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The butterfly *Danaus chrysippus* (Lepidoptera: Nymphalidae) in Kenya is variably infected with respect to genotype and body size by a maternally transmitted male-killing endosymbiont (*Spiroplasma*)

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Abstract. Female-biased sex ratios in *Danaus chrysippus* (Linnaeus) (family Nymphalidae, subfamily Danainae, tribe Danaini) populations are attributed to the action of an endosymbiotic 'male-killer' bacterium of the genus *Spiroplasma*. In stark contrast to the extensive geographic range of their host, the *Spiroplasma* appears to be restricted to East Africa, where four African *D. chrysippus* subspecies exist sympatrically and form a hybrid zone. In this study, specimens collected at three sample sites within the hybrid zone were screened for *Spiroplasma* infection. The findings demonstrate that, within the hybrid zone, the frequency of *Spiroplasma* infection varies both spatially and temporally. Host genotype at three biallelic wing pattern loci, representative of subspecific genetic divergence, is correlated to *Spiroplasma* infection. Linkage between the frequency of the recessive *a* allele and *Spiroplasma* is established, suggesting the presence of a linked allele controlling infection susceptibility. In addition, a negative correlation between *D. chrysippus* forewing length and *Spiroplasma* infection is identified, suggesting that infection has a deleterious effect on body size.

Key words: hybrid zone, male-killing, *Spiroplasma*, *Danaus chrysippus*

Introduction

Cytoplasmically inherited endosymbiotic bacteria-causing reproductive disorders have been described in more than 30 insect species spanning six insect orders (Hurst and Jiggins, 2000). *Wolbachia* has been implicated in the majority of cases, but there is increasing evidence for the importance of other bacterial clades including *Spiroplasma* and *Rickettsia*. Contrasting inheritance patterns of endosymbiotic genetic elements and their host autosomes lead to genetic conflicts. Endosymbionts are transmitted from mother to daughter through egg cytoplasm and are therefore selected on the basis of their effects on the number and fitness of female offspring (Stouthamer *et al.*, 1999). Autosomal genes are generally selected for even investment in either sex (Fisher, 1930). Opposing selective forces generate intergenomic conflict between cytoplasmic and autosomal genes.

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In some cases, opposing selective forces are manifested as a distortion of sex ratios. Endosymbiotic sex ratio-distorting bacteria are reproductive parasites and function as 'selfish genetic elements' (Hurst and Werren, 2001), thus gaining a relative transmission advantage by means that are either neutral with respect to the organism's fitness or detrimental to it.

Variability in the frequency of male-killing between populations of infected host species has been widely reported (Hurst and Jiggins, 2000). Variability in the frequency of male-killing can stem either from the evolution of genetic suppression (Hornett *et al.*, 2006) or ecological factors that may affect their ability and drive to invade (Veneti *et al.*, 2004).

Male-killing in the butterfly *Danaus chrysippus* (Linnaeus) (family Nymphalidae, subfamily Danainae, tribe Danaini) is caused by a *Spiroplasma* bacterium (Jiggins *et al.*, 2000). There is a markedly heterogeneous spatial and temporal distribution of female-biased sex ratios (Smith *et al.*, 1998) and also *Spiroplasma* infection in *D. chrysippus* (Jiggins *et al.*, 2000).

Owen and Chanter (1968) observed female-biased sex ratios in *D. chrysippus* in Uganda. Until the work of Smith *et al.* (1998), this was attributed to the action of meiotic drive for the W chromosome, the female being the heterogametic (ZW) sex in Lepidoptera. Smith *et al.* (1998) showed that all-female (SR) broods in *D. chrysippus* were maternally inherited. High embryo mortality rates found in these SR lines led to the conclusion that death of males was generating sex ratio bias. Jiggins *et al.* (2000) demonstrated that antibiotics could 'cure' all-female lines. DNA from SR individuals was used for PCR, confirming the presence of *Spiroplasma*. The *Spiroplasma* infecting *D. chrysippus* was found by Jiggins *et al.* (2000) to be closely related to two ladybird male-killers identified by Hurst *et al.* (1999).

The infection dynamics of *Spiroplasma* in *D. chrysippus* are complicated by the fact that the species is polytypic in Africa; it is divided into four races with different colour patterns that overlap in a hybrid zone (Fig. 1) in East and Central Africa (Talbot, 1943; Smith *et al.*, 2005). Colour pattern genetics involves three loci (A, B and C) (Owen and Chanter, 1968; Clarke *et al.*, 1973; Smith, 1975a, 1980, 1998; Gordon, 1984; Smith *et al.*, 1998; Lushai *et al.*, 2003). Mitochondrial and nuclear DNA sequencing indicates that the four races are truly genetically divergent (Lushai *et al.*, 2005). Smith (1975a) found a significantly higher frequency of all female broods obtained from *chrysippus* compared with *dorippus* females in Tanzania, and Smith *et al.* (1997) suggested that female-biased sex ratios are associated with particular adult colour patterns.

Jiggins *et al.* (2000) found that 40% of *D. chrysippus* females ($n = 90$) in Uganda were infected with *Spiroplasma*, whereas populations on the eastern coast of Kenya had only 4% infected females ($n = 50$). By deduction from the sex ratio data of Smith *et al.* (1998), 18% of Tanzanian females ($n = 77$) and 69% of Kenyan females ($n = 62$) from the area around Nairobi produce female-biased sex ratios.

On the other hand, female *D. chrysippus* samples from outside the East African hybrid zone, including Ghana ($n = 6$), Oman ($n = 15$), South Africa ($n = 4$) and Zambia ($n = 3$), have all tested negative for *Spiroplasma* (Jiggins *et al.*, 2000). Breeding from wild females and rearing from wild-collected eggs have produced a wealth of data suggesting that *Spiroplasma* may not occur in *D. chrysippus* outside the East African hybrid zone: Gordon from Ghana ($n = 3$ broods) and Zimbabwe ($n = 4$ broods); Smith from India ($n = 2$ broods) and Malaysia ($n = 1$ brood) (all in Smith *et al.*, 1998, Appendix I); Clarke *et al.* (1973) using stock from Sierra Leone, Australia and hybrids between them, broods not separated ($n = 796$ individuals); D. F. Owen (unpublished, 1969) from Sierra Leone ($n = 9$ broods); Gordon and Smith from India (unpublished, 2006) ($n = 5$ broods); Smith from the Cape Verde Islands, wild-collected eggs ($n = 27$), in Lushai *et al.* (2003); Julian P. L. Davies from Mauritius, wild-collected eggs ($n = 110$) (*in litt*. to Smith, 08.10.1978). Whereas negative evidence is never conclusive, it is clear that data from diverse locations outside East Africa have failed to reveal the presence of male-killers. In addition, tracking wild populations over 2 years gave no indication of sex ratio distortions in Ghana (Gordon, 1984).

Relating the distribution of *Spiroplasma* prevalence to subspecific hybridization and nascent speciation in migratory *D. chrysippus* could enhance understanding of the interactions between inherited microorganisms and their hosts. There is a need to test whether *Spiroplasma* infection frequency is higher in particular genetic 'races' of *D. chrysippus*, indicating genetic variation in susceptibility to *Spiroplasma* infection. Alternatively, infection frequency could be solely predicted by geographic region (indicative of ecological factors).

In this paper, we show that (i) *Spiroplasma* infection correlates with a colour pattern locus and (ii) there are differences in body size between infected and uninfected females.

Materials and methods

We have analysed the geographic patterns of male-killer prevalence in *D. chrysippus* within the East African hybrid zone. Adult butterflies were collected from three sites (Fig. 2) in Kenya and

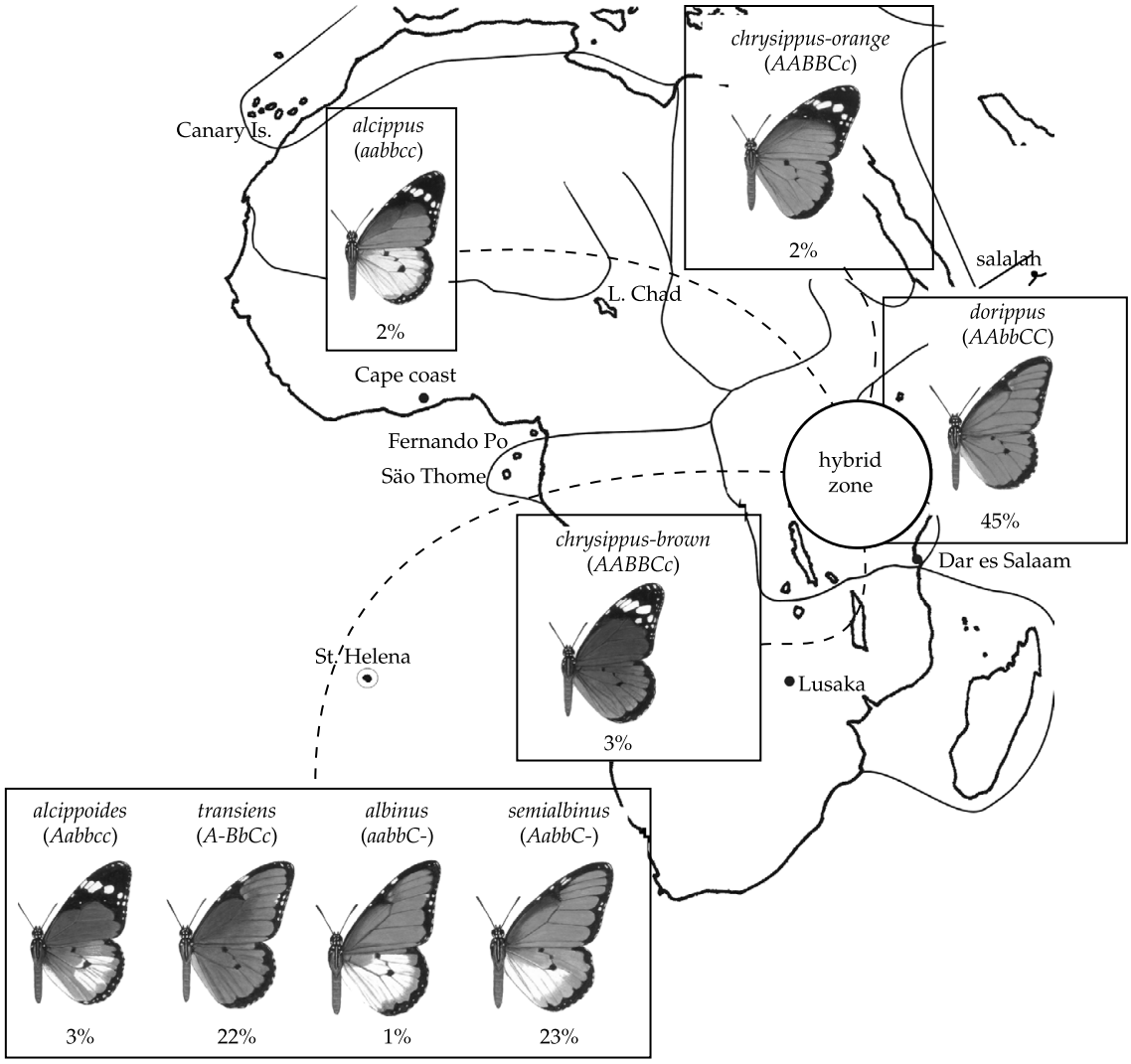

Fig. 1. Distribution of *Danaus chrysippus* subspecies and hybrid forms and frequencies (shown as a percentages) of specimens collected in this study. (Fig. can be viewed in colour in the Supplementary Materials for the online version of the paper.)

subsequently screened for *Spiroplasma* at ICIPE, Kenya and Oxford University, UK.

Collection of butterflies

Adult *D. chrysippus* were collected with a butterfly net from three locations in Kenya (Tsavo, Nguruman and Nakuru, Fig. 2). Sampling sites were chosen to represent a variety of habitats within Kenya, abundance of specimens and availability of data from previously published studies. Specimens TS1 to TS69 from Tsavo were collected in August 2004; the rest were collected between July and September 2005. Butterflies were dissected under sterile conditions and ovaries were stored in 95% ethanol at −20 °C. Voucher specimens (left and right wings) of all butterflies are stored in the collections at ICIPE, Nairobi. Forewing lengths were measured to the nearest millimetre. Genotypes were scored for the detectable alleles at the three colour gene loci as described in Smith *et al.* (1998).

Detection of Spiroplasma *using PCR assays*

DNA from *D. chrysippus* was purified using Chelex-100 in the presence of proteinase K. Small samples (2 mm^3) of ovary tissue were transferred to 1.8 ml microfuge tubes containing an extraction buffer of 100 µl of 5% Chelex-100 solution and 20 µl of 10–20 mg/ml proteinase K and incubated at 55 °C for 2 h. After a 15–10 s vortex, samples were heated at 99 °C for 10 min before another 10–15 s vortex. Samples were then centrifuged at 13,000 rpm in a

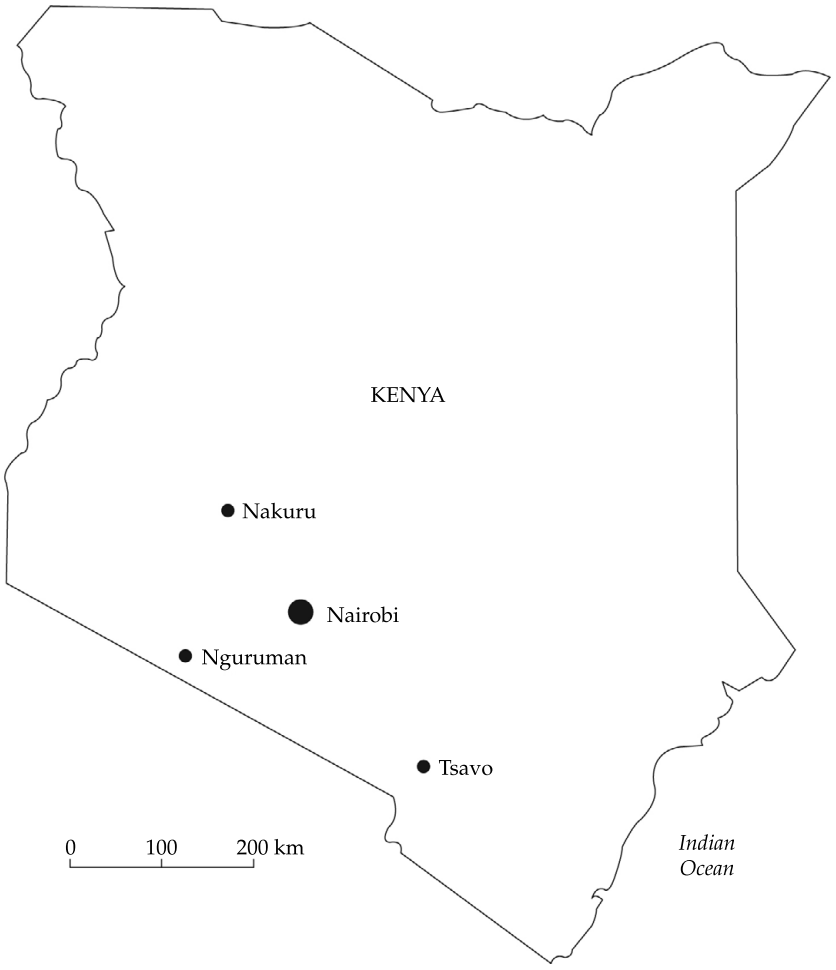

Fig. 2. Map of Kenya showing *Danaus chrysippus* sampling sites

bench-top microcentrifuge for 5 min; the supernatant was used directly for PCR and the remainder was stored at −20 °C.

As a positive control for DNA quality, a region of nuclear 18S ribosomal DNA (rDNA) was amplified using primers 18S(10)F and 18S(10)R (Wada *et al.*, 1996). Ovary samples that did not give positive 18S amplification were discarded or re-extracted from remaining ovary tissue. Positive and negative controls were amplified alongside each set of PCR assays.

Primers Spiro27f and SpiroR were designed during this study to amplify a region of *Spiroplasma* 16S rDNA based on the sequence reported by Jiggins *et al.* (2000): EMBL accession no. AJ245996. SpiroR, (5′-TTC CCT TAC AAC AGA CCT TTA CAA TCC-3′) and Spiro27f (5′GAG AGT TTG ATC CTG GCT CAG-3′) amplify a 436 bp fragment of the *Spiroplasma* 16S rDNA gene.

The PCR was carried out using 1.0 μl of 10 pmol/μl Spiro27F and SpiroR, 2.0 μl extracted DNA, 2.5 μl 10 × buffer, 1.5 μl 25 mM MgCl, 0.5 μl 25 mM dATP/dCTP/dGTP/dTTP, 0.3 μl Taq polymerase and 16.2 μl sterile distilled H_2O in 200 μl PCR tubes. The DNA amplification reactions were carried out in an Omnigene thermocycler (Hybaid®) programmed for an initial denaturation of 94 °C for 5 min, followed by 40 cycles of 49–53 °C for 1 min (primer annealing), 72 °C for 1 min (extension), 94 °C for 1 min (denaturation) and finally an extension step at 72 °C for 10 min.

The identification of amplified DNA in PCR products was conducted by electrophoresis through 1.0% agarose gels containing 0.3 μg/ml ethidium bromide, alongside molecular weight maker (100 bp ladder). For confirmation, a 436 bp band from a positive sample (NG89) was sequenced; BLAST alignment revealed that DNA amplified was from the *Spiroplasma* bacterium.

Results

To investigate the infection dynamics of *Spiroplasma* in *D. chrysippus*, samples representing the four

African subspecies and various hybrid forms (Fig. 1) were collected from three locations (Fig. 2) in Kenya. The total number of butterflies collected was 245, comprising 52 males and 193 females; of the latter, 84 were infected with *Spiroplasma* and 109 were uninfected. As *Spiroplasma* is maternally transmitted, males were not screened. The forewing length data of the females was analysed in a general linear model.

Frequency of infection at sampling sites

Infection frequency (Table 1) varied significantly between sites ($\chi^2_{[3]} = 24.9$, $P < 0.0001$). The Tsavo specimens were collected in consecutive years; in August and September 2004, the infection frequency was 77% ($n = 31$), whereas in August 2005 it was 33% ($n = 15$). The difference in *Spiroplasma* prevalence between years is significant ($\chi^2_{[1]} = 8.4$, $P = 0.004$). Due to the significant difference between the two Tsavo samples, these were considered separately in subsequent analyses. In Nakuru, 57% ($n = 37$) were infected. The lowest levels of infection (31%, $n = 110$) were found at Nguruman. Overall, 44% ($n = 193$) of females were positive for *Spiroplasma* (Table 1, Fig. 3).

Frequency of colour alleles

The frequencies of colour alleles at each location are given in Table 2. The level of variation in the frequency of alleles at the *A*-locus between the four samples is not significant ($\chi^2_{[3]} = 3.0$, $P = 0.391$). Significant variation is restricted to the *C*-locus ($\chi^2_{[3]} = 21.2$, $P < 0.0001$). B-locus data are not included, since Bb heterozygotes cannot be separated reliably from BB homozygotes (Smith, 1998).

Colour alleles and Spiroplasma *infection*

Three of four females homozygous recessive (*aa*) at the A-locus were positive for *Spiroplasma*. Fifty-nine per cent of identifiable heterozygotes at the A-locus ($n = 49$) were infected, compared with 40.6% of butterflies with the dominant phenotype; these are significantly different ($\chi^2_{[1]} = 4.25$, $P = 0.04$). It is likely that the latter value is an overestimate as some heterozygotes (*Aa*) were undetected due to incomplete penetrance of the *a* allele. No correlations between alleles and *Spiroplasma* infection were found at the *B* or *C* loci.

Table 1. The frequency of *Spiroplasma* infection at each sample site

	Tsavo 2004	Tsavo 2005	Nguruman	Nakuru
N	31	15	110	37
% Infection	77.4	33.3	30.9	56.8

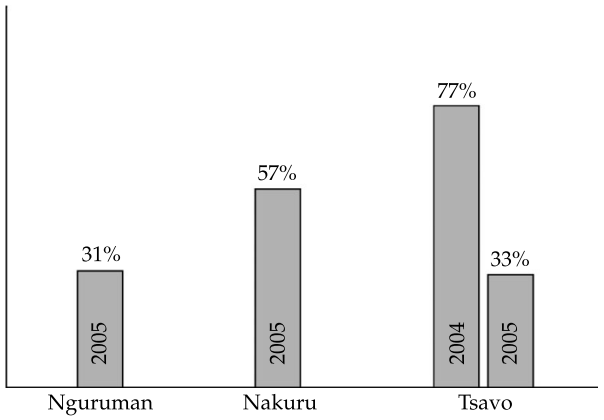

Fig. 3. Spatial and temporal variation in the frequency of *Spiroplasma* infection in *Danaus chrysippus*

Spatial and temporal variation for linkage disequilibrium between *Spiroplasma* and the *A*-locus is shown in Fig. 4. Linkage was highest at Tsavo (2004) with 100% of females with the *a* allele infected ($n = 7$). Tsavo (2005) was 50% ($n = 4$), Nguruman 52% ($n = 27$) and Nakuru 53% ($n = 15$); these are not significantly different ($\chi^2_{[3]} = 3.0$, $P = 0.39$).

Variation in forewing length

Individuals infected with *Spiroplasma* on average had a 0.8 mm shorter forewing length (infected $\overline{x} = 38.3$, SE = 0.32; uninfected $\overline{x} = 39.1$, SE = 0.27). The difference between infected and uninfected individuals is found significant ($P = 0.05$, df = 191, Pooled SE = 0.42) in an unstacked *t*-test. The difference was consistent among samples and in the same direction. The reduction in forewing length associated with *Spiroplasma* infection was 0.59 mm in Nakuru (infected $\overline{x} = 37.5$, SE = 0.58; uninfected $\overline{x} = 38.0$, SE = 0.57), 0.48 mm in Nguruman (infected $\overline{x} = 38.7$, SE = 0.53; uninfected $\overline{x} = 39.2$, SE = 0.36), 1.24 mm in Tsavo 2004 (infected $\overline{x} = 38.0$, SE = 0.66; uninfected $\overline{x} = 39.3$ SE = 0.79) and 0.3 mm in Tsavo 2005 (infected $\overline{x} = 39.8$, SE = 0.73; uninfected $\overline{x} = 40.1$, SE = 0.23) (See Fig. 4).

The determinants of forewing length were analysed in a general linear model. Host genotype at three loci (*A*, *B* and *C*), location and infection status were fitted to the model. All variables with P-values above 0.3 were discarded. There was no correlation between mean forewing length and genotypes for any locus: A-locus, $P = 0.89$, $F = 0.02$, df = 1; B-locus, $P = 0.30$, $F = 1.07$ df = 1; C-locus, $P = 0.57$, $F = 1.07$ df = 2; error df = 184 EMS = 8.4.

Table 2. Genotype at the *A*, *B* and *C* loci of *Danaus chrysippus* individuals in relation to sample site (number of butterflies)

	A-locus			B-locus		C-locus		
	A-	Aa	aa	B-	bb	C-	Cc	cc
Tsavo 2004 ($N = 69$)	45	12	0	12	46	54	15	0
Tsavo 2005 ($N = 16$)	11	15	0	3	12	14	2	0
Nguruman ($N = 110$)	84	23	3	29	81	62	46	2
Nakuru ($N = 55$)	37	16	2	25	30	18	16	21

As previously shown, infection status had a significant effect on forewing length overall.

Discussion

This study shows that the prevalence of the *Spiroplasma* bacterium in the populations of *Danaus chrysippus* varies in both time and space. Spatial heterogeneity of *Spiroplasma* infection within the hybrid zone has previously been directly analysed in two locations (Jiggins *et al.*, 2000), whereas temporal fluctuations have been inferred from sex ratios previously published by Smith (1975b) and Smith *et al.* (1998).

A key question addressed in this study is whether *Spiroplasma* infection frequency varies with respect to the genetic background of butterflies as assessed by scoring the colour polymorphisms at the *A, B* and *C* loci. We find that infection frequency correlates with the presence of the *a* allele. This correlation is probably conservative due to the variable penetrance of the recessive allele, estimated for females at 0.335 by Lushai *et al.* (2003). Therefore, a significant number of phenotypically dominant individuals are actually heterozygous and any linkage between *Spiroplasma* and the *a* allele in these specimens would have gone undetected. Smith (1975a) found significantly higher frequency of all-female broods from *chrysippus* (*cc*) compared with *dorippus* (*C-*) female parents; this study found no significant correlation between C-locus genotype and *Spiroplasma*.

A correlation between colour pattern and *Spiroplasma* infection could be generated and/or maintained in several ways. First, there could be linkage between genotype and susceptibility to (or efficiency of transmission of) *Spiroplasma*. Second, it could result from ongoing immigration of infected individuals with a particular genotype into a largely uninfected population or vice versa. Third, homotypic mate choice (as shown for the A-locus in Uganda by Lushai *et al.*, 2003) could generate and maintain linkage between *Spiroplasma* infection and genotype. The first of these explanations currently appears to be the most probable, but the others cannot be ruled out. The correlation between *Spiroplasma* infection and the *a* allele is somewhat unexpected in view of the apparent absence of both *Spiroplasma* (Jiggins *et al.*, 2000) and all-female broods (Gordon, 1984; this paper) from West Africa where the *a* allele is fixed. However, it is worth noting, and possibly significant, that the occurrence of *Spiroplasma* in our samples is predominantly in the females of *Aa* genotype; hybrids may be especially vulnerable to infection.

An unexpected and novel finding from the present study is that, on average, infected *D. chrysippus* females have a significantly shorter

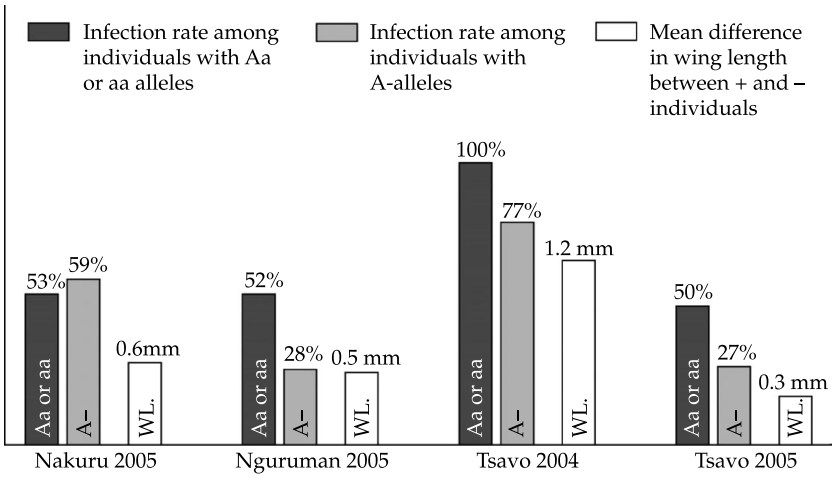

Fig. 4. A-locus linkage to infection, frequency and mean difference in forewing length associated with infection

forewing length than uninfected females. Previously published studies on the effect of *Wolbachia* on host size in *Acraea encedana* (Jiggins *et al.*, 2002) revealed no significant difference in forewing length between infected and uninfected individuals. Furthermore, comparison of forewing length between females from all-female broods ($n = 728$), assumed to be infected with *Spiroplasma*, and from bisexual broods ($n = 236$) reared in a previous laboratory study shows the opposite trend, i.e. the mean forewing length of the former is 1.0 mm greater than the latter ($t = 6.80$, $P < 0.001$, df = 962). However, the butterflies involved were highly heterogeneous with respect to both genotype and geographical origin (they were mostly Ghana × Kenya crosses) and this may have affected the result. The *A*-locus forewing data of Smith (1980) showed no significant difference between *AA* and *aa* genotypes of either sex, but significant heterosis for wing length in the *Aa* genotype in both sexes. Therefore, our finding in this paper that *Aa* heterozygotes are smaller than *A-* suggests that the actual magnitude of the effect of *Spiroplasma* on *Aa* heterozygotes may be greater than that observed.

Three explanations for the reduced forewing lengths of infected individuals in the field samples are possible. First, infected individuals may be essentially a cohort of the population that has shared common ecological conditions in the location where they have developed. Second, and most probably, *Spiroplasma* may have a direct physiological effect on infected females, reducing their growth rates or the durations of their larval instars. Third, *D. chrysippus* females are known to oviposit preferentially on small isolated food plants (Edmunds, 1976; Gordon, personal observation) and frequently lay more eggs on such plants than they are capable of supporting, leading to sibling competition for food among the larvae. In this situation, proportionately more infected larvae are likely to survive than uninfected ones since their male siblings die in the egg stage. Thus, infected females may be more likely to have experienced food shortages in the final instars and therefore be small.

Male insects generally tend to select females on the basis of phenotypic indicators of fecundity; the most common of these is body size (Bonduriansky, 2001). Infected females may, therefore, be subject to adverse sexual selection as well as a potential reduction in fecundity. Future work should evaluate the relative reproductive success of infected and uninfected females, and the implications this may have for *Spiroplasma* infection dynamics.

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Appendix 1: Sequence data

Sequence of 436 bp band from a positive sample (NG89):
H08_EJ-89_050_2005-12-13
NAATNANAGTCNGCGGCATTGCTCCATCAGGCTTTCGCCCATTGGTGAAAAATTCCCTACTGGCTGC-
CTTCCGTAGAAGTTTGGGCCGNGTCTCAGTCCCAATGGTGGCCGTTCATCCTCTCAGATCGGCTACG-
CATCGTTACCTTGGTAAGCCATTACCTTACCAACTAGTTAATGCGCCGCATCCCCATNAATTAGCG-
AAGCAAATGCTCCTTTCATTATTTATCATTGCAAATAAATAACATATGCGGTATTAGCTGTCGTTTCCAA-
CAGTTATTCCCCACTAAATGGTAGGTTAGATACGTGTTACTCACCCGTTCGCCACTAAAGTATTGCT-
ACTTTCGTTCGACTTGCATGTATTAGGCATGCCGCCAGCGTTAATCCTGAGCCAGCNNAAAAACTCT-
NANN