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Micromorphology of ovaries and oogenesis in *Grania postclitellochaeta* (Clitellata: Enchytraeidae)

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ABSTRACT

The genus *Grania* comprises over 70 species of exclusively marine clitellate annelids belonging to the family Enchytraeidae. Morphologically, this genus is well separated from other enchytraeids, with thick cuticles, anterior segments I–IV fused into a "head", chaetal bundles consisting only of one stout chaeta, and reduction of circular musculature. The aim of the present study is to describe the ovary organization and the course of oogenesis in *Grania postclitellochaeta*, and to compare it with other known systems of ovary organization and oogenesis in clitellate annelids, especially in enchytraeids. Generally, oogenesis in *G. postclitellochaeta* can be divided into two phases: (i) early stages of oogenesis, occurring within the paired ovaries – each ovary is similar to a bunch of grapes, where each 'lobe' is a germ-line cyst enveloped by flat somatic cells, and (ii) oogenesis proper, which takes place within the body lumen where each growing oocyte is accompanied by its own group of nurse cells. Germ cells are interconnected by cytoplasmic channels (intercellular bridges, ring canals) and form syncytial cysts. As in other clitellate annelids, the cyst center contains a common cytoplasm (cytophore) to which each cell is connected by one ring canal only. Initially, within the ovary, all interconnected cells develop synchronously and are morphologically similar. At the time when the cysts detach from the ovary, one of the interconnected cells begins to gather nutrients, grows and becomes an oocyte, whereas the rest of the cells (nurse cells) do not continue meiosis and instead seem to provide the oocyte with macromolecules and cell organelles. Analysis of serial sections reveals that cysts are always composed of 16 cells – one oocyte and fifteen nurse cells. A comparative analysis showed that almost all features of oogenesis in *G. postclitellochaeta* are similar to that in other representatives of Enchytraeidae (mainly *Enchytraeus albidus*), suggesting evolutionary conservation of the process across this family.

1. Introduction

During recent years, our knowledge about ovary organization and oogenesis in clitellate annelids has grown considerably. Together with older descriptions, we have acquired quite detailed data on the histological and ultrastructural level about ovary and egg formation in several leech families (see Świątek, 2008 and Bielecki et al., 2014 for details) and in selected representatives of such non-leech clitellate taxa as Lumbricidae (Siekierska, 2003), Lumbriculidae (Urbisz and Świątek, 2013), Tubificinae (Urbisz et al., 2010, 2015), Propappidae (Gorgoń et al., 2015), Naidinae (Gorgoń et al., 2017), Enchytraeidae (Urbisz et al., 2017) and Capilloventridae (Świątek et al., 2016). These studies revealed that the ovarian morphology differs substantially among taxa. In *Stylaria lacustris* (Naidinae), for instance, the ovaries are tiny structures composed of a few dozen cells, where developing germ cells detach early from the gonad and the yolk accumulation takes place within the body cavity (Gorgoń et al., 2017). On the other hand, each of the paired ovaries of *Tubifex tubifex* (Tubificinae) houses more than 2000 cells, and only late, vitellogenic oocytes can be found floating in the coelom (Urbisz et al., 2010, 2015). In clitellates, irrespective of ovarian organization and the location of consecutive stages of oogenesis, the female germ cells form syncytial cysts (Świątek et al., 2012, 2016; Urbisz et al., 2015; , 2017). Generally, the cells forming syncytial cysts are interconnected by stabilized contractile rings which do not close during late cytokinesis, and which allow macromolecules and organelles to be shared between cells (Greenbaum et al., 2011; Haglund et al., 2011). The formation of germ-line cysts seems to be a conservative phase of animal gametogenesis (Pepling et al., 1999), and female cysts have been found in a wide variety of animals including, e.g., insects, annelids and mammals (Büning, 1994; Świątek et al., 2009; Urbisz et al., 2015; Lei and Spradling, 2016). Female cysts are not uniform; their architecture varies between taxa from simple linear

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chains to complicated, branched systems (see, e.g., Büning, 1994; Pepling et al., 1999; Matova and Cooley 2001; Amini et al., 2014; Jaglarz et al., 2014a; Urbisz et al., 2015 for examples). Despite the general theme of variability in female cysts, such cysts in clitellates do have a characteristic pattern of organization − each germ cell has one cytoplasmic channel (intercellular bridge, ring canal − RC) connecting it to the central cytoplasmic mass, the cytophore (Urbisz et al., 2015, 2017). The only known exception is a representative of basal clitellates, *Capilloventer australis* (Capilloventridae), in which RCs and female cysts have not been found (Świątek et al., 2016).

Ovary morphology and oogenesis in Enchytraeidae have been analyzed several times in such species as *Enchytraeus buchholzi* (Vejdovský, 1879) and especially in *Enchytraeus albidus* (Michaelsen, 1928; Paschma, 1962; Dumont, 1969; Urbisz et al., 2017). These studies showed that enchytraeid ovaries may be compared to a bunch of grapes, where groups of germ cells forming ovary subunits (termed as 'ovarial lobes' by Paschma, 1962) detach from the gonads early on and subsequently finalize their development in the body cavity. Older descriptions of enchytraeid oogenesis disagreed on whether oocytes develop in a "solitary" mode, without supportive cells (postulated by Michaelsen, 1928 and Dumont, 1969), or in a "nutrimental" mode, where oocytes are supported by nurse cells (Vejdovský, 1879; Paschma, 1962). Recent analyses (Urbisz et al., 2017) have unambiguously shown that in *E. albidus* each ovarian subunit is made up of one germ-line cyst clustering 15 nurse cells and one oocyte, and that oogenesis should be regarded as nutrimental (meroistic).

To broaden our knowledge about ovarian organization in clitellate annelids and to support or reject the hypothesis that ovarian morphology and the process of oogenesis are conserved at the family/subfamily level (as suggested in the case of leeches by Bielecki et al., 2014), we decided to study these characters at the ultrastructural level in the genus *Grania* (Enchytraeidae). *Grania* Southern 1913 is a monophyletic genus morphologically well separated from other enchytraeids, with, for example, the anterior segments I–IV fused into a "head", chaetal bundles consisting of only one stout chaeta and a reduced circular layer of body muscles resulting in a nematode-like pattern of movement. *Grania* is the only enchytraeid genus which is exclusively found in marine environments, and it has recently been postulated that *Grania* has recolonized the marine habitat from limnic or terrestrial enchytraeid relatives. *Grania* is closely related to the genus *Lumbricillus* (Erséus et al., 2010), which inhabits beaches in non-tropical environments worldwide, with some indications that *Grania* actually might be part of a currently paraphyletic *Lumbricillus* (Klinth et al., 2016). Our analysis revealed that despite these differences, ovary organization and oogenesis in *Grania postclitellochaeta* and *E. albidus* are broadly similar, suggesting evolutionary conservation of this process across the Enchytraeidae.

2. Materials and methods

2.1. Specimen collection and species determination

Shell (amphioxus) sand was collected by the second author in Sweden, Koster area (58°52'34.6"N 11°06'43.8"E), at 4–20 m depth using an Agassiz dredge. The sand was sieved, using the stirring rod technique, through a 250 μm mesh screen, after which *Grania* specimens were collected from the sieve-retained fraction using a stereo microscope.

For accurate and unambiguous species identification each collected specimen was divided into two parts: anterior including segments I–XX and posterior including all the remaining segments. The anterior parts were fixed for morphological studies (see Section 2.3), the posterior ones for DNA sequencing (Section 2.2).

DNA analysis revealed that the majority of collected annelids were *Grania postclitellochaeta* and only the specimens belonging to this species were morphologically analyzed. In total, 20 specimens of *G. postclitellochaeta* were studied.

2.2. DNA sequencing and species identification

Posterior body parts were fixed in 95% ethanol, after which DNA from a total of 21 worms was extracted using a Qiagen DNeasy Blood & Tissue Kit. Polymerase chain reaction (PCR) was performed using universal cytochrome oxidase subunit I (COI) barcoding primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) (forward) and HCO2198 (5′-TAACTTCAGGGTGACCAAAAAATCA-3′) (reverse) (Folmer et al., 1994). The PCR reactions consisted of an initial step of 5 min at 95 °C, followed by 35 cycles, each one consisting of 40 s at 95 °C, 45 s at 45 °C and 60 s at 72 °C. This was followed by a final step of 8 min at 72 °C. The resulting amplicons were sent to Macrogen Europe (Amsterdam, The Netherlands, for Sanger dideoxy sequencing. COI sequences were assembled and aligned to a published dataset of Scandinavian *Grania* COI sequences (De Wit and Erséus, 2010) using Geneious Pro v 4.8.5.

2.3. Light and electron microscopy

Anterior body parts (segments I–XX) were fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH = 7.4 – the buffer solution was prepared by mixing NaH_2PO_4 and Na_2HPO_4 salts in distilled water) at room temperature for two weeks. After washing in a phosphate buffer, the material was post-fixed for 2 h in 1% OsO_4 in a phosphate buffer. Post-fixed material was washed in a graded series of ethanol replaced by acetone and embedded in an epoxy embedding medium kit (Sigma, St. Louis, MO, USA). Semi-thin sections (0.7 μm thick) were cut on a Leica Ultracut ultramicrotome and stained with 1% methylene blue in a 1% sodium biborate solution at room temperature for 30 s. Sections were examined under an Olympus BX60 microscope equipped with an XC50 digital camera (Olympus) and cellSens Standard software (Olympus). Ultra-thin sections (80 nm) were cut on a RMC Power XT ultramicrotome (RMC Boeckeler, Tucson, AZ, USA). Ultra-thin sections were contrasted with uranyl acetate (30 min) and lead citrate (20 min). Contrasted sections were examined using a Hitachi H500 electron microscope at 75 kV.

2.4. 3D reconstructions

To visualize germ-line cysts, we generated three-dimensional (3D) reconstructions of cysts in the different developmental stages. To obtain 3D reconstructions, the anterior body parts of *G. postclitellochaeta* were embedded in epoxy resin as described in Section 2.3, after which they were serially cut into semi-thin sections (1 μm thick) using a diamond knife (Diatome, Nidau, Switzerland). Each section was photographed with an Olympus BX60 microscope using an XC50 digital camera and cellSens Standard software. Following this, margins of germ cells and cytophores (when visible), as well as nuclear outlines were contoured using Fiji ImageJ (Schindelin et al., 2012). The images were processed with Fiji ImageJ software equipped with a 3D viewer and Z-projection plugins. The final movies were saved in avi format.

3. Results

3.1. Ovaries

The paired ovaries were located in segment XII. Each of them was joined to the intersegmental septum between segment XI and XII. The ovaries were lying ventrally just above the nerve cord and close to the blood vessel (Fig. 1A). The ovaries were made up of several subunits and had a shape similar to a bunch of grapes (Fig. 1A). Each subunit was formed by one germ-line cyst (see below) enveloped by flattened somatic cells (Fig. 1A). Somatic cells also formed thin and short ligaments connecting the ovaries to the septum (not shown). The analysis of serial semi-thin sections revealed that in each of the two serially sectioned ovaries, at least five germ cell cysts were present. Along the antero–posterior ovary axis, a developmental gradient of germ cells was