

# Experiments on developing limb buds of the axolotl *Ambystoma mexicanum*

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## SUMMARY

Various experiments were performed on the limb buds of axolotls to compare the behaviour of amphibian limbs with that previously reported for chick limbs. Following removal of the tip or whole limb bud, extensive powers of regulation were observed since complete limbs always formed. Similarly after distal to proximal grafts intercalary regulation occurred to produce perfect limbs and after proximal to distal grafts serial repetitions resulted. Transplantation and rotation of limb buds to reverse either the dorso-ventral, antero-posterior or both axes resulted in the induction of supernumerary limbs in a large proportion of cases. Such regulatory behaviour of axolotl limb buds is in contrast to the mosaic nature of chick limbs and as a result, theories such as the progress-zone theory which have been formulated on the basis of data from chick limbs are not relevant to general principles of vertebrate limb development. Possible reasons for the diverse behaviour between the two systems are discussed.

## INTRODUCTION

Initial investigations into the mechanisms of limb development were performed in the early part of this century mostly using various species of Urodeles. These studies were primarily concerned with the organization of the limb axes and revealed that the antero-posterior, dorso-ventral and proximo-distal axes of the limb bud were independently specified at different times in development (Harrison, 1921; Swett, 1927). Other work investigated the effects of deletion of part or all of the limb rudiment (Harrison, 1918) as a result of which the term 'equipotential self-differentiating system' was coined to reflect the conclusion that the developing Urodele limb was capable of extensive regulation.

Despite a recent revival of interest in Urodeles (Slack, 1977*a, b*; 1980), the contemporary archetype for the study of vertebrate limb development has become the chick. However, the chick limb bud seems to be relatively mosaic in its development and thus exhibits significant differences in behaviour from Urodeles. Concerning the transverse axes, for instance, the classical method of demonstrating the existence of axial specification is to transplant limbs so as to reverse either the dorso-ventral, antero-posterior, or both axes. This operation

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results in the induction of supernumerary limbs at particular positions and of characteristic handedness in regenerating Urodele limbs (Bryant & Iten, 1976; Tank, 1978; Maden & Turner, 1978; Wallace, 1978). The same is almost certainly true of developing Urodele limbs (Harrison, 1921; Swett, 1927; Slack, 1977*a, b*) and the work described here is intended to confirm and extend this conclusion. Yet in the chick limb only opposition of the antero-posterior axis results in the production of supernumerary limbs, opposition of the dorso-ventral axis apparently does not (Saunders, Gasseling & Gfeller, 1958). Consequently much greater attention has been focused upon the organization of the antero-posterior axis and its organizer, the zone of polarizing activity.

Investigations into proximo-distal organization have involved grafting distal tissue onto proximal levels so that part of the presumptive pattern is missing. Although this particular experiment has not, until now, been performed on the limb buds of Urodeles, during regeneration, perfect limbs nevertheless result from such operations following intercalary regeneration (Stocum, 1975; Iten & Bryant, 1975; Maden, 1980*a*). There seems to be no reason, therefore, why intercalation of missing segments should not also occur during limb development. However, the grafting of a distal tip to a proximal stump in the chick limb bud does not result in the intercalation of missing tissue and segmentally deficient limbs result (Summerbell & Lewis, 1975; Kieny & Pautou, 1977) again revealing the relatively mosaic nature of this system

Because of the differences of behaviour outlined above we must be cautious of extrapolating from chick limb data in formulating general theories of vertebrate limb development (Summerbell, Lewis & Wolpert, 1973; Tickle, Summerbell & Wolpert, 1975). In order to clarify how relevant such theories are to other vertebrates, the work reported here involves an extensive series of operations performed on the developing limb of the axolotl, a Urodele.

#### MATERIALS AND METHODS

The experiments were performed on the forelimbs of recently hatched larvae of *Ambystoma mexicanum*, 21–24 days from fertilization. They were kept at 18 °C on a diet of brine shrimps. Limb development in axolotls has not been precisely staged and is retarded compared to other *Ambystoma* species. The limb bud appears as a protrusion around day 16 of development and takes about 12 days to reach the 4-digit stage at this temperature. To clarify the stages at which these operations were done Fig. 1 shows camera lucida drawings and a section through the forelimb bud of a 21-day larva. The levels at which the bud was cut in various series are shown. In order to have a variety of stages available for experiments on any one day some larvae were allowed to develop at the lower temperature of 12 °C.

The larvae were anaesthetized in 1:3000 MS222 and the operations performed in air. The limb buds were severed with tungsten needles or fine scissors

and, for transplantation, placed on the relevant stump. The animals were placed at 4 °C for about 5 min to facilitate sticking of the graft (usually a clot of blood served as glue) and then returned to water. Those larvae whose grafts did not survive were discarded during frequent post-operative observations. After 5 weeks the limbs were fixed and stained with Victoria blue to reveal the skeletal structure.

## RESULTS

### *The proximo-distal axis*

#### *Series I. Tip removal and whole limb-bud removal*

The conical tip was removed (at level A in Fig. 1*a*) from 20 limb buds and the whole limb bud severed at the flank (at level B in Fig. 1*a*) in 14 cases. All 34 limbs were perfectly normal when stained 5 weeks later (Table 1). They were, of course, slightly retarded compared to the controls due to the delay in restoring the lost tissue. Clearly, then, the axolotl limb bud can fully regulate following removal of either a small or a large proportion of the presumptive elements. Similar behaviour has been shown for the earlier limb primordium stages (Harrison, 1918; Slack, 1980).

#### *Series II. Distal tip to proximal stump*

The distal tips of later-stage limb buds (Fig. 1*c*) were grafted to the proximal stumps of younger buds (at level B in Fig. 1*a*). It is not certain that the grafted distal tips contained only presumptive wrist and digit tissue as was intended, since the fate map of the axolotl limb bud has not been described. Nevertheless, a thick slice of tissue was removed by this procedure and if the limb behaved in a mosaic fashion intercalary defects would be expected. In contrast, however, all 13 cases produced perfectly normal limbs (Table 1, Fig. 2). There was no mismatching in the final form of these limbs nor any remaining signs of the level at which the graft was performed, demonstrating that the axolotl limb bud is capable of extensive and perfect intercalary regulation.

#### *Series III. Proximal limb bud to distal tip*

In six cases a limb bud was severed at a proximal level (level B in Fig. 1*a*) and grafted to the distal tip of a later-stage bud (Fig. 1*c*) – the reciprocal experiment to Series II. The resulting limbs all showed various degrees of tandem duplications depending on the precise level of the proximal cut. The most striking cases (Fig. 3) were those in which the whole limb was repeated. In no case was there any interaction between the distal level of the stump (at the carpals in all cases) and the proximal boundary of the graft as has been observed in limb regeneration (Maden, 1980*a*). The possible reason for this difference is considered in the Discussion.

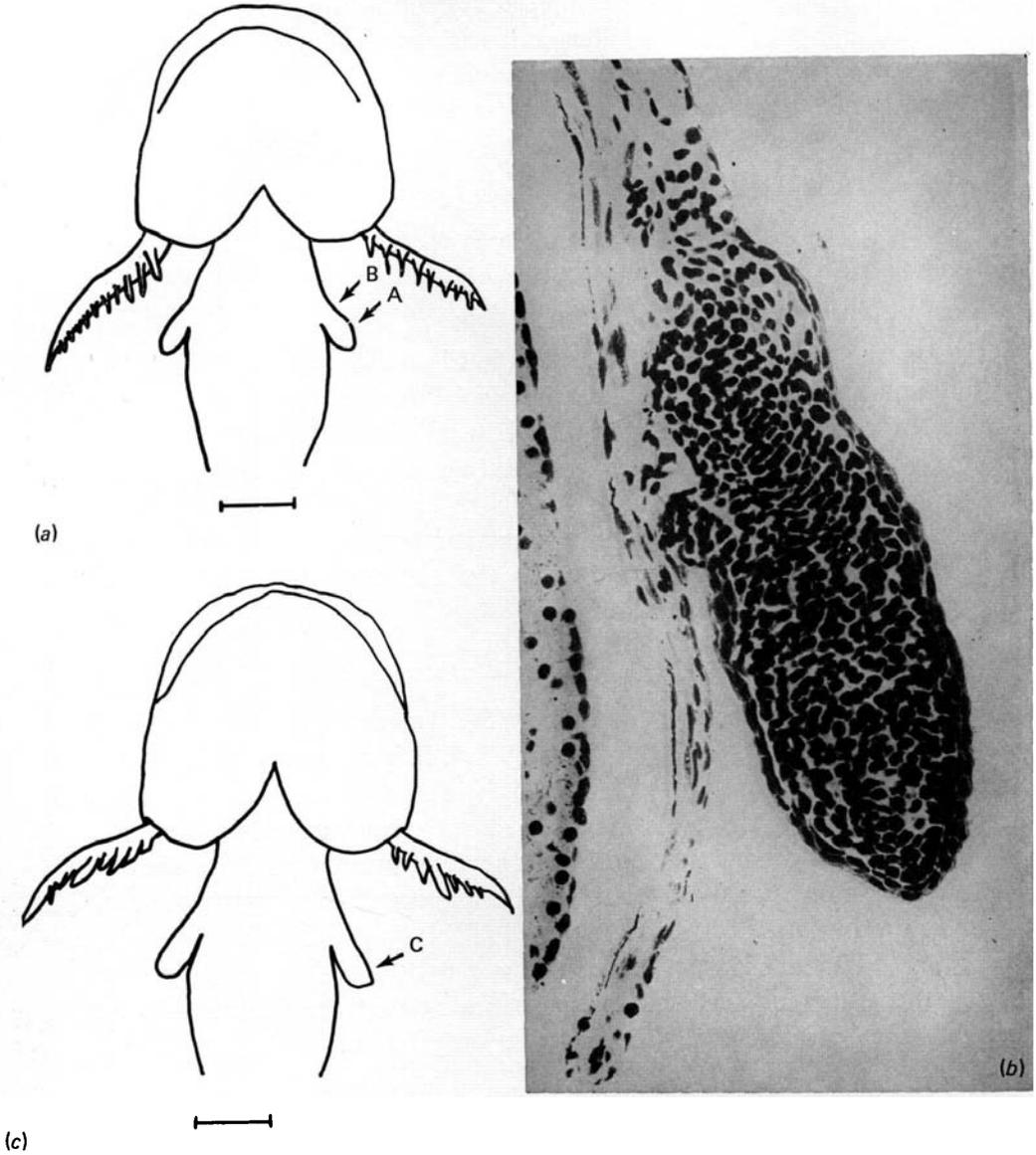


Fig. 1. (a) Camera lucida drawing of the limb buds (ventral view) of a 21-day axolotl larva kept at 18 °C. This is the stage at which the majority of the experiments were performed – Series I, II hosts, III donors, IV, V, VI and VII. A (tip) and B (whole bud) mark the levels at which the buds were cut in various series (see text for details) Bar = 1 mm. (b) A section through the limb bud at the same stage as in (a). Note that no histological differentiation has yet occurred and that there is no apical ectodermal ridge in Urodeles. Mag  $\times 100$ . (c) Camera-lucida drawing of the limb buds (ventral view) of a 24-day axolotl larva kept at 18 °C. This is the stage which provided Series-II donors and Series-III hosts, by cutting at level C. Same scale as (a).

Table 1. *Summary of results of the operations performed on the proximo-distal axis of the axolotl limb bud*

Series	Operation	Number scored	Result
I	Tip removal	20	100% normal limbs
I	Whole bud removal	14	100% normal limbs
II	Dist → prox	13	100% normal limbs
III	Prox → dist	6	Tandem duplications

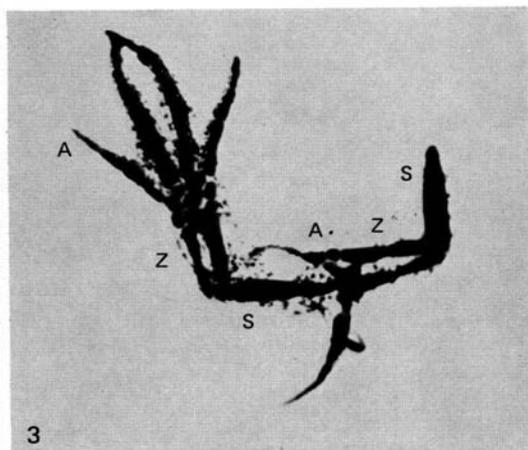
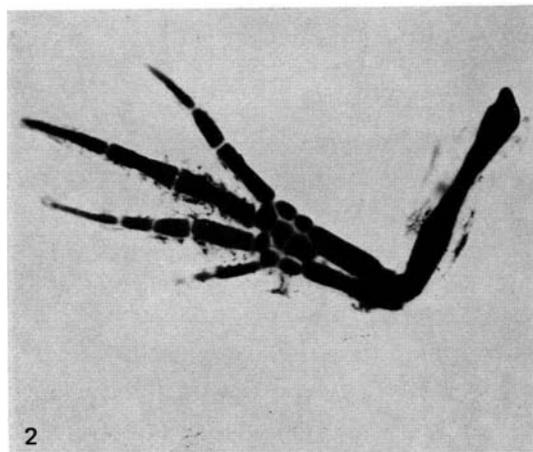


Fig. 2. Series II – the result of grafting a distal tip onto a proximal stump. In all cases the missing part of the presumptive pattern was replaced by intercalation to produce perfect limbs.

Fig. 3. Series III – the result of grafting a whole limb bud onto a distal tip. Two limbs in tandem are produced. S, Stylopodium; Z, zeugopodium; A, autopodium.

### *The transverse axes*

#### *Series IV. Control tip replacement*

The tips of 12 limbs were severed (at level A in Fig. 1a) and then replaced on the same stump without any angular displacement. In 11 cases completely normal limbs developed. The remaining limb had its first digit bifurcated after the metacarpal. Clearly the trauma associated with tip transplantation does not cause great deviations from the normal form of the limb.

#### *Series V. Dorso-ventral opposition*

The tips of limb buds were exchanged between left and right sides and then rotated 180° so that the dorso-ventral axis was inverted, but the antero-posterior axis remained as normal. Forty per cent of both right and left limbs produced supernumerary elements (Table 2). These supernumeraries arose at the carpal

Table 2. *Summary of results of the operations performed on the transverse axes of axolotl limb buds*

Series	Operation	Limb	Number scored	Number producing super-numeraries	%
V	Control		12	0	0
V	D/V opposition	Left	20	8	40
		Right	17	7	40
VI	A/P opposition	Left	15	11	73
		Right	18	12	67
VII	180° rotation	Left	16	6	38
		Right	16	6	38

level (Fig. 4), a consistent observation throughout the rotation series. Of the 15 supernumeraries recorded in Table 2, 6 were double (40%) and 9 single. They were generally not well formed, only five being 4-digit hands with the remainder varying in quality from 2 digits upwards. The extra elements were usually located at the dorsal or ventral poles of the host limb, but three were displaced into the antero-dorsal or antero-ventral quadrant. All supernumeraries whose orientation could be ascertained were of stump-handedness (e.g. Fig. 4).

Despite discarding those animals whose grafts had not healed properly, some of the remainder looked normal rather than being inverted at their distal end as would be expected of a dorso-ventrally inverted graft. The reason for this is not clear: respecification of the graft is unlikely as the operations were performed long after the laying down of limb axes (Harrison, 1921; Swett, 1927). Perhaps the graft was simply repopulated by stump cells. If these limbs are excluded from the analysis the frequency of induction of supernumeraries becomes 60%.

#### *Series VI. Antero-posterior opposition*

In this series, tips were exchanged between left and right limbs and not rotated, thereby opposing only the antero-posterior axis. Seventy per cent of these limbs produced supernumerary elements (Table 2), but if those limbs that looked normal are excluded the frequency becomes nearer 100%. As in the previous series the supernumeraries arose at the carpal level except one limb which had a duplicated radius and ulna (Fig. 5). Of the 23 supernumeraries recorded in Table 2, 8 were double (35%) and 15 single. Again as in Series V, they were not well formed, only 7 of the 23 being 4-digit hands. All supernumeraries which could be characterized were found to be of stump-handedness (e.g. Fig. 5).

The location of these extra elements was not as clear as in Series V: the majority were either at the anterior or posterior poles of the host limb but some had extra digits protruding in the dorso-ventral plane which resembled the

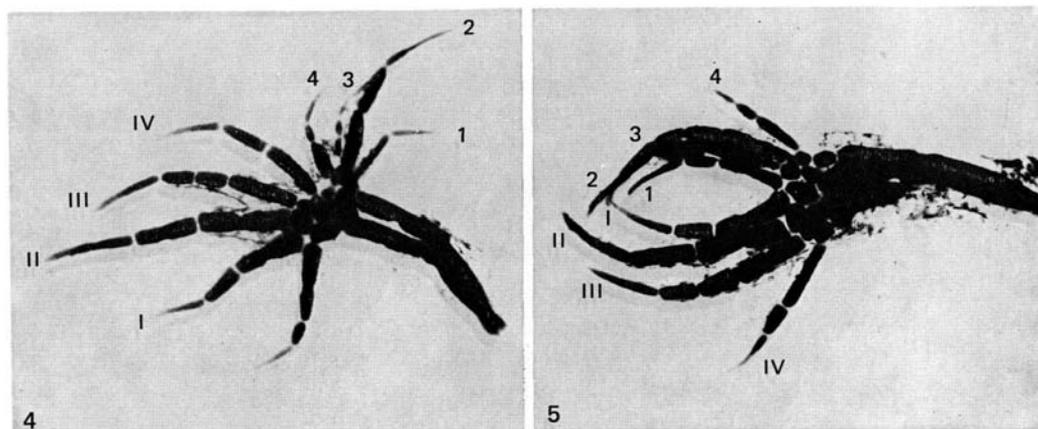


Fig. 4. Series V – the result of grafting a left limb bud on to a right stump so that the dorso-ventral axis is inverted. I-IV are the digits of the left graft which bend upwards. 1-4 are the digits of the supernumerary limb which curve downwards with digit 4 being the nearest in the picture. The two limbs are arranged on top of each other. The supernumerary is thus a right limb which appeared at the ventral pole of the host.

Fig. 5. Series VI – the result of grafting a left limb-bud tip on to a right stump so that the antero-posterior axis is reversed. I-IV are the digits of the left graft which bend downwards. 1-4 are the digits of the supernumerary and since they bend downwards this is a right limb. The two limbs are arranged side by side and the bifurcation occurred in the zeugopodium. The supernumerary appeared at the posterior pole of the host.

supernumeraries produced in the previous series. It is possible that these were caused by derotation of the tip to align the antero-posterior axis, thereby resulting in opposition of the dorso-ventral axis since this phenomenon has been suggested to occur in regeneration blastemas (Wallace & Watson, 1979).

#### *Series VII. Dorso-ventral and antero-posterior opposition*

In this series tips were rotated 180° and replaced on the same stump to oppose both transverse axes. Of these limbs 38% produced supernumerary elements (Table 2) but the remainder looked normal so derotation of the tip probably prevented the frequency from reaching 100%. Bifurcations again arose at the carpal level. Six of the 12 supernumeraries recorded in Table 2 were well formed and these all appeared in the antero-ventral quadrant of the host limb. A total of two-thirds were generated in this position, the remainder being distributed elsewhere. However, none of these supernumeraries were distinctive enough to be able to determine their handedness because the digits did not consistently curve one way or the other. It is thus possible that these are not normal limbs as is the case when supernumerary limbs are generated after 180° rotation of regeneration blastemas (Maden, 1980*b*).

In each of these rotation series the quality of the supernumeraries was poor

and they arose at a very distal level. This is surely due to the fact that the tip was rotated rather than any inability on the part of the axolotl limb bud to generate good supernumeraries since rotating whole limb buds of other amphibians produces excellent limbs which bifurcate in the stylopodium (Maden, 1980c).

#### DISCUSSION

##### *The proximo-distal axis*

Like *Ambystoma punctatum* (Harrison, 1918), the axolotl limb bud is capable of extensive regulation since removal of either the tip or most of the limb-bud results in the development of a complete limb (see also Slack, 1980). This behaviour contrasts markedly with that of the chick limb bud where the same operation results in truncation of the limb and loss of distal parts (Hampé, 1959; Summerbell, pers. comm.). This distinction between axolotl and chick limb buds is also apparent from the results of transplanting a distal tip to a proximal stump. It was revealed here that the axolotl again produces a perfectly normal limb having undergone intercalary regulation, whereas the chick limb is incapable of such behaviour and intercalary deletions result (Summerbell & Lewis, 1975). It is claimed that some intercalary regulation can occur in chicks (Kieny & Pautou, 1977), but the resulting limbs are still grossly deficient compared to the perfect regulation shown here by axolotl limb buds.

On the other hand, when a proximal bud is grafted on to a distal stump the limbs of both axolotls and chicks (Summerbell & Lewis, 1975) exhibit serial repetitions, often two limbs in tandem (Fig. 3). Not all operations resulted in such extreme examples, a spectrum of degree of repetition was produced. This phenomenon can also be seen in chick limbs (Kieny, 1977) but it does not necessarily mean that regulation for excess has occurred. Rather, it could be due to ignorance of the true tissue level at which the limb buds were cut or simply due to loss of grafted tissue at the graft: host junction. Whichever the case may be, axolotl limb buds cannot consistently regulate for excess as they can for defects. Nor can they intercalate missing tissue at the discontinuity to give a continuous field as has been observed in limb regeneration (Maden, 1980a). The period over which such interactions can occur during regeneration is much longer than during development and it may be the speed of development which prevents similar intercalation between the proximal graft and distal stump from taking place.

It is clear therefore that any theory based upon the mosaic nature of the proximo-distal axis of the chick limb, such as the progress zone theory (Summerbell, Lewis & Wolpert, 1973) cannot explain the data presented here and is therefore of doubtful relevance to the general principles of vertebrate limb development. The progress zone is considered to be a region at the distal tip of the limb in which cells become more distal with time. Once they have left this zone they are incapable of further change, thus intercalary regulation

and continued development after tip removal are not permitted in this scheme. However, it may be that the mosaic nature of the chick limb is an illusion created by performing the experiments after the stage of determination (Summerbell, 1977). If they were repeated on earlier stages it is possible that regulative powers equal to those observed here in the axolotl limb would be revealed. Nevertheless, it is perhaps pertinent to make the general point that one must be wary of formulating theories upon the absence of a certain behaviour during development.

*The transverse axes*

After opposing either one or both of the transverse axes, the axolotl limb bud behaved in a similar fashion to the regeneration blastema (Tank, 1978; Maden & Turner, 1978). The chick limb does produce supernumeraries after antero-posterior or 180° rotation but not after dorso-ventral opposition (Saunders *et al.* 1958). More surprising is the report that dorso-ventral opposition in *Xenopus* limb buds does not cause the formation of supernumerary limbs (Cameron & Fallon, 1977). However, we have repeated this experiment and obtained results to the contrary confirming that Urodeles and Anurans behave similarly in this regard (Maden, 1980*c*). It may be that the absence of a dorso-ventral interaction in chick limbs is not a result of a fundamental difference in the organization of the mesoderm, but simply due to the absence of an apical ectodermal ridge (AER) in the appropriate position. Since outgrowth of the limb bud depends upon a continuous ectodermal-mesodermal interaction, the absence of an AER at the dorsal and ventral poles of the graft:host junction after rotation of the tip might prevent the manifestation of supernumerary limbs. If so, then the differences between chick and Urodele limbs could be due to a defect in the ectoderm. The same argument could be applied to the results of tip removal in chick limbs – the AER will not regenerate and therefore regulation cannot occur. However, even if the AER could regenerate the progress zone theory would predict proximal deficiencies rather than perfect limbs. In any case this cannot explain the differences in behaviour after distal to proximal shifts, so it seems that there is indeed a deficiency in the organization of the chick limb mesoderm. Further experimentation such as providing an additional AER after dorso-ventral inversion of the tip, could clarify these points.

The results presented here are perfectly in keeping with there being an antero-posterior polarizing region in Urodele limbs (Slack, 1977*b*) that acts in the same fashion as that described for chick limbs (Tickle *et al.* 1975), but we should surely now be aware of the possible existence of a dorsal (or ventral) polarizing region (Slack, 1980) if indeed this is the appropriate way of thinking about the nature of these axes. An alternative description of the circumferential limb field, which radically departs from the classical concept of two independent axes, is the polar-coordinate model of French, Bryant & Bryant (1976). However, this is not easily reconciled with the early work of Harrison (1921) and

Swett (1927), which revealed that the antero-posterior and the dorso-ventral axes were specified at different times in development. In limb regeneration too, the original form of the polar coordinate model is incapable of explaining the regeneration of mirror-image limbs (Slack & Savage, 1978; Stocum, 1978), the production of supernumerary limbs following blastemal rotations less than 180° (Wallace, 1978; Maden & Turner, 1978) and the unique structure of 180° supernumeraries (Maden, 1980*b*). It thus seems that a model of the limb field is now required which combines the unitary features of the polar coordinate description with the apparent axial decomposability required by experimental observations.

Finally it is worth emphasizing the similar behaviour of developing and regenerating axolotl limbs. They can both regulate following loss of part of the field, for intercalary defects (distal to proximal grafts), and both produce supernumerary limbs after antero-posterior, dorso-ventral or opposition of both axes. The interesting question which then presents itself is – is the behaviour of axolotl limb buds unique to an organism which is capable of limb regeneration? The answer is also relevant to the problem of whether the results presented here are examples of true regulation of the limb bud or regeneration. We have suggested above that the differences between axolotl and chick limb buds could be due to operations on the latter being performed on relatively late stages of development. It is, however, equally conceivable that the development of the chick limb is relatively mosaic at all stages because it is a non-regenerating organism. In an attempt to answer this question, similar experiments on Anuran limb buds, whose adult limbs do not regenerate, will subsequently be reported (Maden, 1980*c*).

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