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Diploidy restoration in *Wolbachia*-infected *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae)

Yuval Gottlieb,[a] Einat Zchori-Fein,[a] John H. Werren,[b] and Timothy L. Karr[c,*]

[a] *Department of Entomology, Faculty of Agricultural, Food, and Environmental Quality Sciences, The Hebrew University, P.O. Box 12, Rehovot 76100, Israel*

[b] *Department of Biology, University of Rochester, Rochester, NY 14627, USA*

[c] *Department of Organismal Biology and Anatomy, The University of Chicago, 1027 East 57th Street, Chicago, IL 60637, USA*

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Abstract

Thelytokous reproduction, where females produce diploid female offspring without fertilization, can be found in many insects. In some Hymenoptera species, thelytoky is induced by *Wolbachia*, a group of cytoplasmically inherited bacteria. We compare and contrast early embryonic development in the thelytokous parthenogenetic species *Muscidifurax uniraptor* with the development of unfertilized eggs of the closely related arrhenotokous species, *Muscidifurax raptorellus*. In the *Wolbachia*-infected parasitic wasp *M. uniraptor*, meiosis and the first mitotic division occur normally. Diploidy restoration is achieved following the completion of the first mitosis. This pattern differs in the timing of diploidy restoration from previously described cases of *Wolbachia*-associated thelytoky. Results presented here suggest that different cytogenetic mechanisms of diploidy restoration may occur in different species with *Wolbachia*-induced thelytoky.

Keywords: Thelytoky; Parthenogenesis; Gamete-duplication; Symbiosis; Haploid-development

1. Introduction

In Hymenoptera, the general rule of sex determination is haplodiploidy in which haploid males are produced from unfertilized eggs while diploid females are usually produced from fertilized eggs (arrhenotoky) (Fig. 1). However, some Hymenoptera are thelytokous. Thelytoky is a mode of reproduction in which individual females produce exclusively female progeny by a process that does not involve fertilization. There are two main types of thelytoky, apomixis (ameiotic thelytoky), and automixis (meiotic thelytoky). In apomixis, the most common form of thelytoky in insects (Suomalainen et al., 1987), meiosis is entirely suppressed, and the maturation division or divisions in the oocyte are mitotic in character (no bivalent formation). In automixis, meiosis occurs in the developing oocyte, and diploidy is restored by the fusion of the meiotic products or by the fusion of the mitotic products (White, 1973).

Restoration of diploidy by automictic thelytoky can occur by either the fusion of meiotic or mitotic products. In the case of meiotic automixis, fusion of the second meiotic division products can occur either between the two sets of chromosomes of the same first daughter nucleus (terminal fusion), or between two sets of chromosomes originating from different daughter nuclei (central fusion). Both cases result in diploid nuclei that enter the first mitotic division. Diploidy restoration by meiotic thelytoky have been described in *Aphtyis mytilaspidis* (Le Baron) (Rossler and DeBach, 1973), *Apis mellifera capensis* Escholtz (Verma and Ruttner, 1983), and *Pristiphora rufipes* Lepeltier (Comrie, 1938). In the case of mitotic automixis, meiosis is completed and the maternal first nucleus undergoes mitosis, but the two sets of chromosomes fuse back together (gamete duplication), resulting in a diploid nucleus that then enters the second mitotic prophase (Crozier, 1975).

* Corresponding author. Fax: 773-834-4669.
E-mail address: tkarr@midway.uchicago.edu (T.L. Karr).

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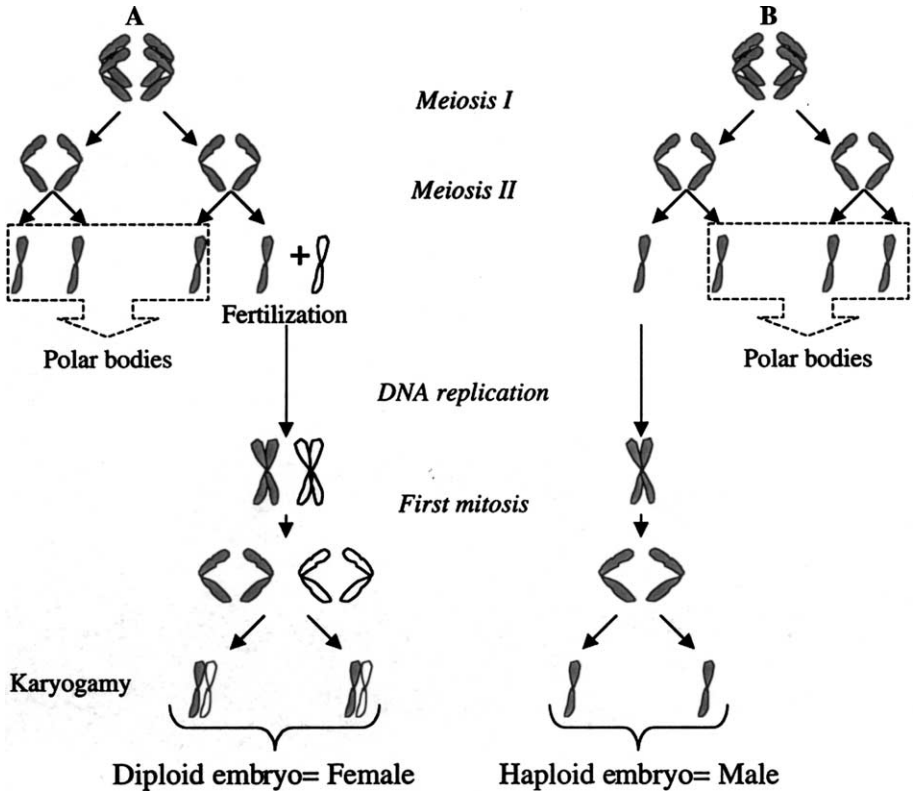

Fig. 1. Schematic depiction of haplo-diploid development. (A) Diploid development by fertilization. (B) Parthenogenetic haploid development. Only one set of chromosomes is presented for simplicity.

Muscidifurax uniraptor Kogan and Legner, a solitary ectoparasitoid of the pupae of various flies (including the housefly), is a thelytokous species (Kogan and Legner, 1970). Thelytoky in these wasps has been found to be caused by the intracellular symbiont *Wolbachia* (Stouthamer et al., 1993). Although at least 40 species of thelytokous parasitic Hymenoptera are known to harbor *Wolbachia* (Stouthamer et al., 1999), the cytological mechanism of diploidy restoration as gamete duplication, has been described only in four species: three *Trichogramma* species (Stouthamer and Kazmer, 1994) and *Diplolepis rosae* (L.) (Stille and Davring, 1980). Legner (1985) suggested that the mechanism of diploidy restoration in *M. uniraptor* is gamete duplication, but no data were presented to support this claim.

Wolbachia is an intracellular bacterium belonging to the alpha sub-division of the purple bacteria, found in many insects and other arthropods (O'Neill et al., 1992). *Wolbachia* are known to alter the reproductive biology of their hosts in a variety of ways that include thelytoky, feminization of genetic males, differential mortality of males, and cytoplasmic incompatibility (for review, see Stouthamer et al., 1999; Werren, 1997). The bacteria are located in the reproductive organs and other tissues of their hosts and are transferred from the female to her offspring through the egg cytoplasm (O'Neill and Karr, 1990; Stouthamer and Werren, 1993; Zchori-Fein et al., 1998). *Wolbachia* have been found in 16–76% of the insects surveyed (Jeyaprakash and Hoy, 2000; Werren et al., 1995b; Werren and Windsor, 2000), suggesting that they have been intimate partners of their hosts over substantial evolutionary periods. Phylogenetic analyses of *Wolbachia* in arthropod hosts has revealed that the bacteria can be divided into at least two different groups: A and B, which are estimated to have diverged from each other approximately 60 MYA. (Werren et al., 1995a; Zhou et al., 1998). Parthenogenesis induction occurs by *Wolbachia* in both A and B groups. Although it is possible to divide the symbionts into distinct groups, no connection has been found between the *Wolbachia* strain and the reproductive alteration it causes.

In this study, we demonstrate that diploidy in *M. uniraptor* is restored following the first mitotic division by fusion of the products of the adjacent first mitotic nuclei, and argue that the cytological mechanisms of *Wolbachia*-induced diploidy restoration differ among host species and *Wolbachia* strains.

2. Materials and methods

2.1. Insect culture

Muscidifurax uniraptor—a thelytokous species was obtained in 1997 from R. Stouthamer. *Muscidifurax*

raptorellus Kogan and Legner—a sexual arrhenotokous species was obtained in 1998 from C.J. Geden, Gainesville, FL. This latter species was used as a control because it is considered to be the most closely related species to *M. uniraptor* as determined by sequencing of mitochondrial DNA genes (Taylor et al., 1997). *Muscidifurax* species were reared in the laboratory on housefly pupae in a rearing chamber under standard conditions of 25 ± 2 °C, 16:8 (L:D) and 50% RH.

2.2. Egg collection

A selective oviposition procedure was designed to obtain eggs from the posterior end of the pupal host (Zchori-Fein et al., 2000). Four 24 h old wasps were placed in a glass vial where the hosts were placed in holes in a foam plug, leaving only their posterior end exposed for oviposition. The hosts were replaced every 2 h and either immediately dissected and processed for cytological observation (see below), or held at 4 °C until egg collection at a later time. The apical end of the puparium was opened and parasite eggs were collected with a fine brush into Grace medium (Sigma).

2.3. Egg fixation

Eggs were dechorionated by soaking them in 50% commercial bleach (3.5% active Chloride) for 10 min, or until they sank. They were then washed in a 0.7% NaCl/0.5 Triton-X100 solution for bleach removal. The eggs were then transferred into a scintillation vial filled with 3 ml heptane and 3 ml methanol that form two layers, and were gently shaken for 5 min. After 10 min of fixation, all the devitellinated eggs sank into the methanol layer (eggs that retained the vitelline membrane were collected at the heptane/methanol interface). Eggs were removed from the methanol layer, washed twice with methanol and transferred to a 1.5 ml tube with methanol, until staining.

2.4. Egg staining

Eggs were rehydrated through a methanol series as follows: 75% methanol/25% TBST (150 mM NaCl, 50 mM Tris, and 0.1% Tween 20); 50% methanol/50% TBST; 25% methanol/75% TBST; and 100% TBST. *M. raptorellus* eggs were treated with 10 U RNase in 0.5 ml TBST overnight in 4 °C, then soaked in 0.001 mM Sytox-Green (Molecular Probes), followed by 1 μg/ml DAPI (Molecular Probes) for 5 min and washed three times in TBST. *M. uniraptor* eggs were rehydrated as above, and stained only with DAPI. Stained eggs were transferred to a microscope slide, mounted under a coverslip with 80% glycerol/TBST×1 with 2% *n*-propylgalate (Sigma), and sealed with clear nail polish.

2.5. Quantitation of DNA content

Images of DAPI stained eggs were obtained using a Zeiss Axioplan II epi-fluorescence microscope equipped with a computer controlled z-stepper motor and a 12-bit research grade CCD camera (Princeton Instruments). Image files were processed with Power Microtome digital deconvolution software (Vaytek). Processed z-series were analyzed with VoxBlast software (Vaytek) designed to display three-dimensional image sets. Sytox-Green stained eggs were examined with a Zeiss LSM 510 confocal microscope. Three-dimensional image datasets were displayed using the "maximum transparency" function. Ploidy values were determined from stained nuclei by measuring the fluorescence intensities of each image using sectoring and threshold functions of IPLab (Sanalytics) image analysis software. These measurements were confirmed with 3–4 images of the same stage. Values for polar nuclei, the products of meiosis II, were considered to represent the haploid DNA content and used to estimate DNA content relative to this value. Values are reported as the ratio of fluorescence intensities of nuclei divided by the DNA value of the polar nucleus. The overall standard error of the means was ±0.1 (data not shown).

2.6. Egg viability assay

To ensure that our cytological observations reflected normal development process, we preformed an egg viability assay for *M. raptorellus* as described for *M. uniraptor* (Zchori-Fein et al., 2000). Egg viability assay was performed with 12 wasps which were placed individually in small glass tubes with about 20 hosts, and allowed to lay eggs for four successive days. Each day the number of laid eggs was determined. To determine survival from egg to adult the above experiment was repeated without egg collection, offspring were counted upon emergence and survival rate to adulthood was calculated. This experiment was replicated three times.

3. Results

In many insects, the mature oocyte is arrested in metaphase of meiosis I. Meiosis I resumes upon oviposition and fertilization, followed by meiosis II to generate four haploid meiotic products (Fig. 1). In normal sexual reproduction, one of the four products of meiosis becomes the pronucleus and diploidy is restored when it fuses with the sperm pronucleus (Fig. 1A). The remaining three polar nuclei migrate to the egg surface and do not participate directly in subsequent embryogenesis. In many insects the first mitosis occurs without physical fusion of the male and female pronuclei, and is thus termed the first gonomeric division. Karyogamy

(i.e., fusion and mixing of paternal chromosomes) occurs following mitosis during subsequent interphase (Fig. 1A; Tram and Sullivan, 2000; Zissler, 1992).

For comparative purposes and as a control of normal haploid development in *Muscidifurax*, over 200 unfertilized *M. raptorellus* eggs were observed for chromosomal content and behavior (Fig. 2). Meiosis results in a haploid pronucleus (Figs. 2A–C). In unfertilized eggs the haploid pronucleus enters interphase, DNA replication occurs (Fig. 2D) followed by metaphase (Fig. 2E) and anaphase (Fig. 2F), producing two haploid nuclei (Fig. 2G). These two nuclei will continue normal haploid development (Figs. 2H–J). Analysis of DNA content in the egg during these stages (Table 1) is consistent with this interpretation. Nuclear DNA content increases two-fold during replication in the prophase of the first mitotic division (Table 1, line 2D), generating two haploid nuclei (Table 1, line 2G).

Unfertilized eggs from *M. raptorellus* mothers continued normal haploid development through the blastoderm stage (data not shown). We determined viability of 77.99% (see Section 2), therefore, we assume that the majority of our observations reflect the actual process of normal early development in *M. raptorellus*.

Early embryonic stages of *M. uniraptor* were next investigated to determine the mechanism of *Wolbachia*-induced thelytokous development and diploidy restoration in unfertilized eggs. Cytological observation of early embryonic stages in over 150 unfertilized *M. uniraptor* eggs was correlated with direct determination of DNA content using fluorescence microscopy (Fig. 3; Table 2). Normal meiosis and normal formation of the pronucleus and three polar nuclei occurs in the same way as in *M. raptorellus* (Figs. 3A–B). In *M. uniraptor*, the female pronucleus enters interphase of the first mitosis, undergoes DNA replication, and otherwise appears to undergo a normal first mitotic division: following DNA replication, chromosomal condensation begins during prophase (Fig. 3C), continues into metaphase where sister chromatids are observed on a metaphase plate (Fig. 3D) after which poleward chromosome movement proceeds during anaphase (Fig. 3E). At the completion of the first mitosis, the two haploid nuclei products of the divided nucleus enter interphase for DNA replication (Fig. 3F), and begin the second mitosis as two diploid nuclei following DNA replication (Fig. 3G). These two diploid nuclear products remain very close to each other and subsequently undergo chromosome condensation (Fig. 3H). These two closely opposed metaphase figures appear to fuse into a single tetraploid nucleus and then decondence (Fig. 3I). A normal mitotic division then ensues in two separate diploid nuclei (Fig. 3J). Subsequently, based upon observations of syncytial embryos (data not shown), normal mitotic divisions of diploid nuclei proceed. Our interpretation was based upon both our cytological examination, and from measurement of DNA content in the developing egg (Table 2). During the developmental stages shown in Fig. 3, DNA content first increased ‘twofold during the first mitotic division (Table 2, line 3C), followed by the appearance of two haploid nuclei (Table 2, line 3E). The haploid nuclei then replicated (Table 2, lines G–H) and formed a tetraploid nucleus (Table 2, line I). Subsequently, two nuclei were observed (Fig. 3J) in the tetraploid state (Table 2, line J), presumably the result of DNA replication during interphase of the next division cycle. Other than the second division, each subsequent mitosis is characterized by the well defined stages of the cell cycle including interphase (DNA replication), prophase (completion of DNA synthesis and beginning of chromosome condensation), metaphase (chromosomes arranged on a metaphase plate), anaphase (chromosome separation), and telophase (completion of chromosome movement, and subsequent chromatin decondensation). In *M. uniraptor*, the two diploid daughter nuclei will continue to divide into four nuclei, and eventually create a syncytial diploid blastoderm (data not shown). Zchori-Fein et al. (2000) showed 65.06% egg viability in the strain we used, therefore, we assume that the majority of our observation reflect the normal course of diploidy restoration in *M. uniraptor*.

4. Discussion

Mitotic thelytoky by gamete duplication has been documented in three species of *Trichogramma* (Stouthamer and Kazmer, 1994), and in *D. rosae* (Stille and Davring, 1980), all of which harbor *Wolbachia* that are believed to cause the thelytokous reproduction (Stouthamer, 1997). Here we describe gamete duplication in the *Wolbachia*-infected wasp *M. uniraptor* and compare and contrast our results with those of previous investigators (summarized diagrammatically in Fig. 4).

Stouthamer and Kazmer (1994) used lacmoid staining and genetic markers to determine that diploidy restoration in *Trichogramma* occurs by gamete duplication. Their observations were confirmed by the segregation pattern of allozymes in the offspring of heterozygous thelytokous *Trichogramma* females, as offspring of uninseminated females were all homozygous. They also found that in the presence of sperm, fertilization can occur normally and suppress *Wolbachia*-induced diploidy restoration. They proposed that gamete duplication occurs in *Trichogramma* by failure of the first mitotic anaphase division. Anaphase failure and subsequent collapse of the spindle results in a single nucleus that is duplicated during subsequent interphase. This nucleus then divides into two diploid nuclei to form a diploid embryo (illustrated in Fig. 4A). This form of gamete duplication is different from what we observed in *M. uniraptor* which is more in line with the mechanism

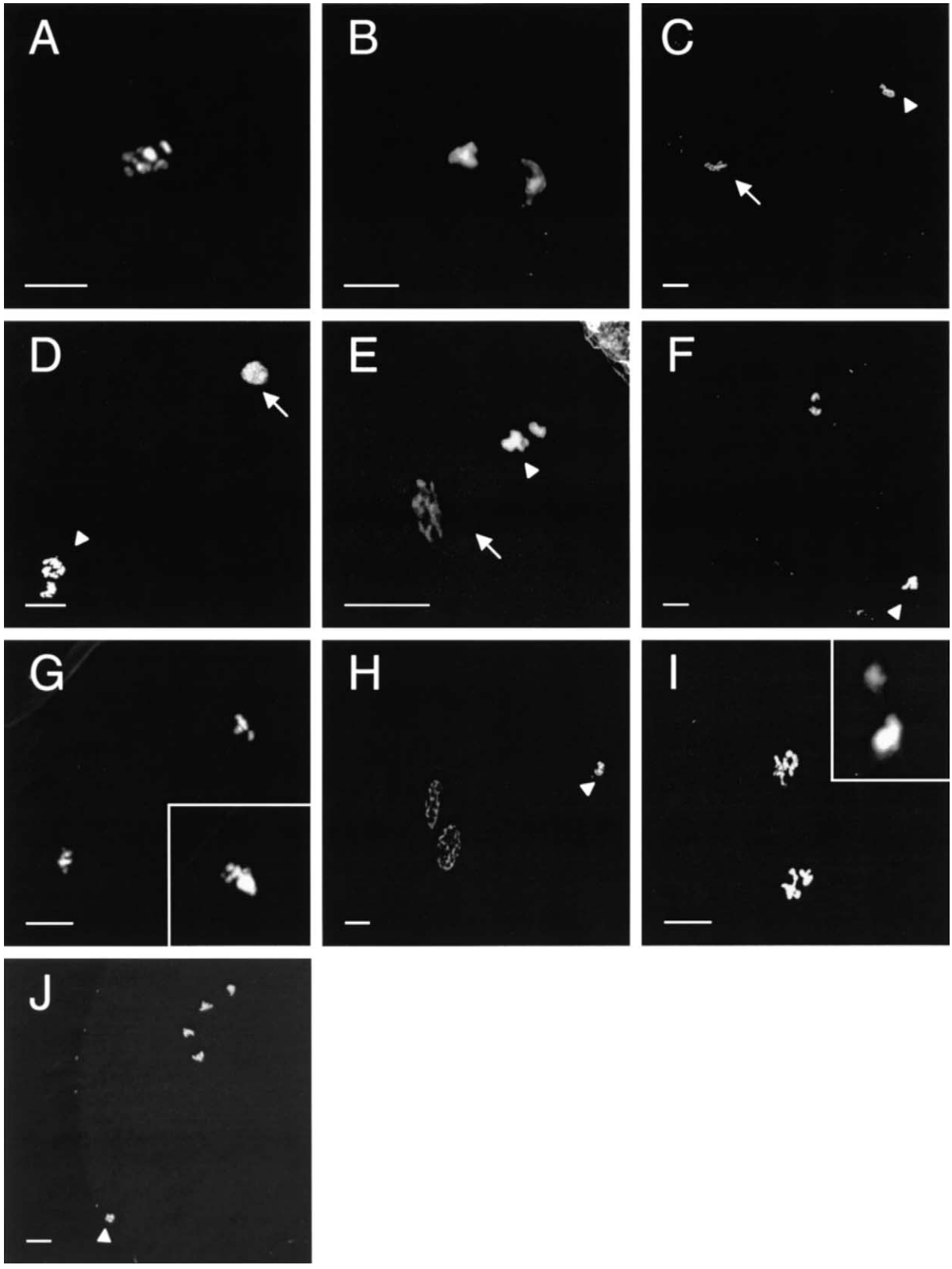

Fig. 2. Haploid development in uninfected *Muscidifurax raptorellus*. (A) Meiosis I, diploid nucleus. (B) Meiosis II division. (C) Migration of the pronucleus. (D) First mitosis prophase. (E) First mitosis metaphase. (F) First mitosis anaphase. (G) First mitosis telophase. (H) Second mitosis prophase. (I) Second mitosis metaphase. (J) Second mitosis anaphase. Arrow, pronucleus; arrow head, polar nuclei. Insets showing polar nuclei, bars indicate 10 μm.

Table 1
Intensity measurements of Fig. 2

Fig.	Cycle stage	Intensity measurements (arbitrary units)[b]		
2A	Meiosis I	Nucleus = 3.8		
2B	Meiosis II	Left nuclei = 2.0	Right nuclei = 1.85	
2C	Pronucleus	Polar nuclei = 2.85	Pronucleus = 1.25	
2D	1st Mitosis propahse	Polar nuclei = 3.0	Nucleus = 2.05	
2E	1st Mitosis metaphase	Polar nuclei = 2.8	Nucleus = 2.05	
2F	1st Mitosis anaphase	Polar nuclei = 3.1	Upper nucleus = 1.1	Lower nucleus = 1.0
2G	1st Mitosis telophase	Polar nuclei = 1.85[a]	Left nucleus = 1.0	Right nucleus = 1.1
2H	2nd Mitosis prophase	Polar nuclei = 1.7[a]	Left nucleus = 2.05	Right nucleus = 2.15
2I	2nd Mitosis metaphase	Polar nuclei = 2.5[a]	Upper nucleus = 1.85	Lower nucleus = 2.1
2J	2nd Mitosis anaphase	Polar nuclei = 1.0[a]	Upper left nucleus = 1.0	Lower left nucleus = 1.1
			Upper right nucleus = 0.95	Lower right nucleus = 1.1

[a] Polar nuclei of these images were not taken into account, as they were too condense and non-informative.
[b] Values obtained as described in Section 2.

proposed by Stille and Davring (1980), (summarized in Fig. 4B).

Our observations for *M. uniraptor* indicate that the first mitotic anaphase is normal, forming two daughter nuclei essentially as occurs in regular haploid development (Figs. 2A–G; 3A–F). Unlike haploid development in *M. raptorellus*, in which two haploid daughter nuclei duplicate and divide into four haploid nuclei (Figs. 2H–J), in *M. uniraptor* the two haploid daughter nuclei duplicate but do not further divide, thus gamete duplication occurs during the second prophase (Figs. 3G–I; 4C). The presence of a single tetraploid nucleus (Fig. 3I) is best explained by the prior fusion of two diploid nuclei. The actual mechanism by which this occurs is yet unknown, and only real-time imaging of live eggs will demonstrate it conclusively. The mechanism of fusion of two nuclei was presumably also found in *D. rosae* (Stille and Davring, 1980). By Feulgen–Giemsa staining these authors suggested that following the completion of the first mitosis, two adjacent daughter nuclei are formed which fuse in the subsequent interphase. The next mitosis prophase shows one nucleus which presumably gave rise to two diploid nuclei (Fig. 4B). Unfortunately, no numerical data for either the ploidy stage of the two daughter nuclei resulting from the pronucleus division, nor the one nucleus that starts the second mitosis division was presented in this study, but a later study on *D. rosae* a survey of 26 enzyme loci revealed very low genetic variation supporting a form of gamete duplication as the operant mechanism for thelytoky (Stille, 1985).

Our data provide direct observation and measurement of the diploid intermediates proposed by Stille and Davring (1980), strongly suggesting this as a main mechanism for gamete duplication in the two wasps. In contrast to diploidy restoration in *Trichogramma*, in *M. uniraptor* the first mitosis appears normal and results in a diploid embryo and no abnormal anaphase. However, the methods used by Stouthamer and Kazmer (1994) were such that they could not exclude other intermediate steps to the 4n nucleus. Therefore, the possibility exists that the mechanism of gamete duplication in *Trichogramma* may be similar or identical to that of *M. uniraptor* and *D. rosae*, thus suggesting a parsimonious explanation that the same specific components are influenced in the same way by *Wolbachia* in these three species. Several cellular structures might be involved in the described chromosome association. The spindle microtubules would be one possible element that might cause insufficient separation. However, Riparbelli et al. (1998) have shown that the poles of the first mitotic spindle have clearly visible astral microtubules, and look very much like subsequent mitotic divisions, and like first mitotic division of other organisms (fruit fly and sea urchin). Although these authors do not describe the first mitotic division in detail, they concluded that the presence of the *Wolbachia* has no influence on the formation of astral array of microtubules. Another possibility could be the failure of the nuclear envelope separation and reformation. Delayed nuclear envelope breakdown was found to be the cause for cytoplasmic incompatibility in *Nasonia* (Tram and Sullivan, 2002). Understanding the mechanisms that control parental chromosomes during the second mitotic division might provide important insights into the mechanism(s) of *Wolbachia*-induced diploidy restoration. A real time description of nuclear behavior similar to that recently described by Tram and Sullivan (2000, 2002), will allow us to more precisely determine the mechanisms involved. Detailed description of *Wolbachia* distribution around, and possible interaction with the dividing nuclei might also provide valuable clues as to the bacteria effect on diploidy restoration.

The *Wolbachia* found in *Trichogramma* spp., *D. rosae*, and *M. uniraptor* are of different phylogenetic groups (B, B, and A, respectively), and appear to have different cytological mechanisms of gamete duplication. Recently, based on microsatellite analysis, Weeks and Breeuwer (2001) found apomictic thelytoky in haplodiploid *Wolbachia*-infected mite species. This is the first recorded case of *Wolbachia*-induced parthenogensis outside the insecta. It is possible that different *Wolbachia*

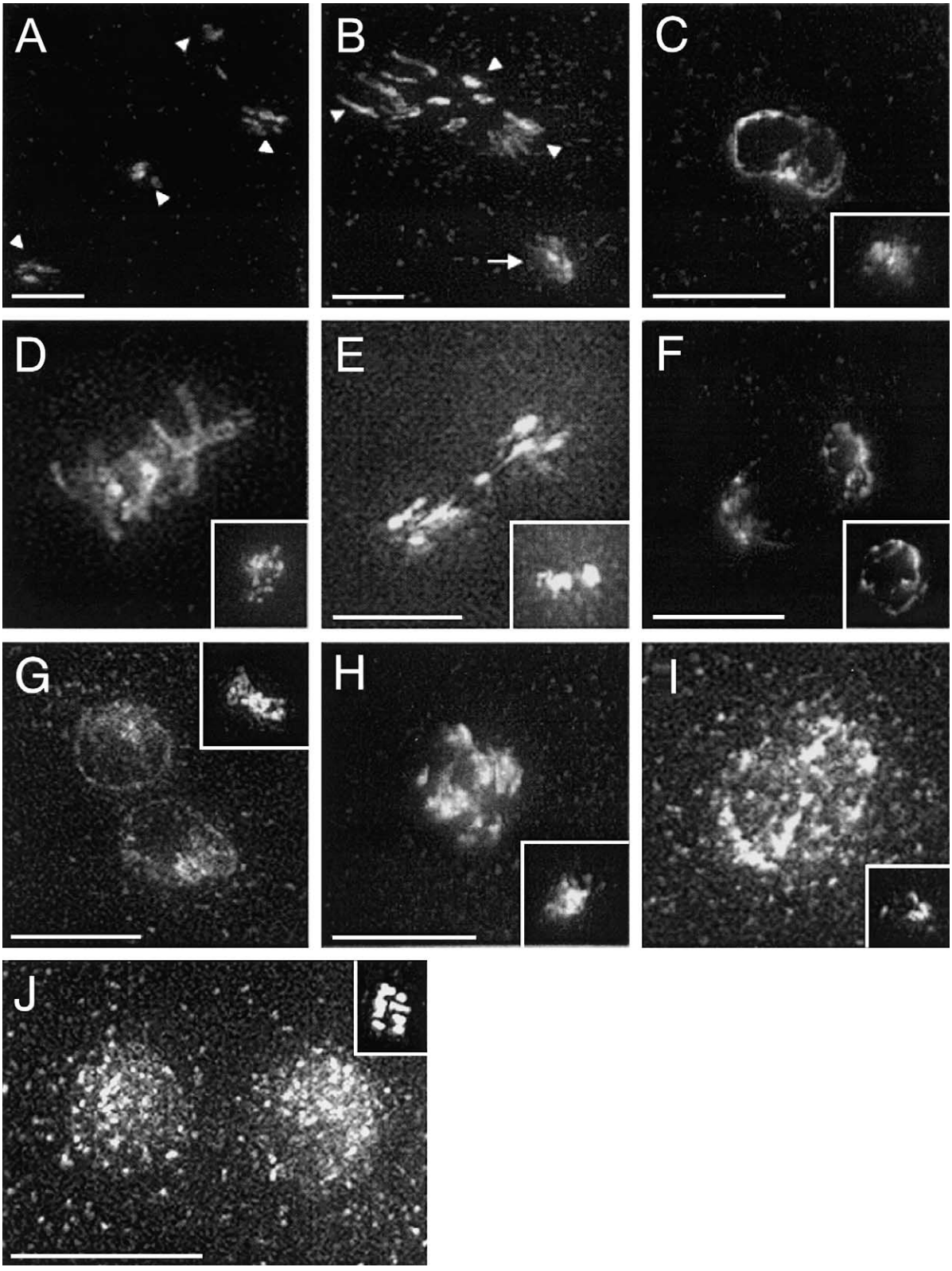

Fig. 3. Diploidy restoration in *Wolbachia*-infected *Muscidifurax uniraptor*. (A) Second meiosis, formation of four haploid nuclei. (B) Migration of the haploid pronucleus. (C) First mitotic division prophase. (D) First mitosis metaphase. (E) First mitosis anaphase. (F) First mitosis telophase. (G) Gonomeric division prophase. (H) Karyogamy of the gonomeric division products. (I) Second mitosis prophase of fused nuclei. (J) Third mitosis prophase. Arrow, pronucleus; arrow head, polar nuclei. Insets showing polar nuclei, bar indicates 10 μm, figures without bars are 10 μm × 10 μm.

Table 2
Intensity measurements of Fig. 3

Fig.	Cycle stage	Intensity measured (arbitrary units)[b]			
3A	Meiosis II	Upper nucleus = 1.0	Right nucleus = 1.1	Middle nucleus = 0.9	Lower nucleus = 0.9
3B	Pronucleus	Pronucleus = 0.95	Right nucleus = 0.9	Middle nucleus = 1.0	Left nucleus = 1.0
3C	1st Mitosis prophase	Nucleus = 2.2	Polar nuclei = 2.85		
3D	1st Mitosis metaphase	Nucleus = 2.3	Polar nuclei = 1.8[a]		
3E	1st Mitosis anaphase	Right nucleus = 0.95	Left nucleus = 0.9	Polar nuclei = 1.0[a]	
3F	1st Mitosis telophase	Right nucleus = 1.1	Left nucleus = 1.0	Polar nuclei = 3.7	
3G	2nd Mitosis prophase	Upper nucleus = 2.1	Lower nucleus = 1.9	Polar nuclei = 1.0[a]	
3H	Collapse into 2nd mitosis prophase	Upper nucleus = 2.2	Lower nucleus = 1.95	Polar nuclei = 2.5[a]	
3I	Tetraploid nucleus	Nucleus = 4.2	Polar nuclei = 1.0[a]		
3J	3rd Mitosis prophase	Left nucleus = 3.8	Right nucleus = 4.3	Polar nuclei = 1.0[a]	

[a] Polar nuclei of these images were not taken into account, as they were too condense and non-informative.
[b] Values obtained as described in Section 2.

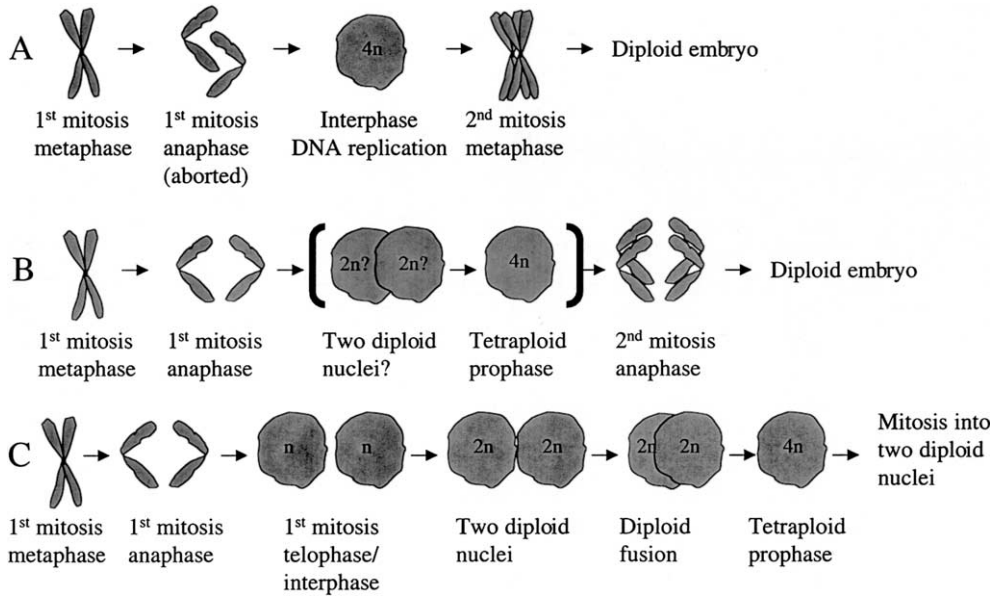

Fig. 4. Models for gamete duplication mechanism according to (A) Stouthamer and Kazmer (1994) in *Trichogramma*; (B) Stille and Davring (1980) in *D. rosae*. Brackets represent the uncertainty of the ploidy state of the nuclei that form the tetraploid prophase nucleus; (C) the present study in *M. uniraptor*. Only one set of chromosomes is presented for simplicity.

have evolved different mechanisms of diploidy restoration, or that different host environments favor one mechanism over another. More detailed studies of the above systems are needed to resolve the differences in mechanisms of diploidy restoration by *Wolbachia*.

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