Effect of Environmental Salt Concentration on Fertilizability of Herring Gametes[1)]

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(*With* 3 *Text-figures*)

I

The experiments of fertilization in fish eggs should be carried out with a medium which allows them to retain their fertilizability for a long time, and to maintain their normal fertilization and further development. Generally the sea water or fresh water have been used in such experiments, but proved to be unsuitable for such a purpose, because in them the gametes lose their fertilizability in a rather short time (cf. Reighard '93, Rutter '02, Gray '20, Kagan '35, Ellis & Johnes '39, K. Yamamoto '51). Yamamoto (T.) (1939–1951) has used successfully isotonic Ringer's solution as the experimental medium in his extensive studies on the mechanism of fertilization in eggs of the fresh water fish, Medaka (*Oryzias latipes*). The results obtained in his experiments indicate that the eggs lose their capacity for normal fertilization within 6 minutes in fresh water, while they remain fertilizable for a considerable length of time, more than three hours, in the Ringer's solution, and further that fertilization and development proceed quite regularly in it.

The prime aim of the present study is to find out the suitable concentration of saline medium to use in subsequent studies on fertilization in the eggs of a marine fish, herring.

Before going further the author wishes to acknowledge here his indebtedness to Asst. Prof. Yasuhiko Kanoh, under whose direction the present study has been carried out. Also his thanks should be extended to the members of Hokkaido Fisheries Scientific Institution for their kind help in many ways, and to Mr. Shizuo Ito for valuable co-operation.

II

The material used was the mature eggs and spermatozoa of the Pacific

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herring (*Clupea pallasii*) captured mainly in the Rumoi-Mashike region on the west coast of Hokkaido (cf. Kanoh '49). They were taken from mature fish by vivisection and stored in glass bowls. In this condition they remained fertilizable for about twelve hours (5°–15° C).

Test solutions prepared were 1/1 (ordinary), 1/2 and 1/4 sea water and Ringer's solutions at various concentrations. 1/2 and 1/4 sea water were made by diluting ordinary sea water (pH 8.2) with non-buffered distilled water, accordingly their pH were 8.1 and 8.0 respectively. A stock Ringer's solution (1M) had the following consitution ; 1M NaCl 100 parts + 1M KCl 3.5 parts + 2/3M $CaCl_2$ 1.5 parts+2/3M $MgCl_2$ 2.4 parts, pH was adjusted to 7.6 by $NaHCO_3$. The other Ringer's solutions variously concentrated were made by diluting this stock solution with distilled water of the same pH-value. As the osmotic pressure of unfertilized egg-contents was found to be nearly osmotically equivalent to M/4.5 NaCl solution by measuring with Barger's capillary method, M/4.5 Ringer's solution was designated as the isotonic Ringer's solution.

III

In the first place, fertilization of eggs inseminated with spermatozoa of the same age in the following media was examined : 1/1 (ordinary), 1/2, and 1/4 sea water ; and M/1, M/2, M/4.5 (isotonic), M/8, M16, and M/32 Ringer's solution.

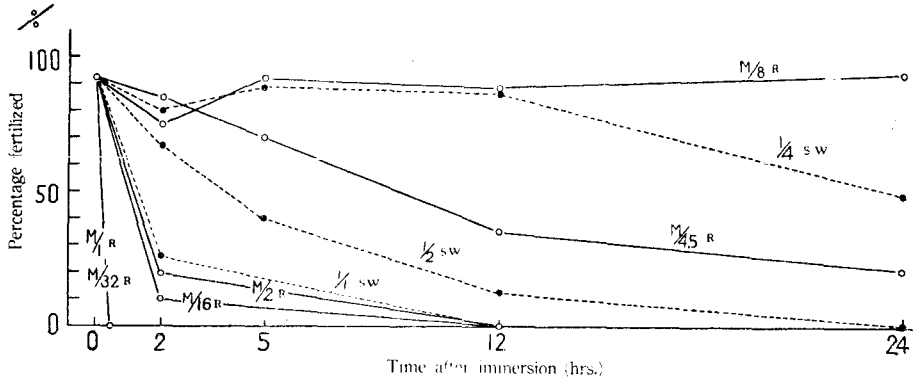

Fig. 1. Durability of capacity for fertilization of eggs inseminated with spermatozoa of the same age in various media (Temp. 8°–10°C). sw .... sea water. R....Ringer's solution.

The procedure was as below ; 50 fresh eggs being separately stuck on a samll slide glass were put into a Petri-dish containing 50 cc of each experimental medium, and after the immersion in it for a desired time they were inseminated with 2 cc of the sperm suspension (the volume ratio of sperm to the medium : 1/100) of the same age, the percentage of fertilization was determined after about twenty

minutes ; fertilized eggs were distinguished from unfertilized ones by the breakdown of cortical alveoli, the formation of perivitelline space, and the bipolar differentiation of cytoplasm (cf. Kanoh '53). In Figure 1 the average percentage of three determinations in each medium is plotted against the duration of immersion.

As is illustrated in this figure, relatively rapid loss of fertilizability takes place in ordinary sea water (and also in M/2 Ringer's solution which is osmotically nearly equivalent to the former) ; on the other hand, in 1/4 sea water and M/8 Ringer's solution the fertilizability retains for the longest time. Thus the maintenance of fertilizablity of the gametes seems to be greatly dependent on the salt concentration of medium, and as to the capacity of preserving the fertilizability one can arrange the media in the following series.

M/8 R or 1/4 sw > M/4.5 R or 1/2 sw > M/2R or 1/1 sw > M/1 R, M/16 R, M/32 R

Then, in order to find out separately the effect of each medium upon fertilizability of eggs and that of spermatozoa, following experiments were performed. Ringer's solutions of various concentrations were mainly adopted as the experimental media in these experiments, because the above experiment made it clear that the sea water and Ringer's solution have similar properties.

(a) *Experiments on the eggs* (cf. Fig. 2)

Eggs exposed for an aimed time to various media (M/1, M/2, M/4.5, M/8, M/16, and M/32 Ringer's solution) were similarly transferred to the isotonic Ringer's solution and inseminated with fresh milt. Data obtained from a typical experiment as represented in Figure 2 bear a conceivable resemblance to those shown in Figure 1, excepting that in M/16 and M/32 Ringer's solution the

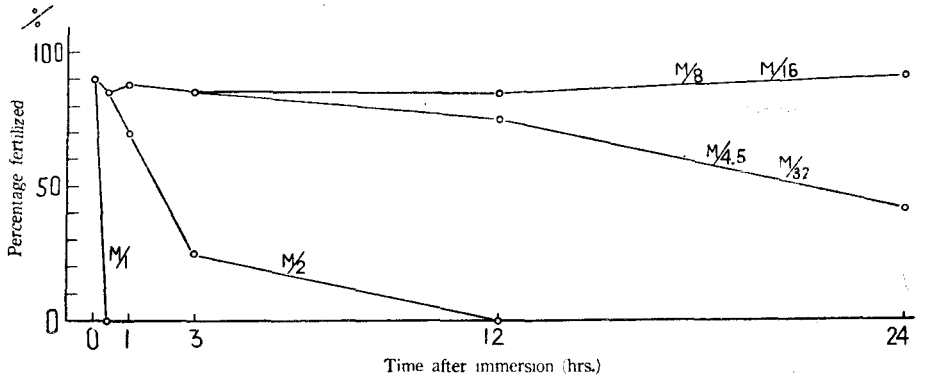

Fig. 2. Effect of Ringer's solution upon the fertilizability of eggs (Temp. 13°–15°C).

fertilizability remains also for a considerable long time as in M/4.5 and M/8 solution : Eggs remain fertilizable for longer time in M/8 or M/16 Ringer's

solution than in other media, while in M/1 or M/2 Ringer's solution the rapid loss of fertilizability takes place. The solutions can be arranged, therefore, in the following order, according to the capacity of preserving the fertilizability in this case.

M/8 R or M/16 R > M/4.5 or M/32 R > M/2 R > M/1 R

The fact that fish eggs lose, in general, rapidly their capacity for normal fertilization after discharge has been interpreted in many ways. For instance, in the case of *Oryzias* eggs, Ishida ('48) has attributed it to the structural-chemical changes brought about in the micropyle, whereas in some other cases such as in *Fundulus* and dog-salmon it has been attributed to activation of egg itself induced immediately on contact with outer medium (Kagan '35, K. Yamamoto '51). However, the rapid loss of fertilizablity of herring egg in M/2 Ringer's solution or in ordinary sea water cannot be attributed to the structural-chemical changes of micropyle or to egg activation, but probably to the damage of egg cortex or to the loss (or inactivation) of fertilization substance on egg surface, because the loss of fertilizability of eggs occurs, roughly in proportion to the decrease in power to respond to parthenogenetic agents (such as pricking or saponin) and also to the decline of sperm activating property of egg surface. An account of sperm activating property of the egg surface will be found in Yanagimachi & Kanoh's paper ('53).

(b) *Experiments on the spermatozoa* (cf. Fig. 3)

2 cc of 1/100 sperm suspension in each medium was added at various intervals to 50cc of the isotonic Ringer's solution or of ordinary sea water in which fresh egg had been immersed, then the percentage of fertilization was estimated fifteen to twenty minutes later. The experiments repeated gave the result that spermatozoa in M/2, M/4.5, and M/8 Ringer's solution remain fertilizable for a

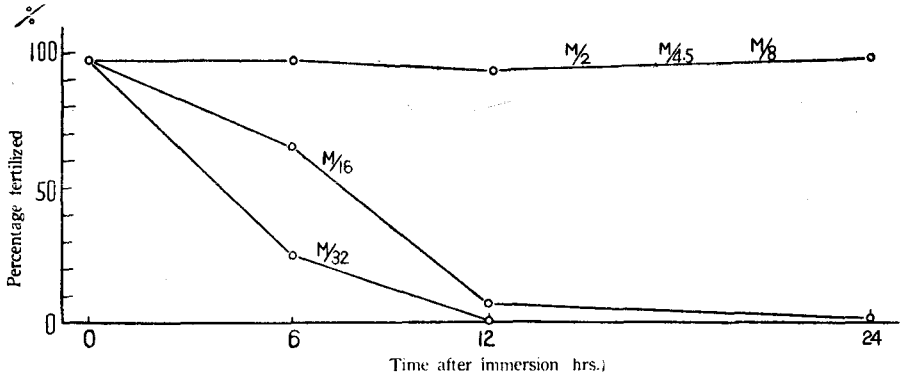

Fig. 3. Effect of Ringer's solution upon fertilizability of spermatozoa (Temp. 9°–11°C).

considerable length of time ; *e.g.*, sperm suspension of twenty-four hours old in each solution mentioned above could fertilize the majority of eggs or all of them,

while the suspension of the same age (even that of twelve hours old) in M/16 and M/32 Ringer's solution could give only low percentage of fertilization (at 10° C).

Now, two questions arise. The one is why gametes become rapidly incapable of being fertilized in M/16 and M/32 Ringer's solution ; in these media the eggs are alive at least for twenty-four hours (cf. Fig. 2) and even spermatozoa for several hours (cf. Fig. 3), nevertheless fertilization is not well assured as is shown in Figure 1. Then, in order to elucidate this phenomenon some additional experiments were made : When unfertilized eggs were thoroughly washed several times with M/16 or M/32 Ringer's solution for ten minutes, and inseminated in each solution with small amount of fresh milt, the percentages of fertilization were 33.5 and 0 respectively. Transferring the same eggs after such treatment to M/4.5 Ringer's solution, however, the majority of them were fertilized at once without further insemination. It may be said, therefore, that the fertilization is reversibly inhibited in these hypotonic media, probably owing to the lack of salts, especially that of calcium. The importance of calcium ions in the process of normal fertilization in herring egg is reported in detail by Yanagimachi & Kanoh ('53).

The other question is whether fertilized eggs are able to develop normally in the isotonic (M/4.5) and also in hypotonic media such as M/8 and M/16 Ringer's solution. In this connection, Isahaya ('32) has examined the development of herring egg in sea water of various concentrations with the interesting result that for the development of herring eggs somewhat diluted sea water (osmotically nearly equivalent to M/2.3–M/4.5 Ringer's solution) is more suitable than ordinary sea water. According to the author's experiment, development proceeds quite normally in the isotonic Ringer's solution, and even in M/8 solution nearly normal development is observed, while development in M/16 solution is not well assured, though it can proceed normally in the early stage. Anyway, it may be stated that herring eggs are able to develop in a considerable wide range of salt concentration.

IV

Summarily the conclusion was drawn from the results that, in stead of ordinary sea water, the Ringer's solution at the concentrations ranging from M/4.5 to M/8 (or 1/2–1/4 sea water) may be adopted as suitable experimental media for the study of fertilization in herring, since in these media gametes retain their fertilizability for a long time, and fertilization and development proceed regularly in them.

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