

Effect of caffeine addition on population growth of *Tetrahymena thermophila*

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Abstract

Tetrahymena thermophila is a unicellular eukaryote commonly used as a model organism in biological research. While there have been several studies on the effect of various substances on the population growth of *T. thermophila*, the effects of caffeine have not been widely studied. The purpose of this study was to characterize the effects of different concentrations of caffeine on the population growth of *T. thermophila* therefore determining if an increased concentration decreases the population growth. After exposing *T. thermophila* to 0 M, 10^{-1} M, 10^{-2} M, 10^{-3} M, and 10^{-4} M caffeine solutions, the population growth was monitored with a haemocytometer-based counting method at time intervals over a 24 hour period. From 4 hours onwards, our results suggest that caffeine concentrations of 10^{-3} M or higher significantly decrease population growth (all P-values < 0.05) when compared to the control which had no caffeine. The long-term investigation of the population sizes (after 24 hours) also suggests a mechanism of adaptation or resistance to caffeine as the populations exposed to the highest caffeine concentration (10^{-1} M) partially recovered after initially decreasing.

Résumé

Tetrahymena thermophila est un organisme eucaryote unicellulaire, souvent utilisé comme organisme modèle en recherche biologique. L'effet de nombreuses substances a été testé sur la croissance démographique de *T. thermophila*, mais les effets de la caféine n'ont été étudiés que partiellement. Le but de cette expérience était de caractériser les effets de différentes concentrations de caféine sur la croissance démographique de *T. thermophila* et ainsi de déterminer si une augmentation de la concentration de caféine engendre une diminution de la croissance. Après avoir exposé *T. thermophila* à des solutions de caféine de concentrations 0 M, 10^{-1} M, 10^{-2} M, 10^{-3} M, et 10^{-4} M, la croissance démographique a été examinée avec une méthode de comptage utilisant une Cellule de Malassez durant une période de 24 heures. Dès 4 heures, nos résultats suggèrent qu'une concentration de caféine supérieure ou égale à 10^{-3} M diminue la croissance démographique de manière significative (toutes valeurs $P > 0.05$). Les mesures de population prises sur le long terme (après 24 heures) suggèrent également un mécanisme d'adaptation ou de résistance à la caféine, puisque les effectifs des populations exposées à la plus haute concentration de caféine (10^{-1} M) ont partiellement ré-augmenté après une réduction initiale.

Introduction

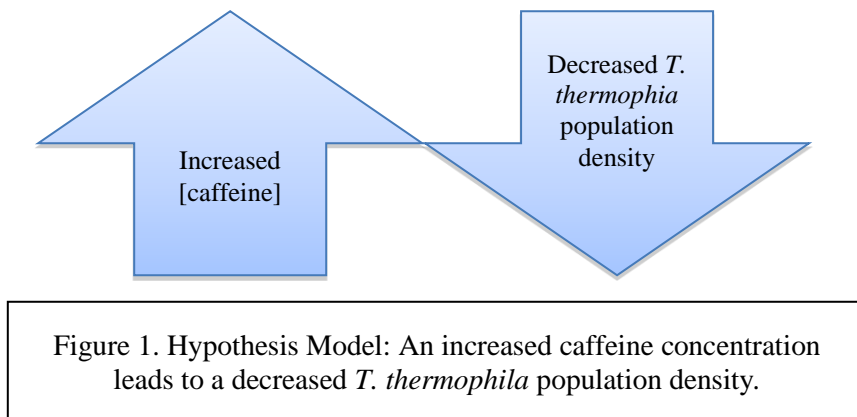
Tetrahymena thermophila is a unicellular, teardrop-shaped ciliate, which grows to be about 50 μm long and lives in freshwater environments (Wloga and Frankel 2012). This organism has been widely used as a powerful model system in population, molecular and cellular biology. As an example, the effect of various highly toxic chemicals has been tested on *T. thermophila*, such as colchicine (Singhal and Wolfe 2005) and arsenic (Yin *et al.* 2011).

However, studying the effect of household chemicals potentially having toxic effects on cellular functions is less common.

Caffeine is an alkaloid that is well known for its stimulating effect on the mammalian nervous system and is widely accessible in various consumable forms. However, its specific effects on human cells remain unclear. Furthermore, it is one of the most common household compounds found in sewage and thus its impact on freshwater organisms should be studied carefully (Gibson *et al.* 2012). For these reasons, it is fitting to examine the effects of this chemical on a simple organism's population and cell physiology.

For a long time, caffeine has been believed to alter various DNA repair processes during the cell cycle (Gibson *et al.* 2012), thus potentially causing DNA to accumulate mutations. Specifically, caffeine has been found to inhibit the G2 and S checkpoints of the cell cycle during the DNA replication of human and ciliate cells and is able to reverse delays in cell proliferation (Kaufmann *et al.* 2003, Kaczanowski and Kiersnowska 2011).

The objective of this experiment was to determine the effect of differing caffeine concentrations on the population growth of *T. thermophila*. It was hypothesized that increased concentrations of caffeine would lead to a decrease in the population growth of *T. thermophila* (see Figure 1). This hypothesis was based on a study conducted by Kaczanowski and Kiersnowska (2011), which showed that *T. thermophila* cells stimulated by caffeine underwent quick division but subsequently lost many major cellular structures such as cilia and oral structures, and finally died. Alternatively, our null hypothesis was that an increase in the concentrations of caffeine would lead to an increase or no change in the population growth of *T. thermophila*.



Methods

Caffeine stock solutions

We obtained four 30 mL caffeine stock solutions (2×10^{-1} M, 2×10^{-2} M, 2×10^{-3} M, and 2×10^{-4} M) through a serial dilution of the initial caffeine solution (2×10^{-1} M) with a standard medium (SSP medium). The SSP medium had a stable pH of 7.5 so that its effect was kept constant. Caffeine concentrations of 2×10^{-1} M showed signs of crystallization at room temperature, and therefore we stored them in a water bath at 40°C to maintain a liquid solution. We stored all other treatments in a 30°C water bath.

Treatments and cell solutions

According to Cassidy-Hanley 2012, an initial cell density of about 5,500 cells/mL is optimal for a *T. thermophila* population to grow for 24 hours without density-related growth inhibition. To obtain this cell density, we first prepared a 120 mL stock solution of 11,000 cells/mL from the medium provided. We then diluted this stock solution to half its concentration (see Figure 2) in order to obtain 5,500 cells/mL for each sample.

We prepared five different caffeine concentration treatments in 15 mL test tubes (control: 0 M and treatments 1 to 4: 10^{-1} M, 10^{-2} M, 10^{-3} M, and 10^{-4} M respectively). For each tube, we took 5 mL from the respective caffeine stock solution, and added 5 mL of *T. thermophila* (1.1×10^4 cells/mL), resulting in an initial population density of about 5,500 cells/mL in the different caffeine concentrations (see

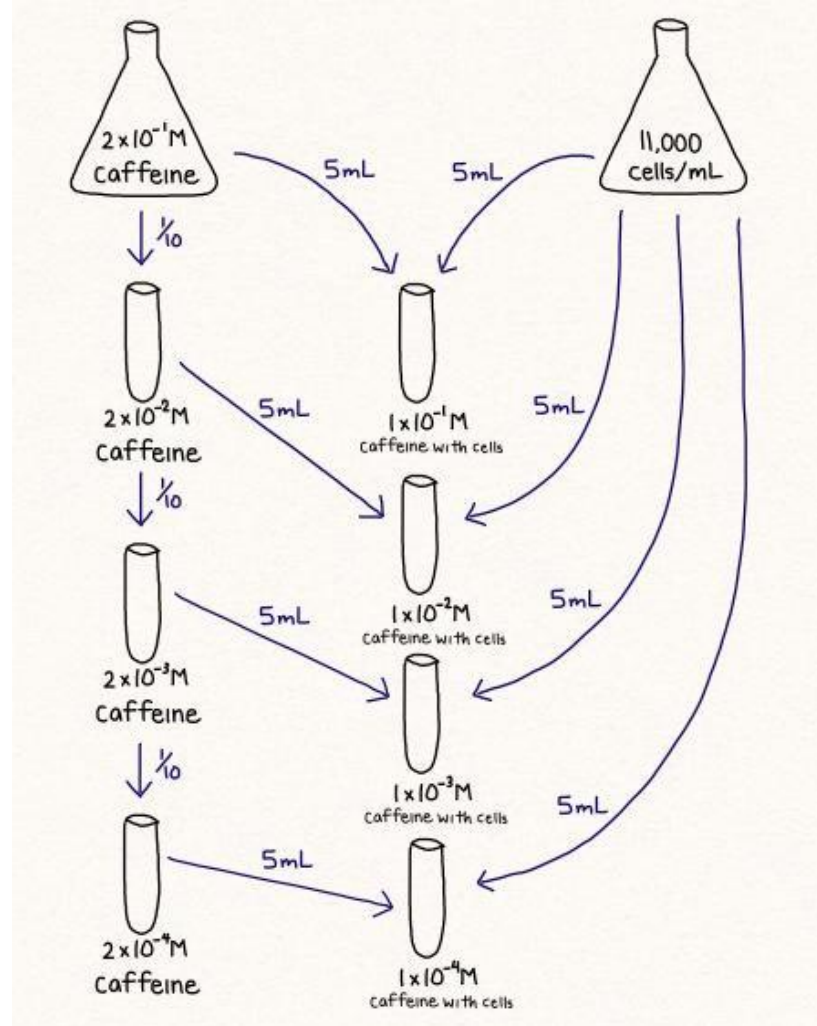


Figure 2). We replicated each

treatment four times, resulting in 20 prepared tubes. The first concentration (0 M) was used as a control to show the expected population growth without caffeine. We expected the highest caffeine concentration to be lethal, as described by Eisen (1961) in his study with *Tetrahymena pyriformis*, a species closely related to our study species.

Incubation conditions

We stored all the tubes in a water bath at a constant temperature of 30°C , as the standard method of Orias *et al.* 1999 describes for a doubling time of two hours. At times of data collection, we set all tubes on tube racks for the duration of the data collection process and

returned them to the water bath at the same time to ensure the replicates of all treatments were subjected to the same conditions.

Data collection and population density estimates

We performed a sterile procedure following the guidelines provided by Orias *et al.* (1999) for every transfer of *T. thermophila* in order to avoid contamination.

We collected data at the beginning of the experiment immediately after the different treatments were set up (this was marked as time 0), then every two hours following, for a total of six hours. We also collected data the next day, 24 hours after the beginning of the experiment to account for long-term trends. To estimate the population density of each solution, we used a Fuchs-Rosenthal haemocytometer-based counting method. We fixed the cells with glutaraldehyde



Figure 2. *T. thermophila* in a 1 mm² grid of a Fuchs-Rosenthal haemocytometer, as photographed with a Dinoscope and Leitz dissecting microscope, at a total magnification of 32x.

(dilution factor 1.1) and observed them under a Zeiss Axiostar compound microscope with a total magnification of 100x, thereby determining their numbers in ten 1 mm² grids (see Figure 3; dilution factor 5×10^3). Then, we calculated the average cell counts for each replicate. To account for the cell dilution, we multiplied the mean number of cells/mm² by $1.1 \times 5 \times 10^3$, the dilution factor of fixative and the dilution factor of the 1mm² grid to obtain the population density (cells/mL). We also observed any morphological changes in the cells under the microscope and measured any recognizable change in cell size.

Statistical Analysis

To allow for a visual comparison of the effect of each concentration on population growth over time, we calculated and graphed the mean population density at time t for each treatment. Then, we calculated the 95% confidence intervals (CIs) of each mean to test for significant differences among the four treatments and the control, and among time intervals. Finally, we performed a t-test when the 95% confidence intervals between treatments did not overlap to determine the level of significance (P value).

Results

General trends in population growth from $t=0$ to $t=6$ hours

Figure 4 is a graph of the mean cell count/mL of *T. thermophila* in each treatment at two-hour time intervals over a total six-hour period. The control without caffeine showed continuous growth with increasing time, with a steady increase of approximately 5,000 cells/mL every two hours from four hours onwards. Treatments 1 and 2 with 10^{-1} M and 10^{-2} M caffeine respectively showed a decrease in mean cell count over time. The population size of treatment 3 of 10^{-3} M caffeine remained stable over time, showing significantly lower population growth than treatment 4 (10^{-4} M caffeine) and the control from 4 hours onwards. Treatment 4 (10^{-4} M caffeine) and the control showed an increase in average cell count over time, with treatment 4 tending to have the highest cell count/mL compared to all of the treatments (the difference was not significant).

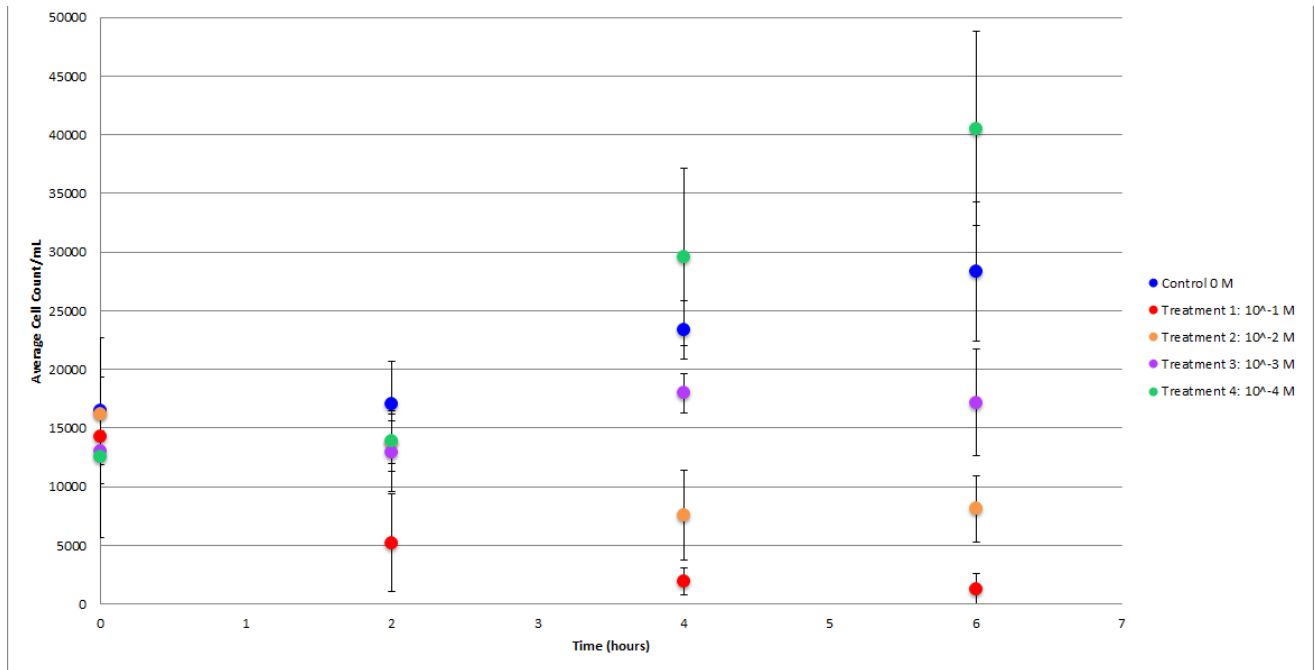


Figure 4. *Tetrahymena thermophila* population growth in caffeine. Increased concentrations of caffeine decreased population growth of *T. thermophila* with 95% confidence intervals (CI).

Sample calculations of the quantitative values

Table 1. Sample calculation of the mean population density (cells/mL) for the control

Replicate I.D.	1	2	3	4	Average
Cell count/mL	22,000	11,000	22,000	11,000	Sum of replicates / number of replicates: (22,000+11,000+22,000+11,000)/4 =16,500 cells/mL

Standard deviation:

$$\sigma = \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n-1}}$$

Where σ is the standard deviation, x_i is the cell count/mL for replicate i

where $i = 1, 2, 3, \text{ or } 4$, \bar{x} is the average of the four replicates,

and n is the number of replicates.

$$\sigma = \sqrt{\frac{[(22000 - 16500)^2 + (11000 - 16500)^2 + (22000 - 16500)^2 + (11000 - 16500)^2]}{4 - 1}}$$

$$= 6350.9$$

Wald confidence interval (CI): $CI = t \times \frac{\sigma}{\sqrt{n}}$,

Where t is 1.96 for a two-sided 95% confidence interval.

$$CI = 1.96 \times \frac{6350.9}{\sqrt{4}} = 6224$$

$$\text{Student t-test: } s = \sqrt{\frac{\sum(x-\bar{x}_1)^2 + \sum(x-\bar{x}_2)^2}{n_1+n_2-2}} \quad t = \frac{\bar{x}_1 - \bar{x}_2}{s \times \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where \bar{x}_1 and \bar{x}_2 are the means of the two samples, n_1 and n_2 are the numbers in each sample and s is the combined standard deviation of the two samples.

If t is higher than the associated value in a Student table, the P-value is lower than 0.05.

Short-term results

The 95% confidence intervals of the average cell counts/mL are shown for each data point in Figure 4. At time $t=0$ hours, all of the CIs overlap for all the treatments. At time $t=2$ hours, a slight separation is visible, as the CI of treatment 1 does not overlap with any other treatment (all P-values <0.05). At time $t=4$ hours and $t=6$ hours, the divergence between treatments is amplified: the CIs overlap between the control and 10^{-4} M caffeine, but the CIs of the rest of the treatments do not overlap (all P-values <0.05).

Long-term results

Another cell count was taken for all treatments at t=24 hours (see Table 2). The data collected at this time showed similar trends of growth for the control and treatment 2. Treatment 3 maintained a stable population density from 12,000-18,000 cells/mL over 24 hours. However, treatment 1 showed a significant increase after the initial decline (P=0.001), while still maintaining the lowest population. Finally, treatment 4 showed a decreasing trend in mean population density over time (P=0.069 between 6 and 24 hours), but its population size after 24 hours still did not differ significantly from the control.

Table 2. Mean Cell Count/mL for Each Treatment After t=24 hours

Treatment	Control	4	3	2	1
Caffeine concentration (M)	0	10^{-4}	10^{-3}	10^{-2}	10^{-1}
Density (cells/mL) \pm SD	33,825 \pm 3,127	29,013 \pm 2,898	18,563 \pm 2,997	9,075 \pm 1,426	9,487 \pm 2,082

Qualitative observations

After 24 hours, the cell size was noticeably smaller (20-30 μm versus 40-50 μm) and more spherical in shape in treatments 1 and 2 than in the control, treatment 3 and treatment 4.

Discussion

General trends

The overlapping of the CIs of the average cell counts/mL in each treatment at t=0 hours was expected, as there may not have been enough time for the caffeine to have any significant effect on the population size. At t=2 hours, the CIs of all treatments overlap except for the 10^{-1} M

caffeine, which diverges from the other treatments. Indeed, the t-test comparing treatment 1 and the control at this time showed a very low p-value of 0.006, indicating a statistically significant effect on the population size. At t=4 hours and t=6 hours, the overlap of confidence intervals of the control and treatment 4 showed a difference that is not statistically significant, suggesting that a low caffeine concentration has no major effect on population growth. Caffeine concentrations of 10^{-3} M, 10^{-2} M, 10^{-1} M do not overlap showing that in comparison to t=0 hours and t=2 hours, as time increases, differences between treatments become more significant, leaving only the control and treatment 4 values similar. Based on the results from t=4 hours onwards, we reject the null hypothesis of increased concentrations of caffeine leading to an increase or no change in the population growth of *T. thermophila*. Further, we support our alternate hypothesis, which states that an increased caffeine concentration leads to a decrease in population growth of *T. thermophila*.

Lowest caffeine concentration (treatment 4)

The general trend from the data presented in Figure 4 shows an increase in population size over time for treatment 4. However, the comparison of this concentration with the control never leads to a significant difference between the two (P= 0.20, 0.11 and 0.069 for t=4 hours, t=6 hours and t=24 hours respectively). This suggests that a 10^{-4} M caffeine concentration can be tolerated and does not have a statistically significant effect on the population growth of *Tetrahymena*. However, the population size with treatment 4 is still higher than the population in the control group at t=4 hours, t=6 hours and t=24 hours and the difference between them increases over time. This suggests that some positive effect of treatment 4 on the population size could be significant in an experiment conducted over a longer time period. This trend could be

explained by the fact that a low concentration of caffeine allows the cells to skip DNA damage checkpoints at the G2 and S phase of the cell cycle, allowing the population to grow continuously without breaks in the short-term (Kaczanowski and Kiersnowska 2011). Further long term studies should be done to know whether a significant positive or negative impact would occur in these populations.

Highest caffeine concentration (treatment 1)

In contrast, the 10^{-1} M caffeine treatment shows a statistically significant decrease in population size over time, as the CIs associated with it at t=4 hours, t=6 hours and t=24 hours do not overlap with the CI at t=0 hours. Moreover, the population size in this treatment is significantly lower than in all other treatments from t=2 hours onwards. These results suggest that treatment 1 may have negative effects on *T. thermophila*, resulting in a population decline over time. This agrees with our alternate hypothesis as well as with conclusions from Eisen (1960) who noted that 10^{-1} M caffeine was found to be deleterious to *Tetrahymena*. This is also supported by Gibson *et al.* (2012), who observed potential DNA mutation accumulations due to caffeine. However, the measurements at t=24 hours (see Table 2) show a significant increase in population density from 1200 cells/mL to about 9,500 cells/mL (P=0.001).

We hypothesize that this partial recovery may be due to a physiological adaptation that occurred because of an initial genetic variation present within the population. The adaptation could have then been passed on to daughter cells in subsequent generations allowing for increased caffeine tolerance. Alternatively, the high concentration of caffeine could have played the role of a strong selective force, causing the cells to maintain spontaneous mutations, favoring a new progeny of caffeine-tolerant mutants. These hypothetical mutants may have survived and

passed the tolerance on to new generations, thus bringing the population back up. It is important to mention that the selection and description of resistance to lethal concentrations of caffeine was already done with the prokaryotic model *E. coli* (Delvaux and Devoret 1969) and thus could occur in higher organisms such as *T. thermophila*.

Long-term results

The results after 24 hours fully support our alternate hypothesis, and allow us to reject the null hypothesis. The gradual differentiation of population sizes into three significantly distinct groups (Table 2) shows how an increased concentration of caffeine has a negative impact on population growth. High concentrations of caffeine (treatments 1 and 2) lead to a significant decrease in population size over time, from t=0 hours to t=24 hours (about 14,300-16,200 cells/mL versus 9,000-9,400 cells/mL). This confirms the deleterious effect of a high caffeine concentration on our organism, similar to that observed by Kaczanowski and Kiersnowska (2011). The intermediate caffeine concentration (treatment 3) shows no significant change in population size over time (see Figure 4 and Table 2), but as the population size is significantly lower than the control from 4 hours onwards, we can conclude that caffeine inhibited population growth in this treatment as well. Finally, little or no caffeine (treatment 4 and control) shows an increase in population size from t=0 hours to t=24 hours (P=0.069).

Morphological changes of cells exposed to high concentrations of caffeine

The morphological changes observed in cells in treatments 1 and 2 may have been due to the caffeine promoting quicker cell division by inhibiting the DNA damage checkpoints during the cell cycle, particularly during the G2 phase. Consequently, the cells would have lacked the

time needed for normal growth (Kaczanowski and Kiersnowska 2011). Alternatively, caffeine resistant cells may have suffered energetic costs due to their ability to tolerate caffeine, thus resulting in their deformed shape.

Sources of error and variation

The experimental results may have been affected by the introduction of other variables at various stages of the experiment. For instance, during the preparation of the 10^{-1} M caffeine solutions, the caffeine began to solidify at room temperature, thus those specific tubes were placed in 40°C water bath to melt the caffeine. As this treatment was only applied to the affected test tubes, the increase in temperature may have altered the cell counts of the affected solutions. Additionally, the water bath used to store the treatments at 30°C was shared with other students, causing irregular opening of the bath, possibly leading to changes in several conditions we may have been unable to control. To avoid large variation in our data, steps were taken to avoid errors by ensuring all group members followed the same criteria for cell counting.

A final and significant source of error was that the starting cell density was 2-3 times greater than expected (15,000 cells/mL vs. 5,500 cells/mL), due to significant delays at the beginning of the experiment. This larger starting population size could have affected the results by providing non-optimal density conditions for cell growth, as well as providing a larger pool of genes, thus a greater chance of having caffeine resistant mutants in the population. However, because we used the same stock solution of cells with our initial population count, all subsequent treatments were standardized.

Conclusion

The long-term results support our alternate hypothesis that increased concentrations of caffeine have a negative impact on population growth of *T. thermophila*, as higher caffeine concentrations of 10^{-1} M and 10^{-2} M led to a significant decrease in population size over a 24 hour period. The intermediate caffeine concentration of 10^{-3} M showed no significant change in population size, while the lowest caffeine concentration of 10^{-4} M and control showed a significant increase in population size.

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