The male gonad of the marine nematode Enoplus: No single distal tip cell but multiple uniform epithelial cells

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ABSTRACT: The distal tip cell (DTC) is a large single cell located at the apex of nematode gonadal arms. It is well investigated in \textit{Caenorhabditis elegans} Maupas, 1900 and described in many other species of class Chromadorea. It has been obtained convincing evidence that in \textit{C. elegans} the DTC plays an essential role in the development of the gonads and regulation of gametogenesis. We have studied the fine structure of the distal part of the testis in free living marine nematodes \textit{Enoplus}. TEM observations revealed the absence of a large specialized DTC at the apex of testes in \textit{E. michaelseni} (Linstow, 1896) and \textit{E. brevis} Bastian, 1865. The testis of both species is lined with numerous uniform testis epithelial cells (TEC) forming an uninterrupted epithelium adjacent to the basal lamina. Besides that, TEC form long outgrowths protruding deeply into the testis between spermatogonia. Unlike a single DTC of \textit{C. elegans}, the multiple TEC in \textit{Enoplus} spp. show hardly any signs of synthetic activity. They possess neither nucleoli nor a rough endoplasmic reticulum in their cytoplasm, but contain thick bundles of filaments giving no evidence for a glandular or a hormonal function. The multicellular epithelium of the distal part of the testis in \textit{Enoplus} spp. complements other plesiomorphic characters of enoplid structure and development (e.g. indeterminate early development, absence of cell constancy, regeneration ability, preservation of the nuclear envelope in spermatozoa) and may be considered as additional evidence for the basal position of the order Enoplida within the phylum Nematoda.


В мужских гонадах морских нематод рода \textit{Enoplus} вместо единственной дистальной концевой клетки обнаружен многоклеточный эпителий

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Introduction

The specialized distal tip cells (DTC) are large single cells positioned at the very distal tip of each arm of the nematode gonads as an integral part of the gonadal somatic epithelium (Chitwood, 1977; Foer, 1983; McKinnon, 1987; Wood, 1988; Bird, Bird, 1991; Wright, 1991; Lints, Hall, 2004). The DTC is an essential component keeping germline stem cells (GSC) in the so-called germline stem cell niche (Kimble, White, 1981; Hubbard 2007; Kimble, Crittenden, 2007; Kimble, 2011; Spradling et al., 2011; Hansen, Schedd, 2013). Cell lineage studies in Caenorhabditis elegans Maupas, 1900 and several other nematodes showed two primordial germ cells (Z2 and Z3) associated with two somatic precursors (Z1 and Z4) forming the gonad primordium (Sulston et al., 1997; Felix, 1999). During morphogenesis Z1 and Z4 give rise to two DTC each controlling gonadal arm elongation during development and the mitosis/meiosis decision of the GSC, both during development and in the adult (Ehrenstein, Schierenberg, 1980; Lints, Hall, 2004; Kimble, Crittenden, 2007). Whether a germ cell self-renews or enters meiotic prophase is determined by its proximity to the DTC. In C. elegans males and hermaphrodites the DTC is a single cell which tightly wraps around the germ cells (Hall et al.,
Proximal to the uniform GSC predominantly germ cells in the early stages of meiotic prophase I (leptotene, zygotene) are found indicating the beginning of the transition zone (Hall et al., 1999; Hansen et al., 2004; Kimble, Crittenden, 2007; Kimble, 2011).

The morphology and function of GSC niche formed by a single DTC has been studied in detail in *C. elegans* (Hall et al., 1999; Lints, Hall, 2004; Kimble, Crittenden, 2007; Hansen, Schedl, 2013), there are also data on DTC in another nematodes from the same order Rhabditida sensu De Ley and Blaxter, 2002 (Foor, 1983; Rudel et al., 2005) and for a free-living marine nematode of the order Chromadorida (Zograf, 2010). The order Enoplida, which includes free-living aquatic forms, is taxon within the phylum Nematoda showing some ancestral peculiarities correlating well with the putative basal position of the order Enoplida (Maggenti, 1963, 1981; Platonova, 1976; Malakhov, 1994, 1998; Voronov, Panchin, 1998; Yushin, Malakhov, 2004). Therefore, detailed data on the structure of the enoplid reproductive system including germ cell structure and development are of particular interest.

The absence of a morphologically distinct DTC in male and female gonads of two *Enoplus* species was mentioned earlier in papers containing data on the ultrastucture of the gonad epithelium (Yushin, Malakhov, 1997, 1998). This striking structural difference from the *C. elegans* gonad together with the great importance of the DTC for GSC niche formation and the control of mitosis/meiosis in GSC justifies a deeper analysis. For this purpose we initiated a detailed investigation of the testis distal tip in two other *Enoplus* species using transmission electron microscopy (TEM). The structure of GSC niche is compared to other more distant nematode taxa.

**Material and methods**

Adult males of *Enoplus michaelseni* (Linstow, 1896) De Man, 1904 (Enoplida, Enoplidae) were extracted from clusters of the bivalve mollusk *Crenomytilus grayanus* (Dunker, 1853) collected at the Marine Biological Station “Vostok” of the Institute of Marine Biology (Vostok Bay, Sea of Japan). Adult males of *Enoplus brevis* Bastian, 1865 were obtained from sand collected in the intertidal zone at White Sea Biological Station of Moscow State University (Kandalaksha Bay, White Sea). Live males of both species were cut transversely into two pieces each containing a distal testis regions.

The specimens were fixed for TEM at 4°C in 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 21 mg/ml NaCl and then postfixed in 2% osmium tetroxide in the same buffer containing 23 mg/ml NaCl. Postfixation was followed by en bloc staining for 2 h in 1% solution of uranyl acetate in distilled water and then the specimens were washed in distilled water and dehydrated in ethanol followed by isopropanol series and embedded in Epon (*E. michaelseni*) and Spurr (*E. brevis*) resin. Serial 0.7 µm or 0.4 µm thick sections stained with methylene blue and mounted in epoxyresin were studied and documented with a Leica DM2500B (Leica Microsystems) and a Biorevo BZ-9000 (Keyence, Japan) microscopes. Thin sections cut with a Leica UC6 ultratome and stained with lead citrate were examined with a Philips EM 300, JEOL JEM 100S and Zeiss Libra 120 transmission electron microscopes. For low magnification panoramic views of sections scanning transmission electron microscopy (STEM) was used with a Zeiss Libra 120 electron microscope. The testes of two males of *E. michaelseni* and four males of *E. brevis* were examined for the present paper.

**Results**

The male reproductive system of *Enoplus* species consists of two opposite testes with dilated proximal parts (seminal vesicles) opening into a common vas deferens (Fig. 1A). Each testis is an epithelial tube filled with spermatogonous cells at successive stages of development. The very distal tip of a testis (germinial zone) contains spermatogonia. Following in distal-proximal direction are zones of spermatocytes, spermatids, and immature spermatozoa filling a seminal vesicle (Fig. 1A).
The distal tip of the testis is a cylinder of 40–50 µm in diameter with a semispherical blind end (Figs. 1B, 2A–C, 3). Serial semithin sections in both species show the testis blind end as a cluster of germ cells delineated by a thin epithelium with no single somatic cell with the characteristics of a specialized DTC (Fig. 2A–C). The closely packed germ cells (spermatogonia) are uniform in size and structure; the occasionally visible nuclei of the testis epithelium are easily recognized on semithin sections (Fig. 2C).

The testis is surrounded a by 75 nm thick basal lamina exposed to the body cavity (Figs. 4A, B, 5A). Internally the basal lamina adjoins the testis epithelial cells (TEC) with their lobated, triangle or flattened nuclei containing dense
Fig. 2. The distal tip of the *Enoplus* spp. testis as seen on the longitudinal semi-thin sections with light microscopy. Note absence of a distal tip cell at the testis blind end (white bordered black *arrows* on A and *asterisk* on C).

A–B — *Enoplus michaelseni*, selection from serial semi-thin sections through the testes of two individuals; A1–A3 — overview of the distal tip; 0.7 µm thick sections with 5 µm interval; B1–B5 — the very distal tip of the testis; 0.4 µm thick sections with 2 µm interval; white *arrows* show mitotic cells. C — *Enoplus brevis*, section through the distal tip showing nuclei of somatic epithelial cells (white *arrows*). Abbreviations: in — intestine; sg — spermatogonia. Scale bars: A1–A3, B1–B5 — 10 µm; C — 5 µm.

Рис. 2. Дистальный конец семенника *Enoplus* spp. на продольных полутонких срезах, световая микроскопия. У слепого конца семенника (отмечен черной стрелкой с белым ободком на A и звездочкой на C) нет дистальной концевой клетки.

A–B — *Enoplus michaelseni*, избранные полутонкие срезы из серийной резки семенников двух разных особей; A1–A3 — обзор дистального участка; срезы толщиной 0,7 мкм выбраны с интервалом 5 мкм; B1–B5 — дистальный конец семенника; срезы толщиной 0,4 мкм выбраны с интервалом 2 мкм; белые стрелки указывают на митотические клетки. C — *Enoplus brevis*, срез дистального конца с хорошо заметными ядрами соматических эпителиальных клеток (белые стрелки). in — кишечник; sg — сперматогонии. Масштаб: A1–A3, B1–B5 — 10 мкм; C — 5 мкм.
clumps of chromatin (Figs. 2C, 3, 4A). The cytoplasm of the TEC is transparent and contains mitochondria, rare Golgi bodies, cisterns of rough endoplasmic reticulum (RER), ribosomes and bundles of filaments (Fig. 4A, B). Between the nucleus-containing cell bodies 0.1 µm thick flattened cytoplasmic outgrowths run along the basal lamina so that the spermatogonia are isolated from the basal lamina throughout the testis (Figs. 1B, 4A, B). Occasional dense clumps are detected under the TEC plasmalemma where it joins intimately the basal lamina (Fig. 5B, insert). Each clump is associated with a bundle of filaments and may be interpreted as epithelial hemidesmosomes.

At the very distal tip of the testis the TEC form long outgrowths protruding deeply into the testis in-between spermatogonia (Figs. 1B, 4A, B). The outgrowths squeezed between spermatogonia may be only 30 nm thick; the structures reminiscent of gap junctions are found occasionally in areas where TEC outgrowths contact spermatogonia (Fig. 4A, B, inserts). The dense bundles of filaments are typical for cytoplasm of TEC and their outgrowths (Fig. 4A, B). The spermatogonia found in the testis germinal zone are uniform polygonal cells of 9–11 µm in size (Figs. 2, 3, 4A, B). The spherical or oval nucleus contains dense clumps of chromatin and a nucleolus; the thin layer of cytoplasm surrounding the nucleus contains clusters of mitochondria, free ribosomes, rare Golgi bodies, and cisterns of RER (Figs. 4A, B, 5A).

The testis germinal zone judged by the uniformity of germ cell nuclei and the occurrence of mitoses in both species is roughly 100–150 µm long. Here metaphase plates are found indicating mitotic proliferation of spermatogonia (Figs. 2B, 5A). Further away from the distal tip a switch to meiosis becomes evident in germ cells. An increase of components with synthetic function (RER, Golgi bodies, ribosomes) and the appearance of synthesis products (numerous vesicles with dense content) marks the stage of early primary spermatocytes (Fig. 5B). The TEC in the area of spermatocytes are flattened and each has a disc-shaped nucleus; the cells form no outgrowths into the testis cavity (Fig. 5B).

### Discussion

The earlier observations on the female and male gonads of two *Enoplus* species (Yushin, Malakhov, 1997, 1998) and new data presented above demonstrate the absence of a morphologically differentiated cell comparable to the DTC in nematodes of the orders Rhabditida and Chromadorida, both from class Chromadorea sensu De Ley and Blaxter, 2002. In *Enoplus* spp., presenting the subclass Enoplea, the continuous epithelium at the distal tip of the testes and ovaries consists of multiple cells with no distinct differences in size and structure. The close morphological association of epithelial and germ cells in *Enoplus* testes is caused by long narrow extensions of epithelial cells penetrating between spermatogonia.

The multiple uniform cells of the distal tip epithelium in the *Enoplus* spp. are in line with many other structural and developmental plesiomorphic features of Enoplida nematodes including early indeterminate cleavage, late establishment of bilateral symmetry and late separation of the germ line (Malakhov, 1994; Voronov, Panchin 1998; Voronov, 1999; Felix, 2004; Joshi, Rothman, 2005). In enoplids no eutely (cell constancy) characteristic of rhabditids like *C. elegans* is found, and they are even capable of limited regeneration (Malakhov, 1994, 1998; Rusin, Malakhov, 1998). In contrast to all other studied nematodes the spermatozoa of enoplids possess a distinct nuclear envelope as a plesiomorphic character (Yushin, Malakhov, 2004, Afanasiev-Grigoriev, Yushin, 2009). The multiple epithelial cells in the distal tip region of the *Enoplus* spp gonad in contrast to the large single DTC of chromadorean species may be considered as one more argument for the basal position of the order Enoplida in the system of the phylum Nematoda. This position is confirmed also by latest molecular phylogenetic analyses of nematode relationships (van Megen *et al*., 2009; Bik *et al*., 2014; Blaxter, Koutsovoulos, 2014).

Important peculiarity in nature of gonadal epithelium is known for several parasitic orders of the class Enoplea: Dioctophymatida, Trichiellida, Marimermitida and Benthimermith—
Fig. 3. *Enoplus brevis*, distal tip of the testis, longitudinal section, STEM. Asterisk marks testis blind end; black arrows show nuclei of testis epithelial cells.
Abbreviations: bc — body cavity; in — intestine; sg — spermatogonia. Scale bar: 5 µm.

ida (Foer, 1983a, b; Bird, Bird, 1991; Miljutin, 2014a, b). They have so called ‘hologonic’ gonads where GSC are proliferated from a series of germinal areas extending the length of the gonad unlike ‘telogonic’ gonads of chromadorean taxa and *Enoplus* where GSC proliferation is confined to the distal end of the testis or ovary. In hologonic gonads development of germ cells is radial across the gonad and structure of GSC niche is obscure. The epithelial cells regulating mitosis/meiosis decision must be multiple and spreading along the gonad.
DTC in the ovaries of the rhabditid nematodes *C. elegans* and *Pristionchus pacificus* Sommer, Carta, Kim et Sternberg, 1996 are relatively large cells with long, protruding processes running along the gonad periphery to envelope the spherical oogonia (Hall et al., 1999; Rudel et al., 2005). The processes are about 20-fold longer than the diameter of an oogonium (Crittenden et al., 1994; Rudel et al., 2005). The processes terminate at the point where the mitotic germinal zone changes over to the transition zone where cells start to enter meiosis (Rudel et al., 2005; Hansen, Schedl, 2013; Kim et al., 2013). Between processes of the DTC the gonadal wall is reduced to a basal lamina. The DTC also form short processes protruding between several most distally positioned germ cells (Kimble, Crittenden, 2007; Hansen, Schedl, 2013). No other epithelial cells are found in the mitotic region. Thus, the single DTC is the only somatic cell at the rhabditid gonad tip. The male gonads (testes) in dioecious species of nematodes possess a DTC as well as a potential GSC niche (Wright, 1991). The study in the free-living marine nematode *Para-cyatholaimus pugettensis* Wiener et Hopper, 1967 (Chromadorida) shows distinct DTC in the germinal zone of the testis as a large elongated cell containing a nucleus with dispersed chromatin and a well developed nucleolus (Zograf, 2010). The DTC of *P. pugettensis* also forms long cytoplasmic outgrowths covering mitotically active GSC.

Ultrastructural data of the rhabditid and chromadorid DTC revealed their synthetic activity (Foor, 1983; Rudel et al., 2005; Kimble, Crittenden, 2007; Zograf, 2010). They possess a nucleus with dispersed chromatin and a well developed nucleolus. The DTC cytoplasm contains ribosomes, cisterns of RER, Golgi bodies and vesiicles. In the DTC of *P. pacificus* large transparent vesicles are concentrated in the cell periphery which contacts the basal lamina (Rudel et al., 2005). It was shown that in *C. elegans* the DTC controls the mitosis/meiosis decision in germ cells via Notch/LIN-12 signalling (Kimble, Crittenden, 2007; Kershner et al., 2013) keeping germ cells near the DTC in a mitotic state (Hansen et al., 2004).

In *Enoplus* the structure of epithelial cells at the distal tip of the testis gives no evidence for high metabolic activity. They rather look as if they had a structural rather than a glandular or hormonal function. At the same time, the consecutive events in the gonads of chromadorean nematodes and *Enoplus* spp. are comparable: the germinal zone of the GSC niche is followed by germ cells at successive stages of meiosis. In both cases the germinal zone contains a small cluster of cells which conserve mitotic activity (Hansen, Schedl, 2013; Kim et al., 2013). In *C. elegans* short DTC processes embrace the distalmost germ cells in the GSC niche (Kimble, Crittenden, 2007). It is surmised that these germ cells receive the strongest signal and that the short processes surrounding these germ cells anchor them within the niche (Hall et al., 1999).

In *Enoplus* spp. the epithelial cells of the distal tip have characteristic outgrowths which run into the testis between spermatogonia (Fig. 1B).

**Fig. 4. Enoplus michaelseni,** distal tip of the testis, longitudinal sections, TEM.

A — epithelial cell with outgrowths (asterisks) protruding between spermatogonia; black arrowheads mark bundles of filaments. B — spermatagonia at the distal tip of the testis are closely associated with testis epithelial cells and their outgrowths (asterisks). Inserts in A and B (high magnification views of areas surrounded by dotted squares): contacts (black arrowheads) of spermatogonium and testis epithelium. Abbreviations: bc — body cavity; bl — basal lamina; mc — mitochondria; ne — nucleus of the testis epithelial cell; ns — nucleus of spermatogonium; nu — nucleolus. Scale bars: A, B — 2 µm; inserts — 0.5 µm.

**Рис. 4. Enoplus michaelseni,** дистальный конец семенника, продольный срез, TEM.

А — эпителиальные клетки с отростками (звездочки) проникающими между сперматогониями; черные наконечники указывают на пучки волокон. Б — у дистального конца семенника сперматогонии находятся в тесном контакте с эпителиальными клетками и их отростками (звездочки). Врезки на А и В (увеличенное изображение участков, отмеченных пунктирными прямоугольниками): контакты (черные наконечники) сперматогониев с эпителием семенника. bc — полость тела; bl — базальная пластинка; mc — митохондрии; ne — ядро эпителиальной клетки семенника; ns — ядро сперматогония; nu — ядрышко. Масштаб: A, B — 2 мкм; врезки — 0.5 мкм.
The male gonad of the marine nematode Enoplus
Fig. 5. *Enoplus michaelseni* testis, longitudinal sections, TEM.
A — germinal zone, the mitotic spermatogonium (asterisk) with chromosomes in mitotic metaphase. B — zone of early spermatocytes; insert — hemidesmosome-like structures (white arrowheads) joining the epithelial cell to basal lamina. Abbreviations: bc — body cavity; bl — basal lamina; ch — chromosomes; er — rough endoplasmic reticulum; Gb — Golgi bodies; mc — mitochondria; nc — nucleus of spermatocyte; ne — nucleus of the testis epithelial cell; ns — nucleus of spermatogonium; nu — nucleolus; tec — testis epithelial cell. Scale bars: A, B — 2 µm; insert — 0.5 µm.

Рис. 5. Семенник *Enoplus michaelseni*, продольные срезы, TEM.
A — гемеринальная зона, митотический сперматогоний (звездочка) с метафазными хромосомами. B — зона ранних сперматоцитов; врезка — полудесмосомы (белые наконечники), присоединяющие эпителиальные клетки к базальной пластинке. bc — полость тела; bl — базальная пластинка; ch — хромосомы; er — шероховатый эндоэлазматический ретикулум; Gb — тельца Гольджи; mc — митохондрии; nc — ядро сперматоцита; ne — ядро эпителиальной клетки семенника; ns — ядро сперматогония; nu — ядрышко; tec — эпителиальной клетки семенника. Масштаб: A, B — 2 мкм; врезка — 0,5 мкм.
While this morphological peculiarity of the TEC is compatible with a role in regulating germ cell differentiation, the absence of visible markers for a secreting function does not support this assumption.

However multiplicity and small size of TEC in *Enoplus* testes do not change general situation with germ cell mitosis/meiosis decision and arrangement of germ cells as successive stages along testis which is the same as in testes of the nematodes with actively functioning large DTC. From morphological point of view TEC must be considered as homologue to DTC and can provide male gonad with the same function. Hormonal activity of *Enoplus* TEC cannot be categorically omitted without molecular studies. Low physiological value of each individual TEC may be compensated by their multiplicity together with their close contacts with GSC. Taking this assumption in account we can hypothesize that single DTC of chromadorean nematodes may be evolved from primarily multicellular gonadal epithelium which may be illustrated by the multiple TEC of the distal tip of *Enoplus* testis. The evolution trend from undetermined multicellularity to highly determined eutely inevitably leads to reduction of cell number and concentration of cellular function in a few or even in a single cell.

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**References**


Blaxter M., Koutsovoulos G. 2014. The evolution of parasitism in Nematoda // Parasitology. DOI: 10.1017/S0031182014000791


Kimble J., Crittenden S.L. 2007. Controls of germline
Kimble J. 2011. Molecular regulation of the mitosis/
Kim S., Spike C., Greenstein D. 2013. Control of oocyte
Kimble J., Crittenden S.L., Friend K., Sorensen E.B.,
Joshi P.M., Rothman J.H. 2005. Nematode gastrulation:
Malakhov V.V. 1994. Nematodes: Structure, Develop-
handbook/reproductivesystem/
MacKinnon B.M. 1987. A light and electron microscopical
investigation of fertilization, chromosome behaviour,
Kimble J.E., White J.G. 1981 On the control of germ cell
development in Caenorhabditis elegans // Develop-
Wright K.A. 1991. Nematoda // F.W. Harrison, E.E. Rup-
Yushin V.V., Malakhov V.V. 2004. Spermatogenesis and
Zograf J.K. 2010. Ultrastructure of the male reproductive

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Joshi P.M., Rothman J.H. 2005. Nematode gastrulation:
Kershner A., Crittenden S.L., Friend K., Sorensen E.B.,
Kim S., Spike C., Greenstein D. 2013. Control of oocyte
Kimble J. 2011. Molecular regulation of the mitosis/
meiosis decision in multicellular organisms // Cold Spring Harbor Perspectives in Biology. 3: a002683.
Kimble J., Crittenden S.L. 2007. Controls of germline stem cells, entry into meiosis, and the sperm/oocyte
Kimble J.E., White J.G. 1981 On the control of germ cell
development in Caenorhabditis elegans // Develop-
handbook/reproductivesystem/
MacKinnon B.M. 1987. A light and electron microscopical
investigation of fertilization, chromosome behaviour,
Kimble J.E., White J.G. 1981 On the control of germ cell
development in Caenorhabditis elegans // Develop-
handbook/reproductivesystem/
MacKinnon B.M. 1987. A light and electron microscopical
investigation of fertilization, chromosome behaviour,