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Initiation of Development in the Egg of Arbacia. III. The Effect of Arbacia Blood on the Fertilization-Reaction

E. E. Just

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INITIATION OF DEVELOPMENT IN THE EGG OF *ARBACIA*.

III. THE EFFECT OF *Arbacia* BLOOD ON THE FERTILIZATION-REACTION.

E. E. JUST,[1]

ROSENWALD FELLOW IN BIOLOGY, NATIONAL RESEARCH COUNCIL.

I.

During May, 1921, *Arbacia* examined at Woods Hole, Mass., were found to be immature. Thus, on May 15, bits of the gonads examined under the microscope contained only immature eggs devoid of color. The testes showed no mature sperm. Toward the end of May a few females were found in which the eggs were undergoing maturation. Early in June (June 1 to 8) the eggs gave very low per cent. fertilization. From this time on eggs exuded through the genital pores steadily increased in fertilizability. Eggs taken from the ovaries, on the other hand, were largely immature; their fertilization per cent. was lower than that of eggs that exuded from the genital pores when the animals were carefully cut around the peristome. Toward the end of July the high fertilization capacity of the shed eggs (*i.e.*, eggs that exuded from the genital pores when the animals were carefully cut around the peristome) fell off. From July 20 to the end of the season the fertilization capacity of shed eggs from a given female was found to be markedly inferior to eggs taken from the same female by cutting up the ovaries in sea-water. There was thus found during the first part of the season in the case of shed eggs a rise in fertilizability followed by a fall. Parallel with this fall was found a rise in fertilizability of eggs from the ovaries which during June were largely immature. It was thus found that eggs of *Arbacia* of high fertilization capacity may be obtained at Woods Hole throughout the season provided shed eggs are used during the early part and eggs from the ovaries during the later part of

[1] Zoölogical Laboratory, Howard University.

the season. Evidence for these statements is included in this paper.

In what follows it will be convenient to consider first those experiments made on shed eggs during the first period of the summer, and second those experiments made on both shed and "ovary" eggs during the second part of the summer.

II.

A word concerning the method used in the experiments here reported may not be amiss. Eggs used were from freshly procured animals whenever these were to be had. In one case animals were collected from the spiles of the dock opposite the laboratory and used immediately after. Results on eggs from animals kept in the laboratory in a few instances for several days, however, showed that such eggs may be as good as those from freshly dredged animals. Each animal was thoroughly cleansed for a minute or two under running tap water. The hands and forearms of the worker were kept under a stream of tap water for about one minute. After the washing under running tap water the urchin was shaken dry and put under a jet of sea-water, then shaken dry again. The animal was thereupon placed in a dry Syracuse watch glass, aboral side down, and its peristome carefully cut with clean, sharp-pointed scissors with very slender blades. Care was exercised to avoid puncturing the gonads. Frequently mere puncture of the peristome was made. As soon as eggs or sperm came through the genital pores as a result of the stimulus of the incision the animal was removed to another dish. Controls of uninseminated eggs were always run and not once was development of an egg discovered. The case may be quite otherwise, however, if the animals are not thoroughly washed in tap water. Among eggs from such unwashed females one may find not only eggs with membranes, but cleavage stages and blastulæ. This is particularly true of animals kept in the tanks where spawning may take place. In such cases eggs caught among the female's spines are fertilized where they remain until the swimming stage.

The blood used in this work was procured in the following manner: The peristome was carefully cut away and the lantern gently removed. The animal was then inverted over a clean Syra-

cuse watch glass and drained of fluid, removed to another watch glass, and allowed to shed. The blood from each animal was used separately or that from several animals united. After clotting, the serum was decanted and set aside until needed. Sea-water in which blood clots were cut up after filtering was used in one experiment. It seemed no different in its action from the serum.

A.

We may begin with the experiments which indicate that during the early part of the season *Arbacia* eggs that exude through the genital pores are of high fertilization capacity. Blood added to these eggs before insemination cuts down the per cent. of fertilization.

THE EXPERIMENTS.

(*a*) July 14, 4:00 P.M. 1 drop of shed eggs from one female in 10 c.c. of sea-water allowed to settle. Water changed three times. Eggs inseminated—Lot *A*.

2 drops of shed eggs from same female in 5 c.c. of sea-water plus 5 c.c. of blood. Allowed to settle. 1 drop of these eggs removed to 10 c.c. of sea-water—Lot *B*—and inseminated. Remainder of the same eggs inseminated—Lot *C*.

7:00 P.M. These eggs give cleavage as follows:

Lot.	*A*.	*B*.	*C*.
Per cent. of cleavage	98	2	0

This experiment is typical of a group of experiments made during the month of June and the first three weeks of July. I always found some inhibitor present in the blood of females, though the amount might vary to a considerable degree. Thus in some cases, particularly in the early experiments, a great deal of blood from several animals had to be used to inhibit fertilization. These experiments, without a single exception, reveal a high capacity for fertilization on the part of shed eggs.

(*b*) July 16, 8:00 P.M. 4 drops of shed eggs from each of three females (*A*, *B*, and *C*) distributed as follows:

*A*1, *B*1, *C*1: 1 drop of shed eggs inseminated in 250 c.c. of sea-water.

A2, *B2*, *C2*: 1 drop of shed eggs plus 2 drops of blood inseminated in sea-water.

A3, *B3*, *C3*: 1 drop of shed eggs plus 4 drops of blood inseminated in sea-water.

A4, *B4*, *C4*: 1 drop of shed eggs plus 8 drops of blood inseminated in sea-water.

10:00 P.M. *A1*, *B1*, *C1* show 96, 98, and 93 per cent. fertilization, respectively. No development in other dishes.

July 17, 10:30 A.M. Nos. *A2*, *B2*, and *C2* show about .01 per cent. development. No development in the others.

Nos. *A2* to *C4*, inclusive, washed and set aside. 3:00 P.M. No cleavage. Samples from each of these inseminated. 3:10 P.M. Good membranes in most eggs of Nos. *A2* to *C3*, inclusive. Nos. *A4* to *C4* only about 40 per cent. membranes each.

3:45 P.M. First cleavage in all eggs that have membranes.

7:30 P.M. Other samples from the eggs *A2* to *C4*, inclusive, which were washed this morning inseminated.

9:00 P.M. About .01 per cent. in two-cell stage in *B2*. No development in others, though numerous sperm attached. All dishes show active sperm.

July 18, 8:00 A.M. One blastula in *B2*.

This experiment and others of this group show several points. They show that shed eggs of high fertilization capacity will not fertilize in the presence of blood. The amount of blood necessary to bring about this inhibition can not be predicted because the inhibitory power of the blood of various females is not exactly the same. In this group of experiments, however, made usually with eggs from different females in samples of the same blood, the results are fairly constant; the inhibition to fertilization is about the same. Finally, the experiment indicates that twenty-six and one half hours after insemination in the presence of blood eggs may on reinsemination be made to develop. Usually such capacity to respond to reinsemination does not persist after twenty-four hours. As Lillie has shown, the presence of blood does not interfere with the action of the sperm agglutinin (fertilizin) produced by *Arbacia*. It would have been interesting to compare the production of fertilizin by these eggs inseminated in blood with that

of an equal quantity of uninseminated eggs from the same female in normal sea-water. Unfortunately this was not done.

(*c*) July 18, 10:00 A.M. 0.5 c.c. of shed eggs (sample of which on insemination gave 98 per cent. membranes) in 10 c.c. of blood—Lot *A*. Following series set up:

No. 1. 5 c.c. of Lot *A* plus 5 c.c. of sea-water.
" 2. " " No. 1 " " " " "
" 3. " " No. 2 " " " " "
" 4. " " No. 3 " " " " "
" 5. " " No. 4 " " " " "
" 6. " " No. 5 " " " " "
" 7. " " No. 6 " " " " "

All inseminated heavily.

12:00 M. Cleavages in these eggs as follows:

No.	2.	3.	4.	5.	6.	7.
Per cent. of cleavage.....	0	0	0	10	20	25

Nos. 2, 3, and 4 killed and sectioned.

The interesting point here is that despite the graded decrease in the quantity of both eggs and blood the inhibitory action of the blood persists. Each lot of eggs was inseminated with the same amount of sperm. This means that the heaviness of insemination steadily increased in the Nos. 2 to 7, for the number of eggs was halved in each successive dilution. It would seem, therefore, that heavy insemination does not necessarily mean an overcoming of the blood block to fertilization. That the failure to cleave was not due to polyspermy we may conclude from the per cent. of cleavage in Nos. 5, 6, and 7, which received relatively to the number of eggs most heavy insemination. However, I should point out that this was the most powerful inhibiting blood encountered in this group of experiments (made during June and to July 20).

(*d*) July 19, 6:30 P.M. 10 c.c. suspension of shed eggs (sample of which gave 98 per cent. membranes) plus 10 c.c. of blood inseminated.

9:00 P.M. No development. Eggs washed in 250 c.c. sea-water. Set aside; on settling, removed to 250 c.c. of sea-water.

July 20, 8:00 to 9:00 A.M. These eggs show a very small per cent. fine top swimmers. Undeveloped eggs show sperm attached. Top swimmers removed. 250 c.c. of fresh sea-water added to remaining eggs.

10:20 A.M. About 1 per cent. of eggs in the 2- and 4-cell stages.

10:25 A.M. Eggs inseminated in 250 c.c. of fresh sea-water.

11:25 A.M. 5 per cent. of eggs in 4-cell stage, 85 per cent. in 2-cell stage. Some others show spindles, remainder no change. Eggs tend to lose membranes and to bud.

4:00 P.M. Small per cent. of eggs in swimming stage.

6:00 P.M. About 90 per cent. swimming.

9:00 P.M. Swimmers at the surface.

The chief point of interest in this experiment is that eggs inseminated in blood may, on washing, develop normally. If portions of such eggs are washed at intervals up to about two hours after insemination, a high per cent. develop perfectly. They lose their power to develop on washing two to three hours after insemination. This seems to be the most significant point of this report (see beyond, page 421). Eggs inseminated in the presence of blood take in sperm; such sperm lie within the cortex. All these eggs show sperm firmly attached to the cortex. These eggs have been studied not only while living, but also in sections and *in toto* mounts properly fixed (Bouin, Lang, Meves, Flemming). In some cases the sections show the egg cortex literally studded with sperm, with other sperm within the egg. The failure of these eggs inseminated in blood to develop is not, therefore, due to failure of sperm attachment or penetration. On washing within about two hours after insemination in the presence of blood the eggs are released from the blood block. The per cent. of eggs that develop depends upon the thoroughness of washing within this two-hour limit as well as the amount of inhibitor present.

This experiment likewise reveals that twenty-eight hours after their insemination in the presence of blood eggs, after washing, will fertilize with a high per cent. of development.

B.

We may now consider those experiments which reveal that during the later half of the season the shed eggs of *Arbacia* are of low fertilization capacity, whereas eggs taken from the ovaries are of high fertilization capacity.

THE EXPERIMENTS.

(*a*) July 23, 8:00 A.M. Portions of shed eggs from 12 *Arbacia* inseminated in turn. All poor; not more than 10 per cent. membranes in any lot.

Ovaries from these same females chopped up separately in sea-water and eggs strained out. Eggs inseminated. Fine membranes (average: around 96 per cent.).

This experiment scarcely needs comment. The eggs that exude through the genital pores are inferior to those procured by cutting up the ovaries in sea-water. And this was found to be true from July 23 to the end of the season in 107 out of 125 females tested on this one point alone.

(*b*) July 25, 9:00 A.M. 9 females opened. Eggs exude through genital pores into dry dishes. 250 c.c. of sea-water added to each lot of eggs. Samples from each inseminated. No membranes in Nos. 1, 2, 4, 6, and 7. 80 per cent. membranes in No. 3. Nos. 5, 8, and 9 give 18, 6, and 14 per cent. membranes, respectively. Effect not due to sperm for inseminations were next made in each case with sperm from 7 different males with essentially the same results.

Eggs removed from these 9 females inseminated in turn. Fine membranes; average around 98 per cent.

To offset any criticism that these results might be due to presence of blood with sperm, great care was exercised in opening all animals. During several days, for example, all instruments were sterilized before using by heating red hot in order to destroy any adherent blood. If animals cut proved to be males, they usually exuded clean sperm into the dry watch glasses. In many cases the males shed spontaneously. Thus only uncontaminated sperm was procured. In addition, sperm from several males was used as mentioned in the experiment cited above. Finally, the results of inseminating the eggs taken from the ovaries would seem to

offset the possibility that the failure of the shed eggs to cleave is due to the presence of blood with the sperm.

(*c*) July 27. Samples of shed eggs from 14 females inseminated in turn. Very high amount of inhibition present in each of Nos. 1 to 11, inclusive. Membrane separation in these eggs as follows:

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Per cent. of membranes..	3	5	0	1	6	8	15	0	4	1	0.1	99	98	100

Remainder of uninseminated eggs of Nos. 1 to 11, inclusive, put in one dish. Washed in five changes of 250 c.c. of sea-water. Inseminated. 13 per cent. cleavage.

Ovaries from these same females chopped up separately in sea-water; eggs strained and collected. Portion of each inseminated. Average fertilization—95 per cent. Remaining eggs added and inseminated in one dish. Fertilization—97 per cent.

Eggs from Nos. 12, 13, 14, added, inseminated. Fertilization close to 100 per cent.

These uninseminated shed eggs gave high agglutinin test. I thought it worth while to add the eggs from several females in order to increase the amount of sperm agglutinin (fertilizin); this, however, failed to improve results. These eggs do not fail to develop because of lack of fertilizin or of failure of sperm entry. They are loaded with blood inhibitor and this suspends the fertilization-reaction.

(*d*) July 30, 9:50 A.M. 9 females opened, gently drained of blood. Each lot of shed eggs then in finger bowls of 200 c.c. of sea-water. Portions of these eggs removed and inseminated at 10:00 A.M., 10:28 A.M., 10:43 A.M., and 11:25 A.M., with the following results:

No.	Amount in c.c. of Shed Eggs at 9:50 A.M.	Per Cent. Membranes Following Insemination.			
		10:00 A.M.	10:28 A.M.	10:50 A.M.	11:25 A.M
1.....	3	0	40	60	95
2.....	.1	3	3	Cytolysis	
3.....	−.1	0	[1]	[1]	
4.....	.2	5	90	98	
5.....	.1	0	70	88	
6.....	.1	0	80	90	
7.....	−.1	6	[1]	[1]	
8.....	.15	10	30	97	
9.....	−.1	0	35	95	

[1] Not sufficient eggs left to make count.

11:48 A.M. Ovaries of each of above females (Nos. 1 to 9, inclusive) separately chopped up in sea-water; strained. Each lot of eggs in 200 c.c. of sea-water.

11:55 A.M. Portions of each lot of eggs inseminated.

2:00 P.M. Beautiful cleavage in all lots—close to 100 per cent.

At times the quantity of eggs that exude from the genital pores is really enormous for the size of the urchin. Such eggs are perfectly beautiful. And yet the bulk of the eggs is no criterion for their fertilizability. I have used eggs from animals fresh from the sea. The results are the same; they are not due to the fact that the animals have deteriorated in the laboratory.

The protocols herewith cited (Sections *A* and *B*) constitute the evidence for the conclusions that eggs of *Arbacia* vary with respect to their fertilization capacity during the summer; during the first part of the season shed eggs possess high fertilization capacity which drops off during the latter part of the season; eggs from the ovary, during the latter part of the season when the shed eggs are poor, possess high fertilization capacity. A simple method is thus indicated for obtaining throughout the summer eggs of high fertilizability. The evidence likewise admits of the conclusion that blood blocks fertilization in shed eggs of high fertilizability. It is likewise true, though no experiments have been cited on this point in the present paper, that fertilization of eggs from the ovary with high fertilization capacity is blocked by blood. The findings of the writer as to this effect of blood on eggs of *Arbacia* are thus in accord with those of Lillie ('14).

The experiments cited above also show that eggs in the presence of blood agglutinate to themselves and even take in sperm, and that such sperm may activate at any time during a dormant period of about two hours if the blood is removed by washing. Finally, the experiments reveal that as long as twenty-eight hours after insemination in the presence of blood eggs failing to develop may do so on reinsemination. In view of Lillie's recent work ('21) on the effect of copper salts in fertilization—especially important in revealing the latent period in the fertilization-reaction—these findings of mine may deserve notice since they lend additional support to Lillie's fertilizin theory.

The protocols here given do not, however, establish that blood

is responsible for the failure during the latter part of the season of shed eggs to fertilize. The failure of these eggs to fertilize may, indeed, be due to other causes. Nevertheless the following points warrant consideration for the assumption that blood is responsible for this inhibition:

First, the failure of fertilization is not due to the absence of fertilizin. For eggs in blood liberate fertilizin (Lillie, '14).[1] Secondly, washing removes the inhibition to fertilization, and this is true of those eggs whose failure to fertilize is indubitably due to the presence of blood. Thirdly, the inhibition decreases after the eggs have remained in sea-water for some time. And, finally, I have observed during several seasons that late in August *Arbacia* females are frequently turgid with eggs from ruptured ovaries. Such blood-soaked eggs are of low fertilization capacity. These considerations point to a blood block, but they certainly do not prove the case.

Indeed, Oshima, likewise working with the egg of *Arbacia* in September, 1921, after I had left Woods Hole, has interpreted the failure of eggs escaping through the genital pores of opened sea-urchins to fertilize as due to what he calls a "dermal secretion." So far as I can determine, Oshima's *sole* criterion for calling this substance a "dermal secretion"—he could get very little of it from *dermal tissues themselves,* it seems—is the fact that he got it from the outside of the urchins. By the same token, eggs and sperm that exude through the genital pores and lodge among the spines—an observation that every worker with sea-urchins has at some time made—are dermal secretions! Oshima's "dermal secretion," I very much suspect, is an *excretion,* if not actually fecal material. And this suspicion is strengthened by the fact, which Oshima points out, that uric acid is found in it.

Moreover, against Oshima's interpretation that the "dermal secretion" inhibits fertilization we have Lillie's extensive experiments to the contrary. I believe with Oshima that he would have reached an entirely different conclusion had he been able to "carry out this series of experiments more fully and accurately." I heartily concur with his conclusion that the action of the "dermal secretion" seems to have no biological significance.

[1] I have additional evidence on this point.

III.

Blood blocks fertilization in the egg of *Arbacia*. Lillie's experiments show this. The findings reported above but confirm his.

If we inquire as to the mode in which blood blocks fertilization, we must recall the rôle of fertilizin in the fertilization-reaction. That the fertilizability of the egg depends upon the presence of fertilizin, the following facts show: Immature eggs, fertilized eggs, and eggs with butyric acid membranes do not secrete fertilizin. They are also incapable of fertilization. Fertilizable eggs washed free of fertilizin lose their capacity for fertilization. But the failure of eggs in the presence of blood does not place these eggs in the same category with fertilizin-free eggs.

The failure of eggs in blood to fertilize is not due to the blocking of the reaction between sperm and fertilizin. It will be recalled that the presence of fertilizin in sea-water is indicated by its agglutinative action on specific sperm. This action is not lost in the presence of blood. "It can not be supposed," says Lillie, "that the plasma operates by preventing the adhesion of the spermatozoön to the egg if this is brought about by agglutination, because it was found that the agglutination of spermatozoa by means of egg secretions takes place as readily in the plasma as in sea-water" (Lillie, '19, page 175). The action of blood is thus on the fertilizin; it constitutes a block between fertilizin and the egg itself. Since eggs of *Arbacia* inseminated in blood are found with sperm attached to or within the cortex, it must be concluded that Lillie's interpretation of the inhibitory action of blood is sustained.

Finally, Lillie's copper experiments ('21) reveal the latent period in the fertilization reaction. If it prove that blood acts similarly, we may have an additional method for study of the latent period. We may thus be able more closely to approach an understanding of the fertilization-reaction.

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