Spermatozoa in spermatheca of the plant-parasitic nematode *Paratylenchus hamatus sensu lato* (Tylenchida, Paratylenchidae)

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Summary. Characterisation of spermatheca as a conspicuous part of the female reproductive system is often included in morphological descriptions of plant-parasitic nematodes from the genus *Paratylenchus*. The minute spermatozoa inside the spermatheca were observed and described using transmission electron microscopy for the first time in the pin nematode *Paratylenchus hamatus sensu lato*. The spermatheca was 16 μm long and 8 μm wide and represented an epithelial sac containing densely packed spermatozoa as uniform rounded cells 1.4 μm in diameter with the nucleus surrounded by a thin layer of cytoplasm. No structures other than occasional small mitochondria and lipid droplets were observed in the cytoplasm. Thus, the spermatozoa of *P. hamatus* are extremely miniaturised, which is most likely related to their small adult body length and width.

Key words: female gonoduct, metazoan miniaturisation, TEM.

The pin nematodes of the genus *Paratylenchus* Micoletzky, 1922 are characterised by their small body size with length varying from 0.2 to 0.6 mm (Solovyeva, 1972; Siddiqi, 2000). Most species of the genus are amphimictic, i.e. their populations have males with a testis and females with a single outstretched ovary joined to prominent spermatheca filled with spermatozoa (Tarjan, 1960; Geraert, 1965; Solovyeva, 1972; Raski, 1975a, b; 1976). Morphological descriptions of *Paratylenchus* species rely on many characters including spermatheca as a conspicuous part of the female reproductive system. Spermathecal characters are a routine part of species descriptions; taxonomical papers on *Paratylenchus* species usually include drawings of spermatheca sometimes supplemented by light microscopy photographs (Bahmani *et al.*, 2014; Wang *et al.*, 2016a, b).

Spermatozoa of many other nematodes usually have prominent cell bodies of at least 4-5 μm in diameter with complicated internal structures (Justine, 2002). However, the spermatozoa from spermatheca in *Paratylenchus* look like densely packed small refringent spheres about 1.5 μm in diameter, as seen from drawings and photographs with scale bars. The small body length and diameter of *Paratylenchus* adults probably result in size restrictions for male and female gametes.

The sperm structure and development was observed in a variety of the nematodes belonging to the large order Rhabditida *sensu* De Ley & Blaxter (2002). As in other nematodes, the spermatozoa of rhabditids represent an aberrant type of male gametes; they are characterised by the absence of an axoneme and an acrosome (Justine & Jamieson, 1999; Justine, 2002; Yushin & Malakhov, 2004, 2014; Yushin *et al.*, 2006, 2007a, b, 2011, 2016; Giblin-Davis *et al.*, 2010; Yushin & Ryss, 2011; Zograf, 2014; Slos *et al.*, 2015; Qing *et al.*, 2017). The basic type of rhabditid spermatozoon is an amoeboid bipolar cell with an anterior pseudopod and posterior main cell body. A prominent

pseudopod based on the unique cytoskeleton protein MSP (major sperm protein) is a very characteristic feature of the rhabditid spermatozoa (Justine, 2002; Yushin & Malakhov, 2014; Yushin *et al.*, 2016). The main cell body includes a condensed nucleus lacking a nuclear envelope, mitochondria and so called 'membranous organelles' (MO), the unique organelles characteristic of developing and mature sperm in many studied nematodes.

The spermatheca, despite being a distinct feature of female morphology in *Paratylenchus*, has never been observed with transmission electron microscopy. This study reveals for the first time the ultrastructure of the minute spermatozoa inside the spermatheca of the pin nematode *Paratylenchus hamatus sensu lato* and provides a brief discussion on miniaturisation of spermatozoa in the rhabditid nematodes.

MATERIAL AND METHODS

Nematode specimens were collected from a willow tree growing in Davis, Yolo County, CA, USA. According to morphological and molecular analysis made by van den Berg *et al.* (2014) the specimens of this population belong to *Paratylenchus* sp. 1 of the *Paratylenchus hamatus* species complex (*P. hamatus sensu stricto*, *Paratylenchus* sp. 1 and sp. 2), named here as *Paratylenchus hamatus sensu lato*.

Nematodes were extracted from the soil using the rapid centrifugal-flotation method (Jenkins, 1964). Light micrographs of live females immobilised by heating were taken with an automatic Infinity 2 camera attached to a compound Olympus BX51 microscope equipped with a Nomarski differential interference contrast.

Fig. 1. *Paratylenchus hamatus sensu lato*, light micrographs of live females. A: general view of the female with empty spermatheca. Insert: empty spermatheca at higher magnification. B: spermatheca containing spermatozoa at high magnification. Abbreviations: asterisk marks vulvar opening; sp – spermatozoa; st – spermatheca. Scale bars: A = 20 μm (insert = 10 μm); B = 10 μm.

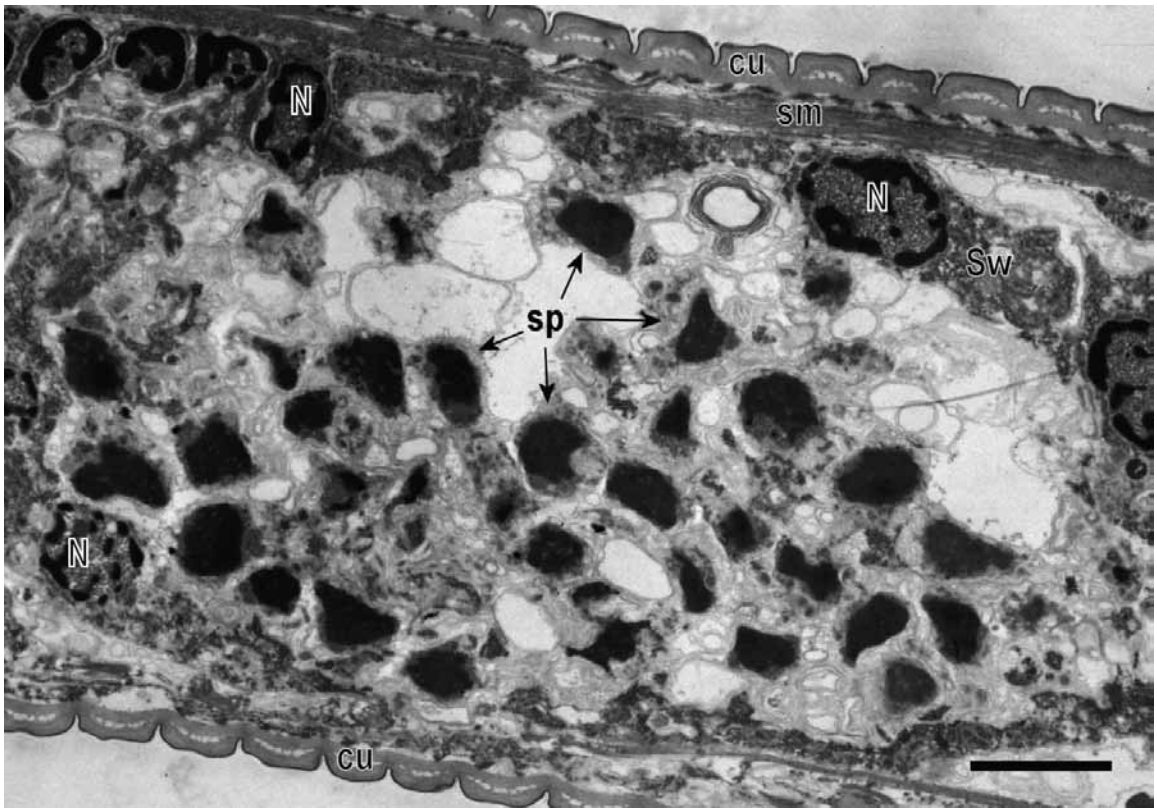

Fig. 2. *Paratylenchus hamatus sensu lato*, general view of the spermatheca on the parasagittal section, TEM. Anterior-posterior direction: left to right. Abbreviations: cu – body cuticle; N – somatic nuclei; sm – somatic muscles; sp – spermatozoa; Sw – spermathecal wall. Scale bar = 2 μm.

For transmission electron microscopy (TEM), live females were cut to obtain the posterior half of the body, containing spermatheca. The specimens were fixed overnight at 4°C in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) with 0.25 mg ml^{-1} $MgCl_2$; post fixation took place 2 h in 1% osmium tetroxide in the same buffer. Postfixation was followed by *en bloc* staining for 1 h in a 1% solution of uranyl acetate in distilled water containing 10% of ethanol. The specimens were dehydrated in ethanol followed by isopropanol series and embedded in Spurr resin. Ultrathin sections cut with both a Leica UC6 and a Leica UC7 ultramicrotome (Leica, Vienna, Austria) were post-stained with uranyl acetate and lead citrate and examined with both a JEOL JEM 1010 and a JEOL JEM 100S electron microscope. Pictures were digitized using a Ditabis system (Pforzheim, Germany).

Six females were examined for the present paper. The structure of the spermatozoa from the spermatheca was studied.

RESULTS

As in the case of other species of the genus, the female reproductive system of *P. hamatus* comprises the single outstretched ovary, spermatheca with or without spermatozoa, uterus and vulva (van den Berg *et al.*, 2014). The collected females of *P. hamatus* were about 350 μm long with maximum body diameter of only 14 μm at the spermatheca level (Fig. 1A). Spermathecae observed from whole-mount specimens were empty or contained spermatozoa observed as densely packed refringent globules (Fig. 1A, B).

The ultrastructure of the spermathecae has been observed on parasagittal thin sections through females (Fig. 2). The size of spermathecae estimated by the outline of cluster of spermatozoa was 16 μm in length and 8 μm in width. Based on spermatheca sections the spermatheca fills the body cavity and is bordered with somatic muscles on both ventral and dorsal side (Figs 2 & 3A). The spermatozoa found in the spermatheca are uniform rounded cells *ca* 1.4 μm in diameter with a highly condensed spherical

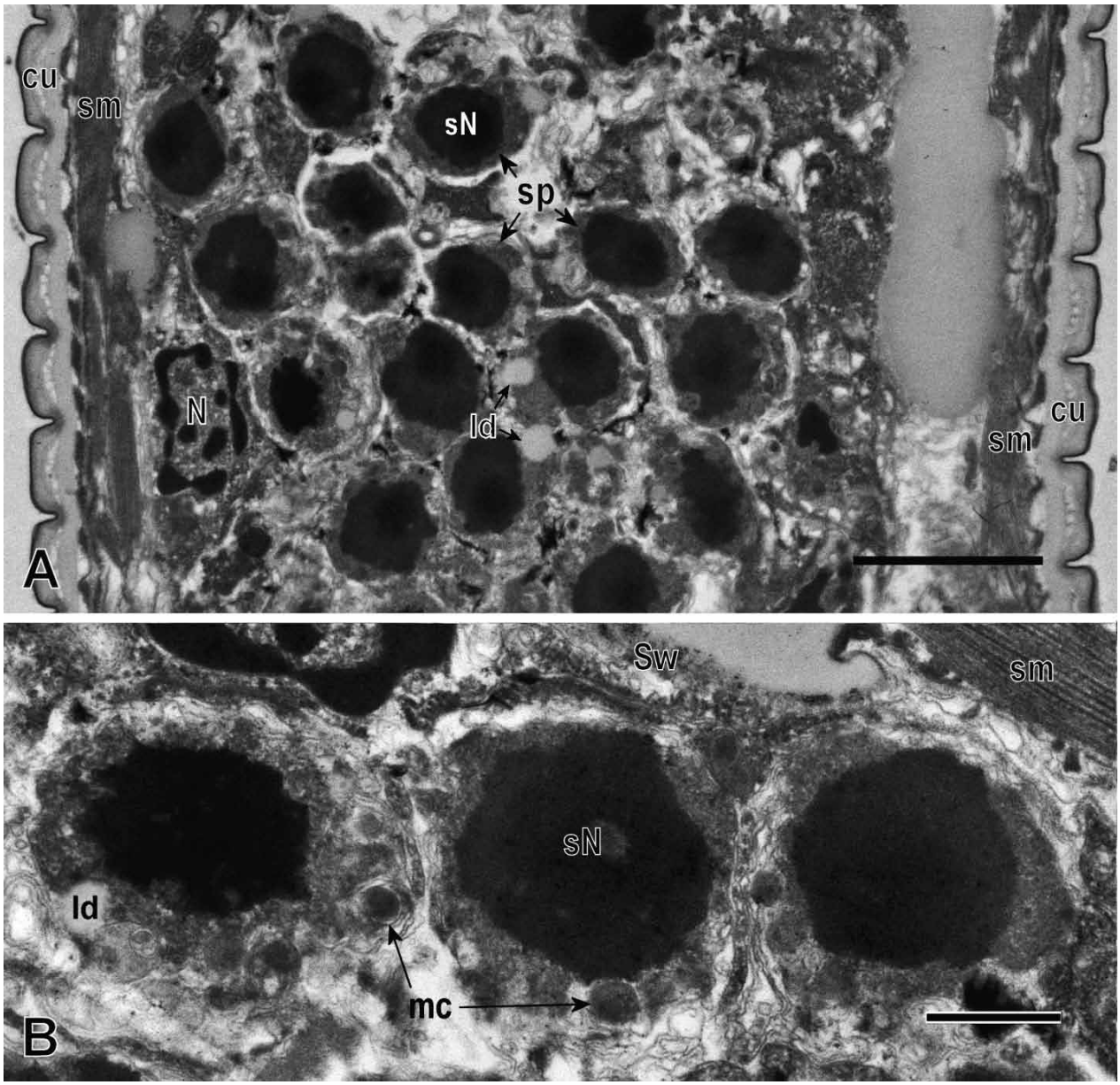

Fig. 3. *Paratylenchus hamatus sensu lato*, spermatheca on longitudinal sections, TEM. A: overview of the sperm cluster in spermatheca. B: spermatozoa stored in spermatheca at high magnification. Abbreviations: cu – body cuticle; ld – lipid droplets; mc – mitochondria; N – somatic nuclei; sm – somatic muscles; sN – sperm nucleus; sp – spermatozoa; Sw – spermathecal wall. Scale bars A = 2 μm; B = 0.5 μm.

nucleus *ca* 0.9 μm in diameter surrounded by a thin layer of electron dense cytoplasm (Fig. 3A, B). The cytoplasm contains several dense membrane bound spheres about 0.2 μm in diameter, which may be interpreted as mitochondria. Occasional lipid droplets also occur in spermatozoan cytoplasm (Fig. 3A, B). No structures similar to membranous organelles of spermatozoa as in other rhabditid nematodes were observed. The spermatozoa bear neither filopodia nor pseudopods but instead have irregular outlines.

DISCUSSION

Observations on *P. hamatus* showed that the spermatheca contains spermatozoa, as has been stated correctly in numerous taxonomic descriptions of species from the genus (Solovyeva, 1972; Siddiqi, 2000). However, these spermatozoa are clearly different from the usual rhabditid spermatozoa due to their small size and simple structure; the spermatozoa are reduced to a nucleus

surrounded by thin layer of cytoplasm with occasional mitochondria.

The unusually small size of spermatozoa in *P. hamatus* may be explained by the minute body size characteristics of this genus of superfamily Criconematoidea. In gonochoristic species, where females produce fertilised eggs, the number of spermatozoa should be at least equivalent to the number of eggs (Singson, 2001). A short body and narrow body diameter result in a volume restriction for the sperm storage organ, spermatheca. The number of spermatozoa necessary for fertilisation may be packed in these spermathecae only by way of radical reduction of size and simplification of structure. The females of the recently described *Protorhabditis hortulana*, one of the smallest free-living soil nematodes known (Rhabditoidea, Protorhabditidae) are only 189-222 μm long; their spermathecae, although not yet investigated by TEM, also contain distinctly miniaturised spermatozoa (Abolafia & Peña-Santiago, 2016). This is another indication that minute body size can result in sperm miniaturisation.

The regular 'rhabditid' type of spermatozoa has been described for representatives of several taxa of the 'rhabditids', such as Spiruromorpha, Ascaridomorpha, Panagrolaimomorpha, Myolaimina, Tylenchomorpha and Rhabditomorpha (Justine & Jamieson, 1999; Justine, 2002; Yushin *et al.*, 2006, 2007a, b, 2011, 2016; Giblin-Davis *et al.*, 2010; Yushin & Ryss, 2011; Zograf, 2014; Slos *et al.*, 2015; Qing *et al.*, 2017). Usually, the spermatozoa are amoeboid cells about 5 μm in diameter with a distinct pseudopod and a main cell body that includes a nucleus, numerous mitochondria and membranous organelles.

However, extremely small spermatozoa or microspermatozoa are well known in several taxa of rhabditids. Males of some Sphaerularioidea (representatives of the genera *Contortylenchus*, *Deladenus* and *Sphaerularia*) produce numerous microspermatozoa and impregnate the infective females. Females store a pool of hundreds of male gametes in voluminous spermatheca until needed for reproduction (Bedding, 1972, 1984; Chitwood & Chitwood, 1977; Kosaka & Ogura, 1993; Siddiqi, 2000). A reduction of cell size results in structural simplification and reduction of cytoplasm volume, together with a reduction in number of cytoplasmic components such as mitochondria, membranous organelles, cytoskeleton fibres and storage inclusions (Yushin *et al.*, 2007a, b; Yushin & Kosaka, 2013).

The spermatozoa found in the spermatheca of *Sphaerularia vespae* (Sphaerulariidae) are only 1.4-1.6 μm in diameter (Yushin & Kosaka, 2013). The microspermatozoa stored in the uterus of infective females of another sphaerularioidid nematode, *Deladenus* sp. (Neotylenchidae), are tiny cells of *ca* 2 μm in diameter with a spherical or oval nucleus and a small amount of cytoplasm containing several mitochondria and membranous organelles (Yushin *et al.*, 2007a).

Spermatozoa of an unusually small size and simplified structure are also known in some species of *Steinernema* (Strongyloidoidea, Steinernematidae), where drastic sperm dimorphism results in the development of giant and minute spermatozoa simultaneously inside a single testis (Spiridonov *et al.*, 1999; Yushin *et al.*, 2007b). The dimorphic spermatozoa of *Steinernema* spp. are united as spermatozeugmata where the giant megaspermatozoon (30-100 μm in diameter) bear numerous tiny microspermatozoa intimately attached to the megaspermatozoon surface (Yushin *et al.*, 2007b).

The genus *Paratylenchu*s may be characterised by the presence of minute spermatozoa as now observed by TEM in *P. hamatus*. Sperm miniaturisation may also be the case in other minute nematodes; however, this remains to be investigated using TEM.

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V.V. Yushin, S.A. Subbotin, M. Claeys and W. Bert. Сперматозоиды в сперматеке фитопаразитической нематоды *Paratylenchus hamatus sensu lato* (Tylenchida, Paratylenchidae).

Резюме. Морфологические описания нематод из рода *Paratylenchus* включают в себя информацию о сперматеках, как заметной части женской половой системы. Впервые изучены сперматозоиды из сперматеки у нематоды *Paratylenchus hamatus sensu lato*. Сперматека – эпителиальный мешок длиной 16 и шириной 8 мкм, содержащий плотно упакованные сперматозоиды, округлые клетки 1.4 мкм в диаметре, с ядром, окруженным тонким слоем цитоплазмы. Никаких структур, кроме нескольких небольших по размеру митохондрий и липидных капель, в цитоплазме сперматозоидов не обнаружено. Сперматозоиды *P. hamatus* имеют предельно миниатюризированные размеры, что может быть связано с миниатюрными для нематод размерами взрослых особей.