

Why is limb regeneration possible in amphibians but not in reptiles, birds, and mammals?

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SUMMARY The capacity to regenerate limbs is very high in amphibians and practically absent in other tetrapods despite the similarities in developmental pathways and ultimate morphology of tetrapod limbs. We propose that limb regeneration is only possible when the limb develops as a semiautonomous module and is not involved in interactions with transient structures. This hypothesis is based on the following two assumptions: To an important extent, limb development uses the same developmental mechanisms as normal limb development and developmental mechanisms that require interactions with transient structures cannot be recapitulated later. In amniotes limb development is early, shortly after neurulation, and requires inductive

interactions with transient structures such as somites. In amphibians limb development is delayed relative to amniotes and has become decoupled from interactions with somites and other transient structures that are no longer present at this stage. The limb develops as a semi-independent module. A comparison of the autonomy and timing of limb development in different vertebrate taxa supports our hypothesis and its assumptions. The data suggest a good correlation between self-organizing and regenerative capacity. Furthermore, they suggest that whatever barriers amphibians overcame in the evolution of metamorphosis, they are the same barriers that need to be overcome to make limb regeneration possible in other taxa.

INTRODUCTION

The capacity to regenerate varies considerably among animals (Goss 1969; Tsonis 2000). Within vertebrates it is highest in amphibians. In amphibians even regeneration of the highly complex limb is possible in many species, as long as a small part of the limb is still present. Regeneration capacity is present in urodeles throughout life, whereas in anurans the regeneration capacity decreases with age and usually disappears after metamorphosis. However, regeneration has been shown to be possible in older tadpoles (complete regeneration) and in adult *Rana* species (partial, Fig. 1) when the wound of the stump is initially kept open or the amputated stump is maltreated in other ways (Liosner 1931; Poležajew 1939, 1946; Rose 1944, 1945). The regeneration program is thus still present in older tadpoles and adult frogs, although not normally executed when a limb is amputated. Curiously, in amniotes limb regeneration is not possible (Lillie 1904; Spurling 1923; Deuchar 1976; Goss 1969; Muneoka and Sassoon 1992; Stephens et al. 1993) despite the fact that limb development of amphibians and amniotes is to a large extent similar and produces highly similar anatomical structures. Even the apical ectoderm cannot be regenerated after removal in the limb bud (Saunders 1948; Summerbell 1974).

To explain the paradoxical discrepancy between amphibians and amniotes we propose the following hypothesis:

Limb regeneration is only possible when the limb develops as a semi-independent module and is not involved in interactions with transient structures.

This hypothesis is based on the following two assumptions:

1. Limb regeneration uses the same developmental mechanisms as normal limb development to an important extent.
2. Developmental mechanisms that require interactions with transient structures cannot be recapitulated later.

We start by discussing evidence that limb regeneration indeed largely uses the same developmental mechanisms as normal limb development. We then show that in amniotes, the limb does not develop as a semi-independent module, and its development requires many interactions with external structures, including transient structures. By contrast, in amphibians limbs develop significantly later than in amniotes and appear to be semi-independent modules with a high self-organizing capacity. Although there are considerable differences in limb development between anurans and urodeles, their development is similar in the element crucial for our

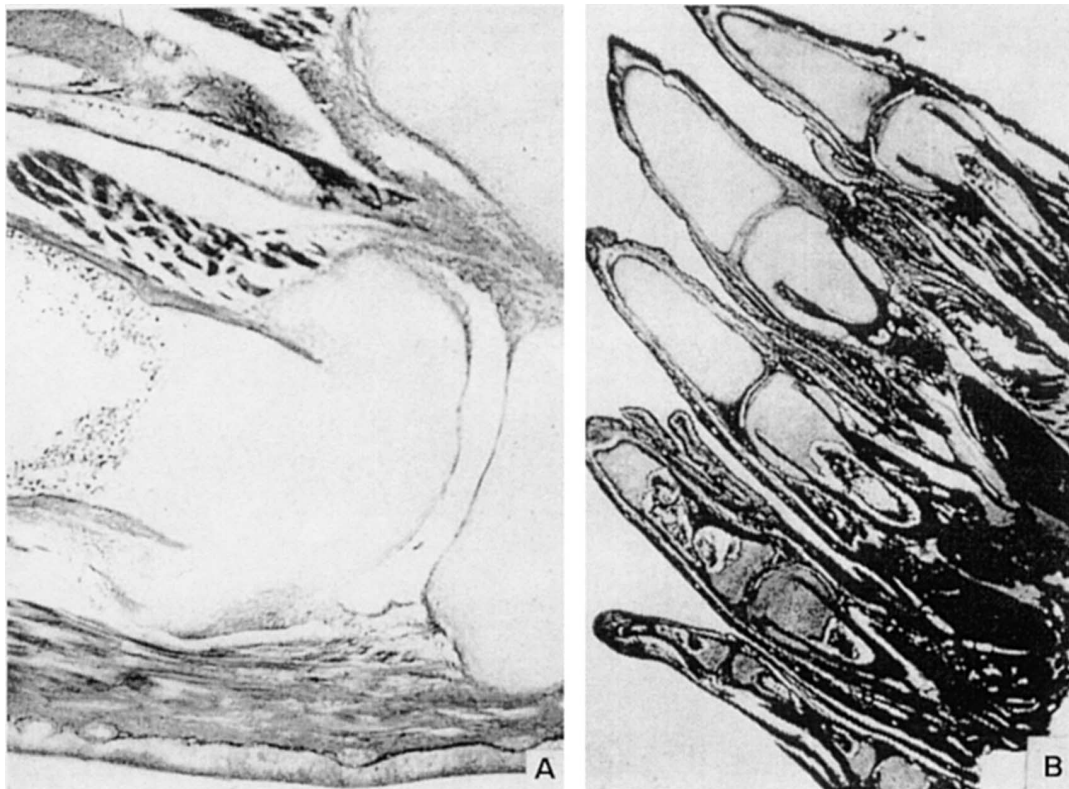


Fig. 1. Recovery of regenerative capacity in adult *Rana clamitans* by daily bathing of amputated stump in a strong NaCl solution. (A) Regenerated wrist region, all tissue in figure is regenerated tissue (left end of forearm, right carpal elements). The limb was amputated halfway along the forearm. The forearm and wrist regenerated completely, but more distal elements regenerated incompletely and irregularly (not shown). (B) Toe regenerants of four toes originally cut off level with the shortest toe which was not amputated (below in figure). Everything distal to level of tip of shortest toe is regenerated tissue (Rose 1944).

hypothesis, whether limb development is autonomous or not. Finally, we present additional comparative developmental data that show a correlation between regenerative ability and independence of development across taxa, including the loss of both of these features in one lineage of amphibians.

REGENERATION RECAPITULATES NORMAL DEVELOPMENTAL PATHWAYS

Epimorphic regeneration, the rebuilding of complex tissues after amputation, starts with wound healing and dedifferentiation of tissue close to the amputation surface to form mesenchyme (Goss 1969). This dedifferentiation phase is followed by growth and redifferentiation of the mesenchyme into a faithful copy of the limb before amputation (Stocum 1968; Bryant and Muneoka 1986). The resemblance between normal limb development and the limb redifferentiation phase of regeneration includes similarities in gene expression patterns, pattern formation mechanisms, and self-organizational capacity.

Similar genes involved

Expression of many genes has been characterized during both normal limb development and regeneration in *Xenopus* and urodele species. In almost all cases, similar expression patterns were found in the regeneration cone and in the limb bud (but see Carlson et al. 2001 on *Hoxc10L*, which seems to have a unique function in regeneration). The genes for which similar expression patterns were found include *Hox a-13*, *Hox c-6*, *Msx1*, *Fgf-8*, *Fgf-10*, *Fgf-4* (virtually absent in both limb development and regeneration of the axolotl although present in the differentiated limb), and *Shh* (e.g., Imokawa and Yoshizato 1997; Carlson et al. 1998; Torok et al. 1999; Endo et al. 2000; Yokoyama et al. 2001a; Christensen et al. 2002).

Similar pattern formation

The pattern formation processes also appear to be similar in normally developing and regenerating limbs (Stocum and Dearlove 1972; Bryant and Muneoka 1986; Muneoka and Sassoon 1992; Torok et al. 1998; Roy et al. 2000). The same signaling centers are present: The epidermis of the limb bud

and the regeneration blastema both have apical ectodermal ridge (AER)-like properties (the apical ectodermal cap [AEC] expressing *Fgf* genes) and play an important role in patterning; the *Shh*-expressing region acts both in the limb bud and in the regeneration blastema as a zone of polarizing activity; and there is a proliferation zone present in both the limb bud and the regeneration cone. In addition, when the axis of the cone-stage regeneration blastema is rotated dorsoventrally, supernumerary limbs are formed, just as in rotated limb buds. Furthermore, polydactyly or even duplicated limbs results from ectopic expression of *Shh* anteriorly in both the regeneration cone and the limb bud (Torok et al. 1999; Roy et al. 2000). Finally, both the regeneration cone and the limb bud can regulate after the loss of part of the field, for intercalary defects (when distal parts are grafted on proximal parts; Maden and Goodwin 1980).

Similar explantation and transplantation capacity

There is similarity between amphibian limb buds and regeneration cones in that both can be successfully transplanted and explanted and, therefore, must have a high self-organizing capacity (see below for data on limb buds; for the regeneration cone see Stocum 1968; De Both 1970; Stocum and Fallon 1982). The first assumption that underlies our hypothesis appears to be fulfilled: To an important extent regeneration uses the same developmental mechanisms as ordinary limb development.

LACK OF SELF-ORGANIZING CAPACITY OF THE AMNIOTE LIMB

In amniotes the timing of limb patterning falls primarily into a highly conserved developmental stage called the phylotypic stage (Fig. 2), a stage characterized by many inductive interactions between different parts of the embryo, including the somites, notochord, neural tube, lateral plate mesoderm, pronephros, mesonephros, and limb bud. The phylotypic stage starts approximately with neurulation and ends when most somites have been formed. In mice, for example, the phylotypic stage is from embryonic days 7 to 11 (Galis and Metz 2001) and the limb buds appear on embryonic day 8. Mesenchymal digit condensations appear on embryonic days 10 (forelimb) and 11 (hindlimb) (Ngo-Muller and Muneoka 2000). The staging tables of Keibel (1897–1937) show that limb development occurs at roughly comparable stages in lapwings, sand lizards, deer, mice, humans, and chicken. It thus appears that in amniotes most of the limb patterning occurs during the phylotypic stage, a period of high interactivity among parts and at a time when many transient structures are present.

Interactions with different modules

The development of the limb appears to be involved in the overall very high interactiveness of development at that time

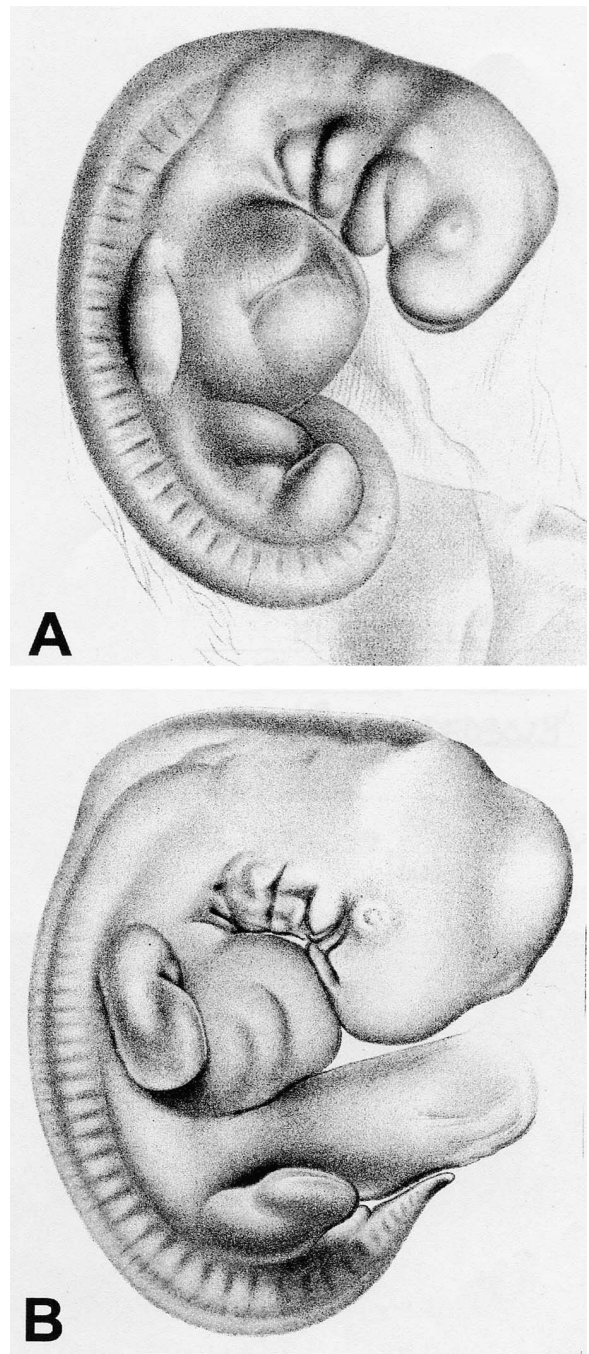


Fig. 2. Embryonal stages of humans. (A) Early limb development during the phylotypic stage. The somites are clearly visible at the level of both the front limb and the hindlimb. (B) A more advanced stage around the end of the phylotypic stage in which the handplate and footplate are visible. In the front limb faint lines between the digits are visible, and the somites at the level of the front limbs are no longer visible. Hindlimb development is less advanced, there are not yet traces of digits visible and the somites are still visible (Keibel 1897–1937).

(Galis et al. 2001). Limb patterning processes that necessarily involve interactions between the limb and other parts of the embryo include (a) migration of muscle progenitor cells from the somites into the limb, (b) migration of neural crest cells (precursors of pigment cells) from the edge of the neural plate into the limb, (c) invasions of axons into the limb, and (d) invasions of blood vessels into the limb. The molecular basis of these processes has not been investigated in as much detail as that of the patterning processes of limb bones; however, some of the signaling has been unraveled. For instance, the migration of muscle precursor cells requires inductive signals from the somites toward the limb bud and from the limb bud toward the somites (e.g., Hayashi and Ozawa 1995; Mennerich et al. 1998; Dietrich et al. 1999). Interactions between *Robo* and *Slit* genes in the lateral plate and the limb bud are involved in the migration of axons into the limb bud (Yuan et al. 1999). Furthermore, inductive signaling from somites is necessary for the dorsoventral polarity in the early chick limb bud (Michaud et al. 1997). At least one more signal from axial structures medial to the limb bud is involved in the dorsoventral patterning of the limb bud and the induction of the AER (Ohuchi et al. 1999). These interactions between the developing limb and surrounding structures suggest that the self-organizing capacity of the amniote limb may be low. The self-organizing capacity has been directly tested in transplantation, explantation, and foil-insertion experiments.

Explantation, transplantation, and foil-insertion experiments

A problem in evaluating the self-organizing capacity in the old explantation and transplantation literature is that the differentiation of limb bones alone, normally or even abnormally, is interpreted as self-differentiation of the limb. The absence of most or all the musculature (derived from the somites) or the presence of disconnected tendons in limbs grown from explants is usually disregarded (but see Hunt 1932). In addition, the inclusion of pieces of somites as landmarks is not seen as the presence of somitic material (Hamburger 1938, 1939). This interpretation of self-differentiation does not suit our purposes. In our interpretation, self-differentiation implies the differentiation of all structures, including muscles, in the absence of external regulatory interactions.

Transplantation experiments on chick limbs show a limited success when the limb bud is grafted to the flank region that contains somites, preferably part of the flank close to the limbs (Hamburger 1938, 1939). No successful transplantations to other areas or to the flank in later stages (without somites) have been reported. In addition, in the transplants to the flank area, small pieces of somites were included. Furthermore, grafting of intact wing bud mesoderm is successful only from stages HH 12 to 17 (Hamburger and Hamilton 1951) to the flank of stages 12 to 17 (Kieny 1968, 1971; Dhouailly and

Kieny 1972; Saunders and Reuss 1974). Older transplants cannot induce an AER in the ectoderm (also not in younger ectoderm). In addition, the flank area is not competent to receive transplants after stage 17. There is, as yet, no evidence that transplantation of chick limb buds is successful except when neighboring tissue is added in the transplant.

Explantation experiments in mice show a low self-organizing capacity for the limb buds: Up to embryonic day 12, which is just after the phylotypic stage when the number of digits has been determined, development of explanted limb buds requires the addition of somitic material. (Fig. 3; also see in Fig. 2A how during early limb bud development the somites are clearly visible in a human embryo). Concordant with a sharp decrease in vulnerability to external interactions on day 12 (Galis and Metz 2001), the self-differentiating capacity increases markedly (Agnish and Kochhar 1977) (also see in Fig. 2B how when traces of the digits can be seen in the front limbs, the somites are no longer visible at the level of the front limbs). Similar results were obtained for chickens, in which the presence of pieces of neural tube, neural crest cells, and mesonephric tissue, as well as somites, improves explanted limb bud development, including skeletal, muscular, and tendon development (Strangeways and Fell 1926; Hunt 1932; Pinot 1970; Strecker and Stephens 1983). The results also improved with the age of the limb bud (Fell and Mellanby 1952; Pinot 1970; Stephens et al. 1993). No normal explanted limbs were obtained without the addition of neighboring tissues to the explanted structure.

The insertion of a foil medial to the limb bud and lateral to the somites shortly before limb bud outgrowth (stage 14/15) inhibits limb development at the level of the foil (Saunders 1948; Stephens and McNulty 1981; Ohuchi et al. 1999). Limb development thus appears well integrated in the general inductive interactiveness of the phylotypic stage and cannot proceed normally without external regulatory interactions.

Inductive interaction with transient structures

Our second assumption is that interactions with transient structures cannot be repeated later. The results described show that during normal development interactions are required with transient structures (i.e., the neural tube, neural crest, somites, lateral plate mesoderm, and possibly mesonephros and pronephros). These structures are only present in the early embryo. When a limb is amputated at a later stage when these structures have differentiated or disappeared, inductive interactions with these structures are not possible. Signaling from axial structures medial to the lateral plate mesoderm (such as somites and intermediate mesoderm) that is necessary for the induction of an AER and for the establishment of dorsoventral polarity may well belong to these interactions that cannot be repeated later. This signaling occurs in chickens around stage 14/15, just before limb bud outgrowth in stage 16 (Kieny 1971; Ohuchi et al.

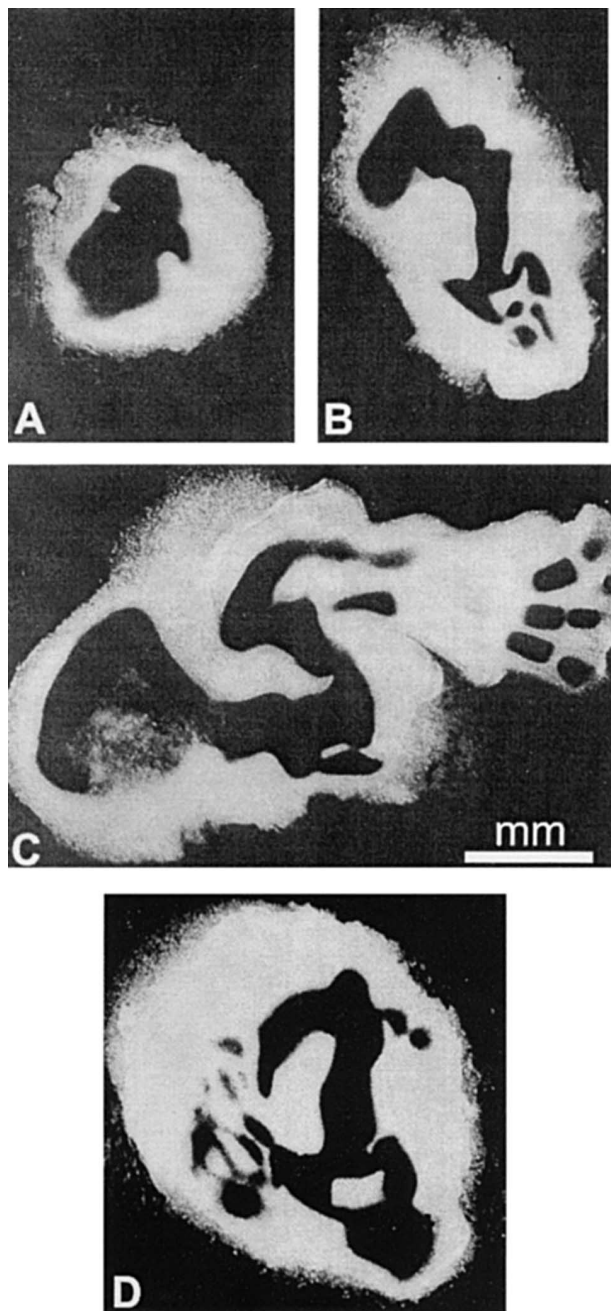


Fig. 3. Explantation of mouse limb buds, explanted at age 11 days p.c. (A), 12 days p.c. (B), 13 days p.c. (C), 11 days p.c. with somites added (D), after 9 (A) or 6 (B–D) days of development. Development improves with the age of the explant and with the addition of somites (Agnish and Kochhar 1977).

1999; Kawakami et al. 2001). Michaud et al. (1997) showed that a signal from the somites is involved in the establishment of dorsoventral polarity in the limb. Ohuchi et al. (1999) showed that the blocking of signaling from paraxial and axial structures with foil from stage 14/15 onward leads

to the absence of Fgf 8 and 10 expression and of ventral and dorsal markers in the ectoderm (*En-1*, *R-fng*, *Wnt7a*, and *Lmx1*). Implantation of *Fgf10* expressing cells lateral to the foil barrier partially rescues AER induction. It rescues *Fgf8* expression in the ectoderm and the expression of the ventral marker genes *En-1* and *R-fng* but not the dorsal marker genes *Wnt7a* and *Lmx1*. Apparently at least one more paraxial or axial signal is necessary for the induction of the AER and the establishment of dorsoventral patterning. As mentioned above, the capacity of the limb bud mesoderm to induce an AER in flank ectoderm is lost a little later at stage 17, even in young ectoderm, which supports the idea that the induction of an AER may not be repeated later. In addition, an interaction with transient structures that cannot be repeated later may be the signaling to the limb bud influencing the early differentiation of muscles. Hunt (1932) found in explanted chick limb buds of various stages that the growth and differentiation of muscular development was very poor compared with skeletal development. When surrounding tissue was added to the explant, including somites, nephric tissue, neural tube, notochord, and lateral plate mesoderm, muscle differentiation markedly improved.

AMPHIBIAN LIMB BUD AS A SELF-ORGANIZING MODULE

Limb development in amphibians is delayed relative to limb development in amniotes (Figs. 4 and 5), especially in anurans with their extreme metamorphosis, and generally takes place after the phylotypic stage. Fossil data indicate that the delay of limb development is a primitive trait of modern amphibians but presumably a derived trait within tetrapods (Milner 1980; Carroll 1987; Laurin 1996; Klembara and Bartik 1999). Late limb development may have been a key event in the evolution of regenerative ability. The delay of limb development means that interactions with transient external structures of the phylotypic stage cannot take place during normal limb development, because the structures are no longer there. The somites, for instance, can no longer be recognized as such but have transformed into other structures such as larval trunk muscles by the time limbs develop (see staging tables of, e.g., *Xenopus*, *Triturus vulgaris*, and *Ambystoma*) (Nieuwkoop and Faber 1956; Bordzilovskaya et al. 1989; Liosner and Dettlaff 1991). This implies that either the inductive interactions necessary for limb development take place between the limb bud and larval structures or the interactions are mainly limited to interactions within the limb bud (i.e., an increase in modularity and self-organizational capacity). It appears from many transplantation and explantation experiments that the limb bud of many anurans and urodeles develops as a largely self-organizing module.

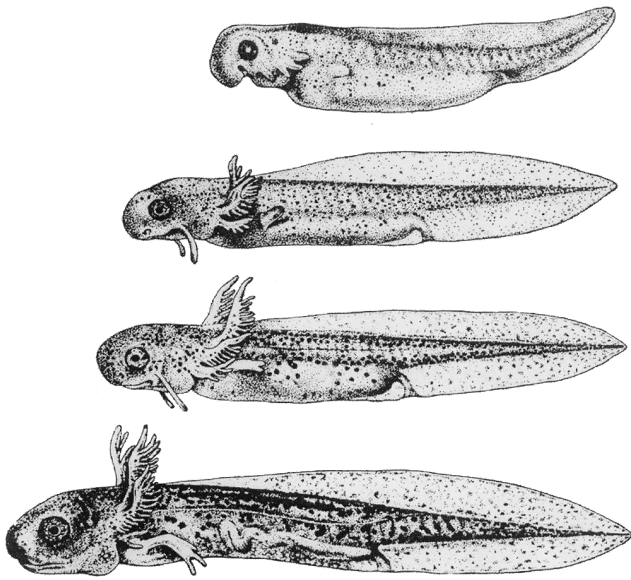


Fig. 4. Front limb development in the salamander *Ambystoma maculatum* (stages 37–46 of Bordzilovskaya et al. 1989.). There are no longer somites at these stages. The hindlimb bud is not yet visible. Limb development is later than in amniotes, after the phylotypic stage and after the differentiation of the somites (Detwiler 1937).

Transplantation and explantation experiments

During the phylotypic stage (neurula and tail bud stage in urodeles), the presumptive limb cells are located in limb anlagen (limb fields) but have not yet formed limb buds. In urodeles transplantation of the limb anlagen is usually suc-

cessful in the flank (Harrison 1918; Detwiler 1933) but always fails outside this region (Amano 1960, Slack 1977) unless flank tissue adjacent to the limb anlage is included in the transplant (Nicholas 1929; Detwiler 1930; Slack 1977, 1980). The flank tissue included in the classic limb field transplantation experiments consisted of splanchnic and somatic mesoderm, intermediate mesoderm (pronephros), and a piece of the gill anlage plus overlying epidermis (Slack 1977, 1980). Slack (1977, 1980) concluded from transplantations to the head and flank that signals from the posterior tissue to the limb anlage were necessary for the establishment of anterodorsal polarity, from dorsal tissue for the induction of growth of the limb bud and possibly also for dorsoventral patterning and anteriorly some gill tissue was necessary to act as a barrier to the passage of signals. In addition, explantation experiments of prebud limb anlagen failed unless somites or pronephric and heart tissue was added, with the best result when somites, pronephric, heart tissue, and neural tube tissue were added (Mangold 1937; Poležajew 1939; Amano 1960). Interactions of the limb anlage with transient external structures during the phylotypic stage are apparently required for normal development, comparable with the situation of the limb bud in amniotes at this stage.

After the phylotypic stage, when the limb fields have formed limb buds, in many amphibian species limb buds and limb bud mesoderm can be very successfully transplanted without adjacent tissue, also when grafted to areas outside the flank (e.g., the head) (Fig. 5) (Braus 1905) or to the flank of later developmental stages (Lauthier 1985). The transplantation and self-organizing capacity of the limb bud appears

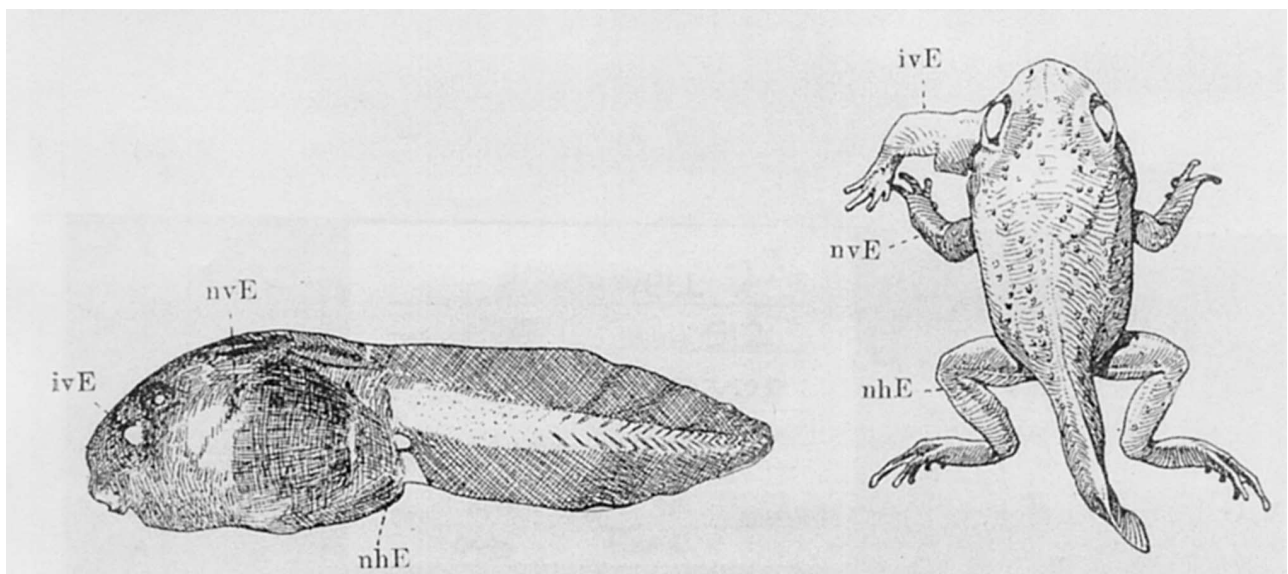


Fig. 5. Transplantation of an early front limb bud to the head just anteriorly and ventrally of the eye of a host of the same age developed into a normal limb. nvE, normal front limb (normale vordere Extremitäten), nhE, normal hindlimb (normale hintere Extremitäten), ivE, transplanted front limb (implantiertes Vorderbein) (Braus 1905).

very much higher than that of amniotes, comparable with that of the above-mentioned regeneration cones in amphibians.

As an aside we address the origin of the muscle cells of the limbs, because a somitic origin for these cells is an ancestral trait of tetrapods, and somites are not present during limb bud development of many amphibians. The developmental origin of the limb muscles in amphibians has not been thoroughly investigated, but in *Xenopus* it appears likely that individual mesenchymal cells originating from the larval muscles dedifferentiate and migrate to the limb bud. Ryke (1953) observed that “practically from the whole latero-ventral portions of the larval myotome cells are proliferated and migrate in the direction of the limb bud,” suggesting migration of individual cells of the larval myotome to the limb bud. In addition, Williamson et al. (1991) showed the presence of cytotactin proteins in fibrils of the extracellular matrix that run from the myotome to the limb bud rudiment. Cytotactin proteins are known to be associated with cell migration and differentiation because they are expressed along the pathway of migrating neural crest cells. The migration appears to occur very early in the development of the limb bud, because Nicolas et al. (1998) found that the myogenic transcription factors *Myf-5* and *MyoD* are expressed very soon after the formation of the limb bud (for a more extensive discussion see Galis 2001). In urodeles the situation is even less clear. Rylkoff (1924) observed that the border between limb bud and larval myotome is never sharply delineated and suggested that this is because of epithelial extension of the larval myotome to the limb bud. Nicholas (1929) and Detwiler (1930) found when transplanting limb anlagen with surrounding flank tissue to the head that limbs developed that were capable of movement. No somites were presumably included in the transplant (Slack 1977, 1980), which implies either that the limb is capable of locally recruiting muscles or that muscle precursor cells had already migrated from the somites to the limb anlage at a prebud stage. The former scenario seems the most plausible one if limb muscle migration is similar to that assumed for *Xenopus*. Further research is urgently needed to clarify the migration of muscle precursor cells in amphibians.

The data from amphibians is in agreement with our hypothesis and assumptions. The limb bud has a high self-organizing capacity and develops as a semi-independent module. Limb bud development after the phylotypic stage does not appear to require interactions with transient structures.

COMPARATIVE TESTING OF THE HYPOTHESIS

To test our hypothesis we investigated whether the correspondence between self-organizing capacity and regenerative capacity of the fin or limb is of a more general nature. In addition, we investigated whether regenerative capacity is correlated with the timing of fin/limb development and thus

the likelihood of interaction with transient structures in other vertebrate groups.

Sharks

In sharks regeneration of the paired fins is not possible (Goss 1969). Fin development takes place during the phylotypic stage (Keibel 1897–1937; Witschi 1956) and includes the migration of myotomes from the somites to the fins. It is thus highly probable that there are interactions between the fin bud and the transiently present somites. No data could be found on transplantation and explantation of fin buds, but our hypothesis predicts they will not be possible.

Teleosts

In teleost fishes regeneration of the fin rays is possible but not of the bony skeleton that is homologous with tetrapod limb bones (Blanc 1949; Goss and Stagg 1957; Wagner and Misof 1992). The pelvic fins are reduced in teleosts and usually lack the bony skeleton distal to the pelvic girdle. Therefore, they are not relevant to our comparison. The development of the pectoral fin buds usually starts during the phylotypic stage (e.g., Ignatieva 1991; Kimmel et al. 1995). Interactions with the somites are necessary for normal fin bud development because transplantation of the fin bud to near the midventral line gives rise to fins without muscles (Lopashov 1950). However, muscles develop if a part of the somite is included in the graft. There is thus agreement in the absence of regenerative capacity and the necessity of interactions with transiently present structures, at least in these teleost species.

Lungfishes

Lungfishes are the only other vertebrate group in which regeneration of the bony central skeleton of the fins is possible (Léger 1897; Conant 1970). The pectoral and pelvic fins develop late, after the somites have started to differentiate and after the phylotypic stage (Fig. 6) (Keibel 1897–1937; Kemp 1982). The late development implies that fin bud development cannot occur in interaction with the transient structures that are only present during the phylotypic stage. Fin development has thus become decoupled from the interactiveness of the phylotypic stage as in most amphibians. This is in agreement with our hypothesis. To further test the hypothesis, the self-organizing capacity of the fins should be investigated using transplantation experiments.

Early limb development in direct-developing amphibians

In amphibians earlier limb development has evolved several times in association with loss of the aquatic larval stage. As direct development is derived, the ancestral development of limbs in these amphibians is as a self-organizing module without interactions with transient structures. This means

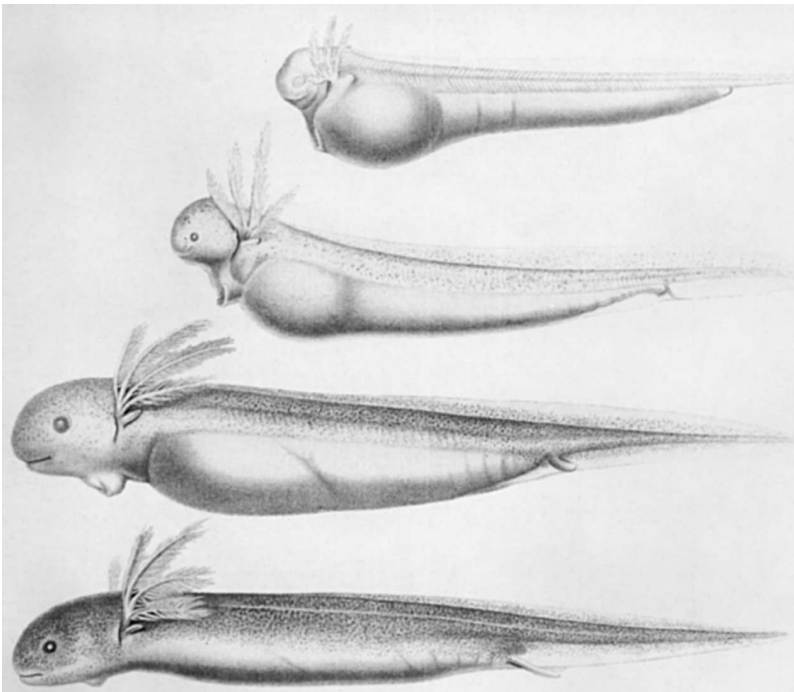


Fig. 6. Fin development in the lungfish *Lepidosiren paradoxa*. Fin development is after the phylotypic stage and at approximately the same stages as limb development in *Ambystoma maculatum* (Fig. 3) (Keibel 1897–1937).

there are two possible scenarios predicted by our hypothesis for the self-organizing and regenerative capacity of the limb. Either limb development has become integrated into the interactiveness of the phylotypic stage, with as a cost a loss of regenerative capacity, or the self-organizational capacity has been preserved to maintain the capacity for regeneration. Both scenarios are in agreement with our hypothesis. If direct-developing amphibians maintain regenerative capacity while their limb development is integrated into the general interactiveness of the phylotypic stage, our hypothesis would be contradicted.

Anurans

In indirectly developing anurans with late limb development, the capacity for limb regeneration is lost during larval life. This is contrary to the prediction of our hypothesis. However, as noted before, the pathway for regeneration is still present in old tadpoles and anurans, although not normally executed. Poležajew (1946) obtained complete regeneration in *Rana temporaria* after traumatization of the amputated stump with a needle, and Rose (1944, 1945) obtained partial regeneration in adult *Rana clamitans* by bathing the amputated stumps in strong NaCl solutions (complete regeneration of the forearm and wrist area and complete regeneration of the digits; Fig 1). Bathing in the salt solution prevented the initial healing of the skin, and Rose (1944, 1945) hypothesized that the success of the regeneration in his and Poležajew's experiments was due to the absence of dermis in the initial phase of regeneration. Regardless of the cause

of the successful regeneration, the capacity to induce an AEC is thus still present in old tadpoles and even adult frogs, although not normally used.

In several independently evolved clades of frogs, limb development starts early. In the speciose direct-developing genus *Eleutherodactylus*, limb development starts as early as in amniotes, just after the closure of the neural tube in the middle of the phylotypic stage (Gardner Lynn 1942; Townsend and Stewart 1985). In *E. martinicensis* both the regeneration and transplantation capacity of developing limb buds have been studied (Hughes 1962). Hughes found that the transplantation capacity was very low. Young limb buds could only be transplanted back to their own amputated stump or to the stump of a similarly amputated individual. Transplantation could also be successful when transplanted right next to the stump. Transplantation to all other areas failed. Transplantation of an older limb bud, even to its own stump, was rarely successful. This is in sharp contrast with the situation of anurans with aquatic larvae. The regeneration capacity of the limbs was similarly found to be reduced in direct developing species. Limb development thus appears to have become integrated into the general interactiveness of the phylotypic stage, with a loss of self-organizational and regeneration capacity.

It is somewhat surprising that a trait as seemingly advantageous as regeneration capacity has been lost. In this sense it is of interest to know whether the integration of limb development in the global inductive interactiveness is itself selectively advantageous and outweighs the cost of the loss

of regenerative capacity or whether the integration is a necessary consequence of early limb development. In the latter case this would represent a developmental constraint, and it would imply that the selective advantage of early limb development outweighs the cost of the loss of regenerative capacity.

Urodeles

In the salamander family Plethodontidae direct development has evolved at least twice (Lombard and Wake 1986; Collazo and Marks 1994; Wake and Hanken 1996). Limb development is somewhat earlier than in plethodontid species with aquatic larvae but not as early as in most amniotes and *Eleutherodactylus* (Collazo and Marks 1994; Vial 1968; Marks and Collazo 1998; E. L. Jockusch, unpublished data on *Batrachoseps attenuatus* and *Oedipina uniformis*). The last pair of limbs that develop start to develop right at the end of or just after the phylotypic stage at the time when the tail somites have either completely developed or are being developed. Furthermore, limb development proceeds more slowly than in amniotes and *Eleutherodactylus*. It appears that at least the last developing limbs cannot develop in interaction with the transient structures of the phylotypic stage. This also holds for most part of the development of the first forming limbs. The expectation is thus that the self-organizing capacity of the limb will be considerably larger than in amniotes. Unfortunately, the self-organizing capacity of the limbs has not been directly tested in transplantation and explantation experiments. The regenerative capacity has been tested and appears large in many genera (Scadding 1977; Dinsmore and Hanken 1986; Sessions and Larson 1987). In the future it will be interesting to test whether the self-organizing capacity is indeed considerable in plethodontids, as predicted by our hypothesis, and whether it has been lost for proximal structures because their development overlaps somewhat with the phylotypic stage, and most tests of regeneration in salamanders leave the proximal and first formed structures intact.

Late limb development in amniotes

The explantation experiments of Agnish and Kochhar (1977) show that in mice, limb development at embryonic day 13 is considerably less dependent on external influences than earlier limb development. Our hypothesis predicts that this allows regeneration of the part of the limb patterned at that time or later. At embryonic day 13 most of the patterning has already occurred, but the phalanges are still being patterned (Kimura and Shiota 1996). In accordance with our hypothesis, regeneration of the distal phalanges is indeed possible prenatally in mice (Wanek et al. 1989; Reginelli et al. 1995). The capacity decreases with time, and immediately after birth only the distal-most phalanx can regenerate in humans and mice.

DISCUSSION

We report associations between regeneration capacity, self-organizing capacity, and timing of limb development. We found that regeneration only occurred when limb (or fin) development is late and proceeds as a largely self-organizing system, without interactions with transient structures. Regeneration of limbs after a dedifferentiation phase appears very similar to normal limb development. When regeneration of limbs or fins is not possible, limb (or fin) development is early, the self-organizational capacity of the developing limb or fin is low, and in many cases there is evidence that normal development requires interactions with transient structures. We would like to stress that the presence of self-organizational capacity and the absence of interactions with transient structures only allows for the possibility of regeneration capacity and does not imply the necessary presence of it. For instance, with our hypothesis we cannot explain why the capacity for limb regeneration is progressively lost with age in anurans. However, as noted before, the pathway for regeneration is still present in old tadpoles and anurans, although not normally executed. It is remarkable that in all investigated vertebrates the presence of self-organizational capacity of limb or fin development was associated with at least temporary regenerative capacity after the initial regulative limb bud stage. Other factors may also play a role in explaining the presence versus absence of regeneration. Research thus far has concentrated on genetic and molecular differences between amphibians and amniotes (e.g., Tsonis 2000; Velloso et al. 2001). It should be kept in mind that once regeneration capacity is lost in a taxon, selection for regeneration capacity will no longer be present. This will lead to increasing differences between taxa in characters associated with regeneration. Such differences are obviously not the cause of the loss of regeneration.

The observed correlation between self-organizing, timing of limb development, and regeneration capacity must be due to independent evolutionary events in at least three lineages, not just to the inheritance of the correlated features from a common ancestor. Delayed limb development and regeneration evolved in lungfishes and tetrapods (either twice independently or once at the base of a lungfish-plus-tetrapod clade with reversals at the base of the amniote clade). Early limb development and loss of regenerative capacity evolved in the direct-developing *Eleutherodactylus* species.

Candidates for interactions that cannot be repeated later in amniotes

Important signaling interactions that probably cannot be repeated later in amniotes are the signals from axial structures medial to the lateral plate mesoderm that leads to the induction of the AER in the early limb bud in at least chickens. In amphibians, limb bud outgrowth and AEC formation are delayed

relative to amniotes and occur either at the end of the phylo-typic stage or later. The transient structures that are medial to the early limb bud in amniotes have either differentiated or disappeared in amphibians at limb bud formation. Because signaling for the initiation of the AER or AEC appears conserved in tetrapods (Yokoyama et al. 2001b), the signals necessary for AEC initiation should either be provided by larval structures close to the limb bud or they should be endogenously produced in the limb. The latter seems the most likely in the light of the capacity of limb bud mesoderm of the newt *Pleurodeles waltlii* to induce an AEC in ectoderm and subsequent limb formation, after transplantation to different places on the flank of larval hosts of varying ages (Lauthier 1985). In limb regeneration in *Xenopus*, the induction of an AEC seems also to be triggered by an endogenously produced signal. Poležajew (1946) amputated limbs of young tadpoles of *Rana temporaria* still capable of regeneration. When the tadpoles had reached a stage where they normally do not regenerate anymore, the regenerated limbs that were delayed developmentally compared with the contralateral limbs (and still at a stage where limb buds can normally regenerate) were again amputated. The contralateral limbs were simultaneously amputated. The limbs that were amputated for the second time regenerated, but the limbs that were amputated for the first time failed to do so. Poležajew (1946) attributed the successful regeneration to the relative younger developmental stage of the regenerated limb buds. If external signals are involved in the initiation of the regeneration, no difference between the once and twice amputated limbs would be expected. It seems therefore likely that initiation of the AEC and of regeneration is in response to an endogenously produced signal.

Among the tetrapods studied there appears to be a good correspondence between the timing at which limb mesoderm loses its capacity to induce an AER/AEC in ectoderm and the timing at which the limb (bud) loses its capacity to regenerate. Fgfs appear to play a key role in the initiation of the AER/AEC in both normal limb development and limb regeneration (Yokoyama et al. 2001a, 2001b). Yokoyama et al. (2001a) found in *Xenopus* of stage 51 (Nieuwkoop and Faber 1956), where limb buds can regenerate, limb mesoderm is capable of inducing an AEC with characteristic gene expression patterns in ectoderm, even in ectoderm of older tadpoles that have lost regenerative capacity. At stage 56 when limb regeneration capacity has virtually completely been lost, limb mesoderm is not capable of inducing an AEC with *Fgf8* and *Fgf10* expression in the ectoderm, also not in stage 51 ectoderm. In chickens limb mesoderm loses the capacity to induce an AER in flank ectoderm at HH stage 17 (Kieny 1968; Dhouailly and Kieny 1972; Saunders and Reuss 1974), whereas the capacity to regenerate the limb bud is lost somewhere between stages 15 and 18 (Cobb and Stephens 1999) and the capacity to regenerate the AER is lost at stage 17 or early 18 (Kieny 1968; Saunders and Reuss 1974). In uro-

deles, limb mesoderm continues to be able to induce an AEC after amputation throughout life.

The key importance of Fgfs in initiating regeneration follows from several experiments in which regenerative capacity was partially or almost completely restored in nonregenerative limb buds. In *Xenopus* tadpoles of stage 56, the addition of *Fgf10* to amputated stumps is able to almost completely restore regenerative capacity in older tadpoles of *Xenopus* (Yokoyama et al. 2001a). Furthermore, *Fgf2* and *Fgf4* were found to induce partial regeneration of chick limb buds of HH stages 25 and 26 (Taylor et al 1994; Kostakopoulou et al. 1996). Finally, in the opossum *Didelphys virginiana* Mizell (1968) succeeded in inducing regeneration of the complete foot of newborns by adding nervous tissue from forebrain of young *Rana pipiens* tadpoles. The limbs already possessed all skeletal elements in cartilaginous condition, including all phalanges. Mizell's (1968) study represents regeneration at the most advanced stage of limb development ever reported in an amniote. Apparently embryonic neural tissues produce Fgfs, at least in the chicken (Kalcheim and Neufeld 1990), and neural implants in regeneration are therefore hypothesized to serve as a source of Fgf, similar to Fgf-soaked beads (Taylor et al. 1994). Possibly the successful regeneration at a very advanced stage of limb development implies that more essential signals were provided by the neural implant.

We propose that the induction of an AER in an amputated amniote limb is not possible because during normal limb development this signaling (including *Fgf10*) is received from transiently present structures medial to the lateral plate mesoderm (such as the somites and intermediate mesoderm) (Ohuchi et al. 1999; Kawakami et al. 2001). In amphibians the necessary signaling for AEC initiation (including *Fgf10*) may well be endogenously produced in the limb bud as seems to also be the case for the reinitiation of an AEC in limb regeneration.

Our hypothesis does not explain the loss of the capacity of limb bud mesoderm of older anuran tadpoles to initiate an AEC in ectoderm, but it should be noted that unlike the experiments that show recovery of regeneration limb buds of chickens, the experiments of Poležajew (1946) and Rose (1944, 1945) showed recovery of regeneration without the addition of beads or cells expressing Fgfs or other genes. The pathway for initiation of an AEC continues to be present within the limb but is normally inhibited.

A further candidate for signaling interactions that cannot be repeated later may represent the external signals that influence the early differentiation of muscles in the limb bud. In addition, it appears that for the formation of marrow cavities in bones external signaling may be necessary (Fell and Mellanby 1952).

Further comparative testing

More comparative testing of the hypothesis is possible on limbs and, importantly, also by comparing the self-organizational and

regenerative capacity of other structures that can be regenerated in vertebrates and invertebrates. The high regeneration capacity of the amphibians within vertebrates suggests a high modularity of its development. In this respect it is of interest that in *Triturus viridescens* regeneration of up to half the myocardium (muscular part of the heart) is possible (Becker et al. 1974). This is in agreement with our hypothesis, as the heart anlage has been shown to have a high self-organizing capacity in other urodele species after inductive interactions with the pharyngeal endoderm during neurula stages (Jacobson and Sater 1988). Furthermore, it is of interest that the lens can be regenerated in some urodeles, whereas the lens is also known to have a self-organizing capacity in the sense that no interactions are required with the retina (Jacobson and Sater 1988). The ability of the lens to form in the absence of the retina has been found in urodele and anuran species. These species are also able to regenerate their lens (e.g., *Triturus pyrrhogaster*, *Taricha torosa*; Reyer 1962). A possible problem is *Triturus cristatus*, which is able to regenerate the lens (Reyer 1962) but, according to Popov et al. (1937), is not able to form the lens in the absence of the retina. However, Popov did not find the self-organizing capacity of the lens in any of his experiments on amphibian species (*Pelobates fuscus*, *Bufo viridis*, *Bombina bombina*, *Triturus cristatus*), whereas other researchers did find positive results for closely related species of three of these genera (*Bombina pachypus*, *Bufo bufo*, *Triturus pyrrhogaster*, and *T. alpestris*; Jacobson and Sater 1988). Popov may have used conditions unfavorable to regeneration, and the question of lens regeneration should be reexamined in *T. cristatus*. Self-organizing capacity appears to be temperature dependent and possibly dependent on other conditions (Jacobson and Sater 1988; Hall 1999). Importantly, the distribution of the self-organizing capacity indicates that its presence is a plesiomorphic characteristic in amphibians (Hall 1999). Finally, in mice and chickens there is also no requirement of interactions with the retina, albeit at a considerably later developmental stage than was found for amphibians (Karkinen-Jääskeläinen 1978). Indeed, when the lens of a chicken is damaged at an early stage, regeneration can be successful, albeit not when the entire lens is destroyed (Barfurth and Dragendorff 1902).

Bely and Wray (2001) concluded that embryonic processes are redeployed during regeneration and fission in annelids. The redeployment of developmental processes during regeneration may well be a general phenomenon, fulfilling one of the assumptions of our hypothesis. Regeneration and self-organizing capacity are high in a large number of invertebrate taxa. Invertebrate taxa thus provide an ideal testing ground to see whether our hypothesis can be applied to epimorphic regeneration in general.

We conclude that, as proposed in our hypothesis, the high capacity for self-organization of normal limb development and the absence of interactions with transient structures in

amphibians allows a recapitulation of the developmental program after the time of original limb development that results in regeneration (after initial wound healing and dedifferentiation). Furthermore, the presence of self-organization and the absence of interactions with transient structures may be a condition for regeneration in general.

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