The Relationship between Growth, Developmental Stage and Postamputation Age of the Regeneration Blastema of the Newt, *Notophthalmus viridescens*

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ABSTRACT The growth of the regeneration blastema of the newt forelimb has been quantitated and analyzed as a function of postamputation age, developmental stage, animal weight, animal length, and cross sectional diameter of the blastemal base in both the anteroposterior and dorsoventral dimensions. Correlation coefficients computed for these variables show that growth of the regenerate in both length and volume is more closely correlated with developmental stage than postamputation age. In addition, the results show a linear relation between the $\log_e$ (regenerate length) and developmental stage, and between regenerate length and volume. Thus, length can be used to assess growth of the regenerate according to a developmental rather than a chronological time scale. There were no significant correlations between regenerate length or volume and animal length, animal weight or cross sectional dimensions of the blastemal base. These results show that one can use a randomly selected population of animals and study the growth of the regeneration blastema by relating to a developmental time scale through a logarithmic transformation of the linear growth data.

The regenerating amphibian limb is a versatile model system for studies of growth, differentiation and morphogenesis. A number of reports have appeared concerning factors influencing the rate of regeneration or the growth of the regenerate (Smith et al., '74; Iten and Bryant, '74; Pritchett and Dent, '72; Schauble, '72; Tweedle, '71; Singer and Craven, '48; Litwiller, '38). In these studies regenerative events were all expressed as a function of time after amputation. There is considerable variation in developmental morphology of limb blastemas with postamputational age (Iten and Bryant, '73; see below). Using the time scale as the main basis for comparison can lead to comparing lengths or volumes of blastemas at totally different developmental stages but of similar chronological age. There have been no studies to date which carefully analyze the progression of blastemas through a series of developmental stages and demonstrate statistically how these two variables are related. Similarly there are no studies which demonstrate statistically the relationship between postamputational age of the regenerate and regenerate length or volume. Many earlier studies have suffered from a lack of accurate methods of ascertaining regenerate growth (Schauble, '72; Pritchett and Dent, '72) or the lack of statistical analysis of the information gathered by relatively accurate methods (Singer and Craven, '48; Litwiller, '38). The present study was undertaken to measure accurately the length and volume of the growing regeneration blastema and statistically to correlate these parameters with: (1) the postamputational age of the blastema; (2) the developmental stage of the regenerate; (3) the length of the animal; (4) the weight of the animal, and (5) cross-sectional diameter of the limb stump.

MATERIALS AND METHODS

Three groups of adult newts (totalling 54 animals) obtained from Bill Lee Newt

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Farm, Oak Ridge, Tennessee at various times of the year (September, and December, 1974 and June, 1975) were anesthetized in ethyl-m-aminobenzoate methansulfonate (Eastman, 1:1000) and subjected to bilateral forelimb amputation just proximal to the wrist. After the soft tissue retracted, protruding bone was trimmed to produce a relatively flat amputation surface perpendicular to the long axis of the limb. No attempt was made to select animals of uniform length or weight. Newts in the sample population averaged 100.94 ± 5.27 mm, (SE) snout to tail tip length and 3.02 ± 0.49 gm in weight. Individual animals were identified by recording the unique spot pattern with which each newt is marked along the dorsolateral body surface. Newts were kept in the dark in dechlorinated tap water in small plastic dishes in an incubator at 25 ± 0.2°C. At intervals from 18 to 44 days after original amputation, forelimbs of several specimens were amputated at the shoulder and fixed in Bouin's fluid.

Following decalcification in 5% nitric acid in 70% ethanol, the limbs were photographed from the dorsal aspect as a permanent record of external morphology. Twenty-nine of these limbs were then embedded in paraplast and sectioned serially (10 μ) perpendicular to the longitudinal axis of the blastema. In a preliminary study several limbs were cut longitudinally but measurements from these limbs proved highly variable, presumably due to problems in properly orienting the limb to the knife edge in sectioning. The cross sections obtained were stained with hematoxylin and eosin and every third section was traced at a magnification of 55× with the aid of a camera lucida mounted on a Wild M-5 stereomicroscope. The most distal section in which osseous tissue could be identified was arbitrarily defined as the base of the blastema (Singer and Craven, '48). The area of each tracing was determined with a K and E compensating polar planimeter (error < 0.1%). The greatest anteroposterior (AP) and dorsoventral (DV) dimensions of each tracing were determined with a Decitrak digital electronic caliper to the nearest 0.1 mm. Data thus obtained were entered into a computer file and reduced to absolute values for length and volume using computer programs (MIDAS) written by the University of Michigan Statistical Research Laboratory. The regenerate volume and length were calculated according to the method of Litwiller ('38).

From the photographic record the developmental stage obtained by each limb in the first group was determined independently by three individuals using the description of stages of newt forelimb regeneration of Iten and Bryant ('73). In joint consultation these three investigators then reached a consensus regarding the stage attained for those limbs where there was some discrepancy in the blind determination. Staging of regenerates in the second and third groups was done by only one person.

Regression analysis, correlation coefficients and scatter plots of the resultant data were produced by MIDAS programs mentioned above. Correlations between length or volume and: (1) times after amputation; (2) stage of regeneration; (3) animal length; (4) animal weight and (5) cross sectional diameter of the base section were sought. In addition, descriptive statistics and correlation coefficients were calculated for relationships between limb diameter (basal cross section dimensions) and animal length and weight.

RESULTS

Figure 1 shows the time after amputation at which developmental stages of regeneration were obtained in the three groups of newts. The great disparity between the times after amputation at which certain developmental stages appeared in these three groups emphasizes the difficulty encountered in obtaining readily reproducible results when a time scale is used. The variability in regeneration progress within a group of animals at any given time point is also obvious from figure 1.

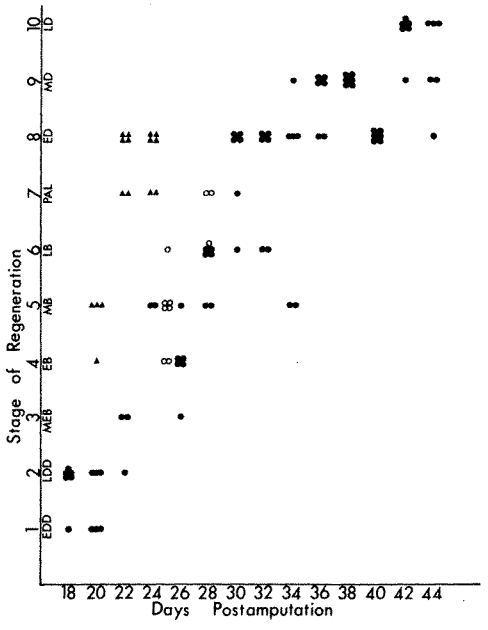

Fig. 1 A graph demonstrating the time after amputation at which the different developmental stages appeared in three groups of newts. Each point on the graph represents one regenerate. The solid circles represent regenerates from the experiment run in September 1974. The open circles represent regenerates from the experiment run in December of 1974 and the triangles represent regenerates from the experiment run in June of 1975. Notice the overlap in stages at times from 18 to 36 days after amputation. In this and following figures the stages are those described by Iten and Bryant ('73). The abbreviations for each stage are: EDD, early dedifferentiation; LDD, late dedifferentiation; MEB, moderate early bud; EB, early bud; MB, medium bud; LB, late bud; PAL, palette; ED, early digits; MD, medium digits; LD, late digits. The numbers beneath the morphological stages were assigned arbitrarily for convenience in manipulating the numerical data.

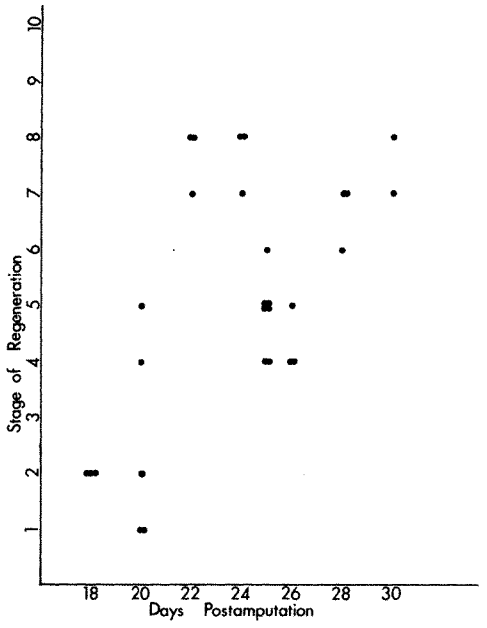

Fig. 2 A graph similar to figure 1 but including only those limbs for which quantitative measurements were made. Each point represents one regenerate. Note the variability in stage attained as a function of time in this graph.

There was at least a three stage overlap at most times between 18 and 36 days after amputation. Quantitative data were calculated on 29 limbs of these three groups from 18 to 30 days postamp. Figure 2 shows the time after amputation at which developmental stages were attained by newts in the quantitative study.

Figure 3 represents regenerate length as a function of time after amputation. The correlation coefficient calculated for these points is 0.2116 and is not sufficiently large to indicate a linear relationship between time after amputation and regenerate length. Figure 4 represents regenerate length as a function of developmental stage. The correlation coefficient for these two variables is 0.8347 which is sufficiently large to indicate a linear relationship between length and regeneration stage. Although the earlier points on the graph don't follow the linear pattern they can be accommodated by a parabolic regression line. Plotting the $\log_e$ of the regenerate length versus developmental stage results in a scatter of points with a correlation coefficient of 0.9079 (fig. 5). Thus, stage is linearly related to the $\log_e$ of regenerate length.

Comparison of volumetric measurements follows the trend for length. Figures 6 and 7 demonstrate that volume of the regenerate correlates most closely with developmental stage (correlation coefficient =

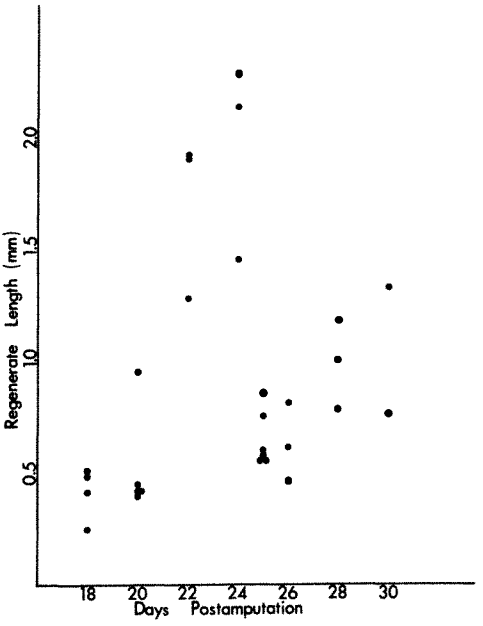

Fig. 3 A plot of regenerate length vs postamputation age. Note the variability in the distribution of these points and compare this with figures 4 and 5. The correlation coefficient for these two variables is 0.2116. Each point represents one regenerate.

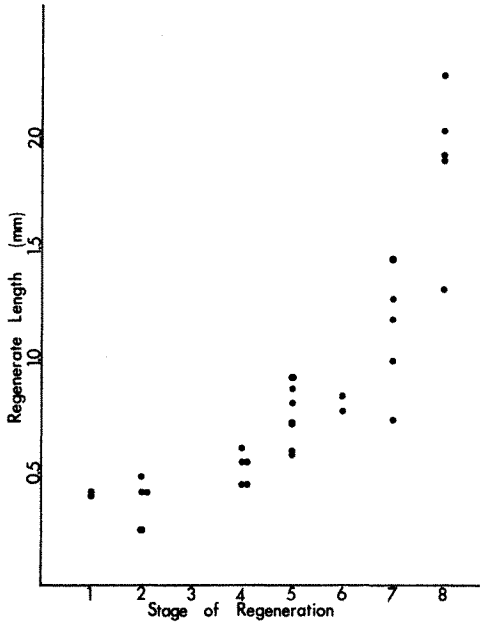

Fig. 4 A plot of regenerate length as a function of the developmental stage. The correlation coefficient between these two variables is 0.8347. Compare the distribution of points in this graph with those of figures 3 and 5. Each point represents one regenerate.

0.7237) rather than with chronological age (correlation coefficient = 0.0611).

A scatter plot of length versus volume (fig. 8) shows a distribution of points with a correlation coefficient of 0.9416. Thus, length and volume are linearly related. The correlation coefficient between stage and time after amputation is 0.6053. This indicates a relationship between the two variables but it is considerably less strong than the correlation between developmental stage and length or volume. There is also no correlation between length or volume of the regenerate and the basal diameter of the regenerate in either the anteroposterior or the dorsoventral axis. There is no correlation between anteroposterior or dorsoventral axis and animal length, but there is a slight negative correlation between dorsoventral axis and animal weight, although it is certainly not large enough to be significant. The basal cross section diameters for limbs of all developmental stages are remarkably similar, regardless of animal length or weight (table 1). Table 2 summarizes the results of the correlation statistics computed for all variables.

DISCUSSION

The results presented above provide statistical evidence relevant to several assumptions commonly accepted in studies of regeneration. For example, it is accepted that after amputation the regenerates which form pass through a sequence of recognizable developmental stages and that during this process the regenerates increase in length and volume. It is assumed that the growth which occurs is closely correlated with time after amputation. The results of this study show that the amputated limbs do pass through a series of recognizable developmental stages. They also show that the growth of the regeneration blastemas is correlated more closely with the developmental stage of the regenerate than with its chronological age. Thus, by comparing lengths of regenerates on a time scale without taking into considera-

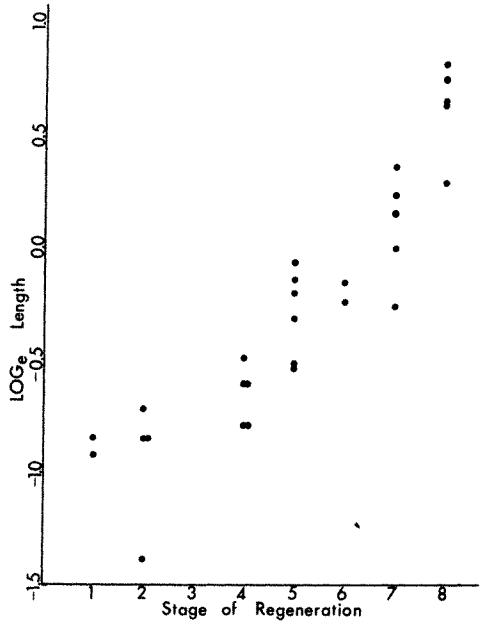

Fig. 5 A plot of the $\log_e$ of the regenerate length vs developmental stage. The correlation coefficient between these two variables is 0.9079. This indicates a strong linear relationship between the $\log_e$ of regenerate length and developmental stage. Compare with figures 3 and 4. Each point represents one regenerate.

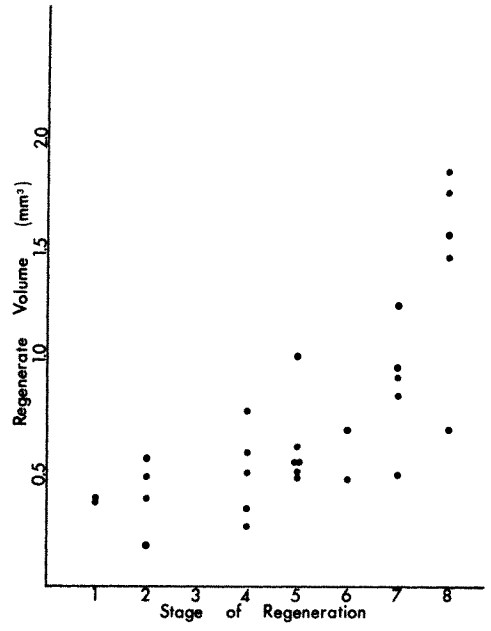

Fig. 6 A plot of regenerate volume versus developmental stage. The correlation coefficient is 0.7237. This indicates a closer correlation between volume and stage than between volume and postamputation age (fig. 7). Each point represents one regenerate.

tion the developmental stage of the regenerates involved may lead to erroneous conclusions about the influence of experimental manipulation on regenerate growth.

The results of this study suggest that there is some predictive value in the relationship demonstrated between the logarithm of the regenerate lengths [$\log_e$ (len)] and developmental stage. Future studies may use this variable as an indicator of the effect of experimental intervention on regeneration. Since $\log_e$ (len) is linearly related to developmental stage rather than time after amputation, much of the variability inherent in using the time scale is reduced. The effects on regeneration would clearly be visible as a breakdown in linearity between the $\log_e$ (len) and developmental stage or as an alteration in the slope of the line. Since length and volume are highly correlated, one could also calculate regenerate volumes for control and experimental groups as a further test for effects of the experimental manipulations. Since there are no correlations between size or weight of animals and regenerate length or volume one could use randomly selected animals, as is commonly done, with little fear of affecting the result.

Accurate length measurements are often difficult to obtain. Other authors (Pritchett and Dent, '72) noted the problems involved in reliably and accurately positioning an ocular micrometer at the amputation plane in serial observations of regenerate growth. Use of cross sectioned limbs and definition of the blastemal base as the most proximal of the regenerate sections with osseous tissue present provides a consistent reference point (Singer and Craven, '48; Chalkley, '54). There can be some argument that due to bone regression this may really not be the blastemal base. Since dedifferentiation takes place for some distance proximal to the cut surface of the bone, and since cells liberated during bone regression certainly contribute to the blastema it may also be argued that this level is

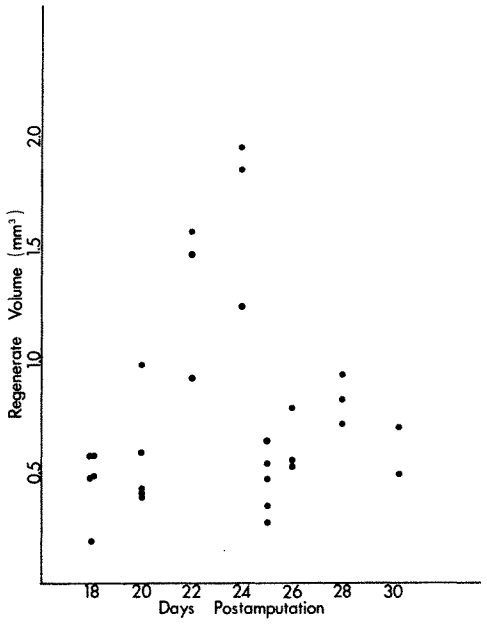

Fig. 7 A plot of regenerate volume as a function of postamputation age. The correlation coefficient for these two variable is 0.0611 and indicates very little relationship between volume and time after amputation. Compare with figure 6 (each point = one regenerate).

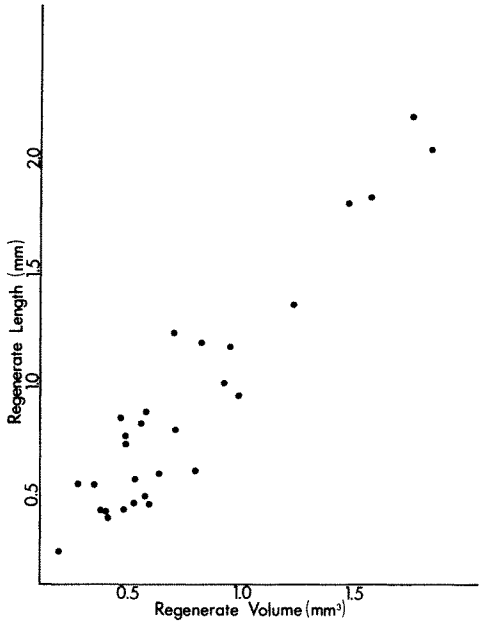

Fig. 8 A plot of length as a function of volume. The correlation coefficient is 0.9416 and indicates a very strong linear relationship between these two variables.

a reasonable compromise for defining the true blastemal base (Chalkley, '54). The presence of the complete circle of dermis cannot be used to define the base of the blastema, since slight variations in orientation of the block to the knife edge result in sections which do not have complete rings of dermis until a considerable proximal distance has been traversed.

Caution has been used in this report when referring to "rate" of regeneration. Since the observations presented here were obtained from specimens fixed at specific times rather than from serial observations of stages on the same animal over the total period of the experiment, our results do not represent a true estimate of the regeneration rate. Under these circumstances progress of regeneration is a more acceptable term to describe the attainment of developmental stages or volume, etc. as a function of time after amputation.

It is recognized that such factors as constant darkness and differences in time of the year could have affected these results (Schauble, '72; Turner and Tipton, '73). It is not possible, at this time, to determine exactly what factors were responsible for the variability in absolute rate of regeneration displayed in figure 1. However, the data herein support the hypothesis that the use of a developmental rather than a chronological time scale reduces the influence of such variables as time of year, photoperiod, hormonal condition of the animals, etc., on quantitative studies of limb regeneration.

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TABLE 1

Descriptive statistics for basal anterior-posterior (BAP) and basal dorsoventral (BDV) diameters according to stage of regeneration

	Stage	N	Mean	SD	Minimum	Maximum
BAP	1 (EDD)	2	1.6044	0.08895	1.5415	1.6673
BDV			1.2445	0.08964	1.1873	1.3018
BAP	2 (LDD)	4	1.7123	0.15643	1.4891	1.8455
BDV			1.2168	0.08169	1.1200	1.3164
BAP	4 (EB)	5	1.6840	0.25372	1.3545	1.9200
BDV			1.2942	0.25782	0.8674	1.5000
BAP	5 (MB)	6	1.6506	0.20634	1.4400	1.9527
BDV			1.2149	0.12593	1.1073	1.4491
BAP	6 (LB)	2	1.6609	0.02446	1.6436	1.6782
BDV			1.0900	0.13506	0.9945	1.0900
BAP	7 (PAL)	5	1.7153	0.12504	1.5018	1.8273
BDV			1.2265	0.10303	1.1236	1.3782
BAP	8 (ED)	5	1.6458	0.16062	1.3673	1.7691
BDV			1.1883	0.13978	0.9782	1.3527

TABLE 2

Correlation coefficients for comparisons between blastemal size, developmental stage, etc.

Postamputation age	vs	Developmental stage	0.6053
Postamputation age	vs	Regenerate length	0.2116
Postamputation age	vs	Regenerate volume	0.0611
Regenerate volume	vs	Developmental stage	0.7237
Regenerate length	vs	Developmental stage	0.8347
Log_e (regenerate length)	vs	Postamputation age	0.3279
Log_e (regenerate length)	vs	Developmental stage	0.9079
Regenerate length	vs	Regenerate volume	0.9416
Regenerate length	vs	Animal length	0.0769
Regenerate volume	vs	Animal length	0.0991
Regenerate length	vs	Animal weight	0.0595
Regenerate volume	vs	Animal weight	0.0565
Basal AP	vs	Regenerate volume	0.3798
Basal AP	vs	Regenerate length	0.1111
Basal DV	vs	Regenerate volume	0.2895
Basal DV	vs	Regenerate length	−0.0019
Basal AP	vs	Animal length	0.0703
Basal AP	vs	Animal weight	−0.1067
Basal DV	vs	Animal length	−0.1602
Basal DV	vs	Animal weight	−0.0282

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