The morphology and histology of the male reproductive system in *Apodiphus amygdali* (Germar, 1817) (Hemiptera: Heteroptera: Pentatomidae)[4]

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Abstract: This paper reveals the morphology and histology of the adult male reproductive system in the stink bug, *Apodiphus amygdali* (Germar, 1817), using light and scanning electron microscopy. Males have a pair of testes situated laterally to the alimentary canal, a pair of elongated cylindrical vasa deferentia and seminal vesicles, which join in a complex bulbus ejaculatorius, a ductus ejaculatorius, a pair of ectodermal sacs, a pair of accessory glands, and the phallus. Each testis consists of seven testicular follicles connected to the vas deferens. Each follicle contains a germarium, followed apically by growth zones where spermatozoa differentiate in the direction of the vasa deferentia. Each testis is connected to the seminal vesicle by a vas deferens. The seminal vesicles are connected to the bulbus ejaculatorius, which is surrounded by the tubularly coiled accessory glands. The bulbus ejaculatorius is continuous with the ductus ejaculatorius and connects to the aedeagus demonstrating its ectodermic origin.

Key Words: Spermatogonia, spermatocytes, spermatids, spermatozoa, light and scanning electron microscope (SEM)

Most insect species in the Heteroptera (Insecta: Hemiptera), including those in the family Pentatomidae, are phytophagous. Pentatomids are called stink bugs or shield bugs. Many of them are considered agricultural pests, because they can create large populations that feed on crops damaging production. Many heteropterans are a threat to cotton, corn, sorghum, soybeans, as well as many native and ornamental trees, shrubs, vines, weeds, and cultivated crops worldwide (Schuh and Slater, 1995; Schaefer and Panizzi 2000).

There are very few studies of the morphology and histology of the reproductive system in pentatomids (Pendergrast, 1956; Ramamurty 1969; Adams 2001; Özyurt et al., 2013a, 2013b). *Apodiphus amygdali* (Germar, 1817) (Pentatominae: Haliini) is known as the Large Tree Pentatomid (Lodos, 1986; Bolu et al., 2006; Candan and Suludere, 2010, also see photo on page 41 this issue). *Apodiphus amygdali***,** which is also known as the Fruit Trees Stink Bug, is distributed in Europe and the Middle East. Among the trees attacked by *A. amygdali* there are fruit trees, such as plum (*Prunus domestica* L., Rosaceae),

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apricot (*Prunus armeniaca* L., Rosaceae), apple (*Malus pumila* Mill., Rosaceae), olive (*Olea europaea* L., Oleaceae), pear (*Pyrus communis* L., Rosaceae) and pistachio (*Pistacia vera* L., Anacardiaceae) as well as non-fruit trees, like poplar (*Populus alba* L., Salicaceae), Turkish pine (*Pinus brutia* Ten., Pinaceae), plane-tree (*Platanus orientalis* L., Platanaceae), alma (*Ulmus minor* Mill., Ulmaceae), and willow bark (*Salix alba* L.,, Salicaceae) (Muhammed and Al-Iraqi, 2010). Considering the economic importance of *A. amygdali,* this study describes the morphology and histology of its reproductive system. Some of the findings contribute new characters for systematic studies of this group.

The male reproductive system of Heteroptera presents five distinct regions (Figure 1): a pair of testes (***t***), two vas deferentia (***t***), two seminal vesicles (***vs***), an ectodermal sac (***es***), a bulbus ejaculatorius (***b***), a ductus ejaculatorius (***d***), and an aedeagus (not shown) (Pendergrast, 1956; Nijhout, 1994; Chapman 1998; Karakaya et al., 2012; Özyurt et al., 2013a, 2013b).

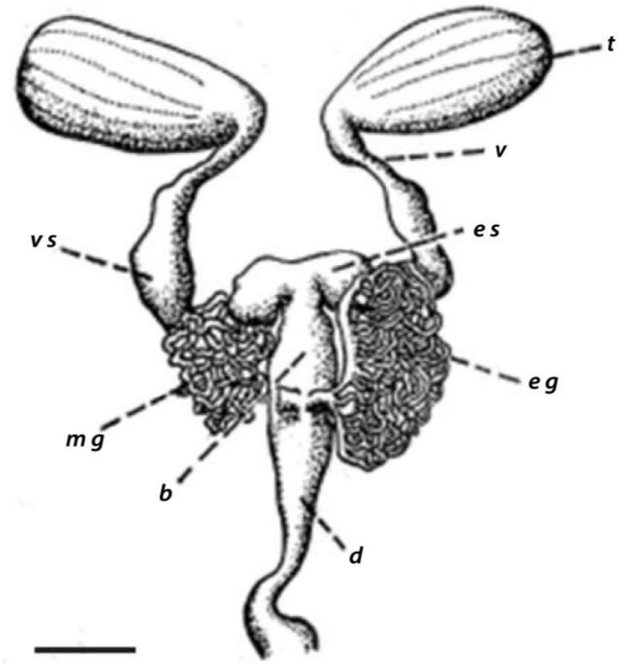


Figure 1. Male reproductive organs, ***t*** - testis, ***v*** - vas deferens, ***vs*** - vesicula seminalis, ***es*** - sac of ectodermal origin, ***eg*** - ectadene accessory gland, ***mg***- mesadene accessory gland, ***b*** - bulbus ejaculatorius, ***d*** - ductus ejaculatorius (Pendergrast, 1956). Scale bar = 1 mm. The image has been reproduced, with small modifications, with permission of Royal Society of New Zealand.

The testes (Figure 1, ***t***) consist of a great number of tubules that enter the vasa deferentia. The vasa deferentia (***v***) extend from the testes to paired seminal vesicles (***vs***). These vesicles insert on the anterior medial portion of the bulbus ejaculatorius (***b***). The bulbus ejaculatorius is covered by irregularly shaped accessory glands (***eg*** and ***mg***). The accessory glands of male insects can be classified into two types according to their mesodermal (***mg***) or ectodermal (***eg***) derivation. Almost all accessory glands that have been studied are of mesodermal origin, while little is known about those that have an ectodermal origin. Secretions of the male accessory glands from the spermatophore contribute to the seminal fluid that nourishes the spermatozoa during transport to the female. These secretions are also involved in the activation of spermatozoa and may alter female behavior (Pendergrast, 1956; Davey and Krieger, 1985; Chapman, 1998). The bulbus ejaculatorius is continuous with that of the ductus ejaculatorius (***d***). In most insects, there is a single ductus ejaculatorius, located medially and bearing a cuticular cover, demonstrating is ectodermic origin (Pendergrast, 1956; Davey and Krieger, 1985; Chapman, 1998; Karakaya et al., 2012; Özyurt et al. 2013a; Özyurt et al. 2013b).

Methods

Gross Anatomy. Adult males *A. amygdali* were collected in July 2011 in Antalya, Turkey. The specimens were killed with ethyl acetate and dissected in 70% ethyl alcohol under a stereomicroscope. For the morphological studies, the dorsal cuticle was first removed from the prothorax to the last full-sized abdominal segments. Subsequently, the epidermis and the digestive system were removed. Two insect pins were inserted laterally through the last full-sized sternite to spread apart the abdomen and to expose the base of the reproductive system attached to the genital segments. The gross morphology of the reproductive systems of the males were examined and photographed with a Leica EZ4D stereomicroscope.

Light Microscopy. For the histological analysis, the reproductive systems of ten males were fixed in Bouin's for 24h. Thereafter, the tissues were washed, dehydrated in a series of ethanol solutions (70%, 80%, 90%, 100%) and finally embedded in paraffin. Paraffin sections were cut into 6-7 µm slices and stained with Hematoxylin-Eosin and Mallory's triple stain for light microscopic examination (Junqueira and Junqueira, 1983). The sections were viewed and photographed by using an Olympus BX51 compound microscope.

Scanning Electron Microscopy (SEM). Cleaned and dried (Polaron CPD 7501 Critical Point Dryer) specimens were mounted using double sided sticky tape on SEM stubs, coated with gold in a Polaron SC 502 sputter coater, and examined with a scanning electron microscope (JEOL JSM 6060 LV) at 5kV.

Results

The internal male reproductive system of *A. amygdali* is formed by a pair of testes, a pair of vas deferentia, a pair of seminal vesicles, a pair of accessory glands, a bulbus ejaculatorius, a ductus ejaculatorius and aedeagus (Figure 2a). The testes are paired, cylindrical and red (testes are already noticed in freshly in emerged adults) (Figure 2a) lie at the sides of the alimentary tract, approximately in the middle of the abdominal cavity. The testis is composed by seven sperm tubes surrounded by tunica propria, peritoneal sheath with embedded tracheoles (Figures 2a, 2b). Sperm develops in the testes and is stored in the seminal vesicles (Figure 2b) until the mating occurs.

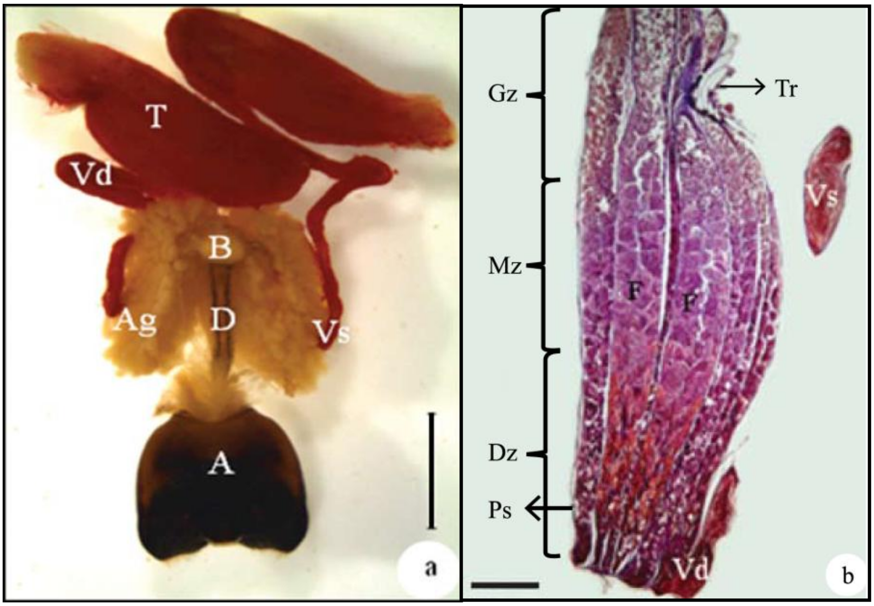


Figure 2. a. Light micrograph of general morphology of the male reproductive system in *Apodiphus amygdali*. Testes (T), vas deferens (Vd), seminal vesicle (Vs), bulbus ejaculatorius (B), ductus ejaculatorius (D), accessory glands (Ag), aedeagus (A). Scale bar = 2 mm. b. Longitudinal section of the testes surrounded by peritoneal sheath (Ps) with embedded tracheoles (Tr) and showing the follicles (F). Growth zone (Gz), maturation zone (Mz), differentiation zone (Dz), vas deferens (Vd), seminal vesicles (Vs). Scale bar = 500 µm.

Spermatogenesis occurs within the lumen of the sperm tubes; different stages of spermatogenesis can be observed across the sperm tubes. Spermatogenesis begins in the germarium, where spermatogonia develop and proliferate within cysts. As both mitotic and meiotic divisions take place, the cysts that contained the spermatogonia degenerate. Spermatogonia differentiate into spermatocytes before becoming spermatids (Figures 3 a-f). The spermatids differentiate into spermatozoa as they approach the vas deferens.

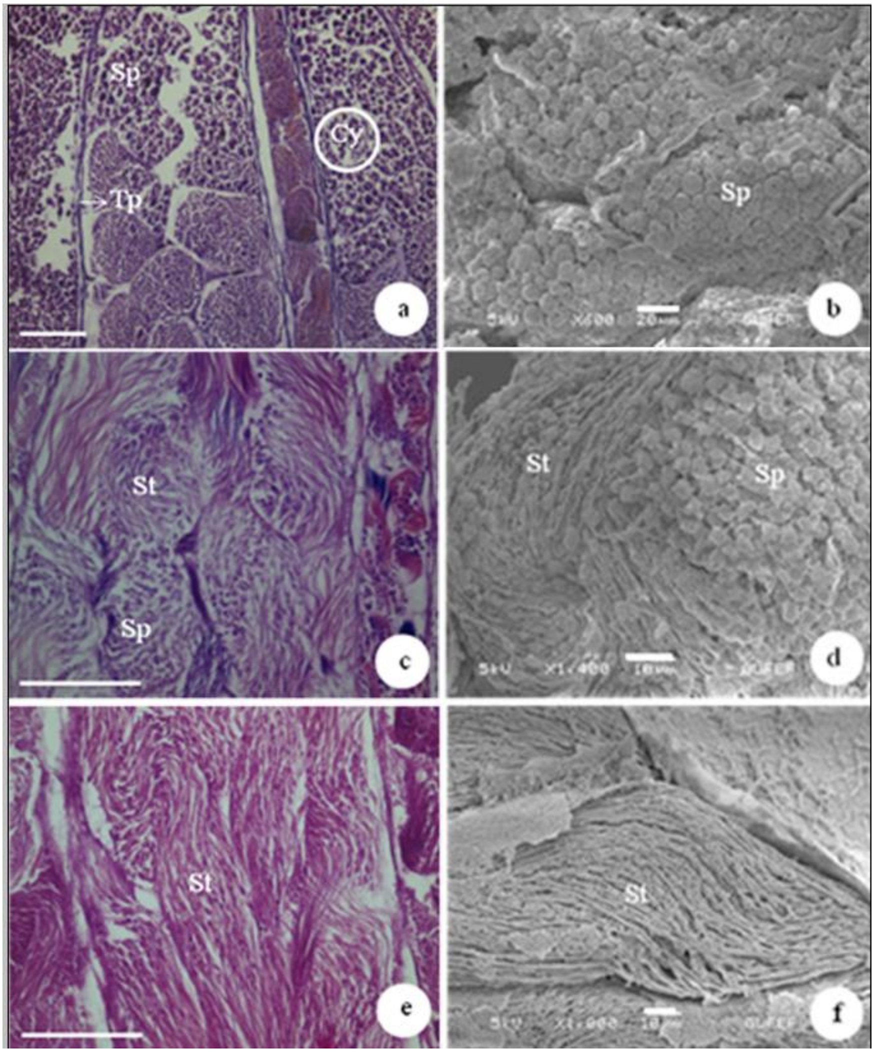
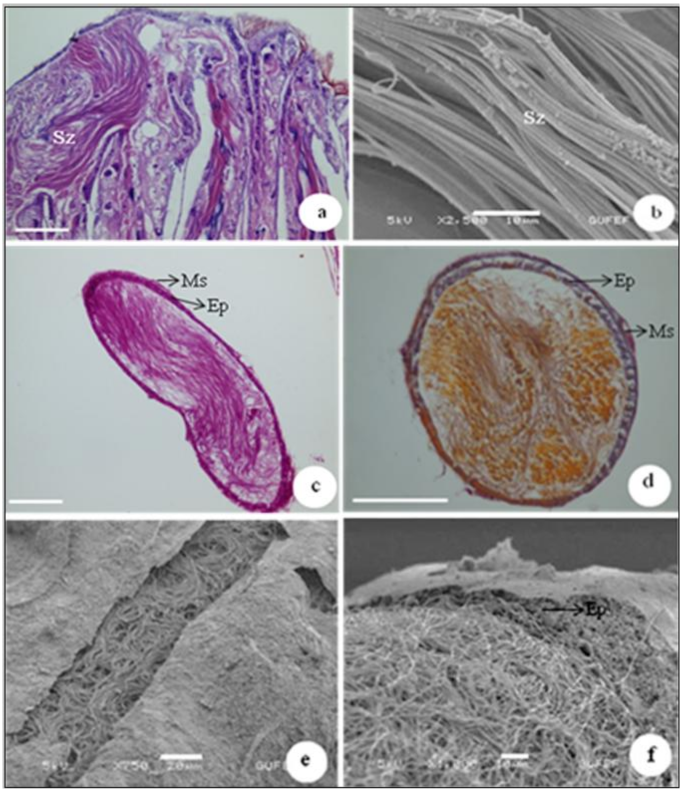
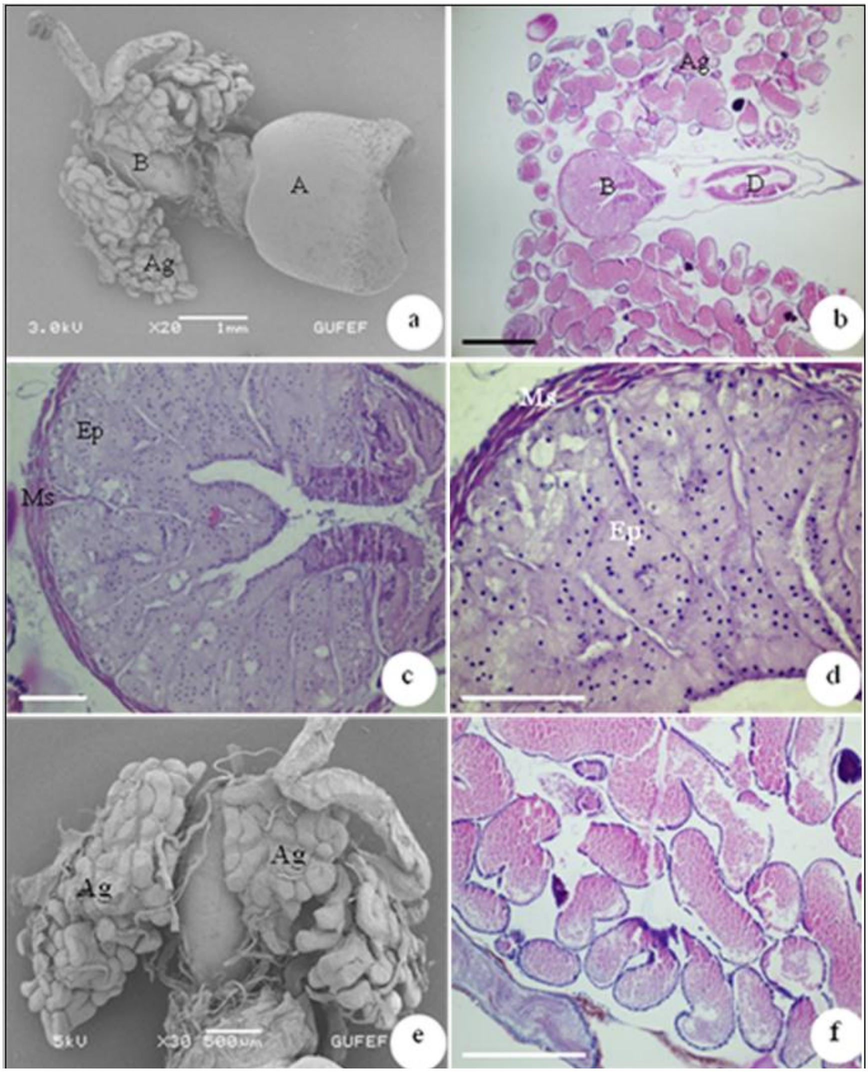


Figure 3. a-b. The presence of the spermatocytes (Sp) forming cysts (Cy) in growth zone of the testes follicles and sperm tubes surrounded by tunica propria Light and scanning electron microscopy (respectively). c-d. Light and SEM photos of the presence of spermatocytes at different developmental stages and differentiated spermatids (St). e-f. Light and SEM photos of the presence of spermatids in maturation zone of the testes follicles. Bar on a, c, and e = 100 µm; on b and d = 20 µm; on f = 10 µm.



Figures 4. a-b. Light and SEM photos of spermatozoa (Sz) bundles in differentiation zones the testes follicles. c-d. Longitudinal (c) and cross sections (d) of seminal vesicles (Vs) and vas deferens (Vd) with large numbers of spermatozoa bundles, epithelium (Ep) and muscle sheath (Ms). e-f. SEM photos of large numbers of spermatozoa bundles in epithelium sheath. Bar on c, c, and d = 100 µm; on b and d = 10 mm; on e = 20 mm.

The spermatozoa are contained in cysts which are grouped together in bundles and from which the spermatozoa are liberated (Figures 4 a, b). The vas deferens is the duct leading from the testes to the seminal vesicle. Within this simple tube spermatozoa are being transported to the seminal vesicles. A portion of each vas deferens and seminal vesicle is dilated to form a tubular chamber. The heads of the spermatozoa are embedded in the epithelial lining of the seminal vesicles and their tails extend posteriorly in a spiral into lumen. There is no outstanding difference between the histology of the vas deferens and the seminal vesicle. The walls of these ducts consist of an inner layer of epithelial cells which is surrounded by a network of muscle fibers extending in various directions. Epithelial cells of the ducts are cuboidal and they contain large spherical and well stainable nuclei (Figures 4c- f).



Figures 5. a-b. SEM and light photos showing of bulbus ejaculatorius (B), ductus ejaculatorius (D) and accessory glands (Ag) in male reproductive system. c-d. Light photos showing of muscle sheath (Ms) and epitelium (Ep) in bulbus ejaculatorius. e-f. SEM and light photo showing of accessory glands. Scale bar on a = 1 mm; on b-d, f = 100 µm; on e = 500 µm.

The seminal vesicle connects the bulbus ejaculatorius which is balloon shaped (Figure 5a). It is partly covered by an epithelium, here termed the investing epithelium. The walls of bulbus ejaculatorius are formed by muscles running concentric to the lumen of the ducts (Figures 5c, d). The ductus ejaculatorius has an inner and outer epithelium, with a band of striated muscle between them. The striated muscle consists of muscle cells that presumably contract in waves to generate a peristaltic movement. This would facilitate the movement of the sperm along the ductus ejaculatorius (Figure 5b). The bulbus ejaculatorius and the ductus ejaculatorius are covered by irregularly shaped accessory glands, which have multi-lobed tubular structures and an abundant supply of tracheae (Figures 5e, f).

Discussion

Studies on the testicular ultrastructure and spermatogenesis must be encouraged to broaden our knowledge of the morphology and histology of the male reproductive system in insects. While in some Pentatomidae the general morphological and histological patterns are similar (Davis, 1956; Pendergrast, 1956; Lemos et al., 2005; Jahnke et al., 2006; Papáček and Soldán, 2008; Rodrigues et al., 2008; Wieczorek and Swiatek, 2009; Freitas et al., 2010; Karakaya et al., 2012; Özyurt et al., 2013a; Özyurt et al., 2013b), the results of this study support the existence of differences with regards location, size, shape and color of *A. amygdali* male reproductive system when compared to other Heteroptera.

Every testis contain the testicular follicles where are produced spermatozoa. The number of follicles varies widely between species and has taxonomic importance (Davis, 1956; Pendergrast, 1956; Lemos et al., 2005; Jahnke et al., 2006; Papáček and Soldán, 2008; Rodrigues et al., 2008; Wieczorek and Swiatek, 2009; Freitas et al., 2010; Karakaya et al., 2012; Özyurt et al., 2013a; Özyurt et al., 2013b). Testes in *A.amygdali* and *Perillus bioculatus* (Fabricius) (Pentatomidae: Asopinae) (Adams, 2001) consists of seven sperm tubes, but in *Nezara viridula* (Linnaeus), *Podisus nigrispinus* (Dallas), *Dolycoris baccarum* (Linnaeus), *Graphosoma lineatum* (Linnaeus) (Pentatomidae), and *Aphelocheirus aestivalis* Fabricius (Heteroptera: Aphelocheiridae) 4-6 sperm tubes are observed (Lemos et al., 2005; Ramamurty, 1969; Özyurt et al., 2013a; Özyurt et al., 2013b). In *Adparaproba gabrieli* Carvalho (Heteroptera: Miridae), two follicles are observed (Uceli et al., 2011). The sperm tubes of *A. amygdali* as in other heteropterans are covered by a peritoneal sheath and a tunica propria (Davis, 1956; Chapman, 1998; Pires et al., 2007; Rodrigues et al., 2008; Karakaya et al., 2012; Özyurt et al., 2013a; Özyurt et al., 2013b).

The testes contain sperm cells at different stages of spermatogenesis across the sperm tubes. The mechanisms of spermatogenesis in *A.amygdali*, including sperm differentiation, are rather similar in other Heteroptera (Bowen, 1922; Davis, 1956; Engelmann, 1970; Chapman, 1998; Lemos et al., 2005; Pires et al., 2007; Rodrigues et al., 2008; Karakaya et al., 2012; Özyurt et al., 2013a; Özyurt et al., 2013b). Once fully developed, the sperm cells travel from the testes to the seminal vesicle via the vas deferens. However, in *Notonecta glauca* (Linnaeus, 1758) (Heteroptera: Notonectidae), the testicular follicles are directly connected to the seminal ducts (vasa deferentia laterals) (Papáček, and Soldán, 2008). In *A.amygdali*, as in other heteropterans, a portion of each vas deferens and seminal vesicle is dilated to form a tubular chamber. The walls of these ducts consist of an inner layer of epithelial cells that is surrounded by network of muscle fibers extending in various directions. Similar ductal structures have been observed in vasa deferentia and seminal vesicles of *D. baccarum*, *G. lineatum*, *N. glauca* (Papáček and Soldán, 2008; Özyurt et al., 2013a; Özyurt et al., 2013b). As within other Heteroptera, the seminal vesicles of *A. amygdali* connect to balloon-shaped bulbus ejaculatorius and are covered by irregularly shaped accessory glands (Özyurt et al., 2013a; Özyurt et al., 2013b). The bulbus ejaculatorius join to form a common ductus ejaculatorius (Bushrow et al., 2006). The terminal portion of the ductus ejaculatorius may be sclerotized to form the intromittent organ, the aedeagus. It differentiates from a pair of ectodermal lobes associated with the ninth abdominal segment and is often concealed within a genital chamber (Klowden, 2007).

The morphological knowledge of insect male reproductive accessory glands of insects is extensive (Papáček, and Soldán, 2008). These structures vary considerably in size, shape, number and embryological origin (Leopold, 1976; Chapman, 1998; Özyurt et al., 2013a; Özyurt et al., 2013b). In most insects, their main function is the formation of seminal fluid and a spermatophore, which is vital for the transfer of sperm. Also the accessory glands are responsible for spermatophore production and produce some active peptides (Chen, 1984; Kaulenas, 1992; Davey and Krieger 1985; Freitas et al., 2010; Uceli et al., 2011). Male *A. amygdali* has a pair of accessory glands, which open into the vas deferens and the ductus ejaculatorius. These accessory glands are similar to those in *D. baccarum* and *G. lineatum* (Heteroptera: Pentatomidae) (Özyurt et al., 2013a; Özyurt et al., 2013b). However, *Triatoma rubrofasciata* (De Geer) and *A. gabrieli* Carvalho have only four accessory glands, and *T. brasiliensis* Neiva as well as *T. melanica* Neiva and Lent (Hemiptera: Reduviidae) have eight accessory glands.

As a result of present study, these goals are followed: a) contribute to the knowledge of the male reproductive system of Pentatomidae; b) reveal the characters that may be useful for future studies; c) obtain the key information for future research into in the reproductive biology, ecology and biological control agents of the Heteroptera.

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Adult *Apodiphus amygdali* (Germar, 1817) (Heteroptera: Pentatomidae). Image from http://upload.wikimedia.org/wikipedia/commons/7/71/Apodiphus_amygdali_(Shieldbug_sp.), Skala_Kalloni,_Lesbos,_Greece.jpg