SCANNING ELECTRON-MICROSCOPICAL AND OTHER OBSERVATIONS OF SPERM FERTILIZATION REACTIONS IN *LIMULUS POLYPHEMUS* L. (MEROSTOMATA: XIPHOSURA)

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SUMMARY

Sperm fertilization reactions of *Limulus polyphemus* were examined by scanning electron and/or light microscopy. The following were considered: sperm motility, attachment of sperm to egg, acrosome reaction, and penetration of the acrosomal filament. The spermatozoa after semination are non-motile and become active only in close proximity to a defined region surrounding the egg. Egg materials diffusing into this region induce sperm motility and stimulate large numbers of spermatozoa to move towards the egg surface. Each sperm initially attaches by the apical tip and undergoes the acrosome reaction which causes a more permanent secondary attachment by the adhesion of acrosomal contents to the egg surface. The acrosome reaction also initiates the penetration of the acrosomal filament through the egg envelope, an event occurring in 70-80% of the attached spermatozoa (about 10⁹). Shortly after this penetration, a secondary reaction occurs which involves a spiralling of the flagellum and an incorporation into the sperm body of the flagellar fibrous components, which then become closely apposed to the sperm nucleus.

These sperm fertilization reactions were performed or initiated with 0.34 M CaCl₂ in whole eggs, egg sections, excised egg envelopes and/or the outer basement lamina of the egg envelope. The *Limulus* fertilization system is very valuable since sperm reactions can be examined biochemically, which may lead to a better understanding of the chemical mechanisms involved in sperm–egg interactions in all animal species.

INTRODUCTION

A need exists to analyse the molecular basis of fertilization in a single species, as a preliminary to establishing a satisfactory biochemical model or mechanism for animal fertilization in general. The *Limulus* fertilization system is convenient for this approach and some studies have already been performed, for example, with immunological techniques (Cooper & Brown, 1972; Mowbray & Brown, 1974). Any supplementary techniques that can be brought to bear on this species are potentially valuable. The present study demonstrates the usefulness of scanning electron microscopy in examining and quantitatively monitoring sperm–egg interactions.

Specimens and gametes of *Limulus polyphemus* are especially suitable for laboratory
studies in fertilization. The species is quite common along the eastern coastline of North America. Its hardiness in shipping has been demonstrated for over a century (Lockwood, 1870) and its survival in the laboratory is quite high. Large quantities of viable gametes can be obtained throughout the year from each sex and used in fertilization and developmental studies (Shoger & Brown, 1970; Brown & Humphreys, 1971; Cooper & Brown, 1972; Brown & Knouse, 1973; Mowbray & Brown, 1974; Bennett, 1974). Significantly, the *Limulus* fertilization system is evolutionarily classified as primitive (Åfzelius, 1972), since fertilization occurs externally and sperm morphology is more or less simple, based on the presence of a 'head' containing the nucleus apically capped with an acrosomal vesicle and a 'tail' or flagellum. The only unique morphological feature is a long, preformed acrosomal rod which is coiled posteriorly to the nucleus and has access to the acrosomal vesicle through an intranuclear canal (André, 1963; Fahrenbach, 1973; Tilney, 1975). The spherical egg (3.0-3.5 mm in diameter) has an exceedingly tough egg envelope which can be removed and manipulated in various manners as described in this present study. This egg envelope consists of 2 basic layers, the outer basement lamina (5 μm) and the inner vitelline envelope (35-40 μm), which surround the egg proper (vitellus) and are responsible for the initiation and control of the sperm interactions with the egg. Upon mixing of the gametes, a large number of spermatozoa (about 10^6) attach to each egg and a high percentage of these spermatozoa undergo the acrosome reactions, projecting acrosomal filaments through the egg envelope (Shoger & Brown, 1970; Brown & Humphreys, 1971). The present study is the first of a series of papers concerning the biochemical aspects of *Limulus* fertilization.

MATERIALS AND METHODS

All specimens of the horseshoe crab, *Limulus polyphemus* L., were obtained from Florida Marine Specimens, Panama City Beach, Florida, and were kept at 15 °C in an Instant Ocean Aquarium in the Electron Microscopy Laboratory at the University of Georgia, Athens, Georgia. Approximately 12 males and 12 females were used in these studies.

Semen was obtained by gentle pressure with a glass centrifuge tube (15 ml) just proximal to the male genital pores, and eggs were obtained by a short electrical stimulation (1 V, 10 A, alternate) proximal to the female genital pores. Collected semen (0.1-1.0 ml) was diluted to a 10% sperm suspension (10^6 spermatozoa/ml) with artificial seawater (Instant Ocean) and generally used within 1 h. Artificially spawned eggs were collected with wooden applicators and were either fertilized immediately, since a delay of 5 min or more caused a drastic decrease in the developing percentage (see Brown & Knouse, 1973), or were experimentally manipulated as described below. Aliquots of eggs used in all experiments were fertilized and observed for development. Experiments were performed at 23-24 °C.

Sperm motility was observed with a Wild M5 Stereomicroscope. Among several procedures, the following was the most satisfactory. A single egg (3.0-3.5 mm diameter) was placed on a glass microslide, washed with seawater, and surrounded on 3 sides by an open ring of Vaseline approximately 0.5-1.0 cm from the egg. A coverslip (22 mm square) was pressed upon the Vaseline ring until contact was made with the egg. Then a 0.1% sperm suspension was gently added through the ring opening and the spermatozoa were immediately observed.

For sperm attachment and acrosome reaction studies, eggs were collected and prepared by one of the following procedures: whole eggs were either: (1) dropped on to 16-mm round glass coverslips to which the eggs readily adhered; (2) dropped on to coverslips and then removed by gently rolling with a sharpened wooden applicator, leaving on the coverslip
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portions of the adhesive basement lamina (outer layer of egg envelope); or (3) frozen in a cryostat, sectioned at 10-30 μm, and attached to a coverslip. The coverslips containing the eggs or egg materials were usually ‘inseminated’ by submerging in 10% sperm suspension in a Syracuse dish for approximately 5 min and then washing gently in seawater. The coverslips in all experiments served as vehicles for transporting the gametes through the various procedures and for affixing to the mounting stub in preparation for scanning electron microscopy.

Acrosome reactions were also readily initiated by treatment of a 10% sperm suspension with 0.34 M CaCl₂ (ratio 1:10). Immediately after treatment, the spermatozoa were gently centrifuged (2 min) and a drop of the resulting pellet was placed on a coverslip for 1 min. A drop of 2% glutaraldehyde was added and the preparation allowed to stand for 5 min, after which it was immersed in fresh fixative and prepared for scanning electron microscopy.

Penetration of the egg envelope by acrosomal filaments was examined by the following method. With watchmaker’s forceps several entire egg envelopes were removed and 3 or 4 were placed on each of several coverslips. Such preparations were submerged in 10% sperm suspension and allowed to remain for 2, 5 or 15 min before removing, washing and preparing for scanning electron microscopy.

Preparation of tissues for scanning electron microscopy involved: either: (1) 2% glutaraldehyde (buffered with 0.1 M cacodylate and 2.7% sodium chloride) fixation, 2% osmium tetroxide postfixation, several washings in distilled water, and freeze-drying by use of liquid-nitrogen-cooled blocks in a vacuum evaporator; or (2) 2% glutaraldehyde fixation followed by ethanol dehydration into amyl acetate and the application of the critical-point technique using liquid carbon dioxide. Both types of specimen preparation were coated with gold and examined on a Cambridge Mark 2A Stereoscan. Throughout this process the egg materials usually readily adhered to the glass coverslips. However, with the use of whole eggs, silver paint was generally applied to their attachment area after the critical-point process was completed. For clarification, Figs. 15-17 demonstrate the only experiments where the freeze-drying method was used.

OBSERVATIONS

The following fertilization reactions were observed: (1) sperm motility, (2) sperm attachment to egg, (3) acrosome reaction, and (4) penetration of acrosomal filaments through the egg envelope. All observations were performed with gametes in which aliquots of fertilized eggs produced a high percentage of development (75-95%).

Sperm motility

Semen examined after collecting contained non-motile spermatozoa. After dilution in seawater to a 10% sperm suspension (10⁹ spermatozoa/ml) or to a lower concentration, spermatozoa were still non-motile. However, when mixed with whole eggs, egg sections, various preparations of the egg envelope or 0.34 M CaCl₂, sperm motility resulted. Interestingly, the site where motility occurred was very specific. For example, when a 0.1% sperm suspension was mixed with a whole egg, sperm motility occurred in a layer-like region (approximately 0.1 mm wide) surrounding the egg. At low magnification, this region gave the appearance of a ‘halo’. At higher magnification, practically all spermatozoa in this region were observed to become motile and to swim directly to the egg surface, where sperm–egg attachment occurred and motility ceased. A few spermatozoa either swam towards the bottom of the medium or ceased motility short of the egg surface. Thus, the halo effect was formed by spermatozoa rapidly leaving this region. As the surrounding spermatozoa (exterior
to the halo region) moved by convection currents into contact with the halo region, motility occurred and each sperm swam towards the egg. In cases where an individual egg was periodically observed, this process continued for several hours, although many of the motile spermatozoa, either failing to attach to the egg or contacting egg surface areas containing a maximum number of attached spermatozoa, eventually drifted to the bottom of the medium. Most spermatozoa observed in the halo region maintained motility for less than 5 s, regardless of whether attachment did or did not occur.

Sperm attachment

Once motile, spermatozoa were observed to attach on initial contact with the egg surface. Attachment occurred with numerous spermatozoa either over the entire egg surface (Figs. 1–3) or to surface components removed from the basement lamina (Figs. 11, 12, 15). Only the sperm apical tip was observed to attach to the egg, and apparently no obvious morphological change resulted in the sperm during this initial attachment (Fig. 6). In the egg, only the basement lamina of the egg envelope contains the components for this initial attachment (Figs. 4–6). However, these components are homogeneously distributed. For example, if an egg is rolled across a glass coverslip, adhering pieces or coating material from the basement lamina are left in an 'upside down position' (Figs. 7, 9), but still have the necessary exposed components for sperm attachment (Figs. 8, 10, 11). Thus an egg once placed on a coverslip and removed leaves an imprint of its former position, which can be demonstrated by placing the preparation in a sperm suspension and observing the areas of sperm attachment (Fig. 13). After this initial sperm attachment, stronger adhesion of the sperm to the egg surface follows. This adhesion or secondary attachment occurs during the acrosome reaction (Figs. 6, 11, 12).

Sperm acrosome reaction

The sperm acrosome reactions were initiated by using several methods: mixing of spermatozoa with (1) whole eggs (Fig. 3), (2) adhering materials on coverslips from rolled eggs (Figs. 11, 12, 15–17), (3) egg sections (Fig. 6), and (4) 0.34 M CaCl₂ (Fig. 14). With the first 3 methods the acrosome reactions occurred although the

Fig. 1. Whole egg which has been inseminated with a 10% sperm suspension for 5 min. The attached spermatozoa are observed over the entire egg surface. The lighter areas on the egg near the bottom of the micrograph are spots of silver paint. × 82.

Fig. 2. Higher magnification of the same egg as in Fig. 1. The high concentration of attached spermatozoa is quite obvious. Note that spermatozoa are attached in practically every conceivable place. Some flagella can also be seen. × 715.

Fig. 3. Higher magnification of the same egg as in Fig. 1. The spermatozoa have attached to the egg surface and undergone the acrosome reaction. Note how flagella are coiled and many are in close association with the posterior region of the sperm posterior end (arrows). This association is indicative of the secondary reaction which occurs after the initiation of the acrosome reaction. Pores on the egg envelope surface are also visible. × 3040.
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projections of the acrosomal filaments were not necessarily observed, particularly if the acrosomal filaments were projected into the egg envelope. The following events, however, can be described in each reacting sperm. Immediately after initial sperm attachment to the egg preparation, the acrosomal vesicle opens and its contents adhere to the egg material (Figs. 6, 11, 12). The acrosomal filament then projects anteriorly through this reacted acrosomal vesicle and through the egg materials (Figs. 11, 16, 17). The acrosome reaction occurred in the majority of spermatozoa within 1 min after insemination. One exception was when egg sections were used. With this method, reacted spermatozoa were in the minority even after 5 min, a result possibly related to the experimental procedure of freezing eggs prior to sectioning. After the acrosome reaction and usually within 5 min, the secondary reaction occurs whereby the flagellar fibrous components are incorporated into the sperm body (Figs. 3, 12, 14). With 0.34 M CaCl₂, acrosome reactions were initiated free from egg materials. Within 3 min of treatment most spermatozoa (> 90%) had undergone the acrosome reaction and many had initiated the secondary reaction (Fig. 14).

Sperm acrosomal filament penetration

The projection of the acrosomal filament is part of the acrosome reaction, while the penetration of the acrosomal filament through the egg envelope is a direct result of this reaction. The latter deserves separate consideration since different mechanisms (e.g. enzymic digestion) are involved. In experiments using excised whole egg envelope (Fig. 18), each acrosomal filament penetrated through the egg envelope perpendicularly to the surface (Figs. 19–21). An actual count of attached spermatozoa on an area of the egg surface and of the protruding acrosomal filaments over a similar area on the inner surface of the egg envelope showed that approximately 70–80% of the spermatozoa were reacted and had successfully projected their acrosomal filaments through the egg envelope. This is based on sperm–egg interactions 15 min after the mixing of gametes, since at 5 min (in micrographs not included in this study), the acrosomal filaments are just beginning to penetrate through, and not easily observed.

Fig. 4. Egg section approximately 25 μm thick. This section was ‘inseminated’ for approximately 1 min with 10% sperm suspension. Spermatozoa are attached to the basement lamina of the egg envelope (ee) and can be observed (arrow) around the periphery of the egg section. x 74.

Fig. 5. Higher magnification of part of Fig. 4 demonstrating area of sperm attachment. Note that all spermatozoa (except an errant one) are attached only to the basement lamina (bl), not to the vitelline envelope (ve). The gap between the vitelline envelope and the vitellus (v) is an artifact, also seen in Fig. 4. x 780.

Fig. 6. Higher magnification of part of Fig. 4 showing several spermatozoa with apical tip of acrosome (a) attached to basement lamina (bl). Other sperm features are the nucleus (n), the region posterior to nucleus (ar) where the mitochondria are located and the acrosomal rod is coiled, and the flagellum (f). Acrosome reactions have occurred noticeably in 2 spermatozoa (arrowed) and are indicated by the flattened appearance of the sperm acrosomes. x 6800.
DISCUSSION

Since the manner of initiating sperm motility is different in Limulus from that in most other species, this phenomenon is briefly discussed before examining other sperm reactions. Contrary to most species which have external fertilization, the spermatozoa of Limulus are non-motile at semination. However, as spermatozoa are brought into close proximity to mature eggs, motility is rapidly initiated. On examining these conditions, initiating factors for sperm motility were found associated with the egg basement lamina and also in the medium immediately surrounding the egg. Interestingly, as sperm motility occurs, a form of chemotaxis is also displayed, since practically all spermatozoa move directly towards the egg. In animals, the phenomenon of chemotaxis is rare and has been observed in only a few species, in particular the fish Clupea (Yanagimachi, 1957) and the hydroid Campanularia (Miller, 1966). In comparing sperm motility and chemotaxis between these species and Limulus a closer correlation is made with Clupea, in which both phenomena occur only in the immediate vicinity of the micropyle (Yanagimachi, 1957). In Campanularia these phenomena differ, since spermatozoa are motile at semination and develop an oriented movement towards the female gonangium and not the egg (Miller, 1966). A final phenomenon involving sperm motility is the duration, which was found to be usually less than 5 s, a very short time compared to other species. Although longer durations of sperm motility in Limulus were observed and have

Fig. 7. Piece of the egg basement lamina which adhered to the glass coverslip when egg was gently rolled across it. The inside surface of the basement lamina is visible. × 475.

Fig. 8. Piece of egg basement lamina similar to that in Fig. 7 but which had been mixed with 10% sperm suspension for 5 min. Note that spermatozoa are attached to an area on basement lamina and to an area on coverslip where no piece of egg layer is obvious. See Figs. 9–12 for explanation. × 480.

Fig. 9. Higher magnification of an adhering piece of the basement lamina. This is composed of matrix-like material (m) and a coating substance which readily adheres to the coverslip and is frequently found in independent patches where egg was rolled past (arrow). As shown in Figs. 10 and 12, the spermatozoa readily attach to this coating substance and to the matrix-like material. × 2500.

Fig. 10. Higher magnification of attached spermatozoa which have been mixed with adhering pieces of basement lamina. Note that spermatozoa (arrows) readily attached to coating substances as well as to the piece of basement lamina. × 2300.

Fig. 11. Spermatozoa attached to coating substance of basement lamina have undergone the acrosome reaction projecting the acrosomal filaments (af), several of which can be observed in the micrograph. These filaments are approximately one half the diameter of the flagella. Note also the network which has been left by coating substance from around the pores on the original surface of the basement lamina. × 5300.

Fig. 12. Spermatozoa which are attached to a piece of the basement lamina and have undergone the acrosome reaction. Note the flattened acrosome (arrow) indicating the acrosome reaction. The secondary reaction (sr), which is the coiling of the flagellum and its eventual incorporation into the sperm body, has been initiated. × 5520.
been previously reported (Mowbray & Brown, 1974) this short duration is presumed
be normal. These 3 phenomena could be acting specifically to conserve energy
in order to aid eventually the sperm nuclear penetration of the egg envelope.

In Limulus, since large numbers of spermatozoa participate in sperm–egg inter-
actions, sperm reactions are easily demonstrated and quantitative analysis can be
used to examine each fertilization process. Earlier, Brown & Knouse (1973) reported
that a high sperm concentration (10^8–10^{10} spermatozoa/ml) was necessary for fertili-
ization, but more recently experiments using a very low sperm concentration have
been successful (Brown, Hopewell & Clapper, 1976). Thus, although the belief
that a high sperm concentration is necessary for fertilization has been changed, the
usage of numerous spermatozoa has been extremely rewarding for the present
study.

Limulus spermatozoa have 2 stages of egg attachment: the initial attachment, as
each sperm reaches the egg surface or egg components; and the secondary attachment,
which is a stronger adhesion of the sperm to the egg surface and occurs only after
the acrosome reaction has been initiated. The initial attachment occurs as the sperm
apical tip contacts the basement lamina, a process similar to that in other species
(e.g. sea urchin, Franklin, 1965). This attachment is species-specific (Mowbray,
Brown & Metz, 1970) and is definitely dependent on the presence of a ‘fertilization
or attachment antigen’ on the basement lamina (Mowbray & Brown, 1974). With
reference to the latter, if the egg is treated with univalent or multivalent anti-egg
envelope antibodies, sperm attachment is prevented. Additional studies (Mowbray
& Brown, in preparation; Mowbray, 1972) on the nature of this surface antigen
by treatment with specific enzymes have demonstrated a protein-lipid component.
The secondary reaction specifically involves binding of the acrosomal materials to
the egg surface. This occurs as the acrosomal vesicle ruptures and releases its contents
directly on the egg surface. As a result each sperm is solidly oriented perpendicular
to the egg surface, an aspect which plays an important role in the direction of penetration of the filament into the egg envelope. If spermatozoa which have undergone the secondary reaction are forcibly removed from the egg surface (not reported in this study) the acrosomal contents either remain adhering to the surface or a deep imprint of the attachment site is left. Elucidation of the nature of the substances responsible for adhesion and their roles in the subsequent sperm reactions would be most rewarding.

The *Limulus* acrosome reaction as described in previous studies (Andre, 1963; Shoger & Brown, 1970; Tilney, 1975) basically involves the fusion of the outer acrosomal membranes to the sperm plasma membrane, release of the acrosomal contents, and projection of the acrosomal filament which is the acrosomal rod covered by the inner acrosomal membrane (all these processes are typical and agree with the *Saccogloccus-Hydroides* model: Colwin & Colwin, 1967). The present need is to examine on the egg the site of the acrosome reaction initiation, its chemical mechanism, and the post-reaction events. Since this reaction occurs shortly after initial sperm attachment to the basement lamina, the site of sperm attachment must also stimulate the acrosome reaction. For if spermatozoa become motile, but fail to attach, they remain unreacted. Since calcium chloride will stimulate the acrosome reaction free of any egg components and produce a very high percentage (80–90%) of reactions, calcium ions are probably also involved in the normal process. The role of calcium ions has recently been examined in the acrosome reaction of sea-urchin spermatozoa and their presence has been demonstrated to be necessary (Gregg & Metz, 1976).

The sperm 'secondary reaction' following the acrosome reaction was observed again (Shoger & Brown, 1970); however, its significance is unknown, except that nuclear transfer through the egg envelope must be involved. Since the flagellar fibrous components are incorporated into the sperm body and surround the nucleus, the flagellar microtubules are probably influencing the nucleus to elongate and eventually to follow the path of the penetrated acrosomal filament. Since microtubules are necessary for the formation and shaping of nuclei in many species during

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**Fig. 16.** Inseminated (5 min) basement lamina prepared by freeze-drying (see Fig. 15 for fuller explanation). The edge of the basement lamina has curled, exposing the acrosomal filaments which have penetrated through the basement lamina. Originally the filaments of the reacting spermatozoa came into contact with the glass coverslip and thus were directed between the basement lamina and the coverslip. The surface being observed is the true outer surface of the egg. The pores are readily visible and seem to be the point of emergence for many filaments. × 6240.

**Fig. 17.** Higher magnification of part of Fig. 16. Note particularly how the acrosomal filaments have passed through the pores of the basement lamina. × 12400.

**Fig. 18.** Whole egg envelope removed from one egg. The egg envelope was allowed to adhere to a coverslip and was inseminated with 10% sperm suspension for 15 min, before preparing for scanning electron microscopy. The outer surface of the egg envelope is observed and is covered with spermatozoa (s) except for obvious bare spots (6) which represent the areas where the basement lamina adhered to the fine forceps during handling of the egg envelope. The inner surface is not visible in this micrograph. × 63.
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Spermatogenesis (Kessel, 1970), a role in transferring the nucleus into the vitellus is also possible.

One amazing aspect of Limulus fertilization reactions is the large number of acrosomal filaments which pass completely through the egg envelope. Two approaches are in need of examination: (1) the mechanisms used by a single filament in its passage; and (2) the reaction of the egg in controlling the numerous filament penetrations and the fusion of a filament tip to the vitelline plasma membrane. Since the filament rotates on its longitudinal axis during penetration (Tilney, 1975), 2 factors could be involved: the physical drilling of the filament and the distribution of digestive enzymes located on the filament membrane. Although acrosomal enzymes are definitely present, their locations and functions are presently unknown. Presumably, during normal fertilization the first acrosomal filament reaching the vitelline plasma membrane elicits sperm-egg membrane fusion and some form of egg activation; both events could either suppress envelope penetration or block the fusion of other acrosomal filaments. In many species, egg cortical reactions occur during egg activation and prevent polyspermy penetration. This may be true in Limulus.

Since filament penetration through the egg envelope takes as long as 5 min, the egg envelope could contain a selective mechanism which allows one of the million reacting spermatozoa to reach initially the vitelline plasma membrane. One such possible mechanism could be the blockage or inhibition of acrosomal enzymes, since such inhibitors have readily been demonstrated in mammalian systems (Williams, 1972). Egg factors controlling this and other sperm-egg interactions are being isolated in our laboratory. In time, the biochemical roles of these factors will be determined and applied towards a mechanism of fertilization in Limulus and other species.

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Fig. 19. Outer surface of the whole egg envelope inseminated for 2 min. Numerous spermatozoa have typically attached to the surface. Observation of the inner layer of this egg envelope revealed no penetrating filaments. × 830.

Fig. 20. Inner surface of whole egg envelope showing acrosomal filaments (arrows) which have penetrated through the entire egg envelope, after insemination for 15 min. Since so many have penetrated, a selection mechanism for sperm penetration into the vitellus probably does not exist in the egg envelope but on the surface of the vitellus instead. × 830.

Fig. 21. Higher magnification of inner surface of egg envelope shown in Fig. 20. Of the spermatozoa attaching to the surface about 70–80% have acrosomal filaments completely penetrating through the egg envelope. The average length of the protruding free end is approximately 15 μm. × 2760.
REFERENCES


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