

Prevalence and penetrance variation of male-killing *Wolbachia* across Indo-Pacific populations of the butterfly *Hypolimnas bolina*

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Abstract

Male-killing bacteria are generally thought to attain low to intermediate prevalence in natural populations, with only mild effects on the host population sex ratio. This view was recently challenged by reports of extremely high infection frequencies in three butterfly species, raising the prospect that male killers, by making males rare, might drive many features of host ecology and evolution. To assess this hypothesis, it is necessary to evaluate how often male killers actually produce a highly female-biased population sex ratio in nature, which requires both high prevalence of infection and high penetrance of action. To this end, we surveyed South Pacific and Southeast Asian populations of *Hypolimnas bolina*, a butterfly in which extreme prevalence of male-killing *Wolbachia* bacteria has recently been recorded. Our results indicate that highly female-biased populations are common in Polynesia, with 6 out of 12 populations studied having in excess of 70% of females infected with a fully efficient male killer. However, heterogeneity is extreme in Polynesia, with the male-killing *Wolbachia* absent from three populations. In contrast to the Polynesian situation, *Wolbachia* does not kill males in any of the three Southeast Asian populations studied, despite its very high prevalence there. We conclude that male killers are likely to have significant ongoing ecological and evolutionary impact in 6 of the 15 populations surveyed. The causes and consequences of the observed spatial variation are discussed with respect to host resistance evolution, host ecology and interference with additional symbionts.

Keywords: evolution, male killing, penetrance, prevalence, selfish genetic elements, *Wolbachia*

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Introduction

Many arthropods are host to intracellular bacteria, which are transmitted vertically from female hosts to their progeny via the egg cytoplasm (Buchner 1965). This maternal inheritance, combined with low levels of horizontal transmission, results in a conflict of interest over sex allocation between the host and microorganism (Werren & O'Neill 1997). Whilst the host benefits from equal allocation to

male and female (in an outbred population), selection on the symbiont favours a bias in allocation towards the survival and production of female progeny. Microorganism-induced sex-ratio distortion is now well known in arthropods, including the induction of parthenogenesis, feminization of genetically male hosts, and selective killing of sons (O'Neill *et al.* 1997). Interest in these interactions derive mainly from the ecological and evolutionary effects that the sex-ratio distorter can have on their hosts (Hurst & Werren 2001; Charlat *et al.* 2003). At the molecular level, selection for resistance can promote evolutionary change in the host-sex determination system (Rigaud 1997). At the

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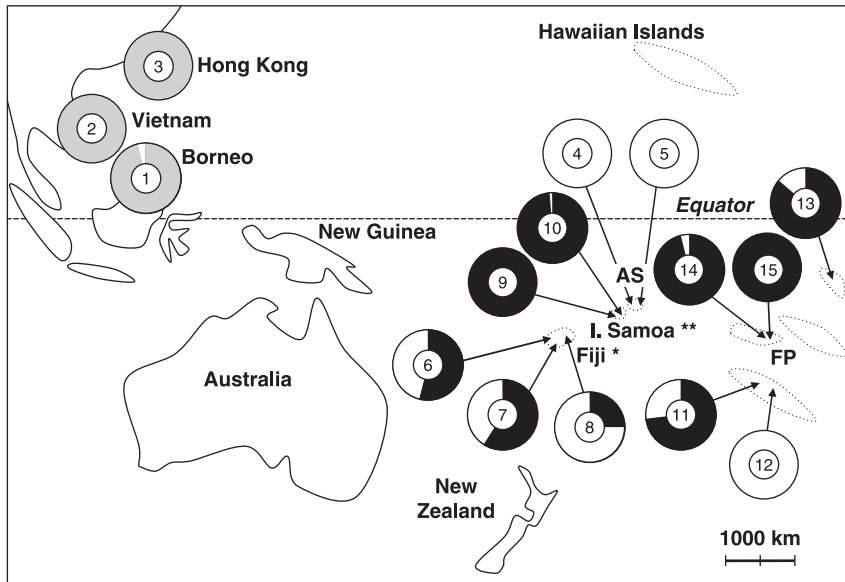


Fig. 1 Prevalence and penetrance of *wBol1* across the populations sampled. The pies show the prevalence of *wBol1* in females (white: uninfected, grey: *wBol1*-infected without male-killing phenotype, black: *wBol1* infection with male-killing phenotype). Numbers represent study sites detailed in Tables 1 and 2. Full lines represent large islands. Dashed lines represent groups of smaller islands (archipelagos). For clarity, most archipelagos from which no sample was obtained are not represented. Details on sample size as well as prevalence in males are given in Table 1. Abbreviations: AS (American Samoa), FP (French Polynesia), I. Samoa (Independent Samoa). *, Previously published data (Dyson *et al.* 2002). **, Previously published data (Dyson & Hurst 2004).

population level, biased sex ratios will alter the dynamics of sexual selection, potentially affecting the design of reproductive systems (Jiggins *et al.* 2000b).

The degree to which sex-ratio distorters drive the ecology and evolution of the host will depend to a large extent upon their frequency. Whilst high prevalence of bacteria-inducing parthenogenesis and feminization is known to be common (Rigaud 1997; Stouthamer 1997), the significance of male killers in this respect is still unclear. Many studies have found only low (< 20%) or intermediate (20–50%) prevalence (reviewed in Hurst & Jiggins 2000). More recently, high prevalence has been reported (Jiggins *et al.* 2000a, b; Dyson & Hurst 2004). In the sibling species *Acraea encedon* and *Acraea encedana*, male-killing *Wolbachia* prevalence as high as 95% was observed. In the most extreme case recorded to date, *Hypolimnas bolina*, over 99% of females were found infected by a male killer in Independent Samoa. In two of these cases, the impact on host populations is clear, with females forming leks in *A. encedon* (Jiggins *et al.* 2000b) and a high rate of failure to find mates in female *H. bolina* (Dyson & Hurst 2004).

In the current study, we investigated whether high prevalence of male-killing bacteria is a localized or widespread phenomenon in *H. bolina*. Individuals of both sexes were collected from across the range of this butterfly, including populations in mainland Southeast Asia (Hong Kong, Vietnam), island populations in Southeast Asia (Malaysian Borneo), and various island populations across Polynesia. In each population, both sexes were assayed for the frequency of *wBol1*, the previously described male-killing *Wolbachia* strain (Dyson 2002; Dyson & Hurst 2004). Additionally, females were bred from a subset of these populations in order to confirm the presence or absence of the male-killing phenotype. We observe that high prevalence

is common in this species. We also note that whilst the male-killing phenotype is expressed in the South Pacific populations, it is not expressed in the three Southeast Asian populations, where the bacterium is present at high prevalence but does not kill males. We conclude that male killers are likely to have significant ongoing ecological and evolutionary impact in numerous host populations.

Materials and methods

Sampling

Adult female and male *Hypolimnas bolina* were collected from the following locations (Fig. 1): Mekong Delta (date: June 2004; country: Vietnam; locations: Can Tho and Soc Trang provinces), Hong Kong (date: July–September 2002; country: China; locations: Tai Po, Cheung Chau, Mui Wo, Big Wave Bay), Malaysian Borneo (date: May 2001; country: Malaysia; locations: Kota Kinabalu, Sabah Province), Tutuila and Olosega islands (date: August 2001; country: American Samoa), Rurutu, Tubuai, Ua Huka, Moorea and Tahiti islands (date: March 2002–March 2004; country: French Polynesia). Samples from previously published studies (Dyson *et al.* 2002; Dyson & Hurst 2004) were also included in our analysis: Viti Levu, Taveuni and Wayalailai islands (Fiji), Savaii and Upolu islands (Samoa).

DNA extraction and PCR

The abdomens of collected individuals were detached and stored in 95% EtOH. DNA was prepared from a small tissue sample (2–5 mm³) using one of four methods: (i) DNeasy kit (QIAGEN) (specimens from Hong Kong); (ii) Promega Wizard Kit (specimens from Vietnam);

(iii) Chelex 100 in the presence of proteinase K (specimens from Ua Huka, specimens from Borneo, 76 specimens from Rurutu, 9 specimens from Moorea, 5 specimens from Tahiti); (iv) phenol–chloroform (specimens from Tubuai, 43 specimens from Rurutu, 34 specimens from Moorea, 15 specimens from Tahiti). In all cases, template was stored at -70°C to avoid degradation. There were no observed differences in the PCR (polymerase chain reaction) amplification stemming from the different extraction protocols.

Detection of *Wolbachia* used two PCR assays. As a positive control for DNA quality, a section of the COI gene of mtDNA was amplified using primer pair COIf/COIr which amplifies COI from insects (Brunton & Hurst 1998). Any material that did not amplify was discarded from further analysis. The presence of the *wBol1* *Wolbachia* strain was assessed using primer pair 81f/522r which specifically amplifies a portion of the *wsp* gene from B clade *Wolbachia* (Zhou *et al.* 1998).

Strain and mtDNA variation

In populations where *Wolbachia* was detected, its relatedness to other strains was ascertained from the sequence of the *wsp* gene, in at least two specimens per population, when two infected specimens were available. Host mtDNA sequences (a 414-bp fragment of COI) were also obtained from a subset of our samples. The sequences were attained directly through both strands from PCR product after purification using Amicon microcolumns. *Wolbachia* sequences were obtained using *wsp* primers 81f and 691r (Zhou *et al.* 1998); COI sequences were obtained using the primer pair COIf and COIr of Brunton & Hurst (1998), and sequenced directly through both strands using the original PCR primers. In addition to our wild-caught samples, COI mtDNA sequence was also ascertained for two infected specimens obtained from commercial breeders, deriving from peninsular Malaysia and Thailand, respectively.

Phenotypic assays

PCR assays of male specimens provide an estimate for the penetrance of the male-killing phenotype: absence of infection in wild males is an indication that males are killed efficiently. To characterize the phenotype more thoroughly, the effect of the infection was directly assessed in seven sampled populations. Wild-caught females were induced to oviposit by being exposed to sunlight, in close proximity to a healthy host plant showing signs of new growth. Depending on location, one of three host plants was used: *Ipomoea batatas* (sweet potato: Convolvulaceae, for samples from Vietnam and Borneo), *Sida rhombifolia* (Malvaceae, for samples from Wayalailai), *Synedrella nodiflora* (Asteraceae, for samples from Rurutu, Tubuai, Moorea and Tahiti). Following Kemp (1998), only very young plants were used in the case of *S. nodiflora*. F_1 larvae were fed on an

excess of the same host plant, except for samples from Rurutu, Tubuai, Moorea and Tahiti, where *Asystasia gangetica* (Acanthaceae) was used instead of *S. nodiflora*, due to its higher nutritive value (Kemp 2000).

In *H. bolina*, fertilized eggs that fail to hatch due to male-killing *Wolbachia* can be distinguished from nonfertilized eggs because dead embryos are visible through the egg chorion (Dyson 2002). We took advantage of this property to include only fertilized eggs (dead embryos) in our hatch rates estimations. Thus, hatch rates are calculated as follows: hatch rate = hatched eggs / (hatched eggs + dead embryos). Sex ratio was measured by counting the total number of males and females at emergence. Chi-squared tests were used to test significant deviation of hatch rates and sex ratio from their expected values.

Results

The results of the assay for *wBol1* in males and females from each population produced the prevalence estimates given in Table 1. The infection was found in 9 of the 12 South Pacific populations sampled, and in all Southeast Asian populations. Eighteen *wsp* sequences were obtained across 9 of the 12 infected populations. All were found to be identical to the previously described sequence from the male-killing *Wolbachia* of *Hypolimnas bolina* in Fiji (Dyson *et al.* 2002) and Japan (Mitsuhashi *et al.* 2004). In addition, we obtained mtDNA sequences from 5 of our infected individuals from Southeast Asia (locations 1, 3) and 17 infected individual from across 8 Polynesian samples (locations 6, 7, 8, 9, 10, 11, 13, 14), as well as two infected individuals from Southeast Asia obtained from commercial breeders. All 24 infected individuals were found to harbour the same mtDNA haplotype (Accession no. AJ844898) suggesting recent flow of *wBol1* across the species range.

The pattern of infection of males and females is obviously dichotomous. Whilst the infection occurs very rarely in males from Polynesian islands, consistent with previous breeding studies indicating this strain acts as a male killer in these areas (Dyson *et al.* 2002; Dyson & Hurst 2004), the infection was present in all males tested in the three Southeast Asian samples (Vietnam, Hong Kong and Malaysian Borneo). This observation suggests that the strain either does not kill males in these populations, or kills them with incomplete penetrance.

This interpretation is corroborated by the breeding data obtained (Table 2). In the broods of infected females from Polynesian populations, a 50% hatch rate reduction was observed (hatch rates significantly differed from 50% in only 2 of the 48 infected broods tested, which is compatible with the rate expected under type I error). In contrast, egg hatch rates were near 100% in infected broods from Southeast Asia. In addition, while infected females from Polynesian populations did not produce F_1 males, the sex ratio

Table 1 Prevalence of *wBol1* in male and female *Hypolimnas bolina* from different populations as detected by PCR assay

Country	Population	Map	Prevalence in females (%)	<i>n</i>	Prevalence in males (%)	<i>n</i>	Reference
Malaysia	Kota Kinabalu	1	96	25	100	19	This study
Vietnam	Mekong Delta	2	100	8	100	10	This study
China	Hong Kong	3	100	3	100	3	This study
American Samoa	Tutuila	4	0	6	0	10	Dyson & Hurst 2004
	Olosega	5	0	23	0	25	This study
Fiji	Wayalailai	6	54	76	0	8	Dyson <i>et al.</i> 2002; this study
	Viti Levu	7	59	34	0	9	Dyson <i>et al.</i> 2002
	Taveuni	8	25	24	—	—	Dyson <i>et al.</i> 2002
Independent Samoa	Savaii	9	100	35	50	2	Dyson & Hurst 2004
	Upolu	10	99	257	33	3	Dyson & Hurst 2004
French Polynesia	Rurutu	11	73	62	0	57	This study
	Tubuai	12	0	19	0	31	This study
	Ua Huka	13	86	43	12	25	This study
	Moorea	14	96	25	0	18	This study
	Tahiti	15	100	14	17	6	This study

Sample sizes are indicated in brackets. (—) indicates no sample obtained. Map (Map reference, Fig. 1).

Table 2 Egg hatch rates and F_1 sex ratio produced by females from different locations with varying infection status

Population	<i>wBol1</i> ?	Map	N_{HR}	n_e	Median HR (IQR)	N_{SR}	n_a	Median SR (IQR)
Borneo	Yes	1	2	33	0.98 (0.95–1.00)	na		
Vietnam	Yes	2	4	1332	1.00 (0.92–1.00)	4	418	0.51 (0.49–0.52)
Wayalailai	Yes	6	5	100	0.50 (0.36–0.53)	5	48	0.00 (0.00–0.25)
Wayalailai	No	6	4	76	0.98 (0.96–1.00)	4	58	0.50 (0.50–0.60)
Rurutu	Yes	11	12	719	0.45 (0.26–0.55)	5	47	0.00 (0.00–0.00)
Rurutu	No	11	4	311	0.91 (0.51–1.00)	3	72	0.69 (0.59–0.73)
Tubuai	No	12	13	806	1.00 (1.00–1.00)	5	59	0.62 (0.31–0.73)
Moorea	Yes	14	12	967	0.53 (0.49–0.54)	9	57	0.00 (0.00–0.00)
Tahiti	Yes	15	5	532	0.42 (0.00–0.49)	3	54	0.00 (0.00–0.00)

Abbreviations: *wBol1*? (presence of *wBol1* *Wolbachia* infection), Map (map reference, Fig. 1), N_{HR} (number of matriline from which hatch rates were measured), n_e (total number of eggs obtained from these crosses), N_{SR} (number of matriline from which F_1 sex ratio was measured), n_a (total number of F_1 adults obtained from these crosses), Median HR (median of egg hatch rates), Median SR (median of sex ratio). Data for egg hatch rate is given as median proportion hatching with interquartile range in parentheses. Data for sex ratio is given as median proportion males, with interquartile range in parentheses.

was in contrast not biased in the offspring of infected females from Vietnam. A small data set from Malaysian Borneo corroborates the absence of the male-killing phenotype in infected individuals in Southeast Asia: two infected females were observed to oviposit in the field, and both clutches produced had a high egg hatch rate (95% on $n = 21$ eggs and 100% on $n = 12$ eggs); the sex ratio of these broods was not ascertained. Thus, the origin of infected males in wild samples from Southeast Asia is clear: females infected with *wBol1* in Southeast Asia produce egg clutches with full egg hatch rate, and an F_1 with a 1:1 sex ratio in which males are infected. In other words, *wBol1* does not seem to act as a male killer in Southeast Asian *H. bolina*.

Another notable feature of the *H. bolina/wBol1* association is the very large variation in prevalence between the Polynesian islands sampled, where the male-killing phenotype is expressed. Whilst *wBol1* is absent in three Polynesian populations, prevalence levels between 25% and 99% are observed elsewhere. Within French Polynesia alone, prevalence varies from absence to near fixation, and is significantly heterogeneous ($\chi^2 = 66$; d.f. = 4; $P < 0.001$). Significant heterogeneity is retained even after exclusion of the population from Tubuai, where the infection is absent ($\chi^2 = 21$, d.f. = 3, $P < 0.001$). It is notable that neighbouring islands can have a very different prevalence of the male killer (e.g. absent in Tubuai, present in 73% of females in Rurutu).

Discussion

Three main conclusions can be derived from this study: (i) *wBol1* does not act as a male killer in Southeast Asian *Hypolimnas bolina*; (ii) prevalence is variable among South Pacific populations, where *wBol1* kills males with high penetrance; (iii) very high male-killer prevalence is a replicated event in this species.

Non-male-killing phenotype in Southeast Asia

Our results provide evidence that *wBol1* does not exhibit the male-killing phenotype in Southeast Asian populations. First, high prevalence was observed in both males and females sampled from this area, suggesting males can survive the infection. Second, breeding data from Vietnam provide direct evidence that infected females produce progeny with a normal hatch rate and 1:1 sex ratio. These results are consistent with a recent report of an *H. bolina* line from Japan where males survive *wBol1* infection (Mitsuhashi *et al.* 2004). Despite the strong contrast between populations from the South Pacific and Southeast Asia, it is notable that no variation of the *wsp* gene is observed.

The phenotypic variation between these populations makes it tempting to hypothesize that the butterflies from South Pacific and Southeast Asia actually represent divergent species. However, two lines of evidence argue against this view. First, crosses recently completed by one of us for an independent study demonstrate that F_1 hybrids are fully viable and fertile, and that the genetic background of the two populations can be introgressed through to an F_3 generation (E.H., unpublished). Thus, it is clear that individuals from Southeast Asia and Polynesia share recent ancestry. Second, identity of the mitochondrial COI sequence obtained from infected individuals from Southeast Asian and Polynesian populations indicate recent flow of cytoplasmic genes in these geographical areas.

Thus, it is safe to conclude that *wBol1* phenotype has changed due to recent shifts in either host or bacterium in either Southeast Asia or in Polynesia. Possible explanations of why *wBol1* exhibits male killing across Polynesia but not in Southeast Asia include (i) that non-male killing was ancestral across the range of the species, and that male killing evolved and spread recently in the Pacific, or (ii) that male killing was present ancestrally over the range of *H. bolina*, but the phenotype has now been lost from Southeast Asian populations. We currently favour the latter hypothesis from historical data. Clarke *et al.* (1975) noted the existence of male killing in eight out of nine females from Sarawak (Borneo) and also the presence of an all-female brood from the single female tested from Hong Kong. The presence of the male-killing phenotype in this earlier report, in contrast to the absence in our surveys from these areas, is compatible with the hypothesis that absence of male killing in Southeast

Asia is a new phenomenon. However, it is difficult to resolve the question of ancestral state without ambiguity because variation in *wBol1* phenotype has been recently reported within Japan (Mitsuhashi *et al.* 2004), and the precise location of sampling could vary between the Clarke study and ours.

Regardless of any evolutionary scenario, the observed penetrance variation among populations must be associated with either host resistance or variation of male-killing ability on the part of the bacterium. These hypotheses will be distinguished via experimental introgression of *Wolbachia* from Southeast Asia and Polynesia onto alternate host genetic backgrounds and observing the presence/absence of male killing following these crosses.

Prevalence variation among South Pacific populations

Male-killer prevalence appears to vary continuously from 0 to near 100% in the South Pacific, with no obvious geographical trend. Indeed, proximal islands such as American and Independent Samoa (100 km apart) and Rurutu and Tubuai (200 km apart) show widely differing prevalence. Current models state that such variation can occur if and only if maternal transmission is imperfect (Hurst 1991; Hurst *et al.* 1997; Engelstadter *et al.* 2004b) unless extreme levels of population structure are assumed (Groenenboom & Hogeweg 2002). However, mitochondrial sequence data demonstrate that transmission efficiency of *wBol1* is perfect or nearly perfect in the populations surveyed in the present study (Dyson, pers. obsn.).

Thus, the prevalence variation observed in this system is something of a paradox, for which we can envisage two solutions. A first possibility is that existing models are missing an important, and yet unidentified parameter. Alternatively, the models may be based upon an assumption that is not met in South Pacific *H. bolina*, namely that populations are at equilibrium. Indeed, the variance in prevalence observed in this system might be associated with extinction following spread of the male killer, to fixation, followed by recolonization events. The later hypothesis could be tested using historical biogeography, with the prediction that high-prevalence populations should derive from old colonization events. In addition, we suggest interference with other selfish genetic elements should also be considered as a potential explanation for the absence of infection in some populations. Theory indicates the presence of an existing reproductive parasite within a locality can impede the spread of later arrivals (Engelstadter *et al.* 2004a, b).

Extreme male-killer prevalence

Male-killer prevalence in excess of 70% of females infected was observed in four of the six Polynesian populations newly surveyed in this study. In two of them (Moorea and Tahiti in French Polynesia), prevalence exceeds 95%. It thus

appears that the previously reported case from *H. bolina* in Independent Samoa, although the most extreme, is not unique and isolated. Our Polynesian data strongly suggest that male-killer infection will have varying ecological or evolutionary consequences on host populations across islands. In Independent Samoa, high female virginity rates were observed, suggesting that sex-ratio bias towards females is such that male capacity for multiple mating becomes a limiting factor (Dyson & Hurst 2004). More generally, the reduction of male–male competition associated with male rareness will impose new selective constraints on reproductive systems. Thus, modifications of sexual traits can be predicted in populations where prevalence is high, in comparison with those where infection is absent or less common. In this system, the range of prevalence variation will allow us to examine the threshold levels for different evolutionary and ecological impact within this species, providing a general framework for understanding the effects of sex-ratio distorters on natural populations.

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