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Protistology

ORIGINAL ARTICLE

Niche partitioning within an insect host: trypanosomatids *Wallacemonas raviniae* and *Trypanosoma* (*Megatrypanum*) sp. in the horsefly *Hybomitra solstitialis*

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Summary

Tabanids (horseflies and deerflies) represent the main vectors of *Trypanosoma theileri* species complex and are frequently infected by them. In these insects, the trypanosomes are transiently present in the midgut and develop predominantly in the ileum. During a survey of infections in tabanids, we encountered a horsefly, in which trypanosomatids were present not only in the ileum but also in the rectum. The analysis of 18S rRNA sequences of the parasites in both locations demonstrated that they represented a *T. theileri*-like trypanosome and the monoxenous species *Wallacemonas raviniae* previously described from a flesh fly, respectively. The investigation using light and transmission electron microscopy showed that the two parasites differed not only in their affinity to distinct hindgut sections, but also in the patterns of attachment to the cuticular lining, interaction between individual cells, and development of extracellular structures. Unlike most monoxenous trypanosomatids inhabiting the rectum, *W. raviniae* was not observed on rectal glands or in the close proximity to them indicating either a peculiarity of the parasite species or specific conditions in the insect host. Our results demonstrated that the two trypanosomatid species partitioned their niches within the horsefly host due to strikingly dissimilar life strategies.

Key words: mixed infection, *Trypanosoma theileri*, ultrastructure

Introduction

The family Trypanosomatidae unites obligate parasites of vertebrates, invertebrates, and plants (Podlipaev, 1990). The members of the family are classified in two non-taxonomical groups: monoxenous and dixenous trypanosomatids, depending on whether one (typically insect) or two alternating hosts (usually an insect and either a vertebrate or plant) are present in their life cycles, respectively (Maslov et al., 2019).

Mixed infections of insects with two or more trypanosomatid species have been repeatedly documented using either molecular or traditional

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morphological methods (Wallace et al., 1965; Westenberger et al., 2004; Maslov et al., 2007; Yurchenko et al., 2009; Malele et al., 2011; Chajbullinova et al., 2012; Votýpka et al., 2012; Frolov et al., 2017; Králová et al., 2019; Maiguashca Sanchez et al., 2020). The unawareness of this phenomenon often causes confusion so that some features of one species are attributed to another (Laird, 1959; Kostygov et al., 2011; Kostygov et al., 2014; Frolov et al., 2019; Frolov et al., 2020). Therefore, studying development of trypanosomatids in insects can be challenging, especially if this concerns natural infections where specific homogeneity should always be checked.

Of special interest are mixed trypanosomatid infections in blood-sucking insects serving as vectors of multiple pathogenic species of the genera *Trypanosoma* and *Leishmania*. In addition, blood-sucking insects are parasitized by various monoxenous flagellates that can be confused with their dixenous relatives, thus complicating studies of natural infections by the latter. For example, *Blastocrithidia triatomae* inhabits triatomine bugs (Cerisola et al., 1971). The members of five genera (*Crithidia, Novymonas, Wallacemonas, Obscuromonas* and *Kentomonas*) have been documented in tsetse flies (Votýpka et al., 2021), *Crithidia mellificae* and *Blastocrithidia* sp. – in tabanids (Votýpka et al., 2019), various species of *Blechomonas, Herpetomonas* and *Leptomonas* – in fleas (Votýpka et al., 2013), and *Paratrypanosoma confusum, C. fasciculata* and *Strigomonas culicis* – in mosquitoes (Novy et al., 1907; Wallace, 1943; Flegontov et al., 2013). Except for a case of two monoxenous species in a single flea (Votýpka et al., 2013), these studies do not report mixed infections. Furthermore, the simultaneous development of two different trypanosomatids has not been studied in bloodsucking or any other insects.

Among numerous articles on trypanosomatid infections in tabanids (horseflies and deerflies), we found only a single report of a mixed infection in *Chrysops longicornis*, which harbored *T. theileri* and *T. evansi* (Abah et al., 2020). However, the latter species is transmitted mechanically and does not undergo any development in the vector (Hoare, 1972). Our previous study of two *T. theileri*-like species developing in tabanids demonstrated that after a transitory stay in the midgut, these flagellates settle in the ileum, the largest section of the hindgut. There, the parasites form a massive continuous carpet of cells attached to the cuticular lining and submerged in a fibrillar matrix. In the next hindgut section, the rectum, only dead trypanosome cells or occasional detached metacyclics could be observed (Kostygov et al., 2022). However, in one of the examined tabanids, attached trypanosomatids were detected not only in the ileum, but also in the rectum suggesting infection by a different species. Here, we describe this case of mixed infection by trypanosomes and a monoxenous flagellate highlighting their different adaptive strategies.

Material and methods

Insect host

The horsefly specimen 193Tab was captured on 12 July 2018 in the Republic of Karelia near the Lakhdenpokhya town (61°31' N; 30°12' E) in the context of a survey of trypanosomatid infections in tabanids. The captured insect was preserved in an individual plastic vial. Several hours later, it was euthanized with chloroform and dissected in normal saline. All sections of the intestine were examined under the Leica DM 2500 microscope (Leica Microsystems GmbH, Wetzlar, Germany). Upon detection of flagellates in the 193Tab specimen hindgut, most of the latter was fixed for transmission electron microscopy (TEM), while the remaining parts were preserved in 96% ethanol for molecular identification.

Microscopy

The hindgut fragments were fixed with 1.5% glutaraldehyde in 0.1 M cacodylate buffer for 1h at 0° C, post-fixed in 2% OsO4 in the same buffer for 1h at 0° C, dehydrated in an ascending alcohol series and propylene oxide and embedded in the Epon-Araldite mixture. Ultrathin 60 nm sections were prepared using the Leica UC-6 ultramicrotome (Leica Microsystems), stained in the saturated aqueous solution of uranyl acetate and lead citrate following a previously described protocol (Reynolds, 1963) and examined in the Morgagni 268-D microscope (FEI Company, Hillsboro, OR).

To determine the localization of trypanosomatids within different parts of the hindgut, semithin 700 nm sections were made with the same ultramicrotome, placed on glass slides, attached by drying on a warm stage at 60° C, and stained with Richardson stain (Richardson et al., 1960). They were examined under the light microscope Leica DM 2500 and photographed using a UCMOS14000KPA 14-Mpx digital camera (ToupTek, Hangzhou, China).

Molecular identification

Genomic DNA was isolated separately from the ileum and rectum material using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. This DNA was used to specifically amplify a 0.8 kb fragment of the trypanosomatid 18S rRNA gene with the primers 1127F and 1958R (Kostygov and Frolov, 2007) as well as the standard barcoding fragment of the mitochondrial cytochrome C oxidase gene (0.65 kb) with the primers LCO1490 and HCO2198 (Folmer et al., 1994). Sanger sequencing of both strands of the resulting PCR fragments was performed commercially with amplification primers. The obtained sequences were used for specific identification of the insect and its parasites using Identification Engine of the Barcode of Life Data Systems (https://boldsystems.org/) and BLAST searches against the Genbank nr database (http://www.ncbi.nlm.nih.gov/), respectively.

Results

Molecular identification

The horsefly COI gene sequence showed a 100% match to that of *Hybomitra solstitialis* from Finland (MZ624570). The trypanosomatid 18S rRNA gene sequences obtained from the ileum and rectum were identical to those of *Trypanosoma* (*Megatrypanum*) sp. Tthα (Kostygov et al., 2022) and *Wallacemonas raviniae* (Yurchenko et al., 2014), respectively. Therefore, all three species were identified unambiguously.

Light microscopy

Examination of the series of semithin hindgut sections of the 193Tab specimen hindgut demonstrated that the trypanosomes were localized only in the ileum, covering most of the surface of its wall with a continuous layer (Fig. 1, A). The distal part of the ileum near the rectal valve was occupied exclusively by symbiotic bacteria. On the border between these two areas, separate aggregates of trypanosomes were surrounded by bacteria (Fig. 1, B). These observations agree with our previous findings (Kostygov et al., 2022).

In the rectal ampulla, containing six rectal glands and situated posteriorly of the valve, aggregates of *W. raviniae* attached to the cuticular lining of this section were detected. Remarkably, the surface of all rectal glands was free of flagellates (Fig. 1, C). In addition to the wall of the rectal ampulla, the parasites also colonized the folds of the rectal valve and those of the post-ampullary part of the rectum (Fig. 1, D). Throughout the length of the rectum, the distribution of the flagellates was patchy. In contrast to the attached trypanosomes from the ileum, the cells of *W. raviniae* were shorter and had not a pyriform, but an oval, rounded, or slightly irregular shape (Fig. 1).

Electron microscopy

More differences between the two trypanosomatids were revealed using TEM. Both species of parasites attached to the cuticular lining of the hindgut using a widened flagellar tip equipped with a zonal hemidesmosome (Figs 2, A–D). The trypanosomes attached individually regardless of their distance to the gut surface; therefore, the length of flagella varied significantly between cells arranged in 2-3 rows (Fig. 2, A). In *W. raviniae*, only cells localized in the proximity to the cuticular lining displayed direct flagellar contact with it (Fig. 2, C). In most wallacemonads, flagella were shortened, exited from a wide flagellar pocket and typically harbored short lateral projections (Figs 2, C, D; 3, A, D), which were never observed in the trypanosomes. However, occasionally cells of this monoxenous trypanosomatid fixed on the cuticle by a lateral surface of a long flagellum also forming a zonal hemidesmosome (Fig. 2, E). Some wallacemonads did not directly attach to the host cuticle but formed flagellar contacts with other cells, thereby creating (attached) rosettes (Fig. 2, F). While trypanosomes formed a well-developed fibrillar matrix uniting the mass of attached parasites (Figs 2, A, B), *W. raviniae* displayed no extracellular structures (Figs 2, C; 3, A).

The ultrastructural organization of *W. raviniae* showed a series of features distinguishing it from trypanosomes. Specifically, the corset of subpellicular microtubules has a peculiar irregular arrangement with breaches, in which mitochondrial branches penetrate (Fig. 3, B). The wide flagellar pocket is dilated in its distal portion to provide more space for the flagellum, which typically shows here a local thickening (Figs 2, C, D; 3, A, C). Fixation of the flagellum in the flagellar pocket is achieved not using an extended zone of desmosomal contacts as in trypanosomes (Fig. 2, B), but by single desmosomes at the very exit from the flagellar pocket as well as at

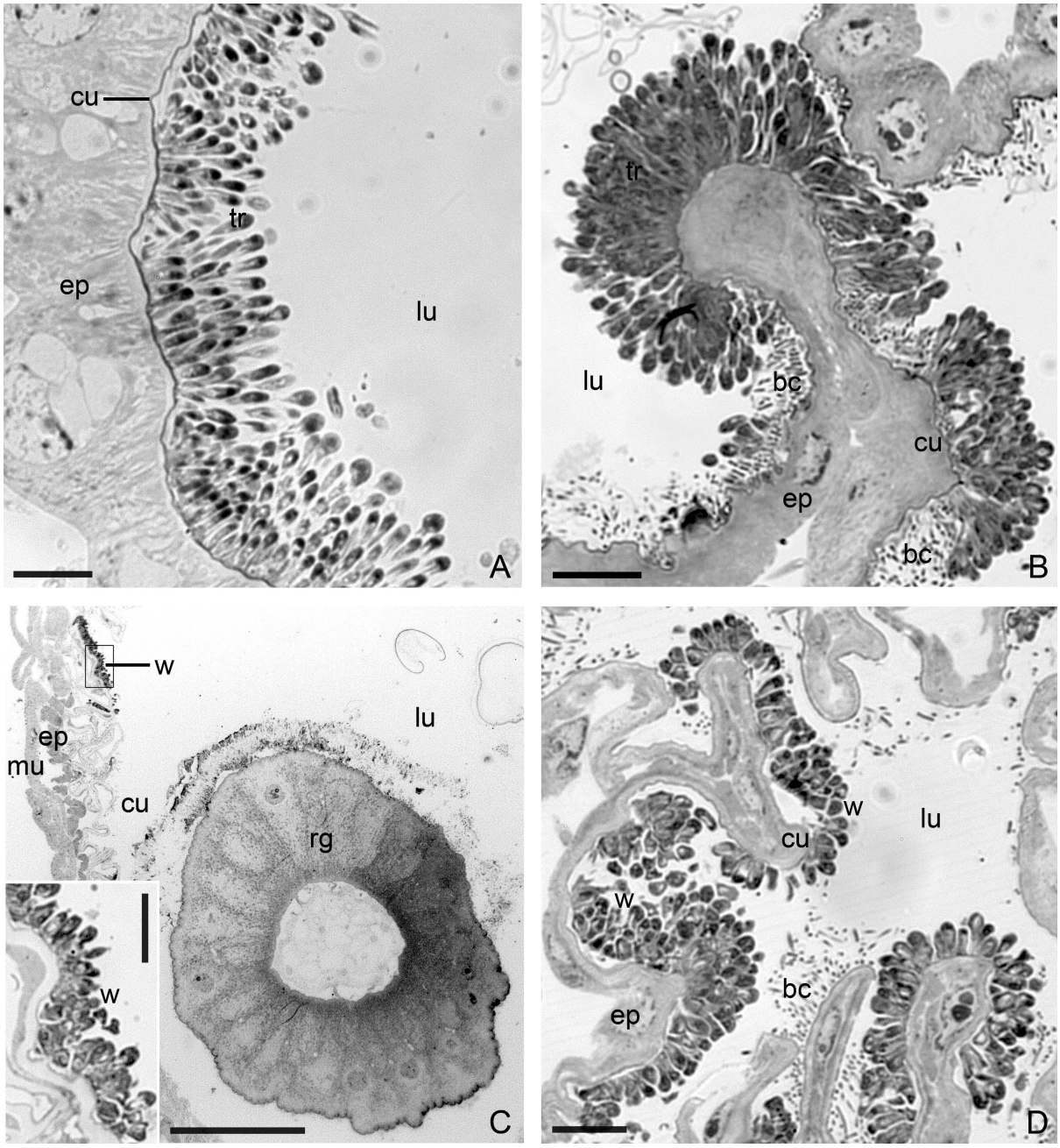

Fig. 1. Semithin sections of the hindgut. A, B – Attached cells of *Trypanosoma* sp. Tthα in the middle and pre-posterior parts of the ileum, respectively; C – *W. raviniae* in the rectal ampulla; inset – zoomed area boxed in C; D – *W. raviniae* in the folds of the distal part of the rectum. *Abbreviations*: bc – bacteria, cu – cuticle, ep – intestinal epithelium, lu – intestinal lumen, mu – muscles, re – rectal gland, tr – trypanosome cells, w – *W. raviniae* cells. Scale bars: A, B, D, inset – 10 μm; C – 100 μm.

the bottom of the pocket's dilation (Fig. 3, C, D). Below the bottom of the dilation, the cytoplasm is free of ribosomes and the corresponding desmosomes are connected to bundles of filaments with a diameter of 20 nm (Fig. 3, D). Immediately below the level of distal desmosome contacts, there is a cytostome associated with a narrow cytopharynx supported by microtubules (Fig. 3, C). In contrast to the irregularly shaped nucleus of *Trypanosoma* sp. (Fig. 2, A), that of *W. raviniae* is rounded (Figs 2, C; 3, A). The monoxenous species has a rod-shaped kinetoplast (0.42 × 0.18 μm in size), whose individual

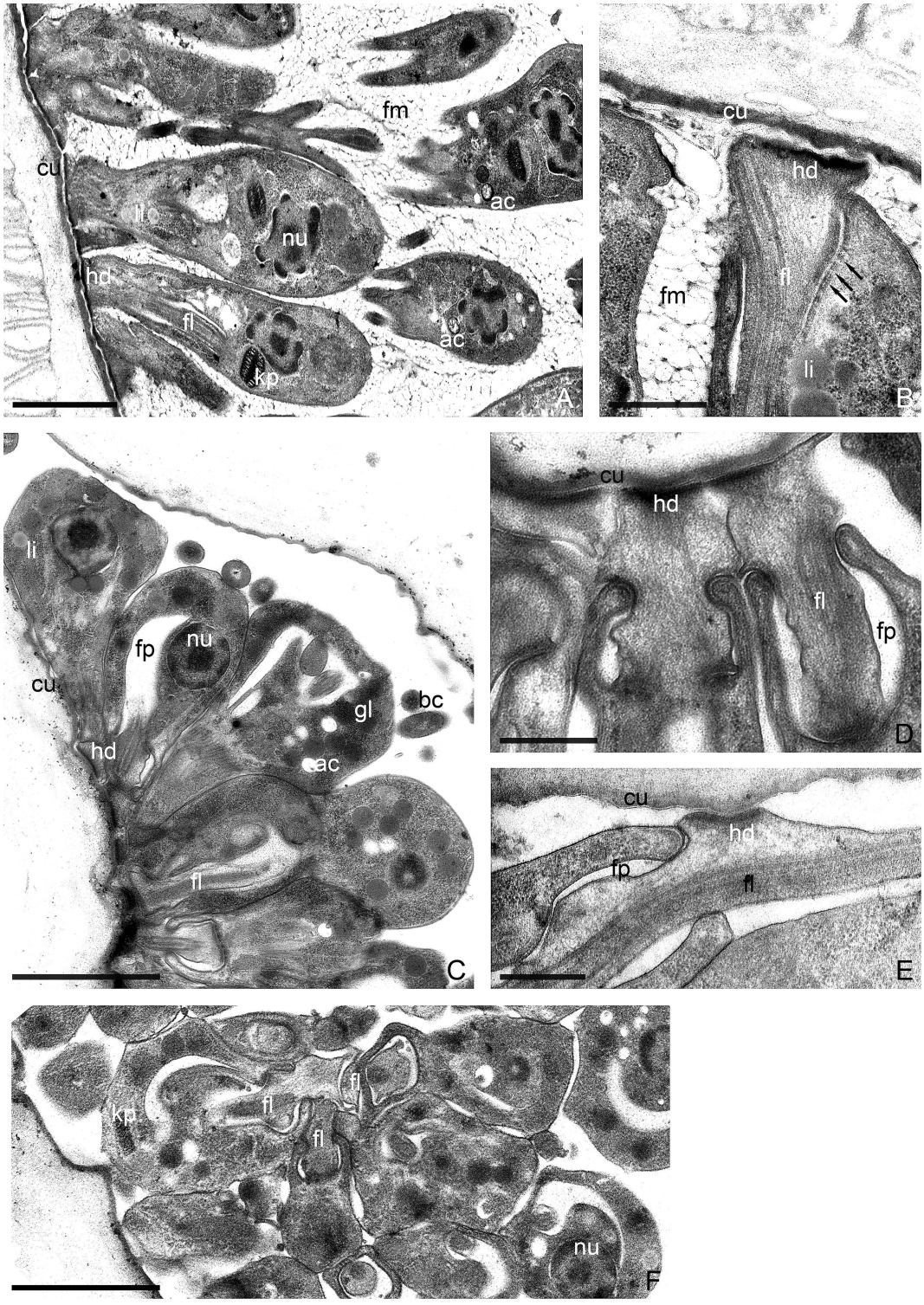

Fig. 2. Attachment of parasites to the cuticular lining of the hindgut. A, B – *Trypanosoma* sp. Tthα general view and the anterior part of an attached cell; C–F – *W. raviniae* in the rectum; C - a group of individually attached parasites; D – the anterior parts of the cells with apical attachment; E – lateral flagellar contact with the cuticle; F – a rosette of cells connected to each other with flagella. *Abbreviations*: ac – acidocalcisome, bc – bacteria, cu -cuticle, fl – flagellum, fp – flagellar pocket, gl – glycosome, hd – hemidesmosome, kp – kinetoplast, li – lipid droplet, nu – nucleus; arrows – desmosomal contacts. Scale bars: A, C – 2 μm; B, D, E – 0.5 μm.

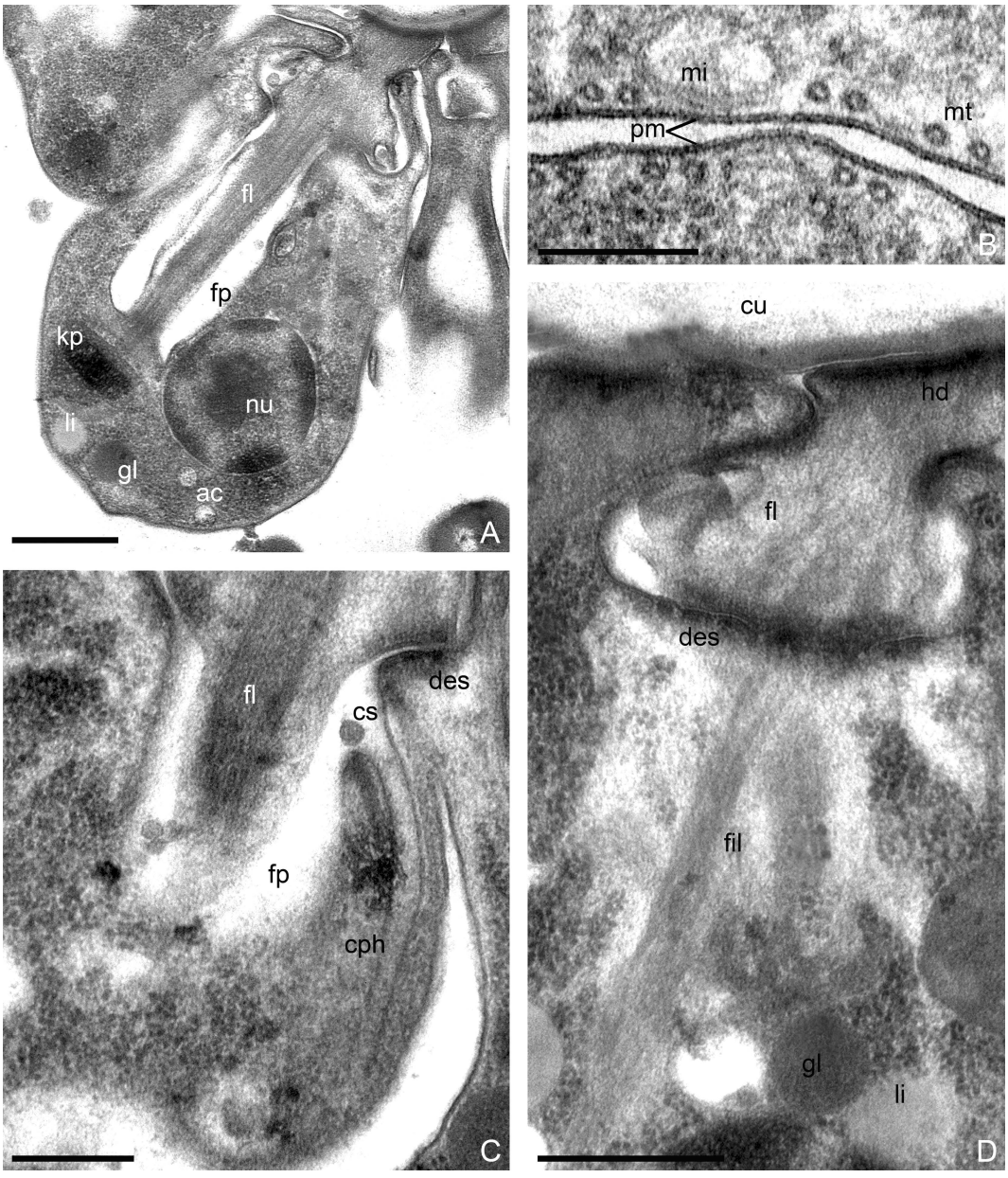

Fig. 3. Ultrastructure of *W. raviniae*. A – General view; B – unevenly spaced subpellicular microtubules; C – cytostome-cytopharyngeal complex; D – desmosome and filaments associated with flagellar pocket dilation. *Abbreviations*: ac – acidocalcisome, cph – cytopharynx, cs – cytostome, cu -cuticle, des - desmosome, fl – flagellum, fm – fibrillar matrix, fp – flagellar pocket, gl – glycosome, hd – hemidesmosome, kp – kinetoplast, li – lipid droplet, mi – mitochondrion, mt – microtubules, nu – nucleus, pm – plasmalemma. Scale bars: A, D – 0.5 μm; B, C – 0.2 μm.

DNA circles are barely distinguishable due to their high and homogeneous condensation (Fig. 3, A). The kinetoplast of trypanosomes is conspicuously thicker and has uneven condensation so that individual DNA fibrils are observable (Fig. 2, A). The hindgut-inhabiting cells of both trypanosomatid species contain lipid droplets in the cytoplasm (Figs 2, A, B, C; 3, A, D). Multiple acidocalcisomes and glycosomes are characteristic of *W. raviniae* (Fig. 2, C), while in the trypanosomes, the former are present only in some cells and the latter are absent (Fig. 2, A).

Discussion

Here we present the first detailed microscopic description of a simultaneous infection of an insect with two different trypanosomatid species. This became possible due to significant differences between *Trypanosoma* sp. Ttha and *Wallacemonas raviniae* in localization within the insect host and morphology/ultrastructure allowing them to be distinguished unambiguously. Our recent study of the development of trypanosomes in tabanids (Kostygov et al., 2022) further facilitated this task by delineating features inherent to these parasites.

Tabanidae represent the main vectors for most members of the *Trypanosoma theileri* species complex, while a few species are transmitted by keds (Hippoboscidae) (Hoare, 1972; Garcia et al., 2020). In addition, trypanosomes of this group have been documented in other dipterans: mosquitoes, sandflies and blackflies (Schoener et al., 2018; Garcia et al., 2020; Brotánková et al., 2022). The presence of monoxenous trypanosomatids (*Crithidia mellificae* and *Blastocrithidia* sp.) in tabanids has been previously reported in the Central African Republic. The authors proposed that those infections were non-specific and could be acquired with sugary liquids such as flower nectar (Votýpka et al., 2019). In the absence of data on the complete life cycle of *W. raviniae*, it is difficult to estimate the specificity of the infection described here, but at least it is obvious that the parasites were able to attach and proliferate in multiple foci. This species has previously been documented in the rectum of a sarcophagid fly (*Ravinia* sp.) from Ecuador and an unidentified fly from Madagascar (Yurchenko et al., 2014; Votýpka et al., 2020). In general, members of the genus *Wallacemonas* have been described from true bugs and dipterans and apparently can use a wide range of hosts, although the available information on this group is scarce (Kostygov et al., 2014; Yurchenko et al., 2014).

Both trypanosomatid species studied here develop in the horsefly hindgut and attach to the intestinal wall. Nonetheless, no interference is observed between them, since they occupy different niches within the host by colonizing two distinct hindgut sections. *Trypanosoma* sp. inhabits the ileum, while *W. raviniae* lives in the rectum. It is surprising that while most trypanosomatids developing in the rectum prefer the surface of the rectal glands (pads), often forming several rows of attached cells (Frolov et al., 2021), *W. raviniae* avoids settling on these organs. However, avoidance of the rectal glands has also been observed for *Herpetomonas samuelpessoai* and two unidentified trypanosomatid isolates that developed in the housefly (Hupperich et al., 1992). The rectal glands perform reabsorption of water, salts, and residual amino acids (Gupta and Berridge, 1966; Wall and Oschman, 1975) and it appears that thereby the conditions in their vicinity should be favorable for parasites. It is unclear whether the behavior manifested by W. raviniae is a specific feature of this trypanosomatid or may vary depending on the host.

Some of the ultrastructural peculiarities of *W. raviniae* highlighted here have not been characterized in the original description (Yurchenko et al., 2014). These include irregular arrangement of the subpellicular microtubules, presence of a cytostome and a well-developed cytopharynx, as well as desmosomes-associated filamentous bundles going deep into the cytoplasm. The peculiar structure of tubulemma with mitochondrial branches spreading into the breaches between microtubules is characteristic of Strigomonadinae, a trypanosomatid subfamily, all members of which bear intracellular bacterial symbionts (Teixeira et al., 2011; Votýpka et al., 2014). In addition, this feature has been observed in one more representative of *Wallacemonas, W. rigidus* (Podlipaev et al., 1991), although bacterial symbionts have not been documented in any species of this genus.

So far, a well-developed cytostome-cytopharyngeal complex has been described only in two monoxenous species: mosquito-parasitizing *Paratrypanosoma confusum* (Skalický et al., 2017) as well as *Herpetomonas nabiculae* inhabiting the oesophagus and stomach of damsel bugs (Shaglina et al., 1995). The former species, which represents the earliest branching trypanosomatid lineage, should have inherited this feature from the common ancestor of the family, whereas in the latter species, such a developed state of the complex is secondary, since in all studied relatives of this flagellate it is reduced. This peculiarity may represent an important adaptation but its significance is still poorly understood (Frolov et al., 2021).

The attachment by the flagellar tip with the formation of a hemidesmosome is the most common mechanism for trypanosomatids developing in the insect hindgut, the walls of which have a cuticular lining (Molyneux, 1977; Frolov et al., 2021), and we observed it in both species studied here. Fixation on the hindgut cuticle by the lateral flagellar sur-

face is rare, but at least two species have been documented to use it along with the regular tip-based attachment mode (both with the formation of hemidesmosomes). These are *Blastocrithidia raabei* developing in the midgut and hindgut of the true bug *Coreus marginatus*, as well as *Angomonas deanei* parasitizing the blowfly *Lucilia sericata* and, similarly to *W. raviniae*, colonizing only the rectum, although it does not ignore the rectal glands (Frolov et al., 2020; Ganyukova et al., 2020). We believe that this mechanism is auxiliary and ensures the attachment of those cells that are localized in narrow spaces between the cuticular folds of the rectum.

The trypanosomes of *T. theileri* group in tabanids colonize all available surfaces of the ileum except for its posterior part, where the epithelium is folded more intensively and forms narrow pockets that favor a more efficient fixation. However, this region is occupied by symbiotic bacteria apparently settling there earlier. Under these conditions, trypanosomes secrete an extracellular fibrillar matrix, which unites all attached flagellates into a single mass and decreases the shearing force of the passing gut contents. Conversely, *W. raviniae* cells inhabiting the rectum take advantage of the high rugosity in this section, preferring rather numerous pockets created by the folds of the cuticular lining than open surfaces. In such conditions, no extracellular matrix is needed. Moreover, it would prevent dissemination of the parasites and infection of new insect hosts. Why the trypanosomes considered here do not settle in the rectum, one of the favorite locations of trypanosomatids? Firstly, they do not need to be discharged from the intestinal tract, since the infection of the ruminants, being the vertebrate hosts of these parasites, occurs after crashing an infected tabanid with tongue or teeth followed by subsequent penetration of flagellates through the oral mucosa (Böse et al., 1987a; Böse et al., 1987b). Secondly, trypanosome cells in the ileum represent mainly metacyclic trypomastigotes (Kostygov et al., 2022), which are resting stages with lowered metabolism levels (De-Simone et al., 2022). Inhabiting the rectum, where the conditions are hyperosmotic, would mean high energetic costs to maintain the homeostasis and, consequently, an intensive metabolism (note the absence of glycosomes in trypanosomes and their presence in wallacemonads).

In sum, the two species of trypanosomadids in the horsefly studied here, due to strikingly different life strategies have evolved distinct adaptations, including localization, mechanisms of attachment, etc. Thus, they have clearly separated their niches within the insect host and do not interfere with each other.

Acknowledgements

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