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Peer Review File

Multi-omics Insight into Cardiac Myofibril Remodeling in Post-Prandial Burmese Pythons

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

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Martin, Suarez-Artiles and colleagues investigate changes in myofibril function and myosin ATPase activity alongside transcriptomic and proteomic analyses in Burmese pythons undergoing different feeding regimes. They report pertinent changes in myofibril function, including increased relaxation rate and passive tension. The discovery that protein synthesis does not keep rate with hypertrophy is also noteworthy and valuable. The experiments are well described and the manuscript is engaging and a good fit for the journal. I have offered a few minor comments.

Regarding identification of changes in MYH genes in the omic analyses, it is now known that different vertebrates exhibit very different repertoires of MYH orthologs with little conservation between species (https://www.biorxiv.org/content/10.1101/2025.10.28.684953v2). Are the authors certain that their nomenclature, which seems based largely on mammalian homologs, is applicable to pythons?

First line: “Burmese pythons (Python molurus bivittatus)”- bivittatus is no longer recognized as a sub-species but a separate species; Python bivittatus

There are some papers in reptilian physiology literature that are overlooked but are highly relevant so should be discussed, including previous reports of increased cardiac contractility in caimans during digestions (https://cdnsciencepub.com/doi/10.1139/cjz-2022-0180) and changes in cardiac filling during the postprandial period that increase stroke volume via Frank-Starling mechanism (i.e. increased sarcomere length) (https://doi.org/10.1242/jeb.142729).

Results are mired by frequent references towards non-statistically significant “trending higher”, “trended toward a decrease in FF-3DPF ($p = 0.102$,”, ” trending increase ($p = 0.089$)”- if there is no statistical support, it is confusing to grasp at unsupported “trends”. Also regarding DRX time constant, $P=0.05000$ is not <0.05, so should not be considered as different.

Fig. 2 title: “Frequent feeding induces faster ATP turnover by myosin heavy chain”. – this is misleading as feeding doesn’t really seem to change anything with ATP turnover, i.e. there are no statistically significant differences (except for borderline case described above)

Fig 2, a minor aesthetic point, but it is confusing in Fig. 2 to use same colors to distinguish different things in different panels (pink/blue for SRX/DRX or different treatment groups of snakes)

Reviewer #3

(Remarks to the Author)
General comments
The purpose of this study was to examine the influence of fasting on the activation of myofilaments from the Burmese python heart and how this may be linked to post translational modifications in the cardiac proteome. This paper is a continuation of previous work on ball pythons demonstrating that feeding state affects both the active and passive properties of the contractile elements. I believe the authors have completed an interesting study that will be relevant to multiple fields; however, the manuscript requires editing for content (there is too much) and focus (there is not enough). Additional methodological details are also required.

Introduction

How does the metabolic rate of a python fed once per month compare with that of one fed eight times per month? The authors wish to link changes in myofibril function and/or the proteome to an increase in metabolic rate.

Is there any evidence suggesting that whole heart function in Burmese pythons changes with overfeeding?

Methods
How old were the animals used, and were they all the same age?

What was the sex of the animals used, and did this differ between treatment groups? THis is relevent as the male of some species demonstrate greater changes in cardiac function/morphology as a result of an increase in workload.

The animals were held at 30 °C, but the myofilament experiments were completed at 15 °C. How is this temperature difference expected to affect the active and passive properties of the myofilaments?

Was the average relative heart mass of the different groups determined, and did this change with feeding regime?

In the figures illustrating myofilament function, what does each data point represent? Does each point correspond to a preparation isolated from a different heart? If so, for example, 16 data points for one treatment would indicate myofilaments isolated from 16 different snakes. This information is relevant to understanding how the data were analyzed for statistical significance.

Myofilaments were activated using solutions delivered from two different pipettes. One pipette released a solution with a pCa of 4.5 and the other a pCa of 9.0. Please explain how six different pCa values were generated for the force–pCa curves and how these concentrations were determined. I reviewed the cited methodological papers, and this information was not provided in either reference.

How long was force measured at each pCa during activation, and did force reach an asymptote before the solution was changed?

How was force generation by the myofilament affected by the protocol overall? For example, what was the percentage decrease in maximum force between the beginning and completion of the protocol? Was this decrease used as a preparation quality criterion (e.g., preparations losing more than 10% of maximum force were excluded)?

How was passive tension measured? Was 2,3 butanedione monoxime used to eliminate any contribution from cross bridge cycling? This detail may be relevant to the discussion of changes in titin and how this relates to passive tension.

I believe it is important that representative raw data traces from the myofilament preparations be included in the figures.

The authors measured tissue density using a method (Lee et al., 2024) originally developed for hydrogels. Has this method previously been applied to biological tissue? I think additional supporting work is required to validate its use in this context. This could be addressed histologically, for example using fluorescent antibodies against proteins such as connexin 43, a gap junction protein in the heart.

With respect to the large format gels, how was the amount of protein loaded per lane standardized? The authors state that 7.5 µL of protein solution was loaded per lane, but it is unclear whether protein concentration (µg/µL) was equivalent across samples.

Results
This section should be rewritten so that results are described rather than interpreted. Doing so would considerably shorten the section.

Overall, a large amount of data is presented in this manuscript, and some of it could be moved to the supplemental material. This includes Figure 3 (RNA sequencing results) and Figure 8. It is unclear how the results summarized in Figure 8 can be meaningfully discussed beyond stating that numerous changes were observed. As the authors note, it is not possible to directly link these changes to specific functional alterations in the proteins. Nonetheless, having these data available as reference material is valuable.

Was there any difference in resting cellular ATP levels in the hearts of the different treatment groups? Differences in ATP availability could affect cross bridge cycling rates and maximum tension development.

In the abstract and throughout the manuscript, the authors describe lower levels of specific proteins in one group as a decrease in “protein expression,” and higher levels of genes as an increase in “expression.” This terminology is not accurate. The methods used only indicate relative concentrations of genes or proteins at the time the animals were killed and do not distinguish between altered synthesis and altered degradation rates. This distinction is important because reducing protein degradation is a relatively low energy strategy for modifying tissue function. Please revise the manuscript accordingly.

Discussion

This section could be edited for length. There is a substantial amount of speculation that could be reduced.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
All of my earlier comments have been satisfactorily addressed. I have no further comments or suggestions.

Reviewer #3

(Remarks to the Author)
The Authors has satisfied my previous comments regarding this MS. It is my opinion that the MS is suitable for publication

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Response to Reviewers
Our initial submission received overall favorable comments from the two reviewers who pointed to the value of the findings and potential appropriate fit in Communications Biology. However, both identified important areas for improvement, including modifications to the discussion and omitted details in the myofibril mechanics experiments. In this revised manuscript, we have fully addressed the critiques of the reviewers, and our point-by-point responses are detailed below (in *italics*). We believe the edits made in this revision have greatly strengthened the quality of the manuscript.

Reviewer #1
Martin, Suarez-Artiles and colleagues investigate changes in myofibril function and myosin ATPase activity alongside transcriptomic and proteomic analyses in Burmese pythons undergoing different feeding regimes. They report pertinent changes in myofibril function, including increased relaxation rate and passive tension. The discovery that protein synthesis does not keep rate with hypertrophy is also noteworthy and valuable. The experiments are well described and the manuscript is engaging and a good fit for the journal. I have offered a few minor comments.

1. Regarding identification of changes in MYH genes in the omic analyses, it is now known that different vertebrates exhibit very different repertoires of MYH orthologs with little conservation between species (https://www.biorxiv.org/content/10.1101/2025.10.28.684953v2). Are the authors certain that their nomenclature, which seems based largely on mammalian homologs, is applicable to pythons?

Response: The reviewer makes a good point regarding myosin conservation. In our experience assessing gene conservation between pythons and mammals, we find that the python Myh15 is more conserved at the protein level with human Myh7 than is the python Myh7 (PMID: 36334627). Therefore, one-to-one matching of myosins between distant vertebrates based on the gene ID alone is not appropriate. Rather than introduce unnecessary complexity, the nomenclature used in the present study is taken directly from the annotations available on NCBI for the Burmese python genome and proteome.

2. First line: “Burmese pythons (Python molurus bivittatus)”- bivittatus is no longer recognized as a sub-species but a separate species; Python bivittatus

Response: Thank you for noting; this is accurate. We have revised accordingly.

3. There are some papers in reptilian physiology literature that are overlooked but are highly relevant so should be discussed, including previous reports of increased cardiac contractility in caimans during digestions (https://cdnsciencepub.com/doi/10.1139/cjz-2022-0180) and changes in cardiac filling during the postprandial period that increase stroke volume via Frank-Starling mechanism (i.e. increased sarcomere length) (https://doi.org/10.1242/jeb.142729).

Response: We thank the reviewer for pointing these studies out and were not familiar with the Caiman study, which is highly relevant. Both studies have been briefly cited in the revised introduction.

4. Results are mired by frequent references towards non-statistically significant “trending higher”, “trended toward a decrease in FF-3DPF (p = 0.102,”, ” trending increase (p = 0.089)”- if there is no statistical support, it is confusing to grasp at unsupported “trends”. Also regarding DRX time constant, P=0.05000 is not <0.05, so should not be considered as different.

Response: We appreciate the reviewer’s point that such references to statistical trends can be confusing and have therefore removed all such references in the revised manuscript.

5. Fig. 2 title: “Frequent feeding induces faster ATP turnover by myosin heavy chain”. – this is misleading as feeding doesn’t really seem to change anything with ATP turnover, i.e. there are no statistically significant differences (except for borderline case described above)

Response: We have modified the title of this figure in the revised manuscript to “Cardiac myosin heavy chain SRX/DRX states are not affected by feeding.”

6. Fig 2, a minor aesthetic point, but it is confusing in Fig. 2 to use same colors to distinguish different things in different panels (pink/blue for SRX/DRX or different treatment groups of snakes)

Response: This is a good point. We have adjusted the color scheme in panels 2A and 2B to provide better figure clarity.

Reviewer #2

The purpose of this study was to examine the influence of fasting on the activation of myofilaments from the Burmese python heart and how this may be linked to post translational modifications in the cardiac proteome. This paper is a continuation of previous work on ball pythons demonstrating that feeding state affects both the active and passive properties of the contractile elements. I believe the authors have completed an interesting study that will be relevant to multiple fields; however, the manuscript requires editing for content (there is too much) and focus (there is not enough). Additional methodological details are also required.

Introduction

1. How does the metabolic rate of a python fed once per month compare with that of one fed eight times per month? The authors wish to link changes in myofibril function and/or the proteome to an increase in metabolic rate.

Response: The metabolic rate in these two feeding paradigms has not been measured. However, based on the metabolic rate increase after a single meal that peaks after three days and then subsides within a week, it is logical to expect that feeding on a similar sized meal once every four days would induce consistently elevated metabolic rate compared to baseline (fasting). We have modified our language in the introduction slightly: "…the latter of which is expected to induce sustained elevation of metabolism due to constant digestion."

2. Is there any evidence suggesting that whole heart function in Burmese pythons changes with overfeeding?

Response: We agree with the reviewer that assessment of whole heart function would be valuable to ascertain feeding paradigm-specific changes. However, no echocardiography analyses have been performed to assess in vivo cardiac function after overfeeding, so we cannot state whether whole heart function changes or not.

Methods

3. How old were the animals used, and were they all the same age?

Response: The pythons used were ~2 years old and were all from the same initial clutch of eggs. These details have been added to the revised manuscript.

4. What was the sex of the animals used, and did this differ between treatment groups? This is relevant as the male of some species demonstrate greater changes in cardiac function/morphology as a result of an increase in workload.

Response: The reviewer brings up an important point, and we were remiss to not include this information in the original methods section. Due to the difficulty in sourcing pythons in sufficient number, the study was not powered to determine potential sex differences and each group included both male and female pythons. The exact breakdown of each group by sex (28DPF: n = 3 male, 2 female; 3DPF: n = 4 male, 1 female; FF28DPF: n = 2 male, 2 female; FF3DPF: n = 3 male, 2 female) for the proteomics studies is now provided in the revised manuscript.

5. The animals were held at 30 °C, but the myofilament experiments were completed at 15 °C. How is this temperature difference expected to affect the active and passive properties of the myofilaments?

Response: The ex vivo myofibril mechanics experiments were completed at 15°C as this allows us to visualize and quantify rapid kinetics associated with cross-bridge cycling because the lower temperature prolongs the timing of the sarcomere mechanics. This allows us to quantify millisecond level changes. Importantly, while

these experiments are not conducted at physiologic temperatures, we are able to draw valuable conclusions about critical differences in disease processes such that we can extrapolate and generate hypotheses for mechanisms of action. Differences in ex vivo mechanics observed at 15°C can elucidate sarcomeric processes and proteins that are impacted by overfeeding.

6. Was the average relative heart mass of the different groups determined, and did this change with feeding regime?

Response: Yes, it was. The organ mass changes for this cohort of pythons have been previously reported in another manuscript (PMID: 40475666). The relative heart mass in both feeding groups increased post-feeding, and this change was particularly pronounced in the frequent feeding group.

7. In the figures illustrating myofilament function, what does each data point represent? Does each point correspond to a preparation isolated from a different heart? If so, for example, 16 data points for one treatment would indicate myofilaments isolated from 16 different snakes. This information is relevant to understanding how the data were analyzed for statistical significance.

Response: Each data point represents a single myofibril, which is the common method for presenting data of this kind. Myofibrils were isolated from 2-4 different python hearts per group. These details are provided in the figure legend of the revised manuscript. Myofibril data points from to each different biological replicate are now denoted by unique shapes to provide additional clarity.

8. Myofilaments were activated using solutions delivered from two different pipettes. One pipette released a solution with a pCa of 4.5 and the other a pCa of 9.0. Please explain how six different pCa values were generated for the force–pCa curves and how these concentrations were determined. I reviewed the cited methodological papers, and this information was not provided in either reference.

Response: We have clarified the methods in the manuscript. This technique was adapted from the Poggesi system (PMID 12937281). Briefly, our system employs a double-barreled perfusion pipette attached to an 8-valve system that allows us to introduce solutions with variable calcium concentrations at a continuous flow individually. The left side is attached to 6 different valves and is the only side with calcium introduced. The right side is attached to 2 different valves to introduce only calcium-free solutions. Solutions of different pCa are made up individually. Our workflow for a single myofibril is as follows, the details of which have now also been added to the revised methods:

a. *Pick up a myofibril using 2 custom microtools, position double barreled pipette at a right angle to the myofibril and within 1mm.*
b. *Our program initiates the start of relaxing solution (pCa 9.0; delivered from the right side of the pipette), the myofibril is within the relaxing solution stream for 3 seconds, at which time passive tension is measured*
c. *At 3 seconds, a stepper-motor-controlled system rapidly switches the pipette so the myofibril is engulfed in the pCa 4.5 activating solution and this remains in place for 17sec. After 17sec, the motor switches the pipette back to relaxation solution and we measure relaxation kinetics.*
d. *Following the return to baseline, we capture the trace. The myofibril remains upright and we maintain the myofibril in relaxation solution for 1 min before switching the calcium solution to a different pCa and complete the measurements again for each of the decreasing pCa solutions (5.6, 5.8, 6.0, 6.2). Following the final pCa measurement, the myofibril is remeasured at pCa 4.5 to account for rundown. If the force collected at the final pCa 4.5 is less than 80% of the force measured at the first pCa 4.5, the entire set of data is excluded.*

9. How long was force measured at each pCa during activation, and did force reach an asymptote before the solution was changed?

Response: For python myofibrils, myofibrils are exposed to calcium solution (of differing pCa) for 17 seconds (see above for detailed workflow). Maximal tension is measured after the force reaches a plateau (which on average occurs within 4 seconds. Reactivation is initiated at 8.5 seconds, and relaxation solution reintroduced after 17 seconds. The total recording ends after 25 seconds, to allow time for the myofibril to return to baseline.

10. How was force generation by the myofilament affected by the protocol overall? For example, what was the percentage decrease in maximum force between the beginning and completion of the protocol? Was this decrease used as a preparation quality criterion (e.g., preparations losing more than 10% of maximum force were excluded)?

Response: Force generation was minorly impacted in our assessment of calcium sensitivity. Our preparation quality criterion is that we excluded data from myofibrils that lost more than 20% of original force measured in the final measurement at pCa 4.5. Using these criteria, we excluded calcium sensitivity curves from fewer than 5% of the measured myofibrils. This lower percentage of myofibrils with rundown is expected as it is postulated that changes in the sarcoplasmic reticulum and not the myofilament play a bigger role in rundown (PMID 25056841). Also, the lower temperatures of our working conditions also protect against protein degradation. However, this is an important consideration, and we have updated our methods so it is clear that measurements from myofibrils that demonstrated rundown were excluded.

11. How was passive tension measured? Was 2,3 butanedione monoxime used to eliminate any contribution from cross bridge cycling? This detail may be relevant to the discussion of changes in titin and how this relates to passive tension.

Response: This is an important point, and we have re-worded this section of the discussion to account for the fact that the passive tension was measured without BDM. Passive tension is measured at pCa 9.0, and the compliance of the myofibril is measured through assessing the deflection of the force probe when the myofibril is pushed 35% of the myofibril length. While it is considered that up to 80% of passive tension is due to titin's contribution (PMID 7756523, 21255582), it is still possible that other contributing factors such as residual crossbridge attachments may contribute to passive tension as we measured it.

12. I believe it is important that representative raw data traces from the myofilament preparations be included in the figures.
Response: We have included representative traces for the myofilament mechanics measurements.

13. The authors measured tissue density using a method (Lee et al., 2024) originally developed for hydrogels. Has this method previously been applied to biological tissue? I think additional supporting work is required to validate its use in this context. This could be addressed histologically, for example using fluorescent antibodies against proteins such as connexin 43, a gap junction protein in the heart.

Response: The method has been validated in biological tissues, and the citation is now updated to include a citation to the initial study that used FRAP to assess diffusion through spheroids and mouse tumor tissues (PMID: 19123683). However, as we agree this approach is not commonly employed outside of the bioengineering field, we have also softened the language regarding the interpretation of these data to "suggest" from "indicate."

14. With respect to the large format gels, how was the amount of protein loaded per lane standardized? The authors state that 7.5 µL of protein solution was loaded per lane, but it is unclear whether protein concentration (µg/µL) was equivalent across samples.

Response: Protein concentrations could not be determined by absorbance-based assays due to the presence of bromophenol blue in the lysis buffer. However, an equivalent mass of each sample was pulverized, and then equivalent volumes loaded onto the gel, meaning samples were approximately the same. This approach is typical for running large format gels to assess titin isoforms (see PMID: 29872636). The value of this gel is not to show comparison of band intensity between lanes, but rather to determine the relative isoform proportions within each lane.

Results
15. This section should be rewritten so that results are described rather than interpreted. Doing so would considerably shorten the section.

Response: We appreciate the reviewer's point and have edited the results to remove unnecessary interpretation.

16. Overall, a large amount of data is presented in this manuscript, and some of it could be moved to the supplemental material. This includes Figure 3 (RNA sequencing results) and Figure 8. It is unclear how the results summarized in Figure 8 can be meaningfully discussed beyond stating that numerous changes were observed. As the authors note, it is not possible to directly link these changes to specific functional alterations in the proteins. Nonetheless, having these data available as reference material is valuable.

Response: We agree that the RNA sequencing data is well-suited to be moved to the supplement, and we have done this in the revised manuscript. While we also agree that the Figure 8 results can only be discussed insofar as they indicate numerous changes occur with feeding, we believe this figure nicely summarizes all of the MYH15 and TTN PTMs together and therefore still provides sufficient value to the reader to warrant its inclusion in the main body of the manuscript.

17. Was there any difference in resting cellular ATP levels in the hearts of the different treatment groups? Differences in ATP availability could affect cross bridge cycling rates and maximum tension development.

Response: Resting cellular ATP levels in the python hearts were not measured; however, the resting ATP levels have no impact on the myofibril experiments as these preps are of permeabilized samples and thus endogenous ATP is washed out prior to the experiments. This allows the experimenter to completely control ATP and calcium in the buffers.

18. In the abstract and throughout the manuscript, the authors describe lower levels of specific proteins in one group as a decrease in "protein expression," and higher levels of genes as an increase in "expression." This terminology is not accurate. The methods used only indicate relative concentrations of genes or proteins at the time the animals were killed and do not distinguish between altered synthesis and altered degradation rates. This distinction is important because reducing protein degradation is a relatively low energy strategy for modifying tissue function. Please revise the manuscript accordingly.

Response: We completely agree with the reviewer and have edited the text throughout to reflect the terminology of "relative protein/mRNA concentration."

Discussion

19. This section could be edited for length. There is a substantial amount of speculation that could be reduced.

Response: We appreciate the reviewer's point and have edited the revised discussion to be more concise.