1. Title; **Abortive meiosis in the oogenesis of parthenogenetic *Daphnia pulex***

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**Abstract**

Most daphnid species adopt parthenogenesis and sexual reproduction differentially in response to varied environmental cues, resulting in the production of diploid progenies in both cases. Previous studies have reportedly suggested that daphnids produce their parthenogenetic eggs via apomixis; the nuclear division of mature oocytes should be an equational division similar to somatic mitosis. However, it seems premature to conclude that this has been unequivocally established in any daphnids. Therefore, the objective of our research was to precisely reveal the process and mechanism of parthenogenetic oogenesis and maintenance of diploidy in *Daphnia pulex* through histology, karyology and immunohistochemistry. We found that when a parthenogenetic egg entered the first meiosis, division was arrested in the early first anaphase. Then, two half-bivalents, which were dismembered from each bivalent, moved back to the equatorial plate and assembled to form a diploid equatorial plate. Finally, the sister chromatids were separated and moved to opposite poles in the same manner as the second meiotic division followed by the extrusion of one extremely small daughter cell (resembling a polar body). These results suggest that parthenogenetic *D. pulex* do not adopt typical apomixis. We hypothesize

that *D. pulex* switches reproductive mode depending on whether the egg is fertilized or not.

## Introduction

Parthenogenesis, which can be caused by a number of cytological mechanisms, is widespread in animals and plants (Suomalainen *et al*. 1987). Although the precise mechanism varies from species to species, patterns of parthenogenesis can generally be classified into a few categories on a cytological basis: Apomixis, meiosis is repressed and the oocyte is produced by single equational division as seen in somatic mitosis; Automixis, meiosis is maintained and the diploid state is restored after meiosis either by the fusion or duplication of meiotic products; and Premeiotic endomitosis, the genome is doubled before meiosis and then segregated. Apomixis and premeiotic endomitosis do not lead to genetic variation of the resulting eggs (Itono *et al.* 2006). However, automixis can lead to offspring with genetic variability because chromosomal recombination could take place between homologous chromosomes, while there is no introduction of new genes from another individual.

In vertebrates, sexual reproduction clearly dominates reproductive modes.

There are, however, several invertebrate species that reproduce by constitutive asexual propagation (Welch and Meselson, 2000). The evolutionary causes and consequences of the shift from sexual reproduction to parthenogenesis remain largely uncertain. Although substantial attention and discussion have been given to the selective advantages and disadvantages associated with meiotic recombination, less clear are the cytogenetic and molecular mechanisms that lead to the conversion of an ancestral meiotic condition to parthenogenesis (Peck 1994, Otto and Barton 2001). For sustainable reproduction, the production of unreduced diploid eggs or the recovery from the reduced haploid eggs is a prerequisite to parthenogenetic reproduction and, therefore, this has been of paramount interest to researchers that are concerned with the evolution of sex.

The aquatic microcrustacean *Daphnia pulex* provides a potentially useful tool for investigating these issues for the following reasons. Most daphnid species reproduce parthenogenetically as well as sexually, resulting in the production of diploid progenies in both cases. In natural populations, parthenogenesis is the common mode of reproduction and parthenogenetic offspring are normally female. However, in response to certain environmental conditions, such as crowding or seasonal change, male offspring are also produced

parthenogenetically, and then sexual reproduction occurs (Hebert, 1978). Previous studies have suggested that daphnids produce their parthenogenetic eggs via apomixis (Kühn, 1908, Lumer, 1937, Zaffagnini and Sabelli, 1972, Ojima, 1954, 1958). Although there has also been a report suggesting the occurrence of endomeiosis (Bacci *et al*., 1961), it is inconsistent with the results of other more extensive cytological studies mentioned above. In any event, the parthenogenetic reproductive mode of these species has not yet been unequivocally demonstrated.

In this study, we closely examined the process and mechanisms of parthenogenetic oogenesis and maintenance of diploidy in *D. pulex* by histology, karyology and immunohistochemistry. Here, we demonstrate that diploidy in *D. pulex* is maintained by meiotic arrest at early first anaphase and subsequent separation of sister choromatids in the same manner as the second meiosis, and also hypothesize the cytological mechanisms of diploidy maintenance, which operate differently between parthenogenesis and sexual reproduction in *D. pulex*.

## Materials and Methods

## Animal

The water flea *Daphnia pulex* collected from a pond on Hokkaido University campus, Japan, was used in the present study. The animals were cultured in freshwater at 18°C under artificial light conditions (14 h light and 10 h dark) to induce and maintain the daily reproductive cycle. They were fed with a concentrated monoculture of green alga (*Chlamydomonas reinhardtii*) every day as described in Sueoka (1960).

## Histological preparation

The animals were fixed in Bouin's fluid (picric acid saturated aqueous solution: formalin: acetic acid = 15: 5: 1) overnight. After dehydration with ethanol and xylene, the specimens were embedded in paraffin and sliced serially into 5 µm thick sections. The sections were deparaffinized with xylene, hydrated with ethanol, and then stained with Delafield's hematoxylin and eosin (HE).

## Antibody and immunostaining

The animals were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH7.4) for 2 h at 4°C. The fixed animals were then embedded in paraffin

and serially sectioned at 5 µm. The sections were rehydrated and washed with 0.1% Triton X-100 in PBS and blocked in blocking solution (10% fetal calf serum in PBS) for 1 h at room temperature. The sections were then treated with anti-phospho-histone H3 [pSer$^{10}$] rabbit antibody (Sigma-Aldrich) at a 1:250 dilution in blocking solution overnight at 4°C. After being washed in PBS, the sections were incubated with Cy3-conjugated anti-rabbit IgG goat antibody (1:500; Chemicon) for 2 h at room temperature. After washing three times in PBS for 5 min, the sections were stained with 100 µg/ml Hoechst 33342 (Molecular Probes, Eugene, OR, USA) in PBS for 5 min to visualize nuclear DNA, followed by a wash three times in PBS for 5 min. Finally, the sections were mounted with glycerol and observed under a fluorescent microscope.

**Chromosome preparation**

Mature oocytes were dissected out from ovaries, and spawned eggs were picked up from the brood chamber. Dissections were made with a pair of forceps (Dumoxel #5 Biologie) under a stereomicroscope. The specimens were put on a glass slide in a drop of 1% sodium citrate and then teased apart with a pair of needles. Accumulated yolk granules and oil droplets were flushed from the eggs.

Immediately after this spreading process, they were fixed in a drop of Carnoy's solution (methanol: glacial acetic acid = 3: 1) and finally a few drops of glacial acetic acid were poured above. After overnight air drying, the slides were carefully rinsed with 50% methanol and stained with 4% Giemsa.

## Results

### Histological observation

Soon after grown juveniles were discharged from the brood chamber, the nuclear membrane of the fully-matured ovarian egg to be spawned gradually disappeared, and finally the breakdown of the germinal vesicle took place just before molting of the mother. The nuclear division apparatus appeared just after molting (at 0 AM: Fig. 1A). We observed the state of nuclei of the eggs at the following timing in the course of parthenogenesis (from egg maturation to early development): 1) the time of molting of the female (Fig. 1A, 0 min after molting (AM)), 2) the interval between molting and 13 minutes after molting. Then the parthenogenetic eggs began to migrate from the ovary to the brood chamber and this process was completed within about 3 minutes, 3) the time when oviposition was completed (Fig. 1A, 0 min post oviposition (PO)), and 4) the time

during which the parthenogenetic eggs in the brood chamber were developed. More than 20 specimens were examined in each stage.

As showed in Fig. 1B, mature ovaries had fully-grown oocytes with oogonia and immature oocytes located in the posteriormost part of the ovary. Both egg cells and nurse cells were derived from immature oocytes; however, only the egg cells developed to form yolk granules and oil droplets during maturation, whereas the nurse cells became smaller in size and finally degenerated. The number of eggs spawned in a clutch depended on the nutritional state and size of the female. Mature eggs and spawned eggs in the same brood were the same size and mostly synchronized in the maturation stage (cell cycle).

The chromosomes co-oriented in a position midway between the poles, and then each bivalent starts to separate into two half-bivalents, one moving to each pole of the spindle by 5 AM (Figs. 2A and 2B). However, the movement of chromosomes from the metaphase plate to the poles was arrested at the early stage of anaphase before 10 AM (Fig. 2C). Egg laying began at 13 AM. To examine whether the division apparatus is placed perpendicular to the cell surface in the periphery for polar body exclusion (or extremely unequal cell division) or not, we measured the distance between the metaphase plate and the

cell surface (Fig. 3). In the ovarian egg, the division apparatus was distributed at random in the egg, not located in the periphery.

The migration of all eggs from the ovary to the brood chamber was completed within about 3 minutes (= 0 PO). At 0 PO, the chromosomes moved back and assembled as diploid equatorial plate around the equator of the spindle in the spawned egg (Fig. 2D). By 5 PO, the division apparatus migrated to the periphery (Fig. 3) and the cell division cycle restarted. Then the sister chromatids moved apart, one going to each pole of the spindle through metaphase and anaphase by 10 PO (Figs. 2E and 2F). The complete set of chromosomes was lifted above the egg surface and eventually one polar body-like small daughter cell was extruded at around 20 PO (Figs. 2G and 2H).

After the completion of oogenesis, the chromosomes left in the egg moved deeper inside the egg (Fig. 2I) and mitosis occurred without cytokinesis, resulting in a polynuclear syncytial embryo (Fig. 2J). Then, the nuclei migrated to the periphery, and a typical superficial cleavage proceeded.

**Immunofluorescence microscopy**

From the above observation, the alignment of chromosomes on the equatorial

plate seemed to occur twice during egg maturation. To verify this possibility, we examined the pattern of histone H3 phosphorylation, a universal marker for mitotic and meiotic metaphase chromosomes, which is applicable to various animal species (Sakai *et al*. 2007, Hendzel 1997, Wei 1998). Chromosomal staining with the anti-phospho-histone H3 at Ser10 antibody could make a precise distinction between metaphase and anaphase. As shown in Fig. 4, histone H3 phosphorylation was lost at the early anaphase (10 AM, Fig. 4B), while the chromosomes were positively stained with the antibody at the stage 0 AM (Fig. 4A), in which the paired homologous chromosomes were arranged around the equatorial plate, and at the stage after the early anaphase (0 PO, Fig. 4C), in which the diploid chromosomes were assembled around the equator of the spindle.

**Karyological observation**

From the above results, it was supposed that the first meiotic division was aborted and subsequently equational division occurred. To clarify this point, we conducted cytogenetic analysis of eggs and embryos, and examined the configuration of meiotic chromosomes in eggs.

Because of the presence of a large amount of yolk and the minute size of chromosomes, it was not easy to obtain good chromosome preparations; however, 5 analyzable images were obtained for ovarian eggs at 0 AM, and 11 images for newly laid eggs at 0 PO. The ovarian eggs exhibited only 12 chromosomes (Fig. 5A), suggesting that the meiotic bivalents comprising four-tetrads were formed at this stage, whereas the newly laid eggs and developing embryos had 24 chromosomes, each with two chromatids (Figs. 5B and 5C). Over one hundred cells were observed and all the cells in the developing embryo had the diploid number of chromosomes (2n=24: Fig. 5C), which is in agreement with Beaton and Hebert (1994).

## Discussion

### The process of parthenogenetic oogenesis in *D. pulex*

It has long been accepted that parthenogenetic *D. pulex* propagates by apomixis (Kühn, 1908, Ojima, 1954, 1958, Zaffagnini and Sabelli, 1972). But the present results suggested that parthenogenetic oogenesis in this species is caused by abortive meiosis (atypical automixis), on the basis of the following observations. Histological and immunohistochemical analyses revealed that two half-bivalents,

which were dismembered from each bivalent and moved to opposite poles in the ovarian eggs, were assembled as diploid equatorial plate after early anaphase in the laid eggs. And the second meiosis-like division, in which chromosomes divided equationally as seen in somatic mitosis, subsequently occurred (Figs. 2 and 4). If extrusion of the first polar body occurred in the ovarian egg, migration of the division apparatus to the egg surface should also have been observed before oviposition. However, Fig. 3 clearly shows that the migration of the division apparatus to the periphery occurred only once after oviposition, suggesting that the extrusion of the polar body, which results in the production of a haploid egg, does not occur in the ovarian egg. The resulting single cell embryos develop by successive mitotic cleavages (Figs. 2I and 2J). Cytogenetic analysis revealed that homologous chromosome pairing, which is the hallmark of meiosis, occurred during the first division (Fig. 5A), and 24 chromosomes, each with two chromatids, were observed at the second division (Fig. 5B).

These results suggest that diploidization in *D. pulex* is maintained by the failure of chromosomal segregation at the first meiotic anaphase. The diploid progeny are produced by this type of non-reductional meiosis (automixis though atypical) in parthenogenesis of *D. pulex* as depicted in Figure 6. Schurko *et al.*

(2009) reported that expression patterns of meiosis-related genes and meiosis-specific genes during sexual reproduction of *D. pulex* are similar to that during parthenogenesis, suggesting that parthenogenetic oogenesis is driven by the meiotic machinery.

A question now arises: does genetic variation arise even in parthenogenetic reproduction in *D. pulex*? These results provide a possibility that *D. pulex* adopts atypical automictic parthenogenesis, giving rise to genetic variability even by parthenogenetic reproduction. However, we have so far failed to observe chiasmata where crossing-over occurred (data not shown). We are planning to clarify whether genetic variation is detected or not by analyzing microsatellite DNA markers.

**The parthenogenetic mode in *D. pulex***

According to Suomalainen *et al*. (1987), the following cases have been categorized as automixis. I) In Trematoda *Fasciola hepatica*, the first meiosis occurs without cytokinesis, giving rise to two haploid nuclei. These nuclei fuse and form a diploid cleavage nucleus (Sanderson, 1952). II) In Lepidoptera *Apterona helix*, the first meiosis is aborted at the end of anaphase and two

metaphase plates, an inner and outer, are formed. Both of these contain the haploid number of chromosomes and have separate spindles. Before the second meiosis the inner metaphase spindle with its chromosome plate moves to the side of the outer spindle. Finally, the two spindles and the metaphase plates lie side by side and fuse (Narbel, 1946). III) In Crustacea *Artemia salina* (Stefani, 1960), Lepidoptera *Solenobia lichenella* (Narbel-Hofstetter, 1950), *Luffia ferchaultella* (Narbel-Hofstetter, 1965) and *L. lapidella* (Nerbel-Hofstetter, 1963), meiosis is interrupted at some stage between the end of the first anaphase and the second metaphase. Then the two haploid plates reunite, forming a new metaphase spindle, and the second diploid meiosis is accomplished.

Taking the automictic cases into consideration, the parthenogenetic *D. pulex* could be categorized into one of the automictic meiosis as described below. As is the case with II) and III), inner haploid chromosomes equal the secondary oocyte nucleus and outer haploid chromosomes equal the first polar body nucleus. Thus, the mode of parthenogenesis in *D. pulex* is regarded as the fusion of meiotic products, although both of the fused products are intermediate because two ‘complete’ haploid nuclei were not formed in the process. As for the stage of

meiotic arrest, which is early first anaphase in *D. pulex*, it differs from any other species so far observed. There has been a report that an atypical course of apomictic oogenesis occurs in parthenogenetic weevils, which are called ‘rudimentary reduction divisions’. The remnants of meiosis, in which chromosomes form bivalents, are not associated with recombination (Suomalainen *et al*. 1987, Lachowska *et al*. 2008). According to later report, however, it is suggested that some degree of recombination occurs (Rożek *et al*. 2009). At any rate, there has so far been no report that oogenesis in parthenogenetic weevils proceeds to first anaphase. Thus, the mode of parthenogenetic oogenesis in *D.pulex* can be thought of as an intermediary mode between ‘apomixis’ representing the remnants of meiosis and ‘automixis’ resulting in the fusion of meiotic products. To gain insight into the mechanisms which cause the arrest of the first meiosis in parthenogenetic *D. pulex*, molecular biological approaches are necessary. In addition, it is worth analyzing functions of meiotic genes (e.g. SMCs, REC8) found in *D. pulex* (Schurko *et al.*, 2009).

**A hypothetical model of the reproductive strategy in *D. pulex***

We hypothesized that *D. pulex* switches its reproductive mode (sexual or

parthenogentic) depending on whether the egg is fertilized or not. It is highly plausible that, if the egg is not fertilized, the first meiosis is aborted and subsequently a second meiosis-like division takes place as observed in the present study. On the other hand, normal meiosis will occur if the ovarian egg is fertilized. In order to verify this hypothesis, we are currently trying to establish methods for *in vitro* maturation of ovarian eggs and artificial insemination.

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## Figure legends

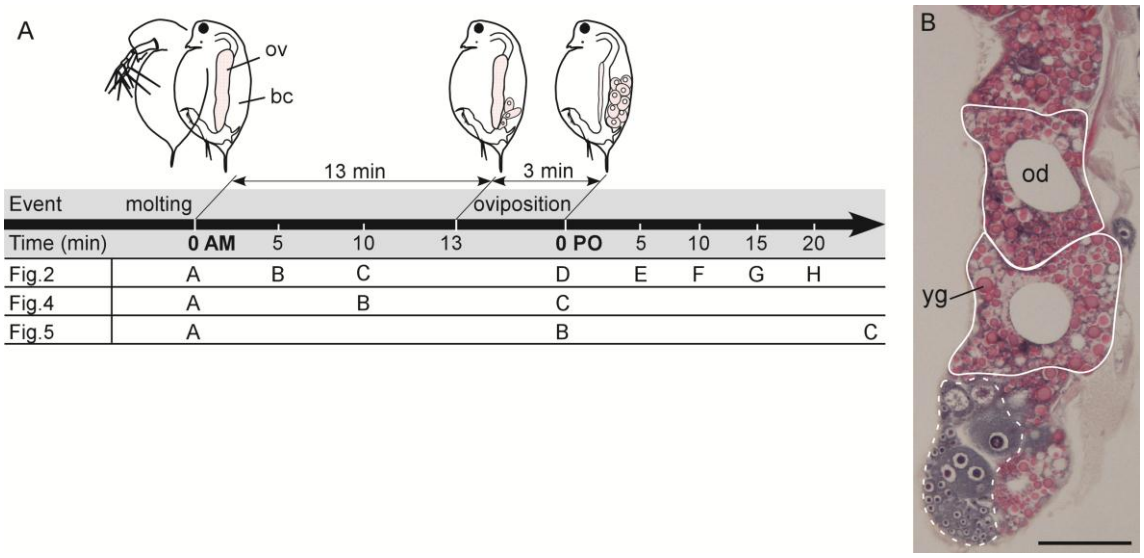


**Fig. 1. The time course of parthenogenesis in *D. pulex***

(A) Usually after releasing all neonates that developed in the dorsal brood chamber, females molt. After this molt occurs at 0 AM (minutes after molting), a strict time course proceeds. The female begins extruding eggs into the brood chamber at 13 AM and this process is completed within about 3 minutes. The point of 0 PO (minutes post oviposition) indicates the time when the female extruded the last egg. Then, the parthenogenetic eggs in the brood chamber develop to juveniles. Ovarian and spawned eggs in a clutch are approximately the same in size and mostly synchronized in the cell cycle. In the lower part of Fig.1A, the capital letters show the point of time when the specimens were observed in the following figures (Figs. 2, 4, 5). (B) The transverse section of

ovary in the fully grown adult. The largest eggs contain a large amount of yolk granules and oil droplets (solid white line). Oogonia and smaller oocytes are located in the most-posterior part of the ovary (dashed white line). Scale bar = 50 µm. ov, ovary; bc, brood chamber; yg, yolk granule; od, oil droplet.

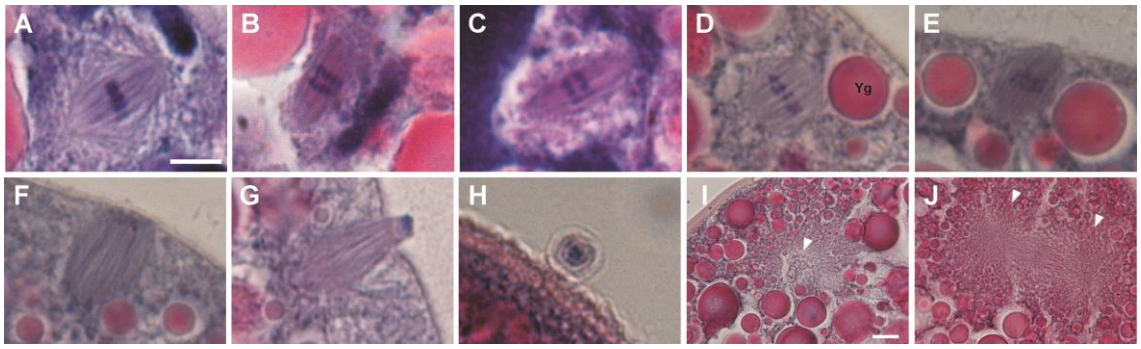


**Fig. 2. Histological observation of oogenesis in parthenogenetic *D. pulex***

(A)-(C) show the mitotic apparatus found in ovarian eggs and (D)-(J) show those in eggs spawned to the brood chamber. (A) 0 AM. Division apparatus appeared and chromosomes aligned at the metaphase plate. (B) 5 AM. Each bivalent is separated into two half-bivalents. (C) 10 AM. The division seemed to stop at early anaphase. (D) 0 PO. The chromosomes moved back and rearranged around the equator of the spindle. (E) 5 PO. The chromosomes started to separate. (F) 10 PO. The division proceeded to anaphase. (G) 15 PO. One complete set of chromosomes was lifted above the egg surface. (H) 20 PO. A

polar body-like small daughter cell was extruded. (I) 30 PO. The swelled chromosomes left in the egg moved to a deeper part (white arrow). (J) 60 PO. The first cleavage proceeded without cytokinesis (white arrows). yg, yolk granule. Scale bars = 5 μm in A-H; 10 μm in I and J.

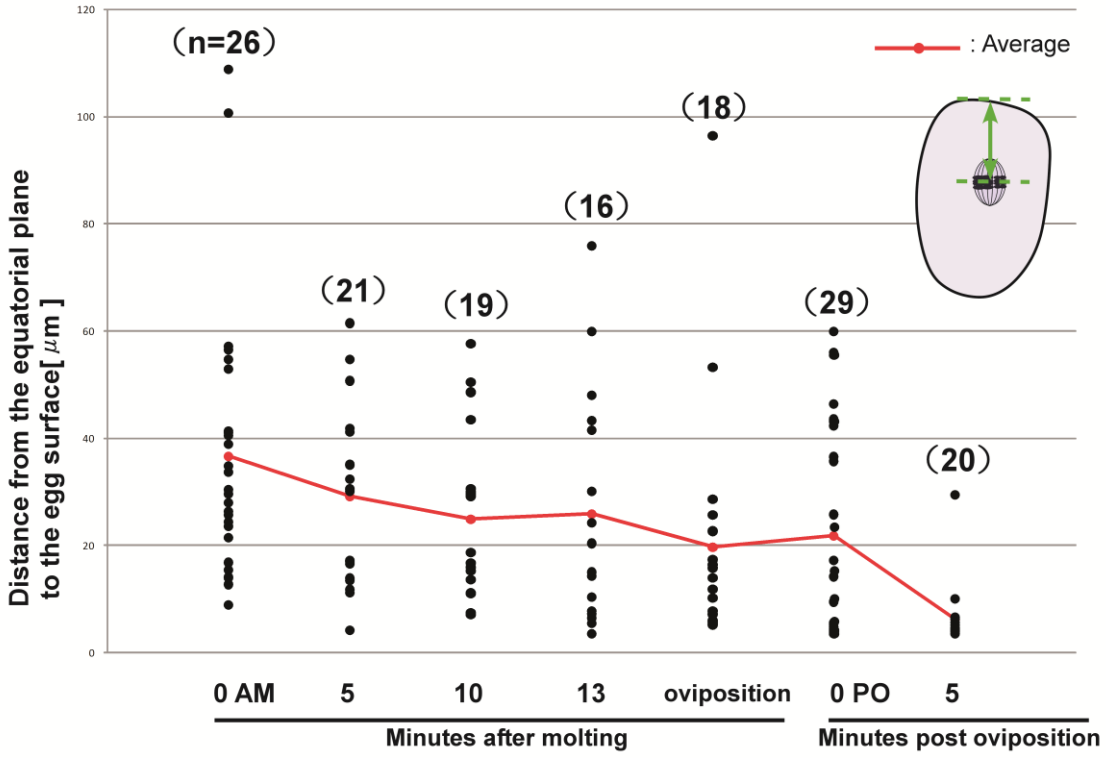


**Fig. 3. Location of division apparatus during oogenesis and development in parthenogenetic *D. pulex*.**

The distance between the metaphase plate and the cell surface was measured with HE-stained specimens (see inset right above). Solid circles indicate each specimen. Numbers in parentheses indicate the number of measured specimens.

The division apparatus in each egg was distributed at random in the ovarian egg. By 5 PO, the division apparatus migrated to the periphery.

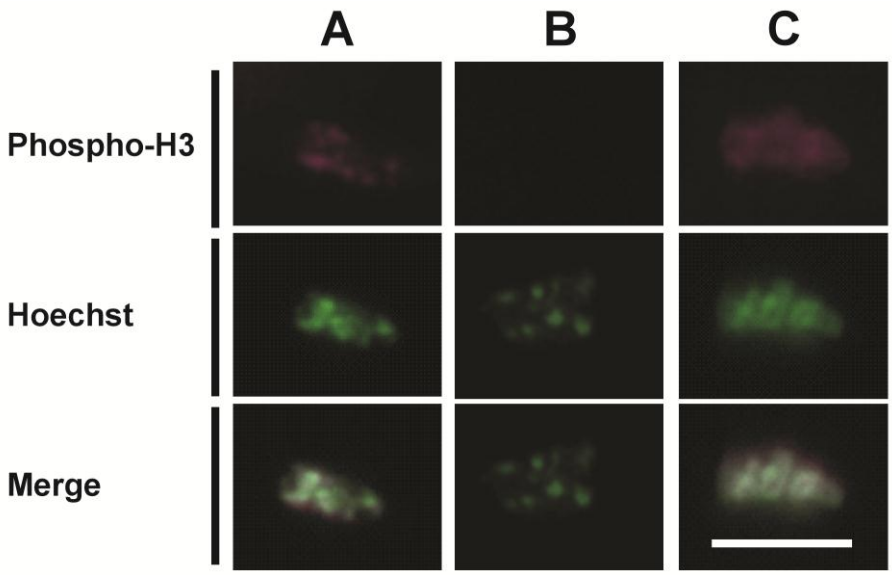


**Fig. 4. Immunohistochemical staining of chromosomes with anti-phospho-histone H3 antibody in parthenogenetic *D. pulex***

All samples were counter-stained with Hoechst 33342. Immunofluorescence of phospho-histone H3 was pseudocolored magenta and fluorescence of Hoechst 33342 was pseudocolored green. Colocalization of phospholylated H3 and DNA staining is white. (A) 0 AM. Histone H3 phosphorylation was detected. (B) 10 AM. Histone H3 phosphorylation was lost from the separating chromosomes. (C) 0 PO. Histone H3 phosphorylation was again detected. Scale bar = 5 μm.

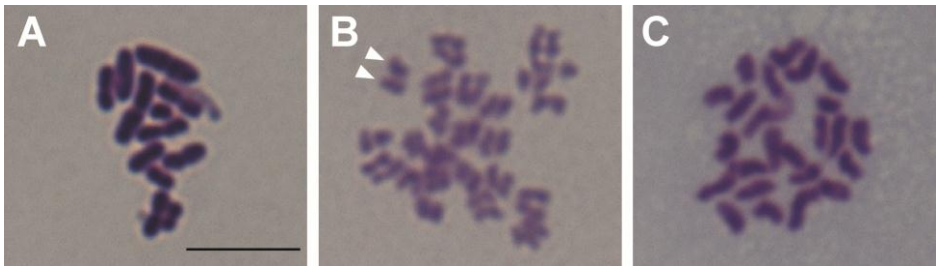


**Fig. 5. Karyological analysis of eggs and embryos in parthengenetic *D. pulex***

(A) 12 bivalents were observed at the first meiotic metaphase (0 AM). (B) 24 chromosomes, consisting of two sister chromatids, were observed at the second division (0 PO). Arrows; sister chromatids. (C) Each nucleus of the embryo had 24 chromosomes. Scale bar = 5 µm.

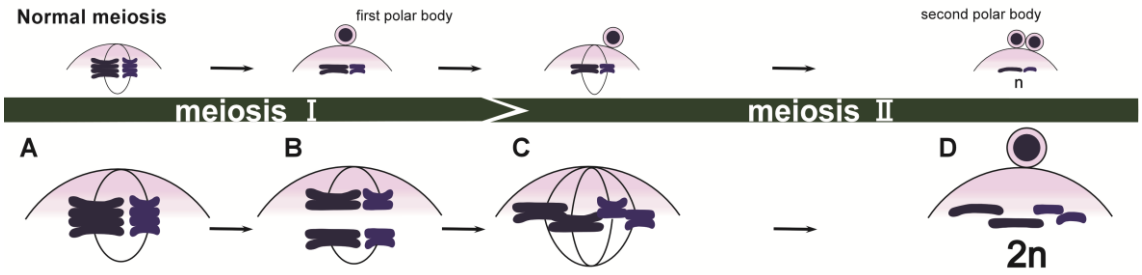


**Fig. 6. Schematic illustration of parthenogenesis in *D. pulex***

The top row shows the process of meiosis. In the first meiosis, bivalents aligned at the metaphase plate and separate into two half-bivalents, producing the first polar body. Then, the second meiosis takes place, producing the second polar body. As a result, a haploid egg is produced. By contrast, a diploid egg is

produced by parthenogenesis in *D. pulex*. (A) In the first meiosis, bivalents align at the equatorial plate and begin to separate into two half-bivalents. (B) However, the division is arrested at early anaphase. (C) Then each half-bivalent moves back and sister chromatids rearrange as diploid equatorial plate around the equator of the spindle. (D) Finally, the second meiosis-like division takes place normally, producing a single polar body-like extremely small daughter cell.