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Review of PhD dissertation of Kyle Coughlan, M.Sc.,
'Oxidative stress biology of nest-box breeding birds'
supervised by dr hab. Ulf Bauchinger, PhD and dr Edyta Teresa Sadowska, PhD, at the Faculty of Biology, Jagiellonian University in Kraków

In the reviewed PhD dissertation, Kyle Coughlan presents results of his studies of the effect of energy expenditure on oxidative stress in wild-living passerine birds. The research project described in this thesis is both very simple and quite complex. On the one hand, author simply compared oxidative damage and antioxidative mechanisms in blood samples collected during two or four time points. On the other hand, author analyzed several indexes of both oxidative damage and antioxidant mechanisms, samples were collected in different way and sometimes combined together etc. Thus, I really appreciate the presence of several summaries like the short chapter 'Thesis structure' (page 12), list of abbreviations, Figure 2.9 (page 53), or Table 4.1 (page 110).

Reviewed thesis begins with an interesting introduction to oxidative damage and antioxidative mechanisms in chapters 1.1 and 1.2. Here, author describes both molecular background of oxidative stress and the potential role of oxidative stress in evolutionary biology and life history trade-offs. Author then proposes a clear hypothesis on page 31: 'Do periods of higher energy requirements necessarily lead to higher oxidative damage?'. Author formulates also alternative hypotheses (page 35) that birds evolved antioxidant mechanisms to compensate for increased ROS production during periods of higher energy expenditure. Here, I do not think that both hypotheses represent two alternatives: I can easily imagine situation when a bird has elevated antioxidant mechanisms but still suffers higher oxidative damage (like in study of clipped Great tits by Vaugoyeau et al 2015 or migrating European robins by Jenni-Eiermann et al 2014 cited on page 27).

The whole thesis consists of 4 studies. In all of them, author intended to compare oxidative stress parameters in blood samples collected from Great tits and Blue tits during periods of supposed higher and lower energy expenditure. All studies were carried out in the forest near Mikołajki in NE Poland. Study I seems to be a kind of pilot study: author compared blood sample collected at day and night (intended to represent periods of high and low metabolic expenditure) at peak chicks feeding intensity in 12 nests of Great tit. Study II was similar to Study I but on a much larger scale and included two species: Great tit and Blue tit. Study III also compared samples collected at day and night but this time during winter. Finally, in Study IV author re-analysed together data from previous studies to compare reproductive and non-reproductive birds. Thus, author was able to analyze variation in different parameters related to oxidative stress at different time scale: periods of higher (daytime) and lower (nighttime) metabolic activity, both during breeding season (summer) and during winter. In doing so, he hoped to investigate changes in oxidative damage and antioxidative mechansism under natural gradient of changes in energy expenditure. I admit this is an interesting approach: to compare effects of both wide- and narrow timescale of variation in energy expenditure. I have little to comment about methodology. Author used standard biochemical methods, based e.g. on Diacron kits. However, it is not explained why antioxidant enzymes were analyzed only during Study I (page 53).

Results of all four studies can be summarized fairly easy: author found no evidence for an increase in oxidative damage during periods of presumed higher energy expenditure, and observed little changes in most antioxidative mechanisms. However, many analyses found higher level of uric acid (potential antioxiodant) during periods of presumed higher energy expenditure. Author concluded that varying level of uric acid can represent mechanism responsible for the lack of expected increase in oxidative damage.

Oxidative stress is one of the most difficult research problems and results of earlier studies have varied widely as author admits on page 30. Sometimes it seems that the more parameters related to oxidative stress we study, the more doubts we have. First, what we call 'oxidative damage' depends on the rate of reactive oxygen species (ROS) production, antioxidative capacity and tissue susceptibility to ROS action. Each of these three 'components' actually represents another complex set of traits: for example, the same factor can downregulate some antioxidant mechanisms and upregulate others. Finally, there is almost endless list of possible confounding factors, and such list is particularly long in studies that are carried out under natural conditions and do not involve any form of experimental manipulation (i.e. they are based on natural variation – like this PhD thesis). Still, author's situation was even worse because he had to deal with mostly non-significant results. What I can do here is to add some general comments and suggestions that perhaps will be helpful during writing publications based on collected data. I am afraid that sometimes I am just pointing out the new potential confounding factors but a few times I thought that author was aware of particular problem but perhaps he did not express it clearly.

Perhaps the single biggest problem is that we do not know the true magnitude of variation in energy expenditure at both time scales i.e. day vs night and breeding vs non breeding (or rather summer vs winter). There are studies (e.g. cited on page 33) that shows no difference in energy expenditure between breeding and winter (I assume this is what word 'seasons' means). The same problem of seasonal variation in energy allocation between summer and winter is discussed on pages 126-129. Here, I do not understand why basal metabolic rate (BMR) is used to distinguish between reallocation and increased demand hypotheses as BMR should not include costs of thermoregulation. Author conclude cautiously on page 129 that results of Study IV (i.e. the same oxidative damage at both seasons) agree rather with reallocation hypothesis i.e. energy expenditure is similar during summer and winter. However, I agree rather with the next sentence that energy management is so complex trait that only direct quantification of energy expenditure can produce any firm conclusions here. I am aware that it was not possible in the present project but author measured both body mass and tarsus length of adult birds (page 52). Thus, body mass or body mass corrected for tarsus length would offer at least some index of condition that could be added as covariate to statistical analyses. Why it was not done? It is striking that adding the mass of chicks/brood size on pages 90-91 was sufficient to produce perhaps more significant results than can be found in the rest of the thesis.

There is no doubt that energy expenditure is higher during daytime that nighttime but this difference also can vary between summer and winter. Both Great and Blue tit show nighttime heterothermy and they reduce body temperature and metabolic rate in order to save energy. I can imagine that such reaction is more pronounced during winter night than during short, warm summer night when adults must be ready to continue chick feeding at dawn.

The only parameter that usually differed between compared time points was the level of uric acid. As Author explains (e.g. on pages 117-8) uric acid is the end product of protein metabolism. Both studied species consume more protein at summer than at winter when they switch mostly to seeds (pages 42, 44, also 124) and author explains on page 124 that blood level of uric acid can react rapidly for change in diet. Author seems to admit on page 118 that uric acid during the winter is based on physical activity and protein turnover rather than diet but I am not sure if he refers here to this diet shift. But can we say that there is higher 'higher activity level of muscles' during winter that can compensate for lower protein content in diet? Once again, it is impossible to conclude anything firm about energy expenditure from variation in oxidative stress. Finally, for me the whole discussion on pages 117-119 sounds like admitting that the real importance of uric acid as antioxidant in birds is still very speculative (because higher level of uric acid is simply an unavoidable effect of higher rate of metabolic rate). Thus, it is a pity that the level of allantoin was not assayed!

I think that firm conclusions about uric acid can be also difficult because of variation between studies in methodology. The time that passed since the last meal and meal's composition are very important predictors of oxidative status (page 124). For example, sample during active phase was taken around 10:20 am in Study I, 4 pm in Study II (page 50), and 1pm in Study III (page 51). Sample during rest phase was taken around 2:30-3 am in Studies I and II and 6 am in Study III (pages 48-49). If we remember that the length of night also differ between February and May-June, then the time from the last meal till rest phase blood sampling was also very different (and also meal composition was likely to be different too!). Thus, this is perhaps not surprising that difference between summer and winter in oxidative damage was found only during rest phase (page 102). In summary, when we compare May-June and February, the presence of reproduction or the lack of thereof is only one of the many differences. I also note very different duration of restraint procedure before blood sampling: almost none during rest phase at breeding (page 48), and sometimes 30 minutes during active phase during the winter (page 51). However, author cites a paper on page 125 that such difference in disturbance does not need to affect results of d-ROM and OXY tests.

I think there is another difference between birds collected during summer and winter. Author specifically explained that only birds that successfully completed their brood were included during assays at summer (e.g. on page 101). In other words, only birds that were fit enough to breed successfully were analyzed. On the other hand, winter Study III analyzed all birds that were caught (including young, first-winter birds that did not breed at all during their life). If we treat winter birds as a kind of 'non-breeding control', then this is the same potential problem as seen during many laboratory experiments on oxidative stress as cost of reproduction: we finish with random sample in control group and non-random sample in experimental group, without weakest individuals. I am aware that author could do little (if anything) to correct this problem but it should be at least mentioned in discussion as another potential confounding factor.

I am aware that statistical analyses of such data was pretty challenging. It must be kept in mind that statistical analyses of complex data collected during field studies is by far more difficult than analysis of results of laboratory experiments. Particularly results of Study II are very complex and sometimes I felt a bit lost here. I think that in many cases author simply should explain better why he did particular analyses. For example, author usually first analyzed his results in model that included all data (like both resting and active state or both species) and then separately for each phase or each species. However, relevant interactions are non-significant: e.g. on page 94 author specifically stated that there was no interaction between species and state but then species were analyzed separately. If there was no interaction in the initial model (like interaction between state and order) there is no reason for doing such analyses. Sometimes I can only guess why it was done. For example, in Study I (page 68) and Study II (page 78) rest and active phase were analyzed separately because data about males were available only for active phase. I think this is never mentioned explicitly and it is not obvious when reading the text for the first time. Similarly, in Study IV significant interaction between state (day vs night) and reproductive period was driven by only one species: Blue tit. However, if this is the reason it should be explained clearly. I also noted that Study IV combines results from summer when daytime and nighttime samples were taken from the same individuals and from winter when daytime and nighttime samples were usually taken from different birds. How it was controlled statistically? I am also curious if factor 'order' could be interpreted as cost of reproduction since this is simply difference in the number of days spent on chick feeding. Finally, I am aware that the usual level of significance $p=0.05$ is purely arbitrary but I think it is too far to state that something is 'slightly higher' when $p=0.43$ (page 96).

The whole thesis is rather well written though some passages were more difficult to follow. For example, I do not understand what is 'this period' when BMR of females of Great tit was higher than during either winter or breeding (page 33)? Perhaps specifically nestling period? Field method section on page 47 first claims that replacement clutches were included in Study II and then that they were not. On pages 42 and 44 author consistently uses 'cm' instead of 'mm' when describing the size of both studied species (they are not so big!). Very important information about time spent by caught birds in hand before blood sampling is given as 'here minutes' (page 52). Sometimes there are sudden 'jumps' in narration like sentence about comparison of daily variation in SOD and CAT in jungle fowl and Study I on page 126 – it is hard to understand. There are also several errors in the use of figures: for example, on page 37, there is legend to figure 3 on the next page but page 38 contains figure 2.1; errors with panel symbols on figure 2.6 (page 46); reference to non-existing figure 6 on page 52.

Sometimes author repeats the same information. I realize that in this way he might try to help a reader but it can be also confusing. Author frequently presents the same or similar results in figures and in tables. I think figures are better. On the other hand, sometimes first and second brood are showed only in tables but not in figures. Moreover, whereas tables present mean values and standard deviation, figures depict median values and quartiles even though, author specifically confirms several times that data did not need to be transformed. Since figures depict additionally all individual points, I think mean values would be more appropriate. Moreover, I think that most of results presented in text/figure/tables is not referred to in discussion (what is not surprising as usually all show similar patterns). This is perhaps justified in PhD thesis but in final publication I would suggest dropping some non-significant results that add nothing new. Finally, it is very frustrating for a reader that author sometimes presents the same, non-significant results twice (in table and on figure) but several significant results on pages 90-91 are not depicted at all!

I am aware that author might be a bit disappointed by his results. However, although I have just added another list of potential problems and confounding factors, the final opinion about reviewed PhD thesis must be definitely positive. Author dealt with very important problem of evolutionary physiology, applied correct methodology and discussed his results in perhaps the best way he could. I admit that most of potential methodological flaws I noted represent inevitable trade-offs of field studies when compared to laboratory experiments: we analyze animals in their natural environment but we have also much less control over the studied system (so we measure what we can measure not what we should measure). As I explained earlier, it is hard to imagine more difficult research problem than analyses of oxidative stress under natural conditions (see also page 130). Author faced problems typical of field studies, including unexpected and non-significant results but presented them and discusses in a sound way. Finally, I can point out that even Study I i.e. by far the smallest and the simplest one, was already published as individual paper, and thus remaining studies should be published soon as well.

To summarize, I think that the reviewed PhD dissertation meets the conditions specified in Article 187 of the Act of July 20, 2018, The Law on Higher Education and Science (Journal of Laws: 2018 number 1668 with subs. changes) and I recommend that the Scientific Council of Biological Sciences of the Jagiellonian University should admit Ph. D. candidate Kyle Coughlan for the subsequent stages of the doctoral proceedings in the field of natural sciences, discipline of biological sciences.

Podsumowując, uważam że przedstawiona do oceny rozprawa doktorska spełnia warunki określone w artykule 187 Ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (Dz. U. z 2018 r. poz. 1668 z późn. zm.) i zwracam się do Rady Dyscypliny Nauki biologiczne Uniwersytetu Jagiellońskiego o dopuszczenie Pana mgr Kyle Coughlan do dalszych etapów postępowania o nadanie stopnia doktora w dziedzinie nauk ścisłych i przyrodniczych w dyscyplinie nauki biologiczne.

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