doi:10.1111/j.1558-5646.2008.00353.x

YOU CAN'T KEEP A GOOD PARASITE DOWN: EVOLUTION OF A MALE-KILLER SUPPRESSOR UNCOVERS CYTOPLASMIC INCOMPATIBILITY

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Received November 16, 2007
Accepted January 22, 2008

Maternally inherited parasites are known to impose a wide variety of reproductive manipulations upon their host. These often produce strong selection on the host to suppress the parasite, resulting in a reduction in the frequency of the parasite. However, in the butterfly *Hypolimnas bolina*, infected with a *Wolbachia* bacterium, field data demonstrate that suppression of the male-killing phenotype does not depress parasite frequency. Here we test and verify one hypothesis to explain this apparent paradox—*Wolbachia* induces a second phenotype, Cytoplasmic Incompatibility (CI), in populations where host suppression has evolved. We further demonstrate that the capacity to induce CI has not evolved de novo, but instead is instantaneously expressed upon the survival of infected males. The significance of these results is threefold: (1) multiple phenotypes can provide *Wolbachia* with the means to maintain itself in a host following suppression of a single manipulative phenotype; (2) the ability to induce CI can remain hidden in systems in which male-killing is observed, just as the ability to induce male-killing may be obscured in strains exhibiting CI; (3) the evolutionary maintenance of CI in a system in which it is not expressed suggests a functional link with male-killing or other traits under selection.

KEY WORDS: ***Hypolimnas bolina*, male-killing, suppression, *Wolbachia*.**

A surprising revelation of the last decade was that many insects carry inherited parasitic bacteria that pass from females to their progeny. One of these bacteria, *Wolbachia*, has attracted particular attention because of the range of ways in which it manipulates the reproduction of its host—including parthenogenesis, feminizing or killing males, and inducing cytoplasmic incompatibility (CI) between infected males and uninfected females (Hurst and Werren 2001). The widespread occurrence of these parasites, present in at least 20% of all insect species (Werren and Windsor 2000), illustrates the evolutionary success that such a large manipulative repertoire might provide.

However, such manipulations produce a conflict with the host—in particular, strains of inherited bacteria that distort the sex ratio of their host can engender strong selection for suppression. Selection can favor modifiers that either prevent the transmission of the parasite to the next generation, or diminish the harmful action of the parasite (Hornett et al. 2006) and these modifiers can spread rapidly through the host population (Charlat et al. 2007).

The expected short-term consequence of suppressor evolution is that the inherited bacterium becomes less common. Impedance of transmission clearly produces a reduction in prevalence. Likewise, the suppression of the phenotype should produce a decline in prevalence, associated with loss of the drive the phenotype produced. Thus, without counter-adaptation on the part of the parasite, host evolution is expected to reduce parasite prevalence. Paradoxically, in the one case in which suppressor evolution has been conclusively documented in the wild, reduction in infection frequency is not observed (Charlat et al. 2005; Hornett et al. 2006). The nymphalid butterfly *Hypolimnas bolina* is found throughout the Indo-Pacific and is known to carry a male-killing strain of *Wolbachia*, called *w*Bol1 (Dyson et al. 2002). Recently, we observed that although *w*Bol1 was fully operational as a male-killer in many Polynesian populations, this was not the case in some SE Asian populations. The spread of a suppressor of male-killing through the SE Asian host populations resulted in an infection that no longer killed males and restored the sex ratio to unity (Hornett et al. 2006). Intriguingly, rather than resulting in a loss of infection, *w*Bol1 occurs at very high prevalence where suppressed (Charlat et al. 2005).

One hypothesis for the retention of *w*Bol1 in *H. bolina* following evolution of a male-killer suppressor is that another phenotype is expressed, producing the drive necessary to maintain the infection in the population. Recent theory and empirical studies have indicated that *Wolbachia* strains can express multiple phenotypes (Hurst et al. 2002). Here we test and demonstrate the hypothesis that infected males, rescued from male-killing through the action of the suppressor, can induce CI, a phenotype where infected male hosts are unable to produce a viable zygote when fertilizing uninfected eggs. We further demonstrate that the capacity to induce CI has not evolved de novo following male-killer suppression, but instead existed prior to suppressor evolution.

Materials and Methods

MATERIALS

Three *H. bolina* populations were used in this investigation: Thailand, the Philippines, and the island of Moorea in French Polynesia. The Moorean population is polymorphic for the presence of *w*Bol1 in females, and does not carry nuclear suppressors of male-killing (Hornett et al. 2006). Individuals from this population were reared from wild females caught locally. The two *H. bolina* stocks from SE Asia were obtained from pupae suppliers in the United Kingdom (Thai pupae from Stratford-upon-Avon Butterfly Farm, and Philippine pupae from London Pupae Supplies). In both cases, *w*Bol1 is found in males and females alike, and does not kill males owing to the presence of a nuclear suppressor of male-killing (Hornett et al. 2006). The SE Asian stocks each represent F2 progeny from wild-caught females from the area indicated. Both populations were sent to Moorea for breeding in semi-natural conditions as described elsewhere (Hornett et al. 2006).

The identity of the *Wolbachia* strain, *w*Bol1, was confirmed as identical in all three populations through *wsp* sequences and four MLST loci (Baldo et al. 2006). Infection status of individuals was confirmed via PCR assays for *w*Bol1 presence based on *Wolbachia*-specific PCR primers, 81F and 522R that amplify the *wsp* gene of B-clade *Wolbachia* (Zhou et al. 1998). Prior to assay for *Wolbachia*, the primer pair CO1F and CO1R were used to confirm the quality of the DNA extraction (Brunton and Hurst 1998).

DO *w*Bol1-INFECTED MALES FROM SE ASIA CAUSE CI IN UNINFECTED FEMALES?

We hypothesized that suppression of male-killing allowed infected males to survive, and that these males induce CI when mated to uninfected females, increasing the infection frequency and preventing loss of the infection. We tested this by crossing *w*Bol1-infected males from SE Asia to uninfected females from Moorea in French Polynesia, a population where suppression has not yet evolved and where infected and uninfected females coexist. Due to the high *w*Bol1 prevalence in SE Asia, no uninfected SE Asian females could be used in this experiment. If CI is expressed this will be revealed as reduced egg hatch rate. In the absence of reliable antibiotic curing, four controls were conducted. First, the fertility of the SE Asian infected males in the above cross was tested by mating SE Asian *w*Bol1-infected males to SE Asian infected females, which should not result in CI. Second, the fertility of the uninfected Moorea females was checked by mating these to uninfected Moorean males. Third, the presence of any nuclear

incompatibility between the populations was ascertained in two different types of crosses. SE Asian *w*Bol1-infected males were crossed to Moorean infected females, and uninfected Moorean males crossed to infected SE Asian females. No CI is expected in these crosses, thus CI-independent incompatibility between nuclear backgrounds can be estimated from any depression in hatch rate.

These crosses were effected in the following way. Adults were individually marked on their wings to permit identification, and crosses conducted in a large outdoor flight cage. Oviposition was encouraged after mating by placing the female into a clear container also containing a young *Syndrella nodiflora* plant. After four days any developing eggs hatch. On day five the total number of eggs laid and the number of hatched eggs (first-instar larvae) were counted in each clutch. Compatibility was measured as egg hatch rate (the proportion of hatched eggs) with a low egg hatch rate indicating the presence of CI. Egg hatch rate estimation for individual crosses was based on sample sizes of more than 50 eggs in all cases.

DOES UNSUPPRESSED *w*Bol1 FROM MOOREA HAVE THE CAPACITY TO CAUSE CI?

We tested the hypothesis that naturally unsuppressed *w*Bol1 could induce CI in *H. bolina* males carrying the Moorean *w*Bol1 infection by examining the compatibility of these males when mated with infected and uninfected females. Males carrying the *w*Bol1 infection from Moorea were generated by crossing infected females from Moorea to males from SE Asia carrying the suppressor of male-killing, resulting in surviving hybrid males with the Moorean *w*Bol1 infection. Larvae were reared in the laboratory under natural light and temperatures and fed on an excess of *Asystasia gangetica*, a larval host plant found in Moorea, until eclosion as adult. The resultant "Moorea *w*Bol1" infected males were crossed with uninfected Moorean females to examine the expression of CI. As a control, these males were also crossed to infected females from both Moorea and SE Asia, and with sister hybrid infected females.

Results

When crossing *w*Bol1-infected males from SE Asia to uninfected females from Moorea there was a complete failure in egg hatch, suggesting that where suppression of male-killing has evolved and infected males survive, these males induce strong CI (Table 1). In contrast, the high egg hatch rates observed in crosses between SE Asian *w*Bol1-infected males and both SE Asian infected females and Moorean infected females, indicate that these males are fertile, and that the *Wolbachia* from both populations make infected females immune to the expression of CI. The high egg hatch rate in the SE Asian infected male × Moorean infected female cross also demonstrates that the incompatibility observed in SE Asian infected male × Moorean uninfected female is not caused by nuclear incompatibilities, a point independently attested by the compatibility of uninfected Moorean males and infected SE Asian females. A further cross between uninfected Moorean males and females demonstrates that uninfected Moorean females are fertile (Table 1). Statistical analysis (Table 2) indicates the SE Asian *w*Bol1-infected male × Moorea uninfected female cross has a significantly lower egg hatch rate than each of the control crosses.

The above results demonstrate that *w*Bol1 in SE Asia can induce and rescue CI; a phenomenon that likely underlies the current high prevalence of infection in male and female *H. bolina* in SE Asia. The CI induction could either have immediately emerged following suppression of male-killing (when infected males are produced in which CI can then be active), or may have evolved as an evolutionary response of *Wolbachia* to the spread of male-killer suppressor genes. We examined this issue by generating surviving *H. bolina* males that carry the naturally male-killing Moorean *w*Bol1 infection but also the suppressor of male-killing, and then crossing these males to infected and uninfected females. As can

Table 1. Crosses conducted to test compatibility between individuals of varying infection status and origin. Compatibility is indicated by egg hatch rates. Sample size (number of crosses) for each cross type is given in parentheses. For each cross, the infection status is given first, followed in parenthesis by the nuclear background of the infection

Male infection status (nuclear origin)	Female infection status (nuclear origin)	Egg Hatch Rate (*n*)
SE Asia *w*Bol1 (SE Asia)	Uninfected (Moorea)	0.00 (11)
SE Asia *w*Bol1 (SE Asia)	SE Asia *w*Bol1 (SE Asia)	0.80 (50)
SE Asia *w*Bol1 (SE Asia)	Moorea *w*Bol1 (Moorea)	0.81 (12)
Uninfected (Moorea)	SE Asia *w*Bol1 (SE Asia)	0.92 (18)
Uninfected (Moorea)	Uninfected (Moorea)	0.81 (22)
Moorea *w*Bol1 (Moorea/SE Asia hybrid)	Uninfected (Moorea)	0.05 (20)
Moorea *w*Bol1 (Moorea/SE Asia hybrid)	Moorea *w*Bol1 (Moorea)	0.72 (2)
Moorea *w*Bol1 (Moorea/SE Asia hybrid)	SE Asia *w*Bol1 (SE Asia)	0.84 (14)
Moorea *w*Bol1 (Moorea/SE Asia hybrid)	Moorea *w*Bol1 (Moorea/SE Asia hybrid)	0.76 (17)

Table 2. Results of Mann–Whitney comparisons of egg hatch rate between different cross-types. Results are transformed into standardized normal deviates (z), with number of crosses of each type given. Parents are described in terms of infection status, with nuclear background in parenthesis.

Cross 1			Cross 2		Mann–Whitney comparison
Male	Female		Male	Female	
SE Asia *w*Bol1 (SE Asia)	Uninfected (Moorea)	vs.	SE Asia *w*Bol1 (SE Asia)	SE Asia *w*Bol1 (SE Asia)	$n_1=11$, $n_2=50$; $z=5.06$, $P<0.001$
SE Asia *w*Bol1 (SE Asia)	Uninfected (Moorea)	vs.	Uninfected (Moorea)	SE Asia *w*Bol1 (SE Asia)	$n_1=11$, $n_2=18$; $z=4.40$, $P<0.001$
SE Asia *w*Bol1 (SE Asia)	Uninfected (Moorea)	vs.	Uninfected (Moorea)	Uninfected (Moorea)	$n_1=11$, $n_2=22$; $z=4.62$, $P<0.001$
Moorea *w*Bol1 (SE Asia/Moorea hybrid)	Uninfected (Moorea)	vs.	Moorea *w*Bol1 (SE Asia/Moorea hybrid)	Moorea *w*Bol1 (SE Asia/Moorea hybrid)	$n_1=20$, $n_2=17$; $z=4.72$, $P<0.001$
Moorea *w*Bol1 (SE Asia/Moorea hybrid)	Uninfected (Moorea)	vs.	Moorea *w*Bol1 (SE Asia/Moorea hybrid)	Moorea *w*Bol1 (Moorea)	$n_1=20$, $n_2=2$; $z=2.17$, $P<0.05$
SE Asia *w*Bol1 (SE Asia)	Moorea *w*Bol1 (Moorea)	vs.	Moorea *w*Bol1 (SE Asia/Moorea hybrid)	Moorea *w*Bol1 (Moorea)	$n_1=12$, $n_2=2$; $z=0.27$, ns
Moorea *w*Bol1 (SE Asia/Moorea hybrid)	SE Asia *w*Bol1 (SE Asia)	vs.	SE Asia *w*Bol1 (SE Asia)	SE Asia *w*Bol1 (SE Asia)	$n_1=14$, $n_2=50$; $z=0.67$, ns

be seen in Table 1, males infected with the Moorean *w*Bol1 induce CI when crossed with uninfected Moorean females, but are compatible with infected females from either Moorea or SE Asia, and with sister hybrid infected females. Statistical analysis again confirms the inference that crosses involving males carrying Moorea *w*Bol1 induce CI when mated to uninfected females (Table 2). This demonstrates the CI phenotype emerges directly following male-killing suppression, rather than evolving afterwards as a response to suppression.

Discussion

Our results demonstrate that the *Wolbachia* strain, *w*Bol1, immediately expresses a second phenotype, CI, when the original phenotype, male-killing, is suppressed by the host. Where male-killing is suppressed, the *Wolbachia* strain is present at near fixation—a likely consequence of the CI revealed following suppression, although it should be noted that we have not directly established that this strain is competent to produce CI against uninfected SE Asian individuals (the test was against uninfected individuals from Moorea). The strain *w*Bol1 shows near-perfect vertical transmission, and theory indicates that fixation is the expected consequence of strong CI in these circumstances (Hurst et al. 2002). Thus, the evolution of suppression does not rid the host of the infection where the strain has more than one phenotype. Rather, it is likely to have caused an increase in prevalence, associated with the drive produced by CI.

It is interesting that the suppressed *Wolbachia* has higher prevalence than the unsuppressed form, indicating that in frequency terms at least, *Wolbachia* does better as a CI strain rather than as a male-killer, a result predicted by population biology models (Hoffmann et al. 1990; Hurst 1991). Despite this, male-killing is retained by *Wolbachia* as its primary phenotype (CI is only expressed when the host suppresses male-killing). This observation is at first paradoxical, but is expected from theory (Hurst et al. 2002). Consider a population of a host, such as *H. bolina*, that carries a strain of *Wolbachia* that can both kill males and, when male-killing (MK) is not expressed, infected males induce CI against uninfected females (a MK + CI strain). Now consider a mutation of this *Wolbachia* strain that causes loss of expression of male-killing (and thus allows males to always survive and express CI) (CI-only strain). This CI-only strain when in males can induce CI against uninfected females, but not against females carrying the ancestral MK + CI form, as the ancestral strain shares the CI competency with the derived form. Thus, in a panmictic population, the derived CI-only strain cannot have higher fitness than the ancestral MK + CI strain as its drive is not effective against it, and thus will not invade, despite of the observation that, on its own, it would achieve higher prevalence than the MK + CI strain.

Wolbachia has been notable for its evolutionary success, measured in terms of the number of host species infected. This has been attributed widely to the range of phenotypes *Wolbachia* induces. It is possible that part of this success is also associated with the ability of single strains to induce multiple phenotypes. The spread of *Wolbachia* through a population depends on the rate of formation of new interactions, and the rate of loss of existing ones. With respect to the longevity of interactions, multiple phenotypes provide resilience against the evolution of suppression that gives additional longevity to the host symbiont interaction. The contribution of possession of multiple phenotypes within a strain to the overall success of *Wolbachia* will depend crucially on the proportion of *Wolbachia* strains that are able to induce multiple phenotypes. Our study adds to the previous finding of Sasaki and coworkers where transinfection experiments demonstrated that a strain of *Wolbachia* could cause male-killing and CI in two different, but closely related, host species (Sasaki et al. 2002). Our data complement the recent finding that *Wolbachia* in *Drosophila recens*, that natively cause CI, induce male-killing when placed in some *D. subquinaria* lines (Jaenike 2007), and the observation of weak CI exhibited by male *D. bifasciata* following thermal rescue from male-killer action (Hurst et al. 2000). These case studies indicate that possession of multiple phenotypes by *Wolbachia* is not rare. However, the precise frequency of the phenomenon of multiple phenotypes awaits further work.

One notable insight of our study pertains to the evolutionary dynamics of *Wolbachia* phenotypes. It has been noted that the phenotype of CI may degrade over time. When all individuals are infected, the ability of *Wolbachia* to produce host modification is no longer positively selected, and becomes an unused trait. Unexpressed traits commonly degrade either by mutation and drift, or mutation combined with selection against a costly nonexpressed function. Thus, the ability to induce and rescue CI is expected to wane over time within a species, ultimately resulting in the possibility of loss of infection (Hurst and McVean 1996). In our case, the hidden phenotype, CI, has been maintained in host populations despite being rarely expressed because of male-killing preventing the formation of infected males. The maintenance of CI is thus in opposition to the theory above, as is the case of the maintenance of male-killing ability by CI-inducing *Wolbachia* in *D. recens* (Jaenike 2007). There are two possible explanations. One is that there has been insufficient time for the CI trait to degrade. The alternative is that maintenance of the CI genetic determinants (both induction and rescue of the incompatibility) in SE Asian as well as Polynesian *H. bolina* host populations indicates a functional, mechanistic link between male-killing and CI. The expression of male-killing would prevent degradation of the hidden CI-induction and rescue ability through mutation. Indeed, further investigations into the suppressor of male-killing may not only reveal information regarding the mechanism of male-killing, but also the determinants of CI.

One further characteristic of the evolution of suppression in this system is potential reproductive isolation against populations infected with different strains of *Wolbachia*. Aside from the "suppressed" *w*Bol1 populations in SE Asia, *H. bolina* populations are of three kinds (Charlat et al. 2005). First, there are populations such as Moorea, where *w*Bol1 is unsuppressed, and females are either infected with *w*Bol1 or uninfected, and males are generally uninfected. These unsuppressed populations will be partially isolated from suppressed populations, as infected migrant males carrying the suppressor are incompatible with uninfected females. Second, there are populations infected by a different strain of *Wolbachia, w*Bol2. This strain can induce CI against both *w*Bol1-infected and uninfected individuals (Charlat et al. 2006). Should *w*Bol1 also be able to induce CI against *w*Bol2-infected individuals, these will be bidirectionally incompatible and reproductive isolation between populations infected with *w*Bol1 but carrying the suppressor and populations infected with *w*Bol2 would be profound. Finally, there are populations apparently uninfected with *Wolbachia*, such as those in Australia. If an infected migrant arrived, this would result in strong unidirectional CI, providing partial reproductive isolation. The evolution of suppression is thus likely associated with alterations in the pattern of gene flow within this species across its range, which may in turn affect the success of each of the infections, and indeed the spread of the suppressor. These effects would add to the effects of asymmetric gene flow associated with sex ratio distortion (Telschow et al. 2006).

Beyond our understanding of the dynamics of infection within a species, the observation that single strains of *Wolbachia* can induce multiple phenotypes in the host will need to be considered in attempts to use comparative genomics to dissect the causes of phenotypic differences between *Wolbachia* strains. It will be important to remember that a strain with a given sequence may have abilities beyond the normally observed phenotype that is characteristic of the strain. *w*Bol1, the strain under investigation in our study, is closely related to *w*Pip, the strain sequenced from *Culex pipiens* (Walker et al. 2007). That *w*Pip may have multiple phenotypes should be born in mind when considering the composition of its genome. Indeed, inferences regarding the cause of different host phenotypes from comparative genomics should, where possible, rely on previous rigorous ascertainment of phenotypes within a single host genetic background.

ACKNOWLEDGMENTS

The authors are grateful to the Government of French Polynesia for permission to work and the BBSRC, NERC (grant no. NE/B503292/1) and NSF (grant no. 0416268) for funding.

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Associate Editor: D. Presgraves