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Some Ultrastructural Aspects of Spermatogenesis and Sperm Morphology in the Brine Shrimp *Artemia salina* Leach (Crustacea: Branchiopoda)

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Although crustacean diversity has stimulated research enormously, sperm morphology and functional aspects have received little attention recently as compared to earlier times (e.g., Gilson, 1886; Retzius, 1909; Wilson, 1928). The exceptions include primarily the malacostracan spermatozoa (e.g., isopods, Reger, 1964; decapods, Moses, 1961; Brown, 1966a; Anderson and Ellis, 1967; Chevaillier, 1966, 1967; Vaughn, 1968), although other representatives have also been studied (e.g., Branchiopods, Fautrez-Firlefyn, 1951; Fautrez-Firlefyn and Fautrez, 1954, 1955; Ostracods, Lowndes, 1935). In recent studies, the author (Brown, 1966a, 1966b; Brown and Metz, 1967) has examined spermatozoa of representative species of seven crustacean subclasses (Ostracoda excepted). These studies involved the comparison of sperm fine structure and relationships to phylogeny. In particular, the sperm structures of two primitive crustaceans, a cephalocarid, *Hutchinsoniella macracantha,* and a mystacocarid, *Derocheilocaris typicus,* were thoroughly examined. In addition, a brief comparison was made with the sperm of the branchiopod, *Artemia salina,* also a primitive crustacean, but phylogenetically divergent from the above two species (Dahl, 1963; Sanders, 1963; Hessler, 1964). This comparison showed the *Artemia* sperm to be quite unique morphologically. Therefore, a more thorough study of the development and function of this cell was undertaken.

The *Artemia* sperm morphology and development has been examined by light microscopy (Fautrez-Firlefyn, 1951; Fautrez-Firlefyn and Fautrez, 1954, 1955). Combining phase-contrast optics with cytochemical methods, the various spermatogenetic stages and mature spermatozoa are described morphologically and cytochemically. The mature sperm is described as a spherical cell with a considerable amount of cytoplasm and an acentral nucleus. Cytoplasmic inclusions consist of an acrosome, mitochondria, and specific granules of mitochondrial origin. These workers could not determine the development of the acrosome, but demonstrated it in the mature sperm by staining with the periodic acid-Schiff meth-

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od. A positive test is one criterium for the presence of an acrosome (Clermont and Leblond, 1955). Fautrez-Firlefyn (1951) briefly observed sperm-egg interactions, noting that many sperm organelles break down or disappear after sperm-egg attachment occurred.

Studies of branchiopod spermatozoa have been performed by Retzius (1909), Longhurst (1954), Zacharias (1884), Fautrez-Firlefyn (1951), and Fautrez-Firlefyn and Fautrez (1954, 1955). At present there are four living orders of branchiopods: Conchostraca, Anostraca, Notostraca, and Cladocera (Tasch, 1963). In addition to *Artemia salina*, an anostracan, spermatozoa representing two other orders have been examined by light microscopy and none with electron microscopy. These include a notostracan, *Triops cancriformis* (Longhurst, 1954) and three cladocerans *Podon intermedius* and *Daphnia pulex* (Retzius, 1909), and *Polyphemus pediculus* (Zacharias, 1884). In general, these authors described a small spherical or oval sperm with one or more dense staining organelles. With one exception these authors failed to observe sperm motility. Zacharias (1884) described an amoeboid sperm in *Polyphemus* which produced long processes and had frequent changes of shape. Retzius, however, observed various forms of the *Podon* sperm in fixed material.

Material and Methods

In the laboratory, specimens of *Artemia salina* Leach were reared from commercial cysts (Brine Shrimp Sales Company, Inc., Hayward, California). At maturity, males which were clasping females and keeping vasa deferentia distended with spermatozoa were selected and used in the following experiments.

The vasa deferentia were dissected and partially squashed under a coverslip in the body fluids of the host. These vasa deferentia would continuously contract, expelling free spermatozoa. These cells were examined with phase-contrast for motility and morphological changes.

Vasa deferentia and testicular tissue were prepared for light and electron microscopy by placing the living specimen into the proper fixative and cutting off most of the anterior segments and the posterior abdominal segments. The remaining tissue, which included the intact reproductory system, was then prepared. For light microscopy, tissue was fixed in Carnoy A (Humason, 1962), and after standard procedures, embedded in paraffin. This tissue was sectioned and stained with the Triple Stain (Himes and Moriber, 1956). For electron microscopy, the tissues were prepared after the Karnovsky's Formaldehyde-Gluteraldehyde Method (Karnovsky, 1965), embedded in Araldite (Fluka), sectioned on a LKBI Ultrotome, double stained with uranyl acetate (Watson,

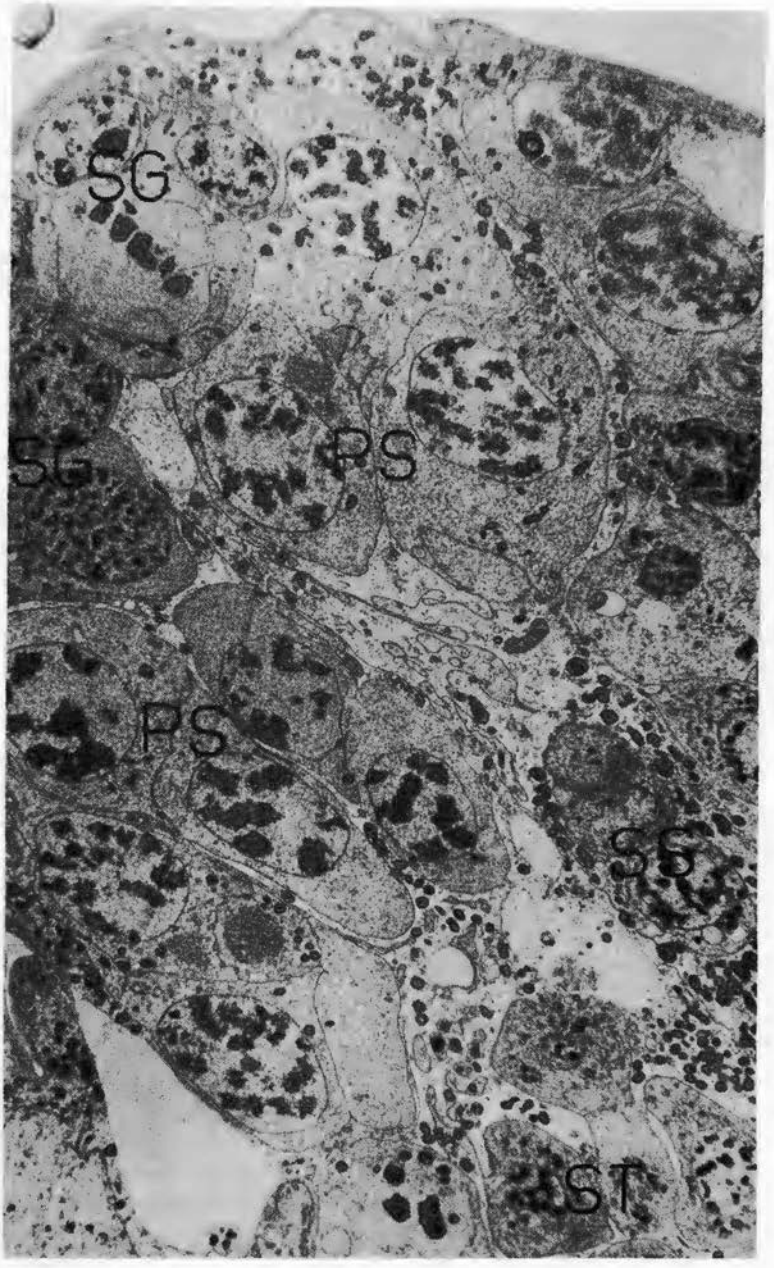

Figure 1. Cross section of testes. The various cells of spermatogenesis represented are spermatogonia (SG), primary spermatocytes (PS), secondary spermatocytes (SS), and spermatid (ST). A dividing spermatogonium (spermatocytogenesis) is observed in the upper left hand corner. X 3,310

1958) and lead citrate (Reynolds, 1963) and examined with Philips 100C, Philips 200, and Hitachi 11C electron microscopes.

Observations

General Description of Testes and Vas Deferens

The testes are long tube-shaped structures found in the dorsal anterior portion of the limbless segments of *Artemia* (Lochhead, 1950). Attached to the anterior end of each testis is a tubular vas deferens which passes posteriorly and then anteriorly into one of two peni in the ventral portion of the genital segment. Each vas deferens in the mature male is distended with spermatozoa. For the purposes of this study, ultrastructural investigations were made on both the testes and vas deferens. The various stages of spermatogenesis are observed in a cross section of the testes passing through the testicular outer wall and lumen (Fig. 1). Spermatogonia are located around the periphery with advanced developmental stages progressively nearer the lumen, where spermatids and developing spermatozoa are found. Other types of cells are also found, in particular, interstitial or nutritive cells. These are characterized by large irregular-shaped nuclei located near the testicular outer wall (Fig. 2). The vas deferens consist of an outer musculature layer surrounding a lumen closely packed with numerous spermatozoa (Fig. 9).

Spermatogonia

Spermatogonia in various stages of mitosis are located along the periphery of the testes (Fig. 5). Intercellular bridges forming a syncytium are common. Each spermatogonial cell is approximately 7 u in diameter with a spherical nucleus approximately 6 u in diameter. Mitochondria are not common and those present are usually rod-shaped. The cytoplasm is quite homogenous, with little agranular reticula but many small vesicles.

Primary Spermatocytes

Adjacent to the spermatogonia are the primary spermatocytes (Figs. 1 and 6). In the early prophase stages, synaptinemal complexes (Moses, 1964) are common and readily observed (Fig. 6). The cytoplasm consists of numerous vesicles and small spherical mitochondria. The cell is approximately 9 u and the nucleus approximately 6 u in diameter. A syncytium is not common, indicating a closing of the intercellular bridges in spermatogonia before or during the formation of primary spermatocytes. Meiotic divisions

Figure 2. A nucleus of an interstitial cell. These organelles are quite large and usually very irregular in shape and are always found near the testiticular periphery. A spermatogonium (SG) is partially observed. X 9,180

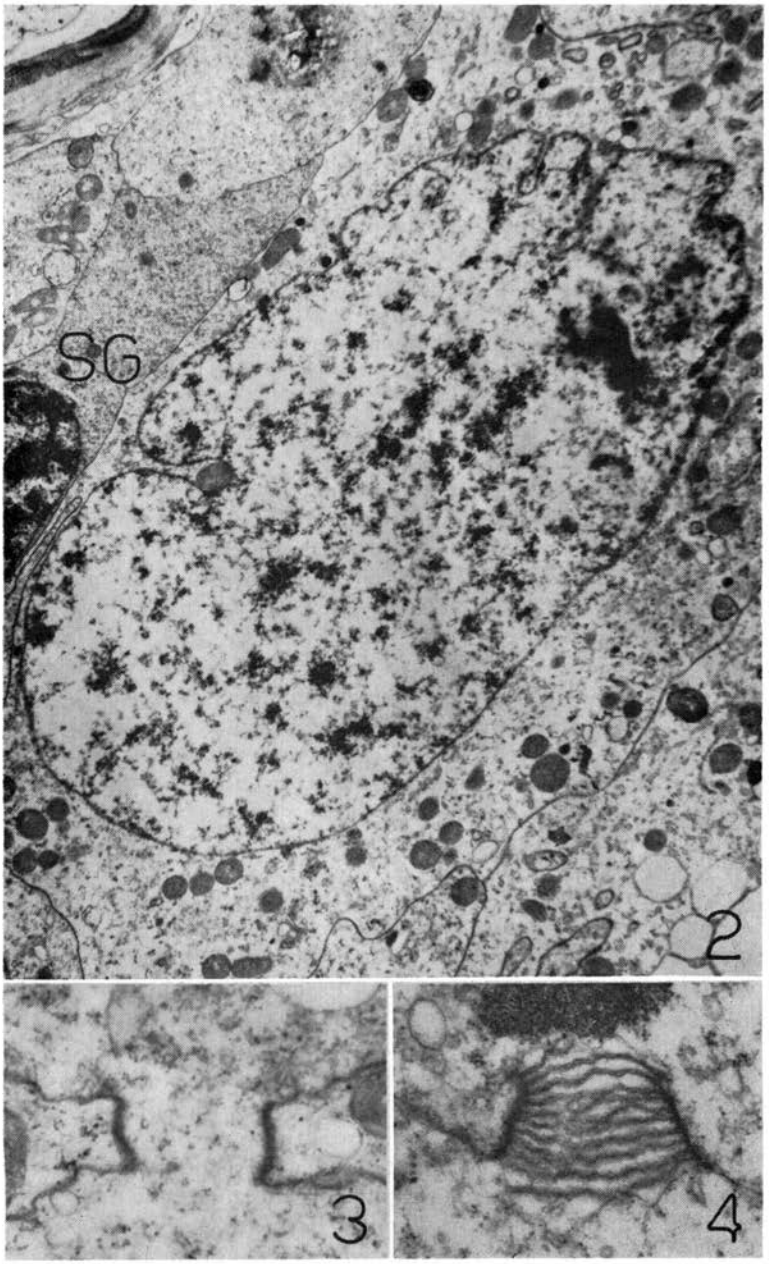

Figure 3. An intercellular bridge between spermatocytes. X 19,360

Figure 4. Between spermatocytes, an intercellular bridge possessing a laminated structure. X 28,480

forming the secondary spermatocytes are commonly observed adjacent to these cells.

Secondary Spermatocytes

Secondary spermatocytes are approximately 7 u in diameter and have spherical nuclei approximately 4 u in diameter (Fig. 7). Dense chromatin material is found in the nucleus. The cytoplasm has no unique structures and few endoplasmic reticula are developing. As in the primary spermatocytes, a few, spherical mitochondria are present. Several types of intercellular bridges are formed by the dividing spermatocytes (Figs. 3, 4, and 7). Continuous cytoplasm is seen between some cells; laminated structures are found in some cells, usually those in which the nuclear envelope is not completely formed (Fig. 4).

Spermatids

Presumably, each secondary spermatocyte eventually divides to form two spermatids; however, such a division has not been readily observed in this study. In any case, spermatids are originally closely associated with interstitial cells. They then become free and move into the testicular lumen (Fig. 8). Here the spermatids are found in various stages of spermiogenesis. However, few have become mature spermatozoa as is the case in the lumen of the vas deferens. Spermatids have more or less spherical nuclei approximately 3 u in diameter, numerous smooth endoplasmic reticula, and several spherical mitochondria (Fig. 8). Small projections which will become the arm projections of the mature sperm extend from the limiting plasma membrane (Figs. 8, 9, and 11).

Mature Sperm

The *Artemia* mature sperm was observed in or from the distended vas deferens of a mature male. The sperm is a non-motile spherical cell, approximately 6 u in diameter. It has a centrally located nucleus approximately 2-3 u in diameter and a considerable amount of cytoplasm enclosing various organelles. Numerous thin projections surround the mature sperm (Fig. 10).

The sperm was examined with phase-contrast and electron optics and by cytochemical methods. The contraction of the dissected vas deferens and gradual pressure on the coverslip forced spermatozoa into *Artemia* body fluid. In phase-contrast, these were at first irregular in shape, but rapidly became more spherical. Numerous arm projections were observed on each sperm (Figs. 9, 10, and 11). The cytoplasm contained several highly refractive organelles and stained positive for proteins (Napthol Yellow-S). The nucleus was Feulgen-positive. No obvious PAS positive structures were observed. No motility was observed.

In ultrastructural examinations, mature spermatozoa were ob-

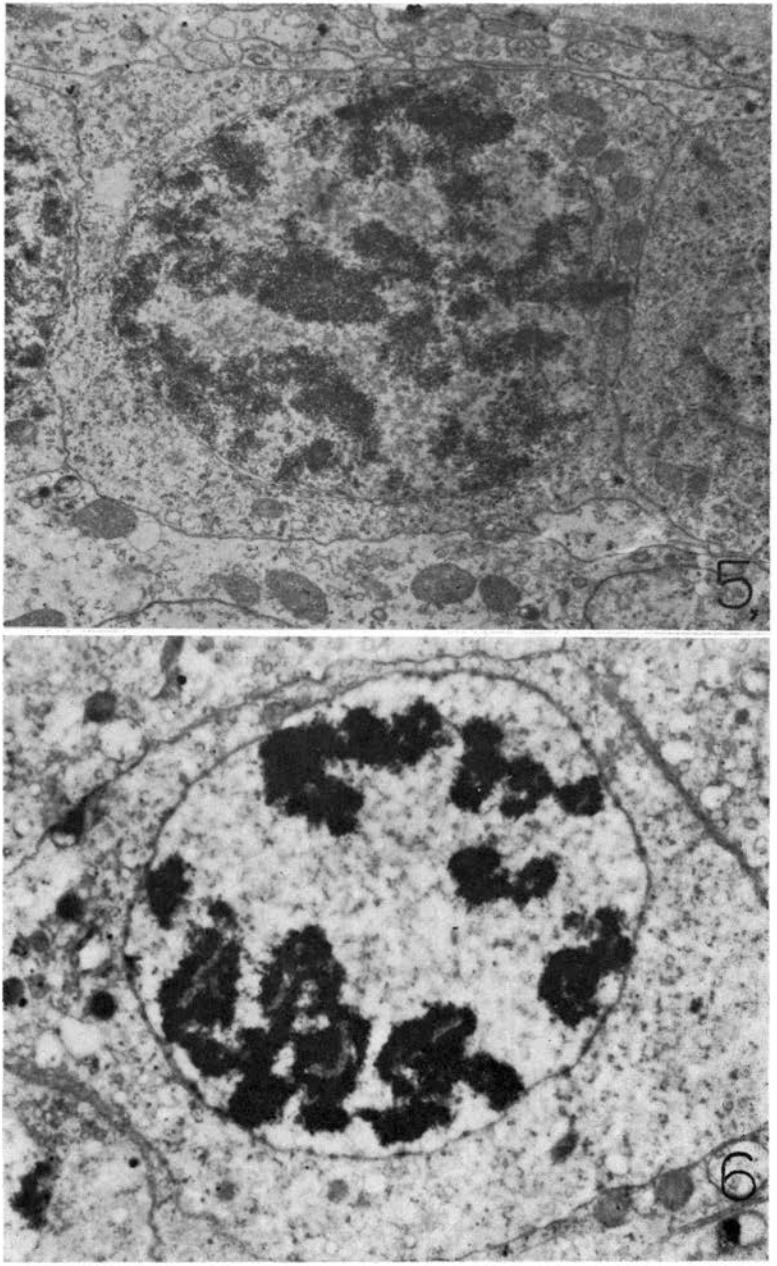

Figure 5. Spermatogonium. X 9,240

Figure 6. Primary spermatocyte. Note particularly the presence of synaptonemal complexes. X 11,800

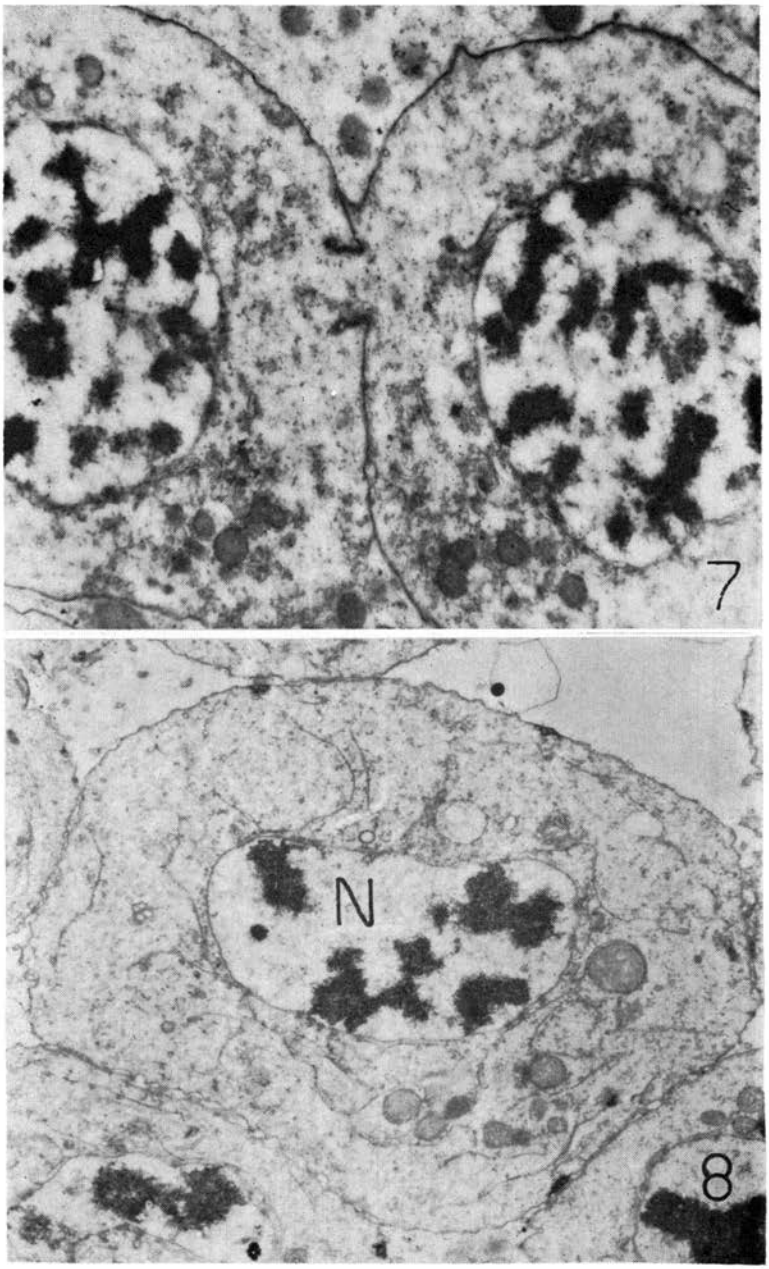

Figure 7. Two secondary spermatocytes connected by an intercellular bridge. X 11,800

Figure 8. Spermatid. Observe the smooth endoplasmic reticula. X 11,950

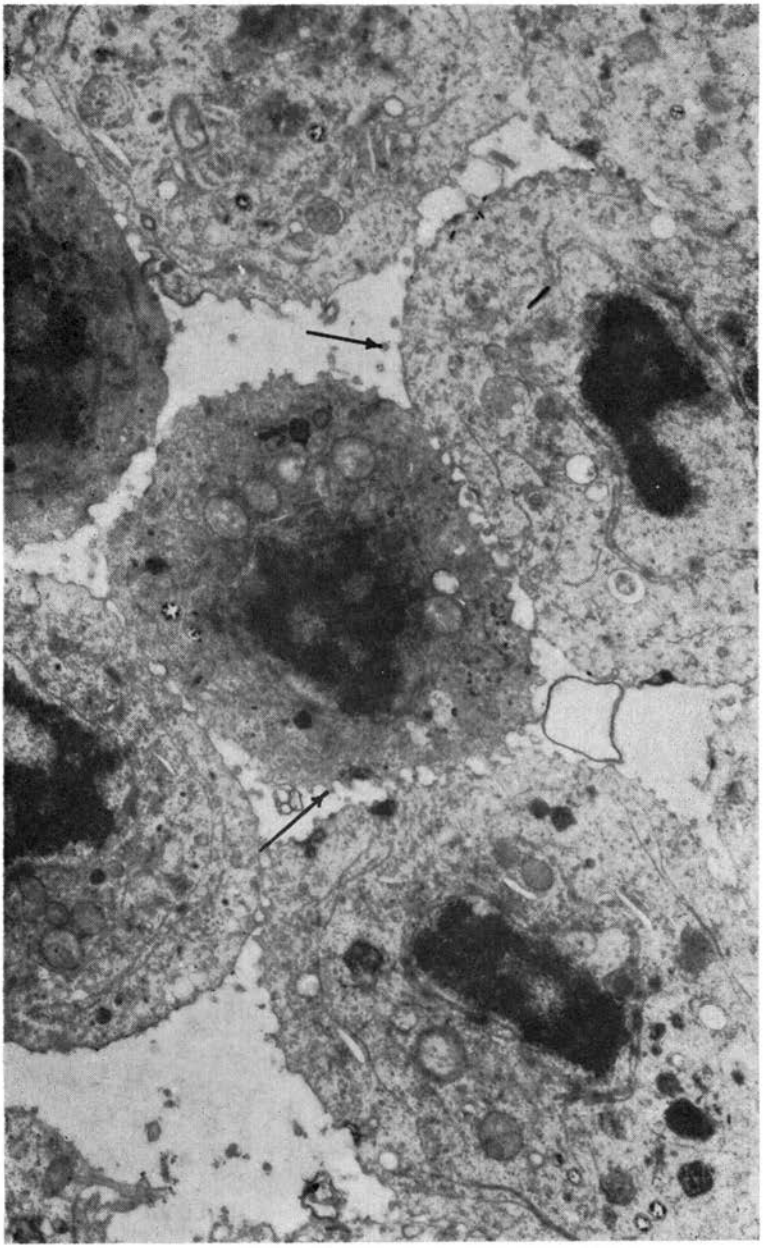

Figure 9. Field of mature spermatozoa in vas deferens. Note particularly the arm projections (arrows). X 9,890

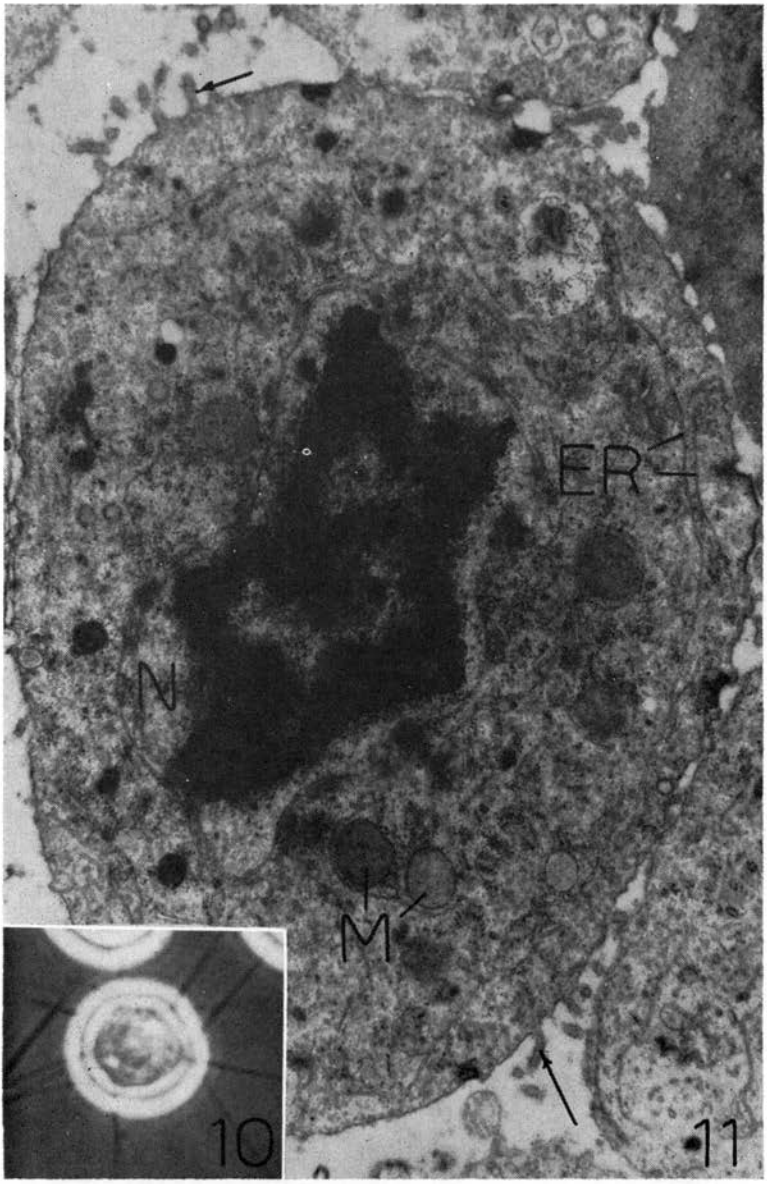

Figure 10. Phase-contrast micrograph of mature sperm. Each of the extensions from the sperm proper probably consist of several arm projections attached together. X 2,375

Figure 11. Mature *Artemia* sperm in vas deferens. Note irregularly shaped nucleus (N), mitochondria in close association with clusters of ribosomes (M), endoplasmic reticula (ER), and arm projections (AP). X 18,230

served in the vas deferens (Figs. 9 and 11), although some late spermatids appeared present. The sperm nucleus is somewhat irregularly shaped and a large proportion is electron dense, probably representing chromatin material. Surrounding the nucleus is a nuclear envelope consisting of double membranes. Scattered typical mitochondria, numerous smooth endoplasmic reticula, and numerous vesicles are found within the cytoplasm. The slender arm projections resolved with phase-contrast optics are observed extending from the periphery of the cell. These consist only of the cytoplasm matrix and the plasma membrane. Their precise position around the cell is unknown but judging from light and electron optical studies, they give the sperm a pin cushion effect. The mitochondria have a typical cristae pattern and are scattered randomly through the cytoplasm. Closely associated with the mitochondria are large numbers of ribosomal-type particles (Fig. 11). Similar sized particles are also scattered throughout the cytoplasm, but in lower concentrations. The smooth endoplasmic reticula run loosely through the cytoplasm and in many areas have a dark core. They may have some relationships to the arm projections.

Discussion

The developmental stages of spermatogenesis consist of spermatocytogenesis, meiosis, and spermiogenesis (Bloom and Fawcett, 1968). In this study these stages have been observed to some degree in *Artemia salina*. The first two stages resemble those of other species, but there are some significant differences. With regard to spermiogenesis and the mature sperm, significant differences are present. These are important for phylogenetic comparisons with other crustacean spermatozoa.

The spermatocytogenetic stages are well described in various species. One feature often observed is the presence of intercellular bridges (Fawcett, 1961), which are also commonly found with oogonia (e.g., rabbit, Zamboni and Gondos, 1967). In *Artemia* spermatogonia, such bridges are readily observed between several closely associated spermatogonia. These cells show every evidence of being synchronized in development. Such relationships in other species have been suggested and discussed in germ cell development by various workers (e.g., Fawcett, 1961; Weakley, 1967). In the present study, the intercellular bridges have been observed between spermatogonia and between secondary spermatocytes, but not between primary spermatocytes. If this is a valid observation, the intercellular bridges are closing as the primary spermatocytes develop.

The spermatocytes of *Artemia* demonstrate little uniqueness, except with regard to a special intercellular bridge. The primary

spermatocytes demonstrate the typical synaptinemal complexes (Fig. 6). As a result of the first meiotic division, intercellular bridges form and probably remain present until the formation of spermatids. One unusual type of bridge observed is a laminated structure (Fig. 4) which has also been demonstrated in spermatocytes of chickens (Nagano, 1961) and oogonia of rabbits (Zamboni and Gondos, 1968). The significance of this structure has not been determined.

Since the mature *Artemia* sperm in many respects resemble a so-called typical cell, speculations about its interaction with the egg surface will be presented. The most unique feature of the sperm is the presence of the arm projections. These projections probably play a significant role in sperm-egg interaction, but no direct evidence is available. Quite possibly the initial contact is the attachment of these projections to the egg surface, thereby stimulating or triggering a biochemical pathway(s) in the egg which ultimately leads to sperm nucleus penetration and pronuclei fusion. The mechanism involved presumably could represent an antigen-antibody reaction. An antigenic study of these projections would be of considerable significance with regard to initial biochemical mechanisms in sperm-egg interactions.

The sperm morphology of *Artemia salina* and presumably other branchiopods are unique to this subclass. They have little resemblances to the spermatozoa of the more primitive crustaceans *Derocheiloceris* and *Hutchinsoniella* (Brown and Metz, 1967) or to the more advanced crustaceans, e.g., malacostracans. If sperm morphology is used as a phylogenetic tool, then an early separation of this group is indicated. Their form of reproduction is highly specialized (as are all crustaceans). It may have released selective pressures on sperm morphology. In this case, the *Artemia* sperm has become morphologically unspecialized, that is the *Artemia* sperm has changed comparatively little from a spermatid when compared with other crustacean spermatozoa.

Acknowledgment

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