

Probability aspects of supernumerary production in the regenerating limbs of the axolotl, *Ambystoma mexicanum*

By R. N. TURNER¹

From the Developmental Biology Group, The University of Sussex

SUMMARY

The applicability of deterministic models, and in particular the polar co-ordinate model of French, Bryant & Bryant (1976), to the regenerative process has been investigated by performing ipsilateral blastemal rotations of varying angle on the fore and hind limbs of the axolotl. The results show that the frequency of supernumerary production rises with increasing angle to reach a peak at 180° and then falls off more rapidly, giving rise to an asymmetric distribution curve. The position of supernumerary outgrowth also suggests a probabilistic event, there being no consistent relationship between this parameter and the angle of rotation. The polar co-ordinate model is incapable of explaining these results and the work suggests that the determinism of current regeneration models may have to be abandoned in favour of a more stochastic theory.

INTRODUCTION

Since the early observations of Bateson (1894) and the formulation of his rule of mirror symmetry, the phenomenon of supernumerary limb production has provoked great interest. During the early part of this century the research focused on the production of reduplicated limbs – the embryonic equivalent of supernumeraries in the mature animal (e.g. Harrison, 1921; Swett, 1927). The results of this research led to the hypothesis that such reduplicates arise as the result of axial misalignment produced by experimental manipulation. This hypothesis was to set a precedent for the current idea of positional disparity and subsequent intercalation which forms the basis of the polar co-ordinate model (French *et al.* 1976). In both its original and modified forms (Bryant, French & Bryant, 1981) the model has made a major contribution to the understanding of pattern regulation not only in regenerating amphibian limbs but also in insect limbs and imaginal discs.

The work presented here is an extension of that reported in an earlier publication (Maden & Turner, 1978) and provides evidence contradictory to current theory. The results obtained from experiments involving ipsilateral blastemal

¹ Author's present address: Anatomy Dept., King's College, University of London, The Strand, London WC2R 2LS, U.K.

Table 1. *Frequency of supernumerary production at different angles*

Angle (°)	Number	No. prod. supers	No. of supers			Super production (%)
			One	Two	Three	
0	25	—	—	—	—	0
45	13	3	2	1	—	23.1
90	24	9	7	2	—	37.5
135	21	12	7	4	1	57.1
180	39	38	18	18	2	97.4
225	12	4	2	2	—	33.3
270	14	4	2	2	—	28.6
315	28	2	2	—	—	7.1

rotations of varying angle strongly suggest that the polar co-ordinate model (and, indeed, similar theories of regeneration) needs to be abandoned, or modified, in favour of a more probabilistic model.

MATERIALS AND METHODS

All experiments were performed on larval axolotls (*Ambystoma mexicanum*), 60–120 mm in length, and anaesthetized with 0.1 % MS 222. Both fore and hind limbs were amputated through the mid-humerus/femur level and allowed to regenerate to the late bud – early palette stage (Tank, Carlson & Connelly, 1976).

On reaching this stage the blastemas were amputated, rotated in an anterior → dorsal → posterior → ventral direction (clockwise for left limbs, anticlockwise for right) and then replaced on the stump. A carmine-marking system previously described (Maden & Turner, 1978) was used to determine the precise degree of rotation. Control grafts were marked with carmine, amputated and then replaced without rotation. During the operations the animals were placed on wet tissues to provide support for the limbs.

Wound healing was started in a cold room at 4 °C for 2 h after which any grafts showing irregularities (e.g. not completely attached to the stump) were discarded. All animals were observed at 2-day intervals subsequent to operation in order to determine whether derotation of the blastema had occurred (as indicated by the carmine mark) and to distinguish between the regenerate and any supernumeraries which formed. After several weeks the limbs were re-amputated, stained in Victoria Blue and analysed in detail.

RESULTS

(1) *Frequency of supernumerary production.* Of the 25 control grafts, 24 produced normal regenerates and one forelimb produced a three-digit, hypomorphic regenerate. Thus, amputation and replacement of the blastema in normal orientation is insufficient to induce supernumerary formation.

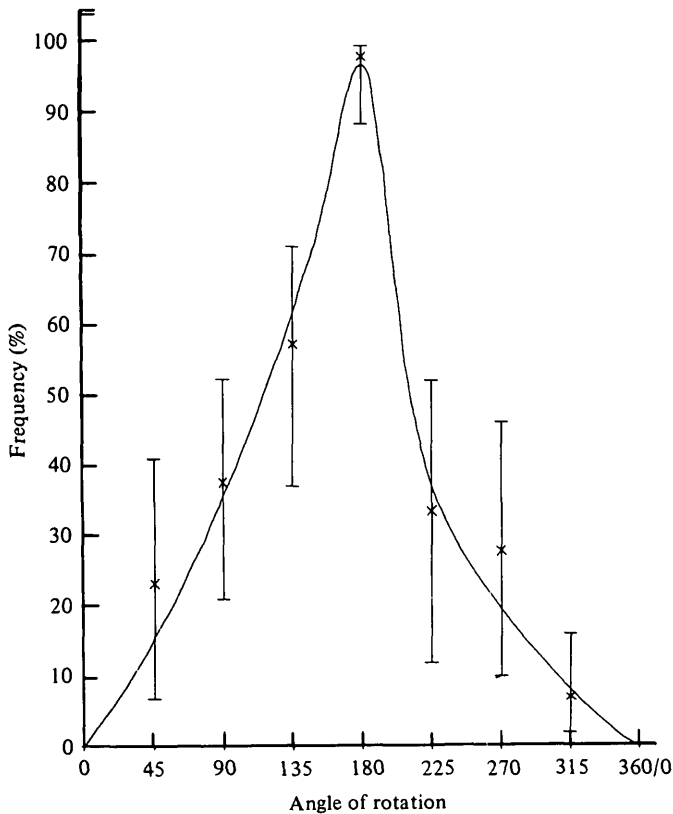


Fig. 1. The frequency distribution of supernumerary production at different angles of blastemal rotation. The bars represent the upper and lower confidence limits computed at 5%.

However, at all angles of rotation from 45° to 315° inclusive, supernumerary limbs were produced at a frequency which varied with the different angles of blastemal rotation (Table 1). Limbs producing a minimum of two digits separated from the main regenerate were classified as having produced supernumerary structures. The relationship between the frequency and angle of rotation is shown in Fig. 1. The frequency of supernumerary production rises steadily with increasing angle of rotation, reaching a peak at 180° , then falls more rapidly for larger angles. This results in the asymmetric distribution curve shown in Fig. 1, though it should be noted that a symmetrical curve could be drawn within the confidence limits shown.

Table 1 also gives the number of supernumeraries forming on any one limb. In the great majority of cases the supernumeraries occur either singly (55.5%) or in pairs (40.3%). Occasionally three supernumeraries were observed (15–17 digits in the whole regenerate). These occurred only at high angular displacements (i.e. 135° and 180°) and exhibited poor separation.

Table 2. *Positions of supernumerary outgrowth*

Angle	No. of Supers	Position of supernumeraries							
		D	P-D	P	P-V	V	A-V	A	A-D
45	4	—	1	—	2	—	—	—	1
90	11	—	3	1	2	1	—	—	4
135	18	1	6	1	2	3	—	3	2
180	60	3	15	5	6	6	9	4	12
225	6	—	3	—	1	—	—	1	1
270	6	1	1	1	—	1	1	1	—
315	2	—	—	—	—	—	—	—	2
Totals		5 (4.7%)	29 (27.1%)	8 (7.5%)	13 (12.1%)	11 (10.3%)	10 (9.3%)	9 (8.4%)	22 (20.6%)

Table 3. Frequency of blastemal derotation

Angle	No. of rotations	No. derotating	Freq. of derotation %
45	13	5	38.5
90	24	4	16.7
135	21	4	19.1
180	39	14	35.9
225	12	3	25.0
270	14	4	28.6
315	28	10	35.7

(2) *Position of supernumerary outgrowth.* The positions used in this analysis, defined relative to the stump, are the four axial poles (anterior, dorsal, posterior and ventral) and the four intervening quadrants. The positions in which supernumerary outgrowth arose is shown in Table 2. It is clear that there is no correspondence between the angle and any one, or small set of positions. When the results from all rotations are combined (bottom line of Table 2) it can be seen that the posterior-dorsal and anterior-dorsal quadrants generate the most supernumeraries (27.1 % and 20.6 % respectively) although all other positions make a significant contribution. As expected from the way in which the positions are geometrically defined, the intervening quadrants contribute significantly more (69.1 %) than the poles themselves.

It is important to note that, in accordance with a previous study (Maden & Turner, 1978), the two members of a pair of supernumeraries do not necessarily form on opposite sides of the regenerate from one another, but can arise only 90° apart.

(3) *Blastemal derotation.* In a number of cases the blastema rotates back to its original position after the ipsilateral rotation. The frequency with which this occurs bears no consistent relationship to the angle of rotation (Table 3) – up to a third of blastemas at all angles derotate. As previously reported (Maden & Turner, 1978) some of these cases in which derotation occurs still produce supernumerary limbs. In such cases it is impossible to determine the time, and thus the angle, during derotation at which supernumerary induction is initiated. Therefore the frequency data have been recalculated excluding all cases of derotation. The distribution for this data fits within the confidence limits computed for Fig. 1 and there is no significant difference between the two distributions (at 5 % level using 2×2 contingency test, with Yates' correction, at each angle). Thus, while derotation is an interesting phenomenon, it does not affect the arguments to be presented here and so will be discussed no further.

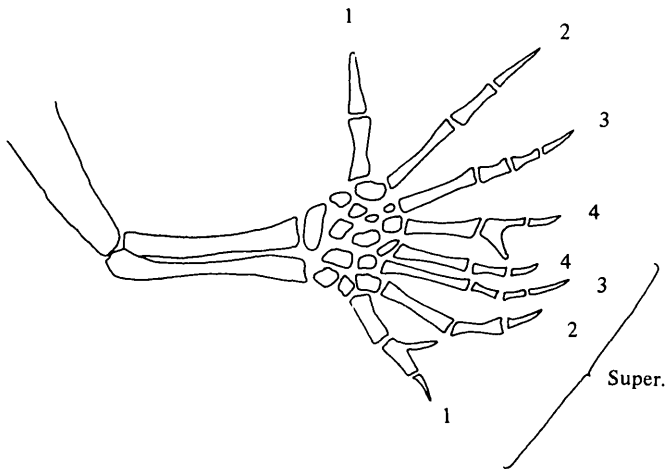


Fig. 2. Camera-lucida drawing of a supernumerary produced following a 45° rotation ($\times 12$).

DISCUSSION

The polar co-ordinate model (French *et al.* 1976; Bryant *et al.* 1981) has been successful in many of its predictions concerning the regenerative behaviour of such systems as cockroach and amphibian limbs and *Drosophila* imaginal discs. For the amphibian, the model explains precisely the outcome of contralateral limb grafts (Bryant & Iten, 1976; Tank, 1978) and the modified version (Bryant *et al.* 1981) goes a considerable way to explaining the behaviour of surgically constructed double half limbs following amputation (e.g. Bryant & Baca, 1978; Krasner & Bryant, 1980; Holder, Tank & Bryant, 1980). However, the results reported here, and in previous publications concerning ipsilateral blastemal rotations (Maden & Turner, 1978; Wallace, 1978; Wallace & Watson, 1979) present great difficulties for this, and any other, deterministic model.

The frequency data in Fig. 1 strongly suggest that supernumerary production is a probabilistic event dependent upon the degree of rotation, or positional disparity, of the blastema with respect to the stump. The greater the disparity the higher the probability of supernumerary formation. This stochastic aspect of regenerative behaviour is reflected further in the data concerning the position of supernumerary outgrowth; this can occur anywhere around the limb circumference and there is no apparent relationship between this position and the angle of rotation.

In terms of the original polar co-ordinate model this randomness of the position of outgrowth is only explicable for 180° rotations and if one assumes that the positional values are evenly spaced around the limb circumference. However, if this assumption is accepted it becomes impossible to explain the generation of supernumeraries following angles of rotation resulting in relatively small positional discrepancies (e.g. 45° and 315°). Conversely, while this latter observation is explicable by redistributing the positional values so that they

become unevenly spaced, this leads to the prediction of specific positions for supernumerary outgrowth (see Bryant & Iten, 1976), which is not upheld by this investigation.

The modified version of the model is really no more successful in attempting to explain these results. Even though it allows distal growth from less than a whole set of positional values, the extent of this distalization should only be slight from less than half of the values. This makes it difficult to explain the generation of whole supernumerary limbs from small angle rotations (e.g. 45° – Fig. 2) where the number of intercalated values, in the light of the previous argument, can only, realistically, be small.

So, can the theory be modified further to accommodate these anomalies? One way would be to incorporate a probabilistic feature into the model which would allow intercalation to occur by the longest route with a frequency dependent upon the degree of positional disparity. That is, the larger the positional discrepancy the higher the probability of longest intercalation. This would result in the kind of distribution curve for supernumerary production, as a function of angular displacement, shown in Fig. 1. But the problem with such a modification is that intercalation by the longest route is totally contrary to the model's most fundamental rule and, furthermore, to view this as a probabilistic event would be unacceptable within such a deterministic framework.

While it is not being suggested that the search for rules of regeneration be abandoned, the observations of this paper do put in question the basis of not only the polar co-ordinate model but also other deterministic theories which rely upon a simple metric (distance measure). It does appear that regenerative behaviour incorporates a sense of distance, demonstrated by a systematic variation in the frequency of supernumerary production as a function of angular discrepancy. However, the concept of distance used in the polar co-ordinate model is essentially one of convenience and, as such, is constrained by the external geometry (i.e. circular cross-section) of the limb. Alternatively, pattern regulation during regeneration may be constrained by an internal geometry which represents the true regeneration field. A model using a distance measure based upon hyperbolic geometry (G. Sawitski – in preparation) incorporates such internal geometrical constraints and is capable of explaining the results presented here. Furthermore, it makes specific predictions concerning the outcome of other manipulations (e.g. variable rotations including a proximo-distal displacement of the blastema) which I am currently testing.

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