# Transinfection of *Wolbachia w*AlbB into *Culex quinquefasciatus* mosquitoes does not alter vector competence for Hawaiian avian malaria (*Plasmodium relictum* GRW4)

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**Keywords:** *Wolbachia pipientis*; *Culex quinquefasciatus*, *Plasmodium relictum*; vector competence; disease control; cytoplasmic incompatibility; incompatible insect technique

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**Abstract**

Avian malaria is expanding upslope with warmer temperatures and driving multiple species of Hawaiian birds towards extinction. Methods to reduce malaria transmission are urgently needed to prevent further declines. Releasing *Wolbachia*-infected incompatible male mosquitoes suppress mosquito populations and releasing *Wolbachia*-infected female mosquitoes could reduce pathogen transmission if the *Wolbachia* strain reduced vector competence. We cleared *Culex quinquefasciatus* of their natural *Wolbachia pipientis* *w*Pip infection and transinfected them with *Wolbachia* *w*AlbB isolated from *Aedes albopictus*. We show that *w*AlbB infection was transmitted transovarially, and demonstrate cytoplasmic incompatibility with wild-type mosquitoes infected with *w*Pip from Oahu and Maui, Hawaii. We measured vector competence for avian malaria, *Plasmodium relictum*, lineage GRW4, of seven mosquito lines (two with *w*AlbB; three with natural *w*Pip infection, and two cleared of *Wolbachia* infection) by allowing them to feed on canaries infected with recently collected field isolates of Hawaiian *P. relictum*. We tested 73 groups ($N_{total}$ = 1176) of mosquitoes for *P. relictum* infection in abdomens and disseminated (thorax) infections 6-14 days after feeding across a range of parasitemias from 0.028% to 2.49%, and a smaller subset of salivary glands. We found no measurable effect of *Wolbachia* on any endpoint, but strong effects of parasitemia, days post feeding, and mosquito strain on both abdomen infection prevalence and disseminated infection prevalence. These results suggest that releasing male *w*AlbB-infected *C. quinquefasciatus* mosquitoes could suppress *w*Pip-infected mosquito populations, but would have little positive or negative impact on mosquito vector competence for *P. relictum* if *w*AlbB became established in local mosquito populations. More broadly, the lack of *Wolbachia* effects on vector competence we observed highlights the variable impacts of both native and transfected *Wolbachia* infections in mosquitoes.

41 **Introduction**

42 Hawaiian birds are experiencing an extinction crisis. Of the 47 species of Hawaiian
43 honeycreepers known to science, forty-one are extinct or federally endangered (Paxton *et al.*
44 2022). One of the key threats to the remaining species is avian malaria, *Plasmodium relictum*
45 (lineage GRW4), transmitted by *Culex quinquefasciatus* mosquitoes (Beadell *et al.* 2006;
46 Paxton *et al.* 2022). Rising global temperatures due to climate change have increased the
47 distribution of mosquito populations and the transmission of malaria in higher elevations on
48 multiple islands, as predicted two decades ago (Benning *et al.* 2002). This has led to further
49 population declines in many species, with two additional species now nearly extinct in the wild
50 (Atkinson *et al.* 2014; Paxton *et al.* 2022). New tools are urgently needed to reduce transmission
51 of avian malaria.

52 One recent breakthrough for reducing transmission of mosquito-borne diseases is the
53 use of *Wolbachia* bacteria either to suppress mosquito populations or to reduce mosquito vector
54 competence (Flores & O'Neill 2018; Ross *et al.* 2019). *Wolbachia* bacteria are intracellular
55 parasites that naturally infect many mosquitoes and insect species (Hilgenboecker *et al.* 2008),
56 and they can be transinfected into new species or populations (Hughes & Rasgon 2014).
57 Different strains of *Wolbachia* can have different effects on mosquitoes, including reducing adult
58 lifespan, reduced vector competence for some pathogens, and a reduction in mosquito
59 populations through cytoplasmic incompatibility (CI) (Iturbe-Ormaetxe *et al.* 2011). CI caused by
60 *Wolbachia* results in inviable embryos unless both male and female mosquitoes have
61 compatible *Wolbachia* strains. Control strategies involving the release of *Wolbachia*-infected
62 incompatible males could be further improved if the *Wolbachia* strain also reduces vector
63 competence in accidentally released female mosquitoes. Similarly, if a *Wolbachia* strain reduces
64 vector competence, then population replacement via large scale releases of female mosquitoes
65 infected with this *Wolbachia* strain could reduce vector competence and transmission, as has
66 been recently demonstrated for dengue virus and *Aedes aegypti* mosquitoes (Utarini *et al.* 2021;

67 Velez *et al.* 2023). The EPA recently approved an emergency exemption to release large
68 numbers of *w*AlbB transinfected male mosquitoes to reduce populations of *C. quinquefasciatus*
69 in Hawaii (US EPA, 2023), making this a viable strategy to reduce transmission of avian malaria
70 in Hawaii.

71 Our goals were threefold: first, to transinfect *Wolbachia w*AlbB into a *C. quinquefasciatus*
72 line to use for incompatible male releases for population suppression in Hawaii; second, to
73 confirm complete maternal transmission of *Wolbachia* infection and CI of transinfected males
74 with wild-type females from Hawaii infected with *Wolbachia w*Pip; and third, to determine if the
75 transfinfected *Wolbachia w*AlbB strain alters the vector competence of transinfected female *C.*
76 *quinquefasciatus* mosquitoes for the avian malaria lineage found in Hawaii, *P. relictum* GRW4.
77 We did not have an *a priori* hypothesis about whether the *w*AlbB strain of *Wolbachia* would
78 increase or reduce vector competence of *C. quinquefasciatus* for *P. relictum* because effects of
79 transinfected *Wolbachia* in other mosquito species have been highly variable (Hughes *et al.*
80 2014; Ross *et al.* 2019) and no studies of the effects of transinfected *Wolbachia* on malaria
81 competence have been done in *C. quinquefasciatus*. Previous studies of *C. quinquefasciatus*
82 naturally infected with *Wolbachia w*Pip found higher susceptibility for *P. relictum* lineage SGS1
83 than mosquitoes cleared of *w*Pip with antibiotics (Zélé *et al.* 2014a). However, there was no
84 effect of *Wolbachia w*Pip on infection prevalence in the field (Zélé *et al.* 2014b). Finally, a
85 previous study that created a *w*AlbB transinfected *C. quinquefasciatus* line didn't quantify the
86 effects on vector competence for any pathogen (Ant *et al.* 2020), and this line has been lost in
87 an insectary malfunction (Steven Sinkins, personal communication).

88

89 **Methods**

90 ***Culex quinquefasciatus* Mosquito Strains**

91 We studied eight *C. quinquefasciatus* mosquito lines (Table S1) which were
92 combinations of four mosquito strains (Palmyra Atoll (abbreviated Palmyra or Palm in figures),

93 Oahu, Maui, Field (Captain Cook, Hawaii)) with one of two *Wolbachia* strains (native *w*Pip or
94 transinfected *w*AlbB) or cleared of *Wolbachia* infection via antibiotic treatment ("None"). We
95 refer to lines using the strain and *Wolbachia* type (e.g. Palmyra-*w*Pip, or Oahu-None). We used
96 subsets of these eight lines for three types of experiments: maternal inheritance of transinfected
97 *w*AlbB *Wolbachia*, cytoplasmic incompatibility, and vector competence for avian malaria, *P.*
98 *relictum* GRW4 (Table S1; Supplemental Material, Methods: Mosquito strains).
99

100 **Clearing *Wolbachia w*Pip and transinfecting *Wolbachia w*Alb**

101 We created two mosquito lines without *Wolbachia*, Palmyra-None and Oahu-None, by clearing
102 native *Wolbachia* wPip with antibiotics and then used these to create two additional mosquito
103 lines transinfected with *Wolbachia* wAlbB (Supplementary material: Methods: Transinfection of
104 *Culex quinquefasciatus* with *Wolbachia w*AlbB). We examined maternal transmission of *w*AlbB
105 in Palmyra-*w*AlbB ("DQB", *D*ebug *q*uinquefasciatus wAlb*B*."), and bi-directional cytoplasmic
106 incompatibility between Palmyra-*w*AlbB and both Oahu-*w*Pip and Maui-*w*Pip (Supplementary
107 material, Methods: Maternal transmission determination and Cytoplasmic incompatibility (CI)
108 testing).
109

110 ***Plasmodium relictum* isolates**

111 We collected two isolates of *Plasmodium relictum* (lineage GRW4) from wild birds at two
112 sites on Hawai'i Island, one from an 'Apapane (*Himatione sanguinea*) from Pu'u Wa'awa'a
113 Forest Reserve (19.738154°N, 155.875234°W, 1,230 m above sea level) in February 2020 and
114 another from a Warbling White-eye (*Zosterops japonicus*) from the same Captain Cook, HI site
115 where Field mosquitoes were collected (Supplemental Material: Methods: *Plasmodium relictum*
116 isolates).
117

118 **Experimental *Plasmodium relictum* infections and mosquito feeding**

119 We inoculated canaries intramuscularly with 50-200 µL of whole blood (parasitemia: 0.1–
120 2.85%) containing an avian malaria isolate that had been passaged 1–7 times and was either a
121 thawed deglycerolized sample, or was fresh blood from another infected canary. Starting on day
122 5 post-infection afterward we took 5-10 µL of blood by brachial venipuncture and screened thin
123 blood smears by microscopy (Supplemental Material: Methods: *Plasmodium relictum* isolates)
124 and by qPCR (Neddermeyer *et al.* 2023; Paxton *et al.* 2023) to detect infection and estimate
125 parasitemia. Once infection was detected, we allowed approximately 50 mosquitoes from each
126 of three mosquito lines to feed on each infected bird simultaneously. We differentiated the three
127 mosquito lines during feedings by spraying each line with a green or red fluorescent marker or
128 leaving it unmarked (Figure S1; Supplementary Material, Methods: Mosquito marking); the line
129 receiving each spray color (or none) was randomly selected. We fed most mosquitoes by
130 restraining an infected canary on top of a container containing the mosquitoes that had holes in
131 the lid that allowed the bird's legs and feet to be inside the container (Figure S2a). Field
132 mosquitoes were more hesitant to feed and were placed in a Bugdorm with an unrestrained bird
133 in a PVC cylinder with a perch and allowed to feed for ~ 8 hrs overnight (Figure S2b).
134 We collected engorged mosquitoes with an aspirator and transferred them into a cage in
135 an incubator set to 26 °C. We provided mosquitoes with cottons soaked in a 10% sucrose
136 solution and held them until dissection. We collected unfed females, knocked them down on ice,
137 and counted the number of each spray color under a UV light to quantify feeding success for
138 each group.
139 We killed engorged mosquitoes 6-14 days after feeding and dissected them by cleanly
140 separating the thorax from the abdomen at the scutellum with sterile dissection needles. We
141 placed abdomens in 96-well DNA extraction plates containing Chemagic DNA lysis buffer
142 (PerkinElmer, Waltham, MA, USA) and placed the head, thorax, and legs of each individual in a
143 second plate to test for a disseminated infection. When DNA extraction plates were not
144 available, abdomens and thoraxes (including the head and legs) were placed in separate 1 mL

145 vials containing 0.5 mL of 70% ethanol. All samples were stored at -20 °C until DNA extraction
146 and processing by qPCR (Supplemental Information, Methods: *Plasmodium relictum* qPCR and
147 ddPCR). We also examined *P. relictum* infection in the salivary glands for a subset of 106 Oahu
148 mosquitoes of all three Wolbachia types (*w*AlbB, *w*Pip, and None) (Supplemental Information,
149 Methods: Salivary gland infection).
150

151 **Statistical analyses**

152 We analyzed the fraction of mosquitoes testing positive for *P. relictum* by qPCR with
153 generalized linear models with a binomial distribution and a log link. We analyzed abdomen and
154 thorax infections separately. We included log(Parasitemia), days post feeding, mosquito strain
155 (Oahu, Palmyra, or Field), and *Wolbachia* strain (*w*AlbB, *w*Pip, or None) as predictors. We
156 excluded small batches of mosquitoes with N<4 (a batch is a unique mosquito strain+*Wolbachia*
157 strain+day post feeding+parasitemia), but results were qualitatively identical if we included all
158 mosquitoes. We examined two and three-way interactions among the predictors
159 $\log_{10}$(Parasitemia), days post feeding, and mosquito strain and used AIC to determine which
160 interactions were best supported by the data. We did not include spray color or *P. relictum*
161 isolate in the final model because neither had support when added to the best fitting model (see
162 Table S4; Spray color: $\square^2$ = 1.47, df = 2, P = 0.48; PUWA Isolate coef.: -0.225, SE = 0.18, Z = -
163 1.23, P = 0.22).

164 To examine salivary gland infection, we log transformed the ratio of *P. relictum* DNA to
165 mosquito DNA in the salivary glands and analyzed it with a linear model with *Wolbachia* type as
166 a predictor. There was heteroscedasticy in the residuals so we performed a robust comparison
167 using the *coeftest* function in the *lmtest* package with a variance-covariance matrix estimated
168 using the *sandwich* package. All analyses were performed in R, v.4.3.1.
169

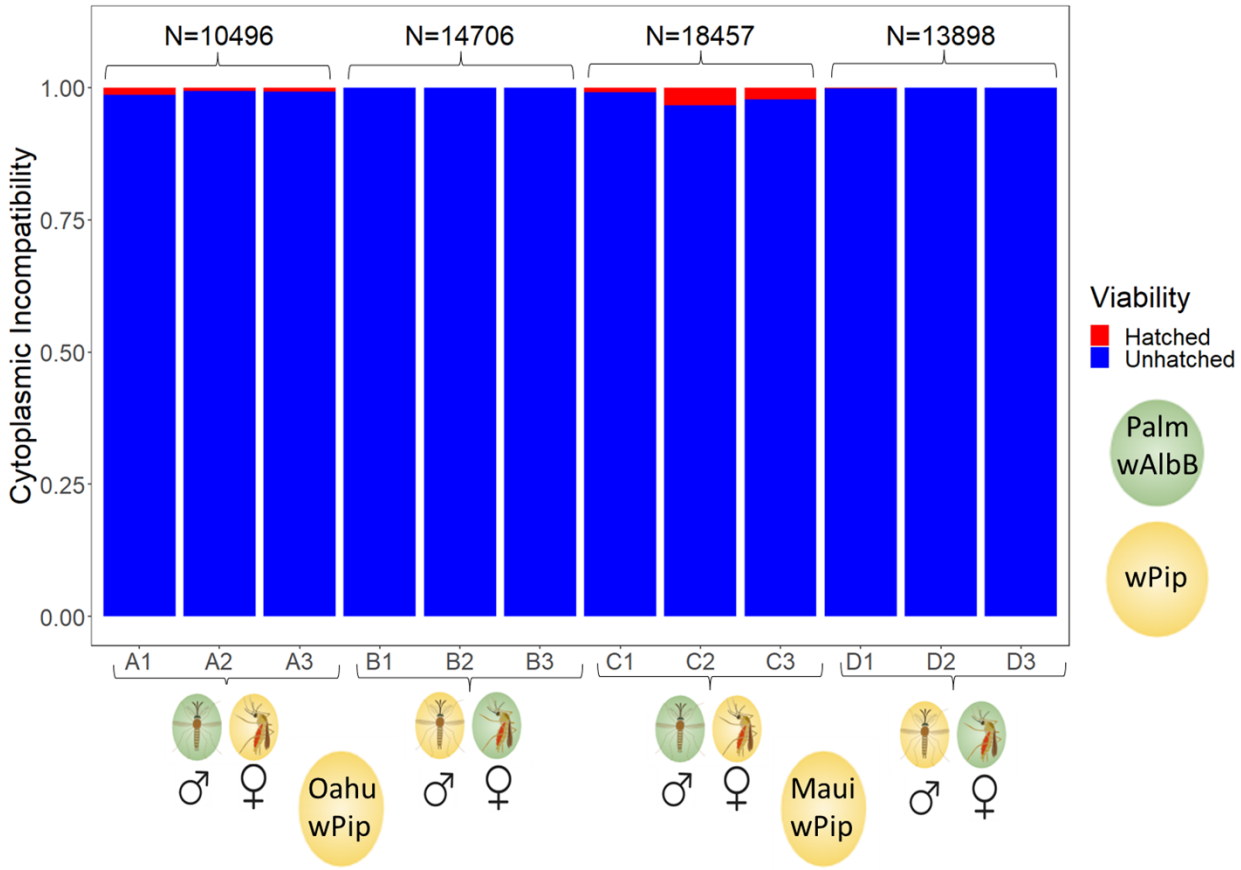
170 **Results**

171 **Transinfected line maternal transmission and Cytoplasmic Incompatibility (CI)**

172 We cleared *w*Pip *Wolbachia* infection from the Palmyra-*w*Pip strain of *C.*
173 *quinquefasciatus* with antibiotics and successfully transinfected a single female with the *w*AlbB
174 strain of *Wolbachia*. We then examined maternal transmission in 991 adults from generations 7
175 thru 13 of the mosquito line from this female ($N_{ave}$ = 188.8 (range 187-192)/generation,
176 excluding generation 9 which had 32 mosquitoes). Every sample from every generation tested
177 positive for *w*AlbB by ddPCR, indicating complete maternal transmission of *Wolbachia* across
178 generations (mean ratio of wAlbB gene copies to *C. quinquefasciatus* gene copies: 2.95; SE =
179 0.077).

180 Bidirectional cytoplasmic incompatibility between mosquitoes infected with *w*AlbB and
181 *w*Pip was nearly complete in both directions. In crosses between Palmyra-*w*AlbB males ("DQB")
182 with Oahu-*w*Pip females, only 0.93% of 18,457 eggs hatched (99.07% were inviable), and with
183 Maui-*w*Pip females, only 1.54% of 13,898 eggs hatched (98.46% were inviable) (Figure 1; Table
184 S2). Similarly, when Palmyra-*w*AlbB females were mated with Oahu-*w*Pip males only 0.03% of
185 10,496 eggs hatched (99.97% inviable), and when crossed with Maui-wPip males only 0.05% of
186 14,706 eggs hatched (99.95% inviable) (Figure 1; Table S3).

187




188

189 **Figure 1. Bi-directional cytoplasmic incompatibility results between Palmyra mosquitoes**
190 **infected with *Wolbachia wAlbB* (green ovals) and two mosquito strains (Oahu and Maui)**
191 **infected with native *Wolbachia wPip* (yellow ovals). Three replicates are shown for each**
192 **of four crosses that include both males and females of each mosquito strain: A1-A3:**
193 **Palmyra-*w*AlbB males X Oahu-*w*Pip females; B1-B3: Oahu-*w*Pip males X Palmyra-*w*AlbB**
194 **females; C1-C3: Palmyra-*w*AlbB males X Maui-*w*Pip females; D1-D3: Maui-*w*Pip males X**
195 **Palmyra-*w*AlbB females. Each of the 12 replicates had $N_{mean}$ = 4796 (range 786-7131); the**
196 **total number of eggs for each cross is shown above the bars.**

197

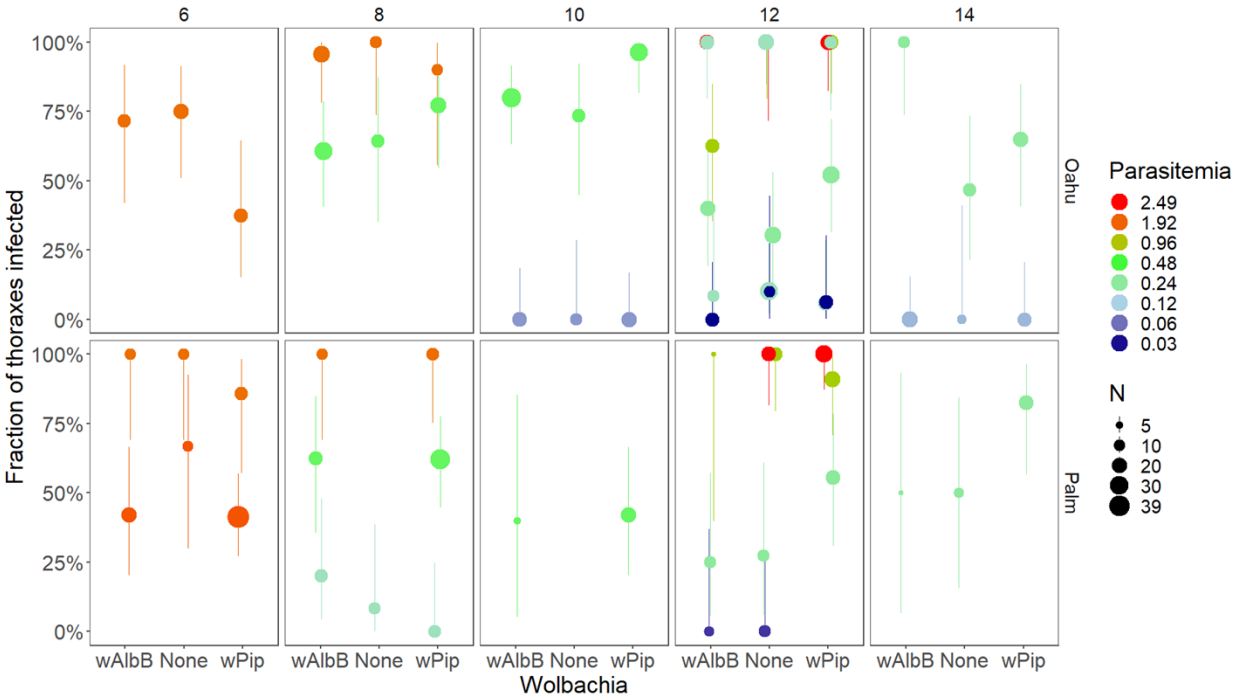
198 **Vector competence measurements**

199 We infected fourteen canaries with *P. relictum* GRW4, which had peak parasitemias
200 averaging 1.22% (range 0.04% - 4.7%; note that we didn't sample birds every day and could

201 have missed individual peak parasitemias). Peak parasitemia increased with passage number
202 (Log(peak parasitemia) = -6.81+ 0.465 (SE 0.19) * Passage number; P = 0.032; N= 13; $R^2$ =
203 35.3%), but was unrelated to the dose injected (# of parasites), or isolate (dose coeff.: -0.39, SE
204 4.02, N = 13, P = 0.925; isolate coef., PUWA vs CACO: -0.042, SE 0.68, N = 13, P = 0.952, in
205 separate models with passage number). Over the course of infection, birds had daily
206 parasitemias of 0.016% to 4.7% and we used parasitemias between 0.028% and 2.49% to
207 measure vector competence of *C. quinquefasciatus* mosquitoes (Figure S3).

208 We fed 68 batches (mean N = 57; SD = 17.9; range 20-130) of *C. quinquefasciatus*
209 mosquitoes on these infected canaries (Figure S4; Table S4; see Supplementary Material:
210 Feeding success). We tested the 1176 fed mosquitoes from these 68 batches in 73 groups for
211 abdomen and thorax infection, by qPCR (mean N = 16/group; SD = 7.5; range 4-46). Despite
212 this enormous number of groups, there was no evidence for differences in disseminated (thorax)
213 infection prevalence among *Wolbachia* strains (*w*AlbB, native *w*Pip, or None) (compare the
214 height of points with the same color in each panel of Figure 2 and height of different colored
215 lines in Figure 3; Table S5).

216 However, the fraction of mosquitoes with disseminated infections increased sharply with
217 parasitemia and days since feeding, and the slope of parasitemia increased over time (Figures
218 2,3, Table S5). Disseminated infection prevalence was slightly higher in the Oahu strain than in
219 the Palmyra strain (Figures S5; Table S5), and highest in the Field strain (Figure S5; Table S5).
220 The Results were qualitatively identical when we analyzed each *Wolbachia*-mosquito strain pair
221 separately (Figure S6; Table S6).




222

223 **Figure 2. Fraction of thoraxes infected (and binomial 95% CI) plotted against *Wolbachia***
224 **type in the mosquitoes (*w*AlbB, *w*Pip, or None).** The color shows the parasitemia (percent of
225 red blood cells infected) of the bird the mosquitoes fed upon (on a $\log_2$ scale), the panel rows
226 show the mosquito strain (Oahu or Palmyra), the panel columns show the days post-feeding
227 when the mosquitoes were dissected (6-14 days), and the size of the points shows the sample
228 size for each point (range 4-39). The fitted model (Table S5) indicates there are no consistent
229 differences among *Wolbachia* types (points of the same color in each panel are, on average, at
230 the same height). Points have been slightly jittered along the x-axis to aid in visualization. Note
231 that in a small number of experiments one of the three *Wolbachia* groups had insufficient
232 mosquitoes that successfully fed and is not shown (e.g. bottom middle panel, Palm-None on
233 day 10).

234

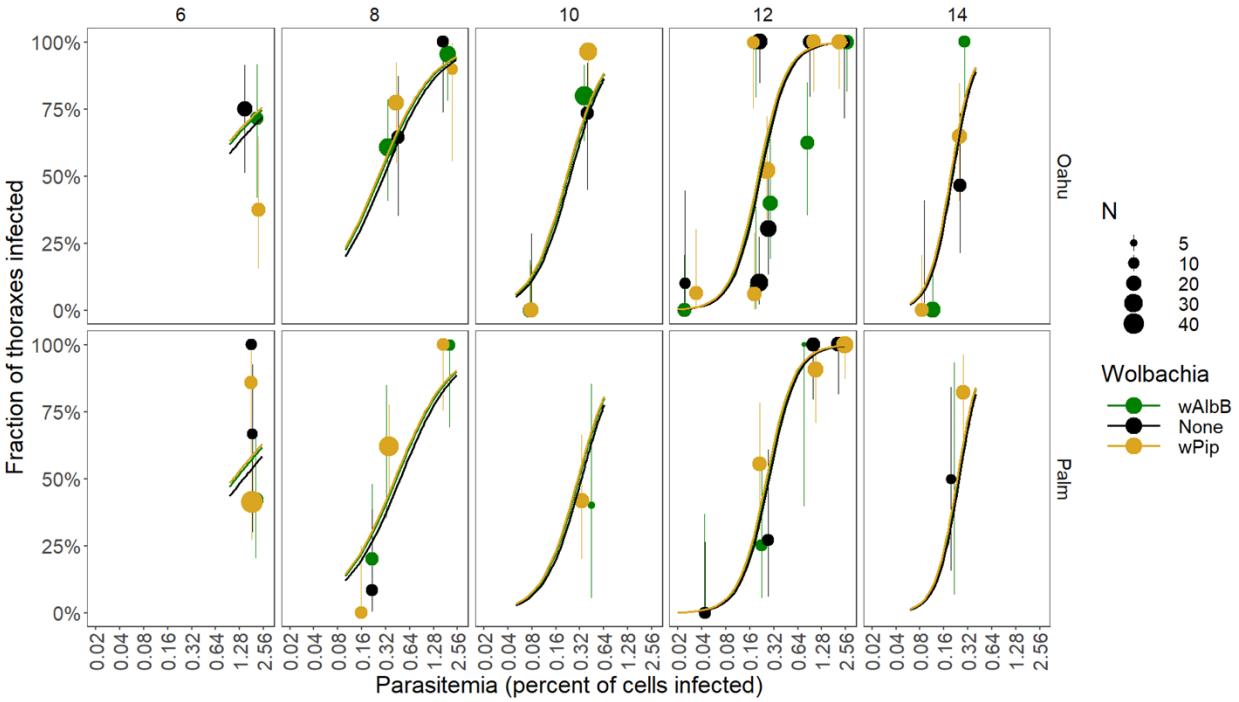
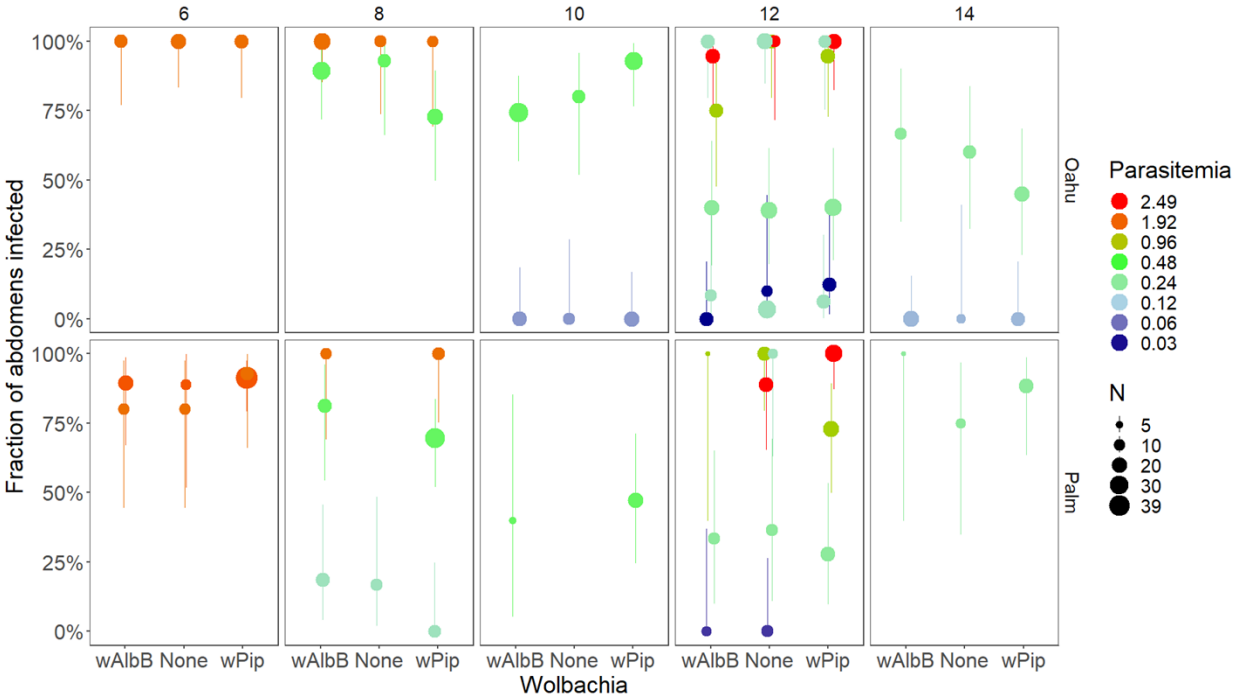



**Figure 3. Fraction of thoraxes infected (and binomial 95% CI) plotted against the parasitemia (percent of red blood cells infected) of the bird the mosquitoes fed upon (on a $\log_2$ scale).** This is the same data as in Figure 2, plotted with a different x-axis. Color shows the *Wolbachia* type in the mosquitoes (*w*AlbB, *w*Pip, or None), the panel rows show the mosquito strain (Oahu or Palmyra), the panel columns show the days post-feeding when the mosquitoes were dissected (6-14 days), and the size of the points shows the sample size (range 4-39). The lines show the fitted model for each of the three *Wolbachia* types, which are on top of each other and difficult to distinguish because there was no measurable differences among *Wolbachia* types (Table S5). Points have been slightly jittered along the x-axis to aid in visualization.

Abdomen infection prevalence was higher than disseminated infection prevalence, but patterns were similar, with there being no detectable effect of *Wolbachia* type, and infection prevalence increased with parasitemia, and differed between the mosquito strains (Figures 4, S7; Table S7). Abdomen infection prevalence increased faster with days since infection for the

251 Palmyra and Field strains than for the Oahu strain, which resulted in differences between the
252 mosquito strains changing with days since feeding (prevalence was higher in Oahu mosquitoes
253 6-10 days after infection but was higher in the Palmyra and Field strains 12-14 days after
254 infection; Figures S7; Table S7).

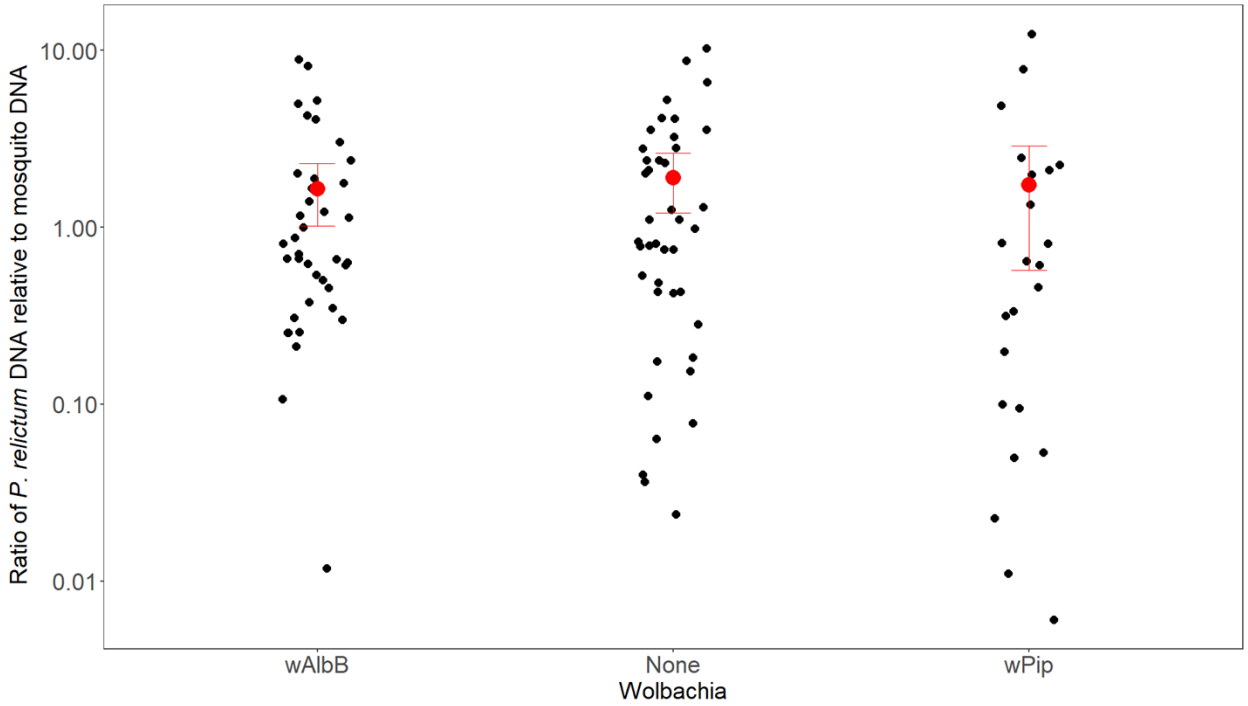



255

256 **Figure 4. Fraction of abdomens infected (and 95% CI) plotted against *Wolbachia* type in**
257 **the mosquitoes (*w*AlbB, *w*Pip, or None).** The color shows the parasitemia (percent of red
258 blood cells infected) of the bird the mosquitoes fed upon (on a $\log_2$ scale), the panel rows show
259 the mosquito strain (Oahu or Palmyra), the panel columns show the days post-feeding when the
260 mosquitoes were dissected (6-14 days), and the size of the points shows the sample size
261 (range 4-39). Points have been slightly jittered along the x-axis to aid in visualization.

262

263 We tested salivary glands from 106 Oahu mosquitoes for *P. relictum* DNA by ddPCR
264 that had fed on a canary with a parasitemia of 0.12% 14 days earlier. Salivary glands from all
265 106 mosquitoes tested positive for *P. relictum* DNA, and there were no differences in the
266 amount of *P. relictum* DNA among the three *Wolbachia* types (Figure 5).




267

268 **Figure 5. *Wolbachia* strain and salivary gland infection.** *P. relictum* DNA measured by
269 ddPCR, on a log scale, in salivary glands from 106 Oahu mosquitoes infected *w*AlbB, *w*Pip, or
270 no *Wolbachia* infection. Red points show mean and 95% CI. There was no difference among
271 *Wolbachia* types (robust F = 1.16, df = 2, P = 0.32).

272

273 **Discussion**

274 We successfully transinfected the *w*AlbB *Wolbachia* strain into *C. quinquefasciatus*
275 mosquitoes originating from Palmyra Atoll after clearing them of their natural *w*Pip *Wolbachia*
276 infection. The resultant Palmyra-*w*AlbB line of mosquitoes exhibited complete maternal
277 transmission of *w*AlbB across generations, and almost 100% bidirectional CI with mosquitoes
278 from Hawaii (Maui and Oahu) infected with *w*Pip. We then created another *w*AlbB-infected line
279 by backcrossing the Palmyra-*w*AlbB into an Oahu strain of *C. quinquefasciatus* that we cleared
280 of natural *w*Pip infection. The creation of these two lines of *w*AlbB-infected *C. quinquefasciatus*
281 mosquitoes makes population suppression of *C. quinquefasciatus* via widespread release of
282 *w*AlbB-infected males possible both in Hawaii and elsewhere. The bidirectional CI we observed

283 between *w*AlbB and *w*Pip mosquitoes would result in any accidentally released *w*AlbB-infected
284 females having inviable offspring if they mate with native *w*Pip males.

285 We then examined the effect of *w*AlbB, *w*Pip or no *Wolbachia* on vector competence in
286 two mosquito strains, Palmyra and Oahu, for *P. relictum* GRW4, the only lineage of avian
287 malaria in Hawaii (Beadell *et al.* 2006). We found infection with *Wolbachia* strains *w*AlbB and
288 *w*Pip had no detectable effect on vector competence of *C. quinquefasciatus* mosquitoes for
289 avian malaria *P. relictum* GRW4. There were no detectable differences among *Wolbachia*
290 groups in thorax infection or abdomen infection in either the Oahu or Palmyra mosquito strains.
291 We also found no difference among *Wolbachia* groups in the amount of *P. relictum* DNA in
292 salivary glands in a subset of mosquitoes from the Oahu strain. This is the first study, to our
293 knowledge, to assess the impact of both natural and stable transinfections of *Wolbachia* on
294 malaria vector competence in a natural mosquito-pathogen system. Taken together these
295 results indicate that the purposeful or accidental introduction of the *w*AlbB strain of *Wolbachia* to
296 mosquito populations in Hawaii would neither help nor hurt conservation efforts to reduce
297 transmission of avian malaria (Wild 2023).

298 Our results differ from two previous studies that found that *Wolbachia* altered
299 *Plasmodium* prevalence in malaria vectors. In *C. quinquefasciatus*, natural *w*Pip infections
300 increased vector competence for *P. relictum* lineage SGS1 compared to those cleared of
301 *Wolbachia* infection (Zélé *et al.* 2014a). In contrast, in *Anopheles stephensi, w*AlbB reduced
302 midgut infection prevalence of *Plasmodium falciparum* (Bian *et al.* 2013). The conflicting results
303 between these two studies suggests that the impacts of *Wolbachia* on *Plasmodium* infection are
304 not uniform, which is consistent with the highly variable effects of transinfected *Wolbachia* on
305 other pathogens in other vectors (Ross *et al.* 2019). We had much larger sample sizes than all
306 past studies combined, and examined seven lines of mosquitoes, including two inbred mosquito
307 strains with two strains of *Wolbachia* or no *Wolbachia* and one Field mosquito strain, suggesting

308 that a lack of an effect of *Wolbachia* on *Plasmodium* vector competence was not due to a lack of
309 power.

310 In contrast to the lack of differences among *Wolbachia* lines, we found large differences
311 among mosquito strains in vector competence, with Field mosquitoes having the highest vector
312 competence, followed by the inbred Oahu strain and then the inbred Palmyra strain. The
313 differences in vector competence among mosquito strains indicates that we had ample power to
314 detect differences in vector competence. They also underline the extensive variability in vector
315 competence among populations of the same mosquito species, which is common for many
316 vector-pathogen pairs (Kilpatrick *et al.* 2010; Reisen *et al.* 2008).

317 We found a relatively steep relationship between parasitemia and both thorax and
318 abdomen infection, and the relationship became steeper with time since feeding. Twelve days
319 after feeding the relationship was very steep, with parasitemias up to 0.1% leading to relatively
320 few disseminated thorax infections (<15.0%), whereas parasitemias only three-fold higher
321 (0.3%) led to most (73.0%) mosquitoes having disseminated infections. In contrast, eight days
322 after feeding, a 10-fold range of parasitemias (0.06% to 0.6%) produced a similar range in
323 thorax infection. The steep relationship between host parasitemia and disseminated infection
324 prevalence made it more challenging to determine the appropriate day to feed mosquitoes on a
325 canary and which day to dissect and test mosquitoes to obtain an intermediate level of infection.
326 In many of our feedings most or almost none of the mosquitoes in all groups had disseminated
327 infections (Figures 2, 3, S5). This steep relationship, if it is also present in wild mosquitoes,
328 would lead to highly heterogeneous infectiousness among birds (and bird species), with birds
329 infecting nearly all or almost none of the mosquitoes that fed on them depending on whether
330 their parasitemia was above or below a relatively narrow threshold parasitemia (e.g. ~0.1%-
331 0.3% on day 12 post feeding). Our data from the Field mosquito strain was limited, with only one
332 day post feeding (day 12) where we had fed mosquitoes on canaries with a range of
333 parasitemias (four parasitemias, ranging from 0.028% to 1.225%; Figure S7). Analysis of just

334 this (small) dataset of wild G0 mosquitoes produced a much less steep relationship (Figure S7),
335 suggesting the steep relationship we observed for the dataset composed of mosquitoes from
336 two inbred lines (Oahu and Palmyra), may be a result of limited genetic variation.

337 In summary, the rapid decline of many species of Hawaiian birds over the last decade,
338 due to a climate-change driven increase in malaria transmission at higher elevations, requires
339 urgent action to prevent extinction. We created two lines of *w*AlbB-infected *C. quinquefasciatus*
340 mosquitoes with high CI with Hawaiian *C. quinquefasciatus* infected with wPip, which could be
341 used to suppress mosquito populations via widespread release of *w*AlbB-infected males. We
342 examined whether the transinfected *Wolbachia* strain *w*AlbB increased or decreased vector
343 competence of these two lines for *P. relictum* GRW4. We found no effect of *Wolbachia* infection
344 on thorax, abdomen, or salivary gland infection, suggesting that replacement of the current
345 *Wolbachia* in Hawaiian mosquitoes (*w*Pip) with the *w*AlbB strain would have little impact on their
346 susceptibility to infection and ability to transmit this parasite. Furthermore, vector competence in
347 one population of mosquitoes collected directly from the field had much higher infection rates
348 than the two transinfected lines. This suggests that accidental releases of small numbers of
349 female mosquitoes with *Wolbachia* strain *w*AlbB with the large numbers of male mosquitoes
350 with *Wolbachia* strain *w*AlbB that is planned for 2024 (Wild 2023) is unlikely to alter the
351 transmission dynamics of malaria beyond the effect of the males greatly reducing mosquito
352 populations.
353

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358

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