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The effects of two retinoids on limb regeneration in *Pleurodeles waltl* and *Triturus vulgaris*

EMILE LHEUREUX, STEPHEN D. THOMS AND FREDERICK CAREY

Laboratoire de Morphogenèse Animale, UA 685, Université des Sciences et Techniques de Lille, F-59655 Villeneuve d'Ascq Cedex, France

SUMMARY

The effects of two vitamin A analogues, retinol palmitate and retinoic acid, on pattern formation during limb regeneration in larvae of two European newts, *P. waltl* and *T. vulgaris* are described. The response of the regenerating limb to retinoid treatment differed according to the larval stage of development for *P. waltl.* Young larval limbs, which were anterior limb buds at the time of amputation, duplicated transversely while limbs of older larvae duplicated proximodistally. Proximodistal duplications were usually limited to the production of supernumerary carpals or a second zeugopod. Complete limbs regenerating from a distal amputation plane were rarely seen. *T. vulgaris* larvae regenerated limbs with either one or the other type of duplication, but never both on the same limb, at all larval stages tested.

When larval *P. waltl* were kept in normal laboratory light during the treatment with retinol palmitate suspended in the rearing water the percentage of limbs which duplicated was very small for young larvae and increased with the age of the larvae used. Keeping the animals in the dark during the treatment period greatly increased the percentage of duplicate limbs obtained on the young larvae but not on the older larvae. This result is discussed in terms of the photodegradation of the retinoid and the length of the sensitive period for the regenerating limb.

A dose–response relationship between the dose of retinol palmitate and either the percentage of limbs duplicated or the degree of duplication was not found. Such a relationship, however, was observed when retinoic acid was injected intraperitoneally into stage-54+ *P. waltl* larvae. Additionally, this technique revealed a peak of sensitivity to retinoic acid on the 6th day after amputation.

Limb regeneration in older larvae was temporarily blocked by retinoid treatment. The limbs showed little or no regression and began blastemal development shortly after the treatment ended. Limbs of young larvae, however, often regressed. Such regressions were followed by blastemal formation and middle- to late-bud blastemas were found at the end of 11 or 14 days treatments with retinol palmitate.

INTRODUCTION

Vitamin A and its analogues, the retinoids, can induce duplications of pattern along all three principle axes of regenerating amphibian limbs. Regenerates with proximodistal serial duplications are those presenting a regeneration of proximal elements from distal amputation levels. They have been observed in adult and larval urodeles and in larval anurans: axolotl (Maden, 1982, 1983*a,b*; Thoms & Stocum, 1984). *Notophthalmus viridescens* (Thoms & Stocum, 1984), *Bufo*

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andersonii (Niazi & Saxena, 1978), *Bufo melanosticus* (Niazi & Ratnasamy, 1984), and *Rana temporaria* (Maden, 1983*c*). Such duplications, violating the rule of distal transformation (Rose, 1970), have been provoked by surgical procedures in only a few exceptional cases (De Both, 1970; Carlson, 1974; Kieny & Pautou, 1977). Transverse duplications after treatment of regenerating limbs with a retinoid are somewhat less frequent but have been encountered in all of the tested amphibian species. A surgical perturbation of the anteroposterior axis before retinoid treatment increases both the frequency and degree of development of anteroposterior duplications (Stocum & Thoms, 1984). Stocum & Thoms (1984) have suggested that the retinoids bring about a change in positional information which completes the pattern along any of the three axes. Whether or not such a change is unidirectional; i.e. distal to proximal or anterior to posterior but not proximal to distal or posterior to anterior; has yet to be determined. It is clear, however, that the retinoids affect the positional information at the level of amputation (Thoms & Stocum, 1984; Wallace & Maden, 1984) and this effect takes place during the early phases of cell dedifferentiation and blastemal formation (Maden, 1983*a*; Niazi & Ratnasamy, 1984; Thoms & Stocum, 1984).

Further research on the mechanism of action by which the retinoids change the positional information expressed by blastemal cells will surely deepen our understanding of pattern formation and pattern regulation during amphibian limb regeneration. Towards this end we have undertaken a study of the effects of two retinoids, retinol palmitate and retinoic acid, on limb regeneration in two European newts. *Pleurodeles waltl* larvae, tested at various ages, show different responses according to their stage of development. Transverse duplications appear when young larvae are used but this result gives way to proximodistal duplication in more mature animals. Young larvae of *Triturus vulgaris*, however, present both transverse and proximodistal duplications.

MATERIALS AND METHODS

Pleurodeles waltl larvae from the laboratory colony and *Triturus vulgaris* larvae from several spawnings by locally collected adults were raised individually in tap water at 23–25 °C and fed brine shrimp or mosquito larvae several times a week.

Trans-retinol palmitate (Type VIII, Sigma) was suspended in the rearing water at various concentrations between 60 mg (15 i.u. ml^{-1}) and 600 mg l^{-1} (150 i.u. ml^{-1}). Young *T. vulgaris* larvae and *P. waltl* larvae at several different defined stages of development, including animals showing anterior limb buds rather than mature limbs (Table 1), were treated with a given concentration of retinol palmitate for 8, 11 or 14 days following amputation at the wrist and ankle or through the middle of the limb bud. At the end of the treatment period the animals were raised separately in tap water until regeneration was completed.

In a few experimental series the animals were kept in darkness during the treatment period to avoid possible photodegradation of the retinol palmitate (Alwood & Plane, 1984). The treatment lasted 11 days for stage-38 larvae and 14 days for older stages. Three times a week the larvae were exposed to the light for 2 h feeding periods, following which they were transferred to fresh retinoid solution and returned to darkness. Doses of retinol palmitate from 2 mg l^{-1} (0·5 i.u. ml^{-1}) to 600 mg ml^{-1} (150 i.u. ml^{-1}) were tested under these conditions.

Additional experiments were carried out on stage-54+ *P. waltl* larvae (45 mm to 48 mm long) using all-trans retinoic acid (type XX, Sigma) according to the method of Thoms & Stocum

(1984). The retinoic acid was dissolved in dimethyl sulphoxide (DMSO, Sigma) at 50 mg ml^{-1} or 100 mg ml^{-1} and injected intraperitoneally at doses from 25 μg/animal to 200 μg/animal. Injections were given 2, 4, 6, or 8 days postamputation to determine the period of maximal sensitivity. In these experiments both anterior and posterior limbs were amputated through the proximal basipod.

When the limbs were completely regenerated they were fixed in 5 % formol, stained with Victoria Blue B and cleared in methyl salicylate.

RESULTS

The results obtained using retinol palmitate are presented before those obtained with retinoic acid. The results for each species are presented separately.

Influence of retinol palmitate on Pleurodeles waltl *limb regeneration*

Influence of retinol palmitate treatment duration

In each series that was carried out in normal light, two lots of animals were treated separately for 8 or 14 days. For stage-35 and -38 larvae an additional third lot was treated for 11 days. No differences in either the percentage or amplitude of the regenerated limb abnormalities were found with the different treatment times. Results are therefore grouped together for all treatment times in Tables 2–4.

Behaviour of the limb stumps during the retinol palmitate treatment

The limb stumps of the older, submetamorphic larvae healed and underwent neither regression nor growth during the treatment period, as has been described for the axolotl (Maden, 1982, 1983*b*). The limb stumps of younger larvae, however, consistently exhibited a noticeable regression followed by blastemal formation and development before the end of the treatment. Limb stump regression was more striking in the very young stages, chiefly stages 35 and 38 where the amputated limb bud often regressed to the presumptive shoulder region. Blastemas then formed on the regressed stumps and medium- to late-bud blastemas were often seen after a 14-day treatment period. In some cases duplicating limb bud blastemas were already present when the treatment ended, demonstrating the

Table 1. *Characterization of the anterior and posterior limbs of* Pleurodeles waltl *of the different stages of development used (derived from Gallien & Durocher, 1957)*

Stage	Length (mm)	Anterior limb	Posterior limb
35	11	Cylindric bud	
38	12	Notch	
40	13	2 digits-elbow	
43	17	3 digits	
45	18	4 digits	Cylindric bud
51	24	ib	3 digits
53	30	ib	4 digits
54	40	ib	5 digits
55b	60	ib	ib

responsiveness of such limbs to retinol palmitate treatment. Duplicating limb bud blastemas were even sometimes found on stage-38 larvae at the end of 11 days of retinol palmitate treatment in the dark. Blastemal development was never noticed at the end of an 8-day treatment.

Nature of the duplications obtained following amputations performed at different stages of larval development

Considering only the stage of development at which the animal was treated with retinol palmitate, ignoring for the moment the dose of the treatment, a striking difference was seen between the duplicated anterior limbs of young and old larvae (Table 2). Transverse duplications were formed when young larvae (principally stages 35 and 38) were used while proximodistal duplications developed in the regenerates of older larvae.

The posterior limbs appear later in larval development than the anterior limbs. It is not until stage 45, the earliest stage at which they were amputated, that the posterior limb buds have the form of an extended cone. Between the stages of 45 and 51; i.e. the period of limb development to the three-toe-bud stage; amputated posterior limbs regenerated normally following a treatment with retinol palmitate. Animals from stage 53 on regenerated posterior limbs with proximodistal duplications (Table 2).

Transverse duplications (Table 3)

The results of treating stage-35 and -38 larvae with retinol palmitate after amputation through the middle of the anterior limb bud can be summarized with five principal observations.

(1) The percentage of anterior limbs which formed transverse duplications is dose-independent for doses from 15 i.u. ml^{-1} to 150 i.u. ml^{-1} when the experiments were done in normal light.

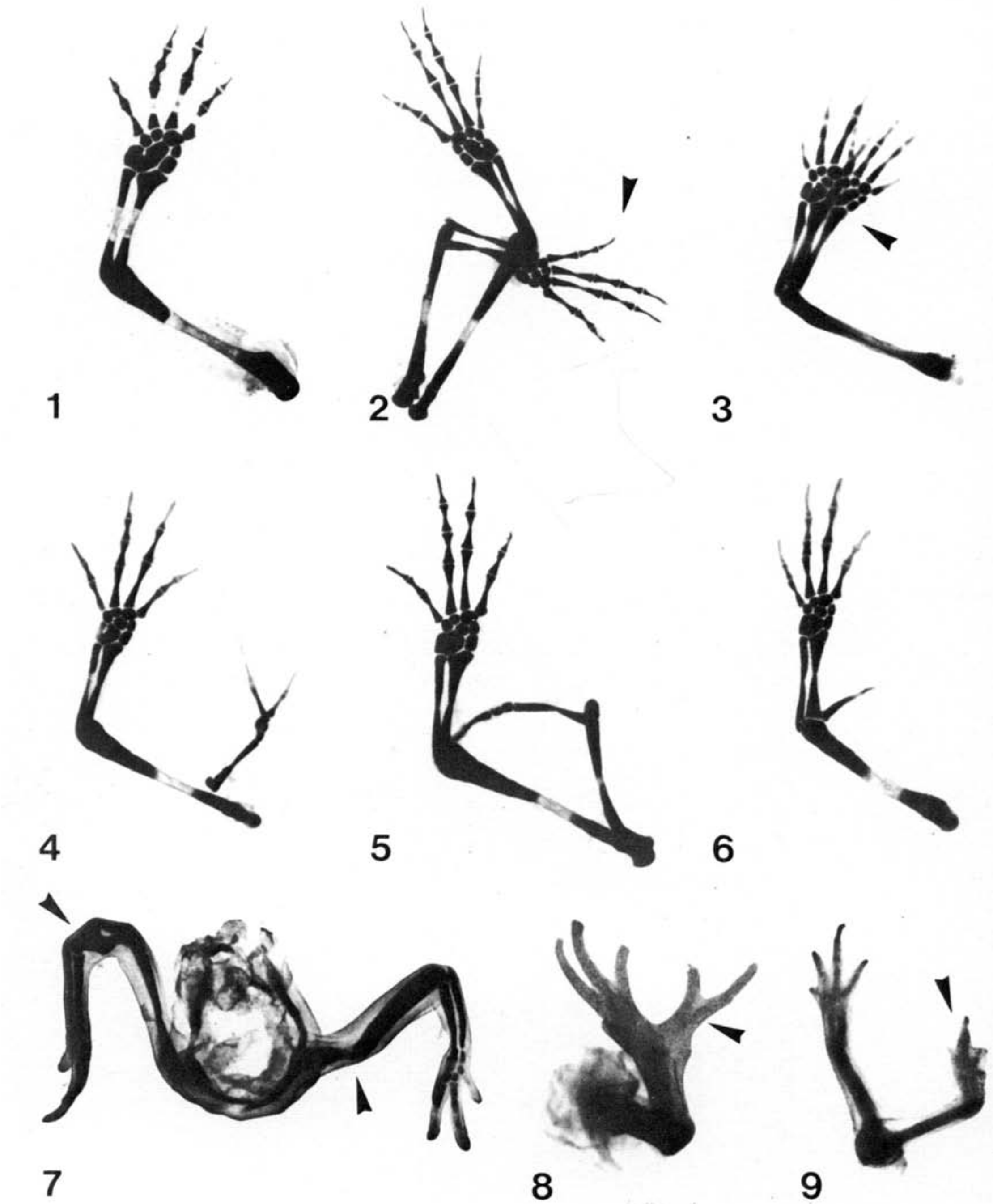
(2) Stage-38 larvae treated in the dark regenerated their forelimbs with a high percentage (67 % to 100 %) of transverse duplications compared to stage-38 larvae treated with similar doses in normal light (0 % to 19 %). In addition, much lower doses of retinol palmitate were required to provoke transverse duplications when the experiments were carried out in the dark than in normal light. In the dark the action of retinol palmitate could be first seen at a dose of 1 i.u. ml^{-1} (8 % of the limbs having a small transverse duplication) and increased to a maximum at 3·75 i.u. ml^{-1} (90 % having a transverse duplication). In normal light transverse duplications were not obtained with doses less than 45 i.u. ml^{-1} for stage-38 larvae.

(3) The pattern or degree of development seen in the supernumerary limbs varied from the formation of a simple spike to an entire second limb (Figs 2–6). These variations do not seem to be dose dependent. When the duplicate was sufficiently complete to determine its handedness it was always a mirror image of the main limb. Sometimes the anterior borders of a duplicated pair were juxtaposed but more often the ventral surfaces were found facing one another

Table 2. *Nature and percentage of duplications of* Pleurodeles waltl *limbs following amputation and retinol palmitate treatment at different stages of larval development and under different lighting conditions**

		Anterior limb					Posterior limb				
Lighting conditions	Stage	Number of limbs	Normal	Hypom.	Transverse duplication	Prox.-distal duplication	Number of limbs	Normal	Hypom.	Transverse duplication	Prox.-distal duplication
Normal light	35	278	233	1	44 (16)						
	38	232	210	8	14 (6)						
	40	58	43	8	2 (3)	5 (9)					
	43	128	118	1	1 (1)	8 (6)					
	45	63	56	4		3 (5)	29	29			
	51	71	52	1		18 (25)	34	32	2		
	53	67	37	1		29 (43)	46	34			12 (26)
	54	66	35			31 (47)	64	44			20 (31)
	55	88	8			80 (91)	75	7		1 (1)	68 (91)
Darkness	38	56	12		44 (79)						
	45	40	22			16 (40)	40	40			
	54	28	2	11		15 (54)	28	10	8		10 (36)

* These results take into account the totality of the treatments using doses of retinol palmitate ranging from 15 to 150 i.u. ml^{-1}. Hypom., hypomorphic in digit number. Numbers in parentheses indicate the percentage of limbs in each category.



(Figs 2, 3). Examination of developing double limbs showed a constant ventral location of the supernumerary bud.

(4) The proximodistal level of origin of the duplicate was also variable and without any relation to either the dose of retinol palmitate or the quality of the

duplicate. Sometimes the duplicate originated from the shoulder, forming a supernumerary which was either a second limb (Fig. 2) or a hypomorphic structure (Fig. 5). Other duplications, however, were found originating from the mid-stylopod (Fig. 4), zeugopod (Figs 3, 6), or autopod. It could have been that the proximodistal level of origin of the duplicate correlates with the level of regression of the stump. However, attentive examination of the limb bud stumps of the stage-38 larvae both during and after the treatment in the dark revealed a complete regression of all the stumps except those treated with the threshold dose ($1\,\text{i.u.}\,\text{ml}^{-1}$). The double limb bud blastemas were all very similar. No morphological criterion allowed the prediction of either the proximodistal level of origin or completeness of the duplicate.

(5) With one exception, the posterior limbs did not form transverse duplications even when amputated at limb bud stages (Table 2). Very surprisingly, the

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Fig. 1. Control left anterior limb regenerate formed after amputation through the wrist of a stage-55 *P. waltl*. Dorsal view. ×7.

Fig. 2. Transverse duplication formed at the shoulder after amputation through the middle of the right anterior limb bud of a stage-35 *P. waltl* and a 14-day treatment with retinol palmitate at $45\,\text{i.u.}\,\text{ml}^{-1}$. The duplicate is located ventrally (arrow) and is a left limb with its dorsoventral axis opposed to the regenerated right limb. Dorsal view. ×8.

Fig. 3. Transverse duplication formed at the elbow after amputation through the middle of the left anterior limb bud of a stage-38 *P. waltl* and an 8-day treatment with retinol palmitate at $75\,\text{i.u.}\,\text{ml}^{-1}$. The duplicate is located ventrally (arrow) and is a right limb having a well-formed zeugopod and hand with its palmar surface facing that of the regenerated left limb. Dorsal view. ×8·3.

Fig. 4. Transverse duplication with two fingers found on the anterior border of the stylopod of the principal regenerate after amputation through the middle of the left anterior limb bud of a stage-35 *P. waltl* and a 14-day treatment with retinol palmitate at $45\,\text{i.u.}\,\text{ml}^{-1}$. Dorsal view. ×8·3.

Fig. 5. Transverse duplication with one finger formed ventrally at the shoulder after amputation through the middle of the left anterior limb bud of a stage-35 *P. waltl* and a 14-day treatment with retinol palmitate at $45\,\text{i.u.}\,\text{ml}^{-1}$. Note that the duplicate is proximodistally complete. Dorsal view. ×10.

Fig. 6. Transverse duplication present as a spike on the anterior border at the elbow of the principal regenerate formed after amputation through the middle of the left anterior limb bud of a stage-35 *P. waltl* and a 14-day treatment with retinol palmitate at $100\,\text{i.u.}\,\text{ml}^{-1}$. Dorsal view. ×8·4.

Fig. 7. Section through the trunk of a *T. vulgaris* at the level of the anterior limbs which show proximodistal duplications. The right (on the left in the photo) and the left (on the right in the photo) limbs were amputated at the wrist at stage 45 and the animal was treated for 11 days with retinol palmitate at $75\,\text{i.u.}\,\text{ml}^{-1}$. The right limb regenerated a distal humerus (arrow) distal to the original zeugopod. The left limb regressed to the mid-stylopod (arrow) from which a complete limb regenerated. Dorsal view. ×13.

Fig. 8. Transverse duplication with two fingers (arrow) found on the anteroventral border of the zeugopod of the principal regenerate after amputation through the wrist of a *T. vulgaris* at stage 51 and a 14-day treatment with retinol palmitate at $150\,\text{i.u.}\,\text{ml}^{-1}$. Ventral view. ×20.

Fig. 9. Transverse duplication formed at the shoulder after amputation through the left anterior wrist of a stage-45 *T. vulgaris* and a 14-day treatment with retinol palmitate at $75\,\text{i.u.}\,\text{ml}^{-1}$. The duplicate is a right limb located anteriorly (arrow). Dorsal view. ×20.

only posterior limb which produced a transverse supernumerary belongs to a 60 mm animal treated for 8 days with a dose of retinol palmitate of 75 i.u. ml^{-1}. Moreover, this limb was also duplicated along the proximodistal axis.

Proximodistal duplications (Table 4)

The percentage of limbs which responded to the retinol palmitate treatment by forming serial duplications along the proximodistal axis increased with the age of the larvae used (Tables 2, 4). This was true for the posterior limbs as well as the anterior limbs. A few cases were observed in anterior limbs of stage-40 (9 %) and -43 (6 %) larvae which did not form transverse duplications. These stages seem to be transition stages during which the type of duplication formed changes from transverse to proximodistal. Stage-45 larvae produced only proximodistal duplications in response to retinol palmitate. The low percentage (5 %) obtained with normal light for this stage was significantly increased to 40 % when the animals were kept in the dark during the treatment period. The percentage of proximodistal duplications obtained in the different series showed no correlation with the different doses used.

An analysis of the degree or extent of proximodistal duplication, however, is complicated by the frequent regressions which displaced the level of amputation in a proximal direction (Figs 16, 17). Such regressions decreased in extent with the increasing age of the animals. This means that a low degree of duplication is seen by supernumerary carpals or tarsals in the older larvae (Fig. 11), where the amputation level remains at the wrist, or ankle while in the young larvae, where regression is the rule, a low degree of duplication is always represented by an elongated zeugopod. Accordingly, we have presented the results in Table 4 using a simplified classification scheme. Two categories of proximodistal duplications are considered; (a) those whose length (L) was less than the normal zeugopod (Z) (Fig. 11) and (b) those whose length was greater than or equal to the normal zeugopod (Figs 13, 14, 16–18). L was obtained by measuring the proximodistal length of the duplicate part.

From Table 4 it can be seen that most of the duplicated limbs showed a low degree of duplication ($L < Z$). However, such a classification does not point out noteworthy cases. In fact, most of the duplicated limbs corresponded to an elongated zeugopod, often with a second elbow bend. In only six cases, two anterior and four posterior limbs (all from stage-55 larvae) was a limb with a duplicated stylopod regenerated from the wrist or ankle level (Fig. 18).

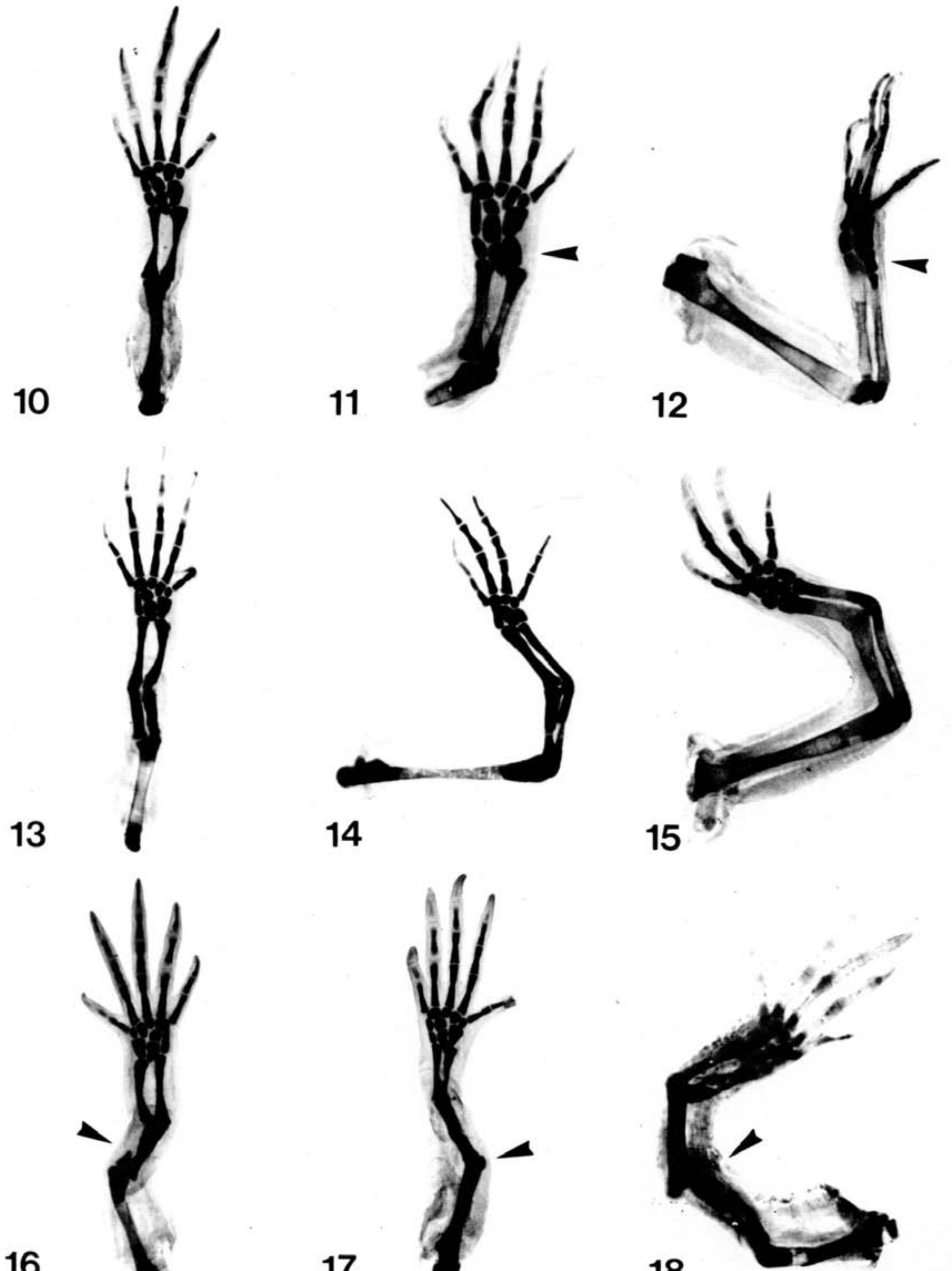
Influence of retinol palmitate on Triturus vulgaris *limb regeneration*

Three stages of larval development were used in experiments with retinol palmitate. These stages correspond to stages 40, 45 and 51 of *P. waltl* development. Observations made during the course and at the end of the treatment revealed a variable frequency and extent of regression of the amputated limbs. After two weeks in the presence of retinol palmitate the limbs had regenerated to

Table 3. *Tranverse duplications induced on regenerating anterior limbs of young larval stages of* Pleurodeles waltl *treated with retinol palmitate**

Lighting conditions	Stage	Doses (i.u. ml^{-1})	Number of limbs	Single +Hypom.	Transverse duplications						
						Tranv. pattern of supern.			Base of supern.		
					Total	Spike	Hypom.	Normal	Sh	St.	Z+H
Normal light	35	15	74	66	8 (11)	5	2	1		5	3
		45	56	46	10 (18)	5	1	4	3	6	1
		75	74	64	10 (14)	3	7			3	7
		100	46	35	11 (24)	3	3	5	1	7	3
		125	12	9	3 (25)	3				1	2
		150	16	14	2 (12)	1	1			2	
Normal light	38	15	48	48							
		45	44	43	1 (2)	1				1	
		75	58	47	11 (19)	1	2	8		6	5
		100	50	48	2 (4)			2	2		
		125	10	10							
		150	22	22							
Darkness	38	0·5	12	12							
		1	26	24	2 (8)	2				1	1
		3·75	20	2	18 (90)	6	5	7	2	8	4
		7·5	8		8 (100)	3	5			4	4
		15	18	6	12 (67)		5	7		10	2
		30	12	2	10 (83)	2		8		6	4
		75	18	3	15 (83)	11	1	3	4	11	
		150	8	1	7 (87)	2	2	3	3	3	1

* Hypom., hypomorphic in digit number; Sh, shoulder; St., stylopodium; Z+H, zeugopodium+hand. Numbers in parentheses indicate the percentage of transverse duplications in each series.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various degrees. Blastemas at the late bud or even two-finger-bud stage were seen as well as some abnormal mass formations.

The results obtained at the end of regeneration are presented in Table 5. A major difference can be seen between the two species in the regenerated limbs obtained after retinol palmitate treatment. Regeneration with transverse or proximodistal duplications of both posterior and anterior limbs was seen in all three series using *T. vulgaris* larvae in contrast to regeneration with either transverse duplications only obtained with stage-35 and -38 *P. waltl* larvae or regeneration with proximodistal duplications only seen with stages 45 and older.

The transverse duplications obtained varied in their degree of development. Some were simple spikes while others were either hypomorphic (Fig. 8) or complete limbs (Fig. 9). No relation between the larval stage or the dose of retinol palmitate and the degree of development of transverse duplication was noticeable. Additionally limbs with transverse duplications did not have proximodistal duplications.

Proximodistal duplications showed little variation in their development. The humerus was sometimes incomplete but present in most cases (Fig. 7). The main difference from one limb to another was the amount of regression which took place during the treatment and therefore the proximodistal level of origin of the

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Fig. 10. Normal right posterior limb of a stage-55. *P. waltl* larva. Dorsal view. ×7·3.

Fig. 11. Right posterior limb regenerate of a *P. waltl* treated for 14 days with retinol palmitate at 45 i.u. ml^{-1} following amputation through the ankle at stage 55. The proximal three tarsals are duplicated in the proximodistal axis (arrow). Dorsal view. ×7·5.

Fig. 12. Right anterior limb regenerate of a *P. waltl* injected with 100 μg of retinoic acid on day 4 after amputation through the wrist at stage 54+. The proximal three carpals are duplicated in the proximodistal axis (arrow). Dorsal view. ×7·7.

Fig. 13. Right posterior limb regenerate of a *P. waltl* treated for 14 days with retinol palmitate at 15 i.u. ml^{-1} following amputation through the ankle at stage 54. A complete zeugopod is duplicated in the proximodistal axis. Dorsal view. ×7.

Fig. 14. Right anterior limb regenerate of a *P. waltl* treated for 14 days with retinol palmitate at 15 i.u. ml^{-1} following amputation through the wrist at stage 54. A complete zeugopod is duplicated in the proximodistal axis. ×7.

Fig. 15. Right anterior limb regenerate of a *P. waltl* injected with 25 μg of retinoic acid on day 6 after amputation through the wrist at stage 54+. A complete zeugopod is duplicated in the proximodistal axis. Dorsal view. ×7·5.

Fig. 16. Left posterior limb regenerate of *P. waltl* treated for 14 days with retinol palmitate at 15 i.u. ml^{-1} following amputation through the ankle at stage 54. The limb regressed to the proximal zeugopod (arrow). The distal femur is duplicated in the proximodistal axis. Dorsal view. ×7.

Fig. 17. Right posterior limb regenerate of a *P. waltl* treated for 8 days with retinol palmitate at 100 i.u. ml^{-1} following amputation through the ankle at stage 53. The limb regressed to the knee. The femur is duplicated in the proximodistal axis. Dorsal view. ×7.

Fig. 18. Left posterior limb regenerate of a *P. waltl* treated for 8 days with retinol palmitate at 75 i.u. ml^{-1} following amputation through the ankle at stage 55. A complete limb has regenerated from the original amputation plane (arrow). Dorsal view. ×6.

Table 4. *Estimation of the proximodistal duplications lengths. Whatever the level of the stabilized regressed limb stump, the length (L) of the supernumerary proximodistal segment is compared with the length of the corresponding full zeugopod (Z)**

		Anterior limb				Posterior limb			
			Proximodistal duplication				Proximodistal duplication		
				Length (L)				Length (L)	
Lighting conditions	Stage	Number of limbs	Total	L<Z	L⩾Z	Number of limbs	Total	L<Z	L⩾Z
Normal daylight	35	278	0						
	38	232	0						
	40	58	5 (9)	5 (9)					
	43	128	8 (6)	8 (6)					
	45	63	3 (5)	3 (5)		29	0		
	51	71	18 (25)	14 (20)	4 (6)	34	0		
	53	67	29 (43)	19 (28)	10 (15)	46	12 (26)	12 (26)	
	54	66	31 (47)	19 (29)	12 (18)	64	20 (31)	14 (22)	6 (9)
	55	88	80 (91)	64 (73)	16 (18)	75	68 (91)	59 (79)	9 (12)
Darkness	38	56	0						
	45	40	16 (40)	11 (28)	5 (12)	40	0		
	54	28	15 (54)	15 (54)	—	28	10 (36)	10 (36)	—

* Numbers in parentheses indicate the percentage of limbs in each category.

Table 5. *Transverse and proximodistal duplications of* Triturus vulgaris *limbs induced by retinol palmitate treatment**

						Transverse duplications				Proximodistal duplication		
							Pattern of supern.				Pattern of supern.	
	Stage	Dose (i.u. ml^{-1})	Number of limbs	Normal	Hypom.	Total	Spike	Hypom.	Normal	Total	Partial	Complete
Anterior limb	(40)	75	16	7		2 (13)	2			7 (44)		7
		150	5	1		1 (20)			1	3 (40)		3
	(45)	75	16	5		1 (6)			1	10 (63)	1	9
		150	16	8						8 (50)	1	7
	(51)	75	14	9						5 (36)		5
		150	16	7		1 (6)		1		8 (50)	5	3
Posterior limb	(45)	75	12	6	2					4 (67)		4
		150	6	3	1	1 (17)			1	1 (17)		1
	(51)	75	12	10	1	1 (8)	1					
		150	16	8	3	3 (19)	1	2		2 (13)		2

* Stage numbers in parentheses do not correspond to the *T. vulgaris* table of development but are equivalent to 40, 45 and 51 stages of *Pleurodeles*. Numbers in parentheses indicate the percentage in each category. Hypom., Hypomorphic in digit number.

second humerus (Fig. 7). In twenty-one cases the regression of the forelimb stump stopped in the zeugopod. Seventeen of these had a duplicated humerus which articulated proximally with the rest of the original zeugopod. In the other four cases a duplicated zeugopod followed the original. In the remaining twenty cases of forelimbs with proximodistal duplications the limb stump had regressed to the stylopod. In these cases a second humerus was formed and a complete limb was regenerated from the regressed stump. The posterior limbs of *T. vulgaris* responded to the retinol palmitate treatment in the same way as the anterior limbs. Nearly two thirds regenerated normal limbs or limbs hypomorphic in digit number. The others were either transversely or proximodistally duplicated. This is in contrast to the results obtained on the posterior limbs of *P. waltl* larvae where only proximodistal duplications were found.

Influence of retinoic acid on Pleurodeles waltl *limb regeneration*

In contrast to our results obtained on *T. vulgaris* larvae and the results reported by other authors on various species of amphibians (Maden, 1982, 1983*a*; Niazi & Saxena, 1978; Thoms & Stocum, 1984) we obtained very few proximodistal duplications of *P. waltl* limbs with a duplicated stylopod distal to the original zeugopod. We therefore decided to check this result using a different retinoid and a different method of administration. The results of intraperitoneal injections of retinoic acid given at doses from 25 to 200 μg at various days after amputation of the autopod are given in Tables 6 and 7.

These limbs resembled those produced with retinol palmitate (Figs 12, 15). The dose dependence of the magnitude and frequency of proximodistal duplications is clearly shown, in contrast to the results with retinol palmitate. An injection on day 6 produced the most significant results compared to earlier injections which give a greater percentage of normal regenerates and carpal level duplications, and later (day-8) injections which yielded hypomorphic regenerates. No transverse duplicates were obtained in these experiments.

Very few cases of duplicated stylopods were found (0 for the forelimb and 4 for the hindlimb). This observation is probably not due to an administration of insufficient retinoic acid. A dose of 50 μg given on the sixth day after amputation (day of amputation = day 0) promoted the formation of duplicated zeugopods in 80 % of the cases for the forelimb and 70 % of the cases for the hindlimb. Increasing the dose two to four times still produced few duplicated stylopods. Additionally, a consistent production of stylopod duplication in axolotl limbs amputated at the wrist has been obtained using similar doses (Thoms & Stocum, 1984). We assume therefore that this is a species characteristic.

DISCUSSION

Larvae of two European newts, *Pleurodeles waltl* and *Triturus vulgaris* were treated with retinol palmitate after limb amputation in order to study the effects of vitamin A analogues on pattern formation during limb regeneration. A second retinoid, retinoic acid was also tested on older larvae of *Pleurodeles waltl.* Retinol palmitate, given during the early stages of limb regeneration, caused the formation of either a transverse or proximodistal duplication on the mature regenerate. Only proximodistal duplications were formed following intraperitoneal injection of retinoic acid during the early stages of limb regeneration. Both types of duplications were found on the regenerates of *T. vulgaris* at each of the larval stages studied. In contrast to the results with anurans (Niazi & Saxena, 1978; Maden, 1983*c*; Niazi & Ratnasamy, 1984) and the American newt (Thoms & Stocum, 1984) both types were not found on the same limb. The regenerated limbs which showed a duplication had either one or the other type.

Similarly, the limbs of *P. waltl* regenerated with either transverse or proximodistal duplications after retinoid treatment. With this species the type of duplication formed was a function of the stage of larval development at the time of limb amputation. Anterior limb buds from very young larvae regenerated with transverse duplications while the anterior limbs of older larvae as well as the posterior limbs having more than three digits regenerated with proximodistal duplications. Posterior limb buds from stages 45 and 51 *P. waltl* showed neither transverse nor proximodistal duplications after retinoid treatment. These limb buds did not completely regress and, therefore, a proximodistal duplication would

Table 6. *Dose-dependent effects on anterior limb regeneration of R.A. injected into stage 54+* Pleurodeles waltl *on various days after wrist level amputation**

					PD duplication	
Dose μg	Day	Number of limbs	Normal	Hypomorphic	Carpals	Zeugopod
25	2	10	7 (70)	—	3 (30)	—
50	2	10	2 (20)	—	2 (20)	6 (60)
100	2	10	—	—	1 (10)	9 (90)
150	2	10	—	—	—	10 (100)
200	2	8	—	—	—	8 (100)
25	4	10	3 (30)	—	4 (40)	3 (30)
50	4	8	5 (62·5)	—	2 (25)	1 (12·5)
100	4	10	—	—	3 (30)	7 (70)
150	4	8	—	—	—	8 (100)
200	4	8	—	—	2 (25)	6 (75)
25	6	10	1 (10)	—	1 (10)	8 (80)
50	6	10	2 (20)	—	—	8 (80)
100	6	10	—	—	—	10 (100)
150	6	10	—	—	—	10 (100)
200	6	10	—	—	—	10 (100)
25	8	10	—	3 (30)	—	7 (70)
50	8	10	—	4 (40)	—	6 (60)
100	8	10	—	1 (10)	2 (20)	7 (70)
150	8	10	—	—	—	10 (100)

* Numbers in parentheses indicate the percentage of limbs in each category.

have been seen in the mature regenerate if the retinol palmitate had provoked a modification of the positional information along this axis. The lack of response by developing posterior limbs remains to be investigated.

When treated in normal light the percentage of limbs regenerating with a duplication increased with the increasing age of the animals used. Treatment in the dark significantly increased the percentage of duplications found on younger larvae but not on older larvae. The degree of development was not changed by keeping the animals in the dark during the treatment period. Light is known to cause a degradation of retinoids (Alwood & Plane, 1984) and it is possible that when keeping the animals in normal light the retinol palmitate is quickly inactivated. This would mean that the amputated limbs were exposed to active retinol palmitate only once every two or three days, when the solution was changed. One possible explanation, therefore, for the low percentage of duplications found on young larvae would be that the sensitive period is less than two or three days long and a number of animals passed through the sensitive period in the presence of inactivated retinol palmitate. Treatment in the dark protected the retinol pamitate and allowed more animals to pass through the sensitive period in the presence of active retinol palmitate. The sensitive period is probably longer in older larvae, increasing the probability that the animals were in the sensitive

Table 7. *Dose dependent effects on posterior limb regeneration of R.A. injected into stage 54+* Pleurodeles waltl *on various days after ankle level amputation**

Dose μg	Day	Number of limbs	Normal	Hypomorphic	PD duplication		
					Carpals	Zeugopod	Stylopod
25	2	10	5 (50)	—	5 (50)	—	—
50	2	10	5 (50)	—	3 (30)	2 (20)	—
100	2	10	1 (10)	—	4 (40)	5 (50)	—
150	2	10	9 (90)	—	1 (10)	—	—
200	2	8	—	—	—	8 (100)	—
25	4	10	5 (50)	—	5 (50)	—	—
50	4	8	5 (62·5)	—	3 (37·5)	—	—
100	4	10	4 (40)	—	2 (20)	4 (40)	—
150	4	8	4 (50)	—	2 (25)	2 (25)	—
200	4	6	—	—	2 (33·3)	4 (66·6)	—
25	6	10	—	—	5 (50)	5 (50)	—
50	6	10	2 (20)	—	1 (10)	7 (70)	—
100	6	10	—	—	—	7 (70)	3 (30)
150	6	10	1 (10)	—	4 (40)	5 (50)	—
200	6	10	—	—	—	9 (90)	1 (10)
25	8	10	2 (20)	8 (80)	—	—	—
50	8	10	—	8 (80)	—	2 (20)	—
100	8	10	—	9 (90)	—	1 (10)	—
150	8	10	—	9 (90)	—	1 (10)	—

* Numbers in parentheses indicate the percentage of limbs in each category.

period when the treatment solution was changed and active retinol palmitate was present. This period of sensitivity would be greater than two or three days for stage-55 larvae which had 91 % of their regenerates duplicated after treatment in normal light.

A correlation between the retinoid dose and either the percentage of duplicated limbs or the degree of duplication was not found when the animals were treated in the light with retinol palmitate. This is in contrast to Maden's (1982, 1983*a,b*) results where the percentage of duplications and their degree of development was in relation to both the dose and duration of treatment. Such a dose–response relationship was found, however, when retinoic acid injections were used. These results are similar to those reported by Thoms & Stocum (1984) using the same technique with axolotls. A possible dose–response curve may exist in the experiments carried out in the dark on young larvae. The curve would be found with doses between $1\,\text{i.u.}\,\text{ml}^{-1}$ and $3{\cdot}75\,\text{i.u.}\,\text{ml}^{-1}$. Our experiments would not allow the determination of such a curve.

Whether the animals were treated in the dark or in the light the amputation stumps of limb buds or mature limbs often regressed. This regression was more extensive with the younger animals, extending to the level of the presumptive shoulder region in the youngest larvae tested. Stage-54 and -55 larval limb stumps generally did not regress. The regression was often followed by blastemal formation and growth. Duplicating blastemas were sometimes observed at the end of the treatment period. This is in contrast to the results reported by Maden (1982, 1983*a*) which demonstrated an inhibition of blastemal formation and a reduction of mitoses during treatment of amputated axolotl limbs with various retinoids. An inhibition of blastemal formation was also found in our experiments with older larvae of *Pleurodeles waltl*, the stages that did not exhibit a regression of the limb stump.

The correlation between the occurrence of extreme regression of the amputation stumps of very young larvae and the development of transverse duplications may not be fortuitous. The type of duplication formed may be in relation to the effective level of amputation, with more proximal levels increasing the tendency to form transverse duplications. If the stump regresses completely then a proximodistal duplication would not be possible. Preliminary experiments with retinoic acid injections have yielded a few limbs with both proximodistal and transverse duplications when the limbs were amputated at more proximal levels (data not shown).

Finally, very few complete limbs were formed from distal amputation planes in our experiments with *Pleurodeles waltl* while such complete proximodistal duplications have been obtained in all other amphibian species tested (Maden, 1982, 1983*a,b*; Niazi & Ratnasamy, 1984; Thoms & Stocum, 1984). This is evidently a characteristic of larval *Pleurodeles waltl* for increasing the dose of retinol palmitate 40 times over the minimal effective dose and that of retinoic acid four times still did not increase the degree of proximodistal duplication.

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