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

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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

60 longevity and developmental time are also known to be compromised. A review of such
61 negative effects of *Wolbachia* on hosts where CI is prevalent is presented in Table 1. In
62 *Trichogramma kaykai and T. deion,* the infected (thelytokous) line shows reduced fecundity and
63 adult emergence rates than the antibiotically cured (arrhenotokous) lines (Hohmann et al.,
64 2001; Tagami et al., 2001). *Leptopilina heterotoma*, a *Drosophila* parasitoid, has adult survival
65 rates, fecundity, and locomotor performance, of both sexes, severely compromised in
66 *Wolbachia* infected strains (Fleury et al., 2000). Preimaginal disc mortality has been observed in
67 both sexes of insecticide-resistant *Wolbachia*-infected strains of *Culex pipiens* (Duron et al.,
68 2006). *Wolbachia* infections can also result in a range of behavioral changes and altered
69 phenotypes in *Aedes aegypti* (Turley et al., 2009).

70 These negative effects of *Wolbachia* on their hosts are not unexpected. The presence of
71 bacteria within a host entails sharing of nutritional and other physiological resources
72 (Kobayashi & Crouch, 2009; Whittle et al., 2021), especially with *Wolbachia* as they are obligate
73 endosymbionts and cannot survive without cellular resources derived from their hosts (Foster
74 et al., 2005., Slatko et al., 2010). For example, *Wolbachia* is known to compete with the host for
75 key resources like cholesterol and amino acids in *A. aegypti* (Caragata et al., 2014). The precise
76 molecular mechanisms of many of these negative effects have not been ascertained and are
77 generally ascribed to partitioning off of host nutrients for its benefit, but what is clear is that
78 *Wolbachia* infections can impose severe nutritional demands on their hosts (Ponton et al.,
79 2014). However, it is also known that *Wolbachia* can elicit antipathogenic responses from their
80 hosts where the host resistance or tolerance to the infection increases (Zug & Hammerstein,
81 2015). For example, *Wolbachia* can utilize the Immune deficiency (IMD) and Toll pathways (Pan

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

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

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

121 **Materials and Methods**

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

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

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

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

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

146 the infected lines were cured again to obtain 'recently cured' lines to minimize the effects of
147 any host divergence that might have accumulated within them. These 'recently cured' lines
148 were named 0(*w*A PU), 0(*w*B PU), and 0(*w*A*w*B PU). All these wasp strains were raised on
149 *Sarcophaga dux* fly pupae with a generation time of 14-15 days at 25°C, 60% humidity, and a
150 continuous daylight cycle.

151 **Sequential mating and sperm depletion of the males**

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

165 **Host longevity, family size, and fecundity**

166 To test whether the presence of *Wolbachia* has any influence on longevity, emerging wasps of
167 both the sexes were kept individually in ria vials at 25°C, without any additional nutrition.
168 Survival following emergence was measured by counting the number of dead individuals every
169 6 hours. Kaplan-Meier analysis followed by Log Rank Statistics was used to identify differences
170 between strains with a significance level of 0.05.

171 To test for the effect of *Wolbachia* infections on the adult family size of virgin and mated
172 females, each female was sorted at the pupal stage and separated into individual ria vials. To
173 enumerate the brood size of mated females, some of these virgins were offered single males
174 from the same strain and observed till mating was successful. All the females were then hosted
175 individually with one fly pupa for 24 hours. These were kept at 25°C for the offspring to emerge
176 which were later counted for family size by randomizing the ria vials in a double-blind assay.
177 The differences were compared using the Mann-Whitney U test with a significance level of
178 0.05.

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

185 **Estimation of relative density of *Wolbachia* infections across different**
186 **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, *w*A(PU), *w*B(PU), and
193 *w*A*w*B(PU), were collected for each developmental stage. DNA extraction was done using the
194 phenol-chloroform extraction method, where samples were crushed in 200 µl of 0.5 M Tris-
195 EDTA buffer with 1% sodium dodecyl sulfate (SDS) and 2 µl 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 µl 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/µl 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 CTTCGCTCTGCTATATTTGCTGC-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 µl of iTaq Universal SYBR® Green supermix (BIO-RAD), 0.05 µl each of 10 µM of
208 forward and reverse primers, and 200 ng of template DNA for a total volume of 10 µl for each

209 reaction. Uninfected *N. vitripennis* DNA was used as negative control while DNase-free water
210 was used as non-template control. Reaction conditions included an initial denaturation step of
211 95°C for 3 minutes followed by 39 cycles of 95°C for 10 seconds, annealing, and amplification at
212 52°C for 30 seconds for 40 cycles. All the reactions were performed in triplicates and included a
213 melt curve to check for non-specific amplification. The relative density of *Wolbachia* was
214 estimated by calculating the mean delta threshold cycle (ΔC_q), using the formula:

215 $$\Delta C_q = 1/3 \sum_{j=1}^{3} [1/3 \sum_{i=1}^{3} hcpA - 1/3 \sum_{i=1}^{3} S6K]$$

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

217 1/ ΔCq was calculated and plotted to show the *Wolbachia* density across different
218 developmental stages of males and females. The relative *Wolbachia* density was compared
219 across the three different strains using the Kruskal Wallis *H* test. Mann Whitney U test was used
220 to compare two different groups with a significance level of 0.05.

221 Results

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

223 *Wolbachia* can compete with the host for available nutrition which can increase nutritional
224 stress resulting in a shortened lifespan of many hosts (McMeniman et al., 2009; Caragata et al.,
225 2014). Therefore, we first investigated the effect of *Wolbachia* infections on the survival of both
226 male and female wasps. As Fig. 1(A), indicates, there is a significant difference in the longevity
227 of the infected males across the three infection types. The double-infected strain, *w*A*w*B(PU),
228 starts to die-off first and has a significantly shorter life span compared to the two single

229 infected strains {Log Rank Test, χ^2=16.8, p < 0.001 for *w*A(PU) and χ^2=33.9, p < 0.001 for
230 *w*B(PU)}. Males from the uninfected strain, 0(PU), lived the longest and showed significantly
231 longer lifespan compared to all the other infected strains {Log Rank Test: χ^2=76.3, p < 0.001 for
232 *w*A*w*B(PU); χ^2=33.0, p < 0.001 for *w*A(PU) and χ^2=16.3, p < 0.001 for *w*B(PU)}. However, there
233 was no significant difference in the lifespan of the two single infected strains *w*A(PU) and
234 *w*B(PU) {Log Rank Test, χ^2=3.84, p = 0.05}. Similarly, infected females (Fig. 1B) also showed a
235 distinct reduction in life span when compared with the uninfected strain. But, unlike the males,
236 the single A infected *w*A(PU) females, showed the shortest lifespan {Log Rank Test: χ^2=11.2, p <
237 0.001 for *w*A*w*B(PU), χ^2=56.9, p < 0.001 for *w*B(PU) and χ^2=31.1, p < 0.001, for 0(PU)} followed
238 by *w*A*w*B(PU) {Log Rank Test: χ^2=20.4, p < 0.001 for *w*B(PU) and χ^2=12.9, p < 0.001 for 0(PU)}.
239 Curiously, 0(PU) and *w*B(PU) females showed similar lifespans {Log Rank Test: χ^2=0.24, p =
240 0.62}. These results indicate a sex-specific variation as *w*A*w*B(PU) show the shortest lifespan
241 among the males but *w*A(PU) showed the shortest among the females. Moreover, the effect of
242 single infections on longevity also varied among the sexes as *w*A(PU) and *w*B(PU) males had
243 similar lifespans, but in the case of females, it was *w*B(PU) and 0(PU) which had similar
244 lifespans. But what is unambiguous from these results is that the uninfected strain lived the
245 longest, irrespective of the sex of the hosts, indicating that the presence of *Wolbachia* is
246 associated with the reduction in lifespan and is thus, costly for *N. vitripennis* to maintain.

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

248 *Wolbachia* is known to be associated with a reduction in the number of mating a male can
249 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 0(PU), this reduction was most
253 pronounced in *w*B(PU) (MWU, U=30, $p < 0.01$), followed by *w*A*w*B(PU) and *w*A(PU) which
254 showed similar copulation capacities (MWU: U=11, $p = 0.49$). The uninfected 0(PU) strain
255 produced males with the highest number of copulations {MWU: U= 32, $p < 0.05$ for *w*A(PU) and
256 U= 27, $p < 0.05$ for *w*A*w*B(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, 0(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, 0(*w*A*w*B PU), increased to similar levels as shown by 0(PU) (an
268 increase of 29%, MWU: U=9.5, $p = 0.2$), while also showing a significant increase from its
269 infected counterpart *w*A*w*B(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, 0(*w*A
271 PU), also increased to the levels of the uninfected strain 0(PU) (an increase of 7%, MWU: U=20,

272 p = 0.76). However, this increase (from 77.5 ± 6.3 to 83.5 ± 12.9) with its infected counterpart
273 was not significant (MWU: U=23, p = 0.48). The re-cured single B supergroup infection, 0(*w*B
274 PU), was the only strain which did not revert to uninfected levels (MWU: U=22, $p < 0.05$)
275 despite showing an increase (from 62.8 ± 6.6 to 78.2 ± 5.1; MWU: U=1, $p < 0.05$). However,
276 what is evident is that the presence of *Wolbachia* is also associated with a reduction in the
277 capability of a male to mate. Furthermore, by curing the infected strains again, we showed that
278 this decrease is not due to the host genotype but is an effect of *Wolbachia* present in these
279 strains.

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

281 *N. vitripennis* males are prospermatogenic (Boivin et al., 2005), where each male emerges with
282 their full complement of mature sperm and have not been reported to produce any more
283 during the rest of their lifespan (Chirault et al., 2016). Thus, if a single male is mated
284 sequentially with as many females it can mate with, it should eventually run out of this full
285 complement of sperm resulting in an all-male brood even after successful copulation. As Fig. 2,
286 indicates, each male did run out of sperm at the tail end of the continuous mating and
287 produced only male progenies (shown by black dots). We looked at the number of mating done
288 by these males before sperm depletion to see if *Wolbachia* affects the sperm production in the
289 males. As shown in Fig. 3, the average number of daughter progenies reduced with the number
290 of mating (shown by the primary Y-axis on the left), indicating sperm depletion. Similar to
291 copulation numbers, *w*B(PU) males were also the quickest to deplete their sperm reserve
292 {MWU: U=27.5, $p < 0.05$ for *w*A(PU) and U=30, $p < 0.01$ compared for 0(PU)}. This was followed

293 by *w*A*w*B(PU) and *w*A(PU) (MWU: U=13, p = 0.7). However, the uninfected males from 0(PU)
294 were the slowest to deplete their sperm {MWU: U= 35, p < 0.01 for *w*A(PU) and U= 24, p < 0.05
295 for *w*A*w*B(PU)}. We again tested whether the host genotype, rather than *Wolbachia*, is causing
296 this rate of sperm depletion, by comparing it with the recently cured strains. As shown in Fig. 3,
297 the number of mating before sperm depletion increased for the recently cured 0(*w*A PU) up to
298 the levels of 0(PU) (an increase of 5%, MWU: U=30, p = 0.06). However, this increase (from
299 48.14 ± 4.94 to 50.57 ± 9.41) was not significantly different from their infected counterpart
300 *w*A(PU) (MWU: U=16, p = 0.8). Rates of depletion for 0(*w*A*w*B PU) also increased up to the
301 levels of 0(PU) (an increase of 15.2%, MWU: U=21, p = 0.66). Again, the cured strain 0(*w*B PU),
302 increased from *w*B(PU) (from 41 ± 1.67 to 47.6 ± 6.0,MWU: U=0, p < 0.05) but was still lower in
303 number than 0(PU) (MWU: U=23, p < 0.05). These results indicate that the presence of
304 *Wolbachia* has a significant negative impact on the number of sperm produced or utilized by
305 the infected strains.

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

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

311 As Fig. 4(A), indicates, there is a significant reduction in the average family sizes of all-male
312 broods produced by the virgin females of the *Wolbachia* infected strains. When compared with
313 the uninfected strain 0(PU), this reduction was most pronounced in *w*A*w*B(PU) (MWU:

314 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)
315 showed similar family size (MWU: U=18582.5, p = 0.29). However, *w*A(PU) showed similar
316 family size when compared with 0(PU) (MWU: U=17191, p = 0.39) and *w*B(PU) (MWU:
317 U=17284, p = 0.26) but had larger all-male broods sizes than *w*A*w*B(PU) (MWU: U=18252, p <
318 0.05). We also compared the recently cured single and double infected strains with the infected
319 parental strains. The recently cured strains 0(*w*B PU) and 0(*w*A*w*B PU) showed marginal
320 increase in the family size which was comparable to the uninfected strain 0(PU) {an increase of
321 1.5%, MWU: U=11554, p = 0.29 for 0(*w*B PU); an increase of 2%, MWU: U=10798, p = 0.21 for
322 0(*w*A*w*B PU)}. However this marginal increase {from 29.84 ± 12.94 to 31.44 ± 10.30 for 0(*w*B
323 PU) and from 28.99 ± 11.60 to 30.97 ± 11.48 for 0(*w*A*w*B PU)} was not significantly different
324 from their infected counterparts *w*B(PU) (MWU: U=9963.5, p = 0.34) and *w*A*w*B(PU) (MWU:
325 U=8650.5, p = 0.09). The recently cured strain 0(*w*A PU) did not show any increase in the family
326 size and was comparable to *w*A(PU) (MWU: U=7085.3, p = 0.63) and 0(PU) (MWU: U=8161, p =
327 0.22).

328 Similar to the virgin females, the reduction was also observed for average family sizes of mated
329 females as shown in Fig. 4(B). When compared with the uninfected strain 0(PU), this reduction
330 is most pronounced in *w*B(PU) (MWU: 15582, p < 0.01) and *w*A*w*B(PU) (MWU: U=16303, p <
331 0.01). However, *w*B(PU) and *w*A*w*B(PU) show similar levels of family sizes (MWU: 13732.5, p =
332 0.55). Interestingly, *w*A(PU) strain shows similar levels of family size as 0(PU) (MWU:
333 U=11396.5, p = 0.86) but had larger family sizes than *w*B(PU) (MWU: U=14080, p < 0.01) and
334 *w*A*w*B(PU) (MWU: U=14682, p < 0.05). Upon curing, the average family sizes of the recently
335 cured 0(*w*A*w*B PU) reverted back to the levels of the uninfected strain 0(PU) (an increase of

336 11.8%, MWU: U= 13295, p = 0.61) showing a significant increase from the infected counterpart
337 *w*A*w*B(PU) (from 29.49 ± 10.67, MWU: U=12023, p < 0.05). The recently cured strain 0(*w*A PU)
338 did not show any significant increase from the infected counterpart *w*A(PU) (MWU: U=8385.5 p
339 = 0.69) and was comparable to 0(PU) (MWU: U=9022.5, p = 0.5). However, the recently cured
340 strain 0(*w*B PU) did not increase to the levels of the uninfected strain 0(PU) (an increase of
341 4.3%, MWU: U=16782, p < 0.05). The marginal increase in the family size (from 28.72 ± 10.46 to
342 29.97 ± 8.59) was not significantly different from the parent strain *w*B(PU) (MWU: U=13854, p =
343 0.47).

344 To understand whether this difference in the family size of the mated females is due to the
345 production of fewer daughters or sons or both, we compared their numbers separately for the
346 four strains (Fig. 4C). No difference was observed in the number of sons produced by the mated
347 females. However, significant differences were observed in the number of daughters produced.
348 When compared to the uninfected strain 0(PU), *w*B(PU) and *w*A*w*B(PU) showed the least
349 number of daughters produced {MWU: 15964, p < 0.01 for *w*B(PU) and U=16283, p < 0.01 for
350 *w*A*w*B(PU)} whereas *w*B(PU) and *w*A*w*B(PU) produced nearly equal number of daughters
351 (MWU: 13392, p = 0.33). Again, *w*A(PU) strain produced equal number of daughters compared
352 to 0(PU) (MWU: U=11543, p = 0.98) but higher in number than *w*B(PU) and *w*A*w*B(PU) {MWU:
353 U=14201, p < 0.01 for *w*B(PU) and MWU: U=14372, p < 0.05 for *w*A*w*B(PU)}. Upon curing, the
354 recently cured 0(*w*A*w*B PU) reverted to the levels of the uninfected strain 0(PU) (MWU: U=
355 13545, p = 0.42) showing a significant increase in the number of daughters from the infected
356 counterpart *w*A*w*B(PU) (MWU: U=12331, p < 0.039). The recently cured strain 0(*w*A PU) did not
357 show any increase in the number of daughters produced from their infected counterpart

358 *w*A(PU) (MWU: U=8468 p = 0.79) and was also comparable to 0(PU) (MWU: U=9330, p = 0.84).
359 However, recently cured strain 0(*w*B PU) did not increase to the levels of the uninfected strain
360 0(PU) (MWU: U=16749.5, p < 0.01).

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

362 To check whether the differences in the family sizes between the different infection strains are
363 due to the number of eggs being laid by the females, we looked at the fecundity of both virgin
364 and mated females across the strains. Among the virgin females (Fig. 5A), *w*A*w*B(PU) had the
365 least fecundity (MWU: U=5042, p < 0.01) when compared to the uninfected strain 0(PU).
366 However, there was no significant difference between 0(PU) and *w*A(PU) (MWU: U=2632.5, p =
367 0.34) and also between 0(PU) and *w*B(PU) (MWU: U=2052.5, p = 0.71).

368 Among the mated females (Fig. 5B), *w*A*w*B(PU) again had the least fecundity (MWU: U=2461.5,
369 p < 0.01) when compared to the uninfected strain 0(PU). This was followed by *w*B(PU) having
370 similar fecundity as that of *w*A*w*B(PU) (MWU: U=1479.5, p = 0.24) and *w*A(PU) (MWU:
371 U=2822.5, p = 0.22). However, *w*A(PU) showed higher fecundity than *w*A*w*B(PU) (MWU:
372 U=2388, p < 0.05) and was similar to the uninfected strain 0(PU) (MWU: U=3799.5, p = 0.75) .

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

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

377 *Wolbachia* density has a major role to play in expressing the effects of the infection on host
378 biology (Hoffmann et al., 1996; Min & Benzer, 1997). An increase in cellular *Wolbachia* density
379 is often associated with a greater expression of their effects (Breeuwer & Werren, 1993). Thus,
380 we estimated *Wolbachia* titers across different developmental stages of *N. vitripennis* males
381 and females. In the case of males (Fig. 6A) *w*A(PU) had the lowest *Wolbachia* density across
382 different larval and pupal developmental stages when compared with *w*B(PU) (MWU: U=11, $p <$
383 0.01) and *w*A*w*B(PU) (MWU: U=12, $p < 0.01$) This difference was more prominent at the adult
384 stage. However, no such differences were found between *w*B(PU) and *w*A*w*B(PU) (MWU: U=51,
385 $p = 0.56$).

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

390 Discussion

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

397 Our results (summarized in Table 2) demonstrate a sex-independent cost of the presence of
398 single and multiple *Wolbachia* infections. Many phenotypes show reduction across the sexes
399 such as longevity (Fig. 1) where infected males and females showed reduced lifespan. When
400 compared with the uninfected strain 0(PU), the *Wolbachia* infected strains *w*B(PU) and
401 *w*A*w*B(PU) have reduced life spans. However, sex-specific variations were also observed among
402 the infected strains where *w*A(PU), in females, had the shortest life span, while in the case of
403 males, had a greater life span than *w*A*w*B(PU).

404 *Wolbachia* affected the reproductive capabilities of the infected males and reduced their
405 copulation capability (Fig. 2) and also led to quicker sperm depletion (Fig. 3). Such negative
406 effects on reproductive traits were also observed in females where the infected females
407 produced fewer progenies (Fig. 4) compared to 0(PU). These differences were elicited at the
408 level of female fecundity where the number of eggs being laid by the infected females was
409 fewer (Fig. 5) indicating that the negative effects of *Wolbachia* manifested themselves even
410 before the egg-laying stage. However, the egg to larval to pupal stage mortality could also have
411 an effect on the brood sizes but these were not assayed.

412 In most cases, these negative effects disappeared with the removal of *Wolbachia*, indicating the
413 role of *Wolbachia* in producing these negative effects and not the host genotype. In phenotypes
414 like family sizes, the recently cured strains showed a significant increase suggesting that the
415 negative effects are due to the presence of *Wolbachia*. However, 0(*w*B PU) did not revert to the
416 levels of 0(PU) in the number of copulations performed and sperm depletion assays. A possible
417 reason can be some residual effects of the parent genotype in 0(*w*B PU) males.

418 Our results also demonstrate supergroup-specific negative effects on the host. Supergroup B
419 *Wolbachia* is costlier to maintain in both the sexes than supergroup A. While *w*B(PU) shows
420 strong effects on all the traits studied across the sexes, *w*A(PU) had significant negative effects
421 only on the reproductive traits of the males and the longevity of females. *w*A(PU) females, as an
422 exception, had their family sizes comparable to 0(PU). These observations are unique as no
423 comprehensive data is available on the supergroup-specific cost of *Wolbachia* infections.

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

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

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

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

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

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

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

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

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

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

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513 **Competing Interests**

514 The authors declare no conflict of interest.

515 **Author Contribution**

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

520 **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-P1pcXBnbA5Ib_HEPo

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761 **Figure legends**

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

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

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

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

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

769 Males from different *Wolbachia* infections were mated sequentially until each of them stopped
770 mating. 0(PU) could perform the highest number of copulations while *w*B(PU) the least. The
771 results show that the presence of *Wolbachia* is associated with the reduction in the number of
772 copulations a male can perform. The figure also shows whether the progenies of these
773 sequential copulations produce any daughters or not, as a measure of sperm depletion. The
774 details of sperm depletion are shown in figure 3. Sample sizes for the strains 0(PU), *w*A(PU),
775 0(*w*A PU), *w*B(PU), 0(*w*B PU), *w*A*w*B(PU) and 0(*w*A*w*B PU) were n=7, n=7, n=7, n=6, n=5, n=6
776 and n=7 respectively.

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

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

782 during each mating. The Y-axis, in grey, on the right, for each figure tallies the average number
783 of copulations that yielded at least one daughter. Thus, it measures the number of mating
784 before a male is depleted of its sperm. The left panel shows the males from *Wolbachia* infected
785 strains whereas the right panel shows their respective cured versions. Data for 0(PU) is
786 repeated at the top for comparison. The statistical significance was tested using the Mann-
787 Whitney U test with $p < 0.05$.

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

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

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

794 The measure of fecundity (number of eggs laid) by females of different infections, with virgin
795 females (A) and mated females (B). For virgin females, 0(PU) shows higher fecundity than
796 *w*A*w*B(PU), while *w*A(PU) and *w*B(PU) strains have intermediate fecundity which is comparable
797 to both 0(PU) and *w*A*w*B(PU). However, for mated females, 0(PU) and *w*A(PU) show higher
798 fecundity than *w*B(PU) and *w*A*w*B(PU). The statistical significance is tested with the Mann-
799 Whitney U test, $p<0.05$.

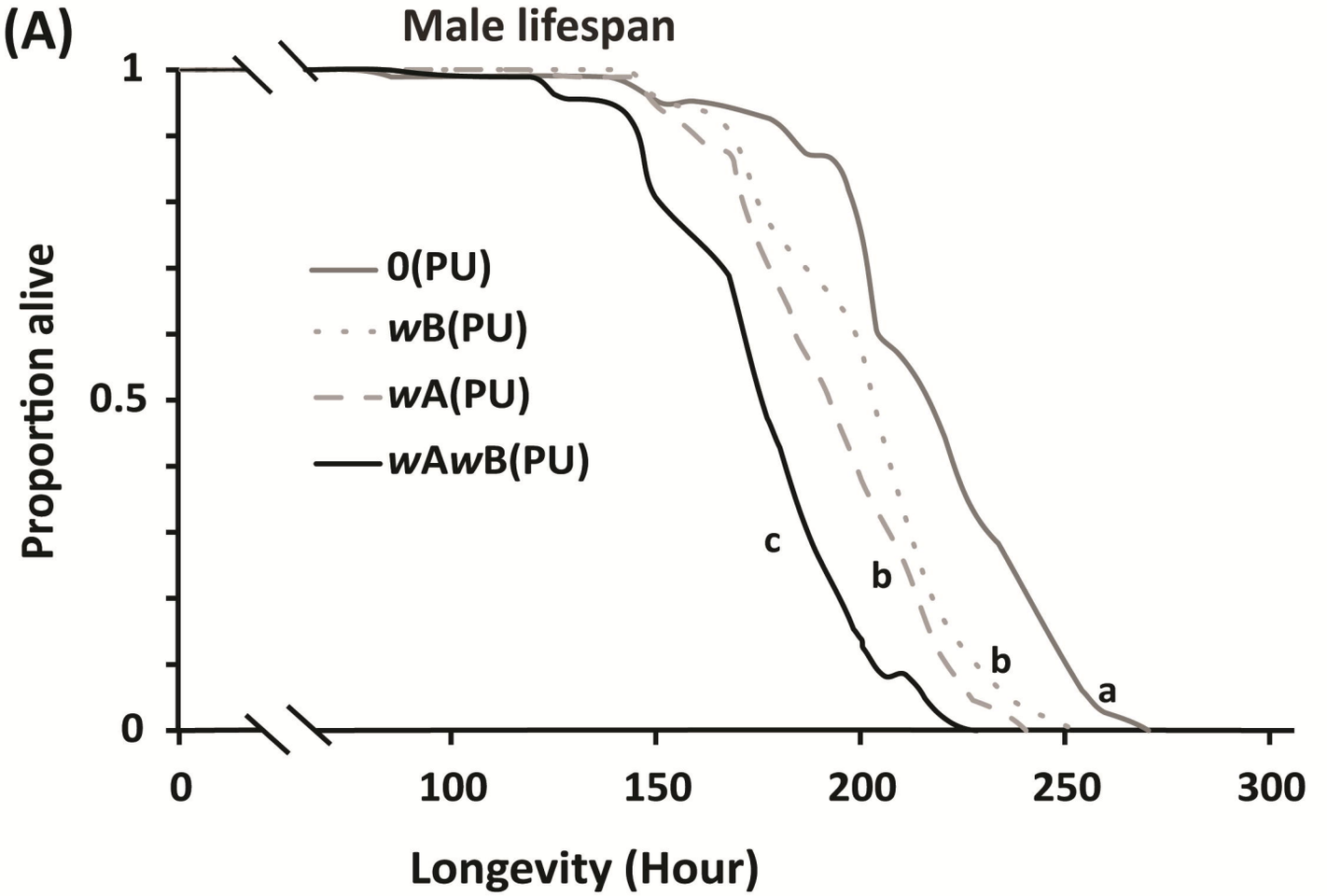
800 **Figure 6. Quantitative estimation of *Wolbachia* across different developmental stages of *N.***

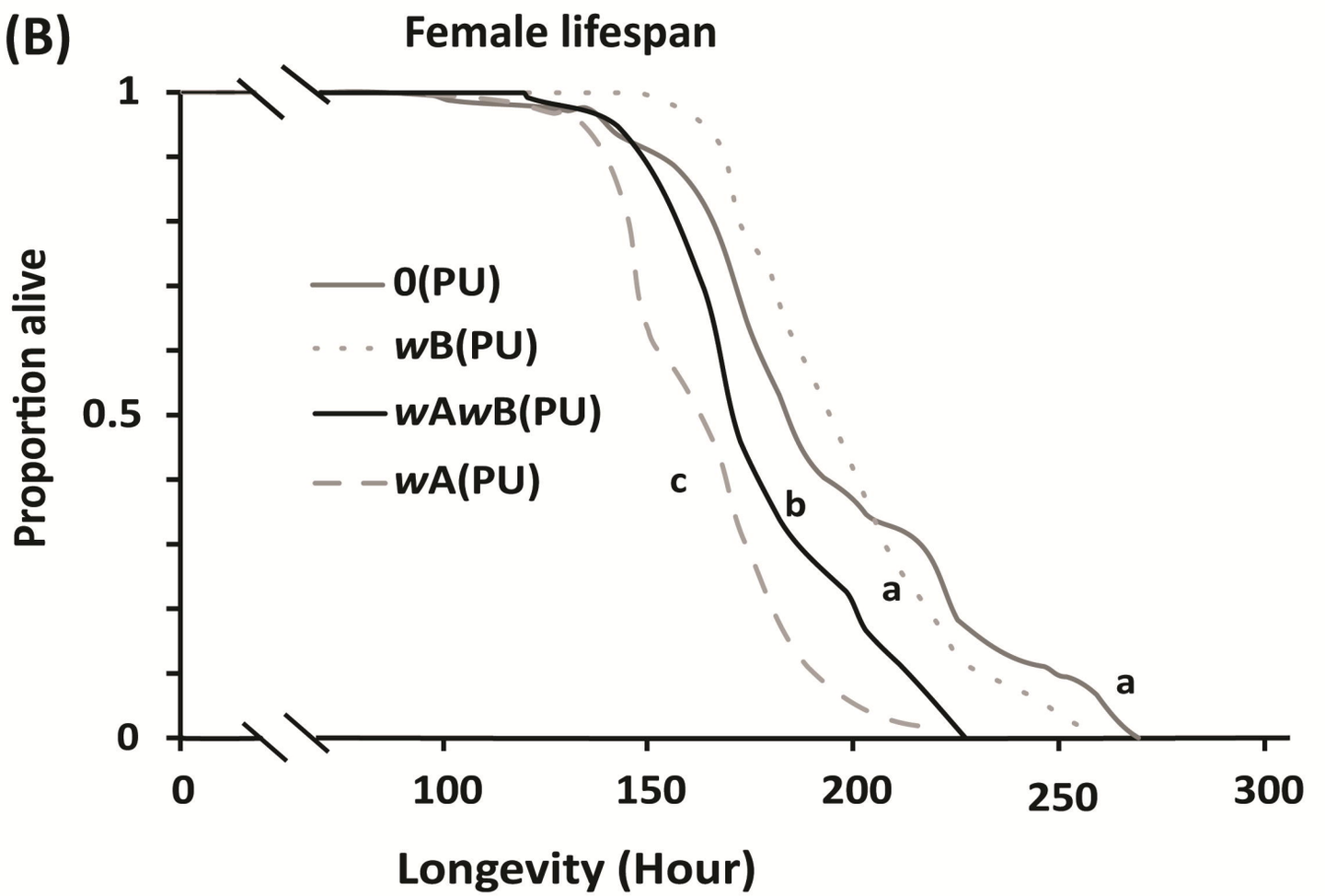
801 ***vitripennis* males (A) and females (B).** The statistical significance between groups is tested

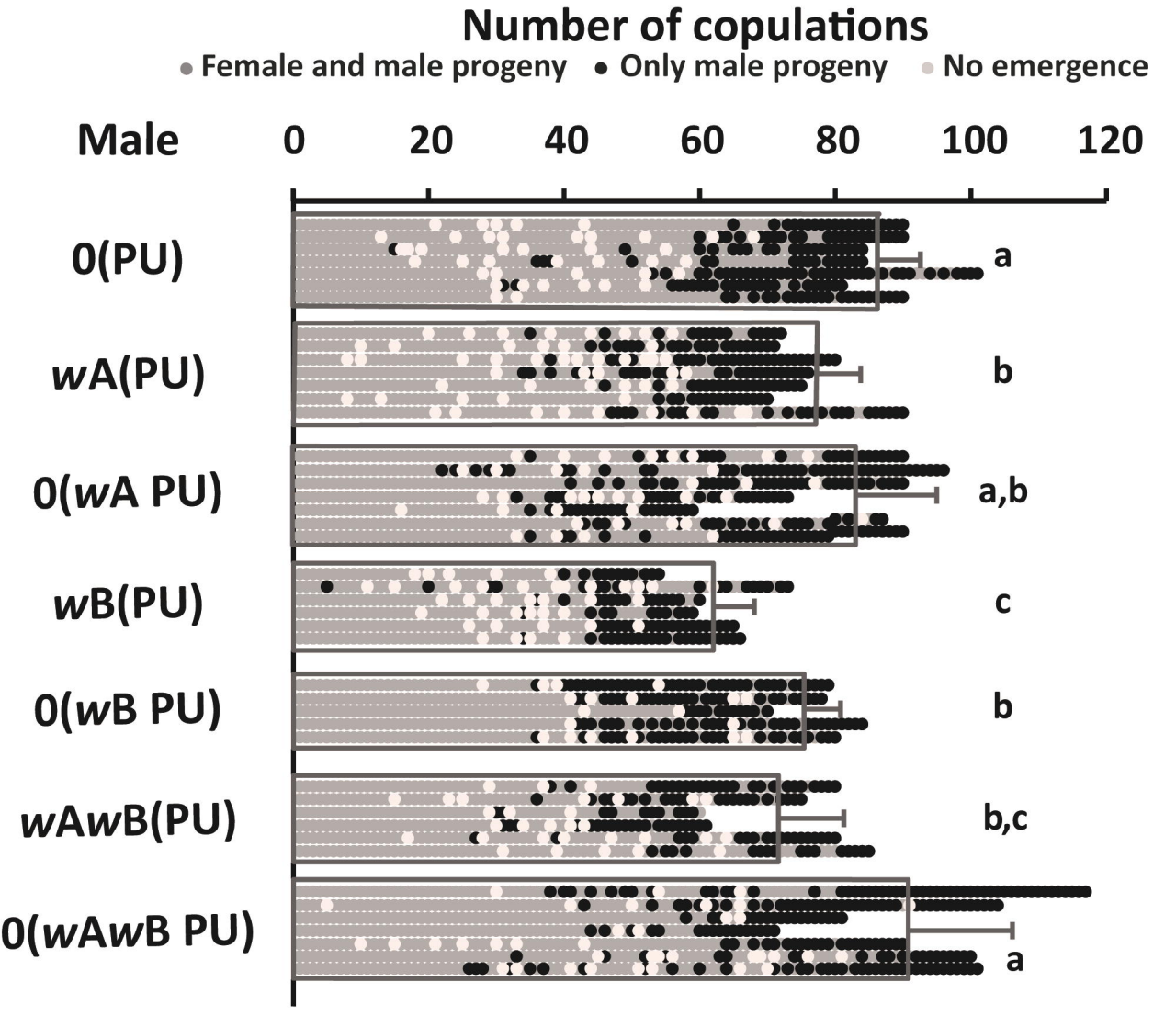
802 using the Mann-Whitney U test, $p < 0.05$.

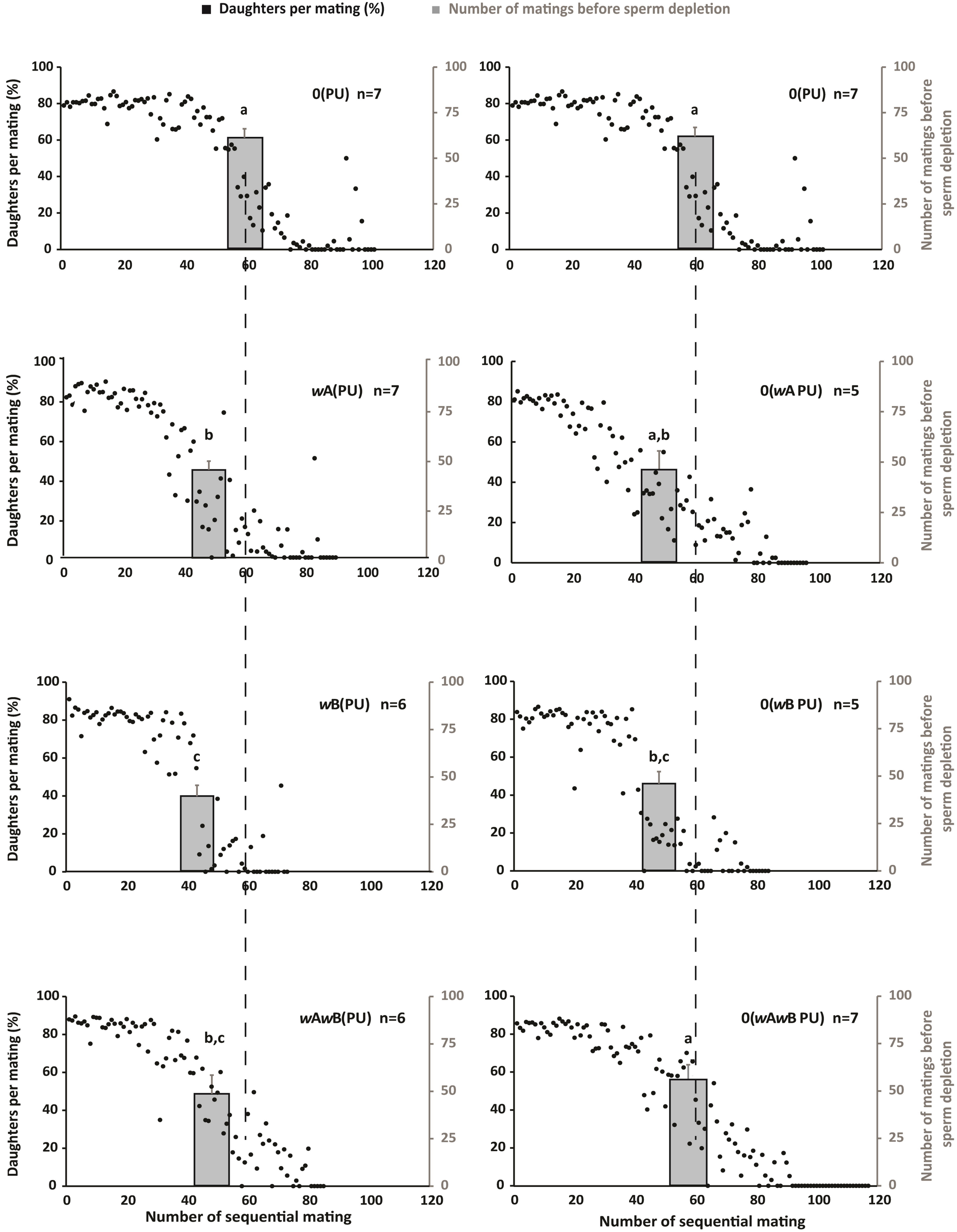
803 **Table 1.** Negative fitness effects of CI-inducing *Wolbachia*

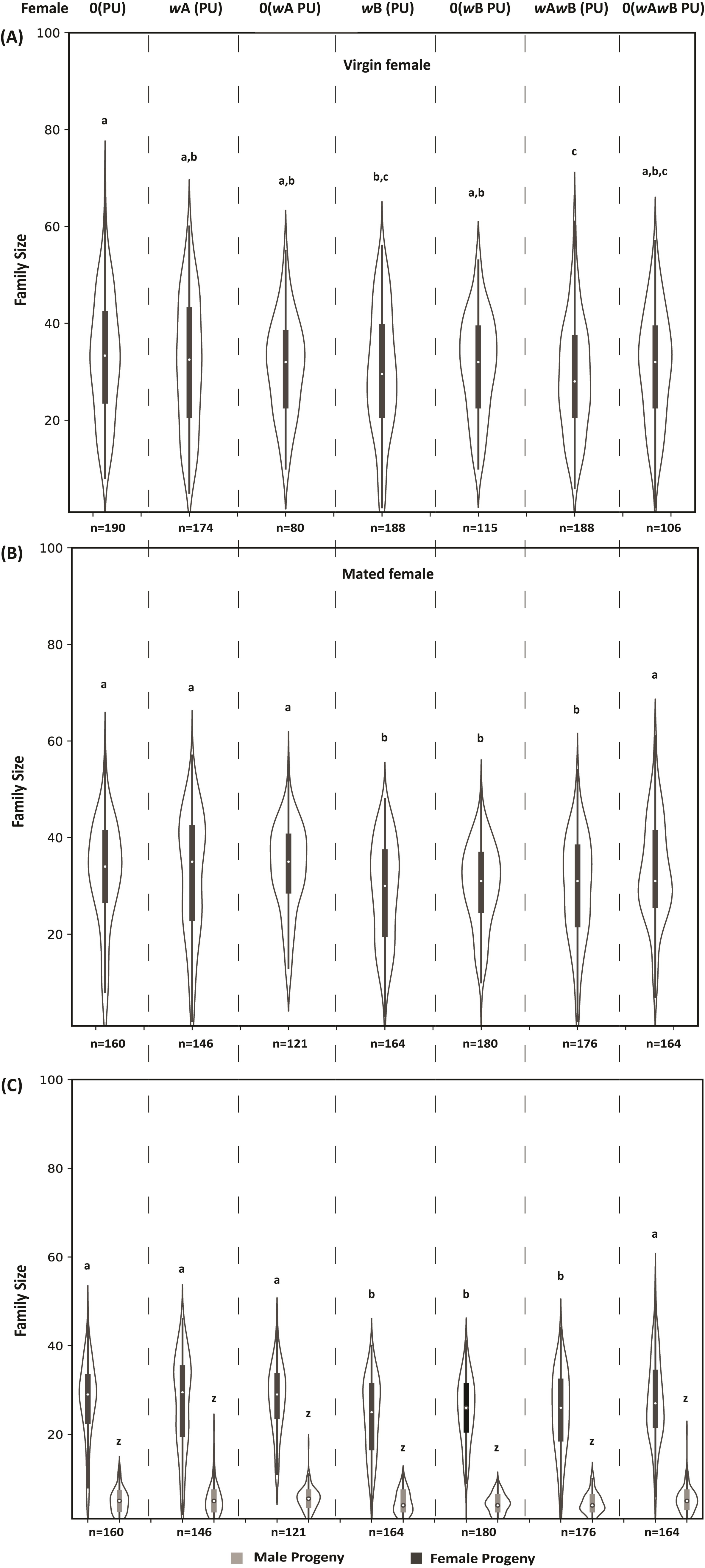
804 **Table 2.** Effect of *Wolbachia* infections on *N. vitripennis* (Summary)

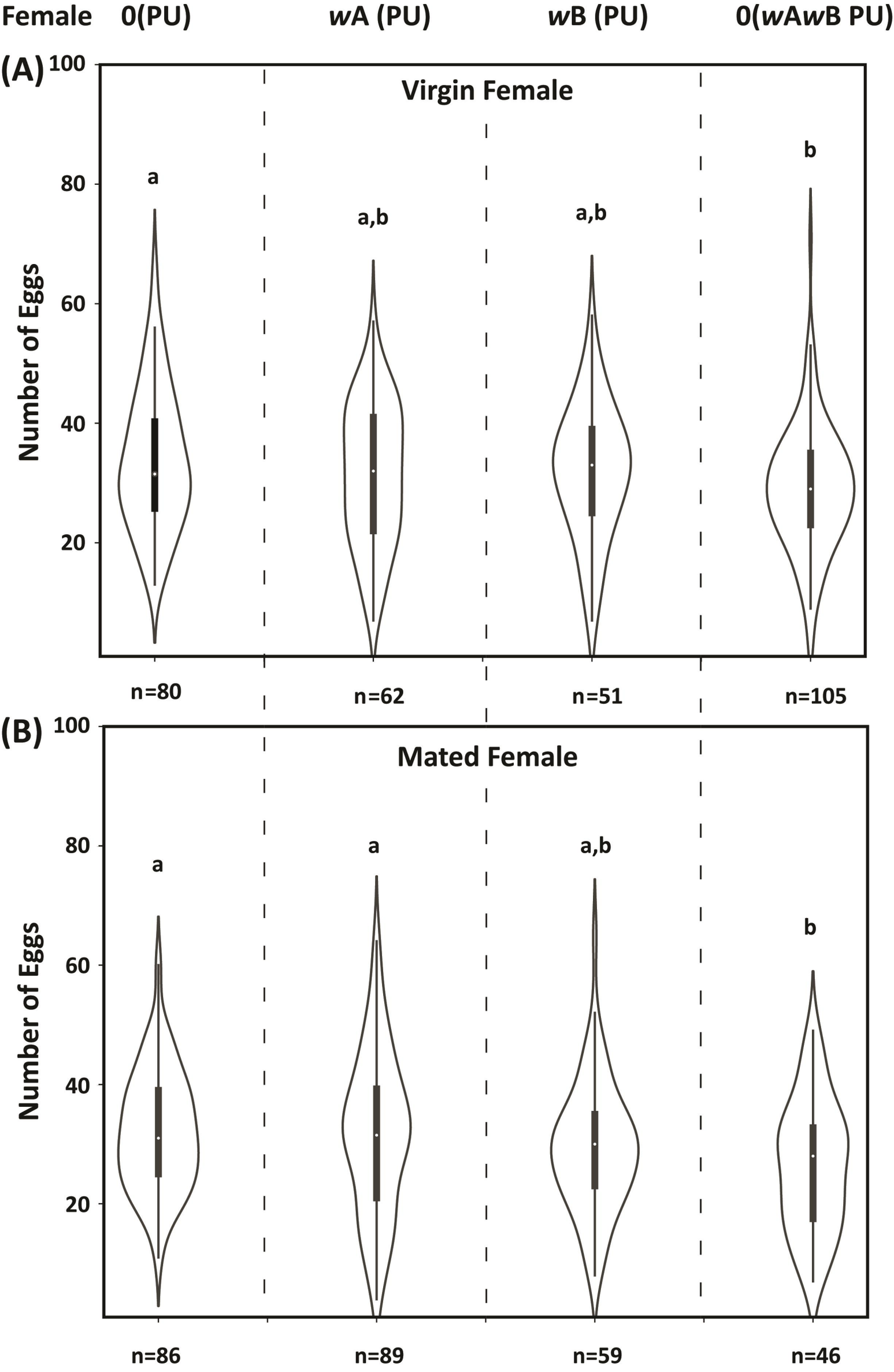

(A)
Male lifespan
Proportion alive
1
0.5
0
0(PU)
wB(PU)
wA(PU)
wAwB(PU)
c
b
b
a
0
100
150
200
250
300
Longevity (Hour)


(B)
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)
(A)
Virgin female
Family Size
a
a,b
a,b
b,c
a,b
c
a,b,c
n=190
n=174
n=80
n=188
n=115
n=188
n=106
(B)
Mated female
Family Size
a
a
a
b
b
b
a
n=160
n=146
n=121
n=164
n=180
n=176
n=164
(C)
Family Size
a
z
a
z
a
z
b
z
b
z
b
z
a
z
n=160
n=146
n=121
n=164
n=180
n=176
n=164
Male Progeny
Female Progeny


Female
0(PU)
wA (PU)
wB (PU)
0(wAwB PU)
(A)
Virgin Female
Number of Eggs
100
80
60
40
20
a
a,b
a,b
b
n=80
n=62
n=51
n=105
(B)
Mated Female
Number of Eggs
100
80
60
40
20
a
a
a,b
b
n=86
n=89
n=59
n=46

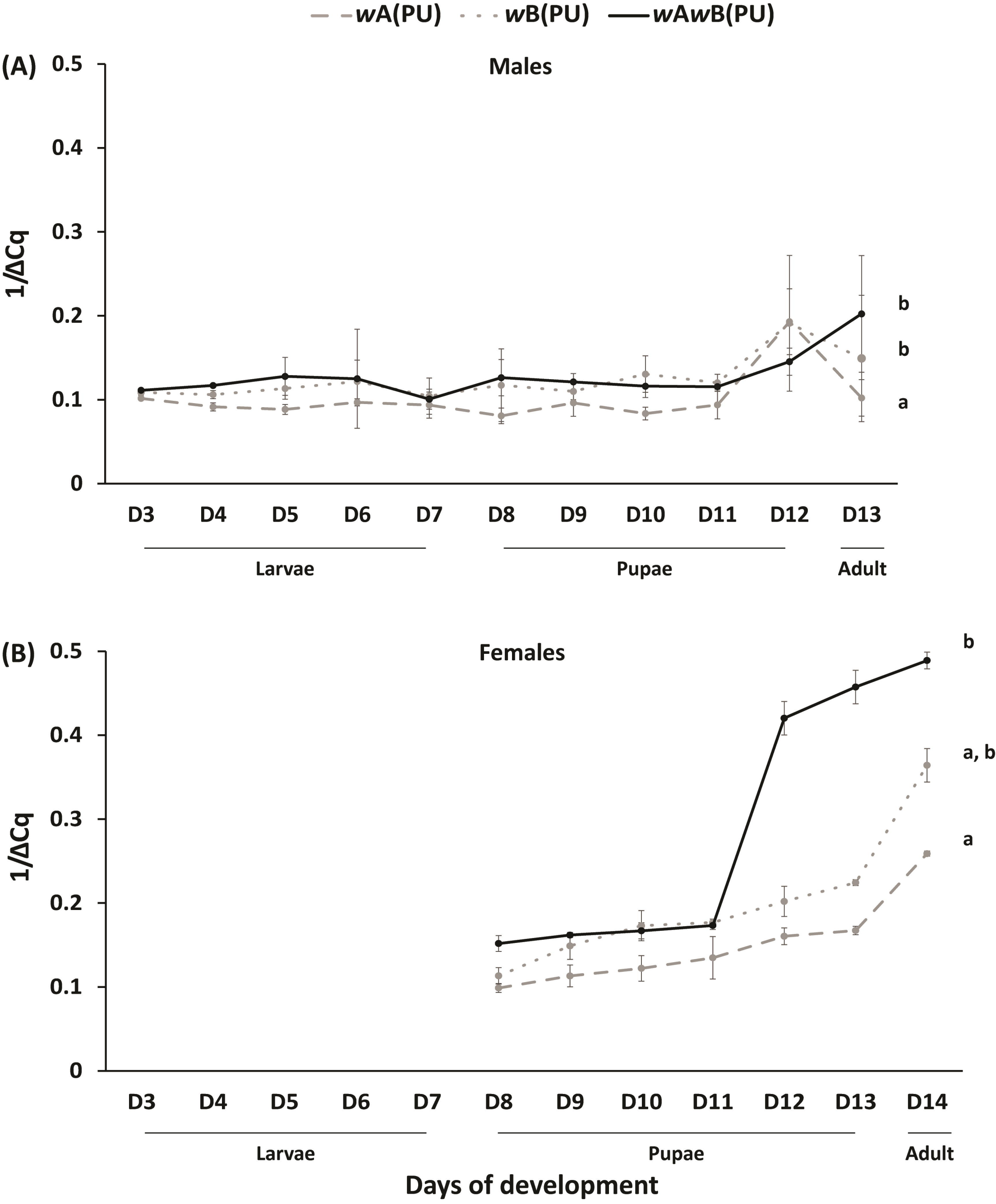

wA(PU)
wB(PU)
wAwB(PU)
(A)
Males
1/ΔCq
0.5
0.4
0.3
0.2
0.1
0
b
b
a
D3
D4
D5
D6
D7
D8
D9
D10
D11
D12
D13
Larvae
Pupae
Adult
(B)
Females
1/ΔCq
0.5
0.4
0.3
0.2
0.1
0
b
a, b
a
D3
D4
D5
D6
D7
D8
D9
D10
D11
D12
D13
D14
Larvae
Pupae
Adult
Days of development

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

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

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

Phenotype	Host Sex	Effect of *Wolbachia*	"Cost" compared to 0(PU)
Lifespan	Male	0(PU) > *w*B(PU) = *w*A(PU) > *w*A*w*B(PU)	*w*A(PU) = 11.1%, *w*B(PU) = 6.5%, *w*A*w*B(PU) = 15.5%
	Female	0(PU) = *w*B(PU) > *w*A*w*B(PU) > *w*A(PU)	*w*A(PU) = 17.7%, *w*A*w*B(PU) = 15.5%
Number of copulations	Male	0(PU) > *w*A(PU) *w*B(PU) and *w*A*w*B(PU) *w*A(PU) = *w*A*w*B(PU), *w*A(PU) > *w*B(PU), *w*A*w*B(PU) = *w*B(PU)	*w*A(PU) = 12.4%, *w*B(PU) = 28.8%, *w*A*w*B(PU) = 16.7%
Sperm depletion	Male	0(PU) > *w*A(PU) *w*B(PU) and *w*A*w*B(PU) *w*A(PU) = *w*A*w*B(PU), *w*A(PU) > *w*B(PU), *w*A*w*B(PU) = *w*B(PU)	*w*A(PU) = 19.4%, *w*B(PU) = 31.3%, *w*A*w*B(PU) = 15.7%
Progeny family size	Female		
	a. Virgin	0(PU) > *w*B(PU) and *w*A*w*B(PU), 0(PU) = *w*A(PU), *w*A(PU) > *w*A*w*B(PU), *w*A(PU) = *w*B(PU), *w*B(PU) = *w*A*w*B(PU)	*w*B(PU) = 10%, *w*A*w*B(PU) = 12.4%
	b. Mated	0(PU) =*w*A(PU) > *w*A*w*B(PU) = *w*B(PU)	*w*B(PU) = 11.5%, *w*A*w*B(PU) = 9.1%
Fecundity	Female		
	a. Virgin	0(PU) = *w*A(PU) = *w*B(PU), 0(PU) > *w*A*w*B(PU)	*w*A*w*B(PU) = 14.1%
	b. Mated	0(PU) = *w*A(PU) > *w*B(PU) = *w*A*w*B(PU)	*w*B(PU) = 9.1%, *w*A*w*B(PU) = 18.2%
***Wolbachia* density**	Male	*w*A*w*B(PU) > *w*B(PU) = *w*A(PU)	
	Female	*w*A*w*B(PU) > *w*A(PU) = *w*B(PU)	