A place for nourishment or a slaughterhouse? Elucidating the role of spermathecae in the terrestrial annelid Hormogaster elisae *(Clitellata: Opisthopora: Hormogastridae)*

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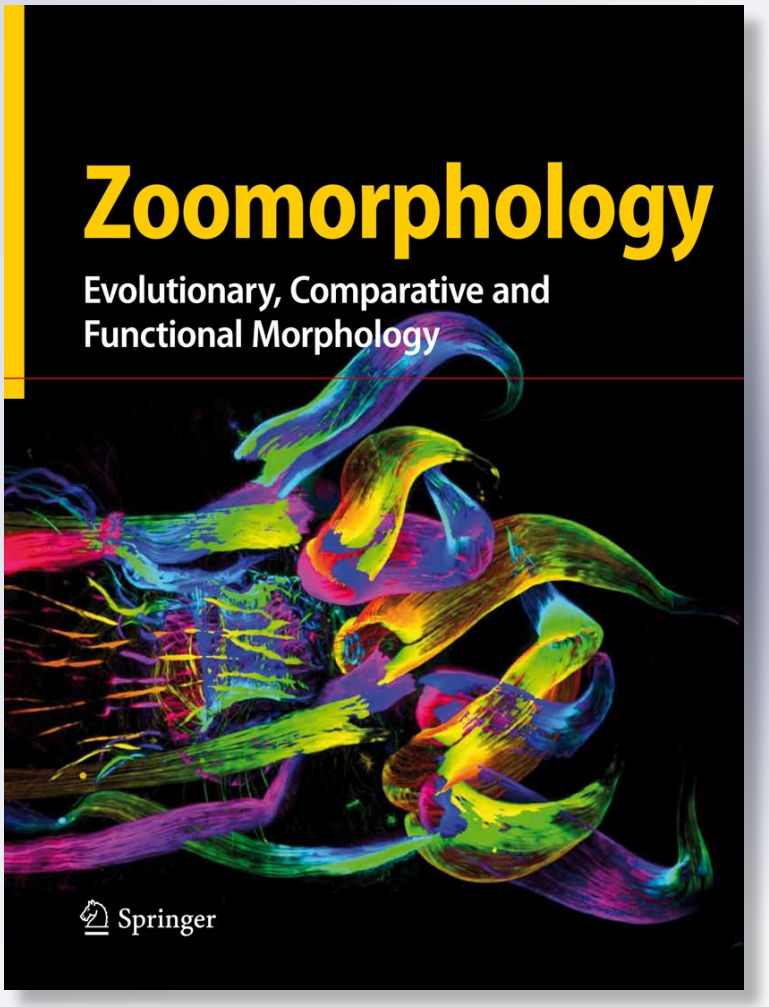

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ORIGINAL PAPER

A place for nourishment or a slaughterhouse? Elucidating the role of spermathecae in the terrestrial annelid *Hormogaster elisae* (Clitellata: Opisthopora: Hormogastridae)

Marta Novo · Ana Riesgo · Carmen Roldán · Gonzalo Giribet · Darío J. Díaz Cosín

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Abstract The capacity of storing sperm within the female reproductive tract occurs widely across vertebrate and invertebrate species. Although the type and position of spermathecae have been commonly used as a taxonomic character in Opisthopora, few studies have focused on the ultrastructural description of these interesting storage organs. This study is the first to report on the ultrastructure of the spermathecae and spermatozoa of *Hormogaster elisae*, an endemism of the central area of the Iberian Peninsula that presents two pairs of tubular spermathecae. Light and electron microscopy showed that the spermathecae are full of highly packed spermatozoa embedded in an electron-dense substance. Two layers constitute the spermathecal wall. The outer layer consists of peritoneal cells, collagenous basal laminae at different levels, several layers of striated muscle, and numerous blood vessels. The inner layer is a monostratified epithelium of prismatic cells presenting long and abundant microvilli probably for the maintenance of a favorable environment for the spermatozoa. The epithelial cells show high activity, and three different types of secretions were detected: holocrine, merocrine, and apocrine, whose hypothetical function on nourishment and/or causing quiescence is discussed here. Although no phagocytotic processes were detected, some sperm cells were observed in digestive vesicles within the cytoplasm of the epithelial cells, and there was also evidence of active sperm entrance into the epithelium. A place for nourishment or a slaughterhouse? Probably both.

Keywords Annelida · Opisthopora · Spermathecae · Spermatozoa · Sperm digestion · Transmission electron microscopy · Secretions

Marta Novo and Ana Riesgo equally contributed to this work.

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Introduction

Female sperm storage has been observed in many different animal groups, both vertebrates (Birkhead and Møller 1993; Holt and Lloyd 2010) and invertebrates, such as Insecta (e.g., Hellriegel and Bernasconi 2000), Mollusca (e.g., Beese et al. 2009), and Annelida (e.g., Hilario et al. 2005; Jamieson 2006), including Opisthopora (e.g., Jamieson 1981; Edwards and Bohlen 1996), but further studies are needed to completely understand this peculiar reproductive strategy. Sperm storage in the female body allows the spermatozoa to be used for fertilization some time after mating, which is particularly useful for Opisthopora living in the soil, because in this cryptic milieu, favorable conditions are discontinuous in time and space. Another advantage of sperm storage is that females may influence paternity by extruding or digesting sperm of different males selectively (Eberhard 1985; Birkhead et al. 1993) and/or by using physiological, anatomical, and functional adaptations of their sperm storage organs (Eberhard 1996).

Although different reproductive strategies are present in soil Opistophora, including parthenogenesis, these animals

are usually thought to be obligate out-crossing simultaneous hermaphrodites (i.e., suing reciprocal insemination, simultaneously transferring and receiving sperm during copulation). At least this is the case of the best-known species, *Lumbricus terrestris* Linnæus 1758 (Díaz Cosín et al. 2011). A similar reproductive strategy is presented by *Hormogaster elisae* Álvarez, 1977, endogeic and endemic to the central area of the Iberian Peninsula. *Hormogaster elisae* represents a complex of cryptic species (Novo et al. 2009, 2010a), which is paraphyletic relative to the remaining species in the genus (Novo et al. 2011). It has a long life cycle, when compared to other terrestrial Opisthopora species, needing, on average, 484 days for clitellum development from hatchling, and presents a cocoon production rate between 0.9 and 2.29 per specimen per year (Díaz Cosín et al. 2009).

As a part of the female reproductive system, terrestrial Opisthopora present spermathecae (of different shapes: tubular, spherical or flattened) of ectodermic origin (Jamieson 1992), where they store the received allosperm. Spermathecae are almost always paired organs: *Lumbricus terrestris* presents two pairs, whereas other species may have more, up to seven pairs in *Octodrilus complanatus* (Dugès, 1828), but some have fewer or none (Edwards and Bohlen 1996). Also, 138 intersegmental sets of spermathecae have been exceptionally cited in *Alma* (Brinkhurst and Jamieson 1971). The spermathecae are attached to the body wall by short ducts, and many species of megascolecids present one or more diverticula arising from these ducts (Jamieson 2001). Moreover, some terrestrial Opisthopora, including some hormogastrid species (Qiu and Bouché 1998), present different sperm loads within a single spermathecae. Inside the spermathecae, sperm cells are usually highly packed among an electron-dense nutritive substance, although in some instances, some species such as *Megascolides australis* McCoy, 1878, present a peculiar sperm grouping known as spermatozeugmata (i.e., sperm in oriented bundles, Vanpraagh 1995).

Hormogaster elisae presents two pairs of large tubular spermathecae, usually full of sperm in mature individuals. The posterior pair of spermathecae has been found to contain more allosperm than the anterior pair (Garvín et al. 2003). It presents no subdivision of the spermathecae, and no differential storage of sperm from distinct partners is performed, as shown by Novo et al. (2010b), who could not identify differences in the origin of the allosperm found in the four spermathecae of *H. elisae* using microsatellite markers. This may indicate that if sperm competition were present in these animals, it should be orchestrated by mechanisms inside each spermatheca. Besides an apparent non-existent differential storage, another possible mechanism of sperm competition in terrestrial Opisthopora is old sperm digestion, as reported in *Dendrobaena subrubicunda* Eisen, 1874 and other lumbricid species by Richards and Fleming (1982) or in *Amynthas* and *Metaphire* species by Teisaire and Roldán (1995).

Long storage of sperm requires the existence of a maintenance mechanism for sperm survival. Some authors have suggested the active contribution of the spermathecal epithelium to the successful preservation of sperm cells by supplying nourishing substances and providing a favorable luminal environment (Grove 1925; Varuta and More 1972; Vyas and Dev 1972; Fleming 1981). Butt and Nuutinen (1998) reported that *L. terrestris* successfully maintained the received sperm up to six months. Meyer and Bowman (1994), even though they were not measuring cocoon viability, observed that *Eisenia fetida* (Savigny, 1826) produced cocoons for up to 12 months after isolation. Garvín et al. (2003) reported spermathecae full of spermatozoa during diapause in *H. elisae*, which suggests that maintenance processes of the sperm within the spermathecae are taking place.

Very few studies have focused on the morphology and ultrastructure of the spermathecae in Opisthopora and Tubificata (Fleming 1981; Jamieson 1981, 1992; Teisaire and Roldán 1995; Vanpraagh 1995). Here, we present a detailed description of morphological features at the ultrastructural level of the spermathecae and spermatozoa of *H. elisae* for the first time using both light and transmission electron microscopy. Our aim was to unravel the role of the spermathecal wall in the maintenance of sperm, as well as the interactions between sperm cells and the spermathecal wall (i.e., the possible presence of sperm degradation, sperm lysis of the spermathecal wall, etc.). We found that the epithelial cells of the wall produce three different types of secretions (i.e., merocrine, holocrine, and apocrine) deduced to nourish the stored sperm. Also, the frequent observations of non-aberrant sperm within the epithelial cells suggest that these cells are actively digesting sperm, although phagocytosis of sperm cells was not clear in this case. In turn, it appeared that spermatozoa lysed the epithelial membranes to enter the cells.

Materials and methods

Specimen sampling

Clitellate specimens of *Hormogaster elisae* were collected by digging and hand sorting in El Molar, Madrid, Spain (GPS: N 40°44′22.9″, W 3°33′53.1″). The climatic and edaphic characteristics of the site are fully described in Valle et al. (1997) and Gutiérrez et al. (2006). Four specimens were collected every 2 months between November 2007 and May 2008, in order to evaluate the differences in the structure of the spermathecae and their contents along

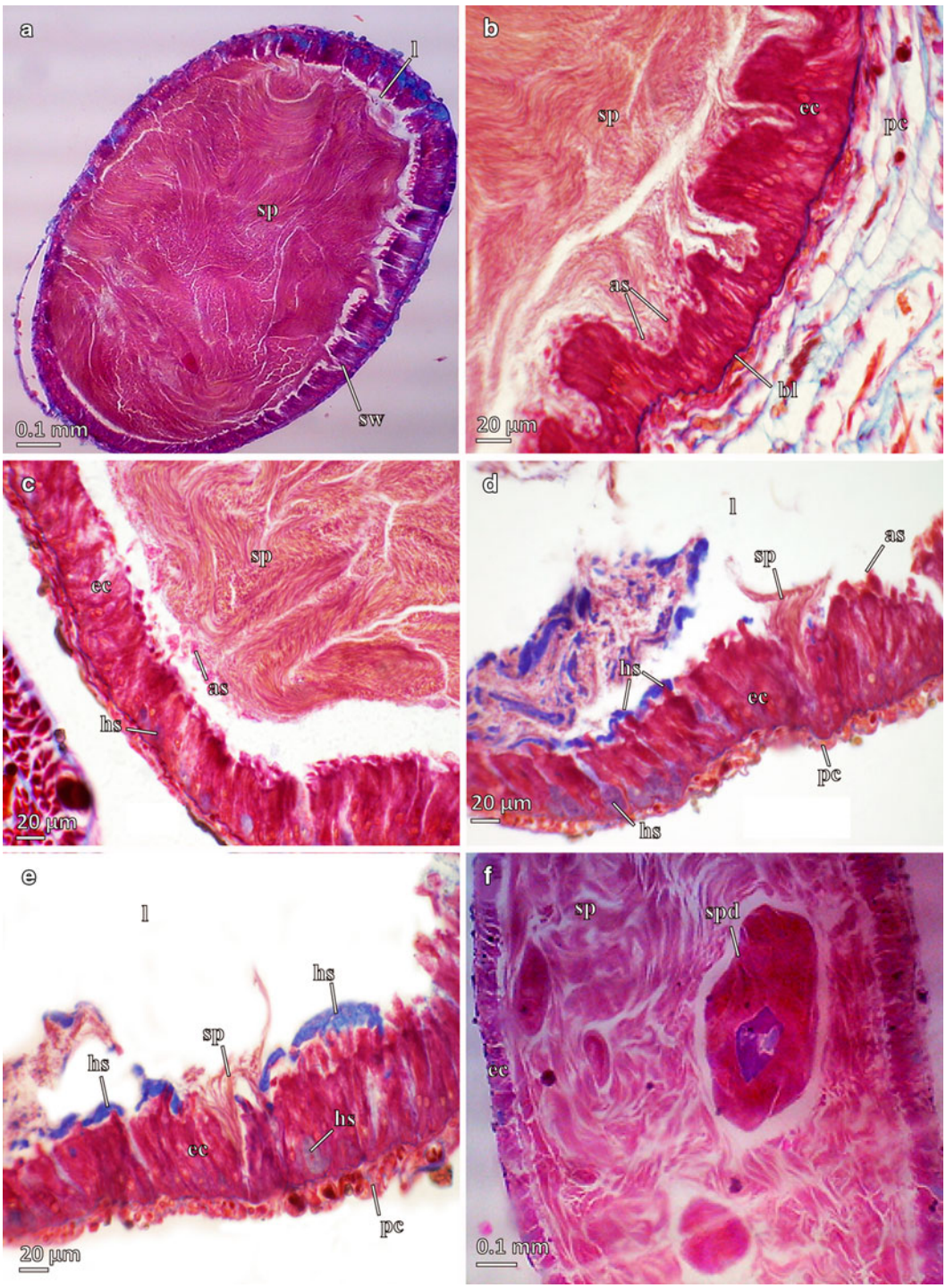

Fig. 1 General structure of the spermathecae of *Hormogaster elisae* using light microscopy. **a**. Overall view of one spermatheca showing its wall and the lumen full of highly packed sperm. **b**. Close-up of the spermathecal wall composed by two layers: outer epithelium with peritoneal cells and the inner epithelium with prismatic epithelial cells, showing the lobulated profile sometimes found due to sperm intrusions. **c**–**e**. Details of different types of secretions produced by the epithelial cells: holocrine secretions and apocrine secretions. In this case, the spermathecal wall shows the commonly observed linear profile although some sperm intrusions are present. **f**. Overview of a spermatheca containing very compacted sperm and a central zone with different affinity for the stain that could be interpreted as sperm degeneration. Abbreviations: *as* apocrine secretions, *bl* basal lamina, *ec* epithelial cells, *hs* holocrine secretions, *l* lumen, *pc* peritoneal cells, *sp* spermatozoa, *spd* sperm degeneration, *sw* spermathecal wall

the reproductive portion of the year. In addition, three aestivating individuals were included in the study, which provided information of the spermathecal content during the driest months when collection of animals is difficult due to the depth at which the animals migrate.

Light microscopy

Live individuals were rinsed in distilled water and immediately killed in Zenker's solution. Specimens were dorsally opened to allow the penetration of the fixative and then dissected out to take only the segments where the spermathecae were located. Fresh Zenker's solution was added to the samples, where they were stored for approximately 3 days. Fixed material was then rinsed with distilled water every 30 min during 1 day and subsequently dehydrated in an ethanol series as follows: 70% (overnight), 80%, 90%, 96% (15 min each), and 100% (twice for 60 min and once for 45 min). The last rinse was performed in toluene (3 times for 1 h). Samples were embedded in paraffin through successive series at 67°C (twice for 1 h, once for 3 h). Histological sections (5–6 μm) were produced using a Jung microtome. Sections were deparaffinized by immersion in absolute toluene and rehydrated in an ethanol series (100%, 96%, 70%), 3 min each rinse. Sections were stained using a standard Mallory's trichromic staining protocol and studied under a compound Zeiss Axioscop microscope. Digital pictures were taken with an Olympus DP70 camera.

Transmission electron microscopy

The ultrastructure of the spermathecae was analyzed using specimens collected in February (2011) by transmission electron microscopy (TEM). The protocol used was modified from Fernández et al. (2011). Two specimens were killed and fixed in 2.5% glutaraldehyde in PBS, and their four spermathecae were removed under the stereomicroscope and preserved in the same solution until further processing. The spermathecae were rinsed three times (10 min each) in 0.05 M HEPES buffer (pH 7.56) and post-fixed first in 1% osmium tetroxide in 0.05 HEPES (pH 7.56) during 1 h. After three rinsing steps (10 min each) with HEPES buffer, the samples were stained in uranyl acetate in distilled water overnight and then dehydrated in a graded ethanol–propylene oxide series as follows: ethanol 30 and 50% 10 min each, ethanol 70% (10 min twice), ethanol 80%, 90%, 95%, 100% (10 min, three times), and propylene oxide 100% (2 min). After dehydration, samples were gradually infiltrated with propylene oxide/resin mixture 3:1 and 1:1 for 90 min each on a rotor, 1:3 in open vials overnight at room temperature and embedded in Embed 812 resin (Electron Microscopy Sciences, PA, USA). Resin polymerization was conducted in a stove at 60°C for 72 h.

Semithin (1 μm) and ultrathin (80 nm) sections were produced using a Leica Ultracut UCT ultramicrotome. Semithin sections were stained with Toluidine's blue dye, and ultrathin sections for TEM were mounted on formvar carbon-coated grids (Electron microscopy Sciences, PA, USA) and counter-stained with uranyl acetate in ethanol 50% during 20 min followed by lead citrate during 12 min. Observations were conducted with a JEOL 2100 (operating at 200 kV) and a Zeiss Libra 120 (operating at 120 kV) transmission electron microscopes and fitted with a Gatan module for acquisition of digital images.

Results

General structure of the spermatheca

The structure and contents of the spermathecae are very similar throughout the year and also in the aestivating specimens, although in the latter, the spermatozoa within the spermathecae are even more packed than in the active individuals. Thus, only individuals from February were studied using TEM. The only difference found between the spermathecae of those individuals was the amount of secretion material within the epithelium. Light microscopy revealed packed spermatozoa embedded in a substance of unknown nature surrounded by a cellular wall of approximately 60 μm in maximum thickness, consisting of two epithelial layers: the peritoneal epithelium and the spermathecal epithelium (Fig. 1). Usually, the wall has a linear profile (Fig. 1c, e), but in some instances, the profile appears lobulated probably due to massive sperm intrusions (Fig. 1b). In some spermathecae, with no relation to seasonality, we found a structure that could be interpreted as sperm degeneration in the center of the lumen (Fig. 1f). This structure is large (ca. 300 μm in max. diameter) and is comprised of highly compacted sperm, and a central zone with different affinity for the stain was noticed (Fig. 1f). However, we were not able to replicate the finding using electron microscopy, and the nature of the structure remains uncertain.

Ultrastructure of the spermathecal wall

The wall of the spermatheca consists of two layers. The outer layer is comprised, in sequence toward the lumen, of a layer of long flat peritoneal cells, a basal lamina of variable thickness (between 0.2 and 0.5 μm), a striated muscle stratum ranging from 1 μm to 2 μm, a second basal lamina of approximately 0.5 μm, blood vessels, another layer of striated muscle, and a third basal lamina of ca. 0.5 μm

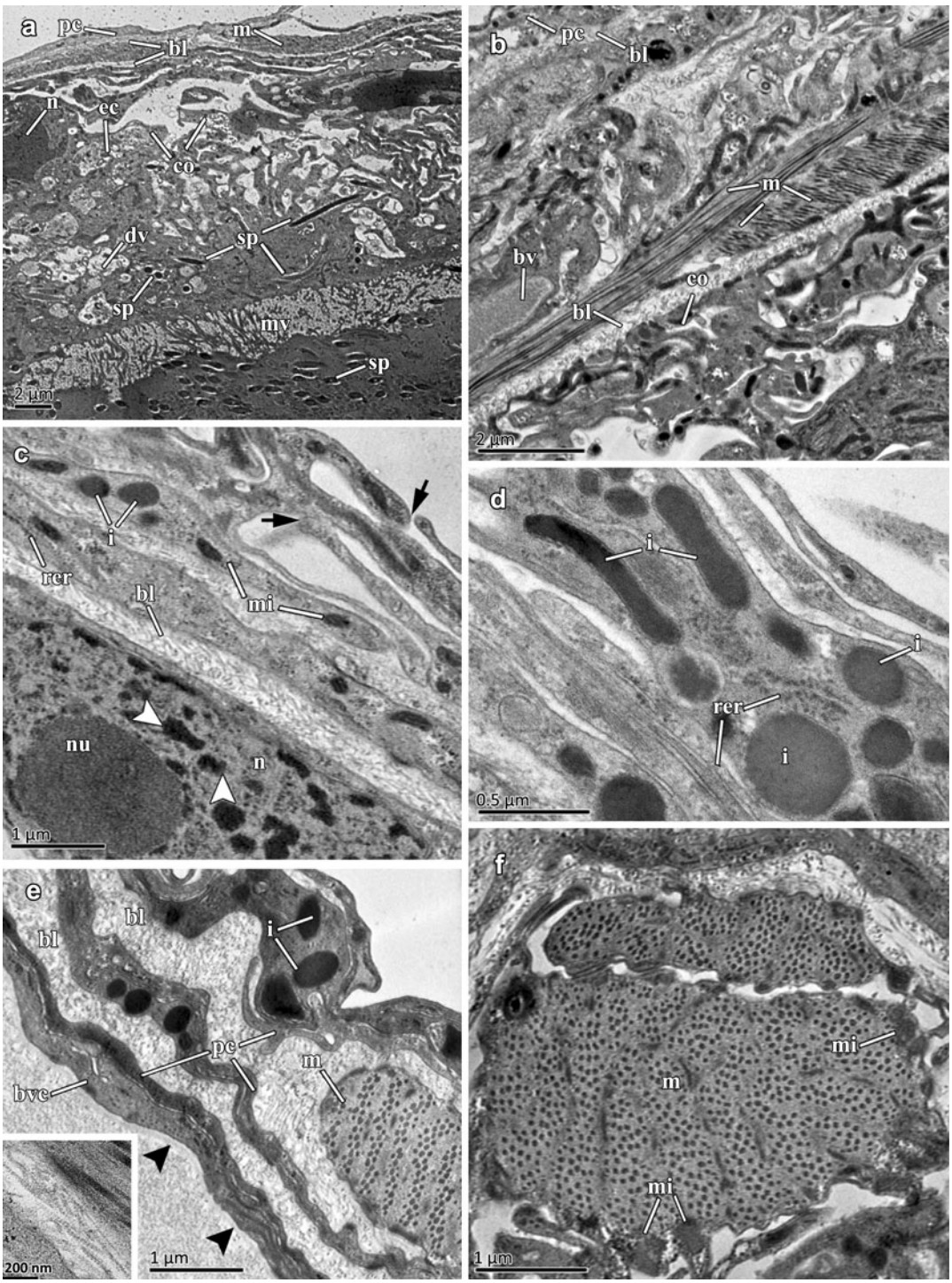

Fig. 2 Ultrastructure of the spermatheca outer epithelium. **a**, **b**. The outer wall of the spermatheca consists, in sequence toward the lumen, of a layer of flat peritoneal cells, a basal lamina, a striated muscle stratum, a second basal lamina, blood vessels, another layer of striated muscle and a third basal lamina. The inner epithelium is comprised of a monolayer of cells with multiple microvilli and convolutions. The lumen of the spermatheca is filled with mature sperm embedded in an electron-dense material. Note the appearance of scanty sperm inside the epithelial cells. **c**, **d**. Peritoneal cells showing the intertwined junctions (*black arrows*), rough endoplasmic reticulum, multiple electron-dense inclusions and a nucleolated nucleus with scattered condensed chromatin (*white arrowheads*). **e**, **f**. Striated muscle surrounded by peritoneal cells and basal laminae. Note the fibrillar vascular lamina (*black arrowheads*) lining the lumen of the vessel. Inset: Details of the striated collagen fibrils in the basal lamina. Abbreviations: *bl* basal lamina, *bv* blood vessel, *bvc* blood vessel epithelial cell, *co* convolutions, *dv* digestive vesicle, *ec* epithelial cells, m striated muscle, *mi* mitochondria, mv microvilli, *n* nucleus, *nu* nucleolus, *pc* peritoneal cells, *i* electron-dense inclusion, *rer* rough endoplasmic reticulum, *sp* spermatozoa

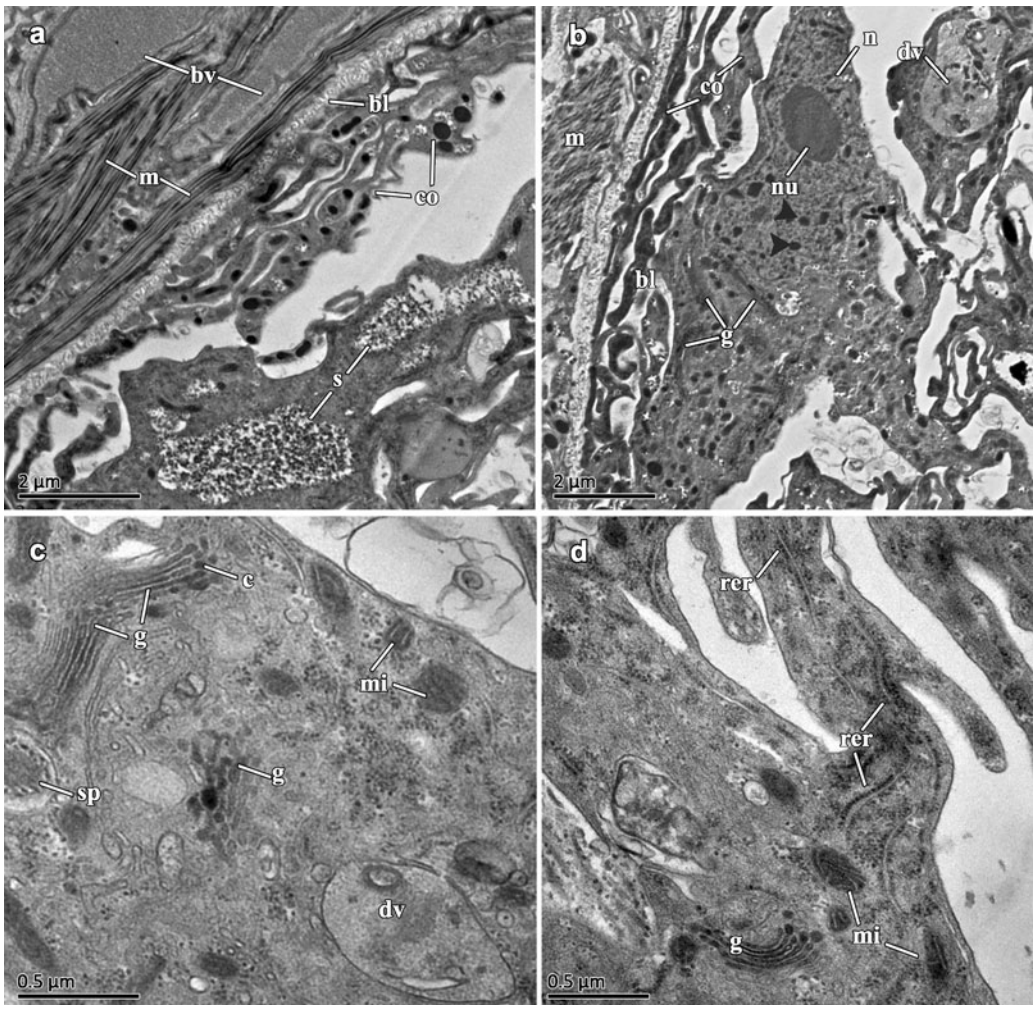

Fig. 3 Basal part of the prismatic epithelial cells. **a**, **b**. The basal part of the prismatic epithelial cells consists of multiple convolutions lying on the basal lamina and a large nucleolated nucleus with scattered condensed chromatin (*black arrowheads*) and several digestive vesicles, some containing secretion material. **c**, **d**. The medial part of the cell shows large Golgi complexes with detaching cisternae, rough endoplasmic reticulum, mitochondria, digestive vesicles, and few spermatozoa inside a vesicle. Abbreviations: *bl* basa lamina, *bv* blood vessels, *c* cisternae, *co* convolutions, *dv* digestive vesicle, *g* Golgi complex, *m* muscle, *mi* mitochondria, *n* nucleus, *nu* nucleolus, *rer* rough endoplasmic reticulum, *s* secretion material, *sp* spermatozoa

(Figs. 2a–f, 3a, b). Peritoneal cells appear highly intertwined and show a large nucleolated nucleus (approximately 7 µm in maximum diameter) (Fig. 2c, d). Their cytoplasm contains electron-dense inclusions (Fig. 2d), mitochondria, and rough endoplasmic reticulum (Fig. 2c, d). Striated collagen fibers form the three basal laminae (Fig. 2b, c, e and inset). The muscle bundles measure between 1 and 2 µm in thickness (Fig. 2b, e, f) and are comprised of highly packed obliquely striated fibers (not shown) and multiple mitochondria located at the edges (Fig. 2f). The blood vessels are lined by a thin layer of intertwined cells, slightly less electron-dense than peritoneal cells (Fig. 2e). The lumen of the blood vessel is lined by a distinctly fibrillar vascular lamina (Fig. 2e).

The inner layer of the wall is a monostratified cellular sheath of prismatic epithelial cells with multiple microvilli in the apical part (Figs. 2a, 4d, f). The basal part of the cells shows a large nucleolated nucleus of 12 µm in maximum diameter (Fig. 3b), and multiple convolutions are observed at both sides of the nucleus, i.e., basal (Figs. 2b, 3a, b) and apical (Figs. 2a, 3b, 4d). The bulk of the cytoplasm, located in the medial part of the cell, contains large Golgi complexes with multiple detaching cisternae, large numbers of rough endoplasmic reticula, electron-dense inclusions, and numerous mitochondria (Figs. 3c, d, 4a). Also, numerous digestive vesicles were found throughout the cytoplasm (Figs. 3b, c, 4b), as well as vesicles filled with secretion material of diverse electron density

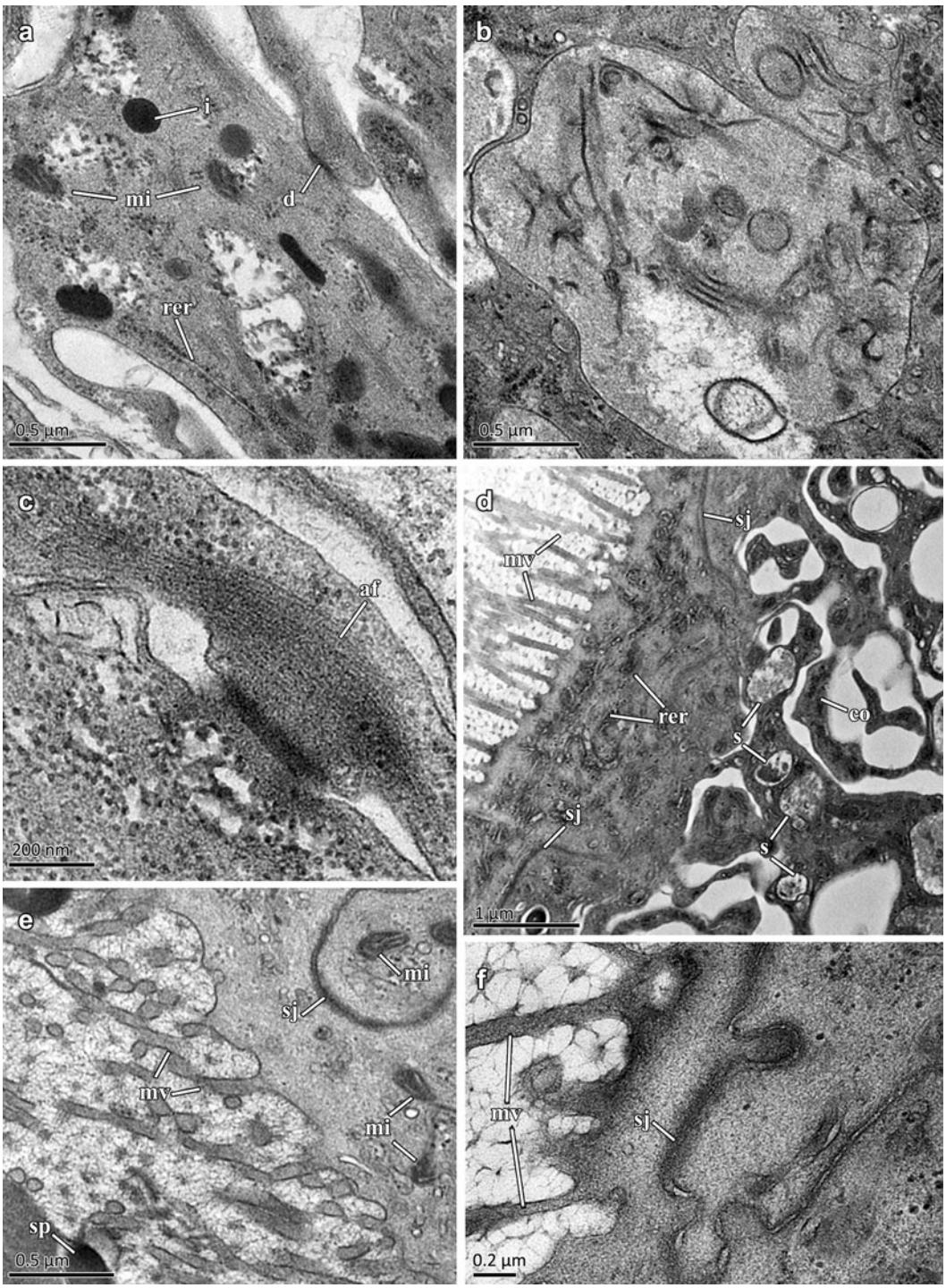

Fig. 4 Medial and apical part of the prismatic epithelial cells. **a**. Medial part of the epithelial cell showing rough endoplasmic reticulum, mitochondria, electron-dense inclusions, and desmosomes connecting adjacent cells. **b**. Details of the digestive vesicle within the cytoplasm. **c**. Details of the desmosome connecting the medial part of adjacent cells, showing bundles of actin filaments. **d**. Apical part of the epithelial cell containing large amounts of rough endoplasmic reticulum, basally located convolutions, septate junctions connecting the apical part of the cells, multiple microvilli, and secretions. **e**. Details of the apical zone of the cell showing thin microvilli contacting the sperm mass. **f**. Close-up of the septate junctions connecting the apical part of adjacent cells. Abbreviations: *af* actin filaments, *co* convolutions, *d* desmosome, *mi* mitochondria, *mv* microvilli, *i* electron-dense inclusions, *rer* rough endoplasmic reticulum, *s* secretions, *sj* septate junctions, *sp* spermatozoa

(Figs. 4d, 5a–f). Epithelial cells are connected laterally at the medial part by desmosomes surrounded by actin fibers (Fig. 4c) and apically by septate junctions (Fig. 4d–f). The apical part of the cells contains large amounts of rough endoplasmic reticulum (Fig. 4d), numerous mitochondria, and numerous microvilli (Fig. 4d–f).

Secretions

The prismatic epithelial cells are actively secreting materials of various natures toward the lumen of the spermatheca (Fig. 5). Finely granulated secretion material was found in both basal and apical parts of the cell (Figs. 3a, 4d, 5a). However, within the apical part of the cells (Fig. 5b), the secretion material appears electron denser. The same electron-dense secretion material was also found toward the lumen of the spermatheca (Fig. 5c) and among the sperm mass in the lumen of the spermatheca (Fig. 5b, c). A less electron-dense type of secretion material was found in large round vesicles of approximately 10 μm in diameter (Fig. 5d, e) and showed affinity for aniline blue in Mallory's staining (Fig. 1c–e). This type of secretion appears to be formed by the release of finely granulated material from small vesicles (Fig. 5e) similar to the secretion substance observed previously (Fig. 5a). Apocrine secretions were detected at the apical part of the cell, when large areas of the cytoplasm are released toward the lumen of the spermatheca (Figs. 5f, 7a) and showed affinity for fuchsin in Mallory's staining (Fig. 1e).

Sperm cells and their interaction with the spermathecal wall

The lumen of the spermatheca is filled with mature sperm embedded in a highly electron-dense material (Figs. 2a, 4e, 5b, c, f, 6a, b, 7b). The nucleus of the spermatozoon measures approximately 250 nm in diameter and contains highly compacted chromatin (Fig. 6a, b, c). The mid-piece of the spermatozoon contains six large densely compacted mitochondria (1.5 μm in maximum length) (Fig. 6b, c). The acrosome is a 2-μm-long membrane-bound organelle comprised of the primary acrosomal vesicle, the acrosome tube with a secondary invagination, and an axial rod (perforatorium) (Fig. 6a, b, e). Between the acrosome and the nucleus, we observed a nuclear pad (Fig. 6e). The flagellum consists of the typical $9 + 2$ arrangement of microtubules (Fig. 7f) surrounded by glycogen strings (Figs. 6a–d, 7f).

Numerous spermatozoa were detected within the wall of the spermatheca (Fig. 7). In some instances, the spermatozoa are very close to the apical part of the prismatic epithelial cells (Fig. 7a–e), while in other occasions, they are detected more basally (Fig. 7e, f). Even though it is unclear whether they are phagocytized or actively enter the cells, in at least one occasion, one spermatozoon was observed penetrating the membrane of a prismatic epithelial cell (Fig. 7b). Also, the position of some of the spermatozoa within the cells suggested active penetration (Fig. 7d, e). Digestion of the sperm cells within the epithelial cells was observed in the apical and medial areas (Fig. 7f).

Discussion

General structure of the spermatheca

The spermatheca of the hormogastrid *H. elisae* shows the typical structure found in other terrestrial Opisthopora with several slight differences. In essence, they are tubular sacs (ampullae without diverticula), without internal tissue subdivisions, containing highly packed spermatozoa in mature individuals. Their walls are comprised of two epithelial layers, being the inner one in close interaction with the sperm cells in the luminal area.

The spermathecae of clitellate individuals do not differ in general structure and processes along the year. Novo et al. (2010b) showed that the sperm contained in the spermathecae of one individual were from one to three paternal individuals, normally from two. This means that even though the animals could copulate through the year (except for aestivating periods), they store the sperm from a certain number of individuals. Maybe the nourishment from the epithelial wall contributes to the invariable appearance of the spermathecal content by ensuring the sperm viability. However, another possibility is that sperm renewal is favored by sperm digestion (see discussion below) or used for fertilization.

The maintenance of the sperm during aestivation (Díaz Cosín et al. 2006), when *H. elisae* enters a period of minimum activity, would require a highly effective system of nourishment and maintenance of the sperm cells, such as that found in the present work (see discussion below).

Ultrastructure of the spermathecal wall

Two epithelial layers constitute the spermathecal wall, as described in other clitellate species (Fleming 1981; Jamieson 1992; Teisaire and Roldán 1995; Vanpraagh 1995; Fernández et al. 2011). Peritoneal cells, several strata of collagenous basal laminae, different layers of striated muscle, and blood vessels comprise the outer layer. Peritoneal cells show a large nucleolated nucleus, which has only been previously shown in *Tubifex tubifex* (Müller 1774) (Fleming 1981).

The inner layer of the spermathecal wall is a monostratified epithelium. In *H. elisae,* epithelial cells show a

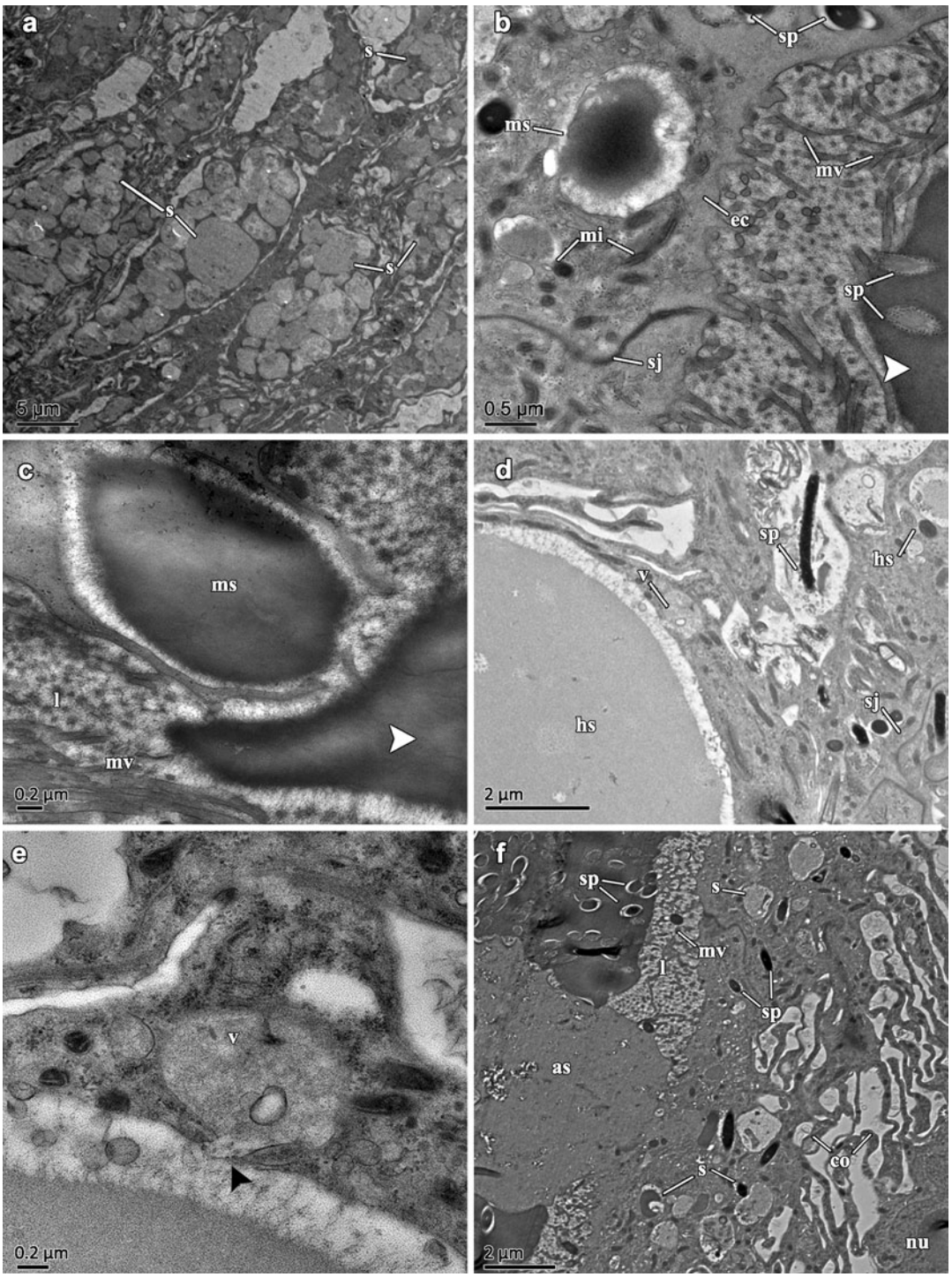

Fig. 5 Secretion types of epithelial cells. **a**. Medial part of the epithelial cell showing vesicles filled with finely granulated secretion material. **b**. Electron-dense merocrine secretion within the apical part of the epithelial cell. Note the similar electron density of the secretion material and the substance (*white arrowhead*) surrounding the sperm mass, and the appearance of spermatozoa within the cells. **c**. Close-up of the secretion process of the electron-dense material toward the lumen, which is occupied by an electron-dense substance similar to the merocrine secretion (*white arrowhead*). **d**, **e**. Holocrine secretion within the medial part of the cell. Note the secretion vesicle shedding material into the large holocrine secretion, and the opening of the vesicle membrane (*black arrowhead*). **f**. Apocrine secretion of the apical part of the cell toward the spermathecal lumen. Abbreviations: *as* apocrine secretion, *co* convolutions, *ec* epithelial cell, *hs* holocrine secretion, *ms* merocrine secretion, *l* lumen, mv microvilli, mi mitochondria, *nu* nucleolus, *s* secretion, *sj* septate junction, *sp* spermatozoa, *v* vesicle

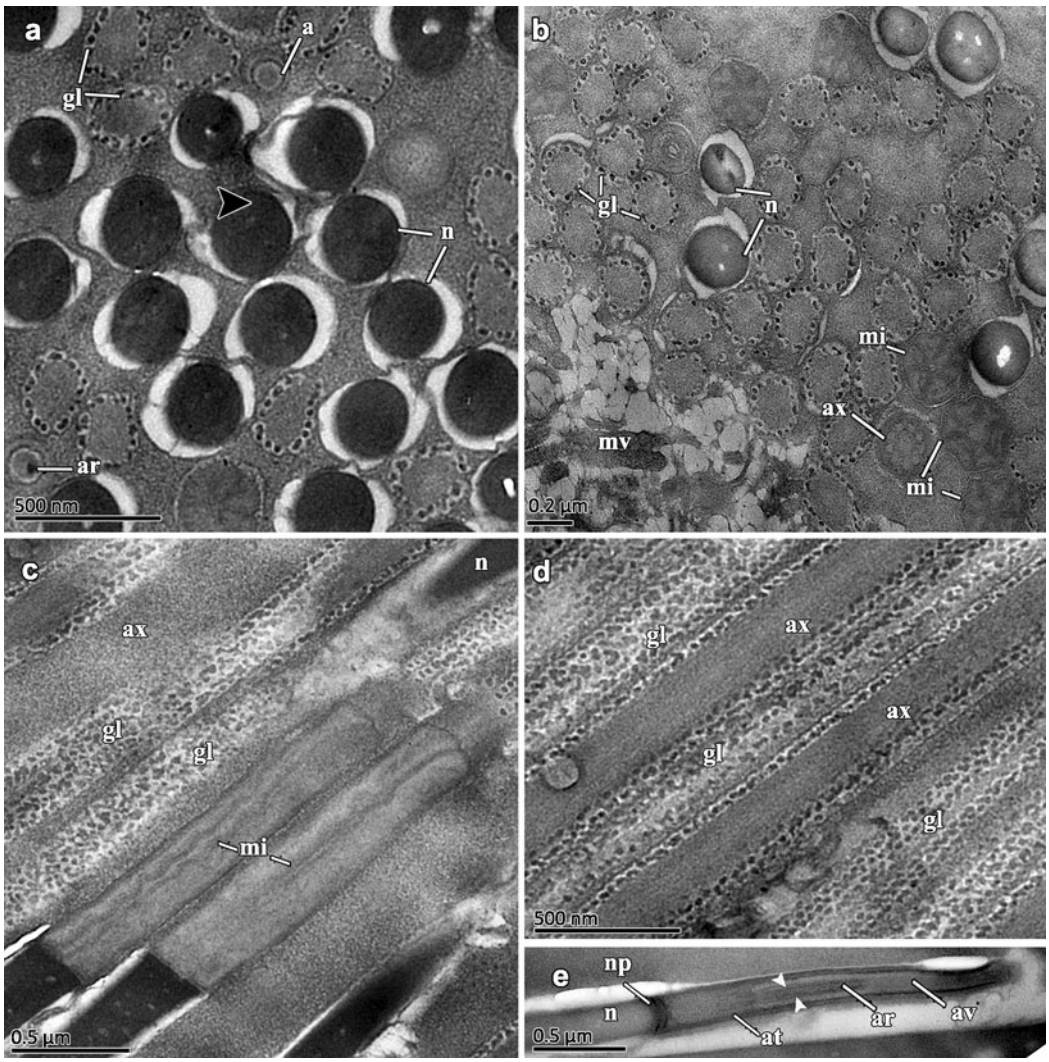

Fig. 6 Ultrastructure of spermatozoa. **a**, **b**. Cross section of mature spermatozoa showing the acrosome, highly condensed chromatin (*black arrowhead*) within the nucleus in the apical part, six highly packed mitochondria in the mid-piece, glycogen strings along the flagellum axis, and the axoneme. **c**, **d**. Longitudinal sections of spermatozoa showing the axoneme, mitochondria, the glycogen strings, and nucleus. **e**. Longitudinal section of the acrosome showing the primary acrosomal vesicle, the axial rod, the acrosome tube, the secondary acrosomal invagination (*white arrowheads*), and the nuclear pad. Abbreviations: *a* acrosome, *ar* axial rod, *at* acrosome tube, *av* primary acrosomal vesicle, *ax* axoneme, *gl* glycogen strings, *mi* mitochondria, *mv* microvilli, *n* nucleus, *np* nuclear pad

prismatic shape, but they are also often referred to as columnar cells in other clitellates (Fleming 1981). Microvilli of epithelial cells are thin and strongly elongated, similar to those of *D. subrubicunda* and other lumbricids (Richards and Fleming 1982). In *T. tubifex* (Fleming 1981), *Eisenia fetida*, *Allolobophora rosea* (Savigny 1826), or *Octolasium cyaneum* (Savigny 1826) (Richards and Fleming 1982), microvilli are thick and regularly spaced, appearing as a brush. In *H. elisae,* microvilli could present various functions, all related with absorption, such as a maintenance of a stabilized environment in the lumen by uptaking waste material of small molecular weight; absorption of water to maintain the osmotic pressure in the lumen after the secretions from the spermathecal wall or control of small ion concentrations in the lumen (Fleming 1981). The basal part of the epithelial cells presents a large nucleolated nucleus and multiple convolutions, indicating that the cell membrane is highly folded in this area. These convolutions could reach more apical areas of the cell, going beyond the nucleus, which could be related to fluid transportation. We detected variation among individuals in the number of convolutions or infoldings, which in some cases could even show an opening to the lumen. A similar organization of the inner epithelium was found by Fleming (1981) in the spermathecal ampulla of *T. tubifex*, as well as in the spermathecal wall of several lumbricids (Richards and Fleming 1982; Fernández et al. 2011), and megascolecids (Vanpraagh 1995; Teisaire and Roldán 1995).

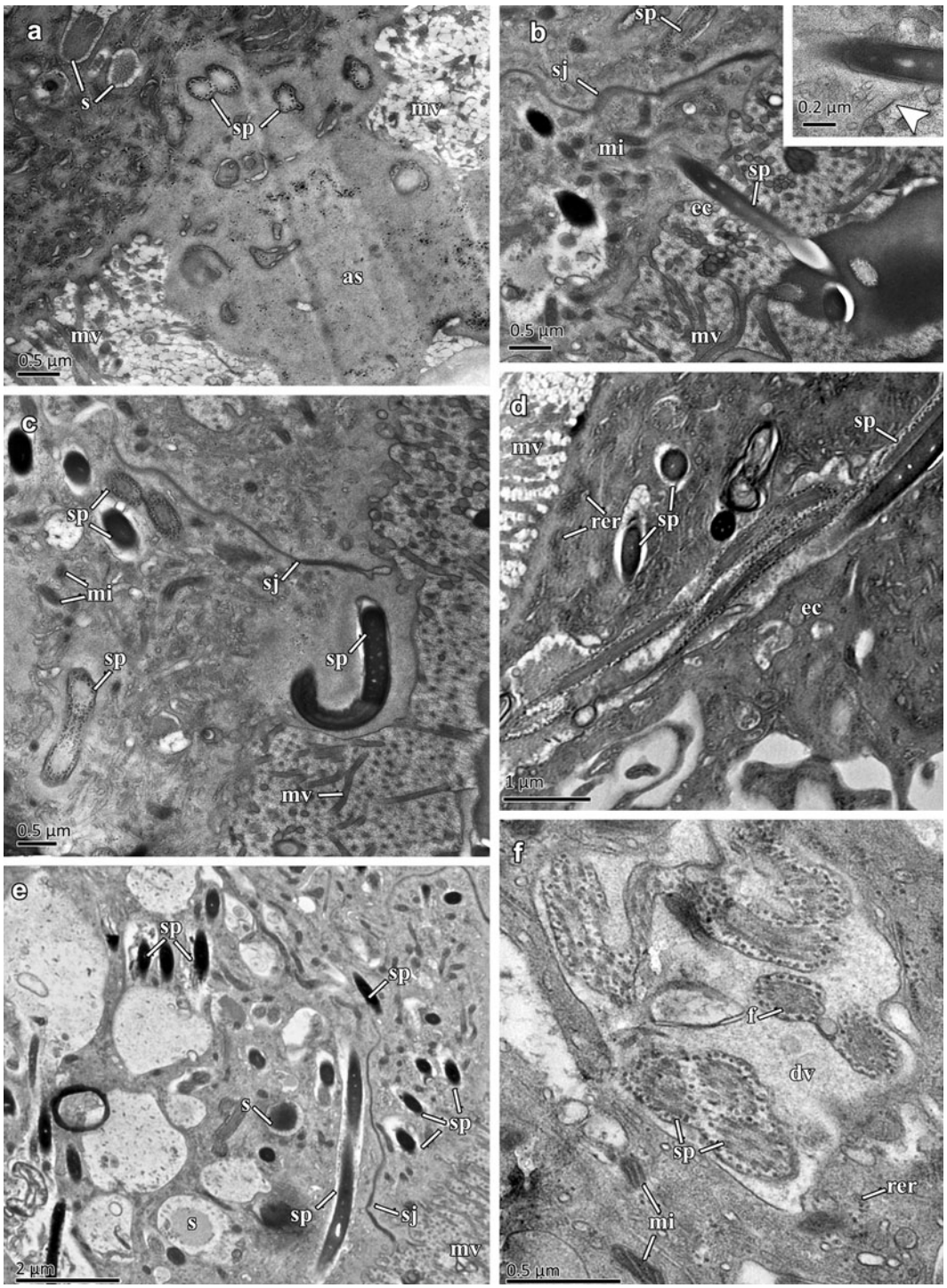

Fig. 7 Sperm intrusions within epithelial cells and subsequent digestion. **a**. Apocrine secretion containing embedded spermatozoa. **b**. Sperm entrance inside the epithelial cell. *Inset*: Note the torn membrane of the apical part of the cell (*white arrowhead*) where the sperm is piercing the cell. **c**, **d**. Apical part of an epithelial cell showing several embedded spermatozoa. **e**. Apical and medial part of an epithelial cell showing numerous embedded spermatozoa. **f**. Details of a digestive vesicle containing partially digested spermatozoa. Note the typical 9 + 2 arrangement of the sperm flagella. Abbreviations: *as* apocrine secretion, *dv* digestive vesicle, *ec* epithelial cell, *f* flagellum, *mv* microvilli, *mi* mitochondria, *rer* rough endoplasmic reticulum, *s* secretion, *sj* septate junction, *sp* spermatozoa

Interestingly, the epithelial cells of the spermathecae of the snail *Arianta arbustorum* Linnæus, 1758 (Pulmonata, Gastropoda) are arranged in a very similar way to those of *H. elisae* and also show numerous basal infoldings, presumably involved in some sort of secretion or storage process (Bojat et al. 2001).

The epithelial cells show an intense activity as indicated by the large quantity of small mitochondria, long and whorled *RER*, and Golgi complexes. All these organelles are present not only in the medial part of the cell but also in the apical zone, and they are probably involved in the production of nutritive material for the nourishment of the sperm cells. This is also supported by the evidence of numerous vesicles filled with secretion material of diverse electron density (see secretions section below). Also, epithelial cells show numerous digestive vesicles (or secondary lysosomes) throughout their cytoplasm, some of them containing partially digested sperm cells (see discussion below), similar to those in *D. subrubicunda* (Richards and Fleming 1982) and *Aporrectodea trapezoides* (Dugès 1828) (Fernández et al. 2011).

Secretions

The epithelial cells constituting the inner epithelium are actively secreting material toward the spermathecal lumen. Our findings regarding this issue are complementary to the few preliminary descriptions of secretion substances for sperm nourishment in other clitellates (Varuta and More 1972; Fleming 1981; Jamieson 1992; Vanpraagh 1995). Nevertheless, we report here three different secretion modes for the epithelial cells of *H. elisae*: merocrine, holocrine, and apocrine, and also three different types of secretion material. Merocrine secretion of *H. elisae* is similar to that found in some Megascolecidae by Teisaire and Roldán (1995). It could serve as nourishment for the sperm cells and/or provide a suitable environment for their survival. Nevertheless, further histochemical studies would be necessary to unravel the unknown nature of this secretion material, although the nutritive function is unquestionable, considering the minimum intrinsic energy of male gametes. Merocrine secretion of granular material has also been reported for *Megascolides australis* (Vanpraagh 1995). Similar to the holocrine secretions of *H. elisae* have also been reported for *Amynthas rodericensis* Grube, 1879 (Jamieson 1992). Using light microscopy, we inferred two different cell types in the spermathecal wall, glandular holocrine cells showing a glandular neck and columnar epithelial cells. However, under electron microscopy, we could not detect such cell diversity. Also, Fleming (1981) only detected one cell type in the spermathecae of *T. tubifex* using transmission electron microscopy, even though Dixon (1915) previously reported two cell types (one glandular and one interstitial) based on histological sections. Finally, the apical part of the epithelial cells releases part of their cytoplasm toward the lumen. It could be also hypothesized that during the period of retention in the spermatheca, some mechanism acts to make the sperm quiescent and thus to reduce their need for nourishment, as suggested by Dent (1970) for the spermathecae of *Notophthalmus viridescens* (Rafinesque 1820), and that some secretions might be quieting factors. That hypothesis could also explain the invariable appearance of the spermathecae and sperm of *H. elisae* when individuals were aestivating.

Sperm cells and their interaction with the spermathecal wall

The sperm cells are highly compacted inside the lumen in a unique mass, as in sexual forms of *A. trapezoides* (ROB population in Fernández et al. 2011) and other lumbricids (Richards and Fleming 1982), and not forming any substructure such as the spematozeugmata (i.e., sperm bundles) described in other clitellates (e.g., *T. tubifex*, Fleming 1981; *M. australis*: Vanpraagh 1995). Sperm cells are embedded in a highly electron-dense material of unknown nature that is secreted by the epithelial cells.

The overall structure of the sperm (Fig. 6e) resembles that already described in *Hormogaster redii* Rosa 1887 (Ferraguti and Jamieson 1984). However due to the compacted arrangement of the sperm cells in the spermathecae of *H. elisae*, its ultrastructure was difficult to elucidate. Since these two species of hormogastrids are distant genetically (Novo et al. 2011), differences in specific features could add more information to their phylogenetic history. So far, the differences on the spermatozoal morphology between both species are related to the size of the mitochondria located in the mid-piece, which are slightly smaller in *H. elisae*.

A large amount of sperm cells, in different digestion stages, were observed within the cytoplasm of the epithelial cells, sometimes being very close to the apical area and others more basally located. Sperm cells actively enter the spermathecal wall, as proved by the observation of a sperm cell piercing the membrane of an epithelial cell. Also, the position of some of the spermatozoa within the cells suggested active entrance. A similar pattern was detected in the diverticulum of *A. rodericensis* (Jamieson 1992), where the acrosomes of spermatozoa were intruding into the degenerating epithelium, suggesting the lysis of the wall by the sperm cells. In other terrestrial Opisthopora, it seems that the sperm is phagocytized, as in several lumbricid species studied by Richards and Fleming (1982), where pseudopodial-like phagocytosis and phagosomes were reported, and by Teisaire and Roldán (1995), who observed cytoplasmic projections enveloping gametes coming from the epithelial cells in megascolecid species. Nevertheless, these authors did not find evidence for phagocytosis in *Eisenia fetida*, and neither did we in *H. elisae*. When sperm cells were observed in digestive vesicles, they presented degenerative morphology similar to that observed for several megascolecids (Teisaire and Roldán 1995). We

observed digestion of the spermatozoa within the apical and medial areas of the cytoplasm of epithelial cells. Also Fernández et al. (2011) found sperm degradation within vesicles throughout the epithelial cytoplasm in a pseudogamic population of *A. trapezoides.*

The only plausible evidence for sperm degeneration in the spermathecal luminal area of *H. elisae* was found in several random spermathecae, where among the very compacted sperm cells there was a central area with different affinity for the staining. Fernández et al. (2011) also found degraded spermatozoa in the lumen of pseudogamic *A. trapezoides*. It is possible that the cells in the core of the sperm mass degenerate to constitute a source of nutrition to assist in long-term storage of the remaining cells, as already hypothesized for central cells in spermatozeugmata of *M. australis* (Vanpraagh 1995).

Conclusions

In summary, the spermathecae of mature individuals of *H. elisae* are full of highly packed spermathozoa embedded in an electron-dense substance. The spermathecal wall comprises two layers; the outer layer is constituted by peritoneal cells, several strata of collagenous basal laminae, different layers of striated muscle, and blood vessels. The inner layer is a monostratified epithelium of prismatic cells with long microvilli. Three different types of secretions were observed: holocrine, merocrine and apocrine of unknown nature and function, although nourishment of stored sperm and/or a role in inducing sperm quiescence are the hypotheses considered. Some sperm cells are digested by secondary lysosomes within the cytoplasm of the epithelial cells, but no phagocytotic processes were detected. Therefore, the active entrance of the sperm cells is becoming the most plausible cause of presence of spermatozoa within the epithelial cells.

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References

Beese K, Armbruster GFJ, Beier K, Baur B (2009) Evolution of female sperm-storage organs in the carrefour of stylommatophoran gastropods. J Zool Syst Evol Res 47:49–60

Birkhead TR, Møller AP (1993) Sexual selection and the temporal separation of reproductive events: sperm storage data from reptiles, birds and mammals. Biol J Linn Soc 50:295–311

Birkhead TR, Møller AP, Sutherland WJ (1993) Why do females make it so difficult for males to fertilize their eggs? J Theor Biol 161:51–60

Bojat NC, Sauder U, Haase M (2001) The spermathecal epithelium, sperm and their interactions in the hermaphroditic land snail *Arianta arbustorum* (Pulmonata, Stylommatophora). Zoomorphology 120:147–157

Brinkhurst RO, Jamieson BGM (1971). Aquatic oligochaeta of the world. Oliver and Boyd, Edinburgh

Butt KR, Nuutinen V (1998) Reproduction of the earthworm *Lumbricus terrestris* Linné after the first mating. Can J Zool 76:104–109

Dent JN (1970) The ultrastructure of the spermatheca in the red spotted newt. J Morphol 132:397–424

Díaz Cosín DJ, Ruiz MP, Ramajo M, Gutiérrez M (2006) Is the aestivation of the earthworm *Hormogaster elisae* a paradiapause? Inv Biol 125:250–255

Díaz Cosín DJ, Hernández P, Trigo D, Fernández R, Novo M (2009) Algunos aspectos del ciclo biológico del endemismo ibérico, *Hormogaster elisae* Álvarez, 1977 (Oligochaeta, Hormogastridae), en cultivos de laboratorio. Bol R Soc Esp Hist Nat Biol 103:49–57

Díaz Cosín J, Novo M, Fernández R (2011) Reproduction of earthworms: sexual selection and parthenogenesis. In: Karaka A (ed) Biology of earthworms. Springer, Berlin, pp 69–86

Dixon G (1915) *Tubifex*. In: Herdman WA (ed) LMBC memoirs on typical British marine plants and animals, vol 23. Williams and Norgate, London, pp 1–100

Eberhard WG (1985) Sexual selection and animal genitalia. Harvard University Press, Cambridge

Eberhard WG (1996) Female control: sexual selection by cryptic female choice. Princeton University Press, Princeton, NJ

Edwards CA, Bohlen PJ (1996) Biology and ecology of earthworms, 3rd edn. Chapman and Hall, London

Eisen G (1874) New Englands och Canadas Lumbricides. Ofversigt af Kongliga Vetenskaps-Akademiens Forhandligar Stockholm 31(2): 41–49

Fernández R, Bergmann P, Almodóvar A, Díaz Cosín DJ, Heethoff M (2011) Ultrastructural and molecular insights into three populations of *Aporrectodea trapezoides* (Dugés, 1828) (Oligochaeta, Lumbricidae) with different reproductive modes. Pedobiologia 54:281–290

Ferraguti M, Jamieson BGM (1984) Spermiogeness and spermatozoal ultrastructure in *Hormogaster* (Hormogastridae, Oligochaeta, Annelida). J Submicrosc cytol 16:307–316

Fleming TP (1981) The ultrastructure of the spermathecae of *Tubifex tubifex* (Annelida: Oligochaeta). J Zool Lond 193:129–145

Garvín MH, Trigo D, Hernández P, Díaz Cosín DJ (2003) Gametogenesis and reproduction in *Hormogaster elisae* (Oligochaeta, Hormogastridae). Inv Biol 122:152–157

Grove AJ (1925) On the reproductive processes of the earthworm, *Lumbricus terrestris*. Q J Microsc Sci 69:245–290

Grube E (1879) Zoology of Rodriguez. Annelida. Phil Trans R Soc London 168:554–556

Gutiérrez M, Jesús JB, Trigo D, Díaz Cosín DJ (2006) Is *Hormogaster elisae* (Oligochaeta, Hormogastridae) a predator of mite and springtails? Eur J Soil Biol 42:S186–S190

Hellriegel B, Bernasconi G (2000) Female-mediated differential sperm storage in a fly with complex spermathecae, *Scatophaga stercoraria*. Anim Behav 59:311–317

Hilario A, Young CM, Tyler PA (2005) Sperm storage, internal fertilization, and embryonic dispersal in vent and seep tubeworms (Polychaeta: Siboglinidae: Vestimentifera). Biol Bull 208:20–28

Holt WV, Lloyd RE (2010) Sperm storage in the vertebrate female reproductive tract: how does it work so well? Theriogenology 73:713–722

Jamieson BGM (1981) The ultrastructure of the oligochaeta. Academic Press, London, p 462

Jamieson BGM (1992) Oligochaeta. In: Harrison FW, Gardiner SL (eds) Microscopic anatomy of invertebrates, vol 7. Wiley-Liss, New York

Jamieson BGM (2001) Native earthworms of Australia (Megascolecidae, Megascolecinae). Science Publishers, Inc., Enfield

Jamieson BGM (2006) Non-leech Clitellata. In: Reproductive biology and phylogeny of annelida. Series Editor Jamieson BGM. Rouse G, Pleijel F (eds), vol 4. Chap. 8. Science Publishers, Enfield

Linnæus C (1758) Systema naturæ per regna tria naturæ, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Tomus I. Editio decima, reformata. pp [1–4], 1–824. Holmiæ. (Salvius)

McCoy F (1878) *Megascolides australis* (McCoy). The giant earthworm. Prodromus Zool Vic 1:21–25

Meyer WJ, Bowman H (1994) Mating period and cocoon production in *Eisenia fetida*. The 5th international symposium on earthworm ecology Columbus Ohio, 5–9, p 128

Novo M, Almodóvar A, Díaz Cosín DJ (2009) High genetic divergence of hormogastrid earthworms (Annelida, Oligochaeta) in the central Iberian Peninsula: evolutionary and demographic implications. Zool Scripta 38:537–552

Novo M, Almodóvar A, Fernández R, Trigo D, Díaz Cosín DJ (2010a) Cryptic speciation of hormogastrid earthworms revealed by mitochondrial and nuclear data. Mol Phylogenet Evol 56:507–512

Novo M, Almodóvar A, Fernández R, Gutiérrez M, Díaz Cosín DJ (2010b) Mate choice of an endogeic earthworm revealed by microsatellite markers. Pedobiologia 53:375–379

Novo M, Almodóvar A, Fernández R, Giribet G, Díaz Cosín DJ (2011) Understanding the biogeography of a group of earthworms in the Mediterranean basin—the phylogenetic puzzle of Hormogastridae (Clitellata: Oligochaeta). Mol Phylogenet Evol 61:125–135

Qiu JP, Bouché MB (1998) Contribution to the taxonomy of Hormogastridae (Annelida: Oligochaeta) with description of new species from Spain. Doc Pedozool Integrol 4:164–177

Rafinesque CS (1820) Ichthyologia Ohiensis. Reprinted edition: Burrows Brothers Co., Cleveland, 1899

Richards KS, Fleming TP (1982) Spermatozoal phagocytosis by the spermathecae of *Dendrobaena subrubicunda* and other lumbricids (Oligochaeta, Annelida). Int J Invert Rep 5:233–241

Rosa D (1887) *Hormogaster redii* n. g., n. *sp*. Bollettino dei Musei di Zoologia ed Anatomia comparata della Universita di Torino 32(II)

Savigny JC (1826) In Cuvier, Analyse des travaux de l'Academie Royale des Sciences pendant l'année 1821, partie physique. Mem Acad Roy Sci Inst Fr 5:176–184

Teisaire ES, Roldán IA (1995) Ultrastructure and histochemistry of the spermatheca of *Amynthas* Kinberg and *Metaphire* Sims and Easton (Oligochaeta: Megascolecidae). Comun Biol 13:169–181

Valle JV, Moro RP, Garvín MH, Trigo D, Díaz Cosín DJ (1997) Annual dynamics of the earthworm *Hormogaster elisae* (Oligochaeta, Hormogastridae) in Central Spain. Soil Biol Biochem 29:309–312

Vanpraagh BD (1995) Reproductive biology of *Megascolides australis* McCoy (Oligochaeta: Megascolecidae). Aust J Zool 43:489–507

Varuta AT, More NK (1972) Cytochemical study of mucus and mucus secreting cells in spermathecae of the earthworms, *Pheretima elongata* (Perrier) and *Hoplochaetella powelli* (Michaelsen). Ind Exp Biol 10:239–241

Vyas I, Dev B (1972) Histochemical localization of alkaline phosphatase in the spermathecae of the earthworm, *Barogaster annandalei* (Stephenson). Acta Histochem 42:344–350