High-prevalence male-killing *Wolbachia* in the butterfly *Acraea encedana*

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Abstract

Inherited bacteria which kill males during early development are widely distributed throughout the insects, but have been little studied outside of a single family of beetles, the Coccinellidae. We have investigated a male-killing bacterium discovered in the butterfly *Acraea encedana*. This bacterium belongs to the genus *Wolbachia* and is identical in *wsp* gene sequence to a male-killer in the closely related butterfly *A. encedon*, suggesting that it has either recently moved between host species or was inherited from a common ancestor of the butterflies. The prevalence of *Wolbachia* is remarkably high, 95% of females are infected and only 6% of wild caught butterflies are male. Measurements of the vertical transmission efficiency were used to calculate that this high prevalence is the result of infected females producing at least 1.79 times the number of surviving daughters as uninfected females (lower confidence limit is 1.25).

Introduction

Maternally inherited genetic elements which kill males during early development are widespread in insects, having been recorded in at least 25 species belonging to five different orders. It is thought that these male-killers have invaded and are maintained in host populations because the death of males benefits their female siblings, who will pass the male-killer vertically onto the next generation. Most of the recent research effort into male-killers has concentrated on the ladybird beetles (Coccinellidae) and in this group several generalizations have become apparent.

Male-killing in ladybirds is thought to be caused by inherited bacteria because, in all of the species investigated, the trait has proved to be curable by either heat or antibiotics. The advent of PCR-based techniques has permitted the taxonomic identification of these bacteria from four species of ladybird. The bacteria belong to four clades: the genus *Spiroplasma* (*Adalia bipunctata* and *Harmonia axyridis*; Hurst *et al*., 1999c; T. M. O. Majerus, unpublished observations), the Flavobacteria (*Adonia variegata* and *Coleomigilla maculata*; Hurst *et al*., 1999a, and Hurst *et al*., 1997a) and two related genera of α-Proteobacteria, *Rickettsia* (*Adalia bipunctata*; Werren *et al*., 1994) and *Wolbachia* (*A. bipunctata*; Hurst *et al*., 1999b). The most studied of the ladybirds, *A. bipunctata*, is itself host to three different male-killing bacteria; a *Spiroplasma*, a *Wolbachia* and a *Rickettsia* (Hurst *et al*., 1999b).

These bacteria have probably invaded their host populations owing to high levels of sibling egg cannibalism in ladybirds; the death of males both provides resources to females which eat the male eggs and prevents the females themselves being eaten by their brothers (Hurst *et al*., 1997b). Despite this selective advantage of the male-killing cytotype relative to the uninfected cytotype, the observed prevalences of ladybird male-killers tend to be between 5% and 50%. The reason for the male-killers not spreading to fixation is that the bacteria have imperfect vertical transmission (Hurst & Majerus, 1993) which can lead to the establishment of stable equilibrium prevalences (Hurst, 1991).

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The extent to which ladybirds can be used as a model for the causes and dynamics of male-killing in other taxa is unknown and further research is needed. A suitable group to study are the Nymphalid butterflies, three species of which are known to be infected with male-killing agents. The recorded prevalences are mostly higher than in ladybirds, 61% of female *Hypolimnas bolina* are infected (Clarke *et al*., 1983), 61–97% in *Acraea encedon* (Chanter & Owen, 1972; Gordon, 1982; Jiggins *et al*., 1998) and 19% (Tanzania) to 60% (Kenya) in *Danaus chrysippus* (Smith *et al*., 1998; F. Jiggins, unpublished observations). The female-biased population sex ratios which will result from such high prevalences are likely to have important consequences for sexual selection in these butterflies. To date, the causative agent of male-killing has been identified only in one butterfly, *A. encedon,* which was found to be infected with a male-killing *Wolbachia* (Hurst *et al*., 1999b).

We chose to investigate *A. encedana* (Lepidoptera: Nymphalidae) as a likely species to be host to male-killing bacteria. This butterfly, which occurs in swampy areas across much of sub-Saharan Africa, is closely related to *A. encedon* (Pierre, 1976), the host of a male-killing *Wolbachia*. In both species, sibling competition and cannibalism is likely to occur as they lay their eggs in clutches of over 100 eggs. This may therefore leave populations of these butterflies vulnerable to invasion by male-killing bacteria. Working in Ghana, Gordon (1984) reported female-biased sex ratios in a population of *A. encedana*. Biased population sex ratios were also suspected in Uganda where Owen & Chanter (1969) recorded predominantly female populations of *A. encedon* which were later realized to be a mixture of both *A. encedon* and *A. encedana* (Owen *et al*., 1994). The aim of this study was to identify the cause of these female-biased sex ratios and compare the nature and prevalence of the element to the sex ratio distorters of *A. encedon* and other insect groups.

Materials and methods

Sex ratio and inheritance

Lines of *Acraea encedana* were established from wild egg batches or wild females collected from around Kampala, Uganda, between February and April 1998. Caterpillars were reared both in Kampala, Uganda and Cambridge, UK, on the host plant *Desmodium salicifolium* (Leguminosae). Females were mated to males from the male-producing lines and maternal lines were continued for up to two subsequent generations. The temperature at which the larvae were reared was not controlled.

Hatch rates and antibiotic treatment

The egg hatch rates were measured by counting eggs from photographs and counting larvae on emergence. Hatch rates from up to 10 females per brood were recorded. Females were antibiotic treated by feeding fifth instar caterpillars for two or more days on leaves painted on one side with 2.5% w/v aqueous tetracycline hydrochloride. The untreated lines were reared alongside the treatments as controls.

Wolbachia-specific PCR

DNA was extracted from butterfly ovaries by phenol-chloroform extraction (Sambrook *et al*., 1989) from all-female, female biased, normal sex ratio and antibiotic-cured matrilines. Using these extractions as templates, PCR was performed with *Wolbachia*-specific *wsp* primers (81F, 691R; Zhou *et al*., 1998). Cycling conditions on a Techne Progene cycler were 95 °C for 2 min followed by 35 cycles of 95 °C for 20 s, 55 °C for 30 s and 72 °C for 30 s and finally 72 °C for 10 min. Prior to the addition of template DNA, all PCR reactions were irradiated with 150 mJ ultraviolet light in a Stratagene UV Stratalinker 2400 so as to cross-link any contaminant DNA and reduce the risk of false positives. To confirm these results and determine to which *Wolbachia* clade the bacterium belongs, PCRs were performed on the same templates using B-group *Wolbachia*-specific *ftsZ* primers (*ftsZ*Bf, *ftsZ*Br; Werren *et al*., 1995) and B-group *Wolbachia*-specific 16S rDNA primers (16SBf, 16SBr; Werren *et al*., 1995) under the same cycling conditions as for the *wsp* primers. To check that the DNA extractions had been successful, PCR was performed using primers which amplify the ITS1 region from all insects (Hillis & Dixon, 1991).

Test for bacteria other than *Wolbachia* in butterfly ovaries

Template from two male-killer matrilines was tested for the presence of bacteria other than *Wolbachia* following the methods of Hurst *et al*. (1999b). PCR was performed using 16S rDNA primer sequences which are conserved throughout the Eubacteria (27f, 1495r; Weisburg *et al*., 1991). The PCR product was then cloned into a T-tailed vector (pGEM, Promega) and transformed into *E. coli* DH5α, the bacteria being grown for only 45 min prior to plating to prevent duplication of clones. The colonies were picked and grown on a clean LB-carbenicillin plate to avoid any contaminating *Wolbachia* DNA on the original plate and then pUC primers were used to determine which colonies bore inserts. These clones were then tested to see if the insert was derived from *Wolbachia* using the B-group *Wolbachia*-specific 16S rDNA primers described above. Blue colonies, which bear a plasmid with no insert, were used as negative controls.

Sequencing the *Wolbachia wsp* gene

The *wsp* gene was amplified as above from two females belonging to different male-killer matrilines using

polymerase with proof-reading activity (Expand High Fidelity PCR system; Boehringer-Mannheim, Germany). The product was purified using Microcon-50 Microconcentrators (Amicon Ltd). Both strands were sequenced in totality direct from the PCR product using the PCR primers. Sequencing was performed via cycle sequencing using the ABI PRISM Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer), visualizing the results on an ABI 384 automated sequencer (Perkin Elmer).

Prevalence of *Wolbachia*

Wild adult butterflies were collected from an area of pasture bordering a swamp at Kajansii, which lies between Kampala and Entebbe in southern Uganda. The butterflies were caught from an aggregation similar to those described by Owen & Chanter (1969). The slow flight of the butterflies and the small area of the colony probably allowed most of the butterflies flying on a given afternoon to be caught, as by the end of each sampling period there were few butterflies left in the study site. Butterflies were collected during the afternoon at fortnightly intervals (or the nearest sunny day) between 15 February 1998 and 17 May 1998. The sex ratio of captured butterflies was recorded. Twenty females from each fortnightly sample were tested for the presence of *Wolbachia* using *wsp* PCR primers as described above. DNA extractions which were negative for *Wolbachia* were tested using PCR primers which amplify the ITS1 region of all insects (Hillis & Dixon, 1991) to confirm the successful extraction of butterfly DNA. Seven butterflies from uninfected lines were used as controls against false positives owing to contamination. Storage, DNA extraction and PCR of these controls were all carried out alongside the wild females being tested.

Vertical transmission rates

Vertical transmission rates in wild butterflies were measured by collecting wild broods of eggs, first or second instar caterpillars. Sibling caterpillars are easily identified as they remain in tight groups. The broods were then reared to second or third instars and nine or 10 caterpillars from each brood were tested as above for infection with *Wolbachia*. Broods which contain a mixture of both infected and uninfected larvae provide evidence for imperfect vertical transmission.

Results

Identification and curing of male-killing lines

Nineteen of 24 broods from the parental generation consisted entirely of females; two were female biased and three were of normal sex ratio (Table 1). Females from all-female broods produced entirely daughters in the F1 and F2 generations whilst females from normal sex ratio broods produced both sons and daughters (Table 1). Females from the two female-biased parental generation broods produced either entirely female offspring or normal sex ratios (Table 1). The overall sex ratio of the nine normal sex ratio broods (Table 1) is significantly male biased ($\chi^2 = 4.97$, d.f. = 1; $P < 0.05$), primarily due to a male bias in two broods from the same matriline. All-female broods had hatch rates approximately half those of normal sex ratio broods (Fig. 1) and were characterized by the presence of unhatched eggs containing fully developed embryos, their tanned head capsules clearly visible. In contrast, the death of larvae during late embryogenesis was rarely recorded in normal sex ratio broods.

Butterflies from all-female matrilines produced sons after being fed on antibiotics. In total, four butterflies were treated with antibiotics and they produced sex ratios (males:females) of 1:0, 1:2, 12:12 and 7:6. Control females reared under the same conditions and at the same time but without antibiotics produced entirely daughters (Table 1, F_2 generation).

Table 1 The inheritance of sex ratio arranged in maternal lines.

	Parental		F_1		F_2	
Sex ratio	Male	Female	Male	Female	Male	Female
All-female	0	22	0	18	0	20
					0	46
All-female	0	28	0	43	0	5
All-female	0	17	0	40	0	2
					0	8
					0	7
All-female	0	13	0	10	0	7
			0	28	0	10
All-female	0	11				
All-female	0	52	0	3		
All-female	0	52	0	13	0	5
All-female	0	17	0	6		
All-female	0	21	0	13		
All-female	0	23	0	8		
All-female	0	29				
All-female	0	17				
All-female	0	47				
All-female	0	12				
All-female	0	68				
All-female	0	11	0	16		
All-female	0	7				
All-female	0	21				
All-female	0	11				
Normal	9	14	20	6	28	13
Normal	12	12	7	4		
Normal	15	16	19	18	3	1
					6	3
Female bias	16	34	25	21	6	1
					12	9
Female bias	3	7	0	32		
			0	10		

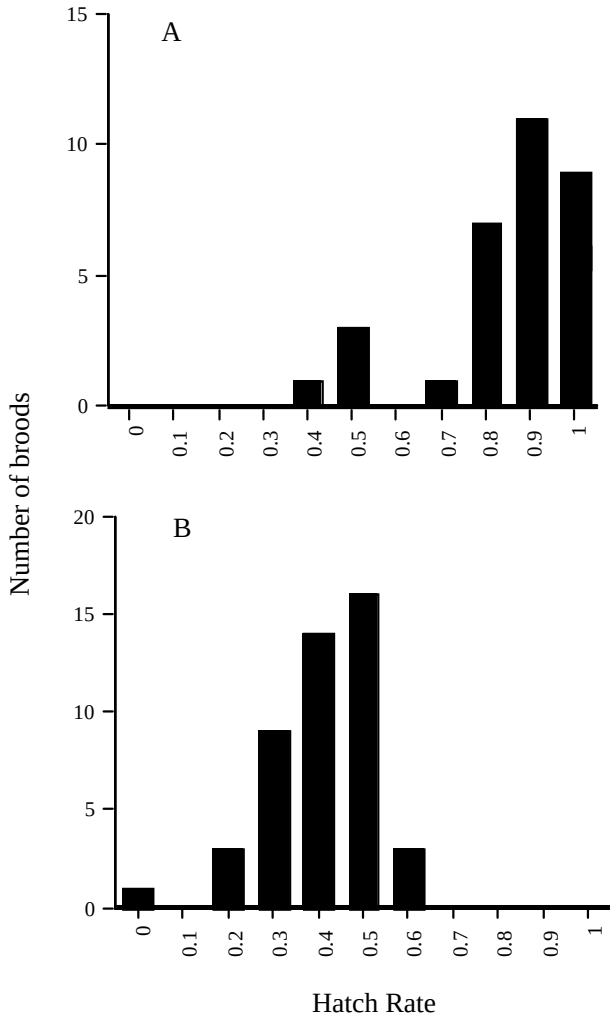

Fig. 1 Histogram showing the proportion of eggs which hatched in normal sex ratio (A) and all-female (B) broods. Clutch sizes range from 21 to 201 eggs. Hatch rates in all-female matrilines are less than those in normal sex-ratio lines (Mann–Whitney *U*-test: $n1 = 46$, $n2 = 32$; $U = 45$; $P < 0.0001$).

Association of *Wolbachia* with male-killing

The presence of *Wolbachia* was initially detected using the *wsp* primers described above. *Wolbachia* was present in each of 14 females taken from different all-female broods of the parental generation (Table 1) but was absent from three females and one male taken from different antibiotic cured broods. The three normal sex ratio parental generation broods containing males (Table 1) were also found to be uninfected with *Wolbachia* (14 females tested in total). The two female-biased parental generation broods (Table 1) consisted of a mixture of infected and uninfected females. Two females were tested from the brood consisting of three males and seven females; one was infected and one uninfected. The infected female produced entirely female progeny in the F1 (0 males and 32 females). Seven females were tested from the brood consisting of 16 males and 34 females; four were infected and three uninfected, and the F1 from one of the uninfected females had a sex ratio of 25 males to 21 females. These results were all replicated using both B group *Wolbachia*-specific 16S rDNA and B group *Wolbachia*-specific *ftsZ* primers. All templates were amplified using general insect primers.

To confirm that *Wolbachia* is the only bacterium associated with the trait, 16S rDNA was amplified and cloned from all Eubacteria present in the ovaries of two butterflies. In total, 83 clones contained inserts and all of these were derived from *Wolbachia* while seven negative controls (blue colonies) tested negative for *Wolbachia* (note this procedure could miss inherited bacteria which are not passed through the ovaries). We can thus conclude that the sex ratio distortions in *A. encedana* are caused by a B group *Wolbachia* that kills males during early embryogenesis and that vertical transmission of the bacterium by wild females is imperfect.

Phylogenetic affiliation of the *Wolbachia*

The *wsp* gene sequence of the *A. encedana Wolbachia* from two butterflies was identical to that of the male-killing *Wolbachia* of *A. encedon* (Accession no. AJ 130716). A phylogeny showing how this male killing *Wolbachia* relates to other *Wolbachia* is given by Hurst *et al.* (1999b).

Prevalence

Ninety-five per cent (133 of 140) of wild female *A. encedana* were infected with *Wolbachia*. Using binomial sampling, this gives a 95% confidence interval for the proportion of females infected with *Wolbachia* as 90–98%. The DNA extractions of the three uninfected butterflies were amplified using a general insect PCR, indicating that these extractions were of good quality. The breeding data (Table 1) are not included in this data set as they are biased towards collections from low-prevalence populations, as breeding butterflies is difficult in the absence of males. Ninety-six per cent (811 of 859) of the butterflies caught at Kajansii during the study period were females. This strong female bias appears to be typical of the Kampala region as butterflies collected opportunistically elsewhere in this area from February until August 1998 had a similar sex ratio (96% females, 289 of 306). These data must be treated with caution as field captures of butterflies are often sex biased, usually towards males, owing to sex differences in behaviour and longevity.

Inheritance rates

Nine or 10 larvae from 17 wild clutches were tested for the presence of *Wolbachia*. One clutch (10 larvae) was entirely uninfected. All the larvae of 15 clutches were

infected (11 clutches of 10 larvae and four clutches of nine larvae). One clutch provided evidence that vertical transmission was imperfect in the wild, consisting of three infected and six uninfected larvae. All the DNA extractions were amplified using general insect primers. The observation that two of 21 parental generation clutches (wild collected adults or eggs) showed imperfect vertical transmission (Table 1) provides further evidence of imperfect vertical transmission of the bacterium.

Summing across laboratory-bred females and wild collected clutches, four of 41 females were uninfected, assuming that the clutch from the wild in which no individuals were infected derives from an uninfected parent. Of the remaining 37 females found to be infected, three (8.1%) showed imperfect vertical transmission. Using binomial sampling, this gives 95% confidence intervals for the proportion of infected females showing inefficient transmission of 1.7–18% of infected females. Amongst the three individuals showing inefficient transmission, around 50% of progeny were uninfected. This means that the 95% confidence interval for mean transmission efficiency is roughly between 0.85% loss per infected female per generation (i.e. 99.15% efficiency) and 9% loss (91% efficiency).

Estimating the effect of the male-killing bacterium on female host fitness

The dynamics of male-killing bacteria are well characterized theoretically (Hurst, 1991). These models assume that the transmission efficiency of the bacterium to offspring is the same in all infected females. However, our data suggest that there is a bimodal distribution of transmission efficiencies: some females pass the bacterium to all their offspring while other females pass the bacterium to only about 50% of their offspring. Below, we present a modified model of the dynamics of male-killing bacteria that takes account of heterogeneity in vertical transmission efficiency and then use this model to estimate the benefit that the survivors of male-killed broods gain from the death of their brothers.

We assume that the bacterium has no direct physiological effect on host fitness, that it does not transmit horizontally and that the host is outbred. The male-killing bacterium exists in the population at a frequency p amongst females. A proportion α of these infected females passes the bacterium to all their eggs while the remainder of females ($1 - \alpha$) transmit the bacterium with a probability of t to their eggs.

The death of males may benefit the survivors in the same brood, for instance by reallocation of resources. In broods in which 100% of the eggs have inherited the bacterium, the relative reproductive output (lifetime egg production) of infected relative to uninfected females is b. However, in the broods showing imperfect vertical transmission, fewer males will die and this may mean that survivors in these broods benefit less. Females in these broods have a reproductive output relative to uninfected females of an unknown function of b and t, $fn(b,t)$.

The frequency of the male-killer in the subsequent generation, p', will be

$$p' = (p\alpha b + p(1-\alpha)tfn(b,t))/ \\ (1 - P + p\alpha b + p(1-\alpha)fn(b,t)).$$

The equilibrium prevalence ($p = p'$) in females, p^*, is given by

$$p^* = (1 - \alpha b - (1-\alpha)tfn(b,t))/(1 - \alpha b - (1-\alpha)fn(b,t)).$$

In the *Acraea* system, we have estimated p, α and t from our data. The estimate for p is 0.95, α is 0.92 and t is 0.50. We then estimated the value of b under a conservative assumption about $fn(b,t)$, assuming that the benefit that surviving females gain from male death is directly proportional to the transmission efficiency, t (linear decline; $fn(b,t) = (bt)$. If $fn(b,t) = bt$, our data produce an estimate $b = 1.79$.

We estimated the 95% confidence space for the proportion of infected individuals in the population and the proportion of infected individuals that showed inefficient transmission jointly, using a maximum likelihood approach. In short, the two values were assumed to be binomial samples, and the joint log likelihood of different combinations calculated. We then calculated the maximum likelihood (that of the observed data: 95% prevalence, 8% of individuals showing inefficient transmission). Following this, we calculated the 95% confidence space as being delineated by pairs of values for prevalence and inefficiency with log likelihood within 0.5 χ^2 ($P = 0.05$) (2 d.f.) of this value (Kalbfleisch, 1985). Along this boundary, we calculated b, and the lower confidence limit for b is the lowest value of b at this boundary. If $fn(b,t) = bt$, our data produce a lower 95% confidence interval $b = 1.25$.

Our model also indicates that variation between broods in their transmission efficiency acts to increase the prevalence of male-killers. For instance, if our data had the same mean transmission efficiency but without any variation between broods ($\alpha = 0$, $t = 0.96$) this would produce an estimate of $b = 5.2$ ($fn(b,t) = bt$; $P = 0.95$).

Discussion

This paper reports the third record of an insect infected by a *Wolbachia* bacterium that kills males. The sequence of the rapidly evolving *wsp* gene is identical in the male-killers of *A. encedon* and *A. encedana*, suggesting that they represent a single evolution of male-killing by *Wolbachia*. This sequence similarity is in striking contrast to the male-killing *Wolbachia* of the beetle *Adalia bipunctata*, which varies at 38 nucleotides in the sequence of its *wsp* gene, implying that this *Wolbachia* evolved to kill males over a million years ago (Hurst *et al.*, 1999b). It is not possible to date accurately the maximum age of the

common ancestor of the *A. encedon* and *A. encedana* male-killers owing to heterogeneity in the rate of *wsp* sequence evolution. A rough approximation can, however, be given as the *wsp* gene has a rate of evolution nearly 10 times that of 16SrDNA (Zhou *et al.*, 1998) which in turn has a substitution rate of 1–2%/50 Myr (Moran *et al.*, 1993). Therefore, the average time for a single *wsp* nucleotide difference to appear in the 555 base pairs sequenced in either of the two lineages following divergence is 450 000 years.

The infection of two different hosts by the same *Wolbachia* strain has three possible explanations. First, the bacterium may have been transmitted horizontally between the two butterfly species or from a third species, perhaps owing to them living in close proximity (*A. encedon* occasionally feeds upon *D. salicifolium,* the host plant of *A. encedana,* F. M. Jiggins, unpublished observation) or because they share vectors for the transmission of *Wolbachia*, such as parasitoids. Second, the *Wolbachia* may have crossed between species by hybridization and subsequent introgression. Matings between the two species are not uncommon in the wild (Owen *et al.*, 1994) but individuals with intermediate genitalia have never been observed and we found matings between male *A. encedon* and female *A. encedana* ($n = 3$) produced inviable eggs. Were hybrid introgression of *Wolbachia* to occur then both the mtDNA and the W chromosome of one species would hitch-hike through populations of the second species, which could potentially confound phylogenies of closely related species reconstructed using mtDNA sequence. Finally, *A. encedon* and *A. encedana* may have only recently speciated and the male-killing bacterium has been inherited from a common ancestor.

Perhaps the most extraordinary aspect of this system is that 95% of wild females are infected by the bacterium. This is comparable with male-killer prevalences observed in *A. encedon* which range from 61% in Tanzania (Owen & Smith, 1991) to 100% in Uganda (Jiggins *et al.*, 2000) and contrasts strikingly with studies in other insect taxa in which male-killers typically infect under 25% of the population. In order to explain this high prevalence we have modelled the dynamics of the *A. encedana* male-killer and measured the relevant parameters in a natural population.

A sex ratio distorter with perfect vertical transmission efficiency and no suppressers is expected to either spread to fixation or be lost from the population (Hamilton, 1967). However, if the element is not transmitted to all the offspring of infected females then this may lead to the maintenance of a stable polymorphism. The equilibrium prevalence of male-killers is thought to depend upon three parameters: the efficiency of transmission, direct physiological effects of the bacterium on female fitness and any increase in female survivorship or fecundity resulting from male death (Hurst, 1991). We have extended earlier models of male-killer dynamics to take account of variation between females in the efficiency with which they transmit the bacterium to their offspring. We then used this model to investigate the dynamics of the *A. encedana* male-killer using measurements of the bacterium's prevalence and vertical transmission efficiency.

The benefit of male-killing behaviour (the increase in lifetime egg production of females from male-killed clutches vs. normal as a result of the death of their brothers) was calculated to be 1.79 (lower 95% confidence interval 1.25). This estimate of the benefit resulting from male-killing is very high. In the only other system in which this has been estimated, the ladybird *Adalia bipunctata* infected with a *Rickettsia*, a ratio of 1.16 was calculated. From this, it is clear that explanations for prevalence incorporating group selection against infection (Heuch, 1978; Heuch & Chanter, 1982) can be rejected. Group selection acts to decrease prevalence, through increasing unmatedness of females in groups with high prevalence. The problem in *A. encedana* is not to see what keeps prevalence down, but in fact what creates such a high prevalence in the face of the observed inefficient vertical transmission.

The benefit of male-killing to females from male-killed clutches is at the upper range of that which is ecologically realistic. Given the high value obtained, future research should focus on measuring the magnitude of b directly, and examining whether the assumptions of the model are violated in anyway such as to cause us to overestimate b. One particular assumption deserves attention in the light of recent work (Bandi *et al.*, 1998; Vavre *et al.*, 1999). This is that the infection has no direct physiological costs or benefits to its host. Violation of this condition would explain a value of b higher than that observed, and test of this assumption is clearly timely.

The high prevalence of infection means that *Wolbachia* will be a powerful force in the evolution of *A. encedana.* The Kajansii population of *A. encedana* contained a significant number of virgin females (F. M. Jiggins, unpublished observation) suggesting that female reproduction may be sperm limited. This could potentially reverse sexual selection and lead to female competition for males and choosy males. Males in the Kajansii population will have an average reproductive success perhaps 20 times that of females, with obviously profound consequences. In particular, this selection will favour the spread of suppressers both of bacterial transmission and the male-killing phenotype. In this respect it is interesting to note that the transmission efficiency of the bacterium shows strong and possibly discrete variation between females, possibly indicating the presence of resistance genes. The sex ratio of one of the three uninfected lines of *A. encedana* was male biased; this could either be an artefact of sex-specific mortality under crowded laboratory conditions or it may suggest genetic variation for sex ratio. Selection favours a 1:1 ratio of investment in the two sexes, so a male-biased primary sex ratio is not expected in response to high male

mortality (Werren, 1987). However, an autosomal gene which biases the primary sex ratio towards males exclusively in broods which are not infected with the male-killer could probably invade a population.

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