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Bolua turkiyae (Orthoptera, Tettigoniidae)'nin Testis ve Yardımcı Bezlerinin Morfoloji, Histoloji ve Sitolojisi

Irmak POLAT[1*], Damla AMUTKAN MUTLU[2], Zekiye SULUDERE[2]

Öne Çıkanlar:
- *Bolua turkiyae*'nin testis ve yardımcı üreme bezlerinin yapısı araştırıldı
- Işık ve elektron mikroskop teknikleri kullanıldı
- Testis ve bezlerin yapıları önceki çalışmalardaki türlerle karşılaştırıldı

Anahtar Kelimeler:
- Endemik tür
- Böcek
- Elektron Mikroskopi
- Morfoloji
- Üreme Sistemi

ÖZET:

Böceklerin dış ve iç organlarının yapısını araştırmak için dünyada birçok morfolojik ve histolojik çalışma bulunmaktadır. Bu çalışmalar sadece böcek türlerinin biyolojisini aydınlatmaya değil, aynı zamanda taksonomik ilişkileri ortaya çıkarmaya da hizmet etmektedir. Bu amaçla, bu çalışmada *Bolua turkiyae* Ünal, 1999 (Orthoptera: Tettigoniidae)'nin erkek üreme sisteminin ana organları olan testis ve yardımcı bezlerin detaylı yapısı ışık mikroskobu, taramalı elektron mikroskobu ve transmisyon elektron mikroskobu teknikleri ile 2019-2020 yıllarında Gazi Üniversitesi, Prof. Dr. Zekiye Suludere Elektron Mikroskop Merkezinde araştırılmıştır. *B. turkiyae* Türkiye'de Bolu İli çevresinde yayılış gösteren endemik bir türdür. Erkek *B. turkiyae* üreme sistemi testis, vas deferens ve yardımcı bezlerden oluşmaktadır. Oval ve sarı testislerin kesitleri incelendiğinde çok sayıda folikül içerdiği gözlenmiştir. Bu foliküllerde spermin gelişim aşamalarını içeren kistlere sahiptir. Foliküllerin distal uçlarından proksimal uçlara kadar sırasıyla germarium, büyüme bölgesi, olgunlaşma bölgesi ve transformasyon bölgesi bulunur. Bu aşamalarda spermatogonium, spermatosit, spermatidler ve son olarak spermatozoa oluşur. Yardımcı bezler, iki farklı boyut ve çapta içi boş boru şeklindeki yapılardır. Her iki yardımcı bez grubunda da tek katmanlı kübik veya piramidal hücreler lümeni çevreler. Bu yeni verilerin, *B. turkiyae*'nin erkek üreme sisteminin yapısı hakkındaki bilgilerimize katkıda bulunacağı umulmaktadır.

Morphology, Histology, and Cytology of the Testis and Accessory Glands of *Bolua turkiyae* (Orthoptera, Tettigoniidae)

Highlights:
- The structure of the testis and assisted reproductive glands of Bolua turkiyae was investigated
- Light and electron microscope techniques were used
- The structure of testis and glands were compared with the species in previous studies

Keywords:
- Endemic species
- İnsect
- Electron Microscopy
- Morphology
- Reproductive System

ABSTRACT:

There are many morphological and histological studies in the world to investigate the structure of the external and internal organs of insects. These studies not only serve to illuminate the biology of insect species, but also to reveal taxonomic relationships. For this purpose, the detailed structure of the testis and accessory glands that are the main organs of the male reproductive system of *Bolua turkiyae* Ünal, 1999 (Orthoptera: Tettigoniidae) was searched with a light microscope, scanning electron microscope, and transmission electron microscope techniques in 2019-2020 in Gazi University, Prof. Dr. Zekiye Suludere Electron Microscope Center in this study. *B. turkiyae* is an endemic species distributed around Bolu province in Turkey. The reproductive system of male *B. turkiyae* is comprised of testis, vas deferens, and accessory glands. When the cross-sections of the oval and yellow testis were examined, it was observed that they contain many follicles. There are cysts in these follicles that have the developmental stages of the sperm. From the distal ends of the follicles to the proximal ends, the germarium, growth zone, maturation zone, and transformation zone are located respectively. In these stages, spermatogonium, spermatocyte, spermatids, and finally spermatozoa are formed. Accessory glands are hollow tubular structures of two different sizes and diameters. Single-layered cubic or pyramidal cells surround the lumen in both groups of accessory glands. It is hoped that these new data will contribute to our understanding of the structure of the male reproductive system of *B. turkiyae*.

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INTRODUCTION

Today, insects are a fairly large group due to making up about 75% of all animal species and their reproductive capacities and abilities (Aslan, 2018; Kotzé et al., 2019). Therefore, insects have significant affect on the ecosystem (Kotzé et al., 2019). Some species are described as beneficial, while others can cause great harm. Orthoptera order is a large group that includes species that can cause various damages in agricultural areas with sudden increases in their population. There are about 734 species of Orthoptera in Turkey. Approximately 370 taxa of this number belong to the Tettigoniidae family (Demir, 2015; Polat, 2016; Ünal, 2019). There are many studies on insects with a wide range of species and distribution areas (Viscuso et al., 2012, 2015; Jones et al., 2013; Silva et al., 2018). In biological control against harmful species, knowing the biology of the relevant species affects directly the success of the struggle. By knowing the histological and ultrastructural structures of the reproductive systems of insects in detail, it can be ensured that the population of these species can be brought under control before it reproduces. In addition, structural differences in reproductive systems can be used as taxonomic characters (Liu et al., 2005; Polat et al., 2019).

The reproductive organs of male insects are generally testis, vas deferens, accessory glands, seminal vesicles, a pair from each, and an ejaculatory duct (Nandchahal, 1972; Happ, 1992; Liu et al., 2005, 2017; Vitale et al., 2011, 2015).

Testis is the central region where sperms are produced with various numbers of insects follicles. The number of follicles where the spermatogenesis occurs can reach up to about 300–400 in Orthopteran males. Follicles comprise numerous cysts characterized by spermatogenesis and spermiogenesis at different stages, from spermatogonia to spermatodesm. The connective tissue covers the follicles from the outside and holds them together (Nelsen, 1931; Bishop & Lawson, 1965; Liu et al., 2005; Sottile et al., 2010; Viscuso et al., 2012; Jones et al., 2013; Polat, 2016; Polat et al., 2019).

The accessory glands play some crucial roles in the reproductive system in insects, such as regulating sperm activity, formation of spermatophores, production of mating plugs, alterations on sperm bundles, promoting female to oviposition, and preventing females from accepting other males (Viscuso et al., 2001; Colonello & Hartfelder, 2005; Braswell et al., 2006; Marchini et al., 2009; King et al., 2011; Lyu & Hua, 2017; Polat et al., 2019, 2020).

Along with the functions of the testis and accessory glands, some qualifications of them, such as shape, size, number, and the area where they connect to the reproductive system can vary among different insect groups (Liu et al., 2005; Gomes et al., 2012; Marchini et al., 2012; Dallai et al., 2014; Gullan & Cranston, 2014; Lyu & Hua, 2017; Polat et al., 2019, 2020). Besides, sperm structure and morphology in insects also show great diversity (Kotzé et al., 2019). Therefore, these features can be taxonomical characters (Liu et al., 2005).

Given the very little data known on the structural and ultrastructural features of the reproductive biology of Orthoptera, it can be said that further work is required on this subject. *Bolua turkiyae* Ünal, 1999 (Orthoptera: Tettigoniidae) is an endemic species distributed around the province of Bolu in Turkey (Ünal, 1999). Since there is no study on the structure of the male reproductive system or testis, which is the main organ of this endemic species in our country, the main goal of this study was to, for the first time, describe the testis and its auxiliary parts called accessory glands of the reproductive system in *B. turkiyae* males. For this purpose, we examined the detailed structure of the testis and accessory glands of *B. turkiyae* with scanning (SEM), transmission (TEM) electron microscopes, and a light microscope (LM).

MATERIALS AND METHODS

Collection and Laboratory Production of *B. turkiyae* Individuals

In this study, adult males of *B. turkiyae* (identified by Prof. Dr. Mustafa ÜNAL) were used. Ten mature individuals were collected from cultivated areas and wild plants around Gölköy area of Bolu province between May and September, 2019. No special permits were necessary when collecting the specimens of *B. turkiyae*. Insects brought to the laboratory environment were fed by branches, shoots, leaves, etc, from the plants were caught on in 1-1.5 L pet containers. No ethics committee permission is required for invertebrate animal experiments in our country.

Dissection of Testis and Accessory Glands Samples

For taking samples, male insects are first anesthetized with ethyl acetate. Then, the testis and related accessory glands were removed from the males in a physiological solution under the stereomicroscope (Leica M125) in the laboratory. Total images were transferred to digital media via a stereomicroscope. Some of the testis and accessory glands were prepared for SEM, some for TEM, and others for the examination under LM.

Preparation of Samples for the LM Examinations

For LM preparation, samples were first washed with distilled water to remove the surrounding tissues and hemolymph on the tissues. Then, they were fixed with 10% formaldehyde for 24 h. Following washing under running tap water, the tissues to remove from the fixative solution, and dehydration processes were performed. For this, the samples were passed through the ascending series of ethanol (%70, %80, %90, and %100) for five minutes each. The samples were blocked in paraffin after clearing in xylol. Sections taken with a microtome at a thickness of 5-6 microns were turned into permanent preparations by covering with entellan after passing through staining series with Mallory's trichrome and hematoxylin-eosin (H&E). Besides, semi-thin sections taken from Araldite blocks prepared for TEM studies were also stained with Methylene blue. (Araldite block preparation procedure is described below under the heading "Preparation of samples for the TEM examinations"). Stained sections were studied with an LM (Olympus BX51, Japan), and the photographs were taken.

Preparation of Samples for the SEM Examinations

For the SEM preparation of the testis and accessory glands, samples were first washed with phosphate buffer (pH: 7.2) to remove the surrounding tissues and hemolymph on the tissues. Afterward, fixation with 5% glutaraldehyde solution (with phosphate buffer, pH: 7.2) was carried out for 24 h. The samples were rinsed again in phosphate buffer to remove the fixative, and dehydration processes in the ascending series of ethanol were performed. Then, the samples were treated in amyl acetate and dried at the critical point with a critical point drier (Polaron, CPD 7501, England), and dried samples were placed on stubs with double-sided tapes. Following the coating process with gold (Polaron SC 502 coater, England), gold-coated samples were investigated in SEM (JEOL JSM 6060, Japan), and their photographs were taken at Gazi University, Prof. Dr. Zekiye Suludere Electron Microscope Center.

Preparation of Samples for the TEM Examinations

For the TEM studies, samples were first washed with phosphate buffer (pH: 7.2) to remove the surrounding tissues and hemolymph on the tissues, then samples were pre-fixed in 5% glutaraldehyde (phosphate buffer, pH: 7.2) for 24 h and subsequently post-fixed in 1% osmium tetroxide for 24 h. After washing with phosphate buffer and dehydration processes with ascending series of ethanol, they were blocked in Araldite embedding medium (Glauert & Glauert, 1958). The ultrathin sections (0,2µm) were taken from the blocks with an ultramicrotome (Leica EM UC6, Austria), and they were stained in lead citrate and uranyl acetate (Reynolds, 1963). The stained sections were examined with TEM (JEOL JEM 1400, Japan), and the photographs were taken in Gazi University, Prof. Dr. Zekiye Suludere Electron Microscope Center.

RESULTS AND DISCUSSION

The male reproductive system of *B. turkiyae* consists of 2 testes responsible for sperm production, the vas deferens that provide the transport of mature sperm from the testis and the accessory glands that take an active role in their secretion in transferring the sperm to the female (Figure 1).

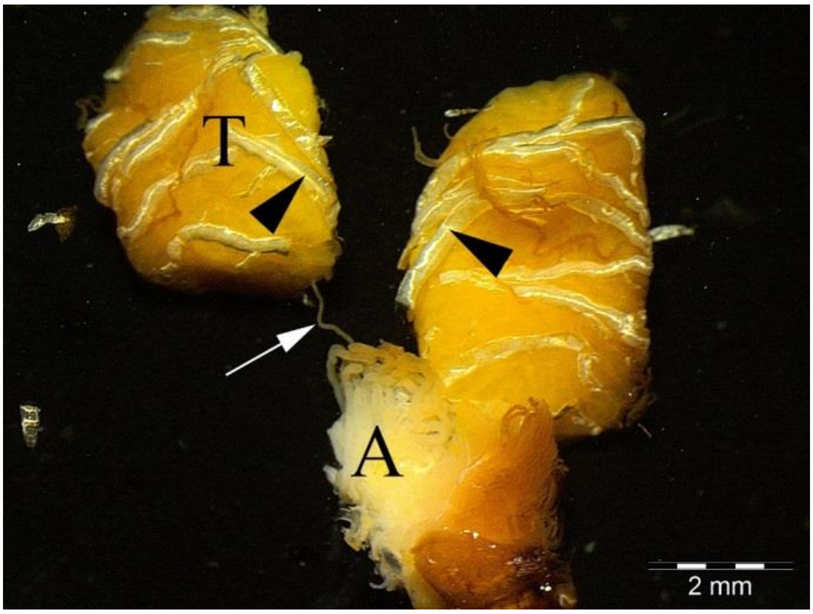

Figure 1. The stereomicroscope image of the male reproductive system in *B. turkiyae*. A: accessory glands, T: testis, arrow: vas deferens, arrowheads: trachea (Scale bar= 2 mm)

When the stereomicroscope images are examined, it is observed that the testis is yellow in mature male individuals. Each testis, oval, flattened from the sides, is surrounded by a web of trachea (Figure 1). In LM studies, many testicular follicles are seen in the sections taken from the testis (Figures 2a, b). The follicles are surrounded by a single layer of epithelial tissue. The epithelial tissue consists of fragile squamous cells, which are called follicle epithelium. There are many cysts in each follicle and all steps of sperm formation and development take place in these follicles. These developmental stages have two stages: spermatogenesis and spermiogenesis. Cysts are separated by a thin tissue similar to follicle epithelium. Each follicle is connected with thin canals called vasa efferentia, which carry the sperm to the vas deferens (Figure 3a). Similar results were obtained by Lyu et al. (2018) on *Bittacus puripennis* Cai & Hua, 2006 (Mecoptera: Bittacidae), Silva et al. (2018) on *Orphulella punctata* (De Geer, 1773) (Orthoptera: Acrididae), and Polat et al. (2019) on *Pseudochorthippus parallelus parallelus* (Zetterstedt, 1821) (Orthoptera: Acrididae). *B. turkiyae* has a significant number of testicular follicles in each testis. This number indicated eight testicular follicles in *O. punctate* (Silva et al., 2018). Each testis in *Chortophaga viridifasciata* (De Geer, 1773) (Orthoptera: Acrididae) has 26-28 testis follicles in the study of Carlson & Handel (1988). *Dione juno* (Cramer, [1779]) (Lepidoptera: Nymphalidae) and *Agraulis vanillae* (L., 1758) (Lepidoptera: Nymphalidae) have four testicular follicle in every testis in the study which was reported by Mari et al. (2018). With the increase in the number of testicular follicles, the number of sperm produced also increases. This situation affects mating success.

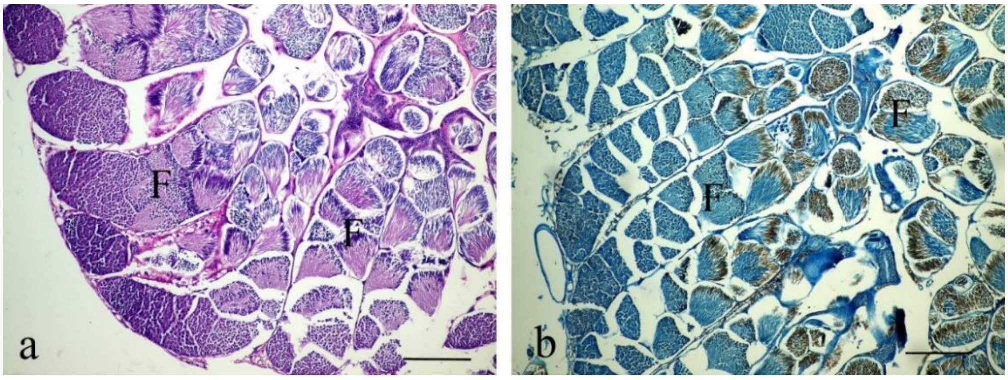

Figure 2. The cross sections of testis with oval follicles (F) (LM image, Scale bar= 200 µm). a. H&E, b. Mallory's trichrome stain

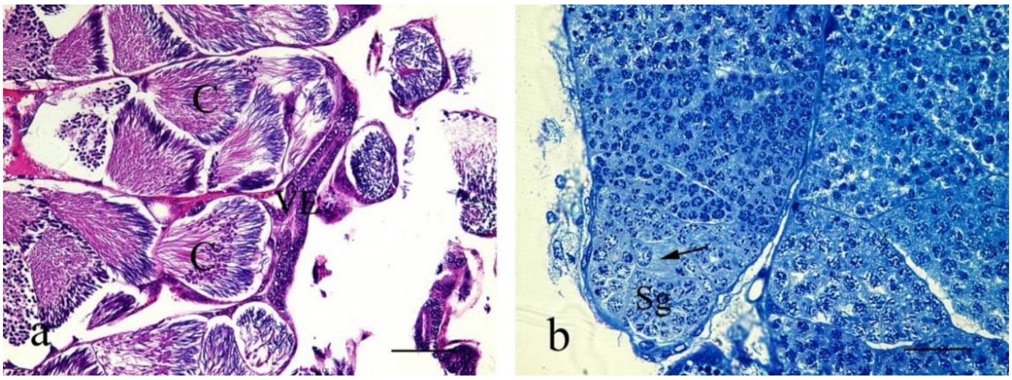

Figure 3. a. The proximal end of the follicles with cysts (C) that contain the final stages of the sperm formation and the connection between the follicle and vasa efferentia (VE) (LM image, H&E, Scale bar= 100 µm). b. The distal end of the follicle with cysts that contain the spermatogonium (Sg) and the apical cell (arrow) in its middle (LM image, Methylene blue, Scale bar= 100 µm)

The first area at the distal end of the testicular follicles is called the germarium. The spermatogonium zone is located in the region of the germarium (Figure 3b). In the middle of the germarium is the apical cell, which appears larger than other cells, with a round shape and no tail (Figure 3b). Round tailless spermatogonium cells form round, large nucleated, and tailless spermatocytes by mitosis. There are many small granules in the cysts of the spermatocyte stage (Figure 4). Spermatids are formed due to maturation and subsequent meiosis of the spermatocytes. First, the tailless early stage of spermatids is observed, and then these cells acquire a tail and form the late stage of spermatids. With the completion of the spermatid stage, the sperms have a roundish-oval head and a short tail (Figures 5a, 6a).

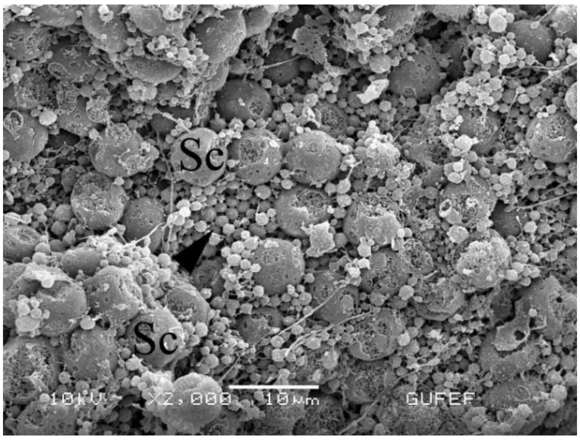

Figure 4. Round-shaped tailles spermatocytes (Sc) and small granules (arrowhead) among them (SEM image, Scale bar= 10 µm)

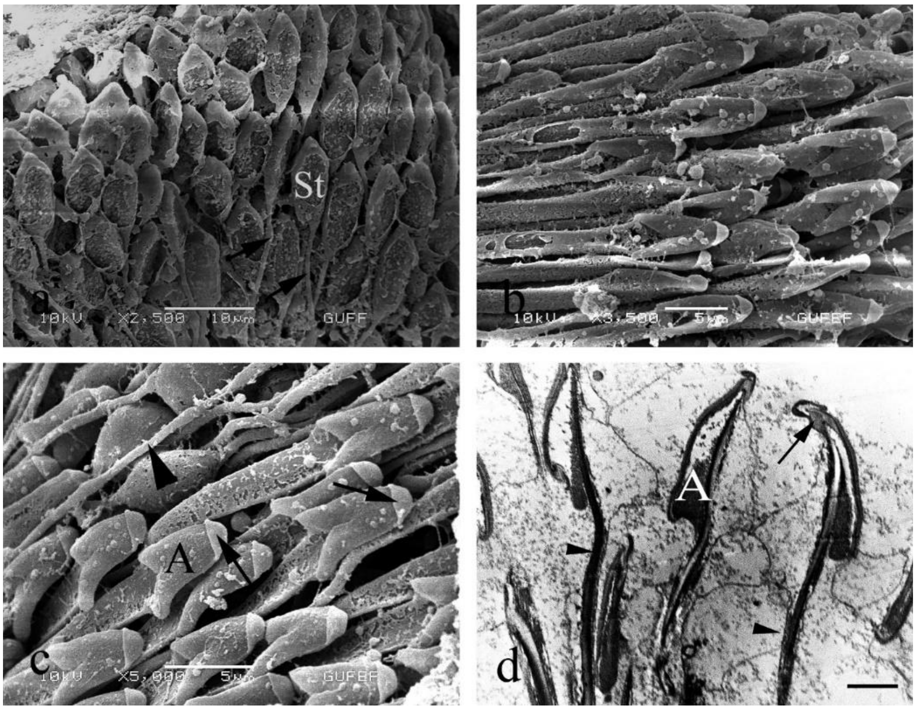

Figure 5. a. The cells at the end of the spermatid stage with roundish-oval head (St) and tails (arrows) (SEM image, Scale bar= 10 µm), b. The final shape of the spermatozoa at the end of the spermiogenesis stage (SEM image, Scale bar= 5 µm), c. The acrosome (A) in the apex of the sperm head and a cap (arrows) on it. Arrowhead: sperm tail (SEM image, Scale bar= 5 µm), d. The acrosome (A)) in the apex of the sperm head and a cap (arrows) on it. Arrowhead: sperm tail (TEM image, Scale bar= 2 µm)

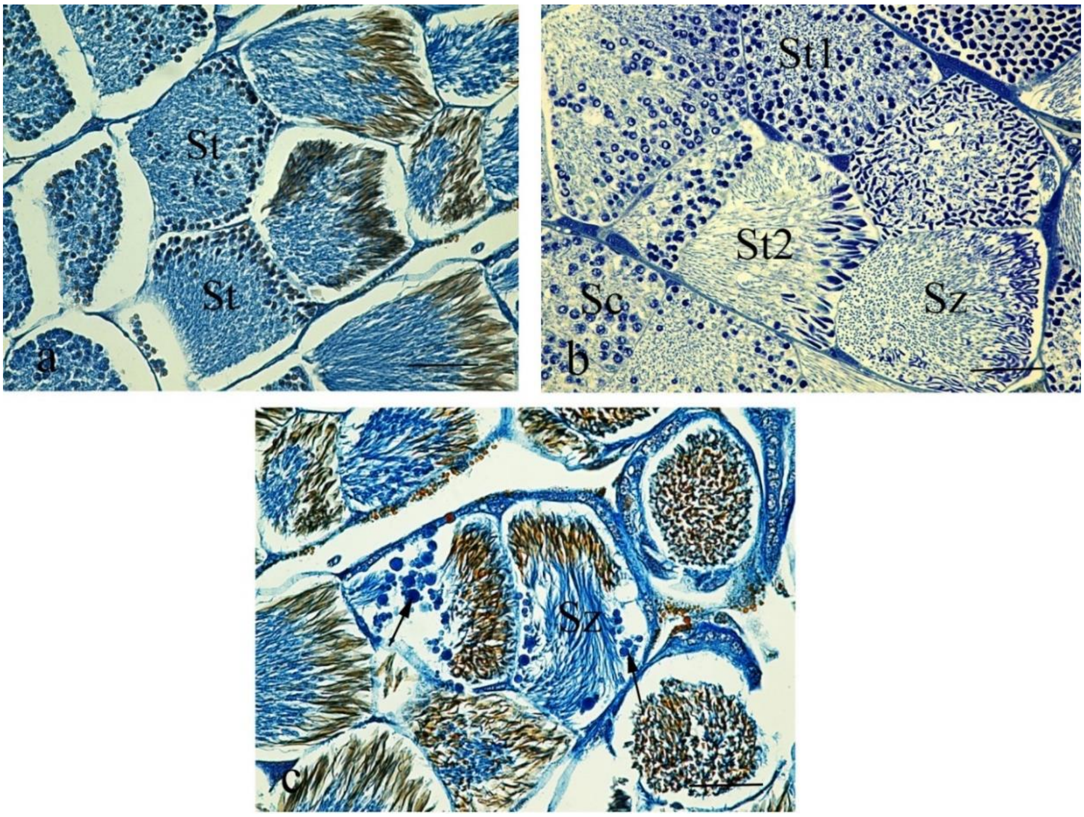

Figure 6. a. The cysts with cells at the end of the spermatid stage (St) (LM image, Mallory's trichrome staining, Scale bar= 100 µm), b. The figure shows the progressive spermiogenesis stage. Sc: spermatocyte, St1: early spermatid, St2: late spermatid, Sz: spermatozoa (LM image, Methylene blue, Scale bar= 50 µm), c. The detailed view of the cyst with spermatozoa stage (Sz) and some granules (arrows) among them (LM image, Mallory's trichrome staining, Scale bar= 100µm)

The head length in spermatids is 10,8 ± 0,6 µm. In the progressive spermiogenesis stage, the head and tail parts of the spermatids elongate and take their final shape to form spermatozoa (Figures 5b, 6b, 6c). The heads of the mature sperm are 22,4 ± 1,8 µm in length. The tail is much longer than the head area. Some granules are also seen among the sperm cells (Figure 5c).

The acrosome is rather apparent in the apex of the mature sperm head. The external appearance of this acrosome in the sperm head resembles a shirt collar, and there is also a cap at its end (Figures 5c, 5d).

The sperm maturation in the testis of *B. turkiyae* is similar to that of most other known grasshoppers (Dumser, 1980; Silva et al., 2018; Polat et al., 2019). The germ cells undergo mitosis in the germarium region in the testicular follicles. As the development progresses, they become mature sperm. The heads of the mature sperm are 22,4 ± 1,8 µm in length in *B. turkiyae*. While this length is 60 µm in *Cylindraustralia kochii* (Saussure, 1877) (Orthoptera: Cylindrachetidae), the head length of the sperm in *Xya variegate* Latreille, 1809 (Orthoptera: Tridactylidae) has 20 µm (Jamienson et al., 1999). In addition to these species, the length of the sperm head is 180 µm in *Orthochtha dimorpha* Miller, 1929 (Orthoptera: Acrididae) (Baccetti, 1987; Silva et al., 2018). However, sperm head length may use as a taxonomic feature among the species.

There are also accessory glands in the male reproductive system in *B. turkiyae*. The accessory glands are composed of many tubular structures with one closed end and the other opening into a common channel (Figure 7). They contain two groups of tubules in different structures: large accessory glands and small accessory glands (Figure 7). When the histological structure of both groups is examined, there is no significant structural difference. The difference is in the diameters and lengths of the tubules. The accessory glands in both groups have epithelial tissue with pyramidal or cuboidal cells with a single layer (Figures 8, 9a, 9b). The nucleus of the epithelial cells is oval. In addition, epithelial cells have scattered microvilli on the free surfaces facing the lumen (Figures 9b, 9c). The cytoplasm of the epithelial cells has well-developed granulated endoplasmic reticulum (GER) (Figure 10).

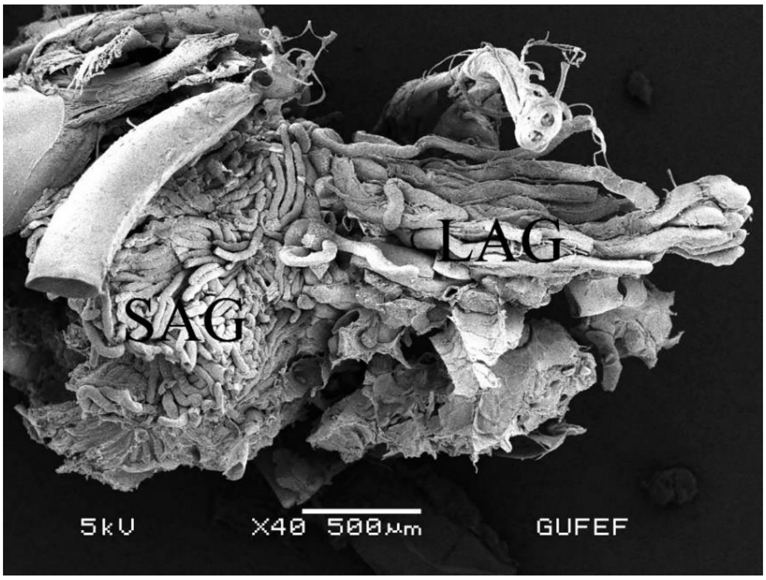

Figure 7. The SEM image of the male accessory glands consisting of large accessory glands (LAG) and small accessory glands (SAG) (Scale bar = 500 µm)

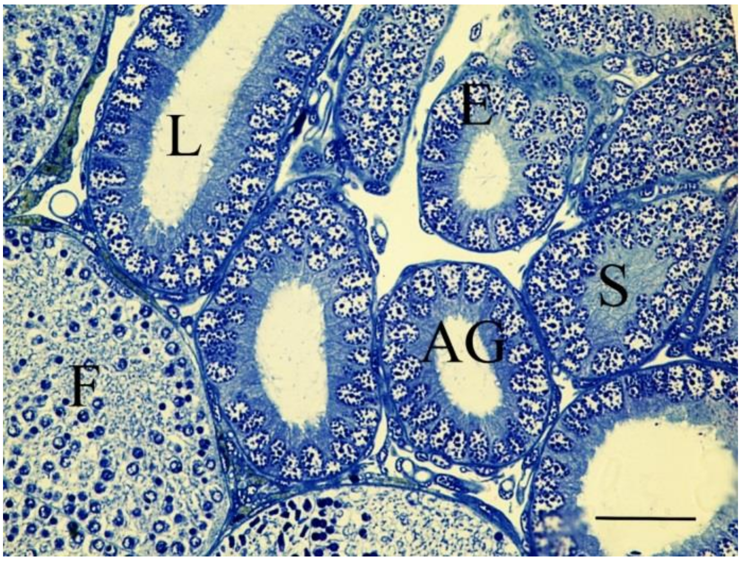

Figure 8. The cross sections of the tubules of accessory glands (AG). E: epithelium of the accessory gland tubules, F: follicles in testis, L: lumen, S: secretion (LM image, Methylene blue, Scale bar = 50 µm)

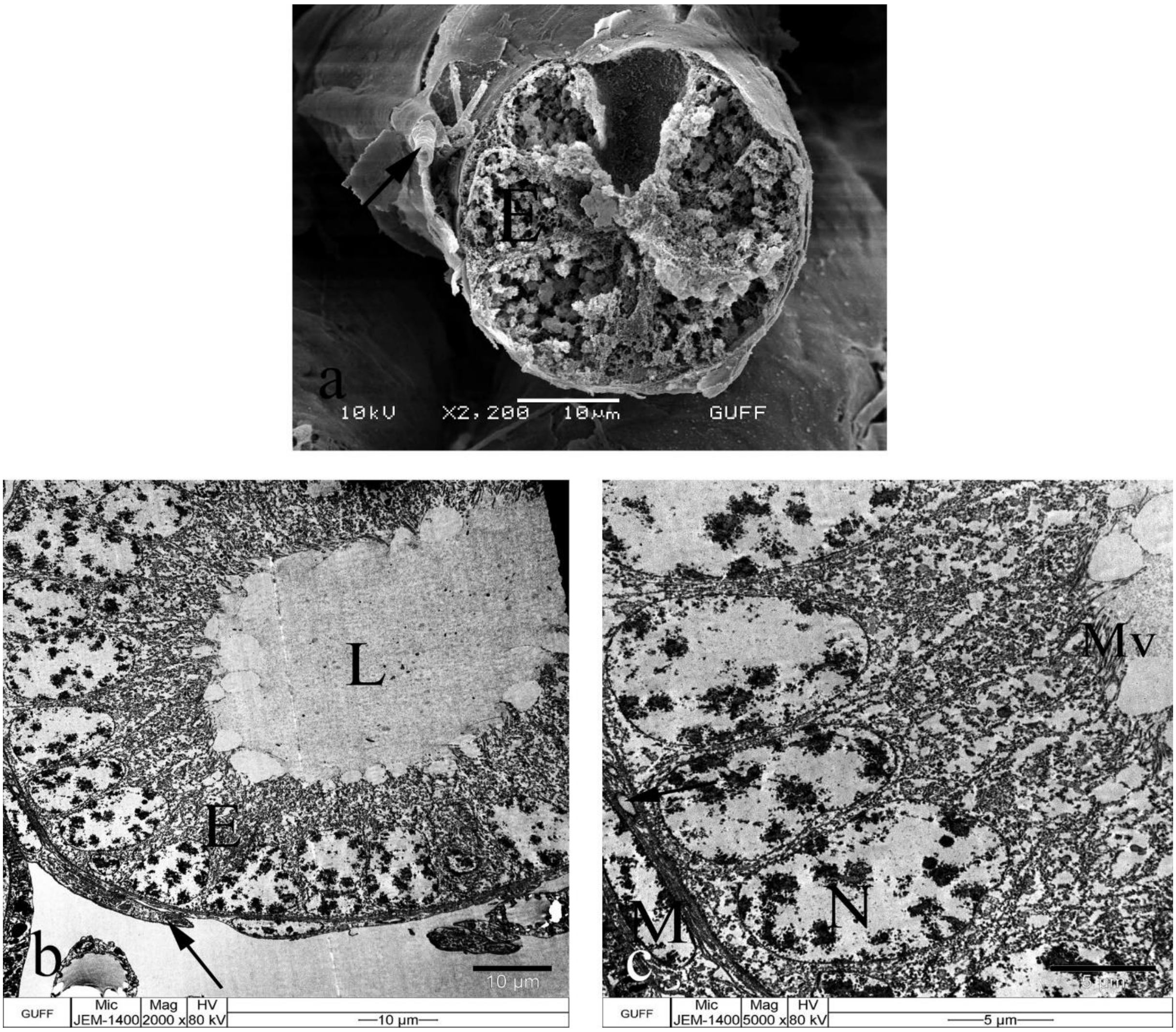

Figure 9. a. The cross-section of an accessory gland tubule. E: epithelium, arrow: trachea (SEM image, Scale bar = 10 µm), b. The TEM image of the cross sections of the accessory gland. E: epithelium, L: lumen, arrow: muscle tissue (Scale bar = 10 µm), c. The detailed view of the epithelial layer. M: muscle tissue, Mv: microvillus, N: nucleus, arrow: trachea (TEM image, Scale bar = 5 µm)

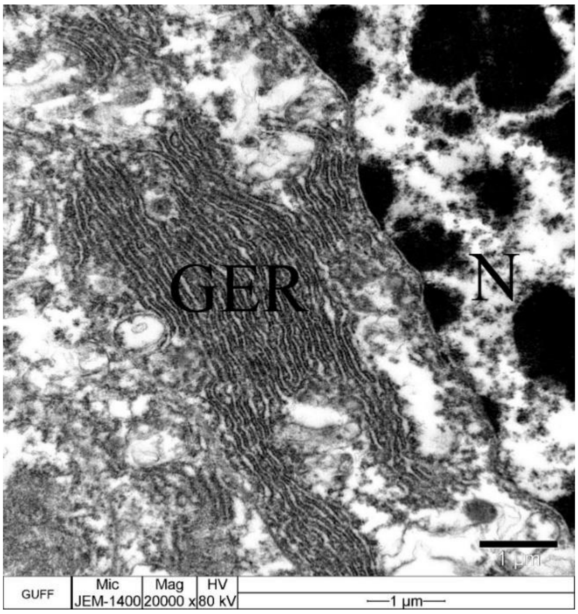

Figure 10. Well-developed GER near the nucleus (N). (TEM image, Scale bar = 1 µm)

The accessory glands serve a significant number of tasks in insects. These functions include producing the seminal fluids, sperm activation, or transferring the sperm to the female reproductive system (Polat et al., 2020; Araújo et al., 2021). As in the testis, a difference in number can be seen in the male accessory glands. For example, Muse & Balogun (1991) reported five types of accessory glands in the accessory glands of male *Zonocerus panegatus* L. (Orthoptera: Pyrgomorphidae). This number is also indicated as five types of accessory glands in Acheta domesticus (L., 1758) (Orthoptera: Gryllidae), too (Klaulenas, 1976). However, two types of glands are classified in the accessory glands of *P. parallelus parallelus* (Polat et al., 2020). In *Sinopanorpa tincta* (Navas, 1931) (Mecoptera: Panorpidae) and *Tropisternus collaris* (Fabricius, 1775) (Coleoptera: Hydrophilidae), there are two types of accessory glands (Lyu & Hua, 2017; Araújo et al., 2021). In this study, we observed two types of accessory glands in *B. turkiyae*.

Ultrastructural examination of accessory glands in different insect species generally shows similar features. They contain a basement membrane and epithelial layer (Muse & Balogun, 1991; Polat et al., 2020). While there are columnar epithelial cells in *S. tincta* (Lyu & Hua, 2017) and *Meloe proscarabaeus* L., 1758 (Coleoptera: Meloidae) (Muzzi et al., 2020), there is a cuboidal epithelium in *P. parallelus parallelus* (Polat et al., 2020). The accessory glands in *B. turkiyae* comprise cuboidal or pyramidal epithelial cells like the epithelium in *P. parallelus parallelus*.

CONCLUSION

In conclusion, although the structure of the reproductive organs of the males we examined is generally similar to the reproductive organs of other species in the Orthoptera order, some features, such as the number of follicles in a testis, the sizes of sperm heads, and the subtypes of accessory glands show differences with some species. The reproductive system is a biological process that ensures the continuity of the species, including cell differentiation and development processes. In addition, knowing the structure of the reproductive systems of insects helps us to have an idea about the reproductive capacity of the species. It allows us to develop methods to combat the species when necessary. With this thought, in this study, the ultrastructure of *B. turkiyae*'s testis and male accessory glands were clarified. It is hoped that these findings will contribute to studies with other species.

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Conflict of Interest

The article authors declare that there is no conflict of interest between them.

Author's Contributions

The authors declare that they have contributed equally to the article.

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