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Self and Non-Self Recognition between Gametes of the Ascidian, *Halocynthia roretzi*

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Summary. The self-sterility of *Halocynthia roretzi* from Mutsu Bay, Japan, was examined. This sterility is strict and not a single egg can be fertilized in self-sterile animals. Less than 2% of the animals were self-fertile (with 100% cross-fertility). All heterologous sperm can fertilize all eggs, although there are pairs of individuals in which the coelomocytes recognize each other as self. Eggs deprived of follicle cells cannot be fertilized by either autologous or heterologous spermatozoa. Detached autologous or heterologous follicle cells can reattach to the chorion in calcium-enriched sea water and the reconstituted eggs recover their ability to be fertilized. A "mosaic egg" can therefore be obtained, which consists of oocyte, test cells and chorion originating from one individual and follicle cells from another. The "mosaic egg" was used to determine the site of recognition of self and non-self. The results indicate that the recognition resides in the chorion and/or test cells, probably the chorion. The relationship between somatic alloreactivity, previously found in coelomocytes of *H. roretzi,* and gamete reactivity is discussed.

Key words: Fertilization – Self and non-self recognition – Follicle cells – Ascidian

Introduction

Ascidians are generally hermaphroditic. Some species are self-sterile, that is, eggs cannot be fertilized by sperm of the same individual in spite of the simultaneous release of eggs and sperm from the same atrial siphon. Morgan (1923, 1942) analysed this phenomenon for the first time using *Ciona intestinalis* and found that naked eggs which are free from their envelopes (follicle cells, chorion and test cells) could be fertilized by spermatozoa of the same individual as well as by those of different animals. He concluded that this self-sterility is due to a block imposed by the egg envelopes. More recently, Rosati et al. (1978a, b; Pinto et al. 1981) found that spermatozoa attached firmly to the chorion whenever fertilization succeeded but did not when fertilization failed. Because of this, they suggested that the chorion was the site of recognition of self and non-self.

In this paper, self-sterility in *Halocynthia roretzi,* an ascidian inhabiting the northern part of Japan, was examined. In *H. roretzi,* both gametes are released at almost the same time under natural and controlled conditions. The present data show that the self-sterility of *H. roretzi* is more strict than that reported of *C. intestinalis* (Rosati et al. 1978a).

Recently, it was shown that the coelomic cells from *H. roretzi* exhibit an allogeneic cellular reaction, denoted 'contact reaction' (Fuke 1980). When isolated coelomocytes from different individuals were brought into contact in vitro, both cells reacted, resulting in their reciprocal lysis. The reactions occurred in some animal combinations, but not all. Therefore, the somatic cells of *H. roretzi* were thought to possess an individuality. Is this somatic individuality related to the selective fusion of germinal cells? Can eggs of one individual be fertilized by all sperm of different individuals, or are there specific pairs of individuals which can not cross-fertilize? To answer these questions, a large number of heterologous fertilizations were examined.

In *H. roretzi,* removal of the egg envelopes, mechanically with a needle, resulted in the breakdown of self-sterility (Hoshi et al. 1981), as did protease treatment (Fuke unpublished data). Therefore, the recognition site for self and non-self in the eggs of *H. roretzi* is thought to be at the egg envelope as in *C. intestinalis.* However the question of which component of the envelope, follicle cells, chorion or test cells, is responsible for self-sterility still remains to be solved. This is the main subject of the present report.

In *H. roretzi,* it was found that eggs deprived of their follicle cells by mechanical force, but still retaining the chorion and test cells, could be fertilized by neither autologous nor heterologous sperm (Ishikawa et al. 1978). The follicle cells seem to be indispensable for fertilization of *H. roretzi* eggs for reasons not yet explained. Follicle cells can also be removed gently by versene and eggs deprived of follicle cells by this method also cannot be fertilized by heterologous or even autologous sperm. Detached follicle cells can be reattached to the chorion under appropriate conditions and these reconstituted eggs recover the ability to be fertilized. As follicle cells obtained from different individuals can attach to the chorion, we obtained a "mosaic egg", which consists of an oocyte, test cells and chorion originating from one individual, and follicle cells from a different individual. This "mosaic egg" was used to determine the site of recognition. The results show that self and non-self recognition in eggs of *H. roretzi* resides in the chorion and/or test cells, probably the chorion.

Materials and Methods

Preparation of Gametes. Individuals of *Halocynthia roretzi* (Drashe) were collected from Mustu Bay, in Aomori prefecture, which is a northern district of Japan. Among three intra-specific variants (Numakunai and Hoshino 1980; Nu-

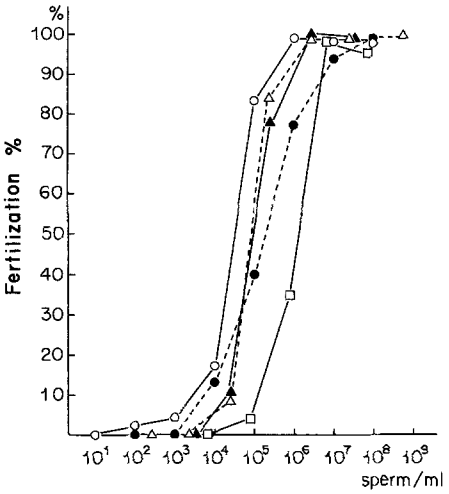

Fig. 1. Curves of % of the heterologous fertilization versus sperm concentrations. The different symbols show five different experiments. *Abscissa:* sperm concentration/ml; *ordinate:* % of fertilized eggs

makunai et al. 1981; Fuke and Numakunai 1982), Type A animals were mostly used. They were collected in October and kept in running sea water until November in the aquarium facilities of the Marine Biological Station, Asamushi Aomori. Numakunai (personal communication) kindly gave directions for controlling the timing of breeding. One day before the experiment, the animals were kept in warm sea water at 10°–15° C for 16–20 h. The next morning from 8:00 to 9:00 a.m., they released their gametes sponaneously. Three methods were used for obtaining gametes. To get naturally spawned gametes, each animal was transferred to an individual container 1 h before the predicted time of spawning. After spawning, gametes were filtered through nylon mesh (NXX13, 94 μ). The filtrate comprised the sperm suspension, while eggs were retained on the mesh and the latter were used after washing with sea water. In the second method, animals were dissected and their digestive organs and gills were removed *during* the natural breeding time. They were first washed with sea water and then put in Petri dishes containing sea water. For 2 or 3 consecutive hours, the eggs and sperm were spawned from the oviduct and spermatic duct of the dissected animals. The third method was a slight modification of the second. To exclude contamination completely by heterologous sperm, the ascidians were dissected about 1–2 h *before* the natural breeding time and washed extensively and very carefully with sea water.

Fertilization. After they were washed with sea water, about 100 eggs from a single animal were suspended in 1 ml of sea water and inseminated with 25–50 μl of diluted sperm suspension, either from the same animal (autologous fertilization) or from different animals (heterologous fertiliza-

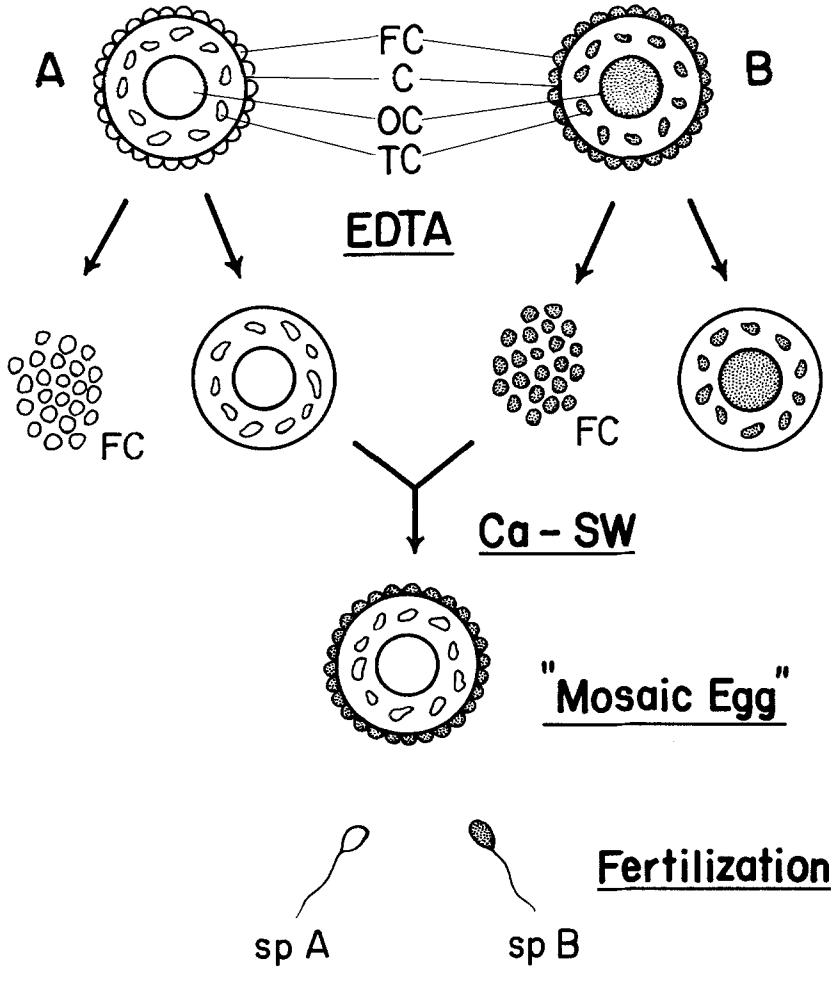

Fig. 2A, B. Diagram showing the design of an experiment which creates "mosaic eggs" and tests their ability to be fertilized with different sperm. The eggs from two individuals **A, B** were treated with 1% EDTA to detach the follicle cells from the chorion. The egg **A** was put in Ca-enriched SW together with the follicle cells obtained from a different egg **B**. The "mosaic egg", which consists of an oocyte, test cells and chorion originating from A and follicle cells from different one **B**, was fertilized with either **A** or **B** sperm

tion). The sperm concentration was estimated by counting sperm using a Burger's hematocytometer under a phase-contrast microscope. In the present experiments, a concentration of 4×10^7 to 1.5×10^8 spermatozoa/ml was used, except in special cases. This concentration is several times the minimum value necessary for fertilization (Fig. 1). The rate of fertilization was represented by the percentage of cleaved eggs, as determined 2–3 h after fertilization.

Preparation of Reconstituted Eggs. Eggs were carefully washed several times, with 5–6 volumes of sea water to remove completely all the autologous sperm. To detach follicle cells, 0.5 ml of eggs (2500–3000 eggs) was treated with 10 ml of 1% EDTA (Sigma Chemical Co., Saint Louis U.S.A.) in 0.56 M NaCl (pH 7.5) for 30 min (Fig. 2). Eggs were pipetted up and down gently, causing all follicle cells to detach from the chorion. The dissociated follicle cells were gathered by centrifugation at 3000 rpm for 15 min and washed twice with sea water. After it was confirmed that no follicle cells remained on the chorion, 800–1,000 eggs were put in 1 ml of Ca-enriched sea water (0.6 g $CaCl_2$ in 100 ml of sea water; Ca-SW). To this solution, 0.5 ml of follicle cells ($4–6 \times 10^6$) in Ca-SW was added. After 1 h of incubation at room temperature (about 10° C), it was confirmed that the follicle cells were again attached to the chorion, irrespective of their origin, that is, from the same individual or a different one (Fig. 3). The reconstituted eggs were used for fertilization experiments as described above.

Results

Fertilization of Intact Eggs by Self and Non-Self Sperm

Intact eggs, with complete envelopes, were released from the oviducts in vitro at the natural spawning time. These were suspended in 1 ml of sea water and inseminated with sperm of different animals (heterologous fertilization). Within the optimum range of sperm concentrations (10^7–10^8/ml), almost all eggs (95%–100%) were fertilized. Below the sperm concentration of 10^3/ml, very few were fertilized. At intermediate concentrations, the fertilization rates differed among the batches examined (Fig. 1).

During November, 1981, 412 heterologous fertilizations were observed at Asamushi Marine Biological Station. In 392 (95%), more than 90% (98.6%, average) of the eggs were fertilized. In the remaining 20 (5%), less than 90% of the eggs were fertilized by heterologous sperm (66% average). This poor fertilization in the latter case was attributed to an insufficiency in the gamete's fertility due to an unknown cause. In 3 individuals, it was due to failure of the sperm and in one, to failure of the eggs. When inadequate sperm was used, all tests yielded results of less than 90% of eggs fertilized, regardless of the origin of the eggs. Similarly, eggs that in some way were bad could not be fertilized with any sperm, even though these sperm could fertilize other eggs normally. There were no specific combinations in which fertilization could not succeed. All gametes of *H. roretzi* could fertilize each other except when physiological conditions were inadequate. The nature of these inadequacies are uncertain, but may be due to immaturity of the gametes.

In another series of experiments, autologous fertilization was tested. During November, 1980, self-fertility was examined using naturally spawned eggs from 22 individuals. One to six per cent of the eggs were found to be fertilized (average, 2.8%). In November 1981, autologous fertilization was tested in 137 experiments using the gametes released in vitro. The results are summarized as follows. In 66% of the animals (90 individuals), no fertilization occurred. In 30% (41 individuals), only a few eggs (1%–10%) appeared to be fertilized. Therefore, 96% of the animals showed autosterility of more than 90%. In 3% (4 individuals), 11%–50% of the eggs were fertilized. Only two individuals had complete self-fertility (100% of eggs fertilized).

The next experiment, using the third method, was carried out to exclude the possibility of contamination by heterologous sperm. Of the ten animals examined by this method, none was self-fertile (Table 1). Moreover, among 2,099 eggs examined not a *single* egg was fertilized by autologous sperm.

Table 1. Auto-sterility and hetero-fertility of gametes obtained from animals, using special care to eliminate contamination by heterologous gametes

Animal no.	Auto-sterility: unfertilized eggs/fertilized eggs		Hetero-fertility: unfertilized eggs/fertilized egg	Hetero-fertility: % of eggs fertilized
	Exp. 1	Exp. 2		
1	104/0	107/0	9/123	93%
2	91/0	151/0	0/91	100%
3	87/0	76/0	20/112	85%
4	132/0	110/0	2/108	98%
5	133/0	92/0	0/103	100%
6	98/0	99/0	0/74	100%
7	122/0	104/0	5/79	94%
8	82/0	128/0	2/88	98%
9	134/0	70/0	0/77	100%
10	85/0	94/0	0/123	100%
Total	2099/0		38/978	96%

Table 2. Fertilization of reconstituted eggs

Eggs treated with:	No. of eggs		Eggs fertilized (%)
	Un-fertilized	Fertilized	
EDTA[a]	298	0	0%
EDTA + follicle cells in SW[b]	305	10	3%
EDTA + follicle cells in Ca-SW[c]	17	194	90%
Intact eggs in SW	11	275	96%
Intact eggs in Ca-SW without sperm	251	0	0%

[a] Eggs from which follicle cells were removed, with 1% EDTA
[b] Reconstituted eggs which were obtained by adding autologous follicle cells to unfertilized eggs lacking follicle cells in natural SW
[c] Eggs reconstituted in Ca-enriched SW. All eggs were inseminated with heterologous sperm, except for the last case

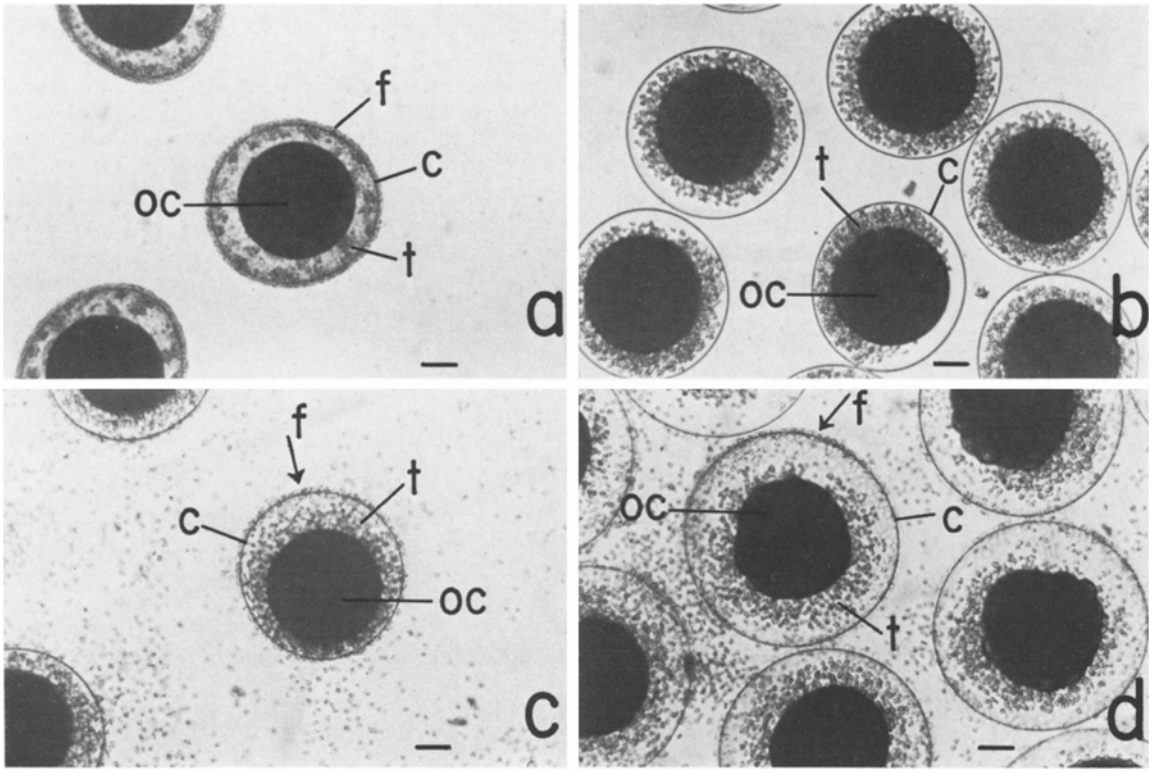

Fig. 3a–d. Fertilization of "mosaic eggs". **a** Normal unfertilized eggs. **b** Eggs from which the follicle cells had been removed were inseminated with the heterologous sperm. Fertilization did not occur. **c** "Mosaic eggs", which consisted of oocytes, test cells and chorion from A and follicle cells from different one, B (Fig. 2). The sperm from A were used for insemination. No fertilization is shown. **d** "Mosaic eggs" were the same as in **c**, but the sperm B used were different from those of experiment c. Fertilization succeeded. The eggs showed an expansion of the perivitteline space as well as cleavage. *f*, follicle cells; *t*, test cells; *c*, chorion; *oc*, oocyte. *Arrows* indicate where follicle cells reattach to the chorion in "mosaic eggs". Bar equals 100 μ

Table 3. Fertilization of "mosaic eggs" with either autologous or heterologous sperm

Eggs	Sperm	No. of unfertilized eggs	No. of fertilized eggs	Eggs fertilized (%)
Intact eggs (A)	(B)	1	256	100
Intact eggs (A)	(A)	157	7	4
Eggs (A) – follicle	(B)	222	1	0
Eggs (A) + follicle (A)	(B)	36	296	89
Eggs (A) + follicle (B)	(A)	248	0	0
Eggs (A) + follicle (B)	(B)	40	272	88

Two individuals, (A) and (B), were used. Eggs (A) – follicle: eggs obtained from (A) were treated with EDTA, which removed the follicle cells. Eggs (A) + follicle cells (B): eggs (A) from which follicle cells were detached were reconstituted by adding follicle cells originating from (B)

Fertilization of Eggs Without Follicle Cells

Eggs enzymatically deprived of their envelopes were fertilized by sperm of the same individual as well as by those of different ones. But follicle-free eggs were not fertilized either by heterologous or autologous sperm (Table 2, Fig. 3b). To exclude the possibility that the chorion and/or oocyte were injured by EDTA, the follicle cells detached from the chorion were reattached, and then the ability of the reconstituted eggs to be fertilized was tested. When sea water was used as the reconstitution medium, only 3% of the eggs recovered the ability to be fertilized (Table 2). The fertilized eggs, without exception, had some follicle cells on the chorion, while almost all uncleaved eggs did not. In Ca-enriched sea water, however, almost all eggs reattached to follicle cells. About 90% of the eggs were fertilized after successful reattachment of follicle cells (Table 2).

Fertilization of Reconstituted Eggs

In the next experiment, the "mosaic eggs" were used to solve the problem of whether the recognition site exists on the chorion (and/or test cells) or the follicle cells. When sperm heterologous to the chorion and test cells were used, about 90% of the eggs were fertilized, while not even one egg was fertilized by autologous sperm, even though the eggs had follicle cells on their chorion (Table 3, Fig. 3c). Therefore, it was concluded that the recognition site for self and non-self was not follicle cells but was the chorion (and/or test cells).

Discussion

In heterologous fertilization of *H. roretzi* during their best breeding season, more than 90% of the eggs were fertilized (average value, 98.5%). Fertilization occurred in all pairs of individuals tested. On the other hand, with autologous fertilization, self-sterility was found in almost all animals. Only 2 of the 137 animals examined had complete self-

fertility. When gametes were collected with special care to exclude contamination by heterologous sperm, none of the eggs was fertilized. When naturally spawned gametes or those from animals dissected without special care were examined, 1%–10% of eggs were found to be fertilized in many cases. These fertilizations may be attributed to contamination by heterologous sperm. With regard to the two individuals with 100% self-fertility, the possibility of contamination cannot be excluded completely. However, self-fertile animals have been found several times in the aquarium of the Marine Biological Station, Asamushi during the past 10 years (Numakunai personal communication).

Therefore, less than 2% of the individuals of *H. roretzi* may be self-fertile. The frequency of self-fertility in *H. roretzi* is lower than that in *Ciona intestinalis*, in which 15% of the animals are self-fertile (Rosati and De Santis 1978a). The pattern of self-fertility in ascidians is thought to be characteristic of each species.

In all heterologous combinations, including those in which the somatic cells did not exibit the "contact reaction", fertilization suceeded. However, this does not necessarily mean that there is no relationship between fertilization and cellular allogeneic reactions. For example, it may be that there are polymorphic elements which govern both fertilization and the somatic reaction. In somatic cells, the reaction is triggered whenever two individuals have no common elements and on the other hand, fertilization succeeds whenever male and female gametes have any different elements. Infact, each individual is thought to have unique elements because no identical individuals were ever found in experiments employing large panels of 30 randomly collected individuals (Fuke and Nakamura, unpublished work).

It is known that the colonial ascidian, *Botryllus primigenus* Oka, has a single multiallelic gene (F) that simultaneously controls gamete compatibility and fusibility between individual colonies. Fusion of two colonies ensues when they share an allele of the gene (F). On the other hand, fertilization takes place only if the single allele carried by the spermatozoa is not shared by the egg donor, even when fusion occurs between the parents. The mechanism of self-recognition in *B. primigenus* was denoted "haploid diploid incompatibility" (Oka and Watanabe 1967; Oka 1970). A similar mechanism is possible in *H. roretzi*. A genetic approach to self-recognition in *H. roretzi*, however, is difficult, because its life cycle is very long compared with that of *B. primigenus*. Biochemical and immunochemical studies on the somatic cell membrane and egg envelopes are in progress to clarify the correlation between gamete and somatic compatibility.

The eggs from which follicle cells were removed with EDTA could not be fertilized with either heterologous or autologous sperm. The eggs recovered the capacity for fertilization after reattachment of the follicle cells. This result strongly supports the conclusion of Ishikawa et al. (1978) that follicle cells are indispensable for fertilization in *H. roretzi*.

There are several reports regarding the roles of follicle cells during the development of ascidians. These roles include egg flotation (Lambert and Lambert 1978), sperm attraction (Miller 1975) and the synthesis of RNAs and proteins (Jaffery 1980). However, as the decisive role of follicle cells in fertilization of this ascidian is not yet known, further intensive studies will be required. From the present studies, it was concluded that the follicle cells are essential for fertilization but are not the site of recognition of self and non-self.

As candidates for the recognition site, the chorion and test cells remain. Whether the chorion or test cells are responsible for the block of self-fertilization cannot be decided because we have not found any effective tool for discriminating between them. However, we regard the chorion as the primary candidate for the recognition site for several reasons. Firstly, it is reasonable to discriminate self and non-self at the outer layer of the egg envelopes before the sperm undertakes the difficult task of passing through the chorion. Secondly, the test cells move about freely in the broad perivitelline space and spaces exist between the test cells. The sperm may be able to pass through the spaces without interference by the test cells.

In *Ciona intestinalis*, Rosati and De Santis (1978a) pointed out the chorion as the site at which the self and non-self recognition occurs, because a firm binding between the chorion and sperm is formed whenever fertilization occurs, but not when fertilization fails. In *H. roretzi*, however, specific binding was not observed in either intact or fixed eggs. Some of the autologous as well as heterologous spermatozoa were seen to attach to the intact chorion or to the glycerinated chorion. There was no difference in either the frequency or strength of binding. The mechanism of discrimination between self and non-self in the chorion of *H. roretzi* remains to be discovered by further studies.

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