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Sperm Activation and Aggregation during Fertilization in Some Fishes
V. Sperm-stimulating Factor on the Vegetal Pole

With 3 Text-figures

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(*Communicated by* T. YAMAMOTO)

In the preceding paper of this series, it was reported that sperm aggregation and activation take place on the egg membrane around the micropyle area of Japanese bitterlings and the fat minnow (1958). Moreover, it was reported that the eggs deprived of the sperm-stimulating factor with acidified Ringer's solution lose the capacity for fertilization in dilute sperm suspension, but if such eggs are inseminated with dense sperm suspension, they are fertilizable (Suzuki, 1960). This fact suggests that the factor plays an important rôle to facilitate the chance of an encounter between the egg and spermatozoon at fertilization in natural condition. This suggestion is substantiated when we take into consideration the spawning behavior of bitterlings as compared with that of other species of fresh-water fishes (cf. Suzuki, 1959b, 1959c).

The present author, however, found that in some eggs of bitterlings the surface of the vegetal pole which has nothing to do with sperm entrance into the egg also aggregated spermatozoa. Since then an analysis of the cause of sperm aggregation in this area has been conducted. Ripe unfertilized eggs used as materials were obtained chiefly artificially using the method reported previously (Suzuki, 1957; Suzuki and Kobayasi, 1958). Occasionally, they were obtained from naturally spawning females. The observations were made at room temperature varying from 18° to 22°C.

Observations and Experimental Data

1) Relation between the sperm-stimulating capacity of vegetal pole and the arrangement of eggs in ovipositor

Sperm activation and aggregation on the vegetal pole were observed in five species of bitterlings, *Rhodeus ocellatus*, *Acheilognathus lanceolata*, *Acheilognathus* *tabira*, *Acheilognathus limbata* and *Acheilognathus cyanostigma*, but not in the fat minnow, *Sarcocheilichthys variegatus*. Typical examples of sperm aggregation on the vegetal pole of *Acheilognathus lanceolata* and *Acheilognathus tabira* are shown in Figure 1*A* and *B*. Such an aggregation on the vegetal pole is less

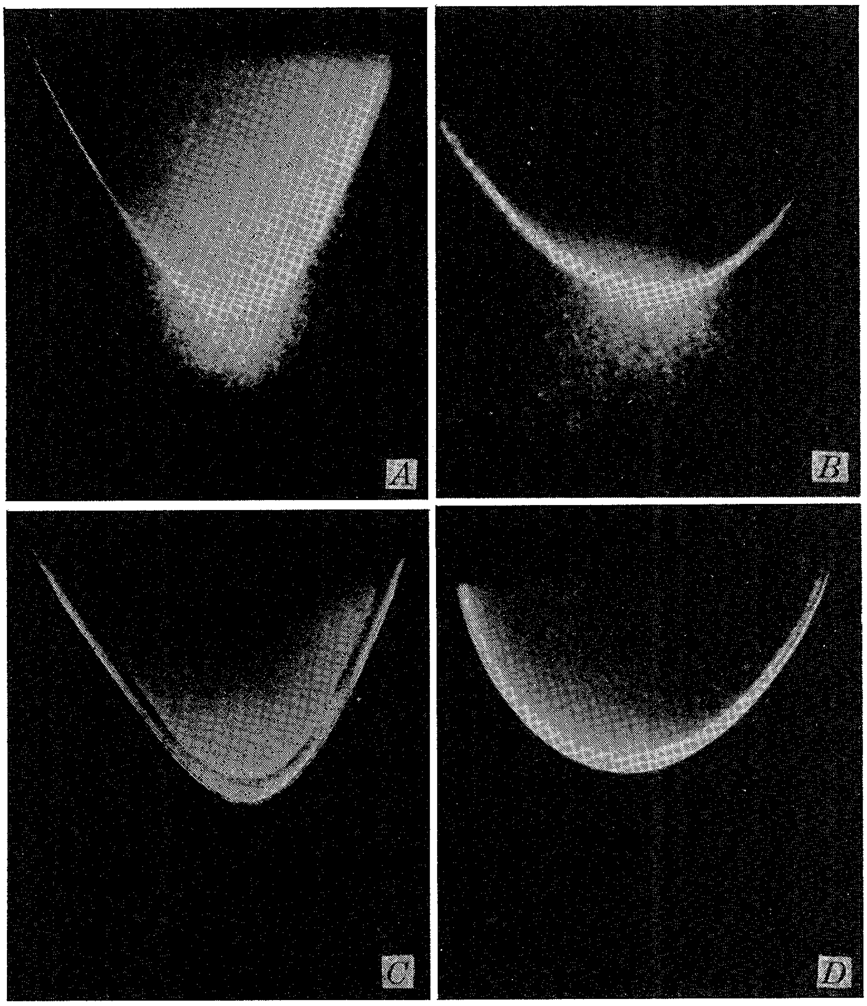

Fig. 1. Sperm aggregation on the vegetal pole of *Acheilognathus lanceolata* (*A*) and *Acheilognathus tabira* (*B*). No sperm aggregation on the vegetal pole of *Acheilognathus lanceolata* (*C*) and *Acheilognathus tabira* (*D*) inseminated after washing with distilled water. Phase contrast photomicrographs.

conspicuous than that on the micropyle area. The phenomenon, however, was observed in eggs obtained either artificially or naturally. On washing eggs with distilled water, the vegetal pole lost completely the capacity for sperm aggregation within a few minutes (Fig. 1*C* and *D*), while the micropyle area retained it conspicuously for 30 minutes or more.

Sperm aggregation on the vegetal pole, however, was not always observable in all eggs. Whether spermatozoa were aggregated or not on the vegetal pole was closely related to the arrangement of eggs within the ovipositor which develops from the belly of the bitterling female during the spawning season. When the belly of females was pressed with the fingers, the eggs were usually produced through the elongated ovipositor making a single row with the vegetal pole of each in front. Sperm activation and aggregation took place on the vegetal pole which had come in contact with the animal pole of the egg adjointed just in front as shown in Figure 2*A*. But no such aggregation was induced by the vegetal pole free from contact with the animal pole of the adjoining egg in front by leaving a space filled with some sort of sap between the two eggs as shown in Figure 2*B*. Furthermore, the vegetal pole of the egg first pushed out of the row failed to attract any spermatozoa at all.

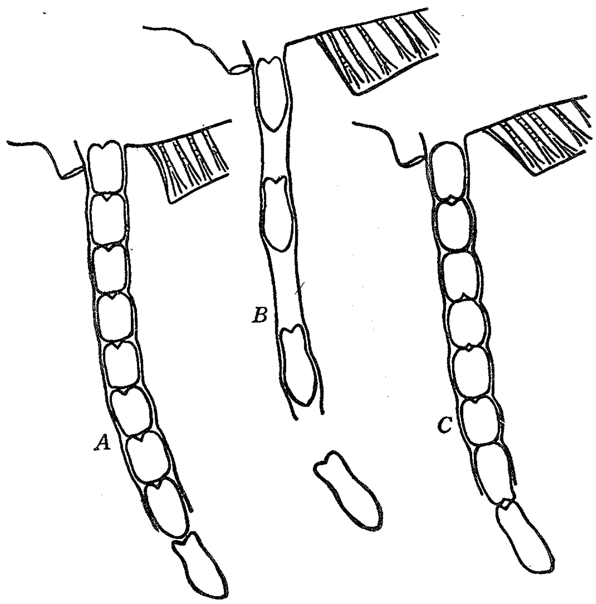

Fig. 2. Diagrams showing three types of egg arrangement within ovipositor when bitterling eggs are laid. *A*: Regular arrangement of eggs with their vegetal poles in front and being contacted with the animal poles of those just in front of them. *B*: Arrangement leaving some space between adjacent eggs. *C*: Irregular arrangement with some eggs with their animal poles in front.

Occasionally, however, an abnormal oviposition was observed in *Acheilognathus lanceolata*; that is, when ripe eggs were laid making a single row, some eggs came out with animal poles of each in front, and the rest, with vegetal poles in front, as shown in Figure 2*C*. In this case sperm activation and aggregation on the vegetal pole also took place only in eggs which had come out in contact with the animal pole of the adjacent egg, but any non-contacted egg failed to aggregate spermatozoa on the vegetal pole.

2) *Artificial transference of sperm-stimulating factor from the micropyle area*

In view of the above-mentioned facts, sperm activation and aggregation on the vegetal pole was considered to be due to a chemical substance which had transferred to the vegetal pole membrane from the micropyle area membrane of the adjoining egg. In order to ascertain this view-point, the following experiments were performed. Some ripe unfertilized eggs of *Rhodeus ocellatus* or *Acheilognathus limbata* were moistened with M/7.5 Ringer's solution adjusted to pH 7.0 with a small quantity of $NaHCO_3$. Then the micropyle area membrane was put in contact several times artificially with the egg membrane of the intermediate area other than both the poles of another fresh ripe unfertilized egg and also to the vegetal pole membrane which had been deprived of the capacity for sperm stimulation by washing with distilled water. Then both contacted egg membranes were tested for the capacity for sperm aggregation.

It was found that spermatozoa did aggregate on these two materials as shown in Figure 3*A* to *D*. On the other hand, when the intermediate area was con-

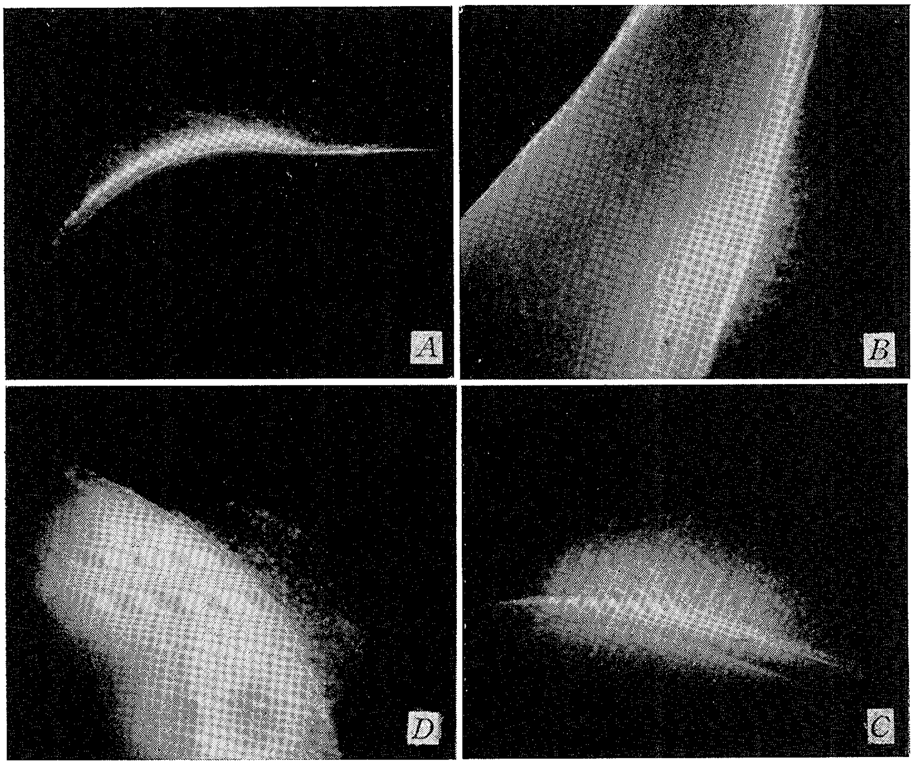

Fig. 3. *A–C*. Sperm aggregation on the intermediate area other than both the poles of *Rhodeus ocellatus* eggs (*A* and *B*) and *Acheilognathus limbata* egg (*C*) which have been made to come in contact artificially with the micropyle area of another fresh ripe unfertilized egg. Phase contrast photomicrographs. *D*. Sperm aggregation on the vegetal pole of *Acheilognathus limbata* egg which has been made to come in contact artificially with the micropyle area of another fresh ripe unfertilized egg after destroying the sperm-stimulating capacity by washing with distilled water. Phase contrast photomicrograph.

tacted artificially with the vegetal pole deprived of the capacity by washing, no aggregation was induced by this material. Of course, no aggregation took place on the intact intermediate area of a ripe unfertilized egg.

3) *Relation between the sperm-stimulating capacity on the vegetal pole and the fertilizability of eggs*

Next, a question arises as to whether or not the sperm-stimulating factor on the vegetal pole plays a rôle for the natural fertilization of bitterlings. In order to solve this question, the following experiment was carried out. Some unfertilized eggs of *Acheilognathus lanceolata* were added to sperm suspension diluted to the density of 10^{-9}; and after having observed the behavior of spermatozoa on

Table 1

Fertilizability of *Acheilognathus lanceolata* eggs (a) with the sperm-stimulating capacity on the vegetal pole, (b) without sperm-stimulating capacity on the vegetal pole

Number of eggs examined	Percentage of fertilized eggs	
	(a) with sperm-stimulating capacity on the vegetal pole	(b) without sperm-stimulating capacity on the vegetal pole
45	100.00	96.97*
68	89.74	89.66
71	87.23	87.50**
Means	92.32	91.38

* Including about 2 per cent cytolysis, no sperm aggregation at all on the whole egg surface. ** Including about 4 per cent cytolysis, no sperm aggregation at all on the whole egg surface.

the vegetal pole, eggs which had the capacity for stimulation were separated from the rest. Then the percentage of fertilization in both cases was calculated by counting the cleaved eggs after about 2 hours. It was found that the fertilizability of eggs with the capacity of stimulation on the vegetal pole and that of those without it were similar to each other. A typical example with *Acheilognathus lanceolata* eggs is given in Table 1.

Discussion and Conclusion

As was pointed out in an earlier paper (Suzuki 1959a), the micropyle region of the bitterling egg membrane keeps a conspicuous capacity for sperm stimulation for 30 minutes, retaining the reduced capacity for 2 hours, when eggs are immersed in distilled water. In the present investigation, however, the vegetal pole lost the capacity completely for sperm stimulation within a few minutes upon washing with distilled water. This rapid loss may have been due to somewhat loose binding of the sperm-stimulating factor on the vegetal pole as compared with the case of the micropyle.

Be that as it may, the fact that spermatozoa aggregated on the intermediate area other than both the poles of egg membrane, which had been put in contact artificially with the micropyle area of another egg, tells us that the sperm aggregation on the vegetal pole is caused by a chemical substance transferred to the vegetal pole membrane from the micropyle area of the adjoining egg when eggs pass through the ovipositor. Likewise, this may be true from the fact that the vegetal pole membrane, which had been deprived of the capacity for sperm stimulation, aggregated spermatozoa when contacted artificially with the micropyle area. When eggs pass through ovipositor leaving a space between each other, the substance is probably unable to be transferred to the vegetal pole from the micropyle area of the neighboring egg. No information, however, is available at present as to why such a space is left between each other when eggs pass through the ovipositor.

Sperm-stimulating factor on the vegetal pole does not play any important rôle for fertilization of bitterling in contrast with the case of the micropyle area. This is obvious from the fact that no significant difference of fertilizability is found between the eggs with the sperm-stimulating capacity on the vegetal pole and those without it. From the point of view of sperm entrance into the egg, spermatozoa may rather be misled by the factor on the vegetal pole which has nothing to do with sperm entrance in natural condition.

Summary

1) When eggs of bitterlings were inseminated with spermatozoa artificially, sperm activation and aggregation were observed not only on the animal pole but on the vegetal pole of some eggs, although less conspicuous on the vegetal pole than on the micropyle area.

2) Such aggregation took place in the egg which had come in contact with the animal pole of the egg adjoined just in front when they were passing through the elongated ovipositor in which they were arranged in a single row with the vegetal pole of each in front. But any non-contacted egg failed to aggregate spermatozoa on the vegetal pole.

3) The factor responsible for sperm stimulation on the micropyle area could be transferred to the intermediate area other than the both poles of the egg membrane by artificial contact with the micropyle area of another fresh ripe unfertilized egg.

4) There was no difference between the fertilizability of the eggs with the sperm-stimulating capacity on the vegetal pole and that of those without it. This fact suggests, therefore, that the factor on the vegetal pole does not play any important rôle for fertilization of bitterling eggs.

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