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Ultrastructure studies on the male testis of milkweed bug, *Spilostethus pandurus* (Scopoli) (Hemiptera: Lygaeidae)

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Abstract
The growth zone, maturation zone and differentiation zone were the three developmental zones observed in the male testis of *Spilostethus pandurus*. In the growth zone, spermatogonia increase by cell mitosis to differentiate into spermatocytes. Two meiotic divisions take place in the maturation zone, and the spermatid differentiation takes place within the cysts. Spermatids grow and alter shape in the differentiation zone, resulting in the formation of spermatozoa. The greater part of the head region of the mature sperm is occupied by the nucleus. The axonemal complex, two mitochondrial derivatives, the reticular appendage, and a centriolar adjunct near to the axoneme constitute the tail region. The present study's objective is to use transmission electron microscopy to examine the reproductive morphology of male *S. pandurus*.

Keywords: male testis, *Spilostethus pandurus*, growth zone, maturation zone, differentiation zone, transmission electron microscopy

Introduction
The Hemiptera are a diverse and significant category of insects from an economic viewpoint. There are about 90 000 species in all climates, from the tropics to the arctic, and it has 75 families.
Because it contains both beneficial (predators) and harmful insects (pests), it is economically important. (Schuh & Slater, 1995) [34].
Hemipteran bugs are one of the dangerous pests that infest the seeds of numerous plants, and they have a long history in Egypt. (Schaefer & Panizzi, 2000 Meguid *et al*, 2013) [33, 25].
The Lygaeidae family is often phytophagous. Because they can grow into large populations that damage crops and harm productivity, many of them are regarded as agricultural pests.
In tropical and subtropical regions, the milkweed insect *Spilostethus pandurus* is common and may cause significant harm (Kugelberg, 1973) [20]. In recent years, their economic significance has expanded rapidly. Their ravages on vegetables and other crops result in annual losses that are occasionally enormous and immeasurable. Numerous crops are infested by it, including seeds of groundnuts, cotton, sorghum, sesame, black-eyed pea, tomato, eggplant, sugarcane, okra, pecans, wheat, and cabbage, as well as the sunflower seeds, water melon, squash, and cantaloupe (Thangavelu, 1979) [35].
On many hemipteran insect species' reproductive systems, there are numerous reports available. Reports on the Hemiptera male reproductive system's structure have been published (Wheeler & Krutzsch, 1992; Jahnke *et al*., 2006; Chapman, 1998 Klowden, 2007. Lemos, 2005 Pendergrast, 1956 Happ, 1992; Karakaya, 2012; Adams, 2001; Freitas *et al*., 2007, 2010).[36, 17, 6, 19, 21, 28, 16, 18, 1]. However, if the family Lygaeidae is not taken into account, all these conducted investigations are insufficient.
Despite *S. pandurus*'s economic significance, morphological research on the growth and maturity of its male reproductive system are uncommon.
The aim of the present study is to investigate the reproductive morphology of male *S. pandurus* by means of transmission electron microscopy.

Material and methods
Insect rearing
According to El-Sherif (1991) [10]. and Elelimy (2012) [8]. the colony of *S. pandurus* was reared in a laboratory and was fed dried sunflower seeds.

Transmission electron microscope (TEM) preparations
Detailed examination of ultrastructure of male reproductive systems was carried out on specimens taken from 5-days old adult.
Milkweed bug adult males were dissected in a drop of prefixation solution (2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.3), and kept for at least two hours at 4° C. Thoroughly rinsed overnight at 4° C in 0.1 M phosphate buffer. Postfixed in 1% osmium tetroxide and in 0.1 M phosphate buffer pH 7.3 for 1 hour at 4° C. Embedded in Epon followed by polymerization for 20 hr at 70˚C. Ultra-thin Sections were stained for 15 minutes with a saturated uranyl acetate solution and counterstained in lead citrate for 20 minutes (Reynolds, 1963). Sections were examined in a Joel JEM-1200 EX II transmission electron microscope.

Results
Ultrastructure of the male S. pandurus testis
Three developmental zones can be found in S. pandurus testicular follicles. Spermatogonia groups separate from the germarium and transform into spherical clusters in the growth zone (a) (Fig. 1). These cell clusters are surrounded by many cells that make up the sperm cyst's wall as shown in Figure (3). Figure (5) show the testis lined by a peritoneal sheath, an outer cellular epithelial layer and inner a cellular layer (tunica propria). Spermatogonia increase by cell mitosis to differentiate into spermatocytes (Figs. 1, 3 and 4). The shape of spermatogonia is not well defined (Fig. 1),

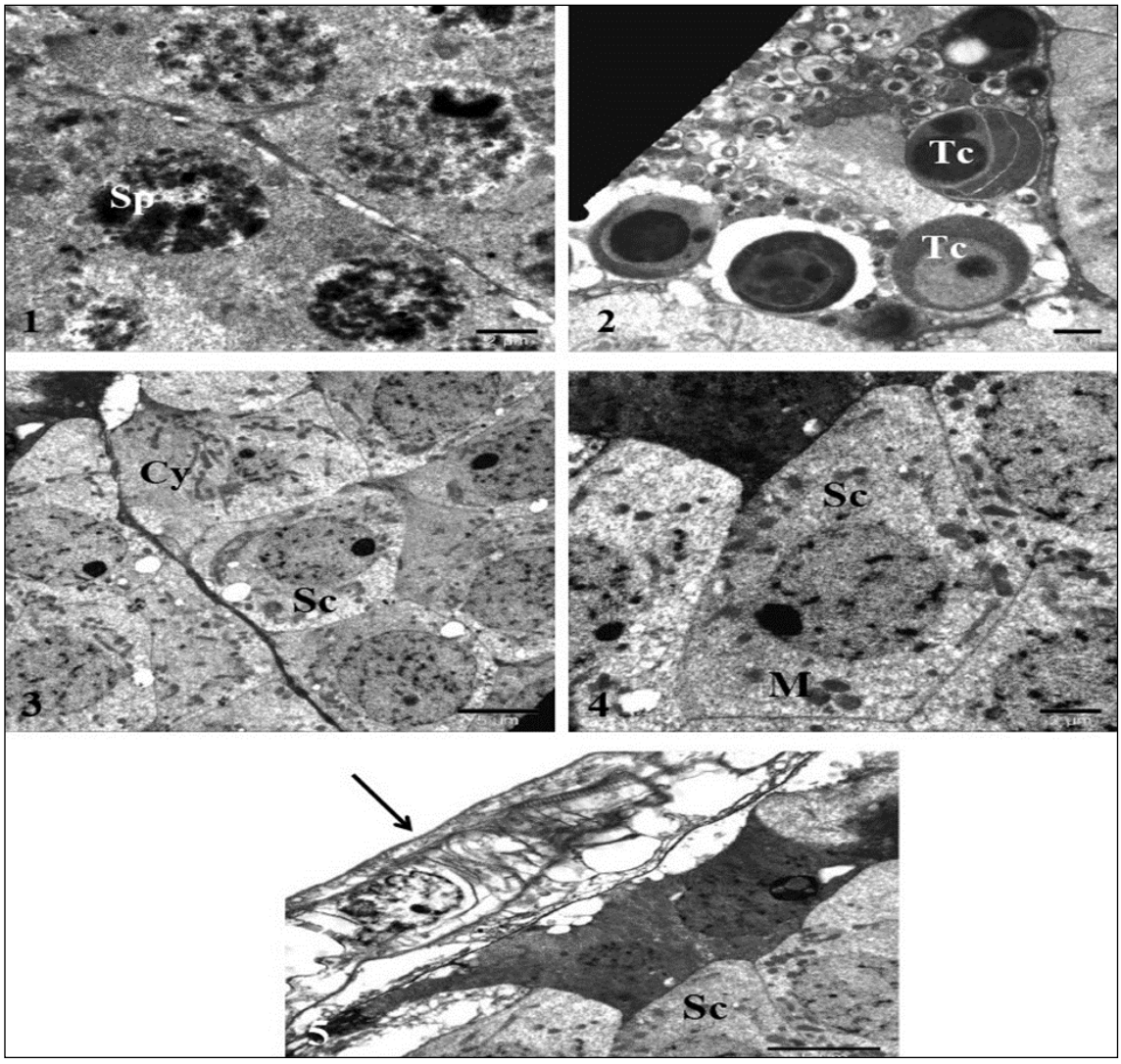
where they appear as large cells. The spermatogonia nuclei are more or less oval, occupying most of the cell, and heterochromatin forms clusters. The cytoplasm contains very small mitochondria, RER, and minute vesicular bodies. The cells are attached by means of vesicular points (Fig. 1). The trophocytes, which are large cells with irregular nuclei, are dispersed among the cysts as shown in Figure (2).

The spermatocytes constitute the sperm head and tail regions in *S.* pandurus' testicular follicles. The sperm head area contained the Golgi complex, mitochondria, euchromatin, and heterochromatin sections of the nucleus. The mitotic divisions are synchronous within a given cyst, aggregation of mitochondria to form 'nebenkern' and the presence of cytoplasmic bridges that result from incomplete cytokinesis in these divisions (Figs. 6 and 7). Figure (7) shows the nuclear envelope surrounded by a relatively dense group of microtubules, the chromatin becomes condensed and peripheral.

The differentiation of the spermatids takes place inside cysts (covered by a somatic cell) in the maturation zone (b), where two meiotic divisions take place. Inside these cysts, all spermatids are in the same stage of maturation (Figs. 6 and 8). Cell elongation, which includes flagellum formation and cytoplasmic sloughing, is a process of differentiation. From the previous zone, mitochondria become increasingly noticeable in the flagellum (Fig. 8).

Spermatids enlarge and alter their shape in the differentiation zone (c), where they develop into spermatozoa. The spermatozoa are housed in cysts that are bundled together and then released from the cysts (Figs. 9 and 10). The spindle-shaped head and long cylindrical structure flagellum are two easily distinguishable regions on the spermatozoa of *S.* pandurus (Fig. 10). The acrosome, nucleus, and Golgi complex make up the head of spermatozoa. The nucleus is elongated and dense. The nucleus occupies the majority of the head area (Figs. 9 and 10).

The axonemal complex, two mitochondrial derivatives, the reticular appendage, and a centriolar adjunct close to the axoneme make up the tail (Fig. 11). The axoneme (axial filament) and the mitochondrial derivatives are separated by an accessory sheath (Fig. 11). The axoneme is made up of (a) central tubules, (b) nine microtubule doublets, and (c) nine accessory tubules that are situated in gaps between consecutive doublets (Fig. 11). The matrix of the derivative is occupied by a paracrystalline material (Fig. 11).



Figs 1-5: TEM micrographs in growth zone of the male testis follicle of *S. pandurus.* Fig. 1. TEM micrographs of spermatogonia (Sp) showing junction between cysts and nucleus surrounded by small vesicles probably microtubules, chromatin condensed in the nucleus. Fig. 2. TEM micrograph of trophocytes cells (Tc) scattered among cysts. Fig. 3. TEM micrograph showing cysts (Cy) of spermatocytes (Sc). Fig. 4. TEM micrograph showing high magnification of spermatocyte (Sc) with numerous mitochondria (M). Figs. 5. TEM micrograph in growth zone showing spermatocytes (Sc) and layer of peritoneal sheath (arrow) which line the male testis of *S. pandurus.*

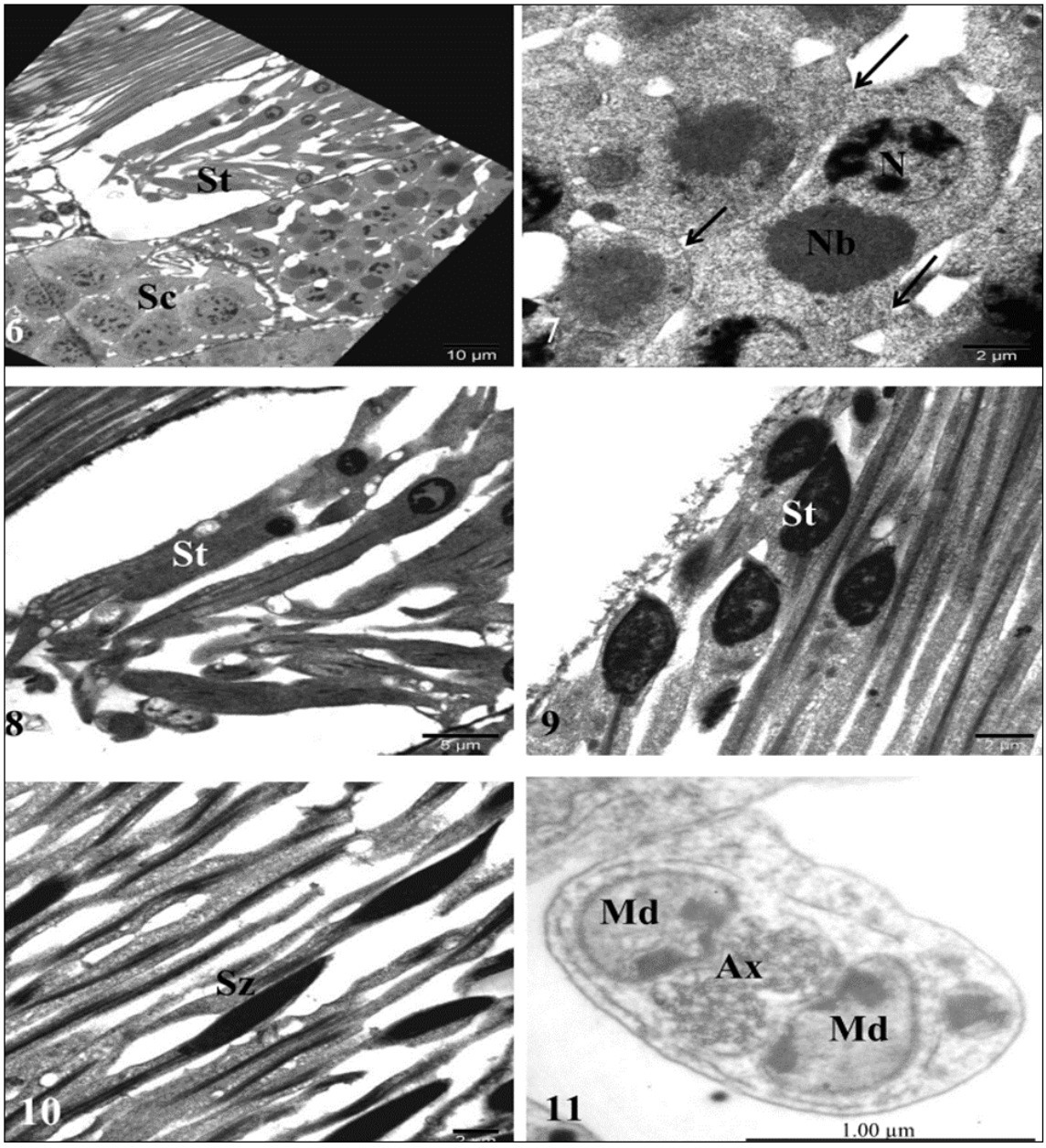


Fig 6: TEM micrograph in maturation zone where spermatocytes (Sc) differentiate to spermatids (St) inside cysts. Fig. 7. TEM micrograph showing nucleus (N) surrounded by microtubules, cytoplasmic bridges (arrows) during cell division in spermatocytes and mitochondrial 'nebenkern' (Nb) derivatives formation. Fig. 8. TEM micrograph of spermatids (St) in testis follicle. Figs. 9-10. TEM micrographs in transformation zone of male testis follicle where spermatid (St) change its shape forming spermatozoa (Sz). Fig. 11. TEM micrograph of sperm tail showing axoneme (Ax) surrounded by microtubules and mitochondrial derivatives (Md), matrix of the derivative is occupied by a paracrystalline material.

Discussion

Numerous insect species' male reproductive systems have been studied, and the histological, ultrastructural, and cytochemical examination of the organs' constituent parts has led to significant advancements (Forbes & Do-Van-Quy, 1965; Bairati, 1968; Louis & Kumar, 1971; Bahadur, 1975; Wheeler & Krutzsch, 1992; Ferreira *et al*., 2004; Mikheyev, 2004; Lemos *et al*., 2005; Freitas *et al*., 2007, 2010; Elelimy *et al*, 2017) [13, 4, 23, 3, 36, 12, 26, 21, 14, 15, 12, 17].

The testis in Hemiptera comprises simple follicles, enclosed within a thin epithelium, sometimes consisting of two layers of cells with a basal lamina (Chapman, 1998) [6]. A layer of cells (peritoneal sheath) and non-cellular layer (tunica propria) line the testis to enclose the testis follicles. Beneath the peritoneal layer a muscle layer surround the testes to enable the organ to perform its peristaltic movement.

The testis follicle typically consists of four regions: the germarium, zone of growth, zone of maturation, and zone of transformation, where the sperm develops in a series of maturational phases during the spermiogenesis process (Chapman, 1998) [6].

Three development zones can be found in *S. pandurus* testicular follicles. Spherical clusters of spermatogonia form in the growth zone, where they split from the germarium. Through mitosis, the quantity of spermatogonia increases, and they subsequently develop into spermatocytes. Two meiotic divisions take place in the maturation zone, where these cells develop into spermatids. Spermatids develop into spermatozoa in the differentiation zone.

The testes of *S.* pandurus contain sperm cells at various stages of spermatogenesis across the sperm tubes, according to the ultrastructure micrographs of this insect. In *S.*

pandurus, the somatic cell that forms the cyst encloses the spermatids that are located in the distal and medial regions of the testes, where they complete the differentiation process. These cells are free in the proximal region of the testes, close to the insertion of the seminal vesicle. Male Heteroptera have large cells with irregular nuclei scattered throughout the cysts of testicular follicle, known as trophocytes, according to Chapman (1998) [6]. He did not mention their function. These cells were never reported by previous authors.

Lygaeidae have same spermatogenesis mechanisms with other insect families, including sperm formation (Bowen, 1922; Davis, 1956; Engelmann, 1970; Chapman, 1998; Pires *et al*., 2007; Rodrigues *et al*., 2008) [5, 7, 11, 6, 29, 32]. Each species has a constant number of spermatids/spermatozoa per cyst, however there may be some variation between species. This number is regulated by the quantity of cell divisions. So, in the systematics of Hymenoptera, this number has been employed as extra information (Zama *et al*., 2007; Lino-Neto *et al*., 2008) [37, 22]. The spermatozoa's heads are embedded in the seminal vesicle's epithelial lining, as they are in many other Lygaeidae, while their tails spiral posteriorly into the lumen (Davis, 1956) [7].

In the zone of transformation, where the spermatids are transformed inside the cyst, the process of spermatogenesis takes place. When the diploid spermatocytes reach the zone of maturation, where each cell undergoes meiotic division to form haploid spermatids, they continue to proliferate inside the cyst (Mahmoud & Shoman, 2009) [24].

During the earlier stages of spermatogenesis the cell organelles of spermatocytes undergo various changes. Sperm morphogenesis reflects the peculiar features of a mature spermatozoon. The transformation process involves several morphological reorganization of the cell. Most insects' mature sperm is composed of a head and a flagellum. The nucleus occupies the majority of the head region. Acrosomes are seen in front of the nucleus, while axial filaments, or axonemes, appear behind the nucleus (Chapman, 1998) [6].

The sperm nucleus is usually elongated spindle shaped, pointed anteriorly and truncated posteriorly. During spermiogenesis the nuclear membrane becomes surrounded by microtubules. Also wide portions of the nuclear membrane form blabs that pinch off in vesicles and disperse into the cytoplasm. Transmission electron micrographs of *S. pandurus* showed clearly the chromatin in the nuclei of sprmatogonia become elongated and tangled. Microtubules surround the nuclei of spermatogonia.

According to Baccetti (1972) [2]. the microtubules compress and elongate the nucleus. The chromatin of the condensing nucleus becomes arranged in labyrinthic laminae.

Anteriorly to the nucleus lies the acrosome. Together they form the head of the sperm. The acrosomal material is derived from the pro-acrosomal granule, a structure secreted by Golgi apparatus.

The mitochondria in the mature spermatocytes become transformed to mitochondrial derivatives (Pratt, 1968) [30]. The mitochondria become numerous, fuse together, and undergo a series of rearrangements. Meiosis leads to an exact division between daughter cell, until the mitochondria back together into a skein. Then begins the metamorphosis of mitochondria into nebenkern. According to Pratt (1968) [30]. two extremely long identical, labyrinthically chondrioconts form.

These eventually lie parallel on either side of the axoneme. Gradually the excess of cytoplasm is eliminated within the spermatocytes.

The axoneme, which is the flagellum's microtubule-based cytoskeleton, one or two mitochondrial derivatives, and one or two accessory bodies, which are extensions of the centriole adjunct that forms a collar at the base of the flagellum, are all components of the flagellum (Nardi *et al*., 2013) [27]. The axoneme which originates from a basal body of a differentiated centriole is arranged in a wheel like structure.

The process of spermiogenesis in *S. pandurus* appears to be typical for insects (Elelimy *et al.*, 2017; Mahmoud & Shoman, 2009) [12, 17].

Conclusion

Within the testicular follicles of *S. pandurus* there are three developmental zones (growth zone, maturation zone and differentiation zone). This study also showed the different developmental stages of sperm maturation in male *S. pandurus*. Comparative studies on the anatomical and ultrastructural of different hemipteran insect species will be carried out in order to determine the different effects of diet on the maturation of sperms.

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