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Introduction

Although in most anuran species the power of limb regeneration is lost during larval life before metamorphosis it is retained at least to a limited extent in some others even in the adult (GOODE, 1967; GOSS, 1969; POLEZHAEV, 1972; NIAZI *et al.* 1979). Recently, RICHARDS *et al.* (1975) discovered that the Kenyan reed frog, *Hyperolius viridiflavus* of the family Hyperoliidae is able to regenerate digits complete with digital pads after amputation through digits even in the adult stage. When amputation is made between the elbow and the wrist only a spike is regenerated at whose tip, however, the digital pad, a terminal structure of the forelimb, does differentiate. The digital pads are adaptations of this frog to cling to the reeds; and according to these authors the ability to restore this structure irrespective of the type of regenerate developing after amputation through a proximal segment of the fore-limb should have a survival value for this species. This finding indicates that the power to regenerate may have adaptive significance at least in some species. The present study of limb regeneration in the tadpoles of spade foot frog was motivated by the finding of RICHARDS *et al.* (1975) mentioned above. *Rana breviceps* is a fossorial frog with a spade-like structure behind the Ist toe which is an adaptation for burrowing. The idea was to study the regeneration capacity at various proximo-distal levels of the hind limbs in young to older tadpoles and

froglets and to see whether the 'spade' is restored irrespective of the type of the regenerate obtained.

Materials and Methods

The tadpoles of *Rana breviceps* were raised in the laboratory from a single spawn, obtained from an amplecting couple. The studies were made on tadpoles of stages VIII/IX, XIII/XIV, XVI/XVII and on 15-20 days old froglets. the tadpoles were staged according to the normal table of TAYLOR and KOLLROS (1946) for *Rana pipiens*. In the tiny hind limbs of stages VIII/IX tadpoles thigh, shank and ankle are recognizable and rudiments of toes 3, 4 and 5 are separated from each other by indentations. Limbs at stages XIII/XIV are well differentiated with distinct foot and ankle regions and are functional. At stages XVI/XVII the hind limbs are fully developed and the tadpoles are about to enter metamorphic climax. In tadpoles and froglets the hind limbs were amputated across the middle of thigh, shank or ankle after narcotizing them in 1:4000 solution of MS 222 (Sandoz) or with diethyl ether, respectively. Only the left or both hind limbs were amputated. The operated tadpoles were reared in tap-water in glass troughs and froglets in a large rectangular glass container with mud in the bottom, a small water pool and planted grass and vegetation around it. The tadpoles were fed boiled spinach and the froglets were provided with small insects. The operated animals were fixed in Bouin's fluid 15-20 days after amputation. The experiment was carried out at room temperature (30-32 °C).

Results

Limb regeneration capacity. Like other anurans the ability to regenerate hind limbs was found to decline proximodistally along the limb axis (table I). At stages VIII/IX regeneration occurred in 100% cases at all the levels (thigh, shank and ankle) through which amputations were performed. At stages XIII/XIV this capacity was completely absent at the thigh level, showed slight reduction at shank-level but at the ankle level 100% cases still regenerated. In the fully grown tadpoles of stages XVI/XVII regeneration

occurred in a little more than 56% cases of shank and in 100% cases at ankle levels. Even in froglets regeneration took place in all cases at ankle level although the regenerates were mere spikes or conical outgrowths. At shank level a little regenerative development was seen at least in 3 out of 10 cases.

With respect to morphological quality, all the thigh and shank level regenerates of stages VIII/IX tadpoles were perfectly normal with 5 toes in each. It was surprising, however, to find that the great majority of ankle level regenerates (23 out of 26) were imperfect with 21 possessing 4 toes and 2 having 3 toes (table 1). At stages XIII/XIV nearly 3/4 of the shank level regenerates were perfect pentadactylous limbs and the remaining were oligodactylous; but in tadpoles of these stages also 38 out of 66 ankle level regenerates were oligodactylous (most having 4 toes) and only 18 (nearly 20%) were perfectly normal limb structures with all 5 toes (table 1). At stages XVI/XVII there was a sharp reduction in the quality of regeneration at shank level; about half the regenerates were only spike-like structures and the great majority of the rest were oligodactylous and defective. At ankle level the regenerates were relatively better formed than those of the shank level although even among them the extent of oligodactyly had increased and in 8 out of 78 cases only flattened spikes had regenerated (table 1).

In froglets the 3 regenerates obtained at shank level were only very small rounded/conical extensions of the stump. Among the ankle level regenerates 8 cases were mere small outgrowths of the stump and 11 were elongated flattened spikes (table 1).

Development of the spade in regenerates. All the regenerates obtained following amputation at all the three levels in the tadpoles and froglets and irrespective of their morphology and size were carefully examined under a stereoscopic binocular microscope for the presence or absence of the spade. With some exceptions it was found to be present in all regenerates including both the pentadactylous

and oligodactylous ones. Even those in which only 1 or 2 toes had developed or only a spike like regenerate had resulted a spade was present in the preaxial region (table 1; figs. 1-4).

The exceptions included shank-level regenerates which possessed 1 to 3 toes or were mere spikes in tadpoles of stages XIII/XIV and XVI/XVII (table 1; fig. 5). Also, when regenerates were only small conical or rounded outgrowths of stump there was no evidence of the formation of a spade.

Some of the regenerates including those with or without the spade were stained *in toto* with victoria blue or alizarin for study of their skeletal elements. The examination of this stained material showed that the spade developed only in those regenerates in which at least both the proximal tarsals were present. In oilgodactylous shank-level regenerates of stage XIII/XIV and XVI/XVII which lacked the spade the regenerated limbs were not only deficient in number of toes but the skeletal elements of their ankle region were also incomplete containing at most only 1 long tarsal bone (most probably the calcaneum). In the spike-like regenerates of this level in stage XVI/XVII tadpoles there was no evidence of differentiation of any tarsal or more distal elements at all. In the ankle-level regenerates irrepective of their morphology both the proximal tarsals were present and in all regenerates of this level spade was present. The regenerates which were small conical outgrowths of the stump contained only a mass of cartilage in which no morphogenesis of separate skeletal elements had occurred.

Discussion

As far as the ability to regenerate at least to some extent is concerned it is found that it is lost at the thigh level completely by the time the tadpoles of *Rana breviceps* attain stage XIII; but at the more distal levels it persists to a fairly high degree until much later up to just before the beginning of metamorphosis. In the shank and ankle, particularly the latter, it is present to a limited extent

even in the froglets. The general pattern of decline is the same as in other anurans which consists of the production of increasing number of defective limb regenerates with less than normal number of toes, spike like structures and then only mere rounded extensions of the stumps and finally simple healing. (DENT, 1962; AGARWAL and NIAZI, 1980; MICHAEL and El MALKH, 1969; MICHAEL and El MEKKAWY, 1977). This species resembles *Rana pipiens* (FRY, 1966) in losing the capacity at thigh level completely at a rather early stage. In possessing a limited capacity to regenerate at least some spike-like structures after amputation through ankle even after metamorphosis it joins the ranks of those ranids and other anurans which can regenerate at least hypomorphic limb structure even in the adult stage.

A surprising feature observed was the regeneration of perfect limbs at ankle level in very few cases even in the youngest tadpoles when at the more proximal levels all the regenerates were normal and pentadactylous. This was also noticed by SHIVPAL (1976) and AGARWAL and NIAZI (1980) in very young tadpoles of *Bufo andersonii* and *Rana tigerina*, respectively. In the present study of *Rana breviceps* tadpoles, however, the difference between the morphological quality of ankle-level regenerates and those of proximal levels was very great in the young stages and this magnitude of difference persisted in tadpoles of later stages also (table 1). All authors who have studied limb regeneration at various proximodistal levels in anuran tadpoles of successive developmental stages have found strictly proximodistal pattern of decline in the capacity to regenerate morphologically perfect limbs. For example, reference may be made to the papers of DENT (1962); MICHAEL and El MEKKAWY (1977); AGARWAL and NIAZI (1980), etc. The present results in *Rana breviceps* tadpoles indicating the reduced ability to regenerate perfect limbs at ankle level as compared to proximal levels militate against the generally observed phenomenon of proximodistal decline in the regenerative capacity in the limbs of anuran larvae. Many years ago VAN STONE (1957) had also found that in *Rana sylvatica* tadpoles regenearation took

place following amputation at the pelvic level even after this ability was lost at the more distal thigh and shank levels. To our knowledge no other report of this kind has been reported so far. Reasons for this discrepancy are not clear.

It was interesting to find. develpment of the spade in all regenerates irrespective of their morphological quality. However, it must be noted that it developed only when both the proximal tarsals were present. The spade is an enlarged and modified prehallux found in some other fossorial frogs and toads also (NOBLE, 1931). It seems its development is in some way connected with the presence of one of the proximal tarsals (astragalus); and in the absence of the latter the prehallux and, consequently, the spade also does not develop at all. So long as the astragalus or its rudiment differentiates the spade like structure also appears in the regenerate irrespective of the number of toes or overall morphogenesis of the foot. The ability to regenerate a spade even with a defective foot may be an adaptation with a survival value related to the habits and habitats of the frog. In this respect it may be placed in the same category as *Hyperolius* which can regenerate digital pads (RICHARDS *et al.* 1975). In *Hyperolius* the digital pad is formed by modification of the epidermis at the tip of the spike-like regenerate at the lower arm level also without fo mationrof digit. The spade in *Rana breviceps,* however, develops only when the astragalus or its rudiment is also present.

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Summary

Hind limb regeneration capacity was investigated in the tadpoles of stages VIII/IX, XIII/XIV, XVI/XVII and froglets of the spade foot frog *Rana breviceps* at thigh, shank and ankle levels. At thigh level perfect regeneration occurs in all cases at stage VIII/IX but this power is completely lost by stage XIII/XIV. At shank level all amputated

limbs regenerate perfectly at stage VIII/IX but by stage XVI/XVII this power is sharply reduced. At ankle level regeneration occurs in all young to older tadpoles but surprisingly the ability to form perfect regenerates is always less than at proximal levels. Limited capacity to regenerate hypomorphic structures is present at ankle level even in the froglets. The spade, an adaptation for burrowing, develops in all regenerates irrespective of their morphology provided they contain proximal tarsals.

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Table 1 Capacity of regeneration in the hind limbs of the frog *Rana breviceps*.

Stage at amputation	Level of amputation	Number of limbs amputated	Limbs regenerating No. (% age)	Morphological quality of regeneration					
				Regenerates with					Simple outgrowths.
				5 toes	4 toes	3 toes	2 toes	1 toe	
VIII/IX	Thigh	20	20 (100%)	20_+	—	—	—	—	—
	Shank	25	25 (100%)	25_+	—	—	—	—	—
	Ankle	26	26 (100%)	3_+	21_+	2_+	—	—	—
XIII/XIV	Thigh	25	nil (0%)	—	—	—	—	—	—
	Shank	26	25 (96.2%)	18_+	3_+	2_-	1_-	1_-	—
	Ankle	66	66 (100%)	18_+	30_+	11_+	6_+	1_+	—
XVI/XVII	Thigh	46	nil (0%)	—	—	—	—	—	—
	Shank	80	45 (56.25%)	3_+	8_+	2_-	5_-	11_-	22^*_-
	Ankle	78	78 (100%)	4_+	28_+	18_+	14_+	6_+	8^*_+
Froglets	Thigh	10	nil (0%)	—	—	—	—	—	—
	Shank	10	3 (30%)	—	—	—	—	—	3^{**}
	Ankle	19	19 (100%)	—	—	—	—	—	$11^*_+;8^{**}$

*flattened spike like regenerates without toe formation; **conical/rounded outgrowth or extension of stump +spade present.

Limb regeneration in spade-foot frog

(see reverse)

Figs. 1-3 : Regenerated limbs with 5 (fig. 1), 4 (fig. 2) and 3 (fig. 3) toes resulting from amputation through ankle in tadpoles of stages VIII/IX.

Fig. 4 : A hypomorphic regenerate with 1 toe resulting from amputation through ankle in a XIII/XIV stage tadpole.
Arrow indicates the regenerated spade.

Fig. 5 : A defective regenerate with 2 toes resulting from amputation through shank in a XVI/XVII stage tadpole. Spade was not formed.