Exposure to predator kairomones after simulated predation affects caudal regeneration in Allegheny Mountain dusky salamanders (*Desmognathus ochrophaeus*)

by
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Abstract: Many prey rely on autotomy to survive encounters with predators. Because the forfeited tissues may serve as a mechanism of defense, organ for energy storage, appendage for locomotion, and indicator of social status, rapid regeneration by autotomized individuals likely confers a fitness benefit. Here I summarize a study to evaluate rates of volumetric caudal regeneration by Allegheny Mountain dusky salamanders, *Desmognathus ochrophaeus* (Cope, 1859), exposed to kairomones from predatory eastern garter snakes, *Thamnophis sirtalis* (Linnaeus, 1758). To that end, I simulated predator attack on salamanders that resulted in caudal autotomy followed by a 12-week experience with three different treatment regimes: acute exposure to predator kairomones (i.e. 30 min per week), chronic exposure (i.e. constant), and control. I used the original SVL as a covariate in our ANCOVA to compare regeneration rates among treatment groups. Our data indicate that the 1) average volumetric caudal regeneration per day, and 2) average regeneration as a percent of the original tail were highest for individuals exposed to the water control versus acute and chronic exposure to kairomones from snakes. Although the mechanism is currently unspecified, it is possible that kairomones from predators elevated stress hormones or metabolic activity in prey, which compromised regeneration.

Introduction

Kairomones from predators or conspecifics may elicit behavioral, physiological and morphological responses in prey species (Petranka et al., 1987; Weider and Pijanowska, 1993; reviewed by Kats and Dill, 1998; Wisenden, 2000; Chivers and Mirza, 2001; Benard, 2004; Ferrari et al., 2010). Behavioral responses are manifest in a variety of ways and may include changes in foraging, mating, and activity. Webster et al. (2007) have observed significantly less prey capture in turbid waters for sticklebacks (*Gasterosteus aculeatus*), but only when coupled with predator cues, suggesting that olfactory cues may aid foraging more so than visual cues. In addition, Maerz et al. (2001) observed a lower tendency to strike at prey in red back salamanders (*Plethodon cinereus*) exposed to substrates laced with cues from garter snakes (*Thamnophis sirtalis*). Furthermore, Poschadel et al. (2006) showed that kairomones could influence mate choice in European pond turtles (*Emys orbicularis*), with males preferentially choosing kairomones from larger females with higher fecundity. Western red back salamanders (*Plethodon vehiculum*) also show a preference for chemical cues from gravid females in laboratory experiments (Marco et al., 1998). Tjossem (1990) has revealed inducible vertical migration patterns in the phantom midge (*Chaoborus flavicans*) exposed to water columns in ponds containing fish predators. Likewise, Turner et al. (2000) also noticed a major change in habitat use among the freshwater snail (*Physella gyrina*) in the presence of caged fish or crayfish predators. In the context of amphibians, Mathis et al. (2003) reported significantly decreased latencies to move in larval ringed salamanders (*Ambystoma annulatum*) when exposed to predator cues from newts in the field (*Notophthalmus viridescens*). Other studies further document the behavioral modifications in amphibians as a result of predator kairomone exposure (Sullivan and Madison, 2002; Gildemeister et al., 2017).

Physiological and morphological responses by prey to predator kairomones are less well-documented, but studies indicate kairomones are capable of inducing changes in morphologies

and physical life-histories (van Buskirk and Schmidt, 2000; Relyea, 2001; Brossman et al., 2014). Crucian carp (*Carassius carassius*) raised in context with predatory pike respond through deeper tails (Brönmark, and Pettersson, 1994). Langkilde (2009) documented that invading fire ants (*Solenopsis invicta*) induced longer hind limbs in Eastern fence lizards (*Sceloporus undulates*) over time, which facilitate to better remove the dangerous ants. Gray tree frog (*Hyla chrysoscelis*) tadpoles that were reared in the presence of predatory dragonflies also exhibited changes, which reflect an increase in swimming ability through deeper tails, and camouflage via black spots (McCollum and Leimberger, 1997). Wood frog (*Rana sylvatica*) tadpoles also respond to predator threats through deeper tails, which can aid in escape (Relyea, 2004; Maher et al., 2013). Lastly, Crane et al. (2011) report increased oxygen consumption in Ozark zigzag salamanders (*Plethodon angusticlavius*) when presented with cues from the predatory nine-banded armadillo. These studies suggest prey are able to modify their phenotypes and physiologies accordingly to best suit environmental pressures. Species that can respond to particular threat levels can employ variable antipredator responses that may ultimately save critical time and energy.

Studies with amphibian models are useful for understanding the complex dynamic of chemically-mediated predator-prey interactions. Because amphibians modify behavioral and morphological tendencies in response to chemical cues from predators, complex interactions in the context of matters like autotomy, regeneration, and stress can be evaluated within an ecological context. Studies confirming the importance of autotomy are well represented in the literature (Labanick, 1984; Whiteman and Wissinger, 1991; reviewed by Maginnis 2006; Marvin, 2010), but studies on regeneration rates or stressed-induced effects are less well represented. However, work has been done to suggest elevated corticosterone levels (which can result from stress) can reduce immune function (McCormick and Langkilde, 2014), impede growth (Barton et al., 1987; Morici et al., 1997), and increase metabolism (Wack et al., 2012). In one study, enhanced corticosterone levels increased activity and foraging in common lizards (*Laverta vivipara*) (Cote et al., 2016). Another study by Small (2004) found that catfish with

supplemental cortisol in their diet had a lower body mass and hepato-somatic index, but increased the number of spawns as compared to control fish. Interestingly, red-legged salamanders (*Plethodon shermani*) exposed to elevated corticosterone levels exhibited a 12% higher oxygen consumption than controls (Wack et al., 2012). Ricciardella et al. (2010) also reported that in dusky salamander (*Desmognathus ochrophaeus*) males, capture and handling increases relative cortisol levels. Finally, Bliley and Woodley (2012) found a decrease in body weight among Ocoee salamanders (*Desmognathus ocoee*) exposed to elevated corticosterone levels and handling. These studies demonstrate the array of effects corticosterone levels may have on organism physiology.

Few studies have explored the effects of these various chemical stimuli on regeneration rates following caudal autotomy. When primary defensive responses fail (e.g., immobility, biting, defensive secretions), caudal autotomy may be utilized to distract the predator and facilitate escape (Labanick, 1984; Whiteman and Wissinger, 1991; Maginnis, 2006; Marvin, 2010). Tails are a valuable resource and can often serve as stores of fat and protein, increase attractiveness to potential mates, and contribute to movement and bursts of speed in addition to their antipredator function (Maginnis, 2006). However, there are trade-offs associated with caudal autotomy. In lizards, autotomy may increase flight initiation distance and time spent in refugia (Cooper and Wilson, 2008; Cromie and Chapple, 2012). In addition, autotomy in lizards has been shown to reduce fecundity, clutch sizes, and social capital (Bateman and Fleming, 2009). In many species, autotomy may reduce the propensity to forage, as a result of increased time spent in refuge and increased use of crypsis. Autotomized individuals may be less attractive to potential mates, and autotomy may thus reduce fitness (Maginnis, 2006). Once autotomy has been implemented, the individual is no longer 'protected' by its tail (i.e. it cannot employ autotomy again), and is now even more vulnerable to predation (Fox and McCoy, 2000). Besides functioning to detach, the tail increases the individuals' length, allowing for more forceful writhing, and the opportunity for the salamander to grasp its own tail with its mouth, increasing its size relative to the predator gape

limit (Whiteman and Wissinger, 1991). Because autotomy poses a trade-off to individual fitness, caudal regeneration rates become undeniably important.

While much is known regarding autotomy, less is known about the relative rates of regeneration. Marvin and Lewis (2013) show variability in the rates of regeneration among Plethodontid salamanders related to temperature. Individual spotted dusky salamanders (*Desmognathus conanti*) maintained at 20°C regenerated four times faster than those held at 10°C. In addition, levels of feeding were shown to restrict regeneration rates, with individuals fed to satiation regenerating slightly (though significantly) faster than those not fed.

In order to compensate for tail loss following autotomy, trade-offs must be endured if regeneration is to occur. Because the presence of predator kairomones is known to induce behavioral and physiological modifications (Kats and Dill, 1998; van Buskirk and Schmidt, 2000; Relyea, 2001; Wack et al., 2012; Brossman et al., 2014), it is reasonable to suggest that kairomones might be able to induce changes in tail regeneration rates. Regeneration is important ecologically because prey must regenerate if caudal autotomy is to be used as an antipredator mechanism. I used Allegheny Mountain dusky salamanders (*Desmognathus ochrophaeus*) as a model for regenerative plasticity because it is well known that these animals respond to kairomones from predators (Cupp, 1994; Johnson and Sullivan, 2014; Gildemeister et al., 2017). This study focused on the rates of regeneration and total regeneration as a percent of the original in three separate treatment groups: acute exposure to predator kairomones (i.e. 30 min per week), chronic exposure (i.e. constant), and control. I hypothesized that groups exposed to predator kairomones would experience higher rates of regeneration than the control. Since the need for a tail is greatest in a predator context, kairomones may drive regeneration rates in order to prepare the individual for the next predation attempt. This non-invasive study is the first to look at the effects of predator kairomones on regeneration rates in salamanders.

Methods

Collection and maintenance of study animals

150 adult (SVL ≥ 3.3 cm, Keen and Orr, 1980) Allegheny Mountain dusky salamanders (*Desmognathus ochrophaeus*) were collected from 1 – 9 September 2016 from the wooded area of the campus of Houghton College (Houghton, NY). I made an effort to collect individuals lacking signs of recent caudal autotomy (i.e., there was no obvious region of discoloration, nor distal caudal bluntness). Once collected, the salamanders were returned to the laboratory where they were housed in 15-cm Petri dishes with acid-free paper towel substrates saturated with reverse-osmosis (RO) water. Individuals were maintained in a climate-controlled chamber between 15.5° C (day) to 12.8° C (night) temperature and on a 12.5:11.5 light: dark photoperiod, (lights on at 0700 h EST). On 17 Sept 2016 (one week prior to the beginning of the experiment,), each individual was provided three flightless *Drosophila* sp. in an effort to standardize the time since last successful foraging. The individuals who did not consume prey were not recorded.

To evaluate the possible impact of predator stimuli on caudal regeneration in salamanders, I collected two predatory eastern garter snakes *Thamnophis sirtalis*, (SVL ≥ 40 cm) from the field site where study salamanders were collected. Snakes were held in 18.9-l glass aquaria and provided with crumpled paper towels for cover, water ad libitum, and one intact salamander weekly (30 min prior to the collection of kairomones).

Preparation of predator kairomones

To collect kairomones, the snakes were placed individually in clean 3.8-l glass jars covered with cheesecloth for 48-72 h. Following collection, the jars were each rinsed with 200 ml RO water and the rinses filtered through glass wool to remove large particulates (feces, skin, etc.) The

rinses from the two snakes were combined. Due to time constraints, three rinses were frozen before use, but the remaining nine were applied fresh.

Experimental bioassay

On 17 September, I induced autotomy in the tails of each individual approximately 1 cm from the posterior of the cloacal vent using a pair of forceps and removed the tail from the dish. Salamanders were haphazardly assigned to one of three treatment groups based on the stimulus to which they would be exposed. Over the course of the study, five individuals died and two were reconsidered based on tail coloration, bringing each treatment group to the following totals: water control (C; n=46), acute exposure to predator kairomones (A; n=48), and chronic exposure to predator kairomones (CH; n=49). Only salamanders that survived for the duration of the experiment are included in the analysis. For twelve weeks from 17 September – 13 December 2016, members of each group received 3 mL of the appropriate stimulus at the beginning of the week (Monday) between 1300h – 1700h. The control group was provided with RO-water as a stimulus during each session over the course of the study, and the CH group was given predator rinse each time (see Sullivan et al. 2002 for long-term activity of kairomones). The individuals in the A group were housed exactly like the control group, but were transferred to separate test dishes containing a 3-mL dose of predator rinse on paper towel substrate for thirty minutes during the session.

Digital photographs of each salamander were taken before application of stimuli using an Olympus TG-4 digital camera for the purpose of recording morphometric data: snout-vent length (SVL), total length (TL), diameter of regenerated portion and regenerated length. Here, I define SVL as the distance from the tip of the snout to the posterior margin of the cloacal vent and total length from the tip of the snout to the tip of the tail. Length and radius of the base of the regenerated tail were identified as the discolored or severed section of the tail and ending at the

tip of the regenerated portion. Pictures were taken with the individual inside the dish positioned directly underneath the camera. An effort was made to ensure the salamanders were lying as flat as possible and no portion of their body size was distorted by contact with the side of the dish.

After photographing individuals, the paper towel substrates in the control and CH groups were changed while each salamander was still inside its home dish. In contrast, home dish substrates for salamanders in the A group were changed during their thirty-minute exposure to predator rinse in test dishes, after which individuals were returned to their clean home dishes. Although the physical nature of the disturbance was different between treatments, all animals were subjected to the same amount of physical movement. While the transfer of group A individuals between test dishes may have been stressful, the control and CH group received equal amounts of potential stress during substrate changes since changing paper towels disturbed the salamander and often required gently nudging the salamander off the substrate.

During the experiment, all individuals were provided with two *Drosophila* prey 30 and 61 days after autotomy was induced. Because Gildemeister et al. (2017) suggest that exposure to kairomones may reduce foraging behavior, I offered flies in small quantities to ensure any differences in caudal regeneration were not due to differences in foraging rates of individuals exposed to predator cues. It is worth noting, however, that Marvin and Lewis (2013) report a small, but significant, difference in regeneration rate between satiated and fasting individuals, where satiated individuals regenerated faster than fasting individuals.

Morphometric and statistical analysis

Images taken during each session were analyzed with the open-source photo-analyzing software ImageJ (Schneider et al., 2012). A small ruler was photographed at the beginning to provide a scale for the measurements. Regeneration became apparent during the third week. The ‘freehand’ line tool was used to carefully trace along the midline around the curvature of the

body. The ‘straight’ line tool was used to measure the tail diameter for regrowth. I estimated volumetric data of the regenerated tail tissue by considering the tail as a cone, which incorporated the diameter and length of the newly regenerated portion of the tail. Each measurement was taken four times with the averages used to calculate volumetric regeneration. Measurements were taken by a single investigator in order to reduce variation in the interpretation of lengths.

To evaluate the effect of stimulus exposure on caudal regeneration in individual salamanders, I performed two analyses of covariance (ANCOVA) using Statistica’s (Statsoft, Inc., 2001, version 6.0) General Linear Model module. In the first, I used average regeneration rate (cm3/day) as the response variable and chemical treatment (control, acute, chronic) as the independent variable. Because the initial (pre-autotomy) volume of the tail was positively correlated with the average volume of regenerated tail per day ($r = 0.658$; $p < 0.001$; Figure 1), I used initial tail volume as a covariate (supported by Marvin, 2011). In the second analysis, I used final tail volume as a percentage of the pre-autotomized tail volume as the response variable with chemical treatment as the independent variable. In this instance, the volume of the pre-autotomized tail was negatively correlated with the final tail volume as percent of the pre-autotomized tail ($r = 0.300$; $p < 0.001$; Figure 2), so I again included initial volume of the tail as a covariate.

Results

There was a significant effect of treatment on the average regeneration rate (cm^3/day) after controlling for the effect of the initial tail volume (F_2, 139 = 11.039; $p < 0.001$; Table 1; Figure 3). Post hoc tests (Unequal N HSD) revealed that salamanders exposed to the control treatment had significantly higher growth rates than salamanders experiencing acute ($p = 0.002$) and chronic ($p = 0.023$) exposure to snake kairomones. Salamanders experiencing acute and chronic exposure to snake kairomones did not differ significantly from one another ($p = 0.683$).

There was a significant effect of treatment on the final tail volume as a percentage of the pre-autotomized tail volume after controlling for the effect of the initial tail volume (F_2, 139 = 10.115; $p < 0.001$; Table 2; Figure 4). Post hoc tests (Unequal N HSD) revealed that salamanders exposed to the control treatment had significantly higher growth rates than salamanders experiencing acute ($p = 0.002$) and chronic ($p < 0.001$) exposure to snake kairomones. Salamanders experiencing acute and chronic exposure to snake kairomones did not differ significantly from one another ($p = 0.788$).

Discussion

In this study, salamanders that were chronically or acutely exposed to kairomones from predatory snakes exhibited significantly lower rates of volumetric caudal regeneration per day compared to the water control. In addition, the volume of tail regrown as a percentage of the original was lower in salamanders exposed to kairomone treatments versus the control. These results differ from my initial prediction that higher rates of regeneration would be exhibited by individuals exposed to kairomones from predators because the benefit of the tail is greatest in a defensive context.

Equivalent handling methods should have minimized any confounding variables related to possible stressors. In addition, because the control and CH group were handled identically, stress due to photography and towel replacement probably does not account for observed differences. Foraging rates were nearly identical among treatments, although five CH group individuals avoided consumption during the second feeding. This may be a result of being fed on a substrate containing kairomones, since both the A and control groups were fed on water substrates. In addition, Marvin and Lewis (2013) reported only slightly significant differences in growth rates for individuals fed to satiation, which makes foraging behavior an unlikely explanation for our observed regeneration rates. Furthermore, because I did not control for sex differences in

treatment groups, the results observed could be due to physiological differences between sexes. However, it is unlikely that this alone accounts for the observed differences.

Kairomones may have the propensity to shift metabolic processes in order that resources may be reallocated to serve other needs. Crane et al. (2001) tested the effects of cues from nine-banded armadillos on oxygen consumption rate in Ozark zigzag salamanders. Consumption increased 12% in individuals exposed to cues when compared to controls. It is possible that modified respiratory levels may have shifted energy away from regeneration, producing the observed results. Consistent with Crane et al. (2001), Steiner and van Buskirk (2009) also observed 16.8% increased oxygen consumption in tadpoles exposed to cues from predatory dragonfly larva. While kairomones clearly impact metabolism in certain species, the mechanism by which they do so is unknown.

Another reason for the observed data may be due to elevated stress levels induced by kairomones. Kairomones may be seen as a threat, and threats may induce stress. Stress has been shown to change cortisol and corticosterone levels and influence metabolic rates (Wack et al., 2012). Visual predator exposure has also been shown to elevate cortisol levels in zebrafish, *Danio rerio* (Barcellos et al., 2007) and handling and capture raises corticosterone in dusky salamanders (*Desmognathus ochrophaeus*) (Ricciardella et al., 2010). Elevated levels of stress hormones may also change wound healing patterns (French et al., 2006; Thomas and Woodley, 2015), or slow growth patterns (Bliley and Woodley, 2012). However, studies show mixed results when comparing growth rates of stress-treated organisms. Small (2004) observed lower growth rates in catfish fed supplemental cortisol, but McCollum and Leimberger (1997) reported faster rates of growth in gray tree frog tadpoles (*Hyla chrysoscelis*) when reared with a dragonfly larvae predator. Furthermore, Hossie et al. (2010) reported lower rates of growth in predator-exposed *Rana pipiens* treated with the corticosterone inhibitor metyrapone. There have also been a number of observed side effects of cortisol on various metabolic and behavioral processes in taxa such as fish (Barton et al., 1987; Small, 2004); and reptiles (Morici et al., 1997). In the bronze

featherback (*Notopterus notopterus*), ovarian activity rates were increased (Shankar and Kulkarni, 2006) following cortisol injections, and spawning was increased by a factor of 1.7 in catfish fed supplemental cortisol (Small, 2004). However, a recent paper by Fonner and Woodley (2015) saw no increase in plasma glucocorticoids in *Desmognathus ochrophaeus* following short-term (45 min and 3 h) and long-term (10 d) exposure to predator cues from spring salamanders (*Gyrinophilus porphyriticus*), even though the cues elicited changes in behavior and activity. Because dusky salamanders exhibit a semi-aquatic life style (Tilley, 1973), and spring salamanders are more associated with aquatic environments, these observations may be due to the specific ecological role spring salamanders play as predators in the life history of dusky salamanders.

From our data and previous studies, it seems reasonable to suggest that exposure to kairomones elicited a physiological response in individuals, prompting stress hormone or oxygen consumption increases, which in turn may have increased metabolic rates or consumed energy otherwise needed for regeneration. However, the effects of predator kairomones are likely specific to different environment and individual situations. Nevertheless, predator kairomones induce physiological and morphological changes in a number of taxa, and it is evident that *D. ochrophaeus* responds to *T. sirtalis* via behavioral modifications. The possibility exists for *T. sirtalis* to modify rates of regeneration through stress elicited by exposure to predator threats.

Future work should explore this possibility using stress hormone levels in order to determine the true modifier of regeneration rates. It would also be interesting to ascertain any affect direct cortisol application may have on regeneration rates. In addition, oxygen consumption levels could be measured in salamanders exposed to cues. Finally, investigating the impacts of other stressors on regenerative rates may help to shed light on these conclusions.

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Table 1. Result of one-way ANCOVA to evaluate differences in the average volume of tail regenerated (cm^3 per day) in Allegheny Mountain dusky salamanders (*Desmognathus ochrophaeus*) exposed to different chemical stimuli (water control, acute exposure to predator kairomones, chronic exposure to predator kairomones) using the initial volume of the tail as a covariate.

Effect	df	*F*	*P*
Intercept	1	17.67	<0.001
Volume of tail (pre-autotomy)	1	138.85	<0.001
Treatment	2	11.03	<0.001

Table 2. Result of one-way ANCOVA to evaluate differences in caudal regeneration (cm^3) as a percent of the pre-autotomized tail in Allegheny Mountain dusky salamanders (*Desmognathus ochrophaeus*) exposed to different chemical stimuli (water control, acute exposure to predator kairomones, chronic exposure to predator kairomones) using the initial volume of the tail as a covariate.

Effect	df	*F*	*P*
Intercept	1	306.74	<0.001
Final volume as % of pre-autotomized tail	1	15.82	<0.001
Treatment	2	10.12	<0.001

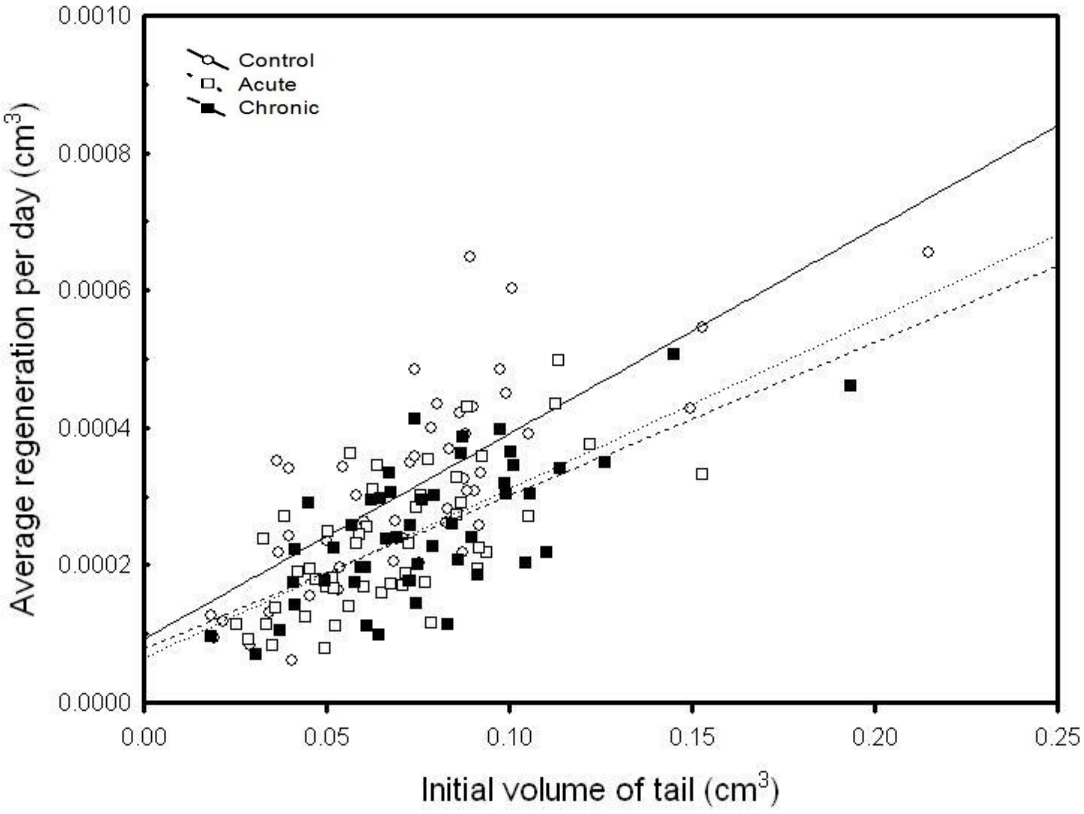

Figure 1. As supported by Marvin (2011), average regeneration per day (cm^3) was positively correlated with initial tail volume ($r = 0.658$; $p < 0.001$). In this way, I was able to use initial tail volume as a covariate for the first analysis.

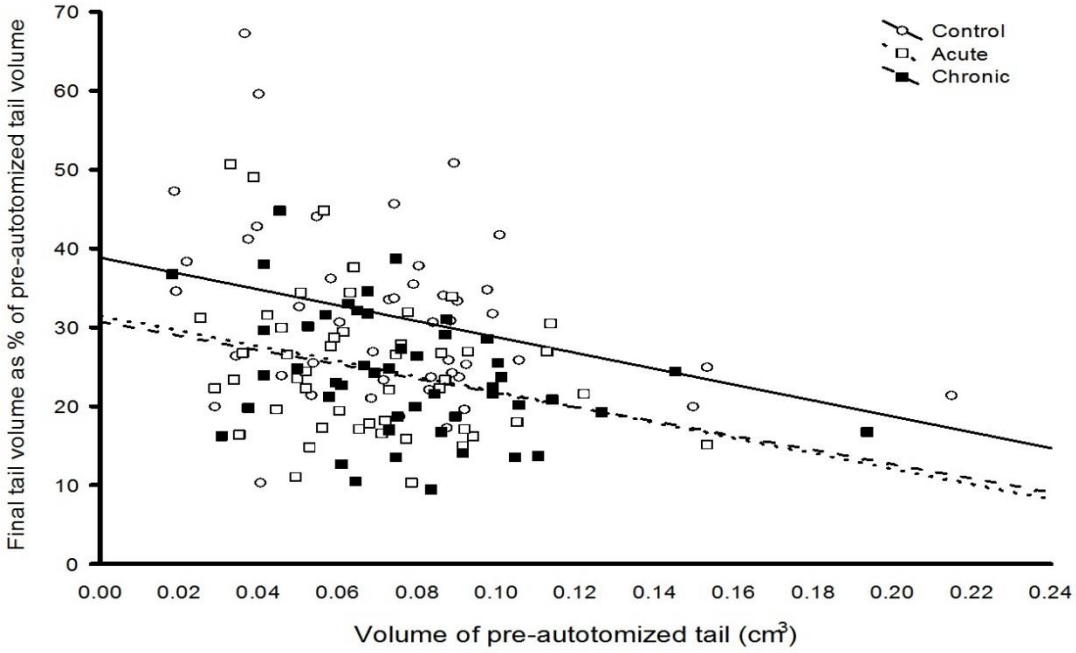

Figure 2. Final volume as a percent of pre-autotomized tail volume was negatively correlated with the volume of pre-autotomized tails (cm^3) ($r = 0.300$; $p < 0.001$). This allowed me to use initial tail volume as a covariate in the second analysis.

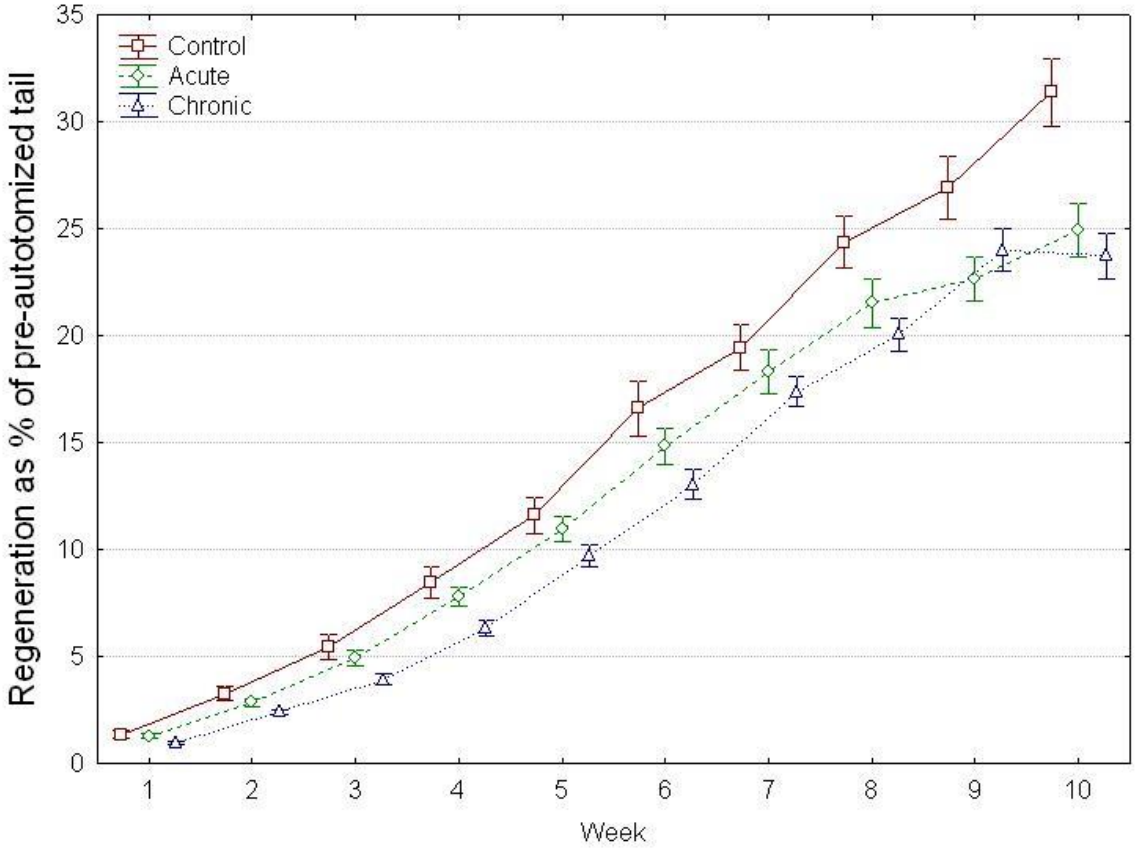

Figure 3. One-way ANCOVA showing regeneration as a percent of the pre-autotomized tail vs. time. Salamanders in the control treatment had higher rates of regeneration than those in the acute and chronic ($F_{2, 139} = 11.039$; $p < 0.001$). Acute and chronic treatments did not vary significantly ($p = 0.683$).

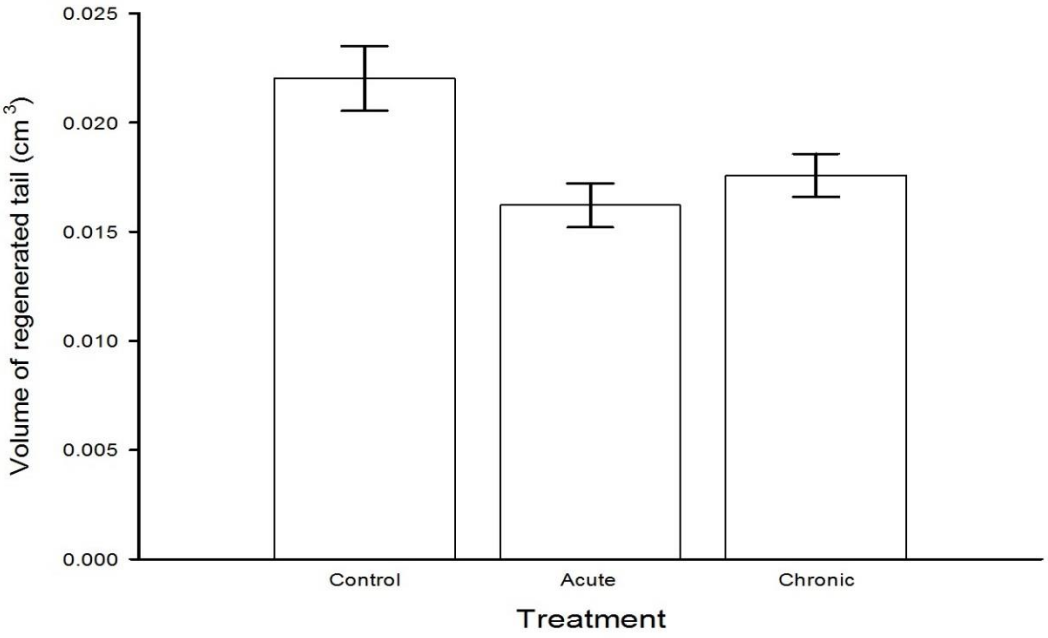

Figure 4. A one-way ANCOVA showed that final volume of regenerated tail (cm^3) was significantly greater in the control treatment ($F_{2, 139} = 10.115$; $p < 0.001$) than the acute or chronic. There was no significant difference in final volumes between acute and chronic treatments ($p = 0.788$).