Manner of Sperm Entry in Herring Egg, with Special Reference to the Role of Calcium Ions in Fertilization[1)]

By

Ryuzo Yanagimachi and Yasuhiko Kanoh

(Zoological Institute, Faculty of Science, Hokkaido University)

(*With* 2 *Text-figures*)

The effect of egg secretions on spermatozoa in fertilization has recently attracted the attention of many authors. But our knowledge has remained very poor in the case of fish gametes. The important role of calcium ions in the process of fertilization has been recognized since Loeb ('14, '15), and it has become clear in sea urchins that the agglutination of spermatozoa and also their oxygen uptake are increased by clacium ions (Loeb '14, Vasseur '49 a & b). Yamamoto (T.) ('44) has also confirmed the importance of calcium ions at fertilization in a fresh water fish, Medaka (*Oryzias latipes*). Before analysing the mechanism of fertilization in fish eggs, it is highly desirable to make clear the manner of sperm entry in the egg, especially behavior of spermatozoa on the egg surface. The present study has been undertaken to learn the behavior of spermatozoa at fertilization in herring, in connection with the role of calcium ions, consulting the studies of Kanoh ('49, '53) on the morphological or cytological changes in the eggs of this fish at the time of fertilization.

Before going further the authors desire to express thanks here to the members of Hokkaido Fisheries Scientific Institution for their supports to this investigation. The authors also wish to acknowledge the valuable cooperation of Mr. S. Ito[2)] and Mr. T. S. Yamamoto. The expenses the of the study were partly defrayed by a grant from the Scientific Research Fund of the Department of Education.

Material and Method

Materials used in the present investigation were all taken from fully matured individuals of the Pacific herring (*Clupea pallasii*) by vivisection. Since eggs in

1) Contribution No. 306 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

2) Present address: the Biological Institute, Kumamoto University.

the isotonic Ringer's solution[1] retain, as stated in the preceding paper (Yanagimachi '53a), their fertilizability for longer period of time than in ordinary sea water and are fertilized normally, the isotonic Ringer's soution was used in stead of sea water as an external medium. In all the cases in which observations and experiments were performed, a certain number of unfertilized eggs were separately stuck on the bottom of Petri-dish or on a slide glass and then immersed in the isotonic Ringer's solution. The insemination was performed with a small amount of sperm suspension, keeping close microscopical observation on the egg surface by means of a water immersion objective lens.

Results

Behavior of Spermatozoa at fertilization, especially on the Egg Surface

The herring spermatozoön belongs to the flagellate type, being composed of a round head (2 μ in diameter), a middle piece (1 μ in diameter), and a long tail (42 μ in length). The acrosome was not distinguishable at least in a living state. Under usual condition they were not very active or almost motionless in either sea water or Ringer's solution, *i.e.*, they vibrated merely their heads, only stiring at times; whereas on the surface of inseminated eggs they showed a peculiar active movement which continued without decline for a considerable long time (for more than two hours after insemination at 10° C). This fact indicates, therefore, that spermatozoa begin to move actively or are activated on contact with egg surface. Careful observations revealed that the most active movement of spermatozoa occurs inside of the depression of adhesive layer around the micropyle; in this area a number of spermatozoa are silding actively on the egg surface, describing circles of varying diameters (in most cases, anticlockwise[2] with reference to the surface of contact). The same movement was also observed at the time of fertilization, finally resulting in the penetration of spermatozoa into the micorpyle, and even just after insemination the micropyle was swarmed with such active spermatozoa (cf. Fig. 1).

No striking movement was found on the surface of the vegetal hemisphere, that is to say, in this area only few spermatozoa were active, but the most of them vibrated merely their heads. Excepting in the depression area around the micropyle, some spermatozoa in the animal hemisphere moved actively as those found inside of the depression, but the others there were almost the same with those found on the sufrace of vegetal hemisphere. Thus it is evident that activation of spermatozoa on egg surface is induced most highly in the vicinity of the micrpyle.

---

1) Constitution of isotonic Ringer's solution : M/4.5 NaCl 100 parts + M/4.5 KCl 3.5 parts + M/6.75 $CaCl_2$ 1.5 parts + M/6.75 $MgCl_2$ 2.4 parts ; pH was adjusted to 7.6 by $NaHCO_3$.

2) Some spermatozoa show a serpentine movement, sliding on the surface.

Manner of Sperm Entry into the Micropyle

The micropyle of an unfertilized egg was brought in view under a water immersion objective lens and a small amount of diluted sperm suspension was added. Within a few seconds after insemination, several spermatozoa reached the vicinity of micropyle to begin the active sliding movement, as described in the preceding paragraph, on contact with egg surface, and entered into micropyle one by one (Fig. 1A). At this time they advanced in a file, since the canal of the

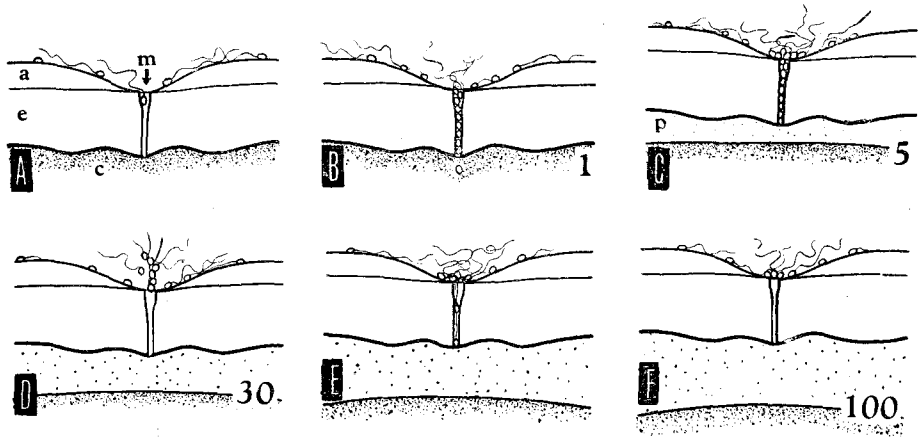


Fig. 1. Successive steps after insemination, at 10°C (Semi-schematic figures). Numbers represent the time in minutes after insemination. A, just after insemination ; B, the first spermatozoön has penetrated into the egg cortex ; C, beginning of formation of the perivitelline space ; D, extrusion of supernumerary spermatozoa from the canal of micropyle ; E, the subsequent step, two spermatozoa have entered the canal again, but cannot pass through it ; F, spermatozoa swarm as ever at the opening of the canal without entry. a, adhesive layer ; c, egg cortex ; e, egg membrane ; m, micropyle ; p, perivitelline space.

micropyle is nearly as wide as the head of spermatozoön. In this way, as a rule, it is only the first one that can reach the egg cortex and takes share in fertilization. The second spermatozoön, even if it follows just behind the first, is not permitted the entry into the egg cortex, coming unavoidably to standstill at the inner end of the canal. The third strikes on the second, and so on. Thus, soon after the insemination the canal of micropyle became to be filled with quiescent supernumerary spermatozoa, whereas the spermatozoa outside the canal were still moving as ever (Fig. 1B & C).

In thirty to forty minutes after insemination, however, all or almost all the supernumerary spermatozoa in the canal were extruded rather suddenly from it (Fig. 1 D), but in the following instance a few or several of them were observed to

advance inwards the canal again, after moving forwards and backwards. They were not able to pass, as a rule, through the inner end of the canal[1] (Fig. 1 E), but were completely extruded again sooner or later and never entered the canal at last. Even in this time it was striking that spermatozoa swarmed actively at the mouth of the canal as usual (Fig. 1 F).

Extrusion of supernumerary spermatozoa from the micropyle has also been reported in the egg of a marine fish, flounder (*Limanda schrenki*) by Yamamoto (K.) ('52) who has observed further that along with the elevation of egg membrane a ball-like body is fequently formed at the mouth part of the micropyle. However, in herring eggs we have not found such a body at least during the course of the present investigation.

In about two minutes after insemination, alveoli embedded in the cortical cytoplasm (cortical alveoli) began to break down, subsequently the formation of perivitelline space and a raised blastodisc occured. Two and half hours later the first cleavage plane divided the blastodisc into two blastomeres (10° C).

In short, it may be concluded from the facts mentioned above that egg has a sperm activating property, accordingly fertilization proceeds in such a way that spermatozoa reached in the vicinity of the micropyle are highly activated and one of them penetrates in the egg cortex through the micropyle.

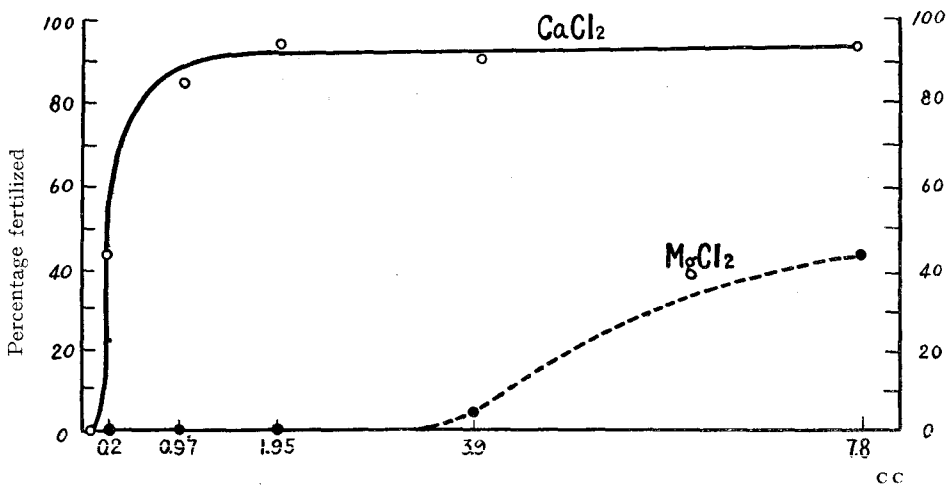
Role of Calcium Ions in the Process of Fertilization or Activation

It has been well known, since the time of Leob ('14, '15), that eggs of some animals lose their capacity for normal fertilization under Ca-free condition. So the authors undertook the experiments to ascertain whether this is true in the case of herring.

When unfertilized eggs were washed several times with an isotonic Ca-free Ringer's solution (pH 7.6) for forty minutes or with an isotonic Na_2-oxalate solution (M/6, pH 7.6) for fifteen minutes and then inseminated with a small amount of fresh milt diluted with Ca-free Ringer's solution, none of them was fertilized (at 10° C). Having put into the normal Ringer's solution, however, they were fertilized at once quite normally. Such failure of fertilization in Ca-free condition was, thus, reversible. The same has been already confirmed accurately in fresh water fish, Medaka (*Oryzias latipes*) by Yamamoto (T.) ('44), who has supposed that calcium ions seem necessary for the union of spermatozoa and egg cortex, because the spermatozoa are found to be quite active in the Ca-free Ringer's solution. As is described above, herring spermatozoa, however, do not usually move actively in Ringer's solution, but are activated when they come in contact with egg surface. Hence, the authors observed the behavior of spermatozoa on egg surface in Ca-free Ringer's solution in which fertilization is inhibited. The observation revealed that the active sliding movement of spermatozoa did not occur on egg surface,

1) On a rare occasion it happens that some of them can pass through the inner end of the canal (cf. Kanoh '53).

i.e., in this condition they were inactive even if they came in contact with egg surface in the vicinity of micropyle, and they vibrated merely their heads and stired at times. Entry in the micropyle, consequently penetration in the egg cortex, did not occur under this condition[1]. On the addition of a small amount of an isotonic $CaCl_2$ solution (M/6.75, pH 7.6), however, the spermatozoa in the vicinity of the micropyle was observed to begin the remarkable active movement and to advance into the canal one by one, in consequence all the eggs were fertilized normally (10° C). It became obvious, therefore, that calcium ions play an extremely important role in the activation process of herring spermatozoa, in other words sperm activating property of the egg surface is reversibly inhibited when calcium ions is deficient in medium.



Amount of isotonic $CaCl_2$ or $MgCl_2$ solution added to 103.5cc of Ca-Mg-free Ringer's solution.

Fig. 2. Percentage of fertilization in mixture of a Ca-Mg-free Ringer's solution and the isotonic $CaCl_2$ or $MgCl_2$ solution in various proportions (10°C).

Then in order to find out the concentration of calcium ions necessary for fertilization and to determine whether or not calcium ions can be replaced by magnecium ions the following experiments were performed. Unferitlized eggs were washed several times for forty minutes with mixture of a Ca-Mg-free Ringer's solution and the isotonic $CaCl_2$ or $MgCl_2$ solution in various proportions, and then

1) In isotonic Na_2-oxalate solution spermatozoa showed in some cases *comparatively* active movement, mode of which was at random in reference to the micropyle, and at least in our observation sperm entry into the canal did not occur, even if they came to the mouth part.

inseminated uniformly with a small amount of fresh milt diluted with Ca-Mg-free Ringer's solution (1/100 sperm suspension). The resultant percentages of fertilization are shown in Figure 2, from which it can be found that fertilization takes place completely when about one cc of the isotonic $CaCl_2$ solution is added to 100 cc of the Ca-Mg-free Ringer's solution, and that calcium ions can be replaced by magnecium ions, though the latter are less effective.

In addition, lack of calcium ions inhibits also the activation of egg itself. Eggs are easily activated parthenogenetically by pricking with glass needle (Kanoh '53), but in the case when they were pricked after repeated washing with the Ca-Mg-free Ringer's solution for forty minutes or with the isotonic Na_2-oxalate solution for fifteen minutes, no egg was activated. If, however, the same eggs were put back into the isotonic Ringer's solution, all of them were activated at once without further pricking. This suggests, therefore, that presence of calcium ions is also of great importance in the activation process of egg itself.

Discussion

Calcium ions seem necessary, according to T. Yamamoto ('44), for the union of spermatozoa and egg cortex, but judging from the results of the present investigation, the failure of fertilization under Ca-free condition may be, partly at least, due to the loss of positive movement of spermatozoa in the direction of the micropyle, and this phenomenon may also happen in other fishes. Therefore, the authors undertook observation in eggs of *Oryzias* and of the dog-salmon (*Oncorhynchus keta*). As was expected, results definitely indicated that in normal fertilization spermatozoa are highly activated in the vicinity of micropyle (at the mouth part of micropyle) and enter into the canal to fill it within few seconds, while it is not the case when clacium ions are absent in medium : i.e., under the latter condition the entry of few spermatozoa into the canal occured, probably owing to their random movement without positive reaction to the egg, and no penetration of spermatozoön into the egg cortex took palce ; and such failure of fertilization was also quite reversible as in case of herring. Thus it may be said that the entrance of fish spermatozoa in the eggs at the time of normal fertilization is not induced by random movement of spermatozoa but by their positive movement towards the egg, and for this movement the presence of calcium ions is of great importance.

Loeb ('14, '15) has stated that sea urchin spermatozoa are active even in Ca-free sea water in which no fertilization occurs. He is of poinion from this fact that the failure of fertilization under this condition may be due to some physical changes (such as surface tension) provoked in the surface of egg and of spermatozoön. This fact has been re-examined and ascertained by many workers, but unfortunately, on the behavior of spermatozoa in these cases careful observation has not been performed. To compare with the case of fish eggs mentioned above

some observations were performed with the eggs of sea urchins, *Strongylocentrotus nudus* and *S. intermedius* (Yanagimachi '53 b). When sea water was free or lacking in calcium ions no boring movement of spermatozoa was found on egg surface and none of the spermatozoa penetrated into the egg. On adding a small amount of $CaCl_2$ solution, however, they began the boring movement *with higher activity* and all the eggs were fertilized. This fact has been more clearly demonstrated when spermatozoa which had already lost their active movemt but had still sufficient power of fertilization, were used for insemination.

The conclution drawn from the case of herring may be, therefore, to an extent generalized; in the process of normal fertilization the positive movement of spermatozoön to the egg, which may be induced by a sperm activating property of the latter, is the prerequisite, in respect to which calcium ions play an important role.

Regarding egg activation, T. Yamamoto ('44) has pointed out in the case of *Oryzias* that a parthenogenetic agent, pricking, when applied to the egg under the Ca-free condition loses its effectiveness, but other agents such as heat or chemicals induce activation under the same condition, and also in the salmon egg it is reported that activation by water occurs under Ca-free condition (Kusa '50, Kanoh '52). In sea urchins, Moser ('39) who studies in the egg of *Arbacia*, has indicated the necessity of calcium ions for egg activation by parthenogenetic agents, whereas in the eggs of *Strongylocentrotus pulcherrimus* and *Heliocidalis crassipina* the opposite result was obtained by Sugiyama ('49). These facts indicate, therefore, that calcium ions are not generally indispensable to egg activation. But since the herring egg was not activated by pricking in the absence of calcium ions, as mentioned already, it may be supposed at least in the case of herring that calcium ions exert certain effect upon the egg cytoplasm, accordingly failure of fertilization of herring egg in Ca-free condition may be partly due to the effect of calcium deficiency upon the egg itself.

Summary

(1) Spermatozoa of herring (*Clupea pallasii*) are not active when they are discharged in sea water or exposed to the Ringer's solution, but highly activated when they come to contact with the egg surface, especially in the vicinity of the micropyle. Then they penetrate into the cortex through the micropyle. This fact suggests that egg possesses a pronounced property to activate the sperm in the vicinity of the micropyle.

(2) Under the Ca-free condition such an activation of spermatozoa in the egg surface is not shown, resulting in the failure of sperm entry, while in addition of a small amount of $CaCl_2$, they begin active movement and penetrate into the egg cortex. Thus it may be said that calcium ions play an important role in activation of spermatozoa. In other words, the sperm activating property of

egg surface is to be inhibited under Ca-free condition. Calcium ions can be replaced by magnecium ions, though the latter are less effective.

(3) From the additional observations made in the eggs of other fishes (*Oryzias latipes* and *Oncorhynchus keta*) and of sea urchins (*Strongylocentrotus nudus* and *S. intermedius*), it is concluded that in the process of normal fertilization the positive movement of the spermatozoön towards the egg is of prerequisite, and calcium ions play an important role.

(4) Furthermore, the failure of fertilization in herring eggs under Ca-free condition may be partly due to the effect of calcium deficiency upon the egg itself, because no egg is activated by parthenogenetic agent (pricking) under the same condition.

Literature cited

Kanoh, Y. 1949. Über den japanischen Hering (*Clupea pallassii* Cuvier et Valenc.). 1. Morphologie des reifen Eies. Cytologia, 15, 138–144.

———— 1952. Calcium problem in the activation of dog-salmon egg. (an abstract in Japanese). Zool. Mag., 61, 94.

———— 1953. Über den japanischen Hering (*Clupea pallasii* Cuvier et Valenc.). 11. Veränderung im Ei bei der Befruchtung oder Aktivierung. Cytologia, 18, 67–79.

Kusa, M. 1950. A preliminary note on the fertilization in egg of salmon (*Oncorhynchus keta*). (Japanese with English Résumé) Zool. Mag., 59, 240–241.

Loeb, J. 1914. On some non-specific factors for the entrance of the spermatozoön into the egg. Scinece, 15, 316–318.

———— 1915. On the nature of conditions which determine or prevent the entrance of the spermatozoön into the egg. Amer. Nat., 49, 257–285.

Moser, F. 1939. Studies on a cortical layer response to stimulating agents in *Arbacia* egg. 11. Response to chemical and physical agents. J. Exp. Zool., 80, 447–472.

Sugiyama, M. 1949. Studies on the artificial parthenogenesis in the sea urchin eggs. XII. Effect of Ca-defect. Zool. Mag., 58, 183–184. (in Japanese with English Résumé).

Vasseur, E. 1949 a. Effect of calcium ions on the agglutination in *Strongylocentrotus droebachiensis* Müll. Arkiv Kemi, 1, 105–116.

———— 1949 b. Effect of sea-urchin jelly-coat solution and calcium ions on the oxygen uptake of sea urchin sperm. Ibid, 1, 393–399.

Yamamoto, K. 1952. Studies on fertilization of the egg of the flounder. 11. The morphological structure of the micropyle and its behavior in response to sperm entry. Cytologia, 16, 302–306.

Yamamoto, T. 1944. Physiological studies on fertilization and activation of fish egg. 1. Response of cortical layer of egg of *Oryzias latipes* to insemination and to artificial stimulations. Annot. Zool. Jap., 22, 109–136.

Yanagimachi, R. 1953a. Effect of environmental salt concentration on fertilizability of herring gametes. J. Fac. Sci. Hokkaido Univ., Ser. VI (Zool.), 11, 139–144.

———— 1953 b. A note on the so-called "boring movement" of sea urchin spermatozoa. (Japanese with English Résumé). Zool. Mag., 62, 220–224.