VERTEBRATE LIMB REGENERATION

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Preface

When I first became interested in this subject about 10 years ago, I badly needed guidance from a book such as this of what was known about limb regeneration and what remained to be discovered. Picking out discrepancies between the most recent reviews allowed me to identify some areas of uncertainty, but it proved much more difficult to distinguish the reliably established aspects of limb regeneration from those which depended more on tradition than evidence. The distinction seemed to be missed, commonly by oversight but also sometimes deliberately obscured in an attempt to avoid controversy, even to the extent of ignoring evidence which conflicted with the accepted tradition. It is this conservative tradition which is mainly reflected in more general surveys of regeneration and which is summarized in the better textbooks of embryology and developmental biology, gaining strength from mere repetition. Consulting the original literature, especially where my own research drove me to do so, gradually convinced me that this traditional dogma contained several gaps and inconsistencies.

So this monograph was written with a certain degree of missionary zeal. It should provide the casual reader with enough detail to appreciate the point of most relevant investigations. It summarizes and compares the results of these investigations in a manner calculated either to clarify and reinforce the traditional view or to expose deficiencies in our knowledge and interpretation of the subject. In particular, it discriminates between alternative interpretations by attempting to justify or refute them. I have not dealt thoroughly with the older histological descriptions of limb regeneration nor with the modern histochemical ones, preferring to concentrate on a few examples which provided most insight into the subject. Apart from that area and a few inevitable oversights, this monograph assesses the merits of all investigations published during the last 50 years and incorporates all the useful information I could extract from the older literature.

Challenging a tradition has inevitably involved explicit criticism of the conclusions reached by several investigators. I trust they will take such criticism in good part. Whether they accept it or not, it should be less

infuriating than an account which blandly ignored their efforts. Many errors and some tactless comments have already been pruned from the text on the advice of several colleagues. I am particularly grateful to Malcolm Maden for helping this book through a long gestation, and to Peggy Egar for acting as an emergency midwife. I shall try to blame them for any fault which has escaped their scrutiny, but cannot pretend they entirely share the opinions expressed here. I do not expect the reader to accept all my opinions either, but I should be disappointed if this book does not provoke some attempts to settle the outstanding issues.

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Chapter 1

Descriptive

Regeneration is the formation of a missing structure by the rest of an organism. Like any other physiological process, it can be studied for its own intrinsic interest and possible medical applications. The replacement of what has been lost often occurs by a process which at least superficially resembles the initial development of the same structure. Consequently, one of the main motivations for investigating regeneration has been the hope of gaining some insight into developmental mechanisms. Amphibian limb regeneration is well suited to this purpose, being a rather precise process which usually ensures that the original and regenerated portions are integrated into an anatomically and functionally normal unit. Sometimes, however, the regenerate shows minor imperfections, most often by a reduction in size or an abnormal number of digits. In extreme cases, particularly after experimental intervention, there only occurs an outgrowth without an identifiable hand or foot. Whether or not such growths are termed regenerates has depended on the discretion of the investigator, a discretion which has been exercised much too freely. Various descriptions contain terms such as 'initiation of regeneration' or 'regenerative process' when the structure formed could equally well represent an abortive attempt at growing a hand, foot, tail, or even antlers. Unless such growths bear a reasonable resemblance to the missing structure, they cannot fairly be called regenerates even though they may share some of the developmental processes typical of true regeneration.

Virtually perfect regeneration occurs routinely after amputation in any limb region of many larval and adult urodeles (newts and salamanders), and in the tadpole larvae of frogs and toads. Nothing approaching the structure of a normal limb has been found after such amputations in postmetamorphic frogs or toads, despite several claims of regenerative tendencies either in natural conditions or after treatments intended to enhance regeneration.

Any regeneration which began during larval life is inhibited at the onset of metamorphosis in anurans, and is noticeably retarded during and after metamorphosis in urodeles. Amputated limbs or tails of adult *Salamandra*

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regenerate very slowly and imperfectly (Roguski, 1961; de Conninck *et al.*, 1955). Schwidefsky (1935) found most *Triturus alpestris* limbs regenerated abnormally, while Scadding (1977) has listed similar cases and recorded that *Necturus maculosus* is incapable of limb regeneration. Several investigators have also remarked on a seasonal influence, in that regeneration occurs relatively slowly during the winter, even under laboratory conditions, and in complete darkness (Maier and Singer, 1977). Such changes in regenerative ability suggest a hormonal influence which will be considered in a later chapter.

The inhibition or interference associated with metamorphosis has caused most studies of regeneration to be performed either on young urodele larvae, whose rapid regeneration can be completed before metamorphosis, or on adult urodeles. The most consistently popular choices have been adults of the european crested newt, *Triturus cristatus*, and the eastern spotted newt of North America, *Notophthalmus viridescens*. These share the virtues of being fairly common and adaptable to laboratory conditions, and seem remarkably tolerant of crude surgery. Perhaps only such relatively aquatic adult urodeles are capable of consistent limb regeneration. Most studies on larvae have involved various species of American mole salamanders, especially the spotted salamander, *Ambystoma maculatum*. The axolotl, *A. mexicanum*, offers a peculiar advantage in having suppressed metamorphosis to retain its larval form and aquatic habit throughout life. Consequently, axolotls have become world-wide curiosities and are maintained routinely as laboratory stocks. Both young axolotls and adults have served in regeneration studies for more than a century (Philipeaux, 1867). Young specimens will regenerate an arm in about three weeks but adults take considerably longer, resembling other adult urodeles in this respect. Regeneration has only rarely been studied in other urodele larvae, even of those species which are used frequently as adults. Larvae of the common European salamander, *Salamandra salamandra*, have been used occasionally and regenerate well, although adults do not do so consistently. The ribbed newt, *Pleurodeles waltl*, is becoming more popular now that several breeding colonies have been established. Larvae of this species also regenerate rapidly and perfectly, but the intervention of metamorphosis impedes the process and sometimes causes structural defects to appear in the regenerate. The few species mentioned above have been reclassified enough times to give them an identity crisis. To avoid confusion, I have consistently translated the names used in the literature to the currently accepted ones by means of the conversion table shown here (Table 1.1).

Most observations on tadpoles and attempts to induce regeneration after their metamorphosis have been conducted on whatever anuran species happened to be available. Limb stumps of adult clawed toads, *Xenopus laevis*, grow quite striking cartilage spurs or spikes which lack articulations (Beetschen, 1952). The contrast between perfect regeneration of larval legs and the spikes produced at and after metamorphosis in *Xenopus*, demonstrated by Dent (1962), dissuade me from considering the latter as

Table 1.1 Correct names of urodeles, and synonyms found in the literature.

Correct name*	Previous names	Distribution	Vernacular names
1. *Salamandridae*			Newts and salamanders
Triturus cristatus	*Triton, Molge*	Europe, Asia	Crested newt, warty newt
Triturus vulgaris	*T. taeniatus*	Europe, Asia	Common newt, smooth newt
Notophthalmus viridescens	*Triturus, Diemyctylus*	N. America	Red-spotted newt
Cynops pyrrhogaster	*Triturus*	Japan	Fire-bellied newt
Pleurodeles waltl	*P. waltlii, P. watlii*	Spain, Morocco	Ribbed or Iberian newt
Salamandra salamandra	*S. maculosa*	Europe, S.W. Asia	Fire salamander
2. *Ambystomidae*			Mole salamanders
Ambystoma maculatum	*Amblystoma punctatum*	N. America	Spotted salamander
Ambystoma tigrinum	*Amblystoma*	N. America	Tiger salamander, Colorado axolotl invented for persistent larvae
Ambystoma mexicanum	*Siredon pisciformis*	Mexico	Axolotl, or Mexican axolotl to avoid confusion with above

*According to opinions 635, 649 and 662 of the International Committee on Zoological Nomenclature (*Bull. Zool. Nomen.* vols 19–20, 1962–3) and Wake (1976).

genuine regeneration. Although commonly described as regeneration, only very limited growth has been recorded for limb stumps of late metamorphic stages of *Rana* and *Bufo* (e.g. Michael *et al.*, 1969, 1970, 1972) and of juvenile or adult specimens of other anurans (Goode, 1967). Considerable efforts to enhance such growth have not revealed any reliable means of causing frogs or toads to regenerate.

Many lizards regenerate tails after autotomy or amputation, to achieve a structurally abnormal but fairly satisfactory functional replacement. Despite that, lizards seem as incapable of limb regeneration as any other amniote. Perhaps the best authenticated case of perfect structural regeneration on a limited scale occurs in man (Illingworth, 1974). Children up to the age of eleven can regenerate most of the terminal phalanx of a finger, including a finger nail, after minimal treatment. There is surprisingly little evidence on which to decide if other amniotes share this ability, or if it can be enhanced or extended in humans. Newly metamorphosed reed frogs, however, also regenerate a terminal phalanx and digital pad much more successfully than a larger arm segment (Richards *et al.*, 1977). Treatments intended to improve regeneration have proved as unsuccessful with lizards and mammals as they were with frogs. Chapters 2 and 5 provide some detail of these treatments and the whole topic will be resumed in the final chapter. Our understanding of vertebrate limb regeneration is necessarily restricted at present to amphibians, and overwhelmingly obtained from half a dozen urodele species.

1.1 Normal regeneration

The sequence of events following simple amputation is essentially the same, whatever the position of amputation on either the arm or leg of larval or adult urodeles (see Figure 1.1). The same sequence has also been described for the legs of tadpoles provided they were able to complete regeneration before the climax of metamorphosis. The end of the limb stump is rapidly covered by a distal migration of the epidermis. This forms a complete transparent sheet over the wound in less than a day for larvae or within a few days for adults. Very little cell division occurs during the epidermal migration and the thickening of the new apical epidermis is achieved initially by continued distal migration without local mitosis. The mesodermal tissues exposed by amputation are soon protected from external conditions, therefore, either by an initial blood clot or by the new epidermal covering. The amputation has damaged local mesoderm cells, however, and degenerative changes occur there during the next few days. Phagocytes accumulate at the tip of the limb stump to remove cell debris and to attack the extracellular matrix of the skeleton and connective tissue. Some fluid often accumulates at the end of the stump, giving it an oedematous or distended appearance. Erosion of the matrix liberates other mesodermal cells which are quite viable. Several investigators have identified these cells as 'activated', usually by the criterion of their enlarged nuclei and nucleoli, and

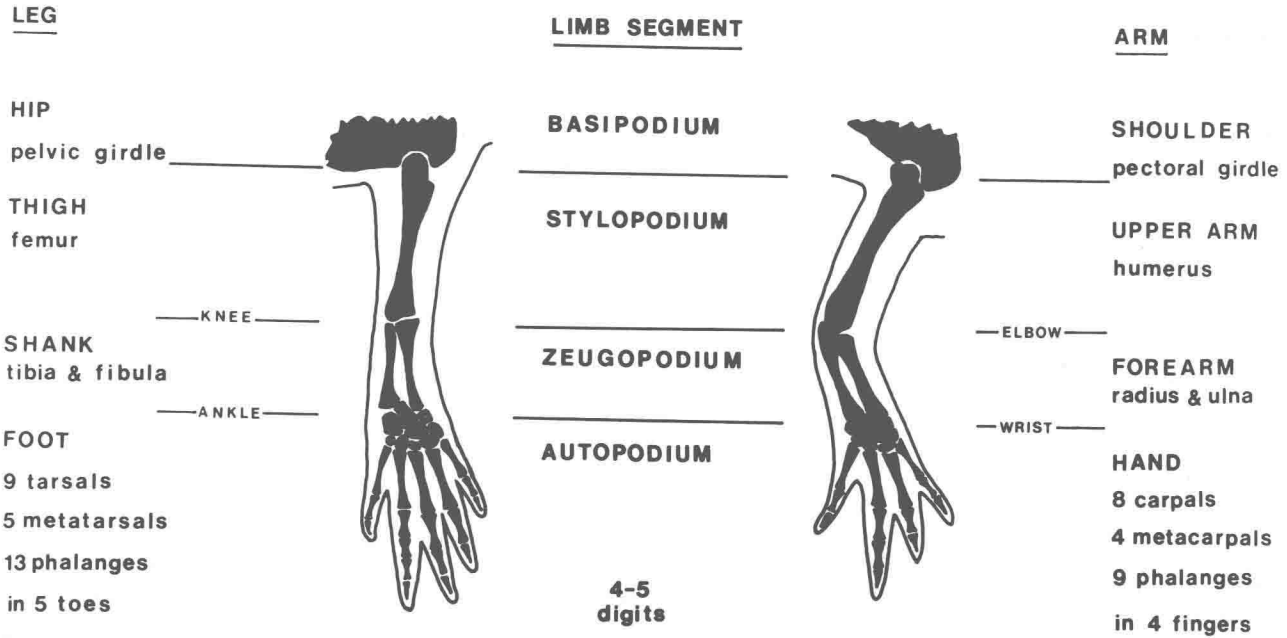

Figure 1.1. Limb skeleton of an axolotl with the conventional terms used to describe different segments and skeletal elements.

defined activation as the first characteristic phase of regeneration. Other investigators see nothing remarkable in activation as much the same process can be recognized in wound healing. As they escape from the stump tissues, these cells quickly lose the histological features which formerly characterized them, and are consequently said to undergo dedifferentiation. The dedifferentiated cells, apparently derived from several or all of the internal tissues close to the site of amputation, accumulate at the apex of the limb-stump under the new epidermal thickening. Their generalized mesenchymatous appearance prevents identification of their former or future tissue type, but collectively they form the initial blastema from which all the internal tissues of the regenerate will be produced. Cell division probably begins even before the cells are liberated from the stump tissues and continues as a massive proliferation, causing the blastema to grow as a colourless protrusion at the tip of the limb. There is apparently some interaction between the blastemal mesenchyme and the overlying apical epidermis, which thickens during this period to form an apical cap without protruding noticeably (Tank *et al.*, 1977). The blastema continues to grow from a slight protrusion to an elongated cone and then becomes flattened or palette-shaped, as the first external indication of the formation of a hand or foot. Blastemata formed after amputation in the upper arm reach this palette stage almost as rapidly as those growing from the fore-arm, and simultaneously develop a bend which marks the position of the elbow. Condensations of blastemal mesenchyme cells occur progressively between the cone and palette stages to produce skeletal cartilage. Additional cartilages are marked out later in the same way as successive digits protrude and elongate from the palette. The form of the regenerate is now essentially complete, while the local differentiation of muscle and the establishment of nervous connections (by axons which extend into the growing blastema) soon render the new limb capable of movement. Growth must continue for considerably longer, however, before the regenerate attains a normal size and becomes a functional replacement.

Several investigators have needed to standardize the rate of regeneration in order to make detailed comparisons during the process. A typical account of such a study was given by Iten and Bryant (1973), who described the changing external appearance and histology of regenerating arms of adult *N. viridescens* at 25°C, and compared their observations to the stages of regeneration distinguished by previous investigators. Analogous staging systems have been prepared for adult axolotls at 21 °C (Tank *et al.*, 1976) and for *A. maculatum* larvae at about the same temperature (Stocum, 1979). These reports have increased the diversity of staging systems to an unreasonable extent. The older descriptive stages of blastema, cone, palette and notch are more universally understood and generally sufficient. I have generally retained these terms, which are illustrated in Figure 1.2 and related to other staging systems in Table 1.2. The rate of regeneration assessed by these recognizable stages is virtually the same whether an arm is amputated

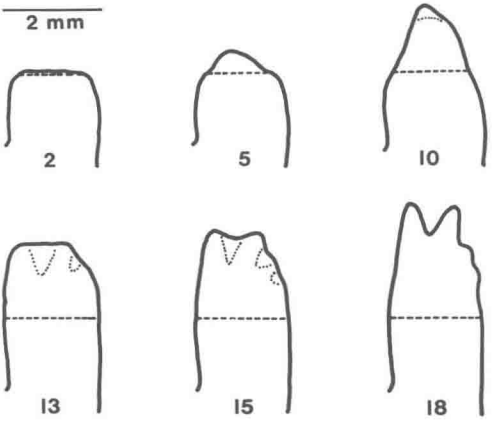

Figure 1.2. Successive stages of regeneration 2–18 days after amputating the arm of a 50 mm axolotl, to show the appearance of a healed stump, early blastema, cone, palette, notch and 2–3 digit regenerate.

through the humerus or through the radius and ulna. In the former case rapid growth continues for longer and thus compensates for the larger missing structure. That observation was first made by Spallanzani (1768) and supported by Iten and Bryant (1973) but may be only approximately true (Smith *et al.*, 1974). Table 1.2 shows that the rate of regeneration is quite sensitive to temperature. Schauble and Nentwig (1974) have investigated the effect of temperature on adult *N. viridescens*, finding a temperature optimum for normal regeneration at 20–25°C and that regeneration virtually ceases at 10°C. We can assume that the rate of limb regeneration shown in Table 1.2 for *N. viridescens* is typical of adult newts, as *T. cristatus* regenerates at a very similar speed (Schwidefsky, 1935; Smith *et al.*, 1974) although *T. vulgaris* and *T. alpestris* tend to regenerate more rapidly and abnormally. Limb regeneration occurs about twice as rapidly in larval urodeles, as shown here for young axolotls and reported in passing for several other species.

Table 1.2 only serves as a rough guide to the rate of regeneration, without predicting accurately the timing in different circumstances. Seasonal changes and conditions of illumination can cause an appreciable difference, as mentioned earlier, and so can experimental conditions. For instance, it is a common experimental practice to compare the regeneration of an operated arm to that of the contralateral 'control' arm. These control arms furnish excellent standards of normal regeneration for most purposes, being subject to the same general conditions as the experimental arms. Tweedle (1971), however, discovered that an arm stump regenerates more slowly if the contralateral arm has been amputated simultaneously. He traced this retardation to a nervous control of regeneration (see Chapter 2), for

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