# Bacterial supergroup specific "Cost" of *Wolbachia* infections in *Nasonia vitripennis*

Alok Tiwary[1], Rahul Babu[1], Ruchira Sen[2], 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. Sri 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

## Abstract

The maternally-inherited endosymbiont, *Wolbachia*, is known to alter the reproductive biology of its arthropod hosts for its own benefit. It is also known to induce both positive and negative fitness effects in many hosts. Here we describe the effect of 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 uninfected, single A, single B, and double infected strains. Contrary to the previous reports, our results suggest that there is a "significant cost" associated with the maintenance of *Wolbachia* infections where traits like progeny family size, fecundity, longevity and rates of male copulation is compromised in *Wolbachia* infected strains. The double infection of *Wolbachia* has more detrimental impacts on the host as compared to single infections, while the supergroup B infections elicits the most pronounced negative effect. 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 found widely 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 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. The most well-known example for this is the obligatory association of *Wolbachia* with its filarial nematode hosts like *Wuchereria bancrofti* (Fenn and Blaxter, 2006) where the mutualistic effect ensures the long-term persistence of *Wolbachia*. This shift from parasitic to mutualistic effect can also happen in facultative associations as seen in *D. 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. In *D. simulans*, *Wolbachia* infected females face a 10-20% reduction in fecundity (Hoffmann et al. 1990) whereas, infected males tend to produce fewer sperm cysts (Snook et al. 2000). *Wolbachia* infections have also been known to negatively impact the lifespan of its hosts in *D. melanogaster* (Min and Benzer, 1997) and *Aedes albopictus* (Kambris et al., 2009; McMeniman et al. 2009). 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

72 (Fleury et al. 2000). Preimaginal mortality, in insecticide-resistant *Wolbachia*-infected strains,
73 has been observed in both sexes of *Culex pipiens* (Duron et al. 2006). *Wolbachia* infections can
74 also result in a range of behavioral changes in *Aedes aegypti* where a significant proportion of
75 them fail to obtain blood meals with increasing age. This complex behavior is associated with an
76 altered phenotype where the infected females have a "bendy proboscis" as compared to their
77 uninfected counterparts (Turley et al. 2009).

78 These negative effects of *Wolbachia* on their hosts are not difficult to explain. The presence of
79 bacteria within a host entails sharing of nutritional and other physiological resources (Kobayashi
80 and Crouch, 2009), especially with *Wolbachia* which are obligate endosymbionts and cannot
81 survive without cellular resources derived from their hosts (Foster et al. 2005; Slatko et al.
82 2010). For example, *Wolbachia* is known to compete with the host for key resources like
83 cholesterol and amino acids (Caragata et al. 2014). Therefore, *Wolbachia* infections can impose
84 severe nutritional demand on the host (Ponton et al. 2014). The precise molecular mechanisms
85 of many of these negative effects have not been ascertained and are generally ascribed to
86 partitioning off-host nutrients for its benefit (Ponton et al. 2014). However, it is known that
87 *Wolbachia* leads to antipathogenic effects in their hosts where the host resistance or tolerance
88 to the infection increases (Zug and Hammerstein, 2015). For example, *Wolbachia* can utilize the
89 IMD and Toll pathways (Pan et al. 2018) and increased ROS (Reactive Oxygen Species) levels in
90 *Wolbachia*-transfected *A. aegypti* mosquitoes, inhibiting the proliferation of Dengue virus (Pan
91 et al. 2012). Moreover, *Wolbachia* is also known to elevate ROS levels leading to tolerance
92 against *Drosophila* C viruses (Wong et al. 2011). Such immune defense requires additional
93 allocation of resources, which can affect other life-history traits of the host. This concept of a
94 "cost of immunity" is a well-established notion, which suggests a trade-off between immunity
95 and other life-history traits (Zuk and Stoehr, 2002). For example, elevated ROS levels negatively
96 effect many host traits like longevity, and fecundity (Dowling and Simmons, 2009; Monaghan et
97 al. 2009; Selman et al. 2012; Moné et al. 2014). Thus, there is sufficient evidence to conclude
98 that *Wolbachia* can have substantial negative effects on the physiology, nutrition, survival, and
99 overall fitness of its host.

100 In this study, we investigate what, if any, are the negative effects on the wasp *N. vitripennis* due
101 to its two resident CI inducing *Wolbachia* infections? *N. vitripennis,* with its cosmopolitan
102 distribution, has already been used to study *Wolbachia* distribution, acquisition, spread and
103 *Wolbachia*-induced reproductive manipulations (Werren *et al.*, 2008; Landmann, 2019).
104 However, the effect of the endosymbiont on the host life-history traits of this wasp remains
105 poorly understood with conflicting reports. In some *N. vitripennis* strains, *Wolbachia* was
106 reported to be the cause of enhanced fecundity (Stolk and Stouthamer, 1996), but a similar
107 effect was not observed in some other strains (Bordenstein and Werren, 2000). *N. vitripennis*
108 harbor two *Wolbachia* supergroup infections, one each from supergroup A and supergroup B
109 (Perrot-Minnot et al. 1996), and the presence of these two infections have been found in all
110 strains of *N. vitripennis* from continental North America to Europe and Russia (Raychoudhury et
111 al. 2010), indicating that it has reached fixation across the distribution of its host. The two
112 *Wolbachia* in *N. vitripennis* cause complete CI when present together, but single infections of
113 supergroup A *Wolbachia* causes incomplete CI while supergroup B infections still show
114 complete CI (Perrot-Minnot et al. 1996). In this study, we investigate the effects of *Wolbachia*
115 infections in a new strain of *N. vitripennis* recently obtained from the field. . This strain, like
116 other *N. vitripennis* strains, has two *Wolbachia* infections with one each from the supergroups A
117 and B. Sequencing of the five alleles from the well-established multi-locus strain typing system
118 (Baldo et al. 2006) reveal no sequence dissimilarity across the two infections (Prazapati,
119 personal communication) indicating this new strain is also infected by the same or very similar
120 *Wolbachia* infections that are present across the distribution of *N. vitripennis* (Raychoudhury et
121 al. 2010b). To compare supergroup specific effects, these two infections have been separated
122 into single infected lines of supergroup A and supergroup B *Wolbachia*. A comparative analysis
123 between the double infected, supergroup A infected, supergroup B infected and uninfected
124 strains revealed a consistent pattern of decreased longevity, quicker sperm depletion, and
125 reduced progeny family size for infected individuals. We have found supergroup specific
126 negative effects on the physiological traits of the host. While supergroup B infection has sever
127 reduction effects on the different traits, supergroup A infection show the negative effcts only on
128 few of the traits. Our results also show a higher *Wolbachia* density of supergroup B and double

129 infection strains across the different developmental stages of *N. vitripennis* males and females
130 as compared to supergroup A *Wolbachia*.

131 **Materials and Methods**

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

133 The *N. vitripennis* strains used NV PU-14 was obtained from Mohali, Punjab, India, in 2015).NV
134 PU-14 was cured of *Wolbachia* by feeding the females with 1 mg/ml tetracycline in 10 mg/ml
135 sucrose solution for at least two generations (Breeuwer and Werren, 1990). The curing was
136 confirmed by PCR using supergroup-specific *ftsZ* primers (Baldo et al. 2006), and CI crosses
137 between the infected and uninfected strains. NV PU-14 also served as the source strain for
138 separating the two *Wolbachia* infections into single A and single B infected strains.

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

147 The preferred method of nomenclature of *Nasonia* strains and their *Wolbachia* infections
148 includes information of both supergroups as well as the host genotype. For example, [*w*NvitA
149 *w*NvitB]V-PU14 indicates that the host species is *N. vitripennis*, with NV-PU14 as the host
150 genotype, which has two *Wolbachia* infections, one each from supergroup A and supergroup B.
151 However, since we used only *N. vitripennis* strains in this study, the nomenclature has been
152 simplified by removing the species name. For example, the same strain will now be denoted as
153 *w*A*w*B(PU), and when cured of these infections, as 0(PU). The single *Wolbachia*-infected strains
154 used were designated as *w*A(PU) for the supergroup A-infected strain while *w*B(PU) for the
155 supergroup B-infected strain. As the cured 0(PU) lines were in culture for three years, many of

156 the infected lines were cured again to obtain 'recently cured' lines to minimize the effects of
157 any host divergence that might have accumulated within them. These 'recently cured' lines
158 were named 0(*w*A PU), 0(*w*B PU), and 0(*w*A*w*B PU).

159 All the wasp strains were raised on *Sarcophaga dux* fly pupae with a generation time of 14-15
160 days at 25$^{o}$C, 60% humidity, and a continuous daylight cycle.

## 161 Sequential mating and sperm depletion of the males

162 To test the effect of *Wolbachia* on male reproductive traits like mating ability, individual males
163 were assayed for the number of copulations it can perform, as well as sperm depletion. As *N.*
164 *vitripennis* is haplodiploid, every successful mating will result in both female and male progenies
165 while an unsuccessful one will result in the production of exclusively male progenies. The males
166 used were obtained from virgin females hosted with one fly pupa for 24 hours and were not
167 given any external sources of nutrition (usually a mixture of sucrose in water) before the
168 experiment. Each male was then mated sequentially with virgin females from the same strain.
169 At the first sign of a male not completing the entire mating behavior (Jachmann and Assem
170 1996) it was given a rest for half an hour and was subjected to mating again until it stopped
171 mating altogether. The mated females were then given a day and each of them were hosted
172 with one fly pupa for 24 hours. The females were removed after 24 hours and the offspring
173 were allowed to emerge and then counted. The average number of copulations and the number
174 of copulations, before sperm depletion, were compared using the Mann-Whitney U test with a
175 significance level of 0.05.

## 176 Host longevity, progeny family size, and fecundity

177 To test whether the presence of *Wolbachia* has any influence on longevity, emerging adult
178 wasps were kept individually in ria vials at 25$^{o}$C, without any additional nutrition. Survival
179 following emergence was measured by counting the number of dead individuals every 6 hours.
180 Log Rank Statistics was used to identify differences between strains with a significance level of
181 0.05

182 To test for the effect of *Wolbachia* infections on the adult progeny family size of virgin and
183 mated females, each female was sorted at the pupal stage and separated into individual ria
184 vials. To enumerate the progeny size of mated females, some of these virgins were offered
185 single males from the same strain and observed till mating was successful. All the females were
186 then hosted individually with one fly pupa for 24 hours. These were kept at 25$^{0}$C for the
187 offspring to emerge which were later counted for progeny size by randomizing the ria vials in a
188 double-blind assay. The differences were compared using the Mann-Whitney U test with a
189 significance level of 0.05.

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

## 195 Estimation of relative density of *Wolbachia* infections across different 196 developmental stages of *N. vitripennis*

197 To collect the different developmental stages, females were hosted for 4 hours, (instead of 24
198 hours in the previous experiments), with one host to narrow down the developmental stages of
199 the broods. The larval and pupal stages (from Day 3 to Day 13 for males and from Day 8 to Day
200 14 for females) were collected every 24 hours. Larval stages for females were not done to avoid
201 any DNA contamination from the males as the two sexes are virtually indistinguishable at the
202 larval stages. Three replicates of ten larvae or pupae from the three strains *w*A(PU), *w*B(PU),
203 and *w*A*w*B(PU) were collected for each developmental stage. DNA extraction was done using
204 the phenol-chloroform extraction method, where samples were crushed in 200 µl of 0.5 M Tris-
205 EDTA buffer with 1% sodium dodecyl sulfate (SDS) and 2 µlof 22 mg/ml Proteinase K and
206 incubated overnight at 37°C. DNA was purified with buffer saturated phenol and chloroform-
207 isoamyl alcohol solution (24:1 ratio) and the DNA was precipitated overnight with isopropanol
208 at -20° C. The precipitated DNA pellet was dissolved in 60 µl nuclease-free water. The DNA
209 concentration of the samples was measured using a spectrophotometer (Nanodrop 2000®,

210 Thermo Scientific). The concentrations of all the samples were normalized to 200 ng/µl across
211 the different male and female developmental stage samples to be used for quantitative PCR.
212 CFX96 C1000® Touch Real-time qRT-PCR machine (BioRad) was used to assay the relative
213 density of *Wolbachia* across the strains. Amplification was done for the *Wolbachia* hcpA gene
214 (Forward Primer: 5'-CTTCGCTCTGCTATATTTGCTGC-3', Reverse Primer: 5'-
215 CGAATAATCGCAACCGAACTG-3'). The primers were tested to amplify both the *Wolbachia*
216 supergroup A and B strains. *Nasonia* S6K was used as the control gene (Bordenstein and
217 Bordenstein, 2011). The total reaction volume contained 5 µl of iTaq Universal SYBR® Green
218 supermix (BIO-RAD), 0.05 µl each of 10 µM forward and reverse primers, and 200 ng of
219 template DNA for a total volume of 10 µl for each reaction. Uninfected *N. vitripennis* DNA was
220 used as negative control while DNase-free water was used as non-template control. Reaction
221 conditions included an initial denaturation step of 95°C for 3 minutes followed by 39 cycles of
222 95°C for 10 seconds, annealing, and amplification at 52°C for 30 seconds for 40 cycles. All the
223 reactions were performed in triplicates and included a melt curve to check for non-specific
224 amplification. The relative density of *Wolbachia* was estimated by calculating the mean delta
225 threshold cycle ($\Delta C_q$), using the formula:

226 $$\Delta C_q = 1/3 \sum_{j=1}^{3} [1/3 \sum_{i=1}^{3} hcpA - 1/3 \sum_{i=1}^{3} S6K]$$

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

228 1/ ΔCq was calculated and plotted to show the *Wolbachia* density across different
229 developmental stages of males and females. The relative *Wolbachia* density was compared
230 across the three different strains using the Kruskal Wallis *H* test. Mann Whitney U test was used
231 to compare two different groups with a significance level of 0.05.

## 232 Results

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

234 *Wolbachia* can compete with the host for available nutrition increasing nutritional stress which
235 can shorten the lifespan of the many hosts (Caragata et al. 2014, Macmenimal et al. 2009).
236 Therefore, we first investigated the effect of *Wolbachia* infections on the survival of both male

237 and female wasps. As figure 1 (top panel) indicates, there is a significant difference in the
238 longevity of the infected males across the three infection types. The double-infected strain,
239 *w*A*w*B(PU), starts to die-off first and has a significantly shorter life span compared to the two
240 single infected strains {Log Rank Test, $\chi^2$=16.8, p < 0.001 with *w*A(PU) and $\chi^2$=33.9, p < 0.001
241 with *w*B(PU)}. Males from the uninfected strain, 0(PU), lived the longest and showed
242 significantly longer lifespan compared to all the other infected strains {(Log Rank Test: $\chi^2$=76.3, p
243 < 0.001 with *w*A*w*B(PU); $\chi^2$=33.0, p < 0.001 with *w*A(PU) and $\chi^2$=16.3, p < 0.001 for *w*B(PU)}.
244 However, there was no significant difference in the lifespan between the two single infected
245 strains *w*A(PU) and *w*B(PU) {(Log Rank Test, $\chi^2$=3.84, p = 0.05)}. Similarly, infected females
246 (bottom panel) also showed a distinct reduction in life span when compared with the uninfected
247 strain. But, unlike the males, the single A infected strain, *w*A(PU), showed the shortest lifespan
248 {Log Rank Test: $\chi^2$=11.2, p < 0.001 with *w*A*w*B(PU), $\chi^2$=56.9, p < 0.001 with *w*B(PU) and $\chi^2$=31.1,
249 p < 0.001, with 0(PU)} followed by *w*A*w*B(PU) {(Log Rank Test: $\chi^2$=20.4, p < 0.001 with *w*B(PU)
250 and $\chi^2$=12.9, p < 0.001 with 0(PU)}. Curiously, 0(PU) and *w*B(PU) females showed similar lifespan
251 {(Log Rank Test: $\chi^2$=0.24, p = 0.62)}. These results indicate a sex-specific variation as *w*A*w*B(PU)
252 show the shortest lifespan among the males but *w*A(PU) showed the shortest among the
253 females. Moreover, the effect of single infections on longevity also varied among the sexes as
254 *w*A(PU) and *w*B(PU) males had similar lifespans, but in the case of females, it was *w*B(PU) and
255 0(PU) which had similar lifespans. But what is unambiguous from these results is that the
256 uninfected strain lived the longest, irrespective of the sex of the hosts, indicating that the
257 presence of *Wolbachia* is associated with the reduction in lifespan and is thus, costly for *N.*
258 *vitripennis* to maintain.

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

260 *Wolbachia* is known to be associated with a reduction in the number of mating a male can
261 perform in *Ephestia kuehniella* (Sumida et al. 2017). To test whether similar effects are seen in
262 *N. vitripennis*, we enumerated the number of copulations an individual male can perform across
263 the infection types. As figure 2 indicates, there is indeed a reduction in the capacity of infected
264 males to mate. When compared with the uninfected strain 0(PU), this reduction was most

265 pronounced in *w*B(PU) (MWU, U=30, $p < 0.01$), followed by *w*A*w*B(PU) and *w*A(PU) which
266 showed similar copulation capacities (MWU: U=11, $p = 0.49$). The uninfected 0(PU) strain
267 produced males with the highest number of copulations {MWU: U= 32, $p < 0.05$ for *w*A(PU) and
268 U= 27, $p < 0.05$ for *w*A*w*B(PU)}. Thus, the presence of *Wolbachia* substantially reduced the
269 number of copulations that a male could perform. However, complex phenotypes like
270 copulation can also be affected by the host genotype. Although all these four strains were
271 derived from the same field-collected isofemale line, but continuous culturing in the laboratory
272 can fix specific alleles within them resulting in interstrain divergence. Moreover, it is also known
273 that in *Nasonia* the effect of *Wolbachia*-induced phenotype is influenced by the hosts' genetic
274 background (Raychoudhury et al. 2012). Therefore, we cured all these infections again and
275 tested whether the host genotype, rather than *Wolbachia*, is causing this reduction in the
276 number of copulations by comparing these newly cured strains back with the previously used
277 uninfected strain, 0(PU). As figure 2 indicates, males from most of these re-cured strains
278 showed a marked and significant increase in the number of copulations that they could
279 perform. This number in the re-cured double infected strain, 0(*w*A*w*B PU), increased to similar
280 levels as shown by 0(PU) (MWU: U=9.5, $p = 0.2$), while also showing a significant increase from
281 its infected counterpart *w*A*w*B(PU) (MWU: U=3, $p < 0.05$). Similarly, the number of copulations
282 for the re-cured single A supergroup infected strain, 0(*w*A PU), also increased to the levels of
283 the uninfected strain 0(PU) (MWU: U=20, $p = 0.76$). However, this increase (from 77.5 ± 6.3 to
284 83.5 ± 12.9) with its infected counterpart was not significant (MWU: U=23, $p = 0.48$). The re-
285 cured single B supergroup infection, 0(*w*B PU), was the only strain which did not revert to
286 uninfected levels (MWU: U=22, $p < 0.05$) despite showing a marginal increase (from 62.8 ± 6.6
287 to 78.2 ± 5.1; MWU: U=1, $p < 0.05$). However, what is evident is that the presence of *Wolbachia*
288 is also associated with a reduction in the capability of a male to mate. Furthermore, by recuring
289 the infected strains, we showed that this decrease is not due to the host genotype but is an
290 effect of *Wolbachia* present in these strains.

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

292 *N. vitripennis* males are prospermatogenic (Boivin et al. 2005), where each male emerges with
293 their full complement of mature sperm and usually does not produce any more during the rest
294 of their lifespan (Chirault et al. 2016). Thus, if a single male is mated sequentially with as many
295 females it can mate with, it should eventually run out of this full complement of sperm resulting
296 in all-male progeny even after successful copulation. Sequential mating of the males from 0(PU),
297 *w*A(PU), *w*B(PU), *w*A*w*B(PU), and also the recently cured single and double infection strains,
298 0(*w*A PU), 0(*w*B PU), 0(*w*A*w*B PU) was done with females from the same strains. As figure 2
299 indicates, each male did run out of sperm at the tail end of the continuous mating and hence
300 producing only male progenies (shown by black dots). We looked at the number of mating done
301 by these males before sperm depletion to see if *Wolbachia* affects the sperm production in the
302 males. As shown in figure 3, the average number of daughter progenies reduced with the
303 number of mating (shown by the primary Y-axis on the left), indicating sperm depletion. Similar
304 to copulation numbers, *w*B(PU) males were also the quickest to deplete the sperm reserve
305 {MWU: $U=27.5$, $p < 0.05$ for *w*A(PU) and $U=30$, $p < 0.01$ compared with 0(PU)}. This was
306 followed by *w*A*w*B(PU) and *w*A(PU) (MWU: $U=13$, $p = 0.7$). However, the uninfected males from
307 0(PU) were the slowest to deplete their sperm {MWU: $U= 35$, $p < 0.01$ for *w*A(PU) and $U= 24$, $p <$
308 $0.05$ for *w*A*w*B(PU)}. We again tested whether the host genotype, rather than *Wolbachia*, is
309 causing this rate of sperm depletion, by comparing it with the recently cured strains. As shown
310 in figure 3, the number of mating before sperms depletion increased for the recently cured
311 0(*w*A PU) up to the levels of 0(PU) (MWU: $U=30$, $p = 0.06$). However, this increase (from 48.14 ±
312 4.94 to 50.57 ± 9.41) was not significantly different from their infected counterpart *w*A(PU)
313 (MWU: $U=16$, $p = 0.8$). Rates of depletion for 0(*w*A*w*B PU) also increased up to the levels of
314 0(PU) (MWU: $U=21$, $p = 0.66$). Again, the cured strain 0(*w*B PU), increased marginally from
315 *w*B(PU) (from 41 ± 1.67 to 47.6 ± 6.0) (MWU: $U=0$, $p < 0.05$) but was still lower in number than
316 0(PU) (MWU: $U=23$, $p < 0.05$). These results indicate that the presence of *Wolbachia* has a
317 negative impact on the sperm number of the infected strains.

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

319 *Wolbachia* is known to have a negative impact on the progeny size of its host (Hoffmann et al.
320 1990; Hohmann et al. 2001). To test whether a similar effect is seen in *N. vitripennis*, we
321 enumerated the adult progeny family sizes for both virgin and mated females for the four
322 different *Wolbachia* infection strains and their recently cured counterparts.

323 As figure 4 (top panel) indicates, there is a significant reduction in the average family sizes of all-
324 male broods produced by the virgin females of the *Wolbachia* infected strains. When compared
325 with the uninfected strain 0(PU), this reduction was most pronounced in *w*A*w*B(PU) (MWU:
326 U=21151.5, $p < 0.01$) followed by *w*B(PU) (MWU: U=19880.5, $p < 0.05$). *w*B(PU) and *w*A*w*B(PU)
327 showed similar progeny family size (MWU: U=18582.5, $p = 0.29$). However, *w*A(PU) showed
328 similar progeny family size when compared with 0(PU) (MWU: U=17191, $p = 0.39$) and *w*B(PU)
329 (MWU: U=17284, $p = 0.26$) but had larger all-male broods sizes than *w*A*w*B(PU) (MWU:
330 U=18252, $p < 0.05$). We also compared the recently cured single and double infection strains
331 with the infected parental strains counterparts. The recently cured strains 0(*w*B PU) and
332 0(*w*A*w*B PU) showed marginal increase in the progeny family size which was comparable to the
333 uninfected strain 0(PU) {(MWU: U=11554, $p = 0.29$ with 0(*w*B PU); MWU: U=10798, $p = 0.21$
334 with 0(*w*A*w*B PU)}. However this marginal increase {from 29.84 ± 12.94 to 31.44 ± 10.30 for
335 0(*w*B PU) and from 28.99 ± 11.60 to 30.97 ± 11.48 for 0(*w*A*w*B PU)} was not significantly
336 different from their infected counterparts *w*B(PU) (MWU: U=9963.5, $p = 0.34$) and *w*A*w*B(PU)
337 (MWU: U=8650.5, $p = 0.09$). The recently cured strain 0(*w*A PU) did not show any increase in the
338 progeny family size and was comparable to *w*A(PU) (MWU: U=7085.3, $p = 0.63$) and 0(PU)
339 (MWU: U=8161, $p = 0.22$).

340 Similar to the virgin females, the reduction in the progeny family size was also observed for
341 average family sizes of mated females as shown by figure 4 (middle panel). When compared
342 with the uninfected strain 0(PU), this reduction is most pronounced in *w*B(PU) (MWU: 15582, p
343 < 0.01) and *w*A*w*B(PU) (MWU: U=16303, $p < 0.01$). However, *w*B(PU) and *w*A*w*B(PU) show
344 similar levels of progeny family sizes (MWU: 13732.5, $p = 0.55$). Interestingly, *w*A(PU) strain
345 shows similar levels of progeny family size as 0(PU) (MWU: U=11396.5, $p = 0.86$) but had larger
346 family sizes than *w*B(PU) (MWU: U=14080, $p < 0.01$) and *w*A*w*B(PU) (MWU: U=14682, $p < 0.05$).

347 Upon curing, the recently cured 0(*w*A*w*B PU) reverts back to the levels of the uninfected strain
348 0(PU) (MWU: U= 13295, p = 0.61) showing a significant increase from the infected counterpart
349 *w*A*w*B(PU) (MWU:U=12023, p < 0.05). The recently cured strain 0(*w*A PU) did not show any
350 increase from the infected counterpart *w*A(PU) (MWU: U=8385.5 p = 0.69) and was comparable
351 to 0(PU) (MWU: U=9022.5, p = 0.5). However, recently cured strain 0(*w*B PU) did not increase to
352 the levels of the uninfected strain 0(PU) (MWU: U=16782, p < 0.05). The marginal increase in
353 the progeny family size (from 28.72 ± 10.46 to 29.97 ± 8.59) was not significantly different from
354 the parent strain *w*B(PU) (MWU: U=13854, p = 0.47).

355 To know whether the difference in progeny family size of the mated females is because of the
356 female or male progenies or both, we compared the number of female and male progenies
357 separately between the four strains (figure 4 bottom panel). No difference was observed in the
358 number of males produced by the mated females. However, the number of female progenies
359 produced followed the same trend as that of the progeny family size of the mated females.
360 When compared to the uninfected strain 0(PU), *w*B(PU) and *w*A*w*B(PU) showed the least
361 number of female progenies {(MWU: 15964, p < 0.01 with *w*B(PU) and U=16283, p < 0.01 with
362 *w*A*w*B(Pu)}. *w*B(PU) and *w*A*w*B(PU) showed similar levels of female progenies (MWU: 13392, p =
363 0.33). Again, *w*A(PU) strain showed similar levels of female progenies as 0(PU) (MWU: U=11543,
364 p = 0.98) but showed higher female progeny number than *w*B(PU) and *w*A*w*B(PU) {(MWU:
365 U=14201, p < 0.01 with *w*B(PU) and MWU: U=14372, p < 0.05 with *w*A*w*B(PU)}. Upon curing,
366 the recently cured 0(*w*A*w*B PU) reverts back to the levels of the uninfected strain 0(PU) (MWU:
367 U= 13545, p = 0.42) showing a significant increase from the infected counterpart *w*A*w*B(PU)
368 (MWU:U=12331, p < 0.039). The recently cured strain 0(*w*A PU) did not show any increase from
369 the infected counterpart *w*A(PU) (MWU: U=8468 p = 0.79) and was also comparable to 0(PU)
370 (MWU: U=9330, p = 0.84). However, recently cured strain 0(*w*B PU) did not increase to the
371 levels of the uninfected strain 0(PU) (MWU: U=16749.5, p < 0.01).

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

373 The difference in progeny family sizes between the different infection strains can either be at
374 the level of eggs being laid by the females or there can be mortality at the larval or pupal stages.

375 To test this, we looked at the fecundity of both virgin and mated females for the four strains.
376 Among the virgin females (figure 5 top panel), when compared to the uninfected strain 0(PU),
377 *w*A*w*B(PU) had the least fecundity (MWU: U=5042, $p < 0.01$). However, there was no significant
378 difference between 0(PU) and *w*A(PU) (MWU: U=2632.5, $p = 0.34$) , and also between 0(PU) and
379 *w*B(PU)(MWU: U=2052.5, $p = 0.71$).

380 Amon the mated females (figure 5 bottom panel), when compared to the uninfected strain
381 0(PU), *w*A*w*B(PU) had the least fecundity (MWU: U=2461.5, $p < 0.01$). This was followed by
382 *w*B(PU) having similar fecundity as that of *w*A*w*B(PU) (MWU: U=1479.5, $p = 0.24$) and *w*A(PU)
383 (MWU: U=2822.5, $p = 0.22$). *w*A(PU) showed higher fecundity than *w*A*w*B(PU) (MWU: U=2388, p
384 $< 0.05$) but was similar to the uninfected strain 0(PU) (MWU: U=3799.5, $p = 0.75$) .

385 The double infection strain *w*A*w*B(PU) showing the least fecundity both in virgin and mated
386 females suggest an effect of *Wolbachia* on egg production in females. The fecundity assay also
387 establishes that the difference in adult progeny size can be because of the difference in the
388 fecundity of the females.

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

390 *Wolbachia* density has a major role to play in expressing the effects of the infection on host
391 biology (Hoffmann et al. 1996; Min and Benzer, 1997). An increase in cellular *Wolbachia* titers
392 are often associated with greater expression of their effects also has an effect on the level of CI
393 as suggested by the bacterial density model of CI (Breeuwer and Werren, 1993). Thus to
394 understand the effect of *Wolbachia* density on the host, once these are present as single and
395 double infections, we estimated the temporal pattern of *Wolbachia* levels across different
396 developmental stages of *N. vitripennis* males and females. In the case of males (figure 6, top
397 panel) *w*A(PU) had the lowest *Wolbachia* density across different larval and pupal
398 developmental stages when compared with *w*B(PU) (MWU: U=11, $p < 0.01$) and *w*A*w*B(PU)
399 (MWU: U=12, $p < 0.01$). However, no difference was observed between *w*B(PU) and *w*A*w*B(PU)
400 (MWU: U=51, $p = 0.56$).

401 In the case of females *w*A(PU), showed lower levels of *Wolbachia* when compared to *w*A*w*B(PU)
402 (MWU: U=8, $p < 0.05$). However, no difference was observed between *w*A(PU) and *w*B(PU)
403 (MWU: U=12, $p = 0.12$) and also *w*B(PU) and *w*A*w*B(PU) (MWU: U=19, $p = 0.5$).

## 404 Discussion

405 *Wolbachia* is known to negatively impact the physiological traits of the vast majority of its host
406 population. The present study suggests similar detrimental effects, or a "cost", associated with
407 the maintenance of *Wolbachia* infection in *N. vitripennis*. This cost is borne by both sexes. Our
408 results (summarized in Supplementary Table S1) suggest that the presence of both single and
409 multiple *Wolbachia* infections can be detrimental for both sexes for traits such as longevity
410 (figure 1) where both infected males and females showed reduced lifespan. In the case of
411 males, the infected strains *w*A(PU), *w*B(PU), and *w*A*w*B(PU) had fewer copulations (figure 2) but
412 quicker sperm depletion (figure 3). In most cases, these negative effects disappeared with the
413 removal of *Wolbachia*, as shown by the males of the recently cured strains 0(*w*A PU) and
414 0(*w*A*w*B PU). However, 0(*w*B PU) did not revert to the levels of 0(PU). Similarly, in the case of
415 females (figure 4) the infected strains *w*B(PU) and *w*A*w*B(PU) had lesser progeny when
416 compared to the 0(PU). This difference was not due to the number of males produced but in the
417 fewer number of daughters. However, *w*A(PU) females, as an exception, had their progeny
418 family sizes comparable to 0(PU). Again, the recently cured strains showed a significant increase
419 in the progeny family sizes suggesting that the negative effects are due to the presence of
420 *Wolbachia*. The differences found in the progeny sizes were at the level of the number of eggs
421 being laid by the infected females (figure 5) indicating that the negative effects of *Wolbachia*
422 manifested themselves even before the egg-laying stage. However, the egg to larval to pupal
423 stage mortality could also have had an effect on the progeny family sizes but these were not
424 assayed.

425 Our data also suggests an additive or synergistic effect of the presence of the two different
426 *Wolbachia* supergroups in the double infection strain *w*A*w*B(PU). Evidence of such effects can
427 be seen in traits like male longevity (additive effect) where the deficit in longevity for *w*A*w*B(PU)
428 is the total of the deficits of *w*A(PU) and *w*B(PU). Similarly, for traits like female longevity, virgin

429 female progeny family size, and female fecundity, reduction effect on strain *w*A*w*B(PU) appears
430 to be a combined effect of *w*A(PU) and *w*B(PU) (synergistic effect).

431 The negative fitness effects of CI-inducing *Wolbachia* (summarized in Supplementary Table S2)
432 have been reported in arthropod hosts like *Drosophila* and mosquitoes. These traits include
433 reproductive traits like fecundity, sperm cyst numbers, etc., and many other physiological traits
434 like longevity and developmental time, which are known to be compromised. In *D. simulans*,
435 *Wolbachia*-infected females face a 10-20% reduction in fecundity especially in laboratory strains
436 (Hoffmann et al. 1990). *D. simulans* males infected with *Wolbachia* tend to produce fewer
437 sperm cysts (Snook et al. 2000). *Wolbachia* infections have also been known to negatively
438 impact the lifespan of its hosts in *D. melanogaster* (Min and Benzer, 1997) and *Aedes albopictus*
439 (Kambris et al. 2009; McMeniman et al. 2009). However, many reports also suggest positive
440 fitness effects because of the presence of *Wolbachia* (Poinsot and Mercot, 1997; Fry et al. 2004;
441 Weeks et al. 2007). Similar host-specific effects have been reported in some strains of *Nasonia*
442 *vitripennis* where the presence of *Wolbachia* led to enhanced fecundity (Stolk and Stouthamer
443 1996). However, a similar effect was not observed in other strains of *N. vitripennis* (Bordenstein
444 and Werren, 2000). This suggests that the varied effects of *Wolbachia* can also be dependent on
445 host strains.

446 *Wolbachia*-induced CI in arthropod host and nutritional competition raises important questions
447 on the maintenance of these endosymbionts over evolutionary time scales. *Wolbachia*
448 competes with the host for nutritional benefits like essential amino acids (Caragata et al. 2014)
449 and hence imposes severe nutritional demands on the host (Ponton et al. 2014). Theoretical
450 studies predict that these maternally inherited reproductive parasites should eventually turn
451 mutualistic for long-term associations with their hosts (Prout 1994; Turelli 1994). Various
452 theories on endosymbiosis also support the vertical transmission of endosymbionts, as the
453 spread of the symbiont depends on the reproductive success of the host itself (Anderson and
454 May, 1982; Ewald 1987; Bull et al. 1991; Lipsitch et al. 1995). Hence mutualism is the preferred
455 way for endosymbionts to ensure their existence. Moreover, if there are indeed some adverse
456 effects of maintaining *Wolbachia,* then hosts would be under strong selection pressure to

457 develop immunity against them. Evidence suggests that there are examples of such emergence
458 of host genetic factors upon *Wolbachia* infection (Zug and Hammerstein, 2015) in hosts like
459 *Drosophila* and mosquitoes. Host suppressor alleles have been identified conferring resistance
460 against feminizing and male-killing *Wolbachia* (Rigaud et al. 1999; Hornett et al. 2006).
461 However, no such host genetic factors have been found for CI-inducing *Wolbachia*, especially in
462 the case of *N. vitripennis*. A possible explanation for the maintenance of these multiple
463 infections comes from the high efficiency of transmission of these infections in *N. vitripennis*,
464 where these infections also show nearly 100% transmission as well as CI (Breeuwer and Werren,
465 1990). For many *Wolbachia*-arthropod associations, the pathogenicity and transmission
466 efficiency is mediated by bacterial titers (Correa and Ballard, 2016). Our results also show higher
467 *Wolbachia* density for double infection strain, which can facilitate its transmission and
468 maintenance in the host.

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

473 Reproductive parasites like *Wolbachia* can also facilitate their transmission by increasing the
474 fitness of the infected individuals in the population against uninfected individuals (Dobson et al.
475 2004; Fry et al. 2004). The double infection strain, which is compatible with all the other strains,
476 can select against single *Wolbachia* infections (Perrot-Minnot et al. 1996). This fitness
477 advantage because of multiple *Wolbachia* infections can also be a plausible explanation for the
478 persistence of the host with *Wolbachia* even though it incurs a cost for maintenance of
479 *Wolbachia* on the host.

480 Our data suggest a significant reduction in the reproductive traits of both male and female
481 hosts. The fitness of adults and offspring depends on the allocation of limiting environmental
482 factors like nutrition (Trivers 1974). Many reports suggest that lack of nutrition results in smaller
483 progenies, a lesser number of eggs, impaired offspring development and metabolism (Telang
484 and Wells, 2004; Matzkin et al. 2013). With the presence of an endosymbiont like *Wolbachia*,

485 competing with the host for nutritional resources, a reduction in the number of female
486 offspring, a reduction in the number of copulations performed by the infected males, etc, can
487 be one of the implications of the nutritional stress caused by *Wolbachia* infection.

488 The cost of *Wolbachia* maintenance is in direct correlation with the density of *Wolbachia* strains
489 present in the host. Thus, in the case of females, the *w*A*w*B(PU), which shows a higher bacterial
490 load than single infections also has more reduced fecundity and longevity. Similarly, in the case
491 of males, the *w*A*w*B(PU) strain shows reduced number of copulations and the number of
492 daughters produced in sequential mating. However, *w*A(PU) showed minimal effect on the host,
493 the longevity of females being an exception. *w*A(PU) females had similar family sizes and
494 fecundities to that of 0(PU). Similarly, in the case of males, *w*A(PU) showed copulation rates
495 comparable to 0(PU) males. A possible explanation for this can be the relatively low density of
496 *w*A(PU) across the different developmental stages (figure 6). Our results also showed relatively
497 low levels of *Wolbachia* in *w*A(PU) strain, as compared to the other infections, which can result
498 in a less severe effect on male and female traits. Moreover, supergroup A infection strains are
499 known to have relatively higher levels of phage density (Bordenstein et al. 2006). According to
500 this phage density model (Bordenstein et al. 2006), higher phage density has an impact on the
501 level of CI caused by supergroup A strains, where it leads to a significant reduction in its CI
502 phenotype (Perrot-Minnot et al. 1996; Bordenstein et al. 2006).

503 Our results conform with the previous reports of the positive correlation between *Wolbachia*
504 abundance and the level of CI induced (Ijichi et al. 2002; Kondo et al. 2002; Bordenstein et al.
505 2006). *w*B(PU) *Wolbachia* showed complete CI and had higher *Wolbachia* titers in both males
506 and females, which was also comparable to that of the *w*A*w*B(PU), whereas *w*A(PU) had the
507 lowest *Wolbachia* titers among the three strains and showed incomplete CI.

508 *w*B(PU) shows strong deleterious effects on the host, similar to the double infection strain. The
509 observations can again be explained by the presence of high *Wolbachia* density in the case of
510 both males and females, which is comparable to that of the *w*A*w*B(PU). This also suggests that
511 the effects of resident *Wolbachia* infection can also be specific to particular *Wolbachia*

supergroup infection. Thus, such supergroup-specific effects of *Wolbachia* can be investigated in further studies.

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## Author Contribution

AT conceived the idea and planned the study. AT did the experiments. AT and RB collected the data. AT analyzed the data. AT, RS and RR contributed to data representation and statistical analysis. AT and RR wrote the manuscript.

## Competing Interests

The authors declare no conflict of interest.

## Data Archiving

The raw data for all the experiments has been archieved at Dryad with DOI: 10.5061/dryad.w0vt4b8s9

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673

674 ## Figure legends

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

676 Top panel: Lifespan of males. Sample sizes for the four infections 0(PU), *w*A(PU), *w*B(PU) and
677 *w*A*w*B(PU) were n=98, n=95, n=94 and n=95 respectively.

678 Bottom Panel: Lifespan of females. Sample sizes for the four infections 0(PU), *w*A(PU), *w*B(PU)
679 and *w*A*w*B(PU) were n=73, n=96, n=95 and n=95 respectively.

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

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

682 Males from different *Wolbachia* infections were mated sequentially until each of them stopped
683 mating (please see Materials and Methods for details). 0(PU) could perform the highest number
684 of copulations while *w*B(PU) the least. The results show that the presence of *Wolbachia* is
685 associated with the reduction in the number of copulations a male can perform. The figure also
686 shows whether the progenies of these sequential copulations produce any daughters or not, as
687 a measure of sperm depletion. The details of sperm depletion are shown in figure 3. Sample
688 sizes for the strains 0(PU), *w*A(PU), 0(*w*A PU), *w*B(PU), 0(*w*B PU), *w*A*w*B(PU) and 0(*w*A*w*B PU)
689 were n=7, n=7, n=7, n=6, n=5, n=6 and n=7 respectively.

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

691 The Y-axis in black on the left of each figure represents the percentage of daughters produced
692 for each mating. The black dots represent the average number of daughters produced for each
693 sequential mating by the males of different *Wolbachia* infections (detailed in figure 2). The
694 number of daughters produced is taken as a measure of the number of sperm transferred
695 during each mating. The Y-axis, in grey, on the right, for each figure tallies the average number
696 of copulations that yielded at least one daughter. Thus, it measures the number of mating
697 before a male is depleted of its sperm. The left panel shows the males from *Wolbachia* infected
698 strains whereas the right panel shows their respective cured versions. Data for 0(PU) is repeated
699 at the top for comparison. The statistical significance was tested using the Mann-Whitney U test
700 with $p < 0.05$.

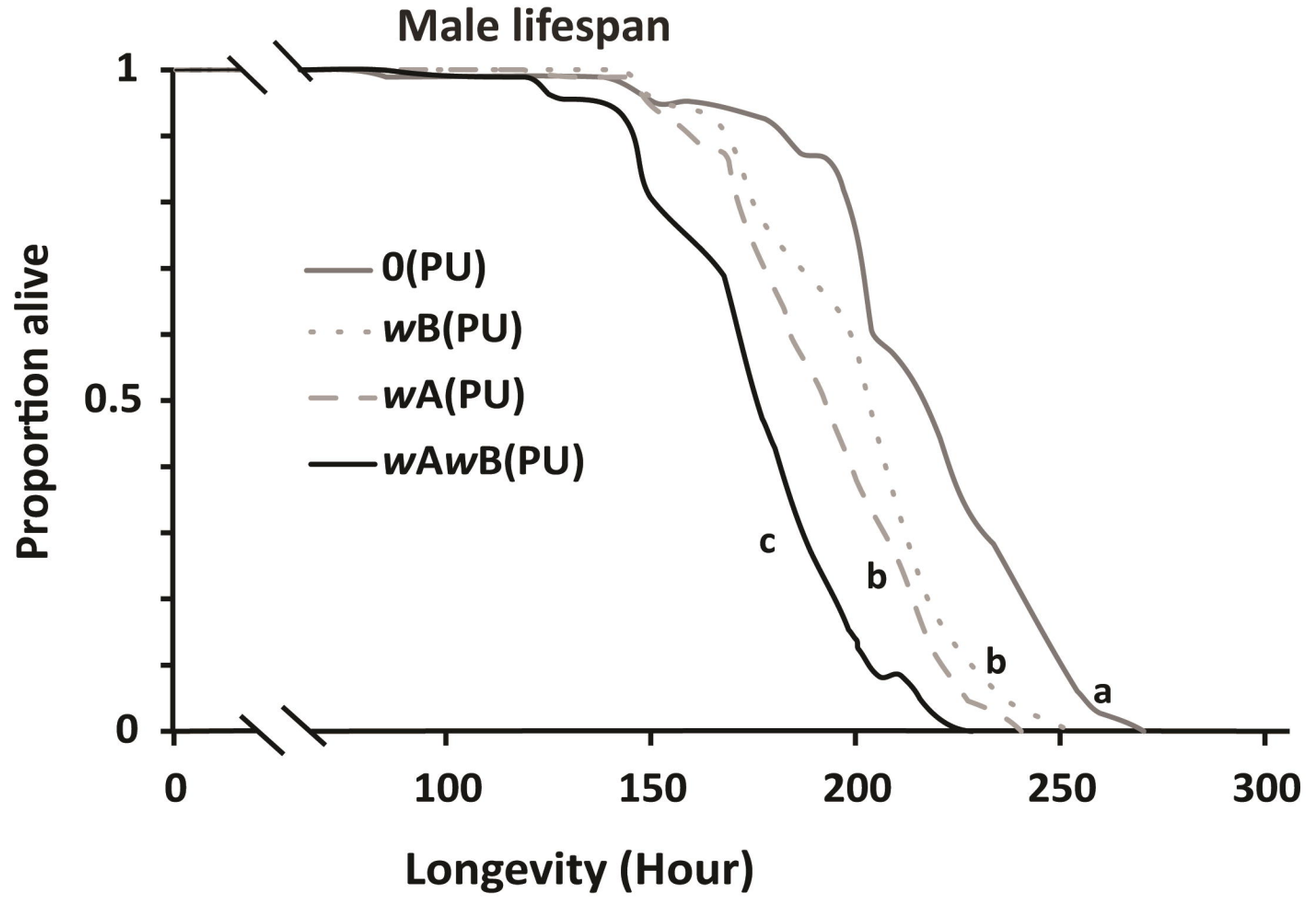
701 **Figure 4. *Wolbachia* infected females produce fewer offspring**

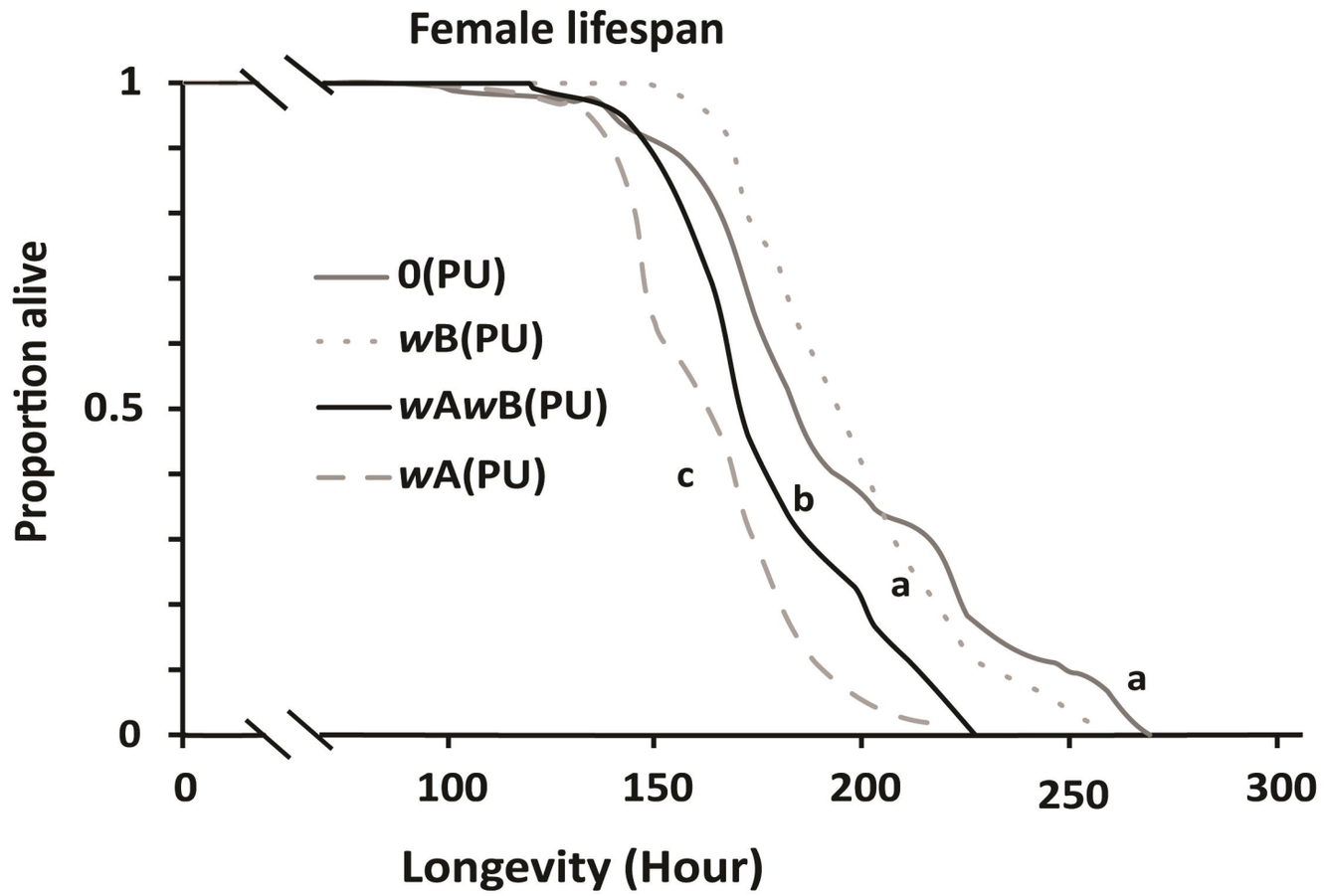
702 Progeny family size produced by females when hosted as virgins (top panel) and mated (middle
703 panel). The difference in the progeny family size of mated females is due to the difference in
704 the number of female progenies (bottom panel) as there is no significant difference in the
705 number of males produced (grey box plots). The statistical significance is tested using the
706 Mann-Whitney U test with $p < 0.05$.

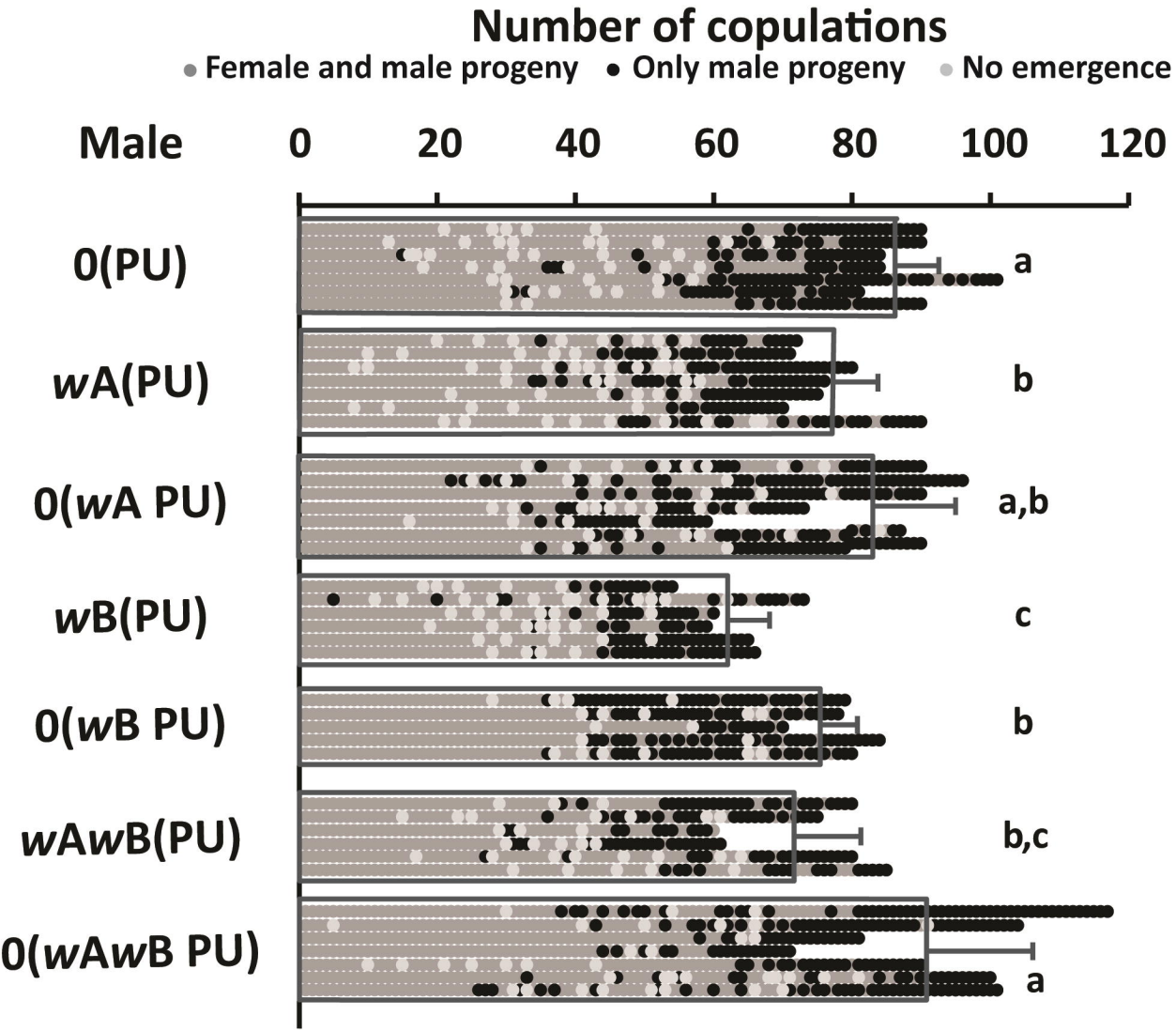
707 **Figure 5. *Wolbachia* infection reduces female fecundity**

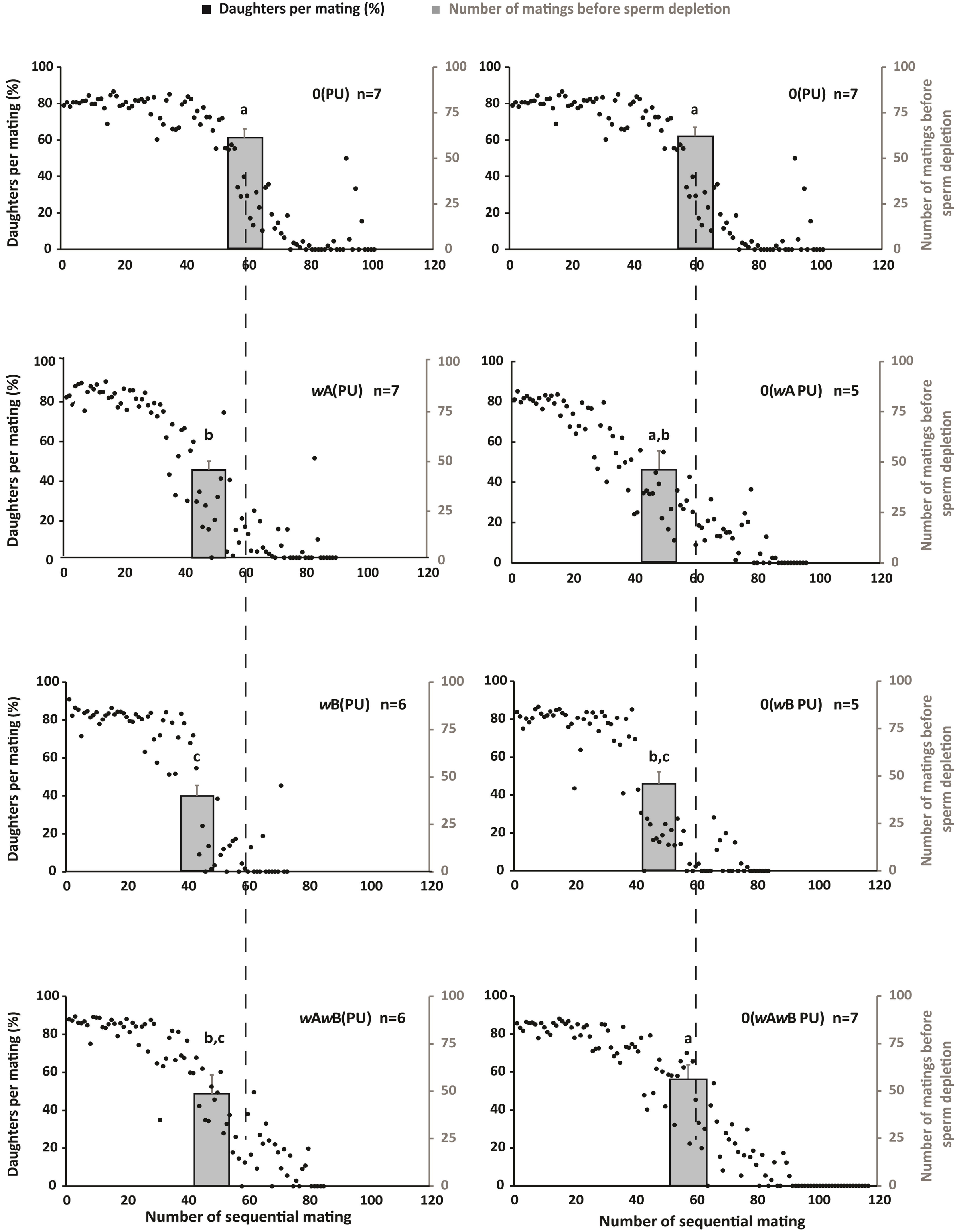
708 The measure of fecundity (number of eggs laid) by females of different infections, with virgin
709 females shown at the top and mated females at the bottom panel. For virgin females, 0(PU)
710 shows higher fecundity than *w*A*w*B(PU), while *w*A(PU) and *w*B(PU) strains have intermediate
711 fecundity which is comparable to both 0(PU) and *w*A*w*B(PU). However, for mated females,
712 0(PU) and *w*A(PU) show higher fecundity than *w*B(PU) and *w*A*w*B(PU). The statistical
713 significance is tested with the Mann-Whitney U test, $p<0.05$.

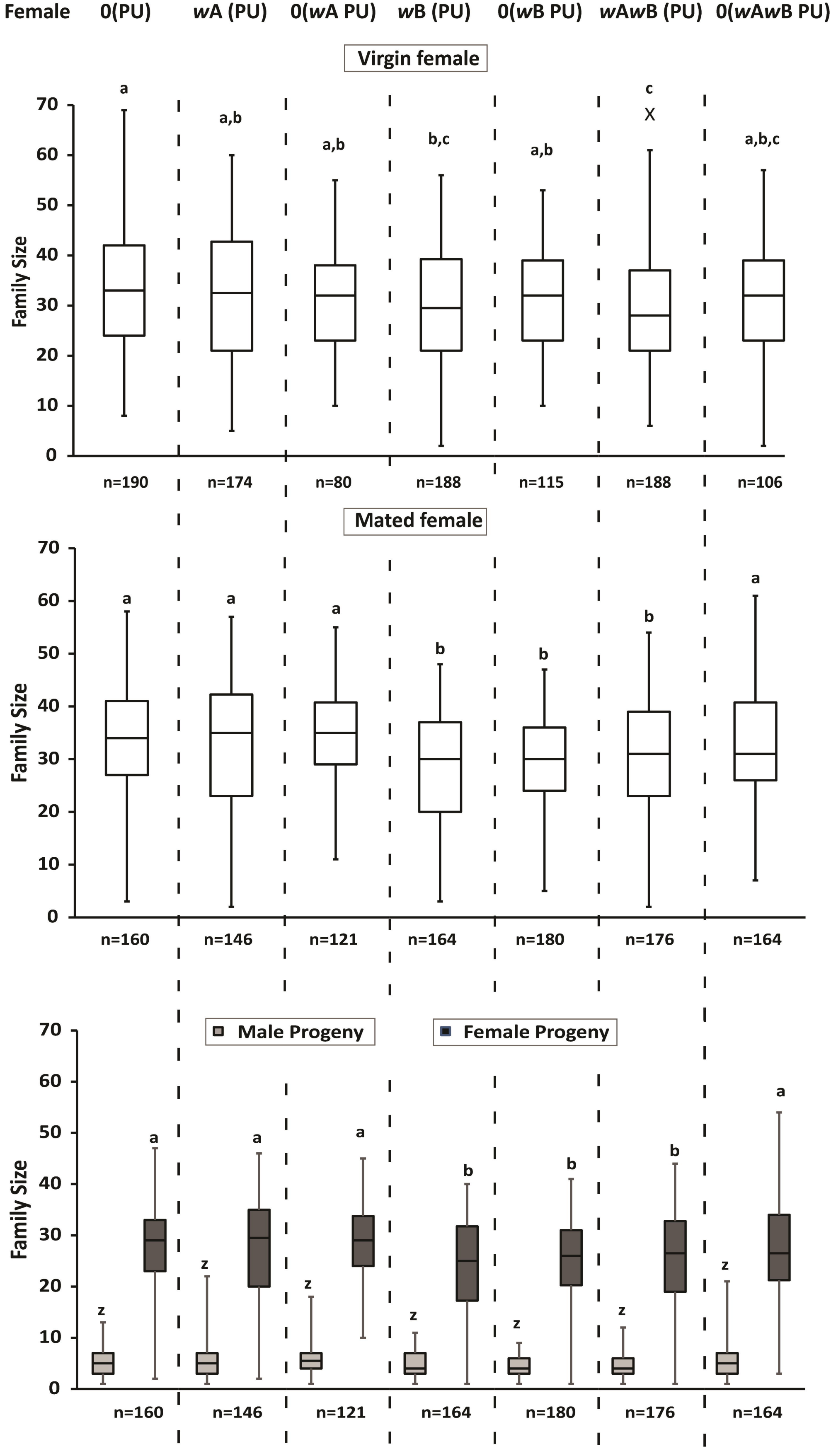
714 **Figure 6. Quantitative estimation of *Wolbachia* across different developmental stages of *N.***
715 ***vitripennis* males (top panel) and females (bottom panel).** The statistical significance between
716 groups is tested using the Mann-Whitney U test, $p<0.05$.

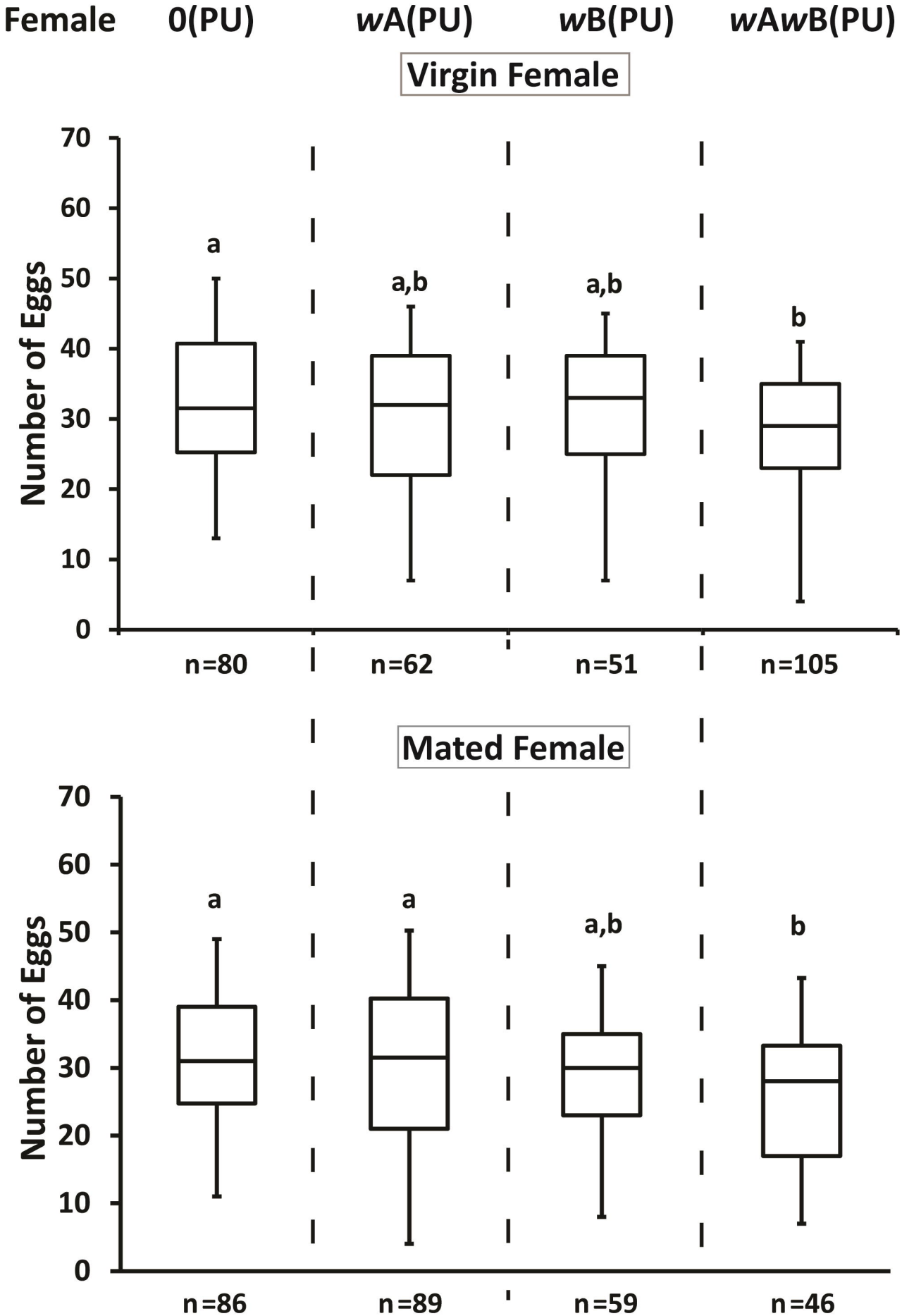

Male lifespan
Proportion alive
1
0.5
0
0(PU)
wB(PU)
wA(PU)
wAwB(PU)
c
b
b
a
0
100
150
200
250
300
Longevity (Hour)

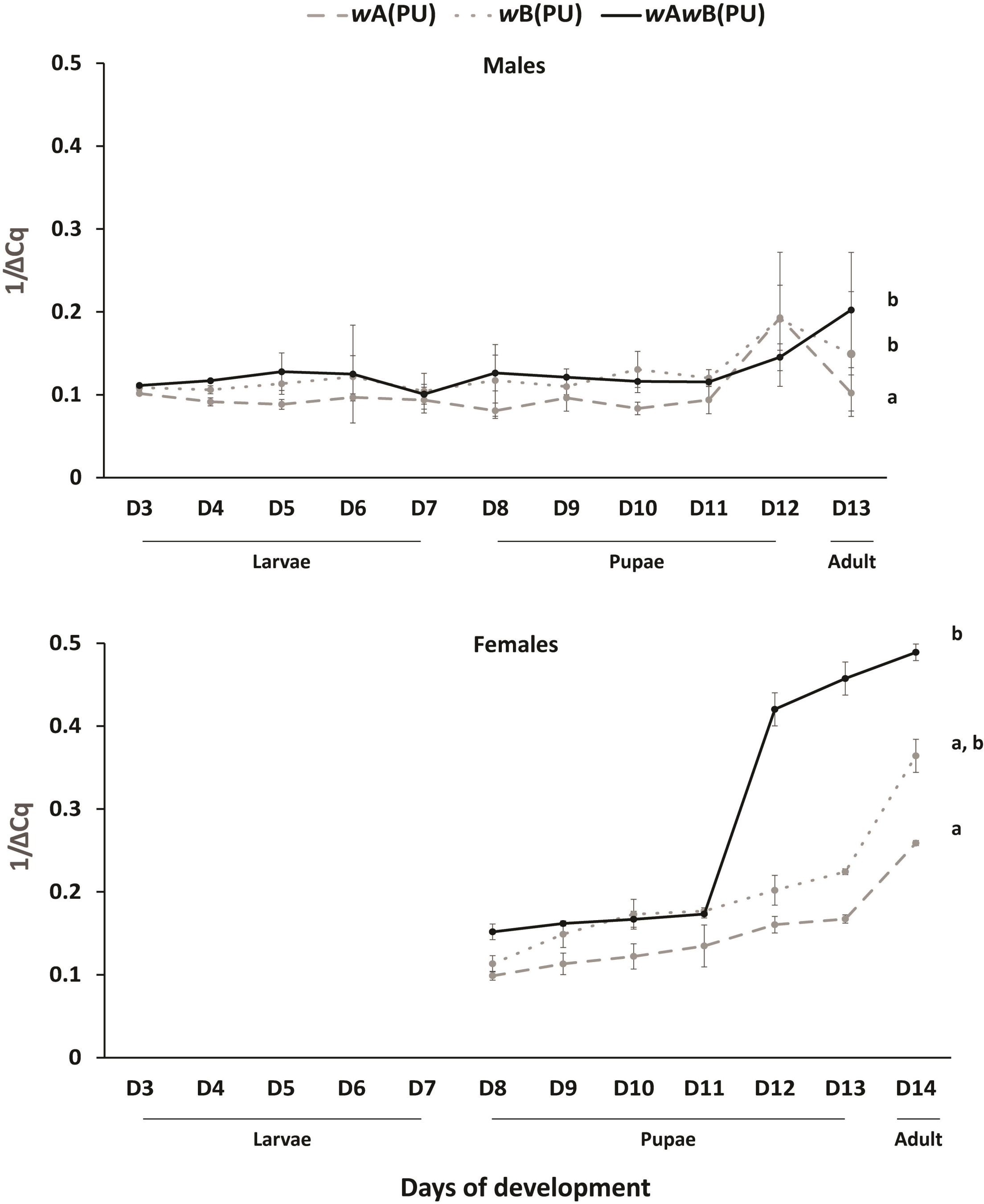

Female lifespan
Proportion alive
1
0.5
0
0(PU)
wB(PU)
wAwB(PU)
wA(PU)
c
b
a
a
0
100
150
200
250
300
Longevity (Hour)


Number of copulations
Female and male progeny
Only male progeny
No emergence
Male
0
20
40
60
80
100
120
0(PU)
wA(PU)
0(wA PU)
wB(PU)
0(wB PU)
wAwB(PU)
0(wAwB PU)
a
b
a,b
c
b
b,c
a


Daughters per mating (%)
Number of matings before sperm depletion
0(PU) n=7
a
0(PU) n=7
a
wA(PU) n=7
b
0(wA PU) n=5
a,b
wB(PU) n=6
c
0(wB PU) n=5
b,c
wAwB(PU) n=6
b,c
0(wAwB PU) n=7
a
Number of sequential mating
Number of sequential mating


Female
0(PU)
wA (PU)
0(wA PU)
wB (PU)
0(wB PU)
wAwB (PU)
0(wAwB PU)
Virgin female
Mated female
Male Progeny
Female Progeny
Family Size
0
10
20
30
40
50
60
70
a
a,b
a,b
b,c
a,b
c
X
a,b,c
n=190
n=174
n=80
n=188
n=115
n=188
n=106
a
a
a
b
b
b
a
n=160
n=146
n=121
n=164
n=180
n=176
n=164
z
a
z
a
z
a
z
b
z
b
z
b
z
a
n=160
n=146
n=121
n=164
n=180
n=176
n=164


Female
0(PU)
wA(PU)
wB(PU)
wAwB(PU)
Virgin Female
Number of Eggs
70
60
50
40
30
20
10
0
a
a,b
a,b
b
n=80
n=62
n=51
n=105
Mated Female
Number of Eggs
70
60
50
40
30
20
10
0
a
a
a,b
b
n=86
n=89
n=59
n=46


wA(PU)
wB(PU)
wAwB(PU)
Males
1/ΔCq
0.5
0.4
0.3
0.2
0.1
0
b
b
a
D3
D4
D5
D6
D7
D8
D9
D10
D11
D12
D13
Larvae
Pupae
Adult
Females
b
a, b
a
D14
Days of development