# Studies on the Fertilization of Pelecypod Gametes—II.

## Facilitation of Fertilization by Attachment to An Egg of Plural Spermatozoa.

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**Abstract**

The Japanese rock oyster, *Crassostrea echinata*, was mainly used as materials. From the morphology of the attached and the once-attached and later expelled spermatozoa, it is inferred that, on attachment, every spermatozoon will effect stimulation to the egg through the acrosome filament. The rate of fertilization was studied with varying sperm concentrations or at different intervals after insemination. It was found that some of the eggs remained unfertilized under circumstances which satisfied attachment of at least a single, probably several, spermatozoa to every one of the eggs. Fertilization in these unfertilized eggs was accomplished on subsequent sperm attachment. This problem was further approached in this paper.

Since an unfertilized egg is a kind of irritable systems which is highly sensitive to sperm stimulation, some excitation should be evoked in it with stimulation from any functional spermatozoon. If the stimulation be subliminal, the excited state will facilitate the initiation of fertilization impulse by an immediately following attachment of another spermatozoon. The writer believes this is the most plausible explanation for the fact in question. Some pieces of supporting evidence have been presented.

It is likely the number of spermatozoa which is needed to achieve fertilization correlates to the grade of maturity of the egg and to the strength of stimulus from spermatozoa, both of which will vary individually.

It is a frequent experience in the course of embryological works that a slight increase above an ordinary concentration of sperm produces a higher rate of fertilization. Mechanisms involved in this phenomenon have been approached in the fertilization of the Japanese rock oyster, *Crassostrea echinata*.

In the fertilization of an oyster egg, spermatozoa become attached to the vitelline membrane on undergoing the acrosome reaction (Dan and Wada, 1955). Although all but one spermatozoon very soon lose their close contact and become separated from the egg membrane, in the initial phase of attachment no differences can be detected between the fertilizing and the non-fertilizing spermatozoa; in both of them, the acrosome filament extruded from the sperm head pierces the egg cytoplasm through the perivitelline layer.

Since the attachment of a fertilizing spermatozoon activates the egg, that of other spermatozoa will very likely induce somewhat similar or other responses in the egg protoplasm. It is possible an excitation thus evoked may facilitate the

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initiation of fertilization impulse by the subsequent attachment of a fertilizing spermatozoon.

From this point of view, experiments were performed to examine whether attachment of spermatozoa prior to a fertilizing one will facilitate fertilization. Although a brief summary of the experiments was published previously in Japanese (Wada, 1956), more detailed accounts will be described in this paper. In addition, some pertinent observations on the behaviour of sperm in fertilization will be included in the paper.

The experiments were carried out at the Misaki Marine Biological Station of Tokyo University, Miura-Shi, Japan. The writer is deeply indebted to the director and other staff of the Station for use of laboratory facilities.

## Materials and Methods

The Japanese rock oyster, *Crassostrea echinata* (Quoy et Gaimard), was mainly used as materials. The animals were obtained in a close vicinity of the laboratory. Eggs expressed from the excised gonad were repeatedly washed with filtered fresh sea water to remove small-sized gametes and other tissues cells as well as the body fluid. The washed eggs were kept standing in sea water for about 20 minutes before insemination, because oyster eggs obtained in this way show maximum fertilizability at this time (Wada, 1961). The egg, when it becomes spherical, measures about 50 microns in diameter. It lacks in any coating structures outside the vitelline membrane.

Expressed sperm from the dissected gonad were used throughout the experiments. The spermatozoon measures about 2.5 microns in the diameter of the head region, with a tail measuring about 45 microns in length. Attachment and other behaviours of spermatozoa were observed with phase-contrast under $\times 97$ and $\times 43$ objectives. Entry of the sperm head region (including the midpiece) into the egg required four to six minutes at a temperature of 25°C.

In order to limit the length of the sperm-egg interaction time, the hypotonic method (Rothschild and Swann, 1951) was adopted. The eggs, after insemination and vigorous stir, were exposed to 10 or 30 per cent sea water for 30 to 60 seconds and transferred to a large volume of ordinary sea water. Preliminary experiments showed that such treatments immobilize spermatozoa through destruction of their tails but impair neither the entry of already-attached sperm nor the fertilizability of the eggs, and that a diluted medium up to 90 per cent sea water does not have any effects on fertilization and cleavage.

Since it was previously found that oyster spermatozoa readily undergo the acrosome reaction on contact with various kinds of objects and that once-reacted spermatozoa are devoid of fertilization capacity, cares were paid to minimize glass- and air-water interface and to make the number of eggs constant per volume of sperm-egg mixtures in a series of experiments.

The rate of fertilization was measured by counting 300 or 400 eggs when the fertilized ones were at the period of cleavage. The sperm concentration in "dry sperm" or "drained sperm" was adopted as unity in expressing the density of sperm suspension.

Besides the rock oyster, *Mactra veneriformis* and *Mytilus edulis* were used to obtain some corroborative evidence on the behaviour of spermatozoa in fertilization. Discharged gametes were employed in the case of *Mytilus*.

## Experiments and Observations

**1. Penetration of acrosome filament into the egg.** Oyster spermatozoa undergo the acrosome reaction when they become attached to the egg membrane. Under living conditions, it is clearly discernible that the attached spermatozoon has lost the acrosome and the radially inward extending, gray-appearing (with dark phase-contrast) conical region under the acrosome has become even more clearly visible and larger. The acrosome filament, as observed in a spermatozoon reacted on contact with glass surface, measures about six microns in length (Dan and Wada, 1955).

Although the state of the filament of a closely attached spermatozoon could not be examined under an intact condition, it seems very certain that the filament penetrates the perivitelline layer into the egg cytoplasm. This is inferable from the following observations.

Eggs, either unfertilized or fertilized, were lightly homogenized with a hand glass-homogenizer and washed a few times with sea water. This procedure gave a bladderlike preparation of egg-membrane about 0.5 to 1 micron thick (the vitelline membrane plus the perivitelline layer; these structures together may be called the vitelline membrane) and almost devoid of the egg cytoplasm. When sperm were added to the preparation, the acrosome filaments of attached spermatozoa were clearly visible extending to the inside through the egg membrane. The filament in this case was nearly as long as or somewhat shorter than in the case of reaction on glass surface.

Furthermore, on an intact egg, of spermatozoa which first made a close attachment to the egg all but one soon became loose and detached. Within ten to twenty seconds after the attachment, the attached supernumerary spermatozoa were pushed off by about three microns from the vitelline membrane, each with a stiff connecting filament between. The filament is doubtless the acrosome filament. It is unlikely the filament is produced as the spermatozoon retreats. It is much more likely the connecting filament was once within the egg cytoplasm.

These observations on spermatozoa attached to the egg-membrane preparation and the intact egg will lead a conclusion that both fertilizing and non-fertilizing spermatozoa extrude the acrosome filament into the egg cytoplasm.

Meanwhile, the expelled spermatozoa, after a few spasmodic twitches, became

completely detached from the egg. This occurred in about a minute after the first-phase detachment. The acrosome filament of the released spermatozoon was found to be decidedly shorter than that of a gamete reacted on glass surface, the former measuring about three microns in length. Presumably the distal part of the filament must have been left and digested in the egg protoplasm.

It has not infrequently been observed that the detached and released spermatozoa become attached to glass surface with the whole length of the filament, leaving the rest of the body freely moving around. This may suggest that the surface of the filament has become adhesive during the stay in the egg. When an egg-membrane preparation was inseminated, some of granular inclusions which may remain inside the egg-membrane bladder were found to become attached to the extended acrosome filaments. Under such circumstances the filaments gradually became faint and finally were dissolved completely. This occurred in from 20 to 40 minutes after insemination. A similar phenomenon was observed with sperm which had once become attached to the intact egg and later released. It seems likely enzymes in the egg cytoplasm can digest the acrosome filament.

In this regard, some corroborative pieces of evidence have been obtained with the supernumerary spermatozoa in the fertilization of *Mactra veneriformis* and of *Mytilus edulis*.

The acrosome filament of *Mactra* sperm is rather short, measuring about three microns in length. The *Mactra* egg is coated with a jelly-hull about 15 microns thick. Supernumerary spermatozoa, after making a close attachment to the egg, became loose and separated by about one micron from the vitelline membrane in about 10 seconds after the attachment. Up to this stage the behaviour is quite the same as in *Crassostrea* sperm. In about 50 seconds after attachment, however, the slightly separated spermatozoa were further pushed off radially and rearwards to the outermost margin of the jelly-hull. A spermatozoon in this state was found to poccess a rigid acrosome filament of about one micron in length, and, in addition, a much longer and even more slender filament was found running straightly from the tip of the just-said acrosome filament to the surface of the vitelline membrane. It is likely the long and slender part of the filament is formed by stretch of a softened part of the original filament. This may suggest the digestion of filament inside the egg.

In *Mytilus edulis*, spermatozoa produce a much longer acrosome filament, measuring about 12 microns in length, and the jelly-hull of the egg is neither so thick, measuring about from five to ten microns in thickness, nor so tenacious as that of the *Mactra* egg. These circumstances with *Mytilus* gametes may reflect the facts that supernumerary spermatozoa are pushed off more markedly in the distance as compared to *Crassostrea* spermatozoa and that the expulsion of the detached spermatozoa by the jelly-hull is barely or hardly recognizable. However, here a similar situation as in *Mactra* holds with the state of the acrosome filament of expelled sperm.

The acrosome filament of an expelled *Mytilus* spermatozoon consists of a proximal rigid part and a distal, pliant and very adhesive, probably partly digested, part. The rigid part of the filament measured about four microns in length, being decidedly shorter than that of sperm reacted on contact with glass surface (*vid.* Figure 1 in Wada, 1955). It seems certain the change in physical properties of the distal part of the filament has been produced by some chemical transformation occurring while it is in the egg cytoplasm.

The foregoing observations, especially those on the state of the acrosome filament of expelled spermatozoa, seem to among other things indicate that spermatozoa which become attached to the egg, if not a fertilizing one, establish some chemical association or combination with the egg protoplasm through their acrosome filaments.

It will not be out of place to mention that expelled spermatozoa become only sluggishly motile and are deprived of the fertilizing capacity, as is indicated by the fact that they never make attachment once more.

**2. The rate of fertilization and the length of sperm-egg interaction time.** If the oyster egg requires attachment of plural spermatozoa to become fertilized, the percentage of fertilization should be increased in accord with the length of sperm-egg interaction time for a given sperm density.

In the experiments performed along this line, the sperm density in sperm-egg mixtures was adapted to be $4 \times 10^{-5}$, i.e. 25,000 time dilution of the dry sperm. In ordinary fertilization, about a quarter of this concentration almost invariably gives a maximum rate of fertilization.

Two sorts of experiments were carried out. In one of them, the sperm-egg interaction time was delimited by the hypotonic method. In the other, further chances of attachment of sperm to eggs were reduced by diluting the mixtures of gametes with ordinary sea water to 400 time volumes at varying lengths (between 5 and 60 seconds) of the sperm-egg interaction time. The latter experiments were performed in order to eliminate any possible injurious effects of the hypotonic treatment on sperm entry and ensuing development, although it was fairly certain that such apprehensions were unnecessary. Results of seven series of experiments along this line are presented in Figure 1.

Even though the procedure, dilution of sperm densities with a bulk of sea water, still permits later sperm attachments, the results show that, at least in certain batches of eggs, some of fertilizable eggs remain unfertilized after five to ten seconds' stay in the denser sperm suspension.

In the following series of experiments, sperm-egg mixtures were exposed to hypotonic (30 per cent) sea water for 30 to 60 seconds to deprive still-free spermatozoa of their fertilizing capacity at varying lengths (from 5 to 60 seconds) of the sperm-egg interaction time. Results of three series of experiments along this line are depicted in Figure 2.

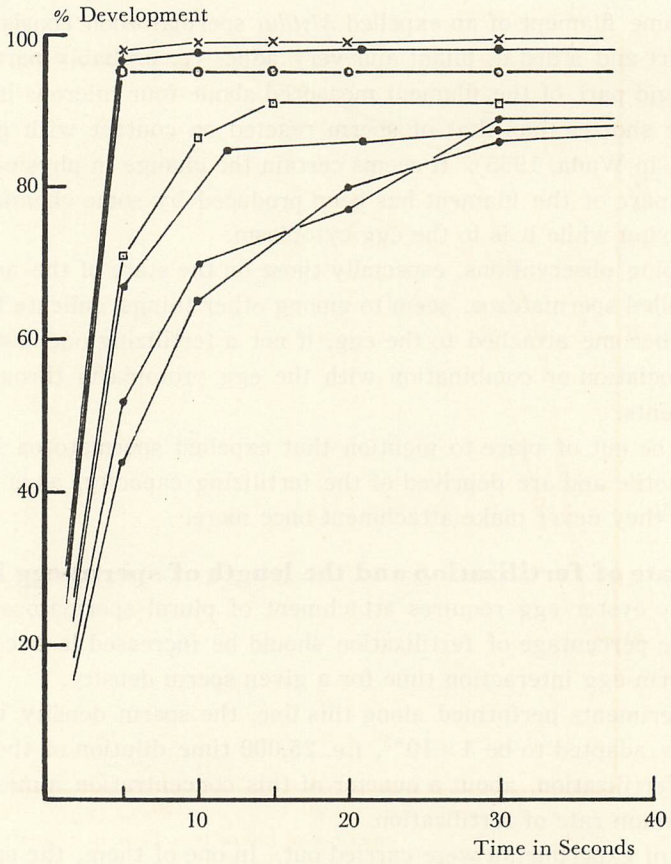


Figure 1. Percentage fertilized eggs as measured by cleavage after various times of contact between unfertilized eggs and spermatozoa (density, $4 \times 10^{-5}$ of dry sperm) of *Crassostrea echinata* followed by dilution with a large volume of fresh sea water.

In the figure, the same symbols as in Figure 1 indicate that the two series of experiments were performed at the same time and with the same batches of eggs and sperm. Comparison of two pairs of experiments represented in the figures by open circles and crosses respectively shows that, in Figure 1, a practically maximum percentage of fertilization was reached in five seconds' exposure of eggs to the original sperm suspension, while the corresponding values in Figure 2 are significantly less. This of course is due to the difference in the treatments.

The results of experiments shown in the two figures clearly indicate that the maximum rate of fertilization is only attainable by a rather long sperm-egg interaction even if with a fairly high sperm density. This will lead to a postulation that a fairly good number of fertilizable eggs will not be fertilized by a single or a few spermatozoa.

In order to examine whether spermatozoa have become attached to every of

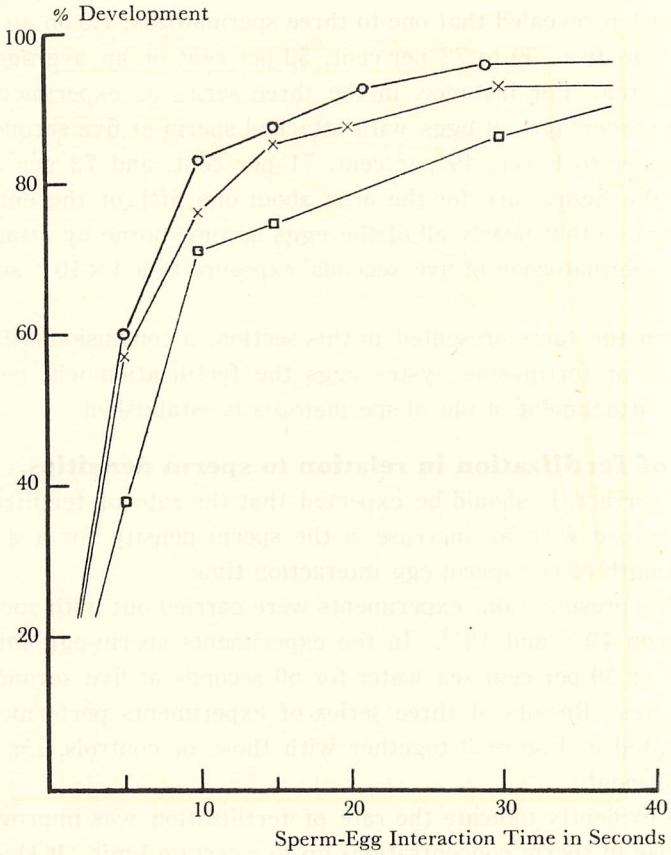


Figure 2. Percentage fertilized eggs as measured by cleavage after various times of contact between unfertilized eggs and spermatozoa (density, $4 \times 10^{-5}$ of dry sperm) of *Crassostrea echinata* before hypotonic treatment. Same symbols as those in Figure 1 denote the two experiments were carried out simultaneously and with the same batches of eggs and sperm.

the eggs employed in the above experimentation before the dilution or the hypotonicity treatment, a following observation was made. Along with the experiments, aliquots of the sperm-egg mixtures used in the experiments were fixed with neutral formalin at five seconds of the interaction. Eggs with firmly attached sperm on their optically equatorial and subequatorial surface were counted together with the number of attached spermatozoa. Properly attached spermatozoa can be identified as such by the acrosome reaction. Since fixation with neutral formalin may effect the sperm to undergo the partial acrosome reaction, only the firmly attached spermatozoa were taken as valid. The examination was made with a phase-contrast $\times 43$ objective, and the surveyed area covered about 20 per cent of the total egg surface.

The observation revealed that one to three spermatozoa, 1.3 on an average, became attached to from 39 to 72 per cent, 53 per cent on an average, of eggs on the surveyed area. For instance, in the three series of experiments shown in Figure 2, the percentages of eggs with attached sperm at five second interaction were, from upper to lower, 49 per cent, 71 per cent, and 72 per cent, respectively. Since the figures are for the area about one fifth of the entire egg surface, it is inferable that nearly all of the eggs become borne by attachment of at least a single spermatozoon in five seconds' exposure to a $4 \times 10^{-5}$ sperm suspension.

Judging from the facts presented in this section, a conclusion will be reached that in certain of fertilizable oyster eggs the fertilization will not be accomplished before attachment of plural spermatozoa is established.

**3. Rates of fertilization in relation to sperm densities.** If the above conclusion is correct, it should be expected that the rate of fertilization will be improved in accord with an increase in the sperm density for a given, neccessarily brief, length of the sperm-egg interaction time.

To prove this presumption, experiments were carried out with sperm densities ranging between $10^{-5}$ and $10^{-3}$. In the experiments sperm-egg mixtures were exposed to 10 or 30 per cent sea water for 60 seconds at five second interaction between gametes. Results of three series of experiments performed along this line are presented in Figure 3 together with those of controls, i.e. without the hypotonic treatment.

The results evidently indicate the rate of fertilization was improved in accord with an increase in sperm concentrations up to a certain limit. It should be noted that about 50 per cent of the fertilizable eggs remained unfertilized after five seconds' exposure to such a rather dense sperm suspension as of $10^{-4.5}$.

These experiments were carried out at temperatures between 28°C. and 29°C. At these temperatures, entry of the sperm head into the egg usually took about four minutes, two minutes at the quickest. An observation revealed that at ten to fifteen seconds after insemination (this is the possible shortest time to bring an inseminated eggs under a high-power objective) the head of a fertilizing spermatozoon was still situated well outside the vitelline membrane. Meanwhile, a treatment of spermatozoa with 10 to 30 per cent sea water caused a swelling of the acrosomal region and particularly a crook of their tails. Most frequently, the tail was bent at the midway upon itself and further showed some convolutions around the proximal part; sometimes it became **V**-shaped with the angle near the midpiece.

During the course of present experimentation, entry of sperm into eggs was often observed with the crooked tail as it stands. Furthermore, as is shown in Figures 2 and 3, high rate of fertilization was obtained in some lots of eggs which had been treated hypotonic sea water at five or ten seconds after insemination. These facts present unequivocal evidence which shows sperm entry can be

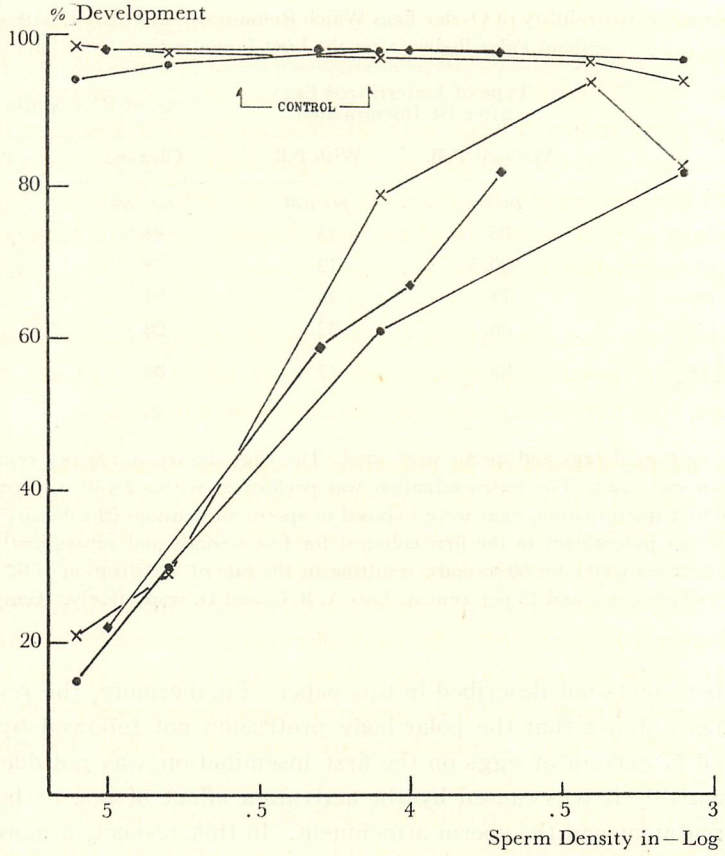


Figure 3. Percentage fertilization as measured by cleavage in *Crassostrea echinata* eggs after five second contact with various concentrations of sperm followed by hypotonic treatment. Upper three lines are for the control where the length of contact time with sperm was not limited (without hypotonic treatment).

accomplished without aid of a driving force of the sperm tail after attachment.

In the next experimentation, eggs which had remained uncleaved in the preceding experiments were re-inseminated with $2 \times 10^{-5}$ sperm. These eggs included those, amounting to from 15 to 30 per cent of a whole, which had extruded one or two polar bodies without further development. Upon the re-insemination the great majority of them, including those with previously extruded polar bodies, were fertilized and showed monospermic cleavage. Results of a typical experiment along this line are presented in Table 1.

The results evidently indicate that eggs not fertilized at the first insemination possessed sufficient fertilizability. This is also inferable from the higher rate of fertilization in the control lots in the preceding series of experiments (Figure 3). The present experiment further proves the hypotonic treatment did not impair fertilizability of the eggs, although this has previously been established in pre-

Table 1. Fertilizability of Oyster Eggs Which Remained Unfertilized, with or without Polar Bodies, after the First Insemination

| Lot No. | Type of Unfertilized Eggs after 1st Insemination | | Type of Re-inseminated Eggs | |
|---|---|---|---|---|
| | Without P.B. | With P.B. | Cleaved | P.B. only |
| | *per cent* | *per cent* | *per cent* | *per cent* |
| A ($8\times10^{-4}$) | 85 | 15 | 84.5 | 3 |
| B ($8\times10^{-5}$) | 80.5 | 19.5 | 89 | 6.5 |
| C ($4\times10^{-5}$) | 74 | 26 | 94 | 2.5 |
| D ($8\times10^{-6}$) | 68 | 32 | 98 | 1 |
| Control 1 (hypotonic treatment only) | 83 | 17 | 88 | 5 |
| Control 2 | — | — | 95 | 0 |

The same batches of eggs and sperm were used. The eggs showed a 100 per cent nuclear breakdown in sea water. The re-insemination was performed with a $2\times10^{-5}$ sperm suspension. In the first insemination, eggs were exposed to sperm suspensions (the density is shown in the figures in parentheses in the first column) for five seconds and subsequently treated with 10 per cent sea water for 60 seconds, resulting in the rate of fertilization of 82 per cent, 61 per cent, 30 per cent, and 15 per cent in Lots A, B, C, and D, respectively. Temperature: 27.5-28.0°C.

liminary experiments not described in this paper. Furthermore, the re-insemination experiment shows that the polar body protrusion not followed by cleavage as it occurred in certain of eggs on the first insemination was not due to sperm entry. Presumably it was caused by the activation effect of one or both of the hypotonic treatment and the sperm attachment. In this respect, a more decisive piece of evidence will be presented in the next section.

**4. Facilitation by sperm attachment of parthenogenetic activation of eggs.** From the foregoing accounts it becomes clear that attachment of a single or a few spermatozoa to an egg does not necessarily establish fertilization impulse in the latter. Since the egg is a kind of irritable system, it seems permissible to assume that stimulation by sperm attachment, if insufficient to initiate fertilization impulse, will to some extent raise the irritability of the egg to make it more readily activated. Experiments were performed to examine whether "infertile" sperm attachment facilitates parthenogenetic activation of the egg.

Because of its being a rather weak activating agent but an effective measure to deprive free sperm of the fertilizing power, an exposure of briefly inseminated eggs to hypotonic sea water was adopted for the purpose. Parallel experiments were made to compare the percentages of activated eggs between the following two types of treatments. In one type, unfertilized eggs were exposed to 10 per cent sea water for one or two minutes. In the other, preceding the same hypotonic treatment, eggs were exposed for three of five seconds to a $5\times10^{-5}$ or $10^{-5}$ sperm suspension. In this case, eggs which underwent cleavage were excluded from the counting. It was previously found that intact eggs would never be ac-

tivated beyond polar body protrusion by an exposure to hypotonic sea water. Results of the present experiments are summarized in Table 2.

Table 2. Comparison between the Intact and the Sperm-Attached Oyster Eggs in Parthenogentic Polar Body Protrusion as Induced by Hypotonic Treatment

| Lot No. | Polar Body Protrusion | | Insemination | |
|---|---|---|---|---|
| | Not Inseminated | Inseminated* | Sperm Density | Exposure Time |
| | *per cent* | *per cent* | | *second* |
| E—a | 17 | 37 | $5 \times 10^{-5}$ | 5 |
| E—b | 17 | 38 | $10^{-5}$ | 5 |
| F | 2 | 32 | $5 \times 10^{-5}$ | 3 |
| G—a | 1 | 13 | $5 \times 10^{-5}$ | 3 |
| G—b | 1 | 12 | $5 \times 10^{-5}$ | 5 |

The eggs were exposed to 10 per cent sea water for one minute in Lots E-a and E-b, and for two minutes in the other lots. The same batch of eggs was used in Lots E-a and E-b or in Lots G-a and G-b.

* Cleaved eggs were excluded from counting.

A higher rate of polar body extrusion in eggs, excluding cleaved ones, treated with brief insemination and hypotonicity seems to indicate that attachment of non-fertilizing sperm renders the eggs activatable with more ease by a parthenogentic agent. In the present experiments there might be eggs which were activated to undergo parthenogenetic cleavage by a cumulative action of sperm attachment and hypotonicity. However, since any cleaved eggs were excluded from the counting, such an incident, if exists, will rather enhance the validity of the conclusion.

## Discussion

It is a well-established fact that a fertilizing spermatozoon on attachment to an egg incites a chain of fertilization reactions in the latter preceding its entry (e. g., see Runnström et al, 1959). It seems, therefore, quite reasonable to assume that non-fertilizing spermatozoa on attachment likewise stimulate an egg in some way and the egg will respond to the stimulation.

The evidence presented in this paper indicate that stimuli from spermatozoa, either fertilizing or supernumerary, are very probably effected by way of the acrosome filaments extruded into the egg cytoplasm. An observation which demonstrates the acrosome filament in the egg is presented by Colwin and Colwin (1956) in a holothurian.

In oysters and other bivalves, the fertilized egg responds to attached supernumerary sperm with an active expulsion of the proximal half of the acrosome filament to the outside; the distal half of the filament is probably digested later, as suggested in this and previous papers (Wada, 1954, 1955; more detailed accounts

on the behaviour of supernumerary sperm will be published later.).

Responses of the unfertilized egg to the acrosome filament of a fertilizing spermatozoon imply the well-known fertilization reactions and the development of force which is exerted to pull the spermatozoon. The fact that the egg plays an active role in sperm entry is obvious from evidence presented in this paper.

It seems certain the unfertilized egg also responds to the attachment of non-fertilizing spermatozoa before fertilization impulse is established. Rothschild (1953) reports sea-urchin eggs can be activated (swelling of the nucleus) but remain unfertilized by attachment of sperm. In this respect, fertilization in starfish presents a more clear-cut picture.

In the starfish fertilization the spermatozoa undergo the acrosome reaction at the outer margin of the egg jelly-hull and become quiescent (Dan, 1954). The subsequent traversal through the jelly-hull is evidently effected by drawing of the acrosome filament in the egg. Under the present knowledge, Chambers's observations (Chambers, 1923, 1930) indicate that the starfish egg forms fertilization cones where the filaments of a fertilizing and non-fertilizing spermatozoa make contact with or, more exactly, are penetrating the egg. Working on *Asterias amurensis*, the present writer has noticed that several spermatozoa were present well within the jelly-hull of the egg when observed between one and three minutes after insemination. This has been observed with both living and fixed materials (unpublished work). Approach of supernumerary spermatozoa to the vitelline membrane was also observed in *Asterias forbesii* and *A. rubens* by Chambers (1923, 1930). Thus, it seems evident the force to draw the acrosome filament exerts not only on fertilizing but also on non-fertilizing spermatozoa.

A like dynamical system seems to be operating in the fertilization of bivalve eggs. When *Crassostrea echinata* eggs were transferred to a citrated Ca-free sea water within five seconds after insemination, polyspermic fertilization very frequently took place. This is also the case with *Mytilus edulis* eggs. Roughly simultaneous entrance of two or three spermatozoa into an egg could readily be observed with phase-contrast. At a stage corresponding to the trefoil stage, the animal half of a polyspermic egg was frequently divided into three or four blastomeres instead of two in the normal cleavage (unpublished work).

These findings in starfish and bivalve fertilization seem to indicate that non-fertilizing spermatozoa at the time of attachment exert a like stimulation to the egg as does a fertilizing spermatozoon. In anyway, it is obvious that some chemical interaction operates between the egg and the non-fertilizing, attached spermatozoa. The aforesaid finding that the attachment of non-fertilizing spermatozoa facilitates the activation of oyster eggs by hypotonic sea water can be interpreted as another indication of this interaction between the gametes.

According to Allen (1958), in sea-urchin fertilization, initiation of two or more cortical reactions (cortical granule breakdown) by different spermatozoa does not take place, although many spermatozoa attach to the egg surface almost simulta-

neously in ordinary insemination. Since the cortical granule breakdown occurs after the establishment of fertilization impulse, the above statment is not contradictory with this Allen's finding.

From the evidence presented in this paper, it is apparent that attachment of plural spermatozoa is necessary to establish fertilization impulse in certain of oyster eggs.

A case in which the fertilizing spermatozoon is not the earliest attached one has been actually observed by the present writer in *Mactra veneriformis* fertilization. The *Mactra* egg is coated with a jelly-hull about 15 microns thick. In the jelly-hull spermatozoa approach the egg membrane always radially. This permits a close observation on the mode of sperm attachment and detachment. On one occasion, two spermatozoa became attached to an egg on undergoing the acrosome reaction, a few microns apart each other. About 60 seconds later, another spermatozoon became attached, the place of attachment being just opposite to that of the previous two spermatozoa. The first two were soon pushed off radially to the outer surface of the jelly-hull, while the one which became attached later entered the egg (an unpublished observation). In fertilization of a sea urchin, Allen (1954) also reports a case in which the earliest arriving spermatozoon did not enter the egg.

The fact that one-to-one relation between eggs and sperm does not always accomplish fertilization has been noticed for a long time. It may be manifested in an incident where some of the eggs remained unfertilized in a sperm suspension which was fairly diluted but still contained an excess number of spermatozoa (Glaser, 1915) or where the rate of fertilization was under the estimation considering sperm densities, sperm-egg interaction time, etc. (Rothschild, 1954). Cases which presumably imply similar facts can be gathered from the data presented in their papers on sea-urchin fertilization by Rothschild and Swann (1951) and by Hagström and Hagström (1954).

Necessity of plural spermatozoa in fertilization can be explained in several, not mutually exclusive, ways. Hitherto proposed and other probable explanations may be classed into three categories according which one among the state of eggs, the capacity of sperm, and the sperm-egg interaction is their main concern.

Working on *Arbacia*, Glaser (1915) claimed that the egg-coverings cannot be changed by a single spermatozoon to permit its entry and thus a mass influence of sperm is needed in softening of the barrier. Under the present knowledge his reasoning seems quite out of place, although still in 1940's similar opinions were proposed by various authors in mammalian fertilization (*vid.* Mann, 1954).

In his studies on block to polyspermy in sea-urchin eggs, Rothschild (1954, 1956) puts forward an opinion that, on a submicroscopic scale, an egg surface is probably a mosaic of sperm-receptive and non-receptive regions. It seems this postulation does not hold in the case of oyster eggs. In the fertilization of oysters, it is inferable that activation of eggs is initiated by some protoplasmic in-

teraction between the egg cortex and the acrosome filament. The acrosome filament of *Crassostrea echinata* sperm measures about six microns in length when the reaction occurs on glass surface. Although the actual length of a filament when extruded into an intact egg is not known, it will be not less than three microns because the filament of once-attached and later separated supernumerary sperm measures about three microns in the length. It seems least conceivable that the cortical layer of an oyster egg has a radially mosaic structure in the thickness comparable to the length of the acrosome filament.

In text-books on embryology there appear frequent descriptions that sperm entry in molluscan eggs occurs only at the vegetal pole. Oyster eggs fresh from the ovary are usually ovoid in shape, the vegetal pole being more convex than the animal pole. Observations on the place of sperm entry have revealed that it frequently occurred near the animal pole. Even though there might be something like a polar gradient as to reactiveness to sperm entry, it is fairly certain that sperm can enter the egg at any place in *Crassostrea*.

According to Runnström (in Runnström et al, 1959), a certain equatorial or subequatorial region of the sea-urchin egg responds more readily to the fertilizing spermatozoon than do other regions.

Being an integral organism, there naturally should exist an individual difference in fertilizability between eggs. Hultin and Hagström (1956), working on *Paracentrotus* eggs, demonstrate that there are marked variations in the fertilizability and fertilization rate even in selected material. Possibly a fully mature egg may be fertilized by a single spermatozoon, while an underripe egg might require some cumulative stimulation from several spermatozoa before it is fertilized by a single spermatozoon. If the latter be the case, it should be of an essential concern in the present discussion.

In most pelecypods including oyster, eggs are stocked in the ovary under an immature condition (at germinal vesicle stage) and nuclear breakdown and other cytoplasmic changes leading to complete maturity abruptly begin in the gonad shortly before discharge. Therefore, eggs obtainable from the excised gonad are always behind the full maturity except those from an animal in the very act of spawning. In the case of *Crassostrea*, the eggs improve maturity while they are kept standing in sea water for some time (Wada, 1961). It is, therefore, likely that oyster eggs will manifest a more marked individual variation in fertilizability as compared to sea-urchin eggs. In fact, if a careful selection was made of parent oysters, an absolutely hundred per cent fertilization was not so frequently achieved when eggs obtained from the dissected gonad were employed.

Turning now to discussions from the side of sperm, Lillie (1919) and Lillie and Just (1924) were of opinion that the conception, as proposed by Glaser (1915), that an excess number of spermatozoa are needed to achieve a maximum rate of fertilization is based on observations where slightly stale sperm suspensions were used.

As is the case with eggs, it is obvious that among spermatozoa there also exists an individual difference in fertilizing capacity. The acrosome reaction, being a morphological representation of "activation" of sperm (used in the same sense as "activation" of eggs), makes it to some extent possible to analyse individual differences in fertilizing power. Oyster spermatozoa show various grades of the acrosome reaction on glass surface (Dan and Wada, 1955). A perfect acrosome reaction was indicated by complete breakdown of the acrosome and extrusion of a long filament. One of the most imperfect cases of the reaction was represented in a displacement of the acrosome to the periphery of the flattened tip of the nucleus to become ring-shaped, not accompanied by the extrusion of a filament. Various grades of partial reaction between these have been observed to occur. Even among spermatozoa which underwent the apparently perfect acrosome reaction, the filaments differed in the length, ranging from 5 to 7 microns, about six microns on an average.

When oyster spermatozoa become attached to an egg, however, all of them will undergo the complete acrosome reaction, since partially reacted spermatozoa have been rarely found in the expelled supernumerary sperm. Although it is likely they still differ in fertilization capacity among themselves, it appears least probable that some of them wholly lack in the capacity to fertilize a fully mature egg.

From the evidence presented in this paper, it is apparent that, on certain occasions, some spermatozoa will make a functional attachment to an egg prior to a fertilizing one and that these non-fertilizing spermatozoa will stimulate the egg. Since an unfertilized egg is an irritable system very sensitive to sperm stimulus, the latter, if subliminal, should evoke some excitation in the former. It seems quite permissible to assume that the excited state facilitates initiation of fertilization impulse by the subsequent stimulus from a fertilizing spermatozoon.

It appears the last-mentioned is the most plausible explanation for the phenomenon in question. This view can explain the hitherto obtained facts without any contradiction. The number of spermatozoa necessary to initiate fertilization impulse in an egg presumably correlates to the grade of maturity of the particular egg. A fully mature egg may be fertilized by a single spermatozoon, while an underripe egg probably requires more than one to become fertilized. In other words, the quality of the eggs in a fertilization mixture will be altered with time by direct contact with the spermatozoa while they are remaining unfertilized. This may be especially the case with *Crassostrea* eggs, because there exists, as stated before, a marked difference in the grade of maturity between the naturally discharged eggs and the artificially obtainable ones from the excised gonad. In addition, the stimulus from sperm probably varies in strength individually; this will also reflect the necessary number of spermatozoa. Furthermore, the time relation between the successive attachments of sperm might be another factor to determine the number, although this is altogether in want of experimental sup-

port.

In their pioneer work on the fertilization rate in sea-urchin eggs, Rothschild and Swann (1951) report that from several to nearly a hundred sperm-egg collisions are necessary to achieve fertilization. The marked variation in the number, which should be nearly constant on the basis of the proposed collision theory, is explained by the authors as probably due to sperm-sperm interaction of a physical nature. A piece of evidence against this assumption has been presented by Allen and Hagström (in Runnström et al, 1959). Hultin and Hagström (1955, 1956) and Hultin (1956) considered the fact a definite proof indicating an inadequateness of the collision hypothesis, which has led Hultin to propose the orbit hypothesis in the place.

In anyway, the variation in number and the large figure obtained by the English authors in the necessary sperm-egg collisions preceding the "successful" one may be, at least partly, explained from the presently proposed view on the role of non-fertilizing spermatozoa.

It seems worth saying that one of the aspects to be considered but hitherto have been neglected in a study of fertilization rate is the irreversible nature of sperm attachment. From the morphology of expelled supernumerary spermatozoa, it is obvious that once-attached spermatozoa are never able to attach again. In fertilization mixtures, functional sperm ever continue to diminish in number, and an equilibrium state between eggs and sperm will not, therefore, be reached before all of the fertile spermatozoa have undergone the acrosome reaction on the eggs or on the interface between water and air or glass.

Finally, it will not be out of place to refer to the well-known fact that use of an extraordinarily dense sperm suspension very often results in an improvement in the rate of inter-species fertilization. It seems here works par excellence a quite different mechanism from straight fertilization. In the cross between *Crassostrea echinata* eggs and *C. gigas* sperm, for example, it was found that the eggs are provided with a substance or property which inhibits the acrosome reaction or attachment of foreign sperm on their surface. However, the inhibition is not perfect. An observation shows the heterologous combination gave an exceedingly low rate of sperm attachment as compared to the combination of homologous gametes (Wada, 1960). These circumstances clearly explain the improved rate of the cross-fertilization with an unusually dense sperm suspension.

Furthermore, since the stimulus from attached spermatozoa must be less effective to foreign eggs than to self-species ones, a much greater number of fertile spermatozoa might be needed in heterologous fertilization than in homologous fertilization.

## Conclusion

The rate of fertilization in *Crassostrea* eggs is improved by attachment of plural spermatozoa. As the most plausible exaplanation for this fact the following wor-

king hypothesis is proposed: Attachment of a non-fertilizing spermatozoon evokes some excitation in an unfertilized egg, the stimulus being effected with the acrosome filament extruded into the egg cytoplasm. This will facilitate the initiation of fertilization impulse by the following attachment of a fertilizing spermatozoon. Grade of maturity of an egg and strength of stimulus from attached sperm will correlate to the number of spermatozoa which is necessary to accomplish fertilization in the particular egg.

## Summary

1. The Japanese rock oyster, *Crassostrea echinata*, was mainly used as materials. Some observations on the behaviour of spermatozoa in fertilization were made also with gametes of *Mactra veneriformis* and *Mytilus edulis*.

2. On undergoing the acrosome reaction on the vitelline membrane, the spermatozoon extrudes a filament into the egg. Pieces of evidence are presented which indicate that the filament probably establishes a protoplasmic connection with the egg. This is the case not only with fertilizing but also with non-fertilizing spermatozoa.

3. The rate of fertilization is improved by attachment of plural spermatozoa to an egg. This is inferable from the fact that some of the fertilizable eggs remain unfertilized under circumstances where the exposure time to a sperm suspension and the sperm density are sufficient or more than sufficient for attachment of at least a single spermatozoon to every one of the eggs.

4. It was found that a brief exposure of eggs to a sperm suspension facilitates the parthenogenetic activation by a subsequent treatment with hypotonic sea water. This seems to indicate the stimulus from attached spermatozoa, though subliminal to initiate fertilization impulse, will to some extent raise the irritability of the egg.

5. Various possible explanations for the fact in question have been discussed. The most plausible one implies a conception that the stimulus from non-fertilizing, attached spermatozoa evokes in the egg some excitation which will facilitate the initiation of fertilization impulse by the immediately following attachment of the fertilizing spermatozoon.

6. The number of spermatozoa needed to accomplish fertilization probably depends mainly on the grade of maturity of the particular egg and, to a less degree, on the amount of stimulus from attached spermatozoa.

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