DO SPERMATOZOA PENETRATE THE MEMBRANE OF SELF-INSEMINATED EGGS OF CIONA AND STYELA?

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In 1935–36 I made many preparations (sections) of unfertilized eggs, and self- and cross-fertilized eggs of *Ciona* to find out whether spermatozoa do or do not penetrate the membrane of their own eggs. The main difficulty in reaching a decision was that the test-cells are so filled with granular materials that it is generally difficult to distinguish these, when stained, from the head of the very small spermatozoön. Nevertheless certain stains were found that could be de-stained to a point where the color of the particles in the test cells was removed leaving the stain in the heads of the sperms. Iron haematoxylin (half an hour) followed by thorough extraction gave good slides. More striking contrasts were obtained in eggs (killed in Bouin or in Carnoy solutions), sectioned, and stained in basic fuchsin (15 minutes); followed by picro-indigo carmine (six minutes), and washed in tap water.

The results showed that the spermatozoa do not as a rule pass the membrane of the eggs of the same individual. In other words, the block to self-fertilization is in the membrane.

During the winter of 1941–42 I have studied the problem again. In addition to the stains mentioned above, Ehrlich's haematoxylin (seven minutes) extracted in acid alcohol (half a minute or less), gave excellent preparations, as also did acetic-orcein (LaCour) followed in some cases by a fast green counterstain. For the study of the sections I had a better set-up than before, especially better illumination. Practically all the sections were examined in collaboration with Dr. Jack Schultz. Each case was examined by both of us. I am grateful to him for help and critical examinations.

Ciona Intestinalis

A few spermatozoa are as a rule found inside the membrane of cross-fertilized eggs, but only one or two in a section, some of them against the surface of the egg, others among the test cells. The sperm are stained

deep red after orcein or haematoxylin. It is difficult to determine whether any of the sperms are inside the test cells or merely between them; the best evidence seems in favor of the latter view. In *Ciona* the test cells form practically a continuous covering around the egg, and it might seem that the sperm are obliged to pass through them to reach the surface of the egg, but the test cells are loosely held together and it may be that sperms can pass between them. The test cells are green after the orcein and fast green, and the sperm red. They are unstained after the haematoxylin if properly extracted. The red-stained heads of the sperms, both outside the membrane and inside it, are strongly contrasted with the contents of the test cells.

The eggs that have been carefully removed from the oviduct, with no sperm added, show in sections, deeply stained particles and strands in the test cells unless the test cells are thoroughly de-stained, or are very little stained. These particles are of a different color from the sperm (orcein and fast green). The contents of the test cells are in these respects exactly like those in the self- and cross-fertilized eggs.

The selfed eggs are like the unfertilized eggs. In one or two cases a sperm has been found inside the membrane. Inasmuch as selfing may occur on a small scale such exceptional cases may be expected to occur, even in sets where no later cleavage happened; for, unless a sperm penetrated the membrane near the antipole, it might not enter the egg.

Certain precautions have to be taken with the sections of these selfed eggs. Occasionally one or more outside sperms may be dragged by the section-knife across the surface of the egg on one side. They will lie above or below the cut surface of the egg and, as a rule, there is no difficulty in detecting their origin. Slight folding of the edge of the section may give the appearance of sperm inside the egg, but such displacements are obvious.

Sections of cross-fertilized eggs show that spermatozoa may penetrate the membrane at all points of the surface, and not simply at the antipole where they also enter to fertilize the eggs. In exceptional cases it is possible that sperms may get in at other points of the surface of the egg before those that enter at or near the antipole, and if they do they may possibly enter the egg, but I have found no evidence of this. If they do, it is possible that abnormal cleavage or development would take place, but I have no grounds for such an assumption.

Styela (Cynthia) partita

At the end of the summer of 1941, five sets of eggs of *S. partita* were preserved. Records of the behavior of the remainder of the same eggs were made. The eggs were taken from the dishes in which they had

been ejaculated (along with the sperm) after 10 to 20 minutes. In some of these cases cross-fertilizations were also made to test the functioning of the sperm, and as a control of the selfed eggs. The sections were stained either in acetic orcein or in haematoxylin (Ehrlich).

1. Eggs and sperm were set free at 8 P.M. Some eggs were later preserved. None of the left-overs segmented, nor were embryos present the next morning. The stained selfed eggs showed no sperm inside the membrane, but stained sperm outside.

2. None of the selfed eggs was observed to segment, but next morning some normal tadpoles were present, presumably from eggs that cleaved late. The stained sections showed a few sperm inside the membrane both in the orcein and in the haematoxylin preparations. Some of the same eggs crossed to sperm of number (1) gave some embryos the next morning, showing that the sperm of (1) was "good."

3. None of the selfed eggs segmented. No sperms were found inside the membrane in the stained preparations with the possible exception of one sperm which was doubtfully inside.

4. Most of the selfed eggs produced abnormal embryos. Sperm were found inside the membrane in some eggs, but only a few in each case. The cross-fertilized eggs, to (3), gave 50 per cent normal tadpoles.

5. No cleavage or embryos were recorded, but the best preparations show a few sperms inside the membrane. No crosses were made.

Discussion

The preceding evidence shows very clearly that in most cases no sperms are found inside the membrane of self-fertilized eggs in cases where no cleavage or embryos were observed. In the last case (5), however, a few sperm were found. Since self-fertilization does take place to a considerable extent in *Styela* it is to be expected that sperm may enter some of the eggs. Cleavage or development would be expected, in some cases at least, provided one of the sperm entered at or near the antipole. If this did not happen it is doubtful if sperm reaching the surface elsewhere would enter the egg, and it is also possible that when a sperm does enter the egg in the antipole region, the surface of the egg may then become resistant to sperm, present at another part of the egg, as is known to be the case in many other eggs.

This examination shows that after suitable staining, i.e., when the sperm are sharply marked off from the contents of the test cells, the sperm are not present or rarely present in or between the test cells after self-insemination. Whether some of the entering sperm pass through or around the test cells is unimportant for the present discus-

sion. I have observed cases where the sperms that were inside the membrane were not inside the test cells, other cases where they appeared to lie on the surface of the test cells, and a few cases where they seemed to be inside these cells. The test cells of *Styela* do not completely surround the egg, as they do in *Ciona*. These cells are often grouped in twos, threes, or more with intervening spaces. Sperm entering these spaces could pass directly to the surface of the egg. There is not the slightest evidence, even after cross-fertilization, that the test cells are filled with sperm. Without suitable staining and extraction the threads and granules might easily be misinterpreted as spermatozoa.

Styela Montereyensis

To date I have not been able to bring about spawning in this species, but abundance of eggs and sperm can be obtained from large individuals by cutting open the animal, removing the digestive tract (including the pharynx), slicing off the ovaries and their ducts, and, necessarily, the testes also and sperm ducts at the same time. A cloudy suspension is obtained, the debris is removed, the eggs concentrated by revolving the dish, the supernatant fluid poured off, or else the eggs removed to sea water. Neither cross- nor self-fertilization takes place (in January) in many cases, but at times cross- and even some self-fertilization occurs, and these are the only ones in the following account that were used to test the entrance, or failure of the sperm to enter. The eggs were killed in Bouin's solution in most cases, cut into sections, and studied under an immersion lens. Active sperm are rarely observed, whether because of the presence of mucus from the body tissues, or because the spermatozoa are sluggish in this species, or are immature. The failure to cross-fertilize seems to be due to failure of the sperm rather than of the eggs. Attempts to activate the sperm by treatment with ether, or with various salts, have failed as a rule, but in a few cases by the use of lemon juice (2.7 pH) self-fertilization took place when it did not occur in eggs fertilized in sea water or had occurred to a smaller extent. A good many preparations of the eggs of this species were examined, both in cases where no cleavage or development took place, and in cases where crossing led to a considerable amount of cleavage and sometimes 100 per cent of embryos. In the former cases no sperm were found inside the membrane. The test cells in this species, as in the other *Styela,* contain yellow-red granules and threads of material that stain, but after proper extraction these lose their color while it remains in the heads of the spermatozoa. The latter stand out conspicuously, being stained deep red (acetic orcein). The sperms here, as in the other species, are large

and can be identified by their shape as well as their color. Nevertheless, I have not found any eggs, even in those that gave a high percentage of cleavage, with sperms inside the membrane, although one sperm at least must have entered at the antipole in the eggs that cleaved. The paucity of active sperms may in this case be the explanation of failure to find any inside the membrane.

Removal of the Test Cells from Most of the Egg's Surface

In order to find out whether the presence of the test cells plays a role in inhibiting the entrance of spermatozoa into their own eggs I centrifuged (1938) the unfertilized eggs of *Ciona* for two hours at a high speed (about 3,500 r.p.m.). The test cells were driven to one pole of the egg where they form a thick mass, or a zone of clear material. The surface of the rest of the egg is brought into contact with the membrane. Such eggs were tested by selfing and crossing. It was found that they do not self but will cross. Control normal eggs were tested in each set and gave corresponding results.

The number of experiments of this kind was small and I repeated the procedure in the autumn of 1941. The first test was negative, the eggs neither selfed nor crossed. I noticed, however, that the centrifuge that I used was quite warm after two hours, and failure of the eggs to cross-fertilize was undoubtedly due to the high temperature. To avoid this I removed the machine to a cold room (4° C.), but after two hours the test cells were not moved. The low temperature had, it appears, stiffened them or produced some other effect such that they were not moved. Then a different make of centrifuge was used that did not warm up during the two hours of rotation. The results in every case were uniformly good; the selfed eggs did not segment while practically all of the crossed eggs segmented.

The results confirm the earlier ones and demonstrate that the test cells are not concerned with the block to self-fertilization. The results do not show, of course, that the block may be in the surface of the egg. Other observations or methods are necessary to demonstrate that it is the membrane around the egg and not its surface that inhibits self-fertilization. Evidence bearing on this point was obtained some years ago (1923) by cutting the membrane which sometimes allows the whole egg either to squeeze out, or to pinch off fragments of different sizes. These exposed eggs and fragments self-fertilized and segmented. It might be argued that the operation itself had affected the surface of the egg so that it lost its power to block the entrance of its own sperm, but this seems to me a rather far-fetched argument, purely theoretical, since such exposed eggs gave normal cleavage and sometimes normal embryos.

Summary

A comparison of unfertilized eggs of *Ciona* with self- and cross-fertilized eggs show that spermatozoa do not, as a rule, penetrate the membrane of their own eggs. Rarely a sperm may be found inside a self-inseminated egg, which is consistent with the fact that occasionally self-fertilization occurs. Even in cross-fertilized eggs only a very few sperm are found inside the membrane of such eggs. The same statement holds for two species of *Styela* examined, except that a few more sperm are likely to be found inside the membrane of the self-inseminated eggs, which is consistent with the fact that self-fertilization is more frequent in these species than in *Ciona*.

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