

Centrosome inheritance in the parthenogenetic egg of the collembolan *Folsomia candida*

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Abstract Unfertilized eggs commonly lack centrioles, which are usually provided by the male gamete at fertilization, and are unable to assemble functional reproducing centrosomes. However, some insect species lay eggs that develop to adulthood without a contribution from sperm. We report that the oocyte of the parthenogenetic collembolan *Folsomia candida* is able to self-assemble microtubule-based asters in the absence of pre-existing maternal centrosomes. The asters, which develop near the innermost pole of the meiotic apparatus, interact with the female chromatin to form the first mitotic spindle. The appearance of microtubule-based asters in the cytoplasm of the activated *Folsomia* oocyte might represent a conserved mechanism for centrosome formation during insect parthenogenesis. We also report that the architecture of the female meiotic apparatus and the structure of the mitotic spindles during the early embryonic divisions are unusual in comparison with that of insects.

Keywords Parthenogenesis · Meiosis · Centrosome inheritance · Mitotic spindle organization · *Folsomia candida* (Hexapoda, Collembola)

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Introduction

The process for the proper chromosome segregation to daughter cells is mediated by the spindle apparatus, a complex bipolar structure mainly composed of anti-parallel microtubule bundles. In mitotic animal cells, the spatial organization and normal function of the spindle machinery requires the activity of the centrosome, a peculiar organelle composed of a pair of centrioles surrounded by a cloud of material from which astral microtubules nucleate. Animal cells usually have only one centrosome at interphase; this doubles once every cell cycle and dictates the bipolar orientation of the spindle microtubules at mitosis. Although mounting evidence suggests that the early steps of spindle formation in both meiotic and mitotic cells reside on common pathways of non-centrosomal microtubule assembly and spatial organization (see Wadsworth and Khodjakov 2004 and references therein), animal cells need a centrosome for successful cell division. Consistent with the crucial role of the centrosomes in ensuring the spatial positioning of the spindle and the correct reproduction of its poles, acentrosomal vertebrate cells progress through the cell cycle but arrest at telophase, and cytokinesis often fails (Hinchcliffe et al. 2001; Piel et al. 2001). In addition, a centrosome-based checkpoint that monitors DNA replication has been described in the early *Drosophila* embryo (Sibon et al. 2000). Thus, the centrosome, via its connection with centrioles, might ensure coordination among nuclear events and could orchestrate accurate spindle positioning by influencing the assembly of astral microtubules. The correct number of centrosomes in mitotic cells thus has to be carefully monitored in order to avoid the formation of multi-polar spindles that would result in the unequal distribution of chromosomes to daughter cells (Delattre and Gonczy 2004). In many malignant tumors, multiple centrosomes can be induced as a result of the mis-

expression of regulatory proteins (see Sluder and Nordberg 2004 and references therein).

The male gamete is generally believed to provide the centrioles during fertilization. Maternal components needed for microtubule nucleation and cell cycle-dependent dynamics of the centrosome are recruited around the centrioles subsequent to fertilization. This mechanism of zygotic centrosome formation is commonly found in animals. However, there are a few exceptions in which the male gamete does not seem to play any role, such as in mice, where sperm lose their centriole during spermiogenesis (Manandhar et al. 2000). However, for most animals, centrosomes exist in both male and female gametes. Thus, the egg cell has evolved means to ensure that only one centrosome is utilized for the organization of the first mitotic spindle. The maternal centrosome is present in echinuran, mollusc, and echinoderm oocytes but is silenced after fertilization, allowing the function of the centrosome provided by the male (Stephano and Gould 1995; Wu and Palazzo 1999; Uetake et al. 2002; Zhang et al. 2004). In insects, this problem is overcome by the loss of the maternal centrosomes during earlier stages of oogenesis (Buning 1994). Parthenogenesis, a peculiar kind of reproduction in which eggs develop to adulthood without a male contribution, has evolved in many animals, commonly insects.

How can unfertilized eggs that successfully develop by means of centrosome-dependent spindles, assemble functional centrosomes, if the cytoplasm in sexually reproducing strains of the same species is usually unable to do so? A peculiar microtubule-organizing center (MTOC) has been reported in the spindle apparatus of some insects during female meiosis II (Callaini et al. 1999), but this structure does not contain centrioles, is unable to duplicate, and disappears at the completion of meiosis.

There is little doubt that insect oocytes lack true centrosomes and that the egg cytoplasm contributes stores of centrosomal material to the male-provided basal body at fertilization. However, whether parthenogenetic development in insects requires functional centrosomes to drive the formation of the zygotic spindle remains unresolved. The careful analysis of parthenogenesis in distantly related insect species might provide insights into the general mechanisms of this peculiar type of reproduction. The *Folsomia candida* parthenogenetic oocyte, offers a new opportunity for the detailed investigation of maternal centrosome silencing and the formation of the zygotic spindle in the absence of a male contribution. Meiosis and early development in the parthenogenetic oocyte of *F. candida* are unclear. To our knowledge, a single published report has dealt with the cytogenetic analysis of these early developmental processes in *F. candida* (Palévody 1973). Palévody (1973) determined that, in this parthenogenetic

species, the oocyte undergoes only one maturation division with the elimination of one polar body, suggesting that the restoration of diploidy is the result of an aberrant meiosis. However, the nature of this process and the manner in which the first zygotic spindle is formed and the first mitotic divisions proceed have not been made clear.

In this study, we show that, as meiosis resumes, unfertilized *F. candida* eggs have the ability to assemble astral arrays of cytoplasmic microtubules. These asters are absent in the fertilized eggs of the sibling species *Folsomia fimetaria* in which the organization of the zygotic spindle is mediated by the sperm-provided basal body.

Materials and methods

Strain

The *Folsomia candida* culture used in this work was collected in forest samples of Japanese red pine, at an altitude of 950 m, on the northeastern slope of Mt. Fuji (Itoh et al. 1995).

Egg collection

Females of *F. candida* were allowed to oviposit in plastic containers on a charcoal/plaster of Paris mixture. The substrate was kept moist by the addition of water. Animals were fed with Brewer's yeast. Eggs were collected from the substrate with a thin brush at various time intervals: immediately subsequent to oviposition, and 15 min, 30 min, 1 h, 2 h, 3 h, 5 h, 10 h, 15 h, and 24 h thereafter. Metaphase I spindles were observed in most newly laid eggs. Progression through meiosis was observed in eggs collected within 3 h. The first mitotic stages were seen in eggs at 3–10 h.

Immunofluorescence preparations

Eggs for whole-mount preparations were placed in a depression slide, dechorionated with a 25% hypochlorite solution for 3–4 min, rinsed in phosphate-buffered saline (PBS), and fixed in 1:1 heptane/methanol, followed by three rinses in methanol. They were then covered in PBS buffer and incubated with RNase for 30 min after which time they were stained for 30 min in 1 µg/ml propidium iodide (PI) and placed on a slide for viewing.

Eggs were also fixed in methanol and kept at -20°C for 1–5 h. The permeable chorion allowed the fixative to penetrate the egg. Eggs were subsequently washed in PBS for 15 min. Groups of five to six eggs were placed in a small drop (2–3 µl) of PBS on a glass slide that had been carefully cleaned with 1N HCl and were gently squashed

under a 20×20 mm coverslip. The slides were then frozen either on a copper bar pre-cooled in liquid nitrogen or directly on liquid nitrogen. After 30 min, the coverslip was removed with a razor blade, and the slides were immersed for 10 min in cold methanol or maintained in methanol at -20°C until the staining step. Fixed eggs were washed in PBS (3×30 min) and incubated for 1 h in PBS containing 0.1% bovine serum albumin (Sigma; PBS/BSA) to block non-specific staining. Microtubules were detected after incubation for 4–5 h at room temperature or overnight at 4°C with either a monoclonal mouse antibody against β -tubulin (Boehringer) diluted 1:200 in PBS/BSA or with the YL1/2 rat antibody (Harlan Sera Lab) diluted 1:100 in PBS/BSA. The samples were then rinsed for 20 min in PBS/BSA and incubated for 1 h in goat anti-mouse (Cappel) or goat anti-rat (Harlan Sera Lab) secondary antibodies (diluted 1:600 with PBS/BSA) coupled to fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate. For simultaneous tubulin and DNA staining, the eggs were first incubated for 4–5 h at room temperature with the anti- β -tubulin antibody. After being washed in PBS-BSA, the eggs were then incubated in the goat anti-mouse antibody to which 1 mg/ml RNase had been added. After further washes in PBS, the eggs were incubated for 30 min in 1 μ g/ml PI. For the simultaneous localization of microtubules and γ -tubulin, the embryos were incubated overnight at 4°C with the anti- γ -tubulin antibody (Sigma) diluted 1:200 in PBS/BSA. The YL1/2 antibody was subsequently added, and the incubation was allowed to proceed for 2 h at room temperature. Samples were then rinsed for 20 min in PBS/BSA and incubated for 1 h in the appropriate secondary antibodies. After being washed in PBS for 20 min, the eggs were mounted in small drops of 90% glycerol containing 2.5% N-propyl-gallate.

Confocal microscopy

Whole-mounts of eggs were examined by using a BioRad MRC 1024ES (Zeiss Microimaging Inc. Thornwood, N.Y.) laser scanning confocal microscope with a mixed gas krypton/argon laser coupled to an Olympus BX50 upright microscope. Digital optical sections of squashed eggs were examined by using a Leica TCS 4D laser scanning confocal microscope equipped with an argon-krypton Laser and coupled to a Leica DMRBE microscope equipped with 63X PL Apo 1.4 objectives (Leica). For double-stained samples, the images of the two fluorochrome distributions were recorded separately by averaging 8–16 scans of a single optical section to improve the signal/noise ratio and by using low laser emission to attenuate photobleaching. Images of chromatin, microtubules, and centrosomes collected at several focal planes were superimposed,

merged into a single file, and imported into Adobe Photoshop to adjust size and contrast. Bacteria were pseudocolored red to improve their localization.

Electron microscopy

For transmission electron microscopy, the oocytes were fixed in glutaraldehyde 2.5% in PBS overnight at 4°C. After washes in PBS for 1 h, the oocytes were postfixed in osmium tetroxide 1% in PBS for 2 h. Following further extensive washes in PBS and distilled water, the samples were then dehydrated in a graded series of alcohols, embedded in an Epon-Araldite mixture, and polymerized at 60°C for 48 h. Sections cut on an LKB Nova ultramicrotome were collected on copper grids, stained with uranyl acetate and lead citrate, and observed with a Philips CM10 electron microscope.

Results

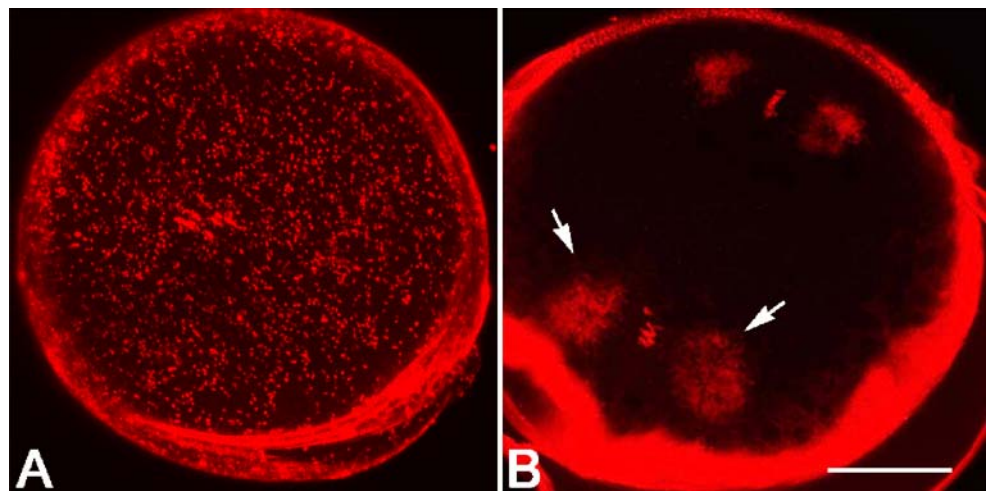
Meiotic apparatus

F. candida eggs are infected by *Wolbachia* bacteria that are easily distinguishable within the cytoplasm following PI staining. We have confirmed the identity of these bacteria as *Wolbachia* by using the polymerase chain reaction and by sequencing of the cell division *FtsZ* gene (R. Giordano et al., unpublished). *Wolbachia* infections in other *F. candida* strains have also been documented by Vandekerckhove et al. (1999) and Czarnetzki and Tebbe (2004). As has been shown in *Drosophila* (Callaini et al. 1994), *Wolbachia* bacteria densely populate the peripheral cytoplasm but are also abundant within the interior of the oocyte (Fig. 1a) and segregate at the spindle poles during chromosome division (Fig. 1b).

Most of the *F. candida* oocytes (61%; $n=43$) examined immediately after laying exhibit a small metaphase spindle. This suggests that meiosis resumes shortly after the transit of the oocyte through the oviduct. The metaphase I spindle is formed by bundles of kinetochore and interpolar microtubules that terminate on opposite poles (Fig. 2a). Although this spindle is tapered and its poles are focused, they are anastral (Fig. 2a). As meiosis resumes, homologous chromosomes position themselves on the metaphase plate and begin to move to the opposite poles. Resumption of meiosis results in a complex pattern of microtubule reorganization. Short microtubules are mainly observed within the area that surrounds the spindle apparatus, whereas the remnant oocyte cytoplasm seems devoid of microtubules (Fig. 2a).

In early anaphase oocytes ($n=19$), the meiotic apparatus appears barrel-shaped with broad frayed spindle

Fig. 1 Whole-mounts of early oocyte (**a**) and embryo (**b**) of *Folsomia candida* stained with propidium iodide. *Wolbachia* bacteria are scattered through the cytoplasm during earlier stages of meiosis (**a**) and are clustered at the spindle poles during mitosis (**b**, arrows). Bar 8 μ m



poles. Some microtubules extend outside of the spindle periphery, and the arms of the chromosomes extend along the whole length of the spindle (Fig. 2b). The latter group of microtubules might represent a population of non-kinetochore microtubules that do not cross-link to assemble the bulk of the meiotic apparatus. Alternatively, they may represent long interpolar microtubules that exceed the length of the anaphase spindle, resembling the peripheral microtubules observed in the early anaphase I spindle of *Drosophila mercatorum* (Riparbelli and Callaini 2003). During anaphase, homologous chromosomes begin to move to the opposite poles (Fig. 2b, inset). At this time, a dense network of microtubules is visible within the cytoplasm of the oocyte, and one or two large microtubule-based asters can be observed (Fig. 2b). These asters are not detected during earlier stages of meiosis. During early anaphase, the spindle apparatus increases in dimensions (Fig. 2c), and the chromosomes extend along the length of the spindle (Fig. 2c, inset).

In oocytes that progress through anaphase ($n=35$), the meiotic spindle shows an extensive reorganization, resulting in an increase in length, whereas the pole regions appear more broad and splayed (Fig. 2d). Chromosome segregation does not appear to occur synchronously, and as the anaphase spindle elongates, some chromosomes move to the opposite spindle poles, whereas others lag behind within the longitudinal spindle axis (Fig. 2d, inset). In late anaphase oocytes ($n=27$), the spindle elongates again, and chromatids migrate at the opposite poles of the spindle as two distinct sets (Fig. 2e). Detailed analysis reveals chromatid pairs at each spindle pole (Fig. 2e, inset). The poles are expanded, and several microtubules can be seen radiating from these regions in all directions. Microtubule density increases at the spindle poles of telophase oocytes ($n=13$), and poles spread to form aster-like structures (Fig. 2f).

Wolbachia bacteria that are present in the cytoplasm of *F. candida* eggs form discrete clusters in association with

cytoplasmic asters (Fig. 2b–e), but not with the anastral poles of the meiotic spindle during metaphase (Fig. 2a) and early anaphase (Fig. 2b).

Zygotic spindle

At the end of meiosis, three to four smaller asters are observed in addition to the two larger asters that are first observed during early anaphase (not shown). At the end of telophase, the bridges of microtubules that connect the two opposite poles are no longer visible. The chromosomes associated with the pole located toward the interior of the egg give rise to the pronucleus, whereas those associated with the more external pole form the polar body (Fig. 2g). After completion of meiosis, the cytoplasmic asters and microtubules associated with the polar body are no longer visible (Fig. 2h). In contrast, the microtubules associated with the pronucleus undergo a complex reorganization and, at the end of meiosis, form a peculiar comet-like structure with a large head and a short tail (Fig. 2i). Serial optical sections show that the head has a clear area at the center as if a hollow spherical region is present. The tail is composed of parallel bundles of microtubules, in part associated with the chromatin.

At the beginning of the first mitosis, the chromosomes of the pronucleus decondense to form large distinct chromatin masses held by a large aster (Fig. 3a). This aster gradually loses its compactness until the hollow core is no longer visible. Microtubules appear to increase in length and mainly converge toward two poorly defined foci of different sizes (Fig. 3b) that become more distinct and compact. Two opposite astral arrays of microtubules form during the prophase of the first mitosis (Fig. 3c). As the chromosomes align at the metaphase plate, two distinct large astral arrays of microtubules are seen at the opposite spindle poles (Fig. 3d). Thus, the metaphase spindle is composed of kinetochore microtubules that contact the

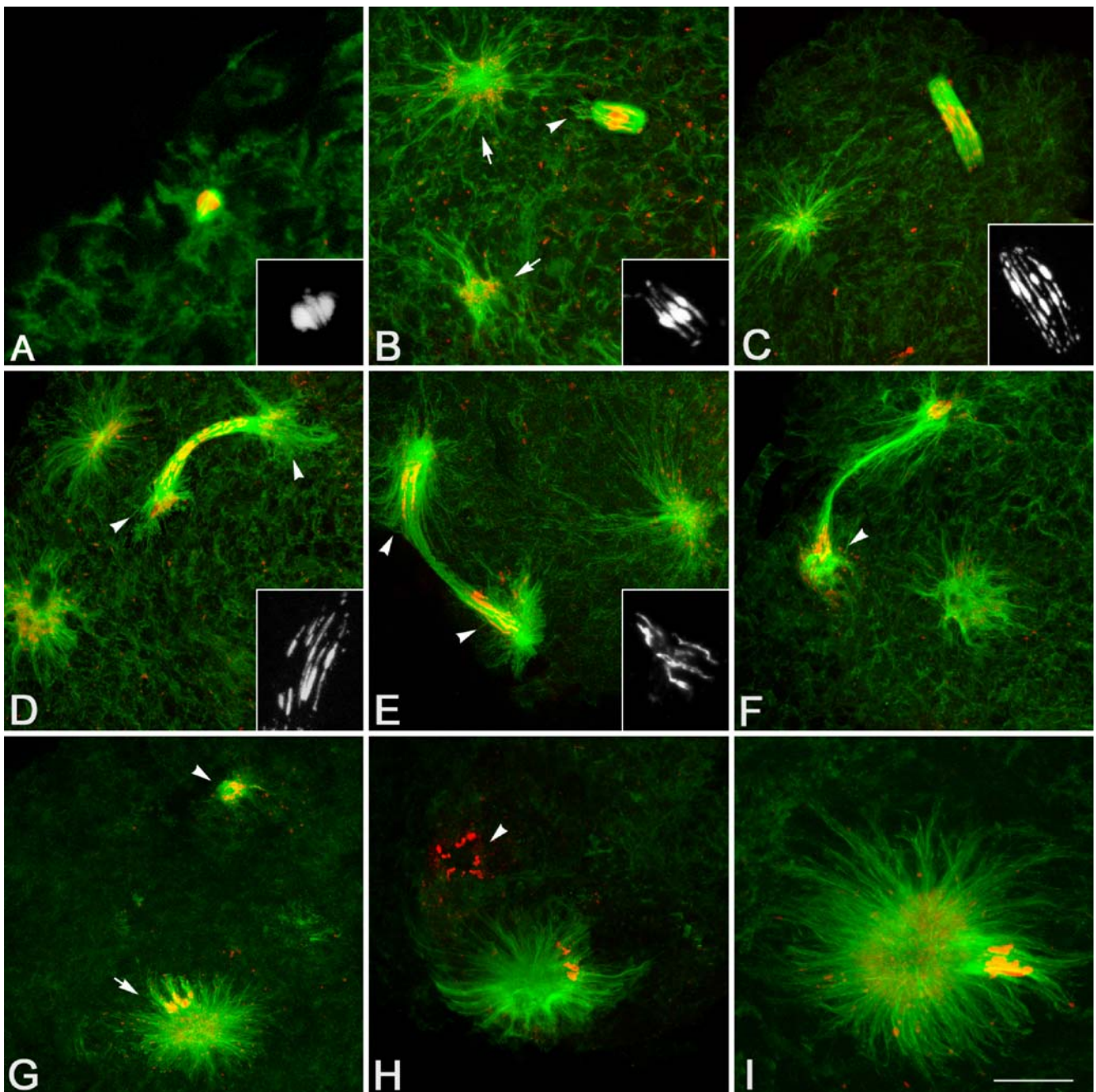


Fig. 2 Female meiosis in *F. candida* (green microtubules, red chromosomes and bacteria; in insets, chromosomes appear white). **a** Metaphase: the spindle poles are anastral but focused. Homologous chromosomes are aligned at the equatorial region (inset). **b** Early anaphase: the spindle poles have enlarged and appear broader. Some microtubules exceed the length of the spindle (arrowhead). Two microtubule-based asters, at which cytoplasmic bacteria are clustered, are visible (arrows). Homologous chromosomes have begun to separate (inset). **c** Early anaphase: the spindle apparatus is larger, and homologous chromosomes span throughout the length of the spindle (inset). **d** Mid-anaphase: the spindle is elongated, and the opposite poles appear broad and frayed. Several microtubules spread from this regions where bacteria accumulate (arrowheads). Chromosome segregation is asynchronous, and some move to the opposite poles, whereas the remnants lag at the equatorial

region (inset). **e** Late anaphase: many microtubules spread out from the spindle poles, which have enlarged. Two separate sets of chromosomes are seen at the opposite extremities of the spindle (arrowheads). Chromosome sets are formed by chromatid pairs (inset). **f** Telophase: the poles of the spindle round up and bear two distinct sets of chromosomes. The innermost will be the presumptive female pronucleus (arrowhead). **g** End of meiosis: the spindle apparatus disappears, except for the inner pole (arrow), which bears the presumptive zygotic nucleus, and the outer pole (arrowhead), which forms the polar body. **h** The microtubules associated with the inner pole increase in number, whereas those associated with the polar body chromosomes (arrowhead) disappear. **i** Detail of the comet-like microtubule configuration associated with the zygotic chromosomes; note the bacteria cluster (right). Bar 15 μm (a–g), 8 μm (h, i), 6 μm (insets)

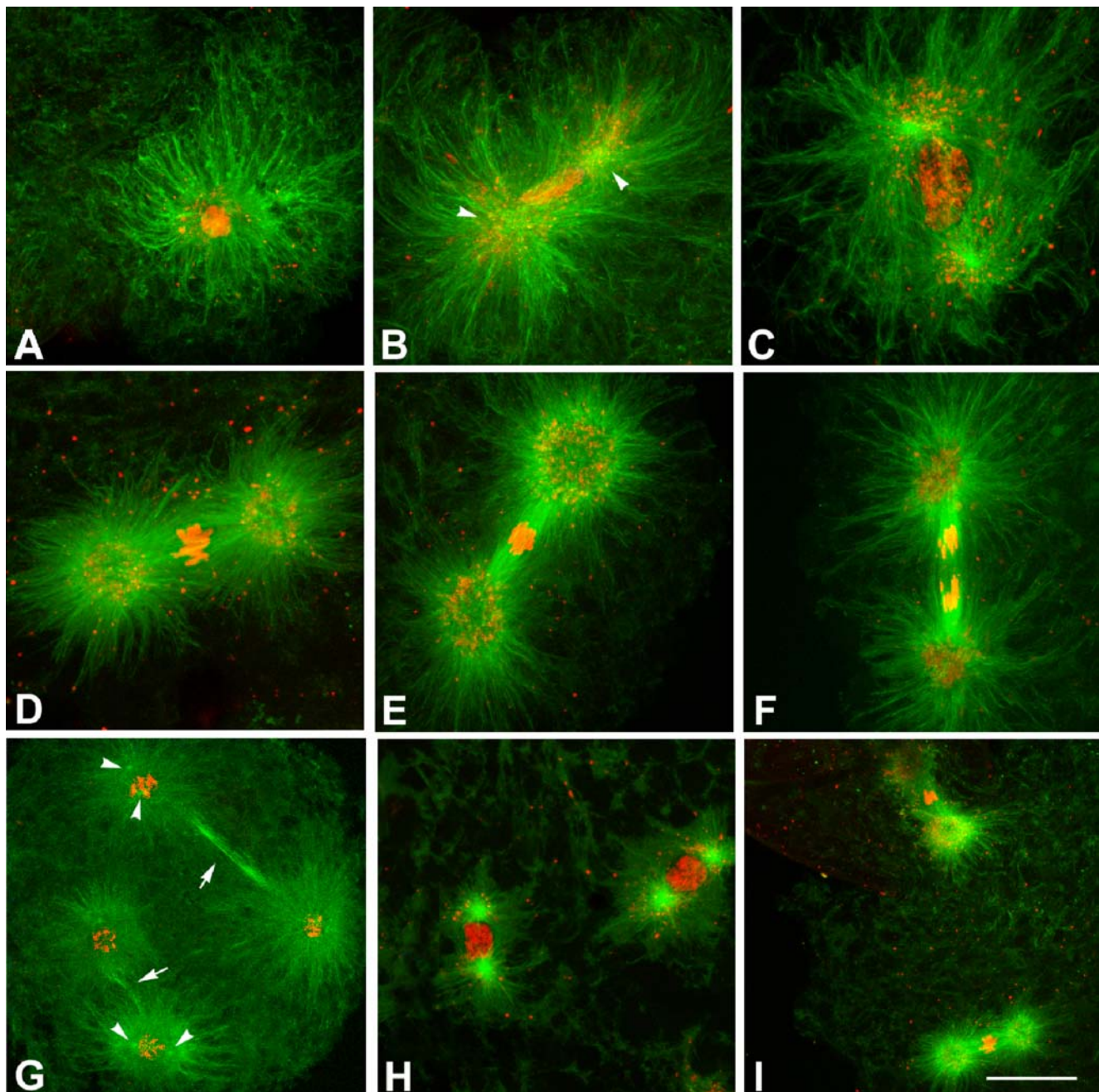


Fig. 3 Mitosis in the early *F. candida* embryo (*green* microtubules, *red* DNA). **a** Detail of the astral array of microtubules associated with the zygotic nucleus. **b** Early prophase: microtubules are focused at two differently sized centers (*arrowheads*). **c** Late prophase: two widely separated microtubule foci organize the mitotic spindle. **d** Metaphase: the chromosomes form a distinct equatorial plate, the asters becoming large. **e** Early anaphase: the sister chromatids start to separate. **f** Late

anaphase: the chromatids have reached the opposite enormous poles. **g** Early interphase of the second mitosis: the poles remain large, and two bright spots are seen inside them (*arrowheads*). Midbody remnants are also visible (*arrows*). **h** Late prophase of the second mitosis. **i** Metaphase of the second mitosis. Bacterial clustering is evident only in the region of the aster near the centrosome (**h, i**). Bar 8 μ m (**a-f**), 15 μ m (**g-i**)

sister chromosomes, interpolar microtubules that overlap through the mid-region of the small spindle, and two large asters.

When chromosome movement to the opposite poles begins during anaphase A, the asters increase in size (Fig. 3e). As

chromosome separation proceeds during anaphase B, the unusual configuration of the asters remains evident (Fig. 3f) and persists until telophase. During early interphase of the second nuclear cycle (Fig. 3g), a remnant of the midbody persists between the daughter nuclei. Interphase nuclei are

usually associated with a large astral array of microtubules similar to those observed in the previous nuclear division cycle. However, here two bright foci become apparent inside the large poles (Fig. 3g). These foci increase in size during the following prophase and move apart, becoming the centers for the poles of the second mitotic spindle (Fig. 3h). Transition to metaphase is still characterized by the dramatic enlargement of the spindle poles to form the large asters (Fig. 3i) that persist until late telophase and reorganize in the interphase astral array of the next mitosis. This complex process of cell-cycle-dependent microtubule rearrangement is clearly visible until the fourth mitosis.

Cytoplasmic asters contain centrioles

The formation of a well-structured astral array of microtubules at the inner pole of the female meiotic spindle indicates the presence of microtubule nucleating material in this region. This conclusion is supported by the accumulation of *Wolbachia* at the broad splayed anastral poles from late anaphase onward. In *Drosophila* (Callaini et al. 1994), *Wolbachia* bacteria are found associated with centrosome-nucleated astral microtubules.

We tested whether the poles of the late meiosis anaphase spindle of *F. candida* contained MTOCs by staining young oocytes with an anti- γ -tubulin antibody, a core component of the microtubule nucleating machinery (Oakley 2000). Our results showed a distinct γ -tubulin label at the foci of the cytoplasmic asters during anaphase, but not at the meiotic spindle poles (Fig. 4a), suggesting a differential regulation of the spindle pole components. Whereas the foci of the cytoplasmic asters were always labeled throughout the progression of meiosis, the poles of the meiotic apparatus lacked γ -tubulin. However, at the end of telophase, when an astral array of microtubules became evident in close association with the inner pole of the meiotic apparatus, a distinct label for γ -tubulin was observed at the focus of this aster (Fig. 4b).

To evaluate the possibility that cytoplasmic asters might be organized by true centrosomes, we sought centrioles at the foci of these structures. Cross sections of six cytoplasmic asters from five different eggs revealed that four of them contained centrioles that were mainly composed of nine single peripheral tubules and one central tubule (Fig. 4c,d); microtubule doublets were occasionally observed in some sections (not shown). γ -Tubulin was found in association with the spindle poles during embryonic mitoses (Fig. 4e,f). Optical sections through the poles of the metaphase spindles showed a γ -tubulin-labeled center from which microtubules radiated outward like spokes in a wheel, reaching a peripheral circular band in which the concentration of tubulin staining increased. Figure 4g illustrates a cross section through this spherical structure.

Optical sections at the level of the central region of the spindle (Fig. 4h) revealed that kinetochore microtubules were arranged in distinct bundles that were focused at their extremities that but did not seem to be connected to the centrosomes.

Zygotic spindle formation in fertilized eggs

To investigate whether the process of zygotic spindle formation observed in parthenogenetic eggs could be modified by the presence of externally provided centrosomes, we examined the process of spindle assembly during the early development of bisexual eggs of the species *F. fimetaria*. In the fertilized eggs of this sexual species, the meiotic spindles resemble those observed in parthenogenetic eggs of *F. candida*. However, a developed sperm aster is visible in fertilized eggs, whereas cytoplasmic asters are absent. The sperm aster is visible in newly laid *F. fimetaria* eggs when the meiotic spindle is in metaphase I (Fig. 5a), indicating that sperm enters at the time that the oocyte resumes meiosis. As meiosis progresses, the sperm aster enlarges, and two close microtubule foci become evident at its center, suggesting that the male-provided centrosomes undergo duplication (Fig. 5b). The zygotic spindle (Fig. 5c) is thus formed by the centrosome associated with the male pronucleus. Mitotic spindles in fertilized eggs appear to have short spindles and large asters similar to those observed in unfertilized eggs (cf. Figs. 3f, 5d).

Discussion

Sexual reproduction encompasses various strategies that insure the inheritance of the necessary components from both parents, together with other strategies that avoid redundancy of organelles that could interfere with normal development. Both parental chromosome complements are needed to form the zygotic nucleus, but only one parental centrosome must be functional at fertilization in order to prevent multipolar spindle formation and abortive development.

Parthenogenesis is a special mode of reproduction in which the male gamete is not required for embryonic development. In this developmental system, a mature egg, which ordinarily represses the assembly and functioning of maternal centrosomes, is able to assemble MTOCs from maternal stores, in the absence of a male contribution. The ability of an unfertilized egg to assemble centrosomes without a basal body provided by a male suggests that the egg cytoplasm contains all the components needed to assemble centrioles and to organize peri-centriolar material, i.e., the true centrosome. What are the changes that occur in

Fig. 4 γ -Tubulin is associated with the focus of the microtubule asters during both meiosis and mitosis (green microtubules, red γ -tubulin). Meiosis. **a** Anaphase: γ -tubulin is associated with the foci of the aster microtubules (arrows), but not with the inner pole of the meiotic apparatus (arrowhead). **b** Late telophase: a distinct γ -tubulin dot is visible at the focus of the microtubule aster associated with the inner pole of the meiotic apparatus (arrowhead). **c** Cross section through the focus of a cytoplasmic aster showing the presence of a centriole (arrow). **d** Detail showing the single tubules forming the centriolar wall. **e, f** Prophase and metaphase, respectively, of the first mitosis: a distinct γ -tubulin accumulation (arrows) is found within the pole regions. **g** Detail of the pole region of a spindle during metaphase of the first mitosis: note that microtubules radiate in a cartwheel-like fashion from a distinct γ -tubulin dot (arrow). **h** Detail of a damaged metaphase spindle showing that kinetochore microtubules are separate units focused at their extremities (arrowheads) and do not show continuity with the centrosome (arrow). Bar 15 μ m (a, b, e, f), 100 nm (d), 200 nm (c), 8 μ m (g, h)

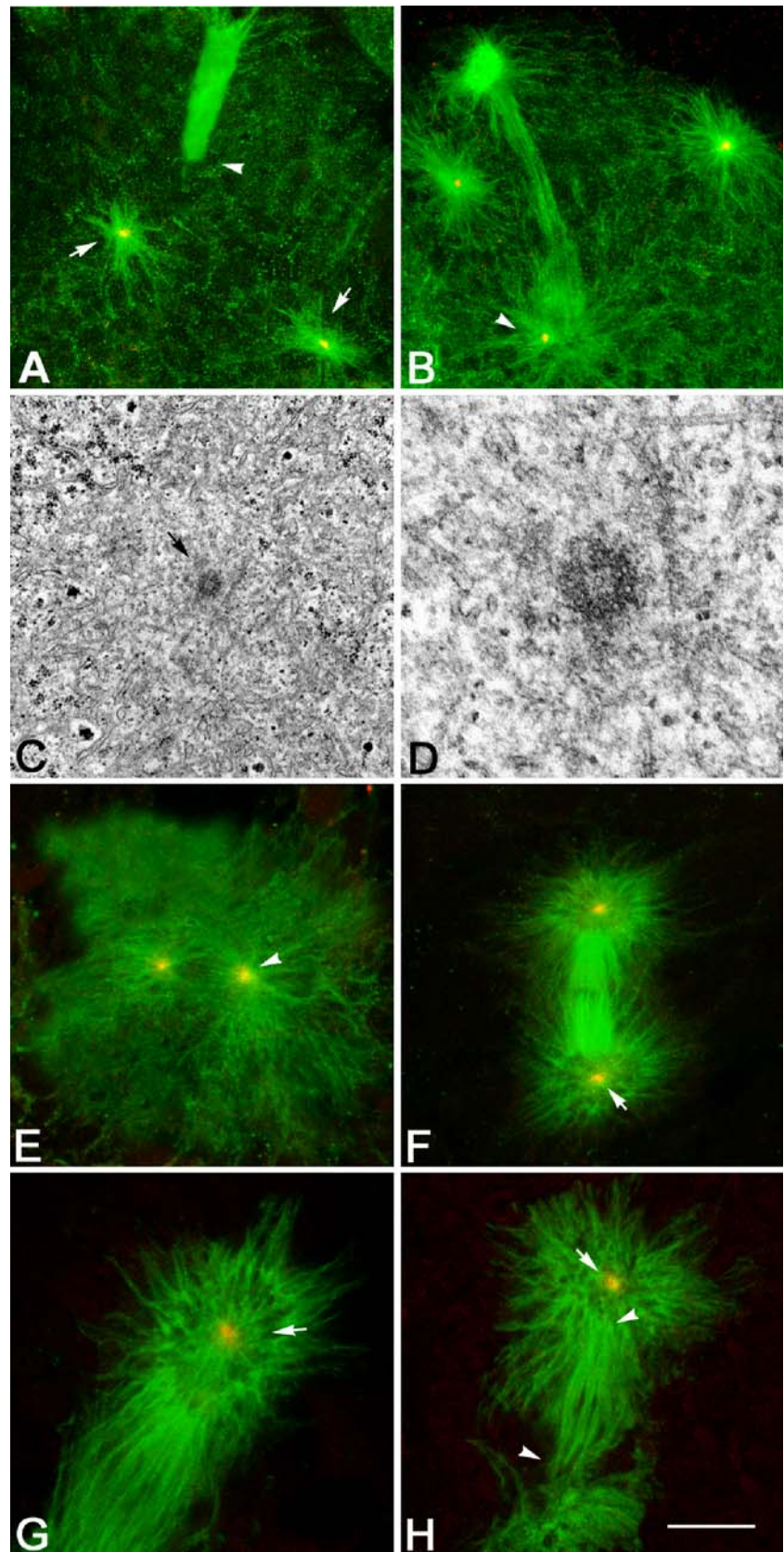
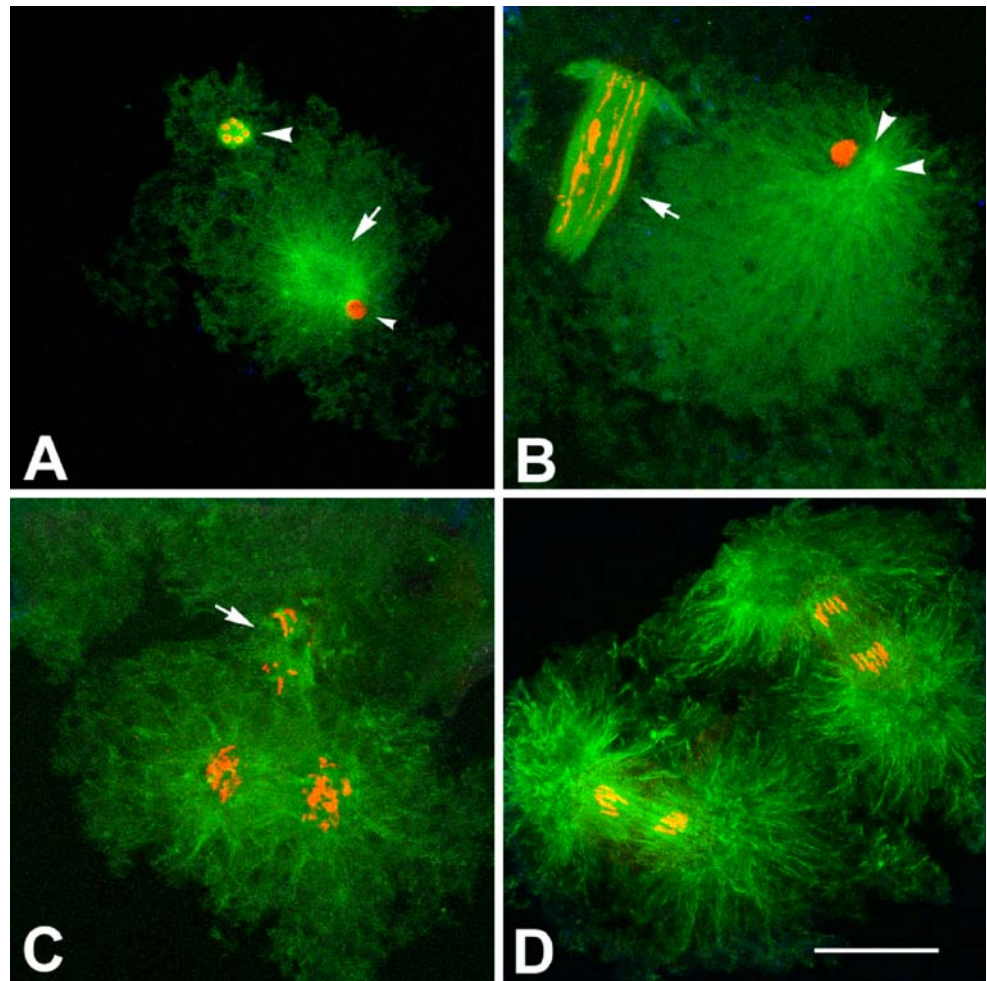


Fig. 5 Fertilization in *F. fimetaria* eggs (green microtubules, red DNA). **a** The sperm aster (arrow) is visible even in the oocyte cytoplasm during late metaphase (large arrowhead meiotic apparatus, small arrowhead sperm head). **b** During anaphase, two bright centers for microtubules of the sperm aster are visible (arrowheads) near the meiotic apparatus (arrow), suggesting that the centrosome has duplicated. **c** Telophase of the first mitosis (arrow polar bodies). **d** Late anaphase of the second mitosis. Bar 10 μ m



the parthenogenetic egg that spontaneously shift centrosome repression to activation? Parthenogenesis in *F. candida* allows a detailed investigation of the formation of the zygotic spindle in the absence of the sperm basal body. Activation of the unfertilized egg triggers the resumption of meiosis and the formation of astral arrays of microtubules. This process of spontaneous aster assembly has been observed in other insect species with parthenogenetic egg development, namely the hymenopterans *Muscidifurax* and *Nasonia* (Riparbelli et al. 1998; Tram and Sullivan 2000), *Drosophila mercatorum* (Riparbelli and Callaini 2003), and the parthenogenetic viviparous pea aphid *Acyrtosiphon pisum* (Riparbelli et al. 2005). The only exception to this common model is represented by parthenogenesis in the stick insect *Bacillus* in which the egg cytoplasm seems unable to assemble cytoplasmic asters, and the mitotic spindles appear anastral during earlier division cycles (Marescalchi et al. 2002).

F. fimetaria reproduces dioically and lays eggs that do not develop without the male gamete and are unable to form cytoplasmic asters. Cytoplasmic asters are not

observed in unfertilized eggs of the dioic strains of *D. mercatorum* (Riparbelli and Callaini 2003) and *A. pisum* (Riparbelli et al. 2005). Therefore, the property of self-organizing cytoplasmic asters is not a default characteristic of all insect oocytes that is switched off by sperm entrance during normal development but rather represents a peculiar feature that parthenogenetic eggs might have evolved under particular conditions. This observation is supported by the finding that fertilized parthenogenetic eggs of *D. mercatorum* can assemble cytoplasmic asters despite the presence of the male centrosome (Riparbelli and Callaini 2003). Therefore, only parthenogenetic oocytes appear to have the capacity to self-organize cytoplasmic asters. Accordingly, the unfertilized eggs produced by the dioic strains of *Sciara* do not possess MTOCs and form bipolar anastral mitotic spindles that do not develop beyond the first intravitelline mitoses (De Saint Phalle and Sullivan 1998). However, artificially activated sea urchin (Dirksen 1961; Kallenbach 1985; Kuriyama and Borisy 1983; Miki-Noumura 1977) and *Drosophila* (Wilson and Borisy 1998) oocytes are able to organize cytoplasmic asters. These data suggest that the

cytoplasm of many eggs has the capability to self-assemble astral arrays of microtubules, but that this property is switched off during normal development.

The conditions that switch on the mechanism leading to the formation of cytoplasmic asters in unfertilized eggs and that result in parthenogenetic development are not clear. Randomly nucleated cytoplasmic microtubules might be focused on common poles by motor proteins, and nucleating material, such as γ -tubulin, could be recruited to the foci of the developing small asters. The progressive accumulation of this protein could lead to the formation of active nucleation centers that determine the origin of microtubule growth, leading to the gradual expansion of the cytoplasmic asters. In vertebrates, oligomeric motor complexes have been proposed to organize randomly disposed microtubules in aster-like structures (Nédélec et al. 2003; Wittmann et al. 2001). Thus, the main prerequisite for parthenogenetic development could be the presence of high numbers of cytoplasmic microtubules in the unfertilized oocyte. Parthenogenesis could be triggered by modifications of the cytoplasmic program that usually prevents spontaneous microtubule self-nucleation, a prerequisite for aster formation.

A peculiar aspect of the early embryonic development of *F. candida* eggs is the organization of the spindle during both meiosis and mitosis. As in other insects, the female meiotic spindle at metaphase is tapered and anastral, but progression throughout the following meiotic stages requires an extensive reorganization of the meiotic apparatus. This profound reorganization, not previously described in insects, could reflect an unusual dynamic of the microtubules that form the spindle apparatus. Since microtubule number and length increase dramatically as meiosis progresses, and since discrete MTOCs are not present in the oocyte cytoplasm, the microtubules might originate from two different sources: randomly dispersed microtubules recruited to the spindle apparatus from the surrounding cytoplasm and/or new microtubules that might nucleate through the spindle region in areas such as the kinetochore regions, where suitable conditions for centrosome-free microtubule nucleation might exist. Kinetochores have been suggested to capture short microtubules, with nucleation probably being promoted by a gradient of Ran guanosine triphosphate (for reviews, see Karsenti and Vernos 2001; Maiato et al. 2004).

The mitotic apparatus of the *F. candida* early embryo is also remarkable in that during interphase and prophase the asters resemble those of other insects; however, they increase in dimensions at metaphase and become large during anaphase-telophase, whereas the central spindle is unusually short. Similar large asters have been observed in several non-insect organisms, such as nematodes (Keating and White 1998), leeches (Cantillana et al. 2000), echino-

derms (Hollebeck and Cande 1985), and ascidians (Sawada and Schatten 1988). The similarity of this type of mitotic apparatus in such distantly related organisms and its absence among higher insects raises an intriguing question as to whether this type of spindle organization is an ancestral trait or a result of convergence. All the above-mentioned species that possess large asters have small eggs with holoblastic development. In these eggs, the large asters could be necessary for contact with the egg cortex in order to trigger the proper signaling for cleavage furrow formation. In echinoderm eggs that contain large asters and a small central spindle, the formation of the contractile ring is mediated by the asters. In contrast, in animal cells that have small asters and a developed central spindle, cytokinesis appears to be triggered by the central spindle (for a review, see Giansanti et al. 1998). *F. candida* displays small eggs that, at the beginning of the embryonic mitoses, cleave totally. In contrast, higher insects have larger eggs in which the nuclei divide through a syncytial cytoplasm, and furrow formation only occurs subsequent to cleavage.

During metaphase of the first mitosis, we have observed a distinct gap between kinetochore microtubule bundles and the aster of microtubules that originate at the centrosome. This suggests that kinetochore microtubules might originate or be captured at the centromeric region of the chromosomes and bundled at the opposite extremity. In this manner, they might interact with the microtubules of the asters to form the mitotic spindle. This hypothesis is supported by the finding that many kinetochore microtubules do not seem to reach the centrosome region and also by the observations that, in some slightly damaged spindles, the kinetochore bundles are clearly detached from the asters. These findings agree with the current hypothesis of mitotic spindle assembly in which acentrosomal and centrosomal pathways of microtubule formation cooperate to build a functional spindle (Wadsworth and Khodjakov 2004).

Perhaps *Wolbachia* symbionts, present in the oocytes, play a role in the restoration of diploidy by modifying the progression of meiosis, resulting in the failed separation of sister chromatids at meiosis II. We have made several unsuccessful attempts to cure *F. candida* of the *Wolbachia* infection. Meiotic oocytes are suggested to undergo only a single equatorial division in the wasp *Trichogramma cacoeciae* (Vavre et al. 2004), which is also infected by *Wolbachia*. These bacteria have been implicated in several cases of thelytoky in haplodiploid insect species (Stouthamer 1997). The influence of this organism on chromosome dynamics has been reported, although the mechanism driving these interactions remain to be elucidated. In the hymenopterans *Trichogramma* ssp. and *Leptopilina clavipes*, diploidization is caused by segregation failure in the first mitotic anaphase (Stouthamer and

Kazmer 1994; Pannebakker et al. 2004), whereas in *Muscidifurax uniraptor*, the first mitosis is normal, and the restoration of diploidy occurs by fusion of the two daughter nuclei (Gottlieb et al. 2002). Parthenogenesis induced by *Wolbachia* seems to be restricted to haplodiploid species, where males are haploid and females diploid. Collembola are not haplodiploid, and there is evidence of other insect species that reproduce parthenogenetically without being infected by *Wolbachia*. An example is the restoration of diploidy and the thelytochous parthenogenesis following failure of the second meiosis that is common in oocytes of aphid species that lack *Wolbachia* but that undergo a single maturation division (Riparbelli et al. 2005). From our data, we cannot conclude that the presence of *Wolbachia* induces parthenogenesis in *F. candida*. A possible alternative hypothesis is that *Wolbachia* is an opportunistic association with an organism that reproduces parthenogenetically in a non-endosymbiont-induced manner.

Concluding remarks

The unfertilized egg of *F. candida* is able to develop without a male contribution by means of newly assembled centrosomes from which the zygotic spindle organizes. The zygotic centrosome appears within the focus of the microtubule aster that forms at the end of meiosis near the inner pole of the spindle apparatus. The occurrence of such asters may be correlated with the cross-linking of self-assembled cytoplasmic microtubules that are present in large numbers in the parthenogenetic oocyte of *F. candida*, but not in the unfertilized oocyte of *F. fimetaria* where they form only after fertilization. The female meiotic apparatus in *F. candida* undergoes previously undescribed dynamics; it begins with a small metaphase-arrested spindle of normal appearance, which, after activation, becomes a large and unusual structure. Mitotic spindles of the early entognathous hexapod *F. candida* embryo have large asters, as reported for holoblastic eggs of other invertebrates. Such asters have not been reported for insects and probably constitute another characteristic that differentiates Collembola from ectognathous hexapods.

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