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Author(s)	KATAGIRI, Chiaki
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On the Fertilizability of the Frog Egg, I¹⁾

By

Chiaki Katagiri

Zoological Institute, Hokkaido University

(With 1 Table and 5 Text-figures)

For the experimental studies on fertilization, it is often needed to use the medium which preserves unfertilized eggs in fertilizable condition for a considerable length of time without causing any harmful effects on their fertilization and subsequent development. In frog eggs, it is well known that eggs tend to lose in relatively short time their fertilizability in water (Bataillon & Tchou '30, Rugh '51, Tchou & Wang '56 *etc.*) and recently in some anuran species structural changes of the cortical cytoplasm of the egg are reported to occur upon fertilization or activation (Motomura '52, Katagiri '59). Relatively little has been studied, however, on the medium for preserving the egg in respect to the mentioned above.

The investigation to be reported here provides a preliminary information concerning fertilizability of the frog egg in various media, particularly in salt solutions, and considerations on this subject.

Material and methods

The material used is mature eggs and spermatozoa of the grass frog, *Rana temporaria*, collected in the vicinity of Sapporo in a breeding season. Eggs were taken directly from uteri by vivisection of the female in amplexic embrace and stored in cold moist chamber at 5°C in refrigerator before use. As a physiological balanced salt solution, De Boer's solution having the following composition was employed; M/8.8 NaCl 100 parts + M/8.8 KCl 1.2 parts + M/13 CaCl₂ 1.8 parts, with pH adjusted to 7.3 by addition of NaHCO₃. In need of diluting the solution, distilled water of pH 7.3 was always used²⁾. Artificial insemination was carried out in the following manner: a pair of testes were macerated in 10 cc of tap water and were allowed to stand for 10 minutes. The sperm suspension thus made was then poured with pipette over eggs in petri-dish. The dish, after being gently shaken for 10 minutes, was filled with a large quantity of tap water. The percentage of fertilization was determined 10 to 18 hours after insemination by counting the eggs undergoing cleavage (morula or blastula stage). All the experiments and observations were performed at room temperatures, 16–20°C.

As shown in Table 1, eggs stored in cold moist chamber for 6 days showed still relatively high fertilizability, but eggs able to develop in high percentage beyond gastrula

1) Contribution No. 526 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

2) 1/2 or 2 De Boer's solution means double diluted or double concentrated solution, respectively.

Table 1. Fertilizability of eggs stored in the cold moist chamber at 5°C.

Day stored	No. of eggs used	No. of eggs cleaved	Percentage cleaved
0	85	71	83.5
1	89	88	99.0
2	79	77	97.5
3	33	32	97.0
4	41	29	70.7
5	61	50	82.0
6	49	36	73.5

stage were only those stored within 5 days. All experiments were therefore performed with the eggs stored in cold moist chamber at longest for 5 days.

Morphological remarks: Besides the translucent vitelline membrane or chorion having about 4μ in thickness, the egg is, as shown in Figure 1, surrounded by jelly envelopes consisting of 4 layers, namely J_1 , J_2 , J_3 and an outermost layer, the adhesive layer. The layers J_1 and J_2 are translucent, and the layer J_3 is opaque. When the egg is immersed in tap water, the jelly, remaining its adhesiveness unchanged, begins to swell, particularly the layer J_3 does so markedly, whereas in De Boer's solution swelling of the jelly is not so conspicuous, though the adhesiveness is lost.

Upon insemination, a great number of spermatozoa come to the surface of the jelly, then a few of them begin to penetrate into the jelly, but being unable to penetrate, most others remain on the surface of the jelly. Until 10 minutes after

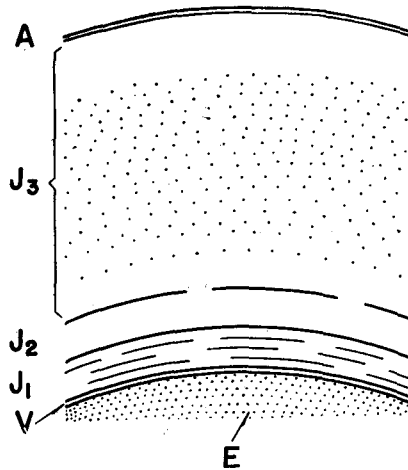


Fig. 1. Diagrammatic illustration of egg envelopes. J_1 , J_2 , J_3 and A represent the layers of jelly. A, adhesive layer; E, egg proper; V, vitelline membrane.

insemination, one cannot find spermatozoa having passed through the jelly and attached themselves to the vitelline membrane. Complete penetration of a spermatozoon into the egg interior through the vitelline membrane takes place a few minutes later, followed by the wave-like progress of membrane elevation from the penetration point. The egg rotation within the perivitelline space at fertilization is completed in about 50 minutes.

Experimental results

In the first place, the effect of media on the fertilizability of egg was studied at room temperature with the following procedure. After treated with tap water, De Boer's solution or with 1/2 De Boer's solution for a desired length of time, unfertilized eggs were inseminated and reared in tap water. As the controls, eggs having been placed in moist chamber at room temperature were inseminated and put into tap water. For each insemination fresh sperm suspensions made of different individuals were used. A typical results are graphically shown in Figure 2. As will be seen in this figure, fertilizability of the egg is lost within 15 minutes

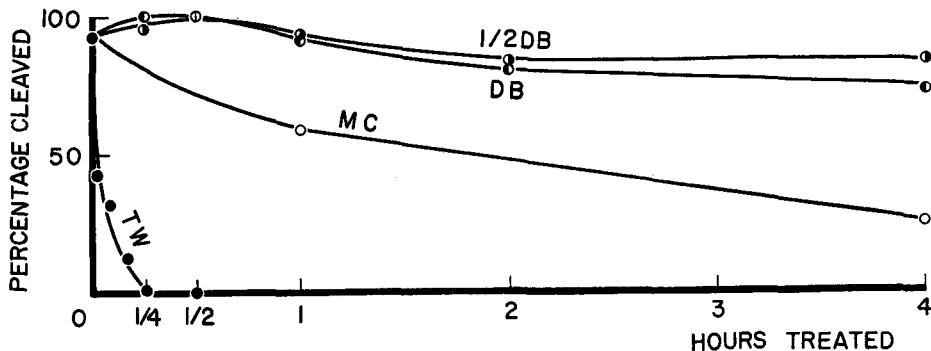


Fig. 2. Fertilizability of eggs treated with various media prior to insemination. DB, De Boer's solution; MC, moist chamber; TW, tap water.

in tap water, whereas it can be retained for a considerable length of time in De Boer's solution, in its 1/2 solution and in moist chamber too. And, comparing salt solutions with moist chamber, solutions such as De Boer's solution or its 1/2 solution are more suitable for the maintenance of fertilizability, at least at room temperature, than moist chamber. Results were always the same in the series of this experiment, though the time of the maintenance of fertilizability in salt solutions varied in each batch. Fertilizability in De Boer's solution fell down, for instance in some cases, to less than 50% in 1 hour³⁾. According to the observa-

3) It is worth-while to note here that fertilizability having lowered in relatively short time in such manner in De Boer's solution could be raised to the initial level by the mechanical removal of an outer part of jelly of the eggs. On this subject, however, further consideration will be given elsewhere.

tion, spermatozoa which penetrate into the jelly were, as stated already, few in number in normal fertilization. But no spermatozoa were found in the jelly of the eggs having lost their fertilizability in tap water or by prolonged treatment with De Boer's solution.

It was evident, furthermore, that the eggs treated with De Boer's solution for 15 and 30 minutes become to show higher fertilizability, as shown in Figure 2, than those before treatment, *i.e.* intact eggs, and that such treatment with De Boer's solution did not exert any harmful effects on development after fertilization. Suggestion arose therefore that short pretreatment with De Boer's solution might be effective for the increase of fertilizability of the eggs. In order to know the time of treatment necessary for such increase of fertilizability, experiments were then carried out. Typical results of the experiments performed with the eggs derived from different females are indicated in Figure 3, from which it can be seen that treatment for 1-5 minutes with De Boer's solution is most effective in these experiments.

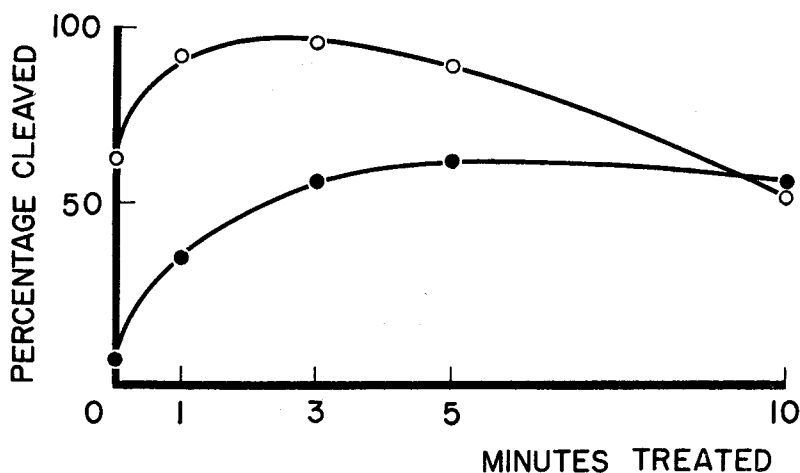


Fig. 3. Fertilizability of eggs treated for a short time with De Boer's solution.

When eggs were previously treated for 1 minute with various concentrations of De Boer's solution or with tap water, as shown in Figure 4, then inseminated by the same sperm suspension and reared in tap water, those treated with De Boer's solution or with its 1/2 solution showed highest percentage of fertilization. A concentration range correspondent to that of M/8.8-M/17.6 NaCl solutions are therefore concluded to be the most suitable concentration for the pretreatment. With the same procedure, effects of various salt solutions of the same concentration (pH 7.3), that is, isotonic with M/8.8 NaCl, were also tested, with the result that Ca and Mg affect rather to decrease fertilizability to some extent when applied

as a single solution (Fig. 5). According to the additional test, treatment with non-electrolytes such as M/4.4 sucrose or urea also proved to decrease fertilizability in a short time. Na and K involved in solution are therefore likely to be important factors for the effectiveness of the pretreatment.

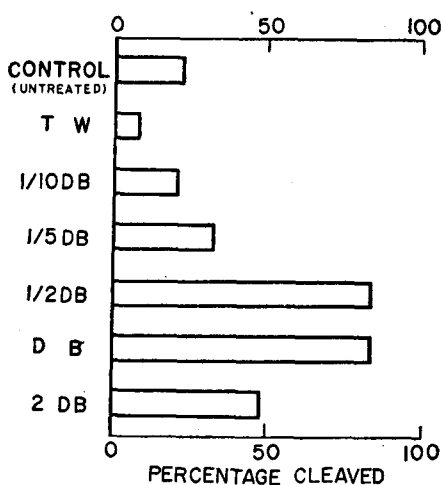


Fig. 4.

Fig. 4. Fertilizability of eggs pretreated for 1 minute with varying concentrations of media. DB, De Boer's solution; TW, tap water.

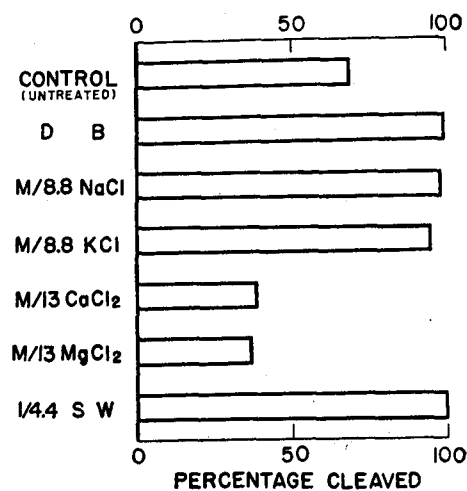


Fig. 5.

Fig. 5. Fertilizability of eggs pretreated for 1 minute with media isotonic with De Boer's solution. DB, De Boer's solution; SW, sea water.

Discussion

Loss of fertilizability of the frog egg in water has already been pointed out by several authors, as cited before, *i.e.* *Hyla* (Bataillon & Tchou '30), *Rana nigromaculata* (Motomura '52) and *Bufo bufo asiaticus* (Tchou & Wang '56). Bataillon & Tchou ('30) have described that a sort of precipitation membrane, a "membrane moyenne", is formed between the outer and the inner layer of the jelly when the egg is immersed in water, so that the penetration of spermatozoa into the egg is prevented. In *R. nigromaculata* the activation of the egg, that is, the breakdown of cortical granules has been reported to take place shortly after immersion of the egg in tap water, accompanied by the rapid loss of fertilizability (Motomura '52). The egg of European *R. temporaria* has been interpreted to be also activated parthenogenetically in tap water, because the egg rotation is observed in tap water without insemination as that in the case of fertilized egg (Picken & Rothschild '48). The fertilizability of toad eggs is also lost in water, and according to Tchou & Wang ('56), a change of the jelly occurring in water is responsible for the loss of fertilizability, but it is inhibited in some manner by 0.3% NaCl solution.

In the present material, any newly formed membrane as described in *Hyla*

egg by Bataillon & Tchou could not be found even in careful observations. On the other hand, as shown in the preceding paper (Katagiri '59), neither the breakdown of cortical granules nor any change of egg nucleus was induced merely by immersing the egg without insemination in tap water or in De Boer's solution, that is, the egg was not activated in these solutions when not inseminated. Even after the egg had been immersed in water for a considerable time and lost its fertilizability, activation could be induced by pricking.

Contrary to the case of tap water, De Boer's solution maintains the egg for a considerable length of time in fertilizable condition, though some physico-chemical change of the jelly must happen with the morphological changes as stated before. Change of the jelly occurred in water, however, differs to some extent and in this case no spermatozoa can penetrate into the jelly, consequently fertilization takes place no more. Therefore it is concluded in the present material that the rapid loss of fertilizability of the egg in tap water has no relation to the egg activation but should be ascribed to some change of the jelly occurred in water. The conclusion is thus in agreement with the view of Tchou & Wang ('56) in the case of the toad egg.

As to the change of the jelly responsible for the prevention of sperm penetration into the egg interior, it may be mainly due to the change of the outermost layer, *i.e.* the adhesive layer, because spermatozoa were never observed to penetrate into the inner part of the jelly beyond the adhesive layer, when the egg had once lost its fertilizability in water. Short pretreatment with De Boer's solution for increase of the percentage of fertilization may also be related to the layer in question. That is to say, the treatment may previously act to retard the change of this layer occurring later in water, and to this action salt concentration and the sort of salt are concerned. Anyway, it can therefore be said that the outermost layer of the jelly envelopes is of importance in the present topics, and a more detailed account of this problem will be given in future.

Summary

Unfertilized eggs of the grass frog, *Rana temporaria*, were treated with various salt solutions and their effects on the fertilizability was studied.

De Boer's solution or its double diluted solution is most suitable for the maintenance of fertilizability. In these solutions fertilizability is well retained at least for 4 hours, whereas it is lost within 15 minutes in tap water. In addition, short pretreatment of the egg with De Boer's solution is effective for increasing the fertilizability and most effective is the treatment for 1-5 minutes, in a concentration range correspondent to that of M/8.8-M/17.6 NaCl. The important factors responsible for this effectiveness are likely to be Na and K involved in the solution.

Discussions and considerations are given on the rapid loss of fertilizability in tap water, in connection with the facts mentioned above and with the morpho-

logical observations leading to the conclusion that the jelly of the egg, particularly its outermost layer, is of importance in the subject concerned.

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