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Research Article

ULTRASTRUCTURAL STUDIES ON TESTES AND SPERMATOZOA OF BLISTER BEETLE MYLABRIS INDICA THUNBERG (COLEOPTERA: MELOIDAE)

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ABSTRACT

The testis and sperm of *Mylabris indica* (Thunberg) were studied histomorphologically using Transmission Electron Microscopy (TEM). On the basis of morphological observations of the testes of *M.indica*, the following structural organizations have been examined: the structure of a pair of testis contained a number of yellowish testicular follicles which are arranged in radials like that of clustered apple. Each testicular follicle is separated into connective tissue similar to that of higher vertebrate animals. Testicular follicles contained a number of small and large size cysts and different stage of germ cells. The spermatozoa develop and mature within the small cyst (each small cyst contain odd number (3 to 9 germ cells)), after maturation, the spermatozoa move to organized to large cyst and each large cyst contained 250 and more number of 7-13 sperm nest. The sperm consisted of a head (nucleus and acrosome) and flagellum (it consist two mitochondrial complex, axoneme 9+9+2 pattern, a pair of accessory bodies and centriolar adjunct was observed near axoneme). The more developmental characters such as structural organizations of testis and spermatozoa of *M. indica* are helpful to trace the phylogenetic evolution of insect species.

INTRODUCTION

The reproductive organs provide a very wide range of variations exists in different insect groups. In their embryonic condition they are similar in both sexes, but variations later found in developmental stages. In male, as a rule, the pair of testes is more or less ovoid, partly or completely separated into a variable number of testicular lobes or follicles and there are so many differentiations in their form and arrangement among different insect species. In coleopteran insects, the peritoneal membrane is covered over the testis as a whole in a common coat or scrotum which is frequently pigmented. The internal organs of reproductive system of the male insect are deceptively simple in their basic plan and consist of testes, vasa diferentia, seminal vesicle, accessory glands and ejaculatory duct.

Morphologically, the testes have a number of testicular follicles in which spermatozoa are produced, with different lengths among families, genera and species (Freitas *et al.*, 2010; El-Bokl *et al.*, 2014; Nurcan ozyurt *et al.*, 2014). Sperm of insects show a great diversity of external shapes and size. The basic architectural feature and specific variation on the basic plan of insect sperm have proved to be useful characters in the study of insect physiology (Nardi *et al*., 2013).

The genesis and development of germinative cells in coleopteran, as in the majority of insects, takes place within cysts, contained in the testicular follicles (Phillips, 1970). In coleopteran, the structural organizations of spermatozoa are characterized by number of accessory bodies, large electron dense fully crystallized mitochondrial derivatives and axoneme (9+9+2 pattern) in the tail region. The head framed by two or three layered acrosome and condensed nucleus (Baccetti and Daccordi, 1988; Almeida and Cruz-Landium, 2000; Nane *et al.*, 2007; Simmons, 2011; Alzahrami *et al.*, 2013; El-Bokl *et al.*, 2014). Variations from this type do occur, predominately in size or number of organelles such as the mitochondrial derivatives and accessory bodies (Baccetti and Daccordi, 1988; Burrini *et al*., 1998; Nane *et al.*, 2007; Alzahrami *et al.*, 2013).

The male reproductive organs of the Meloidae are in more complex than the other families of the coleoptera. This complexity is apparent in the structure of the testicular follicles and in the presence of elaborate and more coiled two ectodermal accessory glands, in addition to more elongated mesodermal accessory glands.

The information about the histological structural features of the testes of meloidae is in scarce. No data available in the literature about the ultrastructure of male gonads or about the spermatogenesis of this pest. Such information might be valuable in finding a suitable insecticide against this pest.

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Hence, the present study is focused to describe the histomorphology of testes and spermatozoa of *Mylabris indica* (Thunberg) by means of Transmission electron microscopy (TEM).

MATERIALS AND METHODS

Selection of experimental animal

The insect used in the present investigation is *Mylabris indica*, coleopteran blister beetle of the family Meloidae. It can be easily maintained in the laboratory at normal temperature and humidity. It is very convenient for dissection as the size of the animal is somewhat larger.

Transmission Electron Microscopic (TEM) studies

For transmission electron microscopy (TEM), the glands were fixed in Karnovsky solution (2% glutaraldehyde plus 4% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4) for 2 h and post-fixed in 0.5 per cent osmium tetroxide containing 0.8 per cent potassium ferrocyanide in the same buffer. Dehydration was done using an increasing acetone series after which the samples were embedded in Epon-Araldite resin that was polymerized at 60°C for 24h. The sections were stained with uranyl acetate for 45 min and lead citrate for 10 min (Reynold, 1963) prior to examination in a Philips Transmission Electron Microscope operated at 80 kv, at Christian Medical College (CMC), Vellore.

RESULTS

The morphology of testes in adult male reproductive system of *M. indica* appeared to be yellowish, custard apple like structure. The testis were located on either side of the mesenteron, each testes composed of number of completely separated follicles covered by a thin follicular membrane. They were tubular, radially arranged and entire structure within a crystalline like layer of a peritoneal connective sheath with yellow pigmentation (Figs.1).

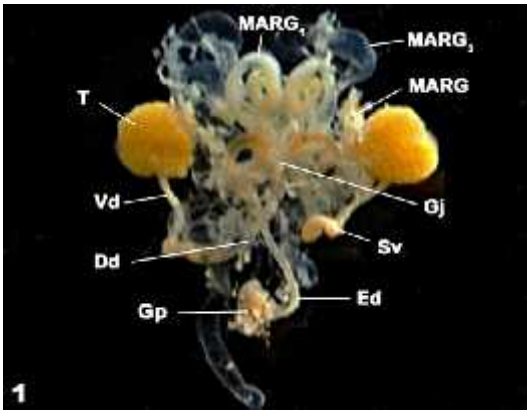


Fig.1 Anatomy of the reproductive system of *Mylabris indica* (Dd-Diferent duct ; Ed- Ejaculatory duct ; Gj- Grand junction ; Gp- Gonopore ; $MARG_1$ -Male accessory reproduction gland 1; $MARG_2$-Male accessory reproduction gland 2; $MARG_3$- Male accessory reproduction gland 3; Sv-Seminal vesicle ; T- Testes ; Vd- vasa deferens).

The peripheral portion of testes was covered by various muscular cells that accompany the basal lamina (Fig. 2). The epithelial cells of the peripheral portion had many folding in their basal surface, with many mitochondria and a well-developed rough endoplasmic reticulum (rER) in the basal cell (Figs. 3-5). Many vesicles were identified similar to a smooth endoplasmic reticulum (sER) near the nucleus (Fig. 4). The nuclei of the epithelial cells were round or oval and located in the center or sometimes towards the peripheral region (Figs. 2-4).

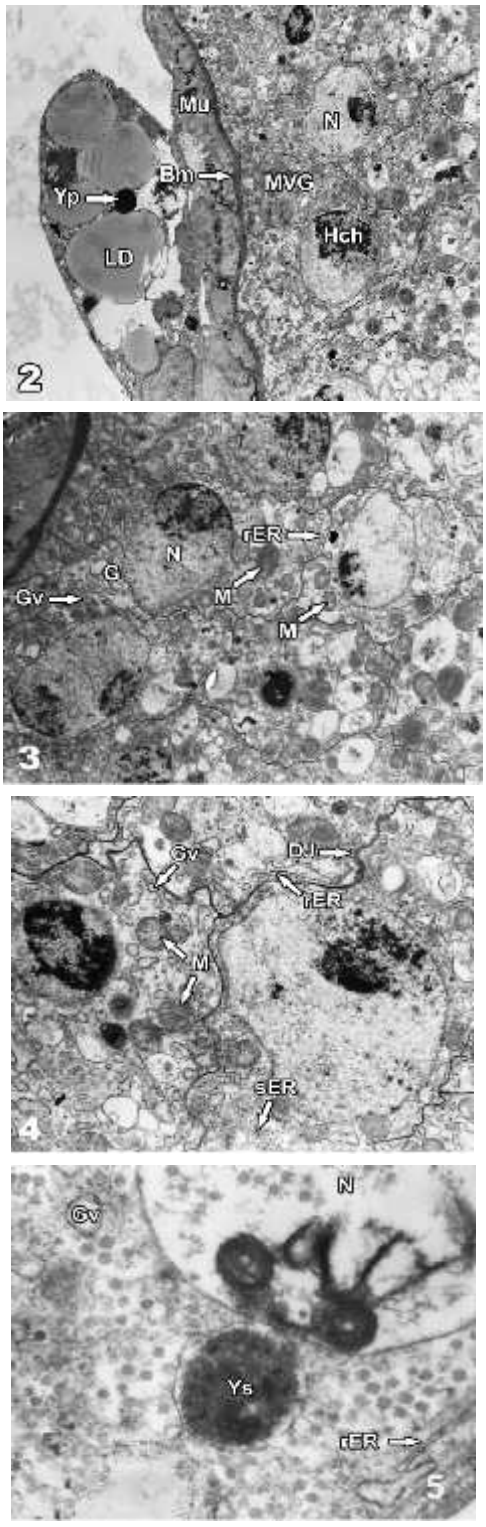
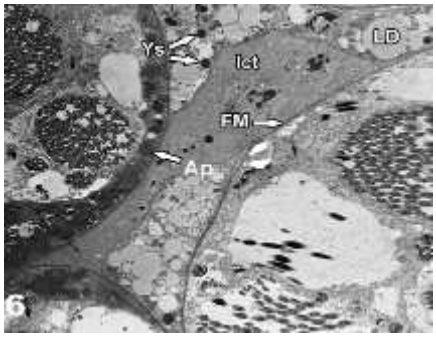
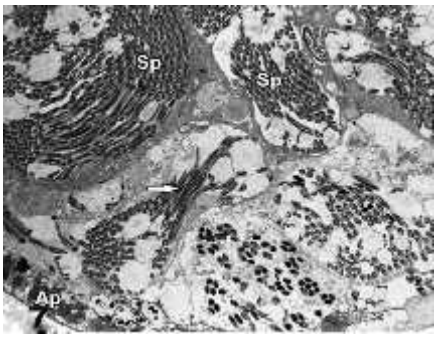
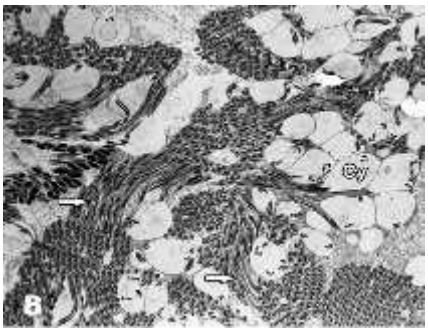


Fig.2-5 Transmission electron micrograph of peripheral portion of testes follicle showing epithelial cells Fig.2. X 3000; Fig.3. X 4500; Fig.4. X 10000; Fig.5. X 30000 (Bm-Basement membrane; DJ-Desmosome junction; Gv-Golgi vesicle; Hch-Hetero chromatids; LD-Lipid droplets; M-Mitochondria; Mu-Muscle; MVG-Multivesicular granules; N-Nucleus; rER-Rough endoplasmic reticulum; sER-Smooth endoplasmic reticulum).







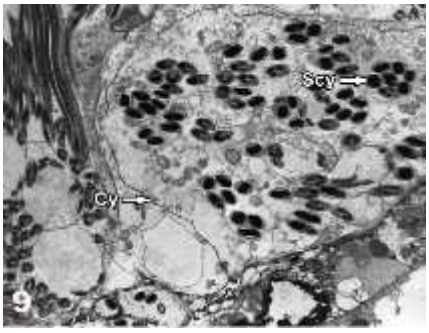


Fig.6-10 Transmission electron micrograph of testes follicle showing the cyst with germ cells Fig.6. X 4500; Fig.7 and 8. X 2000; Fig.9 and 10. X 4500 (Ap-Apical cells ; Cy-Cytoplasm; FM-Follicular membrane; Ict-Interstitial connective tissue; LD-Lipid droplets; Scy-Small cyst; Sp-Spermatozoa; SpN-Spermatozoan nest; →- Migration of spermatozoa from small cyst to form spermatozoan nest).

The epithelial cells showed numerous interdigitations along the cell wall and many desmosomes in the apex region (Fig. 4).

The secretory portion had a large number of secretory vesicles distributed all over the cell. The cells showed interdigitations between them and some microvilli on their free surface in relation to the intermediary portion. Several vesicles were observed being liberated by the cell towards the intermediary portion. A large number of mitochondria and a well-developed rER and Golgi complex were observed all over the cytoplasm. Many myelinic figures were also noted in their cytoplasm near the nucleus.

The ultrastructural section of testes showed the presence of fewer amounts of apical cells. They were actively engaged in synthesis and secrete their nutritive substances for the development of primary and secondary spermatocytes. The initial stages of apical cells with numerous mitochondria began its synthetic and secretory activity contained more quantity of glycogen, protein and fewer amounts of lipids were found (Fig. 6). Each follicular tube consisted with many cysts and germ cells. The occurrence of more vacuoles in terminal part of the testicular tube, the nutritive substances may be utilized for the development of germ cells (Figs. 7 and 8).

Depending on the status of mitosis, the spermatogonia were divided into primary and secondary phases. Primary spematogonia were the largest cells and the ultrastructure of the secondary spermatogonia nucleus and cytoplasm were similar to that of the primary spermatogonia. The secondary sermatogonium became a spermatocyte. The primary spermatocytes were much smaller in size and the secondary spermatocytes transferred into early spermatids. Due to meiotic division, the size of the cells reduced once again and development of the flagellum was the most important alteration in the morphology of spematids. (Figs. 9 and10). Now cyst contained more vacuoles and they might have been utilized the nutrients energy source for the developing sperm (Figs. 9 and 10).

During the early spermatid stage, the nucleus more or less same likes that of somatic cells. It showed dispersed chromatin with some electron dense areas. The proacrosomal vesicle which derived from the Golgi complex was observed near the nuclear envelope (Fig. 11). The nuclear envelope exhibits a more electrondense appearance than that of its other regions. Simultaneously, a large number of mitochondria fuse together forming the mitochondrial complex.

During intermediary stages of spermatic maturity, the nuclear condensation process begins with an increase electron density. In this process, the region near the nuclear envelope having homogeneously condensed chromatin can be distinguished from the central region of the nucleus with a fibrillar aspect (Fig. 11).

The germ cells (spermatozoa) got matured in the various small cysts, and each cyst contained 3 to 9 cells (only odd numbers) (Fig. 9). After transformation of the spermatozoa, it was moved to exact place and form 7 to 13 spermatozoan nests and each nest contained approximately 250 sperms (Fig. 10).

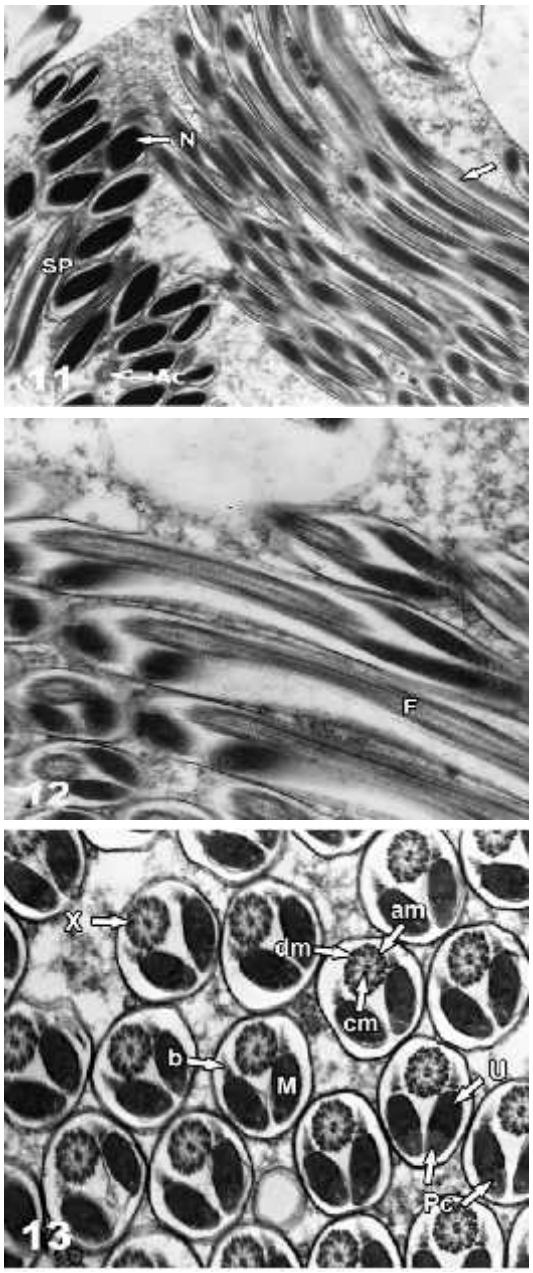


Fig.11-13. Transmission electron micrograph of testes follicle showing the spermatozoa Fig.11. X 10000; Fig.12. X 30000; Fig. 13. X 45000 (Ac-Acrosome; am-accessorymicrotubules; b-Accessory bodies; cm-Central microtubules; dm-Doublets microtubules ; F-Flagellum; M-Mitochontrial derivatives; N-Nucleus; Pc-Para crystalline materials; Sp-Spermatozoa; X-Axoneme; →-Flagellum).

The spermatozoa of *M.indica* were highly differentiated cells and consisted of two morphologically and functionally distinct regions: the head region formed by an acrosomal complex and the nucleus (Figs. 11) and the flagellar region that includes an axoneme, a centriolar adjunct, a pair of more or less symmetrical mitochondrial derivatives and a pair of accessory bodies (Figs. 12). Transverse sections of the head region show a oval and tri-layered acrosome (Fig. 11) followed by the nucleus lateral to the paracrystalline structure. In flagellum, axoneme and two mitochondrial derivatives were organized in left and right side laterally. The triangular shaped accessory bodies were observed in transverse sections of the flagellum (Figs. 13). These stuctures were located at the distal part of mitochondrial complex. The axoneme revealed the typical 9+9+2 microtubule pattern, where the nine single accessory microtubules were the most external followed by nine doublets and a central pair (Fig. 13).

DISCUSSION

Anatomy of testes

The anatomical and morphological studies on male reproductive system has been made in several orders of insects, resulting in considerable improvements with its histological and ultrastructural studies of the structures that make up this organ for reproduction (Mikheyev, 2004; Ferreira *et al.*, 2004; Wieczorek and Swiatek, 2008, 2009; Freitas *et al.*, 2010). Testis of male *M. indica* is typically with completely separated numerous testicular follicles and all the follicles are arranged into a ball like structure which are connected by peritoneal connective tissue. This type of morphological characters of testis is reported to other meloidae insects particularly *M. operta* var. *bioculata* (Vorgelest von and Nikbakhtzadeh, 2004; Nikbakhtzadeh *et al.*, 2007), but the number of testicular follicles varies among other groups. The unpaired testis has been observed in *Eriosoma lanigerun* (Gautam, 1994), some coleopteran species (Chapman and Davies, 2004).

Ultrastructure of testis

Testis of *M. indica*, the testicular follicle was filled with germ cells which could be differentiated into different zones, depending upon various stages of their development. The zones of development can be organized as zone of germanium which consisted of apical cell complex and primordial germ cells; zone of growth where spermatocytes were formed; zone of maturation the primary and secondary spermatocytes were produced; zone of transformation where spermatids enclosed in a cyst and get converted into flagellated spermatozoa. The continuous production of spermatozoa in these species showed the presence of cysts in difference zones of spermatogenesis along the length of the testicular tubes. This pattern is normal for the majority of insect groups (Zama *et al.*, 2007; Moreia *et al.*, 2008;; Winnick, 2009; Wieczorek and Swiatek, 2009) including coleopteran (Nane *et al.*, 2007; Freitas and Fontanetti, 2008; Freitas *et al.*, 2010; Simmons, 2011; Alzahrami *et al.*, 2013;).

On the other hand, the number of sperm contained in each cyst varies in accordance to the number of mitotic events and the variability of the spermatids in initial spermatogenesis. In the testicular follicles the organization in the form of cysts is a common feature for coleopteran insects. However in *M.indica* it was observed that each large cyst contained a number of small cysts with 3-9 germ cells and the mature spermatozoa migrate to form 7-13 spermatozoan bundles, each bundle have approximately 250 spermatozoa. The most coleopteran specialized groups, tend to have the least number of sperm per bundle, especially Alticid beetles (16-256 sperm/bundle), Scarabaeid (128-512 sperm/bundle) and Curculionid (approximately 256 sperm/bundle) (Virkki, 1969 and Name *et al.*, 2007).

The present study TEM revealed that the testes of *M. indica* consisted a number of testicular follicles and all the follicles were covered with peritoneal sheath and for the first time it was

observed that the presence of interstitial connective tissue which connects the follicles. In the present study, the apical portion of follicles has been observed with apical cells which are support to provide nourishment for developing germ cells. Similar apical cells and their nourishment function have been reported in heteropteran insects viz. *Tessartoma javanica* (Shamala devi, 1980), *Spherodema rusticum* (Umamaheswari 2005).

During spermiogenesis, the spermatids undergo specific morphofunctional modifications which involve acrosomal formation, nuclear elongation, chromatin condensation, and are accompanied by flagellar development, with axoneme and mitochondrial derivative formation. These events follow the general pattern described for insect groups (Bao, 1996, Bao *et al*., 1997; Dallai *et al*., 2004; Name *et al*., 2007).

The elongated spermatozoan with axoneme framed by 9+9+2 microtubules, two mitrochondrial derivatives with two accessory bodies and one centrioler adjunt observed in *M.indica* attest the earlier findings in various coleopteran insects (Almeida and Cruz-Landium, 2000; Bao *et al*., 2004; Nane *et al*., 2007; Simmons, 2011; Alzahrami *et al*., 2013).

The participation of Golgi complex in acrosome formation has been reported for some insects (Fernandes and Bao, 2001). In the greater part of the insects where this process has been studied, the proacrosomal vesicle, found in early spermatids, is gradually modified, taking on a characteristic elongated shape in the last stages of spermiogenesis (Ndiaye and Mattei, 1992). Sperm nuclear development is characterized by a change from a spherical to an elongated shape. The nuclear material passes through a stage of conversion from a loose to a more compact form. This structural reorganization and condensation of nuclear material is related to biochemical substitutions. (Loir and Lanneau,1984; Mello, 1987). The acrosome of M.indica displays three layered, similar to that of other coleopteran *Sitophilus zeamais* (M) and *S.oryzea* (L) (Name *et al*., 2007) and *Rhynchophorus furrugineus* (Alzahrami *et al*., 2013). The acrosome, basically made up of a paracrystalline material, has been described for the symphytan *Xyela julii* (Newman and Quicke, 1999a) and who has pointed; the acrosome has a distinct paracrystalline core with a ridge that runs down one side of the acrosome. This ridge itself contained a particulate matter. It reveals granules surrounded by membranes resembling the multilayered coated complex that surrounds the acrosome itself. The acrosome loses the ridge at its posterior end, when it contacts the nucleus and may even be partially enclosed by it.

Despite these exceptions, the acrosome in other insect orders is made up of an acrosomal vesicle and a perforatorium (bilayered pattern) (Lino-Neto and Dolder, 2001a ; Zama *et al*., 2001, 2004, 2005a: Bao *et al*., 2004; Badke *et al*., 2005; Fiorillo *et al*., 2005; Mancini *et al*., 2006) or includes an additional third layer, an extracellular sheath, which covers all the acrosomal vesicle and part of the nucleus. This structure is typical in many coleopteras (Baccetti and Daccordi, 1988; Burrini *et al*., 1988; Name *et al*., 2007; Simmons, 2011).

During spermatid-spermatozoon differentiation, numerous cytoplasmic microtubules are involved in the shaping of the cell, and these microtubules are eliminated simultaneously within the cytoplasmic remains at the end of spermiogenesis. Microtubule participation in spermiogenesis has been described for some invertebrates (Jamieson *et al*., 1999; Chawanji *et al*., 2007), indicating that they may play an important role in the spermiogenesis process.

In *M.indica* development of flagellum has the same morphological pattern as that described for other coleopteran, which involves the formations of axoneme, two mitochondrial deverivatives, a pair of accessory bodies (Gassner at al., 1975). In *M.indica*, the spermatozoan flagellum possesses two axoneme more or less right and left side and each mitochondrial derivative are crystallized and similar size and shape. In this context, the similar observations have pointed out by (Bao and de Souza, 1993). In other hand, the most of the coleopteran and other insects groups possesses two mitochondrial derivatives that are unequal size, with one usually extending further posteriorly or anteriarly then the other (Thomson and Blum, 1967), bigger mitochondrial derivatives is almost long as the axoneme (Name *et al*., 2007) and two unequal mitochondrial derivatives are evident, with advanced crystallization being observed in the larger one (Alzahrani *et al*., 2013).

In *M.indica* the arrangement observed in the axoneme with 9 + 9 + 2 microtubules were the pattern for other coleopteras with the central microtubules and the nine doublets terminating first, followed by the accessory microtubules (Almeida and Cruz-Landium, 2000; Bao *et al*., 2004; Nane *et al*., 2007; Simmons, 2011; Alzahrami *et al*., 2013). This common feature coleopteras spermatozoon is contrast in the family Rhynchitidae (Coleoptera), which appear extremely place apart, because its have a particular features “9+9+0” pattern of axoneme and show, moreover, a limited degree of asymmetry in the tail organelles (Brurini *et al*., 1988).

In *M.indica*, the flagellum has two accessory bodies of equal sizes that are adjacent to the axoneme and connected to the distal part of the mitochondrial derivatives. This pattern was quietly dissimilar to other coleopteran (Name *et al*., 2007). However, the flagellum of *Coelomera lanio* and *Cerotoma arcuata* (Coleoptera: Chrysomelidae) have a single accessory body (Bao, 1996; 1998), differing from the majority of coleopterans, which have a pair of accessory bodies (Baccetti and Daccordi, 1998).

The structure of the *M.indica* spermatozoa is similar to the general description for insect sperm (Lino-Neto and Dolder, 2001a; Zama *et al*., 2004, 2005a, b; Bao *et al*., 2004; Fiorillo *et al*., 2005; Name *et al*., 2007; Simmons, 2011; Alzahrani *et al*., 2013), but dissimilar to arrangement of mitochondrial derivatives and accessory bodies size and shape and orientation. These structural changes and important features have been used for further phylogenetic and reproductive biology studies in meloid beetles.

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