Zoology
Available online 10 August 2023, 126109
In Press, Journal Pre-proof What's this?

Ovary organization and ultrastructure in six species of *Amynthas* and *Metaphire* earthworms (Annelida, Crassiclitellata, Megascolecidae) ☆

Piotr Świątek [a], Rojen Singh Thounaojam [b], Th. Binoy Singh [b], Samuel James [c], Łukasz Gajda [a], Karol Małota [a], Dominika Raś [a], Anna Z. Urbisz [a]

Show more

https://doi.org/10.1016/j.zool.2023.126109
Get rights and content

Highlights

- Paired and fan-shaped ovaries occur in segment XIII
- Oogonia and early meiotic germline cells form syncytial cysts
- During diplotene, germ cells diversify into oocytes and nurse cells
- Growing oocytes, enveloped by follicular cells, form egg strings
- Nurse cells do not gather nutrients and degenerate
- Ovary organization and oogenesis are the same in parthenogenetic and sexual species

Abstract

Ovaries in earthworms belonging to the family Megascolecidae are paired structures attached to the septum in the anterior part of the XIII segment. They are fan to rosette shaped with numerous rows of growing oocytes, known as egg strings, radiating from the ovary center towards the segmental cavity. The histological and ultrastructural ovary organization in megascolecids and the course of oogenesis remain unknown. The paper presents the results of light and electron microscopy analyses of ovaries in six megascolecid species, three from the genus *Amynthas* and three from *Metaphire*. Both parthenogenetic and sexually reproducing species were included in the study. The organization and ultrastructure of ovaries in all studied species are broadly similar. Considering the histological organization of ovaries, they could be divided into two zones. Zone I (proximal, close to the connection with the septum) is tightly packed with germline and somatic cells. Germ cells are interconnected via intercellular bridges and thin strands of the central cytoplasm (known as cytophore) and form syncytial cysts. Cysts unite oogonia, early meiotic cells (till diplotene), and clustering cells develop synchronously. During diplotene, interconnected cells lose developmental synchrony; most probably, one cell per cyst grows faster than others, detaches from the cysts, and becomes an oocyte. The remaining cells grow slightly and are still interconnected via the thin and reticular cytophore; these cells are considered nurse cells. Zone II has a form of egg strings where growing oocytes are isolated one from another by thin somatic cells and form short cords. We present the ultrastructural details of germline and somatic cells. We propose the term "Amynthas" type of ovaries for this ovary organization. We suppose that such ovaries are characteristic of other megascolecids and related families.

Keywords

Clitellata; gonads; gametogenesis; ring canals; reproductive system; histology

1. Introduction

Classical classifications of Clitellata, including earthworms (Crassiclitellata) (Michaelsen, 1928, Michaelsen, 1900, Stephenson, 1930), have been based on the reproductive systems, mainly due to the belief that reproductive structures are more conservative than somatic structures. However, this concept has been criticized many times. Contemporary classifications of earthworms (and other Clitellata) are based on a wide variety of morphological features and molecular data (sequences of selected mitochondrial and/or nuclear genes, transcriptomic data) (e.g., Anderson et al., 2017; Erséus et al., 2020; James and Davidson, 2012). Despite this, scientists still use the features connected with reproductive organs for earthworm taxonomy, species identification, and higher taxa classification (Sims, 1980, Timm and Martin, 2015). Usually, the overall morphology, number, and segmental localization of reproductive organs (e.g., gonads, reproductive tracts, genital pores, and genital markings) are recorded (e.g., Csuzdi et al., 2019, Csuzdi et al., 2015; Lone et al., 2022). Much less attention is paid to the histology and ultrastructure of the reproductive system. The exception is studies devoted to sperm formation and ultrastructure, performed for numerous earthworm (and

other clitellates) taxa and used for some phylogenetic considerations (summarized in Ferraguti, 1999; Jamieson, 2006; Marotta and Ferraguti, 2009). On the contrary, contemporary ovarian histology and ultrastructure analyses are surprisingly rare and fragmentary. It seems that since the time of intensive light microscopy analysis of oogenesis and ovary organization in clitellate annelids at the turn of the 19th and 20th centuries (summarized in Beddard, 1895; Jamieson, 1981; Stephenson, 1930), our knowledge about ovary micromorphology and functioning in Clitellata has not increased significantly. Some ultrastructural and cytochemical analyses were devoted to oogenesis in such lumbricids as *Eisenia fetida* (Lumbricidae) (e.g., Chapron and Relexans, 1971a, Chapron and Relexans, 1971b; Lechenault, 1968) and more recently to *Denrobaena veneta* (Faron et al., 2015, Siekierska, 2003). Occasionally, the analyses of ovaries were used for studies showing the effects of some agents (e.g., pollutants, such as cadmium or insecticides) on earthworm reproduction (Parthasarathi and Ranganathan, 2000, Siekierska, 2007, Siekierska and Urbańska-Jasik, 2002). Our knowledge about ultrastructural aspects of ovary organization and functioning in earthworms is limited mainly to a few representatives of Lumbricidae. In general, ultrastructural analyses of oogenesis in clitellate annelids showed that oocytes, together with accessory (nurse) cells, develop in syncytial groups of cells (cysts) (Eckelbarger and Hodgson, 2021; Gorgoń and Świątek, 2020; Świątek and Urbisz, 2019). The development of oocytes in syncytial cysts seems to be a conservative phase of oogenesis, accelerating oogenesis due to the directional transfer of organelles and macromolecules from nurse cells to oocytes (Chaigne and Brunet, 2022, Gerhold et al., 2022, Lu et al., 2017). In clitellates, the germline cysts have a specific pattern of organization; each cell is connected via a specialized cell junction (intercellular bridge, ring canal) with the central, anuclear cytoplasmic mass, termed cytophore (see Świątek and Urbisz, 2019 for more details and germline cyst classification in other animals; see also Discussion). The abovementioned ultrastructural studies of earthworm oogenesis revealed that also, in these animals, germ cells form syncytial cysts; however, the cytophore is poorly developed (Chapron and Relexans, 1971b, Siekierska, 2003), and the details of germline cyst organization and functioning are limited.

Gates, 1974, Gates, 1976 was the first to see the potential of using ovarian characters in earthworm systematics. He proposed a simple classification of ovaries based on their overall morphology (Gates, 1976). According to Gates, the ovaries of earthworms could be with or without egg strings. Egg strings are separated longitudinal rows of growing oocytes located in the distal part of ovaries from which the gametes are released into the coelomic cavity (Gates, 1976). Gates' ideas were followed by some researchers such as Sims, who proposed the classification of earthworm superfamilies based on these ovarian characteristics (Sims, 1980). On the other hand, as mentioned above, no systematic studies have been devoted to analyses of ovarian organization and function. We recently started a comprehensive project describing ovary histology, ultrastructure, and oogenesis to break this impasse. The first studies described ovarian micromorphology in selected hormogastrids (Świątek et al., 2023). This paper is devoted to ovary organization in six representatives of the family Megascolecidae. We chose three species from the genus *Amynthas* and three from *Metaphire,* which we could collect, identify, and process for histological and ultrastructural analyses. Additionally, both amphimictic and parthenogenetic specimens have been studied. The parthenogenetic morphs were

Amynthas vittatus, *A. divergens, Metaphire agrestis*, *M. hilgendorfi* (Blakemore, 2003, Minamiya et al., 2011; Y. Kamihira and Y. Minamiya personal communication) and *A. hupeiensis* (Gates, 1982). The sexually reproducing specimens were *M. houlleti*.

We used a set of microscopy techniques to describe ovary histology and ultrastructure. We hope these new data are the first step to exploring ovary microorganization in Megascolecidae and closely related families such as Acanthodrilidae, Benhamiidae, and Ocnerodrilidae (Anderson et al., 2017, James and Davidson, 2012, Omodeo, 2000).

2. Material and Methods

2.1. Species collection and determination

Specimens of *Amynthas vittatus* (Goto & Hatai, 1898), *Amynthas divergens* (Michaelsen, 1892), *Metaphire hilgendorfi* (Michaelsen, 1892) and *Metaphire agrestis* (Goto & Hatai, 1899) were collected in Japan in October 2020. Specimens of *Metaphire houlleti* (Perrier, 1872) were collected in Imphal West, Manipur, India, in May and June 2021, *Amynthas hupeiensis* (Michaelsen, 1895) were collected in Iowa, USA, in June 2022. All specimens, except for *M. houlleti*, were identified using morphological characters. Due to uncertainties in identifying specimens collected in India, DNA barcoding was done (see below).

In total, 14 specimens have been taken for analysis. Only mature specimens with well-developed clitellum were collected and analyzed. Table 1 summarizes the data about the studied species, sites of collection, number of analyzed specimens, and methodology.

Table 1. The data about the studied species, sites of collection, number of analyzed specimens, and methods used.

Species	Time and place of collection	Number of analyzed specimens	Stereomicroscopy/ Nomarski contrast	Light microscopy – semi-thin sections	Histochemistry	Transmission electron microscopy	DNA barcoding
Amynthas vittatus	October 2020, Hakodate City, Hokkaido, Japan	2	-	+	-	+	-
Amynthas divergens	October 2020, Hakodate	2	-	+	-	+	-

Species	Time and place of collection	Number of analyzed specimens	Stereomicroscopy/ Nomarski contrast	Light microscopy – semi-thin sections	Histochemistry	Transmission electron microscopy	DNA barcoding
	City, Hokkaido, Japan						
Metaphire hilgendorfi	October 2020, Hakodate City, Hokkaido, Japan	2	-	+	-	+	-
Metaphire agrestis	October 2020, Hakodate City, Hokkaido, Japan	2	-	+	-	+	-
Metaphire houlleti	May and June 2021, Imphal West, Manipur, India,	3	+	+	+	+	COI, 12S, 18S, 28S, histone H3
Amynthas hupeiensis	June 2022, Iowa, USA	3	+	-	-	-	-

2.2. DNA barcoding

Small pieces of body wall were excised from previously dissected specimens of *Metaphire* sp. collected in India. DNA was extracted from ethanol-fixed material using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Fragments of four commonly employed marker genes: mitochondrial cytochrome c oxidase subunit I (COI) and 12S rRNA, nuclear 18S rRNA, and 28S rRNA were amplified. Polymerase chain reactions (PCRs) were

carried out in 50µl reactions consisting of 21µ ddH20; 25µl of Color OptiTaq/tiTaq PCR Master Mix (2x) (EURx, Gdańsk, Poland); 1µl of each primer at 10mM concentration; and 2µl of total genomic DNA. The primers and thermal profiles used for DNA barcoding are listed in Table S1. The amplification products were sent to GenoMed (Warsaw, Poland) and sequenced in both directions. The obtained sequences were analyzed with the BLAST tool available on the NCBI.

2.3. Light microscopy and transmission electron microscopy

Specimens of all studied species were transferred to the laboratory after collection in the field. After narcotization in 50% ethanol for 1min, the body region containing reproductive systems plus some neighboring segments (i.e., body fragment between XI and XVIII segments) was cut out and immediately fixed with 2.5% glutaraldehyde in a 0.1M phosphate buffer (pH 7.4). The vials with fixed body fragments were kept in the fridge for several weeks till transferred to the laboratory in the Institute of Biology, Biotechnology and Environmental Protection, Faculty of Natural Sciences, University of Silesia in Katowice, Poland. In this laboratory, the tissue processing was continued. In the case of *A. vittatus*, *A. divergens*, *M. hilgendorfi,* and *M. agrestis,* the body fragments with reproductive organs were divided into smaller pieces and processed in such form. In the case of *M. houlleti* and *A. hupeiensis*, the body fragments were dissected under a stereomicroscope, and ovaries were extracted. After washing in 0.1M phosphate buffer (pH 7.4), the body fragments or dissected ovaries were postfixed for 2h in 1% OsO_4 in the same buffer, dehydrated in a graded series of ethanol that was replaced by acetone, and embedded in an Epoxy Embedding Medium Kit (Sigma, St. Louis, MO). Semi-thin sections (0.7 µm thick) were cut on an RMC Power XT ultramicrotome (RMC Boeckeler, Tucson, AZ) and stained with 1% methylene blue in a 1% sodium biborate solution at room temperature for 30s. Next, the sections were examined using an Olympus BX60 microscope (Olympus, Tokyo, Japan) equipped with an Olympus XC50 digital camera and cellSens Standard software (Olympus, ver. 1.8.1) and with an Olympus BX63 microscope equipped with an Olympus XC camera and an Olympus cellSens Dimension software (Olympus, ver. 1.8.1). Ultra-thin sections (50-60nm thick) were cut on a Leica 7 ultramicrotome (Leica Microsystems, Wetzlar, Germany). The ultra-thin sections were contrasted with uranyl acetate (30min) and lead citrate (20min). The contrasted sections were examined using a Hitachi H500 transmission electron microscope at 75kV.

2.4. Stereomicroscope and differential interference contrast

The isolated ovaries from *M. houlleti* and *A. hupeiensis,* were placed on Petri dishes and analyzed with a Leica M205C stereomicroscope equipped with an Olympus ZX81 camera and Zeiss Discovery V.8 stereomicroscope. Additionally, in the case of *A. hupeiensis,* ovaries were whole-mounted onto a microscope slide and observed under an Olympus BX63 microscope equipped with a Teledyne Photometrics Prime BSI camera and an Olympus cellSens Dimension software under Nomarski differential interference contrast.

2.5. Histochemistry

For the histochemical staining, semi-thin sections were prepared the same way as described in Section 2.3. Bonhag's method was used to detect proteins. The sections were treated with a 2% solution of periodic acid for 10 min at room temperature to remove the osmium and then stained with bromophenol blue (BPB) for 24 h at 37°C and washed in tap water. Sudan Black B staining was used to detect lipids. The sections were stained with Sudan Black B at room temperature (15 min) and washed in ethanol and water. Additionally, to detect cell components such as polysaccharides and lipids, the epon semi-thin sections were stained with methylene blue–azure II solution and basic fuchsin, according to Humphrey and Pittman (1974). This procedure stains polysaccharides, (e.g., glycogen, pink to red), whereas lipids are stained yellow-brown. After histochemical stainings, the sections were examined using an Olympus BX60 microscope, as described in Section 2.3.

3. Results

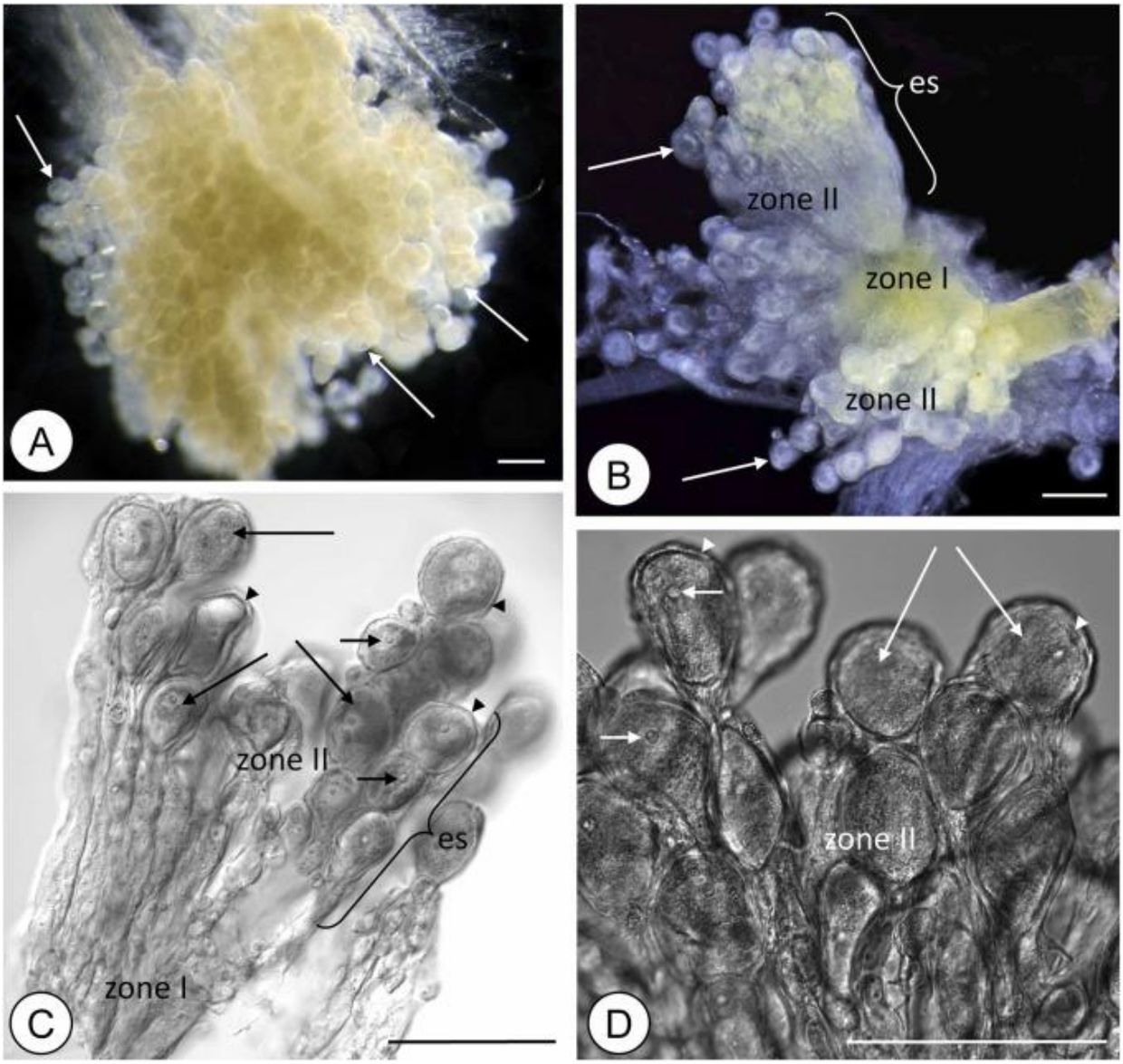
3.1. Molecular analyses

The obtained 658 bp DNA fragment of COI sequence for *Metaphire* sp. from India was identical to *Metaphire houlleti* voucher EW913 (Acc. KU565274). The newly generated sequences for this species: 12S rRNA, 18S rRNA, and 28S rRNA, were deposited in the GenBank database under accession numbers: OQ436964, OQ430852, and OQ430853, respectively.

3.2. General organization of ovaries

Unless otherwise indicated, the following descriptions refer to all species studied.

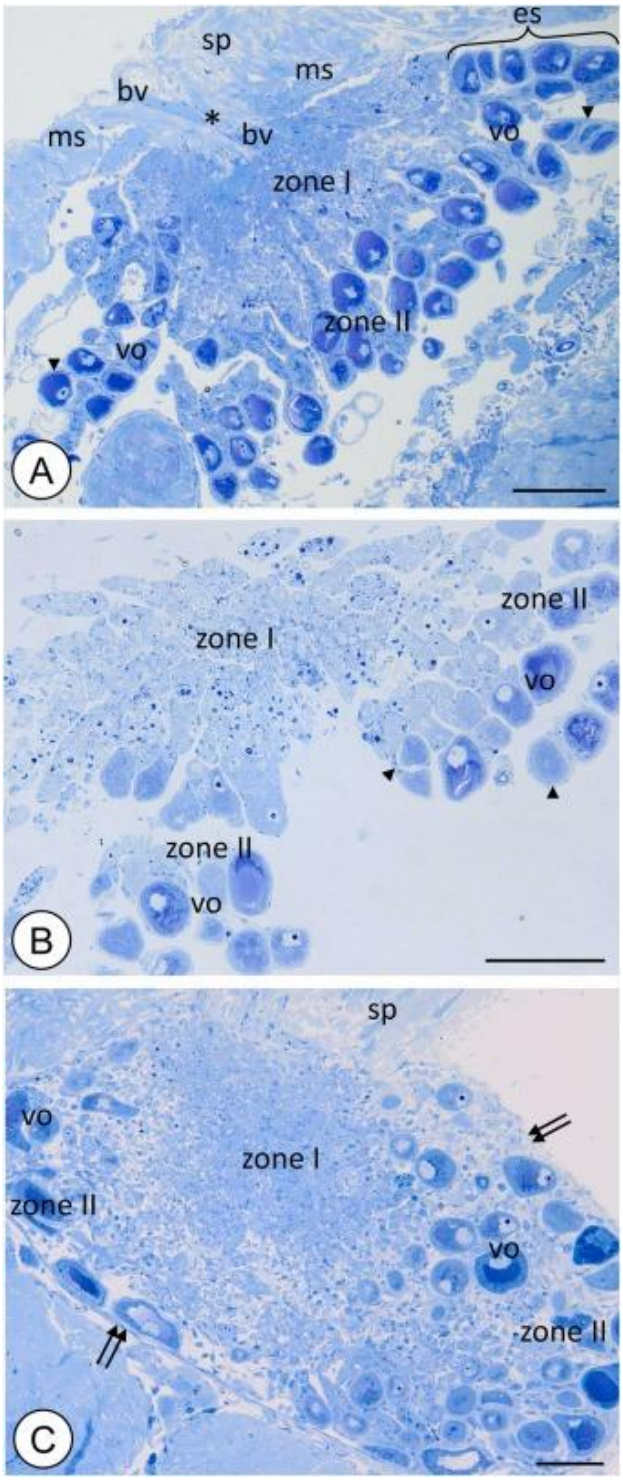
In all species, ovaries are paired and attached to the septum between segments XII and XIII. They are a fan to rosette shape with dozens of short egg strings, which spread out radially from the place of ovary attachment to the septum (Fig. 1, Fig. 2, Fig. 3, Fig. 4). In five of the six studied species, there is no ovary envelope, and ovaries hang freely in the segmental cavity (Figs. 1, 2A, B). In contrast, in *A. divergens,* the whole ovary is ensheathed by a thin somatic envelope (Fig. 2C). The proximal part of each ovary is tightly connected via strands of connective tissue intermingled with muscle cells to the septum (Fig. 2A; for more details, see Section 3.5). In this area, the blood vessels enter the ovary (Fig. 2A). The most proximal part of the ovary, termed zone I, is solid and tightly packed with the mass of germline cells associated with somatic cells (Figs. 1–3A, B). The germ cells here are oogonia and early meiotic (till diplotene) cells. The rest of the ovary has a more loose structure; the cells are not tightly packed, forming strings of growing oocytes enveloped and interconnected by thin somatic cells (the so-called egg strings) (Figs. 1, 2, 3C, D, 4). This is the distal part of the ovary, i.e., zone II. Thus, the developmental gradient of the germ cells can be observed. Oogonia and early meiotic cells occur in zone I, whereas growing oocytes enveloped by somatic cells form numerous short and separated egg strings, which radiate toward the segmental cavity (Fig. 1, Fig. 2, Fig. 3, Fig. 4).



Download : Download high-res image (716KB)

Download : Download full-size image

Fig. 1. Gross morphology of ovaries. A) A general view of *Metaphire houlleti* ovary surface. Growing oocytes in the distal ovary part (zone II) are visible – arrows. Stereomicroscope. B) Ovary of *Amynthas hupeiensis* visualized by stereomicroscope, growing oocytes are marked by arrows. C-D) Portions of *A. hupeiensis* ovaries visualized by Nomarski interference contrast. Germline and somatic cells are tightly packed and hardly visible in the proximal part of the ovary (zone I). Growing oocytes are well-visible (long arrows), and, together with follicular cells (arrowheads), form egg strings (es) in zone II. Within vitellogenic oocytes, nuclei with nucleoli are visible (short arrows). Scale bars = 150µm.

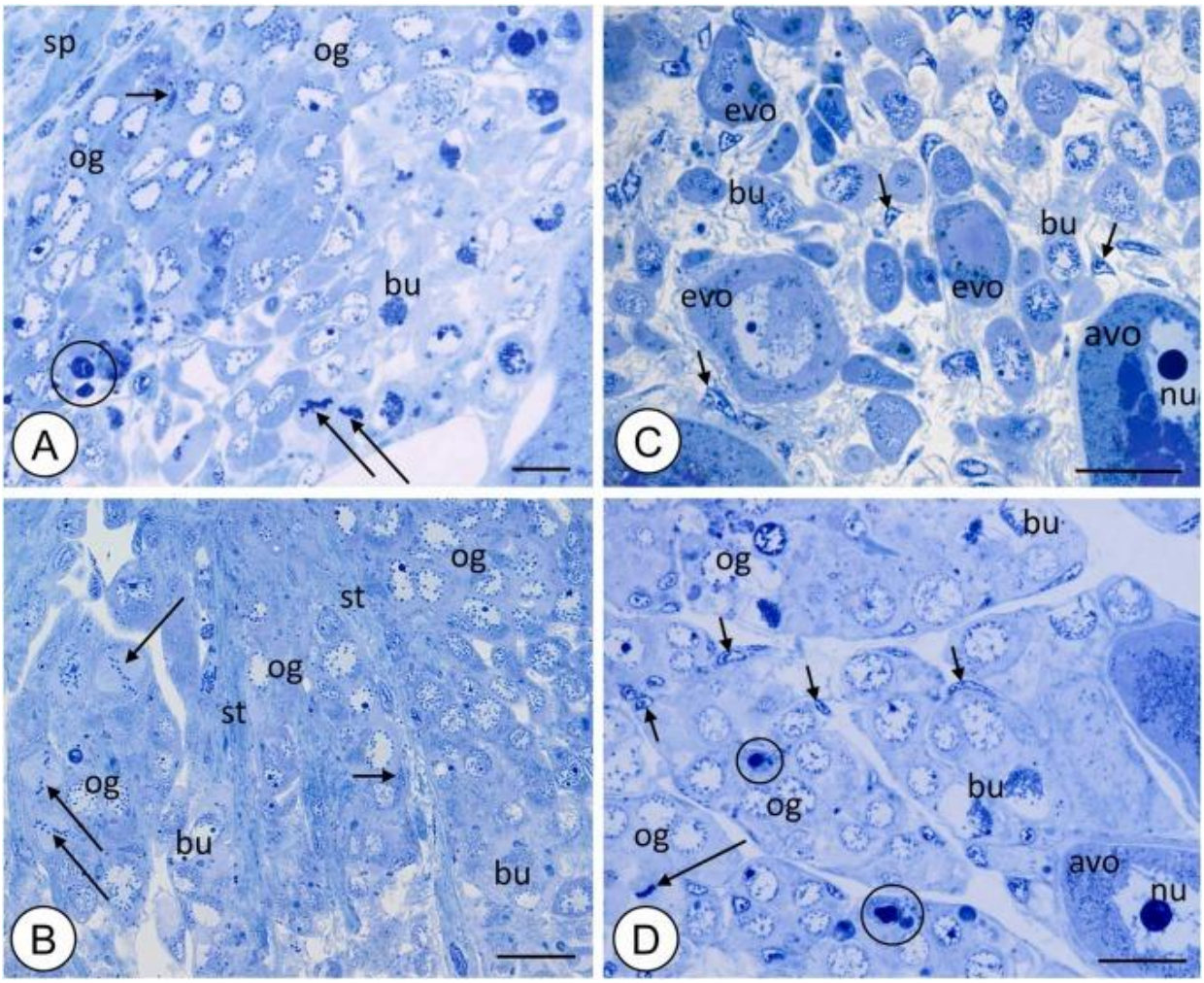


Download : Download high-res image (754KB)

Download : Download full-size image

Fig. 2. General histological organization of ovaries. A) *Metaphire hilgendorfii*; B) *Metaphire houlleti*; C) *Amynthas divergens*. A-B) cross sections, C) oblique section. Zone I contains tightly packed germ and somatic cells; in zone II, vitellogenic oocytes (vo) are ensheathed by somatic cells (arrowheads) and form egg strings (es) which radiate towards the segmental cavity. Ovaries are connected to the septum (sp) via stands of connective tissue (asterisk) and muscles (ms); note blood vessels entering

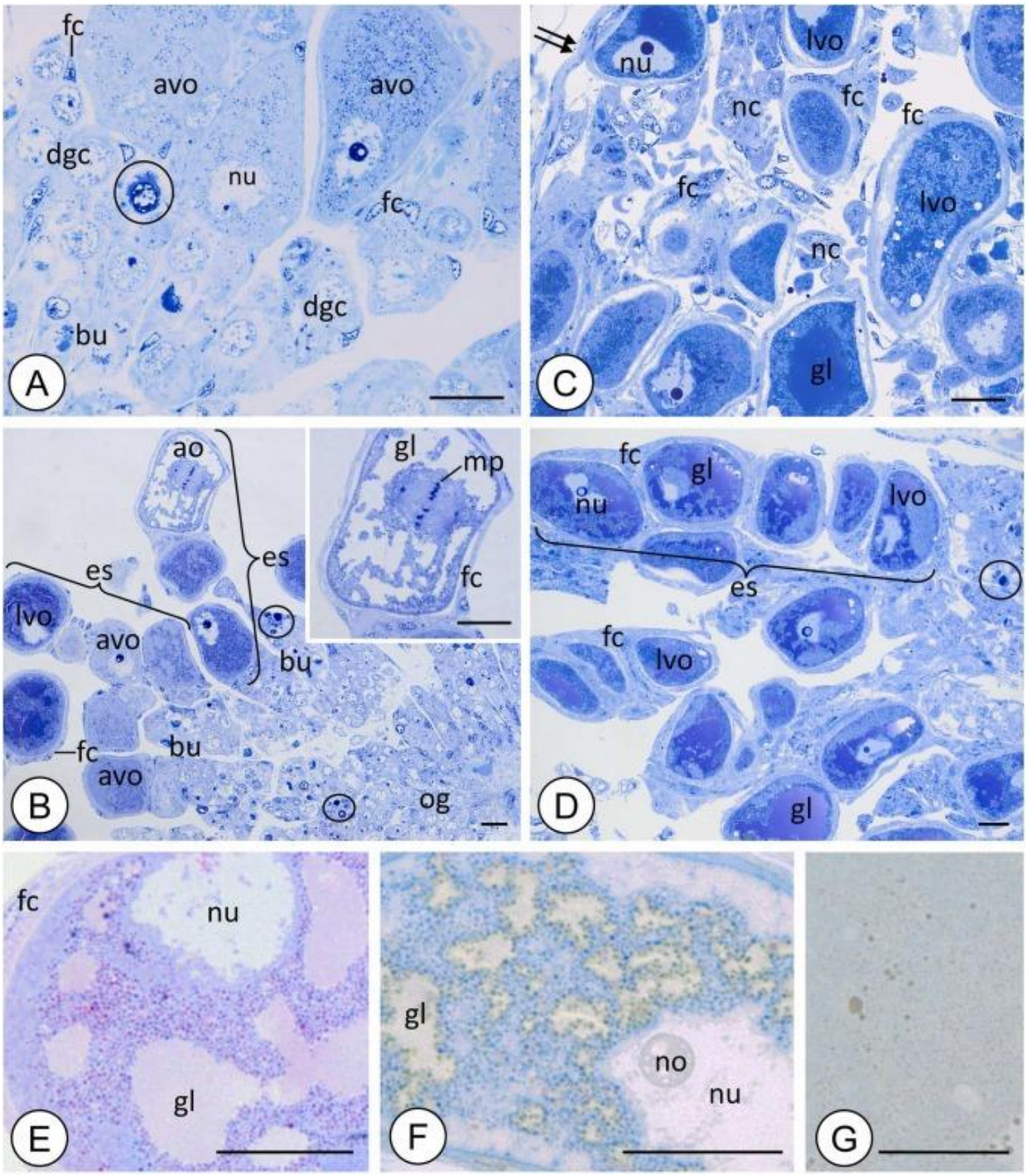
the ovary (bv). Note that ovaries of *A. divergens* are covered by ovarian envelope (double arrows). Epon semi-thin sections stained with methylene blue, light microscopy (LM). Scale bars = 150µm.



Download : Download high-res image (999KB)

Download : Download full-size image

Fig. 3. A-B) Zone I in *Amynthas vittaus* (A) and *Metaphire hilgendorfi* (B). C-D) End of the zone I and the beginning of zone II in *Amynthas divergens* (C) and *Metaphire houlleti* (D). Arrows point to dividing oogonia, short arrows – follicular cells, avo – advanced vitellogenic oocytes, bu – meiotic germ cells in a bouquet stage, evo – early vitellogenic oocytes, nu – oocyte nuclei with nucleolus, og – oogonia, sp – septum, st – strands of somatic tissue entering ovary. Degenerating cells are encircled. Epon semi-thin sections stained with methylene blue, LM. Scale bars = 20µm.



Download : Download high-res image (1MB)

Download : Download full-size image

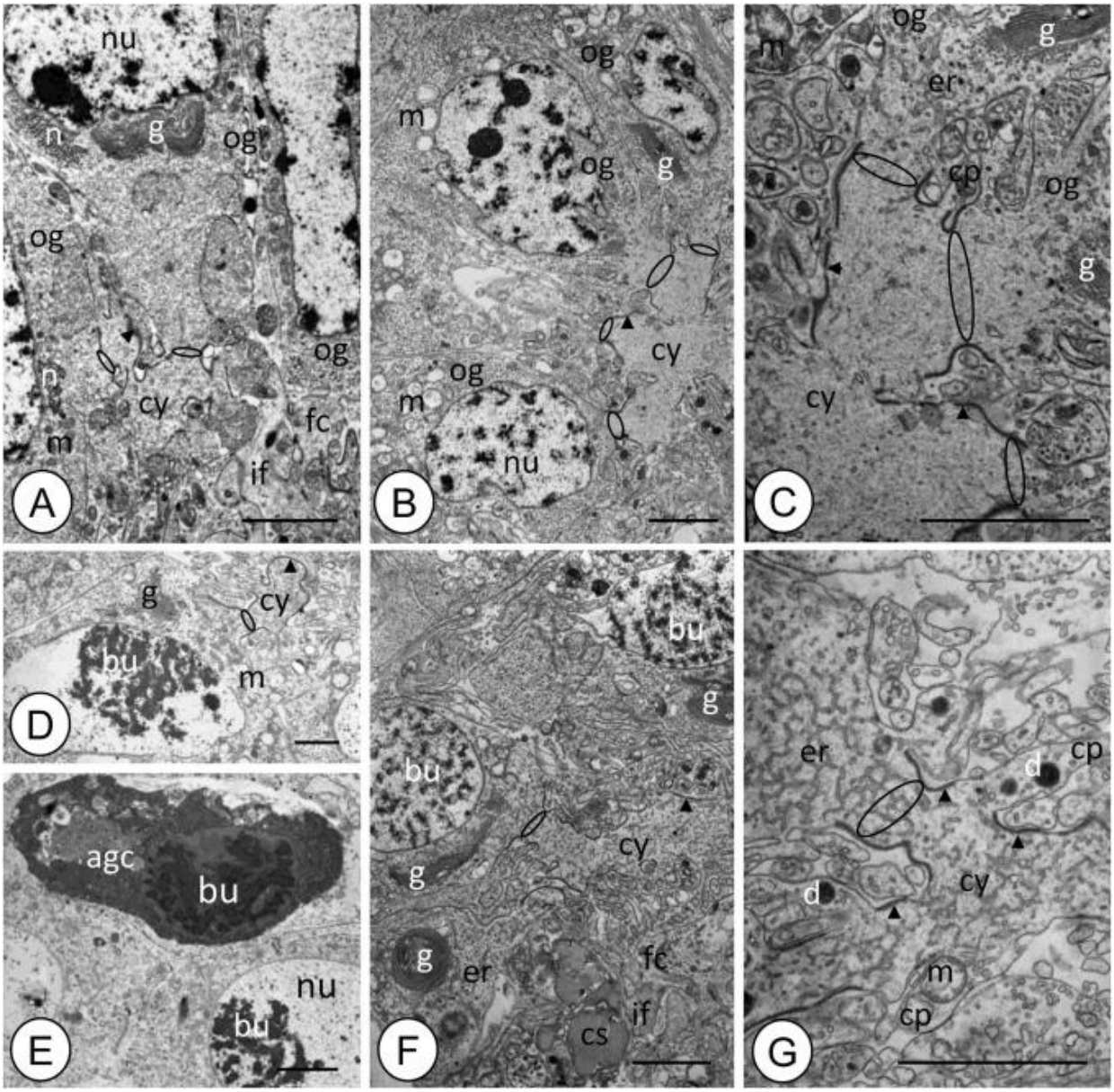
Fig. 4. A) The base of egg string in *Metaphire houlleti*. Egg strings in *M. houlleti* (B), *Amynthas divergens* (C) and *Metaphire hilgendorfi* (D). Inset in B) higher magnification of oocyte arrested in metaphase I. A-D) Epon semi-thin sections stained with methylene blue. E-G) Histochemistry of late vitellogenic oocytes in *M. houlleti*. E) Methylene blue–azure II solution and basic fuchsin staining, pink signals come from polysaccharides, blue from proteins. F) Bromophenol blue staining, blue signals come from proteins. G) Sudan Black B staining, gray signals come from lipid droplets. Double arrows point to ovary envelope in *A. divergens*, circles – degenerating germ cells, avo – advanced vitellogenic

oocytes, ao – arrested oocyte in metaphase I, bu – meiotic germ cells in a bouquet stage, dgc – diplotene germ cells, es – egg strings, fc – follicular cells, gl – glycogen accumulations, lvo – late vitellogenic oocytes, mp – metaphase plate, nu – oocyte nuclei with nucleoli (no), og – oogonia. Scale bars = 20µm.

Below is a detailed description of germline cells in the successive ovarian zones. The somatic components are described separately.

3.3. Zone I – oogonia and early (leptotene-pachytene) germ cells

Oogonia occur close to the connection of the ovary to the septum (Figs. 2–3A, B). They are easily recognizable due to relatively large nuclei with lucent nucleoplasm and dense chromatin patches attached to the nuclear envelope (Fig. 3A, B; 5A, B). Oogonia (and early meiotic germ cells – see below) stay interconnected and form syncytial cysts (Fig. 5A-C). The general pattern of cyst organization found in the studied species is the same as in other clitellate annelids (Świątek and Urbisz, 2019). Namely, each oogonium (and early meiotic germ cell) has one intercellular bridge (ring canal) connecting it to the central and anuclear cytoplasmic mass (Fig. 5A-C). This central cytoplasm, known as the cytophore, has a form of thin and reticular cytoplasmic strands stretched between interconnected cells (Fig. 5A-C). The membranes of ring canals and the cytophore are lined by a thick, electron-dense fibrous material (Fig. 5A-C). This electron-dense material is not continuous; in places where the material is absent, the cytophore cytoplasm forms thin lateral processes (Fig. 5C). The cytoplasm filling ring canals and the cytophore are slightly more electron-lucent than the cytoplasm of oogonia itself; it lacks membranous cell organelles (Fig. 5A-C). Such a cytophore could be considered an initial cytophore because its ultrastructure changes when germ cells enter meiosis (see below). Oogonia, besides having relatively large nuclei, also have accumulations of mitochondria, cisternae of ER, prominent Golgi complexes with electron-dense material filling their trans faces, which suggests their high activity (Fig. 5A-C), some reserve material (glycogen and lipid droplets – not shown), and accumulations of electron-dense membrane-unbound material, known as nuage material (Fig. 5A). In *A. vittatus*, *M. hilgendorfi* and *M. houlleti,* the mitotic divisions of oogonia were observed (Fig. 3A-B, D). Dividing oogonia could be observed in different locations in zone I, and at least two to three cells divide in synchrony (Fig. 3A-B, D). The number of interconnected oogonia per cyst and the number of oogonial cysts occurring in zone I are unknown. The main obstacle was no clear boundaries between the individual cysts. Oogonia are tightly packed and associated with somatic cells, forming a solid mass of cells (Fig. 2, Fig. 3). Thus, no clear borders between cysts were found, even when analyzing serial semi-thin sections. At the ultrastructural level, maximally, four oogonia connected to the cytophore were found on a section (Fig. 5B). It seems that techniques that allow making three-dimensional reconstructions of tissue ultrastructure, such as, e.g., serial block-face scanning electron microscopy (Denk and Horstmann, 2004, Smith and Starborg, 2019), could answer the question of how many cells are interconnected.



Download : Download high-res image (1MB)

Download : Download full-size image

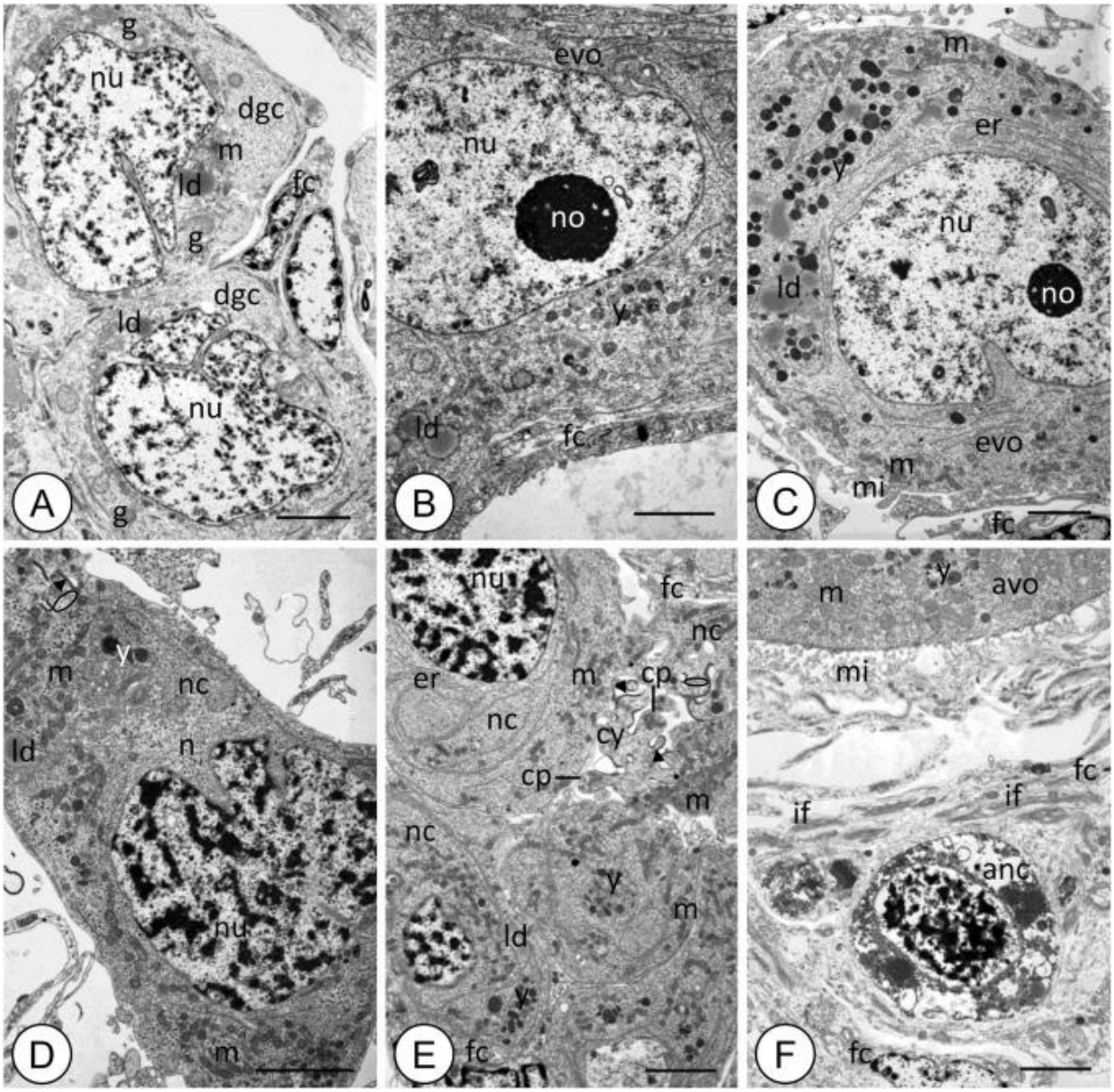
Fig. 5. Ultrastructural details of germline cyst organization in zone I. A-C) Cysts uniting oogonia in *Amynthas vittatus* (A) and *Metaphire hilgendorfi* (B-C). D-G) Cysts uniting zygotene/pachytene cells in a bouquet stage in *A. vittatus* (D-E) and *M. hilgendorfi* (F-G). Arrowheads – electron-dense rim, ellipse – ring canal, agc – apoptotic germ cell, bu – chromosomes organized in a bouquet, cs – crystals, cp – cytoplasmic processes of the cytophore not lined by dense rim, cy – cytophore, d – electron-dense accumulations of unknown character; g – Golgi complex, er – smooth endoplasmic reticulum, fc – somatic cells, if – bunches of cytoskeletal elements interpreted as intermediate filaments; m – mitochondria, n – nuage material, nu – nucleus. Transmission electron microscopy (TEM). Scale bars = 3μm.

Early meiotic germ cells are located more peripherally to oogonia (Fig. 3). They do not form such a compact mass of cells like oogonia (Fig. 3). The interconnected early meiotic germline cells show

developmental synchrony, i.e., all cells in a given cyst are in the same phase of meiosis (Fig. 3, Fig. 5). The nuclei of these cells contain condensing chromosomes (in leptotene) and condensed chromosomes forming a characteristic aggregation on one pole of the nucleus, the so-called bouquet stage (in zygotene/early pachytene) (Figs. 3, 4A-B, 5D-G). Similarly to oogonia, these early meiotic germ cells have prominent accumulations of mitochondria, short cisternae of ER, well-developed Golgi complexes, and some reserved material in the form of lipid droplets (Fig. 5D-G). Early meiotic cells are still interconnected via ring canals and the cytophore and form cysts (Fig. 5D, F-G). Ring canals and the cytophore cell membrane are still lined by the electron-dense material (Fig. 5D, F-G). As with oogonial cysts, the cytophore has a reticular character. In foci with no dense material, the cytophore cytoplasm forms lateral processes (Fig. 5C, G). The cytophore is filled with a well-developed net of smooth ER (Fig. 5F-G); other cell components are rare, e.g., some electron-dense accumulations of unknown character and mitochondria (Fig. 5G). This smooth ER network seems limited to the cytophore; it was not observed within the cell cytoplasm (Fig. 5C, D, F). As in the case of oogonial cysts, the boundaries between cysts are not evident, and the number of interconnected cells is not known. Some early meiotic germline cells undergo apoptosis (Fig. 5E).

3.4. Zone II – diplotene germ cells and growing oocytes – egg strings

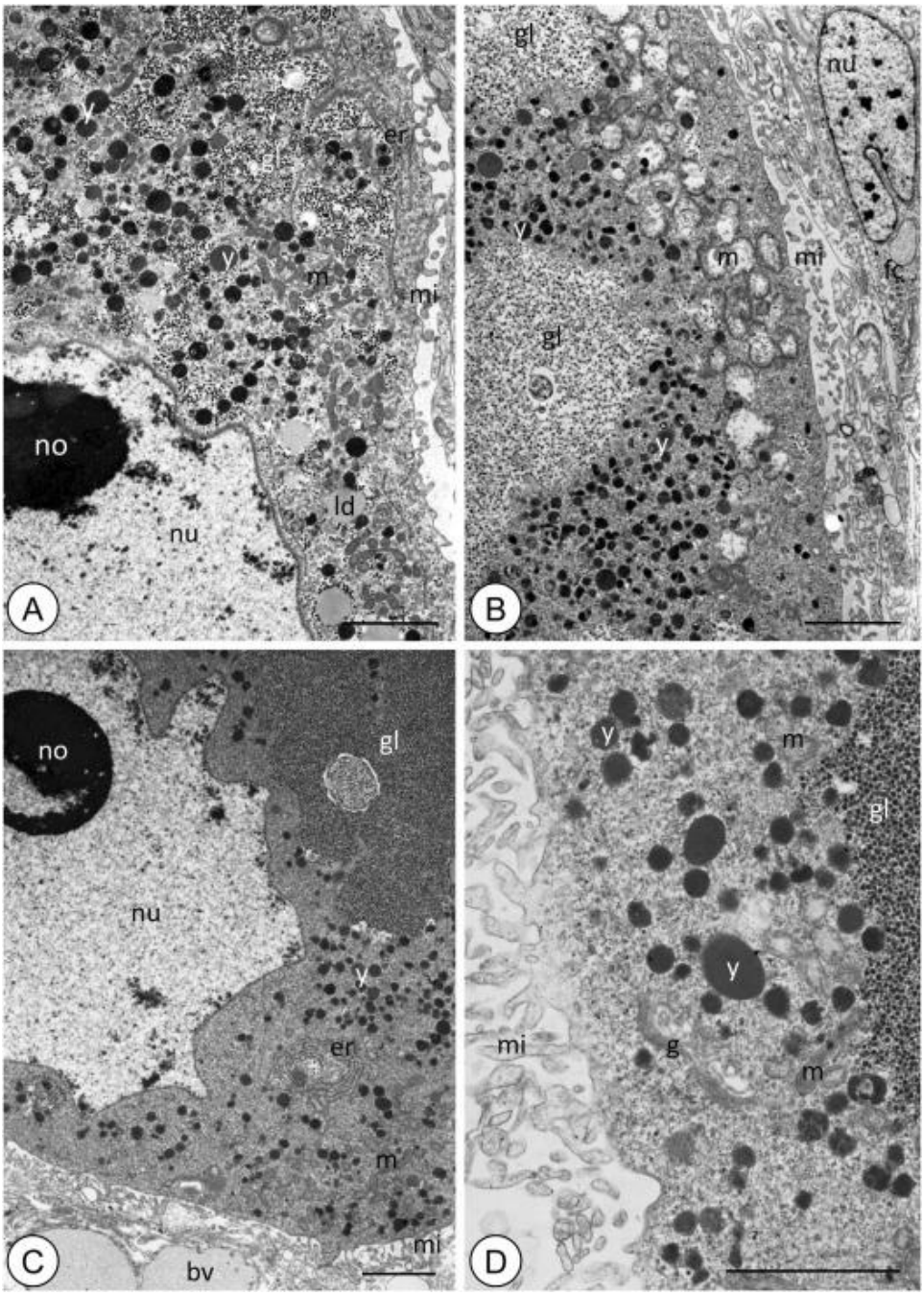
As meiosis progresses, germ cells (interconnected into cysts) are moved to the more distal portions of the ovary (zone II) (Figs. 1–2, 3C–D, 4A–D). Together with somatic cells, they form separated ovarian subunits termed egg strings (Figs. 1–2, 4B, D). Oocytes in a growth phase, gradually accumulating cell components, including reserve material, are the main component of egg strings (Figs. 1–2, 4B, D). However, in the base of egg strings, in direct contact with zone I, there are early diplotene germ cells (Figs. 4A, 6A). They are still interconnected by intercellular bridges and the cytophore (not shown). The chromatin in these cells is decondensed; in the cytoplasm, there are mitochondria, short cisternae of ER, Golgi complexes, and lipid droplets (Fig. 6A). At this stage of meiosis, the synchronous development of interconnected cells is lost. Some diplotene germ cells develop microvilli, accumulate more cytoplasm with organelles and reserve materials than others, and become oocytes (Figs. 3C-D, 4, 6B-C, 7). The second subpopulation of germ cells does not grow significantly; they are regarded as nurse cells. The organization of chromatin in nurse cells resembles interphase; they do not form microvilli and accumulate much fewer reserves than oocytes (Figs. 4C, 6D-E).



Download : Download high-res image (1MB)

Download : Download full-size image

Fig. 6. Ultrastructural details of the base of egg strings – diplotene cells, vitellogenic oocytes, and nurse cells. A) Two diplotene cells in *Metaphire houlleti*. B-C) Early vitellogenic oocytes in *Amynthas vittatus* (B) and *Amynthas divergens* (C). D-E) Nurse cells in *A. vittatus* (D) and *A. divergens* (E). F) Apoptotic nurse cell (anc) close to the advanced vitellogenic oocyte (avo) in *A. divergens*. Cytoplasmic processes of the cytophore not lined by dense rim – cp, arrowheads mark electron-dense rims of ring canals, ellipses – ring canals; cy – cytophore, dgc – diplotene germ cells, er – endoplasmic reticulum, evo – early vitellogenic oocyte, fc – follilular cell, g – Golgi complex, if – bunches of cytoskeletal elements interpreted as intermediate filaments, ld – lipid droplets, m – mitochondria, mi – microvilli, n – nuage material, nc – nurse cells, no – nucleolus, nu – nucleus, y – yolk. TEM. Scale bars = 3µm.



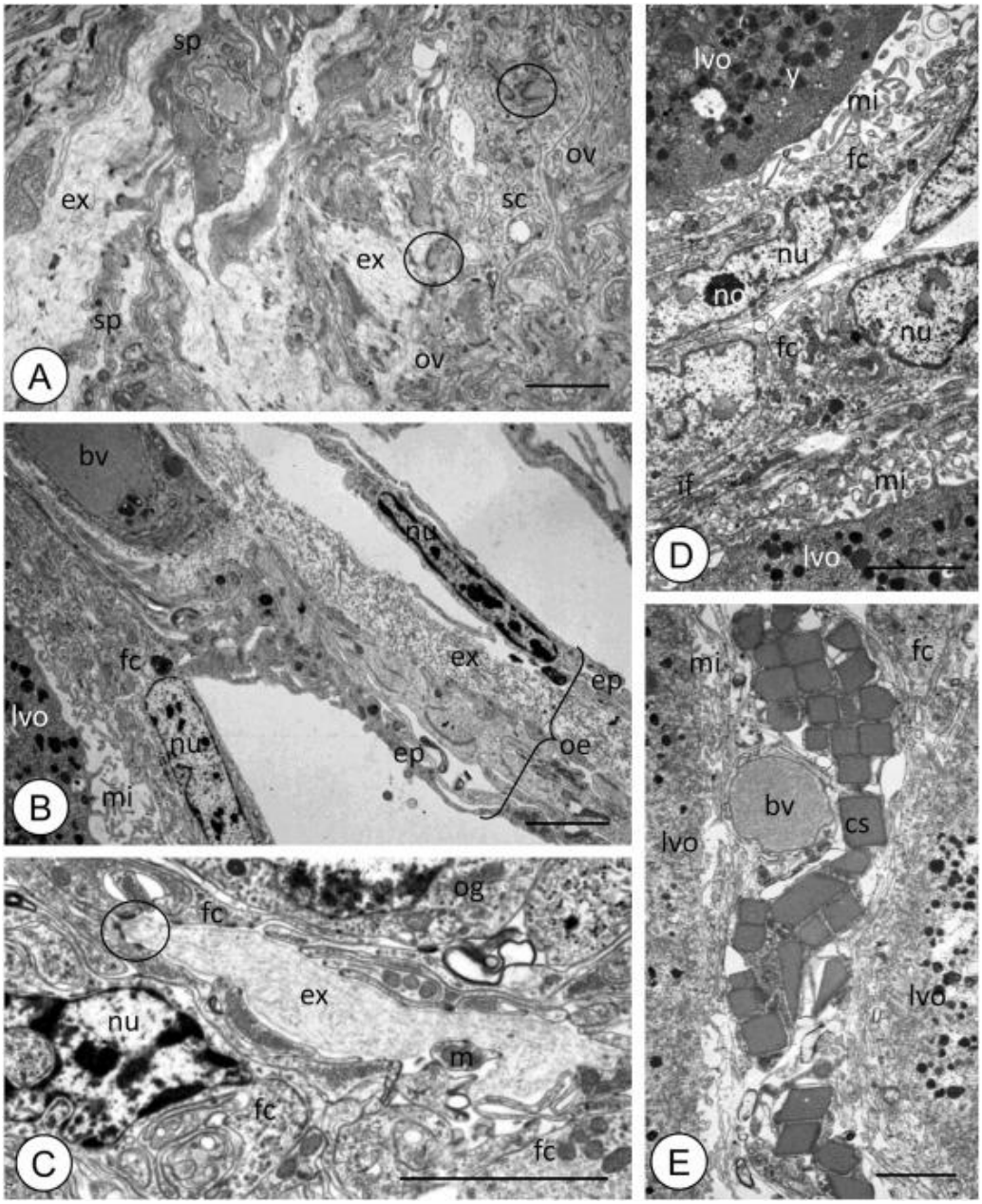
Download : Download high-res image (1MB)

Download : Download full-size image

Fig. 7. Ultrastructure of late vitellogenic oocytes. A) *Amynthas vittatus*, B) *Metaphire hilgendorfii*, C-D) *Amynthas divergens*. bv – blood vessel, er – endoplasmic reticulum, fc – follicular cell, g – Golgi complex, gl – glycogen accumulation, ld – lipid droplet, m – mitochondria, mi – microvilli, no – nucleolus, nu – nucleus, y – yolk. TEM. Scale bars = 3µm.

When becoming morphologically distinguishable from nurse cells, oocytes already contain some reserve materials such as lipids and small accumulations of electron-dense spheres (Fig. 6B-C). Thus, the previtellogenic phase of oogenesis is short and occurs before oocytes and nurse cells become distinguishable. Growing oocytes (vitellogenic phase of oogenesis) have large nuclei with patches of

condensed chromatin, lucent nucleoplasm, and prominent nucleoli (Figs. 2, 3C-D, 4A-F, 6B-C, 7A). For description convenience, we distinguished early, advanced, and late vitellogenic oocytes depending on their volume and amount of accumulated reserve material (Figs. 3C-D, 4, 6B-C, 7, 8). Besides massive accumulations of mitochondria and long cisternae of ER, active Golgi complexes oocytes gather reserve material in the form of vast accumulations of glycogen, numerous electron-dense spheres of yolk, and lipid droplets (Figs. 3C-D, 4C-D, 7). Bromophenol blue staining confirmed that dense yolk spheres contain proteins (Fig. 4F). Sudan Black B staining confirmed the presence of lipids (Fig. 4G), methylene blue–azure II solution and basic fuchsin staining revealed both polysaccharides and proteins (Fig. 4E). The oocyte surface, i.e., oolemma with microvilli, seems not to be covered by a vitelline envelope (Fig. 7). The ring canals connecting the oocytes to the cytophore have never been observed; most probably, oocytes are no longer connected with the rest of the cyst. However, three-dimensional reconstructions should be done to determine if and when oocytes lose connection to the cytophore. Fully grown oocytes are located at the terminal position in the string (Figs. 1C-D, 2, 4B-D). They contain vast glycogen accumulations, usually washed out during material processing (Fig. 4B, E-F). These oocytes are arrested in the metaphase of the first meiotic division, as was observed in the case of *M. houlleti* (Fig. 4B). The following stages of oogenesis, after detaching of oocytes from the ovary, have not been observed.

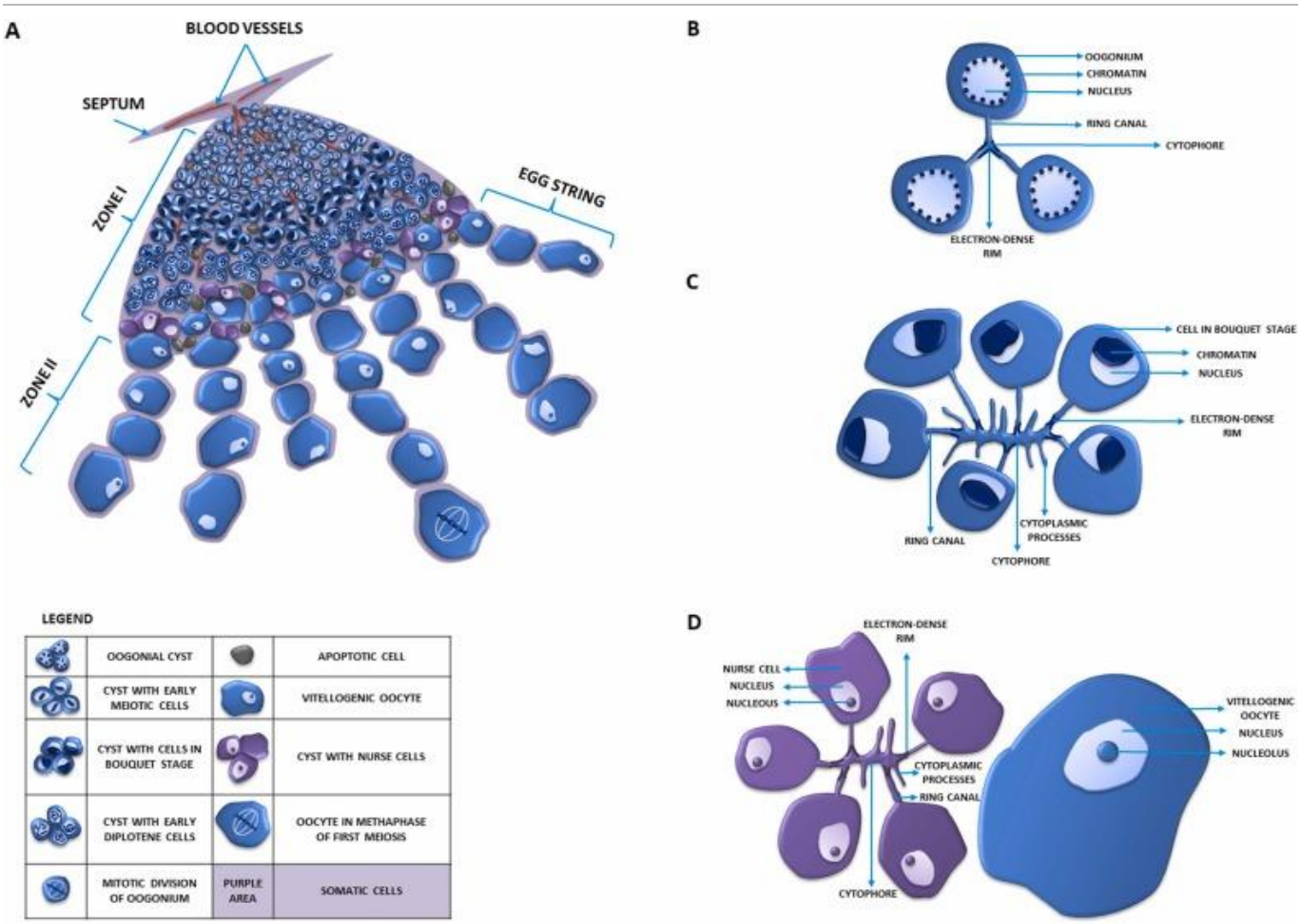


Download : Download high-res image (1MB)

Download : Download full-size image

Fig. 8. Ultrastructural details of somatic components. A) A fragments of ovary connection to the septum in *Metaphire hilgendorfii*. B) Ovary envelope in *Amynthas divergens*. C) Follicular cells and extracellular matrix close to oogonia in *A. divergens*. Hemidesmosome-like cell junctions are encircled. D) Follicular cells enveloping late vitellogenic oocytes in *A. divergens*. E) Crystal-like inclusions in somatic cells in *Metaphire agrestis*. bv – blood vessel, cs – crystal-like inclusions, ep – epithelial cells, ex – extracellular matrix, fc – follicular cells, if – bunches of cytoskeletal elements interpreted as intermediate filaments, lvo – late vitellogenic oocytes, m - mitochondria, mi – microvilli, no – nucleolus, nu – nucleus, oe – ovary envelope, og – oogonia, ov – ovary, sp – septum, y – yolk. TEM. Scale bars = 3µm.

Nurse cells contain large nuclei with patches of dense chromatin and nucleolus (Figs. 4C, 6D-E). Their cytoplasm is enriched in accumulations of mitochondria, cisternae of rough ER, Golgi complexes, nuage accumulations, and a small amount of reserve material in the form of lipid droplets and spheres of proteinaceous yolk (Fig. 6D-E). Nurse cells are still interconnected by ring canals and the cytophore (Fig. 6D-E). Cytophores are poorly developed; they have a form of thin cytoplasmic strands with lateral projections (Fig. 6E). Within the cytophore, dense spheres and some organelles, such as mitochondria, can be observed (Fig. 6E). It seems that nurse cells accompany the growing oocytes for a short time. Analysis of serial semi-thin sections revealed that vitellogenic oocytes are enveloped only by follicular cells (see below), and no accompanying nurse cells were observed within the egg strings. Nurse cells are most probably eliminated when oocytes start the growth phase. The histological and ultrastructural observations suggest that, at least, some nurse cells undergo apoptosis (Figs. 3D, 4A, D, 6F). The scheme showing ovary and germline cyst organization is presented in Fig. 9.



Download : Download high-res image (481KB)

Download : Download full-size image

Fig. 9. A scheme presenting the general organization of “Amynthas” ovaries (panel A) and the details of germline cysts organization (panels B-D). Germ cells in successive stages are not in a scale. A) Ovary is attached to the septum. Small blood vessels enter the ovary. The most proximal part of the ovary (zone I) is solid and tightly packed with the mass of germline cells associated with somatic cells

(details of somatic cells are omitted for clarity). The rest of the ovary (zone II) has a more loose structure, forming strings of growing oocytes (egg strings) enveloped by thin somatic cells. Germ cells in zone I are interconnected and form small syncytial cysts (not depicted in this panel). Germline cysts have never been observed in zone II. B-D) Details of cysts organization. Each cell is interconnected via a thin ring canal to the central cytoplasmic mass, a cytophore. The cytophore has a form of thin and anatomizing cytoplasmic strands stretched between interconnected cells. Ring canals are lined by electron-dense fibrous material (electron-dense rim). In places where the dense material is absent, the cytophore cytoplasm forms thin cytoplasmic processes. The exact number of interconnected cells is not known, however, ultrastructural observations suggest that six to eight cells could be connected. B) Cyst with oogonia; C) Cyst with early meiotic germ cells in a bouquet stage; D) Cyst with interconnected nurse cells. Most probably, vitellogenic oocyte is no longer connected to the cyst.

3.5. Somatic components

No specific, morphologically distinguishable somatic ligament connects ovaries with the septum. Ovaries are tightly interconnected to the septum via a relatively broad connection composed of coelomic epithelium, muscle cells, connective tissue, and blood vessels (Figs. 2A, C, 8A). Muscle cells do not enter into ovaries; they are present only in the connection area (Fig. 2A). Connective tissue has well developed extracellular matrix to which somatic cells are attached via connections resembling hemidesmosomes (Fig. 8A). In five of the six studied species, the coelomic epithelium lining the septum does not extend onto the whole ovary, and there is no outer ovary envelope (Figs. 1, 2A-B). In contrast, in *A. divergens,* the ovary envelope is present (Figs. 2C, 4C, 8B). It comprises two layers of epithelial cells interconnected by an extracellular matrix (Fig. 8B). In all species studied, thin strands of somatic tissue may enter deeply into the ovarian zone I (Fig. 3B). Ultrastructural analysis suggests that thin strands of extracellular matrix occur between germ cells (oogonia), as was shown in the case of *A. divergens* (Fig. 8C).

All categories of germ cells are in direct contact with the somatic cells, regarded as follicular cells. Follicular cells usually tightly adhere to germ cells, as seen in the case of oogonia (Figs. 3A, B, C, 5A) or cysts with early meiotic germ cells (Figs. 3, 4A, 5F). Oocytes are also enveloped by elongated follicular cells, and these two cell categories form egg strings (Figs. 1C-D, 2A, 4A-E, 7B, 8D). Follicular cells are in direct contact with the oocyte microvilli (Figs. 7B, 8B, D), encompass oocytes, and separate one from another (Figs. 1C-D, 4B, D, 8D). Nurse cells are also enveloped by follicular cells (Figs. 4C, 6E). Follicular cells usually have elongated nuclei with dense patches of heterochromatin (Figs. 7B, 8B-D). Their cytoplasm contains mitochondria, short ER cisternae, and electron-dense vesicles (Figs. 7B, 8B-D). The cytoplasm of follicular cells is usually enriched in bunches of cytoskeletal elements interpreted as intermediate filaments (Figs. 5A, F, 6F, 8D). It seems that enriched cytoskeleton is characteristic of follicular cells directly enveloping germ cells, from oogonia till vitellogenic oocytes (Figs. 5A, F, 6F, 8D) and intermediate filaments are anchored by desmosomes and hemidesmosomes (Fig. 8A).

Coelomocytes were not observed inside the ovaries. In *M. agrestis* and *M. hilgendorfi*, some follicular cells are packed with crystal-like elements of medium electron density (Figs. 5F, 8E). Blood vessels are present both within the ovary connection area and within the ovaries (Figs. 2A, 7C, 8B, E).

4. Discussion

4.1. Ovaries of studied megascolecids are composed of germline cysts, growing oocytes, and somatic components

Oogenesis is a complex process with two main stages: the proliferative stage, when oogonia divide mitotically, and the growth phase, when oocytes pass meiosis and grow due to gathering cell components and nutrients necessary for proper embryo development (Gilbert and Barresi, 2016). A growing body of evidence suggests that the conservative aspect of the late proliferative stage and early meiosis is the formation of syncytial germline cysts, also known as clusters, nests, or clones (Chaigne and Brunet, 2022, Gerhold et al., 2022, Matova and Cooley, 2001, Pepling et al., 1999). Mitotic divisions of the last oogonia generations are incomplete, i.e., the contractile rings are not completely closed (there is no abscission) but stabilized by numerous proteins. As a result, the sister cells are interconnected via relatively broad (up to 10μm in diameter) and stable cytoplasmic channels known as intercellular bridges, cytoplasmic bridges, or ring canals (Chaigne and Brunet, 2022, Greenbaum et al., 2011, Haglund et al., 2011, Pepling et al., 1999). Ring canals allow exchanging of cytoplasm with macromolecules (e.g., RNPs, proteins) and organelles between interconnected cells. Such exchange seems crucial for some molecular mechanisms of oogenesis, e.g., oocyte specification (Nashchekin et al., 2021). Female germline cysts are usually polarized, i.e., two cell categories emerge. The first is oocyte or oocytes, which continue meiosis, gather nutrients, and finally become egg cells. The second is accessory cells (nurse cells, trophocytes) which do not continue meiosis, sometimes become polyploid and produce and transfer macromolecules and organelles toward oocytes and eventually die (Lebo and McCall, 2021, Niu and Spradling, 2022, Spradling et al., 2022). This 'nourishing mechanism' is one of the most well-known functional aspects of cyst functioning (Chaigne and Brunet, 2022, Gerhold et al., 2022, Lu et al., 2017, Spradling et al., 2022). Although female cysts are present in numerous animal phyla (Chaigne and Brunet, 2022), our knowledge about the formation and functioning of female germline cysts comes mainly from model species such as *Drosophila melanogaster*, *Caenorhabditis elegans* and *Mus musculus* (Niu and Spradling, 2022).

We found germline cysts in ovaries of all species studied at the ultrastructural level. Oogonia and germ cells in early meiosis (till early diplotene) are interconnected via ring canals and the reticular cytophore. All interconnected cells are in the same cell cycle phase; thus, clustering cells develop synchronously. Then, the synchrony is lost – some cells (prospective egg cells) detach from the cyst, continue meiosis and start to grow. The remaining cells (nurse cells) do not grow markedly and do not continue meiosis; however, they are still interconnected via ring canals and the reticular cytophore. Despite intensive analysis of serial semi-thin and ultra-thin sections, we could not find the ring canals connecting growing oocytes with its group of cells. Most probably, just after the oocyte starts the growth phase, the ring canal is closed, and the cell is no longer connected to the cyst.

During this time, oocytes and somatic cells form egg strings, whereas nurse cells die via apoptosis. Generally, the functioning and final fate of nurse cells in clitellate germline cysts are poorly known (for more details, see, e.g., the paper discussing the fate of nurse cells in *Enchytaeus albidus* - Urbisz et al., 2017). In the case of earthworms, it seems that nurse cells function for a short time as cysts without connection to the growing oocytes and are finally eliminated by apoptosis (this study, Świątek et al., 2023). This phenomenon could be connected with the poorly developed reticular cytophore and the tendency for relatively quick detachment of oocytes from cysts. It implies that the exchange between nurse cells, cytophore, and oocyte is structurally- and time-limited (see Section 4.3). More intense and detailed analyses of cyst organization and functioning in earthworms are planned in selected representatives of lumbricids (Świątek et al., 2023).

Recent, intensive microscopic analysis of numerous clitellates such as leeches and micordriles (Gorgoń and Świątek, 2021, Świątek and Urbisz, 2019) and some older ultrastructural data devoted to earthworms (Chapron and Relexans, 1971b, Siekierska, 2003) clearly show that the formation of female germline cysts does occur and is widespread across Clitellata. What is more, in all clitellates studied to date, the organization of the cysts is the same: each cell has one ring canal connecting it to the central cytoplasmic core (cytophore) (Świątek et al., 2009, Świątek and Urbisz, 2019). Thus, there is a strong evolutionary tendency to retain such cyst organization across Clitellata. It is worth mentioning that cysts equipped with the cytophore are also a conservative evolutionary aspect of spermatogenesis in Clitellata and all Annelida (Jamieson, 2006, Jamieson, 1981, Olive, 1983, Rouse, 2006). On the other hand, female cysts with central cytoplasmatic cores are not widespread in Metazoa. Apart from clitellates, such cysts are known from some echiurans (Leutert, 1974), mites (Liana and Witaliński, 2012), and certain nematodes, including *C. elegans* (Seidel et al., 2018).

It could be concluded that the presence of germline cysts enveloped by somatic cells within the ovaries of the studied megascolecids reflects the conservative evolutionary aspect of animal gametogenesis (Chaigne and Brunet, 2022, Pepling et al., 1999). Specifically, the general pattern of cyst organization found in the studied *Amynthas* and *Metaphire* species is the same as in other clitellates studied to date (Świątek and Urbisz, 2019).

4.2. *Megascolecids have* Amynthas *type of ovaries*

The pattern of ovary organization in the studied megascolecids is the same as in other Clitellata – ovaries are composed of germline cysts equipped with the cytophore and enveloped by somatic cells. However, the ovaries of, e.g., leeches, microdriles, and earthworms are different. Why is this so? There are three possible main reasons: 1) the germline cysts equipped with the cytophore are evolutionary flexible and may differ markedly between taxa, e.g., in the number of interconnected cells or cytophore shape and dimensions (see below); 2) the growing oocytes could be retained within the ovary till the completion of yolk absorption or could be released from the ovary early (before vitellogenesis) and complete oogenesis in the segmental cavity (intraovarian *versus* extraovarian oogenesis *sensu* Eckelbarger, 1983) or 3) the somatic components may differ markedly, e.g., in leeches a massive somatic sheath (ovisac) tightly envelops ovarian cords (for details see Gorgoń and Świątek,

2021), whereas, in microdriles and megadriles, ovaries are loosely suspended in the coelom (Świątek and Urbisz, 2019).

Earthworm ovaries differ in general morphology between taxa. These differences have been recorded from the beginning of studies devoted to the anatomy and histology of earthworms (Beddard, 1895, Stephenson, 1930). Gates (1976) proposed a simple classification of earthworm ovaries based on the overall shape and the presence or absence of egg strings. Ovaries without egg strings are characteristic of *Criodrilus lacuum* and Moniligastridae (Gates, 1976). If egg strings occur, the ovaries could be fan- to rosette-shaped with several or numerous egg strings. Such ovaries are typical for Acanthodrilidae, Megascolecidae, Benhamiidae, and Ocnerodrilidae. In Lumbricoidea, ovaries are discoidal or rectangular to triangular with one egg string only (Gates, 1976). Such classification based only on the general characters does not reflect the diversity and complexity of ovary organization. During our systematic studies of ovaries in clitellate annelids, we proposed the classification based on the above-discussed characters, which, in our opinion, better reflect the state of matter. To date, we have described several types of ovaries in leeches and microdriles, giving them names of the best-studied genera, e.g., ovary types "Glossiphonia", "Piscicola" or "Hirudo" in leeches (Gorgoń and Świątek, 2021), "Tubifex" or "Enchytraeus" ovaries in microdriles (Świątek and Urbisz, 2019). To follow this idea, we propose to term the ovaries found in the studied megascolecids as the ovaries of the "Amynthas" type. The main characters of "Amynthas" ovaries (Fig. 9) are:

1) a fan- to rosette-shape with numerous egg strings arranged radially around the site of ovary connection to the septum;

2) regarding the internal organization, two zones can be distinguished – in zone I (close to the septum), germline cells are interconnected *via* ring canals and form tightly packed and synchronously developing cysts; in zone II (distal part), synchrony is lost, oocytes detach from cysts, grow and form numerous rows of cells (egg strings);

3) germline cysts are equipped with poorly developed reticular cytophore, and growing oocytes quickly detach from cysts;

4) ovaries are directly connected to the septum; the ovary envelope is usually absent, and germline cysts and growing oocytes are enveloped by thin somatic cells.

We studied the micromorphology of ovaries in six *Amynthas* and *Metaphire* species. On the other hand, Megascolecidae is the most speciose family of earthworms (Edwards and Arancon, 2022a, Jamieson et al., 2002). Are "Amynthas" ovaries typical for all Megascolecidae? Considering the evolutionary conservatism of ovary organization in other Clitellata and the observations of ovaries with numerous egg strings in other Megascolecidae (Gates, 1976, Sims, 1980), we suppose this type of ovaries should be typical for all megascolecids. Moreover, we expect the same organization of ovaries in families closely related to Megascolecidae such as Acanthodrilidae, Benhamiidae, or Ocnerodrilidae. Of course, this idea should be proven by detailed microscopical analysis. To date, such analyses are lacking; usually, only the localization of ovaries and gross morphology is given.

Histological studies are scarce; the case of *Eudichogaster kinneari* (Acanthodrilidae) (Lakhani, 2014) is one of the exceptions. These analyses showed that ovaries of *E. kinneari* have overall morphology similar to "Amynthas"; i.e., there are many egg strings. However, these studies are based on light microscopy only, and there is no information about, e.g., the occurrence of germline cysts.

Generally, we expect that in earthworms (regarded as Crassiclitellata + Moniligastridae = Metagynophora) several types of ovaries occur. As mentioned, "Amynthas" ovaries should be characteristic of Megascolecidae and closely related families. "Dendrobaena" ovaries are typical of Lumbricidae, Hormogastridae, and supposedly for other closely related families (Siekierska, 2003, Świątek et al., 2023). "Dendrobaena" ovaries were classically described as ovaries with one egg string, and apart from this difference, they have a very similar internal organization to the "Amynthas" type. In both cases, germline cysts equipped with the reticular cytophore are present, and oocytes quickly detach from cysts (Świątek et al., 2023; this study). Based on general morphological descriptions (Gates, 1976, Sims, 1980), other types of ovaries should be found in, e.g., Glossoscolecidae and Criodrilidae. Ovaries of Eudrilidae most probably also belong to the other type. They could be tiny structures, freely suspended in the coelom and connected via thin oviducts with ovisacs, or ovaries could be wholly enclosed in a celomic sac system (Jamieson, 1967). Such an organization warrants investigation and the ultrastructural analysis of eudrilids ovaries is carried out in our laboratory.

4.3. Selected aspects of ovary organization and oogenesis

Some aspects of ovary organization and oogenesis found in studied megascolecids need additional comments. Generally, the gross morphology and histological organization of ovaries in all studied species are similar. However, some differences have been observed. One is the presence of the ovary envelope directly covering ovaries in *A. divergens*. In contrast, in the remaining species, the ovaries are not enveloped and are suspended freely in the segmental cavity. Surprisingly, such a significant difference is present in closely related species. At the moment, there is no clear explanation for this phenomenon. Generally, ovaries of earthworms are not enveloped by a common somatic sheath, and oocytes are ovulated from them to the segmental cavity and gathered in the ovisac, the extended fragment of the septum between XIII and XIV segments (Edwards and Arancon, 2022b). It is unclear if in *A. divergens* ovulated oocytes are gathered within the ovary envelope or, as in other earthworms, are released from ovaries towards the ovisac. This problem needs further studies involving more megascolecid species and a more detailed analysis of *A. divergens*.

The other details of ovary micromorphology and oogenesis in the studied species are the same, and no apparent differences have been found. It is not surprising that oogenesis is a rather conserved process. An analysis of other clitellates showed that ovary organization and the mode of oogenesis are conserved, at least at the family level (Świątek and Urbisz, 2019). There are also no detectable differences between sexually (amphimictic) reproducing and parthenogenetic specimens. This is an expected result; in both modes of reproduction, oocytes must be produced, and parthenogenesis affects male reproductive organs (Edwards and Arancon, 2022c).

Cytological aspects of oogenesis found in studied genera are similar to other earthworms studied to date (mainly Lumbriculidae and Hormogastridae – "Dendrobaena" ovaries (Lechenault, 1968, Siekierska, 2003, Świątek et al., 2023). As was discussed, germline cysts are formed. The delicate and reticular cytophore and a small number of interconnected cells characterize both "Amynthas" and "Dendrobaena" ovaries. In other Clitellata, e.g., microdriles, cytophores are usually well-developed, large structures which gather a mass of cytoplasm rich in organelles as was described in "Tubifex" ovaries (Urbisz et al., 2021, Urbisz et al., 2015). Poorly developed reticular cytophore characteristic for "Amynthas" and "Dendrobaena" ovaries seems to have a limited contribution to exchanging cell components between sister cells. However, it cannot be excluded that some macromolecules are exchanged between clustering cells before the oocyte detaches. Molecular analyses like those performed on the model species, such as the fruit fly and mouse (Milas and Telley, 2022, Niu and Spradling, 2022, Spradling et al., 2022) could resolve this problem. The number of clustering cells in the "Amynthas" and "Dendrobaena" ovaries is low. In *Carpetania matritensis* (Hormogstridae), which has "Dendrobaena" ovaries (Świątek et al., 2023), eight cells (one oocyte and seven nurse cells) compose one cyst. In studied megascolecids, we could not count interconnected cells due to tightly packed cell mass in zone I and no clear borders between cysts. However, no more than four interconnected cells were present in a single section, suggesting a low number of interconnected cells. Thus, it seems that the reticular cytophore and cyst composed of a few cells are characteristic of "Amynthas" and "Dendrobaena" ovaries. It could be expected that similar features will be found in other earthworms. To prove these predictions, ultrastructural analyses of ovaries in other families are needed because the germline cysts are not detectable at the light microscopy level.

Like other earthworms, the studied megascolecids produce many eggs filled with a relatively small amount of yolk. Fully grown oocytes are characterized by vast accumulations of glycogen, whereas proteinaceous yolk is not abundant. The vitelline envelope in the studied species is absent or so poorly developed and transparent that it was not recognized at the ultrastructural level. In recently studied hormogastrids such as e.g., *C. matritensis,* the vitelline envelope is a thin layer of fibrous electron-lucent material deposited between microvilli (Świątek et al., 2023). Small amounts of proteinaceous yolk and poorly developed egg envelopes are connected with cocoon production, which ensures supplying developing embryos with proteins and their shelter.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Yukiyoshi Kamihira, Emeritus Professor of Hakodate University, Hakodate, Japan, and Akifumi Othaka, Emeritus Professor of Hirosaki University, Hirosaki, Japan, for collecting and fixing earthworms from Japan. We also thank M. Sc. Marzena Zmarzły, Dr. Danuta Urbańska-Jasik,

and Dr. Łukasz Chajec from the Institute of Biology, Biotechnology and Environmental Protection, University of Silesia in Katowice, Poland, for technical assistance.

The research was financed by National Science Centre, Poland, contract number 2020/37/B/NZ4/00560.

Appendix A. Supplementary material

Download : Download Word document (48KB)

Supplementary material

Recommended articles

Data availability

Data will be made available on request.

References

Anderson et al., 2017 F.E. Anderson, B.W. Williams, K.M. Horn, C. Erséus, K.M. Halanych, S.R. Santos, S.W. James

Phylogenomic analyses of Crassiclitellata support major Northern and Southern Hemisphere clades and a Pangaean origin for earthworms

BMC Evol. Biol (2017), p. 17, 10.1186/s12862-017-0973-4

Google Scholar

Beddard, 1895 F.E. Beddard

A monograph of the order of Oligochaeta

Clarendon Press, Oxford (1895), 10.5962/bhl.title.28557

Google Scholar

Blakemore, 2003 R.J. Blakemore

Japanese earthworms (Annelida: Oligochaeta): A review and checklist of species

Org. Divers. Evol., 3 (2003), pp. 241-244, 10.1078/1439-6092-00082

View PDF View article View in Scopus Google Scholar

Chaigne and Brunet, 2022 A. Chaigne, T. Brunet

Incomplete abscission and cytoplasmic bridges in the evolution of eukaryotic multicellularity

Curr. Biol., 32 (2022), pp. 385-397, 10.1016/J.CUB.2022.03.021

Google Scholar

Chapron and Relexans, 1971a C. Chapron, J.C. Relexans

Ultrastructure des gonocytes primordiaux et des gonies chez l'hermaphrodite *Eisenia foetida* (Oligochete Lombricide)

C. R. Acad. Sc. Paris, 272 (1971), pp. 2916-2919

Google Scholar

Chapron and Relexans, 1971b C. Chapron, J.C. Relexans

Connexions intercellulaires et evolution nucleaire au cours de la premeiose ovocytaire. Etude ultrastructurale chez le Lombricien *Eisenia foetida*

CR. Acad. Sci. Paris, 272 (1971), pp. 3307-3310

Google Scholar

Csuzdi et al., 2019 C. Csuzdi, E. Rota, T. Szederjesi, E. Sherlock, G.G. Brown, C.H. Chang, D.D. Cosin, C. Fragoso, B.G.M. Jamieson, Y. Hong, S.W. James, M.G. Paoletti, T. Pavlícek, D. Plisko, V.V. Pop, H.P. Shen

Description of a new Central African earthworm, *Petroscolex centenarius* gen. Et sp. Nov. (Crassiclitellata, Eudrilidae), celebrating the 100th birthday of Pietro Omodeo

Zootaxa, 4674 (2019), pp. 501-508, 10.11646/zootaxa.4674.5.1

View in Scopus Google Scholar

Csuzdi et al., 2015 C. Csuzdi, E. Sherlock, M.T. Kouete, T.M. Doherty-Bone

Four new earthworm species from the highlands of Cameroon with description of a new genus *Okudrilus* gen. N. (Oligochaeta: Eudrilidae & Acanthodrilidae)

African Invertebr, 56 (2015), pp. 25-38, 10.5733/afin.056.0103

View in Scopus Google Scholar

Denk and Horstmann, 2004 W. Denk, H. Horstmann

Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure

PLoS Biol (2004), p. 2, 10.1371/journal.pbio.0020329

Google Scholar

Eckelbarger, 1983 K.J. Eckelbarger

Evolutionary radiation in polychaete ovaries and vitellogenic mechanisms: their possible role in life history patterns

Can. J. Zool, 61 (1983), pp. 487-504, 10.1139/Z83-065

Google Scholar

Eckelbarger and Hodgson, 2021 K.J. Eckelbarger, A.N. Hodgson

Invertebrate oogenesis – a review and synthesis: comparative ovarian morphology, accessory cell function and the origins of yolk precursors

Invertebr. Reprod. Dev., 65 (2021), pp. 71-140, 10.1080/07924259.2021.1927861

View in Scopus ↗ Google Scholar ↗

Edwards and Arancon, 2022a C.A. Edwards, N.Q. Arancon

Earthworm diversity, dispersal and geographical distribution

Biology and Ecology of Earthworms, Springer (2022), pp. 55-80

CrossRef ↗ Google Scholar ↗

Edwards and Arancon, 2022b C.A. Edwards, N.Q. Arancon

Earthworm morphology

Biology and Ecology of Earthworms, Springer, New York (2022), pp. 1-31

Google Scholar ↗

Edwards and Arancon, 2022c C.A. Edwards, N.Q. Arancon

Earthworm life histories and biology

Biology and Ecology of Earthworms, Springer (2022), pp. 81-108

CrossRef ↗ Google Scholar ↗

Erséus et al., 2020 Erséus, C., Williams, B.W., Horn, K.M., Halanych, K.M., Santos, S.R., James, S.W., Creuzé, M., Anderson, F.E., 2020. Phylogenomic analyses reveal a Palaeozoic radiation and support a freshwater origin for clitellate annelids 49, 614–640. ⟨https://doi.org/10.1111/zsc.12426⟩ ↗

Google Scholar ↗

Faron et al., 2015 J. Faron, T. Bernaś, H. Sas-Nowosielska, J. Klag

Analysis of the behavior of mitochondria in the ovaries of the earthworm *Dendrobaena veneta* Rosa 1839

PLoS One, 10 (2015), pp. 1-21, 10.1371/journal.pone.0117187 ↗

Google Scholar ↗

Ferraguti, 1999 M. Ferraguti

Reproductive Biology of Invertebrates

K.G. Adiyodi, R.G. Adiyodi (Eds.), Part B, Progress in Male Gamete Ultrastructure and Phylogeny, Vol IX, John Wiley and Sons, Chichester, New York (1999), pp. 125-182

Google Scholar ↗

Gates, 1982 G.E. Gates

Farewell to North American megadriles

Megadrilogica, 4 (1982), pp. 12-77

Google Scholar ↗

Gates, 1974 G.E. Gates

On oligochaete gonads

Megadrilogica, 1 (1974), pp. 1-4

View in Scopus Google Scholar

Gates, 1976 G.E. Gates

On earthworm ovaries and their importance in megadrile systematics

Megadrilogica, 2 (1976), pp. 1-2

Google Scholar

Gerhold et al., 2022 A.R. Gerhold, J.-C. Labbé, R. Singh

Uncoupling cell division and cytokinesis during germline development in metazoans

Front. Cell Dev. Biol., 10 (2022), pp. 1-16, 10.3389/fcell.2022.1001689

View in Scopus Google Scholar

Gilbert and Barresi, 2016 S.F. Gilbert, M.J.F. Barresi

Developmental Biology (11th ed.), Sinauer Associates, Inc, Sunderland, MA (2016)

Google Scholar

Gorgoń and Świątek, 2021 S. Gorgoń, P. Świątek

The apical cell – An enigmatic somatic cell in leech ovaries – Structure and putative functions

Dev. Biol., 469 (2021), pp. 111-124, 10.1016/j.ydbio.2020.10.004

View PDF View article View in Scopus Google Scholar

Greenbaum et al., 2011 M.P. Greenbaum, T. Iwamori, G.M. Buchold, M.M. Matzuk

Germ cell intercellular bridges

Cold Spring Harb. Perspect. Biol., 3 (2011), Article a005850, 10.1101/cshperspect.a005850

Google Scholar

Haglund et al., 2011 K. Haglund, I.P. Nezis, H. Stenmark

Structure and functions of stable intercellular bridges formed by incomplete cytokinesis during development

Commun. Integr. Biol., 4 (2011), pp. 1-9, 10.4161/cib.4.1.13550

View in Scopus Google Scholar

Humphrey and Pittman, 1974 C.D. Humphrey, F.E. Pittman

A simple methylene blue-azure II-basic fuchsin stain for epoxy-embedded tissue sections

Biotech. Histochem, 49 (1974), pp. 9-14, 10.3109/10520297409116929

View in Scopus Google Scholar

James and Davidson, 2012 S.W. James, S.K. Davidson

Molecular phylogeny of earthworms (Annelida: Crassiclitellata) based on 28S, 18S and 16S gene sequences

Invertebr. Syst., 26 (2012), pp. 213-229, 10.1071/IS11012

View in Scopus ↗ Google Scholar ↗

Jamieson, 2006 B.G.M. Jamieson

Non-leech Clitellata

G. Rouse, F. Pleijel (Eds.), Reproductive Biology and Phylogeny of Annelida, Science Publishers, Plymouth (2006), pp. 235-392

Google Scholar ↗

Jamieson, 1981 B.G.M. Jamieson

The ultrastructure of the Oligochaeta

Academic Press, London, New York, Toronto, Sydney, San Francisco (1981)

Google Scholar ↗

Jamieson, 1967 B.G.M. Jamieson

A taxonomic review of the African megadrile genus *Stuhlmannia* (Eudrilidae, Oligochaeta)

J. Zool., Lond., 152 (1967), pp. 79-126

CrossRef ↗ View in Scopus ↗ Google Scholar ↗

Jamieson et al., 2002 B.G.M. Jamieson, S. Tillier, A. Tillier, J.-L. Justine, E. Ling, S. James, K. McDonald, A.F. Hugall

Phylogeny of the Megascolecidae and Crassiclitellata (Annelida, Oligochaeta): combined versus partitioned analysis using nuclear (28S) and mitochondrial (12S, 16S) rDNA

Zoosystema, 24 (2002), pp. 707-734

Google Scholar ↗

Lakhani, 2014 L. Lakhani

Oogenesis in *Eudichogaster kinneari* (Oligochaeta-Annelida): histological and histochemical profile, 3 (2014), pp. 32-36

Lebo and McCall, 2021 D.P.V. Lebo, K. McCall

Murder on the ovarian express: a tale of non-autonomous cell death in the *Drosophila* ovary

Cells, 10 (2021), p. 1454, 10.3390/cells10061454 ↗

View in Scopus ↗ Google Scholar ↗

Lechenault, 1968 H. Lechenault

Etude cytochimique et uhrastructurale de l'ovocyte d'*Eisenia foetida* (Sav.)

Zeitschrift fiir Zellforsch, 112 (1968), pp. 96-112

View in Scopus ↗ Google Scholar ↗

Leutert, 1974 R. Leutert

Zur geschlechtsbestimmung und gametogenese von *Bonellia viridis* Rolando
Embryol. exp. Morph, 32 (1974), pp. 169-193
CrossRef View in Scopus Google Scholar

Liana and Witaliński, 2012 M. Liana, W. Witaliński
Female and male reproductive systems in the oribatid mite *Hermannia gibba* (Koch, 1839) (Oribatida: Desmonomata)
Int. J. Acarol, 38 (2012), pp. 648-663, 10.1080/01647954.2012.734333
View in Scopus Google Scholar

Lone et al., 2022 A.R. Lone, S.S. Thakur, P. Tiwari, S.W. James, S. Yadav
Phylogenetic relationships in earthworm *Megascolex* species (Oligochaeta: Megascolecidae) with addition of two new species
Diversity, 14 (2022), p. 1006, 10.3390/d14111006
View in Scopus Google Scholar

Lu et al., 2017 K. Lu, L. Jensen, L. Lei, Y.M. Yamashita
Stay connected: a germ cell strategy
Trends Genet, 33 (2017), pp. 971-978, 10.1016/J.TIG.2017.09.001
View PDF View article View in Scopus Google Scholar

Marotta and Ferraguti, 2009 R. Marotta, M. Ferraguti
Sperm ultrastructure in assesing phylogenetic relationships among clitellate annelids
D.H. Shain (Ed.), Annelids in Modern Biology (2009), pp. 314-327
CrossRef View in Scopus Google Scholar

Matova and Cooley, 2001 N. Matova, L. Cooley
Comparative aspects of animal oogenesis
Dev. Biol., 231 (2001), pp. 291-320, 10.1006/dbio.2000.0120
View PDF View article View in Scopus Google Scholar

Michaelsen, 1928 W. Michaelsen
Clitellata
W. Kükenthal, T. Krumbach (Eds.), Handbuch Der Zoologie Bd. 2, Lief. 8, De Gruyter, Berlin (1928), pp. 1-112
View in Scopus Google Scholar

Michaelsen, 1900 W. Michaelsen
Lieferung. Oligochaeta
J.W. Spengel (Ed.), Das Tierreich., Verlag von R. Friedländer und Sohn, Berlin (1900), pp. 1-575
Google Scholar

Milas and Telley, 2022 A. Milas, I.A. Telley

Polarity events in the *Drosophila melanogaster* oocyte

Front. Cell Dev. Biol., 10 (2022), Article 895876, 10.3389/fcell.2022.895876

View in Scopus Google Scholar

Minamiya et al., 2011 Y. Minamiya, H. Hayakawa, K. Ohga, S. Shimano, M.T. Ito, T. Fukuda

Variability of sexual organ possession rates and phylogenetic analyses of a parthenogenetic Japanese earthworm, *Amynthas vittatus* (Oligochaeta: Megascolecidae)

Genes Genet. Syst., 86 (2011), pp. 27-35, 10.1266/ggs.86.27

View in Scopus Google Scholar

Nashchekin et al., 2021 D. Nashchekin, L. Busby, M. Jakobs, I. Squires, D. Johnston St.

Symmetry breaking in the female germline cyst

Science, 374 (80) (2021), pp. 874-879, 10.1126/science.abj3125

View in Scopus Google Scholar

Niu and Spradling, 2022 W. Niu, A.C. Spradling

Mouse oocytes develop in cysts with the help of nurse cells

Cell, 185 (2022), pp. 1-15, 10.1016/j.cell.2022.05.001

Google Scholar

Olive, 1983 P.J.W. Olive

Annelida-Polychaeta

K. Adiyodi, R. Adiyodi (Eds.), Reproductive Biology of Invertebrates, Spermatogenesis and Sperm Function, Wiley, Chichester (1983), pp. 321-342

Google Scholar

Omodeo, 2000 P. Omodeo

Evolution and biogeography of megadriles (Annelida, Clitellata)

Ital. J. Zool, 67 (2000), pp. 179-201

CrossRef View in Scopus Google Scholar

Parthasarathi and Ranganathan, 2000 K. Parthasarathi, L.S. Ranganathan

Influence of pressmud on the development of the ovary, oogenesis and the neurosecretory cells of the earthworm *Eudrilus eugeniae* (Kinberg)

African Zool, 35 (2000), pp. 281-286

CrossRef View in Scopus Google Scholar

Pepling et al., 1999 M.E. Pepling, M. De Cuevas, A.C. Sprading

Germline cysts: A conserved phase of germ cell development?

Trends Cell Biol, 9 (1999), pp. 257-262, 10.1016/S0962-8924(99)01594-9

View PDF View article View in Scopus Google Scholar

Rouse, 2006 G.W. Rouse

Annelid sperm and spermiogenesis

G. Rouse, F. Pleijel (Eds.), Reproductive Biology and Phylogeny of Annelida, Science Publishers, Enfield (2006), pp. 45-76

Google Scholar

Seidel et al., 2018 H.S. Seidel, T.A. Smith, J.K. Evans, J.Q. Stamper, T.G. Mast, J. Kimble

C. elegans germ cells divide and differentiate in a folded tissue

Dev. Biol, 442 (2018), pp. 173-187, 10.1016/J.YDBIO.2018.07.013

View PDF View article View in Scopus Google Scholar

Siekierska, 2007 E. Siekierska

Changes in the ovary cells structure in the earthworm *Dendrobaena veneta* (Rosa) in neurosecretion deficiency and after exposure to cadmium

Eur. J. Soil Biol, 43 (2007), pp. 311-315, 10.1016/j.ejsobi.2007.08.034

Google Scholar

Siekierska, 2003 E. Siekierska

The structure of the ovary and oogenesis in the earthworm, *Dendrobaena veneta* (Annelida, Clitellata)

Tissue Cell, 35 (2003), pp. 252-259, 10.1016/S0040-8166(03)00038-7

View PDF View article View in Scopus Google Scholar

Siekierska and Urbańska-Jasik, 2002 E. Siekierska, D. Urbańska-Jasik

Cadmium effect on the ovarian structure in earthworm *Dendrobaena veneta* (Rosa)

Environ. Pollut., 120 (2002), pp. 289-297, 10.1016/S0269-7491(02)00152-5

View PDF View article View in Scopus Google Scholar

Sims, 1980 R.W. Sims

A classification and the distribution of earthworms, suborder Lumbricina (Haplotaxida: Oligochaeta)

Bull. Br. Museum (Natural Hist. Zool, 39 (1980), pp. 103-124

Google Scholar

Smith and Starborg, 2019 D. Smith, T. Starborg

Serial block face scanning electron microscopy in cell biology: Applications and technology

Tissue Cell, 57 (2019), pp. 111-122, 10.1016/j.tice.2018.08.011

View PDF View article View in Scopus Google Scholar

Spradling et al., 2022 A.C. Spradling, W. Niu, Q. Yin, M. Pathak, B. Maurya

Conservation of oocyte development in germline cysts from *Drosophila* to mouse
Elife (2022), p. 11, 10.7554/eLife.83230 ↗
Google Scholar ↗

Stephenson, 1930 J. Stephenson
The Oligochaeta
Clarendon Press, Oxford (1930)
Google Scholar ↗

Świątek et al., 2009 P. Świątek, J. Kubrakiewicz, J. Klag
Formation of germ-line cysts with a central cytoplasmic core is accompanied by specific orientation of mitotic spindles and partitioning of existing intercellular bridges
Cell Tissue Res, 337 (2009), pp. 137-148, 10.1007/s00441-009-0788-8 ↗
View in Scopus ↗ Google Scholar ↗

Świątek et al., 2023 P. Świątek, M. Novo, D.F. Marchan, Ł. Gajda, K. Małota, A.Z. Urbisz
Ovary micromorphology in hormogastrid earthworms with a particular emphasis on the organization of the germline cysts
Zoology, 158 (2023), Article 126081, 10.1016/j.zool.2023.126081 ↗
View PDF View article View in Scopus ↗ Google Scholar ↗

Świątek and Urbisz, 2019 P. Świątek, A.Z. Urbisz
Architecture and life history of female germ-line cysts in clitellate annelids
in: Results and Problems in Cell Differentiation (2019), pp. 515-551, 10.1007/978-3-030-23459-1_21 ↗
View in Scopus ↗ Google Scholar ↗

Timm and Martin, 2015 T. Timm, P.J. Martin
Clitellata: Oligochaeta
Thorp and Covich's Freshwater Invertebrates, Academic Press (2015), pp. 529-549, 10.1016/B978-0-12-385026-3.00021-8 ↗
View PDF View article View in Scopus ↗ Google Scholar ↗

Urbisz et al., 2017 A.Z. Urbisz, Ł. Chajec, A. Brąszewska-Zalewska, J. Kubrakiewicz, P. Świątek
Ovaries of the white worm (*Enchytraeus albidus*, Annelida, Clitellata) are composed of 16-celled meroistic germ-line cysts
Dev. Biol., 426 (2017), pp. 28-42, 10.1016/j.ydbio.2017.04.009 ↗
View PDF View article View in Scopus ↗ Google Scholar ↗

Urbisz et al., 2015 A.Z. Urbisz, Ł. Chajec, P. Świątek
The ovary of *Tubifex tubifex* (Clitellata, Naididae, Tubificinae) is composed of one, huge germ-line cyst that is enriched with cytoskeletal components

PLoS One, 10 (2015), Article e0126173, 10.1371/journal.pone.0126173

View in Scopus Google Scholar

Urbisz et al., 2021 A.Z. Urbisz, P. Martin, M. Lagnika, Ł. Chajec, P. Świątek

Microorganization of ovaries and oogenesis of *Haplotaxis* sp. (Clitellata: Haplotaxidae)

J. Morphol, 282 (2021), pp. 98-114, 10.1002/jmor.21285

View in Scopus Google Scholar

Cited by (0)

☆ Grant support: This work was financed by National Science Centre, Poland, contract number 2020/37/B/NZ4/00560