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The male reproductive accessory glands of the blister beetle *Meloe proscarabaeus* Linnaeus, 1758 (Coleoptera: Meloidae): Anatomy and ultrastructure of the cantharidin-storing organs

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ABSTRACT

Blister beetles owe their name to their ability to release cantharidin, a blistering terpene, the highest concentration of which is retained in male accessory glands. The anatomy and ultrastructure of the three pairs of male reproductive accessory glands and the glandular region of the two *vasa deferentia* of *Meloe proscarabaeus* were investigated using light, electron and ion beam microscopy. All of the mesodermal glands here analysed share a common structural organization with an outer muscular layer and an inner glandular epithelium facing a broad lumen in which the secretory products are released. Developed rough endoplasmic reticulum, Golgi systems, abundant mitochondria, numerous secretory vesicles and a microvillated apical membrane are commonly found in the cells of different glandular epithelia, suggesting that all accessory gland pairs as well as the *vasa deferentia* are involved in an active synthesis. Nevertheless, each pair of glands appears specialized in the production of a specific set of substances, as suggested by the peculiarities in cellular ultrastructure and by the different aspect of the secretions stored in their glandular lumen. The above cited features of male accessory glands of *M. proscarabaeus* are compared with those of other beetles and some hints on their potential role in producing and/or concentrating cantharidin are provided.

1. Introduction

Male accessory glands are commonly found in insects and play several key roles in their reproduction. One of the main functions of these glands is to secrete the substances involved in the production of spermatophores, structures that protect the sperms and facilitate their transfer from the male to the female (Leopold, 1976; Chen, 1984). Nevertheless, male accessory gland secretions are often involved in many other processes such as the structural organization of spermatozoa bundles (Viscuso et al., 2001) or the promotion of their activation and motility, ensuring its successful storage inside the female genital tract (Davey, 1985; Chen, 1984; Neubaum and Wolfner, 1999; Lung et al., 2001; Mueller et al., 2007). In some other cases, the products of the male accessory glands are directly involved in spermatozoa competition by either inactivating/reducing the effective number of germ cells from the previous mating (Harshman and Prout, 1994) or by participating in the creation of a mating plug (Leopold, 1976; Colonello and Hartfelder, 2005). Other subsidiary effects of male accessory gland secretions include the ability to directly affect and modulate the post mating behaviour of females, causing a vast array of responses that include: increase in egg production and maturation, enhancement of oviposition and decrease in re-mating receptivity (Raabe, 1987; Gillott, 2003; Avila et al., 2011; Hayashi and Takami, 2014; Yu et al., 2014; Yamane et al., 2015; Carmel et al., 2016).

Despite the great diversity of functions performed by the male accessory gland secretions, most of them, especially those involved in physiological and behavioural alterations, are mainly made up of proteins and peptides and consist only in a small percentage of carbohydrates and lipids (Gillot, 2003). To date, proteins and peptides of male accessory glands secretions have been widely

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investigated for their composition, expression, localization and effects (Andrés et al., 2006, 2008; Braswell et al., 2006; Collins et al., 2006; Davies and Chapman, 2006; Baer et al., 2009), especially in Diptera (Dottorini et al., 2007; Sirot et al., 2008, 2011; Rogers et al., 2009; Avila et al., 2011; Mancini et al., 2011).

Nevertheless, male accessory gland secretions can contain many other substances albeit less frequently, including toxic compounds that are transmitted to the female, along with the spermatophore, and are subsequently used to provide protection to the eggs (Hilker and Meiners, 2008). In these cases, males usually sequester toxic substances from an external source and transmit them to the females during mating; examples of this are Noctuoidea butterflies, storing pyrrolizidine alkaloids (Dussourd et al., 1989, 1991; Hartman et al., 2004) or cyanogenic glycosides (Cardoso and Gilbert, 2007), and the spotted cucumber beetle *Diabrotica undecimpunctata howardi* Barber, 1947 seizing cucurbitacins (Tallamy et al., 2000). However, other insects are able to synthesize themselves defensive chemicals, as is the case of the almost 3000 coleopteran species belonging the family Meloidae (Bologna et al., 2008, 2010), also known as blister beetles for their ability to produce and release secretions containing cantharidin, a terpene with well-documented cytotoxic and blistering effects (Bologna, 1991; Carrel et al., 1993). In Meloidae, the main function of cantharidin is to provide defence to adults: in fact, when threatened, blister beetles release small droplets of haemolymphatic exudate containing this toxic substance (Blodgett et al., 1991; Carrel et al., 1993; Nakatani et al., 2004; Verma and Prasad, 2012; Bravo et al., 2017; Gisondi et al., 2019). This typical reflex-bleeding behaviour is usually accompanied by thanatosis and seems extremely effective in discouraging a wide range of potential predators (Carrel and Eisner, 1974; Smedley et al., 1995). On the other hand, cantharidin also plays an important role in reproduction of blister beetles; in fact, although the ability of adult females to synthesize cantharidin has not yet been clearly demonstrated, males are known to produce and transfer large amounts of this terpene as a nuptial gift during copulation to females (Selander, 1964; Bologna, 1991; Carrel et al., 1993; Dettner, 1997; Nikbakhtzadeh et al., 2007a, 2012), which in turn use the transferred compound for protecting eggs from potential predators (Sierra et al., 1976; Carrel et al., 1993; Eisner et al., 2002). In Meloidae the highest concentration of cantharidin are retained in male accessory glands, which have been indicated as important 'reservoirs' in some blister beetle species (Sierra et al., 1976; McCormick and Carrel, 1987; Carrel et al., 1993; Nikbakhtzadeh et al., 2007a). Unfortunately, it is still unclear whether male accessory glands are directly involved in the synthesis of cantharidin or if they only act as compartments to store and concentrate the terpene, likely produced in other organs such as fat bodies (Jiang et al., 2017a, 2019).

Despite male accessory glands of blister beetles have been suggested to have a relevant role in cantharidin biosynthesis and/or absorption, the study of their fine anatomy has long been neglected. In fact, while their general structure is known for many species thanks to the detailed drawings presented in Beauregard's pioneering monograph (1890) and in the papers of Gupta (1965, 1966a, 1966b, 1967), the only study giving a detailed histological information on these systems is the one performed by Gerber et al. (1971a) on the Nearctic species *Lytta nuttalli* Say, 1824, belonging to the subfamily Meloinae, tribe Lyttini. Surprisingly, information on the fine morphology of the accessory glands is still relatively scarce for most of beetles and not only for Meloidae; in fact, as far as we know, ultrastructural analyses are limited to the following species: *Pterostichus nigrita* Paykull, 1790 (Carabidae) (Krüger et al., 2014), *Tenebrio molitor* Linnaeus, 1758 (Tenebrionidae) (Gadzama et al., 1977; Dailey et al., 1980; Grimes and Happ, 1980), *Leptinotarsa decemlineata* Say, 1824 (Chrysomelidae) (De Loof and Lagasse, 1972), *Acanthoscelides obtectus* Say, 1831 and *Bruchidius atrolineatus* Pic, 1921 (Curculionidae Bruchinae) (respectively: Cassier and Huignard, 1979; Glitho and Huignard, 1990).

The aim of this work is to expand the morphological knowledge on male accessory glands of blister beetles by investigating for the first time the ultrastructure of these organs in *Meloe proscarabaeus* Linnaeus, 1758 (Meloinae, Meloini) (Fig. 1) using light, electron and ion beam microscopy. Apart from providing a comprehensive overview of male accessory glands in this blister beetle species, the intra-specific comparison of some interesting features observed in these organs provides clues on their potential role in storing and possibly producing cantharidin in Meloidae.

2. Material and methods

2.1. Material examined

Eight adult males of *Meloe proscarabeus* (Fig. 1) were collected from January to March 2019 in central and northern Italy (Lazio, RM, Roma, Insugherata Park, 41°57′24″N 12°25′51″E 55 m; Abruzzo, AQ, L'Aquila town, 42°21′05″N 13°23′51″E 711 m; Friuli – Venezia Giulia, PN, Fanna, 46°11′25″N 12°21′10″E 247 m). The insects were sampled in pastures and gardens during feeding or courtship behaviour. The specimens were kept alive in plastic fauna-boxes filled with 4 cm of wet coconut fibre substrate, to maintain humidity, and fed daily with fresh lettuce and apple slices.

Samples collected in different locations showed no differences in the morphology of the male accessory glands analysed according to the following methods.

2.2. Light microscopy

Two male specimens of *M. proscarabaeus* were anesthetized with CO_2 and their reproductive systems were rapidly dissected in saline solution under a SZX51 stereo microscope (Olympus, Tokyo, Japan). Pictures of the dissected systems were acquired using an OM-D E-M5 digital camera (Olympus, Tokyo, Japan) mounted on an Axio Zoom V16 microscope (Carl Zeiss AG; Oberkochen, Germany).

For the histological analysis, the same resin embedded samples processed for electron microscopy (according to the methods described below) were cut into 1 μm thin sections using a glass knife on an Ultracut T ultramicrotome (Leica Microsystems, Vienna, Austria). Semi thin sections, stained with 1% toluidine blue, were

Fig. 1. *M. proscarabaeus* male, living specimen in laboratory.

observed and photographed using a BX51 light microscope (Olympus, Tokyo, Japan) equipped with an OM-D E-M5 digital camera.

2.3. Focused ion beam/Scanning electron microscopy

Six live males were euthanized with CO_2 prior to dissection: their abdomens were removed and immediately submerged in cacodylate buffer 0.1 M (pH 7.4) in order to isolate the accessory glands after the removal of ventrites. Each gland was cut in smaller pieces to facilitate the subsequent fixation and staining processes. The small pieces of glandular tissues were immersed in Karnovsky's solution for 12 h at 4 °C, rinsed four times in cacodylate buffer 0.1M (pH 7.4) for 15 min, post-fixed in 1% osmium tetroxide in cacodylate buffer 0.1M (pH 7.4) for 2h at 4 °C and en-bloc stained with 2% aqueous uranyl acetate. Subsequently, the samples were dehydrated in a graded ethanol series (70%, 85%, 95%, 30 min each and 100% for 2h), embedded in epoxy resin and finally polymerized for 72 h at 60 °C.

These resin embedded samples were processed in two different ways, either cut into ultrathin sections of 80 nm with a diamond knife (Diatome Ltd, Bienne, Switzerland) on a Ultracut T ultramicrotome (Leica Microsystems, Vienna, Austria) or cut into thick sequential slices of about 15–20 μm with a glass knife on the same ultramicrotome. The ultrathin sections were collected on TEM grids and examined with the STEM detector of a Dual Beam (FIB/SEM) Helios Nanolab 600 (FEI Company, Hillsboro, USA) at the electron microscopy laboratory of Roma Tre University (LIME, Rome, Italy). The thick sequential slices instead were secured to aluminium stubs with a conductive adhesive carbon disc, sputtered with a thin layer (30 nm) of gold using a K550 sputter coater (Emithech, Kent, UK), and analysed with FIB/SEM following the "Slice&Mill" method (Di Giulio and Muzzi, 2018).

3. Results

3.1. Gross morphology of the male reproductive system

The internal male reproductive system of *M. proscarabeus* consists of a pair of testes, two *vasa deferentia*, three pairs of differently shaped accessory glands and an ejaculatory duct (Fig. 2). The long ejaculatory duct of ectodermal origin (about 30 mm long and 0.7–0.9 mm wide) is anteriorly surmounted by an expanded area of mesodermic origin that receives the secretion of three different pairs of accessory glands and the content of two *vasa deferentia*, each of which is connected to a testis. The oval testis has a diameter of about 5 mm and contains 100–120 follicles, which are tightly packed and radially arranged. Near the testis, the *vas deferens* is a

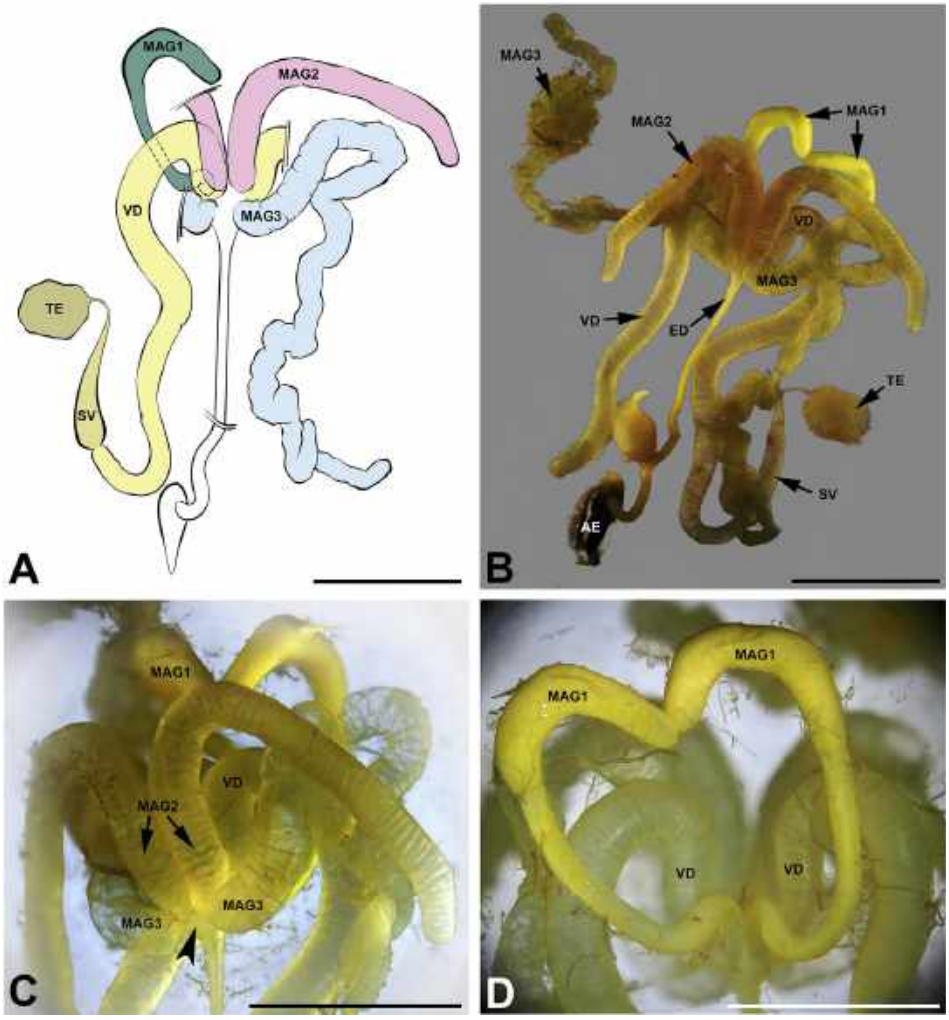


Fig. 2. General structure of the male reproductive system of *M. proscarabaeus*. (a) Schematic drawing of the system in ventral view. (b) Light micrograph of the dissected system in ventral view (c) Close up of the enlarged area (arrowhead) receiving the secretions of the three pairs of accessory glands and the *vasa deferentia*. (d) Close up in dorsal view, showing the insertion of the first pair of accessory glands. AE aedeagus, ED ejaculatory duct, MAG1 male accessory glands of the first pair, MAG2 male accessory glands of the second pair, MAG3 male accessory glands of the third pair, SV Seminal vesicle, TE testicle, VD *vasa deferentia*. Scale bars: A, B = 1 cm; C = 0.75 cm; D = 0.5 cm.

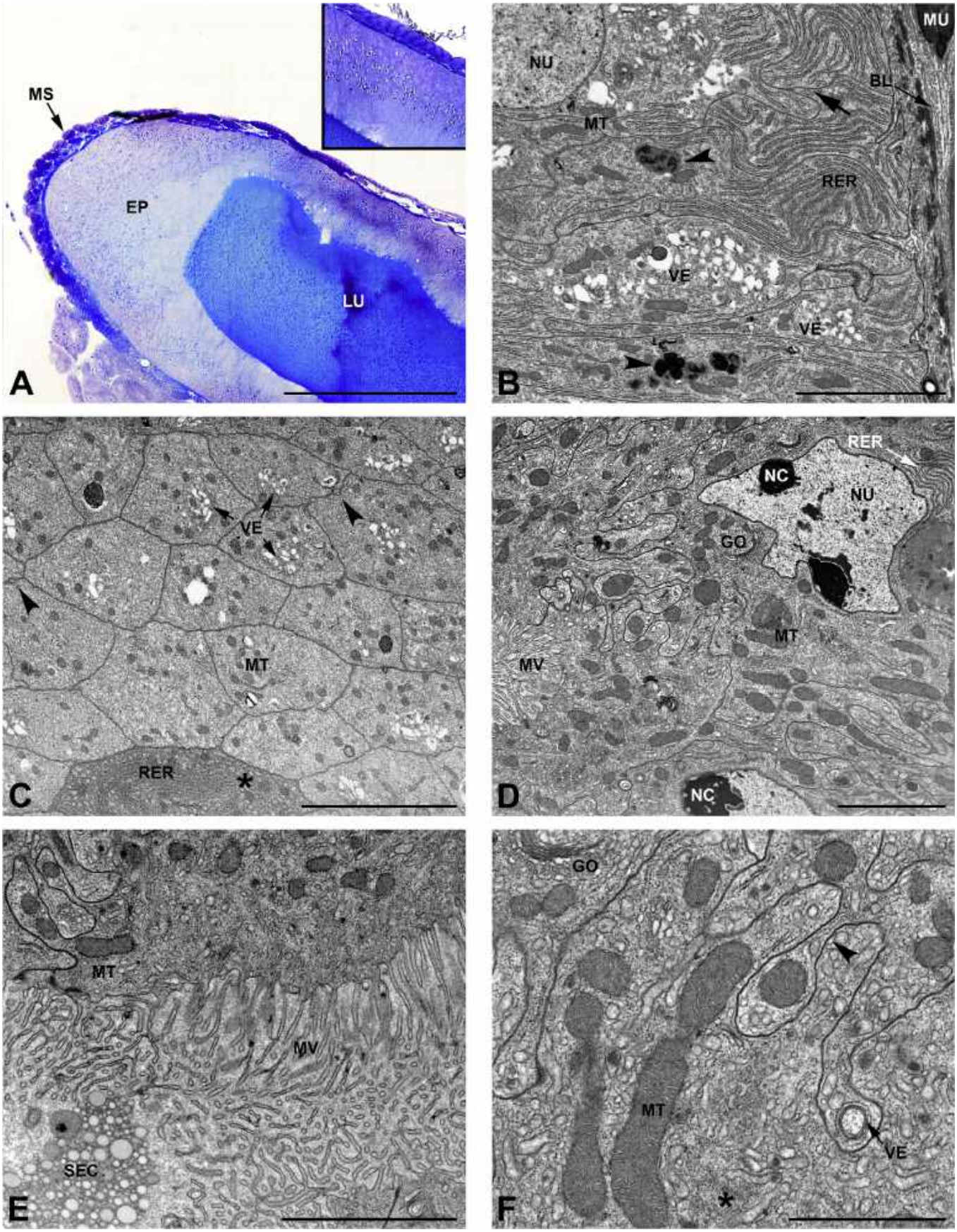


Fig. 3. Morphology of the first pair of male accessory glands in *M. proscarabaeus*. (a) Histological oblique section of the gland showing the pseudostratified epithelium, the developed muscular sheath with the differently oriented muscles and the broad lumen filled with secretions. (b–f) Ultrastructural analysis of the first pair of accessory glands. (b)

short tube with a constant diameter of 0.3 mm and a length of about 3 mm. This tubular region is followed by an expanded and horn-shaped area (about 12 mm long) serving as a seminal vesicle. The latter is followed by a well-developed and long (about 30 mm) glandular region, which is characterized by a serpentine course and an increased diameter (about 3 mm compared to 2 mm of the seminal vesicle). The first pair of accessory glands (Fig. 2d) inserts dorsally into the expanded region above the ejaculatory duct, they appear as C-shaped tubular structures with a blind anterior end that joins that of the symmetrical gland. These glands are 25 mm long and have a diameter ranging from 1.5 to 2 mm, with a progressive thickening near the intermediate region and narrowing at the insertion level in the expanded region. The accessory glands of the second pair are tubular structures (about 30 mm long) with a blind apex and a more or less constant diameter of 2 mm; their insertion in the expanded region above the ejaculatory duct is ventral and posterior to that of the *vasa deferentia*. The third pair of accessory glands has an irregular shape; they appear as sinuous and convoluted structures 65 mm long, often intertwined with the other components of the reproductive system. They are much more delicate and with less turgidity than the other accessory glands and has a variable diameter comprised between 1 and 2.5 mm.

3.2. Morphology of the first pair of male accessory glands

The first pair of male accessory glands of *M. proscarabaeus* is associated with a muscular layer (Fig. 3a) consisting in inner circular muscles and outer longitudinal muscles. These muscles surround a pseudostratified epithelium that delimits a large lumen containing coarse and dense glandular secretions (Fig. 3a). The monolayered epithelium is composed of columnar cells about 80–110 μm tall lying on a basal lamina 0.4–0.6 μm thick (Fig. 3b). A nucleus, approximately 5 μm in diameter, with an evident nucleolus is usually located in the basal region of the cell (Fig. 3a, b) and is only rarely found in the apical region towards the gland lumen (Fig. 3d). The rough endoplasmic reticulum is especially abundant near the nucleus where its cisternae are densely packed and arranged in parallel series (Fig. 3b–d). Evenly distributed in the cytoplasm there are abundant mitochondria of various forms (Fig. 3b–f) and free ribosomes (Fig. 3f). Rare Golgi systems are found near the nucleus (Fig. 3d) while many electron-lucent vesicles are dispersed throughout the whole cytoplasm (Fig. 3b, c, f). Vesicles containing dark spherules are preferentially located in the basal region (Fig. 3b).

The plasma membrane shows an evident and sinuous contour in the basal region of the cell (Fig. 3b), while in the medial regions it has a straight and rectilinear course that gives the cells a more polygonal aspect in cross section (Fig. 3c). In the apical region of the cells (Fig. 3d–f) the plasma membrane appears again more invaginated, showing a high degree of interdigitation while the area near the glandular lumen bears numerous long and thin microvilli, some of which are branched (Fig. 3e). Secretions occupying the glandular lumen appear as spherical structures of various dimensions, immersed in a particulate matrix and showing a variable degree of density that ranges from moderately electron-dense to electron-lucid (Fig. 3e).

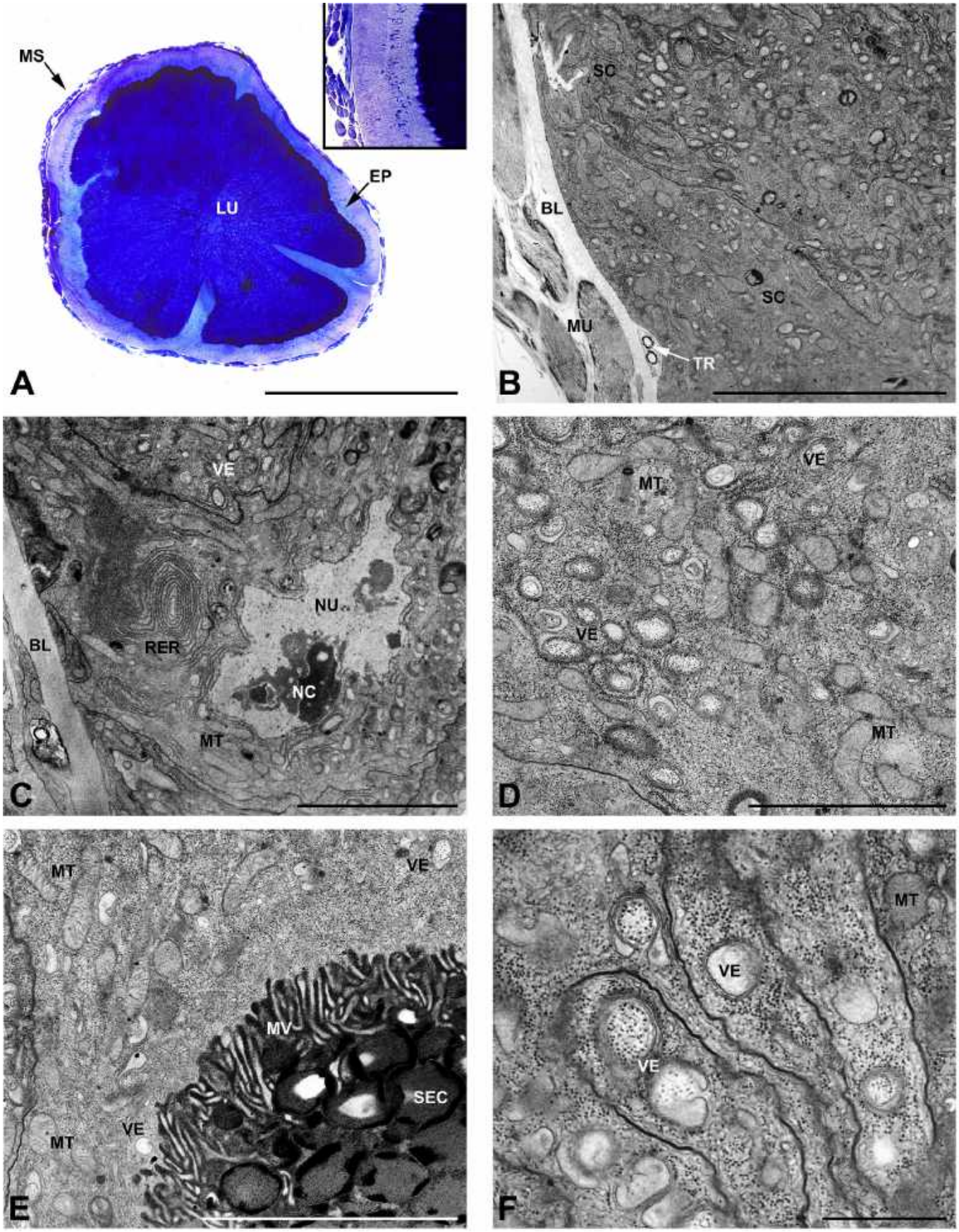
3.3. Morphology of the second pair of male accessory glands

Each gland belonging to the second pair of male accessory glands of *M. proscarabaeus* is enclosed in a muscle layer consisting of inner circular muscles and outer longitudinal muscles (Fig. 4a, b). The glands consist of columnar cells 35–50 μm tall, forming a monolayered epithelium that undergoes several folding and consequent involutions towards a wide lumen receiving the secretions (Fig. 4a). At the base of these cells, the plasma membrane is strictly applied to a basal lamina 0.5 μm thick, even in the folded regions of the epithelium (Fig. 4b, c). Adjacent cells are tightly appressed to one another; their lateral membranes show a moderately sinuous contour, without particular interdigitations (Fig. 4b–d), while in the apical region membranes always bear numerous microvilli (Fig. 4e). A nucleus, presenting a distinct nucleolus and patches of heterochromatin, is usually located medially in the cell (Fig. 4a, c) and is surrounded by a well-developed rough endoplasmic reticulum represented by a series of flattened cisterns, arranged in parallel fashion and appearing as whorls (Fig. 4c). The cytoplasm is filled with many irregularly shaped vesicles of variable diameter having two membranes; a few of them contain electron-lucent material, while the vast majority contains a minute electron-dense particulate immersed in an electro-lucent matrix (Fig. 3d, f). Mitochondria appear moderately abundant and evenly distributed in the cell (Fig. 4b–e), while Golgi complexes are rarely present. The numerous and moderately long microvilli, arising from the apical region of the cell, are closely spaced around the lumen that is filled with a set of spheroidal aggregates having a higher electron density towards the edges and a lighter interior, which in some cases may appear even electron-transparent (Fig. 4e).

3.4. Morphology of the third pair of male accessory glands

The third pair of male accessory glands of *M. proscarabaeus* exhibits a remarkably thin muscular layer of circularly and transversally oriented muscles, loosely encasing a thin monolayered epithelium that define a very large lumen (Fig. 5a). The cells, 15–20 μm tall, are cuboidal or barely columnar and are kept separated from the muscles by a basal lamina 0.6–0.8 μm thick (Fig. 5b). In some gland regions, the plasma membrane is completely adherent to the basal lamina (Fig. 5b); conversely, for most of the gland length, the plasma membrane at the cell base is widely folded, creating a conspicuous lacunar system next to the basal lamina (Fig. 5d, e). The oval nucleus, basally or medially located, contains an evident nucleolus and large clumps of chromatin immersed in the nucleoplasm (Fig. 5b, d). The endoplasmic reticulum is extensively distributed throughout the cell and consists of compressed cisterns, parallel to each other (Fig. 5c, e). Golgi complexes are composed small cisterns scattered throughout the cytoplasm (Fig. 5c, e). This latter is rich in moderately long mitochondria, evenly spread across the cell, and numerous vesicles and inclusions of various appearances (Fig. 5c, e, f). Several vesicles have an electron lucid content while many others are filled by electron-dense flocculent structures, immersed in a low electron-dense matrix; both kinds are widespread throughout the whole cytoplasm and appear smaller near the apical region of the cell (Fig. 5c, e, f). Although to a lesser extent, the cytoplasm shows also

Sections of the basal region of the cells showing a rounded nucleus, developed rough endoplasmic reticulum stacks, abundant mitochondria and vesicles crowding the cytoplasm. Arrow pointing at infolded region of the cell membrane and arrowheads pointing at mitochondria with electrondense inclusions. (c) Transverse section of the medial region of the cells showing their polygonal shape and the interdigitations appearing as small canals (arrowheads). Asterisk marks an exhausted cell with strongly electrondense cytoplasm among the others. (d) Micrographs of apical region of the cells showing an irregular nucleus and widespread mitochondria. (e) Plasma membrane with branched microvilli projected toward a lumen containing secretion spherules. (f) Close up showing long mitochondria, Golgi complex, membrane folding (arrowhead) and free ribosomes (marked by an asterisk). BL basal lamina, EP epithelium, GO Golgi apparatus, LU lumen, MU muscles, MS muscular sheath, MT mitochondria, MV microvilli, NC nucleolus, NU nucleus, RER rough endoplasmic reticulum, SEC secretory products, VE vesicles. Scale bars: A = 250 μm; B-E = 4 μm; F = 2 μm.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multilamellar bodies and electron-dense inclusions, which are located especially in the basal area of the cells (Fig. 5c, e). In the apical region of the cells, several vesicles approach the plasma membrane, which is rich in long and ramified microvilli that are moderately close to each other (Fig. 5d, f). The glandular lumen is occupied by a weakly electron-dense fibrillar secretion (Fig. 5b) and by irregular protrusion and ampullaceus expansion of the microvilli (Fig. 5f).

3.5. Morphology of the glandular regions of the vasa deferentia

Similarly to the three pairs of *mesadaenia* accessory glands presented above, the *vasa deferentia* consist of a developed and continuous muscular layer, which however is formed by parallel muscle fibres which are, obliquely oriented in relation to the cells that form a monolayered epithelium delimiting a broad central lumen (Fig. 6a). Tracheae and tracheoles are located between both the inner and the outer fibres of the external muscular sheath (Fig. 6b). The columnar secretory epithelial cells lie on a basal lamina 1.5 μm thick and are characterized by the well-developed rough endoplasmic reticulum (Fig. 6c). This latter is spread throughout and occupies most of it, occurring both as flattened cisternae and as swollen vesicles (Fig. 6c, e). Large nuclei are located in the medial region of the cells and contain several irregular clumps of heterochromatin arranged in a disorderly manner (Fig. 6a, c, d). Numerous long and slender mitochondria are randomly and evenly distributed in the cytoplasm, while Golgi elements are scarce and seem to be mainly located in the medial and apical region of the cell (Fig. 6d, e). Several electron-dense secretory granules and a few electron-lucent vesicles are dispersed in the medial and apical region of the epithelium (Fig. 6e, f). Their content is released at the level of the apical cell surface, which bears many short and unbranched microvilli, densely packed and projecting toward a lumen that is filled by a homogeneous and electron dense substance (Fig. 6e).

4. Discussion

Our work on male accessory glands of the Palaearctic species *M. proscarabaeus* represents the first contribution on the ultrastructural morphology of these organs in blister beetles. The morpho-anatomical results are discussed in the light of the information available in the literature. Hence, in the following paragraphs, the reproductive system of *M. proscarabaeus* is first compared with those of other blister beetles and, in particular, with that of *L. nuttalli* (Gerber et al., 1971a). Differences and similarities between the three different pairs of male accessory glands within *M. proscarabaeus* are then discussed and the potential role of these structures in the production and/or storing of cantharidin is remarked.

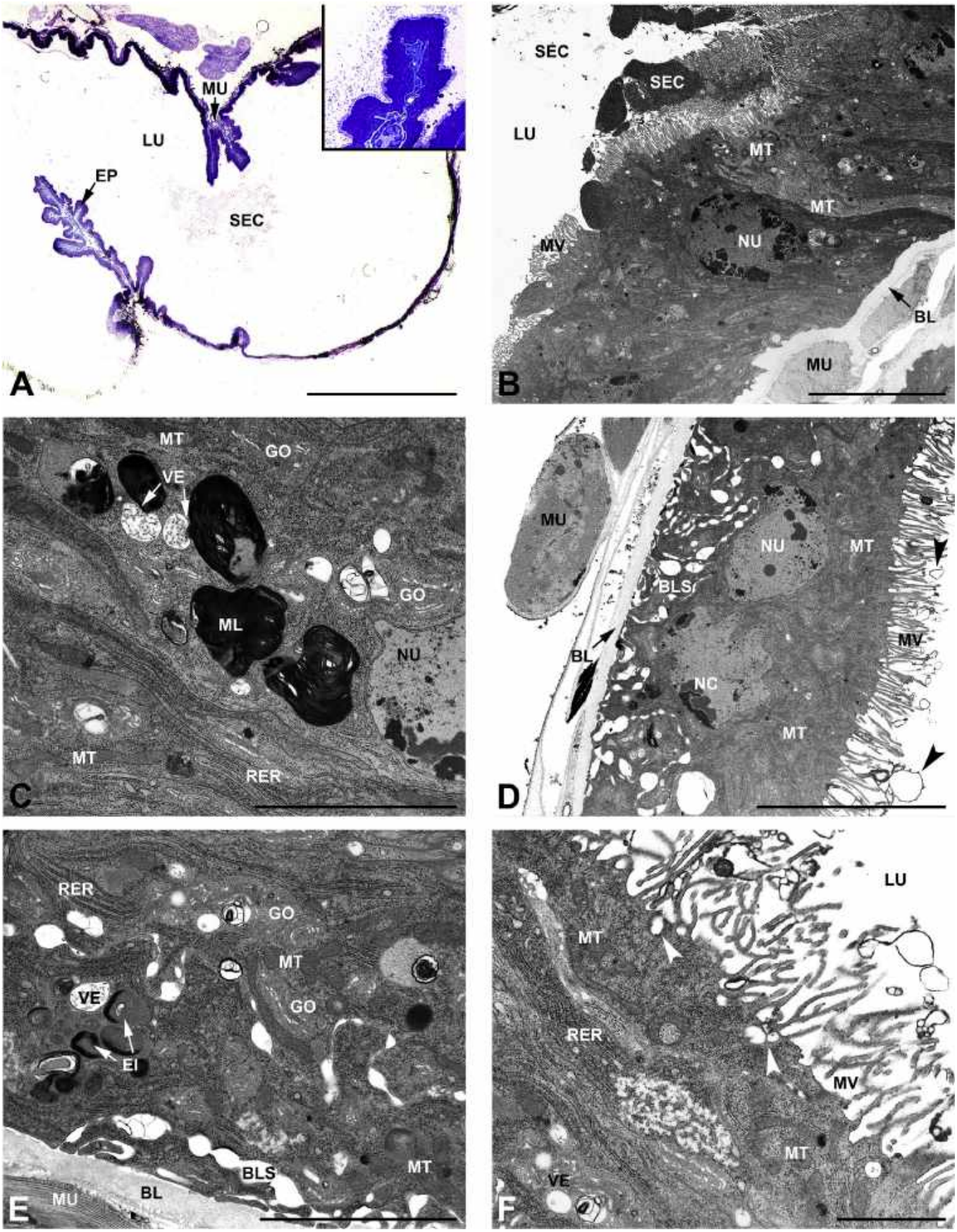
4.1. Reproductive system of Meloe proscarabaeus and of other Meloidae

The reproductive system of *M. proscarabaeus* consists of a pair of round testes, two large *vasa deferentia*, and three pairs of accessory glands of mesodermic derivation that release their secretions into an ectodermal ejaculatory duct (Fig. 2). As illustrated by Beauregard (1890) and Gupta (1965, 1966a, 1966b, 1967), this general organization is shared by many other blister beetle species. Nevertheless, accessory glands with atypical configurations have been reported for the following species: *Horia debyi* (Fairmaire) (as *Cissites testaceus*) (Nemognathinae, Horiini), having a single pair of accessory glands (Bugnion, 1909); *Hycleus phaleratus* Pallas, 1781 (as *Mylabris*) (Meloinae, Mylabrini), having two pairs (Li, 1952), and *Epicauta rufidorsum* Goeze, 1777 (as *Epicauta verticalis*) (Meloinae, Epicautini), having four pairs of accessory glands (Beauregard, 1890). However, according to Gupta (1966a) and Gerber et al. (1971a), some of these old reports might be incorrect rather than true exceptions to the typical configuration comprising three pairs of accessory glands.

Within the exceptional diversity existing in the accessory glands of Coleoptera, those of blister beetles appear to be unique and clearly distinguishable by their number, shape and unusual size. For example many beetle genera of different families belonging to both Adephaga and Polyphaga (e.g. Carabidae: *Limodromus* Motschulsky, 1850; *Pterostichus* Bonelli, 1810; Scarabaeidae: *Popillia* Serville, 1825; Chrysomelidae: *Zygogramma* Chevrolat in Dejean, 1836; *Leptinotarsa* Chevrolat in Dejean, 1836) own only a single pair of tubular and unbranched male accessory glands of moderate dimensions (Anderson, 1950; Gerber et al., 1978; DeLoof and Lagasse, 1972; Krüger et al., 2014; Schubert et al., 2017). Other species, such as the darkling beetle *T. molitor*, have two pairs of differently shaped accessory glands, i.e. a pair of tubular and a pair of bean-shaped structures (Dailey et al., 1980), while five pairs of accessory glands, with a particularly complex shape, are found in the bean weevil genus *Acanthoscelides* Schilsky, 1905 (Cassier and Huignard, 1979). Interestingly, three pairs of accessory glands also characterize the reproductive system of the bean weevil *B. atrolineatus* (Glitho and Huignard, 1990), but their shape and relative dimensions are completely different from those identifying blister beetles.

It is, however, interesting to note that, when compared to the internal reproductive system of many other blister beetles (Beauregard, 1890; Gupta, 1965, 1966a; 1966b, 1967; Gerber et al., 1971a), the one of *M. proscarabaeus* has some uncommon features, in particular with regard to the first and second pair of accessory glands. In fact, while the first pair of glands is usually well developed and spiral-shaped, the one of *M. proscarabaeus* is represented by relatively small C-shaped structures that show only a moderate degree of curvature, which is far from the typical spiral winding characterizing the glands of most blister beetles. Although a small size and limited winding of the first pair of accessory glands have also been previously observed in the Nearctic *Meloe niger* Kirby, 1837 (Gupta, 1965), belonging to the same subgenus, the scarcity of observation do not allow confirming a general rule on such a peculiar feature in this genus. Another notable difference concerns the glands of the second pair; in many Meloidae these structures are usually very small and extremely short (Beauregard, 1890; Gupta, 1965, 1966a, 1697), while in *M. proscarabaeus* these are remarkably developed both in length and width (Fig. 2). As for the third pair of glands, they are well developed in *M. proscarabaeus* and consist of sinuous and twisted structures that do not differ significantly from those of other blister beetles and thus, seem to be a constant within the family. Similarly, the *vasa deferentia* of *M. proscarabaeus* also do not differ from those of other meloid species and have features that are common to the whole family,

Fig. 4. Morphology of the second pair of male accessory glands in *M. proscarabaeus*. (a) Histological cross section of the gland showing the infolding and involution of the monolayered epithelium towards the lumen. (b–f) FIB/SEM micrographs of the gland. (b) Basal region of the cells laying on a basal lamina surrounded by muscles. (c) Irregular nucleus surrounded by mitochondria and rough endoplasmic reticulum. (d) Cytoplasm rich in mitochondria and secretory vesicles. (e) Apical part of the cytoplasm showing microvilli and secretions in the lumen. (f) Close up of the secretory vesicles near membrane infolding. BL basal lamina, EP epithelium, LU lumen, MU muscles, MS muscular sheath, MT mitochondria, MV microvilli, NC nucleolous, NU nucleus, RER rough endoplasmic reticulum, SC secretory cell, SEC secretory products, TR trachea, VE vesicles. Scale bars: A = 400 μm; B = 10 μm; C = 5 μm; D = 3 μm; E = 4 μm; F = 1 μm.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such as the clear presence of a seminal vesicle and the development of a wide and expanded glandular region.

4.2. Comparison between Meloe proscarabaeus and Lytta nuttalli male accessory glands

Despite no other data on the ultrastructure of male accessory glands in Meloidae are available, the detailed histological studies performed on the internal genitalia of *L. nuttalli* and the commendable work on the composition and fate of its spermatophore and accessory glands secretions (Gerber et al., 1971a, 1971b) allow to better evaluate some interesting discrepancies between this species and *M. proscarabeus*.

With regard to the general organization of the tissues, in both *M. proscarabaeus* and *L. nuttalli*, all three pairs of accessory glands and the glandular region of the *vasa deferentia* have a rather similar arrangement that features an outer sheath of muscles encasing a glandular epithelium that delimits a large lumen in which secretions are released and stored. This configuration is commonly found in the male accessory glands of many insects (Dapples et al., 1974; Lai-Fook, 1982; Glitho and Huignard, 1990; Kaulenas, 1992) involving a monolayered epithelium and a muscular layer consisting of an internal area of circular muscles and an external area of longitudinal muscles. The epithelium of the first pair of male accessory glands in both *M. proscarabaeus* and *L. nuttalli* appears slightly different form the one of the other male accessory glands pairs and vasa deferentia in having a pseudostratified epithelium with very tall cells. An interesting difference between the two species concerns the number and type of secretions produced by this first pair of glands. In *L. nuttalli* Gerber et al. (1971a) highlighted as many as six different types of secretions localized in specific glandular regions that suggests the involvement of different cell types or, alternatively, an independent (but unlikely) maturation of the secretion over time. In fact, regardless of the total number of accessory glands in an insect reproductive system, a specific gland may consist of either a single type of epithelial cells or by two or more cell types. In the first case, each cell produces only a single kind of secretion, consisting of a specific group of substances. In the second case, each cell type produces a different kind of secretion and the different types of cells are usually grouped in distinct and specific glandular regions and are only rarely intermingled (Tongu et al., 1972; Ramalingam and Craig, 1978; Dailey et al., 1980; Chen, 1984; Kaulenas, 1992; Krüger et al., 2014). Differently from *L. nuttalli* (Gerber et al., 1971b), only one type of secretion can be recognised in *M. proscarabaeus* and ultrastructural investigations confirm that, throughout its length, the monolayered epithelium of the glands exhibits the same features with a single type of microvillated cells producing and releasing the secretion in the glandular lumen through a simple exocytosis. In the analysed samples we were unable to detect the presence of a holocrine secretion mechanism nor the presence of the associated cell turnover. Assuming that in *M. proscarabeus* the first pair of glands is involved in the production of a posterior spermatophoral tube as in *L. nuttalli* (Gerber et al., 1971b), the lower complexity of the secretion in the former species suggests that this tubular portion of the spermatophore could be structurally much simpler, if not completely absent.

Despite their greater development and unusually large size, the second pair of glands of *M. proscarabaeus* appears structurally similar to that of *L. nuttalli* (Gerber et al., 1971a, 1971b), presenting a typical muscular layer of circular and longitudinal muscles and a monolayered epithelium consisting of a single type of cell, likely to produce only one kind of uniform secretion. Assuming that the second pair of glands in *M. proscarabaeus* has a function similar that of *L. nuttalli* (Gerber et al., 1971b), its glandular products would serve to prevent sperm reflux after mating by sealing the spermatophoral tube and the female genital tracts. In *M. proscarabaeus*, the increase in gland size, not followed by a higher cellular complexity, may be simply related to the need to produce more substance in comparison to blister beetles with smaller glands. In fact, a lower complexity or the total absence of the spermatophoral tube in *M. proscarabaeus*, could lead to the need for a greater quantity of glandular products for plugging a larger and less organized tubular region or the entire female genital tract, in order to avoid sperm backflow at the end of the mating. Anyway, the increase in gland size and the production of more secretion could also be related to the larger size of the species.

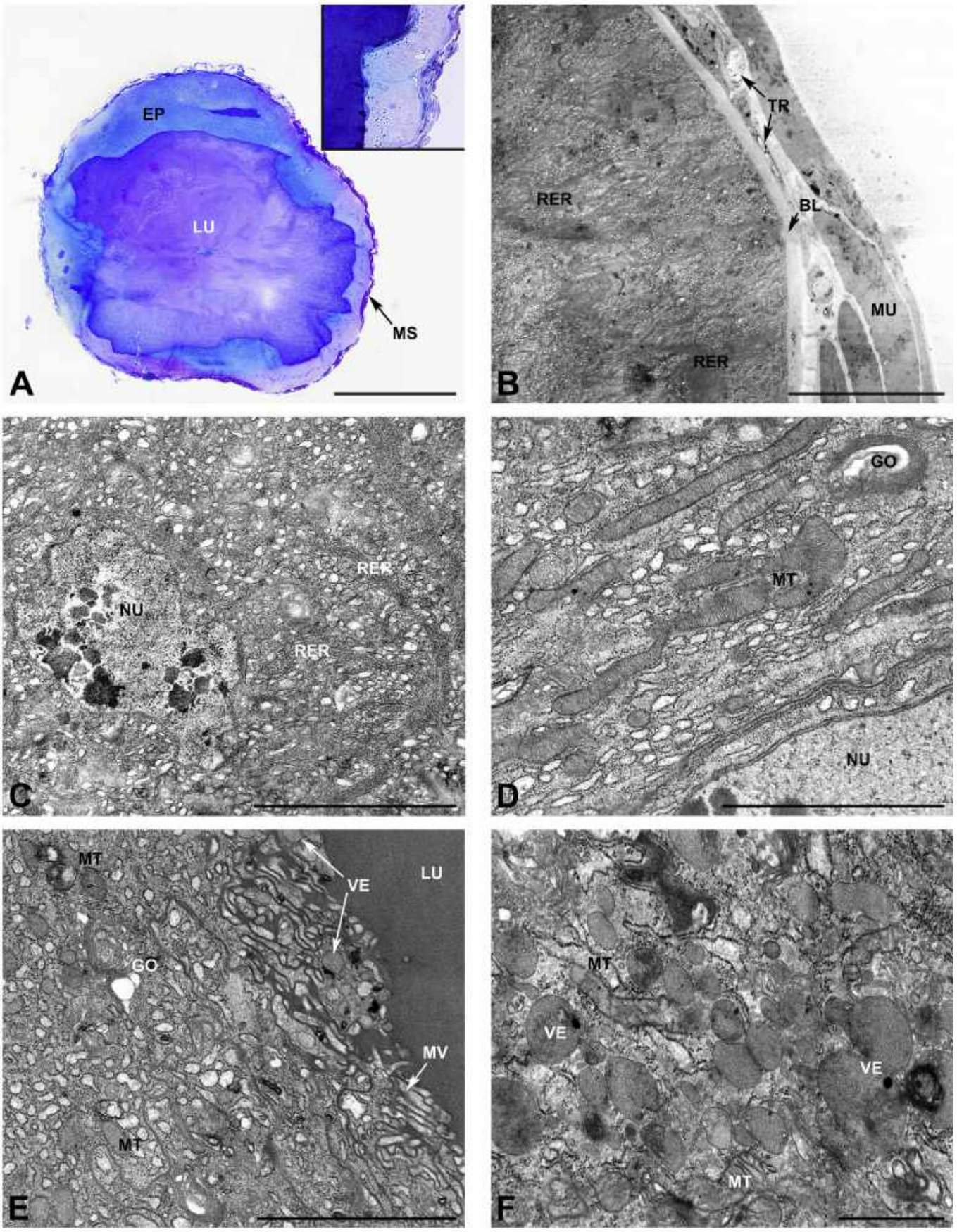
As their gross morphology suggests, the glands of the third pair of *M. proscarabaeus* show a cellular organization that is very similar to that of *L. nuttalli*; in fact, in both cases these voluminous glands have an extremely enlarged lumen surrounded by a rather flattened epithelium. The muscular layer of both species, made up of internal circular muscles and external longitudinal muscles, is less developed than the one found in the other pairs, which always have a thicker epithelium, even in the case of a cellular monolayer. Gerber et al. (1971b) have shown that during the copulation of *L. nuttalli*, the secretions of the third pair of male accessory glands reach the female receptacle where they contribute to the formation of the anterior portion of the spermatophore, which will contain the sperm immersed in the gelatinous substances produced by the glandular region of *vasa deferentia*. Considering the structural similarities and the comparable relative dimension, it is likely that in *M. proscarabaeus* these glands have analogous functions in the formation of the spermatophore.

Vasa deferentia do not show significant differences between *M. proscarabeus* and *L. nuttalli*, in fact, as in all blister beetles, they are enlarged and show an equally expanded glandular region positioned immediately after the seminal vesicles. The only minor differences concern a different arrangement of the muscles, which in *M. proscarabaeus* are arranged obliquely instead of longitudinally and circularly, and the presence of an internal transverse wrinkling that seems to be present only in *L. nuttalli*. Probably in both species, and also in other Meloidae, the *vasa deferentia* maintain the function of nourishing the spermatozoa and create the gelatinous matrix which will incorporate them and which will create the anterior portion of the spermatophore.

4.3. Comparative anatomy of the male accessory glands within Meloe proscarabaeus

The ultrastructural analyses performed on *M. proscarabeus* showed that the different accessory glands and the glandular region of *vasa deferentia* have many characteristics in common. For

Fig. 5. Morphology of the third pair of male accessory glands in *M. proscarabaeus*. (a) Histological section of the gland, note the thinness of epithelium and muscle sheath. (b–f) Ultrastructural features of the glandular epithelium. (b) Micrograph of a rare glandular region where the cells adhere totally to the basal lamina. Note the developed nucleus and the abundant and different secretions in the gland lumen. (c) Basal region of the cell showing abundant rough endoplasmic reticulum, different kind of secretory vesicles and multilamellar bodies. (d) Epithelium showing a basal lacunar system and microvillated apical membrane displaying ampoule-shaped protrusions in the gland lumen (arrowheads). (e) Basal region of the cells displaying labyrinthine system and different secretory vesicles. (f) Apical region bearing numerous microvilli, note small electronlucid vesicles approaching and fusing with the membrane (arrowhead). BL basal lamina, BLS basal lacunar system, EI electrondense inclusions, EP epithelium, GO Golgi apparatus, LU lumen, ML multilamellar bodies, MU muscles, MT mitochondria, MV microvilli, NC nucleolus, NU nucleus, RER rough endoplasmic reticulum, SEC secretory products, VE vesicles. Scale bars: A = 450 μm; B = 10 μm; C = 4 μm; D = 10 μm; E = 4 μm; F = 2 μm.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example, all of them are of mesodermal derivation and are enclosed by a muscle layer composed of internal circular and external longitudinal muscles, except for the *vasa deferentia* in which the muscles show an oblique orientation with respect to the cells. Each pair of accessory glands has an epithelium consisting of a single cell type and seems to be involved in the production of a specific set of substances that constitute a uniform secretion in their lumen. Among the other common features, all the examined epithelia presented an intense metabolic activity testified by large nuclei with well evident nucleoli, abundant rough endoplasmic reticulum and many mitochondria, evenly scattered along the cytoplasm. In addition, the presence of numerous different vesicles which are characteristics for each pair of accessory gland, together with the presence of abundant microvilli on the apical membrane attest the secretory activity of these epithelia, in which, the substances are usually released into the glandular lumen through simple exocytosis or by apocrine secretion as suggested by the ampullaceous protrusions observed in the third pair of accessory glands. The extensive and constant presence of rough endoplasmic reticulum and the abundance of mitochondria suggest that these glands are mainly involved in the synthesis of proteinaceous substances, as observed in many other male accessory glands (De Loof and Lagasse, 1972; Gadzama et al., 1977; Cassier and Huignard, 1979; Davey, 1985; Gillott, 2003). Despite these similarities, the cells of the different accessory glands have their exclusive ultrastructural features, especially with regard to the appearance of the vesicles contained in the cytoplasm and the secretion present in the lumen, showing that each of them is involved in the production of different components that will contribute to the formation of the spermatophore.

4.4. Possible involvement of Meloe proscarabaeus male accessory glands in cantharidin processing

Other than producing the set of substances used in the formation of a spermatophore, accessory glands of Meloidae contain cantharidin and are involved in its transfer from male to female, as already observed back in XIX century by Leidy (1860). In fact, this author was among the first to hypothesize a transfer of defensive substances during copulation after noticing high concentrations of a blistering agent in the male genitalia and in the corresponding tract of the mated females of *Epicauta vittata* (Fabricius, 1775) (as *Lytta*). The transfer of cantharidin from the male to the female as a nuptial gift, has been later confirmed in blister beetles by some chemical investigations that demonstrated a strong increase in female cantharidin content immediately after mating (Selander, 1964; Carrel et al., 1993; Dettner, 1997; Nikbakhtzadeh et al., 2007a, 2012).

Beauregard (1890), based on the higher concentration of this terpene, identified the third pair of male accessory glands as the cantharidin-producing organs, an interpretation that has been disputed by later authors. In fact, Gerber et al. (1971b) considered the third pair of glands as mainly, if not uniquely, involved in the production of the spermatophore, and questioned their role in cantharidin production in the light of the female ability to produce this terpene (Meyer et al., 1968; Schlatter et al., 1968). However, regardless of the controversial and debated ability of females to synthesize cantharidin *denovo* (Sierra et al., 1976; Dettner, 1987; but see also molecular data by Jiang et al., 2017a; Zha et al., 2017), a number of studies have confirmed the presence of a high content of this terpene in male accessory glands in blister beetles (Sierra et al., 1976; Carrel et al., 1993; Nikbakhtzadeh et al., 2007a, 2012; Jiang et al., 2017b, 2019). It is, therefore, reasonable to assume that male accessory glands are involved in the storage of cantharidin and perhaps also in its production, although previous studies have shown that in *Epicauta funebris* (Horn, 1873) (as *Epicauta pestifera*) and *Lytta polita* Say, 1824, these glands are more likely "reservoir" organs (McCormick and Carrel, 1987) and, more recently, fat bodies have been suggested to be involved in the biosynthetic pathway (Jiang et al., 2017a, 2019). Interestingly, in *M. proscarabaeus* only the glands of the third pair exhibit epithelial cells with a strongly folded membrane forming a labyrinthine lacunar system that suggests an absorption of substances from the haemolymph by these cells. Since it is well established that blister beetle haemolymph contains significant amounts of cantharidin (Beauregard, 1890; Nikbakhtzadehet al., 2012, 2007b; Mebs et al., 2009; Bravo et al., 2017; Gisondi et al., 2019), it is possible that the glands of the third pair of *M. proscarabaeus* absorb haemolymphatic cantharidin in order to concentrate this substance inside its lumen.

Although current knowledge does not allow excluding the involvement of the other accessory glands in the transfer and/or storage of cantharidin, it is still interesting to highlight how these accessory glands differ from defensive glands producing terpenoid compounds in other insects. In fact, glands containing terpenes are usually of ectodermal derivation and are often associated with a cuticular reservoir used to store the toxic compounds, a structure that obviously is absent in blister beetle accessory glands due to their mesodermic derivation. For example, Staphylinidae have two pairs of abdominal glands with the smaller one producing secretions containing terpenes, that are released in a cuticular reservoir (Happ and Happ, 1973; Schierling and Dettner, 2013). A similar compartmentalisation of terpenes, although in smaller reservoirs, has also been observed in the mandibular glands of ants and bumblebees (Stein, 1962; Brough, 1977), while bigger storing structures are found in phasmids (Happ et al., 1966). Nevertheless, glands producing terpenes can also directly release their secretions at the level of the integumental cuticle, as is the case of Chrysomelidae and Carabidae immature stages (Bünnige and Hilker, 1999, 2005; Giglio et al., 2011). Nevertheless, in all the reported cases, the terpene glands correspond to class 3 according to the classification of Noirot and Qennedey (1974, 1991) as they are made up of bicellular units, each one consisting of a secretory cell and a duct cell. This organization is widely found in many defensive glands, and it is certainly well-suited for transport and storage of toxic substances, since the cuticular intima allows the isolation of those body regions that could be negatively affected by such chemicals.

It is thus surprising to observe a complete absence of a cuticular partitioning in male accessory glands of blister beetles which suggests that cantharidin can freely move from one body district to another with no apparent cytotoxic effects.

Such an unusual morphological organization of a "defensive gland" – if confirmed in other blister beetle species – remarks the need to thoroughly investigate alternative (cellular, molecular or chemical) mechanisms of self-protection from cantharidin toxicity in these insects. This may be relevant even in applied research: in fact, since the pharmaceutical use of cantharidin is currently strongly

Fig. 6. Morphology of the glandular region of the *vasa deferentia* in *M. proscarabaeus*. (a) Histological cross section of the *vas deferens*. (b–f) Ultrastructural analysis of the *vas deferens*. (b) Muscles surrounding epithelial cells resting on a basal lamina, note the enormously diffused rough endoplasmic reticulum appearing as both flattened and swollen cisternae. (c) Nucleus with heterochromatin patches surrounded by rough endoplasmic reticulum. (d) Slender mitochondria and swollen cisternae. (e) Apical region of the cell with short microvilli. Notice the homogeneous elecrondense secretion occupying the lumen. (f) Close up of electron dense vesicles. BL basal lamina, EP epithelium, GO Golgi apparatus, LU lumen, MU muscles, MS muscular sheath, MT mitochondria, MV microvilli, NU nucleus, RER rough endoplasmic reticulum, SEC secretory products, TR trachea, VE vesicles. Scale bars: A = 400 μm; B = 20 μm; C = 5 μm; D = 3 μm; E = 3 μm; F = 1 μm.

hindered by its high toxicity (Moed et al., 2001), the detection through comparative morphological and/or genomic approaches of autogenous mechanisms of detoxification will certainly represent an invaluable knowledge to set innovative drug-delivery systems for the therapeutic application of cantharidin in the future.

CRediT authorship contribution statement

Maurizio Muzzi: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Resources. **Andrea Di Giulio:** Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Resources, Supervision. **Emiliano Mancini:** Conceptualization, Writing - original draft, Writing - review & editing, Resources, Supervision. **Emiliano Fratini:** Writing - review & editing. **Manuela Cervelli:** Writing - review & editing. **Tecla Gasperi:** Writing - review & editing. **Paolo Mariottini:** Writing - review & editing. **Tiziana Persichini:** Writing - review & editing. **Marco Alberto Bologna:** Conceptualization, Writing - original draft, Writing - review & editing, Resources, Supervision, Project administration.

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