

1 **Transinfection of buffalo flies (*Haematobia exigua*) with *Wolbachia* and effect on host biology**

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16

17 **Abstract**

18 A widespread insect endosymbiont *Wolbachia* is currently of much interest for use in novel strategies
19 for the control of insect pests and blocking transmission of insect-vector diseases. *Wolbachia*-
20 induced effects can vary from beneficial to detrimental depending on host biology and the genetic
21 background of the infecting strains. As a first step towards investigating the potential of *Wolbachia* for
22 use in the biocontrol of buffalo flies (BF), embryos, pupae, and adult female BF were injected with three
23 different *Wolbachia* strains (*wAlbB*, *wMel* and *wMelPop*). BF eggs were not easily injected because of
24 their tough outer chorion and embryos were frequently damaged resulting in less than 1% hatch rate of
25 microinjected eggs. No *Wolbachia* infection was recorded in flies successfully reared from injected
26 eggs. Adult and pupal injection gave a much higher survival rate and resulted in somatic infection and
27 germinal tissue infection in surviving flies with transmission to the succeeding generations on a number
28 of occasions. Investigations of infection dynamics in flies from injected pupae confirmed that *Wolbachia*
29 were increasing in numbers in BF somatic tissues and ovarian infections were confirmed with *wMel* and
30 *wMelPop* in some instances, though not with *wAlbB*. Measurement of fitness traits indicated reduced
31 longevity, decreased and delayed adult emergence, and reduced fecundity in *Wolbachia*-infected flies
32 in comparison to mock-injected flies. Furthermore, fitness effects varied according to the *Wolbachia*
33 strain injected with most marked reductions seen in the *wMelPop*-injected flies and least severe effects
34 seen with the *wAlbB* strain.

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36

37 **Keywords:** *Wolbachia*, *Haematobia*, biocontrol, veterinary ectoparasite, endosymbiont, pest
38 management.

39 **Introduction**

40 Buffalo flies (BF), *Haematobia exigua* are obligate hematophagous ectoparasites of cattle [1]. They are
41 present in the Australasian, Oriental and Palearctic regions of the world [2]. Both female and male BF
42 feed 20-40 times a day on cattle, and the females only leave cattle to oviposit in freshly deposited cattle
43 manure [3]. Their blood-feeding habits result in significant economic losses by reducing milk and meat
44 production and causing defects in cattle leather [4, 5]. Further, BF infestation is a significant welfare
45 issue with biting by flies causing severe irritation and, in association with a filarial nematode transmitted
46 by BF (*Stephanofilaria* sp.), the development of lesions that range from dry, hyperkeratotic and alopecic
47 areas to open suppurating ulcerated sores. BF are tropical and subtropical in their distribution and are
48 mainly pests of cattle in the northern parts of Australia [6]. However, aided by a warming climate and
49 reduced efficiency of control because of the development of chemical resistance, they have been
50 steadily expanding their range southward [2, 6-8].

51 *Wolbachia*, are maternally inherited endosymbionts of insects, that are of much interest for use in the
52 biological control of pests, most particularly as a basis for area-wide integrated control strategies for a
53 range of insect species [9-11]. *Wolbachia* has been used in insect control programs in two main ways.
54 First, it has been used as a means to achieve population replacement, where *Wolbachia*-infected
55 insects impart unique characteristics such as pathogen blocking or fitness deficits, and second, by the
56 incompatible insect technique (IIT) in which *Wolbachia*-infected males released into the population
57 cause the production of non-viable eggs, similar to the sterile male technique [11-14]. Both of these
58 strategies are based on cytoplasmic incompatibility (CI) and the resultant ability of *Wolbachia* to spread
59 through uninfected or differentially infected populations [14]. Some of the novel fitness costs induced by
60 *Wolbachia* include decreased fecundity and male competitiveness, seen in *Anopheles stephensi*
61 infected with wAlbB, lifespan reduction, egg mortality, delayed larval development and altered feeding
62 behaviour seen in *Aedes aegypti* infected with wMelPop [15-20].

63 The first successful field trial of the *Wolbachia*-based IIT technique was in Myanmar in early 1960's to
64 eliminate a native population of *Culex quinquefasciatus* mosquitoes responsible for transmitting
65 filariasis [21]. Following the trial success, this strategy has been widely studied in mosquito species
66 including *Aedes polynesiensis*, *Aedes albopictus*, *Anopheles stephensi*, *Culex pipiens pallens*, and in
67 tsetse flies (*Glossina morsitans*) [10, 22-26]. Presently, wMel-infected *Ae. aegypti* mosquitoes are being
68 released in Australia, Asia (Fiji, India, Sri Lanka, and Vietnam), North America (Mexico), and South

69 America (Colombia, Brazil) to suppress mosquito-transmitted diseases of humans such as dengue
70 fever and Zika virus [27, 28].

71 The first step towards developing *Wolbachia* based control programs is the establishment of *Wolbachia*
72 transinfected lines of the target pest. The most common method used to transinfect new hosts with
73 *Wolbachia* has been embryonic microinjection, although injection into other stages, such as adults and
74 pupae have also given some success [14]. Of the available transinfection procedures, embryonic
75 microinjection is mostly preferred as *Wolbachia* are directly introduced to the pole cells of pre-
76 blastoderm embryos using a fine needle inserted at the posterior end of the egg, desirably resulting in
77 germline and somatic cell infection. In contrast, adult injection is usually carried out into the thoracic or
78 abdominal regions of adults where *Wolbachia* must successfully evade or overcome a number of
79 membrane barriers and the host immune response to become established in the germinal tissues for
80 next-generation transmission [14]. Some instances of successful use of adult microinjection to
81 transinfect new insect strains include the transfer of *wMel* strain to *Drosophila melanogaster*, *wAlbA*
82 and *wAlbB* to *Ae. aegypti*, and *wRi*, *wMel*, *wHa*, and *wNo* to the leafhopper *Laodelphax striatellus* [14,
83 29-31].

84 Buffalo flies collected from twelve locations in Australia and Indonesia were negative for *Wolbachia*
85 infection, and this has been confirmed by more recent testing in our lab (unpublished data) [32].
86 However, *Wolbachia* appears to be ubiquitous in closely related horn flies (*Haematobia irritans*) (HF)
87 suggesting that BF will also be a competent host for *Wolbachia* [32-38]. In previous studies, *Wolbachia*
88 has been mostly sourced from the egg of the infected species for microinjection purposes [14].
89 Nevertheless, using cell lines of the intended host artificially infected with *Wolbachia* as the donor
90 source has been suggested as advantageous for obtaining a high density and host context adapted
91 *Wolbachia*. Hence, we established the HIE-18 cell line from HF to adapt *wAlbB* obtained from mosquito,
92 *wMel*, and *wMelPop* from *Drosophila* into the *Haematobia* spp. context prior to commencing BF
93 microinjection.

94 Here, we report the results of studies towards the establishment of lines of BF sustainably infected with
95 the *wAlbB*, *wMel*, and *wMelPop* strains of *Wolbachia* and the dynamics and kinetics of infection in
96 microinjected flies. The results of preliminary investigations into the related physiological costs of
97 *Wolbachia* infection on the newly infected host BF, which are critical to considerations of the potential
98 for use in biological control programs, are also described.

99

100 **Material and Methods**

101 **Establishment of *Wolbachia*-infected cell cultures**

102 A non-infected *Drosophila* cell line (JW18) was infected with the *wAlbB* (JW18-*wAlbB*), *wMel* (JW18-
103 *wMel*), and *wMelPop* (JW18-*wMelPop*) strains of *Wolbachia* following the protocol of Hebert et al.
104 (2017) to first adapt them in a closely related species [39]. JW18 cell lines infected with the three strains
105 of *Wolbachia* were cultured in a 75 cm² flask in 12 ml Schneider's medium supplemented with 10% FBS
106 at 28 °C (Sigma Aldrich, NSW, Australia). The *Haematobia* embryonic cell line (HIE-18) maintained in
107 our lab without the use of antibiotics were transfected with *wAlbB* (*wAlbB*-HIE-18), *wMel* (*wMel*-HIE-
108 18) and *wMelPop* (*wMelPop*-HIE-18) as above. The infected HIE-18 lines were cultured in 75 cm² flasks
109 containing 12 ml of Schneider's medium supplemented with 10% FBS at 28°C and subcultured every
110 5-6 days by splitting at a ratio of 1:2 into new flasks (Sigma Aldrich, NSW, Australia).

111 ***Wolbachia* isolation**

112 *Wolbachia* were isolated from the cell lines, according to Herbert et al. (2017) [21]. Briefly, *wAlbB*, *wMel*,
113 and *wMelPop* infected cell lines were grown in 75 cm² cell culture flasks for seven days using previously
114 noted methods. Cells were pelleted on the eighth day by spinning at 2000 x g and washed three times
115 with SPG buffer (218 mM sucrose, 3.8 mM KH₂PO₄, 7.2 mM MK₂HPO₄, 4.9 mM L- glutamate, pH 7.5),
116 sonicated on ice for two bursts of 10 sec and cellular debris was removed by spinning at 1000 x g for
117 10 min at 4 °C. The supernatant was passed through 50 µm and 2.7 µm acrodisc syringe filters
118 (Eppendorf, NSW, Australia) and centrifuged at 12000 x g to pellet *Wolbachia*. Finally, the pellet was
119 suspended in 100 µl SPG buffer and used for microinjection.

120 **Embryonic microinjection**

121 Buffalo flies were held in temporary cages for 20-30 min to collect eggs of similar age. Newly laid eggs
122 (40 - 60 min old) were arranged on double-sided sticky tape using a paintbrush and microinjected at
123 the posterior pole of each egg with *wAlbB* (2x10⁸ bacteria/ml) using a FemtoJet microinjector system
124 (Eppendorf, NSW, Australia). The microinjected eggs were then placed on tissue paper on the surface
125 of artificial manure pats to hatch. After eclosion, larvae migrated into the moist manure where they fed
126 until pupation. Pupae were separated from the manure by flotation in water on day 7 post-injection and
127 incubated at room temperature. Flies that emerged from the puparium by day 10 were collected and

128 separated by sex. Females that emerged from microinjected eggs were held singly with two males for
129 mating in small cages made of transparent acrylic pipe (6 cm diameter x 15 cm height) closed with fly
130 mesh and a membrane feeder at the top supplying cattle blood maintained at 26 °C. A 55 cm² petri-dish
131 containing moist filter paper was placed at the base of the cages for collection of eggs deposited by the
132 flies. Females were allowed to oviposit, and the eggs were collected until the death of the flies. Dead
133 flies were collected and tested for the presence of *Wolbachia* using real-time PCR.

134 **Adult microinjection**

135 Approximately 100-150 pupae from the BF colony at the EcoScience Precinct, Brisbane, Australia were
136 held separately from the main colony for collection of freshly emerged female flies (2-3 hrs old) for
137 injection. The female flies were collected within 3-4 h of eclosion from the pupae, anaesthetised using
138 CO₂ for 30-40 s, and then 2 µl of *Wolbachia* suspension (3x10⁹ bacteria/ml) was injected into the
139 metathorax of each fly using a handheld micro-manipulator (Burkard Scientific, London, UK) with
140 hypodermic needles (0.24 X 33 mm). The microinjected flies (G₀) were blood-fed and mated with male
141 flies at the ratio of 1:1 in small cages as described above. On day three after injection, an artificial 100
142 g manure pat was placed onto sand at the base of each cage. Manure pats were removed every second
143 day, and the collected eggs were reared to adults following our standard laboratory protocols. Newly
144 hatched G₁ female flies were mated to potentially infected males, allowed to oviposit until death and the
145 dead G₁ flies then tested by real-time PCR for the presence of *Wolbachia*. Depending on the results of
146 testing, the cycle was repeated.

147 **Pupal microinjection**

148 Approximately 3000-4000 eggs from colony-reared BF were incubated and the larva grown on manure
149 to collect freshly pupated BF for microinjection (1-2 h old). Pupae were aligned on double-sided sticky
150 tape and injected in the third last segment at the posterior end close to germinal tissue using a FemtoJet
151 microinjector system (Eppendorf, NSW, Australia). The microinjected pupae were then placed on moist
152 Whatman filter paper and incubated at 27°C until flies emerged. Freshly emerged flies were separated
153 and placed in a cage with a maximum of five females and five males each. Eggs collected from each
154 cage every day were tested for *Wolbachia* infection. Once infection was detected, female flies were
155 separated into a separate single cage and eggs were collected for the G₁ line until the flies died. Later,
156 dead females were tested for the presence of *Wolbachia* using real-time PCR.

157 ***Wolbachia* diagnostic assay**

158 A modified Chelex extraction protocol from Echeverria-Fonseca et al. (2015) was used for extraction of
 159 DNA from the embryonic and adult microinjected samples [40]. Briefly, flies were homogenised using a
 160 Mini-Beadbeater (Biospec products, Oklahoma, USA) for 5 min in 2 ml screw-cap vials with 2 g of glass
 161 beads (2mm) and 200 µl of buffer containing 1 X TE buffer and Chelex®-100 (Bio-Rad Laboratories,
 162 CA, USA). Samples were then incubated overnight at 56 °C with 10 µl of Proteinase K (20mg/ml) and
 163 dry boiled the next day for 8 min at 99.9 °C. Finally, samples were spun at 13000 X g for 15 min, and
 164 the supernatant was stored at -20 °C until tested. For pupal-injected samples and eggs, DNA was
 165 extracted using an Isolate II Genomic DNA extraction kit (Bioline, NSW, Australia). DNA was amplified
 166 with strain-specific primers using a Rotor-Gene Q machine (Qiagen, NSW, Australia) (Table 1).
 167 Reactions were run in a total of 10 µl having 5 µl PrimeTime® Gene Expression Master Mix (IDT, VIC,
 168 Australia), 0.5 µl each of 10 µM forward and reverse primer, 0.25 µl of 5 µM probe and 3 µl of genomic
 169 DNA. Negative and positive PCR controls were run with every batch of the samples. Optimised
 170 amplification conditions for wMel and wMelPop were 3 min at 95 °C followed by 45 cycles of 10 s at 95
 171 °C, 15 s at 51 °C, and 15 s at 68 °C. For wAlbB, the optimized amplification conditions were 3 min at 95
 172 °C followed by 45 cycles of 20 s at 94 °C, 20 s at 50 °C, and 30 s at 60 °C. To analyse the data, dynamic
 173 tube along with the slope correct was turned on, and the cycle threshold was set at 0.01. Any sample
 174 having CT score < 35 was considered positive, negative in case of no amplification or CT score equal
 175 to zero, and suspicious where CT>35.

176 **Table 1:** List of primers used for the *Wolbachia* Screening in the BF.

Strain	Primer & Probe (5'-3')	Reference
wAlbB	GF_5'-GGTTTTGCTGGTCAAGTA-3' BR_5'-GCTGTAAAGAACGTTGATC-3' FAM_5'-TGT TAG TTA TGA TGT AAC TCC AGAA-TAMRA-3'	[31]
wMel	WD0513_F_5'-CAAATTGCTCTTGTCCTGTGG-3' WD0513_R_5'-GGGTGTTAAGCAGAGTTACGG-3' WD0513_Probe_Cy5'-TGAAATGGAAAATTGGCGAGGTGTAGG-BHQ-3'	[20]
wMelPop	IS5_F_5'-CTCATCTTTACCCGTAATAAAATTC-3' WD1310_R_5'-TCTTCCTCATTAAGAACCTCTATCTTG-3'	[20]

	IS5_Probe_5'-Joe-TAGCCTTTTACTTGTTTCCGGACAACCT-TAMRA-3'	
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178 **Fluorescence *in situ* hybridisation (FISH)**

179 FISH was carried out to visualise *Wolbachia* distribution in female BF post adult microinjection using a
180 method slightly modified from that of Koga et al. (2009) [41]. Briefly, for the whole-mount assay, 10 BF
181 infected with wMel and wMelPop were collected six days post-injection and fixed in Carnoy's solution
182 (a mixture of chloroform, ethanol and acetic acid) at a ratio of 6:3:1 overnight. Flies were washed the
183 next day sequentially in 100% ethanol, 80% ethanol, 70% ethanol and stored in 10% H₂O₂ in 100%
184 ethanol for 30 days to quench the autofluorescence. Preserved flies were subsequently washed three
185 times with 80% ethanol, 70% ethanol, and PBSTx (0.8% NaCl, 0.02% KCl, 0.115% Na₂HPO₄, 0.02%
186 KH₂PO₄, 0.3% Triton X- 100) and pre-hybridised with hybridisation buffer (4 X SSC, 0.2 g/ml dextran
187 sulphate, 50% formamide, 250 µg/ml Poly A, 250 µg/ml salmon sperm DNA, 250 µg/ml tRNA, 100 mM
188 DTT, 0.5x Denhardt's solution) without probe two times for 15 min each. The insects were then
189 incubated with hybridisation buffer and *Wolbachia* 16S rRNA probes overnight [42]. The next morning,
190 samples were washed three times with PBSTx, three times for 15 min each and finally incubated in
191 PBSTx containing DAPI (10 mg/ml) for 30 min. Samples were then rewashed with PBSTx, covered with
192 ProLong Diamond Antifade Mountant (Thermofisher, Australia) and photographed using a confocal
193 microscope.

194 ***Wolbachia* quantification assay**

195 DNA was extracted from whole female BF post adult and pupal injection using an Isolate II Genomic
196 DNA extraction kit (Bioline, NSW, Australia). Six flies were assayed at each point of time for
197 determination of the relative *Wolbachia* density. Real-time PCR assays were carried out in triplicate to
198 amplify the *Wolbachia* *wsp* gene [43] and host reference gene *GAPDH* (378 F_ 5'-
199 CCGGTGGAGGCAGGAATGATGT-3', 445 R_ 5'-CCACCCAAAAGACCGTTGACG-3') on a Rotor-gene
200 Q Instrument (Qiagen, NSW, Australia). Reactions were run in a total volume of 10 µl having 5 µl Rotor-
201 Gene SYBR® Green PCR Kit (Qiagen, NSW, Australia), 0.3 µl each of 10 µM forward and reverse
202 primer and 2 µl of genomic DNA. Negative and positive PCR controls were included in all runs.
203 Amplification was conducted for 5 min at 95 °C followed by 45 cycles of 10 sec at 95 °C, 15 s at 55 °C,

204 and 15 s at 69 °C, acquiring on the green channel at the end of each step. Finally, *Wolbachia* density
205 was calculated relative to host *GAPDH* using the delta-delta CT method [44].

206 **Survival assay**

207 Two to three-hour old female adult BF were injected with *Wolbachia* (*wAlbB*, *wMel*, and *wMelPop*) or
208 SPG buffer (injected control) as described above and placed in triplicate cages containing ten flies each.
209 Flies were cultured under laboratory conditions in small cages, and mortality was noted every 12 hours.
210 Dead flies were later tested for *Wolbachia* infection individually using real-time PCR as described
211 above. The survival assay for microinjected pupae was carried out as per the adult assay except that
212 the number of flies in each cage was 20 (ten male and ten female).

213 **Adult emergence rate post pupal microinjection with *Wolbachia***

214 Data from five independent pupae-microinjected batches were used to analyse the effect of *Wolbachia*
215 on adult emergence. All three *Wolbachia* strains were injected in parallel to the buffer-injected controls.
216 The number of injected pupae varied between batches from 77 to 205 for *wMel*, 98 to 145 for *wAlbB*,
217 and 82 to 148 for *wMelPop*. The emergence of adults was recorded each day and the ratio of total
218 emerged to number of injected pupae was calculated to determine the final percentage of emergence.

219 **Total egg production post pupal microinjection with *Wolbachia***

220 The effect of *Wolbachia* on the number of eggs produced by females after pupal microinjection was
221 assessed in triplicate with ten females per cage. Buffer-injected females were used as controls and
222 number of eggs laid and females surviving were counted every 24 hours to estimate eggs laid per day
223 per female. Dead females were later tested for the presence of *Wolbachia* using real-time PCR.

224

225 **Results**

226

227 **Embryonic microinjection of buffalo flies**

228 Of a total of 2036 eggs microinjected with the *wAlbB* strain only 10 developed through to adult flies (six
229 females and four males) and no infection was detected in any of the adults. Microinjecting buffalo flies
230 is particularly difficult because of the tough chorion surrounding the egg (Fig. 1A). We observed a
231 significant detrimental effect of injection on embryo survival and hatching (one-way ANOVA: $F_{2, 6} =$
232 455.3, $p < 0.0001$) and identified that older eggs (40-60 min) had a better injection survival rate, 21.96%

233 compared to 3.4% for younger eggs (10-30 min) (Tukey's multiple comparison test: $p=0.010$) (Fig. 1B).
234 A number of other variations of the technique were tested to improve the survival rate of eggs post
235 microinjection. These included dechorionation of the eggs with 2.5 % sodium hypochlorite for 30 s to
236 soften the chorion, partial desiccation to reduce hydrostatic pressure in the eggs and increase space
237 for the retention of larger volumes of injectate, and the use of halocarbon oil (2:1 mix of halocarbon 700
238 and 27) to prevent desiccation of the eggs. None of these treatments markedly improved survival post
239 microinjection (2.33%) and they also appeared to reduce egg survival in uninjected eggs (16.33%) (one-
240 way ANOVA: $F_{2,6} = 181.6$, $p < 0.0001$) (Fig. 1C).

241

242 ***Wolbachia* dynamics and tropism post adult injection**

243 The growth kinetics of *Wolbachia* were studied in injected female flies by quantifying *Wolbachia* on days
244 3-11 compared to day zero (day of injection). Overall, the pattern showed an initial significant decrease
245 in *Wolbachia* density to approximately day five followed by subsequent growth and increase in bacterial
246 titre to day eleven in all three strains (Kruskal-Wallis test: $p < 0.0001$) (Fig. 2A-C).

247 Significant variation in *Wolbachia* growth dynamics after injection required a better understanding of
248 tissue tropism. Hence, fluorescence *in situ* hybridisation (FISH) was carried out on whole mounted BF
249 and dissected ovaries to visualise the localisation of *wMel* and *wMelPop* *Wolbachia* six days after
250 injection (Fig. 3). No infection in the germline tissue was evident in any of the six samples analysed
251 from each strain. However, *Wolbachia* was widely distributed in somatic tissues including the thoracic
252 muscle, head, abdominal area, proboscis and legs (Fig 3).

253 The PCR results for *Wolbachia* growth in flies (Fig. 2-3) suggest that the use of FISH at 6 days post-
254 injection was too early to determine the final distribution of *Wolbachia*. Hence, we studied tissue
255 invasion and the detailed distribution of *Wolbachia* in adult flies by real-time PCR after dissecting out
256 the thoracic muscle, midgut, fat bodies, ovary and head at nine days post adult injection (Fig. 4A-C).
257 *Wolbachia* were found to be replicating in all somatic tissues with *wAlbB* having an infection percentage
258 of 33-83 % (N=6) and *wMel* and *wMelPop* between 66-100% (N=6). No infection was found in germline
259 tissues. However, on a few occasions first generation flies from adult injection with *wAlbB*, *wMel*, and
260 *wMelPop* were found positive with infection percentages of 5%, 22%, and 10% respectively, suggesting
261 transmission via the germline tissues in these instances (see Table 2).

262

263 **Table. 2: Summary of pupal and adult injection.** G_0 here represents injected adults and adults
 264 emerged from injected pupae. Infection was determined using real-time strain specific *Wolbachia*
 265 assays.

Injection type	Strain	Total injected	G_0 (infected / total tested) (% infection)	G_1 (infected / total tested) (% infection)	G_2 (infected / total tested) (% infection)
Adult	wAlbB	378 (19 batches)	Adult: 118/126 (95.93%)	Adult: 5/89 (5.6%)	Adult: Not tested; Egg: 0/50 (0%)
Adult	wMel	441 (17 batches)	Adult: 117/123 (95.12%)	Adult: 27/119 (22.68%)	Adult: 0/25 (0%); Eggs: 0/100 (0%)
Adult	wMelPop	417 (15 batches)	Adult: 103/106 (96.26%)	Adult: 10/91 (10.98 %)	Adult: 2/60 = 60 (3.3%)
Pupal	wAlbB	676 (5 batches)	Adult : 82/90 (91.22%); Egg: 4/40 (10%)	Adult: 0/20 (0 %); Egg: 0/50 (0%)	
Pupal	wMel	820 (6 batches)	Adult: 82/82 (100 %)	Adult: 2/9 (22%); Egg: Not tested	
Pupal	wMelPop	741 (5 batches)	Adult: 88/92 (95.65 %);Egg = 0/30 (0%)	Adult: 0/23 (0%); Egg: Not tested	

266

267 **Effect of *Wolbachia* on the survival of flies post adult injection**

268 In order to understand the population dynamics of the flies inside the cage, survival assays were
 269 performed. The results revealed that by day seven less than 20% of the wMelPop and less than 50%
 270 of wMel and wAlbB injected flies were alive (Fig. 5). Both wMelPop (log-rank statistic = 16.92, $p < 0.0001$)
 271 and wMel (log-rank statistic = 11.96, $p = 0.0005$) significantly reduced longevity of female BF. However,
 272 there was no significant effect of the wAlbB strain in comparison to the control injected flies (log-rank
 273 statistic = 0.25, $p = 0.62$).

274 ***Wolbachia* dynamics and tropism post pupal microinjection**

275 A similar quantitative assay to that used for injected adult BF was carried out to track the dynamics and
 276 tropisms of the three *Wolbachia* strains post pupal injection. The extra time in the pupal phase resulted
 277 in 66-100% infection in the somatic tissue with wAlbB and wMel (N=6) and 83-100% with wMelPop
 278 (N=6) 13 days post pupal injection (Fig. 6 A-C). Furthermore, in 16% of cases the ovaries of females
 279 injected with wMel and wMelPop *Wolbachia* were found to be infected. Also, two first generation flies
 280 from wMel-injected pupae and four eggs from wAlbB-injected pupae were found positive for *Wolbachia*
 281 infection (Table 2). Analysis of *Wolbachia* dynamics showed approximately the same pattern as for

282 adult injection, where density initially decreased in the first seven days, then significantly recovered by
283 day nine in *wMel* (Kruskal-Wallis test: $p < 0.0001$), and day 13 in *wMelPop* and *wAlbB* post pupal injection
284 (Kruskal-Wallis test: $p < 0.0001$) (Fig. 6 D-F).

285 **Effect of *Wolbachia* on survival of buffalo flies post pupal microinjection**

286 A significant decrease in the longevity of BF post pupal injection was found in both sexes of *wMelPop*-
287 injected BF (Male: log-rank statistic = 20.25, $p < 0.0001$, Female: log-rank statistic = 29.04, $p < 0.0001$),
288 but the effect was not significant with the two other strains (*wAlbB*: male (log-rank statistic = 2.267,
289 $p = 0.132$), female (log-rank statistic = 3.275, $p = 0.071$)), *wMel*: male (log-rank statistic = 3.027,
290 $p = 0.1545$), female (log-rank statistic = 3.467, $p = 0.063$)) (Fig. 7).

291

292 **Effect of *Wolbachia* on adult emergence rate**

293 Infection of the somatic tissues by *Wolbachia* can have consequences on physiological processes. Non-
294 injected control flies emerged from pupae after 3-7 days, whereas mock-injected control flies emerged
295 from 5-7 days, *wAlbB* after 6-7 days and *wMel* and *wMelPop* injected flies at 5-7 days post injection
296 (Fig. 8A). It is important to note that emergence in *wMel* and *wMelPop* injected flies was less than 2%
297 on day 5. Overall, there was significant decrease in the percent emergence of *wMel* (30.01 ± 3.91)
298 (Tukey's multiple comparison test, $p = 0.0030$) and *wMelPop* (27.98 ± 3.92) (Tukey's multiple
299 comparison host test, $p = 0.0011$) injected flies compared to the control injected flies (46.95 ± 4.15), but
300 no significant difference was observed with the *wAlbB*-injected flies (Tukey's multiple comparison test:
301 $p = 0.77$) (Fig. 8B). Nearly 5% of the flies that emerged from the *wMelPop*-injected pupae were too weak
302 to completely eclose from the pupal case and had deformed wings (Fig. 8 C-D).

303 **Effect of *Wolbachia* on egg production**

304 Difference between infected females and non-infected females in egg production was also analysed
305 following pupal injection with the three different strains of *Wolbachia*. Over 14 days there was a
306 significant reduction in the total eggs laid by females infected with *wAlbB* ($p = 0.012$), *wMel* ($p = 0.0052$),
307 and *wMelPop* ($p = 0.0051$) in comparison with the mock-injected flies (Fig. 9).

308

309 **Discussion**

310 Embryonic microinjection is by far the most frequently used technique to develop *Wolbachia*-
311 transinfected insect lines, mainly because *Wolbachia* injected into the germ cells of the developing
312 embryo provides a direct route for infection of the germ tissues in the early stage of differentiation [14].
313 However, this technique is also the most challenging step because the invasive procedure of egg
314 microinjection can result in high mortality of eggs and optimal methods differ for different insect species
315 [14, 45, 46]. Another disadvantage of this technique is that inability to determine the sex of an embryo
316 prior to injection means that approximately half of the injected flies will be males that do not transmit
317 *Wolbachia* to the next generation [14]. This means that many thousands of eggs must often be
318 microinjected using specialised equipment before successful *Wolbachia* transinfection is achieved [14]
319 and as male embryos cannot be identified, half of this effort is functionally wasted. With BF, less than
320 1% of more than 2000 embryos we injected subsequently hatched because the tough chorion of BF
321 eggs caused difficulties with needle penetration, rapid blunting and high breakage rate of microinjector
322 needles, frequent chorion tearing, and embryo damage. Treatment with sodium hypochlorite to soften
323 the chorion, prior partial desiccation of eggs to reduce hydrostatic pressure, and the use of halocarbon
324 oils to prevent egg desiccation during injection did not markedly improve the survival rate. Similar
325 difficulties were experienced when attempting to use microinjection for gene transfection in closely
326 related *Haematobia irritans* eggs. In this instance, the researchers opted to use electroporation, which
327 is unsuitable for the introduction of bacteria [47].

328 Although embryonic microinjection has been the primary method used to develop transinfected insects,
329 adult microinjection can be advantageous in that females can be selected for injection [14]. Further,
330 adult microinjection can be performed using a simple syringe and small-bore needles delivering higher
331 volumes of *Wolbachia* to overcome the host immunological response [14]. Our results with adult
332 injection of *Wolbachia* were promising. Despite that injections in first few batches were made mainly
333 with *Wolbachia* grown in *D. melanogaster* cells (*wAlbB*, *wMel* and *wMelPop* strain), not previously
334 adapted in *Haematobia* cells, infection rates and persistence in the injected flies were high (generally >
335 90%). In a few batches, transmission to the next generation was confirmed.

336 As oviposition by BF may begin as early as three days after eclosion from the pupae and continue until
337 death, knowledge of *Wolbachia* distribution and dynamics in injected females was critical for us to
338 identify the optimal timing for collecting infected eggs for the establishment of an infected colony (11-
339 15 days). *Wolbachia* density significantly decreased to day five due to host immune response but

340 recovered by day eleven after injection. A similar result was obtained when *wMelPop* and *wAlbB* were
341 injected into *Anopheles gambiae* adult mosquitoes [13]. The initial host immune response was
342 anticipated as the densities of *wAlbB*, *wMel*, and *wMelPop* *Wolbachia* in *Haematobia* cells were also
343 observed to initially decrease, possibly due to an innate immune response mediated by the *Imd* pathway
344 (unpublished data). Real-time PCR analysis of dissected tissues nine days after injection showed
345 *Wolbachia* to be present in all the vital somatic tissues, except for the ovarian tissues, suggesting that
346 *Wolbachia* might need extra time to infect the ovaries. However, injection with *wAlbB*, *wMel* and
347 *wMelPop* *Wolbachia* caused >40% death in flies by day seven post injection, further reducing the
348 likelihood of collecting infected eggs. Therefore, we hypothesised that microinjecting 1-2 h old pupae
349 would give more time than with adult microinjection for *Wolbachia* to multiply, spread and establish in
350 the ovaries. Pupal injection has previously been conducted with *Trichogramma* wasps and resulted in
351 successful ovarian infections and persistence of *Wolbachia* in the wasp colony for 26 generations [48].
352 With BF, *wMel* and *wMelPop* overcame host immune responses and established in both somatic and
353 germline tissues. Further, in two instances, next-generation (G1) BF from *wAlbB* and *wMel* injected
354 pupae were positive for *Wolbachia*, indicating next-generation transmission as a result of pupal
355 injection. The main disadvantages of pupal injection in comparison with adult injection were limitation
356 on the volume of *Wolbachia* that could be injected and inability to distinguish female from male pupae
357 for injection.

358 The *wMelPop* strain is a virulent type of *Wolbachia*, and its over replication in somatic tissues and brain
359 cells, known in other infected insects [49, 50], may have been the reason for the early death of BF.
360 Further, in the studies of *Wolbachia* kinetics we found a higher density of *wMelPop* than with the other
361 two strains following both adult and pupal injection. Reduction in the longevity of infected *Ae. aegypti*
362 mosquitoes caused by infection with *wMelPop*, decreasing the potential extrinsic incubation time for the
363 dengue virus, was one of the characteristics that led to the hypothesis that *wMelPop* infection would
364 reduce dengue spread [51]. Infection with *wMelPop* could also markedly reduce BF lifespan and their
365 ability to transmit *Stephanofilaria* sp. nematodes. These nematodes have been implicated in the
366 development of buffalo fly lesions, a significant production and welfare issue in north-Australian cattle
367 [52]. *Stephanofilaria* has an extrinsic incubation period of up to 3 weeks in *Haematobia* spp. [53] and
368 the life-shortening effects of *Wolbachia* shown in our study could markedly reduce the vector
369 competency of infected flies. There is also the possibility the *Wolbachia* infection could more directly

370 compromise the vector competency of BF for *Stephanofilaria*, as has been seen in the case another
371 filarial nematode, *Brugia pahangi* transmitted by mosquitoes and in the case transmission of the dengue
372 virus by *Ae. Aegypti* [54, 55] .

373 Fecundity of insects has a significant influence on population dynamics of insect populations [56]. The
374 successful establishment of *Wolbachia* in new host populations directly relates to the strong CI, vertical
375 transmission and relatively more fertile egg production by infected females [57]. *Wolbachia* have been
376 found to enhance and reduce egg production depending upon both the strain of the nematode and the
377 host [15, 57-62]. We found that *wAlbB*, *wMel*, and *wMelPop* significantly reduced total egg production
378 in pupal injected flies. Also, *Wolbachia* infection caused delayed and decreased adult emergence of BF
379 post pupal injection. *Wolbachia* being an endosymbiont lacks nutritional biosynthetic pathways and
380 depends on its host for wide range of nutrition [63, 64]. Hence, the fitness costs observed in injected
381 BF could be the result of competition between high density of *Wolbachia* and BF for nutritional resources
382 such as amino acids and lipids [63, 64]. Another possibility could be that as *Wolbachia* was found in all
383 of the critical tissues involved in the endocrine cascades for egg production and maturation in insects
384 (midgut, neuron, fat bodies and ovary), it interfered with egg production by this means [65]. In addition,
385 delayed larval development associated with *wMelPop* infection has been documented in mosquitoes
386 on a number of occasions [17, 19]. If these deleterious effects are a consistent feature of *Wolbachia*
387 infection in BF, they could have a significant impact in altering population dynamics or even crashing
388 BF populations [17, 66]. For instance, female BF lay eggs in fresh cattle manure pats, where eggs take
389 approximately seven days to develop into pupae depending upon the temperature and moisture content
390 of the pat [67]. Prolonged larval development and time to eclosion of *Wolbachia*-infected BF, together
391 with adult lifespan reduction might decrease overwintering and survival of BF, particularly during periods
392 of unfavourable fly conditions and at the edge of the BF range.

393 In this work, we have shown that BF are competent hosts for the growth of *wMel*, *wMelPop* and *wAlbB*
394 *Wolbachia* strains and that infection can induce a number of fitness effects in the injected flies. However,
395 embryonic injection has proven challenging with BF and to date we have not been able to establish a
396 sustainably infected isofemale line using this technique. Pupal and adult microinjection gave much
397 higher fly survival rates, high titres of *Wolbachia* in somatic tissues and ovarian infection and
398 transmission to the next generation in a number of instances. Despite relatively limited testing, this gives

399 hope for the future establishment of *Wolbachia*-infected strains of BF for the future design of *Wolbachia*-
400 based control programs.

401

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407

408 **References**

- 409 1. Urech R, Brown GW, Moore CJ, Green PE (2005) Cuticular hydrocarbons of buffalo fly,
410 *Haematobia exigua*, and chemotaxonomic differentiation from horn fly, *H. irritans*. *J Chem*
411 *Ecol* 31: 2451-2461. doi: 10.1007/s10886-005-7112-1
- 412 2. Oyarzun MP, Quiroz A, Birkett MA (2008) Insecticide resistance in the horn fly: alternative
413 control strategies. *Med Vet Entomol* 22: 188-202. doi: 10.1111/j.1365-2915.2008.00733.x
- 414 3. Sutherst RW, Tozer RS (1995) Control of Buffalo Fly (*Haematobia-Irritans Exigua De Meijere*)
415 on Dairy and Beef-Cattle Using Traps. *Aust J Agr Res* 46: 269-284. doi: Doi 10.1071/Ar9950269
- 416 4. Jonsson NN, Mayer DG (1999) Estimation of the effects of buffalo fly (*Haematobia irritans*
417 *exigua*) on the milk production of dairy cattle based on a meta-analysis of literature data. *Med*
418 *Vet Entomol* 13: 372-376. doi: 10.1046/j.1365-2915.1999.00179.x
- 419 5. Bean KG, Seifert GW, Macqueen A, Doube BM (1987) Effect of Insecticide Treatment for
420 Control of Buffalo Fly on Weight Gains of Steers in Coastal Central Queensland. *Aust J Agr Res*
421 27: 329-334. doi: Doi 10.1071/Ea9870329
- 422 6. Williams JD, Sutherst RW, Maywald GF, Petherbridge CT (1985) The southward spread of
423 buffalo fly (*Haematobia irritans exigua*) in eastern Australia and its survival through a severe
424 winter. *Aust Vet J* 62: 367-369. doi: 10.1111/j.1751-0813.1985.tb14210.x
- 425 7. Schnitzerling H, Noble P, Macqueen A, Dunham R (1982) Resistance of the buffalo fly,
426 *Haematobia irritans exigua* (De Meijere), to two synthetic pyrethroids and DDT. *Aust J*
427 *Entomol* 21: 77-80.
- 428 8. Rothwell JT, Morgan JA, James PJ, Brown GW, Guerrero FD, Jorgensen WK (2011) Mechanism
429 of resistance to synthetic pyrethroids in buffalo flies in south-east Queensland. *Aust Vet J* 89:
430 70-72. doi: 10.1111/j.1751-0813.2010.00685.x
- 431 9. sMateos M, Martinez H, Lanzavecchia SB, Conte C, Guillen K, Moran-Aceves BM, Toledo J,
432 Liedo P, Asimakis ED, Doudoumis V (2018) *Wolbachia pipientis* associated to tephritid fruit fly
433 pests: from basic research to applications. *BioRxiv*: 358333.
- 434 10. O'Connor L, Plichart C, Sang AC, Brelsfoard CL, Bossin HC, Dobson SL (2012) Open release of
435 male mosquitoes infected with a wolbachia biopesticide: field performance and infection
436 containment. *PLoS Negl Trop Dis* 6: e1797. doi: 10.1371/journal.pntd.0001797
- 437 11. Iturbe-Ormaetxe I, Walker T, SL ON (2011) *Wolbachia* and the biological control of mosquito-
438 borne disease. *EMBO Rep* 12: 508-518. doi: 10.1038/embor.2011.84
- 439 12. Zabalou S, Apostolaki A, Livadaras I, Franz G, Robinson AS, Savakis C, Bourtzis K (2009)
440 Incompatible insect technique: incompatible males from a *Ceratitis capitata* genetic sexing
441 strain. *Entomol Exp Appl* 132: 232-240. doi: 10.1111/j.1570-7458.2009.00886.x
- 442 13. Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL (2011) *Wolbachia* infections are virulent and
443 inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS*
444 *Pathog* 7: e1002043. doi: 10.1371/journal.ppat.1002043

- 445 14. Hughes GL, Rasgon JL (2014) Transinfection: a method to investigate Wolbachia–host
446 interactions and control arthropod-borne disease. *Insect Mol Biol* 23: 141-151.
- 447 15. Joshi D, McFadden MJ, Bevins D, Zhang F, Xi Z (2014) Wolbachia strain wAlbB confers both
448 fitness costs and benefit on *Anopheles stephensi*. *Parasit Vectors* 7: 336. doi: 10.1186/1756-
449 3305-7-336
- 450 16. McMeniman CJ, Lane RV, Cass BN, Fong AWC, Sidhu M, Wang YF, O'Neill SL (2009) Stable
451 Introduction of a Life-Shortening Wolbachia Infection into the Mosquito *Aedes aegypti*.
452 *Science* 323: 141-144. doi: 10.1126/science.1165326
- 453 17. McMeniman CJ, O'Neill SL (2010) A virulent Wolbachia infection decreases the viability of the
454 dengue vector *Aedes aegypti* during periods of embryonic quiescence. *PLoS Negl Trop Dis* 4:
455 e748. doi: 10.1371/journal.pntd.0000748
- 456 18. Turley AP, Moreira LA, O'Neill SL, McGraw EA (2009) Wolbachia infection reduces blood-
457 feeding success in the dengue fever mosquito, *Aedes aegypti*. *PLoS Negl Trop Dis* 3: e516. doi:
458 10.1371/journal.pntd.0000516
- 459 19. Ross PA, Endersby NM, Yeap HL, Hoffmann AA (2014) Larval competition extends
460 developmental time and decreases adult size of wMelPop Wolbachia-infected *Aedes aegypti*.
461 *Am J Trop Med Hyg* 91: 198-205. doi: 10.4269/ajtmh.13-0576
- 462 20. Yeap HL, Axford JK, Popovici J, Endersby NM, Iturbe-Ormaetxe I, Ritchie SA, Hoffmann AA
463 (2014) Assessing quality of life-shortening Wolbachia-infected *Aedes aegypti* mosquitoes in
464 the field based on capture rates and morphometric assessments. *Parasit Vectors* 7: 58. doi:
465 10.1186/1756-3305-7-58
- 466 21. Laven H (1967) Eradication of *Culex pipiens fatigans* through Cytoplasmic Incompatibility.
467 *Nature* 216: 383-&. doi: DOI 10.1038/216383a0
- 468 22. Blagrove MS, Arias-Goeta C, Failloux AB, Sinkins SP (2012) Wolbachia strain wMel induces
469 cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. *Proc Natl*
470 *Acad Sci U S A* 109: 255-260. doi: 10.1073/pnas.1112021108
- 471 23. Moretti R, Calvitti M (2013) Male mating performance and cytoplasmic incompatibility in a
472 wPip Wolbachia trans-infected line of *Aedes albopictus* (*Stegomyia albopicta*). *Med Vet*
473 *Entomol* 27: 377-386. doi: 10.1111/j.1365-2915.2012.01061.x
- 474 24. Bian G, Joshi D, Dong Y, Lu P, Zhou G, Pan X, Xu Y, Dimopoulos G, Xi Z (2013) Wolbachia invades
475 *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. *Science*
476 340: 748-751. doi: 10.1126/science.1236192
- 477 25. Chen L, Zhu CL, Zhang DH (2013) Naturally Occurring Incompatibilities between Different
478 *Culex pipiens pallens* Populations as the Basis of Potential Mosquito Control Measures. *Plos*
479 *Neglect Trop Dis* 7: e2030. doi: ARTN e203010.1371/journal.pntd.0002030
- 480 26. Alam U, Medlock J, Brelsfoard C, Pais R, Lohs C, Balmand S, Carnogursky J, Heddi A, Takac P,
481 Galvani A, Aksoy S (2011) Wolbachia symbiont infections induce strong cytoplasmic
482 incompatibility in the tsetse fly *Glossina morsitans*. *PLoS Pathog* 7: e1002415. doi:
483 10.1371/journal.ppat.1002415
- 484 27. Flores HA, O'Neill SL (2018) Controlling vector-borne diseases by releasing modified
485 mosquitoes. *Nat Rev Microbiol* 16: 508-518. doi: 10.1038/s41579-018-0025-0
- 486 28. Ross PA, Ritchie SA, Axford JK, Hoffmann AA (2019) Loss of cytoplasmic incompatibility in
487 Wolbachia-infected *Aedes aegypti* under field conditions. *PLoS Negl Trop Dis* 13: e0007357.
488 doi: 10.1371/journal.pntd.0007357
- 489 29. Kang L, Ma X, Cai L, Liao S, Sun L, Zhu H, Chen X, Shen D, Zhao S, Li C (2003) Superinfection of
490 *Laodelphax striatellus* with Wolbachia from *Drosophila simulans*. *Heredity (Edinb)* 90: 71-76.
491 doi: 10.1038/sj.hdy.6800180
- 492 30. Frydman HM, Li JM, Robson DN, Wieschaus E (2006) Somatic stem cell niche tropism in
493 Wolbachia. *Nature* 441: 509-512. doi: 10.1038/nature04756

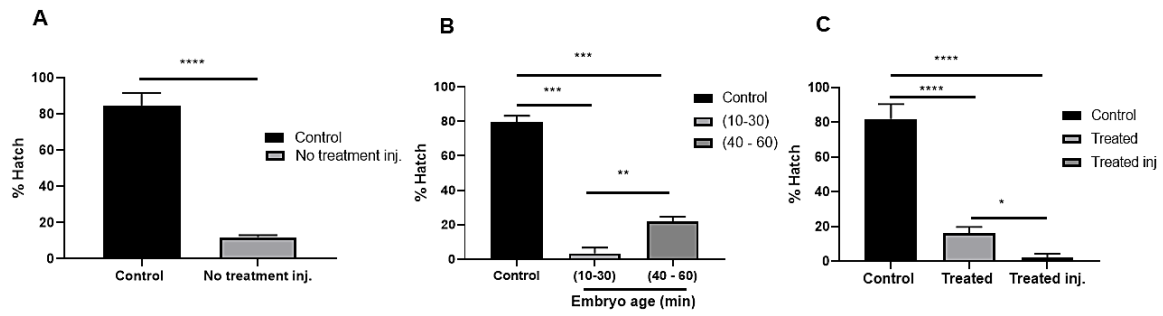
- 494 31. Ruang-Areerate T, Kittayapong P (2006) Wolbachia transinfection in *Aedes aegypti*: a potential
495 gene driver of dengue vectors. *Proc Natl Acad Sci U S A* 103: 12534-12539. doi:
496 10.1073/pnas.0508879103
- 497 32. Zhang B, McGraw E, Floate KD, James P, Jorgensen W, Rothwell J (2009) Wolbachia infection
498 in Australasian and North American populations of *Haematobia irritans* (Diptera: Muscidae).
499 *Vet Parasitol* 162: 350-353. doi: 10.1016/j.vetpar.2009.03.012
- 500 33. Torres L, Almazan C, Ayllon N, Galindo RC, Rosario-Cruz R, Quiroz-Romero H, Gortazar C, de la
501 Fuente J (2012) Identification of microorganisms in partially fed female horn flies, *Haematobia*
502 *irritans*. *Parasitol Res* 111: 1391-1395. doi: 10.1007/s00436-012-2877-y
- 503 34. Palavesam A, Guerrero FD, Heekin AM, Wang J, Dowd SE, Sun Y, Foil LD, Perez de Leon AA
504 (2012) Pyrosequencing-based analysis of the microbiome associated with the horn fly,
505 *Haematobia irritans*. *PLoS One* 7: e44390. doi: 10.1371/journal.pone.0044390
- 506 35. Jeyaprakash A, Hoy MA (2000) Long PCR improves Wolbachia DNA amplification: wsp
507 sequences found in 76% of sixty-three arthropod species. *Insect Mol Biol* 9: 393-405.
- 508 36. Floate KD, Kyei-Poku GK, Coghlin PC (2006) Overview and relevance of Wolbachia bacteria in
509 biocontrol research. *Biocontrol Sci Techn* 16: 767-788. doi: 10.1080/09583150600699606
- 510 37. Torres L, Almazan C, Ayllon N, Galindo RC, Rosario-Cruz R, Quiroz-Romero H, de la Fuente J
511 (2011) Functional genomics of the horn fly, *Haematobia irritans* (Linnaeus, 1758). *BMC*
512 *Genomics* 12: 105. doi: 10.1186/1471-2164-12-105
- 513 38. Hornok S, Foldvari G, Elek V, Naranjo V, Farkas R, de la Fuente J (2008) Molecular identification
514 of *Anaplasma marginale* and rickettsial endosymbionts in blood-sucking flies (Diptera:
515 Tabanidae, Muscidae) and hard ticks (Acari: Ixodidae). *Vet Parasitol* 154: 354-359. doi:
516 10.1016/j.vetpar.2008.03.019
- 517 39. Herbert RI, McGraw EA (2018) The nature of the immune response in novel Wolbachia-host
518 associations. *Symbiosis* 74: 225-236. doi: 10.1007/s13199-017-0503-6
- 519 40. Echeverria-Fonseca G, Mere-Ruiz PA, Carrillo-Toro J, Rodriguez-Hidalgo R (2015) A new DNA
520 extraction protocol for screwworm fly *Cochliomyia* species (Diptera: Calliphoridae). *Front Env*
521 *Sci* 2: 68. doi: ARTN 6810.3389/fenvs.2014.00068
- 522 41. Koga R, Tsuchida T, Fukatsu T (2009) Quenching autofluorescence of insect tissues for in situ
523 detection of endosymbionts. *Appl Entomol Zool* 44: 281-291. doi: 10.1303/aez.2009.281
- 524 42. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, Rocha BC, Hall-Mendelin
525 S, Day A, Riegler M, Hugo LE, Johnson KN, Kay BH, McGraw EA, van den Hurk AF, Ryan PA,
526 O'Neill SL (2009) A Wolbachia symbiont in *Aedes aegypti* limits infection with dengue,
527 Chikungunya, and Plasmodium. *Cell* 139: 1268-1278. doi: 10.1016/j.cell.2009.11.042
- 528 43. Caragata EP, Rances E, Hedges LM, Gofton AW, Johnson KN, O'Neill SL, McGraw EA (2013)
529 Dietary cholesterol modulates pathogen blocking by Wolbachia. *PLoS Pathog* 9: e1003459.
530 doi: 10.1371/journal.ppat.1003459
- 531 44. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method.
532 *Nat Protoc* 3: 1101-1108. doi: 10.1038/nprot.2008.73
- 533 45. Ritchie S (2014) Rear and release: a new paradigm for dengue control. *Austral Entomol* 53:
534 363-367. doi: 10.1111/aen.12127
- 535 46. Caragata EP, Moreira LA (2017) Using an Endosymbiont to Control Mosquito-Transmitted
536 Disease Arthropod Vector: Controller of Disease Transmission. Academic Press, pp. 123-142
- 537 47. Xu Q, Guerrero FD, Palavesam A, Perez de Leon AA (2016) Use of electroporation as an option
538 to transform the horn fly, *Haematobia irritans*: a species recalcitrant to microinjection. *Insect*
539 *Sci* 23: 621-629. doi: 10.1111/1744-7917.12207
- 540 48. Grenier S, Pintureau B, Heddi A, Lassabliere F, Jager C, Louis C, Khatchadourian C (1998)
541 Successful horizontal transfer of Wolbachia symbionts between *Trichogramma* wasps. *P Roy*
542 *Soc B-Biol Sci* 265: 1441-1445. doi: DOI 10.1098/rspb.1998.0455
- 543 49. Pietri JE, DeBruhl H, Sullivan W (2016) The rich somatic life of Wolbachia. *Microbiologyopen*
544 5: 923-936. doi: 10.1002/mbo3.390

- 545 50. Min KT, Benzer S (1997) Wolbachia, normally a symbiont of *Drosophila*, can be virulent,
546 causing degeneration and early death. *P Natl Acad Sci USA* 94: 10792-10796. doi: DOI
547 10.1073/pnas.94.20.10792
- 548 51. McMeniman CJ (2009) Generation and characterization of a life-shortening Wolbachia
549 infection in the dengue vector *Aedes aegypti*., University of Queensland
- 550 52. Shaw SA, Sutherland IA (2006) The prevalence of *Stephanofilaria* sp in buffalo fly, *Haematobia*
551 *irritans exigua*, in Central Queensland. *Aust J Entomol* 45: 198-201. doi: 10.1111/j.1440-
552 6055.2006.00545.x
- 553 53. Hibler CP (1966) Development of *Stephanofilaria stilesi* in the horn fly. *J Parasitol* 52: 890-898.
- 554 54. Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening
555 Wolbachia and reduced filarial competence in mosquitoes. *Science* 326: 134-136. doi:
556 10.1126/science.1177531
- 557 55. Walker T, Johnson P, Moreira L, Iturbe-Ormaetxe I, Frentiu F, McMeniman C, Leong Y, Dong Y,
558 Axford J, Kriesner P (2011) The wMel Wolbachia strain blocks dengue and invades caged
559 *Aedes aegypti* populations. *Nature* 476: 450-453.
- 560 56. Peters TM, Barbosa P (1977) Influence of Population-Density on Size, Fecundity, and
561 Developmental Rate of Insects in Culture. *Annu Rev Entomol* 22: 431-450. doi: DOI
562 10.1146/annurev.en.22.010177.002243
- 563 57. Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA (2007) From parasite to
564 mutualist: rapid evolution of Wolbachia in natural populations of *Drosophila*. *PLoS Biol* 5:
565 e114. doi: 10.1371/journal.pbio.0050114
- 566 58. Dobson SL, Rattanadechakul W, Marsland EJ (2004) Fitness advantage and cytoplasmic
567 incompatibility in Wolbachia single- and superinfected *Aedes albopictus*. *Heredity* 93: 135-
568 142. doi: 10.1038/sj.hdy.6800458
- 569 59. Grenier S, Gomes SM, Pintureau B, Lassbiere F, Bolland P (2002) Use of tetracycline in larval
570 diet to study the effect of Wolbachia on host fecundity and clarify taxonomic status of
571 *Trichogramma* species in cured bisexual lines. *J Invertebr Pathol* 80: 13-21. doi: Pii S0022-
572 2011(02)00039-3
- 573 60. Fleury F, Vavre F, Ris N, Fouillet P, Bouletreau M (2000) Physiological cost induced by the
574 maternally-transmitted endosymbiont Wolbachia in the *Drosophila* parasitoid *Leptopilina*
575 *heterotoma*. *Parasitology* 121: 493-500. doi: 10.1017/s0031182099006599
- 576 61. Rigaud T, Moreau J, Juchault P (1999) Wolbachia infection in the terrestrial isopod *Oniscus*
577 *asellus*: sex ratio distortion and effect on fecundity. *Heredity* 83: 469-475. doi:
578 10.1038/sj.hdy.6885990
- 579 62. Sarakatsanou A, Diamantidis AD, Papanastasiou SA, Bourtzis K, Papadopoulos NT (2011)
580 Effects of Wolbachia on fitness of the Mediterranean fruit fly (Diptera: Tephritidae). *J Appl*
581 *Entomol* 135: 554-563. doi: 10.1111/j.1439-0418.2011.01610.x
- 582 63. Foster J, Ganatra M, Kamal I, Ware J, Makarova K, Ivanova N, Bhattacharyya A, Kapatral V,
583 Kumar S, Posfai J, Vincze T, Ingram J, Moran L, Lapidus A, Omelchenko M, Kyrpides N, Ghedin
584 E, Wang S, Goltsman E, Joukov V, Ostrovskaya O, Tsukerman K, Mazur M, Comb D, Koonin E,
585 Slatko B (2005) The Wolbachia genome of *Brugia malayi*: endosymbiont evolution within a
586 human pathogenic nematode. *PLoS Biol* 3: e121. doi: 10.1371/journal.pbio.0030121
- 587 64. Caragata EP, Rances E, O'Neill SL, McGraw EA (2014) Competition for amino acids between
588 Wolbachia and the mosquito host, *Aedes aegypti*. *Microb Ecol* 67: 205-218. doi:
589 10.1007/s00248-013-0339-4
- 590 65. Negri I (2012) Wolbachia as an "infectious" extrinsic factor manipulating host signaling
591 pathways. *Front Endocrinol* 2: 115.
- 592 66. Ritchie SA, Townsend M, Paton CJ, Callahan AG, Hoffmann AA (2015) Application of wMelPop
593 Wolbachia Strain to Crash Local Populations of *Aedes aegypti*. *PLoS Negl Trop Dis* 9: e0003930.
594 doi: 10.1371/journal.pntd.0003930

595 67. Jones SR, Kunz SE (1996) Effects of immersion in water on survival of preimaginal stages of
596 *Haematobia irritans* (Diptera: Muscidae). *J Med Entomol* 33: 27-31. doi:
597 10.1093/jmedent/33.1.27

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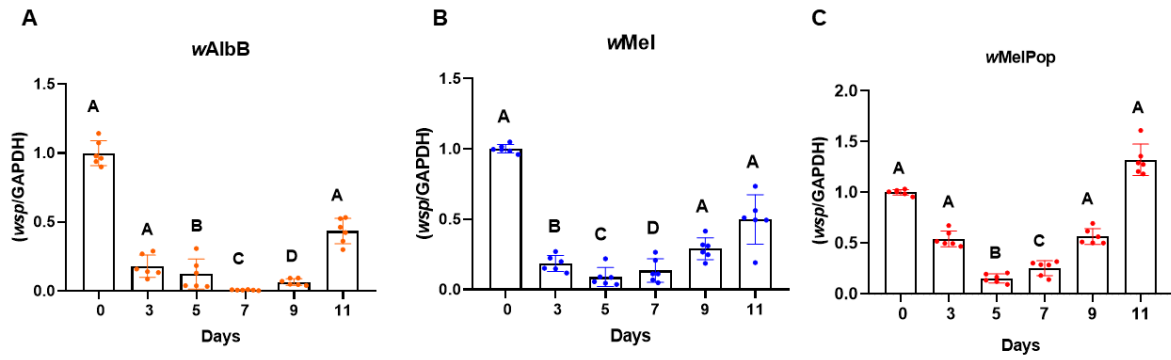
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601 **Fig. 1.** Challenges with buffalo fly embryonic microinjection. **A.** Embryonic microinjection had a
602 detrimental effect on embryo hatching. **B.** 40-60 min old embryos survived injection better than 10 – 30
603 min old embryos. **C.** Eggs were dechorionated by treating with 2.5% sodium hypochlorite for 30 s and
604 covered with 2:1 mix of halocarbon oil 700 and 27 to prevent desiccation. Eggs were sensitive to
605 treatment and survival decreased further with the injection. Error bars are SEM. Analysis was by
606 Student's Unpaired t-test in (A) and Tukey's multiple comparison test in (B) and (C); **** $p < 0.0001$.

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608

609 **Fig. 2.** *Wolbachia* dynamics post adult microinjection of female buffalo flies assessed using real-time

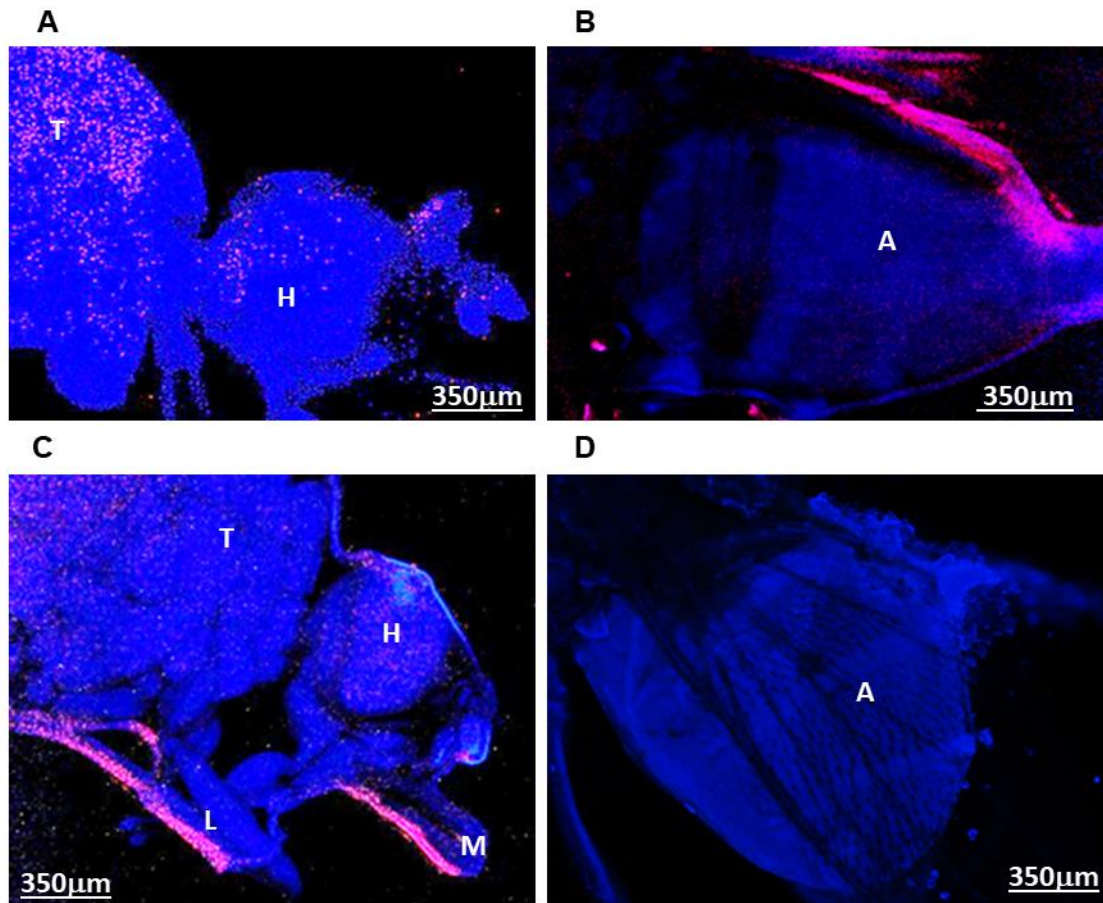
610 PCR. (A-C) *Wolbachia* dynamics measured over eleven days post-injection by analysing N = 6 for each

611 day. Here, *Wolbachia* titre is expressed relative to the host genome. Kruskal – Wallis test and Dunn's

612 multiple comparison test were used to compare titres at day zero. All error bars are SEM. Bars with

613 different letters in each graph are significantly different.

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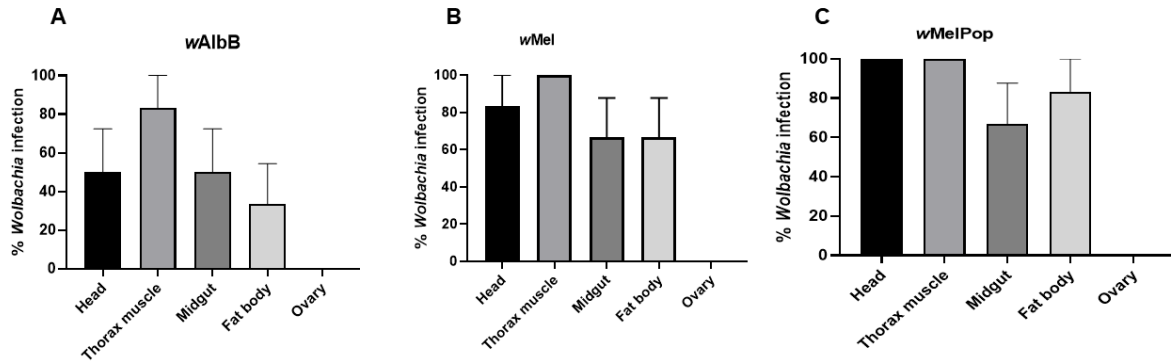
616 **Fig. 3.** Fluorescence *in situ* hybridisation images showing localisation of *Wolbachia* six days post adult

617 injection. *Wolbachia* is distributed throughout the BF (Blue: host, Red: *Wolbachia*). A. wMel in head and

618 thorax. B. wMelPop in the abdominal region. C. wMelPop in the head, mouthparts, thorax and leg. D.

619 Control no probe. T: Thorax, H: Head, A: Abdomen, M: Mouthparts, L: Leg.

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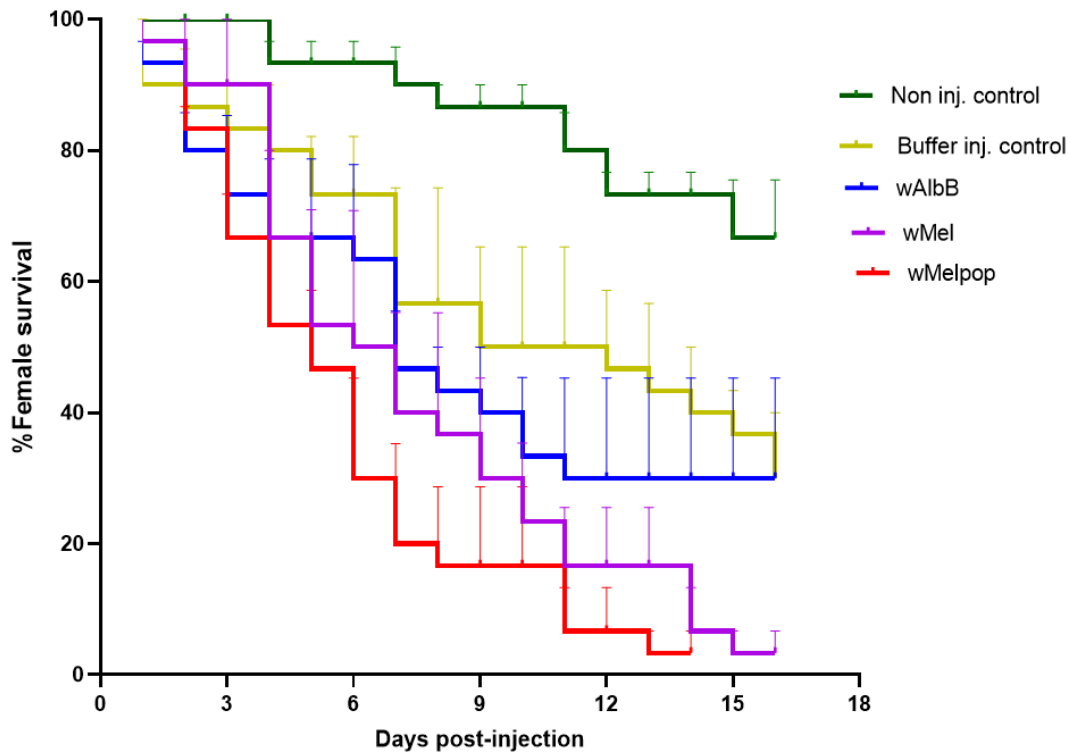
621

622 **Fig. 4. *Wolbachia* tropism post adult microinjection of female buffalo flies assessed using real-**

623 **time PCR. (A-C) shows *Wolbachia* tropism in female (N = 6) nine days post adult injection. None of the**

624 ***Wolbachia* strains was found in the ovaries. Bars represent SEM.**

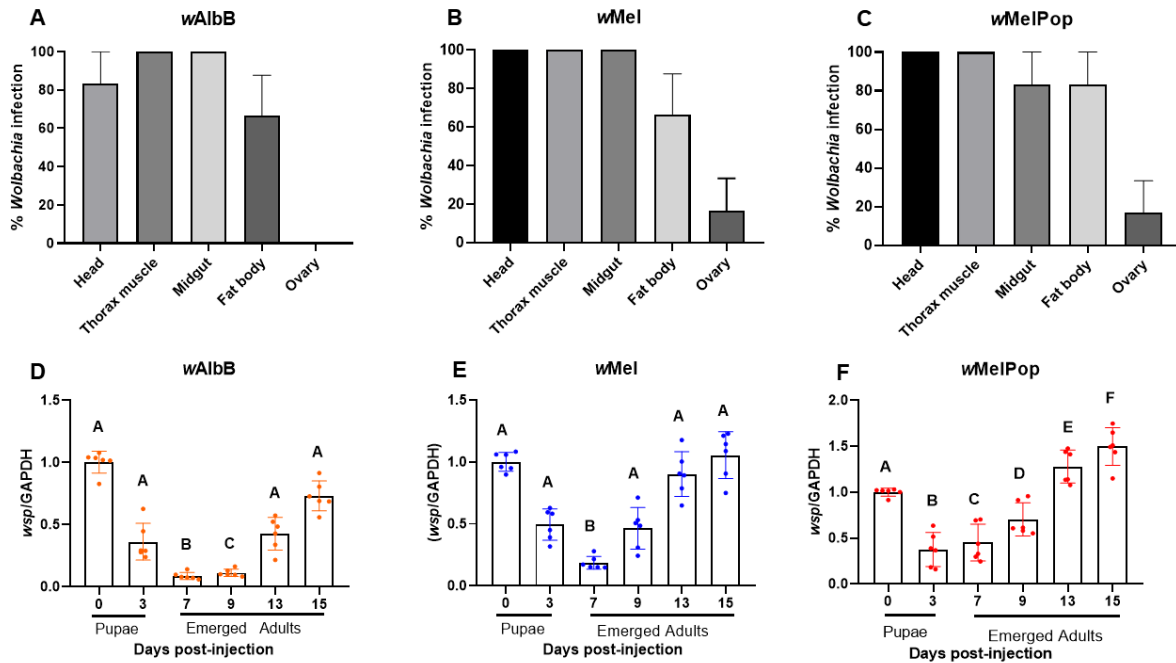
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627 **Fig. 5.** Survival of female buffalo flies post adult injection with *Wolbachia*. Triplicate cages of adult flies
628 each containing ten females were maintained under lab culturing conditions. The number of dead flies
629 were recorded until all died. A significant reduction in survival was observed in wMel ($p < 0.0005$) and
630 wMelPop ($p < 0.0001$) injected flies by Log-rank (Mantel-cox) tests.

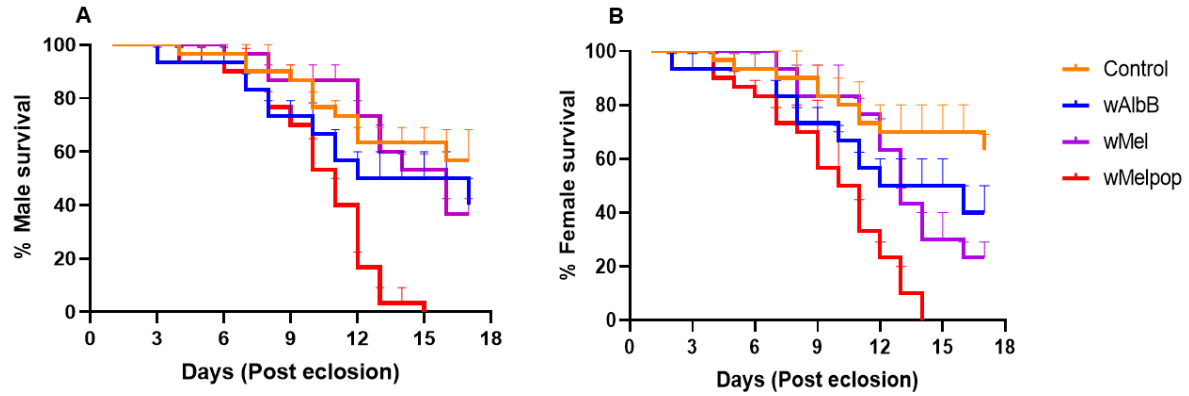
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633 **Fig. 6.** *Wolbachia* tropism and dynamics post pupal microinjection of female buffalo flies assessed using
 634 real-time PCR. A-C show *Wolbachia* tropism in female BF (N = 6) 13 days post pupal injection. Ovary
 635 infection was detected in wMel, and wMelPop injected flies. D-F show *Wolbachia* dynamics measured
 636 over 15 days post-injection. Here, *Wolbachia* density is expressed relative to the host genome. Kruskal-
 637 Wallis and Dunn's multiple comparison tests were used to compare titres to those at day zero. Bars
 638 with different letters are significantly different ($p < 0.05$). Scale on the Y axis for wMelPop (F) is different
 639 to that for the other two strains (D,E) indicating faster growth rate with wMelPop.

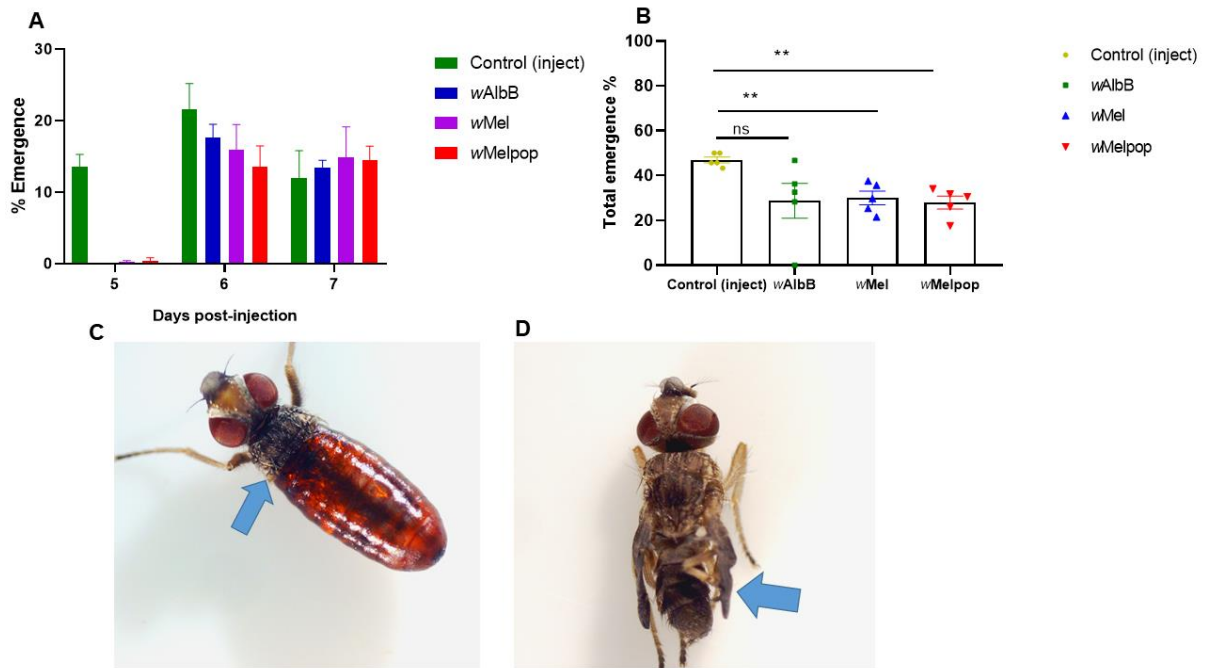
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642 **Fig. 7.** Survival of buffalo flies post pupal injection with *Wolbachia*. Triplicate cages of flies eclosed from
643 pupae on the same day (ten males and ten females per cage) were maintained in lab culturing
644 conditions. Mortality was recorded daily until all flies were dead. Log-rank (Mantel-cox) showed a
645 significant reduction in the male wMelPop ($p < 0.0001$) and female wMelPop ($p < 0.0001$) injected flies.

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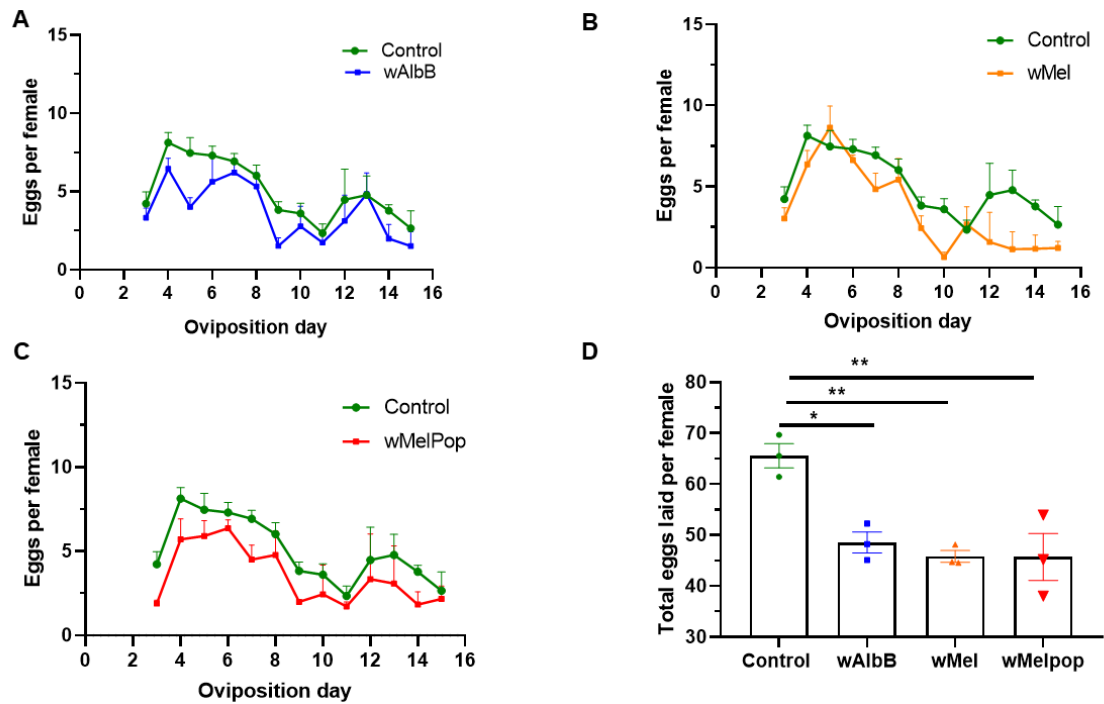
648 **Fig. 8.** Fitness effects on buffalo fly post pupal injection with *Wolbachia*. A. *Wolbachia* delayed adult

649 emergence. B. A significant decrease in adult emergence was observed in wMel ($p=0.0030$) and

650 wMelPop ($p=0.0011$) injected pupae when analysed using Tukey's multiple comparison test. Nearly 5

651 % of wMelPop flies either failed to completely eclose from the pupal case or had deformed wings.

652



653

654 **Fig. 9.** Fecundity of buffalo flies post *Wolbachia* pupal injection. Flies started laying eggs from day three

655 post-emergence and continued until day sixteen. Eggs laid from triplicate cages each having ten

656 females was recorded every day for (A) *wAlbB* (B) *wMel* and (C) *wMelPop*. D. A significant difference

657 between the total number of eggs laid per female over 13 days was found in flies infected with *wAlbB*

658 ($p=0.0123$), *wMel* ($p=0.0052$) and *wMelPop* ($p=0.0051$) (Tukey's multiple comparison test).

659