

## Further evidences of homeotic transformation in anuran tadpoles

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In this communication we report the development of ectopic supernumerary limbs (ESLs) from the cut ends of tails of *Bufo melanostictus* (Anura: Bufonidae), *Microhyla ornata* (Anura: Microhylidae) and *Hoplobatrachus tigerinus* (Anura: Ranidae) tadpoles exposed to various concentrations of vitamin A (Palmitate) solution. In addition, several other deformities (tail abnormality, outgrowth on tail tips, etc.) have also been recorded. Like the previous reports, the ESLs are all hindlimbs and comprise of both normal and abnormal hindlimb elements.

THE effect of tail tissue, its skin epidermis and dermis on the limb regeneration of salamanders *Triturus viridescens*<sup>1</sup> and *Siredon mexicanum*<sup>2</sup> had enough basis to influence researchers working on amphibian regeneration. Based on this, Iten and Bryant<sup>3,4</sup> reported the stages of tail regeneration and regeneration from different levels along the tail of the newt, *Notophthalmus viridescens*. Preceding the above finding was the report by Niazi and Saxena<sup>5</sup> highlighting the effect of vitamin A on tail regeneration of anuran tadpoles. Moreover, the reports on the inhibiting influence of vitamin A on tail regeneration in *Bufo andersonii*<sup>6</sup>, *Xenopus laevis*, *Notophthalmus viridescens* and *Ambystoma mexicanum*

tadpoles<sup>7</sup> confirmed the above report. In contrast, the inhibiting influence of vitamin A was prevented by the use of sulphadiazine<sup>8</sup> showing the antagonistic effects on vitamin A for tail regeneration of *B. melanostictus* tadpoles.

The finding demonstrating the development of ectopic supernumerary limbs (ESLs) at the site of tail amputation in *Uperodon systoma*<sup>9</sup>, was an ultimate breakthrough on vitamin A effect on the regenerating capacity of tails in anuran tadpoles. The phenomenon known as homeotic transformation was the first report in vertebrates, mediated through vitamin A. Thereafter, homeotic transformations have been reported in *Polypedates maculatus*<sup>10-14</sup> (rhacophorid), *Tomopterna rolandae*<sup>13,15,16</sup> (ranid), *Rana limnocharis*<sup>13</sup> (ranid), and *Rana temporaria*<sup>17</sup> (ranid) tadpoles exposed to various concentrations of vitamin A solution. Here we report additional information on homeotic transformation in three other anuran species.

The tadpoles of *B. melanostictus*, *M. ornata* and *H. tigerinus* used in the experiment were reared in the laboratory from egg clutches collected from breeding grounds during July 1994. Amputation was performed on tadpoles at Gosner stage 26 (hindlimb bud stage)<sup>18</sup>. The tadpoles were narcotized in 1:400 solution of MS222 in conditioned water. 128 of *B. melanostictus*, 60 of *M. ornata* and 9 of *H. tigerinus* tadpoles were used in the study. After tail amputation, the tadpoles were transferred to amphibian ringer and kept for about 10 min for recovery from anaesthetic condition and for

Table 1. Effect of vitamin A solution at the site of amputated tail of *Bufo melanostictus*, *Microhyla ornata* and *Hoplobatrachus tigerinus*

Species	Concentration of vitamin A	Hours of exposure	Number of tadpoles	Abnormal tail	Normal tail	Survival time range (days)	Number of tadpoles with ESLs
<i>B. melanostictus</i>	10 IU/ml vitamin A	24	5	2	3	5-96	-
		48	13	9	4	7-75	-
		72	27	20	7	6-46	-
		96	16	15	1	13-48	2
		120	13	9	4	7-50	1
		144	10	9	1	6-50	-
	15 IU/ml vitamin A	24	13	8	5	8-89	-
		48	11	9	2	9-70	-
		72	12	10	2	5-44	1
		96	8	5	3	5-44	-
	Control	-	5	-	5	15*	-
<i>M. ornata</i>	30 IU/ml vitamin A	72	55	19	36	6-86	1
	40 IU/ml vitamin A	48	5	2	3	3-86	1
	Control	-	5	-	5	40*	-
<i>H. tigerinus</i>	20 IU/ml vitamin A	96	9	6	3	20-25	1
	Control	-	5	0	5	25*	-

\*Days for completion of metamorphosis; ESLs, Ectopic supernumerary limbs.



coagulation of blood at the amputation site. For each species, the control group consisted of 5 tadpoles, reared in 500 ml of conditioned water after tail amputation. The rest of the tail amputated tadpoles were treated with various concentrations of vitamin A solution for different time intervals. After exposure to vitamin A solution, the tadpoles were reared in conditioned water

until death or emergence of forelimbs. Table 1 shows the number of tadpoles, concentration of vitamin A solution and hours of exposure.

In *B. melanostictus* (Figure 1 a-f), the control tadpoles metamorphosed within 15 days of amputation and there was no structural abnormality in both the fore- and hindlimbs. Some of the experimental tadpoles survived up to a maximum of 96 days in 24 h exposure to vitamin A solution. However, none of the tadpoles metamorphosed and they died at some stage of development, during the period when a tail stump differentiated into a hindlimb. Out of 128 experimental tadpoles (Table 1), 25% regenerated normal tail. The rest developed abnormal tails with various kinds of morphological abnormalities. The regenerated tails had tail fins that did not cover the tail tip and there was cellular mass protruding from the tip of the axial tissue. In some, the dorsal fin extended along with the tail musculature curving downwards, while the ventral fin narrowed and was suppressed from where the tail tissue began its curve. Others had bent tail fins, ventral fin broader and covering the tail tip, while the dorsal fin narrowing mid way. A swelling that developed in the tadpoles at the amputation site had reduced tail fins. In addition to the abnormal tail tips, the fore- and hindlimbs were also deformed. The hindlimbs had reduced shank and the forelimbs were without distinct humerus, radio-ulna and with 6 fingers. There was also the development of extra paired hindlimbs at the site of the growth of normal hindlimbs.

Ectopic development of limbs at the amputation site was observed in 2.3% of experimental tadpoles. In addition, there was also regeneration of tail with laterally curved axial tissue and tail fins along with the development of ESLs. The ectopic hindlimbs also developed in pairs having distinct thigh, shank, ankle and digits. In some, the shank and ankle were suppressed, thighs fused and the 4th and 5th digits joined (Table 2).

In *M. ornata* (Figure 2 a-d), the control tadpoles metamorphosed within 40 days of amputation, without exhibiting any abnormality. The experimental tadpoles (Table 1) had a maximum survival period of 86 days and 65% of them developed normal tails while the rest regenerated abnormal tails. The abnormalities included

**Figure 1 a-f.** *Bufo melanostictus*. a, Development of cellular outgrowth and a pair of abnormal ESL with suppressed shank and ankle (exposure: 10 IU vitamin A for 120 h); b, Development of three normal ESLs (exposure: 10 IU vitamin A for 96 h); c, Development of a pair of ESL at the limb bud stage, along with a swelling (exposure: 10 IU vitamin A for 96 h); d, Development of a pair of abnormal ESL with jointed thighs, 4th and 5th digits of one ESL fused (exposure: 15 IU vitamin A for 72 h); e, Development of abnormal tail tip, the hindlimbs with reduced shank and the forelimbs without distinct humerus, radio-ulna and with six fingers (exposure: 10 IU vitamin A for 96 h); f, Development of abnormal tail tip and extra pair of hindlimb at the site of the growth of normal hindlimbs (exposure: 10 IU vitamin A for 96 h).

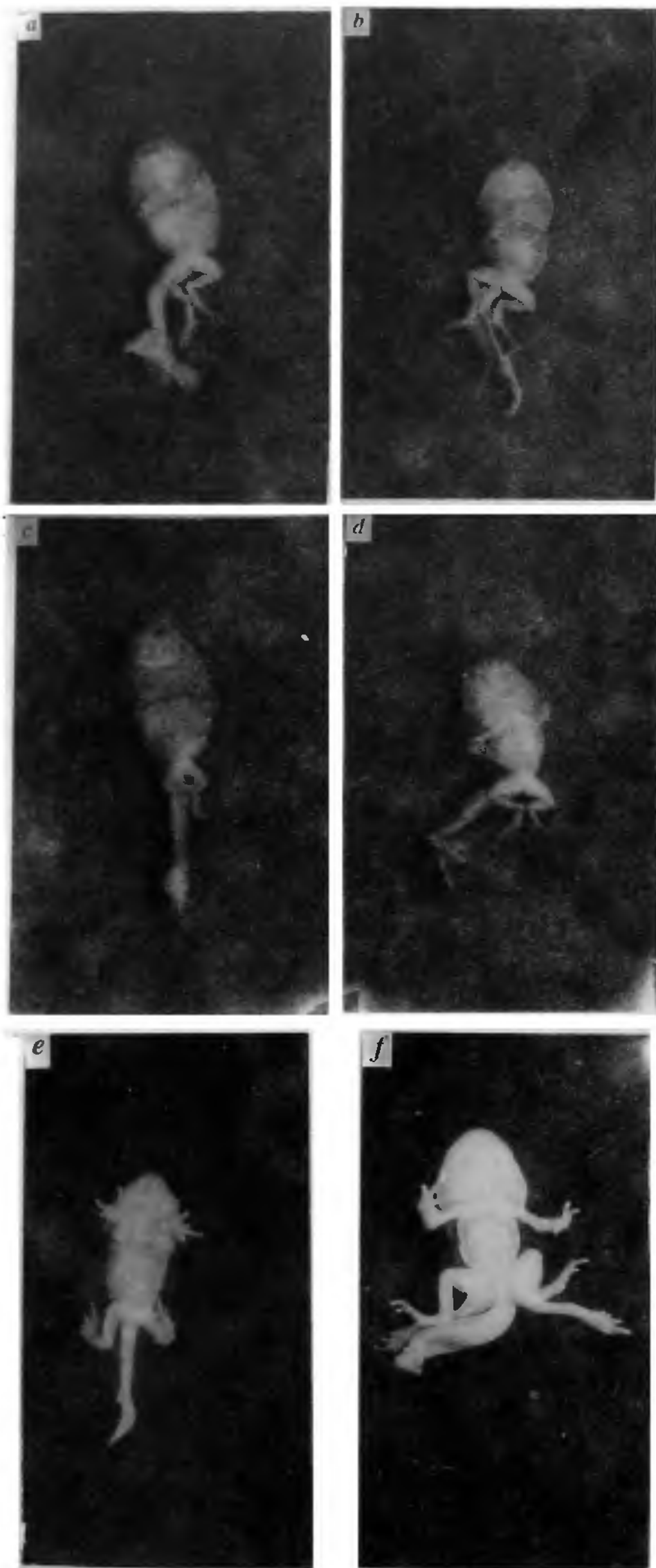
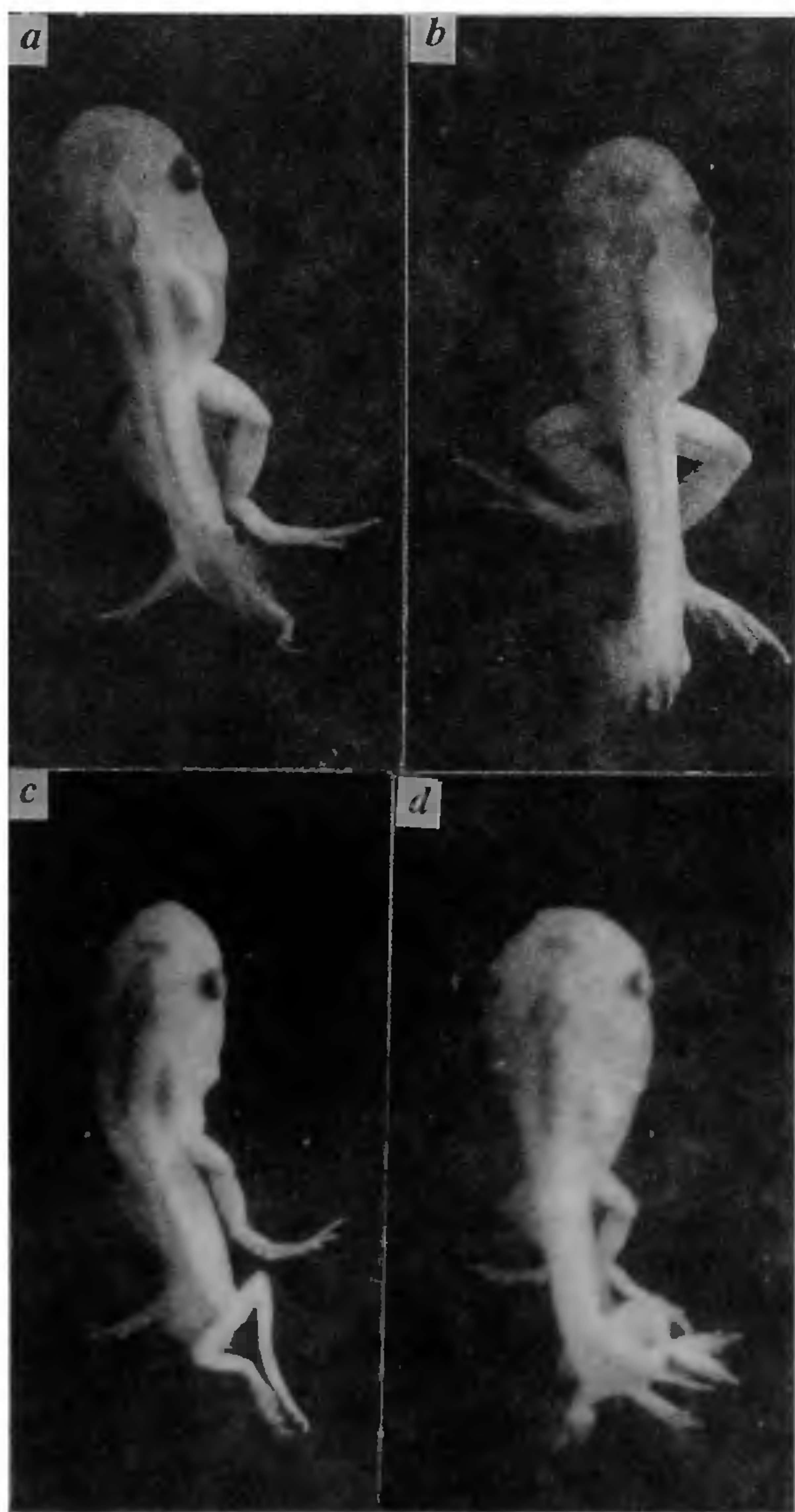




Table 2. Analysis of ESLs of *Bufo melanostictus*, *Microhyla ornata* and *Hoplobatrachus tigerinus*

Species	Group	Number of tadpoles with ESLs	Number of ESLs	Normal ESLs	Abnormal ESLs	Abnormality/normality of the ESLs
<i>B. melanostictus</i>	10 IU (96 h)	2	(i) 1 (ii) 3	— 3	1 —	Limb bud, thigh, shank ankle and digits, well developed
	10 IU (120 h)	1	2	—	2	Shank and ankle, suppressed in both the ESLs
	15 IU (72 h)	1	2	—	2	Jointed thighs digits 4th and 5th attached
<i>M. ornata</i>	30 IU (72 h)	1	4	2	2	Jointed thighs
	40 IU (48 h)	1	2	2	—	Distinct thigh shank, ankle and digits
<i>H. tigerinus</i>	20 IU (96 h)	1	2	—	—	Limb bud



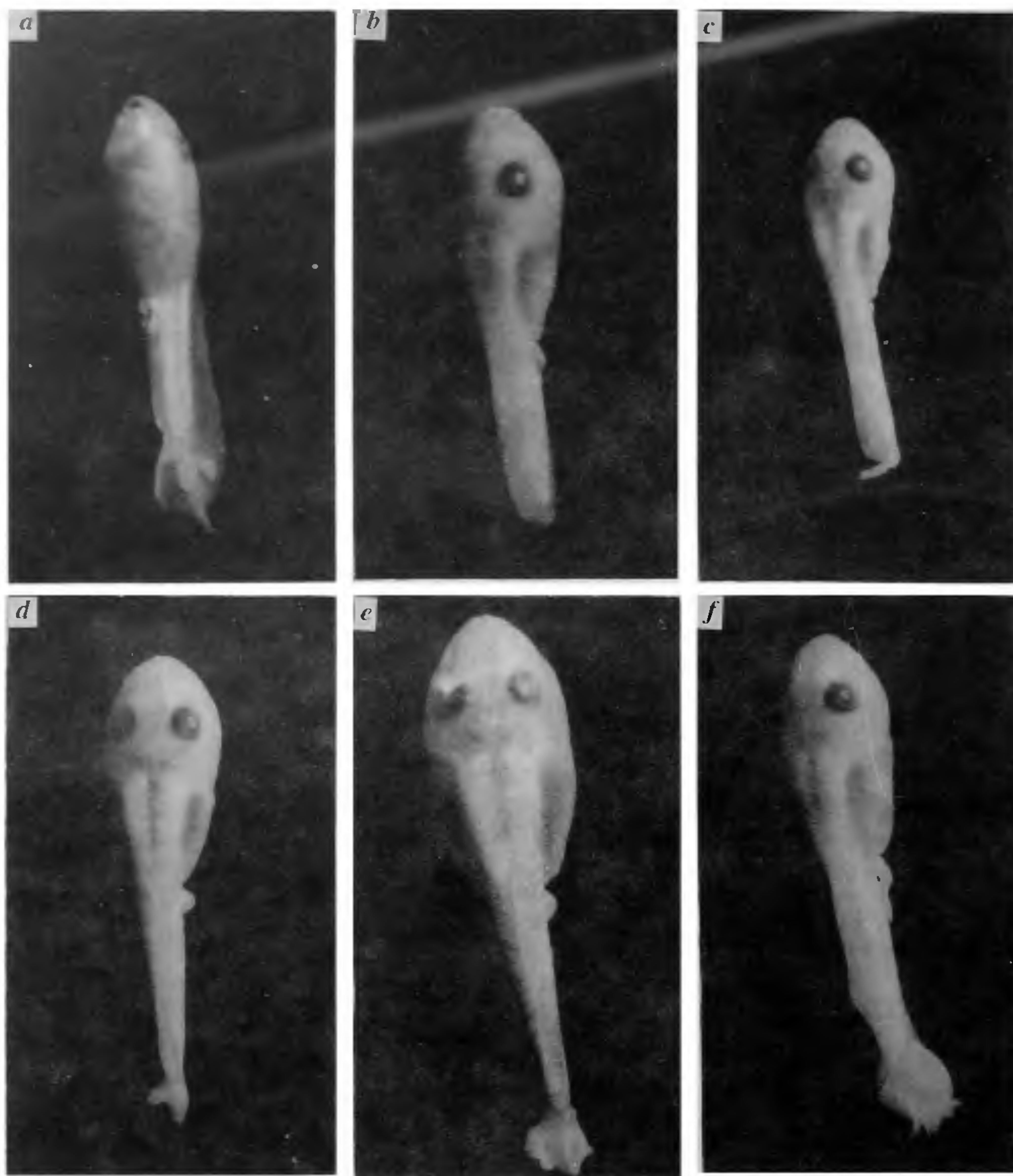
growth of irregular cell mass at the amputation site, deshaped tail fins, loss of fins and small outgrowths ventro-laterally on the regenerating tail, and development of ESLs (3.3%) at the site of amputation. The ESLs besides having distinct thigh, shank, ankle and digits also had fused thigh (Table 2).

In *H. tigerinus* (Figure 3 a-f), the control tadpoles metamorphosed within 25 days of amputation and like the other two species, there was no abnormality in their limb or tail. Like the controls some of the experimental tadpoles survived for a maximum of 25 days, but none of them metamorphosed (Table 1). The abnormalities in tail regeneration included the development of irregular cell mass at the site of amputation, deshaped tail musculature and fins and development of ESLs. The abnormality was 66.7% and out of the total abnormal tadpoles, 11.1% showed ectopic limb development covered externally by the swellings (Table 2).

The above results indicate variation in the time period of completion of metamorphosis in the control groups and this is species-specific. In the experimental tadpoles, the maximum survival time was also recorded for *B. melanostictus* exposed to 10 IU/ml vitamin A solution for 24 h. Besides, the highest percentage (75%) of abnormality was also recorded for the species. The number of ESLs was the highest (4) in *M. ornata*. All the tadpoles of *H. tigerinus* sustained a high dose of vitamin A (20 IU) solution for a longer period and mortality was recorded from the 20th day of treatment.

Figure 2 a-d. *Microhyla ornata*. a, Regeneration of a curved tail and deshaped tail fin (exposure: 30 IU vitamin A for 72 h); b, Cellular outgrowths at the amputation site (exposure: 30 IU vitamin A for 72 h); c, Development of a pair of ESLs (exposure: 40 IU vitamin A for 48 h); d, Development of 4 ESLs; one pair normal and the other pair abnormal with jointed thighs (exposure: 30 IU vitamin A for 72 h).





**Figure 3 a-f.** *Hoplobatrachus tigerinus* tail amputated tadpoles, exposed to 20 IU vitamin A solution for 96 h. **a**, Development of a cell mass and folding of the tail fins at the amputation site; **b**, A small swelling at the amputation site covered by the tail fins; **c**, Loss of fins at the tail tip; **d**, Abnormal tail regeneration, with a small outgrowth at the tail tip; **e**, Development of irregular cell mass at the tail tip; **f**, Development of irregular cell mass with ESLs (minute).

The experimental tadpoles of all three species exhibited delayed growth as reported earlier for *R. temporaria*<sup>17</sup> (ranid) and *P. maculatus*<sup>14</sup> (rhacophorid) tadpoles. In addition, the mortality was not time-dependent and there was no correlation between mortality and the duration of exposure to vitamin A solution, unlike that reported for *P. maculatus*<sup>14</sup>, *B. andersonii*<sup>5,6</sup> and *R. cyanophlyctis*<sup>6,19</sup>.

The experimental tadpoles also regenerated abnormal

tails as reported for *U. systoma*<sup>9</sup>, *B. andersonii*<sup>5,6</sup>, *X. laevis*<sup>7</sup>, *N. viridescens*<sup>7</sup>, *A. mexicanum*<sup>7</sup>, *P. maculatus*<sup>12,14</sup> and *T. rolandae*<sup>15</sup>. The most fascinating finding was the development of ectopic hindlimbs in all the species. However, the percentage (4.1%) was lower than earlier reports<sup>12,14,17</sup>. Vitamin A palmitate administered for at least 48 h showed the induction of limbs at the site of tail amputation in *B. melanostictus*, and *M. ornata* tadpoles. *H. tigerinus* tadpoles needed longer exposure



(72 h) to vitamin A solution. Hence, it is concluded that 48–72 h was the required duration of treatment for the development of ectopic limbs as has also been reported for *R. temporaria*<sup>17</sup> (3 days as the threshold level) and for *P. maculatus*<sup>14</sup> (48 h as the minimum exposure time). However, in *P. maculatus*<sup>12</sup>, *T. rolandae*<sup>15</sup> and *R. limnocharis*<sup>13</sup> ectopic limbs developed when the exposure time was for 24 h. Thus, it can be inferred that the induction of ectopic limbs is not time-dependent as otherwise proposed by Maden<sup>17</sup>.

It has been interpreted that hindlimb development is marked by the rising levels of thyroid hormone (TH)<sup>20</sup>. It is likely that during the regeneration process<sup>21</sup> at the amputated site, the TH combines with vitamin A palmitate and alters the tail hox code while repressing other genes resulting in the formation of limb blastema cells, particularly the ones that give rise to hindlimb fields<sup>17</sup>.

The effect of vitamin A palmitate at the tail amputated site of tadpoles causing homeotic transformation may be attributed to the conversion of the tail blastema cells to that of limbs. This changes the positional values at the tail tip. Subsequently, rearing in conditioned water after exposure to vitamin A, reverses the polarity along the rostral–caudal axis to generate ectopic limbs<sup>22</sup>. Some of the ectopic limbs develop from cellular mass at the site of amputation and mostly in a ventro-lateral direction similar to those observed in *P. maculatus*<sup>12,14</sup> and *T. rolandae*<sup>13,15</sup>. This is comparable to the finding in *B. andersonii*<sup>6</sup> in which continued exposure to vitamin A palmitate resulted in the folding of the fin to form a pouch at the tip of the regenerating tail. The cellular mass which is formed prior to the development of ectopic limbs may act as a reservoir for the transformed cells which needs to be investigated through histology. There was also regeneration of a complete tail that followed the growth of ESLs in *B. melanostictus* tadpoles as reported in *P. maculatus*<sup>12</sup>, *T. rolandae*<sup>15</sup> and *R. limnocharis*<sup>13</sup>. This is expected, in accordance with the interpretation schematically proposed by Bryant and Gardiner<sup>22</sup>. However, the interpretation of development of paired ectopic hindlimbs<sup>22</sup> is contradictory to the present finding as odd number (3) of ectopic limbs has been observed in *B. melanostictus* tadpoles as was also found in *T. rolandae*<sup>15</sup> and *R. limnocharis*<sup>13</sup>. Further, with the growth of an abnormally regenerated tail, there was also the development of extra-paired hindlimbs at the site from where only a single pair of hindlimb was supposed to develop. This observation was previously reported in *B. vulgaris*<sup>23</sup> and again in *U. systoma*<sup>9</sup>. These deformities could be related to the embryonal malformations caused by vitamin A<sup>24</sup>. In addition to the findings on *U. systoma*<sup>9</sup>, *R. temporaria*<sup>17</sup>, *T. rolandae*<sup>15</sup>, *R. limnocharis*<sup>13</sup> and *P. maculatus*<sup>10,12,14</sup>, the present study adds further to our knowledge on homeotic transformation in anuran tadpoles.

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## How does the nocturnal animal, *Mus booduga*, programme its activity in response to varying durations of light and darkness?

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In the temperate zone, animals adjust to various durations of light and dark by scaling the duration of activity with the durations of light (diurnal animals) or dark (nocturnal animals). They are also capable of advancing or delaying their activity depending on the durations of light or darkness. This study examines whether tropical animals which do not normally experience much variations in durations of light and