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1 **Title:**

2 The intracellular symbiont *Wolbachia* enhances recombination in a dose-dependent manner

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6

7 **Article Summary:**

8 The ubiquitous bacterial symbiont *Wolbachia* is known to alter host reproduction through
9 manipulation of host cell biology, protect from pathogens, and supplement host nutrition. In
10 this work we show that *Wolbachia* specifically increases host recombination in a dose
11 dependent manner. Flies harboring *Wolbachia* exhibit elevated rates of recombination across
12 the 2nd and X chromosomes and this increase is proportional to their *Wolbachia* load. In
13 contrast, another intracellular symbiont, *Spiroplasma,* does not lead to an increase in
14 recombination across the intervals tested. Our results point to a specific effect of *Wolbachia*
15 infection that may have a significant effect on infected insect populations.

16 **Key Words:**

17 *Wolbachia,* recombination, *Drosophila*

18 **Abstract**

19 *Wolbachia pipientis* is an intracellular alphaproteobacterium that infects 40-60% of insect
20 species and is well known for host reproductive manipulations. Although *Wolbachia* are
21 primarily maternally transmitted, evidence of horizontal transmission can be found in
22 incongruent host-symbiont phylogenies and recent acquisitions of the same *Wolbachia* strain
23 by distantly related species. Parasitoids and predator-prey interactions may indeed facilitate
24 the transfer of *Wolbachia* between insect lineages but it is likely that *Wolbachia* are acquired
25 via introgression in many cases. Many hypotheses exist as to explain *Wolbachia* prevalence and
26 penetrance such as nutritional supplementation, protection from parasites, protection from
27 viruses, or straight up reproductive parasitism. Using classical genetics we show that *Wolbachia*
28 increase recombination in infected lineages across two genomic intervals. This increase in
29 recombination is titer dependent as the *w*MelPop variant, which infects at higher load in
30 *Drosophila melanogaster,* increases recombination 5% more than the *w*Mel variant. In addition,
31 we also show that *Spiroplasma poulsonii,* the other bacterial intracellular symbiont of
32 *Drosophila melanogaster,* does not induce an increase in recombination. Our results suggest
33 that *Wolbachia* infection specifically alters host recombination landscape in a dose dependent
34 manner.

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35 **Introduction:**

36 Recombination, the exchange of genetic material during meiosis is thought to be largely
37 beneficial, as it increases the efficacy of natural selection [1,2]. Because of chromosome
38 architecture, loci that are physically linked to each other can interfere with selection such that
39 selection at one locus reduces the effective population size, and therefore the efficacy of
40 selection, at linked loci. This phenomenon, termed "Hill-Robertson interference," means that
41 positive or negative selection at one site can interfere with selection at another site. By
42 allowing loci to shuffle between chromosomes, recombination mitigates Hill-Robertson
43 interference [3]. As a result of this re-shuffling, areas of the genome subject to high
44 recombination rates show higher nucleotide diversity, either because of the inherent
45 mutagenic effect of recombination or by the indirect influence of recombination on natural
46 selection in a population. Overall, a large body of literature supports the assertion that
47 recombination increases efficacy of selection and enhances adaptation in animals, as studied in
48 various *Drosophila* species [1-3].

49 One factor that may influence recombination is bacterial infection. For example, injection of
50 flies with the bacterial pathogen *Serratia* increases recombination post infection [4]. Many
51 *Drosophila* species are colonized persistently by *Wolbachia pipientis*, an alpha-proteobacterium
52 within the *Rickettsiales* and the most common infection on the planet, found in 40-60% of all
53 insects. *Wolbachia's* prevalence in populations is likely modulated by its reproductive
54 manipulations, induced to benefit infected females [5]. However, this reproductive parasitism
55 alone is not sufficient to explain *Wolbachia* infection prevalence; indeed there are many
56 recently discovered, insect infecting strains which do not seem to induce any reproductive
57 phenotype at all, suggestive of other potential benefits provided by the symbiont [6-8]. One
58 known benefit is pathogen blocking, where *Wolbachia* repress the virus replication within the
59 insect host [9-11]. This phenomenon has important implications for the use of *Wolbachia* in vector
60 control [12]. In addition to protecting its host from pathogens, *Wolbachia* also generally improves
61 the fitness and fecundity of some hosts, and removal of the endosymbiont can cause a
62 decrease in host fitness (Fry and Rand '02; Fry et al. '04). Finally, *Wolbachia* can rescue
63 oogenesis defects in mutant *Drosophila* strains [13] and has also made itself a necessary
64 component of oogenesis in some wasp species, thereby making the infection indispensable [14].

65 One recently discovered phenotype of *Wolbachia* is that it may increase the amount of
66 recombination events on the X chromosome, but not on the 3rd, in *Drosophila melanogaster*
67 [15,16]. This phenotype contrasts with that observed for *Serratia* infection, where elevated
68 recombination was observed on the 3rd chromosome [4]. Does *Wolbachia* infection actually lead
69 to increased recombination? If so, would any infection of the reproductive tract result in
70 increased recombination? Here we answer these questions using classical genetics in
71 *Drosophila melanogaster* with different *Wolbachia* variants and using another intracellular
72 symbiont, *Spiroplasma poulsonii.* We confirm that *Wolbachia* significantly increases
73 recombination across two intervals, one on the X and one on the 2nd chromosome, but we

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74 could not detect any effect on the 3rd chromosome interval queried. In addition, there is a clear
75 correlation between *Wolbachia* load and recombination events, suggesting *Wolbachia* itself is
76 the cause of the elevated recombination; clearing the host of *Wolbachia* restores
77 recombination rate to a basal level while infection with a high-titer variant increases
78 recombination. Another intracellular symbiont, *Spiroplasma,* does not increase recombination
79 rate, suggesting this phenomenon is not simply due to the presence of a bacterial infection in
80 the gonads, but is *Wolbachia* specific. These results suggest that *Wolbachia* specifically elevates
81 host recombination, providing a previously unknown benefit to its host.

82 **Results:**

83 *Wolbachia infection increases host recombination rate*

84 We reasoned that if *Wolbachia* infected flies exhibited increased recombination rate, this
85 would be evident in natural populations. We took advantage of a set of isogenized flies,
86 sampled from a wild-caught population in North Carolina, the Drosophila Genetic Reference
87 Panel [17]. Virgin females from two backgrounds (DGRP-320, infected with *Wolbachia* and DGRP-
88 83, *Wolbachia*-free) were crossed independently to three different lines carrying chromosomal
89 markers, allowing us to distinguish recombinants along certain genomic intervals based on
90 presence of dominant markers (**Fig. 1**). These lines were y[1] v[1] (BDSC stock #1509) for the X
91 chromosome, carrying *yellow* and *vermillion;* vg[1] bw[1] (stock #433) for the 2nd chromosome,
92 carrying *vestigial wings* and *brown,* and e[4] wo[1] ro[1] (stock #496) for the 3rd chromosome,
93 carrying *ebony* and *rough.*

94 As a control, we also cleared the *Wolbachia* infection from line DGRP-320 by rearing the flies on
95 tetracycline for 3 generations and then repopulating the extracellular microbiome for 1
96 generation. When we compared *Wolbachia* infected and tetracycline cleared individuals,
97 controlling for genetic background, we observed a statistically significant increase in
98 recombination in F2 progeny derived from *Wolbachia*-infected mothers (**Fig. 2**). Specifically, for
99 the X and 2nd chromosomes we observed an increase in mutation rate of 6.4% and 6.1%,
100 respectively. No statistically significant effect of *Wolbachia* infection was observed on the 3rd
101 chromosome (df = 14, χ^2 = 0.080, p = 0.77). It may be that this difference in recombination rate
102 across chromosomes reflects the natural variation in recombination observed across genomic
103 intervals for *Drosophila,* or may be an artifact of the genomic interval sampled and not an
104 influence of *Wolbachia* on specific chromosomes.

105 *Dose dependent effect of Wolbachia on host recombination rate*

106 Because we observed a strong influence of *Wolbachia* infection status on host recombination
107 rate, we sought to modulate infection status by using high titer *Wolbachia* infections in our
108 experiment. *Wolbachia* colonize *Drosophila* at different titers depending on the amplification of
109 a specific genomic interval in the *Wolbachia* genome termed "octomom" [18]. We crossed
110 females carrying the highest titer, pathogenic *Wolbachia* variant, *w*MelPop,
111 (w[1118]/Dp(1;Y)y[+] |Wolbachia-wMelpop; BDSC stock #65284), with males of stock DGRP-

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112 320. Lines were introgressed for 3 generations within the #DGRP-320 genetic background
113 before use in experiments. We looked specifically at the X chromosome intervals as we had
114 already established that *Wolbachia* significantly increased recombination across that genomic
115 interval (**Fig. 2**). Again, we observed a significant effect of *Wolbachia* infection on
116 recombination rate in this experiment (one-way ANOVA; df = 15, $\chi 2$ = 15.14, p = 0.015) (**Fig. 3**).
117 Interestingly, we observed a significant effect of *Wolbachia* titer on recombination rate – the
118 high titer *w*MelPop variant increases recombination on the X chromosome in F2 progeny by
119 9.5% compared to the 6.3% observed for *w*Mel (df = 11, $\chi 2$ = 12.65, p = 0.044) (**Fig. 3**).

120 *Spiroplasma does not increase host recombination rate*

121 We hypothesized that *Wolbachia* may be a stress on the host cell, increasing recombination
122 rate as a result of increased reactive oxygen species or other immune activation pathways. We
123 therefore reasoned that any bacterial infection may increase recombination rate. To test this
124 hypothesis, we procured *Spiroplasma poulsonii* MSRO (a gift from John Jaenike), which we used
125 to infect a *Wolbachia*-free OreR lab stock (Oregon-R-modENCODE, BDSC #25211). We used the
126 same crossing scheme as above to introduce *Spiroplasma* into the y[1] v[1] background,
127 carrying phenotypic markers on the X chromosome. As a genetic control, we used stock
128 #25211. Counter to our hypothesis, we observed no significant increase in recombination based
129 on *Spiroplasma* infection (**Fig. 4**).

130 **Discussion:**

131 For sexually reproducing organisms, recombination is both a source of genetic diversity within a
132 population and a mechanism by which to decouple differential selection on sites across the
133 chromosome. Therefore, recombination is thought to be beneficial. Here, we observed that
134 *Wolbachia* infection significantly increased the recombination rate observed across two
135 genomic intervals (for both the X and the 2nd chromosome). Importantly, two lines of evidence
136 presented here support the hypotheses that this increase is *Wolbachia* specific: we observed a
137 dose dependency to the recombination rate and we did not identify a significant effect of
138 *Spiroplasma* infection on recombination rate. Recombination rates vary dramatically across
139 animals, even within a genus, as best illustrated within the *Drosophila* clade[19,20]. The
140 mechanism behind this difference is not well understood but our data suggest that the
141 symbiont *Wolbachia* may influence recombination rate of infected *Drosophila*. This result
142 suggests a previously unknown benefit to *Wolbachia* infection and may help explain the
143 prevalence of *Wolbachia* in certain insect populations.

144 The mechanism by which *Wolbachia* infection elevates recombination is an active area of
145 inquiry in our lab. *Wolbachia* have an active type IV secretion system that they use to secrete
146 proteins into the host and modulate host cell biology. It is possible that some of these proteins
147 may influence recombination rate directly or indirectly, although no effectors have yet been
148 identified that bind to host DNA. Here we used two different *Wolbachia* variants to support the
149 hypothesis that *Wolbachia* increases host recombination rate. However, it is possible that

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150 strains outside of the *w*Mel clade do not increase host recombination and a comparative
151 genomic framework could be used to identify loci in *Wolbachia* that confer the phenotype.
152 Finally, a recent publication suggested *Wolbachia w*Mel infected *Drosophila* prefer cooler
153 temperatures [21]. Increases in temperature modulate recombination in *Drosophila* [22] and it is
154 possible that *Wolbachia* infection elevates host temperature enough to generate an increase in
155 the number of detected recombinants, in a laboratory setting where flies are kept at a constant
156 temperature.

157 *Wolbachia* is known for its ability to transfer between species. This process can occur through
158 horizontal transmission of the strain or through introgression via hybridization [23]. One
159 particular strain, *w*Ri, has been identified as having globally spread across highly divergent
160 *Drosophila* species, and in a few cases, instances of introgression between species are known to
161 have facilitated this transfer [23]. *Wolbachia* facilitates its own maintenance in populations
162 through reproductive manipulations [5] and potentially through mutualistic benefits offered to
163 the host [6,11]. *Wolbachia* has been shown to facilitate divergence of hosts, through manipulation
164 of sperm-egg compatibility, strengthening species boundaries [24]. It is therefore tempting to
165 suggest, based on these results, that *Wolbachia* may also increase introgression between
166 species to facilitate their own spread. This hypothesis, however, awaits further research.

167

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171 for discussions at earlier stages of data analysis. This work was funded in part by the National
172 Science Foundation (NSF 1456545 to ILGN).

173

174 **Author contributions:**

175 ILGN and KBS conceived of the project, ILGN, and KBS implemented the experiments, and wrote
176 the manuscript.

177

178 **Data and Reagent Availability**

179 *Drosophila* strains used in this work are publicly available through the Bloomington Drosophila
180 Stock Center. *Spiroplasma* infected OreR is available from the Newton laboratory – please
181 contact irnewton@indiana.edu.

182 **Methods:**

183 *Fly Rearing*

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184 Flies were ordered from the Bloomington Drosophila Stock Center. Three marker stocks were
185 selected as recombination trackers for the X, 2nd, and 3rd chromosomes in *Drosophila*
186 *melanogaster*: #1509, which is marked with *yellow* (*y*) and *vermillion* (*v*) on the X chromosome;
187 #433, which is marked with *vestigial wings* (*vg*) and *brown* (*bw*) on the 2nd chromosome; and
188 #496, which is marked with *ebony* (*e*) and *rough* (*ro*) on the 3rd chromosome. Two stocks, one
189 *Wolbachia* infected and one uninfected, were selected at random from the Drosophila Genetic
190 Reference Panel (DGRP): #29654 and #28134, respectively. To modulate infection status, we
191 introduced high titer *Wolbachia* infections into our stock #29654 using the *Wolbachia* variant
192 *w*MelPop from stock #65284. To clear #29654 of its infection, flies were raised on fly food
193 containing 50 ug/mL tetracycline for 3 generations and the allowed to be recolonized by their
194 extracellular microbiome, and recover from tetracycline, for 1 generation. All crosses were
195 conducted at room temperature.

196 *DNA Extraction and Polymerase Chain Reaction*

197 *Wolbachia* infection in #29654 was confirmed by PCR. DNA was extracted using a single-fly
198 extraction method. Whole flies were ground with a pipette tip containing 50 microliters lysis
199 buffer (10mM Tris-HCl pH 8.2, 1 mM EDTA, and 25 mM NaCl) and 5 microliters Proteinase K.
200 They were incubated at room temperature for 20 minutes then heated to 95°C for 2 minutes to
201 deactivate the enzyme. Polymerase chain reaction was performed on standardized quantities of
202 extracted DNA. The cycling conditions are as follows: 98°C for 2 minutes, followed by 30 cycles
203 of 98°C for 30 seconds, 59°C for 45 seconds, and 72°C for 1 minute 30 seconds, then finished
204 with 72°C for 10 minutes. Primers used for this are as follows: wsp F1 5'-GTC CAA TAR STG ATG
205 ARG AAA C -3' and wsp R1 5'- CYG CAC CAA YAG YRC TRT AAA -3'. Amplified *Wolbachia* DNA
206 was visualized using agarose gel electrophoresis. Quantitative PCR was performed to confirm
207 the titer difference in *Wolbachia* infection between *w*Mel to *w*MelPop. Data were collected
208 using an Applied Biosystems StepOne Real-time PCR system and iTaq universal SYBR Green
209 supermix. The *Wolbachia* primers used are as follows: wspF 5'- CATTGGTGTTGGTGTTGGTG -3'
210 and wspR 5'- ACCGAAATAACGAGCTCCAG -3'. The host primers used are as follows: Rpl32F 5'-
211 CCGCTTCAAGGGACAGTATC -3' and Rpl32R 5'- CAATCTCCTTGCGCTTCTTG -3'. The cycling
212 conditions are as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of
213 95°C for 30 seconds and 59°C for 1 minute. The reaction was carried out in a 96-well plate.
214 Gene expression was determined by the Livak and Pfaffl methods.

215 *Recombination Assay*

216 To determine if recombination events had occurred, a two-step crossing method was devised,
217 shown in Figure 1. Ten virgin DGRP females aged 3-5 days were housed with ten phenotypically
218 marked males and were allowed to mate for 10 days, after which parentals were cleared from
219 the bottle. Virgin female F1 progeny were collected and crossed to the male parental line in the
220 same ratio as before and allowed to mate for 10 days before being cleared from the bottle. All
221 F2 progeny from this cross were collected and frozen after 10 days of the clearing. Flies were
222 scored according to their visible phenotypes and sorted into two groups. Significance between

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223 phenotypically normal flies and flies that have undergone recombination was determined
224 through ANOVA.
225

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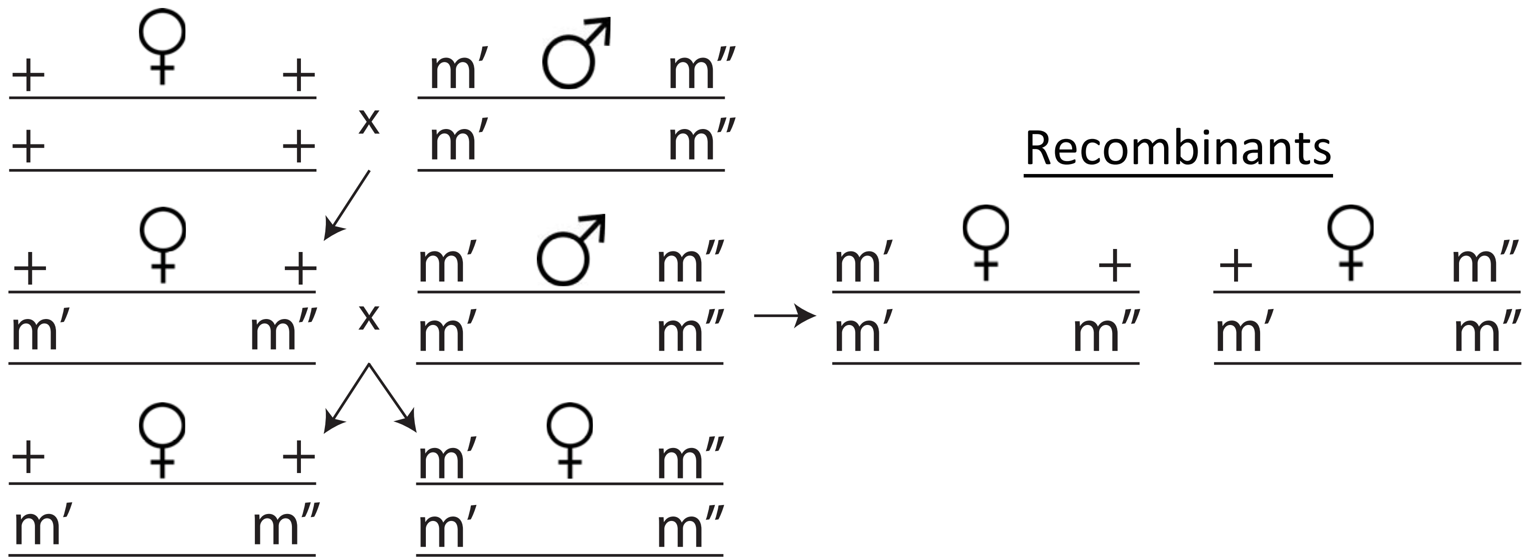

Figure 1. The crossing pattern used to track recombination events. ++ refers to wild type and m' m" refers to genetic markers on each chromosome (*y v* on the X; *vg bw* on the 2nd; *e ro* on the 3rd). Recombination is tracked by looking at the ratio of recombinants to the total number of progeny produced.

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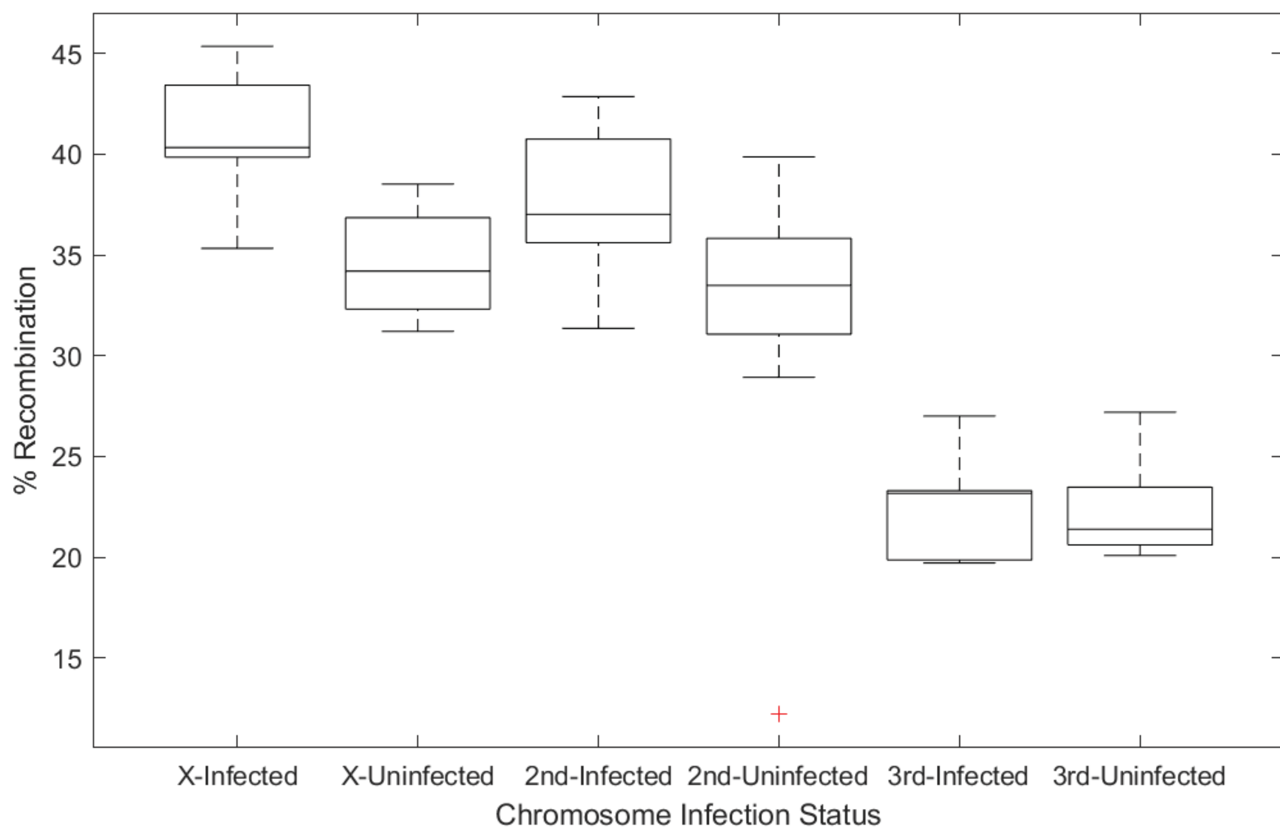

Figure 2. Percentage of recombinants observed, based on genetic markers on the X, 2nd, and 3rd chromosome of *Drosophila*. *Wolbachia* infection significantly increased the recombination rate observed on both the X and 2nd chromosome by 6.4 and 6.1%, respectively. One way ANOVA produced significance for the X (df = 17, $\chi^2 = 16.084$, $p < 6.058e^{-5}$) and the 2nd chromosome (df = 17, $\chi^2 = 7.2357$, $p < 0.007147$), but not on the 3rd chromosome (df = 14, $\chi^2 = 0.080081$, $p = 0.7772$).

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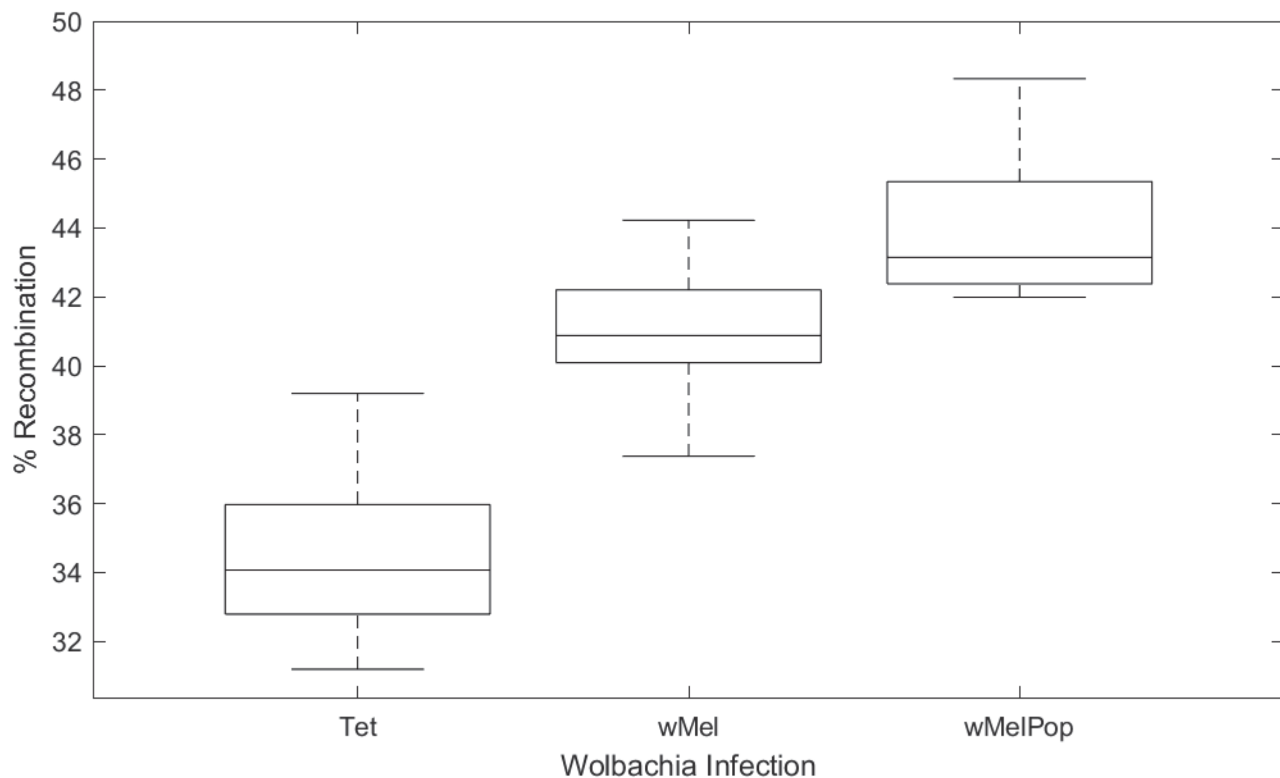

Figure 3. Percentage of recombinants observed, based on genetic markers on the X chromosome of *Drosophila,* when *Wolbachia* titer is varied. A uniform genetic background was used for comparisons across *Wolbachia*-uninfected (Tet) flies, *Wolbachia* infected (*w*Mel) flies, and flies infected with a high-titer variant (*w*MelPop). Increased *Wolbachia* load increased recombination in a load-dependent manner by 6.3% for *w*Mel and 9.5% for *w*MelPop. Significance between titer load was determined by one way ANOVA (df = 15, χ^2 = 15.1495, p = 0.0153). Significance between Tet and *w*Mel (df = 11, χ^2 = 15.536, p < 8.094e^{-5}), *w*Mel and *w*MelPop (df = 11, χ^2 = 12.652, p = 0.0442), and Tet and *w*MelPop (df = 11, χ^2 = 25.994, p < 3.424e^{-7}) was determined by one way ANOVA.

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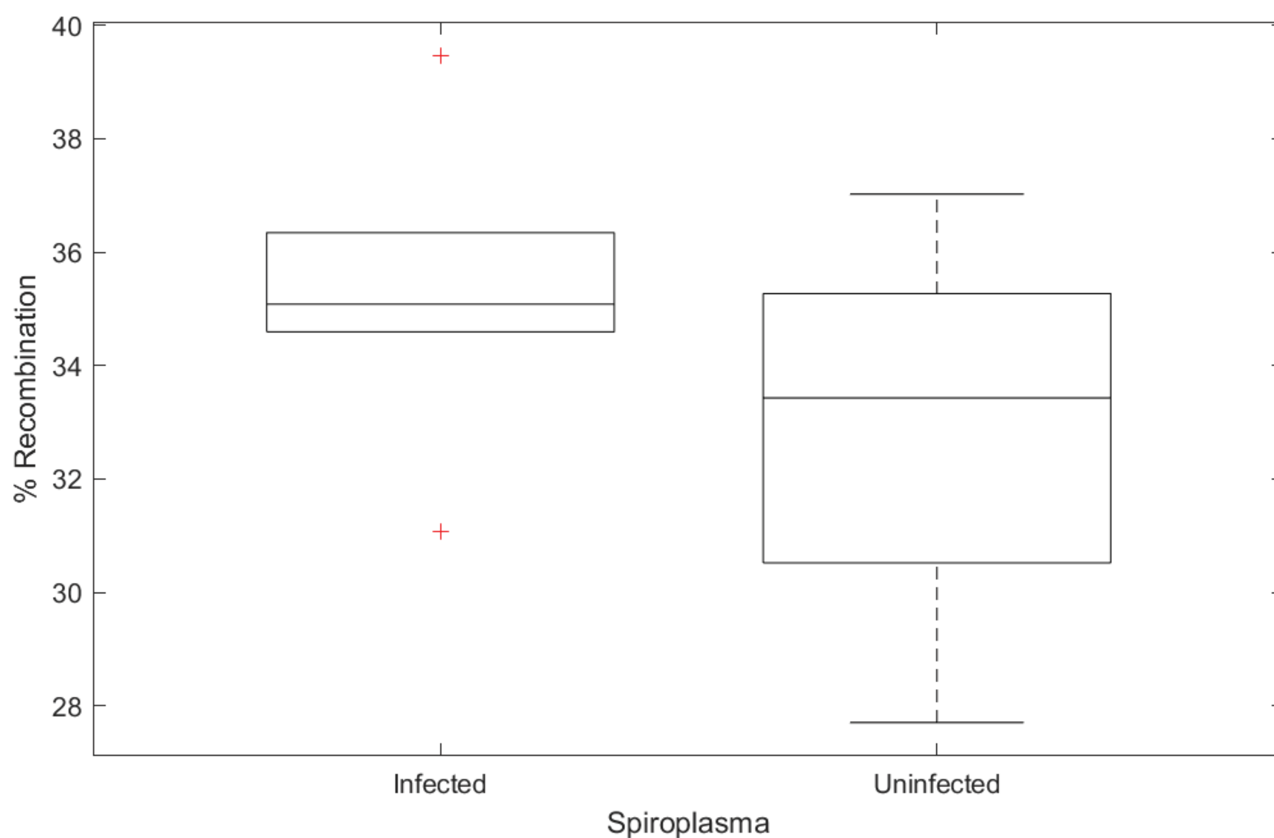

Figure 4. Percentage of recombinants observed based on genetic markers on the X chromosome of *Drosophila*. *Spiroplasma* infection did not significantly increase the observed recombination rate through one way ANOVA (df = 11, $\chi 2$ = 0.03885, p = 0.2153).