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Development of the internal reproductive organs in early nymphal stages of *Archegozetes longisetosus* Aoki (Acari, Oribatida, Trhypochthoniidae) as obtained by synchrotron X-ray microtomography (SR-µCT) and transmission electron microscopy (TEM)

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Abstract

We studied the development of the internal reproductive organs in juvenile stages of *Archegozetes longisetosus*. 3D-renderings of organs were obtained from synchrotron X-ray microtomography (SR-µCT). In addition, transmission electron microscopy (TEM) was used to obtain cytological details. The reproductive organs develop from an unpaired, ventral mass of mesodermal tissue in the larva, and development progresses continuously and largely independent from the development of other organs or moltings. Volume increase of the ovary and a growing number of germ cells indicate proliferation of oogonia in the deutonymph. The oviducts develop from dorso-lateral extensions of mesodermal somatic tissue.

Keywords: Actinotrichida**,** ovary, oviduct, 3D-rendering, premeiotic mitoses

1. Introduction

The Sarcoptiformes are considered an old and highly diversified group of the Acari. This is also reflected by their high diversity of reproductive modes and anatomical layouts of the reproductive organs (Alberti & Coons 1999). In comparison to this diversity, developmental studies are scarce and mainly focus on embryology (Aeschlimann & Hess 1984, Yastrebtsov 1992, Telford & Thomas 1998, Thomas & Telford 1999, Laumann et al. 2010a, b).

Existing studies on postembryonic development of mites mainly cover life history data (e.g. Heethoff et al. 2007, Santhosh et al. 2009, Kaimal & Ramani 2011) or external features (e.g. Köhler et al. 2005, Ermilov et al. 2008, Pfingstl & Krisper 2010.) Little is known about the progress in organogenesis in free living sub-adult stages. *Archegozetes longisetosus* ran (Heethoff et al. 2007) is the most intensely studied oribatid mite so far (e.g. Alberti et al. 2003, 2011; Smrž & Norton 2004 and cited references, Köhler et al. 2005, Laumann et al. 2010a, b; Heethoff et al. 2007, 2011), and anatomical analyses of the genital system have already been conducted on tritonymphal and adult stages (Bergmann et al. 2008). To follow the development of tissues and organs during earlier free-living stages, we combined two methods: synchrotron

X-ray microtomography (SR-µCT) was utilized to obtain structural data of homogeneous quality on the spatial arrangement and overall developmental state of the reproductive organs with a resolution approaching that of conventional light microscopy (Betz et al. 2007). These are represented as three-dimensional renderings of segmented datasets. Additionally, ultra structural data from the respective life stages were obtained by examining exemplary ultrathin sections with transmission electron microscopy (TEM). This combination proved to be useful, as the availability of volumetric data from all stages greatly facilitated the process of sectioning, whereas ultrastructural data could be utilized to validate the segmentation of the tomographies for 3D-models, in addition to clarifying cytological details. The aim of this study was to provide a first outline of the development of genital organs during free living, subadult instars of *A. longisetosus* with respect to both anatomy as well as histology and ultrastructure. In connection with establishing continuity between features found at different developmental stages, a scrutiny of designations derived from the adult state was undertaken. Towards a better understanding of the homologies of acarine reproductive systems, points of interest could be specified, especially concerning the reorganisation of mesodermal tissue and the building mode of lumina. Special interest was laid on providing a framework for the examination of oocyte development during nymphal stages, with the results of this study indicating the bulk of premeiotic mitoses to occur during the deutonymphal stage.

2. Materials and Methods

2.1. Rearing

Specimens were taken from our laboratory culture of *Archegozetes longisetosus* ran (Heethoff et al. 2007).

Freshly laid eggs were transferred singularly into the wells of tissue culture plates (Tissue Culture Cluster[24], Costar), and reared to appropriate age on a moisturized Plaster-of-Paris-charcoal mixture (6:1). The lids of the plates were sealed with Parafilm (Pechiney Plastic Packaging Ltd., Chicago) to prevent desiccation. The wells were kept in constant dark at 23°C and checked daily for moisture, and bark of various deciduous trees, covered with unicellular green algae, was supplied as a food source *ad libitum*. Larvae (one and five days after hatching), protonymphs (two and five days after hatching) and deutonymphs (two days and five days after hatching and during the quiescent period prior to the tritonymph) were removed with a fine brush for further processing.

2.2. Synchrotron X-ray microtomography (SR-µCT)

Specimens were immersed in a 6:3:1 mixture of 80% ethanol, 35–38% formaldehyde (standard solution, Merck) and 100% acetic acid for at least 24 hours for fixation. Dehydration was carried out in a graded ethanol series of 80%, 85%, 90%, 95% and 100% by weight with three times 10 minutes each, followed by critical point drying in liquid carbon dioxide (E 3000 Series II Critical Point Drying Apparatus, Polaron Equipment Limited). Dried specimens were fixed to the tip of PVC stubs of 12mm length and 3mm diameter using cyanacrylate glue.

Tomographies were recorded at the European Synchrotron Radiation Facility (ESRF) on beamline ID19. The selected energy level for all scans was 20.5 keV +/- 0, 5 keV. X-rays were converted to visible light by a scintillator and radiographs were taken on a cooled CCD (ESRF FreLoN) with 2048 x 2048 pixels and an effective pixel size of 0.7 µm for deutonymphs and 0.27 µm for larvae and protonymphs. We recorded 1,300 projections over 180° with 0.35 s

exposure time. For larvae and protonymphs, phase-enhanced tomography was performed at a sample-detector distance of 20 mm.

Deutonymphs were subjected to holotomography (Heethoff & Cloetens 2008), combining separate scans of the same specimen at 10, 20, and 45 mm distances to the detector. Holotomography includes a phase retrieval step, and the grey level in the tomographic slices is proportional to the local electron density (dark corresponding to a higher density in the representation used).

Voxeldata of one specimen per assigned sampling age, as described in paragraph 2.1, were visualized with the software VGStudio Max 2.1 (Volume Graphics, Heidelberg, Germany) and segmented with amira 4 (Mercury Computer Systems, Inc., Berlin, Germany).

2.3. Transmission electron microscopy (TEM)

Specimens were punctured and fixed in modified Karnovsky´s solution, containing 1.33% formaldehyde, 1.66% glutardialdehyde, 4% sucrose and 6.6 µM MgSO4 in 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at 0°C, pH 7.8 for 90 min. Dehydration was conducted in a graded ethanol series, and the samples were embedded in Araldite (Araldite CY212 premix kit 'hard', plano, Wetzlar) according to the method given in greater detail elsewhere (Bergmann et al. 2010). The fixative was prepared using freshly depolymerized paraformaldehyde (Fluka, Buchs, Switzerland) and EM-grade glutardialdehyde 25% (Science Services, Munich, Germany). All aqueous solutions, including rinsing steps, were buffered with HEPES buffer at pH 7.8. In the present preparation, postfixation was conducted using an aqueous solution of 1% osmium tetroxide, and en-bloc staining was included during the dehydration series using a saturated solution of uranyl acetate in 70% ethanol overnight at 4°C.

Ultrathin sections with a thicknes of 50nm were cut on a Reichert Ultracut (Leica-Jung, Vienna, Austria) microtome using diamond knives (Diatome 45°, Biel, Switzerland) and contrasted with ethanolic (50%) uranyl acetate for 12 min and lead citrate for 10 min. TEM was conducted on a Siemens Elmiskop 1A transmission electron microscope at 80 kV.

Micrographs were taken on 6,5 x 9 cm plate negatives. Original negatives were scanned at 1200 dpi in 8-bit RGB, and image processing was conducted using GIMP 2.6.10 (GNU Image Manipulation Program, Copyright © 1995–2008 Spencer Kimball, Peter Mattis and the GIMP development team). The image processing included reduction to grey values, inversion and tonal value and gamma correction. Scaling of the images was done using the program iTEM (Olympus, Tokyo).

3. Results

Putative precursors of the internal reproductive system are discernible as a solid condensation of inconspicuous cells medio-ventrally in the opisthosoma in larval stages 24h after hatching from the eggshell and prelarval exuvia. The condensation is situated close to the ventral body wall just anteriorly of the hindgut. Two lateral extensions are contacting the ventral body wall, and an unpaired portion stretches postero-dorsally between midgut (dorsally), hindgut (caudally) and caeca (laterally) (Fig. 1a,b).

In larvae five days after hatching, tissue structure as recorded by SR-µCT differentiates between the postero-median, unpaired portion and the lateral extensions. The dorsal, postero-median portion increases in volume, developing a kite-shaped appearance. It also

loses contact to the ventral body wall and develops a horizontal rostro-caudal orientation, stretching above and between the lateral portions (Fig. 1c, d).

Early protonymphal stages (two days) retain the solid kite-shape of the dorso-median portion, whereas the ventro-median portion no longer appears paired, but fused in the body midline, contacting the ventral body wall between the pair of protonymphal genital papillae. The dorsal portion appears notably more coarsely grained than the ventral portion in SR-µCT scans (Fig. 2a, b).

Transmission electron micrographs of protonymphs show a massive agglomeration of large (6–8 µm x 2.5 µm) cells in the central part. These contain large nuclei (4–5 µm x 2 µm) with finely grained euchromatin, few small dense particles of heterochromatin and a single prominent nucleolus. The cytoplasm of these cells is denser than that of surrounding tissues, contains numerous ribosomes and a conspicuous, sharply circumscribed region of high electron density, frequently containing an even denser, irregular core. Few, but relatively wide cisternae of the endoplasmic reticulum surround this region and the nucleus and sometimes form concentric figures in the cytoplasm. Numerous mitochondria form clusters in the cytoplasm. These mitochondria are of elongated appearance and relatively small compared to the rounded mitochondria of neighboring fat body cells (Fig. 2c).

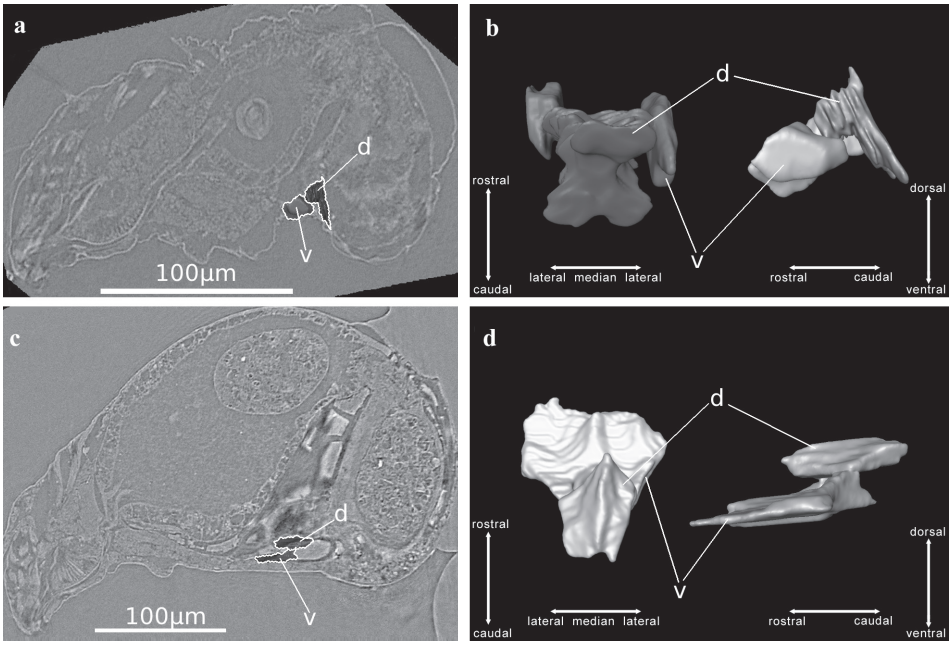

Fig. 1 *Archegozetes longisetosus,* phase enhanced SR-µCT data. Virtual slices and 3D-renderings of the reproductive organs in the larval stage. Top row: Larva 24 h after hatching. **a**: Sagittal virtual slice and 3D rendering outlined in white. **b**: Dorsal (left) and lateral (right) view of 3D rendering obtained from segmentation of voxel data. Two lateral elements are assigned as ventral portion regarding their identification with structures later in development. Bottom row: Larva five days after hatching. **c**: Sagittal virtual slice and 3D rendering outlined in white. **d**: Dorsal (left) and lateral (right) view of 3D rendering obtained from segmentation of voxel data.

Abbr.: d - dorsal, postero-median portion of reproductive organs; v - ventral portion of reproductive organs.

Situated dorsally of these large, dense cells are several smaller, flattened cells with very electron lucent cytoplasm. A strand of small, medium dense cells likely representing the ventro-median portion of the genital organs as obtained from SR-µ-CT (labelled 'vmC' in Fig. 2c) connects the median portion to the ventral body wall.

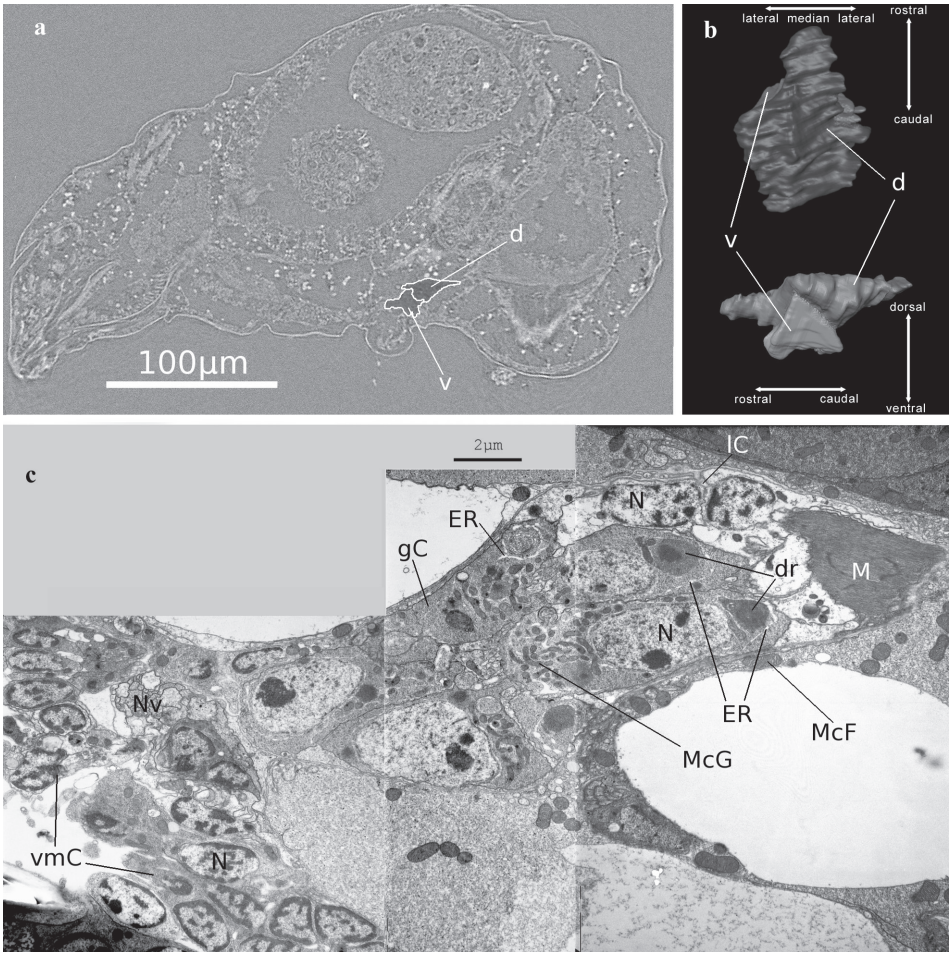

Fig. 2 *Archegozetes longisetosus,* phase enhanced SR-µCT data. Virtual slices and 3D-renderings of the reproductive organs in the protonymphal stage and TEM micrograph of protonymph two days after molting. **a**: Sagittal virtual slice, segmentation area outlined in white with grey shading. **b**: Dorsal (top) and lateral (bottom) view of 3D rendering obtained from segmentation of voxel data. **c**: TEM micrograph combined from three original negatives. Plane of sectioning is parasagittal.

Abbr.: d - dorsal portion of reproductive organs, dr - dense cytoplasmal region of germline cell, ER - cisternae of the endoplasmic reticulum, gC - germ line cell, lC - electron lucent somatic cell, M - muscle, McG - mitochondria of germline cells, McF - mitochondria of fat body cells, N - nucleus, Nv - ventral Nerve, v - ventral portion of reproductive organs, vmC - mesodermal cell of ventral portion of the reproductive organs.

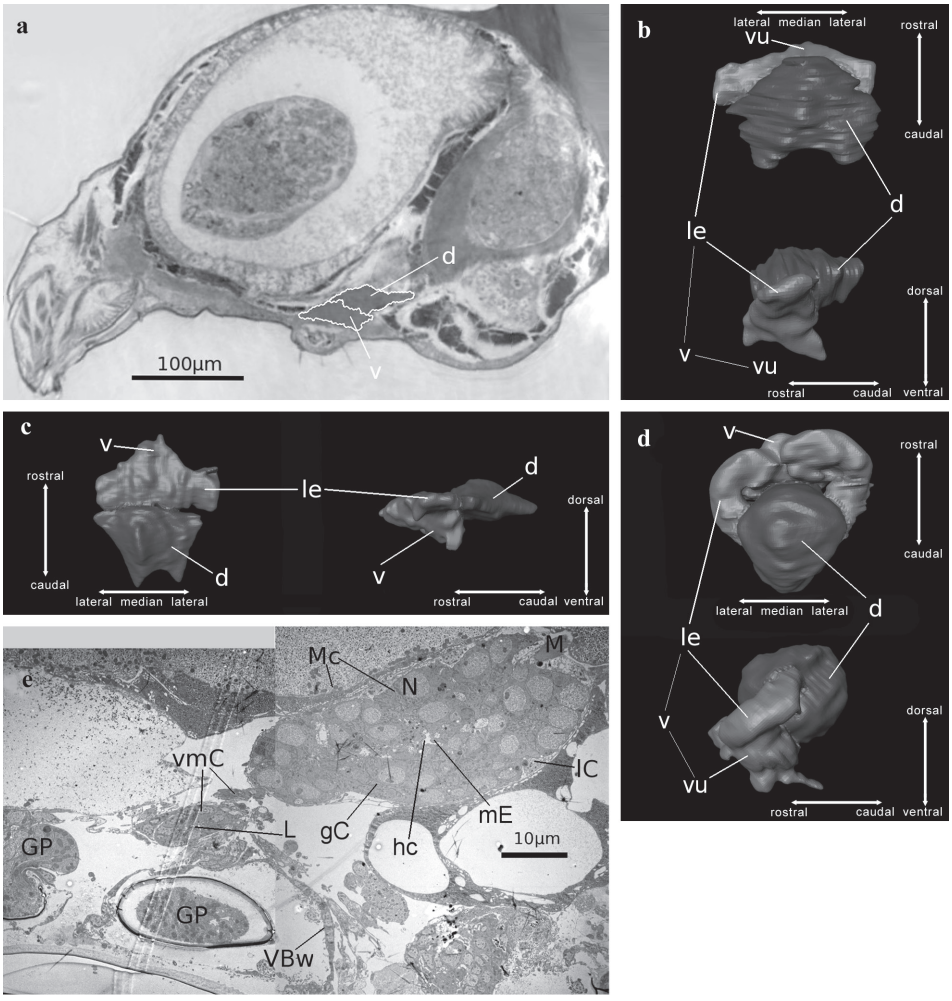

Fig. 3 *Archegozetes longisetosus*, holotomographic SR-µCT data. Virtual slices and 3D-renderings of the reproductive organs in the deutonymphal stage and TEM micrograph of deutonymph. The dorsal portion of the reproductive organs is increasing in volume and acquiring a spherical shape, as the ovary is formed by proliferation of germ line cells. Lateral extensions of the ventral, somatic, mesodermal portion of the reproductive organs develop into oviducts as tubular connections between ovary and ventral, unpaired reproductive duct. **a**: Sagittal virtual slice of deutonymph five days after molting, segmentation area outlined in white with grey shading. **b**: Dorsal (top) and lateral (bottom) view of 3D rendering obtained from segmentation of voxel data of deutonymph two days after molting. **c**: Dorsal (left) and lateral (right) view of 3D rendering obtained from segmentation of voxel data of deutonymph five days after molting. **d**: Dorsal (top) and lateral (bottom) view of 3D rendering obtained from segmentation of voxel data of deutonymph from the quiescent stage prior to molting to the tritonymph. **e**: TEM micrograph combined from two original negatives. Plane of sectioning is parasagittal. (...for abbreviations see next page)

These cells contain only little cytoplasm, so even though their nuclei are only of about roughly half the diameter compared to that of the larger median cells of the central part, their nucleus to cell volume ratio is higher.

The nuclei of the latter two cell types show the aggregation of heterochromatin around their periphery typical for glutardialdehyde fixed interphase nuclei (Fig. 2c).

Deutonymphs two days after hatching show a slightly enlarged genital region (Fig. 3a, b). The dorso-median part is developing a central bulge. Lateral extensions are forming at the dorsal part of the ventral portion, extending dorso-laterally into the haemolymph space. These extensions are flap-like, not tubular, and connected neither to the dorso-median portion nor to the ventral body wall.

In deutonymphs at five days after molting, these extensions appear more prominent (Fig. 3c). The ventro-median part is larger and protrudes more dorsally into the body cavity. The dorsal part appears elongated into two points posteriorly, and shifts caudally.

Deutonymphs from the quiescent phase ready to hatch as tritonymphs exhibit a voluminous, ball shaped dorsal portion with a radially patterned coarse structure (Fig. 3d). The adjacent ventral portion is differentiated into four regions: i) a small, rhombical flattened portion surrounding the prospective genital opening, ii) a barrel shaped unpaired portion reaching dorsally and contacting the dorsal portion of the reproductive organs, and iii) two lateral extensions dorso-laterally connecting the barrel-shaped part to the dorso-caudal portion.

These lateral extensions appear tubular in cross-sections, as indicated in Fig. 3e. TEM micrographs of deutonymphs show three cell types identifiable with the types described for the protonymph (Fig. 3e). The large, medium dense cells corresponding in all aspects to those described before with large nuclei have increased in number and form the bulk of the ball shaped dorso-caudal part. Cells at the periphery of this agglomeration measure 8–10 µm in length, with nucleus diameters of about 3 µm. The central part of this portion contains hollow spaces, partially filled by elongated cell extensions of circular cross section containing several microtubule. Electron lucent, putatively somatic cells of flattened appearance form a single layer surrounding the dorsal part.

The ventral part consists of small, irregular cells of medium electron density very similar to those described for the protonymph in this region. Cross sections reveal a single cell layer surrounding an irregular lumen which is identified as the tubular extensions described above (Fig. 3e).

In young tritonymphs, the medio-ventral part enlarges. An invagination of the ventral body wall is developing into the ovipositor (Fig. 4a). The tubular extensions of the medio-ventral portion extend further dorso-laterally as compared to the deutonymph (Fig. 4b). Ultrathin sections frequently show tubular structures surrounded by a basal lamina and a second layer of flattened cells, which in turn is covered by a basal lamina on the haemolymph side (Fig. 4c).

Fig. 3 (page 464) Abbr.: hc - central hollow of the dorso-median portion, d - dorsal portion of reproductive organs, gC - germ line cell, GP - genital papilla, L - lumen, lC - electron lucent somatic cell, le - lateral extensions of the ventral portion of the reproductive organs, M - muscle, Mc - mitochondria, mE - microtubule-rich extensions of germ cells, N - nucleus, v - ventral portion of reproductive organs, Vbw - ventral body wall, vmC - mesodermal cell of ventral portion of the reproductive organs, vu - ventral unpaired portion of the reproductive organs.

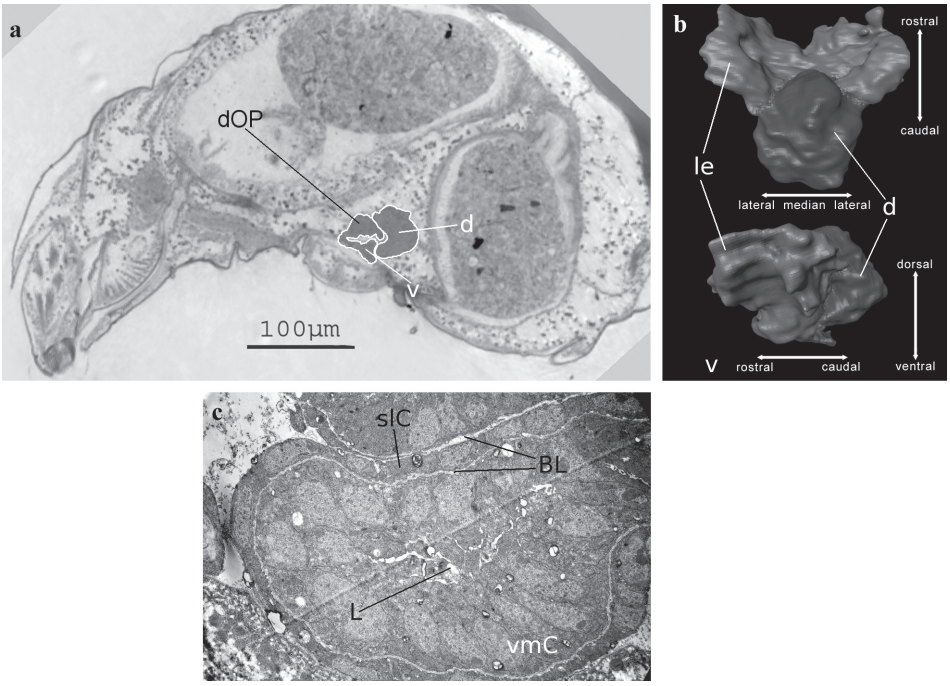

Fig. 4 *Archegozetes longisetosus*, holotomographic SR-µCT data. Virtual slices and 3D-renderings of the reproductive organs in the early tritonymphal stage and TEM micrograph of tritonymph. **a**: Sagittal virtual slice of tritonymph two days after molting, segmentation area outlined in white with grey shading. **b**: Dorsal (top) and lateral (bottom) view of 3D rendering obtained from segmentation of voxel data of tritonymph two days after molting. **c**: TEM micrograph of cross section from the lateral extensions of the ventral portion of the reproductive organs developing into oviducts.

Abbr.: BL - basal lamina, d - dorsal portion of reproductive organs, dOP - developing ovipositor, L - lumen, le - lateral extensions of the ventral portion of the reproductive organs, slC - second layer of somatic cells surrounding the oviduct, sheathed in basal lamina, v - ventral portion of the reproductive organs, vmC: mesodermal cell of ventral portion of the reproductive organs.

4. Discussion

The internal reproductive system of mites generally develops from mesodermal epithelia of the second opisthosomal segment, as is typical in all chelicerates (Anderson 1973). In many groups of chelicerates, reproductive systems in their adult state retain the general, putatively plesiomorphic structure of a folded layer of somatic epithelium, from which germ cells of various or unknown origin bulge into the haemolymph space on a multicellular funnicle, surrounded only by the basal lamina of the ovarian epithelium (Seitz 1971, Alberti & Coons 1999, Talarico et al. 2009), a situation also found, primarily or secondarily, in certain crustaceans, as opposed to the ovariole type of ovaries found in other crustacean groups and hexapods (Ando & Makioka 1998). Although accordant ovarian architectures are present among mites (Saito et al. 2005), acarine ovaries show an astounding variation of specializations overall, leading to a great variety of shapes and setups (Alberti & Coons 1999; Coons & Alberti 1999). In many oribatids, oocytes progress centrifugally from the ovarian

medulla, yet never bulge into the haemolymph space singularly, nor retreat into an ovarian lumen prior to ovulation, but rather progress in solid strands of oocytes and epithelial cells, and finally pass into the oviductal lumen by pervading the surrounding sheet of follicular, or rather ovarian, cells (Woodring & Cook 1962, Witaliński 1986, Taberly 1987).

Comparison of developmental stages enabled by SR-μCT point to the fact that in free-living subadult stages of *A. longisetosus* development of internal organs progresses continuously, and is not synchronized in defined stages with the moltings, including e.g. the development of body appendages. Prior to the molting of the next instar, specimens enter a quiescent phase of 2-3 days (Haq 1978). The state of development is not identical in specimens taken from the same quiescent phase prior to the next molting (pers. obs.), and developmental progression between moltings, i.e. during the same nymphal stage is substantial, especially during the deuto- and tritonymphal stage (Fig. 4).

A similar independence of the developing internal reproductive organs from the time course of development of other organs was also noted by Seitz (1971) for subadult specimens of the spider *Cupiennius salei* Keys.

Neither a segmented ovary precursor as in *C. salei* (Seitz 1971), nor paired structures of the germarium, like in *Hafenrefferia gilvipes* Koch (Witaliński 1986) were found in the development of *A. longisetosus*.

We identified the large cells forming the central, dorso-medial portion of the developing reproductive system as germ line cells. Typical features of germ line cells are large, finely grained nuclei, generally large volume, comparably dense cytoplasm rich in ribosomes, clusters of mitochondria and especially conspicuous areas of dense cytoplasm (Extavour & Akam 2003). The progression from a flattened to a ball shaped structure, simultaneous with an increase in volume during the deutonymphal stage indicates the bulk of premeiotic mitosis during that time, in concordance with the larger number of putative oocytes in sections of deutonymphal organs. As no diagnostic features of ongoing mitosis were found in the sections, we conclude this on indirect evidence only. Thus, in concordance with the findings of Taberly (1987), germ line cells of unknown origin cluster as a massive aggregation in early development, no later than the early protonymphal stage, in the median portion of the mesodermal genital precursor, that is subsequently becoming the germarium of the ovary during the nymphal stages of *A. longisetosus*.

It is surrounded by a thin layer of somatic tissue connected with, but structurally different from the mesodermal tissue which later develops into the mesodermal part of the genital ducts. Two portions of mesodermal tissue in sub adult internal reproductive organs were also described by Seitz (1971): an epithelial sheet, separating the organ from surrounding tissues, and a so-called intermediate tissue between the oocytes in the ovarian proper. The oviducts in *C. salei* are described to develop from rostral portions of the epithelial sheet. Although two types of somatic cells with consistently differing cytological properties were identified in *A. longisetosus* as well, the respective tissue architectures differ. While small, dense cells with high nucleus to volume ratio form the ventro-median part, from which the uterus and oviducts develop, the sheet of somatic cells surrounding the germarium is developing from a group of flattened, electron lucent cells dorso-rostrally adjacent to the germ cell cluster in protonymphs and only later establishing contact to the ventro-median portion.

This distribution of tissues is concordant with the adult stage, in which a massive cluster of oocytes, the rhodoid, is surrounded by follicular epithelium (Bergmann et al. 2008, 2010). Wrapped singularly in this epithelium, oocytes form the meroi of the ovary, progressing towards the oviducts. Oviductal and follicular epithelia differ greatly in terms of tissue

structure, yet share a continuous basal lamina (Bergmann & Heethoff 2012). These adult features let us assume that the electron lucent cells surrounding the germarium, starting from an antero-dorsal cluster in the protonymph, are follicular epithelium precursors derived from the somatic cells of the tip of the mesodermal fold forming the primordial reproductive organs.

Oviducts are generally considered coelomoducts (Anderson 1973) connecting the coelomic interior via the ectoderm with the environment through the primary genital orifice (the tip of the ovipositor, constituting an ectodermal duplicature, forming a secondary, and the closure of the genital plates a tertiary genital opening). In *A. longisetosus,* although it is too early for definite statements, the oviducts seem to form as laterally protruding edges of the mesodermal fold developing into tubular structures. Apparently massive, flattened lateral extensions protrude dorso-laterally from the median, somatic portion of the reproductive system early in development. A lumen develops in the center of these protrusions during the deutonymphal stage, while anterior and posterior ends stay in contact with the ventral and dorsal (ovarian) part, respectively. Whether the oviductal lumen is the primary hollow of the mesodermal fold or forms later, when epithelial cells loose contact on their basal side, remains unclear.

During the deutonymphal stage, starting in late protonymphs, a similar process occurs in the ovary, where hollow spaces develop in the center, partially filled with elongated cell extensions containing microtubules. Whether these are identical with the microtubuli-rich oocytal extensions present in the adult ovarian medulla (Witaliński 1987) could not be demonstrated with certainty. If they are, and the ovarian central lumen is primarily continuous with the oviductal lumen, the site of contact of the oviducts to the ovary in the adult stage is likely to be a secondary one, as no connection of the medulla to the oviductal lumen has been demonstrated in the adult, and developing oocytes in the meroi are propagating outwards after losing contact with the medulla of the rhodoid, and enter the oviductal lumen by passing the follicular epithelium (Bergmann et al. 2008, Bergmann & Heethoff 2012). Further, the 3D-renderings indicate a primary site of contact between ovary and genital tract in the median line. The continuous basal lamina, however, indicates a primary connection site, in which case the massive meroi may be regarded as a secondary feature, indicated by the fact that they develop only as late as in the adult stage, when peripheral oocytes in the ovary start serial vitellogenesis at the contact site. Further studies are essential to assess the progression of tissue-tissue relations as well as the mode of cell movement during development to clarify this point.

So far, the basic organization of the internal reproductive organs of *A. longisetosus* is similar to other chelicerates, and many similarities exist in the adult anatomy of other oribatid genitalia. Developmental tissue reorganization on the other hand seems not to be readily explainable by models applicable to other, non-oribatid groups of Acari (e.g. Shatrov 2002, Saito et al. 2005).

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