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Evolutionary biology

Double trouble: combined action of meiotic drive and *Wolbachia* feminization in *Eurema* butterflies

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Arthropod sex ratios can be manipulated by a diverse range of selfish genetic elements, including maternally inherited *Wolbachia* bacteria. Feminization by *Wolbachia* is rare but has been described for *Eurema mandarina* butterflies. In this species, some phenotypic and functional females, thought to be ZZ genetic males, are infected with a feminizing *Wolbachia* strain, *w*Fem. Meanwhile, heterogametic WZ females are not infected with *w*Fem. Here, we establish a quantitative PCR assay allowing reliable sexing in three *Eurema* species. Against expectation, all *E. mandarina* females, including *w*Fem females, had only one Z chromosome that was paternally inherited. Observation of somatic interphase nuclei confirmed that W chromatin was absent in *w*Fem females, but present in females without *w*Fem. We conclude that the sex bias in *w*Fem lines is due to meiotic drive (MD) that excludes the maternal Z and thus prevents formation of ZZ males. Furthermore, *w*Fem lines may have lost the W chromosome or harbour a dysfunctional version, yet rely on *w*Fem for female development; removal of *w*Fem results in all-male offspring. This is the first study that demonstrates an interaction between MD and *Wolbachia* feminization, and it highlights endosymbionts as potentially confounding factors in MD of sex chromosomes.

1. Introduction

Selfish genetic elements can highjack sex determination systems and distort sex ratios in order to enhance their own transmission in host populations. Examples are meiotic drive (MD) genes of sex chromosomes [1] and endosymbiotic micro-organisms [2] such as *Wolbachia*, a maternally inherited bacterium of arthropods that can induce cytoplasmic incompatibility (CI), thelytokous parthenogenesis, male-killing (MK) and feminization. Feminization is least common and results in female development of individuals with an assumed male chromosome composition [3].

Wolbachia-induced feminization has been reported for several terrestrial crustaceans and is best described for the isopod *Armadillidium vulgare* with presumed heterogametic females (WZ). In this species, *Wolbachia* causes individuals to develop into functional females via manipulation of the androgenic gland [4]. Consequently, the frequency of the W chromosome in infected populations is expected to decline until its eventual elimination, such that female sex is controlled by the presence of *Wolbachia* rather than W, while males may develop when *Wolbachia* transmission is leaky [4].

Wolbachia-induced feminization has also been recorded for three insect species, including two *Eurema* butterfly species [5–7]. *Eurema mandarina* butterfly populations are nearly fixed for *w*CI infections. In some populations, females are co-infected with *w*Fem, a strain thought to cause feminization [6,8]. For example, on Tanegashima Island in Japan, most females harbour both strains and produce

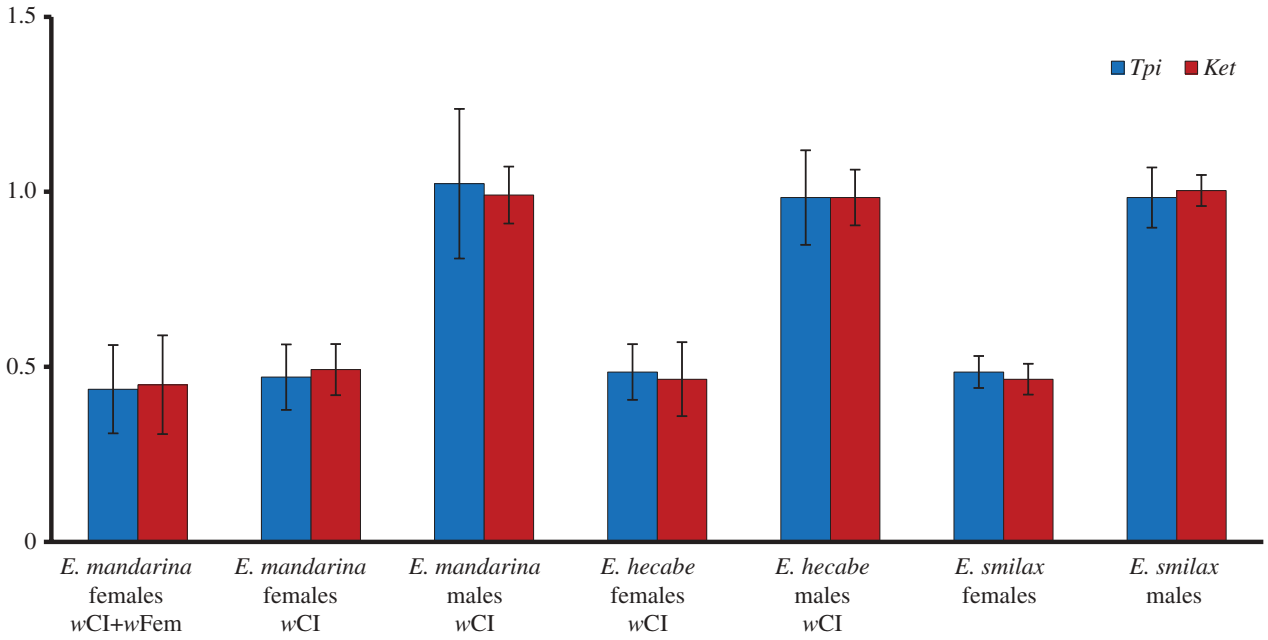

Figure 1. Gene dose ratio of Z-linked *Tpi* and *kettin* normalized to autosomal *Ef-1α* in *Eurema* individuals. Error bars represent s.e. (Online version in colour.)

only daughters with similar offspring numbers to the mixed sex broods produced by *w*CI females [8,9].

Lepidoptera are diplodiploid insects with female heterogamety—females are WZ or 0Z, males are ZZ. As in most Lepidoptera [10], the W chromosome of uninfected and *w*CI *E. mandarina* females forms a heterochromatic body during interphase of somatic cells [6]. However, in *w*Fem females this W chromatin is missing, which has led to the assumption that they have a male ZZ chromosome composition [6,9]. Both MK and MD have previously been excluded as mechanisms for the sex ratio bias, because antibiotic treatment of *w*Fem females did not change offspring numbers and did not restore even sex ratios, but yielded all-male broods [6]. Further antibiotic experiments provided evidence that *w*Fem has a continuous feminizing action on individuals during larval development [11]. Here, we scrutinized the genetic basis of the sex ratio bias in *E. mandarina*, and directly tested the hypothesized ZZ composition of *w*Fem females. We also compared the inheritance of Z in all-female and mixed-sex families to probe them for any segregation distortions.

2. Material and methods

(a) Sampling and *Wolbachia* screening

We tested 57 *E. mandarina* from Tanegashima produced by five and three field-collected mothers that produced all-female and mixed-sex broods, respectively. This number also included six tetracycline-treated individuals from one mixed-sex family. Controls were six *E. mandarina* from Hachijō-jima Island, Japan, as well as 10 individuals each of Australian *Eurema hecabe* and Australian *Eurema smilax*. *Wolbachia* infections were confirmed and sequenced by using strain-specific PCR primers [9] (electronic supplementary material, S1).

(b) W chromatin body assays

After oviposition, the eight field-collected *E. mandarina* females were analysed for presence of the W chromatin body [10,12] using previously established methods for *Eurema* [10,12].

(c) Real-time quantitative PCR

The gene dose ratio (GDR) of Z-linked genes *Tpi* and *kettin* with the autosomal gene *EF-1α* was inferred by quantitative PCR (qPCR) [13]. We tested the offspring of the eight field-collected *E. mandarina* females, six control individuals from Hachijō-jima and 10 individuals each of *E. hecabe* and *E. smilax* (electronic supplementary material, S1).

(d) *Tpi* sequence analysis

Inheritance of the Z chromosome was revealed through *Tpi* sequence analysis of mothers and their offspring. Paternal alleles remained unknown as females were caught after mating.

3. Results

(a) *Wolbachia* infection status

Offspring of all-female families were infected with both *w*CI and *w*Fem. By contrast, offspring of mixed-sex families were only infected with *w*CI. The six tetracycline-treated offspring individuals of a *w*CI female were uninfected. All wild-caught *E. mandarina* from Hachijō-jima and Australian *E. hecabe* were positive for *w*CI, and Australian *E. smilax* were uninfected (electronic supplementary material, S2).

(b) W chromatin body assays

The W chromatin body was detected in the three mothers of *w*CI-infected and *w*CI-cured individuals but not in the five mothers of *w*Fem females (electronic supplementary material, S2). This confirmed previously published absence of W in *w*Fem *E. mandarina* and *w*Fem *E. hecabe* [7,8].

(c) Gene dose ratio of Z-linked genes in males and females

Our qPCR approach correctly determined sex in *Eurema* butterflies, independent of their infection status. Both genes had a GDR close to 1 for all males in all species (figure 1). Females

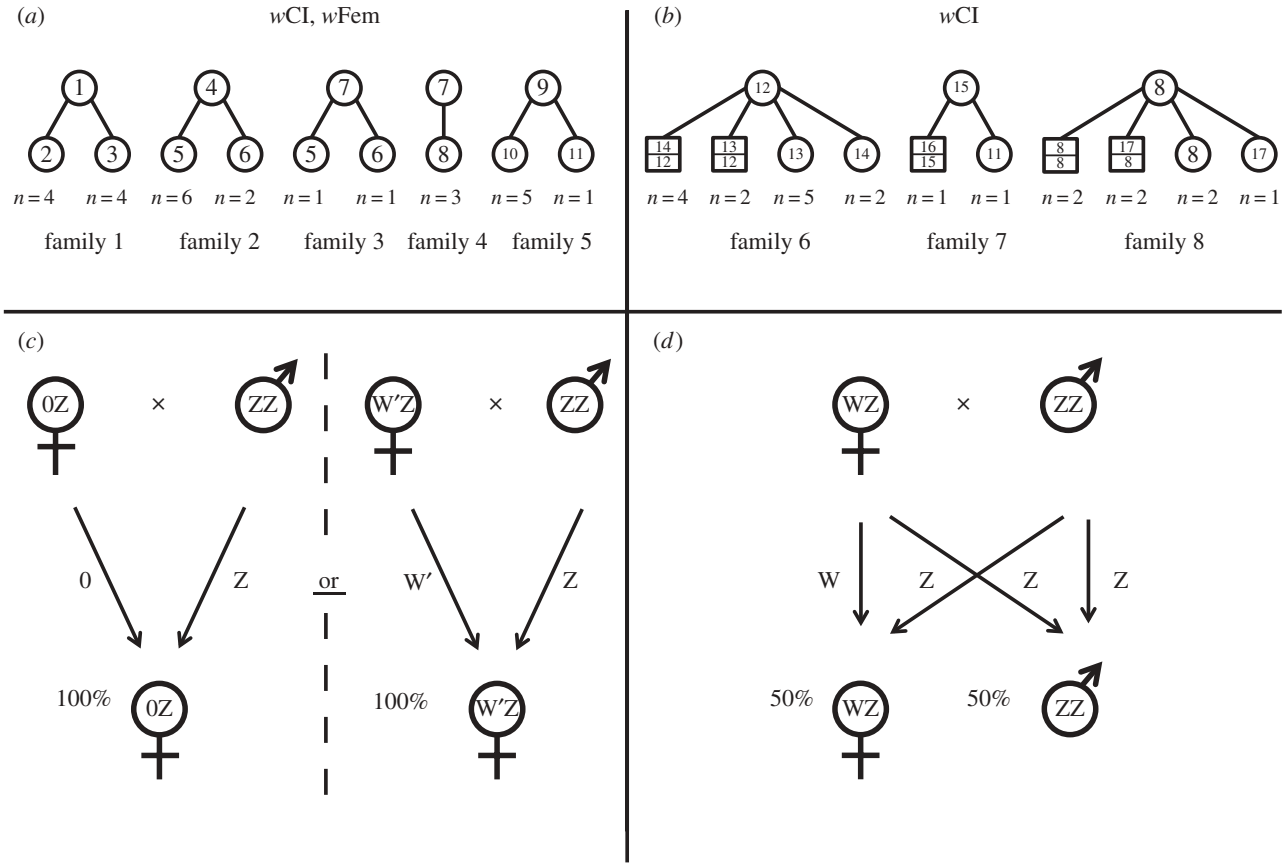

Figure 2. Family pedigrees of *w*Fem co-infected (*a*) and *w*CI-infected (*b*) *Eurema mandarina*. Each number represents a different allele; circles represent females with one Z, squares males with two Z alleles. *w*Fem leads to either a loss of W or a modified W′ chromosome; *w*Fem individuals carry the paternal Z (*c*). In *w*CI-infected lineages with equal sex ratios, sex chromosomes experience Mendelian inheritance (*d*).

of *w*CI *E. hecabe*, uninfected *E. smilax*, *w*CI and uninfected *E. mandarina* had a GDR close to 0.5. Contrary to expectation, GDR of *w*Fem *E. mandarina* females was also 0.5.

(d) Inheritance of the Z chromosome

Sequence analysis of the Z-linked *Tpi* gene provided further evidence that all females had a single Z (figure 2*a*,*b*), while most males were heterozygous with two different alleles (figure 2*b*). In *w*Fem families, the mother's allele was not observed in daughters ($n = 27$ over five families), implying MD against the maternal Z. In families without *w*Fem, normal Mendelian segregation was seen, with maternal Z alleles appearing in sons and not in daughters (figure 2; electronic supplementary material, S3).

4. Discussion

By using qPCR, we accurately identified sex in three *Eurema* species; the GDR of two Z-linked genes in males was twice that in females. This matched the detection of W chromatin in *w*CI-infected *E. mandarina* females but was not in line with the absence of W chromatin in *w*Fem females. Thus, contrary to previous hypotheses, *w*Fem females did not have male ZZ genotypes. We then investigated inheritance of the Z chromosome. Alleles of Z-linked *Tpi* in *w*Fem females always differed from their maternal genotype, revealing paternal inheritance of Z, and more specifically, the exclusion of maternal Z from progeny by a yet unknown MD mechanism.

Based on our findings, we conclude that *w*Fem lineages do not possess a W chromosome, or carry a modified W′ that is dysfunctional and cannot be visualized in W chromatin assays (figure 2*c*). A previous study detected W in just one *w*Fem female [9]; perhaps *w*Fem-infected lineages have a modified W′ that can only occasionally be visualized as W chromatin. Irrespective of whether W is lost or modified, *w*Fem still compensates for it and triggers female development of individuals with a single Z chromosome. This is shown by previous experiments demonstrating that *Wolbachia* must be present in larvae for female development [11].

In addition, MD prevents inheritance of the maternal Z chromosome. MD can polarize the meiotic spindle, leading to a non-random segregation of sex chromosomes [14] where no sex chromosome or W′ may be preferentially inherited while Z may be pulled towards the polar body. It is not yet known whether *Wolbachia* is directly involved in MD of *E. mandarina* or whether MD and *Wolbachia* feminization are two independent mechanisms. The answer depends on the currently unknown Z chromosome composition of the all-male offspring of females cured of *w*Fem; the re-establishment of Mendelian Z inheritance would provide evidence that *Wolbachia* causes the observed MD.

Here, we propose a new conceptual framework in which MD is responsible for the uniform sex chromosome composition within sex-biased lines. *w*Fem does not feminize ZZ males but feminizes individuals with a single Z (0Z or W′Z). *w*Fem compensates for the loss of the female differentiation pathway. Thus, the combined action of MD and feminization may have led to the evolution of 0Z female genotypes, analogous to the loss of the Y chromosome in male heterogametic systems that can result in the evolution of X0 systems [15].

The production of all-male broods after *w*Fem curing could follow the mechanism seen in *Bombyx mori*, where embryos with only one Z chromosome become males when the sex determination signal of the W chromosome, a female-specific piRNA, is silenced [16]. Furthermore, in the moth *Ostrinia scapulalis*, a MK *Wolbachia* strain was found to carry a feminizing factor, while the moth's W chromosome was dysfunctional [17]. How *Wolbachia* induces femaleness in Z individuals remains hidden. One possibility is mimicry of the primary sex determination signal itself. *Wolbachia* has recently been reported to manipulate the host's piRNA machinery in *Aedes aegypti* [18].

While the capacity to induce MD has not yet been demonstrated for endosymbionts, possible interactions of endosymbionts with other selfish genetic elements have previously been discussed [19]. Our study is the first to suggest the combined action of different reproductive manipulations, MD and feminization. It highlights that reproductive manipulations in *Eurema* butterflies are more complex than previously anticipated, and this may apply to current models of *Wolbachia* feminization in general. In addition, our study raises the possibility that endosymbionts might cause MD in their hosts.

Data accessibility. *Tpi* and *kettin* sequences were submitted to GenBank (electronic supplementary material, S1).

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Conflict of interests. We have no conflict of interests.

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