Mammalian Fertilization as Seen With the Scanning Electron Microscope

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ABSTRACT For several years we have been looking at mammalian gametes and their interactions with the scanning electron microscope (SEM). Examining the images produced by the SEM has given us a three-dimensional view of sperm, eggs, and egg investments. We are particularly impressed with the structural variation among gametes of different mammalian species. In this short report we examine the structure of mammalian spermatozoa, eggs, zonae pellucidae, and cumuli. Our observations and those of others have led us to believe that variation in gamete structure and function may have evolved as a mechanism for reproductive isolation of mammalian species.

Of the many techniques that have been employed to analyze fertilization, the scanning electron microscope (SEM) has provided the most vivid and readily interpretable images of mammalian gametes and their interactions. The SEM produces a realistic perception of a third dimension. In contrast, reconstructing three-dimensional relationships based on images obtained with the transmission electron microscope is generally a laborious analytical exercise. During the past 8 years, we have used the SEM to complement physiological and biochemical techniques in studying aspects of mammalian fertilization. We will present here a few micrographs and some of the thoughts that occurred to us in the process of analyzing several thousand SEM images. This paper is not meant to be a review of the extensive literature on mammalian fertilization. The subject has been comprehensively and capably reviewed on several occasions; see, for instance, the recent article by Ryuzo Yana-gimachi (Huang et al., 1981). This issue also contains an excellent short review by Prudence Talbot which the reader may want to read as an aid to placing in context the information we are presenting here.

SPERMATOZOA

Even before the marvellous drawings of Retzius were published at the turn of the century, scientists were well aware of the tremendous variability in the morphology of spermatozoa throughout the animal kingdom. In fact, early biologists were more interested in interspecies variation in sperm morphology than we are today. Morphology of gametes was central to the thrust of what was modern biology of the time, and most biologists in the small scientific community would have been aware of whatever work was published in the field of reproductive biology.

As reproductive biology became a science of specialists, each involved in his or her own system, most workers became less interested in the variations in spermatozoon structure. For instance, a recent review referred to the echinoderm spermatozoon as "the invertebrate spermatozoon." Echinoderm gametes have indeed been by far the most studied invertebrate sperm and egg since Frank Lil-lie, E. B. Wilson, and others gathered hundreds of specimens at Woods Hole many years ago. Grams of gametes are easily obtainable from this source, and external fertilization is readily amenable to experimental manipulation. Moreover, the simple 9 + 2 flagellum of the echinoderm spermatozoon is an appropriate model to study flagellar motility (Fig. 1). The fact that we work on these gametes, however, is hardly a valid reason to equate echinoderm spermatozoon in general to invertebrate spermatozoa or even typical...

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Fig. 1. SEM of a spermatozoon of the starfish *Asterias forbesi* on the surface of an oocyte. Echinoderm gametes have been a favorite system of reproductive biologists for 100 years. ×7,000.

Invertebrate spermatozoa. If one considers only gross morphology, vast variation exists. In the class Decapodia, for example, the aflagellate spermatozoa have various peculiar shapes reminiscent of stars, spaceships, or tiny gastropods (Fig. 2). Annelid sperm have 9 + 1 flagella and complex mitochondrial sheaths, and platyhelminths have biflagellated sperm with distinctive 9 + 1 flagella (Phillips, 1974). Striking variation in spermatozoan morphology has been described among the insects (Phillips, 1969, 1970). There is so much variation in sperm structure in invertebrates that some workers have made elaborate phylogenetic trees based on sperm structure as a phylogenetic trait (Baccetti, 1970; Franzen, 1970).

Impressive variation in sperm structure also occurs among vertebrates. Extensive variation in gross anatomical structure and
ultrastructure of spermatozoa has been described among species of fish, for example (Mattei, 1970). There is also morphological variation among spermatozoa of the Mammalia. Sperm of ruminants have extremely flattened heads, human sperm have unique rounded tapering heads, and sperm of many rodents display hook-shaped heads. The sperm heads of guinea pigs have a shape reminiscent of a horseshoe crab (Fig. 3), and spermatozoa of some marsupials have heads shaped something like arrowheads (Fig. 4).

Mammalian spermatozoa vary considerably in length and mass from species to species. Perhaps surprisingly, larger mammals tend to have smaller spermatozoa, and there has been much speculation about the reason for this (Cummins, 1983).

One might argue that sperm morphology can vary because spermatozoa with different morphologies transverse the female tract and fertilize ova equally well. On the other hand, it is possible that variation in sperm structure might have been generated as one

Fig. 2. SEM of a spermatozoon of the mole crab Emerita talpoida. The spermatozoa of decapods are nonflagellated cells with bizarre shapes. ×8,000.
means to facilitate reproductive isolation of a species. We find this idea attractive, as it is usual to find variation in structure related to variation in function. We do not mean to imply that changes in sperm structure and function are the only or even the major mechanism for speciation. In fact, it has been shown that spermatozoa of some species can fertilize ova of other species (Chang and Hancock, 1967). However, if it is true that speciation can occur by changes in sperm, these changes should be accompanied by evolutionary divergence of the female gamete, its investments, and/or the female reproductive tract. Such divergence may have profound implications as to how we interpret observations on the fertilization process.

Consider a species that has been geographically divided, for example by a mountain range, into two subpopulations, A and B. Members of populations A and B only occasionally come into contact and mate. One might traditionally propose that isolating mechanisms might occur by mutations or chromosomal aberrations that caused sterility in hybrids between populations A and B. This type of isolating mechanism is inefficient in that a female from population A would have to carry and raise infertile offspring if she were to conceive from a mating with a male of population B and vice versa.

Now consider the situation if isolating mechanisms involve a change in sperm structure. In such a case, a genetic change might affect the sperm structure of species A so that spermatozoa from population A could not fertilize eggs of population B. Such a change in sperm structure might dictate an accompanying alteration in the reproductive system of females of population A so that their eggs could be fertilized by the now altered spermatozoa from males of population A. One could envision all sorts of changes in the female reproductive system of population A females: alterations in the vitelline membrane of the oocyte, changes in the mecha-

![Image](image.png)

**Fig. 3.** The heads of guinea pig spermatozoa have a morphology reminiscent of a horseshoe crab. They stack up in a phrase formation. (From Yanagimachi and Philips, unpublished. ×10,000.)

**Fig. 4.** The tapered neck region of this spermatozoon of the long-nosed "rat"-kangaroo, *Potorous*, has been pulled out of its position in the arrowhead-shaped sperm head. (From Bedford and Phillips, unpublished. ×12,000.)

...nism of the block to polyspermy, alterations in the zona pellucida, changes in the cumulus oophorus, differences in oviductal structure or composition of oviduct fluid, differences in mechanisms of capacitation, changes in the uterotubal junction, differences in the composition of cervical mucus, etc. In terms of selection, changes in the females of population A would probably be even more important than changes in males of population A. Consider a female of population A. If she could conceive from a mating with a male of population B, she would have "wasted" those eggs and possibly that whole mating cycle. In the case of a mammal, she would have to carry, nurse, and perhaps raise these offspring. (This argument hinges, of course, on the reproductive behavior of the particular species.)

Thus when we examine the female gamete and the female reproductive tract, we might expect variation comparable to that which occurs among spermatozoa. The degree of interspecies variation we are able to observe, however, will be affected by how the variation is manifested and whether we possess the proper probes to document it. We would suggest that differences in the female tract are more difficult to document than variations in sperm ultrastructure, but the available evidence suggests that they may exist to the same degree. The implication is that we must be very cautious about generalizing from observations on aspects of female gametes or fertilization made in a single species.

**OOCYTE MATURATION**

In mammals, the surge of luteinizing hormone (LH) triggers a complicated series of events in the ripe follicles. Among these changes is the disappearance of the large meiotic prophase oocyte nucleus. In many species this event, termed "germinal vesicle breakdown" or "resumption of meiosis," is easily visualized with the light microscope and therefore has become the basis of assaying oocyte maturation. One simply treats immature oocytes, oocytes, and their investments, or whole follicles in some manner and subsequently quantitates the percent of oocytes in which germinal vesicles cannot be visualized. Using this assay, it was observed some years ago that germinal vesicle breakdown occurred spontaneously when immature oocyte cumulus complexes were removed from their follicles and explanted into a va...
riety of simple media. From these observations it was proposed that an inhibitor might be present in the antral fluid of immature follicles which prevented oocyte maturation. Evidence supporting this theory came from experiments of Channing, Tsafiriri, and their co-workers, who found that a factor existed in pig follicles that in fact inhibited germinal vesicle breakdown (Tsafiriri and Channing, 1975). This idea of an oocyte maturation inhibiting factor (OMI) is an attractive concept, and OMI rapidly made its way into the textbooks. Unfortunately, oocyte maturation is not that simple.

In experiments that demonstrated OMI, it was necessary to score many oocytes, because OMI actually only inhibits some oocytes from maturing. Also several workers have been unable to demonstrate an OMI in follicular fluid (Leibfried and First, 1980; Jagiello et al., 1974; Fleming et al., 1984). Furthermore, Vivarelli et al. (1983) and Schultz et al. (1982) have independently shown that it is a decrease in the level of cAMP in the oocyte that causes germinal vesicle breakdown. The evidence comes from an extensive series of experiments using different types of culture systems, different analytical techniques, and, most recently, very elegant microinjection experiments by Schultz's group (Schultz et al., 1983). How cAMP is related to OMI is at this juncture totally unclear; however, recent unpublished studies by Ep pig suggest that there may be synergism between cAMP and OMI. Germin al vesicle breakdown is only one event in oocyte maturation, and workers in the field of oocyte maturation have probably overemphasized this aspect, because it is the end point that is easily measured. We have used the SEM to measure some of the other important events that take place in the maturation process.

Before the LH surge, cells of the cumulus oophorus of the rat are associated via an extensive network of long microvilli (Fig. 5). Gap junctions are present where the microvilli of one cell associate with another (Amsterdam et al., 1976). Microvilli from cumulus cells nearest the oocyte penetrate the zona pellucida and form gap junctions with the oocyte membrane (Anderson and Albertini, 1976; Gilula et al., 1978). After the LH surge, the interrelationship between cells of the cumulus oophorus, as well as the relationship between these cells and the oocyte, changes drastically. Between the time of the LH surge and ovulation (about 14 hr in the rat), cells of the cumulus oophorus retract their microvilli. The cell surfaces gradually become characterized by blebs (Fig. 6) (Dekel et al., 1976). Mucus is secreted by the cumulus cells. During the course of maturation the mucoid material comes to form an extracellular matrix, which serves to cement the cumulus cells together and sometimes to associate cumuli of different oocytes together after ovulation into an egg mass (Dekel and Phillips, 1979). The structure of the cumulus with cells embedded in a matrix provides a barrier that can be penetrated by motile spermatozoa facilitated by acrosomal enzymes released as a consequence of the acrosome reaction (Huang et al., 1981). We have used the SEM to assay the ability of various agents to cause the transition from a microvillar to a blebbed appearance. Concurrently we examined the effect of these agents on mucus formation.

Using a system of cultured rat proestrus oocyte-cumulus complexes, we observed that a variety of agents including rat and ovine follicle-stimulating hormone and LH, dibuteral cAMP, MIX, chloratoxin, and forskolin induce mucification and also cause cumulus cells to withdraw microvilli, dissociate from junctional complexes, and form blebs. Steroid hormones and prostaglandins have no effect on these processes (Dekel et al., 1976; Dekel and Phillips, 1980). Interestingly, when we cultured whole follicle cells, all agents had the same effect as they did on oocyte-cumulus cultures with the exception of prostaglandins, which caused mucification and blebbing (Phillips and Yanagimachi, 1982). We believe that prostaglandins may act on follicular granulosa cells and/or thecal cells of the follicles which in turn release a substance into the cumulus oophorus. It may appear paradoxical that increasing cAMP levels causes maturation, as it has been clearly shown that levels of cAMP decrease in oocytes during maturation. These data are in fact not incon-

Fig. 5. Before the LH surge, cells of the rat cumulus oophorus have long microvilli which make contact with neighboring cells. ×2,500.

Fig. 6. At the time of ovulation, the blebbing cumulus cells are embedded in a mucous matrix which maintains the integrity of the cumulus oophorus. The transition in the composition of the cumulus is necessary for sperm penetration. ×2,500.
sistent; the explanation is that cAMP causes cumulus cells to retract microvilli through which they transfer cAMP to the oocyte. Thus addition of dibuteral cAMP actually lowers the level of cAMP in the oocyte itself (Lindner et al., 1974; Anderson and Albertini, 1976; Dekel and Beers, 1978).

Our studies on the cumulus have been done on rats. The reason is primarily that the work was begun at Tel Aviv University where at the time the reproductive physiology of rats was being studied. The work is being continued at the Department of Hormone Research at the Weizmann Institute, where there is also an excellent rat colony. The group at Bar Harbor in the United States concentrates on mice, as does the group in Philadelphia. Even among these closely related species there are some basic differences. LH, which causes mucification in the rat cumulus, reportedly has no effect in the mouse (Epplig, 1979). Perhaps these differences, like variation in sperm structure, are related to reproductive isolation.

THE CUMULUS OOPHORUS

At the site of fertilization in the ampulla of the oviduct, there is considerable variation in the morphology of the cumulus oophorus. In some species the cumulus cells adjacent to the zona pellucida, termed the corona radiata, are distinct from the other cumulus cells. In the rabbit cumulus, these cells have almost a carrot shape. Each cell sends a single long process through the zona surface to the vitellus (Fig. 7). As hyaluronidase does not remove the corona radiata even in the ovulated cumulus-oocyte complex, cells of the corona radiata could conceivably transfer small molecules to the oocyte. This is a very different situation from that in the hamster, where there does not appear to be a distinct corona radiata and where all cumulus cells in the ovulated oocyte-cumulus complex are easily dissociated with brief hyaluronidase treatment. In ruminants at the time of fertilization the cumulus is reported to have completely dissociated. The fertilizing spermatozoon may thus be confronted with a cumulus-free zona pellucida (Thibault, 1973). As discussed below this has implications for the role of the acrosome reaction in these species, since the acrosome reaction is presumed to occur in the cumulus.

THE ZONA PELLUCIDA

The zona pellucida varies in thickness among species. The zones of hamster and rabbit oocytes are considerably thicker than those of the rat and mouse. After fixation the zones of the former species are so rigid that they do not become flattened when critical-point dried and sputter-coated with gold (Fig. 8). Viewed in the SEM, the zona surface displays a characteristic morphology reminiscent of a "Swiss cheese" appearance (Phillips and Shalgi, 1980a,b). The inside surface of the zona pellucida shows a more irregular morphology. During fertilization, spermatozoa must first associate with the zona pellucida and then pass through it. In vivo, it is clear that in the rabbit (Bedford, 1972) and hamster (Yanagimachi and Phillips, 1984) spermatozoa undergo the acrosome reaction and lose the acrosomal contents when they pass through the cumulus. When spermatozoa reach the zona pellucida, they are left with an apparently empty, fenestrated acrosomal shell or ghost. It is this ghost that binds to the zona pellucida. In the hamster, the ghost opens up to form a collar which is anchored to the zona pellucida. The spermatozoa pass through the collar formed by the acrosomal ghost as it penetrates the zona pellucida (Fig. 9) (Yanagimachi and Phillips, 1984). Recently Crozet and Dumont (1984) published observations of in vivo fertilization in sheep, and Crozet (1984) reported observations on in vivo fertilization in the cow. Although these workers interpreted their results as showing that the acrosome reaction occurred on the zona surface, their micrographs clearly showed that only the acrosome ghost remained on spermatozoa that were associated with the zona surface. Thus the acrosome reaction must have occurred well before the spermatozoon reached the zona pellucida. The situation in the sheep

Fig. 7. The ovulated cumulus-oocyte complex of the rabbit. This complex was treated with hyaluronidase before fixation, a procedure that removes most of the cumulus cells but leaves cells of the corona radiata. The oocyte is seen in the upper right. The zona pellucida with associated corona cell has been split in half. One half is seen in the center, and the other half is in the lower left. The inset shows the corona cells which extend a single long process through the zona pellucida. (From Dunbar and Phillips, unpublished.) x800, inset, x1,700.
and cow thus appears to be the same as in rabbit and hamster, where the acrosome reaction occurs in the the cumulus and the sperm binds to the zona via the acrosome ghost. There do appear to be species differences, however. In the guinea pig, the large acrosome appears to be totally lost before the spermatozoon reaches the zona pellucida (Huang et al., 1981). In the mouse it has been reported that spermatozoa bind to the zona pellucida with intact acrosomes and subsequently undergo the acrosome reaction on the zona surface (Storey et al., 1984). This is a rather surprising finding, as an impressive amount of evidence has shown that it is the acrosome reaction that allows spermatozoa to penetrate the cumulus and reach the zona pellucida. Whether or not the mouse spermatozoon binds to the zona pellucida with an intact acrosome is especially important as sperm-zona binding in vitro in the mouse is being used in several laboratories as a model system to study the molecular events in sperm binding. Morphological observations suggest that there may be difficulties in applying the results of experiments carried out
on this system to other mammalian systems (Phillips, 1984).

Any experimental system that involves sperm-zona binding must exclude the possibility of nonspecific binding. This is because noncapacitated spermatozoa bind very readily to zonae. In fact, noncapacitated spermatozoa display a strong binding to the zona pellucida of heterologous as well as homologous species (Bedford, 1977). Binding of spermatozoa is by the sperm head just as is the case with sperm-zona binding in vivo (Fig. 10). Noncapacitated mouse spermatozoa, like those of most other species studied with the exception of anthropoid apes (Bedford, 1977), bind to homologous and heterologous zonae (Swenson and Dunbar, 1982). The acrosome reaction is particularly difficult to study in the mouse, because the acrosome is too small to be seen with the light microscope. Observations of acrosome integrity in the spermatozoon reaching the zona pellucida must be

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Fig. 9. In vivo fertilization in the hamster. The empty acrosomal ghost attaches to the zona pellucida and forms a collar through which the spermatozoon passes during zona penetration. (From Yanagimachi and Phillips, 1984.) ×2,200.
Fig. 10. Epididymal spermatozoa readily attach to the zonae pellucidae of heterologous species. Here spermatozoa of the diurnal sand rat, *Psammomys obesus*, are seen associated with a zona pellucida of the laboratory rat. More work must be done before we fully understand the relationships between the mechanism of sperm-zona association in in vitro systems and the mechanism of association in nature. ×6,500.

Based on acrosome staining. Thus it is not entirely clear whether spermatozoa described as "acrosome intact" have totally intact acrosomes. Taken together, the information suggests that mouse sperm binding to cumulus-free mouse zonae in in vitro systems may be a different phenomenon from what takes place in nature.

**SPERM-OOLEmma INTERACTIONS**

Viewed in the SEM, mammalian eggs are seen to be covered with numerous microvilli (Fig. 11). The SEM image is somewhat misleading, because oocytes shrink when they are critical-point dried, and microvilli appear somewhat broader when they are coated with gold. Thus, although the surface of the mam-
malian oocyte is covered with microvilli, the spaces between microvilli are more extensive than they appear even in very good SEMs. Although the egg surface is generally evenly covered with microvilli, regions of the egg surface become microvillus-free in association with events of meiosis and fertilization. In the mouse, there is a microvillus-free bulge which remains after the first meiotic division (Phillips and Shalgi, 1980a). A microvillus-free region also occurs during the second meiotic division as the polar body forms. Microvillus-free zones are also observed on the oolemma, where it overlies the decondensing sperm nucleus (Fig. 11). In rats this microvillus-free zone later disappears, and another microvillus-free bulge termed the "incorporation cone" forms later over the

Fig. 11. In vitro fertilized rabbit oocyte. The zona pellucida was removed after fixation. The tail of the fertilizing spermatozoon (below and right of center) has not yet been incorporated. A microvillus-free region is forming over the region of the decondensing sperm head. (From Dunbar and Phillips, unpublished. ×2,100.)
decondensing sperm nucleus (Shalgi et al., 1978). (See also Longo [this issue] for further discussion of the characteristics of the microvillus-free zone.)

Much of our understanding of the fertilization process comes from experimentation with various types of in vitro fertilization systems, many of which use zona-free oocytes. As is the case with the zona pellucida, sperm associate very strongly with the oolemma. Since in most in vitro systems many thousands of spermatozoa are mixed with a few oocytes, the egg often ends up looking rather like a pincushion (Fig. 12). In the hamster, what appear to be non-acrosome-reacted spermatozoa tend to associate with the oolemma via the surface of the major portion of the acrosome, whereas sperm cells that have lost their acrosomes associate via the equatorial segment of the acrosome (Phil-
lips and Dekel, 1982). We have found that with zona-intact ova, hamster and rat spermatozoa enter the oocyte tip first in vitro (Shalgi and Phillips, 1980, 1982), whereas they penetrate the egg surface with the broad side of the sperm head in vivo (Phillips and Shalgi, 1980a,b). These and other differences between in vivo and in vitro fertilization suggest that we must be cautious when interpreting results of in vitro experiments in terms of natural fertilization.

CONCLUSION

We have presented some SEM observations on mammalian gametes and their interactions. There is an impressive amount of interspecies variation in the morphology of gametes and their interactions that is not observed to this extent among other cell and tissue types. We have suggested that the variation in gametes and their interaction could have a function in speciation. There is also similar variation in physiological aspects of fertilization. For example, the block to polyspermy works by different mechanisms in different species. The way spermatozoa enter the female reproductive tract varies considerably. Some species have cervical mucus, others form vaginal plugs, and some species store spermatozoa in various parts of the reproductive tract.

All the variation that has been observed and the probable variation that has yet to be observed is in a way worrisome, as it suggests that we must be very cautious about generalizing from observations on a single species. It also means that it will take a long time to sort out the fertilization process in mammals.

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LITERATURE CITED


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