Development and function of the genital organs in the parthenogenetic oribatid mite *Archegozetes longisetosus* Aoki 1965

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines

Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von

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Tübingen

2013

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	21. 10. 2013
Dekan:	Prof. Dr. Wolfgang Rosenstiel
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Publications[1]

- **Heethoff, M., Bergmann, P. and Norton, R. A.** (2006) Karyology and Sex determination of Oribatid Mites — Acarologia **46**, 127–131.
- **Heethoff, M., Laumann, M. and Bergmann, P.** (2007) Adding to the Reproductive Biology of the Parthenogenetic Oribatid Mite, *Archegozetes longisetosus* (Acari, Oribatida, Trhypochthoniidae) — Turkish Journal of Zoology **31**, 151–159.
- ***Bergmann, P., Laumann, M., Cloetens, P. and Heethoff, M.** (2008) Morphology of the internal reproductive organs of *Archegozetes longisetosus* Aoki (Acari, Oribatida) — Soil Organisms **80** (2), 171–195.
- ***Laumann, M., Bergmann, P. and Heethoff, M.** (2008) Some remarks on the cytogenetics of oribatid mites — Soil Organisms **80** (2), 223–232.
- ***Bergmann, P., Laumann, M. and Heethoff, M.** (2010a) Ultrastructural aspects of vitellogenesis in *Archegozetes longisetosus* Aoki, 1965 (Acari, Oribatida, Trhypochthoniidae) — Soil Organisms **82** (2), 193–208.
- ***Laumann, M., Bergmann, P., Norton, R. A. and Heethoff, M.** (2010b) First cleavages,preblastula and blastula in the parthenogenetic mite *Archegozetes longisetosus* (Acari,Oribatida) indicate holoblastic rather than superficial cleavage — Arthropod Structure and Development **39**, 276–286.
- **Fernández, R., Bergmann, P., Almodóvar, A., Díaz Cosín, D. J. and Heethoff, M.** (2010). Ultrastructural and molecular insights into three populations *Aporrectodea trapezoides* (Dugès, 1828) (Oligochaeta, Lumbricidae) with different reproductive modes. — Pedobiologia **54**, 281–290.
- ***Bergmann, P. and Heethoff, M.** (2012a). The oviduct is a brood chamber for facultative egg retention in the parthenogenetic oribatid mite *Archegozetes longisetosus* Aoki (Acari, Oribatida). — Tissue&Cell **44**, 342–350.
- ***Bergmann, P. and Heethoff, M.** (2012b) 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) — Soil Organisms **84** (2), 459–470.
- ***Bergmann, P., Laumann, M. And Heethoff, M.** Synaptonemal complexes in early Tritonymphs confirm automictic parthenogenesis in *Archegozetes longisetosus* (Chromosoma, submitted)
- **Heethoff, M., Bergmann, P., Laumann, M., and Norton, R.A.**(2013) The 20th anniversary of a model mite: *Archegozetes longisetosus* ran (Acari, Oribatida) — Acarologia **53**(4), 353-368

[1]Publications marked with an asterisk (*) are part of this thesis

Contributions of authors to the publications included in this thesis

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Abstract

Archegozetes longisetosus Aoki 1965 is a parthenogenetic oribatid mite, that has a pantropical-disjunct distribution. It is a member of the family of Trhypochthoniidae. Its ease of rearing, rapid succession of generations and high fecundity fulfill central requirements of a suitable model organism. **R**oy **A**. **N**orton in 1993 founded a laboratory strain from a single puertorican female, *Archegozetes longisetosus* **ran**, which is since kept in laboratories worldwide. This laboratory strain lead to *A. longisetosus* being the most thoroughly studied oribatid mite. The species is of special interest in studies on evolutionary biology, as it is a member of a cluster of obligatory parthenogenetic species, for which molecular studies have indicated a very old age of probably more than a hundred million years. Detailed insight in the reproduction of *A. longisetosus* therefore does not only broaden our knowledge of a model organism, but may also contribute to a better understanding of the ancillary conditions of uni- vs. bisexual propagation, as well as the differential likelihood of long term stability in different forms of unisexual propagation. One step on the way towards this goal, and a necessary condition for future studies, is the acquisition of anatomical data regarding structure, function and development of organs and tissues. This cognitive interest lay at the starting point of the study at hand. With the aid of high-resolution synchrotron X-ray micro computer tomographies (SR-μCT), three-dimensional models of the genital organs and their precursors were obtained from all freeliving instars (larva to adult). As several models per instar at intervals of several days were obtained, important insight in developmental processes was gained. Already in the larva, germinative and somatic portions of the genital anlage can be distinguished. Further development proceeds continously, and largely independent from cuticular moulting. Precursors of the oviducts start to develop in the protonymphal stage, whereas proliferation of the germcells takes place during the deutonymphal stage. Ectodermal portions of the genital systems start development in the tritonymphal stage. The oviducts apparently do not form as evaginations of a coelomic sac containing germline cells, but either as lateral folds of the coelomic cavity or as lateral evaginations, which retrogradally form secondary contact sites to the germinative portion. Further investigations are needed to establish the mode of oviduct formation unequivocally.

Three-dimensional models also facilitated the planning of semi- and ultrathin serial sectionings, which yielded histological and functional information. Observations made using electron- and lightmicroscopy included the onset of meiosis I in the tritonymphal stage, uptake of yolk precursors from the fat body into the oocyte via microvilli and coated pits, accumulation of egg shell material simultaneous to yolk accumulation, and the solidification of the egg shell upon passing into the oviduct. These observations justified a nomenclature of the genital system and the classification of the ovary as panoistic. Additionally, the oviducts could be described as a sheltered space for embryonal development. In summary, the results indicate that the loss of fertilization in the reproduction of *A. longisetosus* permits both anatomical and temporal compaction of the related processes, enabling relatively high average reproductive rate, even in the face of short-term unstable environmental conditions. The selection of methods permitted to demonstrate the mechanism of thelytoky by terminal fusion automixis with inverted (postreductional) meiosis in a functional context, and to present *A. longisetosus* as a promising model system also for questions beyond the borders of the taxon chelicerata.

Parthenogenesis, reproduction, model organism, SRμCT, electron microscopy

Abstract

Archegozetes longisetosus Aoki 1965 ist eine parthenogenetische Hornmilbe mit pantropisch-disjunkter Verbreitung aus der Familie der Trhypochthoniidae. Durch ihre einfache Haltung, schnelle Generationenfolge und hohe Fruchtbarkeit erfüllt sie wesentliche Merkmale eines geeigneten Modellorganismus. 1993 wurde von **R**oy **A**. **N**orton aus einem einzelnen puertoricanischen Weibchen ein Laborstamm etabliert, *A. longisetosus* **ran**, der inzwischen weltweit in Laboren vertreten ist, was mit dazu führte, dass *A. longisetosus* mittlerweile die insgesamt am besten untersuchte Hornmilbe darstellt. Von besonderem Interesse ist die Art für evolutionsbiologische Studien, Da sie zu einem Schwarm obligat parthenogenetischer Linien gehört, für die molekulare Studien ein sehr hohes Alter von evtl. über hundert Millionen Jahren nahelegen. Ein genaues Verständnis der Fortpflanzungsvorgänge von *A. longisetosus* dient also nicht nur der Erweiterung unserer allgemeinen Kenntnis eines Modellorganismus, sondern darüber hinaus dem besseren Verständnis der Randbedingungen ein- und zweigeschlechtlicher Fortpflanzung, sowie der unterschiedlich langen Überlebenswahrscheinlichkeit der verschiedenen Formen eingeschlechtlicher Fortpflanzung. Ein Schritt auf dem Weg zu diesem Verständnis, und notwendige Bedingung für weiterführende Studien, ist der Aufbau einer anatomischen Datengrundlage zu Aufbau, Funktion und Entwicklung der an der Fortpflanzung beteiligten Organe und Gewebe. Dieses Erkenntnisinteresse lag der vorliegenden Arbeit zugrunde. Anhand hochauflösender Synchrotron- Röntgen-Mikro- Computer- Tomographien (SR-µCT) wurden dreidimensionale Modelle der Genitalorgane und ihrer Anlagen in allen freilebenden Stadien von der Larve bis zum Adultus erstellt. Da pro Entwicklungsstadium mehrere Modelle im Abstand weniger Tage erstellt werden konnten, konnten so wichtige Erkenntnisse über die Entwicklung der Genitalorgane gewonnen werden. So zeigte sich, dass schon in Larvenstadien ein somatischer und ein germinativer Anteil der Genitalanlage unterschieden werden kann. Die weitere Entwicklung dieser Anlage verläuft kontinuierlich, und weitgehend unabhängig von den Häutungen der Körperhülle. Vorläufer der Ovidukte beginnen sich ab der Protonymphe zu entwickeln, während die Vermehrung der Eizellen in der Deutonymphe stattfindet. Ab der Tritonymphe bilden sich auch die ektodermalen Anteile des Genitalsystems aus. Die Ovidukte bilden sich dabei wohl nicht als Evaginationen eines die Eizellen enthaltenden Coelomsackes, sondern entweder als randliche Abfaltungen des Genitalcoeloms,

oder als laterale Evaginationen, die retrograd sekundären Kontakt zum germinativen Teil aufnehmen, eine Beobachtung, die noch weiterer Klärung bedarf. Sehr erleichtert durch die Modellserie wurde auch die Planung von Semi- und Ultradünnschnittserien ausgewählter Strukturen, die histologische und funktionelle Einsichten lieferten. So konnte das Einsetzen der Meiose in der Tritonymphe, die Aufnahme von Dottervorstufen aus dem Fettkörper in die Eizelle durch Mikrovilli und ‘coated pits´, die Ablagerung von Eihüllenmaterial und dessen Verfestigung beim Übergang ins Ovidukt beobachtet werden. Durch diese Erkenntnisse konnte eine Nomenklatur des Genitalsystems begründet werden, das Ovar als funktionell panoistisch klassifiziert und die Ovidukte als geschützter Raum der Embryonalentwicklung beschrieben. Insgesamt ergeben sich Hinweise darauf, dass der Reproduktionsmodus durch Wegfall der Befruchtung die anatomische Verdichtung und Verschränkung der Prozesse erlaubt, was eine konstant hohe Reproduktionsrate auch unter kurzfristig wechselnden Lebensbedingungen ermöglicht. Mit der vorgestellten Methodenauswahl konnte der Reproduktionsmodus der Thelytokie mit terminaler Fusion und invertierter (postreduktionaler) Meiose im funktionalen Zusammenhang dargestellt, und *A. longisetosus* als aussichtsreiches Modellsystem für auch über den Bereich der Cheliceraten hinausweisenden Fragestellungen vorgestellt werden.

Parthenogenesis, reproduction, model organism, SRμCT, electron microscopy

Contents

1 Introduction

1.1 An oribatid mite becomes a model organism

Major leaps in understanding life on earth were achieved following the establishment of model organisms (Heethof et al. 2013). Species like *Arabidopsis thaliana* (L.) Heynh 1842., *Drosophila melanogaster* Meigen 1830, *Danio rerio* (Hamilton 1822) or *Rattus norvegicus* (Berkenhout, 1769) provide starting points to examine the complexity of life in detail (Heethof et al. 2013). Comparison of individual studies with results from model organisms yielded insight in phylogeny, physiology, genetics, evolution, and several other fields of life sciences (Heethof et al. 2013). However, the research fostered by the establishment of model organisms also demonstrated the difficulties of generalising results, especially when using distantly related model organisms (Heethof et al. 2013). Therefore, it seems desirable to expand the number of model taxa to cover most traditionally recognised major metazoan clades (Heethof et al. 2013). The Chelicerata represent a major subgroup of the Arthropoda, that has a long evolutionary history, dating back as long as the Ordovizian era (Weygoldt 1998, Heethof et al. 2013). However, there are few model organisms among the chelicerates that are as generally accepted as *D. melanogaster* is for the Hexapoda. There are certain species of chelicerates that are thoroughly investigated, like the horseshoe crab *Limulus polyphemus* L., the spider *Cupiennius salei* (Keyserling 1877), the spider mite *Tetranychus urticae* Koch 1836, or the hard tick *Amblyomma triste* Koch 1844 (Heethof et al. 2013). However, most of these examples do not easily serve as convenient model species for all chelicerates. This is partly due to the evolutionary age of the chelicerate subdivisions resulting in vast divergences that in turn result in limited comparability. For example, *L. polyphemus* is considered close to the root of euchelicerata (Regier et al. 2010), and represents one of the very few marine forms, whereas *A. triste* is a highly derived ectoparasite of terrestrial mammals. While the range for

which a chelicerate model species can serve as a useful comparison is therefore limited, many major subdivisions of the chelicerates, like for example the Amblypygi, Ricinulei, or Palpigradi, are represented by only few detailed analyses other than their general description (e.g. Rucker 1901, 1903a,b, Alberti 1979). Additionally, the broad range of investigations conducted on *R. norvegicus* or *D. melanogaster* throughout many fields of life sciences is until today rarely reached in any chelicerate model. Combining detailed information on chelicerates therefore often involves only distantly related members. The latter two problems, unrepresented major subgroups and limited fields of investigation, may also be due to the fact that most chelicerates are strictly predatory species, making it more difficult to establish a laboratory strain, since this often includes the establishment of an additional culture of prey species. Model species feasible for comparative analysis would be of special interest for the chelicerates, as the position of this taxon among the well-established monophylum of Arthropoda was not without discussion during recent decades. The existence of a putative taxon "Myriochelata" (Friedrich & Tautz 1995) (i.e., comprising myriapods and chelicerates) remains unclear (Hassanin 2005; Mayer & Whitington 2009; Regier et al. 2010). The concept of myriapods and chelicerates forming a monophylum, however, is conflicting with an older concept of a monophylum Mandibulata, (comprising Myriapoda, Hexapoda and Crustacea). While not contradicting the traditionally recognised "Tracheata" (Myriapoda+Hexapoda) (Wägele 1993), the Mandibulata concept is also supported by recent analyses (e.g. Sombke et al. 2012), of which some propose a different view on relationships within Mandibulata (Regier et al. 2010; Rota-Stabelli et al. 2011). These analyses also found support for the Pancrustacea, comprising Hexapoda nested within the paraphyletic "Crustacea" (e.g., Wheeler et al. 1992; Fahrbach 2004). With new views emerging on the relationships between the major subgroups of Arthropoda, comparative analyses among arthropods might get additional attention in the near future. Hence, this thesis aims at providing additional data from the most species-rich chelicerate group "Acari".

The "Acari" are highly diversified and can be found in almost any habitat from subarctic glacier springs to tropical rainforests (Fig. 1) (Heethof et al. 2013). 50 000 species are described so far, which may represent only a small percentage of the total number of extant species. However, "Acari" is a traditionally recognised taxon, but doubt has repeatedly been cast on its true existence as a monophyletic

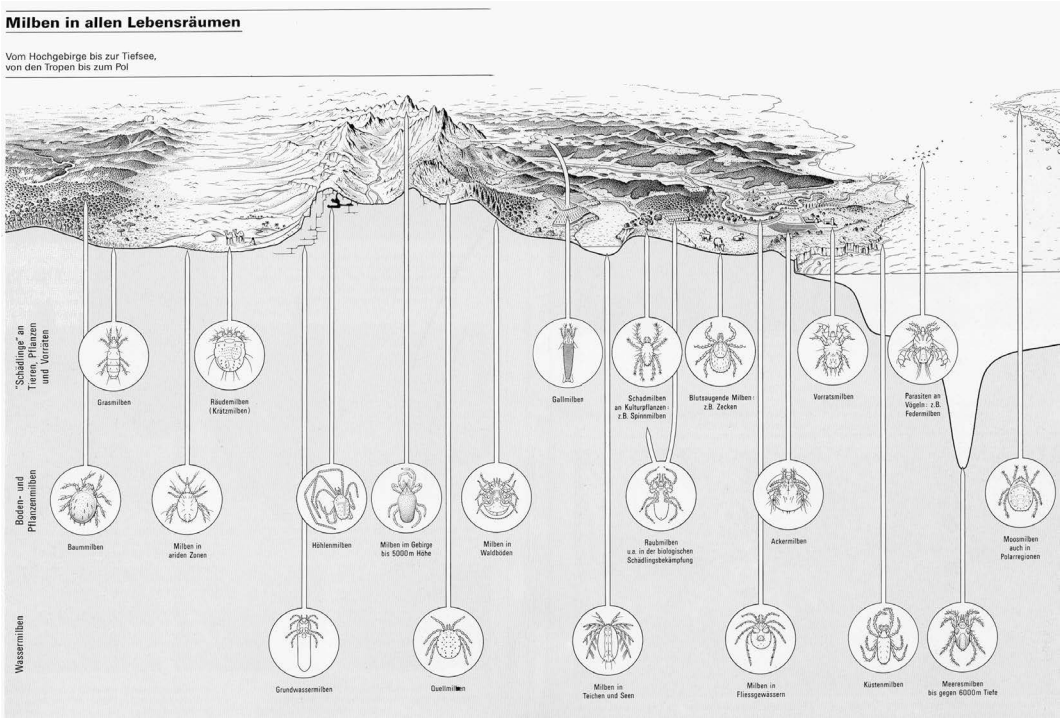


Figure 1.1: Ecology and Diversity of Acari. from: Bader 1989

group (van der Hammen 1972, 1989; Dunlop & Alberti 2007; Alberti & Coons 1999; Dabert et al. 2010). The traditional "Acari" consist of two rather different groups of chelicerate animals, the Actinotrichida and Anactinotrichida (named after the presence or absence of birefringent setae), albeit their sistergroup relationships to other cheliceran taxa varied among different studies (for a comparison, see Wheeler & Hayashi 1998; Shultz 2007). Within the Actinotrichida, the Sarcoptiformes are considered to be an old and highly diversified group comprising of the Astigmata and the Oribatida, although the relationship between these two is debated (Heethof et al. 2013). While a sistergroup relationship was often suggested (e.g. Domes et al. 2007), some authors propose the origin of Astigmata to be nested within "Oribatida" (Norton 1994, 1998; Dabert et al. 2010). The fossil record of oribatid mites is dating back to the Devonian (Shear et al. 1984; Norton et al. 1988; Subias & Arillo 2002). The apparent similarities of these fossil forms with recent groups, as well as the results of molecular analyses, led to estimations, the stem group might be considerably older, dating back to the Silurian (Lindquist 1984), or even Precambrian era (Schaefer et al. 2010). Within the (most probably paraphyletic) "Oribatida" *sensu* "Dugès 1894" (i.e., Sarcoptiformes excluding Astig-

Figure 1.2: *Archegozetes longisetosus*, adult specimen. Of the internal organs, the opisthosomal glands, proventricular glands, ventriculus, caeca, intercolon, Postcolon and ovipositor are visible through the body wall.

mata), about 10.000 species are described, with estimations of the true number of extant species ranging up to 100.000 (Schatz 2002; Subias 2012).

A. longisetosus was referred to as the most-studied oribatid mite under laboratory conditions (Smrž & Norton 2004; Heethoff et al. 2007). Since *A. longisetosus* meets most of the requirements stated for model organisms (Grbić et al. 2007), a laboratory strain was named *Archegozetes longisetosus* **ran** (Heethoff et al. 2007a) in dependence on its founder (**R.A. N**orton). The lineage was started from one single gravid female of this species, which was found in a Tullgren funnel extraction of Puertorican coconut debris in 1993 (Smrž & Norton 2004) and its offspring since spread through numerous laboratories worldwide. The species was introduced as a model organism for chelicerate development (Thomas 2002) and oribatid parthenogenesis (Heethoff et al. 2007a; Laumann 2010), and has been intensely studied by several authors (e.g., Alberti et al. 2003, 2011; Smrž & Norton 2004; Köhler et al. 2005; Heethoff et al. 2007a; Heethoff & Koerner 2007; Laumann et al. 2010a, b; Heethof et al. 2013) As these mites are thelytokous (for an elaboration of terms and concepts, see excursion in paragraph 1.2: "Unisexual reproduction"), daughters are quasi-clonal copies of their mother (Heethoff et al., 2009). This is resulting in a well-defined gene pool (except mutation events, one genome is characterising the culture). In comparison to other oribatid mites, the animals are large with a body length of ca. 850-1200 µm. Their comparably thin cuticle allows the observation of the inner organs in living specimen under the stereomicroscope (cf. Figure 2). Originally living in the tropics, the environmental requirements of the

species can easily be met under the following conditions: saturated air humidity in the culture wells, a constant temperature of 22-24°C and darkness. These criteria result in lively reproduction, with generation times of 40-60 days and up to over 320 offspring per female, which is a very high fecundity among oribatid mites. The animals feed on green algae found on tree bark, for example that of *Platanus spec.* Except for the general morphology of the reproductive organs and information on reproductive rates (Heethoff et al. 2007a), and the thorough examination of meiotic divisions, cleavage, and embryology (Laumann 2010a), the detailed anatomy and development of the reproductive organs of *A. longisetosus* remained largely unknown. This is generally the case in the "Acari", where the evolutionary history is reflected by the high diversity of reproductive modes and anatomical layouts of their reproductive organs (Alberti & Coons 1999). In comparison to this diversity, developmental studies that allude to the reproductive system 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. 2007a; 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 of organogenesis. The interest in anatomy and function of the genital organs was greatly enhanced since *A. longisetosus* belongs to a special group of putatively ancient unisexual lineages. Therefore, a short excursion on unisexual reproduction and its occurrence and consequences in these trhypochthoniid mites is included.

1.2 Unisexual reproduction

Although the majority of living species propagates by sexual reproduction, and most often show two sexes, several groups are known where individuals are able to, or solely, reproduce without the utilisation of gametes from a different individual. Several forms of unisexual reproduction can be distinguished, that differ fundamentally in their mechanisms:

- **Asexual propagation/ Asexuality** includes the separation of somatic tissue and does not involve gametes. Instead it can be interpreted as a special form

of growth. Examples are the budding of *Hydra spec*, or the propagation by runners in several plants, like in strawberries (*Fragaria spec.*).

- **Parthenogenesis** is a form of unisexual reproduction, in which female organisms produce offspring via germline cells. It has to be differentiated from asexual propagation, although the terms are often used interchangeably in literature (Boyden 1950). Parthenogenesis can be differentiated into three modes according to the involved processes:
  - Deuterotoky: unfertilized haploid eggs develop into both haploid males and haploid females
  - Arrhenotoky: oocytes undergo meiosis; unfertilized, haploid eggs hatch into haploid males, while fertilized eggs hatch into diploid females (Suomalainen 1962)
  - Thelytoky: only diploid females exist and produce diploid offspring; different types of thelytoky exist, regarding the presence or absence of meiosis and haploid gametes:
    * Apomixis: diploid females produce diploid offspring solely by mitotic cell divisions among their oocytes; meiosis and haploid phase are absent. Apomixis represents a form of asexuality via germline cells. (Suomalainen 1962)
    * Premeiotic doubling: a second phase of chromatid duplication is accomplished before meiosis, resulting in pseudotetraploid oogonia. Meiosis results in diploid eggs which emerge as embryos (Stenberg & Saura 2009)
    * Automixis: oocytes undergo meiosis, and haploid gametes are produced. Diploidy is restored by fusion of two haploid nuclei. Instead of nuclei of different individuals, like in normal bisexual reproduction (Amphimixis), nuclei of the same individual are utilised, hence the subtypes of automixis are differentiated by the mode of rediploidization that precedes the development of a diploid embryo:
      · *Central fusion automixis*: after completing meiosis, the haploid oocytes fuse with the first polar body that is the divisional product of the first meiotic division. Alternatively, after

fusion of the products of a first reductional meiotic division, the second, equational division occurs, leading to two diploid cells with chromosomes consisting of a single chromatid each (Stenberg & Saura 2009).

- *Terminal fusion automixis*: after completing meiosis, the haploid oocyte fuses with the second polar body that is the divisional product of the second meiotic division (Stenberg & Saura 2009).
- *Gamete duplication:* after normal meiosis, a haploid egg nucleus undergoes an additional division, and the resulting cleavage nuclei fuse. Alternatively, this division stops after reduplication, and the duplicated chromatids remain in the then diploid nucleus. (Stenberg & Saura 2009)

1.3 Parthenogenesis in *Archegozetes longisetosus*

Although parthenogenetic lineages exist in most metazoan taxa, their distribution is normally scattered and they comprise on average ca. 1% of extant species, or even as few as 0.1% if all forms are excluded that perform sexual processes at least once in their lifetime (White 1973). With reference to theories of reproductive biology, these lineages are normally regarded as "accidents", since they are believed to be short-lived evolutionary dead ends. In oribatid mites, however, approximately 10% of the known species are confirmed or suspected of propagating parthenogenetically (Norton & Palmer 1991). Moreover, parthenogenetic lineages form several species-rich clusters among the Oribatida (Palmer & Norton 1990). This indicates long-term stability of parthenogenesis and the potential to radiate parthenogenetically (Maraun et al. 2003, 2004; Heethoff et al. 2007, 2009, 2011; Laumann et al. 2007). These assumptions challenge traditional understandings of evolutionary processes and led to great interest in the exact mode of reproduction that could allow this "evolutionary scandal" (Maynard Smith 1978). One of these parthenogenetic clusters in oribatid mites is the taxon Desmonomata, of which *A. longisetosus* is a member.

1.4 Scientific context of the study

In earlier studies on *A. longisetosus*, an insight on life history parameters (Heethoff et al. 2007a), chromosome type (Heethoff et al. 2006) and the general layout of the adult genital system (Heethoff et al. 2007a) could be gained. However, no detailed investigation of development, anatomy and function of the reproductive organs had yet been performed on *A. longisetosus*. Therefore, the interest to investigate the anatomy and development of the internal genital organs of this species by means of synchrotron X-ray micro-computertomographies (SRμCT) in combination with conventional lightmicroscopy (LM) and transmission electron microscopy (TEM) arose. There are a number of studies available on the internal genital anatomy of several actinotrichid mites (e.g., Woodring & Cook 1962; Heinemann & Hughes 1969, 1970; Alberti 1974; Witte 1975; Vistorin-Theis 1978; Feiertag-Koppen 1980; Taberly 1987 a,b,c; Shatrov 1997, 2002). Focus and scope of these studies vary, not all results are coherent, and differences between the systems are apparent. The nomenclature of internal organs of mites suffered from inconsistencies over the decades, and many denominations are still not based on concepts of homology (Alberti & Coons 1999). One example for the differences in comparing structures is the observable differentiation of the adult genital system. It is not generally established whether the anatomically defined subdivisions reflect the distribution of ectodermal, mesodermal-somatic, and -germinative tissues. This resulted in uncertainty whether the meroi should properly be addressed as proximal oviducts or ovarian extensions, for instance. Another question was whether the ovarial rhodoid constitutes a germarium in the adult state, and whether oogonia persist until the adult state, as no cell divisions could be observed in this region.

Detailed developmental studies on the internal genital organs in nymphal stages of mites are all but absent in recent literature, apart from the investigations on thelytokous reproduction in two species closely related to *A. longisetosus* (Taberly 1987a,b,c,d, 1988). Apart from its reproductive system, *A. longisetosus* is a well examined model system with a very interesting phylogenetic position regarding both higher (Arthropoda, Chelicerata, Acari) as well as lower ranking taxa (Sarcoptiformes, Oribatida, Astigmata, Desmonomata, Trhypochthoniidae). Furthermore, it seems to represents an example for a reproductive mode that is challenging traditional models that predict the prevalence of bisexual reproduction (Kondrashov

1993). In order to understand conditions of long-term stable unisexual reproduction, detailed knowledge of development, anatomy and function of a model system is desirable.

One explanation for the lack of homology concepts in nomenclature of inner structures in mites may be the difficulty of processing these animals for serial sectioning and subsequent 3D-rendering. The occurrence of flexible but durable and impermeable cuticle, and soft and delicate internal tissues results in technical difficulties that make serial sectioning prone to several artefacts (e.g., section loss, distortions). After promising results with phase-enhanced SRμCT on microarthropds and further advance in imaging techniques (e.g., Cloetens et al. 1995; Cloetens et al. 1999; Betz et al. 2007; Heethoff & Cloetens 2008; Weide & Betz 2008), the idea of combining these techniques and conventional microscopy to a mutual benefit arose. The combination of these methods was expected to facilitate the establishment of an anatomical model of the development of the genital organs during sub-adult, free-living instars of *A. longisetosus* with subcellular resolution. Moreover, a description of the adult genital system, including key functional aspects of oogenesis shall be compiled. A number of hypotheses have been addressed, based on the knowledge of developmental processes in other acarines, chelicerates and arthropods:

- I.) The ovary of post embryonic oribatid mites is an unpaired structure, forming together with the gonoducts from mid-ventral mesodermal pouches of anterior opisthosomal segments. This is deduced from the fact that in adult mites oocytes from both oviducts stem from a single ovarian centre. It implies, that
  - a) fusion of the gonocoel precedes the development of genital organs and
  - b) all observed paired structures are secondary developments.
- II.) The ovary is of the panoistic type. This was suspected from the spatial arrangement of oocytes and epithelia in the ovarian meroi. A possible individual association of these tissues, which would indicate a meroistic ovary, had to be scrutinised.
- III.) The mode of oogenesis does not contradict a panoistic ovary.

  - a) It is solitary, i.e. oocytes progress independently from each other and surrounding somatic tissues.
  - b) Vitellogenesis is exogenous, i.e. oocytes actively take up macromolecular components as yolk precursors, which are produced in somatic tissues of the mother.

- IV.) The instant of oviposition is neither a suitable marker for developmental stage of the embryo, nor the generational boundary. The impermeability of the eggshell indicates separation of mother and daughter and the onset of independent development at an earlier instant.

- V) Major events of the developmental processes of internal organs are synchronized with moulting. This is deduced from the progression of the development of externally visible organs in the genital region (plates, bristles, genital papillae), which proceeds in well-defined stages suitable for identifying individual sub-adult stages.

- VI.) Results in closely related species (Taberly 1987 a,b,c) can be confirmed for *A. longisetosus*. Based on the aforementioned studies, the following hypotheses where of special interest for scrutiny:

  - The mode of parthenogenesis is terminal fusion automixis.
  - Oocytes enter prophase I in subadult stages.
  - Vitellogenesis and egg-shell formation are concluded within the ovary.
  - The appearance of a lumen marks the ovary–oviduct transition.

- VII.) The combination of tomographic data with serial sectioning is a suitable way to reduce workload while retaining information of key regions with TEM-resolution from limited numbers of sections and undistorted renderings of histologically correct segmentations of the volumetric data in microarthropods.

2 Summaries

2.1 Publications 1 & 2: Development and anatomy of the genital system

2.1.1 Publication 1: Development of Larval, proto- , deuto- and early tritonymphal nymphal stages:

Bergmann, P. and M. Heethoff (2012). "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)." —Soil Organisms **84**(2): 459–470.

Contribution: Rearing assistance, specimen preparation, assistance with scanning of tomography specimen, fixation, embedding, processing and documentation of histology specimen, discussion of analysis, drafting of the original manuscript

The genital system in the larval stage of *A. longisetosus* is discernible as a mesodermal tissue condensation medioventrally in the opisthosoma, close to the ventral body wall, and between ventral, ventriculus, inter-, and postcolon. In young larvae, tomographic studies show that a portion of dense, coarsely grained tissue is connected to the body wall by tissue strands of lesser density. This denser portion, situated dorsally between inter- and postcolon, is shifting anteriorly to a position directly dorsally of a ventromedian portion of somatic tissue in older larvae. The larval organization of the genital system is continuous over the protonymphal moulting into the early protonymphal stage. Transmission electron microscopy (TEM) analyses indicate a differentiation of three cell types within protonymphal genital organs. The ventromedian portion consists of small somatic cells with small

nuclei. The denser dorsal portion of the genital anlage consists of large cells with cytoplasm of higher density. These cells can be identified as germline cells. A single layer of flattened somatic cells with very light cytoplasm is capping the germ cell cluster anterodorsally. Neither shape nor size of the genital organ precursors significantly changes during the protonymphal stage. During the deutonymphal stage, the ventrally oriented somatic portion develops flap-like lateral extensions. Ultrathin sections show the tubular structure of these extensions. They develop into loops connecting the medioventral somatic and the dorsal germinative portion and form the developing oviducts. The most obvious changes during the deutonymphal stage are observed in the germinative portion of the genital bud. The germcell cluster enlarges both in volume and number of cells. It is developing into a ball-shaped structure that is situated posterodorsally of the somatic portion and on both sides contacted by the oviduct precursors. These characteristic changes (i.e., shape, number of cells, and volume) are regarded as a result of premeiotic mitoses of the oogonia, constituting a germarium. A hollow space develops in the centre of the ball-shaped germarium, into which the germ cells project numerous filiform extensions. In early tritonymphs, the ventral somatic portion enlarges, as well as the tubular lateral extensions. Their wall consists of two layers of cells, separated by a basal lamina.

2.1.2 Publication 2: Tritonymphal and adult stages

Bergmann, P., M. Laumann, P. Cloetens and M. Heethoff (2008). "Morphology of the internal reproductive organs of *Archegozetes longisetosus* Aoki (Acari, Oribatida)." —Soil Organisms **80**(2): 171–195.

Contribution: Rearing assistance, specimen preparation, assistance with scanning of tomography specimen, fixation, embedding, processing and documentation of histology specimen, discussion of analysis, drafting of the original manuscript

In tritonymphs, the ovipositor develops by a ventral invagination of the body wall, located ventrally to the developing genital organs. The invagination starts as a ring-shaped depression of the epidermis growing dorsally into the double-layered tube of the evertible ovipositor. In early tritonymphs, the central part of the developing ovipositor is already differentiating into the three eugenital lobes.

The somatic tissues of the genital system are further differentiating into the unpaired genital duct, covering the anterior face of the ovipositor structure, and the oviducts. The oviducts are extending laterally along the body wall in a double S-shape, connecting the proximal part of the unpaired genital duct with the germarium. A single layer of epithelium is lining the oviduct's lumen, which is delimited from a second layer of flattened cells by a basal lamina. This second layer is delimited by another basal lamina from the haemolymph space. A direct connection of the lumen to the hollow space in the centre of the germarium could not be demonstrated. In older tritonymphs, peripherally situated germ cells grow considerably and show large nuclei with prominent chromatin condensations. This indicates the onset of previtellogenesis in these cells, characterizing them to be oocytes. With the imaginal moult, the ovipositor acquires its cuticularisation. In young adults, two strands of previtellogenetic oocytes wrapped in ovarian epithelium are protruding laterally. They grow outwards from the medulla at the contact site of the oviducts. Now, the differentiating oviductal bulb is taken with them into a more anterior position. Later, the strand of oocytes that are wrapped individually in flattened ovarian epithelium cells, is doubling back dorsally of the previtellogenetic oocytal strand as a single file of vitellogenetic oocytes in posterior direction. The contact site of the oviducts is distinguishable by its coarse and swollen tissue appearance. The folded lateral extensions of the ovary separate the medulla from the oviductal contact site. Additionally to the differentiation of the tissue structure, the onset of a lumen demarcates this point as the ovary–oviduct transition, indicating that the strands of previtellogenetic and vitellogenetic oocytes constitute extensions of the ovary rather than a proximal part of the oviducts. The fully developed ovary thus consists of two structurally different and recognisable subdivisions, i.e., i) an unpaired, radially organized, ventromedian, ball-shaped portion with a flower-like appearance in sections and ii) two folded lateral extensions comprising developing oocytes. As no oogonial mitoses could be observed in the central part, which may therefore already consist exclusively of oocytes, it seemed hazardous to label it as a germarium. To avoid further confusion, two new terms were introduced for the subdivisions of the adult ovary: "rhodoid" for the central part, because of its characteristic appearance in sections, and "meros/meroi" for the lateral extensions. The oviducts contain developing eggs encased in solid egg shells. These eggs have completed vitellogenesis and frequently show signs

of cleavage and embryogenesis. After exiting the ovary, the eggs pass to the distal part of the oviducts, and free lumen remains in the region of the oviductal bulb, which is characterised by its thick epithelial lining The eggs are not entering the unpaired, distal portion of the genital duct until oviposition, as flap-like structures separate the oviducts from the unpaired uterus. These flap-like structures can also be found at the transition of the uterus and the vagina, which extends into the extruded ovipositor. All described findings are in concordance with descriptions of oribatid mites of the Desmonomata (Taberly 1987a,b,c). In contrary to other oribatid mites (Witalinski 1986), no paired arrangement of germaria was found in Desmonomata so far.

2.2 Publication 3: Vitellogenesis and egg shell accumulation

Bergmann, P., M. Laumann and M. Heethoff (2010). "Ultrastructural aspects of vitellogenesis in *Archegozetes longisetosus* Aoki, 1965 (Acari, Oribatida, Trhypochthoniidae)." —Soil Organisms **82**(2): 193–208.

Contribution: Rearing assistance, specimen preparation, fixation, embedding, sectioning and documentation of histology specimen, discussion of analysis, drafting of the original manuscript

As shown before, the ovary of adult *A. longisetosus* is differentiated into several subdivisions, a central rhodoid and two lateral meroi (Bergmann et al. 2008). Ultrastructural investigation of the meroi was done to understand nutrient acquisition of the oocyte, vitellogenesis and eggshell accumulation. The meroi are further subdivided into two parts. The proximal part is oriented anteriorly, containing growing oocytes. It dorsally leads to a distal part, which is oriented caudally. Within this area, oocytes enlarge and accumulate yolk vesicles. The distal part may therefore be referred to as "vitellarium". Previtellogenetic oocytes in the proximal part of the meroi show characteristic cytoplasmal nuage (i.e., granular cytoplasmic material *sensu* Extavour & Akam 2003) and contain numerous ribosomes and polysomes as well as smooth endoplasmic reticulum and groups of mitochondria of the crista-type. After reaching the vitellarium, the close contact between previtellogenetic oocytes and epithelial cells loosens. The perivitelline

space, i.e., an empty zone around these oocytes, develops and the oocytes build up a dense fringe of irregularly arranged microvilli. This is in accordance with descriptions of other oocytes in chelicerates (Witalinski 1986). The epithelial cells surrounding the oocytes contain well-defined Golgi bodies. A granular electron-lucent substance is accumulating in the perivitelline space. Eggshell accumulation and vitellogenesis proceed simultaneously. Microvilli extend into pores and canals in the porose layer of eggshell precursor material. The oocytal cytoplasm forms a uniformly grained electron lucent region throughout most of the cell volume. The former dense cytoplasm only remains close to the nucleus and in the cell periphery. Lipid droplets and proteinaceous yolk vesicles, which are developing in a centripetal manner, are embedded in this dense cytoplasm. During the progress of vitellogenesis, the microvilli become fewer and a growing number of coated pits and vesicles can be observed in the periphery of the oocyte. This indicates active uptake of proteins. Interestingly, the surrounding epithelial cells remain very delicate with electron lucent cytoplasm. These cells do not show signs of metabolic activity. Instead, strong metabolic activity can be observed in the lateral cells neighbouring the genital system (Alberti et al. 2003), which show a peripheral labyrinth and abundant rough endoplasmic reticulum. The ovarian epithelium exhibits mitochondria of the tubular type. This modification of mitochondrial internal structure was earlier described as indicating strong oxygenase metabolism, as associated with lipoprotein turnover and/or steroid production (Sumegi et al. 1988). Steroid hormones are known to mediate vitellogenesis in many arthropods (James and Oliver 1999; Cabrera et al. 2009). Furthermore, coated pits were described as associated with lipoprotein endocytosis in oocytes (Giorgi 1980). The oocytes process yolk precursor substances into yolk vesicles, as represented by the progression of yolk vesicles in the oocyte and the abundant organelles associated with synthetic and/or metabolic activity like endoplasmic reticulum and mitochondria in oocytal cytoplasm. The turnover of microvilli and coated pits indicates different phases associated with different pathways of yolk precursor uptake. This is possibly related to a temporal segregation in the uptake of carbohydrates (see also publication 3), lipids and proteins respectively. This strongly suggests exogenous vitellogenesis as the most important source of yolk in *A. longisetosus*. Therefore, oogenesis is described as solitary, i.e., the oocyte is accumulating nutrients by itself from its surroundings, without cell-cell contacts. Solitary oogenesis was also

described in Microtrombiidae (Shatrov 2002), since nutritive ovarian cells associated with individual oocytes are absent. Nutritive ovarian cells could also not be demonstrated in *A. longisetosus*. On account of the type of oocyte nutrition, the ovary is subsequently classified as panoistic.

2.3 Publication 4: Egg shell solidification at the oviductal bulb and its implications for parity mode and generational succession

Bergmann, P. and M. Heethoff (2012). "The oviduct is a brood chamber for facultative egg retention in the parthenogenetic oribatid mite *Archegozetes longisetosus* Aoki (Acari, Oribatida)." — Tissue&Cell 44(5): 342–350.

Contribution: Rearing assistance, specimen preparation, fixation, embedding, sectioning, processing and documentation of histology specimen, discussion of analysis, drafting of the original manuscript

Generally, the nomenclature of internal genital organs of mites is not based upon sound concepts of homology (Alberti & Coons 1999). This is due to the existing variety of the localization of key processes in oogenesis among different mite taxa. In *A. longisetosus*, the proper localization of the ovary–oviduct transition and its relation to the processes of eggshell formation, embryogenesis and their consequences to the generational boundary was examined. Eggshells in *A. longisetosus* are impermeable to aqueous solutions, a feature common in Acariformes (Aeshlimann & Hess 1984; Walzl 2004), although contradicting evidence exists from the astigmatids (Witalinski 1993). In *A. longisetosus*, even those eggs still enclosed in the oviductal lumen are not penetrated by fixating agents. This indicates a strong limitation for chemical communication between mother and embryo. This is also supported by the fact that embryogenesis was described as beginning independently in eggs prior to oviposition (Laumann et al. 2010). Taberly (1987b) suggested a demarcation point for the ovary–oviduct transition in Desmonomata (Acari) based on the occurrence of a lumen in the oviduct but not the ovary, differences in tissue structure between ovarian and oviductal epithelia, and the completion of vitellogenesis. Further studies (Bergmann et al. 2008, 2010; Laumann et

al. 2010) confirmed these results for *A. longisetosus*. The aforementioned authors described that vitellogenesis, accumulation of egg shell material, as well as meiosis and rediploidisation occur simultaneously in the ovarian meroi prior to the proximal onset of the oviductal lumen at the tip of the ovarian meroi. Ultrastructural investigation yielded histological detail of this region and its related structures and elucidated their functional significance. A continuous basal lamina separates the ovarian somatic tissue and the oviductal wall from the haemolymph space. The individual oocytes are encompassed by the ovarian somatic tissue that is directly neighboured by a thicker and highly vacuolated epithelium under a common basal lamina. This epithelium of merocrine and exocrine glandular cells forms the distalmost wall of the oviduct. The high numbers of secretory vesicles that can be found in this region are interpreted as propagating from a massively developed Golgi apparatus. While this glandular wall epithelium gradually alters to form the smooth delicate walls of the distal oviduct, histological features change suddenly towards the ovarian region. The distalmost epithelial cells of the ovary, although separated from the oocyte surface for the most part, seal the ovarian perivitelline space off from the oviductal lumen. Upon leaving the cover of ovarian tissue and entering the oviductal lumen, the egg shell rapidly solidifies in contact with the secretions that fill the oviductal lumen. Periodic-acid-Schiff (PAS) staining indicated that these secretions originate from the described secretory cells. Additionally, the staining suggested their identity with the mucous substance covering the egg batches after oviposition. No PAS signal has been found in distal oviductal wall cells or ovarian epithelial cells. Instead, another strong PAS signal within the genital system was located in the oocytal cytoplasm and might indicate carbohydrate uptake of the oocytes prior to the accumulation of lipid and proteinaceous yolk. The coincidence of the completion of vitellogenesis, rediploidisation, and accumulation of egg shell material with the change in tissue properties at the proximal onset of the oviductal lumen as well as the subsequent simultaneous egg shell solidification and start of embryogenesis have several implications for the description of reproduction in *A. longisetosus*. The localisation of the ovary–oviduct boundary was confirmed. Hence, the glandular structure has been renamed from “ovarial bulb” (sensu Woodring and Cook, 1962; adopted by Bergmann et al. 2008) into “oviductal bulb”. The generational boundary that was difficult to define in this thelytokous species, can be drawn at this easily recognisable point, from which

on embryos develop independently after the egg shells solidify to an impermeous layer. Early eggshell solidification and independently ensuing embryogenesis within the oviducts indicate an effectively internalised oviposition. This process of early separation of mother and offspring indicate the function of the oviducts as internal brood chambers for the observed facultative egg retention. The parity mode can be described as oviparity with enhanced parental investment, allowing a degree of flexibility and opportunistic behaviour in iteroparity.

2.4 Publication 5: Cleavage and embryonic development

Laumann, M., Bergmann, P., Norton, R. A. and Heethoff, M. (2010). "First cleavages, preblastula and blastula in the parthenogenetic mite *Archegozetes longisetosus* (Acari,Oribatida) indicate holoblastic rather than superficial cleavage." — Arthropod Structure and Development **39**, 276–286

Contribution: Cooperation in the development of preparation techniques and laboratory protocols, assistance with planning of microscopy studies, discussion of results and interpretation.

Literature on the cleavage pattern of Acari is fragmentary and in some cases even contradictory. Generally, superficial or intralecithal cleavage is assumed as the ancestral condition (e.g. Claparède 1868; Anderson 1973; Evans 1992), with the occurrence of mixed cleavage as a secondary condition in some Actinotrichida (e.g. Brucker 1900; Casperson 1986). In this putatively derived mixed cleavage (Dawydoff 1928), an initial phase of holoblastic cleavage preceedes the formation of a superficial layer of cleavage nuclei surrounding the central yolk mass, as it is characteristic for intralecithal cleavage. However, no cleavage data was available so far from the species-rich taxon Oribatida among the Actinotrichida, prohibiting the postulation of a ground plan pattern for this group. To gain insight in early cleavage processes in a middle derivative oribatid mite from the group of Malaconothroidea, embryos from the proximal oviducts of *A. longisetosus* were examined by means of light- and electron microscopy. Cleavage starts right after the completion of vitellogenesis and egg shell formation, in the proximal oviducts. The first division is holoblastic and transversal, resulting in two blastomeres of

equal size. These blastomeres then divide one after another, again in transversal direction, resulting in four blastomeres serially arranged along the long axis of the egg. It is followed by a third holoblastic cleavage that propagates from one pole longitudinally, leading via 5- and 6-cell to an 8-cell stadium. Blastomeres then divide into micro- and macromeres of the preblastula stage (named after Walzl 2004), with micromeres being scattered across the egg surface and containing mitochondria, rER and glycogen, whereas the, not further dividing, macromeres cluster in the central part of the egg and account for most of its volume by their yolk content. Micromeres then divide tangentially and spread over the surface to form the closed superficial cell layer of the blastula. Then, radial divisions of micromeres result in the formation of a germ disc, reaching deeper into the embryo. Cell membranes between individual blastomeres are always clearly traceable in TEM micrographs, but are not detectable in LM. The cleavage pattern of *A. longisetosus* thus is holoblastic, exhibiting several specializations: cell divisions are highly asynchronous, and early equal divisions result in an unusual linear arrangement of blastomeres. Even before reaching a 32-cell stage, which might be omitted, inequal divisions lead to the differentiation of non-segregating macromeres and segregating micromeres. These results challenge the traditional view of intralecithal or mixed cleavage as being the prevalent mode in acari and suggest reinvestigation of reported cases of these cleavage modes (e.g. Sokolov 1952; Edwards 1958; Dittrich 1968) by TEM studies, as all existing TEM studies of acarine embryos indicate holoblastic cleavage. The data also support earlier conclusions (Ungerer & Scholz 2009), that holoblastic cleavage might be the ancestral condition in Chelicerates or even Arthropods.

2.5 Publication 6: Cytogenetics

Laumann, M., Bergmann, P. and Heethoff, M. (2008). "Some remarks on the cytogenetics of oribatid mites." — Soil Organisms **80**, 223–232.

Contribution: Rearing and preparation of material, contribution to data acquisition and analysis, discussion of results

Parthenogenesis in Malaconothroidea is peculiar for a variety of reasons: It appears to be long-term stable (Heethoff et al., 2007b). The cellular mode of reproduction was established as automictic thelytoky (Taberly, 1987c), meaning that

it relies on a meiotic germ line producing haploid gametes, and a rediploidisation using maternal material to constitute a diploid embryo. This rediploidisation is realised by terminal fusion, i.e., the fusion of the haploid products of the second meiotic division, the egg's pronucleus and the second polar body (Taberly, 1987c). In any textbook case of meiosis, this mechanism would result in a completely homozygous embryo (except for crossover regions). However, it does not in *A. longisetosus*: fixed heterozygosity was demonstrated by isozyme techniques (Palmer and Norton, 1992). Molecular analyses further suggested a lack of recombination for nuclear genes (Schaefer et al., 2006). A theoretical model proposed earlier (Wrensch et al., 1994) would be able to unify these seemingly conflicting data: Inverted meiosis, in which the first division is equational, whereas the second division is reductional, contrary to normal meiosis. This inverted meiotic sequence could be enabled by holokinetic chromosomes that have been confirmed for this group. This study presents cytological evidence in *A. longisetosus* in concordance with all cited literature, implying a strengthened likelihood of the model hypothesis to correspond with reality. Holotomographic SR-µCT-data of adult specimen revealed peripheral nuclei and strong indications of the expulsion of a first polar body at the end of the previtellogenetic growth phase. This coincides with the bend between the anteriorly oriented proximal, and the posteriorly oriented distal ovarian meroi. Semithin sectionings of embedded specimen further indicated the condensation of chromatin and the breakdown of the nuclear membrane, indicating a second meiotic division, during vitellogenesis, when the nucleus again is in a central position. An expulsion of a second polar body could not be demonstrated, and, given the circumstances, seems highly unlikely due to the central position of the nucleus, the accumulation of yolk in the peripheral cytoplasm, and the presence of egg shell material on the egg's surface during this time. The data thus strongly suggests terminal fusion automixis by abortive meiosis II, and consequently an inverted meiotic sequence with only the equational first division fully realised in *A. longisetosus*.

2.6 Publication 7: Meiosis, Automixis and Recombination

Bergmann, P., Laumann, M., Norton, R. A. and Heethoff, M. Cytological evidence for automictic thelytoky in parthenogenetic oribatid mites (Acari, Oribatida): Synaptonemal complexes confirm meiosis in *Archegozetes longisetosus* (submitted)

Contribution: Rearing assistance, specimen preparation, fixation, embedding, assistance in sectioning, documentation of histology specimen, discussion of analysis, drafting of the original manuscript

Archegozetes longisetosus is a diplo-diploid parthenogenetic lineage. For its reproduction mechanism, a theoretical model was proposed earlier, involving terminal fusion automixis with an inverted meiotic sequence on the basis of holokinetic chromosomes. In this study, tritonymphal stages of *A. longisetosus* were examined by transmission electron microscopy (TEM). Tritonymphs 2 days after hatching from the last molt showed numerous synaptonemal complexes (SCs) in all oocytes. This observation bears several important implications. It is the first ultrastructural proof of the onset of meiosis I in *A. longisetosus*, therefore automixis can be confirmed for this quasi-clonal lineage, and potentially misinterpreted apomixis can safely be ruled out as an explanation for clonal diplo-diploidy in this species. Additionally, the observation of SCs in all oocytes confirms that the final number of oocytes is already reached in subadult *A. longisetosus*, and oocytes are arrested in late prophase I until sequential oogenesis in the adult. No recombination nodules were found in a total of 75,4 µm of longitudinal sections of pachytene SCs from 221 locations in 4 ultrathin sections 18 µm apart each in the series. This is in concordance with molecular data suggesting supressed recombination and achiasmatic meiosis in *A. longisetosus*. Also, this observation suggests, that SC formation is not dependent on double strand breaks in this case. The nuclei are transcriptionally active in late prophase, as is indicated by numerous nuclear pores throughout prophase I and later, suggesting that chromosomes have a lampbrush configuration during most of meiosis. Fully postreductional meiosis is discussed as a result of the spatial arrangement of chromatid segregation and an altered sequence of transversal element disintegration. A framework for a working scenario of conversion to thelytoky by inverted (postreductional) meiosis terminal fusion automixis is proposed.

3 Conclusions

3.1 General discussion of the results

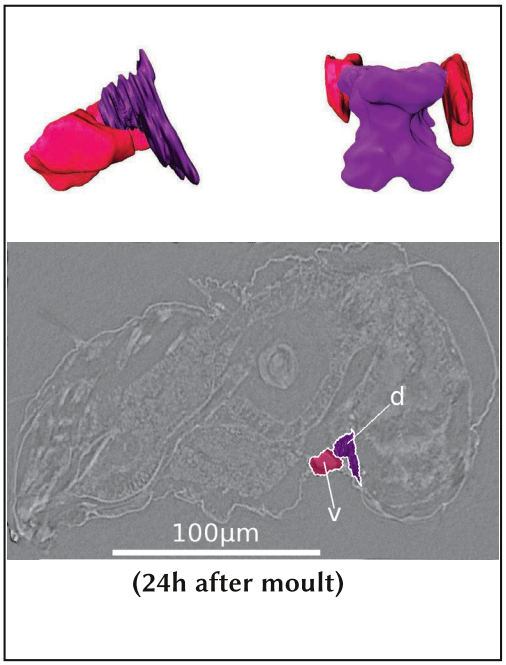
3.1.1 Development of the internal reproductive organs

The development of internal organs systems seems not to be strongly synchronized with moultings in *A. longisetosus*. A peculiar feature of moulting in *A. longisetosus* is that individuals of all instars form large aggregations during the quiescent phase prior to the emergence of the next instar (Haq 1978). Aggregation behaviour has been shown to be mediated by pheromones in many mites (Shimizu 2001). A possible explanation of this phenomenon is that individuals follow a chemical gradient until they reach a certain pheromone concentration and/or come in physical contact with conspecifics. At this point they enter the quiescent phase preceding the emergence of the next instar. This model implies a certain variability of the exact individual moulting date. Individuals show considerable variance in the developmental state of their inner organs, even if taken from the culture at the same time after the last moult. This independence of genital development from the time course of other developmental processes during ontogenesis was also described for the spider *Cupiennius salei* Keys (Seitz 1971). Some freshly moulted tritonymphs show oviducts less developed in e.g., length or lateral extension, than those of deutonymphs about to enter the moult to the tritonymph. The development of internal organs seems to progress continuously during the series of moults. The variability of moult dates seems to be in the range of the duration of the quiescent phase (i.e., 1-3 days) (Haq & Adolph 1981), while the duration of sub-adult instars between moultings is in the range of 10-12 days (Heethoff et al. 2007a). Therefore, certain key events of genital development can nevertheless be assigned with good confidence at least to individual instars, even if this assignment has to be taken as a relative rather than an absolute timeline. Key events in the development of the

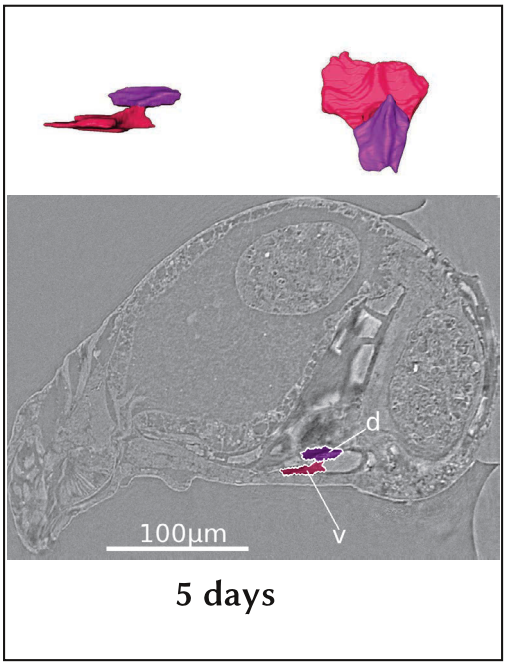
genital organs during the lifetime of *A. longisetosus* (Fig. 3.1) and their association with instars are:

- Larva: The genital anlage as medioventral fold of mesodermal tissue develops into two distinct parts. One is situated at the ventral body wall between the fourth pair of legs and the anal plates, while the other is situated dorsally to the first part, between the mid- and hindgut of the animal.
- Protonymph: The mesodermal genital anlage further differentiates into the germ cell cluster covered by somatic ovarian tissue and the somatic tissue of the genital duct precursor.
- Deutonymph: Lateral extensions of the genital duct precursors develop into tubular oviducts and establish lateral contact sites to the ovary. Premeiotic mitoses within the germ cell cluster lead to the proliferation of oogonia, constituting the germarium. The final number of primary oocytes is reached. Oocytes develop extensions into an ovarian central hollow space that is not connected to the lumen of the oviducts.
- Tritonymph: Oviducts grow longitudinally into tubular ducts, shaped like the letter "S" and extending along the lateral body wall. The ovipositor forms as a circular invagination of the ventral body wall. Hollow spaces in the ovary vanish. Oocytes enter prophase of meiosis I simultaneously as indicated by general abundance of synaptonemal complexes (SCs). Oocytes progress through late prophase centrifugally and peripheral oocytes start previtellogenesis in late tritonymphs. Oocytal extensions into the centre of the ovary become well developed and filled with microtubuli. Oocytal nuclei are active as indicated by numerous nuclear pores, implying lamp brush chromosomes. The radially arranged structure of the rhodoid develops.
- Adult: Cuticularization of ovipositor and vagina is established with the last moulting. Ovarial meroi are formed as lateral progressions of serially developing oocytes. The oviductal bulb forms as a glandular modification of the proximal oviducts at the contact site to the ovary. Oocytes transgress meiosis I and II serially during previtellogenesis and vitellogenesis. Egg-shell accumulation proceeds during vitellogenesis and eggshell solidification is achieved upon entering the oviductal bulb.

Larval stage

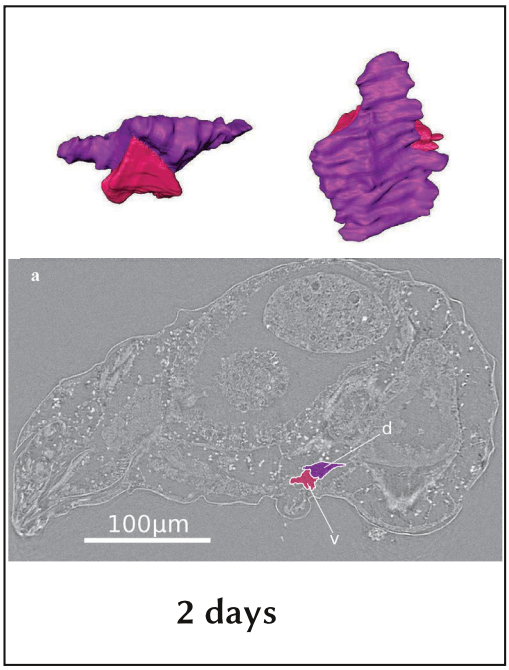


(24h after moult)

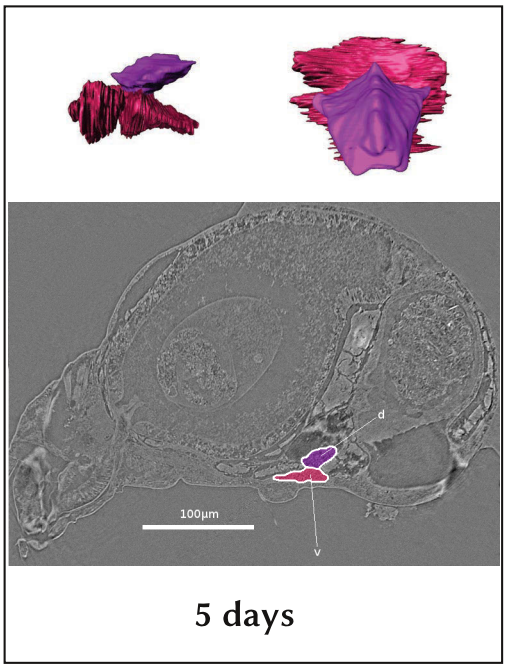


5 days

Protonymphal stage

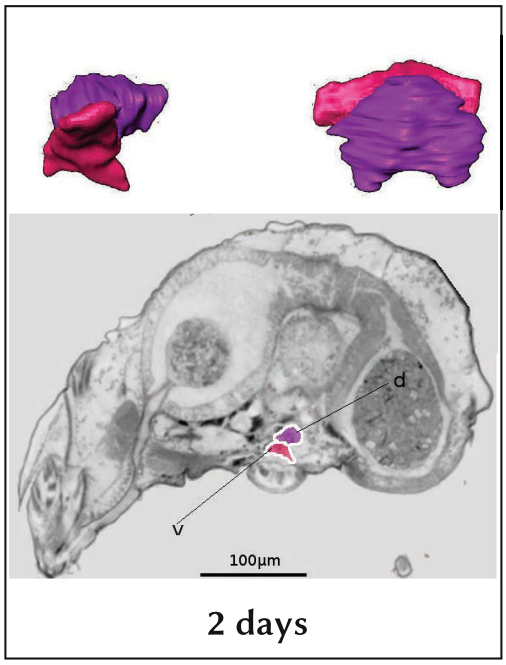


2 days



5 days

Deutonymphal stage



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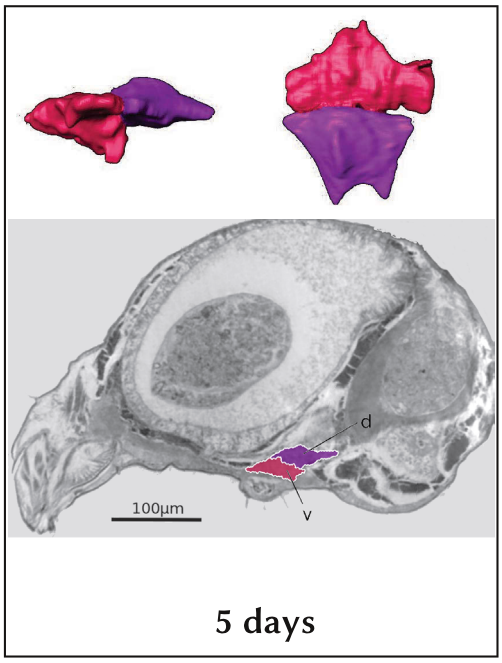

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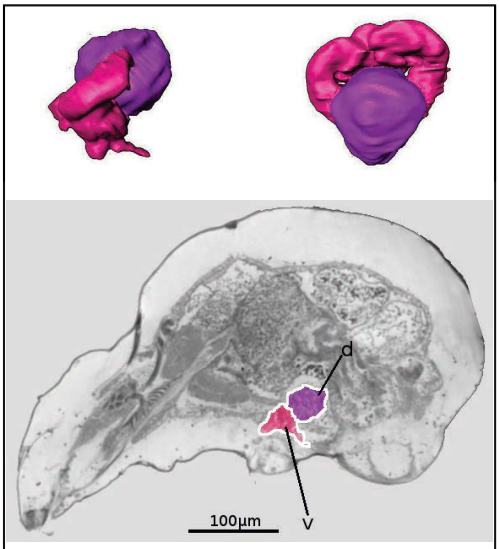
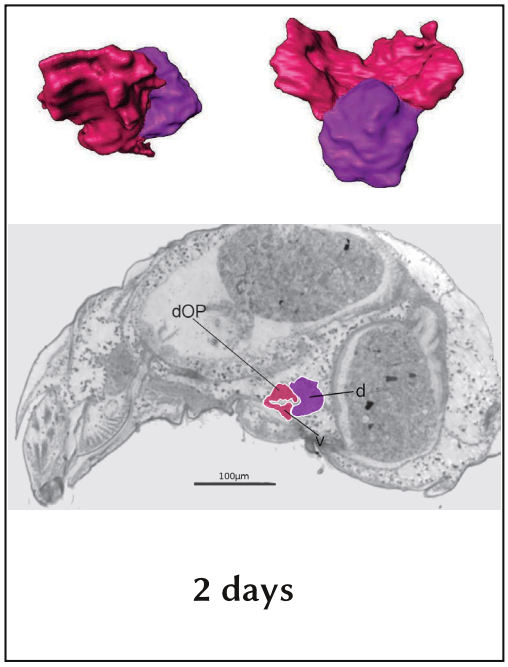
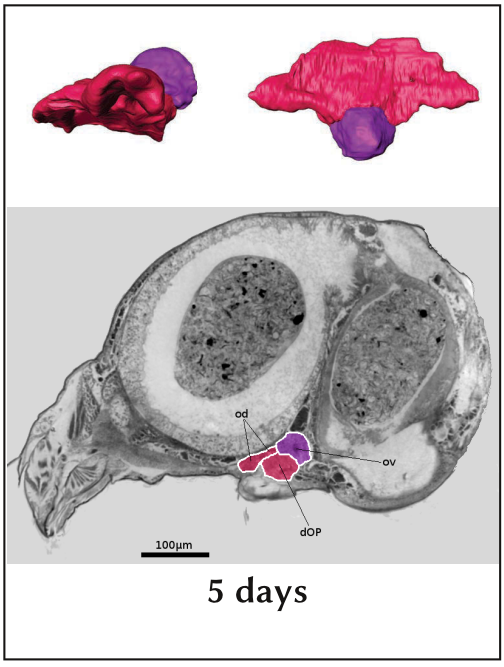
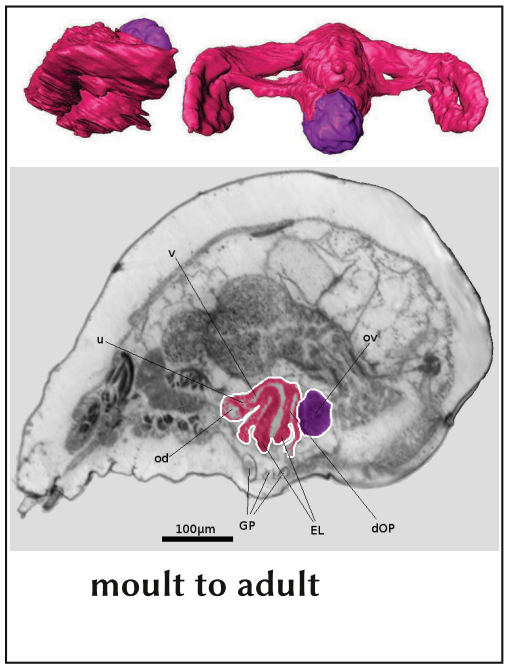


moult to tritonymph

Tritonymphal stage







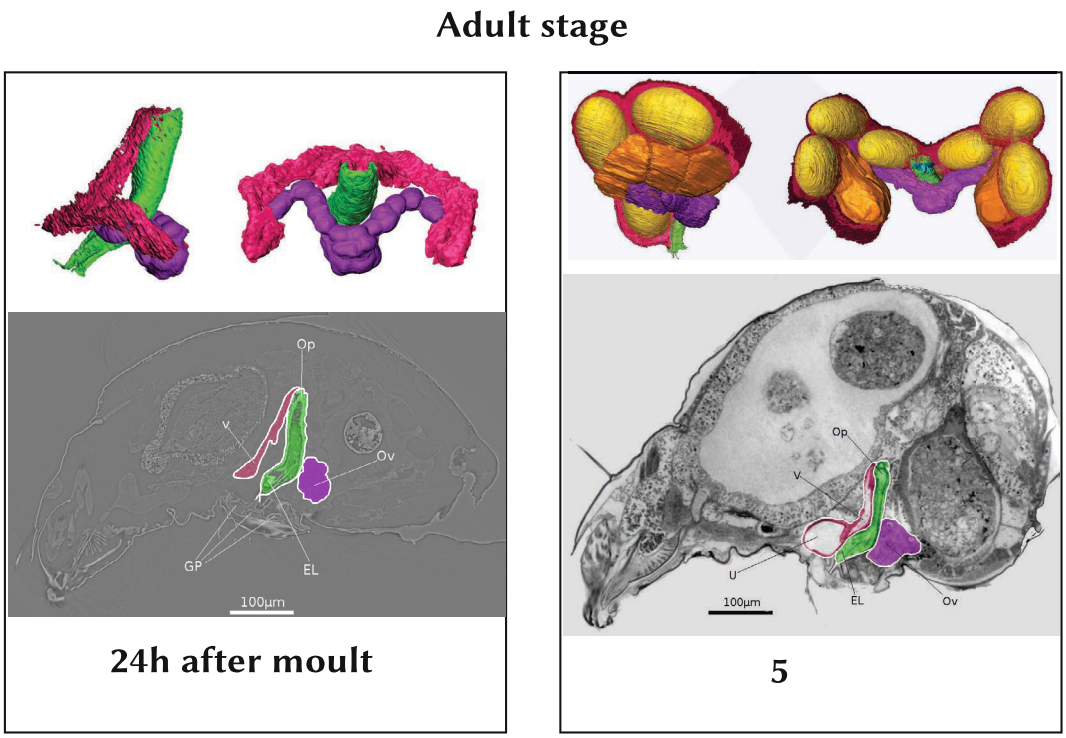


Figure 3.1: Summary of the genital development of *A. longisetosus*. 3D-renderings of segmentations of the genital organs are displayed in chronological order together with exemplary saggital slices of the SR- µCT-volumes from which they were derived. Cross-sections of segmented areas in the slices are highlighted in white and coloured overlay corresponding to the models' colouring. Ovarian tissue is depicted in purple, cuticular structures (ovipositor) in green, and somatic tissues in red. Note that somatic tissues coloured in red may include ectodermal- as well as mesodermal material. Non-sample materials and background noise from the virtual slices were removed by hand for clarity and a homogeneous background applied (refer to supplementary materials for non-adulterated images). Renderings displayed above corresponding virtual slices are saggital view left (left=rostral) and dorsal view (top=rostral) right. Renderings are not to scale. Abbreviations: A= adult state.; D = deutonymphal state; d = dorsal (germinal) portion of the genital Anlage; dOP = developing ovipositor; EL = eugenital lobes; GP = genital papillae; H = sample from the quiescent stage preceeding the moult to the next instar; L = larval stage; Op = ovipositor; Ov = ovary; P = protonymphal stage; U = uterus; V = vagina; v = ventral (somatic) portion of the developing genital Anlage

3.1.2 General considerations on genital development and the anatomy of the genital system

ovary

Given the age and diversity of the group, the diversification of genital organ anatomy among mites (Alberti & Coons 1999) is not surprising. Considering the uncertain position of major acarine clades in the phylogeny of chelicerates (Dunlop & Alberti 2008), this diversity - that is reflected in the extant literature (Alberti & Coons 1999) - poses problems in understanding phylogenetic background and ontogeny of the genital system in mites. According to Anderson (1973), genital organs in chelicerates generally develop from folds of mesodermal tissue of the second opisthosomal segment. In fact, in larval stages of *A. longisetosus*, a fold-like structure of the genital anlagen with strands of mesodermal tissue contacting the ventral body wall can be found. This indicates that the dorsal fusion of the originally paired genital coelomic sacs is already completed early in development, as stated for the taxon Arachnida (Goodrich 1945). Although the contact to the ventral body wall gets lost later during development, serial sections of protonymphs show the ovarian anlage to be organised in a double layer with a continuous basal lamina on the haemolymph side. This is consistent with the fold hypothesis (Anderson 1973). Inside this fold, a hollow space is present as late as in tritonymphs and microtubule-rich extensions of the oocytes form towards and into the hollow space. The hollow space closes during development, but the microtubule-rich extensions form the central part and the medulla of the rhodoid in the adult ovary. The medulla seems to be contacted by a strand of somatic ovarian epithelium anteroventromedially in the region of the original contact area between the somatic and germinative portions of the genital anlage. Oocytes develop outward from the rhodoid centre with the meroi actually forming massive distal extensions pointing outwards from the hollow space of the mesodermal fold. It remains unclear whether these extensions, that contain the vitellarian region of the ovary, correspond to a structurally derived form of the ovarian pouches described in various chelicerates and crustaceans (Ando & Makioka 1999; Talarico et al. 2009). While the ovaries of many Ixodidae (deOliveira et al. 2006) or Prostigmata and Parasitengona (Shatrov 2002, and cited references) feature pouches filled with or sporting oocytes bulging from their surface, as it is also found in Araneae (Michalik et al. 2005)

and other orders of the Chelicerata (e.g. Miyazaki & Biliński 2006; Talarico et al. 2009), this is not common at first glance in Sarcoptiformes. *A. longisetosus*, as well as other sarcoptiform mites (e.g. Woodring & Cook 1962; Taberly 1987b; Walzl et al. 2004) does not feature a folded epithelial layer in the adult state, but a massive, radially arranged unpaired structure from which two strands of serially progressing oocytes protrude laterally towards the oviducts. These ovarial meroi superficially bear functional and even structural resemblance to insect ovarioles of the panoistic type, albeit fused in the body midline. But, as is to be laid out in the following paragraph, the situations in insects and oribatids, respectively, are not to be confused. They both represent independent, highly derived states of arthropod ovaries, merely sporting the convergence of serially progressing oocytes in paired structures. Within Arthropods there seem to exist two basic layouts of genital organs (Fig. 3.2, 3.3). These differ most with regard to the relative positioning of oocytes versus somatic tissues and the path that is taken by developing oocytes. The two layouts are referred to as the "mandibulate" and "chelicerate" types, named after the groups they were first described for (Makioka 1988). The two types of ovarian organisation can be characterised as follows:

Ovaries of the "mandibulate" type are basically organised as paired series of tubular ovarioles (Fig. 3.2). Within each of these tubes, a germarium consisting of germ cells and somatic nutritional cells is enclosed in ovarian epithelium. From this germarium, oocytes wander internally along the ovariole axis towards the oviductal lumen during vitellogenesis. Despite several differing modes of vitellogenesis (Fig. 3.2, A-C), this inward movement of oocytes is a characteristic feature of the hexapod ovary. In the "chelicerate" type, the ovary consists of a folded layer of mesodermal epithelium from which oocytes bulge towards the haemolypmph space during development (Fig. 3.3). They are frequently connected to the ovarian epithelium by a structure that is called "pedicel" (Fig. 3.3), "funniculus" or "stalk" by different authors, through which they are suspected to ovulate back into the lumen for oviposition (Alberti & Coons 1999, and cited references). Although the lumen between the epithelial layers was repeatedly described as oviduct, it seems to not always serve this purpose, since in some cases the oocytes are reported to transgress the ovarian epithelium and accumulate in the haemolymph space (Seitz 1971). Despite different modes of vitellogenesis (see Cabrera et al. 2009), resembling those described for hexapods regarding the role of nutritional

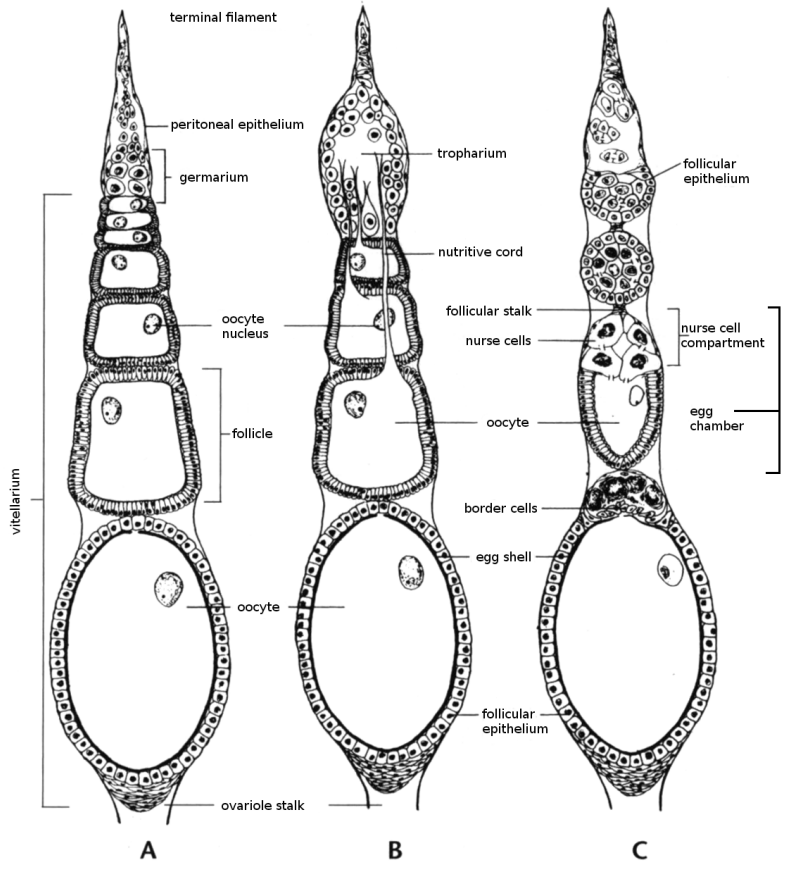


Figure 3.2: Hexapod ovarian types. A) panoistic; B) meroistic-telotrophic; C) meroistic-polytrophic. after F. E. Schwalm 1988, modified

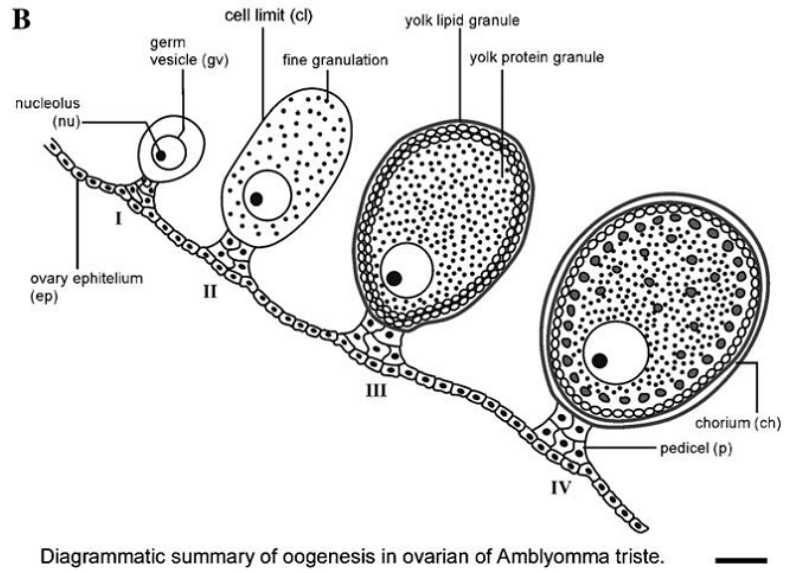


Figure 3.3: Schematic drawing of oogenesis in *Amblyomma triste*, exemplary for Chelicerata. Scale bar = 0.05mm. after Oliveira et al. 2006

cells, this outward orientation (Seitz 1971; Michalik et al. 2005) and movement of oocytes characterises the chelicerate ovary. Regarding vitellogenesis and the origin of yolk precursor substances, or vitellogenins (Vg), fat body derivatives and mid gut epithelia as Vg sources and transport via the hemolymph have been shown or suspected to be involved in both Actinotrichida (Alberti et al. 2003; Bergmann et al. 2010) and Anactinotrichida (Rosell & Coons 1992; Di Palma & Alberti 2001). Bearing the phylogenetic situation in mind, a unifying model for the "Acari" (Cabrera et al. 2009) might thus also appear to be applicable to other groups of Chelicerata in future studies. Although, superficially, the genital organs of *A. longisetosus* bear little resemblance to those of ticks (Oliveira et al. 2006) or web spiders (Michalik et al. 2005), this movement outward from a common centre, which constitutes the tip of a fold of mesodermal tissue, is apparently the principle organisation of the ovary of *A. longisetosus*, if histological and developmental data are taken into account (Bergmann et al. 2008; Bergmann & Heethoff 2012, c.f. preceeding paragraph). The meroi, which do not develop until the adult state, have to be understood as results of this principle of oocytes moving outwards from the germarian centre. Oocytes still within the rhodoid are radially arranged and connected to the medulla via microtubule-rich extensions. As soon as these extensions vanish, further movement of the oocytes is restricted to two lateral, massive strands of oocytes enclosed in somatic ovarian tissue, originating from the initial contact site of the oviductal bulbs, which they apparently take with them to its final, latero-caudo-dorsal positions. Although these strands of serially developing oocytes superficially seem to resemble ovarioles, they constitute fundamentally different developments, as they constitute lateral extensions from an unpaired structure rather than serial, paired structures, converging towards an unpaired duct (Fig. 3.4). Therefore, even the trophic type of the ovary of *A. longisetosus*, that was classified as panoistic (Bergmann et al. 2010) must be regarded as a functionally convergent situation.

While all hexapod ovaries consist of ovarioles (Fig. 3.2), these are not reported from the Chelicerata, all of which show more or less derived versions of the ovary type depicted in Fig. 3.3. Interestingly, keeping in mind that the Hexapoda might be phylogenetically nested within the traditionally recognised "Crustacea" (Pancrustacea hypothesis), both types can be found among crustaceans (Ando & Makioka 1998, 1999; Ikuta & Makioka 2004). Most crustaceans show paired germarian struc-

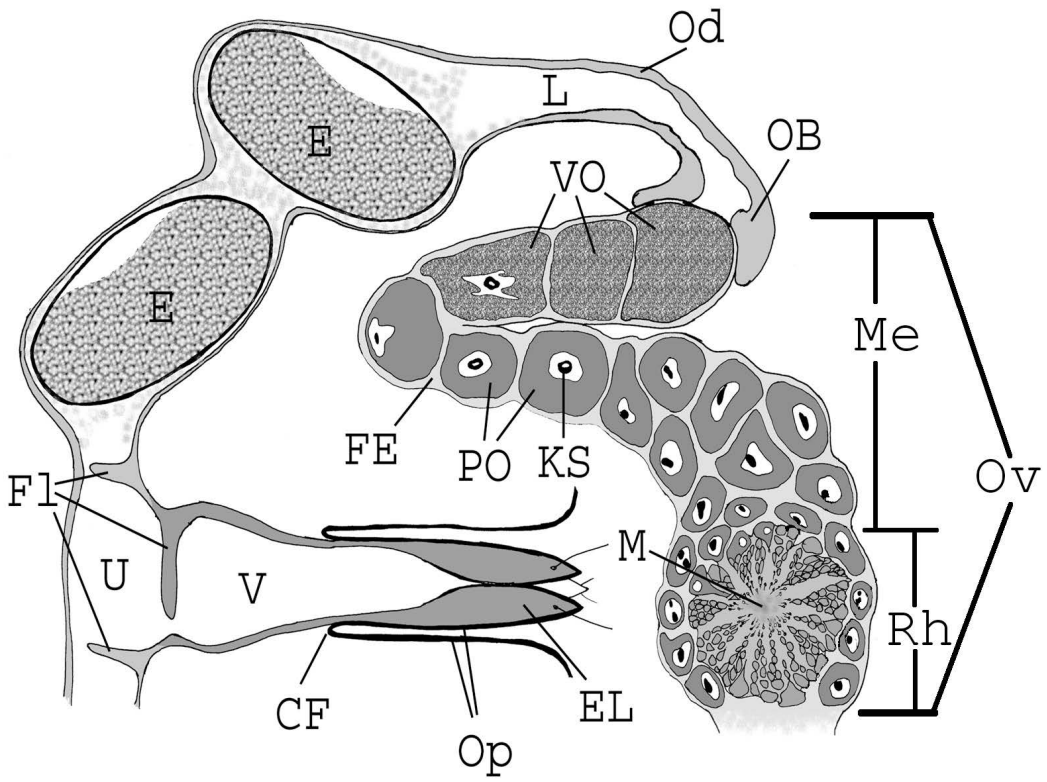


Figure 3.4: Schematic drawing of the genital system of an adult female of *A. longisetosus*. The general arrangement of the major subdivisions is shown along with anatomical features mentioned in this work. Right portion only. Abbreviations: CF: circular fold; E: egg; EL: eugenital lobes; FE: follicular epithelium; Fl: flaps, subdividing the genital tract; GV: germinal vesicle; KS: karyosphere; L: lumen; M: medulla; Me: meros (thigh) of the ovary; OB: oviductal bulb; Od: oviduct; Op: ovipositor; Ov: ovary; PO: previtellogenetic oocyte; Rh: rhodoid (central part) of the ovary; U: uterus; V: vagina; VM: vitellar membrane; VO: vitellogenetic oocyte. after Bergmann et al. 2008, modified

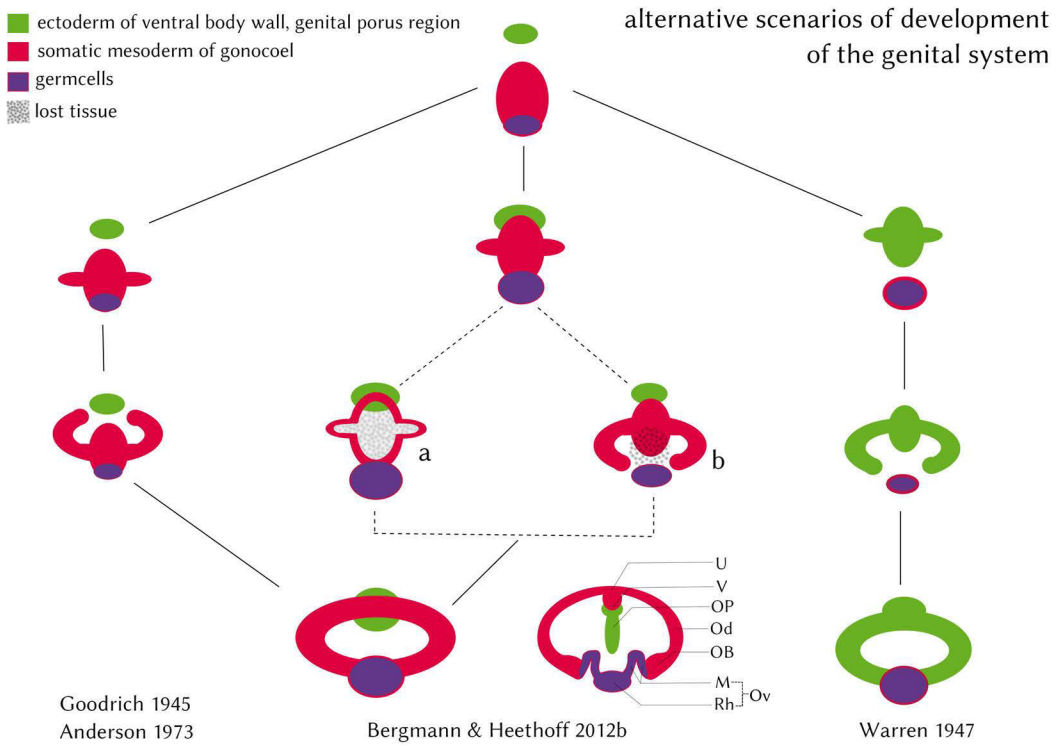


Figure 3.5: : Schematic representation of different scenarios of genital system ontogenesis in *A. longisetosus*. Dorsal view, onogenetic series top to bottom. Left column shows oviducts as anterograd developing coelomoducts following the "classical" view of Goodrich (1945) and Anderson (1973). Right column shows an alternative description by Warren (1947), interpreting the oviducts as retrograd developing ectodermal invaginations. Middle column shows two scenarios derived from the information obtained in this study. It is unclear, whether the oviducts derive from a fold at the rim of the gonocoel with a primary contact site at the ovary (a) or are retrograde developing tubular extensions with a secondary contact site at the ovary (b), and whether their lumen is primary (coelomic cavity) or secondary. Abbreviations: M: meros; OB: oviductal bulb; Od: oviduct; OP: ovipositor; Rh: rhodoid; U: uterus; V: vagina. after Heethoff et al. 2013

tures, which are separated or occasionally connected in the body midline by tubular structures (Ando & Makioka 1998, 1999; Ikuta & Makioka 2004). In contrast, the remipedian *Godzilliognomus frondosus* Yager 1989—the Remipedia being a putative sister group to a monophylum containing Malacostraca and Hexapoda—shows a single unpaired germarium split into two vitellaria that are contacted by oviducts (Kubraskiewicz et al. 2012). This pattern is very similar to that of *A. longisetosus*.

oviducts

The exact nature of the contact between the ovary and the oviducts as well as the nature of the oviductal lumen cannot be fully explained by the data. Oviducts are formed as lateral extensions of the somatic portion of the genital anlage, which is consistent with an origin from the lateral rim of the mesodermal fold (Heethof et al. 2013). A primary contact of ovary and oviducts growing outward from the coelomic sac would require a secondary closing of the primal lumen between medulla and oviductal lumen by growing oocytes and ovarian somatic cells prior to the development of the meroi. Additionally, the somatic portion that is the origin of the buds of the oviducts would either be lost or integrated into the ovarian epithelium. In this study, no evidence was found supporting these situations. Oviducts are understood as coelomoducts of somatic wall material of the genital coelome (gonocoel) of the second opisthosomal segment (Anderson 1973), which grow longitudinally, and contact an unpaired ventral ectodermal invagination housing the primary genital pore (Goodrich 1945). This ectodermal invagination is emerged as the ovipositor and the cuticularised vagina in *A. longisetosus* (Fig. 3.5, left column). Following the traditional understanding of a primary contact site, the oviductal lumen should represent the interior of the coelomic sac. In concordance with the scenario of Goodrich (1945) and Anderson (1973) describing oviducts as protrusions of the gonocoel wall, meroi and oviducts share a continuous basal lamina in *A. longisetosus*. Contrasting the view of Goodrich and Anderson, in nymphal stages oviducts were found protruding laterally as tubular loops from the somatic portion of the genital anlage, contacting the ovarian portion secondarily with their posterior edge (Fig. 3.1). Additionally, a connection of the ovarian medulla to the oviductal lumen could not be demonstrated, since in adult specimen the oviducts begin at the distalmost tips of the ovarian meroi, which grow outward from the ovarian medulla (Fig. 3.1). This outward growth is one of the general principles of chelicerate ovaries. The meroi, which represent massive strands of oocytes wrapped in ovarian epithelium, also grow outward from the original contact zone between generative and somatic portions of the genital coelome. After completing their development, oocytes protrude from their cover of ovarian epithelium at the oviductal bulb. Thereby, ovarian cells seem to yield to the pressure exerted by the oocyte, but stay in contact with its surface at their edges, so that the room between eggshell and ovarian epithelium is not continuous

with the oviductal lumen. By covering parts of the protruding eggs, ovarian cells also extend for some distance into the lumen encompassed by oviductal bulb cells (Publication 2). The oviductal bulb is, according to Taberly (1987) characterised as the proximal onset of the oviducts by structural and functional differences to the ovarian tissues (the latter being involved in vitellogenesis and eggshell accumulation) as well as by encompassing a continuous lumen that is connected to the environment through the genital orifice (Fig. 3.4). However, a secondary contact between ovary and oviducts is explicable considering the observations of this study, although this explanation does not support the traditional understanding of oviductal growth and the formation of the contact as oviductal bulbs (Fig. 3.5, middle column). A secondary contact of ovary and oviducts was already described for an oribatid mite from the suborder Brachipylina, *Cepheus tegeocranus* (Hermann 1804.)(Warren 1947). In the study, oviducts were described as ectodermal structures originating from the invagination of the genital porus (Heethof et al. 2013). The interpretation of oviducts being ectodermal tissue might be due to their apparent retrograde elongation (Fig. 3.5, right column) (Heethof et al. 2013). Within the present study, oviducts are interpreted as mesodermal due to the fact that they never exhibit cuticular lining, whereas the vagina does (Heethof et al. 2013). They furthermore originate from a tissue that is separated from the ventral body wall by an epithelial layer identified as the epidermis (Heethof et al. 2013). A schematic overview over the different scenarios regarding genital development is given in Fig. 3.5. This organisation of the oviducts might be easier to understand in connection with oogenesis and oocyte movement, which also seems to be a derived situation compared to the arachnid ground plan. Developing oocytes bulge out from the ovarian centre. After the connection to the medulla gets lost, the developing meroi can be envisioned as bulging oocytal pouches arranged in a serial manner. Instead of wandering back into the primary lumen through a stalk or funiculus, which is not realised here, oocytes transgress the ovarian epithelium in a similar manner as described in the spider *C. salei* (Seitz 1971). The situation in *A. longisetosus* appears as independently derived due to important differences, however. Instead of dissolving the basal lamina at the site of the oocyte, accumulating in the hemolymph space and extrusion via openly ending oviducts, the oviducts (secondarily?) form defined contact sites, where the oocytes serially leave their epithelial cover, and rapid eggshell solidification is provided at the oviductal bulb.

The ovary–oviduct contact is separated from the hemolymph space by a continous basal lamina which may act as a supporting barrier, putatively facilitating serial ovulation. A functional significance might be envisioned in combination with the large eggs of *A. longisetosus*, its high fecundity and iteroparous oviposition.

3.1.3 Life History

Many basic terms and definitions in evolutionary or developmental biology are derived from bisexually propagating species. In these species, fusion of two haploid gametes leads to the formation of a diploid zygote, with a genomic content that is different from both parents (gonochoristic reproduction). A genetically new individual is established, with the beginning of the next generation in the life cyle starting with zygosis. In parthenogenetic animals like *A. longisetosus* that propagate by postreductional terminal fusion automixis, the definitions of both individuum and generation are indistinct. There is a considerable interval between meiosis and hatching, with oviposition occurring at a variable time in between. Even meiosis itself is difficult to observe directly in these animals, as zygosis of gametes is substituted by terminal fusion automixis, i.e. functionally the reversion of the second meiotic division by the reunion of its products. However, a second polar body could not be observed in *A. longisetosus* and terminal fusion might be realised in a way that, besides a lack of diakinesis, not even a discernible nuclear division might be present in meiosis II (Laumann et al. 2008). It is unclear whether this indicates a derived state of terminal fusion due to the age of the lineage (all steps and processes leading to nuclear division and subsequent diakinesis at the end of meiosis II being no longer protected by selective pressures and hence reduced/lost by deleterious mutations). Alternatively, effective terminal fusion in this case could have been caused by an inability to perform a second meiotic division in the first place. As the only hypothesis combining molecular and histological data (clonal lineage, terminal fusion automixis) without contradiction is a fully postreductional or “inverted” meiosis (Wrensch et al. 1994), suppression of the second meiotic division might result in a situation where, despite meiosis, not a single haploid cell nucleus is present in the life cycle of *A. longisetosus*. Therefore, a specific point is needed that defines the beginning of the next generation. Common biological definitions of the term “generation” are linked to those of the term “individual”, in that “generation” refers to either 1) “individuals of a population originating at

the same time from a common parent or parents or 2) “the developmental phase between one reproductive process and the next” (Scott 1995), whereas an individual is defined as “ a single living organism, defined by its independent existence in time and space, and the uniqueness of its phenotype”(Scott 1995), where “uniqueness” includes both “the particular combination of its genetic material” and “the environmental influences operating at the various stages of its ontogenetic development” (Scott 1995). To pinpoint the demarcation between successive individuals of a reproductive lineage, a change in the combination of alleles in the genome, a change in ploidy level, a developmental independence, a physiological separation, or non-identical environments can therefore be regarded as indicating the generational border (Dittler et al. (eds.) 1934; Kaudewitz 1957). Most of these features are used as landmarks to define generations in gonochoristic species, where they are usually simultaneous. These features may not be simultaneous or even existent in non–gonochoristic lineages. A change in the ploidy level, for instance, is not a general feature, as rediploidisation may be achieved by different mechanisms at different times, or may be lacking altogether (for instance in apomictic or asexual lineages). As a representative for the whole group of Desmonomata, *A. longisetosus* is an iteroparous, thelytokous, egg laying animal, i.e., it produces diploid offspring at several instances during its life. Fertilisation events are nonexistent in these parthenogenetic lineages. Even recombination is thought to be restricted to the telomeric regions of the chromosomes in several parthenogenetic Oribatida (Palmer und Norton 1992; Wrensch et al 1994; Schäfer et al. 2006). As offspring are functionally clone-like in these strains (Palmer & Norton 1992), a potential genetic discontinuity could not be detected either. Hence, these lineages constitute continuous genetic individuals, and generations are no valid concept in these cases from a genetical viewpoint. For the same reason, biological species concepts based on reproductive isolation or coherence have been discarded as not applicable to non–bisexual lineages. However, the evolutionary potential and generational dependence of non–bisexual populations has already been demonstrated (Barraclough et al. 2003; Barraclough & Herniou 2003). Also, parthenogenetic radiation of Desmonomata into morphologically, genetically, and ecologically distinct taxa was already demonstrated (Maraun et al. 2004; Laumann et al. 2007; Heethoff et al. 2011). At least some parthenogenetic lineages, as demonstrated for Desmonomata, are therefore not static entities—or rather, slowly deteriorat-

ing ones, as the classical view predicts the accumulation of deleterious mutations (Muller 1964). Evolutionary processes such as adaptation and speciation do take place in these lineages, dependent on a meiotic germline and mother–daughter relationships, i.e., the succession of generations. Therefore, most if not all of the aforementioned diagnostic features appear as sufficient but not necessary conditions for a generational border. In the case at hand, the anatomically conveniently identifiable environmental isolation of the rediploidised oocyte prior to embryogenesis serves as a justifiable diagnostic feature of the generational border. Oocytes in *A. longisetosus* enter the prophase of meiosis I already in the tritonymph. Previtellogenesis ensues in the adult during the prolonged prophase of meiosis I in the periphery of the rhodoid, while they are still connected to the medulla via microtubuli-rich extensions. These extensions are soon retracted and the oocytes wander along the meroi. While they are encompassed by ovarian epithelium in the meroi, meiosis II is concluded and rediploidisation is achieved by terminal fusion (Laumann et al. 2008). Simultaneously, vitellogenesis and the accumulation of eggshell material proceeds. During this time, intense material exchange of the oocyte with somatic tissues is indicated by numerous microvilli and coated pits. Communication ceases when the eggshell is rendered impermeable (Aeshlimann & Hess 1984) upon passing the oviductal bulb. As soon as the egg is separated from the mother by an impermeous eggshell, embryogenesis starts and the daughter individual is independently developing in an environment that is no longer identical to that of the mother's organism. Subsequently, oviposition is unrelated to the developmental state of the embryo (Laumann et al. 2010a). A biologically valid individual of a successive generation thus originates at the oviductal bulb, which therefore marks the position and instant of the generational border.

3.1.4 Reproductive Ecology

Yastrebtsov (1992) mentioned that, at least in free-living Gamasida, oviparity might prevail in ephemeral habitats. On the other hand, many parasitic gamasids, which can be considered to occupy a fairly stable habitat, exhibit retention up to ovolarviparity and the degree of embryological development in deposited eggs sometimes depends largely on external factors (Yastrebtsov 1992). The model organism *A. longisetosus* might exhibit a mixed mode, as both young and further developed eggs can be observed in the same clutch. This is not surprising, since the pro-

duction rate is 1.3 eggs per day and female, and clutches of 15-35 eggs are laid in varying intervals (Heethoff et al. 2007). Additionally, disturbed animals usually refrain from egg deposition for several days up to two weeks. This indicates the mother's possibility to gradually adjust certain features of her reproductive strategy following environmental leads. A certain degree of control of the mother over the environmental quality into which she releases her offspring may be inferred, as careful probing of the substrate with ovipositor bristles and genital papillae prior to oviposition can frequently be observed (pers. obs.). Clutch size and retention time are obviously interdependent on each other at a given egg production rate, as the storage capacity of the oviducts is limited, ultimately by the rigid cuticle of the opisthosoma. A maximum of 42 eggs, constituting ~1/3 of the opisthosomal volume, could be preparated from the oviducts of a single living female (Bergmann, Diploma thesis). It therefore seems feasible to test whether the mother is actually actively controlling egg laying by following environmental leads, such as temperature, humidity, brightness or the ambient level of volatile products of predators, microbes, or conspecifics. Similar tests, indicating a high variability of reproductive behaviour in correlation with environmental leads, have already been conducted in the two-spotted spider mite *T. urticae* (Clotuche et al. 2013). The potential to retain eggs during short-termed adverse conditions could be envisioned as potentially meliorating offspring survival. Furthermore, it could achieve this without interfering with the constantly high rate of egg production enabled by parthenogenesis, since eggs can develop in rapid succession with vitellogenesis, meiosis and eggshell formation occurring in parallel, and no sperm access is necessary. However, manipulative experimental testing whether active control is exerted, or the mix of young and further developed eggs in one clutch is determined by the function of organs and tissues, is still lacking, as well as testing the effect of variability of hatching dates from a single clutch on offspring survival. Regardless of active control being exerted or not, the specific parity mode of *A. longisetosus* combines elements of oviparity (i.e., solid-shelled eggs rich in yolk, no nutrition beyond choriogenesis, no communication between generations beyond the end of vitellogenesis, potential of rapid successive oviposition) with features of ovilarviparity (e.g., development in sheltered space, potential of egg retention to the stage of larvae, increased and prolonged parental investment) to varying degrees. It is affected by the specialization of the tissues of the proximal oviduct into the

oviductal bulb, ensuring rapid solidification of the eggshell, effectively internalising oviposition and generation boundary, and rendering the oviducts into brood chambers.

3.2 Results of testing the hypotheses

Regarding the aforementioned verbalised hypotheses, the following results were achieved:

- I.) *The ovary of post embryonic oribatid mites is an unpaired structure, forming together with the gonoducts from mid-ventral mesodermal pouches of anterior opisthosomal segments. This is deduced from the fact that in adult mites oocytes from both oviducts stem from a single ovarian centre. It implies, that*
  - *a) fusion of the gonocoel precedes the development of genital organs and*
  - *b) all observed paired structures are secondary developments.*
- During early larval and nymphal development, no paired precursors of the genital system could be observed. A single ventromedian mesodermal structure that contains a cluster of germ cells dorsally, develops into the genital organs. The ovary in the body mid line develops a single medulla in the deutonymphal stage with no indication of paired precursors. The paired oviducts develop from lateral extensions of this structure, when an unpaired ovary is already formed. The paired ovarian meroi develop even later at the ovary–oviduct transition due to outward movement of vitellogenetic oocytes. Therefore,
  - a) fusion of paired gonocoels can be rejected for any stage later than larvae 24h after hatching, i.e. for any stage further developed than the differentiation of a germ cell cluster from mesodermal somatic tissue in the body mid line.
  - b) The sequence of development suggests that oviducts and meroi are not products of a fusion of differentiated organs, but secondary lateral developments of an unpaired gonocoel realised by the earlier fusion of coelomic sacs in the body mid line

- II.) *The ovary is of the panoistic type. This was suspected from the spatial arrangement of oocytes and epithelia in the ovarian meroi. A possible individual association of these tissues, which would indicate a meroistic ovary, had to be scrutinised*

- Premeiotic mitoses during the deutonymphal stage lead to a solid cluster of oocytes. No nutritive cells develop inside the ovary. Somatic tissues are restricted to the surrounding ovarian epithelium, which can be traced back to somatic cells already present in the larva. Oocytes lose the stalks that couple them with the medulla at the onset of previtellogenesis. An empty space between the oocyte and the ovarian epithelium is formed and eggshell material is deposited there during vitellogenesis. As an individual association of oocytes and nutritive cells could not be demonstrated, the alternative hypotheses, that the ovary is meroistic, must be rejected. The ovarian type therefore is described as panoistic.

- III.) *The mode of oogenesis does not contradict a panoistic ovary.*

  - *a) It is solitary, i.e. oocytes progress independently from each other and surrounding somatic tissues.*
  - *b) Vitellogenesis is exogenous, i.e. oocytes actively take up macromolecular components as yolk precursors, which are produced in somatic tissues of the mother.*

- Although of typically chelicerate organisation, the ovarian mode of oocyte nutrition is best described as panoistic (functionally convergent to hexapod ovarioles)

  - a) Ovarian epithelial cells form a single layer between the oocyte and the basal lamina separating the ovary from the haemolymph space. Extensions of these ovarian epithelial cells separate individual oocytes in a way, that frequently the same epithelial cell is neighbouring several oocytes. A continuous layer of cells surrounding individual oocytes was not found. As the existence of true follicles is thus rejected, oogenesis is solitary.
  - b) During vitellogenesis, the oocytes take up nutrients via microvilli and coated pits. Microvilli prevail during early vitellogenesis, while

coated pits take over during the process and dominate late vitellogenesis. Glycane-rich material possibly constituting carbohydrate yolk components accumulate prior to the appearance of lipid droplets and proteinaceous yolk vesicles. No micropyle could be observed. Considering organelle content and signs of synthetic activity, the fat body and lateral cells are more likely responsible for yolk precursor production than the somatic ovarian tissue. Although oocytes also possess numerous mitochondria and abundant rER, evidence of macromolecular substance intake by oocytes, and indications of strong synthesis and product release by lateral and fat body cells suggests the rejection of endogenous vitellogenesis in favour of exogenous vitellogenesis.

- IV.) *The instant of oviposition is neither a suitable marker for developmental stage of the embryo, nor the generational boundary. The impermeability of the eggshell indicates separation of mother and daughter and the onset of independent development at an earlier instant.*

- The generational boundary is located at the ovary–oviduct transition, since there, the eggshell solidification separates the environments of mother and embryo. Embryogenesis proceeds independently. Eggs are laid in varying stages of development, and hatching times vary accordingly. The instant of oviposition is thus rejected as both predictor of developmental stage as well as indicator of the generational boundary.

- V.) *Major events of the developmental processes of internal organs are synchronized with moulting. This is deduced from the progression of the development of externally visible organs in the genital region (plates, bristles, genital papillae), which proceeds in well-defined stages suitable for identifying individual sub-adult stages.*

- During the progression of the larval and three nymphal stages, the internal genital organs develop gradually and continuously. Individual differences in the state of genital development between specimens at corresponding moultings are apparent. Therefore, moultings are imprecise predictors of the state of internal development. The hypothesis, that the development of internal genital organs is synchronized with cuticle moultings is rejected.

- VI.) *Results in closely related species (Taberly 1987 a,b,c) can be confirmed for* A. longisetosus. *Based on the aforementioned studies, the following hypotheses where of special interest for scrutiny:*
  - *The mode of parthenogenesis is terminal fusion automixis.*
  - *Oocytes enter prophase I in subadult stages.*
  - *Vitellogenesis and egg-shell formation are concluded within the ovary.*
  - *The appearance of a lumen marks the ovary–oviduct transition.*
- The situation in *A. longisetosus* closely resembles the findings of Taberly (1987a,b,c) in two closely related species (*P. peltifer* and *T. tectorum*) in Desmonomata.
  - The abundance of synaptonemal complexes in tritonymphs unequivocally confirms a meiotic germline. Hence the mode of parthenogenesis is automictic. As ameiotic modes (Apomixis) are eliminated by these results, the description of the reproductive mode of *A. longisetosus* as terminal fusion automixis is permitted with good confidence in consideration of previous findings (Laumann et al. 2008).
  - During the tritonymphal stage, synaptonemal complexes could be observed in all germ cells of the ovary. Thus, oocytes enter prophase I simultaneously in subadult stages and no oogonia persist into the adult.
  - Previtellogenetic and vitellogenetic oocytes are found in both ovarian extensions that protrude laterally from the ovarian medulla. These extensions consist of oocytes, covered by flattened cells indistinguishable from the somatic ovarian epithelium. The accumulation of eggshell material proceeds simultaneously to vitellogenesis in these extensions. Somatic cells extend between the oocytes, separating them from each other. No lumen can be observed. Hence, the lateral extensions are not proximal oviducts, but part of the ovary and are named “meroi” to distinguish them from the central unpaired “rhodoid” containing the ovarian medulla. Vitellogenesis and eggshell formation are concluded in the ovary.
  - At the proximal-most onset of the lumen of the oviducts, which is continuous with the outer environment via the genital orifice, a sharp tran-

sition of tissue structure and tissue architecture occurs. The lumen is surrounded by a single layer of secretory epithelium. Eggs enter the lumen by transgressing their cover of ovarian epithelium cells, similar to an ovulation event. The eggshell rapidly solidifies after coming in contact with the content of the oviductal lumen. Oviducts acquire a lumen early in development, when contact with a still ball-shaped ovary is established. Meroi do not develop until the adult stage and never exhibit a lumen. No hypothesis alternative to homology of the contact site across life stages could be deduced that does not imply processes of which there is no evidence. The onset of the oviductal lumen, externally discernible as an oviductal bulb, marks the ovary–oviduct transitions as well as the generational border.

- VII.) *The combination of tomographic data with serial sectioning is a suitable way to reduce workload while retaining information of key regions with TEM-resolution from limited numbers of sections and undistorted renderings of histologically correct segmentations of the volumetric data in micro-arthropods.*

- The combination of conventional histological methods and state-of-the-art tomographic techniques yielded useful synergistic effects on the studies for both lines of work. Tomographies resulted in high-quality, three dimensional datasets that permitted reconstruction of overall anatomy and structural arrangement of genital organs of three developmental stages per free-living instar in a comparably manageable time frame. Histological data permitted high-resolution examination and determination of individual tissues and cells along with analysis of histochemical properties. Positive effects of the combination of the aforementioned methods included—in the case of tomography-segmentation—the justification of drawn tissue boundaries by comparison with electron micrographs of corresponding regions. In the case of histology, existing tomographies greatly facilitated the planning of preparation and serial sectioning, resulting in reduced workload for sectioning. Beforehand, regions of interest could be determined with high accuracy relative to the body or other prominent internal organs of the specimens (e.g., caeca). Less sectioning have to be performed per specimen, as only subcellular and ultrastructural data required this technique. During the course of

this study, this combination of methods represented a suitable workflow for the examination of the internal anatomy of a microarthropod.

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List of Figures

5 Publications

5.1 Publication 1

Bergmann, P. and M. Heethoff (2012). "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)". — Soil Organisms **84**(2): 459–470.

Direct link

SOIL ORGANISMS Volume 84 (2) 2012 pp. 459–470

ISSN: 1864-6417

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).

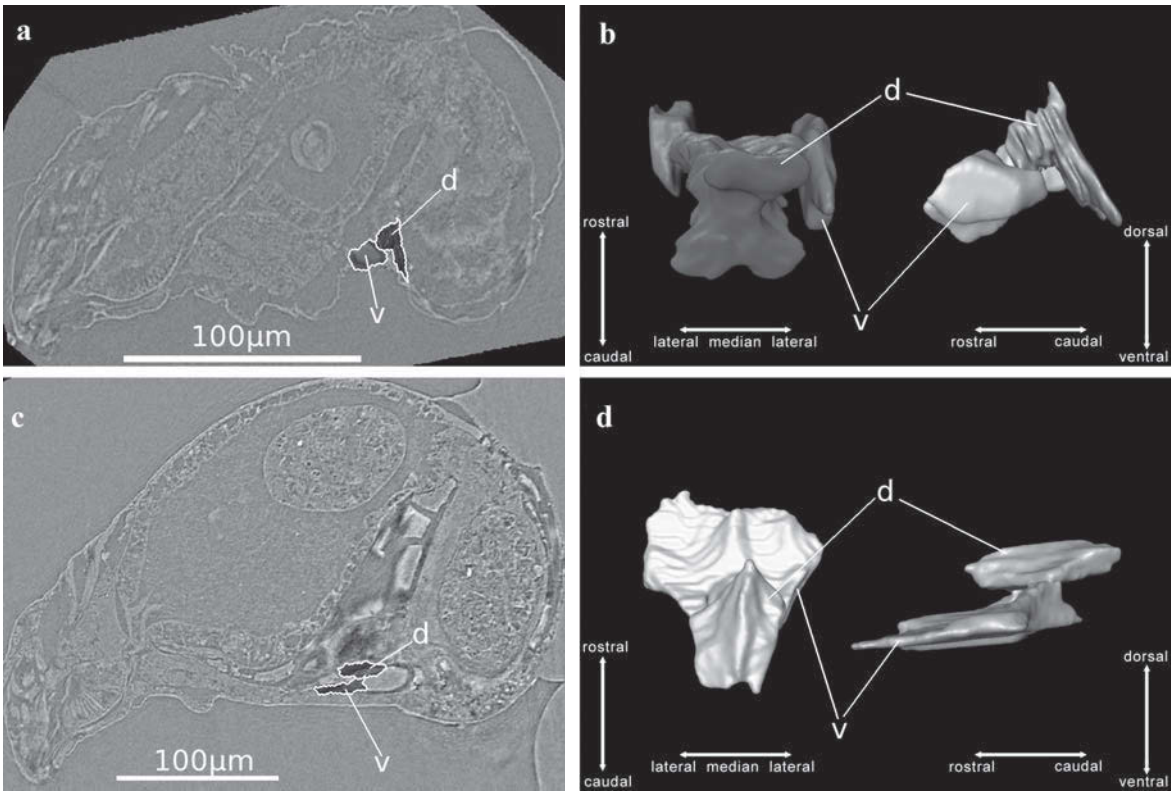

Fig. 1 *Archegozetes longisetosus,* phase enhanced SR-µCT data. Virtual slices and 3D-renderings of the reproductive organs in the larval stage. Top row: Larva 24 h after hatching. **a**: Sagittal virtual slice and 3D rendering outlined in white. **b**: Dorsal (left) and lateral (right) view of 3D rendering 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.

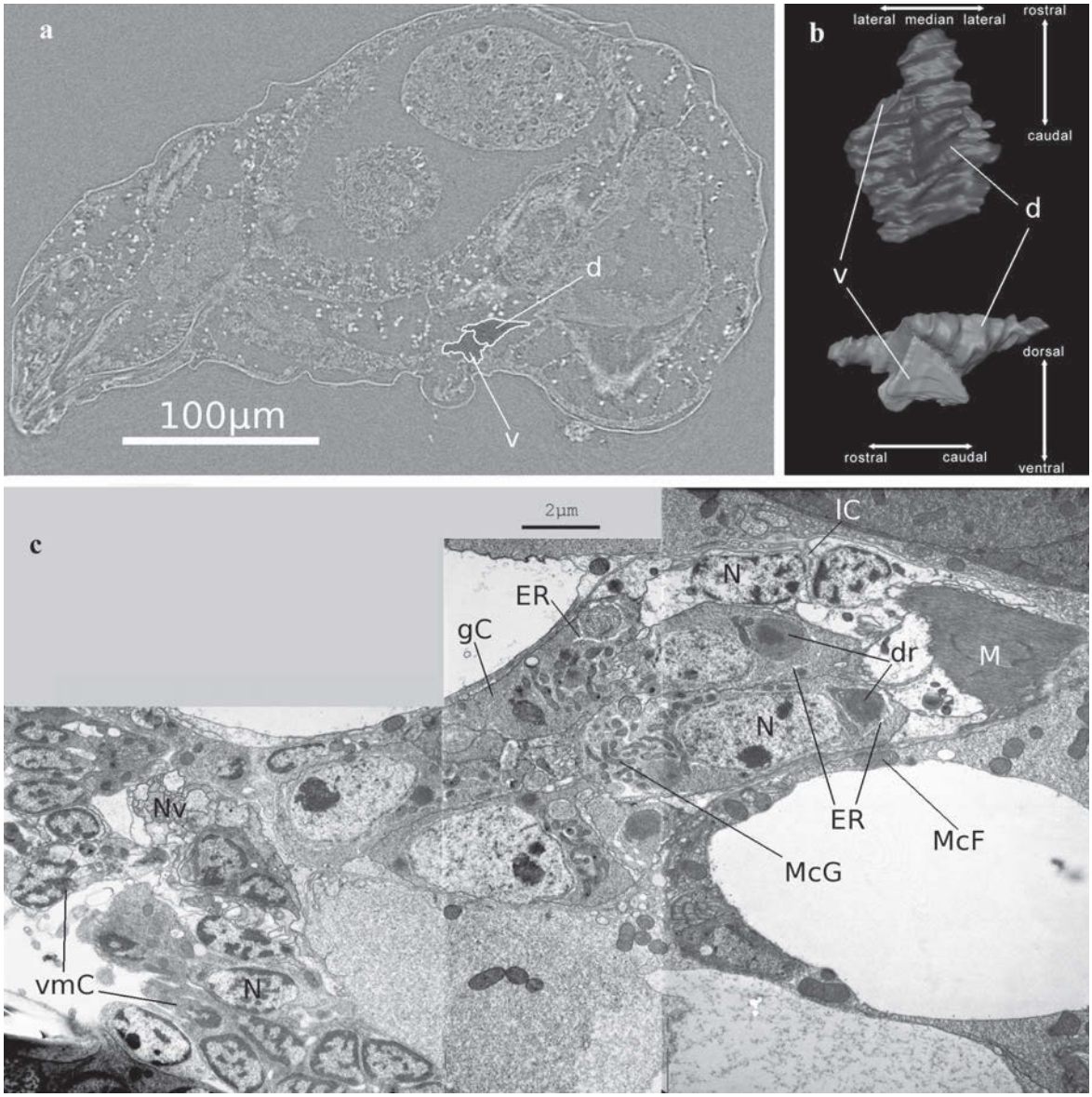

Fig. 2 *Archegozetes longisetosus,* phase enhanced SR-µCT data. Virtual slices and 3D-renderings of the reproductive organs in the protonymphal stage and TEM micrograph of protonymph two days after molting. **a**: Sagittal virtual slice, segmentation area outlined in white with grey shading. **b**: Dorsal (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.

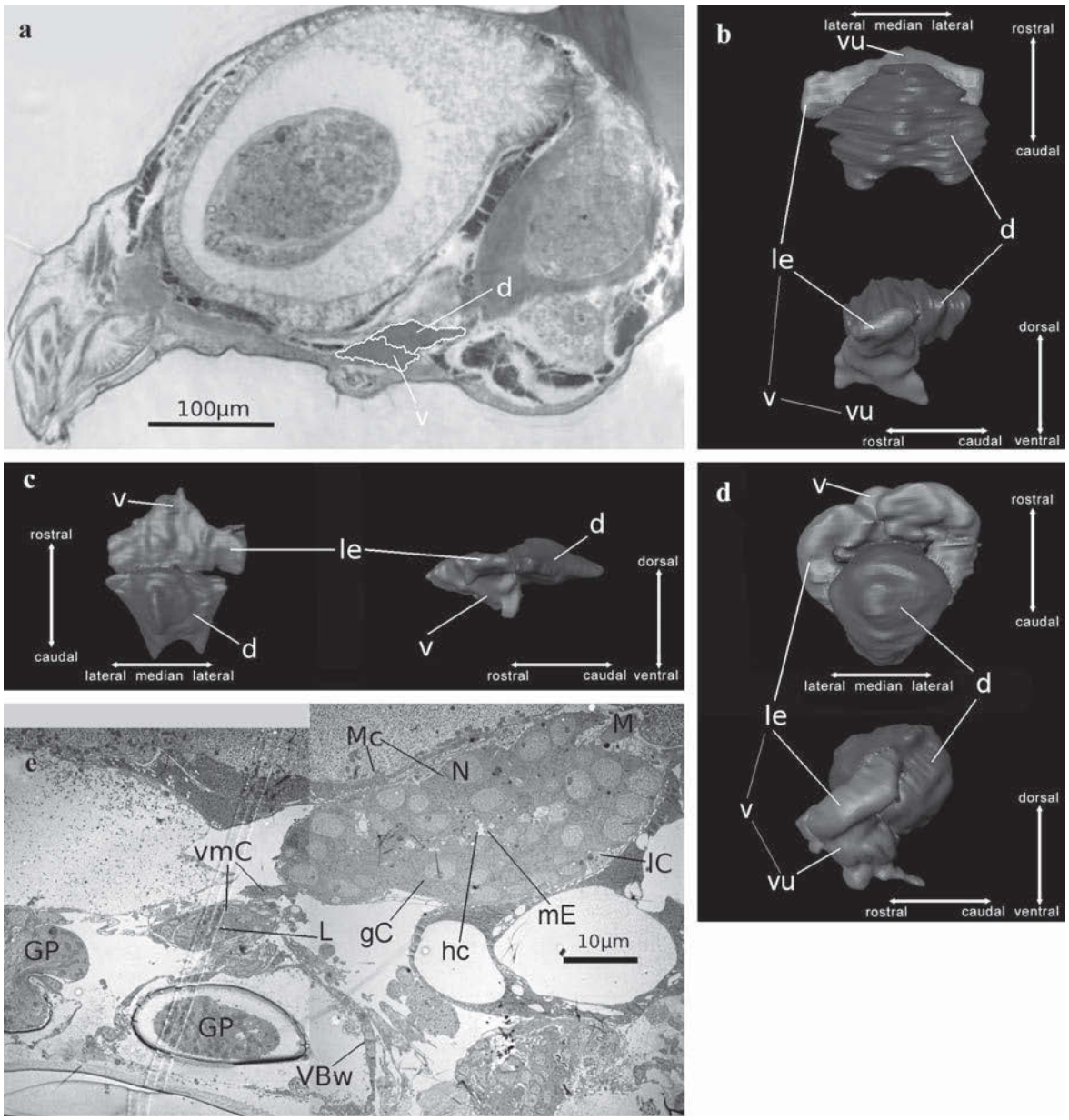

Fig. 3 *Archegozetes longisetosus*, holotomographic SR-µCT data. Virtual slices and 3D-renderings of the reproductive organs in the deutonymphal stage and TEM micrograph of deutonymph. The dorsal portion of the reproductive organs is increasing in volume and acquiring a spherical shape, as the ovary 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.

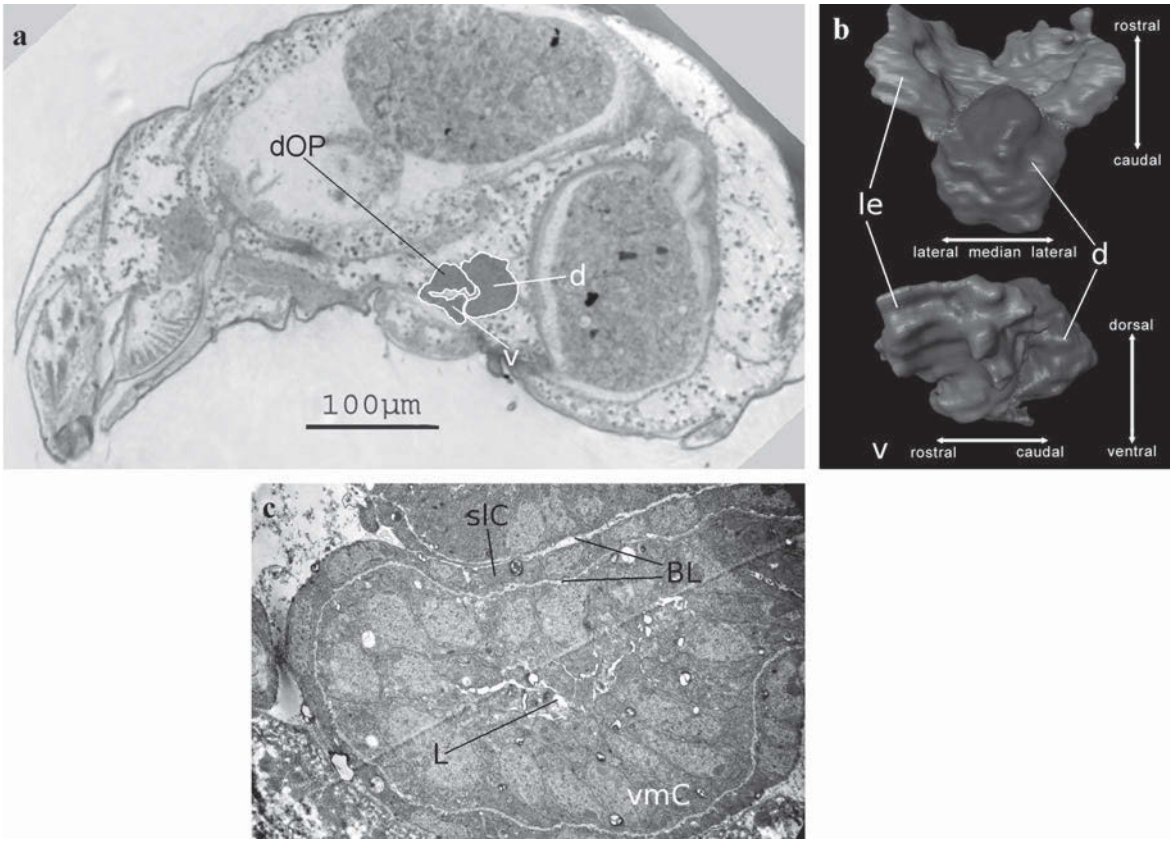

Fig. 4 *Archegozetes longisetosus*, holotomographic SR-µCT data. Virtual slices and 3D-renderings of the reproductive organs in the early tritonymphal stage and TEM micrograph of tritonymph. **a**: Sagittal virtual slice of tritonymph two days after molting, segmentation area outlined in white with grey 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).

5. Acknowledgements

We thank Daniela Weide, Sebastian Schmelzle and Michael Laumann for help with synchrotron measurements and data processing, Monika Meinert, Julia Straube and Karl-Heinz Hellmer for technical assistance, and Roy A. Norton for useful comments on oribatid anatomy and for providing animals to start a hatchery.

This work was supported by the European Synchrotron Radiation Facility Project SC2127 (to Michael Heethoff) through allocation of beam time. We acknowledge the excellent support by Lukas Helfen and Peter Cloetens at the ESRF facilities, beamline ID19. Paavo Bergmann was supported by ‘Evangelisches Studienwerk Villigst e.V.’ through a PhD grant.

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Accepted 15 May 2012

5.2 Publication 2

Bergmann, P., M. Laumann, P. Cloetens and M. Heethoff (2008). "Morphology of the internal reproductive organs of *Archegozetes longisetosus* Aoki (Acari, Oribatida)". — Soil Organisms **80**(2): 171–195.

Direct link

SOIL ORGANISMS Volume 80 (2) 2008 171 – 195

ISSN: 1864 - 6417

Morphology of the internal reproductive organs of *Archegozetes longisetosus* Aoki (Acari, Oribatida)

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Abstract

The morphology and three-dimensional organisation of the internal reproductive system of *Archegozetes longisetosus* is described, using a combination of conventional microscopic methods and the non-invasive technique of synchrotron X-ray microtomography. The reproductive system is described at various points of its development and the differentiation of its components observed. The genital duct forms during tritonymphal stage close to the ventral body wall from different precursors. Previtellogenesis and the differentiation of eugenital lobes start prior to the last moult. Flap-like structures separate the oviducts from the uterus and the uterus from the vagina in the adult stage. We propose a nomenclature for the subdivisions of the ovary.

Keywords: development, ovary, synchrotron X-ray tomography, holotomography

Zusammenfassung

Wir stellen die Morphologie und die dreidimensionale Organisation des internen Reproduktionssystems von *Archegozetes longisetosus* vor. Hierbei kommen konventionelle mikroskopische Methoden und die nicht invasive Technik der Synchrotron-Röntgen-Mikrotomographie zur Anwendung. Das Reproduktionssystem wird zu verschiedenen Zeitpunkten seiner Entwicklung beschrieben und die Differenzierung seiner Komponenten verfolgt. Die Genitaltrakte bilden sich während des Tritonymphen-Stadiums nahe der ventralen Leibeswand aus unterschiedlichen Vorläufern heraus. Die Prävitellogenese und die Differenzierung der eugenitalen Loben beginnen noch vor der letzten Häutung. Klappenartige Strukturen trennen im Adultus die Ovidukte vom Uterus und den Uterus von der Vagina. Wir schlagen eine Nomenklatur für die Untereinheiten des Ovars vor.

1. Introduction

Oribatid mites (Acari, Oribatida) comprise about 9000 described species (Schatz 2002).

They are unique among animals for their exceptionally high rate of parthenogenesis: 10 % of the species reproduce by obligate thelytoky (Norton et al. 1993). These species form several clusters, which presumably radiated while being parthenogenetic (Palmer & Norton 1992, Norton et al. 1993, Maraun et al. 2004, Laumann et al. 2007). According to molecular analyses, this parthenogenetic radiation took place at least 100 million years ago (Heethoff et al. 2007b). Hence, they are excellent examples for so-called 'ancient asexuals', i.e. long-term stable parthenogenetic systems, and it was suggested that the second big group of Sarcoptiformes, the Astigmata, may have emanated from one of the thelytokous clusters (O´Connor 1984, Norton & Kethley 1994, Norton 1998, Maraun et al. 2004), although molecular data on this aspect are contradictory (Murrell et al. 2005, Domes et al. 2007). The suggested stem group of Astigmata within the oribatid mites is the Trhypochthoniidae (Norton et al. 1993), a taxon consisting of more than 50 obligatory parthenogenetic species (Heethoff et al. 2007b). One member of this family is the species *Archegozetes longisetosus* Aoki, 1965 which was referred to as the most-studied oribatid mite under laboratory conditions (Smrž & Norton 2004, Heethoff et al. 2007a). Studies investigated various aspects of its biology such as life history, embryonic development, anatomy of the digestive system, toxicology and functional morphology (Heethoff & Koerner 2007, Heethoff et al. 2007a and cited references). However, except for the gross morphology of the reproductive organs and some information on reproductive rates (Heethoff et al. 2007a), the whole complex of reproductive biology remains largely unknown from its form to its function. The majority of studies on the anatomy of the genital system of mites or their reproductive biology refer to taxa other than Oribatida, with an emphasis on pests, parasites and their possible control agents (Alberti & Coons 1999, and e.g. Di Palma & Alberti 2001, Shatrov 2002, de Oliveira et al. 2007). Comparative studies of Sarcoptiformes are scarce (Alberti & Coons 1999, Walzl et al. 2004).

The genital system of oribatid mites usually consists of a massive, unpaired ovary located posterior to the eugenital orifice and close to the ventral body wall, surrounded by tissue of nutritive nature (Alberti & Coons 1999, Alberti et al. 2003). Adjacent are paired oviducts that fuse to an unpaired uterus, leading to the vagina. The vagina continues into a long, cuticular ovipositor that is double-folded at its insertion and at the circular fold at about half-length inside the idiosoma when not in use. At its tip, it splits into three eugenital lobes that surround the genital orifice. Eugenital lobes and the circular fold that separates the proximal and distal portions of the ovipositor bear setae (Alberti & Coons 1999). As to the adopted nomenclature for the portions of the genital system, please refer to the discussion. In the present study, we used synchrotron X-ray microtomography (Cloetens et al. 1999, Cloetens et al. 2006, Betz et al. 2007) to analyse the spatial organisation of the reproductive system in *A. longisetosus*. To gain insight in developmental processes, whole specimens of various ages were examined, revealing remarkable changes of the organ system during development.

2. Materials and methods

2.1. Rearing

Specimens were taken from our laboratory culture of *Archegozetes longisetosus* ran (Heethoff et al. 2007a). Clutches of eggs were removed from the culture and placed in the wells of tissue culture plates (Tissue Culture Cluster^{24}, Costar) for further development. Wells were filled 1 cm high with plaster-of-paris:charcoal (6:1) mixture. The plates were kept in constant dark; air humidity was kept at 90 % and temperature at 20 °C. Bark of different trees, covered with green algae (mainly *Protococcus*) was supplied as food.

The wells were checked daily for sufficient food supply and moisture, and specimens were removed with a fine brush at appropriate times (tritonymphs: two and five days after moulting to the instar; tritonymph in quiescent period prior to last moult; adults: one and five days after last moult).

2.2. Synchrotron-X-ray-Micro-Computer-Tomography (µCT)

Specimens collected from the culture were fixed in a 6:3:1 mixture of 80 % ethanol, 35 – 38 % formaldehyde (standard solution, Merck) and 100 % acetic acid for at least 24 hours. After dehydration in a graded ethanol series of 80 %, 85 %, 90 %, 95 % and 100 % with three times 10 minutes each, the specimens where subjected to critical point drying in liquid carbon dioxide (E 3000 Series II Critical Point Drying Apparatus, Polaron Equipment Limited). Dried specimens were glued with cyanoacrylate to the tip of plastic stubs (3 mm diameter, 12 mm length) and stored in an exsiccator.

Measurements at the European Synchrotron Radiation Facility (ESRF) were conducted at beamline ID 19 (experiment number SC2127) with an energy level of 20.5 keV. Samples were scanned for holotomography (Cloetens et al. 1999, 2006) at 10, 20, and 45 mm distances to the detector with 1300 projections over 180°. X-Rays were converted to visible light by a scintillator and projections were recorded with 0.35 s exposure time on a cooled CCD (ESRF FreLoN) with 2048 x 2048 pixels and an effective spatial resolution of 0.7 µm. 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).

Phase-enhanced tomography was performed at a sample-detector distance of 20 mm. It enhances all abrupt density changes and boundaries in the sample.

A detailed description of the technique is given by Betz et al. (2007).

Voxeldata were visualised with the software VGStudio Max 2.1 (Volume Graphics, Heidelberg, Germany) and segmented with amira 4 (Mercury Computer Systems, Inc., Berlin, Germany) on a 64bit-Dual-Opteron computer system.

2.3. Light and electron microscopy

Whole specimens and dissected genital organs for serial sectioning were fixed in 2.5 % glutaraldehyde in Na-cacodylate with 1.66 % sucrose at pH 7.2 overnight at 4 °C, and postfixed in 1 % of osmium tetroxide in Na-cacodylate for 2 hours at 0 °C. Samples were dehydrated in a graded ethanol series (AGAR LV) or isopropanol (SPURR) at 70 %, 75 %, 80 %, 85 %, 90 %, 95 % and 100 % for three times ten minutes each and placed in fresh

100 % alcohol overnight. Then, the alcohol was replaced by propylene dioxide (2 x 1 h) and samples were gradually infiltrated (propylene dioxide / resin mixture 1:1, 1:3, 1:7 and pure resin for 1.5 h each, pure resin overnight) and embedded in epoxy resin (SPURR´s medium / AGAR LV). Polymerisation was conducted at 60 °C for 48 h. Semithin (0.5 µm) and ultrathin (70 nm) sections were cut using a Reichert Ultracut microtome and diamond knives (Diatome 45°). Semithin sections were stained in toluidine blue 0.13 % / alkaline fuchsine 1 %, or methylene blue / Azur II according to Richardson et al. (1960) and light microscopy was conducted with a Zeiss Axioplan light microscope and a Zeiss MrC5 digital camera. Ultrathin sections for transmission electron microscopy (TEM) were contrasted with uranyl acetate for 20 min and lead citrate for 10 min. TEM was conducted on a Philips Technai 10 electron microscope. Images were digitally captured by a MegaVision II digital camera.

2.4. Preparations of genital tracts

Fresh specimens were dissected using sharpened tungsten needles (Norton & Sanders 1985) under stereomicroscopic control on a glass slide, either in a drop of glycerol, for immediate observation with phase contrast / DIC light microscopy, or they were dissected submersed in pre-cooled glutaraldehyd-fixative and then processed for serial semithin and ultrathin sectioning as described in the previous paragraph.

3. Results

The results of the analysis are presented in chronological order, although denomination of components and their respective precursors in younger specimens is based upon the interpretation of the adult state, as the features are most clearly distinguishable in reproducing females, and terms are generally defined for adult specimens.

3.1. Tritonymphal stages

Specimens as young as 48 h after moulting to the tritonymph already show a development of the genital bud (Fig. 1a) that allows for observation of the duplicated wall of the ovipositor, precursors of eugenital lobes (Fig. 1b) and precursors of oviducts apparently originating close to the ventral body wall (Fig. 1c). The ovary at this age lacks any structure discernible by µCT, except for numerous small and dense particles that are likely oogonial nuclei (Figs 1a, b)

Specimens collected on day five after moulting of the tritonymph already show a developed genital system with invaginated ovipositor bearing three eugenital lobes as differentiations of its distalmost portion, an unpaired portion (vagina / uterus) (Fig. 2c) and paired oviducts showing a free lumen and making contact to a roughly spherical ovary (Figs 2b, c), although the features are restricted to a more medio-ventral part of the notogaster and are less well defined compared to specimens taken later in development (Fig. 2). The tissue forming the oviducts appears lighter, indicating lesser density, than the tissue of the developing ovipositor. No oocytes had started previtellogenesis at this age.

Specimens collected from the middle of the quiescent phase associated with moulting to the adult already show distinct features of the internal reproductive organs (Fig. 3): the ovipositor is clearly developed, a distinct cuticular intima is not yet recognisable. It already features

distinct eugenital lobes. The unpaired portion of the distal genital tract now appears as two distinct portions, distally the vagina and proximally the uterus. The vagina appears as a hollow structure protruding antero-ventrad from the dorsal rim of the invaginated ovipositor. Its walls are of the same density as the walls of the ovipositor. The lumen of the vagina is not yet continuous with that of the oviducts and uterus (Figs 3b, e). The latter two have wall epithelia of lesser density than the vagina. The paired oviducts are clearly visible, as hollow structures, taking a course from the base of the proximal wall of the ovipositor laterad along the latero-ventral body wall and again mediad to merge with the tissue surrounding the ovary (Fig. 4). The ovary does not show thighs, yet a single layer of previtellogenetic oocytes encompasses the central zone of the medulla and radially organised small cells.

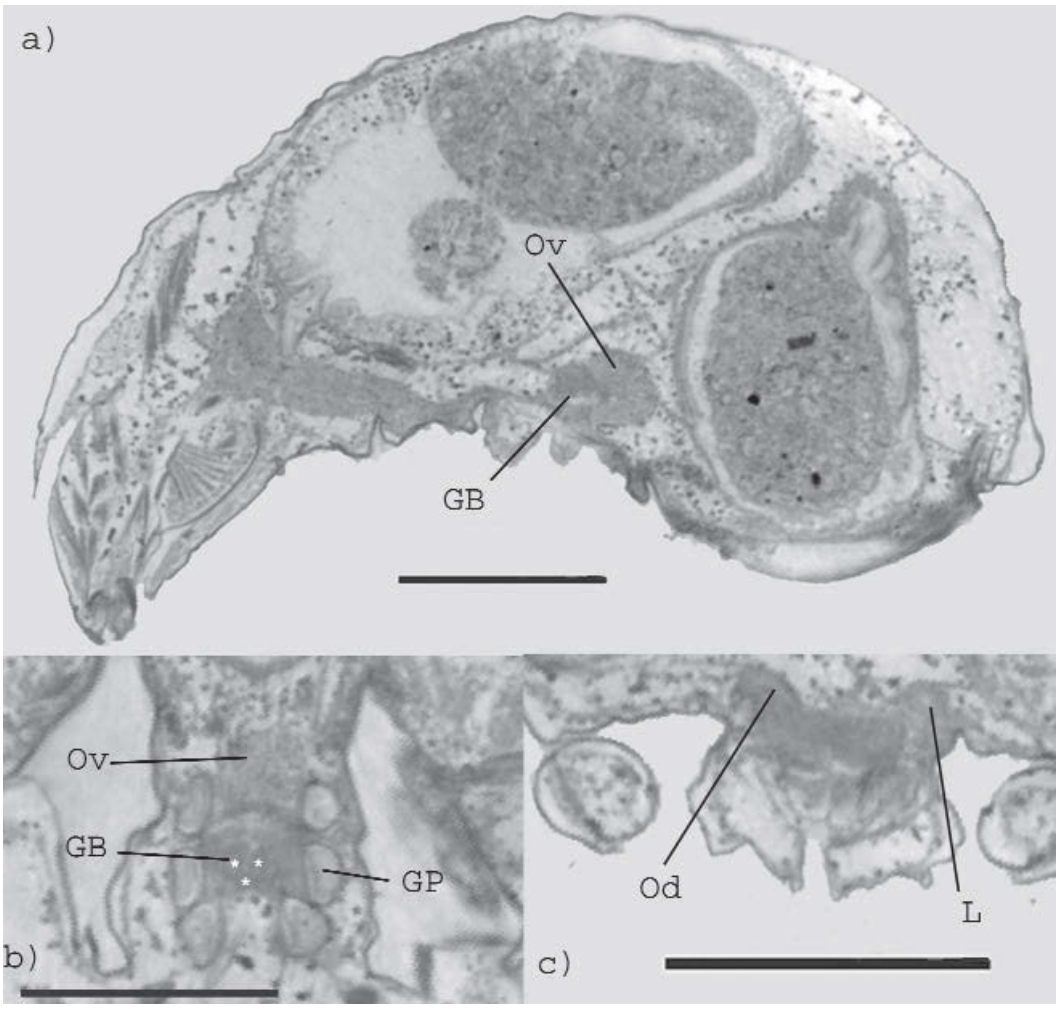

Fig. 1 Tritonymph, two days after moulting. Virtual holotomographic slices, a: sagittal plane; b: horizontal plane; c: cross-sectional plane. The genital bud invaginates into the opisthosoma, the central part shows already the development of eugenital lobes (indicated by white asterisks in b). Oviducts are already being formed as hollow tubes from the rim of the genital bud. The ovary is still undifferentiated. Abbrevations: GB: genital bud; GP: genital papilla; L: lumen of oviduct; Od: oviduct; Ov: ovary. Scale bar = 100 µm.

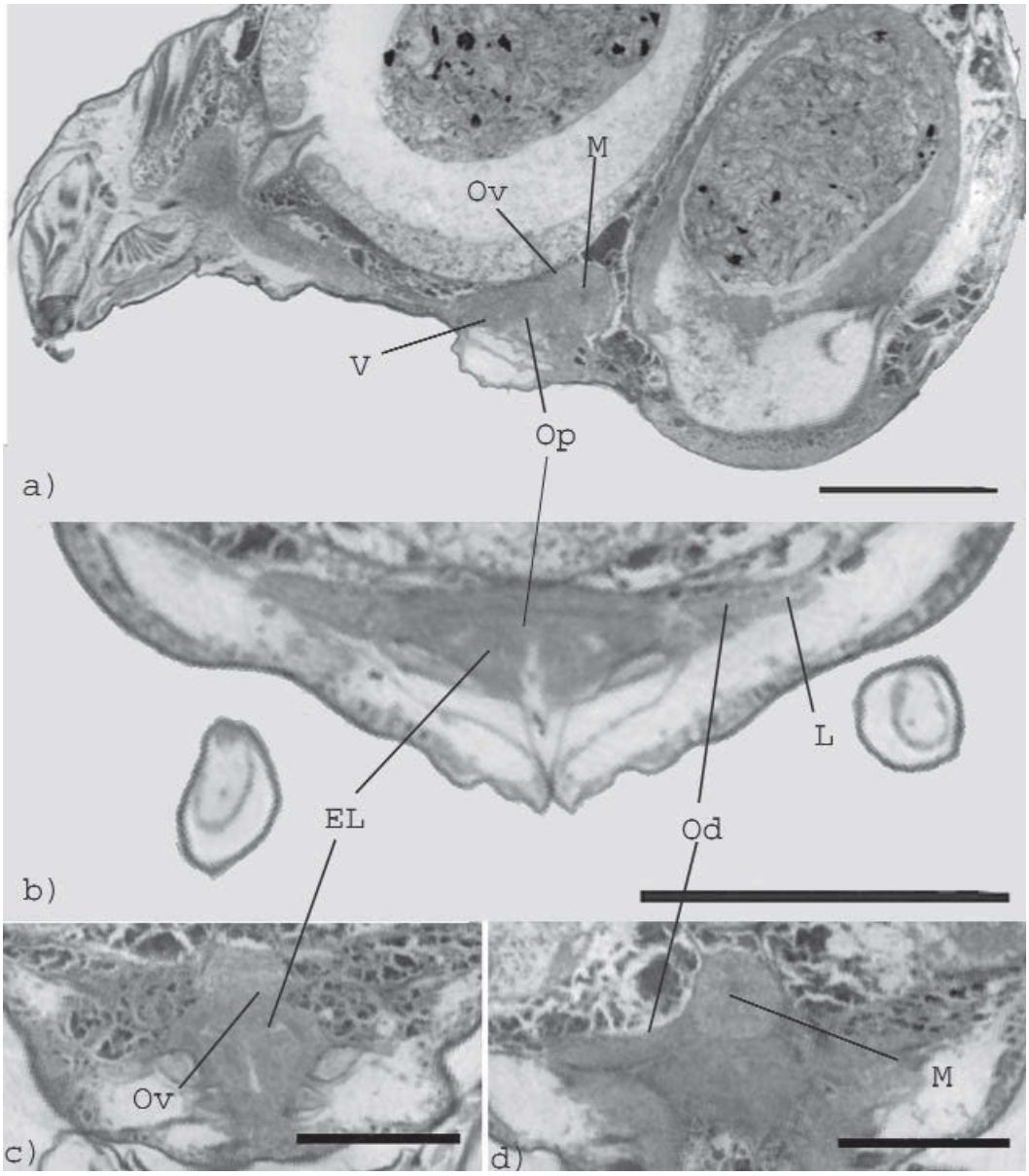

Fig. 2 Tritonymph, five days after moulting. Virtual holotomographic slices: a: sagittal plane; b: cross-sectional plane; c: horizontal plane, close to ventral body wall; d: horizontal plane, more dorsally than c). Ovipositor already with differentiated eugenital lobes, oviducts with free lumen making contact to ball-shaped ovary. No oocytes have started previtellogenesis. EL: eugenital lobes; L: lumen; M: medulla; Od: oviduct; Op: ovipositor; Ov: ovary; V: vagina. Scale bar = 100 μm.

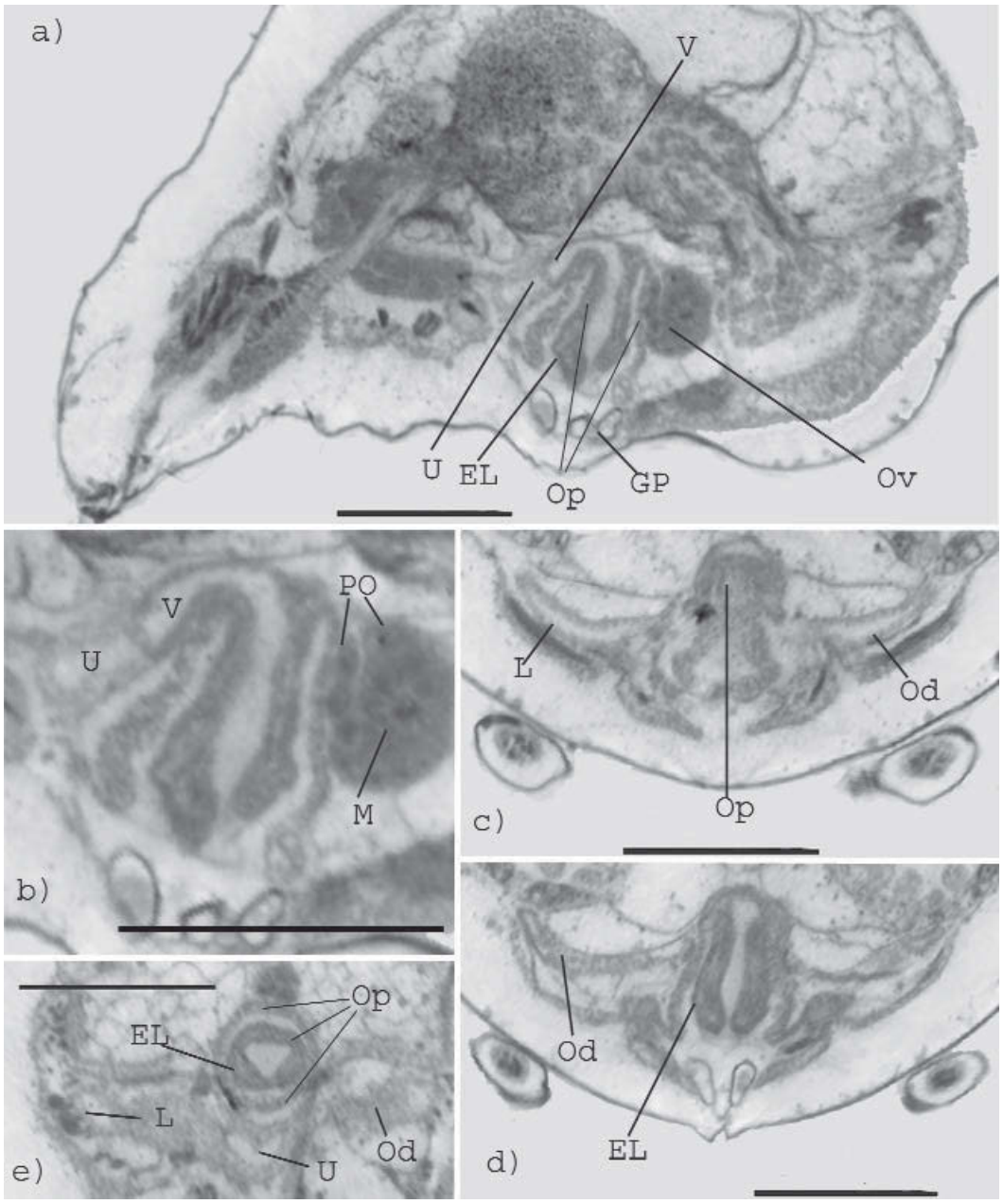

Fig. 3 Tritonymph from the quiescent phase prior to moulting to the adult. Virtual holotomographic slices. a: sagittal plane; b: detail from Fig. a; c: cross-sectional plane, just anterior of genital papilla I; d: cross-sectional plane at genital papilla II; e: horizontal plane. Ovipositor fully developed with three eugenital lobes, yet still lacking a discernible cuticle. Ovary ball shaped, at its periphery are oocytes starting previtellogenesis. Vagina, uterus and oviducts with free lumen. EL: eugenital lobes; GP: genital papilla; L: lumen; M: medulla; Od: oviduct; Op: ovipositor; Ov: Ovary; PO: previtellogenetic oocyte; U: uterus; V: vagina. Scale bar = 100 μm.

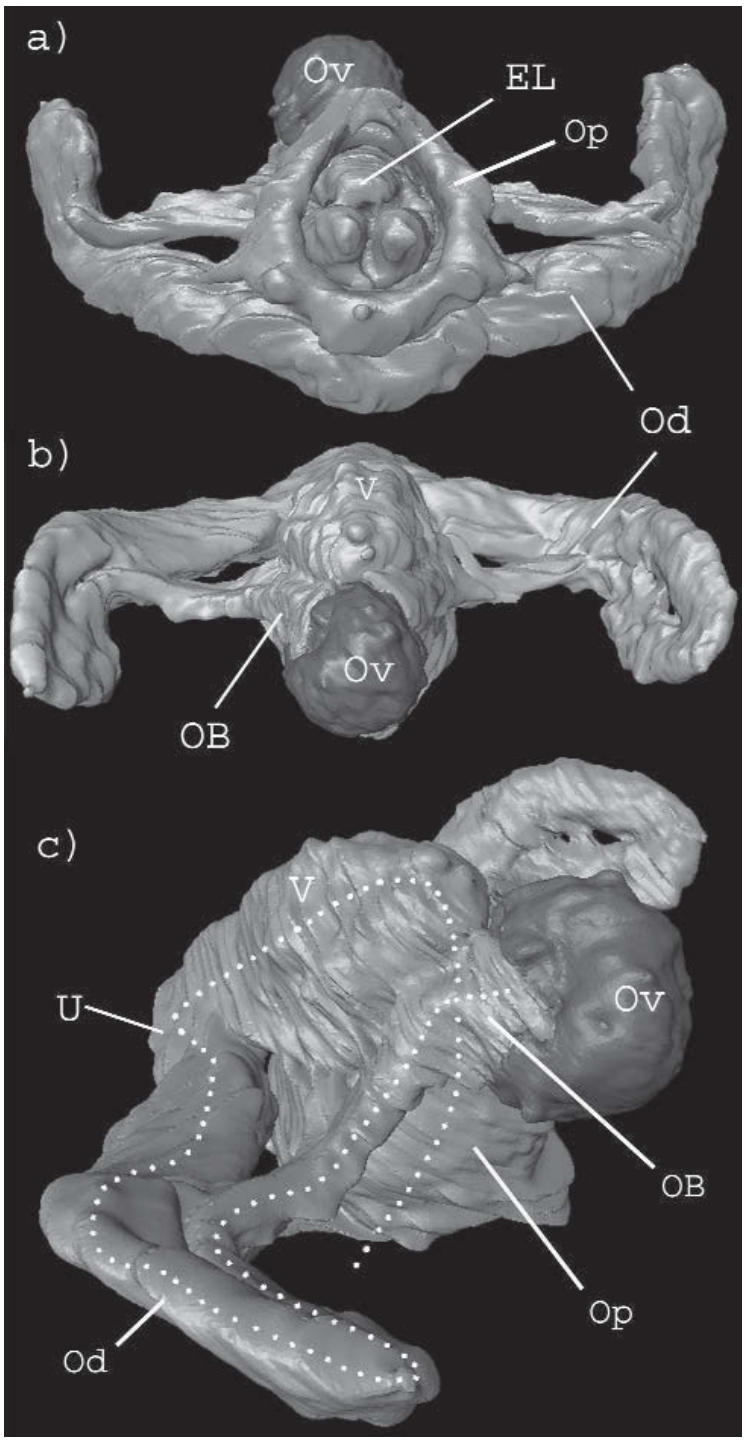

Fig. 4 Tritonymph from the quiescent phase prior to moulting to the adult. Surface model of the genital system, modelled from holotomographic data. a: ventral view; b: dorsal view; c: postero-dorso-lateral view. Ovipositor with three eugenital lobes continues to unpaired uterus and vagina, which leads into paired oviducts. These show lateral S-curvature and make contact to the ball-shaped ovary via ovarial bulbs. Dotted line depicts course of left half of the genital tract. EL: eugenital lobes; OB: ovarial bulb; Od: oviduct; Ov: ovary; Op: ovipositor; U: uterus; V: vagina.

3.2. Adult, on first day after moulting

Phase-enhanced tomography of an adult 24 h after moulting clearly shows the duplicated, corrugated wall of the ovipositor, cuticular structures of the eugenital lobes as well as their setae (Figs 5a, b, c). The epithelium lining the inner surface of the ovipositor continues into the vagina, which proceeds anteriorly ventrad towards the base of the ovipositor, in the region of genital papilla I. The epithelium lining the inner surface of the ovipositor shows no sign of a cuticular intima proximal to the base of the eugenital lobes in µCT scans. It continues into the vagina, which actually forms the inner surface of the proximal portion of the ovipositor in its extended state. The walls of the vagina are of a wrinkled appearance, relatively thin (approx. 5 µm) and envelope a clearly distinguishable lumen (Figs 5a, b, c).

The vagina continues proximally into the uterus from which the paired oviducts take their course close to the ventro-lateral body wall of the idiosoma (Fig. 6). The distal parts of the oviducts show no discernible lumen, but resemble the vagina lining in terms of tissue structure. They project latero-anad until they reach the vicinity of the point where the thighs of the ovary take a turn posteriorly (Fig. 6b). At this point, the proximal oviducts slightly bend anad, accompanying the row of the most distal oocytes until they make contact with the ovary, i.e. the distalmost oocyte and its sheath, forming the ovarial bulb of the oviduct (Woodring & Cook 1962, see discussion). The proximal oviducts, especially the ovarial bulb, show thick walls of a wrinkled, compressed appearance and a clearly discernible, smoothly outlined lumen (Figs 5c, d).

The unpaired ovary is located posterior to the invaginated, cuticular ovipositor. The central zone of the ovary is a distinctive ball-shaped structure. This structure is radially symmetrical in any plane of sectioning. It is surrounded by a layer of previtellogenetic oocytes enveloped in a thin layer of a follicular epithelium. Previtellogenetic oocytes and follicular epithelium form a pair of rostro-laterad protrusions or ‘thighs’ of the ovary, with the oocytes in triple to double row proximally and in single row distally (Fig. 5c). The follicular epithelium wrapping the previtellogenetic oocytes appears smooth and is not continuously discernible in µCT. At this developmental stage, no oocytes have so far reached the oviduct or even accumulated visible yolk vesicles or a vitelline envelope (Fig. 5). The oocytes are spherical, 20 – 30 µm in diameter and possess large, irregularly shaped germinal vesicles (~8 µm) containing a single dense mass of chromatin. The central zone surrounding the medulla shows numerous small, dense nuclei, but no cell borders were discernible in µCT.

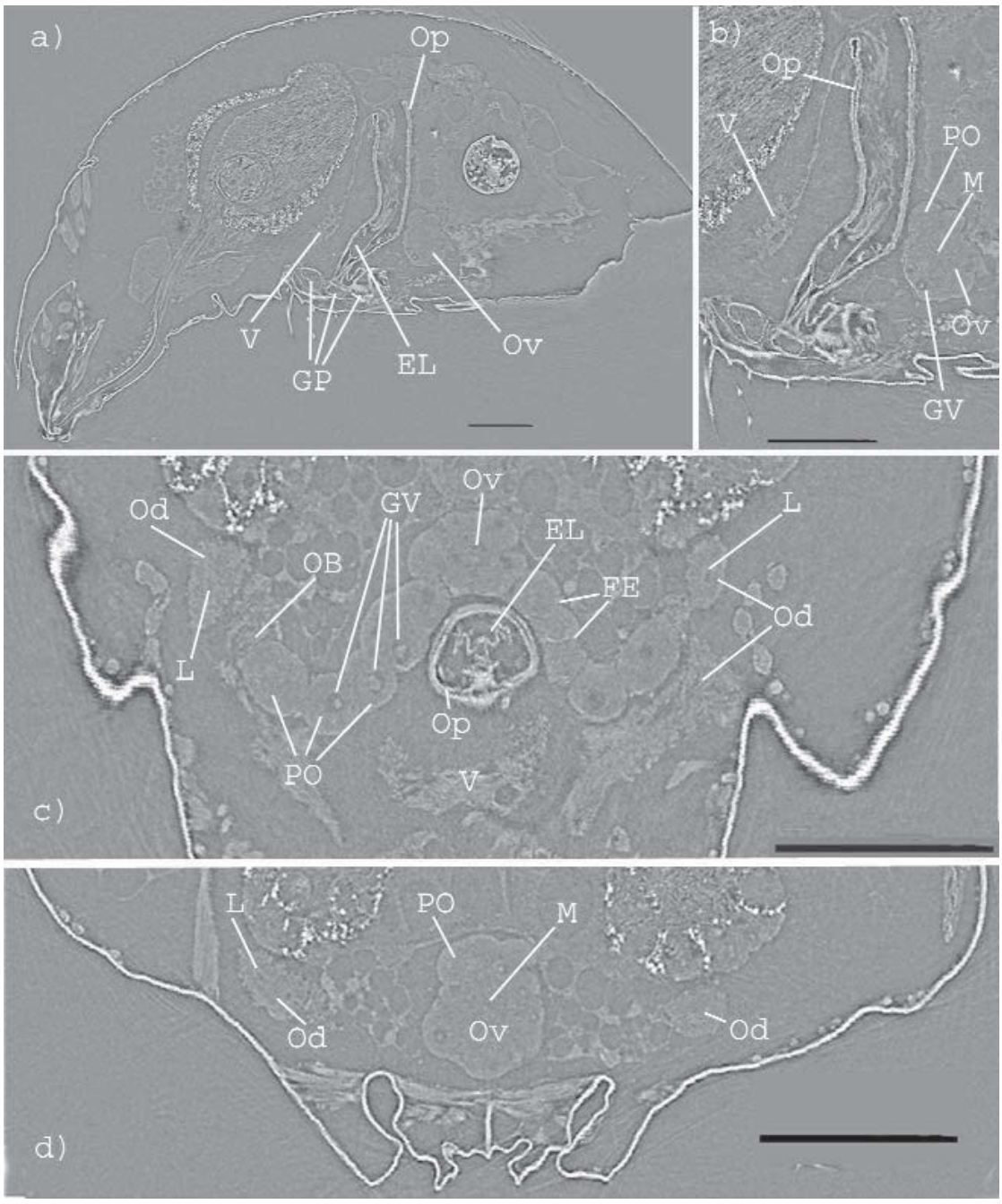

Fig. 5 Adult one day after moulting. Virtual slices from phase-enhanced tomography a: sagittal plane; b: detail from Fig. a; c: horizontal plane; d: cross-sectional plane. Numerous oocytes are in previtellogenesis and show large germinal vesicles. These cells are wrapped in smooth, thin follicular epithelium and form lateral thighs of the ovary. None has started vitellogenesis or passed to the lumen of the oviduct at the ovarial bulb. The oviducts are restricted to an area close to the ventral body wall and are still relatively short. Their walls are thick and have a wrinkled appearance. A fine lumen is smoothly outlined. EL: eugenital lobes; FE: follicular epithelium; GP: genital papilla; GV: germinal vesicle; L: lumen; M: medulla; OB: ovarial bulb; Od: oviduct; Op: ovipositor; Ov: ovary; PO: previtellogenetic oocyte; V: vagina. Scale bar = 100 μm.

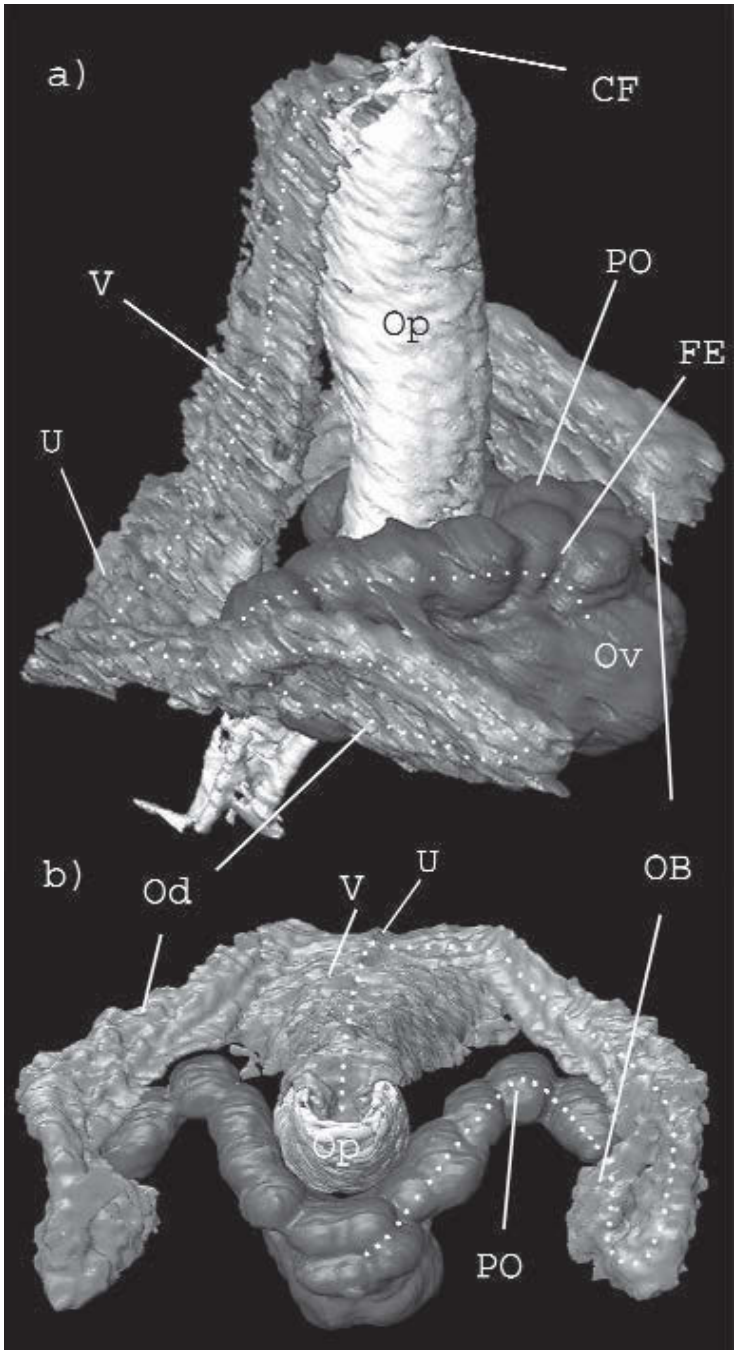

Fig. 6 Adult, one day after moulting. Surface model of the genital system, obtained from phase-enhanced tomographic data: a: dorsolateral view; b: dorsal view. Previtellogenetic oocytes tightly wrapped in follicular epithelium form the lateral thighs of the ovary. These protrude latero-rostrad and then turn to latero-anad. They are contacted by the ovarial bulb of the oviducts, which lead to the vagina. The vagina can be seen to continue into the lining of the ovipositor at the circular fold. The distal portion of the ovipositor bearing the eugenital lobes is situated inside the proximal portion visible in this surface model (see Figs 5a, b, c; 7) Dotted line depicts course of genital tract (left side in Fig. a, right side in Fig. b). CF: circular fold; FE: follicular epithelium; OB: ovarial bulb; Od: oviduct; Op: ovipositor; Ov: ovary; PO: previtellogenetic oocyte; U: Uterus; V: vagina.

3.3. Adult, on fifth day after moulting

The oviducts of a female collected five days after moulting to the adult contained six developed eggs, packed with large, dense yolk vesicles and wrapped in a smooth eggshell (Figs 7b, 8). The analysis of the exact course of eggshell formation, i.e., the origin and formation of vitelline membrane and chorion was not subject of this study, yet resembles closely the situation described by Witalińsky (Witalińsky 1986, 1993. See discussion for further detail).

The eggs considerably stretch the oviduct wall, up to the limit of spatial resolution of the scans (0.7 μm), bulging into the surroundings. Hence, there is some amount of free lumen between and even around the eggs, indicating the presence of an acellular substance that appears in semithin sectionings (Figs 8, 11c). In comparison to freshly moulted adults, the oviduct is shifted to a more dorsal position in the animal, up to the level of the caeca and opisthosomal glands. The most proximal parts of the genital tracts show a peculiar S-curvature (rostrad – anad – rostrad) before reaching dorso-rostrad between the epithelium of the caeca and the body wall (Figs 7b, 8). The eggs are positioned towards the distal part of the paired oviducts, not entering the uterus or vagina and leaving the proximal part of the oviduct with the ovarial bulb yet unstretched and free of eggs (Fig 8). Duplicatures of the wall epithelium form one median and two lateral flap or valve-like structures, providing a marked distinction between the lumina of the paired oviducts, that of the vagina and a short, unpaired section that may represent the uterus (Figs 7a, c, 14). The epithelium forming the distal wall of this section, including the flap at the base of the vagina appears thicker than that of the proximal wall, which in holotomographic scans resembles the thin wall of the oviduct (Fig. 7). A dense substance covers the internal surface of the thicker part of the epithelium that connects to the vagina at the flap mentioned before (Fig. 7a, c). All eggs within the oviduct have completed vitellogenesis and show a rigid eggshell. The ovarial bulb marks the point where the anad curving part of the ovarial thighs contacts the most proximal part of the oviduct. Holotomographic virtual slices show the tissue of the ovarial bulb to be of different structure and considerably less dense than the adjacent follicular epithelium surrounding the vitellogenetic oocytes (Fig. 9). Vitellogenetic oocytes occupy the distal part of the ovarial thighs. No free lumen is visible between or around the densely packed cells in this region. The oocytes show a progression in the accumulation of yolk and at the same time, the formation of the eggshell. Their shape is irregular, occupying virtually all available volume. To a lesser extent, this also applies to the previtellogenetic oocytes in the ventrally adjacent proximal thigh of the ovary (Fig 7b).

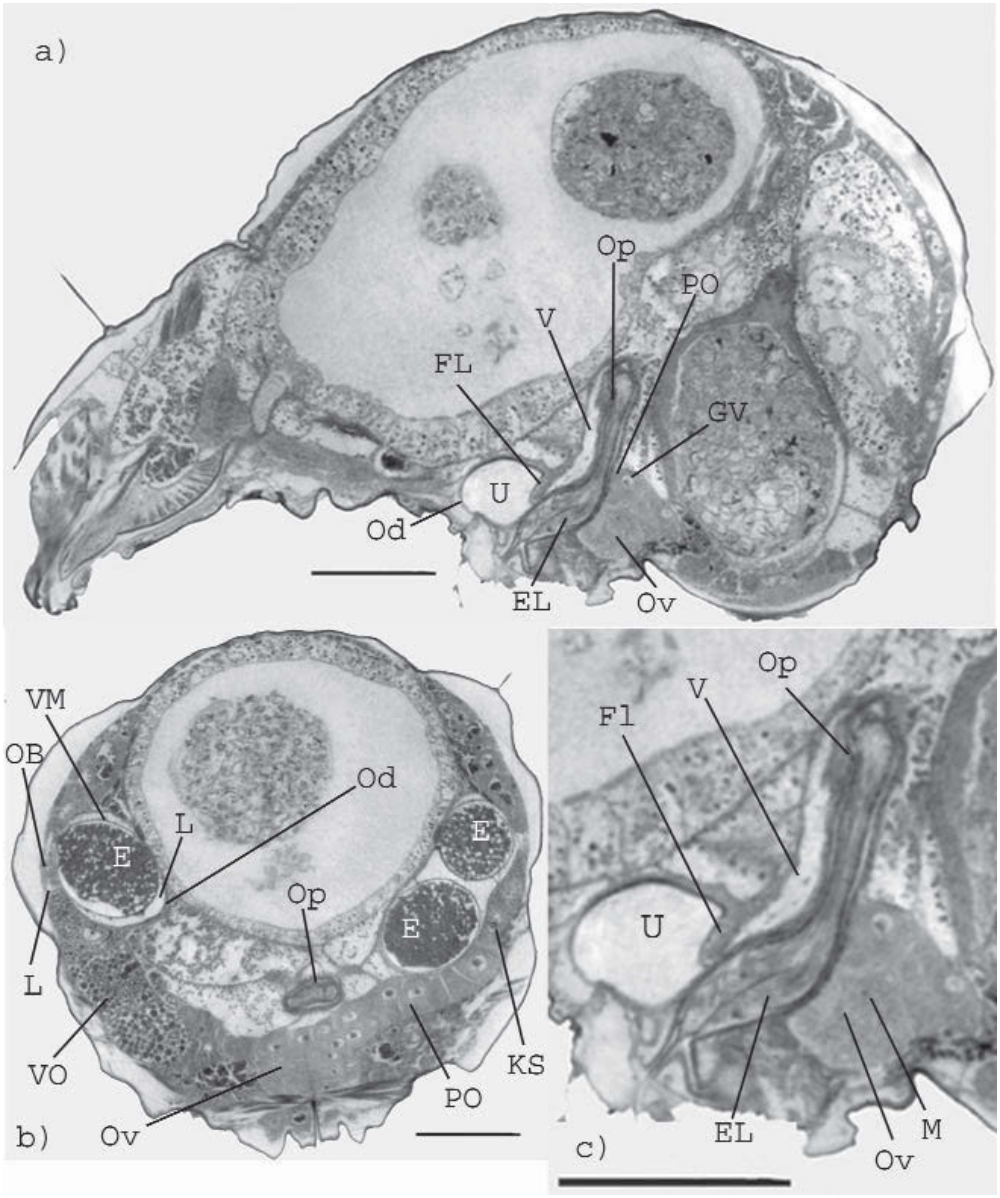

Fig. 7 Adult, five days after last moulting. Virtual holotomographic slices: a: sagittal plane; b: cross-sectional plane; c: detail from Fig. a. Ovary with thighs of previtellogenetic and vitellogenetic oocytes wrapped in thin follicular epithelium (not visible). Karyospheres visible in germinal vesicles as dense, hollow structures. Oviducts stretched and filled with developing eggs. The lumen of the uterus is separated from that of the vagina by a flap-like fold of the wall. Free lumen around chorionated eggs. Folded into the proximal portion of the ovipositor, cuticular structures of the eugenital lobes and their setae are visible, as indicated in a and c. E: egg; EL: eugenital lobes; Fl: flap between uterus and vagina; GV: germinal vesicle; KS: karyosphere; L: lumen; M: medulla; OB: ovarial bulb; Od: oviduct; Op: ovipositor; Ov: ovary (central part); PO: previtellogenetic oocyte in proximal thigh of ovary; U: uterus; V: vagina; VM: vitelline membrane; VO: vitellogenetic oocyte in distal part of ovary. Scale bar = 100 μm.

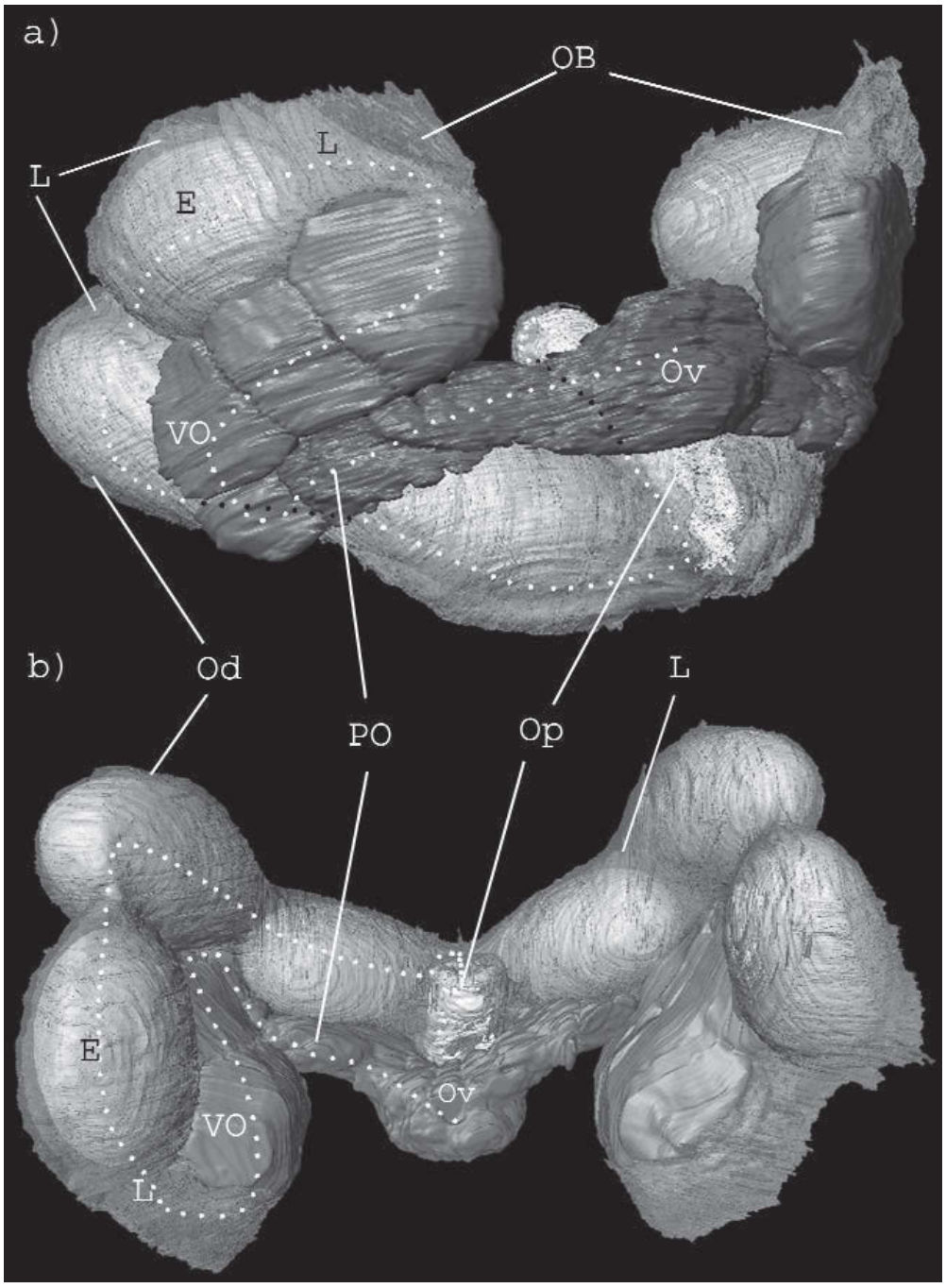

Fig. 8 Adult, five days after last moulting. Surface model of the genital system, obtained from holotomographic data: a: ventrolateral view; b: dorsal view. Four vitellogenetic oocytes and three eggs on either side of the genital tract. The eggs stretch the oviduct walls, yet a free lumen is visible around eggs, and especially in the ovarial bulb, as eggs are collected in the distal part of the oviduct. No eggs have entered the vagina. Dotted line depicts course of the left side of the genital tract. E: egg; L: lumen; Od: oviduct; Op: ovipositor; Ov: ovary (central part); PO: previtellogenetic oocyte; VO: vitellogenetic oocyte.

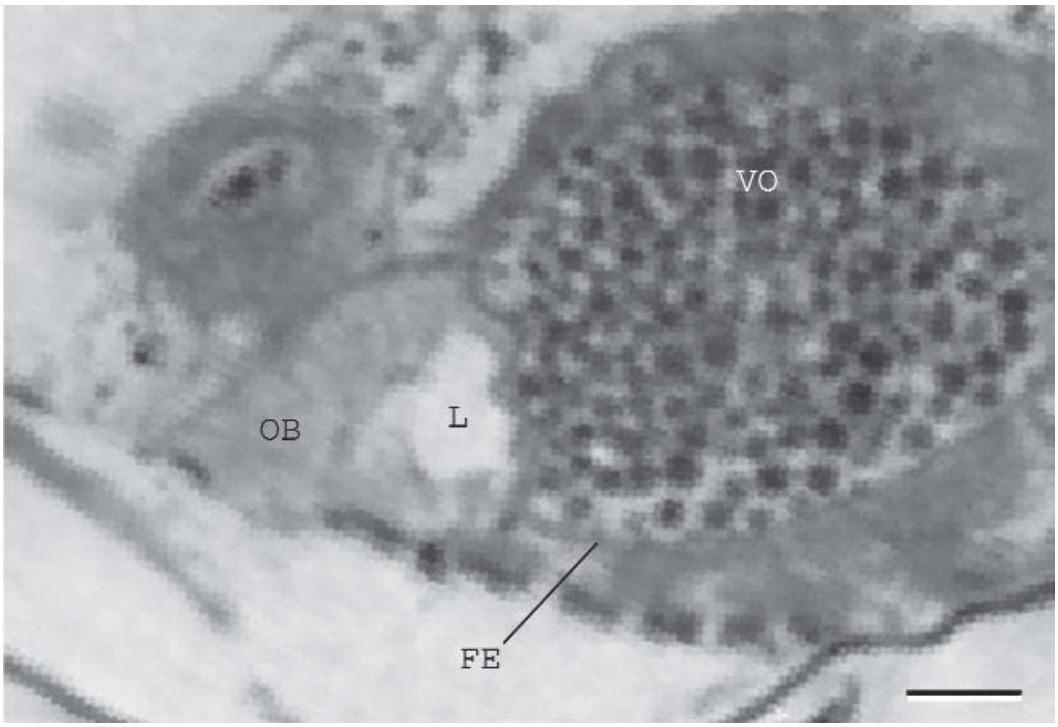

Fig. 9 Adult, five days after last moult. Magnified virtual holotomographic slice. Resolution of image is resolution of scan (0.7 μm). Detail of ovarial bulbs contacting distalmost vitellogenetic oocytes. Note difference in tissue density and structure between OB and FE. L: lumen; OB: ovarial bulb; FE: follicular epithelium; VO: vitellogenetic oocyte. Scale bar = 10 μm.

3.4. Ovarial thighs and oviduct wall

Preparations of the genital tract indicated a continuous resilient lining, and only loose contact to surrounding organs. No oocytes or eggs were found in the open haemolymph space, and developing eggs show a gradual tanning of the eggshell, indicating ongoing development and / or modification of the eggshell (Fig. 10).

A 3-D-model of the thigh of the ovary obtained from semithin sectionings of a prepared genital tract showed the previtellogenetic oocytes tightly wrapped in flattened follicular cells that extend between the individual oocytes (Fig. 11a). Previtellogenetic oocytes start as a single layer surrounding the central zone of the ovary. While they grow in volume and their nuclei develop into a large germinal vesicle with a prominent nucleolus, two ‘streams’ of these cells reach out laterally to form the ovarial thighs. Within the thighs, the ‘stream’ of germ cells narrows down to a single file of previtellogenetic oocytes, one after another (compare Fig. 7b). In this region, dense material likely to be chromatin condenses to a hollow structure within the germinal vesicle, indicating the presence of a karyosphere (Figs 5, 7b, 10, 12, 15). Germinal vesicles of previtellogenetic and vitellogenetic oocytes largely occupy a central position. The follicular cells form a delicate layer of less than 1 μm in thickness, separating the germ cells from the surrounding nutritive tissue as well as from each other

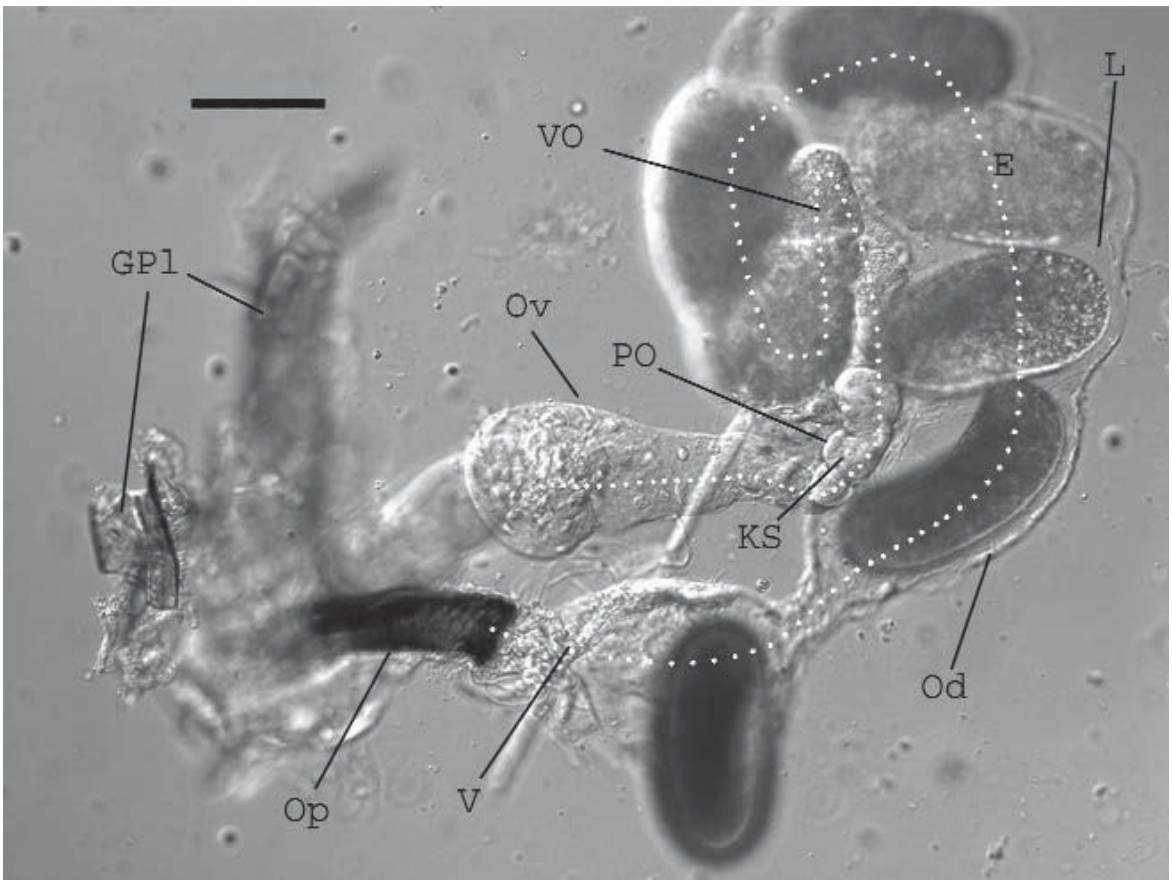

Fig. 10 Adult female. Differential interference contrast. Genital tract, obtained by dissection of fresh specimen. Left branch of oviduct removed. Lumen of oviduct visible around chorionated eggs. Eggshells gradually tanned towards vagina. Previtellogenetic oocytes with karyosphere and vitellogenetic oocytes in S-curvature of genital tract. Dotted line depicts course of genital tract. E: egg; GPl: genital plate; KS: karyosphere; L: Lumen; Od: oviduct; Op: ovipositor; Ov: ovary (central part); PO: previtellogenetic oocyte; V: vagina; VO: vitellogenetic oocyte. Scale bar = 100 μm.

(Figs 11a, b). The follicular cells fill up the spaces between previtellogenetic oocytes and extend proximally to the central zone, that consists of the medulla and radially organised strains of small, polygon cells delineated by lighter staining, nucleus-free cords that originate from the medulla. No cell borders could be traced within the medulla (Figs 5, 12). The cords seem to connect individual cells to the medulla. No tubular arranged tissue or contingent lumen could be observed in this part of the genital tract (Fig. 12). A free lumen appears for the first time, when a change in tissue structure also marks the beginning of the oviduct at the ovarial bulb (Fig. 13).

Fig. 15 gives a schematic overall impression of the features in the adult genital system of *A. longisetosus*.

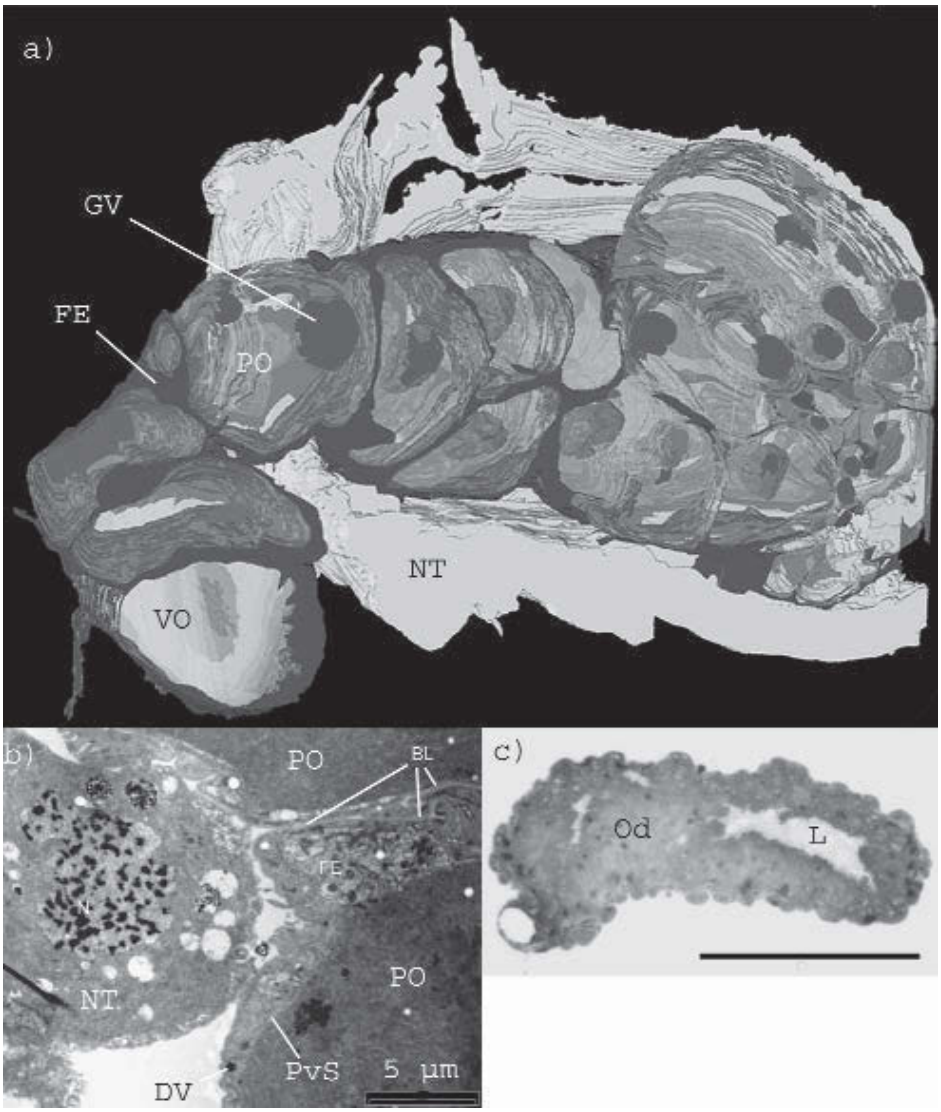

Fig. 11 Adult female. Sections of embedded genital tract obtained from dissection of fresh specimen: a: surface model of the left lateral thigh of the ovary, obtained from semithin sections. The follicular epithelium wraps the individual oocytes and leaves no lumen. Its thickness increases with the onset of vitellogenesis. Germinal vesicles are large and in central position. The ovary is surrounded by nutritive tissue. Previtellogenetic oocytes form a 'stream' that narrows to a single file of oocytes distally. Oocyte surfaces set to transparency; b: TEM micrograph of the same specimen, from the region of the proximal border of the cell labelled 'PO' in Fig. a. Follicular epithelium comprising of flattened cells with folded basal lamina. Distal oocyte developing perivitelline space. Its follicular cells with dense vesicles. c: Semithin sectioning of the same object from an empty part of the oviduct. The oviduct wall appears folded, granulose and vacuolated with rough surfaces. The lumen is filled with an amorphic substance that stains lightly. Stain: Richardson.

BL: basal lamina; DV: dense vesicles; FE: follicular epithelium; GV: germinal vesicle; L: lumen; N: nucleus; NT: nutritive tissue; Od: oviduct; PO: previtellogenetic oocyte; PvS: perivitellin space; VO: vitellogenetic oocyte. Scale bar = 50 µm.

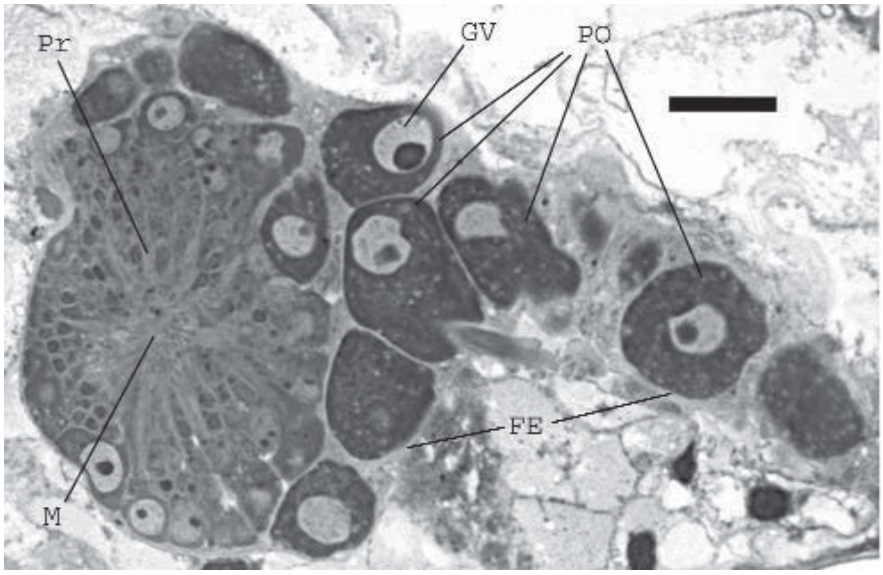

Fig. 12 Adult female. Semithin section of the ovary. Central zone with medulla and radially arranged protrusions and small cells, periphery with previtellogenetic oocytes wrapped in follicular epithelium. These oocytes develop germinal vesicles and reach out to form the lateral thigh of the ovary. FE: follicular epithelium; GV: germinal vesicle; M: medulla; PO: previtellogenetic oocytes; Pr: protrusions of medulla. Slice thickness: 0.5 μm, Stain: toluidine blue / basic fuchsine. Scale bar = 20 μm.

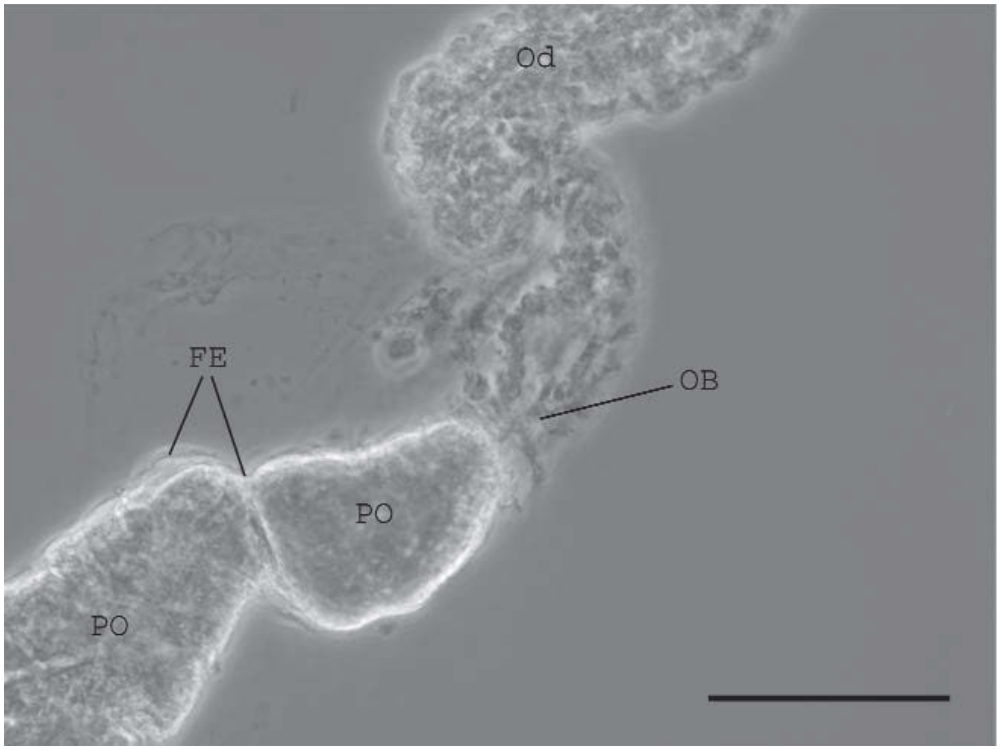

Fig. 13 Adult female. Genital tract, obtained by dissection of fresh specimen. Previtellogenetic oocytes wrapped in smooth follicular epithelium and the ovarial bulb as the beginning of the oviduct. Note differences in tissue structure. PO: previtellogenetic oocyte; FE: follicular epithelium; Od: oviduct; OB: ovarial bulb. Light microscopy; phase contrast. Scale bar = 50 μm.

4. Discussion

In general, the results fit in with the data already available on the gross anatomy of the female reproductive organs of oribatid mites (Michael 1884, Woodring & Cook 1962, Baker 1985, Witaliński 1986, Taberly 1987a, b, c, Witaliński et al. 1990, Witaliński 1993, Heethoff et al. 2007a). All studies cited above describe a massive, unpaired ovary medioventrally in the opisthosoma, connected to paired, tube-like oviducts that converge into an unpaired uterus that leads to an unpaired vagina just anterior to the base of the ovipositor. Differences exist as to what processes take place within which part of the system, as will be discussed below. Ultrastructural analysis as well as additional developmental data are still needed for a sound discrimination of organs and tissues forming the genital duct, especially for the nature of the transition from follicular epithelium to the oviduct wall and from the oviduct wall to the vagina. At the current state, developmental data from younger adult and tritonymphal stages suggest that the paired oviduct forms simultaneously with the unpaired vagina and the invaginating ovipositor at the ventral body wall. As μCT did not reveal a cuticular intima of the ovipositor or the vagina with certainty, ultrastructural analysis of developmental stages is needed to trace the boundary between the tissues forming the unpaired portions of the genital tract, i.e. uterus, vagina and ovipositor. Density differences suggest different physiology that, on the basis of developmental data available so far, may coincide with a different origin of the tissue forming the uterus and vagina, as has already been noted by Taberly 1987b (Fig. 3). The flap-like structure at the basis of the vagina described for the adult would be part of the section of the genital tract that also forms the vagina as well as the roof of the uterus lumen, whereas the rest of the uterus together with the flaps that separate its lumen from those of the oviducts would share its properties and / or origin with the oviduct walls. A structure separating the vagina from the uterus and the uterus from the oviducts is not yet described for the genital systems of any oribatid mite. Its functional significance could lie in proving a guidance system that coordinates the transmission of eggs from the left and right oviduct into the vagina, together with the constrictions between oviducts and uterus (Fig. 14). This is further indicated by the fact that eggs seem to be passed down to the most distal part of the oviducts first, leaving the proximal part free, yet do not enter uterus nor vagina instantaneously (Fig. 8). Histological confirmation of contractile elements would make it possible to develop a functional model of the instant of oviposition. Previtellogenetic and vitellogenetic oocytes show large germinal vesicles in a more or less central position and distinct karyospheres, except for a small portion of cells around the curvature from the proximal rostrad to the distal anad section of the ovarial thigh (see Laumann et al. in this issue). The localisation of vitellogenesis in a lateral s-curvature of the genital duct resembles the situation shown by Walzl et al. (2004) for Astigmata, which possess paired ovaries.

While in certain species structures are reported that suggest a paired predecessor for the generally unpaired oribatid ovary, like the bi-lobated ovary of *Xenillus tegeocranus* (Warren 1947, cited in (Taberly 1987b), or germaria arranged in a two-by-two fashion as in *Hafenrefferia gilvipes* (Witaliński 1986), no traces of a paired origin were found in studies on *Ceratozetes cisalpinus* (Woodring & Cook 1962), or mites of the group Desmonomata, like *Plathynothrus peltifer, Trhypochthonius tectorum* (Taberly 1987b) and *A. longisetosus* (this study).

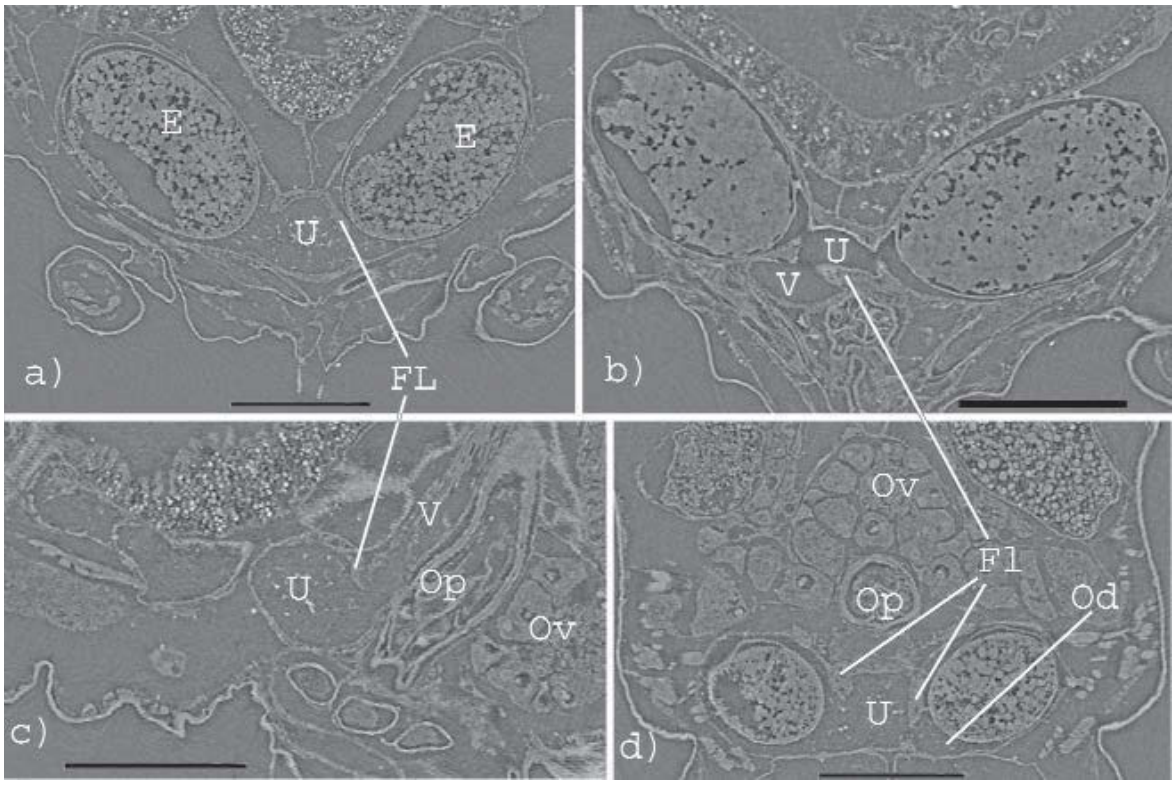

Fig. 14 Adult females, various specimens. Virtual slices of phase-enhanced tomography: a: transversal plane, adult of unknown age, flaps between oviducts and uterus; b: transversal, adult five days after last moult, flap between uterus and vagina; c: Sagittal plane, adult of unknown age, flap between uterus and vagina; d: horizontal plane, flaps between oviducts and uterus. Flap-like structures marking the boundaries between oviducts, uterus and vagina. Fl: flaps; Od: oviduct; Ov: ovary; Op: ovipositor; U: uterus; V: vagina. Scale bar = 100 μm.

A double sheathing of the ovary by a tunica propria and peritoneum, as reported for *C. cisalpinus* (Woodring & Cook 1962) or *Hydrozetes* sp. (Baker 1985) was not revealed in *A. longisetosus*. The authors, however, give no notice as to the nature of these layers. In accordance with Taberly (1987b) and Witaliński (1986), a thin follicle epithelium could be traced by the help of LM and TEM reaching from the periphery of the central zone to the tip of the ovarial thighs closest to the ovarial bulb. This ovarial bulb is described by Woodring & Cook 1962 as the slightly swollen part of the oviduct that makes contact to the ovary, i.e. the sheating of the oocytes. The follicular epithelium is delimited against the haemolymph space by a fine basal lamina, folded in intricate patterns (Fig. 11b). So, potentially the terms 'peritoneum' and 'tunica propria' are mere synonyms for the follicular epithelium and its basal lamina (Shatrov 2002), or may include the surrounding, putatively nutritive (Alberti et al. 2003) tissue. In the developing genital tract, the growing oviduct makes contact as an ovarial bulb at first with the developing central zone of the ovary (Fig. 4b). Later the developing previtellogenetic and vitellogenetic oocytes grow in paired extensions of the ovary, apparently following the path of the dorso-caudad retreating oviducts. Taberly (1987b, c) stated that previtellogenetic oocytes are tightly wrapped in follicular epithelium, and are therefore still being enclosed in the ovary, where vitellogenesis begins. The oocytes are then

passed into the lumen of the oviduct, and the completion of vitellogenesis and choriogenesis take place in the oviduct in *T. tectorum* and *P. peltifer*. In *A. longisetosus*, the follicular epithelium is involved in vitellogenesis and eggshell formation. Although no detailed analysis of eggshell formation was undertaken in this study, the situation found closely resembles the results published by Witaliński (1986) for *H. gilvipes*. Upon formation of a perivitelline space, dense vesicles appear in the follicular epithelium, while a granular vitellar membrane starts to build up and yolk vesicles accumulate and grow in a centripetal manner, as described for Astigmata by Walzl et al. 2004 (Fig. 11b). As described by Witaliński (1986) for *H. gilvipes*, these processes take place in the distalmost parts of the ovary. In discriminating ovary and oviduct, we adopt the nomenclature used by Taberly (1987b), based on the presence of a lumen and differences in tissue structure between follicular epithelium and oviduct wall. In all adult specimens of *A. longisetosus* analysed so far, this demarcation coincides with the presence of smooth, developed eggs in final size that seem to have completed vitellogenesis and the eggshells of which seem to have been already deposited and are merely subject to tanning. As the reported impermeability of the vitelline envelope (Aeshlimann & Hess 1984, Yastrebtsov 1992) would hinder communication between the egg and the mother, this demarcation would probably also mark the beginning of the following generation as separate biological units (Witaliński 1993). Yet this leaves the structure referred to as ‘ovary’ with two easily distinguished subdivisions: First, the central zone, consisting of the medulla and radially arranged nutritive cords and small cells, which have a flower-like appearance in cross sections.

Second, the zone of previtellogenetic and, in the case of *A. longisetosus*, vitellogenetic oocytes tightly wrapped in follicular epithelium and forming two paired lateral extensions (thighs) that reach out along the lateral body wall in rostrad orientation in its proximal and anad orientation in its distal part.

To avoid further confusion, a solution could be to propose useful, not mistakable terms for these subdivisions. With reference to their overall appearance in light microscopy and μCT, the latter portion could be denominated the ‘Meros’ of the ovary (as Greek ‘ό μερός’ = ‘thigh, hip; part, side’), the first one ‘Rhodoid’ (as Greek ‘τό ροδον’ = ‘rose’) (Fig. 15). The term ‘Rhodoid’ does not interfere with ‘Germarium’, as it refers to the persisting structure solely and does not imply the existence of oogonia, which has been denied by Taberly (1987c) for adult specimens of *T. tectorum* and *P. peltifer*.

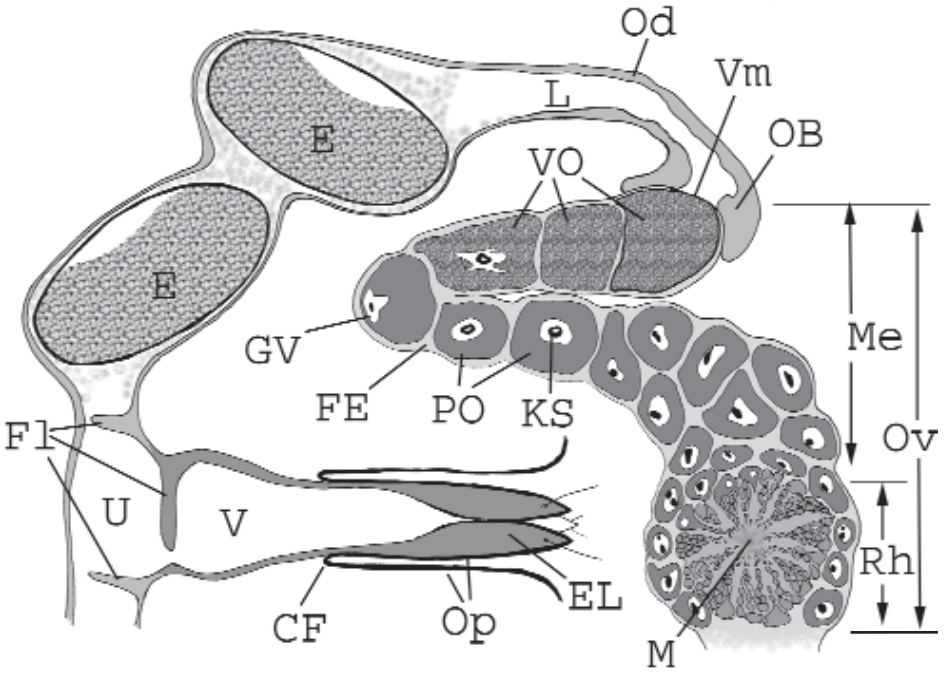

Fig. 15 Schematic drawing of the genital system of an adult female of *A. longisetosus*. The general arrangement of the major subdivisions is shown along with anatomical features mentioned in this work. Right portion only. CF: circular fold; E: egg; EL: eugenital lobes; FE: follicular epithelium; Fl: flaps, subdividing the genital tract; GV: germinal vesicle; KS: karyosphere; L: lumen; M: medulla; Me: meros (thigh) of the ovary; OB: ovarial bulb; Od: oviduct; Op: ovipositor; Ov: ovary; PO: previtellogenetic oocyte; Rh: rhodoid (central part) of the ovary; U: uterus; V: vagina; VM: vitellar membrane; VO: vitellogenetic oocyte.

5. Acknowledgements

We thank Daniela Weide and Sebastian Schmelzle for help with synchrotron measurements and data processing, Julia Straube and Karl-Heinz Hellmer for technical assistance, Petra Föttinger and Reinhart Schuster for kind support of information on oribatid anatomy and Roy A. Norton for useful comments on oribatid anatomy and for providing animals to start a hatchery.

This work was supported by the European Synchrotron Radiation Facility Project SC2127 through allocation of beam time. We acknowledge the excellent support by Lukas Helfen at the ESRF facilities, beamline ID19.

Paavo Bergmann is supported by ‘Evangelisches Studienwerk Villigst e.V.’ through a PhD grant.

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Accepted 28 May 2008

5.3 Publication 3

Bergmann, P., M. Laumann and M. Heethoff (2010). "Ultrastructural aspects of vitellogenesis in *Archegozetes longisetosus* Aoki, 1965 (Acari, Oribatida, Trhypochthoniidae)". — Soil Organisms **82**(2): 193–208.

Direct link

SOIL ORGANISMS Volume 82 (2) August 2010 pp. 193–208

ISSN: 1864 - 6417

Ultrastructural aspects of vitellogenesis in *Archegozetes longisetosus* Aoki, 1965 (Acari, Oribatida, Trhypochthoniidae)

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Abstract

We studied the process of vitellogenesis in the oribatid mite *Archegozetes longisetosus* using light and electron microscopy. Both vitellogenesis and the formation of the first egg shell occur within the meroi of the ovary, starting after detachment from the medulla and completing with ovulation of the egg into the oviductal lumen at the ovarial bulb. Numerous microvilli appear on the surface, and abundant endocytotic pits and coated vesicles occur in the peripheral plasma of the oocyte. Accumulation of fatty yolk does not precede accumulation of proteineous yolk. Differentiation of ooplasm and formation of a perivitelline space beneath a continuous follicular epithelium were observed. Vitelline envelope material appears to be uniform. We compare details of vitellogenesis and propose a classification of the ovarian type as panoistic and the vitellogenesis as exogenic in *A. longisetosus*.

Keywords: mite, oogenesis, yolk, transmission electron microscopy, microvilli, ovary

1. Introduction

The vast diversity of the Acari is reflected by their diversity of reproductive modes and their associated processes such as oogenesis, vitellogenesis and hormonal regulation (Norton et al. 1993). In the last few years, great advances towards a unifying model for vitellogenesis and its hormonal regulation in mites have been achieved (James & Oliver 1999, Cabrera et al. 2009), though the body of basic knowledge still seems heavily biased with respect to the anactinotrichid Ixodidae (ticks), with their life histories greatly diverging from those exhibited by Actinotrichida (Cabrera et al. 2009). Thus, the general model of the evolutionary history of reproduction in mites yet remains unsure, partly due to unresolved phylogenetic issues in such an old clade (Dunlop & Alberti 2008). Within Actinotrichida, studies of oogenesis, albeit seldomly regarding the molecular background, have been undertaken in Trombidiformes (Alberti 1974, Witte 1975, Witaliński 1986, Shatrov 1997, Di Palma & Alberti 2001), Astigmata (Heinemann & Hughes 1970, Walzl et al. 2004), Brachypylina (Woodring & Cook 1962, Baker 1985, Witaliński 1986) and Desmonomata (Taberly 1987a,b, Smrž 1989).

One species from the group of Desmonomata, the thelytokous trhypochthoniid *Archegozetes longisetosus* Aoki, 1965, however, received much attention during recent years as a potentially valuable model organism for a variety of issues, making it the most intensely studied oribatid mite so far (Alberti et al. 2003, Smrž & Norton 2004, Heethoff et al. 2007, and cited references).

We studied the structural aspects of vitellogenesis in *A. longisetosus* by means of light and electron microscopy. Previous studies on the genital system of this species (Bergmann et al. 2008) revealed its composition and yielded first results regarding the time course and location of oogenetical processes. Oocytes start development in a round, radially organised region termed rhodoid. These cells are connected to a central medulla via radially arranged protrusions. Cells detach from the medulla and in the course of oogenesis, including meiosis (Laumann et al. 2008), form paired extensions of the ovary, termed meroi (singular: meros). These are folded in a proximal portion, oriented rostrally and a distal portion dorsally of the proximal and oriented caudally (Fig. 1).

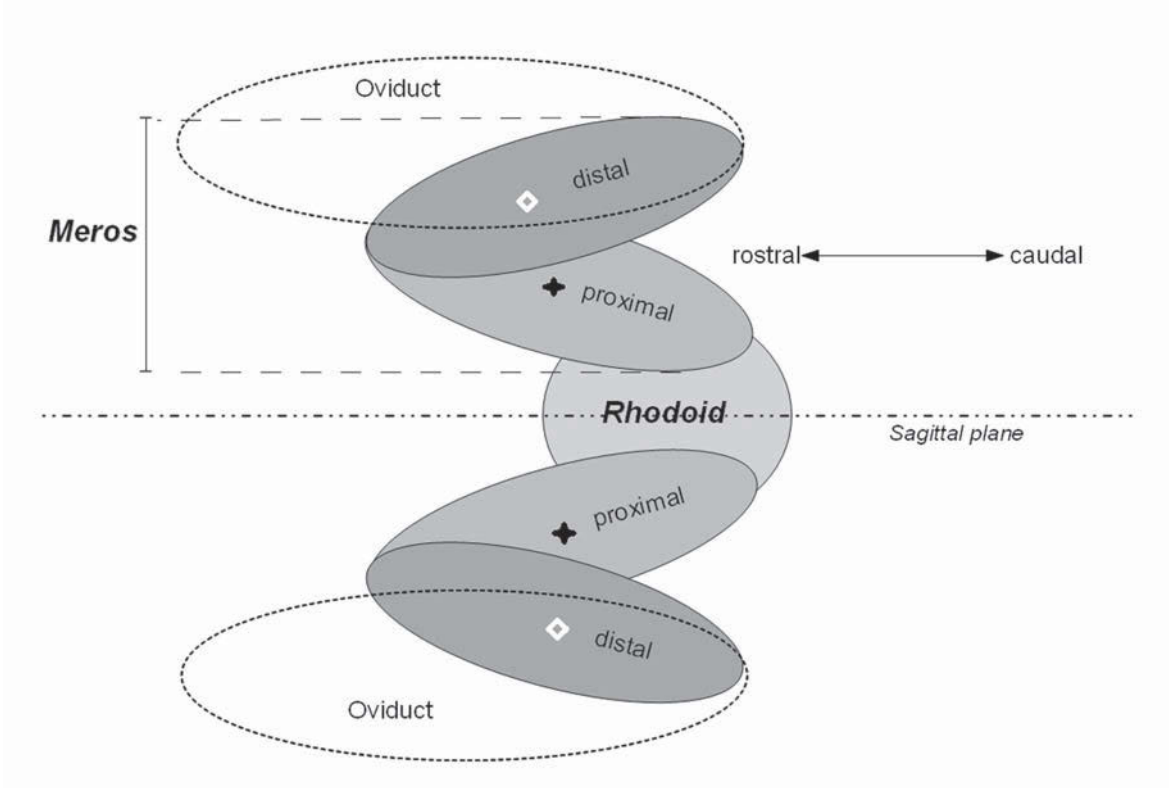


Fig. 1 Schematic representation of the major subdivisions of the ovary in *A. longisetosus* and their spatial arrangement in dorsal view. Black asterisks mark location of previtellogenesis. White diamonds mark location of vitellogenesis, i.e. the vitellarium.

The transition from previtellogenesis to vitellogenesis and the simultaneously ensuing deposition of egg shell material coincides with the passing of the oocytes into the distal part of the meros, as has been previously shown (Bergmann et al. 2008). The oocytes within the ovary are wrapped in and separated from each other by one to few layers of flattened follicular cells (Bergmann et al. 2008).

Here, we show that vitellogenesis is exogenic, with the simultaneously deposited vitelline envelope staying porose until its completion. The oocytes exhibit signs of intense communication with surrounding tissues throughout oogenesis, e.g. a dense microvilli fringe and abundant coated pits and coated vesicles. While microvilli are especially abundant during the early stages of vitellogenesis, coated pits dominate in later stages. Differentiations of the ooplasm are observed during the simultaneous accumulation of proteineous and fatty yolk. We adress differences as well as similarities to existing studies of oogenesis, and describe the ovarian type as panoistic.We further discuss the possible role of follicle cells and surrounding tissues during vitellogenesis and prospects of future research.

2. Materials and methods

2.1. Rearing

Specimens were taken from our laboratory culture of *Archegozetes longisetosus* ran (Heethoff et al. 2007), a lineage established from a single female from Puerto Rico by Roy Norton in 1993, and presently hatched in various laboratories all over the world.

Molting aggregations of immobile individuals were removed from the culture and placed in the wells of tissue culture plates (Tissue Culture Cluster, Nunc, Roskilde, Denmark) for further development. Wells were filled with Plaster-of-Paris:charcoal (6:1) mixture. The plates were kept in constant dark; air humidity was kept at 90 % and temperature at 20 °C.

The wells were checked daily and freshly moulted individuals where removed with a fine brush and transferred to new wells. In these, bark of various trees covered with green algae (mainly *Protococcus* spec.) was supplied as a food source. Adults were collected for further processing 3 and 5 days after hatching.

2.2. Light and electron microscopy

Specimens were submersed in Karnovsky´s fixative diluted in 0,05 M HEPES buffer to 1/3 strength (1.33 % formaldehyde; 1.66 % glutardialdehyde) with 4 % sucrose and 6.6 µM $MgSO_4$ added at pH 7,8 and 0 °C, and punctured with a fine needle (Norton & Sanders 1985), then transferred into 12 ml glass vials with conical bottom filled with fixative prechilled to 0 °C. Fixation time was 80 min at 0 °C and ~ 200 mbar.

The fixative was prepared using freshly depolymerised paraformaldehyde (Fluka, Buchs, Switzerland) and EM -grade glutardialdehyde 25 % (Science Services, Munich, Germany).

After rinsing three times for 10 min in HEPES 0.05 M at 0 °C, postfixation and en-bloc staining was conducted with 1 % OsO_4 and 0.54 % uranyl acetate in 0.05 M HEPES at pH 7.8 and 0 °C for 1h.

After rinsing for 10 min twice in 0.05 M HEPES at 0 °C, dehydration was carried out in a graded acetone series at 30 % and 50 % for 10 min each at 0 °C and at 70 %, 80 %, 95 % and 100 % for three times 10 min each at room temperature. Samples were gradually infiltrated (acetone / resin mixture 3:1 for 90 min and 1:1 for 60 min on a vibratory plate at 100 rpm, 1:3 in open vials overnight) and embedded in epoxy resin (Araldite CY212 Premix Kit, Plano Gmbh Wetzlar, Germany). Polymerisation was conducted at 60 °C for 48 h. Semithin (0.5 µm) and ultrathin (70 nm) sections were cut using a Reichert Ultracut (Leica-Jung, Vienna, Austria) microtome and diamond knives (Diatome 45°, Biel, Switzerland).

Semithin sections were stained with ferric haematoxyline and light microscopy (LM) was conducted with a Zeiss Axioplan light microscope. Photographs were processed in AxioVision 4.0 (Carl Zeiss, Oberkochen, Germany). Ultrathin sections for transmission electron microscopy (TEM) were 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.

3. Results

Two distinct phases were observed during oogenesis. After their detachment from the medulla of the rhodoid, the oocytes migrate to the proximal part of the meros, situated latero-rostrad. Here, the first phase of autogenous growth, multiplication of mitochondria and accumulation of numerous free ribosomes and polysomes ensues, which is termed previtellogenesis (Figs 1, 2). Vitellogenesis, indicated by the accumulation of yolk material from exogenous sources, ensues in the second part of the meros, situated dorsal of the proximal part and oriented caudally. Oocytes entering this part are cuboid, approximately 20–35 μm in diameter and, as a nucleus, contain a large central germinal vesicle with numerous nuclear pores and a prominent reticulate nucleolus. They stain basophilic in light microscopy, usually exhibiting a zone of even darker staining, the nuage, characteristic of previtellogenesis. In electron microscopy, they show a dense, finely grained cytoplasm containing numerous free ribosomes and polysomes, regions of well developed smooth endoplasmic reticulum (ER) containing an electron-lucent material and groups of mitochondria of the crista-type scattered throughout the cell volume.

An overview over a semithin sectioning of *A. longisetosus* is shown in Fig. 2. The follicular cells encompassing the individual oocytes at the onset of vitellogenesis possess a cytoplasm less electron dense than the oocytes and contain large, more or less flattened nuclei, with a single dense nucleolus and several heterochromatin bodies embedded in uniformly grained euchromatin. They generally contain several mitochondria and strands of rough and smooth ER as well as well-developed Golgi bodies. Follicular cells are delimited against the haemolymph space and surrounding tissues by a fine basal lamina (Fig. 3).

At the onset of vitellogenesis, a perivitelline space opens up between the oocyte and the surrounding follicular epithelium (Fig. 3). A coarsely grained, electron-lucent material is deposited in the perivitelline space, apparently constituting the first vitelline envelope. The oolemma develops densely arranged microvilli. These are of irregular shape and orientation, approximately 60–80 nm in diameter, and penetrate the vitelline envelope to come in close contact with the extensions of the follicular cells (Fig. 3).

With ongoing development, the cytoplasm of the oocyte shows an overall loss of basophily, as indicated by lighter staining with ferric haematoxyline (Fig. 2). Closer examination using electron microscopy reveals a complex development of cytoplasmic structures. First yolk vesicles appear in the cortical plasma (Fig. 4). The cytoplasm of the oocyte starts to differentiate into a dense portion, resembling that of the previtellogenetic oocytes, and a portion of reduced density and ribosome content (Fig. 5). The latter at first starts as a network of strands surrounding the germinal vesicle and, with growing total volume of the oocyte, develops into a voluminous zone of electron-lucent cytoplasm devoid of major organelles (Figs 5, 6). This lucent cytoplasm makes up for most of the oocyte volume, leaving areas of

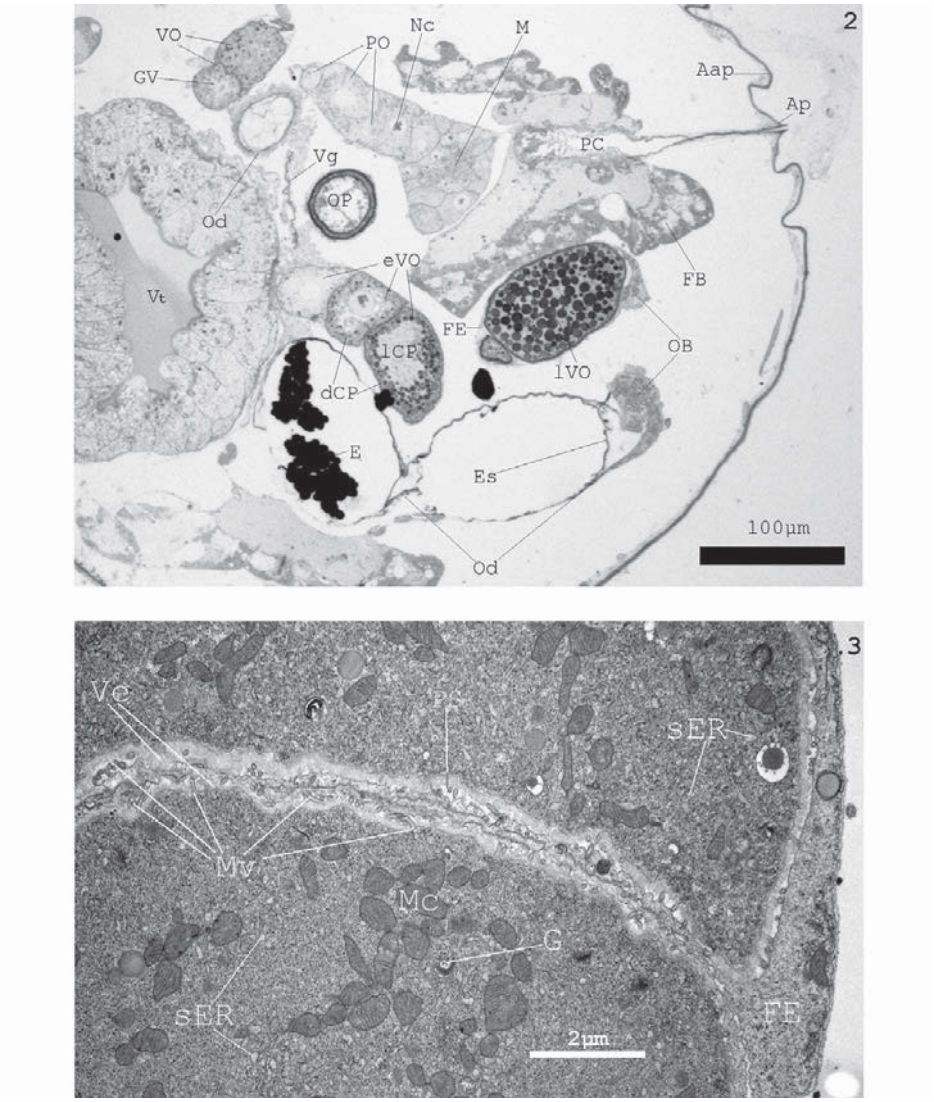
dense cytoplasm only in the vicinity of the germinal vesicle and along the cell periphery. Organelles important for the cell metabolism such as Golgi bodies, mitochondria and ER remain concentrated in this peripheral cytoplasm. Within this region, also new proteineous yolk vesicles and groups of electron lucent droplets, appearing pale greyish-yellow in light microscopy and likely representing lipids, start to emerge and develop independently of each other, generally showing a centripetal progression towards the centre of the oocyte (Fig. 2). Proteineous yolk vesicles appear simultaneously or slightly prior to lipid droplets. They invade the space occupied by the lucent cytoplasm, yet always stay embedded in and connected by strands of dense cytoplasm, leading to a voluminous central mass of yolk with a network of dense and lucent cytoplasm. Individual yolk vesicles start as medium-sized vesicles (1–3 μm), filled with a uniform material of medium electron density. With further development, they enlarge and a regular pattern of higher density starts to emerge from the uniform content. Fully developed yolk vesicles measure 7–10 μm in diameter and contain a central mass of dense material arranged in a crystalline pattern on a relatively clear background, likely to resemble tightly packed yolk protein. One to several (up to ~5) individual crystallites may be present within one yolk vesicle. Towards the vesicle membrane, this mass is surrounded by a finely grained zone of medium electron density (Figs 12–15). On one side, between vesicle membrane and central mass, yolk vesicles generally show a conspicous disk-shaped cap of high electron density and uniformly grained structure (Fig. 9) that apparently emerges from an irregularly shaped precursor (Fig. 7). Lipid droplets develop either singularly or in groups between the proteineous yolk vesicles. They are apparently not surrounded by a phospholipid bilayer membrane. In late vitellogenesis, lipid droplets of about 0.5–1 μm in diameter occupy the space between yolk vesicles leaving only thin strands of dense and lucent cytoplasm in the centre of the oocyte.

The uptake of yolk precursors by the oocyte is indicated by the formation of a large number of coated pits at the base of the microvilli, being incorporated into the oocyte as coated vesicles measuring 120–140 nm in diameter (Fig. 7).

As the oocyte grows, the microvilli fringe gradually becomes less dense, yet more coated vesicles appear in the cell periphery (Figs 3, 7–9, 11).

The vitelline envelope is at the same time increasing both in thickness and apparent electron density, yet retaining its granular appearance. In the latest vitellogenic oocytes, the layer of vitellar envelope material is roughly 1 μm in thickness. In pores and channels not occupied by microvilli as well as in the remaining perivitelline space, a finely grained substance appears. This substance resembles the content of coated pits and coated vesicles found in these stages (Fig. 7). The follicular epithelium in middle vitellogenesis shows well developed Golgi bodies as well as rough and smooth ER and mitochondria with circular cross sections of their cristae in a very lucent ground cytoplasm (Figs 7, 8).

Towards the end of vitellogenesis, the follicular cells tend to lose contact to each other, leaving only the basal lamina as delimitation between the haemolymph space and perivitelline space (Figs 9, 10).

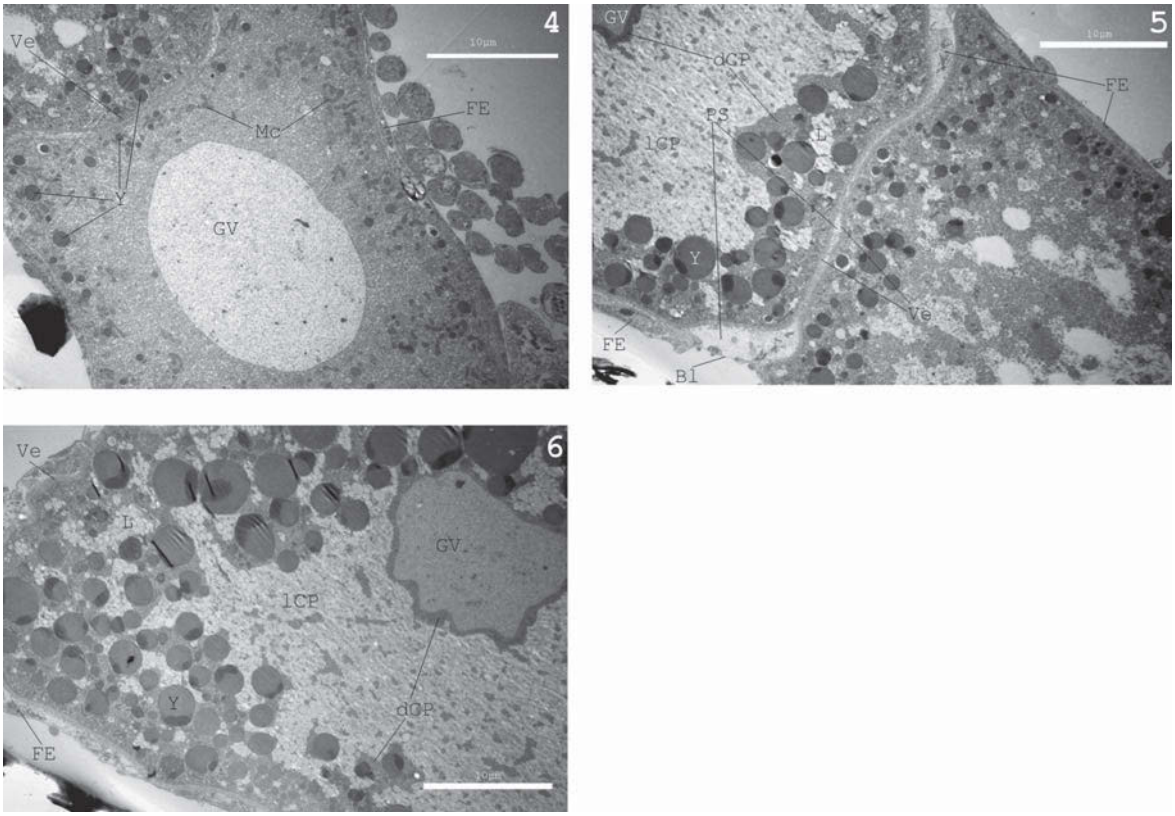


Figs 2–3 2: Light microscopy image of parafrontal semithin sectioning through adult *A. longisetosus*, stained with ferric haematoxyline. The plane of sectioning is slightly tilted to the right and close to the ventral body wall. All major parts of the genital system and oocytes in all major phases. Eggs break out of section due to insufficient fixation and infiltration through egg shell. Apparently empty spaces are artefacts of very large and delicate fat body cells being lost during the sample preparation.

Abbreviations: Aap: Adanal plate; Ap: anal plate; dCP: dense cytoplasm of vitellogenetic oocyte; E: remains of egg within oviduct (partly broken out of section due to improper infiltration via eggshell); Es: egg shell; eVO: early vitellogenetic oocytes; FB: fat body; FE: follicular epithelium; GV: germinal vesicle; lCP: lucent cytoplasm of vitellogenetic oocyte; lVO: late vitellogenetic oocyte; M: medulla; Nc: nucleolus of oocyte; OB: ovarial bulb; Od: oviduct; OP: ovipositor; PC: postcolon; PO: previtellogenetic oocyte; Vg: vagina; VO: vitellogenetic oocyte; Vt: ventriculus.

3: Transmission electron microscopy (TEM) image of ultrathin section from *A. longisetosus*. Proximal meros of an adult ovary. Two oocytes at the onset of vitellogenesis. Numerous microvilli and early vitellar membrane.

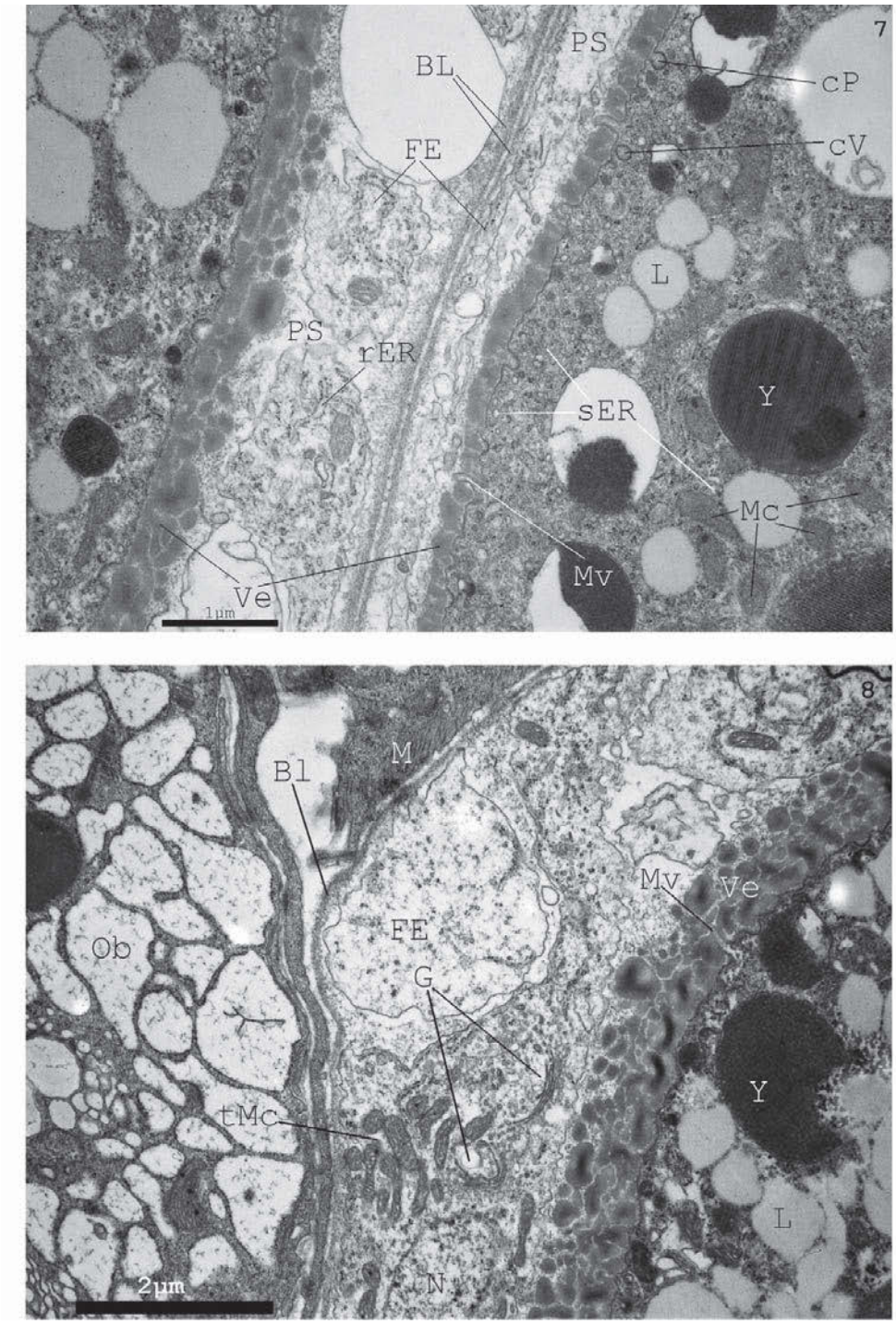
Abbreviations: FE: follicular epithelium; G: Golgi body; Mc: mitochondria; Mv: microvilli; sER: smooth endoplasmic reticulum; PS: perivitelline space; Ve: vitelline envelope.



Figs 4–6 4: TEM image of ultrathin section from *A. longisetosus*. Early vitellogenetic oocyte. Germinal vesicle in central position. Abbreviations: FE: follicular epithelium; GV: germinal vesicle; Mc: mitochondria; NP: nuclear pores; Ve: vitelline envelope; Y: yolk vesicles containing proteineous yolk.

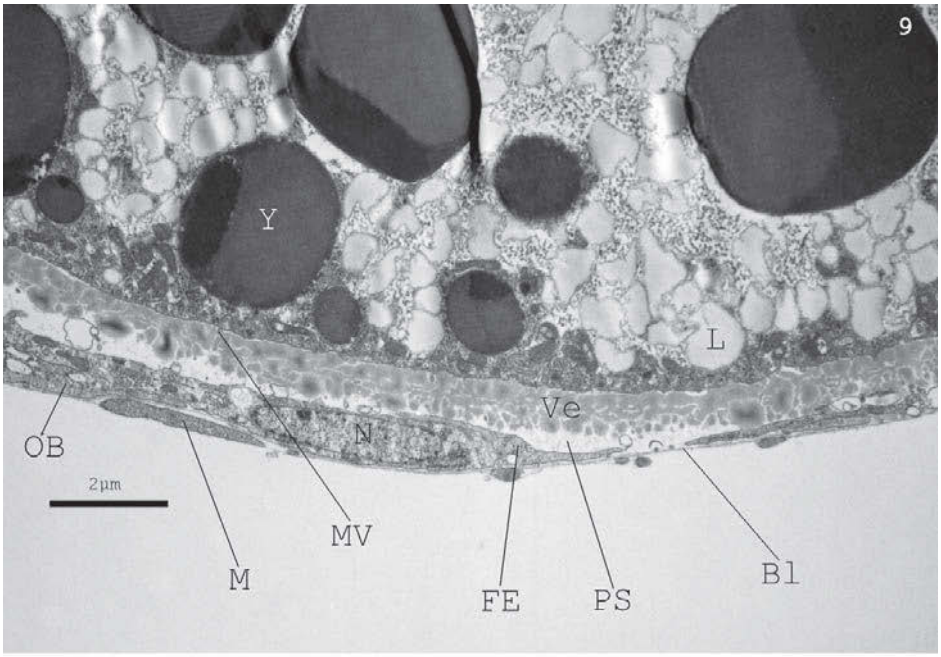
5: TEM image of ultrathin section from *A. longisetosus*. Oocytes later in vitellogenesis, adjacent to cell in Fig. 4. Abbreviations: BL: basal lamina; dCP: dense cytoplasm; FE: folicular epithelium; GV: germinal vesicle of oocyte in central position; L: lipid droplets; lCP: lucent cytoplasm; PS: perivitelline space; Ve: vitelline envelope; Y: yolk vesicle containing proteineous yolk.

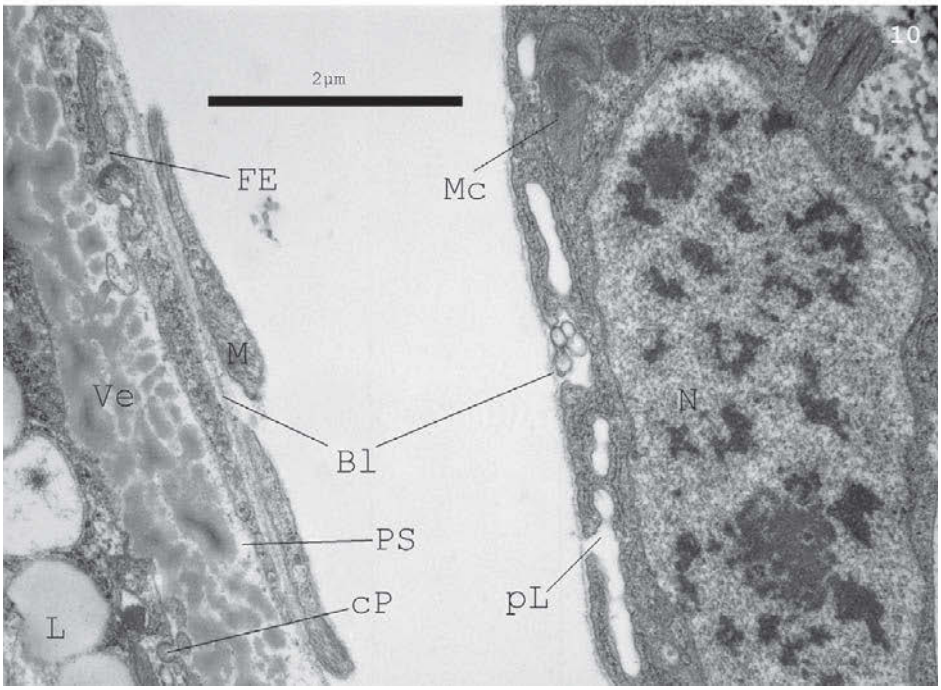
6: TEM image of ultrathin section from *A. longisetosus*. Oocyte still further in vitellogenesis. Figure adjacent to Fig. 5. Abbreviations: dCP: dense cytoplasm; FE: follicular epithelium; GV: germinal vesicle; L: lipid droplets; lCP: lucent cytoplasm; PS: perivitelline space; Ve: vitelline envelope; Y: yolk vesicle containing proteinous yolk. Note ‘caps’ of denser material.



Figs 7–8 7: TEM image of ultrathin section from *A. longisetosus*. Meros of ovary. Two oocytes in vitellogenesis and follicular epithelium. Younger oocyte to the right, older one to the left. Abbreviations: BL: basal lamina; cP: coated pit; cV: coated vesicle; FE: follicular epithelium; L: lipid droplet; Mc: mitochondria; Mv: microvilli; rER: rough endoplasmic reticulum; sER: smooth endoplasmic reticulum; PS: perivitelline space; Ve: vitelline envelope; Y: yolk vesicle containing proteinous yolk.

8: TEM image of ultrathin section from *A. longisetosus*. Follicular epithelium and oocyte in late vitellogenesis at tip of ovarian meros. Abbreviations: Bl: basal lamina; FE: follicular epithelium; G: Golgi body; L: lipid droplet; M: muscle strand; Mv: microvilli; N: nucleus of follicular cell; Ob: epithelium of ovarial bulb (oviduct); tMc: tubular mitochondria; Ve: vitelline envelope; Y: proteineous yolk vesicle.





Figs 9–10 9: TEM image of ultrathin section from *A. longisetosus*. Latest vitellogenesis at tip of ovarian meros, adjacent to the ovarial bulb of the oviduct. Abbreviations: Bl: basal lamina; FE: follicular epithelium; L: lipid droplet; M: muscle; Mv: microvillus; N: nucleus of follicular cell; OB: tissue of ovarial bulb; PS: perivitelline space; Ve: vitelline envelope; Y: proteineous yolk vesicle with characteristic dark 'cap'.

10: TEM image of ultrathin section from *A. longisetosus*. Oocyte in late vitellogenesis adjacent to extraovarian nutritive cell. Abbreviations: BL: basal lamina; cP: coated pit; FE: follicular epithelium; L: lipid droplet; M: muscle strand; Mc: mitochondrium; N: nucleus; pL: peripheral labyrinth; PS: perivitelline space; Ve: vitelline envelope.

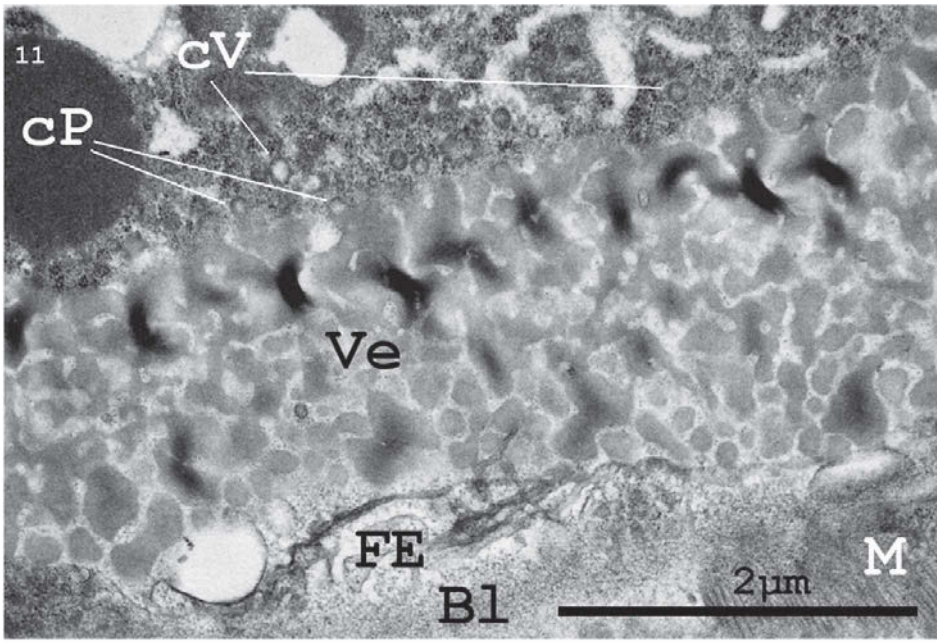
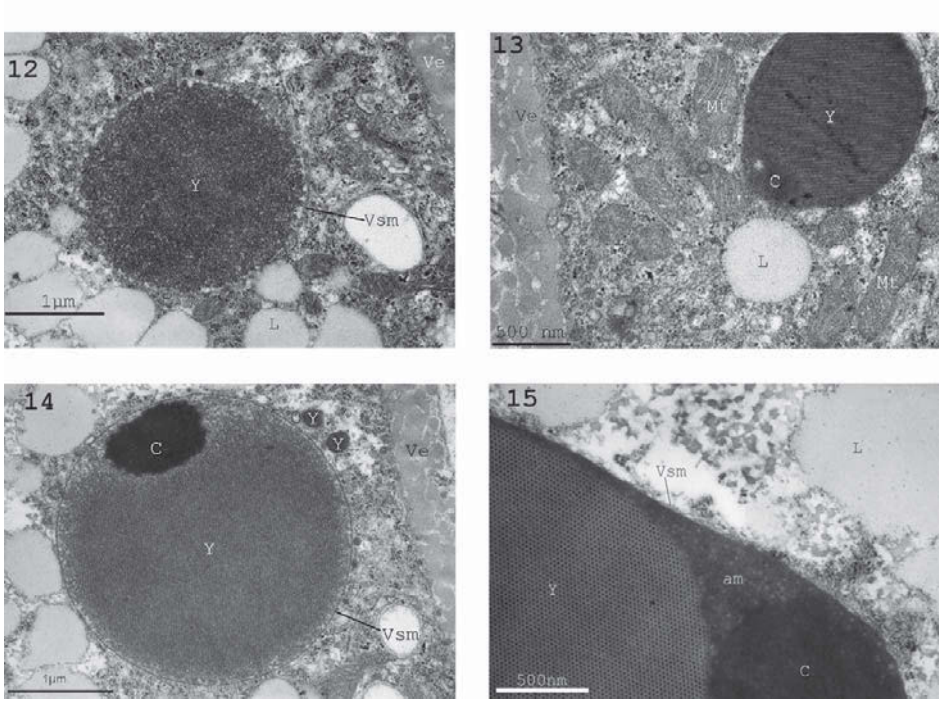


Fig. 11 TEM image of ultrathin section from *A. longisetosus*. Oocyte in late vitellogenesis. Vitellar envelope deposited, yet still porose. Microvilli mainly retracted, numerous coated pits and vesicles. Contact between adjacent follicular cells losened and perivitelline space only delimited against haemolymph by basal lamina. Abbreviations: Bl: basal lamina; cP: coated pits; cV: coated vesicles; FE: follicular epithelium; M: muscle; Ve: vitelline envelope.



Figs 12–15 TEM image of ultrathin section from *A. longisetosus*. Progression (12–15) of individual yolk vesicles and accumulation of crystalline yolk protein. Abbreviations: am: amorphous material in vesicles periphery; C: cap of amorphous material; L: lipid droplet; Mc: mitochondria; ;Ve: vitelline envelope; Vsm: vesicle membrane; Y: yolk protein.

4. Discussion

4.1. Classification of the ovary type

As was already noted before (Heethoff et al. 2007, Bergmann et al. 2008), the overall structure of the ovary in *A. longisetosus* is in accordance with the basic pattern for oribatids (Michael 1884, Alberti & Coons 1999). However, ultrastructural examinations reveal further anatomical and functional features to be discussed.

In contrast to the situation found in many Anactinotrichida, namely in cattle ticks (de Oliveira et al. 2005, Saito et al. 2005), the ovary seems to be structured in readily distinguishable functional regions in correspondance with the time course of oogenesis, a feature present in most arthropods (Saito et al. 2005). In *A. longisetosus*, previtellogenesis takes place in the proximal, ventro-latero-rostrad part of the ovarian meros (marked with black asterisks in Fig. 1). The accumulation of visible yolk vesicles and lipid droplets is restricted to the distal part of the meros, situated latero-caudad and dorsally of the proximal part (Bergmann et al. 2008). This section of the ovary may therefore be termed the vitellarium (marked with white diamonds in Fig. 1).

The oocytes develop outward from the germarium towards the body cavity, a general feature in chelicerates and some maxillopod crustaceans (Ikuta & Makioka 2004), but clearly in contrast to the situation found in insects and most crustaceans, where oocytes develop inside tubular ovarioles towards a lumen (Anderson 1973, Ando & Makioka 1999).

Unfortunately, the typology of ovaries as panoistic, meroistic-polytrophic or meroistic-telotrophic was developed mainly on insect ovaries, and there is ongoing debate which type of ovaries is realised in different groups of mites (Cabrera et al. 2009, and cited references). Consequentely, the transfer of this typology to mites, for want of a better alternative so far, has to rely on the convergently evolved conformance of functional and anatomical definitions and cannot bear implications of homology.

The oocyte in *A. longisetosus* looses contact with the medulla of the germarium early in previtellogenesis. From this event on, the oocyte is completely covered in follicular cells and never ruptures the basal lamina or even bulges individually into surrounding tissues throughout vitellogenesis (Bergmann et al. 2008), in contrast to many arachnid groups studied so far (Alberti 1974, Shatrov 1997, and cited references, Talarico et al. 2009). A situation resembling a meroistic ovary seems unlikely, as neither an individual association of ovarian nutritive cells with one oocyte (polytrophic), nor a connection of vitellogenetic oocytes to the germarium via stalks (telotrophic) could be demonstrated. A nutritive central cell was described for Astigmata (Witaliński et al. 1990, Walzl 2004), which are undergoing nutrimentary oogenesis. This situation is very similar to meroistic-telotrophic ovaries in insects (Schwaha et al. 2008). Although *A. longisetosus* shares many similarities in vitellogenesis (see paragraph 4.2) to the solitary type of oogenesis described in the trombiculid mite *Hirsutiella zachvatkini* Schluger, 1948 (Shatrov 1997), trophic cells within the ovary and associated to oocytes would suggest a meroistic situation in the latter case. In view of the functional arrangement of tissues in the ovary of *A. longisetosus*, it most likely resembles a panoistic ovary. A panoistic ovary has also been described for the brachypyline oribatid mite *Ceratozetes cisalpinus* Berlese, 1908 (Woodring & Cook 1962).

4.2. Vitellogenesis

The process of vitellogenesis during the accumulation of visible yolk vesicles, with a high rate of endocytosis indicated by the dense microvilli fringe in younger and numerous coated pits in later oocytes, resembles the exogenous type in *H. zachvatkini* (Shatrov 1997). This is in contrast to the endogenic vitellogenesis in the brachypyline mite *Hafenrefferia gilvipes* C. L. Koch, 1840 (Witaliński 1986). Accumulation of storage material is putatively accomplished by the uptake of yolk precursors as lipoproteins by the oocyte via the follicular cells. A likely source for these substances is the well-developed nutritive tissue (possibly fat body derivatives) in the region of the genital system of adult females with its abundant rough ER and peripheral labyrinth (Figs 2, 10). These nutritive cells, lying in the same body compartment as the fat body cells, i.e. the haemocoel, are likely to play an intermediate/synthetic role in the process, as the fat body cells in turn are connected to the digestive system via fingerlike protrusions of midgut wall cells (Alberti et al. 2003). The fat body structurally separates the digestive from the genital system, i.e. the calory source from the calory sink, in all specimens examined. Adults of *A. longisetosus* are rather slow-moving animals, yet produce an average of 1.3 eggs per day and up to over 300 eggs during their adult life span (Heethoff et al. 2007). Therefore, this pathway could well account for a major portion of the netto calory flux within the individual mite. An active role of the follicular epithelium in lipoprotein turnover may also be inferred by the presence of mitochondria with circular cross-sections of their cristae in late vitellogenesis (see paragraph 4.4).

Notable in *A. longisetosus* is the well developed microvilli fringe of the oocyte, typical for many chelicerates, but not described in the brachypiline oribatid mite *H. gilvipes* (Witaliński 1986). For example, the combination of a microvilli fringe during vitellogenesis and endocytotic pits in late vitellogenesis with simultaneous deposition of a layered egg shell was also shown for a member of Ricinulei, *Pseudocellus boneti* Bolivar y Pieltain, 1941 (Talarico et al. 2009). The absence of microvilli on the oocyte during vitellogenesis therefore may not be a common oribatid feature, but represent a derived state.

However, the oribatids *A. longisetosus* and *H. gilvipes* share the feature of simultaneous vitellogenesis and deposition of the vitelline envelope, as is characteristic for mites (Witaliński 1986, Shatrov 1997, and cited references). Furthermore, they both exhibit the opening of the perivitelline space, first described in *H. gilvipes* (Witaliński 1986). The former two processes are completed whithin the ovary, as has been described also for the middle-derivative oribatid mites, *Plathynothrus peltifer* C. L. Koch, 1839 and *Trhypochthonius tectorum* Berlese, 1896 (Taberly 1987b), but neither for the brachypyline *C. cisalpinus* (Woodring & Cook, 1962) nor the astigmatic mite *Sancassania berlesei* Michael, 1903 (Walzl et al. 2004).

In *A. longisetosus*, the differentiation of the cytoplasm of the oocyte, the centripetal accumulation of yolk vesicles between the dense peripheral and perinuclear cytoplasm and the development of individual yolk platelets resemble the situation in *S. berlesei* (Walzl et al. 2004).

4.3. Vitelline envelope structure and microvilli fringe

In *A. longisetosus*, the vitelline envelope is neither lamellated as in *S. berlesei* (Walzl, et al. 2004), nor shows a layered structure nor distinct phases of deposition as in *H. zachvatkini* (Shatrov 1997) or *H. gilvipes* (Witaliński 1986). It also does not solidify prior to the end of vitellogenesis as in *H. gilvipes* (Witaliński 1986). By contrast, it stays porose until the completion of vitellogenesis, with oocytal microvilli protruding in the pores, as described for various members of Prostigmata and Mesostigmata (Witaliński 1986). The protruding microvilli of the oocyte in *A. longisetosus* do not exhibit the apical flattening noted in the two latter groups, and, although gradually becoming less electron lucent, the vitelline envelope never reaches the electron density as demonstrated for Mesostigmata and Prostigmata (Witaliński 1986). A porose vitelline envelope during vitellogenesis, but without the protruding oocytal microvilli, has also been shown to exist in *S. berlesei* (Walzl et al. 2004). The decreasing number of microvilli with a simultaneously increasing number of coated pits and vesicles could probably be due to different uptake mechanisms for different compounds in the course of yolk formation in *A. longisetosus*.

4.4. Considerations on molecular processes

As both follicular epithelium and oocytes contain organelles of synthetic activity, such as rough and smooth ER, or the Golgi bodies, it seems quite difficult to infer the origin of vitelline envelope material from ultrastructural analysis only. Vesicles and ER canals with contents matching the electron density of the vitellar envelope could be found in both follicular cells and early vitellogenetic oocytes, yet seem to be missing in late vitellogenetic oocytes. Without specific histochemical markers for the compounds involved in the formation of the vitelline envelope, we can not classify the observed uniform material as a primary or secondary vitelline envelope (*sensu* Raven 1961, Witaliński 1986) with certainty.

A peculiar feature of follicle cells in *A. longisetosus* is the presence of mitochondria that show circular cross-sections of their inner membrane. The internal organisation of mitochondrial membranes may vary significantly between and even within tissues of the same individual due to changing cytoplasmal conditions brought about either by physiological processes or artefacts of the fixation process (Frey & Manella 2000). Although differential reactions of the tissues to the fixation protocol cannot be ruled out in this study, the presence of tubular cristae rather than lamellar ones was found in both vertebrates and arthropods to coincide with a higher activity of oxygenases as opposed to ATP-synthesis. These are involved in lipid metabolism, e.g. the breakdown of cholesterols and steroid production (Sumegi et al. 1988, Blass & Ruthmann 1989, Scheffler 2001).

4.5. Perspectives

Vitellogenesis is a multi-stage process including nutrient uptake, synthesis of vitellogenin, its transformation into vitellin, transport of yolk precursors as lipoproteins and their incorporation into the oocyte as well as the production of regulating hormones. It seems plausible that different stages of the process are distributed among different tissues in *A. longisetosus*. As several of these metabolic events most likely include cytochrome-related enzyme complexes, it seems feasible for future studies to clarify the contribution of candidate tissues such as midgut wall, fat body, nutritive cells and, by view of their mitochondria, follicle cells, using specific markers and labelling techniques.

Histochemical and molecular studies of the process of vitellogenesis and its hormonal regulation by juvenile hormone and/or the steroid ecdysone have already been conducted in a number of acarine taxa, mostly in the Ixodidae (e.g. James & Oliver 1999, Cabrera et al. 2009). Yet, such studies are still lacking in actinotrichid mites. Corresponding examinations could confirm the model inferred from morphology involving the fat body and the nutritive cells surrounding the genital organs, locate synthesis and postranslational processing of the proteins, clarify the role of the follicular epithelium and adjacent tissues, and generally deepen our understanding of vitellogenesis in the Acari and its evolutionary history.

5. Acknowledgements

We thank Julia Straube, Monika Meinert and Klaus Eisler for technical assistance, Ole Riemann, Eike Wilts, York-Dieter Stierhof, Heinz Schwarz and Bruno Humbel for valuable comments on histological techniques and Roy A. Norton for useful comments on oribatid anatomy and for providing animals to start a hatchery.

We are indebted to two anonymous reviewers for their contribution to the improvement of this publication.

Paavo Bergmann is supported by ‘Evangelisches Studienwerk Villigst e.V.’ through a PhD grant.

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Accepted 18 May 2010

5.4 Publication 4

Bergmann, P. and M. Heethoff (2012). "The oviduct is a brood chamber for facultative egg retention in the parthenogenetic oribatid mite *Archegozetes longisetosus* Aoki (Acari, Oribatida)". — Tissue&Cell 44(5): 342–350.

doi:10.1016j.tice.2012.05.002

Direct link

Tissue and Cell 44 (2012) 342–350

Contents lists available at SciVerse ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

The oviduct is a brood chamber for facultative egg retention in the parthenogenetic oribatid mite *Archegozetes longisetosus* AOKI (Acari, Oribatida)

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ARTICLE INFO

Article history:
Received 18 November 2011
Received in revised form 22 May 2012
Accepted 25 May 2012
Available online 16 June 2012

Keywords:
Parity mode
Electron microscopy
Oviposition
Egg shell
Generation
Oviduct

ABSTRACT

Archegozetes longisetosus is a parthenogenetic oribatid mite and a chelicerate model organism. We examined the localisation of processes between vitellogenesis and embryogenesis as well as the anatomy and histology of involved structures by means of light- and electron microscopy. The proximal oviduct is differentiated into an oviductal bulb, exhibiting a strong secretory epithelium. Here, solidification of the egg shell instantaneously occurs upon passing of the egg from the perivitelline space into the oviductal lumen. This is interpreted as an internalised oviposition with the generation boundary being effectively located at the ovary–oviduct transition, rendering the oviducts into functional brood chambers. The parity mode combines elements of oviparity and ovolarviparity with facultative egg retention.

1. Introduction

Despite the vast diversity of mites, studies on their early embryology and reproductive modes are rare (Aeschlimann and Hess, 1984; Walzl et al., 2004; Laumann et al., 2010a,b). Yastrebtsov (1992) listed examples from the suborder Prostigmata that range from ovolarviparity to fertilisation of eggs directly before laying. In this group, such variations in the timing of developmental events or fertilisation with respect to parity seem to track adaptational or environmental factors rather than phylogenetic relationship, while in the suborder Oribatida, some taxonomic correlates are also apparent. In this group, egg retention followed by prelarviposition or even larviposition is especially common in Ptyctima and Desmonomata, while oviposition is the regular case in most other Oribatida (Norton, 1994).

Our general goal was to study the linkage between development and parity in a thelytokous, diplo-diploid member of the oribatid mite group Desmonomata: *Archegozetes longisetosus* AOKI. The genetic strain *A. longisetosus* ran (Heethoff et al., 2007b) has become the most intensely studied oribatid mite and serves as a model organism for investigations on chelicerate developmental biology (Telford and Thomas, 1998; Thomas and Telford, 1999; Heethoff et al., 2007b; Bergmann et al., 2008; Laumann et al., 2010a,b). Unlike the well studied model prostigmatic mite *Tetranychus urticae* KOCH (Grbic et al., 2007), in which eggs are uniformly laid as zygotes and cleavage starts within the first hours after oviposition (Dittrich, 1968), *A. longisetosus* seems to lack a clear-cut starting point. A given female may lay only a few eggs at varying intervals or up to 30 eggs laid together in single clutch over a short time, and hatching time can range from 0 to 17 days (Heethoff et al., 2007b). Embryological studies further confirmed that in this mite oviposition time is irrelevant in terms of generational succession or developmental stage. At one extreme, 2-celled first-cleavage stages were found in laid egg clutches, and at the other extreme progeny developed inside of the gravid mother's oviducts up to the larva, i.e. the second cuticle-encased instar (Laumann et al., 2010a,b). In the astigmatid *Sancassania berlesei* MICHAEL, developmental stages of embryos at the time of oviposition vary depending on the age of the mother, making the comparison of individual stages complicated (Walzl et al., 2004). Further indication of the independence of embryos within a closed egg shell is the occurrence of facultative aparity, where eggs that are already located inside the oviducts complete their development in case of the death of the mother, and the larvae later hatch from the carcass (Michael, 1884; Norton, 1994).

We studied egg shell progression and developmental timing in the anatomical context of maternal tissues by means of semi- and ultra thin sections from specimens embedded in epoxy resin to assess the sequence and location of events between vitellogenesis and embryogenesis. Vitellogenesis is controlled by maternal tissues of the ovary and embryogenesis seems to ensue rather independently from the mother within the oviducts, as indicated by

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http://dx.doi.org/10.1016/j.tice.2012.05.002

the broad range of hatching times and developmental stages in one clutch of eggs. Examination of the ovary–oviduct transition was undertaken to provide morphological evidence as to where and how the separation of mother and daughter comes about. Emphasis was on the communication breakdown between mother and daughter, and whether the passing into the oviductal lumen marks the beginning of a new generation. We demonstrated that the egg shell rapidly solidifies upon passing the highly differentiated oviductal bulb tissue between ovary and oviduct, rendering the latter into functional brood chambers. The parity mode can subsequently be described as oviparity with enhanced parental investment by facultative egg retention.

2. Materials and methods

2.1. Rearing

Specimens were taken from our laboratory culture of *A. longisetosus* ran (Heethoff et al., 2007b). Molting aggregations of immobile individuals were removed from the stock culture and placed in the wells of tissue culture plates (Tissue Culture Cluster^{24} Costar) for further development. Wells were filled 1 cm high with plaster-of-Paris:charcoal (6:1 by volume) mixture. The plates were kept in constant dark at a room temperature of about 25 °C. Air humidity was kept at ~90% by moisturising the substrate.

The wells were checked daily for moisture, and freshly molted individuals were removed with a fine brush and transferred to new wells. In these, tree bark covered with green algae was supplied as a food source and was also checked daily.

Adult specimens, 3, and 5 days after moulting, were collected for further processing.

2.2. Light and electron microscopy

Specimens were punctured and fixed in 1/3 strength Karnovsky's solution diluted in 4-(2-hydroxyethyl)-1-piperazineethanesulphonic (HEPES) acid buffer at 0 °C, pH 7.8 for 90 min.

The fixative contained 1.3% formaldehyde, freshly depolymerised from paraformaldehyde (FLUKA, Buchs) and 1.66% TEM-grade glutardialdehyde (Science Services, Munich) with 4% sucrose and 6.6 μM $MgSO_4$. Postfixation was carried out with osmium tetroxide 1% in HEPES buffer for 1 h at 0 °C. Specimen were dehydrated in a graded ethanol series of 30% and 50% for 10 min each, 70% overnight, two 10 min changes of 70%, and three 10 min changes each of 80%, 90% and 95%. 100% acetone (dried on molecular sieve) was used as intermedium, with two changes of 60 min. Specimens were then gradually infiltrated in acetone-Araldite Cy212 (plano, Wetzlar) mixture 3:1 for 90 min and 1:1 for 90 min on a rotary plate and 1:3 in open block vials overnight, and embedded in Araldite CY212 epoxy resin, prepared with dimethylbenzylamine (BDMA) as accelerator. En-bloc staining was achieved by uranyl acetate saturated in 70% ethanol overnight during the dehydration process.

Some of the TEM-specimens were prepared using acetone as the dehydrating agent in the same manner as stated above. In these cases, 1.7% uranyl acetate was added to the osmium tetroxide solution for en-bloc-staining. Semithin sections were stained with ferric haematoxylin and ultrathin sections were poststained and contrasted with uranyl acetate and lead citrate. All semithin sections were softened in ethanol fumes for 30 min and deosmified in 15% H_2O_2 for 12 min prior to staining.

Light microscopy was carried out on a Zeiss Axioplan microscope and transmission electron microscopy with a Siemens Elmiskop 1A. Original negatives were scanned at 1200 dpi in RGB with 8 bit per colour channel. 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). Images were reduced to grey values and inverted. Empty regions of the images histogram were clipped in tonal value correction, and a sigmoidal curve with its inflexion point at the modus of the histogram was applied to the grey channel curve.

Additional semithin sectionings were stained with periodic-acid-Schiff (PAS) reaction for 1,2-glykanes, rinsed in a mixture of 300 ml aqua bidest, 15 ml 1 N HCl and 18 ml 10% sodium bisulphite prior to rinsing in tap water according to Mc Manus (1948) and counterstained for proteins with 0.2% Fast Green FCF (Merck, Darmstadt) in 50% acetic acid. PAS staining was also conducted on whole-mount preparations of egg clutches.

3. Results

3.1. Anatomical background

As described by Bergmann et al. (2008), the female genital organs of *A. longisetosus* comprise an ovary with an unpaired central structure (rhodoid) and two lateral extensions (meroi), which contain developing oocytes wrapped in flattened follicular cells. These meroi are solid structures, and lead to the tubular paired oviducts. Vitellogenesis is completed in the ovarial meroi, simultaneous with the accumulation of egg shell material as a porose substance (see also Bergmann et al., 2010). The paired oviducts distally converge into a uterus, the lumen of which is separated with muscular flaps from both the oviducts and the unpaired vagina that leads into an eversible ovipositor. The transition point of the solid meros of the ovary into the tubular oviduct is macroscopically discernible as a swollen region of wrinkled appearance. Eggs passing into the oviduct generally cluster at the distal portion, leaving a free lumen in the proximal, wrinkled part.

3.2. Light microscopy

The distinctly differentiated, spongy tissues of each proximal oviduct begin abruptly at a funnel-shaped structure engulfing the tip of the ovarial meroi. It constitutes the region referred to as the "oviductal bulb" ("ovarial bulb" of Bergmann et al., 2008) for its bulky appearance. The oviductal lumen also starts abruptly within the funnel with a diameter of 15–20 μm. The distal face of the oldest vitellogenetic oocyte delimits its proximal onset. This face is the only surface area of ovarial oocytes not wrapped in follicular epithelium. The oviductal bulb is discernible exteriorly by the irregular tissue surface in preparations. Not containing eggs, the lumen appears irregular and tapers to a small channel distally until it opens into the smooth, wide lumen of the distal oviduct that contain eggs ready for release. The region of the oviductal bulb is approximately 200–220 μm long and gradually passes into the smooth, delicate and unvacuolated wall epithelium of the oviduct distally.

Semithin sectionings from the beginning of the oviductal lumen revealed an epithelium of much greater thickness than follicular cells or distal parts of the ovary (Fig. 1). A high proportion of its volume is made up of numerous, irregular vesicles of various sizes. Vesicle-rich portions of the cytoplasm stain differentially into purplish-grey as opposed to the lighter bluish stain of vesicle-free cytoplasm with ferric haematoxylin. These portions of cytoplasm generally appear in the apical part of the elongated cells. The apical portions of the cells bulge irregularly into the oviductal lumen, and sections of filiform extensions (Fig. 2) appear most frequently in the parts directly neighbouring the ovary.

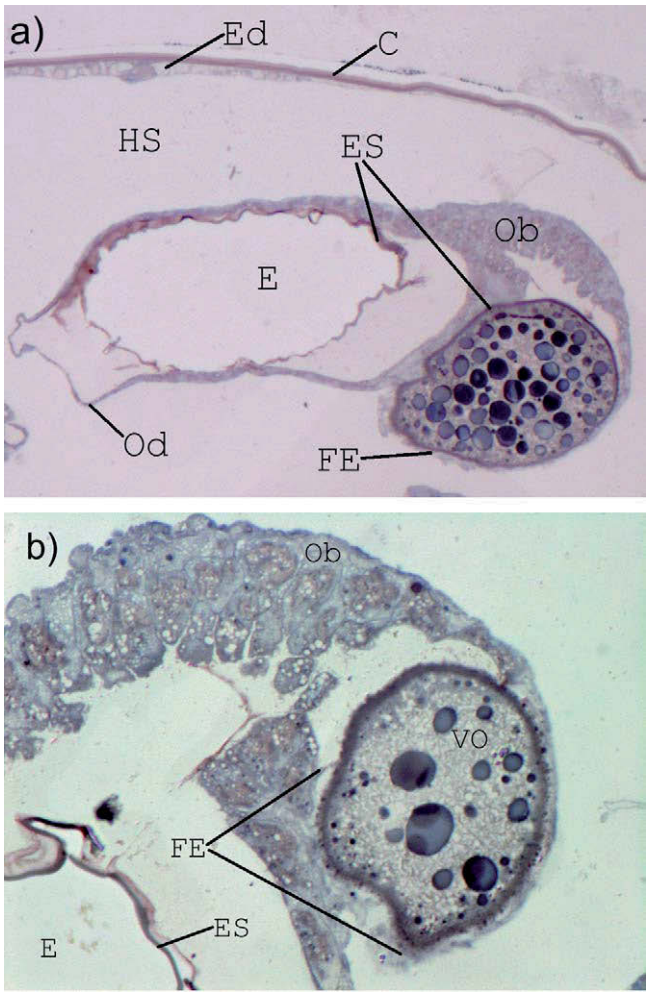

Fig. 1. *Archegozetes longisetosus*, 3 days after molting. Light micrographs of semithin sections of an oviductal bulb. (a) *Overview*: The oviductal bulb appears as a swollen region of cylindrical cells rich in vesicles. They bulge into the lumen of the proximal oviduct. This lumen is delineated by the distalmost ovarial oocyte, which is still wrapped in follicular epithelium. The oviductal bulb epithelium gradually tapers into the delicate oviductal wall comprising of flattened cells devoid of vesicles. Scale bar: 50 μm. (b) *Detail*: Vesicle-rich portions of oviductal bulb cytoplasm stain differentially (purplish-grey) from vesicle-free portions (light blue). Extensions of the follicular epithelium in contact with the oocyte at the transition point. In contrast to oocytes, eggs break from section due to improper fixation and infiltration across solidified egg shell. Scale bar: 20 μm. Stain: ferric haematoxylin. C: cuticle, E: egg (broken from section), Ed: epidermis, ES: egg shell, FE: follicular epithelium, HS: haemolymph space, Ob: oviductal bulb, Od: oviductal epithelium, and VO: vitellogenetic oocyte. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

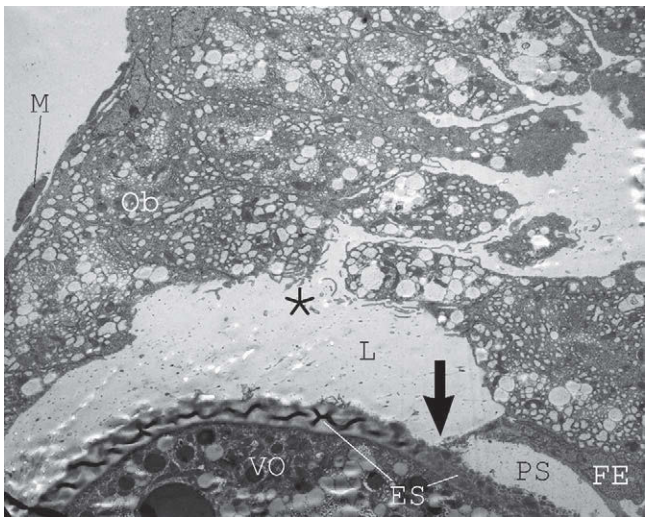

Fig. 2. *Archegozetes longisetosus*, 3 days after molting. TEM micrograph of oviductal bulb. Ultrathin sectioning adjacent to semithin section shown in Fig. 1 Filiform extensions of oviductal bulb epithelium are visible in the lumen. Residues of secretion in vesicles and in the oviductal lumen are discernible. Cell debris in the perivitelline space shows different properties compared to material in vesicles and oviductal lumen. Follicular epithelium is sealing off the perivitelline space from the oviductal lumen, a marked transition of egg shell properties occurs across this region. Scale bar: 5 μm. ES: egg shell, FE: follicular epithelium, L: oviductal lumen, M: muscle strand, Ob: oviductal bulb, PS: perivitelline space, and VO: vitellogenetic oocyte. Asterisk: filiform extensions of oviductal bulb cells. Arrowhead: follicular epithelium contacting microvilli of oocyte.

3.3. Electron microscopy

Electron micrographs reveal that a single layered somatic epithelium forms the wall of the genital duct containing developing eggs. Both egg shell material and epithelial tissue exhibit a marked transition of properties at the onset of the oviductal bulb region. The epithelium is covered by a thin basal lamina, including pouches with fine muscle strands at intervals (Figs. 2 and 3) on the haemolymph side. These features also were found in the follicular epithelium of the ovarial meroi, and the basal lamina appears to be continuous between ovary and oviduct. Under this lamina, oviductal bulb cells directly neighbour follicular cells, and the different cell properties are apparent, as follicular cells are very thin, flattened, electron lucent, and, at this point, contain few organelles other than mitochondria of the tubular type (Figs. 3 and 4). In places, the perivitelline space at the tip of the meroi is separated from the haemolymph space only by the basal lamina, and cell debris indicates degradation of epithelial cells (Fig. 3). Net-like residual traces of an otherwise structureless material of medium electron density are conspicuous in the oviductal lumen, and residuals of very similar appearance appear in the vesicles of the oviductal bulb epithelium (Figs. 2–5). The latter vesicles become larger towards the cell apex and basally converge in dense clusters in the vicinity of the strongly developed Golgi apparatus (Fig. 5). Secretions are putatively released into the lumen by exocytosis, as deep sinuses between the filiform extensions are present in the sections. These match the largest apical vesicles in diameter and content and most likely represent vesicles releasing their content into the lumen. Although the exact nature of cell contacts between adjacent follicular and oviductal cells was not studied in detail, intercellular spaces seem to be slightly larger than those between neighbouring secretory cells of the oviductal bulb (Fig. 5).

In all specimens examined, extensions of the follicular cells sealed the perivitelline space off from the oviductal lumen, making contact with the egg shell at the transition point (black arrow in Figs. 2 and 3). The egg shell material of the individual egg at the transition point is porose and occasionally crossed by microvilli of the oocyte, but quickly turns into a solid, amorphous layer over a distance of a few micrometers upon contact with the oviductal lumen (Figs. 2–5). Traces of the former porosity are no longer detectable in the egg shell facing the oviductal lumen, and the material slightly increases in overall electron density.

A schematic rendition of the region is given in Fig. 6. It provides a graphical overview of the anatomical context of an oocyte in the process of losing contact with the ovary while entering the oviductal lumen.

3.4. Glykane staining

PAS staining of clutches of laid eggs and of semithin sections of the oviductal bulb and oviduct consistently reveals a strong positive signal from distinctive structures. No fuchsine stain appeared in the

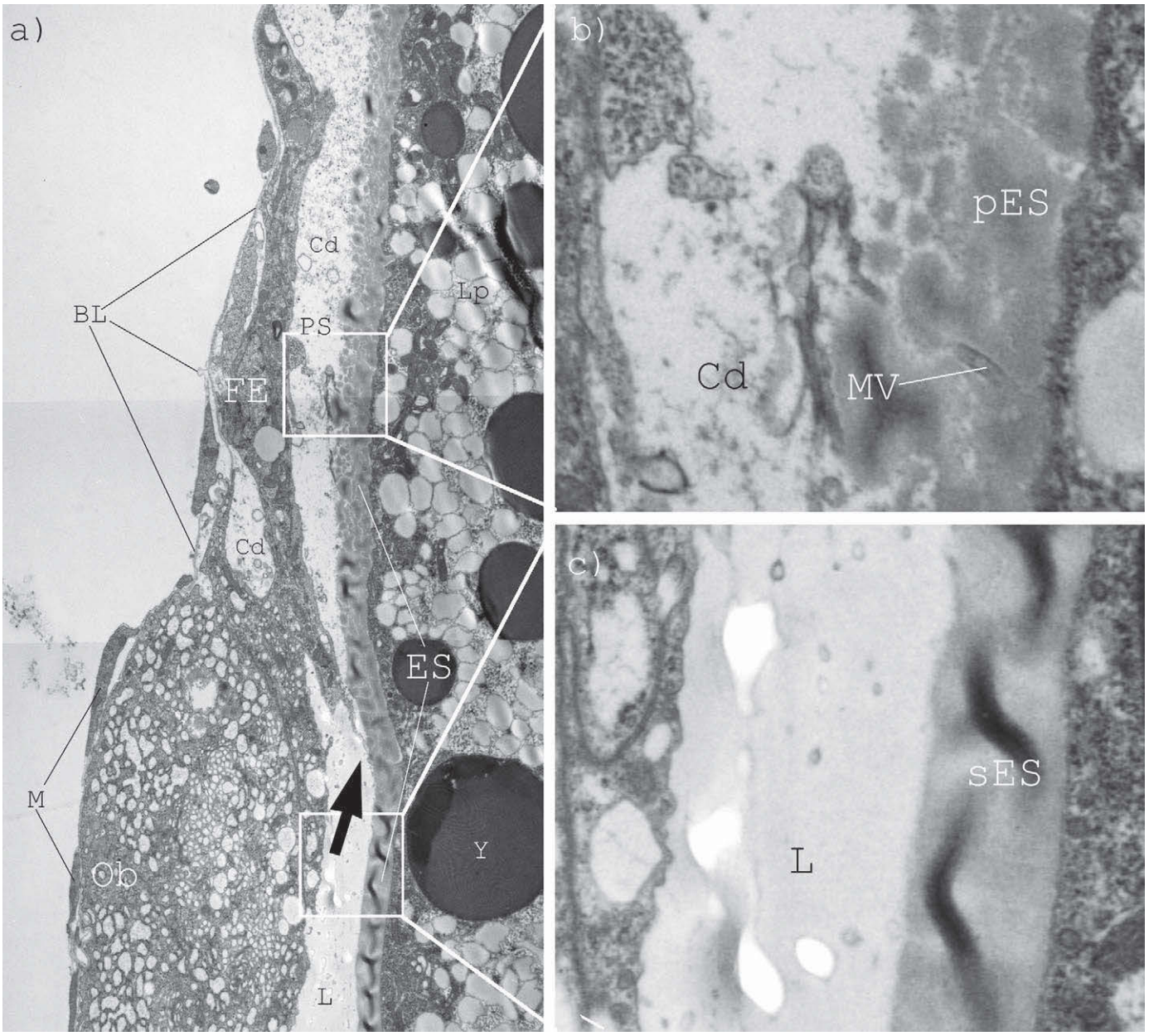

Fig. 3. *Archegozetes longisetosus*, 3 days after molting TEM micrograph of oviductal bulb. (a) Overview combined from scans of three original negatives. Basal lamina continuous between follicular epithelium and oviductal bulb. Muscle strands embedded in basal lamina. Progression of oviductal bulb vesicles towards cell apex as increase in size, confluence and exocytosis into oviductal bulb lumen. Difference in properties between cell debris in perivitelline space and content of oviductal lumen. Scale bar: 2 μm. (b) and (c) Detailed views from the sections as indicated by white outlines in (a). Egg shell is porous and still containing oolemmal microvilli before transition point, whereas it is solid and devoid of microvilli after transition point. Note content of oviductal bulb vesicles in (c). BL: basal lamina, Cd: cell debris, ES: egg shell, FE: follicular epithelium, L: oviductal lumen, Lp: lipid yolk droplet, M: muscle strand, MV: microvillus, Ob: oviductal bulb epithelium, pES: porous egg shell, PS: perivitelline space, sES: solidified egg shell, and Y: Yolk vesicle. Black arrow: ovary–oviduct transition, egg shell solidification, follicular cell extension sealing off perivitelline space from oviductal lumen.

follicular epithelium or the perivitelline space. Fuchsine staining is found in the vesicle clusters in the oviductal bulb epithelium, as traces of signal inside the oviductal lumen with higher densities in the vicinity of eggs, and in a layer of positively staining, amorphous material covering laid eggs (Fig. 7). Vitellogenetic oocytes show strong PAS signal in the cytoplasm prior to the appearance of proteineous yolk vesicles. When fully developed, the latter show different degrees of Fast Green or a mixture of Fast Green and PAS staining, with the non-crystalline portions generally showing stronger fuchsine staining (Fig. 7). Oocytes at the beginning of their centripetal accumulation of proteineous yolk vesicles exhibited strong PAS signal in the central portion of their cytoplasm, which appears granular and electron lucent in TEM.

4. Discussion

4.1. Egg shell transition

Our ultrastructural analyses reveal that the solidification of the accumulated egg shell material, and hence the separation of the developing embryo from its environment, is not a gradual process. This contrasts with most available studies on egg shell formation in various other species of mites (Witalinski, 1986, 1993), which have shown a variety of modes for accumulation of material and its subsequent modification. Although the process of solidification was seldom observed directly in these studies, its gradual nature can be inferred from the fact that usually a considerable length of the

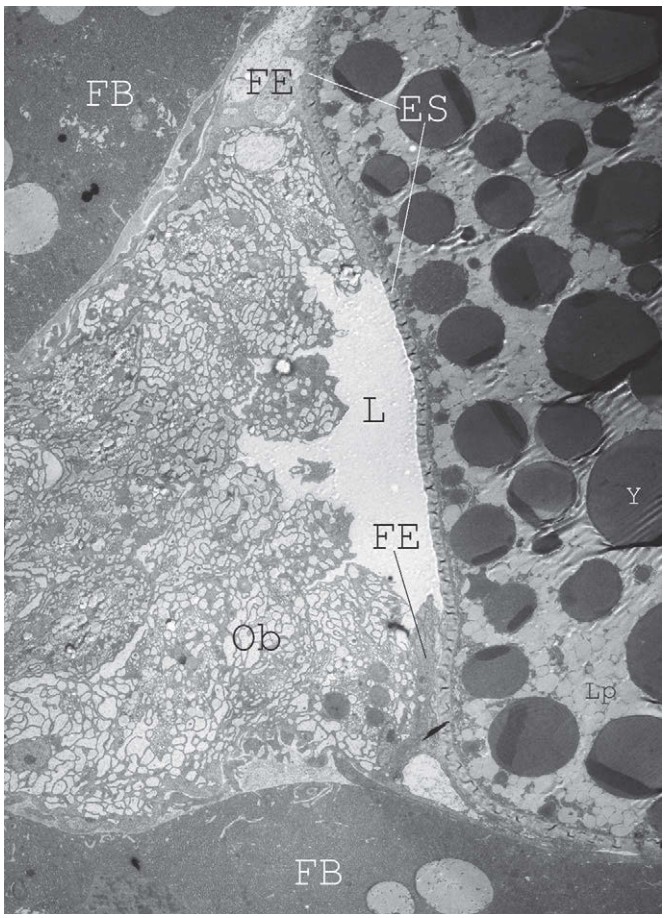

Fig. 4. *Archegozetes longisetosus*, 5 days after molting. TEM micrograph of oviductal bulb. Combined from scans of two original negatives. The egg shell of the distalmost ovarial oocyte is in contact with the follicular epithelium, or with the lumen of the oviduct, but not with oviductal bulb cells. The majority of oviductal bulb cell volume consists of secretory vesicles. Adjacent fat body cells exhibit extensive rough endoplasmic reticulum and peripheral labyrinth. Scale bar: 10 μm. ES: egg shell, FB: fat body, FE: follicular epithelium, L: oviductal lumen, Lp: lipid yolk droplet, Ob: oviductal bulb epithelium, and Y: yolk vesicle.

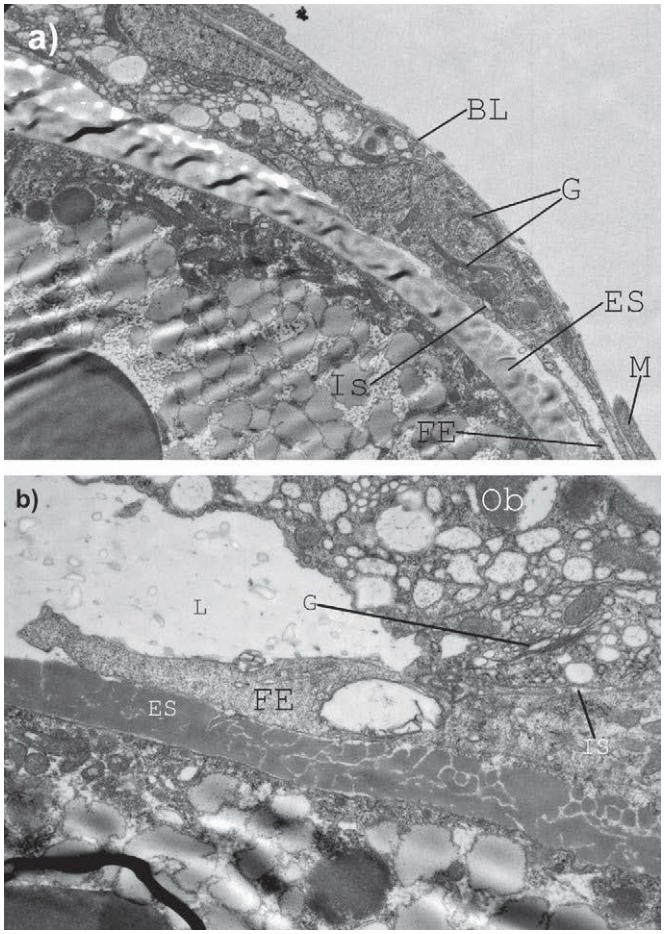

Fig. 5. *Archegozetes longisetosus*, 3 days after molting. TEM micrographs from oviductal bulb. Cell contact between oviductal bulb and follicular epithelium. (a) Strongly developed Golgi apparatus in basal portion of oviductal bulb cell. Intercellular spaces between oviductal bulb cells and follicular epithelium covered by continuous basal lamina. Scale bar: 2 μm. (b) Golgi apparatus giving rise to secretory vesicles of oviductal bulb cells. Intercellular space between oviductal wall epithelium and follicular epithelium cells. Scale bar: 2 μm. BL: basal lamina, ES: egg shell, FE: follicular epithelium, G: Golgi apparatus, Is: intercellular space, L: oviductal lumen, M: muscle strand, and Ob: oviductal bulb epithelium.

oviduct is involved. In Astigmata, a layered structure of the egg shell appears common in Acaroidea, whereas in Psoroptidea, the similarities with oribatid mites are noted, in that a granulose material later compacts into the egg shell after retraction of the oolemmal microvilli (Witalinski, 1993). In the brachypiline oribatid *Hafenrefferia gilvipes* KOCH, a second, compact layer was described to enclose the formerly deposited porose material (Witalinski, 1986). Rapid solidification of a single layer of egg shell material at one specific point, as in *A. longisetosus*, was not previously described. In our study, an individual oocyte might have still been in contact with the maternal tissues via microvilli on its proximal side, but effectively shut off from the oviductal lumen (continuous with the outer environment via the genital orifice) at its distal apex. Once the transition point is passed, the egg shell precludes chemical communication between the embryo and its environment, namely the mother. All eggs contained in the continuous lumen of the oviduct exhibit egg shells that are impermeable to aqueous solutions (Aeschlimann and Hess, 1984; Witalinski, 1993). This impermeability is indicated by the fact that eggs from the oviduct generally do not fix properly, are not well infiltrated with embedding media, and therefore frequently break free during sectioning (Figs. 1 and 6). Application of methods suitable for the processing of acarine eggs on material obtained from oviducts of *A. longisetosus* previously confirmed that embryogenesis starts upon reaching the oviductal lumen (Laumann et al., 2010a).

4.2. Tissue appearance

The appearance of the strongly vacuolated cell type of the oviductal bulb in the genital duct is abrupt, and the sudden change in tissue properties coincides with several significant features. Oocytes at this point have completed meiosis, rediploidisation, vitellogenesis and the accumulation of egg shell material (Bergmann et al., 2010). For the first time, a continuous lumen appears in the genital duct that progresses towards the genital orifice, and the eggs lose any direct contact with maternal somatic tissues.

As vesicle content of oviductal bulb cells seems to be released by exocytosis of secretory vesicles into the lumen, which is continuous with the outer environment, and loss of cytoplasmic material was not observed, we interpret these epithelial cells as merocrine, exocrine glandular cells (Ude and Koch, 2002). The secretory vesicles most likely represent products of a massively developed Golgi apparatus, since they seem to originate in the vicinity of Golgi apparatus and preliminary information about the chemistry of their contents is consistent with such origin.

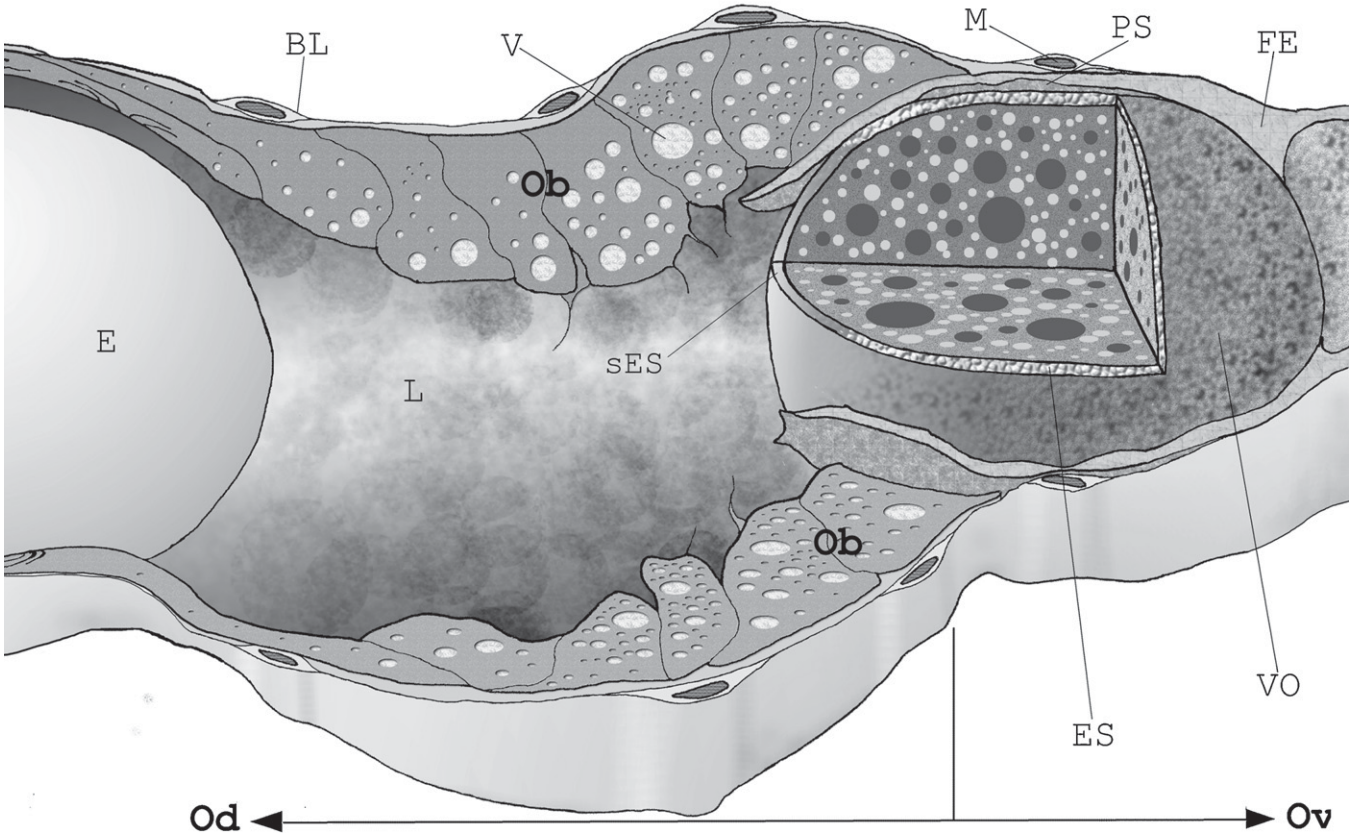

Fig. 6. *Archegozetes longisetosus*. Schematic drawing of oviductal bulb region as the location of the internalised oviposition, and generational boundary. A combined rendition is given of the spatial arrangement of important tissues and features. Oocytes at the distalmost tip of the ovarial meroi leave the covering follicular epithelium and enter the oviductal lumen. The wall epithelium of the proximal oviduct is differentiated as an oviductal bulb of cylindrical cells rich in secretory vesicles. This tissue gradually changes into the delicate wall of the distal oviduct comprising of flattened cells. Upon entering the oviductal lumen, the egg shell material rapidly solidifies. A common basal lamina covers both oviductal wall and follicular epithelium. Embedded within are fine circular muscle strands. The distal oviduct as a functional brood chamber contains eggs with solidified egg shells, already undergoing embryogenesis. BL: basal lamina, E: egg, ES: egg shell, FE: follicular epithelium, L: oviductal lumen, M: muscle strand, Ob: oviductal bulb epithelium, Od: oviduct, Ov: ovary, PS: perivitelline space, sES: solidified egg shell, V: secretion vesicle of ovarial bulb epithelium, and VO: vitellogenetic oocyte.

4.3. Chemistry

The process of egg shell solidification takes place instantaneously upon contact with the oviductal lumen, suggesting that secretions of the oviductal bulb epithelium play an active role in this process.

As mucous epithelial secretions all over the metazoan kingdom commonly comprise mucins (Marin et al., 2007), which are proteoglycanes with a carbohydrate content of 60–95% by mass (Alberts et al., 1983), it is not surprising that the mucous substance surrounding the laid egg batches is rich in glycanes as indicated by positive PAS staining. The origin of these substances among somatic tissues along the genital duct seems clear, because strong PAS signal is located only in the vesicles of the oviductal bulb epithelium. Although it is known that glutardialdehyde fixation may eventually lead to false positive reactions in PAS staining (Hopwood, 1967), processing of the samples for embedding in epoxy resins is known to sufficiently remove excess aldehyde from the sample, thereby retaining specific staining characteristics (Jones et al., 1977). Rinsing the tissues with sodium bisulphite further serves the same purpose (Mc Manus, 1948). The specificity of the observed staining, the generally low background staining in regions strongly counterstained by the unspecific protein stain Fast Green FCR, and the reproducibility of staining results in different specimens are all evidence that the mucous, Schiff-positive substances covering the eggs, filling the oviducts and being present in the vesicles of the oviductal bulb are indeed identical. A high production rate of proteoglycans suggests that the mass of vesicles of different sizes accounting for most of the cell volume in oviductal bulb cells are products of a strongly developed Golgi apparatus in these cells, as TEM images suggest (Fig. 4). The Golgi apparatus generally is involved in the processing of proteoglycans (Alberts et al., 1983). Nothing is known so far about the exact chemistry of the secretion, nor of the chemistry of the egg shells in *A. longisetosus* or any other oribatid mite. Casperson et al. (1986) speculated that egg shells in Tetranychidae are keratinous rather than chitinous. The observed distance of less than 10 μm between the solidified portion of egg shell in one individual egg and its porous precursor, in combination with a production rate of 1.3 eggs per mite per day (Heethoff et al., 2007b) implies a rapid process of solidification. The solidified egg shell of *A. longisetosus* appears homogeneous in TEM micrographs, and over the transition distance, the channels of the formerly porose eggshell gradually become narrower. Witalinski (1993) described the closing of pores due to the retraction of microvilli as being common in mites. This is not the case in *A. longisetosus*, as microvilli are retracted some time before the end of vitellogenesis, while the vitelline envelope is still granular in appearance (Bergmann et al., 2010). We have no evidence that solidification involves the addition of a second component, since no irregularities in TEM sections of the later egg shell are detectable, and since residues of the secretion do not match the egg shell material in terms of structure or electron density (as do some vesicles of the oocytes and the follicular epithelium; Bergmann et al., 2010). Another possible solidification process is a modification

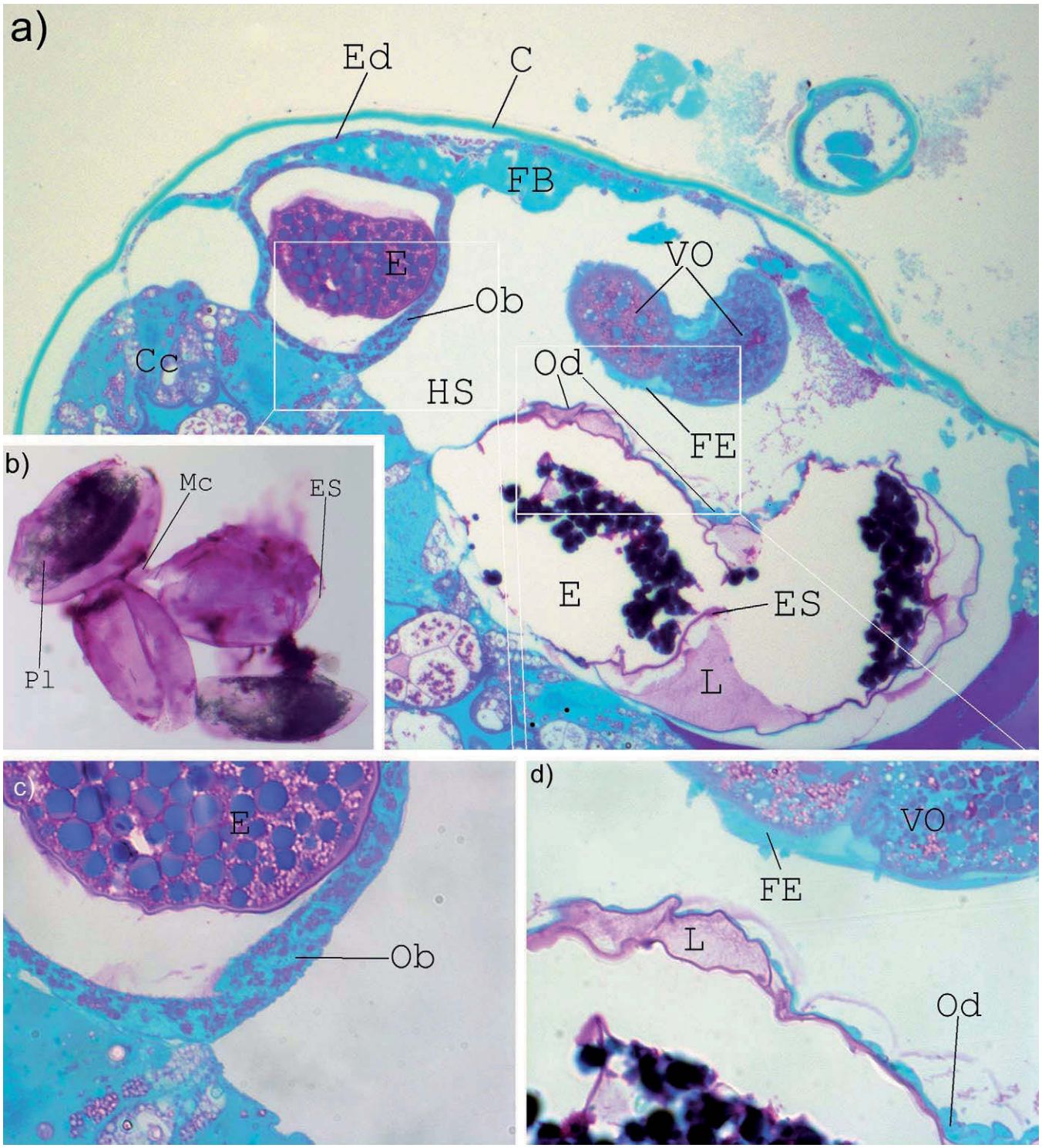

Fig. 7. *Archegozetes longisetosus*, 5 days after molting. Light micrographs. (a) Semithin section of hysterosoma. Stain: PAS/Fast Green. PAS stain in epidermis, yolk, oocyte cytoplasm, oviduct lumen and vesicles of oviductal bulb cells, but neither in follicular epithelium nor in wall epithelium of the distal oviduct. Scale bar: 100 μm. (b) Wholemount preparation of laid eggs. Two eggs already containing cuticulated instars (prelarva). A mucuous, PAS-positive substance is covering the translucent egg shells, effecting cohesion of laid eggs into a tight clutch. Stain: PAS. (c) and (d) Detailed views of the section shown in (a), as indicated by white outlines. Scale bar: 100 μm. (c) Egg within oviductal bulb. PAS signal concentrated in vesicle-rich portions of oviductal bulb cells (compare Fig. 1). Scale bar: 20 μm. (d) Vitellogenetic oocytes wrapped in follicular epithelium and egg within distal oviduct. No PAS signal in oviductal wall epithelium nor in follicular epithelium, positive PAS reaction from oviductal lumen content. Scale bar: 20 μm. C: cuticle, Cc: caecum, E: eggs, (some are broken out of the section), Ed: epidermis, ES: egg shell, FB: fat body, FE: follicular epithelium, HS: haemolymph space, L: lumen of oviduct, Mc: mucus, Ob: oviductal bulb epithelium, Od: oviductal wall epithelium, Pl: prelarva, and VO: vitellogenetic oocytes.

of the material, i.e. the cross-linking of macromolecules. Possible mechanisms are polycondensation of carbohydrates like chitin, and polyaddition like the chinone-tanning of the keratin-like protein arthropodin into sclerotin, and both of which occur during the hardening of insect cuticles (Wigglesworth, 1957). Proteoglycans, however, are not known to be potent tanning agents. We therefore infer the existence of hitherto unidentified additional compounds of the secretion. Glycosilated precursor molecules of

quinones (Wigglesworth, 1957) for instance might even contribute to the positive PAS reaction.

Apparently, the secretion fulfils several additional functions. Moisture expansion of proteoglycans, tentatively accounting for the appearance of the lumen in histological cross sections, together with lubricating properties facilitates egg transport through the oviduct and ovipositor. The mucus further aids cohesion of the laid eggs into a tight clutch. Whether the mucus constitutes an exochorion remains doubtful, as hypotheses of homology in egg shell materials are hazardous (Witalinski, 1986).

4.4. General anatomy

The rapid process of egg shell solidification, as demonstrated in TEM micrographs at the instant of eggs entering the oviductal lumen, strengthens Taberly's (1987) assumption of this structurally easily identifiable point as the ovary–oviduct transition, as we already adopted in earlier work (Bergmann et al., 2008). The proper denomination of anatomical regions of the internal genital organs of mites is so far not consistently based upon sound concepts of homology (Alberti and Coons, 1999). All somatic tissues of the genital organs develop from a ventral fold of mesodermal tissue in chelicerates (Anderson, 1973), as is indicated in this case by the continuous basal lamina with inserted pouches of circular muscle strands. Ovarian nutritive tissue continuous with the oviductal wall was also described in *T. urticae* (Blauvelt, 1945). In case of the term "ovarial bulb" (Bergmann et al., 2008), it was originally borrowed from the anatomical description of the brachypiline *Ceratozetes cisalpinus* BERLESE (Woodring and Cook, 1962) for overall structural similarities. The term "ovarial bulb" was apparently first introduced by these authors as marking the ovary–oviduct transition. It is described as a region of markedly swollen epithelial cells. However, the authors describe the epithelium of the subsequent oviduct as follicular cells, encompassing the oocytes without an apparent lumen and being involved in vitellogenesis. They describe features like follicular epithelium and vitellogenesis as extending beyond this point in *C. cisalpinus*. These processes have been shown to occur in the ovary of *A. longisetosus* (Bergmann et al., 2008, 2010). As the tissue described for *A. longisetosus* in this work is clearly oviductal as discussed above, the homonymy appears misleading, and a hypothesis of homology doubtful at best. We therefore propose to rename this distinguished structure from "ovarial bulb" (as was used by the authors in earlier work, see Bergmann et al., 2008) into "oviductal bulb" for *A. longisetosus*. A glandular appearance of the proximal paired oviducts was already mentioned by Witte (1975) in his description of the anatomy of Erythreidae (Prostigmata).

4.5. Generational border

Although oviposition of embryos into the outer environment may occur at varying states of development, our earlier conclusion is confirmed that a sound generational boundary may be drawn temporally, anatomically and environmentally at the oviductal bulb (Bergmann et al., 2008). Various diagnostic features have been used in gonochoristic species to delimit the generational border, such as genomic constitution, nuclear phase, rediploidisation, fertilisation, developmental independence, physiological separation and non-identical environments. While most appear to be sufficient delineators, not all are necessary conditions. Some cannot be applied to non-gonochoristic lineages, and not all are simultaneous or even existent in different taxa. In the case of *A. longisetosus*—a thelytokous, egg laying strain with clone-like offspring (Palmer and Norton, 1992), with rediploidisation being difficult to observe directly (Laumann et al., 2008)—a traditional understanding of generations seems challenged. On the other hand, parthenogenetic radiations of oribatid mites into morphologically, genetically and ecologically distinct entities have been demonstrated for several taxa (Maraun et al., 2003, 2004; Heethoff et al., 2007a, 2011; Laumann et al., 2007). Evolutionary processes such as adaptation and speciation do take place in these lineages, based upon a meiotic germline and the succession of individual generations. In the case at hand, the anatomically distinct and conveniently identifiable environmental isolation of the rediploidised oocyte prior to embryogenesis serves as a justifiable accessory definition of the generational border.

4.6. Parity mode

The described process hardly qualifies as ovoviviparity, or ovolarviparity sensu strictu, even though some eggs are laid that contain fully developed larvae ready to hatch, since development begins separately and independently, and there is no direct nutrition from the mother. Rather, it constitutes a form of oviparity in which parental investment is enhanced by facultative egg retention. Due to the prolonged retention time, the oviducts not only serve the transport of eggs, as the name implies, but may also be best envisioned as brood chambers in *A. longisetosus*. In this view, the true instant of oviposition is internalised at the oviductal bulb, due to the impermeability of the egg shell. The function of the oviducts as brood chambers for egg retention in the parasitiform mite group Dermanyssina was briefly discussed earlier by Alberti and Coons (1999), but this case is different. The specific histology in *A. longisetosus* does not enable, but rather precludes communication between mother and daughter, and thereby offers the possibility of opportunistic parity behaviour. Egg retention means that the early stages of development occur in a presumably stable environment (i.e. within the mother) with putatively reduced risk of predation or microbial infection as compared with oviposition to the outer environment. It also enables rapid oviposition during favourable circumstances.

Parity in *A. longisetosus* combines elements of different traditionally recognised modes, ranging from oviparity to ovolarviparity, regardless of whether active control is exerted, or whether the potentially wide range of developmental stages laid in one clutch is determined by the function of organs and tissue. As in oviparity, solid-shelled eggs are rich in yolk, there is neither nutrition beyond choriogenesis nor communication between generations beyond the end of vitellogenesis, and there exists a potential for rapid successive oviposition. These traits are combined with the facultative potential for development in a sheltered space, with retention even up to the second instar (larva) and prolonged parental investment, all to varying degrees. This apparently optimal combination is enabled by the specialisation of the tissues of the proximal oviduct into the oviductal bulb, the role of which ensures rapid solidification of the egg shell. This effectively internalises oviposition and the generation boundary, and renders the oviducts into brood chambers that can be emptied when external conditions are favourable.

Acknowledgements

We thank Julia Straube, Monika Meinert and Klaus Eisler for technical assistance, Ole Riemann and Eike Wilts for valuable comments on histological techniques, Michael Laumann for practical help and valuable discussions and Roy Norton for greatly improving this manuscript. Paavo Bergmann was supported by "Evangelisches Studienwerk Villigst e.V." through a PhD grant.

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5.5 Publication 5

Laumann, M., Bergmann, P., Norton, R. A. and Heethoff, M. (2010). "First cleavages, preblastula and blastula in the parthenogenetic mite *Archegozetes longisetosus* (Acari,Oribatida) indicate holoblastic rather than superficial cleavage". — Arthropod Structure and Development **39**, 276–286 doi:10.1016j.asd.2010.02.003

Direct link

Arthropod Structure & Development 39 (2010) 276–286

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

First cleavages, preblastula and blastula in the parthenogenetic mite *Archegozetes longisetosus* (Acari, Oribatida) indicate holoblastic rather than superficial cleavage

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ARTICLE INFO

Article history:
Received 2 October 2009
Accepted 5 February 2010

Keywords:
Chelicerata
Embryology
Transmission electron microscopy (TEM)
Micromere
Macromere

ABSTRACT

The mode of cleavage in the Acari is generalized as superficial or intralecithal, with a preceding phase of total (holoblastic) cleavage, but the knowledge is fragmentary and conclusions have been inconsistent, even when relating to the same species. Since no data about early embryology is available for the speciose group Oribatida, we studied *Archegozetes longisetosus* using transmission electron microscopy. We focused on early cleavages and the formation of the blastula, as these are the important and controversial points in early embryology of the Acari. We expected, as postulated for other acarine eggs, the early cleavages to be holoblastic and followed by a superficial preblastoderm stage. The early cleavages of *A. longisetosus* are holoblastic and blastomeres give rise to yolk-free micromeres and macromeres containing all the yolk. In contrast to expectations, the micromeres do not form a superficial preblastoderm layer. They are scattered along the embryonic surface and form an external, monocellular layer that covers the whole surface of the embryo. Since each of the existing TEM studies of mites shows this same pattern, and since this specialized form of total cleavage seems to be unique in Chelicerata, it may be the general mode of cleavage in Acari. However, the question will require much more investigation, especially since most data relate to the Actinotrichida and very few are currently available for species in the other major group, the Anactinotrichida.

1. Introduction

With about 55,000 named species, mites and ticks (Acari) comprise the most diverse group of chelicerate arthropods, yet detailed information about their cleavage and embryology is sparse, and much of the literature appeared 40–100 or more years ago. Existing data were derived largely from traditional light microscopy (LM). Scanning electron microscopy (SEM) studies are rare (Aeschlimann and Hess, 1984; Walzl, 1988, 1993; Yastrebtsov, 1992; Thomas and Telford, 1999; Walzl et al., 2004) and only three transmission electron microscopic (TEM) studies are available (Casperson et al., 1986; Fagotto et al., 1988; Walzl et al., 2004). The latter is particularly surprising, since TEM is virtually a necessity for the precise analysis of internal structures of the small mite embryos (Fagotto et al., 1988; Walzl et al., 2004).

It is commonly suggested that the usual mode of cleavage in Acari is intralecithal, resembling superficial cleavage (Claparède, 1868; Wagner, 1894; Hughes, 1959; Anderson, 1973; Aeschlimann and Hess, 1984; Evans, 1992). In the small, relatively yolk-poor eggs of some members of the diverse mite taxon Actinotrichida (=Acariformes), a phase of holoblastic cleavage precedes this superficial blastoderm formation (Brucker, 1900; Reuter, 1909; Patau, 1936; Cooper, 1940; Vitzthum, 1943; Sokolov, 1952; Türk and Türk, 1957; Edwards, 1958; Dittrich, 1968; Prasse, 1968; Heinemann and Hughes, 1970; Casperson et al., 1986). Such a mode has been termed "mixed cleavage" (Dawydoff, 1928; Fioroni, 1970) or "combination cleavage" (Johannsen and Butt, 1941). This specialized sequence is assumed to be secondary, with intralecithal cleavage being the plesiomorphic process in Acari (Anderson, 1973). Intralecithal cleavage and mixed/combination cleavage both result in a superficial sheet of cytoplasm containing the cleavage nuclei surrounding a central mass of yolk, thereby forming a syncytial blastoderm or preblastoderm.

Most results concerning embryology of mites originate from publications that focus on other topics and are thus fragmentary,

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doi:10.1016/j.asd.2010.02.003

and this often resulted in inconsistent conclusions and the inability to make generalizations throughout the main subgroups of Acari. For example, work on the actinotrichid mite group Astigmata in the 19th century showed holoblastic cleavage (Leydig, 1848), while studies in the 20th century concluded that cleavage was mixed (Vitzthum, 1943; Türk and Türk, 1957; Prasse, 1968) or superficial (Langenscheidt, 1958). But in 2004 Walzl et al. provided evidence that cleavage is holoblastic in the acarine mite *Sancassania berlesei*. Contradiction and controversy exist even for single species. In 1868 Claparède postulated superficial cleavage for another actinotrichid mite, the prostigmatic *Tetranychus urticae*, but this was later revised to mixed cleavage (Dittrich, 1968; Casperson et al., 1986). Most recently, Dearden et al. (2002) postulated that early embryogenesis in *T. urticae* does not include an early syncytial phase.

Since the recent studies of Dearden et al. (2002) and Walzl et al. (2004) were on very different groups of actinotrichid mites, mixed cleavage with a syncytial phase is certainly not a universal and perhaps not even a normal feature of actinotrichid embryology. Less information exists for the second main group of mites, the Anactinotrichida, but Fagotto et al. (1988) reported total cleavage in an argasid tick, which was previously postulated to have intralecithal cleavage. Therefore, the common generalization that intralecithal cleavage is the usual mode of cleavage in Acari is doubtful, as is the syncytial phase in early embryogenesis.

One major group of actinotrichid mites, for which no cleavage data exist, is the taxon Oribatida (~10,000 species). Thus it is not known if their early embryogenesis involves intralecithal cleavage, as usually assumed for Acari, or if a phase of holoblastic cleavage precedes the superficial blastoderm formation. Cleavage data for an exemplar oribatid mite would contribute to a reevaluation of the ground-plan for mite embryogenesis. As the important points in intralecithal and mixed cleavage are the early divisions of blastomeres or nuclei and the formation of the blastula, we focused on these phases of embryonic development in the middle-derivative species *Archegozetes longisetosus* Aoki 1965 (Trhypochthoniidae). This is one of the oribatid mites most studied under laboratory conditions and is a model organism for developmental, genetic and evolutionary studies of chelicerate arthropods (e.g. Telford and Thomas, 1998; Thomas, 2002; Smrz and Norton, 2004; Heethoff et al., 2006, 2007; Bergmann et al., 2008; Laumann et al., 2008). *A. longisetosus* has a pan-tropical distribution and reproduces by automictic thelytoky (female parthenogenesis; Heethoff et al., 2006), like all studied members of the Malaconothroidea (Palmer and Norton, 1990). This mechanism includes the meiotic production of haploid nuclei and terminal fusion, i.e. the fusion of the egg pronucleus with the second polar body to restore diploidy (Laumann et al., 2008).

2. Materials and methods

2.1. Origin and culture of specimens

Adult *A. longisetosus* are large (approximately 1 mm) and have a relatively high fecundity and short generation time in the laboratory. We used the strain *A. longisetosus* ran (Heethoff et al., 2007), a genetic lineage established in 1993 and used in various subsequent studies (e.g. Smrz and Norton, 2004; Köhler et al., 2005; Heethoff et al., 2006, 2007; Bergmann et al., 2008; Laumann et al., 2008).

Our stock-cultures of *A. longisetosus* were kept in constant climatic conditions (23 °C, relative humidity 90%) in plastic boxes on a plaster-of-Paris/charcoal (6:1) substrate under constant darkness. Cultures were fed twice each week with unicellular green algae (mainly *Protococcus* sp.) growing on bark of various trees. Bark was treated for 45 s at full power in a domestic

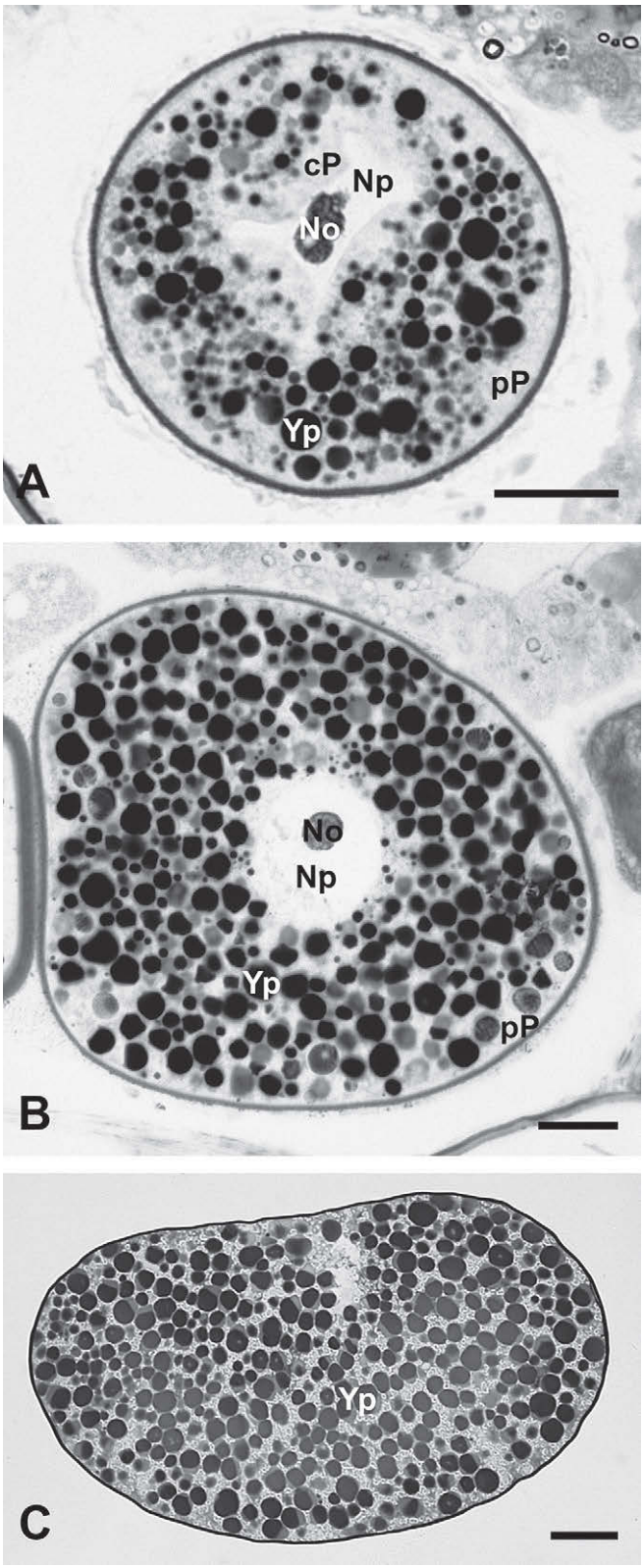

Fig. 1. Early eggs of *A. longisetosus* (LM; chemical fixation). (A) Cross section of an early egg in the ovary. The lobed nucleus increases the contact area between the nucleoplasm and the cytoplasm. (B) Cross section of a growing egg in the ovary. Accumulation of yolk displaces the peripheral and the central plasma, while the lobes of the nucleus are lost. (C) Longitudinal section of an egg from the oviduct after choriogenesis filled with yolk platelets with no remaining peripheral plasma. Abbreviations: cP, central plasma; No, nucleolus; Np, nucleoplasma; pP, peripheral plasma; Yp, yolk platelet. Scale bars = 20 μm.

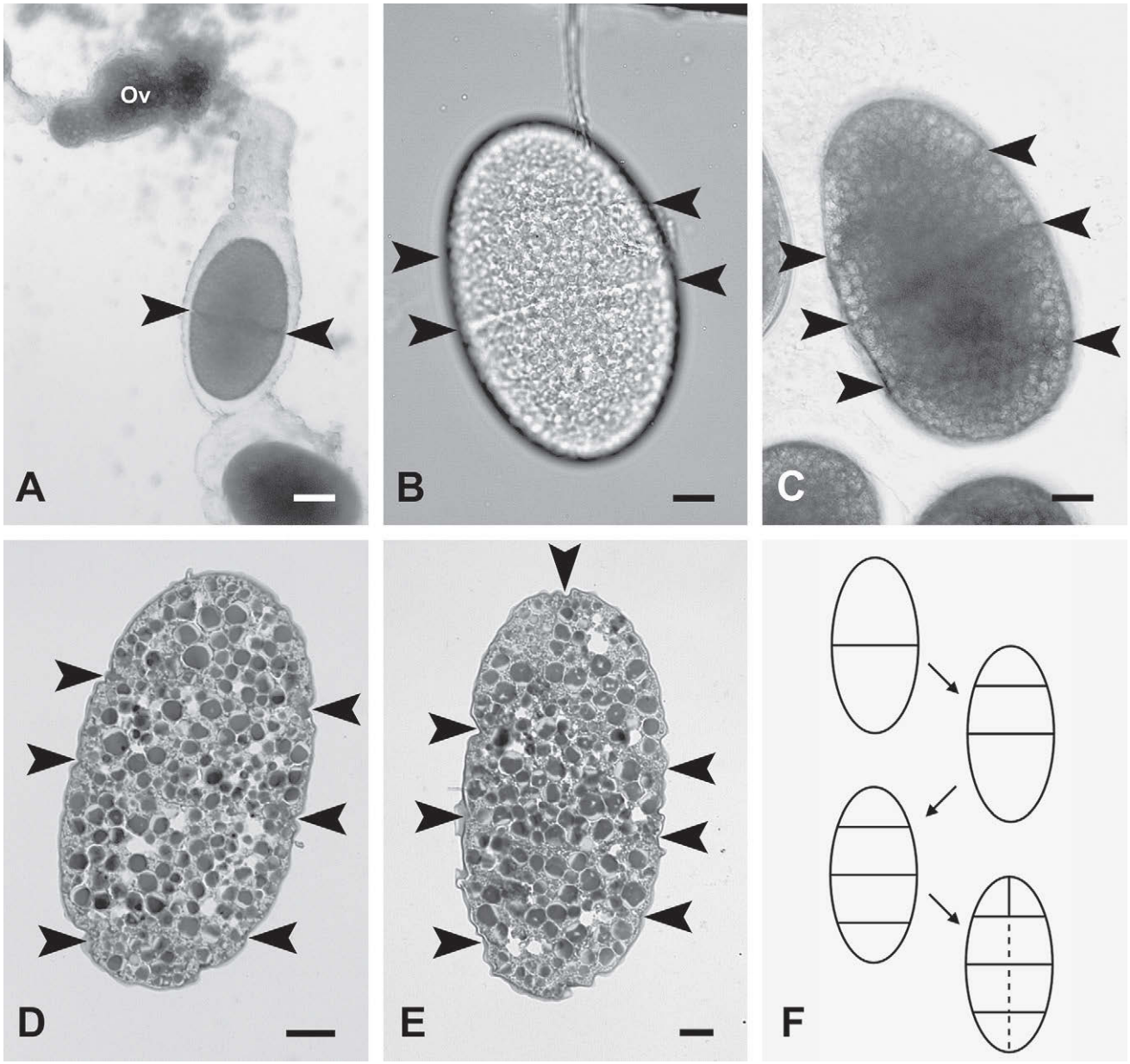

Fig. 2. Early cleavages in dissected eggs of *A. longisetosus* (arrowheads indicate cleavage furrows). (A) Dissected female genital tract showing the ovary, proximal region of an oviduct, and the first cleavage of an egg within it; both blastomeres have reached their finite volume (LM; saline buffer). Scale bar = 50 μm. (B) Asynchronous cleavage of the first two blastomeres resulting in a three-cell stage, as was confirmed by a fluorescent micrograph (LM; lactic acid). Scale bar = 20 μm. (C) Embryo in the four-cell stage with parallel cleavage furrows; yolk platelets visible through the chorion (LM; saline buffer). Scale bar = 20 μm. (D) Longitudinal semi-thin section of the four-cell stage showing the cell membranes crossing the whole diameter; this proves holoblastic cleavage, as was confirmed by TEM. Arrowheads indicate small inversions where the cleavage furrows encounter the chorion. Differences in size of the blastomeres are due to the angle of sectioning (LM; chemical fixation). Scale bar = 15 μm. (E) Longitudinal semi-thin section of the five-cell stage indicating asynchronous cleavage (LM; chemical fixation). Scale bar = 10 μm. (F) Schematic drawing of the consecutive early cleavages resulting in the eight-cell stage. Abbreviation: Ov, ovary.

microwave to prevent contamination with other arthropods. Gravid adult specimens were removed from the culture to obtain eggs in different cleavage stages; eggs were easily seen through the translucent cuticle. Post-vitellogenetic eggs after choriogenesis, which are ready to develop, were dissected from the oviduct of females, as described below. For studying the pre-vitellogenetic stages in the ovary, whole females were processed, because the cell membrane of pre-vitellogenetic eggs does not withstand the chemical preparation process. The stage of development at oviposition ranges from cleavage stages to the larva, which makes it difficult to give precise statements about the age of the embryos.

2.2. Specimen preparation of whole individuals for LM

Gravid adults were collected alive from cultures and fixed by incubation in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for at least 24 h at 4 °C. The samples were washed three times in 0.1 M cacodylate buffer (pH 7.2) and dehydrated through graded concentrations of 70–100% ethanol (5% steps) for 10 min per step; each step was repeated three times. The dehydrated material was transferred to propylene oxide for 60 min and embedded in propylene oxide low-viscosity Agar Resin LV (Plano, Germany) by steps of 1:1, 1:3, and 1:7, for 1 h per step, then in pure resin at 4 °C overnight. Polymerization was allowed to occur at 60 °C for 2 days.

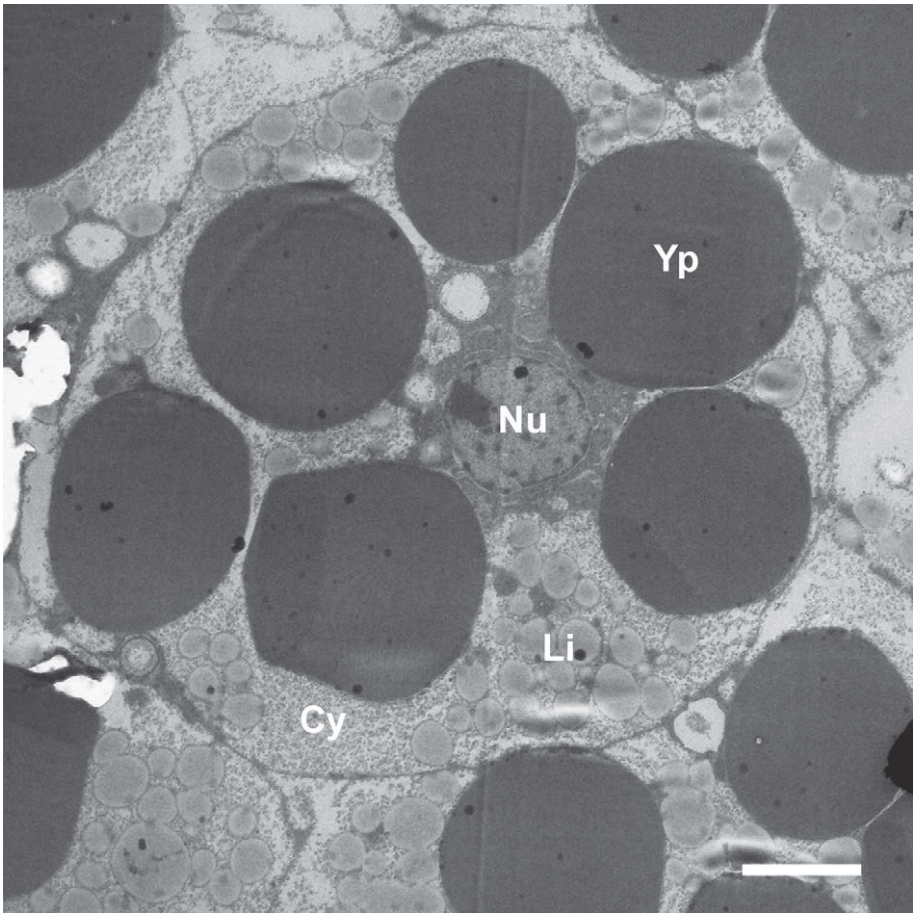

Fig. 3. Macromere packed with yolk platelets and lipid droplets (TEM; chemical fixation). The electron-dense cytoplasm around the nucleus that contains the endoplasmic reticulum is clearly visible. Abbreviations: Cy, cytoplasm; Li, lipid droplet; Nu, nucleus; Yp, yolk platelet. Scale bar = 2 μm.

Semi-thin sections (2 μm) were cut using a Leica Ultracut UCT (Leica, Austria). These sections were stained with 1% toluidine blue, mounted in Entellan (Merck, Germany), observed under a Zeiss Axioplan microscope and documented with a Zeiss MrC5 digital camera (Zeiss, Jena).

2.3. Preparation of eggs

Eggs were removed from gravid females by dissection in saline buffer containing 4% sucrose, using micro-needles (Norton and Sanders, 1985). Whole mount preparations of eggs for LM were made in lactic acid. For serial sectioning, eggs were transferred to special baskets for simultaneously processing. These baskets consist of upside-down BEEM-capsules with the bottom removed and a fine-meshed polyamide gauze (45 μm) inside the pierced lid. Baskets were placed for 120 s in 2.8% sodium hypochloride solution (DanKlorix, Germany) to render the eggshell permeable to aqueous fixatives and rinsed three times with pre-chilled 0.1 M cacodylate buffer containing 4% sucrose (Walzl and Gutweniger, 2002).

2.4. Chemical fixation of eggs for LM and TEM

Chemical fixation was performed at 4 °C unless otherwise noted: primary fixation of prepared eggs was done in half-strength Karnovsky's fixative in 0.1 M cacodylate buffer containing 4% sucrose for 60 min. Samples were washed three times in pre-chilled 0.1 M cacodylate buffer. Secondary fixation was done in 2% osmium tetroxide in 0.1 M cacodylate buffer for 60 min. Samples were washed three times in pre-chilled 0.1 M cacodylate buffer. Specimens were passed through graded concentrations of 30%, 50% and 70% ethanol. Staining with uranyl acetate was done in 70% ethanol for 60 min. Dehydration was continued in 10% steps; finally the ethanol was replaced by propylene oxide for 15 min. Samples were gradually infiltrated in Epon 812 (Serva, Germany) at room temperature, with propylene oxide–resin mixture in the ratios of 3:1, 2:1, 1:1, 1:2, 1:5 and pure resin for 1 h each, and finally with fresh pure resin overnight on a rotary shaker and embedded in epoxy resin in BEEM-capsules. Polymerization was allowed to occur at 45 °C for 12 h followed by 60 °C for 48 h. Semi-thin and ultra-thin sections were cut using a Reichert Ultracut microtome (Leica, Austria).

Semi-thin sections were stained with methylene blue and alkaline fuchsine, embedded in Entellan (Merck, Germany), surveyed in a Zeiss Axioplan light microscope and documented with a Zeiss MrC5 digital camera (Zeiss, Germany). Ultra-thin sections were stained with aqueous lead citrate for 3 min and viewed in a Philips Tecnai 10 transmission electron microscope (FEI, Netherlands) or a LEO 906 transmission electron microscope (Zeiss, Germany) at 80 kV.

2.5. Cryofixation of eggs for LM and TEM

Prepared eggs were transferred to aluminium planchettes (cavity 150 μm) filled with 1-hexadecene. High pressure freezing was done in an HPM 010 (Bal-Tec, Liechtenstein). Samples were freed from 1-hexadecene below −100 °C and transferred in

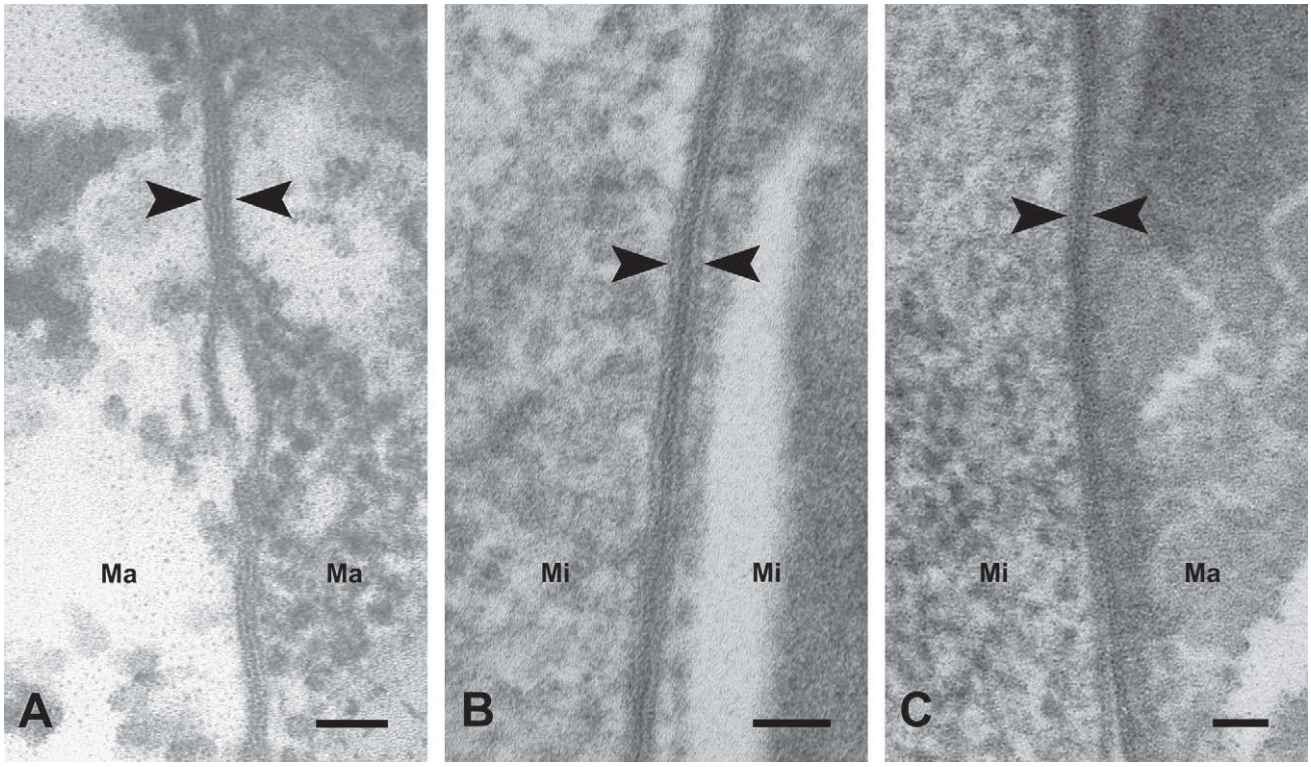

Fig. 4. Cell membranes of micromeres and macromeres in embryos of *A. longisetosus*, with cell membranes of adjacent blastomeres indicated by arrowheads. (A) Detail of the cell membranes of two adjacent macromeres (TEM; chemical fixation). (B) Detail of the cell membranes between two micromeres (TEM; cryofixation). (C) Detail of the cell membranes between a macromere and a micromere (TEM; cryofixation). Abbreviations: Ma, macromere; Mi, micromere. Scale bars = 50 nm.

cryotubes in a Leica FS unit (Leica, Germany) at −90 °C filled with acetone containing 2–4% water, 0.1% osmium tetroxide, 0.5–2.5% methanol and 0.1–0.2% uranyl acetate. After 60 h at −90 °C samples were kept at −60 °C for 8 h, also 0.5% glutaraldehyde in acetone was added. After 10 h at −35 °C samples were washed four times (30 min each) with acetone containing 0.5% glutaraldehyde and 2–4% water. Samples were further warmed to −20 °C for 30 min and thereafter brought to 0 °C for 30 min and washed four times (60 min each) with acetone while further warming to room temperature. Samples were gradually infiltrated in Epon 812 (Serva, Germany) at room temperature, with propylene oxide–resin mixture in the ratios of 9:1, 4:1, 2:1, 1:1, 1:2, 1:5 and pure resin for 2 h each, finally with fresh pure resin overnight on a rotary shaker. Samples were embedded in epoxy resin in BEEM-capsules, and allowed to polymerize at 45 °C for 12 h and 60 °C for 48 h. Semi-thin and ultra-thin sections were cut using a Reichert Ultracut microtome (Leica, Austria).

Semi-thin sections were stained with methylene blue and alkaline fuchsin, embedded in Entellan (Merck, Germany), surveyed in a Zeiss Axioplan light microscope and documented with a Zeiss MrC5 digital camera (Zeiss, Germany). Ultra-thin sections were stained with aqueous lead citrate for 3 min and viewed in a Philips Tecnai 10 transmission electron microscope (FEI, Netherlands) at 80 kV.

3. Results

3.1. Morphology and growth of the oocyte

The diploid oocytes of *A. longisetosus* display a central nucleus surrounded by cytoplasm. After the meiotic divisions in the ovary, including degradation of the first polar body and fusion of the egg pronucleus with the second polar body, yolk vesicles accumulate and vitellogenesis takes place. In early vitellogenesis, the nucleus contains distinct nucleoli and is lobed (Fig. 1A). Due to the lobes the nucleus seems relatively large with a diameter up to 40 μm. During the growing process the yolk precursors and the yolk platelets divide the plasma of the egg into an outer zone of peripheral plasma, which lacks inclusions of yolk and has a thickness of 3–5 μm, and an inner zone of central plasma containing the yolk and the nucleus.

With accumulation of yolk in late vitellogenesis the nucleus becomes spherical with a diameter of 25–30 μm (Fig. 1B). After finishing vitellogenesis the cytoplasm is densely packed with yolk platelets of 5–7 μm diameter, each of which consists of a central part composed of crystalline structures of vitelline surrounded by a less tightly packed peripheral part. As vitellogenesis is completed, the peripheral plasma diminishes and choriogenesis takes place (Fig. 1C).

3.2. Cleavage

After the completion of vitellogenesis, the first cleavage of the embryo takes place in the proximal part of the oviduct. The egg has an ellipsoid shape and the cleavage is transversal and holoblastic (Fig. 2A). Both blastomeres separate partly from each other before they expand again and only the cleavage furrow is visible, resulting in a two-cell stage consisting of two equally sized blastomeres. The second cleavage is not coincidental in both cells. One blastomere of the two-cell stage shows a furrow which is again transversal and holoblastic. The furrow is not perpendicular but parallel to the first cleavage furrow (Fig. 2B). Subsequently the second blastomere divides in the same manner, resulting in four blastomeres. The parallel cleavage furrows are visible through the compact yolk mass

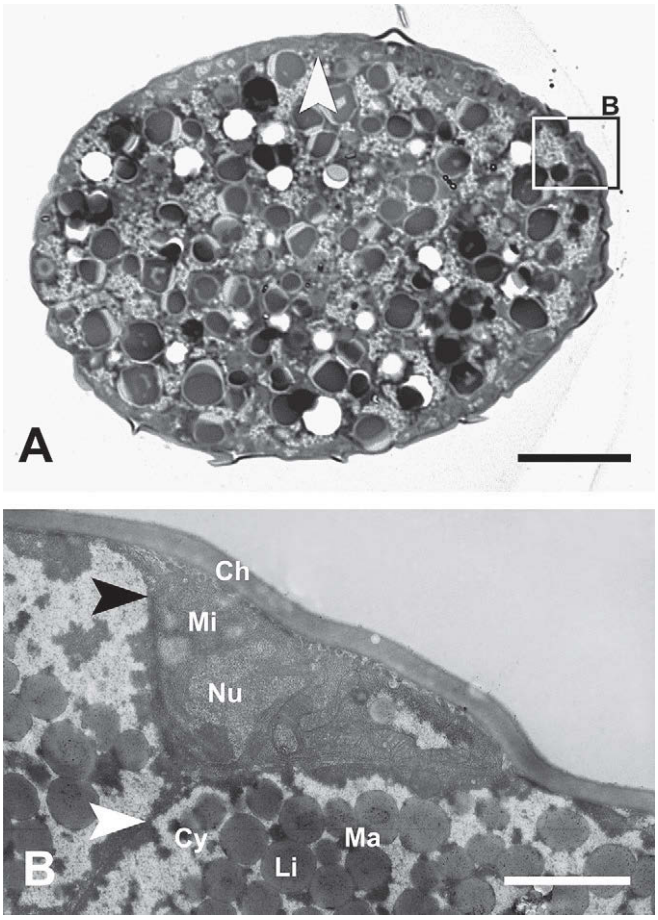

Fig. 5. Preblastula of *A. longisetosus*. (A) Longitudinal section of an embryo (LM; chemical fixation). While some micromeres are clustered beneath the surface of the embryo (indicated by the arrowhead), predominantly scattered single micromeres are embedded at the surface between macromeres. Cell membranes of micromeres are not detectable in this section. Labeled inset marks the region of the embryo which is shown magnified in B. Scale bar = 20 μm. (B) Detail of a single micromere embedded between macromeres of the same embryo (TEM; chemical fixation). The black arrowhead indicates the cell membranes of the micromere and the adjacent macromere, the white arrowhead indicates the cell membrane of adjacent macromeres. The cell membranes surrounding the macromeres and the micromere are clearly visible. Scale bar = 2 μm. Abbreviations: Ch, chorion; Cy, cytoplasm; Li, lipid droplet; Ma, macromere; Mi, micromere; Nu, nucleus.

(Fig. 2C) and each blastomere contains more or less equal parts of the yolk (Fig. 2D). The second cleavage thus results in a situation where only two of the four adjacent blastomeres meet at the poles of the minor axis of the ellipsoid and only one blastomere contacts each pole of the major axis. The third cleavage is also asynchronous; it starts in one of the blastomeres at the poles of the major axis, divides the cell longitudinally to form the five-cell stage (Fig. 2E) and finally results in the eight-cell stage. A schematic view of the cleavage sequence is shown in Fig. 2F. Throughout these early cleavages, the yolk is more or less evenly distributed throughout the embryo.

3.3. Preblastula and blastula

The micromeres contain mitochondria, endoplasmic reticulum, lipid droplets and glycogen. In contrast, most of the volume of macromeres is occupied by yolk platelets and lipid droplets, and their nucleus is surrounded by electron-dense cytoplasm (Fig. 3). The yolky macromeres do not subsequently divide or do so only sporadically; also, the cross-sectional area of their nuclei in the preblastula ($5.8 \pm 3.6\ \mu m^2$; $n = 8$) and in the blastula ($5.4 \pm 1.7\ \mu m^2$; $n = 26$) of several studied specimens was not significantly different, with $F(1,32) = 0.176$, $p = 0.678$ (ANOVA).

Cell borders in the early cleavages are visible in both whole mount preparations and semi-thin sections, but as cell division proceeds – at least after division of the blastomeres in micromeres and macromeres – the delicate cell membranes between macromeres (Fig. 4A), micromeres (Fig. 4B), and between the two types (Fig. 4C) can be seen only by TEM.

Micromeres in the preblastula do not form a continuous layer (Fig. 5A), but are scattered on the embryonic surface as relatively flat cells (Fig. 5B). Their nuclei are larger than those in the blastula, as indicated by a significant difference in their cross-sectional area in several studied preblastulae ($9.5 \pm 6.0\ \mu m^2$; $n = 73$) and blastulae ($3.0 \pm 1.4\ \mu m^2$; $n = 126$) with $F(1,197) = 135.9$, $p < 0.0001$ (ANOVA). The cleavages proceed on the surface of the preblastula, thus micromere divisions have to be tangential.

An external, monocellular layer of micromeres gradually covers the whole surface of the preblastula, surrounding a central sphere of macromeres, to complete the formation of the blastula (Fig. 6A and B). The micromeres may have very long and thin prolongations until they reach their neighbor (Fig. 6C and D). Subsequently, micromeres start to divide radially deeper in the embryo, building a second row of micromeres in the region of the germ disc (Fig. 7A). In this process the cells and the nuclei of the outer row lose their round shape and become more elliptical (Fig. 7B). This elongation is significant with $F(1,159) = 216.909$, $p < 0.0001$ (ANOVA) for the ratio of length:width between outer row micromere nuclei ($2.2 \pm 0.5\ \mu m^2$; $n = 104$) and inner row micromere nuclei ($1.3 \pm 0.2\ \mu m^2$; $n = 57$). In clusters next to this growing zone, the micromeres and their nuclei maintain their round shape (Fig. 7C), and on the dorsal side of the embryo the flattened micromeres with the thin prolongations exhibit no signs of growth (Fig. 7D).

4. Discussion

4.1. Early development of the oocyte

The nuclei of the early oocytes of *A. longisetosus* are typically lobed with many nuclear pores, resulting in an increase in the area of chromatin in contact with the cytoplasm and hence the availability of the DNA to cytoplasmic factors (Haynes and Davies, 1973). A similar pattern of lobed nuclei in early development was shown in the astigmatic mite *S. berlesei* (Walzl et al., 2004). Also the increase and decrease in nuclear size during vitellogenesis, as shown for *A. longisetosus*, is known from other yolky eggs such as those of the tick *Ornithodorus moubata* (Diehl, 1970).

The late egg of *A. longisetosus* is of a perilecithal type with the small zone of peripheral plasma having vanished. This type of egg is also known from the tarsonemid mite *Pediculopsis graminum* (Reuter, 1909). The presence or absence of the periplasm in the eggs of Actinotrichida is not clearly established: some taxa evince peripheral plasma (Hafiz, 1935; Walzl et al., 2004) while others do not (Klumpp, 1954; Langenscheidt, 1958). In the Anactinotrichida, the basic structure of eggs is also not uniform: ticks seem to have centrolecithal eggs (Aeschlimann and Hess, 1984; Fagotto et al., 1988), whereas eggs of the gamasid mite *Spinturnix vespertiliones* are alecithal (Yastrebtsov, 1992). Although data concerning the anatomy of eggs are sparse, existing studies indicate considerable differences in the types of eggs among the major taxa of Acari. Therefore, a general description of acarine eggs as being centrolecithal (Aeschlimann and Hess, 1984) seems inappropriate.

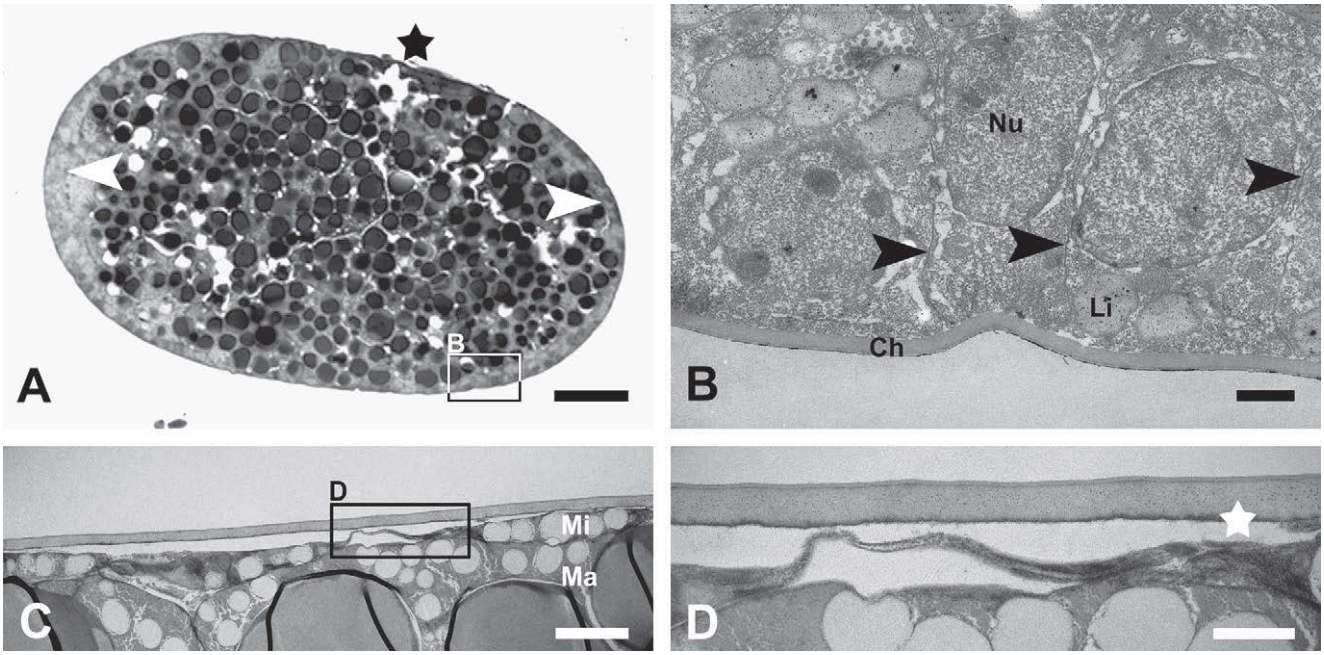

Fig. 6. Blastula of *A. longisetosus*. (A) Longitudinal section of a blastula (LM; chemical fixation). As in the preblastula, the cell membranes of the micromeres cannot be detected in LM. Arrowheads indicate close-packed micromeres near the poles of the ellipsoid embryo; along the longitudinal axis the micromeres are in contact with each other but are misshapen and not detectable in LM (asterisk). Labeled inset marks the region of the embryo which is shown magnified in B. Scale bar = 20 μm. (B) Detail of the same embryo, showing the close-packed micromeres (TEM; chemical fixation). Arrowheads indicate the cell membranes between micromeres. Scale bar = 500 nm. (C) Detail of micromeres along the longitudinal axis of an embryo, corresponding to the area indicated by the asterisk in A (TEM; cryofixation). In contrast to B micromeres are extremely flattened, with prolongations about 1 μm thick. The labeled inset is shown magnified in D. Scale bar = 2 μm. (D) Detail of the cell contact (asterisk) between two micromeres. The margins of the cells are about 50–100 nm thick and contain only cytoplasm. Scale bar = 500 nm. Abbreviations: Ch, chorion; Li, lipid droplet; Ma, macromere; Mi, micromere; Nu, nucleus.

4.2. Pattern of cleavage

The early cleavages in *A. longisetosus* are asynchronous, as is known for other Acari (Reuter, 1909; Edwards, 1958; Prasse, 1968; Walzl et al., 2004; Grbic et al., 2007). A three-cell stage was shown for some other actinotrichid mites – Prostigmata (Reuter, 1909; Edwards, 1958) and Astigmata (Prasse, 1968; Walzl et al., 2004) – with the first and second cleavages of their embryos perpendicular to each other. In contrast, the asynchronous second cleavage in *A. longisetosus* is pivoted, so that the first and second furrows do not contact each other, instead forming a linear array of four blastomeres. We did not find any other arrangement of four blastomeres as a linear array. This rather unusual arrangement of blastomeres has not yet been described for acarine eggs. However, this organization of blastomeres seems not to be critical for normal development, as artificial embryos of the marine shrimp *Sicyonia ingentis* with the first four blastomeres manipulated into a linear array were able to form spherical blastulae and became swimming larvae (Wang et al., 1997). In the Acari the topology of the first blastomeres in the egg seems to be generally variable, as a radial arrangement of blastomeres is known from the eight-cell stage of the argasid tick *O. moubata* (Fagotto et al., 1988) and the four-cell stage of the prostigmatic mite *T. urticae* (Dearden et al., 2002; Grbic et al., 2007), but the second cleavage furrow may lie at different angles to the first cleavage furrow in the latter (Dittrich, 1968). In the five-cell stage of the astigmatic mite *S. berlesei* two blastomeres are located exteriorly at the poles of the major axis and three blastomeres are along the minor axis of the ellipsoid egg (Walzl et al., 2004).

4.3. Preblastula and blastula

In accordance with Walzl et al. (2004) we use the term preblastula to indicate an embryo consisting of scattered micromeres embedded between macromeres, whereas in the blastula a monolayer of micromeres encloses all macromeres. This is in comparison to the syncytial blastoderm in insects, which is referred to as preblastoderm with the cellular blastoderm following (e.g. Zissler, 1992). We use this comparison as an illustrative analogy, with no homology implied. The preblastula of *A. longisetosus* consists of scattered micromeres surrounding a central bulk of yolky macromeres, indicating that in the course of the previous cell divisions total cleavage of each blastomere gives rise to an internal macromere and an external micromere on the surface of the embryo. We did not detect any micromeres detached from the surface of the embryo between the macromeres. The same positioning of micro- and macromeres was shown in the embryo of the argasid tick *O. moubata* (Fagotto et al., 1988). This pattern was also shown by Walzl et al. (2004) for the astigmatic mite *S. berlesei*, although these authors also mentioned some unexplained micromeres lying deeper within the embryo. In general, total cytoplasmic volume does not increase during cleavage; the cells generally progress through a cell cycle consisting of the S-phase (DNA-synthesis) and the M-phase (mitosis) abolishing the growth period (G_1 and G_2 phases) between the cell divisions (Gilbert, 2000). As one consequence, the volume of cytoplasm is divided among increasingly smaller cells, which contain smaller, more compact nuclei (Gossett and Hecht, 1980). In the preblastula of *A. longisetosus* the micromere nuclei are significantly larger than those of the blastula, indicating that cell division has progressed. In contrast, nuclei of the macromeres were of equal size in preblastula and blastula, indicating the absence of division and therefore a delay or suppression of cleavage. This pattern was also suggested for the astigmatic mite *S. berlesei* (Walzl et al., 2004) and shows that at various times single cells or groups of cells may cease to divide or may acquire a cleavage rhythm different from that of their neighbors, as was postulated earlier (Gray, 1927).

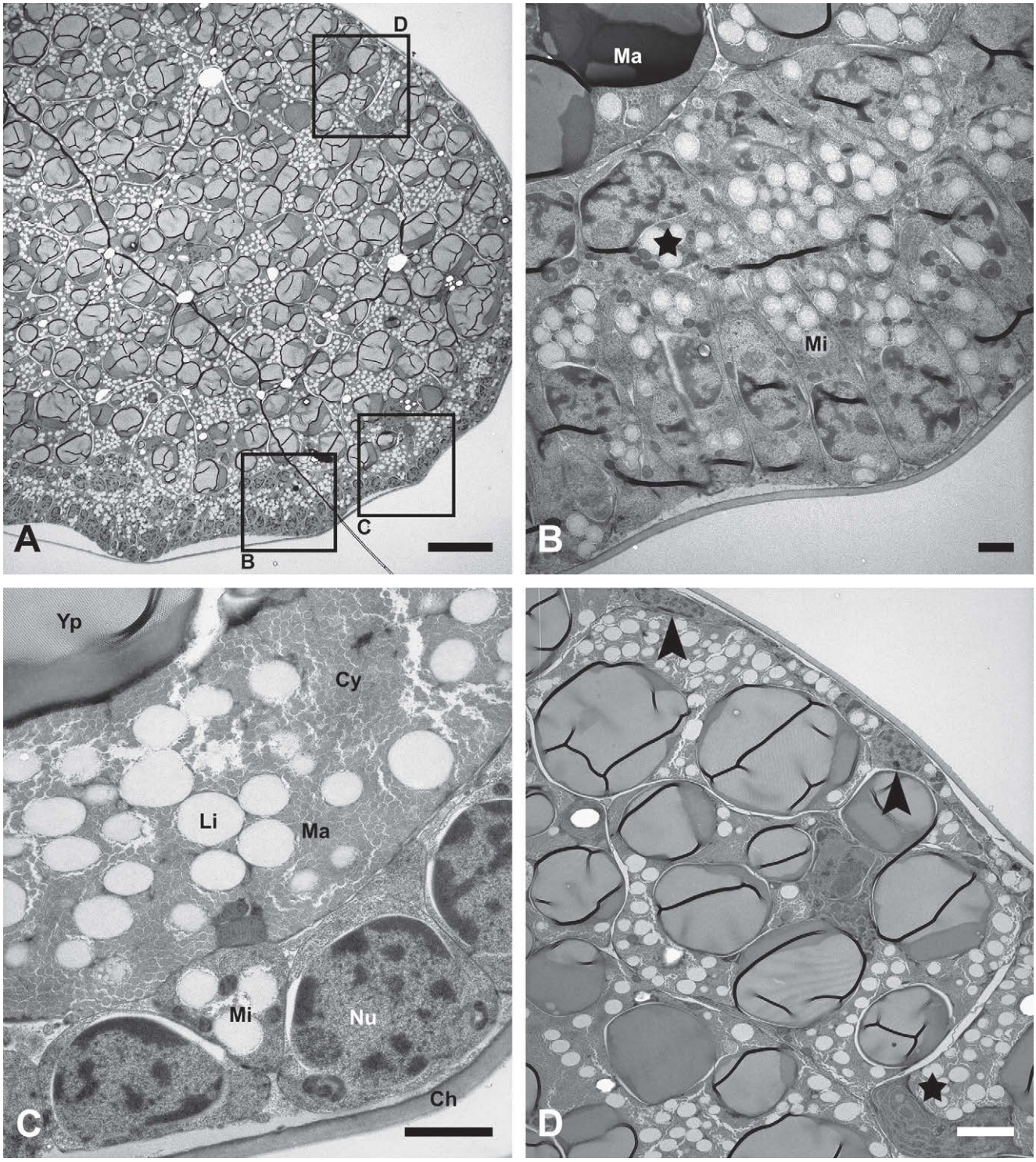

Fig. 7. Germ disc stage of *A. longisetosus* (TEM; cryofixation). (A) Overview of a sectioned embryo. The lower part of the micrograph shows the ventral hemisphere of the embryo with the germ disc and proliferating micromeres; the upper part shows the dorsal hemisphere with flattened and prolonged cells. Labeled insets mark the regions of the embryo which are shown magnified in B-D. Scale bar = 10 μm. (B) Detail of the germ disc with elongated micromeres in the outer row, whereas in the inner row the micromeres (asterisk) are not elongated. Scale bar = 1 μm. (C) Detail of one of the poles of the longitudinal axis of the embryo. The micromeres in this region are single rowed and have not altered their shape. Scale bar = 1 μm. (D) Detail of the dorsal hemisphere of the embryo with flattened micromeres, the arrowheads indicating their nuclei. The electron-dense cytoplasm surrounding the nuclei of a macromere can be seen; their nuclei need not be located in the center of the macromere (asterisk). Scale bar = 2 μm. Abbreviations: Ch, chorion; Cy, cytoplasma; Li, lipid droplet; Ma, macromere; Mi, micromere; Nu, nucleus; Yp, yolk platelet.

Since cleavages are asynchronous and macromere cleavage is mostly delayed or suppressed, at least the generally known 32-cell stage and following stages probably do not exist in *A. longisetosus*. Data concerning the cell stage (mitotic cycle) of blastomere differentiation into micro- and macromeres are scarce. Fagotto et al. (1988) observed cell differentiation to occur in the 256 or 512-cell stage (synchronous cleavage) in the embryo of the argasid tick *O. moubata*, with the radially

arranged blastomeres – they extend from the center of the egg to the periphery – dividing radially into an outer micromere and an inner macromere. In contrast Walzl et al. (2004) showed an embryo of the astigmatic mite *S. berlesei* in 35-cell stage (asynchronous cleavage) with scattered micromeres on the surface embedded between macromeres.

In the blastula a monolayer of cells encloses the yolk without forming a blastocoel. This type of blastula was formerly generalized for the Acari and described as a periblastula with a blastocoel obliterated by an important central yolk mass (Aeschlimann and Hess, 1984). Also, with macromeres building the central yolk mass, vitellophages do not appear in *A. longisetosus*. Whether the macromeres remain unchanged and degenerate, or contribute to the formation of the embryonic midgut, or are involved in germ cell specification is unknown and must be clarified by further investigation (Aeschlimann and Hess, 1984; Fagotto et al., 1988; Dearden et al., 2003; Walzl et al., 2004). The radial proliferation and aggregation of the micromeres indicate the appearance of the germ disc, which may be considered as gastrulation in the embryo of *A. longisetosus*, as described for the Acari in general (Aeschlimann and Hess, 1984; Evans, 1992).

Our results indicate that *A. longisetosus* exhibits an embryonic specialization among arachnids: early holoblastic cleavage, with blastomeres having equal amounts of yolk, is followed by differentiation into non-segregating macromeres that build the yolk mass and segregating micromeres that build the blastoderm. These results question the idea that intralecithal cleavage and the syncytial phase in mixed/combined cleavage are normal features of mite embryology. Therefore, species supposedly with intralecithal cleavage and mixed/combined cleavage, such as the astigmatic mite *Acarus siro* (Sokolov, 1952; Prasse, 1968) and the prostigmatic mites *Cheyletus eruditus* (Hafiz, 1935; Edwards, 1958) and *T. urticae* (Claparède, 1868; Dittrich, 1968; Casperson et al., 1986; Dearden et al., 2002), should be reinvestigated.

4.4. Acari, Chelicerata and Euarthropoda

The monophyly of each distinct group of Acari, i.e. the Actinotrichida and the Anactinotrichida, is supported by morphological data (Alberti, 2006 and references therein) and molecular data (Murrell et al., 2005; Klompen et al., 2007), but whether they are sister taxa forming a monophyletic taxon Acari is controversial. The higher phylogeny of the Acari with respect to the other Arachnida remains also controversial; they have been considered – in whole or in part – sister-group of most other Arachnida, namely the Arachneae, Opiliones, Palpigradi, Solifugae, Ricinulei or Pseudoscorpionida, depending on the molecular and/or morphological characters analyzed (reviewed in Dunlop and Alberti, 2007). Modern phylogenetic results concluded the position of the Acari to be highly dependent on parameter choice of the phylogenetic analysis (Giribet et al., 2002), but current molecular data recovered the sister-groups Actinotrichida–Solifugae and Anactinotrichida–Pseudoscorpionida resulting in diphyletic Acari (Dabert et al., 2010; Regier et al., 2010). A close relationship between these taxa was earlier postulated on the basis of morphological characters by Dunlop (2000).

Total cleavage, as postulated for the oribatid mite *A. longisetosus*, and shown in each of the existing TEM studies of acarine embryology, is rare in the Chelicerata and seems to be limited to the viviparous scorpions, in which total divisions result in a spherical mass of small cells surrounding a central blastocoel, and the pseudoscorpions, which have a mode of cleavage unique among arachnids (Anderson, 1973). In the total cleavage pattern of this latter group the blastomeres differentiate into scattered, external and yolk-free micromeres, which divide further and build the blastoderm, and internal yolky macromeres. These undergo no further divisions and fuse to a nucleated bulk of yolk. A few or only one micromere gives rise to the embryonic envelope, an embryonic membrane beneath the egg membrane (Weygoldt, 1964, 1965, 1968). Despite the presence of the egg membrane, which is not formed in eggs of the Acari, the pattern of cleavage in Pseudoscorpionida, especially the differentiation of external patches of micromeres and internal macromeres, might contain some embryological/morphological evidence for joining them and the Anactinotrichida as closely related, but this comparison is only preliminary. The early embryogenesis of the Solifugae is unknown, making any comparison with the Actinotrichida impossible.

Alterations from intralecithal to total, holoblastic cleavage, or vice versa, occurred in several euarthropod taxa, and reversals back to the former state have produced a patchy distribution of cleavage type in the euarthropods, even within closely related taxa (Scholtz, 1997). To our knowledge the Acari seem to be the largest of the clusters that show a stable, evolutionary long-term holoblastic cleavage pattern. Considering holoblastic cleavage forming part of the ground pattern of the Pycnogonida, as recently indicated by Ungerer and Scholtz (2009), this might reflect the ground pattern of the Chelicerata. They also inferred that the Arthropoda might share an early cleavage pattern characterized by a holoblastic cleavage; with the available cleavage data of the Acari we tentatively support this conclusion and preliminarily contribute to the long-standing question whether the ancestral cleavage mode of euarthropods was a holoblastic or superficial cleavage.

Further investigations on early embryology of Acari promise to provide more evidence for a reconstruction of the ancestral cleavage pattern of this group and might contribute to a reexamination of the traditional interpretations of chelicerate development.

5. Conclusions

Embryological evidence is important to our understanding of metazoan evolution, and if reasonable phylogenetic conclusions are to be drawn from embryology, the ground pattern for the clade in question needs to be established (Wennberg et al., 2008). For the Acari the hypothetical ground pattern in early embryology was considered to be intralecithal or superficial cleavage, because it was thought to be the most widespread type of cleavage in this group. This conclusion was mainly based upon LM techniques and semithin sections in combination with the former assumption that cytokinesis does not progress through dense, yolky cytoplasm (Arnold, 1969). Conversely, recent data using TEM point out that the cleavage pattern in Acari is highly specialized among Chelicerata. These surprising results are revealed only by the high magnifications obtainable by TEM, as the delicate cell membranes of the micromeres and macromeres are not visible with LM. To confirm that the holoblastic cleavage pattern described herein can be considered part of the ground-plan of Acari, it should be sought in a wide range of taxa, including those that were subjects of earlier studies but also a good representation of Anactinotrichida, which are currently represented only by ticks.

Acknowledgements

The authors thank Monika Meinert for excellent technical support and assistance. We also thank Prof. Dr. Oliver Betz and Prof. Dr. Manfred G. Walzl for essential discussions. Dr. York Stierhof is acknowledged for excellent sample preparation and Dagmar Ripper for technical help. We are very grateful to Daniela-Yvonne Weide for useful discussions and Eva-Maria Hoch and Laura Ruoff for technical help. Also we wish to thank two anonymous reviewers for their stimulating and constructive comments on an earlier version of this manuscript.

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5.6 Publication 6

Laumann, M., Bergmann, P. and Heethoff, M. (2008). "Some remarks on the cytogenetics of oribatid mites". — Soil Organisms **80**, 223–32.

Direct link

SOIL ORGANISMS Volume 80 (2) 2008 pp. 223 – 232

ISSN: 1864 - 6417

Some remarks on the cytogenetics of oribatid mites

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Abstract

The behaviour of the meiotic segregations of the parthenogenetic oribatid mite *Archegozetes longisetosus* Aoki were investigated in maturing oocytes. Data about the meiotic mechanisms in parthenogenetic oribatid mites are very sparse, although the mechanism of reproduction is fundamental for the restoration of diploidy and has different consequences for the genotype of the offspring. For our analyses we used a combination of traditional methods and the novel technique of synchrotron X-ray holotomography. We describe the points in maturation at which the meiotic segregations occur and the positions of the oocytes at this time in the female genital tract. As the position of the nuclei in the first segregation is located beneath the cell membrane and in the second one central in the cell, we believe to have evidence for an inverted meiotic sequence.

Keywords: Parthenogenesis, holokinetic chromosomes, inverted meiosis, *Archegozetes longisetosus*, synchrotron X-ray micro-holotomography

Zusammenfassung

In reifenden Oozyten der parthenogenetischen Hornmilbe *Archegozetes longisetosus* Aoki werden die meiotischen Teilungen untersucht. Die Datengrundlage über meiotische Prozesse bei parthenogenetischen Oribatiden ist sehr spärlich, obwohl der Reproduktionsmechanismus fundamental für die Wiederherstellung der Diploidie ist und unterschiedliche Konsequenzen für den Genotyp des Nachwuchses mit sich bringt. Für unsere Untersuchungen benutzten wir eine Kombination aus traditionellen Methoden und Synchrotron Röntgen-Holotomographie. Wir beschreiben die Zeitpunkte und Positionen der meiotischen Teilungen im weiblichen Genitalsystem. Da die erste meiotische Teilung direkt unterhalb der Zellmembran stattfindet, während die zweite zentral in der Zelle stattfindet, meinen wir Hinweise auf eine invertierte meiotische Teilung zu finden.

1. Introduction

The cellular mechanism of meiosis forms the basis of sexual reproduction, whereas asexual reproduction is mostly linked with mitosis. But also a certain type of parthenogenesis includes meiosis as basic process: automixis (Bell 1982, Suomalainen et al. 1987, Hughes 1989, Heethoff 2003). Automictic organisms restore diploidy by fusion of the egg and one of the polar nuclei, which is possible by a number of alternative mechanisms. For example, by duplication of the haploid egg and fusion of these two products to give rise to a complete homozygous diploid embryo, as some crustaceans and insects do (Suomalainen et al. 1987). Also, diploidy can be rebuilt by central fusion. In this mechanism the two central polar nuclei

fuse, restoring the heterozygous state of the mother depending on the segregation of chromosomes in meiosis II. Central fusion is known from some insect species (Suomalainen et al. 1987). A third mechanism is terminal fusion, the fusion of the egg with the second polar nucleus, which leads to complete homozygosity. Some species of nematodes, lumbricids, crustaceans, insects and mites have realised terminal fusion (Suomalainen et al. 1987, Heethoff 2003).

There are a lot more mechanisms for automicts to restore diploidy, many of them incompletely known, but as the eggs of automictic parthenogenetic species have undergone meiosis, they differ considerably from apomicts and asexuals in genetic and evolutionary aspects (Heethoff 2003).

For studying parthenogenesis, oribatid mites (Acari, Oribatida) have become an important group (Norton et al. 1993, Schaefer et al. 2006, Domes et al. 2007, Heethoff et al. 2007b, Laumann et al. 2007, Heethoff et al. in press) since they include species-rich clusters of exclusively thelytokous taxa (Palmer & Norton 1992, Maraun et al. 2004). Recent molecular works have shown that speciation events happened in these parthenogenetic oribatid mite clusters (Maraun et al. 2004, Heethoff et al. 2007b, Laumann et al. 2007). Due to biogeographic evidence it was concluded that these taxa are more than 100 million years old (Heethoff et al. 2007b) and that some taxa even predate the break up of Pangaea (Hammer & Wallwork 1979). This implies that parthenogenesis is not necessarily an evolutionary dead end. And an impressing case displaying this is a group of oribatid mites which indeed re-evolved sex from a parthenogenetic ancestor, a spectacular case of breaking Dollo's law (Domes et al. 2007).

A specific cellular mechanism should be fundamental for the radiation of parthenogenetic oribatid mite species. Only one cytological study of meiosis in parthenogenetic oribatid mites is available (Taberly 1987). Here, automictic thelytoky (including the meiotic production of haploid gametes) and terminal fusion (fusion of the egg pronucleus with the second polar body) were proposed as the cellular mode of reproduction. This mechanism restores diploidy and maintains homozygosity (Suomalainen et al. 1987). Contradictory to these findings, Palmer & Norton (1992) indicated fixed heterozygosity and absence of recombination for parthenogenetic oribatid mites using isozyme techniques. Molecular analyses of nuclear genes also indicated that nuclear recombination is absent in parthenogenetic oribatid mites (Schaefer et al. 2006). However, these incompatible findings can be in unison, if the sequence of reductional and equational divisions in meiosis is inverted. This idea of inverted meiosis, based on the presence of holokinetic chromosomes, as mode of reproduction for parthenogenetic oribatid mites was established by Wrensch et al. (1994).

Holocentric chromosomes have no localised centromer, the microtubules attach over the whole length of the chromosome. These holocentric chromosomes are termed holokinetic, as the kinetochore is the functional part in the movement of the chromosome, not the centromere per se (John 1990, Wrensch et al. 1994, Dernburg 2001, Heethoff 2003).

As the idea of inverted meiosis is based exclusively on theoretical considerations, empirical data of meiotic cleavages in parthenogenetic oribatid mites are urgently needed.

We used X-ray synchrotron micro-holotomography and semi-thin sectioning to study characteristics of the meiotic disjunctions in the parthenogenetic oribatid mite *Archegozetes longisetosus* Aoki, 1965. We show the place and the point in time of the meiotic segregations in the ovary and we trace the fate of the first polar body.

2. Materials and methods

All specimens used in this study belong to the lineage *Archegozetes longisetosus* ran (Heethoff et al. 2007a). This species is rapidly becoming a model organism for a wide spectrum of biological traits in chelicerate animals (Telford & Thomas 1998, Thomas 2002, Heethoff et al. 2007a). Laboratory conditions of the culture are described in Heethoff et al. (2007a).

2.1. Light microscopy

The fixation and processing steps were carried out in a fume cupboard at room temperature unless otherwise noted. Adult specimens were collected alive, and killed and fixed by incubation in 2.5 % glutaraledyde in 0.1 M cacodylate buffer (pH 7.2) for at least 24 h at 4° C. The samples were washed three times in 0.1 M cacodylate buffer (pH 7.2). Specimens were dehydrated through graded concentrations of 70 to 100 % ethanol (5 % steps) for 10 min per step; each step was repeated three times. The 100 % ethanol step was done at 4 °C overnight. The dehydrated material was transferred to propylene oxide for 60 min and embedded in 1:1, 1:3, 1:7 propylene oxide low-viscosity Agar Resin LV (Plano GmbH, Germany) by incubation for 1 h per step. The specimens were finally incubated in pure resin at 4° C overnight and polymerised by incubation at 60 °C for 2 days. Semi-thin sections (2 μm) were cut using a diamond knife and a Leica Ultracut UCT. These sections were stained with 1 % toluidine blue (Richardson et al. 1960), mounted in Entellan® (Merck KGaA, Germany) and observed under a Zeiss Axioplan microscope with a Zeiss MrC5 digital camera.

2.2. X-ray synchrotron microtomography

Adult specimens were collected alive, killed and fixed by incubation in a 6:3:1 mixture of 80 % ethanol, 38 % formaldehyde and 100 % acetic acid for at least 24 h.

The samples were washed and dehydrated as indicated above for light microscopy. The dehydrated specimens were critical point dried with CO_2 (CPD 020, Balzers AG, Liechtenstein), glued with the posterior end of the opisthosoma onto the tips of plastic pins (3.0 mm diameter) and stored in an exsiccator. X-ray synchrotron microtomography was accomplished at the European Synchrotron Radiation Facility (ESRF, Grenoble: beamline ID 19, experiment SC2127) at an energy level of 20.5 keV. The effective pixel size was 0.7 μm and the detector-to-sample distance was 10, 20 and 45 mm. Over the 180° sample rotation, 1300 projections were recorded (ESRF FreLoN camera) with an exposure time of 0.35 s each. Details about this novel technique and case studies of microarthropods are provided in Cloetens et al. (2006), Betz et al. (2007) and Heethoff & Cloetens (this issue). Separation and visualisation of individual structures, such as single cells, was accomplished by using the visualisation software tool amira™ (Mercury Computer Systems, Germany) on a 64-bit Dual-Opteron computer system.

3. Results

The reproductive system of *Archegozetes longisetosus* comprises an unpaired ovary, located posterioventral to the ovipositor, and paired oviducts which emanate from the ovary, form an s-curve and pass in an arc through the body. The oviducts merge anterior to the ovipositor. As a more precise description of the female reproduction system of *A. longisetosus* is introduced by Bergmann et al. in this issue, we will not go into further detail. Consequently, we use the terminology for the different parts of the female genital tract suggested by Bergmann et al. in this issue.

Oocytes are cuboid in form and constantly growing after the disconnection from the rhodoid of the ovary. The nuclei of this previtellogenetic oocytes measure 10 to 20 µm in diameter. In the majority of cases nuclei are in a central position and typically lobed (Fig. 1). As the growing oocytes arrive in the proximal, rostrad-oriented part of the meros of the ovary, the nucleus migrates to the cell membrane and the first meiotic division takes place (Fig. 2). The oocyte nucleus segregates and provides the egg nucleus and the first polar body. The oocyte nucleus migrates into a central position in the cell. Both show nucleoli and completed nuclear membranes. At this stage of maturation the growing oocyte is located in the first bend of an s-curve of the meros (Fig. 3).

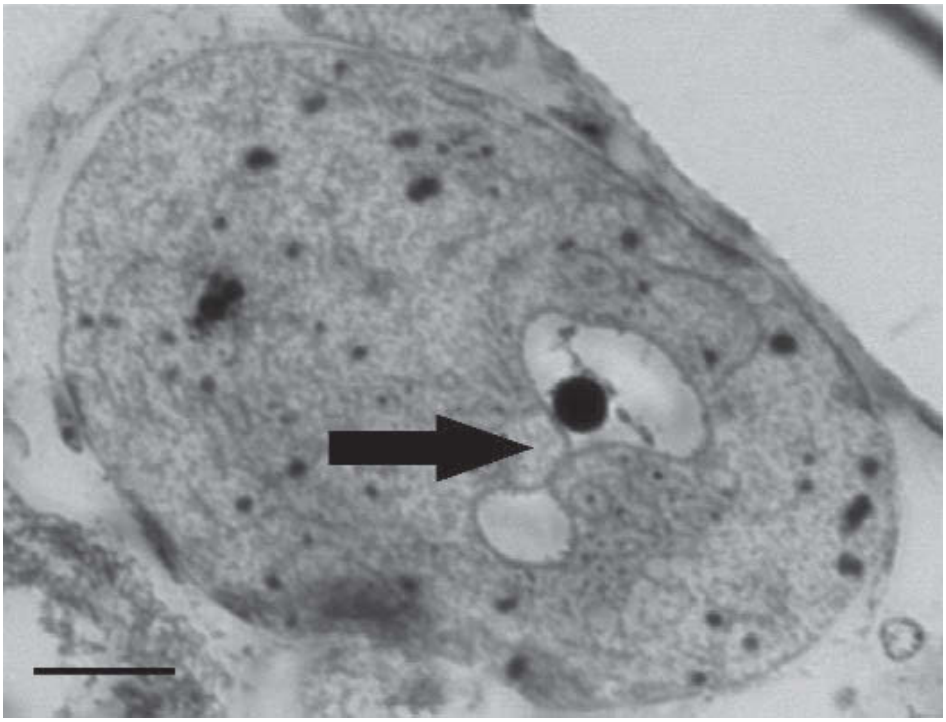

Fig. 1 Semi-thin section showing the lobed nucleus in the centre of a growing oocyte. One nucleolus is visible in the larger nuclear lobe. Both lobes are connected by a cytoplasm-nucleoplasm-cytoplasm sheet (arrow). Bar: 10 µm.

In the following growing process of the oocyte the nuclei remain in central position. The second meiotic division takes place in the distal, anad-oriented part of the meros of the ovary (Fig. 4). The nuclear membrane is disintegrated and the chromosomes are condensed. Here the growing oocyte is located in the second bend of the s-curve of the meros (Fig. 3). Presently after the second meiotic division the growing oocytes are covered with a compact vitelline envelope and further development of the embryo begins.

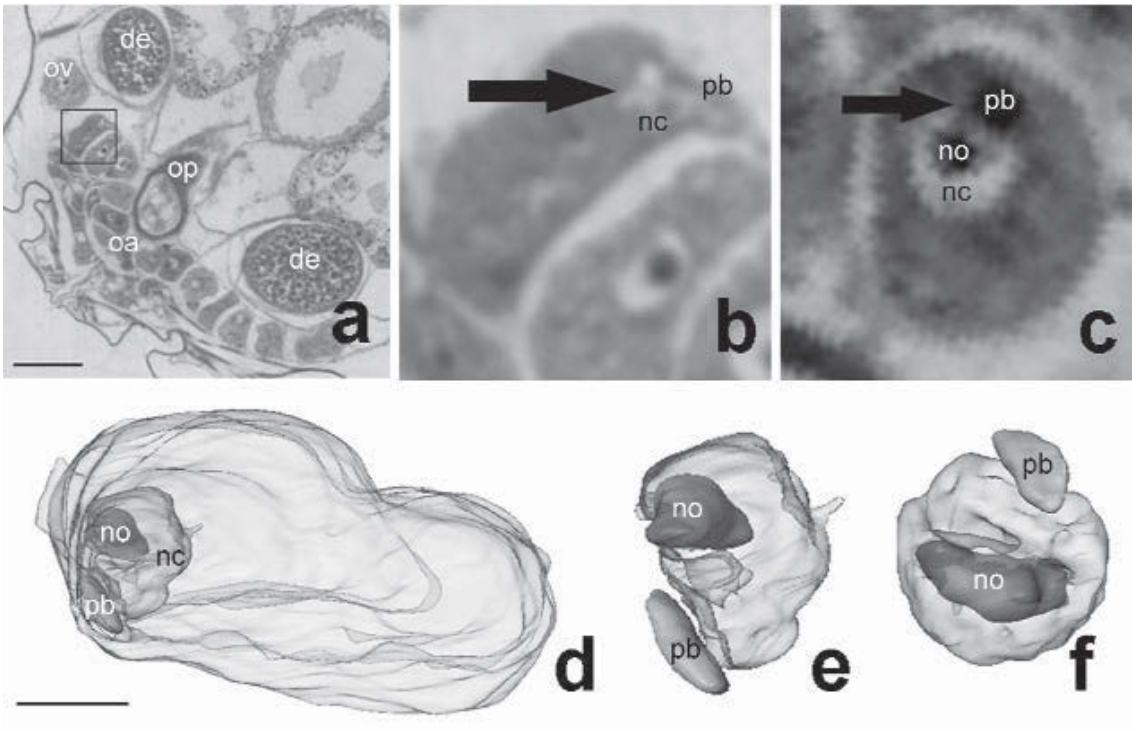

Fig. 2 Holotomographic slices (a to c) and 3D-reconstruction (d to f) of a growing oocyte in meiosis I. Disjunction has taken place, noticeable is the difference in size between the two nuclei. (a) transversal holotomographic slice of the reproductive system showing the ovary (oa), an oocyte in vitellogenesis (ov), two developing eggs (de) and the ovipositor (op). The frame is indicating the detail scaled up in (b) and (c). Bar: 50 µm. (b) transversal holotomographic slice showing the nucleus of the oocyte (nc) and the putative polar body (pb; indicated by the arrow). (c) sagittal holotomographic slice of the same cell, the arrow is indicating the putative polar body (pb), indicating the spatial separation of nucleus and pb. (d) 3D-reconstruction in sagittal view of the growing oocyte. Nucleus (nc), nucleolus (no), polar body (pb). Bar: 10 µm. If the cell membrane of the growing oocyte is removed, in sagittal view (e) and transversal view (f) the spatial separation of the nucleus and the putative polar body is obvious.

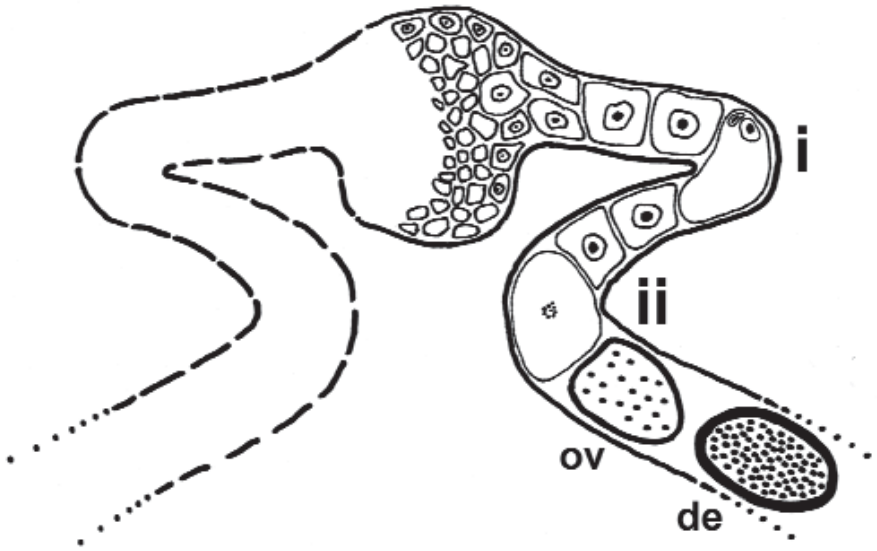

Fig. 3 Schematic diagram of the topology of the ovary showing the unpaired rhodoid and the paired meros of the ovary up to the periphery of the oviduct with an oocyte in vitellogenesis (ov) and a developing egg (de). (i) first bend of the s-curve in which the first meiotic division is taking place. (ii) second bend of the s-curve, the meiotic segregation is completed.

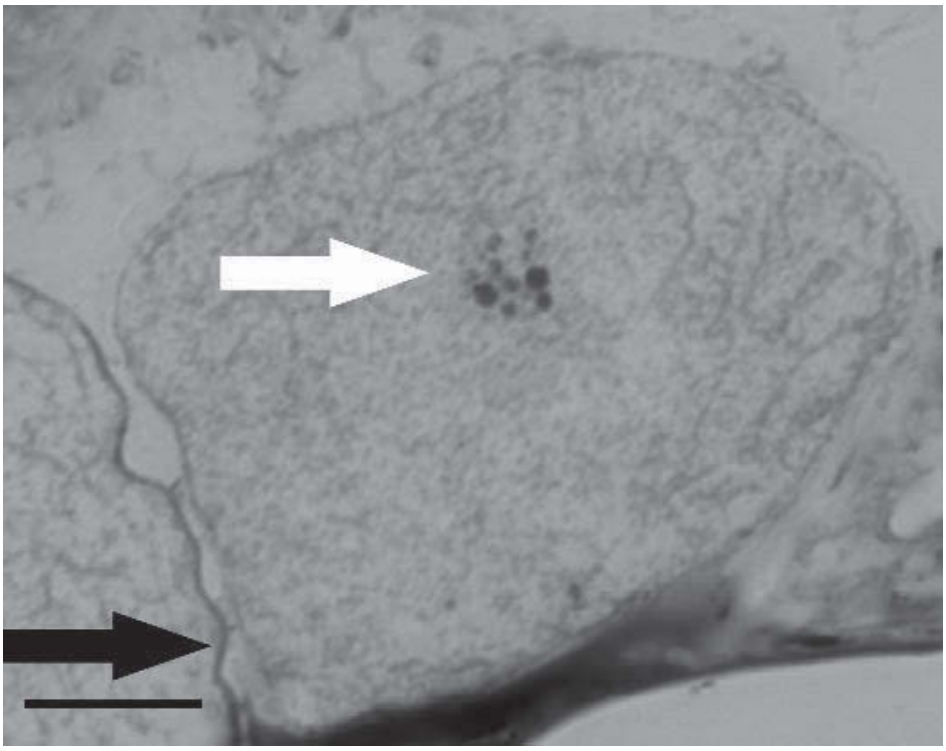

Fig. 4 Semi-thin section showing a growing oocyte in metaphase II, the nuclear membrane is disintegrated; chromosomes are condensed (white arrow). The second meiotic disjunction occurs in the centre of the cell, in contrast to the first meiotic segregation. The black arrow is indicating the onset of the production of the vitelline membrane. Bar: 10 μm.

4. Discussion

The essential premise to accomplish an inverted sequence of meiotic divisions are holokinetic chromosomes (Wrensch et al. 1994), where kinetochore activity is diffused along the entire length of the chromosome. A number of oribatid mite taxa have been analysed, but yet no specific peculiarities in the gross morphology of the chromosomes were found, especially no primary and secondary constrictions, suggesting the oribatid mite species examined do not have a localised centromere (Helle et al. 1984). *Archegozetes longisetosus* also possess holokinetic chromosomes (Heethoff et al. 2006), but the time and mode of reconstitution of diploidy have not been precisely documented yet. Our analysis showed foremost that nuclei in growing oocytes are typically lobed, as are the nuclei of the first blastomeres in acarid mites (Fig. 1, Walzl et al. 2004). The formation of these cytoplasm-nucleoplasm-cytoplasm sheets results in an increase in the area of chromatin in contact with the cytoplasm and hence the availability of the DNA molecules to cytoplasmic factors (Haynes & Davies 1973). Considering their presence in certain oocytes, they are due to the increased growth of the oocyte.

In the proximal, rostrad-oriented part of the meros of the ovary we located what we suppose to represent the nucleus and the first polar body in the first meiotic division. In comparable analysis of Walzl et al. (2004) with astigmatid mites the polar body was also located beneath the cell membrane in vitellogenesis. However, Walzl et al. (2004) showed the polar body after the second meiotic segregation. This discrepancy remains to be clarified in detail.

The actual process of chromosome disjunction has not been detected as such yet, but the cell is elongated as preparation for division and a new membrane surrounds both nucleus and the putative polar body. As this is characteristic for Telophase I, we would expect cytokinesis to complete the creation of two daughter cells. Given the parietal position of both nuclei in the cell a division is rather unlikely. Furthermore we believe the chromosomes of the first polar body to be expelled from the remainder of the oocyte, as was shown for a prostigmatid mite (Feiertag-Koppen 1976). Due to the presumed degeneration of the polar body, diploidy can only be restored by terminal fusion of both sets of chromosomes after meiosis II.

The second meiotic division in the oocyte proceeds in the distal, anad-oriented part of the meros of the ovary. Meiosis is completed before the oocytes are covered with a compact vitelline envelope, as it is also known for prostigmatid and acarid mites (Heinemann & Hughes 1970, Walzl et al. 2004). In contrast to metaphase I, the metaphase II figure is situated near the centre of the ooplasm. This indicates a cytokinesis at telophase II. As diploidy is to be restored, both nuclei have to fuse again after the second meiotic segregation. Unfortunately, figures of telophase II are not available but it seems apparent to accomplish a complete or incomplete meiosis II.

The effect of a terminal fusion automixis with the precondition of inverted meiosis on the embryo would be the same genetic constitution (except for crossing-over regions) as the mother, possibly characterised as functional apomixis (Heethoff 2003).

If *A. longisetosus* exhibits an incomplete meiosis II, the constraints of automixis and apomixis will not only blur in genetic constitution but also in cellular mechanisms. In acarid mites, oogenesis in a thelytokous strain is accomplished by apomixis: during oogenesis pairing of homologous chromosomes does not take place and there is only one pseudo-maturation division (Heinemann & Hughes 1969).

However, with the data available, the assumption of an inverted meiosis with terminal fusion automixis as a cellular mechanism of parthenogenesis can be further supported but not proved without doubt, observations about the chromosomal behaviour in the first and second meiotic division have to be clarified in detail.

5. Acknowledgements

We thank R. A. Norton for providing animals to start our *A. longisetosus* culture and for all kinds of help and support, D. Weide and S. Schmelzle for help with data processing and J. Straube and K.-H. Hellmer for technical assistance.

This work was supported by the European Synchrotron Radiation Facility, Project SC2127. We acknowledge the excellent support by Peter Cloetens and Lukas Helfen at the ESRF facilities, beamline ID19.

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Accepted 30 April 2008

5.7 Publication 7

Bergmann, P., Laumann, M., Norton, R. and Heethoff, M. "Cytological evidence for automictic thelytoky in parthenogenetic oribatid mites (Acari, Oribatida): Synaptonemal complexes confirm meiosis in *Archegozetes longisetosus*". (submitted)

Cytological evidence for automictic thelytoky in parthenogenetic oribatid mites (Acari, Oribatida): Synaptonemal complexes confirm meiosis in *Archegozetes longisetosus*

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Abstract

Diplo-diploid parthenogenesis (thelytoky) is a widespread phenomenon in the mite taxon Sarcoptiformes, and is unusually frequent in the suborder Oribatida, where it characterizes almost 10% of extant species, including whole genera and families. Based on molecular and cytological data, terminal fusion automixis with an inverted meiotic sequence based on holokinetic chromosomes has been suggested as the reproductive mode in these mites. However, unequivocal structural evidence for meiosis is missing. The model organism Archegozetes longisetosus*, a thelytokous member of the parthenogenetic family Trhypochthoniidae, was studied to gain ultrastructural insight in oocyte progression and meiotic processes. In this study, ovarian nuclear organization of its tritonymphal instar was examined by transmission electron microscopy (TEM). Numerous synaptonemal complexes were observed in the ovary, unequivocally confirming automixis (meiotic thelytoky) in oribatid mites for the first time. No recombination nodules were found. The nuclei are transcriptionally active in late prophase. Inverted meiosis is discussed as a result of the spatial arrangement of chromatid segregation.*

Key words: chromatid segregation, achiasmatic, holokinetic chromosomes, ancient asexuals, diakinesis failure

5.7.1 Introduction

The synaptonemal complex (SC) (Moses 1956b) is a highly structured protein aggregation that is observed specifically during the prophase of meiosis I in most eukaryotes (Marec 1996; Page & Hawley 2004). It is associated with the pairing of homologous chromosome bivalents during the zygotene and pachytene prophase stages, which is the prerequisite for all processes linked to meiotic recombination, and thus plays a crucial role in any form of sexual reproduction. Its function is to provide a scaffold for the stable spatial arrangement of corresponding DNA sequences, which enables the action of enzymes effecting recombination as well as segregation (Page & Hawley 2004; Zetka 2009). The 60 years since the discovery of this structure in spermatocysts of crayfish (Moses 1956a) have seen much discussion as to the causal linkage and interdependence of processes like double-strand breakage (DSB), crossing over, recombination and initiation of the formation of the SC. While some studies indicate DSBs to be essential for the formation of the SC (Henderson & Keeney 2004), others point to the SC as being the prerequisite for the initiation of DSBs and subsequently recombination (McKim et al. 1998; McKim et al. 2002; Colaiácovo 2005). Recombination itself seems correlated with the occurrence of electron-dense, ellipsoid protein bodies linked to the central element of the SC, the so-called recombination nodules (Carpenter 1975, 1979a, 1979b). Formation of the SC itself, however, seems not to be directly linked to chiasmata formation and was also reported from achiasmatic meiosis (Welsch 1973; Marec 1996). We report for the first time the occurrence of SCs in a parthenogenetic (thelytokous) species of oribatid mites, a group renowned for having an unusually high proportion (nearly 10%) of suspected or confirmed obligately thelytokous species (Palmer & Norton 1991). Many of these species comprise phylogenetic clusters (clades) of various size, indicating multiple possible radiations in the absence of sex (Maraun et al. 2004; Cianciolo & Norton 2006). Among the largest parthenogenetic clades are in the middle-derivative hyporder Nothrina ('Desmonomata' sensu lato), accounting for nearly 70% of all species in the group. The apparent old age of these clades, as estimated using molecular clock techniques (Heethoff et al. 2007b) led to their designation as putative ancient asexuals, together with bdelloid rotifers and darwinuliid ostracods (Schön & Martens 2002). Considering the different long-term genetic consequences, the specific cytogenetic pathway through which female zygotes are initiated is of crucial importance. The putatively ancient parthenogenetic clade of bdelloid rotifers (Mark Welch & Meselson 2000) are confirmed apomicts. However, the case has been unclear for the various clades of parthenogenetic oribatids. Initially they also were thought to be apomictic, but some data are more consistent with automixy. To better understand oogenesis and the meiotic processes in oribatid mites, we studied the last juvenile instar (tritonymph) of the diplo-diploid

desmonomatan oribatid mite *Archegozetes longisetosus* Aoki, 1965 (Trhypochthoniidae), a model mite species (Heethoff et al. 2013). This parthenogenetic species appears quasi-clonal, with fixed heterozygosity and therefore was first hypothesized to be apomictic or to have a nonsegregative automixis (Palmer & Norton 1992). The type of parthenogenesis in a confamilial species, *Trhypochthonius tectorum* (Berlese, 1896) and in the related *Platynothrus peltifer* (C.L. Koch, 1839; Crotoniidae) appears to be terminal fusion automixis (Taberly 1987; referred to as mixocinèce), albeit solely based upon the interpretation of chromosome segregation in late metaphase using light microscopy. The observed fixed heterozygosity in *A. longisetosus* is incongruent with such a mechanism, except in the case of inverted meiosis (achiasmatic at least for all coding regions, and with an inversed order of meiotic divisions where reductional division is preceeded instead of followed by equational division)(Wrensch et al., 1994). Up to now, proof of unequivocally meiotic ultrastructural features has not been available for this or any other other parthenogenetic oribatid mite. Expulsion of a polar body during late previtellogenesis, as well as transient chromosome condensation indicating a second, incomplete division, indicating terminal fusion automixis, was reported from adult specimens of *A. longisetosus* earlier (Laumann et al., 2008), but distinct characteristic features of early meiotic prophase could not be observed in adult females. Examination of juvenile instars of *A. longisetosus* indicated the proliferation of oocytes from oogonia already during the deutonymphal stage (Bergmann & Heethoff 2012). Microtomographic studies on the development of the genital organs indicated the beginning of previtellogenesis in peripheral oocytes of tritonymphs shortly before molting to the adult stage, but did not permit analysis of nuclear features (Bergmann et al. 2008). Therefore, we studied the nuclei of oocytes in tritonymphal ovaries by transmission electron microscopy (TEM) to test the hypothesis of automixis by attempting to locate and examine ultrastructural features of meiotic prophase during the early stages of oocyte development in *A. longisetosus*. As we show in this study, SC formation in the oocytes of middle aged tritonymphs indicates meiotic prophase to start in the last subadult instar, prior to the onset of previtellogenesis. Further, the presence of SCs in *A. longisetosus* rules out apomixis as the reproductive mechanism underlying clonality.

5.7.2 Materials and Methods

Rearing

Archegozetes longisetosus was cultured on a mixture of plaster-of-Paris and powdered charcoal (~6:1 parts by volume) cast at a thickness of 4cm in 150ml plastic screw cap jars. The vessels were covered with fine-mesh (40µm) synthetic gauze and closed with a perforated

lid. The culture vessels were stored in constant dark at 21-23℃ and checked twice a week for sufficient moisture. Bark of deciduous trees covered with unicellular green algae was provided ad libitum as a food source and replaced regularly with the moisture checks. Animals for processing were collected with a fine brush from molting aggregations prior to the appropriate instar and kept separated under the same conditions as described above in 12-well tissue culture plates until fixation.

Microscopy

Specimens for microscopy were submersed in pre-chilled (0°C) modified Karnovsky´s solution (1.3% formaldehyde, 1.66% glutardialdehyde, 4% sucrose, 6.6µM MgSO4 in 0.05M sodium 4-(2-hydroxyethyl)-1- piperazineethanesulphonic (HEPES) acid buffer at pH 7.8). The fixative was prepared directly beforehand using freshly depolymerised paraformaldehyde (Fluka, Buchs, Switzerland) and EM-grade glutardialdehyde 25% (Science Services, Munich, Germany). Specimens were punctured with a fine tungsten needle (Norton & Sanders 1985) upon submersion and fixed for 90min at 0°C and slight vacuum of approx. 300mbar. After rinsing three times for 10 minutes each in ice-chilled 0,05M HEPES buffer, postfixation was conducted in 1% OsO4 in 0.05M HEPES buffer at pH 7.8 and 0°C for 60min. After another rinsing step, the samples were dehydrated in a graded ethanol series, including en-bloc-staining with uranyl acetate, and embedded in Araldite Cy212 (plano GmbH, Wetzlar, Germany), using acetone as intermedium. A detailed description of the process is given in a preceeding publication (Bergmann et al. 2010). Ultrathin sections of 50nm and 60nm thickness were cut using a Reichert Ultracut (Leica-Jung, Vienna, Austria) microtome and diamond knives (diatome 45°, Biehl, Switzerland), and mounted on single slot copper grids (Science Services, Munich, Germany) coated with polyvinyl formal (Formvar, Monsanto Chemical Company, St Louis, Missouri, USA). The sections were poststained with uranyl acetate for 20-40 min and lead citrate for 90 sec (Venable and Coggeshall 1965), and examined with a Siemens Elmiskop 1A (Siemens & Halske, Berlin, Germany) transmission electron microscope at 80kV. Images were recorded with an attached 6.5x9cm plate camera on SO-163 electron image film (KODAK, Rochester, New York, USA). Original negatives were scanned, and images digitally scaled and processed using the programmes iTEM (Olympus Soft Imaging Solutions GmbH, Münster, Germany), GIMP 2.6.10 (GNU Image Manipulation Program, Copyright © 1995–2008 Spencer Kimball, Peter Mattis and the GIMP development team) and ImageJ (Wayne Rasband, National Institutes of Health, USA; ImageJ is in the public domain). Measurements of SC structures also were taken in iTEM and ImageJ.

5.7.3 Results

Tritonymph, 2-3 days after moulting

Sections from the ovary show a single layer of flattened somatic cells with interphase nuclei surrounding a dense mass of germline cells. Filiform protrusions of the germ cells containing numerous microvilli almost fill a central hollow which apparently is closed by one or few somatic cells ventro-medially (Fig. 1). These somatic cells are separated from the germ cell cluster by a basal lamina (Fig. 1b). Germcells are easily recognizable by the occurrence of prominent synaptonemal complexes (SCs). All germcells show SCs in various stages of development: Centrally located cells tend to be smaller and have smaller nuclei with less prominent SCs, probably representing early stages. Peripherally situated cells are generally larger, and have larger nuclei featuring fully developed SC figures with all typical components. This configuration is diagnostic of the pachytene stage of meiotic prophase I. Large cells in the caudo-dorsal periphery of the ovary possess large, electron lucent nuclei with indistinct or absent SC sections, and frequently one or two dense masses of chromatin. These cells probably represent a further developed diplotene stage, where degradation of the SC already occurs.

The fully developed SCs in pachytene (Fig. 2) consist of central elements, transverse fibers and lateral elements surrounded by dense chromatin masses. Lateral elements of 11-23nm width are approximately 90-130nm apart, and regular transverse elements of 3-6nm thickness in the electron-light space between lateral and central elements occur at intervals of approx. 13-14nm. Central elements stain very electron dense. Longitudinal sections of SC show central elements as more or less parallel lines of dense structures separated by a slightly less electron dense zone (Fig. 2b). In several sections, the central structure exhibits regularly appearing spots of higher density in intervals of 17-35nm. The whole central zone has a width of 24-36nm. Transverse sections of SC appear as electron lucent, sharply defined circles surrounded by a thin, electron dense, fibrous layer, and dense masses of chromatin (Fig. 2c). In the centre of the electron lucent zone, dark spots indicate transverse sections of two, or in several cases, four central elements in a rhombic configuration, indicating the formation and presence of tetrads. Round and ellipsoid transverse sections of SC found in the images could either be due to developmental stages of SC formation, or slanted angles of sectioning. The dimensions of round transverse sections, or the short diameter of ellipsoid ones, respectively, correspond to measurements taken from longitudinally sectioned SCs. In several cells, a close association of SC end with the nuclear envelope is apparent (Fig. 3). SC-like structures associated with the nuclear membrane in centrally located oocytes that seem to lack transverse or lateral elements (Fig. 3b)

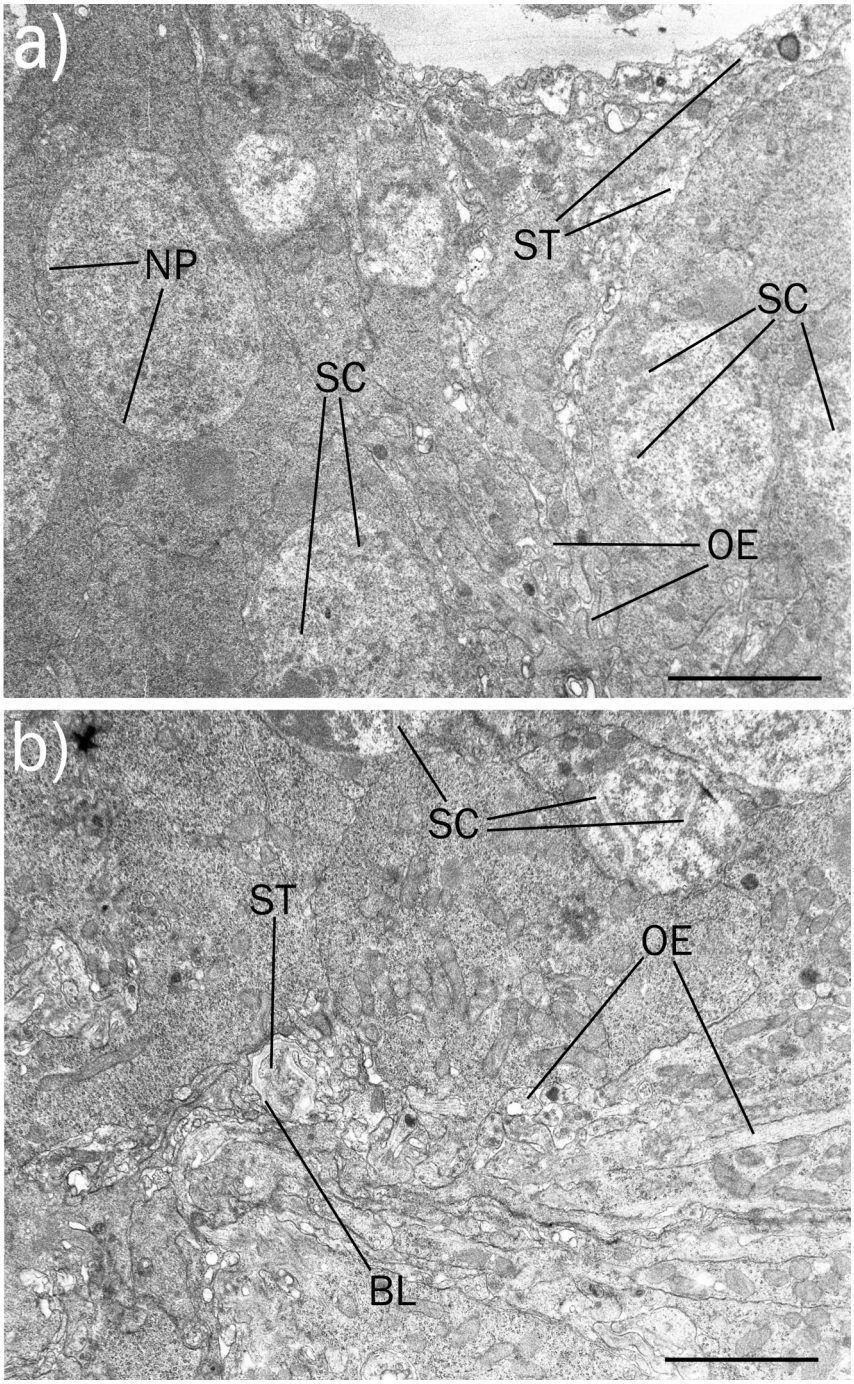

Figure 5.1: *A. longisetosus.* Overview of the arrangement of germcells and somatic tissue in the ovary of a 2 day old tritonymph. a) rostro-medially; b) centrally in the ovary. Abbreviations: BL: basal lamina, NP: nuclear pores, OE: oocyte extension, SC: synaptonemal complex, ST: somatic tissue. Scale bars: 2µm

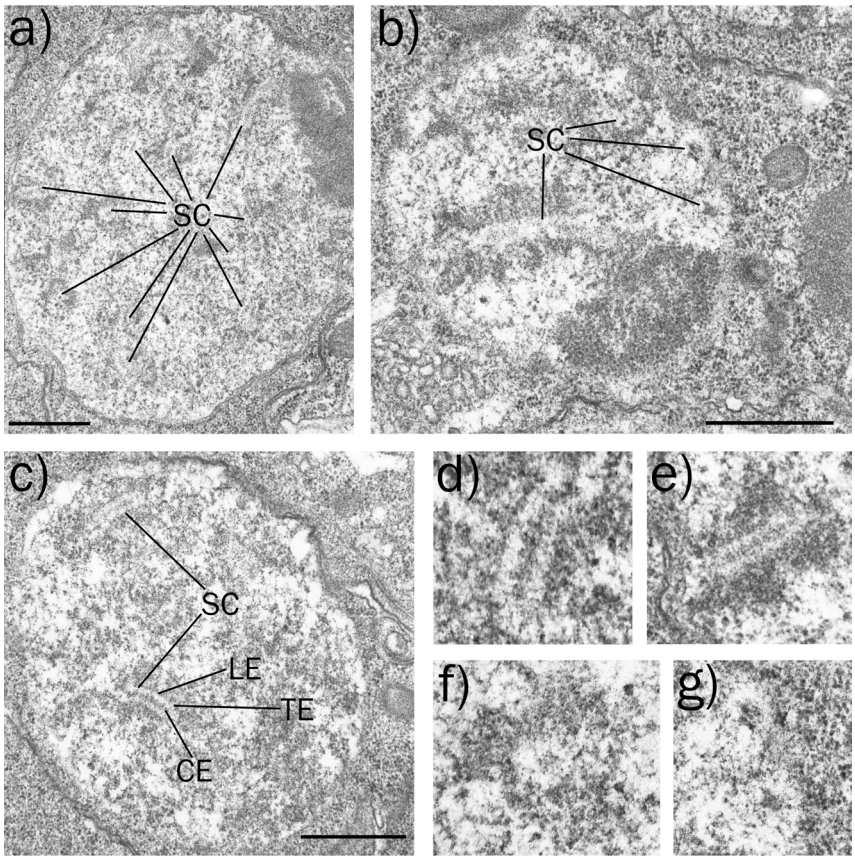

Figure 5.2: *A. longisetosus.* Synaptonemal complexes in oocytes of a 2-day old tritonymph. a)-c): Three pachytene nuclei; d)-g) Details: Synaptonemal complexes in longitudinal (d), e)), and transversal (f), g)) section. Abbreviations: CE: central element, LE: lateral element, SC: synaptonemal complex, TE: transversal element. Scale bars: 500nm

can be interpreted as longitudinally sectioned lateral elements, probably from early stages of SC formation.

No dark structures resembling recombination nodules were observed on a total length of 75.4µm of SC from 221 locations where SCs clearly appear as partially longitudinally sectioned, represented on four sections from 18µm apart each in the sectioning series. Small patches of electron dense materials were observed attached to the central element in several sections.

Tritonymph, 5 days after moulting, and young adults

Middle-aged tritonymphs show large germ cells featuring large, electron-lucent nuclei with prominent single or double nucleoli, and without prominent SCs (Fig. 4). In these specimens, as in young adults, peripheral oocytes are generally larger than those situated centrally in the ovary. Central germ cells are smaller, and their nuclei do not feature nu-

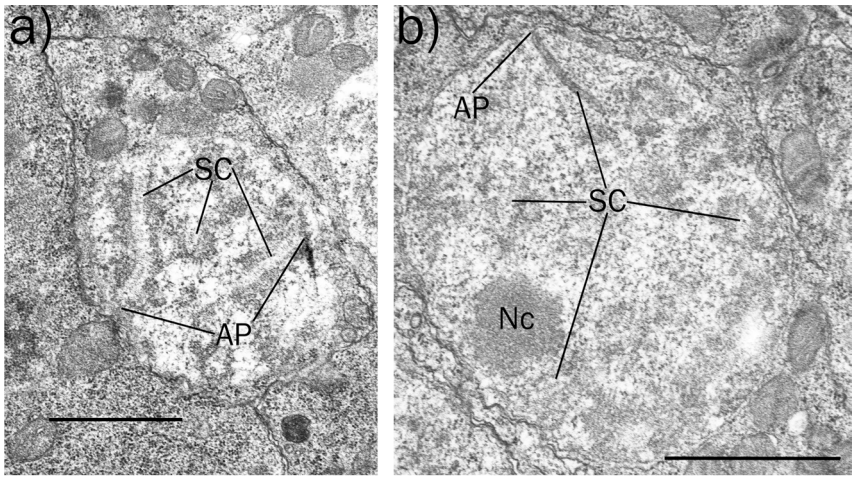

Figure 5.3: *A. longisetosus.* Synaptonemal complexes attached to the nuclear envelope of a 2-day old tritonymph. a) Pachytene cell, b) Putative early stage with developing lateral element in longitudinal section. Abbreviations: AP: attachement plaque, Nc: nucleolus, SC: synaptonemal complex. Scale bars: 1µm

cleoli, but rather a patchy pattern of dense chromatin agglomerations in a semi-lucent, fibrous nuclear content. Both chromatin configurations are thought to represent different stages of diplotene progression. The filiform extensions of growing peripheral germ cells reach between the smaller central germ cells to the central medulla of the ovary (Fig. 4). The content of parallel bundles of microtubules extends towards the nuclear envelope in these cells, with regions of the nuclear envelope neighboring the microtubule bundles being equipped with a dense hexagonal pattern of nuclear pores (Fig. 5). Numerous nuclear pores also appear in oocytes of adult specimen during previtellogenesis and vitellogenesis (Fig. 6). The microtubule-rich extensions connecting oocytes in the rhodoid to the medulla disappear with onset of previtellogenesis and the movement of oocytes towards the ovarial meroi.

5.7.4 Discussion

Exclusively female clades are exceptionally frequent among oribatid mites of the group Nothrina when compared to the average for metazoans (Palmer & Norton 1990). The exact nature of reproduction for any thelytokous oribatid mite has not yet been demonstrated in all details, but molecular data show no apparent signatures of recent recombination and indicate clonality (Palmer & Norton 1992; Heethoff et al. 2000 Schaefer et al. 2006). This is surprising since other molecular data suggest that several clades have propagated unisexually for at least 100 million years (Heethoff 2007b), and even have radiated in this mode (Maraun et al. 2004; Laumann et al. 2007; Heethoff et al. 2011).

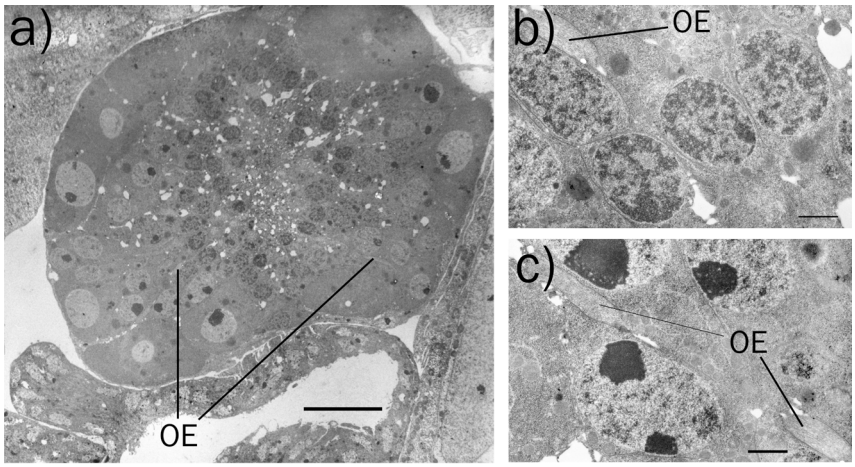

Figure 5.4: *A. longisetosus.* Ovary of a 5 day old tritonymph. a) Overview, parasagittal plane, scale bar: 10µm, b) Detail of central region, scale bar: 1µm, c) detail of peripheral region. Nuclei in different diplotene stages, oocyte extensions of peripheral oocytes reach between central oocytes. Abbreviation: OE: oocyte extensions. Scale bar: 1µm

The existence of prominent synaptonemal complexes throughout the tritonymphal ovarium is interesting because it provides the first direct ultrastructural proof of automixis in oribatid mites. Formation of SCs is seen as an important constituting factor of meiotic prophase I, occurring exclusively in this stage of the germ cell cycle in oocytes and spermatocytes of most species studied so far (Marec 1996; Page & Hawley 2004). Terminal fusion automixis—which was reported earlier from histological studies of related species (Taberly 1987, termed "mixocinèse"; cited in: Wrench et al. 1994)—was proposed in *A. longisetosus* through histological observations of oogenesis (Laumann et al. 2008), although nuclear structures characteristic for meiotic prophase I could not be demonstrated in adult females with certainty. This is explained by the occurrence of SC simultaneously throughout the ovary of middle-aged tritonymphs. While the ovary in the deutonymphal stage indicates proliferation of germ cells (Bergmann & Heethoff 2012), an abundance of SCs indicates that germ cells reach their final number in the tritonymph, enter meiosis early in this last subadult instar, and remain arrested in meiotic prophase during late tritonymphal and early adult stages until their sequential activation during the adult reproductive lifespan. Mediation of the apparent prophasic arrest and sequential activation of oogenesis is not yet understood, but its effects are seen in the mite's life-history. Archegozetes longisetosus exhibits iteroparity, with a recorded maximum of about 320 eggs laid during the lifetime of one individual in batches of about 15-30 eggs at intervals of 10-12 days (Estrada-Venegas et al. 1999; Heethoff et al. 2007a). A further consequence is that the adult ovary no longer constitutes a germarium, as no oogonia in premeiotic mitoses were found. This was spec-

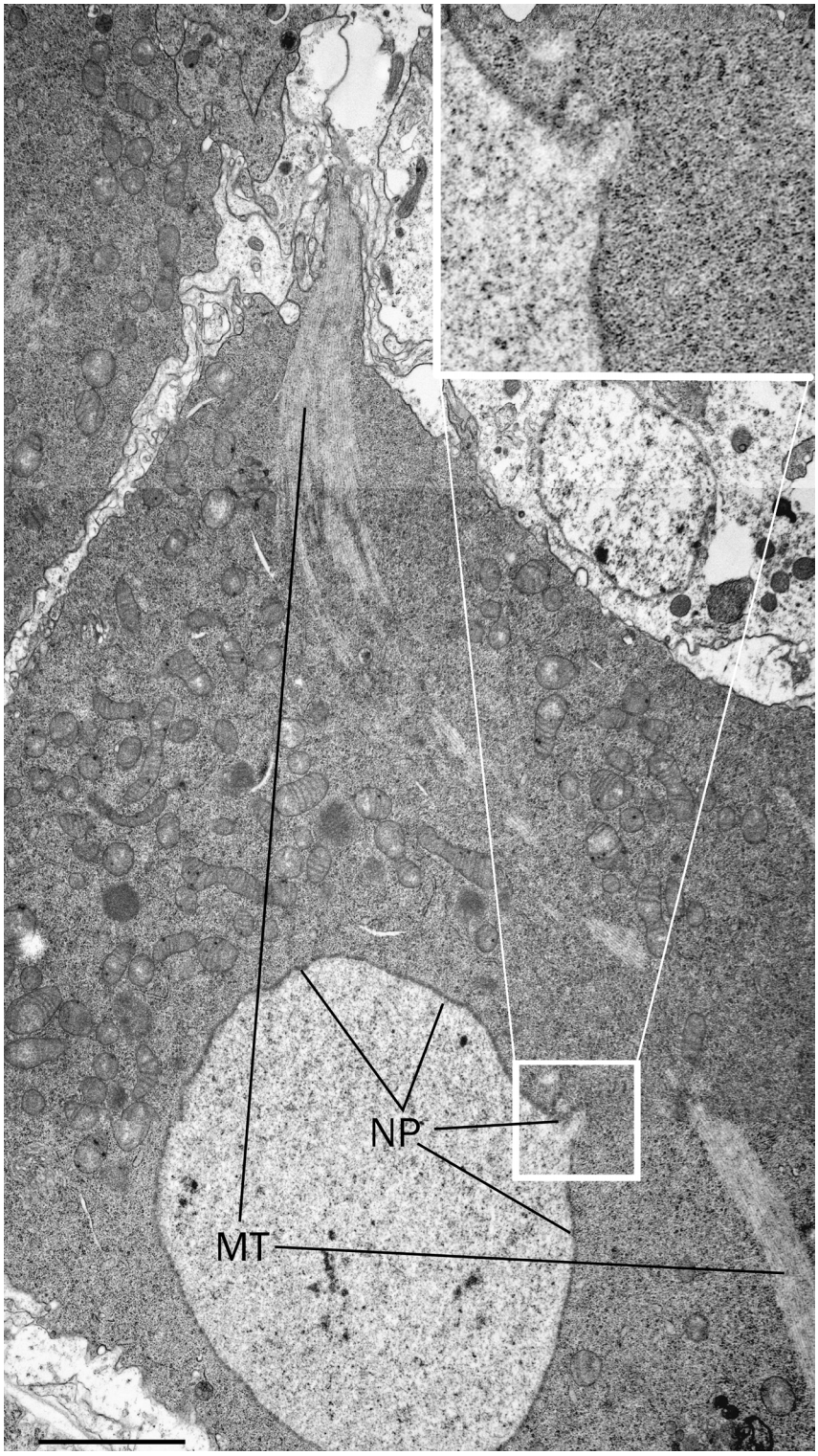

Figure 5.5: *A. longisetosus.* Peripheral oocyte of a tritonymph, showing oocyte extension and dense bundle of microtubuli passing the vicinity of the nuclear envelope. Nuclear envelope with numerous nuclear pores. Abbreviations: MT: microtubuli, NP: nuclear pores Scale bar: 2µm

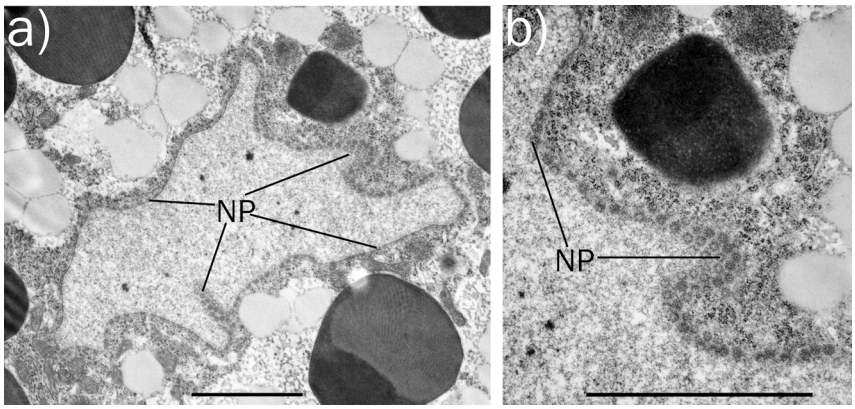

Figure 5.6: *A. longisetosus.* Vitellogenetic oocyte from the ovary of an adult female. Nuclear envelope with numerous nuclear pores. Abbreviation: NP: nuclear pores. Scale bars: 2µm

ulated earlier (Bergmann et al. 2008), based solely on the absence of signs of cell division, such as metaphase plates or breakdown of the nuclear envelope, in the central part of the ovary. The radially patterned central part is not involved in vitellogenesis and was hence termed the rhodoid for its appearance to avoid misleading terminology (Bergmann et al. 2008; Bergmann & Heethoff, 2012).

Transcription and RNA transport

Although ultrathin sections did not provide unequivocal evidence of single-strand DNA loops, the existence of prominent nucleoli between late pachytene and the exclusion of a polar body at the onset of vitellogenesis (Laumann et al. 2008), as well as the abundance of nuclear pores during meiotic stages from late prophase I until the onset of embryogenesis (Fig. 5, 6), suggest the existence of lampbrush chromosomes enabling a high rate of RNA transcription during late prophase. Structural similarities exist with the lampbrush chromosomes described in the insect *Panorpa communis* Linnaeus 1758 (Welsch 1973; Bogolyubov 2007), for example the halo of electron-dense protein agglomerations peripherally of the lateral elements of the bivalent. Despite their structural differences due to the phylogenetically distant relationship to Hexapoda, functionally panoistic ovaries were proposed earlier for *A. longisetosus* based on histological evidence (Bergmann et al. 2010). This latter study also reported a massive build-up of ribosomes during previtellogenesis. Ribosome accumulation during panoistic oogenesis further indicates transcriptional activity, and permits the denomination of single or double dense chromatine structures—exhibiting three structurally distinguishable portions—as true nucleoli. An important indication of transcription coupled with extranuclear RNA export is the observed density of nuclear pores in late prophase oocytes, especially in the vicinity of microtubule bundles

associated with oocyte extensions towards the medulla of the rhodoid (Fig. 5). The abundance of nuclear pores persists throughout previtellogenesis and during vitellogenesis in the adult mite (Fig. 6), indicating transcriptional activity during prophases of meiosis I & II, as meiotic processes are completed during that stage (Bergmann et al. 2010; Laumann et al. 2010). This further supports the idea that chromosomes exhibit a lampbrush configuration during a considerable part of meiosis in *A. longisetosus*. Although microtubules are often described as cytoskeletal elements involved in transport, the function of the microtubule-rich cell extensions common in ovarian cells of oribatid mites is still unclear (Woodring & Cook 1962; Witaliński 1986, 1987; Taberly 1987; Witaliński et al. 1990). Transport vesicles have not been observed in these extensions and, due to scarce information of their destination and the ultrastructure of the sarcoptiform ovarian core, it is not yet clear whether mechanical support or cellular communication is their primary function (Witaliński 1987; Alberti & Coons 1999). In *A. longisetosus*, abundance of nuclear pores is a feature of oocytes throughout previtellogenesis and vitellogenesis (Bergmann et al. 2010), however, the microtubule-rich extensions are lost during diplotene stage, when the oocytes start their centrifugal movement during the previtellogenetic growth phase.

SC formation and achiasmatic meiosis

While recombination events, especially the initiation of DSBs, was demonstrated to be intimately linked to SC formation in *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, 1883 (Henderson & Keeney 2004) and *Arabidopsis thaliana* (L.) Heynh. 1842 (Higgins et al. 2005), this seems not to be the case in *Drosophila melanogaster* Meigen 1830 (McKim et al. 1998) or in *Caenorhabditis elegans* (Maupas, 1900) (Dernburg et al. 1998), which also lacks recombination nodules (Page & Hawley 2004; Colaiácovo 2005). Formation of SCs of structure and dimensions similar to those in *A. longisetosus* was also described in the achiasmatic spermiogenesis of *P. communis* (Welsch 1973). Due to the scarcity of nuclear envelope–SC associations observed in this study, and our inability to directly observe the process of SC formation, we cannot infer the complete absence of DSBs or chiasmata. Chiasmata could still be present in the telomeric region incorporated into the attachment plaque of the mature SC. SC initiation by DSB is still a theoretical possibility, provided that crossovers are restricted to the non-coding telomeric region of the chromosomes, and are therefore genetically meaningless outside their putative structural function (Wrensch et al. 1994). No evidence of chiasmata could be found in the present study. The only site of dense protein masses bridging the SC in *A. longisetosus* were the so-called attachment plaques (Moses 1977). Recombination nodules, believed to be involved in DSB establishment and crossover in many species (Carpenter 1975, 1979a, 1979b) could not be demonstrated. Af-

ter the description of the coincidence of recombination nodules and crossover sites (Carpenter 1975), several types of central-element-associated protein densities were described that apparently are not directly linked to sister-strand exchange sites. Central-element-associated protein densities also appear in recombination-defective mutants (Carpenter 1979b, 2003) or in species with achiasmatic meiosis (Debus 1978). Recombination nodules associated with sister-chromatid exchange are described as spherical bodies that span the complete width of the SC. The dense structures observed in *A. longisetosus* (Fig. 2) most closely resemble so-called ellipsoid bodies (Carpenter 1979b) or sections of "noodles" (Carpenter 2003), in that they are elongated, very dense structures which appear at the central element, but leave electron-lucent space towards the lateral elements in the region of the transverse fibers. As such, they are most likely not an indication of recombination or chiasmatic meiosis. Absence of recombination was also suggested by molecular studies in *A. longisetosus* as well as other parthenogenetic oribatid mites (Palmer & Norton 1992; Schaefer et al. 2006)

Inverted meiosis

The apparent absence of recombination nodules in *A. longisetosus* supports structural evidence that meiosis is achiasmatic, and chromatids retain their genetic identity. As achiasmatic terminal fusion automixis would lead to fixed homozygosity within one generation, only inverted meiosis can reconcile the structural evidence (this study; Laumann et al. 2008) with the molecular evidence of fixed heterozygosity (Palmer & Norton 1992). A model theoretical concept for this case was formulated earlier (Wrensch et al. 1994). It is based on the holokinetic chromosomes found in this group of animals, which also have been confirmed for *A. longisetosus* (Heethoff et al. 2006): If spindle fiber attachment during metaphase is not restricted to the centromere region, a 90° flip of tetrad arrangement at the nuclear envelope can lead to a different sequence of separation of homologous and sister chromatids during the meiotic sequence. This form is called inverted meiosis, referring to the genetical outcome of an inverted sequence of chromatid segregation, leading to an equational first (division of sister chromatids) and reductional second meiotic division (disjunction of sister chromosomes). Although, in situations where chromosomes are monocentric, the terms pre- and postreduction describe a very similar situation, these terms can not easily be applied to situations with holokinetic chromosomes, as they were originally defined regarding the behaviour of the centromeres, which are non-existent in this case (Wrensch et al. 1994). The consequences are equivalent in a genetical sense only, if meiosis is achiasmatic, or if chiasmata are restricted to non-coding telomeric regions (Battaglia & Boyes 1955). Terminalized chiasmata, however, no longer seem to be a

common characteristic in holokinetic chromosomes (Hipp & Escudero 2013). In addition to spatial rearrangement, studies on other organisms exhibiting normal, pre-reductional meiosis, such as *C. elegans* and *S. cerevisiae*, indicate that the inversion of the sequence of the axis-associated protein REC-8´s dissociation from chromatids is required (Buonomo et al. 2000; Pasierbek et al. 2001; Rogers et al. 2002). In the described species, REC-8 is released first from the contact site of homologous chromosomes in meiosis I, and later from the contact site of sister chromosomes during meiosis II, leading to a conventional meiotic sequence. Observation of SCs in prophase I of *A. longisetosus*, however, indicates that inversion of the sequence of meiotic divisions is restricted to the pattern of chromatid segregation in this species, as the formation of the SC is unequivocally associated with the prophase of the first meiotic division in all meiotic systems (Marec 1996; Page & Hawley 2004). Hence, meiosis I in *A. longisetosus*, although equational, may be homologous to meiosis I in non-inverted systems from a cytological point of view. However, inverted meiosis can occur only with holokinetic chromosomes and achiasmatic meiosis (Viera et al. 2009). Both these prerequisites are met in *A. longisetosus*. Thus, a genetically postreductional situation in this case can putatively be achieved with less alteration than would be required for a complete inversion of the meiotic sequence, keeping the complex molecular background and intricate network of transcriptional processes related to meiosis in mind (e.g. Hotta et al. 1995; Perezgasga et al. 2004).

Parthenogenesis

Theoretically, parthenogenesis seems to require three steps in *A. longisetosus*: a) A rearrangement of the SC, effectively a 90° switch relative to the equatorial plane of the cell. b) A subsequent loss of cohesion between sister chromatids first (instead of homologous chromatids) during meiosis I, leading to inverted meiosis. c) Loss of a functional diakinesis at the end of meiosis II. If condition b) is already met, then c) leads to a diploid embryo that is the clone of its mother. This offers a possible explanation for why the SC is retained even though meiosis is achiasmatic, as inferred by the apparent absence of recombination. The SC seems not to be involved with DSB or chiasmata building, but pairing of homologous as well as sister chromatids during prophase I would also enable processes of DNA repair fundamentally different and of far higher fidelity (Cummings & Zolan 1997; Perrot-Minnot & Norton 1997; Schön & Martens 2002) than those associated with mitosis, and thus slow down the accumulation of deleterious mutations and 'Muller's Ratchet' (Muller, 1964). The process described above could extend the longevity of parthenogenetic lineages and, ultimately, parthenogenetic clades, but what explains the apparent frequency of their evolutionary appearance throughout the oribatid mite tree? The clades are widely dispersed

and—to the extent that clade size reflects time since origin—diverse in phylogenetic age (Norton & Palmer 1991; Cianciolo & Norton 2006) The answer seems unrelated to microbial induction. For example, there is no evidence of *Wolbachia* infection in any member of a parthenogenetic oribatid mite clade (Perrot-Minnot & Norton 1997). In addition, the mode of reproduction (gamete duplication) induced by *Wolbachia* is different from what is observed in parthenogenetic oribatid mites (Legner 1985; Stouthamer & Kazmer 1994), in which the easiest imaginable model that is consistent with observations is the failure of diakinesis in meiosis II (Perrot-Minnot & Norton 1997). An understanding will require more general knowledge of the reproductive mechanisms in a larger selection of parthenogenetic clades as well as of bisexual species that are closely related to the parthenogens, i.e. representing their respective stemgroup. If conditions a and b (above) are consistently met in stemgroups, then only the loss of diakinesis II would be needed to initiate a parthenogenetic lineage (and eventual clade). Wrensch et al. (1994) suggested that the genetic system of oribatid mites, with their holokinetic chromosomes, is 'permissive' in this context, setting the stage for multiple evolutions of parthenogenetic clades; but data remain scarce If only the loss of diakinesis is needed to initiate a parthenogenetic lineage or clade of oribatid mites, then conceptually the reestablishment of diakinesis could result in a revolution of bisexuality. This reversal seems to have happened at least once, within the nothrine family Crotoniidae (Domes et al. 2007b), but two other examples are possible. Astigmata, a primarily bisexual mite group, was also putatively derived within a parthenogenetic clade of Nothrina (Norton 1998; but see Domes et al. 2007a; Dabert et al. 2010). In a more primitive oribatid group (infraorder Enarthronota), molecular data (Pachl et al. 2012) support a phylogenetic tree in which the bisexual genera of Mesoplophoridae are more derived, with the basal genus and outgroups of the family being parthenogenetic. We do not know how diakinesis is lost in parthenogenetic oribatid mites, but such reversals may be rare because either repair mechanisms rarely can 'save' the appropriate genes from mutation over long periods of time, or because appropriate back-mutation is rare. In this context, it is an intriguing observation that cyclic parthenogenesis in *Daphnia pulex* Leydig, 1860 was recently shown to be enabled not by apomictic oogenesis as formerly suggested, but by suppression of homologue segregation during meiosis (Hiruta et al. 2010; Hiruta & Tochinai 2012, termed 'abortive meiosis'). In their 2012 publication, the authors also explore several relatively simple deviations from the meiotic program that might lead to the evolution of obligate parthenogenesis (Hiruta & Tochinai 2012). Considering the similarities among distantly related arthropods like Astigmata (Heinemann & Hughes 1969; Okabe & OConnor 2001), bugs (Bongiorni et al. 2004; Nokkala & Nokkala 1996; Viera et al. 2009), fruit flies (Carpenter 1979a, 1979b, 2003), dragonflies (Mola 1994)

and *Daphnia* (Hiruta et al. 2010; Hiruta & Tochinai 2012), automixis by failure of diakinesis during the reductional division might not be an altogether uncommon mode of parthenogenetic reproduction in unisexual arthropods. In view of progress achieved in recent years, incorporating the model species *A. longisetosus* in comparative analyses of meiosis among arthropods, and expanding research into its molecular background, could broaden our understanding of this key process in metazoan evolution.

5.7.5 Acknowledgements

We thank Prof. Oliver Betz and Dr. Klaus Eisler for supporting the electron microscopical studies, and Monika Meinert for excellent assistance with sample preparation and sectioning. Paavo Bergmann expresses his gratitude towards Evangelisches Studienwerk Villigst e.V. for receiving a PhD grant.

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6 Acknowledgements

This project would not have been possible without the kind commitment, support, help, encouragement, criticism and ideas of many people I would like to express my sincere gratitude to.

I am very thankful to Oliver Betz and Michael Heethoff for accepting to give this project a home at the Institute, providing me with a place to work and access to all facilities and supplies I needed, opening up ways for me, teaching me what I needed to know and do, making things possible and being always there for me with support, answers, ideas, perspectives, discussions, advice, patience and encouragement, virtually at all times, and being there for me whenever I needed.

I am indepted to Roy A Norton for providing us with a culture stock of the amazing little animals I grew so fond of during the last years, and for his accessability and support, answering to my questions and giving me valuable comments to manuscripts despite his own tight timetable.

I am very thankful to Klaus Eisler for sharing his knowledge and advice on electron microscopy, filling me in on all the fine details of how to get it right, and spending countless hours keeping the machinery up and running.

Michael Laumann was an extraordinary lab colleauge. I express my thanks for excellent teamwork and many fruitful discussions while we wrestled with machines, animals, protocols and the life of a PhD student.

I thank the European Union for providing the ESRF in Grenoble, and for allocating beamtime for our project. Special thanks go to the staff of beamline ID 19 for simply wonderful support around the clock, namely Peters Cloetens and Lukas Helfen, who solved problems before we even knew they existed, and to one nameless IT support guy who turned up at three a.m. to fix our network problems.

Sebastian Schmelzle and Daniela Weide I thank for being such great office mates and coworkers, making our room a place to live in, with chat, advice, coffee, cookies, caring about each others projects and problems, and helping me out in more than one occasion.

I thank Monika Meinert for her support, sharing her knowledge an histological techniques with me and providing excellent serial sectionings

I thank Reinhard Gerecke for his support, discussions, and for getting me interested in the Acari in the first place

I thank Gerd Alberti, Reinhart Schuster and Petra Föttinger for sharing their experience and insight with me during discussions, and helping me with my research.

York Stierhoff and Dagmar Ripper I thank for discussions on technical details of embedding specimen for electron microscopy

I am thankful to all members of the AG Evolutionsbiologie der Invertebraten, especially Christian Schmitt, Julius Braun, Jan Woyzichovski, Ferdinand Kluge, Daniela Weide, Sebastian Schmelzle, Uwe Kurz, Lars Koerner, Monika Meinert, Anne Jürgens-Hellmer, Karl-Heinz Hellmer and Julia Straube for making this AG such a wonderful place to work, and providing an athmosphere, where people freely shared thoughts, help, support and advice both in professional and everyday life. I also thank Annie Seibert from the lab next door for providing chocolate with expert timing.

I am greatly indepted to Evangelisches Studienwerk Villigst e.V. for providing my support in the form of a generous PhD grant. I also was given the opportunity to meet interesting people from all sorts of academic backgrounds, have a wonderful time at meetings and many stimulating discussions on details of research as well as the big questions of life in general. Special thanks go to Jan Michel for many good discussions, and for keeping my mind open for the broader context of the idea of science. I also thank Ole Riemann, Eike Wilts and Marko Silvestric for sharing their work experience and providing valuable suggestions for my work, and Eberhard Müller for always being there for the stipendiates.

I express my gratitude towards Elsevier, Springer, S. Karger AG Basel, the Naturhistorisches Museum Basel, Serge Kreiter of Acarologia, and Prof. Dr. Willi Xylander, editor in chief of "Soil Organisms", for granting me permission to reuse previously published material in this thesis.

I also thank Jan Prochel, Bianca de Haan and all the Castlearchers for wonderful hours of good company, encouraging me, and reminding me from time to time that there is in fact a world outside the lab. The same goes for Ralph Kindermann, Bernd Köhler, Matthias Herrmann, Nils Muthreich and Jens Hock.

Last but not least, I want to express my thankfulness and gratitude towards my family. You brought me up, got me interested in nature, supported me, encouraged me, endured me and believed in me even in moments when I did not.

I could not have done this without you.