Zebrafish Embryo Lab Investigation:

Effects of Folic Acid on the Development of Zebrafish Embryos

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Abstract:

This experiment was performed in order to investigate the effects of folic acid on the development of a zebrafish embryo. Folic acid is essential to the development of proper bodily structure and organ formation in many species. It was hypothesized that a daily dose of folic acid would aid the zebrafish embryos in proper development and growth. The hypothesis was supported by the experiment.

Zebrafish embryos were placed in a solution equal to the daily dose of folic acid for a zebrafish and 500 times a daily dose of folic acid for a zebrafish. The placement of developing zebrafish embryos in various concentrations of folic acid solution resembles the exposure to folic acid a human embryo would experience if a pregnant mother took supplemental pills or increased her dietary folic acid consumption.

The zebrafish exposed to folic acid were all properly formed and developed according to the expected stage chart (**Figure 2**). By the end of the experiment, the folic acid appeared to have increased the rate of development for most of the zebrafish hatchlings. Also, only three total zebrafish died, so folic acid may have promoted the hatching and survival of early embryos.

Introduction:

In the course of embryo development, many vitamins, minerals, and nutrients from a mother's diet play a key role in the proper formation of certain body parts and certain bodily functions. One such nutrient, folic acid, plays a necessary role in neural and spinal development.

In the folate mediated one-carbon cycle, folic acid “is a cofactor in one-carbon metabolism, acting as a shuttle for methyl groups that will be used in the metabolism of s-adenosyl methionine (SAM), *de novo* synthesis of purines and thymidylate” (Lee et al., 2012).

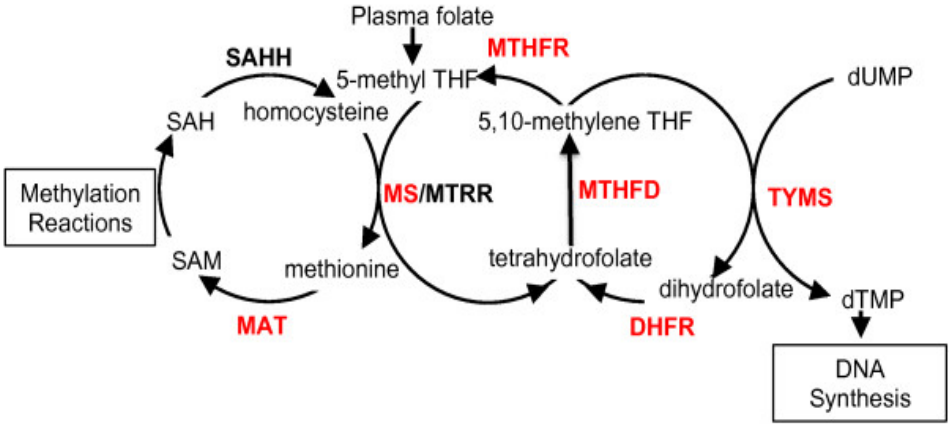


Figure 1: This image shows the complete mediated one-carbon cycle for folic acid in a person. When this cycle is completed, DNA synthesis is initiated (Lee et al., 2012).

As shown in **Figure 1**, folic acid aids in the methylation of DNA, which allows for DNA synthesis. DNA synthesis would lead to the proper expression of genes that are required for spinal formation and neural development.

In humans, folic acid taken by a pregnant mother is known to prevent spina bifida and miscarriages. In studies involving zebrafish, a reduced amount of folic acid has been found to increase the risk of a shorter anterior-posterior axis and cardiovascular system defects, such as incomplete cardiac looping (Sun et al., 2009). A pregnant woman is advised to take 460 micrograms of folic acid to promote normal embryonic development. The average weight of a woman in 2010 was 75.6 kilograms, compared to an average zebrafish weight of 240 micrograms (Stehr, C. M., Linbo, T. L., Incardona, J. P., Scholz, N. L., 2006). The equivalent folic acid dose in zebrafish is 1.46*10^-6 micrograms.

The following equation shows the equation for the daily dose of folic acid for a zebrafish, based upon the dosage for an average human female, given that the average zebrafish weight is 240 micrograms:

$$\frac{0.46 \text{ mg folic acid}}{75.6 \text{ kg}} = \frac{x}{2.4*10^{-7}} \qquad x = 1.46*10^{-6}\mu g$$

The following equation was used to determine the amount of folic acid that must be present in a 500 mL beaker of dechlorinated water, based on the transfer of 3.5 mL of solution into each well on the well tray:

$$\frac{1.46*10^{-6}\ \mu g \text{ folic acid}}{3.5 \text{ mL solution}} = \frac{4.17*10^{-6}\ \mu g}{mL} \times \frac{1 \text{ mg}}{1000\ \mu g} \quad \frac{1000 \text{ mL}}{1} = 4.17*10^{-7} \text{ mg/L}$$

$$\frac{4.17*10^{-7} \text{ mg folic acid}}{L} \quad \frac{1}{2} = \frac{2.09*10^{-7}\text{mg}}{500 \text{ mL}}$$

As an 800 μg folic acid supplement was used, the following dilutions were necessary to create the solution for the daily dose appropriate to the average zebrafish weight. As this solution is equal to the daily dosage of folic acid for a zebrafish, it will be called the 1X solution:

$$800\ \mu g \text{ folic acid} \quad \frac{1 \text{ mg}}{1000\ \mu g} = 0.8 \text{ mg folic acid} \quad \frac{1}{500 \text{ mL}} \quad \frac{1}{500 \text{ mL}} \quad \frac{1}{5 \text{mL}} = 6.4*10^{-7} \text{ mg/L}$$

To create a solution 500 times more concentrated than the approximated daily dosage for a zebrafish, the following dilutions were necessary. As this solution is 500 times the estimated daily dose of folic acid for a zebrafish, it will be called the 500X solution:

$$0.8 \text{ mg folic acid} \quad \frac{1}{500 \text{ mL}} \quad \frac{1}{5 \text{ mL}} = 3.2*10^{-4} \text{ mg/L}$$

Zebrafish (Danio rerio) are found in the Ganges River in India. They are not a common food source nor are they instrumental in fisheries, but they are popular and well-known additions to home aquariums worldwide. Zebrafish have been used as a model organism in experiments since the 1960s. Their bodies are transparent, and they produce hundreds of offspring in one reproduction cycle. They also

grow at a fast rate, making them ideal for both fast production and observation. Zebrafish also share 70% of their genes with humans, and 84% of human diseases have been found to have a zebrafish counterpart. Zebrafish are also cheaper to maintain than other comparable model organisms, making them a cost effective and ideal model organism when simulating human conditions ("Why use the zebrafish in research?", 2014).

During the course of this experiment, the goal will be to investigate and gain insight into how the addition of folic acid to the environment of a maturing zebrafish embryo affects its developmental progress and speed. It was hypothesized that if the appropriate estimated dose (1.46*10^-6 micrograms) is added to the zebrafish embryo environment, neural, spine, and heart development will be completed more successfully, due to folic acid's vital role in the mediated one-carbon cycle, and thus heart and neural development.

Materials and Methods:

The most important material needed in this experiment were the zebrafish embryos. A total of 60 was be needed. In order to begin the procedure, a medium sized tank of dechlorinated water was needed, along with a mixture of 5 mL of instant ocean solution to be added to the solutions that were prepared later on in the procedure. A 500 mL graduated cylinder and a 500 mL plastic beaker were used to measure the dechlorinated water from the tank and into the other 500 mL beakers to perform the dilutions. Two folic acid pills were used to create the solutions. These pills needed to be non-lipid based so that they were able to dissolve in water. Four 500 mL beakers were needed to perform the dilutions of the solutions and store the solutions over the course of the experiment. One 100 mL beaker was also needed for the disposal of used solution each day as well as removal of dead embryos. Plastic well trays containing twelve 7mL wells were needed to contain the zebrafish embryos and solutions they were given as they developed and hatched. Both wide bore and narrow bore pipettes were required to transport small amounts of solution, replace and remove the solution in the well trays of the embryos, and transport the embryos to and from slides. A marker was needed to label the sides of the well tray and split into two

groups each containing six wells for each solution. A stereoscope was used to view the embryos in a general setting, along with a lamp to provide light. It is recommended that white paper or paper towel be placed beneath the well tray containing the zebrafish so as to enhance the experimenter's ability to clearly view the embryos. A compound microscope and single cavity depression slides were used for closer examination of developing zebrafish embryos. A zebrafish development poster was needed for comparison.

The first step in the experiment was to create the folic acid solutions that the zebrafish embryos were placed into. First, a mortar and pestle were used to crush a single folic acid pill into a fine powder. Next, 500 mL of dechlorinated water was measured into a 500 mL beaker from the tank. Another 500 mL of dechlorinated water was also measured into an additional 500 mL beaker. After, 5 mL of dechlorinated water was measured into the 100 mL beaker. The folic acid powder was then added to one of the 500 mL beakers and the solution was stirred well until it appeared that the folic acid was dissolved in the water. Once this was done, 1 mL of the folic acid solution from the first beaker was transferred to the 100 mL beaker ensuring that no particles were deposited on the bottom of the 500 mL beaker. Next, 2.5 mL of dechlorinated water was removed from the remaining 500 mL beaker to a sink. To replace that 2.5 mL, 2.5 mL of instant ocean solution was added to the beaker. To create the final 1X solution of folic acid, 1 mL of the folic acid solution in the 100 mL beaker was transferred to the final 500 mL beaker. This was the beaker containing the instant ocean solution. The completed solution was stirred well.

To create the 500X solution, this process was repeated using only one 500 mL beaker. The crushed folic acid pill was added directly to the 100 mL beaker and the procedure continued as before. 2.5 mL of dechlorinated water was removed from the 500 mL beaker and 2.5 mL of instant ocean solution was added to replace it. Then, 1 mL of solution from the 100 mL beaker was added to the 500 mL beaker with instant ocean.

Once the solutions were finalized, the experiment could begin. Five zebrafish embryos were transported into each well of the plastic well tray, ensuring that solution was transferred along with the embryos. Next, 3.5 mL of either the 1X or concentrated 500X solutions were transferred into each well.

Half of the wells received the 1X solution and the other half received the concentrated 500X solution. The well tray was marked down the middle and labeled with the marker to avoid confusion of the sides. The cover was then set on the plastic well tray and the tray was placed into the incubator at a temperature of 28.5 degrees Celsius until the next day.

For the duration of the experiment the tray was removed from the incubator and general observations of the zebrafish embryos were recorded using the stereoscope and lamp. The developmental stage for each zebrafish embryo was recorded in the data tables and additional and more detailed observations were carried out through the use of a compound microscope. The data collected in the data tables will be compared to the average mortality rate for control group zebrafish embryos in a study performed by S. Ali, H. G. J. van Mil, and M. K. Richardson in a statistical t test to determine if the mortality rates are statistically significant. To view a zebrafish embryo with the compound microscope, a wide bore pipette was used to transfer a single embryo or hatched zebrafish onto a single cavity depression slide that allowed a water droplet to remain in place. It is recommended that a minimal amount of water, approximately one drop, is allowed to accompany the zebrafish embryo or hatchling on the slide. The slide was then placed onto the compound microscope and viewed on scanning before being transferred back into the correct well using the same method as its removal. Each day, after general and detailed observations, the old solution in the wells was removed using a narrow bore pipette and placed into the 100 mL beaker for disposal in the sink. Dead embryos were removed and put into the class disposal. A new 3.5 mL of solution for all wells was then added in coordination with the previous concentration of folic acid present in each well.

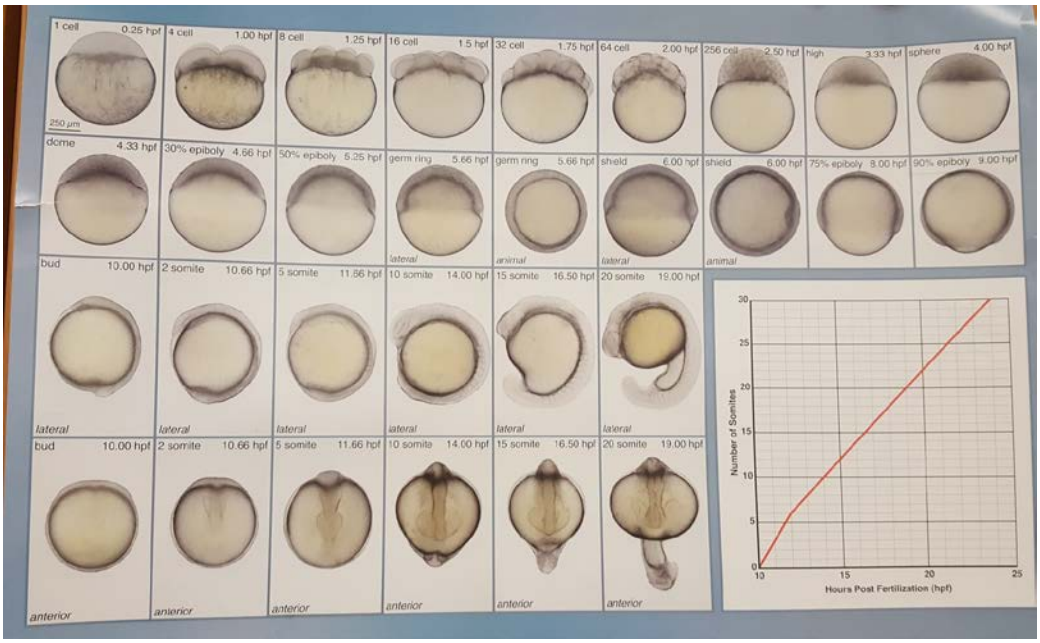
Safety precautions to ensure survival of the zebrafish embryos were essential to the success of this experiment. Zebrafish embryos and hatchlings should be handled with care and delicacy at all times. They should not be exposed to excess light for long periods of time, so breaks must be taken during general observations with the stereoscope and lamp. Also, minimal time needs to be spent viewing the zebrafish embryos and hatchlings under the compound microscope due to the fact that the light is very strong. Care should also be taken to avoid contamination of the solutions by any outside materials and

separate pipettes should be used for each solution to prevent mixing. Also, all beakers should be rinsed with dechlorinated water before use to prevent contamination.

Results:

This experiment was setup to investigate the effects of various levels of folic acid concentration on embryo development. Two solutions with different concentrations of a dissolved folic acid pill were created to simulate a dosage of folic acid appropriate to the reported zebrafish weight and a much higher dosage above what is recommended based on zebrafish weight.

The control group for this lab was not exposed to any folic acid and was allowed to develop with no chemical interference. The experimental groups were exposed to varying amounts of folic acid. The posters below for typical zebrafish growth and development were used to simulate a control group for stage (hpf) comparison of the control and experimental groups.



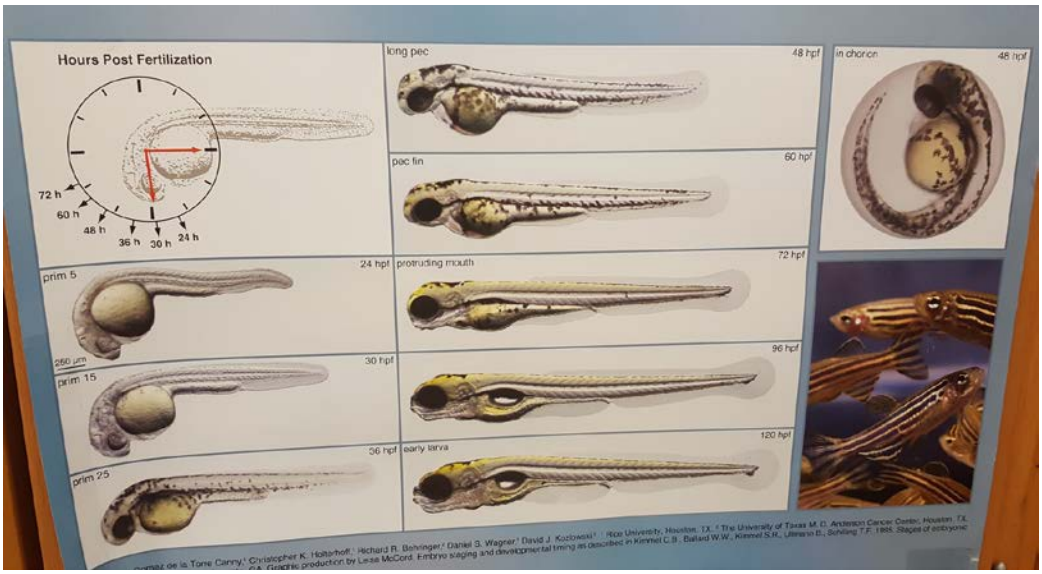


Figure 2: The poster above shows normal zebrafish embryo development. The embryo was stated to remain unhatched until the time between 36 hpf and 48 hpf. This chart was used as a comparison for the graphs below and these images were considered as the control group.

The independent variable in this experiment was the amount of folic acid the zebrafish embryos were exposed to. The dependent variable was the zebrafish spinal development and stage (hpf) progression in response to the solution environment. The constants maintained for the duration of this experiment included the temperature of the environment, the daily care schedule, the exposure to light, the amount of instant ocean present in the solutions, and the incubation environment of the embryos.

All embryos exposed to folic acid experienced a slightly more rapid growth and development rate than those in the control groups. Embryos placed in the 500X folic acid solution progressed only slightly faster than those placed in the 1X folic acid solution. Overall, only three zebrafish embryos failed to hatch and died. No hatched zebrafish had any defects of the spine or bodily structure. These results show that folic acid plays an important role in the proper formation of the zebrafish embryo's spines and overall bodily structure.

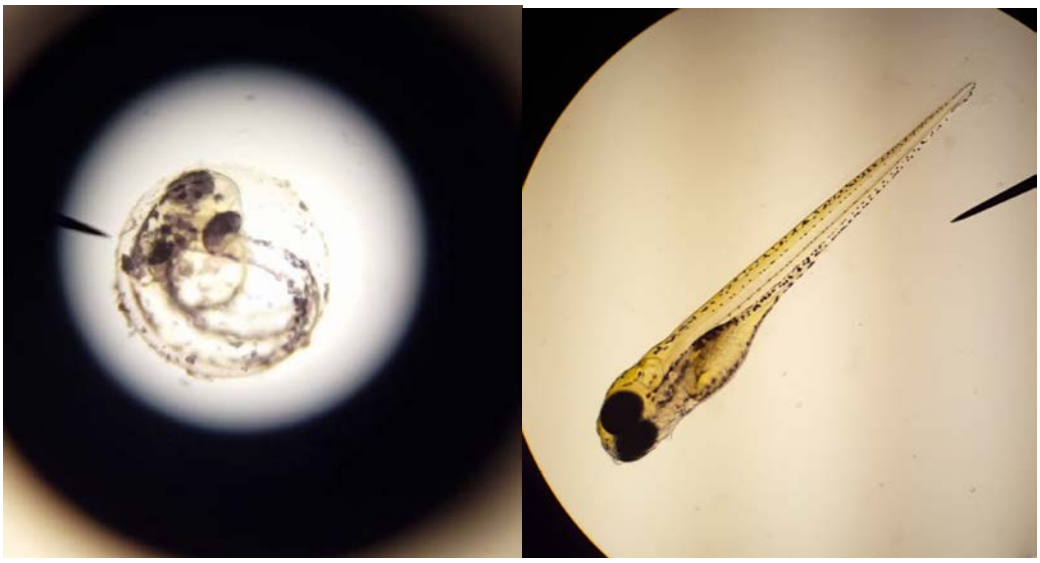


Figure 3: The photos included show a lack of deformities of any kind in both zebrafish embryo and hatchling. The picture on the left shows a living zebrafish embryo that had a visible heartbeat and correct organ formation and structure. The picture to the right shows a zebrafish hatchling that developed a straight spine and proper body structure. The heartbeat was also clearly visible.

Data Presentation:

1X Folic Acid Solution Concentration vs. Zebrafish Embryo Growth and Development Over Four Days

Stage (hpf)	Day 1 (24 hrs)	Day 2 (48 hrs)	Day 3 (72 hrs)	Day 4 (96 hrs)
0.25	-	-	-	-
1	-	-	-	-
1.25	-	-	-	-
1.5	-	-	-	-
1.75	-	-	-	-
2	-	-	-	-
2.5	-	-	-	-

3.33	-	-	-	-
4	-	-	-	-
4.33	-	-	-	-
4.66	-	-	-	-
5.25	-	-	-	-
5.66	-	-	-	-
6	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
10.66	-	-	-	-
11.66	-	-	-	-
14	-	-	-	-
16.5	**1**	-	-	-
19	**13**	-	-	-
24	**19**	-	-	-
30	-	-	-	-
36	-	**15**	**2**	-
48	-	**17**	**1**	-
60	-	**1**	**7**	-
72	-	-	**23**	-
96	-	-	-	**5**
120	-	-	-	**28**
Final	-	-	-	-
Dead	-	-	-	-

500X Folic Acid Solution Concentration vs. Zebrafish Embryo Growth and Development Over Four Days

Stage (hpf)	Day 1 (24 hpf)	Day 2 (48hpf)	Day 3 (72 hpf)	Day 4 (96 hpf)
0.25	-	-	-	-
1	-	-	-	-
1.25	-	-	-	-
1.5	-	-	-	-
1.75	-	-	-	-
2	-	-	-	-
2.5	-	-	-	-
3.33	-	-	-	-
4	-	-	-	-
4.33	-	-	-	-
4.66	-	-	-	-
5.25	-	-	-	-
5.66	-	-	-	-
6	-	-	-	-
8	-	-	-	-
9	-	-	-	-
10	-	-	-	-
10.66	-	-	-	-
11.66	-	-	-	-
14	-	-	-	-
16.5	-	-	-	-
19	17	-	-	-

24	**5**	**-**	**-**	**-**
30	**5**	**-**	**-**	**-**
36	**2**	**5**	**-**	**-**
48	**-**	**20**	**4**	**-**
60	**-**	**4**	**14**	**-**
72	**-**	**-**	**9**	**-**
96	**-**	**-**	**-**	**6**
120	**-**	**-**	**-**	**21**
Final	**-**	**-**	**-**	**-**
Dead	**1**	**-**	**2**	**-**

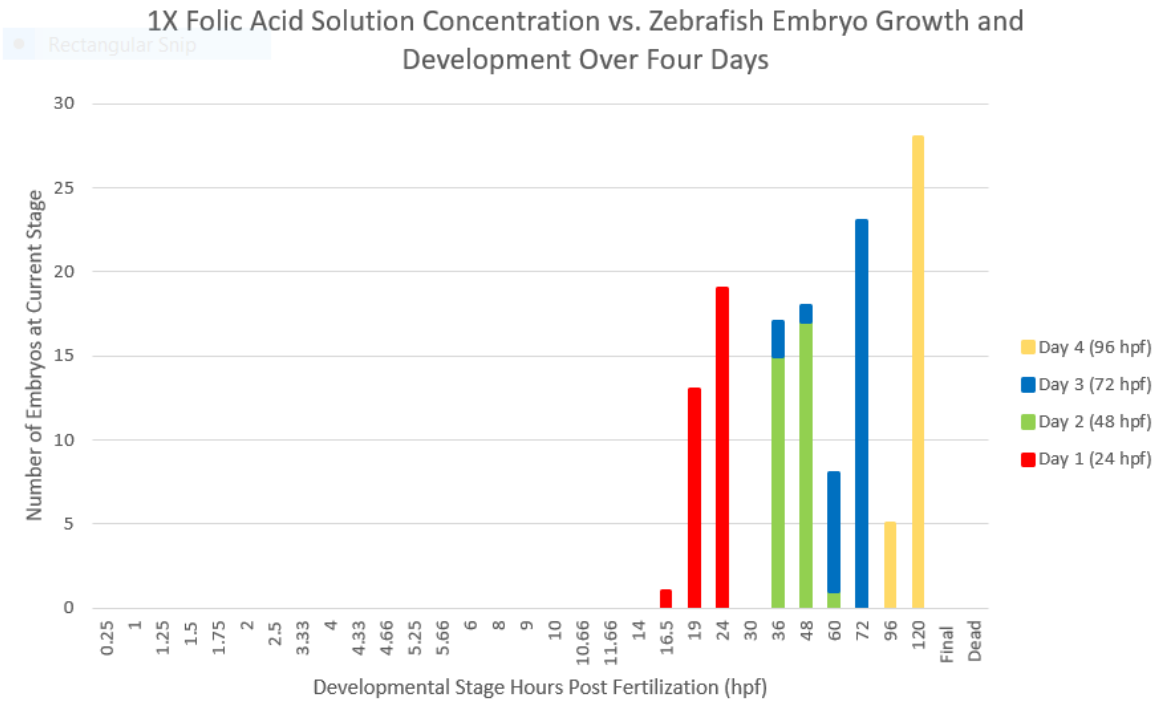


Table 1: 1X Folic Acid Solution Concentration vs. Zebrafish Embryo Growth and Development Over Four Days. The graph above displays the development of the zebrafish embryos in the 1X folic acid

solution equivalent to the estimated daily dose of folic acid for a zebrafish based upon weight. The control is represented by each day. On day one, many of the zebrafish are behind on development. Over days two and three, on average, they appear to reach the control group. On the final day, a majority are ahead of the control group.

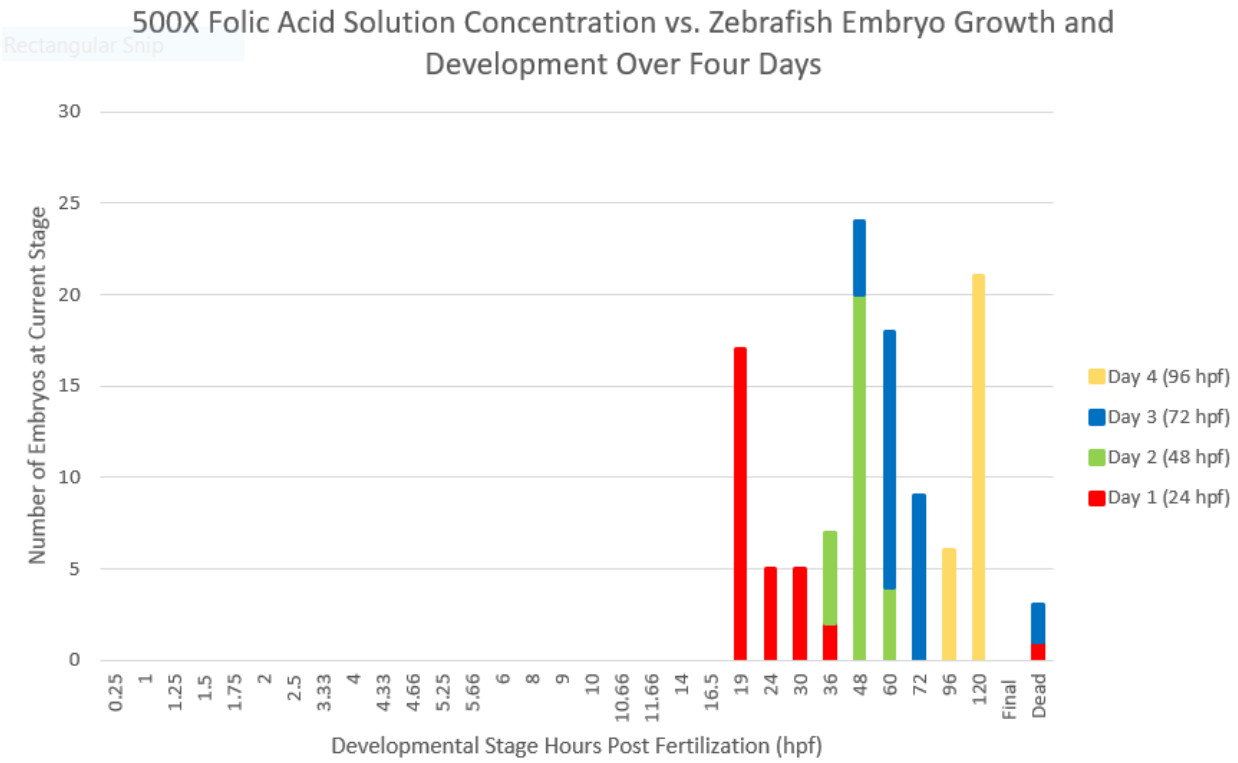
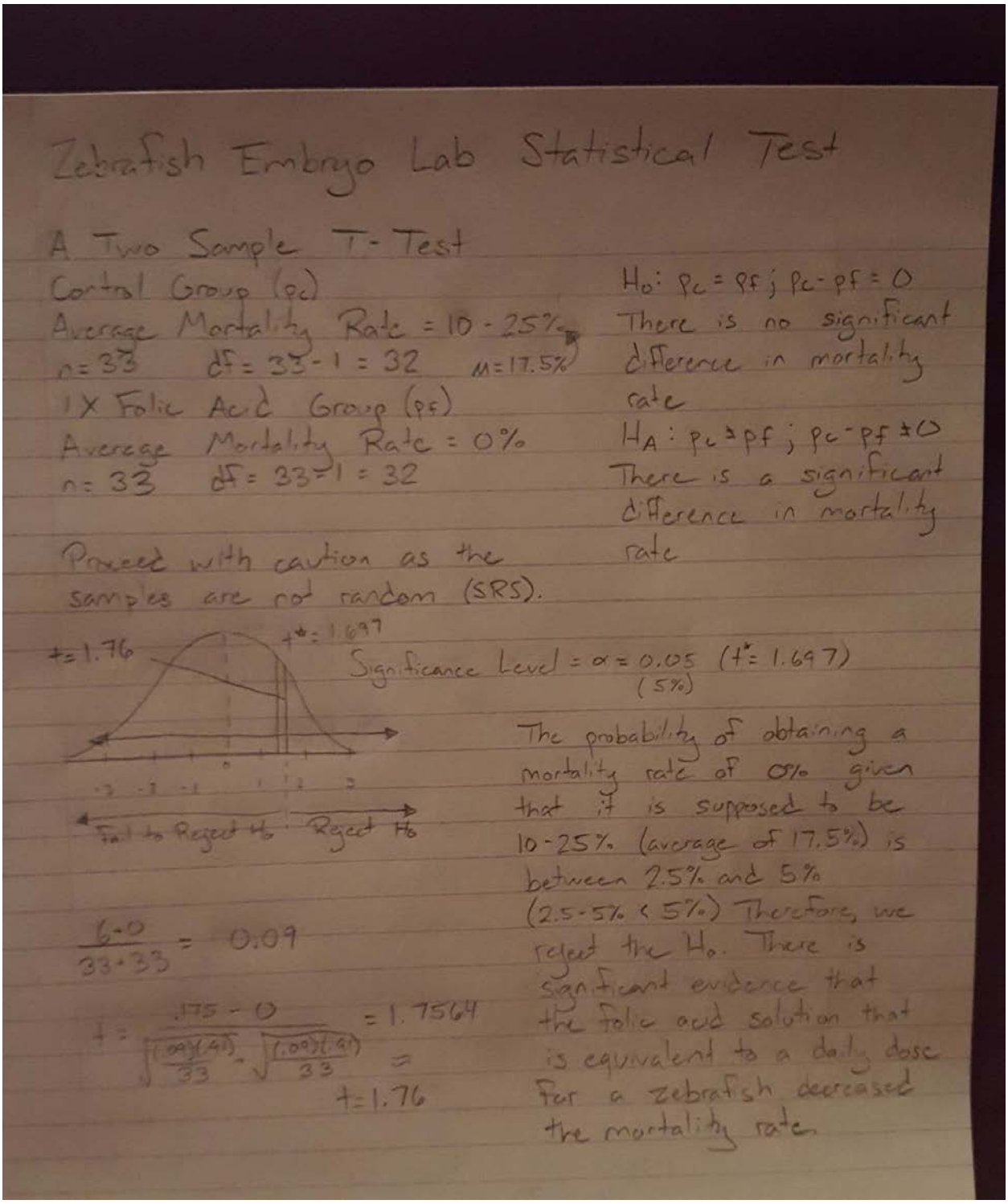


Table 2: 500X Folic Acid Solution vs. Zebrafish Embryo Growth and Development Over Four Days. The graph above shows the development of the zebrafish embryos in the 500X folic acid solution that is 500 times the daily dose of folic acid for a zebrafish. The control is represented by the day. On day one, about half of the embryos are at or above the control group. On day two, the average amount of embryos appear to be at the control group level. On day three, they appear to be slightly below or at the control level. Similar to the 1X Folic Acid Solution, a majority of the zebrafish appear to be above the control group on the fourth day.

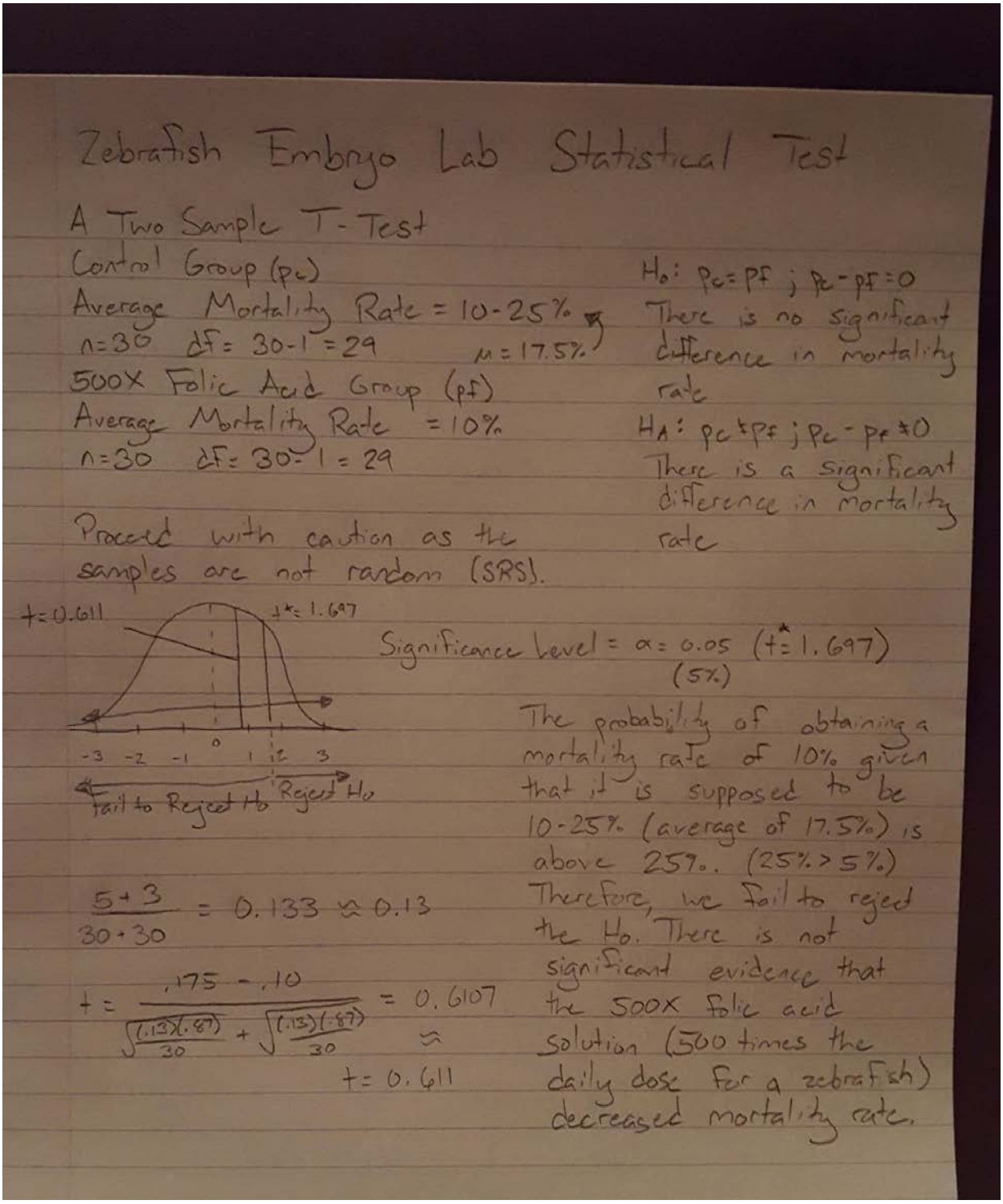
Data Analysis:

In past experiments done by other researchers, the mortality rate for control group zebrafish embryos has been marked at 10-25%. This statistic was determined for control groups that were not exposed to any experimental variables and died spontaneously during the course of the experiment. In the 1X Folic Acid Solution, there was a 0% mortality rate and all 33 embryos hatched and developed without defect. In the 500X Folic Acid Solution, there was a 10% mortality rate. Only 3 out of 30 total embryos did not hatch and died. A two sample t test was used to determine if the difference in mortality rate was statistically significant in each folic acid group compared to the control group. The results show that the 1X Folic Acid Solution had a significant effect by decreasing the mortality rate of the zebrafish embryos.



Test 1 (1X Folic ACid Solution): The probability of obtaining a mortality rate of 0% given that it is supposed to be 10-25% (average of 17.5%) is between 2.5% and 5%. (2.5-5% is less than the significance

level of 5%). Therefore, we reject the null hypothesis (H_0). There is significant evidence that the folic acid solution equivalent to the daily dose for a zebrafish decreased the mortality rate.



Zebrafish Embryo Lab Statistical Test

A Two Sample T-Test

Control Group (p_c)

Average Mortality Rate = 10-25% $\mu = 17.5\%$

$n=30$ $df = 30-1 = 29$

500X Folic Acid Group (p_f)

Average Mortality Rate = 10%

$n=30$ $df = 30-1 = 29$

Proceed with caution as the samples are not random (SRS).

H_0: $p_c = p_f$; $p_c - p_f = 0$
There is no significant difference in mortality rate

H_A: $p_c \neq p_f$; $p_c - p_f \neq 0$
There is a significant difference in mortality rate

$t = 0.611$ $t^* = 1.697$

-3 -2 -1 0 1 t^* 3

Fail to Reject H_0 | Reject H_0

Significance Level = $\alpha = 0.05$ ($t^* = 1.697$) (5%)

$\frac{5+3}{30+30} = 0.133 \approx 0.13$

$t = \frac{.175 - .10}{\sqrt{\frac{(.13)(.87)}{30} + \frac{(.13)(.87)}{30}}} = 0.6107 \approx$

$t = 0.611$

The probability of obtaining a mortality rate of 10% given that it is supposed to be 10-25% (average of 17.5%) is above 25%. (25% > 5%) Therefore, we fail to reject the H_0. There is not significant evidence that the 500X folic acid solution (500 times the daily dose for a zebrafish) decreased mortality rate.

Test 2 (500X Folic Acid Solution): The probability of obtaining a mortality rate of 10% given that it is supposed to be 10-25% (average of 17.5%) is above 25%. (25% is greater than the significance level of 5%). Therefore, we fail to reject the null hypothesis (H_0). There is not significant evidence that the 500X folic acid solution (500 times the daily dose for a zebrafish) decreased mortality rate.

Discussion :

The data collected and the results of the experiment support the hypothesis that a daily dose of folic acid would improve development and growth of zebrafish embryos. Both solutions containing folic acid appeared to aid the embryos in development as only three total embryos died. The three embryo deaths occurred in the 500X Folic Acid Solution and no embryo deaths occurred in the 1X Folic Acid Solution. The 0% mortality rate of the 1X Folic Acid Solution was proven to be statistically significant. No abnormalities were found in either solution. All hatched embryos developed proper spines and body parts. The data also shows that folic acid maintained an average rate of progression through the stages of maturation for the first three days of growth. On the fourth day, folic acid appears to have accelerated the progression of the hatched zebrafish above the normal rate of development for the control group.

Although the experiment yielded satisfactory results, a few minor errors could be corrected to improve the procedure. The experiment would benefit most from the addition of a control group along with the folic acid groups instead of the use of the poster for control data and comparison. Also, data and observations of the embryos should take place on Day 0 when the zebrafish embryos are initially introduced to the solutions to get a baseline of where they started to develop before the experimentation actually began.

The results of this experiment further stress the importance of folic acid in the development of embryos. The introduction of folic acid prevented spinal deformities seen in other group zebrafish and aided the zebrafish in maintaining stable and even accelerated progression of development. This reinforces the vital role that folic acid plays in human embryo and fetus development as well. The daily dosage of folic acid in comparison with that a woman is recommended to take kept all embryos on

schedule for development and caused no fatalities while also preventing any deformities. Folic acid is essential to proper health for both mother and child and measures should be taken to include folic acid in a mother's diet by medical professionals and expecting parents.

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