Peer Community In Evolutionary Biology

The importance of understanding fitness costs associated with drug resistance throughout the life cycle of malaria parasites

Silvie Huijben *based on peer reviews by* ***Sarah Reece*** *and* ***Marianna Szucs***

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Antimalarial resistance is a major hurdle to malaria eradication efforts. The spread of drug resistance follows basic evolutionary principles, with competitive interactions between resistant and susceptible malaria strains being central to the fitness of resistant parasites. These competitive interactions can be used to design resistance management strategies, whereby a fitness cost of resistant parasites can be exploited through maintaining competitive suppression of the more fit drug-susceptible parasites. This can potentially be achieved using lower drug dosages or lower frequency of drug treatments. This approach has been demonstrated to work empirically in a rodent malaria model [1,2] and has been demonstrated to have clinical success in cancer treatments [3]. However, these resistance management approaches assume a fitness cost of the resistant pathogen, and, in the case of malaria parasites in general, and for artemisinin resistant parasites in particular, there is limited information on the presence of such fitness cost. The best suggestive evidence for the presence of fitness costs comes from the discontinuation of the use of the drug, which, in the case of chloroquine, was followed by a gradual drop in resistance frequency over the following decade [see e.g. 4,5]. However, with artemisinin derivative drugs still in use, alternative ways to study the presence of fitness costs need to be undertaken.

There are several good *in vitro* studies demonstrating artemisinin resistant parasites being competitively suppressed by wildtype parasites [see e.g. 6–9], however, these have the limitation that they will only be able to detect the fitness cost during the blood stage of the infection and in an artificial environment. So far,

there have not been animal models that have thoroughly studied the presence of resistance fitness costs for artemisinin resistant parasites. Moreover, in these types of studies, the focus is mostly on the fitness cost as detected in the vertebrate host. However, malaria parasites spent a significant portion of their life cycle in the mosquito host, where fitness costs could also be expressed. Overall, it is the fitness over the entire life cycle of the parasite that would determine if and to what extent a reduction in resistance frequency would be observed when the use of a drug is stopped.

Here, Villa and colleagues present a study to quantify such fitness cost of artesunate-resistant parasites, not only in a vertebrate host, but also in the mosquito vector [10]. They used the underutilized model system of avian malaria species *Plasmodium relictum* in canaries. Villa and colleagues selected for several different resistance strains, which had a similar delayed clearance phenotype as observed in the field. Interestingly, they did not find evidence of a fitness cost in the vertebrate host. In fact, the resistant strains reached greater parasitaemia than the susceptible strains. From this set of experiments it is unclear whether this is an anomaly or a relevant result. Future work should establish this, though fitness benefits associated with drug resistance have been seen before in Leishmania parasites [11]. An important caveat to the present study is that the parasites were grown in the absence of competition and it is feasible that a cost is not detected when growing by themselves, but would become apparent when in competition. However, these types of experiments are technologically more challenging to perform as it would require an accurate quantification methodology able to distinguish based on one SNP. This problem has been circumvented by either using relative peak height in sanger sequencing [12], or via the likely more accurate route of pyrosequencing [7–9], though these methodologies only give relative frequencies rather than absolute densities. The most interesting observation in the study by Villa et al is that the authors detected a fitness cost being played out in the mosquito vector, where the resistant strains had a decreased infectivity compared to the susceptible strain. This finding is important because 1) it demonstrates that the whole life cycle needs to be taken into account when understanding fitness costs, 2) resistance management strategies that are based on treatment within the vertebrate host may not have the intended effect if the cost does not play out in this host, and 3) it opens new research avenues to explore the possibility of exploiting fitness costs in mosquito vector. Future research should focus on incorporating these assays on fitness costs in mosquitoes, particularly for *P. falciparum* parasites. Additionally, it would be interesting to expand this work in a competitive environment, both in the vertebrate host as in the mosquito host. Finally, it would be important to establish the generalizability of such fitness cost in mosquitoes. If it is a significant factor, mathematical models could incorporate this effect in predictions on the spread of resistance.

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Reviews

Evaluation round #1

DOI or URL of the preprint: https://doi.org/10.1101/2022.01.28.478164
Version of the preprint: 1

Authors' reply, 27 September 2022

Download author's reply

Decision by Silvie Huijben, posted 19 April 2022

Dear authors. I have now received two reviews to your manuscript. Both the reviewers and myself agree this is an important piece of work that addresses a critical component of the evolution of drug-resistant malaria parasites. The reviewers' comments are mostly centered around the clarity of the methods used, the data presentation and some of the data interpretation. In addition to the reviewers' comments, please also consider the following:

- Experiment 2: What is the sample size for the data presented in figure 4? Since unfed mosquitoes were removed, how many were remaining that this data is based on? Also, only 29% of females were infected, but this data is, I presume, based on all mosquitoes regardless of infection status. It would be important to address this caveat, as it might be masking a true effect if it were there. Additionally, it seems that for some

strains, it takes longer to establish an infection in the mosquito (AS2, figure 5) and this strain would not have any (detectable?) oocysts at the time of egg laying. It is unclear to me what the hypothesis was to expect to observe differences on day 3-6 when the parasites are unlikely to have an impact on the mosquito at that point in time. - An argument is made that the phenotype of the selected lines is a delayed clearance time, similar as seen in the field. As the data is currently presented, I do not believe we could make this conclusion. In the untreated infections, there is also a 'delayed clearance' where AS1 and AS2 persist for longer. An alternative hypothesis could be that these are just be differences in growth dynamics (e.g. the reference strain peaking and crashing sooner than the resistant strains), and perhaps these differences are enlarged by the treatment? As also mentioned in the review by Sarah Reece, it would be important to show the full parasite dynamics (also before the onset of treatment) to be making any comparisons based on treatment. - Slightly increased fitness was observed in the in selected lines: was the untreated reference strain selected at the same time? Could these differences feasibly have emerged as part of the selection process itself, rather than the pressure that the drug provides? - No fitness cost were found in the vertebrate host in these experiments. It would be useful to compare these to results found in in vitro experiments. While these are referenced as having been conducted, the results from these experiments are not mentioned. In most of these in vitro competition experiments they do demonstrate a fitness cost for ART resistant parasite (in the vertebrate host). It would be useful to discuss this discrepancy as it may throw the extrapolation from this P. relictum model to P. falciparum into question and therefore the conclusion on costs observed in the mosquito host. - It would be nice to see the raw data of the weights and RBC dynamics as well.
Minor comments:
line 108: "will suffer higher fitness costs than their unselected counterparts", change to "suffer fitness costs in comparison to their unselected counterparts" Line 119: "occasional passage through the mosquito", is it possible to give more details on frequency to get a sense on how frequently 'occasional' is? Lines 132-137: Were lines AS2 and AS3 obtained as true replicate lines or isolated from the same birds (somewhere in the passage history)? Were other parasite lines obtained as well or were these the only ones that survived the passages? Some additional information on the process (perhaps in supplementary information) would likely be of interest to some. Line 271: add 'was' Figure 1: Update the caption, it refers to different names of lines (C, R1, R2 and R3) and dpbi instead of pbi, etc) Figure 2: brief explanation of px/pb would be helpful in the figure legend. It would also be helpful if the terminology around the reference line would be consistent, it's mostly referred to as 'reference line', but in figures and sometimes in the text as 'control'. Bibliography: Fix author 'Huijben' initials from 'U' to 'S'

Reviewed by Sarah Reece, 24 February 2022

Overall summary

This manuscript considers a very important but overlooked topic – whether fitness costs of drug resistance exist in the between-host transmission component of the malaria parasite lifecycle. I found the presentation the concepts and the experiments clear and easy to follow on the whole (Fig 1 is very helpful). The study is thorough and finds interesting results, despite small sample sizes. My comments mostly concern clarifications.

General comments

Analysis/results

1) Line 244 – I think more needs to be done with the pre-treatment data. It is important that the dynamics of each line are similar in both the untreated and treated groups so they can be compared without confounding effects of e.g. different parasitaemias at the point of treatment. Thus, combining data across the untreated and treated groups for each line is interesting but not verifying that the key assumption of the experimental design is met.

2) Line 220 – was sampling day also accounted for in the expt 2 oocyst samples? It doesn't appear so from table ST2? I feel this is important given that expt 2 stimulated more detailed consideration of temporal dynamics in expt 3.

Figures

3) Fig 2 – I was expecting this to be presented with lines (connecting mean points and SE bars/shading) to demonstrate the dynamics, not a bar chart. Such data are not normally plotted in this way and I found it hard to connect the day to day patterns.

4) Also, I wonder if the during and after segments for A and B should be split with different y axis scales to aid interpretation?

5) And why not include the pre-treatment dynamics for the reason in line 244?

6) Expt 1 - Given that there are no figures of the virulence data included in main text or SI, then some effect size info should be included in the results text to give a sense of the differences in anaemia (pre and during/after) at least. Its probably worth pointing out there is no evidence that AS3 followed different dynamics due to its 50% lower infective dose?

7) Expt 2 – include some readouts for longevity, e.g what was median / quartiles for lifespan across groups?

Interpretation

8) Line 360 – as well as selection on life history traits that underpin within host replication, AS selected parasites might selected to adopt reproductive restraint (via genetic evolution and /or plasticity), see Schneider & Reece Mol Biochem Para 2021, Schneider et al PLoS Paths 2018.

9) Line 363-70 – I found this rather confusing and sort of contradictory. Maybe first state that the data do not support selection for virulence evolution, and then explain why this is the case?

10) The AS selected lines are not cloned so each passage could contain a mix of sensitive and resistant parasites [unless the number of parasites in each 100ul passage was quantified and found to be very low?]. This means the AS lines used for the experimental infections might contain a mix of AS-altered and ancestral parasites? If so, I imagine this is likely to act to erode the differences between the AS lines and the unselected line, which makes results conservative. This is probably worth pointing out?

11) Furthermore, the number of infections/group for experiment is in line with sample sizes of similar studies, but the transmission experiments rely on only n=2 infections /group. Thus, the differences between the unselected line and the AS lines might be vulnerable to stochastic differences between the 2 unselected line infections in birds and the (4) AS infections rather than the effects of selection. I feel this should be considered in the discussion. Do the unselected line infections used for transmissions follow those of the n=6 in experiment, and previous equivalent infections?

12) The observation of slower developing oocysts in the AS selected lines is really interesting. For a future study, measuring their size would also help understand their developmental rate and fecundity. In the discussion, I was hoping to read some exploration of this result and potential reasons for how DR could link to, for example, reduced ability to acquire/process resources needed for within-vector replication.

Detailed comments

Line 24 -27 – could be more concise and just a single punchy sentence? “research on the costs of DR focusses on interactions with vertebrate host, yet whether they are also expressed in the vector has been overlooked’

Line 70 – could add some more recent observations of costs of DR, such as resource limitation exacerbating poor competitive ability [https://www.pnas.org/content/114/52/13774]?

Line 107 – it’s a bit confusing to refer to both birds and mosquitoes as hosts when in other places, mosquitoes are referred to as vectors. Change to ‘…in untreated hosts (birds) and vectors (mosquitoes)…’ ?

Line 116 – add ‘France’ to the location of collection?

Line 146 – its not accurate to say that parasite density was followed because the data are % parasiteamia. This is an ok metric to use (though subject to being skewed by variation in anaemia), but should be clarified.

Expt 1 - I couldn’t see any info on the sample size of birds used, nor their age or sex, in the main text.

Line 201 and 220 – previously, uninfected birds were referred to as the control, so call the control group in experiment the unselected reference line to clarify its purpose?
Line 222 - Clarify that bird ID (i.e. cage ID) was also fitted as a random effect for the mosquito data
Line 379 – I thought dissections occurred on day 8 and day 9?
Line 552 – ‘were’ is a typo?

Sarah Reece

Reviewed by Marianna Szucs, 13 April 2022

It is a very well-written paper that was easy to follow despite my lack of expertise in disease biology. I am an entomologist with experience carrying out artificial selection experiments. Overall, I found the study well-designed, the methods and analyses sound. The paper appears quite straight forward but some of the data do not align with the main line of interpretation. It is normal to have outlier replicates that do not conform with the main conclusions, but I feel that the discussion largely omits why that might have happened. Firstly, I was wondering why different methods were used to obtain the three replicated artesunate-selected lines. The AS1 replication was obtained using different concentrations of artesunate than AS2 and AS3. For experiment 2 the AS3 line was not used. It is mentioned that ‘following the results of the previous experiment’ it was decided not to use that line. However, I could not fine any explanation in the results why it was dropped. A bit of clarification on these points would help to better understand the reasoning behind these experimental choices.

Given the variation in results among replicates for a few metrics I have kept wondering how the measured parasite density in birds or oocyst density in mosquitoes compare to levels in nature. I think a brief paragraph in the discussion that talks about what kind of variation there is in these metrics in natural populations of birds and mosquitoes would help to place this study in better context. It could also be used to explain the variation seen among replicates that do not necessarily show the expected response.

A few minor comments:
Line 144: were
Line 157: the other half
Line 271: was lower
Lines 552, 574, 597: grammar