Effect of Lipstick on Zebrafish Embryonic Development and Survival and Relating the Results to Humans

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Abstract:

Lipstick is used by people all around the world on a daily basis. As lipstick is worn, it exposes the wearer to harmful chemicals and heavy metals which can potentially cause health problems, especially to pregnant women and their unborn child. Consumption and exposure to these chemicals and heavy metals in the lipstick is known to cause many health problems on developing embryos as well as children and adults. The purpose of this experiment was to investigate the effects of lipstick on Zebrafish embryos and relate results to developing human embryos. This was tested by exposing the Zebrafish embryos to different types of lipstick and observing the survival, and development of the embryos over time. Based on the results, the different types of lipstick used in the experimental trials all caused developmental defects and lower survival rates for the Zebrafish.

Introduction:

As parents or prospective parents become more concerned about the safety and health of an unborn child due to the pregnant mother's exposure to certain chemicals, it is recommended that certain substances are not to be in the environment of, or consumed by a pregnant woman for the duration of the pregnancy. Lipstick is a commonly used cosmetic that could potentially have an effect on the child, raising the question if lipstick is safe to be worn by pregnant women. Recently researchers have studied the ingredients in a wide variety of popular lipstick brands commonly used by millions of women daily after questions about health concerns were brought up. The scientists found that many lipsticks contain unsafe levels of heavy metals such as lead, manganese, aluminum, titanium, chromium, cobalt, nickel, copper, and cadmium (Liu et al., 2013). These heavy metals according to the World Health Organization (WHO) are considered threats to human health (Jarup, 2003, pp. 167-182). While some of these metals have a normal role in the body, the metals accumulate over time. High concentrations of heavy metals such as those found in lipstick can become toxic and dangerous to the body (Lead And Other Heavy Metals, n.d.). Lead specifically is a neurotoxin commonly found in many types of lipstick, and can cause reduced fertility, hormonal changes and irregularities, and many other health issues (Lead In Lipstick, n.d.). Also according to the CDC there is no safe level of lead intake, and for ingested substances such as water, the maximum contaminant level goal in drinking water is zero, while the recommended maximum level of lead in candy commonly consumed by young children is 0.1 ppm. Both water and candy are consumed by humans of all ages and are regulated for lead levels by the FDA for the safety of the consumer.

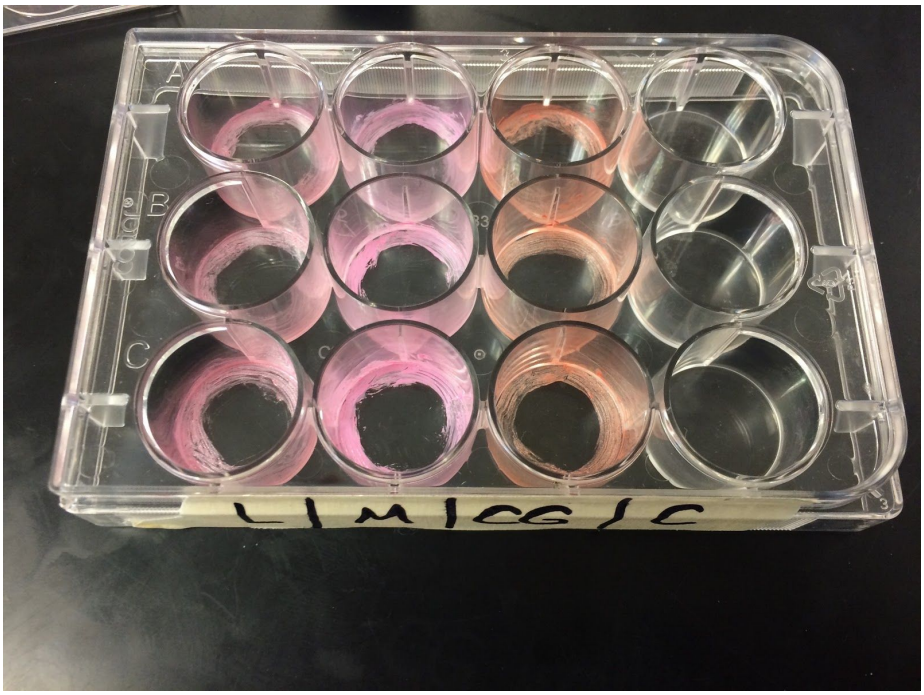
Currently metals in cosmetic products are not regulated in the US, although the FDA and cosmetic companies often argue that lead in cosmetics (specifically lip products) is not a source of concern because the dose is so low per application (Liu et al., 2013)(Lead In Lipstick, n.d.). The problem with this argument is that over time the heavy metals in the lipstick are ingested in small quantities that add up causing potentially dangerous side effects. The dangers of heavy metals including lead pose an even greater risk to pregnant women and their expected child. According to the FDA, "current regulation limits companies to no more than 20 parts per million (ppm) for color additives approved for use in cosmetics according to the FDA, but there is not set limits for lead" (Lipstick & Lead, 2015). On the other hand according to fda.gov, the effect of lead and other heavy metals that are found in lipstick could potentially cause neurological damage and pose a survival risk to embryos. The lack of regulation creates the potential for high amounts of lead in lipstick. The potential for high lead concentrations in lipstick was tested in the expanded survey done by the FDA in 2010. This survey looked at lead content for over 400

commonly used lipsticks. The results of this study found lead concentrations in commonly used lipsticks up to 7.19 parts per million (ppm). From these results the 3 lipsticks used in this experiment to be tested on Zebrafish embryos were chosen because of their varying qualities, brands, and relatively high lead concentrations. The 3 lipsticks in this experiment are as follows; L'oreal (Colour Riche, #165, Tickled Pink) with 4.45 ppm lead, Maybelline (Color Sensational, #125, Pink Petal) with 7.19 ppm lead, which was the highest ppm value of the lipsticks tested in the 2010 FDA study, and the third lipstick used was Covergirl waterproof (Continuous Color, #015, Bronzed Peach) with 2.00 ppm lead (Lipstick & Lead, 2015). By exposing the Zebrafish embryos to the lipstick containing lead and other heavy metals it was predicted that the lipstick would cause a decline in the survival of the Zebrafish embryos and also result in an increase in growth defects such as spinal curves, because of exposure to the chemicals and heavy metals in lipstick. This was tested by exposing Zebrafish embryos to different brands of lipstick with varying amounts of heavy metals including lead to see if it would have an impact on the survival and development of the embryos as they grew from freshly fertilized to a hatched fish. This was done by painting a ring of lipstick around the base of a well plate where the fish would live. Over the course of 5 days, the survival rates and development of the fish was documented over time then analyzed.

Zebrafish embryos are used in this experiment because they can easily be used as a model for embryonic development in vertebrates. They are produced in large numbers, have a rapid and easily observable development rate with clear “morphological indicators of each developmental stage” (Tomasiewicz et al., 2014). Zebrafish also are similar to humans in terms of development making them a good model in terms of growth and development of embryos. The use of Zebrafish embryos is also more ethical than the use of human embryos (Tomasiewicz et al., 2014).

Materials & Methods

-1 Well plate with 12 wells
-Loreal (Colour Riche, #165, Tickled Pink), Maybelline (Color Sensational, #125, Pink Petal), and Covergirl waterproof (Continuous Color, #015, Bronzed Peach) Lipstick
-3 disposable paint brushes
-60 live Zebrafish embryos
-prepared Instant Ocean Solution
-Many small and large tip disposable pipettes
-Dissecting microscope
-Waste Beaker for dead embryos, waste, and old solution
-Sharpie
-Masking tape
-Incubator at 28.5 Degrees Celsius
-Data table and writing utensil



(Figure 1) Visual setup of falcon dish with labels, and lipstick samples on bottom of corresponding well.

Falcon Dish Setup (Table A)

L'oreal Well A	Covergirl Well A	Maybelline Well A	Instant Ocean Well A
L'oreal Well B	Covergirl Well B	Maybelline Well B	Instant Ocean Well B
L'oreal Well C	Covergirl Well C	Maybelline Well C	Instant Ocean Well C

Procedure

The well plate was labeled using the sharpie marker and masking tape according to Figure 1, and Table A. It was labeled by column with 3 wells each for L'oreal lipstick (L), Maybelline lipstick (M), Covergirl lipstick (CG), and control with instant ocean solution only (C). Using the disposable paint brush (one for each type of lipstick to prevent cross contamination), a ring of lipstick corresponding to the column label was painted around the perimeter of the base of each well. Each well was then filled approximately halfway with the prepared instant ocean solution using a large tip disposable pipet.

Each well was filled with 10 Zebrafish embryos each, and was checked under to the dissecting microscope to make sure every embryo was living. All embryos that were not living or had fungus covering them were removed and placed into the waste beaker. Once all wells had 10 living Zebrafish embryos each, data on exactly how many were alive and in what stage of development the embryos were in was recorded in the data table. Observations were taken every 24 hours and the number of alive embryos, dead embryos, alive and hatched Zebrafish, and dead hatched Zebrafish was documented in the

data tables. Observations, and photos were taken through the dissecting microscope. After observations and data of survival was taken, the dead embryos and/or hatched fish were removed using a large tip pipet and put into the waste beaker to be properly disposed of. The old instant ocean solution was also removed from the wells and placed into the waste beaker using a small tip pipet with caution taken not to suck up any alive embryos or hatched fish. A fresh pipet was used for each column to prevent cross contamination of the lipsticks. The wells were then filled halfway with fresh instant ocean solution. The lid was then placed on top of the well plate and taped shut. The well plate was then placed into an incubator kept at 28.5^{0}C until the next observation and cleaning 24hrs later. This process was then repeated for a total of five days.

At the end of the 5 day experiment, the embryos and hatched Zebrafish were placed into a petri dish using a large tip pipet to humanly be disposed of by the teacher. All supplies used was washed, and disposable brushes and pipets were disposed of properly.

The control group in this experiment was the Zebrafish living in the instant ocean solution. The independent variable of this experiment was the type of lipstick the Zebrafish were exposed to, and the dependent variable was the development and survival of the embryos which was measured by looking at survival rates and birth defects. Constants in this experiment were the instant ocean solution used, the type of fish, the age of the embryos, the temperature of the incubator, the amount of solution in each well, the number of embryos per well, the amount of light the embryos were exposed to, the amount of time spent in the incubator, and the time of day that the results of the experiment were checked and the observation/cleaning process took place.

Safety concerns in this experiment were to handle the embryos carefully when moving the plate or placing them into the wells. Another safety concern was to not get the solution in or near your eyes or nose, and to always wash your hands after working with the solution, lipstick, or embryos.

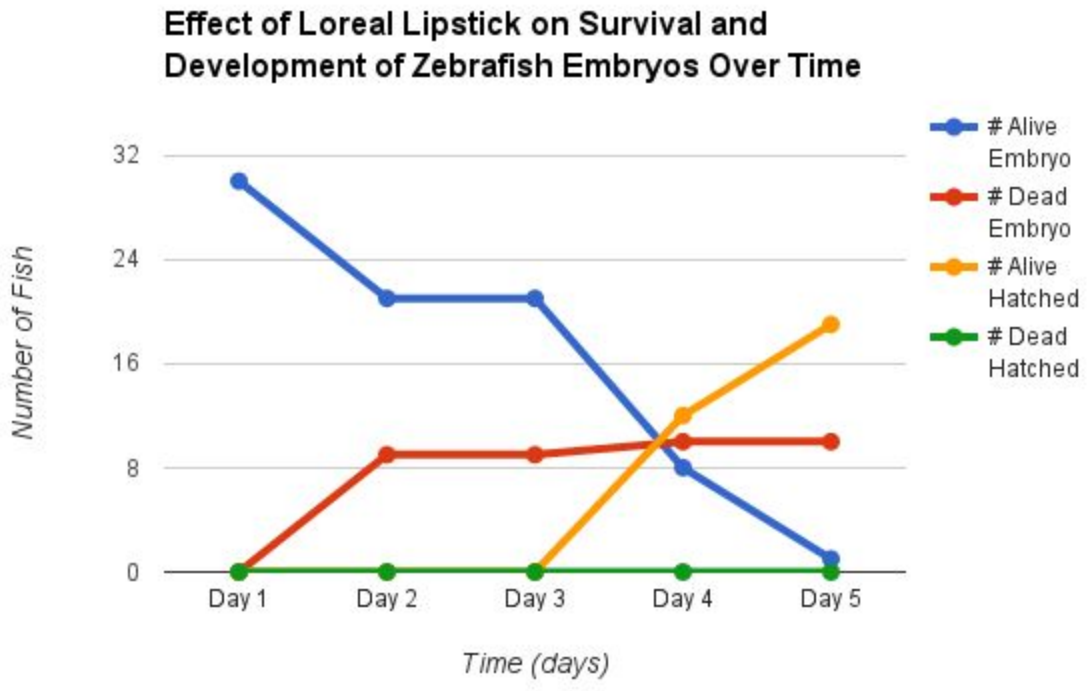
Results:

Developmental changes were seen in the Zebrafish exposed to the lipsticks, specifically curved spines in the Zebrafish exposed to Covergirl and Maybelline Lipstick. Growth abnormalities which can be described as looking like attached yolk sack to the abdomen or stomach area of the hatched Zebrafish in the fish Covergirl and L'oreal lipsticks. Both the growth abnormalities and spinal curves were seen in hatched Zebrafish after exposure to lipstick during the duration of the experiment. The survival rates of the experiment as determined by the T-tests showed the Zebrafish in the Control group had the highest survival rate with a 29.20 average of fish surviving. The Maybelline trial had the second highest survival rate with a 28.80 survival average. Loreal had the third highest average survival rate at 22.4, and the Cover Girl trial had the lowest average survival rate at 19.20. Overall the Covergirl lipstick had the greatest impact on survival and development of the Zebrafish embryos, having the lowest survival rate and both growth abnormalities and spinal curves present.

Table 1: Effect of Loreal Lipstick on Survival and Development of Zebrafish Embryos Over Time

Loreal	Day 1	Day 2	Day 3	Day 4	Day 5
# Alive Embryo	30	21	21	8	1

# Dead Embryo	0	9	9	10	10
# Alive Hatched	0	0	0	12	19
# Dead Hatched	0	0	0	0	0
Additional Observations	All alive with transparent outer shell and circular white inside	N/A	N/A	10 of the hatched fish has a strange growth or morphological difference on underside	8 of the hatched fish have an obvious growth abnormality which looks like an attached yolk sack to the abdomen of the fish

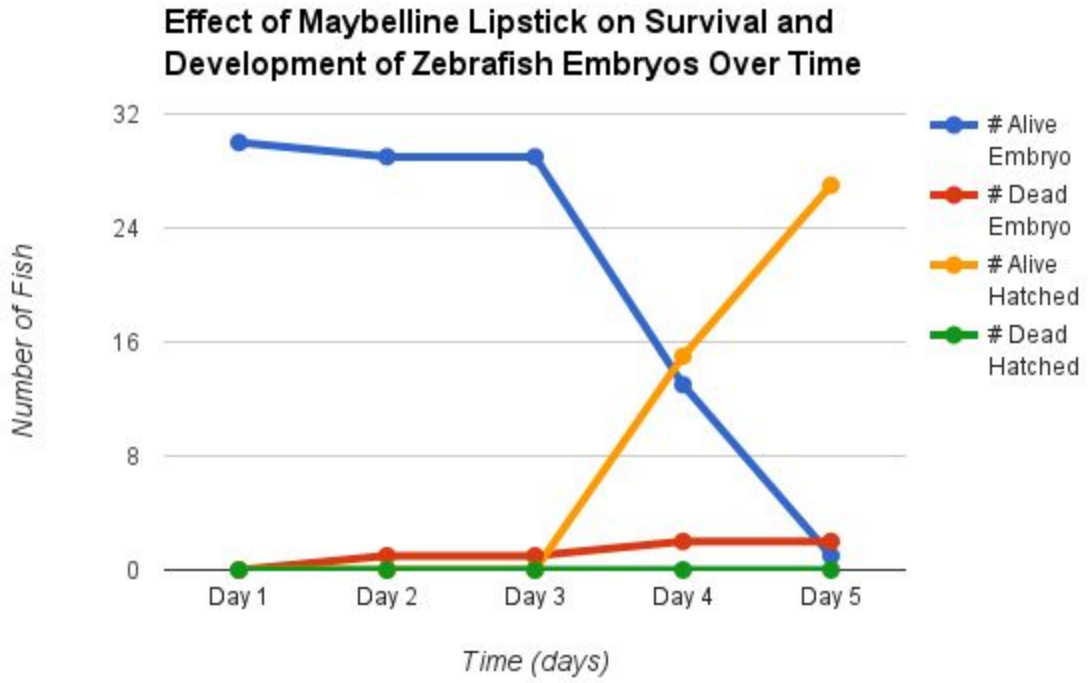


The p=0.0077 shows that the data comparing the number of alive fish in the control group to the number of alive fish in the experimental group of the L'oreal lipstick was statistically significant, because the p-value was very small. The SEM value was was 1.91 for the number of Zebrafish exposed to the L'oreal lipstick over time, and the SEM value for the number of Zebrafish not exposed to anything in the control group was 0.20. The SEM value shows the accuracy of the mean. This value can be compared to

the SD which was 4.28 for the number of Zebrafish exposed to the L'oreal lipstick over time, and 0.45 for the Zebrafish not exposed to lipstick in the control. The SD of the control was lower than the experimental trial which shows that the value of control data was closer to the mean of the data set. For the experimental trial the SD was 4.28 which is much higher than 0.45 and therefore the value of the experimental data was further from the mean of data set. The mean of the data was 22.40 for the experimental group and 29.20 for the control group which shows that on average 22.4 fish survived in the experimental group, and 29.20 fish survived in the control group. This shows that more fish overall survived in the control group rather than the experimental group, showing that the mortality rates of fish exposed to lipstick is higher than that of fish not exposed to lipstick. Finally the N value of the experimental group was 5 and the control group was also 5, so the N value for both the experimental group and control group was the same. Overall, more fish died when exposed to the lipstick in comparison to the fish not exposed to lipstick, showing that lipstick has a negative effect of the survival of the lipstick. For the fish that did survive in the experimental trials, some had growth defects which shows that the lipstick has a negative effect on development as well. For visual of growth abnormalities in L'oreal lipstick exposed Zebrafish see Figure 3.

Table 2: Effect of Maybelline Lipstick on Survival and Development of Zebrafish Embryos Over Time

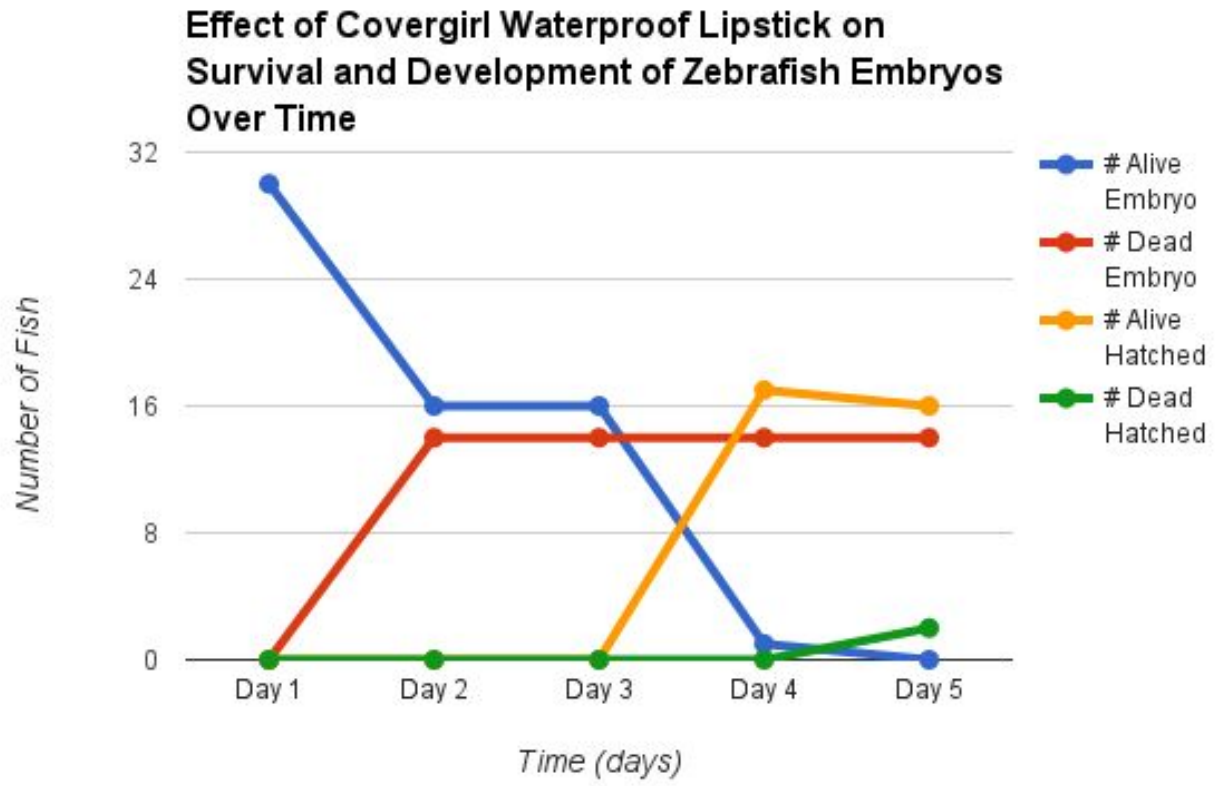
Maybelline	Day 1	Day 2	Day 3	Day 4	Day 5
# Alive Embryo	30	29	29	13	1
# Dead Embryo	0	1	1	2	2
# Alive Hatched	0	0	0	15	27
# Dead Hatched	0	0	0	0	0
Additional Observations	All alive with transparent outer shell and circular white inside	N/A	N/A	2 with curved spines (1 in process of hatching)	3 alive and hatched fish had curved spines



The p=0.3734 shows that the data comparing the number of alive fish in the control group to the number of alive fish in the experimental group of the Maybelline lipstick was not statistically significant, because the p-value was not small enough. The SEM value was was 0.37 for the number of Zebrafish exposed to the Maybelline lipstick over time, and the SEM value for the number of Zebrafish not exposed to anything in the control group was 0.20. The SEM value shows the accuracy of the mean. This value can be compared to the SD which was 0.84 for the number of Zebrafish exposed to the Maybelline lipstick over time, and 0.45 for the Zebrafish not exposed to lipstick in the control. The SD of the control was lower than the experimental trial which shows that the value of control data was closer to the mean of the data set. For the experimental trial the SD was 0.84 which is much higher than 0.45 and therefore the value of the experimental data was further from the mean of data set. The mean of the data was 28.80 for the Maybelline experimental group and 29.20 for the control group which shows that on average 28.80 fish survived in the experimental group, and 29.20 fish survived in the control group. This shows that more fish overall survived in the control group rather than the experimental group, showing that the mortality rates of fish exposed to lipstick is higher than that of fish not exposed to lipstick. Finally the N value of the experimental group was 5 and the control group was also 5, so the N value for both the experimental group and control group was the same. Overall, more fish died when exposed to the lipstick in comparison to the fish not exposed to lipstick, showing that lipstick has a negative effect of the survival of the lipstick. For the fish that did survive in the experimental trials, some had growth defects of curved spines which shows that the lipstick has a negative effect on development as well. For a visual example of the curved spines on Maybelline lipstick exposed Zebrafish see Figures 5, 6, and 8.

Table 3: Effect of Covergirl Lipstick on Survival and Development of Zebrafish Embryos Over Time

Covergirl waterproof	Day 1	Day 2	Day 3	Day 4*	Day 5
# Alive Embryo	30	16	16	1	0
# Dead Embryo	0	14	14	14	14
# Alive Hatched	0	0	0	17	16
# Dead Hatched	0	0	0	0	2
Additional Observations	All alive with transparent outer shell and circular white inside	Colored outer shell of dead embryos	One fish is in the process of hatching	*on day 4 there were 2 extra fish in well C which was unusual and 11 out of the alive hatched fish have abnormal growths and curved spines	6 of the alive and hatched fish have curved spines and some with abnormal growths

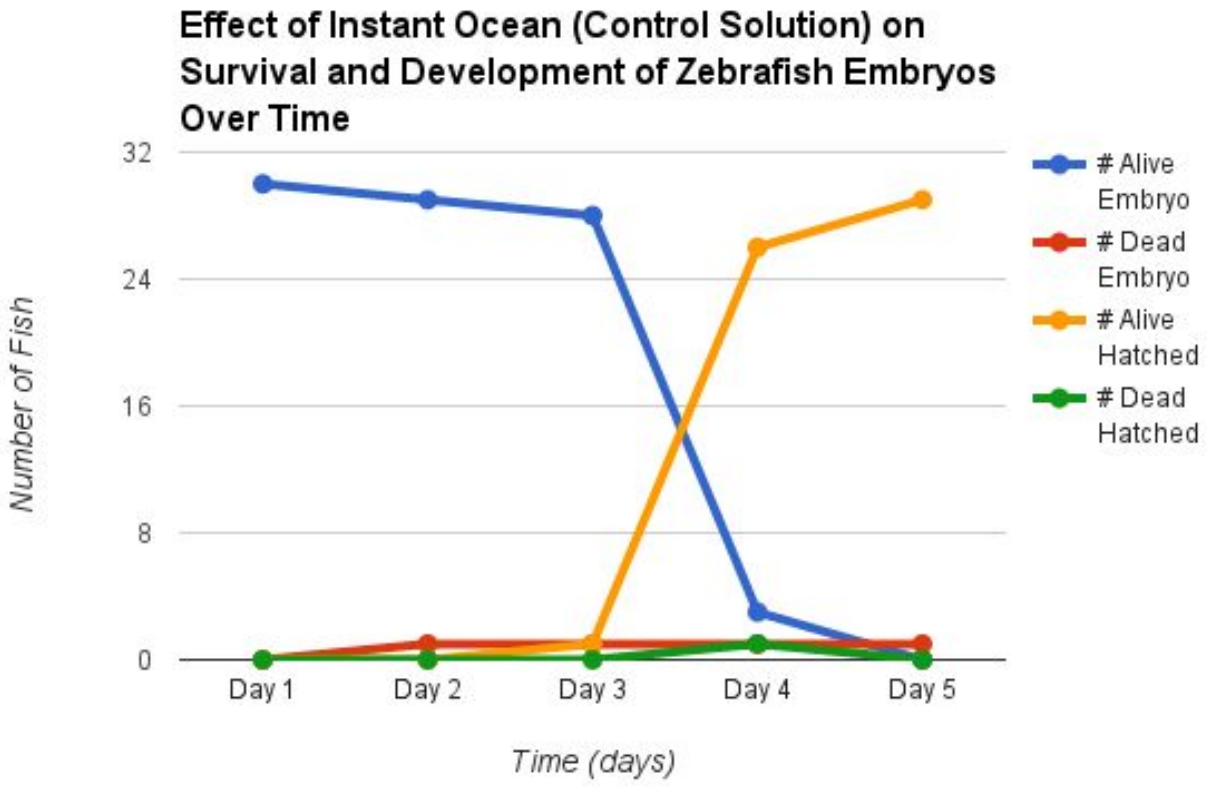


The p=0.0064 shows that the data comparing the number of alive fish in the control group to the number of alive fish in the experimental group of the Covergirl waterproof lipstick was very statistically significant, because the p-value is very small. The SEM value was was 2.72 for the number of Zebrafish exposed to the Covergirl lipstick over time, and the SEM value for the number of Zebrafish not exposed to anything in the control group was 0.20. The SEM value shows the accuracy of the mean, and this value can be compared to the SD which was 6.10 for the Zebrafish exposed to the Covergirl lipstick, and 0.45 for the Zebrafish not exposed to lipstick in the control. The SD of the control was lower than the experimental trial which shows that the value of control data are closer to the mean of the data set. For the experimental trial the SD was 6.10 which is much higher than 0.45 and therefore the value of the experimental data was further from the mean of data set. The mean of the data was 19.20 for the Covergirl experimental group and 29.20 for the control group which shows that on average 19.20 fish survived in the experimental group, and 29.20 fish survived in the control group. This shows that more fish overall survived in the control group rather than the experimental group, showing that the mortality rates of fish exposed to lipstick is higher than that of fish not exposed to lipstick. Finally the N value of the experimental group was 5 and the control group was also 5, so the N value for both the experimental group and control group was the same. Overall, more fish died when exposed to the lipstick in comparison to the fish not exposed to lipstick, showing that lipstick has a negative effect of the survival of the lipstick. Also in the dead embryos the outer shell had a pinkish orange color tint. For the fish that did survive in the experimental trials, many had growth defects of curved spines and growth abnormalities

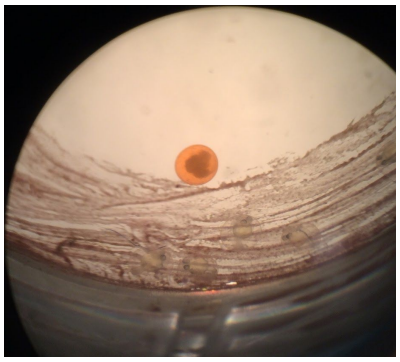
showing that the lipstick has a negative effect on development as well. See Figures 2, 4, and 7 for visual examples of the growth and spine abnormalities.

Table 4: Effect of Control Instant Ocean Solution on Survival and Development of Zebrafish Embryos Over Time

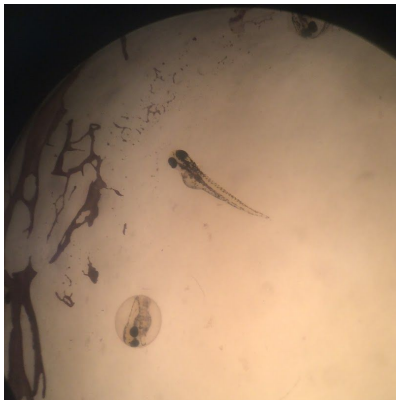
Control Instant Ocean	Day 1	Day 2	Day 3	Day 4**	Day 5
# Alive Embryo	30	29	28	3	0
# Dead Embryo	0	1	1	1	1
# Alive Hatched	0	0	1	26	29
# Dead Hatched	0	0	0	1	0
Additional Observations	All alive with transparent outer shell and circular white inside	N/A	All fish look further developed that those in the experimental trials	**on day 4 there was one extra fish in well C that was hatched, and dead with fungus	N/A



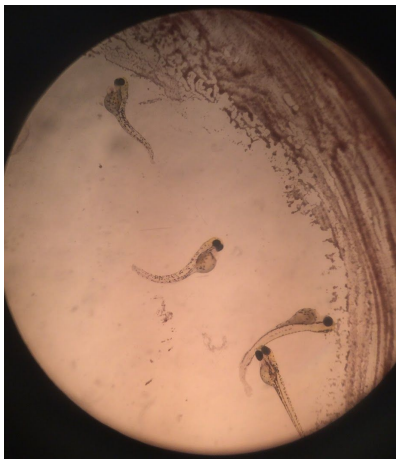
In the controlled setting, all but one of the fish hatched into adulthood showing that the natural survival rate without exposure to lipstick was high. All of the fish in the control setting which was only exposed to the instant ocean solution, developed at a faster rate than fish in the experimental groups. The control group also hatched at an earlier time. The Zebrafish in the control also all had normal spines and no abnormalities in growth.



(Figure 2) Covergirl day 2, dead embryo with pink/orange tint color



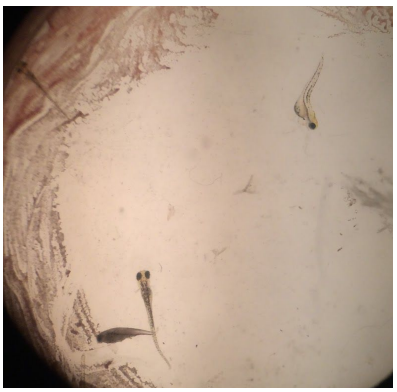
(Figure 3) L'oreal day 4, hatched embryo with strange growth or morphological difference on underside



(Figure 4) Covergirl day 4, abnormal growths and curved spines

(Figure 5) Maybelline day 4, hatching and curved spine

(Figure 6) Maybelline day 4, hatched with curved spine



(Figure 7) Covergirl day 5, 3 hatched, 2 of the hatched with curved spines, one with abnormal growth, and one dead

(Figure 8) Maybelline day 5, hatched fish with spine abnormalities

Discussion

The original hypothesis predicted that the lipstick would cause a decline in the survival of the Zebrafish embryos and also result in an increase in growth defects such as spinal curves, because of exposure to the chemicals and heavy metals in lipstick. In this experiment there were developmental changes in the Zebrafish exposed to the lipsticks as seen by the curved spines as seen in the Covergirl and Maybelline trials, and growth abnormalities which look like an attached yolk sack to the abdomen of the fish in the Covergirl and L'oreal trials. In terms of survival rates, the Control group and the highest survival rate with a 29.20 average. The Maybelline trial had the second highest survival rate with a 28.80 survival average. Loreal had the third highest average survival rate at 22.4, and the Cover Girl trial had the lowest average survival rate at 19.20. Overall the Covergirl lipstick had the greatest impact on survival and development of the Zebrafish embryos with the lowest survival rate and most cases of both growth abnormalities and spinal curves present. Overall the results of this experiment supported the original hypothesis because all of the experimental groups were impacted by the exposure to the lipstick, and this resulted in lower survival rates and increases in developmental abnormalities. According to Liu, S., Hammond, K., and Rojas-Cheatham, A. (2013) the metals such as lead, manganese, aluminum, titanium, chromium, cobalt, nickel, copper, and cadmium found in many lipstick according to the experiment conducted at University of California, Berkeley likely caused these findings due to the negative health effects they have on humans such as being carcinogenic or neurotoxins.

An error in this experiment was that in the control group there was an outlier on day 4. On the fourth day there was one extra fish counted in well C of the control group. This is probably due to the difficulty in counting the embryos because of their small size, or that was accidently moved into the well when changing the solution. There was one hatched fish in that same well that had died due to fungus also on day 4. This increases the probability that the extra fish was accidentally transferred into well C during solution changes, when any dead embryos or fish were being sucked up in order to be removed. It was most likely removed from another well and accidently dropped back into the well plate into well C of the control group. Another error was in the Covergirl group where there was an outlier on day 4 as well because 2 extra fish were counted. This is probably due to the difficulty in counting the embryos because of their small size, and how they blend into the lipstick at the bottom of the well making them difficult to count. There is a chance that these fish were accidently moved into the well while cleaning, when any dead embryos or fish were being sucked up in order to be removed, and solution was being changed. It is possible that these extra fish were removed from another well and accidently dropped back into the well plate. It is also a possibility that this was a miscount or that these extra fish were present from the start but were unable to be seen due to the lipstick creating an environment where they were unable to be seen under the flakes of lipstick around the perimeter of the well.

The findings of this experiment are predicted to be caused by the dangerous chemicals and heavy metals found in the lipstick, although further testing would need to be completed to confirm this prediction. The results of this experiment can be applied to humans in that lipstick is dangerous to fetus and therefore pregnant women should not wear it during pregnancy. This specifically applies to waterproof Covergirl lipstick which had the greatest impact on embryo development and survival. The safest lipstick tested in this experiment was the Maybelline lipstick because it had the lowest impact on survival rates and development of the embryos, but it is still best to avoid lipstick while pregnant. This experiment can be helpful for finding what lipstick to avoid, and which brand of lipstick is the safest to

use. Further research on the effects of lipstick is encouraged to expand on the findings of this experiment. As more research is conducted, updated U.S. standards for heavy metals in lipstick may need to be implemented in order to protect human health, specifically focusing on the protection pregnant woman and the health of their unborn child.

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