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THE FERTILIZATION-REACTION IN *ECHINARACHNIUS PARMA*. VII.

THE INHIBITORY ACTION OF BLOOD.

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The present communication aims to set forth results of experiments made during two seasons (1919 and 1920) at the Marine Biological Laboratory, Woods Hole, Mass., to test that part of Lillie's fertilizin theory which postulates that blood (in *Arbacia*) inhibits fertilization through intervention of the fertilizin and the egg (Lillie, '14). The present writer was firmly of the opinion that this postulated action might be merely a surface effect: that despite the agglutination of *Arbacia* sperm, by *Arbacia* egg-water in the presence of specific blood, the main action of the blood is on the surface of the egg so that sperm can not enter. The results of the experiments here reported, however, show that, in the egg of *Echinarachnius parma* at least, this is not the case: blood blocks fertilization in this egg by interfering with the reaction of fertilizin and egg and not with the sperm and fertilizin at the surface of the egg. For repeated observations reveal that both in straight and cross fertilization, with *Arbacia* sperm, eggs of *Echinarachnius* inseminated in blood, though they fail to develop, nevertheless take in sperm. We may divide the experiments into two groups: those that deal with straight fertilization and those that deal with cross fertilization with *Arbacia* sperm.

I.

Eggs of *Echinarachnius* obtained by cutting up ovaries in sea-water invariably give low fertilization percentages. Thus the early observations—1910, 1914, 1915—made on such eggs gave the impression that this is a poor egg for the study of fertilization. An egg suspension strained from ovaries cut up in sea-water shows a slight turbidity or greater depth in color depending upon the amount of blood and detritus present. My notes indicate that fertilizing power falls off with increasing depth of color. With shed eggs, on the other hand, the case is quite different: they invariably yield 100 per cent. fertilization. If, however, shed eggs

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of maximum fertilization capacity be inseminated in cœlomic fluid, the per cent. of cleavage is decreased. Thus equal parts of cœlomic fluid and sea-water may cut down the per cent. of cleavage to zero; higher proportions of cœlomic fluid, 75 to 100 per cent., invariably permit no fertilization.

In practice it was found extremely difficult to use large quantities of blood owing to its scarcity. Since, however, eggs from one female only were used in any given experiment, this was found no great difficulty, since the number of eggs used was very small in each case.

The method used is about as follows: Equal parts of cœlomic fluid and sea-water made solution No. 1. To half of No. 1 was added a like quantity of sea-water to make No. 2. Thus a series of half dilutions was made. One half of the last member in the series was discarded in order that all numbers would contain the same quantity of solution. Uninseminated eggs were placed in each solution—one drop of an egg suspension to each. Likewise a drop of uninseminated eggs was placed in normal sea-water equal in amount to that of mixture of cœlomic fluid and sea-water. The eggs in all dishes were then inseminated with the same amount of sperm from one male. In general, inseminations were made first in 100 per cent. and in 50 per cent. blood. Unless these gave high percentages of inhibition, I made no further dilutions.

The appended summary (Table I.) gives the results of six experiments made in 1919:

TABLE I.

THE INHIBITORY EFFECT OF SPECIFIC BLOOD ON FERTILIZATION OF THE EGG OF *Echinarachnius parma* AS REVEALED BY THE PER CENT. OF CLEAVAGE IN VARIOUS CONCENTRATIONS OF BLOOD IN SEA-WATER IN 6 EXPERIMENTS OF 1919.

No. .	Per Cent. of Blood in Sea-water.	Per Cent. of Cleavage.					
		Exp. 1.	Exp. 2.	Exp. 3.	Exp. 4.	Exp. 5.	Exp. 6.
1. . . . .	100	0	0	0	0	0	0
2. . . . .	50	0	0	0	0	0	0
3. . . . .	25	0	0	0	3	6	0
4. . . . .	12.5	0	1	0	8	33	0
5. . . . .	6.25	0	12	16	14	50	0
6. . . . .	3.125	49	40	47	33	81	0
7. . . . .	1.5625	89	64	78	90	90	0
8. . . . .	0 (control)	99	100	98	98	99	36

It is thus seen that eggs of high fertilization capacity fail to fertilize if inseminated in the presence of certain concentrations of blood. Not all eggs give results comparable to those in the table. Thus during May, 1921, several samples of blood tested showed very weak inhibitory power. In essentials, however, the results are quite comparable to those obtained by Lillie in his study of fertilization in *Arbacia*. Moreover, Lillie's interpretation of the mode of action of the blood inhibitor is sustained by this work on the egg of *Echinarachnius*, as will now be shown.

Sperm of *Arbacia* readily agglutinate in mixtures of *Arbacia* egg-water and blood as though the blood were absent. Lillie thus concluded from this that the blood does not block the reaction between the sperm-agglutinating substance (fertilizin) and the sperm; the block comes between fertilizin and substances in the egg. But it is at once apparent to the reader that this is not wholly conclusive: the substance in blood that inhibits fertilization may well do so by some action on the surface of the egg rendering sperm attachment and penetration impossible. Thus it might well be that sperm in the presence of blood and egg-water rich with sperm agglutinin of high power agglutinate; but in ordinary insemination this amount of agglutinin is not present, nor is the insemination made as heavy as the sperm suspensions must be to detect the presence of agglutinin. In the inseminations usually employed for fertilizing eggs agglutination of spermatozoa does not occur; instead, the spermatozoa stick to the egg. As a matter of fact, sperm likewise stick to *Echinarachnius* eggs inseminated in blood. The failure of such eggs to fertilize can not, therefore, be attributed to the effect of blood in blocking the agglutination of sperm to the egg.

At first I considered this result as due to the poor quality of the sperm; that it was not so much an inhibition by blood as a failure of fertilization. Subsequently it was found repeatedly that on inseminating in the presence of blood spermatozoa are attached to the eggs. Thus we have evidence to support the postulate offered by Lillie as to the mode of action of blood inhibitor. This is brought out again in the next group of experiments.

II.

It has been shown (Just, '19) that the fertilization of eggs of *Echinarachnius* by *Arbacia* sperm is greatly facilitated by the use of alkali or by heavy insemination. Though giving a lower per cent. of cleavage than alkali, heavy insemination was for several reasons the method adopted in the experiments made to determine the effect of *Echinarachnius* blood on fertilization by *Arbacia* sperm. That this cross is inhibited by blood was suggested in the earlier work. The experiments now cited indicate that this is true. I cite four experiments made in June and in August, 1920.

Uninseminated eggs of *Echinarachnius* are washed in sea-water and allowed to settle. Five drops of this suspension is distributed equally among five dishes as follows: *Lot A*, uninseminated in sea-water; *Lot B*, inseminated in sea-water with *Echinarachnius* sperm; *Lot C*, inseminated in 3 per cent. *Echinarachnius* blood with *Echinarachnius* sperm; *Lot D*, heavily inseminated in 3 per cent. *Echinarachnius* blood with shed *Arbacia* sperm; *Lot E*, heavily inseminated in sea-water with shed *Arbacia* sperm. The results follow:

Lot.	Per Cent. of Cleavage.			
	June 22.	June 24.	August 4.	August 5.
A.....	0	0	0	0
B.....	99	100	78	81
C.....	74	69	54	49
D.....	0	0	0	0
E.....	43	34	21	17

In some cases a concentration of *Echinarachnius* blood that has no effect on the fertilization of *Echinarachnius* eggs by its own sperm will not give a single membrane or cleavage with heavy *Arbacia* sperm suspension. Again, shed eggs of *Echinarachnius* are superior to eggs cut out of the ovaries for cross fertilization. Also, after thorough washing, eggs cut out of the ovaries and fertilized with *Arbacia* sperm yield a higher per cent. of cleavage. I interpret these facts as indicating that it is the presence of blood that makes cross fertilization difficult. Blood thus acts in cross fertilization as it does in straight fertilization; the differences are quantitative only. For *Arbacia* sperm enter *Echinarachnius* eggs

in the presence of blood, but they set up no reaction. In my 1917 series of *Echinarachnius* eggs, heavily inseminated with *Arbacia* sperm, this is clearly shown in the sectioned material. Moreover, these eggs are viable; though literally studded with *Arbacia* sperm, they are capable up to twenty-four hours later on insemination with *Echinarachnius* sperm of giving development of a high degree of normality.

It should be noted that in these experiments clean shed sperm of *Arbacia* was used. If the sperm be obtained from the testes admixed with blood, the per cent. of cross fertilization is reduced. This has been repeatedly observed. In some cases, indeed, the shed sperm may give around 30 per cent. fertilization and the sperm from the testes no fertilization. I believe that this is due to the toxicity of *Arbacia* blood. This toxicity is well known from Lillie's observations. I have likewise mentioned elsewhere that *Arbacia* blood is markedly toxic for *Nereis* eggs.

Similarly, mixtures of shed *Arbacia* sperm and shed *Echinarachnius* sperm exhibit no antagonism; eggs of either form or of both dropped into such sperm mixtures fertilize. With mixtures of sperm cut from the testes the results are different, for such mixtures cut down the per cent. of cleavage. In one experiment made with mixtures of shed *Arbacia* sperm mixed with shed *Nereis* sperm there was no sperm antagonism, since eggs of each form developed upon inseminations from the mixture.

These, then, are the results of inseminating eggs of *Echinarachnius* in its body fluid or own blood.

We may conclude: (1) Blood blocks straight fertilization. (2) Blood blocks cross fertilization. (3) Blood blocks both straight and cross fertilization after the spermatozoa stick to the eggs or enter them and not by preventing the attachment of spermatozoa to the eggs.

These conclusions admit of certain suggestions concerning the nature of specificity in the fertilization reaction. We may discuss these briefly.

### III.

The block to cross fertilization is cortical. As Lillie says: "The various methods used to induce hybrid fertilization—staling of

eggs, high concentration of sperm, use of alkalies or other chemicals—have therefore this one feature in common, that they destroy the chemical or physical integrity of the cortex of the egg” (Lillie, '19, page 219). Specificity in fertilization thus manifests itself in the cortex of the egg.

But specificity in fertilization is not absolute, but relative. This fact would seem to indicate that the results of straight and of cross fertilization are due to quantitative, not qualitative, differences in the cortical response to insemination; species sperm more readily than foreign sperm overcome the same resistance to fertilization set up by some cortical substance or condition. The question, therefore, comes down to this: What in the cortex is responsible for the block to fertilization, whether by species or foreign sperm?

In the first place, most methods used to induce cross fertilization in echinids hasten the loss of fertilizin. Thus staling is an easy method for the removal of fertilizin. Eggs allowed to stand or repeatedly washed lose their fertilizin content. Washing the eggs rapidly with dilute sea-water brings about a loss of fertilizin. Dense sperm suspensions rapidly bind available fertilizin. I venture the opinion that heat hastens the loss of fertilizin also.

If, now, we postulate that specificity in fertilization is wholly due to the presence of fertilizin, then must we also take the next step, namely, that cross fertilization is most successful when the fertilizin is reduced? That is, fertilizin is necessary for straight fertilization, but a block to cross fertilization; certain kinds of artificial parthenogenesis (heat, for example, on *Nereis* egg) depend upon the presence of the fertilizin in maximum concentration; certain eggs lose their capacity for fertilization by species sperm very rapidly (*Platynereis*); but with foreign sperm the case is otherwise—it can fertilize after an egg is no longer capable of response to artificial stimulus or that of species germ. But might not specificity in fertilization be accounted for in part on the basis of the data presented in this paper? This would mean at least with the knowledge at hand that specificity in fertilization is due in part to the blood, since the presence of blood blocks fertilization by species or foreign sperm.

When species sperm comes in contact with an egg, it gains entrance and fertilizes against the blood present. The greater the amount of blood, the more difficult the fertilization. Indeed, the blood may actually inhibit fertilization in every egg. Therefore, dense sperm suspensions must be employed for fertilization in the presence of blood rich in inhibitor. The blood inhibitor acts by binding the fertilizin so that the fertilizin can not react with the egg receptors. Heavy insemination insures fertilization perhaps by increasing the chances of some spermatozoa locating fertilizin free of blood inhibitor. Or in heavy insemination the onslaught of numerous sperm brings it about that the fertilizin shakes free the inhibitor.

The blood slowly leaves the egg as it lies in sea-water. But the fertilizin also goes. Hence while the egg is losing inhibitor it is also losing fertilizing power. The blood is perhaps never an irremovable block to species sperm; however, though present in but a trace, it serves to block foreign sperm. In staling, therefore, what results is not only loss of fertilizin, but also loss of blood. The loss of blood makes possible cross fertilization.

What is true of staling is doubtless true of other methods for obtaining cross fertilization—heat, use of alkali, and of dilute sea-water; they serve to remove the blood block. The fertilizin remains albeit in reduced quantity. Whenever an egg is capable of fertilization it possesses the fertilizable substance. And it is safe to assume that an egg that will not respond to its own sperm will not cross fertilize.

From this point of view, then, fertilizin is not the only factor in specificity. It is specific since it engages species sperm against the inhibition of blood. But the blood is an aid to specificity, since it blocks all sperm, species sperm least of all.

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