The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium

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SUMMARY

Many insects carry maternally inherited bacteria which kill male offspring. Such bacteria will spread if male death benefits the female siblings who transmit the bacterium, and they are therefore expected in insects with antagonistic sibling interactions. We report that the butterfly *Danaus chrysippus* is host to a maternally inherited male-killing bacterium. Using diagnostic PCR and rDNA sequence, the bacterium was identified as a *Spiroplasma* closely related to 2 ladybird beetle male-killers and the tick symbiont *Spiroplasma ixodetis*. The male-killer was found to have a geographically restricted distribution, with up to 40% of females being infected in East Africa, but no detectable infection in small samples from other populations. *Danaus chrysippus* is a surprising host for a male-killer as its eggs are laid singly. This suggests that the ecological conditions permitting male-killers to invade may be more widespread than previously realized.

Key words: selfish genetic elements, *Spiroplasma*, male-killers, Mycoplasma, *Danaus chrysippus*.

INTRODUCTION

Maternally inherited bacteria which kill males during early development have been found in 5 insect orders with varied sex determination systems. Moreover, invasion of a given host by male-killers may occur frequently on an evolutionary time-scale, as in the case of the ladybird *Adalia bipunctata* which has been invaded by at least 3 different male-killers (Hurst *et al*. 1999*a*). Therefore male-killers are likely to have arisen in susceptible hosts. Their distribution is therefore probably determined by whether the ecology of the insect host permits their invasion.

Theory suggests that for a male-killer without horizontal transmission to spread, male death must benefit the surviving female siblings. This may occur if females eat the dead males or if the death of males reduces deleterious sibling interactions such as competition or inbreeding (Hurst, Hurst & Majerus, 1997). Under these ecological conditions the incidence of male-killers may be very high, for example, over half of randomly sampled aphid-eating ladybird species were host to male-killers (G. Hurst and T. Majerus, unpublished observations). This remarkably high incidence probably results from sibling cannibalism, as an extra meal extends the period female larvae can survive before finding their first aphid (Hurst *et al*. 1997). Male-killers may also be widespread beyond this rather unusual ecological situation and there are a growing number of records from phytophagous insects which lay their eggs in clutches (Groeters, 1996; Higashiura, Ishihara & Schaefer, 1999; Jiggins *et al*. 2000; Jiggins, Hurst & Majerus, 1998).

Danaus chrysippus (L.) is one of the commonest and most conspicuous butterflies throughout the old world tropics. It appears, at first sight, to be an unlikely host for a male-killing bacterium as sibling offspring rarely interact. Nonetheless, in East Africa many females produce only daughters due to the death of their sons as embryos or early instar larvae (Smith *et al*. 1998). Despite this trait being maternally inherited, Smith *et al*. (1998) felt they could rule out the possibility of a male-killer because the butterfly lays its eggs singly and widely scattered, making the sibling interactions necessary for the spread of male-killers unlikely. Instead Smith *et al*. (1998) have argued that male death is caused by sex-specific hybrid dysgenesis. They reached this conclusion because the trait is found in an area of colour pattern polymorphism which may indicate a hybrid zone between butterfly races. Intriguingly, Smith *et al*. (1998) also found that all-female broods were associated with sex-linked inheritance of certain colour pattern genes. The aim of this study was to identify the cause of female-biased sex ratios in *D. chrysippus*.

MATERIALS AND METHODS

Sex ratio, inheritance and survivorship of the sexes

Wild adult female butterflies collected from Kampala, Uganda in March 1997, April 1997 and

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July 1998 were taken to either Watamu, Kenya (1997) or Cambridge, UK (1998) for breeding experiments. The milkweeds *Asclepias* spp., *Calotropis* spp., *Pergularia* sp. and *Araujia sericofera* were used for oviposition and larval food. The March 1997 and 1998 collections were made at random while only broods with low egg hatch rates were reared from the April 1997 collection (these broods are excluded from all analyses of hatch rates). Following emergence, males were allowed access to sources of pyralizidine alkaloids which are necessary for sex pheromone production. Females were then mated to these males and occasionally to wild Kenyan males due to a shortage of reared males. Their eggs and newly emerged larvae were counted on the plant to give hatch rates. Because the eggs were laid with the butterfly 'sleeved' on a single plant they often laid several eggs on a single leaf, these were removed so as to prevent egg cannibalism giving artificially low hatch rates. During the 1997 breeding experiments, survival through each larval instar, pupation and emergence were also recorded to give mortality rates at all life-cycle stages.

Antibiotic treatment

Fourth and fifth instar caterpillars from all-female matrilines were fed for 2 or more days on milkweed leaves painted on one side with a 2·5% (w/v) aqueous solution of tetracycline hydrochloride. Broods from these treated females were reared alongside untreated control broods. The egg hatch rates and subsequent sex ratio of their progeny were recorded.

Identification of the bacterium

Male-killer lines were then tested for the presence of bacteria known to cause male-killing in other insects. *Spiroplasma*-specific PCR reactions were used to test female biased and normal sex ratio matrilines for the presence of these bacteria. DNA was extracted from parental generation females using a method modified from Walsh, Metzger & Higuchi (1991). Under sterile conditions a small piece of ovary tissue was dissected from the butterflies and incubated at 56 °C for 2 h with Chelex–100 ion-exchange resin (Sigma), dithiothreitol and proteinase K, boiled and the supernatant used directly for PCR. To test for the presence of *Spiroplasma* bacteria the primers SP-ITS-J04 and SP-ITS-N55 were used. These are known to amplify the rDNA spacer region of a clade of *Spiroplasma* bacteria which contains 2 ladybird male-killers (Majerus *et al*. 1999). In order to cross-link any contaminant DNA and therefore reduce the risk of false positives, all PCR reactions were irradiated with 150 mJ of ultra-violet light using a Stratagene UV Stratalinker 2400 prior to the addition of template DNA. PCR cycling conditions in a Techne thermal cycler for the *Spiroplasma* rDNA spacer region primers were 2 min at 95 °C, 35 cycles of 20 sec 95 °C, 1 min 55 °C and 1 min 72 °C and finally 10 min at 72 °C. PCR which amplifies the rDNA ITS1 region from all insects (Hills & Dixon, 1991) was then used to check that all of the DNA isolations were, in general, suitable for PCR.

Bacterial phylogeny

The phylogenetic relationship of the *D. chrysippus* male-killer to other *Spiroplasmas* was investigated using the sequence of 16S rDNA (1523 bp at the 3′ end), the entire ribosomal spacer region (187 bp) and 63 bp at the 5′ end of the 23S rDNA. This region was amplified from 2 male-killer lineages using the Expand High Fidelity PCR system (Boehringer-Mannheim) which contains an enzyme with proof-reading activity. Two overlapping fragments were amplified using the primer pairs 27f/MGSOr and GPO1f/SP-ITS-N2 (Van Kuppeveld *et al*. 1992; Weisburg *et al*. 1991). The primer SP-ITS-N2 (5′ GGTAGTCACGTCCTTCATCG 3′), which is previously unpublished, is specific to the genus *Spiroplasma*. These PCR products were then purified with Microcon-50 Microconcentrators (Amicon Ltd) and ligated into a T-tailed vector (pGEM, Promega). These plasmids were used to transform *E. coli* DH5α which were subsequently grown on a selective agar medium containing carbenicillin, IPTG and X-Gal. White colonies, which contain the insert, were grown overnight in LB medium and the plasmids purified using the Wizard Miniprep DNA purification system (Promega). The inserts were then sequenced with the M13 plasmid primers and internal sequencing primers using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer). Both forward and reverse strands were sequenced except for about 100 bp at the 3′ end of the 16S gene for which only single-stranded sequence was obtained.

This sequence (EMBL accession no. AJ245996) was then added by eye to an alignment containing all available ribosomal *Spiroplasma* sequences of the same length. Data were thus included for *S. citri* (Accession numbers: M23942, X63781), *S. poulsonii* (M24483, AJ30955), *S. ixodetis* (M24477, AJ130954), and the male-killing symbionts from *Adalia bipunctata* (AJ006775, AJ130952) and *Harmonia axyridis* (AJ132412, AJ130953). The 16S rDNA alignment used was taken from previous studies (Hurst *et al*. 1999*b*; Majerus *et al*. 1999), and combined with that of the *Spiroplasma* ribosomal spacer region available from the EMBL alignment database under accession number DS38158 (ftp://ftp.ebi.ac.uk/pub/database/embl/align/). The most variable part of the spacer region could not be accurately aligned and were excluded from the analysis (positions 172–216, 251–272 and 308–454 in

the spacer region). A second alignment of the sequences most similar to *D. chrysippus* was then constructed which included the entire spacer region.

The phylogenetic tree of the bacteria was then reconstructed from these alignments by maximum likelihood with the program PAUP* v. 4.0.0d55 and v. 4.0b1. Account was taken of rate heterogeneity across sites using 4 discrete gamma-distributed rate categories (Yang, 1996). Rate heterogeneity and transition/transversion ratios were estimated from the data using a starting topology constructed with maximum parsimony. A heuristic search was performed with branch-swapping by nearest-neighbour interchanges and assuming the HKY85 substitution model (Hasegawa, Kishino & Yano, 1985). The resulting tree's robustness was then assessed using non-parametric bootstrapping (100 replicates) (Felsenstein, 1985). The Kishino–Hasegawa test (Kishino & Hasegawa, 1989), as implemented on PAUP*, was used to test whether the 'best' tree topology was significantly more likely than alternatives.

Prevalence

Wild butterflies were collected from locations around Africa and females tested for infection by *Spiroplasma* bacteria as described above.

RESULTS

Sex ratio, inheritance and survivorship of the sexes

Female butterflies tended to produce either approximately equal numbers of sons and daughters (Table 1A) or strongly female biased sex ratios (Table 1B). The trait was maternally inherited, although males were occasionally produced in female biased matrilines. The origin of the male parent (Kenya or Uganda) did not affect the inheritance of the trait.

The proportion of eggs surviving to second instar caterpillars in all-female broods was 41% ($n = 899$) which is approximately half the survival rate of 83% ($n = 481$) in normal sex ratio broods. This results from both lowered hatch rates and first instar mortality. Overall, hatch rates in normal sex ratio matrilines were 89% ($n = 1401$) compared to 50% ($n = 1366$) in all-female lines. Mortality in all-female broods occurred in the final stages of embryonic development when the head capsule was already tanned. Of the eggs that hatched, 91% ($n = 436$) survived to first instar in normal sex ratio broods compared to 76% ($n = 490$) in all-female broods. Both hatch rates and first instar survival were significantly lower in all-female broods (Hatch rates: Table 1; Mann–Whitney U-test: $n_1 = 52$, $n_2 = 51$, $U = 48{\cdot}5$; $P < 0{\cdot}0001$) (First instar survival: data not shown; Mann–Whitney U-test: $n_1 = 37$, $n_2 = 28$, $U = 316$; $P < 0{\cdot}01$).

Antibiotic treatment

The hatch rates in all-female matrilines recovered to between 90 and 100% following antibiotic treatment (Table 2). An outbreak of disease meant that only a small number of offspring were reared to adulthood from these broods but they included males (Table 2). The control broods from all-female lines, which were reared under the same conditions but without antibiotics, had both low egg hatch rates and female biased sex ratios (Table 1B; F2 generation in 1997 and F1 in 1998).

Identification of the bacterium

Females from all-female broods were infected with a *Spiroplasma* bacterium while normal sex ratio matrilines were uninfected (Table 1A and B). Infected females occasionally produced uninfected sons and daughters and the efficiency of vertical transmission varied between female parents (Table 1B). Additionally, infected males occasionally survived to adulthood (Table 1B). All DNA extractions were successfully amplified using insect primers indicating that the negative results obtained when testing for *Spiroplasma* bacteria were not an artefact arising from unsuitable or contaminated DNA extractions.

Bacterial phylogeny

The maximum likelihood tree shown in Fig. 1 provides strong support for a monophyletic group including the male-killer of *D. chrysippus*, 2 ladybird male-killers and a tick symbiont of unknown phenotype. However, the relationships within this clade could not be resolved. Considering all 12 possible arrangements of these 4 taxa, the most probable topology had a log likelihood of $-3494{\cdot}85$ while the least probable had a log likelihood of $-3495{\cdot}00$. This difference was not significant (Kishino–Hasegawa Test: $\tau = 0{\cdot}245$; N.S.).

In an attempt to resolve these relationships, a second maximum likelihood tree was constructed using the entire 16S and ribosomal spacer sequence of these 4 taxa (1734 alignment sites, 32 of which are variable). However, this provided no more information as all the alternative tree topologies had the same log likelihood of -2594.

Bacterial prevalence

In Uganda, 40% of females were found to be infected with the *Spiroplasma* bacterium ($n = 90$; 95% confidence interval assuming binomial sampling: 30–51%). This sample combines the 56 randomly collected females in Table 1 and a further 36 females screened by PCR. A second East African sample was collected from Watamu which lies on the

Table 1. Maternal lines of *Danaus chrysippus* showing their sex ratio, hatch rate and whether individuals of the parental generation brood were infected with *Spiroplasma* bacteria

(The infection status of the butterflies is given as the number of males (m) and females (f) which tested positive (+) or negative (−). Broods marked * were not collected randomly with respect to egg hatch rate. The number of eggs in each brood is given in parentheses after the hatch rate. (A) Contains uninfected lines and (B) contains male-killer lines on the basis of either *Spiroplasma* infection or sex ratio.)

	Parental			F1			F2			F3			Spiroplasma infection	
Line	Male	Female	Hatched	Male	Female	Hatched	Male	Female	Hatched	Male	Female	Hatched	+	−
(A) **Uninfected**														
1,97	4	8	1·00 (16)											1f
2,97	6	1	1·00 (7)											1f
3,97	5	1	0·92 (12)											1f
4,97	4	5	0·80 (15)											1f
5,97	2	4	0·79 (14)											1f
6,97	3	5	0·79 (14)											1f
7,97	6	4	0·93 (14)											1f
8,97	5	5	0·75 (16)											1f
10,97	5	5	0·92 (13)											1f
11,97	5	2	0·94 (16)											1f
12,97	8	5	1·00 (16)											1f
13,97	5	5	1·00 (16)	15	13	0·87 (31)	5	8	0·90 (20)					2f
15,97	5	6	0·88 (16)											1f
17,97	5	2	1·00 (16)											1f
19,97	5	6	1·00 (16)											1f
20,97	4	9	1·00 (16)											1f
21,97	8	5	1·00 (14)											1f
23,97	8	4	0·94 (16)	4	8	0·75 (20)	0	3	0·93 (15)					1f
				7	5	1·00 (14)								
25,97	8	4	0·94 (16)											1f
39,97*	5	6												1f
41,97*	4	4												1f
47,97*	0	2		6	5	0·96 (25)	4	5	0·91 (34)					1f
48,97*	18	2												1f
50,97*	7	4												1f
51,97*	11	10												1f
6,98	3	4	0·57 (7)											1f
7,98	4	4	1·00 (8)											1f
8,98	11	7	1·00 (49)											1f
11,98	7	8	0·81 (27)	11	5	1·00 (43)								1f
12,98	8	10	0·85 (33)											
15,98	5	4	0·68 (19)											
18,98	6	5	0·89 (38)											1f
19,98	8	10	0·79 (38)											1f
21,98	5	8	0·88 (40)	1	4	0·82 (34)								1f

22,98	10	6	0·80 (35)											
25,98	7	2	0·92 (26)											
26,98	11	8	0·95 (40)											1f
27,98	9	7	0·91 (32)											1f
31,98	14	5	0·85 (92)											
34,98	7	6	0·84 (51)											1f
37,98	6	10	0·84 (50)											1f
(B) **Male-killer**														
9,97	1	7	0·81 (16)										4f	1f, 1m
14,97	0	4	0·25 (16)	0	11	0·59 (29)	0	3	0·30 (20)				1f	
				0	10	0·40 (30)								
				0	8	0·60 (25)								
18,97	0	6	0·50 (14)	0	8	0·52 (23)	0	5	0·68 (25)	0	1	0·58 (33)	2f	
				0	6	0·29 (31)	0	3	0·33 (24)					
				0	5	0·52 (29)	0	7	0·45 (20)					
				0	3	0·39 (31)								
22,97	1	10	0·79 (14)	0	14	0·46 (35)	0	16	0·66 (32)	0	3	0·63 (16)	1f	3f, 1m
							0	8	0·71 (24)	0	7	0·53 (30)		
							0	11	0·72 (32)	0	4	0·54 (13)		
							0	7	0·79 (19)					
24,97	0	3	0·81 (16)	0	10	0·50 (30)							1f	
26,97	0	4	0·75 (16)	0	9	0·50 (30)	0	5	0·79 (24)	0	6	0·42 (19)	1f	
							0	6	0·75 (20)					
27,97	6	4	0·94 (16)										4f, 2m	
28,97	0	2		1	9	0·70 (30)	2	8	0·95 (37)				1f	
29,97*	0	4		1	7	0·47 (34)							1f	
34,97	1	10		0	6	0·60 (20)	0	1	0·47 (15)				4f	1m
35,97*	2	10											5f	
42,97*	3	18		1	5	0·31 (26)							3f, 3m	1f
53,97*	1	13											3f	1f, 1m
56,97*	6	6		1	8	0·79 (33)	0	15	0·84 (25)				1f	4f
57,97*	5	11											5f	
59,97	0	9		0	12	0·55 (33)	0	1	0·50 (4)				1f	
				0	11	0·65 (34)	0	3	0·54 (28)					
62,97	0	6											1f	
63,97*	0	5											1f	
1,98	0	4	0·16 (32)											
2,98	0	13	0·57 (21)										1f	
3,98	0	4	0·33 (30)											
4,98	0	9	0·59 (17)	0	1	0·49 (35)							2f	
5,98	0	6	0·46 (13)										1f	
9,98	0	13	0·37 (35)	0	16	0·56 (27)							2f	
14,98	0	11	0·45 (33)										1f	
20,98	0	23	0·41 (64)											
23,98	0	18	0·54 (37)											
24,98	0	18	0·29 (62)										1f	
30,98	0	5	0·26 (19)										1f	
35,98	1	14	0·49 (39)										1f	
36,98	1	14	0·49 (47)										1f	

Table 2. The hatch rates and sex ratios produced by tetracycline-treated females from male-killer matrilines

Line	Male	Female	Hatched
22,97	4	0	1·00 (4)
29,97	1	0	1·00 (25)
23,98	—	—	0·99 (76)
23,98	3	3	0·91 (56)
3,98	0	3	0·94 (35)
9,98	1	2	1·00 (5)

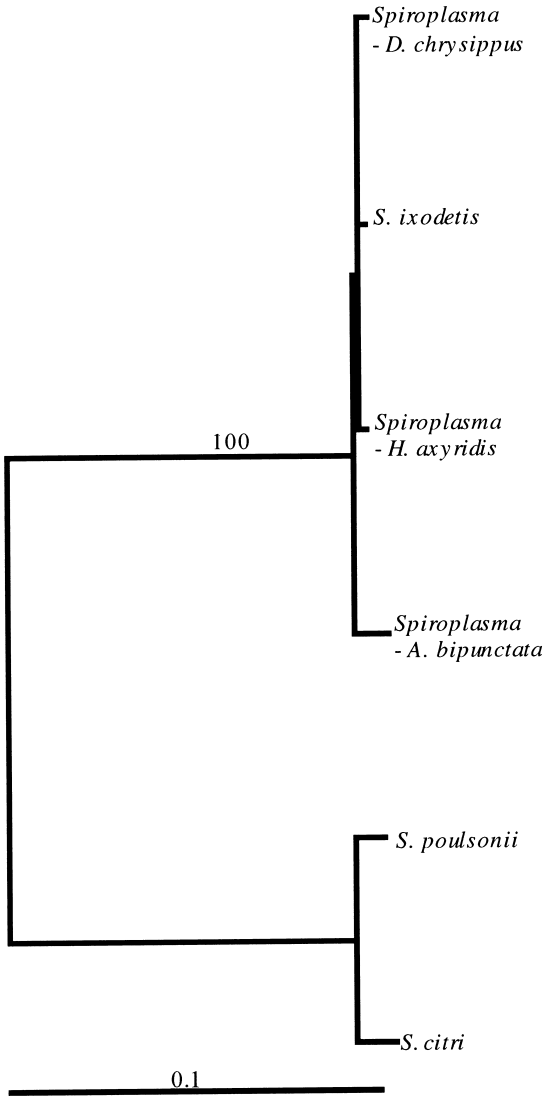

Fig. 1. Maximum likelihood tree inferred from 16S and the rDNA spacer with *Spiroplasma citri* and *S. poulsonii* as outgroups. This data set had 1645 alignment sites of which 264 were variable. The bootstrap values above the branches are only given when they are above 50. The 3 most probable trees all had identical maximum likelihoods and differed only in the branching order of *S. ixodetis*, *Spiroplasma–Harmonia axyridis* and *Spiroplasma–Danaus chrysippus*. The *Spiroplasma* bacteria of the ladybirds *Adalia bipunctata* and *H. axyridis* are both male-killers while the phenotype of the tick symbiont *S. ixodetis* is unknown.

eastern coast of Kenya, here 4% of females were infected ($n = 50$; 95% confidence interval assuming binomial sampling: 0·5–14%).

Estimates of the prevalence of the bacterium elsewhere in East Africa can be obtained from the sex ratios previously published by Smith *et al.* (1998; Appendix 1). They reported that 18% of Tanzanian females ($n = 77$; 95% confidence interval: 10–28%) and 69% of Kenyan females ($n = 62$; 95% confidence interval: 44–80%) produced female biased offspring sex ratios.

In order to check that the bacterium was not present at high prevalences or at fixation elsewhere in Africa, small samples of female *D. chrysippus* were screened for the presence of *Spiroplasma* bacteria from Ghana ($n = 6$), Oman ($n = 15$), South Africa ($n = 4$), and Zambia ($n = 3$). All these specimens tested negative for the presence of *Spiroplasma* bacteria.

DISCUSSION

This study demonstrates that sex ratio distortion in *Danaus chrysippus* is caused by a male-killing bacterium closely related to the male-killing *Spiroplasmas* of ladybird beetles. Biased sex ratios in this species were first discovered in 1968 by Owen & Chanter (1968) who concluded that this was a case of sex chromosome meiotic drive causing the butterflies to lay only female eggs. However, Smith *et al.* (1998) found mortality rates of embryos and early larval stages to be much higher in all-female lines, leading them to conclude that the trait was due to the death of males. In this study we have not only replicated these earlier results, but have also found that all-female lines are cured by antibiotics and are infected with a *Spiroplasma* bacterium. We can therefore conclude that males are not being killed by a mitochondrial gene, as thought by Smith *et al.* (1998), but by a bacterium.

Of course, male death could result from an interaction between the bacterium and a hybrid genetic background. For example, hybrid male sterility between geographical races of *Drosophila paulistorum* is caused by an inherited bacterium which, in the parental genetic background, is probably a beneficial symbiont (Ehrman & Kernaghan, 1971). However, hybrid breakdown seems unlikely in the case of *D. chrysippus*. If the bacterium were a beneficial symbiont, it would be expected to occur at or near fixation in at least one of the parental populations, but the limited data of our surveys of 3 populations failed to find any evidence for the bacterium outside East Africa. Furthermore, other bacteria in the genus *Spiroplasma* are insect and plant pathogens or commensals, which suggests that the *D. chrysippus* symbiont is unlikely to be maintained as a mutualist (Bove, 1997; but see Ebbert & Nault, 1994).

It is therefore most likely that the bacterium benefits from killing male hosts, as imperfect vertical transmission efficiency would otherwise lead to its rapid extinction in the population. This is surprising because *D. chrysippus* lays its eggs singly and scattered between plants, although in the absence of experimental data it is premature to rule out sibling competition or cannibalism. Zalucki & Kitching (1982) investigated the oviposition behaviour of the related butterfly *Danaus plexippus* and found that 12% of the eggs they laid were in 2s or 3s on a single leaf and isolated plants in the wild carried up to 22 eggs. Under these conditions sibling competition and even cannibalism could occur. Moreover, competition may be common among phytophagous insects even where there is little visible defoliation (Denno, McClure & Ott, 1995).

Two additional factors that can maintain male-killers in their host populations are deleterious inbreeding and horizontal (infectious) transmission. Infected females benefiting from avoiding deleterious sibling matings (Werren, 1987) can be ruled out as, not only are adult *D. chrysippus* highly mobile, but males cannot mate for several days after emergence because they must first collect chemical precursors to their sex pheromone (Schneider, 1987). Male-killing microsporidia in mosquitoes are maintained in their host populations because they are liberated from the dead males to infect other hosts (Bechnel & Sweeney, 1990). However, the male-killers which are known to spread through horizontal transmission kill their hosts late in their development as this means more pathogens are liberated into the environment (Hurst, 1991).

The genus *Spiroplasma* consists of 2 major clades which diverged between 200 and 400 million years ago (Hurst *et al*. 1999*b*). Male-killing has evolved in both of these clades, one clade contains the *Drosophila willistoni* male-killer, *Spiroplasma poulsonii*, and the other includes 2 ladybird beetle male-killers and the *D. chrysippus* male-killer (the only other member of this group is a tick symbiont of unknown phenotype) (Hurst *et al*. 1999*b*; Majerus *et al*. 1999). Therefore, this clade of bacteria occurs in 2 arthropod classes and causes male-killing in 2 insect orders. This raises several questions. First, has there been a single evolution of male-killing in this clade? If this is the case then there may be common sex-specific cues in female heterogametic butterflies and male heterogametic beetles that are exploited by the bacteria. The phenotype of other bacteria in this clade would be of particular interest: could these bacteria have evolved a similar diversity of phenotypes as are found in the *Wolbachia* or are they specialist male-killers?

There remain several unanswered questions concerning the *D. chrysippus* male-killer. First, the bacterium has strong geographical variation in its prevalence and is probably restricted to East Africa. In order to maintain this pattern in the face of large-scale butterfly migrations (Smith & Owen, 1997), either the vertical transmission efficiency of the bacterium or the selection pressures favouring the bacterium must vary geographically. For instance, sibling competition might be strongest in East Africa because of some unique aspect of host plant ecology. Second, Smith *et al*. (1997) found that the sex ratio of adults caught varies seasonally in Tanzania, a pattern which was replicated over 3 years. This most likely results from geographical variation in the prevalence of the male-killer coupled with migration of butterflies into and out of the sampling site as they follow the monsoon rains (Smith & Owen, 1997). However, it may also be due to changing selection pressures or vertical transmission rates.

Perhaps most surprisingly of all, Smith *et al*. (1988) reported that the male-killer was associated with particular adult colour patterns. Moreover, these colour pattern elements were always sex linked in all-female broods while in normal sex ratio broods they could be either sex linked or autosomally inherited. We have no explanation for this unusual observation except to note that the W-chromosome will hitchhike with a male-killer and this chromosome could carry polymorphic genes that affect the colour pattern. This could produce an association between the presence of the male-killer and colour pattern inheritance.

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