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RESEARCH ARTICLE

Testisacs microanatomy and spermatogenesis of *Helobdella simplex* (Hirudinida: Glossiphoniidae)

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ABSTRACT. The aim of this study was to describe the testisac microanatomy and spermatogenesis of *Helobdella simplex* (Moore, 1911). The leeches were collected in the Sauce Chico River (Tornquist) during April 2012. The collection of material was carried out manually by checking rocks, logs, leaves and artificial substrates in the river bed. *Helobdella simplex* has six pairs of testisacs located between the gastric caeca. The testisac wall has two structurally and functionally differentiated cell types: the lining mesothelial cells and the germline cells. The lining cells are flat and form a continuous layer. In *H. simplex* no ciliated cells were observed into the testisacs, nor the formation of spermatogonia due to detachment of the testicular wall. Cell types were microscopically characterized, and five spermatogenesis stages are described, taking into account the maturation of germinal cells, the morphological changes in the cytophore and phagocytic activity. Testicular phagocytes actively participate during spermatogenesis. They are involved both in the removal of defective cells during the early stages of spermatogenesis and in the removal of the cytophore during the reacting reabsorption. Our study adds the knowledge of spermatogenesis in *H. simplex* and might be useful to differentiate the stages of development during the reproductive cycle, and to interpret aspects of its population dynamics.

KEY WORDS. Freshwater, gonad, histology, leeches.

INTRODUCTION

Helobdella simplex (Moore, 1911) is an endemic leech of the Neotropical region. Its area of distribution comprises Argentina (Entre Ríos, Córdoba, Buenos Aires, Río Negro, Chubut and Santa Cruz); Uruguay; Chile and Peru (Ringuelet 1985, Cristoffersen 2009). It is typically found in lentic environments around hydrophytes (Gullo 1998, 2007, César et al. 2009) and under rocks in areas protected from the river current (Gullo 2015). Also, it can be found in the *Limnoperna fortunei* (Dunker, 1857) golden mussel byssus (Darrigran et al. 1998) and as an opportunist within the mantle cavity of *Pomacea canaliculata* (Lamarck, 1822) golden apple snail or channeled apple snail (Damborenea and Gullo 1996).

The spermatogenesis and testicular structure have been studied in different Glossiphoniidae leech species: *Glossiphonia complanata* (Linnaeus, 1758) (Damas 1965, 1968); *Haementeria depressa* (É. Blanchard, 1849) (Fernández et al. 1992); *Helobdella stagnalis* (Linnaeus, 1758) (Martínez-Alós and García-Corrales 1988, Gouda 2013); *H. triserialis* (É. Blanchard, 1849); *H. hyalina* Ringuelet, 1942, and *H. michaelseni* Blanchard, 1900 (Gullo 1995, 2004, 2010); *Batracobdella algira* (Moquin-Tandon, 1846) (Ahmed et al. 2019).

The spermatogenesis in leeches occurs within testisacs. The germinal cells undergo meiosis, giving rise to isogenic groups, clones (Fernández et al. 1992), germinal poliplasts (Bonet and Molinas 1988) or cysts (Ahmed et al. 2019). These cells remain connected to an anucleated central mass called cytophore by means of cytoplasmic bridges (Martinucci and Felluga 1977) or intercellular bridges (Ahmed et al. 2019). Divisions are synchronous, without cytokinesis. During spermatogenesis many cysts in development, accompanied by free phagocytes, are observed in the lumen of the testisac during testicular activity (Bonet and Molinas 1985). Once spermiogenesis is complete, the spermatozoa travel through vas deferens to the genital atrium. In the case of Glossiphoniidae, the spermatozoa form spermatophores which are transferred through the male gonopore during copulation (Sawyer 1986, Ferraguti 2000).

As knowledge of gametogenesis in leeches is an important tool to analyze the seasonality of the reproductive cycle (Tillman and Barnes 1973, El-Shimy and Davies 1991, Wilken and Appleton 1993, Gullo 2003), the aim of this work was to describe the testisac microanatomy and spermatogenesis of *H. simplex*, taking into account the maturation of the germinal cells, morphological changes in the cytophore and the phagocytic activity.

MATERIAL AND METHODS

The specimens were collected in the Sauce Chico River, 38°03'S; 62°15'W (Tornquist, Buenos Aires, Argentina) during April 2012. The collection of material was carried out manually by checking rocks, logs, leaves and artificial substrates in the river bed (Kutschera 1988). For the microanatomical examination, 33 individuals were fixed in Carnoy 6:3:1 (Gabe 1968), dehydrated in an ascending series of ethanol and embedded in Paraplast®. Parasagital and cross serial sections (10 µm thick) were cut with a microtome and stained with Haematoxylin and Eosin using standard techniques (Kutschera et al. 2013). Serial sections were examinated under Axiostar Plus Zeiss® microscope equipped with digital camera. Germinal cells sizes were measured with microscope crossline micrometer 10:100, considering the minor diameter of the nuclei. To prepare figures, Adobe Photoshop 7.0 software was used.

RESULTS

Helobdella simplex has six pairs of testisacs located between the gastric caeca. The testisac wall has two structurally and functionally differentiated cell types: the lining mesothelial cells and the germ line cells. The lining cells are flat and form a continuous layer. They have acidophilic cytoplasm and an oval nucleus. During spermatogenesis, cysts of 16, 32, 64, 128, and 256 germinal cells are observed within the testisac, which remain connected to a central anucleated mass (cytophore) by means of intercellular bridges. The testicular funnel is lined by a ciliated cuboidal epithelium, whose cells are characterized by having a spherical nucleus.

Spermatogenesis was divided into five stages, taking into account the morphological changes of the cytophore, the maturation of the germinal cells and the phagocytic activity (Figs 1–9).

Stage I

It is characterized by the proliferation of germinal cells. Spermatogonia divide by mitosis, synchronously, constituting cysts that are released into the testisac lumen. They remain attached to the very small central cytophore. They have an oval nucleus and a prominent nucleolus. The average nuclear size is 4.3 ± 0.4 µm, n = 10.

Stage II

Spermatocytes I initiate the prophase of the first meiotic division. The chromosomes condense and the nucleus shows a marked basophilia. The average size is 5.25 ± 0.6 µm, n = 10. The cytophore increases its size due to the division of the germinal cells.

Stage III

It differs from the previous stage by the presence of spermatids as the most mature cells of the testisac. At the beginning of this stage, the nuclei of the spermatids become elliptical and culminate in the vestigial flagellum. The average size is 1.57 ± 0.24 µm, n = 10. The cytophore size reaches its maximum (50 µm) and subsequently stabilizes. At this stage, cysts reach 256 spermatids and autolytic processes are not observed.

Stage IV

Spermiogenesis is complete. The spermatozoa still remain attached to the cytophore with their flagella facing towards the lumen of the testisac. The elongated nucleus with marked basophilia stands out, with an average size of 1.09 ± 0.17 µm, n = 10. An increase in free phagocytes is observed. Direct reabsorption processes are started inside the cytophore, the presence of acidophilic bodies as a consequence of autolysis is confirmed, and the spermatozoa remain free in the lumen of the testisacs. The cytophore remains are removed by phagocytes during the reacting reabsorption. Phagocytes are cells with an eccentric, spherical, basophilic nucleus, sometimes binucleated; its cytoplasm has eosinophilic granulations and abundant phagocytic vacuoles. The average nuclear size is 23.4 ± 0.56 µm and the average cytoplasmic size is 25 ± 0.52 µm, n = 10.

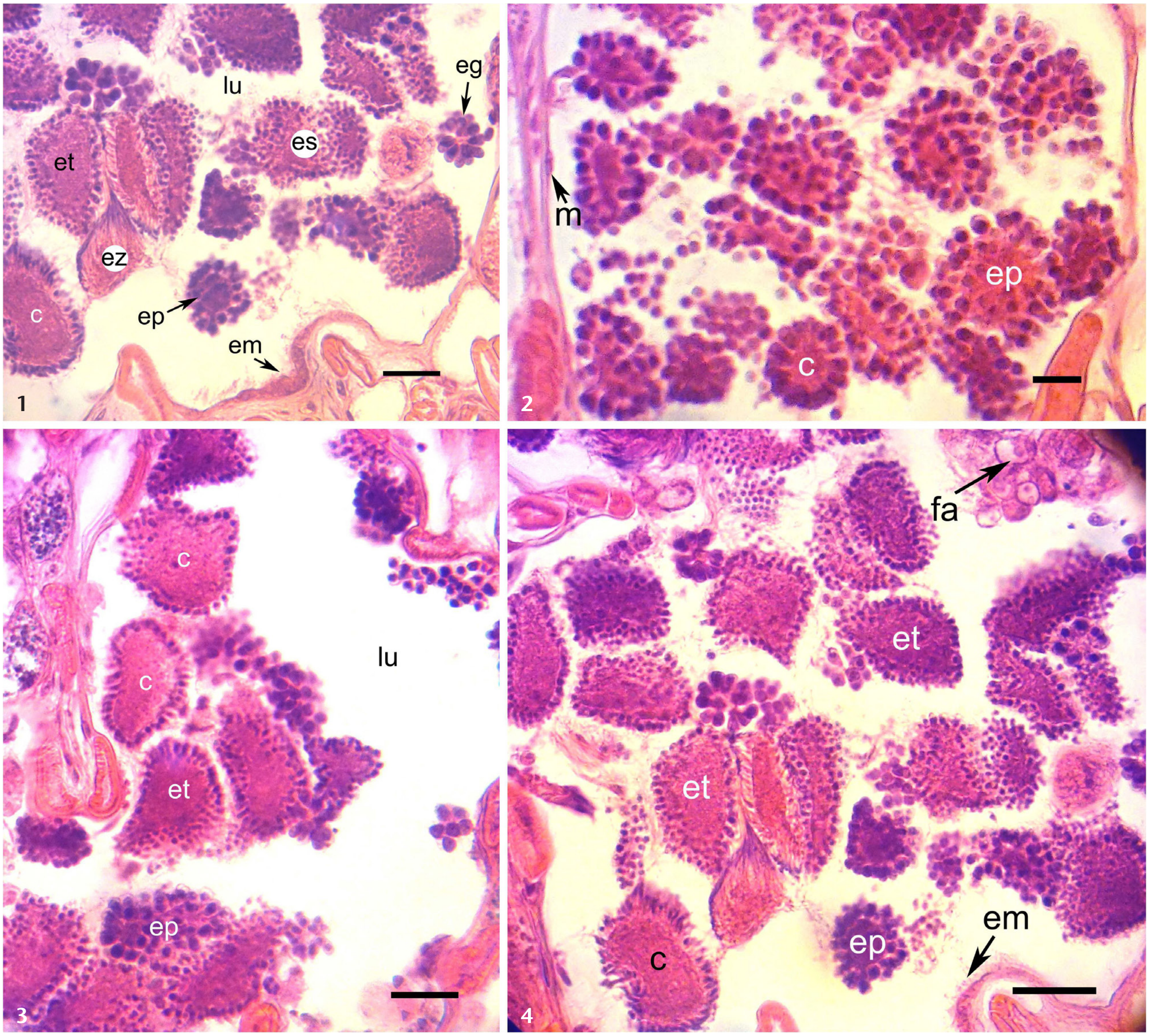
Stage V

It occurs after egg-laying. Testicular activity continues, although new spermatozoa will not form since the cysts are reabsorbed by phagocytes. Germinal cells undergo apoptosis, and it is common to observe nuclei with hyperchromatosis and piknosis, which are also affected by reacting reabsorption processes.

DISCUSSION

Spermatogenesis of *H. simplex* follows the general pattern of sperm production that is found in annelids (Ferraguti 2000) and the early stages of spermatogenesis are basically the same for Clitellata (Sawyer 1986). The spermatogenesis occurs in the lumen of fluid-filled coelomic testisacs. Inside it, the germinal cells are connected to the cytophore by intercellular bridges and develop functional units called cysts. In *H. simplex* we found that the testisac wall has two structurally and functionally differentiated cell types: the lining mesothelial cells and the germline cells. The lining cells are flat and form a continuous layer called coelothelium (Sawyer 1986). The *H. simplex* testisac wall and the germline cells are comparable to those of other Glossiphiniidae species (Gullo 1995, 2004, 2010). Unlike what was observed in *H. stagnalis* by Martínez-Alós and García-Corrales (1988) and *Batracobdella algira* (Ahmed et al. 2019), in *H. simplex* no ciliated cells were observed in the testisacs, nor the formation of spermatogonia due to detachment of the testicular wall.

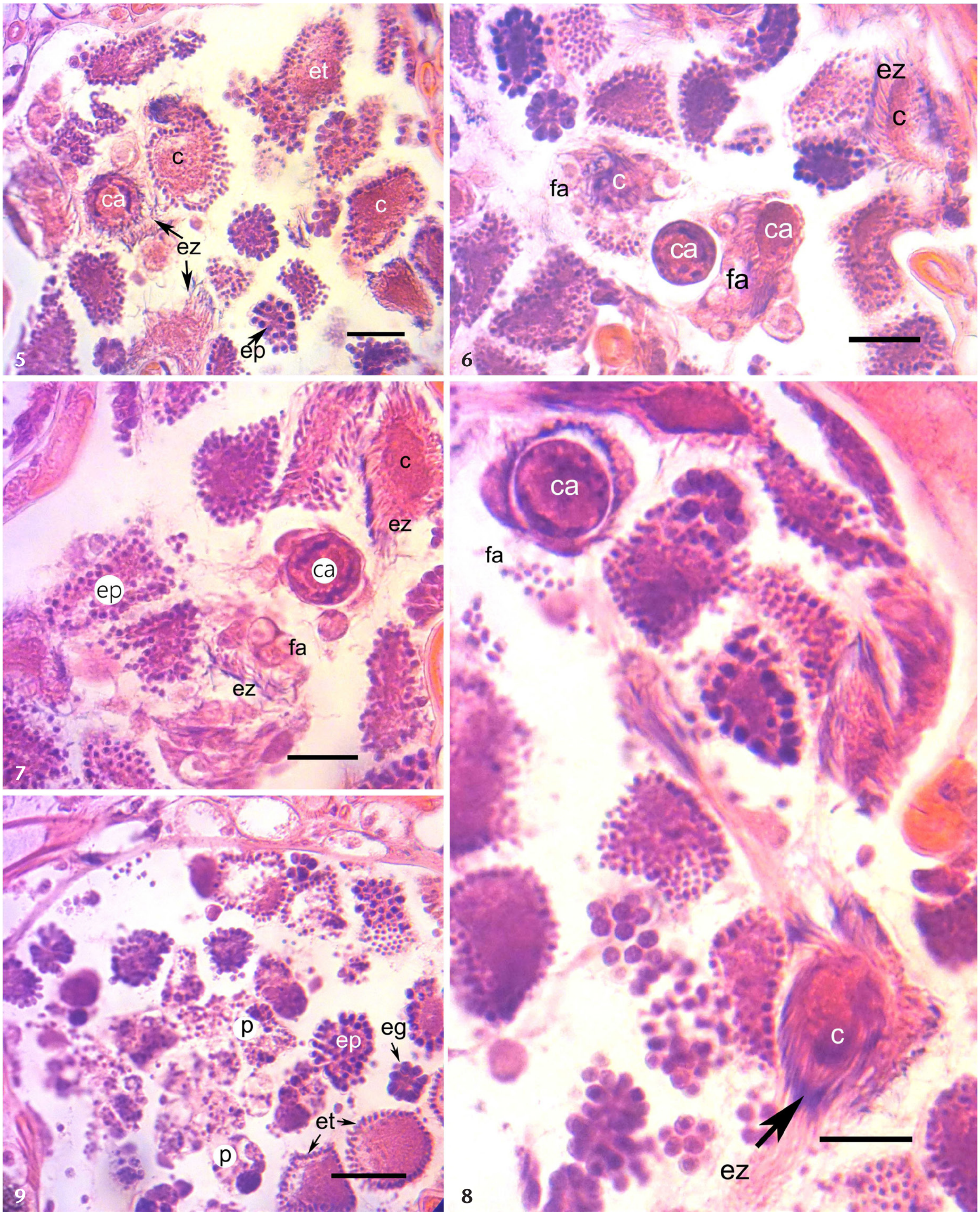
Microanatomical examination about serial sections in testisacs of *H. simplex* revealed sequence of spermatogenic



Figures 1–4. *Helobdella simplex*, cross section of the testisac in stages I, II and III of spermatogenesis: (1) poliplasts in the testisac lumen; (2) Stage II; (3, 4) Stage III of spermatogenesis. (C) Cytophore, (eg) spermatogonia, (em) testicular funnel, (ep) primary spermatocytes, (es) secondary spermatocytes, (et) spermatids, (ez) spermatozoa, (fa) phagocytes, (lu) testisac lumen, (m) mesothelial cell. Scale bars: 1, 3, 4 = 50 µm, 2 = 10 µm.

stages within the testisacs. Spermatogonia divide by mitosis, synchronously, constituting cysts that are released into the testisac lumen. They remain attached to the very small central cytophore. During spermatogenesis, cysts are observed within the testisac, which remain connected to a central cytophore by means of intercellular bridges. The changes in the appearance and volume of the cytophore registered in *H. simplex* have been mentioned for other Rhyncobdellida species, namely: *Glossiphonia complanata* (Damas 1968), *H. stagnalis* (Martínez-Alós and García-Corrales 1988), *H. triserialis*, *H. hyalina* and *H. michaelseni* (Gullo 1995, 2004, 2010), *Helobdella stagnalis* (Gouda 2013) and *Batracobdella algira* (Ahmed et al. 2019).

When the spermiogenesis is complete, the spermatozoa still remain attached to the cytophore with their flagella facing towards the lumen of the testisac. Direct reabsorption processes are started inside the cytophore, and the spermatozoa remain free into the lumen of the testisacs. The cytophore remains are removed by phagocytes during the reacting reabsorption. The



Figures 5–9. *Helobdella simplex*, cross section of the testisac in stages IV and V of spermatogenesis: (5–7) Stage IV, mature spermatozoa attached to the cytophore and free in the testisac lumen; initiation of reabsorption processes. (8) Reacting and direct (autolysis) reabsorption. (9) Stage V, nuclei of germinal cells with piknosis and hyperchromatosis. (C) Cytophore, (ca) cytophore in autolysis, (ep) primary spermatocytes, (et) spermatids, (ez) spermatozoa, (fa) phagocytes, (p) piknosis. Scale bars: 50 µm.

direct reabsorption (autolysis) and reacting processes recorded in this work were described in *H. triserialis*, *H. hyalina*, and *H. michaelseni* (Gullo 1995, 2004, 2010). Likewise, the phagocytic activity during spermatogenesis was confirmed in several *Helobdella* species (Bonet and Molinas 1985, Martínez-Alós and García-Corrales 1988, Fernández et al. 1992).

In *H. simplex*, the processes of hyperchromatosis and pyknosis observed during stage V (after egg-laying) indicate the reabsorption of cysts into the testisacs lumen and therefore gonadal restoration is not possible. This particularity has been observed in other South American *Helobdella* species (Gullo 1995, 2004, 2010) and is consistent with an annual life cycle and semelpary as a reproductive strategy. Despite the importance of direct and reactional resorption processes, these have only been recorded in South American *Helobdella* species.

Testicular phagocytes actively participate during spermatogenesis, developing macrophage activity (Bonet and Molinas 1985, Martínez-Alós and García-Corrales 1988, Fernández et al. 1992). *Helobdella simplex* phagocytes are larger compared to those of other *Helobdella* species (Gullo 1995, 2004, 2010). They are involved both in the removal of defective cells during the early stages of spermatogenesis and in the removal of the cytophore during the reacting reabsorption. In *H. simplex*, the participation of phagocytes in the removal of normal spermatozoa is not excluded.

The criteria proposed to differentiate the stages of spermatogenesis might be useful to interpret the life cycle and the population dynamics of *Helobdella* species.

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