

The relationship between diet, plasma glucose, and cancer prevalence across vertebrates

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript presents a comparative study examining associations between diet, blood glucose, and cancer prevalence among vertebrates. The study is based on a sufficiently large data set and the data are analysed using appropriate statistical methods controlling for phylogenetic non-independence and correcting for a large number of tests performed. The results revealed a rather surprising negative relationship between blood glucose concentration and cancer, and supported an expected higher cancer prevalence in top predators. The study is timely as there has been a growing interest in comparative research of cancer in recent years. The results are of interest to researchers from a wide range of research fields, encompassing life history evolution, comparative physiology, and evolutionary medicine. Overall, I support the publication of this manuscript, although I also have several concerns and comments detailed below.

Hypotheses: To hypothesise the negative relationship between blood glucose concentration and cancer prevalence only because some traits show opposite slopes of relationships within and among species does not seem to be a compelling a priori hypothesis. Or at least not the only possible hypothesis. In my opinion, when proposing such a hypothesis, a potential mechanism should also be proposed, explaining why high glucose concentrations should be associated with low cancer prevalence among species, when a positive correlation is expected within species. Otherwise, a similarly or even more plausible scenario could be the absence of a relationship between blood glucose and cancer among species. Such a hypothesis is more parsimonious than the one proposed in the manuscript because it only assumes that, over macroevolutionary time scales, species with high blood glucose evolved adaptations preventing pro-carcinogenic properties of high blood glucose concentrations, thereby leading to a decoupling of cancer prevalence from blood glucose concentrations, rather than assuming an additional mechanism leading to a somewhat counterintuitive negative correlation between these two variables. Resting metabolic rate-lifespan and blood glucose-oxidative stress relationships may serve as examples of the suggested scenario, as they show either negative or positive direction within species (Duarte & Speakman 2014 Age 36:9731; Brownlee et al. 2001 Nature 6865:819-820) but no association among species (Speakman et al. 2005 J Exp Biol 208:1717-1730; Vágási et al. 2023 bioRxiv doi: 10.1101/2023.05.09.539961). Finally, the third scenario, namely that blood glucose concentration may show a positive association with cancer prevalence, is also plausible as evidenced by the sentence in the Introduction promoting its examination (lines 88-89). After reading this sentence, it is in fact somewhat confusing to the reader why you only predict the opposite direction of correlation. In my opinion, it is better to formulate hypotheses for various scenarios unless at least a general mechanism that should lead to the negative correlation is proposed.

In addition, it should be clarified, whether the predicted correlation between blood glucose concentration and cancer prevalence (or its absence as suggested above) should be mediated by body mass or independent of it. Body mass have been reported to be associated positively with cancer prevalence (e.g., Møller et al. 2017 ref. 4, Compton et al. 2023 ref. 10) and negatively with blood glucose concentration (e.g., Umminger 1975 Comp Biochem Physiol A 52:455-458, Braun & Sweazea 2008 ref. 42, Tomášek et al. 2019 ref. 30, Tomášek et al. 2023 Ecol Lett 25:2203-2216), which would justify proposing a negative association between blood glucose and cancer when body mass is not controlled for. When controlling for body mass, all three scenarios discussed above may be plausible.

Specific comments

Line 50: Please note that Compton et al. 2023 (ref. 10) found a positive relationship between body size and cancer prevalence, not its absence as claimed here.

Line 51: Consider also including Dujon et al. 2023 (Proc R Soc B 290:20230940) and Compton et al. (ref. 10), who supported the relationship between litter size and cancer prevalence on much larger data sets of mammalian species than ref. 6 cited here.

Line 62: Given that only blood glucose-diet associations are discussed below, provide references for the association between blood glucose level and species fecundity here (e.g., Tomášek et al. 2019 ref. 30 and Tomášek et al. 2023 Ecol Lett 25:2203-2216).

Lines 72 and 75: Please note that a higher blood glucose concentration in frugivores and nectarivores was reported recently (Tomášek et al. 2022 Ecol Lett 25:2203-2216).

Lines 83-85 and 88-89: Three lines of reasoning were provided above how blood glucose level can be linked to cancer but, from here, it seems that only the one based on the Warburg effect is considered further. I suggest rewording the closing part of the paragraph starting from this sentence (lines 83-85) to make clear whether your expectation is made on all three lines of reasoning or only on the Warburg effect.

Lines 105: Was liver regarded as a part of the gastrointestinal system?

Lines 106-107: Please explain why the fact that the gastrointestinal tract is the largest organ in contact with the external environment should lead to a steeper slope of the predicted negative relationship between glucose and gastrointestinal cancer.

Line 147: What organs were included in the gastrointestinal system. Did you also include associated internal organs such as liver and pancreas?

Line 148: Change "from previous studies" to "from a previous study".

Line 151: Throughout the text below you use the term "body weight", rather than "body mass". I recommend using that term also here.

Line 151: It should be stated explicitly how was gestation length determined in oviparous species. Is it the incubation period?

Lines 160-162: Have you considered logarithm-based transformation? Data like body mass are usually log-normally distributed. Also note that the statistical models assume normal distribution of residuals, not data.

Lines 163-164: Centring do not change the shape of distribution of the data. Please explain.

Line 167: Specify that "variables" are "dependent variables" here.

Lines 172-173: Proportional variables such as percentage of food type are not normally distributed, and logit or arcsine transformation is used commonly. Why was no transformation used?

Lines 175-176: Please state explicitly whether binomial models were used for modelling prevalence data. In addition, overdispersion is a common problem with binomial models. Was its possible presence checked?

Line 179: Please state whether you used the original Benjamini-Hochberg procedure, or the FDR adjusted P-values. In case of the former, I suggest stating here the highest P-value that passed the FDR threshold for significance for better orientation of which P-values were considered significant. In addition, were the post-hoc tests (e.g., Figure 4A) included in the FDR procedure?

Lines 206-207: The (adjusted?) R^2 is negative in this relationship, when body mass is not controlled for (Table 1, Supp. Fig. 1B), so the explanatory power of the model is minimal if it is not a typo.

Line 207 and elsewhere: What is the rationale behind separating birds for this analysis while lumping mammals together with ectotherms ("non-birds"), when there are clear differences between mammals and ectotherms in blood glucose levels (e.g., Figure 2 showing three clear clusters - ectotherms, mammals, and birds)? In my opinion, it would make more sense to do this analysis while controlling for birds, mammals, and ectotherms, if you want to control for possible effects (on the intercepts of the relationships) of fundamental differences in physiology at high levels of phylogeny.

Line 220: I guess the "positive" relationship is most probably based on visual assessment. But the trophic level was a categorical variable, so I recommend avoiding the term correlation here. Moreover, there is no significant difference between herbivores (primary consumers) and invertivores (secondary consumers). Therefore, I suggest rewording it in a more nuanced way to highlight the fact that only secondary carnivores (i.e., tertiary consumers) had significantly higher cancer prevalence.

Lines 269-308: This part of the Discussion suggests that the results can at least partly be explained by the differences in physiology between birds and other vertebrates (i.e., birds have comparatively high blood glucose concentration and low cancer prevalence). But how do you interpret the fact that the results remained basically the same when birds versus other vertebrates were controlled for and that there was no difference between these two groups (Table 1B, second set of models, P-values of variable B)? Doesn't it indicate that the effect is due to the similar slopes of the glucose-cancer relationship within each group (i.e., within birds and within other vertebrates) rather than due to mean differences between those groups? I think this should be explained here, if the differences in physiology between birds and other vertebrates are proposed as one of the possible explanations.

Line 290: Please cite the primary source.

Line 349: Were the glucose data collected from anaesthetised animals only? This should also be mentioned in the Methods.

Lines 352-354: Capture stress elevates blood glucose concentration also in passerine birds (Tomasek et al. 2022 *Ecol Lett* 25:2203-2216).

Lines 720-721: Incomplete citation

Line 872 and below: Although related, the results here are from the regression analysis, not correlation analysis. Consider rewording the captions.

Table 1F, page 52: Given that the presentation of the results, and particularly those of diet, is quite complex, please use the terms consistently to avoid confusion. Elsewhere in the table, "diet type" is used for categories 100% herbivory vs. omnivory vs. 100% carnivory), whereas the percentage of plant-based and animal-based diet is referred to as "diet".

Figures with regression lines: The regression lines and points are very difficult to see, probably due to being plotted transparent. I recommend avoid transparent feature and using thicker lines.

I hope you will find these comments helpful.

Reviewer #2

(Remarks to the Author)

The present work aims to assess whether diet and mean glucose concentration account for variation in cancer prevalence observed across species. The analyses indicate that mean plasma glucose concentration is not associated with diet but is negatively related to cancer prevalence across vertebrates. While I found the topic to be original and of great relevance, I have several concerns about this work.

First, most results seem to correspond simply to among-class differences in vertebrates, with birds consistently showing both higher glucose concentrations and lower cancer prevalence. There seems to be absolutely no sign of any relationship within vertebrate classes, which would be indicative of functional relationships. This is perfectly illustrated by the Figure 3 where the key result that cancer prevalence is negatively associated with mean plasma glucose concentration is far from convincing. Mean plasma glucose concentration only accounts for a very tiny proportion of variation observed in gastrointestinal malignancy prevalence (only 9%) and the relationship detected seems to come mostly from the combination between a much lower among-species variance in malignancy prevalence and a higher glucose concentration in birds compared to other groups. Within groups, we can hardly see any sign of a relationship. Based on that, in addition to the twelve speculative arguments provided by the authors to explain why birds are different, we could add other speculations such as the fact that birds have feathers while other vertebrates do not have.

Second, I found the variable transformations to be strange at the best. The trait-specific transformations performed before the statistical analyses have indeed no biological support. Allometric rules make the log-scale the natural transformation to perform in comparative analyses of life history traits, as consistently done in the large corpus of papers published since the fifties. What is the rationale to use gestation time raised to the power of 0.2 and the glucose concentration to the power of 0.425? A biologically-driven transformation should be obviously preferred. It is well established since Pearson that statistical tests are robust to deviation to normality. The only exception is bi- or multimodal trait distribution, a problem that will not be solved by the transformations used here.

Lastly, the sources of the data compiled in the excel file (Supplementary Data) are not provided. In particular, it seems from the main text that the authors have used a mixture between Species360 and databases such as AnAge. A clear statement for the origin of the species-specific data is badly needed.

Minor comments:

- l. 71: « detectable » instead of « significant »
- l. 143: « detectable » instead of « significant »
- l. 152: Remove « significantly »
- l. 199: Remove « significantly »
- l. 214: « detectable » instead of « significant »
- l. 223: Remove « significant »

I. 238: Remove « significantly »
 I. 282: Remove « significantly »
 I. 299: « statistically significant » instead of « significant »
 I. 285-304: The twelve statements provided to explain potentially higher plasma glucose and lower cancer prevalence in birds compared to other vertebrates would be better presented in a Table than in the main text.
 I. 407: Remove « significant »
 I. 408: A R² of 0.60 with a sample size of 160 must lead to a P-value well below 0.32. On I. 409-410, a R² slightly lower is associated with a very low P-value despite a much lower sample size. There is obviously something wrong here, which needs to be corrected.
 I. 410: Remove « significant »
 I. 414: Remove « significant »
 I. 416: Remove « significant »
 I. 422: « not statistically significant » instead of « non-significant »
 I. 425: Remove « significant »
 I. 432: Remove « significant »
 I. 440: « statistically significant » instead of « significant »
 I. 440: Remove « significant »
 I. 444: Remove « significantly »
 I. 445: Remove « significant »
 I. 447: Remove « significantly »
 I. 454: Remove « significantly »
 I. 460: Remove « significant »
 I. 466: Remove « significant »
 I. 468: Remove « significantly »
 I. 472: « statistically significant » instead of « significant »
 I. 482: Remove « significantly »
 I. 485: Remove « significantly »
 I. 491: Remove « significant »
 I. 493: « detectable » instead of « significant »
 I. 498: Remove « significant »
 I. 506: Remove « significant »
 I. 508: Remove « significant »
 I. 511: Remove « significant »
 I. 518: « detectable » instead of « significant »
 I. 523: Remove « significant »
 I. 528: Remove « significant »
 I. 531: Remove « significantly »
 I. 533: Remove « significantly »
 I. 539: « detectable » instead of « significant »
 I. 540: Remove « significantly »
 I. 549: « detectable » instead of « significant »
 I. 550: Remove « significant »
 I. 559: Remove « significant »
 I. 561: « detectable » instead of « significant »
 I. 562: Remove « significant »
 I. 571: Remove « significant »
 I. 573: « detectable » instead of « significant »
 I. 574: Remove « significant »
 I. 583: Remove « significant »
 Table 1C: R² values cannot be negative!

Reviewer #3

(Remarks to the Author)

Please find attached as a pdf a detailed review of the manuscript.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed my previous comments thoroughly. However, when checking the code, which they made available in this round of the review, I discovered several errors in the statistical analysis. Most importantly, the authors used Type I (sequential) ANOVA in an incorrect way in models with multiple predictors, namely in a way that did not control for the effects of other predictors. In Type I ANOVA, the order of the predictors in model formulas matters and it seems they used wrong order, at least in some models, when calculating p-values. For the explanation and correct use of Type I ANOVA in R, please see for example <https://www.r-bloggers.com/2011/03/anova-%E2%80%93-type-iiiiii-ss-explained/>.

For example, the p-value of the effect of blood glucose (variable `Mean_Blood_glucose_levels_from_ZIMS..colorimetry.automated...PLASMA.`; using less cumbersome names would be better in my opinion) on gastrointestinal cancer prevalence (`GIMalignancyPrevalence`) controlled for `Birds.vs.Mammals.vs.Ectotherms` according to the Table 1B (the topmost effect on page 44) is $p=0.0009$ with the coefficient -0.001 (btw., -0.0017 should be rounded to -0.002 and there are multiple rounding errors like this one in the paper). However, when this analysis is done correctly, the result is $p=0.26$ (not $p=0.0009$). Note that this is also the exactly same value as the p-value for the t-statistic of the coefficient for the effect of blood glucose in the summary table of the model printed by the function `summary()`. The fact that the association of blood glucose and gastrointestinal cancer is non-significant when the mammal/bird/ectotherm group is controlled for implies that (i) blood glucose and gastrointestinal cancer are not associated with these groups and (ii) their positive association on page 42 is due to differences in values of both variables between birds, mammals, and ectotherms. Similarly, p-value of the effect of blood glucose on general neoplasia prevalence (last model at page 43) is not $p=0.0001$, but $p=0.22$. In other words, there doesn't seem to be a general association between blood glucose and cancer or GI cancer, and the overall pattern is driven by differences among high taxonomic units. This also implies that all p-values from models with multiple predictors need to be checked and ANOVA needs to be reanalysed if done incorrectly.

In addition, as noted by reviewer 2, the combination of $R^2=0.63$ and $p=0.43$ stated in the caption of Figure 1 (currently line 558) is indeed an error. This error most probably resulted from not specifying the reduced model in the function `R2()` from the package `rr2`. When the tested model (`Mean_Blood_glucose_levels_from_ZIMS..colorimetry.automated...PLASMA.~TrophicLevel`) is compared to the reduced model specified as `Mean_Blood_glucose_levels_from_ZIMS..colorimetry.automated...PLASMA.~1`, R^2 obtained by the `R2_resid` method is 0.017. This value makes much more sense with $p=0.43$.

Taken together, the data analysis needs to be thoroughly verified and the errors corrected. I strongly recommend much closer collaboration with a statistician to ensure the robustness of the results.

Specific comments:

Lines 226-228: Do you mean that you simply excluded these diets with small sample sizes (i.e., occurring in less than 10 species, if I understand correctly) and recalculated the percentages based on the percentages of the remaining diets? This could hypothetically lead to a scenario, where a species consuming 90% fish and 10% plants would be assigned as 100% herbivore. Or were these excluded diets replaced by other diets to prevent such scenarios; for example, were proportions of "fish", "scavenger", and "unknown vertebrates" replaced by some other diet based on vertebrates with a larger sample size?

Glucose data collection: Please clarify whether the mean glucose levels reported in ZIMS are derived exclusively from fasted birds, or if they include measurements from both fasted and fed birds.

(Remarks on code availability)

There are several errors in the statistical analysis. Please see details in my comments to the authors.

Reviewer #2

(Remarks to the Author)

This revised version accounts for some problems identified in the previous version. However, I still find that there is only a very weak support for the claim that "mean plasma glucose concentration and trophic level/diet can be viewed as factors affecting cancer prevalence across species". This work convincingly shows that birds have the highest glucose concentrations among vertebrate groups, but fails to provide clear evidence that this is associated with low cancer prevalence. Figures 2 and 3 display a clear between-class variation of cancer prevalence in relation to glucose concentration across vertebrate classes but there is no detectable relationship between cancer prevalence and glucose concentration among species within a vertebrate class. This needs to be explicitly stated.

Detailed comments:

- I. 62: Remove "significantly"
- I. 64: Remove "significantly"
- I. 66: Remove "significantly"
- I. 93: Insert a dot before "However"
- I. 93: "in the third paragraph": what does it mean?
- I. 203: "detectable" instead of "significant"
- I. 240-241: Unclear what "determined the best model fit ($\Delta AIC = 2$)" means. Obviously there is some missing part to link a ΔAIC between 2 models of 2 and the retained model.
- I. 242: "statistically significant" instead of "significant"
- I. 243-244: Same problem as in lines 240-241.
- I. 320: Remove "significantly"
- I. 324: Remove "significantly"
- I. 327: Remove "significantly"
- I. 332: Remove "significantly"
- I. 342: Remove "significantly"
- I. 343: Remove "significant"

I. 352: Remove "significantly"
I. 455: "detectable" instead of "significant"

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)

The updated manuscript shows significant improvements over its previous iteration. The inclusion of additional information is appreciated. The authors have reexamined their dataset, incorporating requested confounding variables such as testing the potential effects of domestication and longevity. Furthermore, they have addressed the verification of assumptions underlying their model and provided clarification on methodological aspects that were ambiguous in the prior version.

In their best model, the mean glucose concentration accounts for a small portion of the dataset's variance, with an R-squared value of approximately 0.10. This suggests relatively weak evidence for a correlation between blood glucose concentration and malignancy prevalence. Upon further analysis, disaggregating the dataset into mammal, bird, or reptile subgroups reveals no significant trends between blood glucose concentration and various neoplasia types within any of the individual groups. Notably, it's only when these groups are amalgamated that the weak association becomes apparent, raising the possibility of it being spurious.

Analyzing neoplasia prevalence data in relation to diet type categorized as herbivore, omnivore, or carnivore, and controlling for blood glucose concentration, yields only slight improvement to the model. However, no significant differences between the groups are observed.

There is some uncertainty in the neoplasia prevalence point estimates (the median number of individuals used to calculate those estimates is 34.5 individuals). The noise in the response variable which is partially accounted for by weighting the estimates by $1/\sqrt{n}$ may explain the weak trend by itself. Additionally, the author highlights various limitations in the discussion section, implying that the introduction of additional unaccounted-for noise in this study could explain the observed pattern. Consequently, replicating this study with an independent dataset is deemed necessary to validate the existence of the trend. This may be difficult because of the scarcity of data on neoplasia prevalence in animals.

Line 389: "Our results also support our third hypothesis that trophic levels are positively correlated with cancer prevalence across tissues and neoplasia prevalence across tissues."

How would you explain that omnivore species have a higher cancer prevalence compared to carnivore species if it is not due to blood glucose concentration?

Line 506: "In almost every case, researchers say they would predict a correlation, but sometimes researchers find a correlation, sometimes they do not."

There could be a publication bias in these studies, as scientists may be less inclined to publish non-significant correlations compared to factors that either increase or decrease cancer risk. Nevertheless, I believe this manuscript, despite offering weak evidence, would still be a valuable addition to the literature once published, even if it doesn't appear in high-impact journals like Nature Communications and as long it acknowledges the limitations.

It would be nice to add the 95% confidence intervals bands around the regression lines on the plot as it helps visualising the uncertainty around those lines.

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In the revised version, the statistical analysis has been corrected, leading to changes in some of the results. Specifically, the association between blood glucose and neoplasia/cancer is now unsupported when controlled for the main taxonomic groups. This result is consistent with the negative results of the analysis done separately in each taxonomic group. Hence, the main issue that precluded the publication was solved. Nevertheless, there are still some specific issues detailed below that should be addressed in my opinion.

The last two sentences of the Abstract about the possible mechanisms linking high blood glucose levels and low neoplasia/cancer prevalence in birds are hypothetical and may make a false impression that these mechanisms are studied in the paper. I recommend either omitting such hypothetical conclusions in the Abstract or at least modifying the wording to explicitly state their hypothetical character.

Line 348: Please be more specific about what is meant by “caloric restriction inside bird cells”. Do you mean that birds may have lower intracellular glucose concentrations? It should be kept in mind that although some findings suggest that some avian tissues may have lower intracellular glucose concentrations, they may rely more on the use of fatty acids compared to mammals. In that context, I am not sure if lower intracellular glucose concentrations can be interpreted as intracellular caloric restriction. Besides, caloric restriction is a temporary state characterised by reduced energy and nutrient intake imposed by external conditions, which elicits a plastic response of the body. In contrast, species-specific intracellular glucose concentrations are genetically determined and were fine-tuned during the evolution of each species. Hence, it is questionable whether it is appropriate to term the latter “caloric restriction”. In addition, the ref. 33 (Holmes & Ottinger 2003) does not seem to contain any mention of intracellular caloric restriction in birds. A relevant reference should be provided.

Moreover, “a possibly higher uptake and faster metabolism of glucose by healthy avian bird cells relative healthy mammalian reptilian cells” is mentioned in Conclusion (lines 429-430). This seems to contradict the suggested intracellular caloric restriction discussed above (and also mentioned in the Abstract). And again, the ref. 94 (Dupont et al. 2004) doesn't seem to contain any mention of the possibly higher glucose uptake and metabolism by avian cells. In addition, remove the duplication in “avian bird cells”.

Lines 117-119: A preprint is cited as a source of data about the primary diet and trophic levels. In my opinion, the source studies or databases should be cited and the procedure at least briefly described here because preprints may change.

Lines 350-353: Given that KEAP1 is missing only in Neoaves but not in the chicken, its loss could not happen during the Permian-Triassic period, but rather around the Cretaceous-Tertiary boundary, when Neoaves originated.

Lines 353-354: There are two hormones – insulin and IGF-1 – rather than a single insulin/IGF-1 hormone and both are active in birds, at least in some tissues. Please clarify what you refer to in this sentence. The relative insulin resistance in birds when compared to mammals? Or lower activity of insulin/IGF-1 signalling (IIS) pathway? In addition, refs 65 (Kenyon et al. 1993) and 66 (Hsu et al. 2003) are about IIS signalling in nematodes. Appropriate citations supporting the claim about birds made in this sentence should be provided instead.

Lines 355-356: This is an outdated information. Avian homologue of TNF-alpha has been identified along with its receptors (please see Rohde et al. 2018, 10.3389/fimmu.2018.00605 and Lu et al. 2022, 10.1016/j.dci.2022.104374).

Lines 357-362: It seems something may have accidentally been deleted or forgotten here as the discussion of the findings is missing in this paragraph about domestication.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

This revised version accounts for some problems I identified when reading the previous version. However, I still have two major problems with this work.

First, I am still left unconvinced « that there is a negative correlation between mean plasma glucose concentrations and neoplasia and cancer prevalence » (as stated in l. 381-383). This work clearly shows that this relationship holds across vertebrate classes with birds showing both the highest glucose concentration and the lowest cancer or neoplasia prevalence, but I did not find any evidence in the current work that such a relationship occurred among species within a given vertebrate class, but reptiles as displayed in Figure 3. I would want to see replicated analyses per vertebrate class to make that point explicit. Not explicitly recognizing this marked difference in the relationships between among-class and among-species within a class leads to a very confusing discussion.

Second, I still have a major concern about the origin of the data analyzed. Note that I was not able to retrieve the dataset including species-specific trait values. Only broad references are provided and the assessment of trait values to a given species cannot be checked, which strongly limits the repeatability of the analyses reported here. An explicit species-specific reference for each trait value in the current work has to be provided. When a same reference is used for several species, it will be easy to repeat the reference for all species concerned.

Detailed comments:

l. 62: Remove »significantly »

l. 64: Remove »significantly »

l. 66: Remove »significantly »

l. 93: A dot is missing before « However »

l. 93: Remove « in the third paragraph »

l. 203: « statistically significant » instead of « significant »

l. 205-206: Whether cube-rooting the dependent variable allows accounting for heteroscedasticity in all cases where the square-rooted dependent variable failed should be explicitly stated.

l. 240-241: the statement « determined the best model fit (DeltaAIC=2) » does not make any sense. By definition, the best model fit is reached by the model including all the tested independent variable. AIC allows to balance model accuracy and model precision and the model retained should be the model with the lowest AIC (not the model with a DeltaAIC of 2!).

Burnham and Anderson's book on model selection published in 2002 provides a thorough user guide. The threshold of 2 for DeltaAIC between competing models is only indicative and should only apply when the model with the lowest AIC is the most complex model. Arnold (J. Wildl. Manage. 2010) provides a thorough justification for doing that.

l. 242: Remove »significant«. When using criteria issued from information theory, the concept of statistical significance is no longer appropriate as well explained by Burnham & Anderson (2002).

l. 243-244: Same problem here as above (l. 240-241)

l. 320: Remove « significantly »

l. 324: Remove « significantly »

l. 327: Remove « significantly »

l. 332: Remove « significantly »

l. 338: Remove « significant»

l. 342: Remove « significantly »

l. 343: « statistically significant » instead of « significant »

l. 352: Remove « significantly »

l. 385: Not « across vertebrates » True across vertebrate classes but not true across species within a vertebrate class! This needs to be explicitly stated.

l. 411: Is it really the case for all reptiles? What about Testudines with very slow pace of life and very long-lived? They seem to have an ageing rate even slower than that of birds (see e.g. da Silva et al. 2022 Science).

l. 428-429: This looks like an abusive generalization. Obviously birds have not increased lifespan relative to all reptiles. Reptiles have been shown to have a much higher diversity of ageing rates than birds or mammals (see Reinke et al. 2022 Science) but some reptile lineages display much longer lifespan than birds (see da Silva et al. 2022 Science). What about cancer prevalence in such very long-lived reptiles?

l. 439-440: Does a lambda of 0.75 mean a very low phylogenetic signal? This needs to be justified. Why should we expect a strong phylogenetic signal only when lambda is 1?

l. 455: Remove « significant »

(Remarks on code availability)

The code looks fine but I was not able to look at the dataset.

Reviewer #3

(Remarks to the Author)

This latest version this manuscript shows significant improvement compared to the previous one. It is much easier to read, the analysis significantly improved, and for me this paper can be accepted. This study will be a useful citation for those interested in the ecology and evolution of cancers.

Line 403: The effect of parasite removal was also tested on zoo mammals using Vincze et al.'s dataset. To the best of my knowledge, this is the only study using comparative analysis to investigate this effect on cancer risk in mammals. Consider citing it here to ensure comprehensiveness since you are listing all risk factors here.

"Cancer hygiene hypothesis: A test from wild captive mammals"

<https://doi.org/10.1002/ece3.10547>

All the best

(Remarks on code availability)

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors satisfactorily addressed all my additional comments, and, in my opinion, the manuscript is suitable for publication. I only have one minor comment to the edited text in the latest version.

In lines 192-193, raising to the power of 1/4, instead of 1/3, is referred to as cube-rooting. Please correct, depending on if cube-root or fourth-root (raising to the power of 1/4) transformation was used.

(Remarks on code availability)

I have already reviewed the code in the previous round of the review.

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Reviewer #1 (Remarks to the Author):

This manuscript presents a comparative study examining associations between diet, blood glucose, and cancer prevalence among vertebrates. The study is based on a sufficiently large data set and the data are analysed using appropriate statistical methods controlling for phylogenetic non-independence and correcting for a large number of tests performed. The results revealed a rather surprising negative relationship between blood glucose concentration and cancer, and supported an expected higher cancer prevalence in top predators. The study is timely as there has been a growing interest in comparative research of cancer in recent years. The results are of interest to researchers from a wide range of research fields, encompassing life history evolution, comparative physiology, and evolutionary medicine. Overall, I support the publication of this manuscript, although I also have several concerns and comments detailed below.

- **Hypotheses:** To hypothesise the negative relationship between blood glucose concentration and cancer prevalence only because some traits show opposite slopes of relationships within and among species does not seem to be a compelling a priori hypothesis. Or at least not the only possible hypothesis. In my opinion, when proposing such a hypothesis, a potential mechanism should also be proposed, explaining why high glucose concentrations should be associated with low cancer prevalence among species, when a positive correlation is expected within species. Otherwise, a similarly or even more plausible scenario could be the absence of a relationship between blood glucose and cancer among species. Such a hypothesis is more parsimonious than the one proposed in the manuscript because it only assumes that, over macroevolutionary time scales, species with high blood glucose evolved adaptations preventing pro-carcinogenic properties of high blood glucose concentrations, thereby leading to a decoupling of cancer prevalence from blood glucose concentrations, rather than assuming an additional mechanism leading to a somewhat counterintuitive negative correlation between these two variables. Resting metabolic rate-lifespan and blood glucose-oxidative stress relationships may serve as examples of the suggested scenario, as they show either negative or positive direction within species (Duarte & Speakman 2014 Age 36:9731; Brownlee et al. 2001 Nature 6865:819-820) but no association among species (Speakman et al. 2005 J Exp Biol 208:1717-1730; Vágási et al. 2023 bioRxiv doi: 10.1101/2023.05.09.539961). Finally, the third scenario, namely that blood glucose concentration may show a positive association with cancer prevalence, is also plausible as evidenced by the sentence in the Introduction promoting its examination (lines 88-89). After reading this sentence, it is in fact somewhat confusing to the reader why you only predict the opposite direction of correlation. In my opinion, it is better to formulate hypotheses for various scenarios unless at least a general mechanism that should lead to the negative correlation is proposed.
 - As the reviewer suggests, we have rephrased the 3rd paragraph of the introduction to explain the various different scenarios:
"Diet appears to correlate with cancer prevalence across... based on data across extant vertebrate species we would expect a negative correlation between plasma glucose levels and cancer prevalence."
- In addition, it should be clarified, whether the predicted correlation between blood glucose concentration and cancer prevalence (or its absence as suggested above) should be mediated by body mass or independent of it. Body mass have been reported to be associated positively with cancer prevalence (e.g., Møller et al. 2017 ref. 4, Compton et al. 2023 ref. 10) and negatively with blood glucose concentration (e.g., Umminger 1975 Comp Biochem Physiol A 52:455-458, Braun & Sweazea 2008 ref. 42, Tomášek et al. 2019 ref. 30, Tomášek et al. 2023 Ecol Lett 25:2203-2216), which would justify proposing a negative association between blood glucose and cancer when body mass is not controlled for. When controlling for body mass, all three scenarios discussed above may be plausible.
 - This is a good point. We now explain the reason we control analyses for body weight in one of our paragraphs before the statistical analyses section:
"Body weight (grams)... species from published resources^{17,71,72"}
 - We have also added this section in the results which explains the best model fit depending on the dependent variable (malignancy or neoplasia prevalence across tissues, gastrointestinal

malignancy or neoplasia prevalence, and non-gastrointestinal malignancy or neoplasia prevalence):

- *"We used cancer and neoplasia prevalence across tissues, gastrointestinal malignancy... significantly change the fit of the model ($\Delta AIC = 1.39$)."*
- We have also added this section in the discussion:
"Our finding that the variation in neoplasia... explained by body weight."

Specific comments

- **Line 50: Please note that Compton et al. 2023 (ref. 10) found a positive relationship between body size and cancer prevalence, not its absence as claimed here.**
 - Thanks for catching that. Compton et al. 2023 find a significant association between adult body weight and neoplasia prevalence (just not quite for malignancy prevalence).
 - We have now amended this section in the text
"The majority of studies testing this hypothesis, however, show that larger and longer-lived animals do not get more cancer^{5–10}."
to
"Body weight has also been significantly positively correlated with neoplasia prevalence across vertebrates¹⁷. The majority of studies testing this hypothesis, however, show that larger and longer-lived animals do not get significantly more cancer^{12–17}."
- **Line 51: Consider also including Dujon et al. 2023 (Proc R Soc B 290:20230940) and Compton et al. (ref. 10), who supported the relationship between litter size and cancer prevalence on much larger data sets of mammalian species than ref. 6 cited here.**
 - We have now amended this sentence in the text
"Other life history traits, such as shorter gestation length (across vertebrates¹⁰), and large litter size (in mammals⁶) or clutch size (in birds⁸), have been correlated with cancer prevalence."
to
"Other life history traits, such as shorter gestation length (across vertebrates¹⁰), and large litter size (in mammals⁶) or clutch size (in birds⁸), have been significantly correlated with cancer prevalence."
- **Line 62: Given that only blood glucose-diet associations are discussed below, provide references for the association between blood glucose level and species fecundity here (e.g., Tomášek et al. 2019 ref. 30 and Tomášek et al. 2023 Ecol Lett 25:2203-2216).**
 - Good point, thank you. We have now added this sentence in the text: *"Studies in birds have found a positive correlation between blood glucose levels and species clutch size^{20,21}."*
- **Lines 72 and 75: Please note that a higher blood glucose concentration in frugivores and nectarivores was reported recently (Tomášek et al. 2022 Ecol Lett 25:2203-2216).**
 - Thank you, good point.

We have now added this sentence in the middle of line 67:
"Across 160 passerine bird species, the species that eat mostly fruit and nectar have higher blood glucose concentrations than other passerine species (Tomášek et al. 2022)."
 - We have also amended line 75
"Diet appears to correlate with cancer prevalence across vertebrates^{13,19}, but does not appear to correlate with glucose levels in the above studies of a few species in the literature."
to

"As mentioned in the first paragraph, diet appears to correlate with cancer prevalence across vertebrates^{6,7}. However, its correlation with glucose levels in the third paragraph of different vertebrate species in the literature is controversial."

- **Lines 83-85 and 88-89: Three lines of reasoning were provided above how blood glucose level can be linked to cancer but, from here, it seems that only the one based on the Warburg effect is considered further. I suggest rewording the closing part of the paragraph starting from this sentence (lines 83-85) to make clear whether your expectation is made on all three lines of reasoning or only on the Warburg effect.**
 - Good point, we now replaced this sentence:
"Thus, an examination of whether the Warburg hypothesis holds across species is warranted."

with this

"Thus, based on data across extant vertebrate species we would expect a negative correlation between plasma glucose levels and cancer prevalence"
 - We have now removed the Warburg expectation.
- **Lines 105: Was liver regarded as a part of the gastrointestinal system?**
 - Yes. In this sentence, *"Data for neoplasia (malignant and benign tumors) ... a previous study⁶."* we mention that the gastrointestinal data were collected from a previous study (Kapsetaki et al. 2022), which states that
"Gastrointestinal malignancy or neoplasia prevalence of each species consists of the malignancy or neoplasia prevalence, respectively, of the oral cavity, esophagus, stomach, gallbladder, bile duct, liver, pancreas, duodenum, small intestine, and colon."
 - We have now also added this section in the methods:
"Specifically, the gastrointestinal tissues included are the oral cavity, esophagus, stomach, gallbladder, bile duct, liver, pancreas, duodenum, small intestine, and colon."
- **Lines 106-107: Please explain why the fact that the gastrointestinal tract is the largest organ in contact with the external environment should lead to a steeper slope of the predicted negative relationship between glucose and gastrointestinal cancer.**
 - We removed this sentence.
- **Line 147: What organs were included in the gastrointestinal system. Did you also include associated internal organs such as liver and pancreas?**
 - Yes. We have now added this section in the methods:
"Specifically, the gastrointestinal tissues included are the oral cavity, esophagus, stomach, gallbladder, bile duct, liver, pancreas, duodenum, small intestine, and colon."
- **Line 148: Change "from previous studies" to "from a previous study".**
 - We have now made this change in the text.
- **Line 151: Throughout the text below you use the term "body weight", rather than "body mass". I recommend using that term also here.**
 - Thanks, we have now changed this to body weight.
- **Line 151: It should be stated explicitly how was gestation length determined in oviparous species. Is it the incubation period?**
 - Yes, we have corrected this in the text. We now mention that we mean incubation length instead of gestation length in the case of oviparous vertebrates.
 - For example in the discussion *"the gestation (incubation in oviparous vertebrates) length"*, Table 1 *"gestation (incubation in oviparous vertebrates) length"*, and methods section *"gestation (incubation in oviparous vertebrates) length"*.
- **Lines 160-162: Have you considered logarithm-based transformation? Data like body mass are usually log-normally distributed. Also note that the statistical models assume normal distribution of residuals, not data.**
 - Since these statistical models assume normal distribution of residuals, not data, in our updated manuscript we have not transformed the dependent or independent variables, unless there is significant heteroscedasticity. If there is significant heteroscedasticity we do the following as we

mention now in the methods section: *"We tested all analyses for the presence of significant heteroscedasticity (Fligner-Killeen test), and if present, we square-rooted the dependent variable. If square-rooting the dependent variable still did not pass the Fligner-Killeen test, we cube-rooted the dependent variable."*

- **Lines 163-164: Centring do not change the shape of distribution of the data. Please explain.**
 - Thanks for this comment. We have now removed all centering from all analyses.
- **Line 167: Specify that “variables” are “dependent variables” here.**
 - We have now made this change in line 167.
- **Lines 172-173: Proportional variables such as percentage of food type are not normally distributed, and logit or arcsine transformation is used commonly. Why was no transformation used?**
 - The statistical models assume a normal distribution of residuals, not of the data, so therefore we only transformed the dependent variable if there was significant heteroscedasticity as we mention in our updated methods section: *"We tested all analyses for the presence of significant heteroscedasticity (Fligner-Killeen test), and if present, we square-rooted the dependent variable. If square-rooting the dependent variable still did not pass the Fligner-Killeen test, we cube-rooted the dependent variable."*
- **Lines 175-176: Please state explicitly whether binomial models were used for modelling prevalence data. In addition, overdispersion is a common problem with binomial models. Was its possible presence checked?**
 - We have now performed the Fligner-Killeen tests across all analyses to test for heteroscedasticity.
 - We have now added this section in the methods:
"We did not use binomial models for modeling the neoplasia prevalence and malignancy prevalence data. We tested all analyses for the presence of significant heteroscedasticity (Fligner-Killeen test), and if present, we square-rooted the dependent variable. If square-rooting the dependent variable still did not pass the Fligner-Killeen test, we cube-rooted the dependent variable."
- **Line 179: Please state whether you used the original Benjamini-Hochberg procedure, or the FDR adjusted P-values.**
 - I added this in the text *"using the Benjamini-Hochberg procedure"*.
- **In case of the former, I suggest stating here the highest P-value that passed the FDR threshold for significance for better orientation of which P-values were considered significant.**
 - We have now added this in the text. For example, in the results section *"In the regressions between diet and mean plasma glucose... corrections is 0.003."*, *"In the regressions between cancer or neoplasia prevalence and mean plasma glucose... FDR corrections was 0.009."*, and *"In the regressions between cancer and trophic level... corrections is 0.01."*
- **In addition, were the post-hoc tests (e.g., Figure 4A) included in the FDR procedure?**
 - We have now added the post-hoc tests in the FDR procedure. We now mention this in the methods section: *"9. post-hoc pairwise analyses comparing mean plasma glucose concentration of species in one trophic level versus another trophic level (Fig. 1A; 6 tests); 10. post-hoc pairwise analyses comparing mean plasma glucose concentration of species having one diet type versus another diet type (Fig. 1D; 3 tests); 11. post-hoc pairwise analyses comparing malignancy prevalence across tissues or gastrointestinal malignancy prevalence of species in one trophic level versus another trophic level (Fig. 4A, Fig. 5A; 12 tests); 12. post-hoc pairwise analyses comparing malignancy or neoplasia prevalence across tissues, gastrointestinal malignancy or neoplasia prevalence, or non-gastrointestinal malignancy or neoplasia prevalence of species having one diet type versus another diet type (Fig. 4D; Fig. 5A; Supp. Fig. 4; 18 tests)"]*
 - There is also a section in the legend of Figure 5 about these pairwise comparisons: *"Omnivores have higher gastrointestinal malignancy prevalence than herbivores (PGLS: P-value = 0.005), and carnivores have lower gastrointestinal malignancy prevalence than*

- omnivores (PGLS: P -value = 0.01). These correlations, however, do not remain statistically significant after applying corrections for multiple testing (Table 1B)."
- This section also refers to the multiple testing corrections in the diet type analyses: "There is no association between diet type and cancer prevalence among 67 species (Fig. 4D), neoplasia prevalence across tissues among 67 species (Supp. Fig. 4A), gastrointestinal cancer prevalence among 44 species (Fig. 5D), gastrointestinal neoplasia prevalence among 44 species (Supp. Fig. 4B), non-gastrointestinal cancer prevalence among 61 species (Supp. Fig. 4C), or non-gastrointestinal neoplasia prevalence among 61 species (Supp. Fig. 4D), with or without controlling for the variance in plasma glucose concentrations and after applying corrections for multiple testing (Table 1F)."
 - We have also added this section in the results: "Specifically, in terms of malignancy prevalence, we see that secondary carnivores have significantly higher malignancy prevalence than herbivores (P -value = 0.004); a result that remained significant after FDR corrections for multiple testing in the post-hoc analyses."
- **Lines 206-207: The (adjusted?) R^2 is negative in this relationship, when body mass is not controlled for (Table 1, Supp. Fig. 1B), so the explanatory power of the model is minimal if it is not a typo.**
 - We have added in the text that "This indicates that the explanatory power of these models is minimal."
 - We have also added this sentence in the methods:
"We obtained R^2 values by running the 'rr2' package (output 'R2.resid' values)⁷⁷ on each PGLS analysis."
 - **Line 207 and elsewhere: What is the rationale behind separating birds for this analysis while lumping mammals together with ectotherms ("non-birds"), when there are clear differences between mammals and ectotherms in blood glucose levels (e.g., Figure 2 showing three clear clusters - ectotherms, mammals, and birds)? In my opinion, it would make more sense to do this analysis while controlling for birds, mammals, and ectotherms, if you want to control for possible effects (on the intercepts of the relationships) of fundamental differences in physiology at high levels of phylogeny.**
 - We have now removed the analyses of birds vs non-birds, and as the reviewer suggests we have added analyses controlling for birds vs. mammals vs. ectotherms.
 - **Line 220: I guess the "positive" relationship is most probably based on visual assessment. But the trophic level was a categorical variable, so I recommend avoiding the term correlation here. Moreover, there is no significant difference between herbivores (primary consumers) and invertivores (secondary consumers). Therefore, I suggest rewording it in a more nuanced way to highlight the fact that only secondary carnivores (i.e., tertiary consumers) had significantly higher cancer prevalence.**
 - We have rephrased this section to "Trophic levels are a significant risk factor of cancer prevalence and neoplasia prevalence across tissues among 160 species, with and without controlling for the variance in species' plasma glucose concentrations (Fig. 4A), even after correcting for multiple testing (Table 1F). Specifically, in terms of malignancy prevalence, we see that secondary carnivores have significantly higher malignancy prevalence than herbivores (P -value = 0.004); a result that remained significant after FDR corrections for multiple testing in the post-hoc analyses."
 - **Lines 269-308: This part of the Discussion suggests that the results can at least partly be explained by the differences in physiology between birds and other vertebrates (i.e., birds have comparatively high blood glucose concentration and low cancer prevalence). But how do you interpret the fact that the results remained basically the same when birds versus other vertebrates were controlled for and that there was no difference between these two groups (Table 1B, second set of models, P -values of variable B)? Doesn't it indicate that the effect is due to the similar slopes of the glucose-cancer relationship within each group (i.e., within birds and within other vertebrates) rather than due to mean differences between those groups? I think this**

should be explained here, if the differences in physiology between birds and other vertebrates are proposed as one of the possible explanations.

- We explain this in the next section: lines 309-327.
- **Line 290: Please cite the primary source.**
 - The paper that we cite (Satoh 2021) mentions that:

"The insulin hypothesis has been accepted with great impact since Kenyon et al.[54] showed that mutations of the dauer formation 2 (DAF2) gene, which encodes an insulin/IGF-1 receptor, double the lifespan of Caenorhabditis elegans. In addition, a later paper showed that inhibiting insulin/IGF-1 signaling changes the lifespan through activating DAF-16 [55], a forkhead box protein O3 (FOXO3) transcription factor [56,57]. Several models of insulin/IGF-1 receptor KO mice [58–60] have lower fat mass and lower body weight than control mice and show an increase in mean lifespan [58–60]. The results of these studies may justify the statement that the birds are in the 'DAF2 mutation' group, whose insulin/IGF-1 receptors are constitutively inactive, doubling the lifespan of the wildtype (mammals)."

Therefore, Satoh 2021 makes this proposal/suggestion in his final sentence here.
 - Instead of *"Third, the constitutive inactivation of the ageing hormone insulin/IGF-181,82 approximately doubles bird lifespan72."* we have updated this sentence in our manuscript to *"Third, the constitutive inactivation of the ageing hormone insulin/IGF-181,82 possibly doubles bird lifespan72."*
- **Line 349: Were the glucose data collected from anaesthetised animals only? This should also be mentioned in the Methods.**
 - We have now added this section in the methods:

"It is not recorded in the ZIMS database whether or not the animals were anaesthetized, however, common veterinary practice suggests that some animals were anaesthetized and some were not. This is a potential source of noise in the data that we acknowledge."
 - We have also rephrased line 348-350:

"For example, the mean plasma glucose concentrations in our study were collected from anaesthetized animals (data from ZIMS), some of which may have"

to

"For example, the mean plasma glucose concentrations in our study were collected from animals (data from ZIMS), some of which may have been anaesthetized for blood withdrawal (information about whether the animals were anaesthetized is not provided in ZIMS), may have"
- **Lines 352-354: Capture stress elevates blood glucose concentration also in passerine birds (Tomasek et al. 2022 Ecol Lett 25:2203-2216).**
 - We have now added this reference in the text.
 - *"Anaesthesia or capture can mildly to moderately elevate the blood glucose levels of felines¹⁰⁸, dogs¹⁰⁹, cattle¹¹⁰, goats¹¹¹, mice¹¹², rats¹¹³, chickens¹¹⁴, pigeons¹¹⁵, passerine birds²⁶, "*
- **Lines 720-721: Incomplete citation**
 - We have updated this to the correct citation.
- **Line 872 and below: Although related, the results here are from the regression analysis, not correlation analysis. Consider rewording the captions.**
 - We have now reworded these parts in the text.
- **Table 1F, page 52: Given that the presentation of the results, and particularly those of diet, is quite complex, please use the terms consistently to avoid confusion. Elsewhere in the table, "diet type" is used for categories 100% herbivory vs. omnivory vs. 100% carnivory), whereas the percentage of plant-based and animal-based diet is referred to as "diet".**

- Thank you for this comment. We have now made this adjustment throughout the manuscript. We have kept the term "diet type" when referring to the categories 100% herbivory vs. omnivory vs. 100% carnivory, and the term "percentage of diet" for the percentages of animal-based vs. plant-based diet as well as the percentages of fruit, plants, invertebrates, seeds, vertebrate endotherms, and vertebrate ectotherms.
- **Figures with regression lines: The regression lines and points are very difficult to see, probably due to being plotted transparent. I recommend avoid transparent feature and using thicker lines.**
 - Thank you for this comment. We have now removed the transparent feature in those figures, and the data points and lines are much clearer.
- **I hope you will find these comments helpful.**
 - Thank you!

Reviewer #2 (Remarks to the Author):

The present work aims to assess whether diet and mean glucose concentration account for variation in cancer prevalence observed across species. The analyses indicate that mean plasma glucose concentration is not associated with diet but is negatively related to cancer prevalence across vertebrates. While I found the topic to be original and of great relevance, I have several concerns about this work.

- **First, most results seem to correspond simply to among-class differences in vertebrates, with birds consistently showing both higher glucose concentrations and lower cancer prevalence. There seems to be absolutely no sign of any relationship within vertebrate classes, which would be indicative of functional relationships. This is perfectly illustrated by the Figure 3 where the key result that cancer prevalence is negatively associated with mean plasma glucose concentration is far from convincing. Mean plasma glucose concentration only accounts for a very tiny proportion of variation observed in gastrointestinal malignancy prevalence (only 9%) and the relationship detected seems to come mostly from the combination between a much lower among-species variance in malignancy prevalence and a higher glucose concentration in birds compared to other groups. Within groups, we can hardly see any sign of a relationship. Based on that, in addition to the twelve speculative arguments provided by the authors to explain why birds are different, we could add other speculations such as the fact that birds have feathers while other vertebrates do not have.**
 - Good point, however, the results between gastrointestinal malignancy and gastrointestinal neoplasia prevalence vs. mean plasma glucose concentration remain significant when we control for birds vs. mammals vs. ectotherms.
So explaining the differences between birds and other vertebrates is one part of trying to explain the observed results, and does not entirely explain the results. In lines 309-327 we provide additional possible explanations for these results that are not entirely based on phylogeny.
 - **"Within groups, we can hardly see any sign of a relationship."** That is correct.
- **Second, I found the variable transformations to be strange at the best. The trait-specific transformations performed before the statistical analyses have indeed no biological support. Allometric rules make the log-scale the natural transformation to perform in comparative analyses of life history traits, as consistently done in the large corpus of papers published since the fifties. What is the rational to use gestation time raised to the power of 0.2 and the glucose concentration to the power of 0.425? A biologically-driven transformation should be obviously preferred. It is well established since Pearson that statistical tests are robust to deviation to normality. The only exception is bi- or multimodal trait distribution, a problem that will not be solved by the transformations used here.**

- We have now removed all transformations and only transformed the dependent variable if there was significant heteroscedasticity as we mention in our updated methods section: *"We tested all analyses for the presence of significant heteroscedasticity (Fligner-Killeen test), and if present, we square-rooted the dependent variable. If square-rooting the dependent variable still did not pass the Fligner-Killeen test, we cube-rooted the dependent variable."*
- **Lastly, the sources of the data compiled in the excel file (Supplementary Data) are not provided. In particular, it seems from the main text that the authors have used a mixture between Species360 and databases such as AnAge. A clear statement for the origin of the species-specific data is badly needed.**

- We mention the sources of our body weight and gestation length data in the text: *"we collected body weight... from published resources^{17,71,72}."*

the malignancy and neoplasia prevalence data:

"Data for neoplasia (malignant and benign tumors) and malignancy prevalence, including gastrointestinal neoplasia and malignancy prevalence as well as non-gastrointestinal neoplasia and malignancy prevalence, were collected from a previous study¹³."

the glucose concentration data:

"We collected records of mean plasma glucose concentrations (measured in mmol/L) for each species as recorded in the ZIMS database (<https://www.species360.org/>)."

and diet/trophic level data:

"We identified trophic levels (herbivore, invertivore, primary carnivore, or secondary carnivore) for n=160 species based on records from previous studies (classified for each species based on their primary diet)¹³. We collected food type percentages (endothermic vertebrates, ectothermic vertebrates, fish, fruit, invertebrates, nectar, plant, scavenger, seed, and unknown vertebrates) from Jim Song et. al. (2020) for n=67 species⁵⁷."

- **Minor comments:**
- **I. 71: « detectable » instead of « significant »**
- **I. 143: « detectable » instead of « significant »**
- **I. 152: Remove « significantly »**
- **I. 199: Remove « significantly »**
- **I. 214: « detectable » instead of « significant »**
- **I. 223: Remove « significant »**
- **I. 238: Remove « significantly »**
- **I. 282: Remove « significantly »**
- **I. 299: « statistically significant » instead of « significant »**
 - Thank you. We have now made these changes.
- **I. 285-304: The twelve statements provided to explain potentially higher plasma glucose and lower cancer prevalence in birds compared to other vertebrates would be better presented in a Table than in the main text.**

- This is a good suggestion that we are considering, however, there are some long descriptive sentences in this section that wouldn't very well fit the style of short descriptions in a table.

e.g. "Eighth, birds have erythrocytes with a nucleus and high metabolic activity, thus increasing their intracellular concentration of haemoglobin relative to mammals and reptiles, and maximizing the use of the limited oxygen in the atmosphere during the Permian-Triassic⁷²."

and

"Twelfth, the antioxidant NRF2 is constitutively expressed in most birds as an evolutionary consequence of the loss of a binding domain in its repressor KEAP1 during the Permian-Triassic period⁷²."

- **I. 407: Remove « significant »**
 - We have now made this change.

- **I. 408: A R² of 0.60 with a sample size of 160 must lead to a P-value well below 0.32. On I. 409-410, a R² slightly lower is associated with a very low P-value despite a much lower sample size. There is obviously something wrong here, which needs to be corrected.**
 - From a conversation with a statistician (Ping) in our team:
"There are some pros and cons of using R²_resid:

Pros:

It is the closest measure to the ordinary least squares, which provides a simple way to interpret the variation.

It provides better results in terms of aligning with the data dispersion.

Cons:

It gives negative R² values (indicating a bad model fit), which to some extent contradicts the significance we see in the p-value (indicating a highly significant single predictor variable in the regression)."
- **I. 410: Remove « significant »**
- I. 414: Remove « significant »**
- I. 416: Remove « significant »**
- I. 422: « not statistically significant » instead of « non-significant »**
- I. 425: Remove « significant »**
- I. 432: Remove « significant »**
- I. 440: « statistically significant » instead of « significant »**
- I. 440: Remove « significant »**
- I. 444: Remove « significantly »**
- I. 445: Remove « significant »**
- I. 447: Remove « significantly »**
- I. 454: Remove « significantly »**
- I. 460: Remove « significant »**
- I. 466: Remove « significant »**
- I. 468: Remove « significantly »**
- I. 472: « statistically significant » instead of « significant »**
- I. 482: Remove « significantly »**
- I. 485: Remove « significantly »**
- I. 491: Remove « significant »**
- I. 493: « detectable » instead of « significant »**
- I. 498: Remove « significant »**
- I. 506: Remove « significant »**
- I. 508: Remove « significant »**
- I. 511: Remove « significant »**
- I. 518: « detectable » instead of « significant »**
- I. 523: Remove « significant »**
- I. 528: Remove « significant »**
- I. 531: Remove « significantly »**
- I. 533: Remove « significantly »**
- I. 539: « detectable » instead of « significant »**
- I. 540: Remove « significantly »**
- I. 549: « detectable » instead of « significant »**
- I. 550: Remove « significant »**
- I. 559: Remove « significant »**
- I. 561: « detectable » instead of « significant »**
- I. 562: Remove « significant »**
- I. 571: Remove « significant »**
- I. 573: « detectable » instead of « significant »**
- I. 574: Remove « significant »**
- I. 583: Remove « significant »**
 - We have now made these changes.
- **Table 1C: R² values cannot be negative!**
 - R² values can be negative.

e.g., from the following article "R-squared can have negative values, which mean that the regression performed poorly." <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8279135/>

Reviewer #3 (Remarks to the Author):

This manuscript conducts a comparative analysis of vertebrate species to explore potential associations between trophic position and blood glucose concentration and their relationship to the prevalence of malignant cancer and neoplasms. This is an interesting hypothesis to investigate, as it is currently unclear which factors primarily contribute to cancer risk across vertebrates. One significant challenge in addressing this question is the scarcity of high-quality, standardized cancer datasets across different species. Existing datasets exhibit substantial heterogeneity in data collection methods and the treatment of censoring.

To examine these potential relationships, the authors employed a phylogenetic least square regression modelling approach to account for the lack of evolutionary independence among closely related species.

Although I believe the idea has merit and should be explored (with results reported and published, even if they turn out to be negative), the statistical analyses conducted in this manuscript do not provide the means to address the initial research questions.

Here are some key points the authors will need to check while they are reanalysing their dataset:

- **Quantify the heterogeneity in the prevalence dataset they used and identify potential sources of bias. If possible, incorporate these factors into the statistical analyses.**
 - Thank you for this comment. We have now controlled for domestication, and both domestication and mean plasma glucose concentration in our analyses.
- **The assumptions of the phylogenetic linear regression models are not always met. Caution should be exercised in interpreting the model results. Some models may exhibit an excessive number of zero values, the response variable deviates from a normal distribution, and there is heteroscedasticity in the data that needs to be addressed.**
 - In our updated manuscript, we test for heteroscedasticity in all analyses, and if there is significant heteroscedasticity we have done the following transformations in the dependent variable as we mention in our updated methods section: *"We tested all analyses for the presence of significant heteroscedasticity (Fligner-Killeen test), and if present, we square-rooted the dependent variable. If square-rooting the dependent variable still did not pass the Fligner-Killeen test, we cube-rooted the dependent variable."*
- **It's crucial to simultaneously assess multiple potential risk factors since species are exposed to various factors throughout their lifetimes. It is unclear from the manuscript whether this was tested for all relevant risk factors. Consider using a model selection procedure, such as AIC based methods, to aid in this task.**

- Thank you for this comment. We have followed this suggestion and performed univariate and multivariate model comparisons, comparing the AIC of different models. We now mention this in the methods section *"In the subset of species that had all the following variables ... an explanatory variable, and determined the best model fit ($\Delta AIC = 2$)."* and in the results section *"We used cancer and neoplasia prevalence across tissues,...change the fit of the model ($\Delta AIC = 1.39$)", "When comparing univariate and multivariate phylogenetic models with malignancy or neoplasia prevalence... malignancy prevalence; $\Delta AIC = 1.95$ in the case of neoplasia prevalence)", and Discussion " In the null models ...(0.75; 0.60; and 0.39, respectively)".*
- **In addition to reporting p-values and R2 values, it's essential to present effect sizes (coefficients of the model) associated with each risk factor. A high R2 value may not necessarily indicate a large effect size, while a model with an intermediate R2 value and a substantial effect size may highlight a more important contributor to cancer risk.**
 - We have now added all the coefficients of the models in Table 1.
- **I recommend including null models and comparing them to models incorporating the various risk factors to assess if they perform better than the null model. This approach helps control for both false positives and false negatives.**
 - We have now compared univariate and multivariate models. We have also added this section in the methods: *"In the subset of species that had all the following variables... determined the best model fit ($\Delta AIC = 2$)."*

You will find below additional comments and suggestions. I strongly encourage the authors to reanalyse their dataset, assess potential biases (and acknowledge them in the manuscript if they cannot be accounted for), and reevaluate whether the data supports their hypothesis. It is highly probable that the results and discussion sections will need substantial revisions once the data is reanalysed.

Introduction

- **Line 61: Please add example of risk factors directly associated with glucose.**
 - In lines 64-68 we provide examples of associations between glucose and diet.
 - Also, in lines 79-81, we mention the direct link between increased glucose levels and increased oxidative stress, DNA damage, glycated proteins, and inflammation.
- **Line 69: There is a lack of consistency in the paper in how species names are reported, sometimes only the common names are reported, sometimes they are reported with the scientific names.**
 - We have now used the common names in the text, and have added the scientific names in parentheses the first time they are mentioned.
- **Line 82: The issue with this prediction lies in the possibility that if blood glucose concentrations are indeed a significant cancer risk factor for species under typical environmental conditions and contribute to mortality during their reproductive period, natural selection may have optimized anti-cancer defences to prevent cancer development linked to glucose. In such a scenario, we would anticipate an absence of a relationship between cancer prevalence and glucose concentrations. This is for example observed in the case of invasive placentation as a cancer risk factor in mammals.**

- This is a good point. We have now added a section about this in the discussion:
- *"There are multiple risk factors... statistical analyses performed."*

- **Line 83-89: This explanation is not clear. What is meant here by "Warburg expectation". I suggest clarifying the hypothesis here.**
 - We have now removed the Warburg expectation.

- **Line 91: Small dogs are the product of artificial selection by Humans. I am not sure it is very relevant to compare it to other species that evolved under mostly under natural selection.**
 - Good point, we have now removed this section.

- **Line 105: Here I would expect natural selection to select for strong anti-cancer defences in those organs, and thus a lack of relationship.**
 - We have now removed this sentence.

- **Line 129: If my understanding of this categorization is correct, an animal that consumes a 95% animal-based diet, with only 5% being plant-based, would be classified as omnivorous and grouped into the same category as an animal with a 50%-50% diet. For instance, in the dataset, *Anas acuta* is labeled as herbivorous, but the table shows a diet consisting of 30% invertebrates and 70% invertebrates. If I interpret the criteria correctly, it should be categorized as omnivorous rather than herbivorous.**
 - The trophic level characterization is based on different criteria than the diet type categorization and percentage of animal-based vs. plant-based foods.
 - The dataset says 30% "Diet_%.of.animal.based" and 70% "Diet_%.of.plant.based".
 - Yes, in the diet type category, *Anas acuta* is classified as an omnivore.

- **If the diet data is accurate, why not use the of percentage of plants and animals in the diet as continuous explanatory variables? It would also allow to include an interaction term between those two variables.**
 - Good point. The percentage of diet variables are continuous and are analysed as such.

- **Line 140: I have reservations about using mean values of glucose concentration in this context. It's essential to consider the typical sample sizes (i.e., the number of glucose measurements) for the species included in the analyses and assess their repeatability.**
 - We have used a sample size of ≥ 20 individuals. We have added this in the text (Methods section).
 - *"We obtained records of mean plasma glucose concentrations (measured in mmol/L) from at least 20 different individuals per species"*
- **To address potential measurement errors in the explanatory variables, especially in cases where repeated measurements were taken for the same species, it's crucial to incorporate them properly into comparative analyses. One of the assumptions of linear models is that all explanatory variables are accurately measured without error. This issue can be addressed, for instance, by employing a Bayesian framework and utilizing the brms R package. <https://paul-buerkner.github.io/brms/>**

- The data that we have are aggregated species-level data from ≥ 20 records of different individuals per species. Our inputs for the PGLS analyses are on the species level, i.e., each row in the entry dataset for the PGLS is species-level data with summarized information from the total number of individuals in that species. Since the PGLS is on the species-level and it is comparing each species independently, the repeated measurements within a species are not an issue.
- **Lines 146: How much do the methods used to determine prevalence in the studies differ?**
 - The methods used to determine the prevalence in this study are the same as the previous study cited in this sentence: *"Data for neoplasia (malignant and benign tumors) and malignancy prevalence, including gastrointestinal neoplasia and malignancy prevalence as well as non-gastrointestinal neoplasia and malignancy prevalence, were collected from previous studies13."*
- **For example, do the prevalence estimates account for both right and left censoring?**
 - Thank you for this comment. The neoplasia and malignancy prevalence data are based on necropsies, not biopsies, so we had neither left nor right censoring.
- **Were cancer cases exclusively identified in necropsied animals, or were they also detected in living ones?**
 - Only necropsies. We have now added in that sentence "from necropsies of at least 20 individuals"
- **What potential biases exist in cancer diagnostics among the different species included in the study? It's crucial to address this heterogeneity, as it can introduce statistical noise into the analyses.**
 - We have tested for heteroscedasticity in all the updated analyses, and controlled for domestication.
 - To avoid any biases such as possibly more cancer being found in species where people have done more necropsies, we also control for the number of necropsies as we mention in the methods: "In analyses where one of the dependent variables was malignancy prevalence or neoplasia prevalence, we weighted the analyses by $1/(\text{square root of the number of necropsies per species})$ (from Revell65)."
 - We have also added this section in the text: *"Some types of cancer may... be a source of noise."*
- **Line 149: Please provide a reason of why the threshold of 20 individuals was selected.**
 - We have now added this in the text: *"The choice of a 20 necropsy threshold is a compromise between accuracy of the prevalence estimates and the number of species in the analysis. A higher threshold would reduce the number of species in the analysis and so reduce our statistical power. A lower threshold would add noise to the dependent variables (malignancy or neoplasia prevalence)."*
- **Line 151: Among mammals, placentation is linked to an elevated risk of cancer mortality, and mammal species with intermediate levels of placentation invasiveness exhibit higher mortality risks, likely because species with invasive placentation evolved stronger anti-cancer defences. If the goal is to incorporate risk factors associated with reproduction, then it should be considered as a factor in the analysis, particularly within the subset of mammals.**

See for example the publications below.

Dujon, A.M., Vincze, O., Lemaitre, J.-F., Alix-Panabières, C., Pujol, P., Giraudeau, M., Ujvari, B., Thomas, F., 2023. The effect of placentation type, litter size, lactation and gestation length on cancer risk in mammals. *Proc. R. Soc. B Biol. Sci.* 290, <https://doi.org/10.1098/rspb.2023.0940>.
<https://doi.org/10.1098/rspb.2023.0940>

Kshitiz, Afzal, J., Maziarz, J.D., Hamidzadeh, A., Liang, C., Erkenbrack, E.M., Nam, H., Haeger, J.D., Pfarrer, C., Hoang, T., Ott, T., Spencer, T., Pavličev, M., Antczak, D.F., Levchenko, A.,

Wagner, G.P., 2019. Evolution of placental invasion and cancer metastasis are causally linked. *Nat. Ecol. Evol.* 3, 1743–1753. <https://doi.org/10.1038/s41559-019-1046-4>

- Thank you. We tested for a role of placentation. Placentation is not predictive in our data, that's why we don't control for it because it is not predictive of increased malignancy or neoplasia prevalence.
- **If body mass is accounted for, then life span should be accounted for too. Vincze et al. showed a negative weak trend between cancer mortality risk and body mass, and a weak positive trend with adult lifetime expectancy.**

Vincze, O., Colchero, F., Lemaître, J.-F., Conde, D.A., Pavard, S., Bieuville, M., Urrutia, A.O., Ujvari, B., Boddy, A.M., Maley, C.C., Thomas, F., Giraudeau, M., 2022. Cancer risk across mammals. *Nature* 601, 263–267. <https://doi.org/10.1038/s41586-021-04224-5>

- Thank you for this comment. We have now controlled the analyses between cancer and neoplasia prevalence and plasma glucose concentration for longevity. We have also added this section in the methods: *"Body weight (grams)... species from published resources^{17,71,72"}*

Statistical analysis

- **Line 156: I may have overlooked it, but it seems that the R code used for analysing the dataset was not provided, making it challenging to verify the results of the statistical analyses conducted by the authors.**
 - We have now added the R code in the supplementary material:

"Supplementary Material

We provide the code used for the statistical analyses."

- **Line 162: Were the variables centered because the model had issues converging otherwise?**

If I understand correctly, the raw prevalence values were just centred. Looking at the raw data provided as supplementary material, the raw prevalence values are positively skewed, which violate that the assumption the response variable should follow a normal distribution. Centring the variable would not remove the skew. Vincze et al. logit transformed their response variable to account for this.

How was it checked that the model assumptions were satisfied, such as the normal distribution of residuals and response variable or the absence of heteroscedasticity in the data (which seems quite very likely here)? This information should be explicitly outlined in the Materials and Methods section of the manuscript.

- We have now removed the centering and performed tests to check for presence of significant heteroscedasticity (Fligner-Killeen test). We have also added this section in the methods: *"We tested all analyses for the presence of significant heteroscedasticity (Fligner-Killeen test), and if present, we square-rooted the dependent variable. If square-rooting the dependent variable still did not pass the Fligner-Killeen test, we cube-rooted the dependent variable."*

- **Line 170: The authors explored the influence of various potential risk factors on cancer prevalence in vertebrates. While they mention the use of both univariate and multivariate models, the details regarding how these models were constructed, whether interactions between risk factors were considered, and how the authors determined the most parsimonious model remain unclear. Additionally, it's not apparent whether a model selection procedure was employed to identify the most parsimonious model.**
 - Thank you for this comment. We have now added analyses on model comparisons (comparing the AIC of different models), and have added this section in the methods: *"In the subset of species that had all the following variables... determined the best model fit ($\Delta AIC = 2$)."*
- **The authors utilized a false-discovery test applied to p-values to assess the impact of a risk factor on cancer prevalence. Although the authors report P-values and R² values for the fitted models, they do not provide coefficient estimates for these models. It's worth noting that P-values may be not very reliable in the context of PGLS analyses. It is crucial to take into account and report effect sizes for each considered risk factor. Relying solely on correlation coefficients is insufficient, as it does not provide information about the magnitude of the regression slope between two variables.**
 - We have added the correlation coefficients of all analyses in Table 1.

Results:

- **I have reservations about the robustness of the results presented in this section. I've identified significant violations of the assumptions underlying the PGLS models employed by the authors. The Materials and Methods section does not provide information on how the authors verified these assumptions or addressed them when they were violated. It is necessary to reanalyze the data to address these issues.**
- **If, following more rigorous statistical analyses, the dataset fails to support the initial analysis, I strongly recommend that the authors publish these findings. Such information is valuable for advancing the field.**
 - We have now performed tests to check for presence of significant heteroscedasticity (Fligner-Killeen test). We have also added this section in the methods: *"We tested all analyses for the presence of significant heteroscedasticity (Fligner-Killeen test), and if present, we square-rooted the dependent variable. If square-rooting the dependent variable still did not pass the Fligner-Killeen test, we cube-rooted the dependent variable."*
- **Figure 1: I find difficult to distinguish between the colours in panels B and C because they have a high transparency. I suggest increasing the contrast of those panels.**
 - Good point. We have now removed the transparency in those panels, and they are much clearer.
- **Figure 2: Several aspects in this figure, as well as in all the other figures presenting regression results in this manuscript, require careful examination to ensure the validity of the model. A significant number of zero values are present in the data, so it is crucial to verify that they do not impact the model (i.e., the model is not zero-inflated). Additionally, heteroscedasticity is noticeable in the data, particularly in cases of high mean plasma glucose concentrations, this must be accounted for. The response variable also appears to be untransformed, and it does not seem to follow a normal distribution.**

Unless the authors accounted for the abovementioned sources of biases, in such cases they need to clarify it in the methods section, I don't think their analyses are very robust.

- Since these statistical models assume normal distribution of residuals, not data, in our updated manuscript we have not transformed the dependent or independent variables, unless there is significant heteroscedasticity. If there is significant heteroscedasticity we do the following as we mention now in the methods section: *"We tested all analyses for the presence of significant heteroscedasticity (Fligner-Killeen test), and if present, we square-rooted the dependent variable. If square-rooting the dependent variable still did not pass the Fligner-Killeen test, we cube-rooted the dependent variable."*
- **I believe it's essential to incorporate confidence error bands on PGLS plots. While I understand that obtaining these bands using the capper package can be challenging, I recommend considering these analyses within a Bayesian framework. While they may be somewhat more complex to conduct, they offer the significant advantage of accurately quantifying the uncertainty for each parameter of the model.**
 - I assume the reviewer refers to the violin plots. In the violin plots, for each category we show the median and every single data point. We have also performed analyses comparing each category, therefore we do not think it's necessary for the aim of this study to perform a Bayesian analysis.
 - The same reviewer also suggests using the Bayesian framework in a previous comment.

"To address potential measurement errors in the explanatory variables, especially in cases where repeated measurements were taken for the same species, it's crucial to incorporate them properly into comparative analyses. One of the assumptions of linear models is that all explanatory variables are accurately measured without error. This issue can be addressed, for instance, by employing a Bayesian framework and utilizing the brms R package."

However, we do not have a case of repeated measurements - each plasma glucose concentration measurement was taken from a different individual within a species, and we have the mean value of those plasma glucose concentrations for each species in our dataset.

Discussion

Line 271:

- **I am not sure whether the pattern will persist once the data is reanalyzed, but even without that, predictions can be possible.**
 - The pattern still exists with the reanalysed data.
- **Given that the divergence in metabolic pathways between birds, mammals, and reptiles occurred millions of years ago, my prediction would be that natural selection has fine-tuned anti-cancer defenses to minimize the emergence of fitness-reducing malignant tumors during their reproductive phase. Consequently, I wouldn't anticipate birds with elevated blood glucose concentrations to exhibit a high prevalence of malignant tumors. The authors have cited various adaptations that, if this hypothesis holds true, could account for this observed pattern.**
 - Yes, we provide possible explanations for the observed pattern in lines 276-327.

- Line 293: Adaptations such as being more physically active compared to what? What is meant here by physically active? In term of energetic expenditure? Distances travelled?**
 - Compared to mammals. We have now added in that sentence *"and have higher energy expenditures than mammals"*.
 - We also mention the higher metabolic rates of birds versus mammals in a later sentence (starting "Ninth, ...").
 - Birds being more physically active is based on this section in previous literature *"What factors might underlie the ability of birds to live long and healthy lives? A striking feature of birds is that they are extremely athletic given that most species (>99%) fly. Birds attain high respiratory efficiencies due to flow-through counter-current pulmonary systems, and their metabolic rates (MRs) and estimated lifetime energy expenditures are 2.5–15 fold greater than that of similar-sized mammals (Harr, 2002)."* <https://www.sciencedirect.com/science/article/pii/S1568163711000742>
- Line 299: The study referenced here (reference 13) is a preprint authored by the same first author. It investigates another interesting hypothesis but employs a very similar statistical approach with comparable limitations to this manuscript. I recommend taking into account my comments on this manuscript for the evaluation of the second manuscript as well.**
 - Thank you - we are going to apply your suggestions to this other manuscript too.
 - In revisions of the other paper, the pattern with trophic levels and low habitat productivity holds true, but the pattern of malignancy or neoplasia prevalence with metabolic rate does not remain significant therefore we have rephrased this section:

"Other variables such as low habitat productivity (i.e., grams of glucose produced in the habitat of species per square meter per year), and low inferred metabolic rates are also correlated with neoplasia prevalence across vertebrates, but not after False Discovery Rate corrections for performing multiple tests13."

to

"Other variables, such as low habitat productivity (i.e., grams of glucose produced in the habitat of species per square meter per year), are correlated with neoplasia prevalence across tissues, gastrointestinal cancer prevalence, and gastrointestinal neoplasia prevalence across vertebrates even after False Discovery Rate corrections for performing multiple tests13."
 - We have also removed this section:

"In a study across vertebrates, including birds and mammals, higher inferred metabolic rates were correlated with lower neoplasia prevalence though these results did not remain statistically significant after multiple testing corrections13."
- Line 313 – 315: The way to test this would be to use a null model (i.e., here a model with no explanatory variable) and determine the proportion of variance in the dataset that is explained by the phylogenetic relationship alone. Overall, it is a good idea to compare all your models to a null model, it helps ensuring that a given risk factor really contributes to the pattern of cancer observed between species.**
 - We have now performed this AIC comparison in the updated manuscript: *"In the subset of species that had all... determined the best model fit ($\Delta AIC = 2$)."*
 - And we mention the results in the results section.
 - We have also added this in line 312.

“In the null model of gastrointestinal neoplasia prevalence and no explanatory variable, the phylogenetic signal lambda is less than 1 (0.75). In the null model of gastrointestinal malignancy prevalence and no explanatory variable, the phylogenetic signal lambda is also less than 1 (0.60).”

- **Line 314: Would the microbiome explanation be the most parsimonious one?**
 - Unfortunately we do not have quantitative microbiome data for each species in our dataset to answer this question.
- **Line 309: Bats have a number of anti-cancer defences that differs from other species that may explain this pattern see the papers bellow**
- <https://www.nature.com/articles/s41467-019-10495-4>
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4764838/>
<https://academic.oup.com/gbe/article/15/9/evad148/7259420>
<https://www.science.org/doi/10.1126/sciadv.aao0926>
- <https://bmcbgenomics.biomedcentral.com/articles/10.1186/s12864-016-3227-8>
 - The point of this sentence and the following (line 309-312) is to show there are not only large differences in glucose levels between mammals vs. birds. But if we look within mammals, there are also large differences in glucose levels among some mammalian species.

I'd rather not propose an explanation for the negative correlation between mean plasma glucose levels and cancer prevalence (across tissues or gastrointestinal) across ≥ 108 species by explaining the anti-cancer defences of bats species because we only have 2 bat species (*Rousettus aegyptiacus* and *Rousettus lanosus*) in our cancer and neoplasia prevalence ~ mean plasma glucose analyses, and only one bat species (*Rousettus aegyptiacus*) in our gastrointestinal cancer or gastrointestinal neoplasia prevalence ~ mean plasma glucose analyses.

- **Line 320: Domestication has an effect on cancer risk, this it must be included in statistical analyses as it is an important confounding factor.**
 - We have now controlled for domestication in our analyses.
- **Lines 348-352: This is, in my opinion, why it is important to include repeated glucose measurements in the analysis to account for that variability (e.g. with the brms package).**
 - We only have one type of variable for glucose, i.e., mean plasma glucose concentration. What exactly is meant by repeated glucose measurements? In the ZIMS database there is the mean value of glucose concentration of each species, but not the exact values of plasma glucose concentration for all individuals in that species.
 - We account for phylogeny using phylogenetic regressions (pgls).
- **Line 361-364: It is important to note in the limitations section that certain taxa commonly regarded as examples of cancer-resistant species, such as elephants, whales, or naked-mole rats, are not included in this study.**
 - *Elephas maximus* is included in this study (e.g., see Supplementary Data) and has 4.5% cancer prevalence. We would like to include whales in this study, however, we did not have cancer or neoplasia prevalence for whales. We would also like to include naked-mole rats, which has 0.6% cancer prevalence (Compton et al. 2023), in this study, but there weren't any plasma or blood glucose data available on Species360 for *Heterocephalus glaber*.

- However, we have 20 species in our dataset with 0% cancer prevalence (e.g., see Supplementary Data).
- We will amend this sentence in line 361-364:

"Including more vertebrate species in the dataset will help draw more robust conclusions about the associations between trophic level (≥ 10 species in every trophic level), diet type or dietary percentage, mean plasma glucose concentration, and cancer prevalence within and across clades."

to

"Including more vertebrate species, such as whales and naked-mole rats, in the dataset will help draw more robust conclusions about the associations between trophic level (≥ 10 species in every trophic level), diet type or dietary percentage, mean plasma glucose concentration, and cancer prevalence within and across clades."

We have also made the following changes to improve clarity in the text.

- We updated this section:
 - *"Even though the associations between trophic level and cancer prevalence have been previously studied across vertebrates"*

to

"Even though the associations between trophic level and cancer prevalence have been previously studied across vertebrates"

- *"We sought to test three... for mean plasma glucose concentration"*

to

"We sought to test three...mean plasma glucose concentration and domestication."

- *"There is conflicting evidence about an association between diet and blood glucose levels in the literature. In humans²⁰, common voles, tundra voles^{21,22}, and vampire bats²³, dietary components can change blood glucose levels."*

to

"There is conflicting evidence about an association between diet and blood glucose levels in the literature. In humans²⁰, common voles, tundra voles^{21,22}, and vampire bats²³, dietary

components, such as micronutrients and macronutrients, or food deprivation, can change blood glucose levels."

- *"There is no association between diet and mean plasma glucose concentration across vertebrate species included in this study after correcting for the false discovery rate of multiple testing (Fig. 1)."*

to

"We found no association between diet and mean plasma glucose concentration in the majority of analyses across vertebrate species (Fig. 1)."

- *"Only the percentage of seeds in the diet is positively correlated with mean plasma glucose concentration across 18 species (Fig. 1B; P-value = 0.003) even after FDR corrections for multiple testing."*

to

"Only the percentage of seeds in the diet is positively correlated with mean plasma glucose concentration across 18 species (Fig. 1B; P-value = 0.003)."

We updated this sentence based on the updated analyses:

- *"Our results do not support our first hypothesis, as we found that diet is not correlated with mean plasma glucose concentration across vertebrates."*

to

"Our results mostly support our first hypothesis, as we found that across the diet versus mean glucose concentration analyses, only in the case of an analysis across 18 species the percentage of fruit in their diet was positively correlated with mean plasma glucose concentration."

We have rephrased the 1st paragraph of the introduction, split it into two paragraphs, and brought the previous literature on diet and cancer to the 1st paragraph, to bring more emphasis on those analyses rather than the life history and cancer analyses (now in the 2nd paragraph of the introduction) which are not as central to the focus of our manuscript.

We have added these sentences in the results for clarification:

- *"We tested for correlations of plasma glucose concentrations with cancer prevalence and neoplasia prevalence across vertebrates."*
- *"We also tested for correlations of plasma glucose concentrations with gastrointestinal and non-gastrointestinal cancer and neoplasia prevalence across vertebrates."*

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

- The authors have addressed my previous comments thoroughly. However, when checking the code, which they made available in this round of the review, I discovered several errors in the statistical analysis. Most importantly, the authors used Type I (sequential) ANOVA in an incorrect way in models with multiple predictors, namely in a way that did not control for the effects of other predictors. In Type I ANOVA, the order of the predictors in model formulas matters and it seems they used wrong order, at least in some models, when calculating p-values. For the explanation and correct use of Type I ANOVA in R, please see for example <https://www.r-bloggers.com/2011/03/anova-%E2%80%93-type-iiii-ss-explained/>.

For example, the p-value of the effect of blood glucose (variable `Mean_Blood_glucose_levels_from_ZIMS..colorimetry.automated...PLASMA`; using less cumbersome names would be better in my opinion) on gastrointestinal cancer prevalence (`GI_MalignancyPrevalence`) controlled for `Birds.vs.Mammals.vs.Ectotherms` according to the Table 1B (the topmost effect on page 44) is $p=0.0009$ with the coefficient -0.001 (btw., -0.0017 should be rounded to -0.002 and there are multiple rounding errors like this one in the paper). However, when this analysis is done correctly, the result is $p=0.26$ (not $p=0.0009$). Note that this is also the exactly same value as the p-value for the t-statistic of the coefficient for the effect of blood glucose in the summary table of the model printed by the function `summary()`. The fact that the association of blood glucose and gastrointestinal cancer is non-significant when the mammal/bird/ectotherm group is controlled for implies that (i) blood glucose and gastrointestinal cancer are not associated within these groups and (ii) their positive association on page 42 is due to differences in values of both variables between birds, mammals, and ectotherms. Similarly, p-value of the effect of blood glucose on general neoplasia prevalence (last model at page 43) is not $p=0.0001$, but $p=0.22$. In other words, there doesn't seem to be a general association between blood glucose and cancer or GI cancer, and the overall pattern is driven by differences among high taxonomic units. This also implies that all p-values from models with multiple predictors need to be checked and ANOVA needs to be reanalysed if done incorrectly.

- Thank you for this comment. In the updated analyses we have now rounded the values appropriately.
- We have also updated the p-values in the manuscript, using the p-values from the summary output (e.g., below shown in the green box, and not the red box).

```
> wildtrophi<-pglsEypagel(asin(sqrt(NonGINEoplasiaPrevalence))~Diet...of.seeds+Mean_Blood_glucose_levels_from_ZIMS..Glucose..colorimetry.automated...PLASMA, data=cutData, tree=pruned.tree, se=SE, method="ML")
> summary(wildtrophi)
Generalized least squares fit by maximum likelihood
Model: model
Data: data
      AIC      BIC    logLik
-16.78566 -13.24541 13.39283

Correlation Structure: corPagel
Formula: ~spp
Parameter estimate(s):
  Lambda
6.610696e-05
Variance function:
Structure: fixed weights
Formula: ~vf

Coefficients:
              Value Std. Error t-value p-value
(Intercept)  0.27342772 0.11812406  2.3147595  0.0210
Diet...of.seeds  0.00234674 0.00254183  0.9232504  0.3757
Mean_Blood_glucose_levels_from_ZIMS..Glucose..colorimetry.automated...PLASMA -0.00839607 0.01009455 -0.8317423  0.4232
factor(Domesticated.or.Semidomesticated)yes  0.17823696 0.08589383  2.0750845  0.0622

Correlation:
Diet...of.seeds (Intr) Dt.... M_B...
Mean_Blood_glucose_levels_from_ZIMS..Glucose..colorimetry.automated...PLASMA -0.155
factor(Domesticated.or.Semidomesticated)yes -0.717 -0.534
-0.118 0.489 -0.321

Standardized residuals:
      Min       Q1      Med       Q3      Max
-1.2554181 -0.7110316 -0.3744187  0.7493747  2.4277726

Residual standard error: 0.000175444
Degrees of freedom: 15 total; 11 residual
> anova(wildtrophi)
Denom. DF: 11
numDF F-value p-value
(Intercept) 1 83.41927 <.0001
Diet...of.seeds 1 0.04279 0.8399
Mean_Blood_glucose_levels_from_ZIMS..Glucose..colorimetry.automated...PLASMA 1 0.03044 0.8647
factor(Domesticated.or.Semidomesticated)yes 1 4.30598 0.0622
```

- To be more clear about the associations between blood glucose and neoplasia/cancer we have now updated the following sections:

- *"To our knowledge, this is the first time associations between plasma glucose levels and gastrointestinal neoplasia prevalence, gastrointestinal cancer prevalence, non-*

gastrointestinal neoplasia prevalence, non-gastrointestinal cancer prevalence, and cancer prevalence across tissues have been reported across vertebrates. The fact that these associations are attributable to the difference between birds, mammals, and ectotherms, suggests that these results may be partly explained by Satoh's framework on the divergence of birds versus mammals and reptiles during the Permian-Triassic boundary"

- *"this appears to be entirely driven... is no correlation between plasma glucose concentrations and malignancy prevalence (Fig. 2B-D) or neoplasia prevalence"*
- *"We found significantly higher MPGLuC and lower cancer prevalence in birds relative to other vertebrates... between MPGLuC and cancer prevalence within birds, mammals, or reptiles"*
- *and we have removed this section "The relationship between higher plasma glucose levels and lower gastrointestinal neoplasia prevalence, gastrointestinal cancer prevalence,. ... in birds and bats."*

- **In addition, as noted by reviewer 2, the combination of $R^2=0.63$ and $p=0.43$ stated in the caption of Figure 1 (currently line 558) is indeed an error. This error most probably resulted from not specifying the reduced model in the function `R2()` from the package `rr2`. When the tested model (`Mean_Blood_glucose_levels_from_ZIMS..colorimetry.automated...PLASMA.~TrophicLevel`) is compared to the reduced model specified as `Mean_Blood_glucose_levels_from_ZIMS..colorimetry.automated...PLASMA.~1`, R^2 obtained by the `R2_resid` method is 0.017. This value makes much more sense with $p=0.43$.**

- Thank you for this comment. In all the updated analyses we're now using the `R2_resid` output of this code that includes the reduced model

- `R2(mod = wildtrophic, mod.r = wildtrophic_reduced, phy = pruned.tree)`

- `R2_lik R2_resid R2_pred`

- We've now added this section in the methods:

- `"(R2(mod = main_model, mod.r = reduced_model, phy = pruned.tree);` where `main_model` refers to the univariate or multivariate model that we are analyzing, and `reduced_model` refers to the model where the independent variable is 1;"

- **Taken together, the data analysis needs to be thoroughly verified and the errors corrected. I strongly recommend much closer collaboration with a statistician to ensure the robustness of the results.**

- Thank you. We have now made the above statistical corrections with further help from a statistician.

Specific comments:

- **Lines 226-228: Do you mean that you simply excluded these diets with small sample sizes (i.e., occurring in less than 10 species, if I understand correctly) and recalculated the percentages based on the percentages of the remaining diets? This could hypothetically lead to a scenario, where a species consuming 90% fish and 10% plants would be assigned as 100% herbivore. Or were these excluded diets replaced by other diets to prevent such scenarios; for example, were proportions of "fish", "scavenger", and "unknown vertebrates" replaced by some other diet based on vertebrates with a larger sample size?**

- Thank you for this comment. We have now updated this section to provide more clarity:

- *"In the analyses of these diet percentages, we only included diets with a percentage*

of $\geq 10\%$. We removed diets (fish, nectar, scavenger, unknown vertebrates) with a

sample size smaller than 10 species to improve the power of the analyses. Upon

removal of these diets, the percentages were not recalculated to add to 100% in order

to keep the percentages consistent with published data."

- **Glucose data collection: Please clarify whether the mean glucose levels reported in ZIMS are derived exclusively from fasted birds, or if they include measurements from both fasted and fed birds.**
 - This information is not provided in the ZIMS database, so we don't know whether the plasma glucose measurements were from fasted or fed animals. We have now added this information in the discussion:
 - *"may have been fasted or recently fed"*
 - *"Information about whether the animals were anesthetized, fasted, their exact health status, and method of capture is not provided in ZIMS."*
 - *"Also, even though the data in ZIMS are from healthy individuals, we do not know the exact health status of every individual at the time of data collection."*
 - We removed the following section from the discussion, since the data in ZIMS are reported to be from healthy individuals:
 - *"may have had infections that affected their blood glucose levels, may have had higher blood glucose levels due to diabetes⁹⁷"*
 - We also added the word healthy in the following section in the methods for clarification about the health status of individuals:
 - *"20 different healthy individuals"*

Reviewer #1 (Remarks on code availability):

- **There are several errors in the statistical analysis. Please see details in my comments to the authors.**
 - Thank you. We have now responded to the comments above about the statistics.

Reviewer #2 (Remarks to the Author):

- **This revised version accounts for some problems identified in the previous version. However, I still find that there is only a very weak support for the claim that "mean plasma glucose concentration and trophic level/diet can be viewed as factors affecting cancer prevalence across species". This work convincingly shows that birds have the highest glucose concentrations among vertebrate groups, but fails to provide clear evidence that this is associated with low cancer prevalence. Figures 2 and 3 display a clear between-class variation of cancer prevalence in relation to glucose concentration across vertebrate classes but there is no detectable relationship between cancer prevalence and glucose concentration among species within a vertebrate class. This needs to be explicitly stated.**
 - We have now replaced this section:
 - *"mean plasma glucose concentration and trophic level/diet can be viewed as factors affecting cancer prevalence across species"*
 - with
 - *"Our analyses did not support our second hypothesis: higher plasma glucose levels were not associated with lower cancer prevalence when we controlled for differences between birds, mammals and ectotherms."*
 - We have also made the following updates to be clearer about the associations between neoplasia/cancer and plasma glucose concentrations across vertebrates:

- *“To our knowledge, this is the first time associations between plasma glucose levels and gastrointestinal neoplasia prevalence, gastrointestinal cancer prevalence, non-gastrointestinal neoplasia prevalence, non-gastrointestinal cancer prevalence, and cancer prevalence across tissues have been reported across vertebrates. The fact that these associations are attributable to the difference between birds, mammals, and ectotherms, suggests that these results may be partly explained by Satoh’s framework on the divergence of birds versus mammals and reptiles during the Permian-Triassic boundary”*
 - *“this appears to be entirely driven... is no correlation between plasma glucose concentrations and malignancy prevalence (Fig. 2B-D) or neoplasia prevalence”*
 - *“We found significantly higher MPGLuC and lower cancer prevalence in birds relative to other vertebrates... between MPGLuC and cancer prevalence within birds, mammals, or reptiles”*
 - We have also added a panel (Figure 2A) comparing the mean plasma glucose concentrations in different groups of vertebrates to emphasize the significant differences in mean plasma glucose concentrations among these groups. We have also added this sentence in the methods section: *“We compared mean plasma glucose concentrations between mammals, birds, and reptiles using the Kruskal-Wallis test.”*
 - We have also moved the within group (mammals vs. birds vs. reptile malignancy prevalence ~ plasma glucose concentration) supplementary figures to figure 2 to bring more emphasis on the finding that within birds, within mammals, and within reptiles, plasma glucose concentration and malignancy prevalence are not correlated.
- **Detailed comments:**
 - I. 62: Remove "significantly"**
Done
 - I. 64: Remove "significantly"**
Done
 - I. 66: Remove "significantly"**
Done
 - I. 93: Insert a dot before "However"**
Done
 - I. 93: "in the third paragraph": what does it mean?**
Thank you. We have now removed the sentence that had this phrase to improve clarity.
 - I. 203: "detectable" instead of "significant"**
Done. We also removed this section *“If square-rooting the dependent variable still did not pass the Fligner-Killeen test, we cube-rooted the dependent variable.”* since in the updated analyses there wasn’t any need for cube-rooting the dependent variable. In cases where there was detectable heteroscedasticity in an analysis, square-rooting the dependent variable led to the analysis then passing the Fligner-Killeen test.
 - I. 240-241: Unclear what "determined the best model fit ($\Delta AIC = 2$)" means. Obviously there is some missing part to link a deltaAIC between 2 models of 2 and the retained model.**
Thank you. We have now rephrased this to *“determined the best model fit ($\Delta AIC > 2$) across those univariate and multivariate models”*.
 - I. 242: "statistically significant" instead of "significant"**
Done
 - I. 243-244: Same problem as in lines 240-241.**
Thank you. We have now rephrased this section to:
“(e.g., model 1: neoplasia prevalence ~ factor(trophic level), versus, model 2: neoplasia prevalence ~ factor(trophic level) + factor(domestication), versus, model 3: neoplasia prevalence ~ factor(trophic level) + plasma glucose concentration, versus, model 4: neoplasia prevalence ~ factor(trophic level) + plasma glucose concentration + factor(domestication)) and the AIC of the null model (e.g., model 0: neoplasia prevalence ~ 1)”
 - I. 320: Remove "significantly"**
Done
 - I. 324: Remove "significantly"**
Done
 - I. 327: Remove "significantly"**
Done

- I. 332: Remove "significantly"
Done
- I. 342: Remove "significantly"
Done
- I. 343: Remove "significant"
Done
- I. 352: Remove "significantly"
Done
- I. 455: "detectable" instead of "significant"
Done

Reviewer #3 (Remarks to the Author):

- The updated manuscript shows significant improvements over its previous iteration. The inclusion of additional information is appreciated. The authors have reexamined their dataset, incorporating requested confounding variables such as testing the potential effects of domestication and longevity. Furthermore, they have addressed the verification of assumptions underlying their model and provided clarification on methodological aspects that were ambiguous in the prior version.
 - Thank you.
- In their best model, the mean glucose concentration accounts for a small portion of the dataset's variance, with an R-squared value of approximately 0.10. This suggests relatively weak evidence for a correlation between blood glucose concentration and malignancy prevalence. Upon further analysis, disaggregating the dataset into mammal, bird, or reptile subgroups reveals no significant trends between blood glucose concentration and various neoplasia types within any of the individual groups. Notably, it's only when these groups are amalgamated that the weak association becomes apparent, raising the possibility of it being spurious.
- Analyzing neoplasia prevalence data in relation to diet type categorized as herbivore, omnivore, or carnivore, and controlling for blood glucose concentration, yields only slight improvement to the model. However, no significant differences between the groups are observed.
 - This is correct.
- There is some uncertainty in the neoplasia prevalence point estimates (the median number of individuals used to calculate those estimates is 34.5 individuals). The noise in the response variable which is partially accounted for by weighting the estimates by $1/\sqrt{n}$ may explain the weak trend by itself. Additionally, the author highlights various limitations in the discussion section, implying that the introduction of additional unaccounted-for noise in this study could explain the observed pattern. Consequently, replicating this study with an independent dataset is deemed necessary to validate the existence of the trend. This may be difficult because of the scarcity of data on neoplasia prevalence in animals.
 - We agree that validation is always important for any new observations such as these. While validation is difficult, it is certainly possible. Bulls et al. have compiled an independent dataset of neoplasia prevalence in animal necropsies (Bulls et al. 2023 <https://www.biorxiv.org/content/10.1101/2022.07.12.499088v2>). Scientists may also apply for access to Species360's cancer mortality dataset of over 8 million animals (<https://species360.org/>). We hope to encourage further efforts in comparative oncology.
 - We have now added this section in the discussion:
 - *"Larger datasets are becoming available. For example, scientists can apply for access to Species360's cancer mortality dataset of over 8 million animals (<https://species360.org/>)."*
- Line 389: "Our results also support our third hypothesis that trophic levels are positively correlated with cancer prevalence across tissues and neoplasia prevalence across tissues." How would you explain that omnivore species have a higher cancer prevalence compared to carnivore species if it is not due to blood glucose concentration?

- In the updated analyses, omnivores do not have a higher cancer prevalence than carnivore species.
- **Line 506: “In almost every case, researchers say they would predict a correlation, but sometimes researchers find a correlation, sometimes they do not.”**
 - We have now rephrased this sentence to:
 - *“A lack of correlation between cancer prevalence and any given factor...”*
- **There could be a publication bias in these studies, as scientists may be less inclined to publish non-significant correlations compared to factors that either increase or decrease cancer risk. Nevertheless, I believe this manuscript, despite offering weak evidence, would still be a valuable addition to the literature once published, even if it doesn't appear in high-impact journals like Nature Communications and as long it acknowledges the limitations. It would be nice to add the 95% confidence intervals bands around the regression lines on the plot as it helps visualising the uncertainty around those lines.**
 - Thank you for this comment. Unfortunately we cannot add 95% CI bands since we are using phylogenetic regressions. Adding 95% CI bands would be possible if the species in our analyses were independent. However, the lambda values in our phylogenetically-controlled analyses vary quite a lot. Therefore, we cannot add 95% CI bands in our figures.

Additional notes:

- We have updated all pgl analyses using TimeTree instead of the NCBI tree, since TimeTree incorporates estimates of divergence times whereas the NCBI tree doesn't.
- We have used “body mass” throughout the manuscript rather than “body weight” as the more appropriate scientific term.
- We also arcsine-square-root-transformed the malignancy and neoplasia prevalence data because those values are on the unit interval and many are close to 0:
 - *“When the dependent variable was malignancy or neoplasia prevalence, we arcsine-square-root-transformed those prevalence data since they are proportions. However, for visualization purposes we display the non-arcsine-square-root-transformed data in the figures.”*
- We removed this section, since the post-hoc analyses are now included in the main groups of analyses.:
 - *“; 9. post-hoc pairwise analyses comparing mean plasma glucose concentration of species in one trophic level versus another trophic level (Fig. 1A; 6 tests); 10. post-hoc pairwise analyses comparing mean plasma glucose concentration of species having one diet type versus another diet type (Fig. 1D; 3 tests); 11. post-hoc pairwise analyses comparing malignancy prevalence across tissues or gastrointestinal malignancy prevalence of species in one trophic level versus another trophic level (Fig. 4A, Fig. 5A; 12 tests); 12. post-hoc pairwise analyses comparing malignancy or neoplasia prevalence across tissues, gastrointestinal malignancy or neoplasia prevalence, or non-gastrointestinal malignancy or neoplasia prevalence of species having one diet type versus another diet type (Fig. 4D; Fig. 5A; Supp. Fig. 4; 18 tests)”*
- We have rephrased this section:
 - *“cancer prevalence data from 160 species that were necropsied under veterinary care.”*
 - to
 - *“cancer prevalence data from up to 273 species”*
 - since our dataset now includes more species and we have already mentioned that these cancer data are from necropsies in the methods section. We added “up to” because not all 273 species in our dataset have data for diet, plasma glucose concentrations, and cancer prevalence.
- Given that we use TimeTree in the updated analyses, there weren't enough species (≥ 10) to conduct the cancer or neoplasia prevalence and “% of Vectotherm in a species diet” regressions. Therefore we removed the cancer or neoplasia prevalence and “% of Vectotherm in a species diet” regressions.
- We have removed the following section in order to improve clarity and bring more focus to the main points of the paper (associations between diet, glucose, and cancer across vertebrates that are mentioned in the remaining paragraphs of the introduction):
 - *“Beyond diet, body size and longevity have been hypothesized to be predictors of cancer prevalence, based on the idea that larger animals (i.e., those with more cells), and long-lived animals (i.e., those with a longer time for mutations to accumulate⁸), should accumulate more*

carcinogenic mutations and thus have a higher cancer prevalence^{9,10}. This has been shown in a study of free-living birds where body weight is positively correlated with tumor prevalence¹¹. Body weight has also been significantly positively correlated with neoplasia prevalence across vertebrates¹². The majority of studies testing this hypothesis, however, show that larger and longer-lived animals do not get significantly more cancer^{12–17}. Other life history traits, such as shorter gestation or incubation length (across vertebrates¹²), and large litter size (in mammals¹⁴) or clutch size (in birds¹⁶), have been significantly correlated with cancer prevalence. The reason for this positive correlation may be that animals may trade off resources between body maintenance (such as protection from DNA damage) and reproduction^{18,19}. Other variables, such as low habitat productivity (i.e., grams of glucose produced in the habitat of species per square meter per year), are correlated with neoplasia prevalence across tissues, gastrointestinal cancer prevalence, and gastrointestinal neoplasia prevalence across vertebrates even after False Discovery Rate corrections for performing multiple tests⁷. “

- We have combined and rephrased the first two paragraphs of the paragraph of the “Relationships Between Plasma Glucose and Cancer” section in the results to simplify the description of the results.
- We have joined this lengthy paragraph “*The reduced import of glucose inside avian cells has multiple intracellular effects that contribute in stabilizing and protecting DNA ...These features may also explain why fowl, which have maintained a functional KEAP1, are amongst the birds with the highest cancer and neoplasia prevalence^{17,68}*” with its previous paragraph and rephrased it to simplify the discussion points in these two paragraphs.
- We moved the gastrointestinal and non-gastrointestinal analyses and the models of close best fit of the paragraph that starts as “*We used cancer and neoplasia prevalence across tissues, gastrointestinal malignancy and neoplasia prevalence (Supplementary Information), and non-gastrointestinal malignancy and neoplasia prevalence (Supplementary Information) as dependent variables.*” to the Supplementary Information in order to make the results section more concise.
- To provide more clarity about the models that best fit the data in the cases where cancer prevalence or neoplasia prevalence across tissues were the dependent variables, we added information in this paragraph
“We used cancer and neoplasia prevalence across tissues, ...by mean plasma glucose concentrations or birds versus mammals versus ectotherms (P-values > 0.05; Supp. Table 1G).”
as well as in Supplementary Table G.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

- In the revised version, the statistical analysis has been corrected, leading to changes in some of the results. Specifically, the association between blood glucose and neoplasia/cancer is now unsupported when controlled for the main taxonomic groups. This result is consistent with the negative results of the analysis done separately in each taxonomic group. Hence, the main issue that precluded the publication was solved. Nevertheless, there are still some specific issues detailed below that should be addressed in my opinion.

The last two sentences of the Abstract about the possible mechanisms linking high blood glucose levels and low neoplasia/cancer prevalence in birds are hypothetical and may make a false impression that these mechanisms are studied in the paper. I recommend either omitting such hypothetical conclusions in the Abstract or at least modifying the wording to explicitly state their hypothetical character.

- Thank you. We have now rephrased these two sentences to:
 - *"A hypothetical explanation for our results may be the evolution of insulin resistance in birds, which selected for loss or downregulation of genes related to insulin-mediated glucose import in cells. This may have led to higher MPGLuC, intracellular caloric restriction, and production of fewer reactive oxygen species and inflammatory cytokines, which may have contributed to lower neoplasia prevalence in extant birds relative to mammals and reptiles."*
- **Line 348: Please be more specific about what is meant by "caloric restriction inside bird cells".** Do you mean that birds may have lower intracellular glucose concentrations? It should be kept in mind that although some findings suggest that some avian tissues may have lower intracellular glucose concentrations, they may rely more on the use of fatty acids compared to mammals. In that context, I am not sure if lower intracellular glucose concentrations can be interpreted as intracellular caloric restriction. Besides, caloric restriction is a temporary state characterised by reduced energy and nutrient intake imposed by external conditions, which elicits a plastic response of the body. In contrast, species-specific intracellular glucose concentrations are genetically determined and were fine-tuned during the evolution of each species. Hence, it is questionable whether it is appropriate to term the latter "caloric restriction". In addition, the ref. 33 (Holmes & Ottinger 2003) does not seem to contain any mention of intracellular caloric restriction in birds. A relevant reference should be provided.
 - Thank you for this comment. We have now removed this phrase "*caloric restriction inside bird cells*³³" and kept the previous phrase "lower intracellular storage of glucose as glycogen⁸" in that sentence given that Braun & Sweazea 2008 mention that "*Birds maintain higher plasma glucose concentrations (PGlu) than other vertebrates of similar body mass and, in most cases, appear to store comparatively very little glucose intracellularly as glycogen.*"
- **Moreover, "a possibly higher uptake and faster metabolism of glucose by healthy avian bird cells relative healthy mammalian reptilian cells" is mentioned in Conclusion (lines 429-430).** This seems to contradict the suggested intracellular caloric restriction discussed above (and also mentioned in the Abstract). And again, the ref. 94 (Dupont et al. 2004) doesn't seem to contain any mention of the possibly higher glucose uptake and metabolism by avian cells. In addition, remove the duplication in "avian bird cells".
 - Thank you for this comment. We have now removed this section "*and/or a possibly higher...and reptilian cells*".
 - In the abstract, we have now replaced this phrase "*intracellular caloric restriction*" with "*relatively low intracellular glucose concentrations in the form of glycogen*".
- **Lines 117-119: A preprint is cited as a source of data about the primary diet and trophic levels.** In my opinion, the source studies or databases should be cited and the procedure at least briefly described here because preprints may change.
 - We now provide the updated citations as excel files (Supplementary references.xls and Supplementary_extended references.xls) in the submission portal.
 - We have also updated this section in the methods: "*their primary diet (Supplementary material). The primary diet was determined following a procedure reported in previous literature⁷. Specifically, the primary diet refers to the primary diet of adult animals in the wild. Herbivores primarily eat plants and/or fungi. Invertivores primarily eat invertebrates. Primary*

carnivores primarily eat vertebrates, that are not primary carnivores. Secondary carnivores primarily eat primary carnivores. In the trophic level analyses, we only included trophic levels that had at least 10 species."

- **Lines 350-353: Given that KEAP1 is missing only in Neoaves but not in the chicken, its loss could not happen during the Permian-Triassic period, but rather around the Cretaceous-Tertiary boundary, when Neoaves originated.**
 - Thank you for this comment. After confirming as well with the cited reference which reports that "Another epoch-making event was constitutive nuclear factor erythroid 2-related factor 2 (NRF2) activation induced by deletion of the C-terminal part of Kelch-like ECH-associated protein (KEAP1), preventing ROS leakage, just in Neoaves but not in the Palaeognathae, thus suggesting that this deletion may have occurred during the early Tertiary period.", we have now rephrased the section in the manuscript to "early in the Tertiary period".
- **Lines 353-354: There are two hormones – insulin and IGF-1 – rather than a single insulin/IGF-1 hormone and both are active in birds, at least in some tissues. Please clarify what you refer to in this sentence. The relative insulin resistance in birds when compared to mammals? Or lower activity of insulin/IGF-1 signalling (IIS) pathway? In addition, refs 65 (Kenyon et al. 1993) and 66 (Hsu et al. 2003) are about IIS signalling in nematodes. Appropriate citations supporting the claim about birds made in this sentence should be provided instead.**
 - Thank you for this comment. We have now replaced the previous section with "The fat tissue and skeletal muscle cells of chickens are less sensitive to insulin than rats⁶⁵⁻⁶⁷, possibly also explaining the higher plasma glucose concentrations found in chickens versus rats^{68,69}".
- **Lines 355-356: This is an outdated information. Avian homologue of TNF-alpha has been identified along with its receptors (please see Rohde et al. 2018, 10.3389/fimmu.2018.00605 and Lu et al. 2022, 10.1016/j.dci.2022.104374).**
 - Thank you for noticing this. We have now removed this sentence "In addition, birds lack certain inflammatory cytokines such as TNF- α ^{67,68}."
- **Lines 357-362: It seems something may have accidentally been deleted or forgotten here as the discussion of the findings is missing in this paragraph about domestication.**
 - Thank you for noticing this. We have now added this section "In multivariate models with the independent variables plasma glucose concentration, gestation/incubation length, maximum lifespan, trophic levels, domestication, and birds versus mammals versus ectotherms, high neoplasia and malignancy prevalence were mostly driven by primary carnivores and domestication (Supplementary Information; Supp. Table 1G). This result indicates that even though domestication is likely linked to many other factors, its association with neoplasia and malignancy prevalence remains statistically significant after accounting for all the above life history factors and differences in taxonomic groups."
 - We also added this phrase "and potentially explaining the observed relatively higher cancer and neoplasia prevalence in domesticated species" in the following sentence.

Reviewer #2 (Remarks to the Author):

This revised version accounts for some problems I identified when reading the previous version. However, I still have two major problems with this work.

Reviewer #2 appears to have reviewed an older version of the manuscript. Where their comments are applicable to the current manuscript, we have addressed them. The other comments we have noted are no longer applicable.

- **First, I am still left unconvinced « that there is a negative correlation between mean plasma glucose concentrations and neoplasia and cancer prevalence » (as stated in I. 381-383). This work clearly shows that this relationship holds across vertebrate classes with birds showing both the highest glucose concentration and the lowest cancer or neoplasia prevalence, but I did not find any evidence in the current work that such a relationship occurred among species within a given vertebrate class, but reptiles as displayed in Figure 3. I would want to see**

replicated analyses per vertebrate class to make that point explicit. Not explicitly recognizing this marked difference in the relationships between among-class and among-species within a class leads to a very confusing discussion.

- We agree and this comment is no longer applicable as the revised analysis and current version of the manuscript does not make such a claim.
- We have now rephrased this in the discussion to be more explicit about the findings “*Our analyses did not support our second hypothesis: higher plasma glucose levels were not associated with lower cancer prevalence when we controlled for differences between birds, mammals and ectotherms.*”.
- We also make this point clear in Figure 2 “*Figure 2. Variation in mean plasma glucose concentration in different vertebrate groups, and its relationship with overall cancer prevalence. Plasma glucose concentrations are highest in Aves and lowest in Amphibia. There is no correlation between mean plasma glucose concentration and malignancy prevalence across tissues for (B) 66 mammalian species, (C) 47 bird species, and (D) 33 reptile species.*”
- **Second, I still have a major concern about the origin of the data analyzed. Note that I was not able to retrieve the dataset including species-specific trait values. Only broad references are provided and the assessment of trait values to a given species cannot be checked, which strongly limits the repeatability of the analyses reported here. An explicit species-specific reference for each trait value in the current work has to be provided. When a same reference is used for several species, it will be easy to repeat the reference for all species concerned.**
 - Regarding the origin of the trophic level data, we now provide the updated citations as excel files (Supplementary references.xls and Supplementary_extended references.xls) in the submission portal.
 - Regarding the domestication data, we mention in the methods section “*We obtained species domestication data from previous literature^{4,53}.*”
 - The glucose data are from the ZIMS database as noted in the methods “*We obtained records of mean plasma glucose concentrations (measured in mmol/L) from at least 20 different healthy individuals per species as recorded in the ZIMS database (<https://www.species360.org/>).*”
 - The neoplasia and malignancy data are from a previous study “*Data for neoplasia (malignant and benign tumors) and malignancy prevalence, including gastrointestinal neoplasia and malignancy prevalence as well as non-gastrointestinal neoplasia and malignancy prevalence, were collected from a previous study⁷.*”
 - And the body mass, gestation or incubation length, and maximum lifespan data are from previous studies “*we collected body mass, gestation length or incubation length, and maximum lifespan data for each species from published resources^{47,50,51}.*”
 - We provide the values for all these variables in Zenodo “*The data used in this study are available in Zenodo⁶⁰.*”
- **Detailed comments:**
- **I. 62: Remove »significantly »**
- I. 64: Remove »significantly »**
- I. 66: Remove »significantly »**
- I. 93; A dot is missing before « However »**
- I. 93: Remove « in the third paragraph »**
- I. 203: « statistically significant » instead of « significant »**
 - These comments appear to be from an old version of the manuscript and are no longer applicable. We have previously addressed them.
- **I. 205-206: Whether cube-rooting the dependent variable allows accounting for heteroscedasticity in all cases where the square-rooted dependent variable failed should be explicitly stated.**
 - We have now added “*If with this transformation the analysis still did not pass the Fligner-Killeen test, we raised the dependent variable to the power of 1/4. This cube-rooting led to the analysis passing the Fligner-Killeen test.*”
- **I. 240-241: the statement « determined the best model fit (DeltaAIC=2) » does not make any sense. By definition, the best model fit is reached by the model including all the tested independent variable. AIC allows to balance model accuracy and model precision and the model retained should be the model with the lowest AIC (not the model with a DeltaAIC of 2!). Burnham and Anderson’s book on model selection published in 2002 provides a thorough user guide. The**

threshold of 2 for DeltaAIC between competing models is only indicative and should only apply when the model with the lowest AIC is the most complex model. Arnold (J. Wildl. Manage. 2010) provides a thorough justification for doing that.

- Thank you. We have now rephrased this to “*determined the best model fit (lowest AIC and $\Delta AIC > 2$) across those univariate and multivariate models*”
- **I. 242: Remove »significant«. When using criteria issued from information theory, the concept of statistical significance is no longer appropriate as well explained by Burnham & Anderson (2002).**
 - This comment appears to be from an old version of the manuscript and is no longer applicable. We have previously removed the word significant from this line.
- **I. 243-244: Same problem here as above (I. 240-241)**
 - We have now rephrased this to “*determined the best model fit (lowest AIC and $\Delta AIC > 2$).*”
- **I. 320: Remove « significantly »**
- I. 324: Remove « significantly »**
- I. 327: Remove « significantly »**
- I. 332: Remove « significantly »**
- I. 338: Remove « significant»**
- I. 342: Remove « significantly »**
- I. 343: « statistically significant » instead of « significant »**
- I. 352: Remove « significantly »**
 - These comments appear to be from an old version of the manuscript and are no longer applicable. We have previously addressed them.
- **I. 385: Not « across vertebrates » True across vertebrate classes but not true across species within a vertebrate class! This needs to be explicitly stated.**
 - We have now rephrased this section to “*In our cross-vertebrates (including species of mammals, birds, and reptiles) diet, plasma glucose, and cancer association studies we found the following. Our results support our first hypothesis, that diet would not be correlated with plasma glucose concentration, as we found no correlation between diet and mean glucose concentration analyses across species after multiple testing corrections. Our analyses did not support our second hypothesis: higher plasma glucose levels were not associated with lower cancer prevalence when we controlled for differences between birds, mammals and ectotherms.*”
- **I. 411: Is it really the case for all reptiles? What about Testudines with very slow pace of life and very long-lived? They seem to have an ageing rate even slower than that of birds (see e.g. da Silva et al. 2022 Science).**
- I. 428-429: This looks like an abusive generalization. Obviously birds have not increased lifespan relative to all reptiles. Reptiles have been shown to have a much higher diversity of ageing rates than birds or mammals (see Reinke et al. 2022 Science) but some reptile lineages display much longer lifespan than birds (see da Silva et al. 2022 Science). What about cancer prevalence in such very long-lived reptiles?**
- I. 439-440: Does a lambda of 0.75 mean a very low phylogenetic signal? This needs to be justified. Why should we expect a strong phylogenetic signal only when lambda is 1?**
- I. 455: Remove « significant »**
 - These comments appear to be from an old version of the manuscript and are no longer applicable. We have previously removed these sections.

Reviewer #2 (Remarks on code availability):

- **The code looks fine but I was not able to look at the dataset.**
 - Thank you. We provide the dataset in Zenodo “*The data used in this study are available in Zenodo⁶⁰.*”

Reviewer #3 (Remarks to the Author):

- This latest version this manuscript shows significant improvement compared to the previous one. It is much easier to read, the analysis significantly improved, and for me this paper can be accepted. This study will be a useful citation for those interested in the ecology and evolution of cancers.

Line 403: The effect of parasite removal was also tested on zoo mammals using Vincze et al.'s dataset. To the best of my knowledge, this is the only study using comparative analysis to investigate this effect on cancer risk in mammals. Consider citing it here to ensure comprehensiveness since you are listing all risk factors here. "Cancer hygiene hypothesis: A test from wild captive mammals" <https://doi.org/10.1002/ece3.10547>

- Thank you for this comment. We have now added this reference in this section "*richness of parasite species in the hosts*⁹⁰,"

All the best

Notes from the corresponding author:

- In the references section, we have now updated the reference description of the Compton et al. paper given that it has been published in the journal *Cancer Discovery*.
- We removed the words "revised version" from the section "Compton et al.⁴⁷ (revised version)", since that preprint has now been published.
- By checking once again through the trophic level references, we corrected some species' trophic levels. Therefore, we have now updated the analyses that include trophic levels as well as provide the new dataset on Zenodo (dataset version 3).
 - By updating these analyses, some did not pass the Fligner test, therefore we added this sentence in the methods "*If with this transformation the analysis still did not pass the Fligner-Killeen test, we raised the dependent variable to the power of 1/4.*"

Point-by-point response letter

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors satisfactorily addressed all my additional comments, and, in my opinion, the manuscript is suitable for publication. I only have one minor comment to the edited text in the latest version.

- In lines 192-193, raising to the power of $1/4$, instead of $1/3$, is referred to as cube-rooting. Please correct, depending on if cube-root or fourth-root (raising to the power of $1/4$) transformation was used.
 - Thank you for catching this typo. We have now updated the sentence to “This transformation of the dependent variable to the fourth root led to the analysis passing the Fligner-Killeen test.”

Additional changes

- We have added two citations in this sentence “*Data for neoplasia (malignant and benign tumors) and malignancy prevalence^{7,12,47}, including gastrointestinal neoplasia and malignancy prevalence as well as non-gastrointestinal neoplasia and malignancy prevalence⁷, were collected from previous studies*”, specifically the citation Compton et al. 2024 and Kapsetaki et al. 2024, since those papers also have the neoplasia and cancer prevalence data that we’ve used, but do not have the gastrointestinal and non-gastrointestinal data that we’ve used in this article.
- We shortened this subheading “*Relationships Between Cancer and Trophic Levels, Diet percentage, and Diet Type*” to “*Relationships Between Cancer and Diet*” since the guidelines (<https://www.nature.com/documents/ncomms-formatting-instructions.pdf>) require subheadings to be maximum 60 characters.
- We have changed anywhere where we mention meat-based to animal-based for consistency.

This manuscript conducts a comparative analysis of vertebrate species to explore potential associations between trophic position and blood glucose concentration and their relationship to the prevalence of malignant cancer and neoplasms. This is an interesting hypothesis to investigate, as it is currently unclear which factors primarily contribute to cancer risk across vertebrates. One significant challenge in addressing this question is the scarcity of high-quality, standardized cancer datasets across different species. Existing datasets exhibit substantial heterogeneity in data collection methods and the treatment of censoring.

To examine these potential relationships, the authors employed a phylogenetic least square regression modelling approach to account for the lack of evolutionary independence among closely related species.

Although I believe the idea has merit and should be explored (with results reported and published, even if they turn out to be negative), the statistical analyses conducted in this manuscript do not provide the means to address the initial research questions.

Here are some key points the authors will need to check while they are reanalysing their dataset:

- Quantify the heterogeneity in the prevalence dataset they used and identify potential sources of bias. If possible, incorporate these factors into the statistical analyses.
- The assumptions of the phylogenetic linear regression models are not always met. Caution should be exercised in interpreting the model results. Some models may exhibit an excessive number of zero values, the response variable deviates from a normal distribution, and there is heteroscedasticity in the data that needs to be addressed.
- It's crucial to simultaneously assess multiple potential risk factors since species are exposed to various factors throughout their lifetimes. It is unclear from the manuscript whether this was tested for all relevant risk factors. Consider using a model selection procedure, such as AIC based methods, to aid in this task.
- In addition to reporting p-values and R² values, it's essential to present effect sizes (coefficients of the model) associated with each risk factor. A high R² value may not necessarily indicate a large effect size, while a model with an intermediate R² value and a substantial effect size may highlight a more important contributor to cancer risk.
- I recommend including null models and comparing them to models incorporating the various risk factors to assess if they perform better than the null model. This approach helps control for both false positives and false negatives.

You will find below additional comments and suggestions. I strongly encourage the authors to reanalyse their dataset, assess potential biases (and acknowledge them in the manuscript if they cannot be accounted for), and reevaluate whether the data supports their hypothesis. It is highly probable that the results and discussion sections will need substantial revisions once the data is reanalysed.

Introduction

Line 61: Please add example of risk factors directly associated with glucose.

Line 69: There is a lack of consistency in the paper in how species names are reported, sometimes only the common names are reported, sometimes they are reported with the scientific names.

Line 82: The issue with this prediction lies in the possibility that if blood glucose concentrations are indeed a significant cancer risk factor for species under typical environmental conditions and contribute to mortality during their reproductive period, natural selection may have optimized anti-cancer defences to prevent cancer development linked to glucose. In such a scenario, we would anticipate an absence of a relationship between cancer prevalence and glucose concentrations. This is for example observed in the case of invasive placentation as a cancer risk factor in mammals.

Line 83-89: This explanation is not clear. What is meant here by “Warburg expectation”. I suggest clarifying the hypothesis here.

Line 91: Small dogs are the product of artificial selection by Humans. I am not sure it is very relevant to compare it to other species that evolved under mostly under natural selection.

Line 105: Here I would expect natural selection to select for strong anti-cancer defences in those organs, and thus a lack of relationship.

Line 129: If my understanding of this categorization is correct, an animal that consumes a 95% animal-based diet, with only 5% being plant-based, would be classified as omnivorous and grouped into the same category as an animal with a 50%-50% diet. For instance, in the dataset, *Anas acuta* is labeled as herbivorous, but the table shows a diet consisting of 30% invertebrates and 70% invertebrates. If I interpret the criteria correctly, it should be categorized as omnivorous rather than herbivorous.

If the diet data is accurate, why not use the of percentage of plants and animals in the diet as continuous explanatory variables? It would also allow to include an interaction term between those two variables.

Line 140: I have reservations about using mean values of glucose concentration in this context. It's essential to consider the typical sample sizes (i.e., the number of glucose measurements) for the species included in the analyses and assess their repeatability. To address potential measurement errors in the explanatory variables, especially in cases where repeated measurements were taken for the same species, it's crucial to incorporate them properly into comparative analyses. One of the assumptions of linear models is that all explanatory variables are accurately measured without error. This issue can be addressed, for instance, by employing a Bayesian framework and utilizing the brms R package.

<https://paul-buerkner.github.io/brms/>

Lines 146: How much do the methods used to determine prevalence in the studies differ? For example, do the prevalence estimates account for both right and left censoring? Were cancer cases exclusively identified in necropsied animals, or were they also detected in living ones? What potential biases exist in cancer diagnostics among the different species included in the

study? It's crucial to address this heterogeneity, as it can introduce statistical noise into the analyses.

Line 149: Please provide a reason of why the threshold of 20 individuals was selected.

Line 151: Among mammals, placentation is linked to an elevated risk of cancer mortality, and mammal species with intermediate levels of placentation invasiveness exhibit higher mortality risks, likely because species with invasive placentation evolved stronger anti-cancer defences. If the goal is to incorporate risk factors associated with reproduction, then it should be considered as a factor in the analysis, particularly within the subset of mammals.

See for example the publications bellow.

Dujon, A.M., Vincze, O., Lemaitre, J.-F., Alix-Panabières, C., Pujol, P., Giraudeau, M., Ujvari, B., Thomas, F., 2023. The effect of placentation type, litter size, lactation and gestation length on cancer risk in mammals. *Proc. R. Soc. B Biol. Sci.* 290, <https://doi.org/10.1098/rspb.2023.0940>. <https://doi.org/10.1098/rspb.2023.0940>

Kshitiz, Afzal, J., Maziarz, J.D., Hamidzadeh, A., Liang, C., Erkenbrack, E.M., Nam, H., Haeger, J.D., Pfarrer, C., Hoang, T., Ott, T., Spencer, T., Pavličev, M., Antczak, D.F., Levchenko, A., Wagner, G.P., 2019. Evolution of placental invasion and cancer metastasis are causally linked. *Nat. Ecol. Evol.* 3, 1743–1753. <https://doi.org/10.1038/s41559-019-1046-4>

If body mass is accounted for, then life span should be accounted for too. Vincze et al. showed a negative weak trend between cancer mortality risk and body mass, and a weak positive trend with adult lifetime expectancy.

Vincze, O., Colchero, F., Lemaître, J.-F., Conde, D.A., Pavard, S., Bieuville, M., Urrutia, A.O., Ujvari, B., Boddy, A.M., Maley, C.C., Thomas, F., Giraudeau, M., 2022. Cancer risk across mammals. *Nature* 601, 263–267. <https://doi.org/10.1038/s41586-021-04224-5>

Statistical analysis

Line 156: I may have overlooked it, but it seems that the R code used for analysing the dataset was not provided, making it challenging to verify the results of the statistical analyses conducted by the authors.

Line 162: Were the variables centered because the model had issues converging otherwise?

If I understand correctly, the raw prevalence values were just centred. Looking at the raw data provided as supplementary material, the raw prevalence values are positively skewed, which violate that the assumption the response variable should follow a normal distribution. Centring the variable would not remove the skew. Vincze et al. logit transformed their response variable to account for this.

How was it checked that the model assumptions were satisfied, such as the normal distribution of residuals and response variable or the absence of heteroscedasticity in the data (which seems quite very likely here)? This information should be explicitly outlined in the Materials and Methods section of the manuscript.

Line 170: The authors explored the influence of various potential risk factors on cancer prevalence in vertebrates. While they mention the use of both univariate and multivariate

models, the details regarding how these models were constructed, whether interactions between risk factors were considered, and how the authors determined the most parsimonious model remain unclear. Additionally, it's not apparent whether a model selection procedure was employed to identify the most parsimonious model.

The authors utilized a false-discovery test applied to p-values to assess the impact of a risk factor on cancer prevalence. Although the authors report P-values and R² values for the fitted models, they do not provide coefficient estimates for these models. It's worth noting that P-values may not be very reliable in the context of PGLS analyses. It is crucial to take into account and report effect sizes for each considered risk factor. Relying solely on correlation coefficients is insufficient, as it does not provide information about the magnitude of the regression slope between two variables.

Results:

I have reservations about the robustness of the results presented in this section. I've identified significant violations of the assumptions underlying the PGLS models employed by the authors. The Materials and Methods section does not provide information on how the authors verified these assumptions or addressed them when they were violated. It is necessary to reanalyze the data to address these issues.

If, following more rigorous statistical analyses, the dataset fails to support the initial analysis, I strongly recommend that the authors publish these findings. Such information is valuable for advancing the field.

Figure 1: I find it difficult to distinguish between the colours in panels B and C because they have a high transparency. I suggest increasing the contrast of those panels.

Figure 2: Several aspects in this figure, as well as in all the other figures presenting regression results in this manuscript, require careful examination to ensure the validity of the model. A significant number of zero values are present in the data, so it is crucial to verify that they do not impact the model (i.e., the model is not zero-inflated). Additionally, heteroscedasticity is noticeable in the data, particularly in cases of high mean plasma glucose concentrations, this must be accounted for. The response variable also appears to be untransformed, and it does not seem to follow a normal distribution.

Unless the authors accounted for the abovementioned sources of biases, in such cases they need to clarify it in the methods section, I don't think their analyses are very robust.

I believe it's essential to incorporate confidence error bands on PGLS plots. While I understand that obtaining these bands using the `caper` package can be challenging, I recommend considering these analyses within a Bayesian framework. While they may be somewhat more complex to conduct, they offer the significant advantage of accurately quantifying the uncertainty for each parameter of the model.

Discussion

Line 271:

I am not sure whether the pattern will persist once the data is reanalyzed, but even without that, predictions can be possible.

Given that the divergence in metabolic pathways between birds, mammals, and reptiles occurred millions of years ago, my prediction would be that natural selection has fine-tuned anti-cancer defenses to minimize the emergence of fitness-reducing malignant tumors during their reproductive phase. Consequently, I wouldn't anticipate birds with elevated blood glucose concentrations to exhibit a high prevalence of malignant tumors. The authors have cited various adaptations that, if this hypothesis holds true, could account for this observed pattern.

Line 293: Adaptations such as being more physically active compared to what? What is meant here by physically active? In term of energetic expenditure? Distances travelled?

Line 299: The study referenced here (reference 13) is a preprint authored by the same first author. It investigates another interesting hypothesis but employs a very similar statistical approach with comparable limitations to this manuscript. I recommend taking into account my comments on this manuscript for the evaluation of the second manuscript as well.

Line 313 – 315: The way to test this would be to use a null model (i.e., here a model with no explanatory variable) and determine the proportion of variance in the dataset that is explained by the phylogenetic relationship alone. Overall, it is a good idea to compare all your models to a null model, it helps ensuring that a given risk factor really contributes to the pattern of cancer observed between species.

Line 314: Would the microbiome explanation be the most parsimonious one?

Line 309: Bats have a number of anti-cancer defences that differs from other species that may explain this pattern see the papers bellow

<https://www.nature.com/articles/s41467-019-10495-4>

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4764838/>

<https://academic.oup.com/gbe/article/15/9/evad148/7259420>

<https://www.science.org/doi/10.1126/sciadv.aao0926>

<https://bmcbgenomics.biomedcentral.com/articles/10.1186/s12864-016-3227-8>

Line 320: Domestication has an effect on cancer risk, this it must be included in statistical analyses as it is an important confounding factor.

Lines 348-352: This is, in my opinion, why it is important to include repeated glucose measurements in the analysis to account for that variability (e.g. with the brms package).

Line 361-364: It is important to note in the limitations section that certain taxa commonly regarded as examples of cancer-resistant species, such as elephants, whales, or naked-mole rats, are not included in this study.