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Investigation of the reproductive system in calanoid copepods: A new approach using 3D reconstructions from serial semi-thin cross-sections in *Calanus glacialis* and *Metridia longa*

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ABSTRACT

The structure of the female reproductive system of the calanoid copepods *Calanus glacialis* and *Metridia longa* from the White Sea was studied using light microscopy, scanning and transmission electron microscopy, as well as confocal laser scanning microscopy. For the first time, we applied also the method of 3D reconstructions from semi-thin cross-sections to visualize the general plan of the reproductive system in both species. The application of a combination of methods provided novel and detailed information on the genital structures and muscles located in the genital double-somite (GDS) as well as structures used for the reception and storage of spermatozoa, fertilization and release of eggs. An unpaired ventral apodeme and associated muscles located in the GDS are described for the first time for calanoid copepods. The role of this structure in copepod reproduction is discussed. Stages of oogenesis and the mechanism of yolk formation in *M. longa* are studied using semi-thin sections for the first time. A combination of non-invasive (LM, CLSM, SEM) and invasive techniques (semi-thin sections and TEM) applied in this study substantially improves our understanding of the functioning of the genital structures in calanoid copepods and could be recommended as a standard set of methods for future research in the reproductive biology of copepods.

1. Introduction

Copepods are among the most abundant organisms on Earth playing an important role in the marine pelagic ecosystems (Humes, 1994). The free-living copepods from the order Calanoida dominate zooplankton almost everywhere in the world's oceans and provide a critical link between pelagic primary producers and organisms of the higher trophic levels (Turner, 2004). Population maintenance requires their successful reproduction based on specific behavioral mechanisms and morphological adaptations that increase the likelihood of meeting potential mates and the fertilization of oocytes.

The general plan of the female reproductive system is similar in all calanoid copepods. The reproductive system consists of an unpaired ovary, which is located dorsally in the first thoracic somite of the prosome (Blades-Eckelbarger and Youngbluth, 1984; Boxshall, 1992). Two pairs of diverticula originate from the ovary, with one pair directed towards the anterior end of the body (anterior diverticula) and the other towards the posterior end (posterior diverticula). The posterior diverticula, or oviducts, converge as they reach the first somite of the urosome, or so-called genital double-somite (GDS) formed by fusion of the last thoracic and first abdominal somites in female copepods (Boxshall, 1992). Here, they serve as egg-laying ducts and terminate in paired gonopores or gonoporal slits covered by gonoporal plates (Cuoc et al., 1997; Barthélémy et al., 1998a; Bradford-Grieve et al., 2010). Structures used for the reception and storage of spermatozoa, subsequent fertilization and release of eggs include the genital atrium – a cavity on the ventral side of GDS, covered with a cuticular fold (genital operculum) and connected to the environment via genital slit and paired spermathecae or seminal receptacles with seminal ducts that open near the gonopores (Boxshall, 1992; Barthélémy et al., 1998a). The genital field is an area on the ventral surface of the female GDS formed by the external genital structures such as genital operculum covering genital atrium, copulatory pores and gonopores (Boxshall, 1992). External and internal morphology of

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GDS in calanoids is highly variable and such characters are frequently used for taxonomic identification (Blades-Eckelbarger, 1991).

There are numerous studies on the morphology of the reproductive system in calanoid copepods, especially for the abundant species. However, these mainly involve light microscopy (LM) examination of whole mount preparations, and scanning electron microscopy (SEM) investigations of the GDS external morphology (Ohtsuka et al., 1994; Cuoc et al., 1997; Barthélémy et al., 1998a; Barthélémy, 1999; Bradford-Grieve et al., 2010). Studies of the internal structure of the reproductive system in calanoid copepods have also been performed thus far primarily based on light microscopy of histological sections and whole mount preparations (Raymont et al., 1974; Arnaud et al., 1982; Blades-Eckelbarger, 1986; Ianora et al., 1989; Niehoff and Hirche, 1996; Niehoff, 1998, 2003; Barthélémy et al., 1998b; Barthélémy, 1999; Ceballos-Vazquez et al., 2009) with only a few TEM studies (Norrbin, 2001).

The large arctic copepod *Calanus glacialis* Jaschnov, 1955, is one of the best studied calanoids in terms of its gross gonad morphology. Yet even for this well-known species and genus, most studies have been performed with light microscopy (Raymont et al., 1974; Tande and Hopkins, 1981; Hirche and Niehoff, 1996; Kosobokova, 1998, 1999; Niehoff, 1998, 2003). Several studies described stages of gonad development and seasonal patterns of gonad maturation in *Calanus* based on stereo microscope examination of gonads of CV and female specimens stained with 2% borax carmine solution (Tande and Hopkins, 1981; Kosobokova, 1998, 1999; Kosobokova and Hirche, 2001; Hatlebakk et al., 2022). Such histological examination has provided detailed information on the oogenesis and morphological changes of gonads of *C. glacialis* during maturation and spawning (Eckelbarger and Blades-Eckelbarger, 2005; Niehoff, 1998, 2007).

In contrast, for another common large-bodied arctic calanoid, *Metridia longa* Lubbock, 1854, only the gross gonad morphology and some features of the reproductive biology have been described (Tande and Grønvik, 1983; Cuoc et al., 1997; Ershova and Kosobokova, 2012). The morphological study of the reproductive system of *M. longa* from the White Sea showed asymmetry in the process of fertilization associated with the presence of two male morphotypes in the population with differing "handedness" (Ershova and Kosobokova, 2012) that suggested an explanation for low fertilization rates and high percentage of non-viable eggs in clutches of this *Metridia*. Such an asymmetry was also reported for several other *Metridia* species from various geographical localities (Andronov and Grudina, 2007; Arima et al., 2015). The position and morphology of the internal genital structures such as spermathecae, gonopores and copulatory pores, as well as the associated muscle bundles, have been studied for two *Metridia* species (*Metridia princeps* and *Metridia lucens*) using SEM (Cuoc et al., 1997), but remained unknown for *M. longa*. Details of oogenesis and patterns of gonad maturation in *Metridia* spp. have not been investigated until now.

In the present paper a set of methods, including light microscopy (LM), scanning and transmission electron microscopy (SEM and TEM), and confocal laser scanning microscopy (CLSM) was applied to study the female reproductive system and oogenesis in *C. glacialis* and *M. longa* from the sub-arctic White Sea. For the first time, the method of 3D reconstruction using semi-thin cross-sections was applied for visualization of the copepod reproductive system general morphology. This method has been recently used for anatomical investigations in various invertebrates (Brenzinger et al., 2010; Boone et al., 2011; Weber et al., 2014; Song et al., 2020; Temereva et al., 2021), as it is found helpful to visualize the relative position of the internal structures. The study of these two prominent Arctic species will help us understand the process of oogenesis and functioning of the genital structures during insemination, fertilization and egg-laying across a broad range of calanoid copepods.

2. Material and methods

2.1. Sampling

Zooplankton samples were collected with a Juday net (mouth opening 0.1 m^2, mesh size 180 μm) in the vicinity of the N.A. Pertsov White Sea Biological Station of the Moscow State University (WSBS MSU) in Velikaya Salma Strait, Kandalaksha Bay (66°51′ N, 33°35′ E), the White Sea, from 0 to 100 m depth layer in September 2019.

2.2. Sorting and fixation of the material

Live zooplankton samples were sorted under the Olympus SZX-9 stereo microscope immediately after collection. Several specimens of adult females of each species were fixed for further morphological examination in 4% formalin (1), 70% ethanol (2), 96% ethanol (3), or 2,5% glutaraldehyde on phosphate-buffered saline (PBS) (4).

2.3. Light microscopy (LM)

The general morphology of the reproductive system was studied using light microscopy. Specimens fixed with 4% formalin were rinsed in distilled water, stained with 2% borax carmine, following Tande and Hopkins (1981), then transferred into glycerin and mounted onto slides. Specimens preserved with 70% ethanol were transferred to lactic acid and mounted onto slides. Whole mount preparations were studied and photographed with a Leica DM5000 microscope to examine ovaries and genital duct structure (Kosobokova, 1999). In addition, the degree of spermatecae fullness was examined.

2.4. Scanning electron microscopy (SEM)

For SEM examination, several individuals of each species fixed with 96% ethanol were washed in distilled water with a drop of liquid detergent. The urosome of each specimen was dissected prior to being dehydrated in a graded ethanol and acetone series according to standard protocol (Hopkins, 1978). Material was critical-point dried, then transferred to stubs with the ventral surface upwards to be sputter-coated with a platinum/palladium mix. The samples were studied at the MSU Center for Collective Use on a scanning electron microscope JEOL JEM-2000 FXII.

2.5. Confocal laser scanning microscopy (CLSM)

For CLSM, specimens fixed with 4% formalin or 96% ethanol were stained with red Congo solution for 6–8 h at room temperature (Kamanli et al., 2017). After staining, material was rinsed in liquid soap solution, washed in distilled water and mounted onto slides using glycerine as mounting medium. The specimens were studied with a Nikon A1 confocal microscope using 561 nm and 640 nm lasers. Images were processed using ImageJ software to obtain maximum intensity Z-projections.

2.6. Transmission electron microscopy (TEM)

For TEM examination, individuals were fixed with 2.5% glutaraldehyde in 0.1 M PBS for 48 h at 4 °C. The material was postfixed in 1% OsO4 for 1 h, washed with 0.1 M PBS and dehydrated in a graded ethanol and acetone series. Specimens were embedded in Araldite

resin according to protocol (Bowlby and Case, 1991; Petrunina et al., 2018). Ultra-thin 60 nm sections were obtained using a diamond knife on a Leica EM UC6 ultra microtome. Sections were stained with aqueous uranyl acetate (45 min at 37 °C) and lead citrate (10 min at room temperature). Material was examined and photographed at the MSU Center for Collective Use on the JEOL JEM-1011 transmission electron microscope.

2.7. 3D reconstructions from serial semi-thin cross-sections

Serial transverse semi-thin sections (1.5 μm) of the specimens embedded into resin using the TEM protocol (see above) were obtained with a LKB V 2088 Ultra microtome using glass knives. Sections were mounted on glass slides to be stained with a mixture of toluidine blue and azure II, and then photographed with an Olympus VS-120-S6 Slide scanner microscope at magnification of x40 (*C. glacialis*) and x60 (*M. longa*). Every fourth (*C. glacialis*) and third (*M. longa*) section was photographed. The series of images were aligned with Amira version 5.2.2 software (Thermo Fisher Scientific, MA). Four stacks were prepared and used for 3D reconstructions using Imaris version 7.1.1 software (Thermo Fisher Scientific, MA). Images were processed with Adobe Photoshop CS3 (Adobe Systems, San Jose, CA).

3. Results

3.1. Morphology of the female reproductive system in Calanus glacialis

3.1.1. 3D reconstructions of the genital system of C. glacialis from serial semi-thin cross-sections

The ovary (ov) of female *C. glacialis* has an elongated shape and is located in the middle of the prosome dorsally to the midgut (g) (Fig. 1). Two pairs of diverticula originate from the anterior end of the ovary. The anterior diverticula (ad) run closely together; they extend to the head along the dorsal side (Fig. 1, B). There is usually a massive lipid sac (ls) between the anterior diverticula and the intestine (Fig. 1, B, C). The posterior ovarian diverticula (oviducts, pd) run along the lateral sides of the body to the urosome (Fig. 1, C, D). At the border with the genital double-somite (GDS) they continue into the egg-laying ducts (eld) that merge just above the genital atrium, the site of fertilization of oocytes (Fig. 1, D, Fig. 2, E). Paired spermathecae (sp) are also associated with the genital atrium (ga) and occupy a central position in the GDS, being located ventral to the hindgut (Fig. 1, A).

3.1.2. Morphology of the genital double-somite of C. glacialis

The genital operculum (go) in *C. glacialis* is smooth and elongated, longer than wide (Fig. 2, A). An atrial slit, an entrance to the genital atrium, is crescent shaped (Fig. 2, G). A pair of muscle bands (m1) runs from the inner side of the cuticle of the operculum to the anterior edge of the GDS (Fig. 2, E) that are used to lift the operculum thus widening the atrial slit. The m1 muscles are shown here for *C. glacialis* for the first time. Two pear-shaped spermathecae are located inside the middle part of the GDS, adjacent to its ventral wall (Fig. 1, A, Fig. 2, E). Their elongated proximal margins reach the genital atrium (Fig. 2, B, C, D). The spermathecae are connected to the atrium through the copulatory pores and with each other via the genital atrium (ga) (Fig. 2, C). As a result of the discharge of the spermatophore into the genital atrium, spermatozoa are distributed between the two spermathecae (Fig. 2, A–B). Transverse sections of the studied *C. glacialis* specimens showed either both spermathecae full (Fig. 2, A, B) or both empty (Fig. 2, F).

At the bottom of the genital atrium, paired gonoporal plates (gpl) (Fig. 2, D) can be seen closing the openings of the oviducts (od) and forming gonoporal slits (gs) through which mature oocytes enter the genital atrium prior fertilization (Fig. 2, C). Paired bundles of dilator muscles (m2) run from the anterior part of GDS to the gonoporal plates (gpl) (Fig. 2, D, E) enabling the opening of the gonoporal slits.

Near the anterior margin of the GDS, an unpaired 15 μm wide depression of the cuticle is observed that continues into internal medial cuticular fold - a ventral apodeme (va) (Fig. 2, G, H). Bundles of transverse striated musculature (m3) running from the ventrolateral walls of the genital somite are attached on both sides of this structure (Fig. 2, I). A ventral apodeme and associated musculature are described here for the first time for *C. glacialis*.

3.2. Morphology of the female reproductive system in Metridia longa

3.2.1. 3D reconstructions of the genital system of M. longa from serial semi-thin cross-sections

The ovary (ov) of mature female *M. longa* is located dorsally at the level of the second and third thoracomeres, the somites bearing the first two pairs of swimming legs (P1, 2) (Fig. 3, A). A pair of wide anterior diverticula (ad) (Fig. 3, A) originates at the anterior end of

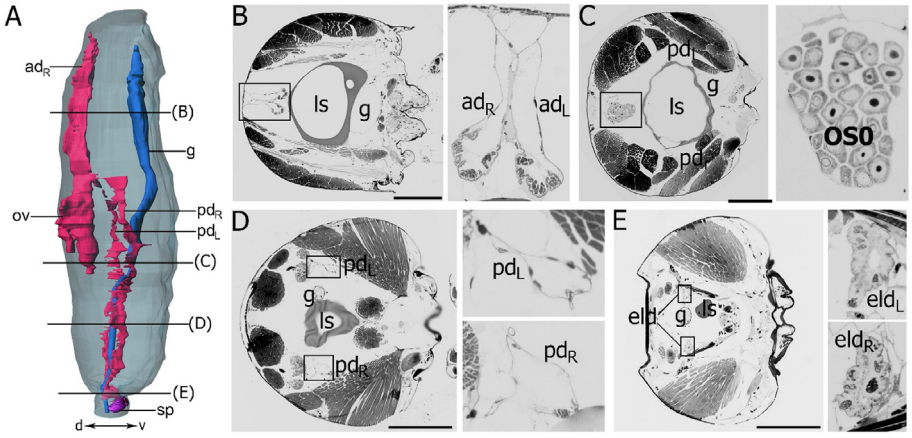

Fig. 1. Anatomy of the female reproductive system of *C. glacialis*. A - 3D reconstruction from serial semi-thin cross-sections, lateral view from the right side; B-E - transverse cross-sections of the body at different levels. Enlarged details of the reproductive system are shown separately to the right of the corresponding section. Abbreviations: d, dorsal side of the body; v, ventral side of the body; ad, anterior diverticulum (ad_L, left anterior diverticulum, ad_R, right anterior diverticulum); eld, egg-laying ducts (eld_L, left egg-laying duct, eld_R, right egg-laying duct); g, gut; ls, lipid storage; pd, posterior diverticula (pd_L, left anterior diverticulum, pd_R, right anterior diverticulum); OS0, previtellogenic oocytes in the ovary. Scale bar = 300 μ.

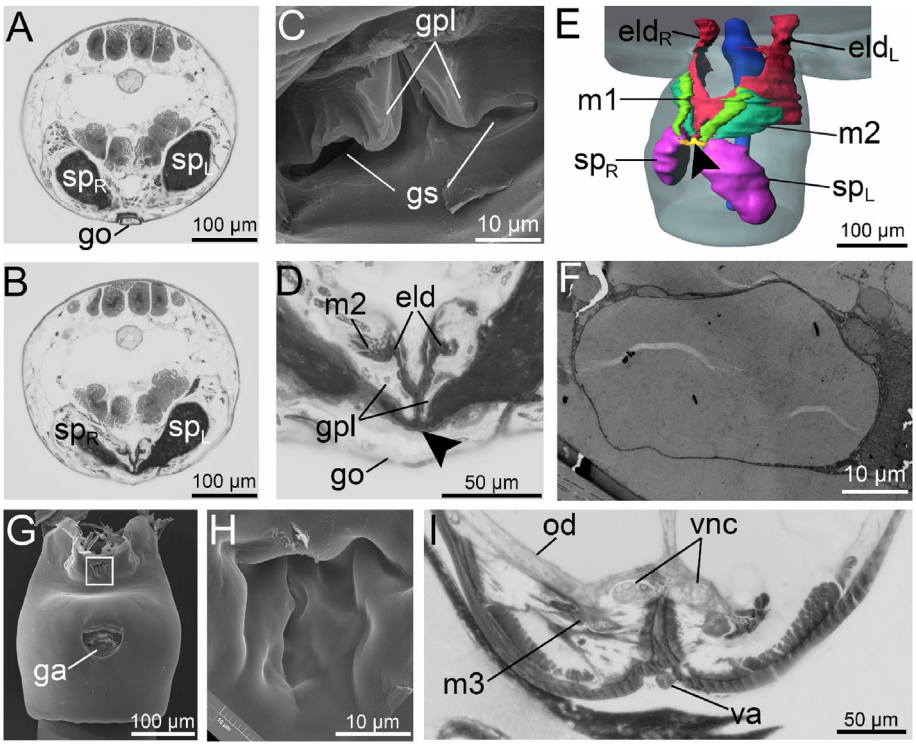

Fig. 2. Morphology of the genital double somite (GDS) of *C. glacialis*. A - transverse cross-section through the genital operculum, LM; B - transverse cross-section through the genital atrium, LM; C - open genital atrium with exposed gonoporal plates, ventral view, SEM; D - transverse cross-section through the genital atrium (black arrow), LM; E - 3D reconstruction of the GDS; F - transverse cross-section through empty spermatheca, TEM; G - genital double somite, ventral view, with invagination of cuticle forming an apodeme SEM; H - invagination of cuticle on the ventral side of the GDS, SEM; I - transverse cross-section through the apodeme, LM. Abbreviations: eld, egg-laying ducts (eld_L, left egg-laying duct, eld_R, right egg-laying duct); ga, genital atrium; go, genital operculum; gpl, gonoporal plate; gs, gonoporal slit; m1, muscle of the genital operculum; m2, muscle of the egg-laying ducts; m3, muscles of the apodeme; od, oviducts; sp, spermatheca (sp_L, left spermatheca, sp_R, right spermatheca); va, ventral apodeme; vnc, ventral nerve cord.

the ovary. Two posterior diverticula (pd) run from the middle part of the ovary extending along the intestine (g) to the posterior end of the prosome (Fig. 3, A) where they form thin oviducts (Fig. 3, A, I). Paired spermathecae (sp) are located inside the GDS ventrally to the intestine (Fig. 3, A).

3.2.2. *Oogenesis in M. longa*

The obtained series of semi-thin sections of a mature *M. longa* female demonstrates the presence of oocytes at different stages of development in the ovary, the diverticula and the oviducts (Figs. 3 and 4). Stages of oogenesis in *M. longa* are described, following the terminology of Niehoff (2007).

- OS0: oogonia and previtellogenic oocytes at early stages of meiosis, located in the posterior part of the ovary (Fig. 3, F, Fig. 4, A);
- OS1: previtellogenic oocytes located in the middle and anterior parts of the ovary, in diverticula; with large nucleus occupying almost entire cell volume, dark-colored yolk granules concentrated around the nucleus (Fig. 3, D, Fig. 4, B, C);
- OS2: early vitellogenic oocytes, with cytoplasm exceeding the nucleus by volume, yolk granules evenly distributed in the ooplasm (Fig. 3, D, G, Fig. 4, D);
- OS3: late vitellogenic oocytes. Larger than OS2, with smaller nuclear-cytoplasmic ratio containing a lot of yolk (Fig. 3, D, G, Fig. 4E, F);
- OS4: mature oocytes of elongated form, the ooplasm filled with yolk and lipid droplets; at this stage the oocytes are ready to be fertilized.

In the *M. longa* female specimens examined, the first four oocytes developmental stages were observed (OS0 to OS3) (Fig. 4). The OS2 and OS3 were found along the entire length of the anterior diverticula (Fig. 3, D, G), with the more mature OS3 cells occupying central and ventral positions relative to OS2. The stage OS4, according to Niehoff (2007) (mature oocytes ready to be fertilized), were not observed in our material. These stages of oogenesis are described for *M. longa* for the first time.

3.2.3. *Morphology of the genital double-somite of M. longa*

The paired bilaterally symmetrical organization of the genital structures in the GDS, in which the left and right parts are not connected to each other, is characteristic for females of *M. longa* (see also Ershova and Kosobokova, 2012). There are paired spermathecae (sp), which are located in the anterior half of the GDS and open with copulatory pores lateroventrally (Fig. 5, A, B). The genital atrium is absent.

The oviducts (od) continue into egg-laying ducts (eld), which do not merge, and each has a separate opening (Fig. 5, A, B, F, G). Paired seminal canals (sd) originating from each spermatheca run towards the anterior edge of the GDS (Fig. 5, A, B, F). The opening of each seminal canal is located close to the related gonopore. These areas are covered by cuticular gonoporal plates (gpl) (Fig. 5, B), to which the dilator muscles (m2) are attached (Fig. 5, A, B, G).

In the anterior part of the GDS, slightly above the gonoporal plates, a 25 μm wide ventral cuticular depression forming an internal apodeme (va) was observed in *M. longa* (Fig. 5, B, white arrow, H, I) similar to that in *C. glacialis*. Two bundles of the transverse striated musculature (m3) run from the apodeme to the ventrolateral wall of the GDS (Fig. 5, I, yellow arrow). A ventral apodeme

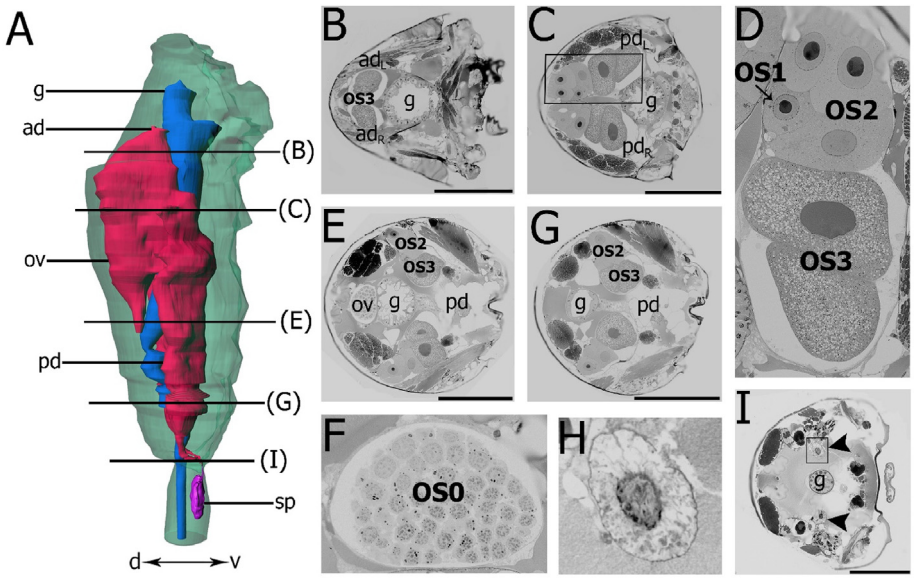

Fig. 3. Anatomy of the reproductive system of the female *M. longa*. A - 3D reconstruction from serial semi-thin cross-sections, view from the right side; B, C, E, G, I - transverse cross-sections at different levels of the body. D - cross-section through the posterior diverticulum (corresponds to the rectangular area in C); F - transverse cross-section through the ovary; H - transverse cross-section through the oviduct (corresponds to the rectangular area in I). Abbreviations: d, dorsal side of the body; v, ventral side of the body; ad, anterior diverticula; g, gut; pd, posterior diverticula (pd_L, left anterior diverticulum, pd_R, right anterior diverticulum); ov, ovary; OS0, OS2, OS3 oocytes developmental stages; black arrowheads in I point to the oviducts. Scale bar = 200μ.

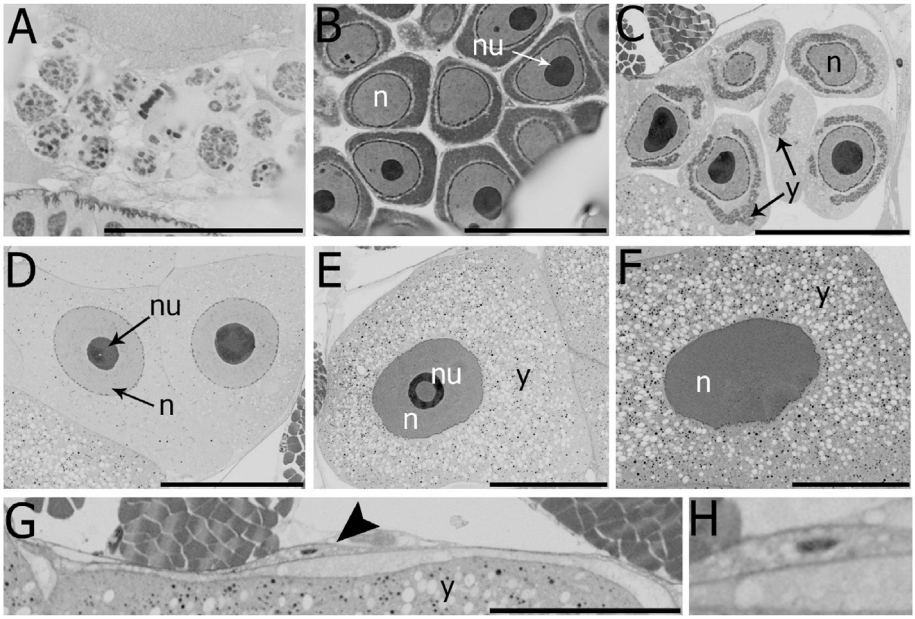

Fig. 4. Stages of the oogenesis in *M. longa*, transverse cross-sections at different levels of the body, LM. A - OS0 previtellogenic oocytes in the posterior part of the ovary; B - young OS1 in the middle part of the ovary; C - late OS1 at the base of diverticula; D, E, F - cross-sections through the posterior diverticula; D OS2, E -young OS3; F- late OS3; G wall of the posterior diverticulum with the follicular cell (black arrow); H - follicular cell. Abbreviations: n, nucleus; nu, nucleolus; y, yolk. Scale bar = 50μ.

and associated musculature are described here for the first time for *M. longa*.

4. Discussion

4.1. General plan of the reproductive system, oogenesis and reproductive status of C. glacialis and M. longa females

The females of the two species studied have similar plans of the reproductive system, classified by Niehoff (2007) as *Calanus*-type gonad. This type is characterized by a dorsally located pear-shaped ovary narrowing towards its anterior end and a dorso-ventral direction of the oocyte maturation. More mature oocytes in the *Calanus*-type gonads could be found within diverticula ventrally compared to less mature ones. The ovary and diverticula contain oocytes at almost all developmental stages simultaneously, and the oocytes of the same stage mature synchronously (Niehoff, 2007).

During our study in the White Sea (early September), the

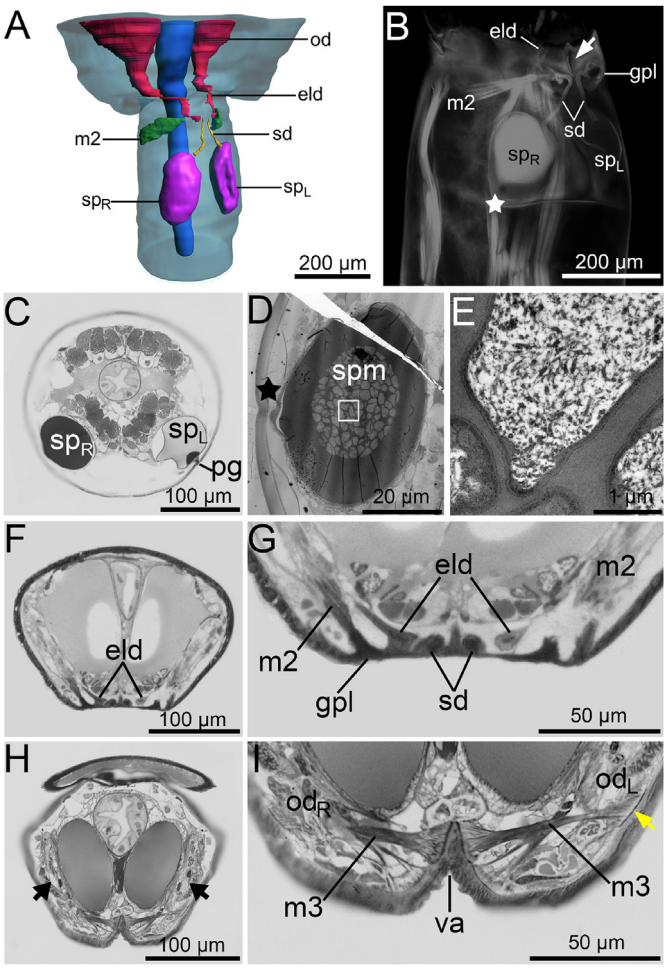

Fig. 5. Morphology of the genital double somite of *M. longa*. A - 3D reconstruction from serial semi-thin cross-sections, B - lateroventral view of the genital double somite, CLSM (copulatory pore indicated with a white asterisk); C - at the level of copulatory pores, LM; D - at the level of the inseminated spermatheca near the copulatory pore (black asterisk), TEM; E - spermatophore mass inside spermatheca, TEM (corresponds to the rectangular area in D); F, G - at the level of the gonoporal plates, LM; H, I - at the level of the ventral apodeme and oviducts (black arrows), LM. Abbreviations: eld, egg-laying ducts; gpl, gonoporal plate; m2, muscle of the egg-laying ducts; m3, muscles of the apodeme attached to the ventrolateral wall of the GDS (yellow arrow); od, oviducts (od_L, left oviduct, od_R, right oviduct); pg spermatophoral plug closing the copulatory pore; sd, seminal ducts; sp, spermatheca (sp_R, right spermatheca, sp_L, left spermatheca); spm, spermatophore mass; va, ventral apodeme.

females of both species were at different stages of gonad maturation which was indicated by the different gonad structure and the state of oocyte development. The *C. glacialis* females had empty diverticula and their ovary with a small number of germ cells at early stage of maturation (Fig. 1), which agrees well with the seasonal pattern of *C. glacialis* gonad development described for the White Sea (Kosobokova, 1999). In this sea, the spawning of *C. glacialis* starts before the ice break in April, and lasts until early July (Kosobokova, 1999; Kosobokova and Pertsova, 2005). Spawning terminates when temperature of the surface water rises to +5 °C forcing females to migrate to deeper, colder water layers (Kosobokova, 1998, 1999; Niehoff and Hirche, 2005), where degradation of the non-released genital products in the ovaries takes place (Kosobokova, 1999). The gonadal stage of the female used for 3D reconstruction in our study indicates that this specimen had already completed spawning and the status of its reproductive system corresponded to a "spent female" (Kosobokova, 1999).

Metridia longa is reported to spawn with varying intensity throughout almost the entire year in different geographical locations (Pertsova, 1974; Tande and Grønvik, 1983; Grønvik and Hopkins, 1984; Kosobokova and Hopcroft, 2010). Here we described stages of oogenesis and the mechanism of yolk formation in *M. longa* for the first time. According to our LM results, the yolk in *M. longa* is formed exogenously at the expense of follicular cells transferring nutrients to oocytes from hemolymph, similar to that in *C. glacialis* (Fig. 4, G, H). In September mature female individuals of *M. longa* were present in the population, with oocytes at four (OS0-OS3) out of five developmental stages, except for the very last stage (OS4) when the eggs are ready to be released (Figs. 3 and 4). The state of oocyte development observed in *M. longa* indicates the onset or ongoing reproduction during our study period.

4.2. Morphology of the genital double-somite

Three types of organization of the GDS have been recognized in calanoid copepods depending on the presence/absence of genital atrium and spermathecae (Bradford-Grieve et al., 2010). The genital atrium, a cavity on the ventral surface covered by the genital operculum is an important part of the reproductive system in copepods because it serves as sperm storage or as a place where fertilization takes place. During copulation, a male attaches a spermatophore under or near the genital operculum, so that the spermatophore content is released into the genital atrium to be transferred to spermathecae for storage. Genital atrium can have different shapes and modifications. According to Bradford-Grieve et al. (2010), the three types of organization of the GDS are as follows:

Type 1: Both genital atrium and spermathecae present;

Type 2: Spermathecae present, genital atrium absent; gonopores open directly to the exterior;

Type 3: Genital atrium present and spermathecae absent.

According to our results, type 1 is characteristic of *C. glacialis* and type 2 is characteristic of *M. longa*.

In females of *C. glacialis*, the spermatophoral mass discharges into the atrium and is subsequently transferred to the paired pear-shaped spermathecae, located in the middle of the GDS (Fig. 2). Their proximal edges lie closely to the genital atrium, so that seminal canals cannot be distinguished on the sections with LM, because the walls of the spermathecae merge with the cuticle of the somite forming the genital atrium. The spermathecae are connected with the atrium via paired copulatory openings that would open to let spermatozoa enter the spermathecae after insemination (discharge of the spermatophore into the atrium). Thus, either two filled spermathecae or two empty ones can be observed in adult females of *C. glacialis*. In one of the specimens examined in this study, both spermathecae were empty (Fig. 2, F). Both the gonadal state ("spent female", see above) and empty spermathecae suggest that at the time of our sampling the female had already finished spawning.

In the *M. longa* female examined, only one, the right spermatheca was filled with heterogeneous spermatophore mass. Transverse sections of the GDS at the level of the copulatory pores show that spermatozoa lie loose in the central part of this spermatheca (Fig. 5, D, E). However, most of the space at the periphery is occupied by seminal fluid, which is also discharged from the spermatophore during insemination. From the cross-sections through the spermathecae, it cannot be assessed exactly when insemination took place and whether spermatozoa from the right spermatheca were used for eggs' fertilization. However, the seminal canal originating from the right spermatheca was brightly stained all along its entire length, and therefore it can be inferred that the spermatophoral mass was moving from the spermatheca toward the eggs

fertilization site.

The left spermatheca on the sections, on the contrary, was empty, and the left copulatory pore was closed with a plug (pg) (Fig. 5, C). It has been shown previously for other *Metridia* species that seminal fluid from the spermatophore forms a plug that closes the copulatory pores of inseminated spermatheca (Cuoc et al., 1997). The left seminal canal was also empty, but its distal part was brightly stained in sections suggesting the presence of remnants of seminal fluid there (Fig. 5, F). Based on this observation and presence of a plug, we assume that the left spermatheca in the examined specimen had been inseminated earlier and the seminal content had been used by this female during a previous spawning.

The CLSM and TEM results obtained for female *M. longa* confirm the cuticular nature of the spermathecae walls, seminal canals and the distal parts of the oviducts (Fig. 5, B, D). Gonoporal plates found both in *C. glacialis* and *M. longa* also contain chitin and are well visualized even without the use of dyes due to autofluorescence (Fig. 5, B).

4.3. Functional role of muscles associated with genital structures

Musculature is an important structural component of the calanoid reproductive system. Depending on their position, the muscle bundles perform various functions to ensure fertilization and release of eggs.

The release of eggs from the oviducts through the gonopores is related to the widening of the gonoporal slit due to contraction of paired m2 muscular bundles. The arrangement of m2 muscles varies slightly between the species studied here. In *C. glacialis*, these muscles are attached closer to the anterior edge of the GDS, while in *M. longa* they are attached to the lateral walls of the somite, running parallel to its anterior margin.

However, the position of these muscles is generally similar to that previously reported for the family Metridinidae by Cuoc et al. (1997). According to the authors, when the dilators are relaxed, the gonoporal plates return to their closed state due to resilience of the cuticular walls of the ducts. The fertilization in *C. glacialis* takes place in the genital atrium where eggs become in contact with spermatozoa released from the attached spermatophore. The spermatophoral mass enters the genital atrium through the atrial slit. The widening of the atrial slit occurs when the genital operculum is lifted due to the contraction of paired muscles, running from the operculum to the anterior edge of the GDS ventrally (m1). The presence of these paired muscular bundles in *C. glacialis* is shown here for the first time.

4.4. Ventral apodeme in GDS of C. glacialis and M. longa

In both *C. glacialis* and *M. longa,* an unpaired ventral depression leading to an internal cuticular fold and located in the anterior part of the GDS, at the border of prosome and urosome - a so called ventral apodeme - was observed for the first time (Figs. 2 and 5, B, G). Paired muscles (m3) were found to be associated with this apodeme, which run to ventrolateral walls of the GDS. Such a structure is observed for the first time in calanoid copepods; information on its origin and function is lacking in the literature. We propose that when the muscles attached to the apodeme contract, the anterior part of the GDS narrows creating pressure to move the eggs inside the oviducts and eventually push them out. Thus, the release of eggs could be facilitated by the contraction of paired muscular bundles m2 and m3 located in the GDS.

4.5. Functional morphology of the internal genital structures of GDS in C. glacialis and M. longa

When the oocytes mature to the OS4 stage in *C. glacialis* and *M. longa*, they move along the oviducts toward the genital somite. The fertilization occurs when oocytes emerge from the gonopores, e.g. in *C. glacialis* when mature oocytes pass through the genital atrium.

In *M. longa* females lacking genital atrium, eggs are fertilized near each gonopore by spermatozoa coming from the corresponding spermatheca. If the spermatheca is empty, the eggs emerging from the respective gonopore will be unfertilized and thus will be non-viable as has been observed in laboratory experiments on eggs viability (Ershova and Kosobokova, 2012; Hopcroft et al., 2005). The release of non-viable eggs was also observed in laboratory experiments in other calanoids (Ianora et al., 1989), presumably resulting from unsuccessful fertilization.

The oviducts of *C. glacialis* open into the genital atrium, so the chances of fertilization of eggs from both of them should be higher in this species compared to *M. longa*, where fertilization occurs outside the GDS. Interestingly, the opening of the gonoporal plates is synchronized by the contraction of both muscle bands, and thus eggs of *M. longa* leave the oviducts simultaneously, regardless of whether they are to be fertilized or not (see also Cuoc et al., 1997). The presence of paired gonopores is a typical feature of calanoid copepod, an arrangement of advantage over unpaired organization with terminal fusion of the oviducts and/or seminal ducts (Cuoc et al., 1997). In the latter case, the oocytes would have to be released one after another, which would double the egg-laying time and, consequently, the associated energy costs.

Morphological differences in the organization of the reproductive system have undoubtedly affected the population structure and reproductive strategies of the copepods we studied, the sex ratio in the populations, mating patterns, success of fertilization and eggs viability. This link between reproductive system morphology and population ecology has been investigated in a number of other species of calanoid copepods (Barthélémy et al., 1998a; Ohtsuka and Huys, 2001; Kiørboe, 2006; Niehoff, 2007). However, a discussion of the ecological aspects of the presented results will be the subject for our future research.

4.6. Methodological aspects of the study of the structure of the reproductive system in the calanoid copepods

The methods applied in this study were each found useful for investigation of morphological characters important for reproduction of calanoids. They can be roughly divided into invasive, which involve partial destruction of the specimens, and non-invasive. The latter include light microscopy (LM) of whole mounts, confocal laser scanning microscopy (CLSM), and scanning electron microscopy (SEM). Preparation of specimens for these non-invasive methods is relatively simple and quick. So called invasive techniques such as LM of semi-thin sections and transmission electron microscopy (TEM) are more time consuming and involve special fixation, complex embedding procedures and ultratome sectioning. However, as a combination of methods applied together and in a particular sequence, all these approaches significantly deepen our understanding of the internal structure and functioning of the reproductive system in calanoids. Here we would like to describe advantages and disadvantages of each method and provide the optimal sequence in which they should be applied to obtain enough data while reducing preparation time and the number of specimens required.

First examination of the specimen could be done using **light microscopy of the whole mount preparations** stained with borax

carmine or other dyes. In large calanoids this method is optimal to determine the type and describe the general plan of the reproductive system, as well as to identify the reproductive status of the female. The same specimen could be further studied with **confocal laser scanning microscopy,** making it possible to visualize both internal and external structures and obtain more precise information on structures hardly visible with LM of whole mounts. CLSM could be used for more detailed examination of the muscle bundles and their attachment sites, combined with general view of the exterior cuticular structures. In large species, such as *C. glacialis* and *M. longa*, CLSM could be used for external morphology examination instead of SEM. However, the small body size of individuals could be a limitation for CLSM. After examination with CLSM, the same specimen could be processed for **Scanning electron microscopy** that allows for a highly detailed study of the external morphology of the genital field. Specific shape and position of gonopores and copulatory pores, morphology of genital operculum, structure of the attached spermatophores could all be described with high precision when examined with SEM. There is no size limitation for this method and particularly in small copepods essential resolution and detail could be achieved only with SEM.

Properly fixed specimens could be used to prepare **semi-thin sections that would be studied under the LM.** This gives enough resolution to observe even fine anatomical characters, such as seminal canals and distal parts of oviducts, as well as to distinguish places of muscle attachment. Some details of the oogenesis and vitellogenesis could be studied based on semi-thin sections. Photographs of serially cut sections could be aligned and used for **three-dimensional (3D) reconstructions** of internal structures. As a result, a volume image is obtained that facilitates analysis of the morphological data. This method is invaluable for proper visualization of the general plan of the reproductive system, but also provides accurate data on fine morphological details. In this study, the 3D reconstructions of the reproductive system of calanoid copepods are presented for the first time.

Sufficient resolution at the ultrastructural level could only be achieved with the help of **transmission electron microscopy**. Such details as some intracellular processes during oogenesis and vitellogenesis, structure of oviduct walls and spermatophore content inside spermathecae could only be effectively studied using TEM. Semi-thin sections of the same specimen could be processed for TEM examination, thereby providing higher resolution pictures of exactly the same structures.

To summarize, the following combination and sequence of techniques could be recommended for detailed investigation of the calanoid copepods reproductive system: LM of the whole mounts stained with borax carmine followed by CLSM observation of the GDS and further SEM of the genital area. Additional properly-fixed specimens should be processed for 3D reconstructions from semi-thin sections, some semi-thin sections should be further prepared for TEM investigation. Such an approach requires a minimal number of specimens and enables obtaining the maximum amount of the highly detailed information.

4.7. Conclusions

Investigations of the gross gonad morphology, morphology of genital structures involved in the reception and storage of spermatozoa, subsequent fertilization and release of eggs, as well as oocyte development, are essential for our understanding of the reproductive biology and reproductive strategies in calanoid copepods. A combination of non-invasive and invasive techniques applied here allowed for the first time to present a highly detailed study of the anatomy of the reproductive system including 3D reconstructions based on series of semi-thin sections in females of *C. glacialis* and *M. longa*. We propose application of this standardized set of morphological methods for future research in the field.

Author contribution statement

Daria A. Yurikova: Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. Ksenia N. Kosobokova: Conceptualization, Validation, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Supervision, Funding acquisition. Alexandra S. Savchenko: Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Visualization, Funding acquisition. DY sampled and processed the material, prepared semi-thin sections, 3D reconstructions, and figures, and wrote the manuscript. KK designed the study and contributed to the writing, editing and revision of the manuscript. AS helped to interpret the results and contributed to the writing, editing and revision of the manuscript.

Data availability statement

The data sets analyzed within this study are available from the authors upon request.

Ethics statement

The field sampling did not involve endangered or protected species. The use of copepods in the laboratory does not raise any ethical issues.

Declaration of competing interest

The authors declare that they have no competing interests.

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