Wolbachia Symbiont Infections Induce Strong Cytoplasmic Incompatibility in the Tsetse Fly Glossina morsitans

Uzma Alam¹, Jan Medlock¹*¹, Corey Brelsfoard¹², Roshan Pais¹⁴, Claudia Lohs²°, Séverine Balmand², Jozef Carnogursky³, Abdelaziz Heddi², Peter Takac³, Alison Galvani¹, Serap Aksoy¹*

¹ Yale University, School of Public Health, Division of Epidemiology of Microbial Diseases, New Haven, Connecticut, United States of America, ² INSA-Lyon, UMR203 BF2I, INRA, Biologie Fonctionnelle Insectes et Interactions, Bat. Louis-Pasteur, Villeurbanne, France, ³ Institute of Zoology, Section of Molecular and Applied Zoology, Slovak Academy of Sciences, Bratislava, Slovakia

Abstract

Tsetse flies are vectors of the protozoan parasite African trypanosomes, which cause sleeping sickness disease in humans and nagana in livestock. Although there are no effective vaccines and efficacious drugs against this parasite, vector reduction methods have been successful in curbing the disease, especially for nagana. Potential vector control methods that do not involve use of chemicals is a genetic modification approach where flies engineered to be parasite resistant are allowed to replace their susceptible natural counterparts, and Sterile Insect technique (SIT) where males sterilized by chemical means are released to suppress female fecundity. The success of genetic modification approaches requires identification of strong drive systems to spread the desirable traits and the efficacy of SIT can be enhanced by identification of natural mating incompatibility. One such drive mechanism results from the cytoplasmic incompatibility (CI) phenomenon induced by the symbiont Wolbachia. CI can also be used to induce natural mating incompatibility between release males and natural populations. Although Wolbachia infections have been reported in tsetse, it has been a challenge to understand their functional biology as attempts to cure tsetse of Wolbachia infections by antibiotic treatment damages the obligate mutualistic symbiont (Wigglesworthia), without which the flies are sterile. Here, we developed aposymbiotic (symbiont-free) and fertile tsetse lines by dietary provisioning of tetracycline supplemented blood meals with yeast extract, which rescues Wigglesworthia-induced sterility. Our results reveal that Wolbachia infections confer strong CI during embryogenesis in Wolbachia-free (GmmWt) females when mated with Wolbachia-infected (GmwWt) males. These results are the first demonstration of the biological significance of Wolbachia infections in tsetse. Furthermore, when incorporated into a mathematical model, our results confirm that Wolbachia can be used successfully as a gene driver. This lays the foundation for new disease control methods including a population replacement approach with parasite resistant flies. Alternatively, the availability of males that are reproductively incompatible with natural populations can enhance the efficacy of the ongoing sterile insect technique (SIT) applications by eliminating the need for chemical irradiation.

Introduction

Tsetse flies are the sole vector of Human African Trypanosomiasis (HAT), also known as sleeping sickness, caused by the protozoan Trypanosoma brucei spp., in sub-Saharan Africa. Recent figures released by the World Health Organization (WHO) indicate that the devastating HAT epidemics, which started in the early 1990s, are coming under control and may no longer represent a major public health crisis [1–3]. While this news is welcoming, about 60 million people continue to live in tsetse infested areas at risk for HAT in 37 countries, and those at high risk are in remote areas where disease control is difficult to implement [2]. Diseases caused by trypanosomes in animals continue to be rampant in Africa and result in severe economic and nutritional losses. The ability to curb infections in animals stands to increase both economic and nutritional status of the continent.

Unfortunately, the disease toolbox remains very limited. To date, no vaccines have been developed for HAT, therapeutic treatments are expensive and have serious side effects, and diagnostic tools are inadequate [1]. Reduction of tsetse populations, however has proven as an effective method for disease control [1]. Although effective, implementation of vector control methods in remote
Author Summary

Infections with the parasitic bacterium Wolbachia are widespread in insects and cause a number of reproductive modifications, including cytoplasmic incompatibility (CI). There is growing interest in Wolbachia, as CI may be able to drive desired phenotypes such as disease resistance traits, into natural populations. Although Wolbachia infections had been reported in the medically and agriculturally important tsetse, their functional role was unknown. This is because attempts to cure tsetse of Wolbachia by antibiotic treatment damages the obligate mutualist Wigglesworthia, without which the flies are sterile. Here we have succeeded in the development of Wolbachia free and still fertile tsetse lines. Mating experiments for the first time provides evidence of strong CI in tsetse. We have incorporated our empirical data in a mathematical model and show that Wolbachia infections can be harnessed in tsetse to drive desirable phenotypes into natural populations in few generations. This finding provides additional support for the application of genetic approaches, which aim to spread parasite resistance traits in natural populations as a novel disease control method. Alternatively, releasing Wolbachia infected males can enhance Sterile Insect applications, as this will reduce the fecundity of natural females either uninfected or carrying a different strain of Wolbachia.

Wolbachia Induces CI in Tsetse

Results

Dietary Supplementation with Yeast Extract Rescues

Ec-fundity in the Absence of the Obligate Wigglesworthia

In many insect systems, tetracycline supplemented diet is used to generate Wolbachia free lines to demonstrate the functional role of Wolbachia through mating experiments. Inseminated tsetse females maintained on tetracycline-supplemented blood meals however do not generate any viable progeny. This is because tetracycline treatment damages the obligate intracellular Wigglesworthia present in the midgut bacteriome structure (Figure S1). These results are similar to prior reports where damage to Wigglesworthia had been found to reduce host fecundity. The fecundity of fertile females maintained on various diets was evaluated (Figure 1A). Specifically, the diet combinations were as follows: a) blood only, b) blood and ampicillin, c) blood and tetracycline, d) blood and yeast, e) blood, ampicillin and yeast, and f) blood, tetracycline and yeast. We monitored the number of

utero. The resulting larva hatches and is carried in the intrauterine environment through three larval instars before being deposited. During its intrauterine life, the larva receives all of its nutrients in the form of milk secreted by the female accessory glands, milk glands. While Wolbachia is transovarially transmitted, the enteric symbionts are maternally transmitted into tsetse's intrauterine larva through mother's milk secretions. By providing ampicillin in the blood meal diet, it has been possible to clear the extracellular Wigglesworthia in the milk without damaging the intracellular Wigglesworthia in the bacteriome. Thus, such females remain fecund but give rise to sterile progeny that lack Wigglesworthia (both bacteriome and milk gland populations) but retain Wolbachia and Sodalis. As a result of the obligate role of Wigglesworthia, it has not been possible to use tetracycline treatment to cure Wolbachia infections, and the biological significance of Wolbachia infections in tsetse has thus remained elusive.

Wolbachia infections associated with various insects have been shown to cause a number of reproductive modifications in their hosts, the most common being CI. CI occurs when a Wolbachia infected male mates with an uninfected female, causing developmental arrest of the embryo. In contrast, Wolbachia infected females can mate with either an uninfected male or a male infected with the same Wolbachia strain and produce viable Wolbachia infected offspring. This reproductive advantage of infected females results in the spread of Wolbachia infections along with other traits that infected insects may exhibit. Empirical studies and previously developed models have shown that the reproductive advantage provided by Wolbachia may be able to drive desired phenotypes along with other maternally inherited genes, organelles and/or symbionts into natural populations. The Wolbachia type found in the tsetse species Glossina morsitans morsitans belongs to the Wolbachia A super group. In a number of insect systems, Wolbachia strains belonging to the A super group have been associated with the CI phenotype in the different hosts they infect.

Here we investigated the possible role of Wolbachia symbionts that can be used to drive desirable tsetse phenotypes into natural populations, or to induce natural reproductive male sterility for field applications. We developed a dietary supplementation method that can restore fecundity of tsetse in the absence of their natural symbiotic fauna, including obligate Wigglesworthia and Wolbachia. We report on the fitness parameters of the engineered symbiont-free lines and on the level of CI expression after wild type and aposymbiotic flies are crossed. A mathematical model was also developed to ascertain whether Wolbachia infections in tsetse could be used to drive a disease refractory phenotype into a natural population.
larva deposited in each group over a 40-day period when females undergo two gonotrophic cycles (defined as time required for the development of a single progeny in-utero). Under optimum conditions the first gonotrophic cycle takes about 20–22 days for development from egg to parturition. In subsequent gonotrophic cycles females produce a larva every 9 to 11 days. As we had previously shown, ampicillin treatment does not reduce fecundity since it does not damage *Wigglesworthia* resident within bacteriocytes in the midgut, unlike tetracycline, which clears all bacteria including *Wigglesworthia* and *Wolbachia* and induces sterility. Accordingly, ampicillin-receiving flies remained fecund while tetracycline receiving flies were rendered sterile.

Figure 1. The effects of antibiotic treatment on *G. m. morsitans*. (A) Effect of yeast supplementation on percent larval deposition over two gonotrophic cycles between wild type flies maintained on normal blood supplemented with antibiotics (ampicillin or tetracycline) compared to flies maintained on yeast supplementation. The sample size (n) is above each column, and is represented as the number of females alive at the beginning of each gonotrophic cycle. (B) PCR analysis shows the *GmmWt* flies are positive for *Wigglesworthia* (Wig Thic), *Sodalis* (Sod Chit) and *Wolbachia* (Wol Groel). In contrast offspring resulting from tetracycline treated females (A and B) lack all three of the symbionts. The bottom panel shows gDNA quality as measured by tsetse β-tubulin. (C) Presence of *Wolbachia* infections in late developing egg chambers of *GmmWt* females. Nuclei are indicated by the blue DAPI stain and *Wolbachia* is shown by the red stain (D&E) Presence and absence of *Wolbachia* infections in early developing egg chambers of *GmmWt* and *GmmApo* females respectively. (F) Comparison of adult longevity between female *GmmWt* and *GmmApo* over a forty-day period on yeast supplemented diet. Error bars are reflective of standard error. Data points are offset for clarity.

doi:10.1371/journal.ppat.1002415.g001
Yeast extract (10% w/v) provisioning of the blood meal rescued fecundity of the females receiving tetracycline to similar levels as that of wild type and ampicillin receiving flies (65%, 55% and 64% over the first gonotrophic cycle and 53%, 58% and 49% over the second gonotrophic cycles, respectively). However, yeast provisioning at 10% w/v had a cost on fecundity when compared to flies maintained on normal blood meals, 92% versus 55% over the first gonotrophic cycle and 92% and 58% over the second gonotrophic cycle, respectively. Nevertheless, yeast supplementation was able to rescue the tetracycline-induced sterility to levels comparable to those observed for GmmWt receiving yeast or ampicillin supplemented blood meals, respectively (Figure 1A). Therefore yeast supplemented blood meals, this line (similar to GmmWt) and another lacking Wigglesworthia but still retaining Wollbachia and Sodalis (GmmWig−) served as the control for the CI cross in tsetse.

The GmmApo progeny resulting from the first and second depositions of tetracycline treated mothers were tested for the presence of Sodalis, Wigglesworthia and Wolbachia by a bacterium-specific PCR-assay. The PCR-assay demonstrated the absence of all three symbionts as early as the first deposition in both the male and female GmmApo adults (Figure 1B). The absence of Wolbachia from the reproductive issues of GmmApo females was also verified by Fluorescent In Situ Hybridization (FISH) analysis (Figure 1E).

To determine if the fertility of GmmApo males is negatively affected, we mated GmmWt females with either GmmWt or GmmApo males and maintained all flies on yeast-supplemented blood meals. Larval deposition and eclosion rates from both crosses were compared using arcsin(sqrt(x)) transformed data to ensure normality. No significant difference was observed between the crosses for two gonotrophic cycles (P > 0.05) (Table 1). The mean larval deposition rate for GmmWt females crossed with GmmWt males was 0.68 and 0.65 for the first and second gonotrophic cycles respectively, while the mean larval deposition rate for GmmApo females crossed with GmmApo males was 0.87 and 0.89 for the first and second gonotrophic cycles, respectively (Table 1). Similarly, no difference in eclosion rates was observed between the two groups (P > 0.05) (Table 2). Of the pupae obtained in the first gonotrophic cycle from the GmmWt cross, 92% underwent eclosion compared to 83% for the cross between GmmWt females and GmmApo males. For the second gonotrophic cycle, we observed 89% average eclosion for pupae from GmmWt crosses and 93% for pupae from GmmWt females crossed with GmmApo males (Table 2). Taken together, these results demonstrate the preservation of reproductive fitness in GmmApo males and rule out possible paternal effects of Wolbachia in tsetse.

Cl Expression

To determine the expression of Wolbachia-induced CI, cage population crosses were setup between GmmWt and GmmApo individuals. Cages were the experimental units and the data were arcsin(sqrt(x)) transformed to ensure normality. To estimate the possible cost of reproductive fitness due to loss of Wigglesworthia, we made use of GmmWig− flies. Since GmmWig− flies still retained Wolbachia infections but lacked Wigglesworthia (as described earlier and in Figure 1A), this line served as the control for the CI cross in order to measure potential fecundity effects due to loss of Wigglesworthia in the GmmApo line and possible yeast-supplementation effects.

Although CI typically manifests itself as embryonic lethality, given the viviparous nature of reproduction in tsetse, we measured larval deposition rates, which are reflective of both successful embryogenesis and larvagenesis (Table 1). Differences in larval deposition rates (number of larva deposited per female) over the two gonotrophic cycles for all crosses were significant by ANOVA on arcsin(sqrt(x)) transformed data [ANOVA; first deposition, F4, 206 = 20.6, P < 0.0001, second deposition, F4, 219 = 21.9, P < 0.0001]. No differences in larval deposition were observed between the crosses GmmWt × GmmWt, GmmWig− × GmmWig− and GmmApo × GmmApo (Table 1). However differences were observed in compar-

Table 1. CI expression shown by average larval deposition rates in crosses between GmmApo females mated with GmmWt males.

| Cross type        | Larval deposition rate 1st gonotrophic cycle | Larval deposition rate 2nd gonotrophic cycle |
|-------------------|---------------------------------------------|---------------------------------------------|
| GmmWt × GmmWt     | 0.68 ± 0.01 ABD; n = 108                    | 0.65 ± 0.07 ABD; n = 89                     |
| GmmApo × GmmWt    | 0.87 ± 0.06 BCD; n = 59                     | 0.89 ± 0.16 BE; n = 48                      |
| GmmWig− × GmmWig− | 0.61 ± 0.20 BCDE; n = 49                    | 0.53 ± 0.18 CDE; n = 26                     |
| GmmApo × GmmWt    | 0.10 ± 0.02 BCD; n = 44                     | 0.00 ± 0.00; n = 38                        |

Larval deposition rates for each gonotrophic cycle and each cross type replicate were determined by dividing the number of larvae deposited per day by the number of remaining females in the cage on the day of larviposition, and summing the values for each gonotrophic cycle. Mean deposition rate values with different superscripted letters are statistically different from each other (P < 0.05) using Tukey-Kramer post hoc multiple comparison tests within each gonotrophic cycle, i.e., a, b, and c are significantly different from each other, c but not a and b are different from ab. n was calculated by combining the total number of females alive when the first larva were deposited for the three replicates of each cross type. GmmWt = Wild-type flies with all three symbions, GmmApo = flies treated with tetracycline that lack Wigglesworthia, Sodalis, and Wolbachia, and GmmWig− = flies treated with ampicillin that lack only Wigglesworthia.

doi:10.1371/journal.ppat.1002415.t001
**Effect of Symbiont Infection on Host Eclosion**

Other than reproductive modifications, *Wolbachia* infections have been shown to affect the fitness of their insect hosts [34,35]. In this study, differences in eclosion rates (Table 2) were observed in the first gonotrophic cycle of crosses of *Gmm*Wt, *Gmm*Apo, and *Gmm*Vg—individuals on arcsin(sqrt(x)) data (ANOVA, first gonotrophic cycle, F2, 11 = 7.5, P = 0.0036, second gonotrophic cycle, F3, 12 = 2.5, P = 0.13) (Table 2). No differences in eclosion rates were observed in single pair crosses for both gonotrophic cycles (Kruskal-Wallis first P = 0.13) (Table S2). No differences in eclosion rates were observed in single-pair crosses (Table S2). Differences were observed in larval deposition rates and pupal eclosion rates showed similar patterns to large cage experiments when measured in single-pair crosses (Table S2). Differences were observed in larval deposition rates and pupal eclosion rates showed similar patterns to large cage experiments when measured in single-pair crosses (Table S2).

**Spread of Wolbachia in Tsetse Populations**

From the experimental data, we estimated the impact of CI on tsetse population biology using a Bayesian Markov chain Monte Carlo method. The transmission failure of *Wolbachia* from mothers to developing oocytes was moderate: 10.7% [0.07%, 22.7%] of progeny produced by *Gmm*Wt mothers was *Wolbachia* uninfected (Table 3). In addition, the incompatibility between *Gmm*Wt males and *Gmm*Apo females was strong: 79.8% [63.0%, 90.3%] of matings between *Gmm*Wt males and *Gmm*Apo females did not result in viable larvae as measured by pupal deposition. There was a significant fecundity (number of larval progeny deposited) benefit for *Wigglesworthia* infection: *Gmm*Wt females had 28.4% [8.5%, 54.2%] higher fecundity than *Gmm*Wt—females. Furthermore, *Wolbachia* infection alone was estimated to give a fecundity benefit of 19.3% [−9.2%, 57.9%]. This is an estimate of the fecundity difference between hypothetical females carrying *Wigglesworthia* and *Sodalis* but not *Wolbachia* and the experimental *Gmm*Wt—females.

Most importantly, our model demonstrates that, given a large enough initial release, *Wolbachia* infected individuals will successfully invade a tsetse population (Table 4). The fixation prevalence of *Wolbachia* is estimated to be 96.9% [83.6%, 99.8%]. There may exist a release threshold, which an initial release must be above in order for *Wolbachia* to invade: the median was no release threshold (i.e., 0%), but the upper end of the 95% credible interval was a release of the size of 39.6% of the native population. The median threshold value is zero because, despite imperfect maternal transmission, the fecundity benefit of *Wolbachia* is strong enough to allow *Wolbachia* to invade a naive tsetse population from any size initial release, no matter how small. In addition, the time to reach fixation from a release of the size of 10% of the native population.
can be relatively short; the median value was 529 days, however the upper end of the 95% credible interval was undefined because in more than 2.5% of samples, 10% initial release was below the release threshold.

Sensitivity analysis showed that the model results are sensitive to both assumed and estimated parameters (supplementary material Text S1). In particular, time to fixation had the largest sensitivity to the time to first deposition and large elasticities to Wolbachia- and Wigglesworthia-related parameters, suggesting that improving the estimates of these parameters would most effectively improve the fidelity of the estimate of time to fixation.

Discussion

Here, we report for the first time on the functional role of Wolbachia infections in tsetse, which support the expression of CI. Microscopic analyses of the CI expressing females show that loss of fecundity results from early embryogenic failure. Essential for our studies we have discovered that we can maintain Wolbachia cured tsetse lines fertile by dietary provisioning of tetracycline supplemented blood meals with yeast extract, despite the fact that such flies lack the obligate mutualist Wigglesworthia, which is essential for tsetse’s fecundity. When incorporated into a mathematical model, our results suggest that Wolbachia can be used successfully as a gene driver and, the time to reach fixation is relatively short given a large enough initial release: on the order of 1 to 2 years. These results provide a first insight into the role of Wolbachia infections in a viviparous insect and indicate that Wolbachia mediated CI can potentially be used to drive desirable tsetse phenotypes into natural populations.

Our data presented here as well as previous results from other studies indicate that in the absence of Wigglesworthia, tsetse females are rendered sterile. Our prior studies where we maintained inseminated flies on ampicillin supplemented blood diets resulted in progeny deposition. This is because ampicillin treatment did not affect the intracellular Wigglesworthia resident in the bacteriome organ in the midgut, which provides essential nutrients to maintain

Table 3. Cytoplasmic-incompatibility parameter estimates.

| Parameter                       | Median     | 95% Credible Interval          |
|---------------------------------|------------|--------------------------------|
| Fecundity Benefit of Wolbachia (sW, W) | 0.1925     | [0.0920, 0.5784]               |
| Fecundity Benefit of Wigglesworthia (sW, W) | 0.2839     | [0.0854, 0.5420]              |
| CI Strength (sh)                | 0.7976     | [0.6295, 0.9025]               |
| Transmission Failure (m)        | 0.1073     | [0.0069, 0.2274]               |

Shown are the posterior median and 95% credible interval from Bayesian Markov chain Monte Carlo estimation.

doi:10.1371/journal.ppat.1002415.t003
tsetse host fecundity [21]. Antibiotic ampicillin treatment however eliminated the extracellular *Wigglesworthia* population present in the milk gland essential for symbiont transmission, and thus the resulting progeny from such females lacked *Wigglesworthia* (*Gmm*$.^{Wt-}$). Such progeny were reproductively sterile although they retained the symbiont *Wolbachia*. The tetracycline diet eliminated both intracellular and extracellular forms of *Wigglesworthia* and thus we did not obtain any viable progeny from inseminated females that were maintained on the tetracycline only diet. Prior studies showed that tetracycline blood meals supplemented with vitamin B1 could partially rescue fertility [15], but in our experiments vitamin supplementation could give rise to at most one progeny deposition, which either did not hatch or did not survive as an adult (data not shown). In sharp contrast, supplementation of the blood meal diet with 10% (w/v) yeast-extract reverted sterility in tetracycline treated flies to levels comparable to *Gmm*$.^{Wt}$ and *Gmm*$.^{Wt-}$ females receiving the same diet (Figure 1A). Although we have compared the fecundity of all three lines for two gonotrophic cycles here, yeast supplemented flies continue to deposit four to five progeny (data not shown). Given the complex nature of the yeast extract (peptides, amino acids, vitamins and other yeast cell components), it is difficult to know the exact nature of the essential nutrients it provides, but we believe that it could be working via supplementation of lipids and/or essential vitamins that are lacking in the strict blood diet of tsetse. However, we did observe some negative effect attributable to the yeast diet when the fecundity of *Gmm*$.^{Wt}$ flies receiving yeast supplemented blood meals is compared to those receiving normal blood diets. As such, we are further investigating the use of different yeast supplementations and/or concentrations in an effort to improve the diet efficiency. Nevertheless the availability of *Wolbachia*-cured flies (*Gmm*$.^{Apo}$) allowed us to begin to understand the functional role of this symbiosis.

In addition to *Wolbachia* symbiont specific PCR amplification, we confirmed the absence of *Wolbachia* from the reproductive tissues of *Gmm*$.^{Apo}$ females by FISH analysis. We show the presence of *Wolbachia* in *Gmm*$.^{Wt}$ females, isolates to a pole late in development (Figure 1C). There are a number of studies in other model systems that have investigated the link between *Wolbachia* localization during spermatogenesis and density effects on CI [36, 37]. However, other studies have found no correlation between *Wolbachia* density and CI during spermatogenesis [38, 39]. There have also been a number of studies investigating *Wolbachia* localization during oogenesis [40–42]. Different *Wolbachia* strains in *Drosophila* embryos display posterior, anterior, or cortical localization congruent with the classification based on the *wcp* gene sequence [39]. A positive correlation between levels of *Wolbachia* at the posterior pole and CI has been suggested, but this has yet to be examined in detail [42]. Notwithstanding, assessing the role of *Wolbachia* during oogenesis is important, given that factors promoting CI rescue are deposited in the egg cytoplasm during oocyte development [43] and bacterial deposition in the oocyte is an essential even for efficient maternal transmission.

Before we could perform crossing experiments to assess for CI, we evaluated the effect of *Wolbachia* clearance on male reproductive capacity. This evaluation is important given that tetracycline has been shown to negatively affect reproductive fitness in *Drosophila simulans* [33]. Additionally, the importance of this finding is highlighted by a study of the mosquito *A. albopictus* system in which the natural *Wolbachia* strains (zaAlB and zaAlB) were cleared and transinfected with the *Wolbachia* strain wK-Ri from *D. simulans* [44]. Their results showed that the wK-Ri transfected males have a reduced mating capacity compared with the wild type super infected males [44]. In contrast, in our system, no decrease in mating capacity was observed in *Gmm*$.^{Apo}$ males compared with *Gmm*$.^{Wt}$ males under the laboratory conditions. Our observation agrees with the evolutionary model proposed by Charlat et al. [45], where *Wolbachia* is exclusively maternally transmitted therefore males may be considered an evolutionary dead end in terms of *Wolbachia* infection [46]. Consequently, no direct selection by *Wolbachia* can be theoretically expected on paternal reproductive fitness.

Loss of fecundity in the cross *Gmm*$.^{Apo} \times \sigma \times *Gmm*$.^{Wt}$ could conceivably arise from loss of *Wigglesworthia*-mediated nutritional benefits in *Gmm*$.^{Apo}$ females rather than to *Wolbachia* mediated CI. To test this possibility, we compared the larval deposition rates in crosses between *Gmm*$.^{Apo} \times \sigma \times *Gmm*$.^{Wt}$ and *Gmm*$.^{Wt-} \times \sigma \times *Gmm*$.^{Wt-}$ flies (Table 1). Our results show no statistically significant differences between these crosses indicating that loss of fecundity in the CI cross is not due to loss of *Wigglesworthia*.

Our empirical results were used to parameterize a population genetic model of the spread of *Wolbachia*. Our model demonstrated that *Gmm*$.^{Wt}$ would successfully invade an uninfected natural population with a large enough release given CI rates. Indeed, uninfected natural populations and natural populations with low infection prevalence have recently been identified for multiple tsetse species [47]. This modeling result is consistent with the natural spread of *Wolbachia* in *Drosophila* populations [48–50]. In addition, the rise to the predicted fixation prevalence of between 86% and 100% is rapid. Apparently, the *Wolbachia*-mediated CI has the potential to rapidly and effectively drive a desirable phenotype into natural populations. We have previously been able to culture and genetically transform the commensal symbiont of tsetse, *Sodalis glossinidius* [51]. It has also been possible to reintroduce the transformed *Sodalis* into tsetse, called a paratransgenic approach [52, 53]. Given that *Sodalis* resides in close proximity to pathogenic trypanosomes in tsetse’s midgut, products expressed in rec*Sodalis* can have an immediate effect on trypanosome biology. The potential paratransgenic strategy in tsetse could harness the *Wolbachia* mediated CI to drive a recombinant *Sodalis* strain that would encode parasite resistance genes into natural populations [6, 10]. Our studies on the maternal transmission dynamics of tsetse’s symbionts in the laboratory indicated perfect transmission of both *Wolbachia* and *Sodalis* into tsetse’s sequential progeny [54]. This high transmission fidelity of the two symbionts, coupled with strong nearly 100% CI caused by *Wolbachia* would serve paratransgenic applications favorably.

An alternative control strategy to paratransgenic population replacement strategy would be use CI as part of an incompatible insect technique (IIT), which is analogous to a SIT approach [29, 55–58]. In a *Wolbachia*-based SIT approach female sterility is artificially sustained by repeated releases of cytoplasmically incompatible males. Similar to SIT, the increasing ratio of incompatible matings over time can lead to population suppression. The benefit of an IIT strategy is that it would not require the use of irradiation or chemosterilants to sterilize males prior to release, which often reduces the fitness of released males, but would rely on the naturally induced sterility of an incompatible *Wolbachia* infection [59]. A *Wolbachia*-based paratransgenic and IIT control strategy for tsetse would rely upon the introduction of a novel infection type into a population with an existing infection that could result in bi-directional CI or the introduction of a novel infection into an uninfected host population. Typically, in other insect systems novel *Wolbachia* infections are established by embryonic microinjections [60, 61]. This would be difficult in tsetse given their viviparous reproductive biology, in that adult females carry and nourish their offspring for their entire larval developmental cycle making injections of embryos difficult. Future
studies however can focus on the introduction of novel infection types via microinjection in aposymbiotic and naturally infected adult flies [62]. Maternal intrathoracic injections of Wolbachia infection establishment has also been successful in Aedes aegypti [63].

There has been a growing interest in understanding the variety of Wolbachia induced phenotypes in arthropods given the impact that Wolbachia infections could potentially have on genetic variation and host speciation impacting evolution of the species. Our data add to this growing field, as this is the first demonstration of the biological significance of Wolbachia infections in tsetse. Interestingly, CI in tsetse appears to be strong in that by the second gonotrophic cycle 0% of the females in an incompatible cross give rise to progeny. This is an exception given that in many insect systems incomplete CI is observed [27,64]. Future studies with natural populations would now be important to confirm some of the parameters we report here including maternal transmission rates, infection prevalence and the maternal linkage efficacy between Wolbachia and other maternally transmitted symbionts such as Sodalis, which is being entertained for paratransgenic applications.

Additionally, the aposymbiotic lines generated in this study are currently being used to address the interactive role of trypanosome transmission in tsetse. The importance of which is highlighted by recent studies that have shown that Wolbachia infections may impact host immune biology, limiting pathogen proliferation in insect hosts [65–70].

Materials and Methods

Fly Rearing

The Glossina morsitans morsitans colony maintained in the insectary at Yale University was originally established from puparia collected in Zimbabwe. Newly emerged flies are separated based on sex and mated at three to four days post eclosion. Flies are maintained at 24±1°C with 50 – 55% relative humidity and fed defibrinated bovine blood (HemoStat Laboratories, CA) every 6uC with 50–55% relative humidity and fed defibrinated bovine blood (HemoStat Laboratories, CA) every forty eight hours using an artificial membrane system [71]. Selective elimination of natural tsetse endosymbionts was obtained as described below.

Tetracycline Treatment

Wild type (GmmWt) fertile females were maintained on blood meals supplemented with 10% (w/v) yeast extract (Becton Dickinson) and 20 ug/ml of tetracycline. The yeast extract was briefly boiled in water before being added the blood meal each time. Flies were fed every 48 h using an artificial membrane feeding system (as above) for the duration of their life span. The resulting progeny are aposymbiotic (GmmApo) in that they lack their natural endosymbionts, Wigglesworthia and Wolbachia. These GmmApo lines were maintained on blood meals supplemented with 10% (w/v) yeast extract without tetracycline.

Ampicillin Treatment

GmmApo flies were maintained on blood meals supplemented with 50 ug/ml of ampicillin. The resulting progeny do not have Wigglesworthia (GmmWig), and were maintained on blood meals supplemented with 10% (w/v) yeast extract without ampicillin.

Monitoring the Fecundity Cost of Yeast-extract Supplementation

Newly eclosed aged matched females and males were divided into six groups and copulation observed. Three of these groups were provided with either normal blood meals (control) or blood meals supplemented with ampicillin at 50 ug/ml or tetracycline at 20 ug/ml. Whereas the remaining three groups received blood meals supplemented with 10% (w/v) yeast extract with either ampicillin (50 ug/ml) or tetracycline (20 ug/ml). The cages were monitored daily for pupal deposition and fly mortality over two gonotrophic cycles (40 days). Fecundity was quantified by determining the number of fecund females relative to total number of females alive at the end of the gonotrophic cycle to give an average percent of females depositing pupae. Each group was setup with 100 females per cage.

Symbiont Prevalence Assay

Total DNA was extracted from adults eight days post eclosion using the Qiagen Blood and Tissue extraction kit under manufacturers conditions (Qiagen Kit #, 69506. CA). The presence of the symbionts Sodalis, Wigglesworthia and Wolbachia was determined by a species-specific PCR amplification assay using the primer sets and conditions described (Table S1). For input DNA quality control, the tsetse gene β-tubulin (GmmTub) specific primer set was used. All PCR reactions were performed in an MJ-Research thermocycler and the amplification products were analyzed by electrophoresis on a 1% agarose gel and visualized using image analysis software.

Wolbachia Infection Status by FISH

Dissected reproductive tracts from GmmWt and GmmApo females were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, cut into 5 mm thick sections and mounted on poly L-lysine coated microscopy slides. After dewaxing in methylocellulose and rehydration the sections were processed using the FISH protocol previously described in Anselme et al. 2006 [72]. Slides were covered with a drop of 70% acetic acid and incubated at 45°C until drop had dried, followed by dehydrolysis and a 10 min deproteinization step in 0.01N HCl/pepsine at 37°C. Slides were then dehydrated again, prehybridized for 30 min at 43°C and hybridized for 3 h at 45°C with 5′ end rhodamine labeled 16S RNA probes (5′-AAT CCG GCC GAR CCG ACC G C-3′) and (5′-CTT CTG TGA GTA CCG TCA TTA TC-3′). Microscopic analyses were conducted using a Zeiss Axioskop2 microscope equipped with an Infinity1 USB 2.0 camera and software (Lumenera Corporation). Fluorescent images were taken using a fluorescent filter set with fluorescein, rhodamine and DAPI specific channels.

Monitoring Longevity of GmmApo and GmmWt Females

GmmApo and GmmWt flies that emerged within a 24-hour period (teneral) were collected, mated with GmmWt males at a ratio of 3:2 and copulation was observed. After six days males were removed from experimental cages. Six independent cages were set-up for both GmmApo and GmmWt groups, comprising of a total of 169 GmmApo and 170 GmmWt females, respectively. Both the males and females used represented offspring acquired from different gonotrophic cycles (1st and 2nd). All flies were maintained on yeast extract supplemented blood meals and fly mortality was monitored daily over a 40-day period.

CI Mating Crosses

To determine the expression of CI, reciprocal crosses were set up between GmmWt, Gmm and GmmWc flies, in triplicate. Cages with a minimum of 15 females and 7 males each were set-up in the following combinations: 1) ♀ GmmWt × ♂ GmmWt, 2) ♀ GmmWt × ♂ GmmApo, 3) ♀ GmmApo × ♂ GmmWt, 4) ♀ GmmApo × ♂ Gmm and 5) ♀ GmmWc × ♂ GmmWc. All flies received yeast
supplemented blood meal diets. Flies were observed over two-gonotrophic cycles with daily recording of mortality, larval deposition dates, pupal eclosion dates and sex of emergent progeny. Larval deposition rates for each gonotrophic cycle were determined by dividing the number of larvae deposited per day by the number of remaining females in the cage on the day of larviposition and summing the values for each gonotrophic cycle. At the conclusion of the experiment, all females were checked for insemination by examination of dissected spermatheca for the presence of sperm microscopically. Additionally, single line crosses consisting of a single female and male per cage were set up (Table S2). For the Q GmmWt × Q GmmWt, a total of 31 crosses were set up. Also set up were 40 crosses for Q GmmWt × GmmAb, 20 for Q GmmAb × GmmAb and 33 for Q GmmAb × Q GmmWt. Both the males and females used in these crosses represented offspring acquired from different gonotrophic cycles to rule out batch effects. Spermathecae of females was also dissected to confirm infection to females (mothers (Wolbachia)). The Wolbachia model was developed for the temporal evolution of tsetse population-genetic quantities fundamental to the invasion of Wolbachia into a novel tsetse population. Again following existing Wolbachia models for Wolbachia-mediated CI trade-off of the fitness cost to male hosts, we estimated with both infected and uninfected males (in addition to direct effects of Wolbachia on fecundity and mortality).

For some values of the mechanistic parameters, these models exhibit a threshold for Wolbachia invasion into the host population: if, in a novel population, the proportion that is initially Wolbachia infected is above the threshold, Wolbachia will continue to stable fixation in the population at a high level. If the proportion infected is below the threshold, Wolbachia will be driven out of the population over time. This threshold level was calculated, along with the prevalence of Wolbachia at fixation, and the time to fixation. For the population-genetic model, several parameters could not be estimated from the data on mating crosses. Thus, we also performed a sensitivity analysis on these parameters, along with the parameters estimated from the mating-cross data.

To estimate both the mechanistic parameters for CI and the population-genetics quantities derived from these parameters, a Bayesian Markov chain Monte Carlo (MCMC) method was used with uninformative prior distributions for the parameters [49].

### Supporting Information

**Figure S1** The effect of antibiotics on the bacteriome of G. m. morsitans. Images of bacteriome sections stained with Giemsa (A) bacteriome organ showing bacteriocytes harboring Wigglesworthia from a female maintained on normal bloodmeals, image taken at 10x magnification (B) bacteriocytes taken 40 magnification from a female maintained on ampicillin supplemented diet. A normal bacteriome structure is retained on the ampicillin diet allowing for continued fertility of such females. (C and D) Bacteriome structure observed in the progeny of ampicillin receiving females (C) and tetracycline and yeast extract receiving females in (D). In these individuals, the bacteriocytes lack Wigglesworthia and these females are reproductively sterile, images taken at 10x magnification.

**Table S1** Symbiont PCR primers.

**Table S2** Larval deposition and pupal eclosion data for single cage crosses. In three separate experiments % larval deposition and % eclosion of the pupa deposited was determined. For each experiment, number of larval deposited for surviving females over two gonotrophic cycles and number of their pupae that hatched were recorded. Larval deposition was used as a measure of CI expression. To analyze for CI in replicate experiments of individual crosses, multiple Wilcoxon tests, with a Bonferroni correction were conducted to compare larval deposition rates. Wilcoxon tests, with a Bonferroni correction were also conducted to compare pupal eclosion. Superscripted letters indicate significant differences, P<0.01.

**Text S1** Mathematical methods.

**Acknowledgments**

We thank Kostas Bourtzis for the WSP antibody. We thank Jeffrey Townsend for statistical advice. We are also grateful to FAO/IAEA Coordinated Research Program on "Improving SIT for Tsetse Flies Through Research on Their Symbionts and Pathogens" and to Slovak Academy of Science, Bratislava, Slovakia for providing puparia for our colony.

**Author Contributions**

Conceived and designed the experiments: SA AG UA JM CB AH PT. Performed the experiments: UA JM CB RP CI, JC SB. Analyzed the data: UA JM CB SA AG SB PT. Contributed reagents/materials/analysis tools: AH PT. Wrote the paper: SA UA CB JM.
63. Ruang-Areerate T, Kittayapong P (2006) Wolbachia transinfection in Aedes aegypti: a potential gene driver of dengue vectors. Proc Natl Acad Sci U S A 103: 12534–12539.
64. Turelli M, Hoffman A (1999) Microbe induced cytoplasmic incompatibility as a mechanism for introducing genes into arthropod populations. Insect Mol Biol 8: 243–255.
65. Osborne SE, Leong YS, O’Neill SL, Johnson KN (2009) Variation in antiviral protection mediated by different Wolbachia strains in Drosophila simulans. PLoS Pathog 5: e1000056.
66. Kambris Z, Blagborough A, Pinto S, Blagrove M, Godfray H, et al. (2010) Wolbachia stimulates immune gene expression and inhibits plasmodium development in Anopheles gambiae. PLoS Pathog 6: e1001143.
67. Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening Wolbachia and reduced filarial competence in mosquitoes. Science 326: 134–136.
68. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, et al. (2009) A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya and Plasmodium. Cell 139: 1261–1278.
69. Bian G, Xu Y, Lu P, Xie Y, Xi Z (2010) The endosymbiotic bacterium Wolbachia induces resistance to dengue virus in Aedes aegypti. PLoS Pathog 6: e1000033.
70. Teixeira L, Ferreira A, Ashburner M (2008) The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. PLoS Biol 6: e2.
71. Moloo SK (1971) An artificial feeding technique for Glossina. Parasitology 63: 507–512.
72. Anselme C, Vallier A, Balmand S, Fauvarque MO, Heddi A (2006) Host PGRP gene expression and bacterial release in endosymbiosis of the weevil Sitophilus zeamais. Appl Environ Microbiol 72: 6766–6772.