Effects of endocrine disruptors Bisphenol A and Diethylstilbestrol on *Ambystoma mexicanum* limb regeneration

By
Elliott M. Bartels
April, 2015



Allegheny College
Departments of Biology and Environmental Science

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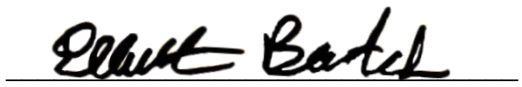
A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.



Pledge

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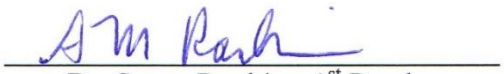
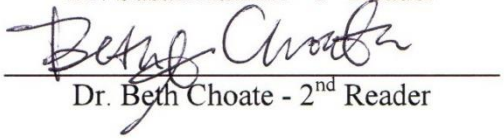


Dr. Susan Rankin - 1st Reader



Dr. Beth Choate - 2nd Reader

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Abstract

With the increasing rate of human health problems around the world in the forms of cancer and birth defects, scientist have focused on a specific group of chemicals called endocrine disrupting contaminates (EDCs). These chronic environmental toxins, including Bisphenol-A (BPA) and Diethylstilbestrol (DES), have been linked to birth defects and cancer in humans. In amphibians EDCs block thyroxine (T4) and other growth hormones that alter development pathways shared with amphibian regeneration. Many amphibians, including the axolotl, *Ambystoma mexicanum,* can regenerate lost limbs through stem cell dedifferentiation.

To examine the effects of EDCs on regeneration, twenty-five juvenile axolotls were exposed to BPA and DES. Axolotls were divided into four groups: (1) untreated control, (2) ethanol carrier control (100 ug/L EtOH), (3) DES (0.00025 mg/ L), and (4) BPA (2 mg/L) with chemicals applied to the water. Prior to treatment, the salamanders were anesthetized and the right forelimb was amputated.

The hypothesis that EDC exposed axolotls would weigh less and have slower or completely inhibited regeneration was partially supported. All groups had complete limb regeneration ($P = 0.68$) and gained weight ($P = 0.42$). However, BPA treatments slowed regeneration when compared to the other groups: ethanol, BPA ($P = 0.0006$); untreated, BPA ($P = 0.0034$); and BPA, DES ($P = 0.0048$).

Smaller salamanders that had previous amputation wounds exhibited much faster regeneration than intact salamanders when initial lengths ($P < 0.001$) were compared to those of week 6 ($P > 0.05$). The ELISA for thyroxine on harvested organs at the termination of the experiment was inconclusive as hormone levels were below detectable limits.

The EDCs in this experiment did not produce deformities, but BPA slowed regeneration. DES, however, did not inhibit regeneration contrary to expectations and further study is necessary to determine how EDCs affect regeneration in amphibians.

Introduction

The rising number of human health cases of cancer, heart disease, birth defects, developmental problems, and mental health disorders has scientists focused on chronic exposure to environmental contaminants ("Bisphenol-A Action Plan," 2010). One group of chemicals found in the environment at large concentrations around the world is endocrine disrupting contaminants (EDCs). Endocrine disruptors affect hormones, such as thyroxine, that are essential in the right amount at crucial stages of development. EDCs mimic or block these essential hormones and prevent normal development, leading to serious health impacts. Two well documented endocrine disruptors are Bisphenol-A (BPA) and Diethylstilbestrol (DES) ("Endocrine Disruptors," 1998). In addition to being harmful endocrine disruptors, these chemicals have been linked to epigenetic histone modifications, which can result in disorders that appear generations later (Crews & McLachlan, 2006). It is clear that further research on endocrine disruptors in the environment is of extreme importance.

Endocrine disruptors leach from nonpoint sources that are hard to pinpoint. Endocrine disruptors DES and BPA are among the most abundantly produced chemicals in the world, with more than six billion pounds manufactured each year (Heimeier & Shi, 2010). These EDCs are found in plastics, epoxy resins, and food cans, which often are improperly discarded and distributed as trash in the environment ("What Are Endocrine," 2011). The United States Environmental Protection Agency (EPA) has conducted numerous studies on levels of BPA in the environment. In the US, the environmental release of BPA exceeds one million pounds per year. Yet, BPA is largely unregulated as the EPA “does not intend to initiate regulatory action under the Toxic Substances Control Act (TSCA) at this time on the basis of human health. The EPA remains committed to protecting human health, but notes that most human exposure, including exposure to children, comes through food packaging materials under the jurisdiction of FDA” ("Bisphenol-A Action Plan," 2010). EPA studies concluded that there are likely endocrine disrupting effects on reproduction and development in lab rats tested at toxic doses >50 mg/kg-bw/day ("What Are Endocrine," 2011). However, government agencies refuse to fully acknowledge its toxicity or agree on intake limits in humans ("Bisphenol-A Action Plan," 2010). The tables below are a compilation of US government-set limitations of BPA and acceptable levels in other countries:

BPA Intake Limits for Human Health Assessments

Authors	Intake Limit (mg/kg/day) [1]	Endpoint (Animal dose in mg/kg/day) And Study
USEPA (Integrated Risk Information System; IRIS (1993))	0.05	Reduced body weight (5) NTP 1982 two year cancer study in both rats and mice (as cited in USEPA 1993)
FDA (2008)	0.005	Systemic – reduced body wt and liver effects (5)
	0.05	Irreversible reproductive effects (50)
	0.5	Reversible reproductive effects (50) (All based on both 2-generation mouse study (Tyl et al., 2008) and 3-generation rat study (Tyl et al., 2002))

Summary of Bisphenol A Ecological Hazard Values

Country	Predicted No Effect Concentrations[1] (µg/L)	Endpoints
European Union	1.5	The predicted no effect concentration (PNEC) for aquatic organisms (derived by using a statistical analysis of data from available data on freshwater and marine aquatic organisms (in this case, 16 different studies, unpublished and published, from 10 different taxonomic groups) to arrive at a value of 7.5 µg/L, which is divided by an uncertainty factor of 5, resulting in a PNEC of 1.5 µg/L (EC, 2008).
Canada	0.175	This PNEC was derived by using a lowest observed effect concentration (LOEC) of 1.75 µg/L for reduced semen quality and delayed ovulation in a published brown trout study (Lahnsteiner et al, 2005) and applying an uncertainty factor of 10 (Canada, 2008).
Japan	1.6	The PNEC was derived by using the 16 µg/L no effect concentration (NOEC) for egg hatchability in fathead minnows from the unpublished 3 generation study by Sumpter, et al. (2001) multigeneration fish study and dividing by an uncertainty factor of 10 (AIST, 2007).

[1] In Europe, Canada, and Japan, a predicted no effect concentration (PNEC) is compared directly with an exposure value to evaluate risk. If the ratio of environmental concentration to PNEC is less than one, the risk is generally considered acceptable. As noted in the table, countries use different approaches for generating PNECs, and the precise values may differ even when based on the same studies.

Figure 1: BPA limitations around the world. US agencies do not agree on acceptable levels, and these differ from acceptable limits set by other governments ("Bisphenol-A Action Plan," 2010, p. 22).

Once in the watershed, EDCs disrupt development in fish, amphibians, and can be traced to the drinking water of humans (Lintelmann et al., 2003). Fish and amphibians are especially affected by environmental toxins since they respire through their skin and/or gills. The presence or absence of species of fish and amphibians can be used to determine overall health of a waterway. Many species of mole salamanders like the Mexican tiger salamander, *Ambystoma mexicanum*, better known as the axolotl, are used as indicator species (Scott, 1995). Their absence in a waterway should be alarming to humans who share the water for drinking and bathing because such absence signals significant environmental issues.

Not only are axolotls important indicator species, but they are one of the few animals that can regenerate lost organs and limbs. Being vertebrates, these salamanders share similar developmental stages and hormones with humans. EDCs that block hormones such as the thyroid hormone, thyroxine (TH), in the developmental stages of salamanders similarly affect humans. TH is essential for development of amphibians and common endocrine disruptors can alter or block TH pathways (Lintelmann et al., 2003).

The research question explored by this study was: Does exposure to the endocrine disrupting chemicals BPA and DES inhibit regeneration in *Ambystoma mexicanum*? It was hypothesized that axolotls exposed to BPA and DES after limb amputation would regenerate more slowly or experience completely inhibited growth. It was further hypothesized that treated animals would weigh more than untreated control animals. Untreated animals were expected to regenerate limbs faster (in an anatomically correct manner) and weigh less by the end of the trial period. Additionally, experimental groups treated with EDCs were expected to have lower serum levels of TH when measured with an ELISA.

Literature Review

Axolotls have been used as model specimens for studying the effects of endocrine disruptors in vertebrate developmental studies because of their ease of breeding and regenerative properties. Axolotls in the wild are listed by the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora as an endangered species and by the International Union for Conservation of Nature (IUCN) as critically endangered in the wild (Scott, 1995).

Axolotls are native to Lake Xochimilco and Lake Chalco of central Mexico. Members of the Phylum *Chordata,* axolotls are vertebrates, meaning they have a cranium and vertebral column. Axolotls are in the class Amphibia ("Welcome to Ambystoma Genetic," n.d.); order *Caudata;* and family *Ambystomatidae*, the mole salamanders. Mole salamanders range from North America to Central America-Mexico. Axolotls are unique within the family *Ambystomatidae* because they retain the larval body form when reproductively mature. This is an adaptation called neoteny. Most salamanders go through complete metamorphosis, beginning with an egg, to a gilled larva, and ending with a semi-terrestrial adult (Scott, 1995). Axolotls develop both hind and front limbs and retain their tails in their adult stage, but remain fully aquatic. Axolotls have large feathery gill extensions on both sides of their head used for gas exchange (Clare, 2011).

The introduction of iodine in the water supply in combination with environmental stressors, such as the lowering of the water level or change in temperature, can induce axolotls into metamorphosing into the terrestrial adult form. However, since axolotls have evolved neotenic, induced metamorphosed adults are usually not as healthy as their neotenic counterparts and only live for three to five years compared to twenty (Scott, 1995).

The biological mechanism that induces adult metamorphosis in axolotls is unknown. T4 released by the thyroid gland stimulates metabolism and development. The anterior pituitary gland regulates the release of T4 through the thyrotrophic stimulating hormone (TSH) (Heimeier & Shi, 2010). The hypothalamus secretes thyrotrophin-releasing factor (TRF), which regulates the use and release of TSH. The combined release of these hormones is thought to be triggered by both internal and external factors such as temperature and the presence of iodine in the water source (Fini et al., 2009). Neotenic species are found in water sources that lack iodine. Iodine plays an import role in the release of the thyroid hormones. The lack of iodine in natural habitats of the axolotl would account for neoteny through evolution (Scott, 1995).

Amphibian Regeneration

All animals possess some ability to regenerate. Mammals cannot regenerate lost limbs, but can regenerate lost skin and hair cells as well as repair broken bones or muscles (Simon & Thompson, 1964). Amphibians have remarkable regeneration possibilities, with larval amphibians having the greatest potential. Young salamanders and frog tadpoles can regrow lost limbs and tails, while the adults usually cannot (Straube & Tanaka, 2006). For example, if the hind limb of a froglet were removed, it would regrow. If the hind leg of an adult frog were removed it would not likely regenerate (Simon & Thompson, 1964). Young axolotls can regrow portions of the tail, arms, gills, and head within two to three months. Once maturity is reached, axolotls lose the ability to regenerate lost body parts; instead, they repair damage through scar tissue. Portions of organs in axolotl larvae such as the liver, spleen, and the retina can regenerate (Roy & Gatien, 2008). Strangely, young axolotls regenerate and heal faster in cooler water than warm. Being coldblooded, axolotls greatly contrast coldblooded fish, where healing is directly proportional to an increase in temperature (Scott, 1995).

The process of regeneration in axolotls at the cellular level has been evaluated. Salamanders can regenerate lost limbs, skin tissues, and several organs identical to the originals. Regeneration follows a well-recognized sequence of events. The loss of a tail or limb activates the following cellular response: After limb loss, the site of amputation constricts and clots to prevent blood loss. Shortly after, epithelial cells migrate to the amputation site and form a wound epithelium. The cells around the wound site lose their specialization (dedifferentiate) (Roy & Gatien, 2008). The dedifferentiated epidermal cells move as a “blanket” or outer covering to protect exposed

mesenchymal tissues and to form the mesenchymal growth zone. The mesenchymal growth zone (blastema) promotes re-differentiation to form the new limb. Blastema cells can become a variety of specialized cells needed in the process. Muscle, cartilage, nervous, and connective tissues can transform into blastema cells (Ord & Stocken, 1984). However, blastema cells originate from dedifferentiating internal cells and not apical limb epidermal cells. After fifteen days, the blastema is fully formed and after twenty days, begins to flatten and form a limb (Straube & Tanaka, 2006). Dedifferentiation and histogenesis continues, resulting in the reformation of bone, muscle, and cartilage (Sicard, 1985). The first to reform are cartilage cells, followed by muscular tissue. Soon after, blood vessels, connective tissues, and nervous tissue appear (Chaar & Tsilfidis, 2006). In tail regeneration, the notochord and spinal cord extend into the new section of tail by the thirty day mark (Kimball, 2011). By the thirtieth day, the arm has fully regenerated with the first few fingers appearing. By the fortieth day, the last few digits appear, completing regeneration. Pigmentation continues to darken after the forty days and muscle continues to grow at the sites of regeneration (Ord & Stocken, 1984).

Hormones in Metamorphosis and Development

Hormones play an important role in the metamorphosis and regeneration of amphibians. Hormones are essential for body regulation, maturation, and sexual development in all chordates. In regeneration, the endocrine system stimulates the dedifferentiation and proliferation of cells at the wound site. As discussed earlier, thyroxine-T4, thyrotrophic stimulating hormone (TSH), and iodine play an integral part in the proper development of amphibians (Brown, 1997). Estradiol, estrone, and estriol are forms of estrogen. Estrogen promotes secondary sex characteristics in developing females as well as growth and mating behavior. Estrogen also maintains pregnancy and prepares the breasts for lactation in mammals. Progesterone, another female sex steroid, regulates changes during menstruation and maintains the development of fetal membranes (Kloas, Lutz, & Einspanier, 1999). These hormones are essential to the proper development of amphibians and regeneration (Lintelmann et al., 2003).

Endocrine Disruptors and Development

Hormonal and environmental factors both affect amphibian regeneration. Environmental contamination increases deformities in amphibians undergoing epimorphic regeneration. These

deformities are caused by chemical interference of the endocrine systems. Endocrine disruptors are present in many common household items ("Endocrine Disruptors," 1998). Many of the compounds are manmade industrial compounds: heavy metals, chemicals, and crop pesticides. Exposure to EDCs, even in small amounts, disrupts normal endocrine processes and can cause cancer, low sperm counts in males, or the inability to reproduce (Lintelmann et al., 2003). EDCs affect the body by binding to receptors and activating or blocking normal functions. Alteration can also occur through the modification of normal hormone levels, halting the production of hormones, or changing the form in which hormones travel through the body (Heimeier & Shi, 2010).

Endocrine Disruptors in the Environment

Exposure to EDCs may occur in several ways: pesticide/insecticide runoff, direct contact, surface water runoff, sewage treatment, rainfall, and the inhalation or ingestion of contaminated water, food, or air. EDCs can enter the air or water as a byproduct of many chemical and manufacturing processes where plastics are burned or formed ("What Are Endocrine," 2011). EDCs also leach out of plastics and cans used in food packaging. Eating fish or other animals contaminated with EDCs can also cause health problems. Several chemicals are known EDCs: diethylstilbesterol (DES), dioxin, polychlorinated biphenyls (PCBs), dichloro-diphenyl-trichloroethane (DDT), bisphenol-a (BPA), as well as other pesticides and plasticizers (Kloas & Lutz, 2006).

The effects of EDCs have been well documented. The Great Lakes are contaminated with polychlorinated biphenyls (PCBs) and other man-made chemicals, like BPA. PCBs are commonly found in coolant fluids, capacitors, motors of industrial machines, and in electronics. EDC presence in the Great Lakes has resulted in reproductive problems and abnormal thyroid glands of resident sick fish. Fish-eating birds in the Great Lakes region, including eagles, terns, and gulls, showed similar dysfunctions because of bioaccumulation (Carlson & Swackhamer, 2006). Another study in Lake Apopka, Florida, found a decline in the alligator population due to sterile reproductive organs. The sterility rendered a large population of alligators unable to breed and was linked to a pesticide spill upstream that occurred several years earlier. Eggs laid by adults following successful breeding did not hatch as they also contained EDCs (Semenza et al., 1997 and "Endocrine Disruptors," 1998).

One study, in the natural habitat of axolotls, the Xochimilco Wetland of Mexico City, examined water samples to determine the levels of several endocrine disruptors. It was believed that these chemicals had an inhibitory effect on the reproductive abilities of the native animals, including axolotls. Monitored areas included agricultural sites, urban sites, and livestock pastures. The EDC levels determined by gas chromatography/mass spectrometry were not high enough to produce endocrine disruption in humans, but could in fish and amphibians. The concern was that humans may be impacted by the EDC presence in the wetland as it provides the majority of Mexico City with its drinking water. In Lake Xochimilco (see figure below) the average concentration of BPA in the wetland was 30 ng/µL. This low level was one of the few studies conducted in the axolotl natural habitat.

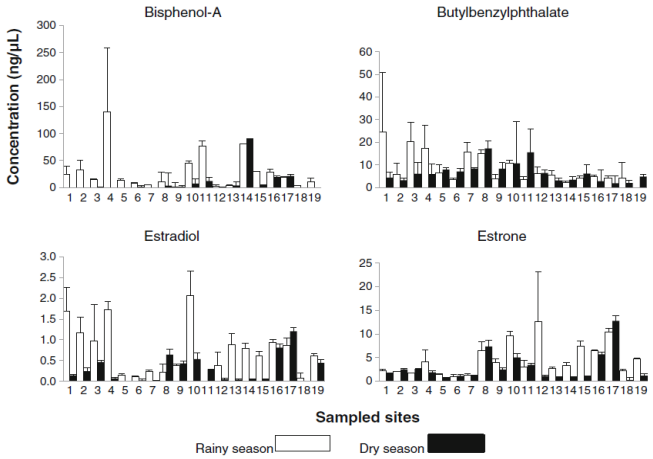

Fig. 3 Average and SD of the concentration of each compound analyzed in Xochimilco wetland water in both seasons: rainy season (*empty bars*) and dry season (*filled bars*)

Figure 2: Portrays results of BPA levels found in the Xochimilco wetland, the natural habitat of axolotls (Díaz-Torres et al., 2013). BPA was reported in the wetland at 30 ng/µL.

EDC levels were correlated with land uses. Areas near farms or heavy industry had the highest levels of EDCs. All of the chemical contaminates were elevated within the wetland samples during the rainy season, likely because runoff from the rain into the watershed increased during the rainy season (Díaz-Torres et al., 2013). The need for further examination of the effects of EDCs on regeneration and metamorphosis processes in amphibians is critical.

Amphibians as Indicator Species for EDCs

Amphibians are highly susceptible to environmental conditions because of their semi-permeable skin and because they spend the majority of their lifecycle in the water. Humans are also susceptible to these chemical compounds. In humans, EDCs like PCB and BPA have been

linked to the disruption of thyroxine (TH) production, which may be a leading cause of disorders in humans globally (Fini et al., 2012). Disruption of TH production, which is necessary for proper vertebrate development and brain maturation, has been linked to human reproductive inabilities and cancers of the breasts and testes. The African Clawed Frog, *Xenopus laevis*, has served as a model organism in several of these tests because it remains aquatic its entire life and is easy to breed (Levy et al., 2004). Clawed frogs exposed to BPA had interrupted TH production, which is necessary in amphibians to trigger metamorphosis. BPA affects the estrogen system, which shares the same pathways with the TH regulating system (Fini et al., 2012). Endogenous and exogenous estrogens affect the regulation of numerous genes during transcription that must be activated by specific hormone promoters. These estrogen response elements (EREs) promote growth factors in the body including fibroblast growth factors and hepatocyte growth factors. These factors are necessary for regeneration and wound healing (Taylor et al., 2011). BPA can bind to both estrogen and TH receptors. When BPA binds to TH receptors, TH is unable to bind and developmental metamorphosis is disrupted. BPA interrupted metamorphosis produced limb deformities in frogs and axolotls. BPA also suppressed thyroid hormone T3, necessary for tail regression of tadpole metamorphosis. Human fetuses depend on TH for proper development as well (Heimeier & Shi, 2010).

The EDC, diethylstilbesterol (DES), has devastating effects on vertebrate development and is a synthetic xenoestrogen. From the late 1940s to the 1970s, DES was medically prescribed to pregnant women to prevent miscarriages. In the 1970s the United States Food and Drug Administration banned DES because doctors reported alarmingly high numbers of vaginal cancers in young females ("DES History," n.d.). Over five million pregnant women were given DES. Problems did not arise until the teenage girls reached puberty. Birth defects, vaginal cancers, and sterility were common in the teenagers whose mothers had been given DES. The government responded by making DES illegal for use in pregnant females. However, DES remained on the market until the late 1990s in other countries as a postcoital contraceptive ("What Are Endocrine," 2011). Today, DES is restricted to laboratory use and is not available to the public.

The Need to Be Concerned

EDCs are well known disruptors of metamorphosis in amphibians, but little is known about

potential effects on limb regeneration. It was expected that axolotls (or any amphibian with regenerative abilities) undergoing amputation while exposed to EDCs would grow/regenerate abnormalities due to hormone (TH) interference. The hypothesis was that limb regeneration would be altered in axolotls exposed to the chemicals BPA and DES. This study is relevant to humans because EDCs cause birth defects and cancer. An estimated ten million Americans were exposed to DES. BPA and DES are structurally similar and have estrogenic effects that alter expression of key developmental hormones, including TH (Doherty et al., 2010). By identifying the effects of EDCs on amphibians we can learn about the potential effects they may have on humans. Manmade objects, like plastics and metals that have endocrine disrupting properties are in our watersheds. An increased awareness of EDCs and their developmental effects is necessary to protect the environment and people from devastating outcomes.

Methods

Applications for the use of living specimens were completed and approved by the Animal Review Committee (ARC) ("ARC Form: Allegheny College," 2003). Axolotls were ordered from the Ambystoma Genetic Stock Center (AGSC) of the Department of Biology at the University of Kentucky. Twenty-five (n=25) 5-8 cm salamanders were ordered for the study with six salamanders for each trial group detailed below ("Welcome to Ambystoma Genetic," n.d.). One extra replacement salamander was kept separately to account for a single untimely death.

On October 15, 2014 the salamanders were delivered. Several of the specimens were smaller than the preferred 5-8 cm size because of issues with low stock. This proved not to be a problem as younger salamanders have an increased chance of morphologically successful and quicker regeneration than older salamanders (Hutchison, Pilote, & Roy, 2007).

Research space in an environmental chamber of the aquatics lab was obtained from the Biology Coordinator, Liz Caskey. The salamanders were allowed to acclimate to the water for a few hours in their plastic shipping bags. Salamanders were randomly selected and placed into one of the four treatment groups (DES, BPA, EtOH, or Untreated). They were randomly selected to eliminate bias in terms of weigh/size preference for treatment selection. Salamanders were kept at 18° C with eight hours of daylight (Makanae & Satoh, 2012).

Multiple flow through tanks made from RubberMaid® containers were constructed and equipped with air bubblers to maintain water quality for six weeks. Six 200 ml cups with holes

drilled through the side were placed per block to allow the water to move through each container and evenly distribute the chemical treatments (Figure 3 below). Axolotls were placed in the individual containers to prevent unintentional injuries during the experiment and eliminate variables in regeneration. See Appendix N for an example of a flow through tank (Scott, 1995).

Groups were: (1) untreated control group, (2) ethanol carrier control (100 ug/L EtOH), (3) DES (0.00025 mg/ L), and (4) BPA (2 mg/L). See Appendix B for chemical calculations. DES is approximately 15,000-fold more potent than BPA (Taylor et al., 2011). There were two blocks for each treatment. Containers were setup in the following manner with a total of twenty-four salamanders:

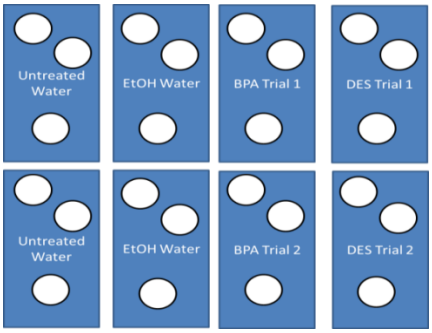

Figure 3: Depicts setup of blocks for each treatment and replicates

The first dosing day the original BPA concentration was based on the findings of Díaz-Torres et al., 2013, in which levels of BPA were 30-50 mg/L. When 50 mg/L of BPA concentration was added to the treatment containers, it immediately clouded, bubbled, and formed a precipitate. Upon review, it became obvious that the units of the graphs published by Díaz-Torres et. al in *Water Air Soil Pollution* were incorrectly labeled, being several times above the solubility of BPA in water (Díaz-Torres et al., 2013). Corrections were made to both the BPA and DES concentrations for them to be water soluble and still expected to have observable EDC effects. See Appendix B for revised concentration calculations.

After a one week quarantine and acclimation period, the salamanders were removed one at a time and anesthetized using a 0.1% solution of MS222 (Sigma), Tricaine Methanesulfonate, for one minute. (Youngs, 2012). The salamanders were weighed (g) and the length of the right arm was measured (mm) prior to amputation. Following measurement, the distal third of the right forelimb was amputated with scissors. Hemostasis, the stoppage of bleeding, occurred almost instantly; regeneration started shortly after. Antibiotics and other post-operative medications were not administered to the wound site. Several of the smaller salamanders were missing

portions of their arms and had started regeneration. Salamanders that already started regeneration of their right arm had the blastema site reopened. After amputation, the salamanders were returned to their individual cups and allowed a week before the treatments were administered.

Treatments were administered to the water in each of the 2 L containers at the pre-determined concentrations starting October 24, 2014. Water was changed and treated appropriately each Tuesday in addition to having weekly arm length (using a dissecting microscope ocular) and weight measurements taken for six weeks. Water was topped off each Friday before the weekend to ensue water levels did not drop below the 2 L line for proper aeration purposes. Axolotls were fed at least twice a week with exception for long weekends or breaks, in which they were fed more than the usual amount the day before extended leave. Two to three pellets of specially formulated axolotl feed was given to each specimen.

In addition to taking weekly measurements, pictures of the regeneration were taken each week using the DinoCapture Microscope Camera. Three specimens from each of the four trial groups were selected and photographed each week. A sampling of pictures from each set can be viewed below in Appendix M. Pictures were not taken at the last measurement period as there was no significant change in regeneration; only continued growth or enlargement of digits occurred.

Week six measurements marked the last recorded data with the forty day regeneration period being reached. Salamanders were humanely euthanized on December 10, 2014 using an increased concentration of MS222. Due to the almost instant hemostasis in axolotls, trunk blood extraction was not effective for collecting blood samples needed for the TH Enzyme-Linked ImmunoSorbant Assay (ELISA) (Levy et al., 2004). Organs rich in TH and blood, including the heart, liver, and spleen, were selectively removed instead under a dissection microscope ("ELISA (Enzyme-Linked ImmunoSorbant Assay)," n.d.). After harvesting, the organs were homogenized in 100 ul of amphibian phosphate buffered saline (PBS) (See Appendix A for formula) and centrifuged for ten minutes at 13,000 RPM. The supernatant was carefully pipetted and frozen in preparation for the ELISA.

A total thyroxine (T4) ELISA kit (GenWay Bio) was used to determine levels of T4 of the organs harvested. Performed in a 96 well plate, the bottom was pre-coated with three main plasma proteins found in blood that reversibly bind to T4 almost 100% of the time. These proteins were thyroxine binding globulin (TBG) (binds ~70%), thyroxine binding pre-albumin

(TBPA) (binds ~20%), and albumin (binds ~10%). The ELISA was then completed following manufacturer's instructions. The Absorbance was read at 450 nm with a microtiter plate reader. The intensity of the yellow color produced by the plate was proportional to the levels of enzyme left (Total Thyroxine (t4) Enzyme, 2015).

Data Analysis

Data were analyzed using an analysis of variance (ANOVA) using the StatView statistical package in conjunction with Microsoft Excel. A standard curve for the ELISA was created in Excel using the standards to determine total T4 levels from the organ samples that fell within the curve.

Results

Limb regeneration and total body weight gain occurred in all groups. There was no statistically significant difference between groups as shown in Figures 4 and 5 below, except for BPA. The ethanol control showed a general trend of having the highest levels of weight gain and fastest regeneration. DES treated animals depicted a trend of the second highest values for both limb regeneration and body weight change, but was not significant.

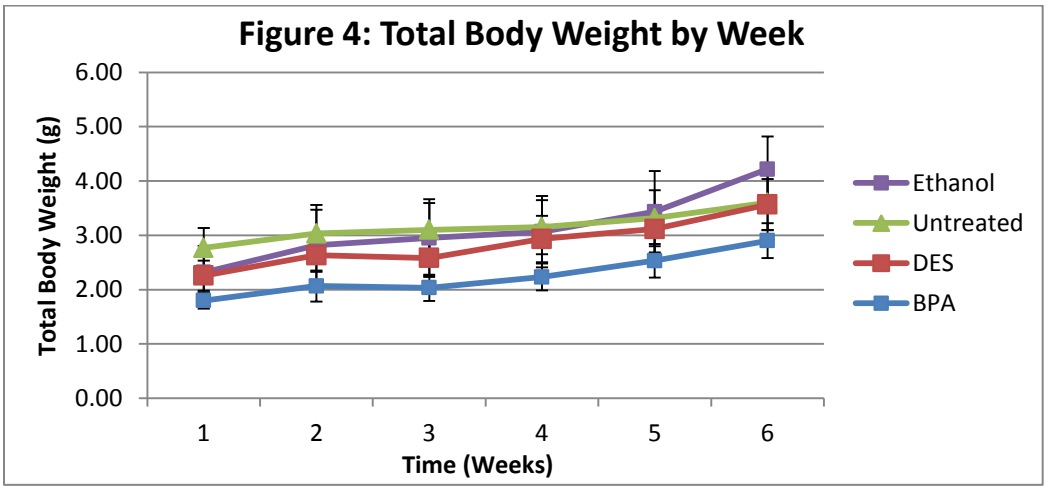

Figure 4: Over a 6 week period, 4 test groups of axolotls of 6 replicates each were exposed to corresponding treatments of BPA, DES, untreated water, and EtOH. Each week total body weight was recorded. At the end of the sixth week final weights were recoded before the salamanders were humanely sacrificed. There was no statistical significance between groups by the 6th week (P = 0.42).

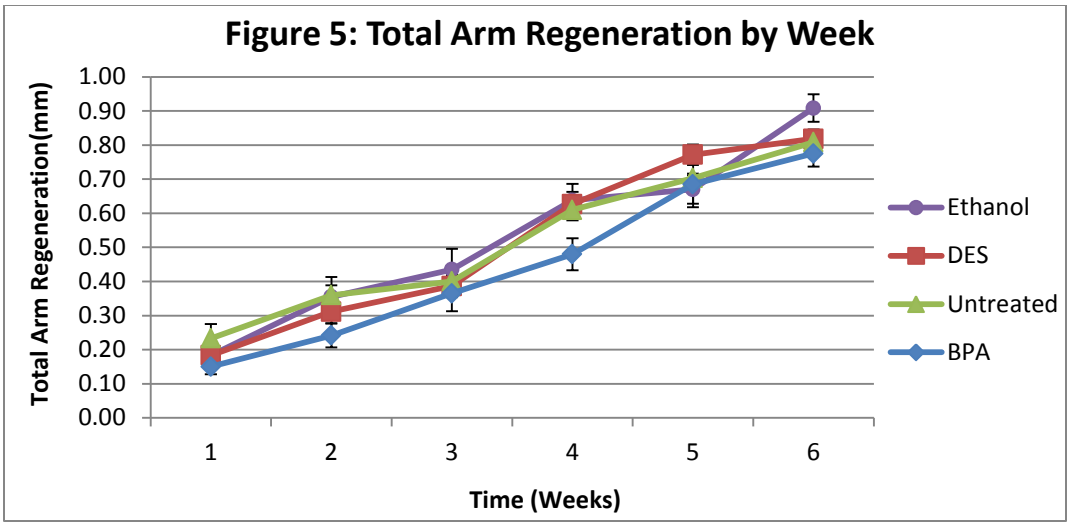

Figure 5: For 6 weeks total arm regeneration was recorded each week under a dissection microscope (1x = mm). Total arm length was measured from the base of the arm to the site of amputation. There were 4 treatment groups with 6 replicates each for DES, BPA, untreated, and EtOH. BPA slowed development when compared to the other groups: ethanol, BPA (P = 0.0006); untreated, BPA (P = 0.0034); and BPA, DES (P = 0.0048). There was no statistical significance between the other test groups for total arm regeneration (P = 0.68).

Severed limbs increased in length each week in all groups (Figure 5 + 6). BPA slowed limb regeneration when compared to the other groups (P < 0.0001). BPA slowed regeneration compared with: ethanol treatment (P = 0.0006), no treatment (P = 0.0034), and DES treatment (P = 0.0048). The final limb lengths of the ethanol treated animals were longer than those of BPA immersed salamanders (P = 0.02).

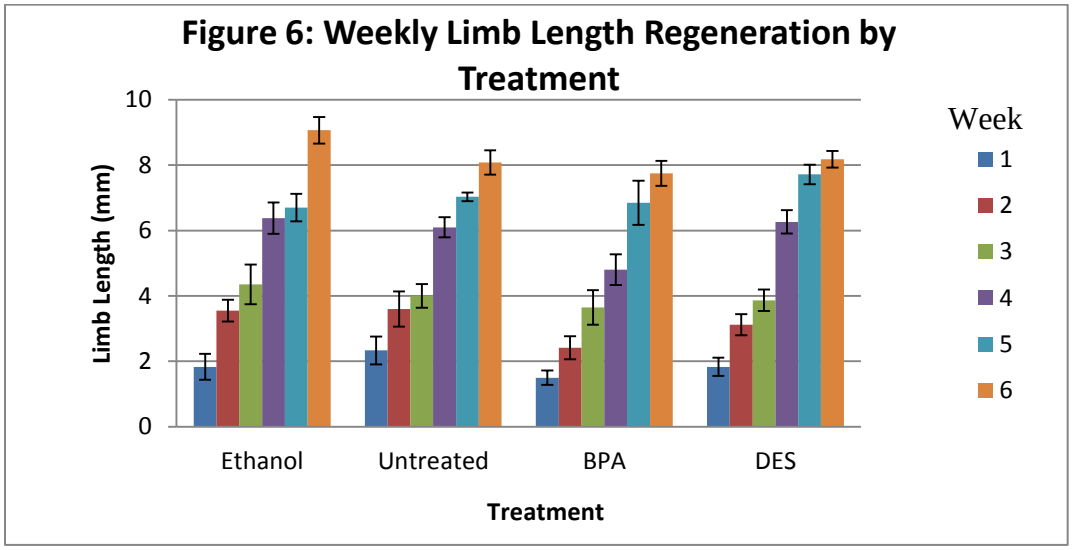

Figure 6: Depicts the comparison of total limb regeneration (mm) for each treatment group (BPA, DES, untreated, and EtOH). Reported values were averaged by week per the six replicates per treatment. Salamanders were given 6 weeks to complete regeneration. Each group completed regeneration within the allotted time frame.

The 10 smallest salamanders that had previous limb amputations had limb length significantly shorter than those of the larger animals with intact limbs before treatments were administered (Figure 7A; $P < 0.001$).

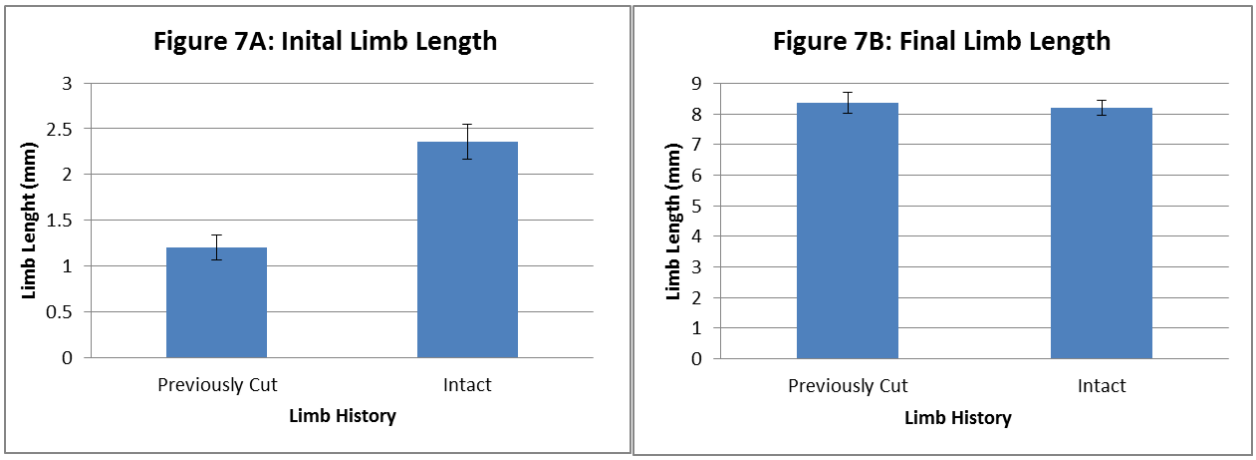

Figure 7: The 10 smallest salamanders that had experienced limb severance prior to arrival at Allegheny College. The remaining 14 larger salamanders had their arms intact upon arrival. **A)** Limb length before amputation ($P < 0.001$). At the initiation of the experiment, the right forelimb of each salamander was severed mid-humorous, leaving a 2 mm stump site. **B)** Limb length at the end of the 6 week experimentation period. There was no statistical significance ($P > 0.05$) between the groups showing that overall, both resulted in similar levels of regeneration.

However, six weeks after limb severance, limb length of both groups of salamanders were not statistically different (Figure 7B; $P = 0.68$). Thus, the severed limbs of the smaller salamanders regenerated more quickly than those of the large animals. Figure 8 shows the differences between the previously cut individuals and the intact salamanders as a weekly comparison ($P < 0.0001$).

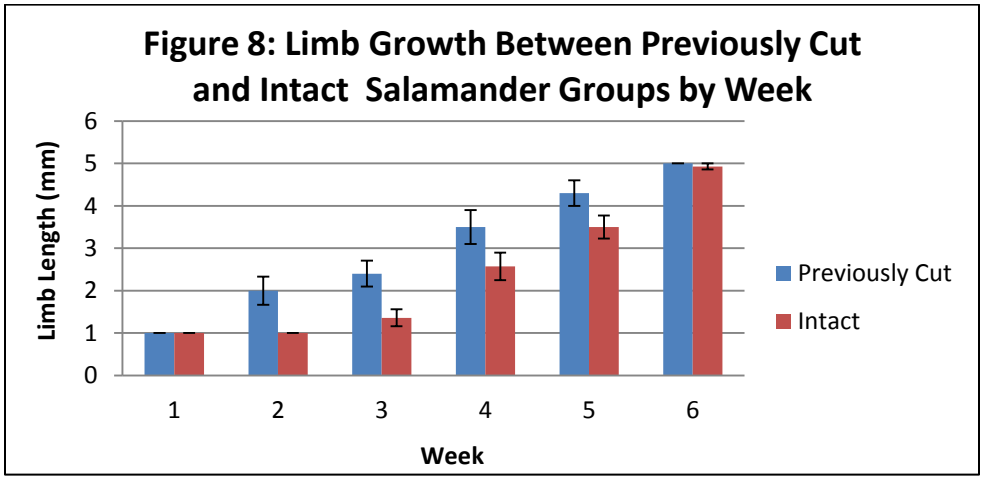

Figure 8: Weekly limb length (mm) comparison between 10 smaller salamanders that had amputation previous to arrival and the 14 intact larger salamanders. At the end of the 6 week period there was no statistical significance between groups ($P = 0.68$).

Thyroxine levels of tissue homogenates were below detection limits for all samples of the ELISA. See Appendix L for more information and data produced by the ELISA.

Discussion

The objective of this study was to determine if endocrine disruptors BPA and DES altered or inhibited regeneration in exposed axolotls. It was hypothesized that animals exposed to the EDCs would not complete regeneration or would develop abnormalities after the distal third of the right forelimb was severed. It was further hypothesized that the rate of regeneration in exposed animals would be slower and that exposed animals would weigh less than the ethanol and untreated groups. The hypothesis that exposed animals would not be able to regenerate or develop abnormally was partially supported as all animals completed limb regeneration by the end of the six week period. The BPA treated animals showed statistically significant slower regeneration when compared to all other treatments ($P < 0.0001$). No abnormalities were observed in this experiment. For both total body weight and total arm regeneration (Figures 4 and 5) the ethanol carrier control exhibited the most rapid rates of regeneration and body weight gain.

One explanation why the BPA-treated salamanders had slowed regeneration compared to the other groups is that BPA blocked estrogen receptors (ER) in the body by directly interacting with them. Tadpoles semi-statically exposed to BPA over a two week period likely take up and metabolize the EDC. Even short term exposure to BPA during sensitive periods of development (or regeneration) can lead to hormone alteration. BPA treatments also altered the sex ratio toward females because it acts as an estrogenic compound at even low concentrations (Levy et al., 2004). Mice exposed to BPA had increased abnormal cell proliferation and altered development timing (Doherty et al., 2010). Since 2005, over 125 studies have found estrogenic endocrine disrupting affects caused by BPA at levels once considered below the no-observed-effect level. Acceptable limits of BPA in humans set by the government resulted from tests in the 1980s by the FDA where rats and mice were given several times (thousands to millions) higher than environmentally relevant levels of BPA. Often, the lowest experimental doses were 1000-fold more potent than predicted safe doses. BPA and DES may have different effects at very different dosages respectively (Welshons, Nagel, & vom Saal, 2006). DES, as discussed below, is better understood than BPA because of decades of research.

Diethylstilbestrol (DES), is structurally similar to BPA; both are non-steroidal, both effect estrogen receptors, and both have been implicated as causing devastating effects in human and animal research models (Doherty et al., 2010). DES is often used as a positive control in studies

of endocrine disruption as it is the best understood EDC within the scientific community (Welshons, Nagel, & vom Saal, 2006). While the method in which EDCs affect the body (by binding to and blocking receptors to disrupt normal bodily functions) has been relatively well identified, the time table in which this process occurs is not well understood (Heimeier & Shi, 2010). When animals in the environment exhibit endocrine disrupting effects it is nearly impossible to determine when exposure occurred or for how long. EDC effects as a result of DES exposure may not have been observed in this experiment simply because the six week period was insufficient time for acute effects to occur at the existing development stage, even though it was enough time for regeneration (Semenza et al., 1997). The salamanders were likely past the early development stages where regeneration and development are most susceptible to EDCs, known as sensitive periods (Hogan et al., 2008). Most documented cases of endocrine disruption occurred after chronic exposure for several years, at an early development stage, and the effects were not identified until several decades later ("Endocrine Disruptors," 1998). One of the most prominent cases of EDCs was the prescription of DES to pregnant females from 1940-1970s to relieve morning sickness and prevent miscarriage. Of the believed 5-10 million pregnant women and the children exposed to the chemical, the observable health effects such as birth defects and infertility were not observed until ten to fifteen years after their mother used DES. Children born with birth defects and later found to be infertile were exposed to the EDCs when they were fetuses. Early stages of development are most vulnerable to hormone changes. The sometimes delayed effects make the study of EDCs hard in laboratory settings where there is not enough time to determine the extended effects of animals exposed, even with short generation times. Our knowledge of DES today is mainly the result of studies conducted by the CDC over thirty years. It is possible that endocrine disrupting effects as a result of DES exposure were not observed because DES takes longer than BPA to affect living tissues ("DES History," n.d.).

Effects of EDCs vary greatly among individuals ("Endocrine Disruptors," 1998). EDCs alter essential hormones such as TH through the modification of normal hormone levels, halting the production of, or changing the form in which the hormone travels through the body, but the effects vary greatly from person to person or between animals of the same species (Heimeier & Shi, 2010). In the case of DES exposure in females, the CDC found that “not all exposed persons will experience DES-related health problems.” Indeed, thousands of patients with confirmed

exposure exhibited little to no changes in health ("DES History," n.d.). The EPA has placed few regulatory limitations on several EDCs, including BPA under the Toxic Substances Control Act (TSCA) ("Bisphenol-A Action Plan," 2010). The salamanders of this study did not show effects from DES, but other axolotls or other salamander species may be more susceptible. Similar results were found in the Lake Apopka, Florida study where EDCs from a chemical plant spill upstream several years earlier left the majority of the alligator population sterile. Some animals however, were not affected and continued breeding normally. Other individuals were fertile, but resulting eggs were unable to hatch or had too thin of shells as a result of EDC exposure. The levels of exposure, lengths of exposure, and differences in hormones/bodily function affected may account for the variation of EDC impacts among individuals (Semenza et al., 1997).

It had been hypothesized that groups treated with EDCs would have lower serum levels of TH when measured with an ELISA. Unfortunately, the T4 ELISA did not yield any indication of hormonal changes in the EDC exposed animals as hoped. ELISAs are one of the few ways to determine if someone was exposed to EDCs. Detecting EDCs without knowing that the subject or person was exposed to them is difficult as there are currently no medical tests (blood, urine, or skin analysis) that can directly determine if someone was exposed ("DES History," n.d.). Studies in other amphibian species (mainly *Xenopus laevis*) found that animals exposed to DES and BPA had altered levels of several hormones (Fini et al., 2012). Exposure to the xenoestrogens also caused feminization, the development of yolk protein in male frogs, and in the reduction of male gonads (Levy et al., 2004). While feminization effects of EDCs were not the focus of my study, the time allotted may not have allowed for the development of these effects. In addition, the majority of the animals in this study were too young to determine sex even when dissected. Further study with additional ELISA tests and larger/older male axolotls may shed more light on the potential effects of EDC exposure in amphibians.

Iodine and the hormones of the thyroid (T3 and T4) are essential to development and regeneration in amphibians (Fini et al., 2009). The natural habitat of axolotls lacks iodine, accounting for their lack of an adult terrestrial metamorphosis stage common to almost all other salamanders (Scott, 1995). In frogs and most salamanders, the exogenous addition of T3 or T4 can prematurely induce metamorphosis, while the removal of either can blocks metamorphosis, leading to giant tadpoles. Made from the amino acid tyrosine, the addition of iodines creates the thyroid hormones thyroxine (T4) and triiodothyronine (T3). The addition of tyrosine and iodine

creates monoiodothyronine (single iodine) and diiodothyronine (two iodine functional groups). When two diiodothyronines combine, T4 is created (four iodines). When a monoiodothyronine and a diiodothyronine combine, T3 is created (three iodines). The thyroid gland takes in iodine from the blood of amphibians to produce both T3 and T4. The release of these hormones triggers development and metamorphosis. The lack of iodine results in the inability to continue development through metamorphosis (Paris & Laudet, 2008). The rate of metamorphosis is directly dependent on the levels of iodine that amphibians receive. When iodine was added to the water or food, tadpoles developed into adult frogs. Metamorphosis was delayed by the lack of iodine. It has been hypothesized that axolotls may not have functional thyroid glands, which may account for neoteny in addition to lack of natural levels of iodine in their habitat (Swingle, 1919).

Exposure to BPA similar to levels found in human infants resulted in delayed tadpole development as a result of certain genes not "turning on". As in humans, thyroid hormones bind to thyroid hormone receptors in amphibians that activate gene transcription. BPA exposure at levels as low as 0.1 - 10 micromolar inhibited T3 response genes from activating, greatly inhibiting the development of the tadpoles into frogs. These levels of interruption are of concern as human infants with similar exposure levels of BPA are likely to display comparable deleterious effects that result in developmental disorders (Heimeier, Buchholz, & Shi, 2009). This study greatly demonstrated the importance of studying in vivo endocrine disruption as human infants are at risk.

One of the most interesting developments from this study was the difference in the rate of limb regeneration between salamanders that had previous amputation and those who were intact. Ten of the smaller salamanders (marked in red Appendices C & D) had quicker regeneration than did the fourteen larger salamanders. Both groups had their limbs severed and the stump site of previous regeneration was reopened on the smaller salamanders. Both groups were provided the forty days necessary for axolotls to regenerate their arms (Makanae & Satoh, 2012). At the end of the sixth week final measurements were taken for all salamanders and compared. There was statistical significance between the smaller (previous regeneration) and larger salamanders (intact) with $P < 0.001$ (Figure 7). By the end of the sixth week when re-measured there was no significance ($P > 0.05$) showing that both resulted with similar levels of successful regeneration. When compared weekly (Figure 8), the smaller salamanders regenerated much faster than the larger intact salamanders, especially those belonging to the EtOH group. This finding has

precedent in other studies where it was found that once regeneration had started interruptions, such as reopening of the stump site, resulted in only minor setbacks (Diogo et al., 2014). Chemical and physical signals, like nerve signals and TH release, trigger the progenitor cells of the stump site to dedifferentiate (Godwin, 2014). Restarting the interrupted process is quicker than the de novo process so the salamander can avoid further tissue damage and permanent disfigurement (McCusker & Gardiner, 2011).

There were several potential sources of error in this experiment. Some occurrences of weight and regeneration loss were recorded. This I attribute to the gram scale's calibration or the movement of the salamanders during weight or arm measurement. The salamanders were hard to measure because they moved, decreasing the accuracy of the measurements. More accurate limb measurements may have been possible by anesthetizing the salamanders at each measuring period. This, however, was not done out of fear that weekly anesthetic may cause health problems. The decline in regeneration by week shown in replicate 4 of BPA (Appendix F) was a result of user error in measuring as no regression of regeneration or loss of weight was observed under the microscope. An important anecdotal observation was that salamanders within the treatment groups of DES and BPA had lower responses times than untreated groups when it came to handling. The untreated and EtOH treated groups were more active and more likely jump out of their containers when it was time to take weekly measurements or change the water. BPA and DES treated animals have been observed to have chronic symptoms of dizziness and sluggishness in vertebrate models of similar studies (Heimeier & Shi, 2010).

Another source of error is the way in which treatments were administered. The concentrations of DES, BPA, and EtOH were diluted past the intended concentration each week as water was "topped off" before the weekend. Water had to be topped off as the bubblers providing the necessary oxygen to the water greatly increased the rate of evaporation. If left until the next treatment dosing period the following Tuesday, the axolotls would not have had enough water to survive the weekend. Either dosing twice a week or increasing the depth of the individual cups would correct this issue.

A third issue to be corrected for a follow-up experiment was the inability to produce accurate results with the ELISA. A standard curve was not produced as a result of several possible errors: pipetting mistakes or from the inability to tap out the water droplets following incubation. A human total T4 ELISA kit was used as an amphibian specific one was not found. Even though

humans and amphibians share the same forms of thyroxine, it is possible that the standards for the kit were beyond the detectable limit for our samples. If time and financial abilities permit, ordering two of the same kits may be beneficial so that one could be used to better determine the standard curve before running the homogenized organ tissue samples.

In conclusion, the hypothesis that limb regeneration would be altered in axolotls exposed to the chemicals BPA and DES was partially supported as DES treatments had no effect on weight change or limb length regeneration, but BPA treatments did. Furthermore, the interesting finding that previously amputated salamanders regenerated much faster than intact salamanders supported similar findings in other research. Additional research will be required before the effects of EDCs can be considered well understood. There is still no definitive test to determine if an organism was exposed to EDCs and for how long. Only continued and persistent research will allow for a better understanding of the effects EDCs have in the living organisms and how long it takes for symptoms to develop, if at all. Amphibians, with their shared hormones and similar organ morphology as humans, are the best hope for a more comprehensive understanding of EDCs as an estimated 10 million people are already contaminated (Doherty et al., 2010). As EDCs continue to leach into the environment from chemical waste and garbage, at a rate exceeding one million pounds per year, they will persist as dangerous environmental contaminates ("Bisphenol-A Action Plan," 2010).

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Appendix

Appendix A:

Amphibian/Xenopus Assay – Phosphate Buffered Saline (PBS) for 2 L

- 16.0 g NaCL
- 0.4 g KH_2PO_4
- 4.4 g Na2HPO4.7H2O dibasic
- 0.4 g KCl
- Bring up to 2L with distilled water, pH to 7.2

Appendix B:

Original Solution Calculations

BPA Solution

$$\frac{50\ ng}{ul}\ in\ 2\ L\ H_2O\ Tank\ \longrightarrow \frac{50\ ng}{ul} = \frac{50\ mg}{L} = \frac{100\ mg}{2\ L}$$

For 2 tanks, add 200ul if stock BPA to each 2L tank

Mixing Steps:

1. Weigh out 0.2g BPA on scale
2. Add 400 ul EtOH to test tube + weighed BPA
3. Vortex 2-3 minutes until soluble
4. Add 200ul of stock #3 to each tank

DES Solution

$$\frac{2.5\ ng}{ul}\ in\ 2\ L\ H_2O\ Tank\ (20x\ less\ than\ BPA)\ \longrightarrow \frac{2.5\ ng}{ul} = \frac{2.5\ mg}{L} = \frac{5\ mg}{2\ L}$$

Mixing Steps:

1. Weigh out 10mg DES on scale (for 4L)
2. Add 400 ul EtOH to test tube + weighed BPA
3. Vortex 2-3 minutes until soluble
4. Add 200 ul of stock #3 to each tank

Revised Calculations

BPA Solution

$$\frac{2000ug}{L}\ in\ 2\ L\ H_2O\ Tank\ \equiv \frac{2\ mg}{L} = \frac{4\ mg}{2\ L} or\ \frac{8\ mg}{4\ L}$$

Mixing Steps:

5. Weigh out 8 mg of BPA on scale (0.008 g on scale)
6. Add 100 ul EtOH to test tube + weighed BPA ($\frac{0.008\ g\ BPA}{100\ ul\ EtOH}\ for\ 4\ L$)
7. Vortex 2-3 minutes until soluble
8. Add 50 ul of stock #3 to each tank of 2 L

DES Solution

$$\frac{2.5\ ug}{1\ L}\ in\ 2\ L\ H_2O\ Tank\ \equiv \frac{0.00025\ mg}{1\ L}$$

Mixing Steps:

5. Weigh out 2.5 ng DES on scale (0.0027 g on scale)
6. Add 1000 ul EtOH to test tube + weighed BPA
7. Vortex 2-3 minutes until soluble
8. Add 2 ul of stock #3 to each tank of 2 L

EtOH

1. Add 400 ul of stock EtOH to each 2 L tank

Appendix C:

10/21/2014		Surgery day						
Treatment	**#**	**Weight (g)**	**Weight Post**		**Arm Initial**	**0.1x**	**Arm Post**	**0.1x**
DES	1	1.91	1.8		3	0.3	1	0.1
	2	2.96	2.87		6.5	0.65	2.5	0.25
	3	2.56	2.48		5.2	0.52	2	0.2
	4	2.2	2.17		4.5	0.45	2	0.2
	5	4.5	3.89		7.2	0.72	2.5	0.25
	6	1.67	1.52		2.1	0.21	1	0.1
								0
BPA	1	1.34	1.3		2	0.2	1	0.1
	2	2.12	2.02		5	0.5	2	0.2
	3	2.17	2.1		3	0.3	1	0.1
	4	1.98	1.78		6	0.6	2	0.2
	5	1.28	1.19		2	0.2	1	0.1
	6	1.83	1.65		4	0.4	2	0.2
Untreated	1	4.09	4.12		6	0.6	4	0.4
	2	2.16	2.14		4	0.4	2	0.2
	3	2.4	2.34		6	0.6	3	0.3
	4	1.7	1.59		3	0.3	2	0.2
	5	3.09	3		5	0.5	2	0.2
	6	1.51	1.47		2	0.2	1	0.1
EtOH	1	3.13	3.07		7	0.7	3	0.3
	2	3.34	3.23		6	0.6	2	0.2
	3	1.4	1.38		2	0.2	1	0.1
	4	1.35	1.1		3	0.3	1	0.1
	5	4.44	4.4		11	1.1	3	0.3
	6	1.71	1.68		2	0.2	1	0.1

Appendix C depicts the initial and final measurements before and after amputation. The pre and post weights were recorded to show the weight loss as a result of limb removal for the 24 salamanders. The replicates marked in red designate the smaller salamanders that already started regeneration. The stump site was reopened if regeneration was already occurring.

Appendix D:

Weekly Weight and Regeneration Data

10/24/2014 Initial treatments first dosing day

Treatment	#	Weight (g)	Arm Post	0.1x
DES	1	2.2	1	0.1
	2	2.9	2.5	0.25
	3	2.1	2	0.2
	4	1.45	2	0.2
	5	3.2	2.5	0.25
	6	1.7	1	0.1
BPA	1	1.4	1	0.1
	2	2.3	2	0.2
	3	2.2	1	0.1
	4	1.8	2	0.2
	5	1.5	1	0.1
	6	1.6	2	0.2
Untreated	1	4.3	4	0.4
	2	2.3	2	0.2
	3	2.4	3	0.3
	4	2.7	2	0.2
	5	3.2	2	0.2
	6	1.7	1	0.1
EtOH	1	2.9	3	0.3
	2	2.8	2	0.2
	3	1.5	1	0.1
	4	1.1	1	0.1
	5	4.2	3	0.3
	6	1.4	1	0.1

11/4/2014 Week 2

Treatment	#	Weight (g)	Arm Meas	0.1x	
DES	1	1.9	3.5	0.35	
	2	3.2	4	0.4	
	3	3.1	2.5	0.25	
	4	2	2	0.2	
	5	3.6	2.7	0.27	
	6	2	4	0.4	
BPA	1	2.5	2.5	0.25	
	2	3	2.5	0.25	
	3	1.2	3	0.3	
	4	2.3	2	0.2	
	5	1.3	3.5	0.35	
	6	2.1	1	0.1	
Untreated	1	4.9	3	0.3	
	2	3.8	3	0.3	
	3	2.5	3.2	0.32	
	4	1.8	4.1	0.41	almost done
	5	3.6	2.3	0.23	
	6	1.6	6	0.6	almost done
EtOH	1	3.8	3	0.3	
	2	4	3	0.3	
	3	1.3	4.5	0.45	almost done
	4	1.2	4.2	0.42	almost done
	5	4.9	2.5	0.25	
	6	1.7	4.1	0.41	almost done

11/11/2014 Week 3

Treatment	#	Weight (g)	Arm Meas	0.1x	
DES	1	2	4	0.4	
	2	2.5	4	0.4	
	3	3.3	2.5	0.25	
	4	2.1	4	0.4	almost done
	5	3.8	3.7	0.37	
	6	1.8	5	0.5	almost done
BPA	1	2.5	2.5	0.25	
	2	2.5	3.1	0.31	
	3	1.1	5.1	0.51	almost done
	4	2.4	4	0.4	
	5	1.5	5.1	0.51	almost done
	6	2.2	2.1	0.21	
Untreated	1	5.2	3	0.3	
	2	3.1	4	0.4	
	3	2.5	5	0.5	budding
	4	2	5	0.5	almost done
	5	3.7	3	0.3	
	6	2.1	4	0.4	almost done
EtOH	1	3.8	3	0.3	
	2	3.9	3.1	0.31	
	3	1.4	6	0.6	almost done
	4	1.3	5	0.5	almost done
	5	5.6	3	0.3	
	6	1.7	6	0.6	almost done

11/18/2014 Week 4

Treatment	#	Weight (g)	Arm Meas	0.1x	
DES	1	2.5	7	0.7	almost done
	2	2.5	6	0.6	
	3	3.5	5	0.5	hand forming
	4	2.3	6	0.6	almost done
	5	4.8	7.5	0.75	budding
	6	2	6.1	0.61	almost done
BPA	1	2.7	4	0.4	almost done
	2	2.8	5.3	0.53	
	3	1.3	5.5	0.55	almost done
	4	2.6	4	0.4	
	5	1.7	6.5	0.65	almost done
	6	2.3	3.5	0.35	almost done
Untreated	1	5.1	5	0.5	
	2	3.1	6	0.6	digits forming
	3	2.7	6.6	0.66	digits forming
	4	2.1	6.5	0.65	digits forming
	5	4	7	0.7	almost done
	6	1.9	5.5	0.55	almost done
EtOH	1	4	6.5	0.65	digits forming
	2	4.1	5.3	0.53	digits forming
	3	1.6	8.2	0.82	digits done
	4	1.5	6.2	0.62	digits done
	5	5.3	5	0.5	slow
	6	1.9	7.1	0.71	digits done

11/25/2014 Week 5

Treatment	#	Weight (g)	Arm Meas	0.1x	
DES	1	2.7	7.1	0.71	done
	2	2.7	7	0.7	digits forming
	3	3.9	8	0.8	hand forming
	4	2.4	7.2	0.72	hand forming
	5	4.9	8.8	0.88	hand forming
	6	2.1	8.2	0.82	hand forming
BPA	1	3	6	0.6	digits forming
	2	3.2	8.5	0.85	digits forming
	3	1.3	4	0.4	almost done
	4	3	7	0.7	digits forming
	5	1.9	8.1	0.81	done
	6	2.8	7.5	0.75	done
Untreated	1	5.4	7	0.7	digits forming
	2	3.3	7	0.7	digits forming
	3	2.8	7.1	0.71	digits forming
	4	2.3	6.5	0.65	digits forming
	5	4.1	7.5	0.75	almost done
	6	2	7.1	0.71	digits forming
EtOH	1	4.7	7	0.7	digits forming
	2	4.7	6	0.6	hand forming
	3	1.8	8.3	0.83	done
	4	1.6	6.5	0.65	done
	5	5.8	5.3	0.53	done
	6	2	7.1	0.71	done

12/2/2014 Week 6

Treatment	#	Weight (g)	Arm Meas	0.1x	
DES	1	3.1	8.1	0.81	done
	2	3.3	7.2	0.72	done
	3	4.5	8.1	0.81	digits forming
	4	2.6	8.1	0.81	done
	5	5.4	8.5	0.85	done
	6	2.5	9.1	0.91	done
BPA	1	3.7	6.3	0.63	done
	2	3.5	7.5	0.75	done
	3	1.6	9.1	0.91	done
	4	3.3	7.5	0.75	done
	5	2.4	8.3	0.83	done
	6	2.9	7.8	0.78	done
Untreated	1	5.6	7.3	0.73	done
	2	3.8	7.1	0.71	done
	3	3.1	9.1	0.91	done
	4	2.6	9	0.9	done
	5	4.3	8.6	0.86	done
	6	2.2	7.4	0.74	done
EtOH	1	5	10	1	done
	2	5.3	7.3	0.73	done
	3	2.2	10	1	done
	4	3.4	9.1	0.91	done
	5	6.1	9.1	0.91	done
	6	3.3	9	0.9	done

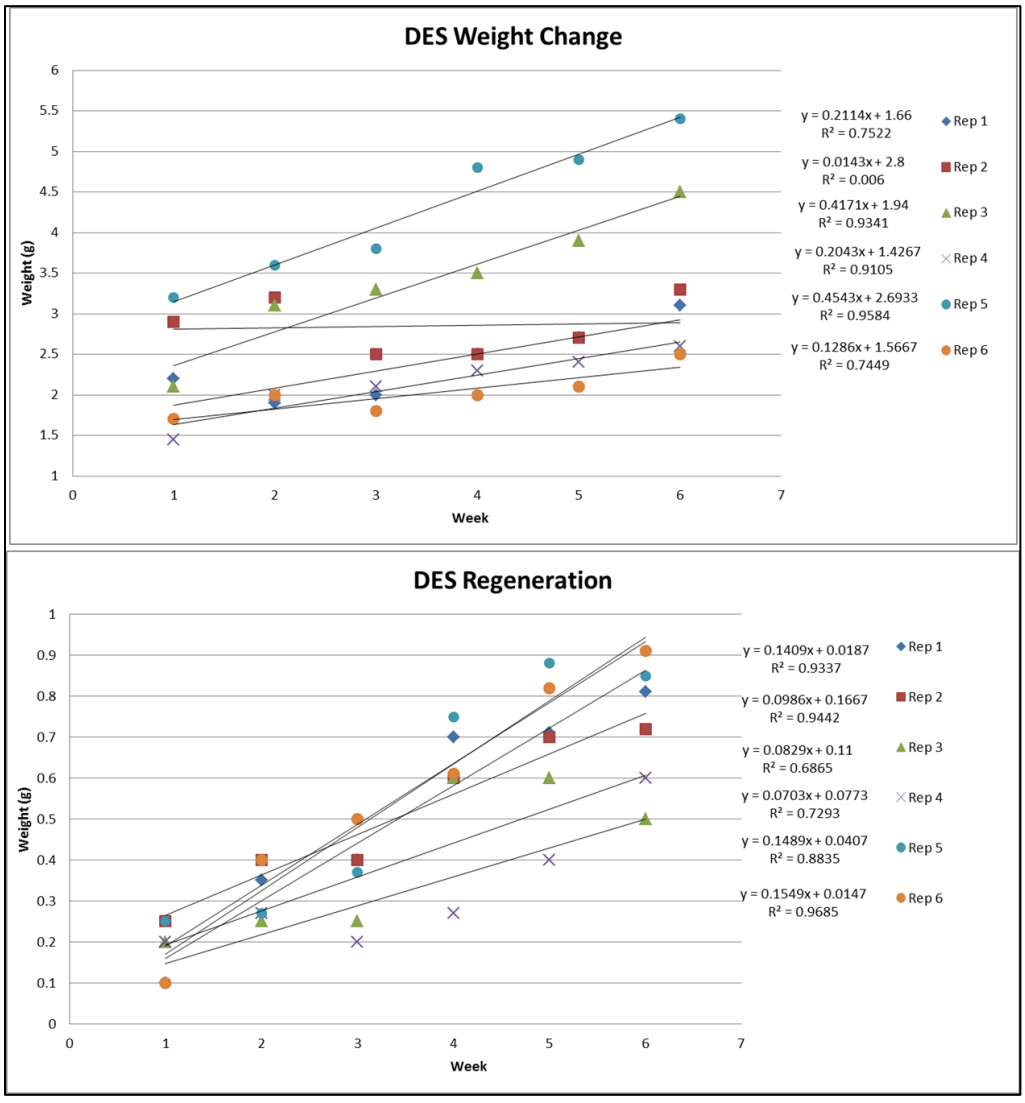
Appendix D depicts the weekly recorded measurements following amputation starting on 10/24/2014. The replicates marked in red designate smaller salamanders that already started regeneration. Arm regeneration was recorded at 1x = millimeters. The stump site was reopened if regeneration was already occurring. The smaller salamanders were found to have quicker regeneration than intact salamanders.

Notes on the right denote observed stages of development. Those marked with budding were at the beginning of regeneration, succeeded by hand forming, digits forming, almost done, and done. Those marked with almost done denote that the hand had regenerated and had significant digit regeneration. This data was used in the formation of Appendix charts E-H below.

Appendix E:

To determine the rates of weight gain/loss and regeneration, scatter plots were created (Appendix E-H) for each treatment replicate based on the weekly data collected from Appendix D. A linear best fit line was added to each graphed replicate to determine the change of slope for regeneration and weight gain. The R^2 values indicated the linear relationship between X and Y as well as the amount of scattering between points. Values closer to 1.0 had a higher linear relationship and less scattering. Groups with greater slopes had a faster regeneration or greater levels of weight gain.

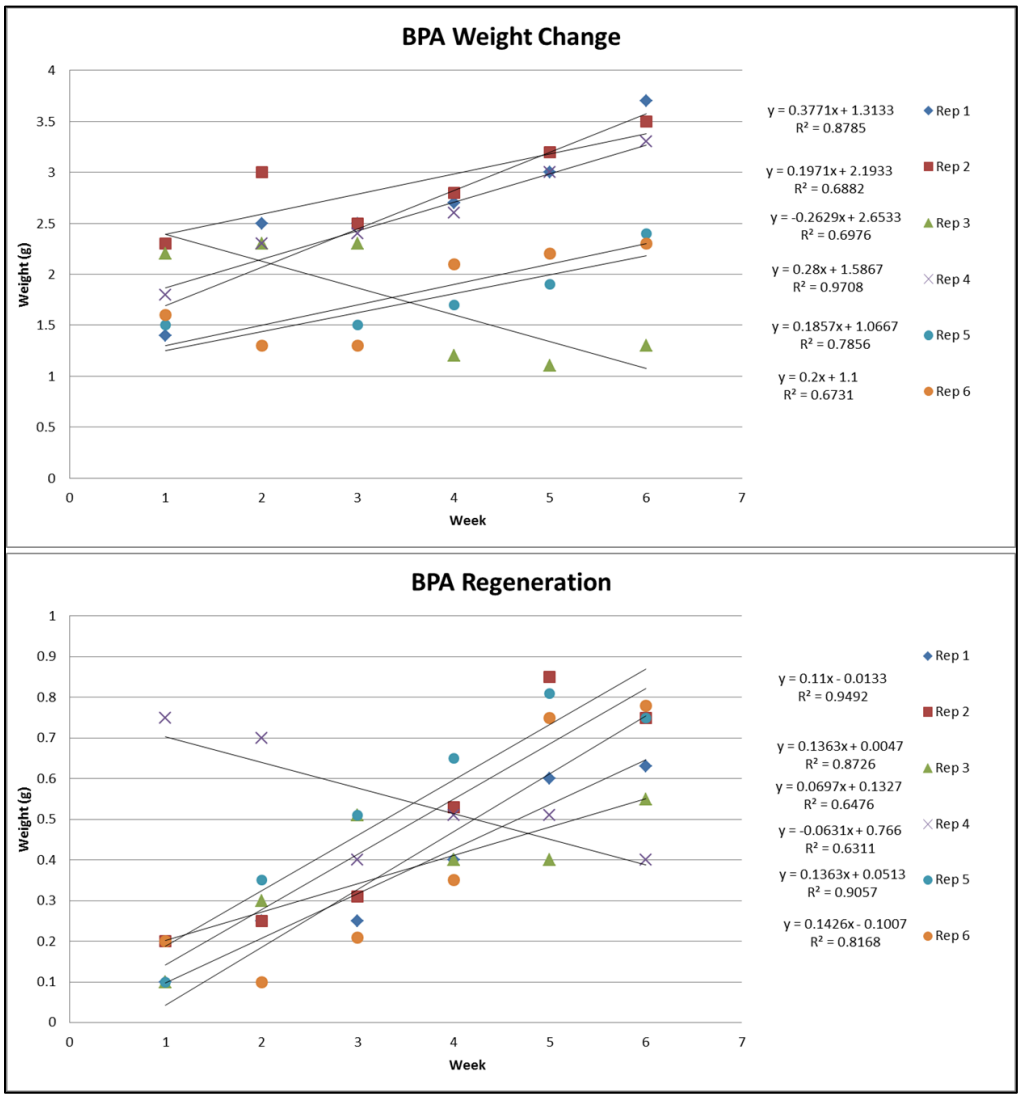
Weekly Weight and Regeneration Data – DES Charts



Appendix E depicts the figures for 6 DES exposed salamanders change in weight and regeneration by week.

Appendix F:

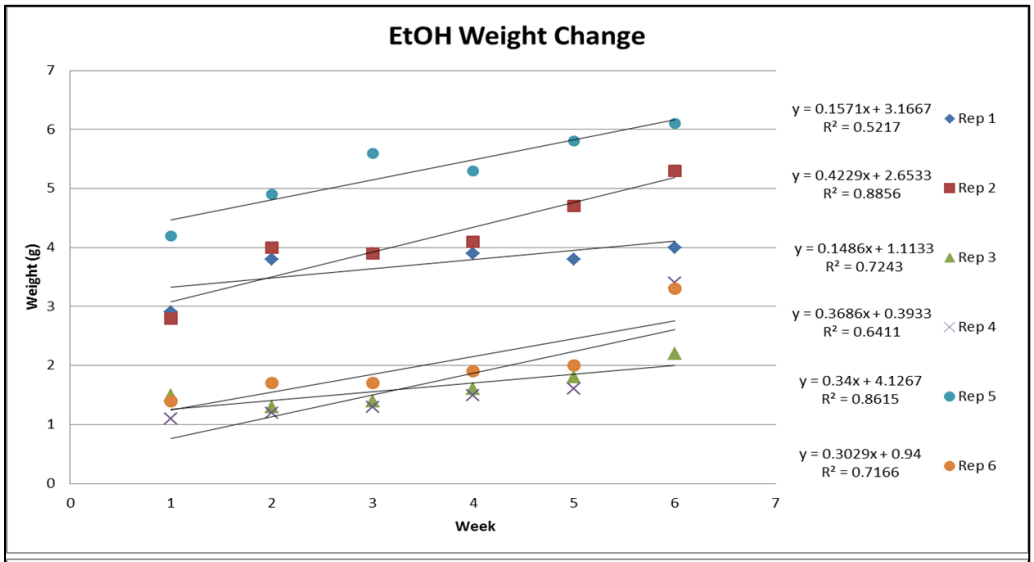
Weekly Weight and Regeneration Data – BPA Charts

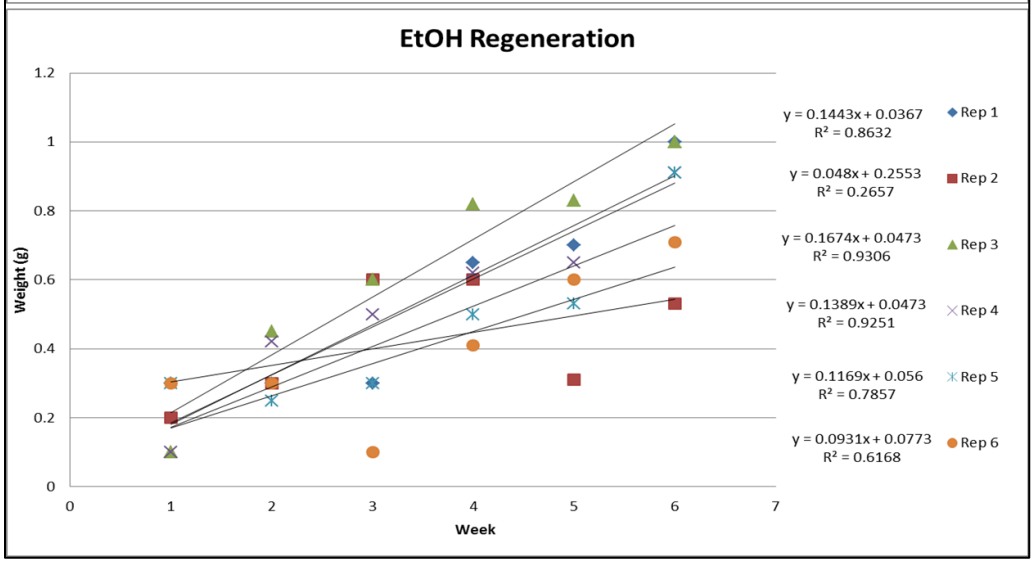


The decline shown in replicate 4 is a result of user error in measuring as no regression of regeneration was observed week to week under the microscope.

Appendix G:

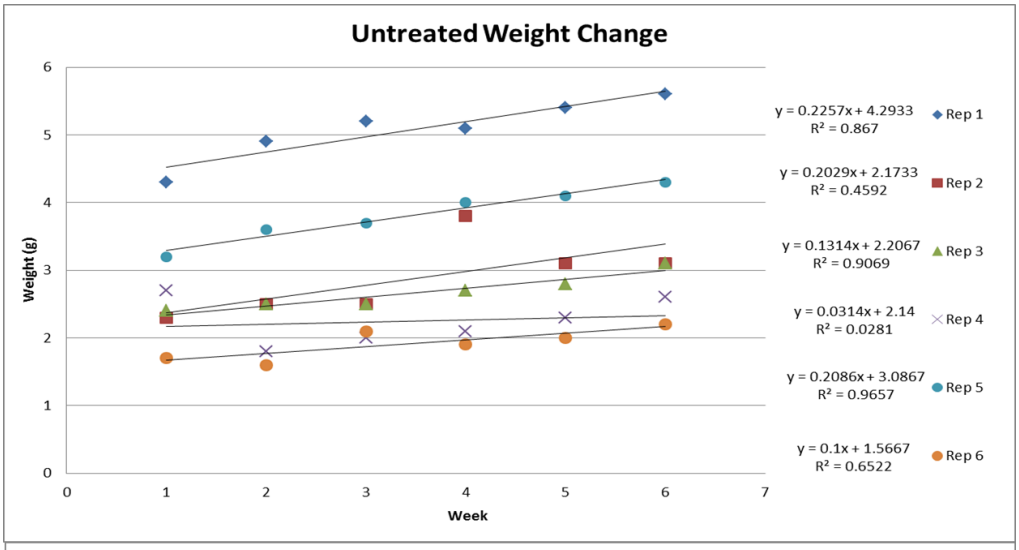
Weekly Weight and Regeneration Data – EtOH Charts

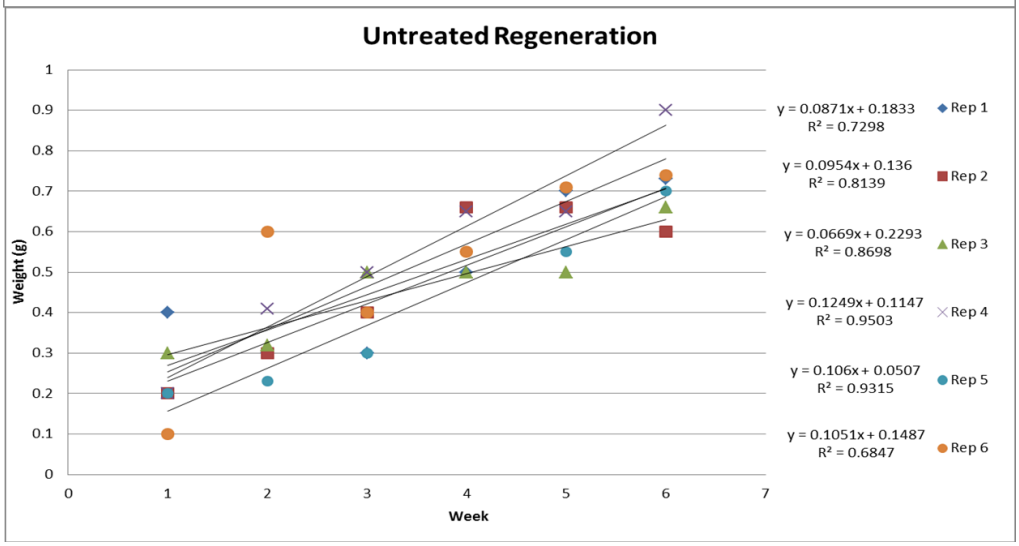




Appendix H:

Weekly Weight and Regeneration Data – Untreated Charts





Appendix I:

DES				**DES**		
Time (Week)	Total Body Weight (g)	Std. Error		Week	Total Arm Regeneration	Std. Error
1	2.26	0.276411		1	0.18	0.027889
2	2.63	0.306232		2	0.31	0.034197
3	2.58	0.326003		3	0.39	0.03283
4	2.93	0.426354		4	0.63	0.035746
5	3.12	0.435444		5	0.77	0.029712
6	3.57	0.468805		6	0.82	0.025353
BPA				**BPA**		
Time (Week)	Total Body Weight (g)	Std. Error		Week	Total Arm Regeneration	Std. Error
1	1.80	0.152753		1	0.15	0.022361
2	2.07	0.285968		2	0.24	0.035158
3	2.03	0.241753		3	0.37	0.052773
4	2.23	0.247207		4	0.48	0.046904
5	2.53	0.30948		5	0.69	0.067268
6	2.90	0.321455		6	0.78	0.038101
Untreated				**Untreated**		
Time (Week)	Total Body Weight (g)	Std. Error		Week	Total Arm Regeneration	Std. Error
1	2.77	0.366667		1	0.23	0.042164
2	3.03	0.52451		2	0.36	0.053479
3	3.10	0.494638		3	0.40	0.036515
4	3.15	0.496488		4	0.61	0.030551
5	3.32	0.516021		5	0.70	0.013081
6	3.60	0.507937		6	0.81	0.037365
EtOH				**EtOH**		
Time (Week)	Total Body Weight (g)	Std. Error		Week	Total Arm Regeneration	Std. Error
1	2.32	0.486769		1	0.18	0.040139
2	2.82	0.654939		2	0.36	0.033342
3	2.95	0.714959		3	0.44	0.060759
4	3.07	0.655574		4	0.64	0.048126
5	3.43	0.750407		5	0.67	0.042032
6	4.22	0.603002		6	0.91	0.040284

Appendix I shows the data used for the comparisons between treatment groups in Results' Figures 4 and 5. The average for each treatment group was taken by week in addition to the calculation of the standard error. The standard error calculations were used in the creation of the error bars for the figures.

Appendix J:

ANOVA Table for limb length by week					
	sum of squa	mean squ	f-value	p value	
treatments	14.796	4.932	5.043	0.0025	
weeks	719.06	143.812	147.049	<.0001	s
treatments * weeks	13.295	0.886	0.906	0.5590	
residual	117.358	0.978			

Fisher's PSLD for limb length by week				
Effect: treatments				
Significance Level: 5%				
	mean diff	Crit. Diff.	P-Value	
ethanol, untreated	0.122	0.462	0.6010	
ethanol, bpa	0.819	0.462	0.0006	s
ethanol, des	0.150	0.462	0.5211	
untreated, bpa	0.697	0.462	0.0034	s
untreated, des	0.028	0.462	0.9053	
bps, des	-0.669	0.462	0.0048	s
Fisher's PSLD for limb length by week				
Effect: week				
Significance Level: 5%				
	mean diff	Crit. Diff.	P-Value	
one, two	-1.296	0.565	<.0001	s
one, three	-2.092	0.565	<.0001	s
one, four	-4.013	0.565	<.0001	s
one, five	-5.200	0.565	<.0001	s
one, six	-6.396	0.565	<.0001	s
two, three	-0.796	0.565	0.0062	s
two, four	-2.717	0.565	<.0001	s
two, five	-3.904	0.565	<.0001	s
two, six	-5.100	0.565	<.0001	s
three, four	-1.921	0.565	<.0001	s
three, five	-3.108	0.565	<.0001	s
three, six	-4.304	0.565	<.0001	s
four, five	-1.188	0.565	<.0001	s
four, six	-2.383	0.565	<.0001	s
five, six	-1.196	0.565	<.0001	s

Appendix J contains the tables produced by the ANOVA from StatView. Comparisons between the weeks were found to be significant demonstrating that limb regeneration occurred each week for all 24 salamanders. Further, the BPA treated groups were deemed significant when compared to the other treatments.

Appendix K:

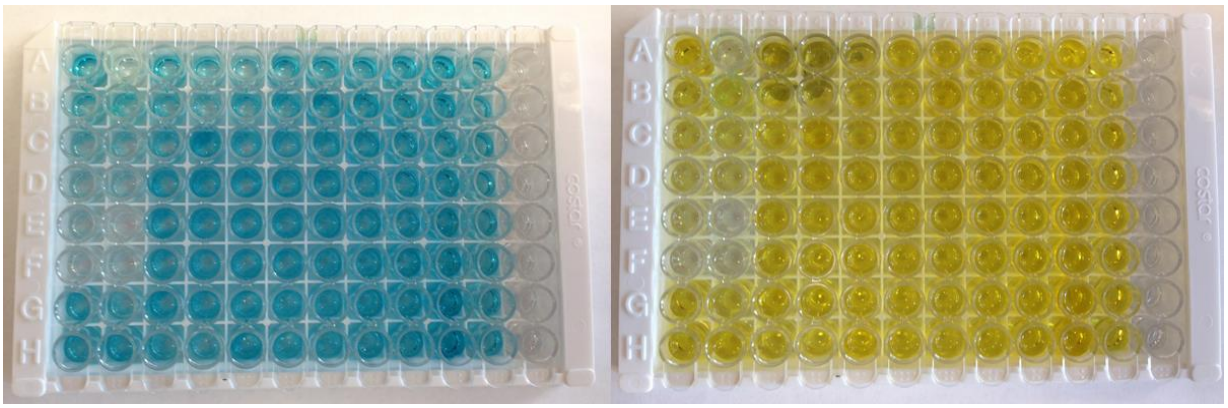
ANOVA Table for Limb Growth Between
Previously Cut and Intact Salamander Groups by Week

	sum of sq	mean squ	f-value	p value
weeks	272.613	54.523	87.692	<.0001
some of limb history	14.357	14.357	23.092	<.0001
weeks * some of limb his	6.613	1.323	2.127	0.661
residual	82.071	0.622		

Fisher's PSLD for Stages				
Effect: weeks				
Significance Level: 5%				
	mean diff	Crit. Diff.	P-Value	
one, two	-0.417	0.450	0.0694	
one, three	-0.792	0.450	0.0007	s
one, four	-1.958	0.450	<.0001	s
one, five	-2.833	0.450	<.0001	s
one, six	-3.958	0.450	<.0001	s
two, three	-0.375	0.450	0.1018	
two, four	-1.542	0.450	<.0001	s
two, five	-2.417	0.450	<.0001	s
two, six	-3.524	0.450	<.0001	s
three, four	-1.167	0.450	<.0001	s
three, five	-2.042	0.450	<.0001	s
three, six	-3.167	0.450	<.0001	s
four, five	-0.875	0.450	0.0002	s
four, six	-2.000	0.450	<.0001	s
five, six	-1.125	0.450	<.0001	s
Fisher's PSLD for Stages				
Effect: some of limb history				
Significance Level: 5%				
	mean diff	Crit. Diff.	P-Value	
previously cut, intact	0.64	0.264	<.0001	s

Appendix K contains the tables produced by the ANOVA from StatView. Comparisons between the weeks were found to be significant demonstrating that limb regeneration occurred each week for all salamanders. Limb history (previously cut or intact) differences were also found to be significant. Smaller salamanders that had previous amputations quickly surpassed the intact salamanders in regeneration.

Appendix L:



Original Plate Read:

Measurement count: 1 Filter: 450

		1	2	3	4	5	6	7	8	9	10	11
A	0ug	0.227	1.171	0.856	0.753	1.223	1.223	1.124	1.368	1.69	1.038	0.046
B	2ug	0.804	0.909	0.702	0.968	1.145	1.646	1.426	1.301	1.46	0.878	0.065
C	5ug	0.549	0.911	1.521	0.971	1.125	1.179	1.357	1.273	1.346	1.128	0.048
D	10ug	0.416	1.12	1.242	1.115	1.17	1.264	1.28	1.327	1.424	0.998	0.072
E	25ug	0.072	1.172	1.087	1.072	1.154	1.258	1.261	1.234	1.834	1.136	0.052
F	bl	0.172	1.057	1.035	1.088	1.174	1.172	1.323	1.599	1.613	1.192	0.047
G		0.699	1.249	1.158	1.16	1.193	1.163	1.379	1.483	2.284	1.138	0.046
H		1.23	0.893	0.842	1.498	1.098	1.122	1.378	1.513	2.217	1.077	0.052
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**

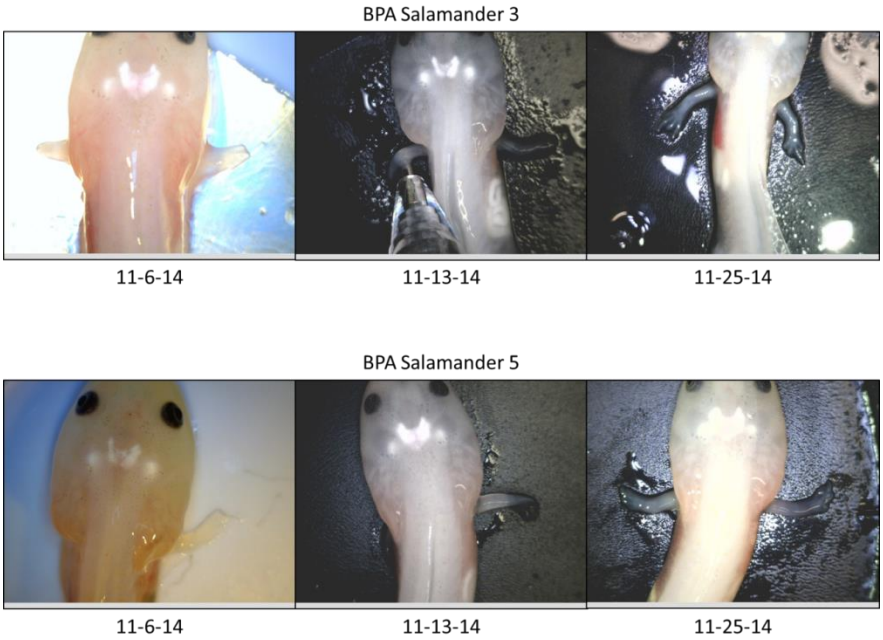
Plate Read Separated by Grouping:

BPA Liver	BL1	0.699		BPA Hearts	BH1	1.179		BPA Spleen	BS1	1.346		ExS	0.052
	BL2	1.23			BH2	1.264			BS2	1.424		ExH	0.047
	BL3	1.249			BH3	1.258			BS3	1.834		ExL	0.046
	BL4	0.893			BH4	1.172			BS4	1.613			
	BL5	0.856			BH5	1.163			BS5	2.284			
	BL6	0.702			BH6	1.122			BS6	2.217			
EtOH Liver	EL1	1.521		EtOH Heats	EH1	1.154		EtOH Spleer	ES1	1.124			
	EL2	1.242			EH2	1.174			ES2	1.426			
	EL3	1.087			EH3	1.193			ES3	1.357			
	EL4	1.035			EH4	1.098			ES4	1.28			
	EL5	1.158			EH5	1.223			ES5	1.261			
	EL6	0.842			EH6	1.646			ES6	1.323			
DES Liver	DL1	0.753		DES Heart	DH1	1.234		DES Spleen	DES 1	1.038			
	DL2	0.968			DH2	1.599			DES2	0.878			
	DL3	0.971			DH3	1.483			DES 3	1.128			
	DL4	1.115			DH4	1.513			DES 4	0.998			
	DL5	1.072			DH5	1.69			DES 5	1.136			
	DL6	1.088			DH6	1.46			DES 6	1.192			
Untr Liver	UL1	1.16		Untr Heart	UH1	1.379		Untr Spleen	US1	1.138			
	UL2	1.498			UH2	1.378			US2	1.077			
	UL3	1.223			UH3	1.368			US3	0.046			
	UL4	1.145			UH4	1.301			US4	0.065			
	UL5	1.125			UH5	1.273			US5	0.048			
	UL6	1.17			UH6	1.327			US6	0.072			

Appendix L (top) is a picture of the change in color before and after the Stop Solution was added to the total T4 ELISA. Below the picture is a chart of the ELISA results separated by grouping. Sample values were below detection limits.

Appendix M:

Picture 1



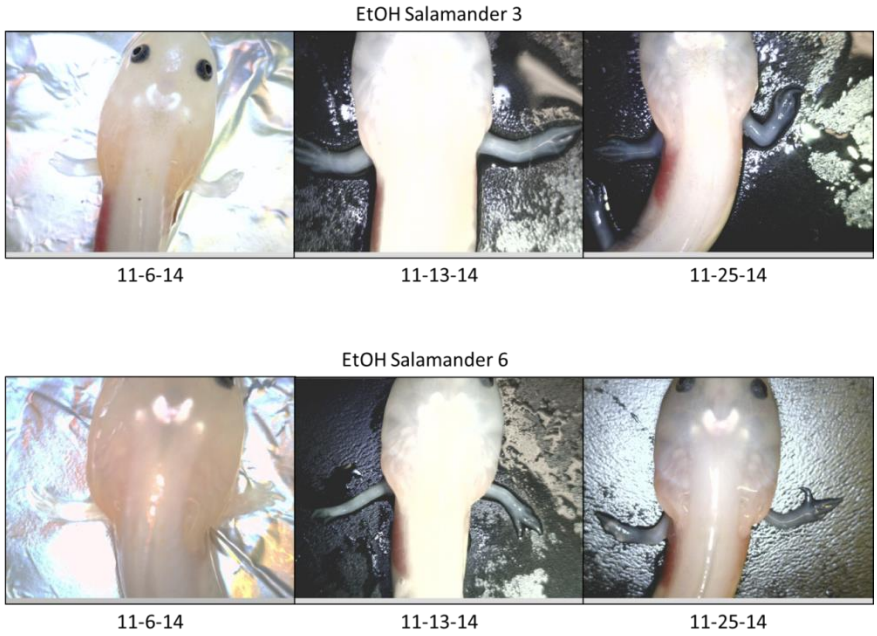
Appendix M: Picture 1 shows the limb regeneration of two specimens within the BPA treatments. By the final week all BPA salamanders had completed successful regeneration.

Picture 2



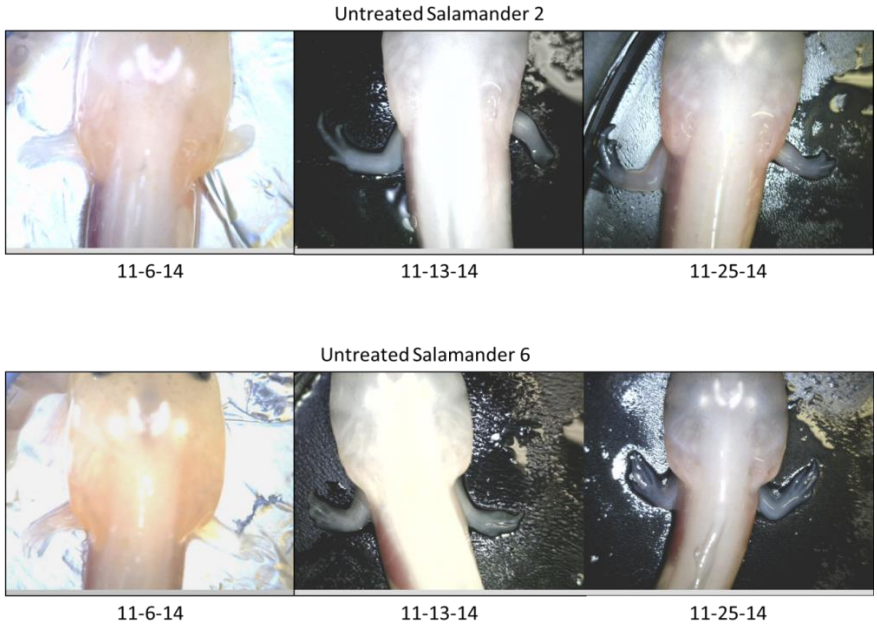
Appendix M: Picture 2 shows the limb regeneration of two specimens within the DES treatments. By the final week all DES salamanders had completed successful regeneration.

Picture 3



Appendix M: Picture 3 shows the limb regeneration of two specimens within the ethanol treatment. By the final week all EtOH salamanders had completed successful regeneration. Ethanol salamanders exhibited the quickest rate of regeneration with many displaying digits by the third week. This was thought to be because many of the EtOH salamanders had previous injury and already started regeneration.

Picture 4



Appendix M: Picture 4 shows the limb regeneration of two specimens within the untreated treatments. By the final week all untreated salamanders had completed successful regeneration.

Appendix N:

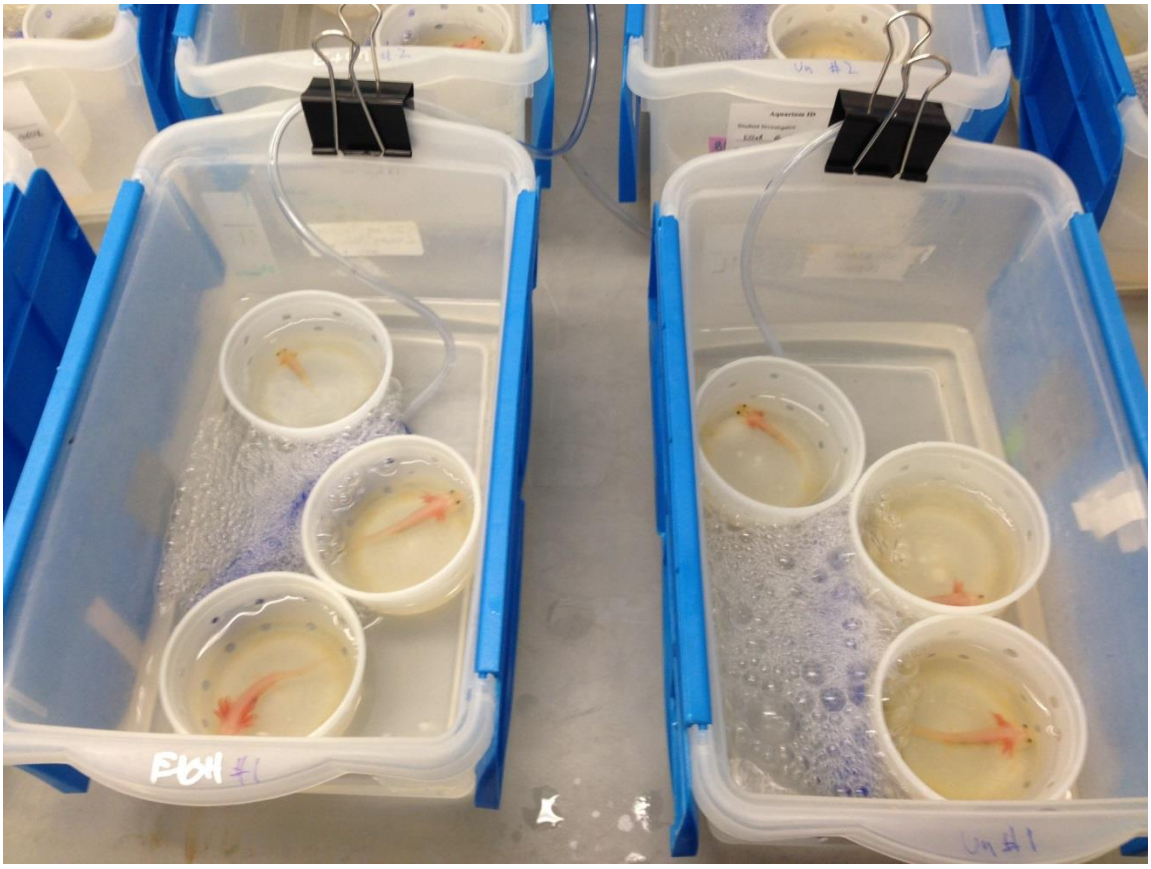
Additional Pictures



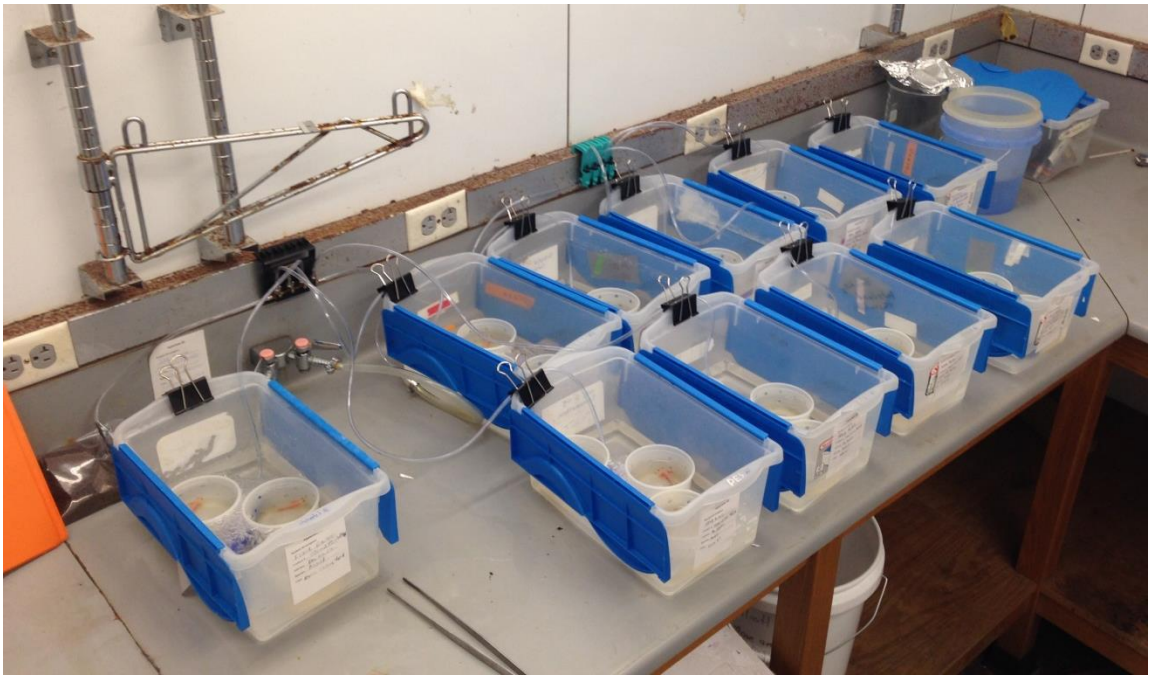
Picture of tanks setup before the arrival of salamanders.



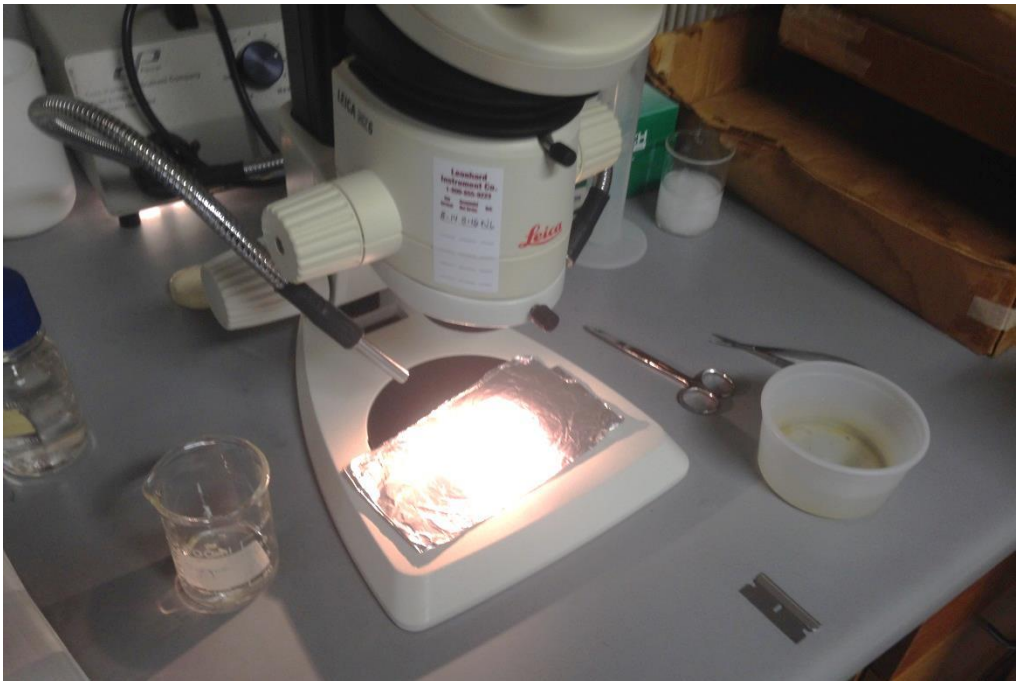
Pictures of salamanders acclimating to tank water temperature.



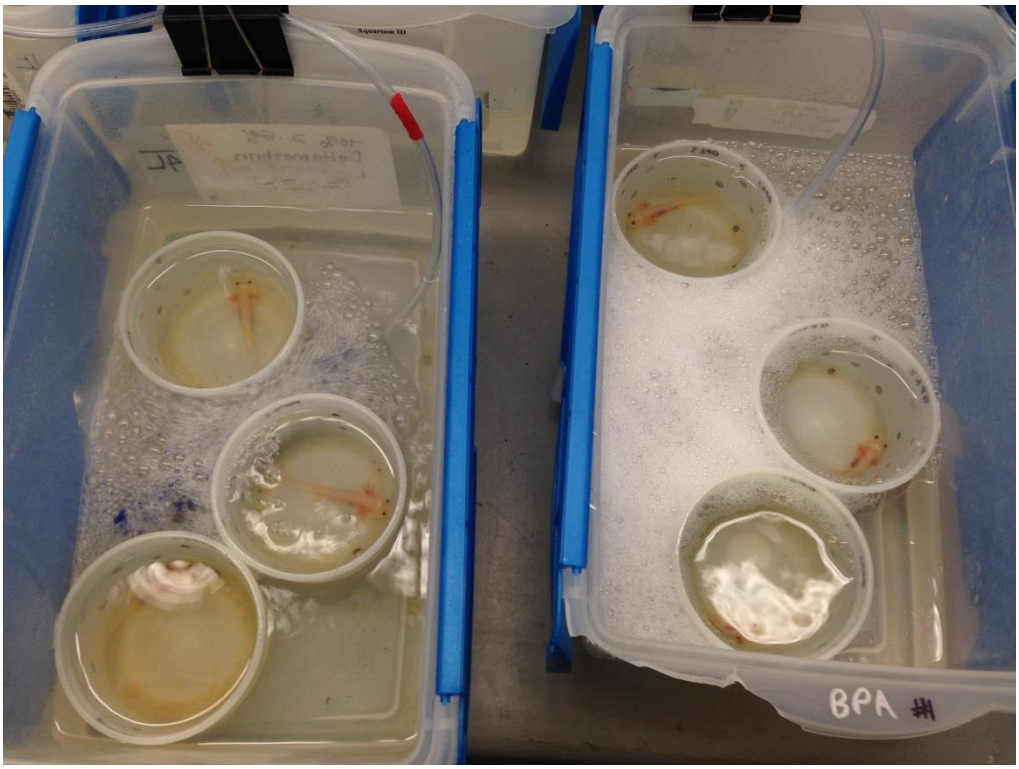
Picture of salamanders once placed in corresponding treatments.



Picture of final setup with extra tanks for 6 non experimental extra axolotls.



Picture of surgical setup for amputations.



Picture depicting issues with initial BPA concentration. Issue of water insolubility caused excessive foaming compared to other treatments as show. Concentrations of BPA and DES were adjusted accordingly.



Pictures of the DinoCapture microscope camera setup.