Required Salt Concentration for Successful Fertilization of *Xenopus laevis*

By

Megumi Moriya[1)]

Zoological Institute, Hokkaido University

(With 2 Figures and 1 Table)

Experimental studies on fertilization of the clawed toad, *Xenopus laevis*, were not made until recently. Wolf and Hedrick (1971) first revealed the properties of the gametes of this toad, and developed a method for artificial insemination. del Pino (1973) modified their method and obtained successful results. In our studies manipulating the gametes of this toad, it was found that a higher salt concentration than previously suspected was suitable for successful fertilization. The basis for this unique salinity requirement will be described below.

Material and Methods

Sexually mature females of *Xenopus laevis* were induced to ovulate by the injection of "Gonatropin" (Teikoku Zôki) into the dorsal lymph sac. Mature eggs were obtained 10 to 14 h after the injection, and the eggs from one female were used for each series of experiments. The sperm suspension was prepared by macerating testes in De Boer's solution (DB, 0.11 M NaCl, 0.0013 M KCl, 0.00044 M $CaCl_2$, pH 7.2 with $NaHCO_3$. *cf.* Katagiri, 1961). Usually the sperm suspension from 2 pairs of testes in 1 ml DB was used as a stock suspension. The suspensions were decanted, and could be stored in an ice-chilled water bath without loss of fertilizing capacity for several hours (*cf.* Wolf and Hedrick, 1971).

Artificial insemination was conducted in Petri dishes (2.5 cm in diameter) by stripping the eggs directly into 1 ml of the insemination medium to be tested, to which one drop (approximately 0.03 ml) of concentrated sperm suspension had been added immediately before the addition of the eggs. The Petri dish was shaken several times to ensure the contact of the sperm suspension with the eggs, and 10 min later 2–3 ml of the test insemination medium was added. The percentage of fertilization was determined by scoring eggs undergoing cleavage 3–4 h after insemination (morula stage). The eggs which had been cytolyzed during manipulation were excluded from the score. The sperm concentration of the insemination

1) Present address: Department of Physiology, Sapporo Medical College, W. 17, S. 1, Sapporo.

mixture was determined by a hemocytometer, and was expressed as the number of sperm per milliliter. All the experiments were carried out at room temperatures, 22°–23°C.

Results

Table 1 summarizes the results from 8 series of experiments in which freshly stripped eggs were inseminated in various dilutions of DB. It appears that in all the series of experiments the insemination in 0.5– and 0.2 DB resulted in a higher percentage of fertilization. In 0.1– and 0.05 DB, the percentages were lower, although occasionally they were as high as in 0.5 DB. Insemination in DB always resulted in extremely low percentages of fertilization. The eggs fertilized in all these solutions underwent normal cleavage and development to the blastula stage. When the solutions were replaced by pond water or 0.05 DB at the blastula stage, normal development beyond metamorphosis ensued. Therefore, the results shown in Table 1 can be explained in terms of a requirement of penetrating spermatozoa for salinity in the surrounding medium.

Table 1. Fertilizability of *Xenopus laevis* eggs inseminated in various dilutions of De Boer's solution (DB)

Expt. No.	Percentage of cleaved eggs (Number of eggs used)				
	Insemination medium				
	DB	0.5DB	0.2DB	0.1DB	0.05DB
1	11.1 (18)	75.0 (20)	87.5 (24)	48.0 (25)	4.5 (22)
2	0 (22)	95.2 (21)	84.8 (33)	83.3 (18)	20.0 (15)
3	2.0 (99)	97.0 (101)	97.7 (88)	98.9 (96)	93.2 (89)
4	11.1 (45)	95.8 (72)	98.6 (73)	98.4 (64)	20.0 (75)
5	0 (24)	85.1 (47)	24.3 (38)	17.9 (28)	0 (21)
6	7.1 (28)	100 (43)	95.2 (42)	70.0 (30)	2.9 (34)
7	3.7 (27)	78.3 (46)	86.7 (30)	21.6 (51)	2.3 (43)
8	0 (37)	88.4 (43)	60.7 (28)	2.9 (34)	0 (39)
Average	4.4	89.4	79.4	55.1	17.9

Sperm concentration; $2–4\times10^6$ cells/ml

The next experiment was carried out to determine the effect of salt concentration on the fertilization rate as a function of sperm concentration. As shown in Fig. 1, the advantage of inseminating in 0.5 DB was again confirmed. To obtain a similarly high percentage of fertilization, a sperm concentration higher than 8×10^6 cells/ml (not shown in Fig. 1) was necessary for 0.1– and 0.05 DB, in contrast with 0.5×10^6 cells/ml for 0.5– and 0.2 DB.

The longevity of spermatozoa in various dilutions of DB was studied in the following way: the suspensions with different salinities but the same sperm

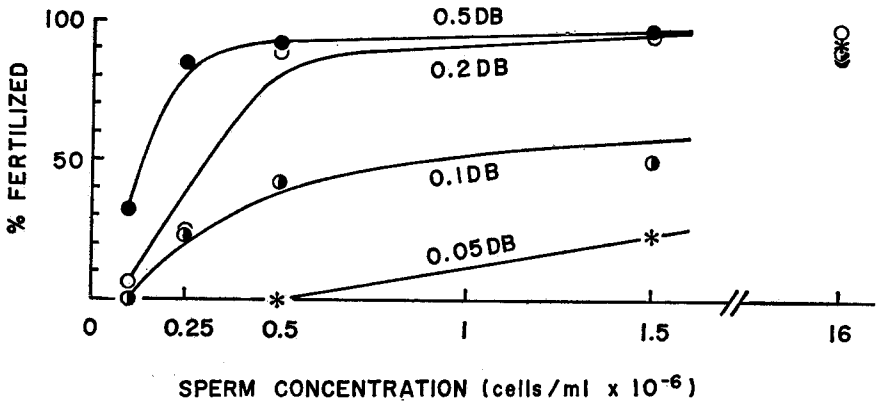

Fig. 1. Percentage of fertilization in *Xenopus laevis* as a function of sperm concentration and various dilutions of De Boer's (DB).

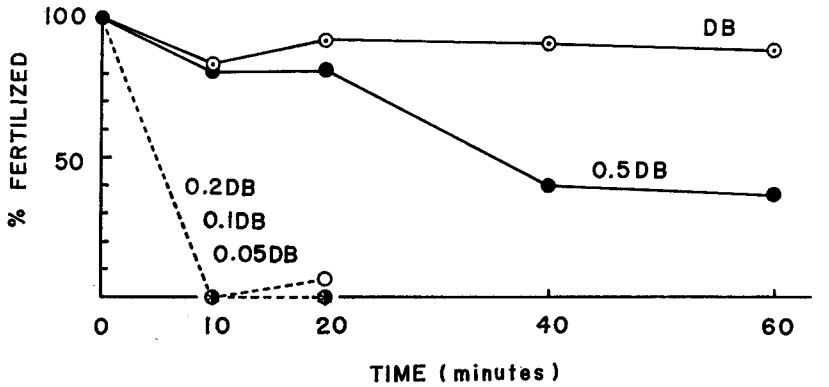

Fig. 2. Longevity of *Xenopus laevis* spermatozoa in various dilutions of De Boer's (DB). Concentration of sperm in insemination medium was 1.6×10^6 cells/ml.

concentrations were allowed to stand for various times at room temperatures (22°–23°C). At the time of insemination, the salinity of the suspensions was equalized to 0.5 DB, then freshly stripped eggs were added. The results shown in Fig. 2 reveal that spermatozoa in DB retain their fertilizing capacity for 60 min although the incidence of fertilization is very low in this medium (see Table 1). In 0.5 DB, the viability of spermatozoa may be gradually lost. It is noticeable that the salinities lower than 0.2 DB cause a rapid loss of the sperm fertilizing capacity in 10 min. The finding that DB is the most suitable medium for storing the sperm viability thus confirms the result of Wolf and Hedrick (1971).

Examination of sperm motility revealed that spermatozoa are immotile in DB, but the motility can be restored by transferring the spermatozoa to 0.5 or lower

concentrations of DB. In 0.5 DB the induced motility was gradually lost. In the much lower concentrations of DB, hardly any motile sperm were visible after 5–10 min.

Discussion

The above experiments explain why artificial fertilization in *Xenopus laevis* is most successful in 0.5 DB, or in Holtfreter's solution as reported by Jones and Jackson (1974). Fertilization may occur in 0.05 DB, but evidently it requires a much higher concentration of spermatozoa in order to obtain a high percentage of fertilization. The success of the fertilization obtained by del Pino (1973), in which the spermatozoa in DB were first put in contact with eggs for 10 min followed by the addition of 0.05 DB, may be explained on the same ground: it is plausible that the salinity of the jelly layers once being left in DB may be brought to that equal to 0.5 DB by the addition of 0.05 DB. The rapid loss of fertilizing capacity of *Xenopus laevis* spermatozoa in a hypotonic medium (Fig. 2) is in contrast with the case of other anurans in which the standard medium for artificial fertilization is osmotically equivalent to 0.05–0.1 DB (*cf.* Rugh, 1962). The sperm fertilizing capacity in *Bufo* and *Rana*s is maintained in 0.05 DB for a long period at room temperature (Katagiri, personal communication).

Environmental salt concentrations will affect not only the viability of sperm, but also the integrity of the jelly coats of eggs. Thus, as in other anurans (Katagiri, 1961, 1962; Elinson, 1971), the hydration and changes of *Xenopus* jelly layers in hypotonic media have been shown to cause a block to penetrating sperm (del Pino, 1973). This loss of egg fertilizability is regained by dipping the eggs in DB for a short period, followed by insemination in 0.05 DB (del Pino, 1973). Wolf and Hedrick (1971) showed that the egg fertilizability is retained in DB for a long period before the eggs are inseminated in 0.05 DB. In the light of the present findings, the imbibition of jelly with DB and its dilution by inseminating in 0.05 DB should have made the salinity of the jelly close to 0.5 DB, so that sperm fertilizing capacity was ensured.

The recognition of the unique salinity requirement for fertilization in *Xenopus laevis* demonstrated here is of practical value for mating these toads. Thus, for the past years a much higher rate of fertilized eggs has been routinely obtained in our laboratory by allowing the hormone injected toads to mate in 0.5 DB or Holtfreter's solution instead of tap water.

Summary

When *Xenopus laevis* eggs are inseminated in various dilutions of De Boer's solution (DB), the percentage of fertilization is extremely low in DB, highest in 0.5 DB, and decreases as the salt concentrations drop below 0.2 DB. Insemination with sperm suspensions of various concentrations revealed that 20 times as many

spermatozoa are required in 0.05 DB to obtain the same rate of fertilization as in 0.5 DB. Spermatozoa are motile with salt concentrations lower than 0.5 DB, but are immotile in DB. Their fertilizing capacity is retained well in DB, lost gradually in 0.5 DB, and lost completely in 10 min in solutions lower than 0.2 DB. It is concluded that artificial fertilization of *Xenopus laevis* is best achieved in a medium with concentration equivalent to 0.5 DB, rather than concentrations of 0.1–0.05 DB used for other anurans.

The author offers her sincere thanks to Dr. Ch. Katagiri for advice and encouragement during the course of this work and for reading through the manuscript. She is also indebted to Dr. H. Hasegawa, Hamamatsu Medical College, who suggested that we use a solution with higher salt concentration for mating *Xenopus*.

References

del Pino, E.M. 1973. Interactions between gametes and environment in the toad *Xenopus laevis* (Daudin) and their relationship to fertilization. J. Exp. Zool. **185**: 121–132.

Elinson, R.P. 1971. Fertilization of partially jellied and jellyless oocytes of the frog *Rana pipiens*. *Ibid.* **176**: 415–428.

Jones, P. and Jackson, H. 1974. Action of antifertility chemicals on *Xenopus laevis* spermatozoa *in vitro*. J. Reprod. Fertil. **38**: 347–357.

Katagiri, Ch. 1961. On the fertilizability of the frog egg, I. J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool. **14**: 607–613.

———— 1962. On the fertilizability of the frog egg, II. Change of the jelly envelopes in water. Jap. J. Zool. **13**: 365–373.

Rugh, R. 1962. Experimental embryology. Burgess Publishing Co., Mineapolis.

Wolf, D.P. and Hedrick, J.L. 1971. A molecular approach to fertilization. II. Viability and artificial fertilization of *Xenopus laevis* gametes. Develop. Biol. **25**: 348–359.