Double trouble: combined action of meiotic drive and \textit{Wolbachia} feminization in \textit{Eurema} butterflies

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

1. Introduction

Selfish genetic elements can hijack 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 microorganisms [2] such as \textit{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].

\textit{Wolbachia}-induced feminization has been reported for several terrestrial crustaceans and is best described for the isopod \textit{Armadillidium vulgare} with presumed heterogametic females (WZ). In this species, \textit{Wolbachia} causes individuals to develop into functional females via manipulation of the androgen 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 \textit{Wolbachia} rather than W, while males may develop when \textit{Wolbachia} transmission is leaky [4].

\textit{Wolbachia}-induced feminization has also been recorded for three insect species, including two \textit{Eurema} butterfly species [5–7]. \textit{Eurema mandarina} butterfly populations are nearly fixed for \textit{wCl} infections. In some populations, females are co-infected with \textit{wFem}, a strain thought to cause feminization [6,8]. For example, on Tanegashima Island in Japan, most females harbour both strains and produce...
only daughters with similar offspring numbers to the mixed sex broods produced by wCI females [8,9].

Lepidoptera are diplodiploid insects with female heterogamy—females are WZ or Z0, males are ZZ. As in most Lepidoptera [10], the W chromosome of uninfected and wCI E. mandarina females forms a heterochromatic body during interphase of somatic cells [6]. However, in wFem 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 wFem 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 wFem 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 wFem 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 Hachijo-jima Island, Japan, as well as 10 individuals each of Australian Eurema hecabe and Australian Eurema smilax (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 Hachijo-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 wCI and wFem. By contrast, offspring of mixed-sex families were only infected with wCI. The six tetracycline-treated offspring individuals of a wCI female were uninfected. All wild-caught E. mandarina from Hachijo-jima and Australian E. hecabe were positive for wCI, 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 wCI-infected and wCI-cured individuals but not in the five mothers of wFem females (electronic supplementary material, S2). This confirmed previously published absence of W in wFem E. mandarina and wFem 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
of *E. hecate*, uninfected *E. smilax*, wCI and uninfected *E. mandarina* had a GDR close to 0.5. Contrary to expectation, GDR of wFem *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 2a,b), while most males were heterozygous with two different alleles (figure 2b). In wFem families, the mother’s allele was not observed in daughters (*n* = 27 over five families), implying MD against the maternal Z. In families without wFem, 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 wCI-infected *E. mandarina* females but was not in line with the absence of W chromatin in wFem females. Thus, contrary to previous hypotheses, wFem females did not have male ZZ genotypes. We then investigated inheritance of the Z chromosome. Alleles of Z-linked Tpi in wFem 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 wFem lineages do not possess a W chromosome, or carry a modified W’ that is dysfunctional and cannot be visualized in W chromatin assays (figure 2c). A previous study detected W in just one wFem female [9]; perhaps wFem-infected lineages have a modified W’ that can only occasionally be visualized as W chromatin. Irrespective of whether W is lost or modified, wFem 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 wFem; 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. wFem does not feminize ZZ males but feminizes individuals with a single Z (0Z or WZ). wFem 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 wFem 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 female reversal 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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