

Metamorphic State in the Axolotl (*Ambystoma mexicanum*) Influences Limb Regenerative Ability

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SUMMARY

Limb regeneration, the ability to regrow an appendage after amputation, is a capability possessed by very few vertebrates. Among species that possess regenerative ability, it is often limited to early embryogenesis in mammals or early and pre-metamorphosis in amphibians. The axolotl, a salamander, is an exception: it retains regenerative ability throughout its adult life. Furthermore, although the axolotl does not naturally metamorphose, it can be experimentally induced to do so. In this experiment, these attributes were utilized in order to determine how metamorphosis impacts limb regeneration capability. The rate and morphology of limb regeneration in axolotls of various metamorphic states were examined and compared to axolotls that had

not been induced to metamorphose. It was found that axolotls in more pronounced metamorphic states demonstrated greater regenerative decline than those in less developed states. Animals in less pronounced metamorphic states presented with only an initial rate delay, whereas further metamorphosed animals presented with an all-around rate delay. Morphological abnormalities were more common and severe in the more progressively metamorphosed axolotls as well. Although the cause of regenerative impairment during metamorphosis remains unknown, this study illustrated that axolotls in differing metamorphic states can be used to gain an understanding of how and why regenerative decline exists in adult vertebrates.

INTRODUCTION

Approximately 19,000 digit amputations occur annually in the United States in the civilian sector alone (Sorock *et al.*, 1993). This statistic, coupled with numerous reports that the tips of fingers regenerate if allowed to heal by secondary intention (i.e. without assisted wound closure), indicate that mammalian regeneration responses—albeit limited—are present (Muller *et al.*, 1999). The digit tip regeneration capability illustrates one example of the limited higher vertebrate regenerative responses. Another example occurs during development. In mouse and rat limb buds, for example, regenerative ability becomes more and more restricted as development progresses until only digit tips can regenerate in adults (similar to the case with humans) (Muneoka and Sassoon, 1992). These two examples of limited limb regeneration, as well as the high incidence of annual amputations, provide an incentive to identify key regeneration requirements for entire limbs.

Similar to mammals, anuran—tailless—amphibians (i.e. frogs and toads) lose regenerative ability as development progresses. This decline manifests itself particularly during

metamorphosis (Brookes, 1989); it can even be viewed as analogous to mammals in that the same biochemical events that occur during amphibian metamorphosis also occur during early mammalian (i.e. embryonic) development (Tata *et al.*, 1991). Due to this link between early mammalian development and amphibian metamorphosis, studying how metamorphosis affects regeneration in amphibians will most likely help us to elucidate why regenerative ability severely declines in higher vertebrates.

Although both metamorphosis and regeneration have been extensively studied, little attention has been devoted to how one influences the other. What has been believed is that urodele—tailed—amphibians (i.e. newts and salamanders) can generally regenerate a perfect replica of a limb throughout the life span (Spallanzani, 1769). Scadding (1977), however, disputed this by studying a survey of anuran and urodele amphibians. He found that no anurans and only some urodeles can post-metamorphically regenerate limbs. Young and coworkers (1983a, 1983b) examined one particular genus of urodeles, *Ambystoma*, and

demonstrated that regenerative capacity persists post-metamorphically as long as proper environmental conditions exist and a significantly longer time to regenerate is allowed. All of these studies have been mainly descriptive, however, and exactly what causes this decline, or exactly when it occurs in urodeles, has not yet been determined.

The axolotl (*Ambystoma mexicanum*) is an ideal amphibian to pursue the combined study of regeneration and metamorphosis. This urodele is a model organism for regenerative studies and its regenerative process has already been staged (Table 1) and correlated with the staging system for the newt, another limb regeneration model organism (Tank *et al.*, 1976). Another characteristic that makes the axolotl ideal for this particular study is that it is an obligatory neotene. That is, the animal can reproduce without undergoing metamorphosis; it retains its larval body form and aquatic behavior. Metamorphosis can be induced, however, with administration of exogenous thyroid hormone (TH or T₄), triiodo-L-thyronine (T₃) or thyroid stimulating hormone (TSH) (Kühn and Jacobs, 1989; Rosenkilde and Ussing, 1996). Thus, the onset of the

metamorphic process as well as the initiation of regeneration can be experimentally controlled.

This experiment utilized the above characteristics of the axolotl to address the general question of how metamorphosis influences regeneration. One of the questions was whether or not the metamorphosis-related surge in thyroid hormone alone causes the hindrance in limb regeneration. A related question was whether the impedance could be dependent upon how much metamorphic progression has occurred. That is, would an animal in a state of incomplete metamorphosis display the same hindrance as an animal that has completely metamorphosed? These questions can be easily examined because axolotls injected with TH exhibit one of three dose-dependent responses: 1) metamorphic resistance (relative lack of response to the TH); 2) incomplete metamorphosis (initiation but not completion of metamorphosis); and 3) complete metamorphosis (*personal observations*). Axolotls in each of the three metamorphic states underwent forelimb amputations and the regenerates were examined with regard to both regeneration rate and morphology.

METHODS AND MATERIALS

Animal Husbandry

Juvenile albino axolotls (*Ambystoma mexicanum*) were obtained from the Indiana University Axolotl Colony. All animals were housed separately in 4 liter HDPE containers that contained 40% Modified Holtfreter's Solution. (A 100% solution consists of 0.35% NaCl, 0.005% KCl, 0.01% CaCl₂, 0.02% NaHCO₃ and 0.02% MgSO₄.) Neotenic and incompletely metamorphosed axolotls were maintained under these conditions while metamorphosed axolotls resided in a semi-aquatic environment.

Thyroid Hormone Injections and Characterization of Metamorphic State

After being anesthetized in a 0.007% benzocaine (Sigma E1501)/40% modified Holtfreter's solution, axolotls were weighed and baseline measurements were taken in accordance with Cano-Martínez and coworkers' parameters (1994). The animals were given a dosage of 1.0 µg or 1.5 µg thyroxin (Aldrich 23, 470-2)/g body weight via intraperitoneal injection. The TH was dissolved in DMSO (Sigma D-8418) and the control animals received an injection of DMSO only (Crawford and Vincenti, 1998). Axolotls that showed no or insignificant metamorphic changes were characterized as "metamorphically resistant." Axolotls that began metamorphosis but then arrested and failed to proceed further were categorized as "incompletely metamorphosed." Finally, animals that presented with all

the changes associated with metamorphosis were characterized as "completely metamorphosed."

Amputations

Animals were anesthetized in a 0.007% benzocaine/40% modified Holtfreter's solution. The forelimb was amputated through the mid-humerus with small scissors. The aquatic animals recuperated in 40% modified Holtfreter's solution. The metamorphosed animals were amputated after complete metamorphosis was attained and they recuperated in a solution of 40% modified Holtfreter's solution and a few drops of methylene blue, a bactericide (Young *et al.*, 1983a). All other axolotls were amputated at the same time as they were injected with either DMSO (controls) or TH. Only after TH injection and weeks to observe the metamorphic progression could the axolotls be classified as metamorphosis resistant or incompletely metamorphosed.

Analysis of the Regenerate

Observations were taken on a daily basis for the first week following amputation and every other day for the remainder of the regenerative process. All limbs were staged according to the parameters outlined by Tank and associates (1976) (Table 1), except that the blastemal stages were not divided. After the animal's limb reached digital outgrowth to the point where an entire miniature limb formed, observations were stopped. These observations were accomplished by placing the anesthetized axolotl under a trinocular dissecting

microscope and both manually recording notes as well as taking pictures with a Nikon 990 CoolPix digital camera. After the experiments were completed, rates of regeneration were compared among the groups. Most of the analysis was in the form of percentage difference. Two standards were compared. The first was the time in days that each animal required to reach a particular stage of regeneration. The second was the amount of time in days that each axolotl spent in each stage. The morphology of the regenerate was also noted.

RESULTS

All control axolotls regenerated normally and in accordance with the stages outlined by Tank and coworkers (1976). The regeneration rate of animals displaying metamorphic resistance, incomplete metamorphosis, or complete metamorphosis were all compared to comparably-sized control animals (Tables 2-4). After examining the data, it became clear that metamorphic state influenced the progression of regeneration. That is, the regeneration rate decreased as the metamorphic state progressed; completely metamorphosed axolotls presented with a more significant delay than the incompletely metamorphosed axolotls, which in turn, displayed a greater delay than the metamorphic resistant axolotls. While metamorphosis resistant and incompletely metamorphosed axolotls both displayed no overall difference in the time needed to reach digital outgrowth (Tables 2A and 3A), the metamorphosis resistant animals spent 30% more time in dedifferentiation (Table 2B) and the incompletely metamorphosed animals spent 75% more time in dedifferentiation than the controls (Table 3B). The completely metamorphosed axolotl presented with a more drastic, overall decrease in regeneration rate (Table 4B). In addition to requiring 200% more time in dedifferentiation, the animal required 70% more time in wound healing, 140% more time in blastema, and almost 200% longer in palette, as compared to controls. The metamorphosed axolotl required 50 days to reach digital outgrowth, as compared to 24 days for the controls (Table 4A).

In addition to the regeneration rate, the morphology of the regenerate was also examined. In 78% (seven of nine) of

Alizarin Red Staining

Select limbs were amputated and stained in order to view the skeletal architecture. Cruz's (1993) staining protocol for the mouse was followed with the following minimal adaptations. The limbs were first placed in either 10% formaldehyde or 10% paraformaldehyde solutions. The skin was not removed prior to staining.

the neotenic control animals, the regenerates formed a perfect replica of the original (Fig. 1). Of those animals that did not form a perfect replica, a very small bifurcation, or "nub" was present on the fourth digit. However, this abnormality did not interfere with the overall structures of the limb. As compared to the controls, axolotls that received TH treatment (regardless of the response) had a 67% (six of nine) normal regeneration morphology. Metamorphosis resistant axolotls grew normal regenerates in all cases (n=3) whereas incompletely metamorphosed axolotls formed abnormal limbs in 50% of the cases (group n=4). The completely metamorphosed axolotl regenerated abnormally as well.

All of the incompletely metamorphosed axolotl regenerates had bifurcated (split) fourth digits and some were hypomorphic: they had fewer digital elements than normal (Figs 2 and 3). One incompletely metamorphosed axolotl bifurcated at the third phalangeal bone. In this case, the bone itself bifurcates; it is not an incidence of soft tissue outgrowth without skeletal structure (Figs 2a and 2b). Another incompletely metamorphosed axolotl also bifurcated, but at the second phalangeal bone. In this case, the bifurcation is not a true one in that the bone itself does not bifurcate, but rather an additional bone outgrows (Figs 2c and 2d). The first digit of this particular axolotl is hypomorphic as well, in that it only has two phalangeal bones rather than three (Fig 2c). The completely metamorphosed axolotl displayed an interesting regenerate in that only three digits are present. The third digit is also bifurcated (Fig 3).

Table 1. Summary of key gross observations used in defining stages of axolotl forelimb regeneration. (From Tank et al., 1976)
WH, Wound Healing; *DD*, Dedifferentiation; *EB*, Early Bud Blastema; *MB*, Mid Bud Blastema; *LB*, Late Bud Blastema; *Pal*, Palette; *DO*, Digital Outgrowth.

Stage	
<i>WH</i>	Begins with amputation; ends with rounding of the edges of the stump.
<i>DD</i>	From rounding of the edges of the stump until the first evidence of blastemal outgrowth.
<i>EB</i>	From the first blastemal outgrowth to formation of a symmetrically contoured, cone-shaped regenerate.
<i>MB</i>	Starts with a symmetrical cone which curves markedly in a postero-dorsal direction. Ends with the first indication of flattening of the cone.
<i>LB</i>	Begins with flattening of the dorsally curved cone and ends with the appearance of the first digital primordium.
<i>Pal</i>	The period beginning with the gross appearance of the first and ending with the appearance of the fourth digital primordium.
<i>DO</i>	The period from appearance of the fourth digital primordium to stabilization of the mature regenerate.

Table 2a: Average Days for Metamorphic Resistant Axolotls and Neotenic Control Axolotls to Reach Each Stage of Regeneration

Each value is the average number of days required to reach each stage (i.e. the number of days post-amputation). The parenthetical number represents the standard deviation for each group. Metamorphic resistant axolotls ranging in size from 8.7 to 9.2 cm were compared to small (6 – 10 cm) controls.

DD, Dedifferentiation; *Blas*, Blastema; *Pal*, Palette; and *DO*, Digital Outgrowth.

	Metamorphosis Resistant (n=3)	Neotenic, Controls (n=3)
<i>DD</i>	2.67 (1.15)	2.00 (0.00)
<i>Blas</i>	9.67 (1.15)	7.33 (1.15)
<i>Pal</i>	17.67 (1.15)	16.00 (0.00)
<i>DO</i>	20.00 (1.73)	20.00 (0.00)

Table 2b: Average Length of Time that Metamorphic Resistant Axolotls and Neotenic Control Axolotls Spent in Each Stage of Regeneration

Each value is the average number of days spent in each stage. The parenthetical number represents the standard deviation for each group. Metamorphic resistant axolotls ranging in size from 8.7 to 9.2 cm were compared to small (6 – 10 cm) controls.

WH, Wound Healing; *DD*, Dedifferentiation; *Blas*, Blastema; and *Pal*, Palette.

	Metamorphosis Resistant (n=3)	Neotenic, Controls (n=3)
<i>WH</i>	2.00 (0.87)	1.50 (0.00)
<i>DD</i>	7.00 (0.00)	5.50 (0.87)
<i>Blas</i>	7.67 (0.58)	8.67 (1.15)
<i>Pal</i>	2.33 (0.58)	4.00 (0.00)

Table 3a: Average Days for Incompletely Metamorphosed Axolotls and Neotenic Control Axolotls to Reach Each Stage of Regeneration

Each value is the average number of days required to reach each stage (i.e. the number of days post-amputation). The parenthetical number represents the standard deviation for each group. Metamorphic resistant axolotls ranging in size from 7.0 to 9.0 cm were compared to small (6 – 10 cm) controls.

DD, Dedifferentiation; *Blas*, Blastema; *Pal*, Palette; and *DO*, Digital Outgrowth.

	Incompletely Metmorphosed (n=4)	Neotenic, Controls (n=3)
<i>DD</i>	2.25 (0.50)	2.00 (0.00)
<i>Blas</i>	9.50 (1.29)	7.33 (1.15)
<i>Pal</i>	17.50 (1.91)	16.00 (0.00)
<i>DO</i>	21.50 (1.73)	20.00 (0.00)

Table 3b: Average Length of Time that Incompletely Metamorphosed Axolotls and Neotenic Control Axolotls Spent in Each Stage of Regeneration

Each value is the average number of days spent in each stage. The parenthetical number represents the standard deviation for each group. Metamorphic resistant axolotls ranging in size from 7.0 to 9.0 cm were compared to small (6 – 10 cm) controls.

WH, Wound Healing; *DD*, Dedifferentiation; *Blas*, Blastema; and *Pal*, Palette.

	Incompletely Metmorphosed (n=4)	Neotenic, Controls (n=3)
<i>WH</i>	2.25 (0.50)	1.50 (0.00)
<i>DD</i>	7.88 (2.02)	5.50 (0.87)
<i>Blas</i>	6.75 (2.36)	8.67 (1.15)
<i>Pal</i>	3.25 (1.89)	4.00 (0.00)

Table 4a. Average Days for Completely Metamorphosed Axolotls & Neotenic Control Axolotls to Reach Each Stage of Regeneration

Two completely metamorphosed axolotls were able to reach DD. One of the axolotls died at day 20, before reaching blastema. The numbers for Pal and DO are from the surviving axolotl. Each value is the average number of days required to reach each stage (i.e. the number of days post-amputation). The parenthetical numbers represent standard deviations.

DD, Dedifferentiation; *Blas*, Blastema; *Pal*, Palette; and *DO*, Digital Outgrowth.

	Completely Metmorphosed (n=2,1)	Neotenic, Controls (n=3)
<i>DD</i>	10.00 (0.00)	6.00 (0.00)
<i>Blas</i>	18+	10.00 (0.00)
<i>Pal</i>	42.00	20.00 (0.00)
<i>DO</i>	50.00	24.00 (0.00)

Table 4b: Average Length of Time that Completely Metamorphosed Axolotls & Neotenic Control Axolotls Spent in Each Stage of Regeneration

Each value is the average number of days spent in each stage. One of the axolotls died at day 20, before reaching blastema. The numbers for Blas and Pal are from the surviving axolotl. The parenthetical number represents the standard deviation for each group. Metamorphic resistant axolotls ranging in size from 14.5 to 15.0 cm were compared to small (14 – 18 cm) controls.

WH, Wound Healing; *DD*, Dedifferentiation; *Blas*, Blastema; and *Pal*, Palette.

	Completely Metmorphosed (n=2,1)	Neotenic, Controls (n=3)
<i>WH</i>	9.00 (0.00)	5.00 (0.00)
<i>DD</i>	7+	4.00 (0.00)
<i>Blas</i>	24.00	10.00 (0.00)
<i>Pal</i>	8.00	4.00 (0.00)

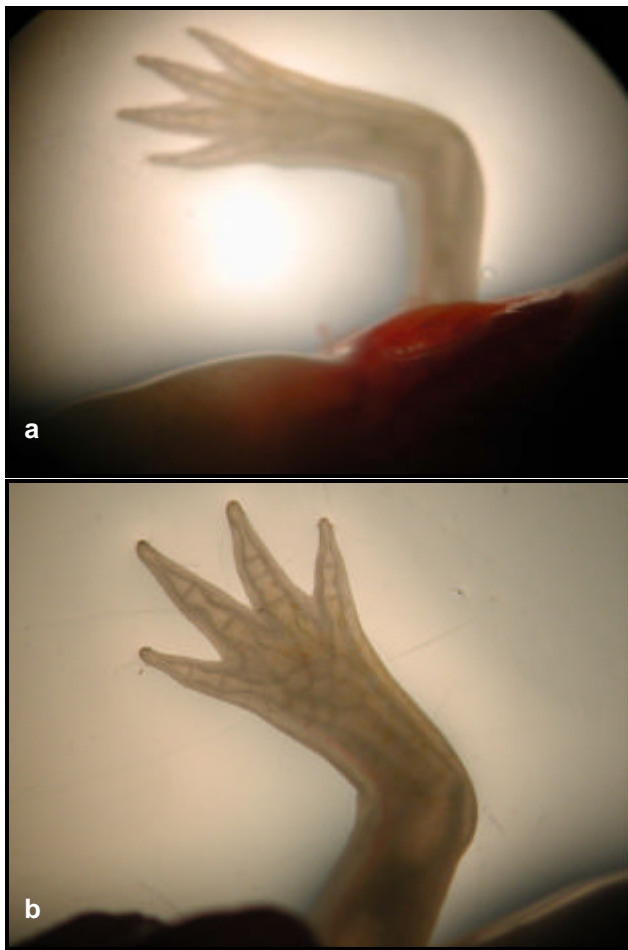


Figure 1. Regenerative Morphology of Neotenic Axolotls Here the pre-amputation picture (a) as well as the 44 days post-amputation picture (b) of a small axolotl are presented. The regenerative process is completed morphologically and the limb has attained the same, or nearly the same size, as the non-amputated limb. Both pictures were taken at 10x.

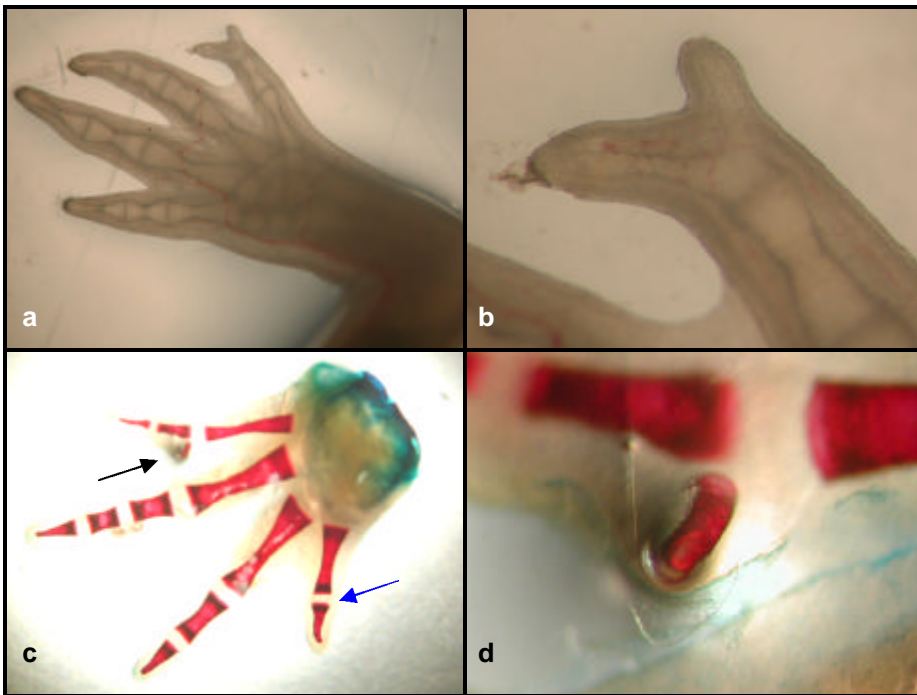


Figure 2. *Bifurcated Regenerates of Incompletely Metamorphosed Axolotls*

(a) The bifurcation of an incompletely metamorphosed axolotl limb at 44 days post-amputation. 15x magnification. (b) A 63x magnified picture of the bifurcation shown in (a). Pictures (c) and (d) are alizarin-red stained photographs. In (c) the bifurcation at the second phalangeal bone of the fourth digit can be seen (black arrow). Due to the red staining, it can be distinguished that the outgrowth is an extra bone and not just soft tissue. In addition, the first digit (blue arrow) lacks a phalangeal bone. 10x magnification. (d) An 80x magnification of the bifurcation shown in (c).

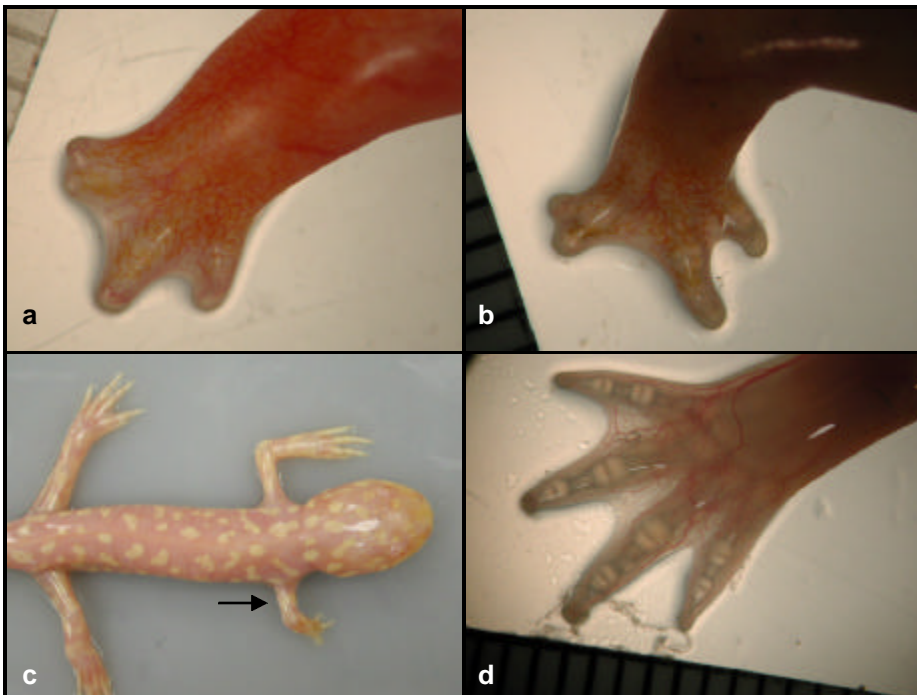


Figure 3. *Regenerates of the Completely Metamorphosed Axolotl*

The picture of the regenerate taken 69 (a) and 117 (b) days post-amputation. Note that not only is the limb hypomorphic in that it has only three digits but that one of the digits is bifurcated as well. (c) A view of the non-amputated and amputated forelimbs. The arrow indicates the approximate plane of amputation. (d) A neotenic control at 117 days post-amputation. The ruler in the photographs represents 1 mm markings.

DISCUSSION

As predicted by comparison with Young and associates' (1983b) study on regeneration in post-metamorphic *Ambystoma*, regeneration rate was retarded after metamorphosis. However, unique to this current experiment and due to its design, is that differing effects were seen depending upon whether or not the animal was resistant to metamorphosis, was in a state of incomplete metamorphosis, or had metamorphosed completely. This indicates that the axolotl can be used as a model organism to further our understanding of how metamorphosis affects limb regeneration ability.

In the metamorphosis resistant animals, the overall rate of regeneration was similar to comparably-sized controls. However, the one significant difference that existed was that the animals spent 30% more time in dedifferentiation. If this were an effect of the TH, or its downstream effects, one would expect a similar hindrance in the incompletely metamorphosed animals. Not only was this the case, but the effect was more pronounced as well. That is, whereas the metamorphic resistant group spent 30% more time in dedifferentiation, the incompletely metamorphosed group spent 75% more time in dedifferentiation, as compared to controls. However, in both cases the overall time needed to reach digital outgrowth was not significantly different since the animals on average spent slightly less time in blastema and palette. This suggests that although TH has the capacity to hinder dedifferentiation, a metamorphosis-associated factor augments this effect. Furthermore, that the extreme hindrance occurred only during dedifferentiation in incompletely metamorphosed axolotls—which only exhibit metamorphic changes initially—indicates that the metamorphosis-associated factor could exert further and more dramatic effects if metamorphosis were to continue.

The most remarkable instance of rate retardation occurred with the axolotl that was completely metamorphosed before amputation. These results agree with the studies done by Young and coworkers in other species (1983a; 1983b) and aid in demonstrating that metamorphosis progressively exerts its hindering effects. This is similar to the case of anurans (Tata *et al.*, 1991), although with most urodeles—including the axolotl—regeneration is not hindered completely (Young *et al.*, 1983a).

Due to the small sample size and nature of this study, it is difficult to pinpoint the reason for the hindrance in regeneration. At least two possibilities, however, can be proffered: an effect of the TH and senescence. Schmidt (1958) found that thyroidectomizing adult newts resulted in a faster regeneration rate but he did not analyze the differences stage-by-stage until the newts reached the equivalent of early bud blastema. These results show concordance with the current experiment in that thyroid hormone treatment resulted in a slowing of regeneration rate

and an absence of thyroid hormone resulted in an acceleration of regeneration rate. Saadi and cohorts (1993) analyzed thyroid hormone's role in the conversion of larval myosin isoforms to adult myosin isoforms. This conversion is thyroid hormone dependent in development, but not in regeneration. Dedifferentiation, therefore, could be delayed in thyroid hormone treated axolotls because their muscle fibers (as well as possibly other tissue cells) have transitioned from a more embryological state to a more adult-like state. In this manner, the dedifferentiative process takes longer since the cells have to “undo” more differentiation than the cells that were not thyroid hormone treated. However, it is very interesting that thyroid hormone is not required for this isoform change during regeneration. This most likely explains how the animals in Schmidt's experiment could regenerate faster without thyroid hormone since it is not needed for some aspects of regenerative muscle differentiation.

Senescence is another idea that one could propose to explain the hindrance in regeneration rate. That it could be induced by metamorphic processes is supported by the overall decrease in proliferative ability that occurs as one ages (Takahashi *et al.*, 2000). However, a distinction arises between senescence—the eventual complete cessation of cell division—and quiescence, the normal physiological withdrawal from the cell cycle. The main distinction between these two processes is that while quiescence is reversible under certain conditions, senescence is irreversible (Sedivy, 1998). Thus, since metamorphosed axolotls still retain regenerative ability, complete senescence could not be the cause. However, since the initiation of senescence involves a decline of cell division, metamorphosis could act synergistically with aging processes to propagate regenerative decline. An equally likely possibility is that the cells enter into a more pronounced quiescent state where reversal becomes more difficult.

In addition to regeneration rate hindrance, morphological changes were also present. The incidence of morphologically normal regeneration is lower in those who received TH (67% vs. 78%), but the sample sizes are too small to determine whether or not this difference is significant. However, potentially significant differences do arise if one looks at the responsiveness to TH.

Even though no TH-treated, metamorphosis-resistant axolotls developed abnormally, 50% of the incompletely metamorphosed animals regenerated with bifurcations and the completely metamorphosed axolotl regenerated with both a bifurcation and a hypomorphism. These bifurcations are incredibly similar to those seen in regenerating limbs that have been treated with low doses of retinoic acid (Crawford and Vincenti, 1998). None of the incompletely

metamorphosed animals nor the metamorphosed animal, however, displayed the proximalization characteristic of retinoic acid treatment. Retinoic acid has also been known to inhibit the rate of regeneration (Maden, 1982). However, even more interesting is that a connection between retinoic acid and thyroid hormone has been established. Their receptors are both members of the steroid/thyroid hormone receptor family (Brockes, 1989) that is a member of the superfamily of ligand dependent transcription factors (Fang and Hillgartner, 2000). The newt limb and limb blastema cells express six different members of this retinoic acid receptor family (Brockes, 1997), one of which has been shown to increase thyroid hormone responsiveness in the regulation of one enzyme (Fang and Hillgartner, 2000). Although this enzyme is not expressed in the limb, that a retinoic acid receptor in other organs can be regulated in any body part by thyroid hormone indicates that this regulation mechanism could occur in the limb. Brockes (1989) also mentions the similarity between retinoic acid and thyroid hormone receptors indicates that one compound could bind to the DNA response element of another compound. In this manner, it is not out of the question to propose that thyroid hormone could have reacted with retinoic acid receptors, thus leading to the bifurcations.

It is also interesting to note that in addition to the bifurcations common to the incompletely meta-morphosed

axolotls and completely metamorphosed axolotl, hypomorphisms were also typical in both. In the case of one of the incompletely metamorphosed axolotls, only one phalangeal bone was missing. However, in the case of the completely metamorphosed axolotl, the regenerate not only lacked a digit, but was bifurcated as well. Similar to the progressive increase in regeneration rate, the morphological effects are also more pronounced as one compares metamorphic resistant axolotls to incompletely metamorphosed axolotls to the metamorphosed axolotls.

The reiterating theme is that more severe abnormalities are seen in the regenerates—be it rates or morphology—as animals in thyroid-treated, metamorphic resistant states are compared against axolotls displaying incomplete metamorphosis. This is also seen when either of these groups are compared to the completely metamorphosed animals. Although the cause for regenerative impairment remains unknown, axolotls in differing metamorphic states can be used to further illustrate why and how this decline occurs. Perhaps future investigations, such as those involving gene expression, will serve to elucidate the mechanisms of this decline and aid us in understanding how mammals progressively lose regenerative ability as development progresses.

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