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REGULAR ARTICLE

Spermatogenesis and lobular cyst type of testes organization in marine gastropod *Littorina saxatilis* (Olivi 1792)

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Abstract

Although individual stages of spermatogenesis in *Littorina saxatilis* are well studied at the electron microscopic level, the gonad structure and the spatial localization of gametes at different stages of maturation remain unclear. Using differential-interference contrast (DIC) for observations of fresh tissue we show that the mature testis consists of numerous ovoid lobules forming larger lobes. The lobules of intact mature testes of *L. saxatilis* are filled with randomly arranged multicellular cysts containing gametes at different stages of maturation. Gametes within a cyst are highly synchronized in respect of the differentiation degree. At the same time, no spatial gradient in the arrangement of cysts according to the maturation degree of gametes in them was observed in any of the studied lobules. The male gonads contain cysts with early spermatids, mid, late spermatids, and spermatozoa. Using silver-staining, DAPI, and chromomycin A3 (CMA3) staining, we identify 20 main types of nucleus organization in differentiating sperm. Premature and mature male gonads contain cysts with a mosaic arrangement as well as rare solitary cyst cells, goniablast cysts, or separate spermatogonia in between them. Our data indicate that the testis structure in *L. saxatilis* cannot be attributed to the tubular type, as previously thought. It corresponds to the lobular cyst type but individual lobules contain cysts with gametes at the same stage of development. It is similar to the testis structure of several fishes, amphibians, and *Drosophila melanogaster*. This type of the gonad organization has never been described in gastropods before.

Keywords Marine snails *Littorina* · Cyst type of testis · Stages of spermatocytogenesis · Cyst cells · Germline cells

Introduction

Two main types of testes organization in animals are known: the tubular type and the cyst type. Testes of the tubular type mostly consist of seminiferous tubules—coiled tubes producing spermatozoa. They are described in many amniotic vertebrates (reptiles, birds, and mammals), some bony fishes, and some invertebrates (Jones and Lin 1993; Franco et al. 2008; Gribbins 2011; Vergilio et al. 2012). Each tubule starts from the external layer of the testis tissue and passes towards the center, becoming tortuous before emptying into a system of collecting tubules. The walls of seminiferous tubules consist of a multilayered germinal epithelium containing spermatogenic cells. If spermatogenic cells are situated locally, a gradient of maturing spermatozoa can be seen in the tubule: all sperms located in the same zone along the tubule are at the same stage of maturation, while adjacent zones contain previous or later stages of sperm development.

The second type of testes organization, the cyst type, is known in cyclostomes and tailed amphibians (Uribe 2009) and has been described in detail in bony fishes (Grier 1981; Uribe et al. 2015) and a model insect, the fruit fly *Drosophila melanogaster* (Gonczy and DiNardo 1996; Spradling et al. 2011). In case of the cyst type, the gonad contains numerous rounded cysts composed of a cohort of isogenic germ cells. In all bony fishes, spermatogonia are associated in the germinal compartment with Sertoli cells (Uribe et al. 2015). Their

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processes envelop the germ cells throughout spermatogenesis, isolating them both from the basement membrane and from the lobular lumen. However, in *D. melanogaster*, the cysts are formed in the apical part of the testes in a special niche of the gonad made of stem and differentiated cells (Kimble 2011). Each cyst consists of two cyst cells of somatic origin accompanying a germline stem cell (Gonczy and DiNardo 1996).

Our knowledge about fine structure of testes in molluscs, the second largest phylum of invertebrates, is fragmentary. They are usually given as additional information in studies of spermatozoan morphology, fertilization mechanisms, or reproductive strategies of species. The resolution of images is often different in different studies (light and electron microscopy), which confounds comparison and precludes conclusions about structural organization of the gonad tissue. The tubular type of the testes organization has been described for some gastropods and many bivalves (Franco et al. 2008; Gimenez 2013; Tos et al. 2016), while the cyst type, to the best of our knowledge, has never been described in any mollusk. *The tubular type of the gonad in periwinkles has been suggested twice (Paviour et al. 1989; Fretter and Graham 1994) but without supporting information*. Apparently, the structural organization of the testis in *Littorina* has never been an object of a special study.

Littorina saxatilis is one of the five closely related North Atlantic species of the genus *Littorina*, *the other four being L. arcana*, *L. compressa*, *L. obtusata*, *and L. fabalis*. *These species largely overlap in distribution in the intertidal zone but their females* have different reproductive strategies (Reid 1996). *L. saxatilis* and *L. arcana* are sibling species, whose males are almost identical morphologically; these two species are capable of interspecies hybridization (Mikhailova et al. 2009; Granovitch et al. 2013).

In this study, we made a morphological and cytogenetic description of the testis and stages of spermatogenesis in *L. saxatilis.* Our data can be used for comparative anatomical analysis of sympatric *Littorina* species. They are also an important contribution to the characteristics of the class Gastropoda since differences between testis types have significant implications for the systematics of molluscan taxa in general.

Materials and methods

Specimens and collecting sites *L. saxatilis* specimens were collected during spring reproductive season at the littoral zone of the Barents Sea at *Tromsø* island, Norway ($n = 25$), from a sympatric population with *L. arcana* and at the Kola Bay, Murmansk, Russia ($n = 15$), where *L. arcana* is not present. Only males with single row penial glands were used for analysis from the Norwegian samples. Males were kept in *aquaria* with running sea water under standard conditions (at 10 °C and an automatic imitation of the tidal cycle with an appropriate water level). Male gonads were studied on *fresh and fixed tissue*.

Microdissection and anatomical studies of gonads For dissection, males were removed from the shell, transferred to a glass Petri dish into a drop of seawater, and dissected under a MBI-10 binocular microscope using standard Dumoxel instruments (Fine Science Tools, USA). Each testicle was isolated and then transferred into a drop of filtered sea water in the center of the slide limited by transversely fixed bars (5 × 26 mm) of a thin double-sided tape. The dissected organ was spread into 120 μl of quail egg white, slowly covered with a cover slip and gently pressed. The adhesive tape formed a chamber which kept for 2–3 h and was sufficient for a light microscope observation of living tissue and cells both at low or high magnification.

Observation of fresh tissue We used the light microscope Leika DM2500 with software for differential-interference contrasting method (DIC). The microscope was equipped with CCD-camera Leika DFC420. Oil immersion objective 100 × 1.25 was used for lifetime DIC observations and imaging preparation.

Cytological preparations Premature and mature male gonads were used for slide preparations. Testicles were incubated in hypotonic solution (0.5 ml of 130 mM KCl solution, 20 min at room temperature (RT)), then fixed in 0.2 ml freshly made 3:1 (*v*/*v*) mixture of methanol and glacial acetic acid solution for 2–4 h at RT and stored at − 20 °C until preparation.

Cytological preparations were obtained using a high-pressure squashing technique (Solovyeva et al. 2016). Fixed testicles were transferred onto a slide in 3:1 fixative solution and divided into small pieces in 25 μl drop of 50% propionic acid, then covered with a 24 × 24-mm siliconized cover slip. The slide was then put in vertical hydraulic vise equipped with a manometer. Approximately 250 kg/cm^2 of pressure through the vise was gradually achieved within a 90–120 s interval. The slides were then placed into liquid nitrogen, and cover slips were removed with a razor blade. They were dehydrated by taking through ethanol series of (70, 80, and 100%), air dried, and stored at − 20 °C.

Staining techniques and fluorescence microscopy We used a standard staining technique with 5% Giemsa solution (pH = 7.0), silver-staining, and staining with 4′,6-diamidino-2-phenylindole (DAPI) and chromomycin A3.

Silver-staining procedure with a protective colloidal developer was performed according to as previously described (Howell and Black 1980).

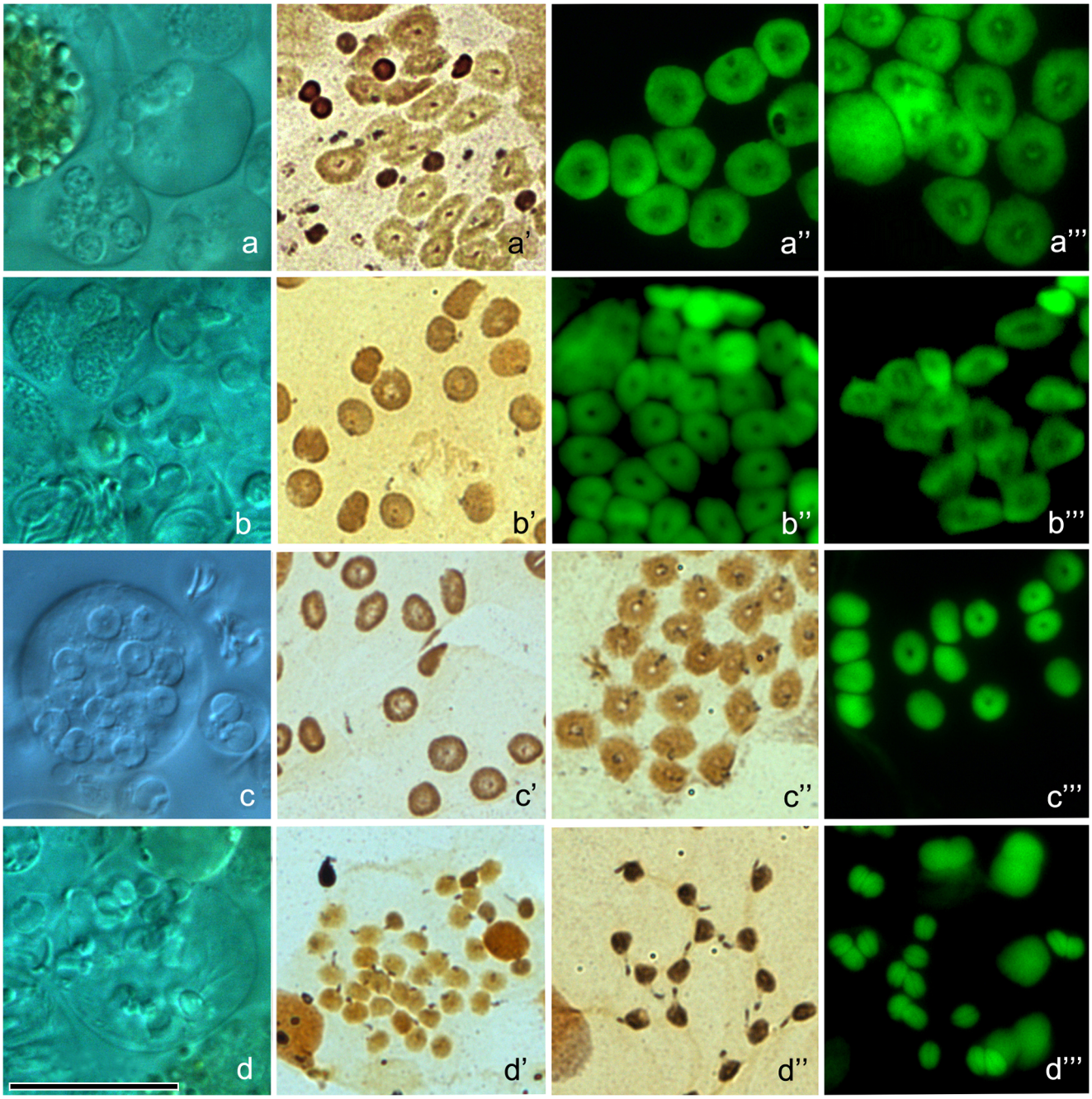

Fig. 1 Early spermatids of *L. saxatilis* (rows from top to bottom): a, a', a", a"'—stage 1; b, b', b", b"'—stage 2; c, c', c", c"'—stage 3; d, d', d", d"'—stage 4. Columns a, b, c, d—living observation; images a', b', c', d', c", d"—cell nuclei silver stained; images a", b", a"', b"', c"', d"'—cell nuclei CMA3 stained. Scale bar, 20 μm

DAPI staining. Freshly prepared 0.5 mg/mL working solution of DAPI in McIlvaine buffer (pH = 7.0) (Schweizer 1980) was used. The preparations were embedded in ProLong® Gold antifade (Invitrogen), sealed, and stored at − 20 °C until use.

Chromomycin A3 (CMA3) staining was carried out according to the method of Schweizer (1976) with some modifications. Stock solution of chromomycin A3 (Sigma-Aldrich) (1 mg/ml) was prepared in deionized water dissolving the compound during several days at 4 °C in the dark without stirring. Working solution of CMA3 (0.5 mg/ml) was obtained by dissolving the stock solution in McIlvaine's buffer (pH = 7.0) with 5 mM $MgCl_2$. Slides with samples were rinsed in McIlvaine's buffer and placed in CMA3 working solution under a coverslip and stained in the dark at RT for 1–2 h. To remove the coverslip slides were briefly washed in McIlvaine's buffer, air dried, and mounted in ProLong® Gold antifade (Invitrogen) and sealed. The stained slides were

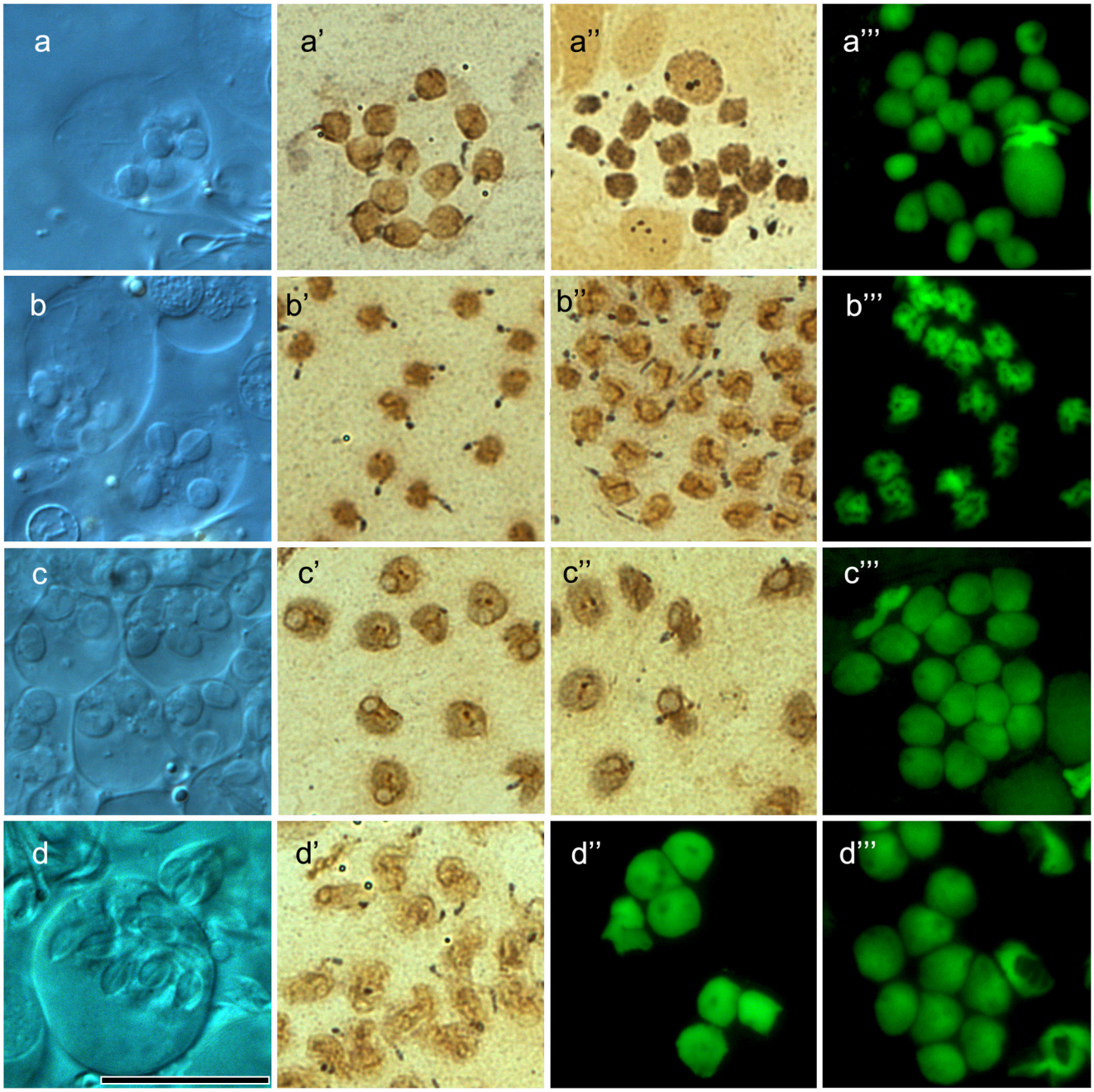

Fig. 2 Mid spermatids of *L. saxatilis* (rows from top to bottom): a, a', a", a"'—ellipsoidal spermatids (stage 5); b, b', b", b"'—smooth spermatids (stage 6); c, c', c", c"'—medium spheroid spermatids (stage 7); d, d', d", d"'—small spheroid spermatids (stage 8). Columns a, b, c, d—living observation; images a', b', c', d', a", b", c"—cell nuclei silver stained; images d", a"', b"', c"', d"'—cell nuclei CMA3 stained. Scale bar, 20 μm

left for 3 to 5 days in the dark at 4 °C to stabilize chromomycin fluorescence before examination. The Leica DMI6000 inverted microscope (Leica Microsystems, Germany) was used for analysis of CMA3- and DAPI-labeled nuclei. Images were processed with the use of ImageJ software.

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Results

Highest resolution observation of an intact testis. Observations of the microsurgically dissected reproductive system of *L. saxatilis* spread on slides showed that the mature testis consisted of numerous ovoid lobules forming larger lobes. The lobules of mature testes were semi-transparent pigmented pinkish structures.

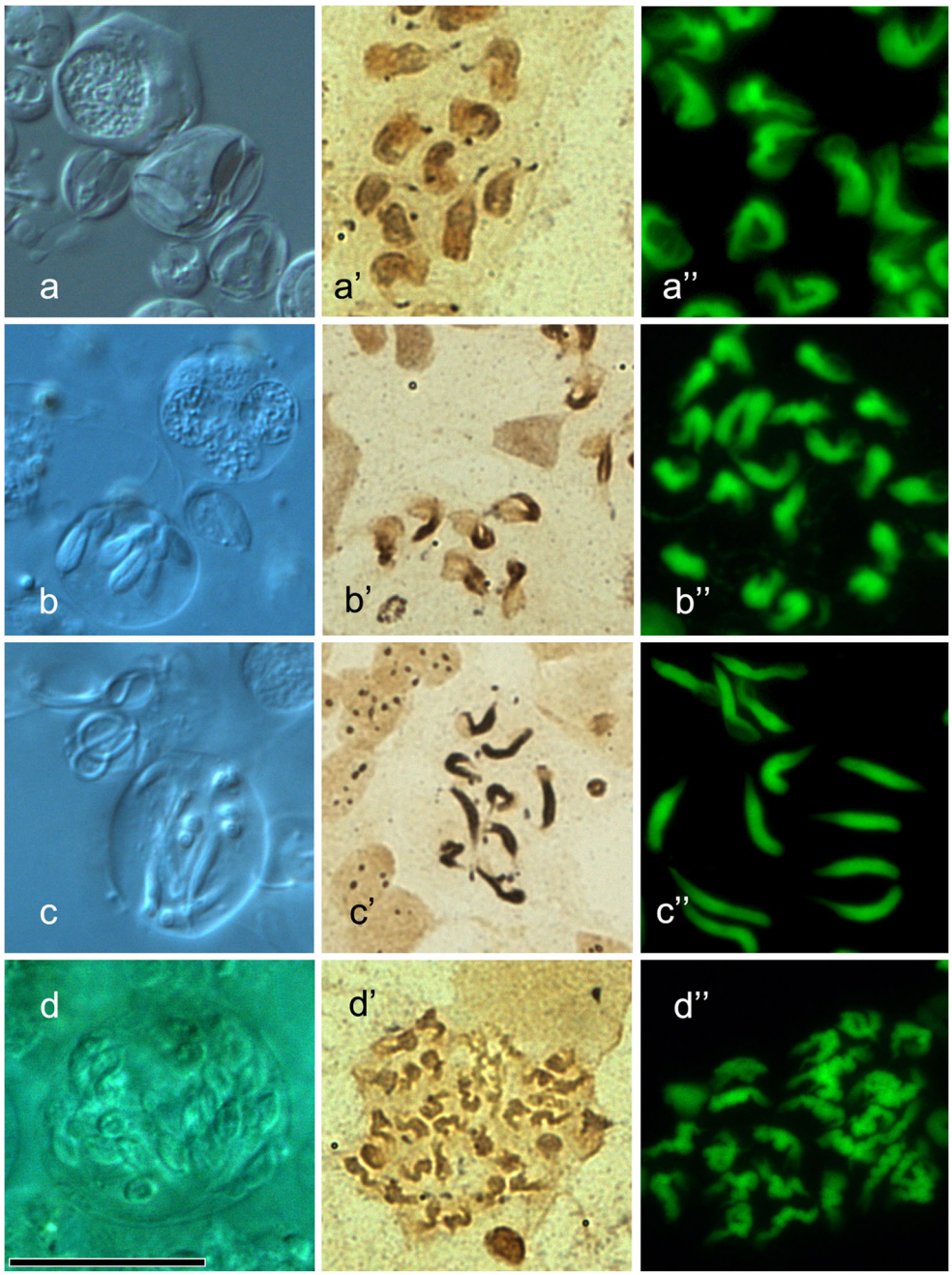

Fig. 3 Mid elongated spermatids of *L. saxatilis* (rows from top to bottom): a, a', a"—spermatids with tailed nucleus (stage 9); b, b', b"—spermatids with distinctive central channel (stage 10); c, c', c"—highly elongated spermatids with head swelling (stage 11); d, d', d"—tadpole shape spermatids (stage 12). Columns a, b, c, d—living observation; images a', b', c', d'—cell nuclei silver stained; images a", b", c", d"—cell nuclei CMA3 stained. Scale bar, 20 μm

The lobules of intact mature testes of *L. saxatilis* were filled with randomly arranged multicellular cysts containing gametes at various stages of maturation (Figs. 1, 2, 3, 4, and 5). Gametes within a cyst were highly synchronized in respect of the differentiation degree, while neighboring cysts varied considerably as to the the size, the number, and the differentiation stage of the gametes in them. Importantly, *gametes of different degrees of maturation were randomly spread in the lobules*. Morphologically, the testes of *L. saxatilis* individuals from the single-species population (Kola Bay, Barents Sea, Russia) were identical to those of *L. saxatilis* individuals from the sympatric population with the sibling species *L. arcana* (Tromsø island, Norway).

During *fresh tissue* observations of the male gonads of *L. saxatilis*, we found cysts containing early spermatids (Fig. 1), mid spermatids (Figs. 2 and 3), late spermatids, and spermatozoa (Figs. 4 and 5). *We described 20 types of nucleus organization in differentiating sperm based on the spreading data* (*20 rows in Figs. 1, 2, 3, 4, and 5*) *and 20 corresponding stages of nucleus reorganization during spermiogenesis based on silver and CMA3 staining*. All these stages occurred within the cysts with the exception of fully matured spermatozoa,

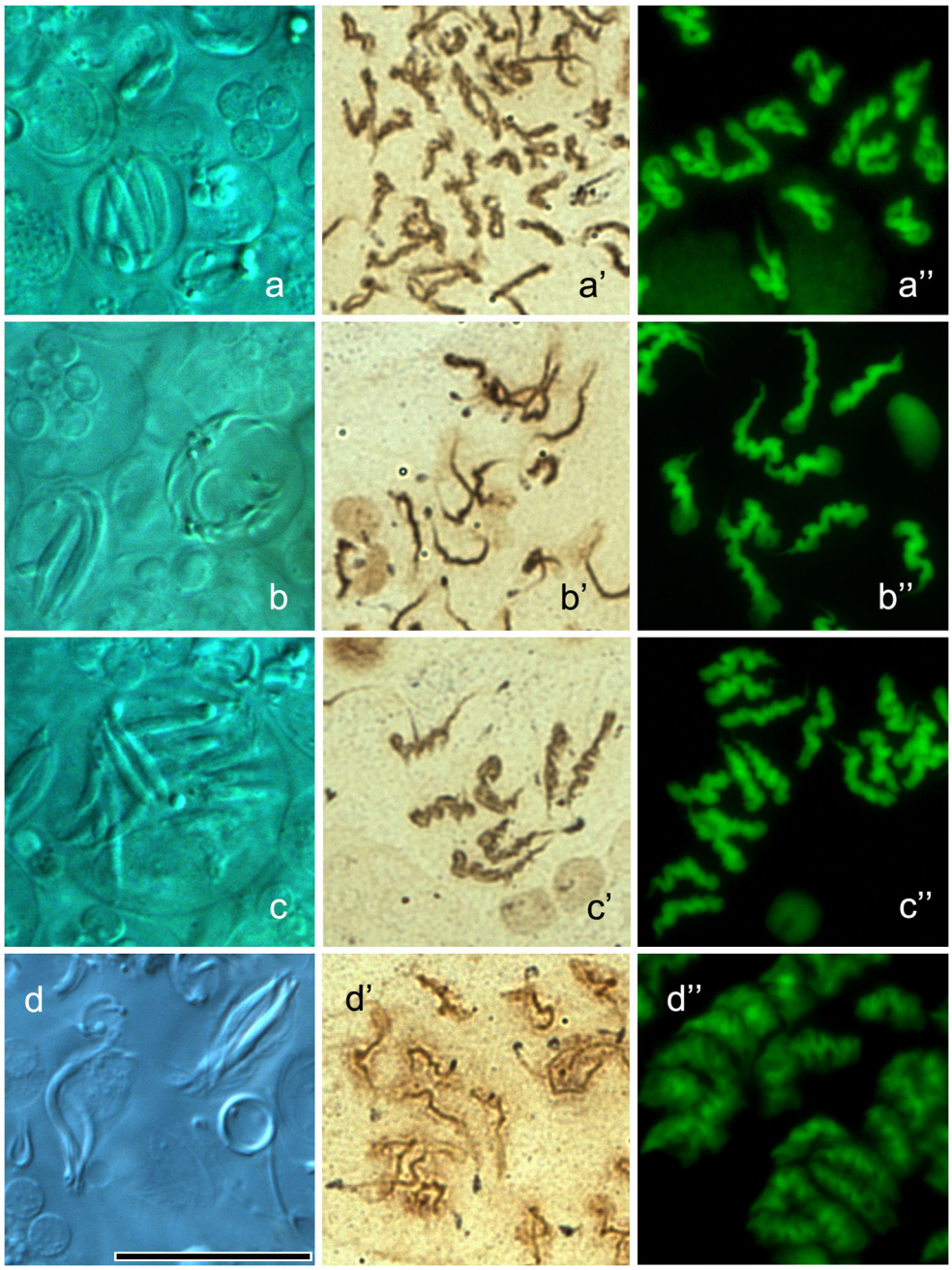

Fig. 4 Late spermatids of *L. saxatilis* (rows from top to bottom): a, a', a"—spermatids without head swelling (stage 13); b, b', b"—elongated spermatids (stage 14); c, c', c"—constricted spermatids with head swelling (stage 15); d, d', d"—twisted spermatids without head swelling (stage 16). Columns a, b, c, d—living observation; images a', b', c', d'—cell nuclei silver stained; images a", b", c", d"—cell nuclei CMA3 stained. Scale bar, 20 μm

which left the cysts and entered the space between them (Fig. 5d, d', d").

We observed living cells from isolated testicles (premature and mature male gonads) (Figs. 6 and 9b, d–i) and microsurgically isolated cysts (Figs. 7, 8, and 9a, c, j–l). The testicles of *L. saxatilis* comprised mosaically arranged spermatocyte, spermatid, and spermatozoa cysts with rare incorporations of solitary spermatogonia, cyst cells, and goniablast cysts. All cysts contained a single cyst cell of somatic origin and differentiating germline cells, whose number varied from cyst to cyst.

Premature and mature male gonads of *Littorina* mainly contained multicellular cysts or, less often, bicellular cysts (Figs. 6, 7, 8, and 9). Unbound spermatozoa and spermatid and spermatozoa cysts dominated in the mature male gonads. Numerous primary and secondary spermatocyte cysts were also present in them (Figs. 6 and 7). In addition, bicellular goniablast cysts (Figs. 6, 7, and 10f–h) and single diploid or polyploid cells (Figs. 6 and 7) were often found. Detached cells rarely clustered within the gonad and in some snails they probably did not cluster at all. The clusters, when present, were composed of testicular cysts and occasionally seminal

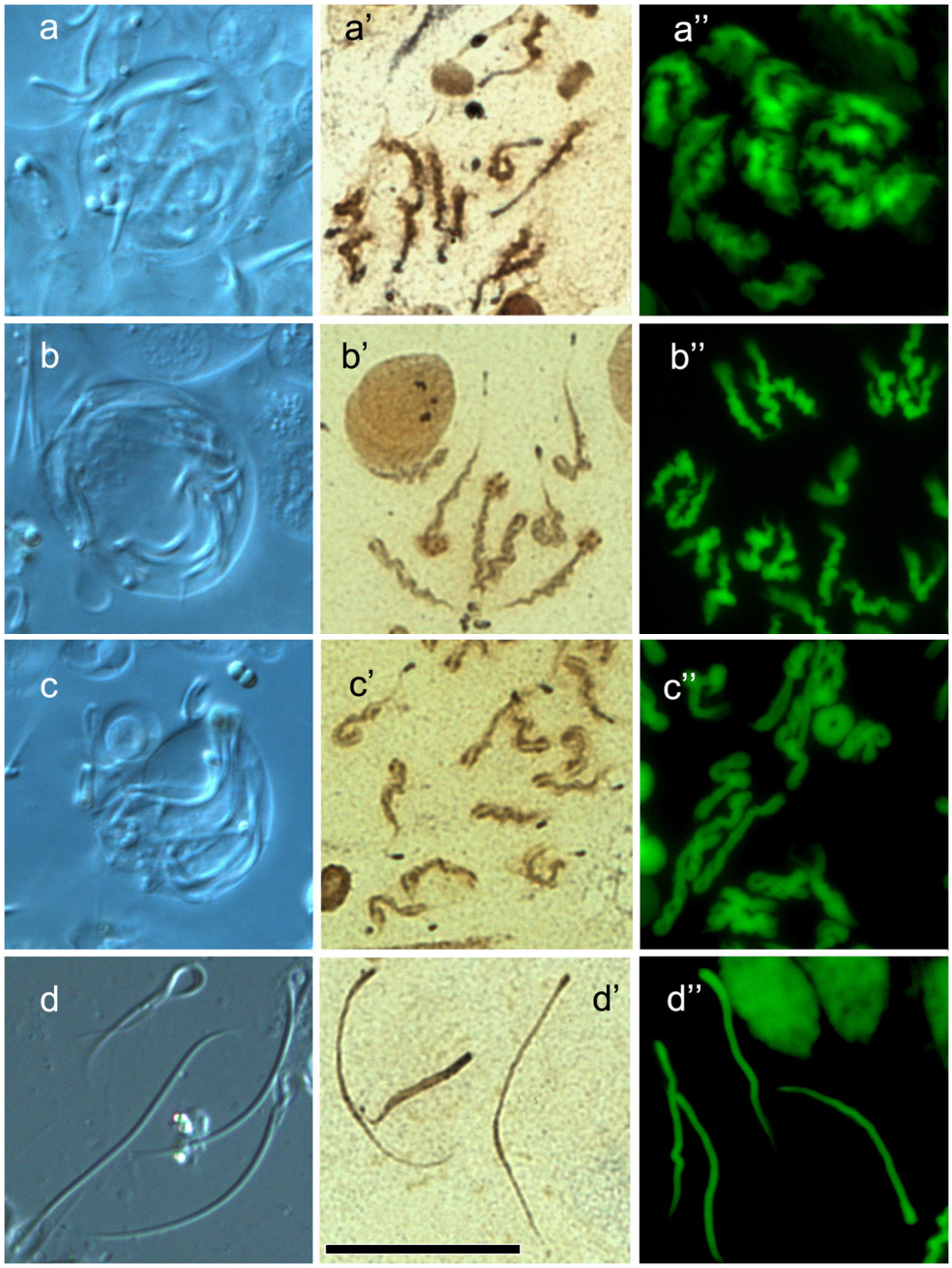

Fig. 5 Late spermatids and spermatozoa of *L. saxatilis* (rows from top to bottom): a, a', a"—twisted highly elongated spermatids with head swelling (stage 17); b, b', b" and c, c', c"—twisted immature spermatozoa with head swelling (stages 18, 19); d, d', d"—mature spermatozoa (stage 20). Columns a, b, c, d—living observation; images a', b', c', d'—cell nuclei silver stained; images a", b", c", d"—cell nuclei CMA3 stained. Scale bar, 20 μm

vesicles (Fig. 8) as well as unbound cyst cells of varying ploidy and spermatogonia. Observing spermatocyte nuclei with a well-defined ploidy number, we established that the doubling of DNA contents in *Littorina* resulted in a 1.4–1.5-fold increase of the spermatocyte nucleus diameter. Extrapolating these data, we could obtain a rough estimate of the ploidy level of the cyst cell nuclei based on measurements of their diameter (Fig. 8).

Descriptions of spermatogenesis stages illustrated with the images of living cells obtained at the threshold of optical resolution (DIC microscopy) are given below (Fig. 9). For the description of stages of male meiosis in *Littorina*, we adopted custom terms used in developmental biology of *D. melanogaster* (FlyBase 2017. http://flybase.org/static_pages/termlink/termlink.html). Stages of meiotic differentiation of *L. saxatilis* spermatocytes are presented in sequential order.

Primary spermatocyte cysts (Fig. 9a–h) contain 1 to 16 nuclei (meiotic chromosome configurations) of spermatocytes and one nucleus of cyst cell. Primary spermatocyte cysts containing two cells occur in male gonads regularly but less frequently than cysts with a greater number of spermatocytes (Fig. 9g, h).

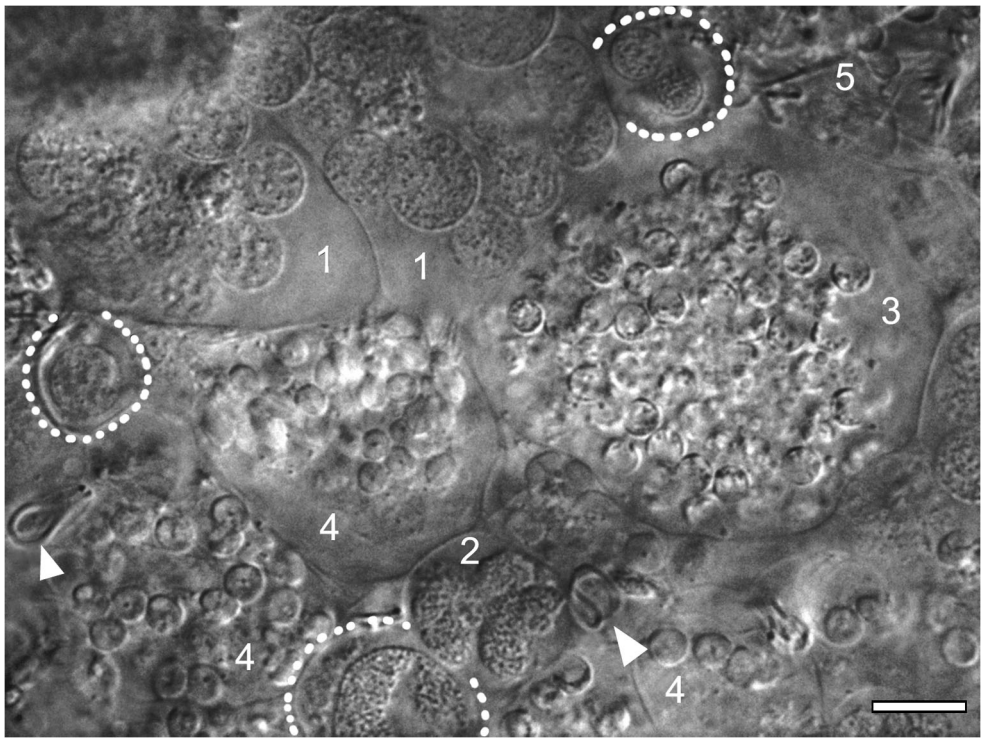

Fig. 6 Optical section through inner part of intact mature testicle of *L. saxatilis*: lifetime DIC image: 1—primary spermatocyte cysts; 2—prophase secondary spermatocyte cyst; 3—telophase secondary spermatocyte cyst; 4—spermatid cysts; 5—spermatozoa cyst. Broken line—circled solitary cells of different size (left and below) and goniablast cyst (top); triangles marked mobile-free spermatozoids in the intercystic slits. Scale bar, 10 μm

Interphase primary spermatocyte cysts (Fig. 9a) contain spermatocytes with a spherical diploid nucleus 8.2–8.6 μm in diameter. Chromatin is represented by a loose network of tiny fibrils and single globules. Nucleoli are invisible, which makes it possible to distinguish this type of cysts from similar spermatogonial cysts. In the latter, one or two prominent nucleoli are always present in the nuclei of spermatogonia.

Leptotene primary spermatocyte cysts (Fig. 9b) have nuclei typical of spermatocytes at this stage. They are evenly filled with long and thin chromatin fibers. There are no chromatin globules in these nuclei, which were shown by DIC microscopy.

Chromatin in spermatocytes at the *zygotene stage of primary* spermatocyte cysts (Fig. 9c) is represented by unevenly compacted chromosome fibers. These regions are probably pairing initiation sites of homologous chromosomes for bivalent forming.

In the spermatocyte nuclei in *pachytene primary* spermatocytes cysts (Figs. 9d and 10b) spatially separated extended regions of pachytene bivalents with well-defined chromomere structure are clearly visible. Homologous chromosomes comprising the bivalent are well discernible. The two homologs are usually arranged in collateral order.

Compact stage primary spermatocytes (Figs. 9e and 10c). At this compact stage of meiosis, the bivalent looks like a fiber of condensed chromatin about a 1 μm thick.

Homologous chromosomes in the nuclei of *diakinesis* primary spermatocytes (Fig. 9f) are composed of a single thread only at the centromere in most of the bivalents. The bivalents in this case resemble mitotic chromosomes rather than classical bivalents typical of vertebrates at this stage.

The nucleus of the cyst cell in *Littorina* is permanently in the interphase state. Observing the live testis tissue, we showed the dimorphism of the nuclei by discovering a bicellular cyst comprising prophase-telophase spermatocyte (Fig. 10a–f). In this case, the nucleus of the cyst cell and the chromosomes of the spermatocyte localized in the same focal plane. The image of double cells in metaphase I primary spermatocyte cyst is used for illustration in Fig. 9g. It can be seen that the cyst contains an interphase nucleus of a diploid size belonging to the cyst cell and haploid metaphase I bivalents located at some distance from it. All 17 bivalents of the set can be easily identified on metaphase I chromosome spreads (Fig. 10d–f).

Double cell cyst with *anaphase-telophase I* chromosome configurations (Fig. 9h) contained daughter sets of segregating chromosomes, which were superimposed to such an extent that they could not be counted. Separate chromosomes were nevertheless visible.

Haploid nuclei of secondary spermatocytes in *interphase secondary spermatocyte cysts* (Fig. 9i) were nearly rounded. Their diameter varies from 6.0 to 6.8 μm. Chromatin structure did not differ from that of interphase nuclei of primary spermatocytes. *Telophase* nuclei of secondary spermatocytes (Fig. 9k) contained round chromatin blobs. Their diameter made up 4.0–4.4 μm. Two or three nucleoli, about 0.5 μm in size, could be seen in these nuclei.

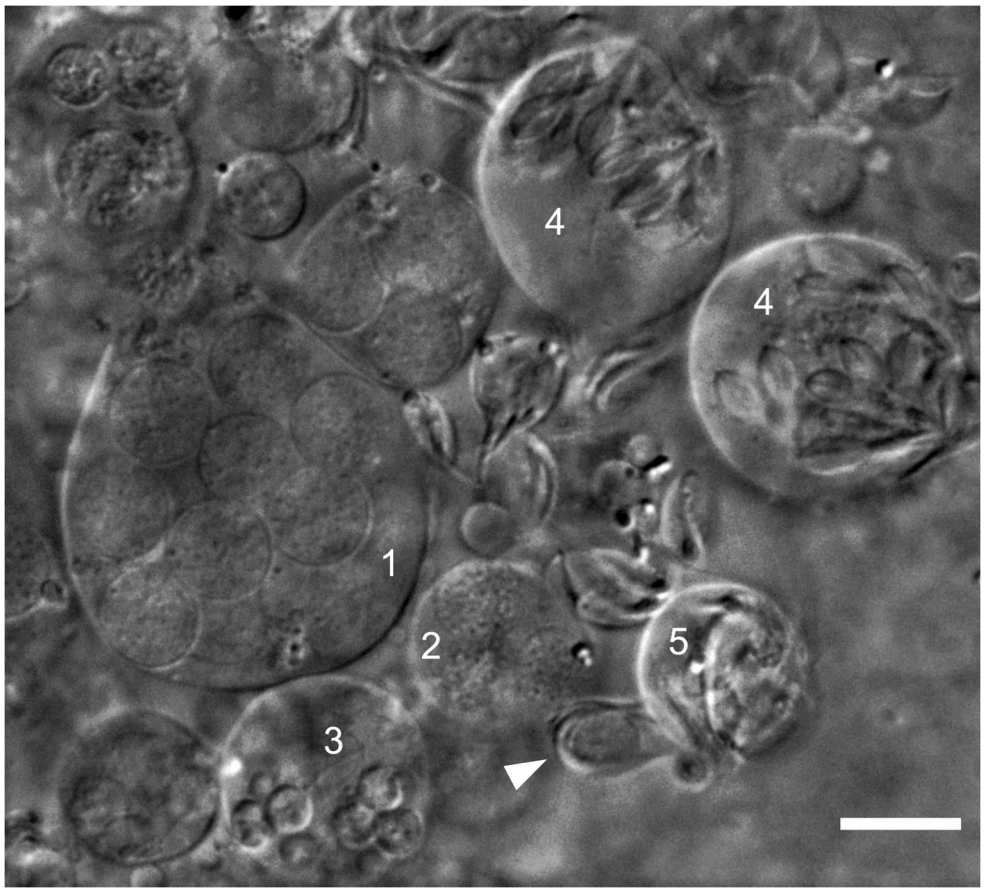

Fig. 7 Lifetime DIC image of the testicular cysts isolated from *L. saxatilis* mature gonads: 1—interphase primary spermatocyte cyst; 2—interphase secondary spermatocyte cyst; 3—early spermatid cyst; 4—late spermatid cysts; 5—spermatozoa cyst. Triangles marked mobile-free spermatozoid. Scale bar, 10 μm

Juvenile spermatid nuclei (Fig. 9l) had the same shape and size as telophase nuclei of secondary spermatocytes. Nucleoli were invisible in them and chromatin was diffused. A continuous central passage 0.5 μm wide was observed in spermatid nuclei. Strictly speaking, this stage of germline cell differentiation does not belong to spermatocytogenesis but is the starting point of the next differentiation stage, spermiogenesis (Figs. 1, 2, 3, 4, and 5).

Discussion

In the process of spermatogenesis, functional sperm is created from undifferentiated germ cells. A typical pathway involves mitotic and meiotic divisions involving spermatogonial cells, primary and secondary spermatocytes, spermatids, and mature spermatozoa. All these stages develop in the testis with support of various accessory cells.

Our data indicate that the gonad of mature males of *L. saxatilis* is organized according to the lobular cyst type since each lobule of an intact mature testis is filled with multicellular cysts and each cyst contains gametes at a certain stage of development. *Observations of fresh tissue* showed that cysts were randomly arranged inside the lobule. There was no gradient of the degree of maturity of their content from the periphery to the center, as it is the case in the seminiferous tubules in the testes of the tubular type. We demonstrated that the testis morphology was identical in individuals of *L. saxatilis* from the single-species population (Russia) and those from the sympatric population with the sibling species *L. arcana* (Norway). It remains unclear whether the lobular cyst type of the testes organization is species-specific or characteristic of other species of periwinkles too. *Curiously, cysts in the male gonad observed with light microscopy of freshly fixed or formalin-preserved material, probably for the first time been found previously by David Reid (Reid 1996, p. 16, Fig. 3) when describing paraspermatozoa in nine Littorina species. These data may indirectly confirm the assumption of the identical cyst type structure of the gonad in periwinkles.* Further, detailed studies are necessary to elucidate this issue.

The structural organization of animal testes has been studied best in the teleost fishes. The morphology of the testes and their cellular composition (germ cells, Sertoli and Leydig cells, and other components) has been described in detail, and several testis types, including the lobular cyst type, were described *earlier* (see review Uribe et al. 2015). Interestingly, summing up abundant experimental material, the authors conclude that the type of organization of the testicle is determined

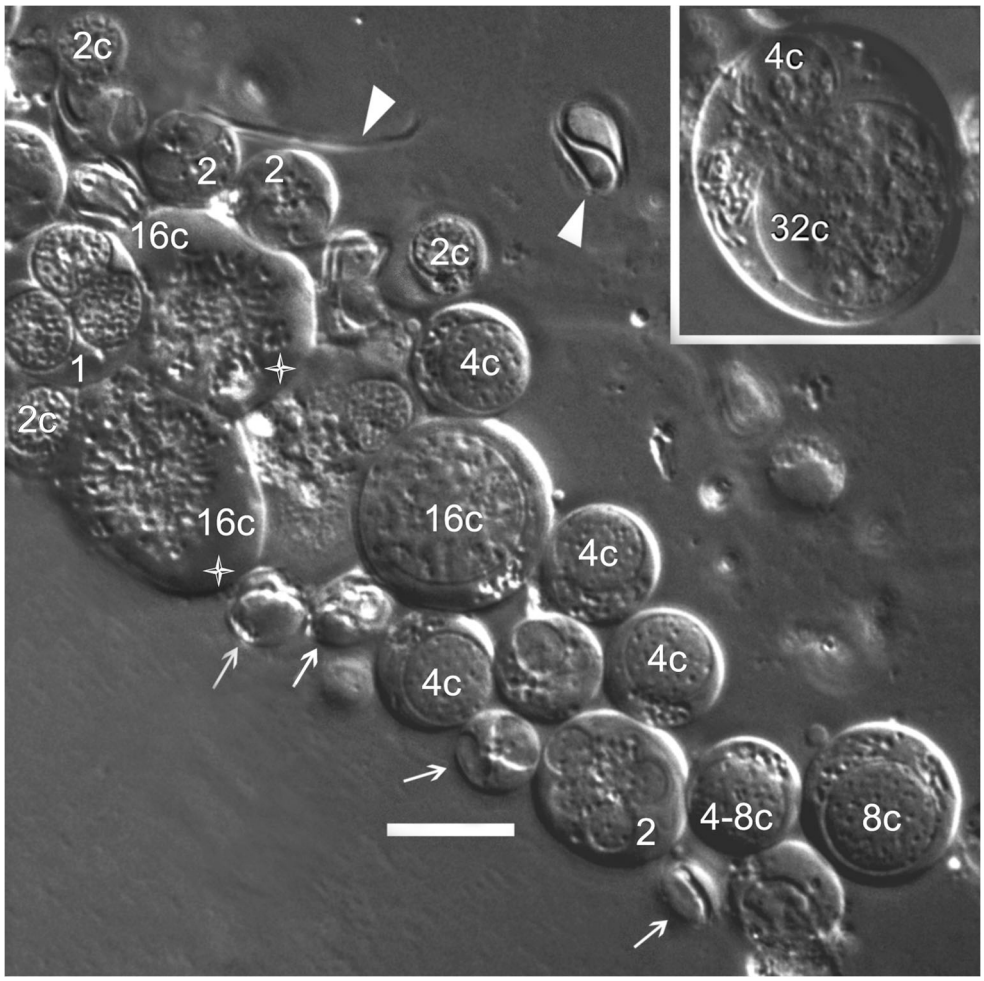

Fig. 8 Lifetime DIC image of cell cluster and primary spermatocyte cyst (insert) isolated from *L. saxatilis* mature gonad. The cluster includes testicular cysts (1 and 2), seminal vesicles (arrows), and solitary cyst cells of different ploidy (2c–32c) with endometaphase cells (16c star). Inserted image of the spermatocyte cyst contains polyploid cyst cell nucleus (32c), near tetraploid spermatocyte nucleus (4c) and cytoplasmic inclusions typical for solitary cyst cells. 1—interphase primary spermatocyte cysts; 2—spermatid cysts; triangles marked mobile-free spermatozoid. Scale bar, 10 μm

by the position of spermatogonia in the germinal layer. Moreover, they note that upon commencement of spermatogenesis, the Sertoli cells form the borders of cysts in which synchronous spermatogenesis occurs (Uribe et al. 2015).

Germline cells and somatic cells accompanying germ cells throughout spermatogenesis also play an important role in the testis of *L. saxatilis* during formation of mature spermatozoa. Accompanying cells in molluscs are Sertoli cells, whose fine structure and functional morphology has been described in detail for three marine prosobranch molluscs including *Littorina sitkana* (Buckland-Nicks and Chia 1986). According to these authors, in each of the three species of prosobranchs that they studied, *Littorina sitkana*, *Fusitriton oregonensis*, and *Ceratostoma foliolatum*, Sertoli cells "extend from the basal lamina to the lumen of each testicular *tubule*" (Buckland-Nicks and Chia 1986, p. 306, italics added). The main functional characteristics of molluscan Sertoli cells are the regulation of the micro-environment of germ cells, the removal of residual cytoplasm, the phagocytosis of waste sperm, and the control of spermiation. In this study, we *conducted observations* at the threshold of optical resolution but failed to find any large Sertoli cells morphologically similar to those described by Buckland-Nicks and Chia (1986). We did find, however, small fragments of cells with numerous granules, which might be fragments of Sertoli cells.

At the same time, the cells of somatic origin found around the cysts in our study are similar to the cyst cells from the testes of *Drosophila* (Gonczy and DiNardo 1996; Spradling et al. 2011). A *Drosophila* testis contains two types of stem cells, germline stem cells (GSCs) and cyst stem cells (CySCs), which give rise to the germline cells and cyst cells, respectively. GSCs and CySCs divide asymmetrically to produce daughter cells (gonialblasts and cyst cells, respectively) that form developmental units, cysts (Gonczy and DiNardo 1996; Zoller and Schulz 2012). The cyst cells do not divide but increase in size as the germ cells enclosed by them develop and grow. In this way, the cyst cells functionally resemble Sertoli cells (Gonczy et al. 1992; Gonczy and DiNardo 1996; Spradling et al. 2011).

Germline stem cells, gonialblasts, and spermatogonia in a single niche at the apical tip of the testis (hub cells) are found in *Drosophila* (Zoller and Schulz 2012) so that the entire testicle looks like a giant "seminiferous tubule." In such a case,

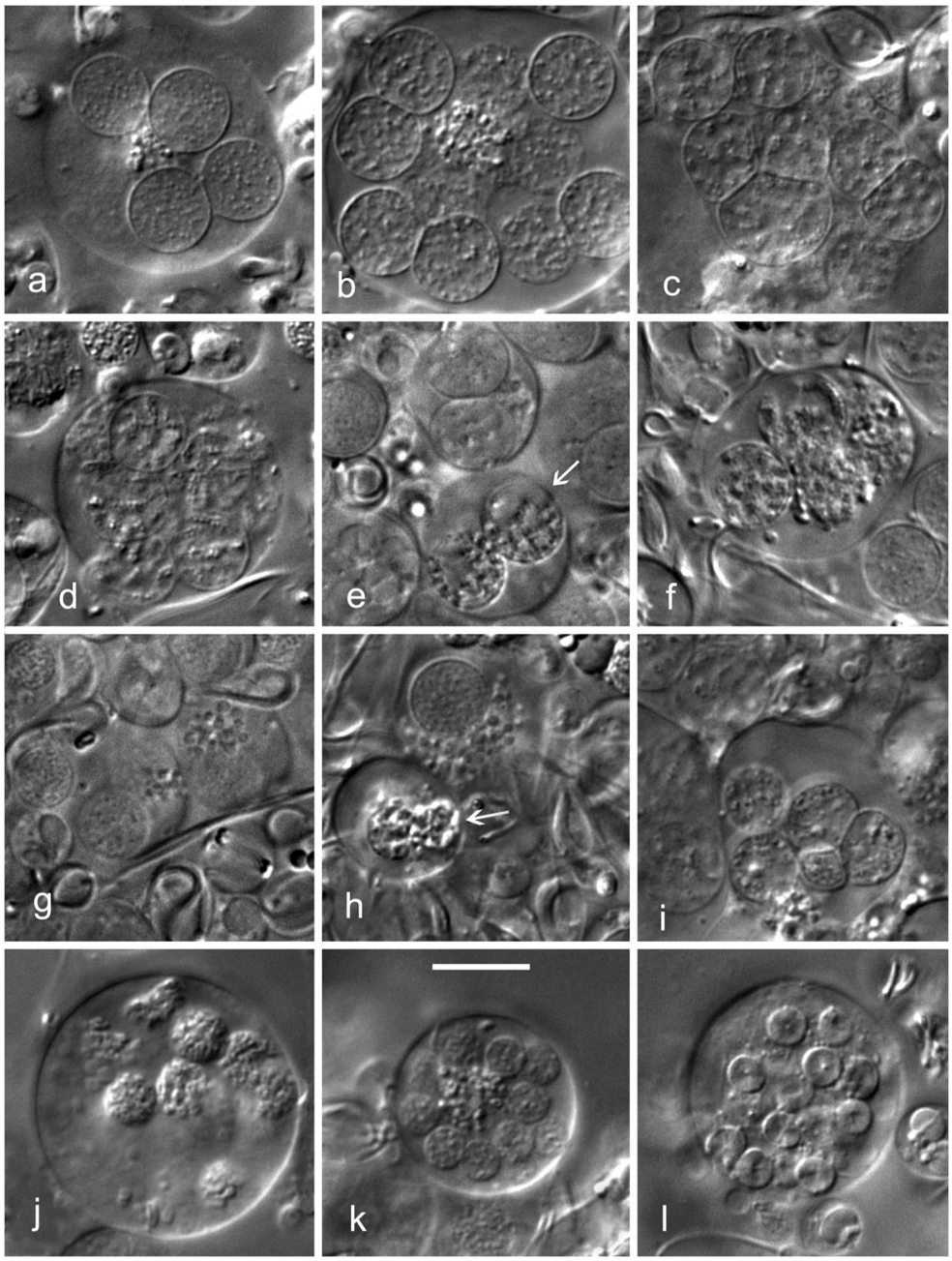

Fig. 9 Sequential stages of spermatocytic meiotic pathway illustrated by lifetime DIC images of the testicular cysts in *L. saxatilis*. Primary spermatocytes in interphase (**a**), leptotene (**b**), zygotene (**c**), pachytene (**d**), compact stage (**e**, arrowed), diakinesis (**f**), metaphase I, and anaphase-telophase I (**g** and **h**, arrowed). Secondary spermatocytes in interphase (**i**), prophase II, metaphase II and anaphase II (**j**), and telophase II (**k**). Early spermatid cysts (**l**). Scale bar, 10 μm

the testis may be said to have a "tubular cyst type" of organization. Niches of germline stem cells are also found at the tips of the distal ends of the gonad in another model object, the nematode *Caenorhabditis elegans* (Kimble and Crittenden 2007; Kimble 2011). At the same time, in marine nematodes, *Enoplus* the distal part of the testis is represented not by a single cell but by numerous cells (Yushin et al. 2014). It was suggested that these differences might be due to the evolutionary time of the origin of the orders within the phylum Nematoda (Yushin et al. 2014). So far, we did not find any hub cells in the testes of *L. saxatilis* but we hypothesize that several such niches should be present in a lobule. This idea is based on the lack of a gradient in the arrangement of the cysts containing gametes at successive stages of maturation. We attributed testes like these to the "lobular cyst type." The structural organization of gonads of different types (tubular, tubular cyst, and lobular cyst) is schematically shown in Fig. 11.

The similarity in cyst type of *Drosophila* and *Littorina* testicle organization gives the opportunity to use comparative approach to accelerate studies of particular traits of molluskan male gonad structure. Our data on germ cell differentiation stages in live testicles of *L. saxatilis* are a solid foundation for further research employing single cell assay and molecular

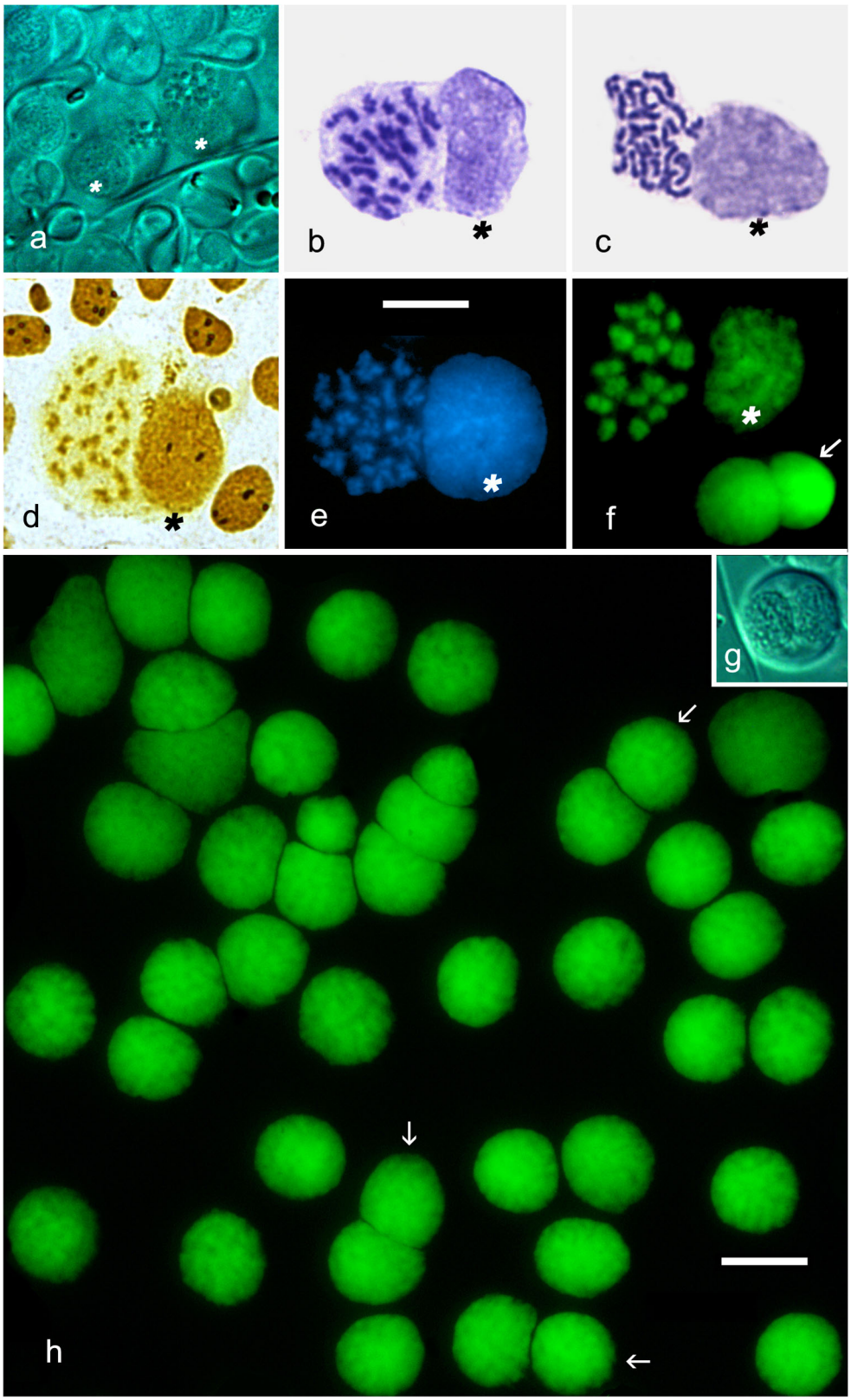

Fig. 10 Bicellular primary spermatocyte (**a**–**f**) and goniablast (**f**, **g**, **h**, arrow) cysts in *L. saxatilis*. Lifetime DIC images of metaphase I primary spermatocyte (**a**) and goniablast (**g**) cysts. Lifetime DIC image (**a**) and spreads of fixed cysts (**b**–**f**) with interphase nucleus belongs of cyst cell (marked with an asterisk); single spermatocyte in pachytene (**b**), compact stage (**c**) and metaphase I (**d**–**f**). **h** Cytological spread of putative hub of stem cells enriched by goniablast cysts (arrowed). Stained by Giemsa (**b**, **c**), silver (**d**), DAPI (**e**), CMA3 (**f**, **h**). Scale bar, 10 μm

identification. To note, molecular markers for germ cell differentiation stages and cyst cells are available for *Drosophila* (Gonczy et al. 1992; Zoller and Schulz 2012) but have not yet been developed for the Mollusca. Moreover, the advance of studies in the field of genomics, transcriptomics, and proteomics using *L. saxatilis* as a newly introduced model

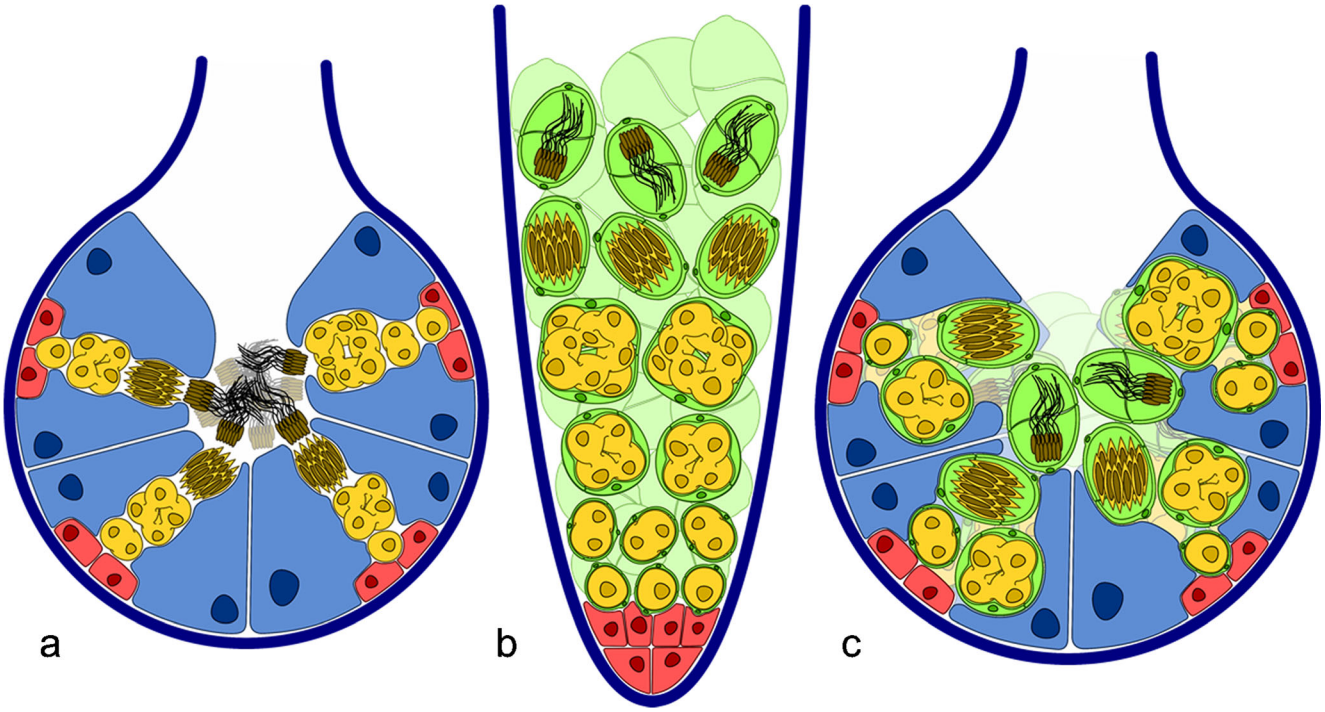

Fig. 11 Graphic depicting of three types of testes organization: **a** – tubular type, **b** – tubular cyst type of Drosophila melanogaster; **c** – lobular cyst type of *Littorina saxatilis*. Spermatogenesis pathway marked by different color: red – germline stem cells, yellow – spermatogonia and spermatocytes, brownish elongated – spermatids; brown with tail – spermatozoa, blue – the Sertoli cells. Cyst cells of somatic origin marked by green color

organism is promising good progress in the practical realization of molecular targeting methods. The cytological guide of spermatocytogenetic stages in molluscs provided in this study allows a quick identification of primary and secondary spermatocytes, which are randomly distributed among other cell types. This guide can also be used to ascertain the connection between a germ cell and a certain differentiation stage in premature and mature male gonads.

It should be noted that the total screening of live and fixed cysts of *L. saxatilis* on glass slides made it possible to ascertain that all types of cysts from spermatogonia to spermatozoa are formed from a single cyst cell, which is always in the interphase stage (90–95% of cysts have one interphase cyst cell nucleus of diploid size). We found only one cyst cell in *Littorina* although *Drosophila* cyst consists of two somatic cyst cells. So far, we cannot offer a satisfactory explanation of this observation. It may be a methodological artifact or an important novel finding. Further studies on testes of closely related species of *Littorina* are needed to verify this.

Based on live observations and spreading data, we identified 20 main types of nucleus organization of differentiating gametes during spermatogenesis (Figs. 1, 2, 3, 4, and 5). All these stages occur within cysts except the last stage (fully formed mature spermatozoa, which have entered the lumen of the lobule). An important result of this study was that we demonstrated successive stages of nucleus compactization and elongation in maturing gametes, supported by two independent methods of nuclei staining (silver-staining and CMA3) and revealed, by silver-staining, the centrioles marking the distal end of the nucleus. These results, which agree well with the earlier data on *L. sitkana* and Polyplacophora (Buckland-Nicks and Chia 1976; Buckland-Nicks et al. 1990), will be discussed in a separate publication.

In recent years, *L. saxatilis* has been broadly used as a model species. In particular, it was employed in state-of-the-art research areas such as whole-genome sequencing (Canbäck et al. 2012), proteomic analysis and the study of protein functions (Lobov et al. 2015; Muraeva et al. 2016; Maltseva et al. 2016; Lobov et al. 2018), and molecular karyotyping (García-Souto et al. 2018). Our study opens new vistas for involving cytogenetic data and new specific molecular markers into studies of spermatogenesis and reproductive biology of sister species. Novel research techniques may elucidate the causes and the molecular mechanisms of speciation in complexes of closely related species. In addition, a new testis type described in our work may have significant implications for the systematic of lower and higher molluscan taxa.

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Compliance with ethical standards

The authors declare that they have no competing interests.

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