Fitness costs and benefits in response to artificial artesunate selection in *Plasmodium*

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Abstract

Drug resistance is a major issue in the control of malaria. Mutations linked to drug resistance often target key metabolic pathways and are therefore expected to be associated with biological costs. The spread of drug resistance depends on the balance between the benefits that these mutations provide in the drug-treated host and the costs they incur in the untreated host. The latter may therefore be expressed both in the vertebrate host and in the vector.

Research on the costs of drug resistance focusses on interactions with vertebrate host, yet whether they are also expressed in the vector has been overlooked. In this study, we aim to identify the costs and benefits of resistance against artesunate (AS), one of the main artemisinin derivatives used in malaria-endemic countries. For this purpose, we compared different AS-selected lines of the avian malaria parasite *Plasmodium relictum* to their ancestral (unselected) counterpart. We tested their within host dynamics and virulence both in the vertebrate host and in its natural vector, the mosquito *Culex quinquefasciatus*.

The within-host dynamics of the AS-selected lines in the treated birds was consistent with the phenotype of resistance described in human *P. falciparum* malaria: a clearance delay during the treatment followed by a recrudescence once the treatment was interrupted. In the absence of treatment, however, we found no significant costs of resistance in the bird. The results of the two experiments to establish the infectivity of the lines to mosquitoes point towards a decreased infectivity of the drug-selected lines as compared to the ancestral, reference one. We discuss the potential implication of these results on the spread of artesunate resistance in the field.

Keywords: *Plasmodium,* drug-resistance, fitness costs, within-host dynamics, virulence, ACTs, artemisinin derivatives.

Introduction

Malaria is a life-threatening disease caused by *Plasmodium* parasites which are transmitted to people through the bites of infected mosquitoes. According to the latest World Health Organisation Malaria Report, in 2020 there were an estimated 240 million episodes of malaria which caused over 500 000 deaths, most of them among African children (WHO 2021).

Currently, four major antimalarial drug classes exist for the treatment of malaria: quinolines, antifolates, atovaquone and artemisinin derivatives. The widespread use of these synthetic anti-malarials has saved thousands of lives, but has also exerted an intense selection pressure for the evolution of resistance in the parasite. Drug resistance in malaria has become a major human health issue because *Plasmodium* parasites have evolved resistance to all classes of anti-malarials that have gone into widespread use. The rapid evolution of drug resistance in *Plasmodium* highlights the urgent need to understand the selective pressures under which drug resistant mutations emerge and spread in the population (Read and Huijben 2009; Huijben et al. 2011).

The fate of mutations responsible for resistance is expected to be governed by the balance between the benefits accrued in the drug-treated hosts and the costs suffered in the untreated hosts. The benefits of resistance are obvious: drug resistant parasites show an improved ability to survive the drug treatment. The costs of resistance, on the other hand, arise from the metabolic costs of detoxification or the reduced biochemical efficiency associated with target site mutations and are expected to become manifest in the absence of the drugs (Sirawaraporn et al. 1997; Hastings and Donnelly 2005). For this reason, these costs can be expressed both in the vertebrate host, but also in the vector. Current views about the impact of drug resistance on parasite fitness are, however, almost entirely based on data obtained from vertebrate hosts. In humans, costs of resistance have been inferred by the decrease in the frequency of drug-resistance alleles when drug use is stopped or discontinued (Laufer and Plowe 2004; Ord et al. 2007; Babiker 2009). The largest contributions to our understanding of the costs of drug resistance in the vertebrate host have, however, come from work carried out in animal models. *In vivo* competition experiments using laboratory selected drug resistant strains of the rodent malaria parasite *Plasmodium chabaudi* have shown that drug resistant strains are competitively suppressed by the sensitive strains in the absence of drug treatment (De Roode et al. 2004; Wargo et al. 2007; Huijben et al. 2010).

The costs of resistance in the mosquito vector have, in contrast, either been ignored entirely or given only cursory attention (Koella 1998). Yet, the passage through a mosquito is an essential step in the life cycle of *Plasmodium*. Within mosquitoes the parasites reproduce sexually, differentiate, proliferate and migrate to the salivary glands to ensure transmission to the next host.

75 Costs of drug resistance may be expressed at any one of these stages, profoundly impacting the
76 probability of transmission of drug resistant strains. These costs may be expressed in two different
77 ways: by altering the ability of the parasite to infect the mosquito vector (infectivity), or by altering
78 the effects that the parasite has on the fitness of the mosquito vector (virulence).

79 Here, we investigate the costs and benefits of resistance to artesunate (one of the main
80 artemisinin derivatives) both in the vertebrate host and in the vector. Artemisinin derivatives are
81 the most potent and effective anti-malarial to date. The precise mode of action of artemisinin
82 derivatives is still controversial, but several lines of evidence indicate that they exert their anti-
83 malarial action by perturbing redox homeostasis and haematin detoxification in the parasite
84 (O'Neill et al. 2010). To prevent the evolution of resistance, the World Health Organisation
85 recommends the use of the so-called artemisinin combination therapies (or ACTs) which combine
86 the highly toxic, but short lived, artemisinin derivatives with a partner drug with a long half-life
87 such as sulfadoxine-pyrimethamine, amodiaquine, or lumefantrine (WHO 2020). Despite the hopes
88 raised by the implementation of ACTs, artemisinin resistance is already present in several countries
89 of the Greater Mekong Subregion (WHO 2020), and worryingly, the *de novo* emergence of
90 artemisinin resistance has been recently detected in Subsaharan Africa (Uwimana et al. 2020).
91 Phenotypically, artemisinin resistant parasites are characterized by (i) a delay in parasite clearance
92 (resistant parasites take an extra 24-48 to be cleared from the blood) followed by (ii) a recrudescence
93 of the parasite at the end of the standard 3-day therapeutic course (Dondorp et al. 2009). This
94 phenotype has been shown to be associated to several mutations in the Kelch 13 (K13) propeller
95 domain of the parasite (Straimer et al. 2014). Despite the ongoing spread of artemisinin resistance
96 across malaria-endemic areas we still lack a clear picture about the costs and benefits associated to
97 these mutations (but see Nair et al. 2018; Tirrell et al. 2019).

98 Selecting for drug resistance in rodent and avian malaria has proven to be a straightforward
99 procedure that provides a powerful tool for testing the effects of a particular drug resistance
100 mutation on parasite fitness *in vivo* (Greenberg 1956; Beaudoin et al. 1967; De Roode et al. 2005;
101 Walliker et al. 2005; Wargo et al. 2007; Huijben et al. 2011). Here we select for artesunate (AS, a
102 widely-used artemisinin derivative) resistance in *Plasmodium relictum* (the most widespread species
103 of avian malaria in the wild) by submitting it to gradually increasing doses of the drug. We then
104 compare the within host dynamics and virulence of the drug-selected lines to the reference line
105 both in the presence and in the absence of an AS treatment in the vertebrate host. We expect that:
106 i) in treated hosts (birds), artesunate-selected lines will exhibit a similar phenotype to human
107 malaria, i.e. delayed clearance followed by recrudescence, ii) in untreated hosts (birds) and vectors

108 (mosquitoes), artesunate-selected parasites suffer higher fitness costs in comparison to their
109 unselected counterparts.

110 Material and Methods

111 **Mosquito and parasite protocols -**

112 *Plasmodium relictum* (lineage SGS1) is the aetiological agent of the most prevalent form of
113 avian malaria in Europe. The biology of this parasite species is similar to that of human malaria,
114 both in the vertebrate host and in the mosquito. Avian malaria has historically played a key role in
115 human malaria research, and in particular in the development and testing of the first anti-malarials
116 (Hewitt 1940; Rivero and Gandon 2018). Our parasite lineage was isolated from blue tits (*Parus*
117 *caeruleous*) collected in the Montpellier area (France) in October 2016 and subsequently passaged to
118 naïve canaries (*Serinus canaria*) by intraperitoneal injection. Since then, it has been maintained by
119 carrying out regular passages between our stock canaries through intraperitoneal injections with
120 the occasional passage through the mosquito (one passage through mosquito every c.a. 15 bird to
121 bird passages, on average).

122 All experiments were carried out using a laboratory line of *Culex quinquefasciatus* (SLAB
123 strain). Vectors in the *Culex pipiens* complex (*Cx pipiens* and *Cx quinquefasciatus*) are the principal
124 natural vector of *Plasmodium relictum* in the wild. The larvae in all the experiments were reared at a
125 constant density per tray (n=300 larvae) following previously published laboratory protocols
126 (Vézilier et al 2010). Larval trays (n=20) were placed individually inside an "emergence cage" (40
127 cm x 28 cm x 31 cm) and emerged adults were allowed to feed *ad libitum* on a 10% glucose water
128 solution.

129 AS-selected parasite lines were obtained by treating infected birds with increasing
130 concentrations of artesunate (Sigma A3731). Birds were infected by injecting them intra-
131 peritoneally with 100 µl of infected blood. The artesunate treatment was initiated 12 days later, to
132 coincide with the peak of the acute phase of the parasite's infection. Artesunate was diluted in
133 bicarbonate (50 mg/Kg) and administered through intra-peritoneal injections carried out twice
134 daily (9am and 6pm) for four consecutive days. Five or six days later, 100 µl of blood from the
135 treated birds was taken via a wing puncture, transferred to new canaries, and the treatment was
136 thus repeated until a total of 5 passages were carried out. Three AS-selected lines were obtained in
137 this way. Line AS1 was obtained by treating birds with 16 mg/kg artesunate for 3 passages followed
138 by 32 mg/kg for a further 2 passages. Lines AS2 and AS3 were obtained by treating birds with 8
139 mg/kg for 3 passages followed by 16 mg for 2 passages. The reference line was maintained in

140 parallel by injecting birds with bicarbonate following the same protocol. Experiments were initiated
141 immediately after the 5th passage.

142

143 **Experiment 1 – Phenotypic characterization of drug resistance in the bird**

144 Artesunate-resistance is expected to be beneficial for the parasite in the presence of the
145 drug, but to incur fitness costs when the drug is absent. In this experiment, we infected birds with
146 either the reference line or one of the drug-selected lines (AS1, AS2, AS3). Half of the birds where
147 then treated with a standard dose of artesunate, while the other half were given a sham
148 (bicarbonate) injection. For each bird we quantified parasitaemia (proportion of red blood cells
149 infected) through time and parasite virulence (bird weight loss and anaemia).

150 The detailed experimental protocol was as follows (**Figure 1**). Birds were inoculated with
151 each of the parasite lines (reference, AS1, AS2 and AS3) by means of an intra-peritoneal injection
152 (6 birds per line, all birds in these experiments were 1-2 years old). Each bird received between
153 70 000 to 90 000 parasites, except for the birds injected with the AS3 line, which received 30 000
154 parasites because the parasitaemia in the donor birds were too low. Bird weight and red blood cell
155 count (RBC: number of red blood cells per ml of blood, Beckman Coulter Counter, Series Z1)
156 were measured immediately before the infection and every two days thereafter. Bird parasitaemia
157 (% red blood cells infected) was monitored every two days from the second day after the
158 inoculation (henceforth "day 2 pbi": post-bird inoculation) onwards, using thin blood smears. On
159 day 12 pbi, to coincide with the acute phase of the parasite infection, half of the birds were injected
160 with 50 µl of 16mg/kg AS (dissolved in 50mg/kg bicarbonate) while the other half were injected
161 with an equivalent amount of bicarbonate (50mg/kg). The injections took place twice a day (9 am
162 and 6 pm) for 4 consecutive days (days 12-15 pbi). During this period, parasitaemia and virulence
163 measurements were taken 24h after each injection (days 13-16 pbi). Bird parasitaemia, weight and
164 RBC were thereafter followed every two days for 16 days after the end of the treatment (days 18-
165 30 pbi, **Figure 1**). To account for the variance in parasitaemia between birds on the day immediately
166 preceding the treatment (day 12 pbi, see Supplementary Table ST1), the parasitaemia on day 13 pbi
167 onwards was calculated as a fold-increase (or decrease) in the number of parasites using the
168 parasitaemia at 12 pbi as the baseline (*px*/*pb*, where *px* is the parasitaemia at a given day, and *pb* the
169 baseline parasitaemia at day 12 pbi).

170 **Experiment 2 – Costs of drug resistance in the vector: parasite infectivity and virulence**

171 The aim of this experiment was to establish whether AS-selected and reference lines differ
172 in their infectivity and virulence to the mosquito. For this purpose, mosquitoes were allowed to
173 blood feed on birds infected with the drug-selected and reference parasite lines. Mosquitoes fed on
174 uninfected birds were used as controls. A subsample of the mosquitoes was used to quantify
175 parasite density, while the rest were followed for several days to measure their fecundity and
176 longevity. Since having too many mosquitoes in the cage risk compromising the wellbeing of the
177 bird, and given that AS3 did not have the expected phenotype of resistance (delayed clearance
178 followed by recrudescence), this experiment was done using only AS1 and AS2.

179 The detailed experimental protocol is as follows (**Figure 1**). Six naïve birds were infected
180 with 100 000 parasites of either the reference, AS1 or AS2 lines (2 birds per line). Two further birds
181 were used as uninfected controls. On day 12 pbi, birds were introduced individually into cages
182 containing 150 12-day old female mosquitoes. The following morning, unfed females were counted
183 and discarded from the study and all cages were provided with *ad libitum* sugar in the form of a
184 10% sugar solution. To quantify fecundity, three days after the blood meal (day 3 pmi, post-
185 mosquito infection), a tray of water was placed in each to allow females to lay eggs. The trays were
186 checked daily for the presence of eggs for 3 consecutive days (days 3-6 pmi). *Culex pipiens* females
187 lay eggs in a single raft containing up to 250 tightly-packed eggs. This facilitates the estimation of
188 the number of laying females and the eggs laid per female. The egg laying date was recorded and
189 the egg rafts were photographed using a binocular microscope equipped with a numeric camera.
190 The eggs counted using the Mesurim Pro freeware (Academie d'Amiens, France). Survival was
191 assessed daily by counting dead individuals lying at the bottom of each cage until all females died.

192 To quantify parasitaemia, on day 8 and 9 pmi, 20 mosquitoes were haphazardly sampled
193 from each cage and immediately dissected under a binocular microscope to extract the oocyst-
194 infected midguts. Midguts were stained with a 5% mercurochrome solution to assess infection rate
195 (oocysts present/absent) and oocyst burden (number of oocysts) under a phase contrast
196 microscope.

197 **Experiment 3 – Costs of drug resistance in the vector: parasite dynamics**

198 The aim of this experiment was to explore whether the results obtained in Experiment 2
199 may be explained by a difference in the within-vector parasite dynamics between AS-selected and
200 reference lines. For this purpose, mosquitoes were allowed to blood feed on birds infected with
201 the drug-selected and reference parasite lines (as above). Mosquitoes were dissected at regular
202 intervals and the number of oocysts and sporozoites were compared between the AS-selected and
203 the unselected reference lines.

204 The detailed experimental protocol is as follows (see **Figure 1**). Six naïve birds were
205 infected with 100 000 parasites of either the reference, AS1 or AS2 lines (2 birds per line). As in
206 the previous experiment, on day 12 pbi, birds were introduced individually into cages containing
207 150 12-day old female mosquitoes. The following morning, unfed females were counted and
208 discarded from the study and all cages were provided with *ad libitum* sugar for the reminder of the
209 experiment. Starting on day 4 post blood meal and every 2 days thereafter (4-20 dpmi), 20
210 mosquitoes were haphazardly sampled from each cage and immediately dissected under a binocular
211 microscope to extract the oocyst-infected midguts and count the oocysts (as above). The mosquito
212 thorax, containing the sporozoite-infected salivary glands, was then severed and frozen at -20°C
213 for the subsequent quantification of parasites via qPCR. Sporozoite quantification was calculated
214 as a ratio between mosquito DNA (*ace2* gene) and *Plasmodium relictum* DNA (*cytb*) as described
215 previously (Zélé et al. 2014).

216 **Ethics statement**

217 Bird manipulations were carried out in strict accordance with the "National Charter on the
218 Ethics of Animal Experimentation" of the French Government. Experiments were approved by
219 the Ethical Committee for Animal Experimentation established by the authors' institution (CNRS)
220 under the auspices of the French Ministry of Education and Research (permit number CEEA- LR-
221 1051).

222 **Statistical analyses**

223 Analyses were carried out using the R statistical package (v3.4.4). The general procedure to
224 build models was as follows: line (reference, AS1, AS2, AS3), sampling day, treatment (AS treated or
225 sham-treated, Experiment 1 only) and parasitaemia were introduced into the model as fixed
226 explanatory variables. Bird ID and the qPCR plates used to quantify sporozoites in the head-thorax
227 fraction of the mosquito (Experiment 3 only) were fitted as random effects. Maximal models,
228 including all higher order interactions, were simplified by sequentially eliminating non-significant
229 terms and interactions to establish a minimal model. The significance of the explanatory variables was
230 established using a likelihood ratio test (LRT) which is approximately distributed as a chi-square
231 distribution (Bolker 2008) and using $p = 0.05$ as a cut-off p-value.

232 Mosquito survival (Experiment 2) was analyzed using Cox proportional hazards mixed
233 effect models (coxme). Proportion data (oocyst and sporozoite prevalence) were analyzed using
234 mixed linear models and a binomial distribution. Count data (eggs per raft) were analyzed using
235 mixed linear models and a Poisson distribution. Response variables that were highly overdispersed
236 (e.g. oocyst burden) were analyzed using mixed negative binomial models (glmmTMB). *A posteriori*

237 contrasts were carried out by aggregating factor levels together and by testing the fit of the
238 simplified model using a LRT (Crawley 2007). Statistical analyses are summarised in Supplementary
239 Table ST2. Two additional standard measurements of survival were obtained in each cage: the
240 median survival (the time at which 50% of the population is still alive) and the proportion of
241 mosquitoes that survived till day 14 (the time at which sporozoites peak in all lines, see below).

242

243 Results

244 **Experiment 1 – Phenotypic characterization of artesunate resistance in bird**

245 We infected birds with either the reference line or one of the drug-selected parasite lines
246 (AS1, AS2, AS3). Twelve days later, half of the birds were then treated with AS, while the other
247 half were given a control (bicarbonate) injection (**Figure 1**). For each bird, we quantified parasite
248 density dynamics (proportion of infected red blood cell through time) and parasite virulence (bird
249 weight loss and anaemia) before, during and after the AS treatment.

250 We started by analysing whether there were differences in parasitaemia between lines *before*
251 the AS-treatment (days 2-12 pbi). We fitted a full model that included day, line and treatment (and
252 bird as a random factor to control for temporal pseudo-replication). There was no significant 3-
253 way interaction and no significant differences in parasitaemia between birds infected with the
254 different lines, or subsequently allocated to the treated or control treatments (although variance
255 between birds was high, **Supplementary Figures SF1A-D**). This model only detected differences
256 in parasitaemia between days (model 1, χ^2= 33.34, p < 0.0001).

257 *During* the treatment (days 13-16 pbi), the parasitaemia of treated and untreated birds
258 followed very different courses. Treatment with AS had a very significant effect on the
259 parasitaemia, and the effect was dependent on both the day and the parasite line (model 2, χ^2=
260 12.088, p = 0.0071, **Figure 2A**). To obtain a more accurate picture of the nature of this interaction,
261 subsequent analyses were done separately for AS-treated and reference birds. Treatment with AS
262 resulted in an overall decrease in parasitaemia in all lines (see Supplementary Table ST3). On the
263 first day after the treatment (day 13 pbi) the reduction in parasitaemia was greater in the reference
264 line (- 93.6% on average) than in either the selected AS1 (- 59.8%), AS2 (- 57.8) or AS3 (- 69,6%)
265 lines. This effect largely disappears in subsequent days (Supplementary Table ST3). The statistical
266 analyses revealed a significant interaction between day and line (model 3, χ^2= 20.563, p= 0.0147).
267 In untreated birds, there was a significant difference between the lines (model 4, χ^2= 9.1288, p=

268 0.0276) due to AS2 having a significantly higher parasitaemia than the other lines. This effect was
269 independent, albeit marginally, of the sampling time (model 4, χ^2= 7.7845, p= 0.0507, **Figure 2B**).

270 *After* the AS treatment, parasitaemia in the treated birds showed a different pattern between
271 the lines (model 5, χ^2= 8.8851, p= 0.0308, **Figure 2A**). Lines AS1 and AS2 had a significantly
272 higher parasitaemia than the reference line. In untreated birds, the parasitaemia decreased gradually
273 with time (model 8, χ^2= 54.449, p < 0.01) and was independent of the parasite line (model 6, *line*:
274 χ^2= 7.9084, p= 0.0479, **Figure 2B**).

275 Virulence was quantified as a change in weight and a change in RBC counts (anaemia) with respect
276 to the weight and RBC counts on the first day of the experiment. Weight changes following the
277 infection were significant and dependent both on the parasite line, on the treatment group (AS-
278 treated or untreated), and on the timing with respect to the treatment (before, during or after,
279 model 7, χ^2 = 34.51, p = 0.001). In untreated birds, there was a weight difference between the lines,
280 with birds infected with AS1 and AS2 (but not AS3) having a significant lower weight than those
281 infected with the reference line throughout the experiment (model 9, χ^2 = 26.56, p = 0.0002, Figure
282 SF2B). In treated birds, however, the weight difference disappeared during the treatment, only to
283 reappear after the treatment ended (model 8, χ^2 = 29.28, p < 0.0001, **Supplementary Figure**
284 **SF2A**). RBC counts were significantly lower during the treatment than before or after the treatment
285 (model 10, χ^2 = 54.55, p < 0.0001), but were independent of the line and were similar in AS-
286 treated and control birds (**Supplementary Figures SF3A and SF3B**).

287 **Experiment 2 – Costs of drug resistance in the vector: parasite infectivity and virulence**

288 Mosquitoes were allowed to blood feed either on uninfected birds or on birds infected with
289 drug-selected and reference parasite lines. A subsample of the mosquitoes fed on the infected birds
290 was used to quantify oocyst density, while the rest were followed for several days to quantify their
291 fecundity and longevity.

292 The overall prevalence of infection was low (29 ± 4% of female mosquitoes had at least 1
293 oocyst in their midgut) and independent of the parasite lines (model 11, χ^2= 0.6017, p= 0.7402,
294 **Figure 3A**). Oocyst burden (number of oocysts in infected females) was, however, significantly
295 different between the lines (model 12, χ^2= 8.0512, p= 0.0179); mosquitoes infected with the
296 reference line had a significantly higher number of oocysts (mean ± s.e: 70 ± 22) than mosquitoes
297 infected with the AS1 (mean ± s.e: 13 ± 5) and AS2 (mean ± s.e: 25 ± 11) lines (**Figure 3B**).

298 There were also significant differences in mosquito fecundity (number of eggs per raft)
299 between lines (model 14, χ^2= 11.1, p= 0.0112). Post-hoc analysis showed that mosquitoes infected

300 with the AS2 line laid significantly fewer eggs (mean ± s.e: 126 ± 4) than uninfected mosquitoes
301 (142 ± 5) and mosquitoes infected with the refence (142 ± 4) and AS1 (148 ± 4) lines (χ^2= 6.5741,
302 p= 0.0104, **Figure 4**). There were no significant differences in longevity between infected and
303 uninfected mosquitoes, or between mosquitoes infected with the different lines (model 13, χ^2=
304 3.7758, p= 0.2867). The median longevity (the time at which 50% of the mosquitoes in each
305 treatment is still alive) and the proportion of mosquitoes that survived till day 14 (peak sporozoite
306 production) are given in Supplementary Table ST4.

307 **Experiment 3 – Costs of drug resistance in the vector: parasite dynamics**

308 This experiment was carried out to investigate whether the differences in oocyst burden
309 between selected and reference lines may be due to a difference in the dynamics of development
310 of the parasite within mosquitoes. **Figure 5** shows the cumulative increase in oocysts in mosquitoes
311 having fed in birds infected with each of the lines (reference or AS-selected). No mosquitoes in
312 any of the lines were infected on day 4 so this time point was taken off the analyses. Mosquitoes
313 infected with the reference line showed a faster cumulative increase in oocysts than mosquitoes
314 infected with the AS-selected ones. As a result, by days 8-9 (corresponding to the dissection time
315 in Experiment 2), mosquitoes fed on birds infected with the reference line had between 60-70%
316 of the cumulative total number of oocysts, while mosquitoes fed from 3 out of the 4 birds infected
317 with AS-selected lines had 0-16% of the cumulative total. Mosquitoes fed from one of the (AS1-
318 infected) bird, however, showed an unusually high number of oocysts on the first dissection day.

319 The mean oocyst prevalence (% mosquitoes containing at least 1 oocyst) averaged over the
320 whole experiment was higher in reference (67.92 ± 6.47%, mean ± s.e.) and AS1 lines (53.65 ±
321 7.88%) than in the AS2 line (36.36 ± 10.49%). The same pattern was found with oocyst burden,
322 which was higher in the reference (32.80 ± 11.95) and AS1 lines (18.90 ± 11.33) than in the AS2
323 line (7.75 ± 4.83). When day was fitted to the model the effect of line, however, disappeared for
324 both oocyst prevalence (model 15, χ^2= 5.3164, p= 0.070 and burden (model 16, χ^2= 0.2313, p=
325 0.8908; **Figure 6A and 6B**).

326 The mean sporozoite prevalence, averaged over the whole experiment, was roughly 2-times
327 higher in the reference (28.30 ± 6.24%) and AS1 lines (27.90 ± 6.92%) than in the AS2 line (13.63
328 ± 7.48%). Sporozoite burden, on the other hand, was 4-5 times higher in the reference line (0.0126
329 ± 0.0067) than in the AS-selected lines (AS1: 0.0026 ± 0.0009, AS2: 0.0034 ± 0.0029). As above,
330 when day was fitted to the model the effect of line on sporozoite prevalence and burden
331 disappeared (model 17, χ^2= 0.1788, p= 0.9145, and model 18, dχ^2= 1.9214, p= 0.3826, respectively
332 **Figure 6C and 6D**).

333

334 ## Discussion

335 The two telltale signs of *P. falciparum* resistance to artemisinin derivatives in the field are a
336 delay in the clearance of parasites during ACT treatment followed by a recrudescence once the
337 treatment is interrupted (Douglas et al. 2021). Artemisinin derivatives are remarkably efficient at
338 clearing susceptible parasites from the blood (typically within 24-48 hours in *P. falciparum*). A slower
339 clearance of parasites after the treatment, typically taking an extra 24-48 hours, is interpreted as
340 evidence of resistance to the artemisinin-derivative component (Witkowski et al. 2013; Mok et al.
341 2015). This delayed clearance phenotype is a far cry from the standard definition of resistance
342 (survival to a drug treatment), which is why artesunate resistance is commonly referred to as a
343 'partial resistance' (Uhlemann et al. 2010; Menard and Dondorp 2017). Partial artemisinin resistance
344 rarely leads to ACT failure, but may select for drug resistance to the partner drug, as larger parasite
345 populations remain in the blood stream for longer periods of time (Fairhurst 2015). In our study,
346 the within-host dynamics of the drug-selected lines in the treated birds was consistent with the
347 phenotype of resistance on *P. falciparum* in the field. AS-selected lines took longer to respond to
348 the treatment than their reference line counterparts. In the first 24h after the beginning of the
349 treatment the parasitaemia of the reference line dropped by over 90%, while that of the AS-selected
350 lines only dropped by between 60% (AS1, AS2) and 70% (AS3). By the following day, there was
351 no discernible difference between the parasitaemia of the reference and drug selected lines, (**Figure**
352 **2A**). There are no significant differences in the pre-treatment dynamics between the lines that may
353 explain this significant difference in the parasitaemias in the first 24h between the treatments.
354 (**Supplementary Figure SF1**). In keeping with previous studies on *P. falciparum* parasites, two of
355 our drug-selected lines (AS1, AS2) also had a pronounced recrudescence after the treatment was
356 interrupted, producing on average more than 20 times as many parasites in the two weeks following
357 drug treatment than did the reference line (**Figure 2A**). One potential explanation of the different
358 dynamic of AS3, may be found in the lower infective dose given to the birds (see above).

359 Drug resistance mutations are known to disrupt the parasite's metabolism, generating
360 fitness costs. In drug-treated hosts these costs are largely compensated by the benefits conferred
361 by the resistance. In untreated hosts, however, the magnitude of these costs will determine whether
362 these mutations will persist and spread in the population. Current views about the impact of drug
363 resistance on parasite fitness are almost entirely based on data obtained from the vertebrate host.
364 In humans, costs of resistance have been inferred by the decrease in drug-resistance alleles when
365 drug use is stopped or discontinued (Laufer and Plowe 2004) and from the decrease in the

366 frequency of drug resistant parasites during the dry season, when there is no transmission and drug
367 use is dramatically reduced . Direct experimental evidence of the costs of artemisinin resistance has
368 been obtained by setting up *in vitro* competition experiments between drug-resistant and reference
369 *Plasmodium* strains (Hott et al. 2015; Nair et al. 2018; Tirrell et al. 2019; Mathieu et al. 2020). To our
370 knowledge the only *in vivo* experiments were carried out with *Plasmodium chabaudi* (a rodent species)
371 and showed no obvious cost of AS in the absence of treatment (Pollitt et al. 2014).

372 We did not observe any costs associated to the AS-selected lines in the absence of
373 treatment. Experimental evidence of the costs of resistance has been obtained by setting up
374 competition experiments between drug-resistant and susceptible *Plasmodium* strains. Using *in vitro*
375 experiments where the erythrocyte stages of a drug resistant *P. falciparum* strain bearing mutations
376 in the *pfmdr1* gene competed against a drug-sensitive strain with the same genetic background,
377 Hayward et al (2005) estimated that the loss of fitness of the resistant strain was ca. 25% per
378 generation. *In vivo* competition experiments using laboratory selected drug resistant strains of the
379 rodent malaria parasite *Plasmodium chabaudi* have confirmed that drug resistant strains are
380 competitively suppressed by the sensitive strains in the absence of drug treatment (de Roode *et al.*
381 2004b; Wargo *et al.* 2007b; Huijben *et al.* 2010). In our experiments the AS-selected lines were not
382 set to compete with the reference line, which may explain the absence of costs. We instead
383 obtained an opposite trend, whereby the drug-selected lines (AS2 in particular) fared better in terms
384 of parasitaemia than its unselected counterparts. One possible explanation for these results, which
385 would warrant further studies, is that drug selection may be expected to select for traits that increase
386 the parasite's survival probabilities in the presence of drugs (Birget et al 2018). Highly replicating
387 parasite lines may be selected for because they can rapidly recover high parasite densities after a
388 bout of drug-induced mortality (Schneider et al. 2008, Schneider et al. 2012). Another possibility
389 is that when confronted with the stressful environment imposed by the drugs, parasites may
390 parasites may exercise a reproductive restraint (Schneider & Reece 2021), that is, sacrifice
391 gametocyte production and prioritize asexual replication to maximise long term survival in the host.
392 Either way, in as much as virulence is related to parasite fitness through high asexual parasite
393 densities and gametocyte production (Mackinnon and Read 2004), this opens the unpalatable
394 possibility of drugs selecting for more resistant but also more virulent parasites. In our experiments,
395 we obtained some indication of a differential virulence between lines. Birds infected with AS1 and
396 AS2 lines had a significantly higher weight loss than those infected with the reference line, but this
397 was not associated to any clear pattern of RBC loss (anaemia). Current evidence for an association
398 between drug resistance alleles and parasite virulence in the field is weak (Tukbasibwe et al. 2017,

399 Cuu et al. 2020), but more work using experimental animal models under controlled conditions
400 are, in our opinion, urgently needed.

401 Despite its crucial implications for the emergence and spread of drug resistant mutations
402 in the field, work on the transmissibility of artemisinin-resistant parasites, is practically non-
403 existent. St. Laurent et al. (2015) showed that artemisinin-resistant strains of *P. falciparum* from
404 Cambodia were able to infect and produce sporozoites in three vector species (*An. dirus*, *An.*
405 *minimus, An. coluzzi*), and Pollitt et al. (2014) showed that in the rodent malaria species *P. chabaudi*,
406 the AS-selected line produced more gametocytes (the blood stages of the parasite that are
407 transmitted to the mosquito) than its reference counterpart. Here, we infected mosquitoes with the
408 drug-selected and reference lines in two different experiments. In Experiment 2, all mosquitoes
409 were dissected on a single day (half of the mosquitoes on day 8 and half on day 9). corresponding
410 to the peak ocystaemia as established in previous experiments with the ancestral, reference line In
411 this experiment we observed no significant difference in prevalence between the lines, but a highly
412 significant difference in the number of oocysts between the reference and the two drug-selected
413 lines : the latter produced between 3-5 times fewer oocysts than the reference line (**Figure 3**). This
414 difference could reflect either a true difference in oocyst burden between the lines, or a shift in the
415 within-mosquito dynamics (peak oocystaemia may have occurred earlier or later in the drug-
416 selected lines). To discriminate between these two options a new experiment (Experiment 3) was
417 launched, where both oocysts and sporozoites were quantified at regular, 2-day, intervals, from day
418 6 to day 20 post infection. This experiment suffered from the caveat of an unusually low infection
419 prevalence (23%), which significantly reduced the statistical power of the burden analyses, and
420 precluded any meaningful analyses of peak oocystaemia. The cumulative frequency of oocysts,
421 however, suggested that oocysts accumulate at a slower rate in mosquitoes infected with the AS-
422 selected lines (**Figure 5**). Further work should explore this issue by, eg measuring potential
423 differences in the growth rate (size) of oocysts issued from reference and AS-resistant lines. The
424 mean overall prevalence and burden of oocysts and sporozoites (i.e. prevalence and burden
425 averaged over all time points) was also in lower AS1 and AS2 mosquitoes. Sporozoite burden, in
426 particular, was 4-5 lower in the drug-selected lines than in the reference line. The results therefore
427 concur with those obtained in the previous experiment in that AS-selected lines were less infective
428 to the mosquitoes than their reference counterparts. Another potential caveat of these experiments
429 was that, for logistical reasons, we only used 2 bird hosts per line. It would be interesting to repeat
430 them with a higher number of hosts to account for potential between-host variability in mosquito
431 infection rates.

432 In conclusion, our data using drug-selected strains of *P. relictum* provides further proof that
433 susceptibility to artesunate can be rapidly lost under drug pressure. The clearance delay and
434 recrudescence observed during and after the AS treatment, respectively, in two of our three AS-
435 selected lines were similar to the resistance phenotype seen in human malaria infections. The
436 genetic underpinnings of artemisinin resistance are extremely complex and not yet entirely
437 elucidated (Behrens et al. 2021). In *P. falciparum* the resistant phenotype is associated with several
438 mutations in the propeller domain of kelch13 (*pfk13*), a protein that is essential for the intra-
439 erythrocytc growth of the parasite. There are currently 33 candidate *pfk13* mutations associated to
440 artemisinin resistance (WHO Report 2018). In addition, non-synonimous mutations in several
441 other (kelch13-independent) proteins, have also been associated with resistance, either alone or in
442 combination with *pfk13* mutations (Behrens et al. 2021). kelch13 is a highly conserved protein
443 amongst Apicomplexans, and orthologous aminoacid sequences have been found in 21 different
444 *Plasmodium* species, including *P. relictum* (Coppée et al. 2019). The phylogenetic relationship between
445 the k13 in *P. relictum* and human malaria has not been clearly established (Coppée et al. 2019), and
446 its role in the resistance to artesunate in avian malaria remains to be demonstrated.

447 The recent *de novo* emergence and clonal expansion of a new *pfk13* mutation (R561H) in
448 Subsaharan Africa (Uwimana et al. 2020) where >90% of malaria cases occur, highlights the urgent
449 need to understand the costs associated to artemisinin-resistant strains both in the vertebrate host
450 and in the mosquito vector. In our avian malaria model, we observed no obvious costs of the AS-
451 selected lines in the vertebrate host in single infections. Further work should aim to develop
452 markers of resistance that would allow us to explore whether the drug-selected lines are suppressed
453 when in competition with the reference line (De Roode et al. 2004; Wargo et al. 2007; Huijben et
454 al. 2010, Wale et al 2017). Arguably, however, since our AS-selection lines were not clonal, they
455 may have contained a mixture of resistant and ancestral parasites.

456 Current knowledge on the transmission potential of artemisin-resistant strains is extremely
457 limited. Our results suggest that, under certain experimental conditions, drug-selected strains may
458 generate lower sporozoite prevalences and burdens in mosquitoes, thus potentially impacting their
459 transmission efficiency. Although the relative costs and benefits of resistance likely differ in
460 magnitude between human and avian malaria parasites, these results provide proof of principle of
461 the need for further studies comparing the infectiousness to mosquitoes of artemisinin-resistant
462 and reference strains of human malaria.
463

464 **Acknowledgments**

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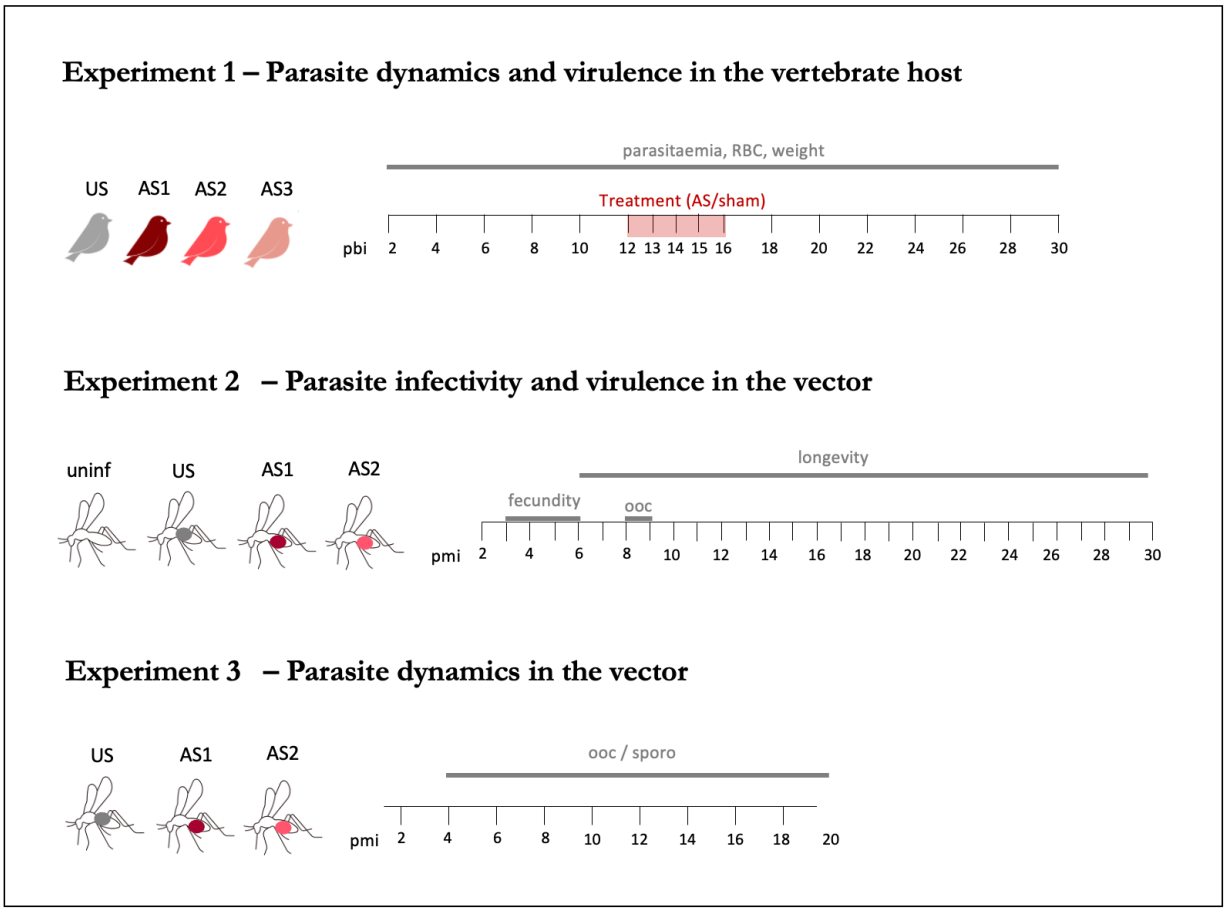
Figures

Figure 1. Experimental design for experiments 1 - 3. In Experiment 1(n= 6 birds per line), samples were taken every two days between day 2 and day 30 post bird inoculation (pbi), except during the treatment course where samples were taken daily. The parameters quantified were : bird parasitaemia, bird weight and RBC count. In Experiment 2 (n = 2 birds per line plus 2 uninfecteed birds) we quantified fecundity (4-7 pmi, ~30 mosquitoes per bird per line), longevity (~150 mosquitoes per bird per line, survival was monitored on a daily basis), and oocyst count (8-9 pmi, 20 mosquitoes per bird). In Experiment 3 (n = 3 birds per line) : we dissected mosquitoes every two days (2-20 pmi) and quantified oocysts (microscopic examination of the midgut) and sporozoites (qPCR of the thorax). pbi = days post bird infection, pmi = days post mosquito infection. US = unselected reference line, AS1, AS2, AS3 = artesunate-treated (putatively resistant) lines.



604 **Figure 2.** Experiment 1: parasitaemia (mean ± s.e) in artesunate-treated (**A**) and untreated (**B**)
605 birds. Shaded area corresponds to the 4-day treatment period (measurements taken 24h after each
606 injection). Untreated birds were sham injected with the artesunate solvent. Dashed line indicates
607 the baseline parasitaemia on the day immediately before the first injection (day 12). Bars
608 above/below the dashed line indicate an increase/decrease in parasitaemia with respect to day 12.

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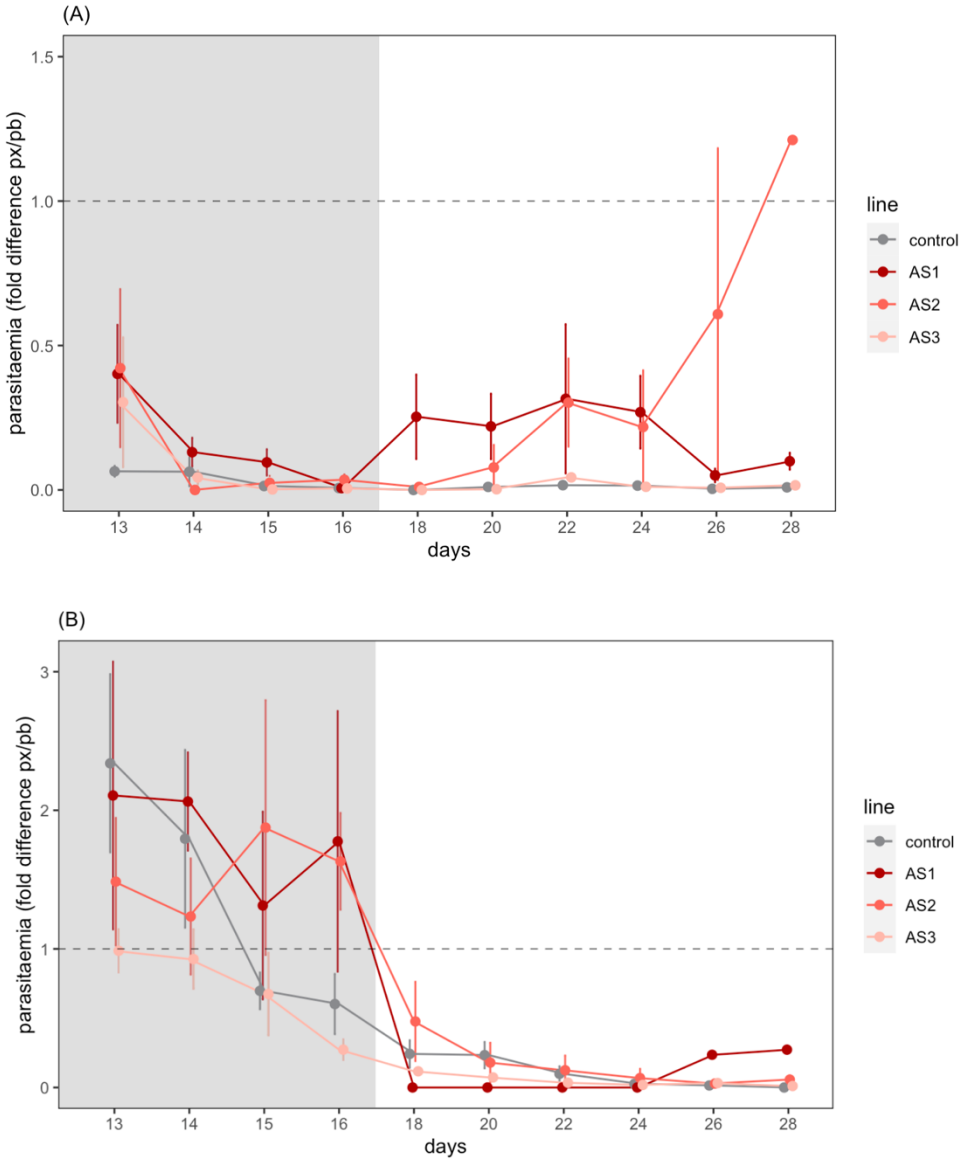
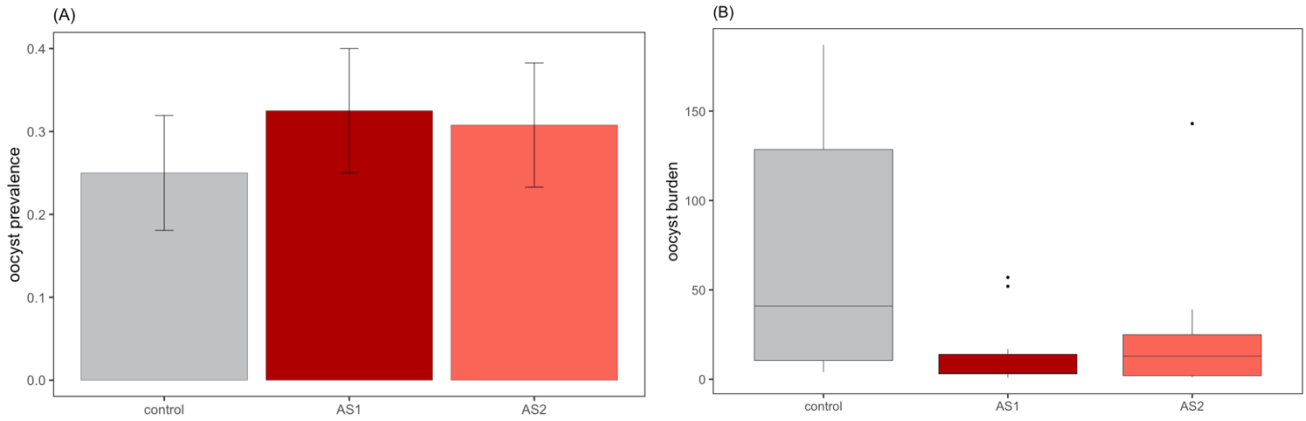


613 **Figure 3.** Experiment 2: mean (± s.e) oocyst prevalence (**A**) and burden (**B**) in mosquitoes infected
614 by each parasite line 8-9 days post infection. Burden is represented as a boxplot showing the median
615 (horizontal lines), and the first and third quartiles (box above and below the medians). Vertical lines
616 delimit 1.5 times the inter-quartile range above which individual counts are considered outliers and
617 marked as circles.



618 **Figure 4.** Experiment 2: Fecundity of *Culex pipiens* females uninfected (n=60) or infected with each
619 of the different parasite lines (n=60 per line). The number of eggs per raft is represented as a boxplot
620 with the median (horizontal lines), first and third quartiles (box above and below the medians). Vertical
621 lines delimit 1.5 times the inter-quartile range above which individual counts are considered outliers
622 and marked as circles.

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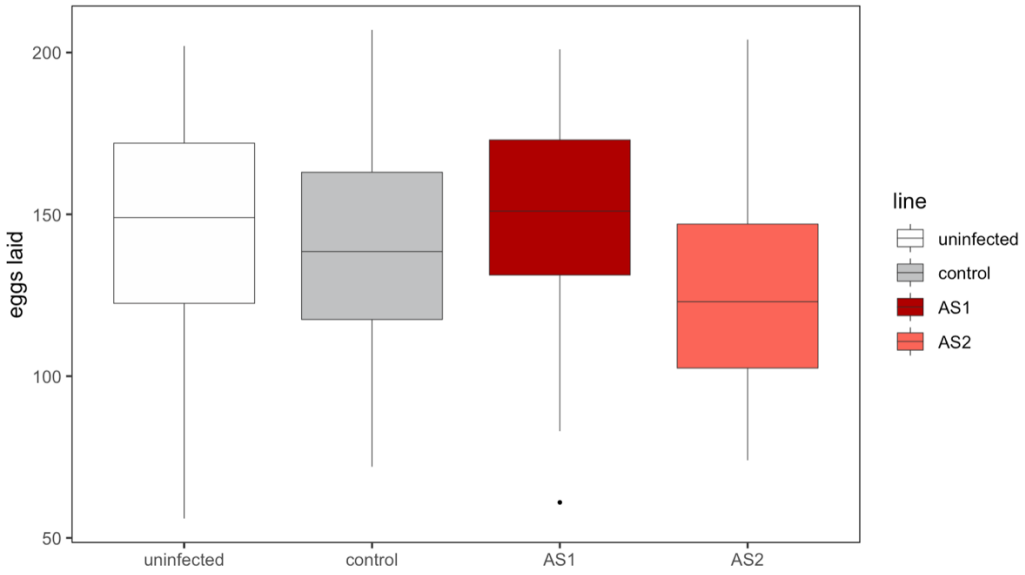
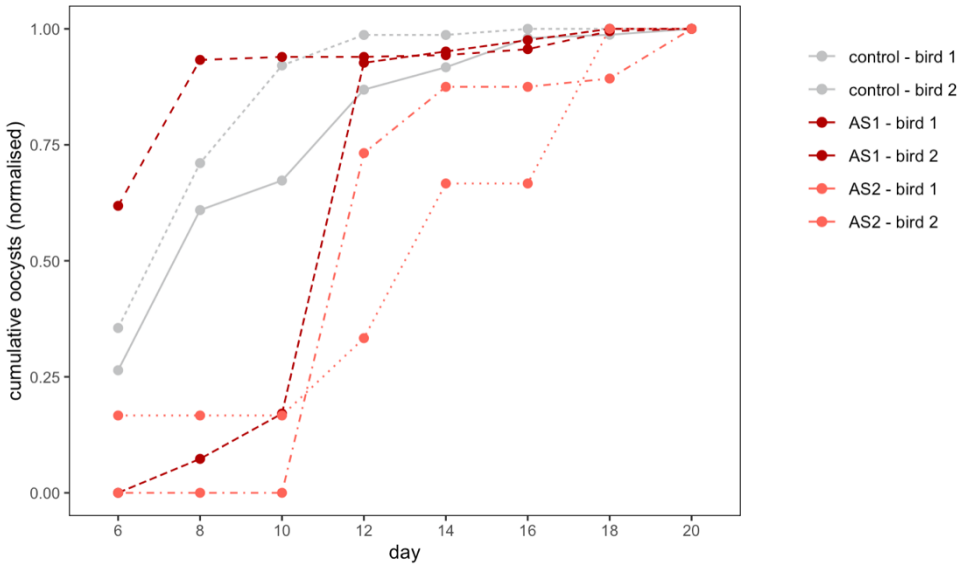


637 **Figure 5.** Experiment 3: Cumulative proportion of oocysts per day in mosquitoes infected with
638 the reference (control) and AS-selected lines. Data is provided separately for each cage/bird.



639 **Figure 6.** Experiment 3: oocyst prevalence (A) and burden (B), sporozoite prevalence (C), and
640 burden (D) of mosquitoes infected by each of the parasite lines for different sampling intervals.
641 Prevalence is represented as the mean ± standard error (calculated as sqrt(pq/n)). Oocyst and
642 sporozoite burden are represented as a boxplot with the median (horizontal lines), first and third
643 quartiles (box above and below the medians). Vertical lines delimit 1.5 times the inter-quartile range
644 above which individual counts are considered outliers and marked as circles.

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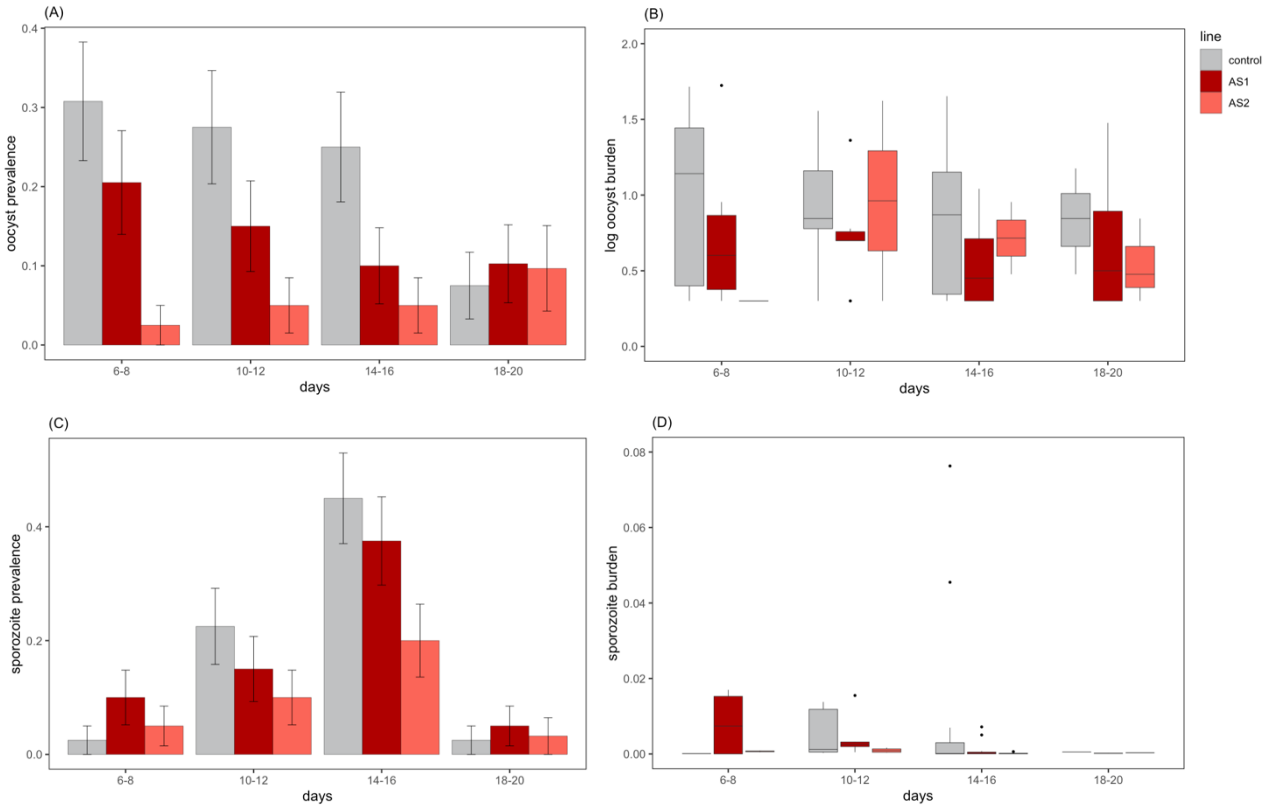


649 # Supplementary Tables

650

651 **ST1: Proportion of blood cells infected (parasitaemia) immediately before the AS-**
652 **treatment**. Table shows the maximum (max) and minimum (min) values, the median (med) and
653 the 25% (Q1) and 75% (Q3) quartiles.

654

	reference	AS1	AS2	AS3
max	6.50	8.92	2,92	9.63
Q3	3.48	5.12	2.25	6.91
med	1.27	0.83	1.48	4.18
Q1	1.17	1.13	0.39	1.54
min	1.14	0.56	1.19	0.83

655

ST2. Description of the statistical models used to analyze the costs of AS-resistance in birds and vectors. N gives the number of mosquitoes or birds included in each analysis. "Maximal model" represents the complete set of explanatory variables (and their interactions) included in the model. "Minimal model" represents the model containing only the significant variables and their interactions. N denotes the total number of observations (nb: which is not always the same as the number of independent replicates). Round brackets indicate that the variable was fitted as a random factor. Square brackets indicate the error structure used (n: normal errors, b: binomial errors), *day*: sampling day, *timing*: whether it's before, during or after the treatment, *status*: alive/dead on sampling day, *eggs*: number of eggs laid, *trt*: treatment (AS or sham-treated), *line*: parasite line, *para*: proportion of infected red blood cells, *rbc*: number of red blood cells per ml.

	Variable of interest	Response variable	Model Nb.	Maximal model	Minimal model	R subrout
Experiment 1 – Bird - parasite dynamics and virulence						
parasitaemia	Before - all birds	bx (para)	1	line*day*treatment + (1|bird)	day + (1|bird)	lmer [n]
	During - all birds	bx(para d_n/d_{12})	2	line*day*trt + (1|bird)	line*day*trt + (1|bird)	lmer [n]
	During - treated birds	bx (para d_n/d_{12})	3	line*day + (1|bird)	line*day + (1|bird)	lmer [n]
	During - untreated birds	log (para d_n/d_{12})	4	line*day + (1|bird)	line+ (1|bird)	lmer [n]
	After - treated birds	bx (para d_n/d_{12})	5	line*day + (1|bird)	line*day + (1|bird)	lmer [n]
	After - untreated birds	bx (para d_n/d_{12})	6	line*day + (1|bird)	day + (1|bird)	lmer [n]
weight	All birds	weight (d_n/d_0)	7	line*timing*trt + (1|bird)	line*timing*trt + (1|bird)	lmer [n]
	Treated - all birds	weight (d_n/d_0)	8	line*timing + (1|bird)	day*tt + (1|bird)	lmer [n]
	Untreated – all birds	weight (d_n/d_0)	9	line*timing + (1|bird)	line*timing + (1|bird)	lmer [n]
rbc	All birds	rbc (d_n/d_0)	10	line*timing*trt + (1|bird)	timing	lmer [n]
Experiment 2 – Mosquito – parasitaemia and virulence						
infection	Number of mosquitos with at least 1 oocyst	cbind (inf, uninf)	11	line*dd + (1| bird)	1 + (1| bird)	glmer [b]
	Number of oocysts per infected mosquito	oocyst	12	line*dd + (1| bird)	line + (1| bird)	glmer [p]
survival	Overall survival	(day, status)	13	line + (1| bird)	1 + (1| bird)	coxme
fecundity	Number of eggs per raft	eggs	14	line + (1| bird)	line + (1| bird)	lmer [n]
Experiment 3 – Mosquito - parasite dynamics						
oocysts	Number of mosquitoes with at least 1 oocyst	cbind (inf, uninf)	15	line*day + (1|bird)	day + (1|bird)	glmer [b]
	Number of oocysts per infected mosquito	oocysts	16	line*day + (1|bird)	day + (1|bird)	glmmTMB
sporozoites	Number of mosquitoes with sporozoites	cbind (inf, uninf)	17	line*day + (1|bird)	day + (1|bird)	glmer [b]
	Sporozoite burden	log(sporozoite)	18	line*day*oocyst + (1|bird)	day + (1|bird)	lmer [n]

ST3: Reduction in parasitaemia as a result of the AS treatment for each of the 3 lines. Treatment was given on days 12-15, parasitaemia was calculated 24h after each treatment (days 13-16). The reduction in parasitaemia is calculated as *(px-pb)/pb * 100* where *px*= parasitaemia at a given day, and *pb* = baseline parasitaemia (parasitaemia immediately before the treatment, day 12). mn=mean, md=median, Q1=25% quartile, Q3= 75% quartile.

	reference	AS1	AS2	AS3
day 13	**mn: - 93.6 %** **md: - 93.9 %** Q1: - 90.2 % Q3: - 96.6 %	**mn: -59.8 %** **md: - 71.6 %** Q1: - 24.6 % Q3: - 85.7 %	**mn: - 57.8 %** **md: - 78.0 %** Q1: - 37.2 % Q3: - 91.8 %	**mn: - 69.6 %** **md: - 85.9 %** Q1: - 25.2 % Q3: - 97.6 %
day 14	**mn: - 93.7 %** **md: - 97.2 %** Q1: - 83.9 % Q3: - 100 %	**mn: - 86.9 %** **md: - 86.1 %** Q1: - 77.7 % Q3: - 98.3 %	**mn: - 100 %** **md: - 100 %** Q1: - 100 % Q3: - 100 %	**mn: - 95.7 %** **md: - 96.1 %** Q1: - 91.41 % Q3: - 96.6 %
day 15	**mn: - 98.6 %** **md: - 100 %** Q1: - 95.9 % Q3: - 100 %	**mn: - 99.3 %** **md: - 91.1 %** Q1: - 81.4 % Q3: - 100 %	**mn: - 97.6 %** **md: - 100 %** Q1: - 92.7 % Q3: - 100 %	**mn: - 99.8 %** **md: - 100 %** Q1: - 99.4 % Q3: - 100 %
day 16	**mn: - 99.3 %** **md: - 100 %** Q1: - 98.0 % Q3: - 100 %	**mn: - 99.3 %** **md: - 100 %** Q1: - 98.0 % Q3: - 100 %	**mn: - 96.5 %** **md: - 95.4 %** Q1: - 94.2 % Q3: - 100 %	**mn: - 99.5 %** **md: - 99.2 %** Q1: - 99 % Q3: - 100 %

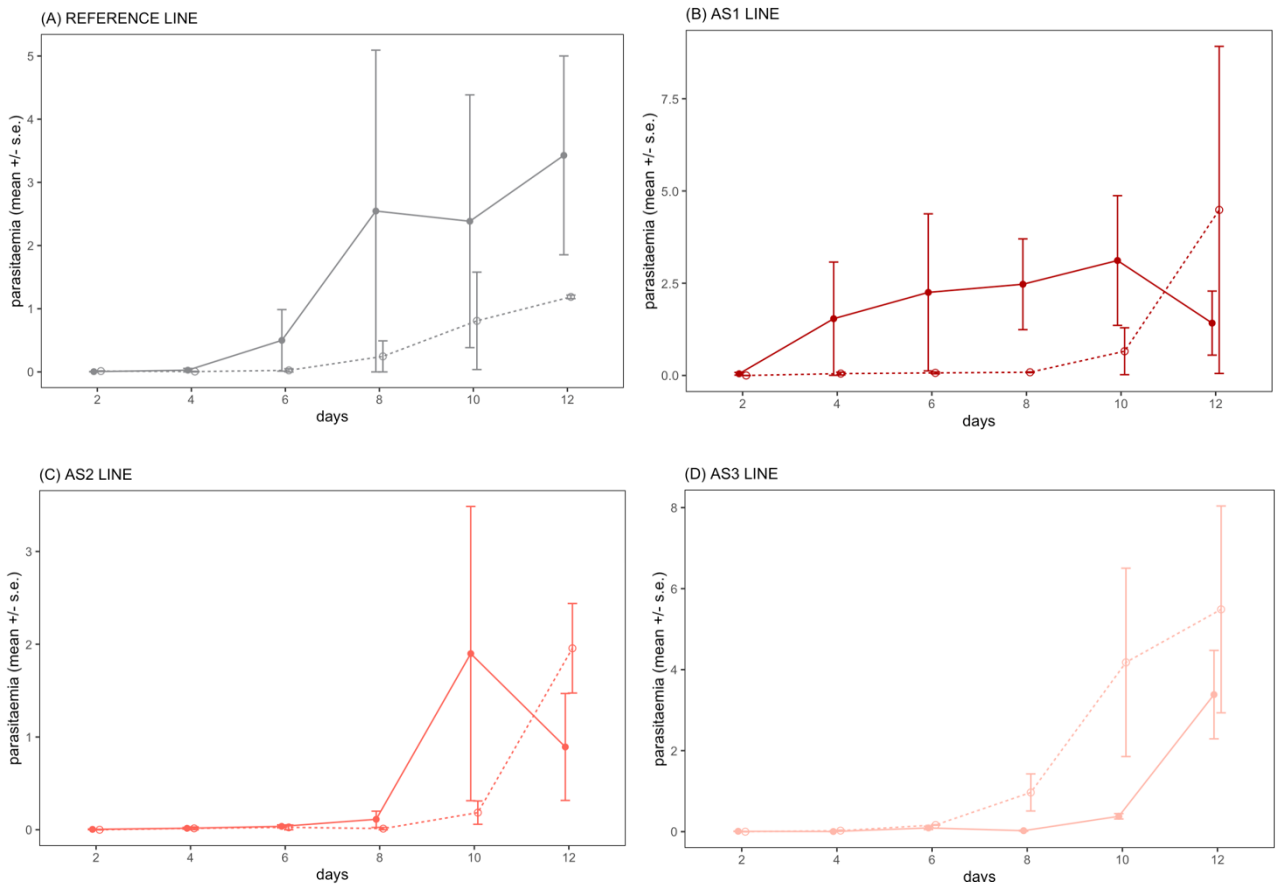
677 **ST4:** Median mosquito longevity and, in parenthesis, proportion of mosquitoes surviving to day
678 14 (peak sporozoite production) for uninfected mosquitoes, and mosquitoes infected with the
679 reference or AS-selected lines. Data are presented separately for each bird.
680

	Median longevity (proportion surviving to day 14)			
	uninfected	reference	AS1	AS2
Bird 1	23 (0.64)	21 (0.56)	21 (0.57)	21 (0.57)
Bird 2	24 (0.62)	26 (0.65)	19 (0.56)	14 (0.41)

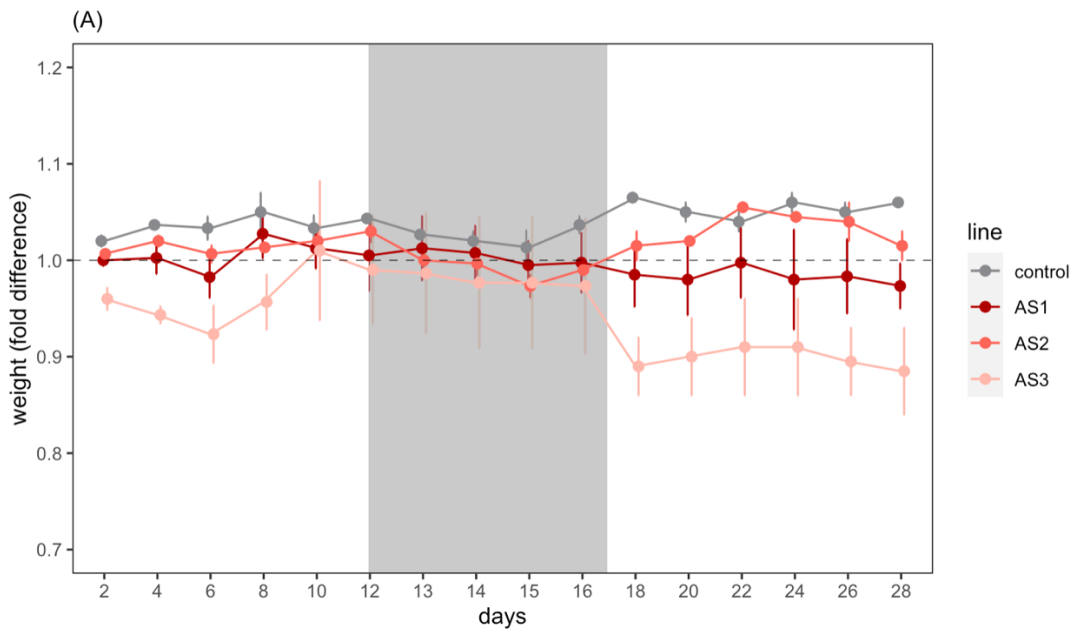
681
682

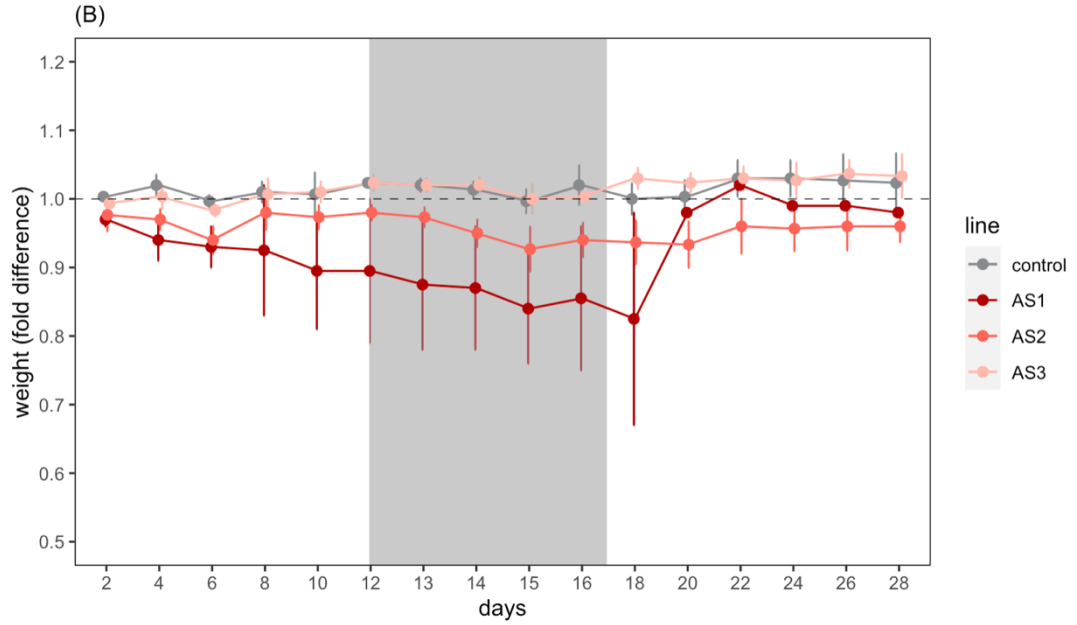
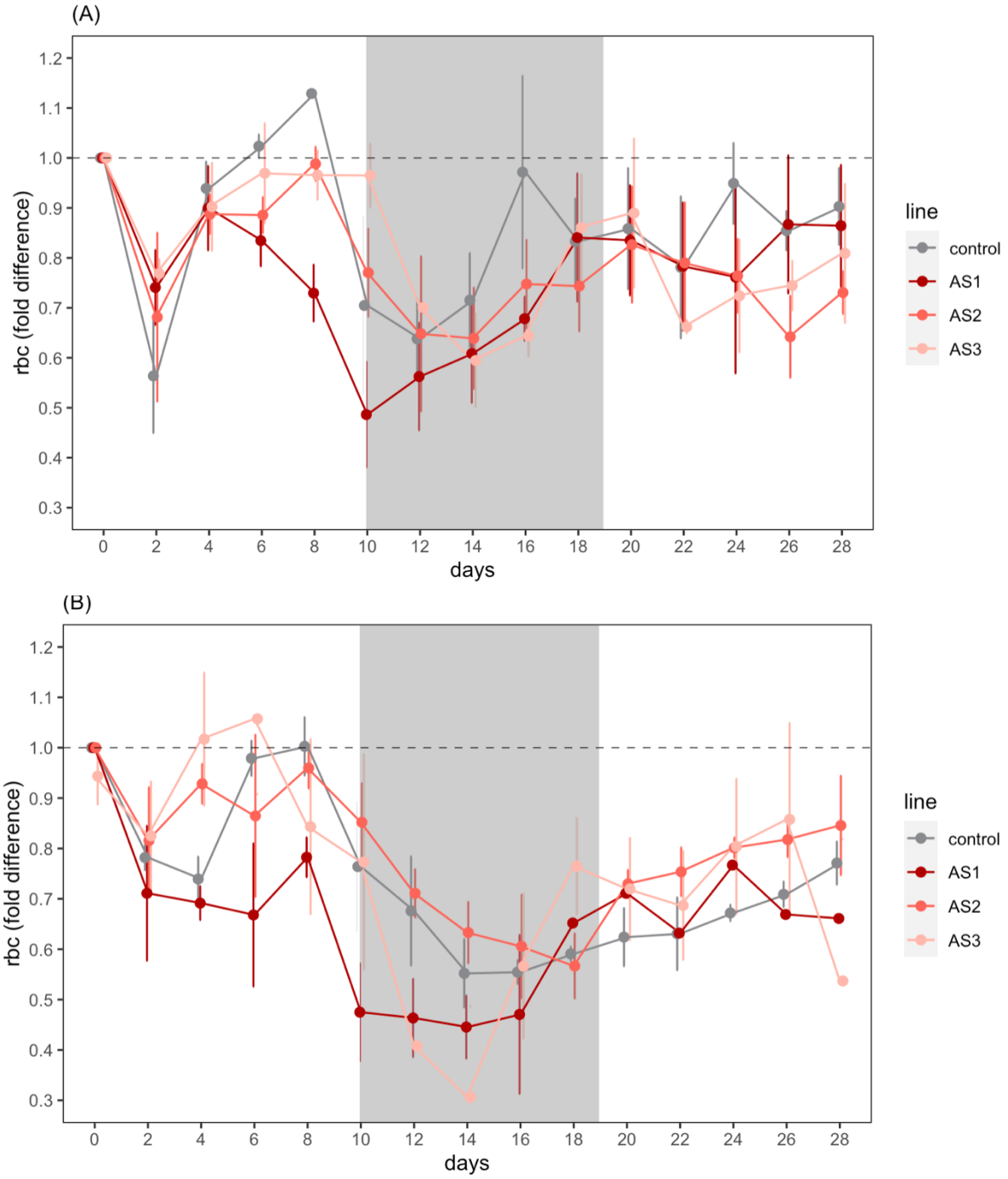
Supplementary Figures

SF1: Parasite dynamics before the treatment. (A) reference line, (B) AS1 line, (C) AS2 line, (D) AS3 line. On day 12, birds were blindly allocated to either the untreated (sham injection, dashed lines and empty circles) or treated (artesunate injection, solid lines and full circles) group.



SF2. Bird weight changes (mean ± s.e) in artesunate-treated (**A**) and untreated (**B**) birds. Shaded area corresponds to the 4-day treatment period. Untreated birds were sham injected with the artesunate solvent. Dashed line indicates the baseline bird weight at the start of the experiment (day 0). Bars above/below the dashed line indicate an increase/decrease in parasitaemia with respect to day 0.





SF3. Bird red blood cell count (rbc) changes (mean ± s.e) in artesunate-treated (**A**) and untreated (**B**) birds. Shaded area corresponds to the 4-day treatment period. Untreated birds were sham injected with the artesunate solvent. Dashed line indicates the baseline rbc at the start of the experiment (day 0). Bars above/below the dashed line indicate an increase/decrease in rbc with respect to day 0.