Examining the induction of limb regeneration through modulation of Stump currents by the sodium ionphore monensin in non-Regenerating post-metamorphic *Rana pipiens* frogs

An honors thesis

Submitted by

David Patterson Hessler Jr.

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1. Abstract

Limb regeneration in mammals has long been a notion better belonging to the science fiction realm than in reality. Humans are able to demonstrate some regenerative capabilities in the liver and in the regenerating fingertips of young children (Illingsworth 1974). Here we demonstrate a study aimed to inducing regeneration of an amputated limb by an adult non-regenerative frog. Regenerative medicine is an incredibly interesting field of research and its potential for therapeutic treatment is unmatched.

While previous studies have focused on electrical stimulation (Smith 1974 & Borgens 1977), we examined a pharmacological means of increasing the stump current. *Rana* are a non-regenerative species of frog that serve as a model organism to study regenerative growth. Previous studies by the Levin Lab have demonstrated that regeneration in vertebrates can be induced by transient sodium current, specifically by the selective sodium ionphore monensin. Frogs were amputated at the right forelimb and allowed to fully heal before treatment was applied. The objective of this project was to examine the regenerative abilities of the *Rana pipiens* frogs in response to monensin treatments in sodium baths. Therefore, this study sought to determine the ability of monensin to induce a regenerative event to post-morphogenic non-regenerative frogs

Although the study is still at an early time point and full analytical data is not yet recorded, early indication shows that treatment with monensin and sodium is capable of inducing a regeneration event in adult *Rana pipiens*. When compared to water treated and monensin only treatment groups, the monensin + sodium treatment produced results that appear indicative of

blastema formation and propagation. After all growth has halted and animals are sacrificed, histological data will reveal what structures, if any, were regenerated.

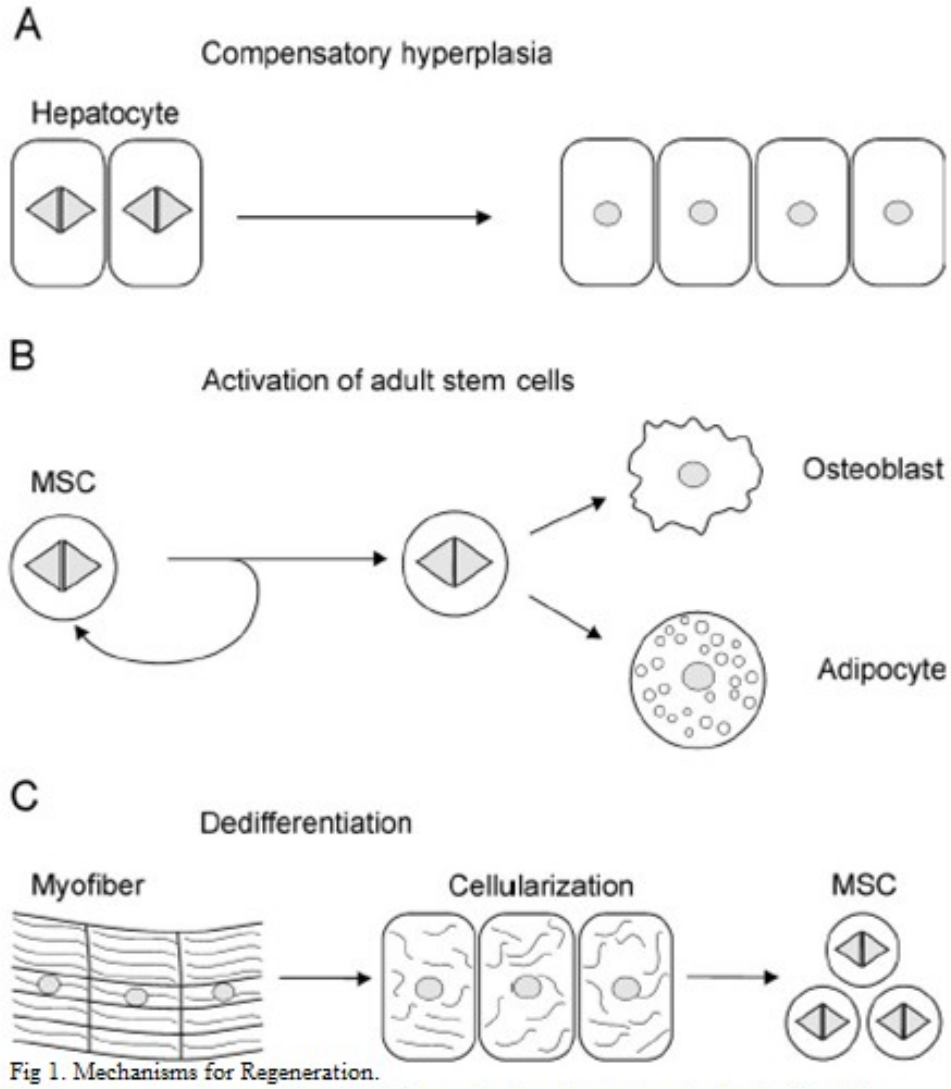

Fig 1. Mechanisms for Regeneration.
A. Compensatory hyperplasia. This is the mechanism for regeneration in the liver. B. Activation of adult stem cells, such as a mesenchymal stem cell. This type of regeneration plays a criticial role in the regrowth of bone after a fracture or break. C. Formation of mesenchymal-like stem cells by dedifferentiation. The myofiber breaks down into mononucleate cells that return to a stem cell-like state.

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2. Background

a. Regenerative process

Regeneration is a ubiquitous process that functions throughout the life cycle in all species. These regenerative processes can be broken down into three distinct events as shown in Figure 1. Our research focuses directly on Dedifferentiation (Fig 1C), which allows for the local cells around the wound site to be transformed into a stem-cell-like state, a critical initial step in the regeneration process and formation of the blastema(Fig 2).

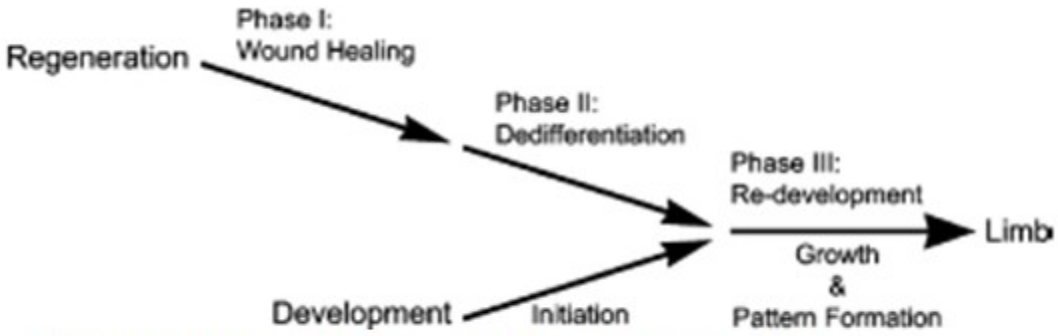

Fig 2. Schematic illustrating the steps of a regenerative event
The bottom half describes the analogous steps during limb development

After amputation, a layer of epithelial cells known as the wound epidermis covers the wound area (Stocum 2006). This wound epidermis does not contain a basement membrane and differs from the epidermis that is seen in normal wound response. This initial process is very critical to regenerative success, as forced wound closure by sewing a skin flap over the amputated area halts regeneration (Illingworth 1974 & Borgens 2002). In addition, this resistance of the wound epithelium can directly alter the stump currents; stump current and

wound epithelium thickness are in an inverse relationship. When the wound epithelium was gently removed from a limb with a low stump current, the current was immediately transformed to a higher level (McGinnis 1986). After the amputated limb is covered in a thin epidermal layer, the blastema begins to form under the surface.

The blastema, a mass of dedifferentiated mesenchymal cells, is formed from underlying tissues, including muscle, cartilage, dermis, and Schwann cells (Tanaka 2003). The tissue cells dedifferentiate into a "stem-cell" like state, allowing them to differentiate into bone, tissue, or nerve. Recent research by Krag *et al.* in 2009 demonstrated that these cells do not need to reach a fully pluripotent state in order to successfully regenerate. They exhibited that positional identity was retained by cartilage-derived blastema cells, but not from Schwann cell-derived blastema cells, indicating that most cell types are restricted to their own tissue identity. These results call indicate that the blastema is a not just a "mass" of dedifferentiated cells, but rather retains some original identity. Many factors are believed to be responsible for signaling these cells to dedifferentiate. It has been demonstrated that the rate of wound healing and the extent of proliferation are directly regulated by electrical cues (Song (2002)).

The final step in this process is the re-development of the amputated limb. This stage is characterized by the growth and differentiation of the blastema. At this stage, studies have indicated that the blastema is functionally equivalent to a developing limb bud, seen in larval development (Gardiner *et al*. 2002). Early stage limb buds can regenerate perfectly; however, by the end of limb development, this regenerative ability is completely lost for mammals. Induced regeneration for organisms such as Xenopus, results in the growth of a structure known as a hypomorphic spike instead of a fully functional limb (Brockes 1997). Histological analysis has shown that muscle, bone, tendons, ligaments and dermis are not contained within the spike, only

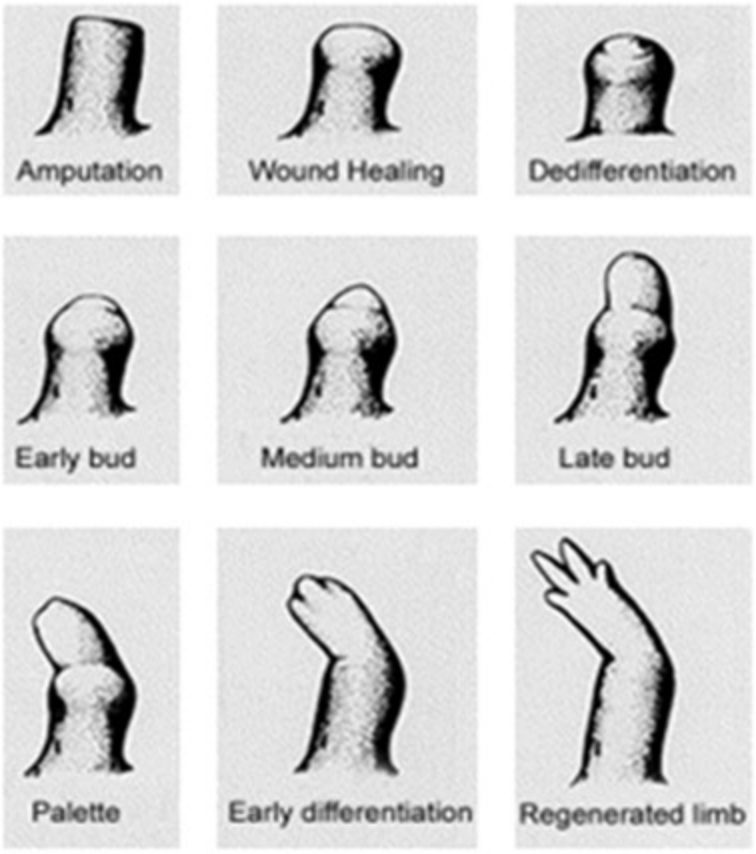

Fig 3.
Illustration depicting the process of limb regeneration.
(Brockes 1997)

skin, nerves and blood vessels reform (Beck *et a.,* 2009). In order for proper reconstruction, these blastema cells must remember information about their original positioning and features of the lost limb. This positional identity is demonstrated in the yearly regeneration of antlers in deer (Levin 2009). Figure 3 depicts the events of regeneration from initial limb loss through to full regeneration.

b. Stump Currents

The alteration of cellular transmembrane potentials, due to ion flows, is thought to be a triggering factor for the cells to dedifferentiate and enter a highly mitotic state (Hechavarria *et al.*) In fact a great deal of research has indicated that stump currents play a crucial role in the regenerative and developmental process (Borgens, McCaig). This current must be pulled out of the stump, as inward current is capable of inducing degeneration (Borgens (1977, 1996)).These stump currents originate in the subdermal area of the organisms, indicating that nervous system signals are not responsible for driving ionic flow (Borgens (1979)) and that the battery is skin driven. Therefore, alteration of the skins permeability can increase the flow of ions. Large steady currents leave the end of the amputated stump in newt limbs for up to two weeks after an

amputation. These currents likely increase the signaling pathway process and expose the amputation site to a variety of factors that signal cells to dedifferrentiate (Levin (2009)). These newts are capable of complete regeneration throughout their lifetime; however, most frog species do not exhibit the same type of regenerative capability. The current density leaving the stump of *Rana pipiens* is greatly depressed (less than a quarter) in comparison to newts. It has been suggested that these depressed currents are due to shunts caused by the subdermal sinuses of the frogs as seen in Figure 4 (Borgens (1979)). *Rana pipiens* tadpoles retain the ability to regenerate until the adult phase, matching the development of the subdermal sinuses (Borgens (1979)). These greatly depressed currents are incapable of inducing regeneration. Therefore, the development of a large and uniform current out of the stump is critical for the regeneration process. Sodium ions play a critical role in this process as they are largely responsible for controlling membrane potentials. In fact, the magnitude of the stump current is often dependent upon the concentration of sodium in the external medium (Borgens (1984)). In addition, adult frogs are more responsive to changes in Sodium concentration than larval stage organisms (Rose (1944)). As such, the focus of this and many other studies are to determine effective methods to increase this stump current.

c. Classical Studies

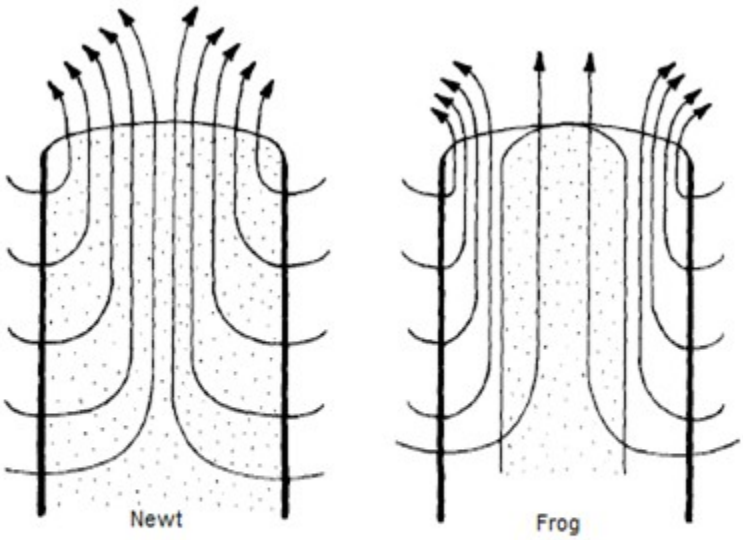

Fig 4. Models of current patterns through newts and frogs. The heavy lines represent mature skin, which drives current inwards. The dotted areas represent core tissue, while clear space represents the subdermal space that causes shunting. (Borgens 1979)

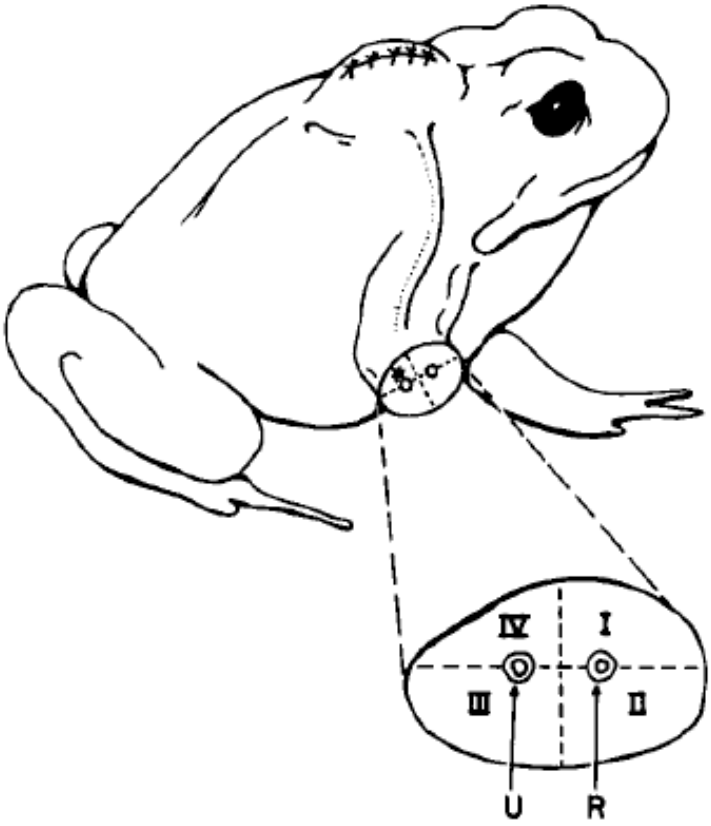

Fig 5. Diagram of frog with the negative electrode represented by (*) at the wound surface. The four quadrants (treatment groups) are as follows: I dorsal-preaxial, II ventral-preaxial, III ventral-postaxial, IV dorsal-postaxial. The U and R stand for ulna and radius. Smith (1974)

High degrees of frog limb regeneration have been produced through research studies. For instance, implantation of an additional adrenal glands in hypophysectomized frogs resulted in a very high degree of regeneration(Schotte & Wilber 1958). Our research is focused on increasing the regenerative abilities of *Rana pipiens* by stimulating the stump to produce a larger stump current. Previous studies have focused on accomplishing this same outcome, mainly through direct electrical stimulation. A study by Dr. Stephen Smith was one of the first to investigate the effects of using an implantable electrode in frogs to stimulate regeneration. He used adult *Rana pipiens* frogs that had entered into the post-metaphoric period of growth. He kept these frogs in individual plastic boxes housed in a 24°C incubator with a twelve hour light cycle. In addition, he fed the animals three times a week on mealworm larvae. Frogs were divided into 4 separated treatment groups of 25 frogs each, anesthetized, and then amputated with scissors at the mid-forearm level. The 1.35 V mercury cell was placed into an incision in the dorsal midline of the frog. Next they fed a Teflon-insulated

The results of Smith's experiment indicated a large amount of regeneration caused by the implantation of this electrode. In all 4 treatment groups the vast majority of frogs demonstrated at least a grade 2 regeneration. In addition, treatment groups II & IV demonstrated several grade 4 regenerates. In fact, all 21 surviving frogs of group IV produced some regenerative response. He reports that one of his grade 4 regenerates produced a completely organized regenerate that

was “absolutely indistinguishable from a normal one.” However, this individual frog represented an extreme case and all other grade-4 regenerates were not nearly to the same degree. Interestingly, both of the postaxial treatment groups produced a higher proportion of regenerates, especially grade 3 and 4 regenerates than the preaxial groups. These results can be seen in Table 1.

TABLE 1

SUMMARY OF RESULTS

Position of Implant	Number of Animals Surviving out of 25	Grade 1	%	Grade 2	%	Grade 3	%	Grade 4	%
1. Dorsal-preaxial	20	6	30	10	50	4	20	0	0
2. Dorsal-postaxial	21	0	0	8	38.1	8	38.1	5	23.8
3. Ventral-preaxial	18	8	44.4	7	38.9	3	16.7	0	0
4. Ventral-postaxial	22	3	13.6	9	40.9	7	31.9	3	13.6

Smith successfully demonstrated that a very small electrical current is capable of having a profound effect on the stimulation of regeneration, but specifically in a non-regenerating species. In addition, the position of the implant played a critical role in the development of the regenerate. Smith concluded that stimulation of the postaxial stump must mimic the embryonic state more accurately than preaxial stimulation. These results provide a great deal of information about the possibility to induce stump regeneration, however there is a large amount of information missing. For instance, Smith introduced no control samples to demonstrate that the regenerative effects were not due to an additional factor. Specifically, he needed two non-growth controls: One group that contained no electrode and was left untreated, as well as an additional group that contained a sham electrode. Controls like these are imperative to provide a means to compare the results. In addition, it is possible that the addition of the antibacterial and antifungal powder could have some measured effect. Although they used only healthy and vigorous frogs,

placing the frogs in individual containers in an incubator is an interesting approach to frog care, as frogs in high stress or uncomfortable situations may be less likely to focus all energy on limb regeneration.

Fig 6. Position of implanted battery and electrodes.
Wick stimulater was implanted on the back with one electrode coiled underneath. The wick bridge exited in the dorsal postaxial quadrant of the stump and was connected to the other electrode

In 1977, three years after Smith's work, a research team led by R. Borgens sought to follow-up on Dr. Smiths initial observations. They used a nearly identical experimental setup, as shown in figure 6. Therefore they were able to improve upon several aspects that greatly improved the quality of their experimental procedure. One of the most important adaptations that Borgens used was the introduction of a wick stimulator that is able to draw current through the stump without introducing significant electrode products into the tissue. This was made possible by using Ag-AgCL electrodes connected via a wick bridge, instead of an electrode made using an unreactive metal such as platinum, as used by Smith. The only toxic product of this system is the Ag+ ions that are released from the interface between the silver electrodes and the AgCl aqueous environment protecting it. Borgens maintained large adult *Rana pipiens* in individual plastic boxes with moistened toweling at the bottom. The frogs were fed three times a week on live mealworm larvae, and were inspected for wellbeing before surgical procedures. Borgens used MS 222, as did Smith, to anesthetize the frogs and amputated at the right forearm. Twenty frogs were implanted with these wick stimulators that were capable of delivering 0.17-0.2 μA of negative current, and ten were implanted with anodal stimulators (positive current). In addition, Borgens included ten more frogs that were implanted with a sham stimulator to act as a control group. All

units were removed after three to four weeks, but frogs were allowed to continue growth for up to one year.

The results from Borgen's experiment showed marked and consistent differences between the three experimental groups; cathode stimulate, anode stimulated, and sham stimulated. Although there were a few atypical responses, cathode stimulated animals showed an organized extension of the radio-ulna. In addition there was some growth of nerve and muscle tissue. Sham stimulated animals showed formation of disorganized calluses, with little to no nerve or muscle growth. In comparison, anode stimulated frogs showed extensive degeneration, a confirmation of their earlier work which suggested current directed into the stump current could decrease or inhibit stump growth. In addition, both sham and anodally stimulated frogs demonstrated no change in external appearance after 5-8 weeks post amputation. Cathodally stimulated frogs showed grossly swollen and red wound sites at 4-6 weeks post amputation, and formed a blastema. These bulbous masses continued to growth and change form for up to 11 months. The Cathode treated frogs were the only ones to exhibit growth of nerve trunks, regenerated muscle and organized bone extension. However, there was one atypical anode stimulated frog that developed some nerve and muscle regeneration. A schematic diagram of the difference between newt skin batteries and implanted frog batteries is shown in figure 7.

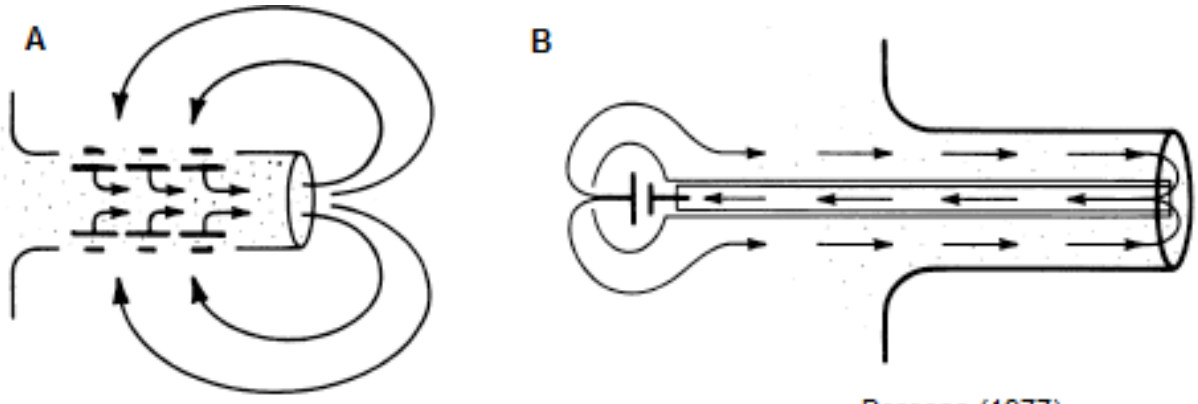

Fig 7. Comparison of stump currents Borgens (1977)
A. Endogenous currents through newt limbs that are driven by the skin batteries
B. Artificially imposed current through frog limbs driven by implanted mercury cell

Although Borgen's results clearly delineated a difference between out flux and influx of current, the frogs in his study did not exhibit nearly as high a degree of regeneration as did Smith's study. Although these differences between the two studies could be due to a variety of factors, it raises some questions about the regeneration of *Rana,* particularly due to the presence of two atypical regenerates, the fully regenerated specimen described by Smith, and Borgen's anodally treated frog that showed minor regeneration. Although both were extreme cases within their treatment groups, it is interesting to note that this atypical event may be a common occurrence. Despite these differences, both studies demonstrated that relatively small currents can evoke limb regeneration in a typically non-regenerative species. These studies demonstrated that direct electrical stimulation is capable of causing this regenerative response; however the implantation of such a device requires two procedures and is very invasive. Therefore, a pharmacological means of inducing a regeneration response would represent an interesting approach to regeneration

d. Current Studies

Recent work by Michael Levin's Developmental Biology lab (A. Tseng 2010) has indicated that vertebrate regeneration can be induced by transient sodium currents into specimens

that grow a nonpermissive wound epithelium, as most non-regenerating species do. This is possible through direct modulation of the voltage-gates sodium channels to increase the sodium transport and thus the stump current. The research team used the larval stage specimens of the aquatic frog species *Xenopus laevis*, which has widely been implicated as a powerful study organism that uses several regeneration pathways conserved by mammals (Beck 2009). In the larval stages it is capable of complete repair of all appendages upon injury, and even shows regenerative ability in adult stages.

The research team first investigated the importance of sodium flux in regeneration by blocking the voltage-gated sodium channels during larval development. In addition, through the use of RNA interferences of the *Xenopus* $Na_V1.2$ gene, they demonstrated that these sodium channels are a requirement for the establishment and outgrowth phases of regeneration in *Xenopus*. This partially explains why increasing sodium concentration of frog baths caused a great increase in the stump currents of the frogs (Borgens 1977). These channels are critically active during the initial stages of the regenerative response, causing a large influx of sodium thusly creating a strong current leaving the stump site. They next investigated the effects of the sodium ionophore monensin to directly modulate sodium transport without genetic manipulation.

To accomplish this task, they amputated larval tails that had entered into the refractory period. During this stage the *Xenopus* tadpoles have lost their regenerative potential and will no longer be able to regenerate a tail. At a similar time-point to the native $Na_v1.2$ expression, the tadpoles were treated with monensin in a medium containing elevated levels of sodium (Sodium Gluconate). Using a CoroNa

Green indicator dye to visualize sodium content, they confirmed that the monensin treatment increased intracellular sodium content (figure 8). In addition, this was capable of returning the

tadpole to a regenerative state in which full regeneration is capable. Their results indicated that it was the effect of the induced sodium influx, rather than monensin or high extracellular sodium treatment alone.

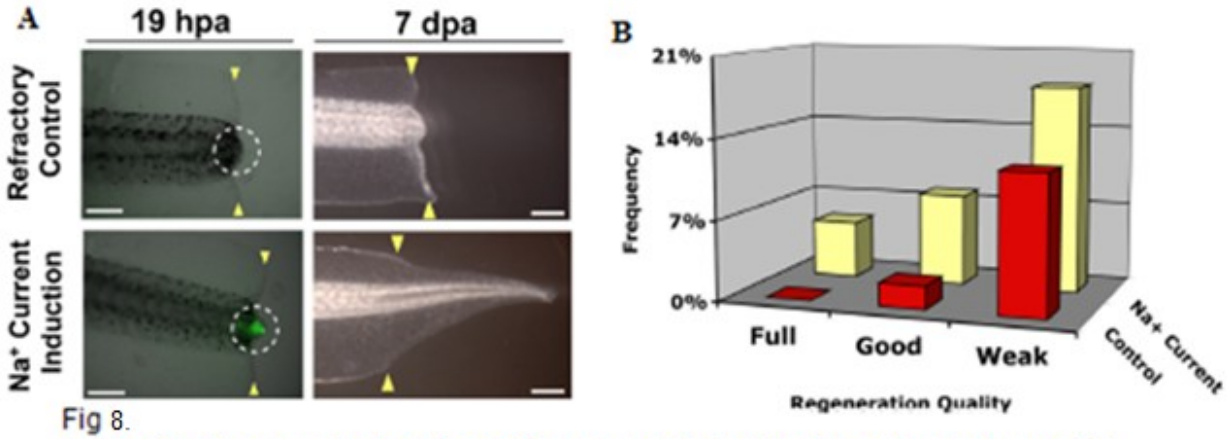

Fig 8.
A. CoroNa Green analysis of the sodium current induction. Amputees that were left untreated failed to regenerate, while those treated with monensin regenerated fully. B. Stimulation of the sodium current increased regeneration quanitity and quality compared with refractory controls. Tseng (2010)

These results represent a novel role for the Voltage-gated sodium channels in the mediation of sodium transport. They were able to demonstrate that Na_v activity was required during the initiating stages of limb regeneration. In addition, monensin has the capability of inducing a large sodium influx that can effectively mimic the action of these channels when they are either blocked or have lost function and guide regenerative outgrowth through cell proliferation and gene expression. It has long been thought that once cells reach the refractive period they lose all ability to regenerate. However, these results indicate that the cells maintain their intrinsic ability and represent an exciting pharmacological approach to restore regeneration.

e. <u>Monensin</u>

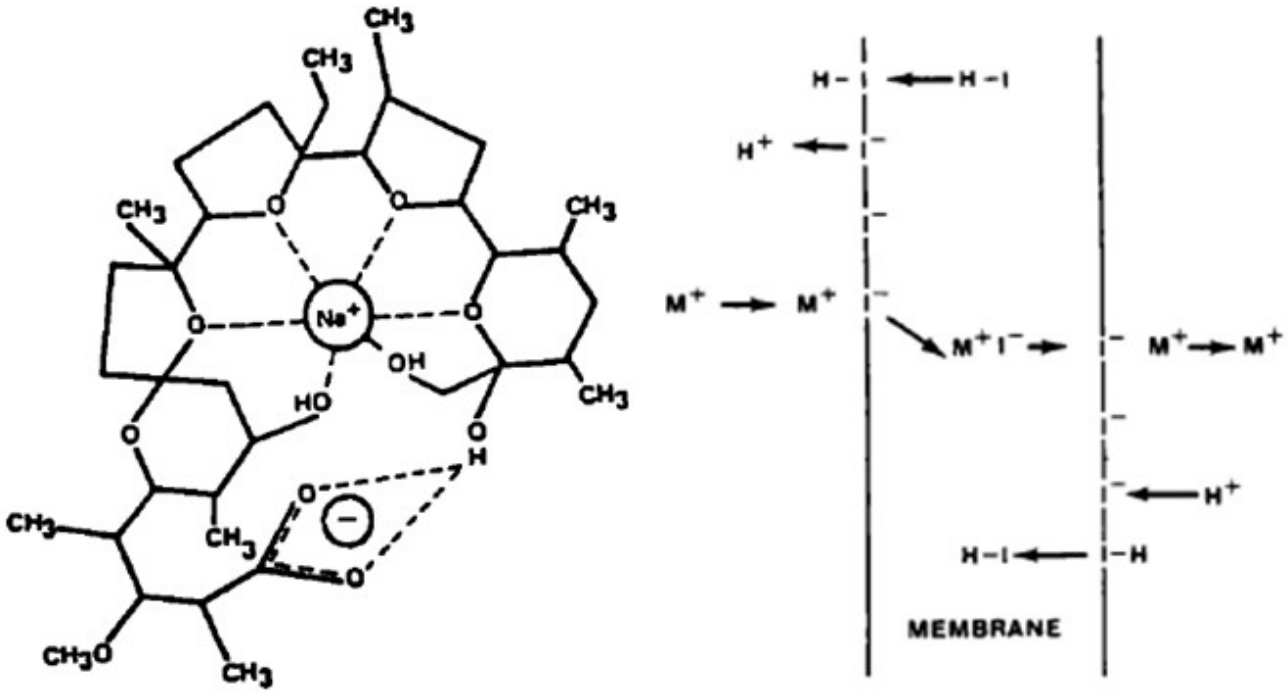

Fig 9.

A. Structure of monensin
B. Transport of sodium and hydrogen through the cell membrane by monensin

Monensin is an ionophore antibiotic that is capable of collapsing Na^+ and H^+ gradients. Monensin is an open chain molecule (Fig. 9), that is capable of selectively binding sodium at an affinity ten times greater than its nearest biological competitor K^+ (Mollenhauer 1990). It is composed of non-polar hydrocarbons which allow it the complex to be freely soluble in the lipid membranes (Mollenhauer (1990)). Through this binding, monensin is capable of transversing the membrane in this monensin-ion complex. Once it reaches the interior membrane, it releases the ion and binds to a proton. It then returns through the membrane and exchanges the proton to the external medium as shown in Figure 9 (Mollenhauer 1990). On a cellular level monensin has

f. Our Approach

The work by the Levin Group demonstrated an intriguing approach to induce stump currents during non-regenerative growth periods. Although they used the aquatic *Xenopus laevis* model which exhibits regenerative capabilities throughout its life cycle, the previous work by Smith and Borgens (Table 2)has suggested that even non-regenerative species such as *Rana pipiens* are capable of a regeneration response. *Xenopus laevis* are a very effective study organism that requires fairly minimal (less involved) care, we wanted to study the effects of Monensin on the *Rana pipiens* frog due to its non-regenerative abilities. This would allow for greater accuracy when determining the degree of regenerative growth of the frogs, because endogenous regeneration will not be confused with induced regeneration. In addition, previous studies that have used *Rana* as a study organism have taken minimal effort into their care and wellbeing. Therefore, it was of paramount importance to our study that we provided the most comfortable and natural habitat for our study organisms. Poor conditions will induce a stress response in the frogs causing lots of excretion and skin shedding. Under these conditions, the frogs will become uncomfortable and less likely to feed properly, decreasing their health and recovery abilities. Table 2 below outlines the results and methods of several previous studies.

Studies	Method	Time Point	Species	Outcome
Borgens (1977)	Implantable Battery	Post-amputation	Adult *Rana*	Slight Regeneration
Cecil (1986)	Vitamin A treatment	Post-amputation	Juvenile *Rana*	Enhanced regeneration
Smith (1974)	Implantable battery	Post-amputation	Adult *Rana*	Some full regeneration *
Tseng (2010)	Monensin	Post-refractory	*Xenopus* tadpoles	full regeneration

Therefore this presents a situation in which the regenerative response of *Rana* can be determined in response to pharmacological treatment instead of direct electrical stimulation requiring implantation and removal operation. In addition, because the monensin treatment is

applied after a normal wound healing response, it represents a new method of regeneration induction that is free of the preconceived regeneration timeframes. Although the direct mechanisms (downstream processes) through which sodium current contributes to the regenerative process is the subject of many current studies, it has clearly been implicated as having an important role in the induction of regeneration (Levin 2010). In addition, because the degree of difference between the regeneration events experienced by the Borgen's study and Dr. Smith's study, additional studies should investigate this regeneration in order to compare the results.

3. Hypothesis

In this study we examine the ability of treatment with monensin and sodium to the amputated forelimbs of non-regenerating post-metamorphic *Rana pipiens*. Previous studies have indicated that regeneration can be induced in *Rana* frogs as demonstrated in Table 2. However, we present a novel timeframe in which regeneration can occur after the normal wound healing response. We hypothesize that treatment with Monensin + sodium will result in a higher degree of regeneration than sham treated and Monensin only treated individuals; however, full regeneration of a functional limb will not occur. Regeneration events in the experimentally treated individuals will indicate that a regeneration event can be induced outside of the refractory period.

4. Specific Aims

The specific aim of this study was to determine the response of *Rana pipiens* to the sodium ionphore monensin. Previous studies by Tseng *et al* demonstrated that monensin was capable of inducing regeneration of a full tail after the refractory period of tadpole tail growth. Previously, regeneration was assumed impossible after the refractory period had passed. Therefore, by examining the ability of monensin and sodium treatment to induce regeneration in a typically non-regenerative species we seek to further develop an understanding of the role of monensin in regeneration. This study seeks to evaluate the regenerative response of these treated specimens in comparison to control treated specimens. These controls should exhibit no outgrowth and confirm that *Rana pipiens* is a non-regenerative species in the adult form. In addition this study seeks to provide the best care conditions for the study organisms. We believe that by providing the most natural habitat and providing full care for the frogs that the most natural response to the treatment will be achieved. Because the frogs will be comfortable in their environment and well-fed, they will be capable of focusing all energy on regeneration instead of towards a stress response and hyperactivity.

5. Methods

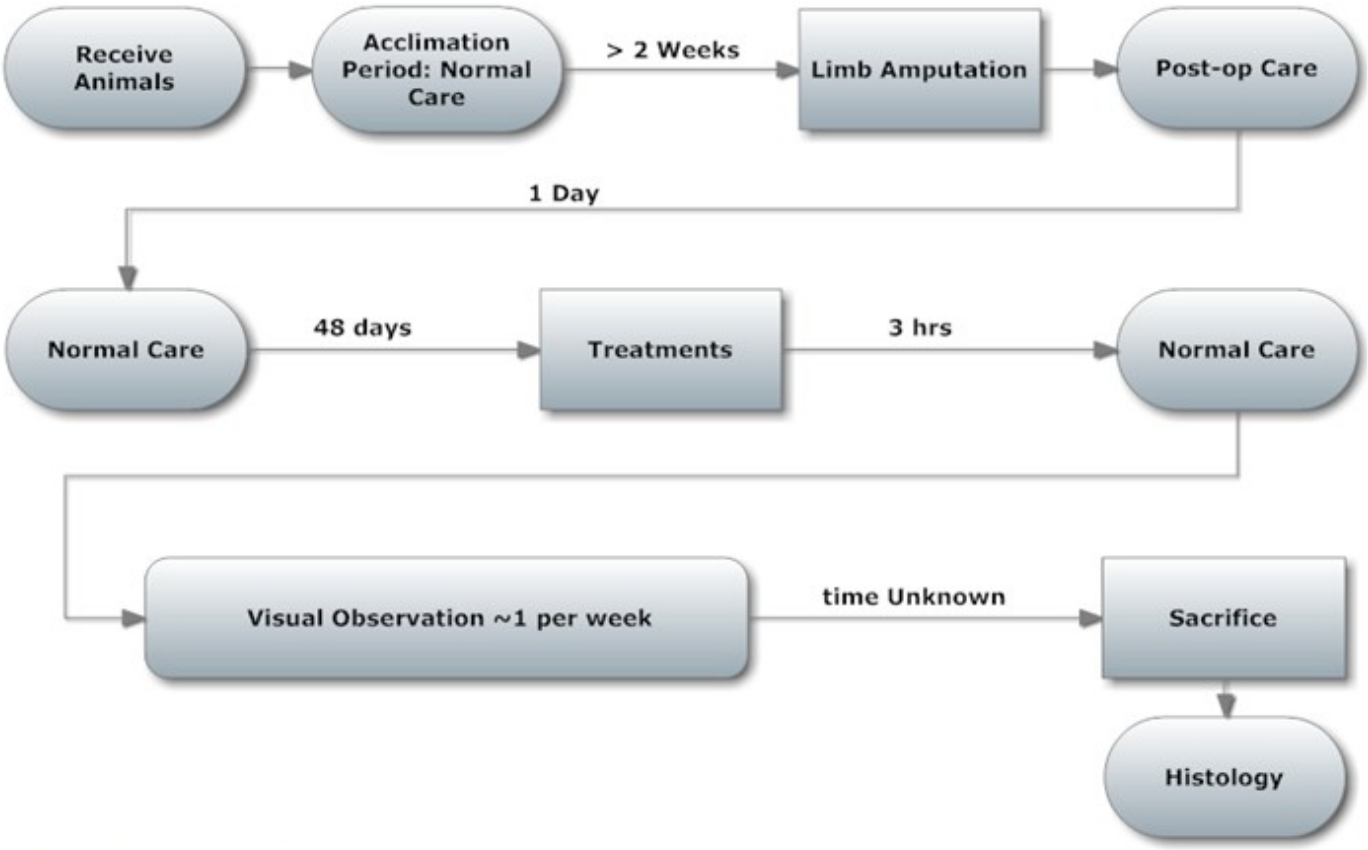

Fig 10. Experimental Process

a. Experimental Process

b. Animal Care

All specimens were cared for in accordance to submitted protocols for IACUC an DLAM.

I. Study Organism –*Rana* pipiens

Rana pipiens is a fairly large species of frog that reaches a length of about 4.5 inches (Fig. 11). They are a member of the Ranidae family of frogs. This family is referred to as "True Frogs" because they have smooth moist skin, webbed feet and powerful hind legs (Halliday 1986). In addition their skin is capable of changing hue to improve its camouflage depending on its environmental surroundings. Their natural habitat is located around permanent sources of water with aquatic vegetation that is abundant. Most of their time will be spend in marshy

or grassy land, but they will return to the water for breeding and hibernation. Although they are opportunistic feeders, they are very well adapted to hunting due to their great jumping ability as well as their sticky tongue which allows them to grab and hold onto prey such as crickets (Hofrichter (2000)). 2.5" frogs were acquired from NASCO. The frogs were rush shipped to our lab and were immediately unpacked and checked for sickness or any noticeable change in appearance. They were then placed into tanks at a maximum of 17 per tank, or else overcrowding can become an issue.

Fig 11. Northern Leopard Frog (*Rana pipiens*)

II. Cage Setup

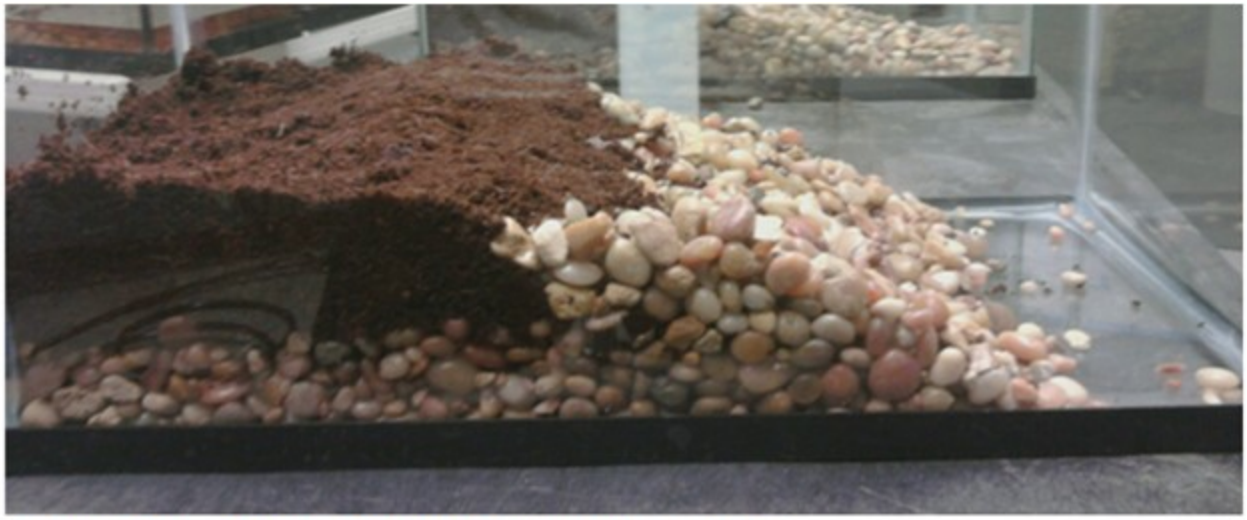

Fig 12. Creation of the frog habitat (shown without water)
Tank is approximately half-land half-aquatic environment. Coconut husk is used as the substrate, and the rocks are used to provide water filtration and keep the substrate from dissolving into the water

TopFin glass aquariums were used to house all of the frogs. Varying aquarium size was used depending on the amount of frogs contained within each tank. Because the frogs' ideal habitat is aquatic based but with lots of surrounding dry terrain, I attempted to create a terrarium setup that included both parameters (Fig 12). Smooth aquarium rocks were first added to the empty tank to provide a base for the substrate. Rocks were placed about an inch thick for the first half of the tank. The substrate used was Zoo-Med's Eco Earth coconut fiber substrate. This substrate has the ability to absorb and breakdown waste products in these amphibian habitats, therefore it keeps the tanks from smelling like frog urine. In addition, this substrate does not allow for bacterial or fungal growth, making it an ideal solution. The substrate comes in compressed bricks that are soaked with water to allow them to separate.

III. Feeding

All frogs are fed live crickets three times a week *ad libitum*. Crickets are removed from the cage and placed into a cricket shaker. Here they were coated with calcium powder to supplement the frog's diet. In addition to providing calcium supplementation, the calcium powder made the normally brownish crickets a pure white color. This eliminated their natural camouflage in the substrate and made them easier to see and therefore catch by the frogs. After the crickets were well coated in calcium, they were dropped into the frog tanks. Typically each frog would eat 3-5 crickets per feeding time, but in some of the larger tanks it is nearly impossible to assure a completely balanced distribution. Crickets were continually

added until frogs did not show any more interest in eating. After the frogs appeared to slow down their eating, any crickets that remained in the tanks were hand fed to individual frogs using tongs. Although most frogs were initially afraid of the tongs, after continued use they became accustomed to it and did not jump away. Frogs were never force fed.

IV. Frog Tank Care

After each feeding, the water in the frog tanks was changed and the tank was gently cleaned. Using a siphon attached to the sink in our room, the majority of the water in the tank was removed. In addition because the siphon was attached to the sink, we were able to create very powerful suction that was useful to remove waste, feces and skin, from the tank. Once the majority of the water had been removed, the tanks were allowed to sit as the frogs finished the remaining crickets. During this time, water and waste that had been absorbed by the coconut substrate was allowed to filter out into the little water than remained in the tank. This remaining water was scooped out via a small capsule so that there was essentially no water remaining. Tongs were used to remove remaining skin and waste that was either on the dry terrain portion or was stuck to/in the rocks. After the tanks had been cleaned, Poland Spring water was added back into the tank to its original water level (Fig. 13)

Fig 13.
Frog tank showing both dry terrain and an aquatic area. Water level on this tank is a little high, hence the erosion of substrate that can be seen in the rocks in the middle of this figure. Frogs spend about an equal time in each area

Often times the activity of the frogs causes the rock slope to degrade and exposes the leading edge of the coconut substrate to the water. The tidal action caused by frogs moving in and out of the water slowly erodes away the substrate, decreasing the amount of dry land space.

When these situations occur, the eroded coconut is removed from the bottom of the tank and the slope is rebuilt. Additional substrate is added to return the land terrain to its original form. After a month of use, tanks were completely cleaned by removing all substrate and the rocks and scrubbing the tank. This ensures that the substrate does not become oversaturated with waste products and keeps the frog habitats relatively fresh.

In the event that a frog shows sign of infection or sickness, it is immediately separated from the other frogs and placed into a solitary cage containing a low level of rocks and substrate but covered completely in water. Antibiotics were administered to the water to improve the infection. Sick frogs were isolated for up to a week, at which time their appearance typically returned to normal. Once they returned to normal appearance, they were returned to their original tank.

V. Cricket Care

Crickets were ordered through and delivered by Ghann's Cricket Farm (www.ghann.com), on a weekly basis. Approximately 1,500 crickets in the size were ordered. The crickets were equally distributed into cricket cages (Fig. 13) to prevent overcrowding. The cricket cages contained two feeding troughs filled with Ghann's cricket chow. In addition, crickets require water, however they can easily drown in large bodies of it, so cricket pillows were used. Once added to water, the pillows rapidly absorb moisture and balloon up. Two to three of these pillows were placed in each cage. Finally, crickets require a dark habitat in which to hide, or else they will stress out and die. Egg crates are commonly used, but for the size of our cages they were rather inconvenient. Therefore we used long black tubes with a covered outside end that had been gauged on the interior to allow crickets to climb into them. Not only did these tubes provide an adequate place to hide, but they also made the feeding

process very easy. If egg-crates were used, it was very difficult to effectively dump them into the cricket shaker. These tubes allowed for easy removal from the cricket cages and then subsequent dumping into the cricket shaker. In addition, this prevented me from having to individually pick up each cricket with tongs, as was necessary when cleaning the cages.

Fig 14. Cricket Cage
4 black tubes provide the main housing for the crickets. The tubes are open ended on the interior of the cage. The cricket pillows and the feeders are both visible.

VI. Post-operative care

Following surgical procedure, as will be discussed next, frogs were maintained in individual weigh boats with a small amount of water. The amputated limb was slightly elevated out of the water so that it could successfully clot. Frogs were periodically sprayed with water to ensure that they would not dry out. When the frogs are anesthetized, they are extremely limp, therefore any movement of the frog must be made carefully or else the wound site could be reopened. Frogs were allowed to recover in their individual areas until the bleeding had stopped and they were capable of supporting themselves. Once they were capable of free

movement, they were returned to a recovery tank with the other frogs that were also recovering.

c. Experimental Setup

I. Control and Experimental Groups

All frogs underwent the same exact surgical and care procedure. About 48 days after the date of amputation the frogs were broken into different treatment groups. The Experimental group of frogs was treated in a sodium and monensin bath. Several control groups were used to not only to ensure that the frogs did not possesses natural regenerative capabilities, but also to ensure that if regeneration occurred in the experimental treatment groups, that it was due to monensin and sodium. In addition, it provides a point of comparison for the experimental treatments, and ensures that monensin itself is not chemically responsible for the regeneration induction, but rather stump current created through the action of the sodium ions. These treatment groups are displayed in Table 3.

Treatment	Number	Group
Monensin + Sodium + Poland Spring	5	Experimental
Monensin + Poland Spring	5	Control
Poland Spring	5	Control

II. Surgery

For each surgery day, a new tank is prepared to house the post-amputation frogs. About 15 frogs that were in good health and had been acclimated to the lab environment for at least 2 weeks were amputated on each surgery day. Frogs were not fed within 24hrs of the surgical procedure. Frogs are removed from their original housing and injected with 2-4mL (depending on size) of tricaine mesylate (MS-222) the same anesthetic used by both Smith and Borgens. Frogs were allowed to go completely under the affects of anesthesia before an operation was to occur. The frogs were placed on their stomach in a sprawled out position and observed for any sort of movement. If the frogs continued to twitch and show slight leg movements, then an additional injection of Tricaine was used. Once frogs were completely anesthetized, typically after a ½ hour, their right forearm was amputated with a single clean cut through tissue and bone with a No. 11 surgical grade scalpel (Fig. 15). Although some specimens exhibited extensive bleeding, most bled relatively minor amounts. After amputation the frogs were placed into their individual weigh boats as outlined by the post-operative care procedure

III. Treatment Baths

All treatments were applied 48 days post amputation, after the frogs have endured a normal wound healing event, and the amputated site is largely covered by non-permissive wound epithelium. The experimental group of frogs is introduced to the monensin treatment. Small containers, capable of comfortably holding 2-3 frogs are filled with about an inch of solution, or enough to cover the wound area. The solution contains about 200 μM Monensin and 180 mM NaG (Sodium Gluconate) in Poland Spring Water. This is an extremely high concentration of sodium compared to the normal salt levels in Poland Spring. Therefore the treatment solution provides the pharmacological ingredient, monensin, as well as the

Fig 15. Amputation site
Position of forearm cut is indicated on the skeleton by the single black line. Cut was made orthogonally across the forearm

necessary ion source of sodium to increase the intracellular concentration and thus drive stump currents. Frogs are allowed to soak for 90 minutes. After the initial soaking, the solution is continually diluted with additional Poland spring. The solution is then poured out, and new Poland Spring water is added to the containers. Frogs are kept in these containers until they begin to move around normally, at which point they are returned to their cage. The two control treatments are performed in the same exact manner.

IV. Visual inspection

Frogs were inspected at least once a week for any change in appearance to the amputation site. Pictures were taken of each frog in three different poses so that the amputation site could be viewed from several angles. The frogs were completely awake and uninhibited during the picture taking, because anesthetizing them for such a short period was unnecessary.

V. Histology

Protocols are currently being submitted so that we can run histology samples on the sacrificed frog. The right forearm will be fixed and histology will be performed looking for growth of tissue, bone and nerves. Many regeneration events do cause native tissue,

bone, and nerves to grow, but rather result in a hypomorphic spike characterized by some tissue surrounding a cartilaginous outgrowth.

6. Results

Although it is still very early in the regenerative process, as the first group of experimentally treated frogs is only two months post treatment, a few initial results have shown the potential for regeneration induction. As expected amputated frogs completed a normal wound healing response that caused the amputation site to slowly close up with regular epithelium. Without any

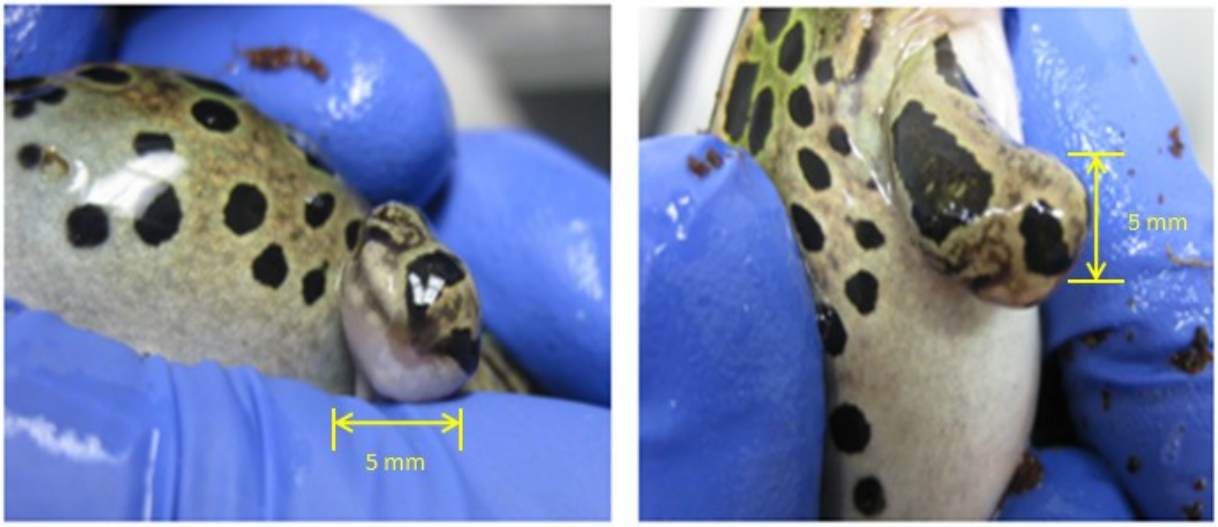

Fig 16. Poland Spring treated frog
Head on view of the poland spring treated frog shows that the amputation site is completely closed. The side view demonstrates that no outgrowth from the amputation site occurs

treatment, the frogs showed no regenerative capability nor did they demonstrate any signs of developing a bud. In control group frogs treated only with Poland spring, the thick epithelium continued to close over the wound site, and 23 days after the treatment (~2 months post amputation) the wound site was completely closed over (Fig 16). This is the typical wound healing response of adult *Rana pipiens*, and confirms that these frogs do not retain their regenerative capabilities. In addition, frogs treated with Monensin without sodium ultimately healed similarly to the untreated frogs. As seen in Figure 17, some redness appears after treatment with monensin, but it never develops into an outgrowth and by Day 56 the wound is completely healed over. This agrees with the results achieved by A. Tseng *et al*. which indicated that treatment with monensin or high levels of sodium alone is incapable of inducing regeneration.

In comparison, frogs subjected to the monensin and sodium treatment have demonstrated a distinct change in appearance of the wound site. A week after treatment the healed wound site became reddish in color and a distinct bulge develops. This small outgrowth , although only a

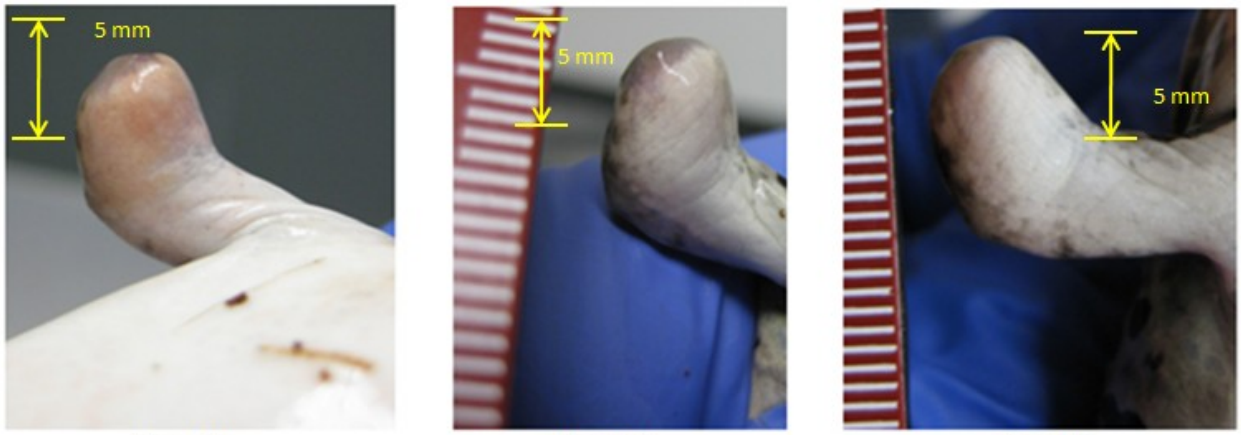

Fig 17. Monensin treated frog
12 days after treatment the amputation site is slightly red as is also seen at 27 days post treatment. However, this slight change in appearance was not accompanied by a regenerative outgrowth. By day 56 the site was completely healed over

few mm in extension from the original plane of amputation, is thought to be a blastema and is representative of a limb development phenomenon, an event not experienced by the control frogs. In Figure 18, this regeneration bud can be clearly seen by Day 15. This outgrowth is clearly absent when compared to the nonregenerative frogs in Figure 16/17. The head-on view used in Figure 19 clearly demonstrates the change in appearance of the amputation site as the regenerative outgrowth matures. After the initial appearance of the regenerative outgrowth, the bud continued to change in structure and appearance as further time passed post amputation. These frogs will continue to be monitored until all growth has halted. At this point, the specimen will be sacrificed and the regenerated arm will undergo histological examination.

Because this study is still at a very early timepoint, we do not currently have any histological or quantitive results in which to present. Solutions to these issues are presented in the discussion and future work sections.

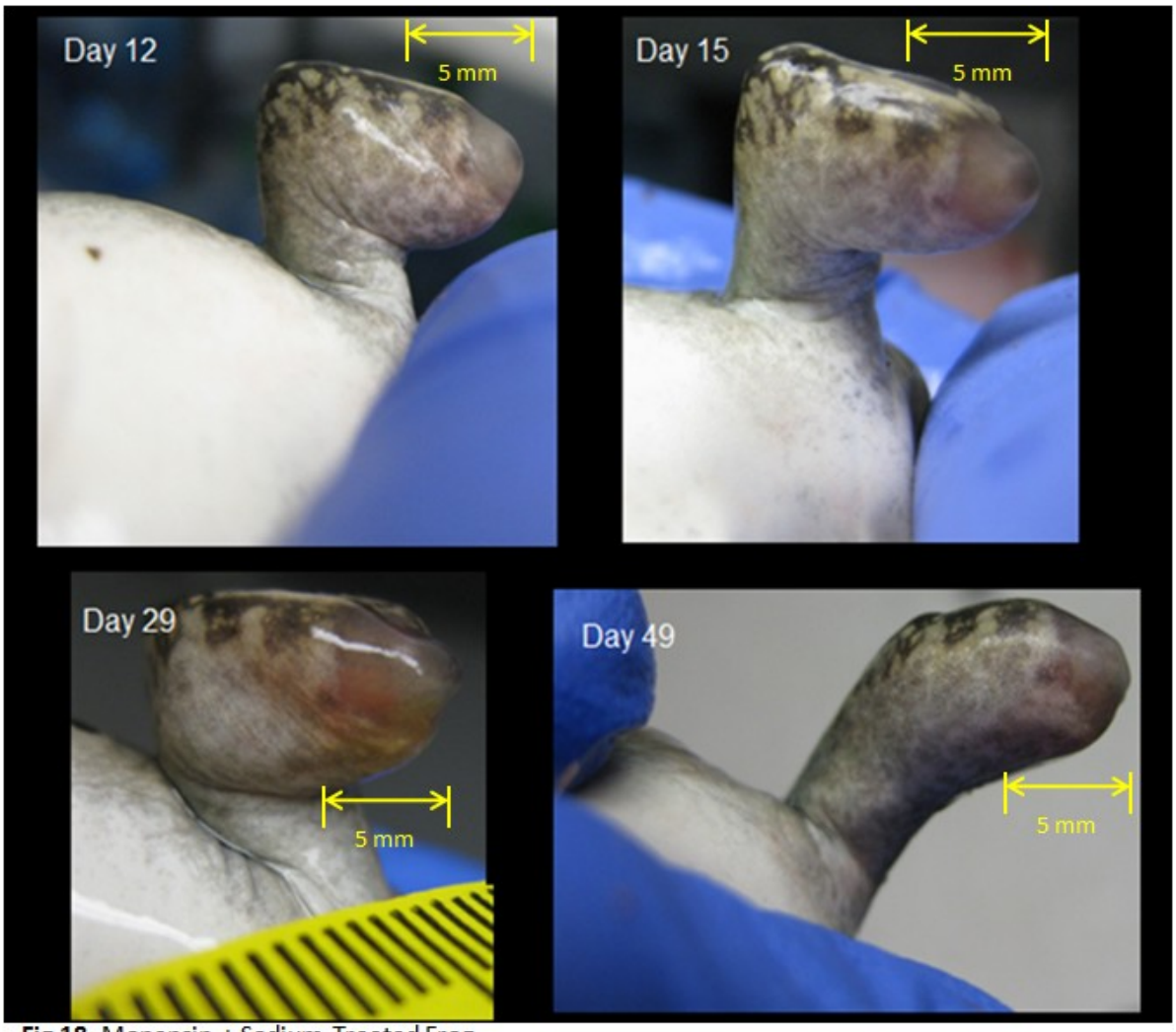

Fig 18. Monensin + Sodium Treated Frog
By day 12 post treatment the amputation site has become red similar to that seen in fig. 17. However, this change in appearance is accompanies by the development of an outgrowth that continues change in morphology.

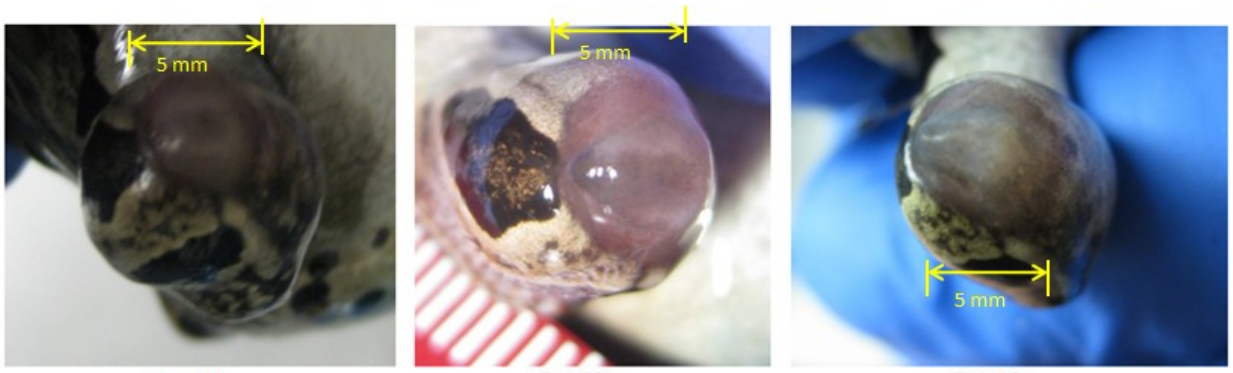

Fig 19. Monensin + Sodium Treated Frog
Top down view of the same frog shown in Fig 18. By day 27 after treatment, the initial amputation site has increased in area and size. A clear substructure can be depicted in the images of Day 27 and Day 46 post treatment.

7. Discussion

Although no definitive conclusions can be drawn at this point due to the lack of histological data as well as the need for more samples for reproducibility, the monensin and sodium treated frogs are clearly demonstrating some type of regenerative outgrowth. This is even more conclusive when compared to the untreated frog (Fig. 16) and the frog that was only subjected to monensin (Fig. 17). Interestingly, the appearance of the monensin treated frog limb was slightly different from the untreated group. This is most likely due to minute amounts of sodium ions that were present in the solution. However, at such small quantities, the wound site only slightly changed in appearance, but did not present any sort of growth. In comparison, the images seen in Figures 18 & 19 demonstrate a clear outgrowth from the amputation site. This blastema formation is indicative of the early stages of a regenerative response. Although it is too early in this study to conclude that these outgrowths are induced by monensin's ability to rapidly transport sodium ions into the cell, the initial results are promising. This regeneration induction is most likely the result of a cascade of signaling that is enhanced by the increased ionic flux

(Levin 2009), where a variety of physiological factors may be acting to signal the cells to dedifferentiate. Additionaly, the role of ionic currents has been demonstrated to play a critical role in the limb development process of mammalian vertebrates. A 2001 study by Altizer *et al.*, as part of Borgens group, demonstrated the importance of these currents in limb development of chick and mouse embryos. Using a vibrating probe they monitored the exterior portion of the embryo for ionic currents. They discovered that the emergance of the murine limb bud was associated with a steady outwardly directed ionic current. In addition, inwardly directed flank currents were observed at flank regions of the embryo indicating the completion of a dc circuit. The researchers were able to induce developmental defects in these embryos by applying an inwardly directed current to the sites of limb development. These results of this research team indicated that ionic currents are crucial to the development of a normal limb. If monensin is capable of inducing regeneration at a timepoint after the normal regeneration response is considered achievable, then it may be acting to create conditions that are very similar to the developmental stages. Although humans are incapable of large scale regeneration, the developmental processes used are similar through most animals. Monensin may be capable of inducing the mammalian cells into a developmental-like state of regeneration.

As previously discussed, proper frog care was considered to be a crucial component of this study. When frogs initially arrived they were very startled by any sort of human interaction and would rapidly jump around their habitat attempting to escape. After a week or two of acclimitization in the lab environment their fear subsided and they were became more comfortable with human interaction. In addition, they became accustomed to the weekly feeding cycle and their activity and awareness were markedly increased shortly before feeding time. During this period leading up to feeding, as well as during and immediately after, the frogs

became very vocal. These sounds seemed to alert the other frogs in the room that food was coming. One key aspect of our approach for frog care was to populate the tanks with 10-15 frogs. Most previous studies have housed each frog in their own individual container. In our experience, frogs sequestered to individual containers did not demonstrate the same voracity and liveliness of the grouped frogs, and often times would go without eating. However, once they were returned to the group they resumed normal activity. Although a more detailed study of this response would be appropriate, it was of potential interest due to the difference in animal care of this study as compared to previous ones.

I was very fortunate to become a part of this investigation at a very early stage in the research process. I joined with Nik Kojic in the Spring of 2010, and it wasn't until the late fall of that same year that our first frog specimens arrived. It has been a very long and time consuming process from starting off with a few *Xenopus laevis* frogs to transitioning to include over 200 *Rana pipiens*. However, the continued effort has led to a fantastic learning experience, specifically for experimentation using live animals, as well as a great opportunity to learn from Nik and Punita. Although my work on this project is shortly coming to a close, there remains a great amount of work and research that will be continue to be conducted on this on-going study.

8. Conclusion

a. This Project

No definitive conclusions can be drawn at this time point because it is still at a very early stage in this study. Although some of the initial results indicate that Monensin and sodium are inducing a “regeneration-like” event in the amputated frogs, a greater degree of reproducibility is required. As has been demonstrated by previous studies, these frogs have demonstrated a wide range of regeneration responses for similar to identical treatments. If Monensin and sodium

treatment are to be implicated in the regeneration of the limbs, similar treatments should result in similar outgrowths from the study organisms. Large variations in the regenerative response would indicate that additional factors are at play. This can be strengthened by further demonstration that sodium treatment or monensin treatment alone is incapable of producing a regenerative response. In addition, histological results will provide information regarding the quality and amount of regeneration that is occurring. Although visual inspection allows for basic information regarding the outgrowth, it is very minimal and is incapable of identifying the mechanisms responsible for this response. Therefore, the monitoring of the change in concentration of sodium and other important ions will provide crucial data at the molecular level (Zhang (2009).

With the promise demonstrated by the initial results, a strong continued effort on this investigation will hopefully indicate the ability of monensin to act as a regeneration inducer. Current work is focused on providing information regarding the effect of environmental and chemical factors on the frog's regeneration. It is expected that these treatments will not demonstrate regeneration and thus will further implicated the role of sodium and monensin.

b. Potential of Regeneration

Using regeneration as a means of therapeutic treatment still remains a distant possibility. Researchers are still working to analyze all the factors that enable many organisms to demonstrate the ability to regenerate lost limbs and organs. Until all these factors can be determined and understood, use on a human model is not possible, although that remains the ultimate goal. Much of these researchers work is spent determining connections and similarities between processes in regenerating species and in humans. A study by A. Altizer *et al*. indicated that the location of flank currents in the larvae of chicks and mouse are indicative of the site of

limb formation. In addition, they were able to produce developmental defects in these limbs by alteration of the outward stump current. Although the processes may not be completely understood, this indicates that stump currents are critical to the development of vertebrate species and thus may represent a unique possibility. If the cells in a non-regenerative vertebrate can be triggered in such a manner to mimic developmental conditions, then regeneration might occur. We hope that our research will provide further information regarding the regenerative pathways and will further our knowledge regarding regeneration.

c. What I have learned

Working on this project has been a unique opportunity and an amazing learning experience. Despite spending close to ten hours a week taking care of the frogs, I feel privileged to have had this opportunity. Although it is disappointing to not have more results after working for over a year a on this research, it has provided several important lessons that I am confident will be beneficial for my future endeavors.

The first of which is flexibility. When I first started to work with Nik, the focus of our efforts was on using Silk fibroin e-gels on the amputated limbs of *Xenopus* frogs; however, that focus was shifted to our current effort on limb regeneration using monensin. In addition, shipments of frogs or crickets could arrive at anytime during the day; therefore it was important to be able to adjust the work schedule to when the shipments arrived.

Patience was another important lesson that was stressed by working on this project. We had to wait for several months before our protocols were accepted. Therefore, although we were ready to start our experimentation last spring, the long waiting process for approval pushed the start until the fall. When our frog room was finally approved in early fall, we were excited to finally begin the study. However, as previously mentioned, *Rana pipiens* are not available

throughout the year, usually they become available by the start of October. However due to declining population and alteration in the environmental conditions, we received our first shipment of frogs by early November. Although our start time was greatly delayed, it is critical not to compound mistakes by attempting to rush through to achieve results. Patience is especially important in live animal studies, where controlling all factors is challenging. In comparison to *in vitro* studies, because the frogs are living organisms they are not always going to respond as desired. It is critical to remain gentle and calm when handling the frogs, as rough handling can cause damage and discomfort to the organism.

This research opportunity has been an amazing experience for scientific learning. Although animal studies are very different from some of my past research experiences, I thoroughly enjoyed the responsibility associated with caring for over 200 animals. Not only did it require lots of hard work but also a commitment to a high standard of quality and care, a necessity when handling live organism. I hope that all my efforts, and the continued work of the research team will work to demonstrate the regenerative abilities induced by monensin and sodium treatment.

9. Future Work

The future of this study appears to be very promising; however, there is still a great deal of work that needs to be conducted. Not until the end of the regrowth process for the intial treatment

group will we have a better understanding of the regenerative capacity induced by treatment with monensin and sodium. In addition in the span of several months our operation size has increased from 30 frogs in early winter to over 200 frogs at the current time. Although this marked increase in size is largely due to the fact that *Rana pipiens* are protected during their spawning time in the summer and are unattainable, it is also out of necessity for reproducible results. For example, Dr. Smith's results should be considered truly remarkable, especially due to the fact that one of his study organisms demonstrated a complete regeneration of a lost limb. However, many details regarding the methods used by Dr. Smith are missing, and he never repeated his experimental process. Therfore, it is critical that we are capable of demonstrating consistent growth with our regenerates. At the current time, additional control treatments are being conducted to ensure that no environmental factors (coconut substrate, rocks, or Poland Spring water) were active in inducing the regeneration event. These controls will help to strengthen the results of the monensin and sodium treated specimens. In addition, repeat experiments subjecting the frogs to treatments of monensin only and sodium only should be conducted to provide additional support. Finally, depending upon the upcoming results, an attempt to repeat the Smith and Borgens experiments should be made to guage what scale of regrowth we can achieve. This electrical stimulation could be used in conjuction with monensin and sodium treatment to create very strong currents, however it is uncertain wether excessive currents would be beneficial or detrimental.

Monitoring of these currents will be important to demonstrate the flux of sodium ions. Borgens used vibrating probes that allows them to measure extracellular voltage differences. This allows for inference about the extracellular current densities, but provides no means for visualization. N. Ozkucur *et al*. have recently demonstrated that this visualization is possible

through the use of ion specific vital dyes. These dyes allow for the *in vivo* monitoring of several specific ions during the regeneration of axolotol limb. This real-time monitoring would enable us to visualize the movement of ions during the regeneration of the *Rana*.

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