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Retinoids and Tail regeneration in frog tadpoles

Abstract

Regeneration is the process of restoration of lost parts of an organism and is wide spread in animal kingdom. Amongst vertebrates, the tadpoles of anuran amphibians regenerate their body parts like limbs and tails. Tadpole is not only an ideal model to study regeneration of organs but also ideal for understanding loss of regenerating capacity as it stops regeneration of organs soon after onset of metamorphosis. Tail amputated tadpoles treated with vitamin A / retinoic acid show an interesting phenomenon of homeotic transformation where tail cells are transdifferentiated into cells of ectopic pelvic girdle and hind limbs. In this review, effects of vitamin A /retinoic acid on tail amputated tadpoles of different species of Indian anurans has been described. Vitamin A treatment leads to regeneration of an abnormal tail in the tail amputated tadpoles. The ectopic hind limbs developed at the cut end of tail follow the path of normal hind limb development. Vitamin A treatment induces oxidative stress in the tail regenerates. Specific activities of two phosphatases, namely acid and alkaline phosphatase are elevated in vitamin A induced abnormal tail regenerates. Immune positive cells for both these enzymes are observed to be more in the treated tail regenerates. Besides, three fibroblast growth factors (FGFs) 1, 2 and 10 are expressed in the tail regenerates.

Introduction

Regeneration, the replacement of lost or damaged tissue(s) or organ(s) of an organism, is a wide spread phenomenon in animals. It can occur by an outgrowth of new tissue from the surface of the wound (epimorphosis) or by remodeling of the remaining parts (mophallaxis). The remarkable phenomenon of regeneration was first observed with the discovery that *Hydra*, a fresh water polyp, could generate a complete body from a small piece by Trembley (Trembley, 1744). Regeneration in vertebrates was reported in the year 1768 by Spallanzani based on the fact that a variety of amphibians could regenerate their legs and tails following amputation. Since Spallanzani's first scientific description of the phenomenon of limb regeneration (Dinsmore, 1991; Tsonis and Fox, 2009), numerous workers have investigated the regenerative capacity in different groups of animals. Among the tetrapods, the amphibians exhibit the highest degree of regenerative ability. Urodele amphibians regenerate different body parts throughout their life where as in anurans, regenerating capacity is restricted to the larval period. Since the capacity to regenerate is lost on the onset of metamorphosis of the anuran tadpole, it is an ideal model to study the mechanism of loss of regeneration. Tadpoles also exhibit the phenomenon of homeotic transformation where tails are converted into hind limbs induced by vitamin A and its derivatives (Mohanty-Hejmadi et al., 1992; Maden, 1993; Muller et al., 1996). It is a process of transdifferentiation of one type of cell to another and regeneration studies

Tadpoles of *Megophrys* sp.
Photo Credit: Abhijit Das

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in anuran tadpoles can give insight into the mechanism of such transformation of cells.

Role of extrinsic factors during regeneration

Regenerative processes in amphibians are known to be influenced by several extrinsic factors. It has been described that X-irradiation inhibits regeneration by preventing cell division (Maden and Wallace, 1976). Nerves have also been described to be essential for limb regeneration in all known species excepting the aneurogenic limbs regeneration of some larval amphibians (Kumar et al. 2011). Extracts from nervous tissues have been shown to have effects on blastema cell synthetic activity. Ultrasonication has shown inhibition of limb regeneration in urodele amphibians (Pizzarello et al. 1975). Actinomycin which inhibits transcription, when applied to the tail amputated tadpoles of *Ambystoma tigrinum* and *Rana pipiens,* greatly reduces the amount of regenerating tissue and at the same time increases the thickness of apical epidermal cap (AEC) which covers the wound after amputation and plays an important role in amphibian regeneration both in anuran and urodele amphibians (Wolsky, 1988). All the extrinsic factors without exception either had no effect or inhibited the process of regeneration. None showed any modulating effect on pattern formation. These factors either permitted or inhibited regeneration but never altered regeneration in any way.

Influence of retinoids on pattern formation

The extrinsic factors that have been shown to directly influence pattern formation in amphibian tail and limb regeneration is vitamin A and its derivatives, the retinoids (Maden 1993; Ju and Kim 1994, 2010; Mahapatra, 1994). The retinoids include retinol, retinal and retinoic acid. The inhibitory and modifying influence of Vitamin A on tail regeneration in the anuran tadpoles of *Bufo andersonii* was reported for the first time by Niazi and Saxena (1968). Dose dependent inhibition of tail regeneration in two urodeles, i.e., *Notopthalmus viridscens, Ambystoma mexicanum* and one anuran *Xenopus laevis* following vitamin A (palmitate) treatment was reported by Scadding (1987). However, there was no evidence of any case of duplication of any part of the tail structure. The finding that vitamin A can induce homeotic transformation of tail to limbs in the marbled frog Uperodon systoma showed another remarkable effect of vitamin A (Mohanty-Hejmadi et al. 1992) i.e., transdifferentiation of cells of the tail into the cells of pelvic girdle and hindlimb.

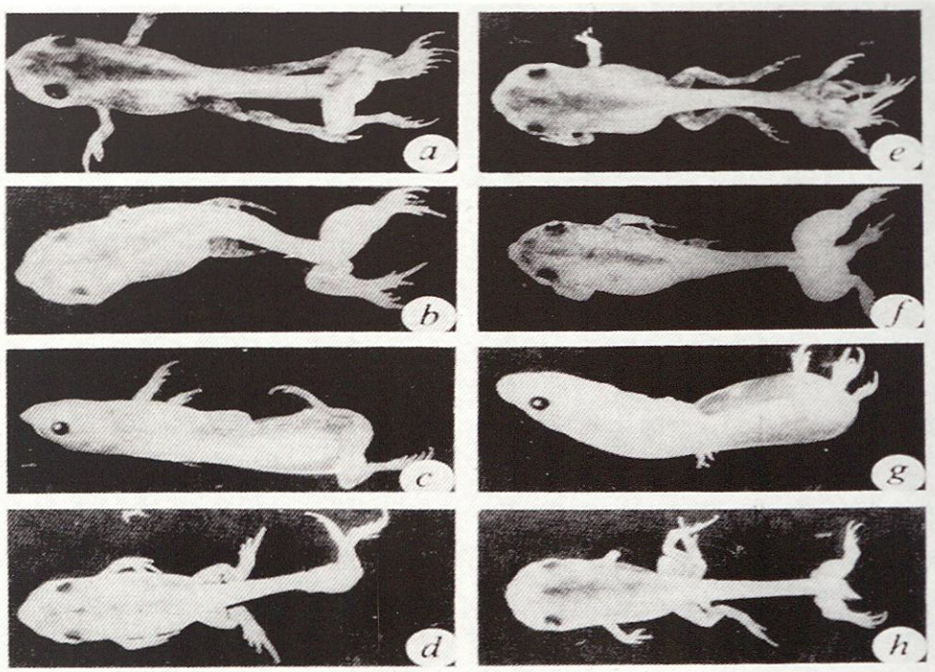


Figure 1: Homeotic Transformation of tails to limbs in the tadpoles of *Uperodon systoma.* (Source: Mohanty Hejmadi et al. 1992)

Following the initial finding, there were several reports on vitamin A induced inhibition of tail regeneration and homeotic transformation in different anuran species namely *Polypedates maculautus*, *Bufo melanostictus* (present name *Duttaphrynus melanostictus*), *Microhyla ornata* (Mahapatra, 1994; Mahapatra and Mohanty-Hejmadi, 1994, Mohanty-Hejmadi and Crawford, 2003) and *Rana tigerina* (Present name *Hoplobatrachus tigerinus*) (Das and Dutta, 1996). This phenomenon of homeotic transformation was also confirmed in two temperate anurans i.e., *R. temporaria* (Maden, 1993; Maden and Corcoran, 1996; Muller et al. 1994, 1996) and *R. ridibunda* (Muller et al. 1994, 1996). Maden (1993) reported induction of ectopic limbs by vitamin A 10IU/72 hours treatment upto the 54 stage (Nieuwkoop and Faber, 1967), which are comparable to Gosner (1960) stage 33-34 (limb paddle stage with toe demarcation) tadpoles. In *R. temporaria* tadpoles, Muller et al., (1996) observed ectopic limb induction from stage 26 to 31 (limb bud to elongated buds with distal paddle-shaped structure) following 10IU/72 hour treatment of vitamin A palmitate. They also reported homeotic duplication of a whole body segment including vertebral elements, pelvic girdle elements and limb buds at the mid tail level.

Ectopic and normal hind limbs follow the same developmental pathway

Normal looking tails regenerated in the control tadpoles within 15 days of tail amputation while abnormal tails regenerated in the treated tadpoles in different anuran species. In more than 20% abnormal tails, bud like structures appeared which subsequently developed into ectopic hind limbs (Fig.2).

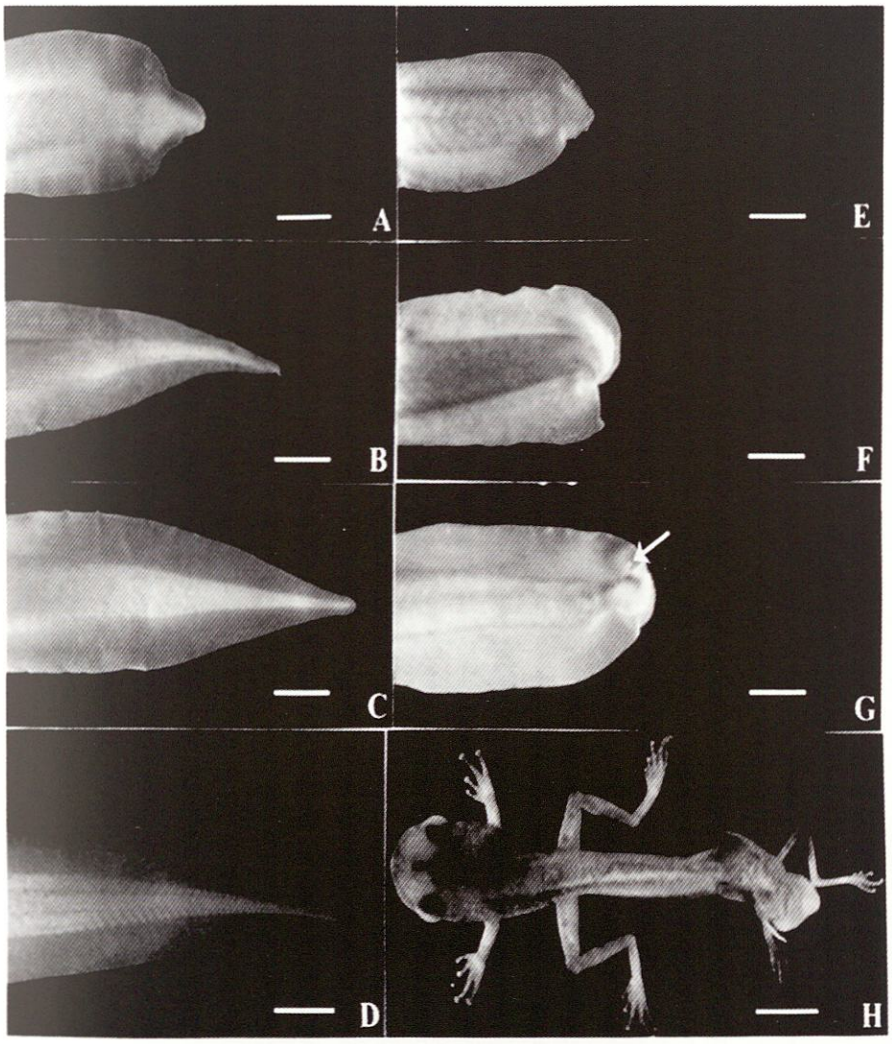


Figure 2: Morphology of the original and regenerated tails of the tadpoles of *Polypedates maculatus*. (A). Regenerated tail of the control group 5 days after amputation.(B). Tail of control group 10 days after amputation. (C). Normal looking tail of the control group 15 days after amputation. (D). Original tail before amputation. (E). Abnormal tail regeneration with a blunt end 5 days after amputation (F). Further enlargement of the abnormal tail 10 days post amputation (G). A bulbular mass in 15 days post amputated tail with limb buds (arrows). (H). A treated tadpole with ectopic limbs at the cut end of tail at the emergence of forelimbs (50 days post amputation). (Scale bar: Fig. A to G=2mm; Fig. H=5mm) (Source, Patnaik et al. 2012)

Metamorph of *Rhacophorus* sp. Photo Credit: Abhijit Das

Marked histological similarities was reported to exist between normal and vitamin A induced ectopic limb buds in the tadpoles of the Indian tree frog, *Polypedates maculates* (Mahapatra et al. 2004). However, close association of nephric tubules and lateral plate mesoderm, as seen in normal hind limb bud did not seem to be essential for ectopic limb development (Figs.3 and 4)

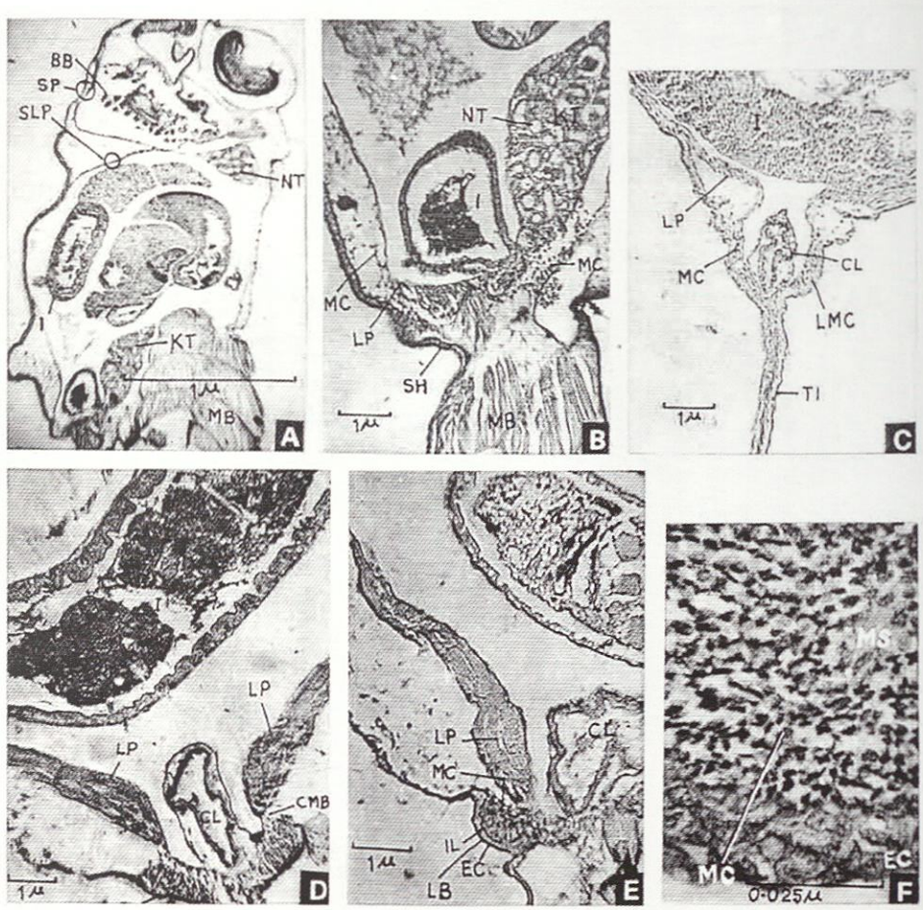


Figure 3: (A) L.S. of Gosner stage 24 showing the trunk with distinct branchial basket (BB), Kidney tubules (KT), muscle bundles (MB), somatopleure (SP) and splanchnopleure (SLP) (B) L.S. of Gosner stage 24, showing migratory mesodermal cells (MC) at the site of hindlimb bud formation (SH) and by the side of lateral plate (LP), nephric tubule (NT) in close association with mesodermal cells (MC). (C) L.S. of early feeding stage (Gosner stage 25), showing loose mesenchymal cellas (LMC) at both sides of cloaca (CL) and prominent lateral mesodermal cells (MC). (D) L.S. of Gosner stage 26, showing the connection of mesodermal band (CMB) to the developing limb bud (LB). (E) L.S. of tadpole at Gosner stage 28 showing outer epidermal layer (EC), inner thickened maesodermal cells (MC) and the intervening layer (IL), (F) L.S. of Gosner stage 28, showing marginal sinus (MS) with in mesodermal cells (MC) and multilayered epidermal cells (EC). (Source: Mahapatra et al. 2004)

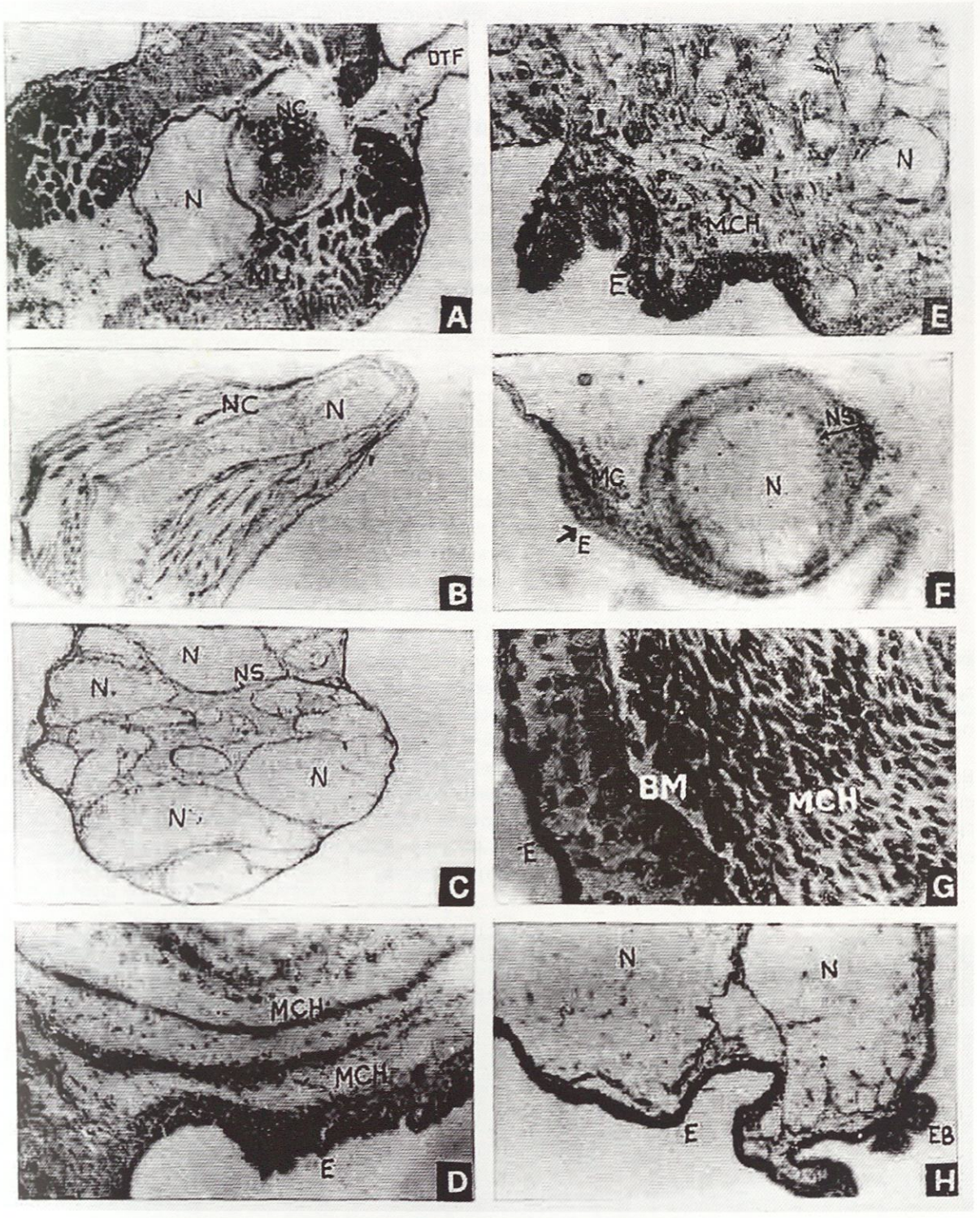


Figure 4: (A) T.S. through tail of a control tadpole, showing notochord (N) spinal cord (SC) and muscle bundle (MU). (B) L.S. through vitamin A treated regenerated tail, showing enlarged notochord (N) and small nerve cord (NC). (C) T.S. through tail, showing notochordal mass (N) in globules surrounded by notochordal sheath (NS). (D) L.S. through tail, showing folded epidermis (E) and two layers of condensed mesenchymal cells (MCH) below the epidermis. (E) L.S. through tail, showing thick epidermis (E), mesemchymal cells (MCH), compact and small globules of notochordal cells (N). (F) L.S. through abnormal tail, showing folded epidermal layer (E), large notochord (N) with thick notochordal sheath (NS) and accumulation of mesodermal cells (MC) beneath the ectoderm (Arrow). (G) L.S. through tail, showing thick epidermal layer (E), dark basement membrane (BM) and inner mesenchymal cells (MHC) with intercellular space. (H) L.S. through abnormal tail, showing vacuolated notochordal cells (N) and two ectopic limb buds (EB) protruding from the epidermal layer (E). (Source: Mahapatra et al. 2004)

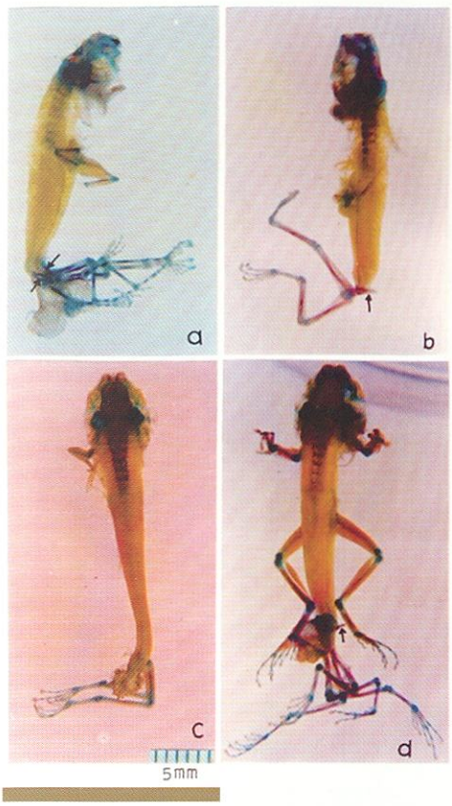


Figure 5: Fig.5 Ectopic hindlimbs showed both bony and cartilaginous elements (a-d). Pelvic girdles indicated as black arrows (a,b,d). (Source: Mahapatra, 1994)

Ectopic limbs were always hindlimbs with distinct thigh, shank, ankle and digits. Generally the ectopic hindlimbs were smaller than the normal hindlimbs and developed in pairs. In more than 50% cases ectopic limbs originated from distinct pelvic girdles.

Role of oxidative stress during vitamin A induced abnormal tail regeneration

Oxidative stress state of the original, regenerating tails of the control and vitamin A treated groups were reported for the first time (Mahapatra et al. 2002) in the tadpoles of the Indian tree frog *Polypedates maculatus*. Lipid peroxidation (LPX) was investigated as an index of oxidative stress. Hydrogen peroxide (H_2O_2) was estimated to quantify level of this potent oxidant. Besides, two enzymatic antioxidants, i.e., superoxide dismutase (SOD) and catalase (CAT) related to normal development of anurans were estimated. The level of a non enzymatic antioxidant, reduced glutathione (GSH) normally expressed during cell division was also estimated. There was always a higher level of oxidative stress in the regenerating tails of the tadpoles. The level of oxidative stress further increased in the regenerated tails of the vitamin A treated tadpoles where there was abnormal tail regeneration. Thus, it was established that a hyper oxidative stress condition prevailed in the abnormal tails which is a pre requisite for ectopic limb development (Table 1).

Polypedates maculatus
Photo Credit: Abhijit Das

Table 1: Table1 Changes in oxidative stress parameters of the regenerated tail of control (C) and vitamin A treated (T) tadpoles of *Polypedates maculatus* (Mahapatra et al. 2002)

Oxidative stress parameters	Group	Days following tail amputation			
		5	10	15	20
LPX1	C	1.16*	1.08	1.8	0.63
	T	1.61	1.91	1.16	1.07
H2O22	C	1.87	1.88	1.82	1.14
	T	3.85	5.37	2.04	1.27
SOD3	C	14.37	8.1	6.9	0.91
	T	9.06	9.38	4.19	0.9
CAT4	C	1	1.21	1.3	1.09
	T	1.6	1.96	1.81	2.12
GSH5	C	2.11	1.4	1.15	1.15
	T	2.11	1.67	1.29	1.18

*Values in fold relative to original tail, C-control, T-treated group

1. Level of lipid peroxidation (nmol MDA formed/mg protein)
2. Level of H_2O_2 (nmol/mg protein)
3. Activity of SOD (units of SOD/mg protein)
4. Activity of Catalase (pmol/mg protein/min)
5. Level of Glutathione (μm/g tissue)

Role of phosphatases during vitamin A induced abnormal tail regeneration

Specific activities of acid and alkaline phosphatase

In the tadpoles of *Polypedates maculatus* and *Duttaphrynus melanostictus*, elevation of acid and alkaline phosphatase has been described during vitamin A induced abnormal tail regeneration, a pre requisite for ectopic organ formation (Patnaik et al. 2012; Mahapatra et al. 2015). Acid phosphatase is a lysosomal marker enzyme and is associated with lytic activities. As lysis of cell has been described during regeneration in anurans and urodeles (Carlson, 2005; Ju and Kim, 2010) this enzyme was estimated during tail regeneration. Alkaline phosphatase, a metalloenzyme attached to plasma membrane of cells (Moss, 1992) is a potent marker for undifferentiated stem cells (O'Connor et al. 2008; Keeling et al. 2009) and is also associated with regeneration in different groups of animals like planarians (Osborne and Miller, 1963) ascidian (Keeling et al. 2009), anurans (Junqueira, 1950) and urodeles (Ghiretti, 1950; Karczmac and Berg, 1951; Schmidt and Weary, 1963; Inoue and Suzuki, 1969). As accumulation of undifferentiated cells takes place during tail regeneration (Mahapatra et al., 2004), activity of this enzyme was studied. Elevation in specific activity of acid phospatase indicated lytic activities during regeneration and rise in specific activity of alkaline phosphatase was correlated with accumulation undifferentiated cells at the site of regeneration.

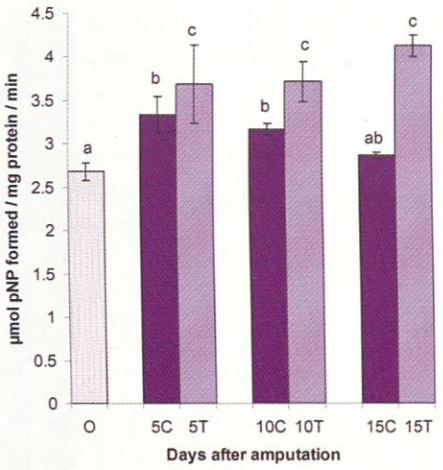


Figure 7: Specific activity of acid phosphatase in µmol p-nitrophenol (pNP) formed/mg protein/min at 370C of the regenerated tails of vitamin A 10IU/ml treated (72h) and control tadpoles of Polypedates maculates. O-original, C-Control, T-treated (Patnaik et al., 2012)

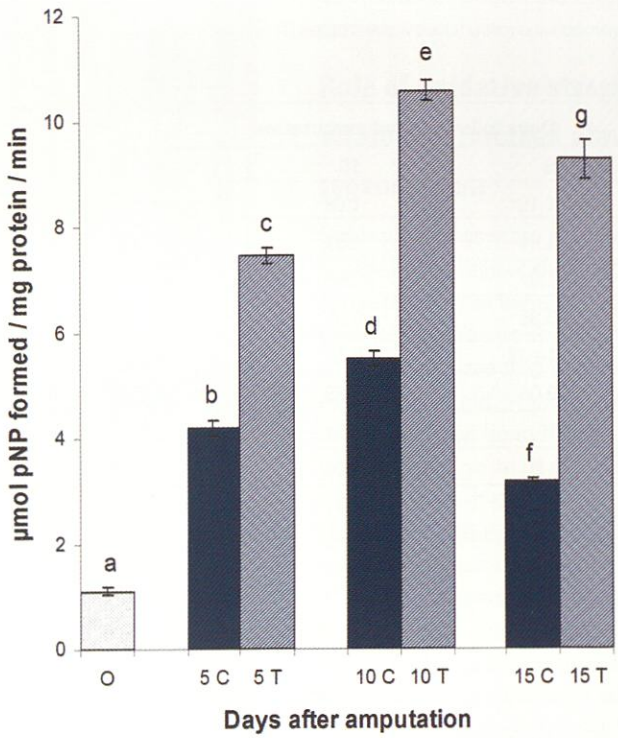


Figure 8: Specific activity of alkaline phosphatase in µmol p-nitrophenol (pNP) formed/mg protein/min at 370C of the regenerated tails of vitamin A 10IU/ml treated (72h) and control tadpoles of Polypedates maculates. O-original, C-Control, T-treated (Patnaik et al., 2012)

Immunohistochemical localization of phosphatases

For immunohistochemical localization of acid and alkaline phosphatases, normal and regenerating tail tissues of control and vitamin A treated groups were considered. In the non-amputated and normally regenerated tails, acid phosphatase was majorly restricted to the epidermis and muscle patches although in the normally regenerated tails, notochordal sheath and spinal cord also stained for this enzyme. In vitamin A treated tails, acid phosphatase was mostly localized in the epidermis, notochord precursor cells and undifferentiated cells of the mesenchyme. Notochordal cells and notochordal sheath also showed positive staining (Fig. 9). Since, acid phosphatase was majorly expressed by tissue forming precursor cells, this enzyme has been suggested to be involved in tissue remodelling processes (Mahapatra et al., 2017).

Epidermis of the uncut tails showed no immunoreactivity while the regenerating tails of both control and treated groups expressed alkaline phosphatise (ALP). In the treated tails, in addition to the epidermis, ALP was expressed in the layer below basement membrane, undifferentiated cells lodged in muscle and mesenchyme, spinal cord, notochordal sheath, notochord precursor cells and also blood vessels (Fig.10). ALP positive cells were more in the treated tails than their corresponding controls. In treated groups, the distal portion of the tails showed higher ALP expression than proximal part. Such differential expression of ALP can be correlated with quantity of undifferentiated cells in the distal portion of the tail where the normal process of regeneration is interrupted and the tail regenerates abnormally, a pre requisite for ectopic organ formation (Unpublished data).

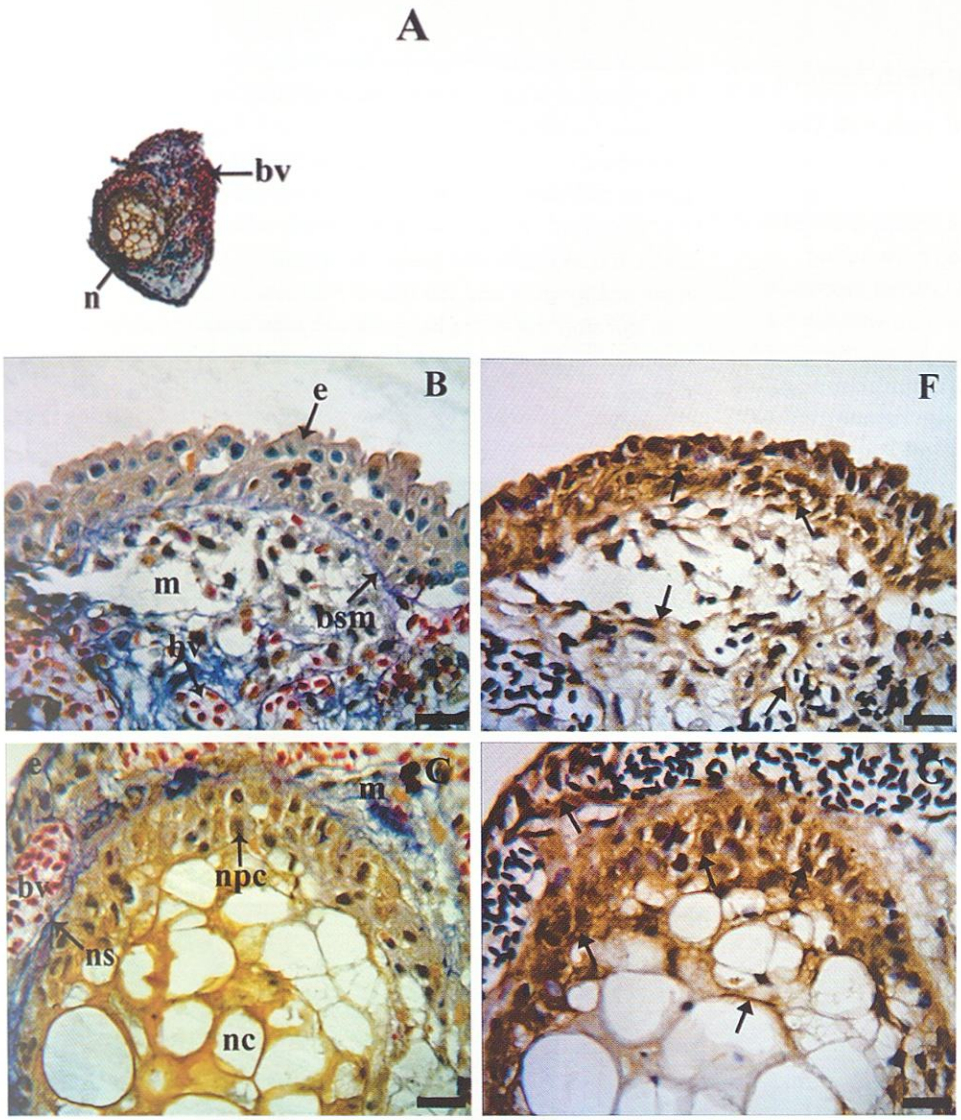
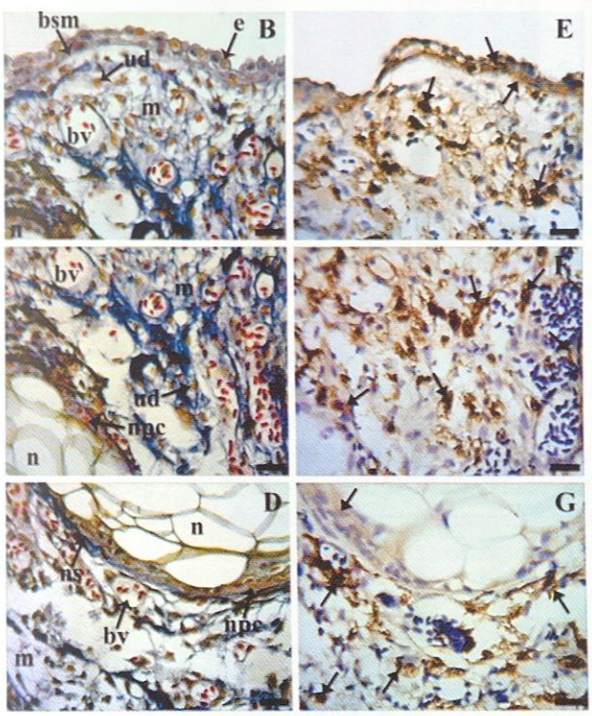


Figure 9: Immunohistochemical localization of acid phosphatase in the cross section of 3 day regenerated tail of *Polypedates maculatus* treated with vitamin A 10IU/ml (A-C) Sections stained with Mallory's triple stain; (D, E) Immunostained sections where black arrows indicate positive staining. Scale bar A=100 µm, B-E= 20 µm Abbreviations: apc - apoptotic cell; bsm - basement membrane; bv - blood vessel; e - epidermis; m - mesenchyme; n - notochord; nc - notochordal cell; npc - notochord precursor cell; ns - notochordal sheath (Source: Mahapatra et al. 2017)



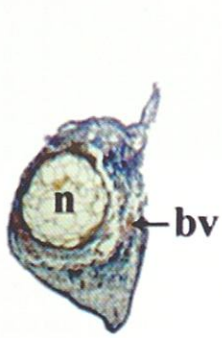


Figure 10: Immunohistochemical localization of alkaline phosphatase in the cross section of 3 day regenerated tail of *Polypedates maculatus* treated with vitamin A 10IU/ml (A-D) Sections stained with Mallory's triple stain; (E-G) Immunostained sections where black arrows indicate positive staining. Scale bars: (A) = 100 µm; (B-G) = 20 µm. Abbreviations: bsm - basement membrane; bv - blood vessel; e - epidermis; m - mesenchyme; n - notochord; npc - notochord precursor cell; ns - notochordal sheath; ud- undifferentiated cell. (Unpublished data)

Role of fibroblast growth factors during retinoic acid induced tail regeneration

Fibroblast growth factors (FGFs) are well known for their diversified roles during proliferation and wound healing processes. These ubiquitously present growth factors are best known to operate across the epithelial- mesenchymal boundaries and such interactions have been reported to be critical during appendage development. Interestingly, when reports of development of several ectopic limbs during tail regeneration in Vitamin A plamitate treated anuran tadpoles of *Uperodon systoma* came into limelight (Mohanty-Hejmadi et al. 1992), the regeneration research became more challenging and the role of FGFs in channelizing the epithelial-mesenchymal interactions have been emphasized in our present study.

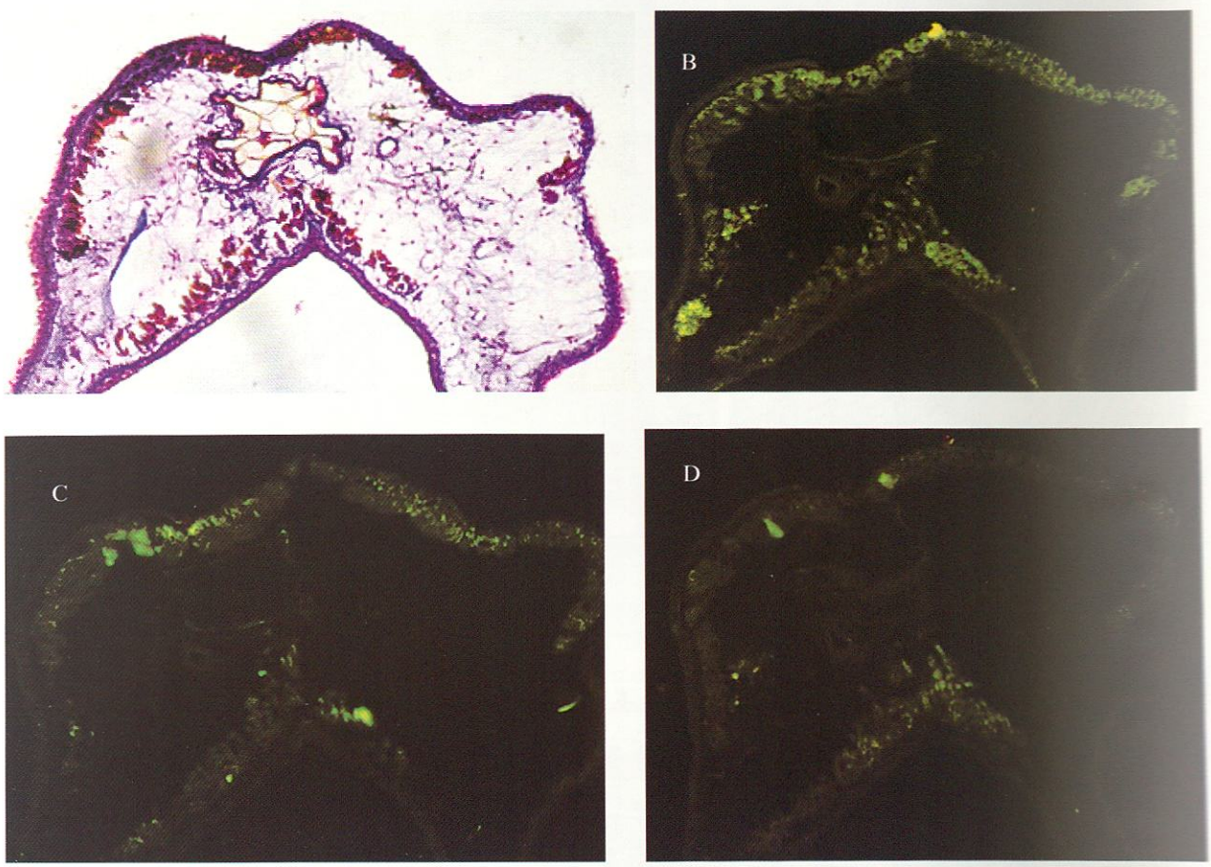


Figure 11: (A) Mallory staining of a retinoic acid treated abnormal tail section; (B,C,D) Immuofluorescence localization of FGF10, 2 and 1, respectively in retinoic acid treated abnormal tail. (Scale bar = 50 µm). (Unpublished data)

In harmony with the earlier reports of the teratogenic effect of Vitamin A palmitate during tail regeneration, retinoic acid (RA) treatment during tail regeneration has also yield the obvious results. A model was proposed by Bryant and Gardiner (1992) to describe vitamin A induced transformation of tail to hind limbs along with pelvic girdle. According to this model, cells at the amputation site change their positional value to flank positional value due to vitamin A treatment. As a result of this change in positional value, from the cut end of tail hind limbs along with pelvic girdle developed instead of tail. FGF1 and FGF2 are potent neurotrophic factors and established mitogens and FGF10 has been known to be an important factor during limb bud initiation. Considering, the above potentialities of these growth factors, immunofluorescence localization was studied in the tissue sections of the regenerating tail and also in the retinoic acid treated regenerates of the tadpoles of *Polypedaus maculatus*.

In the retinoic acid treated tadpoles, stronger immunolocalization of FGF2 and 10 were noted in the tissue regenerates 72 hours onwards. However, FGF1

immunoreactiveity remained low and were mostly found in the regenerating spinal cord. Such cells were also found immunopositive for FGF2 suggesting their neurotrophic action. Since, the immunolocalization of these growth factors was more intense in the retinoic acid treated abnormal tails, their involvement during transdifferentiation of tail cells to limbs is suggested.

Conclusion

Frog tadpoles are easy to rear in laboratory conditions and are excellent model for regeneration studies of an organ. Since the regenerating capacity is lost soon after metamorphosis, there develops a block from regenerating to non-regenerating state. Understanding the mechanism that stops regeneration to occur soon after metamorphosis can unfold the mystery of loss of regenerating capacity in higher vertebrates.

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Lentic tadpole habitat in Evergreen Forest (Jeypore Reserved Forest, Assam). Photo credit: Abhijit Das