Response to Reviewers' Comments

Reference number: RSOS-241265

Title of article: Carcass size, not source or taxon, dictates breeding performance and carcass use in burying beetle

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Dear Dr. Bart Pannebakker,

Thank you for inviting us to submit a revised version of the manuscript. We greatly appreciate the valuable comments and feedback from you and the reviewers. We have carefully considered each comment and incorporated the suggestions. In particular, we have made the following major changes:

- Expanded the discussion on carcass source and taxon as suggested by Reviewer 1.
- Addressed the reviewers' concerns about the statistical analyses (controlling the false discovery rates for multiple comparisons).

Please also see the following section for our detailed point-by-point responses. All line numbers refer to the changes we made in the revised manuscript (with tracked changes). We believe that the revisions have further improved the quality of this manuscript, and we hope that it is now suitable for publication in *Royal Society Open Science*.

Sincerely,

Gen-Chang Hsu (submitting author)

Department of Entomology, Cornell University

Editor's comments

Comment 1 > Thank you for submitting your work to Royal Society Open Science. Your work has now been reviewed by two reviewers. As you can see, both are positive and praise the quality of your science and of the readability of the manuscript, and I can only agree with them. Your work on the relation between carcass size, origin and taxon on the breeding success of *Nicrophorus nepalensis*, is a sound study which would fit Royal Society Open Science well. However, there a number of issues indicated by both reviewers that need to be revised before the paper can be accepted. Besides a list of minor issues, these concern some (small) concerns on the statistics, and a request by Reviewer 1 to extend the interpretation and discussion of the results. He provides some suggestions for papers which are a good starting point.

All in all, these are not big concerns, and I would like to encourage you to use the comments by the reviewers to further improve the paper. I am looking forward to seeing the revised version of your paper.

Response 1 > Thanks for the positive feedback on our manuscript. We greatly appreciate the comments and suggestions from the reviewers and have now revised the manuscript accordingly. We also enhanced our discission on the results of carcass source and taxon as suggested by Reviewer 1 and addressed the statistical concerns raised by Reviewer 2. Please see our point-by-point responses in the following section for more details.

Reviewer 1's comments

Comment 1 > I commend the authors for this interesting study. However, the authors have missed many studies in the scientific literature that test the same hypotheses and provide additional detail for other species of burying beetles. I suggest the authors dig deeper into the literature and revise their interpretation and discussion. Below are specific points that should be addressed.

Response 1 > Thanks for the feedback and suggestions. We have incorporated the relevant studies on carcass size and reproduction of burying beetles. We have also expanded the discussion on the results of carcass source and taxon. Please see our response to Comment 3 and Comment 4 for more details.

Comment 2 > Line 28 and 49 Rather than just using the word "carcasses", you should specify "small vertebrate carcasses".

Response 2 > Revised (Line 28 and 49).

Comment 3 > Lines 63-65 "Such cost-benefit trade-offs suggest that reproductive performance might not necessarily be greater on larger carcasses, yet no study has empirically examined whether there is an optimal carcass size for breeding." This is not true, several papers have addressed this issue in burying beetles. These papers have used single bout and lifetime reproductive success to evaluate optimal carcass size. See:

Belk, Mark C.; Meyers, Peter J.; Creighton, J. Curtis. 2021. Bigger is better sometimes: the interaction between body size and carcass size determines fitness, reproductive strategies, and senescence in two species of burying beetles. Diversity 2021, 13, 662. https://doi.org/10.3390/d13120662.

Quinby, BM, Belk, MC, Creighton, JC. Behavioral constraints on local adaptation and counter-gradient variation: Implications for climate change. EcolEvol. 2020; 10: 6688– 6701. https://doi.org/10.1002/ece3.6399

Creighton, J.C., N.D. Heflin, and M.C. Belk. 2009. Cost of reproduction, resource quality, and terminal investment in a burying beetle. American Naturalist 174:673-684.

Response 3 > Thanks for pointing out this mistake. We have corrected the original statement and included the relevant references for it (Line 65–67).

Comment 4 > Lines 66-78. This is a really important point and a novel part of the study. I suggest the authors enhance the discussion of this point, and I commend them for including it in the study.

Response 4 > Thanks for the positive comment. We have modified the relevant paragraphs in the introduction section (Line 68–82) and the discussion section (Line 319–337) to strengthen our point on carcass source and taxa.

Comment 5 > I notice that all of your experiments and analyses are based on single bouts of reproduction (presumably the first bout, although this should be explained). There is a difference between single bout and lifetime reproductive output. For example, in another species of burying beetle there is a clear difference between single bout reproductive success on 20 and 30 gram carcasses. However, lifetime reproductive output is equivalent on these two sizes. At the very least you should consider this difference in the discussion.

Response 5 > Thanks for the suggestions. The study species *N. nepalensis* does reproduce multiple time throughout the life, and in our study, we recorded only the breeding outcomes during the first reproductive bout. We have now mentioned this in the methods section (Line 148–149). As suggested, we also brought up the difference between single vs. lifetime reproductive output in the discussion section (Line 364–367).

Comment 6 > Line 231. Please provide an explanation for using a type II sums of squares approach. Usually, Type III sums of squares provide a more complete test of the hypothesis with multiple predictors.

<u>Response 6</u> > We used type II sums of squares in the ANOVA because it respects the principle of hierarchy (marginality). For example, in a model with two predictors A and B as well as the interaction A×B, it first tests the interaction term A×B (Y ~ A + B vs. Y ~ A + B + A×B) and drops A×B to test the main effect of A (Y ~ B vs. Y ~ A + B) and B (Y ~ A vs. Y ~ A + B).

On the other hand, type III sums of squares tests the main effect when all other terms are kept in the model. For example, for the same model above, it first tests the interaction term A×B (Y ~ A + B vs. Y ~ A + B + A×B), which is the same as type II sums of squares. However, it tests the main effect of A (Y ~ B + A×B vs. Y ~ A + B + A×B) and B (Y ~ A + A×B vs. Y ~ A + B + A×B) in the presence of A×B. The models Y ~ B + A×B and Y ~ A + A×B are meaningless because they contain the higher order term A×B without the lower-order terms A and B.

Besides the issue of marginality, studies have also shown that type II sums of squares generally has higher statistical power than type III sums of squares and therefore is more appropriate for testing main effects (Langsrud 2003, Smith & Cribbie 2014).

References:

Langsrud, Ø. (2003). ANOVA for unbalanced data: Use Type II instead of Type III sums of squares. *Statistics and computing*, *13*(2), 163-167.

Smith, C. E., & Cribbie, R. (2014). Factorial ANOVA with unbalanced data: a fresh look at the types of sums of squares. *Journal of Data Science, 12*(3), 385-403.

<u>Comment 7</u> > Lines 341-342. I don't think this is an accurate statement. The citation is old, and the level of involvement of both parents varies among species. Do you have information from field data about *N. nepalensis* specifically?

<u>Response 7</u> > Based on the field data collected in a previous study, a group size of 2 is common in *N. nepalensis* (Fig. 2 in Liu et al. 2020). However, the group size can vary substantially across the elevations and with population densities. To avoid confusion, we have now removed the statement (Line 353–354).

Reference:

Liu, M., Chan, S. F., Rubenstein, D. R., Sun, S. J., Chen, B. F., & Shen, S. F. (2020). Ecological transitions in grouping benefits explain the paradox of environmental quality and sociality. *The American Naturalist*, *195*(5), 818-832.

Comment 8 > Line 352. See above comment. This is not the first time this has been shown.

Response 8 > Revised (Line 369).

Comment 9 > Line 359-360. Not quite an accurate statement. The balance between size and number of offspring is more involved than simply carcass size. Many studies not cited here have addressed this relationship in burying beetles.

Response 9 > Thanks for pointing this out. Yes, we agree that there are many factors besides carcass size that can influence the offspring quality-quantity relationship in burying beetles. In fact, we did not intend to conclude that carcass size is the "only" or the "most important" factor. Instead, our study adds to the understanding that carcass size, among many other factors addressed in previous studies, can shape this quality-quantity balance. We have modified our statement to avoid potential misunderstandings (Line 375–378).

Reviewer 2's comments

<u>Comment 1</u> > The authors examine the impacts of carcass size, origin (lab-reared vs wild), and taxon (mammal, bird, or reptile) on various breeding success metrics in captive experiments using a burying beetle (Nicrophorus nepalensis). The authors further test for nutritional differences among carrion, estimate optimal carcass size, and test for a trade-off between larval size and number in the context of carcass size, origin, and taxon. The authors find strong evidence for an optimal size of carcass under laboratory conditions and find little difference in reproductive success between carcass origin and taxon, the latter of which did not differ substantially in protein/fat content. Overall, the study provides important validation of other work that uses lab-reared carcasses in experiments with Nicrophorus, as well as interesting evidence for optimal carcass size and an influence of carcass size on life history strategies.

Overall, the manuscript was very clear and well written. The experiments were well-designed and carefully executed with clear and appropriate statistics, plots, and well-balanced and reasonable interpretations. It was just a really nice, refreshing manuscript to read. I had a few comments, suggestions, and corrections, listed below.

<u>Response 1</u> > We greatly appreciate the positive feedback on this study and we have revised the manuscript based on the comments below.

<u>Comment 2</u> > The authors conduct many statistical tests using non-independent data, which can lead to some p-values that fall below 0.05 simply due to chance. Have you considered controlling for false discovery rates (e.g., Pike 2011, Methods in Ecology and Evolution)? Controlling for false discovery rates wouldn't change the main results of the paper, but would put some of the borderline p-values/results into better context.

<u>Response 2</u> > Thanks for the nice suggestion. Yes, we did conduct multiple tests on various breeding outcomes and carcass use efficiency, each with multiple predictors (Table 1), which could potentially lead to significant results by chance. As suggested, we therefore performed the Benjamini–Hochberg multiplicity adjustment for the *p*-values in Table 1 to control for the false discovery rates at $\alpha = 0.05$:

Model response	n	Predictor		
		Carcass weight	Carcass source	Weight × Source
Clutch size	210	Original: $P < 0.001$ Adjusted: $P < 0.001$	Original: $P = 0.39$ Adjusted: $P = 0.59$	Original: $P = 0.24$ Adjusted: $P = 0.45$
Hatching success	176	Original: $P < 0.001$ Adjusted: $P < 0.001$	Original: $P = 0.37$ Adjusted: $P = 0.59$	Original: $P = 0.88$ Adjusted: $P = 0.96$
Brood size	238	Original: $P < 0.001$ Adjusted: $P < 0.001$	Original: $P = 0.93$ Adjusted: $P = 0.96$	Original: $P = 0.17$ Adjusted: $P = 0.36$
Brood mass	129	Original: $P < 0.001$ Adjusted: $P < 0.001$	Original: $P = 0.99$ Adjusted: $P = 0.96$	Original: $P = 0.004$ Adjusted: $P = 0.01$
Carcass use efficiency	95	Original: $P < 0.001$ Adjusted: $P < 0.001$	Original: $P = 0.96$ Adjusted: $P = 0.96$	Original: $P = 0.57$ Adjusted: $P = 0.77$

In fact, we did not have borderline *p*-values in the original results, so there is less concern about this issue. Since the adjustment did not alter the overall conclusions, we decided to keep the original results.

For the pairwise comparisons between the three wild carcass taxa in Fig. 3 and Fig. 4, we did perform the Tukey multiplicity adjustment in the original analysis to control for the family-wise error rates.

Comment 3 > lines 61-65: What about additional costs of competitive interactions that vary with carcass size? For example, are large carcasses more likely to be found and eaten by vertebrates, thereby reducing fitness? How does microbial competition vary with carcass size and with different environmental temperatures? [I see this discussed on lines 344-350 - great!]

Response 3 > Thanks for bringing these points up. As mentioned, we did talk about how competition from vertebrate scavengers and microbes may influence the optimal carcass size for burying beetles in nature in the discussion section. We have now added a brief explanation to the original sentence in the introduction by saying that larger carcasses might be more difficult to utilize because of greater competition from other carcass-feeding organisms (Line 62–63). Regarding the effect of temperature on optimal carcass size, we have included this in the discussion section. Please see our response to Comment 13 for more details.

Comment 4 > lines 117-120: Were carcasses from the lab versus wild at similar levels of decomposition?

Response 4 > The wild carcasses used in the study were roadkill animals immediately transferred to the −20°C freezers upon discovery. Even though these wild carcasses might not be as fresh as the lab carcasses (which were killed via euthanasia and frozen right away), we selected wild carcasses with decomposition levels as similar to those of the lab carcasses to minimize the potential confounding effects of carcass freshness on breeding outcomes (degraded wild carcasses would not be used in the experiments).

Comment 5 > lines 126-128: I really liked the controlled and paired design.

Response 5 > Thanks for the positive feedback.

Comment 6 > lines 151-153: Isolating viscera and muscle seems to miss independent fat deposits (e.g., often well-developed between the skin and muscle on migratory birds). Why not estimate fat/protein composition of the entire carcass (after removing external hair/feathers)? Wouldn't the entire carcass (excluding bones) be a better representation of what the beetles use?

Response 6 > Thanks for the comment. First, there were not many fat deposits under the skin (subcutaneous fat) for the wild bird carcasses we dissected (we did not have migratory birds in our study). Second, we did include the visible subcutaneous fat as well as fat deposits around the internal organs (visceral fat) in the viscera tissue samples, and the criterion for the inclusion of fat tissues was consistent across the carcasses. Since the muscle and viscera tissues are the main parts of the carcasses that the beetles use, our sampling should fairly characterize the carcass resources for them.

<u>Comment 7</u> > line 208: What is carcass ID? If it relates to only one row of data per analysis, then why include it as a random effect?

<u>Response 7</u> > In the analysis of carcass tissue nutritional composition, we collected three muscle and three viscera tissue samples from each carcass (a total of seven lab carcasses, seven wild mammal carcasses, six wild bird carcasses, and six wild reptile carcasses were dissected and analyzed), and each row in the dataset represents one tissue sample. Since the tissue samples from the same carcass were not independent, we included carcass ID as a random effect to account for this.

<u>Comment 8</u> > lines 219-220: "Dead larvae were excluded from the analysis." How often did larvae die in the experiment?

<u>Response 8</u> > Thanks for the comment. The larval survival rates in the feeding experiments were low (22.3%); only 42 out of 188 larvae survived after the five-day feeding period. We have provided this information in the methods section (Line 224–225). In fact, this indicates the importance of parental care in the reproduction of burying beetles: without parents, larval survival is low, presumably because of competition from microbes or a lack of food provisioning.

<u>Comment 9</u> > lines 262-263 vs Figure 4f: "... although larvae feeding on wild bird carcasses tended to gain more weight compared to those feeding on wild mammals and reptiles (Fig. 4f)." Larval growth was highest for mammal carcasses, according to figure 4f.

<u>Response 9</u> > Thanks for pointing this out. We double checked the raw data and larval growth indeed tended to be higher on wild bird carcasses (although not statistically significant). We also checked the R script for the figure and found a mistake in the axis labels. We have corrected this and updated the figure.

Comment 10 > lines 297-299: Was this evident in your study as well? Or would this only be evident if you use natural soil or conduct the experiments in nature?

Response 10 > We do not have direct evidence for this as we did not analyze the microbial communities on the carcasses, but we proposed it as a potential explanation for why breeding performance did not simply increase with carcass size as one would predict based solely on resource availability. This speculation can be tested via the correlation between carcass size and skin/gut microbial communities (abundance, composition, etc.) either in the field to allow for natural variation in background soil microbes or in the lab settings to control for the background microbes.

Comment 11 > lines 304-307: An interesting idea, and consistent with some suggestions that Yarrowia (in *Nicrophorus secretions*) 'pre-digest' carrion for the young.

Response 11 > Yes, regurgitation is an important part of parent care in burying beetles.

Comment 12 > lines 321-322: Again, I don't see this result in figure 4f.

Response 12 > Please see our response to Comment 9 for more details.

Comment 13 > lines 346-347: I would think that temperature may further interact with carcass size and competitors to shift optimal carcass size in nature.

Response 13 > Thanks for the suggestion. This is a great point and we have now brought it up in the discussion section (Line 359–361).

Comment 14 > lines 344-350: I really appreciated the discussion section acknowledging the potential for different optimal carcass sizes in nature, where other selective pressures (e.g., vertebrate scavengers) could play important roles.

<u>Response 14</u> > Thanks for the positive feedback.