nature portfolio

Peer Review File

Thermal variation influences the transcriptome of the major malaria vector *Anopheles stephensi*.

Corresponding Author: Dr Ashutosh Pathak

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thermal variation influences the transcriptome of the major malaria vector Anopheles stephensi
Pathak et al.

In this manuscript, the authors analyzed the transcriptome of the malaria vector Anopheles stephensi at three different temperatures—20oC, 24oC, and 28oC—in order to determine gene expression changes associated with temperature variations. They monitored gene expression changes in midguts and carcasses at various time points post blood meal. Over 3,100 genes were identified that were tissue- and temperature-specific, with a higher number of genes identified at 20oC and 28oC. They observed two different trends in gene expression patterns at the studied temperatures.

The manuscript could benefit from more careful preparation. Mosquitoes were allowed to feed and then returned to the incubator. Twenty to twenty-five mosquitoes were collected from the cage at the desired time points. However, it is unclear how the authors distinguished between the fed and unfed mosquitoes during collection. It is also not clear whether the mosquitoes were allowed to lay eggs. The study focused on the effect of temperature changes on gene expression, but the rationale for providing a blood meal was not explained. There were three variables involved: temperature, blood meal, and the age of the mosquitoes. Which genes were specifically related to temperature?

Neither Figure 2 nor the text described the number of genes at individual time points; only the total number of genes across all time points was mentioned. Additionally, in Fig. 2, the dotted and solid lines were not visible. In the Fig. 2 legend, ‘1A’ and ‘1B’ should have been ‘2A’ and ‘2B’. The same issue occurred in Fig. 4. It would also be helpful to better understand the results if the time required for blood meal digestion and egg development at each temperature had been described.

The clustering of genes in Figs. 3 and 4 was highly problematic. The text mentioned seven clusters, but Fig. 3 showed only 4. Additionally, did the color codes in Figs. 2A, B, and C represent different clusters? That would have made sense, but if so, I would have suggested using a different color for each new cluster. For example, was cluster 2 in Figs. 3A and C the same? If so, their expression patterns differed between 20o and 28oC. The same issue arose for cluster 3 at 20oC and 24oC. A similar problem existed in Fig. 4. As discussed by authors, Day 1 genes were in response to the blood meal. Cluster 1 genes showed different patterns at 20oC and 24oC on day 1. By days 3-5, egg development should have been complete. Was overexpression of genes (e.g., Cluster 4 in Fig. 3A and C) at later time points related to age? Similarly, why did cluster 2 in Fig. 2C at 28oC show elevated expression at late time points?

Reviewer #2

(Remarks to the Author)
The present work aims to determine the transcriptomic response of malaria vectors to different thermal levels when infected with Plasmodium. The experimental design, as well as the bioinformatic and statistical analyses, are of very high quality. However, I have serious concerns about how the authors have decided to structure the article. Below, I suggest some modifications and recommendations to improve the structure of the article and make it easier for readers to follow. That said, I would like to congratulate the authors on a well-executed experimental work and robust results.

- I have the impression that the last paragraph of the introduction is a mixture of materials and methods (including figure 1) and results (the authors briefly state part of their results in this paragraph). I suggest including this information where appropriate and devoting that paragraph to making clear the gap in knowledge that this article aims to fill. If necessary, and as is sometimes the case, outlines of the materials and methods can be given (for instance indicating that the study is based

on RNA seq), but not on such a specific scale as described in this version of the manuscript. Instead, I suggest including more information in the introduction about the objective/hypothesis, significance of the study (lines 85-102)

- At the introduction, authors have mentioned the importance of identifying physiological mechanisms, but it would be beneficial to clearly state the specific objectives of the experiment. For example, are the authors trying to identify specific genes associated with thermotolerance in Anopheles stephensi under fluctuating temperature conditions? Are they aiming to assess transcriptional responses under different temperature regimes? Clarifying this will help the reader better understand the study's focus from the beginning.

- At the introduction, if possible, the authors should formulate one or more specific hypotheses that the experimental study is designed to test. This will frame the work within a clear scientific structure and provide a guiding thread for the reader throughout the article.

- Although in the introduction the authors present clear points they want to cover (e.g., 'determine whether the gene(s) that ultimately mediate these physiological responses could be selectively inherited in response to current trends in climate change') in the R&D section the authors should provide clearer explanations of the biological implications of their findings in relation to the points made in the introduction.

- I wonder if it is possible to elaborate a bit more on how the statistics are carried out with DESeq2 using the LTR test, do you compare gene expressions between times, how do you conclude that a gene is or is not significant, it is briefly indicated but not enough to fully understand it (lines 168-17).

- In my opinion, the discussion section might benefit from a more structured approach, such as separating it into subsections that address different aspects of the results, such as biological implications, methodological considerations, and future research. This would make the discussion easier to follow

- It seems to me that in lines 282- 289 the authors take many ideas for granted without referencing any of them. I suggest that these ideas (such as the fact that the presence of other tissues in the carcasses could have resulted in a lower signal to noise ratio) should be supported by previous scientific papers. This fact occurs several times during the discussion.

- In my opinion, the discussion could benefit from a more detailed comparative analysis with other studies. How do these results resemble or differ from previous research on Plasmodium transmission by different mosquito species?

- The authors should provide a more detailed discussion of their study's limitations, addressing factors like the sample size, which may affect the robustness and statistical power of their findings, and the generalizability of results to other regions or mosquito species.

- General comment for figures: I really like all the figures. I only suggest changing the colours of the figures to be colour bind safe.

- In addition to discussing the theoretical and practical implications, the authors could highlight how the results of this experimental study might influence future research. For example, could the findings guide the development of new strategies to control mosquito populations or mitigate the spread of diseases?

Reviewer #3

(Remarks to the Author)
The authors raise an important issue and tackle the issue of climatic variations on gene expression in An. stephensi. The data generated by the study will be useful for future research in the field.

There are still a few issues that needs to be addressed before the MS can be published though.

I have given the comments by section.

Abstract

The abstract needs to be improved. It is a bit too brief and lacks a conclusion. It would also be better if you used a specific term rather than "gene product" because it is not clear as to whether you are looking at RNA only or RNA and protein.

Introduction
Line 64-67 is completely unclear. I assume you mean that some species of Anopheles can transmit Plasmodium, but this is extremely confusing.

Line 77-84: I had to read this paragraph quite a few times to understand what you are saying. Please simplify it for ease of reading. Also, are you stating that testing on lab strains is an issue, as this is not clear.

Line 90: The term carcass is not appropriate. This does not tell you enough information. Did you use the remainder of the body once the midgut was dissected. What is the purpose of dissecting the midgut and sequencing that specific tissue, while

combining the rest of the tissues.

Line 93: It is not clear what you mean by chronic

Line 93-102: Why are you giving the results in the introduction?

You put Figure 1 in the introduction, where you should rather have this in the methods. Furthermore, the figure and legend are not useful to improving the readers understanding of how the experiment works.

Methods

You need to start your materials and methods section with the materials. You need to start with the mosquito description first before you give the study description.
There is no explanation as to why the specific time intervals were chosen. I am still not clear on the procedure you used and how your treatment worked.

Results, discussion and conclusion.

In general, I am not a fan of results and discussion combinations as I find it makes it difficult to disentangle. Nevertheless, these are the results. I like the figures as they do the function of clarify the text which I found a little difficult to follow. My major problem with the study is the use of “carcasses” as this is not clear what tissue is being assayed, particularly as it is clear that guts specifically were assayed. If this has been clarified, then it was not clear to me, it probably will not be clear to the readers. The remainder of the tissue will include reproductive tissue, possibly Malpighian tubules, salivary glands, cuticle and all these are going to have vastly different, and this is going to complicate your analysis. I think that this is the greatest methodological flaw, and it must be acknowledged/explained/addressed.
Regarding the conclusion, it is concerning to me that the first conclusion is that tissue makes a difference (which makes sense), but (as far as I understand) tissues were combined. Also, please avoid references in the conclusion.

Therefore, I suggest a revision before accepting the MS.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Reviewer #2

(Remarks to the Author)

Reviewer #3

(Remarks to the Author)
I am satisfied with the changes that the authors have made. They have addressed all the issues I have raised, so I am happy to have this published.

Manuscript number COMMSBIO-24-4198

We would like to thank the reviewers and editor for taking the time to read our manuscript. To address the comments more effectively, we numbered the comments, with minor editing of the format from the original submitted. All changes we have made here are in red text. All minor edits in the revised manuscript are copied here, while these and other major changes included as track changes to provide a reference to the previous version.

We have made extensive changes to our manuscript and provide a point-by-point response below. Amongst the major changes, we provide separate Results and Discussion sections and updated versions of Figure 1, Figure 3, Tables 1 and 2 and Supplementary tables 1 and 2.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Thermal variation influences the transcriptome of the major malaria vector Anopheles stephensi Pathak et al.

In this manuscript, the authors analyzed the transcriptome of the malaria vector Anopheles stephensi at three different temperatures—20°C, 24°C, and 28°C —in order to determine gene expression changes associated with temperature variations. They monitored gene expression changes in midguts and carcasses at various time points post blood meal. Over 3,100 genes were identified that were tissue- and temperature-specific, with a higher number of genes identified at 20°C and 28°C. They observed two different trends in gene expression patterns at the studied temperatures.

1.1 The manuscript could benefit from more careful preparation. Mosquitoes were allowed to feed and then returned to the incubator. Twenty to twenty-five mosquitoes were collected from the cage at the desired time points. However, it is unclear how the authors distinguished between the fed and unfed mosquitoes during collection.

Thank you for pointing out this oversight on our part.

To address the concerns about how the mosquitoes were sorted and collected, we have made **three changes**. First, In lines 101 and 102 of the revised manuscript, we have added text (underlined) stating after the blood meal, engorged mosquitoes were returned to the chamber as follows: *"Mosquitoes were allowed to feed for 15 minutes and fully engorged mosquitoes then returned to the respective temperature regime."* In the second change, we revised the text In lines 105 as follows *"Midguts of 10 gravid individuals were dissected and pooled from each temperature…"* assuming that only blood fed mosquitoes would be gravid at the time of collection. Third, we have also added a brief description of the study design to the legend for Figure 1.

1.2 It is also not clear whether the mosquitoes were allowed to lay eggs.

The mosquitoes were not allowed to lay eggs at any point in the study.

1.3 The study focused on the effect of temperature changes on gene expression, but the rationale for providing a blood meal was not explained.

Imbibing a blood meal is the most critical trait in mosquito life history. This study focused on bloodfed females because of their indispensable contribution to mosquito population dynamics (e.g., intrinsic growth) and disease transmission.

1.4 There were three variables involved: temperature, blood meal, and the age of the mosquitoes. Which genes were specifically related to temperature?

All mosquitoes received a blood meal at the start of this experiment, placed into their temperature treatments and sampled at the same time points (i.e., at the same chronological age). The genes that are shared across all temperature treatments are likely the genes responding to digesting the blood meal and producing eggs, while the genes that are not shared across temperature treatments are likely responding to temperature. Regarding age, this is a bit more difficult to assess. While our mosquitoes sampled at various time points throughout the experiment are the same chronological age, temperature will alter mosquito metabolism and physiological age, which from our perspective, is an important consequence of temperature variation on the ectothermic mosquito. Whether global gene expression is a response to shifts in temperature or changes in physiological age, we are not sure if we can parse these two effects from each other because they are highly related. We would also be uncomfortable assigning effects of specific gene expression patterns to changes in chronological age because a mosquito of a given chronological age at day 5 housed in a cool environment, for example, is likely not the same physiological age as a day 5 mosquito housed in a warm environment.

1.5 Neither Figure 2 nor the text described the number of genes at individual time points; only the total number of genes across all time points was mentioned.

Thank you for pointing this out. This information was included in Tables 1 and 2, however, in the revised manuscript, we add a column to each table and provide additional details in the legend that should make this information more cohesive with that of Figure 2, but also the rest of manuscript.

1.6 Additionally, in Fig. 2, the dotted and solid lines were not visible. In the Fig. 2 legend, ‘1A’ and ‘1B’ should have been ‘2A’ and ‘2B’. The same issue occurred in Fig. 4. It would also be helpful to better understand the results if the time required for blood meal digestion and egg development at each temperature had been described.

As described above, solid lines should only be visible if the genes are shared between 2 or 3 temperatures. We have also corrected our error in the legends to Figure 2 and Figure 4.

1.7 The clustering of genes in Figs. 3 and 4 was highly problematic. The text mentioned seven clusters, but Fig. 3 showed only 4.

The 7 clusters we stated in the text was the total of trend 2 clusters at all three temperatures; in the revised manuscript, we have clarified our statements in lines 416 and 417.

1.8 Additionally, did the color codes in Figs. 2A, B, and C represent different clusters? That would have made sense, but if so, I would have suggested using a different color for each new cluster. For example, was cluster 2 in Figs. 3A and C the same? If so, their expression patterns differed between 20o and 28°C. The same issue arose for cluster 3 at 20°C and 24°C. A similar problem existed in Fig. 4.

Color schemes used in Figure 2 were not meant to represent different clusters, just the different treatment temperatures, as is the case for Figure 4. Figure 3 color codes do represent different clusters, as does the 'inset' in Figure 4, which are already color-matched as closely as possible. Having said this, in Figure 3 aside from the shared 'profiles' of the clusters there are no similarities like gene overlaps. We have recolored Figure 3 to represent this, and modified Figure 4 to better color match the inset's gene expression profiles.

1.9 As discussed by authors, Day 1 genes were in response to the blood meal. Cluster 1 genes showed different patterns at 20°C and 24°C on day 1. By days 3-5, egg development should have been complete. Was overexpression of genes (e.g., Cluster 4 in Fig. 3A and C) at later time points related to age? Similarly, why did cluster 2 in Fig. 2C at 28°C show elevated expression at late time points?

This is a good question. We expand on these points in the "Discussion' section of our manuscript (lines 471 – 490). One thing to note is due to temperature effects on metabolism, the rate of digestion and egg production will vary across our temperature treatments. For example, blood digestion and egg development should be completed in 3-4 days at 24 C. However, this process will be faster at the warmer temperature and slower at the cooler temperature. Whether gene expression is related to variation in age (chronological / physiological?), we do not think so. First, if gene expression was related to physiological age, we imagine that we would see similar patterns of gene expression across the temperature treatments, but delayed dynamics in the cool temperature and fast dynamics in the warm temperature.

Reviewer #2 (Remarks to the Author):

The present work aims to determine the transcriptomic response of malaria vectors to different thermal levels when infected with Plasmodium. The experimental design, as well as the bioinformatic and statistical analyses, are of very high quality. However, I have serious concerns about how the authors have decided to structure the article.

Thank you for your thoughts on our experimental design and bioinformatic analysis. We would like to remind the reviewer that this work was done in uninfected mosquitoes only.

Below, I suggest some modifications and recommendations to improve the structure of the article and make it easier for readers to follow. That said, I would like to congratulate the authors on a well-executed experimental work and robust results.

Thank you!

2.1 - I have the impression that the last paragraph of the introduction is a mixture of materials and methods (including figure 1) and results (the authors briefly state part of their results in this paragraph). I suggest including this information where appropriate and devoting that paragraph to making clear the gap in knowledge that this article aims to fill. If necessary, and as is sometimes the case, outlines of the materials and methods can be given (for instance indicating that the study is based on RNA seq), but not on such a specific scale as described in this version of the manuscript. Instead, I suggest including more information in the introduction about the objective/hypothesis, significance of the study (lines 85-102)

Thank you, we have modified the introduction accordingly. See lines 81-92.

- At the introduction, authors have mentioned the importance of identifying physiological mechanisms, but it would be beneficial to clearly state the specific objectives of the experiment. For example, are the authors trying to identify specific genes associated with thermotolerance in Anopheles stephensi under fluctuating temperature conditions? Are they aiming to assess transcriptional responses under different temperature regimes? Clarifying this will help the reader better understand the study's focus from the beginning.

We have now clarified the questions we are addressing in this study. Thank you. See lines 81-92

2.2 - At the introduction, if possible, the authors should formulate one or more specific hypotheses that the experimental study is designed to test. This will frame the work within a clear scientific structure and provide a guiding thread for the reader throughout the article.

To the best of our knowledge, no study has looked at the effect of temperature at as high of resolution for a medically important vector as this study. Thus, as a first step, this study is descriptive in nature. Based on what we know phenotypically for how mosquito life history traits respond to temperature variation we expect to see significant differences in the transcriptional responses of mosquitoes to relevant temperature variation. However, we do not know enough about this system to be able to formulate specific hypotheses regarding which genes we might expect to see being upregulated / downregulated in response to temperature, how these genes might relate to specific phenotypic traits that respond to temperature variation, etc. This is our first step in this general direction. Please see our response above regarding clarifying our initial questions that we would like to address in this study.

2.3 - Although in the introduction the authors present clear points they want to cover (e.g., 'determine whether the gene(s) that ultimately mediate these physiological responses could be selectively inherited in response to current trends in climate change') in the R&D section the authors should provide clearer explanations of the biological implications of their findings in relation to the points made in the introduction.

Thank you for taking the time to think about these questions. To the best of our knowledge, we are not aware of any study that has looked at the effect of (fluctuating) temperature over such a long duration for a medically important vector. As such, our goal was to provide a broad overview of the possibilities before we proceeded with the goals of our study.

Thus, paragraph 1 argues for understanding the effect of temperature and provides 3 reasons why. Paragraph 2 argues for why studying the effect of temperature is particularly important for a malaria vector. Paragraph 3 begins with arguing for an -omics approach and why fluctuating temperatures are more relevant. Finally, paragraph 4 outlines the key questions we would like to address and the broad implications of our results to the field.

2.4 - I wonder if it is possible to elaborate a bit more on how the statistics are carried out with DESeq2 using the LTR test, do you compare gene expressions between times, how do you conclude that a gene is or is not significant, it is briefly indicated but not enough to fully understand it (lines 168-17).

We have added additional detail in how DESEq2's Likelihood Ratio Test implementation works, line 157-172. To summarize, we are comparing gene expression across time, and the LRT implementation builds two statistical models for each gene being analyzed, one considering time as a factor and one that does not, before comparing expression data for each gene against both models. The null

hypothesis assumes that the expression data is equally applicable to both models, and a rejection indicates that removal of time as an experimental factor causes a significant deviation in the model. We set a standard significance cutoff of $p < 0.05$ after multiple testing correction, and statistically significant genes were used for follow-on expression profile clustering.

2.5 - In my opinion, the discussion section might benefit from a more structured approach, such as separating it into subsections that address different aspects of the results, such as biological implications, methodological considerations, and future research. This would make the discussion easier to follow

Thank you for this suggestion. In the revised version of the manuscript, we have separated the Results from the Discussion, structured the Discussion as you suggest (biological implications, methodological consideration (limitations), and future research (Conclusion).

In addition, we added section headers to the Results to improve its overall structure and help with navigation. For instance, under “Functional profiling of Trend 1 genes in carcasses”, we begin by stating that the first sub-section will discuss “Functional profiling of genes in clusters expressed in (i), carcasses, (ii), unique to each temperature and (iii), following Trend 1”, followed by “Functional profiling of genes in clusters expressed in (i), carcasses, (ii), shared across all three temperatures and (iii), following Trend 1”.

2.6 - It seems to me that in lines 282- 289 the authors take many ideas for granted without referencing any of them. I suggest that these ideas (such as the fact that the presence of other tissues in the carcasses could have resulted in a lower signal to noise ratio) should be supported by previous scientific papers. This fact occurs several times during the discussion.

While we were unable to locate any source that has assessed this quantitatively, we have added references that allude to the limitation listed above in your comment. We have also added references throughout. If the reviewer knows of additional publications that would help support the previous statements that were not well referenced, or think any additional statements need better referencing, please let us know what these are and we will address them.

2.7 - In my opinion, the discussion could benefit from a more detailed comparative analysis with other studies. How do these results resemble or differ from previous research on Plasmodium transmission by different mosquito species?

Although we describe the transcriptomes of un-infected mosquitoes in our manuscript, we do refer to vector-pathogen systems wherever possible. It is worth noting that due to space constraints and avoid making inaccurate claims or conjectures, we restricted our discussion to annotated genes and processes with known functions only (Supplementary table 2). Nonetheless, to place our discussion in the context of vector-parasite/pathogen, we cite references 10, 11, 41, 43, 46, 49, 55, and 62, with references 39, 40, 42, 44, 45, 53, 54, 57, 58, 59, 60, 61, 64, 65 and 66 to explain our results in the context of mosquitoes and finally, references 47, 48, 50, 56 and 67 for other insects when we were unable to locate mosquito-specific citations.

2.8- The authors should provide a more detailed discussion of their study's limitations, addressing factors like the sample size, which may affect the robustness and statistical power of their findings, and the generalizability of results to other regions or mosquito species.

Thank you for the suggestion. In the revised version of the manuscript, we have added a paragraph discussing the Limitations, including how samples sizes affect robustness and statistical power (lines 491 – 511). We also discuss the generalizability of our results in the Discussion and the Conclusions (lines 530 – 535).

2.9- General comment for figures: I really like all the figures. I only suggest changing the colours of the figures to be colour bind safe.

Thank you for the suggestion. We have changed the colors accordingly.

2.10- In addition to discussing the theoretical and practical implications, the authors could highlight how the results of this experimental study might influence future research. For example, could the findings guide the development of new strategies to control mosquito populations or mitigate the spread of diseases?

Thank you for suggesting this possibility. Although we only consider un-infected mosquitoes, we discuss some of the potential applications in the "Discussion" of the revised manuscript and in the "Conclusions". We have added what we feel confident saying considering implications for the control of mosquito populations to the Conclusions section, discuss the implications of doing similar work at constant temperatures (which is mostly what has been done previously), implications for mosquito-malaria interactions, some methodological considerations, etc.

Reviewer #3 (Remarks to the Author):

The authors raise an important issue and tackle the issue of climatic variations on gene expression in An. stephensi. The data generated by the study will be useful for future research in the field.

There are still a few issues that needs to be addressed before the MS can be published though. I have given the comments by section.

Abstract

3.1 The abstract needs to be improved. It is a bit too brief and lacks a conclusion.

We have revised the abstract by clearly stating our conclusions and adding implications. While we agree with the reviewer's suggestion, we would like to add that journal guidelines limit the abstract to 150 words, which constrains the amount of detail.

3.2 It would also be better if you used a specific term rather than "gene product" because it is not clear as to whether you are looking at RNA only or RNA and protein.

We have changed "gene product" to "gene expression".

Introduction

3.3 Line 64-67 is completely unclear. I assume you mean that some species of Anopheles can transmit Plasmodium, but this is extremely confusing.

Thank you for pointing this out. This line has been simplified to “Several species within the genus *Anopheles* are important vectors of human malaria, where it continues to cause significant morbidity and mortality ”.

3.4 Line 77-84: I had to read this paragraph quite a few times to understand what you are saying. Please simplify it for ease of reading. Also, are you stating that testing on lab strains is an issue, as this is not clear.

This line has been simplified to “A few studies have shown the total number of genes and overall profiles can vary significantly between ectotherms housed at constant temperatures compared to fluctuating conditions [25-27]. Resolving responses in realistic environments is essential to the understanding of how ectotherms, like mosquitoes, respond to field relevant variation in temperature.”

3.5 Line 90: The term carcass is not appropriate. This does not tell you enough information. Did you use the remainder of the body once the midgut was dissected. What is the purpose of dissecting the midgut and sequencing that specific tissue, while combining the rest of the tissues.

The description of our study goals has now been expanded in the revised version of the manuscript (lines 81-92). We wanted to differentiate between systemic transcriptional profiles vs. those specific to the midgut, where digestion of the bloodmeal is occurring. The carcass represents everything else other than the midguts. We have added additional text to the methods describing the purpose of this choice as well (Lines 101-108). If the reviewer feels carcass is still not appropriate, can an alternative suggestion be provided please?

3.6 Line 93: It is not clear what you mean by chronic

Chronic refers to the fact that once a mosquito is infected, the infection may persist for most of the vector’s lifetime. However, this portion of the text has now been removed.

3.7 Line 93-102: Why are you giving the results in the introduction?

Our goal here was to help the reader navigate the information-heavy portion of the manuscript. But we have modified the introduction according to reviewer 1’s suggestion, which is like your concern here, so the results have been removed from the introduction.

3.8 You put Figure 1 in the introduction, where you should rather have this in the methods. Furthermore, the figure and legend are not useful to improving the readers understanding of how the experiment works.

We have moved the reference to Figure 1 to the Methods section and added a summary of the study design to the figure legend for Figure 1.

Methods

3.9 You need to start your materials and methods section with the materials. You need to start with the mosquito description first before you give the study description. There is no explanation as to why the specific time intervals were chosen. I am still not clear on the procedure you used and how your treatment worked.

Thank you for the suggestion. We urge the reviewer to consider our extensive revisions the sections describing our study section.. However, we respectfully disagree with the reviewer's suggestion to switch the order of priority of materials and methods; the current sequence as we have presented is commonly used in numerous other studies. As this is an experimental study, the most important information in the methods section is a description of the experimental design, which is why it has been listed first.

Results, discussion and conclusion.

3.10 In general, I am not a fan of results and discussion combinations as I find it makes it difficult to disentangle.

That's ok. The other two reviewers have raised the same point. We have separated Results & Discussion sections in the revised version of our manuscript.

3.11 Nevertheless, these are the results. I like the figures as they do the function of clarify the text which I found a little difficult to follow. My major problem with the study is the use of "carcasses" as this is not clear what tissue is being assayed, particularly as it is clear that guts specifically were assayed. If this has been clarified, then it was not clear to me, it probably will not be clear to the readers. The remainder of the tissue will include reproductive tissue, possibly Malpighian tubules, salivary glands, cuticle and all these are going to have vastly different, and this is going to complicate your analysis. I think that this is the greatest methodological flaw, and it must be acknowledged/explained/addressed.

We request the reviewer to refer to lines 101-108 in the Methods in the revised manuscript. We also discuss the implications of using the whole carcasses (and other tissues) as a potential limitation of our work in the Discussion section (lines 491 – 511)).

3.12 Regarding the conclusion, it is concerning to me that the first conclusion is that tissue makes a difference (which makes sense), but (as far as I understand) tissues were combined.

Assuming the reviewer finds our clarification of the study design above sufficient, they are correct in expecting there to be a difference in gene expression profiles between the midgut and the other tissues combined; however, as we describe further in our conclusion, despite these differences, both midguts and carcasses show reduced number of differentially expressed genes at the intermediate temperature of 24 DTR 9°C, with gene expression profiles that are both overlapping and distinct between temperatures, but with similar trends in how gene expression is enriched or depleted by the blood meal.

3.13 Also, please avoid references in the conclusion.

Well, we are a bit conflicted on how to proceed here. Reviewer 2 had concerns about not referencing statements, some of which occurred in the conclusion. While we generally agree, because this is not a requirement of the journal, we will maintain references with particular statements in the conclusion section, especially if we did not address them explicitly above with references. If this is a very strong feeling, then we can consult with the editor on how best to proceed and address this concern in another revision.

Please accept our gratitude for taking the time to review our manuscript a second time. Below we offer a point-by-point response to each of the concerns. Black text is used to indicate comments from each reviewer, with our responses highlighted with red text. Finally, any changes to the main text are depicted with yellow background.

Reviewer #1 (Remarks to the Author):

This is an improved version of the manuscript. However, I still have a few concerns. The authors did not explain why they selected the specific timepoints, particularly the later ones. I recommend that the authors provide a rationale for their experimental design at the beginning of the Results section.

Thank you for the suggestion. We have added rationale for the time points at the beginning of the Results section, in lines 195 – 201 of the revised manuscript.

Additionally, were there any mosquitoes without a blood meal reared at these temperatures for the duration of the experiment? If so, were they analyzed?

Only blood fed mosquitoes were analyzed here.

The gene expression profiles at early times points reflect processes related to blood meal digestion and egg development across various temperatures. In contrast, later timepoints likely reveal true temperature-dependent gene expression. However, these were not analyzed separately.

We apologize, but we are slightly confused with this concern. Temperature has effects on all metabolic processes, including blood meal digestion and egg development and we feel that our analyses, which look at gene expression dynamically through time, capture these different effects. While genes in Trend 1 were enriched a day after the blood meal and depleted at subsequent time points, genes in Trend 2 were depleted day 1 post-blood meal, with enrichment thereafter. Thus, we feel that the different patterns of gene expression for genes in Trend 1 and 2 support the reviewer's assertion already.

However, as we state in the Discussion later (lines 492 – 496), for the genes that followed Trend 2, and that were unique to each temperature, we were unable to assign GO profiles or annotations to some of the key temperature-specific clusters (Table 1), which limited our ability to offer further insight into temperature-specific gene expression.

Were there any non-coding RNAs observed? I did not see any mention of ncRNAs in the manuscript.

As we stated in all versions of the manuscript, non-coding RNAs were enriched exclusively in the midguts of mosquitoes at 20 DTR 9°C (lines 405 – 409).

Line 350, "…the 111 genes in cluster 3 increased in expression until 7 days post blood meal before beginning to decline." In Fig. 3B, cluster 3 genes showed increased expression until day 13. Please revise and change the line color to blue, as indicated in the text.

Thank you for pointing out this oversight. These figures also have a new and much larger color palette in accordance with another reviewer's recommendations. As such, we have removed any reference to line colors from the text.

Line 408, "…depletion of 238 genes in cluster 2 at 20 DTR 9oC one day after the blood meal was replaced by a sustained increase…." Cluster 2 genes showed increased expression on day 3, followed by a decrease at later timepoints (Fig. 3D). Please revise this for clarity.

Thank you for finding this. The statement has now been revised to more accurately reflect the trends.

Line 484, The authors states that "comparison GO terms enriched 1 day post blood meal from other studies performed at higher constant temperatures (27-28DTR 0oC) revealed overlap with the GO terms identified her at 20 DTR 9oC, but not the two warmer temperatures" . However, the authors did not explain why these two studies produced different results at 28oC.

Our goal here was to highlight the potential pitfalls of studying mosquito biology at constant daily temperatures (i.e., DTR = 0°C). We have revised lines 471 – 476 to add further clarity to our interpretation.

Conclusions. This section reads more like a discussion rather than a conclusion. I suggest restructuring it to focus on summarizing the key findings and their broader implications.

We appreciate the reviewer's suggestion. We have attempted to shorten the conclusion section and focus more on the implications of our results then the results themselves.

Tables. All tables should have clear headings with additional details provided in the footnotes.

We have added footnotes to Tables 1 and 2 and edited the legends accordingly.

Reviewer #2 (Remarks to the Author):

I am pleased to confirm that the authors have performed an excellent job of editing the manuscript. They have also demonstrated their awareness of the limitations of their work and have followed my suggestions.

The structure of the introduction has been modified for a better understanding of the article. In addition, they have modified the conclusions following my instructions, for which I am grateful.

I consider the study ready for publication in the present format.

Thank you for your support.

Reviewer #3 (Remarks to the Author):

I am satisfied with the changes that the authors have made. They have addressed all the issues I have raised, so I am happy to have this published.

Thank you for your support.