

[ORIGINAL PAPER]

# Comparative Morphology of Ovarian Follicle in Two Polydesmid Millipedes: Evolutionary Implication of Folded Structure of Follicle Epithelium in Polydesmida (Myriapoda: Diplopoda)

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## Abstract

In animals, folding of the epithelial tissue plays crucial roles in morphogenesis, development, and diversification. In Polydesmida (Myriapoda: Diplopoda), the ovarian follicle has a folded structure of the epithelium. However, the nature and evolutionary process of the follicle epithelium remain unknown. To elucidate the morphological nature of the folded structure of the follicle epithelium in Polydesmida, we examined the ultrastructure of the ovarian follicle in *Epanerchodus orientalis* (Polydesmidae), which possesses a folded follicle, and *Eutrichodesmus elegans* (Haplodesmidae), which has an unfolded follicle. In both species, the follicle epithelium continued to the ovarian epithelium as a continuous layer of basement membrane. Hemocytes were detected within the follicle in both species. In *Ep. orientalis*, each folded follicle contained a single oocyte, while, in contrast, each unfolded follicle in *Eu. elegans* contained multiple oocytes. To our knowledge, this is the first study to report a single ovarian follicle containing multiple oocytes in animals. Our results also suggest that there are at least three types of ovarian follicle in Diplopoda: unfolded pouch containing a single oocyte, folded pouch containing a single oocyte, and unfolded sac containing multiple oocytes. Here we discuss the evolution of the folded structure of the follicle epithelium in Polydesmida.

## Introduction

Epithelial tissue is an animal-specific tissue that is essential for morphogenesis, development, and morphological evolution, i.e. many organs such as the epidermis, alimentary canal and gonads consist of epithelial tissue in animals. Folding of the epithelial tissue enables the formation of various complex structures (Davidson, 2012). It occurs in the ectodermal and endodermal epithelium of invertebrates and vertebrates: e.g., imaginal discs in some holometabolous insects (Beira and Paro, 2016), neural tube formation in vertebrates (Nikolopoulou et al., 2017), and the inner wall of alimentary canal in some animals (Štorgel et al., 2016). While much is known about these specific structures, the folded structure of the mesodermal epithelium is less understood in general.

An example of folding of the mesodermal epithelial tissues can be found in the myriapod ovary. In Myriapoda, the ovary comprises two mesodermal epithelia: the epithelium of the ovarian wall and the follicle epithelium surrounding the oocyte. The myriapod oocyte grows within the hemocoelic space of the ovarian follicle (Symphyla: Yahata et al., 2018; Chilopoda: Miyachi and Yahata, 2012; Diplopoda: Kubrakiewicz, 1991a). In addition, each myriapod oocyte generally grows individually,

within a single ovarian follicle (Sareen and Adiyodi, 1983; Kubrakiewicz, 1991a). Yahata (2012) histologically analyzed the ovarian structure of polydesmid millipedes and reported morphological diversity of the ovarian follicle in Polydesmida (Myriapoda: Diplopoda). In *Epanerchodus orientalis* (Polydesmida: Polydesmidae), the oocyte within the ovarian follicle develops along the oogenetic stages. The germ zone contains many closely packed ovarian follicles, and is present in the ovarian lumen. Therefore, the follicle epithelium surrounding the oocyte is intricately folded within the germ zone. In *Eutrichodesmus elegans* (Polydesmida: Haplodesmidae), the oocyte develops along the growth stage like *Ep. orientalis*, but the follicle epithelium is an unfolded structure in the germ zone. Yahata (2012) suggested that the folded structure of the ovarian follicle in the germ zone of *Ep. orientalis* was due to the greater number of eggs than *Eu. elegans*.

These polydesmid millipedes are expected to be an efficient model for the morphological evolution of folding of mesodermal epithelium in higher taxa due to the presence of both the folded and the unfolded structures of the follicle epithelium. Nevertheless, our knowledge of the morphological

features of the ovarian follicle in Polydesmida is limited to only a few histological observations (Crane and Cowden, 1968, Yahata, 2012, Pinheiro et al., 2013), and there have been no ultrastructural studies so far.

Therefore, to understand the nature of the folded and the unfolded structures of follicle epithelia in Polydesmida better, we examined the gross morphology and the ultrastructure of the ovarian follicle in *Ep. orientalis* and *Eu. elegans*, and discuss their morphological evolution. The unusual features of the ovarian follicle in these species are also described.

## Materials and Methods

### Animals

Adult *Epanerchodus orientalis* Attems, 1901 were collected from leaf litter at Ueda City, Nagano Prefecture, Japan, in August 2015 by hand sorting. Adult *Eutrichodesmus elegans* (Miyosi, 1956) were collected from leaf litter at Sado City, Niigata Prefecture, Japan, in May 2016 by shifting survey. Animals were kept in plastic containers at room temperature.

### Histological and ultrastructural analysis

In *Ep. orientalis*, ovaries were dissected from the bodies and cut into blocks. In *Eu. elegans*, the whole bodies with the ovaries were cut into blocks. Samples were prefixed with 2.5% glutaraldehyde in phosphate buffer (pH=7.2) for 3 h at room temperature, and post-fixed with 1% OsO<sub>4</sub> in the same buffer for 1 h at 4°C. Fixed samples were dehydrated in a graded acetone series. Dehydrated samples were immersed and embedded in Quetol-651 resin (Nisshin EM, Tokyo, Japan). Resin blocks containing the samples were polymerized for 60 h at 60°C. Next, semi-thin (800 μm in thickness) and ultrathin (50–80 nm) sections were prepared using glass knives and a diamond knife on an ultramicrotome (EM UC7: Leica, Tokyo, Japan). The semi-thin sections were stained with methylene blue and observed under a light microscope (BH-2: Olympus, Tokyo, Japan). Ultrathin sections were observed under a transmission electron microscope (H-7650: Hitachi, Tokyo, Japan) equipped with a CCD camera (Velta 2kx2k: Olympus, Tokyo, Japan) at 80 kV.

## Results

### *Epanerchodus orientalis* (Polydesmidae)

#### Gross morphology of the ovarian follicle

In the ovary of *Ep. orientalis*, each previtellogenic oocyte had large nucleoli in the germinal vesicle and was surrounded by a single layer of follicle epithelium (Fig. 1A, B, D). Each vitellogenic oocyte containing large yolk granules was also covered with follicle epithelium (Fig. 1A, C, E). The female germ cells were closely packed within the folding of the follicle epithelial layer along the germ zone (Fig. 1A). The previtellogenic oocyte was surrounded by a thin layer of homogenous vitelline membrane, 30–100 nm in thickness (Figs. 1D, 2B). The vitellogenic oocyte was surrounded by a double layer of vitelline membrane. The outer layer was a homogenous structure, 70–100 nm in thickness (Figs. 1E, 2C),

while the inner layer was a meshed structure, 0.6–1.8 μm in thickness (Figs. 1E, 2C). These results were identical to those described previously by Yahata (2012).

#### Ultrastructure of the ovarian follicle

The follicle epithelium surrounding the oocyte was a flattened epithelial layer, 0.2–1.5 μm in height. Follicle epithelial cells were connected to each other, with adherence junctions on their apical side (Fig. 2B). In *Ep. orientalis*, the follicle epithelium was in line with its basement membrane along the side of the oocyte (Fig. 2). Many electron-dense materials indicating hemidesmosomes scattered along the contact zone between the epithelial cells and the basement membrane (Fig. 2B, C). Follicle epithelial cells were connected to the ovarian epithelial cells, and the basement membrane was shared between the follicle and the ovarian epithelium (Fig. 2A). Therefore, in *Ep. orientalis*, the follicle epithelium continued to the ovarian epithelium with a continuous layer of basement membrane (Fig. 2A). Some follicle epithelial cells elongated their cytoplasm toward the oocytes (Fig. 2B, C). Hemocytes were detected between the follicle epithelium and the oocyte (Fig. 2D).

The folding of follicle epithelium formed a complex-shaped dent of the ovary containing many oocytes (Fig. 1B–E). Each oocyte in the dent was covered with the follicle epithelium, and was completely separated from the other ones (Fig. 1D, E).

### *Eutrichodesmus elegans* (Haplodesmidae)

#### Gross morphology of the ovarian follicle

In the *Eu. elegans* ovary, previtellogenic and vitellogenic oocytes were surrounded by a single flattened layer of the follicle epithelium (Fig. 3A–C). These female germ cells were closely packed in a paired germ zone (Fig. 3A, B). The previtellogenic oocyte was covered with a single layer of homogenous vitelline membrane of 50–160 nm in thickness (Figs. 3C, D, 4B). The vitellogenic oocyte was covered with a double layer of vitelline membrane. Its outer layer, 50–100 nm in thickness was a homogenous structure (Fig. 4C), in contrast to the meshed structure of its inner layer, 1.0–1.7 μm in thickness (Fig. 4C). These features were identical to those previously described by Yahata (2012).

#### Ultrastructure of the ovarian follicle

The follicle epithelium of *Eu. elegans* was a single flattened layer, 50–650 nm in height. On its apical side, adherence junctions were distributed between the epithelial cells (Fig. 4B). In contrast, on the basal side (the side of the oocyte), the follicle epithelium was in line with the basement membrane, along with hemidesmosomes (Fig. 4B, D). The follicle epithelium continued to the ovarian epithelium with a continuous layer of basement membrane (Fig. 4A). Some follicle epithelial cells elongated their cytoplasm toward the oocytes (Fig. 4B–E). Hemocytes were detected between the follicle epithelium and the oocyte (Fig. 4D).

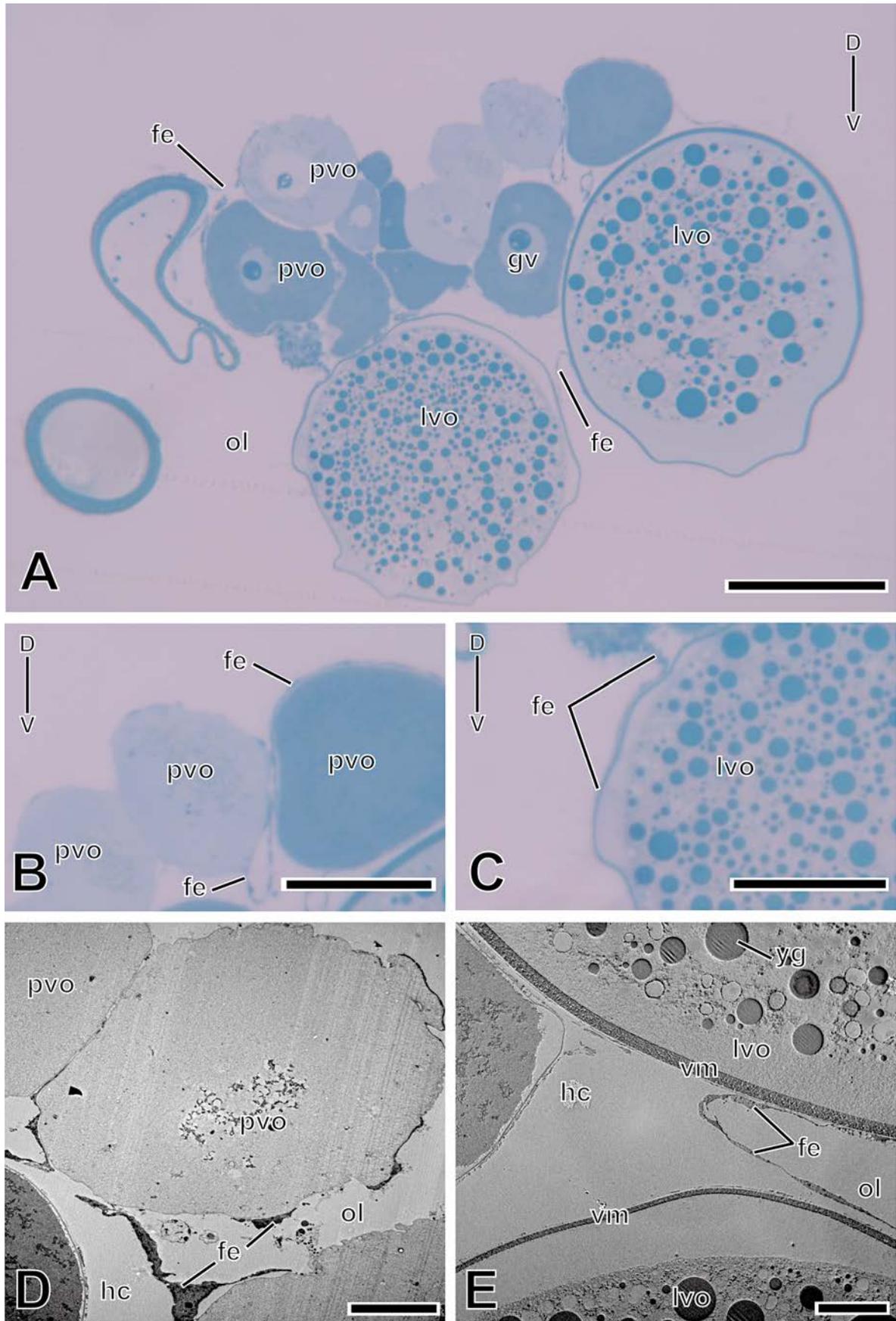


Fig. 1 Gross morphology and ultrastructure of the ovarian follicle in *Epanerchodus orientalis*. (A) Gross morphology of the germ zone. (B–C) Light microscopic image of the folded structure of follicle epithelium in the previtellogenic (B) and vitellogenic (C) stages. (D–E) Ultrastructural image of a folded follicle pouch contained a single oocyte in the previtellogenic (D) and the vitellogenic (E) stages. D: dorsal side, fe: follicle epithelium, gv: germinal vesicle, hc: hemocoel, lvo: late vitellogenic oocyte, ol: ovarian lumen, pvo: previtellogenic oocyte, V: ventral side, vm: vitelline membrane, yg: yolk granule. Scales = A: 100  $\mu$ m, B, C: 50  $\mu$ m, D, E: 10  $\mu$ m.

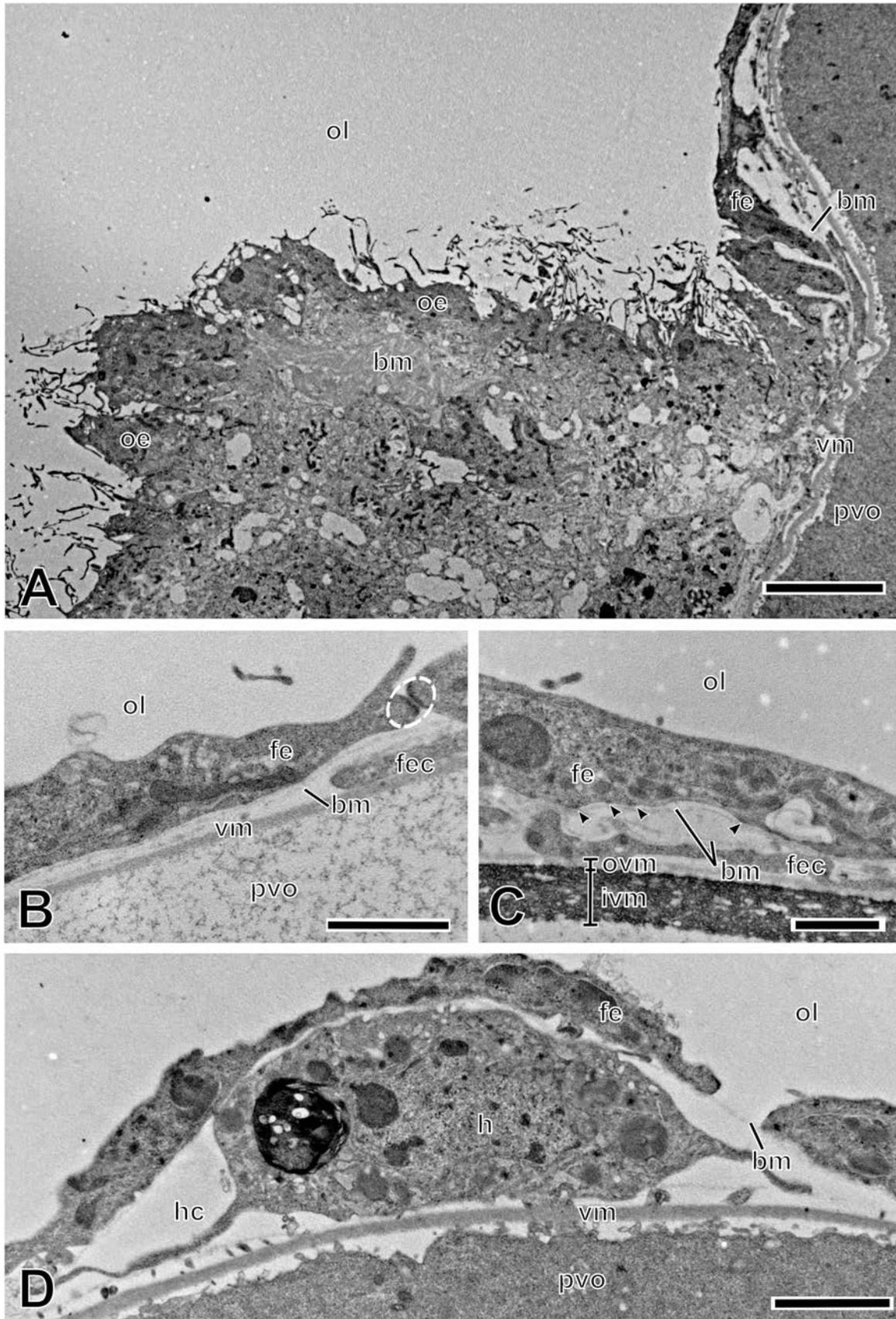


Fig. 2 Ultrastructure of the ovarian follicle in *Epanerchodus orientalis*. (A) Continuity between the ovarian epithelium and the follicle epithelium with a continuous layer of its basement membrane. (B–C) Follicle epithelium in the previtellogenic (B) and vitellogenic (C) stages. (D) Hemocyte within an ovarian follicle. bm: basement membrane, fe: follicle epithelium, fec: elongated cytoplasm of follicle epithelial cell, hc: hemocoel, h: hemocyte, ivm: inner layer of the vitelline membrane, ovm: outer layer of the vitelline membrane, oe: ovarian epithelium, ol: ovarian lumen, pvo: previtellogenic oocyte, vm: vitelline membrane. circle: adherence junction, arrowhead: hemidesmosome. Scales = A: 5  $\mu\text{m}$ , B, C: 1  $\mu\text{m}$ , D: 2  $\mu\text{m}$ .

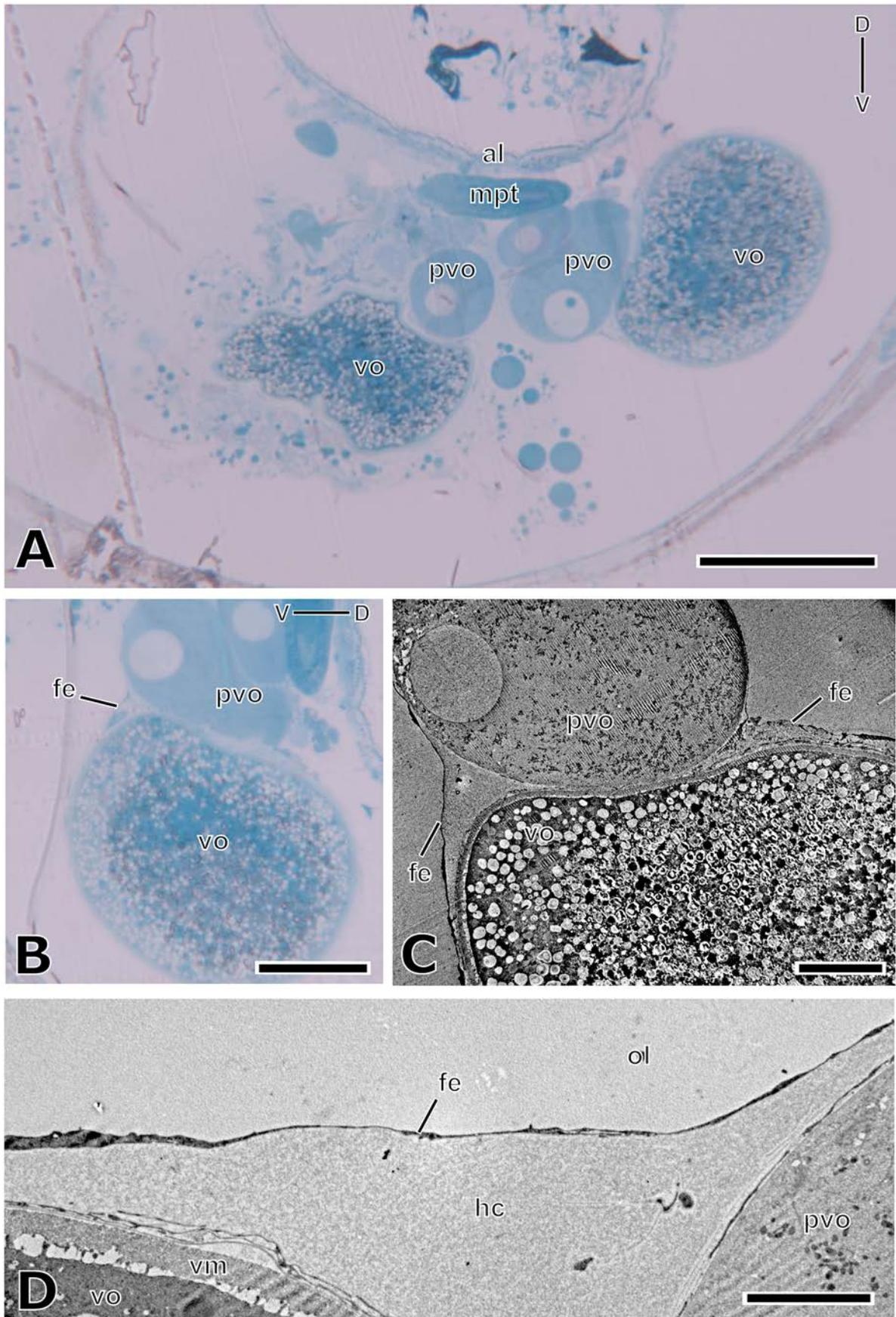


Fig. 3 Gross morphology and ultrastructure of the ovarian follicle in *Eutrichodesmus elegans*. (A) Gross morphology of the germ zone in the ovary. (B) Light microscopic image of the ovarian follicle of the previtellogenic and the vitellogenic stages. (C–D) Ultrastructure of the unfolded ovarian follicle with multiple oocytes at lower (C) and higher (D) magnifications. al: alimentary canal, bm: basement membrane, D: dorsal side, fe: follicle epithelium, hc: hemocoel, mpt: Malpighian tube, ol: ovarian lumen, pvo: previtellogenic oocyte, V: ventral side, vm: vitelline membrane, vo: vitellogenic oocyte. Scales = A: 100  $\mu$ m, B: 50  $\mu$ m, C: 20  $\mu$ m, D: 5  $\mu$ m.

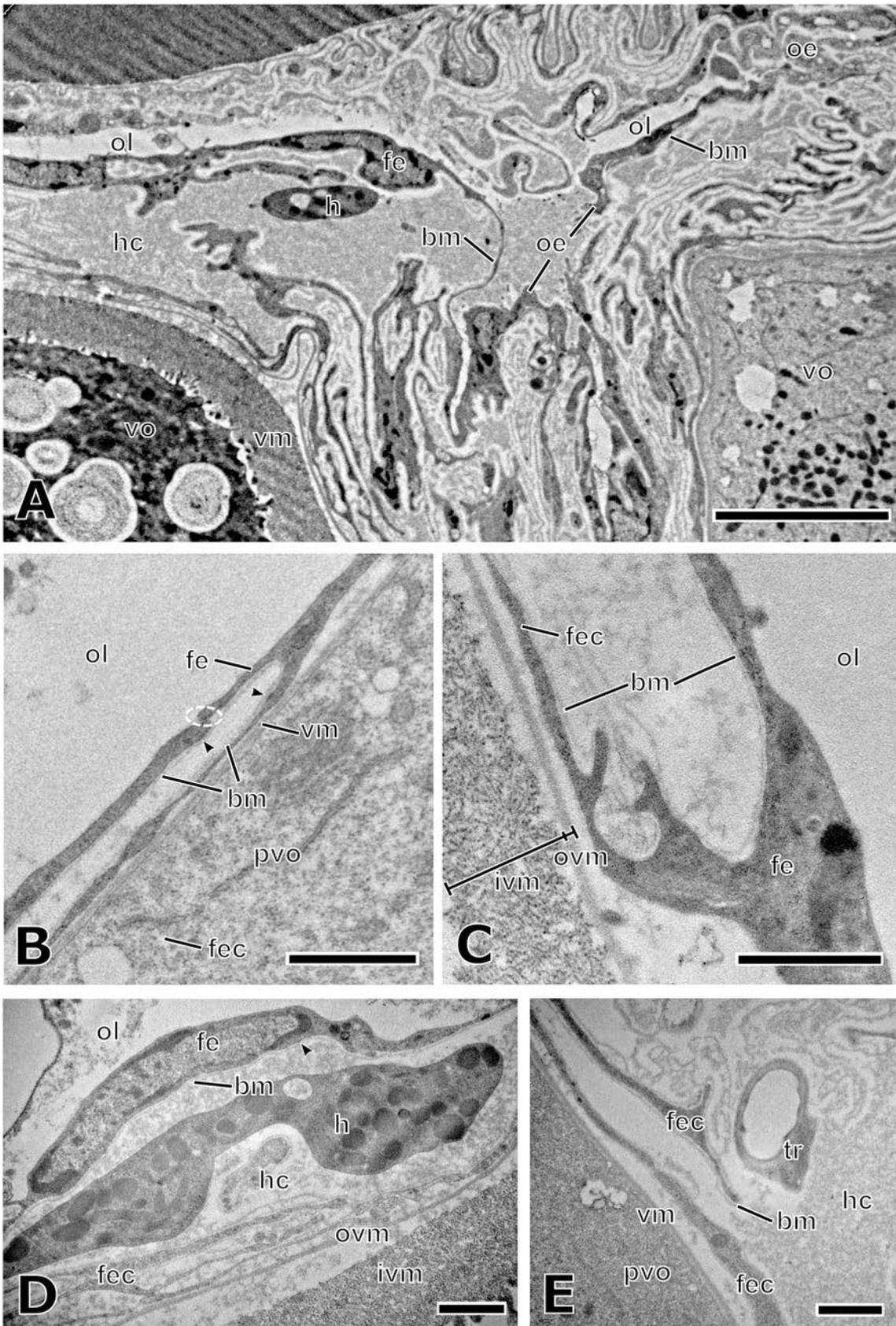


Fig. 4 Ultrastructure of the ovarian follicle in *Eutrichodesmus elegans*. (A) Continuity between the ovarian epithelium and the follicle epithelium with a continuous layer of its basement membrane. (B–C) Follicle epithelium in the previtellogenic (B) and vitellogenic (C) stages. (D) Hemocyte within the ovarian follicle. (E) Tracheole within the follicle. bm: basement membrane, fe: follicle epithelium, fec: elongated cytoplasm of follicle epithelial cell, hc: hemocoel, h: hemocyte, ivm: inner layer of the vitelline membrane, ovm: outer layer of the vitelline membrane, oe: ovarian epithelium, ol: ovarian lumen, pvo: previtellogenic oocyte, tr: tracheole, vm: vitelline membrane, vo: vitellogenic oocyte. broken lined circle: adherence junction, arrowhead: hemidesmosome. Scales = A: 5  $\mu$ m, B, C, D, E: 1  $\mu$ m.

The follicle epithelium of *Eu. elegans* formed a large follicle sac, which was not intricately folded (Fig. 3C, D). Oocytes in the germ zones were enclosed within the large single follicle sac (Fig. 3C, D), but its epithelium did not completely cover each oocyte. Cell-cell junctions or intercellular bridges could not be detected between the oocytes. Some hemocytes and tracheoles were distributed in the large follicle sac (Fig. 4D, E).

## Discussion

### Oocyte-growth position

In *Ep. orientalis* and *Eu. elegans*, the follicle epithelium continued to the ovarian epithelium with a continuous layer of the basement membrane. In addition, hemocytes in both species were detected within the follicle. These results show that the oocyte grows within the hemocoelic space in the follicle in both polydesmid species, like that of other myriapods (Kubrakiewicz 1991a; Miyachi and Yahata, 2012; Yahata et al., 2018).

### Types of ovarian follicle

Our results demonstrate that the Polydesmida has at least two types of structure of the ovarian follicle (Fig. 5B, C). In *Ep. orientalis*, the follicle is an intricately folded structure of the

follicle epithelium, each containing an oocyte (Fig. 5B). This feature revealed by the present ultrastructural observations corresponds to the previous histological one (Yahata, 2012). In *Eu. elegans*, the unfolded structure of the follicle is identical to that in the previous histological study (Yahata, 2012), but the follicle sac containing many oocytes (Fig. 5C) is first reported in the present study.

### Unusual ovarian follicle type in *Eutrichodesmus elegans*

In *Eu. elegans*, each ovarian follicle contains multiple oocytes. In contrast, in many other arthropods, each ovarian follicle contains a single female germ cell surrounded by a single layer of follicle epithelium (e.g., Pseudoscorpiones: Makioka, 1979; Jędrzejowska et al., 2013; Scorpiones: Jędrzejowska et al., 2014, 2016; Symphyla: Tiegs, 1940, 1945; Yahata et al., 2018; Pauropoda: Tiegs, 1947a, b; Chilopoda: Herbaut, 1974; Knoll, 1974; Miyachi and Yahata, 2012; Diplopoda: Kubrakiewicz, 1991a, b; Yahata and Makioka, 1994, 1997; Xenocarida: Kubrakiewicz et al., 2012; Apterygota and most Pterygota: Büning, 1994). The only known exception is the polytrophic-type ovary, seen in some pancrustaceans (Branchiopoda: Jaglarz et al., 2014; Diplura: Biliński, 1993; some Pterygota: King and Büning, 1985). In this type of

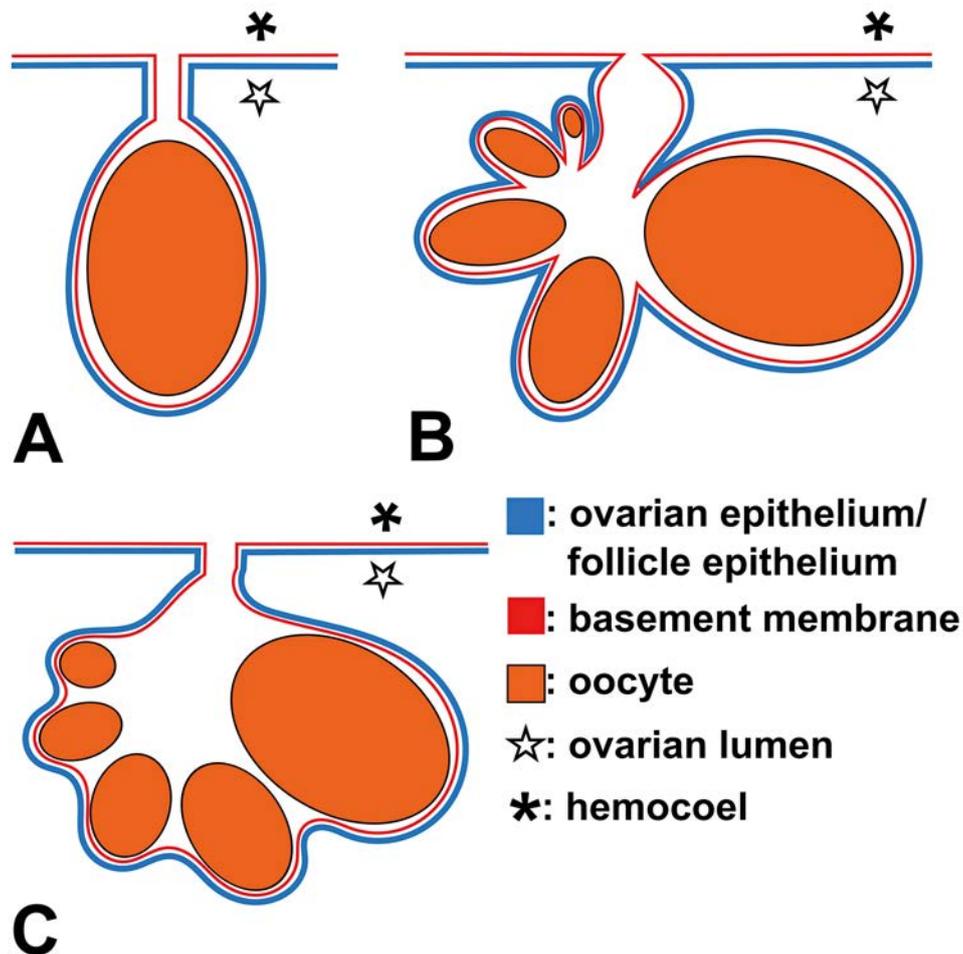


Fig. 5 Schematic pictures of three types of the ovarian follicle in the Diplopoda. (A) Typical unfolded pouch with a single oocyte-type found in many diplopods. (B) Folded pouch with a single oocyte-type found in *Epanerchodus orientalis*. (C) Unfolded sac with multiple oocyte-type found in *Eutrichodesmus elegans*.

ovaries, the nurse cells, derived from the female germ cell, provide nutrient material for each oocyte via a nutrient tube or heterocellular junctions (Büning, 1994). As each oocyte within a single ovarian follicle grows adjacent to nurse cells, each follicle in the polytrophic-type ovary contains multiple female germ cells. In *Eu. elegans*, all female germ cells in one follicle have no heterocellular junction, and are covered with their own vitelline membrane. These architectures suggest that the oocytes grow solitary as seen in other myriapods (Sareen and Adiyodi, 1983; Kubrakiewicz, 1991a, b), and thus the ovary in *Eu. elegans* should not be regarded as the polytrophic-type. To our knowledge, this is the first report of arthropod ovary with multiple solitary oocytes within a single ovarian follicle.

#### *Evolutionary implication of ovarian follicle in Diplopoda*

The present and some previous studies suggested that there are at least three types of diplopod ovarian follicle according to the degree of folding, namely, the unfolded follicle pouch containing a single oocyte (e.g., Polyxenida: Kubrakiewicz, 1991b; Yahata and Makioka, 1994; Glomerida: Yahata and Makioka, 1997; Julida: Kubrakiewicz, 1991a) (Fig. 5A), the folded follicle pouch containing a single oocyte (*Epanerchodus orientalis*: present study) (Fig. 5B), and the unfolded follicle sac containing multiple oocytes (*Eutrichodesmus elegans*: present study) (Fig. 5C).

Recent molecular phylogenetic study shows that the Polydesmida is one of the most derived groups in Diplopoda (Miyazawa et al., 2014). The more basal groups of Diplopoda suggested by Miyazawa et al. (2014) are the Polyxenida and the Pentazonia, whose ovarian follicles are regarded as the unfolded pouch with an oocyte (Yahata and Makioka, 1994, 1997). Therefore, it is likely that the polydesmid ancestor had an unfolded pouch with a single oocyte-type follicle. There are two possible alternative hypotheses on the most derived state of the ovarian follicle in Polydesmida: 1) the folded pouch with a single oocyte-type follicle or 2) the unfolded sac with multiple oocyte-type follicles. The unfolded structure of the follicle epithelium in *Eu. elegans* differs from that of other diplopods due to the oocyte multiplicity. Accordingly, the unfolded follicle is regarded to be acquired secondarily in *Eu. elegans*. In addition, the single oocyte is common in follicles in *Ep. orientalis* as well as other diplopods. Considering these, the latter hypothesis appears to be more likely. In this hypothesis, the unfolded follicle structure in *Eu. elegans* should be a secondary evolved feature. Hence the “restoration” of folding in the follicle epithelium may occur in Polydesmida. Under situation, constraint of the folding of the follicle epithelium may have been lost in *Eu. elegans*.

Further studies into the ovarian morphology of other polydesmids and morphogenesis of folded and unfolded follicle structure are required to verify this hypothesis and elucidate the evolutionary process.

**Acknowledgments:** We are deeply grateful to Mr. R. Nagasawa and Mr. G. Takahashi for their encouragement to

collect *Eutrichodesmus elegans*. We also express sincerely thanks to members of the Sugadaira Research Station, Mountain Science Center, University of Tsukuba. Our analysis of transmission electron microscope was carried out with instrument at Open Facility Center for Science and Technology, University of Tsukuba.

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