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# Spindle Assembly and Spatial Distribution of $\gamma$ -tubulin During Abortive Meiosis and Cleavage Division in the Parthenogenetic Water Flea *Daphnia pulex*

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In most animal species, centrosomes, the main microtubule-organizing centers, usually disintegrate in oocytes during meiosis and are reconstructed from sperm-provided centrioles before the first cleavage division. In parthenogenetic oocytes, however, no sperm-derived centrosome-dependent microtubule nucleation is expected, as fertilization does not occur. The water flea *Daphnia pulex* undergoes parthenogenesis and sexual reproduction differentially in response to environmental cues. We used immunofluorescence microscopy with anti- $\alpha$ -tubulin and anti- $\gamma$ -tubulin antibodies to examine spindle formation and the occurrence of centrosomes during parthenogenetic oogenesis and the subsequent cleavage division in *D. pulex*. The spindle formed in abortive meiosis in parthenogenesis is barrel-shaped and lacks centrosomes, whereas the spindle in the subsequent cleavage division is typically spindle-shaped, with centrosomes. During abortive meiosis,  $\gamma$ -tubulin is localized along the spindle, while in the first cleavage division it is localized only at the spindle poles. Thus, *D. pulex* should provide a useful comparative model system for elucidating mechanisms of spindle formation and improving our understanding of how evolutionary modification of these mechanisms is involved in the switch from sexual to parthenogenetic reproduction.

**Key words:** *Daphnia pulex*, parthenogenesis, barrel-shaped spindle, abortive meiosis, gamma-tubulin, centrosome

## INTRODUCTION

In many animal species, oocytes undergo meiosis without centrosomes, in contrast to most somatic cells, which have centrosomes at the spindle poles during mitosis (Schatten, 1994). Spindles formed without centrosomes are usually barrel-shaped and anastral, quite different from the typical spindle-shaped mitotic spindle. As the primary microtubule organizing center (MTOC) of the cell, the centrosome plays a crucial role in assembly of the bipolar spindle that drives the orderly segregation of the genetic material, and it directs many of the microtubule-based processes within the cell (Schatten, 1994; Doxsey, 2001). The centrosome is defined by having two perpendicularly arranged centrioles surrounded by a cloud of pericentriolar material (PCM).

In sexual reproduction in most animal species, centrioles are inherited paternally, and recruit maternal proteins to form a new centrosome after entering an oocyte (Schatten, 1994; Manandhar et al., 2005). In maturing oocytes, the centrosomes are eventually disassembled, and the centrioles are lost during oogenesis. This means that the first centriole in the zygote is contributed by the sperm. Centriole

inheritance thus constitutes a developmental constraint on the evolution of parthenogenesis (Manandhar et al., 2005; Engelstädter, 2008), which requires assembly of a functional spindle without sperm-provided centrioles. Possible mechanisms to achieve this are: (1) the centrioles are retained in the egg without disintegrating during oogenesis, or (2) new centrioles are formed de novo in the oocyte. The former case has not been reported to date, whereas the latter occurs in insects. For instance, in several insect species, many asters are assembled in the egg, and two of them form centrosomes in association with the female pronucleus, permitting parthenogenesis; the centrosomes are disassembled if the egg is fertilized (Riparbelli et al., 1998; Tram and Sullivan, 2000; Riparbelli and Callaini, 2003; Riparbelli et al., 2005). Comparing independently acquired mechanisms permitting parthenogenesis among representatives of various taxonomic groups provides a powerful tool for understanding the evolution of reproductive modes.

The water flea *Daphnia pulex* (Arthropoda: Crustacea: Order Cladocera) can undergo either sexual reproduction or parthenogenesis, differentially in response to varied environmental cues. Little is known about how the meiotic program has been evolutionarily altered to permit parthenogenesis. Although Schurko et al. (2009) reported the expression patterns of meiosis-related and meiosis-specific genes during parthenogenesis in *D. pulex*, the question remained whether abortive meiosis and subsequent cleavage division are gov-

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erned by the formation of spindles.

The reproductive system in *D. pulex*, which switches between parthenogenesis and sexual reproduction, allows a direct comparison between parthenogenetic and fertilized oocytes, in terms centrosome inheritance and how sperm entry affects spindle formation and the localization of  $\gamma$ -tubulin. In this study, to begin to understand the cytological bases for parthenogenesis in *D. pulex*, we investigated the extent to which spindle structures control abortive meiosis and the subsequent cleavage division (mitosis), using immunofluorescence to examine alpha ( $\alpha$ )-tubulin in microtubules and gamma ( $\gamma$ )-tubulin in centrosomes. In addition, our study establishes a novel model experimental system for investigating the mechanism of de novo centrosome assemblage prior to the first cleavage division.

## MATERIALS AND METHODS

### Time course of abortive meiosis in *D. pulex*

As background to the experimental approach used in this study, we will briefly describe the time course of abortive meiosis in *D. pulex* (Fig. 1) (Hiruta et al., 2010; Hiruta and Tochinai, 2012). After releasing all offspring that have developed in the dorsal brood chamber, females molt. This molt provides a reference time point, 0 AM (minutes after molting), for the beginning of another iteration of the reproductive cycle, which proceeds along a strict time course at 18°C. The female begins to extrude eggs into the brood chamber at 13 AM and completes this process within about 3 min. The time at which a female extrudes the last egg is considered time point 0 PO (minutes post-oviposition). After release, the parthenogenetic eggs develop into juveniles in the brood chamber.

In the first meiosis (Fig. 1, lower part) starting at 0 AM, bivalents align on the equatorial plate and begin to separate into two half-bivalents. However, division is arrested in early anaphase at 10 AM; each half-bivalent moves back, and at 0 PO the sister chromatids are rearranged around the equator of the spindle. No polar body is produced in the aborted first meiosis. The second meiosis-like division takes place normally, producing a small cell analogous to the second polar body at 20 PO.

### Animals and experimental methods

*Daphnia pulex* were obtained from a pond on the campus of Hokkaido University, Sapporo, Japan, and subsequently maintained in culture in freshwater at 18°C, under artificial light conditions of

14 h light and 10 h dark to induce and maintain reproduction. They were fed daily with a concentrated monoculture of the green alga *Chlamydomonas reinhardtii*, as described in Sueoka (1960).

We observed spindle formation in eggs in the course of abortive meiosis, as previously described by Hiruta et al. (2010; Fig. 1). Changes in spindle microtubules during abortive meiosis and cleavage division were examined in oocytes and laid eggs by immunostaining using an anti- $\alpha$ -tubulin antibody.

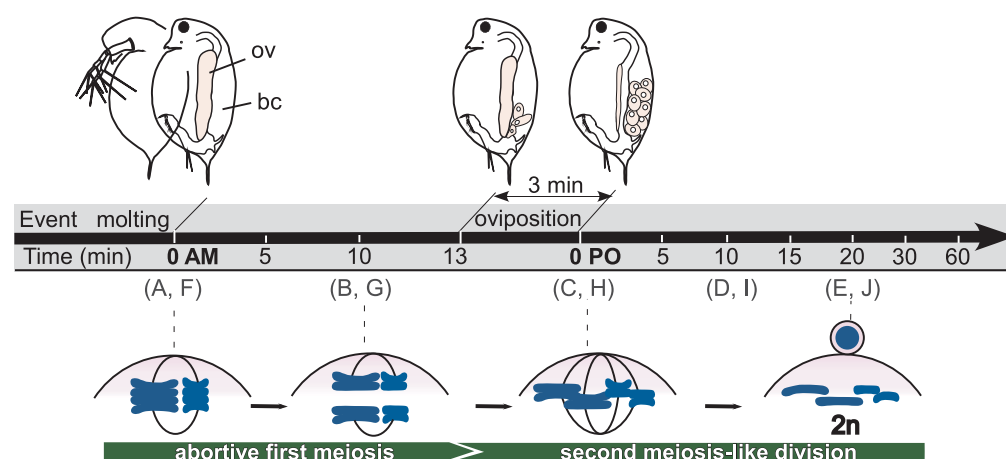
Mature oocytes were dissected from ovaries, using forceps (Dumoxel #5 Biologie) under a stereomicroscope, and spawned eggs were collected from the brood chamber. Specimens were fixed in 99% glacial ethanol and stored at -20°C until use. For immunocytochemistry, fixed animals were washed three times in PBST (0.05% Tween 20 in PBS) for 10-min each. The samples were then treated at 4°C in blocking solution (10% fetal calf serum and 0.5% Tween 20 in PBS) with anti- $\alpha$ -tubulin mouse antibody (MON4009; Monosan) at a 1:40 dilution overnight, or anti- $\gamma$ -tubulin mouse antibody (T6557; Sigma) at a 1:500 dilution for two days. After three 10-min washes in PBST, the samples were incubated with Cy3-conjugated anti-mouse IgG rabbit antibody (1:500; AP160C; Chemicon) overnight at 4°C. After another three 10-min washes in PBST, the samples were stained with 0.5  $\mu$ g/ml DAPI (MP Biomedicals, LLC) in PBS for 25 min to visualize nuclear DNA, followed by two 10-min washes in PBST. Finally, the samples were mounted with Vectashield (H-1000; Vector laboratories) and observed under a fluorescence microscope (BX-50, BX-FLA; Olympus) and confocal laser scanning microscope (LSM510; Zeiss). Spindle axial length and width measurements were taken from electronic images of immunostained specimens by using the measuring tool in the software Adobe Photoshop CS4, and the ratio of axial length to axial width (axial ratio) was calculated.

## RESULTS

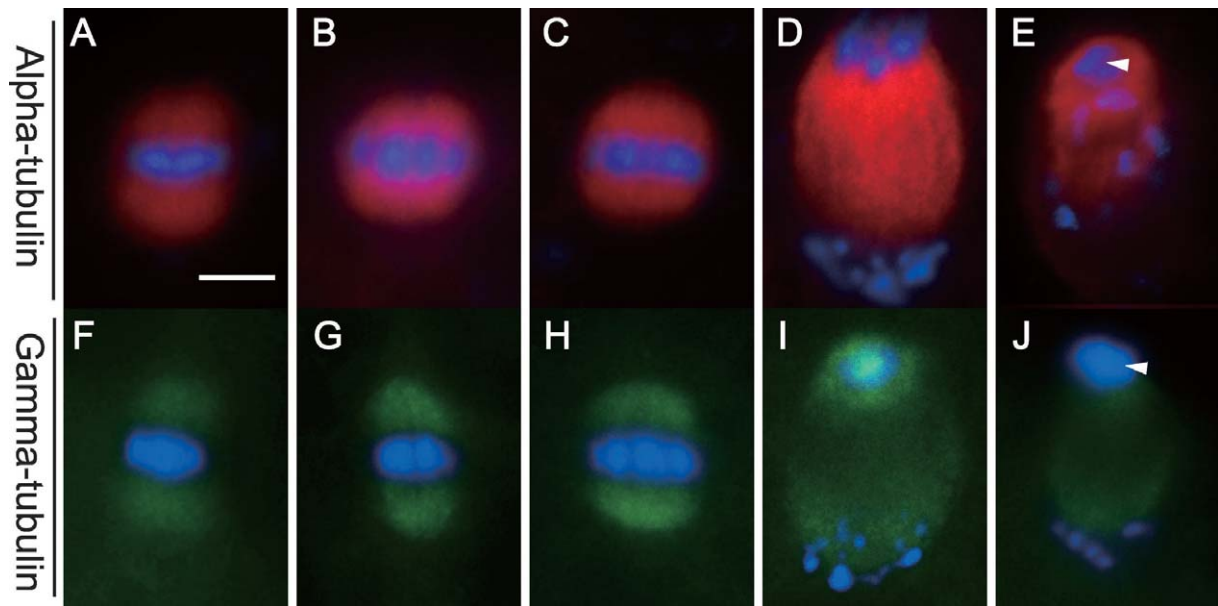
The spindle formed in abortive meiosis is shorter and barrel-shaped, without asters (Fig. 2A–E) or centrosomes (Fig. 2F–J). In contrast, in the first embryonic cleavage following abortive meiosis, the spindle is longer, and of the 'spindle-shaped' form typical of mitosis in most animals, with asters (Fig. 3A, B) and centrosomes (Fig. 3E–H). Figure 4 quantifies the difference in spindle size and shape between abortive meiosis and cleavage division. The spindle in both the first and second meiosis-like divisions of abortive meiosis had a significantly lower spindle axial ratio than in the

cleavage division, and was shorter. In other words, the spindle in the meiosis-like divisions was barrel-shaped, whereas that in the cleavage division was more extended along the longitudinal axis, and spindle-shaped.

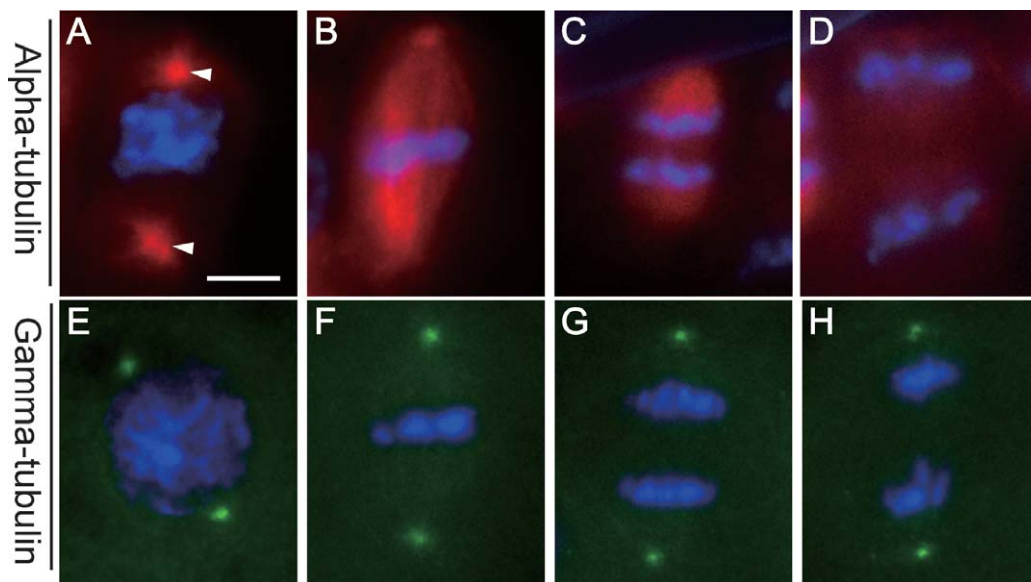
Throughout abortive meiosis, in both the first (Fig. 2F, G) and second (Fig. 2H–J) meiotic-like stages,  $\gamma$ -tubulin was distributed along spindle microtubules, with no apparent centrosomes at the poles. From prophase through telophase in the cleavage division,  $\gamma$ -tubulin



**Fig. 1.** Time course of abortive meiosis in *D. pulex* (see Materials and Methods for a description). The capital letters below the time line correspond to the timing of panels in Fig. 2. ov, ovary; bc, brood chamber. Modified from Hiruta et al. (2010).



**Fig. 2.** Parthenogenetic eggs of *D. pulex* undergoing abortive meiosis, with spindles (pink, top row) indicated by immunofluorescence visualization of  $\alpha$ -tubulin and possibly present centrosomes (green, bottom row) by visualization of  $\gamma$ -tubulin. Chromosomes are counter-stained with DAPI (blue). The spindle is barrel-shaped, anastral, and organized without centrosomes;  $\gamma$ -tubulin is distributed along the spindle microtubules. **(A, F)** 0 AM, first metaphase. **(B, G)** 10 AM, division arrested at first anaphase. **(C, H)** 0 PO, second, meiosis-like metaphase. **(D, I)** 10 PO, second meiosis-like anaphase. **(E, J)** 20 PO, emission of small cell analogous to the second polar body (white arrowhead). Scale bar = 5  $\mu$ m.



**Fig. 3.** Embryos of parthenogenetic *D. pulex* during cleavage division (mitosis), with spindles (pink, top row) indicated by immunofluorescence visualization of  $\alpha$ -tubulin and centrosomes (green, bottom row) by visualization of  $\gamma$ -tubulin. Chromosomes are counter-stained with DAPI (blue). The spindle is spindle-shaped and astral;  $\gamma$ -tubulin is present only at the spindle poles. **(A, E)** Prophase; arrowheads in **(A)** indicate asters. **(B, F)** Metaphase. **(C, G)** Anaphase. **(D, H)** Telophase. Scale bar = 5  $\mu$ m.

was localized in the centrosomes, evident as a distinct spot at each spindle pole (Fig. 3E–H).

### DISCUSSION

We found that the spindle in abortive meiosis in *D. pulex* is barrel-shaped, anastral, and organized without centrosomes (Fig. 5). A barrel-shaped meiotic spindle without

centrosomes has been reported in a number of other animal species (e.g., mouse, pig, nematode; Schatten, 1994; Lee et al., 2000); among facultatively parthenogenetic insects, it occurs in the hymenopterans *Muscidifurax uniraptor* (Riparbelli et al., 1998) and *Nasonia vitripennis* (Tram and Sullivan, 2000); *Drosophila mercatorum* (Riparbelli and Callaini, 2003); and the pea aphid *Acyrtosiphon pisum*



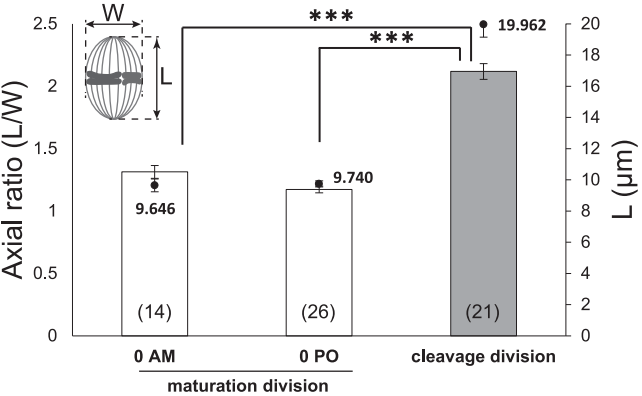
(Riparbelli et al., 2005). Diversity among spindles of this type suggests several different mechanisms may be involved in their production. For example, spindles are assembled from microtubules nucleated near the chromosomes in parthenogenetic *Sciara* embryos (de Saint Phalle and Sullivan, 1998) and in meiotic *Drosophila* oocytes (McKim and Hawley, 1995), whereas the meiotic spindle of the mouse egg has no centrosomes, but exhibits a broad band of pericentriolar material (PCM) at each pole (Calarco-Gillam et al., 1983). We still do not understand how these various systems of spindle formation evolved. A better understanding of mechanisms behind spindle formation in sexually reproduction animals is needed before informative comparisons can be made between normal sexual meiosis and asexual abortive meiosis.

Figure 5 summarizes diagrammatically the differences in the spindle between abortive meiosis and the parthenogenetic cleavage division in *D. pulex*. In abortive meiosis,  $\gamma$ -tubulin is distributed along spindle microtubules, whereas in the cleavage it is present only at the spindle poles. Localization of  $\gamma$ -tubulin to the spindle poles (centrosomes) is the typical condition in mitosis in animals and is highly conserved (Stearns et al., 1991). Unlike mitosis,  $\gamma$ -tubulin local-

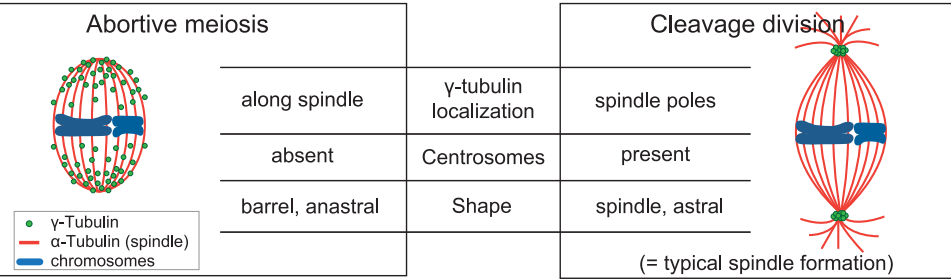
ization during the meiotic maturation of oocytes has not been well studied. In the parthenogenetic pea aphid (*Acyrtosiphon pisum*), Riparbelli et al. (2005) detected no  $\gamma$ -tubulin aggregates at the poles of meiotic spindles, indicating that this spindle formation is not dependent on MTOC function. In contrast, meiotic spindle assembly in *D. pulex* possibly has some relationship to MTOC function, as we detected  $\gamma$ -tubulin along the spindle during abortive meiosis.

We consider PLK1 (Polo-like kinase), which is involved in orienting kinetochores during mitosis and meiosis, to be a candidate protein involved in the localization of  $\gamma$ -tubulin. In mitotic spindle formation in human and *Drosophila* cells, PLK1 controls the localization of  $\gamma$ -tubulin within the spindle through the augmin complex (Goshima et al., 2008; Uehara et al., 2009), and it might be informative to investigate whether any of the PLK paralogs is specifically expressed in abortive meiosis in *D. pulex*. Moreover, the distribution of  $\gamma$ -tubulin in *D. pulex* is quite similar to those in pig oocytes during metaphase I and II, although  $\gamma$ -tubulin is localized in the spindle midzone during anaphase I and telophase II in pig oocytes (Lee et al., 2000). Lee et al. (2000) suggested that the pig meiotic spindle poles are formed by the bundling of microtubules at the minus ends by NuMA (nuclear mitotic apparatus protein). Comparative studies of spindle formation between parthenogenetic and sexual meiosis are needed to elucidate the mechanisms governing meiotic spindle assembly in the absence of preexisting centrosomes, and whether sperm entry affects the localization of  $\gamma$ -tubulin.

Centrosomes are present in the parthenogenetic cleavage division in *D. pulex*. During sexual reproduction in various animals, the sperm supplies the centriole after fertilization (Schatten, 1994). In contrast, in insect parthenogenesis, centrosomes are spontaneously assembled among the mitotic spindle microtubules. Riparbelli et al. (2005) reported that in oocytes of the parthenogenetic pea aphid *A. pisum*, microtubule-based asters self-organize, and in turn interact with the female chromatin to form the first mitotic spindle. Because we did not observe microtubule-based asters, it appears that parthenogenetic eggs in *D. pulex* have another mechanism for rapidly and efficiently assembling mitotic spindles in the absence of pre-existing centrosomes. We speculate that the de-novo assembly of centrosomes is an evolutionary conserved process leading to parthenogenetic development, but how *D. pulex* assembles new centrosomes in the parthenogenetic oocyte remains a mystery.



**Fig. 4.** Spindle axial ratio ( $L/W$ ; see spindle diagram at upper left) and spindle length for abortive meiosis and cleavage division in parthenogenetic *D. pulex* ( $***P < 0.001$ ,  $t$ -test). The large bars represent mean values for the axial ratio (left axis), with range lines indicating the standard deviation. Numbers in parentheses below the large bars indicate the sample size. Small circles with range bars represent mean axial length (right axis) and the standard deviation.



**Fig. 5.** Schematic diagram comparing spindle morphology between abortive meiosis and cleavage division in parthenogenetic *D. pulex*.

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