The development of *Psychodiella sergenti* (Apicomplexa: Eugregarinorida) in *Phlebotomus sergenti* (Diptera: Psychodidae)

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SUMMARY

Psychodiella sergenti is a recently described specific pathogen of the sand fly *Phlebotomus sergenti*, the main vector of *Leishmania tropica*. The aim of this study was to examine the life cycle of *Ps. sergenti* in various developmental stages of the sand fly host. The microscopical methods used include scanning electron microscopy, transmission electron microscopy and light microscopy of native preparations and histological sections stained with periodic acid-Schiff reaction. *Psychodiella sergenti* oocysts were observed on the chorion of sand fly eggs. In 1st instar larvae, sporozoites were located in the ectoperitrophic space of the intestine. No intracellular stages were found. In 4th instar larvae, *Ps. sergenti* was mostly located in the ectoperitrophic space of the intestine of the larvae before defecation and in the intestinal lumen of the larvae after defecation. In adults, the parasite was recorded in the body cavity, where the sexual development was triggered by a bloodmeal intake. *Psychodiella sergenti* has several unique features. It develops sexually exclusively in sand fly females that took a bloodmeal, and its sporozoites bear a distinctive conoid (about 700 nm long), which is more than 4 times longer than conoids of the mosquito gregarines.

Key words: *Psychodiella*, *Psychodiella sergenti*, gregarine, *Phlebotomus sergenti*, sand fly, life cycle, PAS, egg, larva, adult.

INTRODUCTION

Gregarines parasitizing sand flies (Apicomplexa: Eugregarinorida) are aseptate eugregarines recently separated from the mosquito genus *Ascogregarina* Ward, Levine and Craig, 1982 to form a new genus *Psychodiella* Votypka, Lantova and Volf, 2009 (Votypka *et al*. 2009). Despite their high degree of host specificity, only 5 *Psychodiella* species have been described so far. Numerous studies on the mosquito *Ascogregarina* species (e.g. Vavra, 1969; Walsh and Callaway, 1969; Sanders and Poinar, 1973; Munstermann and Levine, 1983; Chen *et al*. 1997*a*) showed that mosquito gregarines differ from sand fly gregarines at two critical points of the life cycle: in mosquito larvae, the gregarines develop intracellularly in the intestinal epithelial cells, and in adults, they are located in the Malpighian tubules.

The life cycle of *Psychodiella* has been studied in detail in *Psychodiella chagasi* (Adler and Mayrink, 1961) by Adler and Mayrink (1961), Coelho and Falcao (1964) and Warburg and Ostrovska (1991). Briefly, the 1st instar larvae are infected by swallowing the gregarine infective stages, the oocysts. Sporozoites released from oocysts reside in the larval midgut and develop into trophozoites. Mature stages of the gregarines, the gamonts, can be found mostly in the larval gut lumen, where they undergo sexual development; 2 gamonts associate into syzygy, which is later enclosed in a cyst wall, forming a gametocyst. Within the gametocyst, each gamont develops into gametes by gamogony, and after fertilization, during sporogony, zygotes differentiate into oocysts with 8 sporozoites. In adults, the gregarines are located in the body cavity, undergoing sexual development. In females, gametocysts attach to the accessory glands, and oocysts are injected into their lumen. During oviposition, the accessory gland fluid containing the oocysts adheres to the chorion of eggs and serves as a source of infection for newly hatched larvae. This general life cycle is modified in other *Psychodiella* species, and differences were described in *Psychodiella mackiei* (Shortt and Swaminath, 1927) and *Psychodiella tobbi* Lantova, Volf and Votypka, 2010.

Psychodiella sergenti Lantova, Volf and Votypka, 2010 is a recently described specific pathogen of the sand fly *Phlebotomus sergenti* Parrot, 1917 (Diptera: Psychodidae), an important vector of *Leishmania tropica* (Wright, 1903) (e.g. Killick-Kendrick *et al*. 1995), which is a causative agent of human cutaneous leishmaniasis. Native preparations of sand fly adults revealed that sexual development of *Ps. sergenti* (formation of syzygies, gametocysts and oocysts) occurs only in female sand flies and is conditioned by a bloodmeal intake (Lantova *et al*. 2010). The aim of

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the present study was to document the *Ps. sergenti* life cycle in various life stages of its host, including the eggs and 1st instar larvae. Several microscopical methods were used: scanning electron microscopy, transmission electron microscopy and light microscopy of native preparations and histological sections stained with PAS reaction (periodic acid-Schiff). Using such a wide variety of microscopical techniques provided a clear overview of the whole parasite's life cycle.

MATERIALS AND METHODS

Sand flies

The *Phlebotomus sergenti* colony infected with *Ps. sergenti* originated from females collected in Sanli Urfa, Turkey. The colony maintenance was described by Volf and Volfova (2011) and included an egg-washing procedure using a series of solutions (Poinar and Thomas, 1984) to reduce the infection intensity of this pathogenic gregarine (Lantova *et al.* 2011). The washing was omitted in a batch of eggs used for this study.

Native preparations

Adults of both sexes and different ages were used, as well as 2 groups of 4th instar larvae: those with a gut filled with larval diet are hereafter referred to as larvae before defecation (BD), and those ready to pupate with an empty gut are hereafter referred to as larvae after defecation (AD). The specimens were immobilized on ice and dissected in phosphate-buffered saline (PBS) under a stereomicroscope SZX-12 (Olympus Corporation, Tokyo, Japan). Micrographs were produced with a DP-70 digital camera (Olympus) connected to an optical microscope BX-51 (Olympus).

Scanning microscopy

Gravid sand fly females were left to oviposit into a plastic cup filled with plaster (commonly used for the colony, see Volf and Volfova, 2011), and 1–2 days later, eggs were transferred using a fine brush onto a double-sided tape. Oocysts were documented on the surface of *Ph. sergenti* eggs using a scanning microscope TM-1000 (Hitachi High-Technologies Corporation, Tokyo, Japan). This scanning microscope does not require any sample preparation and allows direct observation of unfixed and non-desiccated samples.

Electron microscopy

Three-day-old 1st instar larvae were fixed in modified Karnovsky fixative (Karnovsky, 1965) or in 2·5% glutaraldehyde in cacodylate buffer (4 °C), post-fixed in 2% osmium tetroxide in cacodylate buffer (4 °C), dehydrated through an ascending ethanol and acetone series and embedded in Araldite 502/Poly-Bed 812 (Polysciences Inc., Warrington, PA, USA). Thin sections (70 nm) were prepared on a Reichert-Jung Ultracut E ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and stained using uranyl acetate and lead citrate (Reynolds, 1963). The sections were examined and photographed using an electron microscope JEM-1011 (JEOL Ltd, Tokyo, Japan).

Histology

Larvae of 4th instar (BD and AD) and adults at different days post-eclosion were dissected and fixed at 4 °C in AFA fixative (96% ethanol: 38% formaldehyde: acetic acid: distilled water; 12·5:1·5:1:10). The head and the last posterior segments were removed for better penetration of fixatives. Specimens were then washed 3 times in PBS and 70% ethanol and embedded in 2-hydroxyethyl methacrylate (JB-4 Plus Embedding Kit, Polysciences) following the manufacturer's instructions. Histological sections (2–4 μm) were prepared using a Shandon Finesse ME + microtome (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and stained using PAS (periodic acid-Schiff) reaction with Ehrlich's acid haematoxylin: oxidization in 1% periodic acid for 5 min, Schiff's reagent (Sigma-Aldrich Corporation, St Louis, MO, USA) for 28 min, Ehrlich's acid haematoxylin for 4 min (with extensive washing in running water between steps). Stained sections were mounted on glass slides with Plastic UV Mount Mounting Media (Polysciences) and observed and photographed under an optical microscope BX-51 (Olympus) connected to a DP-70 digital camera (Olympus).

RESULTS

Ps. sergenti *oocysts on sand fly eggs*

The exochorion of *Ph. sergenti* eggs was contaminated with *Ps. sergenti* oocysts (Fig. 1). The number of oocysts per egg varied; some eggs appeared without any contamination, others contained dozens of oocysts. The distribution of oocysts on the chorion was not even, they usually concentrated around the tip of the egg (Fig. 1A – D) or along the longitudinal axis of the egg in contact with the exochorion sculpturing ridges (Fig. 1A – C). Manipulation of eggs by a brush occasionally caused detachment of the oocysts from the egg surface. In some cases, distinct imprints of the detached oocysts were visible (Fig. 1E).

Ps. sergenti *sporozoites in 1st instar sand fly larvae*

In 1st instar larvae, *Ps. sergenti* sporozoites were found in the intestine (Fig. 2). They were located

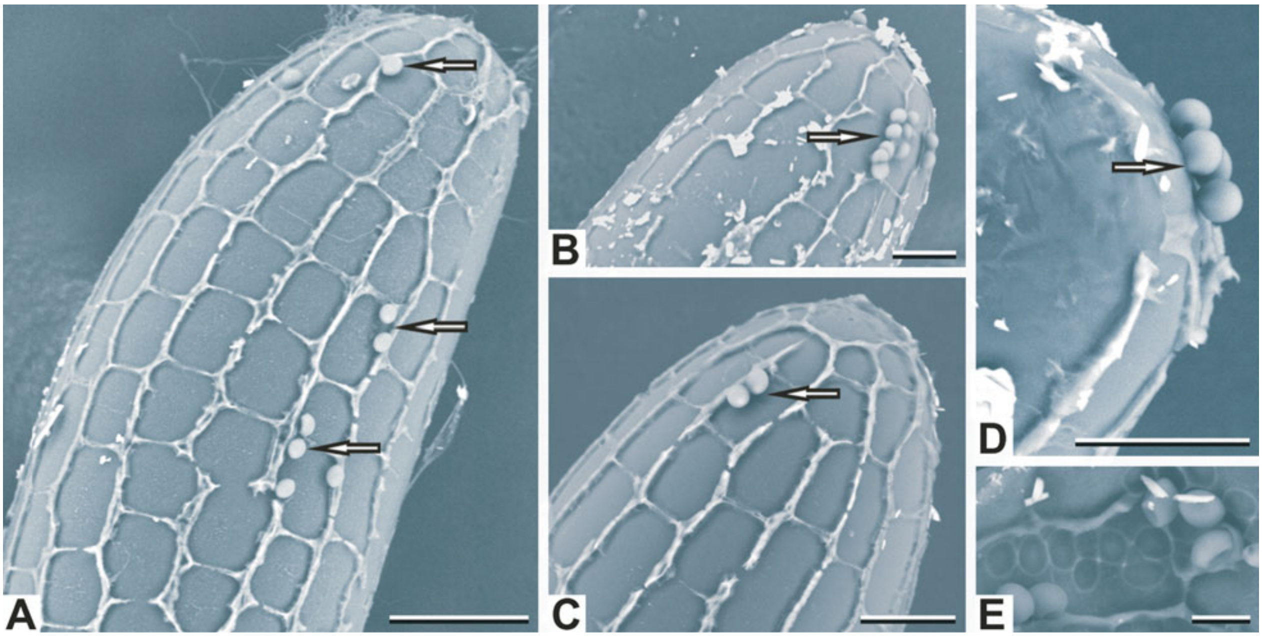

Fig. 1. Scanning electron micrographs of *Phlebotomus sergenti* eggs infected with *Psychodiella sergenti*. (A – D) *Psychodiella sergenti* oocysts (arrows) attached to the chorion of eggs. (E) Imprints of the detached oocysts. Scale bars (A) = 50 μm, (B – D) = 25 μm, (E) = 10 μm.

mostly in the posterior midgut, exclusively in the ectoperitrophic space between the peritrophic matrix and the epithelium (Fig. 2A). The sporozoites appeared to be gliding on microvilli, or they were attached to the epithelial cells with a distinct mucron (Fig. 2B, E). They had a 3-layered pellicle (Fig. 2D), tightly arranged subpellicular microtubules (Fig. 2C) and posteriorly located nucleus (Fig. 2E). A very distinct conoid (approximately 700 nm long) as well as a polar ring and numerous micronemes were observed (Fig. 2E, F). The sporozoites were never located intracellularly.

Ps. sergenti *development in 4th instar sand fly larvae*

The documentation of *Ps. sergenti* in 4th instar larvae was accomplished in native preparations (Fig. 3A, B) and histological sections stained with PAS reaction with Ehrlich's acid haematoxylin (Fig. 3C, D).

The gregarine stages found in 4th instar larvae were mostly gamonts, occasionally also syzygies and gametocysts but never oocysts. In BD larvae, gamonts were documented in the ectoperitrophic space of the intestine, mainly in the posterior midgut (Fig. 3A, C), while in AD larvae the gregarines were located in the midgut lumen along its whole length (Fig. 3B, D). In a few cases, gamonts were found also in the larval body cavity, but no intracellular development or cell damage was detected.

The PAS reaction proved to be very useful in highlighting gregarines in the host tissues. Their PAS-positive amylopectin granulation was typical and easily recognizable from other PAS-positive objects, e.g. from the midgut contents (Fig. 3C).

Ps. sergenti *development in sand fly males*

Even though males up to 13 days post-eclosion were examined, observations of both the native preparations and histological sections recorded only gamonts (Fig. 4A – C). The gamonts were usually round or oval, but in high-intensity infections, some had a shape of an hour-glass or a tear-drop. They were found mostly in the body cavity, in several cases also in the fat body but never in the intestine or elsewhere. The characteristic appearance of the gregarines with distinctive nucleus (Fig. 4B) allowed them to be easily distinguished from rectal papilla, the PAS-positive tissue of adult sand flies (Fig. 4A).

Ps. sergenti *development in sand fly females*

In females that did not take a bloodmeal, no other gregarine stages except gamonts were found, even though the females were dissected up to the age of 13 days. On the other hand, in blood-fed females, the whole sexual development, including syzygies, gametocysts and oocysts, was demonstrated (Figs 4D – F and 5). Gamonts and gametocysts were located in the body cavity and a few gamonts, particularly when the infection intensity was high, in the fat body.

In females 2 days post-bloodmeal, young gametocysts were found still consisting of the 2 original gamonts with their nuclei (Fig. 5A). From 3 to 5 days after a bloodmeal, gametocysts at different stages of maturation were documented (Fig. 5D, E), some of them being attached to the accessory glands (Figs 4D and 5B – E). Around day 5, the gametocysts were

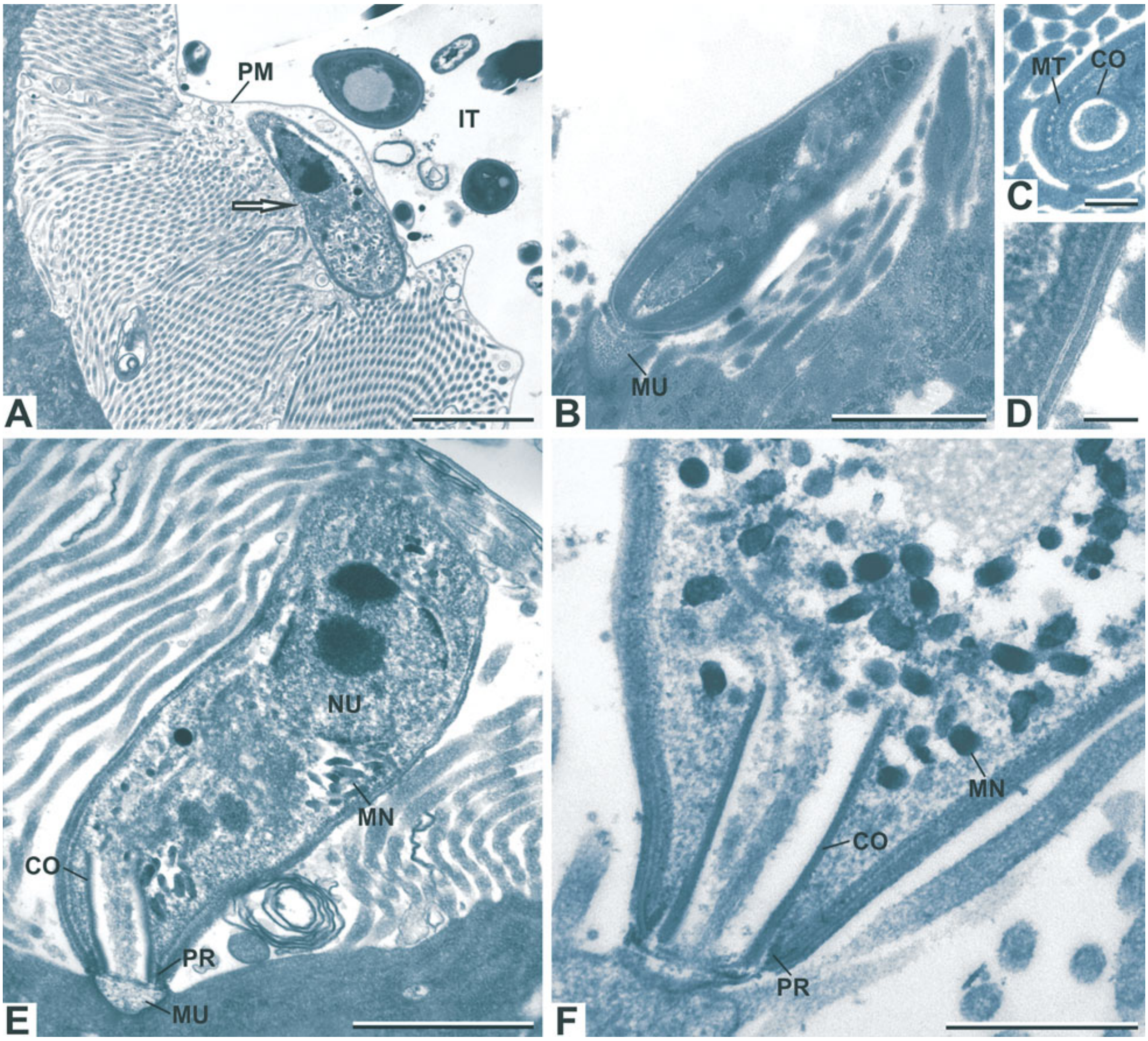

Fig. 2. Transmission electron micrographs of the intestine of 1st instar *Phlebotomus sergenti* larvae infected with *Psychodiella sergenti*. (A) Unattached sporozoite (arrow) in the ectoperitrophic space of the intestine. (B, E, F) Sporozoites attached to the epithelial cells of the intestine. (C) Transverse section of a sporozoite. (D) Detail of a 3-layered pellicle of a sporozoite. CO, conoid; IT, lumen of the larval intestine with organic debris and yeasts; MN, micronemes; MT, subpellicular microtubules; MU, mucron; NU, nucleus; PM, peritrophic matrix; PR, polar ring. Scale bars (A) = 2 μm, (B, E) = 1 μm, (C) = 300 nm, (D) = 100 nm, (F) = 500 nm.

fully matured and, later, the accessory glands of blood-fed females became filled with oocysts (Figs 4E, F and 5F–H).

Rectal papilla (Fig. 5A) and oocytes (Fig. 5D–F) are the strongly PAS-positive tissues found in females; however, gregarines stained with PAS reaction were distinct, particularly in sections not post-stained with Ehrlich's acid haematoxylin (Fig. 5B, C).

DISCUSSION

The main distinctive feature of the *Ps. sergenti* life cycle is the fact that the gregarines develop sexually exclusively in adult females that had a bloodmeal. This is a striking contrast to *Ps. chagasi* and *Ps. tobbi*, in which gametocysts are formed in adults of both sexes, including females that did not take a bloodmeal (Coelho and Falcao, 1964; Lantova *et al.* 2010). The only other sand fly gregarine with the life cycle in adult hosts similar to *Ps. sergenti* is an undescribed parasite reported by Ayala (1971) in *Lutzomyia vexatrix occidentis* Fairchild and Hertig, 1957. Similar to Ayala (1973), we hypothesize that the hormonal changes influenced by the ingestion of blood trigger the sexual cycle of the gregarine.

In *Ps. chagasi*, Coelho and Falcao (1964) and Warburg and Ostrovska (1991) found gregarine oocysts in 4th instar larvae, the former authors even described the formation of oocysts that were being defecated and served as a source of horizontal infection to other larvae. Contrastingly, no oocysts

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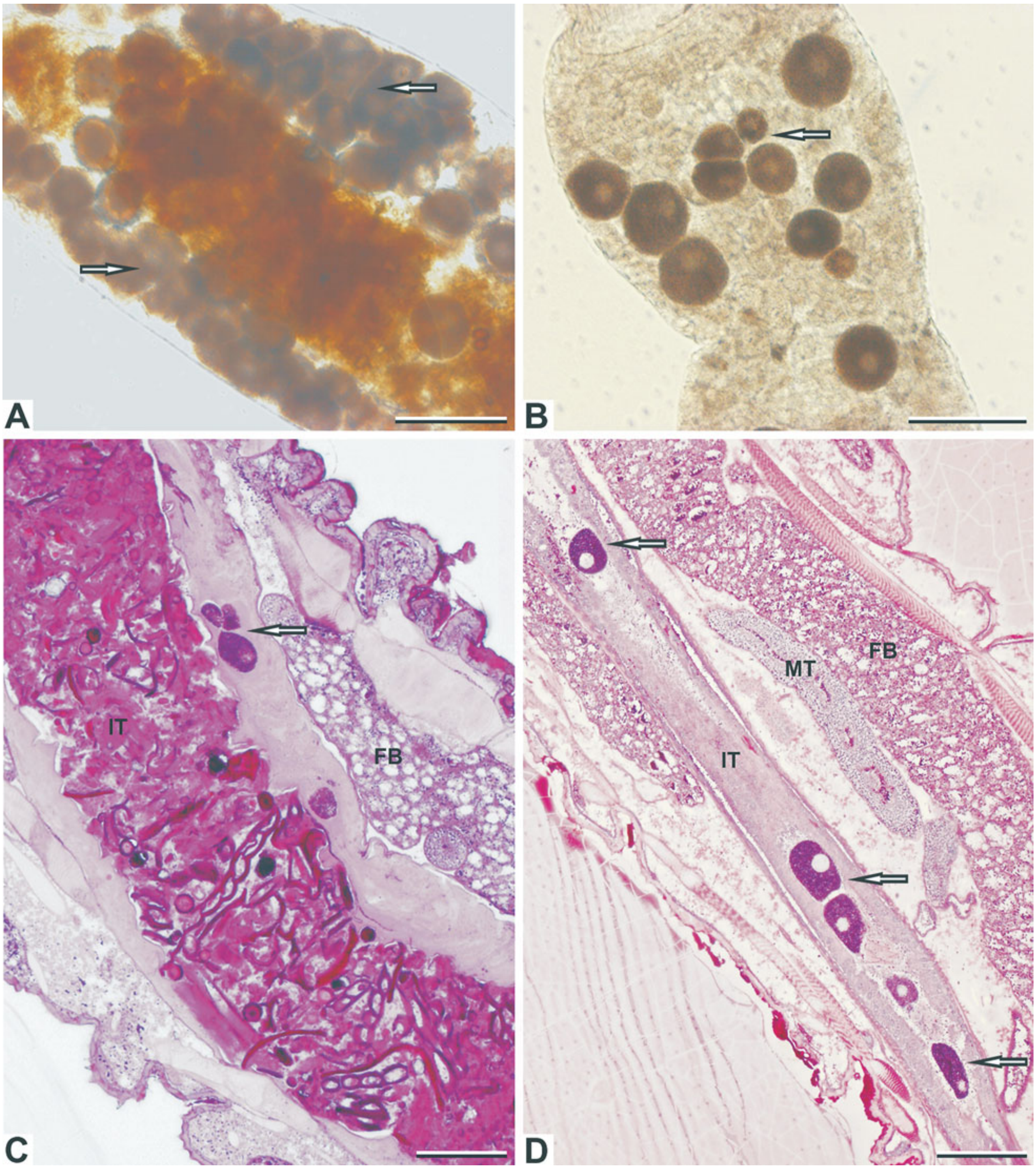

Fig. 3. Native preparations of the intestine (A, B) and histological sections of the whole body stained with PAS reaction with Ehrlich's acid haematoxylin (C, D) of *Phlebotomus sergenti* 4th instar larvae infected with *Psychodiella sergenti*. (A, C) 4th instar larva before defecation. The gregarines (arrows) are located in the ectoperitrophic space of the intestine. (B, D) 4th instar larva after defecation. The gregarines (arrows) are located in the lumen of the intestine. FB, fat body; IT, intestine; MT, Malpighian tubules. Scale bars = 100 μm.

were found in 4th instar larvae of *Ps. tobbi* (Lantova *et al*. 2010) or *Ps. sergenti* in this study. The localization of gregarines in the ectoperitrophic space of 4th instar BD larvae was observed in this study and also by other authors (Shortt and Swaminath, 1927; Coelho and Falcao, 1964; Warburg and Ostrovska, 1991).

In adults, the main location of the gregarines was the body cavity, as also recorded by Shortt and Swaminath (1927), Adler and Mayrink (1961) and Ostrovska *et al*. (1990). In high-intensity infections, we found *Ps. sergenti* also in the fat body. The attachment of the gametocysts to the accessory glands of females observed in *Ps. sergenti* was recorded in all

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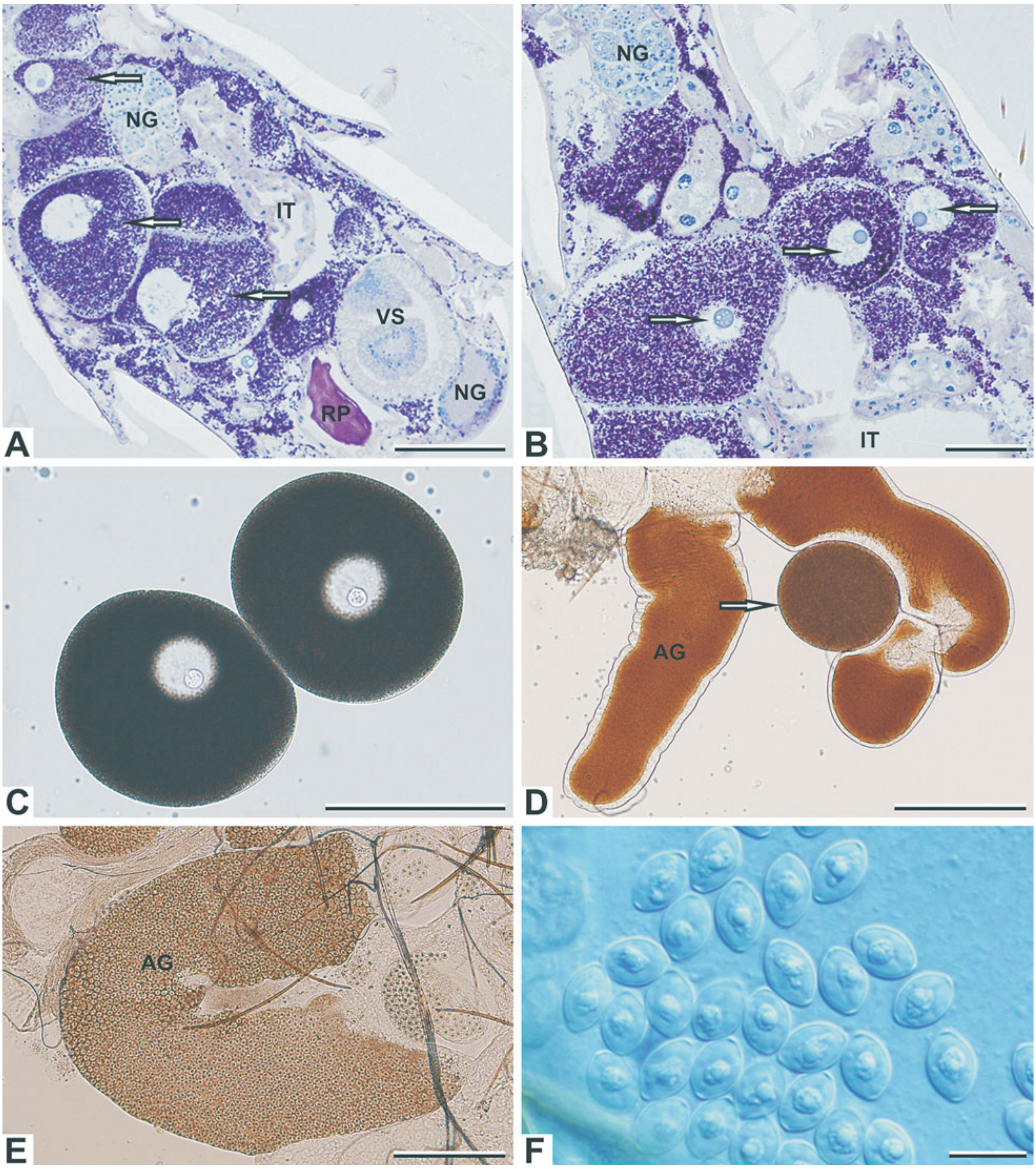

Fig. 4. Histological sections stained with PAS reaction with Ehrlich's acid haematoxylin of *Phlebotomus sergenti* males infected with *Psychodiella sergenti* (A, B) and native preparations of *Ph. sergenti* adults infected with *Ps. sergenti* (C–F). (A) Male body cavity filled with gamonts (arrows). (B) Gamonts with distinctive nuclei in the male body cavity (arrows). (C) Gamonts from the body cavity of a male sand fly. (D) Gametocyst (arrow) attached to the accessory gland of a female 5 days post-bloodmeal. (E) Accessory gland of a female 8 days post-bloodmeal filled with oocysts. (F) Detail of oocysts. AG, accessory glands; IT, intestine; NG, neural ganglion; RP, rectal papilla; VS, vesicular seminalis. Scale bars (A, C–E) = 100 μm, (B) = 50 μm, (F) = 10 μm.

other sand fly gregarine species (reviewed by Ostrovska *et al*. 1990).

Psychodiella sergenti sporozoites in 1st instar larvae were either in contact with the microvilli or attached to the epithelial cells with a mucron. No intracellular development of *Ps. sergenti* was recorded in the sand fly larvae. This is in agreement with findings of various authors on *Ps. chagasi,* but in contrast to the findings of Shortt and Swaminath (1927) on *Ps. mackiei*, where intracellular stages were reported in the gut of 1st instar larvae.

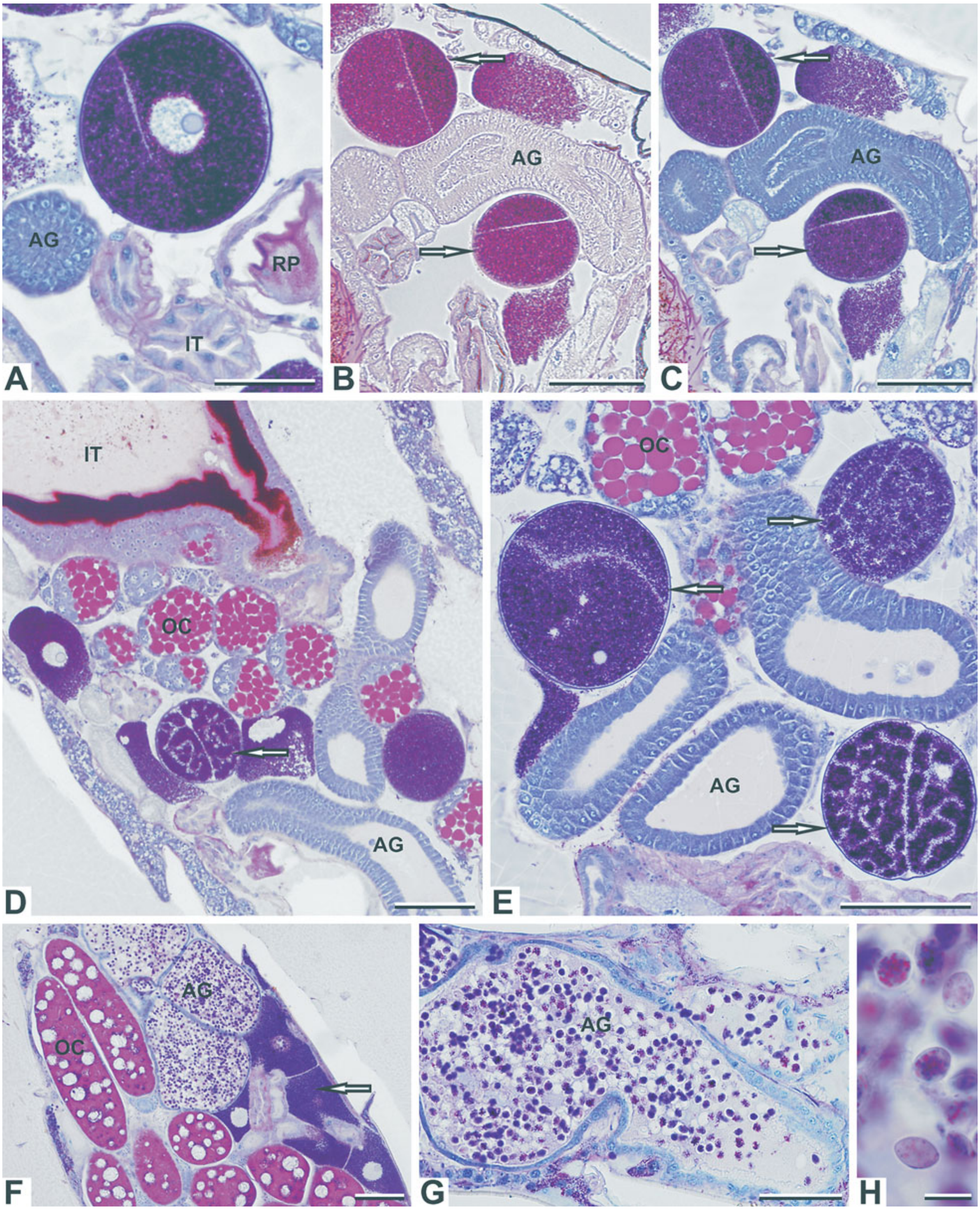

Fig. 5. Histological sections of *Phlebotomus sergenti* females 2 (A – C), 3 (D, E) and 7 (F – H) days post-bloodmeal infected with *Psychodiella sergenti* stained with PAS reaction with Ehrlich's acid haematoxylin. (A) Young gametocyst in the body cavity of a female 2 days post-bloodmeal. (B) Gametocysts (arrows) attached to the accessory glands of a female 2 days post-bloodmeal, stained only with PAS reaction. (C) Section B post-stained with Ehrlich's acid haematoxylin. (D) Gamonts and gametocysts (arrow) in the body cavity of a female 3 days post-bloodmeal. (E) Gametocysts (arrows) at different stages of maturation attached to the accessory glands of a female 3 days post-bloodmeal. (F) Female 7 days post-bloodmeal with gamonts (arrow) in the body cavity and oocysts in the accessory glands. (G) Accessory gland filled with gregarine oocysts. (H) Detail of oocysts. AG, accessory glands; IT, intestine; OC, oocytes; RP, rectal papilla. Scale bars (A, G) = 50 μm, (B – F) = 100 μm, (H) = 10 μm.

The conoid of *Ps. sergenti* sporozoite has a typical coiled appearance and is strikingly long (approximately 700 nm). This is in accordance with observations performed on *Ps. chagasi* (Warburg and Ostrovska, 1991), where the conoid was around 700 nm long. On the other hand, shorter conoids were recorded in mosquito ascogregarines (approximately 150 nm – Sheffield *et al*. 1971; Chen *et al*. 1997*b*) and some other apicomplexans (approximately 250 nm – e.g. Roberts *et al*. 1970; Dubey *et al*. 1998). This suggests that longer conoids might be typical for sand fly gregarines. The organization of the subpellicular microtubules of *Ps. sergenti* resembles *Ps. chagasi* (Warburg and Ostrovska, 1991) and differs from ascogregarines; both *Psychodiella* species have a high number of tightly arranged subpellicular microtubules, while *Ascogregarina culicis* Ross, 1989 possesses 21 of them (Sheffield *et al*. 1971). The surface of *Ps. sergenti* sporozoites consists of a 3-layered pellicle. Previously, a 2-layered pellicle was reported by Warburg and Ostrovska (1991) in *Ps. chagasi* and by Sheffield *et al*. (1971) and Sanders and Poinar (1973) in mosquito gregarine species. As pointed out by Vavra (1969), such a difference might be due to the fact that the 2 inner membranes could sometimes be very close, giving the impression of a single membrane.

Scanning electron microscopy showed *Ps. sergenti* oocysts frequently attached to the longitudinal exochorion sculpturing ridges of the sand fly eggs. In contrast, Adler and Mayrink (1961) recorded *Ps. chagasi* oocysts adhered to the *Lutzomyia longipalpis* (Lutz and Neiva, 1912) egg surface at a right angle to the longitudinal axis. This suggests that, similar to the species-specific chorion ornamentation (e.g. De Almeida *et al*. 2004), also the character of the oocyst contamination might be species specific. The location of oocysts seems to be connected to the process of the exochorion formation during oviposition, when it is secreted by the accessory glands. The viscous consistency of the secretion enables the oocysts to adhere to the egg surface at the site where drying exochorion produces characteristic sculpturing ridges.

The *Psychodiella* life cycle has been studied in detail by a limited number of authors, and only a single report has been published documenting this parasite (*Ps. chagasi*) using electron microscopy (Warburg and Ostrovska, 1991). In the present study, we used various microscopical methods in major sand fly developmental stages giving a self-contained overview of the *Ps. sergenti* life cycle. There are several features suggesting a close relationship between *Ps. sergenti* and its sand fly host, supporting the hypothesis about co-evolution of gregarines and sand flies as mentioned by Ostrovska *et al*. (1990). (1) The attachment of the oocysts to the exochorion is possibly closely related to the exochorion formation, facilitating the vertical transmission of the parasite. (2) The gregarine is protected from the expulsion from the larval intestine during pre-pupal defecation by being located in the ectoperitrophic space. This helps the parasite to sustain a certain level of infection during pupation. (3) The injection of the oocysts into the accessory gland lumen, facilitated by the host immune response (Warburg and Ostrovska, 1989), is a unique mode of vertical transmission. (4) The most remarkable feature is the fact that *Ps. sergenti* does not develop sexually in males or females without a bloodmeal, which is advantageous for the gregarines, as they only invest energy into the sexual development where the vertical transmission is possible, i.e. in blood-fed females.

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