Master manipulation continued: feminizing *Wolbachia* endosymbiont distorts sex chromosome inheritance

Short title: *Wolbachia* distort sex chromosome inheritance

Daisuke Kageyama[a,1], Mizuki Ohno[b], Tatsushi Sasaki[b], Atsuo Yoshido[b], Tatsuro Konagaya[c], Akiya Jouraku[a], Seigo Kuwazaki[a], Hiroyuki Kanamori[d], Yuichi Katayose[d], Satoko Narita[a,e], Mai Miyata[f], Markus Riegler[g], and Ken Sahara[b,1]

[a]Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, 1-2, Owashi, Tsukuba, Ibaraki 305-0854, Japan; [b]Laboratory of Applied Entomology, Faculty of Agriculture, Iwate University, 3-18-8, Ueda, Morioka 020-8550, Japan; [c]Graduate School of Science, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan; [d]Institute of Crop Science, National Agriculture and Food Research Organization, 1-2, Owashi, Tsukuba, Ibaraki 305-0854, Japan; [e]Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Health and Nutrition, 1-1, Hachimandai, Tsukuba, Ibaraki 305-0843, Japan; [f]Graduate School of Horticulture, Chiba University, Matsudo 648, Matsudo, Chiba 271-8510, Japan; and [g]Hawkesbury Institute for the Environment, Western Sydney University, Locked Bag 1797, Penrith, New South Wales 2751, Australia

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[1]Correspondence should be addressed: kagymad@affrc.go.jp (D.K.), sahara@iwate-u.ac.jp (K.S.)

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Abstract

Wolbachia is a maternally inherited ubiquitous endosymbiotic bacterium of arthropods that displays a diverse repertoire of host reproductive manipulations. For the first time, we demonstrate that *Wolbachia* manipulates sex chromosome inheritance in a sexually reproducing insect. *Eurema mandarina* butterfly females on Tanegashima Island, Japan, are infected with the *w*Fem *Wolbachia* strain and produce all-female offspring, while antibiotic treatment results in male offspring. Fluorescence *in situ* hybridization (FISH) revealed that *w*Fem-positive and *w*Fem-negative females have ZO and WZ sex chromosome sets, respectively, demonstrating the predicted loss of the W chromosome from *w*Fem-infected lineages. Genomic quantitative polymerase chain reaction (qPCR) analysis showed that *w*Fem-positive females lay only ZO eggs that carry a paternal Z, whereas females from lineages that are naturally *w*Fem-negative lay both WZ and ZZ eggs. In contrast, antibiotic treatment of adult *w*Fem females resulted in the production of ZO and ZZ eggs, suggesting that this *Wolbachia* strain can induce meiotic drive. Moreover, most male offspring produced by antibiotic-treated *w*Fem females had a ZZ karyotype, implying reduced survival of ZO individuals in the absence of feminizing effects of *Wolbachia*. Antibiotic treatment of *w*Fem-infected larvae induced male-specific splicing of the *doublesex* (*dsx*) gene transcript, causing an intersex phenotype. Thus, the loss of the female-determining W chromosome in ZO individuals is functionally compensated by *Wolbachia* feminization. We discuss how *Wolbachia* may manipulate oogenesis to cause meiotic drive and that *Wolbachia* may have acquired this coordinated dual mode of reproductive manipulation first by the evolution of feminization and then cytoplasmically induced meiotic drive.

Significance Statement

Genomes are vulnerable to selfish genetic elements that enhance their own transmission often at the expense of host fitness. These include cytoplasmic elements such as endosymbiotic bacteria that cause feminization, male-killing, parthenogenesis and cytoplasmic incompatibility. We demonstrate, for the first time, that meiotic drive, a phenomena so far seen only for nuclear genetic elements, can also be caused by the ubiquitous endosymbiotic bacterium *Wolbachia*. In female butterflies with a ZO sex chromosome constitution, *Wolbachia* prevents the production of ZZ zygotes. *Wolbachia* also compensates for the female-determining function of the W chromosome lost from infected lineages, thereby causing the production of all-female progeny. Our findings highlight that cytoplasmic elements play an important role in sex determination systems and sex chromosome evolution.

Introduction

Genomes of sexually reproducing organisms are exposed to genetic conflicts. For example, some genes bias reproduction towards male offspring while other genes within the same genome may favor reproduction of more daughters. Selfish genetic elements (SGEs), such as meiotic drivers, cytoplasmic sex ratio distorters and transposons, are extreme examples, which enhance their own transmission often at the expense of their hosts' fitness (1, 2). There is growing evidence that SGEs, and the resulting genetic conflict, trigger important evolutionary change and innovation in eukaryotes (2).

Meiotic drive is a distortion of Mendelian inheritance as it leads to the more frequent inheritance of one copy of a gene than the expected 50% (3, 4). A meiotic drive factor that sits on a sex chromosome biases the sex ratio. For example, X chromosome drive and Y chromosome drive in flies (Diptera), result in female-biased and male-biased sex ratios, respectively (4). In male-heterogametic species, meiotic drive factors are expected to be encoded in the nuclear genome. In female-heterogametic species, however, W chromosome and cytoplasm behave as a single linkage group and thus W chromosome drive can theoretically also be caused by cytoplasmic elements. Although this possibility has previously been proposed (5, 6), lack of empirical evidence questions whether it is mechanistically possible for cytoplasmic elements to cause meiotic drive.

Wolbachia pipientis (Alphaproteobacteria), a bacterium simply referred to as *Wolbachia*, attracts significant interest in evolutionary and developmental biology but also from applied perspectives such as pest management because it can manipulate reproduction of arthropods in various ways such as cytoplasmic incompatibility, parthenogenesis induction, feminization and male-killing (7). Here we demonstrate for the first time that meiotic drive caused by *Wolbachia* constitutes the underlying mechanism of all-female production in the butterfly *Eurema mandarina*. In most populations, *E. mandarina* is infected with the cytoplasmic-incompatibility (CI)-inducing *Wolbachia* strain *w*CI at a high prevalence of close to 100%. Hiroki et al. (8, 9) first reported an all-female production in *E. mandarina* (then known as *Eurema hecabe* yellow type), which

was considered to be due to the feminization of genetic males (ZZ) by co-infections with the *Wolbachia* strain *w*Fem (hereafter we refer to this double infection status as CF and single infection with *w*CI as C). Three observations about CF lineages supported this view, i.e., (a) antibiotic treatment of adult females led to production of all-male offspring (8), (b) antibiotic treatment of larvae resulted in intersexual adult phenotype (10) and (c) females did not have the W chromatin body (8, 10). However, Kern et al. (11) challenged this notion by demonstrating that CF females have only one Z chromosome and that this Z chromosome always derived from their fathers. Therefore, two hypotheses were formulated, namely that the CF females have either a ZO or a W'Z sex chromosome set (whereby W' cannot be visualized in W chromatin assays and does not have a female-determining function), and that meiotic drive occurs in CF lineages due to *Wolbachia* or another factor.

In a multifaceted approach, by combining chromosome FISH, genome sequencing, quantitative PCR, reverse transcription PCR and antibiotic treatment, we have tested these hypotheses and revealed that *Wolbachia* is the cause for both the meiotic drive and the feminization of ZO individuals which would otherwise be determined as males in the absence of *Wolbachia*. These results highlight that cytoplasmic elements can have profound effects on oogenesis, sex chromosome inheritance and sex determination – fundamental biological processes of eukaryotes.

Results

All-female-producing CF females have a ZO karyotype

We performed FISH on *E. mandarina* chromosomes prepared from CF females, C females, and C males collected on Tanegashima Island (Fig. S1). In the mitotic complement of C females, which harbor a $2n = 62$ karyotype, genomic probes highlighted the W chromosome, with scattered signals on the other chromosomes (Fig. 1A; see Materials and Methods for technical details). A probe for the Z-linked gene *Kettin* (*Ket*) identified the single Z chromosome in C females (Fig. 1A), and also hybridized to the Z chromosome paired with the W chromosome in pachytene bivalents (Fig. 1J). The *Ket* probe identified two Z

chromosomes in the mitotic complement of C males (Fig. 1B; 2*n* = 62). No painted W chromosome was observed in interphase nuclei (Fig. 1H, I), the mitotic complement (Fig. 1C) and pachytene complement (Fig. 1L) of CF females, but the *Ket* signal appeared on the single Z chromosome in the mitotic complement (Fig. 1C) and Z univalent in the pachytene complement (Fig. 1L). Based on the relative read counts homologous to *Bombyx mori* Z-linked and autosomal genes in females and males, our genome sequencing data support the notion that CF and C females have one Z chromosome (Figs. 1M–O; Table S1), which is consistent with genomic qPCR data based on two loci, *Triosephosphate isomerase* (*Tpi*) and *Ket*, relative to the autosomal gene *EF-1α* (11). Thus, our results directly reveal the sex chromosome constitution of C females, C males, and CF females as WZ, ZZ, and ZO, respectively. This conclusion disproves previous interpretations based on the W-body diagnosis alone that C and CF females possess WZ and ZZ sex chromosome constitutions, respectively (8, 10).

CF females deposit exclusively ZO eggs

Previously, the high number of survivors among the offspring of CF females (compared with those of C females) has been the only reason to exclude male-killing during embryonic or later stages as a possible reason for this outcome (10–12). We performed real-time genomic qPCR (to detect Z-linked *Tpi* or *Ket* relative to autosomal *EF-1α*) on individual fertilized eggs, and found that C females oviposited eggs with either one or two Z chromosomes at nearly equal frequencies (Fig. 2A, left; Fig. S2). In contrast, all eggs deposited by CF females were single Z carriers (Fig. 2A, middle; Fig. S2). These findings indicate that the progeny of CF females are exclusively ZO individuals, supporting the view that meiotic drive occurs in CF females.

***Wolbachia* causes the exclusive production of ZO eggs**

To abolish the effects of *Wolbachia*, tetracycline (tet) was administered to adult CF females previously inseminated by antibiotic-treated male offspring of C females. The Z-linked gene dose of eggs produced by these tet-treated females

ranged from approximately 0.5–1.0, indicating that some eggs are ZO and others are ZZ (Fig. 2A, right; Fig. S2). This suggests that the *Wolbachia* strain *w*Fem in CF females causes the exclusive production of ZO embryos. Therefore, our finding is the first empirical evidence that in a female-heterogametic species meiotic drive can also be caused by cytoplasmic elements (5, 6). Furthermore, *Wolbachia*-like structures were observed near the chromosomes in CF females while less apparent in C females and C males, and this may represent different tropism and function of *w*Fem when contrasted with *w*CI (Fig. 1C).

Sixty-nine adults (15 females and 54 males) were obtained from offspring produced by five tet-treated adult CF females (Fig. 2B). Three of these tet-treated females produced only male offspring. Exclusive production of males was previously observed in tet-treated *E. mandarina* females derived from a different population on Okinawa-jima Island, Okinawa Prefecture, Japan (8). In this study, we obtained 15 female offspring from two broods in the first days after tet treatment; however, the mothers produced more males as the duration of tet treatment increased, and eventually produced only males. Examination of the Z-linked gene dose of these offspring by genomic qPCR showed that the females had one Z chromosome, whereas almost all of the males had two Z chromosomes (Fig. 2C). The nucleotide sequences of the introns of the *Tpi* gene strongly suggested that, in brood 19-1, all females (n = 12) were hemizygous and nine out of 10 males were heterozygous (Fig. 2C; Table S2). Curiously, one male (21m) that exhibited the lowest gene dose of *Ket* (0.588) appeared to be hemizygous (Fig. 2C). These results suggest that the emerged females had a ZO sex chromosome constitution, whereas most males had a ZZ sex chromosome constitution, with one exception (21m) of either ZO or ZZ' (Z' represents partial deletion/mutation in Z). These results also demonstrate that, in principle, tet-treated adult CF females can oviposit eggs with either a ZO or ZZ sex chromosome constitutions (Fig. 2A, right). However, ZO individuals appear to have zero or very low survival rates because few emerge as adults.

Involvement of *Wolbachia* in the sex determination of *Eurema mandarina*

Next, we fed CF larvae a tet-containing diet. As previously observed (10), all

individuals treated in this way developed an intersex phenotype at the adult stage, typically represented with male-like wing color and incomplete male-specific structure on wing surface (Fig. 3E and H; Fig. S3). The qPCR assay to assess the Z-linked gene dose revealed that these intersexes (n = 23) had just one Z chromosome (Fig. 3I), and therefore a ZO genotype. Because these ZO individuals were destined to develop as females without tet treatment, *w*Fem is likely to be responsible for female sex determination.

Further evidence in support of this idea was obtained by examining the sex-specific splicing products of *dsx* (Fig. S4), a widely conserved gene responsible for sexual differentiation (13). Similar to *B. mori* (14), C females exhibited a female-specific splicing product of *E. mandarina dsx* (*Emdsx*^{F}), whereas C males a male-specific splicing product of *E. mandarina dsx* (*Emdsx*^{M}; Lanes 1 and 2 in Fig. 3A, respectively; Fig. 3B). Similarly to C females, CF females exhibited exclusive expression of *Emdsx*^{F} (Lanes 3 and 4 in Fig. 3A; Fig 3B). Intersexual butterflies, generated by feeding the larval offspring of CF females a tet-containing larval diet, expressed both *Emdsx*^{F} and *Emdsx*^{M} (Lanes 5 and 6 in Fig. 3A; Table 1).

Discussion

Contrasting two potential underlying mechanisms of cytoplasmically induced female meiotic drive: Selection of gametes lacking sex chromosomes or elimination of the maternal sex chromosomes?

Exclusive production of ZO embryos by CF females suggests that the cytoplasmically induced meiotic drive effect of *w*Fem occurs before oviposition. However, its underlying mechanism remains unclear. Parthenogenesis can be excluded because the Z chromosomes are transmitted from their fathers (11). We believe that two mutually exclusive hypotheses can account for the phenomena observed in *E. mandarina* (Fig. 4). The first assumes that, in CF females, an O gamete that does neither bear a maternal Z chromosome, nor any sex chromosome in general, is always selected to become an egg pronucleus (meiotic drive *sensu stricto*) (Fig. 4A) (15). The second assumes that meiosis itself is normal, and that maternal Z chromosomes, or sex chromosomes in

general, are selectively eliminated from Z-bearing gametes during, or possibly after, meiosis (Fig. 4B). Based on the terminology of meiotic drive established in the population genetics literature, the second scenario can also be referred to as meiotic drive (cf. male meiotic drive in Diptera and *Mus musculus* (3, 4), in which gametes that do not carry a driver are selectively inactivated *after* normal meiosis). At present, it is unclear which of the two hypotheses (meiotic drive *sensu stricto* or elimination of the maternal Z at a later stage) is more plausible. However, it is noteworthy that, in the moth *Abraxas grossulariata*, a matriline consisting of putative ZO females produced only females or a great excess of females, and the underlying mechanism was considered to be the selective elimination of Z chromosomes (16–19). In *A. grossulariata*, the presence of cytoplasmic bacteria such as *Wolbachia* has not yet been examined. However, if we assume that the elimination of the maternal Z chromosome is also the mechanism of the female meiotic drive in *E. mandarina*, the exceptional individual 21m (Fig. 2C) could be viewed as ZZ', possessing a maternal Z chromosome that was only partially eliminated by the incomplete action of *w*Fem. It is possible to further speculate that the presence of *w*Fem results in the elimination of sex chromosomes in general (Z or W chromosomes) and, therefore, the absence of W chromosomes in CF females may also be a direct effect of *w*Fem.

Feminizing effect of *Wolbachia*: Compensation for W chromosome function in ZO individuals?

Generation of intersexes by treating the CF larvae by antibiotics suggests that ZO individuals are male by default, and that *w*Fem *Wolbachia* overrides sex determination so that infected individuals develop as females. The assumption that male sex is default in ZO individuals is congruent with the recent finding in *B. mori* where a W chromosome-located *Fem* piRNA is responsible for female sex determination (20). The morphologically and functionally complete female phenotype of CF individuals (ZO) suggests that *w*Fem compensates for the loss of the female-determining function of the W chromosome in *E. mandarina*.

Reduced survival of ZO individuals resulted from antibiotic treatment on

either ZO adults or larvae may suggest an improper dosage compensation in ZO males. Improper dosage compensation was also suggested to be the cause of male- and female-specific lethality in *Wolbachia*-infected and cured lines of *Ostrinia* moths (21–24).

How did the coordinated dual effects of *Wolbachia* evolve?

In haplodiploid parasitoid wasps, *Wolbachia* and *Cardinium* induce thelytokous parthenogenesis in a two-step mechanism, comprising diploidization of the unfertilized egg followed by feminization (25, 26). Similarly, we determined that *w*Fem causes meiotic drive and feminization in *E. mandarina* in two steps (Fig. 5A).

Next, we speculate about the potential evolutionary scenario that led to the appearance of both effects (Fig. 5B). A WZ female *Eurema* butterfly may have acquired an ancestor of *w*Fem that exerted a feminizing effect on ZZ males. The feminizing effect was lethal to ZZ individuals because of improper dosage compensation, as evident in *Ostrinia* moths (23, 24). This effect may have been similar to a male-killing phenotype (27, 28), but it was redundant in WZ females where the W chromosome acted as a female determiner (20). Subsequent loss of the W chromosome, leading to the generation of ZO females, can then be reasonably assumed due to it becoming redundant. Similarly, in *Ostrinia* moths, a female-determining function is thought to have been lost from the W chromosome in *Wolbachia*-infected matrilines (22). Spontaneous loss of a nonfunctional W chromosome may be easier than expected: in a wild silkmoth *Samia cynthia*, the W chromosome does not have a sex-determining function, and ZO females are frequently obtained by experimental crosses between subspecies (29). It appears that, in *E. mandarina*, *Wolbachia* may be involved in the evolution of sex chromosomes in a manner mechanistically different from that in the woodlouse *Armadillidium vulgare* where *Wolbachia* was involved in the loss and birth of the W chromosome (30, 31).

After the loss of the W chromosome, *Wolbachia* then acquired a novel function that affected female meiosis and resulted in female meiotic drive. The order of these events is unlikely to be the other way around: if the appearance of

meiotic drive were to precede the loss of the W chromosome, the feminizing or female-determining function would become unnecessary for *Wolbachia* because there would be no males. In the short term, female meiotic drive in a female-heterogametic species represents a great advantage to cytoplasmic symbionts because all vertically transmitted symbionts gain the opportunity to survive. However, males are still required for fertilization, and fixation of the symbionts in the host population will inevitably lead to the extinction of both the symbionts and the hosts (32). In the long term, suppressors against sex ratio distortion, as has been observed for the male-killing phenotypes in the butterfly *Hypolimnas bolina* or a ladybird beetle (33, 34), can be expected to evolve in the host. However, the evolutionary outcomes of combined meiotic drive and feminization would be different from that of male-killing, because suppression of the effects of *Wolbachia* would lead to all-male progeny, resulting in the loss of the matriline as well as *Wolbachia*. This situation would increase the frequency of normal WZ females.

Concluding remarks

In summary, we demonstrated for the first time that the manipulation of sex chromosome inheritance and cytoplasmically induced meiotic drive can be added to the repertoire of host manipulations induced by *Wolbachia*. Therefore, the host effects of this bacterium are far more diverse and profound than previously appreciated. Disentangling these complex interactions between insects and *Wolbachia* may provide further exciting discoveries in the areas of host-parasite interactions, endosymbiosis as well as cell and chromosome biology in years to come, and perhaps also provide new avenues for pest population control.

Materials and Methods

Insect collection and rearing

Female adults of *E. mandarina* (Lepidoptera: Pieridae) were collected on Tanegashima Island, Kagoshima, Japan (Fig. S1). In the laboratory, each female was allowed to lay eggs on fresh leaves of *Lespedeza cuneata* (Fabales:

Fabaceae) in a plastic cup with absorbent cotton immersed with 5% honey solution. The artificial diet for larvae was prepared by mixing leaf powder of *Albizia julibrissin* (Fabales: Fabaceae) in the custom-made Silkmate (Nihon-Nosa, Yokohama, Japan) devoid of mulberry leaves. Insects were reared under the 16 h/ 8 h light /dark photoperiod at 25°C.

Antibiotic treatment

We performed antibiotic treatment on two different stages (larval stage and adult stage) of *E. mandarina*. For larval antibiotic treatment, larvae were fed with the artificial diet (shown above) containing 0.05% tetracycline hydrochloride (tet). For adult antibiotic treatment, female adults were fed with 5% honey solution containing 0.1% tet. Specifically, CF females were mated to antibiotic-treated male offspring of C females. Antibiotic treatment of these males was performed in the larval stage and prevented CI in the crossing. After mating, each CF female was allowed to lay eggs on fresh leaves of *L. cuneata* in a plastic cup with absorbent cotton immersed with 5% honey solution containing 0.1% tet. Fresh leaves of *L. cuneata* and cotton with tet-containing honey solution were exchanged daily.

Diagnosis of *Wobachia* strains

To diagnose *Wolbachia* strains in *E. mandarina*, several legs of each adult were homogenized in STE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 150 mM NaCl) and incubated at 56°C for 30 min followed by 92°C for 5 min. After centrifugation at 15,000 rpm for 2 min, the supernatant was used for polymerase chain reaction (PCR) using different primer pairs. The primer pair wsp81F (5'–TGGTCCAATAAGTGATGAAGAAAC–3') and wsp691R (5'–AAAAATTAAACGCTACTCCA–3') amplifies a ca. 610-bp fragment of the *Wolbachia wsp* gene (35). The primer pair wsp81F and HecCIR (5'–ACTAACGTCGTTTTTGTTTAG–3') amplifies specifically a 232-bp fragment of the *wsp* gene of *w*CI, while the primer pair HecFemF (5'–TTACTCACAATTGGCTAAAGAT–3') and the wsp691R amplifies specifically a 398-bp fragment of *wsp* gene of *w*Fem (9).

Whole genome sequencing and de novo assembly

We performed whole genome sequencing for three types of *E. mandarina* individuals (CF females, C females and C males) collected on Tanegashima Island, Japan (Fig. S1). Six genomic DNA libraries (two libraries for each sample type derived from two individuals) were constructed following manufacturer's instructions (http://www.illumina.com). The average insert size of the libraries was approximately 350 bp and each library was multiplexed using a single indexing protocol. The genomic DNA libraries were sequenced by Illumina MiSeq using MiSeq Reagent Kit v3 (600-cycle) (Illumina, San Diego, CA). Generated raw reads (8.31 Gb, 5.34 Gb, and 6.94 Gb for CF females, C females and C males, respectively) were filtered by Trimmomatic (36) and then mapped to the complete genome of *Wolbachia* strain *w*Pip (GenBank: NC_010981.1) by Bowtie2 (37). Mapped reads were discarded and then remaining reads of the three samples were merged and de novo assembled by SGA assembler (38). Generated genome contig sequences were used for further analysis.

Analysis of mapped read counts on chromosomes

To verify that CF and C females have one Z chromosome, we compared normalized mapped read counts of the three samples on Z chromosomes and remaining chromosomes. The filtered reads of each sample were mapped to the genome contigs by Bowtie2 (only concordantly and uniquely mapped reads were counted) and then normalized mapped read count of each sample on each contig was calculated based on the ratio of the number of total mapped reads between the three samples. Nucleotide sequences of relatively long genome contigs (length is 2 kb or more) with enough coverage (20 or more mapped reads) were extracted and compared with the gene set A of *B. mori* (39) by blastx search (cutoff e-value is 1e-50). Genome contigs with blastx hits were extracted and classified into 28 chromosomes based on the location of the homologous *B. mori* genes. For each chromosome, the average number of relative normalized mapped read counts was calculated for each sample (the number of C males was normalized to 1) using the normalized mapped read

counts in the classified genome contigs, respectively.

Sanger sequencing

To genotype Z chromosomes, a highly variable intron of Z-linked triosephosphate isomerase (*Tpi*) gene was PCR amplified using the primers, 5'–GGTCACTCTGAAAGGAGAACCACTTT–3' and 5'–CACAACATTTGCCCAGTTGTTGCAA–3', located in coding regions (40). The PCR products were treated with ExoSAP-IT® (Affymetrix Inc., Santa Clara, CA) and subjected to direct sequencing at Eurofins Genomics K.K. (Tokyo, Japan). No indels or SNPs were observed in sequence chromatograms of females; some males where heterozygous due to detected double peaks and shifts of sequence reads. By sequencing from both sides, it was possible to obtain the genotypes of males and females (Fig. 2C).

FISH analysis

In most lepidopteran species a heterochromatic body, that is rather conspicuous, is exclusively found in female polyploid nuclei. Since W derived-BAC as well as genomic probes have highlighted the W chromosomes and heterochromatin bodies in *B. mori* (41, 42), there is no doubt that the bodies consist of the W chromosomes. The diagnosis however retains unreliable if a species of interest carries a W–autosomal translocation and/or partial deletion of the W (43, 44). Hiroki et al. (8) as well as Narita et al. (10) relied on the W-body diagnosis for C and CF females and concluded that they have WZ and ZZ sex chromosome constitutions, respectively. However, Kern et al. (11) has recently found that, on the basis of genomic qPCR designed to amplify Z-linked gene sequences (*Tpi* and *Ket*) relative to an autosomal gene (*EF-1α*), both CF and C females have only one Z chromosome while males have two Z chromosomes. This finding rejected the previous conclusion that the sex chromosome constitution of CF females is ZZ (8, 10) but was inconclusive about whether CF females have a ZO or ZW' system (with W' as a modified W that has lost the feminization function and cannot be detected by the W-body assay). Hence we carried out more extensive chromosome analysis (other than just the W-body) to prove whether

CF females carry the W or not.

In Lepidoptera, the W chromosome can be highlighted by FISH using probes prepared from whole genomic DNA of males or females. The reason for the W chromosome specificity is considered to be due to the numerous repetitive short sequences occupying the W chromosome, which is then prone to be hybridized by random sequences. Genomic probes also paint repetitive regions scattered across other chromosomes, albeit at a lower density (autosomes and Z chromosome). Here we made mitotic and pachytene chromosome preparations from wing discs and gonads, respectively, in the last instar larvae of C and CF individuals of *E. mandarina* (see (45) for details). Genomic DNA was extracted from tet-treated C female larvae. Insect telomeric repeats were amplified by non-template PCR (46). *Kettin* gene fragments were amplified from adult cDNA synthesized by PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Japan) and cloned by TOPO® TA Cloning® Kit (Thermo Fisher Scientific, Waltham, MA). We used 4 pairs of primers, Em_kettin_F1: 5'–AGGTAATCCAACGCCAGTCG–3' and Em_kettin_R1: 5'–TGCTTGCCCTAAGGCATTGT–3', Em_kettin_F2: 5'–ACAATGCCTTAGGGCAAGCA–3' and Em_kettin_R2: 5'–TGGGCAAAGCCTCTTCATGT–3', Em_kettin_F3: 5'–AGATTCCGCACTACGCATGA–3' and Em_kettin_R3: 5'–TAAATTGTGGTGGGACGGCA–3', Em_kettin_F5: 5'–ACATGAAGAGGCTTTGCCCA–3' and Em_kettin_R5: 5'–TCATGCGTAGTGCGGAATCT–3', for PCR amplification with 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 3 min finalized by 72°C for 10 min. Probe labeling was done by using the Nick Translation Kit (Abbott Molecular, Des Plaines, IL). We selected Green-dUTP, Orange-dUTP (Abbott Molecular Inc.) and Cy5-dUTP (GE Healthcare Japan, Tokyo) fluorochromes for genomic DNA, *Kettin* and insect telomeric repeat (TTAGG)*n* probes respectively. Hybridizations were carried out according to protocols described elsewhere (45). Signal and chromosome images were captured with a DFC350FX CCD camera mounted on a DM 6000B microscope (Leica Microsystems Japan, Tokyo) and processed with Adobe Photoshop CS2. We

454 applied green, red and yellow pseudocolors to signals from Green, Orange and
455 Cy5 respectively.
456
457 **Quantitative polymerase chain reaction (qPCR)**
458 Eggs of mated females were sampled 48 h after the oviposition and stored at –
459 80°C until DNA extraction. Eggs were individually subjected to DNA extraction
460 using DNeasy® Blood & Tissue Kit (Qiagen, Tokyo, Japan). Real-time
461 fluorescence detection quantitative PCR (qPCR) was performed using SYBR
462 Green and a LightCycler® 480 System (Roche Diagnostics K.K., Tokyo, Japan).
463 Z-linked *Tpi* was amplified using TPI-F (5'–GGCCTCAAGGTCATTGCCTGT–3')
464 and TPI-R (5'–ACACGACCTCCTCGGTTTTACC–3'), Z-linked *Ket* was amplified
465 using Ket-F (5'–TCAGTTAAGGCTATTAACGCTCTG–3') and Ket-R (5'–
466 ATACTACCTTTTGCGGTTACTGTC–3'), and autosomal *EF-1α* was amplified
467 using EF-1F (5'–AAATCGGTGGTATCGGTACAGTGC–3') and EF-1R (5'–
468 ACAACAATGGTACCAGGCTTGAGG–3') (11). For each qPCR, a standard
469 dilution series of PCR products (10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 copies per
470 microliter) was included in order to estimate the absolute copy numbers of the
471 target sequence in the samples. To prepare standard samples, PCR products
472 were gel-excised and purified by Wizard® SV (Promega). Copy numbers of the
473 standard samples were estimated by the concentration measured by a
474 spectrophotometer, considering that the molecular weight of a nucleotide is 309
475 g/mol. For each qPCR, two replicates were performed that delivered similar
476 results. All qPCRs were performed using a temperature profile of 40 cycles of
477 95°C for 5 s, 60°C for 10 s, and 72°C for 10 s. The qPCR data were analyzed by
478 the Absolute Quantification analysis using the Second Derivative Maximum
479 method implemented in the LightCycler® 480 Instrument Operator Software
480 Version 1.5 (Roche).
481
482 **RT-PCR**
483 RNA was extracted from adult abdomens that were stored at -80°C using
484 RNeasy® Mini Kit (Qiagen, Tokyo, Japan). The cDNA synthesized by using
485 Superscript™ III (Invitrogen) and Oligo(dT) was used as a template for RT-PCR.

A partial sequence of *dsx* which contains alternative splicing sites was amplified using a primer pair, E520F (5'–GCAACGACCTCGACGAGGCTTCGCGGA–3') and EhdsxR4 (5'–AGGGGCAGCCAGTGCGACGCGTACTCC–3') and a temperature profile of 94°C for 2 min, 30 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min 30 s, followed by 72°C for 7 min. The sequences of seven dsx^F isoforms and a dsx^M isoform were deposited in DDBJ/EMBL/Genbank (LC215389-LC215396).

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References

1. Burt A, Trivers R (2006) *Genes in Conflict: the Biology of Selfish Genetic Elements* (Harvard University Press, Cambridge, MA).
2. Werren JH (2011) Selfish genetic elements, genetic conflict, and evolutionary innovation. *Proc Natl Acad Sci U S A* 108(Suppl 2):10863–10870.
3. Jaenike J (2001) Sex chromosome meiotic drive. *Annu Rev Ecol Syst* 32:25–49.
4. Lindholm AK, et al. (2016) The ecology and evolutionary dynamics of meiotic drive. *Trends Ecol Evol* 31(4):315–326.
5. Hurst LD (1993) The incidences mechanisms and evolution of cytoplasmic sex ratio distorters in animals. *Biol Rev* 68(1):121–194.
6. Beukeboom LW, Perrin N (2014) *The Evolution of Sex Determination* (Oxford University Press).
7. Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol* 6:741–751.
8. Hiroki M, Kato Y, Kamito T, Miura K (2002) Feminization of genetic males by a symbiotic bacterium in a butterfly, *Eurema hecabe* (Lepidoptera: Pieridae). *Naturwissenschaften* 89(4):167–170.
9. Hiroki M, Tagami Y, Miura K, Kato Y (2004) Multiple infection with *Wolbachia* inducing different reproductive manipulations in the butterfly *Eurema hecabe*. *Proc R Soc Lond B* 271(1549):1751–1755.
10. Narita S, Kageyama D, Nomura M, Fukatsu T (2007) Unexpected mechanism of symbiont-induced reversal of insect sex: feminizing *Wolbachia* continuously acts on the butterfly *Eurema hecabe* during larval development. *Appl Environ Microbiol* 73(13):4332–4341.
11. Kern P, Cook JM, Kageyama D, Riegler M (2015) Double trouble: combined action of meiotic drive and *Wolbachia* feminization in *Eurema* butterflies. *Biol Lett* 11(5):20150095.
12. Narita S, Nomura M, Kageyama D (2007) Naturally occurring single and double infection with *Wolbachia* strains in the butterfly *Eurema hecabe*: transmission efficiencies and population density dynamics of each

Wolbachia strain. *FEMS Microbiol Ecol* 61(2):235–245.

13. Bopp D, Saccone G, Beye M (2014) Sex determination in insects: Variations on a common theme. *Sex Dev* 8(1–3):20–28.
14. Ohbayashi F, Suzuki MG, Mita K, Okano K, Shimada T (2001) A homologue of the *Drosophila doublesex* gene is transcribed into sex-specific mRNA isoforms in the silkworm, *Bombyx mori*. *Comp Biochem Physiol - B Biochem Mol Biol* 128(1):145–158.
15. Pardo-Manuel De Villena F, Sapienza C (2001) Nonrandom segregation during meiosis: The unfairness of females. *Mamm Genome* 12(5):331–339.
16. Doncaster L (1913) On an inherited tendency to produce purely female families in *Abraxas grossulariata*, and its relation to an abnormal chromosome number. *J Genet* 3(1):1–10.
17. Doncaster L (1914) On the relations between chromosomes, sex-limited transmission and sex determination in *Abraxas grossulariata*. *J Genet* 4(1):1–21.
18. Doncaster L (1915) The Relation between Chromosomes and Sex-determination in "*Abraxas grossulariata*." *Nature* 95:395.
19. Doncaster L (1922) Further observations on chromosomes and sex-determination in *Abraxas grossulariata*. *Q J Microsc Sci* 66(263):397–406.
20. Kiuchi T, et al. (2014) A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature* 509(7502):4–6.
21. Kageyama D, Traut W (2004) Opposite sex-specific effects of *Wolbachia* and interference with the sex determination of its host *Ostrinia scapulalis*. *Proc Biol Sci* 271(1536):251–258.
22. Sugimoto TN, Ishikawa Y (2012) A male-killing *Wolbachia* carries a feminizing factor and is associated with degradation of the sex-determining system of its host. *Biol Lett* 8(3):412–5.
23. Fukui T, et al. (2015) The endosymbiotic bacterium *Wolbachia* selectively kills male hosts by targeting the masculinizing gene. *PLoS Pathog* 11(7):1–14.

24. Sugimoto TN, Kayukawa T, Shinoda T, Ishikawa Y, Tsuchida T (2015) Misdirection of dosage compensation underlies bidirectional sex-specific death in *Wolbachia*-infected *Ostrinia scapulalis*. *Insect Biochem Mol Biol* 66:72–76.
25. Giorgini M, Monti MM, Caprio E, Stouthamer R, Hunter MS (2009) Feminization and the collapse of haplodiploidy in an asexual parasitoid wasp harboring the bacterial symbiont *Cardinium*. *Heredity (Edinb)* 102(4):365–371.
26. Ma W-J, et al. (2015) Diploid males support a two-step mechanism of endosymbiont-induced thelytoky in a parasitoid wasp. *BMC Evol Biol* 15(1):84.
27. Dyson EA, Kamath MK, Hurst GDD (2002) *Wolbachia* infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera: Nymphalidae): evidence for horizontal transmission of a butterfly male killer. *Heredity (Edinb)* 88(3):166–71.
28. Harumoto T, Anbutsu H, Lemaitre B, Fukatsu T (2016) Male-killing symbiont damages host's dosage-compensated sex chromosome to induce embryonic apoptosis. *Nat Commun* 7:12781.
29. Yoshido A, Marec F, Sahara K (2016) The fate of W chromosomes in hybrids between wild silkmoths, *Samia cynthia* ssp.: no role in sex determination and reproduction. *Heredity (Edinb)* 116(5):424–433.
30. Rigaud T, Juchault P, Mocquard J-P (1997) The evolution of sex determination in isopod crustaceans. *BioEssays* 19(5):409–416.
31. Leclercq S, et al. (2016) Birth of a W sex chromosome by horizontal transfer of *Wolbachia* bacterial symbiont genome. *Proc Natl Acad Sci U S A* 113(52):201608979.
32. Hatcher MJ, Taneyhill DE, Dunn AM, Tofts C (1999) Population dynamics under parasitic sex ratio distortion. *Theor Pop Biol* 56(1):11–28.
33. Charlat S, et al. (2007) Extraordinary flux in sex ratio. *Science* 317(5835):214.
34. Majerus TMO, Majerus MEN (2010) Intergenomic arms races: Detection of a nuclear rescue gene of male-killing in a ladybird. *PLoS Pathog* 6(7):1–7.

35. Braig HR, Zhou W, Dobson SL, O'Neill SL (1998) Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont Wolbachia pipientis. *J Bacteriol* 180(9):2373–2378.
36. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15):2114–2120.
37. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nat Meth* 9(4):357–359.
38. Simpson JT, Durbin R (2012) Efficient de novo assembly of large genomes using compressed data structures. *Genome Res* 22(3):549–556.
39. Suetsugu Y, et al. (2013) Large scale full-length cDNA sequencing reveals a unique genomic landscape in a lepidopteran model insect, Bombyx mori. *G3 Genes|Genomes|Genetics* 3(9):1481–1492.
40. Jiggins CD, et al. (2001) Sex-linked hybrid sterility in a butterfly. *Evolution (N Y)* 55(May 1998):1631–1638.
41. Sahara K, Marec F, Eickhoff U, Traut W (2003) Moth sex chromatin probed by comparative genomic hybridization (CGH). *Genome* 46:339–342.
42. Sahara K, et al. (2003) W-derived BAC probes as a new tool for identification of the W chromosome and its aberrations in Bombyx mori. *Chromosoma* 112(1):48–55.
43. Traut W, Marec F (1996) Sex chromatin in Lepidoptera. *Q Rev Biol* 71(2):239–256.
44. Abe H, et al. (2008) Identification of the female-determining region of the W chromosome in Bombyx mori. *Genetica* 133(3):269–282.
45. Yoshido A, Sahara K, Yasukochi Y (2014) Chapter 6; Silkmoths (Lepidoptera). *Protocols for Cytogenetic Mapping of Arthropod Genomes.*, ed Sharakhov I (CRC Press, Boca Raton, U.S.A.), pp 219–256.
46. Sahara K, Marec F, Traut W (1999) TTAGG telomeric repeats in chromosomes of some insects and other arthropods. *Chromosom Res* 7(6):449–460.

Figure legends

Fig. 1. Fluorescence *in-situ* hybridization and sequence read counts for a C female, C male, and CF female *E. mandarina*. A–C: Mitotic complements hybridized with a genomic probe (green; green arrows) and a Z-linked *Kettin* (*Ket*) probe (red; red arrows) in a C female (2n = 62) (A), C male (2n = 62) (B), and CF female (2n = 61) (C). D–I: Genomic *in situ* hybridization (GISH) and FISH with a Z-linked *Ket* probe performed on interphase nuclei of *E. mandarina* C females (D, E), C males (F, G), and CF females (H, I). J–L: GISH, telomere-FISH and FISH with *Ket* probe performed on pachytene complements of *E. mandarina* C females (G, n = 31), C males (H, n = 31), and CF females (I, n = 31). Green paint signals in A, E and J revealed that C females have the W chromosome. The *Ket* probe signals (red) appeared on the Z pairing to the W in C females (J), the ZZ bivalent in C males (K), and the Z univalent of CF females (L). The single signals were observed both in C and CF female nuclei. The signals in C females (J) and males (K) clearly showed their respective WZ and ZZ chromosome sets, and a ZO chromosome set in CF females (L). W: W chromosome; Z: Z chromosome; white arrows: *Wolbachia*-like structures. A bar represents 10 μm. M–O: Relative normalized sequence read counts in CF females, C females, and C males for 67 contigs homologous to *Bombyx mori* loci on chromosome 1 (Z chromosome; M), 28 contigs homologous to *B. mori* loci on chromosome 4 (N), and 33 contigs homologous to *B. mori* loci on chromosome 16 (O), with relative read counts set to 1 (males). Details about genome sequencing are provided in Materials and Methods.

Fig. 2. Effects of *w*Fem on Z-linked gene dose in *E. mandarina* offspring. (A) Estimate of the gene dose of *Ket* (relative gene copies per copy of *EF-1α*) by genomic quantitative polymerase chain reaction (qPCR) analysis in each of the fertilized eggs laid by C females, CF females, and tetracycline (tet)-treated CF females. Each colored circle represents a single fertilized egg. Sample sizes are given in parentheses. (B) Offspring sex ratio of five females tet-treated prior to oviposition and three non-treated CF females. Numbers to the left of the arrows represent duration (days) of tet treatment. (C) Estimate of the gene dose of *Ket*

(relative gene copies per copy of *EF-1α*) by genomic qPCR in each of the adult offspring produced by CF females that were tet-treated during the adult stage (prior to oviposition). Each circle represents an adult offspring. Z chromosomes of these offspring individuals were genotyped as Z^A, Z^B, Z^C or Z^D on the basis of intron nucleotide sequence of Z-linked *Tpi*. The green arrow points to a male individual (adult) whose karyotype was considered to be ZO but possibly ZZ' (see text for details). f: female, m: male. (D) Estimate of the gene dose of *Ket* (relative gene copies per copy of *EF-1α*) by genomic qPCR in each of the intersexual adults generated by treating CF larvae with tet.

Fig. 3. Effects of *w*Fem on splicing of the *doublesex* gene in *E. mandarina*. (A) Reverse-transcription polymerase chain reaction (RT-PCR) products of *E. mandarina doublesex* (*Emdsx*) run on an agarose gel. Lane 1: C female; lane 2: C male; lanes 3 and 4: CF females; lanes 5 and 6: intersexes generated by tetracycline (tet) treatment of larvae produced by CF females; lane 7: 100-bp ladder. Females have at least seven splicing products, whereas males have a single product. (B) Structures of the splicing products of *Emdsx*. Translated regions are indicated by red and blue bars, untranslated regions by gray bars, and stop codons by triangles. Numbers of clones obtained by cloning the RT-PCR products are shown in the table on the right. C–H: color and morphology of forewings. Females are pale yellow on the dorsal side of the forewings (C) and do not have sex brand on the ventral side of the forewings (F), while males are intense yellow on the dorsal side of the forewings (D) and have sex brand on the ventral side of the forewings (G). Many of the intersexes generated by tet-treating CF larvae are strong yellow on the dorsal side of the forewings (E) and have faint sex brand on the ventral side of the forewings (H).

Fig. 4. Schematic illustration of two alternative mechanistic models of meiotic drive that explain the observed data. (A) The "Selection of O gametes" model assumes that Z-bearing gametes are selected against during meiosis. (B) The "Elimination of maternal Z" model assumes that Z chromosomes are eliminated during or after normal meiosis.

691

692 **Fig. 5.** (A) All-female production explained by *Wolbachia*–host interaction.
693 Effects of *w*Fem on the development and sex determination of *E. mandarina*,
694 and outcomes of larval versus adult tet treatment are illustrated. Asterisk: The
695 majority of ZO males die, but a few survived. (B) Hypothetical evolutionary
696 trajectory of *Wolbachia*–host interaction. See Discussion for details.

Fig. 1

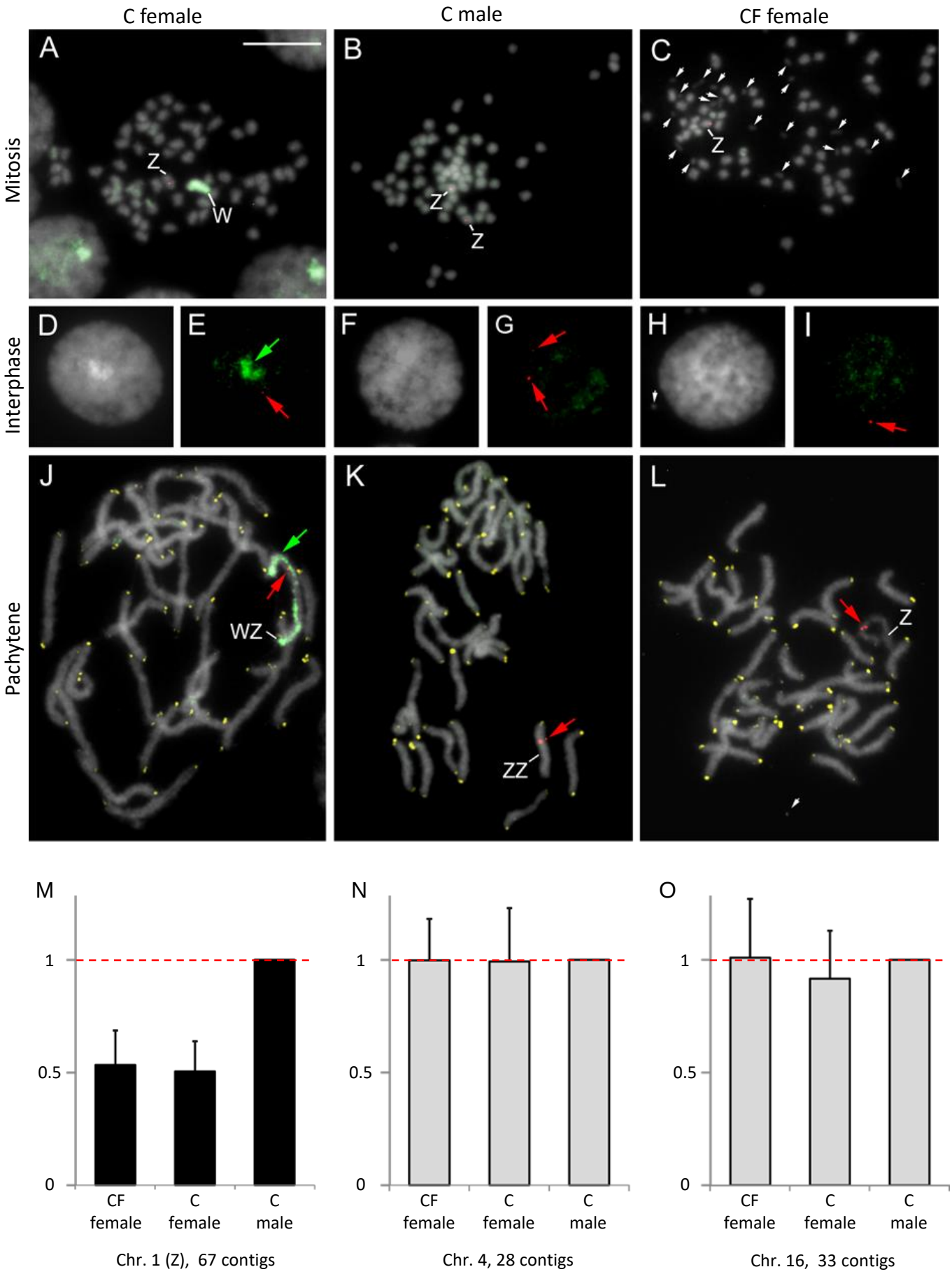

C female
C male
CF female
Mitosis
Interphase
Pachytene
A
B
C
D
E
F
G
H
I
J
K
L
Z
W
Z
Z
Z
WZ
ZZ
Z
M
N
O
1
0.5
0
CF female
C female
C male
Chr. 1 (Z), 67 contigs
Chr. 4, 28 contigs
Chr. 16, 33 contigs

Fig. 2

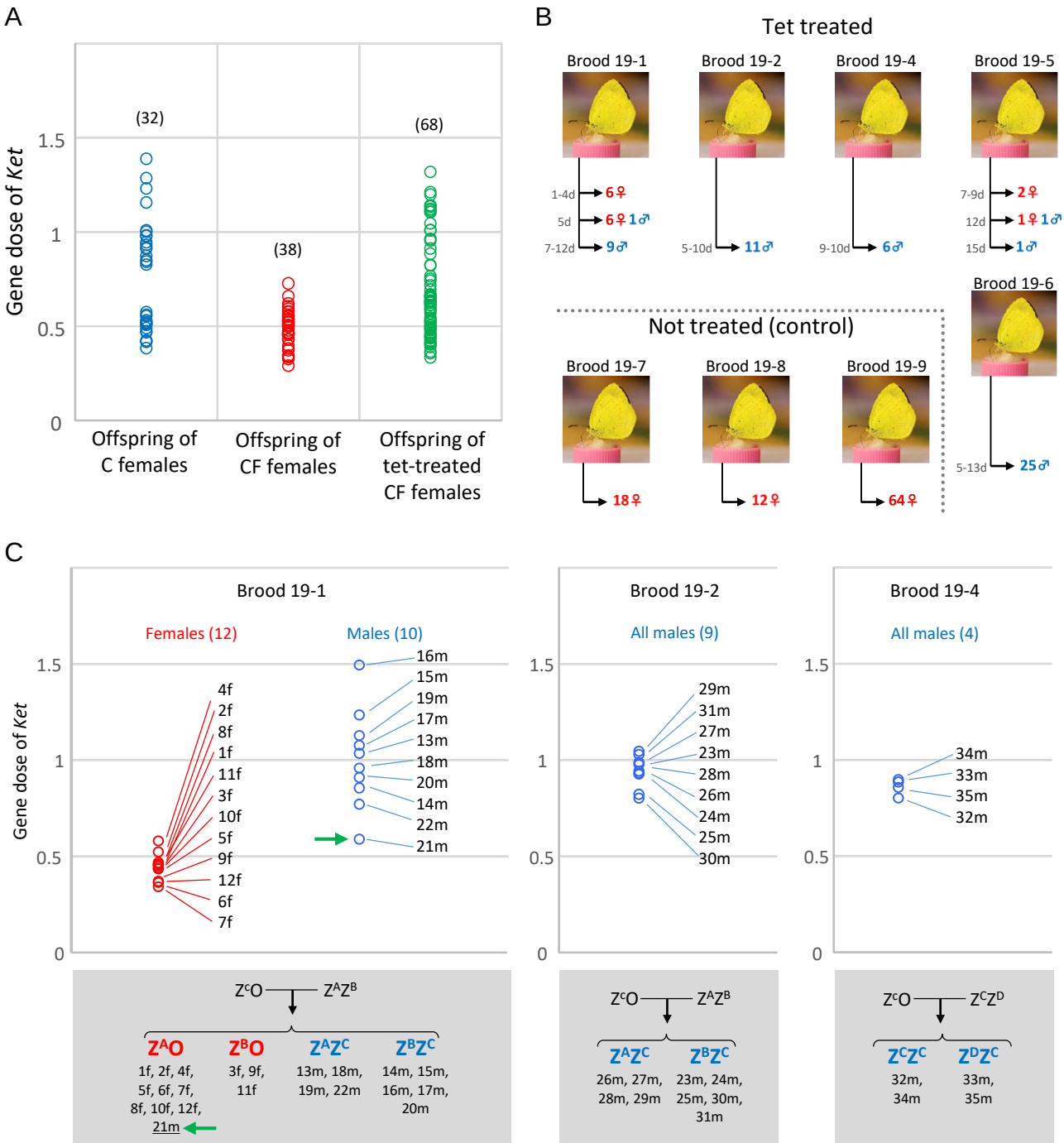
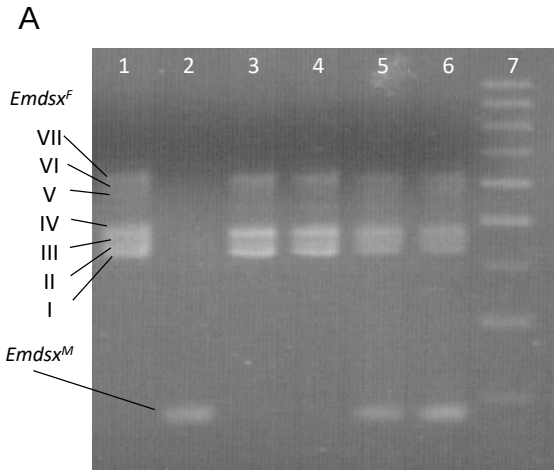
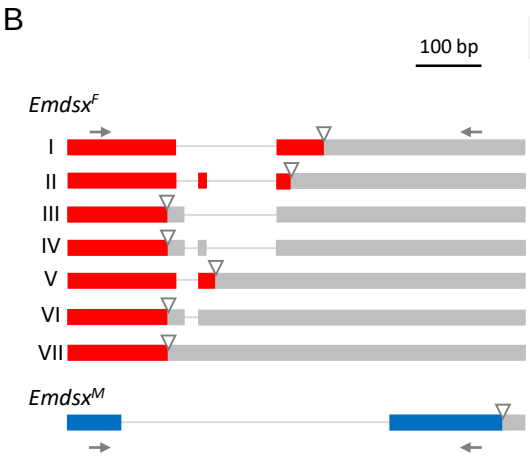
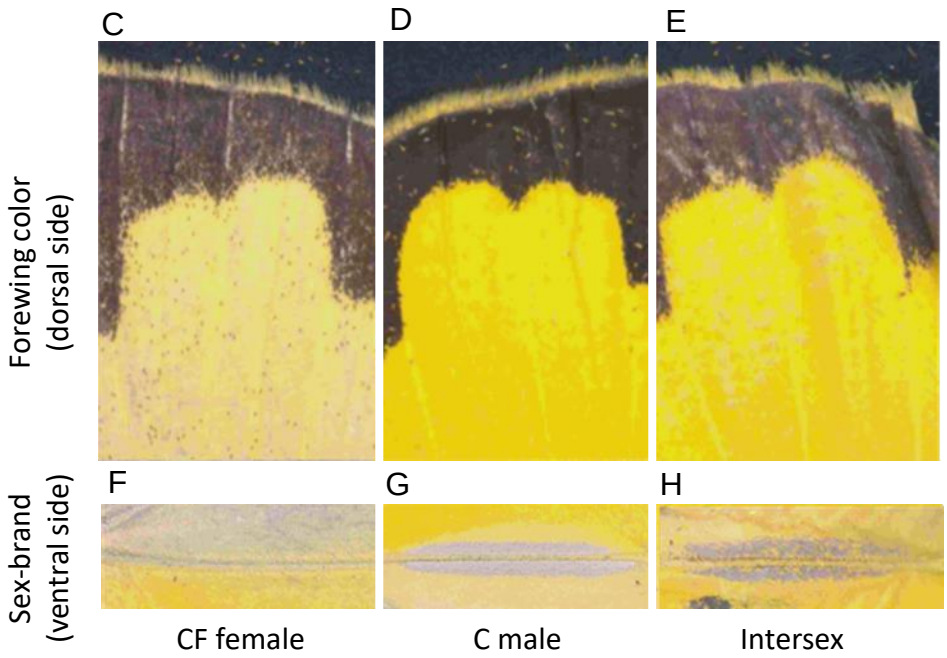

A
Gene dose of Ket
1.5
1
0.5
0
(32)
(38)
(68)
Offspring of C females
Offspring of CF females
Offspring of tet-treated CF females
B
Tet treated
Brood 19-1
Brood 19-2
Brood 19-4
Brood 19-5
1-4d → 6♀
5d → 6♀1♂
7-12d → 9♂
5-10d → 11♂
9-10d → 6♂
7-9d → 2♀
12d → 1♀1♂
15d → 1♂
Brood 19-6
5-13d → 25♂
Not treated (control)
Brood 19-7
Brood 19-8
Brood 19-9
18♀
12♀
64♀
C
Brood 19-1
Females (12)
Males (10)
4f
2f
8f
1f
11f
3f
10f
5f
9f
12f
6f
7f
16m
15m
19m
17m
13m
18m
20m
14m
22m
21m
ZᶜO — ZᴬZᴮ
ZᴬO
1f, 2f, 4f, 5f, 6f, 7f, 8f, 10f, 12f, 21m
ZᴮO
3f, 9f, 11f
ZᴬZᶜ
13m, 18m, 19m, 22m
ZᴮZᶜ
14m, 15m, 16m, 17m, 20m
Brood 19-2
All males (9)
29m
31m
27m
23m
28m
26m
24m
25m
30m
ZᶜO — ZᴬZᴮ
ZᴬZᶜ
26m, 27m, 28m, 29m
ZᴮZᶜ
23m, 24m, 25m, 30m, 31m
Brood 19-4
All males (4)
34m
33m
35m
32m
ZᶜO — ZᶜZᴰ
ZᶜZᶜ
32m, 34m
ZᴰZᶜ
33m, 35m

Fig. 3





	No. of clones		
	C female	C male	CF female
I	12	–	16
II	1	–	–
III	10	–	3
IV	2	–	1
V	4	–	3
VI	6	–	5
VII	1	–	–
Emdsx^M	–	7	–



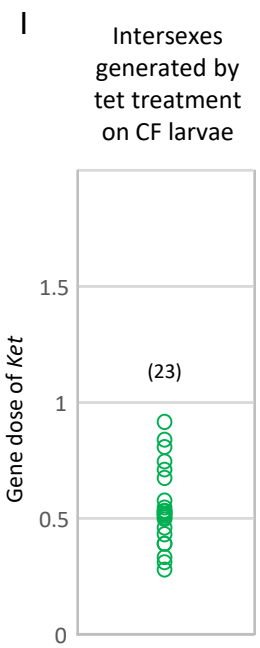


Fig. 4

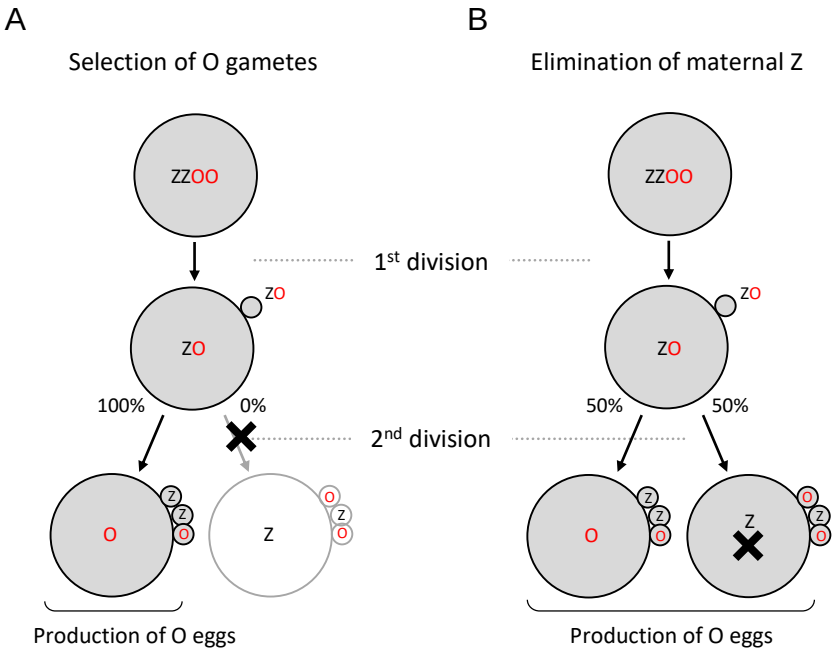
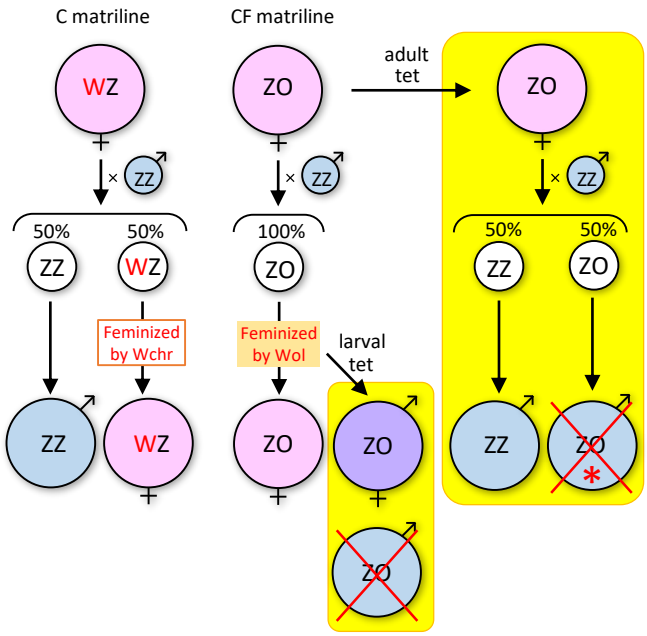

A
Selection of O gametes
B
Elimination of maternal Z
ZZOO
ZZOO
1st division
ZO
ZO
ZO
ZO
100%
0%
50%
50%
2nd division
O
Z
O
Z
Production of O eggs
Production of O eggs

Fig. 5

A **All-female production explained by *Wolbachia*–host interaction**



B **Hypothetical evolutionary trajectory of *Wolbachia*–host interaction**

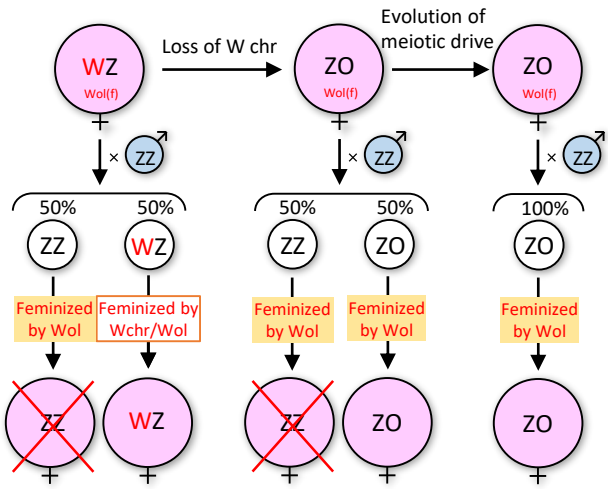


Table 1. Detection of *Emdsx* in adults that were tet-treated during various larval stages

Presence of *Emdsx* transcripts	Offspring of CF females					Offspring of C females
	Non-treated	4th-5th treated	3rd-5th treated	2nd-5th treated	1st-5th treated	Non-treated
$Emdsx^F$ only	46	0	0	0	0	12
Both $Emdsx^F$ and $Emdsx^M$	1	4(1)	19(7)	0	6(3)*	0
$Emdsx^M$ only	0	0	1(1)	2(2)	0	12
Total	47	4(1)	20(8)	2(2)	6(3)	24

The numbers of adults that failed to emerge from their pupal cases are in parentheses. *Signals for $Emdsx^F$ were faint.