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REGENERATIVE ABNORMALITIES IN HIND LIMBS OF *BUFO REGULARIS* INDUCED BY REPEATED AMPUTATIONS I. EARLY LARVAL STAGES

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التشوهات الناتجة من البتر المتكرر في الاطراف الخلفية المتجددة للضفدع بوفو رجيولارس
١ ـ المراحل اليرقية المبكرة

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درس المؤلفان في هذا البحث القدرة على التجدد في الأطراف الخلفية للمراحل اليرقية المبكرة ٥٢ ، ٥٣ ، ٥٤ ، ٥٥ في الضفدع المصري بوفو رجيولارس بعد البتر المتكرر في مستوى رسغ القدم . وقد أظهرت دراسة الأطراف المتجددة مورفولوجيا وفحص تركيبها الهيكلي زيادة في درجة تشوه الأطراف مع تقدم اليرقات في العمر وزيادة عدد مرات البتر . وتتمثل هذه التشوهات في اختزال عدد الاصابع وعدد السلاميات ، قصر الاصابع وفي التوقف الكامل لعملية التجدد .

وقد نتج عن اعادة بتر الطرف في نفس المستوى ظهور أطراف متجددة ذات تشوهات تركيبية تصل نسبتها إلى ٤١ – ٧١٪ من الحالات (في المراحل اليرقية المختلفة) وذلك بعد حدوث البتر مرتين ، أما بعد البتر ثلاث مرات فإن نسبة التشوهات تصل إلى ٧٨ – ٩٦٪ من الحالات . وقد اقترح المؤلفان من هذه النتائج أن حدوث التشوهات للأطراف المتجددة يزيد زيادة مباشرة مع عدد المرات التي تحتاج إليها الأطراف المبتورة (جدعة الطرف) إلى فقد التميز وتكوين البلاستيما لاستعادة الأجزاء المفقودة منها . وقد تم استنتاج أن تكرار البتر في نفس المستوى للأطراف المتجددة كان السبب الوحيد لزيادة نسبة التشوهات والتي ظهر معظمها في المرحلة اليرقية ٥٥ .

Key Words: Bufo, regeneration, amputation, abnormalities.

ABSTRACT

The regenerative response of the hind limbs of early larval stages: 52, 53, 54 and 55 of *Bufo regularis* was studied following repeated amputations at the ankle level. The pattern of morphogenesis as well as skeletal configuration of the regenerates showed increasing degrees of abnormality, with a gradual reduction in the number of toes or phalanges, shortening of toes or complete cessation of regeneration, as the larvae grow older and the number of amputations increases. Reamputations at the same level resulted in structurally abnormal regenerates in 41-71% of the cases (at the different larval stages) following two amputations; and 78-96% of the cases following three amputations. The results suggested that the occurrence of abnormal regeneration was found to increase directly with the number of times the limb stumps were induced to initiate dedifferentiation and repair. The increase percentage of abnormal regenerates mostly established by stage 55, Series III, was the result of repeated amputation at the same level of the regenerating limb, combined with the greater differentiation of the limb tissues.

INTRODUCTION

Various investigators have, for many years, employed urodelean and anuran limb regeneration to examine the process of vertebrate regeneration. It has been recognized for a long time that in the anuran larvae, unlike urodeles, the regenerative ability is gradually lost proximodistally along the limb axis with the onset and further progress of metamorphosis (see Goss, 1969). However not in all species is this capacity lost completely by the end of larval life; and also, the developmental stage at which this ability disappears varies in different species.

Many studies have revealed the production of various categories of regenerative abnormalities from normal regeneration experiments. Defective regenerates were produced from stages 55, 56, 57 and 66 of *Bufo regularis* (Michael and El-Mekkawy, 1977a, b). The distal as well as the proximal skeletal elements were affected through the reduction of phalanges, reduction of number of toes and autopodial elements, fusion of some toes together, or development of two diverging prongs. Some malformed regenerates were earlier recorded at stage 55 of *Xenopus laevis* (Dent, 1962); long bones were shortened, and joints were ankylosed. Observations of Anton *et al.* (1988) on the same species revealed the presence of malformed regenerates after transection through the thigh at stage 53; the tibiofibula showed continuity with the first metatarsal, and autopodium was nearly absent.

There are many reports in the literature on how growth of the regenerating amphibian limb is affected by various natural parameters such as temperature (Schmidt, 1968; Schauble and Nentwig, 1974), body size and age (Goodwin, 1946; Manner *et al.*, 1960; Pritchett and Dent, 1972; Zamaraev, 1974, for review), season of the year (Schauble, 1972), starvation (Twitty and De Lanney, 1939), and level of amputation (Iten and Bryant, 1973; Smith *et al.*, 1974; Abdel-Karim, 1989) Abnormal regenerative responses have been induced by X-irradiation (Butler, 1933, 1935; review by Brunst, 1950; Goss, 1957), skin cuff rotations (Settles, 1967; Carlson, 1974a; Lheureux, 1972, 1975a, b), minced muscle implantation (Carlson, 1975 for review) and treatment with vital dyes (Dearlove *et al.*, 1975). Shift-level grafting procedures in larval *Ambystoma* (Stocum, 1975a) and adult *Notophthalmus* (Iten and Bryant, 1975) have been undertaken to examine the possibility of "positional information" directing the regenerative processes (see also Summerbell *et al.*, 1973; Smith *et al.*, 1974 for review). Such grafting procedures necessitate performing more than one amputation of the limb, since blastemata derived from one level of the proximodistal axis of the limb are removed and transplanted to other levels along the limb axis. The regenerates formed following these grafting procedures were examined for serial duplications or intercalary deletions of skeletal elements (Stocum, 1975a: Iten and Bryant, 1975). By repeated amputations through the same plane of adult newt forelimbs (Dearlove and Dresden, 1976), the following pattern emerged. Interdigit webbing occurred most frequently at each amputation. Autopodial reduction (i.e. distal developmental errors) occurred with the next greatest frequency while major developmental anomalies (e.g., precocious termination of the regenerative response) occurred with less regularity (15-25% of the abnormal cases) Increasing the number of amputations of the newt limb not only resulted in more regenerates with major abnormalities, but also increased the incidence of occurrence of all categories. Kollros (1984) found that repeated amputation of limb buds during the normal period of regenerative decline, considerably prolonged the period of regenerative competence in two genera of anurans (*Rana* and *Pseudacris*). The present study was carried out to investigate the regenerative capacity and the incidence of various types of abnormal regenerates following repeated amputations just distal to the same original plane in hind limbs of early larval stages of *Bufo regularis.*

MATERIAL AND METHODS

Early larval stages of *Bufo regularis* obtained from some ponds in El-Zobara Farm in Doha, Qatar, were maintained at room temperature (24±1°C). Stages numbers 52, 53, 54 and 55 (according to the normal Table of Sedra and Michael, 1961, for the same species) were used in the study. The most distinctive criteria for these stages were:

Stage 52, 22 days: Distal part of hind limb bud paddle-shaped; first indication of ankle constriction.

Stage 53, 24 days: Foot paddle shows fourth and fifth toe protuberances.

Stage 54, 25 days: Foot has toe protuberances 5-3.

Stage 55, 26 days: Margin of foot paddle is indented between all five toes.

As anaesthetic, MS 222 (ethyl-aminobenzoate methane sulfonate) in tap water in a concentration of 1:2000 was used. The experiments were divided into three types: 1) Series I (control group): Amputation, at the ankle level or its anlage, was carried out through the right hind limb or its bud. 2) Series II: limb amputation was carried out as in series I and then by the fourth post-operative day, the larvae were again narcotized and the operated limbs were reamputated at a level just distal to that of the previous amputation (indicated by pigmentation differences). 3) Series III: In this type, limb amputation was carried out as in series I and reamputated as in series II and then the procedure was repeated (a third amputation) on the same plane, on the third day after the second amputation. The operated cases were reared for a period ranging from 3-7 weeks and all had completed their metamorphosis before fixation. The regenerating limbs were examined for gross morphology and were subjected for macroscopical examination of the skeletal configuration after preparing transparencies by using toluidine blue-Alizarine red S stain (double staining technique, modified from Dingerkus and Uhler, 1977 and Kimmel and Trammell, 1981). Total of about 270 limbs or limb buds were used in the experiments. The percentages of the abnormal regenerates produced in each experiment was calculated for the different concerned stages.

RESULTS

Tables 1, 2 and 3 summarize the percentages of abnormal regenerates following repeated amputations of the hind limbs of the concerned stages. A single amputation of the hind limbs (series I) resulted in the regeneration of morphologically and functionally normal replacement appendages in about 85-95% of the cases at the different operated stages (see Table 1). Repeated transections of the hind limbs resulted in the occurrence of abnormal regenerates which increased in direct proportion to the number of times a limb was amputated, and also to the progress in the developmental larval stages. The initial amputation resulted in abnormal regenerates in 5% of cases at stage 52 and 14% of cases at stage 55. Following two amputations, abnormal regenerates in 41% of cases at stage 52 were obtained, whereas at stage 55, 71% of cases showed abnormal regeneration. The incidence of malformations of the regenerating limbs then increased following the third amputation at the same level, recording 78% of cases at stage 52 and 96% at stage 55. The abnormalities appear either as gross morphological deviations from normal hind limb size and shape (Figs. 1-8), and/or as alterations in the normal pattern of skeletal elements expressed by the regenerates (Figs. 5 & 7). The abnormal regenerates obtained could be subdivided into three categories, based on the extent of these malformations: minor, intermediate and major abnormalities.

(a) Minor abnormalities

This category appeared as an incomplete replacement of the distal-most autopodial (foot plate) elements. These animals are characterized as having shortened or partially fused digits while structurally they exhibit deletions or fusions of their autopodial skeletal elements. However, most of the limbs of this category were functionally used.

Table 1
Percentage of abnormal regenerates following a single amputation (Series I).

Stage at operation	Total No. of limbs	Frequency of abnormalities			
		Gross Morphology		Gross Morphology & skeletal configuration	
		No. of abnormal limbs	Percent	No. of abnormal limbs	Percent
.52	20	1	5	1	5
53	26	2	7	3	11
54	22	2	9	2	9
55	28	3	10	4	14

Table 2
Percentage of abnormal regenerates following two amputations on approximately the same plane (Series II).

Stage at operation	Total No. of limbs	Frequency of abnormalities			
		Gross Morphology		Gross Morphology & skeletal configuration	
		No. of abnormal limbs	Percent	No. of abnormal limbs	Percent
52	17	6	35	7	41
53	20	9	45	11	55
54	18	10	55	11	61
55	21	12	57	15	71

Table 3
Percentage of abnormal regenerates following three amputations on approximately the same plane (Series III).

Stage at operation	Total No. of limbs	Frequency of abnormalities			
		Gross Morphology		Gross Morphology & skeletal configuration	
		No. of abnormal limbs	Percent	No. of abnormal limbs	Percent
52	19	12	63	15	78
53	28	23	82	25	89
54	25	21	84	23	92
55	24	22	91	23	96

Table 4

Numbers of minor (M), intermediate (D) and major (J) abnormalities obtained after the first amputation (series I), second amputation (series II) or third amputation (series III) of the hind limbs of larval stages 52, 53, 54 and 55 of *Bufo regularis*. No case of major abnormalities was recorded in series I or II.

Stage at operation	Number of abnormalities								
	Series I			Series II			Series III		
	M	D	J	M	D	J	M	D	J
52	1	-	-	3	4	-	3	6	6
53	2	1	-	4	7	-	5	8	12
54	2	-	-	4	7	-	3	8	12
55	3	1	-	5	10	-	-	7	16

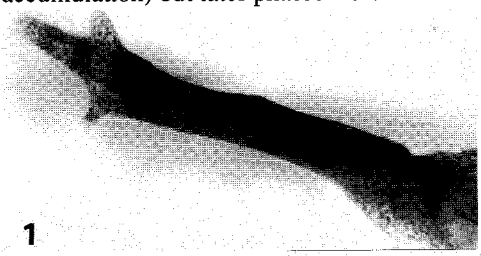
This type of anomaly occurred on limbs which have progressed normally through the early phases of wound healing, blastemal accumulation and growth and begin to display abnormalities during the formation of foot plate. Such abnormal limbs were characterized by distinct morphological malformations in the autopodium. Most defects were localized distally and appeared as limbs with reduced digit numbers (Figs. 1 & 2). The appearance of limbs with these regenerative defects was more prominent in limbs of series I (a single amputation) especially at stage 55, but some limbs were also found in series II (two amputations) which were also more frequent at stage 55 (see Table 4). Some other cases were recorded in series III (three amputations). In these limbs, the toes were foreshortened through the reduction of the number of the phalanges; and fusion of some toes together (syndactyly) was also observed.

(b) Intermediate abnormalities:

Regenerates which fall into this category have greater errors than the minimal distal abnormalities described above but are not severe as to yield a non-regenerating or functionally useless limb. The regenerates exhibited severe malformations in the autopodium region. These abnormalities were most frequent in series II and III, but two cases were also recorded in series I. Mot defects (at the operated stages 53, 54 and 55) appeared as erroes of the skeletal elements in the autopodium. Fusion of some elements was observed, and some regenerates were hemimelic, each having abnormal shank and an atrophied foot with four toe protuberances (Fig. 3). Other regenerates (of the operated stage 55) exhibited some digitlike prominences protruding from the ventral surface of the autopodium. Some other abnormal regenerates appeared as normal limbs in configuration but were greatly atrophied being slender and weak (Fig. 4). In series III, some abnormal regenerates showed misorientation of the toes; some of them were greatly shortened (Fig. 5). Although distal developmental abnormalities are the most commonly occurring errors encountered following repeated amputations, stylopodial (thigh) and zeugopodial (shank) anomalies were also obtained. Some of these abnormalities are shown in Fig. 6, where defects extended to the shank as well as to the thigh regions; the size of stylopodium, zeugopodium (hypomorphic) and autopodium is reduced (Fig. 6a). Other cases developed four short toes, having reduced shank which formed a kink with the ankle (Fig. 6b).

(c) Major abnormalities:

Regenerates belonging to this category were greatly severed producing non-regenerating or functionally useless limbs. These abnormalities were obtained in series III (three amputations) and most frequently at the operated stage 55. No case of series I or II was recorded showing this category of abnormalities. The third amputation of the hind limbs often produced major disruptions of the regeneration process and in addition, the larva progressed through metamorphosis and the hind limb was getting larger and differentiating. These major abnormalities ranged from the production of regenerative spike (Fig. 7) especially at stage 53 through the total deletion of the toes, with part of the autopodium regenerated (with tapering end), to the complete cessation of regeneration. In those latter abnormalities, the stump failed to restore the autopodium, and it was ending with a blunt end (Fig. 8). These limbs appeared to be undergoing normal early regeneration processes (i.e., wound healing and blastemal cell accumulation) but later phases were arrested.



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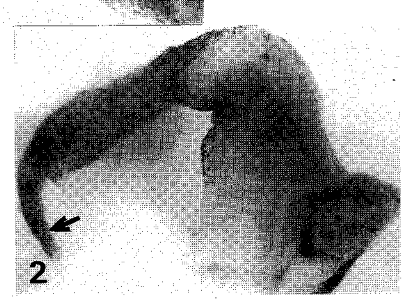
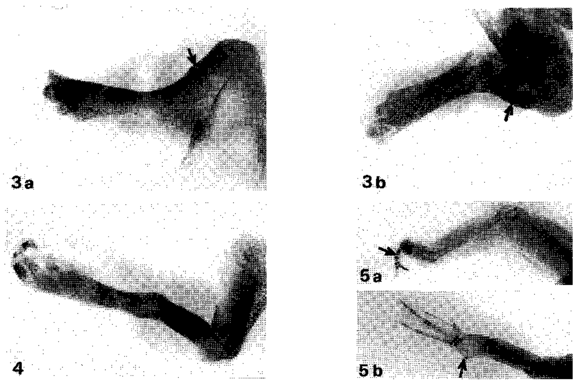


Fig. 1: A regenerate ending in 3 short toes; fixed 7 weeks after the second amputation at stage 52 (minor abnormalities, series II). X 20.

Fig. 2: A regenerate having abnormal shank (reduced), and well developed ankle ending in one toe (arrow); fixed 4 weeks after the second amputation at stage 55 (minor abnormalities, series II). X 20.



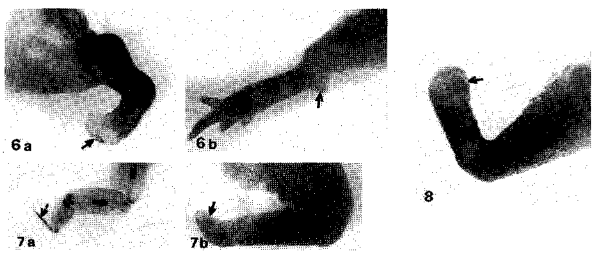


Fig. 3a: A regenerate having reduced abnormal shank (arrow), and ankle with atrophied foot ending in four toe protuberances; fixed 4 weeks after the third amputation at stage 54 (intermediate abnormalities, series III). X 20.

Fig. 3b: A regenerate having reduced abnormal shank (arrow), and ankle with atrophied foot ending in four toe protuberances; fixed 4 weeks after the third amputation at stage 55 (intermediate abnormalities, series III). X 20.

Fig. 4: A regenerate with complete limb segments, but appeared abnormal being slender and weak; fixed 6 weeks after the third amputation at stage 53 (intermediate abnormalities, series III). X 20.

Fig. 5a: A regenerate with normal shank and greatly reduced abnormal ankle (with one bone) ending in one short abnormal toe (arrow); fixed 3 weeks after the third amputation at stage 55 (intermediate abnormalities, series III). Double staining of skeleton. X 20.

Fig. 5b: A regenerate with reduced shank and greatly reduced ankle (arrow), with abnormal skeleton, ending in 3 short toes; number of phalanges reduced; fixed 4 weeks after the third amputation at stage 54 (intermediate abnormalities, series III). Double staining of skeleton. X 20.

Fig. 6a: A regenerate appeared hypomorphic, its limb segments (stylopodium, zeugopodium and autopodium) are reduced in size and autopodium ending in an atrophied foot (arrow); fixed 3 weeks after the third amputation at stage 55 (intermediate abnormalities, series III). X 24.

Fig. 6b: A regenerate with reduced shank forming a kink with ankle (arrow) which ending in 4 short toes; fixed 4 weeks after the third amputation at stage 55 (intermediate abnormalities, series III). X 22.

Fig. 7a: A regenerate with normal shank and reduced ankle ending in a spike-like toe protrusion (arrow); fixed 6 weeks after the third amputation at stage 53 (major abnormalities, series III). Double staining of skeleton. X 18.

Fig. 7b: A regenerate with normal shank and greatly reduced regenerated ankle (arrow), and total absence of toes; fixed 5 weeks after the third amputation at stage 54 (major abnormalities, series III). X 24.

Fig. 8: A regenerate with reduced shank, the stump failed to restore the ankle and having a blunt end (arrow); fixed 3 weeks after the third amputation at stage 55 (major abnormalities, series III). X 24.

DISCUSSION

The present results clearly show that various categories of regenerative abnormalities have been produced by repeated amputations of the hind limbs of *Bufo regularis* at the early larval stages. These abnormalities comprise gross morphologically malformed regenerates and defects in the skeletal structures of the autopodial as well as zeugopodial regions. Many previous investigations on the regenerative capacity of the urodelean and anuran limbs led to the induction of abnormal regenerative responses; stimulated regeneration in tissues normally refractive to regeneration, or prevented a regeneration response in normally regenerating tissues (see Carlson, 1974; Wolsky, 1974, for reviews). Settles (1967), Lheureux (1972, 1975 a, b) and Carlson (1974a) have obtained a complete range of regenerative responses (from inhibition of regeneration to formation of supernumerary regenerates) by surgical manipulations of cuffs of skin grafted to the limb prior to amputation. Removal of the apical skin cover on the third post-operative day, followed by traumatization after further two days in the limb resulted in the enhancement of the regenerative ability in *Bufo regularis* (Michael and Aziz, 1975). Minute electrical currents could enhance limb regeneration in adult *Xenopus* (Borgens *et al.*, 1979). Intercalary regeneration in the hind limbs of larval stages of *Bufo regularis* was revealed by removal of the shank and base of ankle region (Michael and Hassona, 1982). The intercalated skeleton, particularly that of the ankle, showed deformity in its organization by stage 55. Supernumeraries appeared close to the transplantation sites in the operated cases of stages 53, 54 and 55. The present investigations have demonstrated that the occurrence of abnormalities with respect both to gross morphology and to skeletal structure was found to increase directly with the number of times of limb amputations and the limb stumps were induced to initiate dedifferentiation and repair after each transaction. From the literatures on limb regeneration in anurans, it is well known that the ability of the limb to regenerate declines with the progress of the developmental stages, i.e. with the age of the larvae. This shown, in the present work, by Series I. However, the effect of the second amputation is much greater: at age 26 days, Series I (Stage 55) gave 14% abnormals, while Series II (Stage 52) gave 41%. Similar results were obtained by Dearlove and Dresden (1976) when they studied the regenerative response in the adult *Notophthalmus viridescens* following repeated amputations at the lavel of the distal one-third of humerus. They have concluded that the occurrence of abnormal regeneration was directly increased with the number of times the limb is amputated. Schauble and Nentwig (1974) have reported an increased incidence of abnormal regenerates in adult newts resulting from experimental manipulation of ambient temperature and prolactin therapy (27% abnormal at 25°C and 33% abnormal at 30°C with prolactin treatment).

From the present results we may suggest that each time the limb is amputated, the proximal regenerate tissues as well as the adjacent stump tissues may reinitiate dedifferentiation and then cellular proliferation to accumulate a sufficient mass of blastemal cells to replace the missing appendage. Since the morphogenetic informations required to replace the autopodial elements are more complex than those of the stylopodium and zeugopodium (due to the greater degree of the skeletal complexity in these elements) it is suggested that the greater is the chance for errors to occur as the blastemal cells are repeatedly reprogrammed. This interpretation is readily compatible with the hypothesis for regeneration proposed by Iten and Bryant (1975), Stocum (1975) and Dearlove and Dresden (1976).

The most extensive regenerative anomalies were obtained after the third amputation at stage 55. When the operated limbs of such stage were reamputated (the second amputation, four days after the first amputation), early phases of regeneration occurred till the dedifferentiation and beginning of blastema cell accumulation, then by that time, at the third post-operative day, the limbs were amputated for the third time. This may confirm the interpretation of the repeated changes of the morphogenetic informations of the blastema cells to restore the lost autopodial elements. However, at the operated stages 52 and 53, by the fourth day, the apical part of the stump is elongated to form a cone blastema composed of numerous mesenchyme cells. At this time the proximal blastema cells (present close to the level of the original amputation) were then subjected to the second amputation of the limb, giving rise to the limb abnormalities described in the results. The percentage of the abnormal regenerates increased after the third amputation but was much lower than that obtained by the operated stage 55. On stage 52, the 2nd amputation increases the percentage of abnormals over the 1st amputation by 41-5 = 36%. On stage 55, it increases by 71-14 = 57%. Thus the blastema of the older limb is more easily disturbed by cutting than the blastuma of the younger limb. It is suggested that it is due to greater differentiation of the older limb. The effect of the 3rd amputation is also greater on stage 55 than on stage 52, but this is due mainly to the 2nd amputation; the extra abnormals produced by the 3rd cut (compared with the 2nd cut) is 78 - 41 = 37% on stage 52, and 96-71 = 25% on stage 55 (see Fig. 9). We may conclude that the regenerates produced by the severed early blastema cells (stage 55, third amputation) showed more anomalies and errors in the autopdium than those obtained from amputated cone-stage blastema (stage 52-53, third amputation). In addition, the limb tissues of stage 55 are more differentiated than those of stages 52, 53, and this may lead us to state that the greater the differentiation of the limb tissues at the site of amputation, the more extensive are the regenerative abnormalities produced. Also as the larva progresses through developmental stages, the repeated amputations result in greater percentage of anomalies. The number of intermediate and major abnormalities increases with stage of development (age), and also with the number of amputations (see Table 4).

In some cases of the operated stage 55, the third amputation resulted in the failure of the stump to restore the lost autopodium. The repeated amputations disrupted the cell proliferation resulting in cessation of regeneration. An analogous situation was observed by denervation (Tassava *et al.*, 1974; Mescher and Tassava, 1975). Also we may suggest that the repeated amputations result in repetitive dedifferentiation which may disrupt normal programming for proliferation so that an insufficient mass of blastemal cells accumulates and regeneration stops. These conclusions confirm the observations of Dearlove and Stocum (1974) on denervated forelimb regeneration in the adult newt. The present investigations suggest that repeated amputations may result in repeated cell cycling through dedifferentiation, proliferation and dedifferentiation phases of regeneration, and finally giving rise to abnormal regenerates. In the experiments of Iten and Bryant (1975) on the interaction between the blastema and stump in the establishment of the anterior-posterior and proximal-distal organization of the limb regenerate, abnormal regenerates appeared at all stages of regeneration and were evidently the result of incorrect programming of the dedifferentiating cells, or of inability of the blastemal cells to determine where they are, and therefore what they should become. The abnormalities observed by Schauble and Nentwig (1974) following prolactin administration and temperature adjustment were in the distal limb elements. Stinson (1963, 1964) also obtained autopodial deletions in newt limbs which had been amputated three times. Distal skeletal abnormalities were also prevalent in the experiments of Dearlove and Dresden (1976); they have stated that these abnormalities were most probably related to replace the hand and all of its constituent elements.

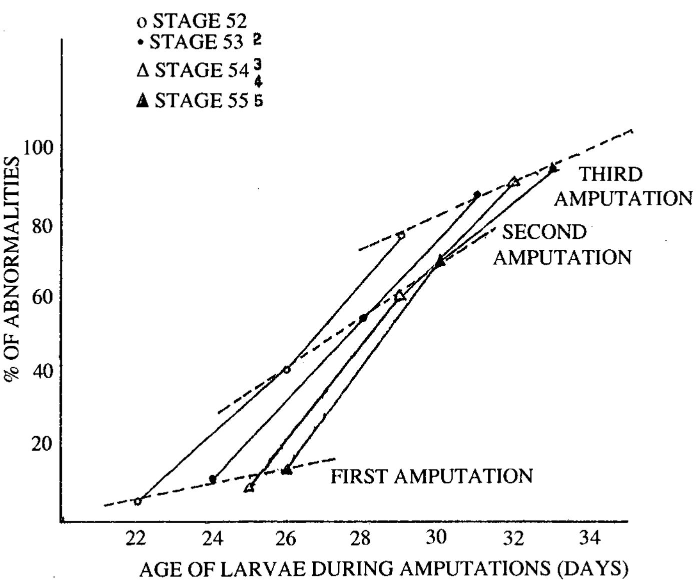


Fig. 9: Percentage of abnormalities (including gross morphology and skeletal configuration) obtained in series I (after one amputation), series II (after two amputations) and series III (after three amputations), taking in consideration the age of the operated larval stages 52, 53, 54 and 55.

The present observations demonstrated that the occurrence of abnormalities extended to include zeugopodium and stylopodium in addition to autopodium (level of all amputations), mostly recorded in series III (see Fig. 6). These results confirm the conclusions of Michael and El-Mekkawy (1977b), in their normal limb regeneration experiments on *Bufo regularis,* that the malformations in the regenerated skeleton extend in a disto-proximal order in hind limbs of late larval and premetamorphic stages. Firstly, the autopodium is affected and in more severe cases abnormality extends to zeugopdium.

Tables 1, 2, 3, & 4 have revealed that the increased percentage of abnormal regenerates (for example from 14% after a single amputation to 96% by the third amputation, at stage 55) is solely the result of repeated surgical removal of the regenerates at the same level of the proximo-distal axis of the regenerating limb. We may conclude that differentiation of the limb is accompanied by reduction of control over the morphogenetic programming of the blastema cells. Repeated amputation, which necessitates repeated reprogramming, greatly increases the probability of erroneous morphogenetic information. Earlier studies by Schotte and Hilfer (1957), Schotte and Liversage (1959), and Stinson (1963, 1964) employed multiple amputations to examine various aspects of regeneration as it relates to recovery from concomitant hypophysectomy, denervation, or X-irradiation, respectively. However, in these studies they did not employ repeated amputation at the same level along the proximo-distal limb axis.

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