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# Fertilization of Dog Salmon Eggs in Ringer's Solution

By

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(With 2 Plates)

In the physiological study of fertilization in fish eggs, T. Yamamoto (1961) found that Ringer's solution is not only excellent for the preservation of ripe unfertilized eggs of medaka, but can be used as an insemination solution. According to him, the medaka eggs are activated by the penetration of spermatozoa and undergo embryonic development in Ringer's solution. In the dog salmon, the ripe eggs retain their fertilizability for several hours in Ringer's solution as in the case of medaka but are not activated by insemination in this solution (K. Yamamoto, 1951). It is possible to assume that such a failure of activation is due to the absence of real penetration of the dog salmon egg by a spermatozoon. To examine this possibility, Kusa (1950) carried out an interesting experiment and concluded that the effective spermatozoon had actually penetrated into the egg in Ringer's solution. However he did not observe the behavior of spermatozoa which had penetrated such eggs. Therefore the fate of spermatozoa after penetrating dog salmon eggs in Ringer's solution was traced cytologically in the present study.

## Materials and Methods

The materials used in the present study were derived from matured individuals of the dog salmon, *Oncorhynchus keta*. Ripe eggs taken from the abdominal cavity of the females were repeatedly washed with Ringer's solution and were artificially inseminated in the same solution. Two minutes later, the eggs were thoroughly washed with a large amount of Ringer's solution to reduce the number of excess spermatozoa adhering to the egg surface. The inseminated eggs were maintained in newly prepared Ringer's solution at 10°C. The Ringer's solution used had the following constitution: M/7 NaCl 100 parts + M/7 KCl 2.8 parts + M/10 CaCl<sub>2</sub> 3.4 parts (pH 7.2).

When the eggs were immersed in Bouin's fluid for the fixation, the membrane enclosing the egg became temporarily transparent but soon turned opaque. There-

fore the perivitelline space was discernible, if present, by observing the eggs horizontally with the naked eye immediately after immersion in Bouin's fluid. For the cytological observation, 10  $\mu$  thick paraffin sections prepared from these fixed eggs were stained with Delafield's hematoxylin. Where necessary, Feulgen's nuclear reaction was also employed.

### Observation

In the eggs inseminated in Ringer's solution, the inner part of the micropylar canal was compactly filled with more than twenty spermatozoa as shown in Figure 1. Since the diameter of this part of the canal nearly corresponded to the size of the sperm head, the spermatozoa packed in this part were arranged in a row. The effective spermatozoon was the first one of the row and occupied a position at the inner opening of the micropylar canal. At this position it was not certain whether the spermatozoon had penetrated into the ooplasm or was merely in contact with the ooplasmic surface. The same picture of the micropylar region was also observed in the eggs fixed at 2 hours after insemination. The situation was however somewhat altered in the eggs 3 hours after insemination. To describe it in detail, the head of the effective spermatozoon was found in the ooplasm about 10  $\mu$  from the ooplasmic surface. The inner part of the micropylar canal was in these eggs no longer compactly filled with spermatozoa. The inner opening of the canal obviously no longer contained the effective spermatozoon. These observations undoubtedly indicate that the actual entrance of the effective spermatozoon into the ooplasm occurs at about 3 hours after insemination.

As shown in Figure 2, the sperm head swelled slightly during the next hour in Ringer's solution and developed a small aster near its base. In the eggs at 5 hours after insemination, a peculiar bipolar structure was detected in the peripheral ooplasm (Fig. 3). It resembled the meiotic spindle and contained Feulgen-positive particles looking like chromosomes. However the arrangement of the particles in the structure was very irregular. Since all of these eggs possessed the distinct metaphase spindle of the second meiotic division in the peripheral ooplasm of the animal pole, the above mentioned bipolar structure was considered to be derived from the penetrated spermatozoon. The fibers constituting the meiotic spindle were rather thick and distinct (Fig. 4). In the bipolar structure derived from the spermatozoon, however, the fibers were thin and indistinct. Owing to the differences found in the properties of the fibers and also in the arrangement of Feulgen-positive particles (or chromosomes), the bipolar structure derived from the penetrated spermatozoon was easily distinguished from the meiotic spindle. In the eggs at 5-6 hours after insemination, the Feulgen-positive particles were all found at the poles of the structure. The distribution of the particles was however unequal between the two poles, so that sometimes no particles were detected at one pole of the structure. At the pole, these particles formed a mass but were never surrounded by a membrane. With the migration of the particles

toward the poles, the bipolar structure itself moved toward the inner part of the ooplasm and was usually found 30–40  $\mu$  from the ooplasmic surface. As time elapsed, one or two poles were newly formed on the lateral sides of the bipolar structure. Accordingly the structure took the form of a tri- or tetra-polar spindle (Fig. 5). The unequal distribution of Feulgen-positive particles in each pole was also detected. Thereafter the poles of structure derived from the penetrated spermatozoon seemed to repel each other leading to the decomposition of tri- or tetra-polar spindle. With this change, the distance of the poles from the ooplasmic surface also increased. Therefore, in the eggs fixed at 48 hours after insemination, 3–4 asters undoubtedly originated from the above-mentioned structure were found to be scattered throughout the ooplasm of a single egg (Fig. 6). This fact indicates that the formation of a new pole did not occur after decomposition of the tri- or tetra-polar spindle.

The alveoli embedded in the cortical ooplasmic layer of the intact egg were found to be unchanged in these eggs inseminated and kept in Ringer's solution (Fig. 6). As for the egg nucleus, the normal metaphase spindle of the second meiotic division occurred at the animal pole of the eggs kept up to 24 hours. However the spindle fibers became indistinct during the next 12 hours in Ringer's solution, though the dyad chromosomes did not move toward the pole and were detectable on the equatorial plate of the spindle (Fig. 7). This observation suggests that the process of degeneration was initiated in the meiotic spindle fibers during the immersion in Ringer's solution. It is clear therefore that activation never occurs in the inseminated eggs immersed in Ringer's solution unless they are treated with the hypotonic solution.

In order to study the ability to develop the embryo of inseminated eggs kept in Ringer's solution, the eggs were transferred into tap water and fixed at 1 and 17 hours after transference. As is known, the eggs were activated by the immersion in tap water; the breakdown of the cortical alveoli, the formation of a perivitelline space and the extrusion of the second polar body occurred. The process of activation proceeded normally in the inseminated eggs previously kept in Ringer's solution for 12 hours. However the breakdown of cortical alveoli was incomplete in the eggs kept for 24 hours in Ringer's solution (Fig. 8). The margin of blastodisc distinctly formed in these eggs contained a small number of unbroken alveoli. In accord with this fact, the perivitelline space was narrower than that which appeared in the intact eggs. The number of cortical alveoli remaining unbroken in the blastodisc increased as the duration of previous immersion in Ringer's solution was prolonged (Figs. 8–10). A great number of cortical alveoli were found in the eggs previously kept in Ringer's solution for 48 hours and transferred into tap water (Figs. 10 and 11). Even in this case, however, a few of the cortical alveoli contained in the unactivated eggs were assumed to be broken down, because a very narrow perivitelline space was also detected in these eggs. As for the extrusion of the second polar body in tap water, it occurred normally in the eggs previously kept in Ringer's solution for 24 hours. If the

immersion in Ringer's solution was prolonged for more than 36 hours, however, the eggs failed to extrude the polar body in tap water. In these eggs, the degenerating metaphase spindle was detected in the peripheral ooplasm of the animal pole.

The ooplasmic division of the eggs in tap water was normal when the duration of previous immersion in Ringer's solution was not more than 24 hours (Fig. 8). However the cleavage was incomplete in the eggs treated for 36 hours and no furrows appeared in the eggs kept in Ringer's solution for 48 hours (Figs. 9 and 10). In this connection it is worth noting that most of the eggs undergoing nuclear division without formation of cleavage furrows (48-hr. eggs) became turbid but none of the cleaving eggs (kept not more than 36 hr.) died within 17 hours in tap water. The blastomere nuclei appeared to be normal in size and consisted of fine granular chromatin enclosed by the nuclear membrane when the duration of immersion in Ringer's solution was not more than 24 hours (Fig. 12). In these eggs, the process of mitotic division appeared to proceed normally (Fig. 13). However in the eggs kept in Ringer's solution for 36 hours and which failed to extrude the second polar body in tap water, the nuclei were not constant in their size and were very indistinct (Fig. 14). They were barely distinguishable from the surrounding ooplasm by Feulgen's reaction and seemed to lack a nuclear membrane.

### Discussion

It is shown in the present study that the effective spermatozoon occupies a position at the inner opening of the micropylar canal until 2-3 hours after insemination in Ringer's solution. At this position, it was not clear whether the effective spermatozoon had penetrated into the ooplasm or was merely in contact with the ooplasmic surface. The presence of this spermatozoon may prevent the penetration of supernumerary spermatozoa into the egg through the micropylar canal. A similar observation has been reported by Ginsburg (1963) who worked with lake trout and salmon eggs. When the insemination of these eggs is performed in Ringer's solution, the effective spermatozoon is at the inner opening of the micropylar canal. She has described that the anterior part of the head of the effective spermatozoon is embedded in the ooplasm whereas the posterior part closes the inner opening of the micropylar canal thus blocking the way to other spermatozoa. The experimental results obtained by Kusa (1950) are somewhat different from these observations. He inseminated dog salmon eggs in Ringer's solution, then immersed them in isotonic KCl solution. By this procedure, Kusa expected that the inseminated eggs would not cleave after transfer into tap water if the effective spermatozoon had not penetrated into the ooplasm in Ringer's solution and had been killed by KCl. When these eggs were transferred into tap water, however, they developed normally. From these results Kusa concluded that the effective spermatozoon could penetrate into the ooplasm

in Ringer's solution. Later he found cytological evidence in the rainbow trout that the effective spermatozoon was embedded at a position  $5 \mu$  from the ooplasmic surface of the eggs immediately after insemination in Ringer's solution (Kusa, 1964). This fact indicates that, in spite of the inner opening of the micropylar canal being unblocked, the penetration of supernumerary spermatozoa into the egg does not occur, and seems to contradict the findings that the unfertilized fish eggs whose surrounding membrane had previously been removed were enabled to receive many spermatozoa (Kanoh and Yamamoto, 1957; Yanagimachi, 1957; T.S. Yamamoto, 1958; Sakai, 1961). In an analytical study of monospermic fertilization in fish eggs, Kanoh (1957) has concluded that supernumerary spermatozoa are restricted in their penetration into the egg primarily by the morphological status and secondarily by the physico-chemical properties of the micropyle. Furthermore he has emphasized the importance of the protective change against the penetration of supernumerary spermatozoa occurring physiologically in the egg cortex itself after penetration by the effective spermatozoon. Considering the experimental results obtained by earlier workers, the mechanism of monospermic fertilization in the dog salmon egg observed in the present study may tentatively be interpreted as follows: up to 2-3 hours after insemination, the effective spermatozoon closes the inner opening of the micropylar canal which prevents mechanically the penetration of supernumerary spermatozoa into the egg. After the actual entrance of the effective spermatozoon, the ooplasmic surface undergoes some physiological changes in the restricted area beneath the micropyle or in the entire egg surface and becomes impermeable to other spermatozoa.

According to T. Yamamoto (1961), the penetration of the effective spermatozoon stimulates the medaka egg and causes an excitation in the ooplasm. The breakdown of the cortical alveoli is a result of the conduction of this excitation in the cortical ooplasmic layer. In the present study, the breakdown of cortical alveoli was not initiated in the eggs undoubtedly penetrated by spermatozoa in Ringer's solution. There are two possibilities as to the cause of failure of alveolar breakdown in Ringer's solution: (1) The excitation is caused by the penetration of the effective spermatozoon but the alveolar breakdown itself is inhibited in Ringer's solution. (2) The stimulation inflicted by the penetration of the spermatozoon is not enough to cause excitation in the dog salmon egg. In the fertilized eggs, the metaphase spindle of the second meiotic division persisted in the animal pole without any change during immersion in Ringer's solution and finally degenerated. On the other hand, the head of the penetrated spermatozoon showed an indication of nuclear division in the ooplasm. In this connection, it should be remembered that the female nucleus of partially fertilized sea urchin eggs is unable to migrate toward the center of the egg if located in unfertilized ooplasm, though it swells spherically (Allen, 1954). This observation suggests that the excitation of the cortical ooplasmic layer causes not only the breakdown of cortical granules but also the migration of the egg nucleus. If this holds true for fish eggs, the excita-

tion is supposed not to be caused by the penetration of the spermatozoon in the dog salmon egg. In a study of the activation of dog salmon eggs with various parthenogenetic agents, the author reported that the eggs do not react to a local stimulation such as pricking with a steel needle but the development is induced by the contact of the entire egg surface with agents (T.S. Yamamoto, 1966). These data also suggest that the stimulation inflicted by the penetration of the spermatozoon is not enough to cause excitation in the dog salmon egg.

The degeneration of the metaphase spindle of the second meiotic division commenced in the eggs 36 hours after insemination in Ringer's solution. When these eggs were transferred into tap water, they underwent incomplete cleavage. Since the maternal chromosomes did not participate in the cleavage process, the blastomere nuclei all originated from the head of the penetrated spermatozoon. Therefore it is possible to regard the development of these eggs as androgenetic, though the materials constituting the blastomere nuclei were variable in amount.

### Summary

The eggs of the dog salmon, *Oncorhynchus keta*, were inseminated in Ringer's solution and kept in this solution for 2 days. The effective spermatozoon occupied a position at the inner opening of the micropylar canal for 2-3 hours and seemed mechanically to prevent the penetration of supernumerary spermatozoa into the egg. Thereafter the head of the spermatozoon entered into the ooplasm, where it showed an indication of nuclear division. Probably owing to the physiological change of the ooplasmic surface, these eggs did not accept supernumerary spermatozoa. Although the effective spermatozoon had penetrated into the egg, no indication of egg activation was observed. These eggs were activated in tap water. The breakdown of cortical alveoli was incomplete in the eggs previously immersed in Ringer's solution for more than 24 hours. The extrusion of the second polar body occurred if the meiotic spindle had not show any sign of degeneration in Ringer's solution. The eggs which failed to extrude the polar body by the degeneration of spindle underwent cleavage in tap water without the participation of the egg nucleus. These observations suggest that the stimulation inflicted by the penetration of spermatozoon in Ringer's solution is not enough to cause excitation in the dog salmon egg.

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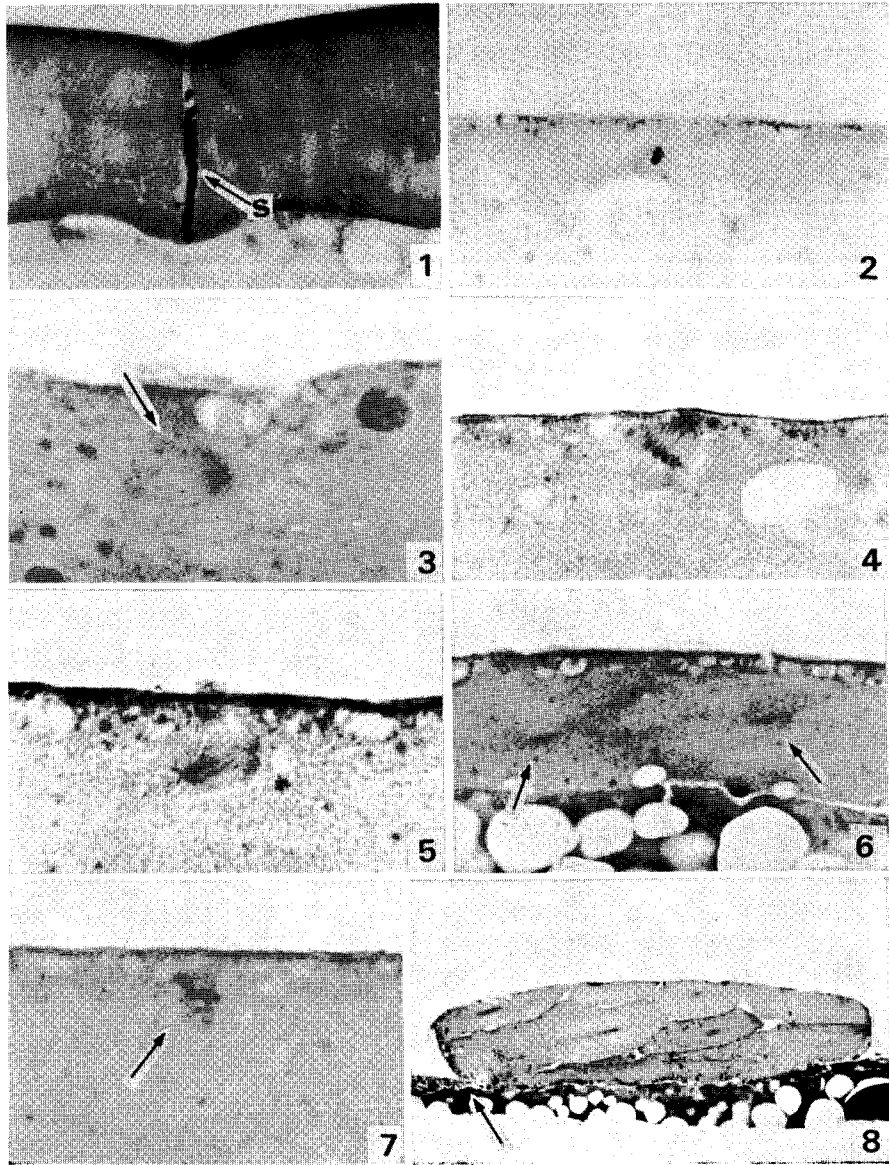
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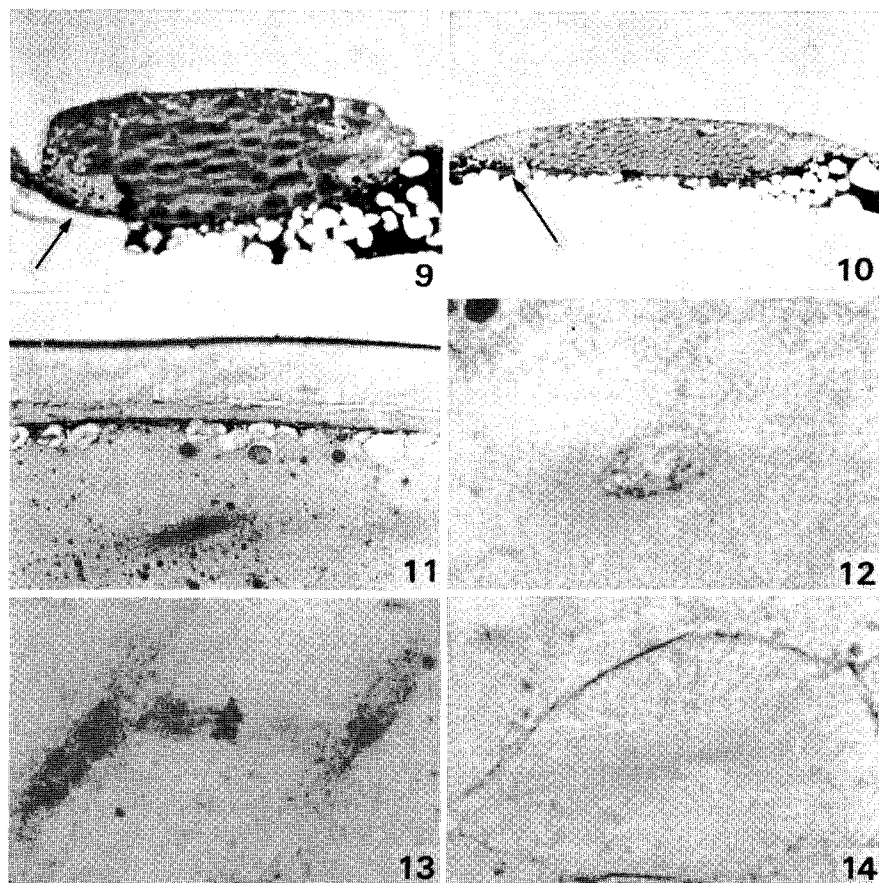
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## Explanation of Plates V-VI

- Fig. 1. Section through the micropyle of an egg inseminated in Ringer's solution and kept for 2 hours. The micropylar canal is filled with many spermatozoa (s).  $\times 650$ .
- Fig. 2. The spermatozoon penetrated into the ooplasm. The egg was kept in Ringer's solution for 4 hours. Note a small aster developed near the base of the sperm head.  $\times 560$ .
- Fig. 3. The bipolar structure (arrow) formed in an egg kept in Ringer's solution for 5 hours. The particles looking like chromosomes are irregularly arranged in the structure.  $\times 560$ .
- Fig. 4. The metaphase spindle of the second meiotic division found in the same egg as shown in Fig. 3. The spindle fibers are thick and distinct.  $\times 560$ .
- Fig. 5. The tripolar structure formed in an egg kept in Ringer's solution for 6 hours. The particles looking like chromosomes are found at the poles.  $\times 560$ .
- Fig. 6. The animal pole of an egg kept in Ringer's solution for 48 hours. Two asters (arrows) originating from the penetrated spermatozoon can be seen.  $\times 140$ .
- Fig. 7. The metaphase spindle of the second meiotic division found in an egg kept in Ringer's solution for 48 hours (arrow). The spindle is indistinct, suggesting the commencement of its degeneration but the chromosomes are still observable on the equatorial plate of the spindle.  $\times 560$ .
- Figs. 8-10. Sections through the blastodisc of eggs kept in Ringer's solution for various lengths of time, then immersed in tap water for 17 hours. The cortical alveoli remaining unbroken are found to be accumulated in the margin of the blastodisc (arrows).
- Fig. 8. An egg kept in Ringer's solution for 24 hours. The cleavage appears to be normal.  $\times 50$ . Fig. 9. An egg kept for 36 hours. The cleavage is incomplete but the region of the blastodisc near the surface is apparently divided by the furrows.  $\times 50$ .
- Fig. 10. An egg kept for 48 hours. No cleavage furrows are found in this egg. A number of small asters are scattered throughout the blastodisc.  $\times 11$ .
- Fig. 11. The cortical region of an egg kept in Ringer's solution for 48 hours, then immersed in tap water for 1 hour indicating that the breakdown of the cortical alveoli has not commenced.  $\times 210$ .
- Figs. 12 and 13. High magnifications of the egg presented in Fig. 8 showing the interphase and metaphase nuclei of the blastomeres.  $\times 560$ .
- Fig. 14. The blastomere of the egg shown in Fig. 9. The nucleus is very indistinct and is smaller than that in the normally dividing egg. Compare with Fig. 12.  $\times 560$ .
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*T.S. Yamamoto: Fertilization of Dog Salmon Eggs*



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