

Comparison of the effects of vitamin A on limb development and regeneration in the axolotl, *Ambystoma mexicanum*

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SUMMARY

The objective of this investigation was to compare the effect of vitamin A on limb development and limb regeneration in the same animal, at the same time, thus eliminating the possibility that species differences or different rates of uptake between animals would influence the results. Axolotl larvae had both right limbs amputated and then were treated with retinol palmitate by immersion at 60 or 300 mg l⁻¹ for 4 or 10 days. Intact left developing limbs at the cone, two-digit, or four-digit stages responded to the treatment by deletion of skeletal elements producing hypomorphic limbs. Severity of the deletions was correlated with higher dose, longer times, and earlier stages of limb development. In contralateral right regenerating limbs, the effect of the same treatment was to cause various degrees of proximodistal duplication as well as occasional hypomorphic regenerates. Thus, there is a marked difference in response to vitamin A between developing and regenerating limbs. The implications of this observation are discussed especially with respect to the underlying morphogenetic mechanisms.

INTRODUCTION

Vitamin A has unique effects on the development and regeneration of limbs of several vertebrates. It is of tremendous interest because of its highly specific effects on pattern formation, first noted by Niazi & Saxena (1978). In regenerating axolotl limbs, treatment by immersion in a suitable dose of vitamin A causes a proximodistal (PD) duplication of limb structures such that a complete limb including girdle and autopodium is regenerated following amputation at a distal level (Maden, 1982). In the chick embryo, localized application of retinoic acid to the anterior edge of the developing limb bud mimics the effect of the zone of polarizing activity and causes an anterior–posterior (AP) duplication of the limb

Key words: vitamin A, retinoids, limb development, limb regeneration, axolotl, *Ambystoma mexicanum*.

such that a second limb develops as an anterior mirror image of the primary limb (Tickle, Alberts, Wolpert & Lee, 1982; Summerbell, 1983). In *Rana temporaria* tadpoles, regenerating hindlimbs can be simultaneously serially duplicated in the PD axis and mirror-image duplicated in the AP axis (Maden, 1983a). Thoms & Stocum (1984) showed that while PD duplications were the most common responses to retinoic acid treatment in the newt, *Notophthalmus viridescens*, partial transverse duplications also occurred although at a low frequency.

Pattern duplication, however, is not the only effect of vitamin A. Treatment of mouse embryos with retinoic acid causes reduction defects in the developing limbs (Kochhar, Penner & Tellone, 1984). Similarly, the effect of retinoic acid on forelimb regeneration in postmetamorphic *Xenopus laevis* is to cause a dose-related inhibition of the regenerative outgrowth (Scadding, 1983). Skeletal deletions or reductions have also been occasionally observed subsequent to some vitamin A treatments in the chick, newt, and axolotl (Maden, 1983b; Summerbell, 1983; Thoms & Stocum, 1984). These deletions and reductions could be due to the known effect of retinoic acid as an inhibitor of the cell cycle (Maden, 1983b). On the other hand, they could also be attributed to an interference of vitamin A with morphogenetic processes.

Why the response of developing and regenerating limbs to vitamin A is so variable is not known. It is puzzling that the same agent should cause both duplication and deletion and in the case of duplications can specifically affect different axes. These differences could be due to differences between species, differences between methods of administration or uptake, or differences between developing and regenerating limbs.

The main objective of this investigation was to compare the effect of vitamin A on both developing and regenerating limbs in the same animal at the same time. Thus, this eliminates the possibility of differences between species or differences in method of administration influencing the response. This can be achieved in the axolotl since one can use one limb to study vitamin A effects on a developing limb while amputating the contralateral limb and using it to observe a regenerating limb simultaneously. The axolotl also allows two different stages of development to be studied simultaneously since the forelimb develops earlier than the hindlimb. One can thus compare the regenerating forelimb to a developing hindlimb which is perhaps at a more comparable stage of development than the contralateral limb. This then avoids one of the problems of previous work in which studies on vitamin A effects on developing limbs were carried out in different species from the studies of effects on regenerating limbs. Using the axolotl, one can investigate vitamin A effects on morphogenesis in both developing and regenerating limbs in the same animal at the same time and at two different stages of development.

Another objective of this study was to investigate the effect of vitamin A on amputated developing limb buds. Thus, hindlimbs were amputated at cone and two-digit stages of embryonic limb development to determine if these regenerating hindlimb buds responded to vitamin A like intact developing limbs or like mature regenerating limbs.

MATERIALS AND METHODS

All experiments were performed on young laboratory-raised axolotl larvae, *Ambystoma mexicanum*, of 2 to 3 cm in total length, maintained during the experiment in groups of six or seven in 1 litre of constantly aerated water in plastic bins and fed chopped *Tubifex* worms daily *ad libitum*. The axolotl larvae were selected for use at two different stages of development: Series I animals were from a single mating and used at the stage where the hindlimb bud was an elongated cone, slightly longer than it was broad, and Series II animals were from a different mating and used when the hindlimb was at the two-digit stage. In both series, the forelimbs were well-developed four-digit limbs at the time of the experiment, with all skeletal elements already present with the occasional exception of the distal phalanges. Any axolotls showing any evidence of previous limb injury or amputation (via cannibalism), e.g. contralateral limbs at different stages, were not used.

The axolotl larvae were anaesthetized in 0.2 g l^{-1} tricaine methane sulphonate (Sigma) neutralized with sodium bicarbonate, and both right limbs were amputated. The forelimb was amputated through the distal radius-ulna while about a half to one third of the hindlimb bud was removed using fine scissors and tungsten needles.

Axolotls were then immediately transferred to a suspension of retinol palmitate (Sigma Type VII – water dispersable) and randomly assigned to a treatment group of six or seven animals according to the following scheme. Series I, composed of groups 1 to 5 with hindlimbs at the cone stage, and Series II composed of groups 6 to 10 with hindlimbs at two-digit stage, were treated with retinol palmitate as follows:

Groups 1, 6: Controls – no retinol palmitate treatment

Groups 2, 7: 60 mg l^{-1} for 4 days

Groups 3, 8: 60 mg l^{-1} for 10 days

Groups 4, 9: 300 mg l^{-1} for 4 days

Groups 5, 10: 300 mg l^{-1} for 10 days

The suspension was changed every 2 or 3 days. The retinol palmitate concentrations and treatment times were known from previous work (Maden, 1983b) to be effective at inducing PD duplications in regenerating limbs of older axolotls. After the 4- or 10-day treatment period, the axolotls were transferred to aerated tap water and subsequently maintained therein for the balance of the 42-day experimental period.

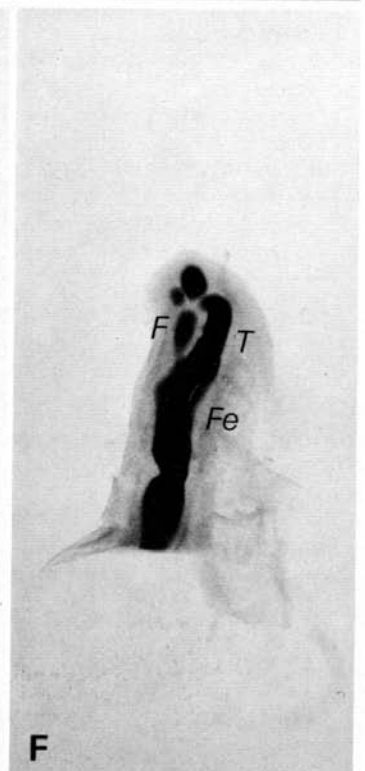
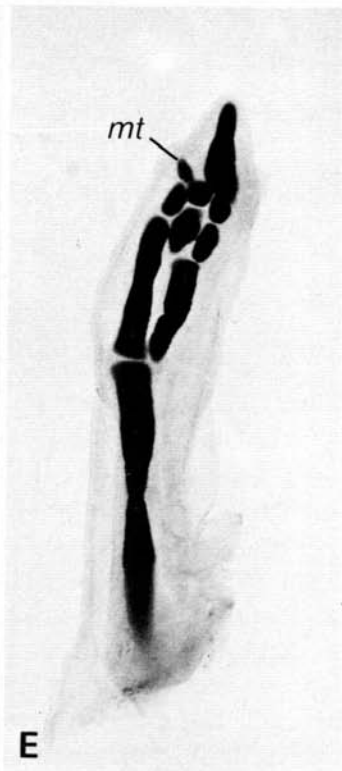
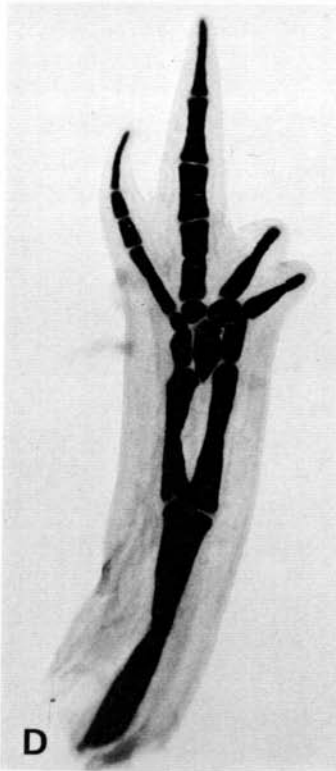
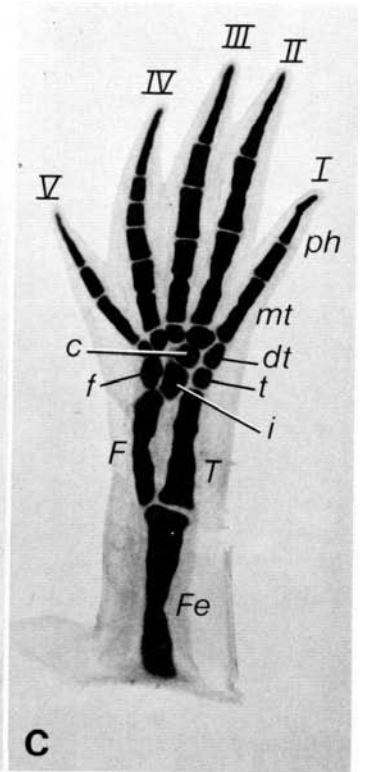
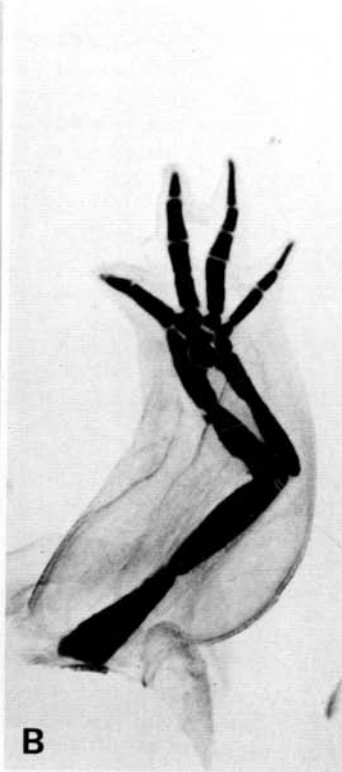
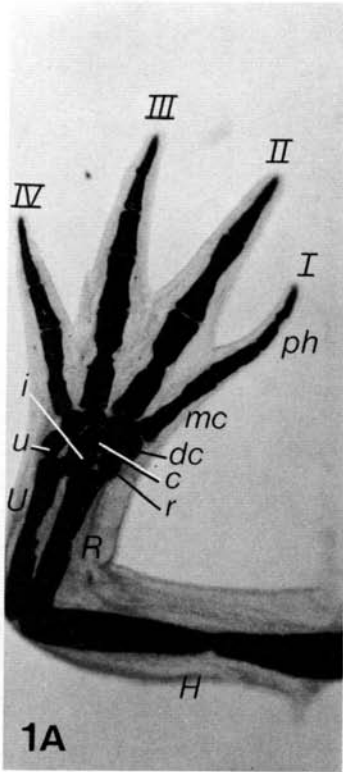
After 42 days, the axolotls were re-anaesthetized and fixed in neutral buffered formalin. The entire animal was then stained with Victoria Blue B and cleared in methyl salicylate for examination of the limb skeleton using a method similar to that of Bryant & Iten (1974).

Of the 68 animals at the start of the experiment four died and were discarded. An additional ten limbs were damaged during the experiment, presumably by the predations of other axolotls. The results were then based on an examination of the remaining 246 limbs.

RESULTS

Normal limb skeleton

It is necessary to assess the variability of the normal limb skeleton as a basis for evaluating the differences observed in regenerating and vitamin A-treated limbs. An untreated intact left forelimb from Group 1 (Fig. 1A) illustrates the basic skeletal pattern of the axolotl forelimb, which consists of a humerus, radius and ulna, eight carpals (radiale, ulnare, intermedium, centrale, and four distal carpals I to IV), four metacarpals, and nine phalanges (arranged on digits I through IV in the pattern 2–2–3–2). This gives a basic set of 21 skeletal elements in the wrist and hand, i.e. eight carpals, four metacarpals, and nine phalanges. However, minor variations from this basic pattern occur frequently. In untreated intact left forelimbs of Groups 1 and 6, the number of carpals varied from five to nine, and the number of phalanges from seven to nine. Thus, although some of these limbs



occasionally had minor skeletal deficiencies, they must be considered as lying in a normal range. Similarly, in untreated regenerated right forelimbs, there was also some variation in the pattern (Fig. 1B). Numbers of carpals varied from five to nine, metacarpals from three to four, phalanges from five to nine. Thus, slightly hypomorphic limbs, even absence of a digit, occurred in the untreated regenerating forelimbs.

Similarly, the untreated intact left hindlimb (Fig. 1C) consisted of a femur, tibia and fibula, nine tarsals (tibiale, fibulare, intermedium, centrale, and distal tarsals I to V), five metatarsals, and 13 phalanges (arranged on digits I to V in the pattern 2-2-3-4-2). This gave a basic set of 27 skeletal elements in the ankle and foot. Both the intact left hindlimbs and regenerated right hindlimbs showed much less variability than the forelimbs with occasionally one extra tarsal or one additional or one less phalange.

Developing limbs

When developing hindlimbs were treated with adequate dose levels of retinol palmitate at the cone stage or two-digit stage, the response was invariably the production of hypomorphic limbs. Table 1 gives the total number of skeletal elements in wrist and hand, or ankle and foot, of the intact left limbs. The marked reduction in number of skeletal elements in the hindlimbs treated with retinol palmitate at either the cone or two-digit stage was obvious. The effect of retinol palmitate on the four-digit forelimbs was significant although much less dramatic, as might be expected since in these cases the development of the limb was closer to completion by the time of treatment.

The developing hindlimbs treated with retinol palmitate at the cone stage exhibited skeletal deletions varying from slightly hypomorphic to complete inhibition of development. Treatment for 4 days at either 60 mg l^{-1} or 300 mg l^{-1} resulted in slightly hypomorphic limbs with 7.5 (mean) tarsals, 4.0 metatarsals, and 7.1 phalanges (Fig. 1D). The 300 mg l^{-1} dose at 4 days of treatment time did

Fig. 1. (A) Group 1, untreated intact left forelimb showing normal skeletal elements: humerus (*H*), radius (*R*), ulna (*U*), radiale (*r*), ulnare (*u*), intermedium (*i*), centrale (*c*), distal carpals (*dc*) I to IV, metacarpals (*mc*) I to IV, and phalanges (*ph*) arranged in 2-2-3-2 pattern on digits I to IV respectively. (B) Group 6, untreated regenerated right forelimb. This limb has only one phalange on digit I and only seven carpals, but is clearly still within the normal range. (C) Group 1, untreated intact left hindlimb showing normal skeletal elements: femur (*Fe*), tibia (*T*), fibula (*F*), tibiale (*t*), fibulare (*f*), intermedium (*i*), centrale (*c*), distal tarsals (*dt*) I to V, metatarsals (*mt*) I to V, and phalanges (*ph*) arranged in 2-2-3-4-2 pattern on digits I to V respectively. (D) Group 4, intact left hindlimb treated with 300 mg l^{-1} retinol palmitate for 4 days, shows only two complete digits, and seven tarsals of uneven size. (E) Group 3, intact left hindlimb treated with 60 mg l^{-1} retinol palmitate for 10 days, shows a digitless limb resulting from deletion of lateral digits and fusion of the central metatarsals into a single triangular cartilage. A vestige of one of the lateral metatarsals (*mt*) is visible. (F) Group 5, intact left hindlimb treated with 300 mg l^{-1} retinol palmitate for 10 days, shows extreme skeletal reduction. Femur (*Fe*) is fused to tibia (*T*) with the fibula (*F*) reduced to a tiny adjacent cartilage nodule. Distal limb skeleton is completely absent except for two small cartilage nodules. Magnification $\times 15$.

Table 1. Total number of skeletal elements in developing hands and feet

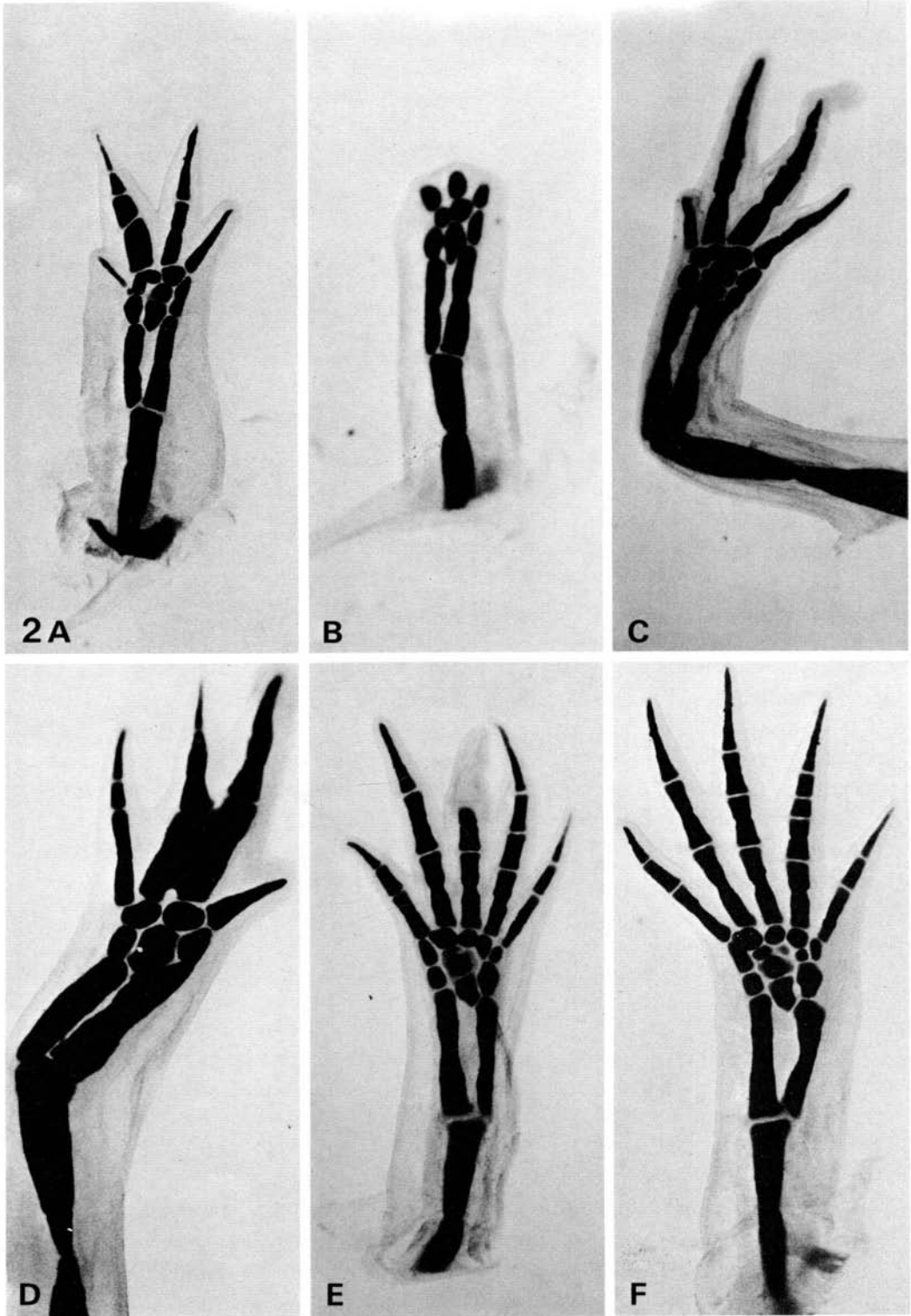
	Untreated controls	Vitamin A treatment			
		60 mg l ⁻¹ 4 days	60 mg l ⁻¹ 10 days	300 mg l ⁻¹ 4 days	300 mg l ⁻¹ 10 days
<i>Series I</i>					
Left forelimb (four-digit stage)	20.7 ± 0.8 (7)	19.2 ± 1.5 (6)	18.4 ± 1.5 (7)	17.8 ± 2.8 (5)	16.2 ± 1.6 (6)
Left hindlimb (cone stage)	27.1 ± 0.4 (7)	17.9 ± 3.9 (7)	7.7 ± 2.6 (7)	19.5 ± 5.1 (6)	5.9 ± 6.2 (7)
<i>Series II</i>					
Left forelimb (four-digit stage)	17.8 ± 1.0 (6)	14.2 ± 1.0 (6)	12.4 ± 0.6 (5)	13.8 ± 1.5 (6)	13.0 ± 1.1 (6)
Left hindlimb (two-digit stage)	27.0 ± 0.0 (6)	10.5 ± 2.6 (6)	8.4 ± 0.6 (5)	10.3 ± 1.5 (6)	9.3 ± 2.7 (6)

Note: The datum in each case is the mean number of skeletal elements in wrist and hand, or ankle and foot, plus or minus the standard deviation, with the number of cases given in brackets.

not increase the degree of skeletal deletions. However, increasing the treatment time to 10 days had a marked effect on the morphology of the developing limb. Those limbs treated for 10 days at 60 mg l⁻¹ had a typical digitless morphology with the distal skeleton usually consisting of five or six tarsals and one triangular metatarsal (Fig. 1E). The femur, tibia and fibula were approximately normal. However, at 300 mg l⁻¹ for 10 days, the effects were even more pronounced. The femur, tibia, and fibula were reduced in size (5/7 cases) or completely absent (2/7 cases) and the distal skeleton was reduced to a very few irregular cartilages or completely absent (Fig. 1F). Thus, at 10 days of treatment, the 300 mg l⁻¹ dose resulted in a much more severe inhibition of limb development than the 60 mg l⁻¹ dose.

The developing hindlimbs treated with retinol palmitate at the two-digit stage exhibited deletion of skeletal elements. Even at 60 mg l⁻¹ for 4 days' treatment, all limbs were hypomorphic, typically with a three-digit hand composed of six tarsals, three metatarsals, and zero to four phalanges (Fig. 2A). In the 60 mg l⁻¹ for 10

Fig. 2. (A) Group 7, intact left hindlimb treated with 60 mg l⁻¹ retinol palmitate for 4 days, showing reduction in number of tarsals, metatarsals, and phalanges. (B) Group 8, intact left hindlimb treated with 60 mg l⁻¹ retinol palmitate for 10 days, showing extreme reduction of foot. Foot skeleton is interpreted as three reduced distal metatarsals and five tarsals. (C) Group 3, intact left forelimb treated with 60 mg l⁻¹ retinol palmitate for 10 days showing reduction of digit IV and indistinct separation between phalanges and between phalanges and metacarpals. (D) Group 10, intact left forelimb treated with 300 mg l⁻¹ retinol palmitate for 10 days showing fusion of metacarpals II and III and serial fusion of digits and metacarpals. (E) Group 2, regenerated right hindlimb treated with 60 mg l⁻¹ retinol palmitate for 4 days showing loss of phalanges in digit III and reduction in size of digit V. (F) Group 4, regenerated right hindlimb treated with 300 mg l⁻¹ retinol palmitate for 4 days showing additional tarsals in an otherwise normal hindlimb. Twelve tarsals are present in place of the usual nine. Magnification × 15.



days treatment group, the effects were slightly more severe. There were five or six tarsals with three metatarsals and no phalanges at all. The metatarsals, however, were often so reduced that they were similar in size to the tarsals and identifying the homologies of the skeletal elements became difficult (Fig. 2B). The severity of the deletions in the 300 mg l^{-1} treatment groups was about the same as the 60 mg l^{-1} groups as these cases had four to eight tarsals, two to four metatarsals, and zero to two phalanges. The increased dosage of retinol palmitate did not seem to cause a significant increase in the extent of the skeletal deletions (cf. Table 1). However, increasing the treatment time from 4 to 10 days resulted in a slight increase in the extent of deletions.

The effects of retinol palmitate on developing forelimbs treated at the four-digit stage are much reduced compared to the hindlimbs in which treatment occurred at an earlier stage. In the 60 mg l^{-1} for 4 days groups, the only effect was a slight reduction in the number of phalanges (mean 6.2). In the 60 mg l^{-1} for 10 days groups, there was a further reduction in phalange number (mean 5.1), but as well the phalanges occasionally showed poor separation from one another and from the metacarpals, and these skeletal elements were occasionally reduced in size (Fig. 2C). Metacarpals II and III were often partially fused. In the 300 mg l^{-1} for 4 days groups, the main observations were reduced phalange number (mean 5.5), and increased fusion of metacarpals II and III (fusion was occasionally complete). In the 300 mg l^{-1} for 10 days groups, the phalange number was further reduced (mean 3.9), and the digits lacking phalanges were most often I and IV. Metacarpal defects were also more obvious, with frequent fusion of metacarpals II and III, and a reduction in size of metacarpals, especially IV (Fig. 2D). The reduction in overall number of skeletal elements in these groups (Table 1) was largely due to reduction in phalange numbers. This is not surprising since by the time the limb had developed to the four-digit stage, all of the skeletal elements except the phalanges had already formed.

In summary, when retinol palmitate had any effect on a developing limb, the effect was invariably to cause inhibition of growth and deletion of skeletal elements. Limb duplication was never observed in developing limbs as a result of retinol palmitate treatment.

Regenerating limbs

When axolotl limbs were amputated and treated with retinol palmitate, a range of responses occurred. These included morphologically normal limbs as well as the range of duplications previously described (Maden, 1983b), i.e. extra carpals, extra partial radius and ulna, extra complete radius and ulna, extra part humerus, and extra complete humerus (and the homologous hindlimb duplications). However, our results included a number of cases in which hypomorphic limbs resulted from the low doses of retinol palmitate, varying from slightly hypomorphic limbs (which could not be readily distinguished from controls), to severely hypomorphic limbs in which the regenerate contained only a few irregular cartilages. This led to

Table 2. Retinol palmitate effects on regenerating axolotl limbs

Treatment	Right hindlimbs amputated at cone stage (Series I - Groups 1-5)			Right hindlimbs amputated at 2-digit stage (Series II - Groups 6-10)			Right forelimbs amputated at 4-digit stage (Series I and II)		
	Group	Individual observations	Mean	Group	Individual observations	Mean	Group	Individual observations	Mean
0	1	0 0 0 0 0 0	0.0 ± 0.0 (7)	6	0 0 0 0 0 0	0.0 ± 0.0 (6)	1	0 0 0 0 0 0	0.15 ± 0.38 (13)
60 mg l ⁻¹ - 4 days	2	0 1 0 0 0 1	0.29 ± 0.49 (7)	7	4 0 0 4 4 0	2.0 ± 2.2 (6)	2	0 0 0 0 0 4 0	1.6 ± 1.7 (13)
60 mg l ⁻¹ - 10 days	3	1 1 1 0 0 0 0	0.43 ± 0.53 (7)	8	4 0 0 4 4	2.4 ± 2.2 (5)	3	6 4 7 5 7 7 7	5.8 ± 1.4 (12)
300 mg l ⁻¹ - 4 days	4	1 4 4 4 4	3.4 ± 1.3 (5)	9	5 4 4 0 4 5	3.7 ± 1.9 (6)	4	0 0 0 0	1.9 ± 2.2 (10)
300 mg l ⁻¹ - 10 days	5	8 8 8 8 8 8 0	6.9 ± 3.0 (7)	10	7 8 6 8 7 7	7.2 ± 0.8 (6)	5	6 5 7 8 9 7 9	6.5 ± 2.0 (13)
							6	6 3 6 6 3 9	

Strength of Activity Index

- 0 - Normal regeneration
- 1 - Slightly hypomorphic (at least 1 metacarpal/metatarsal and 3 phalanges missing)
- 2 - Moderately hypomorphic (2 recognizable digits)
- 3 - Severely hypomorphic (1 or 0 recognizable digits)
- 4 - Extra carpals/tarsals
- 5 - Extra part radius-ulna/tibia-fibula
- 6 - Extra complete radius-ulna/tibia-fibula
- 7 - Extra part humerus/femur
- 8 - Extra complete humerus/femur
- 9 - Stumped limb exhibiting no regeneration

Note: In each case, the mean strength of activity index is followed by the standard deviation, with the number of cases given in brackets.

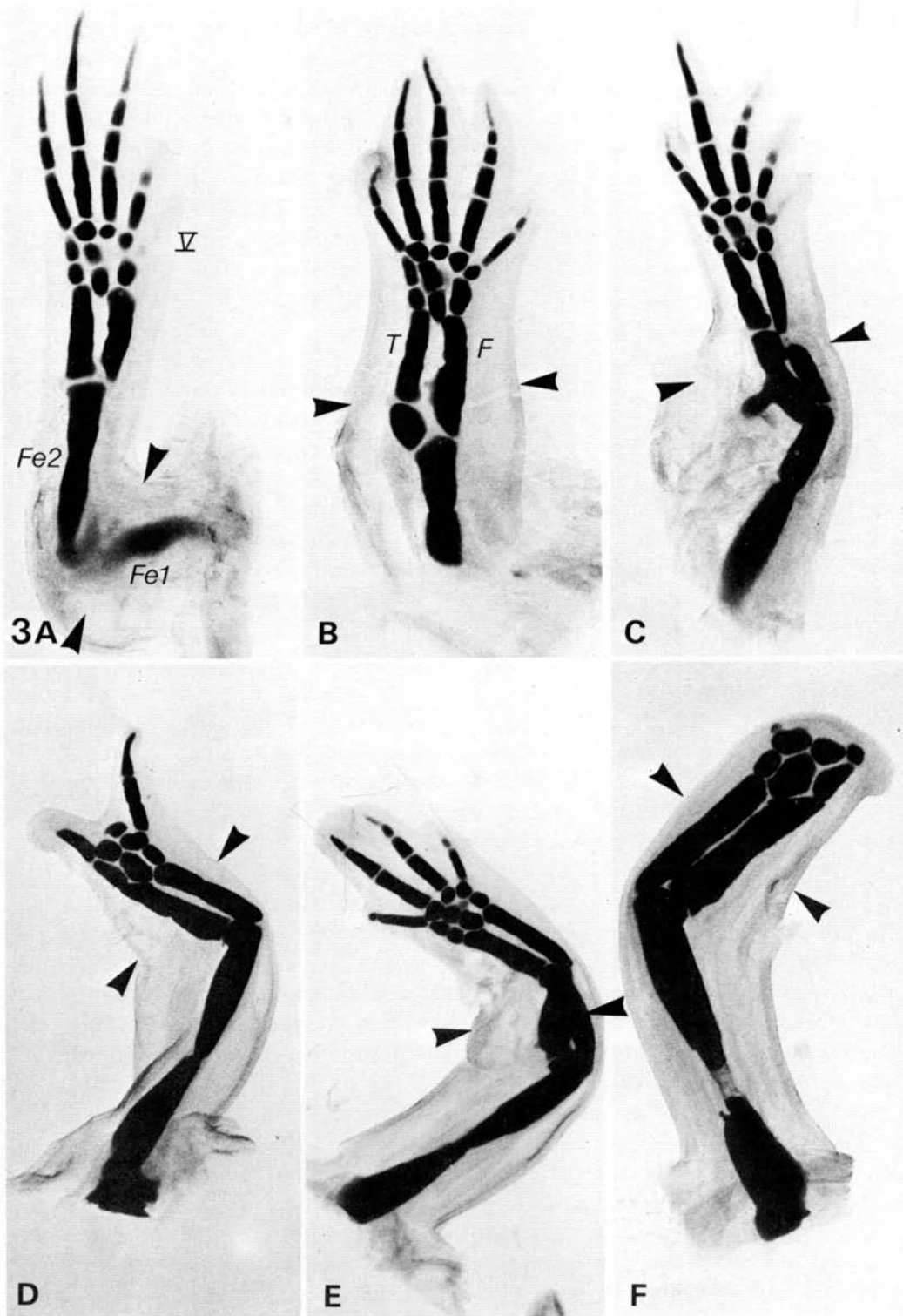
an expansion of the scoring system previously used (Maden, 1983*b*; Maden, Keeble & Cox, 1984) to include these hypomorphic limbs. The strength of activity index (cf. Summerbell, 1983) used in this paper is given in Table 2. Table 2 also includes a summary of all the results obtained from regenerating limbs treated with retinol palmitate.

Right hindlimbs amputated at the cone stage and treated with retinol palmitate at 60 mg l^{-1} for 4 or 10 days usually produced normal regenerates but occasionally these were slightly hypomorphic with one metatarsal and a few phalanges missing (Fig. 2E). When the dose was raised to 300 mg l^{-1} for 4 days, the response was usually to produce a limb containing additional tarsals (Fig. 2F). When the 300 mg l^{-1} treatment was extended to 10 days, the response was to produce a duplicated limb in which a complete femur was formed and articulated with the stump femur at the level of amputation (Fig. 3A).

Right hindlimbs amputated at the two-digit stage and treated with retinol palmitate at 60 mg l^{-1} for 4 or 10 days produced normal regenerates and regenerates with extra tarsals in about equal frequencies. However, when the dose was increased to 300 mg l^{-1} for 4 days, the regenerates began to show PD duplications of part of the tibia–fibula (Fig. 3B). At 300 mg l^{-1} for 10 days, all cases showed PD duplications of complete tibia–fibula (Fig. 3C), part femur, or complete femur.

Right forelimbs amputated at the four-digit stage and treated with retinol palmitate at 60 or 300 mg l^{-1} for 4 days produced limb regenerates that were normal, moderately hypomorphic (Fig. 3D), or had extra carpals. At 60 or 300 mg l^{-1} for 10 days, however, most of the regenerates exhibited varying degrees of PD duplication (Fig. 3E). However, these groups also included a few cases exhibiting severely hypomorphic regenerates (Fig. 3F) or completely stumped non-regenerating limbs (cf. Table 2).

Fig. 3. (A) Group 5, regenerated right hindlimb treated with 300 mg l^{-1} retinol palmitate for 10 days showing complete PD duplication of the femur (*Fe2*) distal to the original amputation level (arrowheads) which passed through the distal part of the original femur (*Fe1*). The regenerate is still incomplete and lacks some phalanges of digit IV as well as cartilages of digit V (*V*) which are just barely visible on the dorsal side of the foot. (B) Group 9, regenerated right hindlimb treated with retinol palmitate for 4 days showing partial duplication of the tibia–fibula distal to the amputation plane (arrowheads) which passes through the proximal tibia–fibula. A complete tibia (*T*) has regenerated; however, the regenerated fibula (*F*) is simply an extension of that in the stump. (C) Group 10, regenerated right hindlimb treated with 300 mg l^{-1} retinol palmitate for 10 days after amputation (arrowheads) through the tibia–fibula showing regeneration of a complete tibia–fibula distal to that in the stump. (D) Group 7, regenerated right forelimb treated with 60 mg l^{-1} retinol palmitate for 4 days after amputation (arrowheads) through the mid radius–ulna. Hand is reduced to only two defective digits. (E) Group 8, regenerated right forelimb treated with 60 mg l^{-1} retinol palmitate for 10 days following amputation (arrowheads) through the mid radius–ulna. The distal ends of the stump radius–ulna have fused to form the distal part of a humerus and this articulates with a completely new distal radius–ulna in the regenerate. (F) Group 10, regenerated right forelimb treated with 300 mg l^{-1} retinol palmitate for 10 days following amputation (arrowheads) through the mid radius–ulna. This regenerate is severely hypomorphic, lacks a hand, and consists of only six cartilage nodules distal to the radius–ulna. Magnification $\times 15$.



At the cone stage, an increase in the concentration of retinol palmitate had more effect on regeneration than an increase in the duration of treatment (cf. Table 2, strength of activity indices), i.e. compared to Group 2, increasing the dose to 300 mg l^{-1} (Group 4) caused a greater increase in effect than increasing the duration of treatment to 10 days (Group 3). Conversely, however, at the four-digit stage, increasing the concentration to 300 mg l^{-1} had relatively little effect (compare Groups 2 & 7 to Groups 4 & 9) while increasing treatment time to 10 days at 60 mg l^{-1} greatly increased the effect (compare Groups 2 & 7 to Groups 3 & 8). The response of two-digit-stage limbs was intermediate, with both increased concentration and increased duration of treatment having comparable effects.

DISCUSSION

The effect of retinol palmitate on regenerating axolotl limbs amputated at three different stages of development, i.e. cone stage, two-digit stage, and four-digit stage, was usually to produce PD duplications in a similar manner to that previously reported for mature axolotl limbs (Maden, 1983*b*). The duplicated regenerates so produced exhibited no abnormalities other than the duplication. In other cases, regenerates were hypomorphic, but duplication and skeletal reductions never occurred simultaneously on the same limb. These seemed to be mutually exclusive alternatives. However, the contralateral developing limbs only gave rise to hypomorphic limbs with skeletal deficiencies, varying across a continuum from production of slightly hypomorphic limbs to complete suppression of limb development. The limbs treated at the cone stage of development were the most severely affected. This is not unexpected since the further development proceeds prior to vitamin A treatment, the less likely will be an effect on pattern regulation because much of the process will already have occurred. The complete absence of any duplications in the developing axolotl limb is surprising, since the doses and treatment times used gave results varying from no effect (normal limb development) to complete inhibition of limb development. Thus, it would seem unlikely that the absence of duplications in our results can be attributed to an inappropriate treatment regime. In regenerating and developing hindlimbs of *Bufo melanostictus* tadpoles a similar phenomenon has been observed. The regenerating limbs produce PD duplications while simultaneously the intact developing limbs became hypomorphic in response to the same vitamin A treatment (Niazi & Ratnasamy, 1984). This basic difference in response of developing limbs and regenerating limbs to vitamin A in both urodeles and anurans suggests that there may be differences in the underlying processes involved.

The responses of the regenerating limbs to retinol palmitate in these experiments did not fall along a single continuum as the response of the developing limbs did. In the developing limbs, one could simply count the number of skeletal elements present and get a reasonably quantifiable estimate of the degree of skeletal reduction which correlated well with morphological observations of reductions and with dose levels employed. By contrast, regenerating limbs gave

several disparate responses to retinol palmitate which did not bear any obvious relationship to one another. (A) Some regenerating limbs treated with retinol palmitate became hypomorphic and these covered a continuum from limbs which were very slightly hypomorphic to those which were severely hypomorphic. 'Slightly hypomorphic' was arbitrarily defined as lacking at least one metacarpal/metatarsal and at least three phalanges. This degree of reduction was never seen in untreated intact limbs, only rarely seen in untreated regenerating limbs (2/26 cases), and commonly seen in some of the low-dose retinol palmitate groups. The severely hypomorphic limbs showed a definite regenerative outgrowth in which the radius-ulna was completed and at least six carpal-like cartilage nodules were also regenerated. We did not observe any regenerative outgrowths containing less than six skeletal elements. (B) Some amputated limbs completely failed to regenerate as a result of retinol palmitate treatment. Complete failure of regeneration was characterized by no outgrowth at all and was observed only in the highest dose groups. (C) Some regenerating limbs intercalated extra carpals/tarsals into the regenerating pattern in response to retinol palmitate. (D) In response to retinol palmitate, some limbs produced PD duplications, that is they regenerated as if the amputation plane was in fact more proximal than it actually was. If the positional coding was shifted to a slightly more proximal value, then an extra-long radius-ulna/tibia-fibula was produced; however, if the positional coding was shifted to a very proximal level, then a complete limb beginning with a complete humerus/femur regenerated from the distal amputation plane.

Thus, with four different categories of response to the retinoid it becomes somewhat difficult to explain the results in the context of a single dose-response relationship. The strength of activity index (SAI) is an attempt to do so (Table 2). The results suggest that the effect of retinol palmitate differs at different dose levels. For example, complete inhibition of regeneration (SAI 9) occurred only in the highest dose group. Hypomorphic limbs (SAI 1 to 3) were more frequent in low-dose groups (six at 60 mg l^{-1} for 4 days, four at 60 mg l^{-1} for 10 days, three at 300 mg l^{-1} for 4 days, two at 300 mg l^{-1} for 10 days; cf. Table 2). PD duplications and extra carpals/tarsals appeared to have intermediate distributions. These observations were then the basis of the SAI selected. In fact, the SAI in Table 2 was selected from several which were tried and this one fitted the data the best. For example, an SAI in which hypomorphic limbs were scored higher than PD duplicated limbs gave mean SAIs with higher standard deviations in each treatment group, and did not have as good a dose-response correlation. Another possible SAI that was tried assigned the same range of values to both hypomorphic and duplicated limbs. This fitted the data as well as the SAI in Table 2, but was discarded because it required an additional hypothesis to explain why the same dose of retinoid sometimes caused duplication and sometimes caused deletions. It is worth noting in passing that PD duplicated limbs were never hypomorphic. If PD duplication was initiated then the resulting regenerate exhibited no abnormalities other than the duplication. It seems that duplication and deletion were mutually exclusive categories. The selection of the SAI in Table 2 was based on its

relative simplicity and good fit with the data. Summerbell (1983) has discussed at length the same problem of selecting a strength of activity index for use in scoring retinoid effects on chick wing bud development. It is worth noting that in chick wing buds the dose-response sequence is different. There with increasing dose the response ran from: no effect, to duplication, to deletion, to absence of wing development.

One possible explanation of the results could be that vitamin A has two (or more) simultaneous and antagonistic effects. One of these effects might be to alter pattern formation (Maden, 1983*b*), the other to interfere with the cell cycle (Schroder, Rapaport & Black, 1983), and hence limit the availability of cells for the construction of a regenerate or especially to interfere with cartilage differentiation (Lewis, Pratt, Pennypacker & Hassell, 1978). However, one difficulty with such an explanation is that it would be difficult to see why inhibition of regeneration occurred at low doses (hypomorphic limbs) and at high doses (absence of regeneration) and yet not at intermediate doses where duplications occurred. It is conceivable that if vitamin A can interfere with positional signalling such that cells are reset to levels proximal to the actual plane of amputation, then it may be that cells could be reset by a lower dose of vitamin A to levels distal to the plane of amputation, resulting in deletions. If this occurred, then deletions might be the result of an effect on pattern formation, rather than an effect on cell-cycle inhibition.

It is puzzling that while a developing limb is inhibited by retinol palmitate and becomes hypomorphic, the contralateral regenerating limb can simultaneously duplicate and produce more regenerated limb than usual. It is possible that even at low doses retinoids interfere with cell proliferation and hence interfere with limb growth. It could be that in regenerating limbs there is sufficient promotion of cell proliferation by the process of wounding, leading to release of mitogenic neurotrophic factors, etc., for the inhibitory effect of vitamin A to be more than compensated for. It may be that only at very high levels of retinol palmitate is the cell-proliferation-inhibiting effect of vitamin A great enough to overcome the cell-proliferation-promoting effects released during regeneration, and at this point all limb regeneration is suddenly arrested.

One could criticize our comparison between developing limbs which were treated with vitamin A beginning at the cone or two-digit stage, and regenerating limbs which were treated immediately after amputation up to the cone stage. One might suggest that a more appropriate comparison would be with a developing limb bud at an earlier stage, before a limb bud has formed or while it is just forming, and that if such a stage were used PD duplications might be induced. However, it is difficult to imagine how one could duplicate a pattern which has not yet developed at all. In mature limbs, or even in a cone-stage limb bud, there is a pattern present (or at least a latent pattern) in the stump which could be duplicated. However, in a very early limb bud before cell differentiation has advanced to the point where a pattern is present at the cellular level, it is difficult to conceive what a PD duplication would entail. Amputation is essential for PD

duplication of the limb to occur in response to vitamin A in all cases reported to date. PD duplication of limbs during regeneration may be quite unrelated to AP duplications as seen in the chick limb buds and regenerating amphibian limbs. AP duplications may arise via a quite independent mechanism and may be more closely related to the induction of accessory limbs.

If there is a dual effect of vitamin A on axolotl limb regeneration or if different mechanisms are involved, it might explain why axolotls do not give AP duplications in response to vitamin A. *Rana temporaria* gives AP duplications only at the highest dose levels (Maden, 1983a). It may be that the induction of AP duplications requires a higher dose level than even complete PD duplication. However, if the cell-proliferation-inhibiting effect of vitamin A sets in before this level is reached, then it may not be possible to induce AP duplication in the axolotl. Cell proliferation in *Rana* may be less sensitive to vitamin A.

If there is, however, a significant influence on cell proliferation then it would be expected to be most marked in the higher dose groups. In this regard, we did note that regeneration was slowed down in the highest dose group. By 42 days, the PD duplicated regenerates had often not proceeded to completion of the skeleton (i.e. distal phalanges and especially digit V were often very small and histologically immature, Fig. 3A), although the pattern regenerated appeared entirely free from any deletions. This slowing of regeneration at higher doses could be due to interference with the cell cycle.

Previous investigations have reported hypomorphic limbs and skeletal deletions in response to vitamin A treatment of regenerating limbs (Maden, 1983a,b; Saxena & Niazi, 1977; Scadding, 1983; Thoms & Stocum, 1984). These cases tend to be given much less attention, since they seem to be of less interest than duplications. However, an understanding of retinoid effects on developing and regenerating limbs requires hypotheses which will cover all categories of responses.

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