Dear Dr. Diaz,

Thank you for giving us the opportunity to revise our paper “Cities as parasitic amplifiers? Malaria prevalence and diversity along an urbanization gradient in great tits”. We are glad that you and the reviewers found merit in our paper, and we are grateful for the constructive criticisms that helped improve the quality of our manuscript. We have completed a thorough revision of our paper in which we addressed each of the reviewers’ comments, as detailed in the point-by-point response below in blue. We have included most of the revised text in this response letter, underlining the text that was newly added. We have also uploaded a Word file that uses “track changes” to clearly show the revisions in context, as well as a clean version in case you prefer to see that version. Please note, the line numbers quoted below in our point-by-point responses refer to the “track changes” version of the revised manuscript.

All authors have read and agreed to the final version of the paper and the response letter. We hope you find our revised paper acceptable for publication in Peer Community in Ecology. Please do not hesitate to contact us with any questions or additional suggestions you might have.

On behalf of all authors,

Dr. Aude E. Caizergues & Benjamin Robira

Dear authors,

Thanks so much for consider your work to be recommended by PCI Ecology.

After carefully revision of myself and based on 3 revisions from reviewers I invited you to read revisions and submitted a new reviewed version of your manuscript. As you can see, the work is important and support new and needed information about diseases ecology and land use. Most of reviewers have expressed concern on methodology and statistical analyses, specially when classified urban vs. non urban sites. This classification was quite artificial and not considered the landscape heterogenity.

Attached you will find reviewers comments.

Best wishes

Reviews

Reviewed by anonymous reviewer, 16 Oct 2023 11:47

This study is very interesting in that it investigates the impact of urbanization on host/parasite interactions beyond the usual but too simplistic « urban vs. non-urban » dichotomy. It relies on a very valuable longitudinal monitoring program of great tits and their parasites in contrasted habitats from Southern France. Although I am not a native English speaker, I have the feeling that the manuscript is well written.

To me, this work provides new insghts into urban ecology, especially urban health ecology, and it clearly deserves to be made available to a wide academic audience. However, I would like to raise a few metholodogical issues which I believe that, once addressed by the authors, may make their conclusions more robust and convincing.

The choice of the study model and the experimental design

I am not an expert about avian malaria and I obviously lack basic knowledge about it. So the following remarks may be poorly pertinent. But in case…

The authors state that the tit – Haemosporidian parasites pathosystem is accurate for exploring host/parasite interactions evolution in regards to urbanization. Although I understand that they can rely on a very valuable long-term monitoring program dedciated to this particular model, I wonder to which extent their statement is true.

(1) Indeed, by essence, vector-transmitted parasites are expected to be drastically impacted by vector(s) spatio-temporal distribution, ecology and evolution, thus adding a critical layer of eco-evolutionary processes at play (i.e., vector-environment, vector-parasite as well as vector-reservoir interactions). As a direct consequence, investigations of the relationships between habitat and parasite characteristics should be complicated by vector-associated confounding factors. In fine, convincing patterns may be retrieved only with very robust datasets (that allow one to take all major components into account), hence, most probably, very large sample sizes. In this paper, although total sample size reaches 386 birds, class-specific sample sizes may drastically drop down when one takes into account sex (N=2), age (2), sites (9) and year (N=6). For instance, this makes an average of <6 individuals (both males and females) per site and year. Yet, it may be possible that each of these factors plays an important role in the evolution of host-parasite evolution in urban vs. non-urban habitats. Could resulting sample sizes be too low to be biologically representative, and statistically sound?

Response: We agree that eco-evolutionary processes which operate in this context can be quite complex. In particular, investigating both the host and the vector is essential to better apprehend the overall epidemiological dynamics. To emphasize that we did not make such an attempt, and only focused on the host, we added in the abstract L28 "*In this study, we focused on a host species and*

investigated the prevalence…" and in the discussion L462 *"In particular, since vector distribution, abundance, and diversity are likely to play a major role in malaria infection patterns observed in bird hosts, disentangling the processes underpinning the parasite prevalence and diversity pattern in different urban conditions will require combining parallel investigation of vectors and hosts along gradients of urbanization."*

Furthermore, we agree with the reviewer that, even in this case, our sample sizes might not permit to obtain a holistic view of the processes at play. However, we do not align with the calculation done by the reviewer for two reasons. First, the variables were not analyzed in interaction with each other and therefore the "exclusion" rules used by the reviewer to estimate the sample size given a set of levels for the different variables is misleading. Second, many of the variables were treated as continuous predictors, hence minimizing negative impact on the degrees of freedom. In fact, although limited (386 individuals in total), our model does not estimate more than 5 fixed effects, and one random effect. As a rule of thumb, it is recommended to have a sample of at the very least 10 points for each estimate. With 386 individuals, our analysis respects this recommendation given the complexity of our models. We thus believe that 386 samples is still a decent sampling to match the study objective which remains descriptive for most, especially knowing the literature gap in this field.

(2) In addition, parasite prevalence in a given host species may be greatly influenced by the presence/absence or relative abundance of alternative host species (not even talking about alternative vector species). For this reasons, and since many readers (including me) may not be familiar with the biological models used, the authors should provide some pieces of information in the introduction about Plasmodium and Leucocytozon specificity to great tits in the studied area. Note that the presence of other (abundant) hosts, even only in some of the studied sites, would strongly weaken the study, and would deserve to be discussed in details.

Response: Despite the growing number of studies investigating avian malaria, we still know little about *Plasmodium* and *Leucocytozoon* strains specificity in great tits. Avian haemosporidian parasites are widely distributed and are known to have a considerable diversity of definitive and intermediate hosts. While some strains seem to be specific to some bird species or families (Ricklefs and Fallon 2002 https://doi.org/10.1098/rspb.2001.1940), some are known generalists (e.g. SGS1 Plasmodium strain, broadly discussed in discussion). As suggested by the reviewer we now provide more insight in the introduction on the biological model in L89.

"Avian malaria parasites belong to Haemoproteus, Plasmodium, or Leucocytozoon genera and are widely studied in the context of host-parasite interactions (Rivero and Gandon 2018). Avian haemosporidians are ubiquitous parasites and encompass a vast diversity of species and strains that can be generalists, and infect a broad number of bird species (e.g. SGS1 Plasmodium relictum strain), or specialist, and infect only one or few species (Valkiunas, 2004). They are vector-borne parasites infecting blood cells and mainly transmitted by five families of Diptera insects: Culicidae, Hippoboscidae, Simuliidae, Ceratopogonidae, and Psychodidae (Valkiunas and Iezhova 2018)."

To this date, we don't have a detailed measure of birds communities compositions along the urbanization gradient, but our results from the zoo align with your remark since some strains found there were previously reported only in Africa, and the zoo hosts multiple African bird species. We added a mention of this matter in the discussion L504:

"A possible explanation could be the difference in bird community composition between habitats, leading to contact with different bird species, each with their own body of specific Haemosporidian parasite lineages as suggested by the occurrence of a rare lineage in the zoo that is hosting African species. Testing this hypothesis would require a thorough scan of Haemosporidian infections in multiple host species from both urban and non-urban habitats in replicated cities and combined with a thorough characterisation of bird community' assemblages and abundances along urbanization gradients."

(3) In the same manner, could variations in distribution and relative abundance of Haemosporidian vector species (Culex pipiens… and others?) exist, so that they may obscure urban vs. non-urban patterns retrieved here? For instance, the authors explain that massive insecticide-based control treatments were implemented against mosquitoes in this region of France (page 5, line 122-124). Were these treatments applied similarly in urban and non-urban areas? Did they impact them in similar ways in terms of mosquito and parasite ecology and population dynamics in urban and non-urban areas? If not, could the contrasted effects of insecticide-based control inside vs. outside cities explain at least partly the urban vs. non-urban patterns described here?

Response: To our knowledge, no mapping of Avian malaria vector distribution/density (*Culex* sp., …) exists for our study area. We agree that multiple biotic and abiotic factors might play a role in vectors distribution and densities. Regarding insecticide treatment, they have been going on in the region for decades but are mostly located on the coastal area (~15 km south from the center of the city of Montpellier), and not in the city or the forest studied here. Hence, it is unlikely that insecticide based control affects the patterns observed here. We have specified this L131:

"*This region of France hosts high densities of avian malaria Plasmodium vectors such as Culex pipiens, for which massive insecticide-based control treatments have been deployed for more than 60 years, with a focus on coastal areas (i.e., ca 15 km from Montpellier historical center, EID, 2020).*"

However, we agree that a combined investigation of host infection and vector distribution/infection is necessary to disentangle the processes at play in link with urbanization and add this perspective in the discussion, see response to comment 1.

As a conclusion, I suggest that these potential caveats/biases/limits are addressed, and that, if realistic, they are made explicit and that the associated conclusions are softened.

Analyses

(5) Similar to the possible sample size issue raised above (see point 1), most of the Results and Discussion sections as well as the Figures deal with prevalence data, hence proportions. However, indicating raw values and sample sizes (e.g., in Figures 2, 3 and 5; in the Results section, page 17, line 334; etc) would allow the readers to better grasp how confident they can be in the interpretation of the data. Dealing with dozens of individuals when interpreting a prevalence is probably not as convincing as dealing with a one or two positive individuals

out of a handsome. As it stands, the paper does not allow one to see clearly what the sample sizes are about (see point 1).

Response: Sample size per age and site are detailed in Figure 2 below each category, we added the information in the caption to attract the reader's attention. We also added them to Table 1.

(6) Page 11, lines 255-256: the authors state that using only lineages for which type II error is below 0.2 and that occur at least 10 times ensures « statistical robustness ». Could they argue (or give a reference) about this approach?

Response: A type II error of 0.2 represents a common choice (reference to Cohen, 2013 was added). To make it clearer, we have now rephrased L268:

"To avoid false negative habitat association in rare lineages, we computed the test only for lineages for which the type II error was below 0.20 (Cohen, 2013), and that occurred at least 10 times overall."

(7) Although I understand from Figure 1 that the zoo site (ZOO) lies inside the limits of Montpellier city per se, it looks like a quite large area between two other large green spaces. In addition, the authors describe it as « the least urbanized urban site » (page 14, line 313). Could it be that this zoo display ecological features that makes it quite different from what is usually perceived as « urban » sites (or even urban parks)? Could this particular zone be more preserved (wild?) than other urban parks, a fortiori other hard-built urban sites? If yes, when performing urban vs. non-urban sites analyses, wouldn't it be pertinent to consider ZOO as a non-urban site (or to remove it from these particular binary analyses)?

Response: Montpellier zoo is located at the edge of the city of Montpellier yet it is really included in the urban matrix (see Figure 1C), as it is surrounded by built up areas on all sides. We have now added the satellite image below in the appendix for a more explicit view (Figure S1).

Overall, the Zoo site thus remains close to other urban sites (about 600 m away from the CEF site ; average distance between all urban sites is 4.4 km). In comparison, the forest site is located 20 km away from the city, in a rural landscape. Knowing that great tits are mobile animals that can easily fly from one site to another to forage or disperse, as suggested by the low FST (<1%) estimates between all city sites (Perrier et al. 2017 https://doi.org/10.1111/eva.12580), we think it would not be appropriate to consider the ZOO separately from the rest of the city sites in the analyses which for now only distinguish between urban *vs* non-urban environment. Furthermore, by representing "parks with a natural-like vegetation", the Zoo site ranges less extreme along the urbanization gradient than some other urban sites (e.g., MAS) and matches more with the non-urban sites in analyses relying on a continuous description of urbanization. In fact, considering the zoo as urban appears even more relevant *a posteriori* as the composition of the samples from the ZOO

were closer to the samples from the urban sites(see Figure 6). Finally, despite also considering a more nuanced gradient of urbanization, the analyses relying on a continuous quantification of urbanization were in agreement with those considering urbanization as a binary trait, suggesting that the ZOO classification as an urban site has little consequence in the end.

To alleviate these doubts, we have now added L161:
"*Among urban sites, the zoo (ZOO; Figure 1C) was the least urbanized site, but was well settled in the urban matrix (Figure S1), with short distances from CEF and FAC allowing great tits from the zoo to interact with birds in these neighboring sites (see low genetic differentiation described in Perrier et al. 2017).*"

Figure S1: Satellite view of the Zoo area | The image was taken from Google Earth Engine (REF) on the 20/11/2023. The Zoo site is located close to urban sites (e.g., about 600 m away from the CEF site; average distance between all urban sites is 4.4 km). In comparison, the forest site is located 20 km away from the city, in a rural landscape.

Minor and wording remarks

(8) In the introduction, when briefly describing urban habitats characteristics, the authors may want to complement their sentence about living organisms communities, and add that animal

and microbial communities are also drastcially modified and usually dominated by exotic species (page 3, line 50).

Response: We rephrased this section to read L55:

"Hence, urban communities are altered and mainly composed of fewer, often generalist and/or exotic, species with higher population densities compared to natural habitats (Shochat et al. 2006; Faeth et al. 2011)."

(9) I am not sure about the term « vector species » as it is used in the Introduction (see e.g. page 3, line 59): do the authors mean « reservoir species », « vector and reservoir species », « host species », etc?

Response: We apologize if this was not clear. In this context we distinguished between the parasite, host species (which you may call reservoir species, here the great tit), and other hosts which may be involved in the spread and transmission of the parasite: the vector species (e.g., some diptera species). We have now rephrased it L61:

"*because of variation in both the occurrence and abundance of species enabling the spread and transmission of the parasite (i.e., the vector species)*"

(10) The positive and negative impacts of urbanization on disease prevalence may not always be exclusive, and the sole dichotomic (i.e. « twofold ») perspective of urbanization / disease prevalence may be a bit simplistic. For instance, urban environment may offer larger quantities of food, but diet of lower quality, thus potentially having both positive and negative effects on hosts' body condition, immune system, hence reproduction and fitness, competency towards parasites, etc. The authors may want to slightly modify their statement to take such a complexity into account.

Response: We are sorry the "twofold" terminology let it believe that it was mutually exclusive. However, we would like to point out that we talked about the "overall" resulting effect, thus encompassing situations where both positive and negative effects were at play. To make it clearer, we have now rephrased L67:

"*First, in cases where urbanization predominantly negatively impacts vector species and/or predominantly favors the host species…*"

(11) I am not sure to have it right: are all Plasmodium lineages more prevalent in urbanized sites (Conclusion, page 24, lines 482-483), or only the YWT4 lineage (Figure 5)?

Response: We reorganized the sentence to make it clear that we found habitat "preference" (for the urban habitat) only for the YWT4 lineage, but no other lineages.

Reviewed by Ana Paula Mansilla, 13 Oct 2023 21:54

The manuscript "*Cities as parasitic amplifiers? Malaria prevalence and diversity along an urbanization gradient in great tits*" analyze the effect of the urbanization on the prevalence and diversity of avian malaria parasit es in the great tit, through two methodological approaches or spatial scale: compares an urban environment (with 8 sites/replicas) vs. a non-urban environment (1 site only) and then, a gradient of urbanization within the urban environment. I find some problems or lack of clarity in this methodological part.
(12) It remains to be described how the data is distributed over the years, and there is a great imbalance between the number of samples from the urban and non-urban environments.

Response: We added a table with the detail of sample size per site, year, and sex ratio (Table S2).

(13) At the same time, I believe that the heterogeneity of the urban environment must be considered in analyzes at the nest scale, including the site as a random variable through generalized linear mixed models, for example.

Response: We added the site as a random variable in the generalized mixed models investigating prevalence as a function of nest-box urbanization level and edited the results section accordingly. Note that while estimates values slightly changed (to the second or third decimal), the overall results remained the same.

(14) The results of the models are not so conclusive (some have enormous confidence intervals, or include 0), to express it that way in their conclusions. You should be much more cautious when providing your conclusions.

Response: For a detailed response to these points, we refer to our reply to comment 29 and 31. We also added in the discussion L513:

"While we found no striking difference in malaria prevalence between urban and non-urban great tits, urbanization was associated with earlier infections in nestlings. In addition, we found a weak tendency for Plasmodium sp. prevalence to increase with urbanization. While our results will need to be replicated with higher number of sampled sites and individuals, they could suggest that urbanization does not decrease parasitic load but may, on the contrary, lead to a parasitic burden for urban great tits."

(15) Although it is a very interesting and necessary work from the point of view of disease ecology and also challenging when studying the environment or context that is in constant change, there are some important methodological issues to review. A proposal or alternative to think about, could be to consider 3 environments: urban, semi-urban and rural or forest with a homogeneous or similar number of samples and explore in greater detail the characteristics of these environments and how the prevalence varies. The semi-urban

environment could consider some of the sites on the periphery of the city and that gather a considerable number of samples.

Response: We refer to our answer to Reviewer 1 in comment 7 to point out the text modifications that deal with this comment in the main text. Overall, the binary view of the urban vs non-urban habitat is secondary and we favored, whenever possible, the use of a continuous quantification of urbanization.
As answered to Reviewer 1, considering such an "intermediate" urban category would be equivalent to considering that "green" and "not green" habitats in cities are independent. In the specific context of our study site, this is clearly not the case (see response to comment 7). Furthermore, this would occult the idea that cities are actually a heterogeneous matrix which includes remnant natural, or artificial, green patches. Here, all the "urban" sites clearly lay within a dense urban matrix, even if some of them display higher local levels of vegetation cover (e.g. ZOO, see response to comment 7). The heterogeneity of the urban matrix, which may vary with the scale under scrutiny, is in fact the reason why we quantified urbanization either at the nest, or at the site, scales. Distinguishing between the two scales led to no overall result difference yet.

(16) **Title:** I suggest change the order of the last part of the title. Cities as parasitic amplifiers? Malaria prevalence and diversity in great tits along an urbanization gradient. Add the scientific name of the great tit and it would also be appropriate to add in Montpellier, France or, at least, France.

Response: We rephrased the title with the change of order suggested, to now read "*Cities as parasitic amplifiers? Malaria prevalence and diversity in great tits along an urban gradient*." We do not think that adding the scientific name of the great tit is necessary, nor the specification of the location in the south of France which would make the title very long, but are happy to make these additions if the editor requests it. To keep this information easily accessible, however, we have added the species scientific name and "*South of France*" in the keyword list.

Abstract

(17) **Line 40**: I consider that it is a somewhat alarmist conclusion and due to the experimental design and/or methodological problems I would not have enough support to say this. Furthermore, it is not correct to use the concept of epidemic if we are talking about avian malaria. Try to be more cautious with the conclusions.

Response: We removed this sentence as suggested.

(18) **Keywords**: I suggest adding France to the list.

Response: We added "*south of France*" as a keyword.

INTRODUCTION

(19) **Line 74 and 81**: Criticizes the binary perspective of urban vs. not urban but presents and analyzes its results in that same way.

Response: We fear we disagree with this comment, which in our opinion, lacks nuance. We tried to move from the binary comparison as much as possible by analyzing the data grouped per nest or site varying in urbanization level and along an urbanization gradient. We were indeed constrained in one analysis to use only the binary urban vs. rural approach (i.e. the strain habitat specificity analyses) as we were not aware of a better statistical way to tackle habitat specificity, particularly given our small sample. Yet, we hope to benefit from a larger sample size in a near future to analyze the diversity of strains along the urbanization gradient. For the rest of the analyses, the binary classification of the habitat was only used for comparative purpose with previous studies, and we always performed the analyses considering a gradient of urbanization (either at the site or the nest scale), and thus presented and discussed the analyses as such.

(20) **Line 85**: Move this sentence. The paragraph could start directly with… "Avian malaria parasites…"

Response: We edited as suggested and moved the first sentence later in the introduction, and this paragraph now starts with L89:

"Avian malaria parasites belong to *Haemoproteus*, *Plasmodium*, or *Leucocytozoon* genera and are widely studied in the context of host-parasite interactions (Rivero and Gandon 2018)."

(21) **Line 103-109**: It's being repetitive and getting confusing. I suggest eliminating the first sentence and starting with the sentence that starts from "Specifically…" pointing out the 3 objectives.
Maybe it starts here with the paragraph and sentence on line 85.

Response: We rephrased this section as recommended to read L109:

" In this study, we investigated the prevalence and diversity of avian malaria parasites in great tits (Parus major) in and around the city of Montpellier, south of France. Specifically, we (1) compared the prevalence in nestlings and adult individuals across different urbanization levels measured at the different scales, (2) characterised parasite molecular lineage richness and diversity along the gradient of urbanization, and (3) assessed the role of urbanization levels on parasite diversity."

(22) **Line 104**: Spatial resolution is another thing; I imagine you mean to refer to a spatial scale.

Response: We rephrased this section, see previous comment. However, in the rest of the text, we now use the word "scale".

METHODS

(23) **Line 128-129:** Wouldn't it also be important to include variables relevant to the biology of the vectors? For example, distance to water bodies or presence and size of water bodies… Think about which variables could influence the ecology of malaria and be masked within the "urbanization" variable.

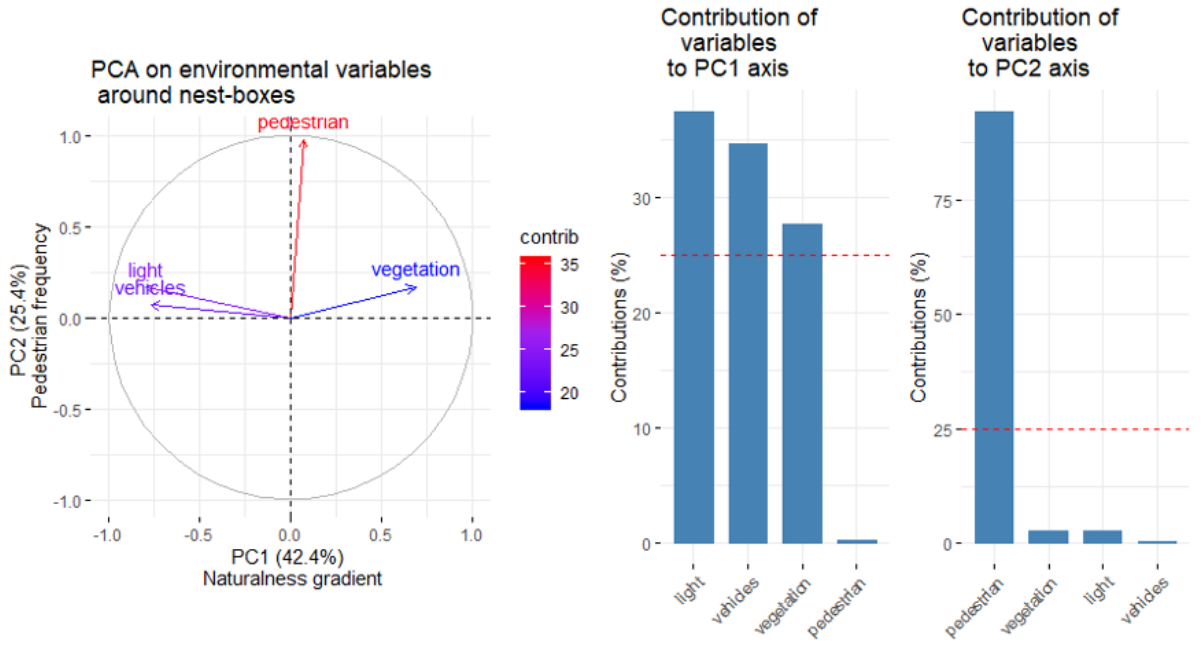
Response: We agree that the biology and distribution of the vector potentially plays a major role in malaria infection in great tits. However, choosing environmentally relevant variables describing vectors distribution is not straightforward. For the case of water bodies, estimating the distance to ponds and river would be largely insufficient since *Culex pipiens* thrives in small water bodies of natural origin such as water filled tree-holes, puddles, ruts but also man-made receptacles such as gutters, flooded cellars, construction sites, road drains and pits, water barrels, metal tanks, ornamental ponds and any type of container (e.g. plant pots in gardens or cemeteries)… Therefore, quantifying the presence and density of water bodies available for mosquitoes in a forest and along an urbanization gradient is virtually impossible with our data. Besides, other vectors of malaria do not necessarily depend on water bodies, for instance *Aedes* mosquitoes lay their eggs outside of water and only require small amounts of water to develop, and dew is sometimes sufficient. Similarly, most of louse flies (*Hippoboscidae*), blackflies (*Simuliidae*) and biting midges (*Ceratopogonidae*) avian malaria vector species do not require water to develop at all. We acknowledge that understanding how the urban environment affects not only the host, but also the vector, is of crucial importance to understand pathogen dynamic difference in urban or non-urban habitat. We introduced this idea as soon as the introduction. However, this falls well beyond the data collection within the frame of this study. To emphasize this point nonetheless, we now state in the discussion L462:

"*In particular, since vector distribution, abundance, and diversity are likely to play a major role in malaria infection patterns observed in bird hosts, disentangling the processes underpining the parasite prevalence and diversity pattern in different urban conditions will require combining parallel investigation of vectors and hosts along gradients of urbanization. In this study, we however only focused on the host.*"

(24) **Line141:** The PCA is usually used when you want to summarize the number of variables, since there are many explanatory variables that are correlated with each other. On the one hand, 4 variables seems like a small number of explanatory variables to me (considering that then there is a glm with only 1 vs. Explanatory), and on the other hand it would be good to incorporate the graph to see how the variables relate to each other (although be in the supplementary material).

Response: We added the figures in supplementary materials as requested. As you can see, they summarize fairly well the naturalness of the environment. As per including the 4 environmental variables in the model, as mentioned by reviewer 1, our sample size is fairly

low and adding them separately would greatly reduce our statistical power. This is why we summarized these variables with a PCA approach.



*"**Figure S2:** (A) Results from the PCA analysis on environmental factors measured around nest-boxes: vegetation cover, vehicle density, pedestrian frequency and light pollution. (B) Histograms showing the contribution of each variable to the first two Principal Component axes. These histograms show that PC1 is interpretable as a naturalness gradient and PC2 as pedestrian disturbance."*

(25) **Line 154**: Blood sample collection… How is the blood sample collection over the years? It would be necessary to incorporate a table specifying all sampling sites, type of habitat, year of sample collection, number of samples (nestlings and adults), etc.

Response: Thank you for this remark. Since the annual sampling was not clear, we have now added a supplementary table (Table S2) with details of yearly sampling. Please note that per site sample sizes are also provided in Figure 1 and Table 1.

(26) **Line 159-160**: How many sites were from the urban environment and how many from the non-urban environment? Specify for both chicks and adults. This should be in the table requested in the previous point.

Response: See response to previous comment.

(27) **Line 214**: Change resolution by spatial scale.

Response: We changed it.

(28) **Line 222**: Fix next by nest box.

Response: We corrected it.

RESULTS

(29) **Line 273-274:** Careful. The value of the estimate and the CIs are very large. It may be because in the non-urban environment there are only 0.

Response: The large confidence intervals are rather due to the small sample sizes we had with the nestlings. To be more cautious in the presentation of this, we now state L286:

"*Prevalence was significantly higher in the urban nestlings compared to non-urban nestlings (16.67% averaged on all urban sites vs. 0% in the non-urban site; $\chi 21 = 9.854$, $P = 0.002$). However, overall small sample sizes and the presence of only one replicate of "non-urban" site potentially inflated these differences*."

(30) **Line 279**: Close parentheses.

Response: We corrected it.

(31) **Line 288-289**: Again, be careful with these results. The confidence interval includes 0 so it would not be significant.

Response: Yes indeed, but as the overlap is not that extensive (hence leading to a p-value close to the arbitrary, and quite restrictive given our sample sizes, threshold of significance 0.05), we speak of a "tendency". To emphasize this point, we have now rephrased (L304) as follows:
"*However, nest-level naturalness gradient was potentially related to Plasmodium sp. prevalence (glmer: est. ± S.E. = -0.616 ± 0.397, $\chi 21 = 2.937$, $P = 0.087$), with a weak tendency for lower prevalence in less urbanized areas*"

DISCUSSION

(32) **Line 429**: Surprisingly however, it would not be a good expression to start the paragraph. I would start directly with..."the diversity of Haemosporidian lineages
Correct Haomosporidian by Haemosporidian.

Response: We edited the text as suggested.

(33) Be much more cautious with your conclusions throughout the discussion. Problems with experimental design and number of samples heterogeneity may be leading to erroneous results. In addition, there are many variables that can affect the host and the vectors that may be masked within the urbanization variable and were not taken into account.

Response: We have now regularly highlighted the possible biases due to our sample sizes and tame down our conclusions in several places in the discussion, see for instance response to comment 14 and text L420:

"In addition, urban nestlings showed higher prevalence than non-urban ones, although the non-urban site had only one replicate, limiting robust generalisation. Replication of the work done here at other sites is therefore crucial to (in)validate this finding. Provided that this is generalisable, one can hypothesize that early malaria infection in urban nestlings might be an indirect result of the heat island effect. Indeed, Paz and Albersheim (2008) showed that higher temperatures in urban areas proved beneficial to Culex pipiens mosquitoes growth…"

We also added emphasize on the importance to study both host and vector distribution in the urban matrix to have a holistic view of the processes at play in urban malaria epidemiology, L467:

"However, such hypothesis remains to be further tested. In particular, since vector distribution, abundance, and diversity are likely to play a major role in malaria infection patterns observed in bird hosts, disentangling the processes underpinning parasite prevalence and diversity patterns in different urban conditions will require combining parallel investigation of vectors and hosts along gradients of urbanization. In this study, we however only focused on the host."

(34) **Figure 1**: Add in the regional scale that are shown the non-urban habitat (ROU) and the urban habitat (Montpellier).

Response: We fear we do not understand the reviewer point. The regional scale, depicted by panel B, shows the location of the non-urban habitat (ROU) with respect to the city of Montpellier. The inset in panel B refers to panel C, where the locations of the different urban study sites are shown. Depicting the locations of the urban sites also in panel B would be very inefficient given the small range of the city overall (compared to the distance to the ROU site).

(35) **Figure 5 and 6**: Change location of figures to before discussion.

Response: We relocated Figures 5 and 6 as suggested.

(36) There are several spelling errors in the supplementary material. The word Plasmodium is misspelled several times. Review.

Response: Thank you for spotting these mistakes, we corrected them.

Reviewed by anonymous reviewer, 21 Oct 2023 14:11
Dear Editor:

(37) I do find the present Article of interest for the scientific community. The topic is interesting and well developed. The data is organized, statistical analyzes are appropriate, and the conclusion is well supported with the results obtained. I includ minor comments or suggestions within the text, which I uploaded.

Best regards,

Response: We corrected all typos and formatting issues (reference and table quality) raised by Reviewer 3.