SPERM-EGG INTERACTIONS OF *LIMULUS POLYPHEMUS* WITH SCANNING ELECTRON MICROSCOPY

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INTRODUCTION

The primitive horseshoe crab, *Limulus polyphemus*, is an excellent species for studies of intial sperm-egg interactions (Shoger and Brown, 1970). The gametes are easily obtained from mature animals, and fertilization and development can be demonstrated year around in the laboratory. Although the egg is comparatively large, quite opaque, and difficult to prepare for experiments involving embedding, much information on sperm-egg attachment is being obtained particularly with the use of frozen egg sections (Brown and Mowbray, in preparation). However, as in many other species there is the necessity of observing fertilization in the whole egg. Thus, with the use of scanning electron microscopy, the study of gamete interactions, especially *Limulus* eggs and spermatozoa before and after their attachment, has been greatly enhanced.

MATERIALS AND METHODS

Specimens of *Limulus polyphemus* L. were obtained from the Marine Biological Laboratory (Woods Hole). Gametes were collected using an electrical stimulator (Shrank et al., 1967). Spermatozoa and eggs were mixed and samples were removed at varying intervals. These samples plus unfertilized eggs were prepared for scanning electron microscopy. The remaining eggs were observed until development was definitely seen.

The gametes were washed in sea water and fixed for several days in 2% glutaraldehyde buffered with 0.1 M cacodylate to pH 7.2. After a rinse in sea water they were postfixed for 3 hr in 2% osmium tetroxide buffered to pH 7.4 with 0.2 M phosphate buffer. They were thoroughly rinsed eight to ten times in glass-distilled water. The fixed and washed eggs were snap-frozen, one at a time, in liquid nitrogen, and transferred onto the flat end of a brass cylinder (6 × 6 cm) that was precooled in liquid nitrogen. The cylinder was placed on an insulated surface under a bell jar, which was immediately evacuated to a pressure of 5×10^{-5} torr. The specimens were left on the cold block overnight to freeze-dry. The freeze-dried eggs were very brittle, and some were fractured with a sharp razor blade in order to expose structures for cross-sectional views. An electrically conductive layer of gold approximately 200 A thick was vaporized onto all specimens before they were viewed in a Cambridge Mark 2A "Stereoscan" scanning electron microscope (Electron Microscopy Laboratory, University of Georgia) using an accelerating voltage of 10 kv.

OBSERVATIONS

Before discussing the initial sperm-egg interactions, a brief description of the mature gametes is necessary. The mature egg is approximately 2.5 mm in diameter, and the egg envelope (Fig. 1a) is composed of an outer layer termed basement lamina and an inner layer termed vitelline envelope (Dumont and Anderson, 1967). The basement lamina (5 μ thick) is perforated perpendicularly with pores which vary in size and which are randomly spaced (Figs. 1a and 1b). The vitelline envelope (approximately 35 μ thick) does not show any evidence of pores. Both layers of the egg envelope uniformly surround each egg and there is no evidence of a micropyle. The mature sperm consists of the four basic components: apical acrosome, nucleus, mitochondria, and centriolar-flagellum apparatus (Fig. 1c). The unique feature is the axial rod coiled posterior to the nucleus and passing through the intranuclear canal to the proximal border of the acrosomal cap (André, 1963).

The initial sperm-egg interactions have been described with light microscopy and transmission electron microscopy (Shoger and Brown, 1970). The following confirms, enhances, and modifies these previous observations. Mixing of eggs with a high concentration of spermatozoa demonstrates large numbers of spermatozoa attaching to the egg surface (Fig. 1d). Assuming that this condition would exist over the entire surface of the egg, the extrapolated figure infers that at least 1,000,000 spermatozoa attach to each egg. Each sperm attaches perpendicularly to the egg surface (Fig. 2a)

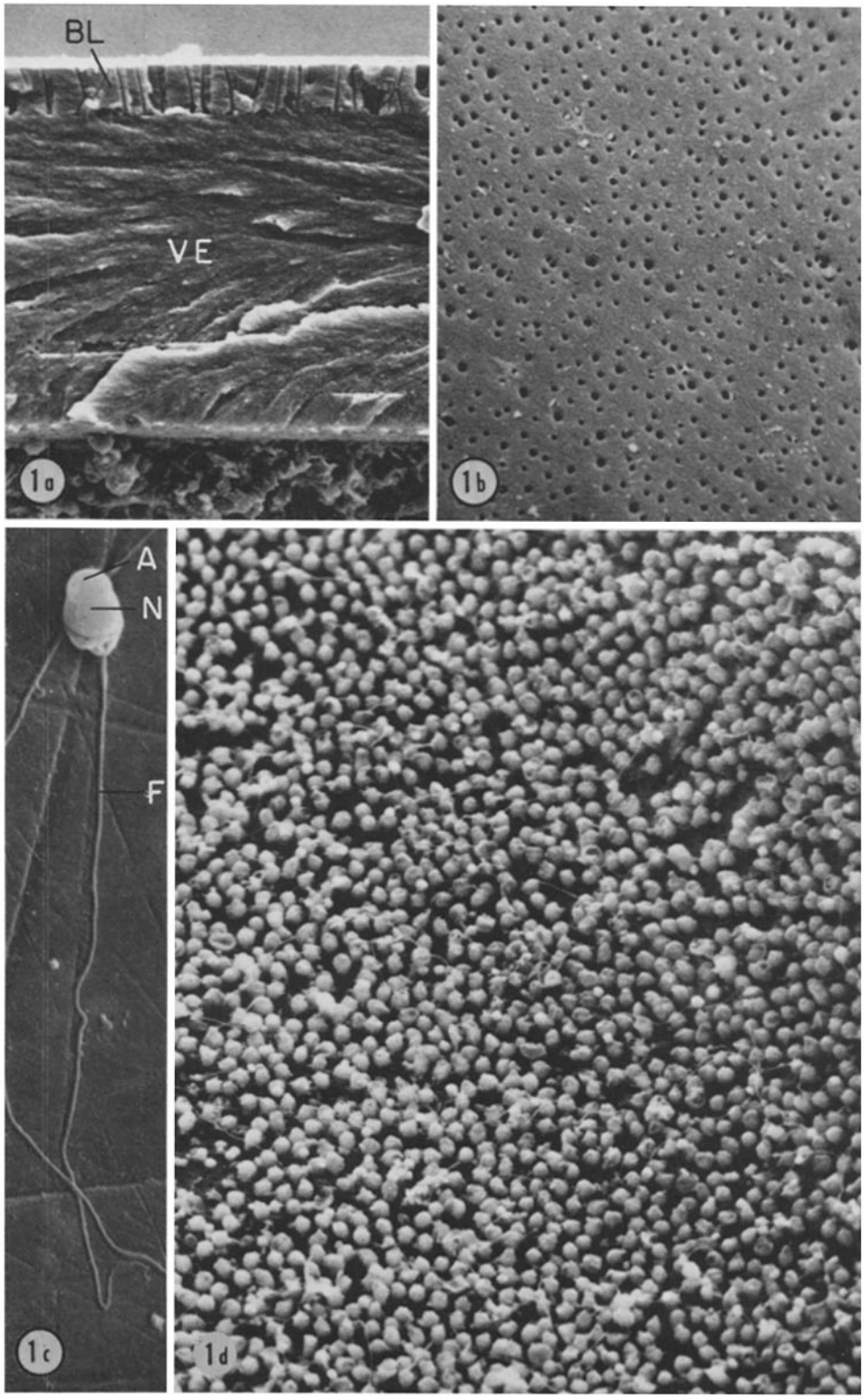

FIGURE 1a Fractured unfertilized egg showing the egg envelope in cross section. Note the two layers, basement lamina (*BL*) and vitelline envelope (*VE*). Pores through the first layer are observed. × 1400.

FIGURE 1b Surface of unfertilized egg demonstrating pores in basement lamina. Note irregular spacing and variable sizes of pores. × 1800.

FIGURE 1c Unreacted sperm mounted on scanning stub. Note acrosome (*A*), nucleus (*N*), and flagellum (*F*). × 3700.

FIGURE 1d Spermatozoa attached to egg surface. Approximately 5 min after mixing of gametes. × 1000.

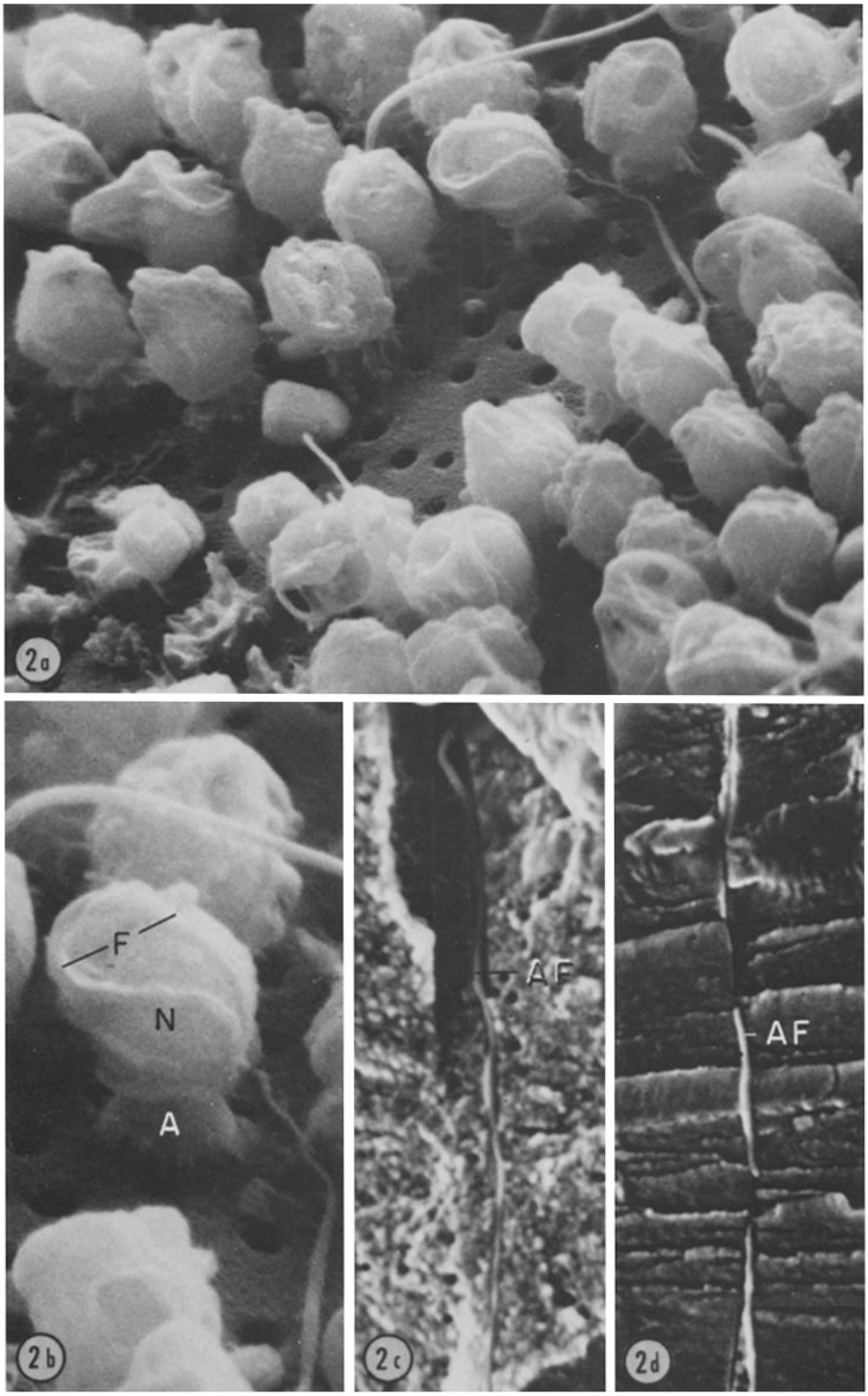

FIGURE 2a Higher magnification of spermatozoa attached to egg surface. Approximately 5 min after mixing of gametes. × 6300.

FIGURE 2b Sperm attached to egg surface. Note acrosome (*A*), nucleus (*N*), and flagellum (*F*). × 8600.

FIGURES 2c and 2d Fractured eggs fixed 5 min after fertilization. The sperm acrosomal filament (*AF*) has penetrated the egg envelope. 2c, × 5800; 2d, × 5000.

by means of the acrosomal material from the ruptured acrosomal cap (Fig. 2b) and immediately undergoes an acrosome reaction, resulting in the projection of the acrosomal filament through the two egg layers (Figs. 2c and 2d) and presumably to the egg plasma membrane. Each acrosomal filament apparently passes through a pore of the basement lamina. These filaments may be digesting a pathway and/or mechanically forcing their way through the vitelline envelope. In all stages observed from 1 min to 60 min after fertilization, the sperm nuclei remained on the egg surface. The next step presumably would be the passage of the nuclear material through the egg envelope and into the egg proper; however, the difficulty of finding the one sperm penetrating is insurmountable.

DISCUSSION

In reference to the large number of spermatozoa attaching to each egg, the significance of such a phenomenon is considered. In laboratory experiments a decrease in the sperm concentration will drastically decrease the per cent of developing embryos even though the number of spermatozoa (100–10,000) per egg is high (Brown, in preparation). In nature, according to the descriptions of Lockwood (1870) and Kingleys (1892), the following events occur. The male *Limulus* attaches by amplexus to the opisthosoma of the female. The female during spawning excavates a shallow pit (1–2 inches) in the sand and deposits approximately 200 eggs. After spawning, the attached male deposits semen directly on the eggs, assuring a large number of spermatozoa in the immediate vicinity of the eggs. Thus, although not actually observed, the occurrence of the above phenomenon in nature is considerably more than a probability.

The question raised is the significance of so many spermatozoa (estimated 100,000–1,000,000) attached to each egg. Although the answer must be speculative there are two possibilities: (*a*) lysin-like chemicals are released from the large number of spermatozoa. This results in the digestion or a chemical change of the egg envelope and/or plasma membrane, thus facilitating the entry of one sperm nucleus. (*b*) On the egg may exist a suitable site which is particularly penetrable by a sperm, the difficulty being the chance attachment of a sperm to this site.

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