

EFFECTS OF GAP JUNCTION BLOCKAGE ON REGENERATING BODY
SEGMENTS OF *LUMBRICULUS VARIEGATUS*

By

Monica K. Richmond

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Committee Membership

Dr. Bruce O’Gara, Committee Chair

Dr. Ethan Gahtan, Committee Member

Dr. John Reiss, Committee Member

Dr. Amy Sprowles, Committee Member

Dr. John Steele, Committee Member

Dr. Paul Bourdeau, Program Graduate Coordinator

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ABSTRACT

EFFECTS OF GAP JUNCTION BLOCKAGE ON REGENERATING BODY SEGMENTS OF *LUMBRICULUS VARIEGATUS*

Monica Richmond

Lumbriculus variegatus is a freshwater annelid that is well known for its regenerative capacity. There are many different factors that affect regeneration in animals. Cell-to-cell communication is a key component of regeneration. Gap junctions are made up of proteins that create a channel that connects the cytoplasm of two cells. Many molecules and ions pass through the gap junctions that can affect regeneration. The purpose of this study was to determine if blocking gap junctions would have an impact on regenerating body segments of *L. variegatus* and what effects it would produce. *L. variegatus* was exposed to a known gap junction blocker, octanol, for differing time periods following transection, then measured and photographed for six days. Worms treated with octanol experienced regenerative growth that was notably slower and also resulted in abnormal morphology of the regenerated tail. Octanol is known to leave the system immediately after treatment is taken away; however, *L. variegatus* did not show immediate recovery of growth when placed back into pond water. It is not clear whether there is a critical time period for exposure to octanol that affects regeneration; however, the duration of the exposure corresponded to the severity of abnormal morphology. The observations of the outcome of gap junction blockage on

regenerative growth will contribute to the on-going research in areas of biology related to regeneration and gap junction communication.

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INTRODUCTION

Lumbriculus variegatus is a freshwater oligochaete annelid that is often studied for its regenerative properties and behavioral reflexes. *L. variegatus* contains neoblasts, which Randolph first described in 1892. The neoblasts occur in every somite in the body and are distinguished by their large size and shape. They are granular with deeply staining nucleoli. These neoblasts contribute to the worm's regenerative capacity (Randolph, 1892).

In general, annelid worms are known to have regenerative qualities, although not all annelids are capable of regenerating lost body parts (Zoran, 2010). Webster's Dictionary (Merriam-Webster.com) defines regeneration as "the renewal, regrowth or restoration of a body or bodily part, tissue or substance after injury or as a normal bodily process". There are two specific types of regeneration in annelids, morphallaxis and epimorphosis (Gilbert, 2014). Morphallaxis is the remodeling of existing tissues and re-establishment of boundaries with very little new growth. Epimorphosis is the dedifferentiation of adult structures to form an undifferentiated mass of cells (a blastema), which then redifferentiate to form a new structure.

Lumbriculus variegatus is well known for its regenerative capacity (Adams et al., 2006). Following amputation, some tissues in the anterior region can be formed without cell migration and that regeneration occurs by dedifferentiation, proliferation and redifferentiation. Unlike head regeneration, the regeneration of the tail relies on migration

of cells to the cut site (Tweeten and Anderson, 2008). The head will always regenerate 7-8 segments while the number of tail segments regenerated is variable (Drewes, 2004).

L. variegatus has a natural autotomy reflex that is initiated when experiencing mechanical stress (Lesiuk and Drewes, 1999). Any segment in the mid-body or tail region is capable of being an autotomy site. This is an all-or-nothing response and the body is quickly separated into two fragments with a rapid closure of the body wall with no blood loss. Within 10 minutes after autotomy, the ends of both the anterior and posterior surface appear rounded and sealed. Regeneration becomes visible within hours of autotomy. The somatic cells aren't the only ones that are capable of fast recovery from amputation. The ventral nerve cord is able to quickly recover its functionality following damage. Transection studies of the ventral nerve cord show that *L. variegatus* is able to again produce the swimming and reversal body escape movements across the transection site in only eight hours post transection (Lesiuk and Drewes, 2001). Following complete ablation of 5-8 segments of the ventral nerve cord, *L. variegatus* is able to fully recover normal behavioral and electrical conduction of action potentials in the giant fibers across the lesioned site by three days post ablation (Lesiuk and Drewes, 2001). In addition to regenerating the ventral nerve cord, studies show that new replacement body segments are fully regenerated with an intact sensory field and escape reflexes within days (Drewes and Fournier, 1990; Lybrand and Zoran, 2012).

L. variegatus has giant nerve fibers in its body called the medial giant fiber (MGF) and the lateral giant fibers (LGF). When the anterior end is touched in a normal worm, the MGF is activated, while posterior touch activates the LGFs. Activation of the

two sets of giant fibers, produce similar, but distinct, rapid shortening behaviors that are used to avoid predators. Regenerating worms undergo a reorganization of the giant fiber sensory fields after amputation of either the anterior end or the posterior end (Drewes and Fournier, 1990). When the anterior portion of the worm has been amputated, the new anterior end of worm that originally was part of the LGF sensory field, reorganizes to become part of the MGF sensory field. In other words, areas of the body wall that activated the LGFs prior to amputation, activate the MGF following amputation and reorganization. Depending on how much of the anterior end was amputated, the MGF was activated in as little as two days up to four days post-amputation.

There are many different factors that affect regeneration in animals, ranging from abiotic factors to various endogenous molecules and ions that flow in and out of cells. Low temperature and fasting have a negative effect on tail regeneration in the salamander, *Desmognathus conanti* (Marvin and Lewis, 2013). Hormones play a role in limb regeneration in the fiddler crab *Uca pugilator* (Hopkins, 2001). There are two phases of regenerative growth in *U. pugilator*: basal growth and proecdysial growth. There are always circulating arthropod molting and growth hormones, called ecdysteroids, in the body. After loss of a limb, basal growth occurs. The autonomy membrane seals the wound immediately. Fibroblast growth factors are known to play a role in repair and regeneration of tissues. Two days post autonomy, the fibroblast growth factor FGF2 homologue in invertebrates is released for early blastema development. Four days post autonomy FGF4 homologue and endogenous retinoic acid are present.

During this time, the ecdysone receptor level increases, but the circulating ecdysteroids remain low. During the proecdysial growth phase, the formed limb bud continues to grow in length. At this time there are multiple pulses of the circulating ecdysteroids which are timed with development. If the level of circulating ecdysteroids varies or the timing is off, regeneration fails to occur.

Neurotransmitters are another factor that can affect regeneration. For example, in planarians, dopamine and serotonin are present and speed regeneration (Villar and Schaeffer, 1993). In *Lumbricus terrestris*, serotonin levels fluctuate in different parts of the nervous system during regeneration (Csoknya et al., 1993). After removing the cerebral ganglion, during the first three days of regeneration, there is a reduction of serotonin levels in intact ganglia, with serotonin levels gradually increasing until there is more serotonin than in control worms. After day 17, the serotonin levels decrease again. Lastly, ion flow in and out of cells can affect regeneration. Bioelectrical mechanisms such as H^+ flux in and out of cells via the V-ATPase pump, is necessary for the *Xenopus laevis* tadpole tail to initiate regeneration and for normal neuronal patterning (Adams et al., 2007).

Bioelectrical signaling is a type of cell to cell communication that involves changes in the resting membrane potential across the plasma membrane due to changes in voltage gradients, ion flows and concentration gradients between cells and tissues (Levin, 2007; Adams and Levin, 2012). Bioelectricity is a component of many different developmental processes including stem cell differentiation, initiation of regeneration, anterior-posterior polarity, and left-right axis patterning. In addition to the example of

Xenopus laevis tadpole tail regeneration noted above, H^+ flux via the V-ATPase pump also is required for left right asymmetry in the chick and zebrafish (Adams et al., 2006). The membrane potential is involved in the regulation of cell proliferation, differentiation and migration during regeneration (Sundelacruz et al., 2009). In addition, the membrane potential plays a role in determining large scale polarity (Adams and Levin, 2012). H^+ , K^+ -ATPase activity is required to regenerate a head in planaria (Beane et al., 2011). Inhibition of H^+ , K^+ -ATPase blocks the anterior regeneration from posterior fragments of transected planaria.

Gap junctions help facilitate the bioelectric flow of ions between cells. In addition, gap junction connectivity is regulated by voltage gradients (Adams and Levin, 2013). Gap junctions are formed by a group of proteins that connect the cytoplasm of adjacent cells. They provide a regulated channel for communication between cells. Ions and small molecules can pass through gap junctions and they are a means of short and long range metabolic and electric communication between cells (Cooper and Hausman, 2013). Long range signaling of positional information during regeneration occurs in part, via gap junctions (Adams and Levin, 2013). Gap junction proteins are connexins and pannexins in vertebrates but in invertebrates they are innexins (Phelan, 2005; Oviedo and Levin, 2007). Nogi and Levin (2005) characterized innexin genes in the planarian *Dugesia japonica* to determine where they are expressed in the body and which genes played a role in anterior-posterior polarity during regeneration. Using the gap junction blocker, heptanol, they produced 2-headed worms during regeneration and found gap junction connections are used for signaling mechanisms that determine polarity. Later, it

was discovered that the gap junction signaling during anterior-posterior regenerative morphogenesis in planarians is mediated via three specific innexin gene products. The use of the gap junction blockers octanol and heptanol consistently alter the anterior-posterior polarity and produce multiple-headed worms (Oviedo et al., 2010).

In addition to regulating anterior-posterior polarity, gap junctions are also recognized as being a key regulator of left-right asymmetry. In *Caenorhabditis elegans*, intercellular calcium signaling, via gap junctions, coordinates the developing left-right asymmetric neuronal network (Chuang et al., 2007; Schumacher et al., 2012).

Gap junctions may also have an effect on cell proliferation. The gap junction protein smedinx-11 in the planarian *Schmidtea mediterranea* was identified to be specifically associated with neoblasts (Oviedo and Levin, 2007). A *smedinx-11* RNAi experiment resulted in reduced blastema formation in regenerating worms, and expression of the neoblast markers declined over time. Not only was there a decline in neoblast markers, but also a decline in the neoblast subpopulation. In the zebrafish short fin mutant, *sof*^{h123}, the *Cx-43* gap junction gene function is related to the number of dividing cells in regenerating fins (Hoptak-Solga et al., 2008). The number of dividing cells is reduced and *Cx-43* is expressed in mitotic cells.

The communication between cells via gap junctions relies on these channels to be open during critical time periods when specific molecules are needed. Oviedo et al. (2010) found that the most significant disruption of regeneration occurred when gap junction blockers were applied within 3-6 hours post-amputation. When treatment was first applied at least 12 hours post-amputation, disruptions in regeneration decreased

significantly. Emmons-Bell et al. (2015) treated amputated planarians with the gap junction blocker octanol for three days and then allowed the worms to regenerate in water for seven additional days, which resulted in worms with different species-specific head morphologies than control flatworms.

The primary objective of this study was to determine if the gap junction blocker, octanol, impeded regeneration following whole body transection of *L. variegatus*, and if there was a critical time period required for open communication through gap junctions for worms to have normal regeneration. As we will see, the data reveal that the gap junction blocker, octanol, did negatively affect the growth rate and morphology of transected worms during regeneration. While the immediate recovery of normal regeneration after placement in pond water was not observed following octanol exposure, the duration of exposure to octanol did appear to negatively affect regeneration in the long term. Worms that were taken out of the octanol solution and placed in pond water plateaued and even slightly declined in growth by the end of the six days.

METHODS

Animal culture

Lumbriculus variegatus were laboratory-reared in aquaria containing aerated artificial pond water with pieces of paper towels to act as a substrate. The asexually reproducing cultures were derived from animals originally purchased from Aquatic Foods (Fresno, CA). Worms were fed commercial fish food one to two times per week (Tetra Pond *Pond Sticks*). The soft artificial pond water had the following composition: 1 mM NaCl, 13 μ M KCl, 4 μ M Ca(NO₃) \cdot 4 H₂O, 17 μ M Mg(SO₄) \cdot 7 H₂O, 71 μ M HEPES buffer with a pH of 7.0 ± 0.05 . The aquarium was cleaned and water was changed once per week. No attempts were made to monitor or adjust the pH once the worms were placed in the water. Water used for the artificial pond water and all other solutions was produced by a Thermo Scientific Barnstead Epure system, using house RO water as the source water.

Treatment solution, concentrations and testing

Worms used in these experiments were randomly chosen and were 2-5 cm in length and lacking any obvious morphological defects as well as any obvious signs of current regeneration.

To create body fragments, the worms were cut with a stainless steel surgical blade. The blade was changed after cutting every six worms. The worms were cut into three equal parts, one third down from the head, and two thirds down from the head.

Only the middle third of the body was used for the experiments. (Figure 1). Immediately after transection, individual worm sections were transferred into solutions outlined below.



Figure 1: Illustration describing body fragments of worms cut into thirds. The worms were cut into three equal parts. Only the middle one third section of the body was used for the experiments. Worms have many more segments than in the illustration and segments are not to scale.

To determine the morphological effects of gap junction blockers on regenerating worms, middle portions of cut worms were placed individually in a solution of 0.01% octanol in 24 well dishes (octanol from Sigma Chemical Company, St. Louis, MO). One worm fragment was placed in each well with 2 mL of solution. The octanol concentration used in these experiments was determined during preliminary experiments based on its ability to create abnormal morphologies on regenerating worms while producing less than 15% mortality.

To determine if there was a critical time period for gap junction blockers to affect regeneration, the middle cut portion of worms were placed in the 0.01% octanol using the treatment schedules shown in Table 1. The middle cut portion of worms were placed in the 0.01% octanol solution in four time frames but maintaining a maximum exposure time of six days. Hexanol (Sigma Chemical Company, St. Louis, MO) is not a gap junction blocker (Chanson et al., 1989) and was used as an additional control treatment to test whether octanol's effect as a gap junction blocker affected regeneration. The 0.01%

hexanol worms were exposed for the entire six days. The control worms were placed in pond water for the duration of treatment. Pond water, octanol and hexanol solutions were replaced with fresh solution each day.

Table 1: Treatment protocols for transected fragments of worms. Immediately following transection (day zero), worms were placed in the following solutions. Group Octanol 0 (n = 40) were placed in 0.01% octanol for day zero followed by pond water for days one through six. Group Octanol 0-1 (n = 34) were placed in 0.01% octanol for day zero through day one followed by pond water for days two through six. Group Octanol 0-2 (n = 30) were placed in 0.01% octanol for day zero through day two followed by pond water for days three through six. Group Octanol 0-6 (n = 26) were placed in 0.01% octanol solution for days zero through six. Group Hexanol (n = 31) were placed in 0.01% hexanol solution for days zero through six. Group Control (n = 47) were placed in pond water for days zero through six.

Treatment	Day 0 Cut Day	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Octanol 0	Octanol						
Octanol 0-1	Octanol	Octanol					
Octanol 0-2	Octanol	Octanol	Octanol				
Octanol 0-6	Octanol	Octanol	Octanol	Octanol	Octanol	Octanol	Octanol
Hexanol	Hexanol						
Control	Pond water - Control						

Determination of abnormal morphological growth

The regenerating worms were viewed and photographed on an Olympus SZX16 microscope with an Olympus camera using *cellSens Standard* 1.6 software. Each day, all the worms were photographed and measured for overall growth of the head and tail. The middle portion of the worm that had regenerated head and tail regions were measured using the ‘measure’ tool on the *cellSens Standard* 1.6 software on post transection days

one through six. The measurement of regenerated tissue, for both heads and tails extended from the tip of the regenerated tissue to where pigmentation occurred (i.e., tissue present of the original worm) (Figure 2). Regenerating tissue on the worm is unpigmented. Head and tail regions appear similar during the early stages of regeneration, therefore, the anterior-posterior axis was determined by observing the pulsatile contraction of the dorsal blood vessel, which flows from the posterior end to the anterior.



Figure 2: Anterior and posterior measurements of regeneration started on the anterior or posterior tip and ended where pigmentation of the worm started (unregenerated tissue). Arrows indicate measured portion of regeneration. Image of typical control worm measured on day one. Length 1 indicates anterior end, Length 2 indicates posterior end. Regenerating tissue is unpigmented.

Growth was determined to be abnormal if the regenerated morphology was different from control worms. Head growth was considered abnormal if there was slow growth. Tail growth was considered abnormal if there were multiple prongs on the tail or if there was slow or no tail growth.

Cell proliferation assay

The effects of gap junction blockers on cell proliferation in *L. variegatus* was assessed using EdU staining in both heads and tails.

Selection of worms for EdU staining

Two worms each day from each treatment were randomly selected. Worms were then photographed and prepped for the EdU assay.

EdU staining protocol for assessment of cellular proliferation

EdU staining was performed by following the manufacturer's instructions for Click-iT® Plus EdU Imaging Kits (Life Technologies, Grand Island, NY) with the modifications described below (Alkhathlan, 2015). Regenerating worm body fragments were maintained individually in 24-well dishes. Two worms per regeneration day from each treatment were stained with a solution consisting of either 500 μ M EdU in pond water, 500 μ M EdU in 0.01% hexanol or 500 μ M EdU in 0.01% octanol. Each worm was exposed to EdU by adding 250 μ l of 500 μ M EdU solution to each well. Following a 1.5 hour exposure time, the EdU solution was removed and the worm in each well was fixed in 1 ml of 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) overnight at 4°C. Fixative was then removed from each well and the worms in each well were washed twice for 15 minutes with 1 ml of 3% bovine serum albumin (BSA) (Fisher

Scientific, Pittsburgh, PA) in phosphate-buffered saline (PBS) (Fisher Scientific, Pittsburgh, PA). The washing solution was removed and the worms in each well were permeabilized by the addition of 1 ml of 0.5 % Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS and incubated for 45 minutes at room temperature. The permeabilization buffer was removed from each well and the worms in each well were washed twice for 5 minutes with 3% BSA in PBS. Click-iT reaction cocktail (250 μ L) consisting of 1x Click-iT reaction buffer, copper protectant, AlexaFluor picolyl azide, and 1x reaction buffer additive was added to each well and incubated at room temperature for 1 hour while protected from light. After 1 hour, the Click-iT reaction cocktail was removed and each well was washed once for 5 minutes with 1 ml 3% BSA in PBS and washed once with 1 mL of PBS for 5 minutes. To visualize nuclei, 250 μ L of 1x Hoechst 33342 in PBS was added to each well and worms were incubated at room temperature for 1 hour protected from light. After 1 hour, each well was washed twice with PBS.

Determination of cell proliferation

Worms were imaged and analyzed using a fluorescence Zeiss microscope and AxionCam MRm camera. To determine cell proliferation, images of worms were analyzed by the Zen 2.3 pro 2012 Blue Edition Image Analysis software. An outline of the worm was traced to calculate area, on both the Alexa fluor images and the Hoechst images. The software analyzed the mean intensity of Alexa Fluor 594 (proliferating cells) and mean intensity of Hoechst 33342 (all neuclei) per area of the worm. To visualize

Alexa fluor 594 dye, the filters used were 590 nm excitation and 615 nm emission filters. To visualize Hoechst 33342, the filters used were 350 nm excitation and 461 nm emission filters. The mean intensity numbers from all the areas were added for the Alexa fluor and Hoechst for each treatment worm. Analysis was done in triplicate. The areas were also analyzed by excluding the outline of the worms in order to rule out autofluorescence of residual liquid pooling at perimeter of worms.

Statistical analysis of regeneration

Appropriate statistical tests were performed using R statistical software 2013. A two-way ANOVA was used to analyze all data, and Tukey's Pairwise comparison was used to determine significance differences between groups. The data was described as the mean \pm the SEM.

RESULTS

Regeneration of control worms

Following transection of the body, *Lumbriculus variegatus* normally regenerates 7-8 head segments (Drewes, 2004). The control worms in this study were able to regenerate 7-8 head segments in six days (Figure 3). Tail segments continue to be added for the duration of the observation period, producing indeterminate growth.

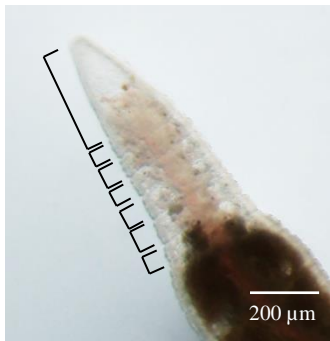


Figure 3: Untreated control worms regenerate 7 segments of head growth. Image of typical untreated control worm on day six following transection showing seven regenerated head segments. Each bracket indicates one segment of growth.

Treatment with 0.01% octanol had a significant negative effect on regeneration compared to untreated control worms.

To determine if regeneration was affected by gap junction function, a solution of 0.01% octanol, a known gap junction blocker, was administered to transected *L. variegatus* as described in Table 1. Worms were exposed to a total of four treatments of 0.01% octanol, one treatment of 0.01% hexanol, and one treatment of pond water.

Octanol produced abnormal morphology and reduced growth of *L. variegatus* regenerated body parts (Figs. 4-9), while hexanol treatments showed less difference from pond water controls.

To investigate if the time period of exposure to octanol affected regeneration, and whether worms recovered normal growth function following transfer back to pond water, the worms were exposed to octanol on different days. The worms were initially exposed to octanol immediately after transection and then taken out of octanol treatments on different days. The different treatments named indicate the different time periods worms were exposed to octanol. The octanol treatments are named for the days that worms were exposed to octanol. For example, Octanol 0-treated worms were exposed to 0.01% octanol only on the cut day (day zero) and placed in pond water on days one through six.

Results of that experiment show either abnormal morphology, regeneration less than 100 μm , regeneration more narrow than the body, or a combination of these morphologies occurred in 13% of the hexanol- worms, 28% of the Octanol 0-treated worms, 29% of the Octanol 0-1-treated worms, 30% of the Octanol 0-2-treated worms and 81% of the Octanol 0-6-treated worms. When considering growth slower than control worm growth as abnormal, then 100% of the octanol-treated worms had abnormal growth. There were no abnormal morphologies of the head, but head growth was considered abnormal if there was slow growth. However, tail growth was considered abnormal if there were multiple prongs on the tail or if there was slow or no tail growth. (Figures 4-9).

Worms in Figures 4-9 show typical growth patterns for that treatment group. They are representative of growth and morphology; however, not all worms in each treatment group displayed the exact morphologies shown. Control worms started regenerating a new head and tail on day one and continued growth through day six. On day four, there was clear segmentation of both head and tail. There was more head regeneration than tail regeneration on day one, but tail growth increased faster than head growth starting on day two and continuing through day six. By day six, head growth of 7-8 segments was complete (Figure 4). Hexanol-treated worms started regeneration on day one and continued growth through day six. However, growth was slower than control worms and overall growth was less than control worms for both heads and tails. Unlike control worms, head and tail growth rate were similar to each other (Figure 5). Octanol 0-treated worms had more head growth than tail growth starting on day one and continuing through day six, unlike control worms. Abnormal morphology of the tail started early, on day three in the case of the worm in Figure 6. The head was considered abnormal due to the slow rate of growth compared to control worms. Also, there was no clear segmentation of the head by day six. (Figure 6). Octanol 0-1-treated worms showed similar growth to Octanol 0-treated worms. There was more head growth than tail growth starting on day one and continuing through day six. Similar to Octanol 0 worms, abnormal morphology was evident after two days. The head was considered abnormal due to the slow rate of growth compared to the control worms and segmentation does not appear until day six. (Figure 7). Octanol 0-2-treated worms had less growth than Octanol 0 and Octanol 0-1-treated worms. Similar to the Octanol 0 and

Octanol 0-1-treated worms, the head started regenerating before the tail and had a faster rate of growth through day six. The head and tail were both considered abnormal due to the slow rate of growth compared to control worms. There was no segmentation in either head or tail by day six. (Figure 8). Octanol 0-6-treated worms had the slowest overall growth and slowest rate of growth of all the treated worms. Growth patterns were most similar to Octanol 0-2-treated worms. Head and tail were considered abnormal due to the slow rate of growth. There was no segmentation in either head or tail by day six. (Figure 9).

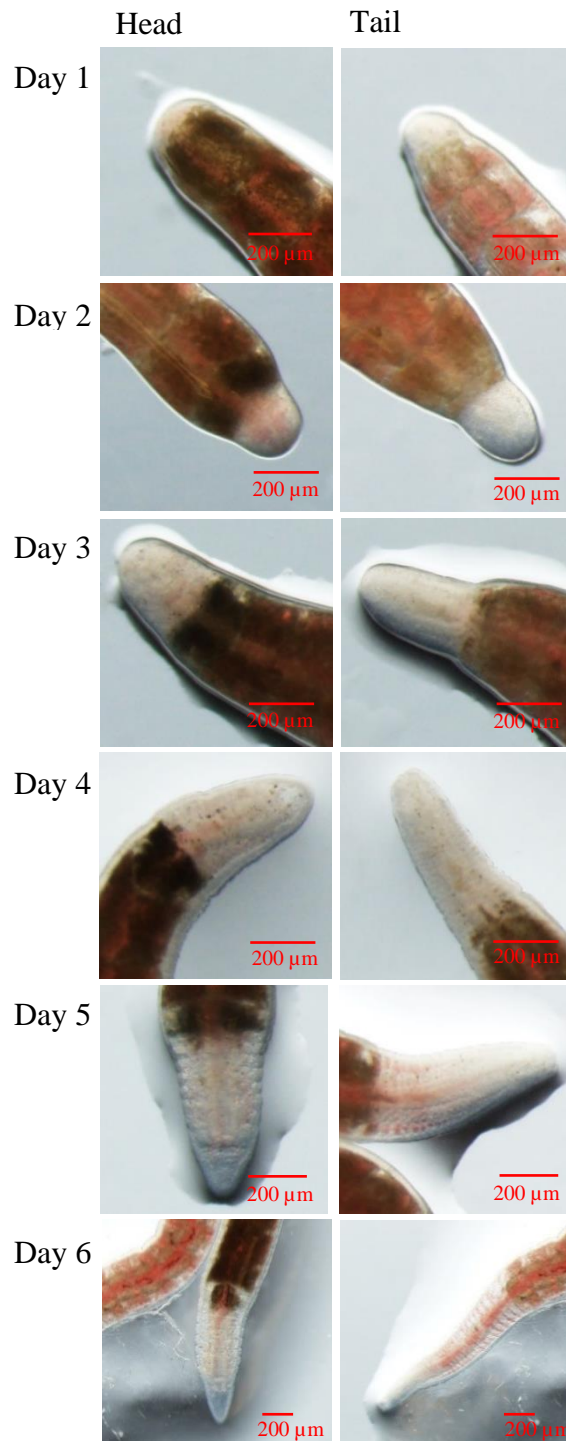


Figure 4: Untreated control worms had more tail growth than head growth as indicated by the length of the regenerated tissue. Images of untreated control worm regeneration head and tail, days 1-6. Regenerated tissue is unpigmented and normal.

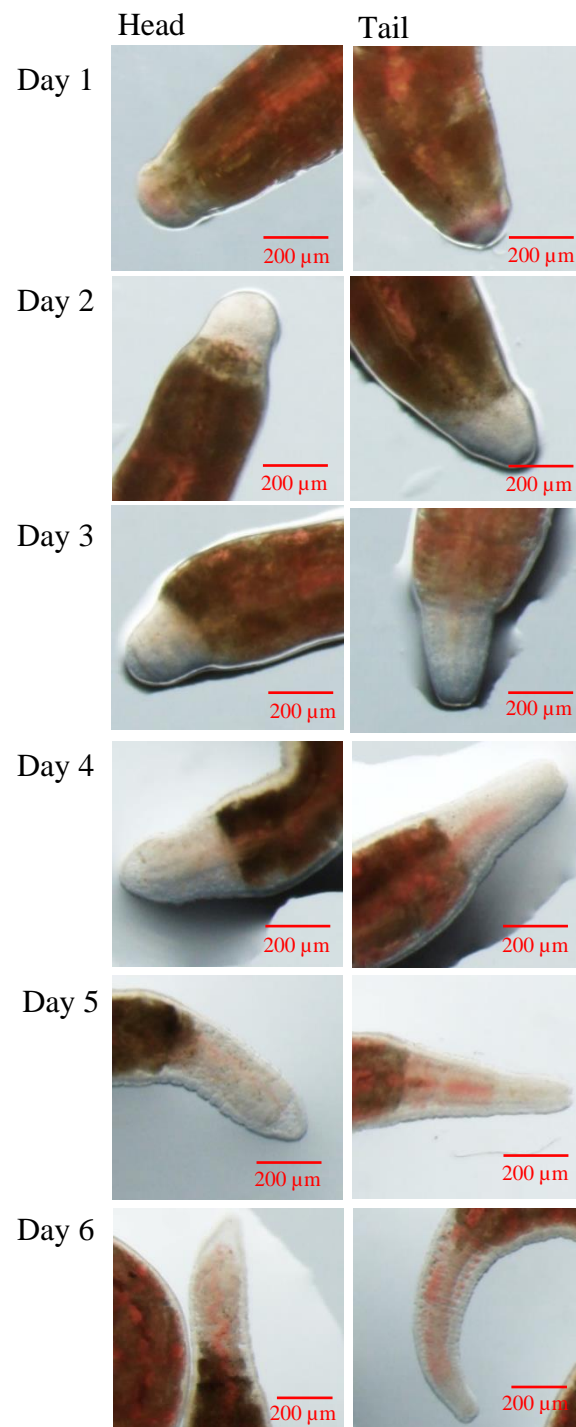


Figure 5: 0.01% Hexanol-treated worms have more tail growth than head growth. Worms were exposed to hexanol days one through six. Growth was slower in hexanol-treated than in control worms. Regenerated tissue is unpigmented.

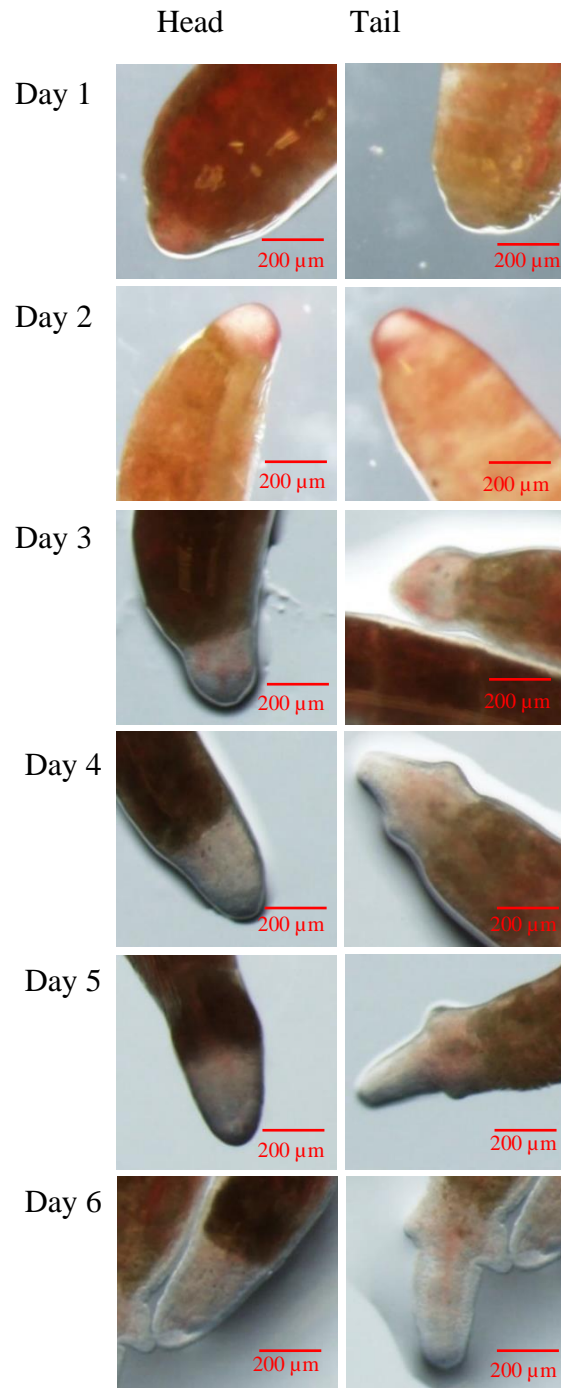


Figure 6: Octanol 0-treated worms (octanol treatment day 0, pond water days 1-6) had more head growth than tail growth and growth at both head and tail was abnormal. Images of Octanol 0-treated worm regeneration head and tail, days 1-6. In these images, head growth was abnormal due to slow rate of growth and tail growth was abnormal due to the two prongs growing out the side at the base of the tail starting on day four along with slow rate of growth. Regenerated tissue is unpigmented.

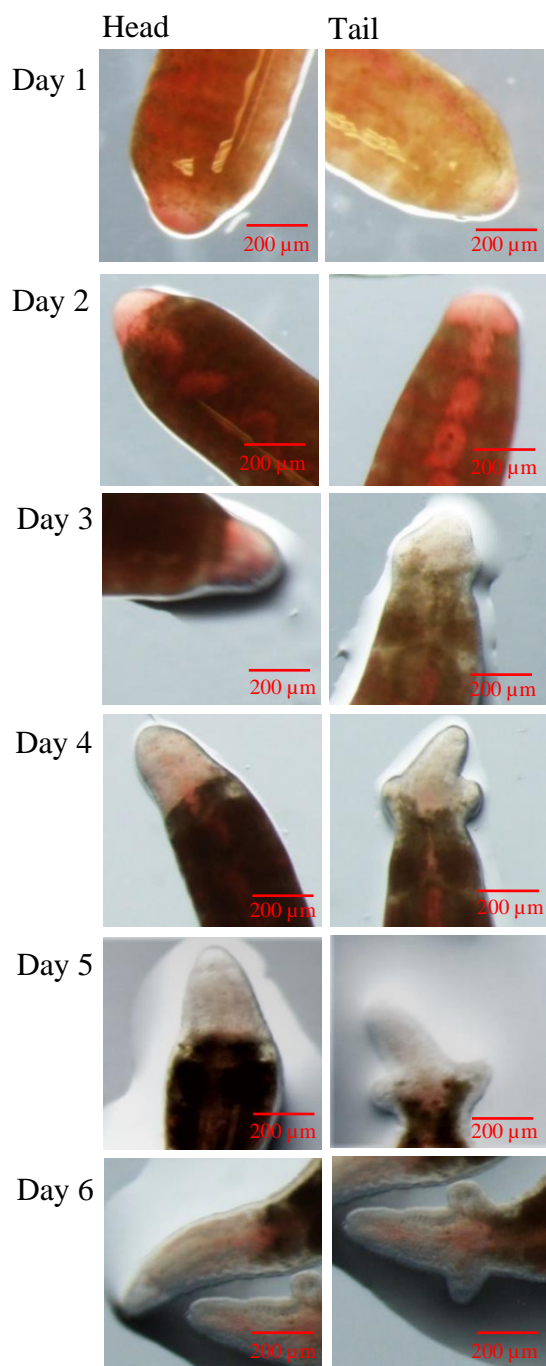


Figure 7: Octanol 0-1-treated worms (octanol treatment days 0-1, pond water days 2-6) had more head growth than tail growth and growth of both the head and tail was abnormal. Images of Octanol 0-1-treated worm regeneration head and tail, days 1-6. In these images, head growth was abnormal due to slow rate of growth and tail growth was abnormal due to the two prongs growing out the side at the base of the tail starting on day three along with slow rate of growth. Regenerated tissue is unpigmented.

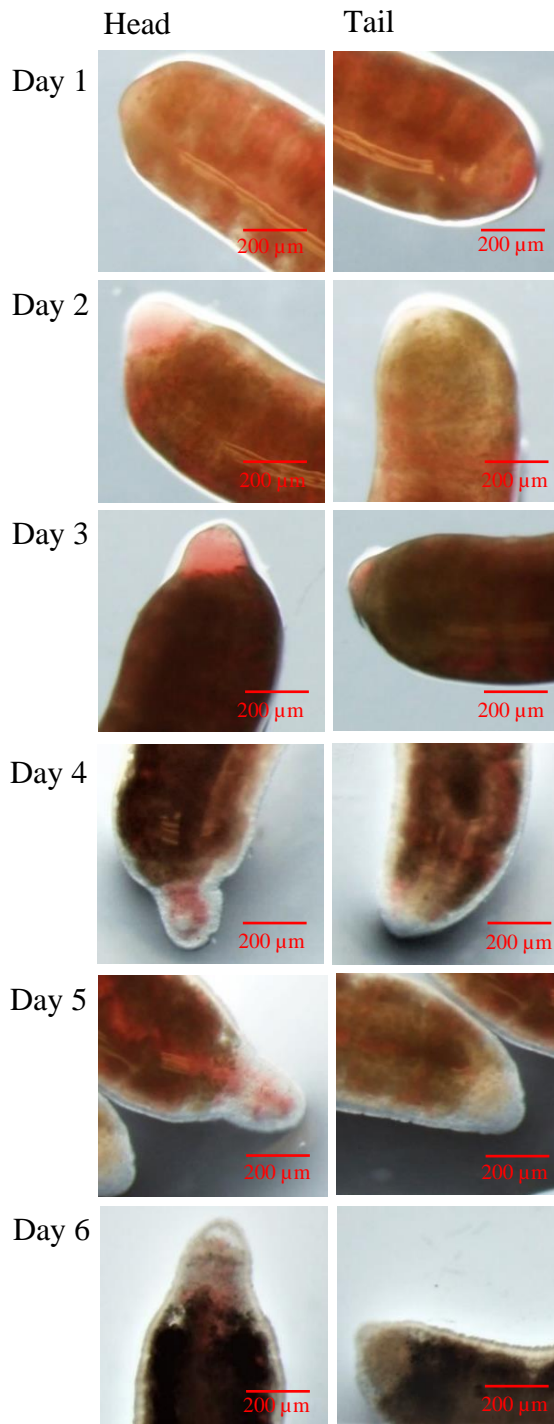


Figure 8: Octanol 0-2-treated worms (octanol treatment days 0-2, pond water days 3-6) had more head growth than tail growth and growth at both head and tail was abnormal. Images of Octanol 0-2-treated worm regeneration head and tail, days 1-6. In these images, head growth was abnormal due to slow rate of growth and incomplete segmentation of the head, and tail growth was abnormal due to the lack of regeneration in the tail by day six along with slow rate of growth. Regenerated tissue is unpigmented.

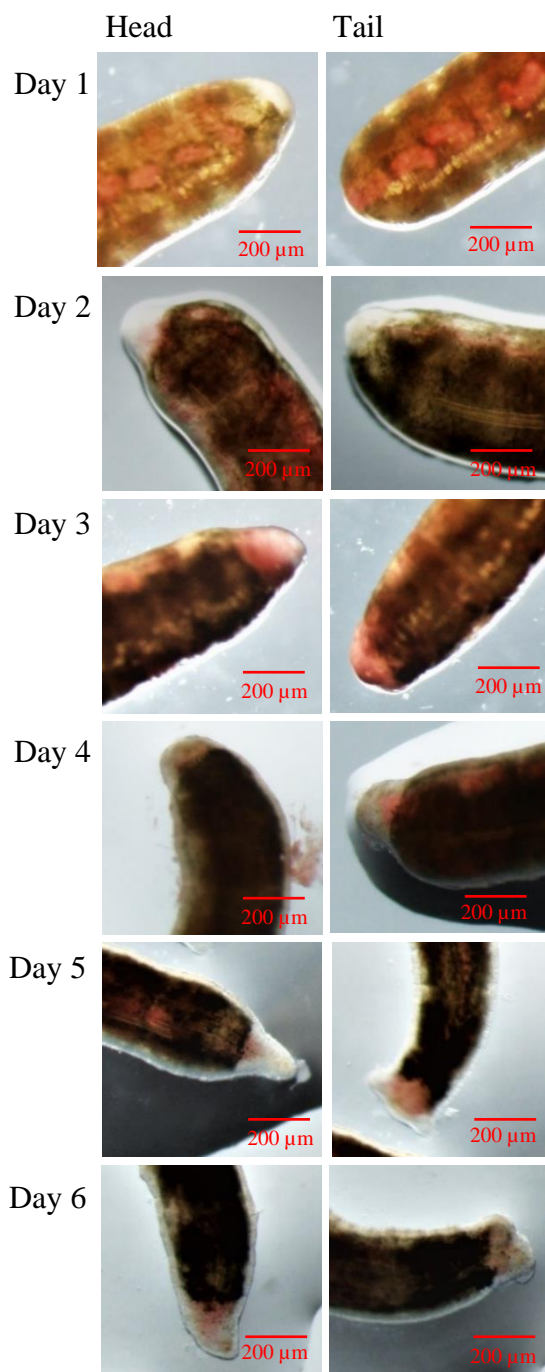
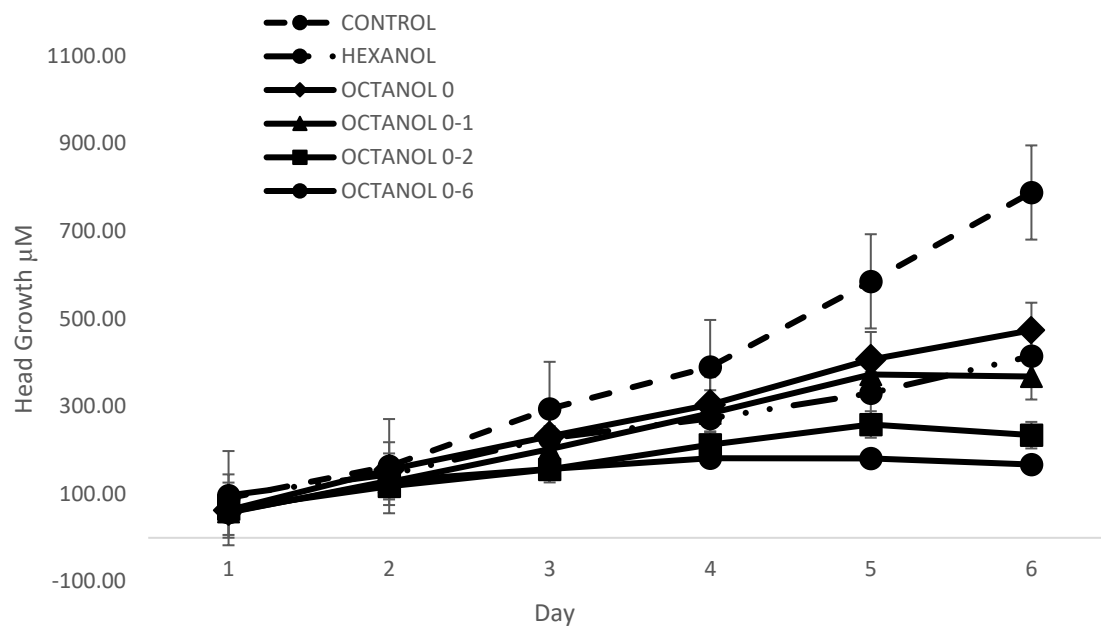


Figure 9: Octanol 0-6-treated worms (octanol treatment days 0-6, no pond water) had more head growth than tail growth and growth of both head and tail was abnormal. Images of Octanol 0-6-treated worm regeneration head and tail, days 1-6. In these images, head growth was abnormal due to slow rate of growth and incomplete segmentation of the head and tail growth is abnormal due to both the two prongs growing out the side at the base of the tail starting on day five and slow rate of growth. Regenerated tissue is unpigmented.

In general, the growth of the regenerated region of the untreated worms steadily increased through day six. The octanol-treated worms displayed less regeneration of both heads and tails than the untreated control worms. The degree of regeneration in octanol 0-6-treated worms was the least of all treatments. Both the octanol treatment and the duration that the worm was exposed to the octanol had an effect on worm regeneration. The longer the worm was exposed, the less the worm regenerated. The data in Figures 10 and 11 show that during the period of exposure to octanol, regeneration was reduced, but that there is no obvious recovery period following return to clean water. If recovery of growth occurred after placement back in pond water, the data would show a greater increase in growth on days after pond water exposure.

To analyze differences in regeneration between untreated control worms and all other treated worms, the data were divided by head and tail regeneration separately and analyzed in two ways; overall growth from transection for days one through six and the rate of growth per day for days one through six. For each of the six treatments, head and tail worm growth on each day was averaged for days one through six (Figures 10A and 10B). In order to determine if different days of treatment were more critical for regeneration, a rate of growth per day was calculated. For each of the six treatments, head and tail worm growth on each day was averaged and subtracted from the previous day's growth to get the rate per day of growth (Figures 11A and 11B).

A.



B

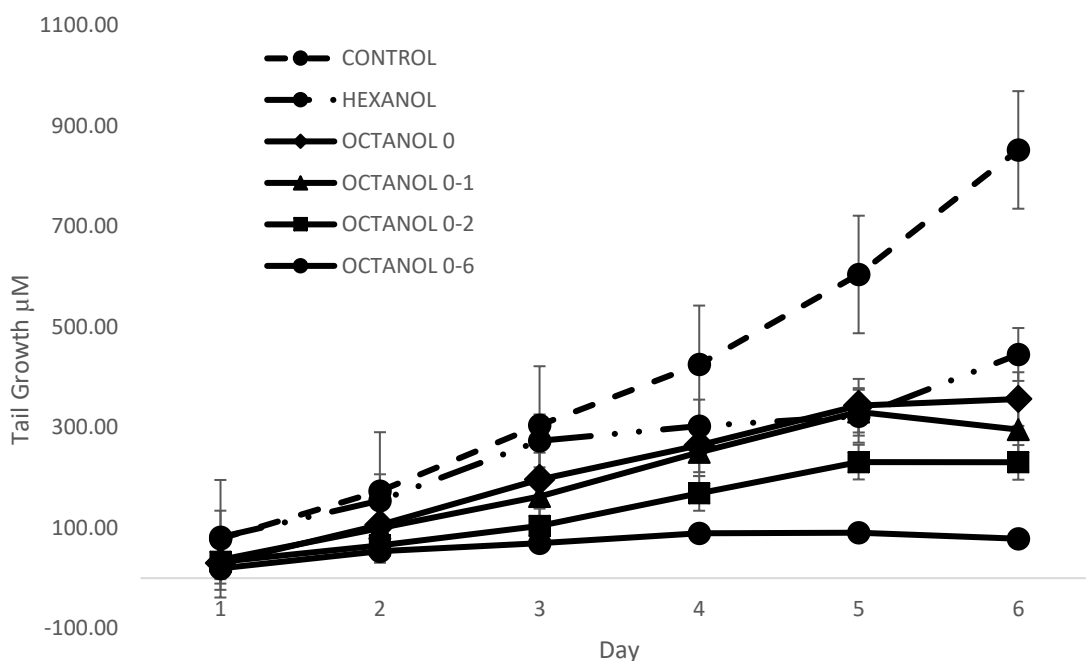
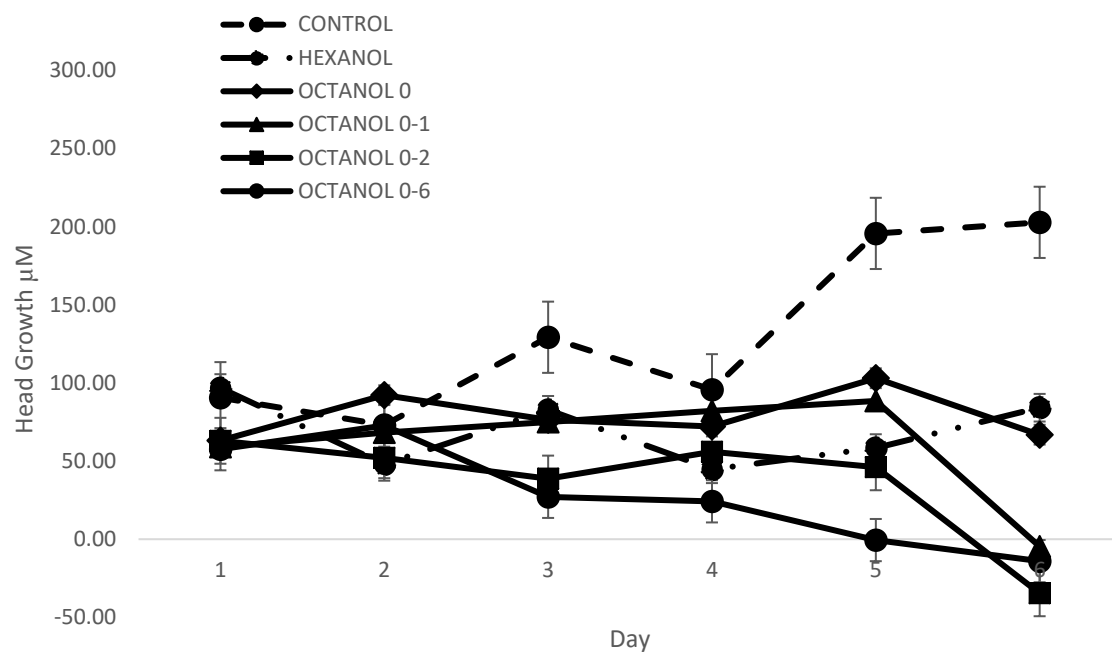


Figure 10: Mean regeneration on days 1 through 6 for heads and tails showed an upward trend in growth for all treatments with untreated control worms having a sharper upward trend than all treated worms. (A) Both the untreated control worms and the hexanol-treated worms had upward growth trends for the head, but untreated control worm growth increased at a faster pace. Mean head regeneration for untreated control worms increased each day until day six. The hexanol-treated worms and all octanol-treated worms had significantly less growth than untreated control worms ($p < 0.05$). The Octanol 0-treated worms were the only octanol-treated worms that did not have a decrease in head growth on any day through day six. The Octanol 0-1, Octanol 0-2 and Octanol 0-6-treated worms all had a decrease in head growth on day five. (B) Untreated control worm and hexanol-treated worm mean regeneration for tails had upward growth trends through day six. But, untreated control worm days five and six was significantly different than hexanol-treated worm growth on days one, two, four and five ($p < 0.05$). All octanol-treated worm tail growth slowly increased over the six days. Starting on day four, Octanol 0-6-treated worm regeneration slowly decreased until day six. Octanol 0, Octanol 0-1, Octanol 0-2-treated worm regeneration continued to increase until day five and then plateaued, but Octanol 0 is the only octanol treatment that continued to increase in growth through day six.

A



B

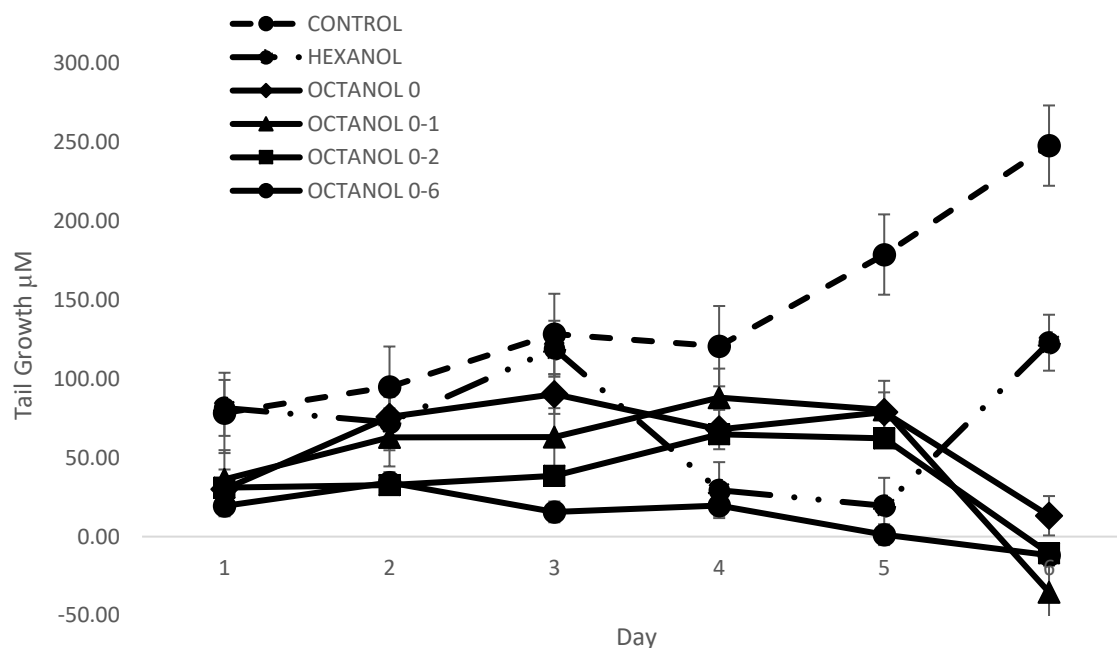


Figure 11: Mean rate of regeneration on days 1 through 6 for heads and tails fluctuated each day for all treatments and showed an upward trend for untreated control worms and a downward trend for all treated worms. (A) Untreated control worm mean rate of head regeneration fluctuated, however, by day six, there was significantly more growth that day than all other treated worms ($p < 0.05$). Untreated control worm head rate of regeneration had a sharp upward growth trend overall, but hexanol-treated worms had a slight downward trend. Octanol 0-treated worms were the only octanol-treated worms that had a slight upward growth trend. Octanol 0-1-treated worms had a slight upward trend until day five but ended with an overall downward trend by day six. Octanol 0-2 and 0-6-treated worm tail rate of regeneration had a steady downward trend. (B) Untreated control worm rate of tail regeneration fluctuated each day, however, by day six, there was significantly more growth that day than all other treated worms ($p < 0.05$). Untreated control tail rate of regeneration had a sharp upward trend overall. Hexanol-treated tail rate of regeneration also had an upward trend, however, less steep than untreated control worms. All octanol-treated worm tail rate of regeneration had a steady downward trend.

The two-way ANOVA comparing total head growth among the six treatments indicated that there was a significant difference in total head growth. The mean growth on at least one of the days post transection for all treatments was significantly different than at least one other day ($p < 0.001$, $F_{5, 1290} = 1108.53$). The overall growth trend for head regeneration was consistent for each treatment; however, the untreated control worms had more regeneration and grew at a faster rate than the treated worms. The two-way ANOVA comparing treatments on regenerating heads indicated that there was a significant difference in the type of treatment given; specifically, the mean growth a worm head of one treatment is significantly different than the mean growth of a worm head of at least one other treatment ($p < 0.001$, $F_{5,1290} = 307.05$). Also, the interaction between day and type of treatment was significant ($p < 0.001$, $F_{25,1290} = 42.18$). The untreated worms had the most daily regeneration and had steady growth each day for the six days. The hexanol-treated worms started day one with similar growth as the untreated control worms, but continued days two through six with variable growth. The untreated control worms had significantly more growth on days five and six than hexanol-treated days one through six ($p < 0.05$). All octanol-treated worms started day one with less growth than the untreated and hexanol-treated worms. The Octanol 0-1, Octanol 0-2 and Octanol 0-6-treated worms all had slow growth, ending with a plateau in growth at six days. (Figure 10A). Tukey's Honest significant difference for pairwise comparisons indicated that that mean day head regeneration for all octanol-treated worms was significantly different than untreated worms ($p < 0.05$).

The two-way ANOVA comparing total tail growth among the six treatments indicated that there was a significant difference in total tail growth ($p < 0.001$, $F_{5,1290} = 470.6$). For each treatment, the overall tail regeneration followed its own specific trend, but in general, the untreated worms had the most overall regeneration and had consistent growth each day for the six days and the treated worms had slower growth with each treatment different from each other. The two-way ANOVA comparing treatments on regenerating tails indicated that there was a significant difference in the type of treatment given, specifically, the mean of tail growth of one treatment was significantly different than the mean of tail growth of at least one other treatment ($p < 0.001$, $F_{5,1290} = 211.1$). Also, the interaction between day and type of treatment was significant ($p < 0.001$, $F_{25,1290} = 20.05$). The untreated worms started day one with more growth than all treated worms and continued the upward growth trend through the six days. The hexanol-treated worms had similar growth as the untreated worms up to day two, but on day three they started to plateau in growth. The hexanol-treated worms ended the six days with an overall increase in growth. All octanol-treated worms started day one with less growth than the untreated worms and the hexanol-treated worms. All octanol-treated worms had very slow growth, ending with an overall minimal growth at the end of the six days. (Figure 10B). Tukey's Honest significant difference for pairwise comparisons indicated that mean day tail regeneration for all octanol-treated worms was significantly different than untreated worms ($p < 0.05$).

Mean rate of regeneration showed that growth varied each day for all treated and untreated worms

The rate of head growth per day was inconsistent. Both the untreated worms and all the treated worms showed variable amounts of head regeneration each day, but the overall patterns were different for the different treatments. The untreated control worms had an upward trend by day five and plateaued at the end of day six (Figure 11A) with significant differences in growth between days ($p < 0.05$) (Table 2). The hexanol-treated worms had little variability each day with an overall slight downward trend in growth at the end of the six days. The Octanol 0-treated worms had small fluctuations in growth through the six days with little change each day and an overall slight upward trend. The Octanol 0-1-treated worms were the only worms that had an upward growth trend until day five then on day six had a sharp decrease in growth. The Octanol 0-2 and Octanol 0-6-treated worms consistently had slow growth each day though day six with an overall downward trend. (Figure 11A). Table 2 shows all of the significant differences for head regeneration between treatments and days. Tukey's Honest significant difference for pairwise comparisons indicated that only the untreated control worms had significant differences over time in head growth. The differences were between days one and six, days one and five, days two and six, days two and five, days four and six and days four and five ($p < 0.05$). There were no treated worms with significant differences within the same treatment over time (Table 2).

Table 2: Table of significant differences for head regeneration between treatments and days. Empty cells do not have significant differences for head regeneration.

Treatment/Day	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Control Day 1						Octanol 0-2 Octanol 0-6
Control Day 3			Octanol 0-2 Octanol 0-6	Octanol 0-6	Octanol 0-6	Octanol 0-1 Octanol 0-2 Octanol 0-6
Control Day 4						Octanol 0-2 Octanol 0-6
Control Day 5	Hexanol Octanol 0 Octanol 0-1 Octanol 0-2 Octanol 0-6 Control	Hexanol Octanol 0 Octanol 0-1 Octanol 0-2 Octanol 0-6 Control	Hexanol Octanol 0 Octanol 0-1 Octanol 0-2 Octanol 0-6	Hexanol Octanol 0 Octanol 0-1 Octanol 0-2 Octanol 0-6 Control	Hexanol Octanol 0-1 Octanol 0-2 Octanol 0-6	Hexanol Octanol 0 Octanol 0-1 Octanol 0-2 Octanol 0-6
Control Day 6	Hexanol Octanol 0 Octanol 0-1 Octanol 0-2 Octanol 0-6 Control	Hexanol Octanol 0 Octanol 0-1 Octanol 0-2 Octanol 0-6 Control	Hexanol Octanol 0 Octanol 0-1 Octanol 0-2 Octanol 0-6	Hexanol Octanol 0 Octanol 0-1 Octanol 0-2 Octanol 0-6 Control	Hexanol Octanol 0-1 Octanol 0-2 Octanol 0-6	Hexanol Octanol 0 Octanol 0-1 Octanol 0-2 Octanol 0-6
Octanol 0-1 Day 6					Octanol 0	
Octanol 0-2 Day 3					Octanol 0	
Octanol 0-2 Day 6	Hexanol	Octanol 0	Octanol 0		Octanol 0 Octanol 0-1	Octanol 0
Octanol 0-6 Day 3					Octanol 0	
Octanol 0-6 Day 4					Octanol 0	
Octanol 0-6 Day 5					Octanol 0	

Treatment/Day	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
					Octanol 0-1	
Octanol 0-6 Day 6	Hexanol	Octanol 0	Octanol 0		Octanol 0 Octanol 0-1	Octanol 0

The rate of tail growth per day for untreated control worms had less fluctuation overall than rate of head growth. The untreated control worms had an overall upward trend of growth each day with significant differences in growth between days ($p < 0.05$) (Table 3). The hexanol-treated worms had large variability in growth each day with an overall upward growth trend. However, there were no significant differences in growth between days for hexanol-treated worms. All of the octanol-treated worms had little variability in growth each day with an overall downward trend. On day six, all of the octanol-treated worms decreased in rate of growth (Figure 11B). Only Octanol 0 and Octanol 0-1-treated worms had significant differences in growth between days (Table 3). Tukey's Honest significant difference for pairwise comparisons indicated that the untreated control worms had significant differences in tail growth between days one and six, days one and five, days two and six, days two and five, days three and six and days four and six ($p < 0.05$). The Octanol 0 treatment had a significant difference in tail growth between days one and five ($p < 0.05$). The Octanol 0-1 treated worms had significant differences in tail growth between days four and six, and days five and six ($p < 0.05$). All other treated worms did not have significant differences within the same treatment over time (Table 3).

Table 3: Table of significant differences for tail regeneration between treatments and days. Empty cells do not have significant differences for tail regeneration.

Treatment/Day	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Control day 1						Octanol 0-6
Control day 2				Octanol 0-6	Octanol 0-6	Octanol 0-6 Octanol 0-1
Control day 3	Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0	Octanol 0-6 Octanol 0-2	Octanol 0-6 Octanol 0-2	Octanol 0-6	Octanol 0-6	Octanol 0-6 Octanol 0-2 Octanol 0-1
Control day 4	Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0	Octanol 0-6 Octanol 0-2	Octanol 0-6 Octanol 0-2	Octanol 0-6	Octanol 0-6	Octanol 0-6 Octanol 0-2 Octanol 0-1
Control day 5	Control Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0 Hexanol	Control Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0 Hexanol	Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0	Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0 Hexanol	Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0 Hexanol	Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0
Control day 6	Control Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0 Hexanol	Control Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0 Hexanol	Control Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0 Hexanol	Control Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0 Hexanol	Control Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0 Hexanol	Octanol 0-6 Octanol 0-2 Octanol 0-1 Octanol 0 Hexanol
Octanol 0 Day 1			Hexanol			Hexanol
Octanol 0 Day 5	Octanol 0					
Octanol 0-1 Day 5	Octanol 0					

Treatment/Day	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Octanol 0-1 Day 6		Octanol 0	Octanol 0 Hexanol	Octanol 0-1	Octanol 0-1 Octanol 0	Hexanol
Octanol 0-2 Day 1					Octanol 0-1 Octanol 0	Hexanol
Octanol 0-2 Day 5						Octanol 0-1
Octanol 0-2 Day 6					Octanol 0-1 Octanol 0	Hexanol
Octanol 0-6 Day 3			Hexanol		Octanol 0-1 Octanol 0	Hexanol
Octanol 0-6 Day 4			Hexanol		Octanol 0-1 Octanol 0	Hexanol
Octanol 0-6 Day 5			Octanol 0 Hexanol	Octanol 0-1	Octanol 0-1 Octanol 0	Hexanol
Octanol 0-6 Day 6		Octanol 0 Hexanol	Octanol 0-1 Octanol 0 Hexanol	Octanol 0-1 Octanol 0	Octanol 0-2 Octanol 0-1 Octanol 0	Octanol 0 Hexanol

Comparison of heads and tails within the same treatment show similar regeneration trends to each other

In general, all untreated control worms and all treated worms followed a similar trend of total regeneration within treatment for both heads and tails. Untreated control worms showed a steady upward growth trend for both head and tail. Even though all treated worm head and tail regeneration was slower than control worms, all treated worms showed similar patterns for both heads and tails. All treated worms had more

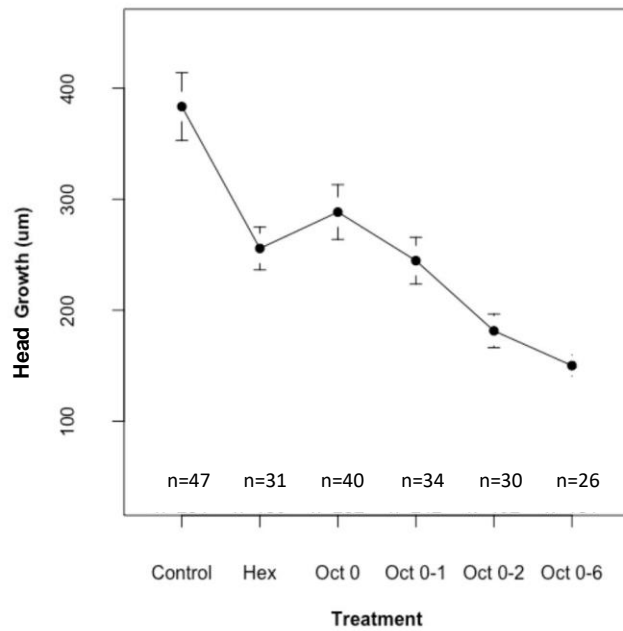
head regeneration than tail regeneration by day six. Unlike the treated worms, the untreated control worms had more tail regeneration than head regeneration by day six (Figures 10A and 10B).

The rate of regeneration also followed a similar trend within treatment for both heads and tails. The untreated control worm head rate of regeneration had more fluctuation each day than the tail rate of regeneration. The head rate of regeneration plateaued towards the end of the six days, but the tail ended with a strong upward trend of growth. Unlike the control worms, the hexanol-treated worm regeneration had more fluctuation each day for the tail than the head, with the head ending day six with a plateau of growth and the tail ending day six with a larger upswing in growth. The octanol-treated worm regeneration was similar for heads and tails. There was a slow rate of growth each day with an overall downward trend and a sharp decrease in growth for head and tail both on day six (Figures 11A and 11B).

The untreated control worm head and tail regeneration was statistically different from all treated worms ($p < 0.05$). The Octanol 0 and Octanol 0-2 treated worm head and tail overall mean growth were statistically different from each other ($p < 0.05$). The Octanol 0 and Octanol 0-6-treated worm head and tail overall mean growth were statistically different from each other ($p < 0.05$). The Octanol 0-1 and Octanol 0-2 treated worm head and tail overall mean growth were statistically different from each other ($p < 0.05$). The Octanol 0-1 and Octanol 0-6 treated worm head and tail overall mean growth were statistically different from each other ($p < 0.05$). (Figure 12A and

12B). Refer to Tables 1 and 2 for specific days of statistical difference between treatments.

A



B

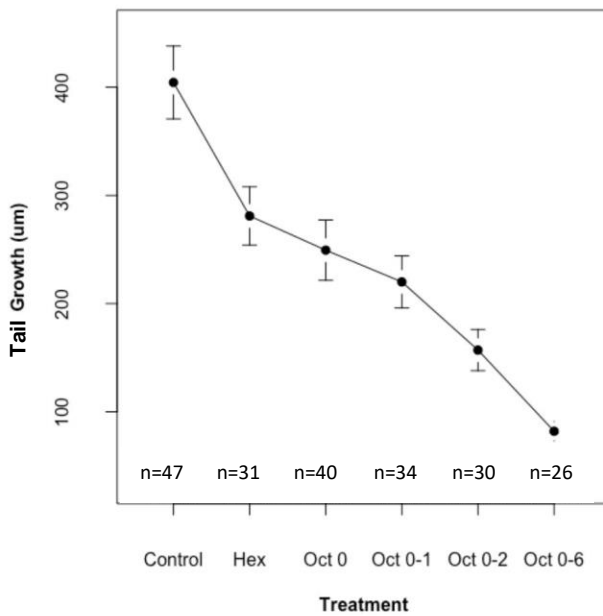


Figure 12: Plot of the means for total growth from transection through day six for total head and tail growth showed more growth for untreated control worms than treated worms. (A) Untreated control worm head growth was significantly greater than treated worms ($p < 0.05$). (B) Untreated control worm tail growth was significantly greater than treated worms ($p < 0.05$).

EdU cell proliferation assay

Images from both the untreated control worms and the Octanol 0-6-treated worms showed proliferating cells throughout the body. The Alexa flour stain used to indicate proliferating cells was not different than the Hoechst stain which is the blue fluorescent stain used to stain DNA. (Figures 13–16).

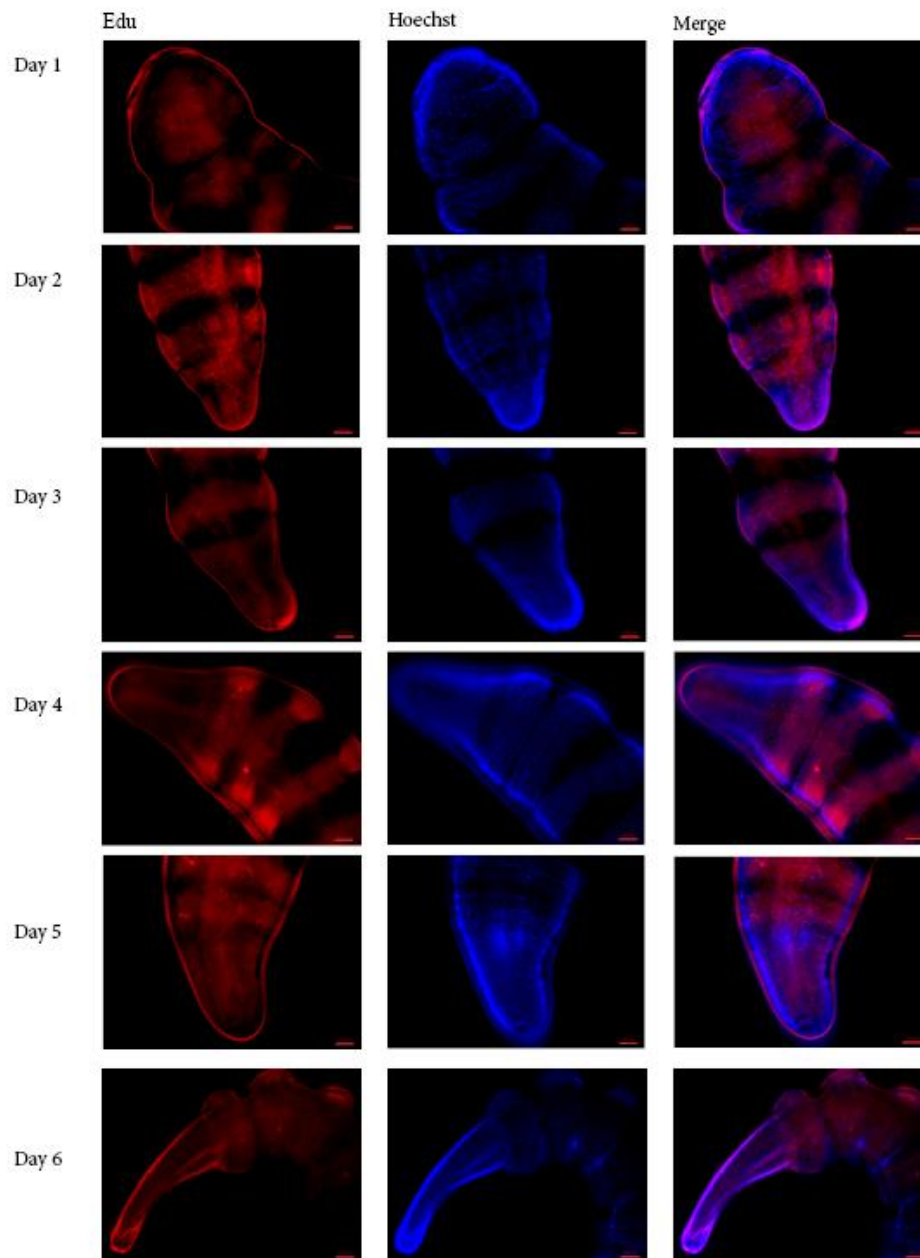


Figure 13: Cell proliferation images of untreated control worm heads. Images of the untreated control worms show proliferating cells throughout the body. The regenerating head shows cells with the Hoechst stain, but not as vividly with the EdU stain. Scale bar = 100 μ

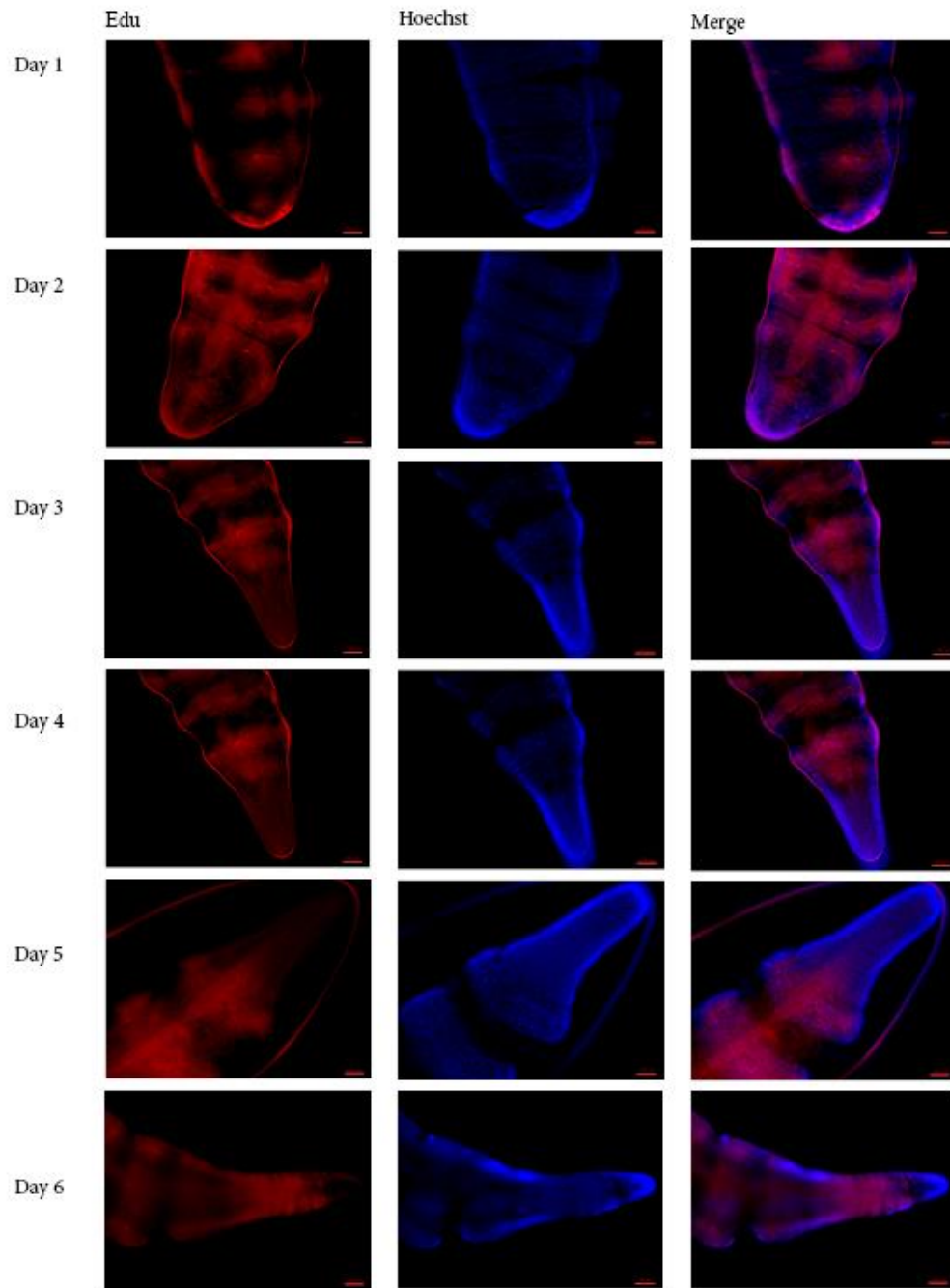


Figure 14: Cell proliferation images of untreated control worm tails. Images of the untreated control worms show proliferating cells throughout the body. The regenerating tails shows cells with the Hoechst stain, but not as vividly with the EdU stain. Scale bar = 100 μm

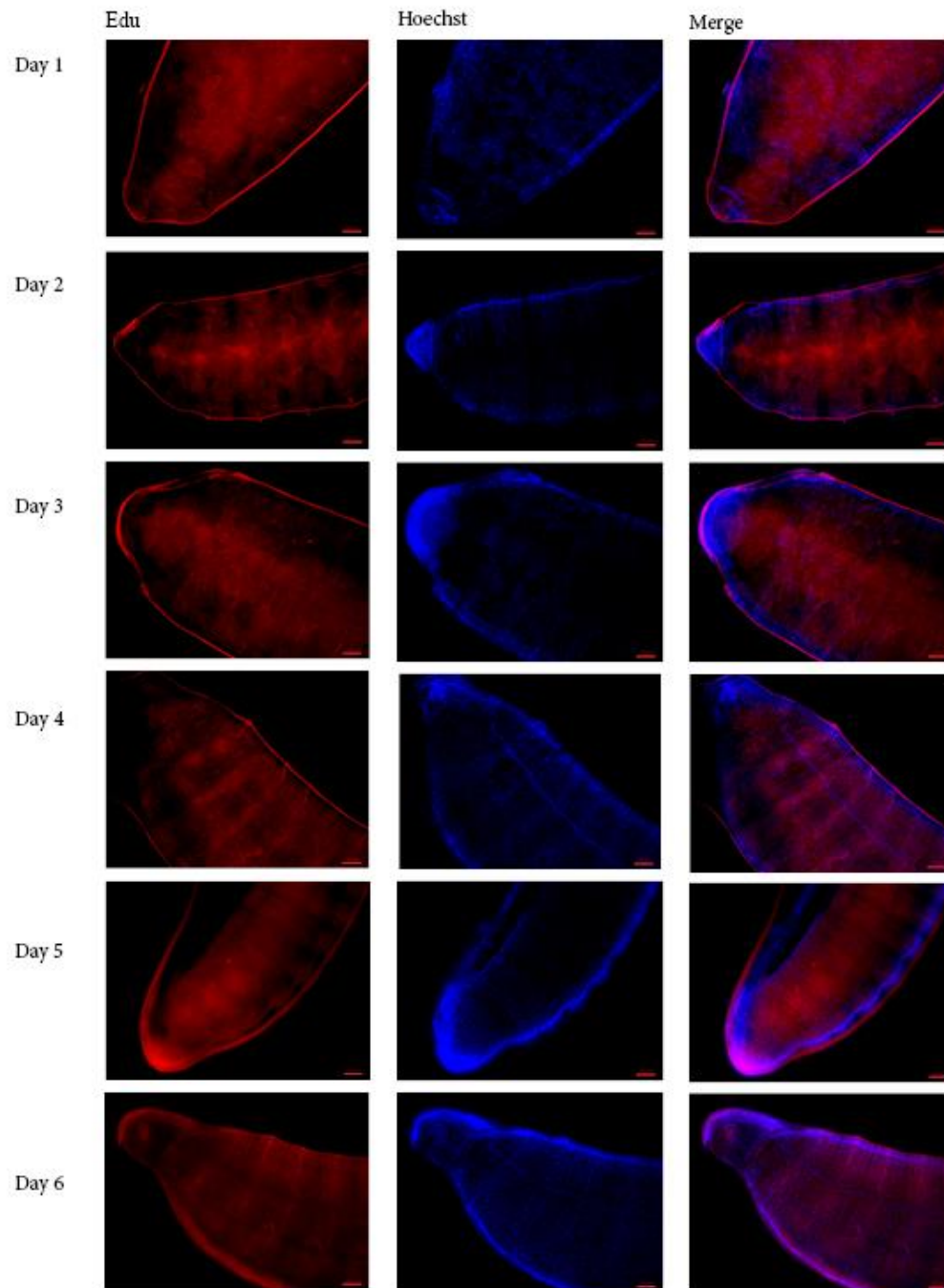


Figure 15: Cell proliferation images of Octanol 0-6-treated worm heads. Images of the Octanol 0-6-treated worms show proliferating cells throughout the body. The regenerating head shows cells with the Hoechst stain, but not as vividly with the EdU stain. Scale bar = 100 μ m

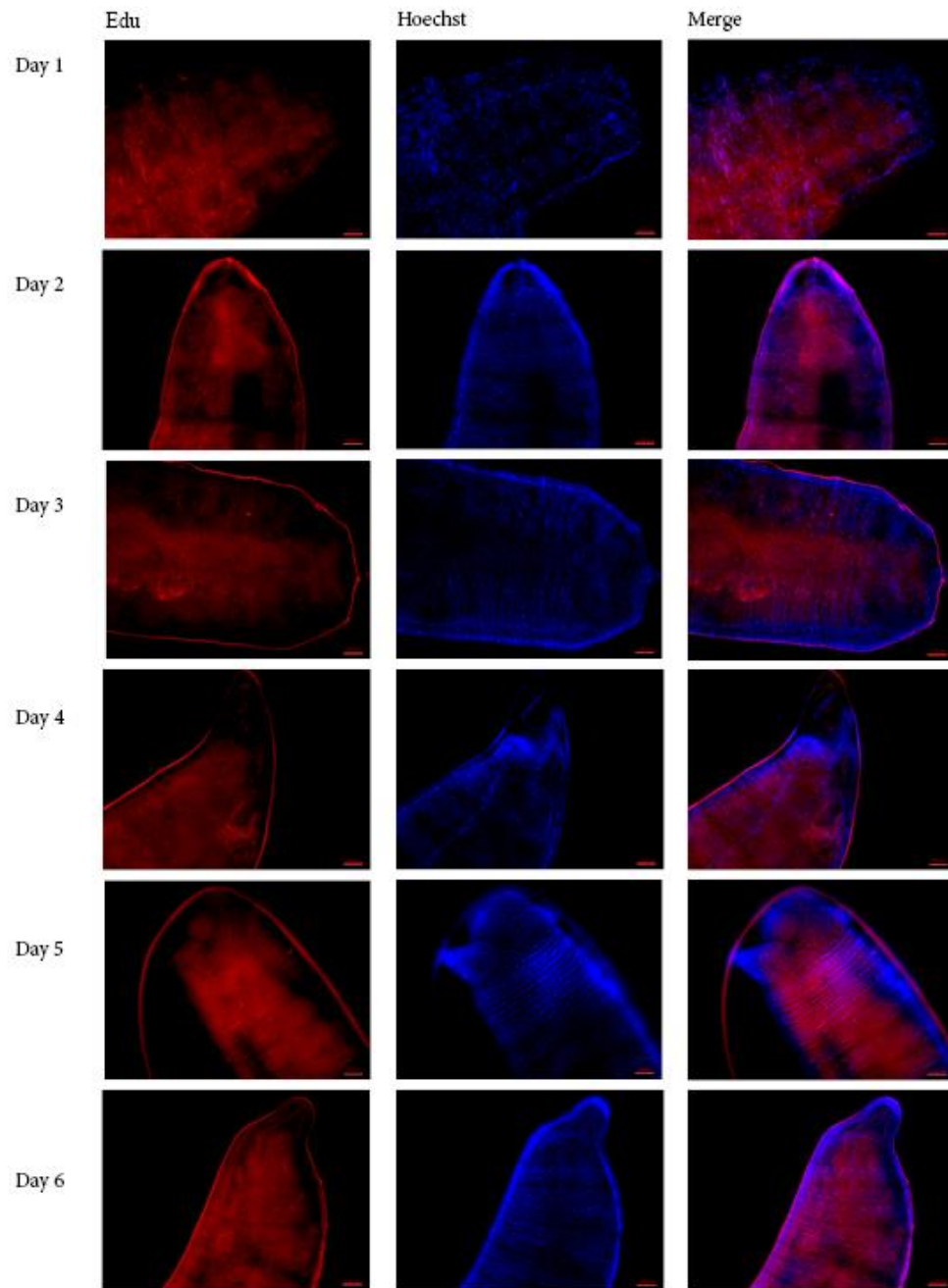


Figure 16: Cell proliferation images of Octanol 0-6-treated worm tails. Images of the Octanol 0-6-treated worms show proliferating cells throughout the body. The regenerating tails shows cells with the Hoechst stain, but not as vividly with the EdU stain. Scale bar = 100 μ m

DISCUSSION

Gap junctions are made up of proteins that differ in vertebrates and invertebrates. They form channels that use gating mechanisms that respond to biological stimuli, but the mechanisms for this response are poorly understood (Hervé, 2013). Blocking gap junctions has many effects on animals including: reducing gap junction coupling conductance in *Aedes albopictus* cell lines (Weingart and Baukaskas, 1998), inducing different species specific head anatomies in the flatworm *Girardia dorocephalia* (Emmons-Bell et al., 2015), affecting amylase secretion in rat pancreatic acinar cells (Chanson et al., 1989), and inducing ectopic head formation in regenerating planaria (Nogi and Levin, 2005; Oviedo, 2010). There are known gap junction blockers such as long chain alcohols including octanol that produce these effects; however, the mechanism by which alcohols such as octanol block gap junctions is not currently known. It's suggested that long chain alcohols act by incorporation into the lipid bilayer of cell membranes. In particular, Weingart and Bukauskas (1998) suggest that the long chain alcohols interfere with the voltage sensor and alter its membrane potential sensitivity. Emmons-Bell et al. (2015) also suggest that octanol disrupts the bioelectric gradients, but a sophisticated analysis method would have to be developed to understand the mechanism. Since the proteins that comprise gap junctions form a channel, they are a means of cell-cell communication that small molecules pass through. Changes in intracellular Ca^{2+} , pH, membrane potential, and phosphorylation/dephosphorylation of

channel proteins can affect communication between cells (Hervé, 2013), and can in turn affect the regeneration process.

The purpose of this study was to determine whether the gap junction blocker octanol had an effect on regeneration of *Lumbriculus variegatus* body parts, and if so, how regeneration was affected. The results indicate that 0.01% octanol solution does have an effect on regeneration of transected *L. variegatus*. Based on photos and measurements taken every day for six consecutive days, there was both slower than normal regeneration and abnormal morphology of the octanol-treated worms. This shows that the gap junction blocker octanol does negatively affect regeneration.

Control worm regeneration steadily increased each day. Head and tail blastemata appeared by day one and growth proceeded through day six. Morphology was normal and a head with 7-8 segments was fully formed by day six.

In the treated worms where regeneration was abnormal there was no segmentation at the tail in some instances (Figures 6, 7, 8, and 9). Segmentation appeared at the head on an earlier day than the tail. In the control and the hexanol-treated worms, segmentation of both the head and tail appeared at day three.

In a study of posterior regeneration of the marine annelid *Platynereis dumerilii*, Planques et al. (2012) characterized posterior regeneration at a morphological, molecular, and cellular level. After amputation of the posterior part of the body, worms were able to heal the wound and regenerate both the differentiated structures of the pygidium and the stem cells of the growth zone which allow for growth of new segments. They refer to that process as posterior regeneration. Following the wound healing and posterior

regeneration phases, there is a phase which they call post-regenerative posterior growth where segment addition occurs to replace the amputated segments. They deduced that there are two stages of regeneration in the annelid; regeneration and post-regenerative growth in which segmentation appeared. Therefore, there is a difference between regeneration and segment addition. In this study, the octanol treatment appeared to not have an effect on regeneration or obvious effect on formation of the blastema, but did hinder the post-regenerative growth where segmentation appears. A more detailed study would be needed to determine the exact effect on the blastema.

Comparison of treated and non-treated worm regeneration

The regeneration and growth of control worms was as predicted for both the head and tail regions. The control worms showed steady growth throughout the six days with the head regenerating the full 7-8 segments within six days as expected. The mean growth of control worms was significantly higher than the mean growth for all octanol-treated worms. However, the control worm mean growth was not significantly higher than the mean growth for the hexanol-treated worms. Because hexanol is not known to block gap junctions (Chanson et al., 1989), it was expected that the hexanol-treated worms would have similar regeneration patterns as control worms. The hexanol-treated worm growth was less each day than the control worm growth but higher than all octanol-treated worms. All octanol-treated worms showed slower growth than the hexanol-treated and control worms (Figures 10A and 10B). However, the Octanol 0 and

0-1-treated worms showed similar growth to the hexanol treated worms for head regeneration (Figure 10A). This suggests that hexanol does have a negative effect on regeneration in *L. variegatus*. A study by Nogi and Levin (2005) found that a long chain alcohol, heptanol, blocked gap junctions and regenerated a bipolar two-headed planaria after bisection. However, after exposure to hexanol, anteriorization of the posterior part did not occur; instead, hexanol inhibited tail regeneration, which they characterize as the weakest class of anteriorization. This inhibition of tail regeneration also occurred in *L. variegatus* after exposure to hexanol (Figures 10B and 11B).

Chanson et al. (1989) found that alcohols with seven, eight and nine carbons blocked gap junctions in pancreatic acinar cells, but alcohols with fewer than seven carbons were not effective in blocking gap junctions. This two-carbon difference between hexanol and octanol may have slight differences in effect in *L. variegatus* because it is clear that hexanol does negatively affect regeneration; however, the Chanson et al. (1989) study did not experiment with invertebrate gap junctions.

Weingart and Buauskas (1998) used an insect cell line from the mosquito *Aedes albopictus* to determine the effect of long chain alcohols on the conductance of gap junctions. For their experiment, they considered hexanol, as well as heptanol, octanol, nonanol and decanol to be a long chain alcohol. They found that pentanol had no effect on gap junctions, but hexanol (15 mM) did have an effect. It is possible that innexons in invertebrates are sensitive to hexanol as a gap junction blocker. Weingart and Buauskas (1998) found that the effects on gap junctions were dependent on the structure of the alcohol. The longer the alcohol chain, the more pronounced were the effects. The

concentration required to have an effect different from hexanol was tens of mM of octanol, tens of μM of nonanol. The concentration required to see a desired outcome decreased with increasing length of the hydrocarbon chain. This could be why hexanol had a negative effect on growth and morphology, but not as great of a negative effect as the octanol treatments in this experiment. The concentration of hexanol (0.98 mM) used in the current experiments was significantly less than that used in the Weingart and Bukauskas study.

Emmons-Bell et al. (2015) used 123 μM octanol to induce species-specific head anatomy in the planarian *Girardia dorocephala*. Using the same method with hexanol (concentration not given) that they used with octanol did not produce the irregular morphologies in the planarian. This could also be evidence that different invertebrates react differently to the long chain alcohols, different concentrations and different exposure times. Also, planaria are more closely related to annelids than arthropods on a phylogenetic tree.

Heads and tails of worms differ in their functional morphology. In annelids the anterior end contains the head with the mouth, pharynx, cephalic ganglia and foregut. The posterior end contains the anus. In terms of regeneration, the anterior end cannot tolerate much morphological variability to function properly, whereas once the posterior end regenerates the anus, function is maintained as segments are added to grow longer (worms normally add posterior segments through time). The hexanol- and octanol-treated worms differed from each other for head and tail regeneration, but both had less overall growth than the control worms. The head regeneration (Figure 10A) for hexanol

and octanol treatments was more similar than tail regeneration (Figure 10B). It appeared that the octanol treatment ultimately affected the tail regeneration more than the head regeneration. This is somewhat surprising given the greater complexity of the head compared to the tail regions. However, the processes underlying indeterminate growth may have a complexity in control not reflected in the differences in morphological complexity. Overall head regeneration for hexanol and octanol-treated worms was faster than tail regeneration. However, overall head regeneration for the control worm was slower than the tail starting on day two. In an unpublished Master's thesis, control worm regeneration showed the same patterns of growth as this study with the head growth slower than the tail growth (Alkhathlan, 2015). The previous study used a different treatment, tricaine, which blocks voltage-gated sodium channels to alter regeneration.

All octanol-treated worms started day one with similar growth to each other for both heads and tails. Starting on day two, Octanol 0 and Octanol 0-1-treated worms had more tail growth than the octanol-treated worms that were still being exposed to the octanol treatment (Figure 10B). This would make sense for the Octanol 0-treated worms because they were taken out of the octanol treatment on day one, but not for the Octanol 0-1 treated worms that were still immersed in the treatment on day one and taken out on day two.

For head regeneration, all octanol-treated worms start day one similar in growth to each other. Starting on day three, the Octanol 0-treated worms differ from the other octanol-treated worms by having a higher mean regeneration per day until through day six (Figure 11A). In comparison, the control worms are steadily increasing in growth

from day one and day three is the first day there is a significant difference in growth between control worms and octanol-treated worms. The control worm growth was significantly higher than both the Octanol 0-2 and Octanol 0-6-treated worms (Table 2).

The octanol-treated worms had slower growth than the controls, as expected. The octanol treatments showed differences between the four different treatment schedules. However, not all of the octanol treatments were significantly different from each other. For both head and tail regeneration, the Octanol 0-treated worms had the most growth. The Octanol 0-6-treated worms had the least growth. The Octanol 0-1 and Octanol 0-2-treated worms had growth lengths between the latter two, with the Octanol 0-1-treated worms most similar to the Octanol 0-treated worms (Figures 10 A and 10B). The octanol-treated worms had more significant differences for mean rate of regeneration within treatments for the tail than the head. This difference between heads and tails is probably due to the sharp decrease in rate of regeneration for tails on day six. But, for both heads and tails, there was slow growth each day until day six which was surprising because it was expected that there would be a recovery period for the worms in Octanol 0, Octanol 0-1, and Octanol 0-2-treatments due to the placement in pond water.

Recovery after octanol exposure

A recovery period is the return to the original state after the treatment is taken away. Chanson et al. (1989) found that there is a rapid recovery period following octanol treatment in pancreatic acinar cells. Pancreatic acinar cells synthesize, store and secrete

digestive enzymes. Chanson et al. (1989) examined the relationship between coupling and secretion using alcohols that uncouple pancreatic acinar cells. Using dye coupling and electrical coupling experiments, they were able to conclude that no modification of cell coupling occurred with hexanol, and cell uncoupling (gap junctions blocked) did occur with octanol. The alcohol induced closure of gap junctions occurred within seconds and was reversible just as quickly after removal of the alcohol.

When *L. variegatus* was taken out of the octanol treatment, the octanol may have left the system immediately, but it still had effects on long term growth. Octanol 0-treated worms were taken out of octanol treatment and placed in pond water on day one. Octanol 0-1-treated worms were taken out of octanol treatment and placed in pond water on day two. Octanol 0-2-treated worms were taken out of octanol treatment and placed in pond water on day three. For both head and tail regeneration, worm daily mean growth steadily increased until day five for Octanol 0, Octanol 0-1, and Octanol 0-2-treated worms. This steady increase shows that if octanol leaves the body, it isn't immediately reflected in growth. Evidence of recovery would be more obvious if there was an immediate increase in growth after removal from the treatment. The mean rate of regeneration per day does not indicate that there is a recovery period on any particular day. When looking at the mean rate of regeneration, it appears that there might be recovery on days after the worm is taken out of the octanol treatment; however, it isn't clear that the increases and decreases on days following the removal of the octanol treatment are due to a recovery period. Regardless of when octanol left the body, there is a sharp decline in growth on day six for both heads and tails, which suggests that there is

not a recovery period and the octanol has a delayed effect. In a study by Emmons-Bell et al. (2015) following a transected head, the flatworm *Girardia dorocephala* regenerated heads with the morphology of other known species of planaria after being immersed in octanol. They found that octanol increased the number of regions in the planarian body with distinct membrane potential (V_{mem}) patterns, and even ten days post octanol treatment, perturbation of gap junction communication altered patterns of voltage distribution in the body. They concluded that even after octanol has left the body, there are prolonged effects of decreased electrical connectivity in the tissues. In the Planques et al. (2019) study of regenerating tails of *Platynereis dumerilii*, they found that blocking cell proliferation during regeneration with hydroxyurea treatment is reversible after removal of the treatment. Regeneration started again and followed normal stages and timelines of regeneration, however the worms treated from day zero to day one post amputation showed delayed segment addition and the worms treated from day zero to day two post amputation had delayed regeneration. In this study, the octanol treatment also seems to have a delayed effect on segment addition on *L. variegatus* even after removal of the treatment.

Critical time period for regeneration

Based on this study, the critical time period required for open communication through gap junctions for worms to have normal regeneration is not known. There may not be a critical time period during the various exposure times of this study. Since

regeneration in the octanol-treated worms slowly increased from day one through day five, it is likely that the duration of exposure is the factor that affects regeneration. The Octanol 0-6-treated worms that were in the octanol treatment all six days had the least regeneration overall. Of all the worms in various octanol treatments, the worms that were in the octanol treatment the least amount of time (Octanol 0) had the most regeneration overall, and overall regeneration decreases as the amount of time exposed increases. Future experiments could stagger days for initial exposure to the octanol treatment to determine if later exposures after transection have an effect on regeneration. These experiments would reveal if different days of initial exposure show different patterns of overall growth, delayed segment addition, delayed overall regeneration, and different abnormal morphologies. Staggering initial exposure to octanol treatment would also help elucidate if there is a critical time period for open communication through gap junctions for normal regeneration. Also, future experiments could study the expression of genes known to be involved in segment, organ and tissue patterning and differentiation during the regeneration process during different periods of octanol treatment. This would elucidate if and when genes are being turned on or off while gap junctions are blocked during regeneration.

Comparison of mean daily overall regeneration and mean rate of regeneration

It is not possible to compare overall regeneration and the rate of growth per day to each other. The rate of growth per day is variable due to some worms having no growth on a particular day. They are different measures of when and how growth is occurring. The rate of growth per day should have been able to show if there was a recovery period, however, the growth was variable each day and did not reveal a particular recovery day. The rate of growth per day did show that there is a decrease of growth on day five and therefore may prove delayed long-term effects of octanol.

EdU results inconclusive

Results of the EdU assay were inconclusive. The EdU stain on proliferating cells was indiscernible as individual cells. (Figures 13-16). It was expected that the regenerating head or tail would be where proliferating cells would be most obvious, however, the EdU stain was not visibly different in the regenerating tissue compared to the rest of the body. The Hoechst stain was more visible at the regenerating tissue than the EdU stain. The expected outcome for the untreated control worm EdU assay was to see fewer proliferating cells at the cut site during the first few days of observation and then to see more proliferating cells around day three as growth was proceeding. It was expected that the octanol treated worms would have fewer proliferating cells than the untreated control worms due to the slower growth and lack of growth in some cases of

treated worms. If the EdU assay showed expected results, there would be a clearer understanding of cell proliferating timelines and where proliferating cells are located.

The Zen 2.3 pro 2012 Blue Edition Image Analysis software that was used to measure the mean intensity of Alexa Fluor and Hoechst gave an output of unitless numbers. To determine if there were any patterns in mean intensity of the overall worm in terms of proliferating cells this data was graphed. This data was also inconclusive because there was not a pattern of more or less proliferating cells per day or per treatment.

CONCLUSIONS

From this study, I conclude that the gap junction blocker, octanol, does have a negative effect on regeneration of *Lumbriculus variegatus*. Worms treated with octanol experience regenerative growth that is notably slower and also results in abnormal morphology of the regenerated tail. Octanol is known to leave the system immediately after treatment is taken away, however, *L. variegatus* did not show immediate recovery of growth when placed in pond water. It is not clear whether there is a critical time period for exposure to octanol to affect regeneration, however; the duration of the exposure corresponds to the severity of abnormal morphology. There is some evidence for delayed effects of octanol around days four and five since the Octanol 0, Octanol 0-1, and Octanol 0-2-treated worms had a sharp decrease in rate of regeneration on day five while having been placed in pond water for previous days. Further experiments monitoring *L. variegatus* post treatment with octanol will help to determine long term effects of octanol. Also, an area for future study would include a more detailed approach to determining if and when there is a critical time point for gap junction communication for normal regeneration.

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APPENDIX

In a study by Emmons-Bell et al. (2015) following a transected head, the flatworm *Girardia dorocephala* regenerated heads with the morphology of other known species of planaria after being immersed in the gap junction blocker octanol. The shape change of the head was not permanent and after regeneration was complete, the animals remodeled back to *G. dorocephala* wildtype head shape after a couple of weeks. They observed that in their study, repair occurred in two phases. Following amputation, head regeneration was completed in less than ten days with a result of different species head morphology, then a subsequent longer remodeling back to normal *G. dorocephala* morphology. They concluded that morphology appears to be a homeostatic parameter and that there is an implication that morphology is consistently reassessed and edited.

In this study, random worms that displayed abnormal morphology from the Octanol 0 (n=3), Octanol 0-1 (n = 1), and Octanol 0-6-treated worms (n = 6) were photographed on day seven through day thirteen, with the exception of day eight. (Figures 17-20). Day eight was not photographed. The worms were all placed in pond water when these photographs were taken. Results showed that two of the worms that did not die before day thirteen remodeled back to normal morphology by day thirteen. (Figures 17 and 18). The morphological remodeling of the two worms may be a similar type of remodeling that *G. dorocephala* experienced but additional experiments are needed for a more conclusive statement. Four out of six of the worms from the Octanol 0-6 treatment died before day ten and the remaining two did not show large increases in

growth. (Figures 19 and 20). This shows potential evidence that the octanol has a delayed effect.



Figure 17: Octanol 0-treated worms days seven through thirteen, excluding day eight. Both Worm A and Worm B had abnormal morphology presenting as dual prongs for Worm A and multiple prongs for Worm B in the tail. Worm A did not recover to normal morphology by day thirteen, however, segment addition did occur in the tail. Worm B recovered to normal morphology of the tail by day ten and continued segment addition by day thirteen. Scale bar = 100 μ m



Figure 18: Octanol 0-1-treated worm days seven through thirteen, excluding day eight. Worm C had abnormal morphology presenting as irregular shape in the tail. Worm B recovered to normal morphology of the tail by day ten and continued segment addition by day thirteen. Scale bar = 100 μ m



Figure 19: Octanol 0-6-treated worms days seven through thirteen, excluding day eight. Worm D, Worm E, and Worm F had abnormal morphology presenting as slow growth and abnormally narrow shape in the head and tail on day seven. Worm D and Worm F died on day 10. Worm E survived until day thirteen, however, did not have any segment growth. Scale bar = 100 μm

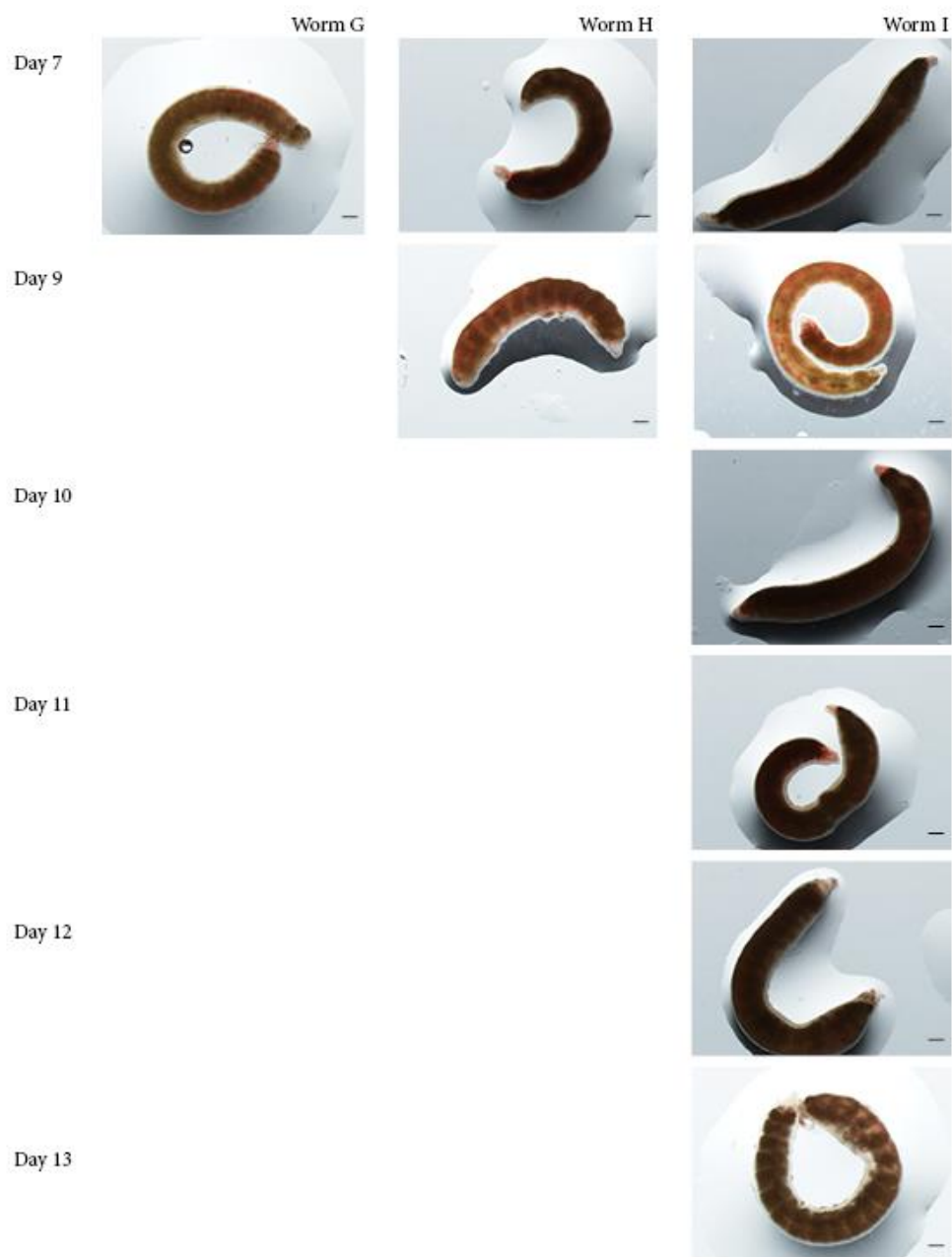


Figure 20: Octanol 0-6-treated worms days seven through thirteen, excluding day eight. Worm G, Worm H, and Worm I had abnormal morphology in the tail on day seven. Worm G did not have any tail growth. Worms H and I had abnormal morphology presenting as slow growth and abnormally narrow shape in the head and tail. Worm G died on day nine. Worm F died on day 10. Worm E survived until day thirteen, however, did not have any segment growth and appears to be dying on day thirteen. Scale bar = 100 μ m