Reviewer: 1  
  
Comments to the Author  
in their ms, the authors provide experimental evidence for changes in metabolic rates and innate immune response in naïve juvenile siskins when being infected with avian malaria. The study adds important facts how resting oxygen consumption in birds is affected by haemosporidian parasites from the transmission to the chronic stage of infection. Such data are widely lacking but fundamental for the understanding how malaria in general and parasite lineages in particular can affect wildlife.  
The study is well designed, and the statistical analysis is sound. However, the ms may improve by a stronger focus and a more concise style in many sections (esp. the discussion).

I personally was happy to see the very similar pattern but different strength of RMR dynamics during the infection. This similarity is one of the most important outcomes of the study, and I would put more emphasis on this nice result; whereas the comparison of var. RMR between the two lineages might be more expectable due to specific virulence.

BMR/RMR: in my opinion the authors measured RMR in all phases of the experiment, and not BMR at DPI<0. Birds were captured during autumn migration and thus in migration physiological stage, which already differs from the basal conditions needed for a BMR (migration induced elevated hemoglobin concentration, increased enzyme activity, and fat and muscle accumulation).

I would like to see some additional information about the sex and size (wing length) of the individuals and the physiological state at the beginning and at the end of the experiment, i.e. body mass, hemoglobin concentration, fat and muscle scores, and finally the date of death (DPI) during the infection experiment (as symbols in fig 1A).  This is not only important for the study itself but also allows to compare the experimental data with other studies of similar species and/or different stages within the annual cycle.

Нужно описать динамику веса! Хотя она и включена в модель.

RMR repeatability: the topic should be removed from the current manuscript (its already mentioned that this will be a core topic of another ms in future (l. 524).

Согласен!

Some additional comments:  
Abstract: I would prefer to read an abstract in its classical form covering an introduction into the topic, few words about the method and then detailed results and their embedding into the wider subject.     
Method section: please reorder the paragraphs with experimental design before DNA sampling and PCR methods.

Fig 1 AB: please change the current arrangement in stacking A above B and allow for widening the x axis to guide the reader towards the temporal changes of parasitemia.

Техническая правка!

Fig 2 and 3:  B the lower figures -> diff RMR/IL6 of SGS1 and GRW2 can be removed, the data can be presented in the result section only.

Не согласен! Эта картинка нужна!

Why is SGS1 line in red (A)? What does the red background grid (A) symbolize? Peak parasitemia period?

Это же было в подписи к рисунку!

References in text and reference list: please check if all statements match the results in the reference (ie l 417 Videvall 2017 did not provide parasitemia measures, and Videvall 2015 used ‘decreasing parasitemia’ at 31dpi as end point but not chronic infection stage). The reference list needs an update.  
  
  
Reviewer: 2  
  
Comments to the Author  
Summary:  
The authors sought out to test for energetic costs associated with experimental Plasmodium infection, interpreted through resting metabolic rate. The authors further inquired whether this (a) differed among Plasmodium lineages that differ in coevolutionary history, (b) if RMR explained by parasitaemia during infection, and (c) if RMR is explained by immune function during infection. Although these are very informative questions to answer to understand how wild birds respond to Plasmodium infection, I have significant/major concerns with the methods and interpretation of their statistics that impede with the interpretation of the data. I do not have experience conducting respirometry experiments, so I hope the other reviewer can speak to this aspect of the methodological design.  
  
1.      The use of IL-6.  
a.      Authors “assume” (line 138) that IL-6 is a marker of immune response. Please specify which immune response is assumed here. The use of IL-6 as a marker of immune investment needs to be rationalized/validated because IL-6 also stimulates the process to produce red blood cells (haematopoiesis). In a disease that targets and destroys RBCs, IL-6 concentrations could be interpreted as an index of haematopoiesis just as well as immunity. How will the authors disentangle these possibly correlated interpretations of IL-6 concentrations (immune function nor haematocrit were quantified).

А что вообще известно про ИЛ у птиц? Он как у человека? Или тут что-то другое.

b.      Method of IL-6 ELISA with respect to blood sampling of siskins and validation.  
i.      A validation for use of this assay in wild siskins is not provided. This is important because the structure of the IL-6 protein may differ in selectively bred domestic poultry vs wild birds that are under selection against pathogen, so sensitivity of the assay may differ in these species. Please provide a validation with evidence of parallelism.

Нужен анализ литературы про ИЛ и прочий иммунитет у птиц.

ii.     Report the intra-and inter-variation of the ELISA plates.  
iii.    Sample replicates: were samples run in replicate as the manufacturer recommends? Please state as such.

А как вообще устроен анализ на ИЛ? Если там действительно должны быть замеры у одной птицы по несколько раз, то это важно! Если в файле с данными приведены средние по нескольким повторностям замеров в один DPI, то лучше бы использовать первичку и сделать nested random effect.

Да и вообще рецензент прав, если тест-система предназанчена для цыплят, то почему она годится для чижей. Кроме того, что известно про аллельный полиморфизм у ИЛ? Антитела из тест-системы могут не работать или работать плохо на других аллелях.

iv.     Volume of blood required to run the ELISA: 200ul of plasma is required to meet the 100ul per well per manufacturer’s instructions (found here: [https://www.cusabio.com/ELISA-Kit/Chicken-Interleukin-6IL-6-ELISA-Kit-84590.html#a01](https://www.cusabio.com/ELISA-Kit/Chicken-Interleukin-6IL-6-ELISA-Kit-84590.html" \l "a01" \t "https://mail.rambler.ru/folder/INBOX/95332/_blank); no dilution of plasma is called for in this manual). 400ul of blood would be required to get 200ul of plasma to run this assay in duplicate (based on 50% haematocrit). This is a lot to take every 6 days from a small songbird, which leads to concern #2.  
  
2.      Ethical concerns of blood sampling.  
a.      IL-6 ELISA plasma requires 400ul of blood to get 200ul of plasma (see concern 1biv), and that is 20-58% of a siskin’s entire blood volume (based on 12-18g masses and 6-12% of their mass as blood). Authors state they followed the “current laws of Russia”, but no reference was provided. I consulted the The Ornithological Council’s “Guidelines to the Use of Wild Birds in Research” (see  
[https://www.research.ucsb.edu/sites/default/files/policies/iacuc/SS\_WildBirds.pdf](https://www.research.ucsb.edu/sites/default/files/policies/iacuc/SS_WildBirds.pdf" \t "https://mail.rambler.ru/folder/INBOX/95332/_blank)), that states no more than 1% of a bird’s body mass may be collected at any one time; no more than 2% in 14 days. Authors sampled every 6 days, so the 2% rule applies. Siskins range 12-18g, so 120-180ul of blood can be collected at the 2% limit, which equals 60-90ul of plasma. This is not enough plasma to run a single well of the IL-6 ELISA and does not include the volume of blood that was used to make blood smears to assess parasitaemia. Thus, as the manuscript is written, the authors have violated the Guidelines for using wild birds in research.

Сильный ход! Тут не очень ясно чем крыть.

b.      Interpretation of data. If excessive volumes of blood were collected, this is a concern for how to interpret the results of this experiment. Excessive losses of RBCs are an insult to injury for a malaria infection that destroys RBCs; the applicability of the results from birds that experience this volume of blood loss during malaria infection might not be applicable to any other bird with a malaria infection. Moreover, considering the predictions of this study state that RBC loss by the parasite could affect BMR & RMR, I worry that the blood draws may also bias the respirometry results.

Тоже справедливо! НО! Можно попробовать в качестве ковариаты вставить суммарную потерю крови. Она правда будет коллинеарна с DPI. Ну или нужны какие-то внешние данные, согласно которым потеря крови не является причиной изменения ИЛ и RMR.   
  
3.      Concerns with interpretations of statistical analyses.  
a.      The number of models and how they are structured was not clear to me. I could not identify the dependent and independent variables for each model. Line 299 states “For each response variable…”, but the response variables have not explicitly been stated. Ideally, the link between each model and how it tests the hypothesis would be clear, i.e., “In order to test the hypothesis/prediction that … we used a … model with … as the independent variable and … as dependent variables.”

Согласен! Это несложно сделать. Надо вставить формальное описание моделей. НО! Для человека, который понимает почему для анализа паразитемии используется, негативный бином это выглядит как подколка.

b.      The authors state “statistical significance” beyond its restrictions.  
i.      Lineages GRW2 & SGS1 are not explicitly contrasted for RMR and IL-6 (Tables 2 & 3), but there are multiple instances of inferring differences between these groups. The models for these tables contrast controls to GRW2 and controls to SGS1, but not GRW2 and SGS1.

Это не так! Таблицы 2 и 3 показывают лишь формальное описание моделей, то есть оценки значимости коэффициентов и смузеров. Это не таблицы, которые позволяют формально сравнить тритменты друг с другом. Теоретически это можно сделать, но это будет не корректно, так как смузеры для DPI для разных тритментов разные (грубо говоря для одного DPI различия между тритментами одни, а для другого DPI они другие). Это аналог проблемы сравнения тритментов при взаимодействии факторов. Если бы не было взаимодействия между DPI и Линией, то мы могли бы сравнить линии друг с другом и с контролем. А в нашей ситуации нет! Вместо этого мы приводим рисунки, где рассматривается смузер РАЗНИЦЫ между всеми парами тритентов. Мы принимаем за значимые те различия, где 0 выходит за пределы CI разницы в соответствующий DPI. Это надо описать не только в материалах и методах, но и в обсуждении тоже. В обсуждении надо сказать, что прямое статистическое сравнение RMR (и ИЛ) между линиями невозможно из-за наличия взаимодействия.

ii.     The authors repeatedly state ‘significantly higher/lower’ for specific timepoints when they describe the trends in the data for parasitaemia, RMR, and IL-6, but the terms in the model do not allow for these specific interpretations. E.g., parasitaemia was not statistically different for parasitaemia between lineages, but authors state significantly higher/lower parasite loads on line 343. This is further interpreted in the discussion as such lines 407 onward yet the authors present no evidence that the long prepatent period is significantly longer for one lineage or the other – these are subjective interpretations by the authors. Statistically test for these differences if such statements are to be made.

Это все про то же! Как и в случае с взаимодействиями мы НЕ МОЖЕМ сравнивать main effects. Возможны лишь «локальные» сравнения. В случае с GAM это делается вот так, как мы сделали.

“The highest point” or “the biggest differences between X & Y occurred during DPIs”, but stating significantly different means for specific timepoints is beyond the statistical analysis completed.

На основе построенной модели мы построили модель, описывающую поведение РАЗНИЦЫ смузеров. Если она выходит в минус, то то, из чего вычитают, оказывается меньше, чем то что вычитают. Короче, дядя не въехал. Значить надо более подробно расписать метод.

To reconcile this, I recommend that the authors split their data by biologically relevant timepoints of infection: acute and recovery. This can be done by including ‘timepoint’ as a factor in the analysis or having separate models for each infection stage.

Он предлагает построить более дорогую и более произвольную модель. Разделение на три фазы будет достаточно произвольным. Кроме того, в модели точно будет взаимодействие факторов Линия и Период. Это возможный ход! НО! Зачем? GAM для того и придумали, чтобы решать подобные задачи. По-моему, дядя знает про линейные модели достаточно, но не знает ничего про GAM.

И уж тем более не надо делать «separate models for each infection stage» это уж совсем не правильно.

Additional Comments  
Line 187 – Were these 5 birds ‘amplifiers’ of infection (their blood to be used in a subsequent passage) or are these experimental birds? This is not clear as written.

А действительно, это как? Не надо ли источник инфекции, если птиц было несколько, рассматривать, как фактор в модели.

Please specify the number of days post-inoculation that amplifier blood was used for experimental inoculations, whether or not blood was pooled from amplifiers of the same lineage, and what the average parasitaemia was in the inoculum for each lineage.

Это хороший вопрос! Надо бы убедить всех, что кровь о разных птиц но с одной линией - это одно и то же. Хотя бы надо проверить связь остатков с этим фактором. Или кровь пулировали?

Explicitly state the number of experimental birds that received inoculation with SGS1, with GRW2, and the number of controls.

Да!

Line 195 – Explicitly state whether this blood collection occurred for amplifiers, or only the experimental birds.

Согласен!

Line 231 – ‘Each day’ is vague. State when measurements began as ‘days pre-inoculation’ and clarify whether this occurred every day post-inoculation for each bird.  
Were birds acclimated to the respirometry chambers prior to the measurement of BMR? Was BMR an average of multiple nights of this acclimation? Without acclimation, how can the authors rationalize that this baseline isn't affected by aversion to the respirometry chambers?

??? Не понял про что это он....

Rationale for placing 4 birds at once into the chambers? Was there a separate chamber for each bird? This isn't clear as written.

Надо дописать, чтобы неспециалисты поняли!

Line 335 – As confirmation of the experimental design, please state the parasite load of the control group during the peaks for the other experimental groups (day 18). Why did 5 control birds die? How does this limit the interpretation of mortality for the experimental groups?

Кстати, неплохо было бы описать процедуру распределеиня птиц по тритментам. И, действительно, почему в контроле такой падеж? Стояли ли клетки с разными тритментами в одном помещении?

Line 337 – ‘Species’ can be the host or the parasite; please clarify.  
  
Positive Feedback  
Predictions were all well rationalized and the authors provided sufficient background information of the question, the study system, and rationale for the lineages in this study. I liked that the authors defined BMR & RMR for their study becuase I am not an expert here. Good choice of negative binomial models for parasite count data.

Ну прям как в том анекдоте :)

- Есть у вас хоть что-то положительное?

- Да, реакция Вассермана!

Все ОК доделок, по-моему, не так много!