The effect of nitrogen-deprivation on mating and reproduction of *Chlamydomonas reinhardtii*

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Abstract:

The importance of *Chlamydomonas reinhardtii* flagella in mating was studied in this experiment using wild-type, biflagellated populations of both mating types (Wt+ and Wt-) and mutant, non-flagellated populations (M-). All populations were grown without nitrogen for 48 hours in continuous light, to provide sufficient time and appropriate conditions for gametes to form. After 48 hours, four sample cultures were created. These included Wt+/Wt- and Wt+/M- combinations which would remain in a nitrogen-rich medium for the duration of the experiment, as well as Wt+/Wt- and Wt+/M- combinations which would remain in a nitrogen-free medium for an additional 120 hours before being re-suspended in regular medium. The cell abundance of each sample type was studied at 24 hour intervals for a total of 216 hours. It was hypothesized that the Wt+/Wt- cell abundance in the samples exposed to nitrogen deprivation would recover sooner than the cell abundance in the Wt+/M- samples which had also been exposed to nitrogen deprivation after returning to regular medium because functional flagella are required for successful protective zygote formation in nitrogen-deprived conditions. The cell abundance in the samples grown in regular medium during the entire experiment were used as a control, showing regular growth curves for each mating type population. It was observed that the Wt+/Wt- which had gone through nitrogen deprivation had significantly higher cell abundance at t=192 hours than the Wt+/M- mating pair in the same conditions (2.21×10^5 cells $\pm$ 1.34×10^4 cells/mL compared to 6.60×10^4 cells $\pm$ 1.63×10^4 cells/mL respectively). Based on these results, zygote formation was successful between Wt+/Wt- in the nitrogen-deprived conditions, due to functional flagella, and this aided in the continuation of reproduction upon return to a nitrogen rich environment.

Introduction:

Chlamydomonas rienhardtii is a green, biflagellated single celled organism. They have light receptors, the capacity to perform sexual and asexual reproduction under certain environmental conditions, and the ability to photosynthesize (Harris 2001). Due to these traits, *Chlamydomonas* is an ideal organism to study for a variety of topics, such as flagella construction and motility, photosynthesis mechanisms, and environmental conditions for sexual reproduction (Silflow and Lefebvre 2001). The topic of reproduction mechanism - sexual compared to asexual – under various environmental conditions is of particular interest in this experiment, and is studied through crosses between wild type and mutant *Chlamydomonas* strains, while manipulating the presence of nitrogen.

Asexual reproduction occurs through the mitotic division of a parent cell into identical daughter cells; this type of mating occurs during favorable conditions (Wilson 2008). Conversely, sexual reproduction is induced by nitrogen deficient environments, and can only occur between opposite mating types, mt+ and mt- (Colegrave *et al*, 2002). This unfavorable condition stimulates gametogenesis, wherein the flagella become agglutinable (have the ability to recognize flagella of the opposite mating type) and the gametes develop mating structures on the plasma membrane (Brawley and Johnson 1992). Sexual reproduction is achieved through the flagellar adhesion of opposite mating types, facilitated by glycoproteins known as adhesins (Brawley and Johnson 1992). The two cells, once fused together, disintegrate their flagella and enter a dormant state in which the fused cells possess a thick wall that is filled with starch and lipids. Referred to as a hypnozygote, the fused cell is diploid, non-motile, and will germinate upon a return to favorable conditions (Chakmak *et al* 2012).

The purpose of this experiment was to explore the role of flagella in sexual reproduction of *Chlamydomonas*. Wild type *Chlamydomonas* strains, with functional flagella, and a mutant *Chlamydomonas* strain, lacking functional flagella, were used in different mating combinations to ascertain the influence of flagellar function on reproductive strategies. It was hypothesized (H_a) that wild type mating pairs of *Chlamydomonas reinhardtii* exposed to a nitrogen free environment will have a higher recovery rate (measured by cell abundance) after re-suspension into a nitrogen rich environment

than the wild type/mutant mating pairs. The null hypothesis (H_o) proposed that wild type mating pairs of *C. reinhardtii* exposed to a nitrogen free environment will have no change or a slower recovery rate in cell abundance after re-suspension into a nitrogen rich environment than the wild type/mutant mating pairs.

Methods:

Chlamydomonas reinhardtii CC-1690-Wt mt+ 21 GR, CC-1927-Wt mt- (labelled Wt+ and Wt-, respectively) and CC-3913-PF9-3 mt- (labelled M-) were obtained from cultures grown by the laboratory technician. These cultures had been grown in *Chlamydomonas* maintenance medium at 17°C prior to use in our lab, and the concentration of cells in each culture was initially unknown. On the first day in the lab, 30 uL of each culture (Wt+, Wt- and M-) were transferred into separate test tubes to be viewed under the microscope for initial observations. 27.5 mL of each culture were then centrifuged on the highest speed for 5 minutes in a 50 mL falcon centrifuge tube. Following decantation, 20 mL of each culture were added to the previously centrifuged tubes, and re-centrifuged for 5 minutes on the highest speed. This method was used to decrease the amount of time each sample would take to centrifuge, rather than trying to centrifuge a 50 mL sample all at once. Each falcon tube was decanted so that only the pellet remained, and was then re-suspended in 75 mL of media without nitrogen. This "nitrogen-free" medium was prepared by the lab technician, using the chemical recipe for *Chlamydomonas* maintenance medium, but without adding nitrogen in the form of ammonium nitrate, NH_4NO_3. However, it still contained the NH_4molybdate trace metal, in case the molybdate was required for *Chlamydomonas* survival.

Samples were left to grow for 48 hours in a constantly lit, 17°C environment to form gametes while mating types were still separate. Upon the completion of this 48 hour time span, 500 uL of each culture (Wt+, Wt- and M-) was removed and mixed with 50 uL iodine solution (IKI) to fix the samples for counting. Cells were counted using haemocytometers. Using the cell numbers, the amount of sample required to obtain the same number of cells from each culture population was determined, and these

volumes were centrifuged for 5 minutes at high speed, then decanted. This was done twice for each culture type; one pellet of each culture type (Wt+, Wt- and M-) was re-suspended in 35 mL of regular *Chlamydomonas* maintenance medium (nitrogen-rich) and one pellet of each culture type was re-suspended in 35 mL of nitrogen-free medium. Once re-suspended, these six tubes were well mixed via finger vortexing to disperse the cells into the solution.

Using these six stock solutions of cells in the nitrogen-rich or nitrogen-free medium, the twelve sample tubes that would be used for the remainder of the experiment were created. Three were made using a combination of 2.5 mL of Wt+ from the nitrogen-rich medium, and 2.5 mL of Wt- also from the nitrogen-rich medium (Treatment A). Three were made using a combination of 2.5 mL of Wt+ from the nitrogen-rich medium, and 2.5 mL of M- also from the nitrogen-rich medium (Treatment B). Three were made using a combination of 2.5 mL of Wt+ from the nitrogen-free medium, and 2.5 mL of Wt- also from the nitrogen-free medium (Treatment C). The final three were made using a combination of 2.5 mL of Wt+ from the nitrogen-free medium, and 2.5 mL of M- also from the nitrogen-free medium (Treatment D). All twelve sampling tubes, at this point, contained the same concentration of cells, and these twelve populations are shown in Figure 1. Samples were observed using an Axiostar microscope until the first evidence of mating was present in the Wt+/Wt- nitrogen-free sample population. Mating was observed as two cells coming together, and all four flagella intertwining. After a moment, the two cells were observed to merge and appeared like one large cell, at which point the flagella were no longer clearly visible. When this occurred (at time = 30 minutes since creation of the twelve samples), a cell concentration count was performed for all twelve populations.

All samples were left in ventilated test tubes, on a shaking rack in a 17°C room for the duration of four days to allow for zygote maturation. On day four (120 hours after the creation of the twelve samples), counts to determine cell concentration in each sample were again completed. Counts were then taken five times at 24 hour intervals. Each time cells were counted, 100 uL of sample were removed from each of the twelve populations, and 10 uL of IKI was used to fix the cells. Data was graphed as cell

abundance in each sample over time, including 95% confidence intervals. Statistical t-tests were performed on the data from the final two sampling periods.

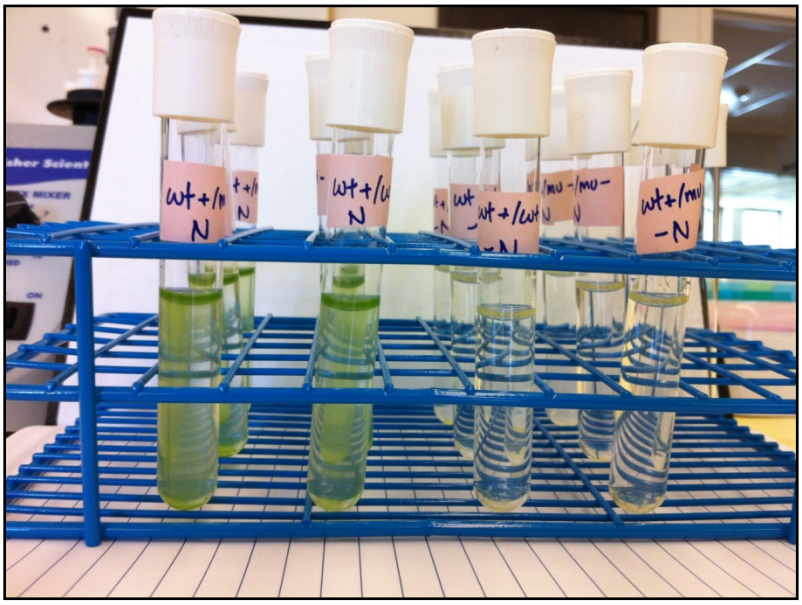

Figure 1. Laboratory set-up of the twelve test tubes to be sampled from at 0, 0.5, 120, 144, 168, 192, and 216 hours. Three tubes were Wt+/Wt- in nitrogen-rich medium for the course of the experiment. Three tubes were Wt+/M- in nitrogen-rich medium for the course of the experiment. Three tubes were Wt+/Wt- in nitrogen-free medium for the first four days of the experiment, and nitrogen-rich medium for the rest of the experiment. Three tubes were Wt+/M- in nitrogen-free medium for the first four days of the experiment, and nitrogen-containing medium for the rest of the experiment.

Results:

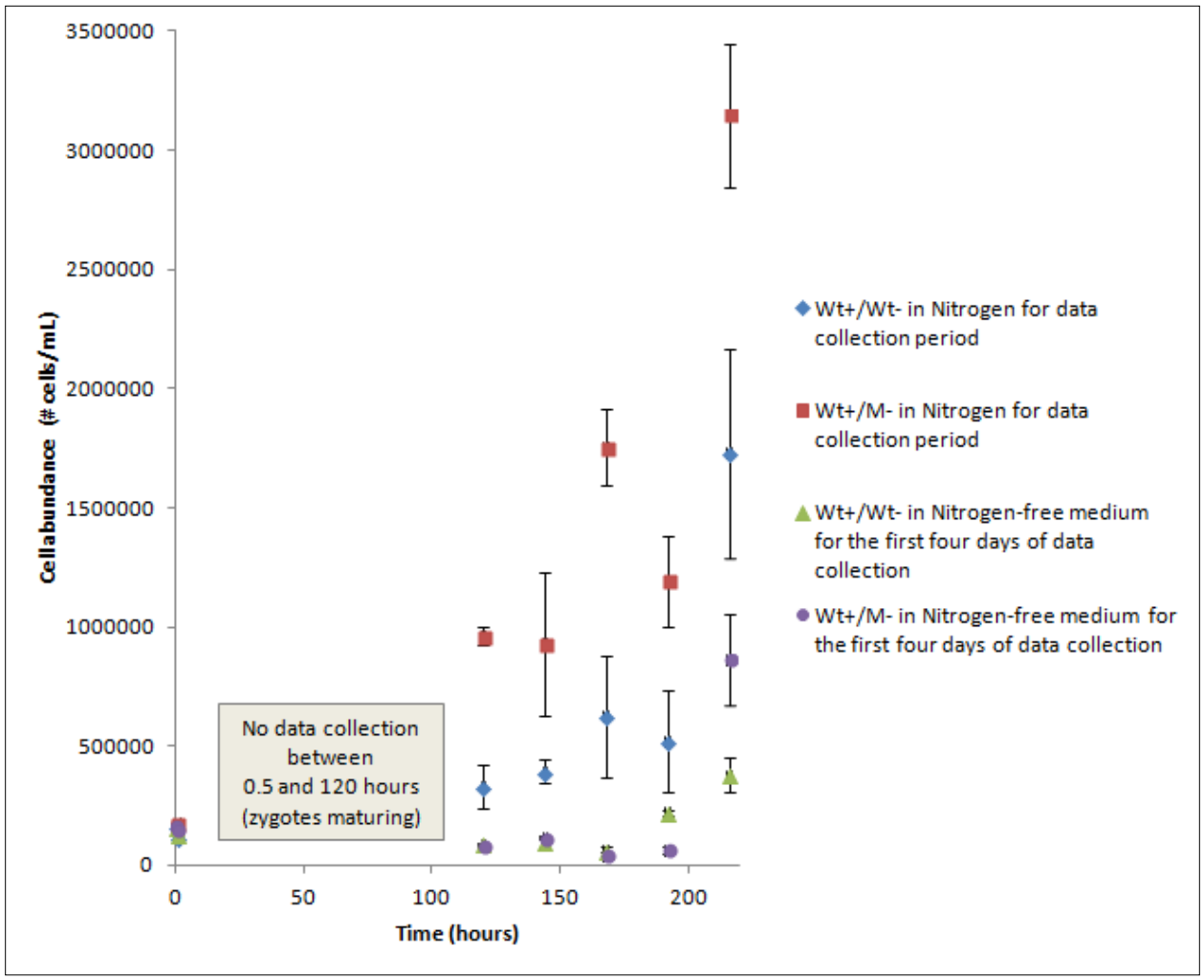

Figure 2. Cell abundance in each culture at time 0, 0.5, 120, 144, 168, 192, and 216 hours after setup of the twelve sample populations. Error bars represent 95% confidence intervals. Data at 120 hours represents the cell abundance directly before transfer of all populations into regular medium with nitrogen. Blue diamonds show data for treatment A. Red squares show data for treatment B. Green triangles show data for treatment C. Purple circles show data for treatment D. N=3.

When the twelve sample populations were setup, all twelve had an equivalent number of cells. After 30 minutes, mating was observed in both types of populations (treatments C and D) in the nitrogen-free media as shown in Figure 2, the abundance of cells in each population at this time was slightly lower than the initial count, but was not significantly less (overlapping 95% confidence intervals). At a time of 120 hours, after four days of zygote maturation for the populations in the nitrogen-free medium, and

regular growth for the populations in the nitrogen-rich medium, there was a difference between the abundance of cells in the Wt+/Wt- and Wt+/M- populations that had been grown in the nitrogen-rich medium (treatments A and B). The Wt+/M- populations in medium with nitrogen had an average of 9.59 x 10^5 ± 3.6 x 10^4 cells/mL while the Wt+/Wt- populations in nitrogen-rich medium had an average of 3.31 x 10^5 ± 8.85 x 10^4 14 cells/mL. The two types of populations which had been growing in the nitrogen-free medium for four days (treatments C and D) did not have cell abundance levels that were significantly different from one other until the counts that were completed at 192 hours (6.01 x 10^4 ± 2.09 x 10^4 cells/mL and 4.11 x 10^4 ± 1.37 x 10^4 cells/mL respectively at t=168 hours).

Until the count at 192 hours, the cells in both of the populations that had spent four days without nitrogen were observed to appear mostly in clumps of many large cells seemingly stuck together, with very few individual cells present in the samples used for counting as shown in Figures 4 and 5. From 192 hours onwards however, these two populations were observed to hold many very small individual cells in combination with the clumps viewed previously. When counted at 192 hours, the Wt+/Wt- nitrogen-free population (treatment C) was significantly larger than the Wt+/M- population (treatment D) (2.21 x 10^5 ± 1.34 x 10^4 cells/mL compared to 6.60 x 10^4 ± 1.63 x 10^4 cells/mL). The difference in cell abundance between these Wt+/M- and Wt+/Wt- groups was found to be statistically significant also using a t-test ($t(4) = 8.302$, $p < 0.05$; Figure 2).

However, at 216 hours, the Wt+/M- nitrogen-free population (treatment D) was larger than the Wt+/Wt- population (treatment C) (3.79 x 10^5 ± 7.20 x 10^4 cells/mL compared to 8.60 x 10^5 ± 1.90 x 10^5 cells/mL). Both types of populations that had been in medium with nitrogen during the four day break period (treatments A and B) were found to have mostly individual cells when observed for counting. The trend of greater cell abundance in the Wt+/M- population with nitrogen (treatment B) compared to the Wt+/Wt population with nitrogen (treatment A) continued over the course of the experiment. At all sampling points, the abundance of cells in the Wt+/M- population with nitrogen (treatment B) was significantly greater than the Wt+/Wt- population with nitrogen (treatment A).

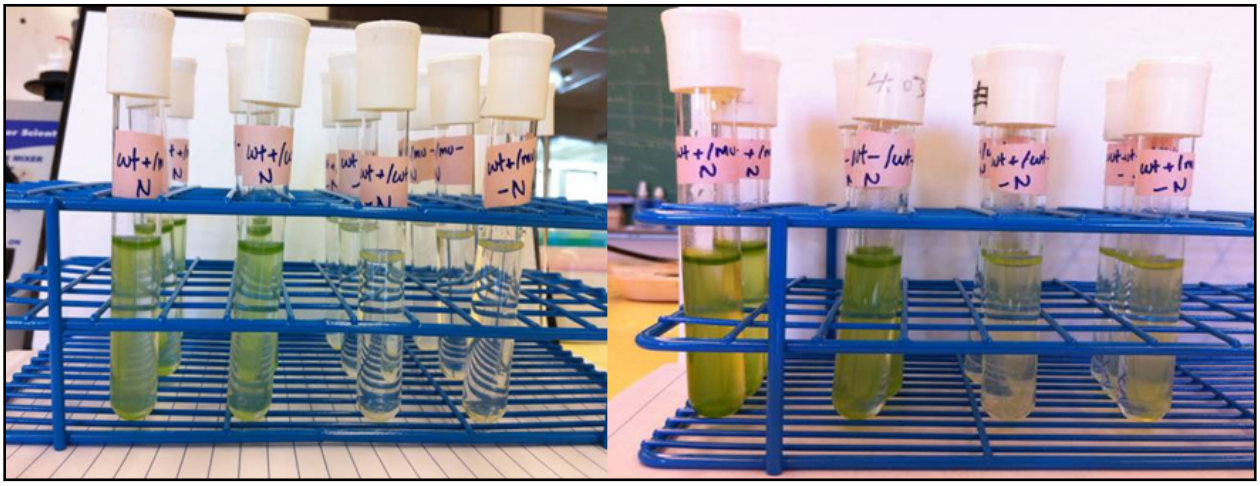

Figure 3. On the left are sample populations at time = 0 hours (first day of mating types mixed together). On the right are sample populations at time = 216 hours (final day of experiment).

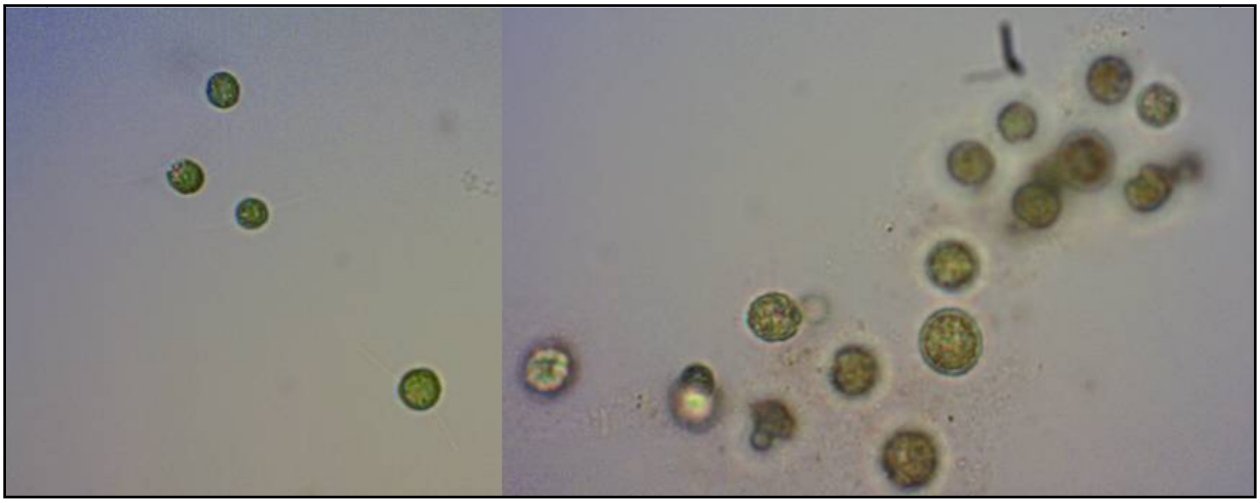

Figure 4. On the left is the Wt+/M- population in an environment with nitrogen at time = 120 hours. On the right is the Wt+/M- population in the nitrogen-free environment at time = 120 hours. Magnification = 400X.

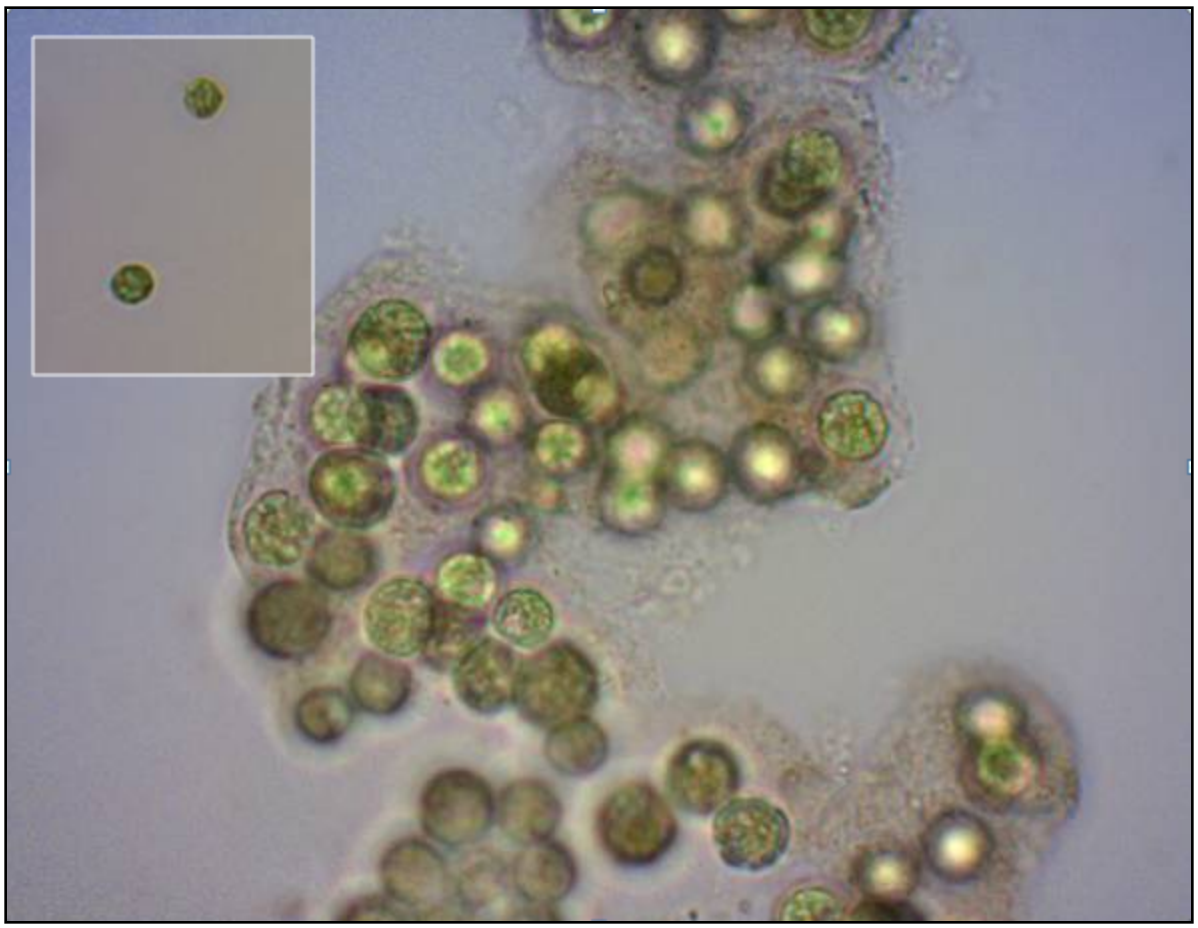

Figure 5. Full photo of Wt+/Wt- in nitrogen-free medium at time = 120 hours. Inlaid is a to-scale photo of the Wt+/Wt- in nitrogen-rich environment at time = 120 hours. Magnification = 400X.

Two Sample T-test for Wt+/M- and Wt+/Wt- mating pair cell abundance, exposed to a nitrogen-deficient environment:

$$s = \sqrt{\frac{\sum (x-\bar{x}_1)^2 + \sum (x-\bar{x}_2)^2}{n_1 + n_2 - 2}}$$

$$s = \sqrt{\frac{(211200-220733)^2 + (244200-220733)^2 + (206800-220733)^2 + (83600-66000)^2 + (77000-66000)^2 + (37400-66000)^2}{3 + 3 - 2}}$$

$$s = 22827.76$$

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{(1/n_1) + (1/n_2)}}$$

$$t = \frac{220733 - 66000}{22827.76\sqrt{(1/3)+(1/3)}}$$

$$t = 8.302$$

Figure 6. Sample calculation for t-test.

$$95\%\ C.I. = mean\ value\ \pm (1.96)\left(\frac{standard\ deviation}{\sqrt{sample\ size}}\right)$$

$$95\%\ C.I. = \bar{x}\ \pm (1.96)\left(\frac{s}{\sqrt{n}}\right)$$

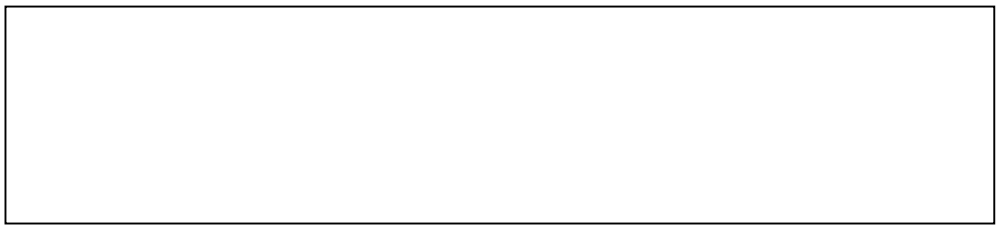

Figure 7. Sample calculation for 95% confidence interval.

Discussion:

The experiment aimed to investigate the change in cell abundance of mutant and wild type strains of *Chlamydomonas reinhardtii,* following a period of environmental nitrogen deficiency. Based on statistical analysis of our results, we reject our null hypothesis and provide support for our alternate hypothesis, that wild type mating pairs of *Chlamydomonas reinhardtii* exposed to a nitrogen free environment will have a higher recovery rate in cell abundance, upon re-suspension in a nitrogen rich environment, than the wild type/mutant mating pairs. These results support previous findings, in that mutant strains without flagella are unable to sexually mate or form zygotes and therefore have a reduced survival rate when exposed to nitrogen-deficient environments, as is reflected by recovery growth curves (Colegrave *et al*. 2002; Kates and Jones 1964; Snell 1984).

As seen in Figure 2, it was observed that 72 hours after re-suspension in a regular nitrogen buffer, there was significantly greater cell abundance in treatment C than in treatment D. As this demonstrates a difference in growth between the two mating conditions, the data suggests that the Wt+/Wt- mating pair had a survival advantage over the Wt+/M- mating pair when previously exposed to the nitrogen-deficient environment. As our mutant *Chlamydomonas reinhardtii* strain was lacking flagella, our results are in accordance with findings from Silflow and Lefebvre (2001), who demonstrated that flagellar interactions are essential for sexual reproduction and the formation of a functional zygote.

Therefore, our data supports the suggestion that mutant cells in treatment D were unable to engage in sexual reproduction due to the absence of flagella, which in turn resulted in an inability to form the protective zygote. As previous evidence from Perrin (2012) suggests, zygotic formation is an essential

mechanism used by *Chlamydomonas reinhardtii* for survival when exposed to stressed environmental conditions. It would then be expected that cells within treatment D, when exposed to the nitrogen-deficient medium, would be at a survival disadvantage when compared to cells of treatment C, which all have functional flagella and would therefore be expected to undergo normal zygotic development. Upon re-suspension in a normal nitrogen medium, findings from previous research would suggest that all dormant zygotes formed under the stressed environment would begin to divide, and growth curves would again be observed (Bell 2005). Therefore, as Figure 2 demonstrates a significant increase in cell abundance in treatment C at 192 hours, but not in treatment D, our data suggest that the mutant cells in treatment D cause abnormal dormancy and an inhibited survival in the nitrogen deficient environment, resulting in a delayed growth curve upon re-suspension in the nitrogen rich environment.

The observed growth curves of treatment C and D in the first 168 hours of the experiment agree with previous research, in that cells placed in a nitrogen-deficient environment go into a dormant phase in which no division or reproduction is observed (Sager and Granick 1954). Furthermore, as the control treatments A and B show increasing cell abundance over the same time intervals, it can be argued that the observed lack of change in cell abundance in treatments C and D results from the manipulated variable, nitrogen availability. Therefore, upon a return back to the normal medium, the altered growth curves observed in treatments C and D are reflective of changes within the population under the nitrogen-deficient environment.

Another interesting observation was made, when a final cell count was taken 96 hours after re-suspension in the nitrogen-containing medium. As seen in Figure 2, the data show significantly greater cell abundance in treatment D than in treatment C, suggesting treatment D cell abundance has increased much faster than treatment C. While this initially appeared contradictory to our expected results, a possible solution is presented in the observations recorded from the experiment. It was noted that while viewing the organisms under the microscope, the mutant cells appeared to be notably smaller than the wild-type cells, possibly indicating an increased rate of cell division. Furthermore, it was also observed that treatment A had a greater cell abundance than treatment B at each counting interval, suggesting that

the mutant strain underwent division at a faster rate than the wild-type strain under normal conditions. Therefore, the increased abundance of the treatment D cells at 216 hours is not particularly disconcerting to our hypotheses, as the surviving Wt+/M- cells, with their possible faster division rate, may have had sufficient time to grow and divide such that their numbers can no longer be used as a representation of the population surviving the nitrogen-starved environment.

Variations were observed among the cells within each culture, which could have led to inconsistencies in our results. The most notable variation was observed among the mutant cells, which were described as having no flagella. When viewing the cells through the Axiostar microscope, it was clear that some of the mutant cells did indeed have flagella and were motile. While these flagella did still appear to be deformed and shorter in length then the wild-type flagella, this could be the reason why some mating amongst the Wt+/M- samples was observed, when none was expected. The behavior of the cells in each treatment also differed, and caused distinctive problems when counting cell abundance. When the zygotes in the nitrogen deprived medium formed, they aggregated in clumps and were not freely moving through the media (Figure 4). This caused counting errors, as the cells were not evenly distributed throughout the counting well, therefore giving a skewed estimation of the true cell abundance within the sample. Aside from these variations, there were also some potential errors in the centrifuge processes. It was observed that while centrifuging, the cells often stuck along the sides of the tubes, and therefore the residue that was discarded with the tubes could have resulted in a lowered cell abundance observed within the samples.

Conclusion:

This experiment found significant statistical differences between treatments C and D at 192 hours, three days after the nitrogen deprived samples were re-suspended in the nitrogen-containing medium. After 192 hours, treatment C had a faster recovery rate in cell abundance than treatment D. As a result of this conclusion, we reject our null hypothesis and support our alternative hypothesis, which states that wild type mating pairs of *Chlamydomonas reinhardtii* exposed to a nitrogen free environment

will have a higher recovery rate in cell abundance after being reintroduced into a nitrogen rich environment than the wild type/mutant mating pairs.

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