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Ovary ultrastructure and oogenesis in *Propappus volki* Michaelsen, 1916 (Annelida: Clitellata)

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ABSTRACT

The paired ovaries of *Propappus volki* are small, conically shaped structures with a narrow end that is connected to the intersegmental septum, while the wide end extends into the coelom. Ultrastructural studies showed that the ovaries are composed of interconnected germ cells that form syncytia and accompanying somatic cells. Each germ cell in a cyst is connected with the central, anuclear cytoplasmic core, the cytophore, via one cytoplasmic bridge. The somatic cells form both a thin ligament that joins the gonad with the septum and also surround the germ cells, thus, forming a thin ovary envelope. The somatic cells that are in immediate contact with growing oocytes, i.e., follicular cells, are more voluminous than other somatic cells. There is a clear gradient in the development of germ cells along the long ovary axis. As a consequence, three zones can be distinguished. In zone I all of the germ cells are in the prophase I of meiosis, while zone II contains undifferentiated germ cells with a similar morphology. In zone III there are two morphologically distinct categories of germ cells – the more numerous but smaller germ cells that are regarded here as nurse cells and several growing oocytes. The growing oocytes gradually accumulate cytoplasm and yolk material and protrude from the ovary into the segment cavity. The late vitellogenic oocytes, which are filled with yolk spheres, lose contact with the gonad and float within the coelom.

The comparison of the results that were obtained with the ovary structure and oogenesis in other Clitellata shows that: (1) the pattern of germ-line cyst organization in *P. volki* and other Clitellata is the same; (2) the course of oogenesis in *P. volki* is similar to other oligochaetous clitellates and (3) the ovary organization in *P. volki* is broadly similar to that which has been described in tubificins, brachiobdellids and lumbriculids but which differs significantly from that found in *Enchytraeus albidus*.

1. Introduction

The genus *Propappus* contains three freshwater species (*Propappus glandulosus*, Michaelsen 1905; *P. volki*, Michaelsen 1916 and *P. arhyncotus*, Sokolskaya 1972) of oligochaetous annelids, which were originally regarded as primitive Enchytraeidae because of several features that were regarded as ancestral and that indicated a freshwater origin of this family (Michaelsen, 1916, 1923; Stephenson, 1930; Černosvitov, 1937; Timm, 1981). However, some authors (Nielsen and Christensen, 1959) questioned the placement of *Propappus* in Enchytraeidae and later Coates (1986) established a new family, Propappidae, for this genus and described its unique character-states. The monophyly of Propappidae was not strongly supported in morphology-based phylogenetic analysis (Coates, 1987). The results of subsequent phylogenetic analyses are not clear. Studies that were based on 18S rRNA genes grouped *P. volki* together with tubificids, haplotaxids and phreodrilids (Erséus and Källersjö, 2004), studies based on a multigene data set placed *P. volki* also outside the Enchytraeidae, as a sister to the haplotaxid *Haplotaxis* cf. *gordioides* (Erséus et al., 2010). On the other hand analyses that were based on three nuclear and one mitochondrial gene placed *P. volki* close to enchytreid *Fridericia tuberosa*, but *P. volki* remains the sister taxon to *H.* cf. *gordioides* in this analysis, and all three of these species are in a clade with the tubificids and the phreodrilid *Antarctodrilus proboscidea* (Rousset et al., 2007). Finally, a Bayesian analysis of the 18S rDNA sequence and 82 morphological characters (Marotta et al., 2008) indicated a close relationship of *P. volki* with enchytraeids. Recent ultrastructural studies that were devoted to cuticle organization and sperm morphology in *P. volki* revealed that both structures share many similarities with enchytraeids (Gustavsson et al., 2008).

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In contrast to sperm morphology, which has been used many times in phylogenetic analyses of clitellate annelids (e.g., Jamieson et al., 1987; Erséus and Ferraguti, 1995; Ferraguti and Erséus, 1999; Ferraguti et al., 1999; Cardini and Ferraguti, 2004; Marotta et al., 2003, 2008), the characters that are connected with ovary structure and oogenesis have rarely been used. Recently, Bielecki et al. (2014) used 22 characters that are connected with ovary structure and oogenesis, together with an additional 27 morphological characters in a parsimony analysis of leech phylogeny. This study showed that the features that are connected with the ovary organization and oogenesis are useful in the phylogenetic reconstruction of Hirudinida at the family level (Bielecki et al., 2014). Female gonad morphology and oogenesis have not been much used in phylogenetic studies of annelids. First there are doubts that ovarian morphology and the course of oogenesis is useful for such an analysis because of the strong correlation between female gametogenesis and life strategy (Eckelbarger, 2006). Second, little data about ovary morphology has been available. This latter point is changing, several ultrastructural papers devoted to ovary organization and oogenesis in Clitellata have been published recently, i.e., a set of papers describing the ovary organization in Hirudinida (see Bielecki et al., 2014 for references): Siekierska (2003) described the ovary in an earthworm, *Dendrobaena veneta*; Urbisz et al., (2010) studied three representatives of Tubificinae; Świątek et al., (2012) studied two species of Branchiobdellida and *Acanthobdella peledina* and Urbisz and Świątek (2013) analyzed two species of Lumbriculida. However, there remains no detailed information about these structures and processes in several clitellate families, including the Capilloventridae, Haplotaxidae, Phreodrilidae, Naidinae and Propappidae.

In this study our aim was to describe ovarian morphology and oogenesis in *P. volki*, providing new characters that may help to clarify the phylogenic relationships of the family Propappidae.

2. Materials and methods

2.1. Material examined

2.1.1. The site of the collection of Propappus volki

P. volki Michaelsen, 1916 was sampled in a spring (50°35′42.66″N 19°33′19.00″E) that is situated in the Białka Zdowska Valley (the Cracow–Częstochowa Upland, Southern Poland). The spring is protected by Polish law as a natural treasure named "The group of springs in Zdów" (the permit for sample collection was provided by the Włodowice Commune – R.V. 6122.001.2014).

P. volki was collected from a coarse substratum with a bottom scraper (15 × 15 cm frame with a 0.2 mm mesh) in June, July, August and September 2014. In the laboratory, mature worms that had a clitellum were picked from the substratum under a stereomicroscope. Seventy to eighty specimens were studied during the presented analyses.

2.2. Light and electron microscopy

Whole specimens were initially fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for several days in a room temperature. After washing in a phosphate buffer, the material was postfixed for 2 h in 1% OsO_4 in the same buffer and temperature, dehydrated in a graded series of ethanol replaced by acetone, and then embedded in an Epoxy Embedding Medium Kit (Sigma, St. Louis, MO). Semithin sections (0.8 μm thick) were stained with 1% methylene blue in 1% sodium biborate solution in a room temperature for 30 s and examined under an Olympus BX60 microscope equipped with a XC50 digital camera (Olympus) and cellSens Standard software (Olympus). Ultra-thin sections (80 nm) were cut on a Leica Ultracut UCT ultramicrotome. After contrasting with 13% uranyl acetate in 50% ethyl alcohol for 15 min and with lead citrate (a mixture of 2.6% aqueous lead nitrate and 3.5% aqueous saline sodium citrate; regulated by use of a 1 N sodium hydroxide to pH 5.0) for 20 min, the sections were examined using a Hitachi H500 electron microscope at 75 kV.

2.3. Fluorescence microscopy

Dissected ovaries of *P. volki* were fixed in 4% formaldehyde (freshly prepared from paraformaldehyde) in PBS (phosphate buffered saline, NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 8 mM; KH_2PO_4, 1.5 mM, pH 7.4) for 30–40 min at room temperature and stained with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) (1 μg/ml; Sigma) in PBS for 45 min at room temperature in darkness. Whole mounted preparations were examined under an Olympus BX60 epifluorescence microscope equipped with the appropriate filters.

3. Results

3.1. General morphology

The bilaterally paired ovaries are small (~300 μm in length) slightly elongated, conically shaped structures (Fig. 1A) with their narrow (proximal) end connected to the intersegmental septum between XII and XIII segment via a thin ligament (Fig. 1B). The outermost part of the ovary (distal end) is wider and extends into the coelom of segment XIII (Fig. 1C). The ovaries are usually twisted or coiled (Fig. 1B and C; Fig. 2A and B).

The ovaries are built of somatic and germline cells. The somatic cells are thin and flattened cells that form a thin ovary envelope and a ligament that connects the ovary to the septum. Somatic cells are barely visible on the semi-thin sections (Fig. 1B and C; Fig. 2A and B); however, their nuclei could be seen on the whole-mounted preparations that had been stained with DAPI (Fig. 1A). The ultrastructural details of somatic cells are given in Section 3.5.

Three types of germ cells were found within the ovaries: undifferentiated germ cells (cystocytes), nurse cells (trophocytes) and previtellogenic and vitellogenic oocytes (Figs. 1–3). Analysis of the ultra-thin sections revealed that all of the germ cells that are found within the ovaries are interconnected through intercellular bridges (usually referred to as a ring canals), and form cysts (clusters, clones nests; see Section 3.2). A developmental progression of germ cells can be seen along the long ovary axis (Fig. 1A, C). To clarify the description, three zones within the ovary were distinguished; however, the borders between zones are not sharp (Fig. 1A–C). In the narrow, proximal part of the ovary (zone I), all of the cystocytes are in meiotic prophase (Fig. 1B and C). Below, in zone II, the meiotic chromosomes can no longer be seen within the nuclei of the cystocytes (Fig. 1B–F). The distal end of the ovary (zone III) contains germ cells that are morphologically differentiated into two categories – a few bigger cells that accumulate reserve material, grow and gradually protrude from the ovary into the coelom (these cells are regarded as growing oocytes) (Fig. 1C, Fig. 2A and B) and numerous cells with a morphology that is broadly similar to those germ cells found in zone II (these cells are regarded as nurse cells) (Fig. 1C, Fig. 2A and B). In zone III a mass of anuclear cytoplasm that is called a cytophore can be seen between the germ cells (Fig. 2A and B). Late vitellogenic oocytes filled with reserve material are not connected to the ovary; they float freely in the coelom (Fig. 3A).