

Mainz, November 20th 2023

Dear Prof. Loiseau

We thank you for the opportunity to re-submit our revised preprint to PCI Infections. We are very grateful to the three referees for their thorough reviews and we appreciated their constructive and interesting comments. We included their suggestions in our manuscript as stated in the responses below (in **bold**). We uploaded the revised version on bioRxiv and joined to this re-submission the text with changes written in **red**.

Kind regards,
Camille-Sophie Cozzarolo, on behalf of all co-authors

Reviews

Reviewed by Luz García-Longoria, 13 Sep 2023 10:16

Firstly, I would like to congratulate the authors for the excellent field and laboratory work they have conducted. In this sense, the review that the authors did, showing the different results in relation to parasite load and reproductive success, is very interesting and well-structured. I like how the authors state the objective of the paper and present their hypothesis. The results are well-presented/structured. I find them very easy to follow. Secondly, I have some comments that hopefully will help improve the interpretation of the results and the structure of the manuscript.

Thank you for these encouraging comments

Line 77: Maybe give some examples explaining this premise.

We added Marzal et al. 2013 as an example. We also reworded next sentences. “On the other hand, as life-history theory assumes that components of reproductive effort are costly, investment in reproduction may have longer-term consequences for the host's ability to clear or at least to control parasite replication rate (Williams, 1966; Stearns, 1989; Sheldon and Verhulst, 1996). This was notably shown in great tits, collared flycatcher and Soay sheeps, in which increased reproductive effort was later associated with higher intensities of infections by haemosporidian parasites and strongyle nematodes, respectively (Richner et al. 1995, Oppliger et al. 1996, Christe et al. 2012, Nordling et al., 1998; Leivesley et al., 2019).”

Line 79: I miss here (or after the next paragraph) a “however” clearly showing the knowledge gap that this study tries to cover.

We added “However, the association between natural reproductive effort and parasite load several months after the reproductive event was rarely investigated.”

Lines 80-109: I find this paragraph quite lengthy. Perhaps the authors could create a separate paragraph specifically focusing on avian malaria parasites and their characteristics, thereby splitting this paragraph into two. There is information related to malaria parasites, their attributes, and the controversies among various studies. It might be beneficial to organize these ideas into two distinct paragraphs.

We split the paragraph between the examples and the potential explanations.

Line 125: Could be possible to show the coordinates?

We added the coordinates.

Line 131: I miss some information about how the authors collected breeding parameters, especially those related to fledging success or laying date. Do you monitored each nest boxes?

Yes, we reformulated this sentence, hopefully making it clearer: “As in Pigeault, Cozzarolo, et al. (2018), we monitored nest-boxes during each breeding season (from March to June), collecting breeding parameters: laying date, clutch size and fledging success.”

Lines 144-156: I wonder if authors check for double infections for Haemoproteus-Plasmodium and they did not find any Haemoproteus infection, or they just focused on Plasmodium. Anyway, this information should be indicated in this section, as I see it.

The nested PCR we used to diagnose birds amplifies both *Plasmodium* and *Haemoproteus* with the same pair of primers. We then used Sanger sequencing on positive samples to identify haemosporidian species. If sequences amplified by the *Plasmodium*/*Haemoproteus* primer pair indicated infection by multiple lineages, we were not able to distinguish between multiple *Plasmodium*, multiple *Haemoproteus* or *Plasmodium*+*Haemoproteus* infections. Thus, we used only individuals infected by a single *Plasmodium* lineage. We made this clearer in the Appendix. We added analyses including infection by *Leucocytozoon* as a covariate.

Line 158: “bird fitted as a random factor” Do you mean bird individual?

Yes, we added the word individual.

Line 166: I do not fully understand if authors only included those individuals captured more than once or all the individuals and correct this “issue” by including in the model bird individual as a random factor. Please, explain this in the Statistical analyses section.

We used all individuals that were captured more than once over the whole study period. However, for a focal year, if an individual bird was captured more than once in the same season (for example, in October and November 2017), we averaged the parasitaemia measurements (within the same season of the focal year) in order to reduce data complexity (that is, we do not have more than one data per bird per season per year). We tried to make this clearer: “we calculated the average parasitemia of individual birds within each season of each year. In fact, some individuals were captured several times in the winter or in autumn of a focal year, while others were captured only once, so we used average seasonal parasitemia to reduce data complexity.”

Line 159: Some Plasmodium words are not in italics. Please, check.

Done.

Line 273: It is difficult to explain the positive/negative correlation between year seasons and parasitemia. However, authors could include some information about vector abundance or diversity in those areas. Of course, parasitemia is not affected directly by vector abundance/diversity but could affect indirectly through prevalence. It is just an idea.

It would be interesting to evaluate annual within-host dynamics with regards to mosquito abundance fluctuations, but we do not have data on mosquito populations for these years.

Line 292-294: Perhaps the authors could also include information about the potential role of these two lineages as specialist or generalist parasites. Although both lineages can infect a high number of host species (SW2=33 and SGS1=147, according to the MalAvi database), studying the infection dynamics in a specific population, such as great tits in Switzerland, should take into account the historical interaction between great tits and these two lineages. In my opinion, SW2 could adopt a more specialist strategy, while SGS1 might exhibit a more generalist one. Of course, it's important to note that this is a hypothesis that cannot be tested directly, but it could be mentioned as a possible

explanation for the observed differences in parasite loads between these two malaria lineages in this population.

Thank you for this suggestion, we added a couple of sentences to include this hypothesis in the discussion: "The fact that *P. relictum* SGS1 is a very generalist and widespread lineage (147 host species and worldwide distribution according to MalAvi database; Bensch et al. 2009) compared to the relatively less common *P. homonucleophilum* SW2 (33 host species, found only in Afro-Eurasia) might correlate with these differences, in line with the hypothesis of a trade-off between generalism and virulence in parasites (Leggett et al. 2013). Peak parasitemia (a proxy for virulence) was negatively correlated with host breadth (a proxy for generalism) in primate malaria parasites (Garamszegi 2006), but, to our knowledge, there is no such evidence for avian haemosporidians. However, there does not seem to be a trade-off between generalism and prevalence in avian *Plasmodium* (Hellgren et al. 2009). Alternatively, *P. relictum* might also have been infecting the Dorigny population for longer than *P. homonucleophilum* has been infecting the Monod population; if this is the case, birds may have had more time to evolve specific immune defenses to limit the virulence of *P. relictum*."

Reviewed by anonymous reviewer, 26 Sep 2023 10:35

The manuscript "Spring Reproductive Success Influences Autumnal Malarial Load in a Passerine Bird" is a very interesting work. Not only it was well-written, but the experiments were well-designed. I appreciated the opportunity to review it and I am looking forward to future manuscripts with a similar approach.

Thank you for these positive comments.

I have a few minor suggestions:

- To include parasite lineages in the abstract
 - **We included parasite lineages in the abstract.**
- Some scientific names are not in italic (i.e., in the Statistical analysis section)
 - **We checked the scientific names and we put them in italics, hoping this time we did not forget any other.**
- I recommend formatting the lineage names and presenting them in capital letters: PARUS1 and TURDUS1. Additionally, I believe the authors could include the morphospecies linked to these lineages.
 - **We added the morphospecies names and formatted the lineage names.**

Reviewed by anonymous reviewer, 27 Sep 2023 10:59

This is an interesting research on the annual variation in Plasmodium infection in peripheral blood from Great tits. The manuscript is well written and the research design is correct. However, at the end of discussion it appears to be clear that authors are treating with two different and isolated populations of Great tits affected by different Plasmodium specires. If that is the case, they are comparing two different

host-parasite interactions. This is also interesting because it is a way to show similarities and differences between both host-parasite interactions but I think it must be clearly stated from the beginning and not at the end of the manuscript. Both host-parasite interactions coincide in the annual pattern of variation although in one population parasitaemia reach higher levels than in the other. From here some differences could occur and should be clarified: Are there differences in reproductive parameters between populations? Are there differences in mass, size, age composition or condition between host populations? In addition, I suppose that there are not mixed infections because you mention that individuals infected by each parasite come from different host population but this should also be clearly stated.

We already discussed the differences between both populations in a previous study (Pigeault, Cozzarolo et al 2018) and we do not wish to extend the introduction with more details, but we added a sentence in the introduction to make this point clearer: “Two great tit populations, characterized by different haemosporidian communities – in particular, the most prevalent *Plasmodium* species in each population is not found in the other one – and different overall reproductive parameters (Pigeault, Cozzarolo et al. 2018), were studied here.” and repeated this in the conclusion: “Interestingly, we found this pattern in two great tit populations, infected by two different *Plasmodium* species.”

Minor comments:

Line 153: I suggest that you make the effort to define and use a single term throughout the entire manuscript.

We reduced the diversity of words, by using “parasite load” when referring to any parasite, and “parasitemia” when referring to haemosporidian parasite load in the peripheral blood.

Line 160, Age was included in analyses but you do not mention how age was calculated.

We added an explanation in the material and methods: “We categorized the birds’ age as “first-year” or “older” based on whether they already had their first post-nuptial molt or not, by looking at their wing feathers.”

Figure 1 and associated results: The relationships between winter, spring and autumn parasitaemia are interesting but, as you include three years of study, I wonder if it would be possible to study also how autumn parasitaemia affects winter parasitaemia the following year. Perhaps you have not enough sample size for that comparison but, if that is the case, you should mention it in the text.

As you guessed, the sample size is too low to evaluate this question. We mentioned it in the text: “We could not look for a correlation between autumn and subsequent winter parasitemia, because of the low sample size (N = 2).”

Line 282: I suggest you analyse infections by other blood parasites in both Great tit populations including *Leucocytozoon*. Interaction among blood parasite infections could affect to your results.

Thank you for your suggestion. We had done this analysis and decided not to include *Leucocytozoon* infection as a covariate as it decreased sample size, but following your suggestion, we included it in the Appendix and added a comment in the material and methods: “As co-infection with *Plasmodium* and *Leucocytozoon* may affect life history traits of great tits (see Figure 2 in Pigeault, Cozzarolo, et al., 2018), birds were also screened for *Leucocytozoon* infection (see Appendix 1, section 1). However, detection of *Leucocytozoon* was not achieved for 12 individuals. Given the relatively small size of our dataset and the fact that including *Leucocytozoon* infection status as an explanatory variable did not change the conclusions of our study, all the analyses with *Leucocytozoon* infection status fitted as an explanatory variable are presented in the Appendix (Section 3).”