

# Validation of metaxin-2 deficient *C. elegans* as a model for MandibuloAcral Dysplasia associated to mtX-2 (MADaM) syndrome

Corresponding Author: Professor Philippe Manivet

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This study presents a novel model for investigating a progeroid syndrome, Mandibuloacral dysplasia associated with MTX2 (MADaM), utilizing the nematode *Caenorhabditis elegans* as an alternative animal model. This choice is particularly relevant as *C. elegans* can recapitulate various molecular and physiological defects observed in human patients, and is globally recognized as a valuable model for studying human diseases.

The authors comprehensively characterized numerous phenotypes and morphological changes using a diverse range of methods. They successfully identified that some of the phenotypes reported in MADaM patients are also evident in the *C. elegans* model.

The study is particularly intriguing due to its comprehensive exploration of different aspects related to the consequences of the absence of metaxin-2. The findings contribute significantly to our understanding of the pathology associated with MADaM, shedding light on potential avenues for further research and therapeutic interventions.

I have some comments:

Minor points:

-Introduction section

1-The authors asserted the utilization of downregulated mtX-2 worms, which is misleading as they employed ko. Please revise the terminology accordingly.

-The authors referenced Page et al. and asserted that the cuticle has been advocated as a model for human skin. However, this article posited the *C. elegans* cuticle as a potential parasitic target. I recommend citing alternative works to support this claim.

Chisholm AD, Hsiao TI. The *Caenorhabditis elegans* epidermis as a model skin. I: development, patterning, and growth. Wiley Interdiscip Rev Dev Biol. 2012 Nov-Dec;1(6):861-78. doi: 10.1002/wdev.79. Epub 2012 Jun 19. PMID: 23539299; PMCID: PMC3607643.

Chisholm AD, Xu S. The *Caenorhabditis elegans* epidermis as a model skin. II: differentiation and physiological roles. Wiley Interdiscip Rev Dev Biol. 2012 Nov-Dec;1(6):879-902. doi: 10.1002/wdev.77. Epub 2012 Jun 19. PMID: 23539358; PMCID: PMC3607645.

2- They proposed using *C. elegans* as a model for MADaM disease, with the mutants replicating several phenotypes observed in patients. It's worth noting that a model organism can be valuable even if it exhibits phenotypes different from those observed in humans. For instance, Alzheimer's disease (AD) models in *C. elegans* have shown affected chemotaxis (Dosanjh LE, Brown MK, Rao G, et al. Behavioral phenotyping of a transgenic *Caenorhabditis elegans* expressing neuronal amyloid-beta. Journal of Alzheimer's disease: JAD. 2010;19(2):681-90.; Wu Y, Wu Z, Butko P, et al. Amyloid-beta-induced pathological behaviors are suppressed by Ginkgo biloba extract EGb 761 and ginkgolides in transgenic *Caenorhabditis elegans*. J Neurosci. 2006 Dec 13;26(50):13102-13.). This assay could serve as a valuable tool for drug screening for AD. Therefore, it is essential to recognize the versatility of model organisms in capturing relevant aspects of diseases, even if the phenotypic manifestations may differ from those observed in human patients.

They also mentioned the potential use of *C. elegans* as a rapid screening tool for treatments. It would be insightful to provide additional details on this aspect, including specific assays that could be employed for drug screening. It is important to note that some assays may be challenging to apply in a high-throughput screening setting. Therefore, elucidating the feasibility

and practicality of certain assays for drug screening in the context of *C. elegans* would enhance the clarity of this proposition.

#### Results Section:

1-The authors stated that the over-expression of *ugt-44* and *ced-7*, as well as the under-expression of *col-12* and *dpy-7*, were confirmed through RT-qPCR (data not shown). It would be valuable to elucidate the rationale behind the selection of these specific genes. While the volcano plot indicates the selection of *ugt-44* and *col-12*, clarity is needed regarding the criteria for choosing *ced-7* and *dpy-7*, especially if they are not prominently featured as selected genes in the plot.

2-The results for the pharyngeal pumping rate in wt animals were observed to be lower compared to findings in other studies (Izquierdo et al., 2021; Croft et al., 2023; Andersen et al., 2022). It would be valuable to provide an explanation for these observed differences. Possible factors contributing to the variation, such as experimental conditions or methodology discrepancies.

#### Discussion Section:

-It would be beneficial to incorporate commentary on the utility of CRISPR as a tool for recapitulating human mutations.

#### Figures:

Figure 4.

To address the exclusion of outlier values, it would be beneficial to provide a description of the methodology employed.

#### Figure 7- Egg laying Assay

1-In the text the author claimed:

Reproductive capacity was evaluated by the number of eggs laid by individual gravid worms placed on NGM agar/OP50 plates for 3h. Significantly fewer eggs were laid by *mtx-2* and *gas-1* worms compared to WT ( $6 \pm 4$  and  $4 \pm 2$  vs  $186 \pm 39$ ,  $p = 0.0004$  and  $p < 0.0001$ , respectively, Fig. 7c).

Fig. 7 Phenotypic characterization and comparison of worm strains. c Reproductive capacity assessed by; number of eggs laid during 3 hours (left) and number of worms after incubating those eggs for 3 days (right).

The legend is unclear and causes confusion when compared to the accompanying text. Clarifying details in the legend will enhance the overall coherence of the figure and its interpretation.

#### Major points:

1-One characteristic of the pathology is its profound impact on patients' lifespan, with individuals not surviving beyond 20 years. However, in the *C. elegans* model, the authors did not observe any differences in this parameter when compared with the WT lifespan. I have a concern regarding the use of FUDR during lifespan assays.

The authors say:

"While the lifespan of *mtx-2* worms is unaffected, this does not provide an assessment of their physiological function, which would better reflect accelerated aging. The longer lifespan of *gas-1* worms is most likely due to the use of FUDR (5-Fluoro-2'-deoxyuridine) to prevent egg laying. FUDR45 has a positive effect on respiratory chain complex I which is normally perturbed in *gas-1* worms and without FUDR, *gas-1* lifespan has been shown to be shorter than WT"

I am curious whether the absence of differences observed between the wild-type (WT) and *mtx-2* in lifespan could be attributed to the use of FUDR. It's possible that *mtx-2* has a shorter lifespan, and FUDR may be prolonging it, similar to what occurs in *gas-1* mutants. I suggest repeating this experiment without FUDR to confirm this conclusion.

2- The authors claimed:

"We previously showed that *mtx-2* worms have an abnormal mitochondrial network and mitochondrial fragmentation, consistent with observations in patient-derived fibroblasts<sup>5</sup>. In this study, we show that *mtx-2* worms accurately display MADaM-related phenotypes including skin abnormalities<sup>53</sup>(rougher, less elastic cuticle), muscle degeneration and dysfunction(reduced pharyngeal pumping), aberrant mitochondrial network morphology, growth retardation (shorter body length), and developmental delay"

While they claim the existence of muscle degeneration, no evaluation of this parameter was conducted in any of the experiments. I would like to propose incorporating experiments to assess body muscle degeneration. This could involve measuring locomotion activity and/or conducting microscopy experiments using a GFP reporter

#### Reviewer #2

(Remarks to the Author)

#### Abstract

-Please use the full definition of MADaM and then give the abbreviation.

-The whole abstract can be further improved. More background information can be included, while the importance of the findings needs to be stressed.

## Introduction

- I suggest splitting the first paragraph into smaller paragraphs (e.g., 1st rare genetic diseases, 2nd introduction to MADaM.....etc).
- The last two paragraphs of the Introduction include results and significance. I suggest not to include this kind of information on the Introduction section.

## Results

- Fig.1 Clarify in the caption that the images in first and last columns are the same.
- On 2D AFM images scale bars are missing (only for Z-axis are included)
- “While the SEM observations support the AFM data” discuss a little the difference that might be observed due to the different imaging modalities and the differences in sample preparation

## Discussion

The final “conclusion” paragraphs are very weak. The authors need to stress the importance of and novelty of their work.

## Materials and Methods

Atomic Force Microscopy (AFM) characterization of *C. elegans*:

- For mitochondrial analysis some references for supporting the selected methods are missing.
- “a glass slide was coated with 0.01% poly-L-lysine solution for 20 min then rinsed with water”-> what kind of water?
- “MLCT-F cantilevers” -> please clarify that MLCT probes were used by using the cantilever F (MLCT of MLCT-Bio were used?)
- How frequently did the cantilevers was changed?
- Write the calibration procedure as a separate (sub-) procedure to be clear
- Clarify the if for analyzing AFM images and mechanical properties the same software was used.

## Reviewer #3

### (Remarks to the Author)

In this work, Talarmin-Gas et al. present a characterization of a *mtx-2* null mutant in *C. elegans* with the aim of establishing an animal model for studying Mandibulo Acral Dysplasia associated with *mtx-2* (MADaM) syndrome. They employ various techniques, including Atomic Force Microscopy (AFM) to study the morphology of mitochondria and the animal's cuticle (SEM was also used), transcriptomics to determine the differential gene expression pattern in these mutants, Oxygen Consumption Rates (OCR) to assess mitochondrial functionality, and basic phenotypic analyses to estimate the fitness of the animals. Overall, I believe the study is well-conceived, innovative, and necessary, given the need to establish multicellular models for such pathologies to understand molecular mechanisms and evaluate initial therapeutic approaches. The experiments are generally well-designed, and advanced techniques are appropriately employed.

However, before publication, I would like the authors to address some key points

### Major points:

1) From the transcriptomic analysis, the authors find that many collagen-encoding genes are differentially expressed in the *mtx-2* mutant. Given this result, they appropriately analyze the structure of the cuticle and compare it with wild-type animals, demonstrating alterations in cuticle structure that likely lead to changes in the size of the animal. At this point, considering the significant impact on cuticle structure in the mutant background, I suggest performing an assay to measure the functionality of the cuticle directly. This can be achieved by assessing permeability using Hoechst 33258 staining in whole animals, as described by Moribe et al., 2004 (<https://doi.org/10.1242/jcs.01403>), or Sandhu et al., 2021 (<https://doi.org/10.1093/genetics/iyaa047>). I believe this approach provides a more direct evaluation of the role of *mtx-2*. While pharyngeal pumping, progeny, and development time may offer some information, any animal with reduced fitness is likely to show alterations in these parameters. In essence, studying the functionality of the affected structure, in this case, the cuticle, is a more robust analysis

2)

Typically, to confirm the association of a gene with a specific phenotype, it would be desirable to test with two different alleles. I understand that there is no other available null allele of *mtx-2* for *C. elegans* to corroborate the findings. Beyond this limitation, especially in a case where only one allele is utilized, it is always advisable to investigate whether the phenotypes observed in *mtx-2* mutants can be rescued by injecting the normal gene. Moreover, it would significantly enhance the value of this study if these defects could be rescued using the human ortholog of *mtx-2*.

Furthermore, I have a question regarding whether, for example, the effect on the cuticle is due to mitochondrial failure in the hypodermis (which secretes the cuticle) or if it is a systemic detrimental phenomenon in the entire animal. Expressing *mtx-2* in the hypodermal cells that secrete the cuticle (for example, *dpy-7*) could help address this question.

### Minor points

1. It is not clear to me the purpose of showing the analysis of the shape and stiffness of mitochondria based on age only for wild-type animals (Figure 1E). Is it to demonstrate changes with age and that the *mtx-2* and *gas-1* mutants are already different at the L4 stage? However, these changes do not always follow the same direction as the *mtx-2* mutants (for example, as they get older, the length decreases in the wild type, but *mtx-2* shows greater length than the wild type at L4). It would be beneficial to observe how these parameters change with age in the mutants. This point and the objective of experiment 1E should be clarified.

2. Since *mtx-2* affects mitochondrial function, and as mentioned by the authors, FUDR activates respiratory chain complex I, it is advisable to conduct lifespan assays without FUDR to obtain a more accurate understanding of the mutant's effect on lifespan.

3. In Figure 7C, there appears to be a mistake. A non-mated hermaphrodite lays 300 eggs in its entire life, making it impossible to lay 186 eggs in three hours (as mentioned in the wild type). Could this be 18.6 eggs? Or did I misunderstand the experiment?

4. In the discussion, as well as in other sections, the authors claim, "In this study, we show that *mtx-2* worms accurately display MADaM-related phenotypes, including skin abnormalities (rougher, less elastic cuticle), muscle degeneration, and dysfunction (reduced pharyngeal pumping)." I did not observe any experiments demonstrating muscle degeneration or dysfunction. A decrease in pharyngeal pumping does not necessarily imply malfunctioning pharyngeal muscles; it could be related to neuronal alterations or changes in the perception of nutritional status. To support these claims, the authors should, at the very least, analyze muscular morphology. They could even perform electrophysiology or optogenetics to study muscular functionality. I understand that this might be beyond the scope of this study, but I believe the claims regarding muscular activity need to be tempered.

5. It would be convenient to include line numbers in the manuscript. This would facilitate the review process.

Author Rebuttal letter:

Response to reviewers' comments

"Validation of metaxin-2 deficient *C. elegans* as a model for MandibuloAcral Dysplasia associated to *mtx-2* (MADaM) syndrome"

We thank all reviewers for their time and thoroughness in their interrogation of our manuscript, and for all their comments/suggestions for improvements. Our responses to each reviewer's comment are in red, directly following each individual point.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This study presents a novel model for investigating a progeroid syndrome, Mandibuloacral dysplasia associated with *MTX2* (MADaM), utilizing the nematode *Caenorhabditis elegans* as an alternative animal model. This choice is particularly relevant as *C. elegans* can recapitulate various molecular and physiological defects observed in human patients, and is globally recognized as a valuable model for studying human diseases.

The authors comprehensively characterized numerous phenotypes and morphological changes using a diverse range of methods. They successfully identified that some of the phenotypes reported in MADaM patients are also evident in the *C. elegans* model.

The study is particularly intriguing due to its comprehensive exploration of different aspects related to the consequences of the absence of metaxin-2. The findings contribute significantly to our understanding of the pathology associated with MADaM, shedding light on potential avenues for further research and therapeutic interventions.

Minor points:

-Introduction section

1-The authors asserted the utilization of downregulated *mtx-2* worms, which is misleading as they employed *ko*. Please revise the terminology accordingly.

We thank the reviewer for bringing this to our attention and have amended terminology accordingly to improve clarity in this regard, for example [line 62] now reads: "*mtx-2 k.o. Caenorhabditis elegans*"

-The authors referenced Page et al. and asserted that the cuticle has been advocated as a model for human skin. However, this article posited the *C. elegans* cuticle as a potential parasitic target. I recommend citing alternative works to support this claim.

Chisholm AD, Hsiao TI. The *Caenorhabditis elegans* epidermis as a model skin. I: development, patterning, and growth. *Wiley Interdiscip Rev Dev Biol*. 2012 Nov-Dec;1(6):861-78. doi: 10.1002/wdev.79. Epub 2012 Jun 19. PMID: 23539299; PMCID: PMC3607643.

Chisholm AD, Xu S. The *Caenorhabditis elegans* epidermis as a model skin. II: differentiation and

We agree with the reviewer that these are more suitable references and have amended the reference list accordingly (references 26 and 27).

2- They proposed using *C. elegans* as a model for MADaM disease, with the mutants replicating several phenotypes observed in patients. It's worth noting that a model organism can be valuable even if it exhibits phenotypes different from those observed in humans. For instance, Alzheimer's disease (AD) models in *C. elegans* have shown affected chemotaxis (Dosanjh LE, Brown MK, Rao G, et al. Behavioral phenotyping of a transgenic *Caenorhabditis elegans* expressing neuronal amyloid-beta. Journal of Alzheimer's disease: JAD. 2010;19(2):681-90.; Wu Y, Wu Z, Butko P, et al. Amyloid-beta-induced pathological behaviors are suppressed by Ginkgo biloba extract EGb 761 and ginkgolides in transgenic *Caenorhabditis elegans*. J Neurosci. 2006 Dec 13;26(50):13102-13.). This assay could serve as a valuable tool for drug screening for AD. Therefore, it is essential to recognize the versatility of model organisms in capturing relevant aspects of diseases, even if the phenotypic manifestations may differ from those observed in human patients.

We thank the reviewer for raising this point and completely agree with their sentiment. As we wrote in the initial paragraph of the discussion [line 423], "A commonly accepted definition of a disease model is an animal or cells which display all or some of the pathological processes that are observed in the human or animal disease". We do not feel we have ignored, dismissed, or in any way underplayed the importance or versatility of other *C. elegans* disease models, and we can only assume our interchangeable usage of phenotype/pathology may not have made this completely clear. To improve clarity, we have substituted "pathology" for "phenotype" where appropriate in the discussion, reworded the first paragraph of the discussion for clarity, and put in new references. This section now reads: - "A commonly accepted definition of a disease model is an animal or cells which display all or some of the pathological processes that are observed in the human or animal disease. Animal models have a number of advantages over cellular models, in particular the presence of human gene orthologs, complex signaling pathways, and interplay between tissue types, all of which are critical for elucidating pathogenic mechanisms, identifying new drug targets, assessing therapeutic effect, and refining drug design. The closer a model mirrors disease pathology, the more confident we can be in extrapolating observations to humans, although valuable information can also be obtained from models designed to allow us to isolate specific aspects of a disease, even if their overall phenotype isn't strictly analogous to the human pathology<sup>54</sup>. *C. elegans* is a well-characterized model animal which has been used to study biological processes in development, cardiology, neurobiology<sup>56</sup>, and aging<sup>57</sup> as well as diseases including Alzheimer's disease and Parkinson's disease<sup>16,58</sup>."

They also mentioned the potential use of *C. elegans* as a rapid screening tool for treatments. It would be insightful to provide additional details on this aspect, including specific assays that could be employed for drug screening. It is important to note that some assays may be challenging to apply in a high-throughput screening setting. Therefore, elucidating the feasibility and practicality of certain assays for drug screening in the context of *C. elegans* would enhance the clarity of this proposition.

We thank the reviewer for raising this point and have reworded a section of the discussion accordingly from line 437 onwards:- "Phenotypic assays can be easily scaled up and automation is now commercially available with equipment such as the SydLab™ One60, allowing rapid, cost-effective assessment of the effects of genetic variations and response to chemical compounds, with continuous progress being made towards using *C. elegans* in true high throughput systems<sup>61</sup>.", and have included references to currently commercially available equipment and assays as well as recent advances from research laboratories. We have not written in detail on this in the text as we feel the references will be sufficient for anyone interested in automation and experimental scale up, and because an extended discussion of these points, while extremely interesting, would be outside the scope of this study.

#### Results Section:

1-The authors stated that the over-expression of *ugt-44* and *ced-7*, as well as the under-expression of *col-12* and *dpy-7*, were confirmed through RT-qPCR (data not shown).

It would be valuable to elucidate the rationale behind the selection of these specific genes. While the volcano plot indicates the selection of *ugt-44* and *col-12*, clarity is needed regarding the criteria for choosing *ced-7* and *dpy-7*, especially if they are not prominently featured as selected genes in the plot.

We thank the reviewer for this comment. We have amended Figure 3b (shown below) where now *dpy-7* and *ced-7* are also marked. The text has also been amended and now reads [line 263]: "Ugt-44 and *col-12* were chosen as the two most significantly over- and under-expressed DEGs respectively (Fig. 3b), while *dpy-7* and *ced-7* were chosen because they encode a structural constituent of collagen- and cuticulin-based cuticle and a key player in apoptosis, respectively<sup>40</sup>."

b Volcano plot of DEGs (n=323). The 10 most statistically significant DEGs and the 2 DEGs further tested by RT-qPCR are indicated on the plot.

2-The results for the pharyngeal pumping rate in wt animals were observed to be lower compared to findings in other studies (Izquierdo et al., 2021; Croft et al., 2023; Andersen et al., 2022). It would be valuable to provide an explanation for these observed differences. Possible factors contributing to the variation, such as experimental conditions or methodology discrepancies.

We thank the reviewer for asking this and have reviewed the suggested articles, along with several more from the same groups and others in the field to get a broader range of published rates to compare ours with. While the average PP rate (n/min) we measured here is indeed slightly lower than the averages of the Izquierdo (~250 vs 229 at L4+1), Croft (~260 vs 240 at D3 of adulthood), and Andersen papers (~250 vs 229 at L4+1), our rate and range is well within the normal ranges that are observed in data published in these papers, the caveat being of course that as we do not have access to the raw data from these papers, we can't perform any statistical analyses to confirm our "by eye" judgment. For reference, the other recently published PP rates we surveyed include; de Oliveira et al. 2024 (~200 at L3), Gowripriya et al. 2024 (~250 at L4+1), Zheng et al. 2024 (~350 at L4+1), Ciccarelli et al. 2024 (~200 at L4+1), Pereira et al. 2024 (~240 at L4), Kuo-Esser et al. 2024 (~120 at L4), and Shanmugham et al. 2023 (~220 at D1, ~260 at D2, ~270 at D3). Any discrepancies could be due to differences in methodology, for example, Izquierdo used manual counting for 3min/worm whereas we used 30s x 3 to obtain our average per worm, Croft and Andersen both digitally recorded worm movements then manually counted slowed down footage for 30s and 15s, respectively, to obtain a PP/min average. Besides experiment specific considerations, we feel that our slightly lower average rate compared to the reviewers selected references may just be a representation of natural variation, as the broad range of published PP rates we noted above shows.

#### Discussion Section:

-It would be beneficial to incorporate commentary on the utility of CRISPR as a tool for recapitulating human mutations.

We thank the reviewer for raising this interesting point. The power and utility of CRISPR for selective genetic manipulation is undeniable, and it could indeed be effectively used to insert the human gene into the *mtx2* worm to both see if this restores normal phenotypes, then make the same mutations as found in human patients. That said, this study focused on characterizing a modified worm that was made with non-CRISPR methods and can be easily obtained from the CGC (VC1064 *mtx2(gk444)*). Because we were not trying to make a new model animal (although if we were, CRISPR-based methods are exactly how we would do so), but to validate the use of a pre-existing mutant as a model, we feel that the inclusion of any commentary on CRISPR-modified organisms would inevitably increase the likelihood of confusion and would draw focus away from the (as we see them) critical points of this manuscript.

#### Figures:

##### Figure 4.

To address the exclusion of outlier values, it would be beneficial to provide a description of the methodology employed.

We thank the reviewer for this comment and have added the following description to the "Image processing, data plotting, and statistical analysis" section in Materials and Methods [line 714]: "The identification of outliers was performed using the Robust regression and OUTlier removal (ROUT) method; in order to control the False Discovery Rate (FDR), the Q coefficient was set at 1% (FDR <1%)."

The following text was also added to the Figure 4 legend [line 276]: "The E values for the *mtx-2* worms identified as outliers but not excluded from the analysis are highlighted."

The following text was also added [line: 292]: "Outlier E values for the *mtx-2* strain were identified (Fig 4d, right), however after performing the analysis with and without the values in question, the level of statistical significance was the same. Thus, these values were considered as true outliers and were not excluded from the dataset."

##### Figure 7- Egg laying Assay

1-In the text the author claimed:

Reproductive capacity was evaluated by the number of eggs laid by individual gravid worms placed on NGM agar/OP50 plates for 3h. Significantly fewer eggs were laid by *mtx-2* and *gas-1* worms compared to WT ( $6 \pm 4$  and  $4 \pm 2$  vs  $186 \pm 39$ ,  $p = 0.0004$  and  $p < 0.0001$ , respectively, Fig. 7c).

Fig. 7 Phenotypic characterization and comparison of worm strains. c Reproductive capacity assessed by; number of eggs laid during 3 hours (left) and number of worms after incubating those eggs for 3 days (right).

The legend is unclear and causes confusion when compared to the accompanying text. Clarifying details in the legend will enhance the overall coherence of the figure and its interpretation.

We thank the reviewer for this comment and have edited the figure and legend to help improve clarity and reduce confusion. In the revised versions of Figure 7c and d (below), the corresponding legend and text were amended to improve clarity:

1) the data in Fig. 7c are now shown in a double axis graph where the left axis refers to the number of eggs counted after an incubation for 3h and the right axis to the number of worms after an incubation for 3d. In Fig. 7d the axis now reads "Stages of worms (% , 3d)" instead of "% of worms".

2) in the legend of Fig 7d, we have replaced in the sentence [line 376]: "Developmental assay showing the percentages of worms at different stages on D1 of adulthood" by "Developmental assay showing the percentages of worms at different stages on D1 of adulthood of WT worms".

c Reproductive capacity assessed by; number of eggs laid during 3 hours (left axis) and number of worms after incubating those eggs for 3 days (right axis). d Developmental assay showing the percentages of worms at different stages on D1 of adulthood of WT worms.  
Major points:

1-One characteristic of the pathology is its profound impact on patients' lifespan, with individuals not surviving beyond 20 years. However, in the *C. elegans* model, the authors did not observe any differences in this parameter when compared with the WT lifespan. I have a concern regarding the use of FUDR during lifespan assays.

The authors say:

"While the lifespan of *mtx-2* worms is unaffected, this does not provide an assessment of their physiological function, which would better reflect accelerated aging. The longer lifespan of *gas-1* worms is most likely due to the use of FUDR (5-Fluoro-2'-deoxyuridine) to prevent egg laying. FUDR45 has a positive effect on respiratory chain complex I which is normally perturbed in *gas-1* worms and without FUDR, *gas-1* lifespan has been shown to be shorter than WT"

I am curious whether the absence of differences observed between the wild-type (WT) and *mtx-2* in lifespan could be attributed to the use of FUDR. It's possible that *mtx-2* has a shorter lifespan, and FUDR may be prolonging it, similar to what occurs in *gas-1* mutants. I suggest repeating this experiment without FUDR to confirm this conclusion.

We thank the reviewer for raising this point and to address their concerns we have repeated the lifespan study of the 3 worm strains without FUDR. In the main body of text, we have replaced Fig. 7a (below) to show the -FUDR data and have moved the previous version +FUDR to the supplemental data section (Supplementary Data Figure 7). As can be seen, in the absence of FUDR, there is no statistical difference in WT (median survival +/- FUDR: 15 vs 14 days,  $p = 0.4710$ ) and *mtx-2* (median survival +/- FUDR: 15 vs 14 days,  $p = 0.6755$ ) lifespans, but there is a notable reduction in *gas-1* lifespan (median survival +/- FUDR: 20 vs 14 days,  $p < 0.0001$ ) [line 381].

Fig. 7 Phenotypic characterization and comparison of worm strains. a Lifespan; Kaplan-Meier survival curves (without FUDR).  
Supplementary Figure 7. Phenotypic characterization and comparison of worm strains. a Lifespan with FUDR; Kaplan-Meier survival curves.

2- The authors claimed:

“We previously showed that *mtx-2* worms have an abnormal mitochondrial network and mitochondrial fragmentation, consistent with observations in patient-derived fibroblasts<sup>5</sup>. In this study, we show that *mtx-2* worms accurately display MADaM-related phenotypes including skin abnormalities<sup>53</sup> (rougher, less elastic cuticle), muscle degeneration and dysfunction (reduced pharyngeal pumping), aberrant mitochondrial network morphology, growth retardation (shorter body length), and developmental delay”

While they claim the existence of muscle degeneration, no evaluation of this parameter was conducted in any of the experiments. I would like to propose incorporating experiments to assess body muscle degeneration. This could involve measuring locomotion activity and/or conducting microscopy experiments using a GFP reporter.

We thank the reviewer for this comment and they are correct that no evaluation of muscle dysfunction was performed in this study. Furthermore, we believe that our phrasing in this section has caused some misunderstanding which has led to the conclusion that we were specifically referring to muscle dysfunction in *C. elegans*. Our intention here was to list the main human clinical symptoms and then have the *C. elegans* phenotype in brackets to show which phenotype (worm) is analogous to which clinical symptom. We now see that our attempted brevity has led to a loss of clarity in this regard and have edited this section accordingly. This section [line 442] now reads:- “We previously showed that *mtx-2* worms have an abnormal mitochondrial network and mitochondrial fragmentation, consistent with observations in patient-derived fibroblasts<sup>5</sup> and here we further show that multiple aspects of the *mtx-2* worm phenotype; rougher, less elastic cuticle, reduced pharyngeal pumping (a sign of aging), shorter body length, and developmental delay are analogous to clinical presentations of MADaM; skin abnormalities<sup>55</sup>, muscle degradation and dysfunction (a sign of aging), growth retardation, and developmental delay”.

While pharyngeal pumping is more than just muscle action, it is a noted sign of aging in *C. elegans*, and in this respect, we believe it is analogous to muscle degradation and dysfunction in humans which is also a sign of aging that is more than just a measure of muscle action.

We completely agree that studies of locomotion and assays of muscular activity and degeneration would provide very interesting data, and it is something we look forward to doing in the near future.  
Reviewer #2 (Remarks to the Author):

## Abstract

1. Please use the full definition of MADaM and then give the abbreviation.

We thank the reviewer for bringing this to our attention and the abstract has been amended accordingly.

2. The whole abstract can be further improved. More background information can be included, while the importance of the findings needs to be stressed.

As per the reviewers' recommendations, the abstract has been edited for clarity and conciseness and to provide more background and highlight the key findings. It now reads as follows: -

“MandibuloAcral Dysplasia associated to MTX2 gene (MADaM) is a recently described progeroid syndrome (accelerated aging disease) whose clinical manifestations include skin abnormalities, growth retardation, and cardiovascular diseases. We previously proposed that *mtx-2*-deficient *C. elegans* could be used as a model for MADaM and to support this, we present here our comprehensive phenotypic characterization of these worms using atomic force microscopy (AFM), transcriptomic, and oxygen consumption rate analyses. AFM analysis showed that young *mtx-2*-less worms had a significantly rougher, less elastic cuticle which becomes significantly rougher and less elastic as they age, and abnormal mitochondrial morphology. *mtx-2* *C. elegans* displayed delayed development, decreased pharyngeal pumping, significantly reduced mitochondrial respiratory capacities, and transcriptomics analysis identified perturbations in the aging, TOR, and WNT-signaling pathways. The phenotypic characteristics of *mtx-2* worms shown here are analogous to many of the human clinical presentations of MADaM and we believe this validates their use as a model which will allow us to uncover the molecular details of the disease and develop new therapeutics and treatments.”

## Introduction

1. I suggest splitting the first paragraph into smaller paragraphs (e.g., 1st rare genetic diseases, 2nd introduction to MADaM.....etc).

This paragraph has been split and edited to help improve clarity.

2. The last two paragraphs of the Introduction include results and significance. I suggest not to include this kind of information on the Introduction section.

We agree with the reviewer that this information is superfluous and have removed these paragraphs from the introduction.

## Results

1. Fig.1 Clarify in the caption that the images in first and last columns are the same.

The legends of Figures 1, 4, and 5 were amended accordingly.

2. On 2D AFM images scale bars are missing (only for Z-axis are included)

Scale bars were added on Figures 1, 4, and 5 and Supplementary Figures 1 and 5.

3. "While the SEM observations support the AFM data" discuss a little the difference that might be observed due to the different imaging modalities and the differences in sample preparation

The following text was added to the discussion [line 520]:

"Loss of skin elasticity and increased roughness are hallmarks of both aging and progeroid syndromes, and the combination of SEM and AFM allows us to clearly see a comparable change in cuticle morphology in *mtx2* worms. While SEM and AFM can be considered complementary with respect to the study of surface morphology in this paper, differences exist between them which could affect observations in other applications. SEM provides better resolution, but worms must be dead and treated before analysis which may result in observation of treatment-generated artifacts. AFM resolution, while lower, is still sufficient to distinguish main surface features, and morphological and mechanical measurements can be carried out in parallel on live, untreated worms. AFM is currently underutilized in biomedical research and here we demonstrate its utility in quantifying biomechanical changes related to disease state and aging at the organismal and organelle level."

## Discussion

The final "conclusion" paragraphs are very weak. The authors need to stress the importance of and novelty of their work.

We have reworded and added to the conclusion paragraph to improve clarity and explicitly highlight the critical findings and conclusions of this study. The final paragraph [line: 531] now reads: -

"In this study, we have expanded upon our previous characterisation of *mtx-2 C. elegans* and shown that the worm's physiological characteristics are analogous to human clinical symptoms. Through AFM, we have been able to show, for the first time, biomechanical changes in *C. elegans* cuticle and mitochondria associated with organismal aging and loss of *mtx2*. With transcriptomics, we have uncovered a range of pathways affected by *mtx2* loss which not only provide a better insight into the molecular mechanisms of MADaM, but also provide critical information for choosing therapeutics based on their known pathway-specific mechanism of action to test their efficacy in treating/managing MADaM. We believe we have provided ample support for the validation of *mtx2 C. elegans* as a functional model of MADaM which can be used to more clearly elucidate the molecular mechanisms of MADaM and to effectively screen potential therapeutics and treatment regimes."

## Materials and Methods

Atomic Force Microscopy (AFM) characterization of *C. elegans*:

1. For mitochondrial analysis some references for supporting the selected methods are missing.

Relevant references have now been added, along with the following [line 95]: "Although AFM has previously been used to study individual mitochondria<sup>29,30</sup>, this is, to the best of our knowledge, the first study to examine mitochondria from *C. elegans*."

The added references are:

29. Layton, B. E. et al. In situ imaging of mitochondrial outer-membrane pores using atomic force microscopy. *Biotechniques* 37, 564–573 (2004).

30. Layton, B. E. & Boyd, M. B. Atomic Force Microscopy of Isolated Mitochondria. in *Methods in molecular biology* (Clifton, N.J.) vol. 736 133–151 (Methods Mol Biol, 2011).

2. “a glass slide was coated with 0.01% poly-L-lysine solution for 20 min then rinsed with water”-> what kind of water?

This clarification was added [line 584]: “Milli-Q ultrapure water”

3. “MLCT-F cantilevers” -> please clarify that MLCT probes were used by using the cantilever F (MLCT of MLCT-Bio were used?)

The following clarification was added [line 566]: “MLCT probes with “F” cantilevers (MLCT-F)”  
Classic MLCT probes were used so no further modification was needed in the text.

4. How frequently did the cantilevers was changed?

The following was added for both mitochondria and cuticle analysis [lines 592 and 606]: “A new cantilever was used for every independent experiment (thus three cantilevers per condition)”.

5. Write the calibration procedure as a separate (sub-) procedure to be clear

We thank the reviewer for this suggestion and to help improve clarity, the calibration steps are now written as a separate subprocedure [line 559 onwards].

6. Clarify the if for analyzing AFM images and mechanical properties the same software was used.

The text was amended accordingly and now reads [line 609]: “All data (topographical and mechanical) was processed with JPK Data processing software (Bruker, version 6.3.23).”

Reviewer #3 (Remarks to the Author):

In this work, Talarmin-Gas et al. present a characterization of a *mtx-2* null mutant in *C. elegans* with the aim of establishing an animal model for studying MandibuloAcral Dysplasia associated with *mtx-2* (MADaM) syndrome. They employ various techniques, including Atomic Force Microscopy (AFM) to study the morphology of mitochondria and the animal's cuticle (SEM was also used), transcriptomics to determine the differential gene expression pattern in these mutants, Oxygen Consumption Rates (OCR) to assess mitochondrial functionality, and basic phenotypic analyses to estimate the fitness of the animals. Overall, I believe the study is well-conceived, innovative, and necessary, given the need to establish multicellular models for such pathologies to understand molecular mechanisms and evaluate initial therapeutic approaches. The experiments are generally well-designed, and advanced techniques are appropriately employed.

However, before publication, I would like the authors to address some key points

Major points:

1. From the transcriptomic analysis, the authors find that many collagen-encoding genes are differentially expressed in the *mtx-2* mutant. Given this result, they appropriately analyze the structure of the cuticle and compare it with wild-type animals, demonstrating alterations in cuticle structure that likely lead to changes in the size of the animal. At this point, considering the significant impact on cuticle structure in the mutant background, I suggest performing an assay to measure the functionality of the cuticle directly. This can be achieved by assessing permeability using Hoechst 33258 staining in whole animals, as described by Moribe et al., 2004 (<https://doi.org/10.1242/jcs.01403>), or Sandhu et al., 2021 (<https://doi.org/10.1093/genetics/iyaa047>). I believe this approach provides a more direct evaluation of the role of *mtx-2*. While pharyngeal pumping, progeny, and development time may offer some information, any animal with reduced fitness is likely to show alterations in these parameters. In essence, studying the functionality of the affected structure, in this case, the cuticle, is a more robust

analysis.

We thank the author for this suggestion and agree that it would tell us more about the cuticle-specific impact resulting from a loss of *mtx2* in *C. elegans*. However, when we attempted to perform these experiments, we found that there was a huge amount of variation between replicates and that they lacked sufficient reproducibility. To attempt to understand why a seemingly straightforward experiment wasn't working in our hands, and why we were seeing Hoechst stained N2 nuclei (or sometimes not) we reviewed a range of papers that reported using the same assay. In brief, there is a range of different Hoechst concentrations, incubation times, wash times, populations used (age and/or synchronization) and types of analysis done. All give different results for N2 worms, some reporting absolutely no stained nuclei, others reporting up to 10%. Some performed statistical analysis, others just a qualitative assessment. Some showed images of the head, others the tail, and others the whole body when describing nuclei staining. The papers we reviewed were; Ward et al. 2014, Li & Paik 2010, Meli et al. 2010, Thein et al. 2009, Hirami et al. 2016, Moribe et al. 2004, Partridge et al. 2008, Fritz & Behn 2009, and Sandhu et al. 2021. In brief, we will work towards a robust cuticle permeability assay for future work, but the time needed to do it for this manuscript would take us well past the deadline for resubmission.

2. Typically, to confirm the association of a gene with a specific phenotype, it would be desirable to test with two different alleles. I understand that there is no other available null allele of *mtx-2* for *C. elegans* to corroborate the findings. Beyond this limitation, especially in a case where only one allele is utilized, it is always advisable to investigate whether the phenotypes observed in *mtx-2* mutants can be rescued by injecting the normal gene. Moreover, it would significantly enhance the value of this study if these defects could be rescued using the human ortholog of *mtx-2*.

Furthermore, I have a question regarding whether, for example, the effect on the cuticle is due to mitochondrial failure in the hypodermis (which secretes the cuticle) or if it is a systemic detrimental phenomenon in the entire animal. Expressing *mtx-2* in the hypodermal cells that secrete the cuticle (for example, *dpy-7*) could help address this question.

We thank the author for these insightful comments, and agree that both restoring function in *mtx2* *C. elegans* and selectively expressing *mtx2* in specific parts of the worm would provide very interesting data, which could very well enhance the overall impact of this article. However, to properly and fully perform these experiments would take a considerable amount of time, well past the deadlines for reassessment of this article. More importantly, the author with the exact skill set necessary to perform them is no longer a permanent member of the laboratory which would require us to either outsource this specific piece of work to either a CRO or a new collaborator, or wait until we can regain this skill set in the lab. Any of these options would again take an unspecified amount of time and for this reason we are unable to include them, although they will be priorities for future follow up projects.

#### Minor points

1. It is not clear to me the purpose of showing the analysis of the shape and stiffness of mitochondria based on age only for wild-type animals (Figure 1E). Is it to demonstrate changes with age and that the *mtx-2* and *gas-1* mutants are already different at the L4 stage? However, these changes do not always follow the same direction as the *mtx-2* mutants (for example, as they get older, the length decreases in the wild type, but *mtx-2* shows greater length than the wild type at L4). It would be beneficial to observe how these parameters change with age in the mutants. This point and the objective of experiment 1E should be clarified.

We thank the reviewer for this suggestion. We performed the corresponding AFM experiments for both aging mutant worms, in order to complete the comparison between all 3 strains of aging worms. The results were added to the Results section [line 126 onwards] and the conclusions to the Discussion section [line 454].

The results are shown in Supplementary Figure 2 (below).

Supplementary Figure 2 Characterization of morphology and mechanical properties of individual mitochondria by AFM. Scatter plots (mean  $\pm$  SD) of (from left to right) length, width, height, aspect ratio, and elastic modulus (E) of mitochondria extracted from a the 3 worm strains at D2, and D8 of adulthood, and b *mtx-2* and *gas-1* worms at L4 stage, D2, and D8 of adulthood. Statistically significant differences are shown (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

2. Since *mtx-2* affects mitochondrial function, and as mentioned by the authors, FUDR activates respiratory chain complex I, it is advisable to conduct lifespan assays without FUDR to obtain a more accurate understanding of the mutant's effect on lifespan.

We thank the reviewer for raising this point and to address their concerns we have repeated the lifespan study of the 3 worm strains without FUdR. In the main body of text, we have replaced Fig. 7a (below) to show the -FUdR data and have moved the previous version +FUdR to the supplemental data section (Supplementary Data Figure 7). As can be seen, in the absence of FUdR, there is no statistical difference in WT (median survival +/- FUdR: 15 vs 14 days,  $p = 0.4710$ ) and *mtx-2* (median survival +/- FUdR: 15 vs 14 days,  $p = 0.6755$ ) lifespans, but there is a notable reduction in *gas-1* lifespan (median survival +/- FUdR: 20 vs 14 days,  $p < 0.0001$ ) [line 381].

Fig. 7 Phenotypic characterization and comparison of worm strains. a Lifespan; Kaplan-Meier survival curves (without FUdR).

Supplementary Figure 7 Phenotypic characterization and comparison of worm strains. a Lifespan with FUdR; Kaplan-Meier survival curves.

3. In Figure 7C, there appears to be a mistake. A non-mated hermaphrodite lays 300 eggs in its entire life, making it impossible to lay 186 eggs in three hours (as mentioned in the wild type). Could this be 18.6 eggs? Or did I misunderstand the experiment?

We thank the reviewer for bringing this to our attention and have amended the Figure 7c and the corresponding legend (below) and text [line 407] to clarify the data: "Reproductive capacity was evaluated by the number of eggs laid by a total of 150 individual gravid worms per strain placed on NGM agar/OP50 plates for 3h". In Materials and Methods section the text now reads [line 707]: "At least three independent experiments were performed and at least 150 worms per strain were used." c Reproductive capacity assessed by; number of eggs laid during 3 hours (left axis) and number of worms after incubating those eggs for 3 days (right axis).

4. In the discussion, as well as in other sections, the authors claim, "In this study, we show that *mtx-2* worms accurately display MADaM-related phenotypes, including skin abnormalities (rougher, less elastic cuticle), muscle degeneration, and dysfunction(reduced pharyngeal pumping)." I did not observe any experiments demonstrating muscle degeneration or dysfunction. A decrease in pharyngeal pumping does not necessarily imply malfunctioning pharyngeal muscles; it could be related to neuronal alterations or changes in the perception of nutritional status. To support these claims, the authors should, at the very least, analyze muscular morphology. They could even perform electrophysiology or optogenetics to study muscular functionality. I understand that this might be beyond the scope of this study, but I believe the claims regarding muscular activity need to be tempered.

We thank the reviewer for this comment and they are correct that no evaluation of muscle dysfunction was performed in this study. Furthermore, we believe that our phrasing in this section has caused some misunderstanding which has led to the conclusion that we were specifically referring to muscle dysfunction in *C. elegans*. Our intention here was to list the main human clinical symptoms and then have the *C. elegans* phenotype in brackets to show which phenotype (worm) is analogous to which clinical symptom. We now see that our attempted brevity has led to a loss of clarity in this regard and have edited this section accordingly. This section [line 442] now reads: - "We previously showed that *mtx-2* worms have an abnormal mitochondrial network and mitochondrial fragmentation, consistent with observations in patient-derived fibroblasts<sup>5</sup> and here we further show that multiple aspects of the *mtx-2* worm phenotype; rougher, less elastic cuticle, reduced pharyngeal pumping (a sign of aging), shorter body length, and developmental delay are analogous to clinical presentations of MADaM; skin abnormalities<sup>55</sup>, muscle degradation and dysfunction(a sign of aging), growth retardation, and developmental delay".

While pharyngeal pumping is more than just muscle action, it is a noted sign of aging in *C. elegans*, and in this respect, we believe it is analogous to muscle degradation and dysfunction in humans which is also a sign of aging that is more than just a measure of muscle action.

We agree that optogenetics and electrophysiology studies of muscular activity and degeneration would provide very interesting data, and it is something we would like to consider for future work. However, we also agree that they are somewhat outside the scope of this current study.

5. It would be convenient to include line numbers in the manuscript. This would facilitate the review process.

Line numbers have now been added.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors responded in a elucidated manner to all of my inquiries.

They not only incorporated new experiments but also clarified certain figure legends that were previously ambiguous.

I recommend this article for publication.

Reviewer #3

(Remarks to the Author)

Although several of my concerns have been addressed, I am not entirely satisfied with the explanation that rescues of MTX-2 cannot be performed because doing so would extend the review time beyond the deadline. It is possible to request an extension. The ability to carry out rescues relatively quickly is an advantage of *C. elegans*, and I believe it is important to do so when attempting to establish a *C. elegans* model of a specific disease

Minor: I still don't understand Figure 7C. It seems that in the cases of MTX-2 and GAS-1, there are more worms at 3 days than the theoretical eggs they would have come from. How is this possible?

Author Rebuttal letter:

Response to reviewers' comments

"Validation of metaxin-2 deficient *C. elegans* as a model for MandibuloAcral Dysplasia associated to mtX-2 (MADaM) syndrome"

Once again, we would like to thank the editors and reviewers for all their hard work in assessing our manuscript. After considering all comments, we have made the following changes. Reviewer comments are in black, our response and manuscript modifications are in red;

Reviewer #3 (Remarks to the Author):

Although several of my concerns have been addressed, I am not entirely satisfied with the explanation that rescues of MTX-2 cannot be performed because doing so would extend the review time beyond the deadline. It is possible to request an extension. The ability to carry out rescues relatively quickly is an advantage of *C. elegans*, and I believe it is important to do so when attempting to establish a *C. elegans* model of a specific disease

After reading and taking into account all comments from the editor and reviewers, the following paragraph was added to the discussion [lines 536-544]. Here, we acknowledge that while the lack of full body rescue experiments (and related phenotype experiments) may be a limitation of this study, rescue experiments have already been performed in other studies using MADaM patient-derived cells and mtX-2 k.o. *C. elegans*, and in both cases, showed complete rescue of the mtX-2-deficient mitochondrial phenotype. We also outline our future work in this area including tissue-specific rescue experiments:

"We recognise that the lack of full body rescue experiments in mtX-2 *C. elegans* using either the *C. elegans* mtX-2 or human MTX2 genes could be seen as a limitation of this study. That said, it should be noted that previous studies have already performed a range of rescue experiments in both MADaM patient-derived cells<sup>5</sup> and mtX-2 K.O. *C. elegans*<sup>23</sup> and in both cases show that the mitochondrial phenotype seen in mtX-2-deficient cells and worms can be completely rescued. Future planned work, including tissue-specific rescue experiments, will allow us to determine the extent of rescue possible in the cuticle and whether the decrease in longevity and the developmental and reproductive delays reported here may be linked to the loss of mtX-2 from a particular tissue/organ or its global loss."

Minor: I still don't understand Figure 7C. It seems that in the cases of MTX-2 and GAS-1, there are more worms at 3 days than the theoretical eggs they would have come from. How is this possible?

We thank the reviewer for bringing this to our attention. To address this discrepancy, we went back to check the original raw data. While doing so, we unfortunately discovered that some of the original data

was missing and thus decided the only course of action was to perform this experiment again. As can be seen in these newly performed experiments, the trends remain the same, although the significance has reduced. This has not led to any changes in the conclusions of the manuscript and the modifications made are as follows:

The figure 7c and d was replaced:

The text was also amended accordingly [lines 410-426]:

“Reproductive capacity was evaluated by the number of eggs laid by a total of 45 individual gravid worms per strain placed on NGM agar/OP50 plates for 3h. Compared to WT, significantly fewer eggs were laid by gas-1 worms ( $245 \pm 46$  vs  $13 \pm 3$ ,  $p = 0.0073$ , Fig. 7c, left axis). A similar trend was seen with the number of worms after 3 days of culture with significantly fewer gas-1 worms than WT ( $12 \pm 4$  vs  $239 \pm 47$ ,  $p = 0.0073$ , Fig. 7c, right axis). These plates were then incubated for 3 days at 20°C (the average time for WT worms to reach adulthood), and developmental capacity was assessed by counting the worms at each developmental stage; larval stages (L1 - L4), young adult (YA) and adult. After 3 days, the majority of WT and mtX-2 worms were in the YA, and gas-1 worms were in the larval stages, respectively (Fig. 7d). Compared to WT, significantly fewer gas-1 worms were in the YA stage ( $77.3 \pm 14.0\%$  vs  $41.5 \pm 14.7\%$ ,  $p = 0.0369$ ), while no significant difference was observed for mtX-2 YA worms ( $76.3 \pm 4.6\%$ ,  $p = 0.8815$ ). This means that after 3 days, the majority of WT and mtX-2 worms are able to lay eggs and reproduce and that the majority of gas-1 worms had not yet reached that stage of maturity. Although the differences between mtX2 and WT worms were not statistically significant (eggs laid -  $163 \pm 11$  vs  $245 \pm 46$ ,  $p = 0.1797$  and worms at 3 days -  $137 \pm 55$  vs  $239 \pm 47$ ,  $p = 0.1797$ ), there is a visible trend towards lower fecundity and slower development in mtX2 worms compared to WT.”

and in [line 377]

“... of a total of 45 individual gravid worms per strain ...”

and finally in [line 721]

“... and 45 worms per strain were used.”

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

Although the authors did not perform the rescue experiment I suggested, I consider it appropriate that they clarified this limitation. I also appreciate that they reviewed the data for figure 7. Therefore, I am satisfied with the responses provided.

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