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Ultrastructure of the male gonad and male gametogenesis in the clam shrimp *Eulimnadia texana* (Crustacea, Branchiopoda, Spinicaudata)

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Abstract. The ultrastructure of the male gonad of *Eulimnadia texana* (Branchiopoda, Spinicaudata) has been observed for the first time to investigate the sexuality of a well-studied case of androdioecy in the animal kingdom. The male gonad is a double structure located in the hemocoel throughout the entire body length on each side of the midgut. Male gametes originate from the wall and mature centripetally toward the lumen; the proliferative activity is very high and continuous and therefore the mature gonad is full of numerous germ cells. Inside the lumen several degenerative stages are found mixed with sperm cells and spermatids, the latter two being not easily distinguishable because of the slight differences between them. The evolutionary meaning of the degenerative process in *E. texana* male gametes is difficult to explain, and we propose some hypotheses about its possible role or cause in the studied population: (a) to help build spermatophores, (b) to act as a trophic component for viable sperm, (c) as a manifestation of inbreeding depression, and/or (d) to regulate the number of sperm cells.

Additional key words: male gametogenesis, degenerative stages, androdioecy

Eulimnadia texana PACKARD 1871 (Spinicaudata, Limnadiidae) is a North American clam shrimp well known for both studies on sex determination (Sassaman & Weeks 1993; Sassaman 1995) and its peculiar mating system, androdioecy (mixtures of males and hermaphrodites). In fact, *E. texana* is one of only 36 species in the kingdom Animalia that show androdioecious sex distribution (S.C. Weeks, L. Benvenuto, & S.K. Reed, unpubl. data). Weeks and coworkers have extensively investigated how this particular sexual system affects the reproductive biology and evolution of this species (Weeks et al. 2000a,b, 2001, 2004; Weeks 2004; Weeks & Bernhardt 2004).

However, to date no ultrastructural analysis on male gametogenesis and gonad structure has been carried out, except for the histological investigation performed by Zucker et al. (1997). A sound ultrastructural knowledge of male sexuality is necessary for future comparison with the male region of the hermaphroditic gonad to determine possible morphological and functional convergences or differences between their respective sperm cells. Our analyses could clarify some obscure constraints linked to low outcrossing rates (Weeks et al. 2004) and thus to the fertilization success and egg viability in this species. Regarding this last point, Strenth (1977) observed in *E. texana* a structure he called a "spermatophore," but which was only found on "female" bodies. This structure has never been photographed but only drawn and, importantly, has never been described from males, where it should be found, even though it has been specifically sought in several studies (Knoll 1995; Weeks et al. 2000b). Scanabissi & Mondini (2000) have observed and described the formation of a so-called "spermatophore" in males of the conchostracan *Leptestheria dahalacensis* RÜPPEL 1837 (Spinicaudata, Leptestheriidae). It was found inside the male gonad, on male bodies before mating, and after mating on female bodies. No such structure has been described in *E. texana*. However, given the absence of a copulatory organ, it is difficult to understand how the ameboid sperm, typical of branchiopods, can penetrate the tertiary eggshell (Belk 1989) during mating.

An important datum is the low fertilization rate found in *E. texana* by Weeks et al. (2004), who studied the pre- and post-mating barriers to outcross-

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ing. They stated that the low fertilization rate is likely due to post-mating barriers to outcrossing that can cause the "highly skewed sex ratio observed" in *E. texana*.

Another important aspect emerging from previous studies is that inbreeding is clearly a predominant character in most populations of *E. texana* (Weeks & Zucker 1999; Weeks et al. 1999, 2000a; Weeks 2004). This could be linked to the possibility of egg–sperm contact along the genital ducts, which could lead to their fusion and thus to self-fertilization. In fact, selfing may account for 44–100% of the offspring (Weeks & Zucker 1999; Weeks et al. 1999), and even when males are abundant in the populations, selfing rates remain very high (45–75%; Weeks & Zucker 1999).

The present article aims to analyze the ultrastructure of the male gonad of *E. texana* and to study all aspects of male gametogenesis to elucidate the complex relationships of the gametes.

Methods

Male adults of *Eulimnadia texana,* recognizable by their claspers (see Olesen et al. 1996; Eder et al. 2000), were hatched in the laboratory from soil collected near Portal in Cochise Co., Arizona (previously reported as WAL in Weeks & Zucker 1999). Males were fixed in 2.5% glutaraldehyde in cacodylate buffer 0.2 mol L^{-1} (pH 7.2) for 2 h at 4°C. The specimens were washed in 0.1 mol L^{-1} phosphate buffer and then post-fixed in 1% OsO_4 in the same buffer for 1 h at 4°C. The samples were processed through a graded acetone series, then propylene oxide, and then embedded in Epon-Araldite. The ultrathin sections were stained with lead citrate and uranyl acetate and they were observed through a Philips EM 410 electron microscope (Philips, Eindhoven, the Netherlands).

Results

The gonad is a tubular, double organ located in the hemocoel, on each side of the midgut of adult males. Males are easily distinguishable from hermaphrodites by the claspers, which are the first two pair of thoracic legs modified for clasping hermaphrodites to facilitate mating. The grasping capability of the claspers is enhanced by the presence of suckers on each endopod (Fig. 1A). The male gonad protrudes into every available space between the midgut and the body wall muscles. The internal pressure forces the gonad into several lateral pouches, which extend centripetally among the muscles so it appears to be a diverticulate sac (Figs. 1B, 2B).

In thin sections, the male gonad is made up of two kinds of cells: the somatic cells and the germ cells (Fig. 2).

The somatic cells constitute the epithelial wall from which sperm cells originate. The gonad's epithelium is made up of elongated cells, in some cases 2–3 μm thick, which includes a thin (0.15 μm) basal lamina (Fig. 2B).

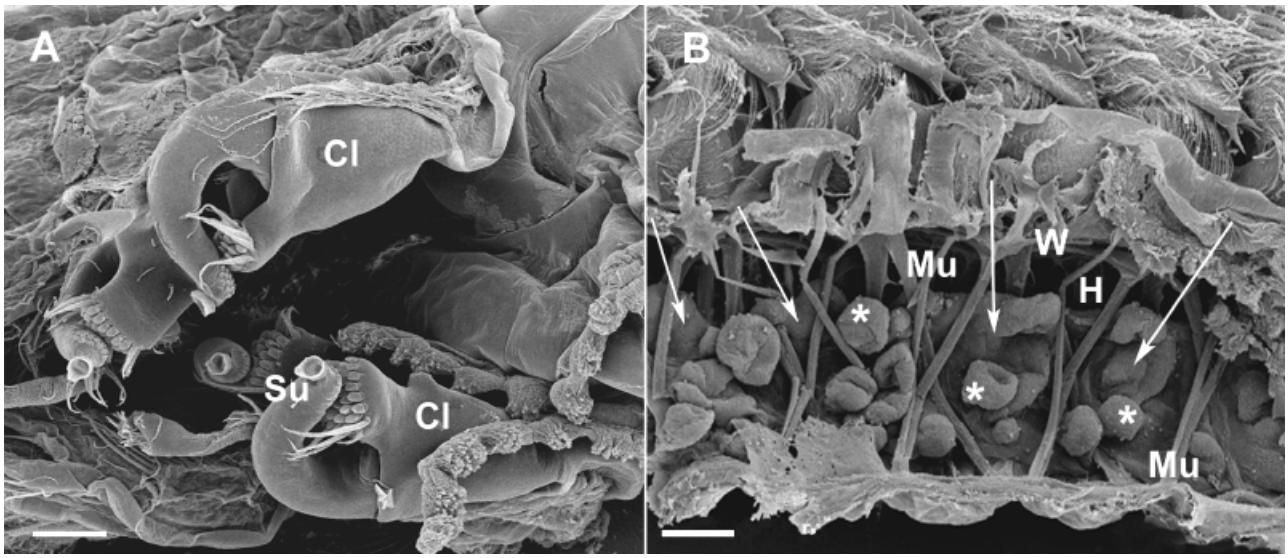

Fig. 1. A. The males are characterized by the first two thoracic legs modified as claspers (Cl), with sucker-like distal projections (Su). Scale bar, 100 μm. **B.** Body section of an adult male showing the gonad (arrows) located in every void space of the hemocoel (H) between the midgut and the body wall (W). Contracted muscles (Mu) enlarge portions of the gonad in sac-like dilations (*). Scale bar, 100 μm.

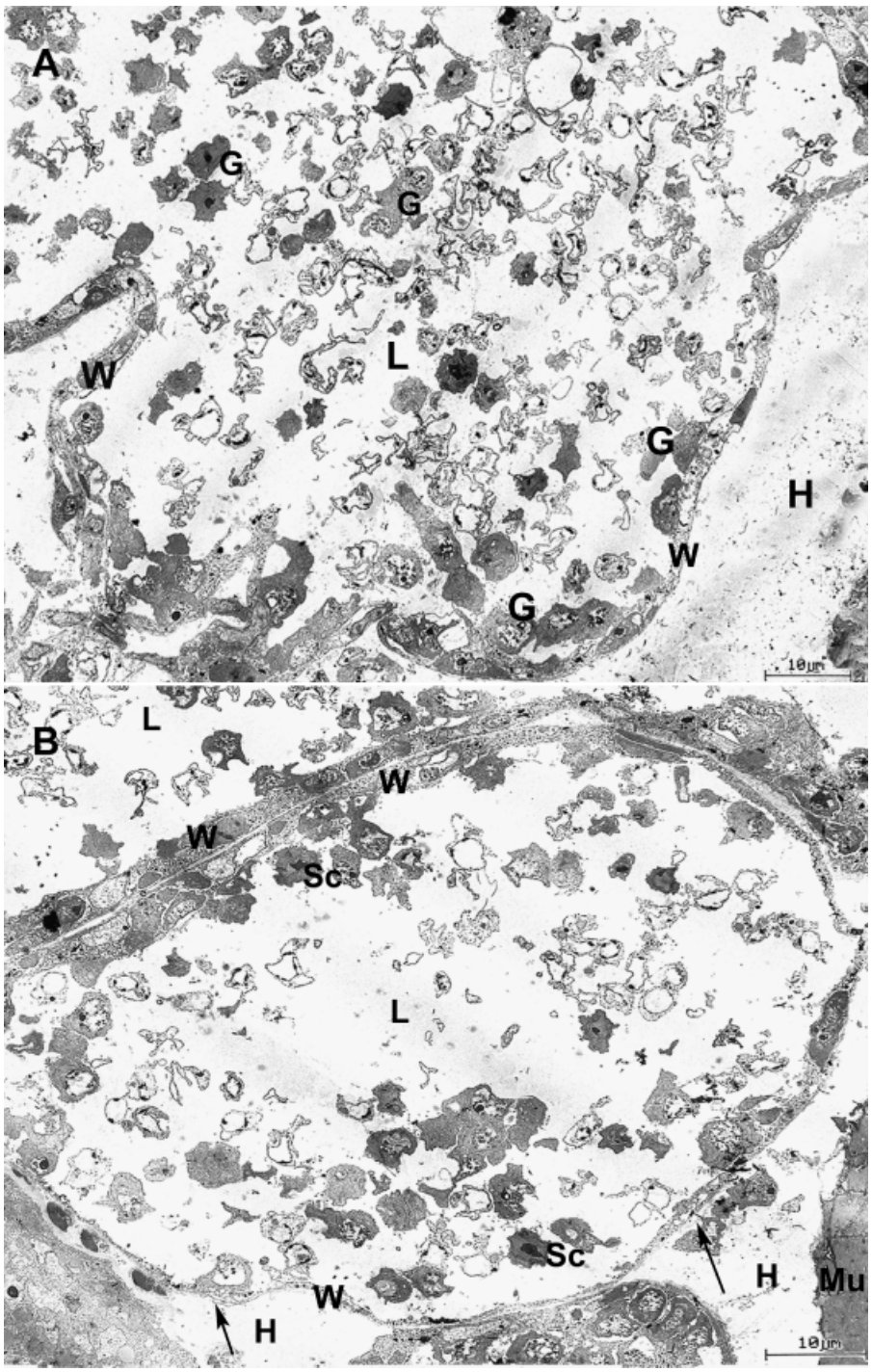

Fig. 2. A. Male gonad. The somatic and germinal districts are evident. The thin, squamous wall (W) envelops the lumen (L), into which male gametes are discharged (G) and in which their maturation takes place. Hemocoel (H). **B.** A sac-like dilation of the male gonad. Muscle (Mu), spermatocytes (Sc), basal lamina (arrows).

A widespread parietal spermatogenesis occurs. Spermatocytes, and in part spermatids, are continuously produced, intermixed with the somatic cells, from the wall to the lumen, where maturation takes place (Fig. 3). Very numerous spermatids are continuously liberated into the lumen where they were frequently found in division, showing clearly evident centrioles and fiber spindles (Fig. 3B). The spermatids often appear linked by means of an intercellular bridge, due to incomplete division (Figs. 3C,D, 4A). Mitochondria are easily recognizable (Figs. 3B–D, 4A,B). Commonly, the nucleoli assume a ring form (Figs. 3C, 4A).

Unlike most taxa, sperm maturation in branchiopods does not involve marked changes in cellular morphology. Therefore, it is quite difficult to

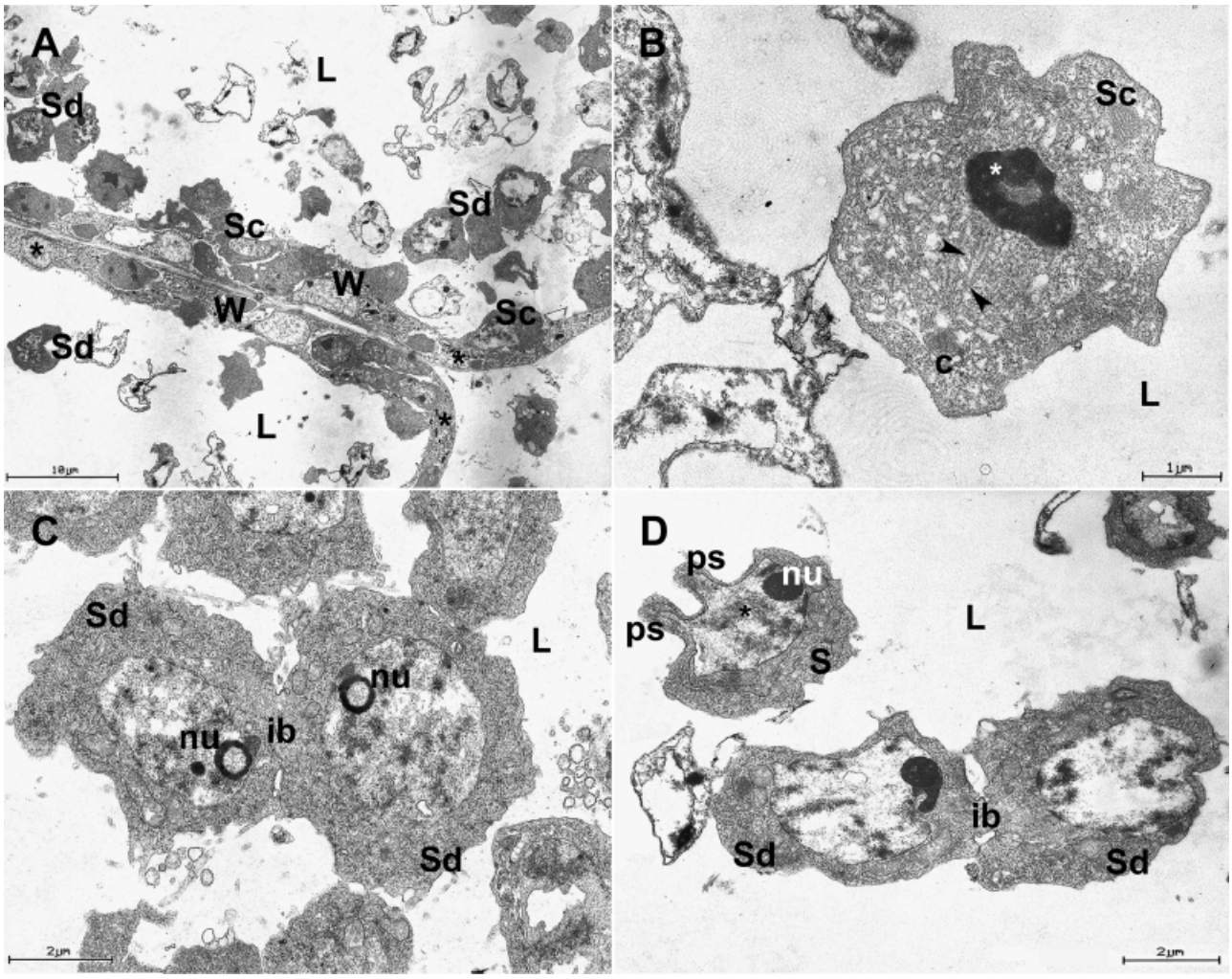

Fig. 3. A. Sperm maturation. The spermatocytes (Sc) originate intermixed with the elongated somatic cells (*). Spermatocytes and spermatids (Sd) are evident near and along the wall (W), so that its thickness seems enlarged. Lumen (L). **B.** Spermatocyte in metaphase in the lumen. Centriole (c), spindle (arrowheads), and condensed chromosomes (*) are evident. **C.** Spermatids in the lumen, still linked by the intercellular bridge (ib). The cytoplasm is very electron dense. The nucleus maintains a large volume and a round shape. Inside, the nucleoli (nu) exhibit a ring form and the chromatin is partially condensed. **D.** Spermatids still linked and mature sperm cells (S). The mature sperm cell is recognizable by pseudopodia (ps) and a reduced volume. The nucleus presents filamentous chromatin (*) and a large, dense nucleolus.

distinguish the spermatocytes and the spermatids from the mature sperm cells. Spermatids and sperm cells can be distinguished by cytoplasmic density and size (spermatocytes: $\sim$5.7 μm diameter; sperm cells: $\sim$4.4 μm diameter), even though the differences are not always easy to detect (Fig. 3D). The sperm cells are ameboid and irregularly shaped, and the plasmic membrane presents thin elongations or pseudopodia. The cytoplasm tends to be reduced and the electron density increases; in the nucleus, the chromatin appears scattered and, even though thoroughly examined, no signs of meiosis were found (Fig. 3D).

Mixed with the sperm cells and spermatids in the gonad lumen of each specimen, a second kind of cell was constantly present. This cell type seemed to be derived from either spermatids or sperm cells. Its development started from the inside of the nucleus with the opening of large spaces (arrows, Fig. 4A,B) while the nuclei maintained their membrane integrity, and later the cytoplasm was affected, too. The end result was the emptying of the cells (Fig. 4C). The voiding process involved all cytoplasmic organelles, except for the residual mitochondria and chromatine attached to the nuclear membrane (Fig. 4D). As can

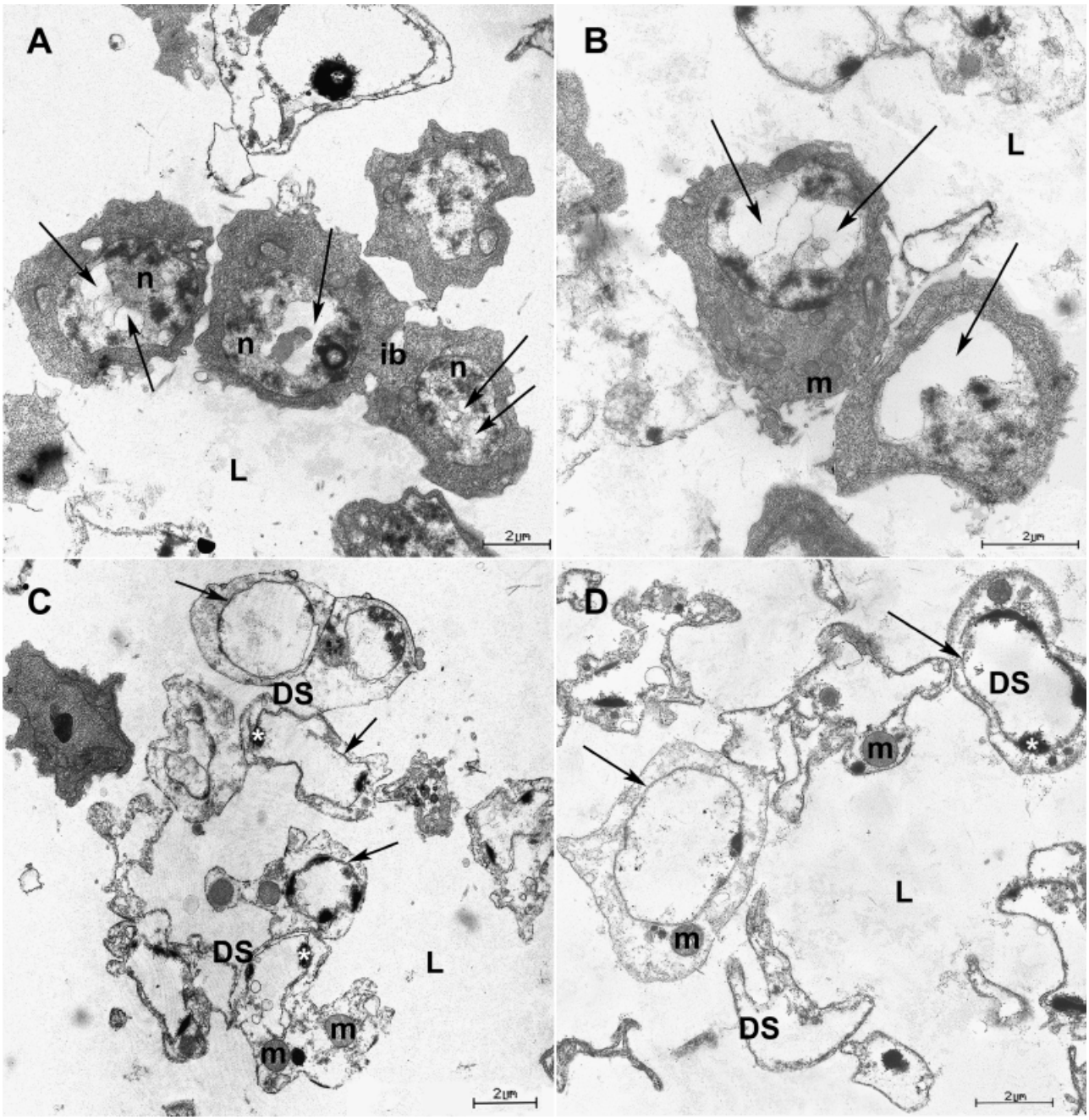

Fig. 4. A. Male gonad. Cell degeneration (arrows) starts inside the spermatid nuclei (n) (here still linked by the intercellular bridge [ib]), while the remaining cytoplasmic features do not appear to be involved with the degenerative process. Lumen (L). **B.** Cell degeneration (arrows) spreading to the entire nuclear volume of spermatids and mature sperm cells. The cytoplasm appears intact. Mitochondria (m). **C.** Degenerate sperm (DS) have lost their electron density, appearing devoid of the normal cytoplasmic components. Mitochondria appear enlarged and more evident. Chromatin blocks (*) are lying on the inner nuclear membrane (arrows). **D.** Completely degenerate sperm cells (DS).

be observed, the degeneration process, already described in branchiopods not belonging to the Spinicaudata, can be found in many cells.

In order to understand the number of cells involved in the degeneration, we counted the normal and degenerate germ cells, reconstructing entire sections of the gonad for different body regions. The percentage of degenerate sperm is substantial in five of the six males analyzed, ranging 55.2–91.6% with a mean of 71.3%. Only "male 2"

Table 1. Percentage of degenerate sperm cells found in males of *Eulimnadia texana.*

	Normal	Degenerate	Degenerate sperm (%)
Male 1	77	118	60.5
Male 2	91	53	36.8
Male 3	40	322	89.0
Male 4	24	260	91.6
Male 5	68	103	60.2
Male 6	90	111	55.2

has an overall low degeneration rate of 36.8% (Table 1).

In summary, the male gonad of *Eulimnadia texana,* like that in other conchostraca, presents a widespread parietal spermatogenesis. The sperm cell maturation does not involve either morphological transformation or apparently meiosis (i.e., absence of synaptonemal complexes) and takes place inside the gonad's lumen, notwithstanding evident degenerative stages.

Discussion

The male gonad of the conchostracan *Eulimnadia texana* appears organized exactly as in the lumen maturation model described by Wingstrand (1978) in the work on comparative branchiopod spermatology. The proliferative zone is a very thin wall, where gametogenesis occurs and where spermatocytes and spermatids are evident. Mature sperm cells are ameboid, with a few pseudopodia, a dense cytoplasm, and a few organelles, very similar to those described in other representatives of the order Branchiopoda. It was not possible to identify meiotic prophase stages, like synaptonemal complexes, as has been found in other branchiopods such as the notostracan *Triops cancriformis* (Scanabissi et al. 2005); only metaphase and anaphase were observed in the lumen.

The most remarkable finding that emerges from our observations is the unequivocal presence of many degenerating and degenerate gametes in the gonad lumen of *E. texana*. This is the first finding ever of such gamete degeneration in the clam shrimps (conchostraca), although similar morphological characteristics have been described in the non-clam shimp branchiopod literature, e.g., as described by Wingstrand (1978): "An unexpected feature is the high frequency of degenerating spermatozoa [...] such cells look empty, their plasm is reduced." He first found sperm degeneration in the notostracan *Lepidurus apus lubbocki,* which was further investigated by Scanabissi & Mondini (2002) in an Italian population. Even though there are a few papers on the ultrastructural morphology of male gametes in conchostracans (Scanabissi & Tommasini 1994; Scanabissi & Mondini 2002), such impressive cases of massive degenerative processes have never been described. Such degeneration affects only gametes in the gonad lumen and never those differentiating in the wall, and so do not represent fixation artifacts. The degenerative process starts inside the nucleus, spreads to the entire nucleus, and then gradually moves to the cytoplasm. In the end, all involved cells retained their normal size, but they exhibit only the plasma membrane, some residual hyaloplasm, and a few round mitochondria with a very dense matrix. The nucleus is surrounded by an evident membrane, which is strewn with chromatin blocks on its inner side. No increase in electron density linked to this process has been observed in the lumen.

The number of degenerate cells is striking (Table 1), and so different hypotheses can be proposed for this unexpected observation:

(1) Degenerate sperm cells could be used by the normal ones as a sort of vehicle/support like a "spermatophore," during emission from the gonad, even though on the male body no spermatophore-like structure has ever been observed. In fact, inside the male gonad no potential spermatophore-producing structure or specialized tissue, such as glands, has been observed. The presence of spermatophores in *E. texana* is cited only by Strenth (1977), even though he only reported it from "females" (which are now known to be hermaphrodites; Sassaman & Weeks 1993). The "spermatophore" presence only on the "female" body, and not on the male one, could be linked to a sperm ejection mechanism by the "female" gonad to avoid selfing. A similar scenario is described by Scanabissi & Tommasini (1994) and Scanabissi & Mondini (2000) for male gonads in individuals of the Leptestheriidae, where the low sperm cell production was probably due to the germ cell's transformation into a "spermatophore" fibrous matrix. Therefore, the degenerate sperm could be involved in the building of a primitive spermatophore-like structure.

(2) Production of trophic substances necessary to maintain the viability of the non-degenerate sperm cells. A similar case, typical of the Sertoli cells, characterizes taxa phylogenetically distant from Crustacea. Morphologically, the structure of the male gonad does not exhibit any specialization, like cells or tissue, involved in this process. Thus, the germ cell depletion, not followed by a significant increase in lumen electron density, could be a mechanism to promote the diffusion of nutritive substances to maintain viability in the residual sperm cells.

(3) The high frequency of degenerate sperm cells could be a result of inbreeding. Populations of *E. texana* are highly inbred, with the average proportion of offspring produced by selfing estimated to be 44–100% (Sassaman 1989; Weeks & Zucker 1999; Weeks et al. 1999). Such high rates of inbreeding have been associated with high inbreeding depression, resulting in reduced growth and survival in both male and hermaphroditic shrimp (Weeks et al. 1999, 2000a, 2001; Weeks 2004; Weeks & Bernhardt 2004). Inbreeding depression is also manifest via reduced egg production in hermaphrodites (Weeks et al. 2000a, 2001), but nothing is known about the effects of inbreeding on sperm cell production in males. It is entirely plausible that inbred males would also have decreased sperm production. The population of *E. texana* used for this experiment (WAL) has a selfing rate of ~65% (Weeks & Zucker 1999), which indicates that the majority of males would be inbred. It is quite possible that male 2 was an outcrossed male, and thus had normal sperm production, while all other sampled males were the products of selfing (Table 1). If inbreeding depression is the cause of the high rates of degenerate sperm, and the males exhibiting high levels of such degeneration can nevertheless successfully pair with hermaphrodites for mating, such low levels of viable sperm production may be the cause of the low levels of outcrossing observed in this population (~32%; Weeks et al. 2004).

(4) Autoregulative spermatogenesis mechanism: Once male gametogenesis has started along the entire wall, in a very short time the entire gonad volume can be filled up by gametes. Given that mating, and therefore the ejection of sperm cells, does not occur continuously throughout a male's life, it could be that the oldest sperm cells will gradually degrade in order to ensure space and viability for the newly produced sperm cells.

As a result of our observations, we plan to further study the hermaphroditic gonad in order to compare male gametes produced by hermaphrodites relative to those produced by males. These studies should identify any possible competition between the male and hermaphroditic sperm cells during fertilization, which is clearly a very strategic phase in the reproductive biology of *E. texana*.

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