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1 **Feminizing *Wolbachia* endosymbiont disrupts maternal sex chromosome**
2 **inheritance in a butterfly species**
3

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23 **Abstract** Genomes are vulnerable to selfish genetic elements that enhance their
24 own transmission often at the expense of host fitness. Examples are cytoplasmic
25 elements such as maternally inherited bacteria that cause feminization,
26 male-killing, parthenogenesis and cytoplasmic incompatibility. We demonstrate,
27 for the first time, that segregation distortion, a phenomenon so far seen only for
28 nuclear genetic elements, can also be caused by a cytoplasmic element, the
29 ubiquitous endosymbiotic bacterium *Wolbachia*. For *Eurema mandarina* butterfly
30 lineages with a Z0 sex chromosome constitution, we provide direct and
31 conclusive evidence that *Wolbachia* induces production of all-female progeny by
32 a dual role: the compensation for the female-determining function that is absent
33 in Z0 lineages (feminization) and the prevention of maternal sex chromosome
34 inheritance to offspring as a specific type of segregation distortion. Therefore,
35 our findings highlight that both sex determination and chromosome inheritance
36 — crucially important developmental processes of higher eukaryotes — can be
37 manipulated by cytoplasmic parasites.

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38 ## Introduction

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

47 Segregation distortion (SD), also referred to as meiotic drive, is a
48 violation of Mendelian law as it leads to the more frequent inheritance of one
49 copy of a gene than the expected 50% [3,4]. A segregation distorter that sits on a
50 sex chromosome biases the sex ratio. For example, X-linked segregation
51 distorter (X drive) and Y-linked segregation distorter (Y drive) in flies (Diptera),
52 result in female-biased and male-biased sex ratios, respectively [4]. In
53 male-heterogametic species, X and Y segregation distorters are expected to be
54 encoded in the nuclear genome. In female-heterogametic species, however, W
55 chromosome and cytoplasm behave as a single linkage group and thus
56 distortion of sex chromosome inheritance in female-heterogametic species can
57 theoretically also be caused by cytoplasmic elements. Although this possibility
58 has previously been proposed [5,6], lack of empirical evidence questions
59 whether it is mechanistically possible for cytoplasmic elements to cause SD.

60 *Wolbachia pipientis* (Alphaproteobacteria), simply referred to as
61 *Wolbachia*, attracts significant interest in evolutionary and developmental biology

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62 but also in applied fields such as pest management because it can manipulate
63 reproduction of arthropods in various ways such as cytoplasmic incompatibility,
64 parthenogenesis induction, feminization and male-killing [7]. Here we
65 demonstrate for the first time that *Wolbachia* is responsible for the disruption of
66 sex chromosome inheritance, which can also be seen as a form of segregation
67 distortion, in any host species. We do this by providing multifaceted and
68 conclusive evidence that in the butterfly *Eurema mandarina Wolbachia*-induced
69 SD is the underlying mechanism for the production of all-female progeny. In
70 most populations, *E. mandarina* is infected with the cytoplasmic-incompatibility
71 (CI)-inducing *Wolbachia* strain *w*CI at a high prevalence of close to 100% [8,9].
72 Hiroki et al. [10,11] first reported all-female offspring production in *E. mandarina*
73 (then known as *Eurema hecabe* yellow type), which was considered to be due to
74 the feminization of genetic males (ZZ) by co-infections with the *Wolbachia* strain
75 *w*Fem (hereafter referred to as double infection CF while single infection with
76 *w*CI is referred to as C). Three observations about CF lineages supported this
77 view, i.e., (a) antibiotic treatment of adult females led to the production of
78 all-male offspring [10], (b) antibiotic treatment of larvae resulted in intersex
79 adults [12] and (c) females did not have the W chromatin body [10,12]. This has
80 recently been challenged, because it was demonstrated that CF females have
81 only one Z chromosome and that this Z chromosome always derived from their
82 fathers implying that a SD mechanism may be in place albeit it was not clear
83 whether *Wolbachia* induced this SD [13]. As a consequence two novel (yet
84 untested) hypotheses were formed, namely, that CF females have either a Z0 or
85 a W’Z sex chromosome set (whereby W’ cannot be visualized in W chromatin

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86 assays and does not have a female-determining function), and that the
87 disruption of Z chromosome inheritance occurs in CF lineages due to *Wolbachia*
88 or another factor, such as those encoded by the host nucleus.

89 In a multifaceted approach, by combining fluorescence in situ
90 hybridization (FISH), genome sequencing, quantitative PCR, reverse
91 transcription PCR and antibiotic treatment, we have tested these two
92 hypotheses and revealed that CF females are Z0, and that *Wolbachia* is the
93 cause for both the disruption of Z chromosome inheritance and the feminization
94 of Z0 individuals. Our results demonstrate, for the first time, *Wolbachia* as the
95 agent that is responsible for distorted sex chromosome inheritance, and thereby
96 highlight that cytoplasmic elements can have profound effects on oogenesis, sex
97 chromosome inheritance and sex determination – fundamental biological
98 processes of eukaryotes.

99

## 100 Results

### 101 All-female-producing CF females have a Z0 sex chromosome constitution

102 We performed FISH on *E. mandarina* chromosomes prepared from CF females,
103 C females, and C males collected on Tanegashima Island (***Figure 1***; ***Figure***
104 ***1—figure supplement 1***). In the mitotic complement of C females, which harbor
105 a $2n$ = 62 karyotype, genomic probes highlighted the W chromosome, with
106 scattered signals on the other chromosomes (***Figure 2A***; see Materials and
107 Methods for technical details). A probe for the Z-linked gene *Kettin* (*Ket*)
108 identified the single Z chromosome in C females (***Figure 2A***), and also
109 hybridized to the Z chromosome paired with the W chromosome in pachytene

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110 bivalents (***Figure 2J***). The *Ket* probe identified two Z chromosomes in the mitotic
111 complement of C males (***Figure 2B***; 2*n* = 62). No painted W chromosome was
112 observed in interphase nuclei (***Figure 2H, I***), the mitotic complement (***Figure***
113 ***2C***) and pachytene complement (***Figure 2L***) of CF females, but the *Ket* signal
114 appeared on the single Z chromosome in the mitotic complement (***Figure 2C***)
115 and Z univalent in the pachytene complement (***Figure 2L***). Based on the relative
116 read counts homologous to *Bombyx mori* Z-linked and autosomal genes in
117 females and males, our genome sequencing data support the notion that CF and
118 C females have one Z chromosome (***Figures 2M–O***; ***Figure 2—figure***
119 ***supplement 1***), which is consistent with genomic qPCR data based on two loci,
120 *Triosephosphate isomerase* (*Tpi*) and *Ket*, relative to the autosomal gene *EF-1α*
121 [13]. Thus, our results directly reveal the sex chromosome constitution of C
122 females, C males, and CF females as WZ, ZZ, and Z0, respectively. This
123 confirms one of two previously suggested sex chromosome constitution of CF
124 females [13] while it disproves another previous interpretation based on W-body
125 diagnosis that CF females are ZZ [10,12].
126

127 **All embryos oviposited by CF females are Z0**

128 We performed real-time genomic qPCR (to detect Z-linked *Tpi* or *Ket* relative to
129 autosomal *EF-1α*) on individual fertilized eggs, and found that C females
130 oviposited embryos with either one or two Z chromosomes at nearly equal
131 frequencies (***Figure 3A left***; ***Figure 3—figure supplement 1***). In contrast, all
132 embryos oviposited by CF females were single Z carriers (***Figure 3A middle***;
133 ***Figure 3—figure supplement 1***). These findings indicate that the progeny of CF

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134 females are exclusively Z0 individuals, supporting the view that the maternal Z
135 chromosomes are not inherited in CF lineages.
136

137 ***Wolbachia* causes the exclusive production of Z0 embryos by CF females**

138 To abolish the effects of *Wolbachia*, tetracycline (tet) was administered to adult
139 CF females previously inseminated by antibiotic-treated male offspring of C
140 females. The Z-linked gene dose of embryos laid by these tet-treated females
141 ranged from approximately 0.5–1.0, indicating that some embryos are Z0 and
142 others are ZZ (***Figure 3A right***; ***Figure 3—figure supplement 1***). This suggests
143 that the *Wolbachia* strain *w*Fem in CF females causes the exclusive production
144 of gametes without sex chromosomes that then develop as Z0 embryos after
145 fertilization. Therefore, our finding is the first empirical evidence that in a
146 female-heterogametic species the sex-specific linkage disequilibrium can be
147 caused by cytoplasmic elements [5,6]. Furthermore, *Wolbachia*-like structures
148 were observed near the chromosomes in CF females while less apparent in C
149 females and C males, and this may represent different tropism and function of
150 *w*Fem when contrasted with *w*CI (***Figure 2C***). Sixty-nine adults (15 females and
151 54 males) were obtained from offspring produced by five tet-treated adult CF
152 females (***Figure 3B***). Three of these tet-treated females produced only male
153 offspring. Exclusive production of males was previously observed in tet-treated *E.*
154 *mandarina* females derived from a different population on Okinawa-jima Island,
155 Okinawa Prefecture, Japan [10]. In this study, we obtained 15 female offspring
156 from two broods in the first days after tet treatment; however, the mothers
157 produced more males as the duration of tet treatment increased, and eventually

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158 produced only males. Examination of the Z-linked gene dose of these offspring
159 by genomic qPCR showed that the females had one Z chromosome, whereas
160 almost all of the males had two Z chromosomes (***Figure 3C***). The nucleotide
161 sequences of the introns of the *Tpi* gene demonstrate that, in brood 19-1, all
162 females (*n* = 12) were hemizygous and nine out of 10 males were heterozygous
163 (***Figure 3C***; ***Figure 3—figure supplement 2***). Curiously, one male (21m) that
164 exhibited the lowest gene dose of *Ket* (0.588) appeared to be hemizygous
165 (***Figure 3C***). These results suggest that the emerged females had a Z0 sex
166 chromosome constitution, whereas most males had a ZZ sex chromosome
167 constitution, with one exception (21m) of either Z0 or ZZ' (Z' represents partial
168 deletion/mutation in Z). These results also demonstrate that, in principle,
169 tet-treated adult CF females can oviposit embryos with either a Z0 or ZZ sex
170 chromosome constitutions (***Figure 3A right***). However, Z0 individuals appear to
171 have zero or very low survival rates because few emerge as adults.
172

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

174 Next, we fed CF larvae a tet-containing diet. As previously observed [12], all
175 individuals treated in this way developed an intersex phenotype at the adult
176 stage, typically represented with male-like wing color and an incomplete
177 male-specific structure on the wing surface (***Figure 4E and H***; ***Figure 4—figure***
178 ***supplement 2***). The qPCR assay to assess the Z-linked gene dose revealed
179 that these intersexes (*n* = 23) had just one Z chromosome (***Figure 4I***), and
180 therefore a Z0 genotype. Because these Z0 individuals were destined to develop
181 as females without tet treatment, *w*Fem is likely to be responsible for female sex

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182 determination. Further evidence in support of this idea was obtained by
183 examining the sex-specific splicing products of *dsx* (***Figure 4—figure***
184 ***supplement 3***), a widely conserved gene responsible for sexual differentiation
185 [14]. Similar to *B. mori* [15], C females exhibited female-specific splicing
186 products of *E. mandarina dsx* ($Emdsx^F$), whereas C males had a male-specific
187 splicing product of *E. mandarina dsx* ($Emdsx^M$; Lanes 1 and 2 in ***Figure 4A***,
188 respectively; ***Figure 4B***). Similarly to C females, CF females exhibited exclusive
189 expression of $Emdsx^F$ (Lanes 3 and 4 in ***Figure 4A***; ***Figure 4B***). Intersexual
190 butterflies, generated by feeding the larval offspring of CF females a
191 tet-containing larval diet, expressed both $Emdsx^F$ and $Emdsx^M$ (Lanes 5 and 6 in
192 ***Figure 4A***; ***Figure 4—figure supplement 1***).
193
194 **Discussion**

195 We provide comprehensive and conclusive indirect (qPCR of Z gene dosage)
196 and direct (W chromosome painting; genomic analyses) evidence for the loss of
197 the W chromosome from CF individuals. Furthermore, we demonstrate that the
198 *Wolbachia* strain *w*Fem is directly responsible for chromosomal segregation
199 distortion (SD) by causing the disruption of sex chromosome inheritance in CF
200 females of *E. mandarina*. This is the first empirical proof for previous theoretical
201 predictions that cytoplasmic SGEs, such as *Wolbachia*, can cause SD. In *E.*
202 *mandarina*, *w*Fem has a dual role in both causing segregation distortion and
203 feminization in Z0 lineages that have lost W chromosome and its feminizing
204 function.
205

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206 ***Wolbachia* disrupts Z chromosome inheritance in Z0 females**

207 Our data provides evidence that the exclusive production of Z0 embryos by CF
208 females is due to a yet unidentified developmental process that leads to the
209 disruption of sex chromosome inheritance in CF females prior to oviposition,
210 thereby the absence of maternal Z chromosome in CF offspring. This process
211 can be referred to as SD, according to established conceptual frameworks [5,6].
212 We believe that two mutually exclusive hypotheses can account for the SD
213 observed in CF individuals (***Figure 5A***). The first assumes that a gamete without
214 the maternal Z chromosome (or without any sex chromosome overall), is always
215 selected to become an egg pronucleus (meiotic drive *sensu stricto*) (***Figure 5A***
216 ***left***) [16]. The second assumes that meiosis itself is normal, and that maternal Z
217 chromosomes (or sex chromosomes in general), are selectively eliminated from
218 Z-bearing gametes during, or possibly after, meiosis (***Figure 5A right***). At
219 present, it is unclear which of the two scenarios (meiotic drive *sensu stricto* or
220 elimination of the maternal Z at a later stage) is more plausible. However, it is
221 noteworthy that, in the moth *Abraxas grossulariata*, a matriline consisting of
222 putative Z0 females was observed to produce only females or a great excess of
223 females, and the underlying mechanism was considered to be the selective
224 elimination of Z chromosomes [17–20]. However, the presence of cytoplasmic
225 bacteria such as *Wolbachia* has not yet been examined for this moth species. If
226 we assume that the elimination of the maternal Z chromosome is the mechanism
227 of the SD in *E. mandarina*, the exceptional individual 21m (***Figure 3C***) could be
228 viewed as ZZ’ rather than Z0, wherein Z’ is a maternal Z chromosome that was
229 only partially deleted in the position including *Tpi* and *Ket* by the incomplete

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230 action of *w*Fem. It is possible to further speculate that the presence of *w*Fem
231 results in the elimination of sex chromosomes in general (Z or W chromosomes)
232 and, therefore, the absence of W chromosomes in CF females may also be a
233 direct effect of *w*Fem.
234
235 **The feminizing effect of *Wolbachia* compensates for the loss of the W**
236 **chromosome in Z0 individuals**
237 In general, lepidopterans species with Z0/ZZ sex chromosome constitution are
238 considered to determine their sexes by Z-counting mechanisms, wherein ZZ is
239 male and Z0 is female [21,22]. However, the appearance of the male phenotype
240 in Z0 individuals of *E. mandarina* after antibiotic treatment suggests that *w*Fem in
241 Z0 individuals compensates for the loss of W and its feminizing function (***Figure***
242 ***5B***). We speculate that the W chromosome of *E. mandarina* acts as an epistatic
243 feminizer. In *B. mori*, the W chromosome – more specifically, a piRNA located on
244 the W chromosome – acts as an epistatic feminizer by silencing *Masculinizer* on
245 the Z chromosome [23].
246 Reduced survival of Z0 individuals or their offspring after antibiotic
247 treatment of larvae or adults, respectively, may suggest improper dosage
248 compensation in Z0 males. Improper dosage compensation was also proposed
249 to be the cause of male- and female-specific lethality in *Wolbachia*-infected and
250 cured lines of *Ostrinia* moths [24–27].
251
252 **How did the coordinated dual effects of *Wolbachia* evolve?**
253 We demonstrated that *w*Fem causes SD and feminization in *E. mandarina* in two

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254 steps (***Figure 5B***). This is similar to the dual role of *Wolbachia* and *Cardinium* in
255 haplodiploid parasitoid wasps where they induce thelytokous parthenogenesis in
256 a two-step mechanism, comprising diploidization of the unfertilized egg followed
257 by feminization [28,29]. Here, we develop the potential evolutionary scenario
258 that led to the appearance of both effects in *E. mandarina* (***Figure 6***). A WZ
259 female *Eurema* butterfly may have acquired *w*Fem that exerted a feminizing
260 effect on ZZ males. The feminizing effect was lethal to ZZ individuals because of
261 improper dosage compensation, as evident in *Wolbachia*-infected *Ostrinia*
262 moths (***Figure 6A***) [26,27]. This could be viewed as a manipulation similar to a
263 male-killing phenotype [30,31]. However, the feminizing effect of *w*Fem was
264 redundant in WZ females where the W chromosome acted as a female
265 determiner [23]. Conversely, the function of W had also become redundant in CF
266 individuals and this could have led to the loss of the W chromosome and the rise
267 of a Z0 lineage (***Figure 6B***). Similarly, in *Ostrinia* moths, a female-determining
268 function is thought to have been lost from the W chromosome in
269 *Wolbachia*-infected matrilines [25]. Spontaneous loss of a nonfunctional W
270 chromosome may be easier than expected: in a wild silkmoth *Samia cynthia*, the
271 W chromosome does not have a sex-determining function, and Z0 females are
272 frequently obtained in experimental crosses between subspecies [32].
273 *Wolbachia* has previously been found to be involved in the loss and birth of W
274 chromosomes in the woodlouse *Armadillidium vulgare* [33,34]. However, in *A.*
275 *vulgare* it has not yet been tested whether *Wolbachia* interferes with
276 chromosome segregation and inheritance as we have mechanistically
277 demonstrated it for *E. mandarina*; i.e., after the loss of the W chromosome in CF

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278 lineages, *Wolbachia* then acquired a novel function that affected female
279 oogenesis and resulted in SD (***Figure 6C***). It is unlikely that SD arose prior to the
280 feminization function of *Wolbachia*: if the appearance of SD were to precede the
281 loss of the W chromosome, the feminizing or female-determining function would
282 become unnecessary for *Wolbachia* because there would be no males. In the
283 short term, disruption of Z chromosome inheritance in females in a
284 female-heterogametic species represents a great advantage to cytoplasmic
285 symbionts because all vertically transmitted symbionts gain the opportunity to
286 survive. However, males are still required for fertilization, and fixation of the
287 symbionts in the host population will inevitably lead to the extinction of both the
288 symbionts and the hosts [35]. In the long term, suppressors against sex ratio
289 distortion, as has been observed for the male-killing phenotypes in the butterfly
290 *Hypolimnas bolina* or a ladybird beetle [36,37], can be expected to evolve in the
291 host. However, the evolutionary outcomes of the suppression of a combined SD
292 and feminization would be different from that of male-killing suppression,
293 because it would lead to all-male progeny, resulting in the loss of the matriline
294 that inherits the feminizing and sex-distorting *Wolbachia*. This process thereby
295 selects for an increased frequency of WZ females.
296

## 297 Concluding remarks

298 In summary, we demonstrate for the first time that the manipulation of sex
299 chromosome inheritance and cytoplasmically induced SD can be added to the
300 repertoire of host manipulations induced by *Wolbachia*. Therefore, the host
301 effects of this bacterium are far more diverse and profound than previously

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302 appreciated. Disentangling these complex interactions between insects and
303 *Wolbachia* may provide further exciting discoveries in the areas of host–parasite
304 interactions, endosymbiosis as well as cell and chromosome biology in years to
305 come, and perhaps also provide new avenues for pest population control.
306

## 307 Materials and methods

### 308 Collection and rearing of *E. mandarina*

309 Female adults of *E. mandarina* (Lepidoptera: Pieridae) were collected on
310 Tanegashima Island, Kagoshima, Japan (***Figure 1—figure supplement 1***). In
311 the laboratory, each female was allowed to lay embryos on fresh leaves of
312 *Lespedeza cuneata* (Fabales: Fabaceae) in a plastic cup with absorbent cotton
313 immersed with 5% honey solution. The artificial diet for larvae was prepared by
314 mixing leaf powder of *Albizia julibrissin* (Fabales: Fabaceae) in the custom-made
315 Silkmate (Nihon-Nosa, Yokohama, Japan) devoid of mulberry leaves. Insects
316 were reared under the 16 h/ 8 h light /dark photoperiod at 25°C.
317

### 318 Antibiotic treatment

319 We performed antibiotic treatment of two different stages (larval stage and adult
320 stage) of *E. mandarina*. For larval antibiotic treatment, larvae were fed with the
321 artificial diet (shown above) containing 0.05% tetracycline hydrochloride (tet).
322 For adult antibiotic treatment, female adults were fed with 5% honey solution
323 containing 0.1% tet. Specifically, CF females were mated to antibiotic-treated
324 male offspring of C females. Antibiotic treatment of these males was performed
325 in the larval stage and prevented CI in the crossing. After mating, each CF

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326 female was allowed to lay embryos on fresh leaves of *L. cuneata* in a plastic cup
327 with absorbent cotton immersed with 5% honey solution containing 0.1% tet.
328 Fresh leaves of *L. cuneata* and cotton with tet-containing honey solution were
329 exchanged daily.
330

331 **Diagnosis of *Wobachia* strains**

332 To diagnose *Wolbachia* strains in *E. mandarina*, several legs of each adult were
333 homogenized in STE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 150
334 mM NaCl) and incubated at 56°C for 30 min followed by 92°C for 5 min. After
335 centrifugation at 15,000 rpm for 2 min, the supernatant was used for polymerase
336 chain reaction (PCR) using different primer pairs. The primer pair wsp81F
337 (5'–TGGTCCAATAAGTGATGAAGAAAC–3') and wsp691R
338 (5'–AAAAATTAAACGCTACTCCA–3') amplifies a ca. 610-bp fragment of the
339 *Wolbachia wsp* gene [38]. The primer pair wsp81F and HecCIR
340 (5'–ACTAACGTCGTTTTTGTTTAG–3') amplifies a 232-bp fragment of the *wsp*
341 gene of *w*CI, while the primer pair HecFemF
342 (5'–TTACTCACAATTGGCTAAAGAT–3') and the wsp691R amplifies a 398-bp
343 fragment of *wsp* gene of *w*Fem [11,39].
344

345 **Whole genome sequencing and de novo assembly**

346 We performed whole genome sequencing for three types of *E. mandarina*
347 individuals (CF females, C females and C males) that were collected on
348 Tanegashima Island, Japan (***Figure 1—figure supplement 1***). Six genomic
349 DNA libraries (two libraries for each sample type derived from two individuals)

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350 were constructed following manufacturer's instructions (http://www.illumina.com).
351 The average insert size of the libraries was approximately 350 bp and each
352 library was multiplexed using a single indexing protocol. The genomic DNA
353 libraries were sequenced by Illumina MiSeq using MiSeq Reagent Kit v3
354 (600-cycle) (Illumina, San Diego, CA). Generated raw reads (8.31 Gb, 5.34 Gb,
355 and 6.94 Gb for CF females, C females and C males, respectively) were filtered
356 by Trimmomatic [40] and then mapped to the complete genome of *Wolbachia*
357 strain *w*Pip (GenBank: NC_010981.1) by Bowtie2 [41]. Mapped reads were
358 discarded and then remaining reads of the three samples were merged and de
359 novo assembled by SGA assembler [42]. Generated genome contig sequences
360 were used for further analysis.
361

362 **Analysis of mapped read counts on chromosomes**

363 To verify that CF and C females have one Z chromosome, we compared
364 normalized mapped read counts of the three samples on Z chromosomes and
365 remaining chromosomes. The filtered reads of each sample were mapped to the
366 genome contigs by Bowtie2 (only concordantly and uniquely mapped reads were
367 counted) and then normalized mapped read count of each sample on each
368 contig was calculated based on the ratio of the number of total mapped reads
369 between the three samples. Nucleotide sequences of relatively long genome
370 contigs (length is 2 kb or more) with enough coverage (20 or more mapped
371 reads) were extracted and compared with the gene set A of *B. mori* [43] by blastx
372 search (cutoff e-value is 1e-50). Genome contigs with blastx hits were extracted
373 and classified into 28 chromosomes based on the location of the homologous *B.*

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374 *mori* genes. For each chromosome, the average number of relative normalized
375 mapped read counts was calculated for each sample (the number of C males
376 was normalized to 1) using the normalized mapped read counts in the classified
377 genome contigs, respectively.
378

379 **Sanger sequencing**

380 To genotype Z chromosomes, a highly variable intron of Z-linked
381 triosephosphate isomerase (*Tpi*) gene was PCR amplified using the primers,
382 5'–GGTCACTCTGAAAGGAGAACCACTTT–3' and
383 5'–CACAACATTTGCCCAGTTGTTGCAA–3', located in coding regions [44]. The
384 PCR products were treated with ExoSAP-IT® (Affymetrix Inc., Santa Clara, CA)
385 and subjected to direct sequencing at Eurofins Genomics K.K. (Tokyo, Japan).
386 No indels or SNPs were observed in sequence chromatograms of females;
387 some males where heterozygous due to detected double peaks and shifts of
388 sequence reads. By sequencing from both sides, it was possible to obtain the
389 genotypes of males and females (***Figure 3—figure supplement 2***).
390

391 **FISH analysis**

392 In most lepidopteran species a conspicuous heterochromatic body is exclusively
393 found in female polyploid nuclei. Since W derived-BAC as well as genomic
394 probes have highlighted the W chromosomes and heterochromatin bodies in *B.*
395 *mori* [45,46], there is no doubt that the bodies consist of the W chromosomes.
396 The diagnosis however retains unreliable if a species of interest carries a
397 W–autosomal translocation and/or partial deletion of the W [47,48]. Hiroki et al.

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398 [10] as well as Narita et al. [12] relied on the W-body diagnosis for C and CF
399 females and concluded that they have WZ and ZZ sex chromosome
400 constitutions, respectively. However, Kern et al. [13] has recently found that, on
401 the basis of genomic qPCR designed to amplify Z-linked gene sequences (*Tpi*
402 and *Ket*) relative to an autosomal gene (*EF-1α*), both CF and C females have
403 only one Z chromosome while males have two Z chromosomes. This finding
404 rejected the previous conclusion that the sex chromosome constitution of CF
405 females is ZZ [10,12] but was inconclusive about whether CF females have a Z0
406 or W'Z system (with W' as a modified W that has lost the feminization function
407 and cannot be detected by the W-body assay). Hence we carried out more
408 extensive chromosome analysis (other than just the W-body) to directly prove
409 whether CF females carry the W or not.

410 In Lepidoptera, the W chromosome can be highlighted by FISH using
411 probes prepared from whole genomic DNA of males or females. The capablity of
412 FISH probes in detecting the W chromosome is due to the numerous repetitive
413 short sequences occupying the W chromosome, which is then prone to be
414 hybridized by random sequences. Genomic probes also paint repetitive regions
415 scattered across other chromosomes, albeit at a lower density (autosomes and
416 Z chromosome). Here we made mitotic and pachytene chromosome
417 preparations from wing discs and gonads, respectively, in the last instar larvae of
418 C and CF individuals of *E. mandarina* (see [49] for details). Genomic DNA was
419 extracted from tet-treated C female larvae. Insect telomeric repeats were
420 amplified by non-template PCR [50]. *Kettin* (*Ket*) gene fragments were amplified
421 from adult cDNA synthesized by PrimeScript™ RT reagent Kit (TaKaRa, Otsu,

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422 Japan) and cloned by TOPO® TA Cloning® Kit (Thermo Fisher Scientific,
423 Waltham, MA). We used 4 pairs of primers, Em_kettin_F1:
424 5'–AGGTAATCCAACGCCAGTCG–3' and Em_kettin_R1:
425 5'–TGCTTGCCCTAAGGCATTGT–3', Em_kettin_F2:
426 5'–ACAATGCCTTAGGGCAAGCA–3' and Em_kettin_R2:
427 5'–TGGGCAAAGCCTCTTCATGT–3', Em_kettin_F3:
428 5'–AGATTCCGCACTACGCATGA–3' and Em_kettin_R3:
429 5'–TAAATTGTGGTGGGACGGCA–3', Em_kettin_F5:
430 5'–ACATGAAGAGGCTTTGCCCA–3' and Em_kettin_R5:
431 5'–TCATGCGTAGTGCGGAATCT–3', for PCR amplification with 94°C for 5 min
432 followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 3 min finalized
433 by 72°C for 10 min. Probe labeling was done by using the Nick Translation Kit
434 (Abbott Molecular, Des Plaines, IL). We selected Green-dUTP, Orange-dUTP
435 (Abbott Molecular Inc.) and Cy5-dUTP (GE Healthcare Japan, Tokyo)
436 fluorochromes for genomic DNA, *Ket* and insect telomeric repeat (TTAGG)*n*
437 probes respectively. Hybridizations were carried out according to protocols
438 described elsewhere [49]. Signal and chromosome images were captured with a
439 DFC350FX CCD camera mounted on a DM 6000B microscope (Leica
440 Microsystems Japan, Tokyo) and processed with Adobe Photoshop CS2. We
441 applied green, red and yellow pseudocolors to signals from Green, Orange and
442 Cy5 respectively.
443
444 **Quantitative polymerase chain reaction (qPCR)**
445 Embryos of mated females were sampled 48 h after the oviposition and stored at

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446 –80°C until DNA extraction. Embryos were individually subjected to DNA
447 extraction using DNeasy® Blood & Tissue Kit (Qiagen, Tokyo, Japan). Real-time
448 fluorescence detection quantitative PCR (qPCR) was performed using SYBR
449 Green and a LightCycler® 480 System (Roche Diagnostics K.K., Tokyo, Japan).
450 Z-linked *Tpi* was amplified using TPI-F (5'–GGCCTCAAGGTCATTGCCTGT–3')
451 and TPI-R (5'–ACACGACCTCCTCGGTTTTACC–3'), Z-linked *Ket* was amplified
452 using Ket-F (5'–TCAGTTAAGGCTATTAACGCTCTG–3') and Ket-R
453 (5'–ATACTACCTTTTGCGGTTACTGTC–3'), and autosomal *EF-1α* was
454 amplified using EF-1F (5'–AAATCGGTGGTATCGGTACAGTGC–3') and EF-1R
455 (5'–ACAACAATGGTACCAGGCTTGAGG–3') [13]. For each qPCR, a standard
456 dilution series of PCR products ($10^8$, $10^7$, $10^6$, $10^5$, $10^4$ and $10^3$ copies per
457 microliter) was included in order to estimate the absolute copy numbers of the
458 target sequence in the samples. To prepare standard samples, PCR products
459 were gel-excised and purified by Wizard® SV (Promega). Copy numbers of the
460 standard samples were estimated by the concentration measured by a
461 spectrophotometer, considering that the molecular weight of a nucleotide is 309
462 g/mol. For each qPCR, two replicates were performed that delivered similar
463 results. All qPCRs were performed using a temperature profile of 40 cycles of
464 95°C for 5 s, 60°C for 10 s, and 72°C for 10 s. The qPCR data were analyzed by
465 the Absolute Quantification analysis using the Second Derivative Maximum
466 method implemented in the LightCycler® 480 Instrument Operator Software
467 Version 1.5 (Roche).
468

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469 **RT-PCR**

470 RNA was extracted from adult abdomens that were stored at -80°C using
471 RNeasy® Mini Kit (Qiagen, Tokyo, Japan). The cDNA synthesized by using
472 Superscript™ III (Invitrogen) and Oligo(dT) was used as a template for RT-PCR.
473 A partial sequence of *dsx* which contains alternative splicing sites was amplified
474 using a primer pair, E520F (5'–GCAACGACCTCGACGAGGCTTCGCGGA–3')
475 and EhdsxR4 (5'–AGGGGCAGCCAGTGCGACGCGTACTCC–3') and a
476 temperature profile of 94°C for 2 min, 30 cycles of 94°C for 1 min, 57°C for 1 min
477 and 72°C for 1 min 30 s, followed by 72°C for 7 min. The sequences of seven
478 $dsx^F$ isoforms and a $dsx^M$ isoform were deposited in DDBJ/EMBL/Genbank
479 (LC215389-LC215396).

480

481

482 **Acknowledgements**

483 We thank Isao Kobayashi for help in collecting butterflies and Ranjit Kumar
484 Sahoo for stimulating discussion. A part of this study is supported by NIAS
485 technical support system at NARO.

486

487 **Additional information**

488 **Competing interests**

489 The authors declare no conflict of interest.

490

491 **Funding**

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| **Funder** | **Grant reference number** | **Author** |
|---|---|---|
| Japan Society for the Promotion of Science | 16K08106 | Daisuke Kageyama<br>Ken Sahara |
| Japan Society for the Promotion of Science | 16H05050 | Ken Sahara |

The funder had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

492

493 **Author contributions**

494 DK, KS, designed the research; DK, MO, TS, AY, TK, SK, HK, YK, SN, MM, MR,
495 KS, performed the research; DK, AJ, KS, analyzed the data; DK, MR, KS, wrote
496 the paper with input from AY.

497

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502

503 **References**

504 1. Burt A, Trivers R. *Genes in Conflict: the Biology of Selfish Genetic Elements.*
505 Cambridge, MA: Harvard University Press; 2006.
506 2. Werren JH. Selfish genetic elements, genetic conflict, and evolutionary
507 innovation. *PNAS* 2011;108:10863–10870. doi:

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508 10.1073/pnas.1102343108

509 3. Jaenike J. Sex chromosome meiotic drive. *Annu. Rev. Ecol. Syst.*
510 2001;32:25–49. doi: 10.1146/annurev.ecolsys.32.081501.113958

511 4. Lindholm AK, Dyer KA, Firman RC, Fishman L, Forstmeier W, Holman L, et al.
512 The ecology and evolutionary dynamics of meiotic drive. *Trends Ecol. Evol.*
513 2016;31:315–326. doi: 10.1016/j.tree.2016.02.001

514 5. Hurst LD. The incidences mechanisms and evolution of cytoplasmic sex ratio
515 distorters in animals. *Biol. Rev.* 1993;68:121–194. doi:
516 10.1111/j.1469-185X.1993.tb00733.x

517 6. Beukeboom LW, Perrin N. *The Evolution of Sex Determination.* Oxford
518 University Press; 2014.

519 7. Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of
520 invertebrate biology. *Nat. Rev. Microbiol.* 2008;6:741–751. doi:
521 10.1038/nrmicro1969

522 8. Hiroki M, Ishii Y, Kato Y. Variation in the prevalence of cytoplasmic
523 incompatibility-inducing *Wolbachia* in the butterfly *Eurema hecabe* across
524 the Japanese archipelago. *Evol. Ecol. Res.* 2005;7:931–942.

525 9. Narita S, Nomura M, Kato Y, Fukatsu T. Genetic structure of sibling butterfly
526 species affected by *Wolbachia* infection sweep: evolutionary and
527 biogeographical implications. *Mol. Ecol.* 2006;15:1095–1108. doi:
528 10.1111/j.1365-294X.2006.02857.x

529 10. Hiroki M, Kato Y, Kamito T, Miura K. Feminization of genetic males by a
530 symbiotic bacterium in a butterfly, *Eurema hecabe* (Lepidoptera: Pieridae).
531 *Naturwissenschaften* 2002;89:167–170. doi: 10.1007/s00114-002-0303-5

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532 11. Hiroki M, Tagami Y, Miura K, Kato Y. Multiple infection with *Wolbachia*
533 inducing different reproductive manipulations in the butterfly *Eurema*
534 *hecabe*. *Proc. Biol. Sci.* 2004;271:1751–1755. doi:
535 10.1098/rspb.2004.2769
536 12. Narita S, Kageyama D, Nomura M, Fukatsu T. Unexpected mechanism of
537 symbiont-induced reversal of insect sex: feminizing *Wolbachia*
538 continuously acts on the butterfly *Eurema hecabe* during larval
539 development. *Appl. Environ. Microbiol.* 2007;73:4332–4341. doi:
540 10.1128/AEM.00145-07
541 13. Kern P, Cook JM, Kageyama D, Riegler M. Double trouble: combined action
542 of meiotic drive and *Wolbachia* feminization in *Eurema* butterflies. *Biol.*
543 *Lett.* 2015;11:20150095. doi: 10.1098/rsbl.2015.0095
544 14. Bopp D, Saccone G, Beye M. Sex determination in insects: Variations on a
545 common theme. *Sex. Dev.* 2014;8:20–28. doi: 10.1159/000356458
546 15. Ohbayashi F, Suzuki MG, Mita K, Okano K, Shimada T. A homologue of the
547 *Drosophila doublesex* gene is transcribed into sex-specific mRNA isoforms
548 in the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol. B Biochem. Mol.*
549 *Biol.* 2001;128:145–158. doi: 10.1016/S1096-4959(00)00304-3
550 16. Pardo-Manuel De Villena F, Sapienza C. Nonrandom segregation during
551 meiosis: The unfairness of females. *Mamm. Genome* 2001;12:331–339.
552 doi: 10.1007/s003350040003
553 17. Doncaster L. On an inherited tendency to produce purely female families in
554 *Abraxas grossulariata*, and its relation to an abnormal chromosome
555 number. *J. Genet.* 1913;3:1–10. doi: 10.1007/BF02981560

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556 18. Doncaster L. On the relations between chromosomes, sex-limited
557 transmission and sex determination in *Abraxas grossulariata*. *J. Genet.*
558 1914;4:1–21. doi: 10.1007/BF02981560
559 19. Doncaster L. The Relation between chromosomes and sex-determination in
560 "*Abraxas grossulariata*." *Nature* 1915;95:395. doi: 10.1038/095395a0
561 20. Doncaster L. Further observations on chromosomes and sex-determination
562 in *Abraxas grossulariata*. *Q. J. Microsc. Sci.* 1922;66:397–406. doi:
563 10.1038/095395a0
564 21. Traut W, Sahara K, Marec F. Sex chromosomes and sex determination in
565 Lepidoptera. *Sex Dev.* 2007;1:332–346. doi: 10.1159/000111765
566 22. Sahara K, Yoshido A, Traut W. Sex chromosome evolution in moths and
567 butterflies. *Chromosom. Res.* 2012;20:83–94. doi:
568 10.1007/s10577-011-9262-z
569 23. Kiuchi T, Koga H, Kawamoto M, Shoji K, Sakai H, Arai Y, et al. A single
570 female-specific piRNA is the primary determiner of sex in the silkworm.
571 *Nature* 2014;509:4–6. doi: 10.1038/nature13315
572 24. Kageyama D, Traut W. Opposite sex-specific effects of *Wolbachia* and
573 interference with the sex determination of its host *Ostrinia scapulalis*. *Proc.*
574 *Biol. Sci.* 2004;271:251–258. doi: 10.1098/rspb.2003.2604
575 25. Sugimoto TN, Ishikawa Y. A male-killing *Wolbachia* carries a feminizing
576 factor and is associated with degradation of the sex-determining system of
577 its host. *Biol. Lett.* 2012;8:412–415. doi: 10.1098/rsbl.2011.1114
578 26. Fukui T, Kawamoto M, Shoji K, Kiuchi T, Sugano S, Shimada T, et al. The
579 endosymbiotic bacterium *Wolbachia* selectively kills male hosts by

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580 targeting the masculinizing gene. *PLoS Pathog.* 2015;11:1–14. doi:
581 10.1371/journal.ppat.1005048
582 27. Sugimoto TN, Kayukawa T, Shinoda T, Ishikawa Y, Tsuchida T. Misdirection
583 of dosage compensation underlies bidirectional sex-specific death in
584 *Wolbachia*-infected *Ostrinia scapulalis*. *Insect Biochem. Mol. Biol.*
585 2015;66:72–76. doi: 10.1016/j.ibmb.2015.10.001
586 28. Giorgini M, Monti MM, Caprio E, Stouthamer R, Hunter MS. Feminization
587 and the collapse of haplodiploidy in an asexual parasitoid wasp harboring
588 the bacterial symbiont *Cardinium*. *Heredity* 2009;102:365–371. doi:
589 10.1038/hdy.2008.135
590 29. Ma W-J, Pannebakker B a., van de Zande L, Schwander T, Wertheim B,
591 Beukeboom LW. Diploid males support a two-step mechanism of
592 endosymbiont-induced thelytoky in a parasitoid wasp. *BMC Evol. Biol.*
593 2015;15:84. doi: 10.1186/s12862-015-0370-9
594 30. Dyson EA, Kamath MK, Hurst GDD. *Wolbachia* infection associated with
595 all-female broods in *Hypolimnas bolina* (Lepidoptera: Nymphalidae):
596 evidence for horizontal transmission of a butterfly male killer. *Heredity*
597 2002;88:166–171. doi: 10.1038/sj.hdy.6800021
598 31. Harumoto T, Anbutsu H, Lemaitre B, Fukatsu T. Male-killing symbiont
599 damages host's dosage-compensated sex chromosome to induce
600 embryonic apoptosis. *Nat. Commun.* 2016;7:12781. doi:
601 10.1038/ncomms12781
602 32. Yoshido A, Marec F, Sahara K. The fate of W chromosomes in hybrids
603 between wild silkmoths, *Samia cynthia* ssp.: no role in sex determination

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604 and reproduction. *Heredity* 2016;116:424–433. doi: 10.1038/hdy.2015.110
605 33. Rigaud T, Juchault P, Mocquard J-P. The evolution of sex determination in
606 isopod crustaceans. *BioEssays* 1997;19:409–416. doi:
607 10.1002/bies.950190508
608 34. Leclercq S, Thézé J, Chebbi MA, Giraud I, Moumen B, Ernenwein L, et al.
609 Birth of a W sex chromosome by horizontal transfer of *Wolbachia* bacterial
610 symbiont genome. *PNAS* 2016;113:201608979. doi:
611 10.1073/pnas.1608979113
612 35. Hatcher MJ, Taneyhill DE, Dunn AM, Tofts C. Population dynamics under
613 parasitic sex ratio distortion. *Theor. Pop. Biol.* 1999;56:11–28. doi:
614 10.1006/tpbi.1998.1410
615 36. Charlat S, Hornett EA, Fullard JH, Davies N, Roderick GK, Wedell N, et al.
616 Extraordinary flux in sex ratio. *Science* 2007;317:214. doi:
617 10.1126/science.1143369
618 37. Majerus TMO, Majerus MEN. Intergenomic arms races: Detection of a
619 nuclear rescue gene of male-killing in a ladybird. *PLoS Pathog.*
620 2010;6:1–7. doi: 10.1371/journal.ppat.1000987
621 38. Braig HR, Zhou W, Dobson SL, O'Neill SL. Cloning and characterization of a
622 gene encoding the major surface protein of the bacterial endosymbiont
623 *Wolbachia pipientis*. *J. Bacteriol.* 1998;180:2373–2378. doi:
624 10.1099/0022-1317-69-1-35
625 39. Narita S, Nomura M, Kageyama D. Naturally occurring single and double
626 infection with *Wolbachia* strains in the butterfly *Eurema hecabe*:
627 transmission efficiencies and population density dynamics of each

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628 *Wolbachia* strain. *FEMS Microbiol. Ecol.* 2007;61:235–245. doi:
629 10.1111/j.1574-6941.2007.00333.x
630 40. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina
631 sequence data. *Bioinformatics* 2014;30:2114–2120. doi:
632 10.1093/bioinformatics/btu170
633 41. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat.*
634 *Meth.* 2012;9:357–359. doi: 10.0.4.14/nmeth.1923
635 42. Simpson JT, Durbin R. Efficient de novo assembly of large genomes using
636 compressed data structures. *Genome Res.* 2012;22:549–556. doi:
637 10.1101/gr.126953.111
638 43. Suetsugu Y, Futahashi R, Kanamori H, Kadono-Okuda K, Sasanuma S,
639 Narukawa J, et al. Large scale full-length cDNA sequencing reveals a
640 unique genomic landscape in a lepidopteran model insect, *Bombyx mori*.
641 *G3 Genes|Genomes|Genetics* 2013;3:1481–1492. doi:
642 10.1534/g3.113.006239
643 44. Jiggins CD, Linares M, Naisbit RE, Salazar C, Yang ZH, Mallet J. Sex-linked
644 hybrid sterility in a butterfly. *Evolution* 2001;55:1631–1638. doi:
645 10.1111/j.0014-3820.2001.tb00682.x
646 45. Sahara K, Marec F, Eickhoff U, Traut W. Moth sex chromatin probed by
647 comparative genomic hybridization (CGH). *Genome* 2003;46:339–342.
648 doi: 10.1139/g03-003
649 46. Sahara K, Yoshido A, Kawamura N, Ohnuma A, Abe H, Mita K, et al.
650 W-derived BAC probes as a new tool for identification of the W
651 chromosome and its aberrations in *Bombyx mori*. *Chromosoma*

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652 2003;112:48–55. doi: 10.1007/s00412-003-0245-5

653 47. Traut W, Marec F. Sex chromatin in Lepidoptera. *Q. Rev. Biol.*
654 1996;71:239–56. doi: 10.1086/419371

655 48. Abe H, Fujii T, Tanaka N, Yokoyama T, Kakehashi H, Ajimura M, et al.
656 Identification of the female-determining region of the W chromosome in
657 *Bombyx mori*. *Genetica* 2008;133:269–282. doi:
658 10.1007/s10709-007-9210-1

659 49. Yoshido A, Sahara K, Yasukochi Y. Chapter 6; Silkmoths (Lepidoptera). In:
660 Sharakhov I, editor. *Protocols for Cytogenetic Mapping of Arthropod*
661 *Genomes.* Boca Raton, U.S.A.: CRC Press; 2014. p. 219–256.

662 50. Sahara K, Marec F, Traut W. TTAGG telomeric repeats in chromosomes of
663 some insects and other arthropods. *Chromosom. Res.* 1999;7:449–460.
664 doi: 10.1023/A:1009297729547

665

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666 **Figure legends**

667

668 **Figure 1.** *E. mandarina* butterflies used in this study. (**A**) A photo of *E.*
669 *mandarina* taken in Tanegashima Island. (**B**) Characteristics of three types of *E.*
670 *mandarina* individuals inhabiting Tanegashima Island.

671

672 **Figure 2.** Fluorescence *in-situ* hybridization and sequence read counts for a C
673 female, C male, and CF female *E. mandarina*. **A**–**C**: Mitotic complements
674 hybridized with a genomic probe (green; green arrows) and a Z-linked *Ket* probe
675 (red; red arrows) in a C female (2n = 62) (**A**), C male (2n = 62) (**B**), and CF
676 female (2n = 61) (**C**). **D**–**I**: Genomic *in situ* hybridization (GISH) and FISH with a
677 Z-linked *Ket* probe performed on interphase nuclei of *E. mandarina* C females (**D**,
678 **E**), C males (**F**, **G**), and CF females (**H**, **I**). **J**–**L**: GISH, telomere-FISH and FISH
679 with *Ket* probe performed on pachytene complements of *E. mandarina* C
680 females (**G**, n = 31), C males (**H**, n = 31), and CF females (**I**, n = 31). Green paint
681 signals in **A**, **E** and **J** revealed that C females have the W chromosome. The *Ket*
682 probe signals (red) appeared on the Z pairing to the W in C females (**J**), the ZZ
683 bivalent in C males (**K**), and the Z univalent of CF females (**L**). The single signals
684 were observed both in C and CF female nuclei. The signals in C females (**J**) and
685 males (**K**) clearly showed their respective WZ and ZZ chromosome sets, and a
686 Z0 chromosome set in CF females (**L**). W: W chromosome; Z: Z chromosome;
687 white arrows: *Wolbachia*-like structures. A bar represents 10 μm. **M**–**O**: Relative
688 normalized sequence read counts in CF females, C females, and C males for 67
689 contigs homologous to *Bombyx mori* loci on chromosome 1 (Z chromosome; **M**),

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690 28 contigs homologous to *B. mori* loci on chromosome 4 (**N**), and 33 contigs
691 homologous to *B. mori* loci on chromosome 16 (**O**), with relative read counts set
692 to 1 (males). Details about genome sequencing are provided in Materials and
693 Methods.

694

695 **Figure 3.** Effects of *w*Fem on Z-linked gene dose in *E. mandarina* offspring. (**A**)
696 Estimate of the gene dose of *Ket* (relative gene copies per copy of *EF-1α*) by
697 genomic quantitative polymerase chain reaction (qPCR) analysis in each of the
698 fertilized eggs laid by C females, CF females, and tetracycline (tet)-treated CF
699 females. Each colored circle represents a single fertilized egg. Sample sizes are
700 given in parentheses. (**B**) Offspring sex ratio of five females tet-treated prior to
701 oviposition and three non-treated CF females. Numbers to the left of the arrows
702 represent duration (days) of tet treatment. Blue dots and red dots represent
703 males and females, respectively. (**C**) Estimate of the gene dose of *Ket* (relative
704 gene copies per copy of *EF-1α*) by genomic qPCR in each of the adult offspring
705 produced by CF females that were tet-treated during the adult stage (prior to
706 oviposition). Each circle represents an adult offspring. Z chromosomes of these
707 offspring individuals were genotyped as $Z^A$, $Z^B$, $Z^C$ or $Z^D$ on the basis of intron
708 nucleotide sequence of Z-linked *Tpi*. The green arrow points to a male individual
709 (adult) whose karyotype was considered to be Z0 but possibly ZZ' (see text for
710 details). f: female, m: male.

711

712 **Figure 4.** Effects of *w*Fem on splicing of the *doublesex* gene in *E. mandarina*.
713 (**A**) Reverse-transcription polymerase chain reaction (RT-PCR) products of *E.*

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714 *mandarina doublesex* (*Emdsx*) run on an agarose gel. Lane 1: C female; lane 2:
715 C male; lanes 3 and 4: CF females; lanes 5 and 6: intersexes generated by
716 tetracycline (tet) treatment of larvae produced by CF females; lane 7: 100-bp
717 ladder. Females have at least seven splicing products, whereas males have a
718 single product. (**B**) Structures of the splicing products of *Emdsx*. Translated
719 regions are indicated by red and blue bars, untranslated regions by gray bars,
720 and stop codons by triangles. Numbers of clones obtained by cloning the
721 RT-PCR products are shown in the table on the right. **C**–**H**: color and
722 morphology of forewings. Females are pale yellow on the dorsal side of the
723 forewings (**C**) and do not have sex brand on the ventral side of the forewings (**F**),
724 while males are intense yellow on the dorsal side of the forewings (**D**) and have
725 sex brand on the ventral side of the forewings (**G**). Many of the intersexes
726 generated by tet-treating CF larvae are strong yellow on the dorsal side of the
727 forewings (**E**) and have faint sex brand on the ventral side of the forewings (**H**).
728

729 **Figure 5.** (**A**) Schematic illustration of two alternative mechanistic models of
730 sex-chromosome segregation distortion that explain the observed data. The
731 "Selection against Z gametes" model assumes that Z-bearing gametes are
732 selected against during meiosis (left). The "Elimination of maternal Z" model
733 assumes that Z chromosomes are eliminated during or after normal meiosis,
734 while all the autosomes being intact (right). (**B**) All-female production explained
735 by *Wolbachia*–host interaction. Effects of *w*Fem on the development and sex
736 determination of *E. mandarina*, and outcomes of larval versus adult tet treatment
737 are illustrated. Asterisk: The majority of Z0 males die, but a few survived.

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738

739 **Figure 6.** Hypothetical evolutionary trajectory of the *Wolbachia*–host interaction

740 in *E. mandarina*. See Discussion for details.

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741 **Legends of figure supplements**

742

743 **Figure 1**

744 **Figure supplement 1.** Habitat of *E. mandarina* in Japanese archipelago. (**A**) In
745 this study, female adults of *E. mandarina* were collected on Tanegashima Island
746 (map), located ca. 40 km from the southern tip of Kyushu, Japan. Within *E.*
747 *mandarina*, the *Wolbachia* strain *w*CI is currently spreading northwards [8]
748 together with the mitochondrial haplotypes introgressed from a sibling species (*E.*
749 *hecabe*) by hybridization (hitchhiking effect; [9]). (**B**) On the basis of *Wolbachia*
750 infection status, *E. mandarina* females can be categorized into three groups:
751 uninfected females, C females (those singly infected with *w*CI), and CF females
752 (those doubly infected with *w*CI and *w*Fem). These designations and their
753 offspring sex ratio are summarized in the table. To date, in *E. mandarina*, CF
754 females have only been found on Okinawa-jima Island [10,11] and Tanegashima
755 Island [12,39].

756

757 **Figure 2**

758 **Figure supplement 1.** Relative normalized sequence read counts for 440
759 contigs of *E. mandarina* that matched to *B. mori* loci on 28 chromosomes. Means
760 and standard errors are shown for CF females and C females while those of C
761 males were set to 1.

762

763 **Figure 3**

764 **Figure supplement 1.** Estimate of Z-linked gene dose of *E. mandarina*.

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765 Estimate of the gene dose of *Ket* (top) and *Tpi* (bottom), relative gene copies per
766 *EF-1α*, by genomic qPCR in each of the fertilized eggs laid by C females, CF
767 females and tet-treated CF females. Each circle represents an egg. Each of the
768 codes along the x-axes indicate the brood produced by a single mother.
769 **Figure supplement 2.** Genotyping of Z chromosome based on nucleotide
770 polymorphism of *Tpi*. (**A**) Sequence polymorphism of *Tpi*. In our experiment, Z
771 chromosomes were categorized into four ($Z^A$, $Z^B$, $Z^C$ and $Z^D$) on the basis of *Tpi*
772 sequence. An en dash represents a gap. (**B**) Examples of genotyping based on
773 *Tpi* sequence data. Red triangles represent polymorphic sites. When $Z^B$ was
774 paired to $Z^A$, $Z^C$ or $Z^D$, sequence gaps resulted in ambiguity from the position 109
775 (shown with a red arrow).
776
777 **Figure 4**
778 **Figure supplement 1.** Detection of *Emdsx* in adults that were tet-treated during
779 various larval stages. The numbers of adults that failed to emerge from their
780 pupal cases are shown with gray.
781 **Figure supplement 2.** (**A**-**B**) Intersexual adults generated by feeding the CF
782 larvae with tet-containing diet. Their wings are often curled or crumpled. Most of
783 them are trembling and cannot stand still. (**C**) Normal females. Their wings are
784 neatly closed.
785 **Figure supplement 3.** (**A**) Amino acid sequences of female splice forms of *dsx*
786 genes derived from *Eurema mandarina* (*Emdsx*$^F$: LC215389) and other
787 lepidopteran species, *Lymantria dispar* (*Lddsx*$^F$: BAN82533), *Ostrinia scapulalis*
788 (*Osdsx*$^F$: BAJ25851) and *Bombyx mori* (*Bmdsx*$^F$: NP_001036871). (**B**) Amino

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789 acid sequences of male splice forms of *dsx* genes derived from *E. mandarina*
790 (*Emdsx*$^M$: LC215396), *L. dispar* (*Lddsx*$^M$: BAN82532), *O. scapulalis* (*Osdsx*$^M$:
791 BAJ25850), and *B. mori* (*Bmdsx*$^M$: AHF81625). (**C**) Unrooted NJ tree of the *dsx*
792 gene based on amino acid sequences. Em: *E. mandarina* (LC215389), Dp:
793 *Danaus plexippus* (EHJ78146), Px: *Papilio xuthus* (XP_013171086), Ob:
794 *Operophtera brumata* (KOB69684), Bm: *B. mori* (NP_001036871), Tv: *Trilocha*
795 *varians* (BAS02078), Amy: *Antheraea mylitta* (ADL40853), At: *Amyelois*
796 *transitella* (XP_013184257), Ha: *Helicoverpa armigera* (AHF81652), Of: *Ostrinia*
797 *furnacalis* (AHF81640), Ld: *L. dispar* (BAN82533), Am: *Apis mellifera*
798 (ABV55180), Nl: *Neodiprion lecontei* (XP_015517992), Ar: *Athalia rosae*
799 (XP_012262273), Cm: *Cyclommatus metallifer* (BAO23810), Td: *Trypoxylus*
800 *dichotomus* (BAM93344), Ot: *Onthophagus taurus* (AEX92939), Tc: *Tribolium*
801 *castaneum* (AFQ62107), Ag: *Anopheles gambiae* (XP_309601), Cq: *Culex*
802 *quinquefasciatus* (AJB28478), Aa: *Aedes aegypti* (ABD96571), Md: *Mayetiola*
803 *destructor* (AGW99160), So: *Sciara ocellaris* (CDN30082), Bc: *Bradysia*
804 *coprophila* (CDN30080).

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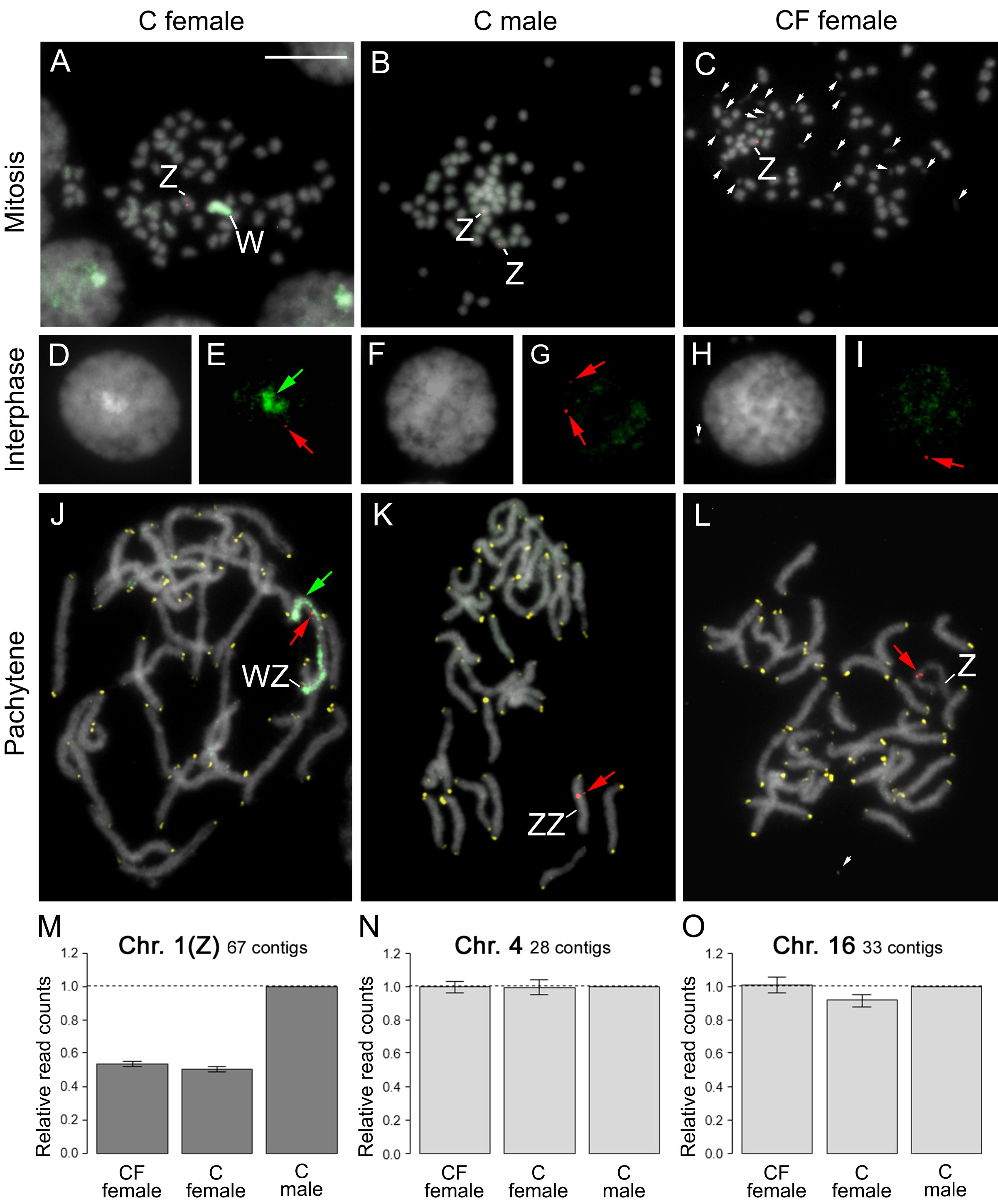
# FIGURE 1

A

B

| Type of individuals | *Wolbachia* | | W body | No. of Z | Predicted karyotype | Offspring sex ratio |
|---|---|---|---|---|---|---|
| | *w*Fem | *w*CI | | | | |
| C females | – | + | + | 1 | WZ | -1:1 |
| C males | – | + | – | 2 | ZZ | -1:1 by mating with C females<br>-All-female by mating with CF females |
| CF females | + | + | – | 1 | W'Z or Z0 | -All-female |

# FIGURE 2



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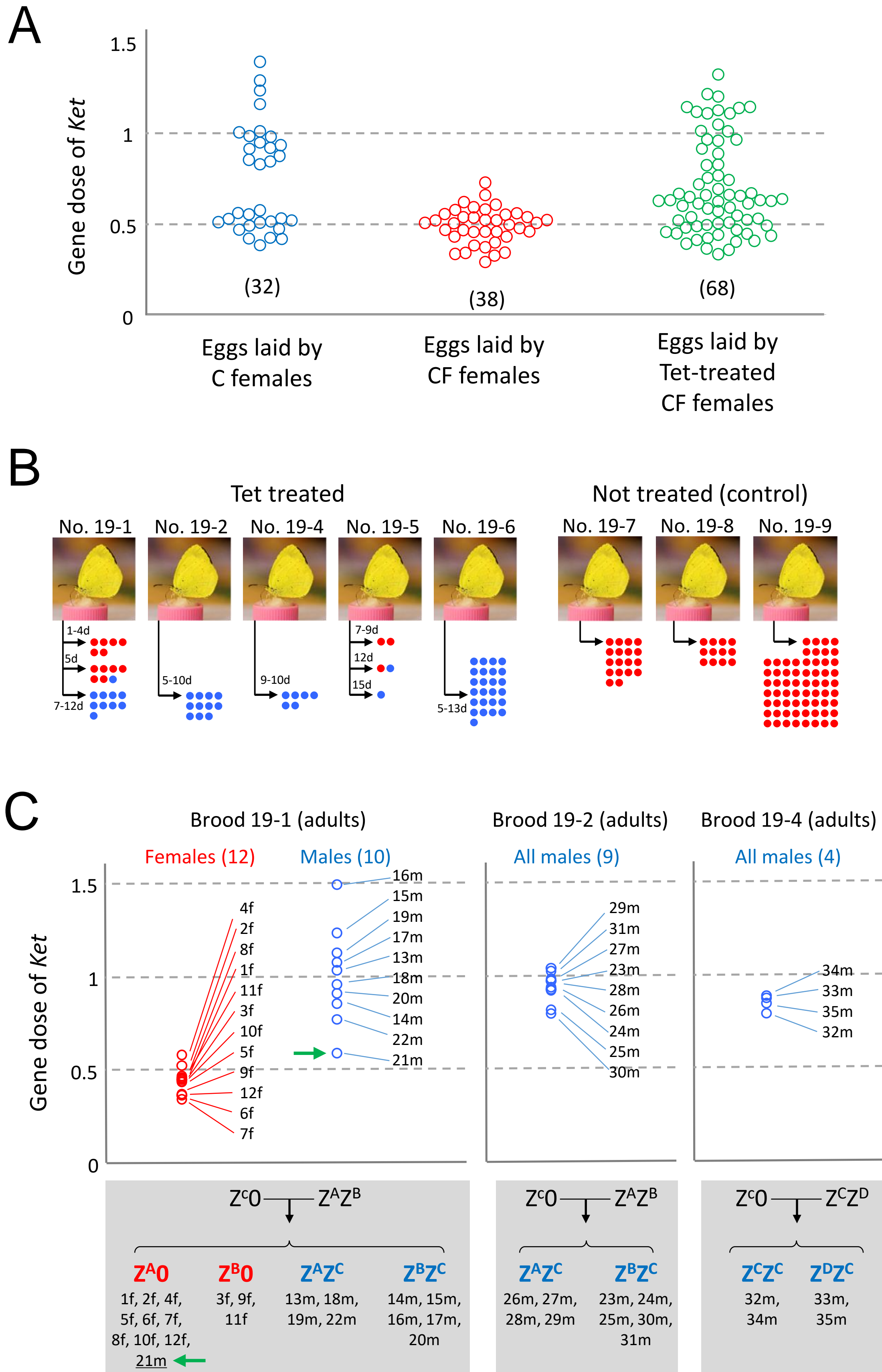
# FIGURE 3



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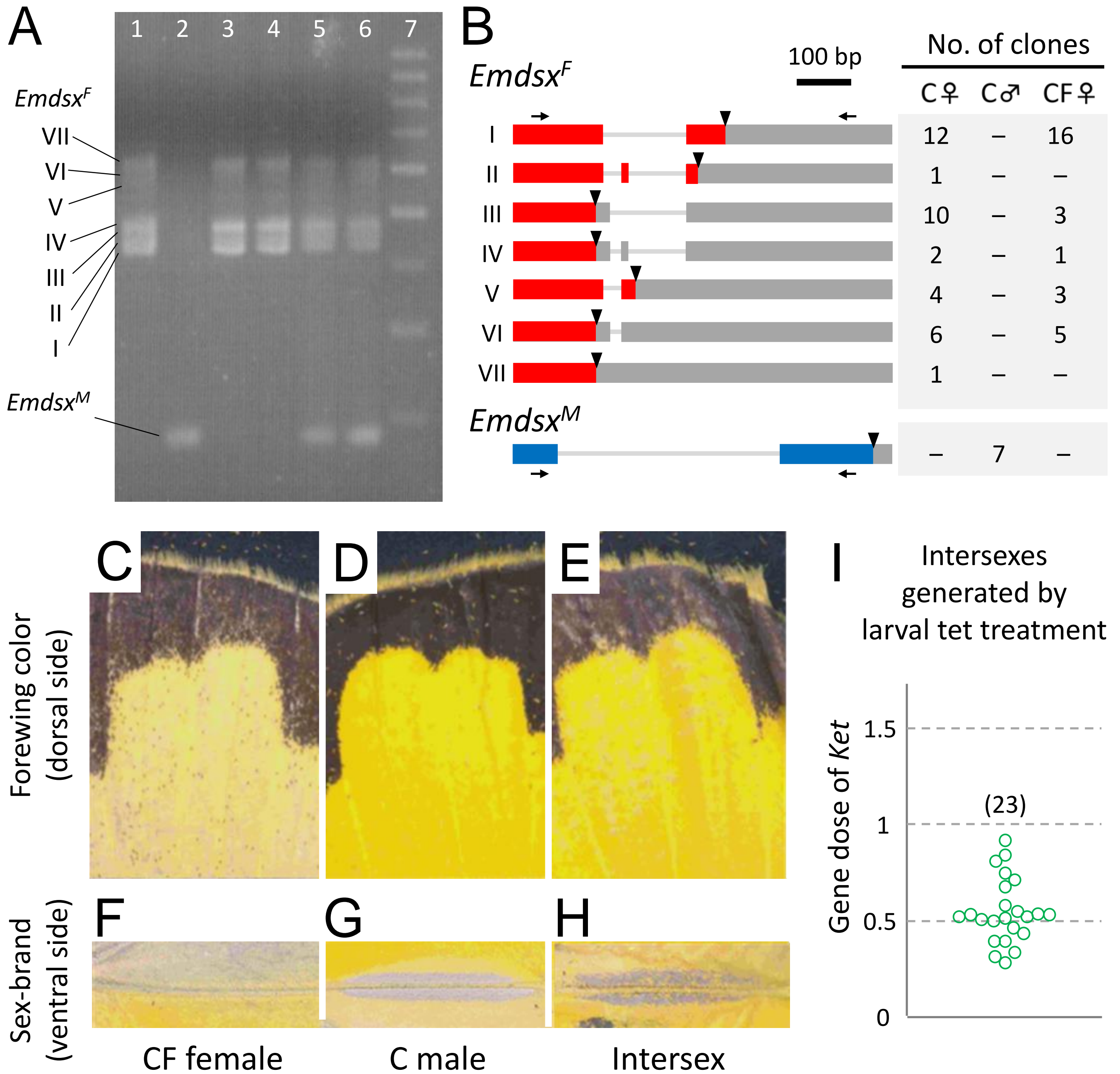
# FIGURE 4



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# FIGURE 5

A

Selection against Z-bearing gametes

Elimination of maternal Z

ZZ00

1st division

Z0

100%

0%

50%

50%

2nd division

0

Z

Production of 0 eggs

Production of 0 eggs

B

C matriline

CF matriline

WZ

Z0

adult tet

ZZ

50%

100%

Feminized by Wchr

Feminized by Wol

larval tet

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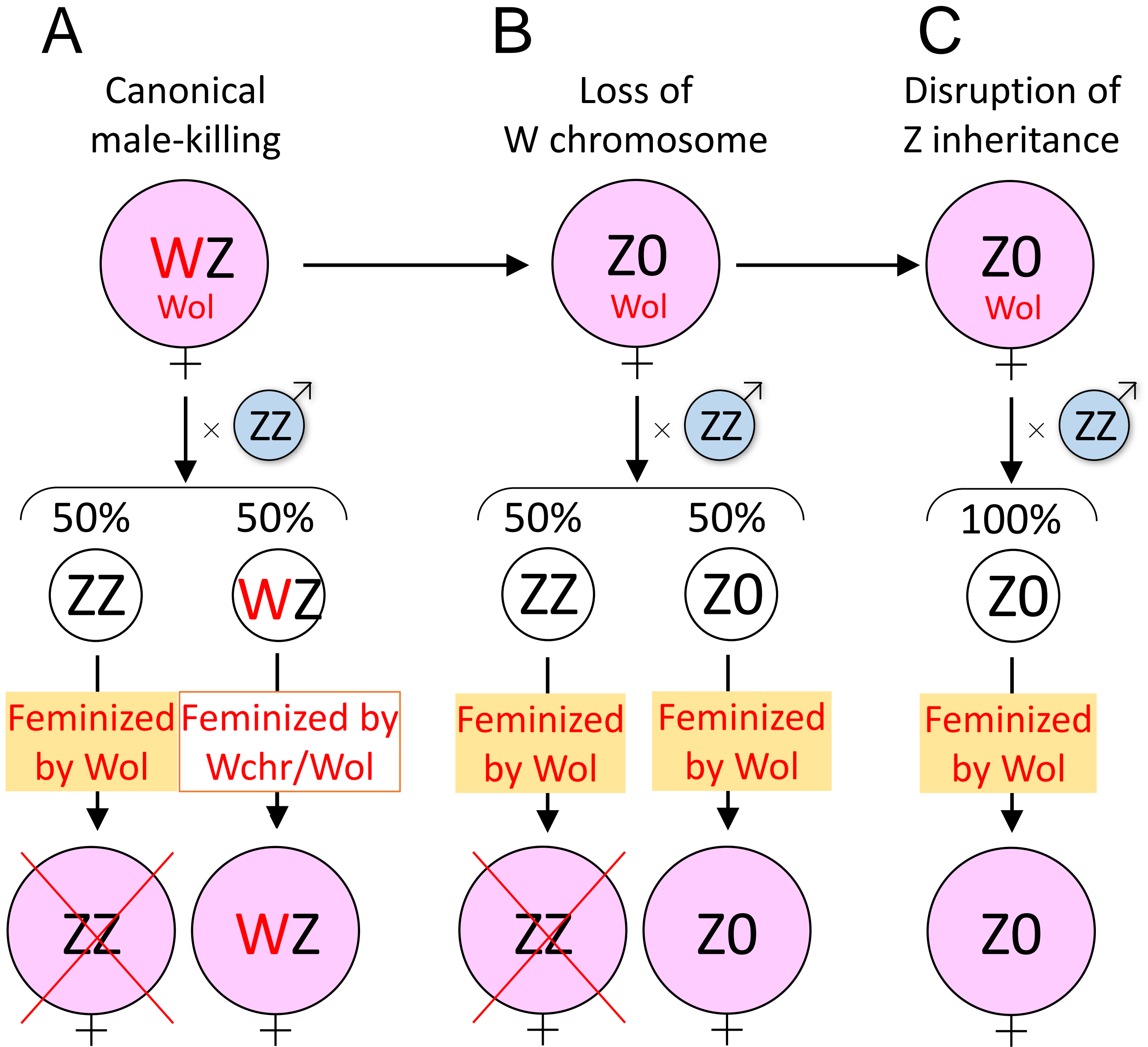
# FIGURE 6



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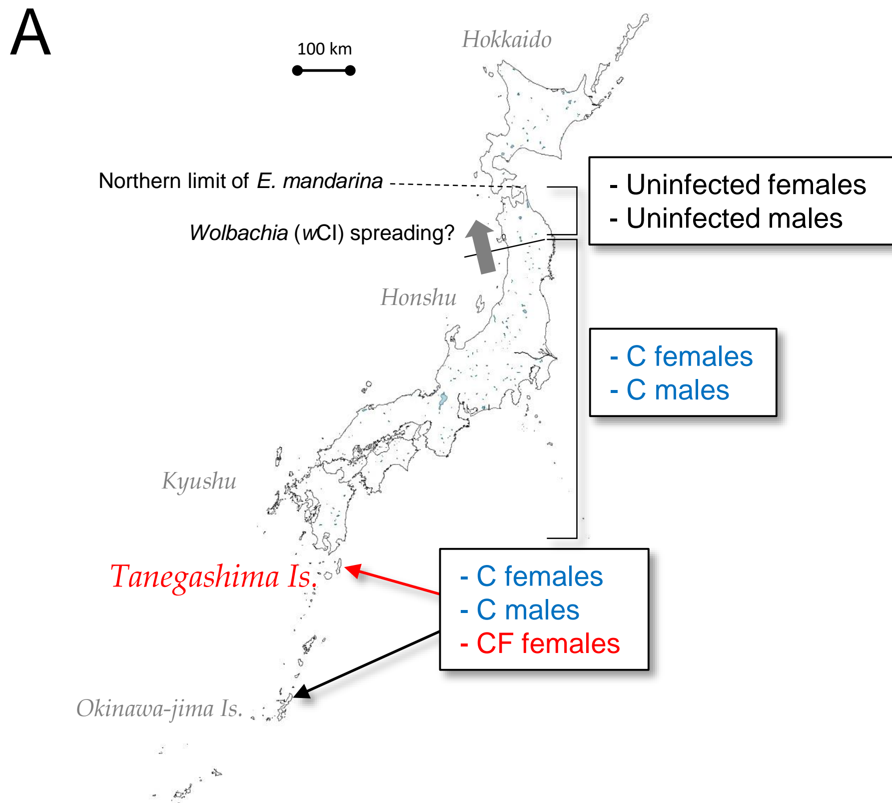
# FIGURE 1—figure supplement 1

A




B

| Type of individuals | *Wolbachia* infection status | Offspring sex ratio | Habitat |
|---|---|---|---|
| Uninfected females and males | – | 1:1 | Northern Honshu |
| C females and males | *w*CI | 1:1 | Everywhere except northern Honshu |
| CF females | *w*CI and *w*Fem | all-female | Found in Tanegashima and Okinawa-jima Islands |

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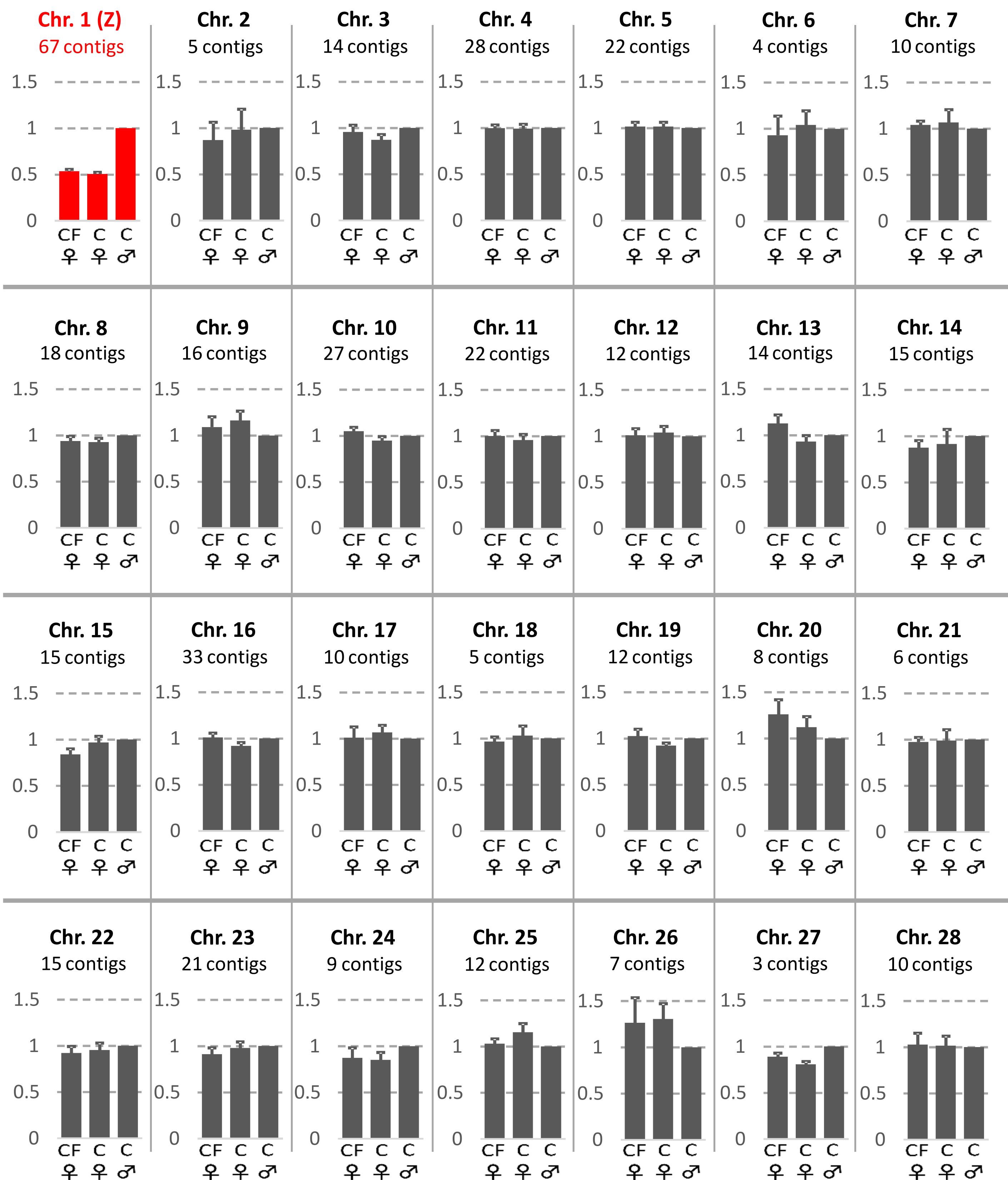
# FIGURE 2—figure supplement 1



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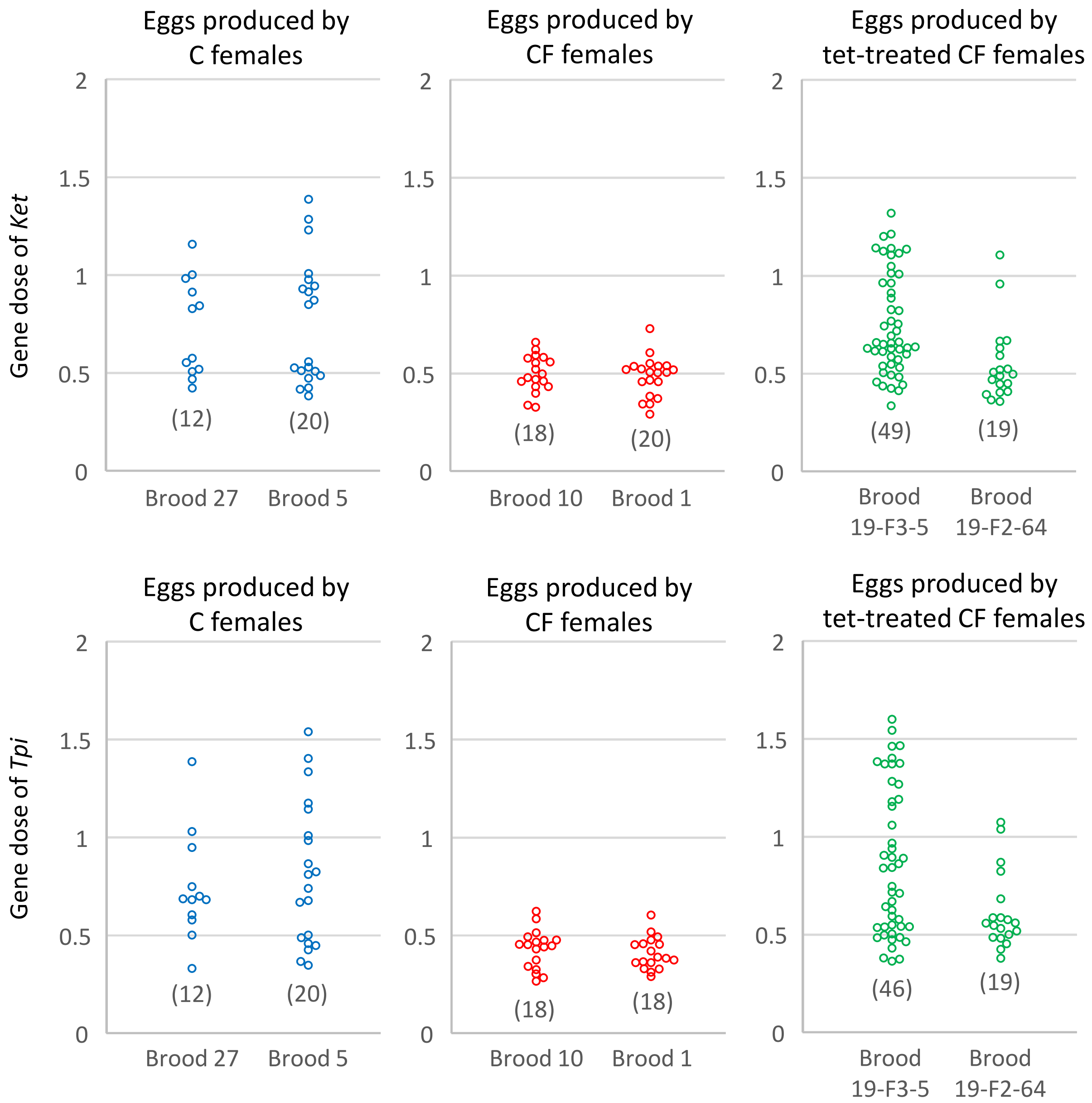
# FIGURE 3—figure supplement 1



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# FIGURE 3—figure supplement 2

## A Sequence polymorphism of Z-linked *Tpi* gene

| Genotype of Z chromosome | Polymorphic sites | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 109 | 110 | 111 | 112 | 135 | 157 | 160 | 173 | 175 | 228 | 233 | 324 | 403 | 425 |
| $Z^A$ | A | T | G | G | A | A | T | C | T | T | T | T | C | G |
| $Z^B$ | – | – | – | – | A | A | C | T | T | A | T | – | T | G |
| $Z^C$ | A | T | G | G | G | A | T | C | T | T | T | T | T | G |
| $Z^D$ | A | T | G | G | A | G | C | T | A | T | G | T | T | A |

## B Examples of genotyping of sex chromosomes based on sequence data

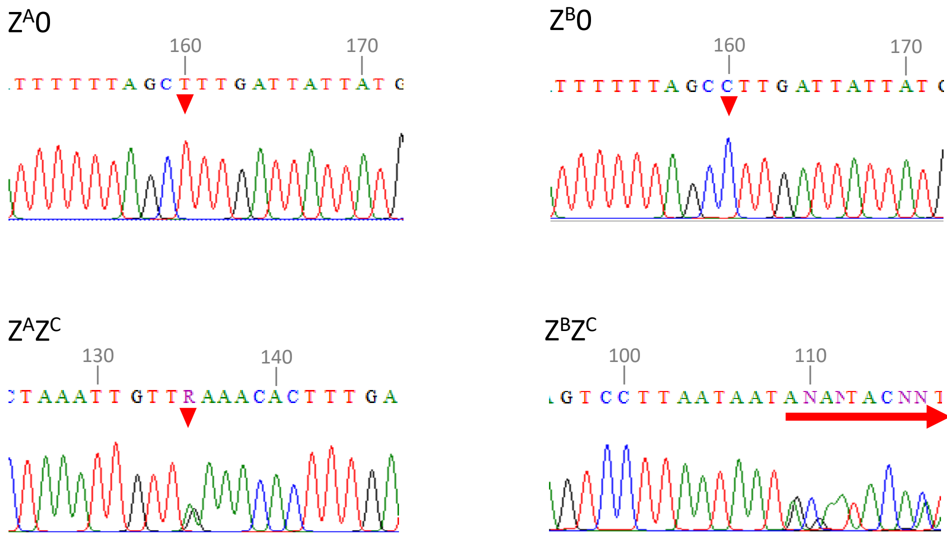


FIGURE 4—figure supplement 1

No. of adult offspring produced by CF females

A Non treated

50
40
30
20
10
0
*Emdsx*$^F$ | *Emdsx*$^F$ + *Emdsx*$^M$ | *Emdsx*$^M$

B Tet treated (during 4th to 5th instar larval stages )

5
4
3
2
1
0
*Emdsx*$^F$ | *Emdsx*$^F$ + *Emdsx*$^M$ | *Emdsx*$^M$

C Tet treated (during 3rd to 5th instar larval stages)

20
15
10
5
0
*Emdsx*$^F$ | *Emdsx*$^F$ + *Emdsx*$^M$ | *Emdsx*$^M$

D Tet treated (during 2nd to 5th instar larval stages)

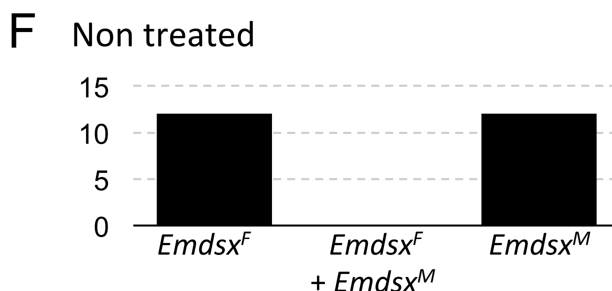
5
4
3
2
1
0
*Emdsx*$^F$ | *Emdsx*$^F$ + *Emdsx*$^M$ | *Emdsx*$^M$

E Tet treated (during 1st to 5th instar larval stages)

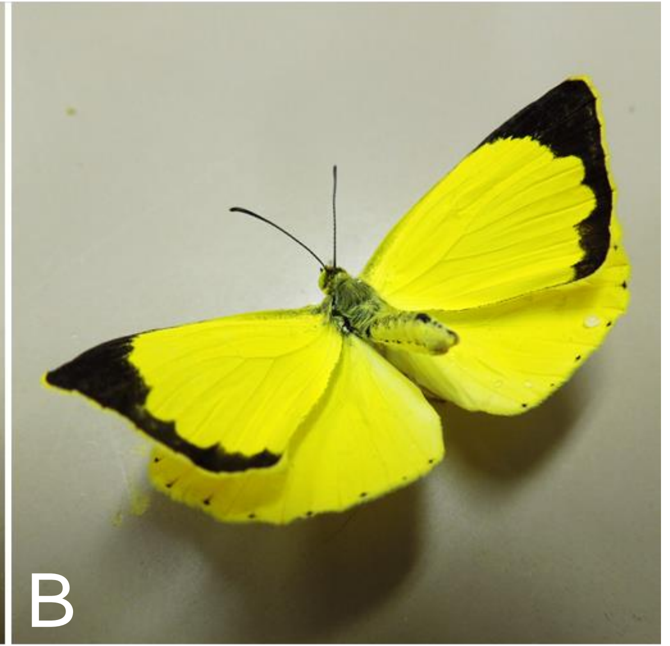
6
4
2
0
*Emdsx*$^F$ | *Emdsx*$^F$ + *Emdsx*$^M$ | *Emdsx*$^M$

No. of adult offspring produced by C females

F Non treated



# FIGURE 4—figure supplement 2



# FIGURE 4—figure supplement 3

A

B

C