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Evaluation by Dr A Nord of "*Avian energy use and its links to oxidative stress*", put forward for the degree of PhD by Ms. Elisavet Zagkle

Background

On May 11, 2022, the undersigned (Dr. Andreas Nord) was asked to serve as external reviewer on the PhD thesis by E. Zagkle at the Jagiellonian University in Kraków, Poland. The relevant documents were sent to Dr. Nord on July 18, 2022.

Below is my evaluation report on the thesis by candidate Zagkle, entitled *"Avian energy use and its links to oxidative stress*"

Lund, Monday 22 August 2022,

Dr. Andreas Nord, Associate Professor
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Reviewer comments

General comments

This is a fine thesis that brings together classic life history theory (the cost of reproduction) with more topically pertinent studies (heat stress as a constraint on reproductive investment) within a context of the balance between energetic investment in breeding, oxidative stress, and offspring phenotypic quality. These studies are compiled as two separate experiments on two model species. Despite a somewhat limited scope in this context, the two experiments are well designed and equally well executed, and results complement existing literature data nicely. Moreover, the topics covered are all close to my own heart and research interest, and so overall I found the thesis a pleasure to read. You will find my general comments below, followed by more detailed feedback pertaining to each of the experiments.

1. The energetic cost of breeding is a recurring theme in the thesis. This, of course, is also a recurring theme in the primary literature. Conceptually, I am interested to know how the candidate defines an energy cost in the context of organismal fitness.
2. The central premise of the thesis is to provide a synthesising account of the relationship between daily energy expenditure and oxidative stress, using avian reproduction as a model system. In the first study, parental energy expenditure was manipulated by increasing brood size, whereby daily energy expenditure increased, antioxidant capacity decreased, and oxidative stress index increased. In the second study, parental heat dissipation capacity was manipulated by a combination of low ambient temperature and feather clipping. This revealed higher resting metabolic rate in clipped birds during cold exposure, but no clear effects on antioxidant capacity or oxidative stress markers (though feather clipping was beneficial for reproductive output in a warm temperature). I am concerned that the choice of two different model systems with different breeding biology, different timing of the reproductive cycle, and two different manipulations with varying degree of clarity with regards to the link to energy expenditure, somewhat constrains the synthesising view of the thesis. Specifically:
    - While it is easy to see how an increase in brood size necessitates increased investment in nestling feeding, hence locomotory activity, that mandates increased daily energy expenditure, it is not as clear to me how the feather clipping (or lack thereof) affects energy expenditure. It is conceivable that all birds will increase energy expenditure in the cold, and that feather-clipped birds will face the highest thermoregulatory costs in that environment, but I am not sure we would expect the link between feather-clipping and reproductive investment in the warm temperature to be paid in a currency of energy if there are no constraints on evaporative cooling. If so, how can we be certain that the driver for any increase in oxidative stress markers is really energy?
    - Conceptually, it is easier to conclude on life history trade-offs in wild systems, where parents pay the full natural cost of any increase or

decrease in reproductive effort. This is more difficult to replicate in captive models. In this case, males were removed from Experiment 2, and there was an also an experimental increase in foraging effort in all groups. This situation undoubtedly made the females work harder, which is appropriate for testing the hypothesis on heat constraints, but it leaves the question as to whether the manipulation was within what we can reasonably expect in a natural breeding situation. That is, is it reasonable that the constraints put onto females in Experiment 2 will be experienced in the wild, where selection can act on different strategies?

- In line with the above two points, we can assume that the manipulations in the two experiments will have affected different organs/tissues/supply chains differently to accommodate experiment-specific increased demands. Yet, oxidative stress was assessed in one tissue only (blood). I realise this is touched upon in the general discussion, but I think a more in-depth account of possible tissue-specific responses is needed in the thesis. Is it possible that lack of treatment effects in some of the studies is because metabolites in the circulation are likely to be less affected by the experiment than those in more relevant tissues?

3. With regards to the premise of manipulating energy expenditure during breeding and linking this to redox balance, some additional justification may be warranted:
    - In Experiment 1, brood-enlarged females increased DEE, reduced non-enzymatic antioxidants, and showed an increased oxidative stress index, largely in line with the predictions. Here, higher DEE is confounded by the change in brood size. How can we be certain that the change in redox status is causally related to higher DEE, and not to any other changes brought about by the experiment, such as reduced dietary intake of antioxidants in the brood-enlarged females?
    - In Experiment 2, feather clipping increased the energy cost of staying warm (Fig. 16) in sub-thermoneutral temperature. However, it is not known if clipped females paid lower energy (or water) costs of staying warm during physical work or when exposed to hot temperatures. Thus, which predictions should we make for the effects of energy expenditure on redox balance here? The subcellular changes recorded were mostly evident in lower circulating uric acid in hot/unclipped females, but by and large these females should be those with the lowest RMR in your experiment. Perhaps it is worthwhile reconsidering the proximate link here?
4. I think a solid justification for working on females only is missing from the manuscript. In Experiment 1, males were not considered despite presumably being present at the nest and in Experiment 2, males were considered during the reproductive stage but were subsequently removed from the study. This raises two questions:
    a. a) Can we extrapolate results obtained from females to effects on males, despite the roles of the sexes being very different during the reproductive cycle? Should we rather view these results as pertaining to females only?

   b. Was the cost of reproduction adequate, over-, or underestimated in Experiment 2? In zebra finches, it is common for both sexes to partake in nest building and incubation, but also in chick rearing. Would the same costs arise to the unclipped/hot females had the males been present?

5. By and large, there was stronger evidence for a general decrease in antioxidants and increase in oxidative stress markers from the beginning to end of the reproductive cycle, or in line with the main effects of metabolic rate, scope, or daily energy expenditure. Comparatively speaking, the addition of the experimental treatments led to few alterations in redox balance. If increased reproductive effort generally does not increase oxidative stress, then how should we interpret oxidative stress as a currency for life history trade-offs?
6. I was missing a clearer account of the mechanisms linking increased daily energy expenditure to generation of ROS and, unless quenched, oxidative stress. The predictions we make will vary depending on whether ROS is produced primarily by the mitochondria or primarily by other cell components. It is also not necessarily so that increased organismal demand for energy will be coupled to increased ROS production in all cases, e.g., if such needs can be compensated for by improved coupling of electron transport to ATP production without a concomitant increase in protonmotive force across the inner mitochondrial membrane. Regardless of the mechanisms involved, it would be good to address putative candidate pathways in the thesis because this would allow for more informed predictions in the two experiments.

Experiment 1

1. I am curious to know more about the oxidative stress index. This is a seemingly useful metric, but it is also difficult to interpret because the variables used to derive the index are on vastly different scales. Thus, is a higher or lower value really a measure of oxidative stress, or rather a sign of a shift in redox balance that may, or may not, be associated with oxidative stress? Information on how to interpret this index is needed to make results clearer.
2. There was no relationship between DEE and BMR, contrasting both the increased intake and compensation hypotheses. Is it possible that this result can be explained by the lack of a proximate link between the constituents comprising BMR and DEE, respectively, the former being driven mostly by metabolism of the viscera (delivery demands) and the latter more by the locomotor apparatus and/or changes in behaviour (e.g., decreased resting periods)? I think what is missing from the discussion here is some reasoning around how ‘strong’ the manipulation of brood size really was for the females: perhaps working at 2.6 or 3.1 multiples of BMR can be accommodated by flexible adjustments to energy metabolism without the need for concomitant growth of supply/delivery organs? For example, we could envision that suddenly increased demands for energy could be met by improved coupling of electron transport to ATP synthesis that could supply energy for a harder-working body without a cost to BMR at the possible of increased ROS production.

3. I found the interaction between brood size category and BMR on oxidative stress index an interesting and unexpected result, wherein brood-enlarged females with lower BMR incurred higher oxidative stress and control females showing a positive relationship. This is not discussed in the thesis, which I think is somewhat of an oversight. It is noteworthy that this relationship was not present when DEE was considered (no effect of DEE on oxidative stress index), which means that the significant, positive, association between oxidative stress index and sustained metabolic scope must be driven by variation in BMR?
4. I am curious to know more about the tight metal cage that was placed inside the metabolic chambers to keep the birds from flapping: did you affirm if this type of restraint was associated with any stress-induced increase in metabolic heat production, and whether presence of the metal cage impacted air mixing inside the metabolic chamber negatively?
5. Sample sizes on page 45 do not match those in Table 1.
6. For the statistics in Experiment 1, I am concerned that the inclusion of the number of chicks brought collinearity issues to your model, because this variable will by necessity convey largely the same information as "treatment". I appreciate you undertook checks of parametric assumptions, but did you also test for multicollinearity before deciding on original model structure?
7. It is posited (page 101) that the reduction in total antioxidant capacity reflects reduced energetic investment in self maintenance. This raises two questions:
    a. Is energy the most appropriate currency in which to pay for OXY? Perhaps this is more of a nutritional cost than anything else?
    b. Is a reduction in OXY always negative, or is it negative only under some threshold value? That is, is the effect of reduced antioxidant capacity on the risk of incurring oxidative stress linear (all is bad) or non-linear (all is good until some threshold, then all is bad)?

Experiment 2

1. It is fascinating that you find evidence of heat dissipation constraints of reproduction at a thermoneutral temperature. However, I would be curious to know more about your justification for the choice of temperatures.
2. I appreciate the care taken to undertake measurement of thermal conductance in your subjects. However, I am concerned you might have underestimated the effect size here. We expect thermal conductance to be minimal at temperatures below thermoneutrality to minimize heat loss ("we want to close all windows before putting another log on the fire"). If so, then the measured conductance need not inform on heat tolerance in the clipped treatment (i.e., the anticipated effect), because when heat stressed or in situations with high metabolic heat production (such as during foraging flights), we expect birds to *maximize* dry conductance by massive peripheral vasodilation. I still expect your clipped birds to have higher heat loss potential in the warmth, but the present metric probably informs more on those birds feeling colder in the cold (and not colder in the warmth, as intended).

3. Did you undertake any power analyses before testing nestling traits in Experiment 2? I am concerned that a model including a three-way interaction between treatment (2 levels), feather clipping (1 level) and chick age (8 levels) will consume largely all degrees of freedom when based on a sample comprising about 40 nests (data from Table 8 on page 91). It might be more straightforward to let chick age be a continuous variable and test for a difference in slope in the models.
4. The dROM result in Fig. 19C indicates there were more reactive oxygen metabolites, and more oxidative stress (Fig. 19D) in the circulation before reproduction than during the nestling-feeding stage, despite the latter presumably being associated with increased female workload. There were no corresponding changes in antioxidant capacity. Does this mean we should interpret pre-reproductive activities as more strenuous than post-hatching activities, in keeping with the main hypothesis?
5. I like the idea that increased mass loss in unclipped, hot, females in Experiment 2 was a strategy to reduce metabolic heat production (page 98). If this argument is correct, then would it not be able to test the idea by portioning RMR data in Fig. 16 on the four treatment combinations instead of only clipped or unclipped? If you are right, then we would expect RMR in hot, unclipped, females to be lower than in cold, unclipped, females? If not, then maybe the reduction in mass was a reduction in fat stores or gut fill, more than any change in the mass of metabolically active tissue.
6. If females in Experiment 2 did not encounter oxidative stress during reproduction (page 103), then why would they upregulate antioxidant defences to protect from further costs? Which costs?