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Wolbachia infection associated with all-female broods in *Hypolimnas bolina* (Lepidoptera: Nymphalidae): evidence for horizontal transmission of a butterfly male killer

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Inherited bacteria that kill male hosts during embryogenesis infect a wide range of insect species. In order to ascertain if there are patterns to host infection, with particular male killing bacteria specialising on particular taxa, we investigated the male killing trait in the butterfly *Hypolimnas bolina*. All-female broods were first reported in this species in the 1920s. Investigation of this system in the Fiji Islands revealed the causal agent of sex ratio distortion in *H. bolina* to be a male killing *Wolbachia* bacterium. This bacterium is identical in *wsp* and *ftsZ* sequence to a male killer in the butterfly *Acraea encedon* in Tanzania, suggesting it has moved between host species, yet retained its phenotype. The prevalence of the *Wolbachia* was calculated for three different island groups of Fiji, and found to vary significantly across the country. Antibiotics failed to cure either the male killing trait or the *Wolbachia* infection. The implications of these results are discussed.
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Introduction

Inherited microorganisms are carried by more than one-fifth of all insects (Werren *et al*, 1995). These bacteria and protists typically inhabit host cell cytoplasm and show maternal transmission. As their fitness in males is zero, selection favours strains of these microbes that produce a female bias to their host's sex ratio. Three different forms of sex ratio manipulation have been recognised: parthenogenesis induction, feminisation and embryonic male killing (Stouthamer *et al*, 2001). Of these a single bacterium, *Wolbachia*, is the only documented causal agent of parthenogenesis induction (Stouthamer, 1997). Feminisation is also associated with a single bacterium, again *Wolbachia*, as well as a range of protists (Rigaud, 1997). Embryonic male killers, in contrast, are much more diverse, deriving from five different bacterial clades separated by 2000 million years (Hurst *et al*, 1997). The lineages are: members of the genera *Rickettsia* and *Spiroplasma*, strains of *Wolbachia pipientis*, the gamma proteobacterium *Arsenophonus nasoniae*, and un-named strains from the Bacteroides – Flavobacteria division. To date, the Rickettsia and Flavobacteria solely infect beetles (Werren *et al*, 1994; Hurst *et al*, 1999a). Male killing *A. nasoniae* have only been recorded from the wasp *Nasonia vitripennis* (Werren *et al*, 1986). *Wolbachia* and spiroplasmas show much more host diversity. Five *Wolbachia* and three *Spiroplasma* male killers have been found within the insect orders Coleoptera, Diptera and Lepidoptera (Williamson and Poulson, 1979; Hurst *et al*, 1999b, c, 2000; Fialho and Stevens, 2000; Jiggins *et al*, 2000a, b).

Clearly some male killers are present in many different host groups. What is unclear, as yet, is whether some strains are restricted to particular host taxa. Restrictions might arise following specialisation on a particular host sex determination system: *Wolbachia* and spiroplasmas maybe the only male killing clades that can cross this boundary. Additionally, the relative incidence of these two genera, that can infect different host taxa, is unknown.

In order to study these patterns of host infection, we need to establish two factors: which male killers affect which taxa, and how many independent evolutions of male killing there are within each clade. An example of the latter is found within the clade *Wolbachia*; the male killer infecting *Drosophila bifasciata* is in the A sub-group, the other male killing *Wolbachia* are all members of the B sub-group (Hurst *et al*, 1999c, 2000), representing different evolutions of male killing. Similarly, there are at least two evolutions of male killing in the spiroplasmas (Schulenburg *et al*, 2000).

To resolve the above controversies requires systematic identification of a wider range of male killing agents. With this in mind, one of the oldest recorded cases of sex ratio distortion in an arthropod group, that of *Hypolimnas bolina* was re-examined.

In the early 1920s, HW Simmonds bred *Hypolimnas bolina* in the Fiji Islands, discovering the occurrence of all-

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female broods in this species. (Poulton, 1923, 1927, 1928; Simmonds, 1926). He reported that the 'all-female' trait was passed from mother to daughter, and was not due to parthenogenesis. Some 50 years later, Clarke and Sheppard (1975a) studied the genetics of the female wing polymorphism in this species, collecting and breeding butterflies from all over the Indo-Pacific. During their course of study, they recorded all-female broods in matrilines from Borneo, Sri Lanka and Hong Kong, reinforcing Simmonds' findings that the trait was passed through the female line. Cytological sexing of larvae and embryos indicated that the deficiency of males was due to their very high mortality in the pre-adult stage (Clarke *et al*, 1975b). Despite obtaining negative results when testing infected females for viral particles and spirochaetes, they suggested a cytoplasmic factor to be the cause of the observed sex ratio distortion. Clarke *et al* carried out a re-survey of *H. bolina* in Fiji in 1983. They demonstrated, by breeding experiments, the persistence of the phenomenon some 150 generations after it was originally recorded (Clarke *et al*, 1983) concluding that, 'no entirely satisfactory explanation has yet been given for the persistence of all-female broods'. In this paper, *H. bolina* in Fiji is re-examined and the causal agent of all-female broods identified.

Methods

Identification of male-killing lines

In July and August 1999, adult *Hypolimnas bolina* samples were collected from Suva and Nadi on the main Island of Viti Levu, Fiji. Butterflies were taken to Colo-I-Suva (eastern coast), caged, and the females encouraged to oviposit on a host plant, *Ipomoea batatus* (Vane-Wright *et al*, 1977). Adults were provided with 'false flowers' containing 0.4 M sugar solution on which to feed. Eggs laid in the laboratory were removed from the plant and each clutch stored in a Petri dish. The total number of eggs laid, number of eggs that hatched, and numbers of grey and yellow unhatched eggs were recorded in all cases. Each clutch was coded according to matriline.

An F1 generation was reared, larvae being fed on an excess of *I. batatus* leaves. Caterpillars were initially reared in Petri dishes, and transferred to large plastic pots after the second larval moult. No more than 10 larvae were reared per pot to avoid overcrowding. Sex ratio was recorded on emergence.

Adults from the F1 generation were mated in an 8 m^3 outdoor flying cage. Outbreeding was ensured by only allowing interaction between unrelated F1 males and females. The F2 generation was reared from each matriline following the methods previously outlined.

Association of *Wolbachia* with the all-female trait

DNA was extracted from the ovaries of female butterflies and prepared for PCR analysis using the Qiagen DNA preparation kit, following the manufacturer's protocol. PCR, using primers wsp81f (5′-TGG TCC AAT AAG TGA TGA AGA AAC) and wsp691r (5′-AAA AAT TAA ACG CTA CTC CA-3′) that amplify the bacterium's *wsp* gene (Zhou *et al*, 1998), was used to assay for the presence of *Wolbachia*. All females that had been bred in the laboratory, and their progeny, were tested. Specimens that gave negative results with *wsp* primers were tested to check the DNA extractions had been successful using PCR with two oligonucleotide primers for the mitochondrial CO 1 block (Brunton and Hurst, 1998) (5′-GGA TCA CCT GAT ATA GCA TTC CC-3′) and (5′CCG GTA AAA TTA AAA TAT AAA CTT C-3′). If DNA was present (positive result with CO 1) samples were retested for *Wolbachia* presence using *wsp* primers, as indicated above. If there was no DNA (negative result with CO 1) the DNA sample was re-prepared, and the process repeated.

Sequence analysis and phylogenetic position

Bacterial *ftsZ* and *wsp* DNA was amplified from two individual infected female *H. bolina* from Fiji. The *Wolbachia ftsZ* gene was amplified using the PCR primers ftsZf1 (5′-GTT GTC GCA AAT ACC GAT GC-3′) and ftsZr1 (5′-CTT AAG TAA GCT GGT ATA TC-3′) (Werren *et al*, 1995). The PCR primers wsp81f and wsp 691r (see above) were used to amplify the *wsp* gene (Zhou *et al*, 1998). In each case, PCR product was purified using Microcon-50 Micro concentrators (Amicon Ltd). Both strands were sequenced in totality direct from the PCR product using the PCR primers. Due to the greater size of the *ftsZ* gene, internal primers were designed and used for sequence analysis: ftsZ-IntR (5′-ATC GGC GAG TTG AAA TGC-3′) and ftsZ-IntF (5′-ATA TTG GCA TAA GAG GAG-3′). The *ftsZ* and *wsp* sequences were manually aligned to previously determined *Wolbachia* sequences of these genes, and the phylogenetic affiliation of the *H. bolina Wolbachia* recorded.

Antibiotic treatment

Antibiotics (tetracycline or rifampicin) of known concentration were painted onto fresh *I. batatus* leaves and fed to different groups of fourth instar larvae. Both antibiotics have been previously demonstrated to cure insects infected with cytoplasmic bacteria (Stouthamer *et al*, 1990). Treatment was commenced when larvae reached fourth larval instar, and continued to pupation. A known amount of antibiotic was presented to larvae under each of seven different treatment regimes. The leaf area consumed per day for each treatment regime was recorded. This data, together with the concentration of antibiotic and the number of larvae in the experiment, was used to calculate the mean amount of antibiotic consumed by each larva (Table 1). Control groups from the same matrilines were fed on untreated plants. Progeny from females reared on each of the antibiotic regimes were tested for *Wolbachia* presence as previously described.

EM analysis for infecting microorganisms

Fresh F2 adult *H. bolina* females from all-female lines were dissected, and their ovaries removed. Following primary fixation in a 2% glutaraldehyde-based fixative and washing in 0.1 M sodium cacodylate buffer, the ovarian tissue was post-fixed in 1% osmium tetraoxide in 0.1 M sodium cacodylate buffer and refrigerated for 1 h. The tissue was stained with 2% uranyl acetate then dehydrated in a series of graded alcohol before being embedded in Araldite resin. Following polymerisation at 60°C overnight, specimens were cut into 70–90 nm sections and examined in a Jeol 1010 transmission electron microscope at 80 Kb.

Prevalence

Female *H. bolina* were collected from three island locations within the Fijian archipelago: Viti Levu,

Table 1 Mean amount of antibiotic consumed by larvae in infected and uninfected lines, following different treatment regimes

Antibiotic treatment regime	*Mean amount of antibiotic per larva (mg)*	
	Uninfected matrilines (normal sex ratio)	*Infected matrilines (all-female)*
0.1% Rifampicin Once	0.040 (20)	0.064 (16)
0.1% Rifampicin Alternate days	0.190 (20)	0.246 (19)
1% Rifampicin Once	0.475 (20)	0.708 (16)
1% Rifampicin Every third day	1.440 (20)	1.974 (19)
1% Tetracycline Once	0.540 (16)	0.887 (10)
1% Tetracycline Alternate days	2.443 (18)	2.417 (10)
1% Tetracycline Every day	8.43 (6)	4.07 (8)

Numbers in brackets indicate the number of larvae subjected to the regime. Treatments were commenced at fourth instar and continued till pupation (8 to 10 days).

Taveuni and Wayalailai. The specimens were preserved in 95% ethanol immediately following death. DNA was extracted from each specimen and the supernatant used directly in PCR assay using the *Wolbachia* B-group specific *wsp* primers, 522r (5′-ACC AGC TTT TGC TTG ATA-3′) and 81f (see above) (Zhou *et al*, 1998). The sequence of the wsp gene was obtained from a single infected individual from each island location to ensure the infection was the same.

Results

Identification of male-killing lines

The prevalence of the all-female trait was found to be 0.58% (n = 12) among broods reared from wild females (binomial 95% CI: lower 0.28, upper 0.85). The all-female trait showed matrilineal inheritance, reinforcing previous findings (Poulton, 1923; Clarke *et al*, 1975b) (Table 2). The adult sex ratio, in both the F1 and F2 generations, was not significantly different from 1:1 in the normal broods (χ^2 = 4.737; df = 2; NS (test for homogeneity between lines: χ^2 = 0.79; df = 10; NS)). Males only occurred in the F1 generation in one all-female matriline, in a single brood, but even here there was a strong female bias.

The hatch rates of clutches of eggs from all-female matrilines are significantly lower than those from normal matrilines (Mann–Whitney U test: n1 = 5; n2 = 7; $P <$ 0.01). No instances of sibling egg cannibalism were recorded, even in cases where newly hatched larvae were confined with their siblings and with no alternative food source.

Association of *Wolbachia* with all-female trait

PCR analysis of the laboratory reared females revealed a correlation with the presence of *Wolbachia* and the all-female trait. Template from all seven all-female matrilines gave amplification products with *wsp* primers, whereas template from five normal sex ratio matrilines did not.

Sequence analysis and phylogeny

Sequence analysis of the *wsp* and *ftsZ* genes of the *H. bolina Wolbachia* revealed them to be identical in sequence to the male killing *Wolbachia* strain from the butterfly *Acraea encedon* in Tanzania (accession numbers: *ftsZ*, AJ307075; *wsp*, AJ307076). Thus the phylogenetic position of the male killing *Wolbachia* in *H. bolina* is the same as that of the Tanzanian *A. encedon*. A phylogeny showing how this male killing *Wolbachia* relates to other *Wolbachia* is given by Jiggins *et al* (2001a).

Antibiotic treatments

Neither the sex ratio bias nor the half-hatch rate observed in all-female matrilines were affected by treatment with antibiotics. In fact no effect from antibiotic treatment was observed in either all-female or control lines. This is surprising when it is considered that some of the adults that were bred consumed 2.85 mg rifampicin, and others 8.23 mg tetracycline as larvae. PCR assay for the presence of *Wolbachia*, using template from F2 adult females who's infected mother had consumed antibiotics as a larva, revealed that the antibiotics had failed to cure the *Wolbachia* bacteria, as well as the all-female trait.

EM analysis for infecting microorganisms

Analysis of ovarian tissue from infected individuals revealed presence of double membraned prokaryotic cells approximately two microns long, each enclosed within a host vacuole (Figure 1). These prokaryotes were not present in uninfected strains. No eukaryotes were observed in either type of tissue.

Prevalence

Prevalence of the male-killer was assayed in three different areas of Fiji, as shown in Table 3. Chi-squared analysis rejects the null hypothesis of homogeneity between islands (χ^2 = 7.778, df = 2; P = 0.02) indicating variation in prevalence of the male killing trait across the Fiji Islands.

Discussion

Correlation between the presence of *Wolbachia* and the all-female trait across different matrilines, as revealed by PCR assay, indicates that *Wolbachia* is the cause of male-killing in the butterfly *H. bolina*. *Wolbachia* bacteria represent one of the most widespread causal agents of embryonic male killing, being responsible for one third of reported cases to date. Knowing this, and given the frequency of *Wolbachia* in insects and other arthropods (Stouthamer *et al*, 1999) it is perhaps unsurprising to find that we are dealing with another *Wolbachia* male killer. These findings support the view that there will be many unreported cases of *Wolbachia* male-killing in the wild, and that male-killing will be a common phenotype within the clade *Wolbachia* (Jiggins *et al*, 2001b).

The result that a male-killing *Wolbachia* is responsible for all-female broods in *H. bolina* is further reinforced by the fact that the sequences of the *wsp* and *ftsZ* genes of this *Wolbachia* are identical to those in a known causal agent of male killing. The two host species *H. bolina* and

Table 2 Hatch rates and sex ratios produced by wild (parental) female *Hypolimnas bolina* and in the subsequent F1 generation

Matriline number	*Parental Females*			*F1 Females*		
	% Hatch (n)	*Male progeny*	*Female progeny*	*% Hatch (n)*	*Male progeny*	*Female progeny*
(a) All-female broods						
1	50.96 (104)	0	37	46.88 (32)	0	12
				48.15 (189)	0	35
				53.55 (183)	0	53
2	48.48 (33)	0	15			
3	53.16 (79)	5	33	50.00 (6)	0	2
				49.40 (336)	0	38
4	41.22 (148)	0	54	45.61 (57)	0	19
				55.56 (27)	0	12
				44.44 (9)	0	4
				46.24 (93)	0	23
				40.91 (22)	0	3
5	39.44 (71)	0	16			
6	38.46 (13)	0	5			
7	39.66 (58)	0	40	43.33 (90)	0	24
				45.45 (33)	0	12
				51.02 (49)	0	17
(b) Normal sex ratio broods						
8	88.89 (341)	54	53	100.00 (33)	10	7
				100.00 (56)	11	7
				97.47 (79)	8	5
				96.34 (82)	6	9
9	93.69 (111)	38	33	100.00 (36)	8	4
				100.00 (3)	2	1
10	98.94 (282)	13	11			
11	100.00 (12)	3	5			
12	97.71 (131)	18	20			

A. encedon are only distantly related (they are in different tribes within the family Nymphalidae) but share an identical male killing *Wolbachia*, strongly implicating horizontal transmission of the male killing element. This suggests that certain *Wolbachia* strains specialise on certain host sex determination systems. In butterflies, the male is the homogametic sex, in contrast to the majority of other insects. Perhaps only particular strains of male killer are able to act on host systems of this type. More lepidopteran male killers need to be examined in order to verify this.

One notable result from this study is the failure of anti-

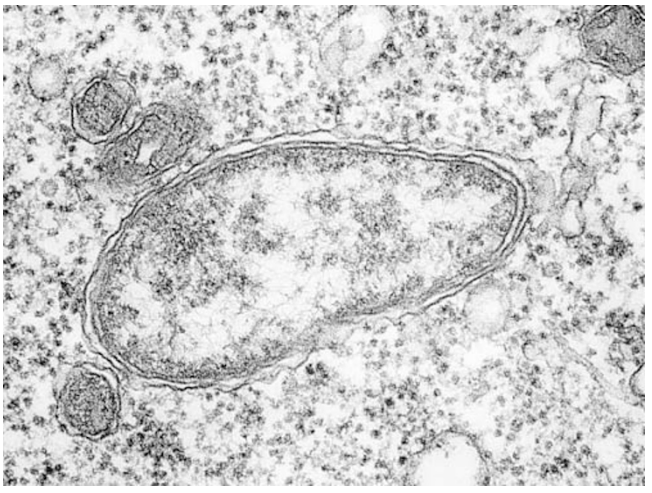

Figure 1 Transmission electron micrograph showing a prokaryotic cell enclosed in a vacuole, in ovarian tissue taken from an F2 female *Hypolimnas bolina* from an all-female line. (Original size: 2 microns).

Table 3 Percentage prevalence of *Wolbachia* male killer in *Hypolimnas bolina* females across different islands of Fiji

Area of Fiji	*Prevalence (%)*	*n*
Viti Levu Island	58.82	34
Wayalailai Island	56	50
Taveuni Island	25	24

biotics to cure the all-female trait. This does not rule out *Wolbachia* as the cause of male killing in *H. bolina* as antibiotics did not cure the *Wolbachia* infection either. There are many examples of cases in which just a single treatment with antibiotic will cure infected arthropods of sex-ratio distorting bacteria (Stouthamer *et al*, 1990). Why this should not be the case in *H. bolina* remains a mystery for further investigation. Potential explanations of the cause of failure of two different antibiotics to cure both the *Wolbachia* infection and the all-female trait in *H. bolina* are that either the *Wolbachia* are antibiotic-resistant, or that the host did not sequester the antibiotics. Antibiotic-resistant *Wolbachia* would be a very useful tool for manipulation of this bacterium, and thus the investigation of the lack of response of the trait and bacterium to antibiotics should be carried further. However, there are huge implications on other *Wolbachia*/host systems if these bacteria can develop antibiotic resistance: *Wolbachia* is thought to play an essential role in the biology and metabolism of filarial worms that cause diseases such as river blindness in humans. Elimination of *Wolbachia* using tetracycline will also kill the adult nematodes (Langworthy *et al*, 2000). The ability of *Wolbachia* to evolve antibiotic resistance, as suggested in this paper, may lower the long-term utility of antibiotics in drug treatment of filiariasis.

Wolbachia prevalence is well understood in theory, but not in practice. Data from Clarke *et al* (1975b) indicates a high degree of variation in prevalence of the male-killer in *H. bolina* across different populations. In this study, prevalence is seen to vary across the different islands of Fiji. The cause of this variation will be an important topic for future work, allowing insight into the factors producing differences in prevalence within a single host-parasite interaction. It should be possible to examine genetic and ecological characteristics in different populations to see if there is any evidence for the presence of resistance genes on certain islands, or if any correlates with life history traits and male-killer prevalence exist in this species. Notably, *H. bolina* lays eggs in clutches of 10–12 eggs in the Fiji Islands (personal observation) whereas in Australia (where Clarke found no evidence for the presence of a male killer) the species lays eggs singly or in pairs. Given the importance of sibling competition in the spread of male-killers (Hurst and Majerus, 1993), further investigation into this phenomenon across a range of populations may give an insight into possible causes and maintenance of a male killing cytoplasmic factor within a host population.

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