration on the swimming speed of *T. thermophila.* Based on a Kruskal-Wallis test result, we failed to reject the null hypothesis that caffeine concentration has no impact on the swimming speed of *T. thermophila.* Furthermore, there appears to be a general trend in increased swimming velocity of *T. thermophila* as the cells are exposed to

increasing caffeine concentrations, although this upward trend is not significant and is not consistent across all treatment groups, as the medium treatment group showed cells with the lowest swimming velocity. As a result, our prediction that as the concentration of caffeine *T. thermophila* are exposed to increases, the mean swimming velocity of the cells will also decrease, is not supported.

A main source of error in this experiment was likely to be caused by the high level of variation between the average velocities across cells in the same treatment group. For example, in the high caffeine concentration treatment group, one cell was found to have an average velocity of 214.48 μm/s, while the velocity of another cell in the same treatment was 34.63 μm/s. This high variance in swimming speed may be the result of *T. thermophila* cells' ability to morph into elongated cells with many more cilia, which function in long distance dispersal (Fjerdingstad et al., 2007). The elongated *T. thermophila* morphs have been seen to have much greater swimming speeds than the typical *T. thermophila* cells and they are likely to contribute to the large variance in data. Since larger variance in data leads to higher uncertainty in the result, the calculated average swimming velocity for each treatment group may not accurately reflect the true average swimming velocity. To minimize the impact caused by large variance, we should include more cells in data as increasing the sample size can decrease the standard error and increase the precision of the result .

A study by Hartfiel and Amrhein (1976) examined the effects of various methylxanthines, including caffeine, upon species of flagellated algae. They found that caffeine was the most potent methylxanthine tested, as after exposure to 1 mM of caffeine, the flagellar movement of *Chlamydomonas reinhardtii* was inhibited, and after exposure to 3 mM of caffeine, *C. reinhardtii* experienced a complete inhibition of cell movement (Hartfiel & Amrhein, 1976). However, the inhibitory impacts of caffeine as observed in *C. reinhardtii* were found to be greatly reduced when other algal species were exposed to similar levels of caffeine (Hartfiel & Amrhein, 1976). Further, Wolff (1973) also found that when exposed to high caffeine concentrations, *Tetrahymena* experiences decreases in swimming speeds. These findings do not align with the trend in our data, wherein the swimming speeds of *T. thermophila* increased with exposure to increasing caffeine concentrations (except in the medium treatment). However, during

analysis of the videos, we observed that some *T. thermophila* cells at high caffeine concentration demonstrated a decrease in movement levels or stopped moving completely, however this observation is not reflected in our results because the cells that were randomly selected exhibited movement. We noted that in comparison to the control treatment, at the high caffeine concentration cells tended to be stationary more frequently, and many cells also appeared to aggregate together and stop moving. This observation that *T. thermophila* cells tended to aggregate together and exhibit slower swimming speeds at high caffeine concentrations does in fact coincide with the findings of Hartfiel and Amrhein (1976) and Wolff (1973) that the movement of the cells decreases as the caffeine concentration increases.

There are several additional sources of uncertainty and error within our experiment. It has been found that the concentration of cAMP within the cells, the factor that impacts the ciliary beating frequency, changes as the *T. thermophila* cells undergo different growth stages (Kariya et al., 1973). As we do not know what growth stage the cells were in at the time the experiment took place, the cells may have had varied levels of cAMP due to their age, which impacts the swimming speed in a manner that is independent of the effects of caffeine that we are trying to observe. Additionally, while the selection of the cells that would be tracked was randomized, cells that were moving faster or slower than the true average movement speed may have been inadvertently selected. Coupled with the relatively small sample size, the random selection of cells with movement speeds unrepresentative of the overall group would result in variation in the swimming speeds that do not necessarily represent the true average swimming speed. There is also variation present through the nature of the movement-tracking software. If the cell did not have a great change in location between two time points, it is difficult to estimate the actual distance traveled by the cell as the software prohibits selecting pixels that are too close to each other precisely. This error can be minimized by tracking the cell through a longer period of time for them to travel sufficient distance. Lastly, there is bias against cells that are moving extraordinarily fast because they often move out of the field of view too quickly for their movement to be tracked, as well as a bias against cells that were stationary because the movement of the cell could not be tracked if the cell was not moving. Thus, the swimming speeds of both fast and stationary cells are excluded from the results, and a

bias towards cells moving at a moderate velocity is introduced. To minimize this bias, a wider field of view could be utilized such that fast moving cells are visible for longer and their movement can be tracked, and the zero velocity of stationary cells could be included in the calculations of average swimming speeds.

Further studies may focus on other chemicals' impact upon the physiology of *T. thermophila*. There are many chemicals released into the water system, and their impacts on the microbe community remain unknown. Common household items like cleaners (detergents/bleach), food items (carbonated beverages/fruit juices), and gardening items (pesticides/fertilizer) that contain various chemicals and heavy metals constantly enter the water systems as the result of human activities. Through studying their effects on *T. thermophila*, we can gain a better understanding on how these substances affect the microbe community and ecosystem in general.

Conclusion

Based on the result of this study, our prediction was not supported. We failed to reject our null hypothesis and thus failed to support our alternative hypothesis that caffeine concentration has an impact on the swimming speed of *T. thermophila*. We observed that the swimming speeds of *T. thermophila* generally increases with increasing caffeine concentration. However, the result of a Kruskal-Wallis statistical test suggested no significant differences among the average swimming speed of *T. thermophila* in different caffeine concentration treatments. Also, the observed trend was inconsistent as the medium treatment group demonstrated the lowest average swimming speed. Our results do not align with previous studies possibly due to the small sample size.

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Appendix A

Table 1. Table of statistics of each treatment sample (control, high (1000 μM), medium (100 μM), and low (10 μM)).

	Control 0 M	High conc. 1000 μM	Medium conc. 100 μM	Low conc. 10 μM
Sample size (n)	18	18	18	18
Sum (Σx)	1849.4545	2839.7675	1831.0571	2016.5648
Mean (μ)	102.7475	157.7649	101.7254	112.0314
Variance (σ^2)	5499.6011	14617.4151	5359.7264	5936.5047
Standard deviation (σ)	74.1593	120.9025	73.2102	77.0487

Table 2. The Kruskal-Wallis rank sum test results with alpha 0.05 for the four treatment groups (control, high (1000 μM), medium (100 μM), and low (10 μM)).

Source	Kruskal-Wallis chi-squared	Degrees of freedom	*p*-value
Velocity by treatment	3.1441	3	0.3699