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# Activation of the Egg of the Dog-Salmon by Water and the Associated Phenomena[1)]

By

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*(With 5 Text-figures and 1 Plate)*

## 1. Introduction

In 1939 Just pointed out as follows, "the fertilizable condition in eggs of sea-urchin may endure for hours or even for three days, depending upon the species and for any given species upon the temperature ; eggs of other animals, fertilized outside of the female body, as those of ascidians and of vertebrates may retain their fertilizable capacity for hours (p. 189)". On the other hand, fish eggs have attracted our attention as the most interesting case illustrative of a brief duration of fertilizability. The unfertlized eggs of fish lose the fertilizability within a short time when they have been put into water. Early in 1873, His reported that the fertilizable period of *Salmo* eggs in fresh water was from 15 to 30 minutes, and afterwards Reinghard ('93) confirmed that 60% of the eggs of the wall-eyed pike, when kept for ten minutes in water, were found to lose the fetilizable capacity. Besides them, the previous studies working with several fishes of other kinds agreed that in the eggs of fihes the fertilizable condition is, at its height, immediately after shedding, as shown by Kagan ('35) in eggs of *Fundulus*, Yamamoto ('44) in eggs of *Oryzias*, Handa ('09) and Yamamoto ('47) in eggs of *Oncorhynchus* and *etc.* The reason why the fish egg loses the fertilizability within a short time in water has been explained by many investigators as follows ; the principal cause of this phenomenon is due to the closure of the micropyle, resulted from swelling of the chorion in water (His, '73, Handa, '09, Runnström, '20, Ishida, '48). Kagan ('35) reported that when activated by sea water the unfertilized eggs of *Fundulus* develop up to the blastodisc stage, covering the disappearance of the platelets and the formation of the perivitelline space, and further that the fertilizable capacity of the eggs decreases conversely with the increase of eggs in which the perivitelline space begins to appear. Recently, the present writer ('48) observed the quite similar phenomena

1) Contributions to the Akkeshi Marine Biological Station, No. 55.

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for the dog-salmon, *Oncorhynchus keta*, together with the extrusion of the second polocyte. It thanks to the following reports that the unfertilized eggs of fishes are activated in water, such as K. Yamamoto ('49) on the salmons (*O. nerka* and *O. masou*), the trout (*S. irideus*) and roach, T. Yamamoto ('49) on carp, smelt and gold fish, and Kawamura ('47) on loach.

On the other hand, Runnstrôm ('20) reported that unfertilized eggs of *S. salvelinus* retained the fertilizability for three days in the isotonic Ringer's solution. T. Yamamoto ('44) obtained also the same results in *Oryzias* eggs. The unfertilized eggs of the dog-salmon can retain also the fertilizable capacity for a long time in the isotonic salt solution, but when the eggs were inseminated in the isotonic salt solution, no egg could cleave, although the spermatozoa remain active in the medium (K. Yamamoto, '48). In 1949, K. Yamamoto attributed the failure of fertilization in isotonic salt solutions to blocking of "fertilization-wave" conduction but not to inactivity of sperm. In sea-urchins the suitable condition retaining the fertilizability of unfertilized eggs is also suitable for the fertilization. On the contrary, in the dog-salmon the most suitable condition retaining the fertilizable capacity of unfertilized egg is unsuitable for the fertilization ; namely in that medium eggs are not fertilizable. Therefore, it is very interesting that water activates unfertilized eggs of the dog-salmon and that the inseminated eggs can not start in development in the isotonic salt solution in spite of ingress of the spermatozoon into the egg, while unfertilized eggs retain the fertilizable capacity for a long time in the medium. The aim of the present experiments lies in an analysis of the changes of the dog-salmon egg immersed into water. Most of the experiments have been carried on the Hokkaido Central Fish Hatchery since the winter, 1946.

Before going further, I wish to express my gratitude to Professor T. Inukai and Professor K. Aoki of the Hokkaido University for helpful suggestions and criticisms during the progress of this work. It is also my pleasant duty to acknowledge my great indebtedness to Professor T. Uchida for his kind service in reading the manuscript for publication. Thanks are also due to all members of the Hokkaido Central Fish Hatchery for their kind aid in performing the experiments.

## 2. Material and Method

The material used in this investigation was the dog-salmon, *Oncorhynchus keta*, which was fished at the Chitose Hatchery in Hokkaido. Mature males and females of dog-salmon run up the Chitose river from early October to late January every year for spawning. A long cut was made along the belly and ripe eggs were taken in Dewar vessel. When these were remained untouched to water at 10°C or below, the fertilizability of the eggs and sperm was found to be retained for several hours or a day. In all experiments the eggs taken from a single female only were used for a series of experiments. The balanced salt solution (b.s.s.) used here shows the following constituents ; 1 M NaCl 100 parts+1 M KCl 2.8

parts+2/3 M $CaCl_2$ 3.2 parts, PH of which is regulated 7.0 by adding of n/10 $NaHCO_3$ solution. This solution was diluted by buffered distilled water and concentration of diluted solution was expressed as 1/1, 1/2, ....., according to the grade of dilution. The freezing point of whole unfertilized eggs of dog-salmon is $-0.62°$, that is osmotically equivalent to ca M/6 NaCl. But unfertilized eggs of dog-salmon retain their weight unchangeable in 1/8 b.s.s. (Aoki, '39). Therefore in this experiment 1/7.5 b.s.s. was used as the isotonic solution.

### 3. Experiments

1) *Fertilizable period in fresh water and in the balanced salt solution*

It is well known that the salmon and trout eggs quickly lose their fertilizability in fresh water. After His ('73) the fertilizable period of the *Salmo* egg ranges from 15 to 30 minutes in fresh water. The same results were obtained in eggs of the dog-salmon by Handa ('09). Runnström ('20) reported the same phenomenon in the *Salmo* eggs and further he ascertained that unfertilized eggs of S. *salvelinus* retained the fertilizability for three days in isotonic Ringer's solution.

The unfertilized eggs were previously treated with both fresh water and the b.s.s. for 2, 5, 10, ....., 300 minutes respectively and then put into fresh water after insemination by "dry method". Water temperature ranged from 5° to 7°C during the experiment. After about a day the eggs were fixed with Bouin's solution and the number of cleft eggs was counted in about 150 eggs in each experiment. The control eggs were found to reach the 4~16 cell stage. Fig. 1 shows the graphical representation of the data obtained. The curves clearly designate that in fresh water the fertilizable period of the dog-salmon eggs is very short, ranging from 15 to 30 minutes, while in 1/7.5 b.s.s. the fertilizability of the eggs is kept as that of fresh eggs even after five hours. These results are accordant with the data of Handa ('09) and of Runnström ('20). The fertilizable capacity of the eggs treated with 1/4 b.s.s. is kept intact for about 180 minutes and then decreases very gradually. In 1/32 b.s.s., eggs lose their fertilizability as quickly as in fresh water in 15~30 minutes. In the case of 1/16 b.s.s., distinctly different cleavage curves are seen in each lot. In the lot a, it drops gradually and in the lot b in a fair steep. The fertilization rate of the lot a shows 90 percent after 60 minutes and 76 percent even after 5 hours, but in the lot b the fertilizability fairly rapidly decreases to about 4 percent after 60 minutes. This difference occurring between the lot a and b seems to be due to the difference of mother fishes.

From these results the conclusion may be made that the unfertilized eggs of the dog-salmon quickly lose the fertilizability in fresh water and fertilizable period is prolonged with increase of the concentration of the balanced salt solution up to the isotonic one, in which the fertilizability is retained for the longest time. But in more concentrated ones, it becomes gradually shorter again (K. Yamamoto, '47).

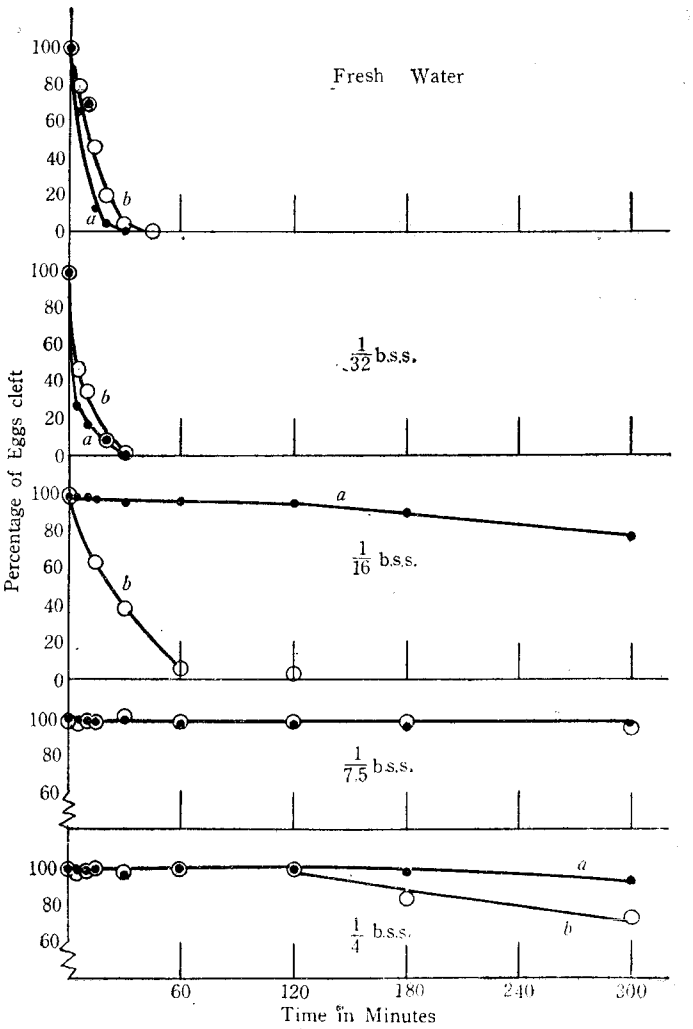


Fig. 1. Fertilizable period in fresh water and in b.s.s. The ordinate represents the percentage of eggs cleft. The abscissa indicates treatment-duration in minutes before insemination.

2) *Morphological changes of unfertilized eggs initiated to develop in tap water*

As mentioned above, the fertilizable period of the dog-salmon egg is very short in fresh water. This result seems to show that in contact with fresh water some changes have taken place in the unfertilized eggs. According to Kagan ('35),

the unfertilized eggs of *Fundulus heteroclitus* submerged in sea water developed up to the balstodisc stage, covering the disappearance of platelets and formation of the perivitelline space. Since the chorion of the dog-salmon eggs is thick and untransparent, such changes are not observable in detail externally. Therefore, the early changes of the egg were studied in detail by sections prepared according to the usual paraffin method.

a) Breakdown of the cortical alveoli : In unfertilized ripe eggs of *Fudulus* the platelets lie in the outer plasma layer. After fertilization or by activation by sea water, the perivitelline space becomes formed and the platelets disappear along with the formation of a blastodisc (Kagan, '35). In unfertilized *Oryzias* eggs the cortical alveoli are evenly embedded in the cortical protoplasm, leaving a small area at the animal pole. T. Yamamoto ('44) made a physiological study on the cortical changes of the egg at fertilization and activation. On his careful observations it has been revealed that the first change at fertilization lies in the breakdown of the cortical alveoli, which proceed from the animal pole toward the vegetable pole, and that the breakdown of the cortical alveoli is also caused by parthenogenetic agents. Furthermore, he postulated that a reaction of some kind or "impulse" may be provoked by the sperm or activating agents, and that the impulse causing the breakdown of alveoli is conducted in wave-like fashion. This invisible wave is termed the "fertilization-wave". So far as the writer is aware[1], there is no report concerning the cortical alveoli in the egg of the dog-salmon, in spite of the considerable amount of work on the early development of its egg. As mentioned above, due to the presence of the chorion one can hardly observe the interior changes of eggs externally. There is considerable difficulty in obtaining suitable figure for study in sections, but fortunately I could ascertain the cortical alveoli in the cortical protoplasm of the unfertilized egg. The cortical alveiol, each measuring $12\sim25\mu$ in diameter, are found uniformly embedded in the outer plasmic layer of the unfertilized egg (Pl. XIV, Fig. 1). These alveoli show vacuolation in thin sections. After fertilization, the cortical alveoli break down within a few minutes. In these eggs, the breakdown of the cortical alveoli is not caused by fertilization alone. Even in unfertilized eggs, when put into fresh water, the cortical alveoli disappear as quickly as in the case of fertilization (Pl. XIV, Fig. 2) and their breakdown proceeds from the animal pole toward the vegetable pole. The elevation of the chorion follows the breakdown of the cortical alveoli as occured in *Oryzias* egg.

b) Extrusion of the second polocyte : When the unfertilized eggs are put into fresh water, the cortical alveoli begin to breakdown and disappear within a few minutes. Then the extrusion of the second polocyte follows. Ripe eggs of the dog-salmon directly taken from the oviduct are, without exception, at the

1) Recently, Kanoh ascertained this existence by "striping method" and his results were read before the Annual Meeting of Zoological Society of Japan in October, 1949.

metaphase stage of the second polar division as generally occurred in other fish eggs (Pl. XIV, Fig. 3). The polar spindle, however, perisists in the stage of metaphase for 1~10 minutes after the breakdown of the cortical alveoli (Pl. XIV, Fig. 4). As the thin protoplasmic sheath inclosing yolk moves towards the animal pole of the egg, the maturation division proceeds further on. About 30 minutes after exposure to water, the division goes on to late anaphase as shown in Fig. 5, which illustrates the separation of the chromosomes and the formation of the polar body in the form of a disc from the egg surface. After the extrusion of the second polocyte, the sister chromosomes left in the egg converge and from a female pronucleus. The female pronucleus of the unfertilized eggs which have been placed in water for 3 hours is of an ellipsoidal shape with fairly smooth contour and contains deeply stained chromatic bodies (Pl. XIV, Fig. 7). Fig. 8 shows a faintly stained, seemingly degenerating female pronucleus of an egg which has been exposed to water for 6.5 hours. The maturation division in the unfertilized egg proceeds almost in a similar manner to that of the fertilized eggs in water.

c) Formation of the "Scheidewand": Spek ('33) observed that in most teleost eggs bipolar differentiation is present and a blastodisc is distinctly formed in the ovary. The blastodisc enlarges strongly when touched to water. In the egg of the rainbow trout, Behrens ('98) reported that the "Scheidewand", deeply stained with haematoxylin, appeared beneath the blastodisc about three hours

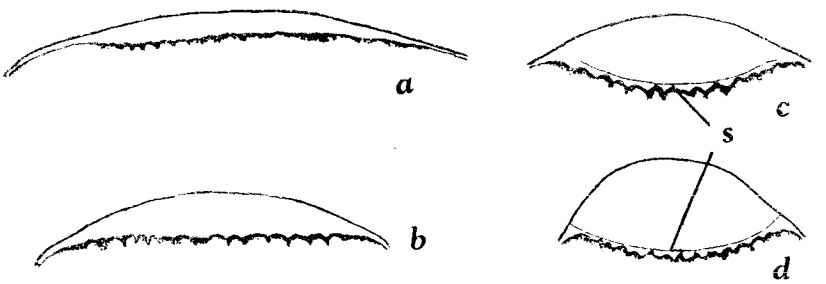


Fig. 2. Formation of the "Scheidewand" in an unfertilized egg put into tap water. a. Blastodisc of eggs taken from oviduct. b. Blastodisc of eggs put in water for one hour. c. Blastodisc of eggs, five hours after exposure to tap water. d. Blastodisc of eggs, 24 hours after exposure to water. s: "Scheidewand"

after insemination. Along with the enlargement of balstodisc, the "Scheidewand" spreads over and finally separates the blastodisc from the protoplasm enclosing the yolk. Even in unfertilized eggs of the dog-salmon which have never been in contact with water, bipolar differentiation occurs. When unfertilized eggs of the dog-salmon are put into water, the breakdown of cortical alveoli firstly occurs and then the extrusion of the second polocyte follows. Following these changes, the

"Scheidewand" is formed along with the enlargement of blastodisc. About three hours after exposing to water, the "Scheidewand" becomes visible under the blastodisc (Text-fig. 2) and about five hours afterwards the blastodisc becomes to be completely separated by the "Scheidewand" from the protoplasm (Text-fig. 2).

3) *Fertilizable period and intervals of activation*

Kagan ('35) observed that sea water initiates development of unfertilized eggs of *Fundulus* up to the blastodisc stage, and their fertilizable capacity gradually falls off with the increase of number of eggs which have formed the perivitelline space. Unfertilized eggs of the dog-salmon are also activated quickly in fresh water and there occurs several changes above described in succession. It seems to be probable that the similar relation exists also between fertilizability and activation of the egg in water as in *Fundulus* egg. As observed already, unfertilized eggs of the dog-salmon quickly lose fertilizability in fresh water and the higher is the concentration of balanced salt solutions, the longer is the fertilizable period. In the isotonic solution the fertilizability remains active for the longest time, but its time becomes short by and by with the increase of salt concentration of hypertonic solution.

Therefore, a relation between fertilizable period and duration necessary for the "Scheidewand" formation of the egg was examined in both fresh water and balanced salt solution. Unfertilized eggs were put into tap water, 1/32, 1/16, 1/7.5, 1/4 b.s.s. respectively. As a control fertilized eggs were immersed into tap water. Every twenty eggs were fixed in Bouin's solution at definite time-intervals and sections were stained with Heidenhain's iron-haematoxylin. The

Table 1. The "Scheidewand" formation in tap water and b.s.s.

| Immersing medium \ Immersing time | Percent of eggs formed the "Scheidewand" | | | | |
|---|---|---|---|---|---|
| | 3 hours | 5 hours | 8 hours | 12 hours | 24 hours |
| Tap water | 75% | 100% | | | |
| 1/32 b.s.s. | 30% | 65 | 95% | | |
| 1/16 b.s.s. | | 50 | | 95% | |
| 1/7.5 b.s.s. | | | | 0 | 0% |
| 1/4 b.s.s. | | | | 60 | 85 |
| Fertilized egg in tap water | 80 | 100 | | | |

Water temperature at 11°~15 C.

results are shown in Table 1. As noted in the table, the "Scheidewand" appears in the eggs exposed to water synchronously with the control eggs and in no eggs immersed in the isotonic solution even after a day. The "Scheidewand" also appears in the eggs exposed to 1/16 and 1/32 b.s.s., but the percentage of the

eggs forming the "Scheidewand" is 65 percent after 5 hours, and 95 percent after 8 hours in 1/32 b.s.s. respectively, but decrease further in 1/16 b.s.s. From this fact it is evident that the duration necessary for the "Scheidewand" formation becomes longer with the increase of salt concentration of the medium. Though the "Scheidewand" was formed also in eggs put into 1/4 b.s.s.,[1] much longer time was required in this case, even after a day some eggs failed to form the "Scheidewand". From these observations it is evident that the fertilizable period of the egg is intimately correlated with the duration necessary for the "Scheidewand" formation (K. Yamamoto, '49). It is, however, more convenient to choose an earlier change as an indicator for this purpose. It has already been confirmed that the first visible change is the wave-like breakdown of the cortical alveoli. Therefore, the velocity of break-down of the cortical alveoli was measured in tap water and in 1/7.5 b.s.s. respectively. In sections it is shown that the cortical alveoli in eggs exposed to tap water were almost broken down at five minutes, but those in eggs immersed into 1/7.5 b.s.s. still remain unchanged for about a day (Pl. XIV, Fig. 9.).

As shown in Fig. 1, fertilization rates of the eggs immersed in tap water before insemination for five minutes and for 10 minutes are about 70 percent and about 25 percent respectively. Accordingly, it can be supposed that the eggs with the broken cortical alveoli still retain the fertilizable capacity for a few minutes. The same phenomenon has been already observed by T. Yamamoto ('49) in the egg of gold fish. Activated quickly in tap water, the cortical alveoli of the unfertilized eggs of gold fish begin to break down and then the membrane elevation follows. But the eggs, in which the cortical alveoli have already broken down, are still fertilizable, if they are inseminated within five minutes after the breakdown of the cortical alveoli. From these evidences, it is clear that the "fertilization-wave", occurring in the unfertilized egg which has been activated in fresh water, does not immunized the egg for fertilization. In *Echinarachinus*, Just ('19) found that some cortical changes occurred before the actual elevation of the membrane immunize the eggs for other sperm. The same phenomenon occurs when the egg is artificially activated. He termed this cortical change as the "wave of negativity". The "fertilization-wave" is possibly not identical with the "wave of negativity" in *Echinarachinus* egg. The reason why the fertilizability of dog-salmon egg is lost immediately after shedding will be discussed here. From the data mentioned above, it has been clear that unfertilized eggs activated and with the blastodisc completely formed in water, can not be fertilized any longer; whereas the eggs put into the isotonic salt solution, though not activated, still retain fertilizability. Therefore some changes which have been occurred during blastodisc formation possibly prevent the entry of spermatozoa into the eggs. From the fact that the

1) In another experiment described in the section 4, the "Scheidewand" was not formed in the eggs exposed to 1/4 b.s.s. even after about a day.

fertilizable capacity of *Fundulus* eggs decreases gradually as the eggs with the perivitelline space increase, Kagan ('35) concluded that the failure of fertilization lies in closure of micropyle by membrane elevation. Unfertilized eggs of the dog-salmon are also activated very quickly in fresh water ; the cortical alveoli break down and then elevation of the chorion is observed. Therefore, the deficiency of fertilizability of unfertilized eggs of dog-salmon in fresh water immediately after shedding, is possibly attributed to the membrane elevation accompanying the closure of microphyle.

4) *Fertilizable period and fertilization rate in b.s.s.*

The fertilizability of the flounder egg (*Limanda schrenki*) is retained for the longest in 1/4 b.s.s., but about a half of the eggs only are fertilized in that medium, though spermatozoa move actively in it. The fact seems to be due to some defects preventing fertilization in the medium (K. Yamamoto. '49). The egg of the dog-salmon is also the case. To ascertain the phenomenon the following experiment was carried out. The eggs were inseminated by dry method, then divided into several groups and placed in tap water, 1/512, 1/256,. . . . , 1/1 b.s.s., respectively. After about a day the eggs were fixed with Bouin's solution and the percentage of the cleft eggs was calculated. As seen in Fig. 3, the eggs placed in lower concentrated media than 1/16 b.s.s. cleft in almost 100 percent. But those immersed into 1/7 and 1/4 b.s.s. neither formed blastodisc nor cleft. All the eggs exposed to 1/2 and 1/1 b.s.s. were found to be in cytolysis. Though the eggs inseminated in 1/7 and 1/4 b.s.s. are not fertilizable, unfertilized eggs retain the fertilizability for a long time, whilst the spermatozoa move activly in these media. The failure of fertillzation in these cases, therefore, may be attributable to either the failure of penetration of spermatozoa into the eggs or the in-

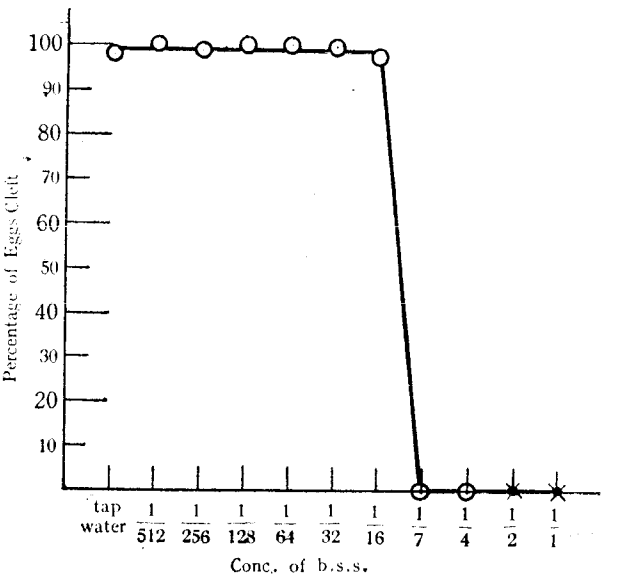


Fig. 3. Fertilization rate in fresh water and in b.s.s. The ordinate represents the percentage of eggs cleft. The abscissa indicates concentration of solution.

hibition of the cortical change in the eggs.

5) *Failure of fertilization in isotnoic salt solution*

a) In order to make clear whether the factors necessary for entry of spermatozoa act as inhibition for fertilization in isotonic salt solution or not, the following experiments were undertaken. A set of the unfertilized eggs was immersed in 1/7 b.s.s. and inseminated in it. Then the eggs were transferred from the solution to tap water after three or five hours. Each lot was divided into two groups, one in tap water and another in 1/7 b.s.s. About twenty hours afterwards they were fixed with Bouin's solution and were examined whether they had cleft or not. The results are summarized in Table 2. Even a single egg in 1/7 b.s.s. showed no

Table 2. Fertilization rate of eggs which have been inseminated in 1/7 b.s.s. and then transferred to tap water.

| Time-duration in 1/7 b.s.s. until transferred to tap water | percent of eggs cleft | | |
|---|---|---|---|
| Hours | No. 1 | No. 2 | No. 3 |
| 3 | 100 | 100 | 99 |
| 5 | 99 | 99 | 99 |
| Controls not transferred to tap water | 0 | 0 | 0 |

changes. But most of the eggs transferred to tap water indicated cleavage. When dry sperm were diluted with 1/7 b.s.s., they were found actively moved, but the movement ceased for a minute. From this fact it seems impossible to consider that the spermatozoa retain the fertilizability for more than three hours in 1/7 b.s.s. The fertilizable capacity of spermatozoa in old sperm-suspension was examined in the following manner. The fresh dry sperm firstly diluted by the admixture of 1/7 b.s.s. with the same concentration as in the above experiment. After three or five hours, fresh eggs were inseminated with those old sperm-suspensions and then a few minutes afterwards were transferred to tap water. These eggs never developed up to the cleavage-stage. From this fact it becomes clear that the spermatozoa suspended in 1/7 b.s.s. do not retain the fertilizability for three hours. It is probable, therefore, that before the eggs insimenated in isotonic solution were removed to tap water, the spermatozoa had already penetrated into the eggs.

b) As is described, eggs of the dog-salmon inseminated in isotonic salt solution and remained there, do not undergo developmental changes and the cortical alveoli remain unchanged for about a day in spite of ingress of the spermatozoon. There are two possible causes for the failure of the development in the above case. Firstly the "fertilization-wave" did not occur and the cortical alveoli were not broken down ; namely the stimulus caused by the entry of spermatozoon under this experimental condition, might be subliminal in strength for activation of the egg.

Secondary, the "fertilization-wave" did not propagate all over the egg, though it occurred in the point of sperm entry.

Eggs inseminated by dry method were treated with tap water for 1, 5, 15, 30 minutes and then immersed in 1/7 b.s.s. As the control, untreated eggs were preserved in 1/7 b.s.s. About 24 hours afterwards they were fixed and the number of cleft eggs was counted. The results were summarized in Table 3. The eggs

Table 3. Percent of cleft eggs inseminated in 1/7 b.s.s. and treated in tap water at difinite intervals, and then transferred to 1/7 b.s.s. again.

| | Percent of eggs cleft | | | | |
|---|---|---|---|---|---|
| | 0 min. | 1 min. | 5 min. | 15 min. | 30 min. |
| No. 1 | 0 | 83 | 100 | 100 | 100 |
| No. 2 | 0 | 93 | 92 | 99 | |

immersed in tap water for a minute showed cleavage in 83 and 93 percent, and those for five minutes cleavage in 100 and 92 percent. The eggs treated with tap water for more than 15 minutes all cleft, while the control eggs showed no cleavage. On examination of the uncleft eggs treated for one minute, there were observed various changes in the eggs. Some eggs were scarcely different from the control eggs, while

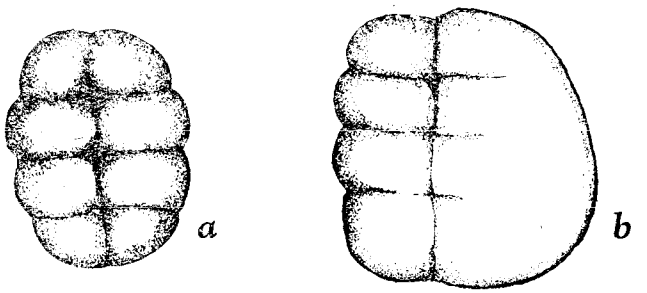


Fig. 4. Abnormally cleft eggs which were inseminated in 1/7 b.s.s., then placed in tap water for one minutes, and transferred to 1/7 b.s.s. again. a, Normally cleft blastoderm. b, Abnormally cleft blastoderm.

some others showed enlargement of the blastodisc, though not complete. The eggs formed a fairly heaped blastodisc were also found. Some of cleft eggs showed abnormal cleavage as shown in Fig. 4. In these eggs protoplasm is not rich in the animal pole and the blastodisc is more sunken and larger than the normal one. Moreover, cleavage-grooves were confined only in part of the blastodisc. In this

experiment the development of eggs was retarded in 1/7 b.s.s. Most of the eggs removed to 1/7 b.s.s. after treatment in tap water for one and five minutes showed the 4-cell stage, while the control eggs cleft to the 16-cell stage at the corresponding time. The shorter was the duration of treatment in tap water before transporting to isotonic solution, the more remarkable was retardation of the development. From the facts described above, the failure of development mainly lies in the inactivity of the "fertilization-wave". The wave, even if occur in the limited point of the sperm entry, possibly does not propagate all over the egg, hence no breakdown of the cortical alveoli takes place (K. Yamamoto, '49).

6) *The conduction of the "fertilization-wave"*

As stated abvoe the conduction of the "fertilization-wave" seems to be most important for further development of eggs. It is already shown in the section 4 that no eggs immersed in 1/7 b.s.s. were fertilized, but in 1/16 b.s.s., all the eggs were fertilized. The difference between these two solution lies in the concentration of salts contained. There can be supposed two probable effects of the solution to eggs, i. e., osmotic action and ionic action. Then the following experiments were made. The eggs inseminated in 1/7 b.s.s. were put into the following two series of solutions, 1/1, 1/2, ....., 1/512 b.s.s. and 1/1, 1/2, ....., 1/256 M sucrose solutions[1]. About 24 hours later they were fixed with Bouin s solution and the number of cleft eggs was counted. The results obtained from the eggs immersed in b.s.s. were nearly similar to that shown in Fig. 3. The eggs placed in the media of lower concentration than 1/16 b.s.s. cleft in almost 100 percent. In 1/7 and 1/4 solutions no egg formed blastodisc, and those exposed to 1/2 and 1/1 solutions all were found to be in cytolysis. The eggs placed in more diluted sucrose solutions than 1/2 M mostly cleft and those immersed in 1/1 solution all died. From these observations, it can be surmised that the osmotic pressure itself of the medium is nothing to do with the conduction of the "fertilization-wave". Therefore, the following experiments were performed. Two series of solutions were prepared. The one was prepared by adding buffered distilled water as follows ; 90 parts 1/7 b.s.s.+10 parts distilled water, 80 parts 1/7 b.s.s.+20 parts distilled water and so on. To the other, M/3.7 sucrose solution was added instead of distilled water in the same way. The eggs inseminated in 1/7 b.s.s. were transferred to these solutions and number of cleft eggs was counted. Each solution of the former series differs both in osmotic pressure and in ionic concentration. Though the ionic concentration progressively decreases in the latter series, the osmotic pressure remains almost constant in all solutions. Therefore, if the ionic concentration activates the conduction of the "fertilization-wave", the number of cleft eggs in the solutions must be subject to salt concentration of the medium. This was actually the case.

1) 1/1 M sucrose solution was prepared as follows ; 1 molecular gram sucrose was dissolved into 1000 cc of tap water, and 1/2 sucrose solution was prepared as 50 parts 1/1 sucrose +50 parts tap water. 1/4, 1/8, ...., 1/256 M solutions were prepared so on.

Table 4 illustrates this relation. In A solution, no eggs exposed to both 90 and 80 percent solution of 1/7 b.s.s. cleft, but in the concentrated solutions lower than 70 percent, cleft eggs were always found. The results obtained from the eggs immersed in B solution showed almost the similar tendency. The cleaving capacity corresponds to the salt concentration of medium in spite of different osmolar concentrations. From the above abservations, it can be concluded that the conduction of the "fertilization-wave" is subjected to the ionic concentration of medium and further that the critical value is

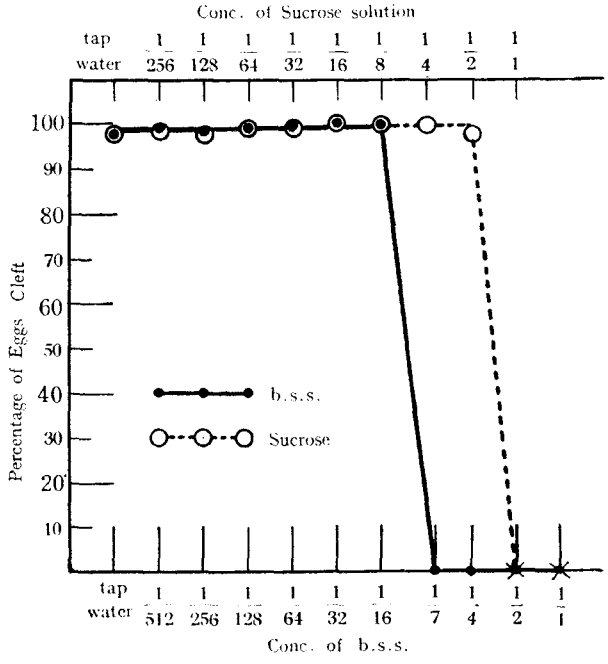


Fig. 5. Fertilization in b.s.s. and sucrose solutions. The ordinate represents the percentage of eggs cleft. The lower abscissa indicates concentration of b.s.s. and the upper one represents the concentration of sucrose solution.

Table 4. Fertilization in two kinds of b.s.s.

| | Per cent. of eggs cleft | | | | |
|---|---|---|---|---|---|
| | A solution (diluted by distilled water) | | | | |
| | 90% | 80% | 70% | 60% | 50% |
| No. 1 | 0 | 0 | 67 | 98 | 98 |
| No. 2 | 0 | 0 | 35 | 79 | 91 |
| | B solution (diluted by M/3.7 sucrose solution) | | | | |
| | 90% | 80% | 70% | 60% | 50% |
| No. 1 | 0 | 0 | 44 | 95 | 98 |
| No. 2 | 0 | 0 | 36 | 77 | 94 |

approximately M/10 in the case of b.s.s. Futhermore, when the eggs inseminated by dry method had been put into distilled water, they were found to be all cleft. The results show that the existence of salts in the medium is not particularly necessary for the conduction of the "fertilization-wave" (K. Yamamoto, '50).

## 4. Discussion

The unfertilized eggs of the dog-salmon activated by water show successively the breakdown of the cortical alveoli, the freeing of the chorion from the egg surface and the extrusion of the second polocyte. Following these changes, the formation of the "Scheidewand" takes place along with enlargement of blastodisc. The eggs which have been thrown in these changes are fertilized no more. Besides the unfertilized eggs of dog-salmon and of brackish minnow, those of many other fresh water fishes are easily activated in water and lose fertilizable capacity quickly. Somewhat similar phenomenon has been recorded in other animals, such as *Chaetopterus*, *Sabellaria*, and *Asterina*. But the eggs of these invertebrates are remarkably different from that of the dog-salmon on the point of fertilizability after the completion of these changes. The eggs of dog-salmon quickly lose the fertilizable capacity in water, which is attribuable to the membrane elevation accompanying the closure of micropyle. In the egg of *Sabellaria*, the separation of the vitelline membrane from egg surface takes place with the changes initiated by the contact with sea water. But the spermatozoa of the annelid can penetrate through the elevated vitelline membrane into the egg (Novikov, '39). The developmental changes in the eggs of *Chaetopterus* and of *Sabellaria*, initiated by sea water, consists of breakdown of the germinal vesicle, migration of its fragments to the animal pole and the occurrence of the first maturation division at metaphase. These karyokinetic processes will be arrested unless the egg be fertilized. Miss Allyn (1912) who observed the changes in *Chaetopterus* in various isosmotic salts solutions and also in its own body fluid, came to the conclusion that the egg needs not be stimulated exteriorly to undergo the changes but only to be released to go on the process; namely, in the egg of *Chaetopterus* the maturation proceeds spontaneously after the egg has been shed into sea water. In the egg of fish, the extrusion of the first polar body is completed in the ovary, and the egg, when laid, reaches the stage of metaphase of the second maturation division. As noted above, the egg of dog-salmon shed into water are activated immediately and subjects to the changes including the extrusion of the second polocyte. But the egg kept in isotonic salt solution or body fluid of mother fish being left unchanged for a long time, it is probable that the maturation does not proceed spontaneously in water as in the case of *Chaetopterus*, but is awaked to start by some inciting cause. The freezing point of unfertilized eggs of the dog-salmon is $-0.62°C$ in value. The eggs, when laid, are subjected to effeccts of hypotonicity of fresh water. In this point eggs of fishes are remarkably different from those of other marine animals which

are isosmotic with sea water. The effect of hypotonicity of fresh water is attributed to direct action of different ionic concentrations between the egg interior and outer medium. The difference of ion-concentrations seems to act as the determined factor for the changes of cortical layer. According to Heilbrunn ('39) the gell→sol transformation in *Amoeba* is inhibited by ions. If the cortical gel of fish eggs is influenced by ions as in amoeba plasma, the mechanism of the ionic action on the changes of cortical layer may be explained by his theory.

## 5. Summary

To analyse the changes of the eggs of the dog-salmon when put in water, experiments were carried out with the following results.

1. When the unfertilized eggs are activated in fresh water, the breakdown of the cortical alveoli, elevation of the chrion, and extrusion of the second polocyte occur in succession. The formation of the "Scheidewand" takes place along with enlagement of the blastodisc.

2. The eggs shed in water lose fertilizability at 15~30 minutes. This is attributable to the membrane elevation accompanying closure of the micropyle.

3. In isotonic salt solution the eggs can not be fertilized even though inseminated. By experiments it is surmised that even if the "fertilizttion-wave" is provoked in the area of entry of a spermatozoon, it fails to progpogate all over the egg in the isotonic salt solutions.

4. Ionic concentration of the medium plays an important role in the conduction of the "fertilization-wave". The critical value is approximately M/10 in the case of b.s.s.

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## Explanation of plates XIV

**Fig. 1.** Section of a ripe unfertilized egg taken from oviduct, showing the cortical alveoli embedded in the cortical layer. ca. ×200.

**Fig. 2.** Cortical protoplasm showing the break-down of the cortical alveoli. From the section of an unfertilized egg placed into water for 5 minutes. ca. ×200.

**Fig. 3.** Section of a ripe unfertilized egg taken from oviduct, showing the metaphase of the second polar division, oblique view. ca. ×630.

**Fig. 4.** A part of the cortical layer in which the cortical alveoli had dissoluted. The egg nucleus remains unaltered. From a section of an unfertilized egg immersed in water for five minutes, side view. ca. ×630.

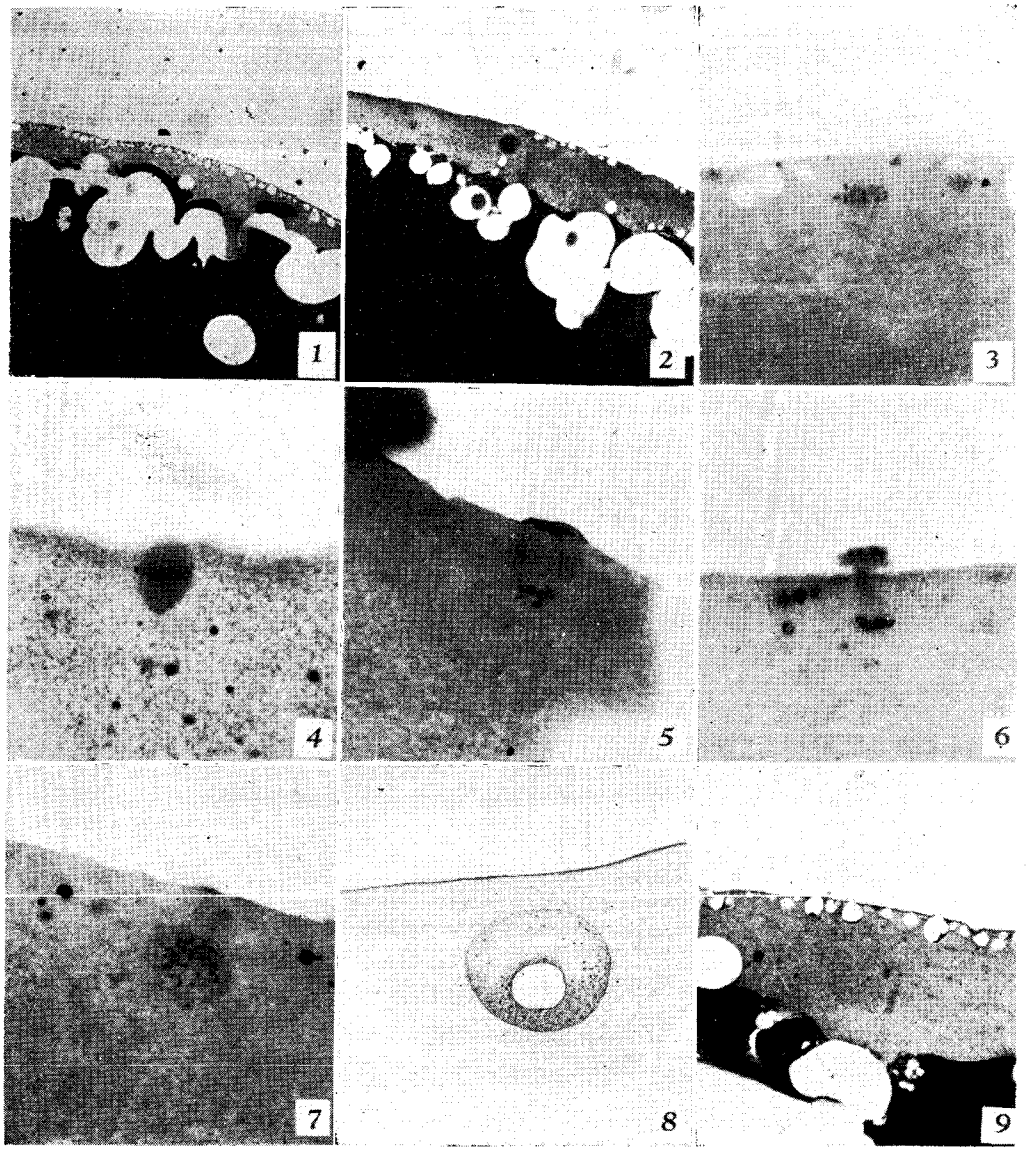
**Fig. 5.** Late anaphase of the second polar division, side view. From an egg about 30 minutes after insemination. ca. ×630.

**Fig. 6.** Successive stage to Fig. 5, showing the second polocyte just separated off. From an unfertilized egg immersed in water for 60 minutes. ca. ×630.

**Fig. 7.** The female pronucleus. From an unfertilized egg about 3 hours after exposure to water. ca. ×630.

**Fig. 8.** A seemingly degenerating female pronucleus of an unfertilized egg which has been immersed in tap water for 6.5 hours. ca. ×660.

**Fig. 9.** Section of an egg which has been inseminated in isotonic salt solution and kept in it for 18 hours, showing the cortical alveoli remain unchanged. ca. ×400.




*Yamamoto, K.: Activation of the Egg of the Dog-Salmon by Water.*