02-Jul-2024  
  
Dear Miss Erokhina:

Manuscript ID CZ-2024-0085 entitled "Dynamics of resting metabolic rate and innate immune response in malaria-infected Eurasian siskins" which you submitted to Current Zoology, has been reviewed.  The comments from reviewer(s) are included at the bottom of this letter.  
  
I have below concerns:

1. Do you have the other data in addition to the RMR and IL-6 level with the sample issues?

**Response:** In addition to RMR and IL-6 levels, we studied the dynamics of a crucial individual characteristic – body mass. It was included in the GAMMs as a covariate. We respectfully disagree with the second reviewer that it was necessary for us to assess the maximum metabolic rate of the birds (see our response below).

2. The manuscript should be language proofed.

**Response:**

In view of the criticisms of the reviewer(s), I must decline the manuscript for publication in Current Zoology at this time.  However, a new manuscript may be submitted which takes into consideration these comments.

**Reviewer: 1**

**Comments to the Author**

The authors studied the physiological response of individuals of siskin to infection with two different strains of parasites that produce malaria in the birds. The authors measured parasitemia, levels of IL-6 and metabolic rates in three groups of birds: control birds, and two groups of siskins infected with each of the two parasite strains. I think the manuscript would be much improved if the authors established connections between the intensity of the infection and metabolic rates. As it is, the authors are discussing changes in the variables one by one, but I think they can be related and that will help the interpretation of the results Specific comments are shown below.

**Response:** We value the reviewer's thoughtful feedback and the provided suggestions. It helped us to see that the role of DPI (day post infection) in GAMMs presented in the manuscript were not explicitly described.

DPI and parasitemia are closely linked, reflecting the natural progression of infection. Early DPIs correspond to low parasitemia (prepatent period), mid DPIs to high parasitemia (acute phase), and late DPIs to lower but persistent parasitemia (chronic phase). This relationship forms a bell-shaped curve on a graph of parasitemia versus DPI, making each a predictor of the other. However, since parasitemia follows a non-linear, bell-shaped pattern, it isn't an ideal predictor. For example, a 15% parasitemia early in infection will likely elicit a different RMR response than the same 15% later, during recovery. Due to this non-linearity and interdependence, using both parameters in a model would cause multicollinearity, so only one should be used. To illustrate the acute phase, we applied a red gradient to the modeled curves, with intensity reflecting parasitemia levels.

Taking into account the reviewers’ remarks, we made a concerted effort to explicitly explain the use of DPI in our models:

**Lines 340-346:** We did not include parasitemia as a predictor because the DPI predictor serves as a more consistent representation of infection progression. DPI provides a consistent measure of the infection timeline and helps avoid multicollinearity, which could occur if parasitemia were used directly due to its non-linear, bell-shaped pattern. By using DPI, we are better able to capture the dynamic changes in the response variable throughout the course of the infection.

**Response (continuation):**

In fact, initially we did consider to analyze the association between parasitemia levels and metabolic rate, as well as between parasitemia levels and IL-6 parameters. However, while working with the raw data, we encountered a challenge: the design of our experiment did not permit a direct link between the measured metabolic values and the calculated parasitemia levels. Our respirometry setup allowed us to test only four birds simultaneously, and blood samples for IL-6 analysis, along with the smears, were taken every six days from the entire experimental or control group at once. To estimate the level of parasitemia on the day of RMR measurement, we calculated the parasitemia's dependence on the date, assuming that over relatively short time intervals (six days), the parasitemia development curve could be approximated as linear. This equation was derived for each RMR measurement from parasitemia estimation on the two days closest to it (one day before and one after the metabolic trial). This approximately estimated parasitemia was utilized to compute the cumulative parasitemia (integral of the parasitemia curve) for our model investigating the association between RMR and parasitemia and between IL-6 levels and cumulative parasitemia levels using, again, the GAMM. In this model, log10-transformed IL-6 or RMR served as the dependent variable, while cumulative parasitemia level, experimental group, and log10-transformed body mass was employed as independent variables. As with all other models, individual bird ring number was included as a random effect variable.

Below, we present our reasoning and analysis of the relationship between RMR, IL-6, and parasitemia.

**Analysis of the relationship between RMR and cumulative parasitemia levels**

For the analysis of RMR and associated approximate cumulative parasitemia, the optimal GAMM was the one with a shared smoother for both experimental groups (based on AIC). This suggested that birds in both infected groups exhibited similar changes in their RMR during the accumulation of parasitemia. From this model, it can be concluded that during the early stages of infection, when active proliferation of the parasite occurs, the resting metabolic rate of infected birds from both experimental groups increases and that no significant differences in RMR levels are observed between birds with SGS1 and GRW2 through all the experiment. We identify several disadvantages of this method of data analysis compared to the one presented in the original manuscript. Due to the small number of measured RMR values, this model fails to distinguish between the experimental groups effectively, especially at the early stages of infection. Some of the initial RMR values post-infection are lost with this method, as not all birds had completed the prepatent period, despite the parasite already being present in their bodies and capable of affecting the host's energy metabolism. Furthermore, in the graph depicting the relationship between RMR and accumulated parasitemia, the number of RMR values for birds with SGS1 clearly fall outside the confidence interval predicted by the model. Conversely, the graph of the model showing RMR dependence on DPI clearly displays a decrease in RMR in birds from this group at the onset of parasitemia development—values that the accumulated parasitemia RMR model fails to detect. Therefore, this analysis does not provide new information and, in fact, overlooks interesting results obtained from the RMR dependence on DPI model at the early stages of RMR measurements, when not in all birds the parasite had completed its prepatent period.

Figure 1. Predicted by GAMM values (curve, gray areas around the lines represent 95% CI) of the connection of RMR and cumulated approximate parasitemia. Dots represent the real values for RMR. Exp 1 – birds with SGS1, Exp 2 – birds with GRW2

**Analysis of the relationship between IL-6 and cumulative parasitemia**

For the analysis of the relationship between IL-6 and cumulative parasitemia, once again, the GAMM with the same smoother function performed better (based on AIC), indicating generally similar trends for both groups. As the accumulated parasitemia value increases for each bird, the IL-6 level remains relatively constant, as indicated by a smoother which is equal to zero (curve appears as a straight line). However, within SGS1 infected birds, the average IL-6 level is higher than in the group infected with the GRW2 parasite (Fig. 2).

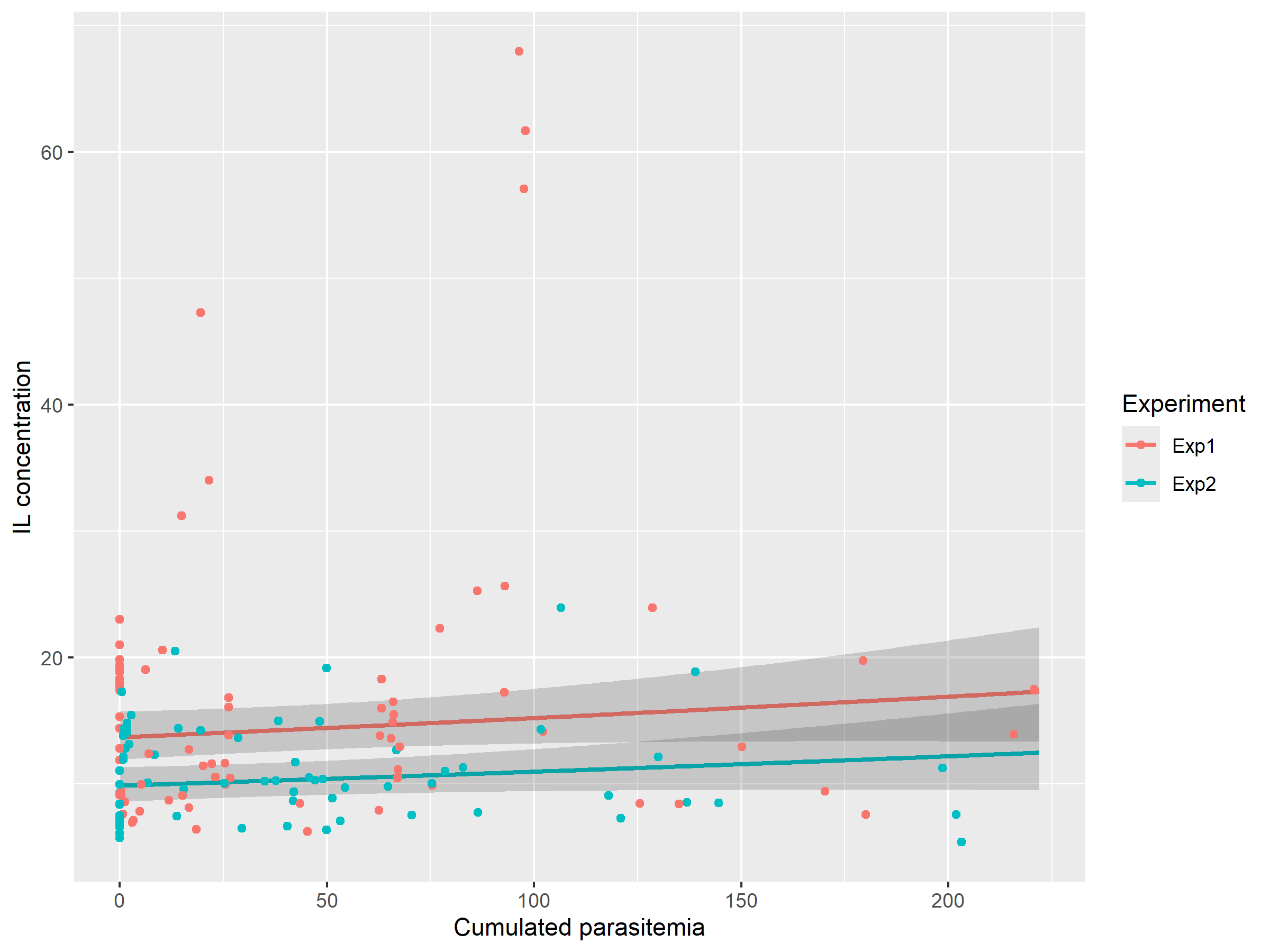


Figure 2. Predicted by GAMM values (lines, gray areas around the lines represent 95% CI) of connection of IL-6 levels and cumulated approximate parasitemia. Dots represent the real values for RMR. Exp 1 – birds with SGS1, Exp 2 – birds with GRW2

All our previously mentioned comments regarding the earlier model also apply to this one. The primary issue is the absence of fundamentally new conclusions about the studied parameters and the partial loss of data.

**Analysis of the relationship between IL-6 and parasitemia levels**

In addition to the GAMMs, we applied a Linear Mixed Model (LMM) analysis to examine the relationship between infection intensity and IL-6 levels. For this analysis, no approximation was needed, as both parasitemia and IL-6 were measured on the same day post-infection (DPI). The LMM revealed differences between the two experimental groups. While no clear correlation was observed among GRW2-infected birds, a negative relationship was evident for SGS1-infected siskins (Fig. 3). This negative relationship indicates that higher parasitemia levels correspond to lower IL-6 levels in the SGS1 group. This finding aligns with our initial speculations based on the GAMM, which showed a decrease in IL-6 levels at the onset of the acute phase of parasitemia for this group of birds. However, this LMM analysis does not provide new information about the studied parameters in infected siskins. Moreover, it does not distinguish between low parasitemia levels at the beginning of parasitemia development and low parasitemia levels after the acute stage of malaria, leading to a loss of data.

Alternatively, it's possible that high parasitemia levels are only attainable when IL-6 levels are low.



Fig. 3. Linear relationship between the parasitemia and IL-6 for SGS1 (red) and GRW4 (blue) infected birds. Exp 1 – birds with SGS1, Exp 2 – birds with GRW2

**Summary on RMR and IL-6 association with cumulated parasitemia level**

To link the measurements of infected birds' RMR with parasitemia, we employed an estimation of the cumulative parasitemia timed to the RMR measurements. Given the imperfections in our experimental design, we had to manipulate the parasitemia approximation. We implied that this approximately calculated cumulative parasitemia, an uncommon parameter in avian malaria studies, would provide a rough estimate of the parasite burden that each bird had been experienced in total up to the moment of RMR measurement. However, as in the case of parasitemia measurements as they are, accumulated parasitemia essentially represents a time-based measure. We have already analyzed the relationship of all response variables with DPI (days post-infection). Therefore, examining accumulated parasitemia would only duplicate this information. These findings indicate that RMR was indeed influenced by cumulative parasitemia in both groups of infected birds. According to the best-fitting GAMM, the trends in RMR changes were similar between the two infected groups, albeit with a slight difference in RMR levels. Specifically, GRW2 infected birds exhibited slightly higher RMR compared to SGS1 infected siskins. However, it's noteworthy that the confidence intervals of the modeled curves overlapped, suggesting some degree of uncertainty in this observation. Remarkably, according to this model, both SGS1 and GRW2 infected siskins exhibited an increase in RMR as the cumulative parasitemia levels rose. While this trend aligns with the previously discussed modeled dynamics of RMR changes for the GRW2 infected group, though the model still indicated a brief initial decrease in RMR, which was promptly followed by an increase, it seems to contradict with our conclusions for the SGS1 infected birds, where the initial decrease in RMR was clearly visible on the model`s curve. The cumulative parasitemia parameter does not directly reflect the time passed since the moment of infection, unlike the DPI. However, with the progression of infection, cumulative parasitemia consistently increased over time. The absence of RMR measurements, particularly during the acute phase of parasitemia, while the most measurements occurred after peak parasitemia, may have contributed to the emergence of a trend indicating increased oxygen consumption within all infected birds.

The analysis of the relationship between IL-6 and cumulative parasitemia within both groups exhibited overall similar trends. Interestingly, as the accumulated parasitemia increased for each bird, the IL-6 level remained relatively constant, as indicated by a smoother that is equal to zero. Again, as in the previous analysis, the GAMM`s curve for IL-6 association with cumulative parasitemia reflects the general trend of IL-6 level within the both infected group of birds. And, according to this model, the IL-6 level appeared to be more or less constant with growing cumulative level of experienced by each bird parasitemia. However, within the SGS1 infected birds, the average IL-6 level was notably higher compared to the group infected with the GRW2 parasite.

The analysis of the LMM relationship between parasitemia and IL-6 levels measured on the same DPI revealed differences between the two experimental groups. While no clear correlation was observed among GRW2 infected birds, a negative relationship can be traced for SGS1 infected siskins. This negative relationship implies that when the parasitemia levels were high in this group of birds, the IL-6 levels were low. This confirms with our speculations based on the model curve of the IL-6 changes over time of the experiment, where we described a decrease in IL-6 level during the beginning of the acute phase of parasitemia. Though, as it was already discussed in original manuscript (**Lines 617-620 revised MS**), we cannot provide clear mechanism of this depletion.

In summary, interpreting these models of the relationship between RMR, IL-6, and cumulative parasitemia level can be challenging. It's possible that accumulated parasitemia better reflects the individual course of the disease, while DPI characterizes a more generalized group response, which may vary depending on the bird species. Further research into these relationships is warranted to better understand the complexities of the host-parasite interaction.

The GAMMs analyzing the relation between RMR or IL-6 and accumulated parasitemia, as well as the linear model of IL-6 and parasitemia levels, do not provide any additional information beyond what is already presented in our manuscript. Additionally, our primary goal was not to study the physiology of the immune response in passerine birds by examining the link between RMR and IL-6. This is a complex issue that lies beyond our current capabilities. Given the limited information on wild bird immunology in the literature, we only speculated that IL-6 might indicate the onset of the acute immune response in birds, similar to its role in mammals.

Lines 73-81. These costs are not directly related with the immune response, but they cannot be disentangled from the process of infection. If there is a way to just quantify the energy expenditure of the immune response, separated from collateral costs, I don’t think that you would have a complete picture of the whole-organismal response.

**Response:** we are a bit confused with this reviewer`s notion. This is exactly what we wrote in the manuscript, that in addition to the energy costs of the immune response, we evaluate the costs of tissue reparation, hematopoiesis, etc. and that we cannot separate these processes:

**Lines 75-81** **in original MS:** However, measuring the precise metabolic cost of immunity is challenging due to its intricate integration with other physiological systems within the organism (Lochmiller and Deerenberg 2000). That implies that RMR measured in our study reflected not only the immune response itself but also the energetic consequences of collateral damages caused by malaria, such as free hemoglobin and heme utilization, replenishment of destroyed red blood cells (RBCs) and tissue damages, and other related factors.

However, we decided to review this section to simplify the understanding of what we meant:

**Lines 77-89 in revised MS:** Directly measuring the energetic cost of immunity is challenging due to the immune system's complexity. However, assessing metabolic rate can provide valuable insights into the energetic costs associated with immune responses. In particular, measuring resting metabolic rate (RMR) during infection and comparing it with pre-infection baseline levels provides insights into the additional energy required for mounting and sustaining an immune response (Demas and Nelson 2012; Ricklefs et al. 1996). However, measuring the precise metabolic cost of immunity is challenging due to its intricate integration with other physiological systems within the organism (Lochmiller and Deerenberg 2000). That implies that RMR measured in our study reflected not only the immune response itself but also the energetic consequences of collateral damages caused by malaria, such as free hemoglobin and heme utilization, replenishment of destroyed red blood cells (RBCs) and tissue damages, and other related factors (Lochmiller and Deerenberg 2000).

Lines 116 and on. I don’t think this is a good way to present your hypotheses and predictions. Basically, you are covering your bases, so no matter what you find, there will be an explanation. But the approach is experimental, so that cannot be formulated like this. It seems to me that you thought about a mechanistic explanation of the infection, so you just have to follow the consequences of the mechanism. If IL-6 is an indicator of the severity of the infection, what I would predict is a negative association between the levels of IL-6 and RMR, since the more severe the infection is, the less RBCs the birds will have, and oxygen delivery to tissues will be compromised. And also, I would change the statement in the last paragraph. It seems to me that you expect that one parasite is going to affect the birds more than the other, but the mechanism is the same. In summary, you predict that there will be a negative correlation between the levels of IL-6 and RMR, and that the slope (or the trajectory) of the relationship will be larger when the birds are infected with the African parasite.

**Response:** We acknowledge the reviewer's feedback and agree that our initial formulation of hypotheses and predictions could be improved. However, we are uncertain about the specific mechanistic explanation suggested. Currently, there are very few studies measuring the metabolism of birds infected with malaria, and even fewer that measure IL-6. We aimed to summarize all available information on both parameters, but found that existing data is largely contradictory. We attempted to cover all possible options, which resulted in the hypotheses appearing confusing. Following the reviewer's suggestion, we have revised the formulation of our hypotheses. In summary, we decided to adhere to our third hypothesis from the original version of the manuscript, as it encompasses the first two in original manuscript. We also slightly improved and extend it:

**Lines 127-167:** Given the contradictory data on metabolic responses in birds during the parasitic infections (Robar et al. 2011), especially malaria, we cannot definitively predict whether relationship between RMR and parasitemia level will be positive or negative. RMR might increase in all infected juvenile siskins due to the development of an acute immune response against proliferating malaria parasites. This is supported by experiments involving the innate immune challenges in passerine birds (Eraud et al. 2005; Ots et al. 2001). On the other hand, RMR might decrease during the acute stage, characterized by the active destruction of erythrocytes and digestion of hemoglobin (Hb) by multiplying parasites. Progressing anemia could reduce the blood's oxygen-binding capacity, disrupting oxygen transportation. This view is supported by Hayworth et al. (1987) and aligns with the general knowledge of the *Plasmodium* parasite life cycle , although some studies have not confirmed it (Hahn et al. 2018; Stager et al. 2021). Another significant factor that can impact on infected siskins` RMR is that erythrocytes can transport much more oxygen than is required for basic maintenance. The maximum energy that birds can obtain from food and expend over long periods (i.e., without losing body mass) is at least four times the BMR (Gavrilov 2014; Lindström and Kvist 1995). Therefore, at low or moderate levels of parasitemia, the remaining intact erythrocytes can not only support the basal metabolic rate but also handle the additional energy demands associated with immune responses, tissue repair, hematopoiesis, etc.

Given the energy demands associated with acute immune activation during infection, the destructive impact of Plasmodium parasites on oxygen-carrying erythrocytes, and the compensatory mechanisms in affected birds, we predict that resting metabolic rate (RMR) will initially increase in response to rising parasitemia. This increase will continue up to a certain parasitemia threshold, beyond which the remaining healthy erythrocytes will be insufficient to sustain a high RMR. Additionally, the development of anemia and hypoxemia, exacerbated by lactic acidosis, further complicates the situation. As blood pH decreases, oxygen saturation of hemoglobin declines (Rigdon and Rostorfer 1946; Rostorfer and McGee 1946) and at elevated parasitemia levels, glycolysis becomes a critical energy source due to its oxygen-independent nature (Cumnock et al. 2018).

Regarding IL-6, we expect its levels to rise before or at the onset of the acute phase of malaria, and then decrease in birds that survive this crisis and develop a chronic infection. Consequently, we anticipate a positive correlation between IL-6 levels and RMR during the early stages of the disease, which may shift to a negative relationship as parasitemia peaks. Additionally, we expected different average responses to P. relictum SGS1 and P. ashfordi GRW2, with potentially more severe outcomes (possibly higher peak IL-6 and more pronounced change in RMR) for birds infected with the latter. This expectation is based on the distinct co-evolutionary histories of the host and these two parasite species.

**Response (continuation):** We appreciate the reviewer’s feedback. In our study, we intentionally refrained from making explicit predictions about the correlation between RMR and IL-6 due to the limited existing literature and the constraints of our data. We felt that making definitive statements in this area would be premature. Instead, our approach was to cautiously explore and describe potential trends and relationships based on the available evidence. The only thing we assumed was that:

**Lines 120-122 (original manuscript):** Moreover, the intensity of the immune reaction could be higher in birds infected with GRW2 – an evolutionary unfamiliar malarial parasite for European resident bird species like siskins. Consequently, this could lead to elevated RMR levels.

 Line 182. Add “C” to the temperature.

**Response:** changed as suggested.

 Lines 215-218. And non-reproductive.

**Response:** changed as suggested.

Line 227. You kept the animals in captivity at a constant temperature of 23 ℃, which I guess is not within the TNZ?

**Response:** Ambient temperatures in the range of 20-25°C are standard for most experiments involving the aviary housing of small birds. According to Gavrilov (2014) and Gavrilov and Gavrilov (2019), 23°C falls within the thermoneutral zone (TNZ) of the common siskin during both winter and summer periods (see Table 1 in the first paper or Table 2 in the second).

Lines 247-256. It is not clear what are the data you took to calculate oxygen consumption. Was it the average of the last 5 minutes of each trial? You started the measurements at 9pm, and finished at 7am the next day. This means that measurements took 600 minutes. You used 20 minutes per bird and 10 minutes for the reference chambers in between birds, with a total of 120 minutes per cycle, this means a total of 5 cycles. So, you took the data from the last 5 minutes of each cycle, and averaged it for each bird? This means you averaged 25 minutes of data per bird, is that what was done? But if you did that, I don’t think you can call that BMR. To estimate BMR, you should select the cycle with the lowest oxygen consumption, and average the last five minutes only for that cycle

**Response:** We thank the Reviewer for this comment. We have provided a more detailed description of the procedure for determining the minimum VO2:

**Lines 283-290:** The fractional concentrations of O2 were recorded with a sampling interval of 4 seconds. In each of the 20-min measurements of each bird, we discarded the first five min as a wash-out time. To estimate the minimum oxygen consumption of each bird (VO2-min), we used minimum running average procedure (Withers 2001). Using this procedure, for each bird in each cycle, among all possible 5-min average VO2 values, we found the minimum one. Subsequently, among all such VO2-min (their number was equal to the number of cycles), we found the lowest one and used it as an estimate of BMR and RMR.

**Response (continuation):** To calculate BMR (and RMR), we did not take the last 5 minutes of each cycle but used the moving average method. This method allowed us to find the interval with the lowest oxygen consumption among all possible 5-minute intervals over the entire measurement period for each individual (Line 249 in original manuscript). The moving average method is a simple and effective smoothing technique for noise reduction and is a standard method for finding the interval with the lowest oxygen consumption (Withers 2001). It is used in numerous avian studies based on respirometry (Bech et al. 1999; Bouwhuis et al. 2011; Briga and Verhulst 2021; Buttemer et al. 2021; Hahn et al. 2022; Jacobs and McKechnie 2014; Nilsson et al. 2011; Østnes et al. 2001; Pacioni et al. 2024; Rønning et al. 2016; Tieleman et al. 2009; Welcker et al. 2013). For each bird in each cycle, we discarded the first 5 minutes as wash-out time. These 5 minutes were excluded as the time needed to flush the respirometer of air from the previous bird and to reach a new equilibrium in oxygen concentration in the chamber for the bird currently being measured, caused by a slight decrease in flow rate after switching channels to the current bird. Thus, for each cycle, we had 15-minute VO2 data for each individual. Over the course of a night, there were 4-5 cycles. For each cycle, we found the interval with the lowest VO2 among all possible 5-minute intervals. Since we recorded gas concentrations and flow rates every 4 seconds, the moving average window shifted by 4 seconds. Therefore, for each cycle for each bird, there were 150 possible 5-minute intervals (150 = (15-5) \* 60 / 4). Among these 150 intervals, we identified the one with the lowest VO2. Then, among all such 4 or 5 minimum VO2 values (the lowest VO2 values in each cycle), we selected the lowest one.

Sample sizes were 20 per group, right? The four birds measured in every trial were selected at random, I am assuming? What I mean is that you were not measuring birds belonging to the same group each trial. Also, you measured BMR for all the birds, and then RMR one week, more or less, after infection (prepatent period), and then again after more or less 18 days (infection peak), right? So, you have three measurements of MR per bird, a baseline value for BMR and two values of RMR postinfection? This is not clear.

**Response:** We appreciate the reviewer’s comment. We maintained a detailed list that included each bird's individual ring number and group assignment (experimental or control). Testing began with birds from the SGS1 group at the top of the list and proceeded sequentially in groups of four. Once all birds in the list had been tested, the cycle started again. This approach generally ensured that birds from the same group were tested together. However, due to bird mortality, this rule was not always strictly followed, and occasionally birds from different groups were tested simultaneously. The number of RMR measurements for surviving birds ranged from five to six times. We have provided a more detailed description of the process for metabolic measurements into manuscript text:

**Lines 261-267:** Each our experimental group consisted of 20 birds. To avoid overlapping metabolic measurements, we staggered the inoculation procedure across different days for each group. The SGS1 group was inoculated first, followed by the GRW2 group four days later, and the Control group received their injections five days after the GRW2 group. The initial metabolic measurement for the SGS1 group was conducted on the tenth day post-inoculation, and subsequent measurements continued regularly until the end of the experiment.

Lines 259-254. These are results, move this to results.

**Response:** changed as suggested.

Lines 266-275. Did you do baseline IL-6 levels for all the birds pre-infection? Or the values are all after infection, and then you considered the control group as your baseline? Please, clarify.

**Response:** Thank you for your comment. We collected plasma from all of the experimental birds before the inoculation procedure to establish baseline values for each birds` group. We have now included the line in our graphs representing the mean baseline value of RMR or IL-6 for each group (see our revised figures).

− Line 283. Incorporate, not incorporates.

**Response:** changed as suggested.

− Line 301. Dynamics OF parasitemia.

**Response:** changed as suggested.

−       Lines 318-319. Check the parentheses ().

**Response:** In response to the reviewer’s comments, we would like to clarify that using empty parentheses to denote a function is a common practice in R documents. However, we have replaced them with apostrophes to avoid confusion for readers unfamiliar with this practice.

−       Line 329. 1e4? Use superscripts or subscripts?

**Response:** changed as suggested. Я не знаю что с этим делать – вот я вроде поменяла, а АБ обратно все вернул (см текст манускрипта).

−       Lines 340-342, “survived WHEN parasitemia”…

**Response:** changed as suggested.

−       Any ideas of why 25 % of the control birds died?

**Response:** This aspect of our work remains challenging. We addressed this concern in the original manuscript and have now added additional details about the control birds to further clarification.

**Lines 667-676:** Our experiments involved wild birds. Despite implementing quarantine measures for all birds prior to the experiments, we were unable to completely rule out or diagnose concurrent infections that could potentially influence the results. We assume that the observed elevation of IL-6 in some birds from the SGS1 group may indicate the recrudescence of an unidentified chronic disease, which manifested following malaria-induced immune dysregulation. Additionally, this could also explain the mortality observed in the control group, where two out of five birds died on the 3rd and 9th days after inoculation with uninfected donor blood. Although the donor for the control group was not infected with malaria, it is possible that it carried another infection beyond our control.

*статьи, где тоже помирали??*

−       Lines 356-357. The GAMM model with different smoothers was better than…

**Response:** changed as suggested.

−       Line 363. Use “significant” instead of “dramatic”.

**Response:** changed as suggested.

−       Line 366. Use “increase”, instead of “growth”.

**Response:** changed as suggested.

−       Figure 2. What are the units for RMR?

**Response:** Thank you for your observation. We have added units to the graphs accordingly.

−       Line 378. “Was” instead of “were”.

**Response:** changed as suggested.

−       Figure 3. What are the units for the concentration of IL-6?

**Response:** Thank you for your observation. We have added units to the graphs accordingly.

−       Results. I would correlate RMR with parasitemia and IL-6. See prediction above.

**Response:** We have provided a detailed response to this comment above.

−       Lines 485-488. This is a very vague statement, you measured IL-6 and RMR, you can conclude that metabolism was affected, but to say that the parasite “affected substantially the physiological state” of the birds is not very accurate. Again, if you correlate the intensity of the infection with RMR in both experimental groups, you can have a better understanding of how the infection affects metabolic rates and provide a mechanistic explanation of your results. But just to say that the parasite affected the “physiology” of the birds is not satisfactory to me.

**Response:** We would like to clarify that, in the original manuscript, this paragraph does not discuss our results but rather cites published data demonstrating that both parasites from our study potentially can significantly affect the physiological state of siskins. However, we have revised this paragraph to enhance its clarity:

**Lines 538-541:** Given the high levels of parasitemia observed for both *P. relictum* SGS1 and *P. ashfordi* GRW2 in our experiment, we conclude that both parasites are highly virulent and can substantially impact the measured physiological traits of experimental siskins.

−       Lines 505-531. Again, with a correlation between parasitemia and RMR you could have a better idea of what’s going on here.

**Response:** We have provided a detailed response to this comment above.

−       The discussion can be shorter, you go back and forth with IL-6 in the last couple of pages.

**Response: мы благодарим ревьюера за это замечание. Мы сократили эту часть дискуссии (см строки … …) еще не сократила ((((( а точно надо сократить??? Может, не надо… (я не хочу)**

**Reviewer: 2**

Comments to the Author

I have read through the manuscript entitled as ‘Dynamics of resting metabolic rate and innate immune response in malaria-infected Eurasian siskins’. Although the manuscript can provide some very useful information. I think Current Zoology might not a proper outlet for this manuscript. My main concern is that too limited data measured. I suggest the authors at least measure energetic parameters such as maximum metabolic rate and aerobic scope as most of the previous studies did. RMR is a consequence of all kinds of physiological and biochemical processes, and cannot reflects the energy cost of disease without proper control. The MMR and aerobic scope as indicators of metabolic capacity or respiratory capacity might be more proper to be selected as relevant parameters in the present study. I also wonder why the authors only measured the IL-6 levels. With the sampled tissues, they can measure much more relevant parameters. With only two variables, it is difficult to draw any solid conclusion.

**Response:** We agree that RMR is an integrative trait reflecting the energetic contributions of numerous physiological processes. It is precisely for this reason that we used this characteristic, as we were interested in the differences in the total energetic cost of infection by two different species of *Plasmodium*. In this regard, our work is similar to the study by Hahn et al. (2018) who concluded that ‘BMR and RMR are pertinent reflections of the energetic costs of physiological processes at particular life-history stages and, thus, can be used to quantify the contemporaneous energy costs of parasite infections.’ Given that the number of functioning oxygen carriers (erythrocytes) decreases as the disease progresses, we hypothesized that the relationship between parasitemia levels and RMR could be complex. Early in the disease, anemia might not lead to a significant decrease in RMR; on the contrary, RMR should increase, reflecting the energy costs of the immune response, hematopoiesis, tissue repair, and so on.

We did not measure the maximum metabolic rate for several reasons. There are two characteristics of maximum metabolic rate. The first is the maximum oxygen consumption rate (MMR) during intense physical exercise (e.g., in a wind tunnel or hop-flutter wheel). The second is the maximum thermogenic rate, previously also called MMR (or peak metabolic rate, PMR), but now commonly referred to as summit metabolic rate (Msum). Both characteristics have been used in studies assessing the maximum energy expenditure in malaria-infected birds (MMR in Hahn et al., 2018, and Msum in Stager et al., 2021). The main reason we did not assess the maximum rate of thermogenesis or maximum locomotor performance in infected birds is that we were interested in the energy expenditure for maintenance rather than maximum capabilities (maximum performance). We believe that the effect of parasitemia on MMR is more predictable and less interesting: since malaria causes some erythrocytes to lose their oxygen-carrying capacity, we would likely have found a negative relationship between MMR and parasitemia. In any case, studying the effect of malaria on the peak capabilities of birds (both thermoregulatory and locomotor) was beyond the scope of our research.

Another reason we avoided measuring MMR or Msum is the significant impact of the experimental procedure itself on the physiological state of the birds. Compared to the measurement of RMR (where the most stressful impact is handling when placing the bird in the metabolic chamber), measuring any of these maximum metabolic rates is highly stressful. This is especially true for Msum, as the measurement process ends when the bird becomes hypothermic. Our experience in measuring MMR in various free-living bird species shows that even in healthy birds, MMR measurement causes weight loss and a noticeable decrease in breast muscle score, after which birds need several days to recover. Since to obtain a dynamic of metabolic rate, we would need to measure MMR in the same individuals (including sick ones) quite frequently, and also take significant blood volumes, such procedures would inevitably affect the dynamics of our primary traits of interest - RMR and body mass. With the required measurement frequency, the birds would not have time to recover from the previous measurement (especially concerning Msum) before the next measurement. Thus, the severity of the MMR measurement procedure itself would affect subsequent MMR and RMR values. Specifically, in our experience, severe cooling of birds in an atmosphere of an oxygen-helium mixture during Msum measurement leads to a significant increase in their BMR after several days (apparently, this is a manifestation of the acclimation effect on BMR, a characteristic with high phenotypic plasticity). Finally, the use of two highly stressful procedures (blood sampling and MMR measurement) raises ethical concerns, as they would inevitably increase mortality among the experimental birds.

I think Current Zoology might not a proper outlet for this manuscript.

**Response:** Formally, the article aligns with the journal’s format. A search for ‘*Plasmodium’* on the journal’s website reveals numerous articles on the topic, including many specialized ones. Similarly, the journal features articles on BMR.

My main concern is that too limited data measured; I also wonder why the authors only measured the IL-6 levels. With the sampled tissues, they can measure much more relevant parameters.

**Response:** We do not believe that the data we obtained is insufficient. However, we would have appreciated the opportunity to gather more details. As it is often the case, we were limited by financial and time resources. Additionally, the only tissue available to us were the remaining RBC precipitates left after plasma separation, as we could not draw more fresh blood from the experimental birds.

I suggest the authors at least measure energetic parameters such as maximum metabolic rate and aerobic scope as most of the previous studies did.

**Response:** Aerobic scope represents an animal’s capacity to increase its aerobic metabolic rate above maintenance levels, defined MMR/BMR or MMR - BMR (Bishop 1999). Since it includes measurements of MMR, the same restrictions discussed earlier apply to this parameter.

**Reviewer: 3**

Comments to the Author

Thank for your manuscript, I enjoyed reading it. Best of luck on your research. Attached you will find a document with all my corrections. My two biggest concerns was:

1) the way the current manuscript is written gives a narrow understanding of basal metabolic rate measurements and standardization. I have provided references to help.

2) the majority of the discussion and argument that is built to support the hypothesis is that birds infected with parasites have higher MR due to erythrocyte destruction however no blood parameters were measured in this study and offer no support to this line of logic. Therefore the argument needs to be reframed as an increase in MR can be tied to an immune response activation as measured by interleukin levels. That would be as stronger argument (still weaker consider only one immune response activator was measured). I think your work has scientific merit it just needs work to uncover the significance. All the best.

**Response:** Thank you for your feedback. We did evaluate the percentage of destroyed red blood cells, which is indirectly measured through parasitemia. However, we do not believe that the relationship between metabolic rate (MR) and parasitemia should be positive. Instead, our predictions and results suggest that this relationship is more complex (see **lines 148-158 in revised manuscript**).

Review for CZ-2024-0085

**Abstract**:

Overall impression is a solid abstract that introduces the topics and does discuss the results and important findings of the study.

Minor correction: Line 26 change from energy cost to energetic cost

**Response:** changed as suggested.

**Introduction:**

Overall solid introduction and does a good job introducing the relevant literature.

Minor correction: Line 39 change from breeding to breeding sites.

**Response:** changed as suggested.

Minor correction: Line 81 this sentence should have a reference at the end 10

**Response:**  Thank you for your comment. We have added a references to this sentence as suggested:

**Lines 85-89:** That implies that RMR measured in our study reflected not only the immune response itself but also the energetic consequences of collateral damages caused by malaria, such as free hemoglobin and heme utilization, replenishment of destroyed red blood cells (RBCs) and tissue damages, and other related factors (Lochmiller and Deerenberg 2000; Sun et al. 2020).

Minor correction: Line 101 change from depending both on to depending on both

**Response:** changed as suggested.

Minor correction: Line 107 change from this lineage to the SGS1 lineage (for better specificity)

**Response:** changed as suggested.

Minor correction: Line 114 add a coma to the sentence- …to its natural host, the great reed warbler (), and to Eurasian siskins.

**Response:** changed as suggested.

Major correction: Line 118-132: This entire sentence is one paragraph and can be confusing to read, especially when this paragraph stops and it transitions to the next topic. I recommend stating there are three possible variants in RMR of infected siskins: I) increase in RMR, II) decrease in RMR, and III) changes in RMR correlate with infection levels. Then following that statement have a sentence that explains why each of those variants are possible. RMR might increase with infection because immune response ….(references). RMR might decrease with infection because…(references). RMR might correlate to infection level because…(references).

**Response:** We deeply appreciate the reviewer's constructive recommendations. In response to the feedback from two reviewers regarding the formulation of our hypotheses, we have revised them to address the concerns and enhance clarity (see **Lines 127-167 in revised manuscript**)

**Materials and Methods:**

Overall the methods are okay and pretty lengthy in places (especially the statistics section) but also lacking on some important details in regards to measuring metabolic rate (see below).

Minor correction: Line 191-197 move to either the top of the material and method section, so it is the first thing started or move to the end of the material method section. Stuck in the middle, it disrupts the flow of the text and feels out of place.

**Response:** Thank you for the helpful advice. We have moved this part to the end of the Materials and Methods section.

Minor correction: Line 232 do not use conjunctions in formal writing. Change to would not

**Response:** Thank you for the very useful advice. We have revised the sentence accordingly.

**Lines 243-245:** The air pumps were connected to the uninterruptible power supply system to prevent suffocation of the birds in chambers while possible power outages.

Minor correction: Lines 259-264 remove and place in the results section

**Response:** changed as suggested.

Minor correction: Line 280, add that because the data set was non-parametric (which assumptions did it fail, normality or unequal variances or both) a GAMM was chosen rather than the parametric GLM.

**Response:** Thank you for your insightful comment. The dataset was indeed non-parametric, failing both the normality and equal variance assumptions. Because of these violations, we opted to use a Generalized Additive Mixed Model (GAMM) instead of a parametric Generalized Linear Model (GLM). The flexibility of GAMM allowed us to better model the non-linear relationships and handle the repeated measures for each bird effectively. We have now clarified this in the manuscript:

**Lines 314-317:** Due to the non-linear nature of our dataset, which violated both normality and equal variance assumptions, we opted to use a Generalized Additive Mixed Model (GAMM). The GAMM approach allowed us to appropriately model the non-linear relationships and handle the repeated measures for each bird throughout the experiment.

Question: Line 212 how long before the metabolic reading were the birds fasted? BMR needs to be conducted under fasting conditions and to avoid the effects of specific dynamic action. The following reference is very handy for explaining all the criteria for reporting metabolic studies event though the reference focuses on aquatic, ectothermic animals, it is still relevant here. “Guidelines for reporting methods to estimate metabolic rates by aquatic intermittent flow respirometry. J Exp Biol 224(18): jeb242522. doi:10.1242/jeb.242522.”

**Response:** Thank you for your comment. We have expanded the description of the conditions for measuring metabolic rates in the manuscript:

**Lines 232-237:** We designate it as BMR because it fulfills all the criteria for BMR measurement (McNab 1997). This trait represents the minimal energetic metabolism necessary to maintain normothermia in a resting inactive nonreproductive adult endotherm in the postabsorptive state, measured during the ρ-phase (for day-active species it is nighttime) and within the thermoneutral zone of ambient temperatures (McNab 1997).

And:

**Lines 259-250:** Birds were deprived of access to food for at least two hours before experiments to ensure that they were in a post-absorptive state during metabolic rate measurements.

**Response (continuation):** Additionally, the effect of specific dynamic action (SDA) is relatively weak for carbohydrate digestion (see references in Karasov and del Rio (2007)). While we could not find information on the duration of SDA in any granivorous passerines, SDA lasts only 1.1 hours in a small insectivorous bird (the wren), 1.4 hours in a large piscivorous bird (the guillemot), and 1.7 hours in a grain-fed mallard (Secor 2009).

In any case, SDA is not an issue in our study, as we estimate the oxygen consumption level throughout the night. The effect of SDA always results in an increased oxygen consumption rate, so the minimum moving average algorithm simply does not select the VO2 interval during the SDA effect as the minimum interval. The criterion of the postabsorptive state is crucial for short-term RMR measurements when there is a risk that the animal has not fully digested and absorbed the food. In our case, measurements continued throughout the night (until dawn the next day), far longer than the duration of the SDA effect. Our experience shows that even if small passerine birds enter the respirometer without food deprivation, the minimal stable part of the oxygen consumption curve is still observed in the middle of the night, coinciding with the time when it is observed in birds that had prior food deprivation.

Major Correction: Line 220 Basal and Resting metabolic rate are not the right terms to use here. Basal metabolic rate (prior to inoculation) is: fasted, thermoneutral zone, rested, restrained, in a stimulus free/darkened environment, and in a healthy state. Some of the criteria are not discussed in the method section, so the more appropriate term would be resting metabolic rate.

**Response:** Thank you for your comment. Previously, to reduce the manuscript's length, we did not list all seven conditions for measuring BMR, instead referencing a publication that discusses these conditions in detail (McNab 1997). We have now edited the manuscript to include all the necessary conditions, clarifying that our VO2 measurement conditions fully met the strictest BMR measurement standards (see our response above).

(Discussions on the redundancy of some of these conditions and possible relaxations are provided in (Ellis and Gabrielsen 2019)).

Then taking the measurements after infection would not be referred to as resting metabolic rate because they are not healthy. The best course of action would be to change what you refer to as basal metabolic rate to MRmin minimum metabolic rate and change what you refer to as resting metabolic rate to MRPI post-inoculation metabolic rate. Resting and basal metabolic rate have very specific and defined criteria in order to be labeled as such.

**Response:** We believe the term RMR is standard in such cases and much more familiar to readers compared to MRPI or other designations. The term RMR is currently used extensively, as measuring RMR requires much less stringent conditions compared to SMR or BMR. In fact, of all the BMR measurement conditions, only one remains for RMR measurement - the animal must be at rest. Thus, RMR is measured in growing animals (e.g., chicks or molting birds (Bech and Østnes 1999; Eichhorn et al. 2019; Moe et al. 2004; Weathers and Sullivan 1991), in animals during the reproductive period (e.g., female birds during oogenesis (Nilsson and Råberg 2001; Vézina and Williams 2005), during the active period of the species (e.g., during the day for diurnal birds (Eichhorn et al. 2019; Wolf and Walsberg 1996), not in the post-absorptive state (Brzorad et al. 2021; Careau et al. 2015; Weathers and Sullivan 1991), at ambient temperatures outside the TNZ (Andreasson et al. 2023; Cabello-Vergel et al. 2022; O’Connor et al. 2017), and so on. It is not surprising that we could not find a single definition of RMR that specified the measured animal must be healthy. Moreover, the term RMR is commonly used in studies on energy expenditure in infected animals, including those infected with various parasites (Delahay et al. 1995; Devevey et al. 2008; Magnanou et al. 2006; Robar et al. 2011; Sun et al. 2020), including blood parasites (Hahn et al. 2018; Schall 1990; Stager et al. 2021).

The introduction to the work by Hahn et al. (2018), which addresses the energetic consequences of malaria infection, outlines the distinction between BMR and RMR terms, which fully aligns with their interpretation in our study: “Endothermic animals have a basal, or minimal, rate of metabolism (basal metabolic rate; BMR) when they are post-absorptive, asleep in the rest-phase of their daily cycle, exposed to thermoneutral temperatures, and not engaged in energetically demanding life-history stages. Thus, the BMR represents an individual's baseline costs of maintaining vital functions. Metabolic measurements made under the same conditions but at times associated with elevated maintenance costs, such as during moult, during the day, or at temperatures outside the thermoneutral zone are termed RMR.”

To clarify the term RMR we extended the description of its application in our study:

**Lines 237-243:** The term RMR is currently used extensively, as measuring RMR requires much less stringent conditions compared to BMR: the only necessary condition is that the animal must be at rest. The term RMR is commonly used in studies on energy expenditure in infected animals, including those infected with various parasites (Delahay et al. 1995; Devevey et al. 2008; Magnanou et al. 2006; Robar et al. 2011; Sun et al. 2020), including blood parasites (Hahn et al. 2018; Schall 1990; Stager et al. 2021).

Question: Line 224 how long where the birds allowed to habituate to the chambers and recover from handling stress before you took their minimum metabolic reading? Most studies recommend approximately 4-8 hours but it depends on the species.

**Response:** Habituating animals to metabolic chambers is primarily used in studies with small mammals, especially rodents. These animals exhibit sporadic activity and lack long periods of rest, making it much more challenging to assess BMR compared to birds. In rodent studies, animals are often introduced to the chambers several days before the metabolic measurements (see, for example, Devevey et al. (2008)). Birds, especially small ones, have very distinct daily rhythms. Small birds settle down in the dark during their rest period very quickly, making it standard practice to start recording the O2 consumption soon after placing them in metabolic chambers. Based on the VO2 graphs, our siskins also settled quickly in the metabolic chambers (within half an hour), and their VO2 levels stabilized rapidly. This quick settling time might be partly due to the siskins living in cages for an average of 25 days before the first BMR measurement. The VO2 measurement procedure was then regularly repeated, so they became accustomed to it (and to the metabolic chambers).

Anyway, exactly as in the case with SDA, it does not really matter whether the recovery period was excluded from the data or not, because we used prolonged measurements (lasting longer than the recovery period), and handling stress always increases VO2. BMR (or RMR), on the other hand, is the minimum part of the oxygen consumption curve, so the moving average algorithm will not use the period when the bird is still under handling stress as an estimate of VO2min.

**Results**:

Overall results discuss the trends of the data but does not provide actual numbers in text for MR or interleukin levels.

**Response: ???**

Question: Line 339 So if the GRW2 group exceeded 6 days, what was the actual number of days for the prepatent period?

**Response:** We are unable to determine the exact duration of the prepatent period for the group of birds infected with GRW2 (as well, as with SGS1), as blood samples were collected only every sixth day. Therefore, we can only estimate that the prepatent period falls between the 6th and 12th day post-inoculation. On the 6th day for the GRW2 group, parasitemia was undetectable in 6 out of 20 birds, and among the remaining birds, parasitemia levels were below 0.1, with an average of 0.03 accustomed.

Question: Line 343 why was there mortality in the control group?? In this typical?

**Response:** <https://www.youtube.com/watch?v=pBUs2R9JV5M>

Question: Line 362-364 is there data/figure that support this statement?

**Response:** We have included graphs in the manuscript where the time periods during which the difference between smoothers is significantly different from zero are marked by black rectangles (see Methods for details).

Этого ему достаточно? Просто отослать его к методам и материалам?

Lines 332-344: To assess the significant differences between our GAMMs for each studied physiological parameter, we employed a pairwise comparison of the estimated smoothers and their associated confidence intervals (CI). This approach enabled us to compare trends across different factor levels for all pairs of treatment groups over time. By examining the difference between trends, we could determine if any observed discrepancies were statistically significant. If the difference between trends for compared groups is negligible, it will be indistinguishable from zero, suggesting that the treatment (infection with one of the two lineages of parasites and the control group) did not induce a discernible change in response within either compared group. In contrast, when the shifts in trends occur, the CI excludes zero, and this allows us to draw conclusions about the differences between the groups without the necessity of calculating the P-value. For a more detailed description of the applied method see Mundo et al. (2022) and Simpson (2017).

Minor correction: Consider adding a table and discussing that table in the results over the mass and sex of the birds used in the experiment. both factors can impact MR and therefore need to be reported

**Response:** we have attached the Excel table, which includes all the information on experiment, including sex and body mass. А мы же проверяли их влияние на модели - оно вроде не значимо

Major correction: Consider adding a figure that compares the just BMR reading prior to infection and the RMR post infection. This way the readers can determine if infection had an effect on metabolic rate. Rather than a figure showing continuous MR readings post infection over the course of a month. Obviously there is a difference between control and experimental birds post injection, but what about their MR prior to injection, did it follow a similar pattern over the course of a month. MR is highly variable day in and day out. It would be good to see this comparison.

**Response:** Thank you for your comment. Our experimental design involved a single measurement of BMR, and we did not intend to estimate potential BMR dynamics prior to the experimental procedures.

**Discussion**:

Minor correction: Line 433 the references refers to basal metabolic rate and not resting metabolic rate, which again are not interchangeable and have specific definitions.

**Response:** Thank you for your comment. See **Lines 237-243** and our previous response regarding the usage the RMR term (page 21). We acknowledge that the measurement of Basal Metabolic Rate (BMR) in infected birds is not feasible, as BMR is defined as the metabolic rate of a resting animal in a post-absorptive state, typically measured under ideal conditions of thermal neutrality and complete rest. In contrast, the resting metabolic rate (RMR) is a practical measure used in our study as it reflects the energy expenditure of birds under variable conditions, including the effects of infection, the activation of immune responses, and associated pathological processes.

Minor correction: Line 466 add a s to the end of parasite

**Response:** changed as suggested

Minor correction: Line 487 Restate the sentence to state that both parasite strains differentially altered the physiological state of siskins after infection.

**Response:** Thank you for your comment. We would like to respectfully disagree. We are referring to the research by Videvall et al. (2020), which demonstrated that the functions of the expressed genes in birds infected with SGS1 and GRW2 are similar. In the previous study (Videvall et al. 2015) the author showed that the highly virulent SGS1 causes a more significant transcriptome response compared to the low-virulent GRW4. Given this information, along with the observation that both parasites in our study exhibited high parasitemia values, we conclude that both parasites can indeed have a significant impact on the physiology of the experimental birds.

However, we made a few adjustments to the sentence, and we hope it is now clearer and easier to understand what we intended to convey:

**Lines 546-549:** According to this data and given the high levels of parasitemia observed for both *P. relictum* SGS1 and *P. ashfordi* GRW2 in our experiment, we conclude that both parasites are highly virulent and can substantially impact the measured physiological traits of experimental siskins.

Minor correction: Line 498 change to the higher the parasitemia the more detrimental impact it has on the bird’s health and would impact metabolic rate.

**Response:** changed as suggested.

Major correction: Lines 489-531 (approximate) A big portion of the argument that the writers make is that increased MR is due to erythrocyte destruction…but they did not study erythrocyte damage from infection. The authors should build their argument about what MR is increased with infection, like the energetic cost of the immune system to fight off active infection. As the discussion stands now, your argument and hypothesis that increased MR is due to erythrocyte destruction is unsupported with your current dataset. Focus instead on interleukin levels and immune response activation, which would be weak considering you only measured one immune activator and not a few others to get a more complete picture.

**Response:** We respectfully disagree with the reviewer's interpretation. The **lines** **531-571** (489-531 in original manuscript) attempt to explain the observed changes in the resting metabolism of siskins by highlighting a synchronicity with changes in the parasitemia curve. However, there is no assertion in the original manuscript that the increase in resting metabolism in birds infected with GRW2 is associated with the destruction of erythrocytes, as this would contradict our previous reasoning (lines 123-126 in original manuscript). Following the reviewers' advice on refining our hypothesis, we have merged, developed, and enhanced them into a single, unified statement. This revised hypothesis suggests that the increase in resting metabolic rate possible in birds as a result of immune response activation do not contradict with the fact that *Plasmodium* parasite destroys red blood cells to a certain level of parasitemia, as explained in our earlier comments ()

As stated in the manuscript (**lines 545-546**), for birds infected with GRW2 (unlike those infected with SGS1, where the decrease in resting metabolic rate (RMR) coincides with the acute phase) we observed an increase in resting metabolism that coincides with the onset of active parasite multiplication. We have proposed two possible explanations for this (lines 546-670), in brief:

a) The number of destroyed erythrocytes may not have been sufficient to significantly impair oxygen transport, allowing the birds to increase their metabolic rate. This is consistent with observations in immunization or infection studies across various animal species, where increased metabolic rates are often seen in response to immune activation (referencing our earlier discussion about the “safety margin” of metabolism in birds).

b) A more intense immune response to an evolutionarily unfamiliar species of malaria parasite.

We acknowledge that we do not have sufficient data to establish a connection between RMR and birds` immune response, but this was not our primary objective. Our work aims to approach the complex issue of the immune response mechanism of birds to malaria parasite. A more in-depth exploration of this intricate issue is a task for future research.

We believe we have been quite careful in explaining our findings. However, we have added a few sentences to further clarify our points and enhance understanding:

**Lines 563-570:** These two explanations do not contradict each other and may indeed be interrelated. It is possible that we are observing the effect hypothesized initially – that up to a certain level of parasitemia, healthy red blood cells can maintain the basal metabolic rate and even allow the bird to increase oxygen consumption to mount an immune response and fight the parasite. While our current data do not allow us to state this with certainty, future studies should aim to compare oxygen consumption levels and various indices of immune response among birds infected with the same parasite but exhibiting different parasitemia levels.

Minor correction: Line 562 do you mean the author of the previous reference? Or the author of the current manuscript? This is confusing since you did not introduce interleukin 15 from the previous reference or study interleukin 15, but you measured interleukin 6. More clarification is needed.

**Response:** Thank you for pointing that out. We were referring to the work of the author mentioned in the previous sentence. We have revised the text for clarity to reflect this:

**Lines 608-613:** Our observations appear to be somewhat similar to the findings from Henao's 2019 master's degree project, which also studied siskins infected with *Plasmodium relictum* SGS1. Henao's research demonstrated that the expression level of IL-15 on Day 8 post-infection (DPI) was negatively correlated with the rapidly increasing parasitemia levels. Specifically, birds with high parasitemia expressed less IL-15, approaching the expression levels observed in uninfected birds.

Table and Figures: You add the tables 1-3 twice Figure 2: missing RMR units Figure 3: missing interleukin 6 concentration units

**Response:** Thank you for your comment. We have removed the extraneous tables and added units to the graph as suggested.

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