Proc. Indian natn Sci Acad. **B69** No. 5 pp 673-690 (2003)

Vitamin A, Regeneration and Homeotic Transformation in Anurans

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(Received on 12 April 2001; Accepted after revision on 26 September 2001)

Vitamin A has profound effects on development and regeneration. Out of all the effects the most surprising is its ability to convert amputated tails into limbs in anurans considered to be the first report of complete homeotic transformation in vertebrates. In this paper I present a comprehensive account of the effects of vitamin A with special reference to homeotic transformation in several species of anurans. In general, vitamin A increased mortality, interfered with growth and regeneration however, it also caused homeotic transformation in a species specific manner. Morphological, skeletal and histological studies showed that the ectopic limbs at tail are comparable to the normal hind limbs although they developed away from the tissues normally associated with limb development. Obviously, vitamin A respecified the tail tissue into limb tissue probably by switching off the tail specific Hox genes followed by activation of limb specific Hox genes. The whole segment with axial elements, pelvic girdle and the limbs are induced. Therefore, this being the first repeatable homeosis in Batesonian sense, the model can be used for molecular and genetic studies of homeosis for the first time in vertebrates.

Key Words: Regeneration, Vitamin A, Homeotic transformation, Anurans, Morphology, Histology

Introduction

It has been acknowledged internationally that some of the breakthrough work, as far as vitamin A related regeneration research is concerned, has been done by scientists in India. The laboratories who have contributed significantly are Niazi's laboratory in University of Rajasthan and our laboratory in Utkal University.

According to Okada (1996), Niazi's discovery on the effect of vitamin A on amphibian limb regeneration is a milestone on the long tradition of regeneration research which revitalised the research on replication of pattern. He also stated that the discovery of heteromorphic regeneration of tadpoles elicitated by vitamin A (Mohanty-Hejmadi et al. 1992) is also of great historical significance. Further, Maden (1993) has also expressed the view that this phenomenon of homeotic transformation of tadpole tails into legs clearly represents a major advance in the study of the genetic basis of pattern formation in vertebrates because it presents us with the opportunity to identify the genes that are activated or repressed by retinoid treatment of tail cells during their respecification into limb cells.

With this preamble I would like to discuss the background on regeneration research leading to our discovery of homeotic transformation of tails into legs after vitamin A treatment and the follow up work in our laboratory as well as those of others.

In the middle of the 18th century the phenomenon of regeneration was first observed with the discovery that *Hydra*, a freshwater polyp, could generate a complete body from a small piece (Trembley 1744). Subsequently, Spallanzani (1768) reported that a variety of amphibians could regenerate their legs and tails following amputation. As reported by Maden (1994) over the period of 200 years since Spallanzani first scientifically described the phenomenon of limb regeneration, many substances have been applied to regenerating limbs. All of them without exception, either had no effect or inhibited the process of regeneration. Therefore, the discovery of the remarkable property of vitamin A to influence pattern formation in a specific manner

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is considered as one of the significant discoveries on regeneration studies in the field of developmental biology. Vitamin A is a generic term referring to all compounds from animal sources that exhibit the biological activity of vitamin A. They are retinol, retinal and retinoic acid. In terms of efficacy in inducing pattern abnormalities, Maden (1982) has reported the following hierarchy: retinol acetate<retinyl palmitate<retinoic acid. Out of these retinyl palmitate (vitamin A) and retinoic acid (RA) are commonly used in regeneration research.

The history of regeneration research took a dramatic turn with the discovery of Niazi and Saxena (1978) that when the hind limb buds of the toad *Bufo andersonii* were amputated through the shank and treated with retinyl palmitate (vitamin A), instead of growing just the missing parts, the regenerates seemed to contain extra elements in the proximo-distal axis: often two regenerates appeared instead of one. As described by Niazi (1996) theirs was perhaps the only laboratory working on the effect of vitamin A on regeneration of amphibians from 1967 to 1980. It was later found that vitamin A can induce an alternation of pattern in both developing and regenerating limbs. In regenerating amphibian limbs, retinoids (RA) can lead to pattern duplication in the proximo-distal, antero-posterior and dorso-ventral axes (Niazi & Saxena 1978, Maden 1982, 1983, Ludolph et al. 1990). Local application of RA to the anterior side of a developing limb bud causes duplications in the antero-posterior axis of the limb (Tickle et al. 1982, Summerbell 1983). The diversity of retinoid effects on differentiation and morphogenesis reflects not only different effects on different tissues but also different effects on the same during different stages of development (Johnson & Scadding 1991). That endogenous RA may actually be involved as a morphogen during limb development, has gained ground during the last few years. Thaller and Eichele (1987) even reported on the presence of endogenous RA in chick limb buds. They reported further that the level of retinal, a precursor of RA, is uniform and high in limb buds but RA itself is differentially distributed with a 2.5 fold enrichment in the posterior three-fourths. They reported later (Thaller & Eichele 1988) that posterior limb cells are capable of synthesizing RA from retinal. Furthermore, the discovery of several nuclear receptors for RA belonging to the steroid hormone receptor family (Mendelsohn et al. 1992) and homologous receptors with unknown ligands (Mangelsdorf et al. 1990) went in favour of endogenous RA levels leading to differential gene expression leading to pattern formation. Tabin (1991) has provided an extensive list of the limb bud expression patterns of retinoic acids and their binding proteins during limb development. Further Krumlauf (1994) has elaborated on the colinear response of Hox genes to RA and multiple examples of RA-induced alterations to Hox expression in a variety of vertebrate embryos.

There are several review articles now available on limb regeneration under the influence of vitamin A (Bryant & Gardiner 1992, Maden 1982, 1996, Niazi 1996) as well as on its implications (Okada 1996). Some of the important findings in regeneration have been cited by Okada (1996). Therefore, only pertinent material for tail regeneration is discussed here. It is interesting to note that while extensive work has been done on the effects of vitamin A on the regeneration of limbs, regeneration of tail has received very little attention. This is inspite of the fact that Niazi started his work on tail regeneration. They observed that vitamin A has an inhibitory and modifying influence on tail regeneration in *B. andersonii* tadpoles (Niazi & Saxena 1968). A positive relationship was later found between the inhibitory influence of vitamin A and the developmental stages of the regenerating tails of the same species (Niazi & Saxena 1979). Scadding (1987) found that vitamin A inhibits tail regeneration in *Xenopus laevis, Notophthalmus viridescens* and *Ambystoma mexicanum*. Since vitamin A inhibits tail regeneration but induces duplication in regenerating limb, Scadding (1987) has opined that the morphogenetic processes involved in tail regeneration are atleast in some way different from those occurring in limbs. Iten and Bryant (1976a) have examined tail regeneration by amputating the newt tail at different levels such that three-fourths, half or one-fourth of the tail was removed in order to examine the influence of the level of amputation on the growth of tail regenerates through different phases of tail regeneration. During the first two phases and the beginning of the third phase of regeneration, the specific growth of regenerates from different levels are not significantly different.

However, later when extensive differentiataion and morphogenesis occur in the regenerate, the rate is higher at more proximal level of amputation. Similar observations have been made by Mufti (1973) during tail regeneration in *Triturus viridescens.* Further, Niazi (1970) has demonstrated that the rate of tail regeneration in *Rana sylvatica* tadpoles decreases with age and the retardation is due to the degree of differentiation. Mohanty-Hejmadi and Parida (1984) have shown that the rate of regeneration under normal conditions without any treatment slowed down in the late hindlimb bud stage tadpoles of *B. melanostictus*, causing a prolongation of life history in experimental ones. At the initiation of metamorphosis there was no regeneration perhaps due to the inhibitory effect of thyroxine.

Thus, treatment of regenerating tail with retinoids leads to inhibition of regeneration in *B. andersonii* (Niazi & Saxena 1968), *N. viridescens, A. mexicanum*, and *X. laevis* (Scadding 1987). Further, we showed that in addition to the inhibition of tail regeneration, limbs were generated at the site of amputation in *Uperodon systoma* (Mohanty-Hejmadi et al. 1992). This was the first clear demonstration of homeotic transformation in any vertebrate. Further, we have reported inhibition of tail regeneration and homeotic transformation in several species namely in *Polypedates maculatus; B. melanostictus, Microhyla ornata* and *R. tigerina* (Mahapatra 1993, Mahapatra & Mohanty-Hejmadi 1994, Das 1998) which have been confirmed by Das and Dutta (1996). The skeleton of the ectopic limbs were also examined (Mohanty-Hejmadi et al. 1992, Mahapatra 1993, Mahapatra & Mohanty-Hejmadi 1994 and Das 1998). The study was extended to histological changes during homeotic transformation (Das & Mohanty-Hejmadi 1995, 1998a, 1998b, 1999). Our observations have been confirmed in *R. temporaria* (Maden 1993, Müller et al. 1994,1996) and in *R. ridibunda* (Müller et al. 1994). Since our discovery of homeotic transformation of tails to limbs in several species of anurans under the influence of vitamin A has opened up a new dimension and has confirmed the critical role of vitamin A in pattern re-specification, I have tried to put together the important information available and their implications in this paper. In this process, much of the data presented in this paper is an attempt to consolidate the available information in the field.

Materials and Methods

The materials and methods have been given in detail in our earlier papers. Hence, only a brief version is given here. The egg mass of all anurans were collected from nature during the rainy season and raised in our hatchery upto the hindlimb bud stage. Only full siblings derived from the same batch of eggs were used for each set of experiment to avoid genetic variability. The tadpoles were anaesthetised in MS 222 and their tails were amputated in the middle following which they were immersed in water containing vitamin A (Arovit; Roche) of desired concentration for different time periods. The controls were also reared in a similar manner but without vitamin A treatment. The tadpoles were allowed to grow till the emergence of forelimbs or till their death, whichever was earlier. One advantage was that we had access to several species of anurans occupying different niches. Hence, we could observe regeneration in a variety of species and picked up many interesting species specific features.

For histological study, the tadpoles were treated for 72 hr and fixed at 24 hr intervals from days 1-6, and on such days when any morphological abnormality was noticed. They were later processed, sectioned and stained using Mallory's triple stain for examination in light microscope. Scanning electron microscopic studies were done in *Institut de recherches clinique de Montreal* by standard method.

For skeletal preparation, the preserved tadpoles were stained differentially by alcian blue and alizarin red technique (Wassersug 1976). For nerve preparation, the tadpoles were subjected to Sihler's differential staining technique (Williams 1943).

Effects of Vitamin A

Vitamin A had diverse effects on tadpoles. It affected mortality, growth and regeneration of tail. However, in some cases it caused heteromorphosis of tail into limbs. The following is a summation of the results.

Mortality

In general, a positive relationship was found between mortality and the duration of exposure by vitamin A. In *U. systoma* 30 to 70% of the tadpoles died before

the emergence of forelimbs maximum being with 72 hr treatment (Mohanty-Hejmadi et. al. 1992); in *P. maculatus* mortality was only 30% with 120 hr and 144 hr treatment (Mahapatra & Mohanty-Hejmadi 1994); in *B. melanostictus*, there was 10% to 60% mortality with maximum being with 72 hr of treatment (Mahapatra et al. 2001a). The tadpoles did not survive beyond 72 hr perhaps because of the small size in comparison to other species. In *M. ornata* the mortality ranged from 60% to 100%, the maximum being with 144 hr of treatment (Mahapatra et al. 2001a). However, if one takes total mortality into account irrespective of length of treatment, maximum death was observed in *M. ornata* (90%) and the minimum in *P. maculatus* (figure 1).

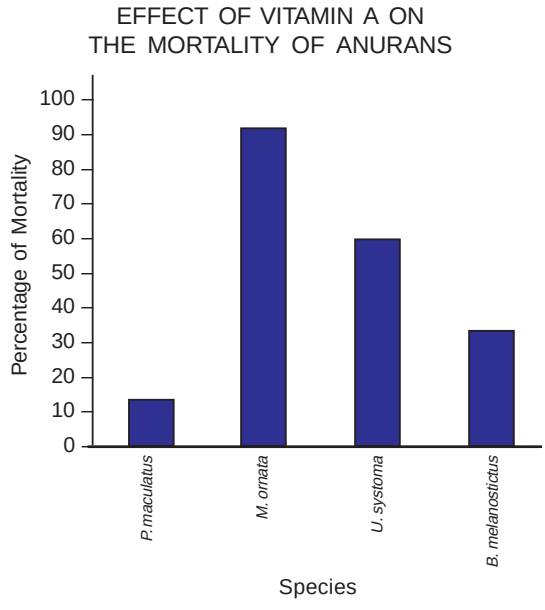


Figure 1 Cumulative effect of vitamin A on the mortality of anurans.

Similar exposure-related mortality has been reported in *B. andersoni* (Niazi & Saxena 1968, Saxena & Niazi 1977) and in *R. cyanophlyctis* (Niazi & Saxena 1972). Based on the results of exposure to 10 IU/ml retinyl palmitate from 1 to 7 d, Maden (1993) has also reported mortality directly related to exposure time in *R. temporaria*. Further when *R. temporaria* tadpoles of different stages were exposed to same concentration of vitamin A, there was higher mortality with younger tadpoles (Müller et al. 1996 see table 1).

Life Cycle

In general, metamorphosis was delayed by vitamin A treatment (figure 2). In *U. systoma*, metamorphosis occurred within 13.6 d in the control which was extended upto 24.8 d with 144 hr treatment (Mohanty-Hejmadi et al.1992). In the control tadpoles of *P.maculatus*, metamorphosis was completed in 27.4 d which was extended upto 50.4 d after 72 hr of treatment (Mahapatra & Mohanty-Hejmadi 1994). Similarly, in *B. melanostictus* metamorphosis was completed in 13.4 d in control but was extended upto 23.7 d with 72 hr treatment and in *M. ornata* metamorphosis was completed in 33.4 d extending upto 51.8 d with 120 hr of treatment (Mahapatra et al. 2001a).

Prolongation of life cycle has also been reported in *R. cyanophlyctis* (Niazi & Saxena 1972) and *R. breviceps* (Sharma & Niazi 1983) on exposure to vitamin A.

Size (Snout to Tail Tip Length)

Vitamin A retarded growth in all the species. The size *i.e.* snout to tail tip (S-T) length at metamorphosis was reduced with vitamin A

Table 1 *Tail abnormalities in different anurans (in percent) after vitamin A treatment*

Type of abnormalities	*P. maculatus*	*M. ornata*	*U. systoma*	*B. melanostictus*	*R. tigerina*
Normal tail	8.33	26.6	1.6	0	16
Blunt tail	0	16.6	19.2	3.3	19.5
Pouched tail	0	25	31.6	3.3	0
Bulbular mass at the tail	16.6	2.2	3.3	7.3	11.5
Upwardly curved tail	5	5.8	5.8	11.3	1.1
Downwardly curved tail	16.7	3.3	3.3	9.6	0
Laterally curved tail	3.3	0	0	0	0
Suppression of ventral fin	0	1.6	0	20	10.5
Suppression of dorsal fin	4.5	0	0	4.5	2.5
Suppression of both fins	11.6	1.6	0	0	2
Development of EHLs	11.6	5	21.6	6.6	6.6

treatment in comparison to the controls (figure 3), the extent of reduction being directly related to the period of treatment. In *U. systoma* the S-T length for control was 31.4 mm which was reduced to 17.8 mm with 144 hr of treatment (Mahapatra et al. 2001a). In *P. maculatus* the same were 38.6 mm and 25.1 mm with 144 hr treatment, respectively (Mahapatra & Mohanty-Hejmadi 1994). In *B. melanostictus* the S-T length for the control was 18.7 mm while in the treated it was 14.3 mm at 72 hr of treatment and in *M. ornata* the S-T length was 22 mm in the control and 17.3 mm at 96 hr of treatment.

These results are consistent with the observations for *B. andersonii* (Niazi & Saxena 1968), *R. cyanophlyctis* (Niazi & Saxena 1972), and *R. breviceps* (Sharma & Niazi 1983). They found a retardation in the overall body growth and a reduction in the size of the thyroid gland after treatment with vitamin A. Vitamin A is known to have an indirect influence on the pituitary gland (Sadhu 1948) as a result of which the development of the thyroid follicles is impaired due to improper secretion of TSH. This results in the improper secretion of thyroxine thereby inhibiting the growth and metamorphosis in frog larvae (Niazi & Saxena 1972). Prolongation of the life cycle and retardation in growth after treatment with vitamin A in the anurans studied by us might also be due to suppression of thyroid gland development.

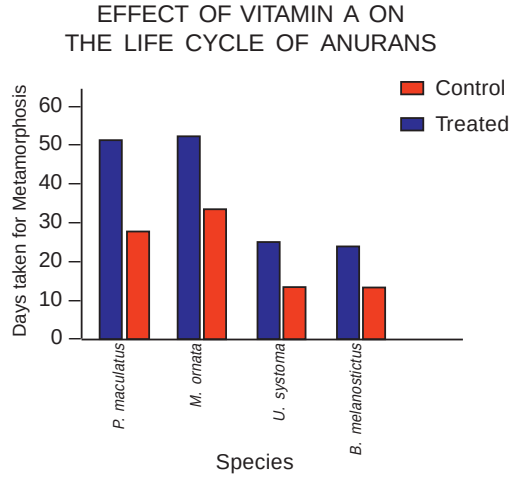


Figure 2 Cumulative effect of vitamin A on the life cycle of anurans.

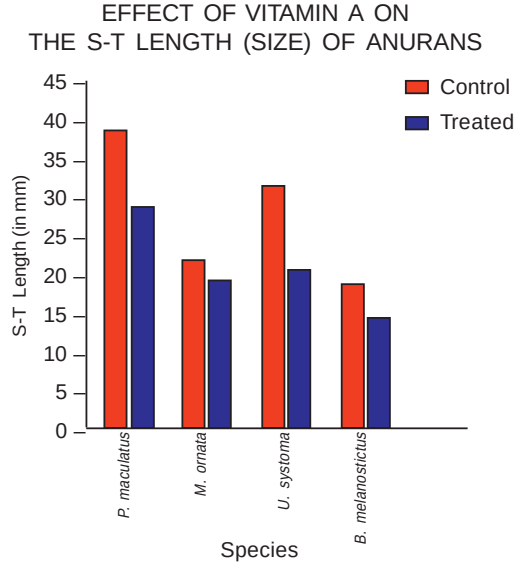


Figure 3 Cumulative effect of vitamin A on the size (S-T length) of anurans.

Tail Abnormalities

There was direct correlation between abnormal tail regeneration and duration of exposure. Tail abnormality included suppression of regeneration, bulbular mass formation at the amputated tail end, suppression of dorsal, ventral or both fins, and upward, downward or lateral curvature of axial tissues. Most unexpected effect was the development of hind limbs at ectopic sites (figure 4) which is dealt separately. Formation of ectopic hind limbs (EHLs) did not interfere with metamorphosis. However, the tail was not absorbed in most cases (figures 5-8). Occasionally, the tail was absorbed resulting in the EHLs connectiong to the rump at the end of metamorphosis (figure 9). These EHLs were

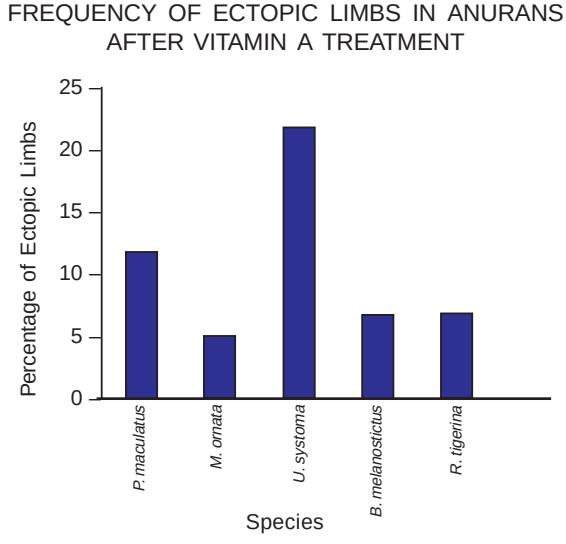
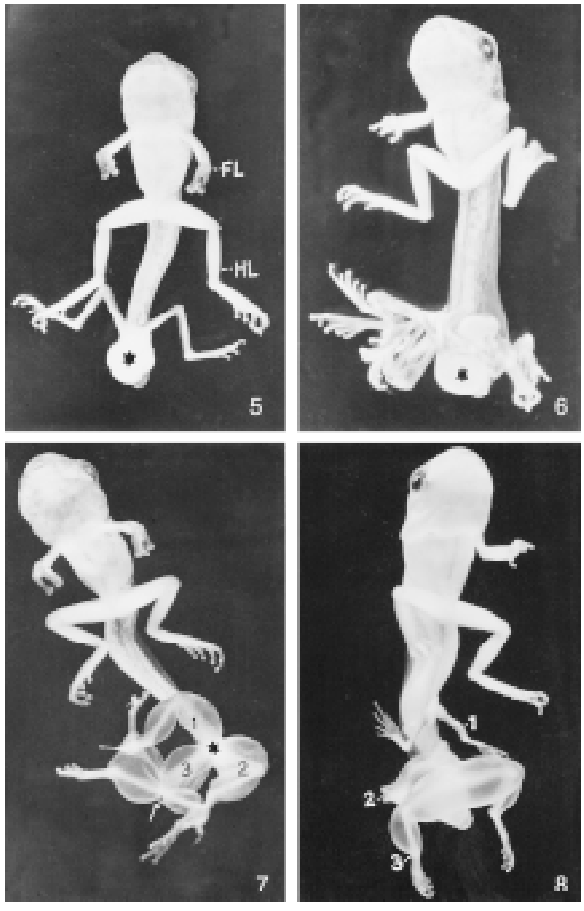


Figure 4 Cumulative data on the frequency of Ectopic Hind Limbs (EHLs) in anurans after vitamin A treatment.



Figures 5-8.5, Paired EHLs in *P. maculatus* (treated) seen arising proximal to the bulbular mass (asterisk), normal hind limbs (HL) and fore limbs (FL); **6** Multiple EHLs in *P. maculatus* (treated) also seen proximal to the bulbular mass (asterisk); **7** 3 EHLs (1,2,3) are seen arising from a common origin (asterisk) on the ventral side of the tail in *P. maculatus* (treated). EHL 1 is upwardly directed. EHL 2 is downwardly directed. EHL 3 arises from the middle and shows proximo-distal duplication (arrow) at the shank level. All the EHLs are without muscles and are oedematous; **8** EHLs arising in tandem from the ventral side of the tail in *P. maculatus*(treated). EHLs 1 and 3 are paired; EHL 2 is single and EHLs 2 and 3 are oedematous.

later shed from the body. This shows that in frogs it will not be possible to detect homeotic transformation under natural conditions as the EHLs will be shed after metamorphosis and hence, only tadpoles in nature may show this phenomenon. This is vindicated by the fact that Dr. Amano of Yoshizato Morphomatrix Project in Tsukuba, Japan has brought to my notice a 300-year old sketch of a "Three legged frog" (*Bufo japonica*) with two hindlimbs and one at the tip of the tail, collected from Tokyo area.

In *U. systoma*, it has been reported that tail regeneration was suppressed with longer treatment (Mohanty-Hejmadi et al. 1992). In *P. maculatus*, vitamin A treatment inhibited normal tail regeneration (Mahapatra & Mohanty-Hejmadi 1994). There was direct correlation between abnormal tail regeneration and duration of exposure. With 24 hr exposure, 60% of the tadpoles developed abnormal tail which increased to 100% by 96 hr of treatment. In *M. ornata*, 24 hr treatment had no adverse effect on tail regeneration. With 48hr and 72 hr treatment, 60% and 80% regenerated abnormal tails, respectively. Beyond 72 hr, tail regeneration was abnormal in 100% tadpoles (Mahapatra et al. 2001a). There was 98.4% tail abnormality in *U. systoma*, 91.7% in *P. maculatus*, 78.4% in *M. ornata*, 100% in *B. melanostictus* (Mahapatra et al. 2001a) and 80.8% in *R. tigerina* (Das 1998). A summary of all types of tail abnormalities is presented in table 1.

Similar suppression of tail regeneration after vitamin A treatment has been reported in *B. andersonii* (Niazi & Saxena 1968). When tadpoles were exposed to 20 or 30 IU/ml vitamin A, the regeneration was totally suppressed and at lower concentrations regeneration of axial tissue was affected, the severity being directly proportional to the concentration. At concentration of 10 IU/ml and above, regeneration of the axial tissue was suppressed irrespective of duration of treatment. When these tissues did regenerate they either turned posterodorsally or posteroventrally similar to our observations. They also observed that at

← **Figure 9** Multiple EHLs in *P. maculatus* (treated). The tail has been resorbed completely and the EHLs are attached to the regressed tail stump (arrow). These EHLs were shed after 3-4 d of metamorphosis (scale bar represents 5mm).

higher concentration the tadpoles were deficient in pigmentation. In correlating the relationship between inhibition of vitamin A and developmental stages in the species, they observed that when four-day old tadpoles were tail amputated in the middle and were exposed during 1-6 d after amputation, susceptibility of cells of regenerating tail to damaging action of vitamin A was greatest during first two days after amputation (Niazi & Saxena 1979). During this period the cells liberated from the integrating stump tissues dedifferentiate and proliferate prior to the redifferentiation of tissues. Similar inhibition after treatment with vitamin A has also been observed in *X. laevis, N. viridescens, A. mexicanum* (Scadding 1987) and in *R. temporaria* (Maden 1993, Müller et al. 1996).

In all the species a pouch-like structure developed in the cut end of the tail early during regeneration in experimental tadpoles. Formation of similar pouch-like structure has also been observed by Niazi and Saxena (1968, 1979) in *B. andersonii*. In our study many of the tadpoles which developed such a structure generated ectopic limbs. Thus, pouch formation seems to be a pre-requisite for homeotic transformation of tail into limbs. Since Niazi and Saxena fixed their tadpoles before emergence of limbs there was no scope for limb development in their experiments.

Homeotic Transformation (Ectopic Limb Formation)

Ectopic hind limb formation occurred in all the species (figure 4) with the highest in *U. systoma* (21.6%), followed by *P. maculatus* (11.6%), *B. melanostictus* (6.6%), and *R. tigerina* (6.6%) and lowest in *M. ornata* (5%) (Mahapatra et al. 2001a). The EHLs were always hindlimbs with distinct thigh, shank, ankle and digits. The EHLs were well developed in *U. systoma, P. maculatus and M. ornata* whereas in *B. melanostictus and R. tigerina*, they were not well differentiated. In *B. melanostictus* the small size of the tadpoles with the resulting minute EHLs and in *R. tigerina* the small size of EHLs, made it difficult to discern parts of the EHLs.

The EHLs were not always oriented along the anteroposterior (AP) axis in all the species. They were oriented normally in 15.3% in *U. systoma*, 57.1% in *P. maculatus* and 66.3% in *M. ornata*. In 28.6% tadpoles of *P. maculatus and in 1.6% in M. ornata* (Mahapatra et al. 2001a), there was reversal of AP axis in the EHLs (figure 10). In rare cases, there was a cascading effect with a series of bilateral limbs forming with some interspersed tail tissue (figure 8). At times EHLs were devoid of muscles and were oedematous. In 1.6% tadpole of *M. ornata* a normal looking tail regenerated beyond EHL (figure 10).

The threshold for optimal concentration and duration for EHL formation was 10 IU/ml of vitamin A treatment for 72 hr in *U. systoma* (Mohanty-Hejmadi et al. 1992). This was also true for *P. maculatus*. But, this concentration was not sufficient for induction of EHL in *R. tigerina* although the tadpoles in this species are comparable in size to that of *U. systoma* and *P. maculatus*. Apparently, a higher dosage was necessary to induce the EHLs but the EHLs were small and the different parts of the EHLs could not be discerned demonstrating a species specificity in induction of EHLs. The tadpoles of *B. melanostictus* are very small and they could survive in only upto 10 IU/ml of vitamin A for 72 hr of treatment. Thus, for the determination of optimal concentration of vitamin A for survival and EHL induction, the size of the tadpoles has to be taken into consideration.

In all the species studied, The EHLs mostly arose in pairs in the flank region although

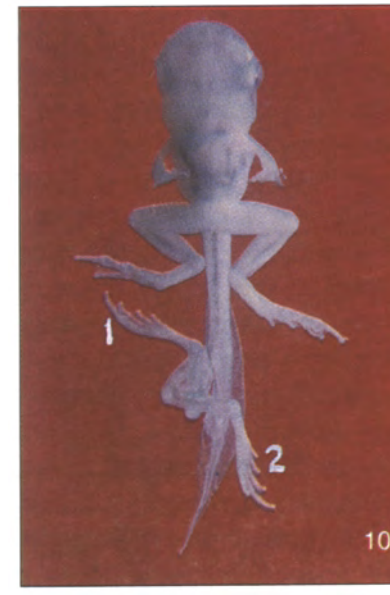


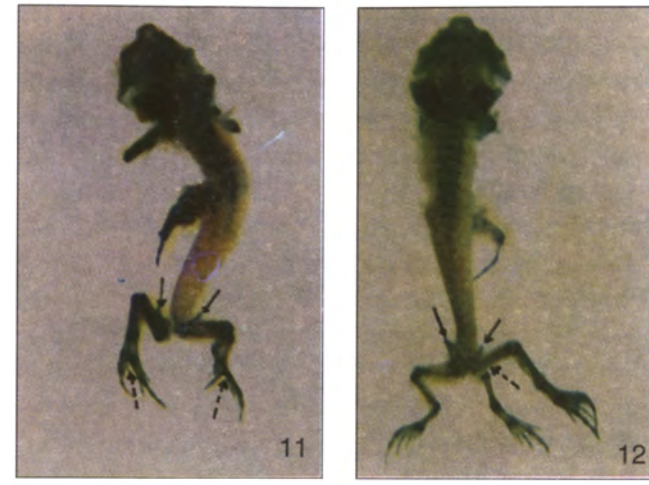
Figure 10 Paired EHLs in *M. ornata* (treated) in association with a normally regenerated tail which is in accordance with the proposed model of Bryant and Gardiner (1992). EHL 1 shows reverse orientation while EHL 2 is normally (downwardly) directed (scale bar represents 5mm).

occasionally odd numbers were observed (Mohanty-Hejmadi et al. 1992, Mahapatra et al. 2001a). The size of the ectopic limbs ranged from normal to smaller size but never larger than the normal limbs (figures 5-8). At times there was secondary duplications at different levels as discussed later.

Maden (1993) has also reported that 10 IU/ml of retinyl palmitate for 3 d is the threshold for a high percentage of limb inductions and additionally the best choice for survival in *R. temporaria.* He also found them arising in singles, pairs, and double-posterior limbs in various combinations, but mostly in pairs. He has observed that in some muscle patterns were abnormal which affected the orientation of digits or limbs. Maden (1993) has indicated that attempts to induce homeotic transformation of tail into limbs has not succeeded in many anurans and has attributed it to the level of thyroid hormone (TH) and thyroid hormone receptors (TRS) in the species.

Homeotic transformation of amputated tails into hind limbs has also been reported for *R. ridibunda* (Müller et al. 1994) and confirmed for *R. temporaria* (Müller et al. 1994, 1996) after treatment with 10 IU/ml vitamin A for 3 d.

Skeletal Pattern and Nerve Supply: There was differentiation of bone and cartilage in the EHLs of *U. systoma* (Mohanty-Hejmadi et al. 1992) and in *P. maculatus* (Mahapatra & Mohanty-Hejmadi 1994). The patterns of bone and cartilage formation in the EHL were comparable with those in normal hind limbs. However, there was occasional duplication of limbs at the distal or ankle (figure 11, see also figure 19), middle or shank (figure 7) and proximal or thigh (figure 12) levels. The number of skeletal elements were reduced in distal parts (digits) in EHLs. As reported earlier (Mahapatra & Mohanty-Hejmadi 1994) sometimes there was mirror-image duplication or posteriorisation (figure 20). In general, vitamin A interfered with the development of distal as well as anterior elements of EHLs. Maden (1993) has also reported such duplications in EHLs of *R. temporaria.* Formation of pelvic girdle was observed in 61.5% tadpoles of *U. systoma* (figures 11 and 12) and 71.4% tadpoles of *P. maculatus* (Mohanty-Hejmadi et al. 1992, Mahapatra & Mohanty-Hejmadi 1994 and Mahapatra et al. 2001a). Pelvic girdle formation has



Figures 11-12.11, Alcian blue and Alizarin red preparation of *U. systoma* tadpoles with 2 EHLs showing distal duplications (dotted arrow) and pelvic girdle on both sides (solid arrow); **12** Alcian blue and Alizarin red preparation of *U. systoma* tadpoles with 3 EHLs showing proximal duplication (dotted arrow) and pelvic girdle on both sides (solid arrow). Figs. 11-12, scale bar represents 5mm.

also been reported in *R. temporaria* (Maden 1993) and confirmed by Müller et al. (1996). According to Müller et al. (1996), since anuran tails do not contain a skeleton, the most striking result in homeotic transformation is the presence of skeletal elements in retinoid-treated regenerates. By an elegant 3D reconstruction of histological sections, they have shown that the first precartilagenous cell condensations of these elements appear before limb bud formation and predominantly form in bilateral pairs. The condensations are located in a dorso-lateral position, slightly rostral from the later-forming ventral limb buds, but there is no connection with the prospective limb bud regions. The location and independent origin of these ectopic elements indicate that they belong to the axial skeleton. Later stages of development show the condensations to become cartilagenous and comparison with sections through normal pelvic regions reveal a remarkable similarity in position and histological structure with the paired rudiments of the sacral vertebra. Soon after the formation of the dorsal cartilages, a second pair of cartilagenous elements appear in a more lateral and ventral position. These rudiments correspond with the position of the first pelvic elements appearing in the controls. In frogs, only one vertebra (the sacrum) is in contact with an element of the pelvic girdle (the ilium); the cartilages inside the regenerate can be

individually identified as the rudiments of the sacrum and ilia. The concurrent presence of elements from the axial skeleton with those from the pelvic girdle and with limb buds show that retinoid-treated tail regenerates are transformed across their entire axis. A whole supernumerary body section is formed at the mid-tail level. The axiality of the regenerate is further supported by the predominantly paired arrangement of all its elements.

The supply of nerves in the EHLs was comparable to that in the normal developing hindlimbs (Das & Mohanty-Hejmadi 1998a). In general, during the development of normal limbs nerves grow out to connect to the limb buds. However, nerves could not be discerned at early stages of EHL formation although they were conspicuous at late stages.

Histology: Histological studies revealed a remarkable difference between the control and experimental tadpoles. Morphologically, a normal compressed tail was regenerated in the control but the regenerating tail after treatment lost its characteristic shape and became more rounded (figure 13). A thick Apical ectodermal cap (AEC) was formed in the control and treated tadpoles of all the species within 24 hr postamputation. It was relatively thick (6-7 layers) in *P. maculatus* and thin (2-3 layers) in *B. melanostictus* and *R. tigerina*. This persisted upto 3 d. The thick transitional AEC is

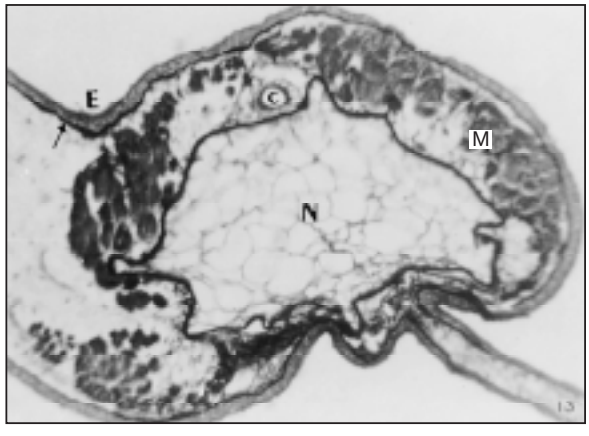


Figure 13 Transverse section of tail of *P. maculatus* (treated)14 d postamputation. The notochord (N) is enlarged with a thick notochordal sheath; The epidermis (E) is thick and multilayered; The basement membrane (arrow) is thick and continuous; Muscles (M) are disoraganised and not arranged in bundles beneath the epidermis; the nerve cord (C) is also enlarged. X150.

believed to contribute to the cellular processes in the regenerating stump (Wolsky 1988). It is concerned with the removal of cellular debris from the wound either by providing proteolytic enzymes or with phagocytic activities. AEC makes further contribution to the process of regeneration, perhaps influencing or initiating cellular differentiation or stimulating cell division (Hay 1966, Carlson 1974).

A blastema was visible in both the control and treated tadpoles within 3 d postamputation in all the species except *in B. melanostictus.* By the end of day 3, blastema disappeared and a normal looking tail regeneration occurred in control. In the treated tadpoles of *P. maculatus, R. tigerina* and *U. systoma*, the blastema persisted for 6 d. A blastema was not formed in *B.melanostictus* confirming the observations for *B. andersonii* (Niazi & Saxena 1979). According to Müller et al. (1996) formation of blastema was delayed for several weeks in *R. temporaria*.

After 3 d postamputation, the epidermis (figure 13) of the treated tadpoles of all the species became thick and mutilayered (6-7 layers). In contrast, epidermis of the control tadpoles remained single layered becoming thick (3-4 layers) only during metamorphic climax, a normal feature for postmetamorphic condition. In *R. catesbiena* (Yoshizato et al. 1993) in the earlier developmental stages, the tail epidermis consists of two types of epidermal cell populations, apical and skein. The basal cells, characteristic of adult epidermis appear among the dividing skein cells at the approach of metamorphosis. It was beyond the scope of our study to examine the exact type of cells in the multilayered epidermis in the treated tadpoles.

The basement membrane was thick in the treated tadpoles and appeared earlier in contrast to the controls where it was relatively thin (figure 13). In the earlier stages of its development, it was discontinuous in the treated tadpoles but became continuous towards the later part of development. The thickening of the basement membrane has been attributed to the synthesis of a mucopolysacharide switched on by vitamin A (Maden 1983a). Further, Hardy et al. (1978) have suggested that a discontinuous basal lamina is related to vitamin A induced mucus metaplasia as vitamin A can act via the dermis and exert its effect on the epidermis.

Just after amputation, the notochord dedifferentiated in both the control and treated tadpoles of all the species. In the treated tadpoles the notochord extended to the bulbular mass, a common morphological abnormality as mentioned earlier; and occupied the whole of the swollen mass. The chorda cells in the bulbular mass were large, thin-walled and more vacuolated than the cells of the tail region where they were condensed, compact, and thick-walled. The notochord was covered by a thick notochordal sheath (figure 13). Maden (1993) has also observed similar enlargement of notochord after vitamin A treatment in the regenerating tail of *R. temporaria*.

Scanning electron microscopic studies revealed interesting enlargement of notochord in the bulbular mass and even in parts quite proximal to the level of amputation . As shown in figures 16 and 17, the notochord is enlar the homeotic transformation.

The nerve cord also dedifferentiated after amputation. Initially the nerve cord at the site of amputation became vesicular and enlarged in both the control and treated tadpoles of all the species studied. In control the original shape of the nerve cord was restored whereas in the treated, it remained enlarged and vesicular (figure 13). It was covered by a thick sheath in contrast to the thin sheath enveloping the nerve cord in the control tadpoles. In *N. viridescens*, Iten and Bryant (1976a) have also observed an ependymal vesicle formation within 3-5 days after amputation. Thus, vesicle formation seems to be a common feature after amputation. In the present study the vesicle is retained probably because vitamin A interfered in its closing.

The muscles were dedifferentaited following amputation in the control and treated tadpoles of all the species. By day 3 postamputaion they had redifferentiated in the controls and were arranged in their characteristic bundles on lateral sides just beneath the epidermis whereas in the treated they remained scattered and unorganized occupying the whole of the section. Iten and Bryant (1976a) have also observed similar dedifferentiation of the muscles in the adult newt *N. viridescens* after amputation. Müller et al. (1996) have observed the musculature being arranged in segmental packages in the amputated tails of *R. temporaria.*

Interestingly enough profuse yolk platelets (figure 18) were observed near the amputation site (unpublished results). It is difficult to comment on this at the moment but we speculate that this may be very significant for mobilisation of vitamin A and needs to be explored in future.

Quite interestingly, in the histological section of *U. systoma*, limb bud could be discerned as early as 72 hr. Externally however, the EHLs were first seen as tiny limb buds on the lateral side of the tail within 13 d in *R. tigerina,* 15 d in *U. systoma* and by 20 d in *P. maculatus* (figure 14). The limb buds developed gradually into full limbs comparable to the normal limbs. Histologically, the EHLs consisted of an inner core of developing cartilage cells, surrounded by differentiating muscle cells with intervening blood vessels and blood sinuses (figure 15). The entire limb was enveloped by an epidermis, 7-8 layers thick, with a thick basement membrane.

Based on our results, a model for homeotic transformation has been proposed by Bryant and Gardiner (1992). Apparently, homeotic change of the tails to limbs in regenerating frogs can be understood in terms of the effect of RA in changing pattern formation competent cells to a posterior ventral-proximal (i.e. flank) positional value, followed by interaction along the rostral-caudal axis of the body to generate two additional pairs of hindlimb sites on the tail. Although it is difficult to determine whether during homeotic transformation the tail tissue is directly converted to limbs or transformed into flank tissues first as suggested by Bryant and Gardiner (1992), there is circumstantial evidence in favour of the latter possibility.

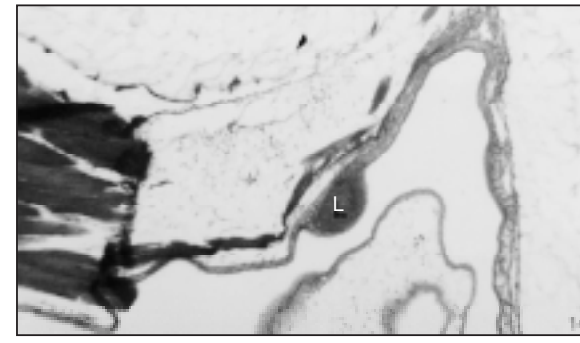
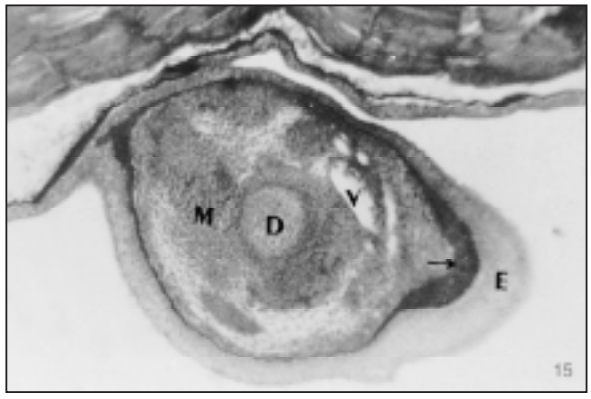
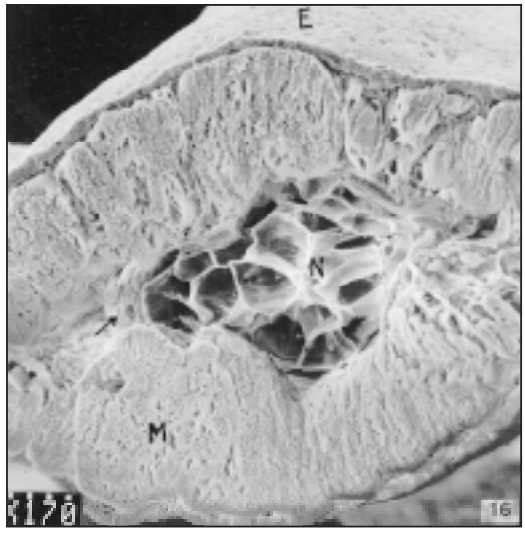


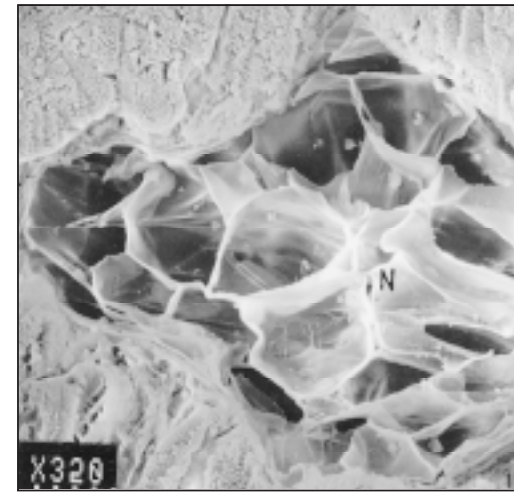
Figure 14 Longitudinal section of the tail of *P. maculatus* (treated) 22 d postamputation. The ectopic limb bud (L) is in its initial stage of development. X100.

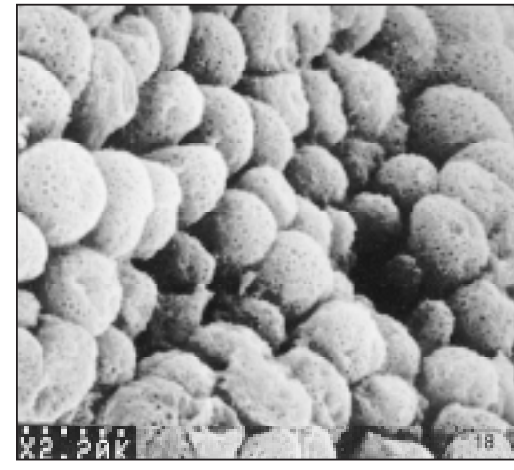




Figures 15-16.16 Longitudinal section of the EHL of *P. maculatus* (treated) 48 d postamputation. The developing ectopic limb consists of a central core of differentiating cartilage (D), surrounded by differentiating muscle fibres (M) with intervening blood vessels (V). The whole limb is enveloped by a thick epidermis (E) with a thick basement membrane (arrow). X150; **16,** Scanning electron micrograpgh (SEM) of the tail proximal to the level of amputation in *P. maculatus* after vitamin A treatment 14 d postamputation. Section showing notochord (N), nerve cord (arrow), muscles (M) and the thick epidermis (E).

Invariably, before generation of limbs, condensation of tissues take place in a position anterior and lateral to the amputated tail tip. The ectopic limbs grow out of this mass in a ventrolateral direction, mostly in pairs, comparable and at times, synchronous to the normal hind limbs. Observations of condensation of tissues expected in the flank region in *R. temporaria* also supports this model. Sometimes, a whole new tail regenerates posterior to the ectopic hind limb (figure 10) showing that two distinct pathways can be operative during regeneration process as proposed by Bryant and Gardiner (1992).





Figures 17-18.18 SEM of the notochord (N) only in higher magnification. The individual chorda cells are enlarged and thin-walled**; 18,** SEM of tail of *P. maculatus* showing yolk platelets.

Effect on Normal Limbs

Although the normal limb buds were left intact during the experiment, they were also affected in the experimental tadpoles where amputation was followed by vitamin A exposure. The most noteworthy are suppression or duplication of limbs. There was also species specificity in these effects.

Limb Suppression

Vitamin A had inhibitory effect on normal limb development in *U. systoma*, *P. maculatus, and M. ornata* (Mahapatra & Mohanty-Hejmadi 2000, Mahapatra et al. 2001a). In *U. systoma* (figures 19 and 20), 10%, 20% and 30% tadpoles showed partial limb suppression after 48 hr, 72 hr and 120 hr of treatment, respectively (Mohanty-Hejmadi et al. 1992). In all the species, limb suppression ranged from partial to

total (Mahapatra & Mohanty-Hejmadi 1994, Mahapatra et al. 2001a). Maximum suppression was seen in the tadpoles with 72 hr of treatment. In *M. ornata*, 10% tadpoles showed partial limb suppression after 72 hr of treatment. In *B. melanostictus* partial and total limb suppression of the developing limbs were seen in 8.33% and 7.5%, respectively. Suppression of the normal limbs was not seen in *R. tigerina*. Thus, there was a species specificity as far as suppression is concerned.

Suppression of limb development after treatment with vitamin A has also been reported in *R. cyanophlyctis* (Niazi & Saxena 1972) and in *R. breviceps* (Sharma & Niazi 1983). According to them, the development of hindlimbs in 10 and 15 IU/ml vitamin A treated tadpoles did not advance beyond the limb buds becoming elongated and conical. Treatment with decreasing amount of vitamin A resulted in hind limbs at various intermediate stages of morphogenesis. In *R. temporaria*, Maden (1993) has also reported that after treatment with retinyl palmitate, almost all the tadpoles (nearly 300) had defective hypomorphic endogenous hind limbs. Similar suppression of limb development by retinol palmitate has also been observed in *A. mexicanum*

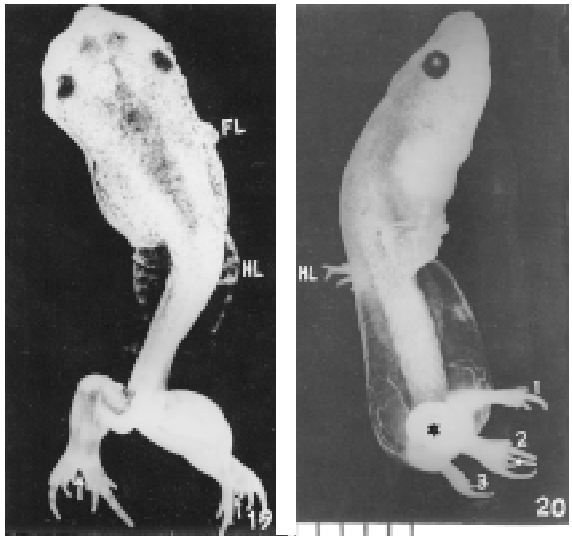


Figure 19 Paired EHLs in *U. systoma* (treated). Both show distal duplication (arrow). FLs normal, HLs partially suppressed; **20,** 3 EHLs (1,2,3) arising from the bulbular mass (asterisk) in *U. systoma.* EHL 2 shows mirror-image duplication of the digits(arrow). FLs are totally suppressed and HLs are partially suppressed. Figs.19-20, scale bar represents 5mm.

(Scadding & Maden 1986a) and larval *X. laevis* (Scadding & Maden 1986b). According to them the suppression of limb development is due to the increase in the signal of RA above the normal threshold level that can naturally occur. We have observed that in *P. maculatus* that the total suppression of hind limbs and fore limbs was maximum (40%) after exposure to vitamin A for 48 or 72 hr, but was less in shorter as well as longer exposure (Mahapatra & Mohanty-Hejmadi 1994). We have proposed that this is due to the level of RA going beyond the threshold level because of additive effects of endogenous and exogenous RA. Since, the tadpoles recovered with longer treatment, it is reasonable to propose that there was a drop in the endogenous level of RA bringing the level to below the threshold level promoting limb development. This also showed that retinoids can have opposite effects on the same cell types in the same animal at the same dose. There is usually a range of suppressions involving fore limbs and hind limbs probably due to differential uptake or differential susceptibility by the cells. Suppression of both limbs may be due to sufficient mobilisation leading to the interference of vitamin A with the Hox genes responsible for limb differentiation.

According to Bryant and Gardiner (1992) the application of retinoic acid in developing limb buds causes all cells to be reprogrammed towards uniform positional values. Lack of positional diversity in the progress zone leads to the failure of growth and pattern formation and to the formation of reduced or truncated limbs. In developing limbs that are unwounded, vitamin A and other chemical agents also produce deficiencies in the limb pattern (Bryant & Simpson 1984). It is observed that RA inhibits chondrogenesis in culture of mouse limb bud cells (Lewis et al. 1978). Vitamin A also has been reported to interfere in condensation and formation of bones leading to the formation of mesomelic limb with various anomalies in bones (Alles & Sulik 1989). Therefore, the suppression as well as anomalies leading to hypomorphic limbs can be explained as a loss of proper positional values in the developing limbs and also due to interference in chondrogenesis.

Limb Duplication

Limb duplication occurred in the tadpoles of *U. systoma* (Mohanty-Hejmadi et al. 1992) *and*

B. melanostictus (Mahapatra et al. 2001b) only. Duplication was unilateral or bilateral (figure 21) and occurred either in the fore limbs or hind limbs or both.

There was 1.66% unilateral duplication in the hind limb in *U. systoma* (Mohanty-Hejmadi et al. 1992). In *B. melanostictus*, there was a wide variation depending on batches of eggs from different individuals. Earlier we had reported 6.6% (Das & Mohanty-Hejmadi 2000), but later it increased to 40% (Mahapatra et al. 2001b) with the same concentration and duration of treatment from 24 to 72 hr which included unilateral, bilateral fore limb, hind limbs or both. Das and Dutta (1996) have also reported hind limb duplication in *B. melanostictus*.

Similar type of hind limb duplication after systemic exposure of *B. vulgaris* tadpoles from early tail bud to the development of opercular folds to vitamin A, has also been reported by Bruschelli and Rosi (1971). Since, hind limb has already been established by this stage, they have speculated that duplication is caused by the division of the cartilage of the limb due to lysosomal activities.

Supernumerary legs or *polymely* in natural population of amphibians is known from centuries (reviewed by Van Valen 1974). It could be due to intrinsic (genetic) factors and more commonly extrinsic (environmental) factors. There are a number of populations of amphibians, specially frogs in which individuals with supernumerary legs occur at relatively high frequencies. Even

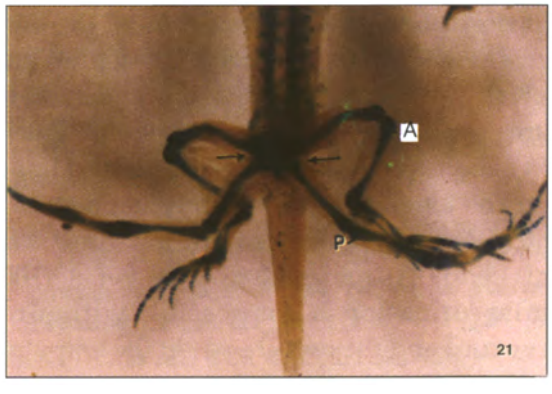


Figure 21 Alcian blue and alizarin red preparation of the skeleton in *B. melanostictus* (treated) showing duplication of the normal hind limb. The posterior limbs (P) are longer with hypomorphic skeleton compared to anterior limbs (A). The pelvic girdle has also duplicated (arrows) to give rise to an entirely separate limb.

implantation of frog kidney, cartilage or liver into forelimbs of adults of salamander *Triturus* leads to *polymely* (Carlson 1968).

There is evidence of specific environmental control and virally induced *polymely* as discussed by Van Valen (1974). Further, naturally occurring supernumerary limbs in *Hyla regila* are as a result of mechanical disruption by trematode eggs (Sessions & Ruth 1990). In *R. catesbeiana*, it is even attributed to chemical treatments in agricultural fields (Lopez & Maxson 1990). All the reports on naturally occurring cases are in agreement that *polymelia* is more common in hind limbs as compared to fore limbs which is consistent with our observations under experimental conditions.

Conclusions and Future Directions

There are two aspects that are remarkable in the homeotic transformation of tail into limbs, first that the EHLs are always hind limbs and the second that the whole pelvic segment with skeletal elements which normally do not occur in the tail, are generated.

First, the vertebrate limb is an extremely complex organ with an asymmetrical pattern of parts. It is formed by a complex series of interactions during development. Under normal circumstances limbs originate as a consequence of the differential growth of cells from the lateral plate mesoderm at specific axial level. Two sources of mesenchyme contribute to the formation of limb. Cells migrating from the somites give rise to all muscle cells. Cells from lateral plate mesoderm give rise to connective tissue and cartilage. Limb development begins when mesenchymal cells are released from the somatic layer of the limb field lateral plate mesoderm followed by cells from the somite. The cells migrate laterally and accumulate under the epidermal tissue of the neurula. The circular bulge on the surface of the embryo is called the limb bud. The initial stage of this proliferation appears to be under regulation of the mesonephros (see Gilbert 1994). There is enough evidence that the three axes of limb (dorsoventral, proximodistal and anteriorposterior) are linked by the respective signals WNT7a, Fibroblast growth factor (FGF4) and Sonic hedgehog (Shh) during limb outgrowth and patterning

(Yang & Niswander 1995). In chick, ectopic limb induction by FGF family has shown that the phenotype of the ectopic limb depends on the somite level at which it is formed; limbs in the anterior flank region resemble wings, whereas those in the posterior flank resemble legs. Hox code and FGF expression in the lateral plate mesoderm specify and select the expression of Tbx5 and Tbx4/Pitx 1 in the prospective wing and leg buds, respectively. Pitx1 acts as a Tbx4 inducer, and Tbx 5 represses expression of Tbx 4. Tbx 4 induces expression of Hoxd 9 which is specific for hind limb (Ohuchi et al. 1998, Takeuchi et al. 1999 and Rodriguez-Esteban et al. 1999).

During homeotic transformation of tail into hind limbs, it is reasonable to speculate that under the influence of vitamin A, mesodermal cells in the tail region induce a limb bud in the presence of a mesenchymal FGF which in turn induces Pitx1 which acts as a Tbx 4 inducer to form the hind limbs. Pitx and Tbx proteins are transcription factors and their targets either direct or indirect, include genes of the Hox homeodomain cluster. Tbx 4 induces hind limb specific Hox genes (Hoxc9-Hoxc11) and suppresses the fore limb specific Hoxd9 genes (Niswander 1999). Further, the genes that play important roles in establishment of axes and regeneration of limbs are the Hox-4, Hox-3 and Hox-1 clusters (Simon & Tabin 1993). The Hox-3 cluster is expressed specifically in normal and regenerating posterior appendages and apparently, provides positional memory for differentiation of hindlimbs from forelimbs. So, it is reasonable to propose that a gene of the Hox-3 cluster may also be involved in the development of the tail to legs (Mahapatra & Mohanty-Hejmadi 1994). The molecular mechanism relating to the role of retinoids in the regulation of Hox genes and influence in the Shh have been discused by Johnson and Tabin (1997). It will be interesting to see if the same processes are repeated during EHL formation from tail tissue.

Second, coming to the transformation of tail tissue into a pelvic or anterior segment under the influence of vitamin A, considerable information is available on the establishment of cranio-caudal axis which is pertinent here. Christ et al. (2000) have presented a comprehensive account of the segmentation of the paraxial mesoderm leading to somite formation and the role of Hox genes in cranio-caudal identity based on observations in chick embryo applicable to other vertebrates. The vertebral column develops from somites, segmental units of the paraxial mesoderm. The regionalization of the paraxial mesoderm and the determination of the axial identity are achieved by Hox genes which include atleast 38 members representing 13 paralogous groups aligned in four clusters (a-d). Hox genes show a cranial-to-caudal pattern of expression with a sequence of cranial expression boundaries that corresponds to their alignment on the chromosomes. The development of vertebral column is a consequence of segment-specific balance between proliferation, apoptosis and differentiation of cells under normal circumstances.

During homeotic limb induction, the sources of the cells and their association are completely different. Cells differentiate away from the normal inducer, mesonephros. But, during this transformation, most noteworthy is the enlargement of the notochord in the treated group. Within 72 hr the control group regenerates a normal notochord, but in the treated group there is considerable enlargement of notochord which remains in this condition for long time. This has also has been noted by Maden (1993). At times, a limb bud can be discerned within 72 hr after treatment in the flank region of the tail . There is a strong possibility that the notochordal cells may play a role in induction of the pelvic segment leading to the formation of limb buds. In chick and mouse, notochord is known to be a source of polarising factors such as shh which in turn encodes a signal that is implicated in both short and long-range interactions that pattern the central nervous system, somite and limb (Marti et al. 1995). As reviewed by Christ et al. (2000), somite formation is not possible without the nerve cord and the notochord. At the amputated tail, both the nerve cord and notochord are present. There is considerable enlargement of notochord after amputation and vitamin A treatment. Nerves grow out to innervate the EHLs. Therefore, we speculate that under the

influence of vitamin A, notochord mediates the expression of the key Hox genes for the establishment of the pelvic region including the vertebral elements and nerve supply by re-specification and proximalisation, in the tail tissue.

As pointed out by Maden (1993), during homeotic transformation, large amounts of transformed tail tissues are generated and molecular analyses of this tissue may lead to the identification of the genes involved in homeotic transformation. Probing the tissue during this unique transformation may lead to identification not only of the cells which contribute to limb formation, but also the mechanism by which tail specific genes are suppressed and limb specific genes are activated. Once the origin of the limb cells are traced in the tail region, these cells can be cultured to see if limbs can be induced *in vitro* which will have academic as well as applied implications.

Last but not the least, as pointed out by several workers (Maden 1993, Okada 1996 and Müller et al. 1996) this phenomenon has considerable evolutionary significance and now provides an opportunity for genetic studies similar to the ones which have enriched our knowledge in pattern formation in invertebrates, for the first time in vertebrates. In describing our work, Maden (1993) says that this was the first clear demonstration of homeotic transformation in the sense Bateson originally described in vertebrates. As pointed out by Müller et al. (1996) this phenomenon is also indicative of retension of segmentality as far as induction and pattern formation is concerned even in a downhill transitory structure like the tail. This is now a powerful model to work as we finally have segments on which we can work in vertebrates like those in invertebrates like *Drosophila.* People like Van Valen (1994) who tried to speculate on the Batesonian implication on *polymely* in anurans can be happy that in vertebrates we have a model to test out Batesonian *"Homeosis"*. It is expected that this model can be used for understanding *homeosis* from molecular to organismic levels in vertebrates. We agree with Okada (1996) that it has opened a way to investigate the most mysterious phenomenon known from early 20th century in modern eyes, most probably in terms of epigenetic switch of homeotic genes.

Acknowledgements

The work on homeotic transformation was done in collaboration of my colleague Dr S K Dutta and much of the information included in this paper are based on the research in my laboratory by Dr Pravati Kumari Mahapatra (now CSIR Pool Officer) and Dr Pragnya Das (now Fast Track Fellow of DST). I thank all of them. We thank Paul Khan for technical assistance and Dr R A Liversage for providing the facilities for SEM om *institut de recherches Clinique de Montreal*. I also would like to thank Dr Pragnya Das for her help in preparation of the manuscript. Part of the research was funded by the UGC under DRS programme to Department of Zoology, Utkal University.

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