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Report

Rapidly Shifting Sex Ratio across a Species Range

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Summary

Sex ratios are subject to distortion by a range of inherited parasites [1]. Although it has been predicted that the presence of these elements will result in spatial and temporal variation in host sex ratio [2–4], testing of this hypothesis has been constrained by availability of historical data. We here determine spatial and temporal variation in sex ratio in a interaction between a butterfly and male-killing *Wolbachia* bacteria [5] by assaying infection presence in museum specimens, and from this inferring infection prevalence and phenotype in historical populations. Comparison of contemporary and museum samples revealed profound change in four of five populations examined. Two populations become extremely female biased, associated with spread of the male-killer bacterium. One evolved from extremely female biased to a sex ratio near parity, resulting from the infection losing male-killing activity. The final population fluctuated widely in sex ratio, associated with varying frequency of the male killer. We conclude that asynchronous invasion and decline of sex-ratio distorters combines with the evolution of host suppressors to produce a rapidly changing mosaic of sex ratio. As a consequence, the reproductive ecology of the host species is likely to be fundamentally altered over short time scales [6]. Further, the study demonstrates the utility of museum specimens as "silent witnesses" of evolutionary change.

Results

Between the 1870s and 1930s, the highly variable female wing color pattern led the tropical butterfly *Hypolimnas bolina* to be intensively collected by traveling entomologists. Many of these specimens were deposited in museum collections alongside accompanying field notes. With these samples, we developed and validated a combination of PCR assays to determine the presence of the inherited bacterial symbiont *Wolbachia* *w*Bol1, which kills male offspring of infected females (male killing) (see Experimental Procedures). The first PCR assay directly tested for *Wolbachia* *w*Bol1 presence, whereas the second indirectly inferred *Wolbachia* presence from a strong association between mitochondrial haplotype and infection status found in this species [7]. After validation, these assays were then used to infer the status of the interaction in historical populations. Comparison of the data from a historical sample to its contemporary sympatric equivalent [8–10] gives a measure of change over time. Repeating this for collections of *H. bolina* from different populations allows ascertainment of the pattern of change in the interaction with *Wolbachia* over the species range.

We first demonstrated that the results of our PCR assays for infection status were repeatable between laboratories, by using specimens maintained in different museums, collected by a variety of people, and from different locations and time periods (Table S1 available online). After this, we validated our ability to determine infection status accurately by examining whether our results corroborated the reported sex ratio. This utilized collections made by H.W. Simmonds from the Fiji archipelago in the 1920s and 1930s. He observed one of the first cases of maternally inherited all-female ("unisexual") broods [11], and detailed sex-ratio data therefore exists for 33 independent families in the material maintained in the Hope collection of Entomology, Oxford, UK [11–13]. In all cases, the blind PCR assays correctly predicted the sex ratio produced by females, with mothers of reported unisexual broods scoring positive for male-killer infection and mothers of bisexual broods negative (Table S2). The association between mitochondrial haplotype and infection presence observed in current specimens was also complete in these museum specimens. Of the 33 mothers, 21 carried haplotype "one," and each of these were recorded as having produced unisexual broods (Fisher's exact test of association between haplotype one presence and production of unisexual broods in Fijian 1920–1930 samples: $p < 0.0001$). Further evidence of this association within historical samples is provided by the fact that all 19 specimens assayed from a 1924–1925 Independent Samoan sample (known to have extreme sex-ratio bias [14]) were *w*Bol1 infected (determined by direct *Wolbachia* PCR assay) and carried haplotype one. We therefore conclude that this haplotype is efficiently diagnostic of infection in museum material.

Having verified the robustness of the PCR assays, we then investigated the *Wolbachia* infection status of *H. bolina* from five populations collected across the species' range from 1878 to 1934. In order to examine the dynamics in the sex-ratio distorter/host interaction temporally as well as spatially, we compared the results from historical specimens with contemporary data from the same populations. The time span between historical and contemporary samples varies from 73 to 123 years, which (assuming 8 generations of *H. bolina* per year) represents c. 500 to 1000 generations. Infection prevalence in female specimens estimates the proportion of females in the population that could produce all-female broods. The infection status of males was used to ascertain whether a host suppressor of male-killing activity had invaded the population, and thus whether infected females from that population would produce all-female broods or a normal (1:1) sex ratio brood.

Overall, the results indicate that the interaction between the male-killing *Wolbachia* infection and the butterfly *H. bolina* is extremely dynamic across the host's geographic range, with

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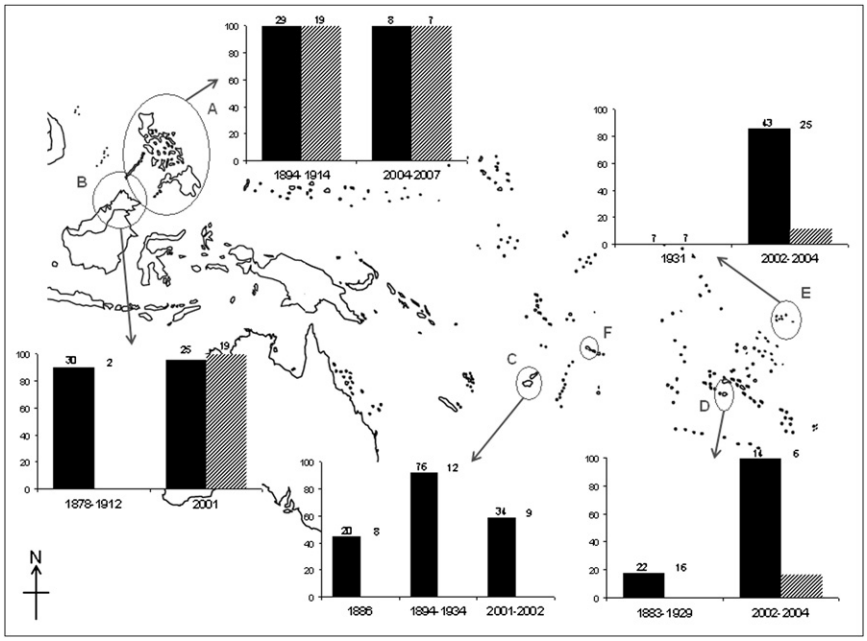

Figure 1. The Change in Frequency over Time of *Wolbachia* in Male and Female *H. bolina* in Five Populations

Populations: (A) The Philippines (Mindanao and Luzon); (B) Sabah, Malaysian Borneo; (C) Viti Levu, Fiji; (D) Tahiti, French Polynesia; and (E) Ua Huka, French Polynesia. *x* axis is time period over which the samples were collected. *y* axis is percentage of *Wolbachia* infection frequency, segregated into male (diagonal hatch) and female (solid black). Sample size is given above column. Infection status of museum specimens was determined by a positive PCR assay for *Wolbachia w*Bol1 or presence of a diagnostic mtDNA haplotype, with uninfected individuals being negative on both these criteria. Fijian historical data were split into two time periods as statistical testing rejected the hypothesis of homogeneity over time. Population (F), Independent Samoa, has been previously described as showing the change observed in population (B) in this study, over the period 2001–2007 [13]. Data of recent populations taken from [5, 8, 9].

populations experiencing rises, falls, or fluctuations in the frequency of the male killer, and host evolution of suppression of male-killing activity (Figure 1; Table S3). These changes will have been associated with substantial alterations in host population sex ratio and therefore would have had profound implications upon many aspects of host biology.

Host suppression of male-killing activity has been observed in contemporary Southeast Asian and Independent Samoan *H. bolina* populations [10, 15] and is suggested in Japan [16]. Among the five historical populations investigated here, infected males (the hallmark of suppression) were commonly observed in only one—the Philippines. Museum collections from this locality dated 1894–1914 carried approximately equal numbers of males and females, and all male and female specimens tested were infected with *Wolbachia w*Bol1. Thus, host suppression of male-killing activity existed 115 years ago, but in only one of the three populations in which it is currently known to occur.

In the four remaining populations investigated, we observed either the spread of host suppression of male-killing activity, an increase in frequency of the *Wolbachia* infection followed by a fall, or a rise to extreme infection prevalence levels. The first pattern, the spread of host suppression of male-killing activity through a population, was observed in Malaysian Borneo. In this locality, investigation of historical samples demonstrate the presence of an unsuppressed male-killing *Wolbachia* at high prevalence, whereas contemporary breeding records show that host suppression of this bacterium's action has evolved and infected males are now common [9]. The high frequency of male-killing *Wolbachia* in the historical population as determined by PCR assay is also reflected in the paucity of male butterflies in the collections sampled. This process of invasion and spread of host suppressors of male-killing parallels the recent "real time" event observed in Independent Samoa [15].

The only population to show a decrease in *Wolbachia* infection prevalence during the time period sampled was Viti Levu, Fiji. During the historical transect taken in Viti Levu, male-killer prevalence was moderate in 1886, rose to very high levels between 1894 and 1934 (Fisher's exact test, two-tailed, prevalence in females 1886 versus 1894–1934: $p < 0.0001$), but has subsequently fallen to 59% in contemporary samples [5]. The fluctuations in male-killing *Wolbachia* prevalence demonstrated in Viti Levu corroborate written notes on the population sex ratio [11–13, 17–19]. The population sex ratio, as estimated from male-killer frequency, moves from c. 2 females/male (1886) to c. 12 females/male (1894–1934) before returning to c. 2 females/male (1999).

Finally, the eastern-most *H. bolina* populations of Ua Huka and Tahiti in French Polynesia currently carry high-prevalence *Wolbachia* infections with full male-killing ability. In contrast, the historical populations either had no male-killing infection present (Ua Huka 1931) or present only at very low prevalence (Tahiti 1883–1929). These data are corroborated by an abundance of male butterflies noted in written records of Tahiti from this time [11, 19]. This suggests that these islands have varied from a near-normal sex ratio to the extreme female bias seen today and may represent cases of invasion of *w*Bol1 male-killing bacterium.

Discussion

Our results broadly vindicate the theoretical prediction that the dynamics of a host/sex-ratio distorter interaction can vary dramatically over relatively short periods of time [2–4]. Outside of the Philippines, variation in either *Wolbachia* prevalence or host-suppressor presence has caused the population sex ratio in all populations to fluctuate between parity of males and females (infection absent or its activity suppressed) and extreme female bias (extreme infection prevalence, unsuppressed). With respect to the fluctuations in the frequency of male-killing infection, it should be noted that the pattern of widely fluctuating frequency of active male-killing *Wolbachia*, as observed in Viti Levu, Fiji, may be a feature of the *Wolbachia* dynamics in many populations across the range of *H. bolina*.

The comparison of contemporary with historical specimens also reveals patterns not expected from theory. First, we expected the suppressor to be of very recent origin. This follows

from the very high level of selection in favor of the host suppressor predicted by theory [20] and observed within populations [15]. However, we observed that the suppressor was already present 115 years ago in the Philippines. Clearly, geographic structure of the metapopulation and patterns of butterfly migration strongly influence the evolutionary dynamics in this system, slowing the spread of a gene under strong selection and producing a geographic mosaic in the host/*Wolbachia* interaction. This contrasts with the dynamics within a population, as evidenced by Independent Samoa, where the host suppressor of male-killing activity can spread very rapidly [15]. This difference in dynamics is likely due to *H. bolina* being highly dispersive and able to traverse freely across land, but being impeded by the significant distance between island groups within the Pacific.

Second, we had no theoretical reason to expect a decrease in male-killer prevalence in the absence of host evolution. The dramatic reduction in frequency of the infection observed in Fiji thus also represents a pattern not predicted from theory. Extinction of the host population caused by the rise to high prevalence of the male-killing *Wolbachia* infection, followed by recolonization by the butterfly, is a potential explanation for this pattern. The change observed in the Fijian population between 1886 (45% infection prevalence) and 1894–1934 (92% infection prevalence) also demonstrates that male killers can climb rapidly in frequency, and thus that the "drive" possessed by these infections is higher than would have been envisaged from the ecology of the species and "benefits" to male killing. The fluctuations observed on Viti Levu also make it clear that the complete past history of a population can only be reliably inferred when there are samples from multiple time points.

Rapid changes in sex ratio will have dramatic effects on many aspects of host species biology, including the intensity and direction of sexual selection [6, 21, 22], population growth rates, and effective population size [23]. Perhaps most notable is the extreme reduction in effective population size seen in male-killer-infected species [24]. Where effective population size varies over time, the smallest population size has a disproportionate influence on levels of genetic variability [25]. All but one of our study populations has experienced extreme sex ratio conditions over the last 130 years, which is therefore likely to lead to reduced genetic variation and a lower capacity to respond to environmental change or coevolving antagonists. Even where suppression of male-killing activity returned host population sex ratio to parity, the impact of the past sex ratio bias will continue to influence standing genetic variation for generations to come. The frequency with which sex-ratio-distorting activity is observed in hybrids or interpopulation crosses or after transinfection [26, 27] suggests that processes of distorter spread and suppression are common in both animals and plants [28, 29] but often remain undetected because spread and suppression can occur quickly.

What is not clear is how commonly the dynamics observed in this system are typical of sex-ratio distorter/host interactions. Aside the *H. bolina* system [9], spatial mosaics of current sex ratio, associated with variation in the frequency of sex-ratio distorter/host suppressors of distortion, are seen in a number of other cases [30, 31]. This geographical variation is the expected product of the asynchronous rise and fall of sex-ratio distorters observed directly in this paper. However, flux over time as well as space is not necessarily a universal feature of these interactions. Indeed, there are cases of cytoplasmic sex-ratio distorters (e.g., [32]) and sex-ratio meiotic drive [33, 34], where there is no evidence of a host "suppression" response despite molecular diversity data, indicating the interactions are ancient. Why some interactions appear stable and others vary rapidly over space and time as predicted by Hamilton [2] awaits further research.

To conclude, evolution can be observed by comparing fossil and current species or inferred from variation between extant species placed on a phylogeny. However, it is rare to directly observe evolution over short time periods. Traditionally, direct observation of evolution has required records over time from long-term study populations [35–37]. Resurrection ecology, where viable propagule stages of known age are retrieved from sediment cores and compared to current specimens, represents a new technique with which to observe evolution directly [38]. However, both of these approaches are obviously limited to the few species for which this type of data or sample is available. We predict that the increasing availability of methods that make DNA from museum specimens accessible will lead to an escalating use of such collections to answer evolutionary questions. With the advent of high-throughput DNA sequencing, the worth of museum collections to future generations of evolutionary biologists is invaluable and inestimable.

Experimental Procedures

Sampling

Leg tissue was dissected from male and female *Hypolimnas bolina* specimens housed in the Natural History Museum, London, and Oxford University Museum of Natural History, UK. For testing of repeatability, two legs were taken at different times and processed through different laboratories. In other cases, a single leg was removed. Leg tissue was used in this study in order to preserve the utility of museum specimens for future morphometric studies. Preliminary work demonstrated the ability of *Wolbachia* to be detected in DNA obtained from leg tissue.

DNA Extraction and Assertion of DNA Quality

Genomic DNA was extracted with the QIAGEN QIAmp DNA Micro kit on ground frozen tissue. We ascertained the quality of historical DNA through a PCR assay based on the *H. bolina* mtDNA COI gene. A 197 bp section of this gene was targeted in order to maximize amplification success from degraded DNA (primer pair COI71F/COI268R). The amplicons were sequenced and then checked; they were of *H. bolina* origin.

Wolbachia Infection Presence PCR Assays

For samples that were positive for DNA quality, the presence of the male-killing *Wolbachia* (strain *w*Bol1) was assessed by two methods. First, we assayed for *Wolbachia* directly. To this end, we developed a PCR assay based on a short section (192 bp) of the *Wolbachia* surface protein (*wsp*) gene from the *H. bolina Wolbachia* strain *w*Bol1 (primer pair *wsp*183F [39]/EH5R). This amplification was designed to produce a product with similar amplicon size to that in the mtDNA amplification, and thus parallel it in terms of efficacy on museum specimens. Second, we ascertained the mitochondrial haplotype of the butterfly sample through sequencing. A recent study on contemporary samples has demonstrated the presence of an mtDNA haplotype (haplotype one) private to *w*Bol1-infected individuals [7]. In short, specimens carrying haplotype one are infected with *w*Bol1. A minority of infections are also found in other haplotypes that are not private to infected individuals, and in these cases only the *w*Bol1 PCR above can be diagnostic. The association between haplotype one and *w*Bol1 was tested in Table S2 via a subset of the Fijian museum sample, with the result that there was perfect concordance. This demonstrates that the association found in recently sampled populations is also present in museum samples. Overall, specimens were scored as infected if they were positive on direct *Wolbachia* assay or if they carried mtDNA haplotype one. It should be noted that this gives a conservative estimate of infection prevalence, because the *Wolbachia* PCR assay suffers from a low level of false negative results on museum material (c. 19% of "known" infected individuals come out as PCR negative on *Wolbachia* assay). False negatives will occur for nonhaplotype one individuals that are infected, but where

Wolbachia PCR shows no product. Given that the direct *w*Bol1 PCR assay recovers 81% of infected individuals from an infected sample, our estimate of 5 of 103 nonhaplotype one specimens being infected with *Wolbachia* will miss one or two infected individuals. Primer information is given in Table S4.

Supplemental Data

Supplemental Data include four tables and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)01588-7.

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