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Position and development of oocytes in velvet worms shed light on the evolution of the ovary in Onychophora and Arthropoda

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Onychophora or velvet worms are of considerable importance in current reconstructions of animal phylogeny. Despite their otherwise conservative morphology, the detailed anatomy of the onychophoran ovary displays significant variation. However, the evolutionary significance of this variation is not well understood. We recognize three major ovarian types in the Onychophora: (1) the exogenous ovary; (2) the pseudoendogenous ovary; and (3) the endogenous ovary. The germ cells in all three ovarian types are intraepithelial in that they occur between the basal lamina and the epithelial cells that line the cavity of the gonad. This is the condition found in the endogenous ovary. Even in the exogenous ovary, with stalked oocytes projecting into the haemocoel, the maturating oocytes are still covered by a basal lamina. Stalked oocytes that are similar to those found in the exogenous ovary, but retain their intra-ovarian position, characterize the pseudoendogenous ovary. This and additional observations support the assumption that the pseudoendogenous ovary is derived from an exogenous type and the similarities with the endogenous ovary are superficial. Embryological data and an outgroup comparison with arthropods suggest that the exogenous ovary is the ancestral condition in velvet worms and a synapomorphy of Onychophora and Arthropoda. The embryonic origin, growth, and position of oocytes outside the ovarian lumen in Onychophora and various groups of Arthropoda do not support the Articulata hypothesis, which proposes a sister-group relationship of Panarthropoda and Annelida.

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ADDITIONAL KEYWORDS: Articulata – endogenous – exogenous – oogenesis – Panarthropoda – phylogeny – pseudoendogenous.

INTRODUCTION

Current hypotheses on animal phylogeny commonly unite Onychophora, Tardigrada, and Arthropoda in a monophyletic Panarthropoda (Budd, 2001; Nielsen, 2001; Dunn *et al.*, 2008; Mayer & Harzsch, 2008). Because of their close relationship to the arthropods and an external anatomy that resembles fossil stem-lineage panarthropods (Ramsköld & Hou, 1991; Bergström & Hou, 2001; Maas *et al.*, 2007), Onychophora play an important role in understanding the evolution and diversification of the arthropod body plan. Despite the generally conservative morphology of onychophorans, their ovaries show considerable structural diversity (Fig. 1A–G). This diversity might be related to the various modes of reproduction displayed by onychophorans, which range from oviparous and ovoviviparous species with yolky eggs to ovoviviparous and viviparous species with relatively yolk-free or yolkless eggs (see reviews Korschelt & Heider, 1899; Anderson, 1966, 1973).

Ovarian morphology in onychophorans differs mainly in the relationship of the ovarian tissues to each other, the distribution of germ cells, and the variously fused ovarian tubes. Despite the apparently high morphological diversity, two major ovarian types have long been recognized in Onychophora, for which the terms 'exogenous' and 'endogenous' have been coined to describe the position of maturating oocytes either on the ovarian surface or inside the ovary,

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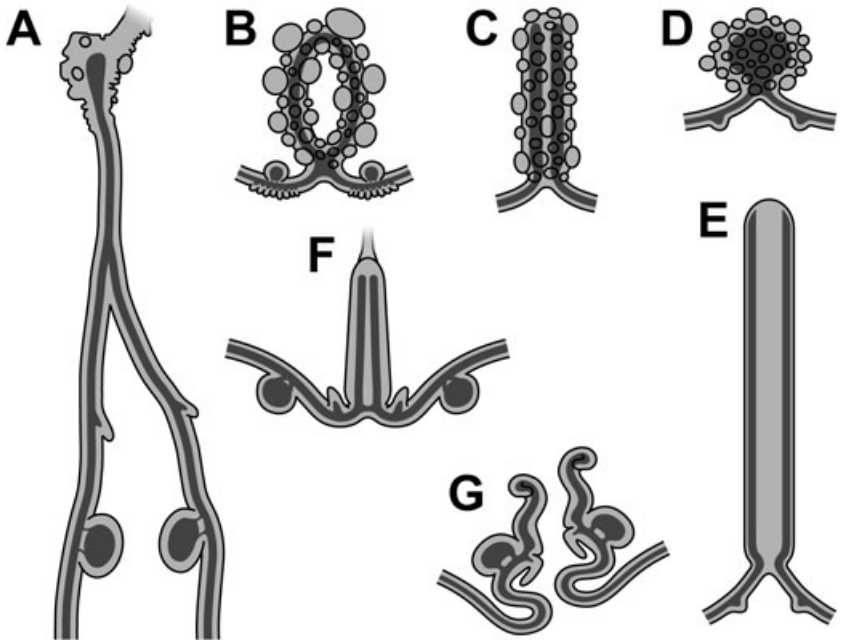

Figure 1. Simplified diagrams of paired versus variously fused structure of the ovarian tubes in Onychophora. Tissues are represented in light grey, ovarian lumen in dark grey. A, exogenous ovary of *Typhloperipatus williamsoni* (South-East Asian Peripatidae). Modified and complemented according to descriptions given by Kemp (1914). B–D, exogenous ovaries in three species of Peripatopsidae. B, *Euperipatoides rowelli* (Australia). Note the completely separate ovarian tubes in the middle of the ovary and the fused ovarian lumens at the anterior and posterior ends. A similar ovarian organization has been described in *Peripatoides novaezealandiae* from New Zealand (Sheldon, 1890: fig. 26). C, *Peripatopsis balfouri* (South Africa, cf. Fig. 2A). Although the ovarian tubes are fused, their lumens are separate along their entire length. D, *Opisthopatus roseus* (South Africa). Note the unpaired ovarian structure with a single lumen. E, pseudoendogenous ovary of *Metaperipatus inae* (Peripatopsidae, Chile, cf. Fig. 2B) with fused ovarian tubes but separate lumens. F, endogenous ovary of *Epiperipatus biolleyi* (Neotropical Peripatidae, cf. Fig. 2C) with lumens communicating only at the posterior end. G, endogenous ovary of *Mesoperipatus tholloni* (Peripatidae, Tropical Africa), modified and complemented after Bouvier (1905). Each ovarian tube is completely separate in this species.

respectively (Moseley, 1874; Gaffron, 1885; Kennel, 1885; Willey, 1898; Bouvier, 1905; Sedgwick, 1908). However, a recent description of a novel ovarian type in representatives of *Metaperipatus* from Chile blurs this traditional distinction. Originally, the ovary of *Metaperipatus blainvillei* was assigned to the endogenous type, based on the internal position of oocytes (Bouvier, 1901, 1905). Yet, a more detailed study revealed that this ovary might be derived from the exogenous type (Mayer, 2007). We, therefore, refer to this ovarian type as pseudoendogenous because its relationship to the endogenous ovary appears to be superficial.

Ultrastructural studies on the onychophoran ovary are scarce and corresponding data on their development are lacking. These studies either focus on various aspects of oogenesis, or the accessory ovarian structures (Herzberg, Ruhberg & Storch, 1980; Walker, 1992; Huebner & Lococo, 1994; Walker & Campiglia, 1998; Brockmann *et al.*, 1999, 2001; Walker *et al.*, 2006). The remaining elements of the ovary, especially the spatial relationship of the various ovarian tissues and germ cells to each other, have not been analysed in depth and require clarification (see Manton, 1938; Huebner & Lococo, 1994; Brockmann *et al.*, 1999, 2001). Our current knowledge of the internal organization of the onychophoran ovary is insufficient to provide insights into its evolution. The main objective of this study is, therefore, to provide comparative data on the three major ovarian types in Onychophora and to compare them with the corresponding data from arthropods. In addition, some aspects of the embryonic fate of primordial germ cells and ovarian morphogenesis are presented.

MATERIAL AND METHODS

COLLECTION OF SPECIMENS

Females of *Epiperipatus biolleyi* (Bouvier, 1902), *Metaperipatus inae* Mayer, 2007, *Met. blainvillei* (Gervais, 1837), and *Opisthopatus roseus* Lawrence,

1947 were obtained as described previously (see Mayer, Ruhberg & Bartolomaeus, 2004; Mayer, Bartolomaeus & Ruhberg, 2005; Mayer, 2006, 2007). Specimens of *Peripatopsis balfouri* (Sedgwick, 1885) were collected from a rotten log in Kirstenbosch Botanical Gardens (Table Mountain, Cape Town, South Africa) in January 2004. Specimens of *Epiperipatus isthmicola* (Bouvier, 1905) were found under and within decaying wood and in leaf litter in the Reserva Biologica Hitoy-Cerere (south of Puerto Limón, Costa Rica) in October 2005. Specimens of *Euperipatoides rowelli* Reid, 1996 and *Phallocephale tallagandensis* Reid, 1996 were obtained from rotten logs in the Tallaganda State Forest (New South Wales, Australia) in March and September 2007. Specimens of *Ooperipatellus insignis* (Dendy, 1890) were found in leaf litter and under moss on Mt Macedon (Victoria, Australia) in March 2008.

Scanning electron microscopy

For scanning electron microscopy (SEM), specimens were either fixed in Bouin's fixative, 70% ethanol, 4% formaldehyde, or 2.5% glutaraldehyde. After dehydration in an ethanol series, the specimens were dried in a critical-point dryer (BAL-TEC, CPD 030), coated with gold in a sputter coater (BALZERS, SCD 040), and examined in a scanning electron microscope (Philips, Quanta 200).

Light and transmission electron microscopy

For light microscopy (LM) and transmission electron microscopy (TEM), specimens were treated as described previously (Mayer *et al.*, 2004; Mayer & Koch, 2005; Mayer, 2006, 2007). The material was cut with a diamond knife into a series of either semi-thin (1 μm) or silver interference-coloured (55–65 nm) sections on a Reichert Ultracut microtome. The semi-thin sections were placed on glass slides, stained with Toluidine blue and viewed with a light microscope (Olympus BX61 equipped with an F-View II digital camera, SIS). The ultra-thin sections were mounted on formvar-covered, single slot copper grids, automatically stained with uranyl acetate and lead citrate in an ultrostainer (Nanofilm TEM Stainer, Nanofilm Technologies GmbH, Göttingen), and imaged on a transmission electron microscope (Philips CM 120).

Image processing

Optimal quality light, transmission, and scanning electron micrographs were achieved by using the AnalySIS software package ('Multiple Image Alignment') and Adobe (San Jose, Ca) Photoshop CS2. Final plates were composed with Adobe Illustrator CS2.

RESULTS

General anatomy of the ovary

The onychophoran ovary is situated in the posterior end of the body cavity where it is either suspended along its entire length from the pericardial floor or is attached to it via an anterior or anterodorsal ligament. The ovary typically consists of a pair of tubes that may be variously fused and which give rise posteriorly to a pair of oviducts (Figs 1A–G, 2A–C).

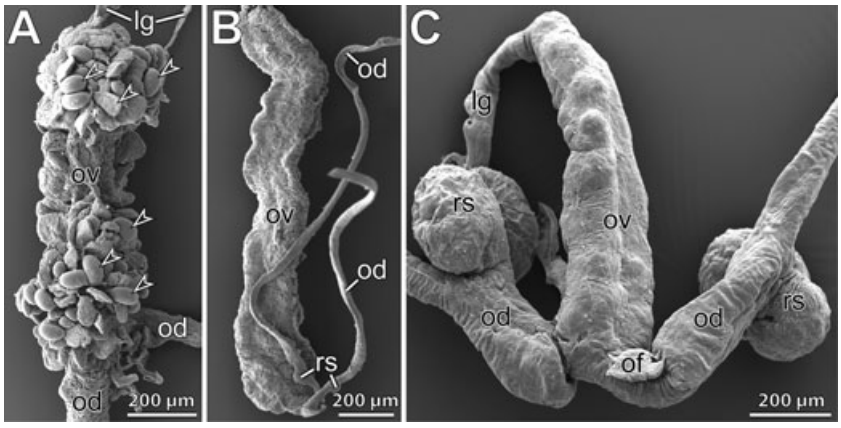

Figure 2. External organization of the three major types of ovaries and associated structures in Onychophora. Scanning electron micrographs, ventral view, anterior upmost. A, exogenous ovary of *Peripatopsis balfouri* (Peripatopsidae, South Africa). Arrowheads indicate 'grape-like', stalked oocytes projecting from the ovarian surface. B, pseudoendogenous ovary of *Metaperipatus inae* (Peripatopsidae, Chile). Note the absence of stalked oocytes on the ovarian surface, which consequently appears smooth. C, endogenous ovary of *Epiperipatus biolleyi* (Peripatidae, Costa Rica) with a smooth ovarian surface. Abbreviations: lg, suspensory ligament; od, oviduct; of, ovarian funnel; ov, ovary; rs, seminal receptacle.

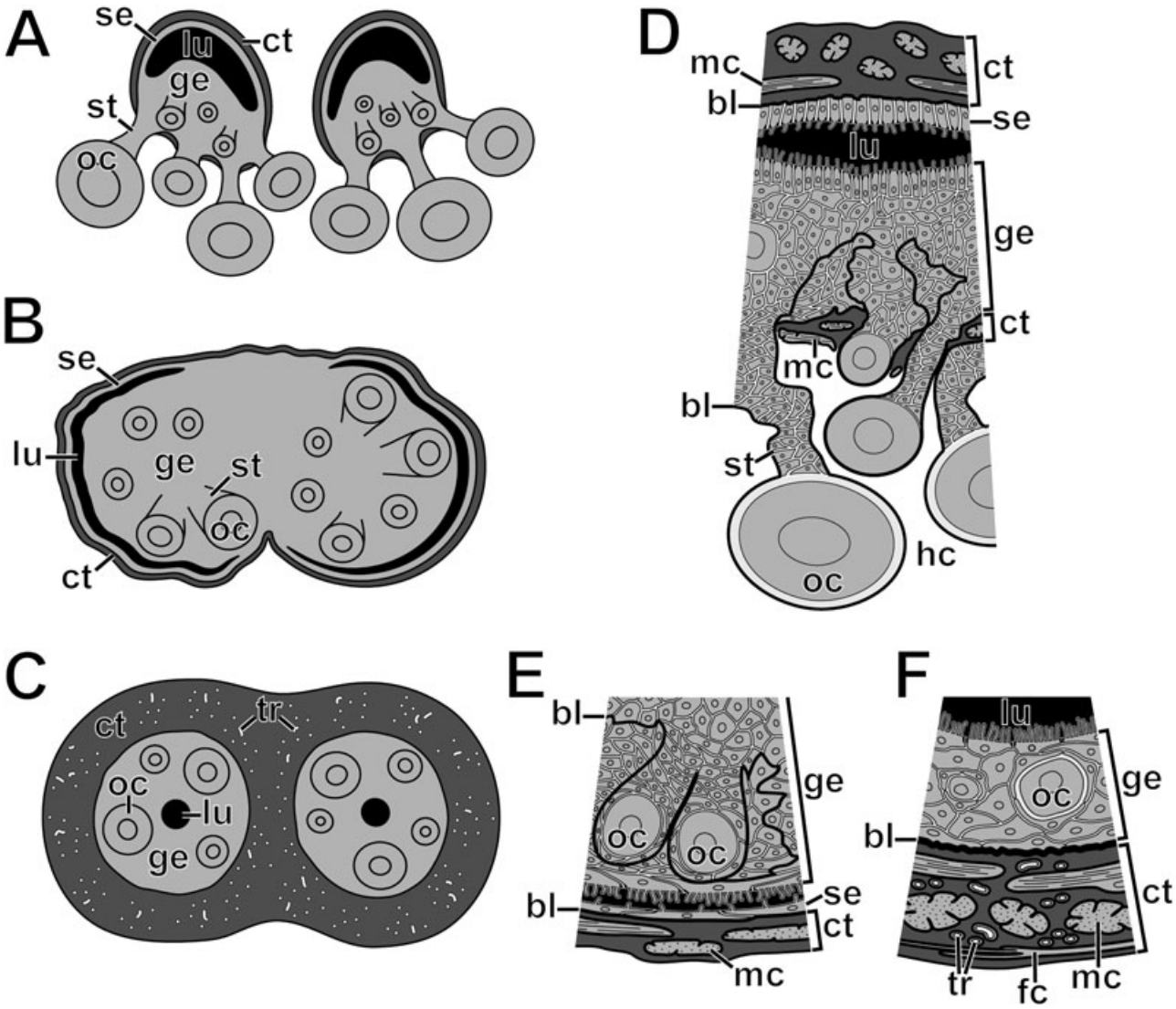

Figure 3. Diagram of internal organization of the three major ovarian types in Onychophora: exogenous ovary (A, D), pseudoendogenous ovary (B, E), and endogenous ovary (C, F). A, cross-section of the exogenous ovary with separate ovarian tubes that occur, e.g. in *Euperipatoides rowelli*, *Phallocephale tallagandensis*, and *Ooperipatellus insignis* (Australian Peripatopsidae). B, cross-section of the pseudoendogenous ovary of *Metaperipatus inae* (Peripatopsidae, Chile). C, cross-section of the endogenous ovary of *Epiperipatus biolleyi* (Peripatidae, Costa Rica). Note the complete lack of a sterile epithelium. D, detail of the composition of the ovarian wall in *Opisthopatus roseus* (Peripatopsidae, South Africa). E, detail of the ovary in *Met. inae*. F, detail of the ovarian wall in *Ep. biolleyi*. Abbreviations: bl, basal lamina; ct, connective tissue; fc, flattened cell; ge, germinal epithelium; hc, haemocoel; lu, ovarian lumen; mc, muscle cell; oc, oocyte; se, sterile epithelium; st, stalk; tr, tracheae.

The ovarian lumen is surrounded by a number of tissues (Fig. 3A–F). The most prominent is the ovarian epithelium, which, towards the haemocoel, is covered by connective tissue containing muscle fibres. A specialized, thickened region of the ovarian epithelium, the germinal epithelium, bears oogonia and oocytes. These cells are lacking in the sterile region of the epithelium, which may or may not be present (Fig. 3A–F).

Despite considerable variation in the details of ovarian anatomy, three major ovarian types can be distinguished in Onychophora, based largely on the position of the late maturating oocytes: (1) the exogenous ovary with stalked oocytes projecting from the ovarian surface into the haemocoel; (2) the pseudoendogenous ovary with stalked oocytes confined to the germinal epithelium; and (3) the endogenous ovary with nonstalked oocytes situated within the germinal

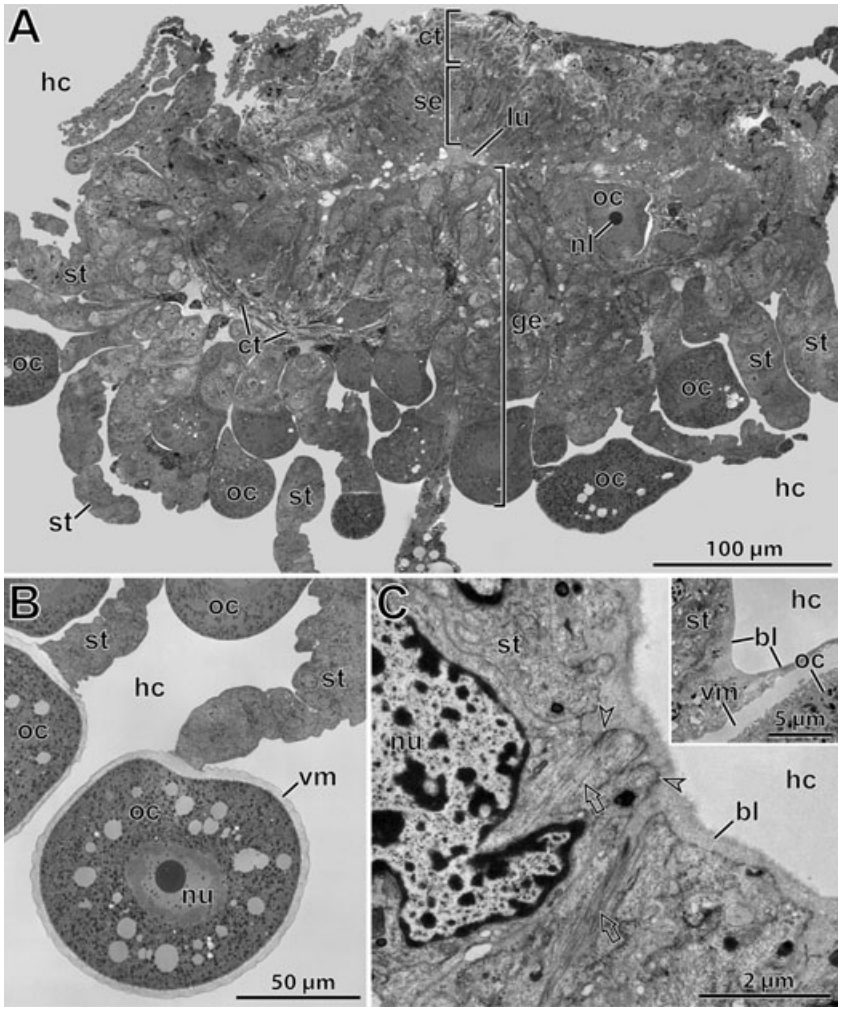

Figure 4. Internal organization of the exogenous ovary in *Opisthopatus roseus* (A, B) and *Peripatopsis balfouri* (C). Transmission electron micrographs. A, cross-section through the middle of the ovary. Dorsal is up. Note the unpaired lumen (lu) and the separation of the ovarian epithelium into a dorsal sterile epithelium (se) and a ventral germinal epithelium (ge). B, stalked oocytes enclosed by a vitelline membrane (= primary egg membrane). C, detail of a stalk. Arrows indicate intermediate filaments, arrowheads point to hemidesmosomes. Inset shows continuity of the basal lamina covering a stalk and a maturating oocyte that bulges into the haemocoel of the female. Abbreviations: bl, basal lamina; ct, connective tissue; ge, germinal epithelium; hc, haemocoel; lu, ovarian lumen; nl, nucleolus; nu, nucleus; oc, maturating oocytes; se, sterile epithelium; st, stalk; vm, vitelline membrane.

epithelium (Figs 2A–C, 3A–F). In the following, the detailed ultrastructure of each of the three major ovarian types is described.

THE EXOGENOUS OVARY

The exogenous ovary occurs in the South-East Asian Peripatidae and members of the Peripatopsidae, except for two species of *Metaperipatus* from Chile. The exogenous ovary displays considerable variability regarding the fusion of its paired tubes. In the Australian Peripatopsidae (e.g. *Euperipatoides rowelli*, *Phallocephale tallagandensis*, and *Ooperipatellus insignis*), the ovarian tubes are joined only at the anterior and posterior ends, at which the ovarian lumen is fused as well (Fig. 1B). In the South African species of *Peripatopsis*, the ovarian tubes are fused along their entire length, but each lumen is separate, contrary to the unpaired external appearance of the ovary (Figs 1C, 2A). Yet in another representative of the South African Peripatopsidae, *Opisthopatus roseus*, the paired ovarian structure is not recognizable any longer and the ovarian lumen is fused along its entire length (Figs 1D, 3D, 4A). A similar situation occurs in the exogenous ovary of the South-East Asian Peripatidae, in which even the proximal portion of the

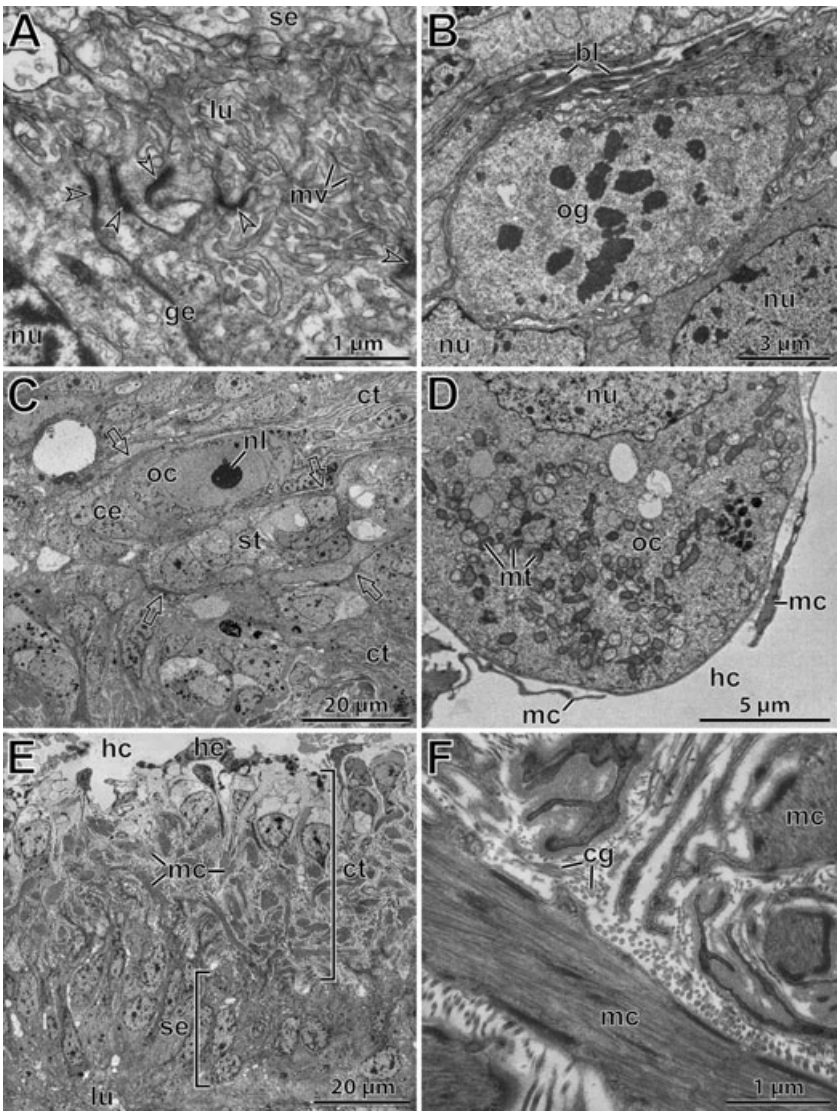

Figure 5. Internal organization of the exogenous ovary in *Opisthopatus roseus* (A–C, E, F) and *Peripatopsis balfouri* (D). Transmission electron micrographs. A, detail of ovarian epithelia and lumen. Arrowheads indicate apical junctions connecting epithelial cells. B, mitotically dividing oogonium (og) from the germinal epithelium. C, maturating oocyte (oc) with associated cells (ce) of modified germinal epithelium. Arrows point to the basal lamina which is strongly folded. D, maturating oc in the process of breaking through the connective tissue and growing out into the haemocoel. E, detail of dorsal ovarian wall showing the sterile epithelium (se) and the connective tissue (ct), which contains the musculature (mc). F, higher magnification of connective tissue showing muscle cells and collagen fibres. Abbreviations: bl, basal lamina; ce, cells associated with an oocyte (sometimes referred to as 'follicle cells' in the literature); cg, collagen fibres; ct, connective tissue; ge, germinal epithelium; hc, haemocoel; he, haemocyte; lu, ovarian lumen; mc, muscle cell; mt, mitochondria; mv, microvilli; nl, nucleolus; nu, nucleus; oc, maturating oocyte; og, oogonium; se, sterile epithelium; st, growing stalk.

oviducts may also be fused (Fig. 1A; see Evans, 1901a; Kemp, 1914).

Irrespective of these differences regarding the various degree of fusion of the ovarian tubes, the internal organization of the exogenous ovary is similar amongst the species studied thus far. The germinal epithelium is situated ventrally, whereas the sterile epithelium occurs dorsally (Figs 3A, D, 4A, 5A–E). Both the sterile and germinal epithelia give rise to numerous microvilli projecting into the ovarian lumen (Fig. 5A). The sterile epithelium consists of only a single cell layer resting on a basal lamina, whereas the germinal epithelium is organized in a more complex way (Figs 3A, D, 4A, 5B–E). The germinal epithelium is thickened considerably, hence only the cells facing the ovarian lumen bear apical

junctions and microvilli. These cells do not rest on a basal lamina, as one might expect for typical epithelial cells, but are instead underlain by other cells, which form a thick cell layer without any apparent stratification (Figs 3D, 4A, 5C). Most of these cells do not show any apicobasal polarity as they neither bear apical junctions nor rest on a basal lamina. Only those cells of the germinal epithelium that are situated in deeper layers or form stalks are supported by a basal lamina (Figs 3D, 4C, 5B, C). The germinal epithelium thus appears highly modified as it is thickened considerably and thrown into folds that are recognized by the distribution of the basal lamina. Towards the haemocoel, the ovarian epithelium is surrounded by a sheath of connective tissue, which is composed of a collagenous matrix with an embedded musculature (Fig. 5E, F). Haemocytes with numerous electron-dense vesicles were frequently seen attached to the ovarian surface (Fig. 5E). Tracheal tubes and nerve processes were not found in the ovarian tissue.

The major characteristic of the exogenous ovary is the projection of late maturating oocytes on stalks into the haemocoel (Figs 1A–D, 2A, 3A, D, 4A–C). Initially, the oocytes originate from oogonia that do not have a direct contact to the ovarian lumen but are instead surrounded by other cells of the germinal epithelium. Some of the oogonia were seen undergoing mitosis (Fig. 5B). The oogonia give rise to cells that begin meioses and become early maturating oocytes. The early oocytes are first situated within the germinal epithelium and are distinguished from other cells by their increased size and a large nucleus with a prominent nucleolus. At a more advanced stage of oogenesis, the oocytes become surrounded by cells that are grouped into elongated structures enclosed by a basal lamina (Fig. 5C). These structures are the future stalks that will elongate further and push the maturating oocytes towards the haemocoel (Fig. 4A, B). During this process, the oocytes break through the connective tissue and musculature that enclose the ovary (Fig. 5D). After their extrusion into the haemocoel, the maturating oocytes remain associated with stalks, thus being covered by the basal lamina of the germinal epithelium (Figs 3D, 4B, C). The stalk cells are stabilized by bundles of intermediate filaments (Fig. 4C). The late maturating oocytes are at least 100 μm in diameter in *Peripatopsis balfouri* and *Op. roseus* (Figs 2A, 4B) but measure over 300 μm in *Euperipatoides rowelli* and *Phallocephale tallagandensis*, and over 500 μm in *Ooperipatellus insignis*. However, the diameter of oocytes might vary seasonally with maturity stage of the female.

The pseudoendogenous ovary

The pseudoendogenous ovary occurs only in representatives of *Metaperipatus* from Chile. The paired ovarian tubes show a fused external appearance, but within the ovary the lumen of each side are separate along their entire length (Figs 1E, 2B, 3B). Each lumen appears crescent-shaped in cross-sections and occupies mainly a lateral position within each ovarian tube (Figs 3B, 6A). Numerous microvilli from the ovarian epithelium project into the ovarian lumen (Fig. 6E). The surface of the pseudoendogenous ovary appears smooth (Figs 1E, 2B), which is in contrast to the 'grape-like' exogenous structure of ovaries in all other species of Peripatopsidae (Figs 1A–D, 2A). The external ovarian sheath is formed by connective tissue, which, apart from numerous collagen fibres, contains muscle cells, haemocytes, and sperm (Fig. 6E, F). Tracheae and nerve processes were not detected in the ovarian tissue.

According to the distribution of oogonia and maturating oocytes in the pseudoendogenous ovary, the germinal epithelium occupies a central position within each ovarian tube, whereas the sterile epithelium is restricted to the outer ovarian wall (Figs 3B, E, 6A, F). The sterile epithelium forms only a single cell layer, which rests on a basal lamina (Figs 3E, 6E, F). In contrast, the germinal epithelium shows a more complex organization, comparable to that found in the exogenous ovary. Within the germinal epithelium, the oogonia and younger oocytes are located mainly in the central portion, whereas the larger oocytes occur predominantly towards the ovarian lumen and the outer periphery of each ovarian tube (Fig. 6A). The maturating oocytes are associated with cells of the germinal epithelium that form short stalks (Figs 3E, 6B). As in the exogenous ovary, the stalk cells are stabilized by intermediate filaments (Fig. 6D). However, the oocytes and stalks of the pseudoendogenous ovary do not grow out from the ovarian surface into the haemocoel during oogenesis but instead remain within each ovarian tube (Figs 3B, E, 6A, B). The maturating oocytes retain their position within the germinal epithelium and are, together with their stalks, separated from the remaining germinal tissue by a basal lamina (Figs 3E, 6B–D). The diameter of the largest oocytes in *Met. inae* is 100 μm (Fig. 6A, B), whereas they measure only about 40 μm in *Met. blainvillei* (cf. Mayer, 2007: fig. 25), which might be the result of differences in the maturity of the females examined.

The endogenous ovary

The endogenous ovarian type occurs in the neotropical Peripatidae, which in *Ep. biolleyi* and *Ep. isthmicola* consists of paired, fused tubes, each with a central lumen enclosed by the germinal epithelium (Figs 1F, 3C, 7A). An endogenous ovary might also be present in *Mesoperipatus tholloni*, from tropical

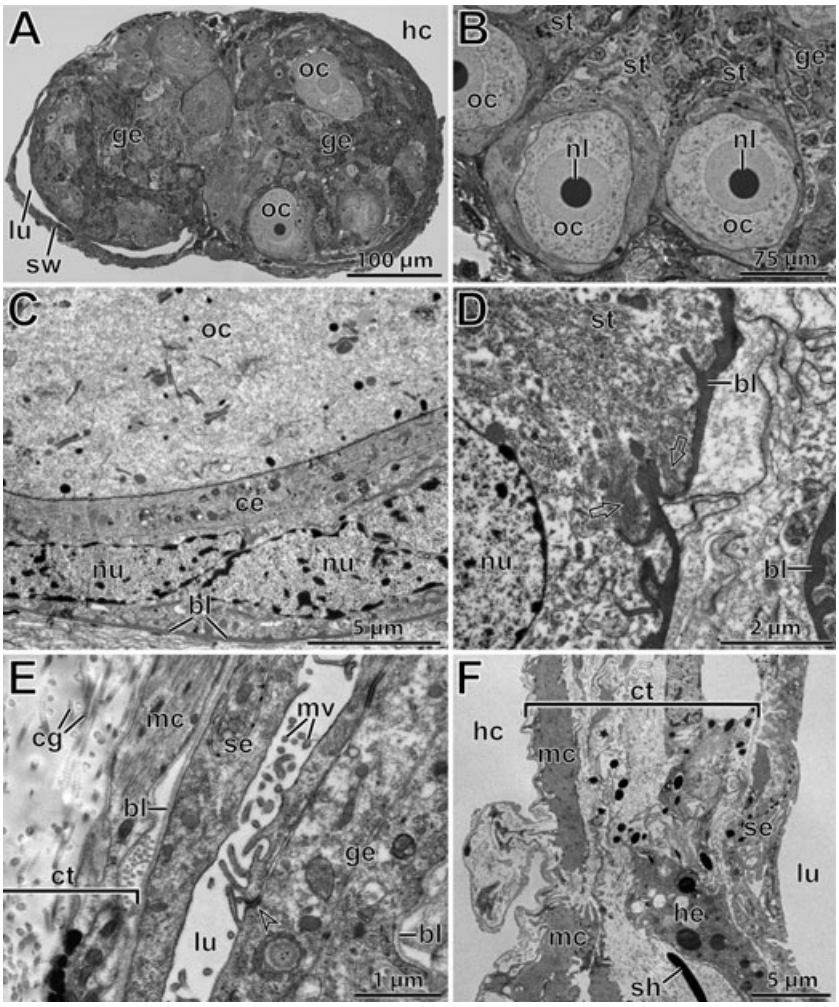

Figure 6. Internal organization of the pseudoendogenous ovary in *Metaperipatus inae* (A–E) and *Metaperipatus blainvillei* (F). Light micrographs (A, B) and transmission electron micrographs (C–F). A, cross-section of the ovary (dorsal is up). Note the lateral position of the crescent-shaped ovarian lumen (lu) and the central position of the germinal epithelium (ge). B, detail of stalked oocytes (oc) from the germinal epithelium, which do not bulge into the haemocoel but retain their position within the ovary. C, detail of cells (ce) surrounding a maturating oocytes in the germinal epithelium. D, detail of a stalk. Arrows indicate the intermediate filaments. E, detail of the lumen and ovarian epithelia. The epithelial cells are connected by an apical junction (arrowhead). F, lateral ovarian wall consisting of the sterile epithelium (se) and connective tissue containing muscle cells (mc), a haemocyte (he), and sperm (sh). Abbreviations: bl, basal lamina; ce, cells associated with an oocyte; cg, collagen fibres; ct, connective tissue; ge, germinal epithelium; hc, haemocoel; he, haemocyte; lu, ovarian lumen; mc, muscle cell; mv, microvilli; nl, nucleolus; nu, nucleus; oc, maturating oocyte; se, sterile epithelium; sh, sperm head; st, stalk; sw, sterile ovarian wall.

West Africa, with its two completely separate ovarian tubes (Fig. 1G; see Bouvier, 1905). The lumen in the neotropical species sometimes appears collapsed but is always visible at the ultrastructural level and contains numerous microvilli originating from cells of the ovarian epithelium (Fig. 7C). The external ovarian surface is smooth because of the internal position of oocytes (Figs 1F, 2C). The connective tissue surrounding each ovarian tube forms a prominent layer containing collagen fibres, muscle cells, a large number of tracheae, and an outer layer of flattened non-epithelial, electron-dense cells (Figs 3C, F, 7A, D–F). Nerve processes were not found in the ovarian tissue.

The germinal epithelium of the endogenous ovary is neither folded nor twisted, which is in contrast to the exogenous and pseudoendogenous ovarian types. It instead forms a thick layer of cells, which contains oogonia and maturating oocytes and rests on a basal lamina (Figs 3C, F, 7A–E). According to the

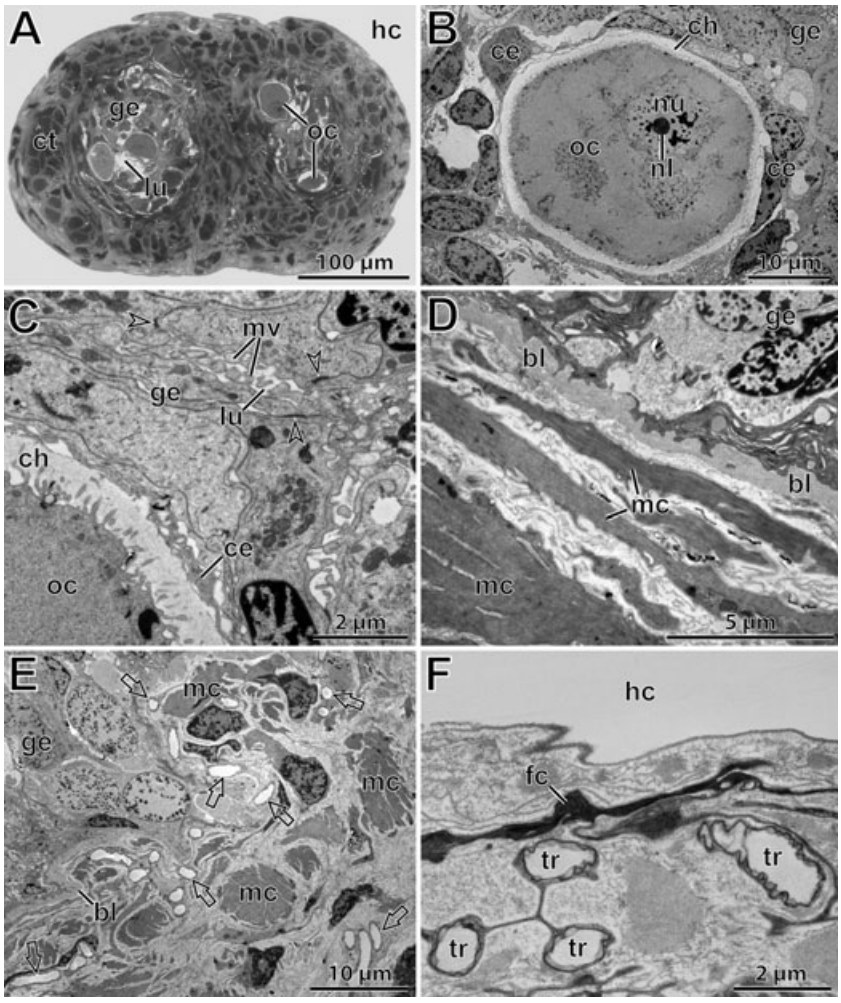

Figure 7. Internal organization of the endogenous ovary in *Epiperipatus biolleyi*. Light micrograph (A) and transmission electron micrographs (B–F). A, cross-section of the ovary (dorsal is up). Note the central position of ovarian lumen (lu) surrounded by germinal epithelium (ge) and the lack of a sterile epithelium. B, maturating oocyte (oc) from the germinal epithelium lying within a spherical chamber (ch), which is formed by specific cells (ce) of the germinal epithelium. C, detail of epithelial cells and an oocyte. Arrowheads indicate apical junctions. D, detail of the basal portion of the germinal epithelium with surrounding connective tissue containing muscle cells (mc). E, lower magnification of connective tissue showing numerous tracheal tubes (arrows). F, peripheral region of connective tissue containing tracheal tubes (tr) and flattened cells with an electron-dense content (fc). Abbreviations: bl, basal lamina; ce, cells of germinal epithelium that form the oocyte chamber; ch, oocyte chamber; ct, connective tissue; fc, flattened cell; ge, germinal epithelium; hc, haemocoel; lu, ovarian lumen; mc, muscle cells; mv, microvilli; nl, nucleolus; nu, nucleus; oc, maturating oocytes; tr, tracheal tubes.

distribution of oogonia and oocytes, which is not restricted to a specific region, the sterile epithelium is lacking completely in the endogenous ovary. Within the germinal epithelium, the late maturating oocytes are situated inside spherical chambers formed by specialized cells of the germinal epithelium (Fig. 7B, C). In contrast to the exogenous and pseudoendogenous ovary, however, these cells do not form stalks at any stage of oogenesis in the neotropical Peripatidae. The largest oocytes in the ovary of *Ep. biolleyi* (Fig. 7A, B) and *Ep. isthmicola* measure 30–40 µm in diameter.

Origin and fate of primordial germ cells

The primordial germ cells (PGCs) arise early during onychophoran development (Manton, 1949; Pflugfelder, 1968, 1980). Their early embryonic fate was investigated in this study in embryos of *Ep. biolleyi* (neotropical Peripatidae) and *Op. roseus* (South

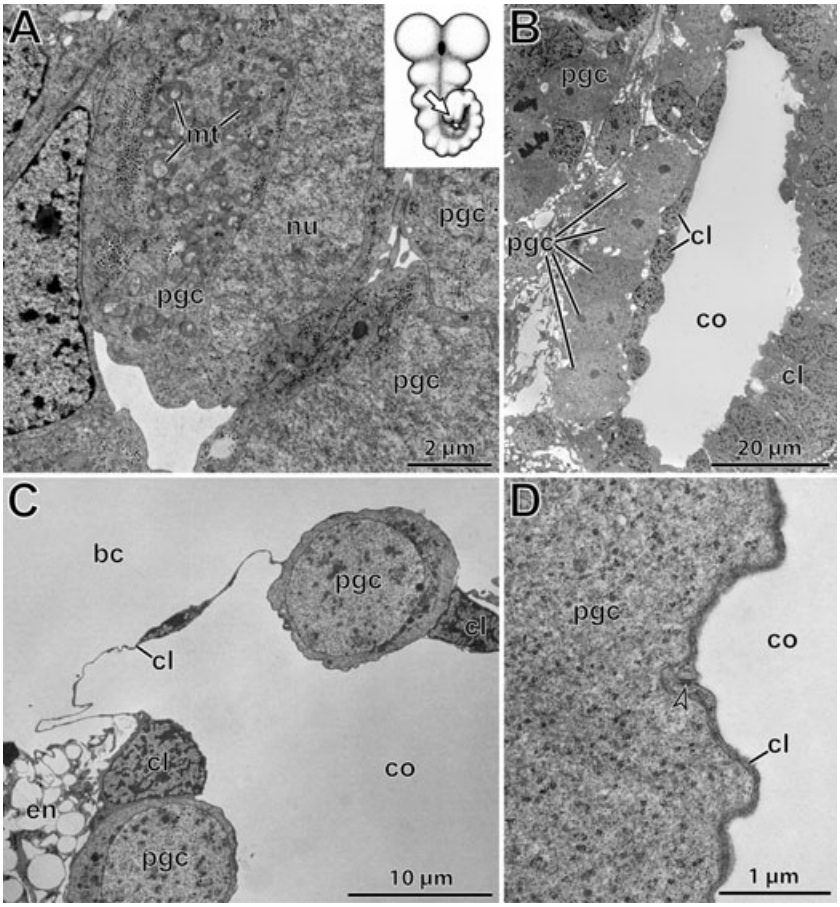

Figure 8. Origin and fate of primordial germ cells in *Epiperipatus biolleyi* (A, B) and *Opisthopatus roseus* (C, D). Transmission electron micrographs. A, primordial germ cells (pgc) that occur as an unpaired cluster of cells at the posterior end of an early segmenting embryo. Inset shows the position of the cluster (arrow) in a slightly older elongating embryo. B, horizontal section of a coelomic cavity with primordial germ cells associated with the visceral coelomic lining (cl) in an embryo with a full number of somites developed. Anterior is up, mid-line is left. C, horizontal section of a coelomic lining with associated primordial germ cells in an embryo with the full number of somites. D, detail of a primordial germ cell, which is covered by slender processes of coelomic lining cells towards the coelomic cavity (co). Arrowhead indicates an apical junction. Abbreviations: bc, primary body cavity; cl, coelomic lining cells; co, coelomic cavity; en, endoderm; mt, mitochondria; nu, nucleus; pgc, primordial germ cells.

African Peripatopsidae). Initially, the PGCs arise as an unpaired cluster of cells at the posterior end of the early segmenting embryo, prior to the formation of coelomic cavities in this body region (Fig. 8A). The PGCs are large, spherical cells that have a large nucleus with sparsely distributed chromatin and one or two prominent nucleoli. The granular cytoplasm contains an aggregation of numerous mitochondria, condensations of electron-dense material (= nuage) around the nuclear membrane, and a few Golgi complexes.

After the establishment of the full number of somites, the PGCs become attached to the visceral portions of the embryonic coelomic walls that will contribute to the future gonad (Fig. 8B–D). In neither of the two species studied do the PGCs migrate into the coelomic cavity or the ovarian lumen. Even in embryos of *Op. roseus*, in which the coelomic linings are extremely thin and hardly visible at the light microscopic level (see Mayer *et al.*, 2005), the PGCs are clearly separated from the coelomic cavity by coelomic lining cells (Fig. 8C, D).

Development of the endogenous ovary

Further ovarian development is described only for the endogenous ovary of *Ep. biolleyi*. In this species, the male and female gonads do not differ in structure at early developmental stages but can be distinguished unambiguously after the establishment of the full

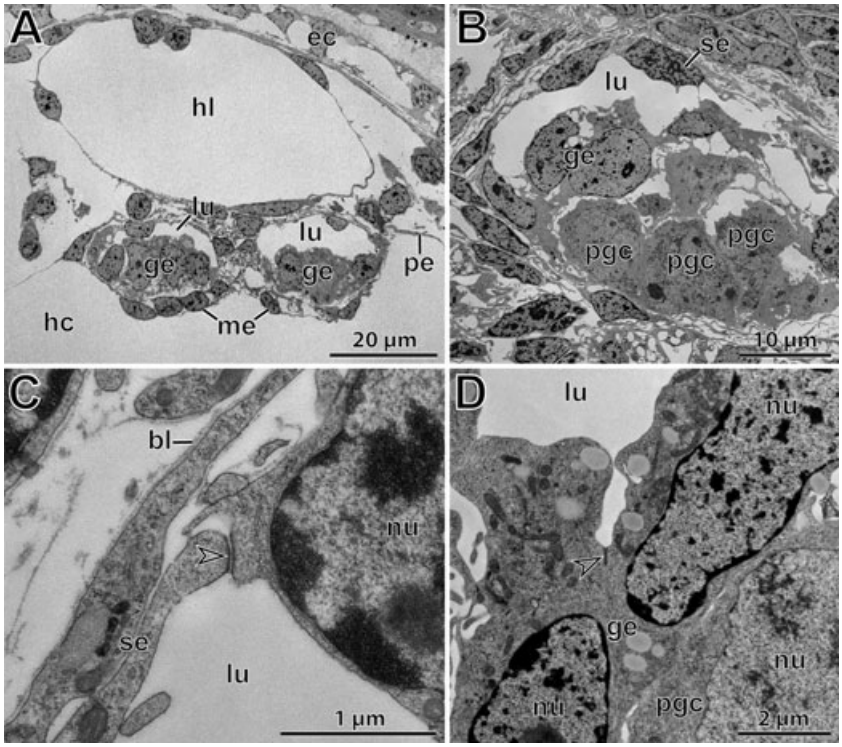

Figure 9. Development of the endogenous ovary in *Epiperipatus biolleyi*. Transmission electron micrographs. A, position of the ovarian anlage beneath the presumptive heart and attached to the pericardial floor (pe) in an embryo with a full number of somites. Cross-section of the dorsal body. B, cross-section of an ovarian tube. Note the distinct separation into a germinal epithelium (ge), which contains the primordial germ cells (pgc), and a sterile epithelium (se). C, detail of the sterile epithelium. D, detail of the germinal epithelium. Arrowheads indicate apical junctions. Abbreviations: bl, basal lamina; ec, ectoderm; ge, germinal ovarian epithelium; hc, haemocoel; hl, lumen of the presumptive heart; lu, lumen of the ovarian anlage; me, outer layer of mesodermal cells that will later form the ovarian musculature; nu, nucleus; pe, presumptive pericardial floor; pgc, primordial germ cells; se, sterile ovarian epithelium.

number of somites, which is higher in females. The ovarian walls are formed by the dorsal and visceral portions of the embryonic coelomic linings within the genital and few pregenital segments. The walls become attached to the pericardial floor during further development (Fig. 9A). At this stage, the internal structure of each presumptive ovarian tube shows a clear spatial separation into a dorsal sterile epithelium and a ventral germinal epithelium (Fig. 9B). The germinal epithelium is thick and contains PGCs, whereas the sterile epithelium is thin and composed of a monolayer of flattened cells that are joined by apical junctions and rest on a basal lamina (Fig. 9B, C). The PGCs do not show any direct contact to the ovarian lumen but are separated from it by the uppermost cells of the germinal epithelium facing the lumen (Fig. 9D). Such a spatial separation into a sterile and a germinal epithelium in *Ep. biolleyi* is remarkable because the fully developed ovary in this species does not show any sterile epithelial region (Figs 3C, 7A). Accordingly, the sterile epithelium must be abolished during further ovarian development. However, this has not been followed in the present study.

DISCUSSION

Ancestral ovarian structure in Onychophora and Arthropoda

In this paper, the structural and spatial relationships of tissues and cells in the three major types of onychophoran ovaries are described. The structural composition is basically similar in all three. The ovarian epithelium, which encloses a variously shaped lumen, is surrounded by a layer of connective tissue containing the musculature. In the exogenous and pseudoendogenous ovaries, the oogonia and immature oocytes originate in a specific region, the germinal epithelium, whereas the remaining epithelium is sterile and devoid of germinal cells. In the exogenous and pseudoendogenous ovaries, the maturating oocytes become

associated with stalks that are formed by specialized cells of the germinal epithelium. These cells have been referred to as 'follicle' cells (see Brockmann *et al.*, 2001), but there is no evidence that they provide nutrients to the developing oocytes as this name would imply. In the exogenous ovary, the oocytes project out into the haemocoel supported by these stalk cells and where they undergo further oogenesis to become mature primary oocytes. In this position, the oocytes are supported at the tip of the stalks and become physically isolated from the stalk cells by the developing primary egg membrane. In the pseudo-exogenous ovary, the oocytes become associated with stalk cells but they retain their intraovarian position. In contrast to the exogenous and pseudoendogenous ovaries, stalks are completely lacking in the endogenous ovary, in which the oogonia and developing oocytes are distributed in the germinal epithelium that completely surrounds the ovarian lumen. The sterile epithelium is absent from this ovarian type.

However, data presented here on the ovarian development in the neotropical peripatid *Ep. biolleyi* show that the endogenous ovarian type passes through a stage in which both germinal and sterile epithelia occur. A similar separation of germinal and sterile epithelia occurs during development in species of Peripatopsidae and South-East Asian Peripatidae (Sedgwick, 1887, 1888; Willey, 1898; Evans, 1901b). The separation persists in the adult ovary at least in Peripatopsidae (Manton, 1938; Brockmann *et al.*, 2001), whereas details of the internal ovarian organization have not been studied yet in South-East Asian Peripatidae. Nothing is known on the development of the pseudoexogenous ovary, but the adult ovarian structure shows a distinct separation into a sterile and a germinal epithelium (Mayer, 2007; present study). A division of the ovarian epithelium into a sterile and a germinal region, therefore, appears to be an ancestral feature of the onychophoran ovary. Accordingly, either the exogenous or the pseudoendogenous type displays the ancestral ovarian architecture because the sterile epithelium is lacking in the fully developed endogenous ovary (Huebner & Lococo, 1994; Brockmann *et al.*, 1999; present study).

Both the exogenous and pseudoendogenous ovaries bear stalked oocytes. However, only in the exogenous ovary do the developing oocytes bulge out into the haemocoel (Manton, 1938; Herzberg *et al.*, 1980; Brockmann *et al.*, 2001; Mayer, 2007). The question of whether the exogenous or the pseudoendogenous ovary displays the ancestral feature for the Onychophora can be addressed by an outgroup comparison with Arthropoda. Chelicerates have stalked oocytes that maturate within the body cavity in a comparable way to that seen in onychophorans (Dumont & Anderson, 1967; Makioka, 1978, 1988; Miyazaki & Makioka, 1989, 1990, 1991, 1994; Miyazaki, Ueshima & Makioka, 2001; Morishita *et al.*, 2003; Alberti & Michalik, 2004; Denardi *et al.*, 2004; Michalik *et al.*, 2005; Saito *et al.*, 2005; Miyazaki & Biliński, 2006; Talarico *et al.*, 2009). Furthermore, oocytes project into the haemocoel in some crustaceans, such as Ostracoda, Branchiura, Notostraca, and Pentastomida (Nørrevang, 1972, 1983; Ando & Makioka, 1992; Storch, 1993; Ikuta & Makioka, 1997, 1999). A number of studies have reported that the oocytes of myriapods are accompanied by stalks or extensions of the ovarian epithelium (Versluys & Demoll, 1922; Tiegs, 1940, 1945, 1947; Jangi, 1957; Knoll, 1974; Kubrakiewicz, 1987; Yahata & Makioka, 1991). However, it has been suggested that stalked oocytes in myriapods have a different origin from those in other arthropods and onychophorans (Yahata & Makioka, 1991). Although more detailed ultrastructural studies on myriapod oogenesis are necessary, it would seem that vitellogenesis takes place in the haemocoel outside the ovarian epithelium, at least in the Diplopoda, Symphyla, and Pauropoda (Tiegs, 1940, 1947; Kubrakiewicz, 1991; Yahata & Makioka, 1991). This is in contrast to the ovary in hexapods and some crustaceans, in which oocyte growth takes place within the ovarian lumen (e.g. Adiyodi & Subramoniam, 1983; Biliński & Szklarzewicz, 1992; Büning, 1994; Ikuta & Makioka, 1996; Fausto *et al.*, 2005; Kugler *et al.*, 2006). Oocyte maturation within the ovarian lumen, however, may be regarded as a derived feature as it does not occur in other arthropods or in onychophorans. Accordingly, an ovary with stalked oocytes projecting into the haemocoel may represent the ancestral organization in Onychophora and hence be a synapomorphy of Onychophora and Arthropoda.

Evolution of three major ovarian types in Onychophora

The exogenous ovary as the ancestral feature of Onychophora is consistent with recent phylogenetic reconstructions and is the most parsimonious assumption (Fig. 10). The endogenous and pseudoendogenous ovaries are considered to be derived from the exogenous type. According to data presented here, an evolutionary scenario can be drawn on how this might have happened. As the first step, the stalks failed to grow out beyond the ovarian surface in the pseudoendogenous ovary (heterochronic shift in oogenesis), but disappeared completely in the endogenous ovary. A detailed examination of the relationships of the ovarian tissues suggests that the endogenous and the pseudoendogenous ovaries diverged along separate

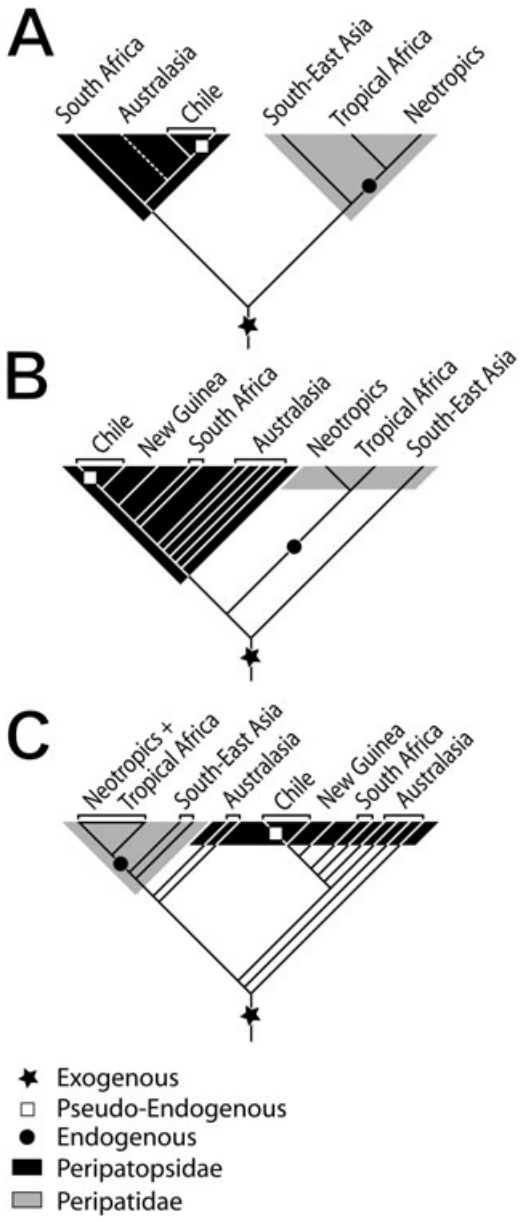

Figure 10. Mapping of three major types of onychophoran ovaries on simplified trees representing three different hypotheses on the phylogenetic relationships of Onychophora. Onychophoran subgroups are designated by their geographical distribution. Note that the suggestion of the exogenous type as an ancestral feature of Onychophora is consistent with all three phylogenetic hypotheses. A, both Peripatopsidae and Peripatidae are monophyletic (phylogeny modified from Monge-Nájera, 1995: fig. 10). B, Peripatidae are nonmonophyletic (phylogeny simplified from Reid, 1996: fig. 29). C, Peripatopsidae are nonmonophyletic (phylogeny simplified from Reid, 1996: fig. 28).

evolutionary pathways. In the pseudoendogenous ovary, the sterile epithelium has been retained and even extended laterally. In contrast to this, the germinal epithelium has obliterated the sterile epithelium in the endogenous ovary and has extended dorsally around a central lumen. Thus, the pseudoendogenous ovary does not simply represent a modified 'endogenous' type, as suggested by Bouvier (1901, 1902a, 1905), but instead evolved from an exogenous ovary by an independent pathway.

The occurrence of an endogenous ovary in the neotropical Peripatidae and, most likely, in the peripatid *Mes. tholloni* from tropical West Africa, supports the sister-group relationship of these two taxa (Kemp, 1914; Monge-Nájera, 1995; Reid, 1996). However, the internal structure of the ovary remains to be studied in *Mes. tholloni* before any final conclusions can be drawn. Special attention should be paid to the distribution of tracheae. Our data show that tracheae are present in the ovarian wall of the neotropical Peripatidae but are absent in the Peripatopsidae. Depending on their presence/absence in *Mes. tholloni* and the South-East Asian Peripatidae, this feature might prove phylogenetically relevant.

The occurrence of the pseudoendogenous ovary in *Met. inae* and *Met. blainvillei* suggests that *Metaperipatus* is a monophyletic taxon. Amongst the three ovarian types in the Onychophora, the exogenous ovary shows the greatest diversity with regard to the degree of fusion of the ovarian tubes (cf. Fig. 1). This diversity might provide additional information for future reconstructions of onychophoran phylogeny. Currently, however, the taxon sampling is too small to provide any meaningful phylogenetic arguments. In addition, some previous reports require verification, e.g. the description of anteriorly separate ovarian lumens in *Paraperipatus amboinensis* (Ambon Island, Indonesia) by Pflugfelder (1968). Future studies should focus not only on the external anatomy of the onychophoran ovary but also provide data on its internal organization.

Site of oocyte maturation does not support the monophyly of Articulata

In postembryonic onychophorans, the oogonia lie within the germinal epithelium, where they give rise to oocytes. This study has shown that neither the oogonia nor the oocytes are exposed to the ovarian lumen at any stage of oogenesis. Instead, they lie deep within the ovarian tissue between the adluminal cell layer and the basal lamina and, thus, belong to the ovarian epithelium. Even in the exogenous ovary, with oocytes bulging into the body cavity, the maturating oocytes are enclosed by the basal lamina of the germinal epithelium. Yet, the germ cells are atypical

epithelial cells as they do not show any apicobasal polarity. The onychophoran oogonia, therefore, differ from 'truly' epithelial germ cells (see Holland & Holland, 1991; Frick & Ruppert, 1996, 1997; Frick, Ruppert & Wourms, 1996).

The development, position, and growth of the oocytes separate from the ovarian lumen, a derivative of the embryonic coelomic cavity, are inconsistent with the traditional Articulata hypothesis that suggests a sister-group relationship between annelids and panarthropods (Nielsen, 1997, 2001; Wägele *et al.*, 1999; Ax, 2000; Wägele & Misof, 2001; Scholtz, 2002, 2003). According to this hypothesis, the 'gametes maturing within coelomic sacs' is one of the 'apomorphies shared with polychaetes' (Wägele & Misof, 2001: 170). However, this contrasts with the mode of oogenesis in Onychophora because the female germ cells are not exposed to the embryonic coelomic cavity or the ovarian lumen at any time during embryogenesis and oogenesis. Many studies on oogenesis in arthropods, including chelicerates, myriapods, and various entomostracan crustaceans and pentastomids, show that the oocytes do not maturate within the ovarian lumen (Nørrevang, 1972; Kubrakiewicz, 1991; Ikuta & Makioka, 1997, 1999; Morishita *et al.*, 2003; Denardi *et al.*, 2004; Michalik *et al.*, 2005; Talarico *et al.*, 2009). Thus, contrary to the suggestion of Wägele & Misof (2001), the site of origin and maturation of at least the female gametes in Panarthropoda does not support the monophyly of Articulata.

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