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Studies on the Fertilization of the Egg of the Flounder

1. Effects of Salt Concentration in the Fertilization[1]

By

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(*With 2 Text-figures*)

The series of studies to be published hereafter has been undertaken with the purpose to contribute something to the physiology of fertilization in marine fishes because of the poor state of knowledge in this field. In fresh water fishes, however, several excellent works have been published on related subjects, such as those by T. Yamamoto ('39-'50) concerning the cortical change during fertilization of the eggs of *Orizias* and that by Kagan ('35) on the fertilizable period of the eggs of *Fundulus*, also of brackish water.

Before proceeding further, the writer wishes to express his hearty thanks to Professor K. Aoki for his helpful suggestions and criticisms in the course of the present study, as also to Professor T. Uchida for his kind help in preparing the paper.

Material and Method

The flounder, *Limanda schrenki* Schmidt, is common around the coasts of Hokkaido. Its spawning season in the vicinity of Akkeshi lasts from late April to late May. The ripe eggs and sperm stripped out from the fish are very healthy. One can obtain some tens of thousands of ripe eggs from a single female of moderate size and several cubic centimeters of milt from a single male, so that a whole series of experiments can be performed without difficulty with the gametes from a single mother fish. The eggs are demersal and adhesive, being spherical in form. They measure 0.68 to 0.75 mm in diameter. The colour of the egg is light brown on the whole ; especially the protoplasm is tinted in more dark tone. The egg membrane is transparent and so thin that the characteristic dipolar differentiation of this egg is easily discernible externally. The constituents and the preparing procedure

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of the balanced salt solution used in this experiment have already been given in another paper (K. Yamamoto '51). The fertilization of the eggs was judged from the evidence of cleavage. Water temperature ranged from 6°C to 8°C throughout the experiments, and under this condition the eggs were observed to develop up to the 8~16 cell stage within 7~8 hours after insemination. In the experiments the eggs of this stage were generally used, but those of later stages for special purpose. Approximately 200~300 eggs were used for each experiment, but fertilization rate has been calculated from the results obtained from 100 eggs among them.

Experiments

1. *Fertilization rate in sea water and b.s.s.*: The eggs were inseminated by "dry method". As soon as the eggs were immersed into experimental solutions they were gently stirred up with a feather. Seven or eight hours later, the rate of fertilization was examined and then they were fixed with Stockard's solution for further detailed observations. The experimental solution in which the eggs were immersed was changed once at 30 minutes after insemination, and then left unchanged.

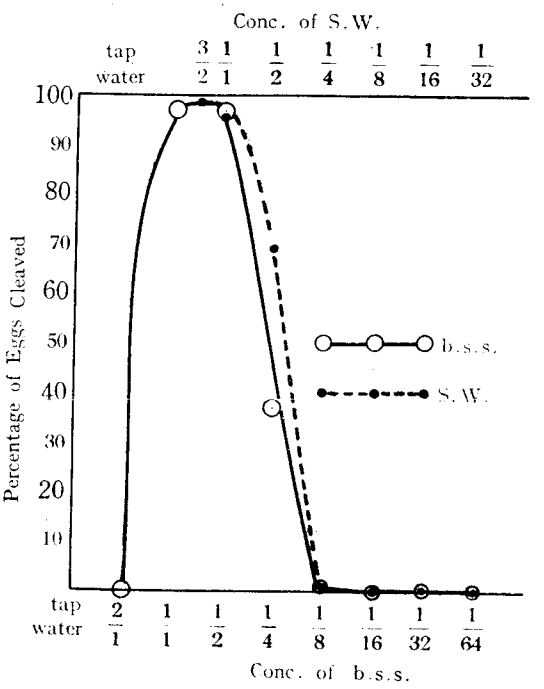

Fig. 1. Fertilization rate in sea water and b. s. s. The ordinate represents the percentage of eggs cleft; the lower abscissa, concentration of b.s.s., and the upper abscissa represnts the concentration of sea water.

The results have been summarized in Fig. 1. From the figure, it can easily be seen that two curves of the fertilization rate obtained from the eggs treated with sea water and from those with b. s. s. show an almost similar tendency. The eggs immersed in the concentrated sea water (S. 42.8) and 1/1 s.w. show a very good fertilization rate, above 95 per cent on the averages. In 1/2 s.w., eggs are still fertilizable, but the fertilization rate is lower than in the former, showing 69 per cent on the average. In concentrated solutions lower than 1/4 s. w.,

no fertilization of eggs occurred with the exception of eggs treated with 1/4 s.w. in which a few eggs were found cleft. The results obtained from the eggs in b.s.s. show a quite similar tendency. There has been observed no egg cleft in the diluted solutions of more than 1/8 b.s.s. and also in 2/1 b.s.s., while those immersed in 1/2 and 1/1 b.s.s. show the good fertilization rate up to 97 per cent. In 1/4 b.s.s., though the eggs are still fertilizable, the average value of the fertilization rate reaches over 38 per cent. Both 1/2 b.s.s. and 1/1 s.w. have almost the same effects on the fertilization in spite of the difference of their constituents and pH. Judging from the fact that 1/2 b.s.s. is isosmotic with 1/1 s.w. in the main, it can be surmised that the rate of fertilization in general is influenced by salt concentration of solution only, though some differences exist between the results obtained from the eggs in 1/4 b.s.s. and those from 1/2 s.w. With regard to the fertilization rate, there is recognizable no difference between the effects of 1/2 and 1/1 b.s.s., but among the eggs treated with 1/1 b.s.s. then were found some abnormally cleft ones. From these observations, it may be concluded that for the fertilization the optimum salt concentration of the medium ranges from 1/1 to 1/4 b.s.s.

2. *Activity of the sperm* : Activity of the spermatozoa was examined by the following procedure ; a touch of dry sperm was taken on a cleaned slide glass with a thin glass rod and diluted with a drop of experimental solution. Then, as rapidly as possible, observations were performed under the microscope, within the period from 15 to 20 seconds after treatment. All spermatozoa moved actively in 1/2 and 1/4 b.s.s., but ceased movement within about a minute. In the case of dilution by 1/1 b.s.s., some of the spermatozoa moved but were seemingly less vigorous than in the former case. In sperm suspension diluted by concentrated solutions lower than 1/8 b.s.s. and by 2/1 b.s.s., spermatozoa showed no movement. The activity of the sperm suspended in s.w. was exactly the same as in the sperm suspended in b.s.s., being mainly subjected to salt concentration of medium. The sperm moves actively in 1/1 and 1/2 s.w., but never in more diluted sea water than 1/4 s.w.

3. *Fertilizable period in b.s.s.* : After the eggs had been placed in the following solutions, 1/2, 1/4, ... , 1/64 b.s.s., each with different durations as shown in Table 1, they were inseminated according to the "dry method". Then they were transferred to the normal sea water, and 7~8 hours later the fertilization rate was examined. The results are also summarized in Table 1. It is very interesting that the eggs which remained exposed to more diluted solutions than 1/8 b.s.s., were found still to retain fertilizability for a fairly long time, whereas they could not be fertilized at all in these solutions themselves. The eggs placed in 1/2 b.s.s., i. e. in the most suitable environment for fertilization, even lose the fertilizable capacity very soon ; the eggs submerged for two hours in the medium lost fertilizability entirely. The fertilizability of the egg was observed to have continued

for the longest time in 1/8 b.s.s., showing the fertilization rate of 78 per cent after 5 hours' treatment. In the diluted solutions more than 1/16 b.s.s., the lower the salt concentration of the media, the shorter was the duration of fertilizability of the egg. But even in 1/64 b.s.s. the lowest salt concentration among the experi-

Table 1. Fertilizable period in b. s. s.

Immersing solution \ Immersing duration	Per cent of fertilized eggs						
	5 min.	10 min.	15 min.	30 nim.	60 min.	120 min.	300 min.
1/2 b. s. s.	95	97	98	81	56	4	0
1/4 b. s. s.	96	98	97	98	92	91	78
1/8 b. s. s.	98	96	96	94	91	92	82
1/16 b. s. s.	91	92		78	71	76	56
1/32 b. s. s.	76	78		74	64	68	46
1/64 b. s. s.	84	72		68	56	48	26

mental solutions, half the number of eggs were fertilized after treatment with that solution for two hours. In general, it may be said the eggs of fish lose fertilizability within a short time after they were put into normal sea water. For instance, the fertilizable period of the egg of *Fundulus* in sea water is recorded as only 15 to 30 minutes (Kagan, '35). The same phenomenon was also found in many other fishes, especially in eggs of fresh water fishes. Judging from the above observations the fertilizable capacity of the eggs of the present fish seems to be retained comparatively longer in sea water.

4. *Morphological changes of the egg immersed in b.s.s.*: Before entering a consideration of the morphological change, it is necessary to give a brief description of an unfertilized egg taken fresh from the oviduct. The form, size and colour of an egg have already been described in this paper. In the living state a membrane is found at the outermost part of the egg consisting of two layers, namely the outer adhesive layer and the inner membrane, the chorion. Beneath the membrane the egg proper lies very colsely, leaving no perivitelline space between them. A micropyle is found at the animal pole. From its characteristic coloration, one can readily detect the protoplasmic layer enclosing the yolk. The protoplasm is fairly thick at the animal pole and becomes thin toward the opposite pole. Thus, the egg clearly shows a remarkable dipolar differentiation. The outer layer of the protoplasm contains mumerous oil drops. The cortical alveoli embedded in the cortical layer are also revealed on closer observation (Fig. 2, A and B). In ferilization the change is initiated by the breakdown of the cortical alveoli and subsequently followed by the elevation of the chorion. Following these changes the protoplasm begins to accumulate at the animal pole gradually, and finally the blastodisc is formed. By immersing the unfertilized egg in b.s.s., a swelling of the adhesive

layer is caused. This swelling is recognized in both fertilized and unfertilized eggs, but is not observable in eggs immersed in concentrated solutions lower than 1/8 b.s.s. The change occurs immediately after the egg came in contact with sea water. In 1/2 b.s.s., the layer swelled up to about 60μ. In addition to these changes, at about 1~2 hours later, more remarkable changes are observable in the eggs exposed to b.s.s. These changes are the breakdown of the cortical alveoli, elevation

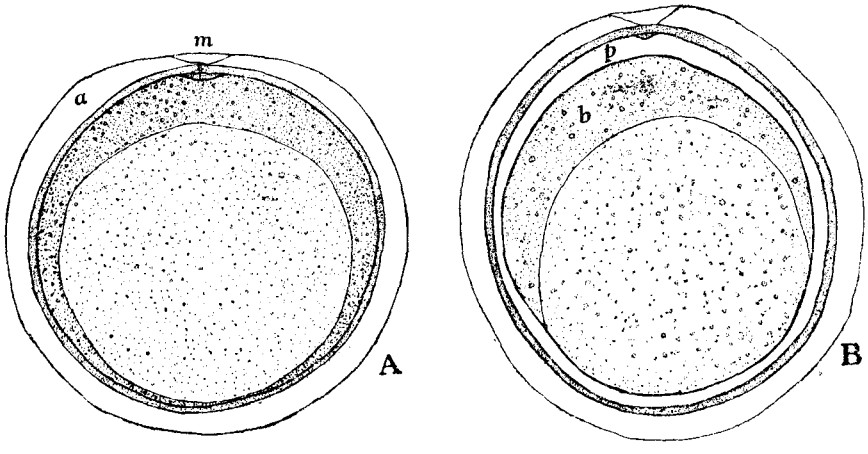

Fig. 2. **A.** An unfertilized egg soon after contact with sea water. **B.** An unfertilized egg treated in sea water for 3 hours. *a*. adhesive layer. *b*. blastodisc. *m*. micropyle. *p*. perivitelline space.

of membrane and formation of blastodisc. The larger the concentration of the medium in which the eggs are immersed, the sooner the change occurs as shown in Table 2. The eggs which have completed these changes are no longer fertilizable. The same phenomenon has already been reported by Kagan ('35) on the eggs of *Fundulus* and by Yamamoto ('49) on the eggs of *Oncorhynchus*. When the unfertilized eggs of *Oncorhynchus* were put into fresh water, the breakdown of the

Table 2. Per cent of unfertilized eggs activated by b. s. s.

Immersing solution / Immersing duration	Per cent of the eggs formed blastodisc				
	1/1 b. s. s.	1/2 b. s. s.	1/4 b. s. s.	1/16 b. s. s.	1/32 b. s. s.
Hours 5	98	98	5	0	0

cortical alveoli, the liberation of the chorion from the egg surface and the extrusion of the second polocyte occur almost synchronously, and the eggs rapidly lose their fertilizability. There is a difference between *Limanda* and *Oncorhynchus* egg as to the duration necessary for the induction of these changes. In the former the changes occur soon after being placed in contact with water, while in the latter there is needed a longer time before these changes.

Discussion

For the fertilization of the egg the optimum salts concentration of the environment is limited to a comparatively narrow range. The range is from 1/1 to 1/4 b.s.s. In the concentrated solutions higher than 2/1 b.s.s. or in the diluted solutions more than 1/8 b.s.s., the eggs could not be fertilized. The fact that the fertilization does not occur in the diluted solutions is possibly due to the inactivity of the spermatozoa, because the fertilizability of the eggs themselves is retained for a long time. It has been already pointed out by Loeb ('14) for the sea urchin that the activity of the spermatozoa is one of the factors necessary for the entrance of the spermatozoa into the eggs. In fish, this may also be the case. The failure of fertilization in the concentrated solutions higher than 2/1 b.s.s. does not depend only on the inactivity of the spermatozoa, but also on the harmful effect suffered by the eggs from the solutions. In the concentrated solution, eggs seem to die sooner or later. Though the spermatozoa could move fairly actively and the fertilizability of the eggs was retained for a long time in 1/4 b.s.s., the fertilization rate was very low. It was 38 per cent on the average. This observation seems to suggest that some factors necessary for fertilization itself were defective. The same phenomenon has already been seen in te fertilization of the dog-salmon. Though in the dog-salmon the spermatozoa moved actively and the eggs retained fertilizable capacity for a long time in the isotonic salt solution, no eggs were fertilizable in the medium itself. In experiments on the dog-salmon, the failure of fertiliazation at that time was not attributed to the lack of activity of sperm to enter the egg, but to the impossibility of the "fertilization-wave" conduction (K. Yamamoto '51). In the flounder, the same explanation may also be applicable, because unfertilized eggs are activated quickly in the condition suitable for fertilization, whereas the condition suitable for keeping the eggs fretilizable is unsuitable for fertilization, as in the case of the eggs of the dog-salmon. To clarify this problem, further experiments are highly desirable.

Summary

1. The optimum salt concentration of environment for the fertilization of the egg of the flounder is limited between 1/1 and 1/4 b.s.s.

2. The spermatozoa can move actively only in the suspension prepared by

adding 1/2 or 1/4 b.s.s., and some can move fairly in 1/1 b.s.s. suspension.

3. Fertilizability of the eggs is retained for the longest time in 1/8 b.s.s. In normal sea water or in 1/2 b.s.s., the eggs lose the fertilizable capacity within two hours.

4. Sea water activates the unfertilized egg and causes subsequently the breakdown of the cortical alveoli, elevation of membrane and formation of blastodisc. In normal sea water the changes occur at one or two hours after the eggs have been placed contact with the water. The eggs which have completed the changes can not be fertilized.

5. Though the spermatozoa can move fairly actively and the fertilizability of the egg is retained for a long time in 1/4 b.s.s., the fertilization rate is reduced to 38 per cent.

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