Experimental Tetraploidy in Newts

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WITH ONE PLATE

INTRODUCTION

It is very easy to change the number of whole chromosome-sets in Amphibia. Amphibia are better suited for this kind of work than other vertebrates. The technique of changing the number of chromosome-sets, combined with hybridization, which is also easily achieved, can contribute to a wide range of problems. In the course of certain investigations it is of importance to know the origin of the chromosome-sets present. In this case newts should be used in preference to anurans, because the contribution of chromosome-sets seems to be more regular in Urodela than in some species of Anura, where in experiments on artificial parthenogenesis larvae with from one to six chromosome-sets were produced (Kawamura, 1939). In this paper only the Urodela will be discussed.

Methods are known for producing haploid gynogenetic and androgenetic newt embryos or larvae. It is also possible to produce haploid androgenetic hybrids which possess only the cytoplasm from one species and one chromosome-set from another. Unfortunately, all these haploid types are not viable. Recently a method has been developed to produce diploid gynogenetic newts (Fischberg, 1947). These animals develop from eggs containing a diploid set of maternal chromosomes and are able to survive much longer than the haploids.

In 1939 Fankhauser & Griffiths discovered a simple method which enabled them to produce triploid newts in great numbers. Although some biologists have been working on polyploidy in amphibians since 1940, no efficient method had been found which made it possible to obtain tetraploid newts in considerable numbers. In order to study the effects of tetraploidy one had to rely on the very few tetraploid larvae which occurred in experiments designed to produce triploidy. But this number only amounted to about 0.7 per cent. of larvae which developed from temperature-treated eggs, or only about 0.15 per cent. of eggs treated in earlier experiments (Fischberg, 1948). The rate of spontaneous occurrence of tetraploidy is still lower.

The highest percentage of tetraploidy that had been obtained so far was from

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1 This paper has been written as a tribute to Professor F. Baltzer.

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the cross of triploid Mexican axolotl females with diploid males. Fankhauser & Humphrey (1950) observed that from 2,027 fertile eggs, 48 tetraploids or near tetraploids developed. This amounts to 2.4 per cent. of all fertile eggs and to 10.79 per cent. of all individuals of which the chromosome number was determined. This second percentage is, according to Fankhauser & Humphrey, artificially high, as these authors think that they were able to recognize all tetraploids but not all hyperdiploids \((2N + 1 \text{ to } 3N - 1)\), the frequency of which they judge as being close to 95 per cent. of all surviving larvae.

This method of producing tetraploid larvae is rather laborious and gives one no freedom to choose the urodele species, as the only urodele species so far found fertile in the triploid state is the Mexican axolotl. Kawamura (1951) showed for a second species \((T. \text{ pyrrhogaster})\) that offspring from triploid individuals crossed with diploids can be obtained in small numbers by means of artificial insemination. But no tetraploid larvae were found amongst them.

The first attempts to produce tetraploidy using a technique similar to that by which triploidy is produced, i.e. by temperature treatment of newt eggs at the appropriate stage, were carried out by Böök (1943), by Fankhauser & Watson (Fankhauser, 1945), and by Sanada (1951). Böök subjected the fertilized eggs, after the 2nd maturation division but before the first cleavage, to low temperatures. He thinks that this treatment inhibits the anaphase movements of the chromosomes of the first cleavage division and thus creates a tetraploid nucleus in the uncleaved egg. All embryos were complex mosaics or, as Böök calls them, 'multiform aneuploids'. They arose, in the opinion of this author, as a consequence of a multipolar mitosis caused by the presence of 4 centrosomes.

Fankhauser treated his eggs shortly before the first cleavage division with a temperature of 36°C. He was able to suppress the first cleavage of the egg, but at the time of the second cleavage these eggs divided simultaneously into 4 cells and gave rise to normal diploid larvae. Watson treated the eggs with colchicine solutions but was not successful either.

Sanada was the only one who actually found a method which produces tetraploid Amphibia without having to rely on fertile triploid newts. He exposed the eggs of \(T. \text{ pyrrhogaster}\) from 6 hours to 7 hours 50 minutes after artificial insemination for 15–31 hours to a temperature of 0° to –1°C. He states that 'the mortality of the treated eggs was considerably high' and that only a 'small number of them developed into larvae' (Sanada, 1951, p. 35). Ten of these (or 6.3 per cent.) were tetraploids. It is quite clear that the practical value of this method cannot be determined unless one knows the mortality. The 10 tetraploids might correspond to anything between 3 per cent. and 0.5 per cent. of the eggs treated.

**PRELIMINARY EXPERIMENTS**

Encouraged by the fact that it was possible to produce tetraploid pre-implantation mouse embryos by heat treatment (Fischberg & Beatty, 1950; Beatty &
Fischberg, 1952), I began some preliminary experiments on newts in 1950. Eggs of *T. palmatus* (*helveticus*) and some eggs of *T. vulgaris* were exposed to temperatures ranging from 35° to 38° C., for a duration of 10–12 minutes. Every quarter of an hour from 3 to 6 3/4 hours after insemination a different series of eggs were treated in this way.

All eggs were artificially inseminated. The eggs of one female were therefore of exactly the same age and were always kept at the same temperature and in the same Petri dish. Nevertheless, even before the treatment the eggs of each female did not develop at exactly the same rate. At the time when the first cleavage division should occur I always found some 2-cell stages, some eggs which showed the first signs of cleavage and some entirely uncleaved (but definitely fertilized) eggs in the same Petri dish.

1,060 eggs were treated before the first sign of cleavage appeared in any of these series of eggs. Only 4 embryos reached advanced developmental stages, but their chromosome number could not be counted. From 1,019 other eggs, of which at least one in each Petri dish was a 2-cell stage or showed the first cutting in of the cleavage furrow, 25 advanced embryos or larvae were obtained. The chromosome number could be determined in the epidermis of the amputated tail tips in 15 of these: 4 were diploid, 5 were tetraploid, 4 were diplo-tetraploid mosaics, 2 were multiform aneuploid.

In spite of the very high mortality, this result indicated that tetraploidy can be produced in newts by application of a 'hot-shock'. All the 5 tetraploid embryos or larvae developed from eggs which were treated at a temperature of 36°–37° C.

**FINAL EXPERIMENTS**

In order to improve the technique we had now to find out which of the three treated stages of the eggs present in each of the Petri dishes gave rise to the tetraploid larvae. We divided the eggs of each female into three groups which were of the same age but at different developmental stages: pre-cleavage stages, beginning of 1st cleavage stages, 2-cell stages.

All the eggs of one female were treated simultaneously at the same temperature. The mortality was still high in all three groups, but we see from Table 1 that the mortality was highest in the pre-cleavage stages, a little lower in the eggs which had begun to cleave and still lower in the 2-cell stages.

Table 2 demonstrates the result of the chromosome counts in the epidermis of the tail tips. The 82 analysed embryos or larvae, characterized by their chromosome number, belong to five classes: normal diploids, triploids, tetraploids, diplo-tetraploid, and triplo-tetraploid mosaics. The triploid and the mosaic larvae are so rare that we can neglect them for the time being and only consider the rows for diploid and tetraploid individuals. On the left of the number of larvae we find the percentage of all treated eggs which developed into diploids or tetraploids. On the right of the same number the percentage of analysable newts which were diploid or tetraploid is indicated.
The high mortality of the treated pre-cleavage stages, and also the rather low percentage of analysable newts which were tetraploid, exclude the treatment of pre-cleavage stages as a working method for the production of tetraploidy. The same holds for the eggs which were treated when beginning cleavage.

### Table 1

*Mortality of eggs and embryos of *T. palmatus* and *T. vulgaris* after treatment with heat*

(Stages Gl. 32 and 41 refer to Glaesner, 1925)

<table>
<thead>
<tr>
<th>TREATED STAGE</th>
<th>TRITURUS PALMATUS</th>
<th>TRITURUS VULGARIS</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PRE-CLEAVAGE STAGE</td>
<td>BEGINNING CLEAVAGE</td>
<td>2-CELL STAGE</td>
</tr>
<tr>
<td>TOTAL EGGS TREATED</td>
<td>681</td>
<td>100</td>
<td>132</td>
</tr>
<tr>
<td>DEAD BEFORE STAGE GL. 32</td>
<td>671</td>
<td>98-53</td>
<td>114</td>
</tr>
<tr>
<td>DEAD BETWEEN STAGES GL. 32-41.</td>
<td>3</td>
<td>0-44</td>
<td>3</td>
</tr>
<tr>
<td>HATCHED LARVAE</td>
<td>7</td>
<td>1-03</td>
<td>15</td>
</tr>
</tbody>
</table>

### Table 2

*Effect of heat-shock treatment of the eggs of *T. palmatus* and *T. vulgaris* on the chromosome number of the resulting embryos*

<table>
<thead>
<tr>
<th>Treated stage</th>
<th><em>T. palmatus</em></th>
<th><em>T. vulgaris</em></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-cleavage stage</td>
<td>Beginning cleavage</td>
<td>2-cell stage</td>
</tr>
<tr>
<td>Total eggs treated</td>
<td>100</td>
<td>61</td>
<td>(%)</td>
</tr>
<tr>
<td>Analysable newts</td>
<td>0-9</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Diploid</td>
<td>0-6</td>
<td>4</td>
<td>66-7</td>
</tr>
<tr>
<td>Triploid</td>
<td>0-2</td>
<td>1</td>
<td>16-7</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>0-2</td>
<td>1</td>
<td>16-7</td>
</tr>
<tr>
<td>Diplo-tetraploid mosaic eggs</td>
<td>..</td>
<td>..</td>
<td>..</td>
</tr>
<tr>
<td>Tripl-o-tetraploid mosaic eggs</td>
<td>..</td>
<td>..</td>
<td>..</td>
</tr>
</tbody>
</table>

But the results of the experiments in which eggs were treated at the 2-cell stage are satisfactory. Seventeen per cent. of the treated eggs survive long enough to be analysed by the tail-tip method and 35-5 per cent. of these are tetraploid. This corresponds to 6 per cent. of all the treated eggs. In the experiments with *T. vulgaris*, which were carried out in 1952 by the late Miss E. Pendleton, the effect of the treatment was even more successful. Twenty-four per cent. of all treated eggs, or 93-7 per cent. of the analysable individuals, were tetraploid.
The method would be ideal if all surviving larvae were tetraploid and no diploids occurred. Then one would not lose time in counting the chromosomes of diploids. It is therefore necessary to make a compromise between relatively low mortality of the eggs and a high proportion of tetraploids compared with diploids.

![Text-fig. Effect of temperature of heat-shock on mortality (4 upper curves) and on the production of tetraploidy (4 lower curves). The numbers on the 4 upper curves represent the total number of eggs treated at the particular temperature, while numbers on lower curves represent the actual number of tetraploid embryos and larvae resulting from the treatment. Gl. 32 is a developmental stage according to Glaesner (1925). ••••• = T. palmatus eggs treated before cleavage began; ••••• = T. palmatus eggs treated at beginning of cleavage; ——— = T. palmatus eggs treated at 2-cell stage; ••••••••• = T. vulgaris eggs treated at 2-cell stage.]

The Text-fig. shows the effect of temperature on mortality and on the frequency of the occurrence of tetraploidy. Many numbers in these curves are too small to be of significance, but it is possible to get a general picture of the temperature effect as 1,137 eggs are included in this figure.

If we look first at the four top curves, we see that the mortality rises with increasing temperature. This holds for the eggs of T. palmatus which have been treated at the pre-cleavage stage, as well as for those treated during the beginning of cleavage or during the 2-cell stage, and also for those of T. vulgaris, treated at the 2-cell stage. The mortality is highest after treatment of pre-cleavage stages
and lowest after treatment of 2-cell stages. *T. vulgaris* shows a considerably lower mortality than *T. palmatus*. It is further evident that treatment with a temperature of 35·6°–36·0° C. is the most effective, the mortality not being too high and the number of tetraploid newts being almost equal to the number of survivors.

**THE MECHANISM**

Previously, it was generally believed that tetraploidy in Amphibia could best be produced by inhibition of the anaphase movements of the chromosomes due to a treatment during the metaphase of the first cleavage division, and hand in hand with this, or as a consequence of it, the cytoplasmic cleavage would be inhibited. Tetraploidy would then result by doubling the chromosome number in the uncleaved egg.

The facts, however, are quite different. As we have seen, the eggs are best treated immediately after completion of the first cytoplasmic cleavage. Cytological examination showed that at this stage of cytoplasmic cleavage each of the two cells contains an interphase nucleus (Plate, figs. A, B).

During heat treatment, or very soon afterwards, the cytoplasmic cleavage is reversed in most eggs. An uncleaved egg with two interphase nuclei results. About 3 hours after the heat treatment the egg divides again into two cells. In most cases the new furrow does not correspond with the position of the primary, vanished furrow. Within these 3 hours the egg becomes tetraploid. We do not yet know if the two interphase nuclei fuse, or if two mitoses fuse at metaphase, or if the chromosomes of the two mitoses migrate during the second anaphase to only two poles instead of four. The presence of diplo-tetraploid mosaic newts (6 in all, 4 from the preliminary experiments and 2 shown in Table 2) suggests a fourth possibility. These mosaic larvae seem to originate from eggs in which only one of the two nuclei, present at the time of treatment, became tetraploid while the other remained diploid. Development would then proceed with both the diploid and the tetraploid nuclei. This seems to indicate that tetraploidy results from the independent doubling of chromosomes of both nuclei during the second mitotic division. This could be brought about by the failure of anaphase movements resulting from inhibition of centrosome doubling or inhibition of centrosome movements. The mechanism which leads to tetraploidy is under cytological investigation.

**THE TETRAPLOID NEWTS**

Tetraploid urodeles have previously been described in some detail (Fankhauser, 1945; Fischberg, 1948; Sanada, 1951). I will therefore give only a sketch of their main characteristics. Diploid individuals of *T. palmatus* and *T. vulgaris* both have 24 chromosomes in each cell and tetraploid individuals have 48 chromosomes (Plate, figs. C, D). The volume of the nuclei and cells of tetraploids is about twice the volume of diploids. However, organ and body size remain more or less normal. This is achieved by a reduction of the cell number in poly-
ploids, proportional to the increase in cell size (Plate, figs. E, F). Each organ therefore contains only about half the number of cells as compared with diploids.

The viability of tetraploids is generally lower than that of diploid or triploid newts. Many of them die as old embryos or larvae, but many may survive for some years, as shown in the case of the Mexican axolotl (Humphrey & Fankhauser, 1949; Fankhauser & Humphrey, 1950) and for T. pyrrhogaster (Sanada, 1951). Apart from an occasional axial curvature and reduced growth rate in many of the older animals the tetraploids are of normal appearance.

**DISCUSSION AND OUTLOOK**

We have seen that the technique for producing tetraploid T. palmatus and T. vulgaris is an extremely simple one. We can neglect the time after fertilization and the temperature at which the eggs are kept. We have only to pick out the early 2-cell stages, which are recognizable with the naked eye, and to transfer them to water at 35.6°–36.0°C. for 10 minutes, and tetraploid newts will be produced.

I am certain that it will now be possible to produce tetraploidy in most other urodele species. We have, however, to consider two factors which might be of importance if experiments of this kind are carried out with other species. Firstly, the result of the experiment depends on treatment at a specific temperature. The Text-fig. demonstrates that the eggs of the two species used in the experiments show a different mortality when exposed to the same temperature. T. vulgaris has the lower mortality, which might perhaps be expected from the geographical distribution of this species, which is as a whole more exposed to extremes of temperature than T. palmatus. The difference in mortality and the fact that different species live in different climates indicates that for the production of tetraploidy the effective temperature is likely to vary from species to species.

Secondly, the stage at which the eggs have to be treated is also very specific. In our species the first cleavage furrow has to close, or almost to close, at the vegetative pole of the egg. Cytological observation has proved that this externally visible stage of the egg corresponds with the presence of two nuclei, which are in middle or late interphase. I believe that it is important that the later interphase stages of the nuclei are treated in order to obtain tetraploidy. It can be assumed that not all species of Amphibia produce eggs in which this specific stage of the nuclei corresponds with the described stage of externally visible cleavage. The size of the eggs and other factors might be of importance.

The reason why I described the method of producing tetraploidy in newts in considerable detail is not because the technique, as such, is of special interest, but because I think this method might be of great help in the investigation of a wide range of problems. It is now possible to produce tetraploid newts in great enough numbers to carry out physiological research. Cell size and relative surface of cells are believed to be of importance for physiological processes. With
the possibility of producing polyploid individuals in Amphibia, such theories can be tested experimentally on ideal material and the physiology of polyploid animals can be investigated (Douglas, in manuscript). Tetraploid organisms can be of the same species and age as diploids, they can have the same body and organ size as the diploid controls, but they are composed of much fewer, larger cells.

It is now possible to produce tetraploid hybrids in Amphibia (Fischberg, unpublished) which are of genetic interest and might also help to throw some light on the part hybridization plays in vertebrate evolution.

It can be assumed that if a method enables us to make tetraploid animals out of diploids the same method will allow us to make diploids out of haploids. This would be of great interest because all haploids so far produced were non-viable or of reduced viability. We shall now be able to produce diploid androgenetic urodeles which will be isogenic. If they prove to be viable the problem of haploid lethality would be much nearer its solution. It would show us that the small nuclei and cells are the reason for the feeble viability and not genetic lethal factors. If the viability of these haploids could not be improved by making them diploid then genetic lethal factors would be the cause of early death. A longer survival of diploid androgenetic individuals would also quickly solve the problem of sex determination in urodeles.

Perhaps the production of diploid androgenetic hybrids, which possess the cytoplasm only of one species and the chromosomes from another species, would be of great importance. The study of such abnormal nucleo-cytoplasmic combinations in amphibia has been very difficult because of the early death of the haploids (Baltzer, 1930, 1940, 1949; Hadorn, 1932, 1934, 1937). If the viability of these androgenetic hybrids could be improved, the species specificity of the cytoplasm and also its possible function in the transmission of phenes, or characters (see Hadorn, 1936), could be investigated. Astaurov & Ostrikova-Varshaver’s excellent investigation (1957) on the silkworm might then be applied to amphibia.

**SUMMARY**

1. A simple method has been devised for producing tetraploid newts by means of a ‘heat’ treatment.

2. The eggs of *T. palmatus (helveticus)* and *T. vulgaris* were at different stages, varying from 3 hours to $6\frac{3}{4}$ hours after artificial insemination, exposed to warm water of 34°–38° C. for a duration of 10–12 minutes.

3. A temperature treatment of 35·6°–36·0° C. proved to be the most successful for the production of tetraploid embryos and larvae. Treatment at the stage when the first cleavage furrow closes at the vegetative pole was found to be the only effective one. The eggs contain at this stage two interphase nuclei. The described treatment causes up to 24 per cent. of all treated eggs, or up to 93·7 per cent. of all analysed newts to develop into tetraploids.
4. The probable cytological mechanism which leads to tetraploidy is discussed.

5. Factors which seem to be of importance for the production of tetraploidy in other amphibian species, and some important problems which might be investigated with the help of this method, are mentioned.

REFERENCES


DOUGLAS, R. (In manuscript). A comparison of erythrocytes and oxygen consumption of diploid and triploid frogs (Rana temporaria temporaria L.).


**EXPLANATION OF PLATE**

**Fig. A.** Section of 2-cell stage of *T. vulgaris* containing 2 interphase nuclei.  ×38.

**Fig. B.** Medium to late interphase nucleus of 2-cell stage, demonstrating the stage of the nuclei at which heat treatment should be applied.  ×455.

**Fig. C.** Diploid mitosis from tail fin of *T. vulgaris*.  ×455.

**Fig. D.** Tetraploid mitosis from tail fin of *T. vulgaris*.  ×455.

**Fig. E.** Tail tip preparation of a diploid *T. vulgaris*.  ×38.

**Fig. F.** Tail tip preparation of a tetraploid *T. vulgaris*. Note large and few nuclei in this preparation.  ×38.

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