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A neo-W chromosome in a tropical butterfly links colour pattern, male killing and speciation

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Sexually antagonistic selection can drive both the evolution of sex chromosomes and speciation itself. The tropical butterfly the African Queen, *Danaus chrysippus*, shows two such sexually antagonistic phenotypes, sex-linked colour polymorphism and susceptibility to a male-killing mollicute *Spiroplasma ixodetis*. In East Africa male-killing is prevalent in a narrow hybrid zone centred on Nairobi. This hybrid zone separates otherwise allopatric subspecies with different colour patterns. Here we show that a neo-W chromosome, a fusion between the W (female) chromosome and an autosome that controls colour pattern, links susceptibility to male-killing with a change in colour pattern. Studies of the population genetics of the neo-W around Nairobi show that the interaction between colour pattern and male-killer susceptibility restricts gene flow between the two subspecies of *D. chrysippus*. Our results demonstrate how a complex interplay between sex, colour pattern, male-killing and a neo-W chromosome, has set up a genetic 'firewall' that keeps the two subspecies apart. The association between the neo-W and male-killing thus provides a 'smoking gun' for an ongoing speciation process.

Keywords: *Danaus chrysippus*; male-killing; neo-W chromosome; colour pattern; speciation; *Spiroplasma*.

INTRODUCTION

Ever since Poulton [1] noted that two butterfly colour morphs could be bred from the same parents, the African Queen, *Danaus chrysippus* (L.), has been considered 'polymorphic'. However, our long-term studies at three sites around Nairobi, Kenya (figure S1), have established that in this region the butterfly comprises two essentially parapatric (though migratory) subspecies. *D. c. chrysippus* and *D. c. dorippus* (henceforth termed *chrysippus* and *dorippus*, figure 1*a*-*c*). Each subspecies has an individual colour pattern controlled by a single autosomal locus *C* [2-4], *dorippus* being homozygous for *CC* and *chrysippus* homozygous for *cc*. The F_1 hybrid *transiens* (the heterozygote *Cc*) closely resembles *dorippus* but is usually phenotypically detectable (figure 1*e*) [5]. The subspecies make frequent contact across East Africa creating colour polymorphisms within a mosaic of hybrid clines centred on Nairobi but reaching south to Tanzania [2], east into Uganda [6-9], and north to Sudan [8], Ethiopia [10] and Oman [9]. Outside the hybrid zone, both subspecies are monomorphic for the *C* locus through their separate ranges [figure 1S, 11-12].

Previous investigations have explored relationships between colour pattern, male-killing and sex linkage. Throughout the hybrid zone female-biased sex ratios, driven by male-killing and marked by hybrid excess, predominate [2-4]. Two phenotypes are associated, the first being colour pattern, which may be sex-linked or independent of sex and the second susceptibility to the male-killing intracellular mollicute *Spiroplasma ixodetis* [13-14]. We have previously found that the *c* bearing autosome of *chrysippus* females strictly segregates with the W chromosome within the hybrid zone and have therefore postulated that a W-autosomal fusion ($W\overline{c}$) has occurred [11-12], physically linking the female determining W chromosome with the colour pattern locus *C*. Further, we have also found within the hybrid zone that *chrysippus* females and their *transiens* daughters are all infected with the male-killing *Spiroplasma* and thus produce all-female broods, whereas, in contrast, *dorippus* females are largely uninfected by *Spiroplasma* and therefore produce both males and females. The aims of the present study were therefore to look at five specific factors likely to govern the population genetics of the hybrid zone. 1) To demonstrate, using cytogenetics, that an autosome has indeed become fused to the female-specific W-chromosome, as previously postulated. 2) To document the frequency of *Spiroplasma* infection in the hybrid zone and to try to understand how this fusion maintains the highly female-biased sex ratios found. 3) To investigate any possible relationship between population density and sex ratio. 4) To document, via spermatophore count per female, potentially different rates of mating between hybrids and pure-bred individuals

in the hybrid population. 5) Finally, to examine whether sexual selection or mate choice (assortative, random or disassortative) within the hybrid zone affects sex ratio.

Here, in a cytogenetic analysis using fluorescent *in situ* hybridisation (FISH) to visualise telomeres, we show that in *chrysippus* females the W chromosome is fused to an autosome and forms a trivalent with the Z chromosome and the autosome in meiosis, whereas in *dorippus* females W/Z and autosomes pair independently, as do Z and autosomes in all males. We also show that in a female-dominated hybrid zone site at Kitengela (figure S1) immigrant *dorippus* males are relatively immune to male-killing and that nearly all females are inseminated despite outnumbering males by around 5:1. Paradoxically, the female-biased hybrid population is an evolutionary stable strategy (or ESS) which can withstand perturbations induced by weather, population density or immigration and reinforces a barrier to gene flow between two nascent species that has endured for at least 40 years around Nairobi [2-3]. The extraordinary effectiveness of this barrier is demonstrated by field data, presented here, that shows a rapid return to the prevailing female biased sex ratios after a seasonal influx of males that are *Spiroplasma*-infected but resistant to male-killing.

2. MATERIAL AND METHODS

(*a*) *Cytogenetics*

Male metaphase I and II cells were obtained from testis cysts of pupae and last-instar larvae, female pachytene cells from the tips of ovarioles of adult females. Fixation in Carnoy's fluid (ethanol:chloroform:acetic acid, 6:3:1) was followed by spreading in 60% acetic acid and staining with 4'6-diamidino-2-phenylindole (DAPI). Female metaphase I was prepared from mature eggs by fixation in Carnoy's fluid, squashing in 60% acetic acid, freezing in liquid N_2, flipping off the coverslip with a scalpel ('dry ice method'), post-fixation in ethanol and staining with DAPI. Telomeres were visualised by FISH with a (TTAGG)n probe generated by substrate-free PCR (primers: TAGGTTAGGTTAGGTTAGGT and CTAACCTAACCTAACCTAAC) and labelled with Orange-dUTP.

(*b*) Spiroplasma *screening*

DNA from *D. chrysippus* was extracted from a 2 mm^2 piece of thorax tissue using the Puragene DNA purification kit (Quiagen, Valencia, CA). DNA was extracted using three times the suggested volume for a single *Drosophila* and then hydrated in 70 µL ddH_2O. Previously described *ixodetus*-clade specific primers (SpixoF

and SpixoR) and PCR cycling conditions [15] were used to amplify an 810 bp region of *Spiroplasma* 16S rDNA to confirm the presence of *Spiroplasma*. As a positive control for DNA quality, a region of host mitochondrial DNA (mtDNA) was amplified using primers LCO1490 and HCO2198 [16].

(*c*) ***Field sampling***

The new field data from Kitengela comprise samples of flying adult butterflies collected by net in 19 two hour sessions from May 2013 to September 2015 (Table S2). As most butterflies seen were caught, the samples are ± random. All butterflies were date marked and released at the point of capture. Mated pairs were recorded. 260 wild females of random genotype were dissected to count spermatophores.

3. RESULTS

(a) Cytogenetics

Previous cross-breeding has suggested that the autosome carrying the *C* locus may have become linked to the female-specific W (and not the Z) chromosome via a previously undocumented fusion [11-12, Table S1]. To test this hypothesis we examined the cytogenetics of butterflies of known descent for colour pattern from within the Nairobi hybrid zone. We found that the diploid chromosome complement in F_1 *transiens* (*Cc*) females from Nairobi contains a novel fusion chromosome (a neo-W): 28 bivalents and a trivalent are present in female meiosis ($2n = 59$), whereas in meiosis of females from outside the hybrid zone 30 bivalents ($2n = 60$) are present (figure 1*b*, *d*, Table S3). This confirms our earlier prediction and explains the strict sex-linkage found only in the hybrid zone (figure 1*c*, *e*). As the neo-W pairs in meiosis I of oögenesis with Z and the *C* bearing autosome, the former becomes Z_1 and the latter Z_2 in a W/Z_1Z_2 trivalent. All males from several sources had 30 bivalents ($2n = 60$), in agreement with older data [17, 18]. The data-set therefore directly supports our earlier hypothesis that an autosome carrying the *C* locus has indeed become directly fused to the female-specific W-chromosome.

(*b*) Spiroplasma *screening*

In order to understand how infection with the male-killing *Spiroplasma* drives the female-biased sex ratios found within the hybrid zone we typed 87 butterflies (72 females and 15 males) from the Kitengela field site for *Spiroplasma* (Table S4). We paid particular attention to infection rates in the rarer males which appear after the rains and then vanish from the population over time. The high infection rate in females (87.9%), equally across all genotypes, is expected from the high rate of male-killing and is similar that observed in 2009-10 at Kasarani

[19] (χ^2_1 = 1.997, p = 0.158). However, Kitengela male samples had the highest ever recorded infection rates (66.7%) [cf. 8, 13, 14, 19] and significantly so (χ^2_1 = 23.356, p < 0.0001) compared to the 2009-10 Kasarani samples [19]. Comparing male infection rates for June (40.0%) and July (81.3%), the increase approaches significance (Exact $p_{\text{(one-tailed)}}$ = 0.115). The increasing frequency of *Spiroplasma*-infected but male-killer resistant males is reflected in a significant change in sex ratio from 86.6% female in May , through 82.1% in June down to 72.4% in July (χ^2_2 = 10.415, p = 0.005) (Table S5). The infected males were found only when butterflies were atypically abundant following heavy rainfall across the region. It is therefore clear that the high frequency of infected males (which can only exist if they are resistant) at this time was unusual and most likely the result of substantial immigration. Prior to this period, 91% (n = 76) of broods obtained from wild *Cc* males mated to *cc* females (Table S1) had produced all-female progeny. Two months later (in September) all males had vanished from the hybrid zone and the sample was once again 100% female (Table S5). The all-female population was therefore restored and all males eliminated in as little as two butterfly generations. The summary statistics of the *D. chrysippus* populations which have been sampled for *Spiroplasma* and/or the male-killing phenotype, on the one hand, and hybridism on the other (Table S6) shows that the two phenomena are far from independent (χ^2_1 = 22.562, p < 0.0001). In conclusion, the high frequency of male-killing *Spiroplasma* and its association with the neo-W fusion within the hybrid zone rapidly cleanses the population of males. Following an unusual immigration of large numbers of males, they can be removed from the population within a staggering two generations.

(***c***) ***Field data***

To rule out the possibility of any association between sex ratio and population density, we estimated the density of the Kitengela population on several occasions. Bailey Index estimates for population size [20] at the 5 ha site varied between extremes of 27 ± 16.7 (5.4 per ha, 100.0% female) in October 2014 and 344 ± 229.5 (68.8 per ha, 86.6% female) in May 2015. Despite a ~13-fold difference in density, the sex ratio was unaffected (Exact p = 0.063). The Kitengela sex ratio data (Table S2) conform to the historical (1986-2010) norm for the region (figure 2S*b* I-III). The mean sex ratio of 84.1% female (figure 2S*b*, IV) – the highest recorded in the Nairobi region – is nonetheless within the C_{95} limits for the regional mean (figure S2*b*, V).

(*d*) ***Spermatophore counts***

To test if females of different colour morphs experienced different rates of mating success we counted the number of the male-transferred spermatophores found per female. Spermatophores per female at Kitengela (2013-14, $n = 260$) averaged 1.73 ± 1.24 (range 0-6) with only 19 (7.3%) unmated; thus all females are expected eventually to mate. The sexual histories of *Cc* ($n = 227$) and *cc* females ($n = 23$) did not differ ($t_{258} = 0.073$, $p > 0.99$). The spermatophore count is significantly less than 3.50 ± 1.36 ($n = 20$) for a population in Ghana with a sex ratio of 1:1 ($t_{278} = 6.126$, $p < 1.0 \times 10^{-4}$). In conclusion, overall spermatophore counts per female do not differ between colour morphs within the hybrid zone.

(*d*) ***Sexual selection***

Finally, we used a period of unusually high male frequency to study the potential effects of sexual selection and to test if mating between colour morphs was assortative, random or disassortative. In May-July 2015 the Kitengela population irrupted during heavy rains after a long period of drought. For the first time males were relatively numerous, and male-female courtship and successful mating was observed in the field. We seized the corresponding opportunity to study sexual selection (Table S7). The frequency of males caught *in copula* was 63.4% compared to 13.4% for females. In other words males were nearly five times more likely to mate. This remarkable statistic attests to the unrelenting pressure to mate on males. Sexual selection for colour genotype was not significant for either sex ($\chi^2_{1\,(♂)} = 0.191$, $p = 0.662$; $\chi^2_{1\,(♀)} = 2.071$, $p = 0.150$). However, with numbers corrected for penetrance (Table S7), the majority of males *in copula* (57.7%) were *CC*, whereas among females 75.0% were *Cc*, 25% *cc* and 0% *CC*; hence pairings were substantially disassortative for genotype. As mate choice in polymorphic populations is normally assortative for colour pattern [21, 22], enforced disassortative mating must invoke sexual conflict [23-25]. Matrilineal descent of neo-W, combined with male-killing, would ensure that the progenies from such disparate pairings are overwhelmingly females of genotype W$\bar{c}$/Z,*C* (Table S1) [3-4].

DISCUSSION

This paper shows for the first time that a neo-W chromosome, a W-autosome fusion, is indeed responsible for the previously observed non-random segregation of colour pattern and sex and that it promotes the genetic separation of two incipient butterfly species across a hybrid zone, as will be shown below. The data on morph

frequencies and mated pairs also confirm for the first time that mating is disassortative within this hybrid zone despite being assortative outside it [21, 22]. This paradoxical situation is entirely due to the restricted mate choice that results from male killing and the neo-W fusion. At this stage we are uncertain as to exactly which autosome is fused to the female-specific W chromosome. However what is clear is that this autosome carries loci controlling both colour pattern (the *C* locus) and susceptibility to the male-killing *Spiroplasma*. The high frequency of the neo-W fusion in the Kitengela population ensures that butterflies within the hybrid zone are predominantly female and that the sex ratio is stable despite seasonal influxes of *Spiroplasma*-infected but male-killer resistant males. If these males carry a Z-linked suppressor gene, as seems likely, the suppressor would be lost in dead male offspring, thus protecting the female-biased sex ratio.

Remarkably, all females obtain at least one mating although their spermatophore count is significantly less than in a population with a 1:1 sex ratio. Thus, females in populations susceptible to male-killing receive fewer (and possibly smaller) spermatophores from their hard-pressed male partners. As spermatophores contain nutrients and defensive pyrrolizidine alkaloids in addition to sperm [26], hybrid zone females are inevitably disadvantaged. Moreover, as females outnumber males ~5:1, to achieve 1.7 pairings per female (as found at Kitengela) each male must mate an astonishing 8.5 times. Males will quickly be seduced by females, become entrapped and eventually exhausted [27]. As all are expected to mate many times, sexual selection on males is relaxed but their life expectancy must be curtailed. Furthermore, the paucity of males reduces effective population size (N_e) by ± 50% and severe inbreeding is the likely outcome from an average of 4.3 females sharing each male partner.

In figure S2 we show the karyotypes that result after a founding cross between unfused *D. c. dorippus* males with 30 bivalents and *D. c. chrysippus* females with 28 bivalents and a W/Z_1Z_2 trivalent. The array is comprehensive for a hybrid population after the first two rounds of mating. Most of the rare recombinants are heterozygotes for either *C* bearing autosomes, sex chromosomes or both, and arguably unfit. However, karyotype J with an unfused W chromosome is probably the standard karyotype in *chrysippus* populations outside the hybrid zone, which, in the absence of the neo-W and associated male-killing, should be subject to Fisherian selection for a balanced sex ratio [28]. The shuffling of chromosomes in females that produces karyotype J suggests that once free from the hybrid zone, *chrysippus* populations could quickly revert to 1:1 sex ratios.

The neo-W fusion is apparently able to avoid suppression and ensures that male immigration does not reduce the high frequencies of females, except in the very short term; in effect the neo-W fusion imposes an ESS on the

Kitengela population. The influx of *dorippus* males in July 2015 reduced the sex ratio only for the duration of their life span ($\chi^2_1 = 4.083$, $p = 0.043$) and by September 2015 the female-biased ESS had been regained within just two generations ($\chi^2_1 = 13.203$; $p = 0.0002$) (Table S5). Given the great selective advantage gained by male-killing suppressor genes [29] this is an extraordinary result and is most easily explained if these genes are carried on the Z_2 chromosome (~C-autosome) introduced by the immigrant males. This chromosome will be automatically eliminated in dead males within two generations, exactly as observed. Immigration by outside females does, however, have the potential to disrupt the ESS as it will always change the W_u: neo-W ratio (figure 2, figure S2*b*). Ratios in the range 1:2 to 3:1 contain a transition point between two adaptive peaks wherein the sex ratio switches rapidly from near normal to 80% female. When the neo-W is less common (30% or lower), the sex ratio remains relatively normal despite the presence of male killing, and in practice (particularly in samples of active adults) will show no significant departures from 1:1. At Kitengela, the high frequency of the neo-W maintains sex ratios well above the tipping point and, even when males were most abundant in July 2015, adult female frequencies were never less than 70%. Moreover, all founding W_u: neo-W ratios in the range 1:1 to 1:4 fall inside C_{95} of the empirical mean for the Nairobi area (figure S2*b*, V), confirming the ESS status of the Kitengela sex ratios.

Within the hybrid mosaic the neo-W and the male-killing phenotype are probably obligatorily linked as neither has ever been observed in the known absence of the other [11, 12]. We speculate that the effect of the neo-W associated phenotypes is the erection of a genetic 'firewall' which diverts both *chrysippus* and *dorippus* male genomes into a 'black hole', thus erasing their Z chromosomes and *C* bearing autosomes; these are precisely the expected locations of 'speciation' genes [30-33] that would damage fitness in alien gene pools. The effectiveness of this firewall is dramatically illustrated by the rapid return to all-female sex ratios observed at Kitengela in September after the introduction of male-killer suppressors in May-July 2015.

Finally, infected male hosts (Table S4) are the hallmark of suppression of the male-killer phenotype [29]. If those *Spiroplasma*-infected males which survive infection mate with uninfected females, *CC* (*dorippus*) or *cc* (*chrysippus*), cytoplasmic incompatibility could cause their progeny to die during embryogenesis [34]. This could explain why female *CC* and *cc* genotypes, unlike their male homologues, are otherwise invariably and inexplicably rare at Kitengela (Table S2). We hope to test this hypothesis but suitable material has so far been elusive. A Red Queen scenario is predictable [35, 36], the protagonists being the neo-W chromosome and a suppressor of male-killing gene [4].

Our results are striking for several reasons. First, they break Haldane's rule [37] which states: '*When in the offspring of two animal races one sex is absent, rare or sterile, that sex is the heterozygous* [heterogametic] *one.*' Haldane's rule does not apply here because in *D. chrysippus* it is the *homogametic* males that die in the hybrid zone. Second, albeit via a different mechanism, the evolutionary outcome is similar to other animal hybrid zones between incipient species such as stickleback fish [38], *Drosophila* or *Anopheles* [39], *Podisma* grasshoppers [40], where gene flow is obstructed by hybrid male sterility, or in *Heliconius* butterflies by hybrid female sterility [41]. Third, unlike all the foregoing, the *D. chrysippus* genetic 'firewall' comprises susceptibility to male-killing and a neo-W conserved by linkage in heterogametic females. The neoW of the African Queen is therefore a reinforcement mechanism of a type not previously in speciation.

Finally, while we have yet to probe the molecular basis of this story, we note some striking parallels with other butterflies and moths in which micro-organisms affect sex ratios. The most notable being *Eurema* butterflies carrying feminising *Wolbachia* (*w*Fem) which lack W chromatin and therefore appear to have actually lost their W chromosome [42]. At this stage we cannot tell if the African Queen is also on the way to loosing its W chromosome but we do know that the neoW now also controls colour pattern conferred by the *C* bearing autosome that is now fused to the W chromosome. Whilst we can only guess as to the genetic nature of the C locus we note that *doublesex* has recently been shown to control not only colour polymorphism in another butterfly, *Papilio polytes* [43, 44], but can also change the penetrance of male-killing via manipulation of alternative splicing within the gene itself [45]. We will therefore now test if *dsx* is indeed carried on the autosome now fused to the W chromosome in the neoW or if some other colour pattern gene is encoded by the *C* locus. Taken together, our data support a hypothesis that two sexually antagonistic traits have been translocated to the newly formed female specific neo-W chromosome of this butterfly and are now driving its speciation around Nairobi. In closing, however, we also note that any local advantage enjoyed by the neo-W may be transitory and gradually erode as Muller's ratchet may well ensure the pervasive loss of autosomal genes and cause rapid subsequent degeneration of the neo-W chromosome [46-48].

AUTHOR CONTRIBUTIONS

D.A.S.S., I.J.G. and R.ff.-C. conceived the project and wrote the first and second drafts of the paper. I.J.G., S.C. and K.S. oversaw the breeding. I.J.G., D.A.S.S., K.S. and D.J.M. planned and supervised the field work. W.T. performed the cytogenetic investigations, J.H. scanned for *Spiroplasma*, and P.I. counted spermatophores. All authors contributed to further revisions of the manuscript.

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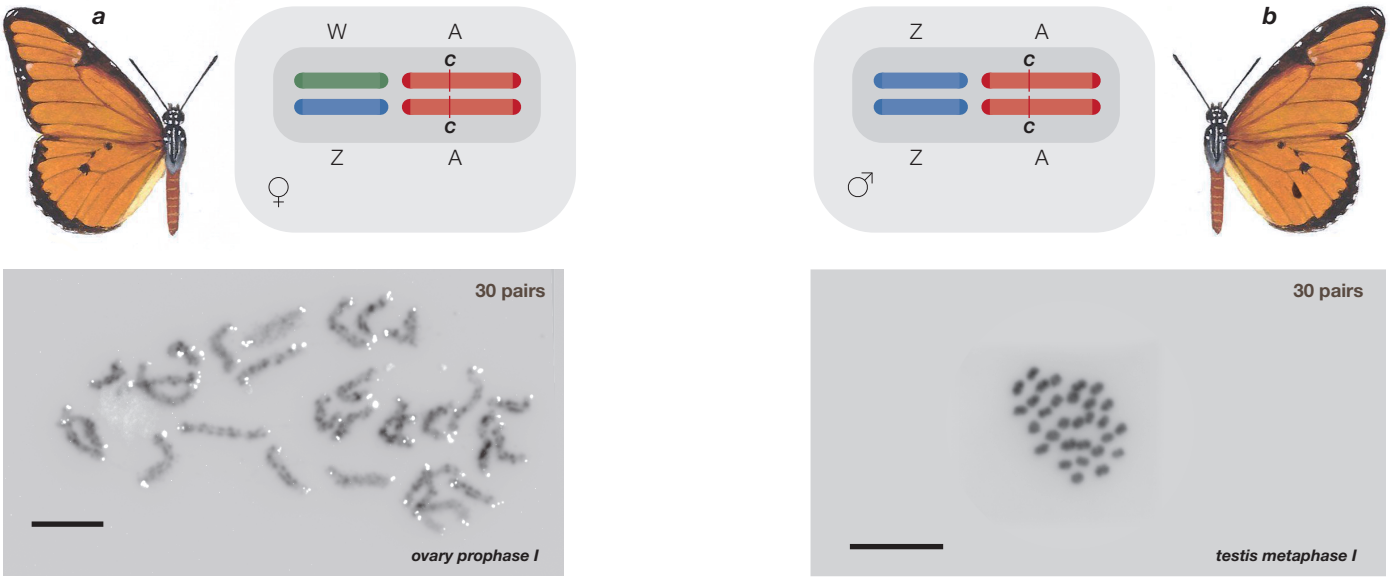
Figure legends

Figure 1. Colour phenotypes, genotypes and karyotypes of subspecies *D. c. chrysippus* (*cc*), *D. c. dorippus* (*CC*) and the hybrid form *transiens* (*Cc*) in the Nairobi region. *a-d*. Meiosis prophase I pachytene chromosome pairs from female ovaries (*a, c*) and metaphase I pairs from male testes (*b, d*): *Danaus chrysippus dorippus*, *a* female, *b* male, 30 bivalents (2*n* = 60) in both sexes; *D. c. chrysippus*, *c* female, 28 bivalents, 1 trivalent (2*n* = 59); *d* male, (2*n* = 60) as *b*; the white dots in the female micrographs *a* and *c* are telomere signals. Scale bars represent 10 μm. ***e-f*.** F_1 progeny, female (*e*) and male (*f*) from a cross between a *Spiroplasma*-positive *D. c. chrysippus* female (*c*) and a *Spiroplasma*-negative *D. c. dorippus* male (*d*). The provenance of chromosomes is shown as red, *dorippus C*-autosome; pink, *chrysippus c*-autosome; dark blue, *dorippus* Z chromosome; light blue, *chrysippus* Z chromosome; green, W chromosome unfused; yellow, W chromosome fused. Wild type bivalents (in *a, b, d*) comprise, respectively, two sex chromosomes (Z/Z or Z/W) with two autosomes carrying the C locus, whereas the $W\overline{c}/Z,c$ trivalent is found only in the mutant females *c* and *e*. Chromosomes in square brackets in females *c*, *e* and male *f* are lost in successive generations of dead sons (marked with a cross in *f*). A few *f* males escape death by either immunity (suppression of male-killing) or failed transmission of *Spiroplasma* [13]; most male survivors have the *transiens* (*Cc*) phenotype detectable by the white spots (arrowed in *e-f*) on the underside and/or scattered black scales on the upper side of the forewing apex.

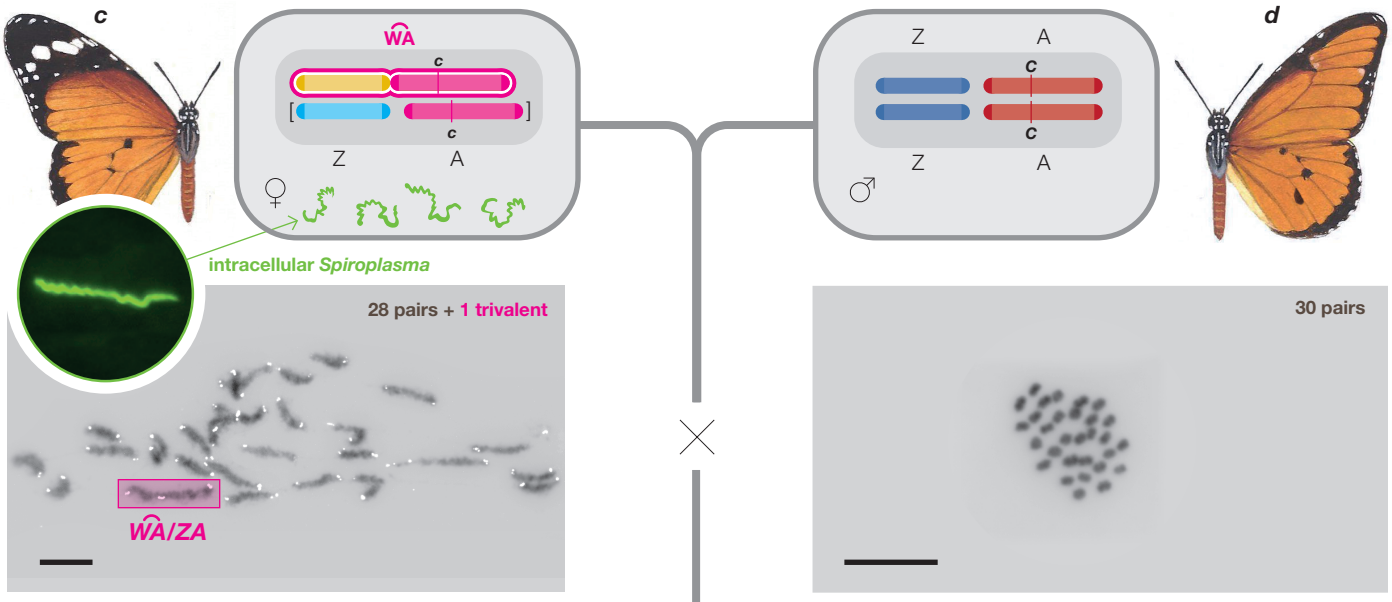
Figure 2. Graph showing the predicted frequency of females (*y*) in a hybrid population of *D. chrysippus* in the Nairobi district of Kenya as a function of the ratio W_u: neo-W (*x*) in the founder females, where $y = (a + b)/(2a + b)$, and $a = fW_u$ (range 10-1), $b = f$neo-W (range 1-10). The model assumes neutrality and absence of migration by females (but not by males).

Danaus chrysippus dorippus (wild type)

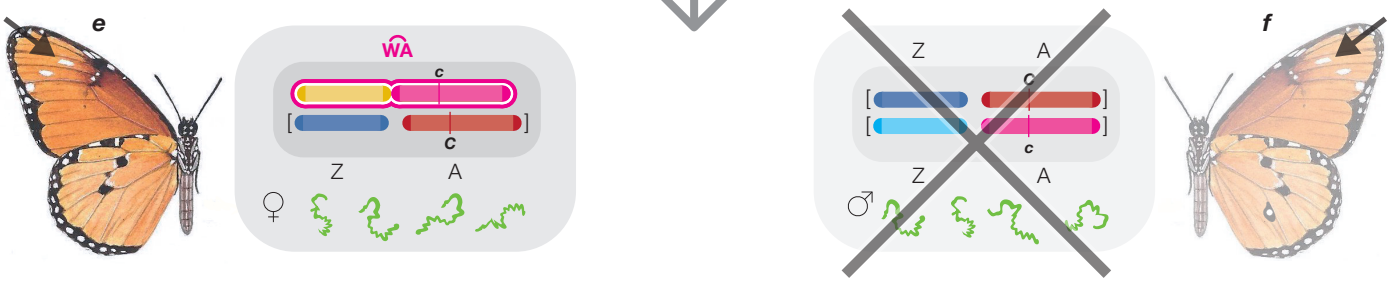


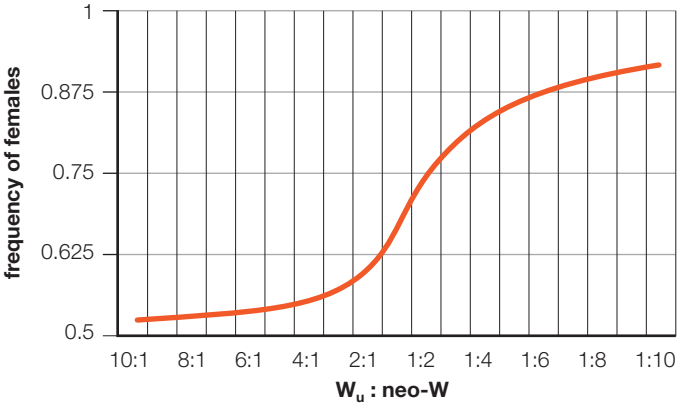
Danaus chrysippus chrysippus ♀ (neoW-BC autosome fusion type)

Danaus chrysippus dorippus ♂



Danaus chrysippus transiens F1
from the cross S+ *D. c. chrysippus* ♀ × S– *D. c. dorippus* ♂




1
0.875
0.75
0.625
0.5
frequency of females
10:1
8:1
6:1
4:1
2:1
1:2
1:4
1:6
1:8
1:10
W_u : neo-W