THE FINE STRUCTURE OF FERTILIZATION IN THE FERN MARSILEA VESTITA

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SUMMARY

The ultrastructural details of fertilization in the fern Marsilea vestita, including gamete approach and fusion, the fate of the spermatozoid organelles and the development of a possible block to polyspermy are described. The spermatozoid approaches the egg through layers of mucilage that surround the megaspores. It moves down the neck of the archegonium into the cavity above the egg. In order to reach the egg, it must move through a small hole in the thick wall that lies across the top of the egg. The fusion of the plasma membranes of the gametes results in an outflow of egg cytoplasm into the clear space under the sperm plasma membrane, creating a fertilization cone. All the organelles of the fertilizing spermatozoid, including nucleus, mitochondrion, microtubule ribbon, multilayered structure, and flagellar band, with approximately 150 flagella, enter the egg cytoplasm. The nucleus enters as a condensed rod of chromatin with no nuclear envelope. The chromatin begins to disperse immediately and a new nuclear envelope is formed around the chromatin by egg endoplasmic reticulum. The mitochondrion and the microtubules of the ribbon and flagella are broken down, but the fates of the flagellar band and the multilayered structure have not been determined. After spermatozoid penetration, a new extracellular layer appears above the surface of the egg, beginning in the region of sperm penetration and spreading across the top of the egg. This layer may be important in preventing other spermatozoids from fusing with the egg.

INTRODUCTION

Among the basic phenomena of the fertilization process are the approach and fusion of the gametes, the fate of the sperm nucleus and other organelles in the egg cytoplasm, and the prevention of polyspermy. This study is an investigation of these phenomena in the fern Marsilea, a representative of a group of plants for which there is comparatively little information about the fine structure of fertilization.

The reproductive biology of Marsilea differs from that of most other ferns in the formation of gametophytes. Upon hydration, megaspores and microspores are released from a sporocarp; the megaspores divide to form an egg-containing archegonium, and the microspores form a reduced gametophyte with 2 antheridia and 32 spermatozoids. The ultrastructure of microspore divisions (Hepler, 1976) and of the subsequent stages in spermigenesis have been described (Myles & Hepler, 1977), as well as the structural details of the mature sperm (Myles & Bell, 1975; Rice & Laetsch, 1967). After their release from the microspores, multiflagellate spermatozoids swim towards the megaspores and become entrapped in layers of mucilage surround-
ing each megaspore. They move through the mucilage and down the archegonial neck to reach the egg and finally fertilize it.

Previous descriptions of fertilization in *Marsilea* include reports by Hanstein (1865-66) and Atkinson (1943).

**MATERIALS AND METHODS**

Sporocarps of *Marsilea vestita* Hook. et Grev., collected from Lake Lagunita on the Stanford University campus, California, were cut open and placed in pond or tap water at room temperature. Megaspores were fixed at hourly intervals before, during and after fertilization with 3% (v/v) glutaraldehyde (TAAB Laboratories, Reading, England) in 0.05 M cacodylate buffer (pH 6.8) for 6 h, followed by 2 h post fixation in 2% (w/v) OsO4 in distilled water. Specimens were dehydrated in acetone followed by propylene oxide and infiltrated with increasing concentrations of Epoxy resin over a period of 3 to 4 days. The final embedding was in Epon or Durcupan ACM (Fluka AG, Switzerland). Blocks embedded in Durcupan were first sectioned at 5 µm and examined with phase-contrast optics. Sections containing spermatozoids in appropriate orientations were remounted in Epon (Woodcock & Bell, 1967) and thin-sectioned. Silver sections were picked up on Formvar-coated grids and stained for 30 min with saturated aqueous uranyl acetate and for 15 min with lead citrate, and examined on a Zeiss EM9 or Siemens Elmiskop 1 or 1A.

**OBSERVATIONS**

*The egg*

The young egg is spheroidal and approximately 70 µm in diameter. A small depression is formed in the top of the egg by the partitioning of 2 small cells out of this region (Fig. 1A). The innermost of these 2 cells, the ventral canal cell, is separated from the egg by a thickened, polysaccharide (PAS-positive) wall. As the egg matures, the ventral canal and neck canal cells are lost and the 8 cells of the archegonial neck separate, making a canal through which the sperm reaches the egg (Fig. 1B).

The cytoplasm of the egg is densely packed with organelles that show a non-random distribution. Plastids are mostly absent from the centre of the egg above the nucleus, where sperm penetrates, but mitochondria, endoplasmic reticulum, and lipid droplets are distributed throughout the egg (Fig. 2). In the nucleus are several, irregularly shaped, vacuolate nucleoli (Fig. 3) and other nuclear inclusions, including nuclear bodies, characterized by dense and loosely granular regions (Fig. 4). Prior to fertilization the egg and its nucleus change from spheroidal to cup-shaped (Fig. 1B, 13).

*The spermatozoid*

The structure of the spermatozoid has been previously described in detail (Myles & Bell, 1975). When it approaches the egg, the spermatozoid is reduced to a coil of organelles with over 150 flagella (Figs. 5, 6A, B). The organelle coil is composed of a nucleus, a single elongate mitochondrion, a microtubule ribbon, a flagellar band and a multilayered structure. The coil makes a spiral of 9 to 10 gyres around the edge of the cell, but the nucleus, reduced to a dense rod of chromatin and lacking a nuclear envelope, is confined to the posterior 4 to 5 gyres and the multilayered structure to the anteriormost gyre.
Fig. 1. Diagram of the archegonium at 3 different stages in development. A, the spheroidal egg with ventral canal and neck canal cells still intact. A thick wall with a hole in its centre separates the egg and the ventral canal cell. The neck cells (4 of 8 are shown) are still close together so that the neck is closed. B, the egg and its nucleus have become cup-shaped. The 2 canal cells have disintegrated or been extruded through the open neck. The neck cells have moved apart and spermatozoids are able to move through the open neck and into the archegonial cavity. One spermatozoid is passing through the hole in the thick wall. C, the zygote after it has regained the spheroidal shape of the egg. A new extracellular layer is found across the top of the cell underneath the original thick wall.
Gamete approach

After the spermatozoids penetrate the megaspore mucilage they move down the open neck canal (Figs. 1B, 5) into the cavity above the egg. Many sperm were observed inside this cavity at one time. The top of the egg is covered by the thick wall that originally lay between the egg and the ventral canal cell (Figs. 1A, 19). The approach of spermatozoids to the egg is limited to a hole in this wall that is only slightly larger in diameter than the spermatozoid (Figs. 6A, 7). When the spermatozoid moves through this hole, its anteriormost tip contacts the central region of the egg plasma membrane (Figs. 6B, 7).

Fusion

The plasma membrane of the sperm fuses with the plasma membrane of the egg, creating a continuity between the egg cytoplasm and the clear space under the sperm plasma membrane (Fig. 8). The egg cytoplasm, including mitochondria, endoplasmic reticulum and ribosomes, flows out into this space and fills it, creating a fertilization cone (Figs. 6B, 8). The entire spermatozoid, including all the organelles of the coil and the flagella, become incorporated into the egg cytoplasm proper (Figs. 6C, 9) and eventually the zygote regains the spheroidal shape of the egg (Figs. 1C, 21).

The spermatozoid after fusion

When the spermatozoid is first incorporated into the egg cytoplasm, the organelles of the coil retain their original spatial relationship to one another. In Fig. 9 the coiled mitochondrion lies adjacent to the nucleus, and is separated by the microtubule ribbon from the flagellar band and the axonemes of the flagella. No plasma membrane was observed around any part of the sperm once it entered the egg (Fig. 9). Even the approximately 150 flagellar axonemes that enter with the sperm have no flagellar membrane surrounding them (Figs. 9, 10).

The spermatozoid nucleus undergoes upon fertilization the most rapid structural changes of all the male organelles. The solid rod of chromatin seen in the mature spermatozoid prior to fertilization (Myles & Bell, 1975) begins to decondense immediately after contacting the egg cytoplasm while still in the fertilization cone (Fig. 11) and continues to decondense as the sperm penetrates deeper into the egg (Figs. 9, 10, 12). Since the nuclear envelope is shed prior to fertilization, the male chromatin enters the egg cytoplasm without a surrounding membrane but, shortly after penetration, egg endoplasmic reticulum forms a new nuclear envelope around the

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Fig. 2. A young unfertilized egg. Both the cell and its nucleus (n) are still spheroidal. Starch-containing plastids (p) are excluded from the central top region of the egg. ×4500.

Fig. 3. Several nucleoli in the nucleus, some with vacuoles. ×10600.

Fig. 4. Two nuclear inclusions showing dense and loosely granular regions. ×46800.

Fig. 5. A section through 4 of the 8 neck cells of the archegonium. Two spermatozoids are in the open neck. ×2800.
Fig. 6. Drawings of the top centre part of the egg during sperm penetration. A, the spermatozoid passing through the hole in the thick wall above the egg. B, the spermatozoid fusing with the egg cell. The 2 plasma membranes have fused, and the egg cytoplasm is flowing into the clear space between the organelle coils, forming a fertilization cone. C, the spermatozoid has moved deeper into the egg cytoplasm.

Fig. 7. A view of a spermatozoid penetrating the egg. A small hole in the centre of the thickened wall (zv) over the egg limits the access of the spermatozoid to the egg. × 7600.

Fig. 8. Another section of the same cell showing that the anterior coils of the spermatozoid have penetrated the egg cytoplasm. Nine gyres of the organelle coil are cross-sectioned. The anterior-most gyre of the coil includes the multilayered structure (mils), and subsequent gyres show the cross-sectioned coil mitochondrion (cm). The sperm nucleus (sn) is confined to the posterior gyres. Egg cytoplasm, including mitochondria (m) and endoplasmic reticulum (er) have penetrated into the region between the spermatozoid coils. The plasma membranes of the 2 cells have become confluent (arrows). × 15600.
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partially decondensed male chromatin (Figs. 9–11). Decondensation was never observed to progress beyond a fibrous stage (Figs. 12, 14). The nucleus eventually becomes dissociated from the other sperm organelles and is found in contact with the egg nuclear envelope (Figs. 13, 14). Subsequent stages of the presumed nuclear fusion were not observed.

The sperm mitochondrion is easily distinguished from the egg mitochondria, by the extra membranes surrounding its double membrane (Fig. 10). The sperm mitochondrion dissociates from the sperm nucleus, but stays close to the flagellar band (Fig. 15) until both mitochondrion and flagellar band are located next to the egg nucleus. Before the first division of the zygote, the sperm mitochondrion degenerates.

The first structural change in the sperm mitochondrion is the detachment and expansion of the membrane sacs that surround it (Figs. 15, 16). Occasionally inflated cristae were also observed (Fig. 16). The sperm mitochondrion then loses its outer membrane (Fig. 17) and its cristae progressively flatten until the mitochondrion is scarcely recognizable (Fig. 18). Egg mitochondria, although located near the sperm mitochondrion, generally remain unchanged during degeneration of the sperm mitochondrion (Figs. 17, 18).

The sperm brings with it, into the egg cytoplasm, a great number of microtubules, including those of the microtubule ribbon and the 150 or more flagella (Fig. 9). The flagellar band maintains its original spiral shape longer than the other coil organelles, but after a short time there is no trace of the basal bodies originally attached to it (Fig. 15). The striated substructure seen along its outer edge, is also retained, although it loses its attachment to basal bodies (Fig. 15). Eventually, the flagellar band becomes closely associated with the nuclear envelope, and it remains in the egg cytoplasm after the first division of the zygote (Myles, 1975).

Shortly after the egg is fertilized, a thin extracellular layer is elevated above the upper surface of the zygote, between the plasma membrane and the thick wall that originally was formed between the egg and the ventral canal cell (Fig. 19). Its appearance begins at the point of sperm entry (Figs. 6c, 20) and spreads just beyond the perimeter of the zygote. A clear space forms between the egg plasma membrane and this wall as the zygote regains its spherical shape (Fig. 21). This process pushes the original thick wall and degenerating spermatozoids in the cavity against the jacket cells of the archegonium (Fig. 21). About 1 h after fertilization, the zygote divides with the cell plate oriented longitudinal to the axis of the megaspore.

Fig. 9. A spermatozoid in the egg cytoplasm shortly after fusion. The relative positions of the coil mitochondrion (cm), flagellar band (fb), microtubule ribbon (mtr) and sperm nucleus (sn), have been retained. Cross-sections of flagellar axonemes (arrows), are seen along the edges of the coil. Egg endoplasmic reticulum (er) is surrounding the sperm chromatin to form a new nuclear envelope. × 21 500.

Fig. 10. A higher magnification of the same cell as in Fig. 9. Extra membranes can be distinguished surrounding the double membrane of the coil mitochondrion (cm). Egg endoplasmic reticulum (er) is forming a new nuclear envelope around the decondensing chromatin. × 45 600.
DISCUSSION

Fertilization in *Marsilea* involves the fusion of a highly differentiated, flagellated sperm with a larger, immotile egg. This type of fertilization is characteristic of the archegoniate plants, where the ultrastructure of some of the events has been described for the fern, *Pteridium* (Bell, 1975; Bell & Duckett, 1976) as well as most animals, and has also been described for the brown alga, *Fucus* (Brawley, Wetherbee & Quatrano, 1976). This study of *Marsilea* has revealed some important details of the process of fertilization including the transformation of the male nucleus and the fate of other spermatozoid organelles. It also points to a possible mechanism for the prevention of polyspermy.

The fate of spermatozoid organelles

In *Marsilea* most of the male cytoplasm is shed before the spermatozoid fuses with the egg. This is accomplished in 2 stages. The first elimination occurs during the final stages of spermiogenesis, when approximately half the cytoplasm forms a cap around the anterior end of the sperm. This cap of cytoplasm contains numerous mitochondria and ribosomes, lipid droplets, and endoplasmic reticulum and is pinched off from the sperm as it becomes motile (Myles & Hepler, 1977). The second stage of elimination occurs after the swimming spermatozoid becomes entrapped in the mucilage surrounding the megaspore. As the spermatozoid moves through the mucilage towards the egg, it loses its posterior vesicle of cytoplasm, including all of the plastids and the nuclear envelope (Myles & Bell, 1975). Hence, the spermatozoid that fuses with the egg contains only the coil organelles, including the nucleus, mitochondrion, flagellar band, microtubule ribbon and the multilayered structure.

Because the spermatozoid has already lost its nuclear envelope, the male chromatin enters the egg with no surrounding membrane and is in immediate contact with the egg cytoplasm. The loss of the nuclear envelope in the mature sperm, prior to fertilization, has been described in several animal species (see Baccetti & Afzelius, 1976, for review), but in many cases the nuclear envelope is lost only after gamete fusion (e.g. Brawley et al. 1976; see Longo, 1973, for review). Dissolution of the nuclear envelope may be important to allow factors in the egg which may be responsible for decondensing the male chromatin (Longo, 1973; Usui & Yanagimachi, 1976) to contact the chromatin and may also provide space for the chromatin to disperse. In organisms in which part of the sperm nuclear envelope is retained, the regions of the chromatin that

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Fig. 11. A part of the fertilization cone during spermatozoid penetration. The coil mitochondrion (cm) and other organelles of the spermatozoid are coiled around the edge of the cone. The sperm chromatin (c) is already beginning to decondense. The remainder of the space is filled with egg cytoplasm, including mitochondria (m), endoplasmic reticulum (er), lipid droplets (l) and ribosomes. \(\times 27000\).

Fig. 12. A portion of the spermatozoid in the egg cytoplasm, including nucleus (sn), the coil mitochondrion (cm), flagellar band (fb), microtubule ribbon and flagellar axonemes (arrows). The fibres of the decondensing chromatin can be distinguished. \(\times 38000\).
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remain in contact with it are slower to disperse (Longo, 1973). In *Marsilea*, however, decondensation is rapid and uniform throughout the chromatin, beginning while the spermatozoid is still in the fertilization cone.

In some organisms egg endoplasmic reticulum has been indirectly implicated in the formation of the new nuclear envelope around the decondensing chromatin (e.g., Brawley *et al.* 1976; Longo, 1976), although the sperm nuclear envelope may also contribute to the new membrane (Longo, 1976). The origin of the sperm nuclear envelope in many cases is unclear since it appears to come from small vesicles, the origin of which is difficult to determine (Longo, 1973). In *Marsilea*, however, it is clear that a major portion of the new nuclear envelope comes from sheets of endoplasmic reticulum.

The fate of the sperm mitochondrion in the egg cytoplasm is important in terms of its potential contribution to the genetic makeup of the future mitochondrial population and the possibility of its being metabolically active in the zygote. In tunicates, sperm mitochondria are lost prior to sperm penetration (Ursprung & Schabtach, 1965). In other organisms, they may be retained in the egg cytoplasm for some time and in sea urchin, Anderson & Perotti (1975) have shown that the sperm mitochondrion can remain intact and retain its cytochrome oxidase activity into the 8-cell stage. In *Marsilea*, where the mitochondrion is destroyed within an hour after sperm penetration, it seems unlikely that the mitochondrion plays any metabolic or genetic role. However, the possibility of one part of the mitochondrion budding off and being retained in the egg has not been disproved.

The destruction of the sperm mitochondrion is apparently selective, since surrounding egg mitochondria are not structurally affected. It is possible that the sperm brings with it into the egg its own lytic enzymes in the membrane sacs that surround the mitochondrion in the mature sperm (Myles & Bell, 1975). Alternatively, enzymes from the egg may attack the sperm mitochondrion. In either case the selective lysis of the sperm mitochondrion could result from topographical confinement or from some type of enzyme specificity.

Because the spermatozoid has so many flagella, a great number of microtubules enter the egg cytoplasm at fertilization. Since axonemes and ribbon microtubules were not found in the egg cytoplasm shortly after fusion, it is assumed that the microtubules depolymerize. This could provide a large pool of tubulin to be used in spindle formation during embryogenesis if, in fact, flagellar and spindle tubulin are inter-changeable.

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Fig. 13. The cup-shaped zygote. The thick wall (w) is visible across the top of the cell, and portions of some of the archegonial cells are visible below. Cross-sections through the spermatozoid nucleus (arrows) are seen next to the egg nucleus. × 4100.

Fig. 14. The spermatozoid nucleus (sn) is in contact with the egg nucleus (en). × 46 000.

Fig. 15. Part of the coil mitochondrion (cm) and flagellar band (fb) of the spermatozoid. The flagellar band has a striated substructure (arrows) along its outer edge. The extra membrane surrounding the sperm mitochondrion is beginning to swell away from it. × 30 000.
Polyspermy and the restrictions on sperm-egg fusion

There are 2 structures reported in this study that may prevent polyspermy in *Marsilea*. Before fertilization the top of the egg is covered by a thickened polysaccharide wall. Although many spermatozoids enter the cavity above the egg, they are able to reach the egg only through a small hole in the central region of the wall, assuring that only one spermatozoid is able to make contact with the egg plasma membrane at a time. A short time after sperm penetration, a new extracellular layer appears in the region of sperm penetration and then spreads along the entire top of the zygote, extending just beyond its perimeter. This could either be the elevation of a pre-existing structure or the formation of a new one. This structure may function to prevent other spermatozoids from reaching the egg plasma membrane. Since only one spermatozoid at a time is able to make contact with the egg plasma membrane, the formation of a barrier to spermatozoid penetration, even if relatively slow, may be sufficient to prevent polyspermy.

This restriction on the approach of the spermatozoid to the egg also means that only a specific region of sperm and egg plasma membrane can contact each other and fuse. It has been shown in many cases that sperm membranes are restricted topographically in their ability to fuse (Austin, 1975). In *Marsilea* both egg and sperm may have regions specialized for membrane fusion, as do gametes of *Chlamydomonas* (Weiss, Goodenough & Goodenough, 1977), and the limited access of the spermatozoid to the egg would ensure that these regions come in contact. Restriction of the penetration site could also be important in establishing polarity in the zygote, which always divides longitudinally with respect to the archegonium.

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Figs. 16–18. The coil mitochondrion of the spermatozoid in the cytoplasm of the zygote.

Fig. 16. The extra membranes surrounding the mitochondrion are inflating and some of the cristae are swollen. × 33,000.

Fig. 17. The outer membrane of the coil mitochondrion is gone, although parts of the inner membranes and stroma are still intact. Nearby egg mitochondria (m) and plastids (p) are not affected. × 19,000.

Fig. 18. The coil mitochondrion is in the final stages of disintegration. A few membranes are still visible (*). The stroma has mostly dispersed. The egg mitochondria and plastids (p) remain intact. A part of the flagellar band (arrows) is visible. × 16,000.
REFERENCES


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Fig. 19. A young egg with portions of the ventral canal cell (vcc) and neck canal cell (ncc). A thick wall (w) lies between the ventral canal cell and the top of the egg. ×10000.

Fig. 20. The top region of the zygote just after penetration of the spermatozoid. The thick wall (w) has a narrow hole in the centre through which the spermatozoid reaches the egg cell. A new extracellular layer (arrow) appears above the egg in this region. ×12800.

Fig. 21. The zygote after it has regained the spheroidal shape of the egg. The new extracellular layer (arrow) has now separated from the surface of the zygote, and the outer thick wall (w) is pushed up against the disintegrating spermatozoids that remained in the archegonial cavity. ×3600.