Infectious Speciation Revisited: Impact of Symbiont-Depletion on Female Fitness and Mating Behavior of Drosophila paulistorum

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Abstract

The neotropical Drosophila paulistorum superspecies, consisting of at least six geographically overlapping but reproductively isolated semispecies, has been the object of extensive research since at least 1955, when it was initially trapped mid-evolution in flagrant statu nascendi. In this classic system females express strong premating isolation patterns against mates belonging to any other semispecies, and yet uncharacterized microbial reproductive tract symbionts were described triggering hybrid inviability and male sterility. Based on theoretical models and limited experimental data, prime candidates fostering symbiont-driven speciation in arthropods are intracellular bacteria belonging to the genus Wolbachia. They are maternally inherited symbionts of many arthropods capable of manipulating host reproductive biology for their own benefits. However, it is an ongoing debate as to whether or not reproductive symbionts are capable of driving host speciation in nature and if so, to what extent. Here we have reevaluated this classic case of infectious speciation by means of present day molecular approaches and artificial symbiont depletion experiments. We have isolated the α-proteobacteria Wolbachia as the maternally transmitted core endosymbionts of all D. paulistorum semispecies that have coevolved towards obligate mutualism with their respective native hosts. In hybrids, however, these mutualists transform into pathogens by overreplication causing embryonic inviability and male sterility. We show that experimental reduction in native Wolbachia titer causes alterations in sex ratio, fecundity, and mate discrimination. Our results indicate that formerly designated Mycoplasma-like organisms are most likely Wolbachia that have evolved by becoming essential mutualistic symbionts in their respective natural hosts; they have the potential to trigger pre- and postmating isolation. Furthermore, in light of our new findings, we revisit the concept of infectious speciation and discuss potential mechanisms that can restrict or promote symbiont-induced speciation at post- and prezygotic levels in nature and under artificial laboratory conditions.

Citation: Miller WJ, Ehrman L, Schneider D (2010) Infectious Speciation Revisited: Impact of Symbiont-Depletion on Female Fitness and Mating Behavior of Drosophila paulistorum. PLoS Pathog 6(12): e1001214. doi:10.1371/journal.ppat.1001214

Editor: Colin Parrish, Cornell University, United States of America

Received November 9, 2009; Accepted October 27, 2010; Published December 2, 2010

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Funding: WJM and DS were partly supported by the research grant FWF P19206-B17 and P22634-B17 from the Austrian Science Fund and the EU-COST Action FA0701 "Arthropod Symbiosis: From Fundamental Studies to Pest and Disease Management". The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Nuclear vs. Symbiotic Speciation Mechanisms

In contrast to prezygotic reproductive isolating mechanisms acting before fertilization via mating behavior, postzygotic isolation arises after mating when hybrids are less fit than their parents [1]. In the latter case, the Dobzhansky-Muller model proposes that hybrid incompatibilities, contributing to speciation, are caused by the interaction between nuclear genes that have functionally diverged over time in their respective hybridizing species [2,3]. Initial direct molecular and genetic evidence for the existence of such Dobzhansky-Muller incompatibility genes recently came from the two sister-species, D. melanogaster and D. simulans [4]. In this case one of the two incompatibility genes localizes to centromeric heterochromatin - an inherently dynamic part of all eukaryotic genomes known for its accelerated genomic turnover and molecular drive [5,6]. Genes located within or functionally interacting with hyperdynamic genomic regions, such as nuclear heterochromatin, evolve rapidly and so contribute to hybrid incompatibilities and thereby to speciation [4–6]. In this speciation model, however, incompatibility genes are both nuclear genes that have evolved differently after an ancestral populations split.

A second class of rapidly evolving genes possessive of high potential for driving accelerated functional divergence are those genes that are under antagonistic coevolution versus parasites, i.e. the reciprocal evolution of host defense and parasite counter-defense mechanisms [7–10]. Accordingly, one or more incompatibility genes is encoded by the host nucleus, and the second set by a parasite. In a consistent arms race between invading cytoplasmatically inherited reproductive parasites and a local host population, interacting gene products encoded separately in both organisms evolve rapidly under strong diversifying selection driven by ongoing cyto-nuclear conflicts [11–13]. Dense interrelationships between a parasite and its host could functionally weld them together by favoring evolution towards a compensatory partnership where both biological systems finally depend on the presence of the other, and both progress evolutionarily from an antagonistic
Author Summary

The Drosophila paulistorum species complex serves as a well-studied model system for evaluating the impact of symbiosis on host speciation since they evolve rapidly and comprise an ancestral, but highly dynamic, reservoir of microbial symbionts. Theory and some experimental evidence suggest that in evolutionary long-term host-symbiont interactions, reproductive parasites might evolve a more benign lifestyle towards mutualism, manipulate sexual mating behavior, and foster host speciation. However, it is an ongoing debate as to whether or not microbial symbionts are capable of driving host speciation in nature and if so, to what extent. Prime candidates are Wolbachia, inherited, endosymbiotic bacteria of many arthropods, presently attracting attention as potential biocontrol agents since they affect host reproductive biology. Here we document that all D. paulistorum semispecies harbor Wolbachia that provide significant fitness benefits to their natural hosts. In semispecies hybrids, however, mutualistic Wolbachia turn into pathogens, triggering embryonic lethality and male sterility via overreproduction. Besides their impacts on post-mating isolation, we show that in their native D. paulistorum hosts Wolbachia manipulate sexual behavior by triggering pre-mating isolation via selective mate avoidance, i.e. avoiding mates harboring another, incompatible symbiont variant. Our study reveals that endosymbionts can coevolve rapidly with their native hosts and play a significant role in driving natural host speciation.

parasitic to a compensatory mutualist relationship [14,15]. So it can be expected that repeated, localized episodes of antagonistic and compensatory coevolution may have the potential to diversify and to substructure host populations up to the point where hybrid incompatibilities emerge between them [16,17]. Increasing empirical evidence and numerous theoretical models predict that transovarially transmitted microbial symbionts that cause cytoplasmic incompatibilities (CI), can have significant impacts as drivers of speciation processes in their natural hosts [12,16–22].

Over the last decades main CI work was focused on the β-proteobacteria Wolbachia, a frequent maternally-transmitted endosymbiont of many arthropods and filamentar nematodes [reviewed by [23–25]]. As deduced from classical theoretical models, for example, Wolbachia cause unstable equilibrium in the prevalence of the symbiotic infection, rather than stable persistence of infected and uninfected populations [26,27]. More recent models, however, propose that under specific genetic and environmental conditions Wolbachia can promote host speciation in nature [19,21]. In addition, there are only a few empirical model systems from nature available in literature such as parasitoid wasps of the Nasonia species group [28–30], and mushroom feeding Drosophila species [30,31]. These data provide clear evidence that Wolbachia-induced CI can promote speciation in nature acting in concert with other genetic and/or geographic isolation mechanisms (see Discussion).

A recent experimental study, however, shows that artificial loss of Wolbachia by antibiotic treatment can have a significant impact on assortative mating behavior of some selected laboratory strains of D. melanogaster females, but the mechanism and biological significance in nature remains obscure [31]. Hence the actual role of endosymbionts in speciation in nature must be debated, since we lack appropriate natural and experimentally accessible model systems in early stages of speciation, where conditions that affect reinforcement can be directly tested in laboratories, as well as observed in nature. Finally, with the exception of so-called mycoplasma-like organisms (MLOs) of D. paulistorum [32] and this study, no empirical cases have been reported that endosymbionts are able to cause hybrid male sterility, a hallmark of an early stage in the evolution of postzygotic isolation [1].

Considering the ubiquity of symbiotic interactions and their inherent coevolutionary potential, one could reasonably expect to find experimentally accessible model systems presently under incipient speciation in nature [17]. Based on theoretical considerations [1,12,16,17,19,20,29,33,34], requisite for experimentally testing this hypothesis, it is essential to choose a symbiotic model system where both biological components, the host and the symbiont, should fulfill the following main criteria: Firstly, an evolutionary young host species complex in statu nascendi ought to be chosen for studying the speciation process in nature. Secondly, this host system should be associated with a fixed endosymbiont with a perfect transmission rate capable of inducing strong bidirectional, postzygotic incompatibilities in hybrids. Finally, this endosymbiont should exert the capacity to trigger prezygotic isolation in order to avoid potential wasteful gene flow between different host infection types. Such candidate model systems are extensively documented in early literature on speciation mechanisms observed in neotropical Drosophila hosts [35–38].

The Neotropical Drosophila paulistorum Species Complex, a Classic Case of Speciation in statu nascendi

The first case in literature suggesting that microbial reproductive parasites can play a pivotal role in driving host-speciation dates back to the late 1960s when Ehrman and coworkers discovered endosymbionts that trigger incipient speciation via hybrid inviability and male sterility in a neotropical Drosophila species group [39–41] reviewed in [42]. The D. paulistorum species complex comprises at least six semispecies showing pronounced sexual isolation; matings between these semispecies succeed significantly less frequently than do those within a semispecies [35–38]. These are: Amazonian (AM), Andean-Brazilian (AB), Centroamerican (CA), Interior (IN), Orinocan (OR), and Transitional (TR) semispecies. The primary extrinsic mechanism differentiating the D. paulistorum semispecies is geographic isolation, albeit incompletely because of consistently overlapping distributions [43]. Three intrinsic isolation mechanisms are operative here - sexual isolation via behavior and hybrid male sterility both in reciprocal crosses [43]. In addition to hybrid male sterility, abnormal pole cell development in early F1 hybrid embryos cause considerable high hybrid inviability [44,45]; depicted in Figure 1. Since mortality takes place during early stages of embryogenesis [44,46] and occurs at high frequencies in all hybrids derived from reciprocal matings between the six D. paulistorum semispecies [45,47], this phenotype can be best described as bidirectional cytoplasmic incompatibility, initially described by Laven in the Calecti pipiens species complex [48].

Transmission studies have shown that extracts of testes from sterile hybrid males induce a similar sterility syndrome when injected into females of the paternal strain [32]. In 1970 pleomorphic cytoplasmatic symbionts were identified via ultrastructural analyses, and, based on morphological criteria, they were initially designated as mycoplasma-like microorganisms, MLOs [41]. These intracellular bacteria possess two and sometimes three or four enveloping membranes and were always found in testes, ovaries, and early embryos of both hybrid and nonhybrid D. paulistorum, though fewer were counted in nonhybrids. In the male reproductive organs of sterile F1 hybrids however, MLOs exist in greater numbers than they do in nonhybrid males [46,49]. It has been postulated that while each of these semispecies possessed its own MLO in a normally benign relationship, in the testes of the
hybrid male there is a rapid proliferation of MLO and a concomitant breakdown of spermatogenesis [49]. Indeed, hybrid male testes are “sperm-less” microorganismal factories.

Numerous efforts have failed to establish “MLO-free” D. paulistorum strains using diverse sets of antibiotic treatments [39,41]. Under mild antibiotic conditions however, Ehrman and colleagues were able to diminish MLO titer levels significantly and to partially rescue hybrid male sterility. Based on the observed hypersensitivity of D. paulistorum semispecies to different antibiotics, these authors suggested that MLOs are maternally transmitted, obligatory microbial symbionts of all D. paulistorum semispecies since no truly bacteria-free strain has or had ever been established [39,41].

Here we report the isolation of close-related α-proteobacteria Wolbachia, belonging to the A supergroup, from the six D. paulistorum semispecies. Based on their intriguing morphological likeness with MLOs, their tight germline associations, and their striking similarities in infection-titer dynamics in hybrids, Wolbachia are most likely identical to formerly designated “MLOs” by Ehrman and coworkers [41]. Furthermore repeated attempts via microbial universal 16S rRNA screening and direct sequencing have failed to isolate any other germline-associated microbes besides Wolbachia. Hence such “MLOs” of previous D. paulistorum literature should be reconsidered, from now onward, as members of the invertebrate-colonizing genus Wolbachia. We have furthermore reevaluated symbiotic interactions between Wolbachia and their natural D. paulistorum semispecies hosts by studying phenotypic traits appearing upon partial symbiont-depletion via antibiotics, traits such as female fecundity, sex ratios, and most significantly, female mating behavior.

Results

Isolation and Characterization of Wolbachia as the Unique Endosymbiont Associated with the D. paulistorum Germline

Since “MLOs” have been diagnosed as maternally-transmitted endosymbionts that accumulate in testes, ovaries and early embryos [42,43,46,49] we have surveyed the germline-associated microbial diversity of the D. paulistorum OR semispecies by applying universal 16S rRNA PCR [50] on DNA of fertilized early embryos derived from sterilized eggs. This approach avoids contaminations with gut bacteria, focusing our survey on vertically-transmitted endosymbionts. In total, twenty amplified 16S rRNA consensus fragments of 437 bp derived from five independent PCR reactions were cloned and sequenced. All reads from early embryos of the OR semispecies were identical to the Wolbachia 16S rRNA gene of the sibling species D. willistoni (GenBank accession number DQ412086).

Furthermore “MLOs” have been described earlier as consistently associated symbionts within adult reproductive tissues of all D. paulistorum semispecies, specifically over-replicating in testes of reciprocal hybrids [46,49]. Hence we have dissected under sterile conditions ovaries and testes from mature AM and OR semispecies, plus AxO hybrid flies for DNA extraction. Gonad-specific PCRs were performed with two sets of universal 16S rRNA primer [50,51] (for details see Materials and Methods) on DNA of fertilized early embryos derived from sterilized eggs. This approach avoids contaminations with gut bacteria, focusing our survey on vertically-transmitted endosymbionts. In total, twenty amplified 16S rRNA consensus fragments of 437 bp derived from five independent PCR reactions were cloned and sequenced. All reads from early embryos of the OR semispecies were identical to the Wolbachia 16S rRNA gene of the sibling species D. willistoni (GenBank accession number DQ412086).

Consensus 16S PCR fragments of 25 independent reactions derived from reproductive organs of native and hybrid samples (6, 14 and 5 reads for AM, OR, and hybrids, respectively) were direct-sequenced. In the six reads derived from AM ovaries and testes double peaks were detected from which one sequence type was found with 100% homology to the 16S rRNA of wWil of D. willistoni (GenBank accession number HQ185362 and HQ185363, respectively) and no other bacteria could be recovered. The identification of Acetobacter in dissected gonads from AM samples demonstrates the sensitivity of our detection system since this semispecies harbors Wolbachia at extreme low density compared to...
OR and AxO hybrids (see below). In the latter two samples we never amplified Acetobacter from dissected gonads. Furthermore Acetobacter are free-living bacteria generally found as part of the gut flora in insects that have never been associated with any reproductive phenotype in literature. We therefore assume that the Acetobacter detected in AM samples can be regarded most likely as gut contaminant from gonad dissections. These data strongly implicate that Wolbachia are the unique germline associated symbiont in D. paulistorum semispecies.

In order to correlate quantitative shifts of universal microbial rRNA titer levels in hybrid testes with our candidate Wolbachia, PCRs were performed on same DNA samples with a diagnostic wsp primer set. This approach is known as a robust and reliable detection method. However, ultrastructural analyses and whole mount immunostainings were performed applying the Wolbachia-specific WSP antibody on fixed embryos, testes and ovaries of native AM and OR hosts and AxO hybrids (Figure 3). Similar to data obtained from 10-day-old females (f) and males (m). DNA extractions and PCR reactions were set up under sterile conditions [54,55], on at least fifty single fly reactions per semispecies. DNA extractions were performed from each 15 pairs of ovaries (f) and testes (m) from ten day-old Amazonian (AM), Orinocan (OR) semispecies, and from hybrids (AxO) derived from matings between AM females and OR males. Controls are testes of D. simulans infected with wRi Wolbachia (+) and D. melanogaster strain w118 uninfected (−). PCRs were performed with universal 16S rRNA (upper gel) and Wolbachia-specific wsp (lower gel) primer. (B) Wolbachia-specific wsp PCR on total DNA from ten flies of D. paulistorum semispecies and hybrids of Amazonian (AM), Centroamerican (CA), Orinocan (OR) semispecies, and F1 hybrids derived from crossings of AM females to OR males (AxO) and CA females to OR males (CxO). PCR controls are adults of D. simulans infected with wRi Wolbachia (+) and two uninfected strains (−), i.e., D. simulans STC and D. melanogaster strain w118. DNA was extracted from 10-day-old females (f) and males (m).

Figure 2. Presence of germline-associated microbes in D. paulistorum semispecies and their sterile hybrids. (A) PCR on total DNA extracted from each 15 pairs of ovaries (f) and testes (m) from ten day-old Amazonian (AM), Orinocan (OR) semispecies, and from hybrids (AxO) derived from matings between AM females and OR males. Controls are testes of D. simulans infected with wRi Wolbachia (+) and D. melanogaster strain w118 uninfected (−). PCRs were performed with universal 16S rRNA (upper gel) and Wolbachia-specific wsp (lower gel) primer. (B) Wolbachia-specific wsp PCR on total DNA from ten flies of D. paulistorum semispecies and hybrids of Amazonian (AM), Centroamerican (CA), Orinocan (OR) semispecies, and F1 hybrids derived from crossings of AM females to OR males (AxO) and CA females to OR males (CxO). PCR controls are adults of D. simulans infected with wRi Wolbachia (+) and two uninfected strains (−), i.e., D. simulans STC and D. melanogaster strain w118. DNA was extracted from 10-day-old females (f) and males (m).

doi:10.1371/journal.ppat.1001214.g002
from 16S rRNA and wsp PCR assays, OR and AM semispecies differ significantly in their respective symbiont titer levels during development. Compared to OR semispecies that exert strong Wolbachia signals in early blastodermal stages (Figure 3A), but further on concentrate specifically in primordial germine cells, PGC (Figure 3B), AM semispecies show only faint but universal staining signals during embryogenesis (Figure 3D,3E). Presence of Wolbachia was also undoubtedly detected in AM ovaries (Figure 3L). In contrast, in AxO hybrids, however, where the symbiont was inherited from low-titer AM mothers (Figure 3E), Wolbachia densities were significantly higher in blastodermal stages (Figure 3G,3H), and clear signals were detectable in hybrid testes within spermatids (Figure 3J,3K). Furthermore ultrastructural analyses on testes of OR (Figure 3C) AM (Figure 3F) and AxO hybrids (Figure 3I) confirmed the general presence of endosymbionts during spermatogenesis with multiple membrane layers morphologically resembling Wolbachia and earlier descriptions [41,49]. Hence we conclude, that intracellular Wolbachia are identical to earlier designated “MLO”, the germline-associated core endosymbionts of D. paulistorum semispecies, and therefore pose most likely the unique microbial candidate as the causative agent driving incipient speciation in this Drosophila host system.

For Wolbachia strain typing, wsp sequence data were obtained by PCRs from multiple independent samples per semispecies (embryonic DNA, adults and hybrids) of AM, CA, OR and TR semispecies. Within a semispecies group no sequence polymorphism could be detected. The wsp locus of Wolbachia is encoding a rapidly evolving outer membrane protein that is under strong diversifying selection and hence permits distinguishing between even closely related Wolbachia strains [11,56,57,58]. Based on wsp data, CA, TR and OR Wolbachia (GenBank accession numbers GQ924888 to GQ924890) are almost identical to the uAu strain of D. simulans and to ancestral infections found in closely related neotropical Drosophila hosts, D. willistoni, D. prosaltans, and D. septentriosaltans [59]. As shown in Table S1 Wolbachia of CA and TR differ from OR at one unique diagnostic site in the hypervariable region HVR1 of the WSP protein [58]. Although wsp of OR semispecies is identical to the orthologous locus wsp BC11 of D. septentriosaltans and uCer2 of Rhiagoletis cerasi, the OR infection harbours a unique variant of the hypervariable VNTR-105 [57] locus (GenBank accession number GQ924884; and unpublished data).

In AM semispecies however, a different Wolbachia wsp sequence identical to the one of uRi of D. simulans was found (GenBank accession number GQ924887). Canonical uRi Wolbachia were originally isolated from natural populations of D. simulans able to induce strong CI when infected males are mated to uninfected females [60]. The close phylogenetic relationship between uRi-like Wolbachia of AM D. paulistorum and uRi of D. simulans was corroborated by sequence data obtained from the non-coding VNTR-105 locus [57] with diagnostic base signatures characteristic of the uRi infection (GenBank accession number GQ924885). The phylogenetic relationships between Drosophila hosts and their respective Wolbachia symbionts are shown in Figure S2.

Effect of Mild Antibiotic Treatments on D. paulistorum Female Fecundity

Currently, applying Tetracycline at 0.03% final concentration in regular food over up to three generations is the standard procedure for successfully curing a diverse range of Drosophila species of their natural symbionts without inducing significant negative fitness effects in their respective hosts [59,61,62]; but also see [63]). In contrast, D. paulistorum semispecies are hypersensitive to standard antibiotic treatments since we have never obtained a single F1 progeny from curing assays of adults at 0.03% Tetracycline (unpublished data). Such results are in agreement with earlier observations that “MLOs” are obligatory microbial symbionts of all D. paulistorum semispecies and no true bacteria-free strain has ever been established [39,41,46,49,64,65].

On the other hand, mild antibiotic assay conditions, 0.01% Tetracycline or 0.01 to 0.2% Rifampicin, allowed us to establish lines of AM, CA, and OR semispecies for more than three generations on antibiotics. Furthermore, we have managed to generate hybrid lines between different D. paulistorum semispecies that are stable on consistent antibiotic media (0.2% Rifampicin) for more than 12 consecutive generations under sibling mating conditions. These hybrid lines were originated from crosses between antibiotic-treated virgin AM and OR parents producing F1 hybrids of partially fertile males and fertile females. F1 progeny were allowed to mate randomly and have reached F12 at the time this manuscript was written. In contrast, control matings between untreated AM and OR semispecies have never resulted in any F2 hybrids. Detailed description of these “stabilized” hybrid lines will be presented elsewhere in a separate publication as soon as more generations are assayed.

For evaluating the potential impact of artificial symbiont-depletion on rescuing hybrid mortality, we have crossed OR females and AM males. This combination is known to induce complete CI since we have never obtained a single F1 hybrid in repeated attempts in both of our laboratories. Antibiotic treatment of both parents, however, was sufficient to overcome complete CI and to partially rescue embryonic mortality. In these experiments interstrain matings were set up in three replicates of twenty virgin OR females and AM males each, allowed to lay hybrid eggs for 48 hrs. Parents were then discarded and hatching F1 adults were counted after two weeks. Whereas no single F1 fly emerged from control matings (both untreated) between 32 and 39 adult hybrids of both sexes emerged per assay from parents that were derived from artificially symbiont-depleted lines.

Hence similar to earlier reports [39,41,49,65] longterm treatments at 0.01% Tetracycline and up to 0.2% Rifampicin in our laboratory are sufficient to accomplish partial rescue of hybrid male sterility and also to increase hatching rates among these D. paulistorum semispecies.

Having determined sublethal dosages of these two antibiotics, Tetracycline and Rifampicin, we have observed that treated D. paulistorum females suffer low fecundity. In order to rule out unspecific side effects of antibiotics on fly oogenesis, we have applied the same treatment conditions to their closely related sister species D. willistoni, naturally infected with facultative Wolbachia [59]. Treated females of all D. paulistorum semispecies showed a significant reduction in mature egg numbers in their ovaries, whereas similar longterm treatments at 0.01% Tetracycline of closely related D. willistoni controls (Pan98) had no effect on oogenesis (Figure 4A). Scoring of mature eggs followed [66]. In D. willistoni average numbers of mature eggs per ovary before and after treatment were 18.8±1.30 and 19.6±2.69, respectively, but in the pool of the seven D. paulistorum strains tested numbers dropped down dramatically from averaged 16.63±4.61 in untreated, to 4.61±2.14 in treated ones. Furthermore, Rifampicin treatment at 0.1% final concentration over three generations also resulted in a severe decrease of mature egg numbers in D. paulistorum where the vast majority of egg chambers were underdeveloped (Figure 4B, C). Closer inspections, using DAPI staining, showed abnormally shaped, and highly degenerate nurse cell nuclei within treated egg chambers (Figure 4D,4E), suggesting that the low fecundity observed in treated D. paulistorum females has been already provoked by defects in cell cycle and/or...
Figure 3. Distribution of Wolbachia during development of *D. paulistorum* semispecies. OR (A–C), AM (D–F, L) and AxO hybrids (G–K) of AM females and OR males. Whole-mount immunostainings with the Wolbachia-specific anti-WSP antibody (yellow/green) were performed on embryos (A, B, D, E, and G, H), testes (J, K), and ovaries (L); DNA is counter-stained with DAPI (blue), or propidium iodide (red) following the protocol.
of [59]. Transmission electron microscopy of rod-shaped pleomorphic Wolbachia cells (arrows) in the testes of fertile OR (C) and AM semispecies (F), plus sterile AxO hybrid males (I). Symbiont morphology and density in reproductive host tissues corroborate earlier pictures published in [41,49]. In OR semispecies high-titer Wolbachia accumulate in early blastodermal stages (A); in further differentiating embryos symbionts selectively target the primordial germ cells of OR (B). Presence of Wolbachia in OR testes associated with developing spermatids (C). In AM semispecies Wolbachia are present at very low levels presumably in somatic and germline cells during blastodermal, gastrulating and late embryonic development (D). Higher magnification of bastodermal AM embryos clearly shows low density of the symbiont (E). Signal intensities are clearly enhanced in F1s of AxO derived from matings between AM females and OR males, suggesting overreplication of the maternally-transmitted symbiont in hybrids (G,H). Wolbachia immunostainings on cryosections of testes of AxO hybrids in transversal sections (J), and during spermatid development (K). Presence of Wolbachia during D. paulistorum oogenesis (L).

doi:10.1371/journal.ppat.1001214.g003

Figure 4. Effect of mild antibiotic treatment on female Drosophila fecundity. (A) Mean numbers and standard deviations (SD) of mature eggs per pair of ovaries of eight-day-old females (blue bars) and after 15 generations of mild Tetracycline of 0.01% (red bars). Total n = 80. Seven D. paulistorum strains were assayed belonging to the six described semispecies Amazonian (AM), Centroamerican (CA), two Interior (IN and LI), Andean-Brazilian (AB), Orinocan (OR), and Transitional (TR). A
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Emergence of Sex Ratio Distortions in Favor of Males upon Partial Symbiont Depletion

In the course of our D. paulistorum semispecies depletion assays we have observed that mild antibiotic treatment conditions at 0.01% Tetracycline and 0.01% Rifampicin are capable of skewing sex ratios towards males in AM but not in OR semispecies. In accordance with earlier reports [42], untreated AM and OR semispecies have the usual 1:1 sex ratio (Table 1). Under mild Tetracycline treatment (0.01% over five generations) however, the AM semispecies produces a highly significant sex bias towards males (ratio males to females = 1.79, $\chi^2 = 43.27$; d.f. = 1; $P<0.0001$), whereas the OR semispecies did not deviate from the usual 1:1 sex ratio (Table 1). Significant excess of males was also observed in AM flies kept for five generations on 0.01% Rifampicin (ratio males to females = 1.46, $\chi^2 = 26.60$; d.f. = 1; $P<0.0001$). Like mild Tetracycline treatments, there was no effect of Rifampicin on sex ratios in OR semispecies (ratio males to females = 1.01, $\chi^2 = 0.012$; d.f. = 1; $P=0.91$). However, higher dosages of Rifampicin (0.03% to 0.1%) were sufficient for inducing statistically significant male sex biases ($P<0.0001$) also in OR semispecies (Table 1).

Hence, the two D. paulistorum semispecies tested on antibiotics are both highly permissive in shifting sex ratios towards males, but in drug dosage-dependent manners. Whereas sex ratio distortion becomes manifested in AM semispecies already at low concentrations of antibiotics, OR semispecies require more severe treatment conditions for inducing a similar phenotype. Dosage-dependent induction of this sex ratio phenotype is directly correlated with our finding that OR semispecies harbor high-titer Wolbachia, while AM semispecies are only infected at low densities. We thus assume that in OR semispecies, higher dosages of antibiotics are necessary to overcome a specific threshold level, critical to affecting symbiont-density significantly, and capable for inducing sex ratio distortions in favor of males. Excess of adult males in treated D. paulistorum lines can potentially be explained by higher sensitivities of females than males to partial symbiont depletion, resulting in enhanced embryonic or larval mortality of females. Similar male-biased distortion phenotypes upon artificial microbial depletion have been reported earlier in host - symbiont systems where obligate,
maternally-transmitted endosymbionts serve obligate mutualistic functions in filarial nematodes [67,68]; and see Discussion. Hence, this report is, to our knowledge, the first case in literature demonstrating the manifestation of a male-biased sex ratio distortion phenotype upon artificial symbiont depletion in arthropods.

Since all tested strains covering the six D. paulistorum semispecies are old laboratory lines, collected before the 1960s, it is possible that in the course of their long term rearing conditions these lines might have picked up Wolbachia artificially and/or that relaxed selection in the laboratory has led to artificial co-adaptations between host and symbiont. In order to overcome this criticism we have included two more recent D. paulistorum lines that were collected in Porto Alegre, Rio Grande. These two lines, POA1 and POA10 were established from isofemales collected in Southern Brazil in 2003, (see Materials and Methods). These two lines, POA1 and POA10 were established from isofemales collected in Porto Alegre, Rio Grande. Both lines belong to the Andean-Brazilian (AB) semispecies group, since they give rise to fertile male hybrids when mated reciprocally to the Mesitas strain (AB); unpublished data. Based on wsp marker sequences both POA strains are infected with the same Wolbachia type (data not shown), but differ significantly in their respective titer levels (Figure S3). Whereas POA1 adults harbor high-titer infections similar to Orinocan semispecies, Wolbachia levels are very low in POA10 adults, similar to most other D. paulistorum semispecies. We therefore conclude that both low- and high-titer Wolbachia are natural symbionts of D. paulistorum semispecies. More data from present-day samples from wild populations will be important to deepen our understanding in the temporal and spatial host-symbiont dynamics and their present impact on incipient speciation in nature.

Effects of Wolbachia-Depletion on Premating Isolation and Female Mate Avoidance

Reinforcement is the process by which postmating isolation acts as a direct selective pressure fostering the evolution of premating isolation in areas of sympathy [2,69]. In this respect, reinforcement is judged the final most efficient stage in speciation processes that block gene flow to incipient species via mating discrimination. Understanding how sexual isolation evolves requires that we capture the process before it has reached completion. Therefore, the D. paulistorum species complex, a cluster of semispecies in statu nascendi, serves as a perfect model system for elucidating the mechanisms and dynamics of incipient sexual isolation [42].

Based on our data, we propose that different semispecies of D. paulistorum harbor mutually incompatible fixed Wolbachia strains that cause postzygotic isolation by strong bidirectional CI and hybrid male sterility in the laboratory [42]. In reciprocal crosses between any of the six semispecies, F1 hybrid females are fertile and - under laboratory conditions - gene flow is possible between semispecies. In nature, however, incompatible matings between semispecies are avoided by female mating choice and courtship behavior [65,70]. In the D. paulistorum species-complex sexual isolation between sympatric semispecific strains is significantly greater than sexual isolation between allopatric semispecific strains [37,71].

In order to evaluate the potential role of Wolbachia mutualists on sexual isolation we have performed mating choice assays between naturally infected and artificially depleted D. paulistorum strains of AB, AM, CA and OR semispecies. Sexual isolation indices (SIIs) were measured between combinations of heterogametic pairs of AM, CA, OR and AB (line POA1 and POA10) semispecies [71,72], before and after Rifampicin treatments for at least five consecutive generations. For AM x OR interstrain assays, lines derived from three different concentrations of the antibiotic, i.e., low-Rif at 0.01% and high-Rif at 0.1% and 0.2%, whereas AM x CA and AB x CA assays were performed at high-Rif only. As shown in Figure S5, control assays between untreated (U), naturally infected, pairs of AM and OR semispecies document high sexual isolation (assay 1, SIIAM U = 0.70 ± 0.004). Current SII of these two allopatric strains is slightly higher but similar to the index obtained in earlier assays (0.61 ± 0.07, in [37]).

Infectious Speciation in Drosophila

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In contrast, sexual isolation of present-day mating choice assays between untreated AM and CA semispecies (assay 10, SIIAM x CA U = 0.90 ± 0.002) is significantly stronger (P = 0.0164) than observed in earlier assays in the mid 1960s (0.70 ± 0.07, in [37]).

Assays performed on AM and OR semispecies, where both partners were treated (AM x OR U), resulted in a weak enhancement of SII from 0.70 ± 0.004 in both untreated to 0.86 ± 0.002 and 0.84 ± 0.003 under low- and high-Rif conditions, respectively (assays 1 to 3). However, this slight effect on sexual isolation...
isolation is not statistically significant under both treatment conditions \( (z = 1.88 \text{ for 0.01\% Rif and 1.41 \text{ for 0.1\% Rif, both } P>0.05}) \). Under 0.2\% Rifampicin, however, double treatment of both partners induced almost random mating (assay 4; and Table S2, \( z = 3.89, P<0.0001 \)).

Unidirectional selective treatment of OR semispecies at low-Rif conditions slightly reduced sexual isolation when OR was mated with untreated AM semispecies, from \( SII_{AM \times OR}^U = 0.70 \pm 0.004 \) to \( SII_{AM \times OR}^T = 0.54 \pm 0.004 \) (assay 5), although this reduction is not statistically significant \( (z = 0.18, P>0.05) \). More intense treatments of OR semispecies with 0.1\% Rifampicin, however, revealed a 33\% reduction of assortative matings (assay 6), compared to both untreated controls \( (SII_{AM \times OR}^U = 0.48 \pm 0.007 \text{ vs. } SII_{AM \times OR}^T = 0.70 \pm 0.004) \). Although increased dosages of antibiotics in OR semispecies further reduce sexual isolation in AM \( \times OR \) assays, Fisher’s Exact tests and t-tests show only weak statistical significance \( (z = 1.93; P = 0.0539) \). Similar to double treatment assays (assays 2–4), in OR semispecies higher doses of Rifampicin up to 0.2\% were necessary to significantly reduce sexual isolation \( (SII = 0.33 \pm 0.007) \) in heterogamic mating choice assays (assay 7; Table S2, \( z = 5.19, P<0.0014 \)).

In contrast to OR harboring Wolbachia at high-titer, selective antibiotic treatments of low-titer AM semispecies trigger dramatic effects on assortative matings in AM \( \times OR \) mating choice assays (assays 8 and 9). Combinations between AM treated and OR untreated lines resulted in the complete breakdown of sexual isolation towards random mating between these two \( D. paulistorum \) semispecies. Random mating was observed in combinations of AM \( \times OR \) under conditions of low- \( (0.06 \pm 0.008) \) and high Rifampicin \( (0.18 \pm 0.008) \), where, in both cases, reductions were statistically highly significant (for both treatment conditions: \( z = 3.89, P<0.0001 \)).

Combinations between the two Wolbachia low-titer semispecies CA and AM \( (10–13) \) showed that partial depletion of the symbiont via 0.1\% Rifampicin decreases levels of mate discrimination by about 50\% in all three directions, i.e., in double and both unilateral treatments. SII dropped from an original 0.90\pm 0.002 (both untreated) to 0.38\pm 0.007 \( (AM^T \times CA^T) \), 0.46\pm 0.007 \( (AM^T \times CA^U) \), and 0.44\pm 0.007 \( (AM^U \times CA^T) \), respectively. For all three combinations reductions in mate discrimination were highly significant \( (z = 3.89, P<0.0001) \).

Finally we have assayed the effect of partial Wolbachia depletion on assortative mating behavior in our most recently collected \( D. paulistorum \) samples belonging to AB semispecies, i.e., high-titer POA1 and low-titer POA10 lines (Figure S3). Mating choice assays were performed in heterogamic combinations between untreated CA semispecies and both POA lines under high Rifampicin treatment conditions (Figure 5, assays 14–17). Similar to “old” laboratory strains of AM, CA and OR semispecies, both AB lines obtained from more recent collections showed statistically significant reduction in their assortative mating behavior after partial symbiont depletion in combinations with untreated CA semispecies (Table S2, \( z = 3.89, P<0.0001 \)). SII dropped from original 0.92\pm 0.001 \( (POA1 \text{ in assay 14}) \), and 0.95\pm 0.001 \( (POA10 \text{ in assay 16}) \) to 0.49\pm 0.006 \( (assay 15) \), and to 0.35\pm 0.007 \( (assay 17) \), respectively.

In order to rule out potential toxic side effects of antibiotics on Drosophila mating behavior and sexual isolation, we have generated symbiont-depleted lines of the \( D. simulans \) Riverside strain naturally infected with \( \alpha Ri Wolbachia \) [60]. 0.2\% Rifampicin treatments were performed for five consecutive generations in accordance to our depletion protocol for \( D. paulistorum \) lines (Materials and Methods). Standard mating choice assays were performed between untreated \( (D. sim^U) \) and treated \( (D. sim^T) \)
samples. As shown in Figure 5, high doses of Rifampicin have no effect on random mating behavior in native D. simulans-Wolbachia associations (assay 10; and Table S2). These data implicate that loss of assortative mating behavior in D. paulistorum semispecies was most likely caused by disturbing natural host-symbiont associations, rather than by any unspecific toxic drug-effect.

In addition there is a direct correlation between the natural Wolbachia titer level in a defined semispecies and their sensitivity to alterations in assortative mating behavior upon partial symbiont depletion. OR semispecies, harboring high-titer levels of their core-endosymbiont, seem more recalcitrant to antibiotic treatments than AM and CA. Similar to the sex ratio distortion phenotype (Table 1), OR semispecies need higher dosages of antibiotics (up to 0.2% Rifampicin) in order to reach the critical threshold level sufficient for triggering statistically significant changes of mating behavior. A similar but statistically not significant correlation between natural symbiont titer level and drug competence was observed in POA lines belonging to AB semispecies. Whereas the high-titer line POA1 showed a moderate 46.7% reduction in mate avoidance after partial symbiont depletion, in the low-titer POA10 line a 63.2% reduction was observed (Figure 5, assays 14–17).

Summing up, the mating choice assays presented here imply strong effects of Wolbachia on pre-mating isolation between D. paulistorum semispecies in a titer-dependent manner. Next we asked the question as to whether artificial symbiont depletion modifies mate avoidance patterns on the part of D. paulistorum females. For that purpose we have determined female mating frequencies of successful heterogamic matings obtained from mating choice assays itemized in Figure 5 and Table S2. As shown in Figure 6, untreated females of AB, AM, CA and OR semispecies strongly oppose heterogamic males, resulting in rare heterogamic mating events under our assay conditions. Under low-Rif conditions (0.01%) only AM-treated females lose mate discrimination by accepting heterogamic OR males (Figure 6A), whereas no significant alterations were observed in treated high-titer OR females (Figure 6B).

Under 0.1%-Rif conditions, however, treated AM and CA females (Figure 6C–6E) accepted reciprocal heterogamic males of untreated (T/U) and treated origins (T/T) significantly more often than do untreated females (P<0.01). In addition, treated OR females mate with untreated AM males (T/U) significantly more frequently (P<0.05) than do untreated females (Figure 6F); higher concentrations of the antibiotic (0.2%) further enhanced effects on male recognition patterns of OR females in a dose-dependent manner (Figure 6G). Similar to the “old” laboratory strains mentioned above, treated females of the two more recently established AB lines showed similar behavioral patterns (Figure 6H,I). To sum up, our data imply that partial depletions of Wolbachia in females of AB, AM, CA and OR semispecies significantly impair their mate avoidance behavior; they lose proper mate recognition signals and finally accept improper heterogamic males.

Besides this effect observed in treated females, partial Wolbachia-depletion also affects male behavior. As shown in Figure 6C,6D treated AM and CA males succeed in copulating significantly more frequently with untreated heterogamic females (U/T) than do untreated males. This enhanced mating frequency on the part of treated males is statistically significant (P<0.05 for CA and P<0.001 for AM males, respectively). Enhanced mating success of treated AM males (P<0.05) was also observed in combinations with untreated OR females (Figure 6F), suggesting that partial symbiont-depletion is capable of also changing male sexual behavior in both low-titer semispecies. Our current Rifampicin conditions up to 0.2% applied to OR semispecies, however, were not sufficient to induce a similar change in male mating behavior, since mating success of treated OR males (U/T) equals the one observed for untreated males (U/U) in heterogamic AM assays (Figure 6E,6G). These data suggest that even our current 0.2% treatment conditions are not sufficient for altering mating behavior of OR males that naturally harbor high-titer Wolbachia.

Figure 6. Frequencies of successful heterogamic matings of D. paulistorum females. Box plots represent distribution of mating frequencies obtained from interstrain mating choice assays with Amazonian (AM), Centroamerican (CA), Orinocan (OR) and Andean-Brazilian (AB; lines POA1 and POA10, respectively) semispecies (females are first named). Tested lines were kept on normal food, or on 0.01% (A,B), 0.1% (C–F) and 0.2% Rifampicin (G–I). Mating frequency was determined by number of successful heterogamic matings out of twelve females in five replicas each (Table S2). Combinations of heterogamic mating choice pairs are indicated by U/U = both partners untreated; T/T = both treated; T/U = females treated, males untreated; and U/T = females untreated, males treated. Statistical significant values are indicated by one, two or three asterisks, i.e., P<0.05, P<0.01 and P<0.001, respectively; and statistical outliers by black stars.

doi:10.1371/journal.ppat.1001214.g006
Discussion

Uncovering Hidden Wolbachia Diversity in D. paulistorum Semispecies

The presence of low-titer Wolbachia in five out of the six D. paulistorum semispecies corroborates earlier ultrastructural observations, showing that “MLOs” reside at low densities in the reproductive organs of their natural hosts, but heavily over-replicate in hybrids [49]. This quantitative shift of Wolbachia densities from extremely low in native hosts to intermediate in interspecies hybrids, strongly suggests that the causative agent of incipient D. paulistorum speciation are Wolbachia. Moreover, no other germ line-associated microbes were isolated by different means of experimental 16S consensus PCR approaches.

In adult flies of AB, AM, CA, IN and TR semispecies, densities of native endosymbionts are too low for unambiguous detection by standard wsp PCR protocols. As such, earlier attempts have failed to isolate Wolbachia from members of the D. paulistorum species complex and hence were diagnosed as Wolbachia-free [39,73]. Importantly, none of these earlier studies reported surveys neither of the high-titer OR semispecies, nor of F1 interspecies hybrids, where Wolbachia is ostensibly present. In a recent publication we have applied our PCR-wsp-hybridization strategy [53] to uncovering multiple low-titer Wolbachia strains in the cherry fruit fly, Rhagoletis cerasi, not accessible by standard PCR methods in earlier studies. We hence encourage considering the broader existence of low-titer Wolbachia in nature, commonly escaping conventional detection approaches, before designating a sample or even a whole group under study as uninfected.

Based on wsp and VNTR sequence analyses, Wolbachia of CA, OR and TR semispecies are host-specific and are closely related with, but not identical to, other wAu-like strains isolated from phylogenetically geographically neotropical Drosophila hosts [59] that harbor unique diagnostic sites (Table S1). As shown in Figure S3, apparent conspecificity between native hosts and defined Wolbachia strains of D. paulistorum semispecies and their related sister species suggests an ancestral association assisted by stable vertical transmission. In order to elucidate the coevolutionary history of this host-symbiont interaction, more detailed molecular Wolbachia-strain-typing analysis will be necessary for D. paulistorum semispecies, from strains kept since the 1950s in the laboratory, as well as from recent samples of natural populations, by means of applying more informative, preferentially non-coding Wolbachia markers other than the wsp locus or other protein coding genes. As such, there are a growing number of cases in recent Wolbachia literature, where strains sharing identical wsp sequences induce clearly distinguishable phenotypes [74–77]. Importantly, these reported cases of phenotypic transitions of Wolbachia in fruit flies, aphids and butterflies, took place in extremely short evolutionary time scales, implying that these symbiotic associations are highly dynamic and rapidly evolving in natural and laboratory populations.

In contrast to the wAu-like Wolbachia of CA, OR and TR, the core-endosymbiont of AM semispecies is unique in that it groups closely with the wRi infection of D. simulans, suggesting a more recent acquisition via horizontal transmission from a yet uncharacterized external source. Potentially, this new Wolbachia strain has replaced an ancestral wAu-like symbiont, similar to CA, OR and TR Wolbachia, already present in the AM progenitor lineage. The uniqueness of wRi-like Wolbachia in AM corroborates with earlier phylogenetic studies, designating AM as the most distal semispecies of the D. paulistorum complex [70,79].

Compensatory Evolution between Wolbachia and Their Natural D. paulistorum Hosts

In agreement with earlier reports, D. paulistorum semispecies are highly sensitive to antibiotics [41]. In addition, ultrastructural analyses have shown that no D. paulistorum fly has ever been observed is that devoid of endosymbionts [39,41,49]. Since complete clearance of Wolbachia is obviously lethal for all D. paulistorum semispecies, it has been suggested that core-endosymbionts of D. paulistorum are well-adapted obligate mutualists that serve essential vital functions benefiting to their hosts (reviewed in [42,65]). In this study we note that partial symbiont depletion under mild antibiotic condition is capable of triggering alterations in (i) female fecundity, (ii) male-biased sex ratio, and (iii) most significantly, female mating behavior in D. paulistorum.

The female fecundity phenotype, induced by artificial depletion of Wolbachia (Figure 4), engenders similar effects on oogenesis that have been detected in the parasitoid wasp Amyama tabida, where Wolbachia is an obligate symbiotic partner [80,81]. Female wasps that are cured of their Wolbachia, fail to produce any mature oocytes, rendering Wolbachia infection essential for female reproduction. In this case of mutualism, Wolbachia influence programmed cell death processes in A. tabida by modulating apoptosis during oogenesis [15]. The authors speculate that after a loss-of-function mutation in a key regulator gene of the A. tabida apoptosis network system, Wolbachia were capable of permanently taking over control of the host apoptotic pathway. Another example of the compensatory effect of Wolbachia on a host loss-of-function mutation, comes from laboratory strains of D. melanogaster, where the presence of Wolbachia is sufficient to restore the ovarian phenotype in Sx$f^4$ mutants [66]. The most recent example showing that Wolbachia can transform even in an obligate nutritional mutualist residing in a bacteriome was found in the bedbug Cimex lectularius, where artificial depletion of its symbiont results in sterility and retarded growth [82].

The second phenotype, uncovered by mild antibiotic D. paulistorum treatments (Table 1), is, to our knowledge, the first report on a symbiont-induced male-biased sex ratio distortion in arthropods. In filarial nematodes, significant male-biased sex ratio distortion was observed after partial depletion of mutualistic Wolbachia by Tetracycline [67,68]. These authors suggest that if Wolbachia play a more active role in females than in males, this may imply a more biosynthetic activity of Wolbachia in female hosts and thus greater susceptibility to antibiotics. This assumption is based on evolutionary models [83,84], asserting that in a mutualistic relationship, selection on inherited microorganisms should (i) select directly for beneficial effects towards the host sex responsible for their transmission (in general, the female), and (ii) favor the spread of phenotypes that are detrimental to those hosts not involved in their transmission (males or uninfected females). In this respect, the profound sensitivity of D. paulistorum females to Wolbachia-depletion (loss-of-function) plus the hybrid male sterility phenotype caused by Wolbachia overreplication in testes (gain-of-function) corroborates this evolutionary model.

Both phenotypes, low female fecundity and male-biased sex ratio distortions, induced by mild antibiotic treatments of D. paulistorum semispecies (Figure 4 and Table 1), can be explained by strong obligate mutualistic interactions between Wolbachia and their natural neotropical hosts, i.e., suppression of enhanced cellular mortality during female development or a direct effect on the Insulin/IGF-like signaling cascade.

The first model assumes that the presence of Wolbachia above a specific threshold level plays an essential role in female, but not male, development by suppressing female lethality. For example, female larvae or pupae require a nutrient vitamin or hormone
surface protein WSP is capable of inhibiting apoptosis in D. paulistorum hosts is likely to be the result of a tight coevolution between an ancestral host responding to infection with apoptosis, the symbiont suppressing it, and the host compensating for this suppression because apoptosis is required for development. Alternatively but not exclusively, depletion of Wolbachia in D. paulistorum semispecies by mild antibiotic treatment might have unleashed a cryptic, but female-biased loss of function phenotype associated with the Insulin/IGF-like signaling (IIS) pathway. Mutations that reduce the activity of the IIS signaling cascade have pleiotropic effects on many traits, such as development, metabolic homeostasis, adult lifespan and fecundity in nematode worms, fruit flies, and mice (reviewed by [87]). As was recently shown, removal of symbiotic Wolbachia in naturally infected D. melanogaster reduces IIS and hence enhances mutant IIS phenotypes, suggesting that Wolbachia normally act to increase insulin signaling [88]. Since some of the key downstream effectors of the IIS cascade are also functionally implicated in regulating apoptosis [89,90], one may assume that these two models explaining the evolution of compensatory mutualistic Wolbachia in D. paulistorum semispecies, are not mutually exclusive. Under both evolutionary scenarios, however, closely related but host-specific Wolbachia strains might act as general suppressors of yet undefined, cryptic, female-biased mutations already present in the common ancestor of all the D. paulistorum superspecies. This assumption is corroborated by our findings that at least three out of the six infected semispecies (CA, OR and TR), from which we have already obtained sufficient information on Wolbachia-strain diagnostics, harbor strain-specific and closely related wAu-like endosymbionts (Table S1). In addition, the TR semispecies is regarded as the relict ancestral semispecies of the D. paulistorum superspecies [78,79] further supporting their long-term relationship and common ancestry with wAu-like Wolbachia symbionts.

As such, regulation of host-programmed cell death during female development, as well as suppression of a cryptic IIS mutation in D. paulistorum semispecies, are both plausible mechanisms that can explain the evolutionary transition of Wolbachia from facultative parasitism towards obligate mutualism via compensatory evolution. Under both models, D. paulistorum Wolbachia can be regarded as obligate mutualists by compensating reduced female-specific fitness effects that were originally caused, either by a suppressor of IIS mutation, or by reproductive parasites such as Wolbachia. In each of the two scenarios, a former reproductive parasite became domesticated by its host system, thereby providing more recently evolved immunity against genomic loss of function mutations - and intracellular parasites, respectively. In summary, the coevolutionary interaction between D. paulistorum semispecies and Wolbachia infection has changed from antagonistic into reciprocally beneficial and thereby into an obligate mutualistic interaction, where the both systems are dependent on each other now.

The Impact of Wolbachia on Infectious Speciation

Increasing empirical evidence and numerous theoretical models predict that transovarially transmitted microbial symbionts can have significant impacts as drivers of speciation processes in their natural hosts [12,17–21]. However, there are substantial difficulties with speciation based solely on symbionts capable of inducing incompatibilities [1,22]. First, as deduced from theoretical models, CI causes unstable equilibria in prevalence and transmission rate of symbiotic infections [16,26,27], but more recent models show that such stabilities can exist, depending on parameters such as host migration rate, CI induced gene flow reduction and the invasion success of mutants at a mate preference locus [21]. Moreover, this criticism has been refuted in Bordenstein 2003 and is not Wolbachia-specific since it applies to any type of postzygotic incompatibility [12]. In our Wolbachia-D. paulistorum model system however, theoretical caveats opposing most speciation models can be overruled by evolving ancestrally fixed, obligate mutualistic interrelations between the endosymbiont and its natural host, vital for both partners. So, even partial tier depletion of the obligate core symbiont below a critical Wolbachia threshold, results in severe pleiotropic and primarily female-biased phenotypes, which natural selection acts against.

Second, the few empirical systems from present literature concerning roles of symbionts in speciation, such as parasitoid wasps of the Nasonia sibling species group, are highly informative in elucidating the evolutionary implications of endosymbionts [28,29]. Bordenstein and colleagues showed that in the youngest Nasonia species pair, N. giraulti and N. longicornis, Wolbachia-induced bidirectional CI was the primary form of reproductive isolation prior to the evolution of other isolating barriers. However, these species are not known to occur sympatrically in nature and preexisting isolation tested in the laboratory was weak and asymmetric [12,29]. Hence it has been suggested to focus on natural symbiotic systems of sympatric, hybridizing host species that are isolated because of CI [12]. Indeed, the mushroom feeding Drosophilida species D. reeves and D. sucina are found in a hybrid zone in central Canada. Moreover they express strong unidirectional CI triggered by Wolbachia in D. reeves, plus asymmetrical premating isolation and hybrid male sterility. The latter two isolation mechanisms, however, are genetically based [20,30]. The assortment of symbiotic and genetically based isolation barriers provides clear evidence that Wolbachia can act in concert with genetic and/or geographic isolation mechanisms in nature. Our incipient speciation system presented here is in flagrant nascendi, occurs sympathetically in consistently overlapping geographic distributions in Middle and South America [43], exerts strong bidirectional CI [44–46], plus pronounced premating isolation in a symbiont-dependent manner (this study).

Finally, no empirical cases have been reported suggesting that endosymbionts are able to cause hybrid-male sterility, a hallmark of an early stage in the evolution of postzygotic isolation [1], but also see [32], and this study. However, the novel Wolbachia-induced phenotype of symbiont-induced hybrid male sterility reported here, can be regarded as currently residual and supportive, since all D. paulistorum semispecies have evolved strong prezygotic isolation mechanisms and intriguing female mate avoidance patterns by successfully preventing inter-semispecific matings [37]. Furthermore, natural hybrids between sympatric semispecies have never been reported in the field, nor has a trapped sperm-storing gravid female ever borne alien sperm (L.E. personal communication).

After having demonstrated that Wolbachia are ancestrally fixed, obligate entities of all D. paulistorum semispecies, perfectly transmitted by the mother and vital for female oogenesis and female development, we have evaluated effects of mild symbiont depletion via antibiotics on female mating choice. Currently a growing number of reports emerge in our literature comparing specific behavioral traits possessed by symbiont-infected and uninfected individuals, such as mate avoidance, male promiscuity, longevity, pathogenic fungal resistance, and olfactory-cued
locomotion [31,91–95]. In spider mites, for just one example, Wolbachia-associated unidirectional CI can be avoided by females at the premating level [91]; they show spider-mite females exhibiting both precopulatory and ovipositional behaviors that increase odds of successful compatible matings. Furthermore, in mating choice experiments, uninfected females preferably mate with uninfected males, and in doing so, directly reduce opportunities for Wolbachia-induced unidirectional CI expression [91].

So according to the scenario observed in spider mites, we will propose the following model for understanding the biological role of Wolbachia driving sexual isolation between D. paulistorum semispecies: Obligate mutualistic Wolbachia, having evolved tight compensatory interrelations with their natural hosts, are capable of triggering female mate recognition patterns in favor of preferentially accepting males harboring the same type of Wolbachia - i.e., members of their natal semispecies - and to reject improper mates carrying an alien version of their obligate symbiont. In the mixed genetic background of artificially generated hybrids however, maternally transmitted, naturally benign mutualistic Wolbachia turn into reproductive parasites, capable of inducing strong embryonic bidirectional CI and complete male hybrid sterility via loss of their controlled replication. Although F1 hybrid females are fertile under laboratory conditions, they show an intriguingly altered sexual “old maid” behavior by hardly accepting any kind of mating partner [96]. Hence we assume that symbiont-directed mate recognition could have evolved in order to prevent strong bidirectional CI and reduced sexual success of potential hybrids, thereby ensuring their continuing vertical transmission. Alternatively, native hosts might have evolved behavioral avoidance patterns that recognize potential mates carrying distinctive but incompatible Wolbachia variants [91]. As suggested by Koukou and colleagues Wolbachia might have evolved the capacity to modulate host pheromone expression and/or perception [31]. Recently, Albertson and colleagues [97] have shown that Wolbachia significantly concentrate in specific brain regions (i.e., the subesophageal ganglia, superior protocerebra and antennal lobe) of D. melanogaster and D. simulans; speculating that the presence of the endosymbiont could potentially affect insect behavior, such as olfactory-cued locomotion and mating behavior [95]. By analytical organic methods it was recently discovered that within the D. paulistorum complex the active male and female pheromonal compounds differ in proportions present in each of the six D. paulistorum semispecies - that is, quantitatively not qualitatively [79,98]. In accordance with these data, we revealed that Wolbachia are also tightly associated with the antennal lobe of D. paulistorum semispecies (unpublished data). Hence, our D. paulistorum-Wolbachia symbiosis system, affecting premating isolation between natural semispecies, provides a unique and excellent model system for studying the global impact of microbial endosymbionts on pheromone production, olfaction, and sexual behavior in insects.

Materials and Methods

Fly Strains

The D. paulistorum species complex [99] is neotropical and comprises at least six semispecies in statu nascendi [33]. They are morphologically indistinguishable, but can be identified by allozymes, chromosomes and courtship behavior [42]. For this study, the following four strains, belonging to Amazonian (AM), Centroamerican (CA), Orinocan (OR), and Transitional (TR) D. paulistorum semispecies were used: Belem, Brazil, AM; Lancetilla, Honduras, CA; Georgetown, Guyana, OR; and Santa Marta, Colombia, TR. For full descriptions of origins and maintenance of all Drosophila paulistorum strains, see [42], and references therein. POA1 and POA10 strains, belonging to the Andean Brazilian (AB) semispecies are isofemale lines that were generated from collections in April 2003 in Porto Alegre City, Rio Grande do Sul State Brazil. Both lines were kindly provided by Yong-Kyu Kim, Emory University, Atlanta, GA, USA. The control strain Pan90, collected in Panama in 1990, belongs to the closely related sister species D. willistoni, naturally infected with wWill Wolbachia [59]; and two D. simulans lines, Riverside (RI) infected with wRi Wolbachia [60] plus the uninfected strain STC [100] serve as positive and negative controls, respectively.

Generation of D. paulistorum Interspecies Hybrids

Sexually mature virgin females of AB, AM, CA and TR semispecies were forced to mated en masse with a twofold excess of heterogamic OR males, respectively. These males had been isolated from females for several days. Flies were transferred into fresh vials every 40 hrs over a period of two weeks.

Molecular Isolation and Strain Typing of D. paulistorum Semispecies Wolbachia

High quality genomic DNA was extracted from a pool of ten adult flies, 15 ovaries/testes or from 30–50 mg embryos using the QiAamp DNA Mini Kit (Qiagen, Hilden, Germany). Eggs were collected from apple juice agar plates in 24 hrs intervals and sterilized by extensive washes with 70% EtOH before DNA extraction. Testes and ovaries were dissected in sterile 1x PBS. All DNA samples were stored at −20°C until use.

Germline-Associated 16S rRNA PCR Screen

In order to determine the microbial diversity of germline associated symbionts of D. paulistorum OR semispecies, we have performed 16S rRNA consensus PCRs after [50] on five independent DNA samples, derived from sterilized 0–24 hrs embryo. From each sample, at least four amplified 16S rRNA consensus fragments were cloned and sequenced. All twenty sequence-reads were identical with the 16S rRNA gene of Wolbachia pipens of D. willistoni (accession number DQ412086), and no other bacterial sequence was detected. To further confirm the presence of Wolbachia in reproductive organs of D. paulistorum we performed two independent 16S rRNA universal PCRs after [50,51] on sterile-dissected ovaries and testes of AM, OR semispecies and AxO hybrids derived from crossings of AM females with OR males. Two different primer sets amplifying overlapping universal microbial 16S rRNA fragments between 0.4 and 0.6 kb were used. Amplicons were purified using the Promega Wizard SV Gel PCR Clean-up system. Sequences were obtained via cycle sequencing with BigDye Terminator v3.1 performed at the Department of Marine Biology, University of Vienna, Austria. Sequences were analyzed using ApE, plasmid editor v1.10.4 (M. Wayne Davis), ClustalX, (www.clustal.org), Mesquite v2.6 (www.mesquiteproject.org), and the BLAST algorithm (www.ncbi.nlm.nih.gov).

Diagnostic Wolbachia PCR, Cloning and Sequencing

wPau Wolbachia was detected using primer specifically targeting the wsp locus, the transposon IS8 as well as VNTR-105 and VNTR-141 locus. wsp-primer sets were taken from [52] and primers for VNTRs and IS8 PCR were taken from [57]. In all reactions the uninfected D. simulans strain STC was included as negative controls. Diagnostic wsp-PCR reactions were performed under the following conditions: 10 μl reactions containing 1 μl of genomic
DNA template in 1x polymerase reaction buffer (Promega, Madison, USA), 4.5 mM MgCl₂, 0.3 mM of each primer, 150 μM of each dNTP and 0.4 U of Taq polymerase (Promega, Madison, USA). Diagnostic VNTR-PCRs were performed as follows: 10 μl reactions containing 1 μl of genomic DNA template in 1x polymerase reaction buffer (Promega, Madison, USA), 2.5 and 4 mM MgCl₂ for VNTR-141 and -105, respectively, 0.3 mM of each primer, 35 μM of each dNTP and 0.4 U of Taq polymerase (Promega, Madison, USA). PCR-profiles for wsp amplifications were taken from [52]. For VNTR-141 locus, amplifications consisted of 5 min at 94°C followed by 34 cycles of 1 min at 94°C, 1 min at 50°C and 1 min 30 sec at 72°C. Final elongation step was performed at 74°C for 8 min. For VNTR-105 a higher annealing temperature of 61°C was used and elongation was performed for 3 min instead of 1 min 30 sec. ZS PCRs were applied as described in [53]. For all PCR reactions a Biometra T3000 Thermocycler (Biometra, Goettingen, Germany) was used. PCR products were purified using the pEQGOLD Gel Extraction Kit (pEqLab, Erlangen, Germany). For cloning purposes, probes were inserted into the pTZ57R/T vector (Fermentas, St. Leon-Rot, Germany), and used to transform competent DH5α Escherichia coli cells. Clones containing the insert were cycle sequenced with BigDye Terminator v3.1 at the Department of Marine Biology, University of Vienna, Austria. Sequences were analyzed as described above.

Detection of Low-Titer Wolbachia via Southern Hybridization

In order to detect low-titer Wolbachia infections in D. paulistorum semispecies, a digoxigenin (DIG, Roche Diagnostics, Germany) labeled probe, binding to the core sequence of the wsp gene, was applied on blots of standard wsp DNA-DNA hybridization. For probe design, hybridization conditions and signal detection see our detailed protocol in [53].

Antibiotic Treatment Conditions

Stock solutions of antibiotics were made by diluting Tetracycline (5 mg/ml) and Rifampicin (30 mg/ml) into ethanol 90%, stored at −20°C. Artificially Wolbachia-depleted strains of AB, AM, CA and OR D. paulistorum semispecies were established by adding aliquots of the respective stocks to regular fly food at final concentrations of 0.01 and 0.03% of Tetracycline and 0.01, 0.03, 0.1 and 0.2% of Rifampicin. Treated lines were kept for at least three generations on antibiotics for bioassays.

Ovary Phenotype Assay

Analysis of ovary phenotype followed [66]. Females were raised on standard food with or without antibiotics (0.01% Tetracycline for fifteen generations and 0.1% Rifampicin for three generations) at 25°C and were dissected ten days after eclosion. Only eggs reaching stage 13 (after [101], indicated by filaments) were counted as mature eggs. For DAPI staining, ovaries were dissected ten days after eclosion in sterile Drosophila Ringer’s Solution. Ovaries were stained in a DAPI-TBST solution (1 mg/l) for 5 minutes under constant agitation and mounted in Câllflor Glycerol/PBS solution (Groâl, Austria). Ovaries were analyzed using a Nicon Eclipse E800 fluorescent microscope.

Immunohistochemistry

Wolbachia density and tissue tropism in D. paulistorum semispecies were determined using the polyclonal WSP (Wolbachia surface protein) antibody [102]. WSP protein expression was analyzed in whole mount immunostainings on ovaries, staged embryos and on sections of male tissues after [103]. Rabbit anti-wsp antibody was used at a 1:500 dilution overnight at 4°C and detected after incubation with a 1:500 dilution of Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Molecular Probes) at room temperature for 1 hour. Slides were stained for 3 min with 1 μg/ml DAPI (Molecular Probes), rinsed and stained with 5 μg/ml propidium iodide (PI) (Molecular Probes) for 20 minutes, rinsed again and mounted with ProLong Antifade medium (Molecular Probes). Immunostainings of embryos and ovaries were examined by using a Zeiss Axiosom 2 Epifluorescence microscope. Images were processed using Photoshop 9.0.2 (Adobe).

Sex Ratio Assays

Sex ratio in the D. paulistorum semispecies Amazonian (AM) and Orinocan (OR) emerging from regular food (untreated) and two independent antibiotic-treatments with tetracycline (Tet) and Rifampicin (Rif). Total number of flies counted for two weeks from initial day of hatching was 8,693 divided into 4,851 males and 3,842 females. Untreated AM and OR semispecies have the usual 1:1 sex ratio (also see [42]; and references therein). Both antibiotic treatments were performed either over five generations on 0.01% Tetracycline (Tet) or 0.01% Rifampicin (Rif); or over three generations on 0.03% and 0.1% Rifampicin (Rif).

Measuring Sexual Isolation

Mating choice assays, monitoring the effect of artificial symbiont depletion via Rifampicin at 0.01% and 0.1% for at least five generations, involved direct mating observations. All details concerning our experimental protocols have been itemized by [37], and reviewed and adjudicated by [104]. Briefly: All replicas were conducted mornings at room temperature in daylight facing north. Beforehand, virgin flies were aged two to three days after light ether anesthetization during which they were sexed (there are no sex differences in abdominal banding in this superspecies); and half were marked via minute distal wing clips (controlled in bioassays). These marks were rotated (wing to wing and treated to untreated). Such abrasions have tested neutral regarding behavioral influences [105] in this superspecies.

For each of the eighteen interstrain mating choice assays monitoring the impact of Rifampicin treatment on mate avoidance between AB, AM, CA and OR semispecies and D. simulans controls, five replicas and 120 matings were scored (12A Q+ Q+2B QQ +12A ♂♂ +12B ♂♂ differentiated by rotated wing clips) totaling 2,160 matings. Without repeated anesthetization at any time, 2-to-3-day old flies were then placed into glass mating chambers [37,106]. A four-power hand lens was employed for scoring partners: the time (from start of observations) each mating takes place; its sequence among other copulae which occur; where in the chamber the mating pair is located (for this purpose a grid constitutes the floor); the kind of female involved; and the kind of male involved, until all flies copulated, in approximately 30–40 minutes. If undisturbed, each copulation lasts approximately 15–17 minutes; females mate only once in these arenas. Recorded location of copula, even upside down, prevented scoring a copula more than once.

Sexual isolation index (SII) and standard error (SE) were measured as in previous studies using multiple mating choice assays, following two calculations, i.e., the isolation index after [72] and the joint isolation index of [107]. In our assays, both calculations result in very similar SII values (Table S2). In general, SII values range from −1.00 (preference for unlikes, heterogamy) through 0 (random mating) to +1.00 (preference for likes, homogamy) between D. paulistorum semispecies.
Statistical Testing
All statistical tests were performed using SPSS 16.0 for Mac and GraphPad Software (www.graphpad.com). Statistical significance of effects of antibiotic treatment on sex ratios in D. paulistorum semispecies was tested calculating single degree-of-freedom Chi-squares. We applied an unpaired t-test to mating frequencies in D. paulistorum combinations, and compared the SIs obtained from mating choice assays using Fisher’s Exact test. Boxplot diagrams of mating frequency distributions were generated using SPSS.

Detection of Wolbachia in D. paulistorum Hosts via Transmission Electron Microscopy
To determine presence of Wolbachia within D. paulistorum hosts, living adult flies were sectioned using a vibrating blade microtome (Leica VT1000 S). Sections of 400–500 μm thickness were fixed in 2% glutaraldehyde, buffered with 0.1 M PO4 at pH 7.1. After postfixation with 1% OsO4, samples were dehydrated via an ethanol series [108]. Samples were then embedded in Epon and 70 nm ultrathin sections were produced. Following uranyl acetate and lead citrate staining of the sections, presence of Wolbachia was observed using a JEOL TEM.

Accession Numbers
Sequences were deposited in GenBank under the accession numbers GQ924884-GQ924891 and HQ185362-63.

Supporting Information
Figure S1 Wolbachia infection in adults of Drosophila paulistorum semispecies. Amazonian (AM); Centroamerican (CA); Interior (IN); Andean-Brazilian (AB); Transitional (TR); Orinocan (OR) semispecies. 2% glutaraldehyde, buffered with 0.1 M PO4 at pH 7.1. After postfixation with 1% OsO4, samples were dehydrated via an ethanol series [108]. Samples were then embedded in Epon and 70 nm ultrathin sections were produced. Following uranyl acetate and lead citrate staining of the sections, presence of Wolbachia was observed using a JEOL TEM.

Table S1 Variable nucleotide (A) and amino acid (B) sites in the wsp sequence of the closely related wAu-like Wolbachia strains of Drosophila and the Cherry fruit fly Rhagoletis cerasi (wCer2). Position number 1 of the consensus sequence corresponds to position number 164 in the wsp sequence of wAu of D. simulans (AF20067). Consensus wsp sequence obtained from the following Wolbachia strains: wAu of D. simulans Coffs Harbour [56]; wAu of D. willistoni; wAu Pro SG1 & 2 of D. pseudosarcina; wPf PLR1, 2 & BL1 of D. septentrionalis; wAu of D. paulistorum CA TR and OR semispecies (yellow, this study); wCer2 of Rhagoletis cerasi [109]; and wMel of D. melanogaster [56]. Designation of hypervariable regions (HVRs) and conserved regions (CR) of the WSP protein after [55].

Table S2 Mating choice assays performed on D. paulistorum semispecies before and after Wolbachia depletion via rifampicin. Mating choice assays performed on Amazonian (AM), Centraoamerican (CA), Orinocan (OR) and the Andean Brazilian (AB; POA1 & POA10) D. paulistorum semispecies before and after Wolbachia depletion via rifampicin.

Acknowledgments
We thank Bertha Green, Traude Kehrer and Monika Imb for excellent technical support and fly work; Ira Perelle for help with statistics and Silvia Bugleresi, Harald Gruber and Johannes Rathi for kindly providing sequencing facilities; and Michael Turelli and Markus Rieger for helpful discussions and encouragement.

Author Contributions
Conceived and designed the experiments: WJM LE. Performed the experiments: WJM LE DS. Analyzed the data: WJM LE DS. Contributed reagents/materials/analysis tools: WJM LE. Wrote the paper: WJM LE.

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