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Report

Male-Killing Bacteria Trigger a Cycle of Increasing Male Fatigue and Female Promiscuity

Sylvain Charlat,[1,2,*] Max Reuter,[1] Emily A. Dyson,[1] Emily A. Hornett,[1] Anne Duplouy,[2,3] Neil Davies,[2] George K. Roderick,[2] Nina Wedell,[4] and Gregory D.D. Hurst[1]

[1] Department of Biology
University College London
4 Stephenson Way
London NW1 2HE
United Kingdom

[2] Richard B. Gump South Pacific Research Station
University of California Berkeley
BP 244 Maharepa
98728 Moorea
French Polynesia

[3] School of Integrative Biology
University of Queensland, St. Lucia
Queensland 4072
Australia

[4] School of Biosciences
University of Exeter
Cornwall Campus
Tremough
Penryn TR10 9EZ
United Kingdom

Summary

Sex-ratio distorters are found in numerous species and can reach high frequencies within populations. Here, we address the compelling, but poorly tested, hypothesis that the sex ratio bias caused by such elements profoundly alters their host's mating system. We compare aspects of female and male reproductive biology between island populations of the butterfly *Hypolimnas bolina* that show varying degrees of female bias, because of a male-killing *Wolbachia* infection. Contrary to expectation, female bias leads to an increase in female mating frequency, up to a point where male mating capacity becomes limiting. We show that increased female mating frequency can be explained as a facultative response to the depleted male mating resources in female biased populations. In other words, this system is one where male-killing bacteria trigger a vicious circle of increasing male fatigue and female promiscuity.

Results and Discussion

The mating system of a species is largely shaped by the operational sex ratio (OSR), the number of males and females willing to mate at any given time [1, 2]. The OSR determines both the level of competition within each sex with respect to gaining matings and the level of conflict between the sexes as to whether mating should take place. Because of the fundamental importance of the OSR, it has been proposed that sex-ratio distorters play a major role in the reproductive evolution of their host [3]. Many sex-ratio distorters, such as maternally inherited or X-linked selfish genetic elements, bias the sex ratio of their hosts toward females [4, 5]. The excess of females is expected to lead to an increase of mating opportunities for males and a decrease in the average number of matings per female. As a result, competition between male hosts for access to mates and fertilization is reduced, as is the intensity of conflict between the sexes over mating and fertilization. Despite the widespread occurrence of sex-ratio distorters, their impact on the ecology and evolution of their host's mating system is to a large extent unknown and has been limited to one observation of role reversal [6].

In this study, we examine alterations of the mating system and reproductive strategy driven by a male-killing *Wolbachia* infecting Pacific Island and southeast Asian populations of the butterfly *Hypolimnas bolina*. This species represents an ideal study system for our purpose because discrete local populations differ naturally in *Wolbachia* infection frequency [7, 8]. We assessed the natural sex ratio in 20 populations (Figure 1) based on estimates of prevalence and penetrance of the male-killer [9] (Table 1 and Tables S1 and S2 in the Supplemental Data available with this article online; see also Experimental Procedures). We combined these data with measures of female mating frequency and male mating investment. In Lepidoptera, ejaculates are enclosed in physical structures called spermatophores, which are deposited within the female bursa copulatrix and remain there once the sperm have been moved to the female's spermathecae. This peculiarity enables us to assess the mating history of wild-caught females by scoring the number of spermatophores found per female.

We first tested the hypothesis that an increasing female bias in the population leads to a decrease in female mating frequency (the number of copulations per female) as males become increasingly rare. Surprisingly, this prediction is not met by the empirical data. The change in female mating frequency with population sex ratio is not a gradual decrease but instead has a strong quadratic component (Table 1 and Figure 2). Female mating frequency first increases with female bias before decreasing at extreme female bias where the rarity of males finally becomes limiting and results in elevated female virginity rates (Table S1). In the two most female-biased populations, mated females lay a high proportion of unfertilized eggs (Table S1), indicating that sperm is limiting even if a female manages to secure a mate [8].

The field data demonstrate that in *H. bolina*, moderate sex-ratio distortion does not lead to a shift in the mating system toward reduced female mating frequency. Rather, female mating frequency increases with female bias in the population until male mating capacity

*Correspondence: s.charlat@ucl.ac.uk

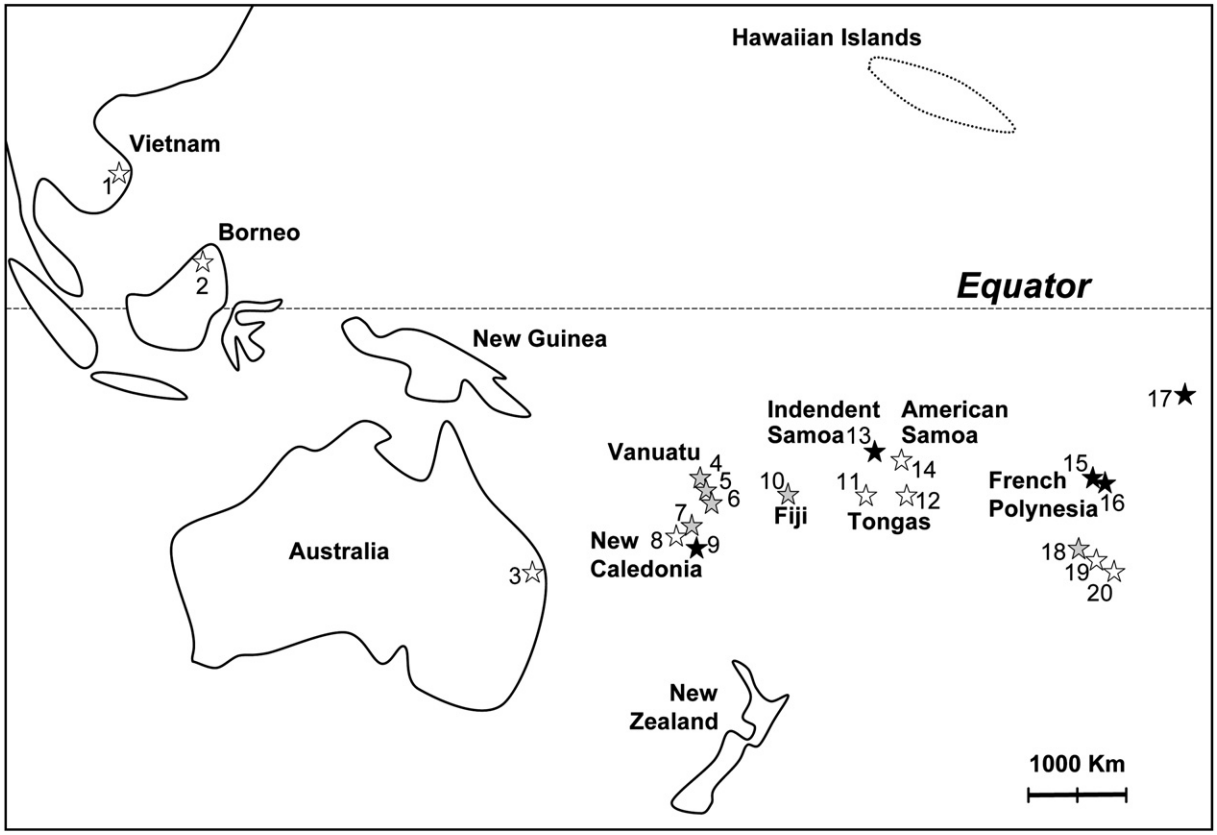

Figure 1. Geographic Distribution and Sex Ratio of the Populations Sampled

Detailed information on each study site is given in Table 1, Table S1, and the Experimental Procedures. Colors provide a simplified view of sex-ratio variation: white (even sex ratio), gray (slightly biased: from 1.2 to 2.9 females per male), and black (highly biased: from 4.9 to 38.7 females per male).

becomes limiting. In butterflies, the decision to mate is mainly under female control [10, 11]. Moreover, smaller spermatophores make females sexually unreceptive for shorter periods [12], suggesting that the increased female mating frequency seen in *H. bolina* could result from reduced male mating investment in female-biased populations. Consistent with this hypothesis, we found a negative correlation between male mating investment (spermatophore size) and the average number of matings per male (the product of sex ratio and female mating rate) (Figure 3). Furthermore, excluding the two populations where female mating rate is constrained by lack of access to males, female mating rate and male spermatophore size are correlated (Spearman's rank correlation test, rho = −0.49, S = 1447, p = 0.037). Thus, in *H. bolina*, just as in other butterfly species, females appear to adjust mating frequency in accordance with the decreasing male investment per copulation.

The decrease in spermatophore size with increasing female bias could result from two non-mutually exclusive processes: (1) With increasing mating rate, males deplete resources needed for the production of spermatophores and hence are unable to maintain a large investment (resource depletion), and (2) the increased number of mating opportunities for males in female-biased populations leads to an adaptive change in male investment per mating toward a more even distribution of reproductive resources between females and maximization of fertilization returns (ejaculate partitioning) [13]. We evaluated the importance of these two processes by measuring male reproductive investment over two successive copulations for initially virgin males from three populations differing in sex ratio: Tubuai (non-female biased: 0.89 females per male), Rurutu (2.89 females per male), and Moorea (4.94 females per male) (Table 1). In order to eliminate confounding factors, we took a "common garden" approach and used wild individuals' offspring, reared synchronously under seminatural conditions on the island of Moorea. This experiment provides compelling evidence for the "resource depletion" hypothesis (Tables S3 and S4). Mating investment in experimental males was strongly affected by resource depletion in that spermatophore diameter decreased significantly in successive matings (main effect "mating": $F_{1,48} = 28.98$, $p < 0.0001$; linear coefficient $b = -0.34$, $t_{48} = -5.38$, $p < 0.0001$) and more so the shorter the time period between successive matings (interaction "time between matings" × "mating": $F_{1,48} = 5.79$, $p = 0.02$; $b = -0.0025$, $t_{48} = 2.41$, $p = 0.02$). In contrast, there was no evidence for reduced mating investment in males originating from female-biased populations. In the overall analysis of variance, the island of male origin did not have a significant effect on either the size of the spermatophore (main effect "island": $F_{2,46} = 2.48$, $p = 0.09$) or the extent of the size reduction between first and second mating (interaction

Table 1. Sex Ratio, Mating Rates, and Spermatophore Size in Natural Populations

Map	Population	F/M Ratio	Female Mating Rate (SE)	Male Mating Rate	Sp. Diam. (SE)
1	Mekong Delta	0.89	1.13 (0.13)	1.01	1.56 (0.05)
2	Kota Kinabalu	0.89	0.96 (0.04)	0.86	2.03 (0.06)
3	Australia	0.89	0.87 (0.13)	0.78	1.78 (0.12)
4	Efate	1.55	0.71 (0.18)	1.10	1.54 (0.07)
5	Tanna	1.19	1.25 (0.63)	1.48	1.61 (0.11)
6	Aneityum	1.17	1.19 (0.11)	1.39	1.64 (0.04)
7	Lifou	1.29	1.38 (0.15)	1.78	1.53 (0.06)
8	Grande Terre	0.89	0.80 (0.13)	0.72	1.55 (0.09)
9	Ile des pins	4.94	1.43 (0.11)	7.06	1.44 (0.04)
10	Viti Levu	2.14	1.02 (0.04)	2.18	2.04 (0.05)
11	Kapa	0.89	1.35 (0.14)	1.21	1.59 (0.05)
12	Niue	0.89	1.10 (0.10)	0.98	1.64 (0.04)
13	Upolu	38.66	0.54 (0.03)	20.87	1.15 (0.03)
14	Olosega	0.89	1.00 (0.05)	0.89	2.09 (0.09)
15	Moorea	4.94	2.18 (0.20)	10.77	1.40 (0.02)
16	Tahiti	16.95	1.29 (0.19)	21.87	1.23 (0.03)
17	Ua Huka	5.90	1.33 (0.12)	7.85	1.44 (0.03)
18	Rurutu	2.89	1.78 (0.09)	5.14	1.58 (0.01)
19	Tubuai	0.89	1.33 (0.08)	1.19	1.70 (0.02)
20	Raivavae	0.89	1.31 (0.14)	1.17	1.74 (0.03)

Column headings are described as follows: map: reference number in Figure 1; F/M ratio: estimated population sex ratio, given as number of females per male; female mating rate (SE): the mean number of spermatophores per female (standard error); male mating rate: the estimated male mating rate (female mating rate × F/M ratio); sp. diam. (SE): the mean spermatophore diameter in mm (standard error). Additional details are provided in the Supplemental Data (Table S1).

"island" × "mating": $F_{2,48} = 1.41$, $p = 0.25$). Indeed, the raw data (Table S3) suggest that mating investment is, if anything, lower and more constant in males from the population with an even sex ratio compared to those from female-biased populations. It remains to be investigated whether the spermatophore composition (sperm type, sperm amount, and nutritional resources) varies with the population sex ratio.

The results of our study present a counterintuitive scenario for the effects of sex-ratio distortion on the mating system of *H. bolina*. Contrary to expectation, increasing female bias in the population leads to an increase in female mating frequency as a response to the decreasing size of spermatophores transferred by males. As demonstrated in our experiment, the decrease in male investment per mating in female-biased populations is not due to the evolution of different ejaculate delivery strategies but rather to the depletion of male reproductive reserves. The female response to the diminishing male resources reinforces the effects of the male-killing bacteria on sexual selection: Female bias in itself shifts the OSR from male bias to female bias; the resulting depletion of male mating resources leads to decreased spermatophore size, which in turn causes an increase of the females' willingness to mate.

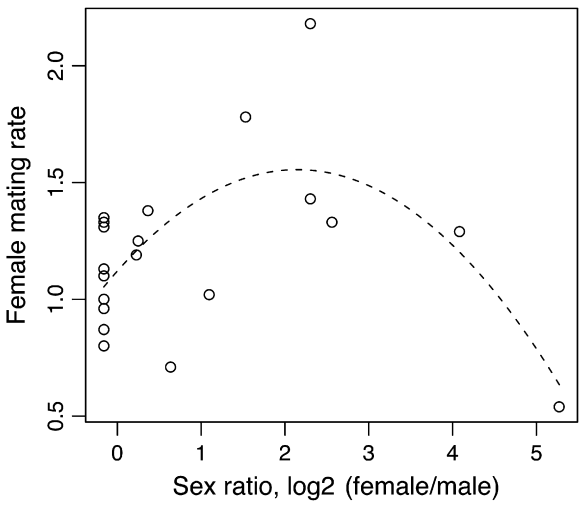

Figure 2. Female Mating Rate as a Function of Population Sex Ratio

Model comparison showed that a regression containing both a linear and a quadratic term (dashed curve) fits the data significantly better than a purely linear one ($F1,17 = 12.10$, $p = 0.002$) or a purely quadratic one ($F1,17 = 11.34$, $p = 0.004$). Female mating rate was estimated from the mean number of spermatophores per female. Sex-ratio is given as $\log_2$ of number of females per male.

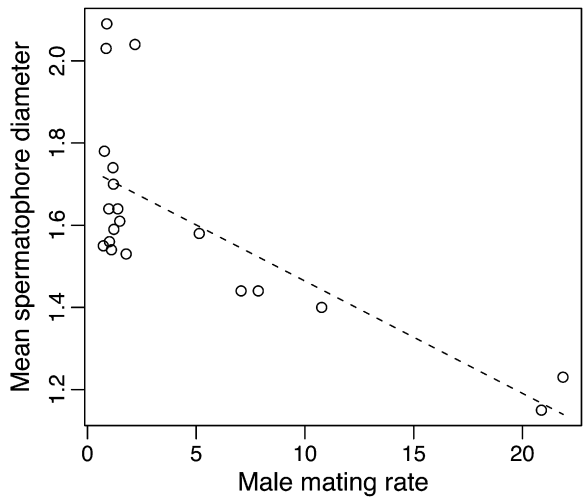

Figure 3. Spermatophore Size as a Function of Male Mating Rate

Negative correlation is highly significant (Spearman's rank correlation test, rho = −0.66, S = 2206, p = 0.002).

The resulting positive feedback between male fatigue and female promiscuity is only broken when female bias becomes so extreme that male mating capacity is a limiting factor, as observed on the islands of Tahiti and Upolu. The unexpected consequence of this positive feedback is that moderate female bias tends to increase the intensity of sexual selection that males experience through sperm competition because females become increasingly promiscuous as long as male mating capacity is not yet limiting. Our experiment suggests that under these circumstances, the evolution of reduced male mating investment in female-biased populations is prevented. The present study thus demonstrates that predictions concerning the effects of sex-ratio distorters on their host's mating system can be misleading if they fail to consider the simultaneous changes in both male and female mating strategies, which in *H. bolina* lead to surprising outcomes.

Experimental Procedures

Sampling

Adult female and male *Hypolimnas bolina* were collected from the following locations (sample ID as given in Figure 1): sample 1 (date: June 2004; country: Vietnam; locations: Mekong Delta, Can Tho, and Soc Trang provinces); sample 2 (date: May 2001; country: Malaysia; locations: Malaysian Borneo, Kota Kinabalu, Sabah Province), sample 3 (date: March 2004 and 2006; country: Australia; locations: Brisbane, Cairns, Coffs Harbour); samples 4, 5, and 6 (date: July 2005; country: Vanuatu; locations: islands of Efate, Tanna, and Aneityum, respectively), samples 7, 8, and 9 (date: August 2005; country: New Caledonia; locations: islands of Lifou, Grande Terre, and Iles des Pins, respectively); sample 10 (date: July and August 1999; country: Fiji; location: Suva and Nadi, island of Viti Levu); sample 11 (date: October 2004; country: Kingdom of Tonga; location: island of Kapa); sample 12 (date: October 2004; country: Niue; location: island of Niue); sample 13 (date: July–August 2000 and July–August 2001; country: Independent Samoa; location: Apia, island of Upolu); sample 14 (date: August 2001; country: American Samoa; location: island of Olosega); samples 15–20 (date: regular sampling from March 2002 to March 2006; except for sample 17 obtained on a single expedition in April 2003; country: French Polynesia; locations: islands of Moorea, Tahiti, Ua Huka, Rurutu, Tubuai, and Raivavae, respectively).

Variation in the size of our samples (Table S1) results from differences in the number of days we spent sampling in the different populations (Spearman's rank correlation between sample size and the number of days spent in the field: rho = 0.58, S = 559, p = 0.008). Sample size therefore does not reflect biological differences, such as density or catchability, between populations.

DNA Extraction, PCR, and Sequencing

DNA was prepared from a small tissue sample (2–5 mm^3) with Qiagen DNeasy tissue kits. Prior to *Wolbachia* PCR assays, DNA extracts were diluted 10×, and their quality was assessed with a general "metazoan" PCR of the COI mitochondrial gene (primer pair LCO/HCO) [14]. Nonamplifiable material was discarded from the analysis. The presence of the male-killing *Wolbachia* (strain *w*Bol1) was assessed by amplification of the *Wolbachia* surface protein gene (*wsp*) with primer pair 81f/522r, which specifically amplifies a portion of the *wsp* gene from B clade *Wolbachia* [15]. Strain identity was confirmed by the obtainment of identical *wsp* sequences from 27 individuals where a B-clade *Wolbachia* was detected. Sequences were attained directly from PCR product with primer 81F, after amplification with the 81F/691R primer pair [15].

Breeding Data: Hatch Rates and Adult Sex Ratio

Wild-caught females were induced to oviposit under sunlight, on very young *Synedrella nodiflora* (Asteraceae). Five to six days after oviposition, eggs were classified as follows: those that hatch successfully, those that do not hatch but show development (a gray embryo is seen through the chorion), and those that do not show signs of development (unfertilized). Unmated females and unfertilized eggs were discarded for hatch-rate measurements. Larvae were reared through to adulthood on *Asystasia gangetica* (Acanthaceae), and adults were sexed based on wing color patterns.

Estimation of Sex Ratio in Natural Populations

H. bolina males are territorial and more conspicuous than females [16], precluding a direct estimation of population sex ratio from field observations. Sex ratio in natural populations was thus estimated on the basis of prevalence and penetrance of male killing with a compilation of previously published breeding data together with the present study [7, 8, 17] (Table S2). In these studies, wild infected females produced a total of only 12 males for 1657 females (0.7% males, sum of 120 crosses). In contrast, uninfected females produced 807 males and 721 females (52.8% males, sum of 50 crosses). The population sex ratio can be estimated from this breeding data as females/males = $0.472/((1 - P_f) \times 0.528 + P_f \times 0.007)$, where P_f stands for the infection prevalence in females. This estimation of sex ratio relies on the following assumptions: (1) equal fecundity and survival of infected and uninfected females; (2) equal survival of male and female as adults; (3) infected and uninfected females producing the same number of adult daughters (no local competition among larvae). However, because the same method was used throughout the populations under study, our overall analysis and conclusions do not rely on these assumptions. The southeast Asian populations, where male killing is fully suppressed [9], were treated as though they were uninfected ($P_f = 0$). In all other populations, male killing was unsuppressed, as evidenced by breeding experiments and the dearth of infected males in the wild [7] (Tables S1 and S2).

It is important to note that our method for estimating population sex ratio precludes any bias due to different capture rates of males and females because only one sex is sampled. Furthermore, the PCR-based detection of infection renders sampling of females blind because infection status is detected a posteriori.

Mating Frequency and Spermatophore Size

Female mating frequency was estimated by the dissection of wild caught females and extraction of spermatophores from the bursa copulatrix. Spermatophores were photographed with a digital camera (DFC 280) connected to a stereomicroscope (Leica MZ6). Millimeter paper was used for calibrating images, and spermatophore diameter was measured with ImageJ version 1.33u.

Spermatophore Size in Standardized Conditions

The size of spermatophores produced by laboratory-reared males was measured as above. The experiment involved comparisons between males from the islands of Moorea, Rurutu, and Tubuai, took place in Moorea (Gump Research Station, University of California Berkeley in French Polynesia), and involved the offspring of wild parents, with the following number of lines: Moorea (six lines), Rurutu (six lines), Tubuai (eight lines). Upon emergence, adults were weighed on a Mettler Toledo balance (Excellence Plus XP203S) and labeled individually with Tough Tags (USA Scientific) stuck on the ventral side of front wings (after scales were removed with a wet cotton stick). After labeling, adults were placed in an outdoor cage (1.80 m × 1.80 m × 3.60 m, Bioquip, model 1412A) exposed to sunlight, with a bright yellow synthetic sponge impregnated with 15% w/v sugar solution available for feeding; the sponge was isolated from ants with Tanglefoot. The cage was split in two parts to isolate males from females. When at least half of the emerged females reached sexual maturity (circa 4 days after emergence [18]), the mating experiment was initiated on sunny days by the mixing of males and females from 9:00 to 14:00. Mating pairs were retrieved every 15 min and isolated in a small cage. After mating, mated males were placed back in the large cage, and mated females were isolated and frozen prior to dissection. The experiment as described was carried out in two blocks in November 2004 and November 2005.

Statistical Analysis

All analyses were performed in R [19]. Data from the mating experiment were analyzed with a linear mixed-effect model with the *nlme* package [20]. Mixed-effect models assume that the individual

measures of a response variable are determined by a number of predictor variables with fixed effects (as in a straightforward linear model) but allow for random variation in the coefficients of all or a subset of the predictor variables between groups of data points. Variation in coefficients is included as additional random effects in the linear model and can hence be separated from the estimates of coefficients of the fixed effect common to all groups. Mixed-effect models are appropriate in cases where data points are clustered and dependent. In our case, dependencies exist between repeated measures involving the same male. Our statistical model therefore included "male" as a random grouping variable, and the intercept (mean spermatophore diameter) was allowed to vary between males. The fixed part of the model was determined by the underlying biological questions of our study and included "male weight" as a covariate, the main effects "time between matings" (a continuous variable), "mating" (with levels "first" and "second"), and "island" (of male origin), as well as the interactions "time between matings" × "mating" and "island" × "mating." A model comparison revealed that adding additional interaction terms did not significantly reduce the residual variance. Data from the two experimental blocks were pooled because prior analysis revealed neither a significant block effect nor significant interactions between the block and any other predictor included in the final model. The final model was fitted with the restricted maximum-likelihood method implemented in *nlme* for analysis of variance and estimation of linear coefficients.

Supplemental Data

Supplemental Data include four tables and can be found with this article online at http://www.current-biology.com/cgi/content/full/17/3/273/DC1/.

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