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Developmental Biology Laboratory, Department of Zoology,
University of Rajasthan, Jaipur, India

The pattern of decline and loss of the capacity for hind limb regeneration in the tadpoles of *Bufo melanostictus* (Schneider)

SHAHEEN ALAM AND I. A. NIAZI

Introduction

Generally the limb regeneration capacity in the anurans is lost proximodistally during the larval life as they approach matamorphosis (SCHMIDT, 1968; GOSS, 1969; SCADDING, 1977). However, a limited capcity to regene rate at least hypomorphic limb structures is present even in the post-metamorphic stage of some anurans of primitive families like Pipidae and Discoglossidae (GOODE, 1967) and also in some species of the phylogenetically advanced families Ranidae and Hyperoliidae (MICHAEL and AL SAMMAK, 1970; POLEZHAEV, 1972; RICHARDS *et al.* 1975; NIAZI *et al.*, 1979). In other species also the loss of this capacity dose not occur at the same rate or time in the larval period (DENT, 1962; FRY, 1966; MICHAEL and EL MALKH, 1969; SHIVPAL, 1976; MICHAEL and EL MEKKAWY, 1977; AGARWAL and NIAZI, 1980). The causes for such variations within the same order of amphibians are not understood. More systematic and comprehensive studies of limb regeneration are needed in a number of anurans of different families and varying in their habitats, habits, rate of growth etc. before any generalization can be made. The present study is concerned with the hind limb regeneration capacity in the young and advanced larvae of the common Indian toad, *Bufo melanostictus*. This toad has a short larval period of about 4 weeks and becomes strictly terrestrial after metamorphosis. The

young tadpoles of this toad have been used in this laboratory for a number of investigations on the influence of vitamin A, thyroxine and hydrocortisone on hind limb regeneration which have provided important information (JANGIR and NIAZI, 1978; JANGIR, 1979). It was, therefore, considered useful to find out the normal capacity for limb regeneration in older tadpoles also.

Material and Methods

The tadpoles were raised in the laboratory at room temperature (30-32°C) from a spawn obtained from a couple of the toad, *Bufo melanostictus,* caught in amplexus in July, 1979. Tadpoles of required developmental stages according to KHAN (1965) were selected from the stock population. The experiments were made on tadpoles of stages 30/31, 34, 36 and 38. At stage 30/31 the hind limb rudiment is paddle shaped; at stage 34 rudiments of all five toes are present and separated from each other; at stage 36 the hind limbs are well formed and elongated and at stage 38 they are fully developed and functional. Tadpoles were maximally fed with boiled spinach and reared in water which was changed every alternate day. After anaesthetisation in 1:4000 MS 222 (Sandoz) the left hind limb of each tadpole was amputated through thigh, shank or ankle with a blade under a stereoscopic binocular microscope (fig. 1). In case of younger stages the operated tadpoles were fixed in Bouin's solution 15 days after amputation; but the stage 38 tadpoles could be kept alive only for 10 days following amputation by which time they had metamorphosed to become toadlets and could not be kept alive thereafter. Bouin's fixed tadpoles were examined for morphological quality of regenerates which were analysed with reference to three parameters : (a) percentage of cases regenerating; (b) percentage of cases regenerating normal pentadactylous limb and (c) percentage of oligodactylous and defective regenerates. Representative cases were photographed.

Results and Discussion

The results are presented in table I. The data shows that with respect to the above parameters the regenerative

capacity at the three proximodistal levels (thigh, shank and ankle) varies in the young and older tadpoles. At stages 30/31 and 34 regeneration occurred in 100% cases at all three levels. At stage 36 this capacity was reduced and the percentages of amputated limbs regenerating were 80% at ankle, 66.6% at shank and 40% at thigh level. It is obvious that the reduction occurred proximodistally at thigh, shank and ankle levels in this order. In the fully grown tadpoles of stage 38 no regeneration occurred at thigh and shank levels; and even at ankle level in only 3 out of 15 cases a very small, formless outgrowth from the cut end of the stump indicated some regenerative development. These results conform to the general pattern of decline of the regenerative capacity in all anurans studied since the pioneering discovery of this phenomenon by SPALLANZANI (1769).

Although regeneration took place throughout the limb in 100% cases at stage 30/31 not all the regenerates were structurally perfect pentadactylous limbs. The percentage of perfect regenerates at all the three levels was, however, approximately the same (table I; fig. 2). Already at this very young stage the ability to regenerate perfect structures was less than 100% throughout the limb axis. This capacity was found to be reduced further at stages 34 and 36. At these stages, however, there were seen very obvious differences in the capacity to regenerate perfect limbs at the thigh, shank and ankle levels. Reference to table I and fig. 2 would show that the decline occurred most rapidly at the thigh level and relatively slowly at the more distal levels. At stage 34 only 26.7% of regenerates obtained after amputation through thigh were morphologically normal and at stage 36 all the thigh level regenerates were defective and severely oligodactylous or mere formless outgrowths. At stage 36, on the other hand, 30% and 33.3% of regenerates at shank and ankle levels, respectively, were still normally formed with five toes in their feet. The difference between the percentages of normal pentadactylous regenerates obtained at the shank and ankle levels at stage 36 was almost insignificant (table I; fig. 2). Fall in the regenerative capacity at the ankle level upto stage 34 was relatively less

than at the shank level; but subsequently, the decline was very rapid at this level also. In fact, it appeared to be more rapid at ankle level than at shank level. The pattern of proximodistal decline in the morphological quality of regeneration as seen in this species is similar to that for other anurans in general (DENT, 1962; MICHAEL and EL MALKH, 1969; SHIVPAL, 1976; AGARWAL and NIAZI, 1979). However, the rate at which this decline occurs is faster in this species as compared to ranids and *Xenopus laevis*. In this respect it resembles *Bufo andersonii* (SHIVPAL, 1976). In case of *Bufo americana* tadpoles also the loss is stated to be rapid and sudden at all levels of the hind limb (MICHAEL and NIAZI, 1972).

The results clearly showed that the reduction in regenerative capacity follows the same pattern throughout the limb. More and more regenerates are found to be structurally sub-normal and defective; severity of oligodactyly increases progressively and ultimately only formless outgrowths are regenerated (figs. 3-8). Finally, amputation results in healing of the wound and no regeneration occurs at all. This process also occurs proximodistally. It is obvious from table I that at stage 34 and 36 the number of morphologically defective oligodactylous regenerates is greater at the thigh and least at the ankle levels.

The result show that the regenerative capacity in the toad, *Bufo melanostictus,* is lost up to ankle level during the larval life much before metamorphosis which begins at stage 39 and the loss is rapid at all levels. In this respect also it is similar to the toad, *Bufo andersonii*. The frogs, *Rana tigerina* (AGARWAL and NIAZI, 1980), *Rana pipiens* (FRY, 1966) and *Rana catesbeiana* (WEIS and BLEIER, 1973) are reported to retain some regenerative capacity until quite late in the larval life. The difference may be related to the rate of growth and duration of larval life. The duration of larval life of these frogs is much longer than that of *Bufo melanostictus* or *Bufo andersonii*. In case of *Rana catesbeiana* it may be as much as 3 years (DENT, 1968).

It is known that limb regeneration is adversely affected by increase in the thyroid hormone (HAY, 1955; SCHMIDT, 1968; SHIVPAL, 1976; JANGIR, 1979). The production of this hormone in amphibian larvae progressively increases as metamorphosis approaches (ETKIN, 1968). It is likely that the thyroid glands become functional at an earlier stage and their activity subsequently increases more rapidly in certain species than in others resulting in differences in the rate of growth and duration of larval life. In fact, a rather early development of this endocrine gland has been observed in several species of the genus *Bufo* as compared to ranids (ALLEN, 1919; ETKIN, 1930; DENT, 1968; MICHAEL and ADHAMI, 1974; SHIVPAL, 1976; AGARWAL and NIAZI, 1979). This may be one of the factors responsible for the rapid and early loss of regenerative capacity of limbs in *Bufo* tadpoles. It has been observed that the thyroid glands develop earlier and their activity increases more rapidly and consistantly in the larvae of *Bufo andersonii* as compared to those of *Rana tigerina;* and while in the former limb regeneration capacity declines rapidly and is lost much before metamorphosis, in the latter species its fall is more gradual and the final loss occurs much later during the larval life (SHIVPAL, 1976; AGARWAL and NIAZI, 1979, 1980).

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Summary

Hind limb regeneration was investigated in *Bufo melanostictus* tadpoles of developmental stages 30/31, 34, 36 and 38 at thigh, shank and ankle levels. Regeneration occurred in 100% cases at all levels upto stage 34. At stage 36 it occurred in 40% cases at thigh, 66.6% cases at shank and 80% cases at ankle levels. At stage 38 this capacity was absent at all levels. Decline in this capacity was manifested in production of progressively decreasing number of pentadactylous and increasing number of oligodactylous and defective regenerates at thigh, shank and ankle levels in this order. Even in the youngest

tadpoles not all regenerates were morphologically perfect. The capacity to regenerate limbs is lost rapidly and much before metamorphosis in this species.

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Authors' address : SHAHEEN ALAM and DR. I.A. NIAZI, Developmental Biology Laboratory, Department of Zoology, University of Rajasthan, Jaipur-302004.

Table I Capacity of hind limb regeneration at thigh, shank and ankle levels in *Bufo melanostictus* tadpoles of different developmental stages.

Stage	Amputation level	Limbs amputated No.	Limbs regenerating No. (%)	Morphological quality of regeneration No. of regenerates with						% age of pentadactylous regenerates
				5 toes	4 toes	3 toes	2 toes	1 toe	Formless regenerates	
30/31	Thigh	25	25 (100%)	21	3	—	—	1	—	84.0
	Shank	15	15 (100%)	13	2	—	—	—	—	86.7
	Ankle	20	20 (100%)	17	3	—	—	—	—	85.0
34	Thigh	30	30 (100%)	8	3	5	6	1	7	26.7
	Shank	30	30 (100%)	13	10	5	—	2	—	43.3
	Ankle	35	35 (100%)	24	9	2	—	—	—	68.6
36	Thigh	15	6 (40%)	—	1	1	2	—	2	0
	Shank	15	10 (66.6%)	3	2	2	3	—	—	30
	Ankle	15	12 (80%)	4	5	3	—	—	—	33.3
38	Thigh	15	Nil	—	—	—	—	—	—	0
	Shank	12	Nil	—	—	—	—	—	—	0
	Ankle	15	3 (20%)	—	—	—	—	—	3*	0

*These regenerates were narrow spike like outgrowths or short extensions of the stump.

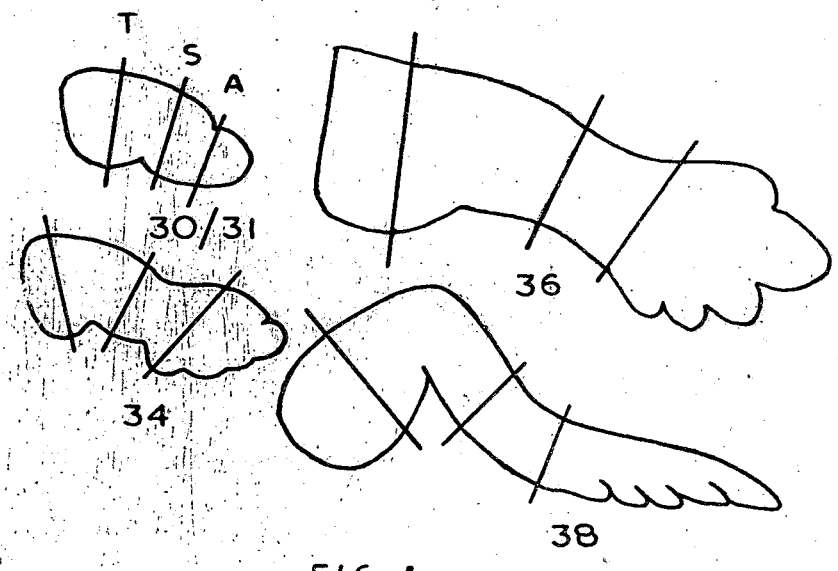


Fig. 1. Outline drawings of hind limbs of stage 30/31, 34, 36 and 38 tadpoles. Transverse lines show planes of amputation through thigh (T), shank (S) and ankle (A).

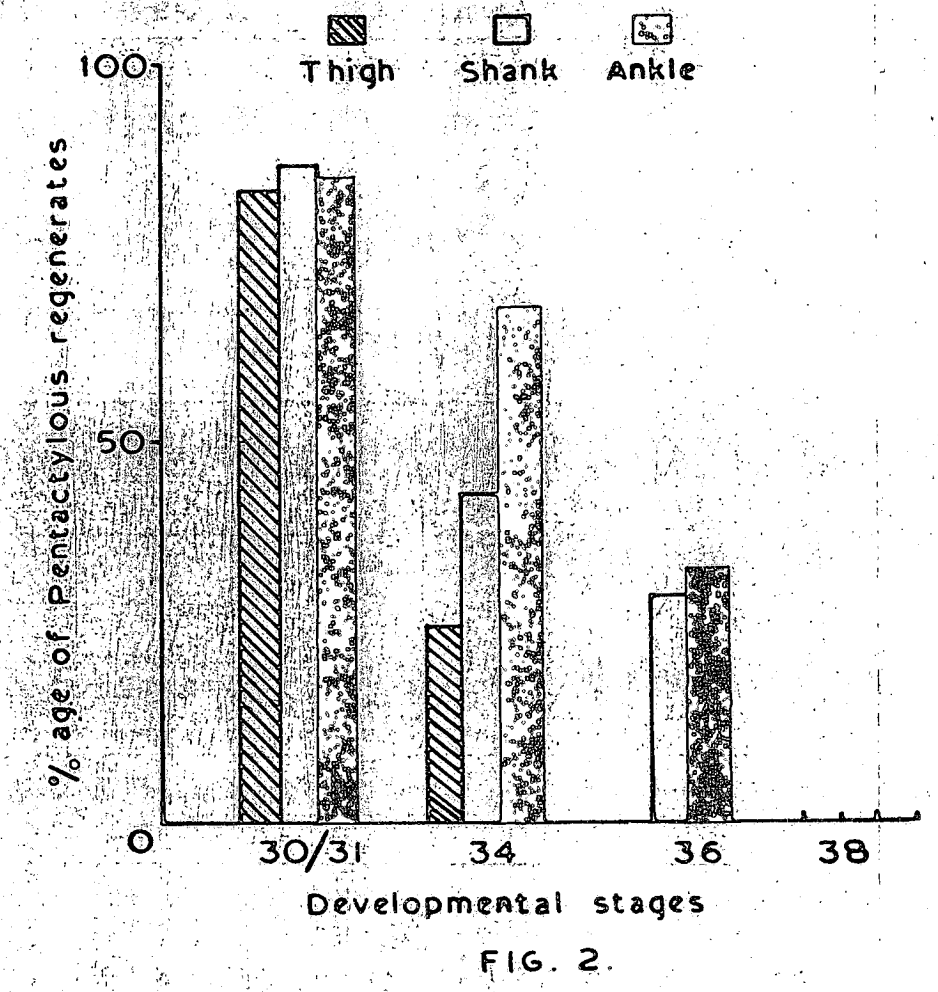
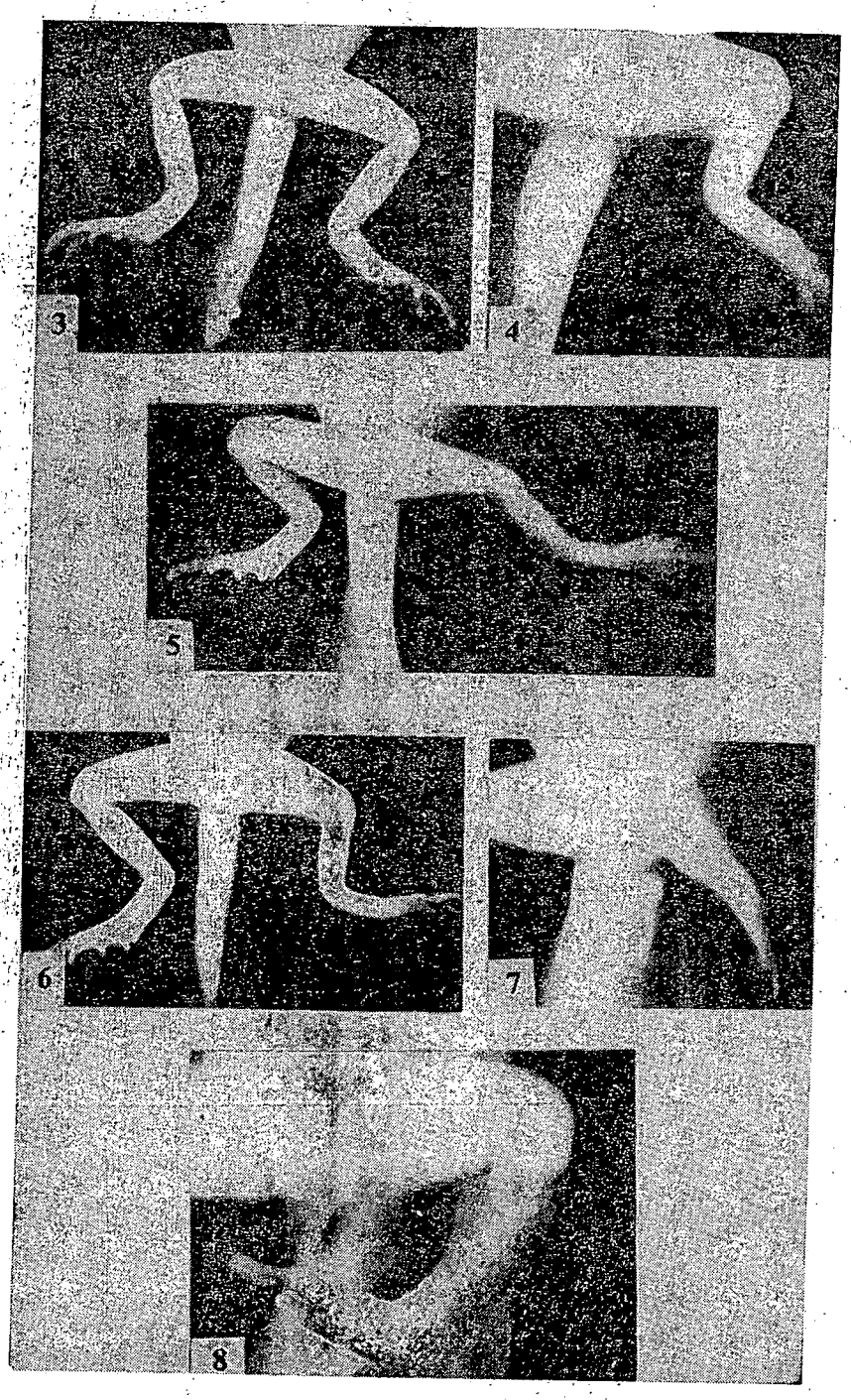


FIG. 2.

Fig. 2. Histograms of percentages of normal pentadactylous limb regenerates obtained in tadpoles of stages 30/31, 34, 36 and 38.

Hind limb regeneration in *Bufo melanostictus* tadpoles



(see reverse)

Figs. 3-7. Ventral views of the various types of regenerates of stage 34 resulting from amputation through thigh of the left hind limbs : Normal, pentadactylous (fig. 3), pentadactylous but defective (fig. 4), oligodactylous with 4 toes (fig. 5), with 3 toes (fig. 6) and highly malformed with 2 toes (fig. 7).

Fig. 8. A malformed ankle level regenerate of a tadpole of stage 36 with 3 toes.