Bacterial supergroup specific “Cost” of *Wolbachia* infections in *Nasonia vitripennis*

Alok Tiwary¹, Rahul Babu^{1, 2}, Ruchira Sen³, Rhitoban Raychoudhury^{1*}

1. Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali, Knowledge City, Sector 81, SAS Nagar, Manauli, PO 140306, Punjab, India.

2. Zoological Survey of India, Kolkata, West Bengal-700053, India

3. Guru Gobind Singh College, Sector 26, Chandigarh-160019, India.

*Corresponding author: Rhitoban Raychoudhury, Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali, Sector 81, Knowledge City, SAS Nagar, Manauli, PO 140306 (Punjab), India

Email: rhitoban@iisermohali.ac.in

Key Words: *Wolbachia*, *Nasonia*, life-history traits, Quantitative PCR, Mann-Whitney U, Prospermatogeny, Cytoplasmic incompatibility

Abstract

The maternally-inherited endosymbiont, *Wolbachia*, is known to alter the reproductive biology of its arthropod hosts for its benefit and can induce both positive and negative fitness effects in many hosts. Here we describe the effects of the maintenance of two distinct *Wolbachia* infections, one each from supergroups A and B, on the parasitoid host *Nasonia vitripennis*. We compare the effect of *Wolbachia* infections on various traits between the uninfected, single A infected, single B infected, and the double infected strains with their cured versions. Contrary to the previous reports, our results suggest that there is a significant cost associated with the maintenance of *Wolbachia* infections where traits like family size, fecundity, longevity, and rates of male copulation are compromised in *Wolbachia* infected strains. The double infected and supergroup B infection strains show higher *Wolbachia* titer than supergroup A. The double infected *Wolbachia* strain has the most detrimental impact on the host as compared to single infections. Moreover, there is a supergroup-specific negative impact on these wasps as the supergroup B infections elicit the most pronounced negative effects. These findings raise important questions on the mechanism of survival and maintenance of these reproductive parasites in arthropod hosts.

Introduction

Wolbachia are maternally-inherited, obligatory intracellular endosymbionts of the order Rickettsiales (Hertig et al., 1927) that are widely found in arthropods and filarial nematodes (Bandi et al. 1992; Rousset et al. 1992; Weinert et al., 2015). These bacteria often alter host reproductive biology with mechanisms like male-killing, feminization, parthenogenesis, and cytoplasmic incompatibility (CI), to enhance their own transmission (Werren et al., 2008). While CI leads to an increase in the number of infected individuals in the population, male-killing, and feminization shifts the offspring sex ratio towards females, which is the transmitting sex for *Wolbachia*. Thus, *Wolbachia* increases the fitness of the infected hosts as it increases its own rate of transmission. While these cases highlight a parasitic effect of *Wolbachia*, there are several examples where no such effect is discernible (Hoffmann et al., 1996). Moreover, there are also examples where *Wolbachia* has now become a mutualist and offers specific and quantifiable benefits to its host. One such example of an obligate mutualism with *Wolbachia* has been reported in *Asobara tabida* where females cured of *Wolbachia* are unable to produce mature oocytes (Dedeine et al., 2001). Such examples of arthropod-*Wolbachia* mutualism have now been reported from various arthropod taxa (Pike & Kingcombe, 2009; Miller et al., 2010). This shift from parasitic to mutualistic effect can also happen in facultative associations as seen in *Drosophila simulans*, where within a span of just two decades, *Wolbachia* has evolved from a parasite to a mutualist (Weeks et al., 2007). However, for the majority of *Wolbachia*-host associations, no such reports of mutualistic benefits exist and instead reveal many negative effects on the host. In addition to reproductive traits, many other life-history traits like

longevity and developmental time are also known to be compromised. A review of such negative effects of *Wolbachia* on hosts where CI is prevalent is presented in Table 1. In *Trichogramma kaykai* and *T. deion*, the infected (thelytokous) line shows reduced fecundity and adult emergence rates than the antibiotically cured (arrhenotokous) lines (Hohmann et al., 2001; Tagami et al., 2001). *Leptopilina heterotoma*, a *Drosophila* parasitoid, has adult survival rates, fecundity, and locomotor performance, of both sexes, severely compromised in *Wolbachia* infected strains (Fleury et al., 2000). Preimaginal disc mortality has been observed in both sexes of insecticide-resistant *Wolbachia*-infected strains of *Culex pipiens* (Duron et al., 2006). *Wolbachia* infections can also result in a range of behavioral changes and altered phenotypes in *Aedes aegypti* (Turley et al., 2009).

These negative effects of *Wolbachia* on their hosts are not unexpected. The presence of bacteria within a host entails sharing of nutritional and other physiological resources (Kobayashi & Crouch, 2009; Whittle et al., 2021), especially with *Wolbachia* as they are obligate endosymbionts and cannot survive without cellular resources derived from their hosts (Foster et al., 2005., Slatko et al., 2010). For example, *Wolbachia* is known to compete with the host for key resources like cholesterol and amino acids in *A. aegypti* (Caragata et al., 2014). The precise molecular mechanisms of many of these negative effects have not been ascertained and are generally ascribed to partitioning off of host nutrients for its benefit, but what is clear is that *Wolbachia* infections can impose severe nutritional demands on their hosts (Ponton et al., 2014). However, it is also known that *Wolbachia* can elicit antipathogenic responses from their hosts where the host resistance or tolerance to the infection increases (Zug & Hammerstein, 2015). For example, *Wolbachia* can utilize the Immune deficiency (IMD) and Toll pathways (Pan

et al., 2018) and increased ROS (Reactive Oxygen Species) levels in *Wolbachia*-transfected *A. aegypti* mosquitoes, inhibiting the proliferation of the Dengue virus (Pan et al., 2012) as well as against *Drosophila* C viruses (Teixeira et al., 2008). Such immune responses require additional allocation of resources, which can further affect other physiological traits of the host. This concept of a “cost of immunity” is well-established and suggests a trade-off between immunity and other life-history traits (Zuk & Stoehr, 2002). For example, elevated ROS levels negatively affect many host traits like longevity, and fecundity (Dowling & Simmons, 2009; Monaghan et al., 2009; Selman et al., 2012; Moné et al., 2014). Thus, there is sufficient evidence to conclude that *Wolbachia* can have substantial negative effects on the overall fitness of its host.

In this study, we investigate what, if any, are the negative effects on the wasp *N. vitripennis* due to its two resident CI inducing *Wolbachia* infections? *N. vitripennis*, being cosmopolitan, has been used to study *Wolbachia* distribution, acquisition, spread, and *Wolbachia*-induced reproductive manipulations (Werren et al., 2008; Landmann, 2019). However, the effect of the endosymbiont on the host life-history traits of this wasp remains poorly understood with conflicting reports. In some *N. vitripennis* strains, *Wolbachia* was reported to be the cause of enhanced fecundity (Stolk & Stouthamer, 1996), but a similar effect was not observed in some other strains (Bordenstein & Werren, 2000). *N. vitripennis* harbor two *Wolbachia* supergroup infections, one each from supergroup A and supergroup B (Perrot-Minnot et al., 1996), and the presence of these two infections have been found in all strains of *N. vitripennis* from continental North America to Europe (Raychoudhury et al., 2010), indicating that it has reached fixation across the distribution of its host. The two *Wolbachia* in *N. vitripennis* together cause complete CI, but single infections of supergroup A *Wolbachia* causes incomplete CI while

supergroup B infections still show complete CI (Perrot-Minnot et al., 1996). In this study, we investigate the effects of *Wolbachia* infections in a new strain of *N. vitripennis* recently obtained from the field. This strain, like other *N. vitripennis* strains, has two *Wolbachia* infections, one each from the supergroup A and B. Sequencing of the five alleles from the well-established multi-locus strain typing system (Baldo et al., 2006) reveal no sequence dissimilarity across the two infections (Prazapati, personal communication) indicating this new strain is also infected by the same or very similar *Wolbachia* strains that are present across the distribution of *N. vitripennis* (Raychoudhury et al., 2010b). To compare supergroup specific effects, these two infections have been separated into single infected strains. A comparative analysis between the double infected, supergroup A infected, supergroup B infected and uninfected strains revealed a consistent pattern of decreased longevity, quicker sperm depletion and reduced family size for infected individuals. While supergroup B infection has a more pronounced negative effect on most of the traits investigated, supergroup A infection show such negative effects only for a few of those traits. By testing for differential titer of bacteria by qRT-PCR, we also show a higher density of supergroup B and double infected strains across the different developmental stages of *N. vitripennis* males and females as compared to the supergroup A infected ones.

Materials and Methods

N. vitripennis strains used, their *Wolbachia* infections, and strain nomenclature

The *N. vitripennis* strains used NV PU-14 were obtained from Mohali, Punjab, India, in 2015. NV PU-14 was cured of *Wolbachia* by feeding the females with 1 mg/ml tetracycline in 10 mg/ml

sucrose solution for at least two generations (Breeuwer & Werren, 1990). The curing was confirmed by PCR using supergroup-specific *ftsZ* primers (Baldo et al., 2006), and CI crosses between the infected and uninfected strains. NV PU-14 also served as the source strain for separating the two *Wolbachia* infections into single A and single B infected strains.

To separate the strains, we utilized relaxed selection on the females by repeatedly mating them with uninfected males which were obtained by antibiotic curing of the same NV PU-14 strain. Uninfected males do not have any sperm modification by *Wolbachia* which results in the removal of any selection pressure on the females to maintain their *Wolbachia* infections. Repeated mating with uninfected males was continued for ten generations till some of the progenies were found to be infected with either single A or single B supergroup infections. The single infection status of these strains was confirmed by using supergroup-specific *ftsZ* gene PCR primers (Baldo et al., 2006).

The preferred method of nomenclature of *Nasonia* strains and their *Wolbachia* infections includes information on supergroups as well as the host genotype. For example, [wNvitA wNvitB]V-PU14 indicates that the host species is *N. vitripennis*, with NV-PU14 as the host genotype, which has two *Wolbachia* infections, one each from supergroup A and supergroup B. However, since we used only *N. vitripennis* strains in this study, the nomenclature has been simplified by removing the species name. For example, the same strain will now be denoted as wAwB(PU), and when cured of these infections, as O(PU). The single *Wolbachia*-infected strains used were designated as wA(PU) for the supergroup A-infected strain while wB(PU) for the supergroup B-infected strain. As the cured O(PU) lines were in culture for three years, many of

the infected lines were cured again to obtain ‘recently cured’ lines to minimize the effects of any host divergence that might have accumulated within them. These ‘recently cured’ lines were named O(wA PU), O(wB PU), and O(wAwB PU). All these wasp strains were raised on *Sarcophaga dux* fly pupae with a generation time of 14-15 days at 25°C, 60% humidity, and a continuous daylight cycle.

Sequential mating and sperm depletion of the males

To test the effect of *Wolbachia* on male reproductive traits like mating ability, individual males were assayed for the number of copulations it can perform, as well as sperm depletion. As *N. vitripennis* is haplodiploid, every successful mating will result in both female and male progenies while an unsuccessful one will result in the production of exclusively male progenies. The males used were obtained from virgin females hosted with one fly pupa for 24 hours and were not given any external sources of nutrition (usually a mixture of sucrose in water) before the experiment. Each male was then mated sequentially with virgin females from the same strain. At the first sign of a male not completing the entire mating behavior (Jachmann & Assem, 1996), it was given a rest for half an hour and was subjected to mating again until it stopped mating altogether. The mated females were hosted after a day with one fly pupa for 24 hours. The females were then removed and the offspring were allowed to emerge and then counted. The average number of copulations and the number of copulations before sperm depletion were compared using the Mann-Whitney U test with a significance level of 0.05.

Host longevity, family size, and fecundity

To test whether the presence of *Wolbachia* has any influence on longevity, emerging wasps of both the sexes were kept individually in ria vials at 25°C, without any additional nutrition. Survival following emergence was measured by counting the number of dead individuals every 6 hours. Kaplan-Meier analysis followed by Log Rank Statistics was used to identify differences between strains with a significance level of 0.05.

To test for the effect of *Wolbachia* infections on the adult family size of virgin and mated females, each female was sorted at the pupal stage and separated into individual ria vials. To enumerate the brood size of mated females, some of these virgins were offered single males from the same strain and observed till mating was successful. All the females were then hosted individually with one fly pupa for 24 hours. These were kept at 25°C for the offspring to emerge which were later counted for family size by randomizing the ria vials in a double-blind assay. The differences were compared using the Mann-Whitney U test with a significance level of 0.05.

To investigate if *Wolbachia* affects the female fecundity, emerged females were hosted with one host for 24 hours. The host pupa was placed in a foam plug so that only the head portion of the pupa was exposed and available for the females to lay eggs. They were removed after 24 hours and the eggs laid were counted under a stereomicroscope (Leica M205 C). The differences in fecundity were compared using the Mann-Whitney U test with a significance level of 0.05.

Estimation of relative density of *Wolbachia* infections across different developmental stages of *N. vitripennis*

187 To collect the different developmental stages, females were hosted for 4 hours, (instead of 24
188 hours in the previous experiments), with one host to narrow down the developmental stages of
189 the broods. The larval and pupal stages (from Day 3 to Day 13 for males and from Day 8 to Day
190 14 for females) were collected every 24 hours. Larval stages for females were not done to avoid
191 any DNA contamination from the males as the two sexes are virtually indistinguishable at these
192 stages. Three replicates of ten larvae or pupae from the three strains, wA(PU), wB(PU), and
193 wAwB(PU), were collected for each developmental stage. DNA extraction was done using the
194 phenol-chloroform extraction method, where samples were crushed in 200 μ l of 0.5 M Tris-
195 EDTA buffer with 1% sodium dodecyl sulfate (SDS) and 2 μ l of 22 mg/ml Proteinase K and
196 incubated overnight at 37°C. DNA was purified with buffer saturated phenol and chloroform-
197 isoamyl alcohol solution (24:1) and precipitated overnight with isopropanol at -20° C. The
198 precipitated DNA pellet was dissolved in 60 μ l nuclease-free water. The DNA concentration of
199 the samples was measured using the Nanodrop 2000® spectrophotometer (Thermo Scientific).
200 The concentrations of all the samples were normalized to 200 ng/ μ l across the different male
201 and female developmental stages to be used for quantitative PCR. CFX96 C1000® Touch Real-
202 time qRT-PCR machine (BioRad) was used to assay the relative density of *Wolbachia* across the
203 strains. Amplification was done for the *Wolbachia* hcpA gene (Forward Primer: 5'-
204 CTTCGCTCTGCTATATTGCTGC-3', Reverse Primer: 5'-CGAATAATCGCAACCGAACTG-3'). The
205 primers were tested to amplify both the *Wolbachia* supergroup A and B strains. *Nasonia* S6K
206 was used as the control gene (Bordenstein and Bordenstein, 2011). The total reaction volume
207 contained 5 μ l of iTaq Universal SYBR® Green supermix (BIO-RAD), 0.05 μ l each of 10 μ M of
208 forward and reverse primers, and 200 ng of template DNA for a total volume of 10 μ l for each

reaction. Uninfected *N. vitripennis* DNA was used as negative control while DNase-free water was used as non-template control. Reaction conditions included an initial denaturation step of 95°C for 3 minutes followed by 39 cycles of 95°C for 10 seconds, annealing, and amplification at 52°C for 30 seconds for 40 cycles. All the reactions were performed in triplicates and included a melt curve to check for non-specific amplification. The relative density of *Wolbachia* was estimated by calculating the mean delta threshold cycle (ΔC_q), using the formula:

$$\Delta C_q = 1/3 \sum_{j=1}^3 [1/3 \sum_{i=1}^3 hcpA - 1/3 \sum_{i=1}^3 S6K]$$

where i = number of technical replicates and j = number of biological replicates.

1/ ΔC_q was calculated and plotted to show the *Wolbachia* density across different developmental stages of males and females. The relative *Wolbachia* density was compared across the three different strains using the Kruskal Wallis *H* test. Mann Whitney U test was used to compare two different groups with a significance level of 0.05.

Results

The presence of *Wolbachia* reduces the lifespan of both males and females

Wolbachia can compete with the host for available nutrition which can increase nutritional stress resulting in a shortened lifespan of many hosts (McMeniman et al., 2009; Caragata et al., 2014). Therefore, we first investigated the effect of *Wolbachia* infections on the survival of both male and female wasps. As Fig. 1(A), indicates, there is a significant difference in the longevity of the infected males across the three infection types. The double-infected strain, wAwB(PU), starts to die-off first and has a significantly shorter life span compared to the two single

infected strains {Log Rank Test, $\chi^2=16.8$, $p < 0.001$ for wA(PU) and $\chi^2=33.9$, $p < 0.001$ for wB(PU)}. Males from the uninfected strain, O(PU), lived the longest and showed significantly longer lifespan compared to all the other infected strains {Log Rank Test: $\chi^2=76.3$, $p < 0.001$ for wAwB(PU); $\chi^2=33.0$, $p < 0.001$ for wA(PU) and $\chi^2=16.3$, $p < 0.001$ for wB(PU)}. However, there was no significant difference in the lifespan of the two single infected strains wA(PU) and wB(PU) {Log Rank Test, $\chi^2=3.84$, $p = 0.05$ }. Similarly, infected females (Fig. 1B) also showed a distinct reduction in life span when compared with the uninfected strain. But, unlike the males, the single A infected wA(PU) females, showed the shortest lifespan {Log Rank Test: $\chi^2=11.2$, $p < 0.001$ for wAwB(PU), $\chi^2=56.9$, $p < 0.001$ for wB(PU) and $\chi^2=31.1$, $p < 0.001$, for O(PU)} followed by wAwB(PU) {Log Rank Test: $\chi^2=20.4$, $p < 0.001$ for wB(PU) and $\chi^2=12.9$, $p < 0.001$ for O(PU)}. Curiously, O(PU) and wB(PU) females showed similar lifespans {Log Rank Test: $\chi^2=0.24$, $p = 0.62$ }. These results indicate a sex-specific variation as wAwB(PU) show the shortest lifespan among the males but wA(PU) showed the shortest among the females. Moreover, the effect of single infections on longevity also varied among the sexes as wA(PU) and wB(PU) males had similar lifespans, but in the case of females, it was wB(PU) and O(PU) which had similar lifespans. But what is unambiguous from these results is that the uninfected strain lived the longest, irrespective of the sex of the hosts, indicating that the presence of *Wolbachia* is associated with the reduction in lifespan and is thus, costly for *N. vitripennis* to maintain.

The presence of *Wolbachia* reduces the number of copulations a male can perform

Wolbachia is known to be associated with a reduction in the number of mating a male can perform in *Ephestia kuehniella* (Sumida et al., 2017). To test whether similar effects are seen in

250 *N. vitripennis*, we enumerated the number of copulations an individual male can perform across
 251 the infection types. As Fig. 2, indicates, there is indeed a reduction in the capacity of infected
 252 males to mate. When compared with the uninfected strain O(PU), this reduction was most
 253 pronounced in *wB*(PU) (MWU, $U=30$, $p < 0.01$), followed by *wAwB*(PU) and *wA*(PU) which
 254 showed similar copulation capacities (MWU: $U=11$, $p = 0.49$). The uninfected O(PU) strain
 255 produced males with the highest number of copulations {MWU: $U= 32$, $p < 0.05$ for *wA*(PU) and
 256 $U= 27$, $p < 0.05$ for *wAwB*(PU)}. Thus, the presence of *Wolbachia* substantially reduced the
 257 number of copulations that a male could perform. However, complex phenotypes like
 258 copulation can also be affected by the host genotype. Although all these four strains were
 259 derived from the same field-collected isofemale line, continuous culturing in the laboratory can
 260 fix specific alleles within them resulting in interstrain divergence. Moreover, it is also known
 261 that in *Nasonia* the effect of *Wolbachia*-induced phenotype is influenced by the hosts' genetic
 262 background (Raychoudhury et al., 2012). Therefore, we cured all these infections again and
 263 tested whether the host genotype, rather than *Wolbachia*, is causing this reduction in the
 264 number of copulations by comparing these newly cured strains back with the previously used
 265 uninfected strain, O(PU). As Fig. 2, indicates, males from most of these re-cured strains showed
 266 a marked and significant increase in the number of copulations performed. This number in the
 267 re-cured double infected strain, O(*wAwB* PU), increased to similar levels as shown by O(PU) (an
 268 increase of 29%, MWU: $U=9.5$, $p = 0.2$), while also showing a significant increase from its
 269 infected counterpart *wAwB*(PU) (from 73.5 ± 10.5 to 94.8 ± 15.39 , MWU: $U=3$, $p < 0.05$).
 270 Similarly, the number of copulations for the re-cured single A supergroup infected strain, O(*wA*
 271 PU), also increased to the levels of the uninfected strain O(PU) (an increase of 7%, MWU: $U=20$,

p = 0.76). However, this increase (from 77.5 ± 6.3 to 83.5 ± 12.9) with its infected counterpart was not significant (MWU: U=23, p = 0.48). The re-cured single B supergroup infection, O(wB PU), was the only strain which did not revert to uninfected levels (MWU: U=22, p < 0.05) despite showing an increase (from 62.8 ± 6.6 to 78.2 ± 5.1 ; MWU: U=1, p < 0.05). However, what is evident is that the presence of *Wolbachia* is also associated with a reduction in the capability of a male to mate. Furthermore, by curing the infected strains again, we showed that this decrease is not due to the host genotype but is an effect of *Wolbachia* present in these strains.

***Wolbachia* infected males deplete their sperm reserves faster than the uninfected ones**

N. vitripennis males are prospermatogenic (Boivin et al., 2005), where each male emerges with their full complement of mature sperm and have not been reported to produce any more during the rest of their lifespan (Chirault et al., 2016). Thus, if a single male is mated sequentially with as many females it can mate with, it should eventually run out of this full complement of sperm resulting in an all-male brood even after successful copulation. As Fig. 2, indicates, each male did run out of sperm at the tail end of the continuous mating and produced only male progenies (shown by black dots). We looked at the number of mating done by these males before sperm depletion to see if *Wolbachia* affects the sperm production in the males. As shown in Fig. 3, the average number of daughter progenies reduced with the number of mating (shown by the primary Y-axis on the left), indicating sperm depletion. Similar to copulation numbers, wB(PU) males were also the quickest to deplete their sperm reserve {MWU: U=27.5, p < 0.05 for wA(PU) and U=30, p < 0.01 compared for O(PU)}. This was followed

by *wAwB*(PU) and *wA*(PU) (MWU: $U=13$, $p = 0.7$). However, the uninfected males from *O*(PU) were the slowest to deplete their sperm {MWU: $U= 35$, $p < 0.01$ for *wA*(PU) and $U= 24$, $p < 0.05$ for *wAwB*(PU)}. We again tested whether the host genotype, rather than *Wolbachia*, is causing this rate of sperm depletion, by comparing it with the recently cured strains. As shown in Fig. 3, the number of mating before sperm depletion increased for the recently cured *O*(*wA* PU) up to the levels of *O*(PU) (an increase of 5%, MWU: $U=30$, $p = 0.06$). However, this increase (from 48.14 ± 4.94 to 50.57 ± 9.41) was not significantly different from their infected counterpart *wA*(PU) (MWU: $U=16$, $p = 0.8$). Rates of depletion for *O*(*wAwB* PU) also increased up to the levels of *O*(PU) (an increase of 15.2%, MWU: $U=21$, $p = 0.66$). Again, the cured strain *O*(*wB* PU), increased from *wB*(PU) (from 41 ± 1.67 to 47.6 ± 6.0 , MWU: $U=0$, $p < 0.05$) but was still lower in number than *O*(PU) (MWU: $U=23$, $p < 0.05$). These results indicate that the presence of *Wolbachia* has a significant negative impact on the number of sperm produced or utilized by the infected strains.

***Wolbachia* infected females produce fewer offspring**

Wolbachia is known to have a negative impact on the progeny family size of its host (Hoffmann et al., 1990; Hohmann et al., 2001). To test whether a similar effect is seen in *N. vitripennis*, we enumerated the family sizes for both virgin and mated females for the four different *Wolbachia* infection strains and their recently cured counterparts.

As Fig. 4(A), indicates, there is a significant reduction in the average family sizes of all-male broods produced by the virgin females of the *Wolbachia* infected strains. When compared with the uninfected strain *O*(PU), this reduction was most pronounced in *wAwB*(PU) (MWU:

U=21151.5, $p < 0.01$) followed by *wB*(PU) (MWU: U=19880.5, $p < 0.05$). *wB*(PU) and *wAwB*(PU) showed similar family size (MWU: U=18582.5, $p = 0.29$). However, *wA*(PU) showed similar family size when compared with *O*(PU) (MWU: U=17191, $p = 0.39$) and *wB*(PU) (MWU: U=17284, $p = 0.26$) but had larger all-male broods sizes than *wAwB*(PU) (MWU: U=18252, $p < 0.05$). We also compared the recently cured single and double infected strains with the infected parental strains. The recently cured strains *O*(*wB* PU) and *O*(*wAwB* PU) showed marginal increase in the family size which was comparable to the uninfected strain *O*(PU) {an increase of 1.5%, MWU: U=11554, $p = 0.29$ for *O*(*wB* PU); an increase of 2%, MWU: U=10798, $p = 0.21$ for *O*(*wAwB* PU)}. However this marginal increase {from 29.84 ± 12.94 to 31.44 ± 10.30 for *O*(*wB* PU) and from 28.99 ± 11.60 to 30.97 ± 11.48 for *O*(*wAwB* PU)} was not significantly different from their infected counterparts *wB*(PU) (MWU: U=9963.5, $p = 0.34$) and *wAwB*(PU) (MWU: U=8650.5, $p = 0.09$). The recently cured strain *O*(*wA* PU) did not show any increase in the family size and was comparable to *wA*(PU) (MWU: U=7085.3, $p = 0.63$) and *O*(PU) (MWU: U=8161, $p = 0.22$).

Similar to the virgin females, the reduction was also observed for average family sizes of mated females as shown in Fig. 4(B). When compared with the uninfected strain *O*(PU), this reduction is most pronounced in *wB*(PU) (MWU: 15582, $p < 0.01$) and *wAwB*(PU) (MWU: U=16303, $p < 0.01$). However, *wB*(PU) and *wAwB*(PU) show similar levels of family sizes (MWU: 13732.5, $p = 0.55$). Interestingly, *wA*(PU) strain shows similar levels of family size as *O*(PU) (MWU: U=11396.5, $p = 0.86$) but had larger family sizes than *wB*(PU) (MWU: U=14080, $p < 0.01$) and *wAwB*(PU) (MWU: U=14682, $p < 0.05$). Upon curing, the average family sizes of the recently cured *O*(*wAwB* PU) reverted back to the levels of the uninfected strain *O*(PU) (an increase of

11.8%, MWU: $U = 13295$, $p = 0.61$) showing a significant increase from the infected counterpart $wAwB(PU)$ (from 29.49 ± 10.67 , MWU: $U = 12023$, $p < 0.05$). The recently cured strain $O(wA\ PU)$ did not show any significant increase from the infected counterpart $wA(PU)$ (MWU: $U = 8385.5$, $p = 0.69$) and was comparable to $O(PU)$ (MWU: $U = 9022.5$, $p = 0.5$). However, the recently cured strain $O(wB\ PU)$ did not increase to the levels of the uninfected strain $O(PU)$ (an increase of 4.3%, MWU: $U = 16782$, $p < 0.05$). The marginal increase in the family size (from 28.72 ± 10.46 to 29.97 ± 8.59) was not significantly different from the parent strain $wB(PU)$ (MWU: $U = 13854$, $p = 0.47$).

To understand whether this difference in the family size of the mated females is due to the production of fewer daughters or sons or both, we compared their numbers separately for the four strains (Fig. 4C). No difference was observed in the number of sons produced by the mated females. However, significant differences were observed in the number of daughters produced. When compared to the uninfected strain $O(PU)$, $wB(PU)$ and $wAwB(PU)$ showed the least number of daughters produced {MWU: 15964, $p < 0.01$ for $wB(PU)$ and $U = 16283$, $p < 0.01$ for $wAwB(PU)$ } whereas $wB(PU)$ and $wAwB(PU)$ produced nearly equal number of daughters (MWU: 13392, $p = 0.33$). Again, $wA(PU)$ strain produced equal number of daughters compared to $O(PU)$ (MWU: $U = 11543$, $p = 0.98$) but higher in number than $wB(PU)$ and $wAwB(PU)$ {MWU: $U = 14201$, $p < 0.01$ for $wB(PU)$ and MWU: $U = 14372$, $p < 0.05$ for $wAwB(PU)$ }. Upon curing, the recently cured $O(wAwB\ PU)$ reverted to the levels of the uninfected strain $O(PU)$ (MWU: $U = 13545$, $p = 0.42$) showing a significant increase in the number of daughters from the infected counterpart $wAwB(PU)$ (MWU: $U = 12331$, $p < 0.039$). The recently cured strain $O(wA\ PU)$ did not show any increase in the number of daughters produced from their infected counterpart

wA(PU) (MWU: $U=8468$ $p = 0.79$) and was also comparable to *O*(PU) (MWU: $U=9330$, $p = 0.84$). However, recently cured strain *O*(*wB* PU) did not increase to the levels of the uninfected strain *O*(PU) (MWU: $U=16749.5$, $p < 0.01$).

***Wolbachia* negatively impacts the fecundity of infected females**

To check whether the differences in the family sizes between the different infection strains are due to the number of eggs being laid by the females, we looked at the fecundity of both virgin and mated females across the strains. Among the virgin females (Fig. 5A), *wAwB*(PU) had the least fecundity (MWU: $U=5042$, $p < 0.01$) when compared to the uninfected strain *O*(PU). However, there was no significant difference between *O*(PU) and *wA*(PU) (MWU: $U=2632.5$, $p = 0.34$) and also between *O*(PU) and *wB*(PU) (MWU: $U=2052.5$, $p = 0.71$).

Among the mated females (Fig. 5B), *wAwB*(PU) again had the least fecundity (MWU: $U=2461.5$, $p < 0.01$) when compared to the uninfected strain *O*(PU). This was followed by *wB*(PU) having similar fecundity as that of *wAwB*(PU) (MWU: $U=1479.5$, $p = 0.24$) and *wA*(PU) (MWU: $U=2822.5$, $p = 0.22$). However, *wA*(PU) showed higher fecundity than *wAwB*(PU) (MWU: $U=2388$, $p < 0.05$) and was similar to the uninfected strain *O*(PU) (MWU: $U=3799.5$, $p = 0.75$).

The double infected strain showed the least fecundity, both for the virgin and mated females, suggesting an effect of *Wolbachia* on egg production in females. The assay also established that the difference in family sizes is due to the differences in the fecundity of the females.

Relative *Wolbachia* density in single and multiple *Wolbachia* infection strains

Wolbachia density has a major role to play in expressing the effects of the infection on host biology (Hoffmann et al., 1996; Min & Benzer, 1997). An increase in cellular *Wolbachia* density is often associated with a greater expression of their effects (Breeuwer & Werren, 1993). Thus, we estimated *Wolbachia* titers across different developmental stages of *N. vitripennis* males and females. In the case of males (Fig. 6A) *wA*(PU) had the lowest *Wolbachia* density across different larval and pupal developmental stages when compared with *wB*(PU) (MWU: $U=11$, $p < 0.01$) and *wAwB*(PU) (MWU: $U=12$, $p < 0.01$) This difference was more prominent at the adult stage. However, no such differences were found between *wB*(PU) and *wAwB*(PU) (MWU: $U=51$, $p = 0.56$).

In the case of females (Fig. 6B), *wA*(PU) showed lower levels of *Wolbachia* when compared to *wAwB*(PU) (MWU: $U=8$, $p < 0.05$) again at the late pupal and adult stage. However, no difference was observed between *wA*(PU) and *wB*(PU) (MWU: $U=12$, $p = 0.12$) and also *wB*(PU) and *wAwB*(PU) (MWU: $U=19$, $p = 0.5$).

Discussion

CI-inducing *Wolbachia* are known to have negative effects on various physiological traits in the vast majority of its host population (summarized in Table 1). The present study also suggests such effects, or a “cost”, associated with the maintenance of *Wolbachia* infection in *N. vitripennis*. This is in contrast to the previous reports suggesting positive fitness effects (Stolk C & Stouthamer R, 1996) and no fitness effects (Bordenstein & Werren, 2000) of *Wolbachia* on *N. vitripennis*. However, several unique conclusions emerge from the study.

Our results (summarized in Table 2) demonstrate a sex-independent cost of the presence of single and multiple *Wolbachia* infections. Many phenotypes show reduction across the sexes such as longevity (Fig. 1) where infected males and females showed reduced lifespan. When compared with the uninfected strain O(PU), the *Wolbachia* infected strains wB(PU) and wAwB(PU) have reduced life spans. However, sex-specific variations were also observed among the infected strains where wA(PU), in females, had the shortest life span, while in the case of males, had a greater life span than wAwB(PU).

Wolbachia affected the reproductive capabilities of the infected males and reduced their copulation capability (Fig. 2) and also led to quicker sperm depletion (Fig. 3). Such negative effects on reproductive traits were also observed in females where the infected females produced fewer progenies (Fig. 4) compared to O(PU). These differences were elicited at the level of female fecundity where the number of eggs being laid by the infected females was fewer (Fig. 5) indicating that the negative effects of *Wolbachia* manifested themselves even before the egg-laying stage. However, the egg to larval to pupal stage mortality could also have an effect on the brood sizes but these were not assayed.

In most cases, these negative effects disappeared with the removal of *Wolbachia*, indicating the role of *Wolbachia* in producing these negative effects and not the host genotype. In phenotypes like family sizes, the recently cured strains showed a significant increase suggesting that the negative effects are due to the presence of *Wolbachia*. However, O(wB PU) did not revert to the levels of O(PU) in the number of copulations performed and sperm depletion assays. A possible reason can be some residual effects of the parent genotype in O(wB PU) males.

Our results also demonstrate supergroup-specific negative effects on the host. Supergroup B *Wolbachia* is costlier to maintain in both the sexes than supergroup A. While *wB*(PU) shows strong effects on all the traits studied across the sexes, *wA*(PU) had significant negative effects only on the reproductive traits of the males and the longevity of females. *wA*(PU) females, as an exception, had their family sizes comparable to *O*(PU). These observations are unique as no comprehensive data is available on the supergroup-specific cost of *Wolbachia* infections.

The higher cost of maintenance of supergroup B *Wolbachia* can be an attribute of the CI phenotype induced by supergroup B *Wolbachia*. Complete CI (i.e, nearly 100% CI) are rare events reported mainly for supergroup B *Wolbachia* in *Culex pipiens*, *Aedes aegypti* (Sinkins et al., 2005; Xi et al., 2005), and in *N. vitripennis* as well (supplementary Fig. 1 and Bordenstein et al., 2006a). This essentially means that nearly the entire sperm complement of each male has the *Wolbachia*-induced CI modification and correspondingly, nearly all the eggs from the females have the rescue effect (Werren et al., 2008). Introducing 100% modification and rescue would necessitate relatively high *Wolbachia* titers to be maintained in both sexes which in turn can cause an elevated nutritional burden, eventually resulting in negative effects on the physiological traits of the host.

Previous reports have suggested a direct correlation between *Wolbachia* density and the level of CI (Breeuwer & Werren, 1993; Noda et al., 2001; Ikeda et al., 2003; Dutton & Sinkins, 2004; Ruang-Areerate & Kittayapong, 2006). Our results also suggest that the cost of *Wolbachia* maintenance is correlated with the density of *Wolbachia* strains present in the host. *Wolbachia* density in both the sexes differs in the late pupal stages (Day 11, Day 12) and adult stages. Thus,

in the case of females, *wAwB*(PU), which shows a high bacterial load, has reduced fecundity and longevity. Similarly, in the case of males, the *wAwB*(PU) strain shows a reduced number of copulations and the number of sperm produced/transferred. *wB*(PU), therefore causes substantial detrimental effects on both the sexes of the host which is similar to the levels found in *wAwB*(PU). This again can be explained by the high *Wolbachia* load in both sexes. In contrast, *wA*(PU) showed minimal effect on the female hosts, with the longevity of females being an exception. A possible explanation for this can be the relatively low density of *wA*(PU) across the different developmental stages (Fig. 6) as compared to the other infections. Moreover, supergroup A infected strains are known to have relatively higher levels of phage density (Bordenstein et al., 2006). According to this phage density model, higher phage density has an impact on the level of CI caused by supergroup A strains, where it leads to a significant reduction in *Wolbachia* titer and hence has a reduction effect on CI phenotype. Our results also conform with the previous reports of the positive correlation between *Wolbachia* abundance and the level of CI induced not only in *N. vitripennis* (Bordenstein et al., 2006b) but in other insect taxa as well (Ijichi et al., 2002; Kondo et al., 2002). *wB*(PU) *Wolbachia* showed complete CI and had higher *Wolbachia* titers in both males and females, which was also comparable to that of the *wAwB*(PU), whereas *wA*(PU) had the lowest *Wolbachia* titers among the three strains and showed incomplete CI (supplementary Fig. 1). Thus, higher levels of *wB*(PU) than *wA*(PU) also explain the more severe effects of *wB*(PU) than *wA*(PU).

The low levels of *wA*(PU) and the associated negative effects on host physiology raise questions on the maintenance of *wA*(PU) infection in *N. vitripennis*. Since supergroup B appears to be a “stronger” *Wolbachia* than supergroup A, any competition for nutritional resources and niche

habitation between them should drive out supergroup A *Wolbachia* from the hosts. But *wA*(PU) is observed to have less severe effects on the females with longevity being the only factor to have any negative effects. Supergroup A *Wolbachia* in *N. vitripennis* is closely related to other A supergroup *Wolbachia* like *wMel* in *D. melanogaster* and *wHa*, *wAu*, and *wRi* in *D. simulans* (Díaz-Nieto et al., 2021). The presence of supergroup A *Wolbachia* in *N. vitripennis* could be due to such defenses against viral infections (Teixeira et al., 2008; Bhattacharya et al., 2017; Pimentel et al., 2021) but remain untested.

The varied effects of *Wolbachia* can be dependent on host strain as well as on a particular *Wolbachia* supergroup. Our data suggest that supergroup B *Wolbachia* behaves more like a parasite in both the sexes while supergroup A *Wolbachia* had detrimental effects mostly in the case of males. This is contrary to what is expected as the two *Wolbachia* supergroups being maternally transmitted, should affect the males more in comparison to the females as males are a dead-end for *Wolbachia* transmission.

Our experiments indicate an additive or synergistic effect of the presence of the two different *Wolbachia* supergroups in the double infected strain *wAwB*(PU). Evidence of such effects can be seen in traits like male longevity (additive effect) where the deficit in longevity for *wAwB*(PU) is equal to the total deficits caused by *wA*(PU) and *wB*(PU). Similarly, for traits like female longevity, virgin female family size, and female fecundity, the negative effects on strain *wAwB*(PU) appear to be a combined effect of *wA*(PU) and *wB*(PU) (synergistic effect). Since the two supergroup infections are bidirectionally incompatible with each other, it is plausible that

they are also competing for the host nutrition, which can further enhance the negative impacts of these infections.

The negative fitness effects of CI-inducing *Wolbachia*, and nutritional competition raises important questions on the maintenance of these endosymbionts over long evolutionary time scales. Theoretical studies predict that for the long-term persistence of these maternally inherited reproductive parasites, they can eventually evolve towards mutualism (Prout, 1994; Turelli, 1994). Various theories on endosymbiosis also support the vertical transmission of endosymbionts, as the spread of the symbiont depends on the reproductive success of the host itself (Anderson & May 1982; Ewald, 1987; Bull et al., 1991; Lipsitch et al., 1995). Hence, mutualism can be a preferred way for endosymbionts to ensure their persistence. Moreover, if there are indeed some adverse effects of maintaining *Wolbachia*, then hosts would be under strong selection pressure to develop immunity against them. Evidence suggests that there are examples of such emergence of host genetic factors upon *Wolbachia* infection in hosts *Drosophila* and mosquitoes (Zug & Hammerstein, 2015). Host suppressor alleles have been identified conferring resistance against feminizing (Rigaud et al., 1999) and male-killing *Wolbachia* (Hornett et al., 2006). However, no such host genetic factors have been found for CI-inducing *Wolbachia*, especially in *N. vitripennis*. A possible explanation for the maintenance of these multiple infections comes from the high efficiency of transmission of these infections in *N. vitripennis*, where these infections also show nearly 100% transmission as well as CI (Breeuwer and Werren, 1990). For many *Wolbachia*-arthropod associations, the pathogenicity and transmission efficiency is mediated by bacterial titers (Correa & Ballard, 2016). Our results also show higher *Wolbachia* density for double infected strain, which can facilitate its

transmission and maintenance in the host. Another possibility can be that these *Wolbachia* infections in *N. vitripennis* are relatively recent, the evidence of which comes from the rapid spread of *Wolbachia* in populations of *N. vitripennis* across North America and Europe (Raychoudhury et al., 2010b). These recent infections, although bearing a cost on the host at present, might eventually lead to the host immunity against the infection.

Acknowledgments

We thank the Indian Institute of Science Education and Research (IISER) Mohali for the funding and graduate fellowship for AT. Partial funding was obtained from Grant No. BT/PR14278/BRB/10/1417/2015, Department of Biotechnology, Government of India, awarded to Nagaraj Guru Prasad (Associate Professor, Biological Sciences, IISER Mohali) and RR.

Competing Interests

The authors declare no conflict of interest.

Author Contribution

AT conceived the idea and planned the study. AT performed the experiments. AT collected the data. RB performed the pilot experiments for female fecundity. AT analyzed the data. AT, RS, and RR were involved in data representation and statistical analysis. AT and RR wrote the manuscript.

Data Archiving

521 The raw data for all the experiments have been archived at Dryad with DOI:
522 10.5061/dryad.w0vt4b8s9

523 URL: https://datadryad.org/stash/share/gKvBkEcYjmrNULX4F53Ktu5Pt-P1pcXBnbA5lb_HEPo
524

525 **References**

526 Anderson RM, May RM (1982). Coevolution of hosts and parasites, *Parasitology*, 85, 411-42
527 <https://doi.org/10.1017/S0031182000055360>

528 Bandi C, Anderson TJC, Genchi C, Blaxter ML (1998). Phylogeny of *Wolbachia* in filarial
529 nematodes. *Proc. R. Soc. Lond. B*, 265, 2407-2413
530 <https://dx.doi.org/10.1098%2Frspb.1998.0591>

531 Bhattacharya T, Newton ILG, Hardy RW (2017). *Wolbachia* elevates host methyltransferase
532 expression to block an RNA virus early during infection. *PLoS Pathog*, 13
533 <https://doi.org/10.1371/journal.ppat.1006427>

534 Boivin G, Jacob S, Damiens D (2005). Spermatogeny as a life-history index in parasitoid wasps.
535 *Oecologia*, 143, 198–202.
536 <https://www.jstor.org/stable/20062237>

537 Bordenstein SR, Bordenstein SR (2011). Temperature affects the tripartite interactions between
538 bacteriophage WO, *Wolbachia*, and cytoplasmic incompatibility. *PLoS One*, 6
539 <https://doi.org/10.1371/journal.pone.0029106>

540 Bordenstein SR, Marshall ML, Fry AJ, Kim U, Wernegreen JJ (2006a). The tripartite associations
541 between bacteriophage, *Wolbachia*, and arthropods. *PLoS Pathogen*, 2, 384–393.

542 <https://doi.org/10.1371/journal.ppat.0020043>

543 Bordenstein SR, Marshall ML, Fry AJ, Kim U, Wernegreen JJ (2006b). The tripartite associations
544 between bacteriophage, *Wolbachia*, and arthropods. *PLoS Pathogen*, 2, 384–393.

545 <https://doi.org/10.1371/journal.ppat.0020043>

546 Bordenstein SR, Werren JH (2000). Do *Wolbachia* influence fecundity in *Nasonia vitripennis*?
547 *Heredity (Edinb)*, 84, 54–62.

548 <https://doi.org/10.1046/j.1365-2540.2000.00637.x>

549 Breeuwer JAJ, Werren JH (1990). Microorganisms associated with chromosome destruction and
550 reproductive isolation between two insect species. *Nature*, 346, 558–560.

551 <https://doi.org/10.1038/346558a0>

552 Breeuwer JAJ, Werren JH (1993). Cytoplasmic Incompatibility and Bacterial Density in *Nasonia*
553 *vitripennis*. *Genetics*, 135(2), 565–574.

554 <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1205656/>

555 Bull JJ, Molineux IJ, Rice WR (1991). Selection of benevolence in a host-parasite system.
556 *Evolution (N Y)*, 45, 875–882.

557 <https://doi.org/10.1111/j.1558-5646.1991.tb04356.x>

558 Caragata EP, Rancès E, O’Neill SL, McGraw EA (2014). Competition for Amino Acids Between
559 *Wolbachia* and the Mosquito Host, *Aedes aegypti*. *Microb Ecol*, 67, 205–218.

560 <https://doi.org/10.1007/s00248-013-0339-4>

561 Caspari E, Watson GS (1959). On the Evolutionary Importance of Cytoplasmic Sterility in
562 Mosquitoes, *JSTOR*, 13, 568–570.

563 <https://doi.org/10.2307/2405922>

Chirault M, van de Zande L, Hidalgo K, Chevrier C, Bressac C, Lécureuil C (2016). The spatio-temporal partitioning of sperm by males of the prospermatogenic parasitoid *Nasonia vitripennis* is in line with its gregarious lifestyle. *J Insect Physiol*, 91–92, 10–17.
<https://doi.org/10.1016/j.jinsphys.2016.06.002>

Correa CC, Ballard JWO (2016). *Wolbachia* Associations with Insects: Winning or Losing Against a Master Manipulator. *Front Ecol Evol*, 3
<https://doi.org/10.3389/fevo.2015.00153>

D JF and AJ V (1996). A Causal Ethological Analysis of the Courtship Behaviour of an Insect (The Parasitic Wasp *Nasonia vitripennis* , Hym ., Pteromalidae). *Behaviour*, 133, 1051–1075.
<https://www.jstor.org/stable/4535410>

Dedene F, Vavre F, Fleury F, Loppin B, Hochberg ME, Boulétreau M (2001). Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc Natl Acad Sci U S A*, 98, 6247–6252.
<https://doi.org/10.1073/pnas.101304298>

Díaz-Nieto LM, Gil MF, Lazarte JN, Perotti MA, Berón CM (2021). *Culex quinquefasciatus* carrying *Wolbachia* is less susceptible to entomopathogenic bacteria. *Sci Rep*, 11, 1–9.
<https://doi.org/10.1038/s41598-020-80034-5>

Dobson SL, Rattanadechakul W, Marsland EJ (2004). Fitness advantage and cytoplasmic incompatibility in *Wolbachia* single- and superinfected *Aedes albopictus*. *Heredity (Edinb)*, 93, 135–142.
<https://doi.org/10.1038/sj.hdy.6800458>

Dowling DK, Simmons LW (2009). Reactive oxygen species as universal constraints in life-history

evolution. *Proc R Soc B Biol Sci*, 276, 1737–1745.

<https://doi.org/10.1098/rspb.2008.1791>

Duron O, Labbé P, Berticat C, Rousset F, Guillot S, Raymond M, *et al.* (2006). High *Wolbachia*

Density Correlates With Cost of Infection for Insecticide Resistant *Culex Pipiens*

Mosquitoes. *Evolution (N Y)*, 60, 303.

<https://pubmed.ncbi.nlm.nih.gov/16610322/>

Dutton TJ, Sinkins SP (2004). Strain-specific quantification of *Wolbachia* density in *Aedes*

albopictus and effects of larval rearing conditions. *Insect Mol Biol*, 13, 317–322.

<https://doi.org/10.1111/j.0962-1075.2004.00490.x>

Ewald PW (1987). Transmission Modes and Evolution of the Parasitism-Mutualism Continuum.

Ann N Y Acad Sci, 503, 295–306.

<https://doi.org/10.1111/j.1749-6632.1987.tb40616.x>

Fleury F, Vavre F, Ris N, Fouillet P, Boulétreau M (2000). Physiological cost induced by the

maternally-transmitted endosymbiont *Wolbachia* in the *Drosophila* parasitoid *Leptopilina*

heterotoma. *Parasitology*, 121, 493–500.

<https://doi.org/10.1017/s0031182099006599>

Foster J, Ganatra M, Kamal I, Ware J, Makarova K, Ivanova N, *et al.* (2005). The *Wolbachia*

genome of *Brugia malayi*: Endosymbiont evolution within a human pathogenic nematode

(NA Moran, Ed.). *PLoS Biol*, 3, 0599–0614.

<https://doi.org/10.1371/journal.pbio.0030121>

Fry AJ, Palmer MR, Rand DM (2004). Variable fitness effects of *Wolbachia* infection in

Drosophila melanogaster. *Heredity (Edinb)*, 93, 379–389.

608 <https://doi.org/10.1038/sj.hdy.6800514>

609 Hertig M and Wolbach S B (1924). Studies on Rickettsia-like micro-organisms in insects. *JMed*

610 *Res*, 329–374.7.

611 <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2041761/>

612 Hoffmann AA, Clancy D, Duncan J (1996). Naturally-occurring *Wolbachia* infection in *Drosophila*

613 *simulans* that does not cause cytoplasmic incompatibility. *Heredity (Edinb)*, 76, 1–8.

614 <https://doi.org/10.1038/hdy.1996.1>

615 Hohmann CL, Luck RF, Stouthamer R (2001). Effect of *Wolbachia* on the Survival and

616 Reproduction of *Trichogramma kaykai* Pinto & Stouthamer (*Hymenoptera*:

617 *Trichogrammatidae*). *Neotropical Entomology*, 30(4), 607-612

618 <https://doi.org/10.1590/S1519-566X2001000400015>

619 Hornett EA, Charlat S, Duplouy AMR, Davies N, Roderick GK, Wedell N, *et al.* (2006). Evolution

620 of male-killer suppression in a natural population. *PLoS Biol*, 4, 1643–1648.

621 <https://doi.org/10.1371/journal.pbio.0040283>

622 Ijichi N, Kondo N, Matsumoto R, Shimada M, Ishikawa H, Fukatsu T (2002). Internal

623 spatiotemporal population dynamics of infection with three *Wolbachia* strains in the

624 adzuki bean beetle, *Callosobruchus chinensis* (Coleoptera: Bruchidae). *Appl Environ*

625 *Microbiol*, 68, 4074–4080.

626 <https://dx.doi.org/10.1128%2FAEM.68.8.4074-4080.2002>

627 Ikeda T, Ishikawa H, Sasaki T (2003). Infection density of *Wolbachia* and level of cytoplasmic

628 incompatibility in the Mediterranean flour moth, *Ephestia kuehniella*. *J Invertebr Pathol*,

629 84, 1–5.

630 [https://doi.org/10.1016/s0022-2011\(03\)00106-x](https://doi.org/10.1016/s0022-2011(03)00106-x)

631 Kobayashi DY, Crouch JA (2009). Bacterial/Fungal interactions: from pathogenes to mutualistic
632 endosymbionts. *Annu Rev Phytopathol*, 47, 63–82.

633 <https://doi.org/10.1146/annurev-phyto-080508-081729>

634 Kondo N, Ijichi N, Shimada M, Fukatsu T (2002). Prevailing triple infection with *Wolbachia* in
635 *Callosobruchus chinensis* (Coleoptera: Bruchidae). *Mol Ecol*, 11, 167–180.

636 <https://doi.org/10.1046/j.0962-1083.2001.01432.x>

637 Landmann F (2019). The *Wolbachia* Endosymbionts. *Microb Spectrum*, Am Soc Microbiol, 7,
638 139–153.

639 <https://doi.org/10.1128/microbiolspec.bai-0018-2019>

640 Lipsitch M, Nowak MA, Ebert D, May RM (1995). The population dynamics of vertically and
641 horizontally transmitted parasites, *Proc. R. Soc. Lond. B*, 260, 321-327

642 <https://doi.org/10.1098/rspb.1995.0099>

643 McMeniman CJ, Lane R V., Cass BN, Fong AWC, Sidhu M, Wang YF, *et al.* (2009). Stable
644 introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*.
645 *Science*, 323, 141–144.

646 <https://doi.org/10.1126/science.1165326>

647 Miller WJ, Ehrman L, Schneider D (2010). Infectious speciation revisited: Impact of symbiont-
648 depletion on female fitness and mating behavior of *Drosophila paulistorum*. *PLoS Pathog*
649 6.

650 <https://doi.org/10.1371/journal.ppat.1001214>

651 Min KT, Benzer S (1997). *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing

degeneration and early death. *Proc Natl Acad Sci U S A*, 94, 10792–10796.

<https://doi.org/10.1073/pnas.94.20.10792>

Monaghan P, Metcalfe NB, Torres R (2009). Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. *Ecol Lett*, 12, 75–92.

<https://doi.org/10.1111/j.1461-0248.2008.01258.x>

Moné Y, Monnin D, Kremer N (2014). The oxidative environment: A mediator of interspecies communication that drives symbiosis evolution. *Proc R Soc B Biol Sci*, 281.

<https://dx.doi.org/10.1098/rspb.2013.3112>

Noda H, Koizumi Y, Zhang Q, Deng K (2001). Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*. *Insect Biochem Mol Biol*, 31, 727–737.

[https://doi.org/10.1016/s0965-1748\(00\)00180-6](https://doi.org/10.1016/s0965-1748(00)00180-6)

Olsen K, Reynolds KT, Hoffmann AA (2001). A field cage test of the effects of the endosymbiont *Wolbachia* on *Drosophila melanogaster*. *Heredity (Edinb)*, 86, 731–737.

<https://doi.org/10.1046/j.1365-2540.2001.00892.x>

Pan X, Pike A, Joshi D, Bian G, McFadden MJ, Lu P, *et al.* (2018). The bacterium *Wolbachia* exploits host innate immunity to establish a symbiotic relationship with the dengue vector mosquito *Aedes aegypti*. *ISME J*, 12, 277–288.

<https://doi.org/10.1038/ismej.2017.174>

Pan X, Zhou G, Wu J, Bian G, Lu P, Raikhel AS, *et al.* (2012). *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*. *Proc Natl Acad Sci U S A*, 109.

674 <https://doi.org/10.1073/pnas.1116932108>

675 Perrot-Minnot M-JJ, Guo LR, Werren JH (1996). Double Infections with *Wolbachia* in the

676 Parasitic Wasp *Nasonia vitripennis*: Effects on Compatibility. *Genetics*, 143, 961–972.

677 <https://doi.org/10.1093/genetics/143.2.961>

678 Pike N, Kingcombe R (2009). Antibiotic treatment leads to the elimination of *Wolbachia*

679 endosymbionts and sterility in the diploid collembolan *Folsomia candida*. *BMC Biol*, 7,

680 54.

681 <https://bmcbiol.biomedcentral.com/articles/10.1186/1741-7007-7-54#citeas>

682 Pimentel AC, Cesar CS, Martins M, Cogni R (2021). The Antiviral Effects of the Symbiont Bacteria

683 *Wolbachia* in Insects. *Front Immunol*, 11, 1–10.

684 <https://doi.org/10.3389/fimmu.2020.626329>

685 Poinot D, Merçot H (1997). *Wolbachia* infection in *Drosophila simulans*: Does the female host

686 bear a physiological cost? *Evolution (N Y)*, 51, 180–186.

687 <https://doi.org/10.1111/j.1558-5646.1997.tb02399.x>

688 Ponton F, Wilson K, Holmes A, Raubenheimer D, Robinson KL, Simpson SJ (2014).

689 Macronutrients mediate the functional relationship between *Drosophila* and *Wolbachia*.

690 *Proc R Soc B Biol Sci*, 282.

691 <https://doi.org/10.1098/rspb.2014.2029>

692 Prout T (1994). Some evolutionary possibilities for a microbe that causes incompatibility in its

693 host. *Evolution (N Y)*, 48, 909–911.

694 <https://doi.org/10.2307/2410244>

695 Raychoudhury R, Grillenberger BK, Gadau J, Bijlsma R, Van De Zande L, Werren JH, *et al.* (2010).

696 Phylogeography of *Nasonia vitripennis* (Hymenoptera) indicates a mitochondrial-
697 *Wolbachia* sweep in North America. *Heredity (Edinb)*, 104, 318–326.
698 <https://doi.org/10.1038/hdy.2009.160>
699 Rigaud T, Moreau J, Juchault P (1999). *Wolbachia* infection in the terrestrial isopod *Oniscus*
700 *asellus*: Sex ratio distortion and effect on fecundity. *Heredity (Edinb)*, 83, 469–475.
701 <https://www.nature.com/articles/6885990#citeas>
702 Rousset F, Bouchon D, Pintureau B, Juchault P, Solignac M (1992). *Wolbachia* endosymbionts
703 responsible for various alterations of sexuality in arthropods. *Proc R Soc B Biol Sci*, 250, 91–
704 98.
705 <https://doi.org/10.1098/rspb.1992.0135>
706 Ruang-Areerate T, Kittayapong P (2006). *Wolbachia* transinfection in *Aedes aegypti*: A potential
707 gene driver of dengue vectors. *Proc Natl Acad Sci U S A*, 103, 12534–12539.
708 <https://dx.doi.org/10.1073%2Fpnas.0508879103>
709 Selman C, Blount JD, Nussey DH, Speakman JR (2012). Oxidative damage, ageing, and life-
710 history evolution: Where now? *Trends Ecol Evol*, 27, 570–577.
711 <https://doi.org/10.1016/j.tree.2012.06.006>
712 Sinkins SP, Walker T, Lynd AR, Steven AR, Makepeace BL, Godfray HCJ, *et al.* (2005). *Wolbachia*
713 variability and host effects on crossing type in *Culex mosquitoes*. *Nature*, 436, 257–260.
714 <https://doi.org/10.1038/nature03629>
715 Slatko BE, Taylor MJ, Foster JM (2010). The *Wolbachia* endosymbiont as an anti-filarial
716 nematode target. *Symbiosis*, 51, 55–65.
717 <https://doi.org/10.1007/s13199-010-0067-1>

718 Stolk C and Stouthamer R (1996). Influence of a Cytoplasmic Incompatibility-Inducing
719 Wolbachia on the fitness of the parasitoid wasp *Nasonia vitripennis*. *Proc Exper and Appl*
720 *Entomol NEV Amsterdam*, 7.
721 [https://research.wur.nl/en/publications/influence-of-a-cytoplasmic-incompatibility-inducing-](https://research.wur.nl/en/publications/influence-of-a-cytoplasmic-incompatibility-inducing-wolbachia-on-)
722 [wolbachia-on-](https://research.wur.nl/en/publications/influence-of-a-cytoplasmic-incompatibility-inducing-wolbachia-on-)
723 Sumida Y, Katsuki M, Okada K, Okayama K, Lewis Z (2017). *Wolbachia* induces costs to life-
724 history and reproductive traits in the moth, *Ephestia kuehniella*. *J Stored Prod Res*, 71, 93–
725 98.
726 [10.1016/j.jspr.2017.02.003](https://doi.org/10.1016/j.jspr.2017.02.003)
727 Tagami Y, Miura K, Stouthamer R (2001). How does infection with parthenogenesis-inducing
728 *Wolbachia* reduce the fitness of *Trichogramma*? *J Invertebr Pathol*, 78, 267–271.
729 <https://doi.org/10.1006/jipa.2002.5080>
730 Teixeira L, Ferreira Á, Ashburner M (2008). The bacterial symbiont *Wolbachia* induces
731 resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol*, 6, 2753–2763.
732 <https://doi.org/10.1371/journal.pbio.1000002>
733 Turelli M (1994). Evolution of Incompatibility-Inducing Microbes and Their Hosts. *Evolution (N*
734 *Y)*, 48, 1500–1513.
735 <https://doi.org/10.1111/j.1558-5646.1994.tb02192.x>
736 Turley AP, Moreira LA, O'Neill SL, McGraw EA (2009). *Wolbachia* infection reduces blood-
737 feeding success in the dengue fever mosquito, *Aedes aegypti*. *PLoS Negl Trop Dis*, 3, 1–6.
738 <https://doi.org/10.1371/journal.pntd.0000516>
739 Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA (2007). From parasite to

mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLoS Biol*, 5, 0997–1005.

<https://doi.org/10.1371/journal.pbio.0050114>

Weinert LA, Araujo-Jnr E V., Ahmed MZ, Welch JJ (2015). The incidence of bacterial endosymbionts in terrestrial arthropods. *Proc R Soc B Biol Sci*, 282.

<https://doi.org/10.1098/rspb.2015.0249>

Werren JH, Baldo L, Clark ME (2008). *Wolbachia*: master manipulators of invertebrate biology. *Nat Rev Microbiol*, 6, 741–51.

<https://doi.org/10.1038/nrmicro1969>

Whittle M, Barreaux AMG, Bonsall MB, Whittle M (2021). Insect-host control of obligate , intracellular symbiont density. *Proc R Soc B*, 288.

<https://doi.org/10.1098/rspb.2021.1993>

Xi Z, Khoo CCH, Dobson SL (2005). Ecology: *Wolbachia* establishment and invasion in an *Aedes Aegypti* laboratory population. *Science*, 310, 326–328.

<https://doi.org/10.1126/science.1117607>

Zug R, Hammerstein P (2015). *Wolbachia* and the insect immune system: What reactive oxygen species can tell us about the mechanisms of *Wolbachia*-host interactions. *Front Microbiol*, 6, 1–16.

<https://doi.org/10.3389/fmicb.2015.01201>

Zuk M, Stoehr AM (2002). Immune defense and host life history. *Am Nat*, 160.

<https://doi.org/10.1086/342131>

Figure legends

Figure 1. *Wolbachia* infected males and females show reduced lifespan

(A): Lifespan of males. Sample sizes for the four infections O(PU), wA(PU), wB(PU) and wAwB(PU) were n=98, n=95, n=94 and n=95 respectively.

(B): Lifespan of females. Sample sizes for the four infections O(PU), wA(PU), wB(PU) and wAwB(PU) were n=73, n=96, n=95 and n=95 respectively.

Statistical significance was tested using Log Rank statistics with $p < 0.05$.

Figure 2. *Wolbachia* infected males show a reduction in the number of copulations

Males from different *Wolbachia* infections were mated sequentially until each of them stopped mating. O(PU) could perform the highest number of copulations while wB(PU) the least. The results show that the presence of *Wolbachia* is associated with the reduction in the number of copulations a male can perform. The figure also shows whether the progenies of these sequential copulations produce any daughters or not, as a measure of sperm depletion. The details of sperm depletion are shown in figure 3. Sample sizes for the strains O(PU), wA(PU), O(wA PU), wB(PU), O(wB PU), wAwB(PU) and O(wAwB PU) were n=7, n=7, n=7, n=6, n=5, n=6 and n=7 respectively.

Figure 3. *Wolbachia* infected males deplete their sperm faster than the uninfected males

The Y-axis in black on the left of each figure represents the percentage of daughters produced for each mating. The black dots represent the average number of daughters produced for each sequential mating by the males of different *Wolbachia* infections (detailed in figure 2). The number of daughters produced is taken as a measure of the number of sperm transferred

during each mating. The Y-axis, in grey, on the right, for each figure tallies the average number of copulations that yielded at least one daughter. Thus, it measures the number of mating before a male is depleted of its sperm. The left panel shows the males from *Wolbachia* infected strains whereas the right panel shows their respective cured versions. Data for O(PU) is repeated at the top for comparison. The statistical significance was tested using the Mann-Whitney U test with $p < 0.05$.

Figure 4. *Wolbachia* infected females produce fewer offspring

Family size produced by females when hosted as virgins (A) and mated (B). The difference in the family size of mated females is due to the difference in the number of daughters (C) as there is no significant difference in the number of males produced. The statistical significance is tested using the Mann-Whitney U test with $p < 0.05$.

Figure 5. *Wolbachia* infection reduces female fecundity

The measure of fecundity (number of eggs laid) by females of different infections, with virgin females (A) and mated females (B). For virgin females, O(PU) shows higher fecundity than *wAwB*(PU), while *wA*(PU) and *wB*(PU) strains have intermediate fecundity which is comparable to both O(PU) and *wAwB*(PU). However, for mated females, O(PU) and *wA*(PU) show higher fecundity than *wB*(PU) and *wAwB*(PU). The statistical significance is tested with the Mann-Whitney U test, $p < 0.05$.

800 **Figure 6. Quantitative estimation of *Wolbachia* across different developmental stages of *N.***
 801 ***vitripennis* males (A) and females (B).** The statistical significance between groups is tested
 802 using the Mann-Whitney U test, $p < 0.05$.

803 **Table 1.** Negative fitness effects of CI-inducing *Wolbachia*

804 **Table 2.** Effect of *Wolbachia* infections on *N. vitripennis* (Summary)

Number of copulations

● Female and male progeny ● Only male progeny ● No emergence

■ Daughters per mating (%)

■ Number of matings before sperm depletion

bioRxiv preprint doi: <https://doi.org/10.1101/2021.09.10.459769>; this version posted December 23, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Table 1. Negative fitness effects of CI-inducing *Wolbachia*

<i>Wolbachia</i> Supergroup	Genera	Species	<i>Wolbachia</i> Strain	Host Sex	Negative effect	Reference
A	<i>Drosophila</i>	<i>D. melanogaster</i>	A-wMelPop	Female/Male	Tissue degeneration, reduced lifespan	Min et al. 1997; Reynolds et al. 2003
			A-wMelPop	Female/Male	Decreased response to food cues	Peng et al. 2008
			A-wMel	Female	Reduced body size	Hoffmann et al. 1997
			A-wMel	Female	Reduced fecundity after a dormancy period	Kriesner et al. 2016
		<i>D. simulans</i>	A-wRi	Female	Reduction in fecundity	Hoffmann et al., 1988, 1990
			A-wRi	Female	Reduction in fecundity	Snook et al. 2000
			A-wRi	Male	Lesser sperm cysts, reduced fertility	Snook et al. 2000
			A-wHa	Female	Reduction in fecundity	Fytrou et al. 2006
			A-wHa	Female/Male	Reduced thorax length, reduction in an immune response against parasitoid infection	Fytrou et al. 2006
			A-wRi	Male	Reduced sperm competition	Crespigny et al. 2006
		<i>D. sukukii</i>	A-wSuz	Female	Reduced progeny family size	Hamm et al. 2014
B	<i>Aedes</i>	<i>A. albopictus</i>	A-wAlb, B-wAlb	Female	Reduced lifespan, reduction in fecundity	Islam et al. 2006; Sun et al. 2009
		<i>A. aegypti</i>	A-wMelPop	Female	Reduced lifespan, reduction in fecundity	Ross et al. 2019
			A-wMelPop	Female/Male	Reduced lifespan	Mcmeniman et al. 2009
			A-wMelPop	Female	Reduction in fecundity, reduced blood-feeding success	Turley et al. 2009, 2013; Allman et al. 2020
	<i>Culex</i>	<i>C. pipiens</i>	B-wPip	Female/Male	Embryonic Mortality	Duron et al. 2005
		<i>C. quinquefasciatus</i>	B-wPip	Female	Reduced fecundity	Almeida et al. 2011

Table 2. Effect of *Wolbachia* infections on *N. vitripennis* (Summary)

Phenotype	Host Sex	Effect of <i>Wolbachia</i>	"Cost" compared to 0(PU)
Lifespan	Male	$O(PU) > wB(PU) = wA(PU) > wAwB(PU)$	$wA(PU) = 11.1\%$, $wB(PU) = 6.5\%$, $wAwB(PU) = 15.5\%$
	Female	$O(PU) = wB(PU) > wAwB(PU) > wA(PU)$	$wA(PU) = 17.7\%$, $wAwB(PU) = 15.5\%$
Number of copulations	Male	$O(PU) > wA(PU) \ wB(PU)$ and $wAwB(PU)$ $wA(PU) = wAwB(PU)$, $wA(PU) > wB(PU)$, $wAwB(PU) = wB(PU)$	$wA(PU) = 12.4\%$, $wB(PU) = 28.8\%$, $wAwB(PU) = 16.7\%$
Sperm depletion	Male	$O(PU) > wA(PU) \ wB(PU)$ and $wAwB(PU)$ $wA(PU) = wAwB(PU)$, $wA(PU) > wB(PU)$, $wAwB(PU) = wB(PU)$	$wA(PU) = 19.4\%$, $wB(PU) = 31.3\%$, $wAwB(PU) = 15.7\%$
Progeny family size	Female		
	a. Virgin	$O(PU) > wB(PU)$ and $wAwB(PU)$, $O(PU) = wA(PU)$, $wA(PU) > wAwB(PU)$, $wA(PU) = wB(PU)$, $wB(PU) = wAwB(PU)$	$wB(PU) = 10\%$, $wAwB(PU) = 12.4\%$
	b. Mated	$O(PU) = wA(PU) > wAwB(PU) = wB(PU)$	$wB(PU) = 11.5\%$, $wAwB(PU) = 9.1\%$
Fecundity	Female		
	a. Virgin	$O(PU) = wA(PU) = wB(PU)$, $O(PU) > wAwB(PU)$	$wAwB(PU) = 14.1\%$
	b. Mated	$O(PU) = wA(PU) > wB(PU) = wAwB(PU)$	$wB(PU) = 9.1\%$, $wAwB(PU) = 18.2\%$
<i>Wolbachia</i> density	Male	$wAwB(PU) > wB(PU) = wA(PU)$	
	Female	$wAwB(PU) > wA(PU) = wB(PU)$	