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.

Current information about the costs of drug resistance mutations comes almost exclusively from vertebrate hosts. The potential for these costs to be expressed also in mosquitoes has been given only cursory attention. 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.

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

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

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

Material and Methods

Mosquito and parasite protocols -

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

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

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

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

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

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

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

168 The aim of this experiment was to establish whether AS-selected and reference lines differ
169 in their infectivity and virulence to the mosquito. For this purpose, mosquitoes were allowed to
170 blood feed on birds infected with the drug-selected and reference parasite lines. Mosquitoes fed on
171 uninfected birds were used as controls. A subsample of the mosquitoes was used to quantify
172 parasite density, while the rest were followed for several days to measure their fecundity and

longevity. Following the results of the previous experiment, no infection with AS3 was carried out in this experiment.

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

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

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

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

The detailed experimental protocol is as follows (see **Figure 1**). Six naïve birds were infected with 100 000 parasites of either the control, AS1 or AS2 lines (2 birds per line). As in the previous experiment, on day 12 pbi, birds were introduced individually into cages containing 150 12-day old female mosquitoes. The following morning, unfed females were counted and discarded from the study and all cages were provided with *ad libitum* sugar for the reminder of the experiment. Starting on day 4 post blood meal and every 2 days thereafter (4-20 dpmi), 20 mosquitoes were

haphazardly sampled from each cage and immediately dissected under a binocular microscope to extract the oocyst-infected midguts and count the oocysts (as above). The mosquito thorax, containing the sporozoite-infected salivary glands, was then severed and frozen at -20°C for the subsequent quantification of parasites via qPCR. Sporozoite quantification was calculated as a ratio between mosquito DNA (*ace2* gene) and *Plasmodium relictum* DNA (*cytb*) as described previously (Zélé et al. 2014).

Ethics statement

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

Statistical analyses

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

Mosquito survival (Experiment 2) was analyzed using Cox proportional hazards mixed effect models (coxme). Proportion data (oocyst and sporozoite prevalence) were analyzed using mixed linear models and a binomial distribution. Count data (eggs per raft) were analyzed using mixed linear models and a Poisson distribution. Response variables that were highly overdispersed (e.g. oocyst burden) were analyzed using mixed negative binomial models (glmmTMB). *A posteriori* contrasts were carried out by aggregating factor levels together and by testing the fit of the simplified model using a LRT (Crawley 2007). Statistical analyses are summarised in Supplementary Table ST2.

Results

Experiment 1 – Phenotypic characterization of artesunate resistance in bird

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

We started by analysing whether there were differences between lines in their virulence or parasitaemia *before* the treatment (days 2-12 pbi). There was a marginally statistically significant difference between the lines: the parasitaemia of AS2 increased at a slightly lower rate than that of the other AS-selected lines (model 1, χ^2= 8.3134, p= 0.04, Supplementary Materials Figure SF1). The red blood cell counts (RBC) were contingent on both parasitaemia and day (model 2, χ^2= 12,887, p= 0.0015), but were independent of the line (model 2, χ^2= 7.3716, p= 0.06). Bird weight, on the other hand, was not explained by any of the fitted explanatory variables (model 3, line: χ^2= 0.3201, p= 0.9562, day: $\chi^2 < 0.01$, p= 0.993, parasitaemia: χ^2= 0.8511, p= 0.3562).

During the treatment (days 13-16 pbi), the parasitaemia of treated and untreated birds followed very different courses. Treatment with AS had a very significant effect on the parasitaemia, and the effect was dependent on both the day and the parasite line (model 4, χ^2= 12.088, p = 0.0071, **Figure 2A**). To obtain a more accurate picture of the nature of this interaction, subsequent analyses were done separately for AS-treated and control birds. Treatment with AS resulted in an overall decrease in parasitaemia in all lines (see Supplementary Table ST3). On the first day after the treatment (day 13 pbi) the reduction in parasitaemia was greater in the reference line (- 93.6% on average) than in either the selected AS1 (- 59.8%), AS2 (- 57.8) or AS3 (- 69,6%) lines. This effect largely disappears in subsequent days (Supplementary Table ST3). The statistical analyses revealed a significant interaction between day and line (model 5, χ^2= 20.563, p= 0.0147). In untreated birds, there was a significant difference between the lines (model 6, χ^2= 9.1288, p= 0.0276) due to AS2 having a significantly higher parasitaemia than the other lines. This effect was independent, albeit marginally, of the sampling time (model 6, χ^2= 7.7845, p= 0.0507, **Figure 2B**).

After the AS treatment, parasitaemia in the treated birds showed a different pattern between the lines (model 7, χ^2= 8.8851, p= 0.0308, **Figure 2A**). Lines AS1 and AS2 had a significantly higher parasitaemia than the reference control line. In untreated birds, the parasitaemia decreased gradually with time (model 8, χ^2= 54.449, $p < 0.01$) and was independent of the parasite line (model 8, *line*: χ^2= 7.9084, p= 0.0479, **Figure 2B**).

The analysis of RBC and weight were done concomitantly for the during and after treatment periods. As expected, anaemia (quantified as RBC counts) lower in AS-treated birds than in their untreated counterparts (model 9, χ^2= 20.407, p= 0.0089). The weight of untreated birds increased with time (model 10, χ^2= 7,99, p= 0.005) but was independent of all of the other measured variables, including the line (model 10, line: χ^2= 1,622, p= 0.65, parasitaemia: χ^2= 0.27, p= 0.60). In treated birds, weight only increased with time in birds infected with the AS2 line (model 11, χ^2= 15,47, p= 0.002).

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

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

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

There were also significant differences in mosquito fecundity (number of eggs per raft) between lines (model 14, χ^2= 11.1, p= 0.0112). Post-hoc analysis showed that mosquitoes infected with the AS2 line laid significantly fewer eggs (mean ± s.e: 126 ± 4) than uninfected mosquitoes (142 ± 5) and mosquitoes infected with the control (142 ± 4) and AS1 (148 ± 4) lines (χ^2= 6.5741, p= 0.0104, **Figure 4**). There were no significant differences in longevity between infected and uninfected mosquitoes, or between mosquitoes infected with the different lines (model 15, χ^2= 3.7758, p= 0.2867).

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

This experiment was carried out to investigate whether the differences in oocyst burden between selected and reference lines may be due to a difference in the dynamics of development of the parasite within mosquitoes. **Figure 5** shows the cumulative increase in oocysts in mosquitoes having fed in birds infected with each of the lines (control or AS-selected). Mosquitoes infected with the control line showed a faster cumulative increase in oocysts than mosquitoes infected with the AS-selected ones. As a result, by days 8-9 (corresponding to the dissection time in Experiment

2), mosquitoes fed on birds infected with the control line had between 60-70% of the cumulative total number of oocysts, while mosquitoes fed from 3 out of the 4 birds infected with AS-selected lines had 0-16% of the cumulative total. Mosquitoes fed from one of the (AS1-infected) bird, however, showed an unusually high number of oocysts on the first dissection day.

The mean oocyst prevalence (% mosquitoes containing at least 1 oocyst) averaged over the whole experiment was higher in control (67.92 ± 6.47%, mean ± s.e.) and AS1 lines (53.65 ± 7.88%) than in the AS2 line (36.36 ± 10.49%). The same pattern was found with oocyst burden, which was higher in the control (32.80 ± 11.95) and AS1 lines (18.90 ± 11.33) than in the AS2 line (7.75 ± 4.83). When day was fitted to the model the effect of line, however, disappeared for both oocyst prevalence (model 16, χ^2= 5.3164, p= 0.070 and burden (model 17, χ^2= 0.2313, p= 0.8908; **Figure 6A and 6B**).

The mean sporozoite prevalence, averaged over the whole experiment, was roughly 2-times higher in the control (28.30 ± 6.24%) and AS1 lines (27.90 ± 6.92%) than in the AS2 line (13.63 ± 7.48%). Sporozoite burden, on the other hand, was 4-5 times higher in the control line (0.0126 ± 0.0067) than in the AS-selected lines (AS1: 0.0026 ± 0.0009, AS2: 0.0034 ± 0.0029). As above, when day was fitted to the model the effect of line on sporozoite prevalence and burden disappeared (model 18, χ^2= 0.1788, p= 0.9145, and model 19, dχ^2= 1.9214, p= 0.3826, respectively **Figure 6C and 6D**).

Discussion

The two telltale signs of *P. falciparum* resistance to artemisinin derivatives in the field are a delay in the clearance of parasites during ACT treatment followed by a recrudescence once the treatment is interrupted (Douglas et al. 2021). Artemisinin derivatives are remarkably efficient at clearing susceptible parasites from the blood (typically within 24-48 hours in *P. falciparum*). A slower clearance of parasites after the treatment, typically taking an extra 24-48 hours, is interpreted as evidence of resistance to the artemisinin-derivative component (Witkowski et al. 2013; Mok et al. 2015). This delayed clearance phenotype is a far cry from the standard definition of resistance (survival to a drug treatment), which is why artesunate resistance is commonly referred to as a 'partial resistance' (Uhlemann et al. 2010; Menard and Dondorp 2017). Partial artemisinin resistance rarely leads to ACT failure, but may select for drug resistance to the partner drug, as larger parasite populations remain in the blood stream for longer periods of time (Fairhurst 2015). In our study, the within-host dynamics of the drug-selected lines in the treated birds was consistent with the

phenotype of resistance on *P. falciparum* in the field. AS-selected lines took longer to respond to the treatment than their control counterparts. In the first 24h after the beginning of the treatment the parasitaemia of the control line dropped by over 90%, while that of the AS-selected lines only dropped by between 60% (AS1, AS2) and 70% (AS3). By the following day, there was no discernible difference between the parasitaemia of control and drug selected lines, (**Figure 2A**). In keeping with previous studies on *P. falciparum* parasites, two of our drug-selected lines (AS1, AS2) also had a pronounced recrudescence after the treatment was interrupted, producing on average more than 20 times as many parasites in the two weeks following drug treatment than did the control line (**Figure 2A**).

Drug resistance mutations are known to disrupt the parasite's metabolism, generating fitness costs. In drug-treated hosts these costs are largely compensated by the benefits conferred by the resistance. In untreated hosts, however, the magnitude of these costs will determine whether these mutations will persist and spread in the population. Current views about the impact of drug resistance on parasite fitness are almost entirely based on data obtained from the vertebrate host. In humans, costs of resistance have been inferred by the decrease in drug-resistance alleles when drug use is stopped or discontinued (Laufer and Plowe 2004) and from the decrease in the frequency of drug resistant parasites during the dry season, when there is no transmission and drug use is dramatically reduced . Direct experimental evidence of the costs of artemisinin resistance has been obtained by setting up *in vitro* competition experiments between drug-resistant and reference *Plasmodium* strains (Hott et al. 2015; Nair et al. 2018; Tirrell et al. 2019; Mathieu et al. 2020). To our knowledge the only *in vivo* experiments were carried out with *Plasmodium chabaudi* (a rodent species) and showed no obvious cost of AS in the absence of treatment (Pollitt et al. 2014).

We did not observe any costs associated to the AS-selected lines in the absence of treatment. In fact, an opposite trend was found, whereby the drug-selected lines (AS2 in particular) fared better in terms of parasitaemia than its unselected counterparts. One possible explanation for these results, which would warrant further studies, is that drug selection may be expected to select for traits that increase the parasite's survival probabilities in the presence of drugs (Birget et al 2018). Highly replicating parasite lines may be selected for because they can rapidly recover high parasite densities after a bout of drug-induced mortality (Schneider et al. 2008, Schneider et al. 2012). In as much as virulence is related to parasite fitness through high asexual parasite densities and gametocyte production (Mackinnon and Read 2004), this opens the unpalatable possibility of drugs selecting for more resistant but also more virulent parasites. Evidence for an association between drug resistance alleles and parasite virulence is however weak (Tukbasibwe et al. 2017, Cuu et al. 2020). In our experiment, AS-selected lines were not associated to any of the commonly

368 measured parameters of parasite virulence (anaemia, weight loss). These results confirm previous
369 studies showing that artesunate resistance treatment does not drive the evolution of virulence
370 (Pollitt et al. 2014).

371 Despite its crucial implications for the emergence and spread of drug resistant mutations
372 in the field, work on the transmissibility of artemisinin-resistant parasites, is practically non-
373 existent. St. Laurent et al. (2015) showed that artemisinin-resistant strains of *P. falciparum* from
374 Cambodia were able to infect and produce sporozoites in three vector species (*An. dirus*, *An.*
375 *minimus, An. coluzzi*), and Pollitt et al. (2014) showed that in the rodent malaria species *P. chabaudi*,
376 the AS-selected line produced more gametocytes (the blood stages of the parasite that are
377 transmitted to the mosquito) than its reference counterpart. Here, we infected mosquitoes with the
378 drug-selected and control lines in two different experiments. In Experiment 2, all mosquitoes were
379 dissected on a single day corresponding to the peak ocystaemia as established in previous
380 experiments with the ancestral, reference line (day 8-9). In this experiment we observed no
381 significant difference in prevalence between the lines, but a highly significant difference in the
382 number of oocysts between the control and the two drug-selected lines : the latter produced
383 between 3-5 times fewer oocysts than the control line (**Figure 3**). This difference could reflect
384 either a true difference in oocyst burden between the lines, or a shift in the within-mosquito
385 dynamics (peak oocystaemia may have occurred earlier or later in the drug-selected lines). To
386 discriminate between these two options a new experiment (Experiment 3) was launched, where
387 both oocysts and sporozoites were quantified at regular, 2-day, intervals, from day 6 to day 20 post
388 infection. This experiment suffered from the caveat of an unusually low infection prevalence
389 (23%), which significantly reduced the statistical power of the burden analyses, and precluded any
390 meaningful analyses of peak oocystaemia. The cumulative frequency of oocysts, however,
391 suggested that oocysts accumulate at a slower rate in mosquitoes infected with the AS-selected lines
392 (**Figure 5**). The mean overall prevalence and burden of oocysts and sporozoites (i.e. prevalence
393 and burden averaged over all time points) was also in lower AS1 and AS2 mosquitoes. Sporozoite
394 burden, in particular, was 4-5 lower in the drug-selected lines than in the control line. The results
395 therefore concur with those obtained in the previous experiment in that AS-selected lines were less
396 infective to the mosquitoes than their control counterparts.

397 In conclusion, our data using drug-selected strains of *P. relictum* provides further proof that
398 susceptibility to artesunate can be rapidly lost under drug pressure. The clearance delay and
399 recrudescence observed during and after the AS treatment, respectively, in two of our three AS-
400 selected lines were similar to the resistance phenotype seen in human malaria infections. The
401 genetic underpinnings of artemisinin resistance are extremely complex and not yet entirely

elucidated (Behrens et al. 2021). In *P. falciparum* the resistant phenotype is associated with several mutations in the propeller domain of kelch13 (*pfk13*), a protein that is essential for the intra-erythrocytc growth of the parasite. There are currently 33 candidate *pfk13* mutations associated to artemisinin resistance (WHO Report 2018). In addition, non-synonimous mutations in several other (kelch13-independent) proteins, have also been associated with resistance, either alone or in combination with *pfk13* mutations (Behrens et al. 2021). kelch13 is a highly conserved protein amongst Apicomplexans, and orthologous aminoacid sequences have been found in 21 different *Plasmodium* species, including *P. relictum* (Coppée et al. 2019). The phylogenetic relationship between the k13 in *P. relictum* and human malaria has not been clearly established (Coppée et al. 2019), and its role in the resistance to artesunate in avian malaria remains to be demonstrated.

The recent *de novo* emergence and clonal expansion of a new *pfk13* mutation (R561H) in Subsaharan Africa (Uwimana et al. 2020) where >90% of malaria cases occur, highlights the urgent need to understand the costs associated to artemisinin-resistant strains both in the vertebrate host and in the mosquito vector. In our avian malaria model, we observed no obvious costs of the AS-selected lines in the vertebrate host in single infections. Further work should aim to develop markers of resistance that would allow us to explore whether the drug-selected lines are suppressed when in competition with the reference line (De Roode et al. 2004; Wargo et al. 2007; Huijben et al. 2010). Current knowledge on the transmission potential of artemisin-resistant strains is extremely limited. Our results suggest that, under certain experimental conditions, drug-selected strains may generate lower sporozoite prevalences and burdens in mosquitoes, thus potentially impacting their transmission efficiency. Although the relative costs and benefits of resistance likely differ in magnitude between human and avian malaria parasites, these results provide proof of principle of the need for further studies comparing the infectiousness to mosquitoes of artemisinin-resistant and reference strains of human malaria.

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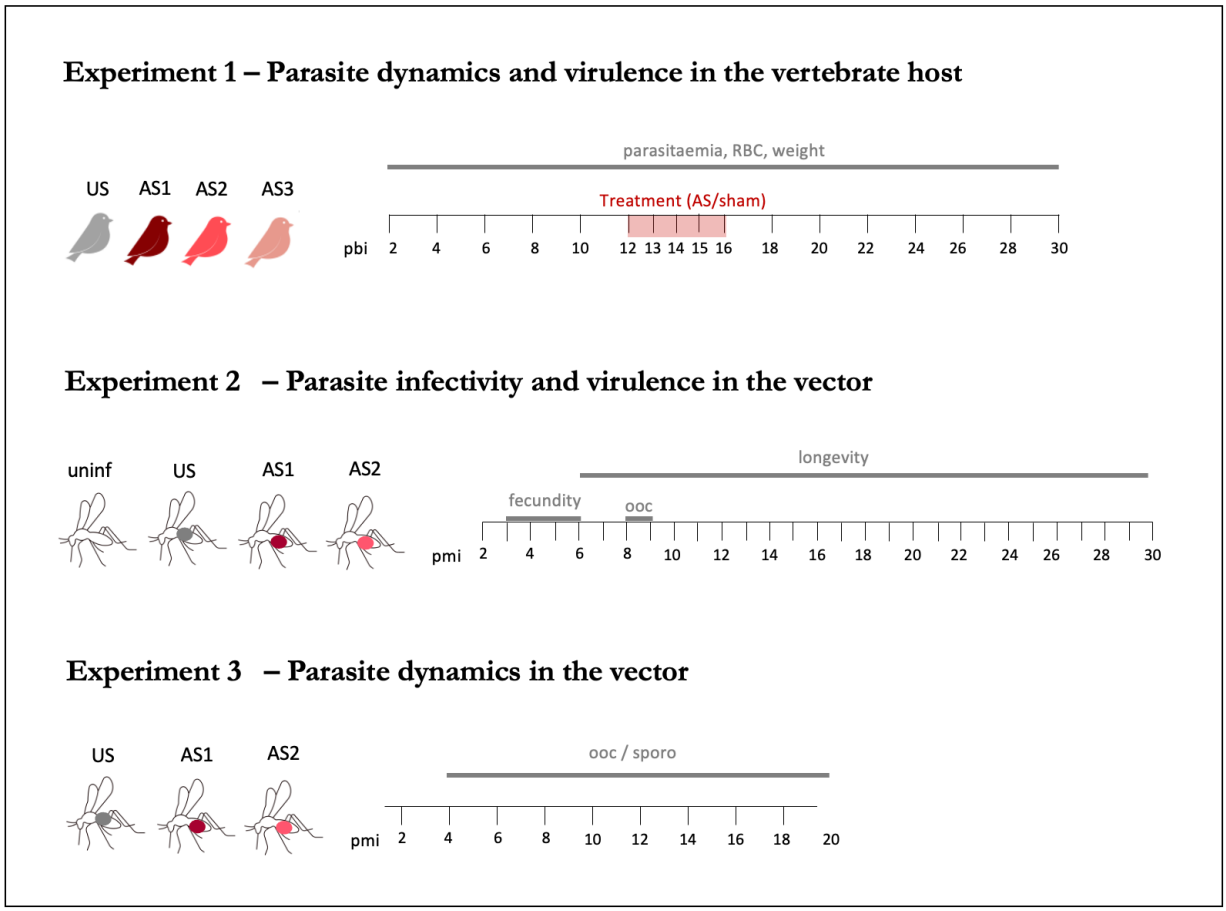
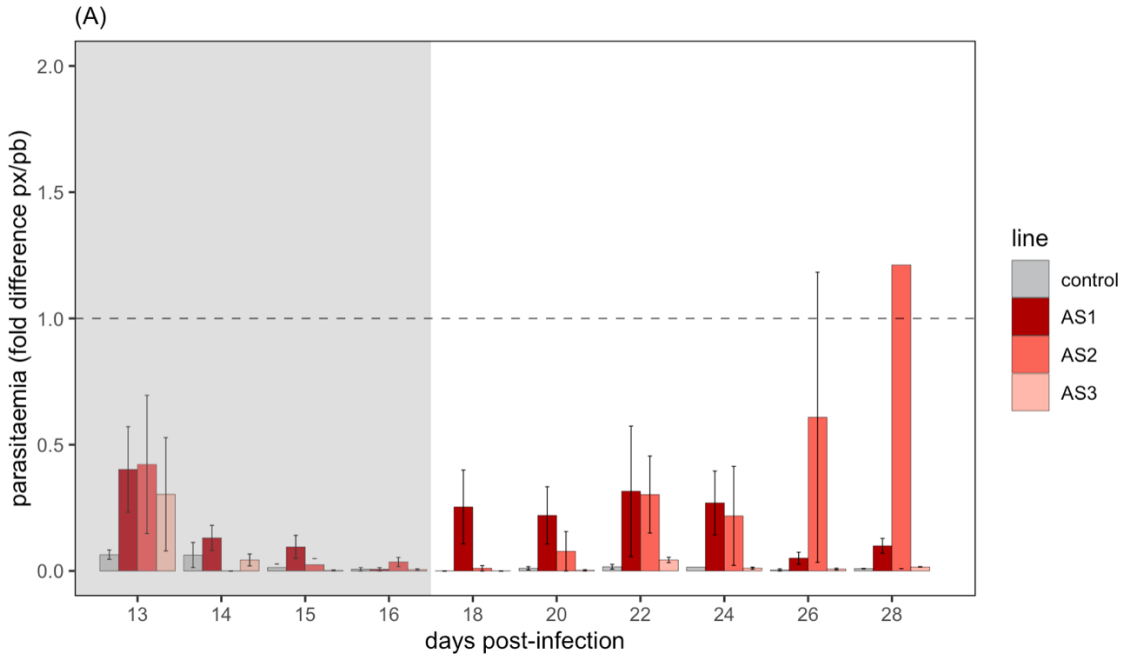
Figures

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) fecundity was quantified between day 4-7 post mosquito infection (pmi), were oocyst (abdomen) and sporozoite (head-thorax) prevalence and intensity within mosquitoes. Experiment 3 (n = 2 birds per line) : fecundity (4-7 dpmi, ~150 mosquitoes per bird), longevity (~150 mosquitoes per bird, survival was monitored on a daily basis), and oocyst count (8-9 dpmi, 20 mosquitoes per bird). dpbi = days post bird infection, dpmi = days post mosquito infection. C = Control line, R1, R2, R3 = artesunate-treated (putatively resistant) lines.



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



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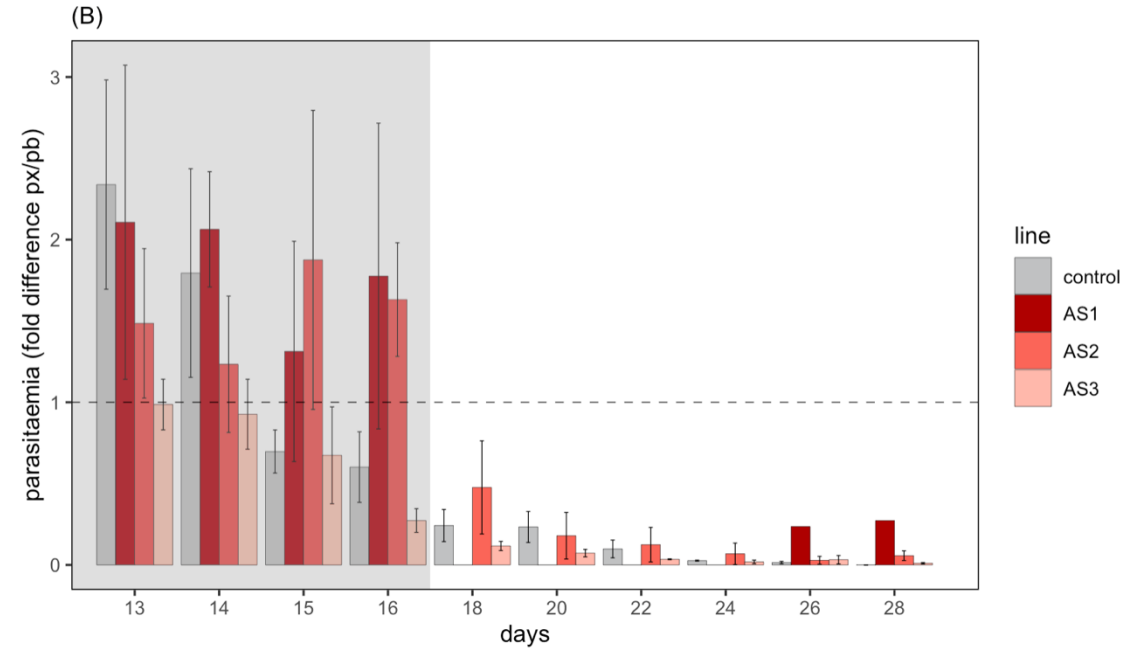

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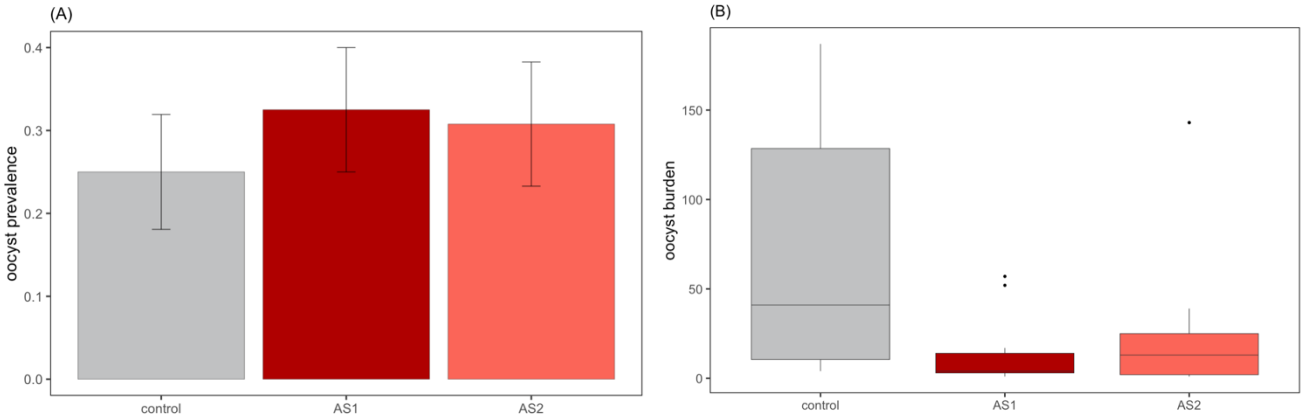
568 **Figure 3.** Experiment 2: mean (± s.e) oocyst prevalence (**A**) and burden (**B**) in mosquitoes infected
569 by each parasite line 8-9 days post infection. Burden is represented as a boxplot showing the median
570 (horizontal lines), and the first and third quartiles (box above and below the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



573 **Figure 4.** Experiment 2: Fecundity of *Culex pipiens* females uninfected or infected with each of the
574 different parasite lines. The number of eggs per raft is represented as a boxplot where with the median
575 (horizontal lines), first and third quartiles (box above and below the medians). Vertical lines delimit
576 1.5 times the inter-quartile range above which individual counts are considered outliers and marked
577 as circles.

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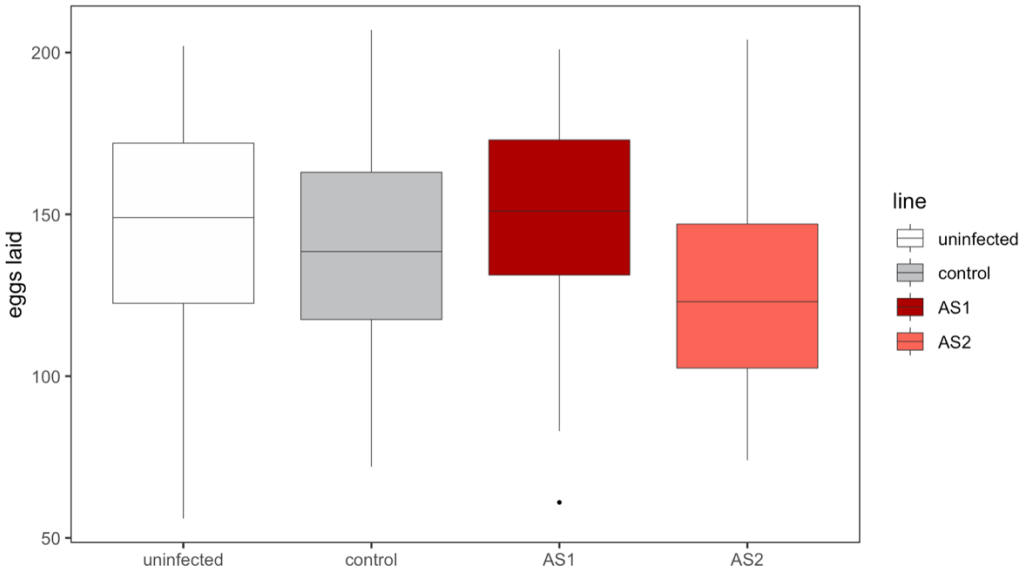


592 **Figure 5.** Experiment 3: Cumulative proportion of oocysts per day in mosquitoes infected with
593 the control and AS-selected lines. Data is provided separately for each cage/bird.

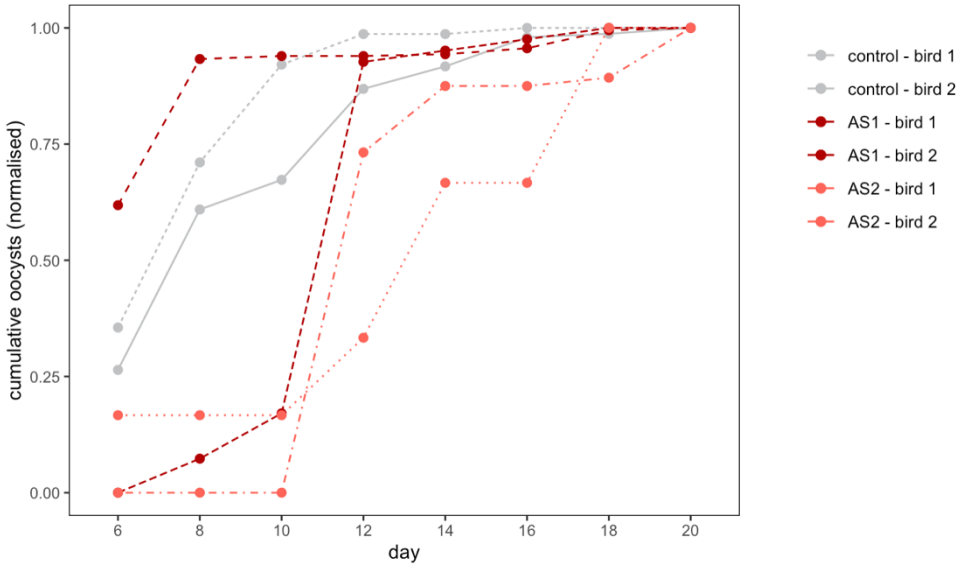
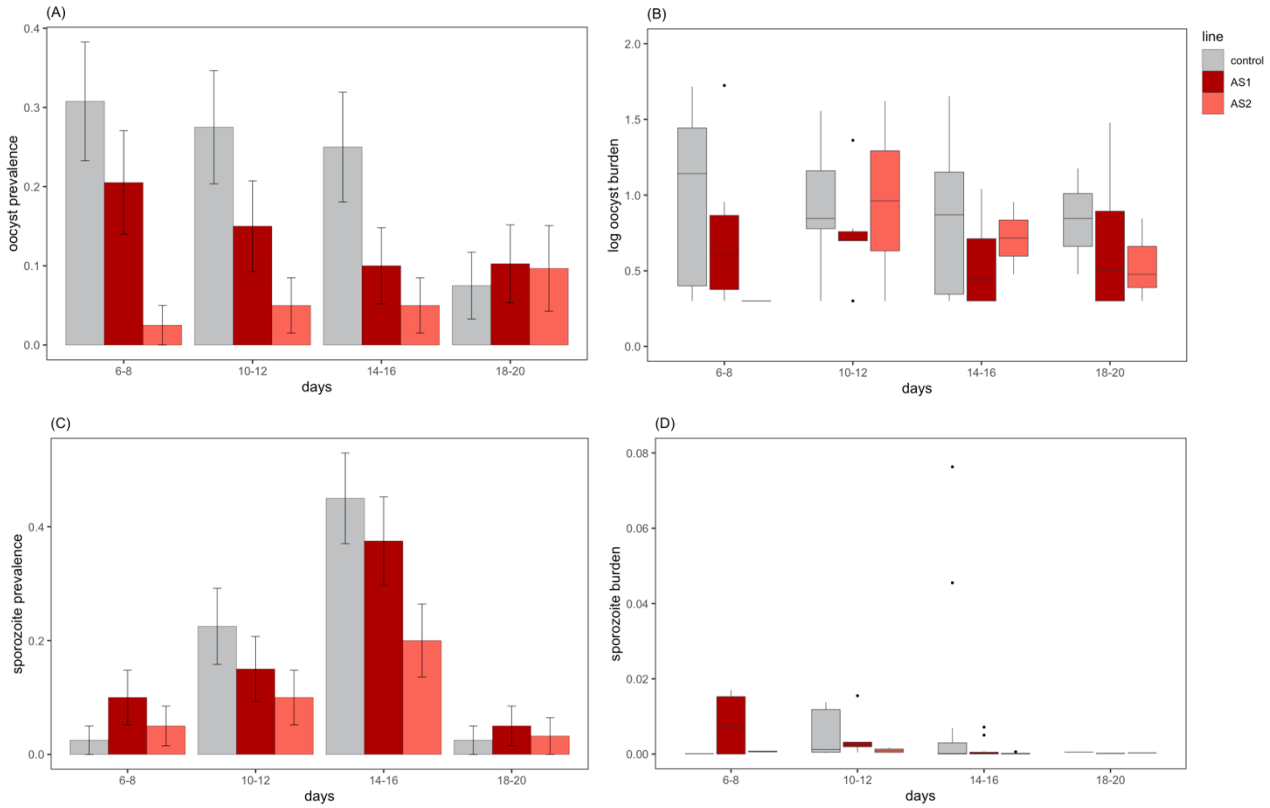


Figure 6. Experiment 3: oocyst prevalence (A) and burden (B), sporozoite prevalence (C), and burden (D) of mosquitoes infected by each of the parasite lines for different sampling intervals. Prevalence is represented as the mean ± standard error (calculated as sqrt(pq/n)). Oocyst and sporozoite burden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



Supplementary Tables

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

	Control	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

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. 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, *status*: alive/dead on sampling day, *eggs*: number of eggs laid, *trt*: treatment (AS or control), *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.	N	Maximal model	Minimal model	R subrout
Experiment 1 – Bird - parasite dynamics and virulence							
parasitaemia	All birds - before	bx (para)	1	116	line*day + (1|bird)	line*day + (1|bird)	lmer [n]
	All birds - during	bx(para d_n/d_{12})	4	95	line*day*trt+ (1|bird)	line*day*trt+ (1|bird)	lmer [n]
	Treated birds - during	bx (para d_n/d_{12})	5	51	line*day + (1|bird)	line*day + (1|bird)	lmer [n]
	Treated birds - after	bx (para d_n/d_{12})	7	67	line*day + (1|bird)	line*day + (1|bird)	lmer [n]
	Control birds - during	log (para d_n/d_{12})	6	44	line*day + (1|bird)	line+ (1|bird)	lmer [n]
	Control birds - after	bx (para d_n/d_{12})	8	114	line*day + day^2 + (1|bird)	line + day + day^2 + (1|bird)	lmer [n]
rbc	All birds - before	rbc	2	140	para*line+para*day + (1|bird)	day*para + (1|bird)	lmer [n]
	All birds - during/after	bx (rbc d_n/d_{12})	9	184	line*day*trt+line*trt*(para d_n/d_{12}) + (1|bird)	day*tt +(1|bird)	lmer [n]
weight	All birds - before	weight	3	116	line*day*para+(1|bird)	1+(1|bird)	lmer [n]
	Treated birds - during/after	bx (weight dn/d_{12})	11		line*day*(para d_n/d_{12}) + (1|bird)	day+ (1|bird)	lmer [n]
	Control birds - during/after	bx (weight dn/d_{12})	10	232	line*day*(para d_n /d_{12}) + (1|bird)	line*day+ (1|bird)	lmer [n]
Experiment 2 – Mosquito – parasitaemia and virulence							
infection	Number of mosquitos with at least 1 oocyst	cbind (inf, uninf)	12	119	line + (1| bird)	1 + (1| bird)	glmer [b]
	Number of oocysts per infected mosquito	oocyst	13	35	line + (1| bird)	line + (1| bird)	glmer [p]
survival	Overall survival	(day, status)	15	734	line + (1| bird)	1 + (1| bird)	coxme
fecundity	Number of eggs per raft	eggs	14	236	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)	16	468	line*day + (1|bird)	day + (1|bird)	glmer [b]
	Number of oocysts per infected mosquito	oocyst	17	66	line*day + (1|bird)	day + (1|bird)	glmmTMB
sporozoites	Number of mosquitoes with sporozoites	cbind (inf, uninf)	18	289	line*day + (1|bird)	day + (1|bird)	glmer [b]
	Sporozoite burden	log(sporozoite)	19	71	line*day*oocyst + (1|bird)	line*oocyst + day*oocyst + (1|bird)	lmer [n]

622 **ST3: Reduction in parasitaemia as a result of the AS treatment for each of the 3 lines**.
623 Treatment was given on days 12-15, parasitaemia was calculated 24h after each treatment (days 13-
624 16). The reduction in parasitaemia is calculated as *(px-pb)/pb * 100* where *px*= parasitaemia at a
625 given day, and *pb* = baseline parasitaemia (parasitaemia immediately before the treatment, day 12).
626 mn=mean, md=median, Q1=25% quartile, Q3= 75% quartile.
627
628

	control	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 %

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631 # Supplementary Figure

632 **SF1: Bird parasitaemia before the treatment**. Shades around the lines represent the 95%
633 confidence intervals.

634