EXPOSURE TO RETINOIC ACID INFLUENCES THE DEVELOPMENT AND STURCTURE OF PLANARIAN (*Dugesia tigrina*) EYES

A Report of a Senior Study

by

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Fall, 2017

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ABSTRACT

Planaria have become model specimens for studying eye development and eye diseases in vertebrates because of their ability to regenerate. The objective of this study is to observe the eye structure and development of planarian eyes as well as the width of the heads when exposed to retinoic acid (RA). Twenty planaria were decapitated, exposed to 5nM retinoic acid for 14 days, and assessed at day 0, day 7, and day 14. There was a statistical difference in pigmentation of the controls vs experimentals at day 0 (p-value= 0.0431) and day 7 (p-value= 0.0496), but no difference on day 14 (p-value= 0.2085). There was no statistical difference in head width of the controls vs experimentals at day 0 (p-value= 0.1492) or day 7 (p-value= 0.0924), but heads of retinoic-acid exposed planaria were significantly day 14 ($p<0.001$). These results suggest that the retinoic acid had an effect on the width of the heads, but the effects of eye pigmentation when exposed to RA could not be determined.

ACKNOWLEDGEMENTS

I would first off like to thank Dr. Crain for his patience, time, advice, guidance, and humor throughout this experiment and writing process. I would also like to thank Brenda Eingle, Dr. Swann, and Kathleen Staller for always giving me positive thoughts, words of affirmation and chocolate. I would like to thank Maryville College's Division of Natural Sciences for their financial support and use of their facilities. Lastly, I would like to thank my friends, family, and roommates for their pep talks, love, and support throughout this process.

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CHAPTER I

INTRODUCTION

Planaria have become model specimens for studying eye development and eye diseases in vertebrates because of their ability to regenerate. Regeneration of eye tissues is controlled by an eye stem cell and relies on genes such as ospin, rhodopsin and Pax-6. All of which are found in the iris and light sensing photoreceptors. Retinoic acid signaling in the developing eye is dependent on Pax-6. Finding the effects retinoic acid has on the iris of planaria could lead to discoveries in the human eye such as the prevention of blindness.

REGENERATION

Regenerative abilities range from restoration of the spinal cord, lens and heart, regeneration of whole limbs or fins, or regeneration of the whole body. The cells that contribute to regeneration differ among animals and tissues. In all types of regeneration, cells are the most contributing factor (Owlarn and Bartscherer 2016). The cells typically form a blastema and each organism have systems to record positional information and detect what tissues are missing. The blastema specification and the formation of local signaling centers in each organism allow blastema growth and pattering, resulting in regeneration (Owlarn and Bartscherer 2016).

Regeneration of organs in humans is a medical need, but has only been accomplished a few times worldwide because of the vast difficulties associated with it. Although cosmetic

and functional prostheses are a good alternative, amputees do not use their prostheses for more than about half of the activities of daily living (Ostile et al. 2012). Because of this, scientists are finding ways to recreate limbs and digits in laboratories using the regenerative approaches of developmental biology and tissue engineering (Shieh and Cheng 2015). Furthermore, regeneration will help with diabetics. The regeneration of mescnchymal stem cells, which are uniquely capable of crossing germinative layer borders, are promising cells for regeneration in many diseases including Type 1 diabetes (Anzalone et al. 2011). Researchers hope that with the regeneration of these cells, there will be a lower rate of rejection and amputation.

STEM CELL RENENERATION

Stem cells have been a focus of much regeneration research because of their potential to develop into many different cell types in the body during early growth stages. Stem cells have two important characteristics that set them apart from other cells: (1) they are unspecialized cells and can regenerate themselves through cell division and (2) under certain conditions, they can become tissue or organ specific cells with special functions (Tsien 2006). When a stem cell divides, each of the new cells can either remain a stem cell or become another type of sell with a specialized function, like a red blood cell or a muscle cell. They are regulated by a combination of shared and tissue-specific mechanisms and are distinguished by differences in transcriptional and epigenetic regulation (Nakada 2011).

Stem cells can divide to repair and replace damaged tissues, and they are able to mitotically renew themselves for long periods of time. For example, if stem cells are grown

for many months in a laboratory, they are able to generate millions of cells. Because stem cells are unspecialized, they are able to be channeled into many different specialized functions by specific growth factors. When unspecialized cells give rise to specialized cells, they undergo a process called differentiation. Differentiation is a process where unspecialized embryonic cells gain the features of a specialized cell and is controlled by the interaction of a cell's genes with the physical and chemical conditions outside the cell, usually through signaling pathways involving proteins embedded in the cell surface (Tsien 2006). As a cell differentiates, it goes through multiple stages and becomes more specialized with each step.

There are two types of stem cells: embryonic and adult. Human embryonic cells come from embryos that develop from eggs that have been fertilized in vitro then donated for scientific research. These cells are not derived from eggs that are fertilized in a women's body. Instead, they are pluripotent stem cells coming from various stages of embryonic development and are capable of differentiating into all tissue cell types (Yabut 2011). In order to generate cultures of specific types of differentiated cells, researchers control the differentiation of the embryonic stem cells via changing their chemical composition or modifying the cells by inserting specific genes. It is hoped that medical researchers can create differentiated cells to treat certain diseases in the future.

Adult stem cells, or somatic stem cells, are found among differentiated cells in an organ or tissue (Tsien 2006). They can renew themselves and can yield most all specialized cell types of organs and tissues. The role of the adult stem cell is to maintain and repair the tissues. In many tissues, evidence suggests that some types of adult stem cells are pericytes, cells that make up the outer most layer of blood vessels (Tsien 2006). Unlike embryotic stem

cells, adult stem cells can't regenerate in large numbers. This is because there are a limited number of cells in each tissue and their capacity to divide is compromised once the cells are removed from the body. For many years, adult stem cells have been used in transplants, specifically bone marrow transplants because researchers found that bone marrow contains at least two different types of adult stem cells- hematopoietic stem cells, forms the blood cells, and bone marrow stromal stem cells, a small population of cells found in bone marrow (Tsien 2006). Adult stem cells are also found to be less likely to initiate rejection after a transplant.

Neoblasts are population of small mesenchymal cells that are the only dividing cells of the adult animal. Following an injury to a planarian, neoblasts divide and form an unpigmented bud of regenerated tissue (Scimone 2014). In the flatworm *Planaria sp.*, neoblasts can give rise to the entire range of cell types and organs found in the planarian body plan (Rink 2012). Planaria have the ability to regenerate and this is in large part because they have an abundance of adult stem cells. Indeed, transplantation of a single neoblast into a treated worm rescued and gave way to a perfectly healthy animal of the donor genotype, demonstrating the pluripotency of neoblasts (Wagner et al. 2011).

PLANARIA

Planaria are free-living flatworms from the class Turbellaria of the phylum Platyhelminthes. They are bilaterally symmetric metazoans found in streams and ponds, and have been a model species used for regeneration based on their ability to regenerate any segment of their body plan (Reddien and Sánchez Alvarado 2004). Planaria can self-renew their entire body within a week or two because of progenitors and the turnover of differentiated cells (Rink 2012). Planaria are acoelomates that have three tissue layers:

mesoderm, endoderm, and ectoderm. Planaria have a simple organ system made up of a nervous system and a digestive system (Figure 1).

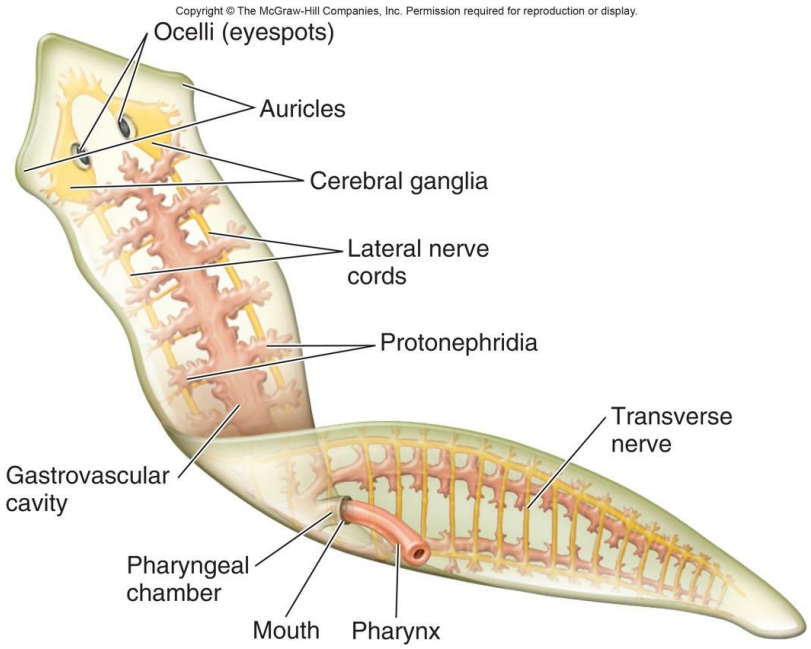

Figure 1: The anatomy of a planarian.

Planaria use the cilia on their ventral side to move over surfaces and are most commonly a light or dark brown color. They feed mainly on insects, insect larvae, and other invertebrates digesting food through an extensible, muscular pharynx that acts as both the mouth and the anus (Reddien and Sánchez Alvarado). The pharynx connects to the three-branched digestive system that consists of two posterior branches and one anterior branch (Sánchez Alvarado and Newmark 1999). They are hermaphroditic and either reproduce sexually by cross fertilizing or reproduce asexually by binary fission where they split their body from top to bottom and regenerate the left and right halves (Gilbert 2014).

Most planaria live in freshwater, but some live in saltwater streams, rivers, and ponds. The most common planaria that is researched, because of its molecular genetics of

regeneration, is the *Dugesia japonica.* This species is light brown and are found in many parts of East Asia including Taiwan, China, Korea, and Japan. They are the most common freshwater planarian in Japan. Another common species is the *Girardia tigrina*. They average 10mm in length, light brown and are native to the Americas, but have distributed to parts of Europe and Japan where they are now an invasive species (Ball 1974).

EYE DEVELOPMENT AND REGENERATION

A main characteristic of planaria is their triangular head that has two ocelli and two auricles that give them the appearance of being "cross-eyed" (Figure 2).

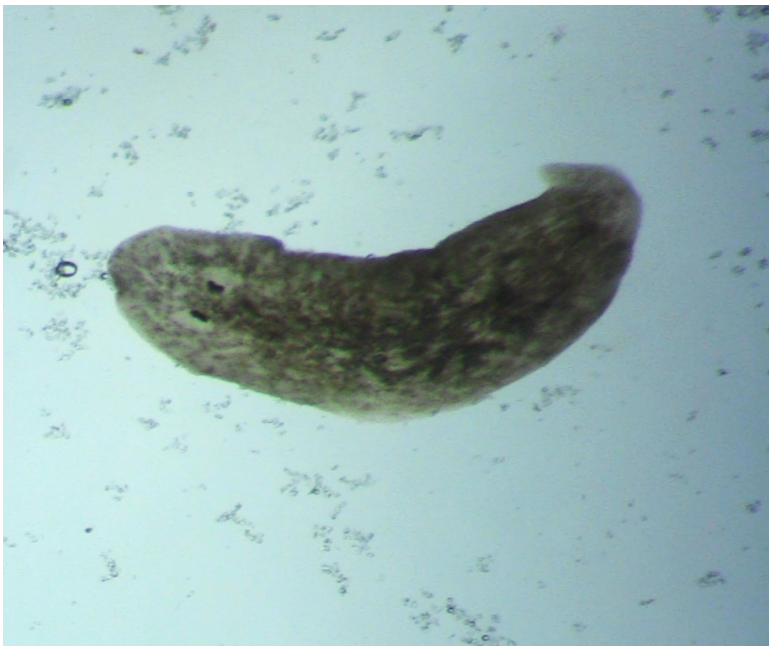

Figure 2: The eye spots and auricles are visible in this close up of a brown speckled planarian (*Dugesia tigrina)*

The ocellus is a type of simple eye that is common in vertebrates; it consists of retinal cells, nerve fibers and pigments. In the case of planaria, it is another name for a light-sensing eyespot. The photoreceptor neurons found in the eyespots are used to transform photons into signals and send them to the brain (Paskin 2014). They also are capable for detecting light in

shadow and are grouped into eyespots defining a simple ancestral visual system consisting of bipolar retinal neurons whose dendrites project into a cup shaped structure composed of pigment cells. The pigment cells form a semi-lunar pattern on the proximal side of the optic cup (Kishida 1967, Sakai et al. 2000 & Pineda 2002). The bipolar photoreceptor neurons project their dendrites into the optic cup, forming stacks of photosensitive microvilli known as rhabdomeres, while their axons innervate the underlying brain both contralaterally and ipsilaterally via a true optic chiasm (Okamoto et al. 2005.) Regeneration of both eye tissues is controlled by an eye stem cell and relies on genes such as ospin and rhodopsin which are shown in the light-sensing photoreceptors (Deochand et al. 2016). Planarian eyes are unable to focus the light patterns into images, as they don't have focusing lenses, they serve essentially the same function, receiving and transducing light into neuronal signals.

Planaria have become model specimens for studying eye development and eye diseases in vertebrates because of their ability to regenerate. Four critical genes that effect eye development and eye structure are Ovo, Pax-6, Tryptophan hydroxylase, and Sp6-9 (see table 1).

Table 1. Four critical genes that effect eye development and eye structure in *Planaria sp.*

Gene	Function	Reference
Pax6	Lens morphogenesis	Callaerts et al (1999)
Ovo	Activates the expression of other genes	Lapan and Riddien (2012)
Tryptophan hydroxylase (tph)	Essential for melanin production in the pigment cups of the photoreceptors	Lambrus et al (2015)
Sp6-9 and dlx	Essential for formation of optic cup progenitors	Lapan et al (2011)

The Pax6 gene is required for eye morphogenesis, the development of olfactory sense organs, and the development of parts of the central nervous system. It is found in many species suggesting that it may be a universal control gene for eye morphogenesis (Callaerts 1999). Pax6 is a master control gene for eye development and is a common genetic program for making eyes. It is characterized in a large number of triploblastic metazoans from planaria to humans (Pineda 2002). The gene, specifically in DtPax-6, is expressed in pigmented cells and in the photoreceptors proving that Pax6 is important for eye evolution. Specifically, the Pax6A gene is expressed in the central nervous system and is activated during cephalic regeneration. Both the Pax6A and Pax6B gene transcripts are detected in only visual cells (Pineda 2002). In some vertebrates, Pax6 is required for the development of head sensory organs or other structures such as the pancreas and the pituitary glands. In *Drosophila,* Pax6 is responsible for inducing the formation of ectopic eyes, but in frogs, the misexpression of Pax6 can lead to ectopic eyes.

Ovo activates the expression of many genes as the eye develops. Ovo is vital for eye regeneration and eye development because when the gene is turned off, an amputated planarian can not regenerate their eyes (Lapan and Riddien 2011). Cells expressing Ovo can be found elsewhere in the body including the female germline (Mével-Ninio 1991).

Tryptophan hydroxylase (tph) contains melanin that is an important pigment found in multiple species of animals. Melanin is responsible for determining the color of hair, skin and eyes of animals (Lambrus 2015). Planaria have two distinct types of pigmentation in their body- the brown pigmentation in their skin and the black pigmentation in their eyes. Tph catalyzes the rate-limiting step of serotonin synthesis and after interrupting the sequence of

tph, planaria's regenerated eyes lacked the pigment cup giving no color to their eyes (Lambrus 2015).

Sp6-9 and dlx are essential for the optic cup progenitors. The optic cup in planarians is formed entirely of pigment cells and photoreceptor neurons project rhabdomeres into the cup (Sato 2005). A primary function of pigmented optic cups is to absorb incoming light, before the detection of light by photoreceptors, creating shade allowing the eye and brain to resolve the direction of incoming light (Lapan 2011). Sp6-9 cells co-express other markers of the optic cup, including dlx. Both dlx and sp6-9 are required in eye regeneration but is not found in planaria that lose their eyes via decapitation (Lapan 2011).

One question raised during eye regeneration of planaria is if different injuries change the timeline of eye regeneration. If decapitated, an adult planarian can regenerate its head and eyes in about 28 days (Deochand et al. 2016). Tissue loss had no effect on the time of eye regeneration. The surgical ablation removed the optic cup, which has pigment cells, and dendrites/cell bodies of the photoreceptor neurons, without disturbing surrounding tissues. The planaria were equally able to regenerate eyes following a surgical ablation (Deochand et al 2016). The temporal regulation of eye regeneration is not affected by the location or the size of the tissue removed. The metabolic rates also had no effect on the timing of eye regeneration. At day 14, a starved planaria had a significantly smaller pigment spot size but by day 28, it was again back to normal size (Deochand et al. 2016). These data suggest that both eye tissue types are regenerated simultaneously following eye loss. While loss of the photoreceptor neurons doesn't affect pigment cell regeneration, loss of pigment cells does result in defective axonal patterning of the photoreceptors (Lapan & Reddien 2011). Thus,

pigment cells may be required for maintenance of optic chiasm patterning and show a relationship between the optic neurons and pigment cells during regeneration.

RETINOIC ACID AND GENE EXPRESSION

Retinoic acid, a metabolite of Vitamin A, regulates over 500 genes in all animals. For some genes, the control is direct, driven by a liganded heterodimer of retinoid receptors bound to a DNA response element. For others, its indirect and reflects the action of intermediate transcription factors, non-classical association of receptors with other proteins (Balmer and Blomhoff 2002). In 1977, Blalock and Gifford were the first to provide evidence that interferon synthesis can be suppressed at a transcriptional level by protein induced by all-trans retinoic action. They showed that vitamin A acid restores density-dependent growth control to a transformed cell line. This suggests that the action of vitamin A may result from a direct effect of vitamin that restores normal functions to transformed cells (Blalock and Gifford 1977).

Vitamin A is a name of a group of fat-soluble retinoids including retinol, retinal, and retina esters. It is involved in immune function, reproduction and cellular communications. Vitamin A is vital for vision as it is an essential component of rhodopsin. The presence of rhodopsin-like proteins have been found in the eyes and auricle of planaria (Asano 1998) where it supports normal differentiation and functioning of the cornea and conjunctival membranes in the eye (Coates 2010). Aside from the eyes, vitamin A effects cell growth and differentiation and plays a crucial role in the normal formation and maintenance of multiple organs including the lungs, kidneys and heart.

During eye development, retinoic acid is synthesized in the retina and controls the fate of the cells comprising the neural retina. Retinoic acid signaling is initially required for reciprocal interactions between the optic vesicle and invaginating lens placode and promotes normal development of the ventral retina and optic nerve through its activities in the neural crest cell-derived periocular mesenchyme (Cvekl 2009). Retinoids are synthesized asymmetrically, a method for preparation of chemical compounds which aims to bias the synthesis in favor of producing one stereoisomer over another stereoisomer, in the developing retina as has been determined by the expression of the enzymes responsible for their synthesis (McCaffery et al 1992). The activation of certain retinoic acid receptors has been found to be a crucial part of retina morphogenesis. Retinoic acid treatment on mollusks during development causes dose-dependent effects ranging from minor defects in eye size to developmental arrest as trocophora larva (Créton et al. 1993). Exogenous retinoic acid disrupts anterior regeneration in planaria. The period of maximum sensitivity in planarian anterior regeneration corresponds with the period of maximum sensitivity in regeneration. This suggests that the regional specification during regeneration is disto-proxmial and takes place by intercalation of the central region between the anterior and posterior ones. (Romero & Bueno 2001). Retinoic acid is essential in the development of the central nervous system and during differentiation in chicken and mice, in the pattering of limbs and the formation of the myocardium in *Xenopus* (Romero & Bueno 2001). A specimen being exposed to too little or too much retinoic acid during embryogenesis causes severe eye defects.

The retinoid receptors activated the expression of alphaB-crystallin gene by targeting the lens-specific regulatory regions found it its promoter, the same region used by Pax-6 (Kastner et al 1994 and Enwright and Grainer 2000). These properties of retinoids and their

receptors during eye and lens development as well as involvement of retinoids in regulation of genes that might be important for lens regeneration and have prompted further examination of their role in lens regeneration. Pax-6 have been found to be specifically expressed in the iris during dedifferentiation of the pigment epithelium and the formation of the lens vesicle. Retinoic acid signaling in the developing eye is dependent on Pax-6. Mice that carry the Pax-6 mutation show a decrease in the retinoid signaling in the eye and the lens anlage cannot respond to exogenous retinoic acid (Enwright and Grainer 2000).

QUESTION AND HYPOTHESIS

The objective of this study is to observe the eye structure and development of planarian eyes as well as the width of the heads. The goal is to see if the structure and pigmentation of a planarian's eye is changed when exposed to retinoic acid after a 14-day period. The null hypothesis is that there will be no change in the eye structure of the planaria when exposed to the retinoic acid. This data collected is important for understanding how silencing a gene will impact the structure and development of an organ. Finding the effects retinoic acid has on eyes could lead to discoveries in the human eye such as the prevention of blindness.

CHAPTER II

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN

This experiment occurred over a period of two weeks. Four separate 100ml beakers were set up in the student laboratory in the Sutton Science Center at Maryville College in Maryville, TN. A total of 100 adult brown planaria were purchased from Carolina Biological Supply Company (2700 York Road, Burlington, NC 27215). The planaria were maintained in the lab at 23ºC and fed hard-boiled egg yolk once a week.

The planaria were first placed into a petri dish and the heads were detached with a straight horizontal cut behind their auricles (Figure 3). A photo of the eye structure was taken using a dissection microscope (at 10x magnification) with a Canon VIXIA HF G20 camera. Once complete, 20 individual petri dishes were each filled with one head and one body. In total, there were 20 heads and 20 bodies. Each petri dish, labeled 1-10 control and 1-10 experimental were filled with 10mL of spring water (collected from Fort Craig Spring, Pistol Creek, Maryville, TN). Then, 100% ethanol was added to the controls and retinoic acid was added to the experimentals.

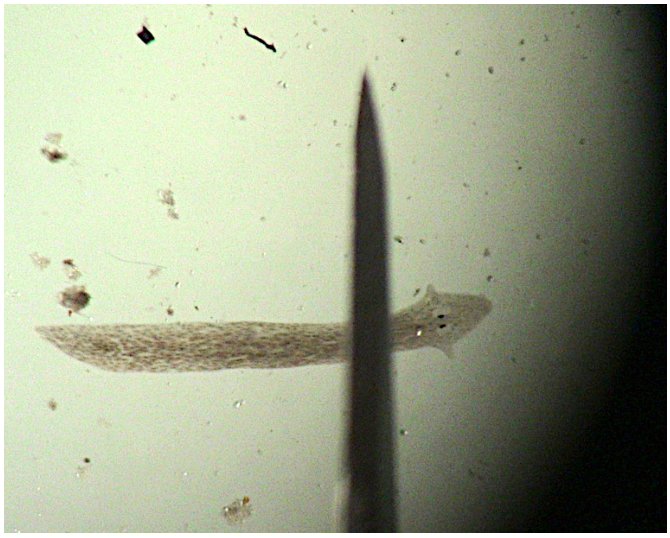

Figure 3: Using a scapula, a straight horizontal cut was made behind the auricles of the planaria. Magnification 15x.

A 50mg bottle of retinoic acid powder (MW 300.44) was purchased from Sigma-Aldrich (St. Louis, MO) and a stock concentration of 1.0M was created using 100% ethanol. Four trials were needed to find a non-lethal concentration of retinoic acid. During trial one (figure 4), the final concentration was 0.5 mM, trial two used 5µM, and trial three used 50nM. During each of these trials, the concentrations were added to a final volume of 100mL of spring water. These resulted in 100% death within 12 hours.

Figure 4: The two beakers (one with heads and one with bodies) on the left shows the experimentals during trial one. The planaria were placed in 100mL of spring water and a final concentration of 0.5mM of retinoic acid was added. The two beakers on the right were the controls.

Trial 4 used a concentration of 50pM, and there was 0% death. These planaria heads and bodies were individually exposed for 14 days to either retinoic acid (5 Microliters of 50pM concentration) or ethanol (5 Microliters) to spring water for a total volume of 10 ml.

DATA COLLECTED

At day 7 and day 14, the eye structure of the planaria was again photographed. ImageJ software (National Institutes of Health) was used to analyze photos from day 0, day 7, and day 14. Pictures of the eyes were uploaded and the area of the whole eye, in pixels, was rerecorded as well as the area of the pigmented section of both eyes. The width of the head was also measured. A scale of centimeters was calibrated using ImageJ and was used to measure the width of the head right behind the auricles (figure 5).

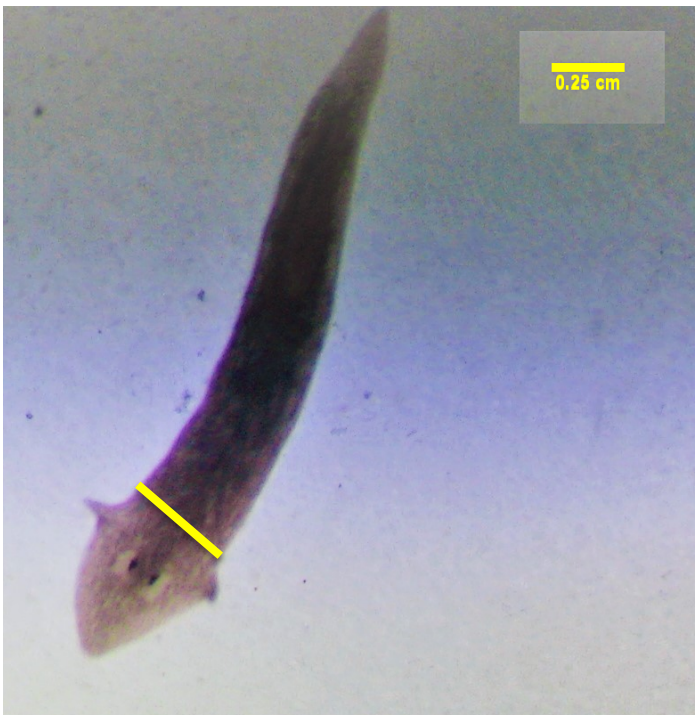

Figure 5: The yellow line represents the place the width of the head was measured in centimeters. Scale bar, 0.25cm

STATISTICAL ANALYSIS

An ANOVA with a Fisher's post-hoc test was conducted to compare day 0, 7, and 14 pigmentation and head width for control and experimental planaria. A standard t-test was created to compare the controls to the experimentals at each of the three time periods and the p-value was recorded showing if any of the three time periods were statically significance. The averages of each were calculated and recorded.

CHAPTER III

RESULTS

Each of the planaria showed regrowth over the 14-days. The planaria that had their heads cut off grew tails and the planaria that were headless and only had a body, grew new heads. The planaria heads analyzed were the heads that grew tails. There was a decrease of the eye pigmentation in both the controls (n=20) and the experimental (n=20) groups (figure 6). The averages were taken from each group at three different time periods: day 0, day 7, day 14. An ANOVA test showed a decrease in pigmentation between each day of both the controls ($p<0.001$) and the experimentals ($p<0.001$).

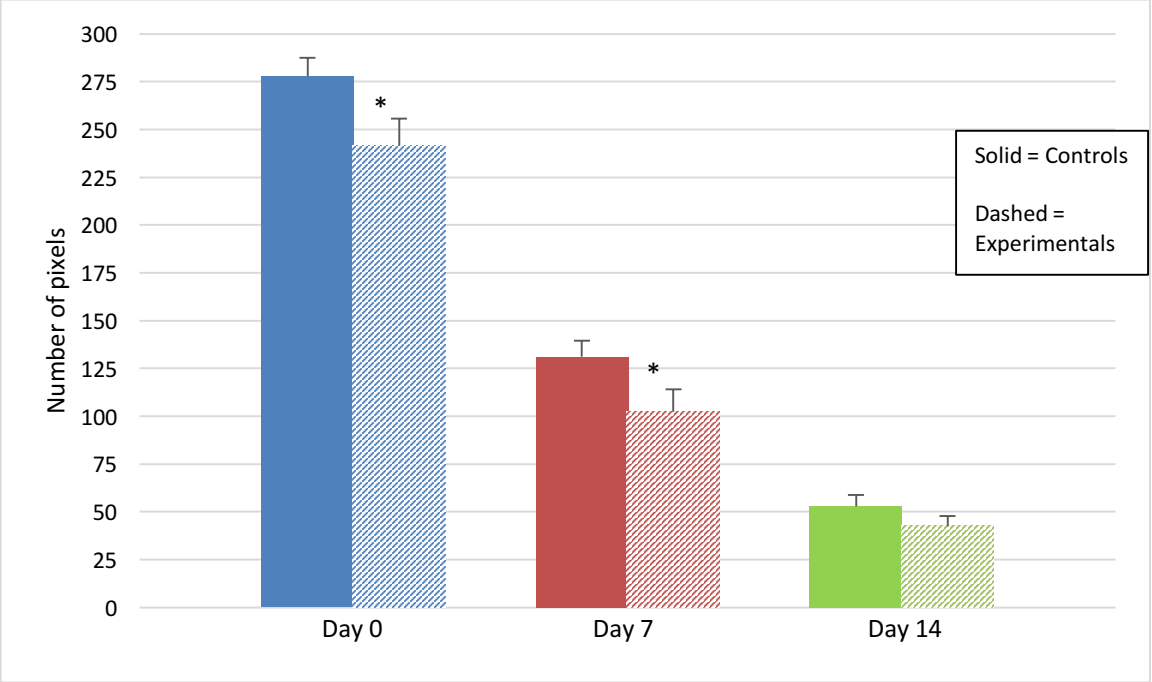

Figure 6: A side by side comparison of the average pigmentation in planarian eyes (left and right eye) of the control and experimental groups calculated at three different time periods. * indicates significance between control and retinoic acid at $p<0.001$.

At each time period, the photographs taken show a decrease in pigmentation for both the controls ($p <0.001$) and the experimentals ($p <0.001$). The pigmentation of the planaria eyes from day 0 (figure 7), day 7 (figure 8), and day 14 (figure 9) were put in a side by side comparison prior to analyzing. Figure 10 shows the same two planaria (control 1 and experimental 1) at each of the three time periods.

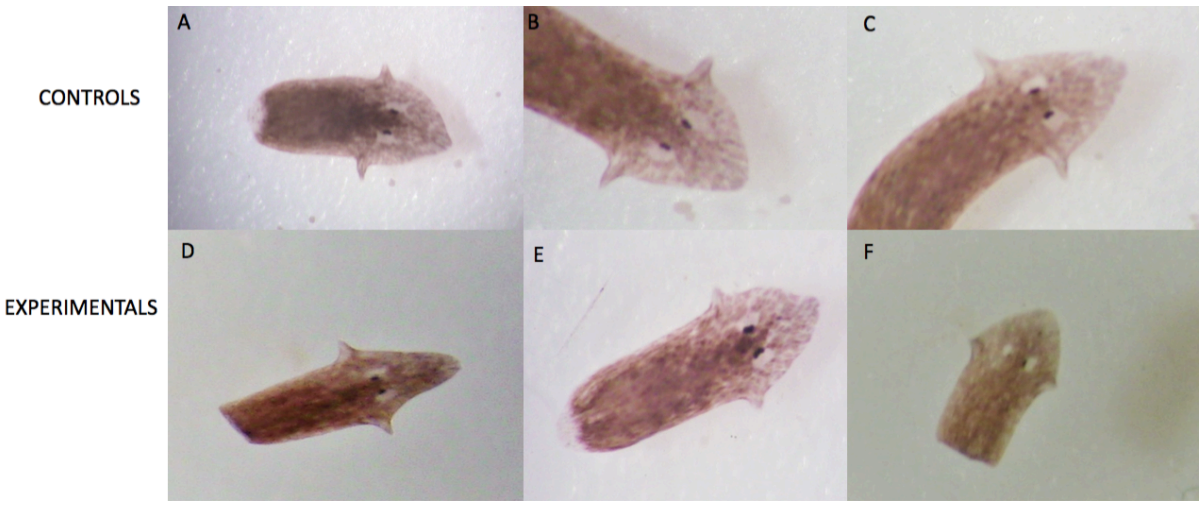

Figure 7: Photographs taken on day 0 of the experiment of three random planaria from the control group and the experimental group. (**A)** Head from control 2. **(B)** Head from control 5. **(C)** Head from control 10. **(D)** Head from experimental planarian 3. **(E)** Head from experimental planarian 4. **(F)** Head from experimental 9. Magnification 10x.

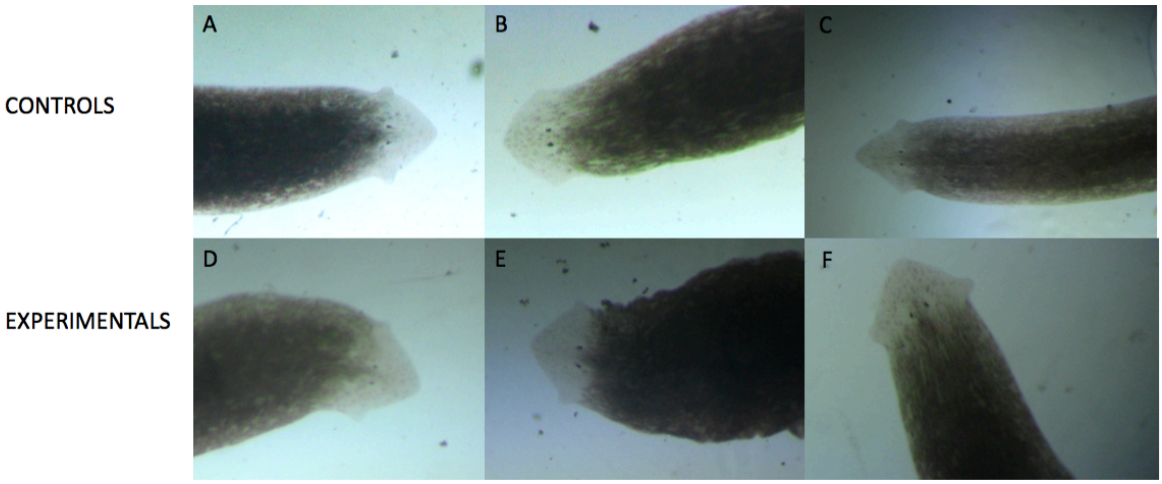

Figure 8: Photographs taken on day 7 of the experiment of three random planaria from the control group and the experimental group. (**A)** Head from control 3. **(B)** Head from control 7. **(C)** Head from control 8. **(D)** Head from experimental planarian 1. **(E)** Head from experimental planarian 5. **(F)** Head from experimental 6. Magnification 10x.

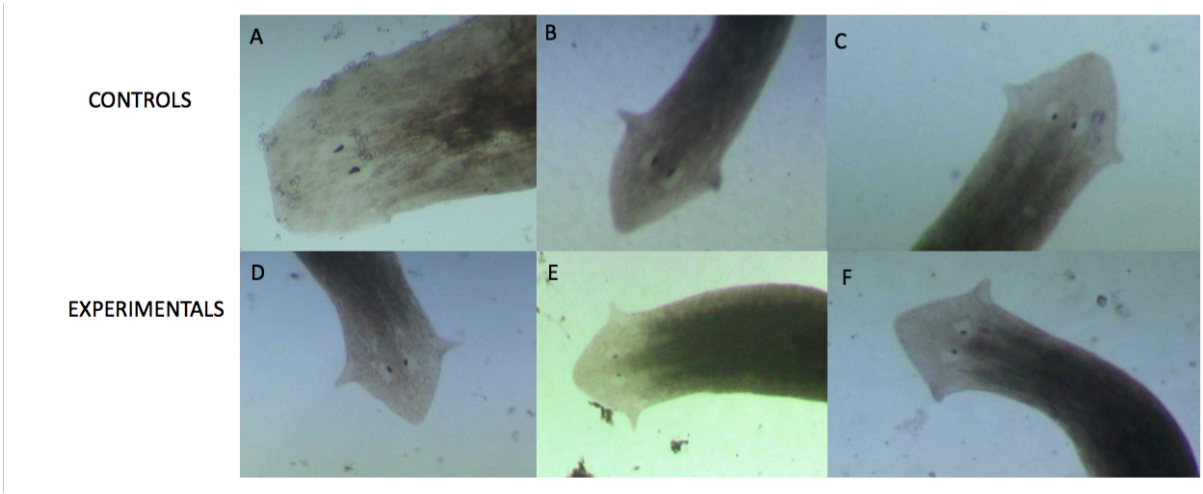

Figure 9: Photographs taken on day 14 of the experiment of three random planaria from the control group and the experimental group. (**A)** Head from control 6. **(B)** Head from control 1. **(C)** Head from control 9. **(D)** Head from experimental planarian 2. **(E)** Head from experimental planarian 8. **(F)** Head from experimental 10. Magnification 10x.

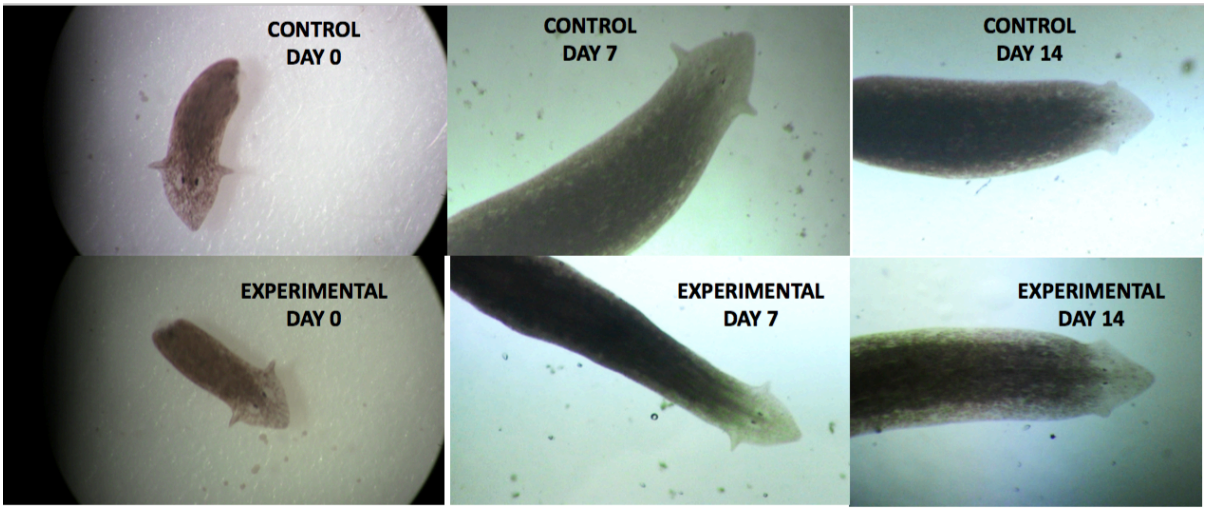

Figure 10: A sequence of photographs of the same two planaria (control 1 and experimental 1) at each of the three time periods. Magnification 10x.

Although there is a statistical difference in pigmentation of the controls vs experimentals at day 0 (p-value= 0.0431) and day 7 (p-value= 0.0496), there was no statistical difference on day 14 (p-value= 0.2085). The averages showed a decrease in pigmentation in both the experimental and controls over the two weeks.

The width of the heads were also measured. The was a decrease in width for both the controls and the experimentals over the 2-week exposure period. The averages were again taken from each of the three time periods (figure 11).

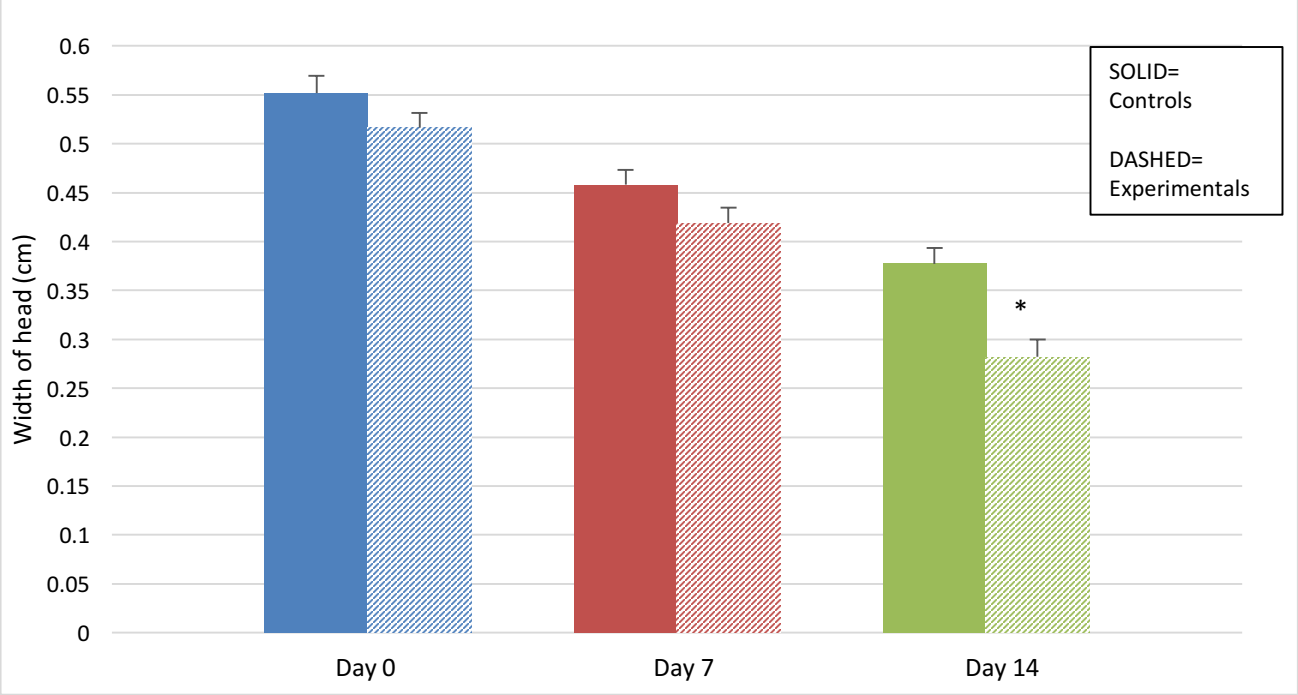

Figure 11: A side by side comparison of the average width of planarian heads of the control and experimental groups calculated at three different time periods. * indicates significantly different at $p<0.001$.

There was no statistical difference in head width of the controls vs experimentals at day 0 (p-value= 0.1492) and day 7 (p-value= 0.0924), there was a statistical difference on day 14 ($p<0.001$). The averages showed a decrease in head width in both the experimental and controls over the two weeks exposure period. An ANOVA test showed a decrease in head width between day 0, 7, and 14 of the control (p-value= 2.436e-07) and experimental (p-value=4.758e-10) groups.

CHAPTER IV

DISCUSSION

SUMMARY

Because there was a significant difference in pigmentation between the controls and the experimentals at day 0, no conclusion can be made between retinoic acid and eye pigmentation. There was a decrease in pigmentation from week to week when comparing the day 0 controls to the day 7 and day 14 controls as well as comparing day 0 experimentals to day 7 and 14. It can be interpreted that 100% ethanol caused a decrease in the pigmentation of the planarian eyes.

There was no statistical significance in day 0 and day 7 of the controls versus experimental when measuring the width of the head. At day 14 there was a statistical significance between the controls and the experimentals meaning that over time, the retinoic acid decreased the width of the planarian heads.

It is interesting to note that the planaria treated at day 0 (just after amputation), when the wound was recent and fresh which allowed for more retinoic acid to get in, showed a smaller difference in head width when compared to the same planaria at day 7. The planaria at day 14, when the wound was sealed and it was harder for external substances to enter, showed a larger difference in compared to day 7. This suggests that the effects are not due to a non-specific toxic effect. It is possible that the high concentration of retinoic acid needed

may be due to the hypothetical receptors present in planarians, as retinoid receptors found in other invertebrates (Jones, 1997) do not display high affinity for retinoic acid.

The data supports the null hypothesis mainly in part to there being a statistical significance between the eye pigmentation of the controls at day 0 compared to the eye pigmentation of the experimentals at day 0. Because of this, it can't be said that the retinoic acid solely had an effect on the regeneration of the head and the eye pigmentation.

MORTALITY

The first three of the four trials resulted in 100% death of the planaria. A possible cause of this was a very high concentration of retinoic acid. This caused the planaria to die within 12 hours of exposure. Even when the concentration was lowered to 0.1mM, it was still too concentrated for the time of exposure tested. The calculated concentration was based on an experiment done in 2001 by Romero and Bueno. They treated their planaria with a final concentration of 0.5 mM in spring water diluted from a 0.1M stock solution of retinoic acid in ethanol. They conducted two separate experiments. In the first experiment, they exposed their planaria to daily retinoic acid treatments of two hours per day for 10 days. In their second experiment, the planaria received a single two-hour treatment ranging from 0-7 days after amputation (Romero and Bueno 2001). After each of the two-hour treatments, regenerates were washed several times in 2ml of spring water. Unlike Romero and Bueno (2001), the present study exposed planaria continuously, and apparently exposure to these high doses of retinoic acid for longer than 2 hours is lethal.

In the fourth trial, the concentration was decreased to a two-week non-lethal concentration. A 50pM concentration was low enough for the planaria to survive past two

weeks. In addition, individual housing of the planaria into separate petri dishes allowed could have contributed to survival. In both experiments done by Romeno and Bruno (2001), their planaria were maintained in a 24-well plate with 5 planaria per well. Each well contained 150µl of retinoic acid solution. In this experiment, the planaria were isolated to one head and one tail per petri dish that contained 10 ml final volume. This experiment had a larger retinoic acid to spring water ratio than the one done by Romeo and Bruno.

Romeo and Bruno (2001) kept their planaria in a dark 4-6˚C room and fed them once a month. They also starved the planaria for at least 15 days prior to use. After that, the new cut heads were maintained in spring water at 17˚C until they were ready for use. In this experiment, the planaria were fed hours before amputation and exposed to the retinoic acid right after amputation. They were also kept at a temperature of 23˚C throughout the entire experiment.

In this experiment, the heads ranged from 0.46cm to 0.68cm at day 0. This shows that the planaria used in this experiment were slightly larger on average and this might have had an effect on the width throughout.

FUTURE STUDIES

Further research should expose planaria to 0.5nM for a longer exposure period to evaluate the effect on complete head regeneration. It takes an average of 28 days for a planarian to fully develop its head back (Deochand et al. 2016). Looking at the pigmentation after 28 days might show the same amount or more pigmentation than that of day 0. Unfortunately, because there was no conclusion of the effects that retinoic acid had on the pigmentation it was difficult to see if there was an effect on the genes expressed in planaria

eyes, Ovo, Pax-6, Tryptophan hydroxylase, and Sp6-9. It would be interesting to see if finding the highest concentration of retinoic acid, that is not lethal and a longer exposure period would show a change in the gene expression of the eyes. This could be done by running a gene expression analysis before exposure and after a certain period of time.

APPENDICES

APPENDIX 1: The number of pixels from the black pigmentation of each eye. The mean of each column is in the last row.

Contol Initial	Control 1wk	Control 2wk	Exp Initial	Exp 1wk	Exp 2wk
212	128	28	212	36	22
297	85	64	297	114	49
290	78	8	290	112	42
245	118	28	245	70	36
320	142	48	320	139	42
283	136	50	283	144	88
281	224	42	281	115	35
278	132	87	278	94	45
240	40	30	240	126	38
257	148	60	257	76	42
392	121	24	180	45	29
255	152	96	304	146	27
268	108	41	130	92	36
295	136	28	285	134	37
287	152	32	182	66	6
275	128	88	184	28	24
273	184	77	319	264	115
361	130	49	267	98	61
235	132	70	175	64	28
211	148	107	105	87	48
277.75	131.10	52.85	241.7	102.5	42.5

APPENDIX 2: Two-Sample Assuming Equal Variances t-Tests of the eye pigmentations during each of the three time periods.

	Contol Initial	*Exp Initial*
Mean	277.75	241.7
Variance	1941.460526	3994.957895
Observations	20	20
Pooled Variance	2968.209211	
Hypothesized Mean Difference	0	
df	38	
t Stat	2.092464096	
P(T<=t) one-tail	0.02156451	
t Critical one-tail	1.68595446	
P(T<=t) two-tail	0.04312902	
t Critical two-tail	2.024394164	

	Control 1wk	*Exp 1wk*
Mean	131.1	102.5
Variance	1427.042105	2697.210526
Observations	20	20
Pooled Variance	2062.126316	
Hypothesized Mean Difference	0	
df	38	
t Stat	1.991628814	
P(T<=t) one-tail	0.026817472	
t Critical one-tail	1.68595446	
P(T<=t) two-tail	0.053634944	
t Critical two-tail	2.024394164	

	Control 2wk	*Exp 2wk*
Mean	52.85	42.5
Variance	747.7131579	561.4210526
Observations	20	20
Pooled Variance	654.5671053	
Hypothesized Mean Difference	0	
df	38	
t Stat	1.279273082	
P(T<=t) one-tail	0.104278752	
t Critical one-tail	1.68595446	
P(T<=t) two-tail	0.208557503	
t Critical two-tail	2.024394164	

APPENDIX 3: An ANOVA test comparing the controls to controls and the experimentals to the experimentals of eye pigmentation.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	521395.3	2	260697.65	190.0029032	6.14537E-26	3.158842719
Within Groups	78208.1	57	1372.07193			
Total	599603.4	59				

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	417715.2	2	208857.6	86.38106723	5.57182E-18	3.158842719
Within Groups	137818.2	57	2417.863158			
Total	555533.4	59				

APPENDIX 4: The width in centimeters of the planarian heads.

Day 0		Day 7		Day 14	
	Length (cm)		Length (cm)		Length (cm)
Control 1	0.529	Control 1	0.412	Control 1	0.35
Control 2	0.54	Control 2	0.467	Control 2	0.417
Control 3	0.656	Control 3	0.504	Control 3	0.404
Control 4	0.465	Control 4	0.353	Control 4	0.266
Control 5	0.593	Control 5	0.5	Control 5	0.45
Control 6	0.486	Control 6	0.477	Control 6	0.381
Control 7	0.541	Control 7	0.475	Control 7	0.365
Control 8	0.588	Control 8	0.42	Control 8	0.385
Control 9	0.591	Control 9	0.484	Control 9	0.342
Control 10	0.529	Control 10	0.489	Control 10	0.414
Exp 1	0.449	Exp 1	0.322	Exp 1	0.259
Exp 2	0.504	Exp 2	0.433	Exp 2	0.378
Exp 3	0.606	Exp 3	0.448	Exp 3	0.324
Exp 4	0.559	Exp 4	0.481	Exp 4	0.244
Exp 5	0.528	Exp 5	0.423	Exp 5	0.226
Exp 6	0.512	Exp 6	0.481	Exp 6	0.232
Exp 7	0.459	Exp 7	0.362	Exp 7	0.21
Exp 8	0.541	Exp 8	0.444	Exp 8	0.336
Exp 9	0.529	Exp 9	0.41	Exp 9	0.31
Exp 10	0.481	Exp 10	0.383	Exp 10	0.301

AEEPENDIX 5: Two-Sample Assuming Equal Variances t-Tests of the head width during each of the three time periods.

	Contol Initial	*Exp Initial*
Mean	277.75	241.7
Variance	1941.460526	3994.957895
Observations	20	20
Pooled Variance	2968.209211	
Hypothesized Mean Difference	0	
df	38	
t Stat	2.092464096	
P(T<=t) one-tail	0.02156451	
t Critical one-tail	1.68595446	
P(T<=t) two-tail	0.04312902	
t Critical two-tail	2.024394164	

	Control 1wk	*Exp 1wk*
Mean	131.1	102.5
Variance	1427.042105	2697.210526
Observations	20	20
Pooled Variance	2062.126316	
Hypothesized Mean Difference	0	
df	38	
t Stat	1.991628814	
P(T<=t) one-tail	0.026817472	
t Critical one-tail	1.68595446	
P(T<=t) two-tail	0.053634944	
t Critical two-tail	2.024394164	

	Control 2wk	*Exp 2wk*
Mean	52.85	42.5
Variance	747.7131579	561.4210526
Observations	20	20
Pooled Variance	654.5671053	
Hypothesized Mean Difference	0	
df	38	
t Stat	1.279273082	
P(T<=t) one-tail	0.104278752	
t Critical one-tail	1.68595446	
P(T<=t) two-tail	0.208557503	
t Critical two-tail	2.024394164	

APPENDIX 6: An ANOVA test comparing the controls to controls and the experimentals to the experimentals of head widths.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	521395.3	2	260697.65	190.0029032	6.14537E-26	3.158842719
Within Groups	78208.1	57	1372.07193			
Total	599603.4	59				

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	417715.2	2	208857.6	86.38106723	5.57182E-18	3.158842719
Within Groups	137818.2	57	2417.863158			
Total	555533.4	59				

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