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Ovarian structure in *Milnesium tardigradum* (Tardigrada, Milnesiidae) during early vitellogenesis

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Abstract

The ultrastructure of the ovary of *Milnesium tardigradum* during early vitellogenesis is described. Within the ovary, there were large multinuclear cells surrounded by many mononuclear oocytes. Observation of serial sections revealed four multinuclear cells that were connected to each other by cytoplasmic bridges. Each peripheral oocyte was connected to the multinuclear cell. An enormous ER-like structure was conspicuous in the centre of the multinuclear cell. The presence of large numbers of lipid droplets and yolk granules in both multinuclear cells and many mononuclear oocytes suggested a role as nurse cells. A small number of these oocytes grow to be eggs. The structural features of the multinuclear nurse cell were compared with other known examples.

Introduction

Milnesium tardigradum Doyère, 1840 is a carnivorous tardigrade living in moss and lichen. This species grows into mature adults after the second moulting (Baumann, 1964; Suzuki, 2003). Egg laying accompanies the moulting process, and the eggs are laid before ecdysis in the space between the old and new cuticles. The female remains within the old cuticle, along with the developing embryos, for several hours. Under a rearing environment, the egg-laying/moulting intervals are around 6–10 days, and up to five clutches occur in the average life history. The number of eggs in a clutch ranges from 1 to 18 (Marcus, 1928; Suzuki, 2003).

A possible factor that influences clutch size is the nutritional condition of the female. The clutch size is possibly determined during vitellogenesis, and the reproductive strategy of tardigrades is to maximise their offspring. Hence, the relationship between oocytes and other ovarian cells is to be clarified but ultrastructural data from tardigrade ovaries are limited (Weglarska, 1979, 1987; Dewel et al., 1993). In this study, the fine structures of the ovary in *M. tardigradum* during early vitellogenesis are described and newly discovered characteristics are discussed.

Materials and methods

A parthenogenetic strain of *Milnesium tardigradum* was kept in continuous culture and fed on rotifers as previously described (Suzuki, 2003). Tardigrades were anaesthetised with 0.3% tricaine and partially cut at the anterior region with a fine tungsten needle in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. They were fixed in the same solution for 2 h at room temperature. After being rinsed, specimens were postfixed in 1% osmium tetroxide in the same buffer for 1 h at room temperature. They were dehydrated through a graded series of acetone and embedded in Epon.

For light microscopy, serial semithin sections (200–300 nm thick) were collected and stained with 1% toluidine blue in 1% sodium borate. A three-dimensional shape was reconstructed from these sections using the DeltaViewer program (http://vivaldi.ics.nara-wu.ac.jp/~wada/DeltaViewer/). For transmission electron microscopy (TEM), ultrathin sections were collected on single slot grids supported by Formvar film. They were stained with uranyl acetate and lead citrate, and observed in a JEOL 1010 electron microscope at 80 kV.

Results

The translucent body of tardigrades permitted direct observation of the maturing ovary (Fig. 1). On the day after oviposition, the ovary was so transparent and small that it was difficult to discriminate it from other organs. By day 2, decreasing transparency and increasing size of the ovary implied the beginning of vitellogenesis. The animal shown in Figure 1 ejected the sclerified structures of the buccopharyngeal apparatus and entered the 'simplex' stage at day 6; she laid the next clutch at day 7. Preliminary observations of thin sections from several stages suggested that several multinuclear large cells were present in each ovary (data not shown.). Thin sections were then collected at several points of serial semi-thin sections of an ovary, the condition of which was similar to the day 3 ovary of Figure 1 and similar to the stage II gonad according to Rebecchi & Bertolani (1994). Figures 2–4 show the TEM pictures of these sections. The ovary was delineated by a basal lamina lined by a single discontinuous layer of thin epithelial cells. The anterior part of the ovary continued to terminal filaments (Fig. 4 tf) that suspended it from the dorsal body wall, and the posterior part of the oviduct (Fig. 4, od) led towards the rectum. Many germ cells were present in the ovary and are described in the following sections.

In the central region of the ovary, there were large multinuclear cells surrounded by many mononuclear oocytes. Observation of serial semithin sections revealed that there were four multinuclear cells, which were connected to each other through cytoplasmic bridges (Figs. 2 and 4, arrows). The cytoplasmic bridges were approximately 4 μm wide and characteristic ring canal structures were observed as in Figure 5 (asterisks). The other ring canal structures in this section of

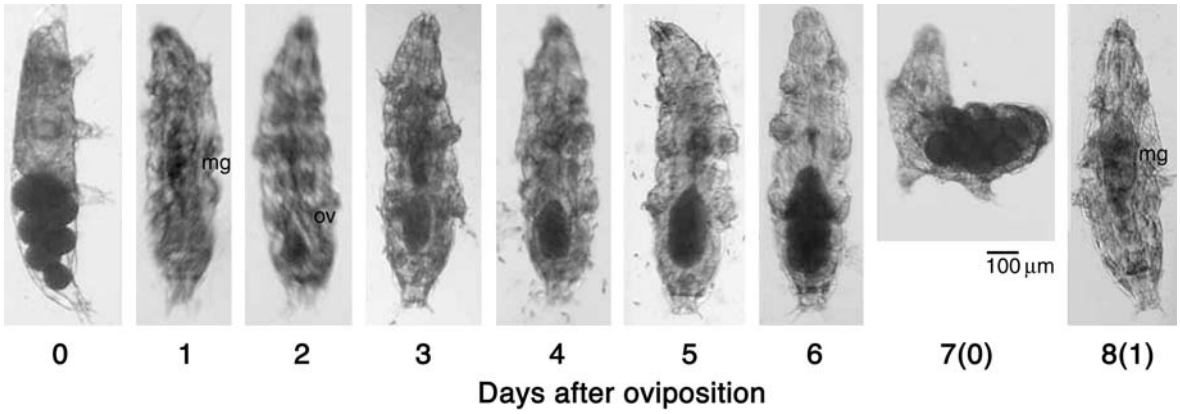
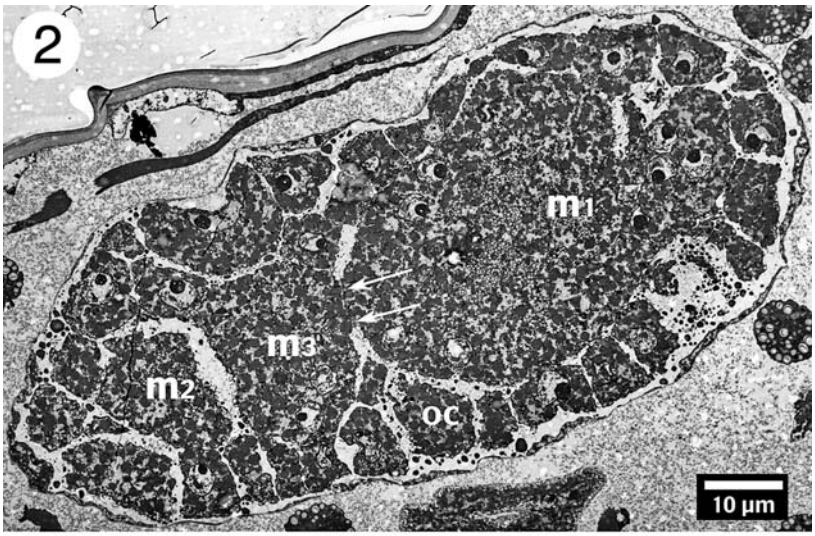


Figure 1. Ovarian maturation of an adult individual of *Milnesium tardigradum*. Images of a live animal under a dissecting microscope were captured daily. Each numeral under the pictures indicates the day after the previous oviposition. mg, midgut; ov, ovary.

Figure 2–4. Ovarian structure of *Milnesium tardigradum* at early vitellogensis. TEM images of sagittal thin sections of an ovary, similar in condition to the day 3 ovary in Figure 1. Anterior is to the right. Multinuclear cells (m1–m4) are surrounded by many mononuclear oocytes. *Figure 2*. Two multinuclear cells (m1 and m3) are connected to each other through a cytoplasmic bridge (arrows). Growing oocyte (oc) is surrounded by electron-dense bodies. *Figure 3*. An enormous ER-like structure (arrow) occupied the centre of the multinuclear cell (m1). A large number of lipid droplets accumulate around it. *Figure 4*. Arrows indicate the cytoplasmic bridge connecting two multinuclear cells (m2 and m4). The anterior part of the ovary continued to the terminal filament (tf). At the posterior end of the ovary, the epithelial cells form the oviduct (od). oc, growing oocyte.


2
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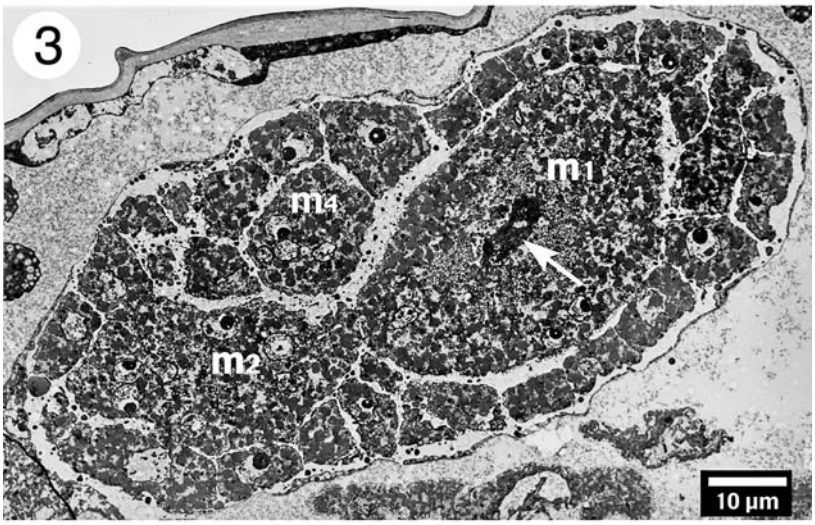
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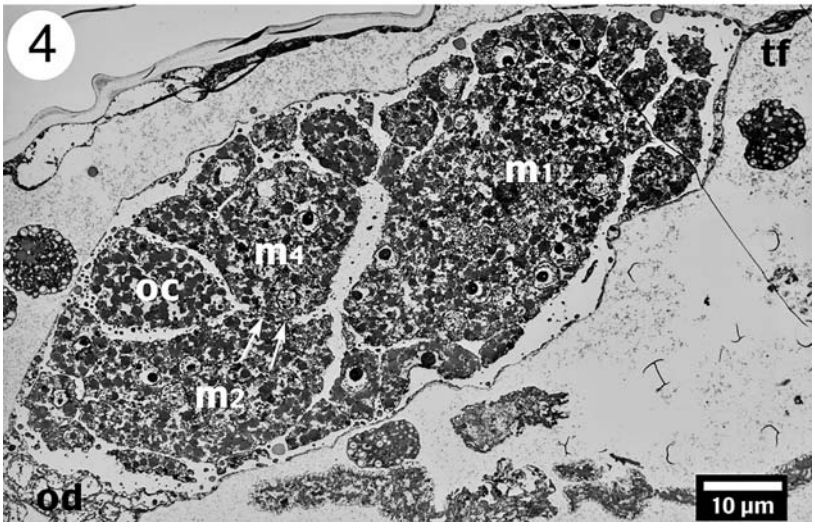
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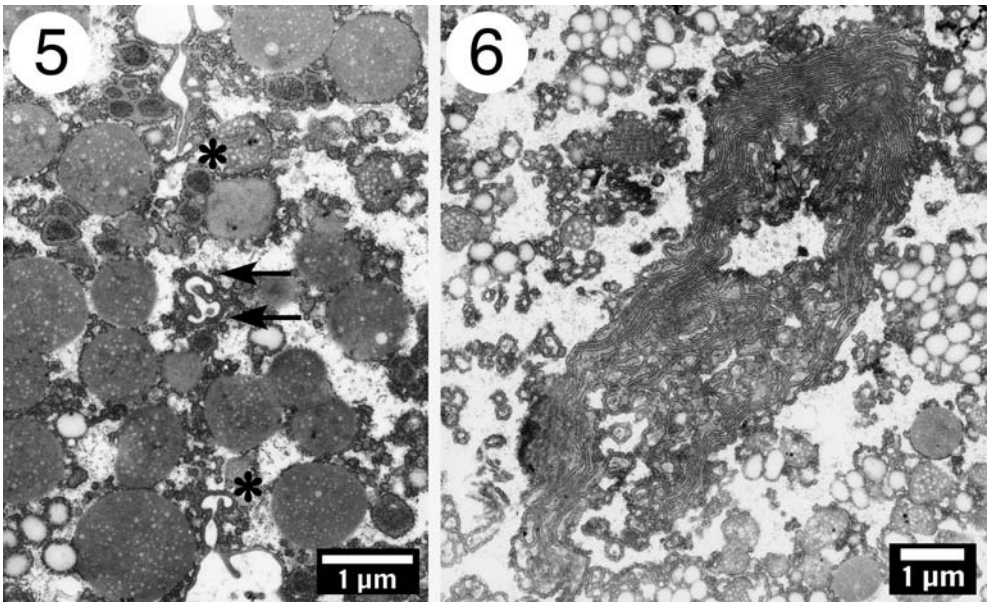


Figure 5. Cytoplasmic bridge connecting multinuclear cells in the ovary of *Milnesium tardigradum*. Higher magnification of the cytoplasmic bridge seen in Figure 2. Two pairs of ring canal structures (asterisks and arrows) suggest an undulate form of the bridge. *Figure 6*. ER-like structure in the centre of the multinuclear cell of *Milnesium tardigradum*. Higher magnification of the structure seen in Figure 3. A large number of small vesicles and lipid droplets are recognized around the structure.

the cytoplasmic bridge (Fig. 5 arrows) suggested an undulate form of the canal. The multinuclear cells were connected as schematically shown in Figure 7; the cytoplasmic bridges between multinuclear cells 1 and 2, 1 and 3, and 2 and 4 were visible. A three-dimensional image of the multinuclear cells (Fig. 8) was obtained by reconstruction of drawings of the outline traced at every 5th section (ca. 1.25 μm thick).

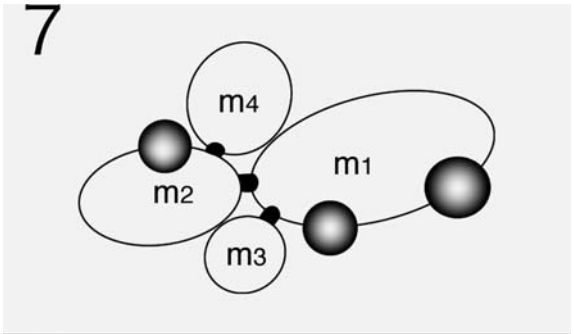
Nuclei were located near the cell surface and the number of nuclei counted in multinuclear cells 1, 2, 3 and 4 were 74, 22, 12 and 21, respectively. Each nucleus, the diameter of which was about 3.5 μm, had a prominent nucleolus.

An enormous ER-like structure occupied the centre of the multinuclear cells (Fig. 3, arrow; Fig. 6), around which a large number of lipid droplets accumulated. Fragments of membranous structures and mitochondria were scattered in the cells. Electron-dense yolk granules were mostly situated in the outer zone of the cytoplasm.

Each mononuclear oocyte connected to one of the multinuclear cells via cytoplasmic bridges that were 1.6–1.8 μm wide. The numbers of oocytes counted around multinuclear cells 1, 2, 3 and 4 were 52, 12, 16 and 24, respectively. The nuclei and cytoplasm of most oocytes appeared similar to those of the multinuclear cells. However, several larger oocytes were distinguishable from the others as they had larger nuclei of about 7 μm in diameter. Microvilli were observed at the cell surface of these large oocytes as well as in the multinuclear cells. Electron-dense granules surrounded three of the large oocytes (Figs. 2 and 4, oc; Fig. 9). This suggests that these three cells were growing oocytes, as the electron-dense bodies may be future chorion (Figs. 10 and 11). The relationship between the growing oocytes and the multinuclear cells is shown schematically in Figure 7.

Discussion

Many studies, especially on arthropods, have observed phylogenetic patterns in the features of ovarian structures and in modes of oogenesis. For example in insects, the structural diversity of the ovary has been categorised into three types – panoistic, polytrophic meroistic, and telotrophic meroistic, and their phylogenetic correlation has been discussed (King & Büning, 1985; Büning, 1994). The structures of the ovary of chelicerates have been found to be very different from those of other arthropods (Makioka, 1988).



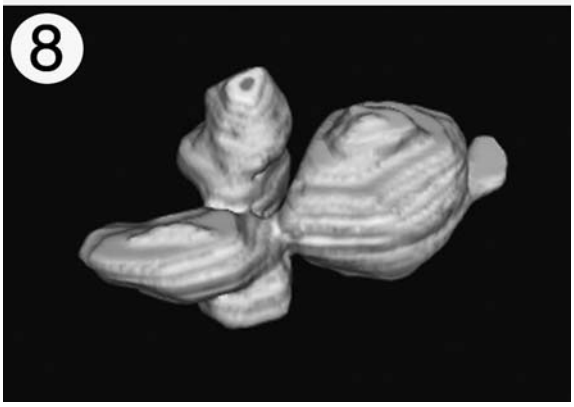
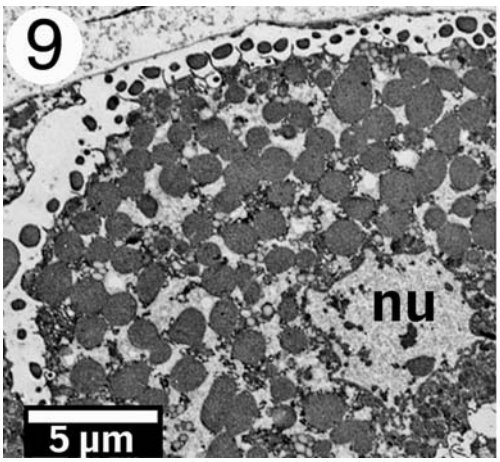
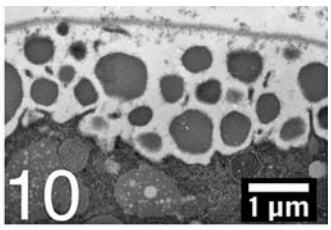


Figure 7. Schematic illustration of the multinuclear cells (m1–m4) interpreted from serial sections of the ovary of *Milnesium tardigradum*. Although the multinuclear cells were surrounded by many mononuclear oocytes, each of which connected to one of the multinuclear cells via cytoplasmic bridge, only growing oocytes are shown as gray balls in this figure.
Figure 8. Computer-reconstructed 3D image of the multinuclear cells in the ovary of *Milnesium tardigradum*. This image was obtained by reconstruction of drawings of the outline traced at every 5th semi-thin section.

With regard to tardigrades, few studies have described the ultrastructure of the ovaries. Weglarska (1979) studied the oogenesis of *Macrobiotus richtersi* Murray, 1911 by TEM and reported on the polytrophic meroistic nature of the ovary, in which a form of cystocyte, which consists of seven nurse cells attached to one oocyte, is formed during oogenesis. However, the description of the cystocyte was based on observation of a fresh specimen by light microscopy without ultrastructural data. Weglarska (1987) reported the same mode of oogenesis for *Isohypsibius granulifer* (Thulin, 1928), also lacking the ultrastructural data on the issue. Dewel et al. (1993) described the ultrastructure of the ovaries of *Milnesium tardigradum* and marine heterotardigrade *Halechiniscus perfectus* Schulz, 1955. In these species, there is a central multinucleate mass surrounded by peripheral mononuclear cells, which are nurse cells and oocytes. Whether or not the difference between these reports reflects the phylogenetic divergence of tardigrades has yet to be examined. In the present study, four multinuclear cells were found in the ovary of *Milnesium tardigradum*. The difference in *M. tardigradum* gives rise to the question whether it reflects diversity between strains of the species, but there are not enough data to answer this question.





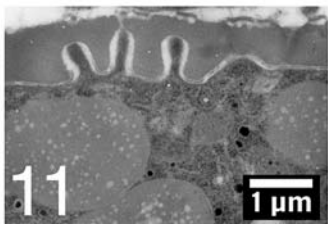


Figure 9–11. Electron-dense materials around the growing oocytes of *Milnesium tardigradum*. *Figure 9*. A growing oocyte seen in a section of the same series as Figures 2–4. Many microvillous projections as well as electron-dense bodies are seen at the cell surface. nu, nucleus. *Figure 10*. A part of a growing oocyte in a more matured ovary. Electron-dense bodies are accumulated around the cell surface. *Figure 11* The electron-dense materials are unified to become chorion in the animal at simplex stage.

Multinuclear nurse cells are also found in other animals, for example, in Arthropoda (Matsuzaki & Ando, 1977; Walzl, 1992) and Rotifera (Bentfeld, 1971; Amsellem & Ricci, 1982). King & Büning (1985) presented a model of cluster formation as the origin of the syncytium in telotrophic meroistic types of ovaries. In this model, many central oogonia may fuse to form a syncytium. As for *M. tardigradum*, however, the unique construction in which four distinct multinuclear cells are connected by cytoplasmic bridges makes it difficult to consider the cell fusion model. A possible explanation for the origin of the multinuclear cells of *M. tardigradum* is that an oogonium might divide twice, incompletely, to create a 4-cell complex. Cell development and multinucleation may occur subsequently. Nevertheless the cell fusion model cannot be discarded because of the complex shape of the ring canal structures.

The peripheral oocytes are also connected to multinuclear cells. It is not known how these oocytes are generated. In the rotifer *Asplancna*, oocytes appear to have budded out from the multinuclear vitellarium, and the bridges joining oocytes to vitellarium are believed to be formed secondarily (Bentfeld, 1971). However, there is no evidence that such secondary formation of the bridges actually takes place. It is an interesting issue to consider the cell-cycle regulatory mechanism during differentiation of oocytes and multinuclear cells. During early vitellogenesis as observed in the present study, the nuclei of oocytes and multinuclear cells, as well as the cytoplasm, mostly exhibited stage synchrony. Although the enormous ER-like structure with abundant lipid droplets and yolk granules in the multinuclear cells shows its dominant role in vitellogenesis, many mononuclear oocytes can also function as nurse cells. Some of these develop to be eggs. What decides the fate of these oocytes, and how are materials selectively delivered to the growing oocytes? As this mechanism may also determine the clutch size, it may have an important role in the reproductive strategy of tardigrades and merits further investigation.

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