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Schmidtea mediterranea planaris as a novel animal model for investigating the dynamics of nervous system development in fetal alcohol spectrum disorder

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SCHMIDTEA MEDITERRANEA PLANARIA AS A NOVEL ANIMAL MODEL FOR INVESTIGATING THE DYNAMICS OF NERVOUS SYSTEM DEVELOPMENT IN FETAL ALCOHOL SPECTRUM DISORDER

By
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Abstract

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Fetal Alcohol Spectrum Disorder (FASD) is a prevalent developmental disease that is caused by excess *in utero* exposure to ethanol. The importance of timing and dosage of ethanol has been elucidated by previous investigations conducted with humans, rodents, and other model organisms, but challenges associated with determining functional deficiencies have necessitated the introduction of a novel animal model for investigating the dynamics of FASD. In this thesis, we propose that the freshwater flatworm planarian species, *Schmidtea mediterranea*, has the capability to fulfill this need. Planaria have the ability to completely regenerate fragments into intact, functional animals, and in these studies, we exploited this property to characterize nervous system development in the presence of ethanol. Functional testing of head-regenerating planaria included planarian locomotor velocity testing to assess locomotor capabilities and light avoidance testing to assess development of innate negative phototaxic behavior. The results indicate that ethanol exposure during development leads to dose-dependent effects, including delayed onset of nervous system functionality in low concentrations and overall nervous system attenuation in high concentrations. This suggests that ethanol exposure in head-regenerating planaria can be used as a novel model for the functional dynamics of FASD, enabling future studies that compare molecular and functional deficiencies in FASD.

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Chapter 1

Introduction

1.1 Background

Fetal alcohol spectrum disorder (FASD) arises from prenatal ethanol exposure and affects roughly 10 to 40 per 1,000 live births worldwide.[1] FASD can be characterized by various congenital anomalies, including: central nervous system (CNS) developmental delays, malformation of the CNS, loss or impairment of fine motor skills, disruption of normal cranial morphogenesis, and learning disabilities.[2,3] However, despite more than a century of research, the underlying mechanisms leading to FASD are not understood fully.

Investigations of FASD have been conducted using humans, rodents, and other model organisms.[4,5,6,7] These studies have elucidated the importance of timing and dosage of alcohol during prenatal development on the severity of postnatal FASD symptoms. However, although the dynamic nature of FASD development is well-established, the retrospective necessity of any functional study of FASD prohibits the simultaneous investigation of molecular dynamics and CNS functional deficiencies during FASD onset in the current animal models. To address this, and to further characterize the dynamics of FASD inception, we propose an alternative animal model of FASD.

1.2 Purpose of Experiment

This study examines the potential of the planarian species *Schmidtea Mediterranea* as an alternative animal model for FASD. Functional testing was used to determine what effect alcohol exposure would have on the development of the CNS for planaria exposed to varying concentrations of alcohol. The purpose of this work is to determine whether alcohol exposure during development of the CNS has an adverse effect on development, making planaria a novel animal model for FASD. In this thesis, we show that ethanol exposure during CNS development exhibits a dose dependent response on CNS function. In Chapter 2, a literary review of the current relevant information regarding FASD and planaria is provided. Chapter 3 provides a description of the materials and methods used to test the hypotheses. Chapter 4 contains all of the results obtained over the course of experimentation, as well as an in-depth discussion of the results. In Chapter 5, the conclusions for the thesis are presented, with recommendations for future research directions.

Chapter 2

Literary Review

One complication during pregnancy occurs following excess consumption of alcohol by the mother during fetal development. This can lead to developmental deficiencies when the child is born, typically observed through morphological dysmorphology and central nervous system (CNS) and intellectual dysfunction. This collection of symptoms describes the signs of Fetal Alcohol Syndrome (FAS), one condition in a spectrum of alcohol related complications known as Fetal Alcohol Spectrum Disorders (FASD). FAS is the most common identifiable cause of mental retardation, and affects an estimated 10 to 40 per 1000 live births.[2]

2.1 Symptoms of Fetal Alcohol Syndrome

Morphological dysmorphology are prevalent in cases of FAS, and children afflicted with FAS often have a distinct set of characteristic features. FAS patients typically have small crania, and are usually in the tenth percentile in height and/or weight.[1] As shown in Figure 1, there are also a collection of minor abnormalities that occur in FAS patients' faces.[1]

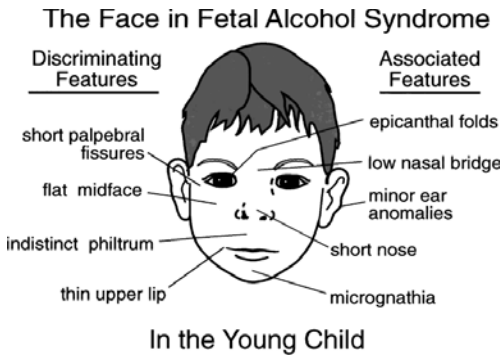


Figure 1: Facial Abnormalities Characteristic of FAS. Image from [1]

Researchers studying FAS have also reported several intellectual deficiencies. Children with FAS are prone to hearing difficulties, including conductive hearing loss.[8] Problems with linguistic capabilities have been reported as well, including voice dysfunction, articulation disorders, fluency problems, and language impairment.[9] While linguistic difficulties usually resolve by early adulthood, there is a delay in speech and language capabilities compared to normally developing peers.[9] IQ scores for the majority of infants, children, and adults with FAS are between 40 and 80, with a mean of 60-65.[2]

In the CNS, alcohol can interfere with neurotransmitter production, leading to malfunction of the hypothalamus and suppression of growth hormone release.[11] Fetal metabolic, physiologic, and endocrine functions can be affected as well due to increased maternal and fetal hypothalamic-pituitary-adrenal (HPA) activity.[2] Brain size and shape is also heavily affected by FAS, with an overall reduction of the cranial vault and brain size.[12] The regions that are affected the most are the cerebellum, corpus callosum, and the basal ganglia.[12] In the cerebellum, size reduction in FAS cases was more than 15%.[12] Of the two major regions of the cerebellum, the anterior vemis is reduced in size

relative to controls, while the posterior vemis remains largely unaffected.[12] In the corpus callosum, it is believed that FAS may be one of the leading causes of agenesis, the absence or failed development of a body part.[12] Most FAS patients do not undergo full agenesis, but instead have significant changes in size and shape of the structure. The basal ganglia are especially sensitive, and typically reduce in size as well. Of the two major regions of the basal ganglia, the caudate decreases in size more than the lenticular nucleus.[12] While these effects can be observed in most FAS patients, the severity of each symptom can depend on when during fetal development ethanol exposure occurred.

2.2 The Role of Timing and Dosage on FAS

Research has determined that timing and dosage of ethanol play a significant role in the etiology of FAS. Most FAS studies utilize two dosing regimens, acute dosage regimens and chronic dosage regimens, both of which were designed to model binge drinking. These models apply to both human and animal models. Acute dosage regimens involve 2.9-6.0 g/kg alcohol administered on one or two occasions within the same day. Chronic dosage regimens involve smaller ($\leq$3 g/kg) doses of ethanol throughout the developmental period of interest. There are three developmental periods that are typically investigated for timing studies: the preconception period, the preimplantation period, and the gastrulation and cellular differentiation period. The preconception period refers to the alcohol consumption prior to conception by either parent. Results found that the alcohol consumption during this period by either parent can have an adverse effect on fetal development.[3] The effect alcohol consumption has on development will differ depending on which parent is consuming alcohol. On the paternal side of alcohol consumption, mice raised on 35% alcohol liquid diets from 25 days old to adulthood

were found to produce smaller litters when paired with non-alcohol treated females than mice that were unexposed to alcohol.[3] Offspring from alcohol-treated mice also had a greater number of malformations, including cranial fissures, hydronephrosis, microphthalmia, microcephalus, and a reduction in fetal weights. Rats exposed to a similar diet over 60 days in the preconception period were found to sire fewer offspring.[3] The role of the maternal consumption of alcohol during preconception on fetal development in is harder to determine, because of the possibility of confounding factors, such as malnutrition and generally reduced vitality in alcoholic mothers. Attempts to study these effects have found that alcoholic mothers produced offspring that had significantly retarded growth in comparison to controls.

The preimplantation period refers to the first 4-6 days of mouse development, which is analogous to the first 2 weeks of human pregnancy. Studies with mice suggest that alcohol exposure in this period tends to produce extreme FAS symptoms. In one study, it was shown that introduction of ethanol to the uterine environment on any of the days of the preimplantation period resulted in severe malformations and growth retardation in 80-100% of viable embryos. However, this effect relies on the interaction between mother and offspring. Mice embryos exposed to ethanol in vitro during the preimplantation period did not have an abnormal rate of morphological defects when transferred to a foster mother and allowed to complete development.[13] It is suggested that the negative consequences of ethanol exposure during this period relate to the production of toxic metabolites in the mother that are transferred to the offspring. Evidence has been discovered that changes in DNA caused by methylation of specific genes may be a cause for birth defects.[14] The genes affected by methylation would be expressed incorrectly,

which bring about the physical and behavioral malformations. The genes are expressed in late gestation, defects caused by improper expression would not be expected in the preimplantation period, but the late gestation period.

Gastrulation and cellular differentiation is the period where ethanol exposure is considered to have the strongest effect on the developing fetus. The corresponding deficiencies depend on which dosage method is used, acute or chronic. With acute dosage regimens, it has been observed that the period during gestation that ethanol exposure occurs in determines the FAS symptoms that occur in the offspring. Ethanol exposure on gestational days 7, 8, and 9 results in craniofacial abnormalities, ocular deficiencies occur on gestational days 7, 8, 9, and 10, brain abnormalities occur from exposure on gestational days 7 and 8, and skeletal abnormalities arise from exposure on days 9, 10, and 11 of gestation.[15] Chronic dosage regimens have also been shown to affect different FAS symptoms differently depending on developmental timing of exposure. On gestational days 4-12, ocular, cardiovascular, and skeletal systems are particularly sensitive to malformation. Growth retardation is associated with chronic alcohol exposure from gestational days 12-17.[15] The CNS is sensitive to deformation due to ethanol exposure throughout the gastrulation period, with symptoms including exencephaly, hydrocephaly, microcephaly, dilated ventricles, and various structural defects.[15] Regardless of dosage regimen, alcohol exposure was shown to reduce proliferation of neural progenitors in the hippocampus in vivo with mice models.[16]

In human models, there is a lack of control in dose, exposure duration, and response measures as a result of patients not following a dosage regimen. Since analysis of living patients with FAS requires birth, there is a retrospective element to human tests.[4] This

element relies on recall from the parents of the child, which can be unreliable. In human models, there is the potential for a collection of confounding effects as well, including previous drug use and poor prenatal care.[4]

2.3 Existing Animal Models for Studying FAS

Due to limitations in human models, as well as ethical and legal problems associated with conducting FAS research on a developing human fetus, the use of animal models is necessary. The most common animal models are mice, with alternative models including rats, zebrafish, chickens, pigs, ferrets, nonhuman primates, and dogs.[8,5,4,6] One study in mice established typical facial characteristics for control animals to provide a comparison to variations observed in FASD-affected mice. In this study, facial morphometric measures were derived from previous studies of human facial dysmorphology.[8] FAS was induced by liquid diet alcohol intake from 16 days pre-pregnancy and continuing into pregnancy. Three treatment groups were utilized: an alcohol group being fed 4.8% v/v alcohol solutions; a prepregnancy, pair-fed group (given alcohol and food until pregnancy); and a chow group (fed food and water throughout). It was found that pair-fed groups had significant facial dysmorphology compared to the chow group, while the alcohol group differed from each respective control group for all features. This study elucidated the importance of defining expected behavior from unaffected control animals to provide a comparison for results obtained for affected animals.

In addition to mice, another popular model is zebrafish. Zebrafish have been used to model morphological and neurobehavior deficiencies caused by ethanol exposure. Carvan et al. showed that zebrafish exposed to alcohol in their fetal environment show a

dose-dependent reduction in memory and learning capabilities when compared to control groups.[6] This dose-dependent relationship also applies to cell death in the CNS of zebrafish embryos. Zebrafish exposed to 10, 30, 100, and 300 mM ethanol concentrations showed differing amounts of cell death, defined as the number of embryonic nervous system cells stained by a cellular dye, Acridine Orange. Significant cell death results were observed only at the highest concentration of ethanol, however malformation of the embryo was present in both the 100 and 300 mM ethanol exposed embryos. As in the mouse model discussed previously, Carvan et al. established normal standard skeletal characteristics for the zebrafish model. Skeletal analysis of embryos exposed to ethanol show that the zebrafish skeletal structure is generally very sensitive to chronic ethanol exposure, with some skeletal elements being affected by concentrations as low as 3 mM.[6]

Due to the severe effect ethanol exposure has on zebrafish, further investigation was conducted to determine at what point in post-fertilization ethanol has the strongest effect on development.[5] A recent study examined the effect of adding various concentrations of alcohol for different exposure periods. In this study, control, 1.5% and 2.9% v/v alcohol solutions were exposed to developing zebrafish fetuses during 0-8, 0-12, 0-24, 6-24, 24-48, and 48-72 hours postfertilization (hpf). Zebrafish exposed to 1.5% alcohol from 6-24 hpf exhibited 60% of the possible skeletal physical abnormalities established in the previous research upon examination, compared to 15% in zebrafish exposed to 1.5% alcohol from 0-12 hpf. This suggested that alcohol significantly attenuated physical development between hours 12 and 24. The effect was magnified when zebrafish were exposed to 2.9% alcohol.[5]

In animal models, there is a greater experimental control than in human models. However, there are different problems with these models as well. In rat or mouse models, drawing blood to quantify blood alcohol content puts stress on the mother, confounding whether or not the results are based on stress or ethanol exposure. Also, pups born with abnormalities may be ignored or killed by their mothers, which prevents long-term FAS research.[4] In these models, it is also impossible to view the skeletal morphological development of the fetus without terminating it first. Zebrafish are able to bypass this limitation, since their embryos are translucent.[5] With this model, fetal development can be observed from a morphological standpoint. However, with none of these models is central nervous system development and functionality quantifiable, since testing of functionality is not possible in the uterine environment. A model that would allow for functional testing would allow researchers to relate abnormal functional development to specific CNS developmental problems. As a result, there is room for a new model for fetal alcohol syndrome. The novel model we propose in this thesis is the planarian species *Schmidtea mediterranea*.

2.4 An Alternative Model: Planaria

Planaria (as shown in Figure 2) are flatworms widely considered to be the simplest organism alive with a central nervous system.[22] These worms can be found in both saltwater and freshwater. Overall length varies depending on the species, but most are between 1 and 20 millimeters.[19] The planarian body has three tissue layers, distinct organs, and a bilateral symmetry. Planaria do not have blood in their system, so measuring the blood alcohol content is not an option for these animal models.

Figure 2: A photographic representation of a *Schmidtea mediterranea* planaria. The total length of this planaria is 18 mm.

The planarian CNS (as shown in Figure 3) consists of a bi-lobed cephalic ganglia, which serves as the planarian brain, two ventral nerve cords, a sub-muscular nervous plexus, photoreceptors and chemoreceptors.[18] The cephalic ganglia consists of nine separate branches on each side exhibiting bilateral symmetry.[18] The ventral nerve cords extend from the posterior to the anterior of the body and are attached to the cephalic ganglia. The sub-muscular nervous plexus runs beneath the body wall musculature and connects to the two ventral nerve cords.[19] The chemoreceptors and photoreceptors send projections to the cephalic ganglia which influence behavioral responses to chemical stimulants and light, respectively.[19] Photoreceptors attach to the cephalic ganglia at the visual center located on the third branch from the posterior of each side of the cephalic ganglia, while the chemoreceptors arise from the 6-9th branches.[18] The planarian brain also consists of a large cluster of neural cells on the dorsal side of the ventral nerve cords.[20] The cephalic ganglia is composed of a core of axons and a cortex of nerve cells, and has lateral branches crossing from lobe to lobe.[20]

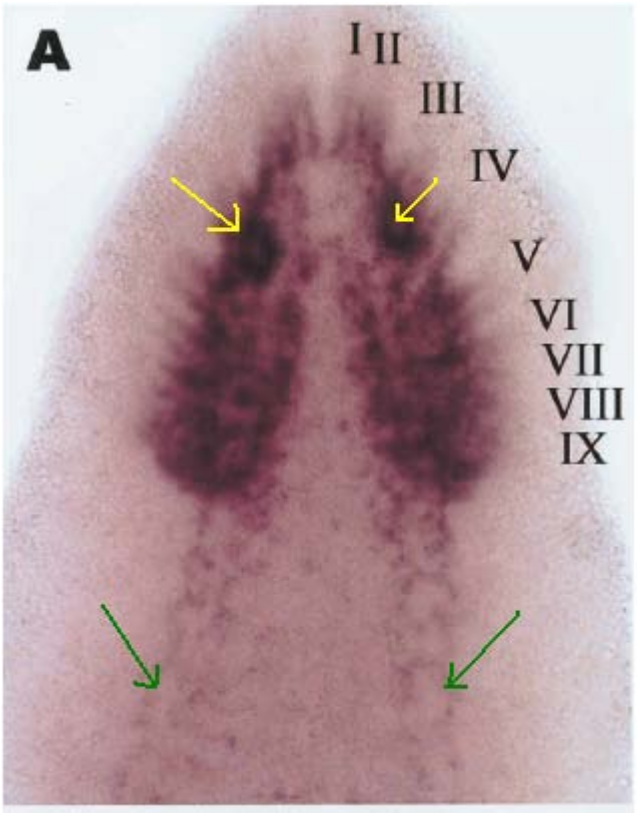


Figure 3: Picture of the planarian CNS adapted from Agata, et al. The dark red portion represents the cephalic ganglia and the photoreceptors (yellow arrows). The roman numerals highlight the separate branches of the cephalic ganglia. The ventral nerve cords (green arrows) extend posterior of the cephalic ganglia.

2.5 Regenerative Capabilities of Planaria

Planaria are most known and studied for their ability to regenerate. When cut in half, both fragments will regenerate into complete, functional animals. In fact, a planarian fragment that is 1/279th the original size of the animal will regenerate fully.[36] The complete mechanism for initiating regeneration is unknown, but it has been suggested that an interaction between the dorsal and ventral sides of the planaria that can only occur

during injury is the catalyst for regeneration.[21] This ability is mediated by a collection of neoblasts scattered throughout the anatomy of the planaria. Neoblasts are embryonic-stem-cell-like cells that exist in large numbers (25-30% of all cells) in planaria and are responsible for cell proliferation.[21] They appear as highly undifferentiated cells with large nuclei and very little cytoplasm.[21] Upon injury, neoblasts will migrate to the wound site and begin the formation of a blastema. Regenerating planaria are able to exhibit tissue polarity as well. For the posterior fragment of a planaria, neoblasts will differentiate into a new head, complete with CNS. For the anterior fragment, neoblasts will differentiate into the completion of the two ventral nerve cords and pharynx, depending on the location of the initial injury. Planaria are also able to regenerate with lateral symmetry. The mechanisms for these processes are not fully understood. It has been observed that the completion of planarian regeneration takes 7 days. Figure 4, from Alvarado, et al., shows a daily progression of blastema formation in a head regenerating planarian.[22]

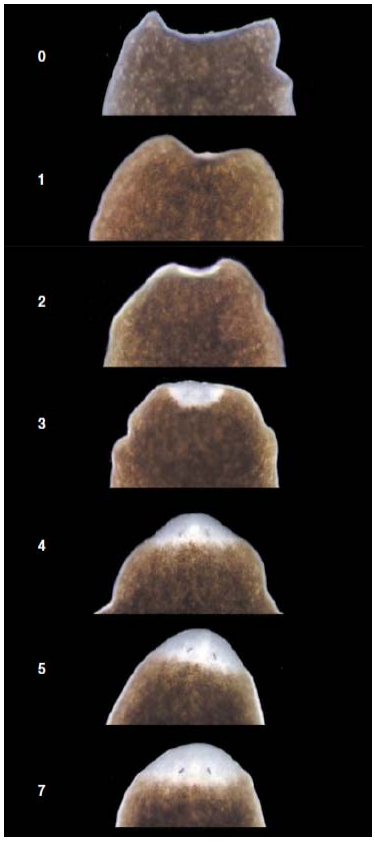


Figure 4: Daily progression of blastema formation in a head regenerating planaria, adapted from Sanchez-Alvarado and Reddien.[22] Each cell represents a day of regeneration from day 0-7. The head is removed immediately before the picture on day 0 was taken.

When the posterior fragment is regenerating a head blastema, as in Figure 4, specific CNS functions will develop at different points in the duration of regeneration. Planaria regain functional locomotor capabilities on day 2 of blastema regeneration, when the developing brain begins to reattach with the ventral nerve cords.[18] Photoreceptors begin developing on day 4, but onset of functionality is not immediate. To determine when onset of photoreceptor functionality occurs, Inoue et al. utilized the negative phototaxic

behavior exhibited by planaria.[23] Negative phototaxis refers to the tendency of planaria to avoid light sources. Planaria exhibit negative phototaxis as an innate built-in behavior because they are bottom dwellers in their natural environment. Therefore, to test for the onset of photoreceptor functionality, Inoue et al. developed a light avoidance test.[23] For these studies, head regenerating planaria were exposed to a light source in a testing apparatus to monitor their sensitivity to light. These tests were conducted each day during the 7 day developmental period. The testing apparatus was divided into 4 quadrants, and light avoidance was quantified as the percentage of experimental time spent in the target quadrant, the quadrant furthest from the light source.[23] As shown in Figure 5, significant photoreceptor capabilities were not obtained until day 5 of head regeneration.[23] When paired with gene expression research performed on planaria, these results can help elucidate which genes are essential to photoreceptor function and the dynamics of development of the functionality.

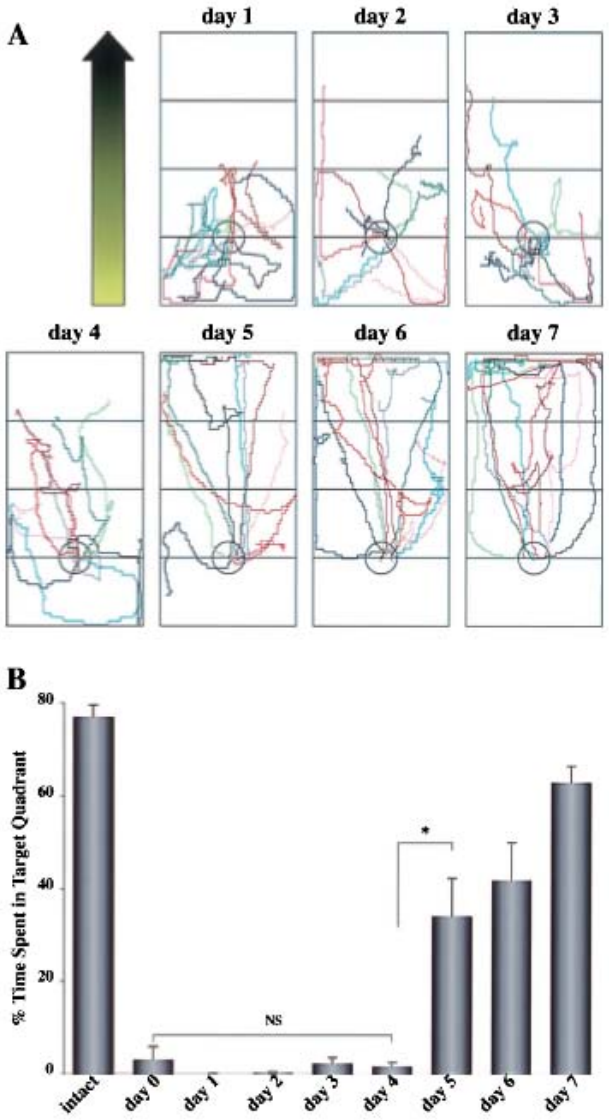


Figure 5: Light avoidance testing results for regenerating planaria. A) Daily progression of results for 10 planaria. Each day refers to a day of CNS regeneration in a head-regenerating planaria. Each colored line represents the path of a single planarian subject on that day of testing. The arrow represents the light gradient in the testing apparatus. B) Graphical representation of data collected from experiment. The target quadrant refers to the testing apparatus quadrant furthest from the light source. Error bars represent differences in each animal sample on a given day. Figure from Inoue, et al.[23]

The ability of planaria to regenerate offers researchers a unique ability to monitor functional development of an animal model's CNS. Since planaria offer the potential to adapt planarian regeneration to human development, their genome has been fully mapped.[37] Analysis of the complete genome elucidated that there were numerous planarian genes that had human analogues, further adding to the hope that their role in regeneration could be applied to human development. As a result, a large portion of planaria research is dedicated to cell-dependent regeneration, which requires the analysis of gene expression patterns. The most common technique utilized for this analysis is whole-mount in-situ hybridization (WISH).

WISH is a method of hybridization that utilizes a labeled complementary DNA or RNA strand to localize and stain a targeted RNA or DNA sequence in an entire tissue. It has been utilized in many biological settings, and the first description of WISH applications in planaria was provided by Umesono, et al.[17] Umesono, et al. used WISH to elucidate the expression pattern of *Djotp*, a homeobox-containing gene of planaria which is closely related to the human gene *Otp*.[17] Since this first WISH application, there have been several revisions to the original protocol, leading to an accepted formaldehyde-based WISH protocol that can be adapted to each lab's specific uses.[44]

RNA interference, or RNAi, is another method of determining characteristics of planarian genes. Specifically, RNAi is used to discover the functionality of genes. RNAi involves the introduction of a specific double stranded RNA (dsRNA) that inhibits the target gene.[18] Therefore, by comparing differences in structure or function among control regenerating planaria and RNAi-treated planaria, researchers can determine the specific role and/or function of individual genes. These analyses, applied in various stages of

regeneration, provide the possibility of uncovering the roles of specific genes in regeneration.[18] One example of this technique in planaria involves the characterization of the noudarake (*ndk*) gene in planaria.[20] By inhibiting the *ndk* gene with RNAi, Umesono and Agata were able to induce a planaria to develop brain formations throughout its anatomy, as shown in Figure 6.[20]

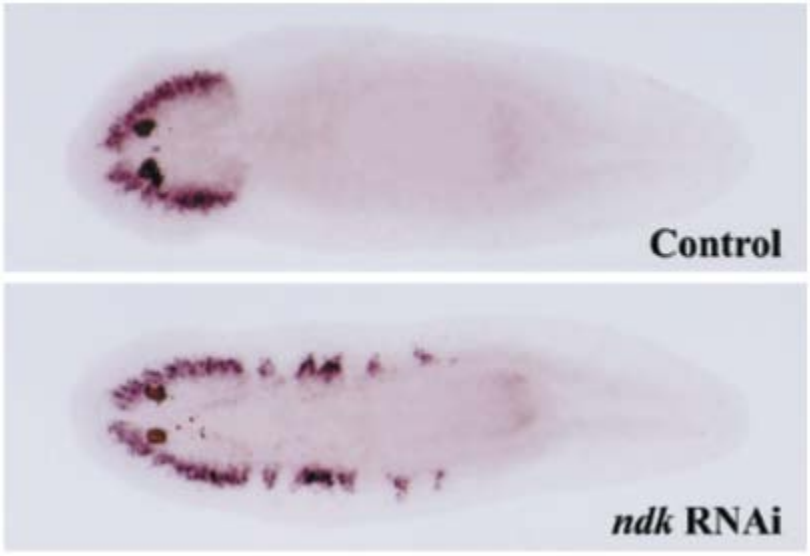


Figure 6: Effect of using RNAi to inhibit the ndk gene. The control planaria displays normal brain anatomy (colored portion), with brain formation only in the anterior of the planaria. However, when *ndk* is inhibited, abnormal brain formation is observed towards the midline of the planaria. Figure from Umesono and Agata[20]

The *ndk* gene is assumed to provide positional information to neoblasts forming the brain to influence brain growth only in the appropriate anterior section of the planaria.[20] Extensive large-scale RNAi screening has started to elucidate the role of additional

individual genes in regeneration, which will provide a deeper understanding of the cellular mechanisms involved in the planarian regeneration process.[23]

2.6 Drug Dependence Studies in Planaria

Gene expression can be used to compare results from functional testing under the influence of negative stimuli with adverse gene expression patterns, elucidating the role of some genes in CNS function. Currently, research into the planarian response to adverse stimuli has been limited to drug dependence studies. Several studies have investigated the effect of marijuana substitutes, cocaine, and opioid-dopamines on planarian CNS function.[24-30] In studies investigating the effect of drug exposure on planarian movement, a protocol developed by Raffa et al. referred to as a planarian locomotor velocity (pLMV) test is used to quantify movement.[29] An example of their results can be seen in Figure 7.

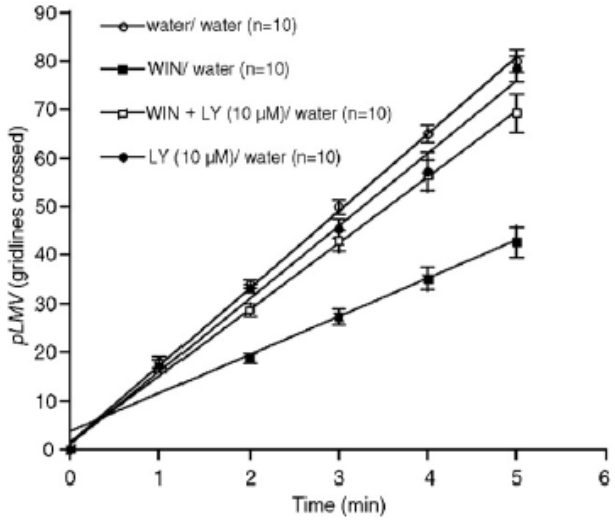


Figure 7: Example of pLMV Results[30]

In one study, it was shown that planaria exhibit statistically significant withdrawal response to the marijuana substitute WIN 52212-2. When planaria were maintained in WIN 52212-2 for 60 minutes and then exposed to water and tested for pLMV, they performed significantly worse than planaria that were maintained in WIN 52212-2 throughout experimentation.[30] Building on this research, another study demonstrated that withdrawal-like behavior was dependent on the duration of drug exposure with cocaine hydrochloride, methamphetamine hydrochloride, and caffeine at 10 µM concentrations.[28] This study showed that duration of drug exposure plays a role in the severity of withdrawal-like symptoms, with samples exposed to drugs longer exhibiting more severe symptoms.[28]

Although planaria are routinely tested in drug dependence studies, alcohol research has been a largely unexplored area. The first study discussing the effects of alcohol on planaria was published in 1944.[32] In this study, Rulon showed that alcohol exposure during regeneration can cause dysmorphology in the resulting intact planaria.[32] The severity of dysmorphology was dependent on the alcohol concentration regenerating planaria were exposed to.[32] Other alcohol research associated with planaria has been focused on the use of ethanol as an immobilization agent for planaria experimentation. Stephenson et al. showed that a one hour treatment of 3% v/v ethanol solutions significantly inhibits planarian motion, with complete recovery occurring 3-4 hours after exposure.[33] While the severity of planarian immobilization depended on the size of the planaria exposed, the recovery time was unchanged.[33] No research has been performed to determine what effect alcohol exposure would have on CNS development in head-regenerating planaria.

2.7 Determination of a Representative Planarian Species

In order to promote continuity and collaboration between laboratories, a representative model species, *Schmidtea mediterranea*, was proposed among the planarian community.[18] Properties that contributed to this selection include: relative ease of culture in the laboratory, developmental plasticity, and a comparatively small, diploid genome. The genome of *S. mediterranea* planaria has been completely mapped.[18] As an additional benefit of *S. mediterranea*, there is also a distinction between sexual and asexual strains in this species, which allows for easy recognition and characterization of progeny.[18] As a result, all the planaria used for our studies in this thesis are *S. mediterranea* to ensure comparability with other laboratories and results.

In this thesis, we show that *Schmidtea mediterranea* planaria are a viable new model for studying the dynamics of FASD. In order to demonstrate this, we perform tests on planaria to ensure that they fulfill the requirements of a new model for FASD dynamics: a model that has the ability to test functionality during development, and a model where ethanol has an observable effect on development of functionality. To assess these requirements, we will use the head-regenerating planarian's ability to regenerate a CNS to mimic the development of the CNS in an FASD-like environment. Light avoidance and pLMV tests described previously are used as functional assessments of CNS development. The experimental design for these functional assessments will be described in Chapter 3.

Chapter 3

Materials and Methods

The materials, methods and equipment used for all experiments are described below. An approved job safety analysis was performed before starting experimentation. All experiments followed standard operating procedures including a protocol for the safe disposal of hazardous materials. All animals were maintained as instructed by the Sanchez Alvarado lab.

3.1 Planaria

A population of the planarian species *Schmidtea mediterranea* was graciously provided by the Sanchez Alvarado laboratory at the University of Utah. These planaria were used for all regeneration experiments. For the initial preliminary alcohol viability testing, "brown" planaria from Carolina Biological (Item #132954) were used. The specific species provided appears to be *Dugesia tigrina* planaria.

3.2 Colony Care

Planaria are maintained in a solution known as a Montjuic solution. The components and concentrations of this solution can be found in Table 1.

Table 1: Final Salt Concentrations in Montjuic Solution. All solutions are made from deionized H_20.

Chemical	Molarity (mM)
NaCl	1.6
$CaCl_2$	1.0
$MgSO_4$	1.0
$MgCl_2$	0.1
$NaHCO_3$	1.2

Planaria were kept in plastic containers at 22°C in an incubator, and the containers were kept partially open to allow fresh air to reach the planaria. Each plastic container was limited to approximately 100 planaria. Plastic containers were not cleaned with soaps, detergents, or other chemicals to avoid poisoning the planaria.

Planaria were fed pureed organic calf liver once per week. Preparation of the liver involved removing all vasculature from the planaria prior to processing. Liver was aliquoted and stored at -20°C. To begin the feeding process, a pureed liver aliquot was thawed at room temperature. Excess Montjuic solution was removed from each planaria container to reduce the volume available for planaria to feed, thereby increasing the likelihood that all animals have a chance to feed each week. A pea-sized portion of liver was placed in each container, and the planaria were allowed to feed for at least an hour. During this process, the spoon used to transfer liver is never placed in the water of any container, since this may promote cross-contamination. After the planaria have fed for an

hour, they are transferred to a new container with fresh Montjuic solution. After two days, the planaria are transferred to fresh Montjuic solution because of waste buildup. To increase the planarian population, planaria were severed into two fragments using a scalpel after feeding and were given a week to regenerate.

3.3 Alcohol Survival Testing

It is unknown if planaria can metabolize alcohol when exposed, although they do possess genes homologous to both alcohol- and aldehyde-dehydrogenase.[37] To determine intact *Schmidtea mediterranea* planarian tolerance to alcohol exposure, planaria were exposed to a dilution series of different alcohol concentrations over the course of 5 weeks. Two planaria each were placed into 10 cm petri dishes with a specific concentration of molecular grade alcohol (Sigma Aldrich, 459844-500ML) diluted into Montjuic solution. The concentrations used were 0.1%-1.0% v/v alcohol solutions in 0.1% increments, 2%, 3%, 4%, and 5% v/v alcohol solutions. Montjuic control solution was also prepared for the dilution series. After being fed, planaria were maintained in the alcohol concentration being tested. Survival was quantified as the percentage of planaria that survived after the completion of a 5 week trial.

3.4 Planarian Locomotor Velocity (pLMV)

Experimentation with pLMV was designed after a procedure established by Rawls et al.[30] pLMV testing is used to assess a planarian's locomotor capabilities. Briefly, a single planaria is placed in the center of a 35 mm petri dish with 5 mL of testing solution (either alcohol-containing or control Montjuic solution). The petri dish is placed over grid paper with lines spaced 0.5 cm apart, as shown in Figure 8. During testing, all planaria were

exposed to a consistent light source to remove light as a variable for error. The pLMV is quantified as the number of times the planaria's head crosses a gridline throughout the full duration of the test, with line intersections scored as two. The overall test duration for all pLMV tests was 5 minutes, as described by Rawls et al. Two variations were made from Rawls' procedure: using different sized petri dishes and monitoring pLMV cumulatively across the entire 5-minute window rather than minute by minute because the purpose of the test was to determine total pLMV performance over several days. Tabulating this data as a minute by minute data set would not fit this experimental design. All pLMV results were plotted with 95% confidence interval error bars. Standard Student's t-tests were performed to determine statistically significant differences in pLMV performance.

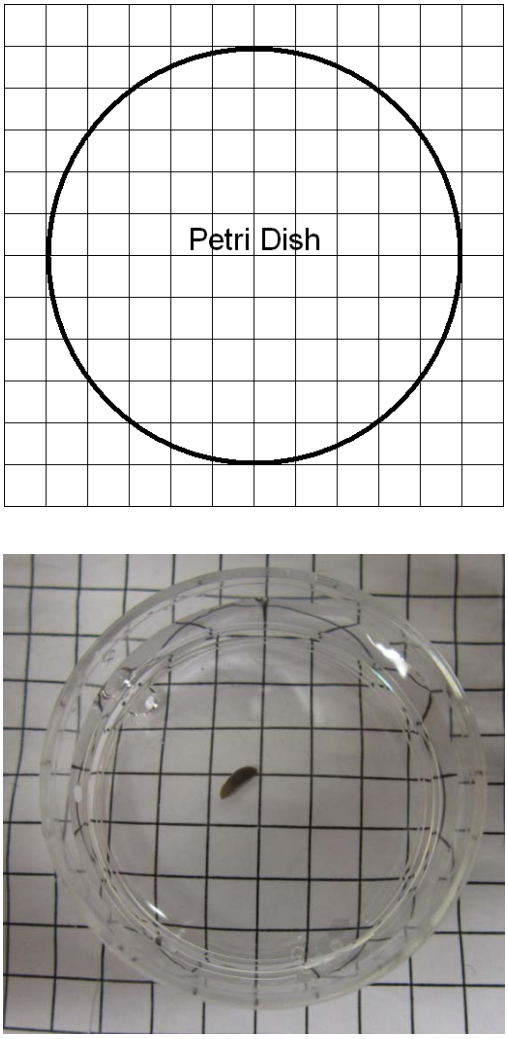


Figure 8 a, b: (A) pLMV testing apparatus (Schematic) with petri dish placed over 0.5 cm gridlines and (B) pLMV testing apparatus (Photograph) displaying a pLMV test in progress.

3.5 Light Avoidance

Light avoidance experimentation allows for analysis of photoreceptor functionality in regenerating and intact planaria. Light avoidance protocols were modeled after the procedure developed by Inoue et al.[23] Briefly, for light avoidance testing, a clear

polypropylene testing apparatus was constructed with the dimensions 60 x 30 x 10 mm. This apparatus can be seen in Figure 9. Each side, with the exception of the side exposed to the light source, was painted black to ensure that light entered from only one side. During testing, the light in the room was turned off to maintain the light gradient from the light source. The only light source was placed at a distance that produced a light gradient from 500 lux to 350 lux across the apparatus. The testing apparatus was positioned over paper with 4 clearly demarcated quadrants and a starting area marker as shown in Figure 9b. A Canon Powershot SD1300 IS digital camera was positioned directly over the testing apparatus to record each planarian's movement in the apparatus throughout the duration of the test.

Prior to each test, the apparatus is filled with the solution being tested. It is important to note that at the beginning of each experiment, each alcohol testing solution is made in bulk and kept in a tightly sealed container to prevent alcohol evaporation. This ensures that for each day of testing, there is no variation in the alcohol solution being tested. To begin testing, a single planarian is placed in the starting area and allowed time to uncurl so that the planarian can start moving as soon as the test begins. (Planaria tend to be curled after being dispensed from the transfer pipette into the testing apparatus, which impedes their ability to move right away.) Once uncurled, the camera recording is started for a 90 second period. Light avoidance is quantified as the percentage of time spent in the target quadrant (see Figure 9b) during the 90 second test. The percentage of time spent in the target quadrant relates to the planaria's ability to detect light and display negative phototaxis. Planaria that have higher scores in light avoidance testing have more fully developed photoreceptors than those with lower scores. Results were plotted

with 95% confidence interval error bars. Standard Student's t-tests were performed to determine statistically significant differences in light avoidance scores.

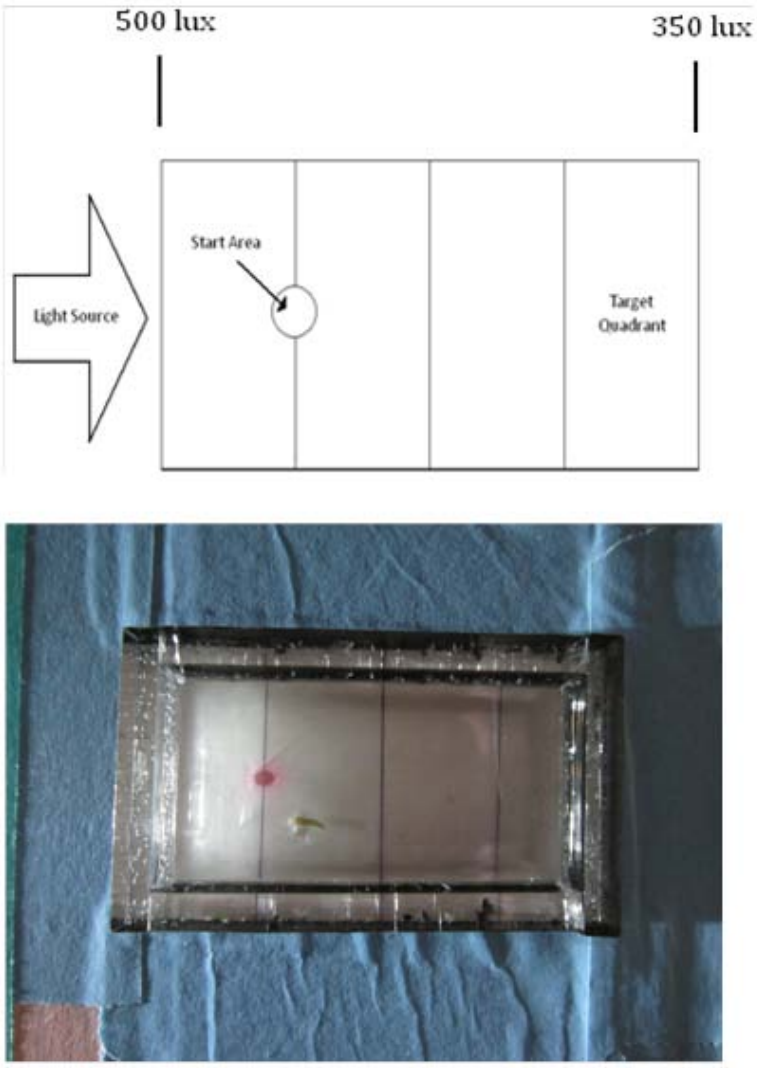


Figure 9 a,b: A) Light Avoidance Apparatus (Schematic) B) Light Avoidance Apparatus (Photograph)

3.6 pH Testing

To determine if exposure to fluctuations in pH caused by the addition of alcohol to Montjuic solution would have an adverse effect on planaria, pH effect tests were performed. The pH of control Montjuic solution and a serial dilution of alcohol solutions (1-4% v/v in 1% v/v increments) were measured using an Orion model 410 pH meter with a Thermo Orion Low Maintenance pH Triode (#9107BN). pH meter measurements were confirmed with colorpHast© pH indicator strips (Cat. 9588). New pH matched solutions were made by adjusting the pH with pellets of sodium hydroxide (Sigma Aldrich S5881-500G). The pH values used to simulate each alcohol concentration are given in Table 2. Five planaria were then placed into each solution and maintained in the incubator at 22°C for one week.

Table 2: pH's used to simulate alcohol pH fluctuations. Alcohol concentrations refer to the alcohol testing solution being simulated. pH represents the pH measured in alcohol testing solutions. Simulated pH represents the pH measured in Montjuic solution with sodium hydroxide adjusted pH.

Alcohol Concentration	pH	Simulated pH
Control Solution	8.84	8.82
1%	8.9	8.93
2%	8.97	8.99
3%	9.03	9.04
4%	9.14	9.18

3.7 Experimental Design

Several variations of experimental trials were conducted. Each experimental trial consisted of a collection of planaria subjected to various concentrations of alcohol or a control Montjuic solution. The purpose of these experiments was to elucidate the effect of alcohol exposure on planaria in varying stages of CNS development. Previous testing has also shown that planaria can show signs of conditioning and memory retention in repetitive testing environments.[31] In these tests, planaria exposed to the same incentive-based Y-testing environment 10 times a day became increasingly directionally biased as the number of trials increased.[31] To ensure that our planaria did not undergo conditioning, each planaria was only tested once a day with pLMV and light avoidance, and new groups of planaria were used for each new set of tests.

All planaria used in experimentation were starved for one week prior to experimentation.[20] This is the accepted experimental convention to ensure that digested food does not confound whole mount in-situ hybridization (WISH) and immunostaining results. Although these planaria were not exposed to WISH or immunostaining, starving prior to experimentation ensures comparability between present and future experiments. The details and motivation for each individual experiment is further explained in the following sections.

3.7.1 Functional Testing for Intact Planaria

Before any experimentation on regenerating planaria could begin, it was important to establish what effect ethanol would have on intact, fully-functional planaria. By determining baseline scores for intact planaria in various solutions, we could compare

functional scores obtained for regenerated planaria to scores obtained for previously developed intact planaria. Also, if ethanol were to have a strong effect on intact planaria, that could confound regeneration results. To begin, alcohol solutions (control, 1% v/v, and 2% v/v alcohol) were each placed into 10 separate petri dishes. One intact planaria was then placed in each petri dish. Intact planaria were tested once per day for 9 days to ensure comparability with regenerating testing, which required 9 days to ensure full regeneration of the CNS. A total of 10 planaria were tested for each solution.

3.7.2 Functional Testing for Regenerating Planaria

Once the effect of alcohol on intact planaria had been quantified, testing could begin on planaria regenerating the CNS. Regenerating planaria testing was performed to determine the effect of alcohol exposure on CNS development and functionality. By comparing these results to those obtained from intact planaria testing, it could be determined which alcohol percentages attenuate or delay normal CNS development. To begin, each solution (control, 1%, 2%, 3%, and 4% v/v alcohol) was placed into 10 separate petri dishes. Planaria were then placed under an Olympus SZ61 Stereo Microscope and their heads were removed with a scalpel just below the photoreceptors, as demarcated with the red line shown in Figure 10. Since we described CNS development as the regrowth of cephalic ganglia and the attachment of the ventral nerve cords to the cephalic ganglia, removing the heads any lower than this was not necessary. One head-regenerating planaria was placed into each petri dish.

Figure 10: Schematic of head removal site. The red demarcation represents where each planaria's head was severed prior to regeneration testing.

Functional testing on planaria began within 2 hours of transfer to petri dishes, and functional testing on subsequent days occurred at roughly the same time of day. The duration of regeneration testing was 9 days.

3.7.3 Withdrawal testing

Once it was determined that alcohol could delay and attenuate CNS function in regenerating planaria (see Chapter 4), it was important to see if the effect would be maintained after planaria were removed from alcohol solutions. Withdrawal testing was performed to determine if planaria exhibited any withdrawal effects once returned to control solution after extended alcohol exposure. To begin, each solution (control, 1%, and 2% v/v alcohol) was placed into separate petri dishes. For each solution, 5 intact planaria were placed in individual petri dishes, and 5 head-regenerating planaria were placed in separate individual petri dishes. Prior to testing, planaria were starved as described for previous experiments. Head-regenerating planaria were prepared as described for regenerating planaria testing. Planaria were maintained in these solutions

for 9 days before withdrawal testing began. After 9 days, all planaria were transferred to new petri dishes containing control solution. Fifteen minutes after transfer, each planarian was subjected to pLMV and light avoidance testing. Planaria were further subjected to pLMV and light avoidance testing at 24 and 48 hours after transfer from ethanol.

3.7.4 Targeted Alcohol Exposure

To determine at what point in planarian CNS development ethanol causes a delay, targeted alcohol exposure experiments were designed. In these experiments, regenerating planaria were subjected to alcohol for targeted periods of time during development. All regenerating planaria were severed as described in section 3.7.2. Three experiments were designed to assess alcohol's effect on CNS development: shock testing, day 0-2 testing, and day 3-5 testing.

Shock targeted ethanol exposure experiments were performed by placing 10 planaria in 3% v/v alcohol solution for one hour immediately after head removal. After one hour, planaria were transferred to individual petri dishes with Montjuic solution and experimentation continued as described in Section 3.7.2. In conjunction with the 10 planaria subjected to the shock testing, 10 more planaria were maintained only in Montjuic solution to act as a control group. This experiment continued with daily testing until day 8.

In day 0-2 targeted alcohol exposure experiments, 10 planaria were maintained in 1% v/v alcohol from immediately after head removal until the end of functional testing on day 2. After the end of testing on day 2, the planaria were returned to a control Montjuic

solution and allowed to finish regenerating. Ten additional planaria were maintained only in Montjuic solution to as a control group. Both groups of planaria were allowed to regenerate for 8 days.

In day 3-5 targeted alcohol exposure experiments, two sets of 10 planaria were maintained in individual petri dishes with a Montjuic control solution. At the end of functional testing on day 2, planaria from one group were transferred to 1% v/v alcohol solutions. These planaria were maintained and tested in 1% v/v alcohol until the end of testing on day 5, when they were transferred back to Montjuic solution. The other group of planaria was maintained in Montjuic solution throughout. Both groups of planaria were allowed to regenerate for 8 days.

3.8 First Order Plus Time Delay Estimation

Results from the initial regeneration experiments were fit to a first order plus time delay model in Microsoft Excel© to determine functional delays in development due to alcohol exposure. pLMV and light avoidance data obtained from each solution tested were fit to the parameters shown in Equation 1

(1)

where K is the gain in lines crossed (pLMV) or % time spent in target quadrant (light avoidance), is the time delay in days, τ is the time constant, and t is the elapsed time in days. Parameters were adjusted until they were able to best fit the results obtained from experimentation. Figure 11 provides a graphical example of the parameters shown in Equation 1.

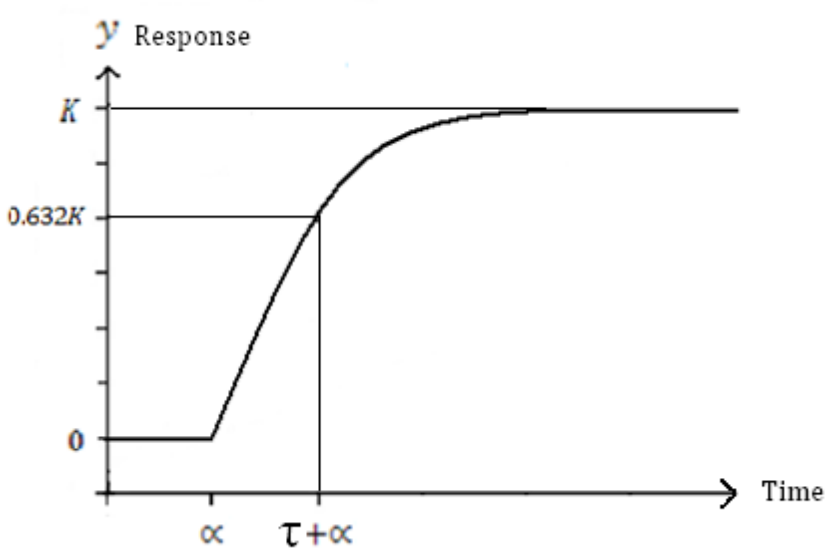


Figure 11: Example of FOPTD with key parameters shown. K is equal to the gain in lines crossed (pLMV) or % time spent in target quadrant (light avoidance), is the time delay in days, τ is the time constant, and t is the elapsed time in days.

The materials and methods outlined in this chapter were used to obtain the results presented in the next chapter. The results of these functional tests were used to determine what effect alcohol had on intact planaria, how it attenuated and delayed CNS development in head regenerating planaria, when normal function could be achieved again, and at what point in the developmental process the CNS is most sensitive to the effects of alcohol exposure.

Chapter 4

Results and Discussion

The full compilation of raw data is presented in Appendix A. Survival curves, pLMV, light avoidance, and first order plus time delay data were obtained using the methods described in Chapter 3. All data are expressed with 95% confidence intervals.

It was observed throughout experimentation that there were typically sizable standard deviations on each day of testing. This high degree of variability could be attributed to several factors. First, there were as many as 10 planaria per solution per experiment, which creates a large base of performance scores to skew data. Also, there is a degree of natural variability when dealing with animal models. It cannot be expected that two individual animals will react exactly the same during experimentation. This could be due to rates of growth or by how much the planaria was exhibiting a starvation response. It could also be a function of planarian length. A slightly smaller planaria might take longer to achieve a high pLMV score or reach the target quadrant in light avoidance testing.

4.1 Initial Alcohol Testing

Since it was unknown how planaria would be affected by alcohol solutions of varying concentrations, the viability of various alcohol concentrations for experimental analysis with planaria were determined with an initial series of testing. Testing the alcohol concentration threshold at which planaria could survive was essential to developing subsequent experimental designs. It was important to determine at what alcohol concentrations the planaria could be expected to survive for at least a week since the

duration of the experiment was 8-9 days. For these experiments, planaria were maintained in varying concentrations of alcohol (1%, 2%, 3%, 4%, and 5% v/v) for up to 5 weeks. Other investigators noted that planaria were immobilized, but not terminated, by 3% v/v ethanol solutions for short periods of time.[33] This was the basis of determining what ethanol percentages were to be tested. Figure 12 shows the results for this initial alcohol testing.

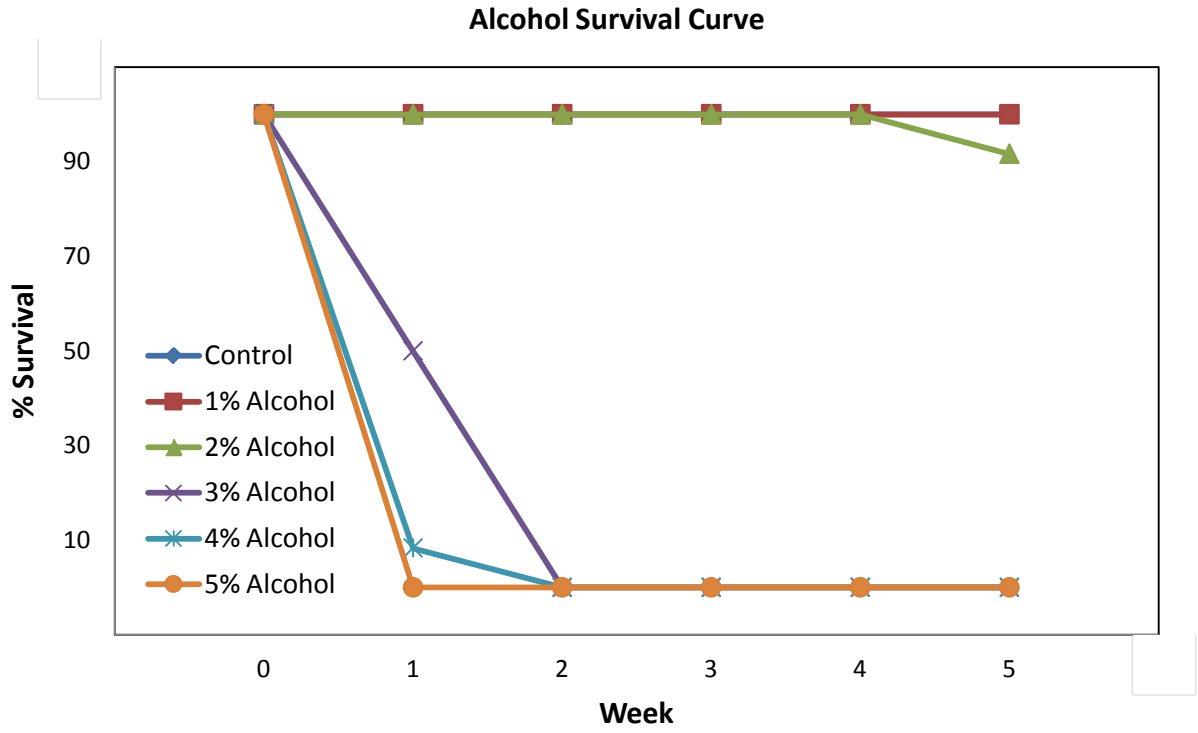


Figure 12: Survival curve for varying concentrations (% v/v) of Alcohol. All animals were individually housed in each alcohol solution. Planaria exposed to 5% alcohol were all dead within 24 hours, while planaria exposed to control-4% v/v alcohol experienced a dose-dependent fatality response.

Control and 1% alcohol exposed planaria survived throughout the duration of the experiment, with 2% planaria only experiencing fatality in the final week of observation. The remaining alcohol concentrations displayed a dose-dependent fatality curve, with increased fatality rate as alcohol concentration increased (Figure 12). Consequently, control and 1-2% alcohol solutions were studied for regeneration experiments. Since the literature review indicated that planaria require at least 7 days to regenerate, we could not use 3-5% solutions because planaria in those solutions might not survive the duration of the experiment. We felt that while the planaria might survive the duration of experimentation in 3% v/v alcohol, it would be difficult to determine a difference between an immobilization effect and actual CNS attenuation.

To ensure that alcohol exposure was the cause of planarian fatality, not fluctuations in pH caused by the addition of alcohol, a pH survival curve experiment was also completed as described in Section 3.6. The adjusted pH values of Montjuic solution representing each alcohol concentration can be seen in Table 2. No animals perished in these experiments, indicating that the change in pH alone does not cause animal fatality, thereby suggesting that alcohol, and not pH change, is the cause of fatality.

4.2 Alcohol Has An Immobilization Effect on Intact Planaria

The determination of optimal alcohol testing concentrations allowed us to begin functional testing on planaria. Before we could test the effect of alcohol on head-regenerating planaria, we needed to determine what effect alcohol has on intact planarian functionality. Therefore, we commenced with functional tests (pLMV and light avoidance) involving intact planaria. All planaria (n=15 each for control, 1%, and 2% v/v

alcohol) survived throughout this experiment. Figure 13 shows the effect of various concentrations of ethanol on intact planaria's ability to move. Treatment of intact planaria with 2% ethanol significantly reduced planarian functionality in pLMV testing. 2% ethanol-exposed planaria displayed a statistically significant difference ($p<0.05$) from both 1% ethanol and control solution planaria each day. 1% ethanol-exposed planaria performed statistically differently from control planaria only on day 0 ($p<0.05$).

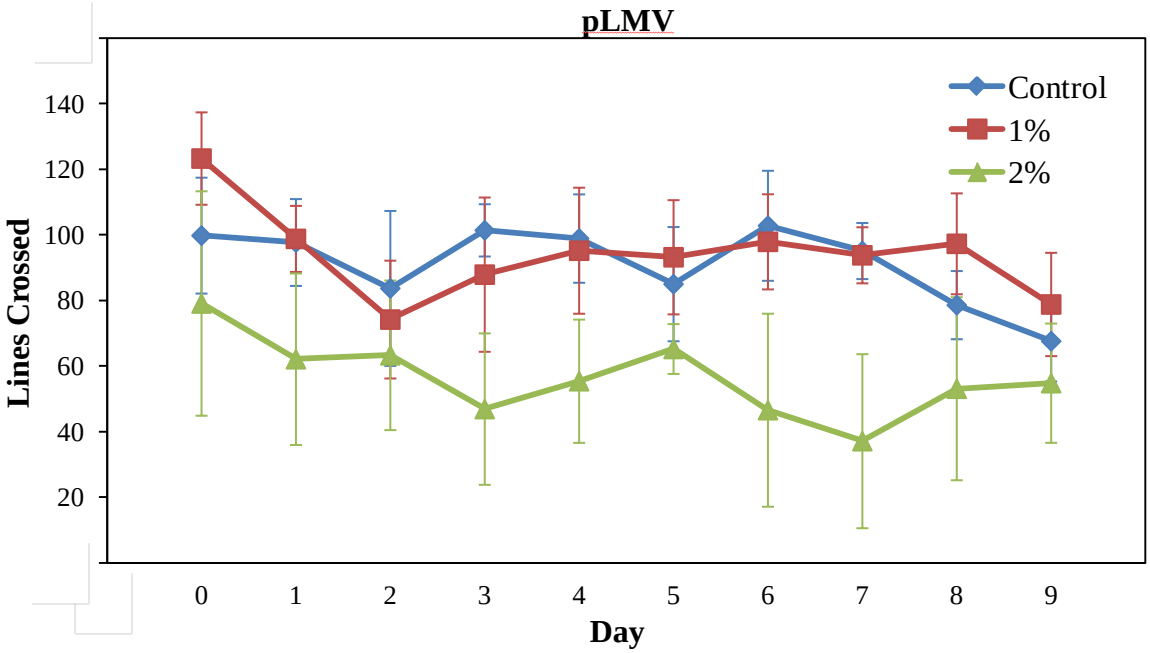


Figure 13: pLMV results for intact planaria. n=15 for all sets of data. 2% ethanol-exposed planaria exhibited statistical differences from control and 1% ethanol-exposed planaria on each day ($p<0.05$). All error bars are expressed with 95% confidence interval.

Planarian performance showed a negative trend throughout the duration of pLMV experimentation, with control averages falling from 99.8 to 67.6 lines crossed, 1%

planaria averages falling from 123.3 to 78.8 lines crossed, and 2% planaria averages falling from 79.1 to 54.8 lines crossed. This negative trend signifies a possible starvation effect on the planaria as the week-long testing progressed. As a result, we hypothesize that as the planaria are further removed from their last feeding, their functional capabilities begin to diminish. However, we cannot substantiate this claim at this time.

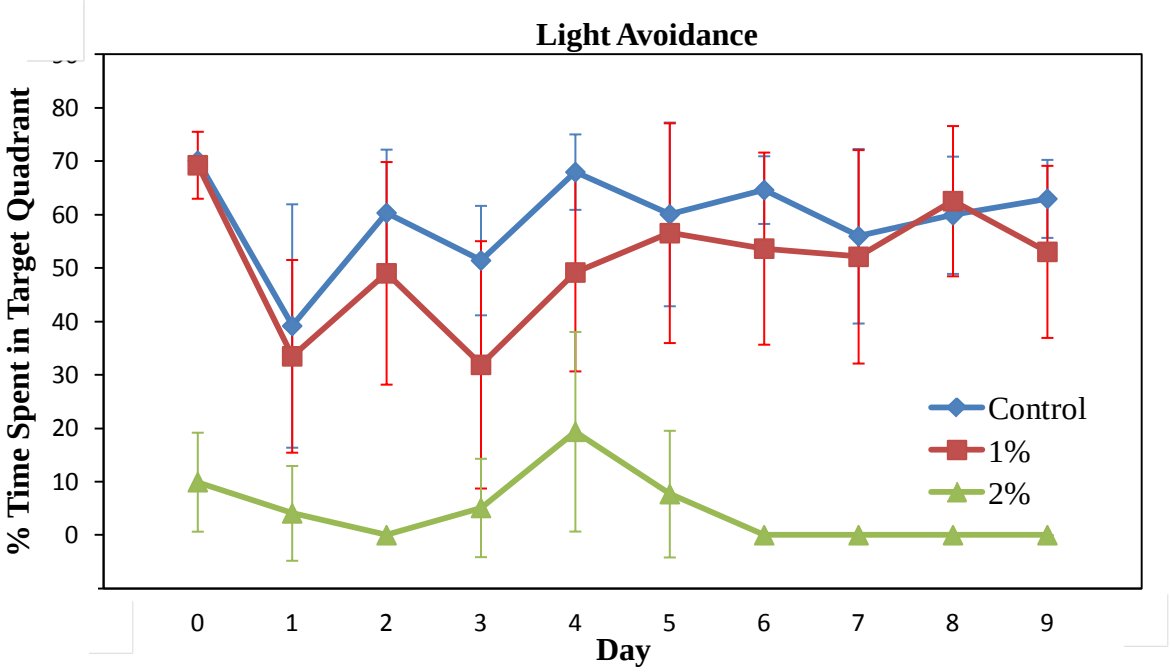


Figure 14: Light avoidance results for intact planaria. n=15 for all experiments. 2% alcohol-exposed planaria performed statistically differently from control and 1% alcohol-exposed planaria on each day. All error bars are expressed with 95% confidence interval.

Figure 14 displays the effect of varying concentrations of ethanol exposure on intact planaria light avoidance. Treatment of intact planaria with 2% ethanol significantly

reduced planarian functionality in light avoidance testing, mirroring the results obtained from pLMV testing. 2% alcohol-exposed planaria displayed a statistically significant difference ($p<0.05$) from both 1% alcohol-exposed and control solution planaria each day. After day 5, 2% ethanol-exposed planaria never reach the target quadrant again, even though they are still moving in pLMV testing (Figure 13). 1% ethanol-exposed planaria responded to functional testing no differently than control planaria with the exceptions of day 3 in light avoidance testing, where they displayed a statistically significant difference ($p<0.05$).

The purpose of intact planaria testing was to establish the immobilization effect various concentrations of alcohol would have on planaria to compare with regenerating experimentation. The results show that control and 1% alcohol-exposed planaria are not different statistically, which suggests that any statistically different data obtained during regeneration testing can be attributed to attenuation or delayed development of the planarian CNS in the alcohol solutions, and not the direct effect of alcohol alone.

The results obtained with the 2% v/v alcohol-exposed planaria suggest that planaria experience an immobilization effect at that concentration. The effect was not as severe as that described in previous studies for planaria exposed to 3% v/v alcohol, where planaria were completely immobilized for several minutes[33], yet it was more severe than the 1% ethanol-exposed planaria, which did not show evidence of immobilization effects. This suggests that alcohol exhibits a dose-dependent immobilization response in planaria. However, it is not clear if alcohol affects both locomotor function and photoreceptor functionality. Since 2% ethanol-exposed planaria are moving at a slower rate than both control and 1% ethanol-exposed planaria, it is expected that they would take longer to

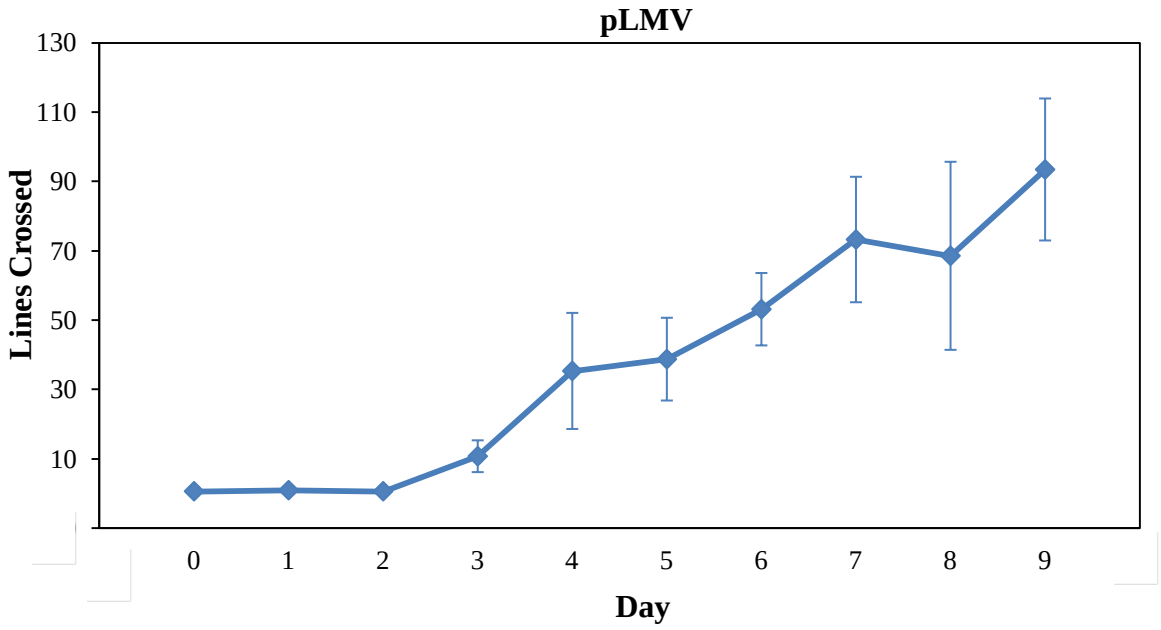
reach the target quadrant in light avoidance testing. If this is the case, then depressed light avoidance capabilities can be attributed to reduced pLMV function, not diminished photoreceptor capabilities.

On its own, the fact that 2% ethanol-exposed planaria show an immobilization effect due to alcohol exposure would confound any scores obtained during regeneration testing, since it could be argued that 2% planaria do not experience attenuated CNS development, but rather suffer from the immobilization effects of the high dose of alcohol. As a result, to test whether planarian development was affected in these conditions, we test planaria after a return to control solution (withdrawal testing, see Section 4.6). Once alcohol's effect on intact planaria had been established, we could move forward with determining what functional effects ethanol has on CNS development and functionality in head regenerating planaria.

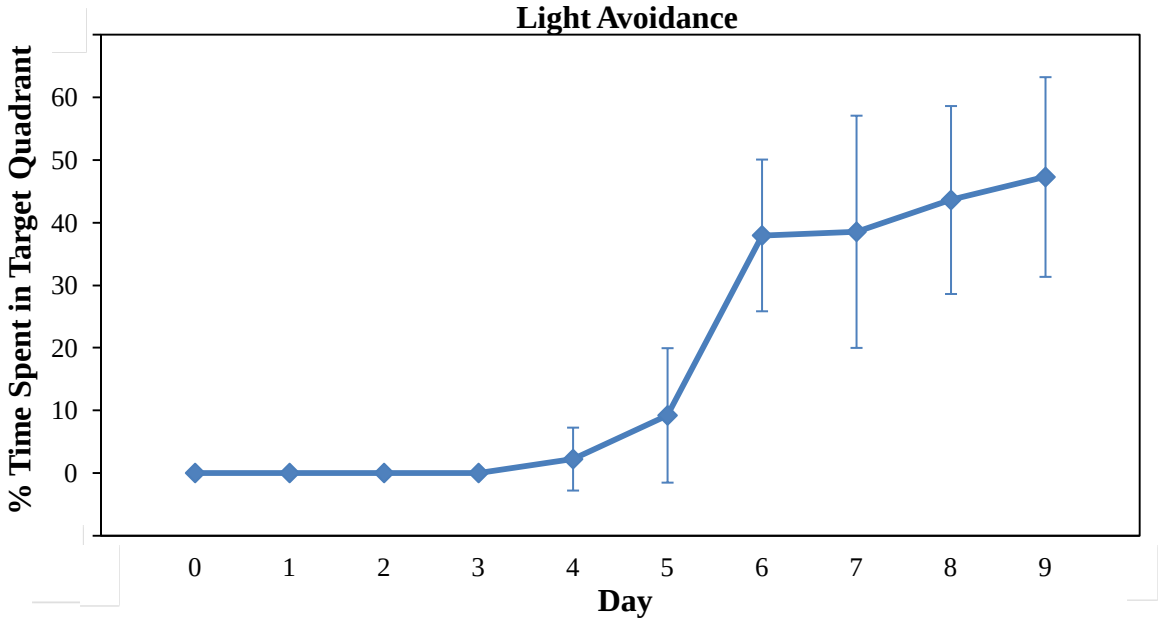
4.3 Regeneration Dynamics of Control Planaria Can Be Established With a FOPTD

In order to determine the effect alcohol has on CNS development in planaria, we exposed head-regenerating planaria to varying alcohol concentrations and tested their functionality. To quantify differences in CNS functional development across alcohol conditions, a series of visual First Order Plus Time Delay (FOPTD) models were developed. To collect the data for these models, functional tests (pLMV and light avoidance) were performed on head-regenerating planaria treated with control, 1% and 2% ethanol solutions. To reduce experimental variability, regeneration experimentation for all groups was performed simultaneously, with the same experimental conditions, including ambient temperature changes and measurement timing. To develop a baseline

model for the dynamics of planarian head regeneration, we began with planaria regenerating in control Monjuic solution. Figure 15a shows the pLMV results obtained for head-regenerating control planaria from days 0-9, while Figure 15b shows the analogous light avoidance results.



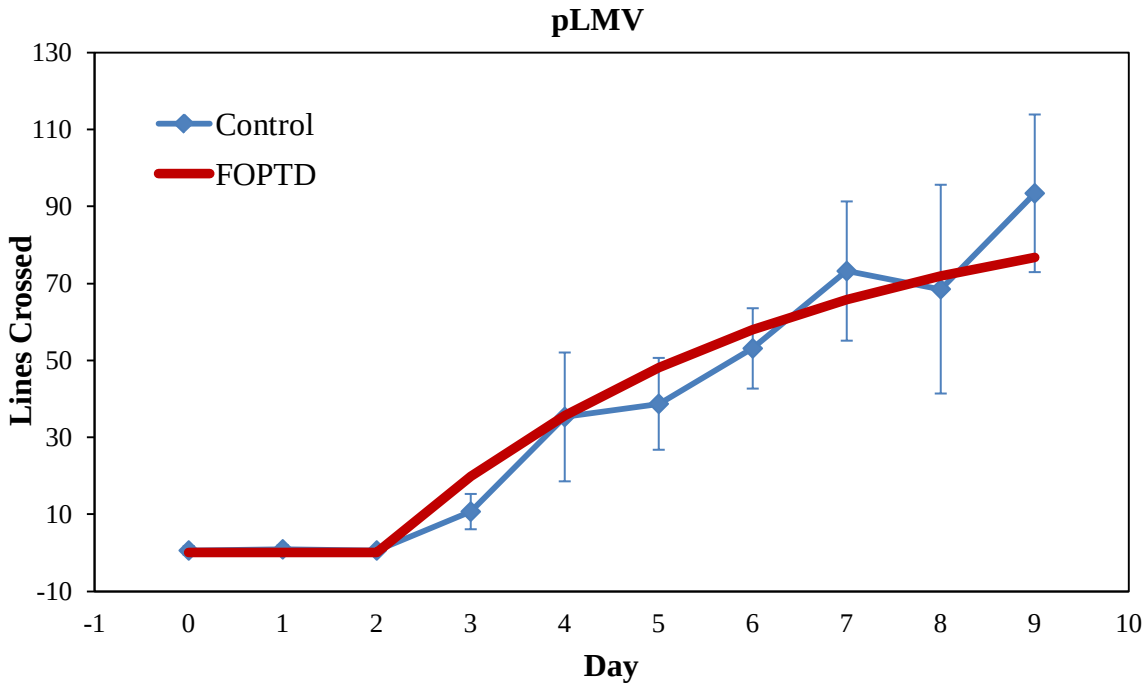
A) pLMV results for head-regenerating control planaria, n=10. Heads were removed from planaria at day 0. The lines crossed on each day represent the total lines crossed in a 5 minute testing duration



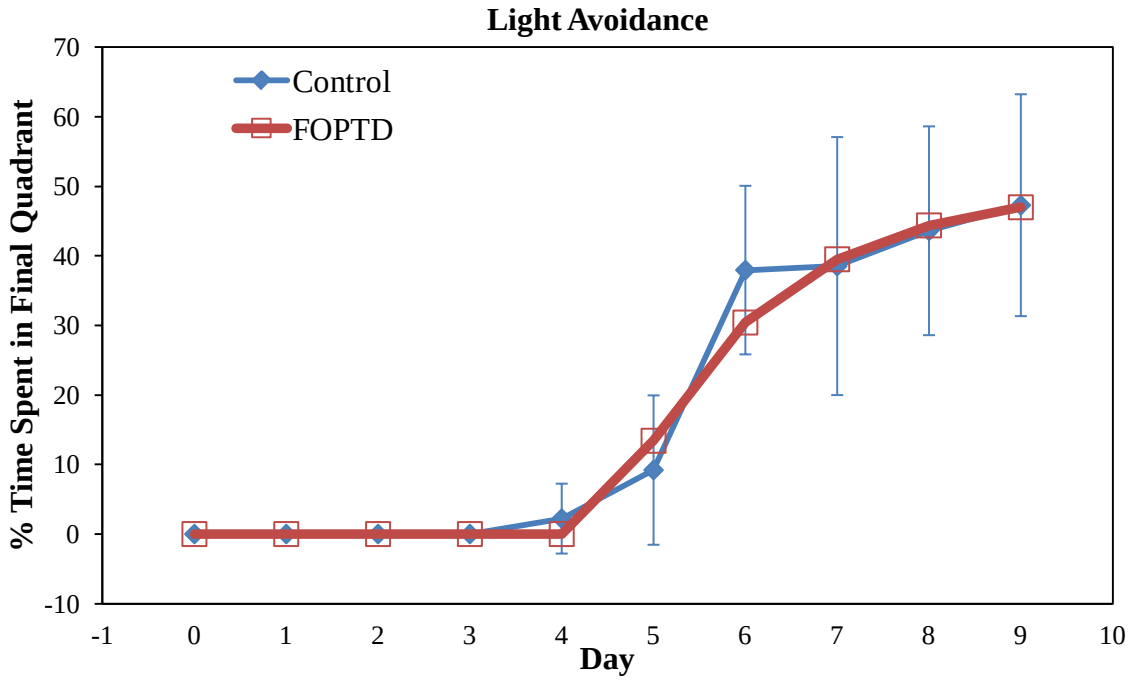
b) Light avoidance results for regenerating control planaria. Heads were removed from planaria at day 0. The percent time spent in target quadrant represents the time spent in a 90 second testing duration.

Figure 15 a & b: Experimental Results for regenerating control planaria. All error bars are expressed with 95% confidence interval.

As seen in Figure 15, planaria were able to regain locomotor functionality between days 2 and 3, while photoreceptor functionality expressed between days 4 and 5, with significant function returning on day 6. This compares favorably with previous light avoidance testing performed by Inoue et al., who also showed that planaria began reaching the target quadrant on day 5, with increased light avoidance performance on each subsequent day.[23] To quantify the regeneration dynamics, a FOPTD model was fit to the pLMV and light avoidance data. These models are shown in Figure 16a and b.



a) pLMV FOPTD model for regenerating control planaria. The model was developed visually, and has a K of 95 lines crossed, α of 2 days, and τ of 4.25 days.



b) Light avoidance FOPTD model for head-regenerating control planaria. The model was developed visually, and has a K of 50% time spent in target quadrant, α of 4.5 days, and τ of 1.6 days.

Figure 16 a&b: FOPTD models for control regenerating planaria. Both models were developed visually by manipulating K (gain), α (time delay), and τ (time constant). The figures suggest that light avoidance capabilities develop at a quicker rate (lower time constant) but later time (higher time delay) than locomotor function.

Equations 2 and 3 correspond to the FOPTD models shown in Figure 16.

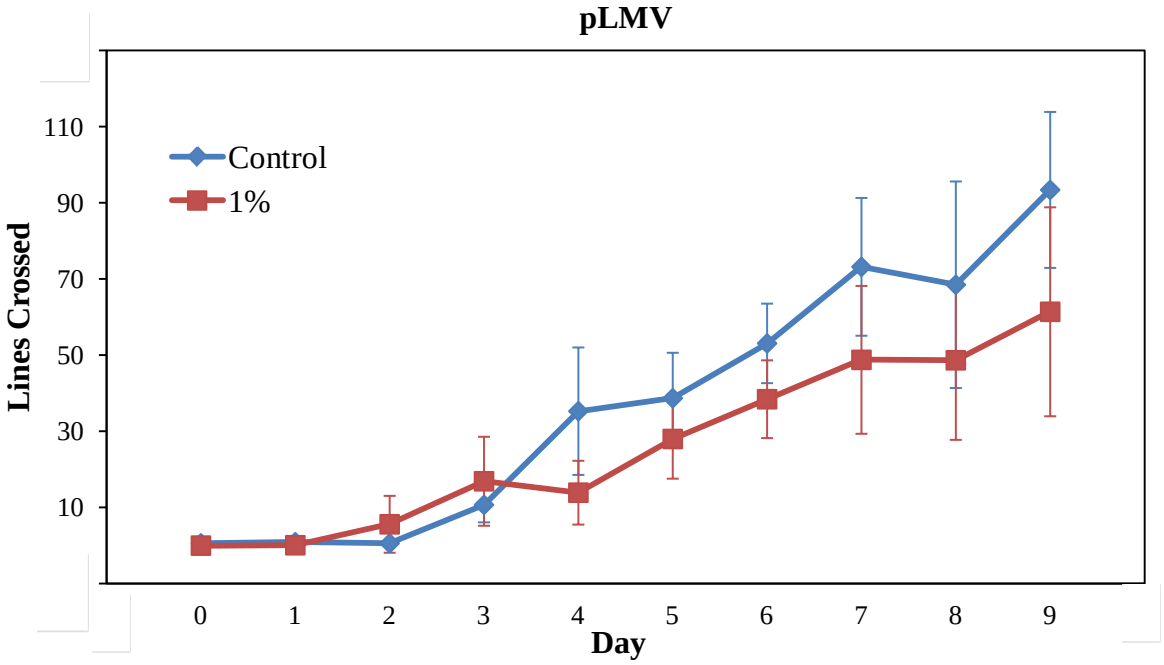
Control pLMV FOPTD equation: (2)

Control light avoidance FOPTD equation: (3)

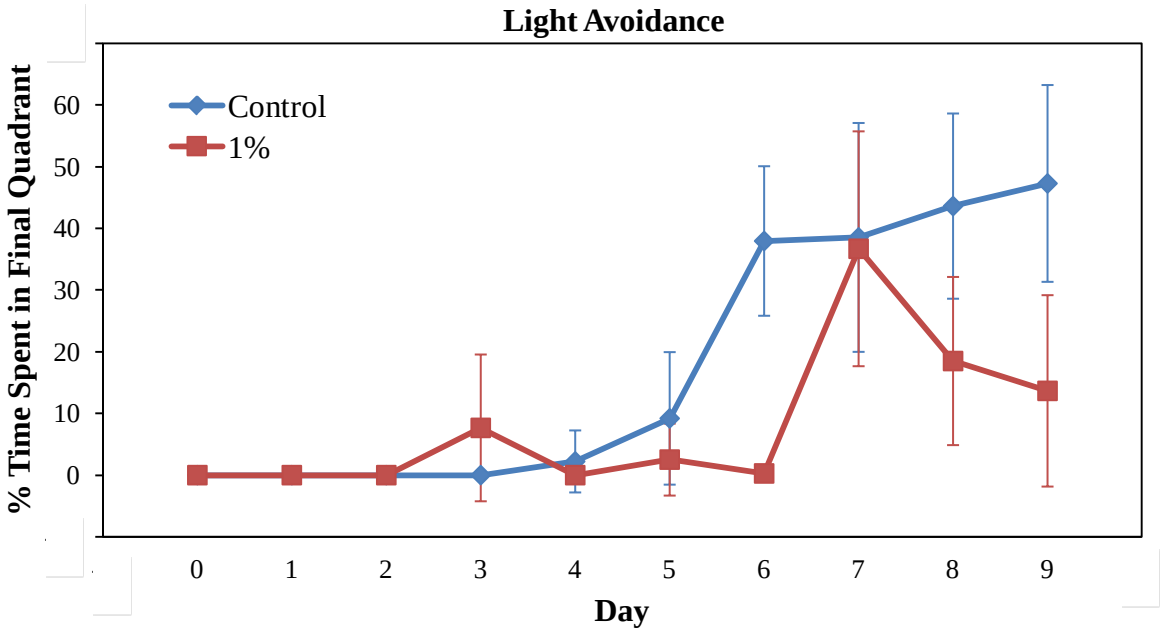
In equation 2, the gain term is 95 lines crossed, and the time delay term is 2 days, and the time constant is 4.25 days. In equation 3, the gain term is 50% time spent in the target quadrant, the time delay term is 4.5 days, and the time constant is 1.6 days. The gain represents the maximum amount to which the measurements will plateau. The time delay represents the amount of time before the system will show a response. This model suggests that control planaria will begin to exhibit movement after day 2 and significant light avoidance after 4.5 days. The time constant is a measure of how sluggish the reaction is. This suggests that while regenerating planaria will develop locomotor capabilities before light avoidance functionality, the development of light avoidance function is much quicker than that of locomotor function.

4.4 1% Alcohol Delays Functional Development

Once the baseline dynamics and functional performance for control planaria were established, we could analyze the effect of 1% v/v alcohol exposure on CNS developmental dynamics. We determined that exposure to 1% alcohol during head-regeneration alters the dynamics of development of movement (pLMV, Figure 17a) and light avoidance (Figure 17b) when compared to control planaria.



a) pLMV results for head-regenerating planaria exposed to control solution and 1% v/v alcohol. All error bars are reported with 95% confidence interval, n=10. 1% alcohol-exposed planaria cross less lines overall throughout CNS development.

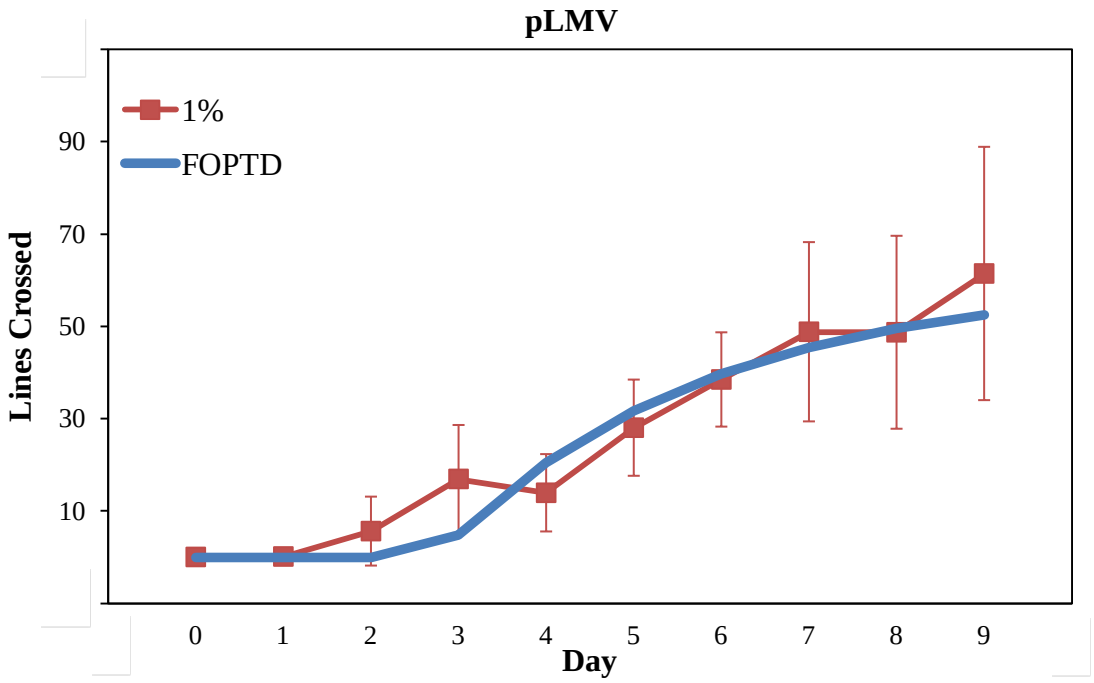


b) Light avoidance results for head-regenerating planaria exposed to control and 1% v/v alcohol solutions. All error bars are expressed with 95% confidence interval, n=10. 1% alcohol-exposed planaria exhibit a delay in onset of light avoidance functionality.

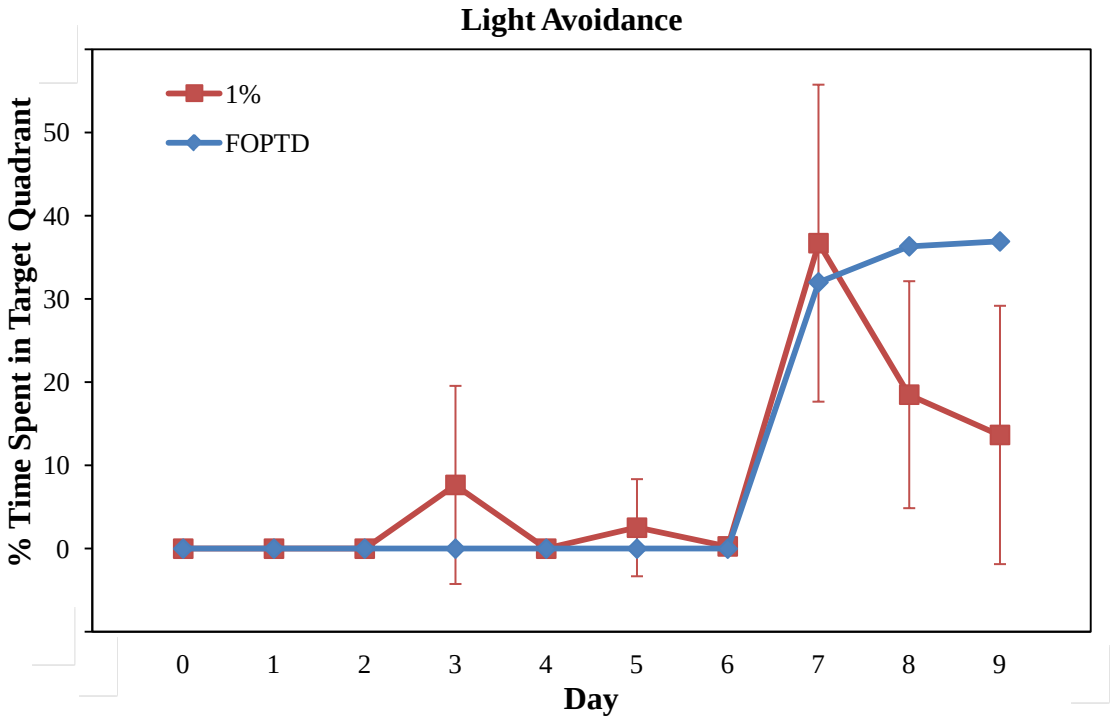
Figure 15 a & b: Experimental results for head-regenerating planaria exposed to control and 1% v/v alcohol solutions. Both a) and b) show that 1% alcohol-exposed planaria have a delay in onset of functionality. All error bars are expressed with 95% confidence interval.

As shown in Figure 17a, planaria begin developing significant motor function between days 2 and 3, but experience an overall delay in functionality when compared to control regenerating planaria. A gradual increase in lines crossed is observed following onset of functionality. Figure 17b shows that planaria do not develop significant photoreceptor functionality until between days 6 and 7, an apparent delay in functionality when

compared to regenerating planaria. To quantify the differences in dynamics between head-regenerating planaria exposed to control and 1% v/v alcohol, Figure 18 shows the FOPTD models developed for pLMV and light avoidance testing with head-regenerating 1% alcohol-exposed planaria.



a) pLMV FOPTD model fit for regenerating 1% alcohol planaria. The model was developed visually, and has a K of 60 lines crossed, α of 2.75 days, and τ of 3 days.



b) Light avoidance FOPTD model fit for regenerating 1% alcohol planaria. The model was developed visually, and has a K of 37% time spent in the target quadrant, α of 6 days, and τ of 0.5 days.

Figure 18a & b: FOPTD models for regenerating 1% alcohol planaria. Both models were developed visually by manipulating K (gain), α (time delay), and τ (time constant). The figures show a 0.75 day delay in onset of functionality for pLMV and a 1.5 day delay in onset of light avoidance functionality when compared to control planaria dynamics. All error bars are expressed with 95% confidence interval.

The equations that correspond to the FOPTD models developed in Figure 18 can be found below.

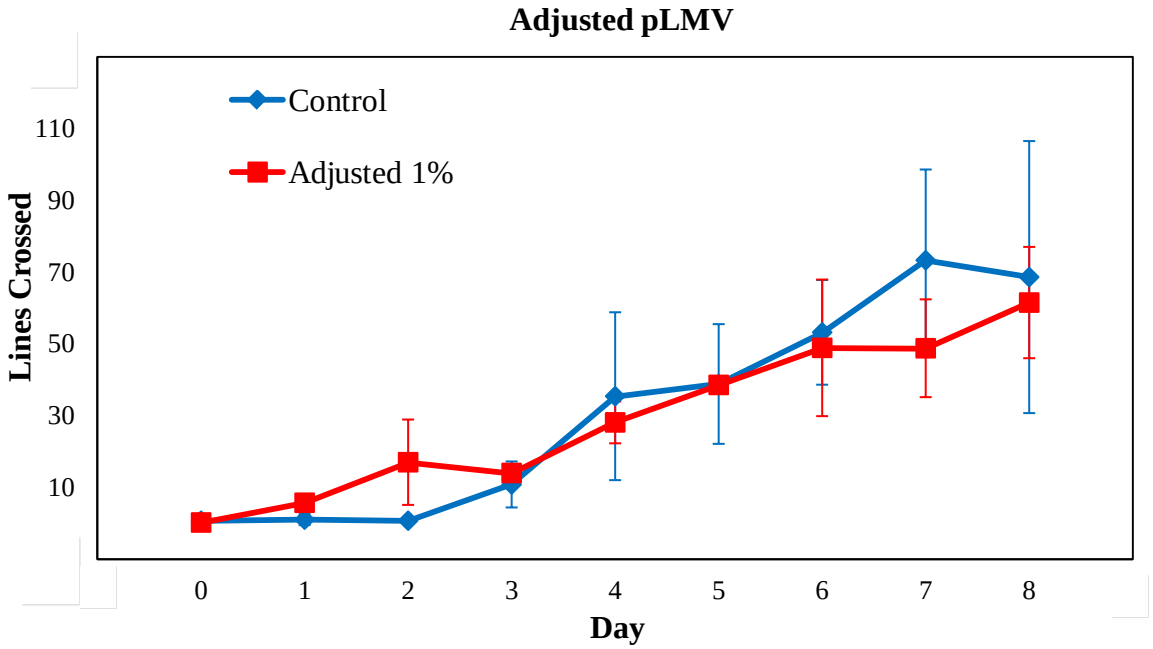
1% alcohol pLMV FOPTD equation: (4)

1% alcohol light avoidance FOPTD equation: (5)

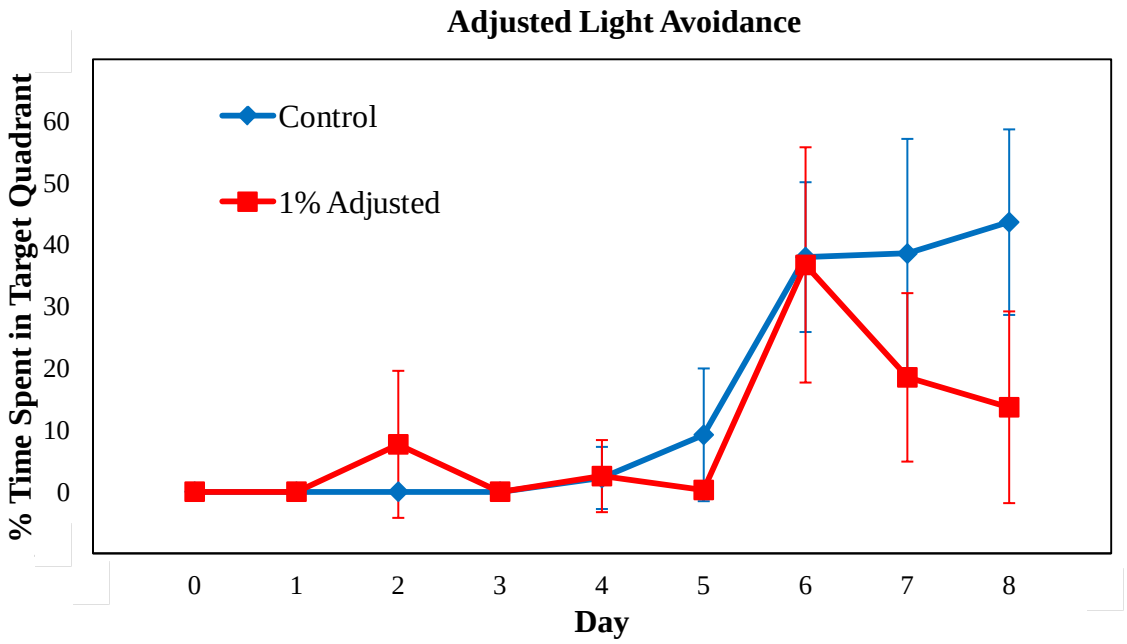
In equation 4, the gain term is 60 lines crossed, the time delay term is 2.75 days, and the time constant is 3 days. When compared to the dynamics observed in control planaria development, 1% alcohol exposed planaria display a lower gain and time constant, and a higher time delay. This suggests that 1% alcohol-exposed planaria will achieve a lower number of lines crossed and will show onset of functionality 0.75 days later. While the time constant obtained suggests that they will improve in functionality more quickly than control planaria, we feel that this is due to the lower gain, and is therefore not indicative of 1% alcohol-exposed planaria developing faster than control planaria after onset of functionality.

In equation 5, the gain term is 37% time spent in the target quadrant, the time delay term is 6 days, and the time constant is 0.5 days. When compared to the dynamics observed in control planaria light avoidance, the 1% alcohol-exposed planaria experience a lower percentage of time spent in the target quadrant, a 1.5 day delay in onset of functionality, and a quicker time constant. Since there is roughly one day delay in onset of functionality in both pLMV and light avoidance for 1% alcohol-exposed planaria, it can be concluded that, overall, there is a one day delay in CNS development in these planaria. As further evidence of this delay, Figure 19 shows that exposure to 1% ethanol induces a 1 day delay, but does not appear to otherwise affect development. In Figure 19, the results obtained for 1% alcohol regenerating planaria were shifted one day earlier and compared

to the control regenerating planaria results. This is done to show that if we could remove the one day developmental delay, the 1% alcohol-exposed planaria would perform statistically the same as the control planaria.



a) pLMV 1% alcohol planaria one day adjusted results. See text for a description of the day adjustment. N=10 for both experiments, and error bars are expressed with a 95% confidence interval.



b) Light avoidance 1% alcohol planaria one day adjusted results. N=10 for both experiments, and error bars are expressed with a 95% confidence interval.

Figure 19a & b) Adjusted 1% alcohol planaria results. Both figures show that when 1% alcohol-exposed planaria are adjusted one day earlier, they match the control results more closely, suggesting the existence of a one day delay in onset of functionality

Figure 19a clearly shows that there is a one day delay in the onset of locomotor function between head regenerating planaria exposed to control and 1% v/v alcohol solutions. While the control planaria developed light sensing capabilities between days 4 and 5 (as expected by results obtained previously[23]), the adjusted 1% results shown in Figure 19b clearly show that there was a one day delay in the onset of photoreceptor functionality. These results suggest that the effect alcohol has on CNS development in planaria is to induce a one day delay in the restoration of function.

4.5 2% Alcohol Attenuates CNS Functionality

In addition to control and 1% v/v alcohol solutions, we also exposed head-regenerating planaria to 2% v/v alcohol. Figure 20 shows the pLMV results obtained for planaria regenerating in 2% ethanol compared to control regenerating planaria. Planaria exposed to 2% alcohol never show normal levels locomotor functionality, but the data suggest that the onset of functionality is between days 3 and 4. We hypothesize that the lack of functionality is due to an immobilization effect of 2% ethanol exposure.

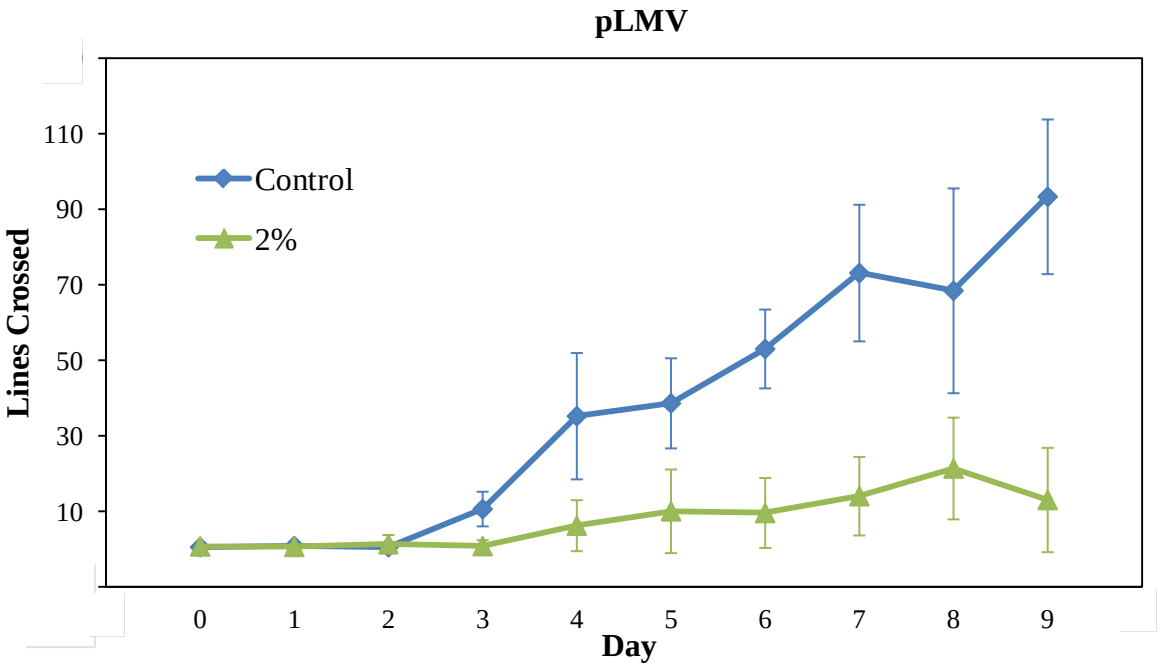
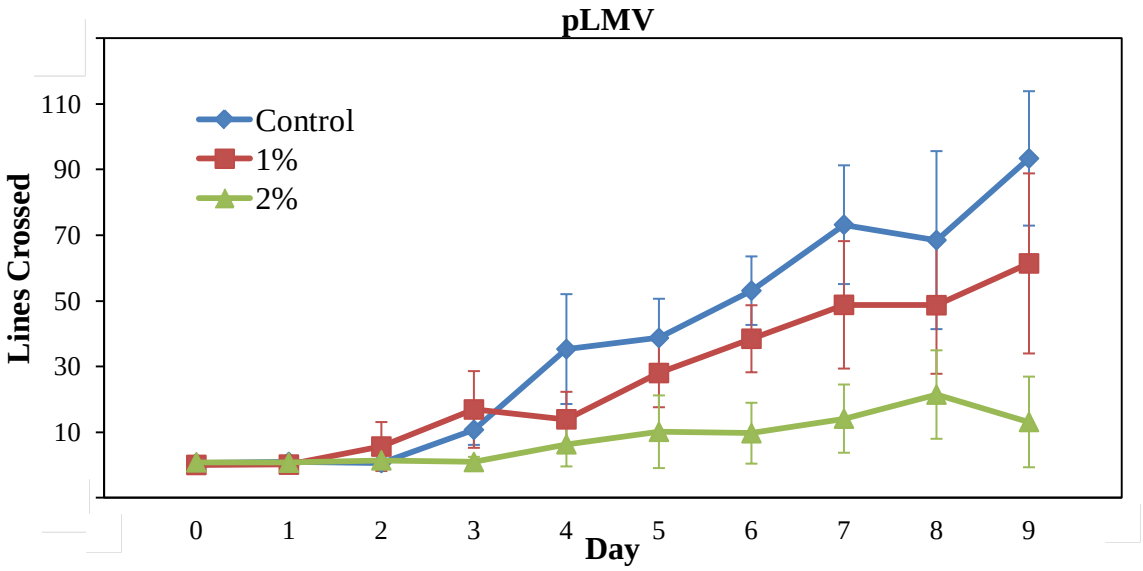
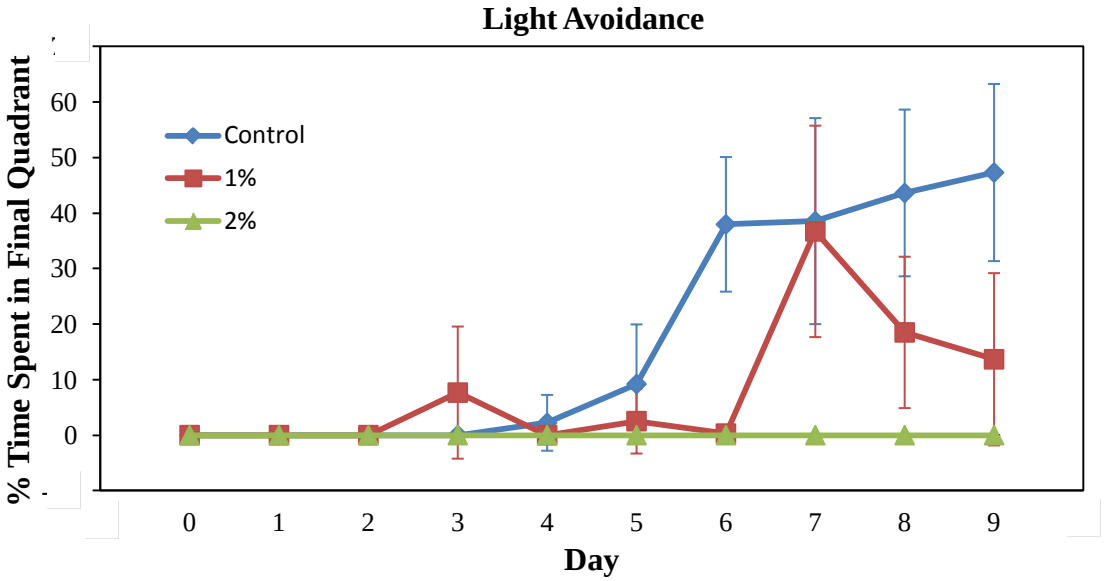


Figure 20: pLMV results for regenerating 2% planaria, n=10 for both sets of data, and error bars are reported with a 95% confidence interval. It is theorized that the low number of lines crossed by the 2% alcohol-exposed planaria is a result of an immobilization effect of alcohol. The data for control planaria is repeated from Figure 15a for comparison.

Because pLMV scores never exceeded 25 lines crossed, light avoidance is not a valid test of planarian functionality and poor light avoidance scores cannot be interpreted as functional deficits. Expectedly, these planaria were unable to reach the target quadrant during testing and consequently, the light avoidance data is not shown. Since 2% alcohol exhibited a clear immobilization effect on intact planaria, this lack of locomotor function cannot be described as either CNS developmental attenuation or alcohol immobilization singularly. As a result, it was necessary to perform withdrawal testing to determine whether or not 2% alcohol-exposed head-regenerating planaria would regain full function instantly, suggesting that CNS attenuation is due to alcohol immobilization, or display attenuated function after removal from alcohol, suggesting that CNS development was strongly affected by alcohol exposure. Figure 21 shows a comparison of the different experimental groups. Taken together these results show definitively that alcohol delays nervous system regeneration in a dose dependent manner.



a) Summary of pLMV results for head regenerating planaria. All error bars are reported with a 95% confidence interval, and n=10 for all sets of data.



b) Summary of light avoidance results for head-regenerating planaria. All error bars are reported with a 95% confidence interval, and n=10 for all sets of data.

Figure 21 a & b (Previous Page): Summary of experimental results for regenerating planaria. A clear dose-dependent response to alcohol can be seen in both pLMV and light avoidance functionality. It was concluded that lower concentrations of alcohol delay CNS development, while higher concentrations attenuate CNS development.

4.6 Planaria Do Not Exhibit Withdrawal-Like Behavior From Alcohol Exposure

In order to determine if planaria exposed to various alcohol concentrations would display long term functional deficiencies when removed from alcohol, withdrawal tests were developed. These tests would be able to determine if intact planaria experienced any withdrawal-like behavior, and would also determine if delayed pLMV and light avoidance performance exhibited by 1% and 2% ethanol-exposed planaria were a function of CNS developmental delays or immobilization effects from ethanol. Previous experimentation has shown that the duration of exposure to drugs such as opioids, cannabinoids, stimulants and benzodiazepines, has a duration-dependent effect on withdrawal-like behavior.[28] In these tests, it was shown that some drugs require less exposure time to produce greater withdrawal-like behavior. All effects were tested immediately after exposure, with the longest being 24 hours. To account for our 8 days of testing, withdrawal-like behavior was tested 15 minutes, 24 hours, and 48 hours after removal from ethanol solutions.

Figure 22 shows the pLMV withdrawal response from both regenerating and control worms in varying solutions. Only head-regenerating planaria exposed to 2% v/v alcohol displayed significantly different and depressed pLMV results, with lower performance at

each time point after return to control solution. This suggests the possible permanent attenuation of locomotor functionality in planaria exposed to 2% ethanol during development. Since locomotor functionality is attenuated for 2% ethanol-treated planaria after return to control solution, it also suggests that the values obtained in regenerating planaria testing for 2% ethanol-exposed planaria is not due to only an immobilization response.

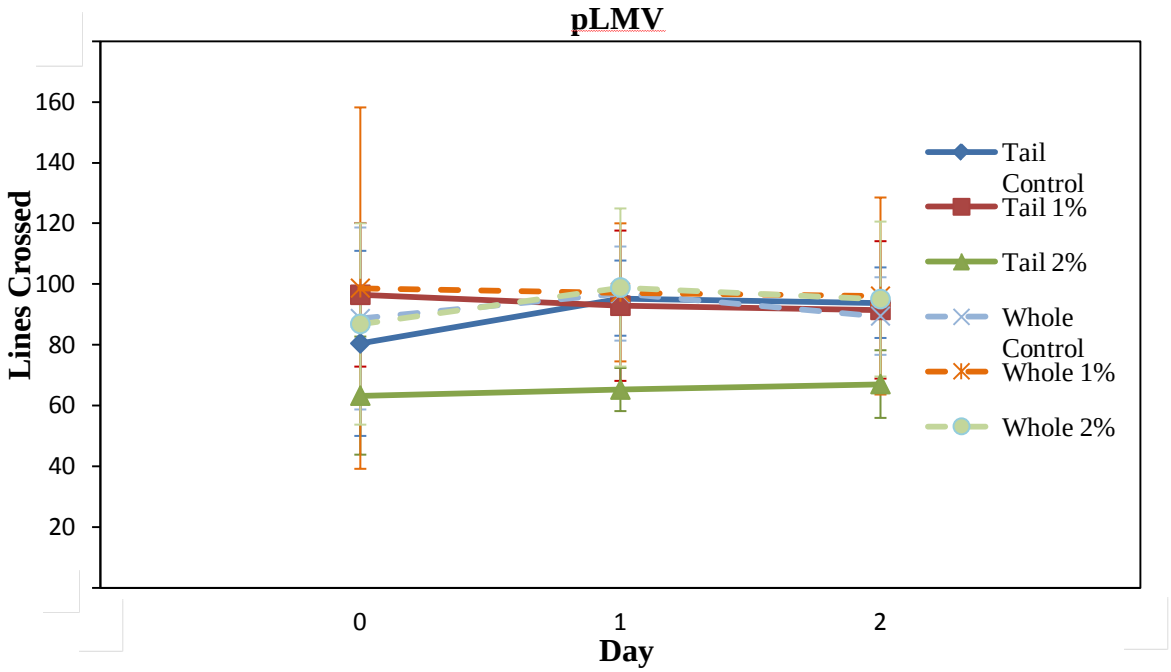


Figure 22: Withdrawal results for intact and regenerating planaria. n=5 for all experiments. 2% ethanol-exposed planaria are the only subjects that are significantly different from the other sets of samples ($p<0.05$).

The light avoidance for this same experiment can be found in Figure 23. Interestingly, while pLMV shows a marked attenuation of movement for planaria exposed to 2%

ethanol, there are no statistically significant differences observed in light avoidance response at any time point. This suggests that the planaria may be exhibiting a hyper-sensitivity to light after being removed from 2% alcohol concentration.

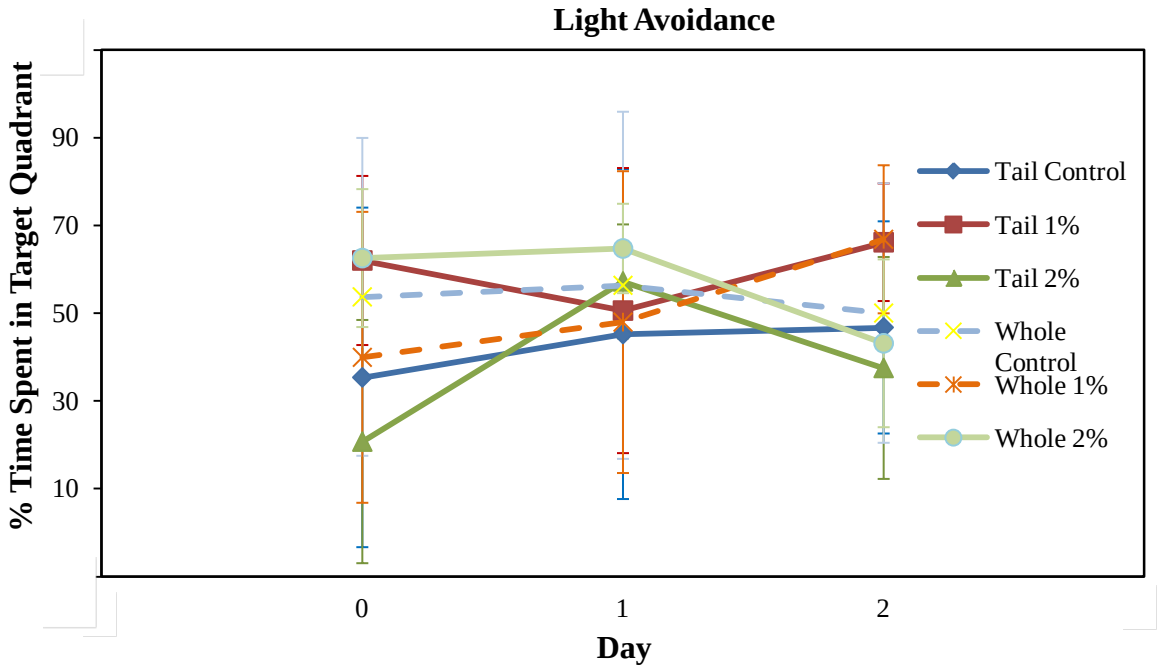


Figure 23: Light avoidance withdrawal results for intact and regenerating planaria. n = 5 for all experiments. No significantly statistical differences ($P<0.05$) were observed between the 6 sets of samples.

When applied to regenerating planaria, the withdrawal tests show that the delay observed in 1% regenerating planaria (discussed earlier) does not correlate to a permanent attenuation of CNS development. This can be inferred through the lack of any statistically significant differences between the control and 1% regenerating planaria in experimentation. Prior to removal from ethanol, regenerating 2% alcohol-exposed

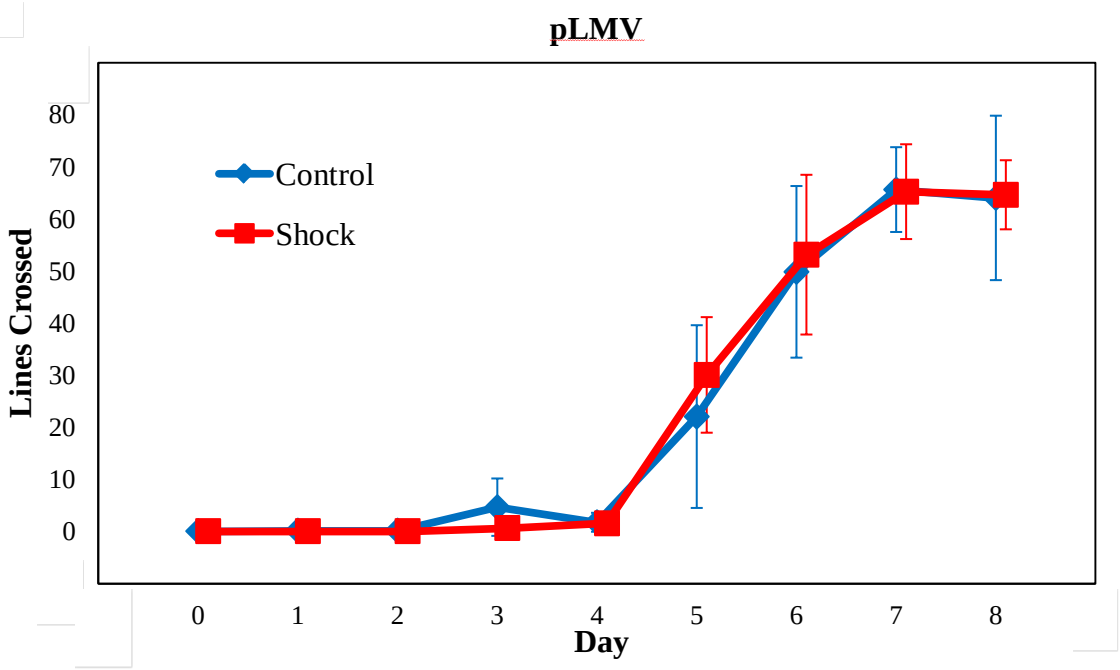
planaria obtained lower scores during regenerating testing for both light avoidance and pLMV, and it was hypothesized that these functional deficiencies can be attributed to both attenuation and immobilization effects. Once the 2% planaria were placed into a control solution, however, their locomotor functionality increased, and they were able to display negative phototaxis by moving to the target quadrant. The regenerated 2% planaria still were significantly slower in pLMV testing 48 hours after removal from 2% ethanol, suggesting that ethanol permanently attenuated locomotor function during the development of the regenerating CNS. Our hypothesis is that this locomotor function will eventually return to normal levels due to the continual regeneration planaria undergo. However, since we did not test past 48 hours, we cannot prove conclusively that locomotor function returned.

4.7 Timing of Ethanol Exposure Effects Severity of CNS Attenuation

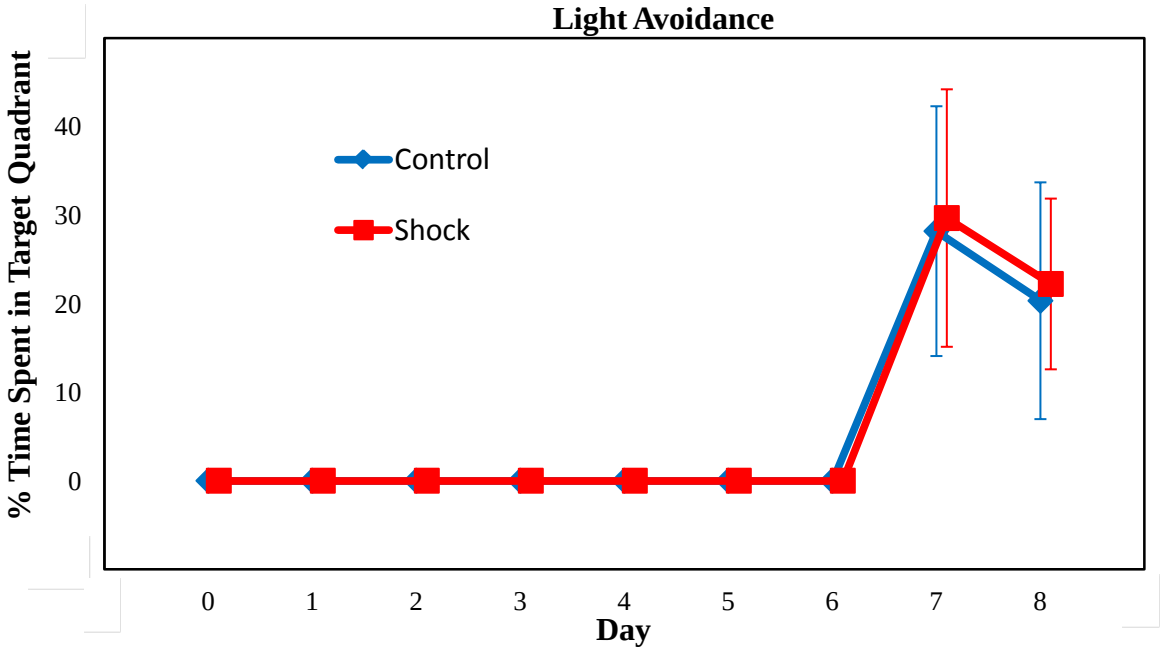
Once it was shown that alcohol has an effect on planarian CNS development, we wanted to determine at what point in the dosage regimen alcohol was having the strongest effect. Also, in terms of introducing planaria as a model for FASD, continuous exposure to alcohol over the duration of development is an unrealistic expectation. A more realistic model involves targeted alcohol exposure, where treated planaria are only exposed to alcohol for a small period of head-regeneration and CNS development.

Initial targeted alcohol exposure testing focused on the effect of applying a "shock" of 3% v/v ethanol one hour prior to regeneration in Montjuic solution. The purpose of this test is to investigate the initial processes. Shock testing would show if alcohol affects the planarian's ability to detect the severing of the head (which could lead to the delay noted

above), or initiate developmental processes. The 3% v/v alcohol concentration was selected because if ethanol had any effect on early processes, a high concentration of alcohol would clearly elucidate it. Figure 24 shows the comparison between control regenerating planaria and planaria undergoing shock treatment prior to regeneration.



a) pLMV results for control and shock head-regenerating planaria. There are no statistically significant differences between control and shock planaria. All error bars are expressed with 95% confidence interval, and n=10 for both sets of samples.



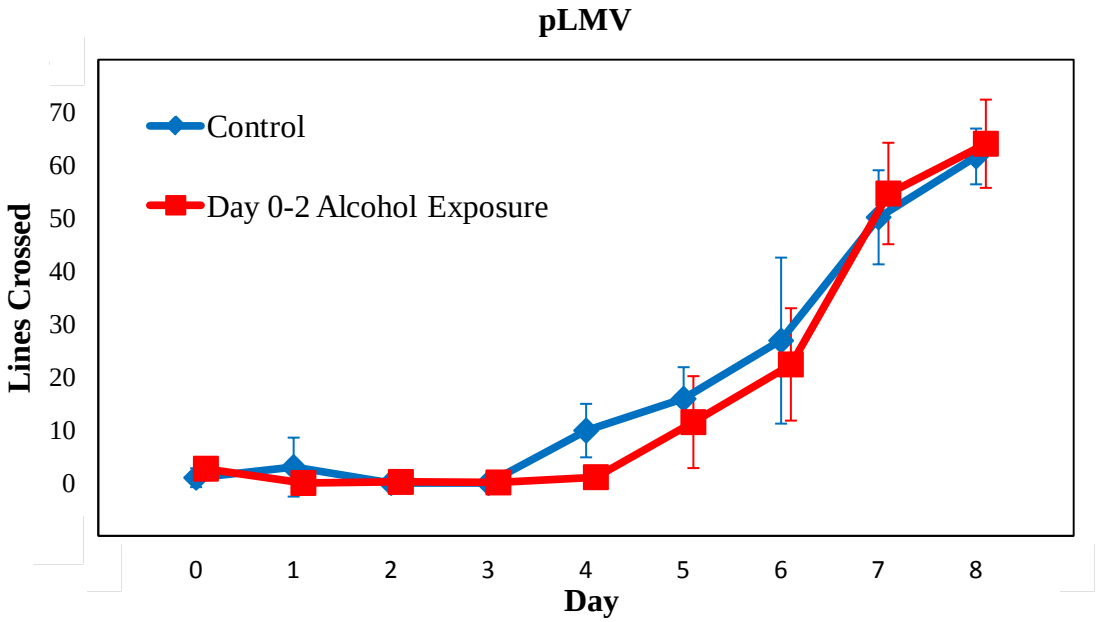
b) Light avoidance results for regenerating control and shock planaria. There are no statistically significant differences between control and shock planaria. All error bars are expressed with 95% confidence interval, and n=10 for both sets of samples.

Figure 16 a and b: Experimental results for control and shock planaria. Shock head-regenerating planaria represent planaria exposed to 3% v/v alcohol for one hour after head removal before being returned to control solution.

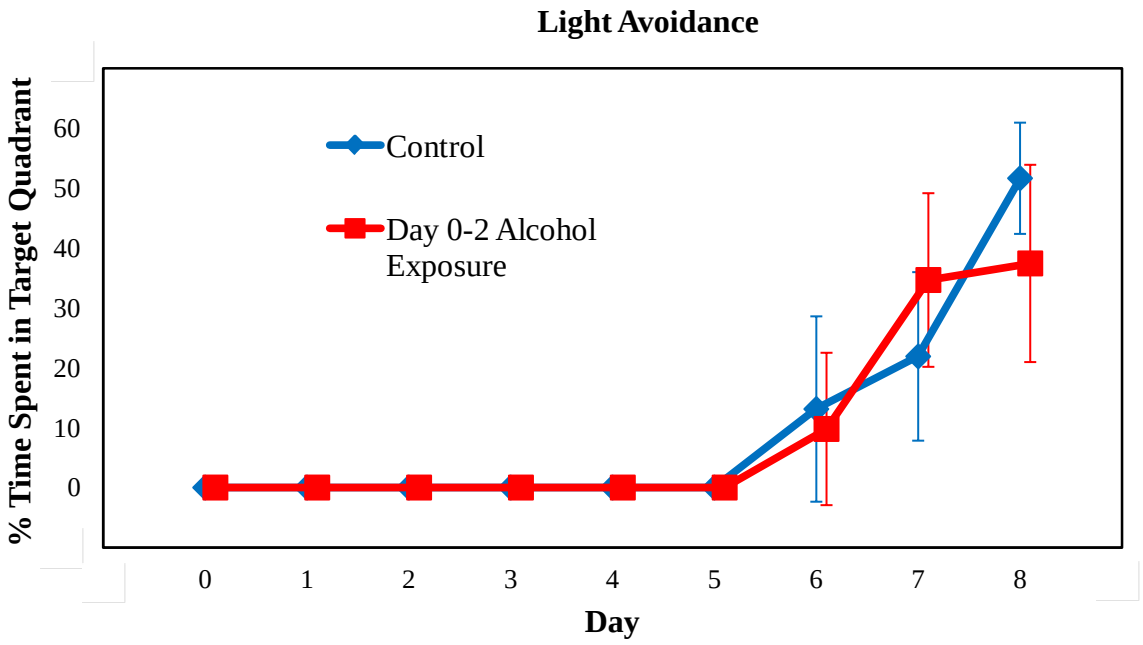
Figure 24 shows that there are no statistically significant differences in either test. Interestingly, both experimental groups experienced unexpected delays in both onset of locomotor and light avoidance functionality, 2 days later than predicted by the literature. This could possibly be caused by undocumented changes in environmental conditions. However, since the delay is seen in both treatment conditions, it does not change the

interpretation of the data. Ethanol exposure does not affect CNS development when applied as an immediate treatment for one hour after head removal, suggesting that ethanol has no effect on the planarian's ability to register head removal or initial developmental processes.

The next dosage regime that was investigated involved placing planaria in 1% v/v alcohol immediately after head removal, and were removed from alcohol after the completion of observation on day 2, a 48 hour exposure. Once removed, treated head-regenerating planaria were placed into control solution to complete CNS development normally. These planaria were compared to a control group undergoing functional regeneration analysis simultaneously. Figure 25 shows the pLMV and light avoidance data obtained from this experiment.



a) pLMV results for regenerating control and day 0-2 ethanol treated planaria. The two treatment groups only have statistically significant differences ($p<0.05$) on Day 4. All error bars are expressed with 95% confidence interval, and n=10 for both sets of samples.



b) Light avoidance results for regenerating control and day 0-2 ethanol treated planaria. There are no statistically significant differences between control and shock planaria. All error bars are expressed with 95% confidence interval, and n=10 for both sets of samples.

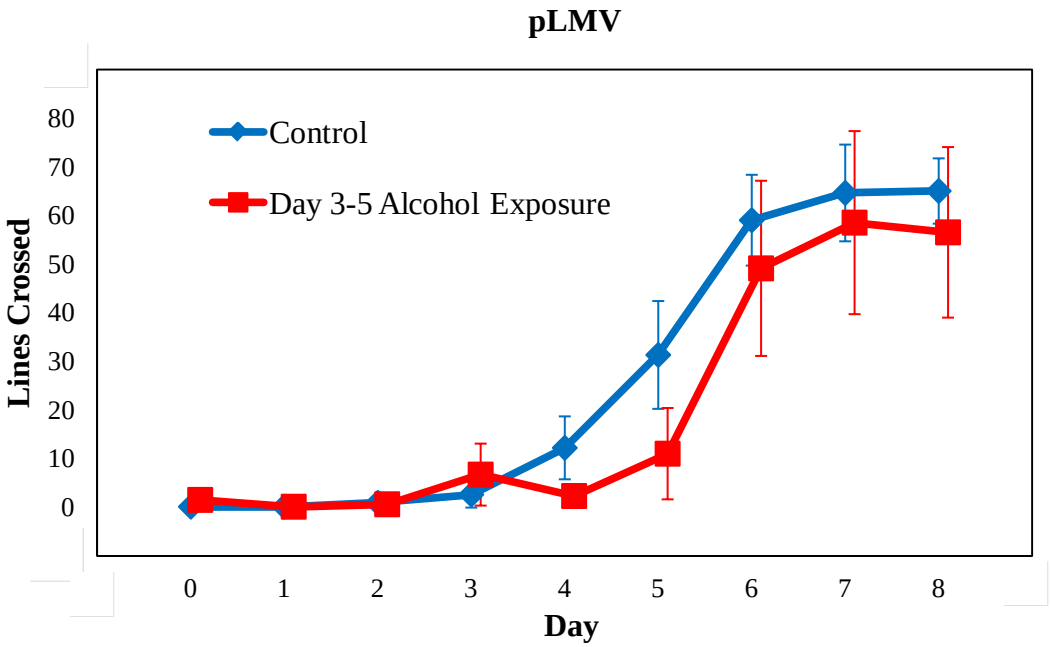
Figure 17 a and b: experimental results for regenerating control and day 0-2 ethanol treated planaria. All day 0-2 ethanol treated planaria were exposed to 1% v/v alcohol for 48 hours after head removal. It was determined that this dosage regime does not have an effect on CNS development in planaria.

The data in Figure 25a suggests that there may be a one day delay in the onset of pLMV functionality for planaria exposed to 1% ethanol from days 0-2, with control regenerating planaria showing onset between days 3 and 4, and treated planaria displaying onset between days 4 and 5. However, because of the low number of lines crossed at this point,

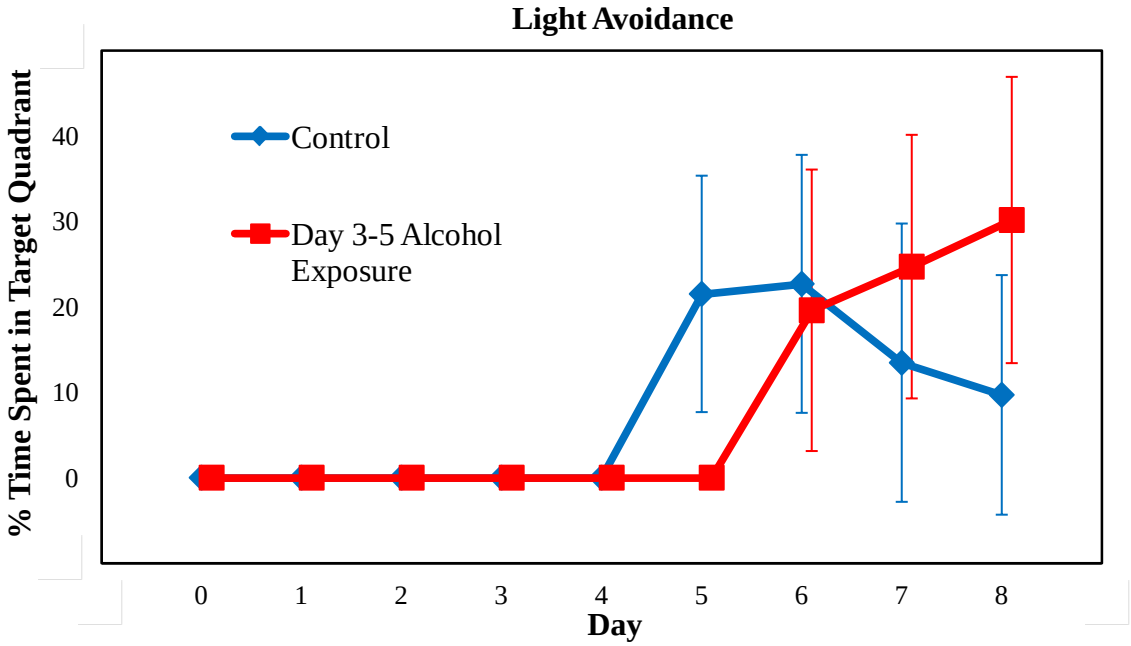
it is more likely that the planaria are just slightly repressed in the day 0-2 ethanol treated group, not necessarily delayed. The only statistically significant difference between the two data sets occurs at day 4. Figure 25b shows that there were no statistically significant differences between the groups in light avoidance testing, with all planaria displaying photoreceptor functionality between day 5 and 6. This suggests that ethanol also has no effect on the first 48 hours of CNS development in head-regenerating planaria.

In this experiment, both treatment groups were delayed by one day from their expected onset of functionality in both pLMV and light avoidance. This is the same behavior observed in the previous testing for control and shock treated planaria. As before, we assumed that since this delay is present in both treatment groups, it does not change the interpretation of the data.

In the final targeted ethanol exposure experiment, one set of regenerating planaria were exposed to 1% ethanol from the end of observation on day 2 to the end of observation on day 5, a 72 hour exposure that spans the expected development of photoreceptor functionality. If a delay in development was observed during this time period, it would suggest that ethanol has some effect on the direct development of locomotor and photoreceptor functionality. It could be possible that the alcohol exposure could be delaying the attachment of the ventral nerve cords to the cephalic ganglia, delaying locomotor function. Alcohol exposure could also be delaying the expression of the genes necessary for photoreceptor development. The results from these experiments are displayed in Figure 26.



a) pLMV results for regenerating control and day 3-5 ethanol treated planaria. A clear one day delay can be observed between the control and day 3-5 alcohol exposure treatment groups, suggesting that ethanol has an effect on the CNS development associated with locomotor function. All error bars are expressed with 95% confidence interval, and n=10 for both sets of samples.



b) Light avoidance results for regenerating control and day 3-5 ethanol treated planaria. A clear one day delay can be observed between the control and day 3-5 alcohol exposure treatment groups, suggesting that ethanol has an effect on the CNS development associated with photoreceptor function. All error bars are expressed with 95% confidence interval, and n=10 for both sets of samples.

Figure 18 a and b: Experimental results for regenerating control and day 3-5 ethanol treated planaria. All day 3-5 ethanol treated planaria were exposed to 1% v/v alcohol for 72 hours after the completion of testing on Day 2. It was determined that this dosage regime has an effect on CNS development in planaria, with both locomotor and photoreceptor function delayed.

Planaria exposed to 1% alcohol in these experiments had a clear one day delay in the onset of pLMV functionality, as shown in Figure 26a, with control planaria displaying onset of functionality between days 3 and 4, and alcohol-treated planaria displaying between day 4 and 5. A similar result is observed in light avoidance testing, shown in Figure 26b, where control planaria exhibit photoreceptor functionality between day 4 and 5, whereas treated planaria exhibit the same functionality between days 5 and 6. Since 1% ethanol exposure was shown to have no effect on immobilization, this cannot be attributed to alcohol acting as an immobilization agent, but instead suggests that alcohol affects developmental processes that occur during this time period.

This one day delay in day 3-5 ethanol treated planaria matches the one day delay observed in 1% v/v ethanol treated planaria tested earlier, and also represents a one day delay from the expected onset of light avoidance behavior as determined by Inoue et al.[23] While it is clear that there is a one day delay, it is impossible at this time to determine whether the delay involves more than a movement delay. Since light avoidance testing is dependent on the planaria having developed locomotor capabilities, if the planaria is not moving in pLMV testing, it cannot be expected to reach the target quadrant in light avoidance testing.

The alcohol-induced delay in the onset of pLMV and light avoidance functionality seen in Day 3-5 ethanol-treated planaria is due to alcohol exposure during this time frame. This leads to two possible suggestions on what effect alcohol exposure during this period has on CNS development. First, it suggests that the molecular mechanisms of development that occur during this time frame are affected by alcohol. Alternately, it could suggest that exposure to the alcohol itself retards movement, as the

delayed/reduced movement occurs only during the time that alcohol is present for testing. We can disregard the latter suggestion due to the intact planarian testing that we performed earlier. In this testing, we showed that planaria exposed to 1% v/v alcohol do not exhibit immobilization effects via diminished pLMV performance, which allows us to conclusively say that alcohol is not retarding movement from days 3-5, but is affecting the developmental processes.

Chapter 5

Conclusions and Future Recommendations

The research performed and described in this thesis supports our hypothesis that planaria can be used as a novel model for studying the dynamics of FASD. We were able to demonstrate that ethanol exposure exhibits a dose-dependent immobilization response in intact planaria. This was supported by Stephenson et al., who used 3% v/v ethanol to temporarily immobilize planaria for imaging purposes.[33]

Once it had been determined that ethanol has an effect on intact planaria, we showed that planaria exhibit a dose-dependent response to ethanol exposure during CNS development in head-regenerating planaria. Testing showed that lower concentrations (1% v/v) of alcohol show a delay in development. The one day delays observed in both pLMV and light avoidance were modeled using a visually fit FOPTD model. For both functional tests, 1% alcohol-exposed planaria had a time delay term that was approximately one day longer than control planaria. Higher concentrations (2% v/v) of alcohol attenuated CNS functionality throughout the CNS development period. Whether alcohol was attenuating only pLMV function or both pLMV and light avoidance function is impossible to say at the moment, since reduced locomotor function measured in pLMV testing would prevent planaria from reaching the target quadrant in light avoidance testing.

Withdrawal testing determined that the functional attenuation exhibited by 2% alcohol-exposed planaria in head-regeneration is not caused by the immobilization effect of ethanol on planaria. Planaria exposed to 2% v/v alcohol throughout head-regeneration still exhibited attenuated functionality compared to control and 1% v/v alcohol-exposed

planaria when returned to control solution and tested 15 minutes, 24 hours, and 48 hours afterwards. This result led to two conclusions about the results obtained in head-regeneration studies. First, while head-regenerating planaria exposed to 1% v/v alcohol show a delay in the onset of functionality, they will still achieve full functionality. Second, the functional attenuation observed in head-regenerating planaria exposed to 2% v/v alcohol is not caused by alcohol immobilization, and will extend at least 48 hours past removal from alcohol solution.

We can also conclude that the timing of alcohol exposure plays a role in alcohol's effect on CNS development in planaria. It was determined that applying an immediate shock of 3% v/v alcohol had no effect on planarian CNS development, suggesting that ethanol does not affect initial molecular mechanisms associated with CNS regeneration. 1% v/v alcohol exposure for the first 48 hours of development also has no effect on planarian CNS development. However, alcohol exposure for a 72 hour period from days 3-5 attenuates CNS function, showing the same one day delay observed in head-regenerating 1% alcohol-exposed planaria. This suggests that the developmental delays observed in 1% alcohol-exposed planaria result from alcohol's effect on developmental processes that occur within the 72 hours from days 3-5.

The requirements for a novel animal model in the area of FASD include an animal that is cost effective, ethical, and easy to use. The most important requirements, however, are that the novel model allows for the ability to test functionality during development, and that ethanol has an observable effect on the development of functionality in the animal model. In this thesis, we were able to conclusively show that planaria fits these two most important requirements. Their regenerative capabilities allow us to test the functionality

of their CNS development with pLMV and light avoidance experiments. The head-regenerating trials described in Chapter 4 show that alcohol exposure has a dose-dependent response on CNS functionality in both pLMV and light avoidance testing, with a one day delay evident at lower concentrations and permanent attenuation at higher concentrations.

An added benefit to planaria as a model for FASD is the lack of interaction between mother and fetus. We discussed in Chapter 2 how there are several parental effects observed in FASD that can confound current FASD research. Since planaria reproduce asexually through regeneration, we can isolate ethanol's effect on the development of the CNS, without having to consider confounding effects from a parental interaction.

In conclusion, we have shown that planaria fulfill all the requirements necessary for a novel animal model for FASD. As a result, we propose the species *Schmidtea mediterranea* as a viable model for the dynamics of FASD. This research will bring a new model for members of the alcohol research community to study the dynamics of ethanol's effect on CNS development. Also, it will provide a new focus into the uses for planarian research into CNS regeneration.

Moving forward, the Staehle lab will continue to work with determining the dynamics of ethanol's effect on planarian CNS development. One research aim is to further narrow the time period investigated for targeted ethanol exposure. 72 hours is a long time for molecular activities, so narrowing the time period down to a more precise time period will allow for a more precise determination of what molecular processes are being affected by ethanol exposure.

The second research aim is to identify what problems are occurring in the physiology of the planaria as a result of ethanol exposure. It is postulated that ethanol exposure is affecting morphological and molecular physiology. The Staehle lab has already commenced research into this area by adapting an existing protocol for whole mount in-situ hybridization (WISH), included in Appendix B.[34] The goal of WISH studies is to compare molecular deficiencies observed in alcohol-exposed planaria to functional deficiencies observed in pLMV and light avoidance testing, proving that alcohol has altered normal planarian physiology. Initial WISH investigations have focused on using general planarian nervous system markers to determine if there is any dysmorphology between alcohol-exposed head-regenerating planaria and control head-regenerating planaria.

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APPENDICES

Appendix A

Experimental Data

In naming and labeling individual worms, the following naming conventions were applied: “trial #” refers to which chronological trial it is, “regenerating or not” refers to whether the planaria is intact (“W” for whole) or regenerating (“T” for tail), “Solution” refers to the solution it is being maintained in (“C” for control, “1-2” for various alcohol concentrations), and “Planaria letter” for that individual planaria’s identification (“A-J” for each solution).

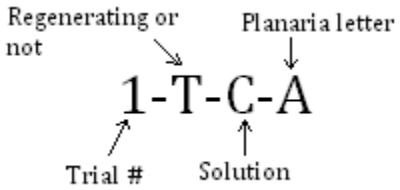

Figure A1: Example of Naming Nomenclature

The following tables include the raw data for all experiments.

Table 3; Experimental Data for Control Intact Planaria

PLMV Testing (Lines Crossed)														
Worm	1-T-C-A	1-T-C-B	1-T-C-C	1-T-C-D	1-T-C-E	1-T-C-F	1-T-C-G	1-T-C-H	1-T-C-I	1-T-C-J	AVG	Std.Dev	n	95% CI
Day 0	0	5	0	0	0	0	0	1	0	0	0.6	1.58	10.00	1.13
Day 1	5	1	0	0	1	0	0	1	0	1	0.9	1.52	10.00	1.09
Day 2	0	0	0	0	2	2	1	0	1	0	0.6	0.84	10.00	0.60
Day 3	17	18	1	13	13	9	18	11	6	1	10.7	6.41	10.00	4.59
Day 4	73	69	19	50	22	1	37	17	23	42	35.3	23.40	10.00	16.74
Day 5	70	26	41	36	56	37	15	21	51	34	38.7	16.68	10.00	11.93
Day 6	80	72	42	51	42	49	31	49	54	61	53.1	14.59	10.00	10.44
Day 7	84	78	86	39	77	66	24	72	100	106	73.2	25.29	10.00	18.09
Day 8	102	49	78	45	80	77	12	15	98	129	68.5	37.91	10.00	27.12
Day 9	125	70	108	64	111	95	37	95	101	128	93.4	28.62	10.00	20.48

Light Avoidance Testing														
Worm	1-T-C-A	1-T-C-B	1-T-C-C	1-T-C-D	1-T-C-E	1-T-C-F	1-T-C-G	1-T-C-H	1-T-C-I	1-T-C-J	AVG	Std. Dev	n	95% CI
Day 0	0	0	0	0	0	0	0	0	0	0	0	0.00	10.00	0
Day 1	0	0	0	0	0	0	0	0	0	0	0	0.00	10.00	
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0.00	10.00	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0.00	10.00	
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0.00	10.00	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0.00	10.00	
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0.00	10.00	0
Day 4	20	0	0	0	0	0	0	0	0	0	2	6.32	10.00	
% Time in Quadrant	22.22	0	0	0	0	0	0	0	0	0	2.22	7.03	10.00	5.03
Day 5	41.07	0	0	12.57	20.04	0	0	0	9.14	0	8.28	13.52	10.00	
% Time in Quadrant	45.63	0	0	13.97	22.27	0	0	0	10.16	0	9.20	15.02	10.00	10.74
Day 6	43.33	35.81	24.57	36.87	34.74	35.64	0	36.47	33.03	61.2	34.17	15.25	10.00	
% Time in Quadrant	48.14	39.79	27.30	40.97	38.60	39.60	0.00	40.52	36.70	68.00	37.96	16.95	10.00	12.12
Day 7	52.29	58.69	20.57	0	21.72	37.58	0	37.62	55.7	62.71	34.69	23.34	10.00	
% Time in Quadrant	58.10	65.21	22.86	0	24.13	41.76	0	41.8	61.89	69.68	38.54	25.94	10.00	18.55
Day 8	59.3	63.07	44.62	17.93	49.17	32.17	0	42.53	46.65	37.13	39.26	18.89	10.00	
% Time in Quadrant	65.88888889	70.07777778	49.58	19.92	54.63	35.74	0	47.26	51.83	41.26	43.62	20.99	10.00	15.02
Day 9	45.9	61.72	62.13	54.91	43.8	53.76	0	15.49	39.65	48.29	42.57	20.07	10.00	
% Time in Quadrant	51.00	68.58	69.03	61.01	48.67	59.73	0.00	17.21	44.06	53.66	47.29	22.30	10.00	15.95

Table 4; Experimental Data for 1% Intact Planaria

PLMV Testing (lines crossed)														
Worm	1-T-01-A	1-T-01-B	1-T-01-C	1-T-01-D	1-T-01-E	1-T-01-F	1-T-01-G	1-T-01-H	1-T-01-I	1-T-01-J	AVG	Std. Dev	n	95% CI
Day 0	0	0	0	0	0	0	0	0	0	0	0	0	10	0.00
Day 1	0	0	0	0	1	0	0	0	0	0	0.1	0.32	10	0.23
Day 2	1	0	9	11	0	1	0	1	0	33	5.6	10.44	10	7.47
Day 3	1	9	7	16	5	44	8	10	21	48	16.9	16.33	10	11.68
Day 4	18	32	13	32	9	19	8	7	1	---	13.9	10.90	9	8.38
Day 5	24	12	35	37	35	1	31	44	33	---	28.0	13.56	9	10.42
Day 6	66	25	39	29	26	34	33	43	51	---	38.4	13.29	9	10.21
Day 7	77	21	47	37	61	0	68	68	60	---	48.8	25.26	9	19.41
Day 8	72	35	58	61	56	3	75	70	8	---	48.7	27.18	9	20.90
Day 9	125	34	48	95	86	7	87	55	77	---	61.4	35.69	9	27.43

Light Avoidance Testing															
Worm	1-T-01-A	1-T-01-B	1-T-01-C	1-T-01-D	1-T-01-E	1-T-01-F	1-T-01-G	1-T-01-H	1-T-01-I	1-T-01-J	AVG	Std. Dev	Day	%	95% CI
Day 0	0	0	0	0	0	0	0	0	0	0	0.0	0.00	0	0.0	0.0
Day 1	0	0	0	0	0	0	0	0	0	0	0.0	0.00	1	0.0	0.0
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0.0	0.00	2	0.0	0.0
Day 2	0	0	0	0	0	0	0	0	0	0	0.0	0.00	3	7.7	11.9
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0.0	0.00	4	0.0	0.0
Day 3	0	0	0	0	0	42	0	0	0	27	6.9	14.97	5	2.5	5.8
% Time in Quadrant	0	0	0	0	0	46.67	0	0	0	30	7.7	16.63	6	0.3	0.6
Day 4	0	0	0	0	0	0	0	0	0	---	0.0	0.00	7	37.0	19.0
% Time in Quadrant	0	0	0	0	0	0	0	0	0	---	0.0	0.00	8	18.5	13.6
Day 5	0	0	0	0	0	0	0	0	20.49	---	2.3	6.83	9	15.2	15.5
% Time in Quadrant	0	0	0	0	0	0	0	0	22.77	---	2.5	7.59			
Day 6	0	0	0	0	0	0	2.23	0		---	0.3	0.79			
% Time in Quadrant	0	0	0	0	0	0	2.478	0	0	---	0.3	0.83			
Day 7	56.73	0	55.23	20.99	27.14	0	45.45	43.9	49.99	---	33.3	22.29			
% Time in Quadrant	63.03	0.00	61.37	23.32	30.16	0.00	50.50	48.78	55.54	---	37.0	24.77			
Day 8	39.44	23.22	7.46	0	23.78	0	39.2	17.08	0	---	16.7	15.96			
% Time in Quadrant	43.82	25.80	8.29	0.00	26.42	0.00	43.56	18.98	0.00	---	18.5	17.74			
Day 9	31.27	0	0	10.18	10.46	0	52.92	0	18.13	---	13.7	18.17			
% Time in Quadrant	34.74	0.00	0.00	11.31	11.62	0.00	58.80	0.00	20.14	---	15.2	20.18			

Table 5; Experimental Data for 2% Intact Planaria

PLMV Testing (Lines Crossed)														
Worm	1-T-02-A	1-T-02-B	1-T-02-C	1-T-02-D	1-T-02-E	1-T-02-F	1-T-02-G	1-T-02-H	1-T-02-I	1-T-02-J	AVG	SD	N	95% CI
Day 0	0	2	1	2	2	1	0	0	0	0	0.80	0.92	10	0.66
Day 1	0	1	0	0	0	0	3	0	3	0	0.70	1.25	10	0.90
Day 2	1	0	1	0	0	0	0	0	11	1	1.40	3.41	10	2.44
Day 3	1	0	0	0	0	0	0	0	7	1	0.90	2.18	10	1.56
Day 4	6	0	0	0	0	0	3	19	27	8	6.30	9.44	10	6.75
Day 5	0	0	4	3	1	16	3	4	48	12	10.11	15.48	10	11.08
Day 6	2	2	1	1	0	34	1	0	26	20	9.67	12.96	10	9.27
Day 7	0	0	0	8	1	1	4	3	45	65	14.11	14.53	10	10.39
Day 8	23	1	7	14	37	39	3	18	51	---------	21.44	17.54	9	13.48
Day 9	3	0	3	3	19	14	40	48	1	---------	13.10	17.97	9	13.81

Light Avoidance Testing															
Worm	1-T-02-A	1-T-02-B	1-T-02-C	1-T-02-D	1-T-02-E	1-T-02-F	1-T-02-G	1-T-02-H	1-T-02-I	1-T-02-J	AVG	SD			
Day 0	0	0	0	0	0	0	0	0	0	0	0	0	Day	%	95% CI
Day 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0
Day 4	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0
Day 5	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0
Day 6	0	0	0	0	0	0	0	0	0	0	0	0			
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0			
Day 7	0	0	0	0	0	0	0	0	0	0	0	0			
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0			
Day 8	0	0	0	0	0	0	0	0	0	----------	0	0			
% Time in Quadrant	0	0	0	0	0	0	0	0	0	--------	0	0			
Day 9	0	0	0	0	0	0	0	0	0	---------	0	0			
% Time in Quadrant	0	0	0	0	0	0	0	0	0	----------	0	0			

Table 6; Experimental Data for Control Regenerating Planaria

pLMV Testing (Lines Crossed)														
Worm	2-W-C-A	2-W-C-B	2-W-C-C	2-W-C-D	2-W-C-E	2-W-C-F	2-W-C-G	2-W-C-H	2-W-C-I	2-W-C-J	AVG	Std.Dev	n	95% CI
Day 0	101	113	54	112	98	148	98	81	110	83	99.8	24.70	10.00	17.67
Day 1	110	117	83	91	58	120	112	97	94	95	97.7	18.51	10.00	13.24
Day 2	71	55	114	99	61	22	134	75	105	101	83.7	33.03	10.00	23.63
Day 3	126	112	93	90	101	105	102	90	101	94	101.4	11.14	10.00	7.97
Day 4	132	118	61	98	92	107	95	94	104	88	98.9	18.84	10.00	13.48
Day 5	106	95	82	86	62	95	114	75	85	50	85	24.34	10.00	17.41
Day 6	116	114	87	107	121	113	132	68	60	110	102.8	23.45	10.00	16.78
Day 7	81	98	107	70	105	104	94	106	94	92	95.1	11.96	10.00	8.55
Day 8	64	85	75	75	83	109	91	75	57	72	78.6	14.53	10.00	10.39
Day 9	62	81	67	75	60	92	92	57	45	45	67.6	17.15	10.00	12.27

Light Avoidance Testing														
Worm	2-W-C-A	2-W-C-B	2-W-C-C	2-W-C-D	2-W-C-E	2-W-C-F	2-W-C-G	2-W-C-H	2-W-C-I	2-W-C-J	AVG	Std. Dev	n	95% CI
Day 0	60.81	60.23	54.68	71.53	51.85	69.67	68.28	62.34	62.40	69.37	63.12	6.60	10.00	0.00
% Time in Quadrant	67.57	66.92	60.76	79.48	57.61	77.41	75.87	69.27	69.33	77.08				
Day 1	45.97	67.68	44.59	59.43	0.00	44.29	58.84	8.53	0.00	69.25	39.86	27.12	10.00	
% Time in Quadrant	0.51	0.75	0.50	0.66	0.00	0.49	0.65	0.09	0.00	0.77	0.39	0.32	10.00	0.23
Day 2	31.78	58.64	64.13	50.69	38.27	73.28	68.19	54.47	35.31	68.37	54.31	14.92	10.00	
% Time in Quadrant	0.35	0.65	0.71	0.56	0.43	0.81	0.76	0.61	0.39	0.76	0.60	0.17	10.00	0.12
Day 3	64.40	36.73	31.40	51.97	36.29	53.57	54.46	33.64	64.56	35.84	46.29	12.89	10.00	
% Time in Quadrant	0.72	0.41	0.35	0.58	0.40	0.60	0.61	0.37	0.72	0.40	0.51	0.14	10.00	0.10
Day 4	54.64	66.77	68.37	67.68	50.29	74.05	58.90	55.63	47.81	67.83	61.20	8.89	10.00	
% Time in Quadrant	0.61	0.74	0.76	0.75	0.56	0.82	0.65	0.62	0.53	0.75	0.68	0.10	10.00	0.07
Day 5	62.87	70.62	40.83	33.16	66.09	70.57	71.11	58.48	4.52	62.45	54.07	21.64	10.00	
% Time in Quadrant	0.70	0.78	0.45	0.37	0.73	0.78	0.79	0.65	0.05	0.69	0.60	0.24	10.00	0.17
Day 6	57.80	61.56	62.21	62.68	50.18	39.23	67.11	58.57	59.90	62.41	58.17	7.97	10.00	
% Time in Quadrant	0.64	0.68	0.69	0.70	0.56	0.44	0.75	0.65	0.67	0.69	0.65	0.09	10.00	0.06
Day 7	51.88	53.77	0.00	62.79	66.32	58.21	62.63	68.84	45.30	34.08	50.38	20.56	10.00	
% Time in Quadrant	0.58	0.60	0.00	0.70	0.74	0.65	0.70	0.76	0.50	0.38	0.56	0.23	10.00	0.16
Day 8	56.97	61.90	61.74	25.95	37.88	59.12	65.48	58.76	42.42	69.02	53.92	13.82	10.00	
% Time in Quadrant	0.63	0.69	0.69	0.29	0.42	0.66	0.73	0.65	0.47	0.77	0.60	0.15	10.00	0.11
Day 9	57.00	41.05	55.48	51.25	65.97	66.92	68.93	49.55	48.69	62.03	56.69	9.19	10.00	
% Time in Quadrant	0.63	0.46	0.62	0.57	0.73	0.74	0.77	0.55	0.54	0.69	0.63	0.10	10.00	0.07

Table 7; Experimental Data for 1% Regenerating Planaria

PLMV Testing (Lines Crossed)														
Worm	2-W-01-A	2-W-01-B	2-W-01-C	2-W-01-D	2-W-01-E	2-W-01-F	2-W-01-G	2-W-01-H	2-W-01-I	2-W-01-J	AVG	Std. Dev	n	95% CI
Day 0	100	148	104	119	149	136	135	116	132	94	123.3	19.692	10	14.09
Day 1	80	123	90	101	114	97	91	80	108	104	98.8	14.069	10	10.06
Day 2	75	62	36	66	69	111	82	102	40	99	74.2	25.112	10	17.96
Day 3	22	90	126	130	100	71	55	109	78	98	87.9	32.881	10	23.52
Day 4	95	51	139	108	104	131	72	91	88	73	95.2	26.865	10	19.22
Day 5	96	116	144	91	76	103	57	72	92	85	93.2	24.343	10	17.41
Day 6	98	123	115	129	68	93	107	87	77	82	97.9	20.272	10	14.50
Day 7	97	105	113	90	86	109	84	77	93	84	93.8	11.951	10	8.55
Day 8	84	78	134	95	128	91	107	102	64	90	97.3	21.504	10	15.38
Day 9	98	98	83	67	105	45	90	81	40	81	78.8	22.019	10	15.75

Light Avoidance Testing															
Worm	2-W-01-A	2-W-01-B	2-W-01-C	2-W-01-D	2-W-01-E	2-W-01-F	2-W-01-G	2-W-01-H	2-W-01-I	2-W-01-J	AVG	Std. Dev	Day	%	95% CI
Day 0	57.90	40.84	66.47	68.82	57.80	68.39	65.22	66.61	61.08	70.40	62.35	8.76	0	0.69	6.27
% Time in Quadrant	64.33	45.38	73.86	76.47	64.22	75.99	72.47	74.01	67.87	78.22			1	0.00	18.05
Day 1	0.00	36.69	29.06	51.06	68.02	54.87	22.65	16.93	22.19	0.00	30.15	22.70	2	0.33	20.84
% Time in Quadrant	0.00	40.77	32.29	56.73	75.58	60.97	25.17	18.81	24.66	0.00	33.50	25.23	3	0.37	23.16
Day 2	8.61	49.30	68.77	63.13	71.23	64.94	64.25	49.81	1.32	0.00	44.14	29.14	4	0.49	18.52
% Time in Quadrant	9.57	54.78	76.41	70.14	79.14	72.16	71.39	55.34	1.47	0.00	49.04	32.38	5	0.54	20.58
Day 3	0.00	0.00	0.00	52.90	0.00	31.42	59.49	41.42	55.69	46.19	28.71	25.89	6	0.32	17.99
% Time in Quadrant	0.00	0.00	0.00	58.78	0.00	34.91	66.10	46.02	61.88	51.32	31.90	28.77	7	0.35	19.99
Day 4	0.00	67.99	9.66	16.76	62.42	51.99	61.76	57.23	58.82	56.13	44.28	25.15	8	0.49	14.07
% Time in Quadrant	0.00	75.54	10.73	18.62	69.36	57.77	68.62	63.59	65.36	62.37	49.20	27.94	9	0.55	16.11
Day 5	59.29	64.37	0.00	41.94	62.37	59.03	62.99	54.53	53.62	65.57	52.37	19.67			
% Time in Quadrant	65.88	71.52	0.00	46.60	69.30	65.59	69.99	60.59	59.58	72.86	56.56	22.52			
Day 6	58.90	61.53	61.83	21.44	29.98	50.72	36.56	53.45	60.30	60.93	49.56	14.85			
% Time in Quadrant	65.44	68.37	68.70	23.82	33.31	56.36	40.62	59.39	67.00	67.70	53.67	16.86			
Day 7	0.00	61.28	52.48	64.69	57.89	45.58	35.48	57.84	47.05	30.84	45.31	19.32			
% Time in Quadrant	0.00	68.09	58.31	71.88	64.32	50.64	39.42	64.27	52.28	34.27	52.13	21.97			
Day 8	47.23	46.30	70.63	12.87	63.87	69.67	59.73	70.73	65.67	16.74	52.34	21.67			
% Time in Quadrant	52.48	51.44	78.48	14.30	70.97	77.41	66.37	78.59	72.97	18.60	62.56	20.85			
Day 9	61.37	44.21	17.07	52.24	64.54	16.85	53.37	58.31	61.82	42.51	47.23	17.52			
% Time in Quadrant	68.19	49.12	18.97	58.04	71.71	18.72	59.30	64.79	68.69	47.23	53.06	20.55			

Table 8; Experimental Data for 2% Regenerating Planaria

PLMV Testing (Lines Crossed)														
Worm	2-W-02-A	2-W-02-B	2-W-02-C	2-W-02-D	2-W-02-E	2-W-02-F	2-W-02-G	2-W-02-H	2-W-02-I	2-W-02-J	AVG	SD	N	95% CI
Day 0	56.00	147.00	124.00	38.00	69.00	60.00	46.00	152.00	89.00	10.00	79.10	47.84	10.00	34.22
Day 1	43.00	109.00	64.00	104.00	31.00	39.00	54.00	65.00	111.00	1.00	62.10	36.55	10.00	26.15
Day 2	72.00	57.00	104.00	29.00	86.00	45.00	83.00	60.00	96.00	1.00	63.30	31.88	10.00	22.81
Day 3	67.00	33.00	17.00	24.00	22.00	52.00	80.00	97.00	77.00	0.00	46.90	32.27	10.00	23.09
Day 4	63.00	60.00	32.00	86.00	58.00	30.00	72.00	81.00	70.00	2.00	55.40	26.27	10.00	18.79
Day 5	64.00	74.00	59.00	62.00	77.00	42.00	60.00	68.00	53.00	28.00	65.22	10.62	10.00	7.60
Day 6	58.00	98.00	61.00	107.00	41.00	5.00	2.00	4.00	8.00	35.00	46.56	41.15	10.00	29.43
Day 7	41.00	85.00	33.00	96.00	2.00	2.00	2.00	0.00	51.00	22.00	37.11	37.09	10.00	26.54
Day 8	67.00	80.00	79.00	100.00	4.00	10.00	70.00	13.00	27.00	28.00	53.11	36.30	9.00	27.91
Day 9	88.00	76.00	72.00	66.00	30.00	42.00	51.00	15.00	43.00	65.00	54.80	23.66	9.00	18.19

Light Avoidance Testing															
Worm	2-W-02-A	2-W-02-B	2-W-02-C	2-W-02-D	2-W-02-E	2-W-02-F	2-W-02-G	2-W-02-H	2-W-02-I	2-W-02-J	AVG	Std. Dev	Day	%	95% CI
Day 0	0.00	0.00	0.00	0.00	15.75	0.00	36.50	23.09	13.99	0.00	8.93	12.96	0	9.93	9.27
% Time in Quadrant	0.00	0.00	0.00	0.00	17.50	0.00	40.56	25.66	15.54	0.00			1	4.08	8.87
Day 1	0.00	0.00	1.32	0.00	35.43	0.00	0.00	0.00	0.00	0.00	3.68	11.17	2	0.00	0.00
% Time in Quadrant	0.00	0.00	1.47	0.00	39.37	0.00	0.00	0.00	0.00	0.00	4.08	12.41	3	5.10	9.21
Day 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4	19.37	18.69
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5	7.69	11.86
Day 3	0.00	0.00	9.42	0.00	0.00	0.00	36.47	0.00	0.00	0.00	4.59	11.59	6	0.00	0.00
% Time in Quadrant	0.00	0.00	10.47	0.00	0.00	0.00	40.52	0.00	0.00	0.00	5.10	12.87	7	0.00	0.00
Day 4	33.73	0.00	0.00	0.00	0.00	0.00	52.26	55.27	33.06	0.00	17.43	23.52	8	0.00	0.00
% Time in Quadrant	37.48	0.00	0.00	0.00	0.00	0.00	58.07	61.41	36.73	0.00	19.37	26.13	9	0.00	0.00
Day 5	0.00	0.00	0.00	0.00	3.84	0.00	0.00	45.57	19.80	0.00	6.92	14.92			
% Time in Quadrant	0.00	0.00	0.00	0.00	4.27	0.00	0.00	50.63	22.00	0.00	7.69	16.58			
Day 6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 8	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 9	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			

Table 9; Experimental Data for Control Withdrawal Planaria

PLMV Testing (Lines Crossed)											Tail				Whole			
Worm	1-T-C-A	1-T-C-B	1-T-C-C	1-T-C-D	1-T-C-E	1-W-C-A	1-W-C-B	1-W-C-C	1-W-C-D	1-W-C-E	AVG	Std.Dev	n	95% CI	avg	stdev	n	95% CI
Day 0	97.00	76.00	101.00	40.00	88.00	91.00	122.00	98.00	73.00	59.00	80.40	24.54	5.00	30.47	88.60	24.13	5.00	29.96
Day 1	106.00	94.00	92.00	84.00	93.00	99.00	105.00	110.00	92.00	78.00	95.30	9.97	5.00	12.38	96.80	12.48	5.00	15.49
Day 2	89.00	105.00	94.00	102.00	101.00	84.00	96.00	79.00	104.00	84.00	93.80	9.35	5.00	11.62	89.40	10.29	5.00	12.77

Light Avoidance Testing											tail		whole			Tail		Whole	
Worm	1-T-C-A	1-T-C-B	1-T-C-C	1-T-C-D	1-T-C-E	1-W-C-A	1-W-C-B	1-W-C-C	1-W-C-D	1-W-C-E	AVG	Std. Dev	avg	stdev	Day	%	95% CI	%	95% CI
Day 0	49.11	0.00	70.57	0.00	39.29	64.47	51.42	49.13	76.52	0.00	31.79	31.15	48.31	29.16	0	35.33	38.68	53.68	36.21
%Time in Quadrant	54.57	0.00	78.41	0.00	43.66	71.63	57.13	54.59	85.02	0.00	35.33	34.61	53.68	32.40	1	45.20	37.59	56.31	39.54
Day 1	37.27	24.77	67.19	0.00	24.19	70.10	64.23	59.52	59.53	0.00	40.68	27.24	50.68	28.66	2	46.71	24.17	49.99	29.57
% Time in Quadrant	41.41	27.52	74.66	0.00	26.88	77.89	71.37	66.13	66.14	0.00	45.20	30.27	56.31	31.84					
Day 2	55.15	24.83	50.75	41.04	23.69	70.89	26.48	47.88	59.38	20.33	42.04	17.52	44.99	21.43					
% Time in Quadrant	61.28	27.59	56.39	45.60	26.32	78.77	29.42	53.20	65.98	22.59	46.71	19.47	49.99	23.81					

Table 10; Experimental Data for 1% Withdrawal Planaria

PLMV Testing (Lines Crossed)											Tail				Whole			
Worm	1-T-01-A	1-T-01-B	1-T-01-C	1-T-01-D	1-T-01-E	1-W-01-A	1-W-01-B	1-W-01-C	1-W-01-D	1-W-01-E	AVG	Std. Dev	n	95% CI	avg	sdev	n	95% CI
Day 0	74.00	98.00	124.00	102.00	84.00	127.00	110.00	136.00	16.00	104.00	96.40	19.05	5.00	23.65	98.60	47.92	5.00	59.51
Day 1	82.00	65.00	115.00	107.00	95.00	104.00	99.00	121.00	71.00	91.00	92.80	19.93	5.00	24.75	97.20	18.31	5.00	22.73
Day 2	98.00	75.00	104.00	94.00	70.00	121.00	78.00	127.00	70.00	84.00	91.43	18.22	5.00	22.62	96.00	26.12	5.00	32.44

Light Avoidance Testing											tail		whole			Tail		Whole	
Worm	1-T-01-A	1-T-01-B	1-T-01-C	1-T-01-D	1-T-01-E	1-W-01-A	1-W-01-B	1-W-01-C	1-W-01-D	1-W-01-E	AVG	Std. Dev	avg	stdev	Day	%	95% CI	%	95% CI
Day 0	58.45	30.07	72.26	59.99	58.05	62.64	62.28	26.28	0.00	28.34	55.76	15.51	35.91	26.69	0	61.96	19.26	39.90	33.14
% Time in Quadrant	64.94	33.41	80.29	66.66	64.50	69.60	69.20	29.20	0.00	31.49	61.96	17.24	39.90	29.66	1	50.55	32.47	47.92	34.38
Day 1	53.70	53.19	7.02	74.45	50.94	53.99	53.43	45.21	0.00	63.01	45.49	23.54	43.13	24.92	2	66.11	13.39	66.79	16.87
% Time in Quadrant	59.67	59.10	7.80	82.72	56.60	59.99	59.37	50.23	0.00	70.01	50.55	26.15	47.92	27.69					
Day 2	67.44	61.99	53.19	48.39	63.47	65.76	43.10	65.37	52.43	73.89	59.50	9.71	60.11	12.22					
% Time in Quadrant	74.93	68.88	59.10	53.77	70.52	73.07	47.89	72.63	58.26	82.10	66.11	10.78	66.79	13.58					

Table 11; Experimental Data for 2% Withdrawal Planaria

PLMV Testing											Tail				Whole			
Worm	3-T-02-A	3-T-02-B	3-T-02-C	3-T-02-D	3-T-02-E	3-W-02-A	3-W-02-B	3-W-02-C	3-W-02-D	3-W-02-E	AVG	Std. Dev	n	95% CI	avg	sdev	n	95% CI
Day 0	55.00	49.00	89.00	66.00	57.00	124.00	57.00	95.00	65.00	93.00	63.20	15.66	5.00	19.44	86.80	26.71	5.00	33.16
Day 1	58.00	73.00	65.00	68.00	62.00	111.00	90.00	117.00	66.00	110.00	65.20	5.72	5.00	7.10	98.80	20.97	5.00	26.04
Day 2	75.00	60.00	71.00	74.00	55.00	102.00	74.00	121.00	74.00	104.00	67.00	8.97	5.00	11.14	95.00	20.54	5.00	25.51

Light Avoidance Testing											tail		whole			Tail		Whole	
Worm	3-T-02-A	3-T-02-B	3-T-02-C	3-T-02-D	3-T-02-E	3-W-02-A	3-W-02-B	3-W-02-C	3-W-02-D	3-W-02-E	AVG	Std. Dev	avg	stdev	Day	%	95% CI	%	95% CI
Day 0	2.69	31.42	51.77	7.29	0.00	67.13	69.50	53.89	52.61	38.15	18.63	22.31	56.26	12.65	0	20.70	27.71	62.51	15.71
% Time in Quadrant	2.99	34.91	57.52	8.10	0.00	74.59	77.22	59.88	58.46	42.39	20.70	24.79	62.51	14.06	1	57.20	12.98	64.70	10.18
Day 1	48.65	38.83	39.55	45.09	51.51	61.17	55.42	63.51	46.61	64.43	51.48	9.41	58.23	7.38	2	37.48	25.28	43.09	19.12
% Time in Quadrant	54.06	43.14	43.94	50.10	57.23	67.97	61.58	70.57	51.79	71.59	57.20	10.45	64.70	8.20					
Day 2	20.47	62.82	1.43	32.64	26.03	29.34	57.08	24.60	33.43	49.47	33.73	18.32	38.78	13.86					
% Time in Quadrant	22.74	69.80	1.59	36.27	28.92	32.60	63.42	27.33	37.14	54.97	37.48	20.36	43.09	15.40					

Table 12; Experimental Data for Control Immediate Targeted Ethanol Exposure Testing

Worm	4-T-C-A	4-T-C-B	4-T-C-C	4-T-C-D	4-T-C-E	4-T-C-F	4-T-C-G	4-T-C-H	4-T-C-I	4-T-C-J	AVG	Std.Dev	n	95% CI
Day 0	0	0	0	0	0	0	0	0	0	0	0	0	10	0.00
Day 1	0	1	0	0	0	0	0	0	0	0	0.1	0.32	10	0.23
Day 2	0	1	0	0	0	0	0	0	0	0	0.1	0.32	10	0.23
Day 3	0	0	0	0	11	20	0	0	15	0	4.6	7.71	10	5.51
Day 4	2	0	0	8	4	1	0	0	1	1	1.7	2.54	10	1.82
Day 5	27	0	0	0	59	41	0	0	42	51	22	24.53	10	17.55
Day 6	57	48	4	65	20	78	59	41	73	53	49.8	23.04	10	16.48
Day 7	57	68	48	67	63	84	63	54	82	70	65.6	11.38	10	8.14
Day 8	13	70	64	91	66	85	43	66	75	67	64	22.08	10	15.79

Light Avoidance Testing															
Worm	4-T-C-A	4-T-C-B	4-T-C-C	4-T-C-D	4-T-C-E	4-T-C-F	4-T-C-G	4-T-C-H	4-T-C-I	4-T-C-J	AVG	Std. Dev	Day	%	95% CI
Day 0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	1	0	0
Day 1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
Day 2	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0
Day 3	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0	7	28.19	14.11
Day 4	0	0	0	0	0	0	0	0	0	0	0	0	8	29.67	13.37
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0			
Day 5	0	0	0	0	0	0	0	0	0	0	0	0			
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0			
Day 6	0	0	0	0	0	0	0	0	0	0	0	0			
% Time in Quadrant	0	0	0	0	0	0	0	0	0	0	0	0			
Day 7	48.80	0.00	28.80	10.10	17.20	23.90	29.00	55.00	6.50	34.40	25.37	17.75			
% Time in Quadrant	54.22	0.00	32.00	11.22	19.11	26.56	32.22	61.11	7.22	38.22	28.19	19.72			
Day 8	0.00	0.00	15.70	39.10	35.90	37.80	26.30	27.10	50.80	34.30	26.70	16.82			
% Time in Quadrant	0.00	0.00	17.44	43.44	39.89	42.00	29.22	30.11	56.44	38.11	29.67	18.69			

Table 13; Experimental Data for Shock Immediate Targeted Ethanol Testing

PLMV Testing (Lines Crossed)														
Worm	4-T-S-A	4-T-S-B	4-T-S-C	4-T-S-D	4-T-S-E	4-T-S-F	4-T-S-G	4-T-S-H	4-T-S-I	4-T-S-J	AVG	Std. Dev	n	95% CI
Day 0	0	0	0	0	0	0	0	0	0	0	0	0	10	0.00
Day 1	0	0	0	0	0	0	0	0	0	0	0	0	10	0.00
Day 2	0	0	0	0	0	0	0	0	0	0	0	0	10	0.00
Day 3	0	2	1	0	0	2	1	0	0	0	0.60	0.84	10	0.60
Day 4	6	0	0	5	0	0	1	0	3	0	1.50	2.32	10	1.66
Day 5	46	37	0	31	35	8	46	29	43	25	30.00	15.51	10	11.10
Day 6	76	64	67	50	50	73	45	0	54	52	53.10	21.44	10	15.34
Day 7	60	68	47	56	65	96	60	67	63	70	65.20	12.73	10	9.10
Day 8	63	66	53	69	69	86	57	56	62	65	64.60	9.28	10	6.64

Light Avoidance Testing															
Worm	4-T-S-A	4-T-S-B	4-T-S-C	4-T-S-D	4-T-S-E	4-T-S-F	4-T-S-G	4-T-S-H	4-T-S-I	4-T-S-J	AVG	Std. Dev	Day	%	95% CI
Day 0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0	0
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1	0	0
Day 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2	0	0
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3	0	0
Day 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4	0	0
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5	0	0
Day 3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	6	0	0
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7	27.30	14.54
Day 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8	22.22	9.64
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
% Time in Quadrant	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 7	5.80	31.40	9.20	50.60	30.00	26.80	7.30	0.00	50.50	34.10	24.57	18.29			
% Time in Quadrant	6.44	34.89	10.22	56.22	33.33	29.78	8.11	0.00	56.11	37.89	27.30	20.33			
Day 8	43.30	8.20	17.20	32.10	12.10	8.50	14.60	27.40	28.40	8.20	20.00	12.13			
% Time in Quadrant	48.11	9.11	19.11	35.67	13.44	9.44	16.22	30.44	31.56	9.11	22.22	13.48			

Table 14; Experimental Data for Control Day 0-2 Targeted Testing

PLMV Testing (Lines Crossed)														
Worm	5-T-C-A	5-T-C-B	5-T-C-C	5-T-C-D	5-T-C-E	5-T-C-F	5-T-C-G	5-T-C-H	5-T-C-I	5-T-C-J	AVG	Std.Dev	n	95% CI
Day 0	0	8	0	0	1	0	1	0	0	0	1	2.49	10	1.78
Day 1	1	0	3	0	1	0	0	0	25	0	3	7.79	10	5.57
Day 2	0	0	0	0	0	0	0	0	0	0	0	0.00	10	0.00
Day 3	0	0	0	0	0	0	0	0	0	0	0	0.00	10	0.00
Day 4	9	10	14	12	5	9	15	0	24	1	9.9	7.06	10	5.05
Day 5	9	16	36	11	21	11	14	14	7	20	15.9	8.36	10	5.98
Day 6	9	33	48	33	38	13	25	1	69	0	26.9	21.93	10	15.69
Day 7	47	52	67	66	45	57	47	32	58	31	50.2	12.43	10	8.89
Day 8	63	64	70	49	69	56	72	58	61	55	61.7	7.36	10	5.27

Light Avoidance Testing															
Worm	5-T-C-A	5-T-C-B	5-T-C-C	5-T-C-D	5-T-C-E	5-T-C-F	5-T-C-G	5-T-C-H	5-T-C-I	5-T-C-J	AVG	Std. Dev	Day	Q4 %	95% CI
Day 0	0	0	0	0	0	0	0	0	0	0	0.00	0.00	0	0.00	0.00
% Time in Quadrant 4	0	0	0	0	0	0	0	0	0	0	0	0	1	0.00	0.00
Day 1	0	0	0	0	0	0	0	0	0	0	0.00	0.00	2	0.00	0.00
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3	0.00	0.00
Day 2	0	0	0	0	0	0	0	0	0	0	0.00	0.00	4	0.00	0.00
% Time in Quadrant 4	0	0	0	0	0	0	0	0	0	0	0.00	0.00	5	0.00	0.00
Day 3	0	0	0	0	0	0	0	0	0	0	0.00	0.00	6	13.00	15.45
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7	21.44	13.97
Day 4	0	0	0	0	0	0	0	0	0	0	0.00	0.00	8	50.12	10.19
% Time in Quadrant 4	0	0	0	0	0	0	0	0	0	0	0.00	0.00			
Day 5	0	0	0	0	0	0	0	0	0	0	0.00	0.00			
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 6	0	0	56	0	36	9	16	0	0	0	11.70	19.44			
% Time in Quadrant 4	0.00	0.00	62.22	0.00	40.00	10.00	17.78	0.00	0.00	0.00	13.00	21.60			
Day 7	29	11	53	12	28	0	0	0	28	32	19.30	17.58			
% Time in Quadrant 4	32.22	12.22	58.89	13.33	31.11	0.00	0.00	0.00	31.11	35.56	21.44	19.54			
Day 8	37.3	39.7	51.4	51.7	18.5	47.6	64.5	46.4	36.8	57.2	45.11	12.83			
% Time in Quadrant 4	41.44	44.11	57.11	57.44	20.56	52.89	71.67	51.56	40.89	63.56	50.12	14.25			

Table 15; Experimental Data for Treated 0-2 Targeted Testing

PLMV Testing														
Worm	5-T-S-A	5-T-S-B	5-T-S-C	5-T-S-D	5-T-S-E	5-T-S-F	5-T-S-G	5-T-S-H	5-T-S-I	5-T-S-J	AVG	Std. Dev	n	95% CI
Day 0	6	8	0	6	0	2	4	0	1	0	2.7	3.06	10	2.19
Day 1	0	0	0	0	0	0	0	0	0	0	0	0.00	10	0.00
Day 2	0	0	0	0	0	2	0	0	0	0	0.2	0.63	10	0.45
Day 3	0	0	0	0	0	0	0	0	0	1	0.1	0.32	10	0.23
Day 4	0	0	1	0	3	0	0	3	2	2	1.1	1.29	10	0.92
Day 5	7	1	34	0	27	3	18	19	4	2	11.5	12.14	10	8.68
Day 6	10	15	27	23	40	4	26	0	41	38	22.4	14.84	10	10.62
Day 7	42	57	44	56	64	31	77	49	63	64	54.7	13.40	10	9.59
Day 8	50	52	71	56	82	66	78	50	65	71	64.1	11.66	10	8.34

Light Avoidance Testing															
Worm	5-T-S-A	5-T-S-B	5-T-S-C	5-T-S-D	5-T-S-E	5-T-S-F	5-T-S-G	5-T-S-H	5-T-S-I	5-T-S-J	AVG	Std. Dev	Day	Q4 %	95% CI
Day 0	0	0	0	0	0	0	0	0	0	0	0.00	0.00	0	0.00	0.00
% Time in Quadrant 4	0	0	0	0	0	0	0	0	0	0	0	0	1	0.00	0.00
Day 1	0	0	0	0	0	0	0	0	0	0	0.00	0.00	2	0.00	0.00
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3	0.00	0.00
Day 2	0	0	0	0	0	0	0	0	0	0	0.00	0.00	4	0.00	0.00
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5	0.00	0.00
Day 3	0	0	0	0	0	0	0	0	0	0	0.00	0.00	6	9.67	12.71
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7	34.22	14.45
Day 4	0	0	0	0	0	0	0	0	0	0	0.00	0.00	8	34.24	15.88
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 5	0	0	0	0	0	0	0	0	0	0	0.00	0.00			
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 6	0	0	0	0	0	0	36	41	9	1	8.70	15.99			
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	40.00	45.56	10.00	1.11	9.67	17.77			
Day 7	0	38	36	0	36	26	50	53	40	29	30.80	18.18			
% Time in Quadrant 4	0.00	42.22	40.00	0.00	40.00	28.89	55.56	58.89	44.44	32.22	34.22	20.20			
Day 8	0	32.7	50	38.2	57	20	51.1	0	24.6	34.6	30.82	19.98			
% Time in Quadrant 4	0.00	36.33	55.56	42.44	63.33	22.22	56.78	0.00	27.33	38.44	34.24	22.20			

Table 16; Experimental Data for Control Day 3-5 Targeted Testing

PLMV Testing (Lines Crossed)														
Worm	6-T-C-A	6-T-C-B	6-T-C-C	6-T-C-D	6-T-C-E	6-T-C-F	6-T-C-G	6-T-C-H	6-T-C-I	6-T-C-J	AVG	Std.Dev	n	95% CI
Day 0	0	0	0	0	0	0	1	0	0	0	0.1	0.32	10	0.23
Day 1	0	0	0	0	0	1	0	0	0	0	0.1	0.32	10	0.23
Day 2	4	0	0	0	0	0	2	0	3	0	0.9	1.52	10	1.09
Day 3	1	2	8	5	10	0	0	0	0	0	2.6	3.75	10	2.68
Day 4	7	4	17	6	11	17	14	6	6	34	12.2	9.04	10	6.47
Day 5	35	4	40	38	21	47	29	26	16	57	31.3	15.49	10	11.08
Day 6	56	62	79	42	68	66	54	74	48	41	59	13.05	10	9.33
Day 7	83	51	69	69	74	76	60	35	59	70	64.6	13.90	10	9.94
Day 8	54	51	54	71	79	62	73	67	68	71	65	9.38	10	6.71

Light Avoidance Testing															
Worm	6-T-C-A	6-T-C-B	6-T-C-C	6-T-C-D	6-T-C-E	6-T-C-F	6-T-C-G	6-T-C-H	6-T-C-I	6-T-C-J	AVG	Std. Dev	Day	Q4 %	95% CI
Day 0	0	0	0	0	0	0	0	0	0	0	0.00	0.00	0	0.00	0.00
% Time in Quadrant 4	0	0	0	0	0	0	0	0	0	0	0	0	1	0.00	0.00
Day 1	0	0	0	0	0	0	0	0	0	0	0.00	0.00	2	0.00	0.00
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3	0.00	0.00
Day 2	0	0	0	0	0	0	0	0	0	0	0.00	0.00	4	0.00	0.00
% Time in Quadrant 4	0	0	0	0	0	0	0	0	0	0	0.00	0.00	5	21.49	13.82
Day 3	0	0	0	0	0	0	0	0	0	0	0.00	0.00	6	22.66	15.09
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7	13.44	16.27
Day 4	0	0	0	0	0	0	0	0	0	0	0.00	0.00			
% Time in Quadrant 4	0	0	0	0	0	0	0	0	0	0	0.00	0.00			
Day 5	0	0	0	15	29.5	13	15.4	45.3	31.2	44	19.34	17.39			
% Time in Quadrant 4	0.00	0.00	0.00	16.67	32.78	14.44	17.11	50.33	34.67	48.89	21.49	19.33			
Day 6	0	1.13	0	33.32	51.28	24.06	40.3	28.81	25.07	0	20.40	18.98			
% Time in Quadrant 4	0.00	1.26	0.00	37.02	56.98	26.73	44.78	32.01	27.86	0.00	22.66	21.09			
Day 7	0	46.6	25	0	49.4	0	0	0	0	0	12.10	20.47			
% Time in Quadrant 4	0.00	51.78	27.78	0.00	54.89	0.00	0.00	0.00	0.00	0.00	13.44	22.75			
Day 8	0	48.1	0	4	35	0	0	0	0	0	8.71	17.63			
% Time in Quadrant 4	0.00	53.44	0.00	4.44	38.89	0.00	0.00	0.00	0.00	0.00	9.68	19.58			

Table 17; Experimental Data for Treated Day 3-5 Targeted Testing

PLMV Testing (Lines Crossed)														
Worm	6-T-S-A	6-T-S-B	6-T-S-C	6-T-S-D	6-T-S-E	6-T-S-F	6-T-S-G	6-T-S-H	6-T-S-I	6-T-S-J	AVG	Std. Dev	n	95% CI
Day 0	3	6	0	0	0	6	0	0	0	0	1.5	2.55	10	1.82
Day 1	0	0	1	0	0	0	0	0	0	0	0.1	0.32	10	0.23
Day 2	0	6	0	0	0	0	0	0	0	0	0.6	1.90	10	1.36
Day 3	2	6	0	0	7	5	30	10	7	0	6.7	8.91	10	6.37
Day 4	3	5	1	1	4	1	3	2	3	0	2.3	1.57	10	1.12
Day 5	41	0	14	16	3	23	0	5	8	0	11	13.12	10	9.39
Day 6	40	2	20	54	61	59	68	88	64	35	49.1	25.17	10	18.01
Day 7	54	33	64	68	49	12	103	90	66	46	58.5	26.31	10	18.82
Day 8	62	20	17	52	65	70	46	100	68	65	56.5	24.51	10	17.53

Light Avoidance Testing															
Worm	6-T-S-A	6-T-S-B	6-T-S-C	6-T-S-D	6-T-S-E	6-T-S-F	6-T-S-G	6-T-S-H	6-T-S-I	6-T-S-J	AVG	Std. Dev	Day	Q4 %	95% CI
Day 0	0	0	0	0	0	0	0	0	0	0	0.00	0.00	0	0.00	0.00
% Time in Quadrant 4	0	0	0	0	0	0	0	0	0	0	0	0	1	0.00	0.00
Day 1	0	0	0	0	0	0	0	0	0	0	0.00	0.00	2	0.00	0.00
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3	0.00	0.00
Day 2	0	0	0	0	0	0	0	0	0	0	0.00	0.00	4	0.00	0.00
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5	0.00	0.00
Day 3	0	0	0	0	0	0	0	0	0	0	0.00	0.00	6	19.57	16.46
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7	24.68	15.41
Day 4	0	0	0	0	0	0	0	0	0	0	0.00	0.00	8	30.12	16.75
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 5	0	0	0	0	0	0	0	0	0	0	0.00	0.00			
% Time in Quadrant 4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			
Day 6	0	0	0	0	29.44	37.5	0	57	19.88	32.32	17.61	20.71			
% Time in Quadrant 4	0.00	0.00	0.00	0.00	32.71	41.67	0.00	63.33	22.09	35.91	19.57	23.01			
Day 7	42.6	0	5	21	41.1	52.5	0	26	30	3.9	22.21	19.39			
% Time in Quadrant 4	47.33	0.00	5.56	23.33	45.67	58.33	0.00	28.89	33.33	4.33	24.68	21.55			
Day 8	6.5	31.6	0	57.6	0	46.7	12.5	44.8	28.9	42.5	27.11	21.07			
% Time in Quadrant 4	7.22	35.11	0.00	64.00	0.00	51.89	13.89	49.78	32.11	47.22	30.12	23.41			

Appendix B

Whole Mount In-Situ Protocol with Explanation

Below is a detailed WISH protocol for future work with detailing gene expression in planaria. The experimental step is numbered and the explanation for each step is italicized afterwards. Experimental steps are separated by days of experimentation.

Day 1 – kill, remove the mucus, fix, reduce/permeabilize, dehydrate, bleach)

1) Worms are starved for one week and are of a length between 2 and 6 mm. *The worms are starved so that no undigested food is in their system when the staining begins.*

2) Reduction Solution is prepared and placed in the incubated shaker to allow it to heat to 37^{o}C. *Any more than 5-10 worms, and there starts to be too many in the tube to work well without fracturing a few of them.*

3) 5-10 planaria are placed into a seal-locked 1.5 mL Eppendorf tube with water. *The worms are killed with the 5% NAC solution because the NAC will remove the mucus layer that is secreted around the worms' outer body. The mucus acts as a barrier against the exchange of fluids and large molecules needed during whole-mount in situ hybridization.*

4) Planaria water is replaced with 5% NAC using a micropipettor for 5-10 minutes at room temperature. *A formaldehyde fixative is used because it promotes cross linking, which in turn allows higher cellular resolution.*

5) NAC solution is removed from the Eppendorf tubes using a micropipettor.

6) Planaria are transferred to a 10 cm Petri dish with 4% fixative. Petri dishes are placed on the nutator for 15-20 minutes at room temperature. *A formaldehyde fixative is used because it promotes cross linking, which in turn allows higher cellular resolution.*

7) Petri dishes are returned to the RNAase-free zone and the planaria are placed back into their 1.5 mL vials from the 10 cm Petri dishes using transfer pipettes.

8) Worms are rinsed 1X with PBSTx. *This step is meant to remove the fixative solution before the next chemical solution is added.*

9) Reduction solution is removed from the incubated shaker. PBSTx in tubes is replaced with Reduction solution using a micropipettor. Tubes are placed in incubated shaker for 5-10 minutes at 37^{o}C. *The reduction solution is meant to take the place of the more common SDS permeabilization step in a typical whole mount in situ hybridization (WISH) step. The SDS step is meant to increase staining intensity and specificity. With the standard SDS step, the pre-pharyngeal region is refractory. The reduction solution instead allowed staining in the impermeable pre-pharyngeal region.*

10) Tubes are removed from incubated shaker and reduction solution is removed using a micropipettor. Worms are rinsed 1X with PBSTx. *This step is meant to remove the Reduction solution from the specimen prior to dehydration.*

11) PBSTx is replaced with 50% methanol solution for 5-10 minutes at room temperature. *The methanol is meant to dehydrate the specimen to increase staining intensity. This is a common practice in WISH protocols.*

12) 50% methanol solution is replaced with 100% methanol solution for 5-10 minutes at room temperature. *This step is meant to continue the dehydration process.*

13) Tubes were then stored at -20 ^{o}C for at least one hour. At this point, specimens can be saved for several months.

14) Tubes are removed from -20 ^{o}C storage, and the 100% methanol solution is replaced with 6% bleach solution. Tubes are then placed under direct light from a lamp or the light source from the microscope overnight at room temperature. *This step removes pigments from the animal to help with visualization of the signal.*

Day 2 – Rehydrate, Proteinase K, post-fix, and hybridization

15) Start the incubated shaker and set the temperature to 80 ^{o}C.

16) Riboprobe mix is placed at 80 ^{o}C for 5 minutes in the incubated shaker. *The riboprobe mix needs to be denatured between 72-90 ^{o}C.*

17) Riboprobe mix and PreHyb are placed in incubated shaker at 56 ^{o}C.

18) 6% bleach solution from previous night is removed and specimens are rinsed 2X with 100% methanol. Specimens can then be stored at -20 ^{o}C or used immediately. *This removes the bleach and returns the specimen to their dehydrated state, which is the state they should be stored at. If the sample is to be stored long-term, it would be better to use ethanol, but this results in weaker overall staining.*

19) 100% methanol is replaced with 50% methanol using a micropipettor for 5-10 minutes at room temperature. *Continuation of the rehydration step back to PBSTx.*

20) 50% methanol solution is replaced with PBSTx using a micropipettor for 5-10 minutes at room temperature. *Continuation of the rehydration step back to PBSTx.*

21) PBSTx is replaced with Proteinase K solution for 10 minutes at room temperature. *The introduction of Proteinase K is meant to increase mRNA accessibility for hybridization. It accomplishes this by removing nucleases. It is also meant to increase staining intensity. Proteinase K works with this because we aren't using Carnoy's solution. If Proteinase K were used with a non cross linking fixative, it could disrupt associations between protein and RNA molecules, which would cause weaker staining.*

22) Planaria are transferred to a 10 cm Petri dish with 4% fixative for 10 minutes at room temperature. *When coupled with the Proteinase K solution, this post-fixation increase staining intensity as well.*

23) Worms are placed back into the appropriate 1.5 mL tubes from the 10 cm Petri dish. *The fixative is removed to prepare the specimen for hybridization.*

24) Specimens are rinsed 2X with PBSTx. *Rinses fixative solution from the planaria.*

25) Planaria are washed in 1:1 PBSTx:PreHyb for 10 minutes at room temperature. *Prepares the specimen for introduction of probes. The PreHyb, consisting of formamide, is an RNA stabilizing agent that works through deionizing RNA.*

26) 1:1 mix is replaced with Prehyb for 2 hours at 56 ^{o}C. *Further prepares the specimen for introduction of probes.*

27) Prehyb was replaced with Riboprobe mix using a micropipettor in the incubated shaker for at least 16 hours at 56 ^{o}C. *Introduction of riboprobes. The probes will attach to a target RNA sequence to produce the staining. The conditions selected are set to favor the binding of the probe to the target sequence.*

Day 3 – Washing and antibody incubation

28) Preheat 1:1 [Wash hyb: (2X SSC + 0.1% Triton-X)], 2X SSC + 0.1% Triton-X, and 0.2X SSC + 0.1% Triton-X solutions to 56 ^{o}C in the incubated shaker.

29) Remove riboprobe mix from tubes and store at -20 ^{o}C. Wash specimens with 1:1 [Wash hyb: (2X SSC + 0.1% Triton-X)] 2X for 30 minutes, 2X SSC + 0.1% Triton-X twice for 30 minutes, and 0.2X SSC + 0.1% Triton-X twice for 30 minutes. *These washes work to remove non-selectively and partially bound probe. The varied salt concentrations are used to accommodate different lengths of probe.*

30) Planaria were allowed to adjust to room temperature. Then, they were washed with MABT 2X for 10 minutes at room temperature. *Another wash used to remove non-selectively and partially bound probe.*

31) Place planaria in a blocking solution for 1-2 hours at room temperature (or overnight at 4 ^{o}C if time is an issue). *The blocking solution will bind to the probes and allow binding of antibody.*

32) Replace blocking solution with Antibody solution using a micropipettor for 4 hours at room temperature or overnight at 4 ^{o}C. *This floods the specimen with the antibody, and the antibody binds to wherever the block and probe combinations are.*

Day 4 – Antibody washes and development

33) Antibody solution is removed and specimens are washed with MABT times, 20 minutes per wash. The MABT is used to remove any excess antibody.

34) MABT is replaced with AP buffer (prepared fresh) using a micropipettor. This is allowed to sit out for 10 minutes at room temperature. *This is meant to flood the specimen with alkaline phosphate. PVA is used to increase AP activity and help stain weaker probes.*

35) AP buffer is replaced with development buffer using a micropipettor and lab lights are turned off to ensure planaria are in the dark. *The development buffer is what will stain the antibody. The NBT and BCIP are the staining agents of the development buffer.*

36) Rate and extent of development is monitored under the Olympus microscope and stopped (by replacing development buffer with PBSTx) once an optimal signal-to-background ratio is reached. *This step ensures that a clearly distinguishable and visible amount of staining has occurred, and it is obvious what is stained antibody and what is background staining of other tissues. Using PBSTx stops increasing the fluorescence of the labeled antibodies.*

37) Planaria are placed into 10 cm Petri dish with 4% fixative for 10 minutes at room temperature. *This step removes non-specific background staining.*

38) PBSTx and fixative are replaced with 100% ethanol solution for 20 minutes at room temperature. *A gradual step-down procedure to go from the 100% ethanol to the PBSTx again.*

39) 100% ethanol solution is replaced with 50% ethanol solution for 5 minutes at room temperature. *Continuation of the step-down procedure from 100% ethanol to PBSTx.*

40) 50% ethanol solution is replaced with PBSTx. *The completion of the transfer of the specimen from 100% ethanol to PBSTx.*

41) PBSTx is replaced with an 80% Glycerol solution and stored at 4 ^{o}C *Changes to Glycerol because the Glycerol will preserve the staining for long periods of time when stored properly.*

42) Planaria are transferred to a slide and mounted under a #1 weight coverslip, stored at 4^{o}C.