Studies on the Fertilization of Pelecypod Gametes—I.

Increase in Maturity and Accomplishment of Fertilization of Pearl Oyster Gametes in Ammoniacal Sea Water.

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Abstract

Two species of pearl oysters, *Pinctada maxima* and *P. fucata*, were mainly used as materials. When gametes obtained from the excised gonads are employed, fertilization is never achieved in the former and usually fails in the latter form. If, however, the eggs which had been kept for a while in an ammoniacal sea water with around 0.5×10^{-3} N NH_3 were inseminated in the hyperalkaline sea water, fertilization was readily accomplished, and a normal development ensued if the eggs were transferred to plain sea water shortly after the fertilization.

In the case of *P. maxima* an exposure for about half an hour to ammoniacal sea water was necessary to make the eggs fertilizable, while *P. fucata* eggs could become fertilizable in a few minutes' exposure to the hyperalkaline medium. The sperm, which were very slightly moving when released in sea water, became intensely active and obtained fertilizing capacity when suspended in ammoniacal sea water. It is concluded that ammonia induces an increase in physiological maturity in both eggs and sperm and thus facilitates the fertilization.

Hyperalkaline sea water with NaOH or KOH in some cases showed an improving effect on the fertilization of *P. fucata* eggs. This effect, however, is barely recognizable in *P. maxima*. Ammonium chloride-sea water, not only with a hyperalkaline but also with a normal pH value, induced the nuclear breakdown of eggs and a marked increase in sperm motility just as ammoniacal sea water does, resulting in an improvement in the rate of fertilization. These facts suggest that the facilitation of fertilization by ammoniacal sea water is mainly caused by a specific action of ammonia which is known to very readily penetrate a cell.

The use of ammoniacal sea water is applicable to other forms of pelecypods where an ordinary method of artificial fertilization will fail. The method, which was first developed by the present writer some twenty years ago, is now being adopted in a practical propagation of two forms of economically important pearl oysters, *Pteria penguin* and *Pinctada maxima*.

In pelecypods generally the gametes, especially the eggs, are stocked in the gonads under a somewhat underripe condition. In certain forms, for instance *Crassostrea*, gametes expressed from the excised gonads increase the maturity when they enter sea water (Wada, 1961), and fertilization is more or less readily accomplished by merely mixing both kinds of gametes. In pearl oysters and some other forms, however, the maturity of the gametes will not improve to an extent sufficient to permit normal fertilization in plain sea water, and fertilization is never achieved by an ordinary method or, if the eggs are fertilized, normal em-

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bryos are never obtained (Wada, 1941, 1961).

In the previous papers (Wada, 1941, 1947, 1953; Wada and Wada, 1953), it has briefly been reported that fertilization in several species of pearl oysters is rendered successful if the eggs are exposed for a while to ammoniacal sea water and inseminated in the medium. The rate of cleaved eggs not infrequently amounts to practically a hundred per cent. In ammoniacal sea water, the nuclear breakdown as well as the protoplasmic maturation are induced in the eggs, and the sperm which are very sluggish become intensely active and obtain fertilizing power. In this paper experiments performed along this line will be described in a more detail.

Experiments with the Japanese pearl oyster and some other bivalves were carried out at the Misaki Marine Biological Station of Tokyo University; fertilization in the Australian pearl oysters was studied at Thursday Island, Australia, in the laboratory provided by the Queensland Government and maintained by the C.S.I.R.O. and later in the laboratory of the Cape York Pearling Co. Pty., Ltd. Complementary experiments with the winged pearl oyster were made at the Pearl Culture Station of the Amami Pearl Culture Co., Ltd., Amami-Oshima, Japan.

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Materials and Methods

The Japanese pearl oyster, *Pinctada fucata* (GOULD) (syn.: *P. martensii* (DUNKER)), and the maxima pearl oyster, *P. maxima* (JAMESON), were mainly used. Several other forms, *P. albina sugillata*, *Pteria penguin*, *Venerupis variegata*, *Crassostrea echinata*, *Caecella chinensis* and *Mactra veneriformis*, were also used in supplementary experiments.

Eggs obtained from the excised gonads were thoroughly washed with filtered sea water before use. The concentration of sperm in the fertilization medium was so adjusted as to be about 2×10^{-5} of the dry sperm; cares were paid so that the sperm density was approximately equal in a series of experiments.

Ammoniacal sea water was prepared by an addition of a required quantity of 0.1 N ammonia solution to 200 ml of filtered fresh sea water. The percentage of fertilization was measured by counting 300 or 400 eggs when fertilized ones were at cleavage stages.

Experiments

Increase in maturity of eggs. The nuclear breakdown and the cytoplasmic (cortical) maturation, though they are probably not linked with a strict cause-and-effect relation (Wada, 1961), are closely associated each other. Therefore, it is permissible with some reservations to regard the nuclear breakdown as a visual criterion indicating the cytoplasmic maturation of oocytes as far as fertilization concerns.

Contrary to the case of *Mactra* or *Crassostrea* eggs, *P. maxima* eggs obtained from the excised gonads will not undergo the nuclear breakdown when they enter sea water. Of nearly one hundred batches so far observed, the nuclear breakdown occurred only in one batch in less than one per cent of the eggs after a long sojourn in sea water.

If they, however, are exposed to ammoniacal sea water in which the ammonia concentration is over $0.25 \times 10^{-3} N$, they undergo the nuclear breakdown and sometimes exclude the first and, less frequently, the second polar body. Although percentages of nuclear breakdown at a low concentration of ammonia varied with the batches of eggs, nearly all of the eggs usually underwent the nuclear breakdown when the ammonia concentration was over $0.75 \times 10^{-3} N$ (Table 1).

Table 1. Nuclear Breakdown of Pearl Oyster Eggs Induced by Exposure to Ammoniacal Sea Water

Batch of Eggs		Concentration of Ammonia ($\times 10^{-3} N$)						
		0	0.25	0.5	0.75	1.0	1.25	1.5
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
P. maxima:	A	0.5	1	96.5	100	100	100	100
	B	0	22	74	95	98	99	
	C	0	29	98	100	100	100	
	D	0		68	100	100	100	
	E*	0	12	62	94	100		
	E*	(0)	(10)	(60)	(90)	(50)		
	F*	0		84		100	98	
	F*	(0)		(76)	(80)	(42)	(27)	
	G*	0		81	95	100	100	
	G*	(0)		(76)	(67)	(24)	(17)	
P. albina:	A	0		42	95	93	95	98
	B	4.5		5		84		99
	C	0		1		33.5		84
P. fucata:	A	26.5		61		97		100
	B*	40.5		66		98		100
	B*	(0)		(62)		(56)		(39)

* Inseminated. Figures in parentheses show percentage development.

The required time of exposure to ammoniacal sea water for the breakdown varied also with the batches of eggs, as well as temperatures and concentrations of ammonia; within $2.0 \times 10^{-3} N$, the breakdown occurred the earlier the higher the concentration was. With $1.0 \times 10^{-3} N\ NH_3$ it took from 25 to 50 minutes at a temperature of 30°C.

In these respects, *Pteria penguin* eggs behaved quite alike as *P. maxima* eggs. Eggs of the winged pearl oyster did not undergo the nuclear breakdown in plain sea water; about half an hour's exposure to ammoniacal sea water was necessary for inducing the breakdown of the germinal vesicle. A similar phenomenon was also observed in *P. albina sugillata* eggs (Table 1).

In their readiness for the nuclear breakdown, *P. fucata* eggs somewhat differ from those of the above-stated forms. *P. fucata* eggs expressed from the excised ovaries frequently undergo the nuclear breakdown while they are exposed to plain sea water; in certain batches the percentages of the breakdown amounted to practically a hundred (see Table 1 in Wada, 1961). In ammoniacal sea water the breakdown occurred more quickly and/or in higher percentages than in plain sea water (Table 1).

It will not be out of place to report that the just-stated facts which were first observed in *P. fucata* from Japanese waters were later confirmed with animals from Australian waters, because there might be some opinions as to an identification of the Japanese pearl oyster, which has been for years designated *P. martensii* (DUNKER), as *P. fucata* (GOULD) as is adopted in this paper.

Eggs of pearl oysters obtained from the excised gonads are ovoid in shape with an irregularly indented contour. On activation the indentation disappeared and the eggs took a spherical shape. Although a similar change in shape occurred after a long stay in plain sea water, it took place much more quickly in ammoniacal sea water and usually before the breakdown of the germinal vesicle. It has been noted that eggs, especially of *P. maxima*, first showed a marked increase in indentations before they became spherical in ammoniacal sea water.

The fact that eggs are stimulated to undergo the nuclear breakdown by an exposure to ammoniacal sea water has been confirmed in several other forms of pelecypods. In certain batches of *Venerupis variegata* eggs in which the nuclear breakdown occurred in only a few percentages in plain sea water, the percentage was found to increase to about 80 to 100 in $1 \times 10^{-3} N\ NH_3$-sea water. In this form the artificial fertilization was facilitated markedly with use of ammoniacal sea water.

In *Caecella chinensis*, in which artificial fertilization can be accomplished with ease in plain sea water before the eggs undergo the nuclear breakdown, an exposure of unfertilized eggs to ammoniacal ($0.5 \times 10^{-3} N$) sea water caused the nuclear dissolution, and this occurred decidedly earlier than the case in which the eggs were inseminated in plain sea water.

Eggs of *Crassostrea echinata* will undergo the nuclear breakdown when they enter sea water. The percentage of the breakdown increases as the breeding season elapses. If eggs are obtained in the later part of the season, nearly all of them usually undergo the nuclear dissolution. It was found that the nuclear breakdown as well as the fertilization in plain sea water were impeded to some extent by a presence of a small quantity of the body fluid. An addition of ammonia to the sea water plus body fluid mixture was effective to obliterate the action of the body fluid, as is shown in Table 2.

Table 2. Antagonism between Ammonia and Body Fluid on Nuclear Breakdown of Crassostrea echinata Eggs

Batch of Eggs	Constitution of Medium			Nuclear Breakdown
	Sea Water	Body Fluid	Ammonia	
	part	*part*	*N*	*per cent*
CE-A	50	50	0	0
	60	40	0.02	87
	70	30	0	2
	95	5	0	30
	100	0	0	100
CE-B	98	2	0	21
	98	2	0.002	100
	100	0	0	100

It has been proved that the eggs of *Mactra veneriformis* very readily undergo the nuclear breakdown when they enter sea water and that the breakdown is suppressed if a small quantity of the body fluid is added to the medium (Iwata, 1951; Sawada, 1952). It was found that an addition of ammonia induced the nuclear breakdown of *Mactra* eggs in the presence of the body fluid even in a very high concentration.

In previous papers (Wada, 1941, 1953), it was reported that hyperalkaline sea water with sodium or potassium hydroxide was far less effective in inducing the nuclear breakdown of *Pinctada maxima* eggs as compared to that with ammonia. In the former types of hyperalkaline sea water containing $0.5 \times 10^{-3}N$ added alkali,

Table 3. Effect of Hyperalkaline Sea Water on the Nuclear Breakdown of Pinctada fucata Eggs

Batch of Eggs	Type of Sea Water	Concentration of Alkali ($\times 10^{-3}N$)			
		0	0.5	1.0	1.5
		per cent	*per cent*	*per cent*	*per cent*
PF-A	NH_4OH	26.5	61	97	100
	NaOH	26.5	23.5	41.5	50
PF-C	NH_4OH	27		100	
	NaOH	27	32	33	

The figures show percentages of nuclear breakdown.

less than one per cent of the eggs underwent the nuclear dissolution, while the ammoniacal sea water of the same alkali concentration gave from 60 to 100 per cent dissolution. A similar fact, though less marked, was also observed with *P. fucata* eggs (Table 3).

In eggs of a venerid clam, *Venerupis variegata*, the nuclear breakdown was induced in a high percentage in ammonium chloride-sea water in which the hydrogen ion concentration is equal with (or even higher than) that in plain sea water, while it was not induced with such ease by a long exposure to plain sea water (Table 4).

Table 4. Effect of Ammonium Chloride on the Nuclear Breakdown of Venerupis variegata Eggs

Batch No.	Type of Sea Water			
	Plain pH 8.2	NH_4Cl-NaOH* pH 8.2	HCl pH 7.8	NH_4Cl* pH 7.8
	per cent	*per cent*	*per cent*	*per cent*
VV-A	0	100	2	29
VV-B	33	99	29	66

* Concentration of NH_4Cl: 0.0025 *M*. Figures show percentage nuclear breakdown.

These facts suggest that the nuclear dissolution as is induced by an exposure of eggs to ammoniacal sea water is mainly due to the action of ammonia. Hyperalkalinity of the outside medium, while it may favour the nuclear breakdown in some way, does not seem a direct factor of importance in bringing about an improvement in maturity.

Increase in maturity of spermatozoa. In all the species of pearl oysters hitherto studied, the sperm expressed from the dissected testes usually were very slightly moving on release in sea water. Unlike the *Mytilus edulis* sperm, they remained inactive even after a long stay in sea water. In the course of a study on the sexuality of pearl oysters (Wada, 1957), the spermary sperm of over five hundred animals of *P. fucata* were examined with microscope. Intensely active spermatozoa were not observed in any of them, although in certain batches some spermatozoa became somewhat active in the presence of eggs.

The immotile sperm became intensely active in a hyperalkaline sea water not only with ammonia but also with sodium or potassium hydroxide. They became immotile again on being transferred to plain sea water. In *P. maxima*, it was found that sperm were rendered intensely active in hyperalkaline sea water with ammonia in a less concentration than that which is necessary for inducing the nuclear breakdown of the eggs.

The sperm motility was increased also in ammonium chloride-sea water. In sea water the pH of which had been lowered to around 7 by an addition of isotonic NH_4Cl solution, *P. fucata* sperm showed a marked increase in motility though of a fleeting occurrence. In case the pH of NH_4Cl-sea water was raised to 8.3 by an addition of NaOH, the sperm were activated at and over a concentration of $0.35 \times 10^{-3} M NH_4Cl$. At a concentration of $5 \times 10^{-3} M$, however, the motility decreased markedly in 6 to 8 minutes at a temperature of 28°C.

It has been known that sperm of some marine invertebrates including bivalves become more excitable to undergo the acrosome reaction in ammoniacal sea water (Dan and Wada, 1955; Dan, 1956). In pearl oyster sperm the acrosome reaction was also observed to occur in the fertilizing as well as the supernumerary spermatozoa attaching to the egg.

Crassostrea echinata sperm, which are intensely active in plain sea water, were rendered to undergo the acrosome reaction on contact with glass surface with more ease when suspended in NH_4Cl-sea water of pH 8.3 as compared with the case in which they were suspended in plain sea water.

It seems evident that ammoniacal sea water improves a physiological maturity of pearl oyster spermatozoa. This occurs partly owing to the hyperalkalinity and partly, rather primarily, to the presence of ammonia molecules.

Artificial fertilization. In pearl oysters and many other bivalves, artificial fertilization cannot be achieved in plain sea water if gametes obtained from the excised gonads are employed. This naturally is to be attributed to an underripeness of one or both kinds of gametes.

Fertilization in pearl oysters is readily accomplished if the eggs after kept standing a while in ammoniacal sea water are inseminated in the medium. As reported in the first section of this paper, the percentage of the nuclear breakdown increases as the concentration of ammonia becomes higher. However, if the ammoniacal concentration exceeded a certain level, the inseminated eggs did not cleave or, if cleaved, the pattern was abnormal (Table 1).

From the viewpoint of the percentage of normally cleaved eggs, the optimum concentration of ammonia, though it varied with baches of eggs, nearly always lay between $0.4 \times 10^{-3} N$ and $0.8 \times 10^{-3} N$ in every of the three species of pearl oysters, *P. fucata*, *P. maxima* and *Pteria penguin*. The optimum roughly coincided with the lowest concentration which was still sufficiently high to induce the nuclear breakdown in the majority of the eggs. In this respect it should be noted that pearl oyster eggs are very sinsitive to the concentration of ammonia.

Eggs of *P. maxima* and *Pteria penguin* do not become fertilizable before the onset of nuclear breakdown. In these forms, therefore, about half an hour's exposure to ammoniacal sea water was necessary to achieve fertilization.

In *P. fucata*, on the contrary, the sperm can enter the egg whose germinal vesicle is still intact (Wada, 1961). Eggs of this form could be fertilized in a few minutes' exposure to ammoniacal sea water. A result of a typical experiment along this line follows:

Length of exposure time to NH_3-sea water ($10^{-3}N$)	$0^{sec.}$	$10^{sec.}$	$20^{sec.}$	$30^{sec.}$	$1^{min.}$	$2^{min.}$
% Development	0	26	37	42	72	75

(Temperature: 28°C)

In *P. maxima*, the highest rate of normal development was obtained in cases in which the insemination was made after the dissolution of the germinal vesicle but while the nucleoplasm was still recognizable as a hyaline mass in the central part of the egg. A later insemination resulted in more or less abnormal pattern in development. A too early insemination brought about a waste of capable spermatozoa since they would undergo the acrosome reaction before the egg became fertilizable.

When *P. maxima* eggs obtained from the well-ripened ovary were employed, they developed into normal veliger larvae if they were transferred to plain sea water before the extrusion of the first or the second polar body. A longer sojourn in ammoniacal sea water induced a delay in sequences of the following cleavages, resulting in the production of abnormal veligers. This fact suggests that an exposure to ammoniacal sea water is necessary only up to an early phase of activation.

In this connection, it is worth while to emphasize that eggs fertilized in ammoniacal sea water cleave and continue to develop quite normally in plain sea water in spite of the artificial treatment. This becomes more evident from the success to rear the veligers obtained in this way up to spat and young animals in *P. maxima* and even to adults in *Pteria penguin*. Details of the rearing experiments will be published elsewhere.

In certain batches of *P. maxima* eggs, however, a longer stay in ammoniacal sea water gave a better percentage of development as compared with the case in which the fertilized eggs were removed to plain sea water before the first cleavage was expected to occur (Table 5).

Table 5. Rate of Development in Pinctada maxima Eggs from Underdeveloped Gonads in relation with the Time of Transfer from Ammoniacal Medium to Plain Sea Water

Batch No.	Transferred		NH_3 Conc.	Nuclear Breakdown
	Before Cleavage	After Cleavage		
	per cent	*per cent*	$\times 10^{-3}N$	*per cent*
PM-H	4	53	1.25	100
PM-H	19	60	1.0	100
PM-I	13	41	1.0	66
PM-J	2*	86	1.0	100
PM-J	2*	49	0.5	50

* The transfer was made before the second polar body protrusion; in other lots, after the second polar body was extruded.

This was the case with batches of eggs from the rather underdeveloped gonads.

The resultant embryos never developed into normal veligers. It seems in this case either that ammonia is in some way incorporated with events involved in the later phase of activation and the cleavage, or that an abrupt change in the ammonia content or pH of cytoplasm before the cleavage suppresses the subsequent development.

Abnormalities in the early veligers were most evidently indicated by a stunted growth of the **D**-shaped shell, for instance, a crooked condition of the hinge line and an imperfection in antero-posterior symmetry. Such veligers sooner or later perished down without developing the umbone. In order to obtain a healthy brood of larvae, it was found to be of prime importance first to make a choice of parent animals, especially of females. A good batch of eggs usually contains the spheroidal to ovoid oocytes in a great number when fresh from the ovary.

As stated before, pearl oyster sperm become intensely active in hyperalkaline sea water with sodium or potassium hydroxide. Meanwhile, certain batches of *P. fucata* eggs undergo the nuclear breakdown in a high percentage when they enter sea water. Insemination to such eggs in NaOH- or KOH-sea water sometimes gave a decidedly lower percentage of fertilization as compared with the case in which the eggs were inseminated in NH_3-sea water (see Table 1 in Wada, 1961).

Series of experiments were carried out with batches of *P. fucata* eggs where the final percentage of development was roughly equal between NaOH and NH_3 treatments. It was found that the eggs became fertilizable markedly earlier in NH_3-sea water than in NaOH-sea water (Table 6).

Table 6. Development of Fertilizability in Hyperalkaline Sea Water of Pinctada fucata Eggs

Exp. & Batch No.	Type of Medium	Length of Exposure Time to Hyperalkaline Medium (in minutes)											
		0	1	1.5	2	3	4	5	10	15	20	30	60
Exp. 1:													
PF-D	NH_3	0*		79*			89*		87		89		90
PF-D	NaOH	0*		6*			4*		21*		85		87
Exp. 2:													
PF-E	NH_3	0*	5*		16*	23*		32*	40	46	45	50	64
PF-E	NaOH	0*	0*		0*	0*		0*	0*	0	0	3	50
Exp. 3:													
PF-E	NH_3	0	15		22	23		21	28	36	35	37	
PF-E	NaOH	0	0		0	0		1	19	26	34	40	

The figures represent percentage development. Concentrations of added alkalis were $0.5 \times 10^{-3}N$ in all three series of experiments. Eggs were deposited to sperm-containing hyperalkaline sea water while with the intact germinal vesicle in Exps. 1 and 2 and after the nuclear breakdown in Exp. 3. In the lots marked with an asterisk the transfer to plain sea water was made before the nuclear breakdown occurred. Temperature: 29°–29.5°C. Nuclear breakdown in plain sea water: 88 per cent in Batch PF-D and 53 per cent in Batch PF-E.

In another experiment, *P. fucata* eggs which had underwent the nuclear breakdown in plain sea water were inseminated in sodium hydroxide-sea water and then transferred to plain sea water at varying times after the insemination. The result is shown in the following:

Length of exposure time to $0.5 \times 10^{-3} N$ NaOH-sea water plus sperm (in minutes)	0	1	2	3	5	10	15	20	30
% Development	0	0	0	2	5	24	26	28	69

(Temperature: 28.5°C)

This batch of eggs underwent the nuclear breakdown in 98 per cent in plain sea water and gave 81 per cent of fertilization in five minutes' exposure to $0.5 \times 10^{-3} N$ NH_3-sea water plus sperm while the germinal vesicle was intact.

The foregoing facts with regard to *P. fucata* evidently indicate that hyperalkalinity itself of outside medium facilitates fertilization to some extent and that, among other things, a treatment with ammoniacal sea water is outstandingly effective in achieving fertilization.

The difference between the treatments with NH_3-sea water and with NaOH- or KOH-sea water is much more marked in the case of *P. maxima*. As was briefly reported in previous papers (Wada, 1941, 1953), *P. maxima* eggs gave less than one per cent fertilization when inseminated in $0.5 \times 10^{-3} N$ NaOH- or KOH-sea water, while the same batches of eggs gave far better percentage development, amounting to from 60 to 80, when treated with $0.5 \times 10^{-3} N$ NH_3-sea water.

In another series of experiments with *P. fucata* gametes it was found that the fertilization was facilitated in ammonium chloride-sea water. In some cases fertilization was accomplished in a high percentage in ammonium chloride-sea water of the equal pH with that of plain sea water. The facilitation was more pronounced if the pH value was slightly raised by a further addition of sodium hydroxide (Table 7).

Table 7. Fertilization of Pinctada fucata Eggs in Ammonium Chloride-Sea Water

Batch No.	Type of Medium				
	Plain S. W. pH 8.3	NH_4Cl-S.W.* pH 8.3	NH_4Cl-S.W.* pH 8.8	NaOH-S.W. pH 8.8	NH_3-S.W. pH 8.8
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
PF-F	0	1	63	4	69
PF-G	0	0	25	4	24
PF-H	0	63			86
PF-I	0	3			85

* Concentration of NH_4Cl: $0.5 \times 10^{-3} M$; a required quantity of NaOH solution was added to raise the pH.

The figures represent percentage development as measured at cleavage stages. The concentration of alkali in NaOH- or NH_3-sea water is $0.5 \times 10^{-3} N$.

This and earlier stated facts suggest that ammonia (or its hydroxide) molecules are mainly responsible for the facilitation of fertilization. Furthermore, it seems evident that an ammoniacal sea water improves not only the fertilizability of underripe eggs but also the fertilizing capacity of physiologically immature spermatozoa and thus facilitates the fertilization.

Discussion

In pelecypod molluscs generally the gametes are stocked in the gonads under a physiologically underripe condition and they suddenly become fully mature immediately before the discharge. It is highly probable that the so-called latent period in the experimentally induced spawning (e.g. Galtsoff, 1938) is in the main the time which is required for the gametes to achieve a physiological maturity that should occur in the gonads before shedding, as is the case with *Mytilus edulis* (Iwata, 1952). In some cases, for instance, *Crassostrea* eggs and *Mytilus edulis* sperm, such a maturity can be accomplished while the gametes are kept standing in sea water.

Artificial fertilization, where the gametes obtained from the excised gonads are employed, is usually successful in bivalves belonging to the following genera or families: *Crassostrea*, Mactridae, Chamidae, Amphidesmatidae and Pholadidae. In these forms, presumably both kinds of gametes achieve the maturity when they enter sea water. It should be, however, noted that even in these forms much more excellent results, with respect to the conformity of developmental sequences, the morphology of embryos and larvae, etc., have been obtained when the discharged gametes are employed (unpublished data).

In the other forms which make up a great majority of pelecypods, one or both kinds of gametes obtained from the excised gonads will rarely become mature in plain sea water to an extent which will permit fertilization. In the following statements, "eggs" and "sperm" should refer, if not otherwise stated, to those which are obtainable from the excised gonads, and the artificial fertilization means the fertilization where such gametes are employed.

Pearl oysters belonging to the genera, *Pinctada* and *Pteria*, to some extent differ in the readiness of artificial fertilization between the species. In *Pinctada maxima* and *Pteria penguin*, attempts to achieve the artificial fertilization in plain sea water have failed without exception. A similar failure is reported by Galtsoff (1950) in the Panamanian pearl oyster, *Pinctada mazatlanica*. On the other hand, there are some cases with *P. fucata* in which eggs become fertilizable in a fairly good percentage when they enter sea water (Wada, 1961), although the eggs fertilized in this way will not cleave normally.

Eggs of *P. maxima* and *Pteria penguin* rarely undergo the nuclear breakdown while they are kept standing in sea water, while the great majority of *P. fucata* eggs not infrequently undergo the nuclear dissolution in fifteen to thirty minutes' exposure to sea water. As is reported in this paper, *P. fucata* eggs are rendered fertilizable in ammoniacal sea water in a much shorter time as compared with the case of *P. maxima* or *Pteria penguin*. Judging from these facts, it may be permissible to conclude that *P. fucata* eggs are stocked in the ovary at a more advanced stage of maturity than those of *P. maxima* or *Pteria penguin*.

Working on the Hawaiian pearl oyster, *Pinctada galtsoffii* BARTCH, Galtsoff (1933) reported that eggs could be fertilized "by adding to them a few drops of freshly obtained sperm and in a few hours typical swimming larvae developed". It appears that artificial fertilization in this form can be accomplished with much more ease than in other forms of pearl oysters, although he did not state clearly whether the gametes obtained from the excised gonads or the discharged ones were employed.

The Hawaiian pearl oyster is believed to be very closely related to or probably belong to the same species as the black-lip pearl oyster, *P. margaritifera* (LINNÉ). Working on the black-lip from the Palau Islands, West Calorines, and from Tanegashima Island, Japan, the present writer has found that artificial fertilization is successful only by use of ammoniacal sea water (Wada, 1953, and unpublished data). This is also the case with the Australian black-lip (verbal communication from Mr. D. J. Tranter).

It has been known for a long time that alkalis cause a marked increase in the activity of spermatozoa. As early as 1856, Koelliker decribed this effect for the vertebrate spermatozoa. He noticed that if a drop of a fairly concentrated solution of potassium hydroxide is mixed with a drop of semen, there is usually a sudden outburst of activity before the spermatozoa are rendered motionless. Mann (1954) is of the opinion that such a period of short-lived stimulation is rather characteristic of sperm-paralysing agents ("Todeszuckung" after Schlenk) and there is a fundamental difference between short and prolonged activation phenomena. In ammoniacal sea water with about $0.5 \times 10^{-3} N$ NH_3, pearl oyster sperm maintain the state of activity for at least a few hours. The effect on the sperm motility of hyperalkaline sea water is reversible in the sense that the activated spermatozoa become quiescent on being transferred to plain sea water.

The use of a hyperalkaline medium to facilitate artificial fertilization in marine animals was first made by Loeb (1903, 1904) in a successful attempt to produce heterologous hybridization between sea urchin eggs and starfish sperm, and has since been widely adopted by various authors for the same purpose in not only heterologous but also homologous fertilization. Usually sodium hydroxide has been used to make sea water hyperalkaline.

It has been known strong bases as sodium hydroxide in a dilute solution hardly penetrate the cell membrane. Since the fertilization is favoured only in the actual presence of the alkali, it has been claimed that the facilitation probably involves a rapidly reversible modification of the surface of one or both kinds of gametes (Loeb, 1915; Lillie, 1919; Lillie and Just, 1924).

Besides the reversible, prolonged activation of the sperm motility, a hyperalkaline medium favours the acrosome reaction of spermatozoa (Colwin and Colwin, 1956; Dan, 1952, 1956; Dan and Wada, 1955). In the fertilization of *Crassostrea* eggs, particularly where somewhat underripe eggs are involved, there are some pieces of evidence which suggest that the attachment to the egg of plural spermatozoa facilitate the fertilization (Wada, 1956, 1963). It seems probable that an initial stimulation by the sperm attachment, if not successful to activate the egg to achieve fertilization, increases the irritability of the activation system of the egg. These circumstances in respect of spermatozoa may be able to explain some of the cases in which artificial fertilization is facilitated by the use of a hyperalkaline sea water.

The fact that a hyperalkaline sea water induces the nuclear breakdown of oocytes and thus makes them fertilizable was first found by Loeb (1905) in *Lottia*, a limpet. He reported that immature oocytes of the gastropod become fertilizable in from four to five hours' exposure to 0.002*N* NaOH-sea water. This was confirmed by Wolfsohn (1907) in four other forms of limpets belonging to the genus *Acmaea*. Wolfsohn found that the limpet eggs underwent the nuclear breakdown in one hour's exposure to 0.003*N* NaOH-sea water and became fertilizable, while they would remain immature if they were kept standing in plain sea water for two days.

As an agent for the causation of artificial parthenogenesis, Loeb (1912) found that weak bases such as NH_4OH are more effective than strong bases such as NaOH or KOH. Working on the echiuroid eggs, Hiraiwa and Kawakami (1936) and Tyler and Bauer (1937) were successful to induce the nuclear breakdown and further to produce parthenogenetic development with ammoniacal (0.02–0.002*N*) sea water. Inaba (1936) reported that *Crassostrea* eggs could be activated parthenogenetically to undergo cleavages by a treatment with ammoniacal (about 0.036 *N*) sea water. It should be noted the ammoniacal sea water used by these authors is much more alkaline than that which has been revealed optimum for achieving fertilization of pearl oyster eggs. During the present experimentation it was frequently observed that *P. fucata* and *P. maxima* eggs treated with the ammoniacal sea water were activated up to the polar body protrusion. Allen (1953) reports that an exposure of *Spisula* eggs to NH_3 (10^{-3}–$10^{-4}N$) resulted in good activation.

Since the present writer's finding that ammoniacal sea water is strikingly effective in accomplishing fertilization in several species of pearl oysters and related animals (Wada, 1941, 1947, 1953; Wada and Wada, 1953), the method has widely been adopted for the same purpose in pelecypod molluscs in which artificial fertilization has been found otherwise impossible except with naturally discharged gametes (Hatanaka et al, 1943; Yamamoto and Nishioka, 1943; Dan and Wada, 1955). In pearl oysters veliger larvae obtained in this way have successfully been reared up to spat. This is the case with the following species: *P. fucata* (Kobayashi and Yuki, 1952); *Pteria penguin* (Shinmura et al, 1959), *Pinctada margaritifera* (unpublished work by Mr. I. Setoguchi of the Kagoshima Prefectural Fisheries

Experimental Station). Working at Thursday Island, Australia, the present writer has succeeded in obtaining several hundred spat of *P. maxima* in the laboratory (unpublished work).

In the case of the winged pearl oyster, *Pteria penguin*, a cultivation of young animals obtained from the eggs thus fertilized is now being undertaken on a quasi-industrial scale. In Amami-Oshima Island located in the southernmost part of Japan, a once-thriving pearl culture industry of *Pteria* has become threatened to be on the verge of dissolution because of shortage of animals due to overfishing. At present, however, yearly attempts to obtain young animals from the eggs fertilized in ammoniacal sea water have been successful and in 1962 culture pearls were first harvested from the artificially propagated Pteria. It is now very hopeful to re-establish the industry. This work has been and is being performed by the co-operation of the Amami Pearl Culture Co. and the Kagoshima Prefectural Fisheries Experimental Station under the present writer's instruction.

Besides such a practical application, these facts among other things demonstrate that the treatment of pearl oyster gametes with ammoniacal sea water, though very artificial in itself, produces an improvement in maturity very similar to that which will occur in the gonads immediately before natural spawning.

Since NaOH-sea water, in the case of *P. fucata*, to some extent facilitates fertilization, an increase in alkalinity of the outside medium must be one of the actions of ammoniacal sea water. In the case of *P. maxima*, however, the hyperalkalinity alone shows far less marked effects on the nuclear breakdown and the fertilization than ammoniacal sea water does. This and the experiments with NH_4Cl clearly indicate that the presence of ammonia molecules is mainly responsible for the actions of ammoniacal sea water in causing the physiological maturation of pearl oyster gametes.

Iwata (1952a, 1952b) reports that the nuclear breakdown of *Mytilus edulis* eggs can be caused by ammoniacal sea water but not by NaOH-sea water. A similar fact is also reported by Shirase (1955) in *Venerupis philippinarus* eggs.

It is a well-established fact that ammonia is one of the substances which most readily penetrate into the cell. It is also known the cytoplasm is rendered alkaline when the cell is exposed to an ammoniacal medium but not when exposed to NaOH-sea water (see Davson and Danielli, 1952, for the mechanism of ammonia penetration).

It is quite obscure how ammonia works in inducing a protoplasmic maturation in underripe gametes. Nevertheless, use of ammoniacal sea water will be a very effective method for achieving artificial fertilization in a number of bivalves where the ordinary method will end in failure.

Conclusion

In pearl oysters as in many other pelecypods, artificial fertilization cannot be accomplished in plain sea water if gametes from the excised gonads are employed. This is due to an underripeness of both kinds of the gametes. If eggs are, however, exposed to ammoniacal sea water, the physiological maturity is improved accompanied by the nuclear breakdown and the eggs become fertilizable. The sperm which are very sluggish in plain sea water become intensely active and acquire the fertilizing capacity in ammoniacal sea water. In this way, artificial fertilization is very readily achieved in the ammoniacal medium.

Judging from the fact that the eggs thus fertilized develop normally and the resulting larvae can be grown up to spat and further to adult forms, it is concluded that the treatment, though artificial in itself, causes an improvement in maturity of the gametes of the same nature as will occur in normal reproduction.

This effect of ammoniacal sea water probably is mainly due to the action of ammonia molecules inside the sex cells. Sodium hydroxide-sea water is far less effective than ammoniacal sea water. It is suggested that the well-known phenomenon that NaOH- or KOH-sea water facilitates fertilization may be interpreted mostly through its improving effect on proper attachments of spermatozoa.

Summary

1. Two species of pearl oysters, *Pinctada fucata* and *P. maxima*, were used as materials. Two other species of pearl oysters and five more species of other bivalveswere involved in comlementary experiments.

2. An exposure of *P. maxima* eggs to ammoniacal sea water induces the nuclear breakdown which will not occur in plain sea water. In certain batches of *P. fucata* eggs, a great majority may undergo the nuclear breakdown when they enter plain sea water; but the cytoplasmic maturity will not be increased to an extent sufficient to achieve normal fertilization.

3. Spermary spermatozoa when released into plain sea water are not actively motile. They become intensely active and acquire fertilizing capacity in ammoniacal sea water.

4. Artificial fertilization which will not occur in plain sea water is very readily accomplished in ammoniacal sea water, the optimum concentration of ammonia being about $0.5 \times 10^{-3} N$ for both species of pearl oysters.

5. Eggs of *P. fucata* become fertilizable in a few minutes' stay in ammoniacal sea water, whereas *P. maxima* eggs require a much longer exposure to the ammoniacal medium. This and another fact stated above (2) may indicate that *P. fucata* eggs are stocked in the ovary at a more advanced stage of maturity than those of *P. maxima*.

6. To obtain a healthy brood of veligers, it is recommended to make a choice of batch of eggs and the eggs inseminated in ammoniacal sea water should be transferred to plain sea water shortly after the sperm entry has taken place.

7. Veligers thus obtained are able to grow to spat and even to adults. This evidently indicates that the treatment of gametes with ammonia induces an in-

crease in maturity very similar to that which will occur in the gonads preceding natural spawning.

8. Eggs from the underdeveloped gonad can be fertilized and developed if they are transferred to plain sea water at (not before) cleavage stages, although the embryos will not grow to normal veligers.

9. In facilitation of fertilization ammoniacal sea water is far more effective than other hyperalkaline media with strong bases. NH_4Cl-sea water with the same pH as that of ordinary sea water is effective in achieving the nuclear breakdown, the sperm activation and the fertilization. It is inferred that ammonia penetrating into the cell interior causes the increase in maturity of gametes.

10. In the case of *P. fucata*, insemination in NaOH-sea water sometimes induces a marked improvement in the rate of fertilization. It is suggested the hyperalkaline sea water in this case may act mainly on spermatozoa towards an increase in number of those which will make a proper attachment (i.e. undergo the acrosome reaction) to the egg.

11. Use of ammoniacal sea water to achieve fertilization will be applicable to a number of pelecypods where the ordinary method does not work. It has found a fruitful application in the artificial propagation of *Pteria penguin* on a quasi-industrial scale; some culture pearls have been harvested from the animals grown from the eggs fertilized in ammoniacal sea water.

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