

## Development of the lateral line system in *Xenopus laevis*

### III. Development of the supraorbital system in triploid embryos and larvae

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#### SUMMARY

During normal development of the supraorbital lateral line system of *Xenopus*, an elongated streak of primordial cells becomes subdivided into a linear series of cell groups containing only about eight cells each, thus forming a row of primary lateral line organs (Winklbauer & Hausen, 1983*a, b*). In triploid *Xenopus* embryos, cell size is  $1.5 \times$  normal. When the formation of lateral line organs occurs in triploid primordia, the nascent organs contain only about five or six cells each, i.e. about two thirds of normal. Thus, the increase in cell size is compensated for by a corresponding reduction in cell number, keeping constant the organ size in terms of total cell mass or volume. This result excludes a cell counting mechanism for determining organ size. In diploids, the primary organs, although being of equal size initially, differ vastly in their final size and exhibit a peculiar frequency distribution of organ sizes. A detailed quantitative model for supraorbital lateral line development has been proposed, which accounts for this characteristic frequency distribution (Winklbauer & Hausen, 1983*b*). This model makes precise predictions as to the frequency distribution of the final size of triploid lateral line organs, where the initial organ size is reduced to five or six cells. These predictions were verified experimentally.

#### INTRODUCTION

Because of its lucid structure and its small cell number, the lateral line system of *Xenopus* is well suited for a quantitative analysis of morphogenesis at the cellular level. In the previous communications of this series, we described the normal development of the supraorbital (SO) lateral line system in *Xenopus laevis*. The whole system originates from the SO lateral line primordium, a local thickening of the inner epidermal layer in the ear region of the embryo. This primordium elongates by cell migration and multiplication along the dorsal margin of the eye. The streak of cells thus formed is subdivided into the primary lateral line organs of the supraorbital and parietal line. On the basis of data concerning the cell proliferation kinetics and the size distribution of the primary organs, we proposed a detailed quantitative model for SO lateral line development (Winklbauer & Hausen, 1983*a, b*).

The model assumes that the SO system grows by the asymmetric division of stem cells. At the beginning of SO line development, only stem cells are present. Each

*Key words:* *Xenopus laevis*, lateral line system, supraorbital system, triploidy.

stem cell is capable of producing eight non-dividing, terminal cells by successive unequal cell divisions before becoming terminal itself. After one round of cell divisions, when stem cells and terminal cells are about equal in number, organ segregation commences, and the streak-like primordium is divided into a linear series of small cell groups consisting of 8 cells each. Stem cells and terminal cells are randomly allocated to these groups, which are the nascent SO organs. If an organ, by chance, obtains no stem cell at all, it cannot grow further and remains at a size of 8 cells. If an organ obtains one stem cell, this cell will still divide seven times to produce 7 terminal cells, until growth is arrested at stage 47½. The final size of that organ will be 8+7 cells. If an organ contains two stem cells, the final organ size will be 8+2×7 cells, and so on. So the frequency distribution of organ sizes should exhibit nine discrete peaks, with an interval of 7 cells/organ between neighbouring peaks, with the first peak at 8 cells/organ, and with the size of the peaks approximating a fairly symmetrical binominal distribution. This was actually found and provided the strongest evidence for our model. Moreover, many other independent findings, e.g. the linear cell kinetics, the progressive divergence of organ sizes, the time of organ segregation etc., could be explained or calculated by the model (Winklbaauer & Hausen, 1983b). In the present article, we describe the development of the SO system in triploid embryos of *Xenopus laevis*.

In amphibians, viable triploid individuals may occur either spontaneously or after experimental treatment of fertilized eggs (see review by Fankhauser, 1945a; and Fankhauser, 1941; Fankhauser, Crotta & Perrot, 1942; Briggs, 1947; Fischberg, 1949; Smith, 1958; Di Berardino, 1962; Kawahara, 1978; Tompkins, 1978; Blackler & Cassidy, 1980; Elinson & Briedis, 1981; Tashiro, Misumi, Shiokawa & Yamana, 1983). The primary effect of a triploid set of chromosomes per nucleus is a proportional increase in nuclear and cell size, the volume of triploid nuclei and cells being 1.5 times that of diploid ones. In general, however, triploid amphibians are of normal size and proportions. Thus, cell number must be reduced to two thirds to compensate for the increase in cell size (Fankhauser, 1941, 1945a,b, 1952; Briggs, 1947; Fischberg, 1949).

However, exceptions to this rule may occur (Artom, 1926, 1928; Ellinger, 1979; Held, 1979; Santamaria, 1983), and it apparently depends on the specific mode of development whether the size of an organ or its cell number is kept constant. Thus, a comparison between the structures of organs in the diploid and the triploid condition may help to infer developmental mechanisms.

Our results show that in triploid SO systems of *Xenopus laevis*, cell number is indeed reduced to compensate for the increase in cell size. This being so, our theoretical model makes specific predictions as to the organ size distribution of triploid SO primary organs. We show that the empirical distribution agrees well with this prediction, lending further support to our model.

#### MATERIALS AND METHODS

Laboratory-bred *Xenopus laevis* females were injected with human chorionic gonadotropin (Serva; 50 units, 6 h later 150–250 units). About 10 h later, eggs were stripped from the females

and fertilized with fresh macerated testes in one tenth-strength Modified Barth's Solution (MBS-H) (Elsdale, Gurdon & Fischberg, 1960). Triploid embryos were produced according to the method of Kawahara (1978) and Tashiro *et al.* (1983). 15 min after fertilization, eggs were exposed to a temperature of 1–2°C for 15 min. Cold-shocked and untreated control embryos were raised in one tenth-strength MBS-H at 22.5°C and staged according to Nieuwkoop & Faber (1967).

To visualize epidermal nuclei, animals fixed in 4% formaldehyde were stained for 10 min in an aqueous solution containing  $1\ \mu\text{g ml}^{-1}$  4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Boehringer Mannheim). The head of an animal was then cut in the median plane, and the skin of each half was peeled off and mounted in distilled water between two coverslips. With the fluorescent DNA-binding dye, DAPI, epidermal nuclei appear only weakly fluorescent in such skin preparations, in contrast to the tightly packed, bright and condensed lateral line nuclei. This allows the identification of lateral line cells within the epidermis (Winklbauer & Hausen, 1983a,b).

Diploid and triploid epidermal and lateral line organ cells can be distinguished by their different nuclear sizes (Fankhauser, 1945a), and by the maximum number of nucleoli per nucleus in a given skin preparation (Briggs, 1947; Kawahara, 1978; Blackler & Cassidy, 1980; Tashiro *et al.* 1983). To visualize nucleoli, a piece of skin was silver stained by incubation for 2.5 h in 96% ethanol containing two or three drops of ammonium hydroxide  $10\ \text{ml}^{-1}$ , followed by 3 h in 4%  $\text{AgNO}_3$  at 37°C and a subsequent wash in distilled water. The preparations were then treated for 1 h with one quarter-strength Ilford microphen developing solution. Skin preparations were scored as triploid when the maximum number of nucleoli per nucleus was three, and as diploid when it was two. Some spontaneous triploids were detected among the control embryos. This is common among amphibians (Fankhauser *et al.* 1942; Fankhauser, 1945a; Briggs, 1947; Michalski, 1948; Uzzell, 1963; Humphries, 1966; Richards & Nace, 1977).

## RESULTS

### *Cell number during development in diploid and triploid SO systems*

In normal SO system development, cell number increases linearly between stage 35/36, the beginning of primordial elongation, and stage 47½. In starving larvae, development is arrested between stages 47 and 48. Concomitantly, the growth of the SO system ceases, and the cell number reaches a plateau and remains constant for at least 5 days (Winklbauer & Hausen, 1983b).

When the cell numbers of diploid and triploid SO systems are compared between stages 35/36 and 47½, it is found that the triploid condition is always correlated with a significant and constant reduction in cell number to about two thirds of the diploid number, or even slightly less (Table 1, also Table 3). As a consequence, the mitotic rate must be approximately the same in diploid and triploid SO systems.

### *Cell size and the dimensions of the primordium in diploid and triploid embryos*

The area occupied per SO cell in the plane of the epidermis remains nearly constant between stages 35/36 and 41 in normal development (Winklbauer & Hausen, 1983a). The area per cell in triploid SO primordia at stages 35/36 and 40 is 1.5 times that of diploid ones (Table 2). As one can estimate by focussing through skin preparations at high magnification, the epidermis is equally thick in diploid and triploid animals. This is in agreement with the results of Fankhauser (1945a,b, 1952). Thus, the increase in area per cell in triploid SO primordia corresponds

Table 1. Cell number during development in diploid and triploid SO systems

	Developmental stage (Nieuwkoop & Faber, 1967)			
	35/36	40	45	arrested 47½
Time after fertilization	50 h	66 h	98 h	240 h
Morphology of SO system	beginning of elongation	fragmentation	primary organs separated	primary organ formation completed
Cell number/SO system (number of SO systems examined)	diploid 86 ± 1 (n = 2) 47 ± 11 (n = 6)	diploid 166 ± 35 (n = 5) 99 ± 25 (n = 6)	diploid 325 ± 41 (n = 8) 217 ± 28 (n = 6)	diploid 538 ± 39 (n = 6) 334 ± 50 (n = 10)
Ratio triploid/diploid cell number	0.55*	0.60	0.67	0.62

Average ratio triploid/diploid cell number (weighted according to n): 0.62 (n = 49).  
 Increase in cell number 3.5 cells/h between stages 35/36 and 45 (regression analysis; correlation coefficient r = 0.96).  
 \* Significantly smaller than 2/3 at significance level  $\alpha = 0.05$ .  
 Cells were counted in DAPI-stained skin preparations of the respective stages.

exactly to the expected increase of the cell volume in triploids as compared to diploids.

Because of the reduction of the cell number in triploids (Table 1), the area per SO primordium is nearly equal in diploid and triploid embryos, given the measured increase in area per cell in triploids (Table 2). Also, the width of the primordia is equal in diploid and triploid SO systems at comparable stages (Table 2). Thus, the dimensions of diploid and triploid SO primordia are very similar.

*Rate of development in diploid and triploid SO systems*

As was to be expected (Fankhauser *et al.* 1942; Fankhauser, 1945a; Briggs, 1947; Fischberg, 1949), no difference in the overall rate of development between diploid and triploid *Xenopus* embryos and larvae could be detected, as judged from the external criteria given by Nieuwkoop & Faber (1967). At identical embryonic stages, which were attained at identical times after fertilization, diploid and triploid SO primordia always showed a very similar morphology (Fig. 1). Thus, there is no conspicuous difference in the rate of development of diploid and triploid SO systems either.

*Organ number and average organ size in diploid and triploid SO systems*

Between stages 47 and 48, primary organ formation is completed in the SO system. In feeding larvae, the increase in SO cell number is accelerated at this stage, and accessory organs are formed by the primary organs. In starving larvae, development is arrested, and the growth of the SO system ceases between stages 47 and 48. Up to these stages, the organ number in the SO system is essentially constant (Winklbauer & Hausen, 1983a,b).

The number of primary organs formed and their final size was compared between diploid and triploid SO systems in developmentally arrested larvae. The average number of cells per organ decreases in the triploid state to nearly two thirds of the diploid (Table 3). This would be expected if the increase in cell volume in triploids were compensated in each individual organ by a corresponding decrease in cell number. If the organ number were exactly the same in diploids and

Table 2. *Cell size and width of primordium in diploid and triploid SO systems*

	area/cell ( $\mu\text{m}^2$ ) stages 35/36 and 40	width of primordium ( $\mu\text{m}$ ) stage 40	width of primordium ( $\mu\text{m}$ ) stage 35/36
diploid (no. of SO systems)	74.4 $\pm$ 8.5 (n = 7)	26.4 $\pm$ 3.1 (n = 5)	38.8 $\pm$ 1.8 (n = 2)
triploid (no. of SO systems)	110.8 $\pm$ 15.1 (n = 12)	25.3 $\pm$ 1.0 (n = 6)	40.0 $\pm$ 5.4 (n = 6)
ratio triploid/diploid	1.49	0.96	1.03

The area and the width of the primordia were determined from photographs of DAPI-stained skin preparations. The area of epidermis occupied on the average by a single primordial cell was calculated by dividing the area of a primordium by its cell number.

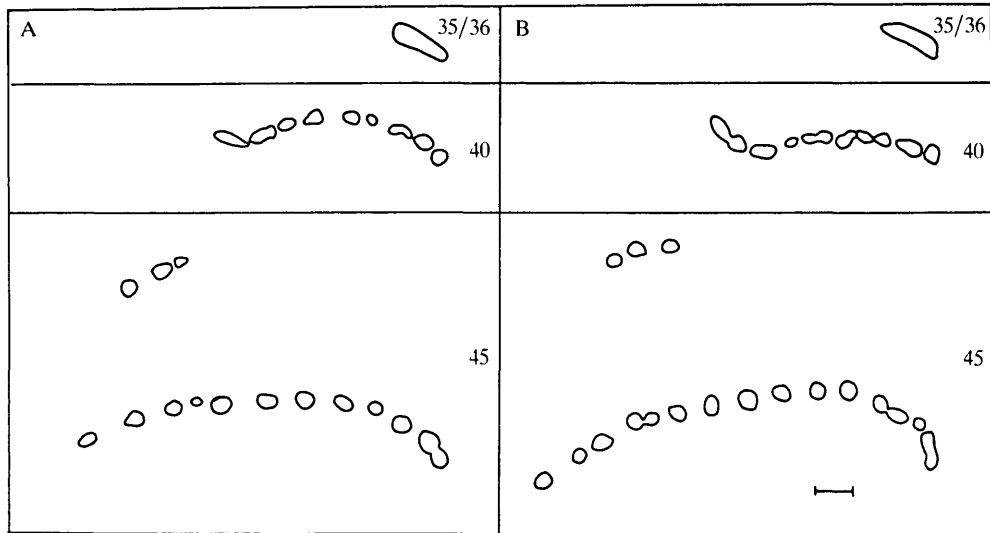


Fig. 1. Morphology of the supraorbital lateral line system at different stages of development in triploid (A) and diploid control (B) embryos. The outline of the supraorbital system was redrawn from photographs of skin preparations representative of the respective stages. Embryonic stages are indicated. Bar equals 100  $\mu\text{m}$ .

Table 3. Cell number, organ number, and organ size in diploid and triploid SO systems after growth arrest

		cell no./SO system (n = no. of SO systems exam.)	organ no./SO system	cells/organ
Experiment I†	diploid	648 $\pm$ 79 (n = 5)	18.8 $\pm$ 1.5	34.6 $\pm$ 5.5
	triploid	354 $\pm$ 68 (n = 4)	14.8 $\pm$ 2.2	24.1 $\pm$ 2.8
	ratio triploid/diploid	0.55*	0.79**	0.69
Experiment II§	diploid	538 $\pm$ 39 (n = 6)	18.0 $\pm$ 1.8	29.9 $\pm$ 2.1
	triploid	334 $\pm$ 50 (n = 10)	15.6 $\pm$ 1.3	21.4 $\pm$ 2.1
	ratio triploid/diploid	0.62	0.89**	0.72

\* Significantly smaller than 2/3 at significance level  $\alpha = 0.05$ .

\*\* Significantly smaller than 1 at significance level  $\alpha = 0.01$ .

† Data from the triploid sides of haploid/triploid chimaerae and from triploids and from diploid controls. Embryos produced by a female from a batch of animals which was used in our earlier experiments (Winklbauer & Hausen, 1983a,b).

§ Data from same batch of embryos from which all other data of this article were derived. Embryos produced by a female from a batch of animals which was used also for the experiments of the accompanying article.

Cells and organs were counted in DAPI-stained skin preparations.

triploids, the total cell number per SO system should also be reduced in triploids to nearly two thirds of normal. The actual reduction seems to be even greater, to less than two thirds, and this is met by a slight, but significant reduction in the organ number of triploids (Tables 1, 3).

*Frequency distribution of organ sizes in triploids after growth arrest*

Fig. 2A shows the frequency distribution of the sizes of primary organs, measured as the number of cells per organ, from growth-arrested triploid larvae. The organ size ranges from 3 to 41 cells per organ. The frequency of organ sizes forms a series of distinct peaks rather than a smooth distribution. Six peaks occur at a regular interval of 7 cells/organ, the first peak being positioned between 5 and 6 cells/organ, the second between 12 and 13 cells/organ, etc. If the six peaks are superimposed to equalize random irregularities, the most symmetrical peak is

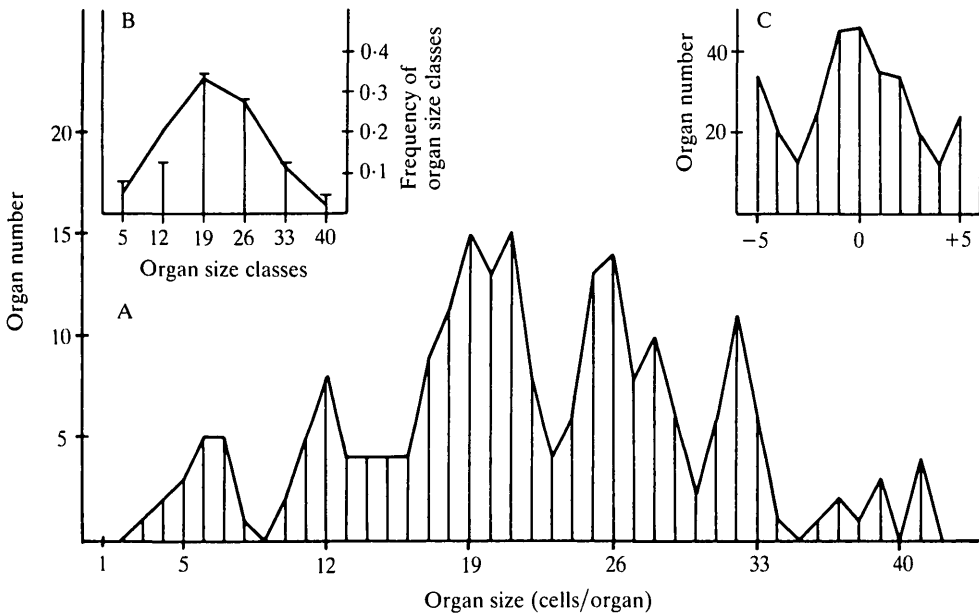


Fig. 2. Frequency distribution of organ sizes. (A) The cell number per organ from 217 triploid SO organs was determined from DAPI-stained skin preparations of growth-arrested larvae (starvation). (B) Frequency of organ size classes. According to the frame indicated below, organ size classes can be defined, each ranging over an organ size difference of 7 cells/organ, with 5, 12, 19, etc. cells/organ as the approximate medians of the respective classes. The frequency of the organ size classes is indicated by bars. This empirical distribution approximates a binomial distribution with number of trials  $n=6$  and probability of success  $P=0.45$  (solid line). (C) Superposition of peaks. It is assumed that the first peak is, as a first approximation, at 5 cells/organ, and that the difference between neighbouring peaks is 7 cells/organ. Organ numbers at 5, 12, 19, etc. cells/organ of (A) are summed and give the organ number at zero in (C). Organ numbers at 6, 13, 20, etc. cells/organ are added to give the organ number at +1 in (C), etc. The centre of gravity of the ensuing peak is at +0.2.

obtained when this regular frame is applied (Fig. 2C). The empirical distribution approximates a slightly asymmetrical binominal distribution ( $n = 5$  and  $P = 0.45$ ) (Fig. 2B). These findings bear a striking resemblance to the corresponding results from diploid larvae (Winklbauer & Hausen, 1983b).

#### DISCUSSION

The effect of cell size on SO lateral line development was studied in triploid *Xenopus* embryos. In triploids, the number of cells per SO system is reduced to about two thirds of normal during all stages of development, but the rate of development, and hence the time of organ formation, seems to be unaltered; furthermore, a nearly normal number of organs is formed. Therefore, each newly forming organ must contain only two thirds of the normal number of cells. This normal number was deduced from the frequency distribution of organ sizes to be 8 cells/organ in our earlier experiments (Winklbauer & Hausen, 1983b). In the present experiments, this number was reproduced in diploid control embryos (accompanying communication). If the proposed model of organ formation is correct, the number of cells per newly forming organ should be two thirds of 8, that is 5.3 cells, in triploids, and the frequency distribution of organ sizes should exhibit six main peaks, separated by regular intervals of 7 cells/organ, with the size of the peaks approximately fitting into a fairly symmetrical binominal distribution. This is indeed found (Fig. 2).

Two main conclusions can be drawn from this result. First, the frequency distribution of the size (in cells/organ) of triploid SO organs is in support of our model of cell multiplication and organ segregation in the SO system (Winklbauer & Hausen, 1983b), since the special features of this distribution can be accurately predicted from our model.

On the basis of the model, the number of cells at the time of organ formation in triploid SO systems can be calculated, and from this one can determine by means of the cell kinetics (Table 1) the time of organ segregation to be stage 39½. The same stage is obtained for the diploid controls (not shown) and in earlier investigations with diploids (Winklbauer & Hausen, 1983b). These calculations confirm, as is evident already from the morphological comparison of diploid and triploid SO systems during development (Fig. 1), that the timing of development is not altered in triploids. This is in agreement with the results of Fankhauser *et al.* (1942), Fankhauser (1945a), Briggs (1947), and Fischberg (1949).

Also on the basis of the model, the length of the cell cycle can be calculated to be 10.6 h in triploids. This is close to the 11.1 h calculated for the diploid controls. As expected (Briggs, 1947), the length of the cell cycle is not significantly changed in triploid SO systems. Likewise, the number of divisions a stem cell performs in that system is not influenced by the triploid state (otherwise the skewness of the frequency distribution or the interval between peaks would be changed).

A second conclusion, which is partially independent of the model, can be drawn from our results. Since the number of cells contained in a nascent organ is reduced



in triploids, the mechanism which determines the size of the forming organs cannot be a strict cell counting mechanism. Alternative mechanisms must be considered and in the accompanying publication this problem is analysed further.

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(Accepted 19 February 1985)