

A study of embryonic development in eriophyoid mites (Acariformes, Eriophyoidea) with the use of the fluorochrome DAPI and confocal microscopy

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Abstract The embryonic development of four eriophyoid mite species, *Cecidophyopsis ribis*, *Phytoptus avellanae*, *Oziella liroi* and *Loboquintus subsquamatus*, has been studied with the use of fluorochrome DAPI and confocal microscopy. The first three nuclear divisions occur on the egg periphery (the groups of 2, 4, and 6 nuclei have been recorded), while the biggest part of yolk remains undivided. After four or five nuclear divisions all nuclei are situated only in one sector of the embryo, while other sectors contain only yolk suggesting possible meroblastic cleavage. Later, the formation of superficial blastoderm takes place. A few large yolk cells are situated inside the embryo. Germ band formation initiates as funnel-like cell invagination and leads to formation of a typical stage with four paired prosomal buds (cheliceræ, palps, legs I and II). Each palp contains two lobes (anterior and posterior), the adult subcapitulum is presumably a fusion product of the anterior pair of the lobes. Neither rudiments of legs III and IV, traces of opisthosomal segments nor remnants of the prelarval exuvium under the egg shell were detected. Overall, the pattern of embryonic development in eriophyoids re-emphasizes the peculiarity of this ancient group of miniaturized phytoparasitic animals, and invites researches to pursue a deeper investigation of various fundamental aspects of this aberrant group of Acari. Further studies using various fluorescent dyes and transmission electron microscopy are needed to visualize plasma membranes and clarify the pattern of early cleavage of eriophyoids.

Keywords Cleavage · Blastoderm · Prosomal limb buds · Arthropod development

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Introduction

Up to now, several acarine species have been studied with respect to early embryology. Among them are trombidiform mites—*Cheyletus eruditus* (Hafiz 1935; Edwards 1958), *Knemidocoptes mutans* (Langenscheidt 1958), *Tetranychus urticae* (Dittrich 1968; Dearden et al. 2002; Grbic et al. 2007)—and sarcoptiform mites, *Tyroglyphus farinae* (Hughes 1950; Sokolov 1952) and *Archegozetes longisetosus* (Laumann et al. 2010a, b). Early development of ticks *Boophilus calcaratus* (Wagner 1894), *Ornithodoros moubata* (Aeschlimann 1958; Fagotto et al. 1988), *Hyalomma dromedarii* (El Kammah et al. 1982), *Rhipicephalus (Boophilus) microplus* (Santos et al. 2013), and a few other ixodid and gamasid species (Yastrebtsov 1992), were also analyzed.

Traditionally, there were two points of view on the general pattern of early cleavage in the embryos of mites and ticks. Several authors (Hafiz 1935; Aeschlimann 1958; Langenscheidt 1958) discovered the occurrence of a superficial meroblastic cleavage, while the others (Hughes 1950; Sokolov 1952; Edwards 1958; Dittrich 1968) described the earliest two or three cleavage divisions as total (holoblastic) followed by a later series of superficial cleavage divisions on the periphery of inner yolk mass. This discrepancy stimulated further investigations (e.g., El Kammah et al. 1982; Fagotto et al. 1988; Yastrebtsov 1992; Gotoh et al. 1994), and recent publications (Laumann et al. 2010a, b; Scholtz and Wolff 2013) have favored the holoblastic cleavage pattern as a general character of Acari. Therefore, these recent works suggest that there is no diversity of cleavage patterns among mites and ticks. Laumann et al. (2010b) noted that acariform mites exhibit a specialized form of total cleavage with differentiation of blastomeres into micro- and macromeres, and involving unequal yolk distribution into micromeres and macromeres. They also assumed that total cleavage with blastomere differentiation may represent the cleavage ground-plan for all Acari.

It should be remarked, though, that among Acari there are a number of aberrant taxa, strongly different in their organization from other acarine groups. The phytoparasitic eriophyoid mites (Acariformes: Trombidiformes: Eupodina: Eriophyoidea) represent one of those aberrant taxa. Their evolution was characterized by miniaturization and great structural simplification (Nuzzaci and Alberti 1996; Lindquist 1996a). As a result, they acquired a morphology that is absolutely untypical for acariform mites: microscopic worm-like animals with two legs of 100–300 µm length and mouth parts highly adapted for piercing epidermal cells and sucking plant sap (Lindquist 1996a, b; Chetverikov and Craemer 2015). Although these mites are economically important (Lindquist and Amrine 1996), many fundamental aspects, including their early embryonic development, have never been studied. In the meantime, research in this direction might be helpful for elucidating the origin of their strange morphology and understanding the evolutionary trends in the group.

In this paper we were primarily aimed to describe the early development of eriophyoid mites and answer the question: does it correspond or not to the recently proposed ground-plan by Laumann et al. (2010b)? We were also focused on finding typical and chronologically distant embryonic stages to compare those in representatives of phylogenetically remote lineages of Eriophyoidea in order to estimate the uniformity of the developmental patterns among this group of mites. Finally, we were interested to trace the steps of formation of the prosomal limbs (cheliceræ, palps, legs I and II) including possible buds of legs III or IV and check the presence of the putative prelarval exuvia (see Shevchenko 1961; Lindquist 1996b for detail).

Table 1 Material studied

Mite species	Collecting data	Number of fixed eggs	Number of appropriately stained eggs	Number of eggs scanned with CLSM
<i>Cecidophyopsis ribis</i> (Westwood)	14 March 2015, RUSSIA: Saint-Petersburg, Mozhajskeye Lake, Dudergoff-hill, from big buds of <i>Ribes alpinum</i> L., 59°69'84"N, 30°12'43"E, coll. P. E. Chetverikov and C. Craemer	>5000 (from 12 buds)	≈ 700	98
<i>Loboquintus subsquamatus</i> (Chetverikov and Petanović)	14 February, 3 March and 20 April 2015, ISRAEL: Jerusalem, Herzl Blvd, under bark scales on young twigs of <i>Cupressus sempervirens</i> L., coll. A.V. Novikova	≈ 150	28	22
<i>Oziella liroi</i> (Roiveinen)	10 June 2015, RUSSIA: Saint-Petersburg, near main building of Saint-Petersburg State University, from leave sheaths of <i>Carex leporina</i> L., coll. P.E. Chetverikov	38	14	14
<i>Phytoptus avellanae</i> (Nalepa)	11 April 2015, RUSSIA: Saint-Petersburg, Mozhajskeye Lake, Dudergoff-hill, from big buds of <i>Corylus avellana</i> L., 59°69'84"N, 30°12'43"E, coll. P. E. Chetverikov	≈ 1000 (from 7 buds)	≈ 250	34

Materials and methods

Sampling (Table 1)

We studied early development of four species: *Cecidophyopsis ribis* (Westwood) from buds of *Ribes alpinum* L., *Phytoptus avellanae* Nalepa from buds of *Corylus avellana* L., *Oziella liroi* (Roiveinen) from leave sheaths of *Carex leporina* L., and early derivative mite *Loboquintus subsquamatus* Chetverikov and Petanović from under bark scale of young twigs of *Cupressus sempervirens* L. These species belong to the three different lineages of eriophyoids, which were revealed through a recent molecular phylogenetic analysis (Chetverikov et al. 2015b). Main data on the embryonic development of eriophyoids were obtained from a study of *C. ribis*, whereas the three other species were primarily used to confirm the presence of the typical developmental stages revealed in the embryos of *C. ribis*. It should be noted that we had no possible way of obtaining precise information on the age of the embryos; we also had no information on the location of the pole of the egg.

Collecting mites

Buds of alpine currant and hazel nut (containing mites *C. ribis* and *P. avellanae*, respectively) were cut into 2–3 parts by a blade, dissected by pincers, put in Eppendorf tubes containing 1 ml PBT¹ and slightly shaken by hand for 2–3 min. Thereafter the material was poured through a sieve (0.01 mm diameter) in a small cup and large pieces of

¹ =PBS (1 tablet of phosphate buffer saline P4417 Sigma dissolved in 200 ml of purified water) with 4–5 drops of 0.1 % Triton X-100.

plant tissue were removed. Applying this technique, we collected thousands of eggs (Table 1). The eggs of *Loboquintus* and *Oziella* were less numerous. We used a stereomicroscope, for detailed observation, and a fine needle to collect them from under the bark twig scales of cypress or within leaf sheaths of the sedge. As with *C. ribis* and *P. avellanae*, the eggs of *Loboquintus* and *Oziella* were transferred in a small cup with PBT and incubated at room temperature for about 10 min until all the eggs settled at the bottom.

DAPI staining

Prior to fixation, all the PBT was sucked by pipette and 10–15 drops of commercial bleach were added for 2–3 min to dechorionize the eggs (as was suggested by Laumann et al. 2010a and Barnett and Thomas 2013). After that the eggs were rinsed twice (at intervals of 20 min) in PBT. After the first rinsing all the eggs are usually clustered on the bottom of a cup, forming a rounded “ruft”. The eggs were fixed for 24 h in two ways: #1 most of the samples were fixed in 8 % PFA and kept at +4 °C; #2 several samples were fixed in cold methanol (−25 °C) in a freezer. After that, the eggs were twice rinsed in PBT and incubated for 3 h with DAPI solution (0.001 % in PBT). The stained eggs were thereafter rinsed once in PBT for 30–40 min, mounted on microscopic slides in pure glycerine, sealed with nail polish to avoid evaporating of glycerin, and kept in a refrigerator (+4 °C) prior to observation with a confocal laser scanning microscope.

CLSM technique and image processing

CLSM acquisition was carried out using Spectral Confocal and Multiphoton System Leica TCS SP2 with following adjustments: excitation wavelength of 405 nm, emission wavelength range of 415–470 nm, blue laser 405 nm at 90 % intensity. Both transmitted and excited (fluorescent) light was recorded in two different channels. Acquisition resolution was 1024 × 1024 pixels, level of gain 650–750, frame averaging of 1 (no averaging, i.e. a single frame) to scan images, and zoom range of 1.5–2.9 times. Stacks of 22–92 optical slices were recorded digitally from each of the 168 studied embryos. Most images showing different stages of embryonic development are maximum intensity projections (MIP) obtained with *ImageJ* free software (<http://rsb.info.nih.gov/ij/>); in the overlay images gray colour corresponds to transmission light and cyan to DAPI staining. Volume rendering and reconstructions of the advanced embryos of *Loboquintus* were carried out using Amira[®] 5.3.2 software and images were recorded using the “Snapshot” command in Amira[®] 5.3.2. Those images were obtained applying “volrenred” colormap of Amira[®], the colour corresponds to DAPI signal.

Results

Earliest cleavage stages (Fig. 1)

The eggs of all the studied species are slightly elongated and evenly filled with numerous yolk droplets. The eggs of *L. subsquamatus* are notably larger than those of the three other species: about twice as long as the eggs of *C. ribis* and about 1.5 time as long as the eggs of *P. avellanae* and *O. liroi* (Table 2). The earliest cleavage stages observed have 2, 4, and 6

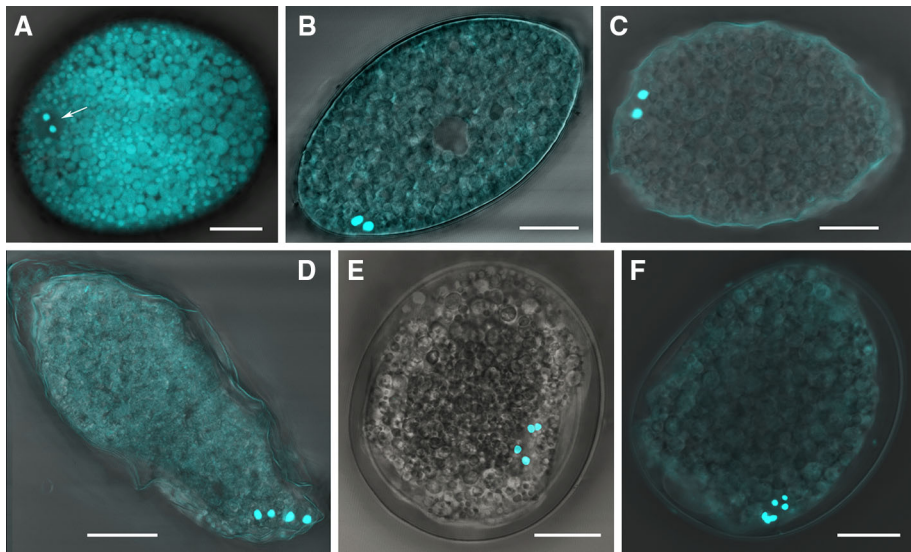
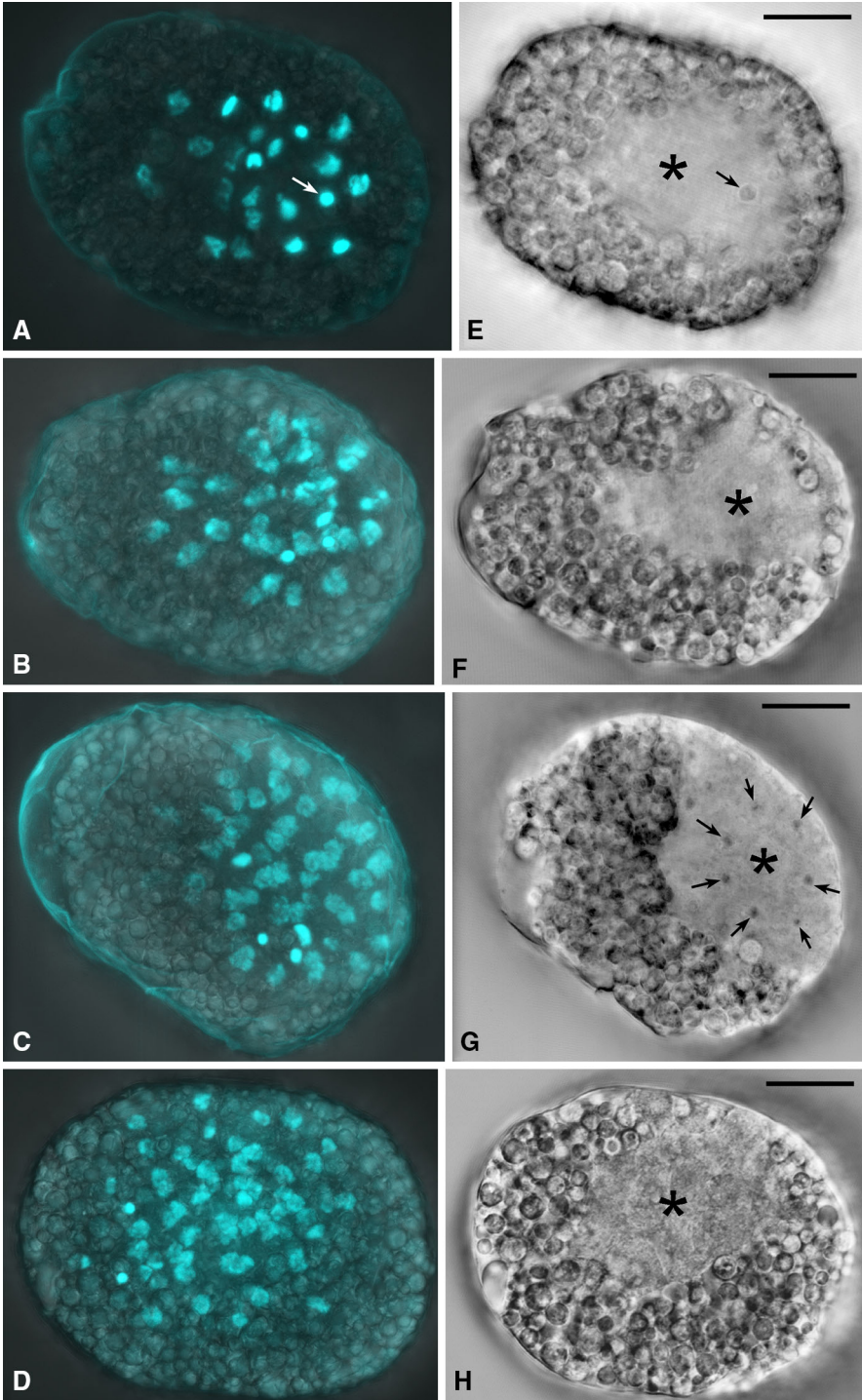


Fig. 1 CLSM MIP images showing the earliest cleavage stages of *Phytoptus avellanae* (a) and *Oziella liroi* (b, d) and *Cecidophyopsis ribis* (c, e, f). Scale bar 10 μm . Note that the peripheral position of nuclei during the early cleavage in these three species is in accord with our data (Chetverikov et al. 2015a, Fig. 7) concerning the peripheral position of the nucleus in mature eggs of another eriophyoid mite, *Neoprothrix hibiscus* Reis and Navia

Table 2 Morphometrics of the fresh laid eggs of the four studied eriophyoid species

Character	<i>Oziella liroi</i> (n = 14)		<i>Cecidophyopsis ribis</i> (n = 30)		<i>Phytoptus avellanae</i> (n = 30)		<i>Loboquintus subsquamatus</i> (n = 22)	
	Mean \pm SD	Min–Max	Mean \pm SD	Min–Max	Mean \pm SD	Min–Max	Mean \pm SD	Min–Max
Length (μm)	66 \pm 6.2	57–74	47 \pm 3.8	41–54	60 \pm 4.2	50–64	93 \pm 7.1	80–105
Width (μm)	34 \pm 2.4	32–38	36 \pm 3.6	29–43	48 \pm 3.9	40–52	54 \pm 7.4	42–66

small nuclei (Fig. 1) situated on the periphery of the embryos. Quite expectedly, cell borders were not visible during early cleavage, because DAPI does not stain plasma membranes. However, these embryos were not abundant: eight, four and three embryos of *Cecidophyopsis*, *Phytoptus* and *Oziella*, respectively; such early stages were not found in the case of *Loboquintus*. The occurrence of the stage with three (not shown) and six nuclei (Fig. 1f) was observed suggesting an early loss of division synchrony. However, additional material and observations would be necessary to finally prove this point. We did not see mitotic figures during the early cleavage stages (perhaps due to the brevity of nuclear divisions). The stained nuclei are bright, small (about 1.6 long and 1.2 wide in average), usually elongated, and with distinct margins. A dark halo (an area devoid of yolk droplets) around the nuclei were observed in *P. avellanae* (Fig. 1a, arrow) and *C. ribis* (not shown).



◀ **Fig. 2** CLSM overlay (**a, b, c, d**) and corresponding transmission light (**e, f, g, h**) images showing different stages of cleavage in four embryos of *Cecidophyopsis ribis*. **a** 22 nuclei, **b** 32–34 nuclei, **c** 48–50 nuclei; **d** 60–64 nuclei; images on the *right* (**e–h**) are MIPs of a single slice from the middle of the same embryos shown in the images on the *left* (**a–d**). The *asterisks* on the transmission light images indicate the homogenous area devoid of yolk droplets where the cleavage presumably appears; the *arrows* indicate nuclei. Scale bar 10 μ m

Sectoral growth of blastomeres inside yolk and problem of cellularization (Figs. 2, 3)

We did not observe embryos with between 6 and 20 nuclei. Further divisions result in the formation of a relatively compact group of nuclei (up to 50) situated closer to the egg surface than to the egg center (Fig. 2). At the stage of 60–80 nuclei, they grow into the yolk, and at a later stage (>100 nuclei) they start to spread under the surface so that the strands of nuclei envelop the yolk. After at least four or five nuclear divisions (a stage of 20–30 nuclei), the putative cell borders are visible in some embryos fixed in PFA (Fig. 3e, f, h, i); the yolk is mainly outside the cells and not within the homogeneously stained cytoplasm surrounding the nuclei. Several arguments favor the meroblastic pattern of cleavage: #1 in all observations, all nuclei are situated only in one sector of the embryo, while several other sectors of the same embryo contain only yolk with no trace of cleavage furrows (Figs. 2, 3); #2 under transmission light the sector of the embryo containing nuclei is homogeneously grey and without any bubbles or droplets (Fig. 2e, f, g, h *asterisk*) whereas the remaining part of the egg is heterogeneous and contains a lot of yolk droplets; #3 when observing the CLSM stacks in 3D editor, the number of cells separated by dark furrows (putatively indicate cell membranes), each containing a stained nucleus in DAPI images, directly correlates with the number of cells observable on the transmission light images (e.g. Figs. 3e, f, g, 4a, b, c). Additionally, we observed several embryos with quite distinct borders between blastomeres (Fig. 3h, i). Cell borders in Fig. 3 might have become visible because chromatin stained with DAPI partially leaked to the cytoplasm making it glowing. Another possible explanation is that in PFA fixed material DAPI sometimes marks not only chromatin but also brightly stains cytoplasm. However it does not contradict to the assumption that the lipid droplets are not included in the blastomeres, because the space between the droplets is not homogeneously stained (like the putative cytoplasm around the nuclei in the Fig. 3) suggesting absence of such a material (cytoplasm) between the droplets.

Superficial blastoderm, yolk cells and the “funnel” (Figs. 4, 5)

Cells are tightly packed, forming a single superficial cellular layer (blastoderm), surrounding the yolk (Fig. 4). The superficial borders of blastodermal cells are distinctly visible under a transmission light microscope (Fig. 4b). Among blastodermal nuclei, there are groups of compacted elongated brightly stained nuclei (probably engaged in mitotic divisions) (Fig. 4a, d, e) hinting at a possibility of “proliferation waves”. Note that mitotic waves have been detected in *Drosophila melanogaster* embryos during syncytial blastoderm development (Foe and Alberts 1983; Tomer et al. 2012).

Inside blastulae of all three species, there are individual large nuclei among yolk droplets (Fig. 5), while cell borders cannot be seen. These cells seemed to penetrate inside the embryo at the stage of advanced cleavage (Fig. 2a–d), and they can be seen at all subsequent stages up to the formed larva within the egg (not shown here). According to our

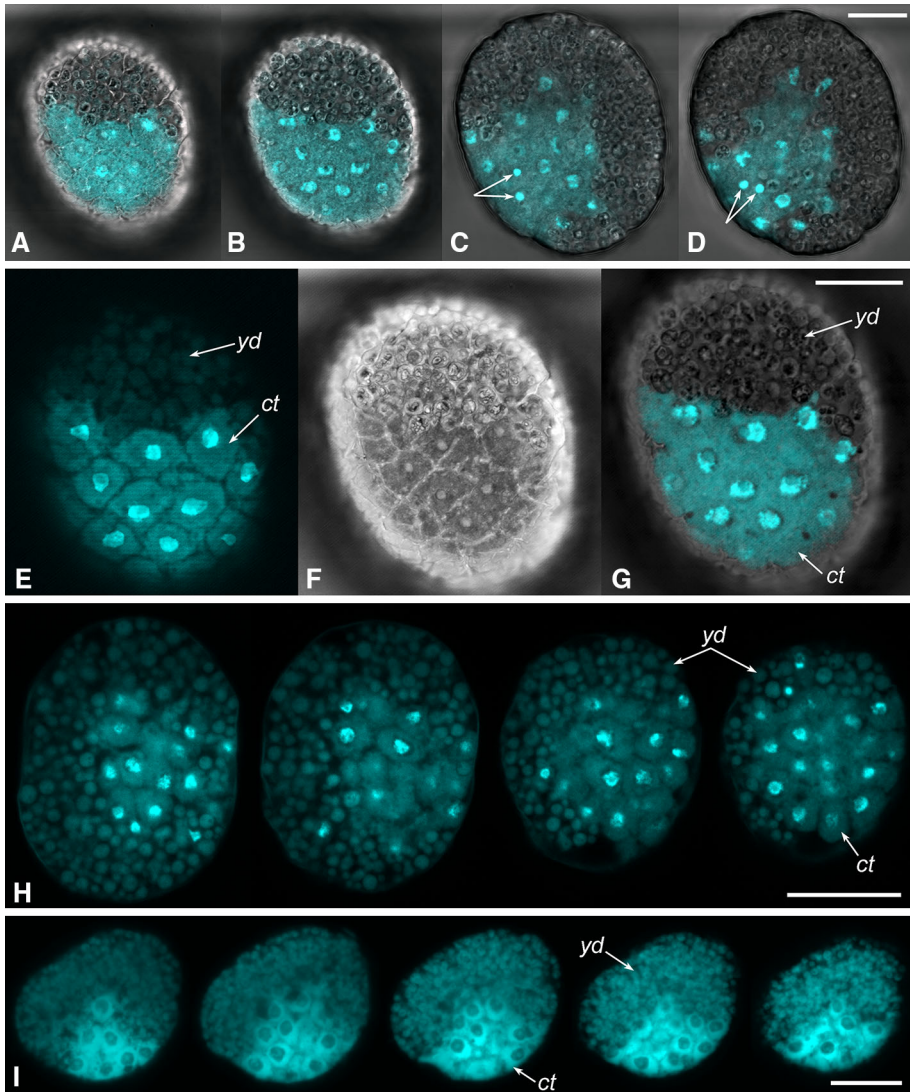


Fig. 3 CLSM images (**a, b, c, d, g** overlay, **e, h, i** fluorescence; **f** transmission channel) showing putative blastomeres with glowing cytoplasm (*ct*) and yolk droplets (*yd*) inside the cleaving eggs of *Cecidophyopsis ribis* (**a–h**) and *Phytoptus avellanae* (**i**). Scale bar **a–g** 10 μm , **h, i** 20 μm . Images **a, b, c, d** are MIPs of single slices (##3, 7, 28, 34) from one stack. Images **e** and **g** are composite images to show the glowing cytoplasm (obtained from more superficial slices) and corresponding nuclei (obtained from deeper slices) from the same stack as for images **a–d**. Images **h** and **i** represent two series of slices from deeper part of two embryos to more superficial part showing a sector of cleaving blastomeres. Paired arrows in **c** and **d** indicate two pairs of bright round young nuclei (similar to those observed in earlier stages, see Fig. 1) underwent division

preliminary data, the number of these cells is essentially the same during embryonic and larval development of all studied species: approximately 20–25. We will conventionally call them the “yolk cells”. At a later stage, a funnel-shaped invagination appears (Fig. 5c–f). In all studied species it forms near one end of the elongated egg, at first as a small group

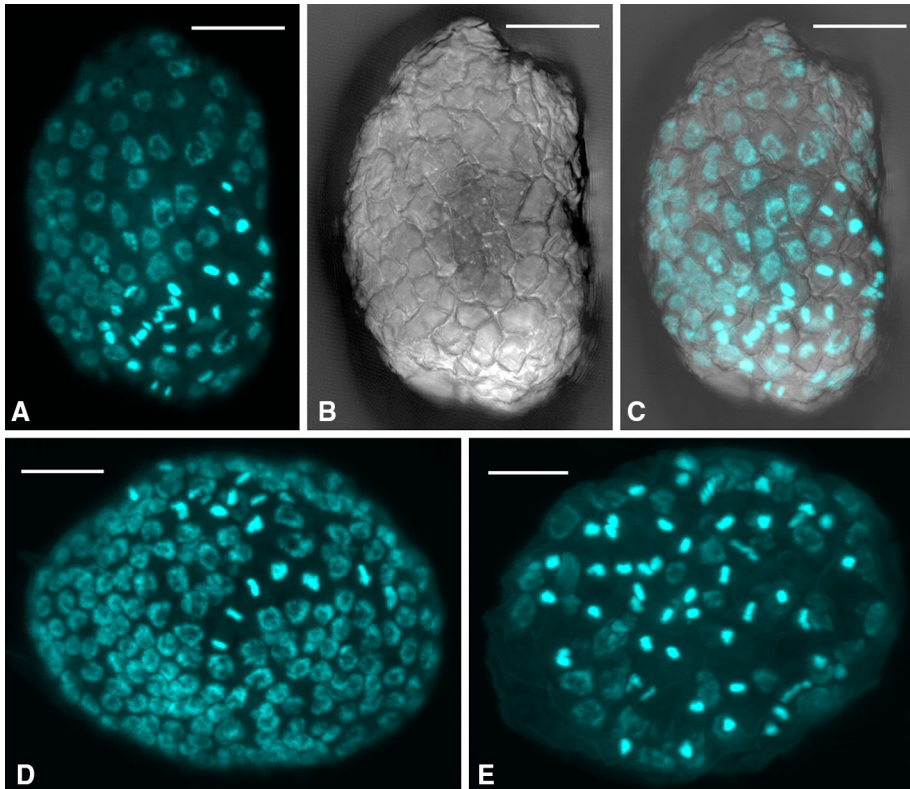


Fig. 4 DAPI fluorescence (a, d, e), transmission light (b) and overlay (c) CLSM MIP images showing blastula of *Cecidophyopsis ribis* (a, b, c same egg), *Loboquintus subsquamatus* (d) and *Phytoptus avellanae* (e). Scale bar 10 μ m

of cells (Fig. 5b), and later as a funnel-shaped invagination (Fig. 5c, d, e). Finally, in one egg of *C. ribis* and two eggs of *P. avellanae*, we have seen that this structure is already rod-shaped and connects the opposite sides of the blastoderm (Fig. 5f). When viewed with the 3D editor, the rod seemed to be hollow and with thin walls.

Germ band, furrows and limb buds (Fig. 6)

Further embryo development includes the formation of the germ band and prosoma appendages. In the eriophyoid mites, germ band development occurs in a close link with the above mentioned funnel. After the funnel reaches the opposite end of the egg, several cellular clusters are formed, which connect the funnel with the superficial embryonic tissue (not shown here). Among these clusters, the conglomerations of cells appear which grow and form the paired bulges on the ventral surface of embryo. Already at this stage, a middle longitudinal furrow is distinct and easy to see. This structure seems to correspond to the anterior/posterior embryonic axis (Fig. 6a, b *arrow*). Several transverse furrows, dividing the paired buds of prosomal limbs (Fig. 6c *arrows*), are also clearly visible at this stage. The differentiation of appendages seems to proceed from front to back: at first, the anlagen of chelicerae and palps, and then the anlagen of legs I and II (Fig. 6f). We did not observe

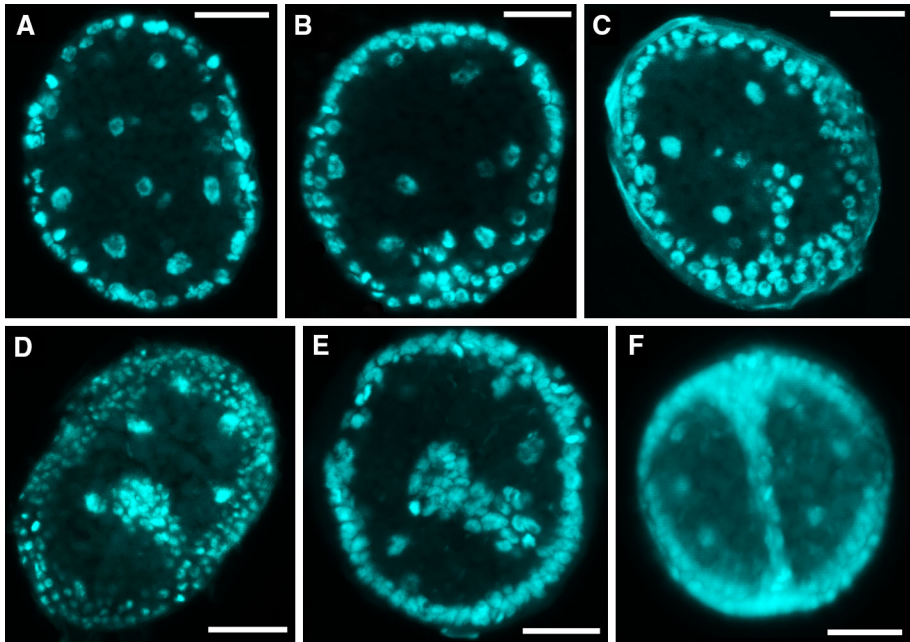


Fig. 5 Yolk cells and the “funnel” in *Cecidophyopsis ribis* (a, b, e), *Phytoptus avellanae* (c, f) and *Loboquintus subsquamatus* (d). a Yolk cell within blastula; b early “funnel” formation; c middle funnel; d, e “funnel” up to the middle of the embryo; f a through “funnel”. Scale bar 10 μ m

any anlagen of legs III, IV or any protuberances, which might be considered rudiments of these legs (Fig. 6g, h, i). The longitudinal sections definitely show that a minimum of one large (“yolk”) cell (Fig. 6d arrows) resides within each prosomal bud (corresponding to a chelicera, a palp, and legs I, II). In the light of the recent work on *Drosophila* (Huelsmann et al. 2006), it is possible that these yolk cells are migrating embryonic macrophages. Nonetheless, the yolk cells inside blastulae (see above) seem to be vitellophages. In the beginning, each palp bud is entire (Fig. 6f), but later two small protuberances appear (Fig. 6g, asterisks), and soon the palp resembles a spanner as it bears two lobes: anterior (Fig. 6h lobe) and posterior (Fig. 6h black arrow).

Body growth, folding the larva inside an egg

Intensive growth of the posterior part of the body (opisthosoma) begins after the formation of prosomal limbs. No external segmentation of the opisthosoma can be observed (Fig. 6i). The growth of the embryonic body occurs inside a restricted volume of the oblong egg, which leads to the folding and straightforward growth of the posterior body region. As a result, the embryo happens to be arranged in two layers and its legs embrace the posterior end of the body (e.g. CLSM image from Chetverikov 2012, p. 60, Fig. 2). A completely formed larva resides in the egg at this stage. Interestingly, we have never found any additional moulting exuvia inside the eggs. We did not plan to analyze organogenesis. Nonetheless, it is appropriate to remark that inside the DAPI stained embryos that have fully formed limbs and a mouth apparatus, 90 % of the anterior body region is represented by the putative CNS, which has similar structure to that in adult

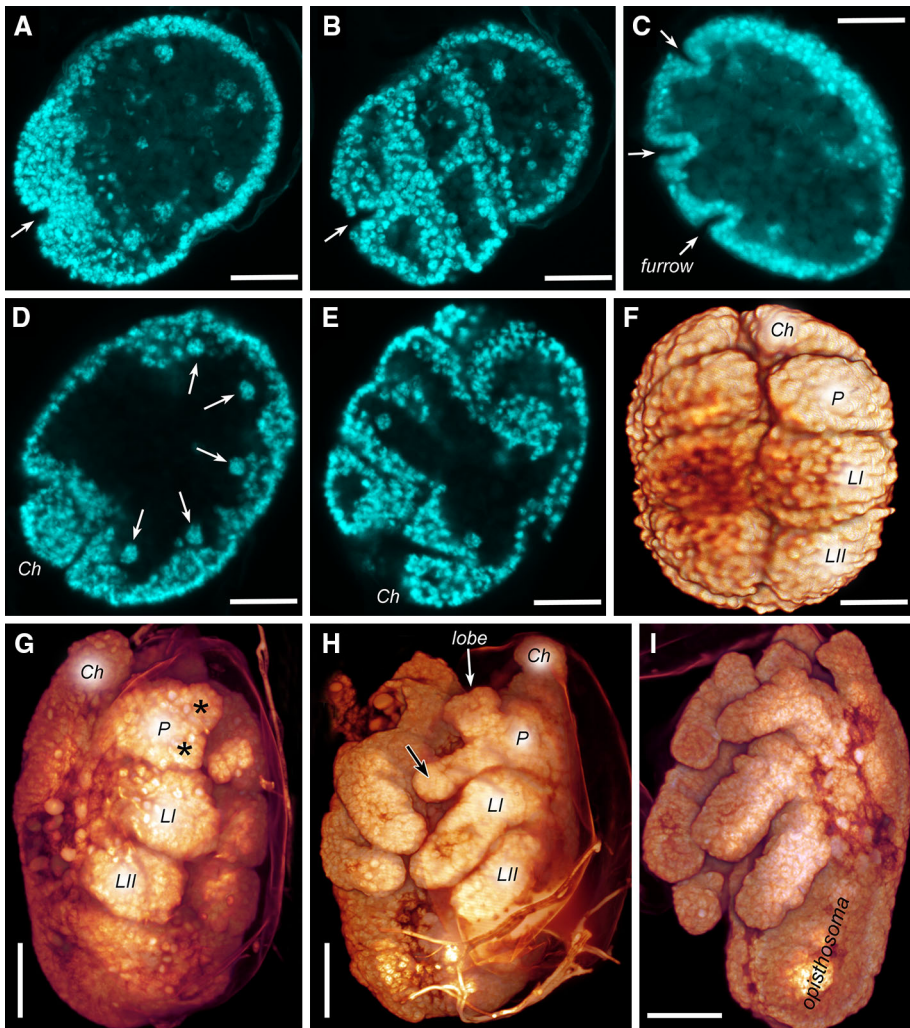


Fig. 6 Partial maximum intensity projections (**a–e**) and volume rendering reconstructions (**g–i**) showing formation of prosomal limbs in *Cecidophyopsis ribis* (**a, b, d, e, f**), *Phytoptus avellanae* (**c**) and *Loboquintus subsquamatus* (**g–i**). Notation: *Ch* chelicera, *P* palp, *LI* leg I, *LII* leg II, *lobe* anterior lobe of the palp, *furrow* one of the transverse furrows delimiting limb buds. *Arrows* in **a, b** indicate longitudinal furrow, arrows on the **d** indicate the putative yolk cells within limb buds. *Scale bar a–f* 10 μm , **g–i** 20 μm

individuals (Nuzzaci and Alberti 1996). For all the species we have studied it is typical for larval eriophyoids to contain a huge amount of the yolk droplets. Large cells with shapeless nuclei are situated among these droplets. They seem to take part in the assimilation of “maternal” nutrient materials. This event might compensate for the frequent phenomenon of weak exotrophic nutrition of eriophyoid larvae after hatching and, in turn, enable the larva to accumulate enough metabolic resources for the nymphal transformation.

Discussion

Uniformity and atypical traits of eriophyoid embryonic development

Our study shows that eriophyoid mites possess an uncommon pattern of early development among the Acari. Additionally, this pattern demonstrates remarkable uniformity among the main phylogenetic lineages of the Eriophyoidea. The early cleavage pattern in eriophyoid mites (based on DAPI stained nuclei and confocal microscopy) seems to be meroblastic. This is in contrast with recent data on other groups of Acari (Grbic et al. 2007; Laumann et al. 2010a, b). The difference may represent a trait that is unique to eriophyoid mites. The evidence, so far, therefore indicates that there may be substantial variation in the early ontogenetic patterns within the Acari, which may represent comparable levels of developmental diversity to those observed within other groups of invertebrates, such as sea urchins (Raff 1987), ascidians (Jeffery 1997), and parasitic wasps (Mancini et al. 2013).

Certainly, further analyses of the embryonic sectors with nuclei and the parts containing only yolk with transmission electron microscopy would be of great importance for decisive elucidation of cleavage pattern. However, presuming that the cleavage of eriophyoids might be meroblastic it would be interesting to consider it in the context of the general scheme of arthropod cleavage phylogeny (Scholtz and Wolff 2013, Fig. 4.3, p. 70). In the future, phylogenetic data on the Acari will be helpful for discriminating between two hypotheses. #1 Despite a contemporary consensus on the phylogenetic position and origin of Eriophyoidea (Lindquist 1996b), our embryological data admit that the eriophyoid lineage might have diverged from very primitive Acari before the latter underwent a transition from superficial to total cleavage. As a result, the eriophyoid mites might have acquired their unusual meroblastic pattern distinguishing them from all other arthropods. #2 In accordance with the current view on eupodine origin of eriophyoids (Lindquist 1996b), the transition from the total cleavage (basic for all Acari) to the atypical meroblastic cleavage might have occurred during the lengthy divergence of eriophyoid mites from other eupodine mites, which might have been associated with the process of miniaturization.

Enygmatic eriophyoid prelarva

In general, Acari may pass through up to six instars after eclosion: prelarva, larva, protonymph, deutonymph, tritonymph and adult (Walter and Krantz 2009, p. 59). Typically, the prelarva is a nonfeeding form that occurs not only in the Acari but in other arachnids as well. Contemporary known acarine prelarvae are quiescent and sequestered within the egg chorion (Walter and Proctor 1999). Shevchenko (1961) reported that he observed indistinct remnants in the eggshell of an eriophyoid mite *Eriophyes laevis*, which he interpreted as a putative prelarval exuvium. Similar observation belongs to Prof J. Amrine (*pers. comm.* 2013) who indicated indistinct squashed material in the egg shell of a hatching eriophyoid larva as prelarval vestiges. This was observed on one of the LTSEM images provided by Dr. R. Ochoa when they taught students attending the Acarology Summer Program at The Ohio State University (USA). The retention of the prelarval stage in the life cycle would reinforce the ancestry of the eriophyoid stalk, especially in the light of the recent finding of the eriophyoid-like triasacaroid mites in Triassic amber (Sidorchuk et al. 2015). Under CLSM we inspected hundreds of eggs containing differently developed embryos, including larvae which have already started slowly moving their legs under the egg shell. However,

we did not detect any prelarval remnants, which implies that this stage is probably missed or greatly suppressed in Eriophyoidea.

Germ band, subcapitulum and reduction of legs III and IV in eriophyoids

The “funnel” (Fig. 5) observed in the eriophyoid embryo is one of the hard structures for interpretation. However, according to Walter (2009, p. 55), this might be a mark of gastrulation. He reports that «...during the early stages of gastrulation, a ventral germ disc ... appears and develops as a funnel-shaped band in the yolk mass, eventually giving rise to the endo-mesoderm layers. The germ band elongates and gives rise to the cephalic lobes, after which the prosomatic segments with limb buds become distinct». Although we did not observe a distinct “ventral germ disc”, the short description by Walter precisely corresponds to what we observed in eriophyoid embryos, reinforcing similarities in the formation of the germ band in various groups of Acari. In all of the studied eriophyoid embryos, the early prosomal limbs appear as uniform symmetrical buds delimited by a longitudinal furrow, which starts between the bases of the chelicerae and passes between the bilobed palps and legs I and II (Fig. 6f, g). We suggest that, when the chelicerae elongate and take a terminal position (Fig. 6h, i), the anterior lobes of the palps appress to each other and presumably fuse medially, forming the subcapitulum enclosing the anterior portion of the gnathosomal stylets (see for details in Chetverikov and Craemer 2015). Further basal parts of the palps fused, forming the subcapitular plate, whereas each posterior lobe develops into a segmented palpal appendage. Overall, this draft scenario of the formation of the larval gnathosoma in eriophyoids is likely to be in accordance with the brief description by Walter (2009, p. 56) on the formation of subcapitulum in Acari and previous findings of the bilobed palps in the embryos of other chelicerates (e.g. Aeschlimann and Hess 1984; Grbic et al. 2007; Liu et al. 2009; Barnett and Thomas 2013).

Typically adult and nymphal Acari have four paired legs while the larva possesses only three pairs. However, the suppression of legs III and IV is characteristic of all stages of Eriophyoidea. Recent studies indicate that in spite of the 6-legged larval condition in embryos of acariform mites, the limb buds for legs IV are present (Barnett and Thomas 2013, Fig. 1), though external evidence for their existence soon disappears. One could expect to find the same situation in eriophyoids: limb buds for legs III develop early on but later dissolve. Our results suggest that in eriophyoid mites, only legs I and II develop, while no rudiments of limb buds III and IV have been visualized in CLSM reconstructions. The presence of the large cells migrating into each of the four prosomal limb buds (and thus “indicating” them, Fig. 6d) provide additional support for this hypothesis. If this is true, the question arises as to whether the formation of legs III and IV is only suppressed in eriophyoids, or is it that the metapodosomal segments do not develop at all? Precise comparative myoanatomy and studies of groups of genes involved in segmentation development, including *Hox* gene expression, could elucidate this question.

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