Sterility and partial sterility in the South African clawed toad following the pricking of the egg

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Bounoure (1934) first described the presence in the egg of the frog *Rana temporaria* of a special cytoplasmic differentiation, which he termed the ‘germinal cytoplasm’. He was able to trace the developmental history of this plasm as it moved from its original position just under the cortex of the vegetal pole of the fertilized egg, to its inclusion in a number of cells of the early gastrula endoderm, and also mapped the migration of these cells into the gonad rudiments. Bounoure believed that the germinal cytoplasm is the definitive germ-cell determinant, and that its presence in a cell is a prerequisite for the cell to undergo meiosis and differentiate into a gamete.

Since Bounoure’s discovery, germinal cytoplasm has been described in the eggs of a number of other anuran species (Nieuwkoop, 1956; Blackler, 1958; DiBerardino, 1961; Gipouloux, 1962) and a number of techniques have been used to test Bounoure’s determinant hypothesis. Several workers (Bounoure, 1937; Bounoure, Aubry & Huck, 1954; Padoa, 1963, 1964; Smith, 1966; A. W. Blackler, unpublished) have obtained varying degrees of sterility in tadpoles and metamorphosed frogs derived from eggs whose germinal cytoplasm had been irradiated with ultraviolet light at the earliest stages of development. Attempts to remove the germinal cytoplasm from the egg, and to examine the effects of that removal, have been limited. Nieuwkoop & Suminski (1959) pricked the vegetal pole of the four-cell stage in the South African clawed toad, *Xenopus laevis*, and thereby provoked the formation of an exudate that presumably contained the germinal cytoplasm (which, according to histological studies, is located immediately under the vegetal pole cortex at this stage). Tadpoles grown from eggs which had been subjected to pricking showed no significant reduction in number of germ cells as compared with the number present in control animals. Nieuwkoop & Suminski concluded that the germinal cytoplasm was not indispensable for the formation of gonocytes, and therefore that the germinal cytoplasm was not a germ-cell determinant. A little later, Fischiarolo (1960) removed cells from the vegetal region of blastulae of the frog *Disco-...
glossus with a view to extirpating cells containing germinal cytoplasm. Animals which developed from blastulae surviving the ablation failed to show any diminution in the number of their germ cells as compared with control animals.

Two other deletion studies have led to opposite conclusions. Monroy (1939) removed part of the endoderm of the neurula of Discoglossus (the region removed being that part expected to contain the germ cells) and obtained some experimental survivors that were sterile. Later, Librera (1964) reported the results of experiments in which the vegetal pole of the 4-cell stage of Discoglossus was pricked, using the same technique as that of Nieuwkoop & Suminski. She found that complete and some partial sterility occurred in animals reared from pricked eggs; similar results were also obtained from pricked fertilized, but uncleaved eggs.

None of the experiments commented on above incorporated proof that pricking the egg removes germinal cytoplasm, or that removal of parts of the blastula and neurula involved removal of cells containing germinal cytoplasm. In view of this missing feature, as well as the direct conflict between the results of Librera (1964) and Nieuwkoop & Suminski (1959) (which may have reflected some basic difference in the origin of the germ line between Discoglossus and Xenopus), we have considered it advisable to repeat the technique of deletion-by-pricking, coupling it with an histological study to determine if pricking is indeed efficient in removing germinal cytoplasm from the egg.

**MATERIALS AND METHODS**

Fertilized eggs were obtained from pairs of Xenopus laevis following stimulation of the toads with human chorionic gonadotropin. All eggs, experimental and control, were manually stripped of their outer jelly coat and kept in full-strength Steinberg saline until they reached the advanced blastula stage, at which time they were transferred to 10 % saline.

At the 2- or 4-cell stage, when the first or second cleavage furrow was seen to circumscribe the egg completely, experimental eggs were quickly inverted and a small X-shaped incision made at the vegetal pole with a glass needle. In the case of 4-cell stages, care was taken to ensure that each blastomere was incised. For the 2-cell stage, the vegetal pole was located with some accuracy by means of the pale grey ring that encircles it in X. laevis. After operation, experimental eggs were permitted to develop to the late blastula or middle tadpole stages (stages 9 and 49–52 of the Normal Table of Nieuwkoop & Faber, 1956). The average survival rate of experimental eggs was 25 %, but varied from 0–40 % according to the quality of the egg batch used; control eggs developed normally at the customary 80–90 %.

Late blastulae from experimental and control groups were fixed in Smith's fluid, sectioned in paraffin wax, stained with Heidenhain Azan, and examined for the presence of germinal cytoplasm. A few eggs were fixed within 20 min of the
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FIGURE 1

(A) Pricked 2-cell stage photographed within a few minutes of treatment. Egg diameter 1.4 mm.

(B) Pricked 2-cell stage as it appears 20 min after treatment. Note the increase in the size of the exudate over that depicted in Fig. 1 A. Egg diameter 1.4 mm.

(C) Pricked 2-cell stage during the second cleavage division. The exudate is approaching its maximum size. Egg diameter 1.4 mm.

(D) Pricked 2-cell stage which had developed as far as the mid-blastula stage. Note that the exudate has moved to an equatorial position below the vitelline membrane, and has not affected the normal cell divisions in the rest of the egg. Egg diameter 1.4 mm.
incision for examination of the immediate effects of the pricking. Tadpoles of stages 49–52 (the stage varying between experimental series) were fixed in Smith’s fluid, sectioned in paraffin wax, stained with Mayer’s Haemalum and Eosin, and direct counts made of the number of gonocytes present in the gonads.

RESULTS

Twelve series of eggs were subjected to the experimental treatment. Of these, five series were unusable because of sterility in the controls or excessive mortality following the operation; it is a feature of *Xenopus* eggs, following hor-

![Diagram](image)

**Figure 2**

Drawings of sections through pricked and cleaving eggs of *X. laevis* to show the size of the exudate and its relation to the germinal cytoplasm. Exudates are signified by arrows and patches of germinal cytoplasm are shown in black.

(A) 2-cell stage immediately after pricking. Germinal cytoplasm is near the exudate but not included in it. Inclusion in whole or in part may take place within the next 20 min.

(B) 4-cell stage (egg had been pricked at 2-cell stage). The exudate has increased to maximal size and contains all of the germinal cytoplasm; other sections did not reveal plasm other than in the exudate.

(C) 8-cell stage (egg had been pricked at 2-cell stage). The exudate contains a single patch of germinal cytoplasm in this section; elsewhere a second patch is found, but the egg retains the rest of the plasm.

(D) 16-cell stage (egg had been pricked at 2-cell stage). No germinal cytoplasm has entered the exudate in any section.
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monal stimulation of mating, that they vary widely from mating to mating in their ability to withstand manipulation and to form fertile animals. The results below are based on the seven usable series.

1. The effects of pricking. The amount of vegetal cytoplasm exuded by an experimental egg increases from the moment of pricking throughout cleavage; the initial exudate is about one-half the size of the definitive exudate (see Fig. 1). The effect of the incision on the germinal cytoplasm can be seen by sectioning eggs at various times following the incision; some 20 min following incision, the germinal cytoplasm ‘slides’ along the vegetal cortex and enters the exudate. This latter becomes detached from the egg by the late blastula or early gastrula stages. An examination of sections from 8- or 16-cell stages reveals that germinal plasm may partially or wholly enter the exudate or may fail to enter the exudate (see Fig. 2).

Table 1. Results of examination of control and experimental animals for the presence of germinal cytoplasm (blastulae) and gonocytes (tadpoles)

<table>
<thead>
<tr>
<th>Blastulae</th>
<th>Tadpoles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Controls</td>
<td>39</td>
</tr>
<tr>
<td>Experimental</td>
<td>54</td>
</tr>
</tbody>
</table>

The examination of blastula sections (see Table 1) showed that only one blastula derived from unpricked control eggs failed to contain germinal cytoplasm. On the other hand, 18 (or 33%) of 54 blastulae derived from pricked eggs were completely deficient in germinal cytoplasm. There is some slight indication that pricking the 4-cell stage, as opposed to the 2-cell, is more efficient in depriving the egg of all germinal cytoplasm.

2. The examination of tadpole gonads. As has already been noted above, tadpoles from the seven acceptable series were not killed at the same stage, but for the purposes of Table 1, gonocyte counts from all the series have been combined in order to obtain an index of total sterility. Of 45 control tadpoles, two bore gonads completely deficient in gonocytes, and of 63 experimental tadpoles 20 were completely sterile.

In making the counts for total sterility, it became apparent that many of the experimental gonads contained diminished numbers of gonocytes (partial sterility) as compared with control gonads of the same stage. Thus pricking, while not being always effective in removing all of the germinal cytoplasm from the egg, often affects the number of gonocytes present at a later stage. The average number of gonocytes in normal tadpoles of X. laevis varies from stage to stage and in order to remove this variable from the analysis of partial sterility,
we quote only the counts of one of the experimental series, in which control and experimental tadpoles were killed at stage 52 (see Fig. 3).

Of 21 control tadpoles in the selected series, none were sterile. The number of sex cells/tadpole of stage 52 ranged from 40 to 199, with an average of 122. Of 21 tadpoles derived from eggs which had been pricked at the 2-cell stage, 5 were sterile; the remaining 16 tadpoles contained gonocytes ranging from 4 to 105 in number, with an average of 58 cells. Finally, of 16 tadpoles obtained from eggs which had been pricked at the 4-cell stage, 5 were sterile, and gonocytes ranged from 1 to 138 in the remaining eleven tadpoles, with an average of 43.

The above diminishations in germ-cell number occur only when the vegetal pole is pricked; tadpoles raised from a small sample of eggs which had been pricked at the animal pole at the 4-cell stage showed no difference in gonocyte counts from control animals.
DISCUSSION

1. Total sterility. Although the percentage total sterility varied between the seven experimental series, in each series the percentage was higher than in the control group. Moreover, there is an excellent correlation, which stands up to a statistical test, between the number of blastulae derived from pricked eggs which no longer contain germinal cytoplasm (33 %) and the number of tadpoles whose gonads are completely sterile (31.7 %).

Some sterility was found among the control animals (2.5 % of blastulae and 4.4 % of tadpoles), but sterility of this order is not unusual in embryos obtained from matings of X. laevis which have been hormonally induced. In any case, this sterility is very much below that of experimental groups.

2. Partial sterility. The experimental data show clearly that even when an animal derived from a pricked egg is not totally sterile, the number of gonocytes in its gonads tends to be reduced in comparison with the control average for the particular developmental stage. Reference to Fig. 3 shows that the numbers of gonocytes in both control and experimental groups tend to occur in more-or-less normal distributions, the difference between the distributions being that the peak of the experimental curve is moved to the left: that is, the average number of gonocytes is lower in the experimental tadpoles. It is worth emphasizing that the average number of gonocytes has been computed only on the basis of gonads containing gonocytes, and totally sterile gonads have been excluded. The averages reflect, therefore, a tendency towards a reduction in fertility even in those animals classified as ‘fertile’.

Moreover, the range of gonocytes in experimental animals overlaps, to an extent, the range in control animals. It seems likely, therefore, that pricking the egg is not universally effective in removing some or all of the germinal cytoplasm. Although the number of animals in the largest series is not great, this consequence is brought out in the histogram. About one-quarter of tadpoles derived from pricked 2-cell eggs were totally sterile, while the numbers of gonocytes in the remainder tended to be clustered around an average which was about half the average for the control group. On the other hand, for those tadpoles which developed from pricked 4-cell eggs, 11 of the 16 tadpoles were either totally sterile, or contained less than 20 gonocytes. Two of the remaining animals were indistinguishable from control animals as regards the number of gonocytes in the gonads, and these animals were probably derived from eggs in which the pricking had been totally ineffective in removing germinal cytoplasm.

The smaller reduction in gonocytes following pricking of the 2-cell stage, as opposed to the reduction after pricking of the 4-cell stage, can be explained by reference to the normal distribution of the germinal cytoplasm in the egg of X. laevis at these stages (Blackler, 1958). At the 2-cell stage, the germinal cytoplasm is found scattered in small subcortical patches in the vicinity of the
vegetal pole. By the time the egg is in the 4-cell stage, these patches have begun to coalesce and to converge upon the vegetal pole. It is likely, therefore, that the pricking of the egg at the 2-cell stage tends to cause some, but not necessarily all, of the scattered and separate patches of germinal cytoplasm to be lost in the exudate. At the 4-cell stage in contrast, where there is coalescence of the plasm in fewer and larger patches, it is much more likely that the plasm will be mostly lost from the egg.

Partial sterility of tadpoles was observed in every series of experimental origin. It would have been more conclusive if it had been possible to correlate the extent of partial sterility with a reduced quantity of germinal cytoplasm in blastulae derived from pricked eggs. The evaluation of the amount of germinal cytoplasm in such blastulae proved, however, to be impossible to perform on a truly quantitative basis, and scoring on presence or absence of germinal cytoplasm alone was possible with certainty.

3. Comparison with other studies. It will be appreciated that our results agree with those of Librera (1964), with the addition of proof that pricking of the egg can result in deletion of germinal cytoplasm. At the same time, our results are diametrically opposed to those obtained by Nieuwkoop & Suminski (1959), who used the same species as we employed. We feel that the discrepancy may be due to several reasons. One should note that in the experiments reported here, a larger amount of vegetal cytoplasm was removed from the egg than in the experiments of Nieuwkoop & Suminski. There is, therefore, the greater probability for success in removing the germinal cytoplasm. It is unfortunate that the Dutch authors did not undertake the histological analysis to determine if their attempted deletions were indeed effective. Further, their results were based on a smaller number of experimental animals than we employed, and thus this small number may not have given a true picture of the potential of the technique.

In this present study, it seems abundantly clear that removal of germinal cytoplasm from the egg at the 2- or 4-cell stage results in sterility in the tadpole that develops from the egg. In consequence, it would appear that the germinal cytoplasm is a determinant whose inclusion within an embryonic cell is a prerequisite for that cell's entering the germinal line. This conclusion is substantiated by other studies which have sought to destroy the germinal cytoplasm in situ (Smith, 1966; A. W. Blackler, unpublished).

**SUMMARY**

When eggs of *Xenopus laevis* are pricked at the vegetal pole of 2- or 4-cell stages, some or all of the 'germinal cytoplasm' is contained in the resulting exudate. Tadpoles which develop from pricked eggs are either totally sterile or bear a reduced number of gonocytes in their gonads. This result supports the hypothesis that the germinal cytoplasm is a germ-cell determinant in that its presence in the egg is necessary for the production of reproductive cells, that
reproductive cells are not derived from secondary sources, and that there is a quantitative relationship between the amount of germinal cytoplasm and the number of gonocytes present in the gonads at any particular tadpole stage.

Résumé
Stérilité et stérilité partielle du Xénope après piqûre de l’œuf

Quand les œufs de *Xénopus laevis* sont piqués au pôle végétatif, aux stades 2 ou 4 blastomères, une partie ou la totalité du ‘cytoplasme germinal’ se trouve dans le matériel exsudé. Les têtards qui se développent à partir des œufs piqués sont totalement stériles ou montrent un nombre réduit de gonocytes dans leurs gonades.

Ces résultats sont en accord avec l’hypothèse selon laquelle le cytoplasme germinal est le déterminant de la cellule germinale. Le cytoplasme germinal est nécessaire pour la formation des cellules reproductrices. Ces dernières ne proviennent donc pas de sources secondaires. D’autre part, il existe une relation entre la quantité de cytoplasme germinal et le nombre de gonocytes retrouvés dans les gonades de têtards ayant atteint un stade déterminé.

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