THE EFFECTS OF HOMOGENIZED HIND LIMB BUDS ON THE REGENERATION OF SCIATIC NERVE AXONS IN ADULT *RANA PIPENS*

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

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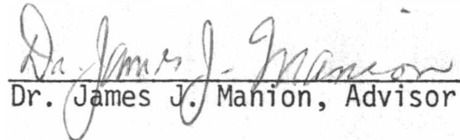
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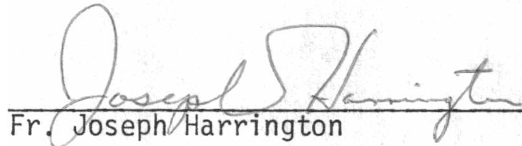
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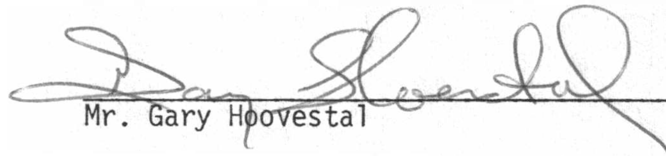
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ABSTRACT

A solution of homogenized hind limb buds was used as a source of an inducing agent(s). This was placed in an artificial sheath, bridging the ends of the severed sciatic nerve axon of which a small portion was removed and which also served to maintain localization of the inducing agent(s). These experimentals were compared with a control group whose sheath contained amphibian ringer's solution. No correlation could be found from the data obtained. However, it is believed that these results do not directly reflect the hypothesis because of the number of variables involved.

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INTRODUCTION

Research has uncovered many inhibitors of regeneration and defined more clearly conditions which promote regrowth. Elements such as directing the pathway and the use of nerve growth factor have been shown to enhance the growth of axons. Further investigation involving application of this research in the regeneration of severed axons would be of great clinical importance.

The tadpole limb bud has a complex inducing system working in differentiation. Some of the inducing agent(s) are involved in promoting nerve differentiation and eventual synapsis with a definite organ. It is possible that the inducing agent(s) found in the limb bud, which are responsible for the sciatic nerve differentiation, might also enhance the regeneration of the sciatic nerve in the adult.

This paper is concerned with the effects of homogenized limb bud solution on regeneration of the sciatic nerve axon as a possible source of inducing agent(s).

LITERATURE REVIEW

The literature which specifically encompasses regeneration of the amphibian sciatic nerve is limited. However, the general topic of neural regeneration has been extensively explored. These investigations involve nerves of the central nervous system (CNS) and of some peripheral nerves where the nerve cell body (NCB) serves as the focal point. Many aspects of these studies are applicable to the axonal regeneration of the amphibian sciatic nerve.

This review has two main sections: the first summarizes the characteristic changes that occur in the nerve, with emphasis on the axon, in response to axotomy; the second gives evidence of an active ingredient(s) in the hind limb bud of tadpoles and some nerve response to nerve trophic factors.

The nerve cell body of a severed nerve undergoes tremendous changes which seem imperative to the regeneration of the axon.[1] It is therefore necessary to discuss the response of the nerve cell body, one of which is an increase in the size of the NCB. This increase is believed to be twofold: firstly, the immediate swelling is due to the uptake of water, thus diluting the contents of the NCB; and secondly, later expansion results from the accumulation of dry mass needed for axonal regrowth.[2] Studies show that the initial swelling of the cell is possibly caused by the increased water uptake elicited by an apparent alteration of membrane potential which may cause an

abnormal movement of ions across the membrane.[3] The dry mass, the second swelling in cell size, is due to the increase in RNA synthesis,[4] protein synthesis,[5] lipid synthesis[6] and other cellular products necessary for regeneration. In general, there seems to be a shift from the production of transmitter substance to the synthesis of structural material necessary for restoration of the axon.[7]

Alterations caused by axotomy also occur on the axonal level. Although the axon is cut and a large portion of the membrane is separated from the cell body (the degenerating end), the defect produced in the cell membrane has a relatively small area, with respect to the remaining area of membrane still intact. Within a few hours after the severing of the axon, the open end is "plugged up," due to accumulation of cellular material from the cell body, and the membrane is repaired.[8] This accumulation results because axonal transport is not immediately affected. It is not until the initial regrowth of the axon that transport is noticeably affected. Here, there is a decrease in the amount of transmitter substance and an increase in the amount of overall protein transported.[9]

Research shows that immediately after the nerve has been severed, the axon is slightly retracted and the myelin of this de-innervated area dies. This finding is verified by other studies which demonstrate that the myelin is maintained by the axon and that the myelin of the degenerating axon (portion not in contact with the cell body) disintegrates.[10] After this initial retraction and the alterations of the cell body associated with axonal regeneration, the axon grows outwards, deprived of a myelin sheath for an undetermined amount of time. If the axon was initially myelinated, myelin is gradually generated from

the proximal end to the distal end. This is due to stimulation by the still-intact neurilemma which appears to direct growth along the same path wherever possible.[11]

Up to this point, only the response of the nerve to axotomy has been discussed. Also pertinent to this review are the possible ways by which the responses are initiated. There is no direct information pertaining to this; however, the available facts indicate that the initiation involves an intraneuronal mechanism; the transmission of a signal along the axon from the site of injury to the cell body, since the time required for the cell body response is proportional to the length of the axon still intact.[12]

The accepted hypothesis of the initiation of the signal is that of Cragg, who has postulated that a number of mechanisms are involved, as stated by Bernice Grafstein.[13]

> Among the factors to which Cragg assigned a possible role in the initiation of the response are: depolarization of the membrane, loss of action potentials, depletion of axonally transported constituents (such as transmitter-associated materials or a substance which would act as a repressor of the genes regulating protein synthesis, loss of axoplasm and mitochondria, and loss of a trophic substance coming from the periphery.[14]

According to Cragg, these factors may be stimulated to different degrees, which accounts for the variety of responses.

However, a number of recent observations have arisen contrary to Cragg's view, although they themselves have not been sufficiently proven. One such study performed on cells undergoing collateral sprouting shows that depolarization and loss of axoplasm may inhibit the cell body's response.[15] Another study suggests that extracellular materials at the site of injury can reach the cell body in a proportional amount of time to elicit the response--example, horse-radish peroxidase--

however, these only generate some of the responses.[16] Many other findings are recorded but as mentioned have not been substantiated sufficiently to reject Cragg's hypothesis.

Other factors which affect the regenerativity of nerves, although on a more general scale, are the age of the animal, and the site and nature of injury.[17] Younger animals have a higher cell death of axotomized nerves,[18] possibly due to the fact that immature neurons lack the homeostatic capacity of mature neurons.[19] Also, axons severed close to cell bodies have a higher death count.[20] Liberman suggested that ". . . there is an earlier cutoff of the increase in RNA synthesis, which would result in a smaller increase in cytoplasmic RNA content."[21] If the remaining portion of the axon is highly branched, the nerve is more likely to survive the injury.[22]

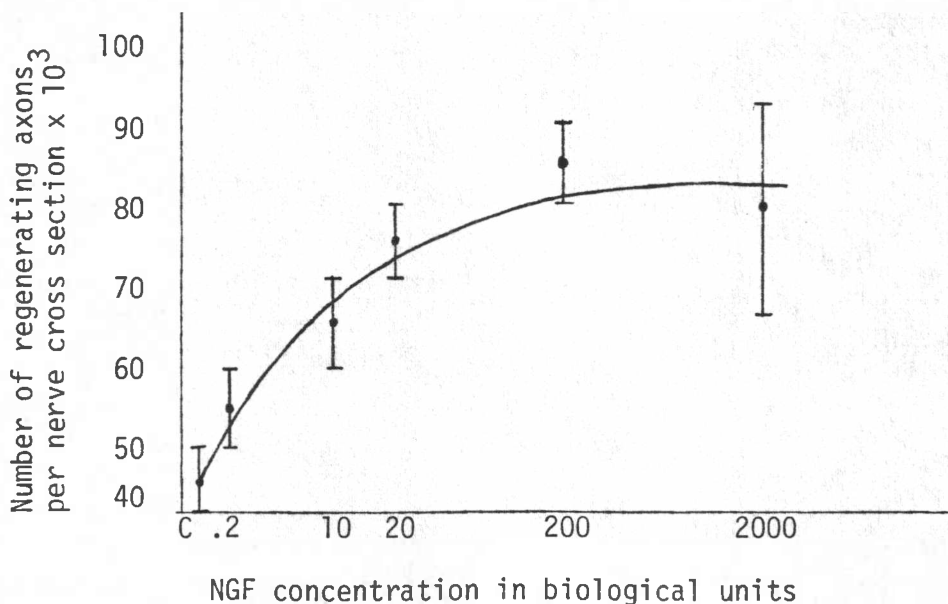
The nature of injury can also play a major role in the regenerative property (by freezing, cutting, crushing, etc.) because it is possible that the initial sprouting is important for the intensity of the response.[23]

The second major portion of this literature review deals with evidence of nerve trophic factors that stimulate regeneration of axons.

Research demonstrates that the limb buds of amphibians contain inducing agents of growth such as nerve trophic factor(s).[24] Marcus Singer proposed that all limb tissues synthesize trophic factors, but that nerves synthesize much more than the others (nerve trophic factor[s]) which spills over into the neighboring tissues and prevents them from synthesizing this same factor. This creates a dependence of the tissues on their continued supply of nerves.[25] Other studies show that the more nerve fibers innervating the limb, the more trophic factor(s) secreted and the greater the regeneration.[26]

A particular nerve trophic factor has been isolated, the nerve growth-promoting factor (NGF) from a few sources such as the mouse submaxillary gland,[27] some snake venoms,[28] tumoral tissue, [29] neuroglia cells [30] and some evidence leans toward the optic nerve in newts.[31]

There are numerous experiments cited in literature concerning the effect of NGF on regeneration. One of these involved the severed optic nerve of the newt (Triturus viridescens) by J. E. Turner and K. A. Glaze. They found that the nerve growth factor had a definite stimulating effect on the regenerativity of the optic nerve.[38] This conclusion was reached after a clear dose-response relationship was demonstrated by varying doses from 2 to 2000 biological units (BU) administered at the time of injury and growth was allowed for a 14-day period. Their study showed that there was a rapid increase in the number of regenerated axons in animals treated with 2 to 20 BU concentrations, but that values plateaued from 20-2000 BU. Here is a graph to illustrate this:[33]



NGF concentration in biological units

A dose-response curve showing the log of various NGF concentrations, administered as single intraocular injections at the time of nerve transection, plotted against numbers of regenerating axons per nerve cross section at 14 days postlesion. Points (filled circles) represent mean values (N=5) with ventrical lines indicating the standard error. The letter C on the abscissa is the control value.

Other studies have also shown that NGF has a stimulatory effect on the developing and regenerating amphibian nervous system. Radeva and Taxi[34] reported that the NGF stimulated the maturation of the newt nervous system by stimulation of the general synthetic process, such as protein synthesis. They also demonstrated acceleration of formation of synaptic and non-synaptic contacts between neighboring neurons. In another study, Robinson and Allenby[35] indicated the stimulatory effect of NGF on hind limb regeneration in _Xenopus laevis_.

More recent studies lean toward the effect of NGF as a guidance for regenerating axons. This was investigated by R. W. Gunderses and J. N. Barrett.[36] They used a micropipette filled with NGF as a localized source which was placed near the growth cone of the regenerating axon, about 25 um away. A small concentration of NGF was also placed in the media, to be used as a background concentration and a flow (at a rate of 25 ml/hour) of media was set up to oppose the direction of initial growth. This flow carried the NGF that flowed from the micropipette at a much slower rate (1 to 2 ul/hour) which produced a high concentration of NGF near the micropipette. They found that the regenerating axons grew in the direction of the NGF source, thus reversing its direction. Gunderses and Barrett then carried out another experiment. They bathed forty axons in a background solution of NGF and observed that all growth cones turned and grew toward the concentrated source in the micropipette. The controls were exposed to a background concentration by the same method as the first experimentals were exposed to the NGF by way of the micropipette. In these the axon showed only slight and random displacement. No significant change in turning was noticed even if the source was placed in the direction of initial growth. They concluded that the

turning of the axon toward elevated concentrations of NGF was not due to a drag elicited by the pipette or the flow of NGF from it, and that the turning response shows no relationship between itself and an increase in growth rate because both control and experimental axons grew at almost the same rates.[37] Another concluding remark was that the response was specific because no turning was observed when other types of proteins were used, such as bovine serum albumin or fetal calf serum. Also, concentrations of NGF as low as 1 BU above the background concentration, which was 1 BU, demonstrates the response. However, axons did not turn toward the micropipette which contained only 1 BU of NGF per ml when the background contains no NGF, as in the controls of the second experiment. The final general conclusion made was that the response was not an enhancement of growth rate or survival, as other experiments indicate; instead it resulted from chemotactic guidance.

MATERIALS AND METHODS

Subjects

Mature Rana pipens were used and each was identified by characteristic spot patterns on their back. They were fed meal worms once a week.

Inducing Agent (Experimental medium)

The hind limb buds of Xenopus sp. tadpoles (approximately 7 weeks in development) were extracted and weighed. This yielded .15397 g and was homogenized in 1.5 ml of amphibian ringer's. This solution was used as the source of inducing agent(s).

Amphibian Ringer's Solution (Control medium)

A pure amphibian ringer's solution was used as the controlling medium.

Artificial Sheath

Tylene intra-medic tubing was used with an outer diameter of 210 um and an inner diameter of 120 um. It was sterilized in an autoclave in foil to prevent moisturization of the tube.

Anesthetic

A one-percent solution of tricane methanesulfonate (MS-222) in amphibian ringer's was injected intraperitoneally in the amount of 1 ml per 100 g of frog.

Surgical Equipment

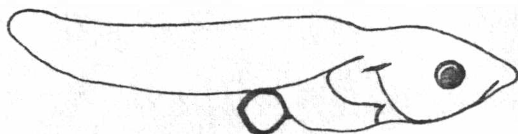
Stainless steel equipment and nonabsorbable, silk, braided, surgical suture, size 4-0, were used.

Tank System

Large plastic tubs with plastic tubes bringing tap water in and a tube at the opposite end, draining water out, were used as a tank. The tubs were tilted at a slight angle to promote better drainage so water could be run through the tanks continually. The tanks could then be cleaned frequently to decrease the possibility of disease (red leg). Copper pennies were also placed in the tank for this purpose. A fiberglass screen was fastened over the top of the tanks.

Procedure

Upon arrival, the frogs were placed in a common tank for an adjustment period of several weeks, and the tadpoles in a small tank of pond water where they were allowed to develop for approximately 7 weeks, into the hind limb bud stage (see Illustration 1).



Hind limb bud

Illustration 1. Hind limb bud

At this time the limb buds were surgically removed with aid of a dissecting microscope. They were immediately weighed and placed in a small tissue grinder with 1.5 ml of amphibian ringer's solution and the mixture was homogenized. The solution was then kept refrigerated for about 10 hours until surgery began the following day.

An incision through the skin along the back length of the right thigh of the adult frog was made in the anesthetized animal. The cut continued through two layers of membrane to expose the sciatic nerve which is embedded between muscles. The nerve was then carefully separated from any membranous attachment with the muscles on the sciatic artery and slightly elevated with a probe to allow for the removal of a small portion about 3.0 mm to 7.6 mm. The cut was made with a pair of stainless steel surgical scissors at approximately mid-thigh.

The intramedic tubing was cut at a length approximately equal to the length of the incision, and filled with homogenized hind limb bud solution in the case of the experimentals and amphibian ringer's solution for the controls. With the aid of a small hook, the nerve endings were lifted and carefully guided into the tubing, thus forming a bridge between the two endings (see Illustration 2).

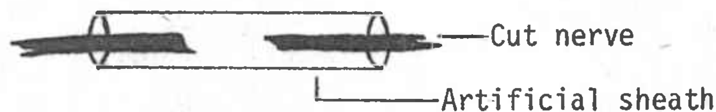


Illustration 2.

The tubing was placed between the muscles where the nerve normally lies, then the skin was sutured together. After 2 days a random sample of frogs were re-opened to verify that the nerve remained in the sleeve.

The frogs were placed in a small recovery tank of fresh water, under close surveillance, with their heads elevated on a petri dish to prevent drowning until the effects of the anesthetic wore off. After recovery, the frogs were placed in identical tanks of running water containing either experimentals or controls.

After a period of time had elapsed, the frogs were sacrificed and placed in the freezer (many of the frogs died of red leg and at that point were frozen). Later they were placed in a 10-percent solution of formalin until the nerve could be removed for examination.

The nerve was removed and placed on a slide for examination. It was examined under oil immersion by both light microscopy (1000x) and phase contrast microscopy (1250x) for verification of results. From these studies, two forms of data were collected: first, whether or not the nerve ending atrophied. We considered atrophy as the deterioration of the nerve ending leaving only a glassy-transparent membrane-like material. The second form of data was whether or not there was any sign of axonal regeneration. The criterion for this was the presence or absence of axonal fibers at the nerve ending.

RESULTS

Surgery was completed and data obtained on 20 adult frogs; 9 of these were experimentals and 11 served as controls. The original length of time allowed for regeneration was 16 days. Red leg spread through the tanks, however, killing many of the frogs and this caused the time period from the point after the completion of surgery to death to vary among the frogs.

The results for the control group were as follows:

The first frog showed no nerve atrophy but there was no sign of axonal regeneration. The nerve ending was shredded and opaque with a slight reddish color. The time allowed for regeneration was 16 days (that is, the time period from the point after completion of surgery to death) and the frog died of red leg.

The second frog showed nerve atrophy and no axonal regeneration. The nerve ending was rough with a glassy-transparency and reddish in color. The time allowed for regeneration was 15 days and the frog died of red leg.

The third frog showed no nerve atrophy but there was no sign of axonal regeneration. The nerve ending was shredded and opaque with no discoloration. The time allowed for regeneration was 9 days and the frog died of red leg.

The fourth frog showed no nerve atrophy but there was no sign of axonal regeneration. The nerve ending was shredded and opaque with no

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discoloration. The time allowed for regeneration was 7 days and the frog died of red leg.

The fifth frog showed no nerve atrophy and signs of axonal regeneration. The nerve ending was shredded and opaque with no discoloration. The time allowed for regeneration was 7 days and the frog died of red leg.

The sixth frog showed no nerve atrophy and signs of axonal regeneration. The nerve ending was blunt and opaque with a reddish color at the tip. The time allowed for regeneration was 7 days and the frog died of red leg.

The seventh frog showed no nerve atrophy but no signs of axonal regeneration. The nerve ending was rough and opaque with no discoloration. The time allowed for regeneration was 9 days and the frog died of red leg.

The eighth frog showed nerve atrophy and no sign of axonal regeneration. The nerve ending was rough and had a glassy-transparency with reddish discoloration. The time allowed for regeneration was 4 days and the frog died of red leg.

The ninth frog showed no nerve atrophy and signs of axonal regeneration. The nerve ending was shredded and opaque with a slight bluish discoloration. The time allowed for regeneration was 7 days and the frog died of red leg.

The tenth frog showed no nerve atrophy and signs of axonal regenerations. The nerve ending was blunt and opaque with a reddish coloration. The time allowed for regeneration was 9 days and the frog died of red leg.

The eleventh frog showed nerve atrophy and no sign of axonal regeneration. The nerve ending was rough and had a glassy-transparency with a reddish discoloration. The time allowed for regeneration was 10 days and the frog died of red leg.

The results for the experimental group were as follows:

The first frog showed no nerve atrophy but no sign of axonal regeneration. The nerve ending was shredded and opaque with a red discoloration. The time allowed for regeneration was 9 days and the frog died of red leg.

The second frog showed no nerve atrophy and signs of axonal regeneration. The nerve ending was shredded and opaque with no discoloration. The time allowed for regeneration was 9 days and the frog did not die of red leg.

The third frog showed nerve atrophy and no sign of axonal regeneration. The nerve ending was blunt but had a slight glassy-transparency and red discoloration. The time allowed for regeneration was 9 days and the frog died of red leg.

The fourth frog showed no nerve atrophy but no sign of axonal regeneration. The nerve ending was blunt and opaque with red discoloration. The time allowed for regeneration was 9 days and the frog did not die of red leg.

The fifth frog showed no sign of nerve atrophy and signs of axonal regeneration. The nerve ending was shredded and opaque with some reddish discoloration. The time allowed for regeneration was 9 days and the frog did not die of red leg.

The sixth frog showed nerve atrophy and no signs of axonal regeneration. The nerve ending was blunt but had a slight glassy-transparency with reddish discoloration. The time allowed for regenerations was 15 days and the frog did not die of red leg.

The seventh frog showed no nerve atrophy but no signs of axonal regeneration. The nerve ending was rough and opaque with some reddish discoloration. The time allowed for regeneration was 8 days and the frog died of red leg.

The eighth frog showed no nerve atrophy but no signs of axonal regeneration. The nerve ending was shredded and opaque with some reddish discoloration. The time allowed for regeneration was 9 days and the frog did not die of red leg.

The ninth frog showed nerve atrophy and no sign of axonal regeneration. The nerve ending was rough with a glassy-transparency and had some reddish discoloration. The time allowed for regeneration was 16 days and the frog did not die of red leg.

TABLE 1. CONTROL GROUP:
RELATIONSHIP OF GROWTH TO TIME ALLOWED FOR REGENERATION

Time allowed for regeneration (days)	Atrophy or no atrophy	Presence of nerve endings
16	+	-
15	-	-
10	-	-
9	+	-
9	+	-
9	+	+
7	+	-
7	+	+
7	+	+
7	+	+
4	-	-

Key: (+) represents no atrophy or the presence of axon endings
(-) represents atrophy or no sign of axon endings

TABLE 2. EXPERIMENTAL GROUP:
RELATIONSHIP OF GROWTH TO TIME ALLOWED FOR REGENERATION

Time allowed for regeneration (days)	Atrophy or no atrophy	Presence of nerve endings
16	-	-
15	-	-
9	+	-
9	+	+
9	-	-
9	+	-
9	+	+
9	+	-
8	+	-

Key: (+) represents no atrophy or the presence of axon endings

(-) represents atrophy or no sign of axon endings

TABLE 3. RELATIONSHIP OF GROWTH TO RED LEG

In the frogs where death caused by red leg			In the frogs which were sacrificed		
Time allowed for regeneration (days)	Atrophy or no atrophy	Presence of nerve endings	Time allowed for regeneration (days)	Atrophy or no atrophy	Presence of nerve endings
16	+	-	16	-	-
15	-	-	15	-	-
10	-	-	9	+	+
9	+	-	9	+	-
9	+	-	9	+	+
9	+	+	9	+	-
9	+	-			
9	-	-			
8	+	-			
7	+	-			
7	+	+			
7	+	+			
7	+	+			
4	-	-			

Key: (+) represents no atrophy or the presence of axon endings
(-) represents atrophy or no sign of axon endings

DISCUSSION

As mentioned in the abstract, no correlation can be drawn from the data obtained. Therefore, our hypothesis of whether the inducing agent(s) of the hind limb bud has any effect on the regenerative capacity of the adult severed sciatic nerve axon cannot be ascertained from our results.

The data is inconclusive because of the different times involved. The initial time that was meant to be allowed for regeneration was 16 days. This was arbitrary, although studies done by Turner and Singer[38] on optic nerves in newts showed that at 14 days postlesion, the nerves were relatively free of degenerating debris and were filled with fasicles of regenerating axons. However, many of the frogs died of red leg before this time and at varying intervals (see Tables 1 and 2), resulting in a scattered array of time periods allowed for regeneration.

The data collected was based on the criterion mentioned in the procedure: the determination of atrophy and the presence of any nerve axons. The verification of this criterion is greatly enhanced by the fact that all of the regenerated axons were found in the nerve endings that showed no atrophy.

There were many factors that could possibly have affected the regeneration of the axon: firstly, the hind limb bud of _Xenopus sp_. used; secondly, the stage of the hind limb bud; and thirdly, possible effect of red leg.

Since _Xenopus sp._ tadpoles were used as the source of inducing agent(s), it is possible that the nerve of the adult _Rana pipens_ (a different genus) responded negatively to the foreign material in it (such as cells, cellular organelles, and other cellular components).

A second chance of error could have been that the stage of the limb bud chosen did not have as high concentration of inducing agent(s) as a later stage might possess.

The third aspect of possible error is the disease red leg, from which most of the frogs perished. It is logical to assume that any agent as detrimental to the health of the animal as red leg can disrupt one or many of the processes necessary for regeneration (see first part of literature review).

There are many factors that must be considered if successful nerve regeneration is to be obtained. We tried to control most of these as mentioned in the procedure and verified in the first part of the literature review. However, there were a few beyond our control, such as these mentioned in this discussion.

Despite our unfortunate dilemmas, the thesis was a success. It was successful because it gave us the opportunity to carry out extensive research on a topic that interests us and an experience we will never forget.

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