Fertilizability of Crassostrea and Pinctada Eggs as Related to Germinal Vesicle Breakdown

Seiji K. WADA*

Abstract

1. Rock oyster, *Grassostrea echinata*, and three species of pearl oyster, *Pinctada fucata*, *P. maxima* and *Pteria penguin*, were used as materials.

2. The discharged eggs of *C. echinat* and *P. fucata* are at the first maturation prophase. The eggs of these species obtained from the excised gonads, though they are underripe when stocked in the ovaries, can be rendered fertilizable before the nucleus shows any signs of breaking down.

3. The oyster eggs are progressively improved in fertilizability while kept standing in sea water. A similar situation has been obtained in *P. fucata* eggs if they are exposed to ammoniacal sea water. The pearl oyster eggs which have undergone the nuclear breakdown in plain sea water are not always fertilizable.

4. The eggs of *Pinctada maxima* and *Pteria penguin* which are obtainable from the excised gonads become mature if they are kept standing in ammoniacal sea water but not in plain sea water. The eggs of these forms are not rendered fertilizable until the beginning of the nuclear breakdown.

5. Relationships between the cortex maturation and the nuclear breakdown have been discussed. It has been postulated that these two phenomena, though very probably they are started by some identical reaction(s), are not linked with a strict cause-and-effect relation, but that each is accomplished through a divergent way in the later part. This view seems to be able to explain the known facts on a much simpler assumption as compared with the classic notion which implies the cortex maturation is induced by some substances liberated from the nucleus.

In a majority of marine invertebrates the eggs are fertilized after the breakdown of germinal vesicle but before the extrusion of the first polar body. In these forms, it has been believed that mixing of nuclear substances with cytoplasm in the egg is prerequisite to normal sperm entry. In *Crassostrea* (common oyster) and *Pinctada* (pearl oyster), the eggs discharged under quasi-natural stimuli such as sperm suspension plus warm sea water are at a stage where the nuclear membrane has just completely been dissolved. In other words, natural sperm entry takes place at this stage.

An intact germinal vesicle is, on the other hand, always preset in the egg which is obtainable by expressing the excised gonad, except in the case in which the animal is in the very act of spawning. It is, therefore, permissible to say that the eggs are usually stocked in the ovary under a somewhat underripe condition. Such eggs are, however, rendered fertilizable by an exposure to plain sea water or ammoniacal sea water even while the germinal vesicle remains intact.

* From the Faculty of Fisheries, Kagoshima University, Kagoshima-Shi, Japan.

This and other facts which will be described in this paper seem to indicate that a protoplasmic maturation of egg cortex which permits normal sperm entry can be achieved more or less, if not entirely, independently of the nuclear breakdown.

The experiments were mainly carried out at the Misaki Marine Biological Station of Tokyo University. The writer is deeply indebted to the director and other staff of the station for use of laboratory facilities. Some additional experiments were performed at Thursday Island, Queensland, Australia and at Amami-Oshima, Kagoshima Prefecture. The writer's indebtedness is also due to Mr. B. A. Norman, Dr. J. S. Hynd, Mr. T. Kuribayashi, and Mr. K. Kozuka for kindly providing him opportunities to do experiments in these places.

Materials and Methods

The Japanese common rock oyster, *Crassostrea echinata* (Quoy et Germard), and the Japanese pearl oyster, *Pinctada fucata* (Gould) (syn.: *P. martensii* (Dunker)), were mainly used as materials; some experiments were also made with other species of pearl oysters, *Pinctada maxima* (Jameson) and *Pteria penguin* (Roding).

Eggs obtained from the excised gonads were thoroughly washed with filtered sea water before use. Hyperalkaline sea water in which the concentration of added alkali was 0.5×10^{-3}N was, unless otherwise stated, used throughout the experiments. Rates of fertilization were calculated by counting from 200 to 400 eggs when the normally developing eggs were at cleavage stages. A special care was paid to make sperm concentrations roughly equal in all of the fertilization dishes in a series of experiments.

Fertilizability of Crassostrea Eggs with Intact Germinal Vesicles

In the eggs of *C. echinata* when discharged or immediately before the shedding, the hyaline nucleoplasm is observable still occupying the central area, but the nuclear membrane has been completely dissolved. Eggs are, however, fertilizable before the nucleus shows any signs of breakdown. This will be affirmed by the following facts.

When a batch of eggs fresh from the excised gonad were inseminated, the nuclear breakdown very frequently occurred in less time and in higher percentages as compared with the case in which the eggs were kept without addition of sperm. Similarly, the shape of eggs become spherical more quickly in the inseminated lot than in the non-inseminated.

It will not be out of place to state that, although the times of first polar body formation after the deposition in sea water become progressively the shorter the earlier the insemination is made, the times between those of insemination and of first polar body formation are shorter when inseminated after the nuclear breakdown than when inseminated before.

Eggs obtained from the excised gonads may or may not undergo the nuclear breakdown while they are kept standing in sea water. Batches of eggs obtained in an earlier part of the breeding season generally show small percentages of the nuclear dissolution, while in those obtained later in the season practically a hundred per cent of the eggs usually undergo the breakdown in about 15 to 25 minutes' exposure to sea water at a temperature of 25°C to 28°C.

A more clear-cut piece of evidence which will show the *Crassostrea* eggs become fertilizable before the nuclear breakdown has been obtained from the following experiments. The eggs were inseminated at varying times after deposition in sea water, and following two minutes' interaction with sperm, each lot was exposed to hypotonic (5 per cent) sea water for two minutes.

Preliminary experiments show that such hypotonic treatment deprives free sperm of their fertilizing capacity but does not affect the fertilizability of eggs. It should be noted that an attached fertilizing spermatozoon, though the tail has been greatly injured, readily enters the egg.

Results of a typical experiment are presented in the following. In this batch of eggs the nuclear breakdown occurred at about 18 minutes after deposition and in 89 per cent when the eggs were kept in sea water without addition of sperm.

Time of transfer to hypotonic sea water after deposition (in minutes)	4	6	10	15	20	30
% Development	2	15	58	77	87	94

The results indicate that the *Crassostrea* eggs progressively increase the fertilizability during the sojourn in sea water and that, since most of the eggs have developed to normal veligers, the eggs, even while the germinal vesicle remains intact, can well attain a certain level of cortex maturity which is enough to permit normal sperm entry.

Fertilizability and Nuclear Breakdown in Pearl Oyster Eggs

Three species of pearl oysters, *Pinctada fucata*, *P. maxima*, and *Pteria penguin*, were used.

(1) ***Pinctada fucata.*** As regards the state of nucleus, the discharged eggs of this form show a similar situation as those of *Crassostrea.* The eggs obtained from the excised gonad may undergo the nuclear breakdown when they enter sea water; the percentages of breakdown vary with batches of eggs, not infrequently amounting to practically a hundred.

While the discharged sperm are intensely active, the gametes obtainable from the dissected testes invariably are only slightly motile when suspended in sea water. Although the rather immotile sperm at times become moderately active in the presence of eggs, attempts of fertilization in plain sea water have resulted in failure if gametes from the excised gonads were employed. Among scores of such trials, only about 30 per cent of the eggs at best showed some cleavages but the pattern was unexceptionally more or less abnormal.

As was reported in earlier papers (Wada, 1941, 1947, 1953; Wada & Wada, 1953), artificial fertilization in this and other forms of pearl oysters is readily accomplished if gametes are ripened and mixed in ammoniacal sea water with $0.5-1.0 \times 10^{-3}$N NH_3. The rate of fertilization not infrequently amounts to practically a hundred per cent.

In ammoniacal sea water the nuclear breakdown of unripe eggs is markedly accelerated and immotile sperm are rendered intensely active. This effect on sperm is reversible in the sense that they become quiescent on being transferred to plain sea water. Hyperalkaline sea water with sodium hydroxide or potassium hydroxide shows a similar effect on the motility of sperm, but its effect on the eggs is far less marked.

In the experiments summarized in Table 1, batches of *P. fucata* eggs where the nuclear breakdown occurred in the great majority in plain sea water were inseminated with discharged sperm or with spermary sperm activatable in the presence of eggs. As is shown in the table, there occurred several cases in which most of the eggs remained unfertilized in plain sea water or NaOH– (or KOH–) sea water while fertilization was achieved in high percentages in ammoniacal sea water.

Table 1 Fertilizability of *P. fucata* Eggs Which Have Undergone the Nuclear Breakdown

	% Nuclear Breakdown in Plain Sea Water	% Development			
		Plain S. W.	NaOH-S. W.	KOH-S. W.	NH_3-S. W.
Exp. No.:		With Discharged Sperm			
1	79	0	2	4	66
2	80	0	70	—	76
3	80	75	79	67	82
4	99	28	69	80	—
		With Activatable Sperm			
5	90	0	9	—	90
6	95	0	0	—	92
7	100	2	80	82	81
8	97	24	71	72	—

Besides a favorable effect of ammonia on increasing the physiological maturity of gametes, these results seem to show that the nuclear breakdown as is induced by exposure to plain sea water does not necessarily result in making the eggs fertilizable.

In another series of experiments, the eggs fresh from the excised gonads were exposed to ammoniacal sea water; they were inseminated in situ then transferred to plain sea water at varying times before and after the nuclear breakdown. Results of a typical experiment along this line are presented in the following:

Time of transfer to plain sea water after deposition in ammoniacal sea water (in minutes)	5.5	6.5	7.5	10	17
% Development	5	23	52	81	84

In this batch the nuclear dissolution took place about 15 minutes after deposition in ammoniacal sea water.

Since sperm entry does not occur in plain sea water, the results indicate that fertilizability of the eggs is progressively improved in ammoniacal sea water, reaching the maximum even before the nuclear breakdown takes place.

(2) ***Pinctada maxima and Pteria penguin.*** The eggs of these two forms which are obtainable from the excised gonads do not undergo the nuclear breakdown how long they are kept standing in plain sea water. In this respect, they differ markedly from those of *Crassostrea* and *Pinctada fucata.* It may be permissible to say that eggs of the former forms are stocked in the ovary under somewhat more undermature condition than those of the latter.

The eggs of *Pinctada maxima* and *Pteria penguin*, however, inaugurate the nuclear maturation in from 20 to 45 minutes' exposure to ammoniacal sea water with $0.4–1.0 \times 10^{-3}$N NH_3 and sometimes result in the formation of the first polar body. If the eggs from the well-developed gonad are inseminated at the time of the onset of nuclear breakdown while kept in ammoniacal sea water, practically a hundred per cent of the eggs are not infrequently fertilized.

If insemination is made while the germinal vesicle is still intact, sperm do not enter the egg until the onset of breakdown. In other words, the eggs in ammoniacal sea water are rendered fertilizable first at a stage which is heralded by the dissolution of the nuclear membrane or, at least, disappearance of the nucleolus. This can be deduced from the following two observations: (1) if the inseminated eggs were transferred to plain sea water from ammoniacal sea water before the nucleus showed any signs of breakdown, the germinal vesicle remained intact; (2) In a less concentrated (0.3×10^{-3}N NH_3) ammoniacal sea water, the eggs did not undergo the nuclear breakdown, and, if inseminated, the eggs remained intact although sperm were rendered intensely active in such hyperalkaline sea water.

Discussion

As regards the stage of nuclear maturation the egg has reached before natural sperm entry occurs i.e. when discharged, pelecypod molluscs may be classified into the following three classes:

(1) The eggs are discharged while the germinal vesicle is intact. Examples: *Mactra*, *Spisula*, *Lutaria*, and probably other forms belonging to the family Mactridae. In these forms, the eggs become unfertilizable after the nuclear breakdown.

(2) The eggs are discharged at the first maturation prophase. Examples: *Crassostrea echinata*, *C. gigas*, *Pinctada fucata*, and presumably other species of pearl oysters. Unpublished experimental evidence shows that *Crassostrea* eggs are still fertilizable after formation of the first polar body.

(3) The eggs are discharged at the first maturation metaphase. Examples: *Mytilus*, *Cumingia*, and probably the great majority of forms not mentioned above.

Although the eggs of marine invertebrate animals are diverse among species in the stage of nuclear maturation at the time of sperm entry, a great majority fall under above stated Class 3. It is a well-established belief that normal fertilization in any form requires a certain level of physiological maturation of the egg cortical layor.

Fol (1877, 1879) was probably the first to associate the maturation of the ovum with the breakdown of the germinal vesicle. Since then, a body of evidence which suggests a close association between the two phenomena has been accumulated, for instance, the classic experiments on *Cerebratulus* by Wilson (1903) and on *Asterias* by Delage (1901) and Chambers (1921).

Such evidence has built up an opinion that changes incurred in the egg cytoplasm through the admixture of nuclear material is prerequisite to fertilization. Thus, Lillie and Just (1929) state that substances from the germinal vesicle bring about a protoplasmic maturation that is necessary for fertilization. As to the case of *Nereis* eggs which are fertilizable only at the germinal vesicle stage, they claim that "it may be supposed that the substances necessary for such protoplasmic maturation diffuse through the wall of the germinal vesicle." It seems to me this view has been too much extraporated; there remains another and probably more appropriate interpretation.

It is a well-established fact that a sperm aster does not develop in the egg cytoplasm while the germinal vesicle is intact. Chambers and Chambers (1949), working on *Asterias* eggs, have demonstrated that the sperm aster never appears until after the second polar body has been given off. However, if we herewith confine the term "cortex maturation" and the like to denote a maturation of the egg cortex which is enough to permit sperm entry followed by normal development, at least in the eggs of certain forms of animals such as *C. echinata* and *P. fucata*, such cortex maturation is perfect even while the germinal vesicle is intact.

Facts along this line are most evidently presented by *Mactra* eggs. As far as the present writer's observations go, sperm entry has never been observed to occur in the eggs of *M. sulcataria* and *M. veneriformis* after the breakdown of germinal vesicle, although it is still obscure whether this is due to an overripeness of the cortex or to some change incurred in the perivitelline layer. Moreover, as is stated in this paper, *P. fucata* eggs which have completed the nuclear breakdown are not always fertilizable.

These facts suggest that the cortex maturation can be completed more or less independently of the nuclear breakdown. On the other hand, the view that substances from the germinal vesicle are responsible for the cortex maturation will be met with some difficulties in explaining these facts.

Breakdown of the germinal vesicle is induced by external agent. Naturally these agents will first affect the cortical layer before they influence the nucleus. From his works on the *Chaetopterus* egg, GOLDSTEIN (1953) has proposed a hypothesis that, as soon as the egg enters sea water, a proteolytic enzyme system is activated by the entrance of calcium into the cell interior and this protease is responsible for the breakdown of germinal vesicle. HEILBRUNN and WILSON (1955) show that in *Chaetopterus* eggs after the entrance into sea water the protoplasm shows a liquefaction preceded by a very brief clotting reaction which occurs before the nucleus shows any signs of breaking down. They believe that both the gelation and the subsequent liquefaction can be caused by the calcium-activated protease described by GOLDSTEIN (1953).

In pearl oyster eggs, when they are exposed to ammoniacal sea water, the first visible change to occur before the nuclear breakdown is a rounding-up of the egg which has been more or less irregularly ovoid. This change in the shape is very probably due to a liquefaction of the cortex. According to WILSON and HEILBRUNN (1952), all agents which have been found to activate the *Chaetopterus* egg cause a liquefaction of the cortex. These findings provide another piece of evidence which will support the view proposed in the second last paragraph.

Although the external agents, as just stated, first affect the cortical layer, the same agents also induce the breakdown of germinal vesicle. In addition, in the eggs of *Pinctada maxima* and *Pteria penguin*, the cortex maturation becomes perfect concurrently with the nuclear breakdown. In *Mytilus* and others, the egg cortex does not become mature until after the dissolution of nucleus.

Judging from these facts, it may be permissible to state that the two pathways, one leading to the cortex maturation and the other to the nuclear breakdown, initially have some reaction or reactions in common.

While it is quite obscure what reactions cause the cortex maturation, it should be noted that ammonia, very probably owing to its high penetrating power, is strikingly efficacious in producing the maturation in pearl oyster eggs at such a low concentration as $0.4–0.5 \times 10^{-}$N.

Conclusion

The nuclear breakdown and the cortex maturation as indicated by fertilizability, though it appears they are closely associated, are not in a strict cause-and-effect relation. Probably they are initiated with some reaction(s) in common, but it may be supposed that each has a divergent path in the later part.

References

Chambers, R., 1921 : Microdissection studies. III. Some problems in the maturation and fertilization of the echinoderm egg. Biol. Bull., **41**, 318–350.

Chambers, R., and E. L. Chambers, 1949 : Nuclear and cytoplasmic interrelations in the fertilization of the Asterias egg. Biol. Bull., **96**, 270–282.

Delage, Y., 1901 : Etudes experimentales sur la maturation cytoplasmique et sur la parthenogenese artificielle chez les echinodermes. Arch. de Zool. exp. et gen., 3e serie, **9**, 285–326.

Fol, H., 1877 : Sur le commencement de l'henogenie chez divers animaux. Arch. de Zool. exp. et gen., **6**, 145–169.

Fol, H., 1879 : Recherches sur la fecondation, et le commencement de l'henogenie chez divers animaux. Mem. Soc. Physique et Hist. nat. (Geneve), **26**, 89–397.

Heilbrunn, L. V., and W. L. Wilson, 1955 : Changes in the protoplasm during maturation. Biol. Bull., **109**, 271–275.

Goldstein, L., 1953 : A study of the mechanism of activation and nuclear breakdown in the Chaetopterus egg. Biol. Bull., **105**, 87–102.

Lillie, F. R., and E. E. Just, 1924 : Fertilization. (General Cytology, ed. E. V. Cowdry, Section VIII.) Chicago: University of Chicago Press.

Wada, S. K., 1941 : On the artificial fertilization and development of the silver-lip pearl oyster, Pinctada maxima (Jameson). Kagaku Nan'yo (Science in the South Seas), **4**, 202–208 (in Japanese).

Wada, S. K., 1947 : Artificial fertilization of the Japanese pearl oyster in ammoniacal sea water. Bull. Japan. Soc. Sci. Fish., **13**, 59 (in Japanese).

Wada, S. K., 1953 : Biology of the silver-lip pearl oyster, Pinctada maxima (Jameson). 1. Artificial fertilization and development. Margarita (Tokyo), **1**, 3–15.

Wada, S. K., and R. Wada, 1953 : On a new pearl oyster from the Pacific coast of Japan, with special reference to the cross-fertilization with another pearl oyster, Pinctada martensii (Dunker). Jour. Oceanogr. Soc. Japan, **8**, 127–138.

Wilson, E. B., 1903 : Experiments on cleavage and localization in nemertean egg. Arch. f. Entw.-mech., **16**, 411–460.

Wilson, W. L., and L. V. Heilbrunn, 1952 : The protoplasmic cortex in relation to stimulation. Biol. Bull., **103**, 139–144.