The Journal of Physiology

Regulation of metabolic health by dietary histidine in mice

Victoria Flores, Alexandra B Spicer, Michelle Sonsalla, Nicole E Richardson, Deyang Yu, Grace E Sheridan, Michaela E Trautman, Reji Babygirija, Eunhae P Cheng, Jennifer M Rojas, Shany E Yang, Matthew H Wakai, Ryan Hubbell, Ildiko Kasza, Jay L Tomasiewicz, Cara L Green, Claudia Dantoin, Caroline Alexander, Joseph A Baur, Kristen C Malecki, and Dudley Lamming
DOI: 10.1113/JP283261

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The following individual(s) involved in review of this submission have agreed to reveal their identity: Adam Salmon (Referee #1); Troy L Merry (Referee #2)

Review Timeline:

Submission Date:	26-Apr-2022
Editorial Decision:	12-May-2022
Revision Received:	18-Jul-2022
Editorial Decision:	12-Aug-2022
Revision Received:	15-Aug-2022
Accepted:	01-Sep-2022

Senior Editor: Scott Powers

Reviewing Editor: Lykke Sylow

Transaction Report:

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Dear Dr Lamming,

Re: JP-RP-2022-283261 "Regulation of metabolic health by dietary histidine in mice" by Victoria Flores, Alexandra B Spicer, Michelle Sonsalla, Nicole E Richardson, Deyang Yu, Grace E Sheridan, Michaela E Trautman, Reji Babygirija, Eunhae P Cheng, Jennifer M Rojas, Shany E Yang, Matthew H Wakai, Ryan Hubbell, Ildiko Kasza, Jay L Tomasiewicz, Cara L Green, Claudia Dantoin, Caroline Alexander, Joseph A Baur, Kristen C Malecki, and Dudley Lamming

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Please advise your co-authors of this decision as soon as possible.

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If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

Scott K. Powers
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-You must start the Methods section with a paragraph headed Ethical Approval. A detailed explanation of journal policy and regulations on animal experimentation is given in Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818.). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: https://physoc.onlinelibrary.wiley.com/hub/animal-experiments. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015). The Methods section must contain details of the anaesthetic regime: anaesthetic used, dose and route of administration and method of killing the experimental animals.

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Please add the raw data to all histograms (if feasible) to comply with the Journal of Physiology statistics policy.

REFEREE COMMENTS

Referee #1:

In this study, the authors build on theirs and others previous work to understand the mechanisms regulating benefits (metabolic and longevity) of low protein diets. They use short and long-term studies focused on low histidine and its metabolic effects (or lack of) in mice. They then expand in a short discussion of data on potential implications in humans. While mostly well controlled studies are well described, there are a few concerns. Some have been brought up by the authors but might need more discussion and others need addressed to better understand these outcomes.

1. Concerned about the relevance of the restricted diets due to the composition of AA available. Yes, targets are essential AA, but cases of their metabolic pathways compensated through others. Ex. cysteine (which these diets have lots of) serves as feedstock for many of methionine's metabolic pathways. Authors point this out for methionine in discussion, but other AA

have similar relationships. Example, Aspartate can be converted to threonine, others. Without measuring changes in AA concentrations in vivo, hard to tell whether the "negative" outcomes of these effects are due to direct impact or the fact that they weren't really restricted per se.

2. Regarding #1, it would be beneficial to examine AA content in plasma/tissues of animals to confirm a real reduction of these effects in diet. Understandable not exciting in those diets that "didn't work" but it might also explain why met restriction didn't work (as authors note this is in contrast to 30+ yr of study). That is, if Met concentration in vivo were unchanged, this is an easy explanation. Similarly, if His levels are lower, but not in any other diet, that might explain why this diet showed benefit.

3. With reduced fat and lean mass in Phe and His diets, one might argue this is growth restriction rather than decreased adiposity (also reflected by no change in ratio fat/lean). Would authors argue that reduced lean mass would be good for metabolic effects? Assessment of growth-associated hormones (IGF-1, GH, etc.) would be appropriate to address the endocrinological impact of these diets.

4. The significance of FGF21 and Ucp1 data would be strengthened by showing similar from Low Ph diet. I.e., does Low Phe diet look like Low AA or Low His. Don't particularly understand the inclusion of FGF21 KO mouse data. Would be more informative to include the low AA diet in the FGF21 KO based on the gene expression data presented before in this study (Fig 6)

5. Hepatic steatosis examination is a bit under-discussed mechanistically. Authors show reduced liver fat, but molecular markers are essentially the same low AA v low His suggesting this isn't the main reason.

6. The body temp/cold exposure experiments are a bit confusing in light of the energy expenditure and fat compostition data. Low His increases heat generation, but lowers core body temp, without UCP1 expression and still protects from cold? It's not straightforward to me and authors should dicuss what seem like discrepancies.

7. His is a precursor to alpha keto-glutarate - which has been shown to have similar metabolic effects only in the reverse direction where increaseing alpha keto-glutarate improves metabolism.. Again, would expect low His = low alpha keto glutarate and in discrepance with these studies. Would be an important metabolite to measure.

8. Somewhat surprising that GTT in WD low His animals has small relative (though significant) effect despite "complete protection from adiposity". But, WD vs control is not significant according to graph. More detailed metabolic analysis? ITT? Clamps?

9. In older animal studies, would authors contend the main effect is it reduced adiposity? Lean mass also decreased - what is fat/lean ratio at this point. One might argue loss of lean mass at old age could be detrimental though not reflected in frailty assessments.

10. SHOW data have specifically looked at His. But, if data also have all AA (or even those essential tested here) it would be important to show those relationships as well. I.e., Does a single percentage point increase in met or trp or phe also have a significant association with BMI rise. I.e., is this really specific for His or can be extrapolated for any AA?

11. Small point - authors state "Similar to our findings in lean mice and previous observations of WD Low AA-fed mice, we observed that RER was increased in mice consuming the WD Low AA and WD Low His diets". But not entirely correct, found RER in light cycle only increased in Low AA not Low His.

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Flores et al. examined the impact of reducing levels of the dietary amino acids Thr, Phe, His, Met, Lys, Trp on body composition, energy expenditure and glucose homeostasis in mice. This is an interesting question given the debates around the benefits of low/high protein diets in weight loss, and what amio acids are of particular importance.

They report that reducing Phe and His attenuate normal weight gain in mice, and in particular, reducing dietary His attenuated diet induced obesity and metabolic dysfunction. This appears not to be the result of modulating FGF21 levels. Some of these effects on metabolism were reproduced an aging male mouse model. Overall the study design is simple, effective and appear to be well conducted. The manuscript is well written and easy to follow, and while of don't have any major issues with the current form, the authors may wish to address to following comments/edits. I hope these improve your manuscript.

1. The manuscript is written in a way to suggest the authors wanted to test the impact of reducing amino acids Thr, Phe, His, Met, Lys, Trp on obesity/weight loss. However, the first two experiments (Figure 1-4) are conducted in chow fed mice who were not obese/didn't have excess fat mass. Is there a reason for this and is it possible that reducing some of these amino acids may only have a metabolic impact under conditions of obesity?

2. Figure 9. Figure legend indicates n=3 in some groups, which groups? This is quite a low N, and perhaps authors might considering softening their interpretation of this figure since:

- Energy expenditure (heat) data is only expressed relative to body weight, and this can be miss leading (https://doi.org/10.1038/s42255-021-00451-2). Same may applied for food intake.

- It appears this experiment was undertaken in chow rather than western diet (where effect were more pounced) conditions

- No body weight or body composition data is presented. It seems imperative this is done as the argument would be low His may promote FGF1 to ultimately result in greater weight loss.

3. Figure 10. Both males and female mice are used here, but it appears both sex's were not used in other experiments. Is this correct? This may be important given that there is not diet effect in females in figure 10. If only male were used in other experiments than this, at minimum needs to be explicated stated in results and discussed in abstract and discussion.

4. Figure 10. Am I right in stating that loss of lean mass with aging is probably not a good thing, as seen in male low Hist mice?

5. With the low n's in the life span study (Figure 10H-I) how confident are the authors in this data set. Perhaps its not important to the overall story here.

6. Its up to the authors discretion but it would make more sense to me if Figure 11 directly followed figure 9, given its using a WD paradigm.

7. Figure 11. WD had a very limited impact weight gain (with no difference in % fat compared to chow control) and no impact GTT area under the curve. Do the authors know why this is? It appears the effects here seem to be effect of age-related weight gain rather than the WD per se.

8. Figure 11I is not convincing. It appears the positive association is being driven by a few participants with very low His in their diet. With these removed is the association still seen? You may like to consider:

- analysis separately for male and female (given sex-specific effects in mouse data).

- Comparing hist in diet (or BMI) for different quartiles of BMI (or Hist in diet).

- Is BMI the right measure here given low hist is effecting both lean and fat mass in mice?

As such references to evidence for his in human body comp may need to be removed from abstract and discussion/conclusion

9. Would the author like to speculate on how they believe low His in diet is increasing EE? Do they think it is a result of WAT "browning" independent of FGF21 (although UCP1 data doesn't support)? What do you think is driving cool challenge data (Figure 8L).

END OF COMMENTS

Confidential Review **26-Apr-2022**

We thank the reviewers and editors for their comprehensive review of our manuscript and detailed comments. We thank the reviewers for noting that “Overall the study design is simple, effective and appear to be well conducted” and that “The manuscript is well written and easy to follow.” The detailed comments of the reviewers below have assisted us in refining our paper, and we have endeavored to address all the concerns highlighted by the editors and reviewers below.

Referee #1:

In this study, the authors build on theirs and others previous work to understand the mechanisms regulating benefits (metabolic and longevity) of low protein diets. They use short and long-term studies focused on low histidine and its metabolic effects (or lack of) in mice. They then expand in a short discussion of data on potential implications in humans. While mostly well controlled studies are well described, there are a few concerns. Some have been brought up by the authors but might need more discussion and others need addressed to better understand these outcomes.

We thank the reviewer for appreciating the importance of the questions we have asked; we also appreciate the thoroughness of this review and hope the changes (as detailed in this response) fully address the specific issues raised below.

1. Concerned about the relevance of the restricted diets due to the composition of AA available. Yes, targets are essential AA, but cases of their metabolic pathways compensated through others. Ex. cysteine (which these diets have lots of) serves as feedstock for many of methionine's metabolic pathways. Authors point this out for methionine in discussion, but other AA have similar relationships. Example, Aspartate can be converted to threonine, others. Without measuring changes in AA concentrations in vivo, hard to tell whether the "negative" outcomes of these effects are due to direct impact or the fact that they weren't really restricted per se.

2. Regarding #1, it would be beneficial to examine AA content in plasma/tissues of animals to confirm a real reduction of these effects in diet. Understandable not exciting in those diets that "didn't work" but it might also explain why met restriction didn't work (as authors note this is in contrast to 30+ yr of study). That is, if Met concentration in vivo were unchanged, this is an easy explanation. Similarly, if His levels are lower, but not in any other diet, that might explain why this diet showed benefit.

This is an excellent point, and we have expanded our discussion to make it clear that that there may be compensation between pathways that helps to mask the effects of restricting individual amino acids. Thus, other combinations of amino acid restriction may also contribute to the beneficial effects of a low protein diet. In the specific case of methionine, methionine restriction is “well known” within the methionine restriction field to only work when methionine is restricted by over 80%, and the Gettys lab showed that dietary cysteine blocks the effects of MR by reducing MR-induced oxidative stress. We have expanded our discussion of this issue as well.

As the Simpson lab and others have shown, blood levels of amino acids differ between portal vein and circulation, and as we have shown with restriction of individual BCAAs, the effects in the blood are minor while levels in tissues differ, and also differ when looking at fed and fasting animals. We now include as a limitation that we did not measure the levels of specific amino acids; we feel a comprehensive analysis is beyond the scope of the present study.

3. With reduced fat and lean mass in Phe and His diets, one might argue this is growth restriction rather than decreased adiposity (also reflected by no change in ratio fat/lean). Would authors argue that reduced lean mass would be good for metabolic effects? Assessment of growth-associated hormones (IGF-1, GH, etc.) would be appropriate to address the endocrinological impact of these diets.

As shown in Figure 1C-E, while both lean and fat mass accretion are affected, the overall effect is a reduction in adiposity. We have explained thus more clearly in the discussion as well as noting the desirability of further investigating the endocrinological impacts of these diets. Since the WD experiments began at 4.5 months of age, and the experiments in aged mice begun at 16 months of age, both of which are in fully developed mice, it is likely that this is not due to growth restriction. **However, we now discuss this**, and have now included examination of IGF-1 and GH as work that should be done in the future

4. The significance of FGF21 and Ucp1 data would be strengthened by showing similar from Low Ph diet. I.e., does Low Phe diet look like Low AA or Low His. Don't particularly understand the inclusion of FGF21 KO mouse data. Would be more informative to include the low AA diet in the FGF21 KO based on the gene expression data presented before in this study (Fig 6).

Many amino acid restricted diets activate the FGF21-UCP1 axis, and we and others have shown that the effects of protein or methionine restricted diets on energy balance are dependent upon FGF21 using FGF21 KO mice. Surprisingly, the data we present here suggest that the FGF21 axis is NOT required for the effects of histidine restriction, which is consistent with the results we find in Figure 6, where we see no significant induction of FGF21 or UCP1 by a Low His diet. We respectfully suggest that the role of FGF21 in the response to a low protein/Low AA diet has been comprehensively addressed by Cristal Hill and Christopher Morrison in a series of manuscripts, who we cite, as well as work from our own lab. We agree that further study of this diet is warranted and we have elaborated on this in the discussion.

5. Hepatic steatosis examination is a bit under-discussed mechanistically. Authors show reduced liver fat, but molecular markers are essentially the same low AA v low His suggesting this isn't the main reason.

This is an excellent point, and we have now expanded our discussion, including the need for future work to address how restriction of histidine helps to clear liver fat.

6. The body temp/cold exposure experiments are a bit confusing in light of the energy expenditure and fat compostition data. Low His increases heat generation, but lowers core body temp, without UCP1 expression and still protects from cold? It's not straightforward to me and authors should dicuss what seem like discrepancies.

We have expanded our discussion of this issue – in brief, our data is consistent with the possibility that histidine restriction promotes heat loss, perhaps through decreasing skin insulation, as we see decreased dWAT thickness in mice fed a WD Low His diet. This would be expected to lower core body temperature slightly; and further, might prime the mice to be able to better respond to cold stress (e.g., by more quickly inducing Ucp1). However, proving this hypothesis is beyond the scope of the current work, and is likely to be quite difficult as the insulative properties of skin are still the focus of intense study.

7. His is a precursor to alpha keto-glutarate - which has been shown to have similar metabolic effects only in the reverse direction where increaseing alpha keto-glutarate improves

metabolism.. Again, would expect low His = low alpha keto glutarate and in discrepance with these studies. Would be an important metabolite to measure.

This is an interesting point. While we feel these additional metabolomic experiments are beyond the scope of the present manuscript, we now cite the work on aKg and metabolism and discuss that we don't know how the limited amount of histidine is now being utilized for protein synthesis and catabolism.

8. Somewhat surprising that GTT in WD low His animals has small relative (though significant) effect despite "complete protection from adiposity". But, WD vs control is not significant according to graph. More detailed metabolic analysis? ITT? Clamps?

That is correct – the p value is 0.09, which we have added to the graph. Likely the main reason for the relatively small difference is that the animals had only been on the diets for 3 weeks, with and the WD Ctrl and Ctrl AA -fed mice were of identical weight. We were not able to collect GTTs at later timepoints due to a COVID research shutdown. We have updated the figure legend to clarify this time point.

9. In older animal studies, would authors contend the main effect is it reduced adiposity? Lean mass also decreased - what is fat/lean ratio at this point. One might argue loss of lean mass at old age could be detrimental though not reflected in frailty assessments.

This is an excellent point and though adiposity is reduced in males, which may be beneficial, it makes a great deal of sense to carefully consider the effects of histidine restriction on muscle function and health. We now discuss this at greater length making this exact point.

10. SHOW data have specifically looked at His. But, if data also have all AA (or even those essential tested here) it would be important to show those relationships as well. I.e., Does a single percentage point increase in met or trp or phe also have a significant association with BMI rise. I.e., is this really specific for His or can be extrapolated for any AA?

While we have not been able to perform all these analyses, we previously reported that there is a weaker (and smaller effect size – B equals 2.46) of Isoleucine restriction on BMI, while there is not a relationship between BMI and either leucine or valine. Thus, it is not true for all amino acids.

11. Small point - authors state "Similar to our findings in lean mice and previous observations of WD Low AA-fed mice, we observed that RER was increased in mice consuming the WD Low AA and WD Low His diets". But not entirely correct, found RER in light cycle only increased in Low AA not Low His.

Good point – we have now noted that this is true only during the dark cycle.

Referee #2:

Flores et al. examined the impact of reducing levels of the dietary amino acids Thr, Phe, His, Met, Lys, Trp on body composition, energy expenditure and glucose homeostasis in mice. This is an interesting question given the debates around the benefits of low/high protein diets in weight loss, and what amio acids are of particular importance.

They report that reducing Phe and His attenuate normal weight gain in mice, and in particular, reducing dietary His attenuated diet induced obesity and metabolic dysfunction. This appears

not to be the result of modulating FGF21 levels. Some of these effects on metabolism were reproduced an aging male mouse model. Overall the study design is simple, effective and appear to be well conducted. The manuscript is well written and easy to follow, and while of don't have any major issues with the current form, the authors may wish to address to following comments/edits. I hope these improve your manuscript.

We thank the reviewer for their kind remarks and insightful critiques, and we have addressed the individual points below.

1. The manuscript is written in a way to suggest the authors wanted to test the impact of reducing amino acids Thr, Phe, His, Met, Lys, Trp on obesity/weight loss. However, the first two experiments (Figure 1-4) are conducted in chow fed mice who were not obese/didn't have excess fat mass. Is there a reason for this and is it possible that reducing some of these amino acids may only have a metabolic impact under conditions of obesity?

This is a slight misunderstanding – our goal was to determine which of the non-BCAA essential amino acids contributed to the effects of a low protein diet, as our published work suggested that EAAs other than the BCAAs played a role but we had not identified which of these amino acids were important. We have revised the abstract and text to reflect this, and noted in the discussion that other amino acids might have different effects in the context of obesity.

2. Figure 9. Figure legend indicates n=3 in some groups, which groups? This is quite a low N, and perhaps authors might considering softening their interpretation of this figure since:
- Energy expenditure (heat) data is only expressed relative to body weight, and this can be miss leading (https://doi.org/10.1038/s42255-021-00451-2). Same may applied for food intake.
- It appears this experiment was undertaken in chow rather than western diet (where effect were more pounced) conditions
- No body weight or body composition data is presented. It seems imperative this is done as the argument would be low His may promote FGF1 to ultimately result in greater weight loss.

We have been able to include new data from additional cohorts of mice, and we now include body weight and composition data as well as metabolic chamber data for a larger number of mice. We attempted ANCOVA analysis as the reviewers suggested, but we found that the assumptions of the ANCOVA were violated for most pairwise analyses of interest. As suggested, we have revised the text to reflect both the new data the limitations of our experiments and analyses.

3. Figure 10. Both males and female mice are used here, but it appears both sex's were not used in other experiments. Is this correct? This may be important given that there is not diet effect in females in figure 10. If only male were used in other experiments than this, at minimum needs to be explicated stated in results and discussed in abstract and discussion.

We have revised the abstract results and discussion to clarify that the effects observed are in male mice with the exception of the work in Figure 10, and that studying these diets in females will be necessary.

4. Figure 10. Am I right in stating that loss of lean mass with aging is probably not a good thing, as seen in male low Hist mice?

Correct, and as noted in the response to reviewer 1 we now discuss this in the discussion section

5. With the low n's in the life span study (Figure 10H-I) how confident are the authors in this data set. Perhaps its not important to the overall story here.

The n ranges from 18-24, which gives approximately 85% power to detect a 15% change in lifespan and 98% power to detect a 20% change in lifespan based on the power analysis of Liang et al 2003 for a n of 20 in B6 mice. In comparison, we observed low protein and Low BCAA diets extending male lifespan by over 30% with similar group sizes (Richardson et al, 2021, Nature Aging). Thus, the study is well powered for the degree of restriction we have previously observed.

6. Its up to the authors discretion but it would make more sense to me if Figure 11 directly followed figure 9, given its using a WD paradigm.

We thank the author for the suggestion; we felt it was more appropriate to end with it as it less of an intervention paradigm than a prevention/exposure paradigm.

7. Figure 11. WD had a very limited impact weight gain (with no difference in % fat compared to chow control) and no impact GTT area under the curve. Do the authors know why this is? It appears the effects here seem to be effect of age-related weight gain rather than the WD per se.

Weight and adiposity gain is a bit impaired on our Western AA diet vs a natural source western diet. Notably the WD-fed mice do start to gain weight and fat mass more quickly after the first 6 weeks on the diet, and by 12 weeks they have significantly higher adiposity. We have replaced our initial figure with a corrected line graph matching the other figure panels to make it easier to understand.

8. Figure 11I is not convincing. It appears the positive association is being driven by a few participants with very low His in their diet. With these removed is the association still seen? You may like to consider:
- analysis separately for male and female (given sex-specific effects in mouse data).
- Comparing hist in diet (or BMI) for different quartiles of BMI (or Hist in diet).
- Is BMI the right measure here given low hist is effecting both lean and fat mass in mice?
As such references to evidence for his in human body comp may need to be removed from abstract and discussion/conclusion

We have provided an updated analysis, demographics and figure. With respect to the specific questions, we re-analyzed the sexes separately and see a similar and strongly significant effect in each sex, and we have added this to the discussion. We have also run an analysis of BMI vs quartile, and our analysis showing that BMI is still strongly associated with BMI by quartile. A one unit increase in percent of total histidine in protein in diet is associated with a .92 ($p<.001$) increase in quartile of BMI. Ordinal logistic regression found a similar increase in percent histidine ($p<.001$) for increasing quartile.

While BMI is not a perfect proxy for adiposity, it is the best available data we have access to. A number of studies have compared BMI and adiposity in humans, and the results have suggested that in the general population BMI is a good proxy for fat mass and percent body fat in both men and women. We have added some of these references to the paper to support our use of BMI.

9. Would the author like to speculate on how they believe low His in diet is increasing EE? Do they think it is a result of WAT "browning" independent of FGF21 (although UCP1 data doesn't support)? What do you think is driving cool challenge data (Figure 8L).

We have expanded our discussion of possible mechanisms surrounding the increased energy expenditure, but we believe our results are generally consistent with thermal loss driving the increased energy loss, although there are also other possibilities which could contribute. We have expanded our discussion about these possible mechanisms.

Dear Dr Lamming,

Re: JP-RP-2022-283261R1 "Regulation of metabolic health by dietary histidine in mice" by Victoria Flores, Alexandra B Spicer, Michelle Sonsalla, Nicole E Richardson, Deyang Yu, Grace E Sheridan, Michaela E Trautman, Reji Babygirija, Eunhae P Cheng, Jennifer M Rojas, Shany E Yang, Matthew H Wakai, Ryan Hubbell, Ildiko Kasza, Jay L Tomasiewicz, Cara L Green, Claudia Dantoin, Caroline Alexander, Joseph A Baur, Kristen C Malecki, and Dudley Lamming

Thank you for submitting your manuscript to The Journal of Physiology. It has been assessed by a Reviewing Editor and by 2 expert Referees and I am pleased to tell you that it is considered to be acceptable for publication following satisfactory revision.

Please advise your co-authors of this decision as soon as possible.

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If you have any queries please reply to this email and staff will be happy to assist.

Yours sincerely,

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- If n â‰¤ 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

- If n > 30, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

- 'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

- All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision).

- The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

- Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

- Statistics Summary Document completed appropriately upon revision.

EDITOR COMMENTS

Reviewing Editor:

I am pleased to tell you that your manuscript has been accepted for publication in The Journal of Physiology.

Senior Editor:

Thank you for revising your manuscript according to reviewer comments. Your revised report has been reviewed by the referees and both referees are pleased with your revisions. Nonetheless, the reviewers and review editor have requested to two final changes to the manuscript (i.e., include sample size for the lifespan studies in the methods and add a statement in the methods confirming that the human studies comply with the Declaration of Helsinki). We look forward to receiving your revised report.

REFEREE COMMENTS

Referee #1:

Authors have addressed previous concerns.

Referee #2:

The authors have done an excellent job responding to my initial comments, and I only have the following minor additional comment for further clarification:

1. In response to comment 2, it was stated that the assumption of ANCOVA were violated and thus this analysis could not be done. There are multiple different statically approaches, including a simple linear regression, which can be used to correct/adjust for body weight and avoid dividing EE by body weight. In dividing by body weight you are assuming a 1:1 relationship between EE and body weight which is almost never the case and leads to miss-leading interpretation of EE data as outlined in the reference provided in initial review.

2. Please insert the sample size analysis provided for lifespan studies into methods. This is important as sample size decisions are being made a very large effect size (15%), and the assumption of similar impact in males and females (which is surprisingly rare).

I congratulate the authors on a very interesting and complete manuscript.

_____

END OF COMMENTS

1st Confidential Review **18-Jul-2022**

We thank the reviewers and editors for their comprehensive review of our manuscript and detailed comments. We thank reviewer 2 in particular for noting that “The authors have done an excellent job responding to my initial comments.” The detailed comments of the reviewers below have assisted us in refining our paper, and we have endeavored to address the remaining issues.

Editors

You must start the Methods section with a paragraph headed Ethical Approval. If experiments were conducted on humans confirmation that informed consent was obtained, preferably in writing, that the studies conformed to the standards set by the latest revision of the Declaration of Helsinki, and that the procedures were approved by a properly constituted ethics committee, which should be named, must be included in the article file. If the research study was registered (clause 35 of the Declaration of Helsinki) the registration database should be indicated, otherwise the lack of registration should be noted as an exception (e.g., The study conformed to the standards set by the Declaration of Helsinki, except for registration in a database). For further information see: https://physoc.onlinelibrary.wiley.com/hub/human-experiments.

> We have added an “Ethical Approval” subheading at the beginning of the methods, and now note that “The Survey of the Health of Wisconsin (SHOW) study was approved by the University of Wisconsin Health Sciences Institutional Review Board 2013-0251. All participants provided written consent before any data and or biological sample collection. All policies and procedures used in the collection, analysis, and dissemination are in accordance with the Institutional Review Board requirements and in accordance with the Declaration of Helsinki, except for registration in a database. Data used in this study were coded, and all study team members followed human subject guidelines to approve confidentiality and security of data.”

- Papers must comply with the Statistics Policy: https://jp.msubmit.net/cgi-bin/main.plex?form_type=display_requirements#statistics.

Our paper fully complies with the Statistics Policy –

- If n <= 30, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.

> This has been done.

- If n > 30, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository e.g. FigShare, with access details provided in the manuscript.

> The full raw data is available as Supporting information

- 'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.

> N throughout refers to biologically independent animals, and this is noted in the methods

- All relevant 'n' values must be clearly stated in the main text, figures and tables, and the Statistical Summary Document (required upon revision).

> N values are clearly stated with exact N for every group in every panel included in the Statistical Summary Document

- The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.

Mean and standard deviation for every experiment is listed in the Statistical Summary Document

- Exact p values must be stated. Authors must not use 'greater than' or 'less than'. Exact p values must be stated to three significant figures even when 'no statistical significance' is claimed.

All calculated p values are listed in the Statistical Summary Document

- Statistics Summary Document completed appropriately upon revision.

Done.

Referee #2:

Please insert the sample size analysis provided for lifespan studies into methods. This is important as sample size decisions are being made a very large effect size (15%), and the assumption of similar impact in males and females (which is surprisingly rare).

This has now been included in the methods.

Dear Dr Lamming,

Re: JP-RP-2022-283261R2 "Regulation of metabolic health by dietary histidine in mice" by Victoria Flores, Alexandra B Spicer, Michelle Sonsalla, Nicole E Richardson, Deyang Yu, Grace E Sheridan, Michaela E Trautman, Reji Babygirija, Eunhae P Cheng, Jennifer M Rojas, Shany E Yang, Matthew H Wakai, Ryan Hubbell, Ildiko Kasza, Jay L Tomasiewicz, Cara L Green, Claudia Dantoin, Caroline Alexander, Joseph A Baur, Kristen C Malecki, and Dudley Lamming

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Yours sincerely,

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EDITOR COMMENTS

Reviewing Editor:

We are happy to inform you that your manuscript has been accepted for publication in JP. Please consider providing an Author Translational Perspective paragraph, as detailed above.

Senior Editor:

Congratulations on the completion of an outstanding study.

2nd Confidential Review **15-Aug-2022**