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The Fertilization-Reaction in Echinarachnius Parma. VI. The Necessity of the Egg Cortex for Fertilization

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

THE NECESSITY OF THE EGG CORTEX FOR FERTILIZATION.

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If we define fertilization as an instantaneous irreversible reaction at or in the cortex of the egg between an ovogenous substance, fertilizin, and the spermatozoön, it must follow (1) that an egg, once fertilized, is incapable of response to additional insemination, and (2) that fragments of fertilized eggs are likewise incapable of fertilization. If, moreover, the fertilization-reaction be limited to the cortex, then it must likewise be shown (3) that uninseminated eggs, or fragments thereof, devoid of cortex are not fertilizable. The present paper aims to set forth certain observations made at the Marine Biological Laboratory, Woods Hole, Mass., on the egg of *Echinarachnius parma* which indicate that fertilized eggs, or fragments thereof, are unfertilizable; and that uninseminated eggs, or fragments thereof, devoid of cortex are likewise unfertilizable. It is therefore concluded that the cortex of the uninseminated egg is necessary for fertilization.

II.

The fertilized egg does not react to additional insemination; sperm do not enter fertilized eggs. In order to test the validity of this generally accepted statement, fertilized eggs after removal of their membranes have been inseminated two, three, and four minutes after insemination and at later stages during development to gastrulation. Such eggs have been sectioned.

In no case have sperm been found in the egg or blastomeres after even the heaviest insemination. Rupture of the blastomeres

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with outflow of cytoplasm does not facilitate sperm entry. To offset the possibility that fixation might be a source of error, most diverse fluids were used. In the living egg, in addition, it was easy to see that spermatozoa do not react with fertilized eggs. There is here, certainly, no evidence in support of Kohlbrugge's results.

Many experiments have likewise been made thus: Eggs are lightly inseminated and at five-second intervals up to the time of membrane separation are given an additional heavy insemination. Such eggs fail to reveal polyspermy in higher per cent. than eggs that have but one insemination.

Thus, June 28, 1918, eggs of *Echinarachnius* were inseminated and at five-second intervals up to time of membrane separation were reinseminated. After membrane separation, the eggs were gently shaken to remove the membranes; samples of these were reinseminated at six, thirteen, sixteen, and twenty minutes after the original insemination. Samples of these eggs were fixed in corrosive sublimate-acetic two minutes after each insemination. No evidence of a reaction was found in any of the sectioned material.

In addition, the bulk of the evidence (Lillie, '19) shows that artificially activated eggs fail to react with sperm.

Wilson found that fragments of fertilized eggs of *Cerebratulus* are incapable of fertilization. Conklin has shown that the unusually large polar bodies produced by the egg of *Crepidula* through centrifugal force do not fertilize. Since in this egg polar-body formation follows insemination, Conklin's results are capable of the interpretation that here, too, parts of fertilized eggs do not refertilize.

In *Echinarachnius* the situation is the same. If inseminated eggs of *Echinarachnius* be gently shaken in a vial with bits of broken coverslips and the fragments thus obtained be divided into two lots, one of which is inseminated, the per cent. of development in the two lots is the same. The insemination of these fragments, even if made *twenty seconds* after the insemination of the intact eggs, does not increase the per cent. of development.

These observations indicate that fertilization is irreversible, eggs

completely activated can not respond to additional insemination; and fragments of inseminated eggs behave similarly.

Through the well-known experiments of the Hertwigs, Boveri, Morgan, and others it has been shown that enucleated fragments from uninseminated eggs of sea-urchins are fertilizable as are nucleated fragments. Wilson has shown that the enucleated fragments taken from the egg of *Cerebratulus* after dissolution of the germinal vesicle are fertilizable. In a very cautiously worded paper appearing posthumously Boveri maintained his original position as to the fertilizability of enucleated fragments of uninseminated eggs.

I find that fragments from uninseminated eggs of *Echinarachnius* obtained by gently shaking the eggs in a vial with bits of a broken coverslip are capable of fertilization and development. The development of these fragments does not depend upon the presence of the egg nucleus. Some fragments without egg nuclei fail to respond to insemination. A fragment of large size and with a nucleus may not fertilize. Very small fragments with no egg nucleus develop. *I believe that the failure of fragments to fertilize is due to the absence of cortical material.* This belief is based on results which may now be considered.

III.

Toward the end of the season of 1917 I frequently found that fertilized eggs of the *Echinarachnius* gave rise to abnormal gastrulæ which I took to be ordinary exogastrulæ. They prove to be gastrulæ with masses of undifferentiated protoplasm attached.[1] The breaking up of these masses simulates cleavage. A careful study of these eggs was made and the history of this condition revealed.

I found that among various lots of eggs kept for some time in shallow dishes with little sea-water were some eggs which on return to a larger quantity of normal sea-water underwent a fragmentation. Under the microscope this process is easily followed. The eggs give off a bud or form an exovate that slowly increases in size and drops off. Thus in a given lot of eggs there are in-

[1] These masses are always located at the vegetative pole. This may be significant for the problem of polarity.

numerable cases each with a bud of varying size still attached to or just detached from the egg. If insemination takes place before the bud drops off, a membrane separates from the "egg" and never from the bud. Repeated observation puts this statement beyond doubt. I have never seen two membranes on such eggs, nor a single membrane enclosing both egg and bud. The portion within the membrane alone cleaves and develops. The portion outside the membrane never develops; it remains attached to the gastrula until completely disintegrated. In some cases the bud is so much larger than the "egg" that membrane separation takes place from a relatively small disk; the cleavage of such eggs is discoidal; such eggs never give rise to swimmers.

If the observer inseminate eggs after the buds drop off, only one member of a pair separates a membrane, cleaves, and gastrulates, though it may be the smaller. The presence or absence of the egg nucleus is of no consequence for the development of these fragments.

Any one of three possibilities was thought of as responsible for this phenomenon of bud formation in the egg of *Echinarachnius:* (1) staleness of the eggs, (2) the presence of blood, (3) the general deterioration of the sexual products toward the end of the season. Accordingly, in 1918 attempts were made to ascertain which of these factors is responsible for this bud formation. And we may state at the outset that though each factor may contribute to the production of buds, *the essential factor is the hypertonicity of the medium.*

If eggs of *Echinarachnius* are allowed to stand in sea-water for several hours, they slowly undergo changes that eventually lead to their complete disintegration. A portion of these eggs upon insemination separate membranes many of which are stuck to the swollen cortex. If previous to insemination these eggs be gently shaken, buds are formed from those with swollen cortex. Such inseminated eggs with buds separate membranes only from the "eggs" and never from the buds. These eggs cleave and gastrulate, but the per cent. is always low. Late in the season buds are more easily produced. And throughout the season the presence of blood increases the number of buds formed.

By far the easiest method for the production of a high per cent.

of buds at any time during the season is to allow the uninseminated eggs to stand in a small quantity of sea-water in a shallow dish, thus permitting evaporation; or, better, to place uninseminated eggs in hypertonic sea-water (6 parts of 2½ *M* NaCl plus 50 parts sea-water). On transfer of the eggs to normal sea-water they are gently shaken or squirted through a pipette. Large numbers of such eggs produce buds.

On insemination membranes separate from but one component of these budded eggs. Only that portion of the egg within the membrane divides and gastrulates. The gastrulæ swim attached to the undifferentiated mass of budded cytoplasm which eventually disintegrates. The process of bud formation is easily followed under the microscope and insemination made at any stage. Insemination made after complete separation of the bud gives the same result: in any two given masses of egg material separated by constriction of a bud one only develops, regardless of size or the presence of the egg nucleus.

The explanation of these results on budded eggs of *Echinarachnius* is as follows: The cortex of the eggs changes under various forms of treatment. As the uninseminated egg of *Echinarachnius* lies in sea-water it slowly deteriorates. A distinguishing mark of this deterioration is the physical change in the cortex: the cortex is thick and practically transparent. Late in the season also many eggs are found with thick cortices. Blood, too, will frequently hasten this change in the cortex. Now, hypertonic sea-water very clearly brings about a physical change in the cortex. After exposure to hypertonic sea-water the cortex may be readily seen as a thick jelly-like hull enclosing the egg. It is from this jellied cortex that the membrane separates on insemination.

If an egg with a thick cortex be gently shaken on transferal to normal sea-water, the cortex breaks and the contents of the egg flows out. Indeed, merely the transfer from hypertonic sea-water to normal sea-water will tend to produce this outflow in some eggs, as they rapidly take up water. The bud is thus made up of endoplasm and is without cortical material. In favorable cases this is readily determined.

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And only that component of the budded egg which has the clear rim of cortex is fertilized on insemination as revealed by the pres-

ence of the membrane, cleavage, and gastrulation. The naked mass of endoplasm rounds up still attached to the developing egg. It never reacts with sperm whether inseminated while attached to the egg or after separated from the egg. Thus the presence of the egg cortex is necessary for fertilization. Many observations make this interpretation certain.

In the egg of *Arbacia* the results are the same; indeed, if anything, they are more clear-cut.

Hypertonic sea-water is not the only agent that will bring about this outflow of endoplasm. Frequently shaking will bring it about in a few eggs of a given lot. Hypertonic sea-water is best, however. first because it produces a high per cent. of budded eggs, and second because it makes very clear that the cortex is on the egg and not on the endoplasmic mass.

One additional method may be mentioned now because its use has in turn led to some interesting experiments along another line. This method involves the use of bolting silk, soft filter paper, and lens paper. We may briefly consider this method.

Uninseminated eggs of *Echinarachnius* are dropped on bolting silk (in focus under low power of the microscope), the mesh of which has a diameter less than that of the egg, stretched above the surface of sea-water in a stender dish. If the concentration of eggs in the drop of sea-water is just right, some eggs rupture as they flow through the meshes of the silk. If the observer work rapidly, he can after trial inseminate these eggs just as they burst. The silk is then quickly thrust into the dish of sea-water. Some of the eggs form membranes with naked buds attached.

With filter paper the method is much the same. Soft moist filter paper on which is placed a drop of eggs is mounted under the microscope above sea-water in a low stender dish. The eggs flow beneath the fibers of the filter paper and thus burst because of pressure and slight drying. As they burst they are inseminated and the paper plunged into sea-water. Some of these eggs later show buds without membranes attached to cleaving eggs within membranes. Intact eggs inseminated among the fibers of filter paper in sea-water on insemination will develop normally. I have kept such eggs through to the pluteus stage. With lens paper one may obtain much the same results; the lens paper, in addition, is

much easier to handle: the endoplasmic outflow is more readily followed.

With a little care one may induce flow of endoplasm through the cortex. The naked endoplasm rounds up and in appearance is like the remaining part of the egg. But the endoplasm does not fertilize; it fails to react with sperm.

Here, again, eggs of *Arbacia* give comparable results.

While my observations were under way in 1918, Dr. Robert Chambers informed me that by the method of microdissection he was able to remove the cortex from the egg of the starfish (Lillie, '19). Such eggs are incapable of fertilization. Portions of the egg with cortical material, on the other hand, readily fertilize.

We may conclude from these observations that certain forms of treatment so alter the cortex as to facilitate endoplasmic outflow. By such treatment the fertilization capacity of the egg is not lost; it is, however, localized in only that part of the egg enclosed by cortical material. It thus follows that the inner substance of the egg is non-fertilizable in fertilized eggs not because of progressive centripetal changes set up at the cortex on insemination, but because the endoplasm is inherently non-fertilizable. Again, it is not necessary to postulate that the development of fragments from uninseminated eggs following fertilization may be due to the presence of some nuclear material of the egg (cf. Boveri). If the interpretation of the observations here reported be correct, fragments of uninseminated eggs, whether nucleated or not, are fertilizable if they possess cortical material. The egg cortex is thus necessary for the fertilization-reaction.

IV.

In any attempt at defining fertilization we must consider several facts.

In the first place, animal ova vary with respect to the stage in their development in which they are fertilized. Thus some reach the fertilizable condition before the germinal vesicle breaks down, others in the mesophase of the first maturation, still others during the second maturation, and many after maturation is complete. Starfish eggs may be fertilized at any time from the dissolution of the germinal vesicle to a short time after complete maturation.

Nor, again, is mere sperm penetration fertilization, since sperm normally penetrate ova (*Dinophilus, Saccocirrus,* etc.) some time before fertilization ensues. There are thus all possible types of fertilization with respect to the maturation stage of the egg when normally inseminated. No definition of fertilization is worth while if based on one type of egg alone.

In the second place, though the end result of fertilization is cleavage, there are here, too, many differences among animal ova. Thus the zygote nucleus may at first divide without cytoplasmic division (*Renilla*); the germ nuclei may fuse or appose merely; the cleavage spindle may be homodynamic, or heterodynamic; the sperm amphiaster may be homo- or heterodynamic, its second aster arising before or after union with the egg nucleus; and the cleavage centers may arise about the sperm nucleus or the egg nucleus or in part about each. A definition of fertilization in terms of the behavior of the germ nuclei or of the origin of the cleavage centers is manifestly inadequate.

If, for example, we consider the classic theory of Boveri that fertilization is due to the introduction of centrosomes by the spermatozoön, we realize its inadequacy at once, since it demands that the middle-piece enter the egg. It is true that the whole spermatozoön enters certain eggs whose maturation spindles are without centrosomes or asters, thus apparently supplying a deficiency. But in many other cases the middle-piece does not enter the egg, and where it does as in echinid ova the identity of its so-called centrosomes is wholly mistaken. To support the Boveri hypothesis we must shift the position of the potent centrosomes to fit those cases where the middle-piece does not enter the egg, or on entering takes no part in aster formation.

Because of failure to recall these simple facts purely morphological theories of fertilization fail. Indeed, many studies on fertilization are but studies of cell division; they deal with structures and phenomena in cell division in no wise restricted to egg cells. Nor yet have many physical or chemical theories been more fortunate. These theories are based on the study of physiological changes incident to cell division. But cell division is not fertilization.

An approach to the fertilization problem can be made only

through study of fertilization in the most diverse types of ova and by rejection of the incidental phenomena for the basic and common. What is the common factor in fertilization? So far as we know, it is some type of cortical change. But by cortical change we do not mean that the sign of the thing is the thing itself. Thus membrane separation in the sea-urchin egg is an easily visible sign of cortical change. Membrane separation, however, is not fertilization. It is here that an error lies in much of the work on experimental parthenogenesis.

Though we may doubtless gain knowledge of the nature of the cortical changes following insemination through study of these changes experimentally induced, we can not rely wholly on such work to explain fertilization. A far more simple mode of attack is to study fertilization itself. And if, in addition, the theory of the action of the agent in experimental parthenogenesis is erroneous, such a theory for fertilization can but hinder the solution of our problem. If it be true that cell division is not fertilization, it is equally true that experimental parthenogenesis is not fertilization. We must, therefore, study the fertilization process itself, the common factor of which is some kind of cortical change.

The evidence at hand indicates that the cortical changes in fertilization are due to an instantaneous, irreversible reaction between an ovogenous substance, fertilizin, and the spermatozoön. Stated in these terms the theory almost demands that the cortex is necessary for fertilization. The evidence herewith submitted points to this conclusion. The primary, if not, indeed, the whole event in fertilization, is the cortical reaction. The succeeding events with concomitant physical and chemical changes leading to cell division and development are the consequence of a complete cortical reaction between fertilizin and spermatozoön.

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