A low sugar diet enhances *Drosophila* body size in males and females via sex-specific mechanisms

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Original submission decision letter

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MS TITLE: A low sugar diet enhances Drosophila body size in males and females via sex-specific mechanisms
AUTHORS: Jason W Millington, Lianna W Wat, Ziwei Sun, Paige J Basner-Collins, George P Brownrigg, and Elizabeth J Rideout

Dear Dr. Rideout, dear Liz,

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. I should say, however, that in this particular case the revision will require new experiments that address the reviewers' concerns in full. These must confirm and expand the differential regulation of IIS/TOR in males/females by the low sugar diet at the molecular level, and provide some insight into the sex specificity of the metabolic phenotypes.

Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns.

Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The study of Millington and coworkers analyze the sex specific effects of dietary sugar on animal growth control, using Drosophila as a model system. While dietary sugar is known to inhibit growth of Drosophila larvae, the possible sex differences have not been explored. In general, sex specificity of animal physiology represents a field, which has been, until recently, understudied. Therefore, studies filling this gap in the literature are warranted. A previous study of the Rideout group has shown clear sex specific differences in the growth regulation by dietary protein and dissected the underlying molecular mechanisms (PMID: 33448263). In contrast to protein, sugar feeding (studied here) shows no sex specificity on growth control. Despite this lack of difference the authors molecularly analyze the activity of insulin and TOR signaling in females vs. males. They conclude that low sugar diet activates insulin signaling in males, but not in females. This conclusion is supported by a very modest downregulation of insulin-repressed genes in males, but not in females. The authors also conclude that low sugar activates TOR signaling more in females than in males.

To test the functional relevance of insulin and TOR signaling the authors use genetic experiments to partially inhibit insulin and TOR signaling. They conclude that there is a sex-biased requirement for insulin and TOR signaling in sugar-dependent growth control. Finally, sex-specific changes in metabolism (including glycogen and trehalose) and gene expression in response to sugar are shown.

The experiments are generally well performed, the manuscript is clearly written, and the figures are mostly of good quality. However, some main conclusions are not, in my opinion, robustly supported by the data (see major points #1 and #2). Moreover, the gene expression and metabolic data seems disconnected from the rest of the manuscript, detailed in major point #3. These concerns should be properly addressed prior to publication. Even if successfully addressed, I do not think this study makes a significant enough contribution to our understanding to merit publication in Development. This conclusion is based on the lack of sex specific growth phenotype by dietary sugar, the very small sex-specific effect size in growth-related phenotypes, and lack of mechanistic insight to explain the sex specificity of the metabolic phenotypes.

Comments for the author

Major comments:
1. One of the main conclusions of this manuscript is that low dietary sugar activates TOR signaling more strongly in females than in males. This conclusion is based on one Western blot (and its replica). I do not agree that the data sufficiently supports this conclusion, based on the following justification. The loading of Western blot of Figure 2F is unevenly loaded, the Actin levels on lanes #1 and #4 are significantly lower than on lanes #2 and #3. Therefore, it is possible that the difference of pS6K signal between females and males on 0S (lanes #2 vs. #4) is due to uneven loading rather than a real difference in signaling activity. This concern is strengthened by looking at the data of the replicate experiment (Figure S1). There the loading between lanes #2 and #4 seems more even and the pS6K signal is also at a similar level between lanes #2 and #4. In Figure S1, Actin loading is clearly lower on lane #1, which likely explains the low pS6K signal on the female 1S sample.
In general, conventional Western blotting is a semiquantitative method, and therefore unreliable (although often used) to detect such minute differences. To overcome these technical limitations and more reliably analyze the TOR signaling activity, quantitative Western blotting with several replicate samples should be used. In addition, total levels of S6K should also be measured to rule out the possibility that the possible differences truly reflect signaling activity rather than S6K expression. Further reliability would be achieved by analyzing phosphorylation of another TOR downstream target, such as 4EBP.

2. Figure 3 displays the data of experiments addressing the role of insulin and TOR signaling on the sugar-dependent size differences. The experiments have been carefully conducted and statistically analyzed by ANOVA (mentioned only in a supplementary table). These experiments convincingly show that genetic inhibition of Insulin and TOR signaling blunt the growth response to low dietary sugar, especially in the case of InR and TOR heterozygotes (Figure 3 A, B and 3E, F). Based on the statistical analyses (sex:diet:genotype interaction) the authors then conclude that Insulin and TOR signaling differentially influence the sugar-dependent growth phenotype in females vs. males, stating for example: "This reveals a previously unrecognized female-biased requirement for TOR activity in regulating body size in a low sugar context". If we critically look at the data leading to this conclusion (Figures 3E, F), we observe that in females of w1118 the size difference between 1S and 0S conditions is 9.87% and in TOR heterozygotes it is 2.57%. In males the corresponding values are 9% to 3.84%. While I do not question the outcome of the statistical test (I am not an expert there) I do not think that such an effect size is biologically meaningful. Similarly, the effect sizes in the insulin pathway mutants are very small. My concerns are strengthened by the fact that in panels 3C and D there are two replicates of the same genotype (w1118), showing a difference in females (18.54% vs. 12.49%), but less so in males (13.85% vs. 12.51%). So the sex-specific difference between these two experimental groups with identical genotypes (w1118 vs. w1118) is much larger than that observed in the case of w1118 vs. TORdelta/+ comparison. To me this implies that the experimental variation is greater than the minute differences observed between the genotypes. In conclusion, I regret that I do not agree with the authors that the data robustly supports the conclusion that there is sex-biased requirement for Insulin and TOR signaling in the context of sugar-mediated growth regulation.

3. Figure 4 shows sex specific differences in gene expression as well as on the levels of several metabolites in 1S vs. 0S diets. Some of the metabolic changes, such as differences in glycogen and trehalose are potentially interesting and certainly worth reporting. However, their relationship to the observed gene expression changes, growth control, or insulin/TOR signaling remains unclear.

4. The presentation of gene expression data in Figure 4A should be improved. In the current figure, it remains unclear, which genes are upregulated and which are downregulated on 0S. A small subset of genes belonging to each biological category (e.g. lipid synthesis) is shown, but many of these genes display no significant differences in any condition. Therefore, it remains unclear why these genes were selected for the figure, while many other genes belonging to these categories were left out. I would recommend the authors to first perform an unbiased global analysis, like Gene Set Enrichment Analysis, to show which pathways are significantly differently regulated by sugar in females vs. males. Then show expression of representative genes from these pathways in a way that clearly displays the changes of their expression levels (for example bar plots or box plots). This would allow the reader to get a more comprehensive and precise view on the relevant gene expression changes.

Minor comments:

5. The statistical tests should be properly described in the Materials and Methods and mentioned in the Figure legends.

6. The number of biological replicates should be mentioned for each experiment in the Figure legends.

7. The data is often displayed at very high accuracy (at the level of 0.01%). I do not think the decimal fractions are meaningful, considering the inherent imprecision of the experimental measurements.

Reviewer 2

Advance summary and potential significance to field

Overall, the topic of sex-specific differences in the metabolism and growth of organisms in response to diet is an important issue that needs to be considered and in the Drosophila field this

study is one of the first to examine this issue in any detail. The data suggest that males and females may use different molecular and metabolic mechanisms to achieve similar phenotypic outcomes which is quite interesting an important.

Comments for the author

There are a number of issues that need to be addressed before publication. Some require additional experimentation while others may simply require some additional discussion or explanation.

1) Is pupal volume a good proxy for body size? Although this is a widely used metric, there is a bit of a disconnect in this case. In figure 1A the percent increase in female pupal volume is close to the percent increase in adult weight (~13% V vs ~12W). However this is not true for males where pupal volume is increased by ~18% on 0S whereas adult body weight was only 5% increased. Any thoughts as to why this might be?

2) There seems to be a significant variance in the degree to which pupal volume changes depending on the experiment. For example, in Fig 1 A and 3D (is 3D showing the same data as shown in 1A? - if so, that should be stated) female volume is 13.8% higher on 0S compared to 1S while in an equivalent experiment Fig 3E (first panel) it is only 9.8 % different. Similarly, for males Figure 1B and 3F (first panel) show 18.5% increase in male body size on 0S compared to 1S while in Figure 3F the difference is only 9%. These are very large variances in size between similar experiments and calls into question if there really is any sex- specific effect for example in the experiments to examine if loss of particular dilps attenuates the pupal volume increases since the magnitude of the effects are similar to the variance seen in wildtype volumes in the different replicative experiments.

3) Throughout the manuscript it is a bit difficult to determine how many replicates were done and the umber of animals used in each replicate. For example, in Figure 1 is each dot = one animal? For the qPCR data in Fig2 the methods say there were 3-4 biological replicates each consisting of 10 larva and each was done twice. However when examining the raw the data in Sup table 4 it is not clear what each value in the mRNA levels table represents. For example, there are 7-8 values given for each sugar level of Nlaz and puc mRNA levels for females and 6-7 for males. What does each value represent? Is each a different biological replicate or technical replicate? Likewise, for the transcriptome data in Sup 4 Figure 4 what does each number represent in terms of replicate number and animals used?

4) The data shown in the western blots in Fig2F and S1 does not really fit with the description given in the results where it says that the phosphorylated S6k was higher in females but not in males on 0S. This does not look to be the case. In both blots, it appears that pS6k is higher in both sexes on 0S. The blots need to be done in triplicate using different biological replicates and then normalized to actin levels.

5) Is it known whether there are sex-specific differences in organ size relative to overall body volume or weight in larva? For example, if there is more fat, muscle or cuticle relative to volume or weight in one sex versus another then this might skew the metabolic and gene expression analysis data.

6) In this study, caloric content of the food does not appear to be accounted for. What happens if caloric level is held constant while sugar concentration is varied? Might it be that the effects are due to changes in caloric content and not sugar?

minor issues

1) At what time point where larva sexed? What was the error rate in the sorting (as determined by actual males versus females that eclosed from each group)?

2) At what stage where larva picked for biochemical and gene expression studies?

Reviewer 3

Advance summary and potential significance to field

The manuscript by Millington and colleagues investigates the effects of nutrients on larval growth in Drosophila, specifically focusing on differences between the sexes in response to a diet low in sugar. The authors start by confirming that reducing the levels of sugar in the diet results in larger body size for both males and females. Interestingly, the authors present evidence that the reduction in sugar activates the growth promoting signalling pathways, IIS and TOR, in a sex specific manner. Indeed, using genetic tools, the authors show that IIS activity is required for a low sugar diet to increase body size in males, while TOR is required for the same effect in females. Additionally, the authors identify differences in gene expression and metabolite content between males and females under low sugar conditions.

Overall, the manuscript presents a very interesting observation that substantially advances the field: despite an apparent lack of sexual dimorphism in the wild-type's growth response to dietary sugar, the underlying mechanisms ensuring growth differ between males and females. The experiments are generally well conducted, appropriately analysed, interpreted and presented. I believe the paper would be a very strong candidate for publication in Development after the authors address the comments below.

I thank the authors for their acknowledgement of the First Nations.

Comments for the author

1) Differential regulation of IIS/TOR in males/females by the low sugar diet is an important part of the manuscript, supporting the main conclusion. However, I think that the analysis of pathway activation, as it stands in the manuscript, has important limitations that should be addressed. Firstly, with respect to IIS activation, the authors measure the levels of Foxo targets to infer IIS activity. However, are the authors certain that the chosen genes are targets of Foxo in both sexes? More direct evidence of IIS activation could be obtained by quantifying total and phosphorylated AKT, for example. I suggest authors do this additional experiment.
With respect to TOR, the authors only quantify the levels of phosphorylated S6K (not total) and present a single blot. The increased levels of pS6K could be due to changes in the total levels of the S6K and not TORC1 activity. For this reason, I think that the authors should look at both phospho and total S6K, and present experimental repeats.
2) The dietary condition used is presented as "low sugar" but it can also be framed as high protein/sugar ratio. This is an important point to discuss.
3) The authors appropriately use 2-/3-way ANOVA for the testing of effects of genotype/diet/sex on growth. I think they can extend this statistical approach to other data presented in the manuscript, where relevant.
4) Can the authors speculate on why these sex differences in how growth is promoted under low sugar exist and what effect they may have on the adult, if any?

Original submission

First decision letter

MS ID#: DEVELOP/2021/200491

MS TITLE: A low sugar diet enhances Drosophila body size in males and females via sex-specific mechanisms

AUTHORS: Jason W Millington, Puja Biswas, Charlotte Chao, Yi Han Xia, Lianna W Wat, George P Brownrigg, Ziwei Sun, Paige J Basner-Collins, Ramon I Klein Geltink, and Elizabeth J Rideout

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development. I would only ask that you consider the two outstanding issues raised by two reviewers, which should not require additional experiments. If you do not agree with their suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have essentially addressed all of my main concerns. I think that the data on IIS are now more solid and the study overall of a high enough quality and interest to be accepted for publication in Development - by demostrating sex differences in mechanisms that regulate growth in response to nutrition the paper contributes in a new and significant way to our understanding of development.

Comments for the author

I still have one small remark: in the rebuttal the authors mention doing westerns for total S6K but I could not find them in the manuscript – please include them and the phosphor/total ratio for S6K. I do not think that lack of observed changes in the ratio of phospho/total S6K detracts from the importance of the manuscript. The authors can still claim a near-significant increase in pS6K that is likely due to an increase in the expression of the enzyme. Including the data would ensure completeness and transparency.

Reviewer 2

Advance summary and potential significance to field

The authors have very thoroughly addressed my initial concerns and the manuscript is significantly improved both in terms of data and conclusions as well as general advance of the field. Therefore, I am happy to recommend publication in Development.

Comments for the author

I think some of the most interesting findings are presented in Figure 4 (sex specific differences in glycogen and protein levels, female-specific differences in requirement of TOR activity). I encourage the authors to consider whether to mention these findings in the abstract.

Reviewer 3

Advance summary and potential significance to field

This manuscript shows that while the levels of dietary sugar affect both male and female body sizes, the mechanisms responsible do not fully overlap. In males, a low sugar diet promotes growth through enhanced IIS signaling sensitivity, while in females IIS activity and sensitivity were unaffected on a low sugar diet and could not account for the metabolic and body size responses observed in females. These studies further demonstrate that sex-specific differences need to be considered when examining environment vs. genotype interactions.

Comments for the author

The authors have added a great deal of new data, additional explanations of methodology, and demonstrated increased reproducibility to fortify their main point that there are sex-specific

differences in the way that Drosophila males and females change there metabolic responses to achieve a similar outcome in terms of body size adjustments to dietary sugar concentration. This is an important finding that needs to be communicated to the field. While a complete description of the molecular mechanism used in females to adjust body size in response to dietary sugar is not yet available, I agree with the authors that this should be the goal of future studies since the data provided clearly demonstrates that there are differences in how the sex, environment and genotype interact during development to adjust overall body size and this needs to be communicated to the field. Therefore I have no additional comments and am in favor of publication of this manuscript as is pending addressing any additional concerns from other reviewers.

First revision

Author response to reviewers' comments

We thank the Reviewers for their suggestions on how to improve our manuscript. Below, we provide detailed information on additional experiments and text changes we made to address individual concerns that were raised.

Reviewer 1: Advance Summary and Potential Significance to Field:
The study of Millington and coworkers analyze the sex specific effects of dietary sugar on animal growth control, using Drosophila as a model system. While dietary sugar is known to inhibit growth of Drosophila larvae, the possible sex differences have not been explored. In general, sex specificity of animal physiology represents a field, which has been, until recently, understudied. Therefore, studies filling this gap in the literature are warranted. A previous study of the Rideout group has shown clear sex specific differences in the growth regulation by dietary protein and dissected the underlying molecular mechanisms (PMID: 33448263). In contrast to protein, sugar feeding (studied here) shows no sex specificity on growth control. Despite this lack of difference the authors molecularly analyze the activity of insulin and TOR signaling in females vs. males. They conclude that low sugar diet activates insulin signaling in males, but not in females. This conclusion is supported by a very modest downregulation of insulin-repressed genes in males, but not in females. The authors also conclude that low sugar activates TOR signaling more in females than in males. To test the functional relevance of insulin and TOR signaling the authors use genetic experiments to partially inhibit insulin and TOR signaling. They conclude that there is a sex-biased requirement for insulin and TOR signaling in sugar-dependent growth control. Finally, sex-specific changes in metabolism (including glycogen and trehalose) and gene expression in response to sugar are shown.

The experiments are generally well performed, the manuscript is clearly written, and the figures are mostly of good quality. However, some main conclusions are not, in my opinion, robustly supported by the data (see major points #1 and #2). Moreover, the gene expression and metabolic data seems disconnected from the rest of the manuscript, detailed in major point #3. These concerns should be properly addressed prior to publication. Even if successfully addressed, I do not think this study makes a significant enough contribution to our understanding to merit publication in Development. This conclusion is based on the lack of sex specific growth phenotype by dietary sugar, the very small sex-specific effect size in growth-related phenotypes, and lack of mechanistic insight to explain the sex specificity of the metabolic phenotypes.

We thank Reviewer 1 for their positive comments on the general quality of the experiments, writing, and figures. To address Reviewer concerns, we added a significant amount of new data, made substantial changes to the text, and clarified the broad significance of identifying sex-specific mechanisms that contribute to equivalent phenotypes between males and females.

Major changes include:

- Adding new data on sex-specific regulation of IIS activity between 1S and 0S.
- Identifying the mechanism that accounts for the sex-specific IIS regulation.
- Unbiased analysis of gene expression between 1S and 0S.

- Linking sex-specific IIS regulation with sex-limited metabolic changes in 1S and 0S.
- Refocusing the manuscript on the male-specific changes in IIS activity.

Together, our new data confirms the male-specific IIS increase in 0S (Fig. 2B,D), demonstrates increased IIS in males was caused by improved insulin sensitivity in 0S (Fig. 2G,H), and shows that IIS function was required for the male-limited increase in glycogen in 0S (Fig. 4H). Given that we do not observe any change to IIS activity or insulin sensitivity in females (Fig. 2A,C,F,H), these new data further support our overall conclusion that the mechanisms underlying the low sugar-induced increase in body size are not shared between the sexes.

This conclusion is significant, as it challenges a common assumption in many fields that mechanisms discovered in a single- or mixed-sex population will apply to both sexes if the phenotype is shared between males and females. Our findings will therefore have broad relevance across the field of larval growth, a point we now more clearly communicate at the end of the Introduction and Results and Discussion sections:

[Introduction] “Taken together, our data suggest that male and female larvae achieve a larger body size in 0S via mechanisms that are not fully shared. This highlights the importance of including both sexes in larval growth studies, as distinct mechanisms may operate even when male and female phenotypic outcomes are similar.”

[Results and Discussion] “Together with the differential transcriptional responses in 0S between males and females, these data suggest that the mechanisms underlying the low sugar-induced increase in body size are not fully shared between the sexes. This highlights the importance of including both males and females in larval growth studies, as not all mechanisms will be shared between the sexes even in contexts where the phenotype is equivalent.”

Reviewer 1 Comments for the Author:
Major comments:
1. One of the main conclusions of this manuscript is that low dietary sugar activates TOR signaling more strongly in females than in males. This conclusion is based on one Western blot (and its replica). I do not agree that the data sufficiently supports this conclusion, based on the following justification. The loading of Western blot of Figure 2F is unevenly loaded, the Actin levels on lanes #1 and #4 are significantly lower than on lanes #2 and #3. Therefore, it is possible that the difference of pS6K signal between females and males on 0S (lanes #2 vs. #4) is due to uneven loading rather than a real difference in signaling activity. This concern is strengthened by looking at the data of the replicate experiment (Figure S1). There the loading between lanes #2 and #4 seems more even and the pS6K signal is also at a similar level between lanes #2 and #4. In Figure S1, Actin loading is clearly lower on lane #1, which likely explains the low pS6K signal on the female 1S sample.

In general, conventional Western blotting is a semiquantitative method, and therefore unreliable (although often used) to detect such minute differences. To overcome these technical limitations and more reliably analyze the TOR signaling activity, quantitative Western blotting with several replicate samples should be used. In addition, total levels of S6K should also be measured to rule out the possibility that the possible differences truly reflect signaling activity rather than S6K expression. Further reliability would be achieved by analyzing phosphorylation of another TOR downstream target, such as 4EBP.

We thank the Reviewer for the suggestions on how to improve our conclusions regarding TOR pathway activity. We used many approaches to investigate TOR regulation in males and females in 1S and 0S, and to determine whether there is a role for TOR function in regulating the sugar-induced changes to body size and metabolism.

While I provide detailed comments on measuring TOR activity in 1S and 0S below (see “Detailed comments on measuring TOR activity”), here is a brief summary of the new TOR-related data we include in the revised manuscript:

- RNAseq data showed that mRNA levels of two known TOR-responsive genes *unk* and *cbt* were altered only in females and not in males in 0S (Fig. 4L).

- Reduced TOR function blocked the female-specific increase in whole-body protein levels in 0S (Fig. 4I).
- Lower TOR function caused a reproducible female-biased effect on the low sugar-induced increase in body size in 0S across multiple experiments (Fig. 4J,K; Fig. S9A-D).
- Levels of p-S6k showed a trend (p=0.0625) toward increased levels in both sexes in 0S compared with 1S (Fig. S10A,B).
- RNAseq data shows altered mRNA levels of anabolic processes known to be regulated by TOR.

A potential sex-biased role for TOR is supported by our new genetic data confirming a female-biased requirement for TOR function in regulating the body size and metabolic responses to a low sugar diet. Dr. Ramon Klein Geltink, an expert in TOR-mediated metabolic changes and author on our revised manuscript, further highlighted that the female-specific regulation of several cellular processes in 0S (e.g. lysosomes, biosynthesis of amino acids, pentose phosphate pathway) are consistent with known TOR-regulated processes. Indeed, Dr. Klein Geltink pointed out that TOR acts through multiple effectors beyond S6k to regulate these diverse cellular processes, suggesting more studies will be needed to monitor sex-specific regulation of TOR targets in addition to S6k.

To present all the TOR data together, and to reflect the complexity between the TOR readouts we used (*unk*/*cbt* and p-S6k), we made several changes to the manuscript:

- We refocused the first three figures of the manuscript on our discoveries regarding the male-specific regulation and function of IIS in regulating the male body size and metabolic response to a low sugar diet.
- We limited our conclusions regarding a potential female-biased role for TOR to the metabolic and body size phenotypes.
- Because TOR is known to affect cellular metabolism via multiple downstream effectors, we state that future studies will need to monitor additional TOR targets.
- We made changes to the text to suggest more knowledge is needed about TOR biology in both sexes.

Importantly, our TOR data, when taken together with our findings on the sex-biased regulation and function of IIS in regulating metabolism and body size in a low sugar context, supports our overall conclusion that the mechanisms underlying the low sugar-induced increase in body size are not fully shared between the sexes.

Detailed comments on measuring TOR activity

4E-BP. Unfortunately, when we tested a commercial phospho-4E-BP antibody, no bands were lost in *4E-BP* null mutant larvae. Because we could not be sure which of the many bands detected by this antibody corresponded to 4E-BP, we were unable to draw any conclusions from this series of experiments.

TOR gene expression readouts. Our RNAseq data allowed us to monitor mRNA levels of known TOR-responsive genes *unk* and *cbt* (Guertin et al., 2006; Ingaramo et al., 2020; Tiebe et al., 2015) (Supplementary file 2). We found that *unk* and *cbt* were significantly downregulated in female, but not male, larvae reared in 0S compared with larvae raised in 1S (Fig. 4L). Because both genes are repressed by TOR, this suggests a female-specific upregulation of TOR in 0S.

Additional S6k Western blotting. To improve the S6k blot quantification, we were fortunate to receive a small aliquot of total S6k antibody. Unfortunately, we observed significant diet-induced changes to total S6k levels in 1S and 0S. Because drawing accurate conclusions about TOR activity using the ratio of phospho:total S6k requires that levels of total S6k do not vary with the experimental manipulation, we were concerned about the sugar-dependent effect on total S6k levels. In consultation with Dr. Hilla Weidberg, an expert in Western blotting, we normalized phospho-S6k levels to the total amount of protein loaded in each lane using the stain-free labeling system from BioRad (Cat# 1610185). This avoids overreliance on loading levels of one protein which may be subject to regulation by nutrients. We found that levels of p-S6k showed a trend (p=0.0625) toward increased levels in both sexes in 0S compared with 1S (Fig. S10A,B).

2. Figure 3 displays the data of experiments addressing the role of insulin and TOR signaling on the sugar-dependent size differences. The experiments have been carefully conducted and statistically analyzed by ANOVA (mentioned only in a supplementary table). These experiments convincingly show that genetic inhibition of Insulin and TOR signaling blunt the growth response to low dietary sugar, especially in the case of InR and TOR heterozygotes (Figure 3 A, B and 3E, F). Based on the statistical analyses (sex:diet:genotype interaction) the authors then conclude that Insulin and TOR signaling differentially influence the sugar-dependent growth phenotype in females vs. males, stating for example: "This reveals a previously unrecognized female-biased requirement for TOR activity in regulating body size in a low sugar context". If we critically look at the data leading to this conclusion (Figures 3E, F), we observe that in females of w1118 the size difference between 1S and 0S conditions is 9.87% and in TOR heterozygotes it is 2.57%. In males the corresponding values are 9% to 3.84%. While I do not question the outcome of the statistical test (I am not an expert there) I do not think that such an effect size is biologically meaningful. Similarly, the effect sizes in the insulin pathway mutants are very small. My concerns are strengthened by the fact that in panels 3C and D there are two replicates of the same genotype (w1118), showing a difference in females (18.54% vs. 12.49%), but less so in males (13.85% vs. 12.51%). So the sex-specific difference between these two experimental groups with identical genotypes (w1118 vs. w1118) is much larger than that observed in the case of w1118 vs. TORdelta/+ comparison. To me this implies that the experimental variation is greater than the minute differences observed between the genotypes. In conclusion, I regret that I do not agree with the authors that the data robustly supports the conclusion that there is sex-biased requirement for Insulin and TOR signaling in the context of sugar-mediated growth regulation.

We thank the Reviewer for their comment. To address concerns about experimental variation versus smaller effects of genotypes on body size (*Tor*/+; *InR*/+), we repeated the experiments several more times. The sex:genotype:diet interactions we observed in our initial manuscript persist across all independent replicates (Fig. 3A,B and S2A-D [InR/+]; Fig. 4J,K and Fig. S9A-D [Tor/+]): loss of one *Tor* allele had a female-biased effect on the low sugar-induced growth and loss of one *InR* allele had a male-biased phenotypic effect.

These results indicate the sex-biased effect of genotype on the body size response to a low sugar diet is robust, and that it persists despite interexperiment variation in the magnitude of low sugar-induced growth. To more clearly communicate the magnitude of the growth-reducing effects that arise due to genetic disruption of IIS/TOR, in the revised manuscript we added a graph showing the % growth blocked for each genotype and text to reflect this growth effect:

[IIS] "Body size was larger in w^{1118} male larvae reared on 0S compared with males cultured on 1S (Fig. 3A); however, 100% of the low sugar-induced increase in body size was blocked in InR^{E19}/+ male larvae (Fig. 3A; genotype:diet interaction $p<0.0001$). This suggests the elevated IIS activity we observed in males reared in 0S was required to achieve a larger body size. In females, only 48% of the low sugar-induced increase in body size was blocked (Fig. 3B), where the magnitude of genotype effects on the body size response to a low sugar diet was larger in males than in females (sex:diet:genotype interaction $p=0.0114$)."
[TOR] "In w^{1118} females, larvae reared on 0S were significantly larger than genotype-matched larvae raised on 1S (Fig. 4J); however, 100% of the low sugar-induced increase in body size was blocked in $Tor^{\Delta P}$/+ female larvae (Fig. 4J; genotype:diet interaction $p<0.0001$). This suggests TOR function in females contributes to their larger body size in 0S. In males, while the low sugar-induced increase in body size was 50% blocked in $Tor^{\Delta P}$/+ larvae (Fig. 4K; genotype:diet $p<0.0001$), the magnitude of genotype effects on the diet-induced increase in body size was greater in females than in males (sex:diet:genotype interaction $p=0.0303$)."

We also included new text to comment on the reproducibility and effect size of the sex:genotype:diet interactions:

[IIS] "Genetically reducing IIS function therefore had a male-biased impact on the low sugar-induced increase in body size, an effect we replicated across multiple independent experiments despite modest interexperiment variation in the magnitude of the low sugar-induced increase in body size (Fig. S2A-D)."

[TOR] “This female-biased effect was robust, as we reproduced the sex:diet:genotype interaction in *Tor*$^{\Delta P}$/+ larvae across multiple biological replicates (Fig. S9A-D).”

3. Figure 4 shows sex specific differences in gene expression as well as on the levels of several metabolites in 1S vs. 0S diets. Some of the metabolic changes, such as differences in glycogen and trehalose are potentially interesting and certainly worth reporting. However, their relationship to the observed gene expression changes, growth control, or insulin/TOR signaling remains unclear.

We thank the Reviewer for this suggestion. To address this comment we made several changes to the revised manuscript:

i) Text changes to discuss why we examined gene expression in males and females raised in the 0S or 1S diets.

“IIS influences body size by triggering profound changes in gene expression (Alic et al., 2011; Bülow et al., 2010; Grewal, 2009; Guertin et al., 2006; Li, Edgar, and Grewal 2010; Musselman and Kühnlein, 2018; Teleman et al., 2008; Tiebe et al., 2015; Webb et al., 2016; Zinke et al., 2002). Because we observed sex-specific regulation of IIS in a low sugar diet, we performed an unbiased analysis of transcriptional changes in males and females reared on 1S and 0S.”

ii) Unbiased analysis of low sugar-induced gene expression changes in each sex.

We generated an RNAseq dataset and used KEGG pathway analysis to identify key pathways that were altered in each sex in response to a change in diet. This analysis showed distinct gene expression responses to a low sugar diet in each sex (Fig. 4A-D, Fig. S4A,B). These data further support our finding of sex-biased changes to IIS between 1S and 0S by identifying a male-specific downregulation of genes in the “FoxO signaling pathway” category.

iii) Text changes to connect gene expression changes with metabolic phenotypes.

These RNAseq data showed that 80.7% of differentially regulated genes between 1S and 0S belonged to the “Metabolic Regulation” category, providing a strong rationale for investigating low sugar-induced metabolic changes that occur in each sex. In our revised manuscript we use this unbiased RNAseq analysis to justify our studies on whole-body macronutrient storage:

“One biological process that was overrepresented in both sexes between 1S and 0S was metabolic regulation: 80.7% of genes differentially expressed in a low sugar diet were linked with metabolism (Fig. 4D). We therefore examined several metabolic parameters in male and female larvae in 1S and 0S to examine the physiological significance of these sex-specific gene expression responses.”

iv) New data to analyze diet-induced metabolic changes in InR/+ or Tor/+ larvae.

To connect the sex-specific metabolic changes with IIS in males, or with TOR in females, we measured diet-induced changes to whole-body macronutrient storage in male and female *InR*/+ and *Tor*/+ larvae. The male-specific increase in glycogen in 0S was blunted in *InR*/+ male larvae (Fig. 4H), whereas the female-specific increase in whole-body protein levels was blocked in *Tor*/+ female larvae (Fig. 4I).

Taken together, this new data and the accompanying text changes more clearly outline the relationship between IIS and TOR and metabolic phenotypes in males and females, respectively.

4. The presentation of gene expression data in Figure 4A should be improved. In the current figure, it remains unclear, which genes are upregulated and which are downregulated on 0S. A small subset of genes belonging to each biological category (e.g. lipid synthesis) is shown, but many of these genes display no significant differences in any condition. Therefore, it remains unclear why these genes were selected for the figure, while many other genes belonging to these categories were left out. I would recommend the authors to first perform an unbiased global analysis, like Gene Set Enrichment Analysis, to show which pathways are significantly differently regulated by sugar in females vs. males. Then show expression of representative genes from these pathways in a way that clearly displays the changes of their expression levels (for example bar

plots or box plots). This would allow the reader to get a more comprehensive and precise view on the relevant gene expression changes.

We thank the Reviewer for this suggestion. In the revised manuscript we generated an RNAseq dataset and used KEGG pathway analysis to reveal pathways that are differentially regulated in each sex in response to a low sugar diet. Overall, this RNAseq data supports the existence of distinct gene expression and metabolic responses to a low sugar diet in male and female larvae. Indeed, the majority (58%) of biological processes that were differentially regulated between 1S and 0S were different between the sexes (Fig. 4D; Fig. S4A,B). To ensure the significance of these new findings is clearly communicated to the reader, we added several additional figures (Fig. 4A-D; Fig. S4A,B), a table with all RNAseq data (Supplemental file 2), and text to the revised manuscript.

Minor comments:
5. The statistical tests should be properly described in the Materials and Methods and mentioned in the Figure legends.

The Methods section has been updated to include detailed descriptions of the statistical tests we performed, and statistical tests are now listed in all Figure Legends.

6. The number of biological replicates should be mentioned for each experiment in the Figure legends.

We adjusted all Figure Legends to include the number of biological replicates.

7. The data is often displayed at very high accuracy (at the level of 0.01%). I do not think the decimal fractions are meaningful, considering the inherent imprecision of the experimental measurements.

We adjusted the body size data to display only whole numbers.

Reviewer 2 Advance Summary and Potential Significance to Field:

Overall, the topic of sex-specific differences in the metabolism and growth of organisms in response to diet is an important issue that needs to be considered and in the Drosophila field this study is one of the first to examine this issue in any detail. The data suggest that males and females may use different molecular and metabolic mechanisms to achieve similar phenotypic outcomes which is quite interesting an important.

We thank the Reviewer for their careful reading of the manuscript, and for their suggestions on improving our manuscript.

Reviewer 2 Comments for the Author:
There are a number of issues that need to be addressed before publication. Some require additional experimentation while others may simply require some additional discussion or explanation.

1) Is pupal volume a good proxy for body size? Although this is a widely used metric, there is a bit of a disconnect in this case. In figure 1A the percent increase in female pupal volume is close to the percent increase in adult weight (~13% V vs ~12W). However this is not true for males where pupal volume is increased by ~18% on 0S whereas adult body weight was only 5% increased. Any thoughts as to why this might be?

We also wonder about the occasional discrepancy we see between pupal volume and adult weight, which we also observed in our previous manuscripts (Millington et al. 2021 *G3*: *Genes, Genomes, Genetics*; Millington et al. 2021 *eLife*). One possibility is that there may be differential effects of signaling pathways on endoreplicating and mitotic tissues, but we do not have data in hand to support this speculation.

In our revised manuscript we therefore comment on the discrepancy in our study, and suggest further studies will be needed to resolve this very interesting topic:

“We reproduced this finding using adult weight (Fig. 1D); however, we note that the magnitude of the low sugar-induced increase in adult weight was smaller than we observed with pupal volume, a discrepancy that will require further studies to resolve.”

2) There seems to be a significant variance in the degree to which pupal volume changes depending on the experiment. For example, in Fig 1A and 3D (is 3D showing the same data as shown in 1A? - if so, that should be stated) female volume is 13.8% higher on 0S compared to 1S while in an equivalent experiment Fig 3E (first panel) it is only 9.8 % different. Similarly, for males Figure 1B and 3F (first panel) show 18.5% increase in male body size on 0S compared to 1S while in Figure 3F the difference is only 9%. These are very large variances in size between similar experiments and calls into question if there really is any sex-specific effect for example in the experiments to examine if loss of particular dilps attenuates the pupal volume increases since the magnitude of the effects are similar to the variance seen in wildtype volumes in the different replicative experiments.

We thank the Reviewer for their comment. To address concerns about the relative importance of the effects of genotype on the body size response to a low sugar diet when there was variation in the magnitude of the body size response between experiments, we repeated the experiments using the *Tor*/+ and *InR*/+ larvae several more times.

While we found modest differences between experiments in the magnitude of the low sugar-induced increase in body size, the sex:genotype:diet interactions we observed in our initial manuscript persist across all independent replicates (Fig. 3A,B and S2A-D [InR/+]; Fig. 4J,K and Fig. S9A-D [Tor/+]): loss of one *Tor* allele had a female-biased effect on the low sugar-induced growth and loss of one *InR* allele had a male-biased phenotypic effect.

Thus, no matter how much the low sugar diet augmented body size, loss of one *Tor* allele had a female-biased effect on the low sugar-induced growth and loss of one *InR* allele had a male-biased phenotypic effect. This suggests the sex-biased effect of genotype on the body size response to a low sugar diet is not simply a random fluctuation between experiments; rather, it is a robust sex-biased effect of genotype on the response to a low sugar diet.

In the revised manuscript, we included all our additional replicates (Fig. S2A-D [InR/+] and S9A-D [Tor/+]) and adjusted the text in line with our new data. To more clearly communicate the magnitude of the growth-reducing effects that arise due to genetic disruption of IIS/TOR, in the revised manuscript we added a graph showing the % growth blocked for each genotype and text to reflect this growth effect:

[IIS] “Body size was larger in w^{1118} male larvae reared on 0S compared with males cultured on 1S (Fig. 3A); however, 100% of the low sugar-induced increase in body size was blocked in InR^{E19}/+ male larvae (Fig. 3A; genotype:diet interaction $p<0.0001$). This suggests the elevated IIS activity we observed in males reared in 0S was required to achieve a larger body size. In females, only 48% of the low sugar-induced increase in body size was blocked (Fig. 3B), where the magnitude of genotype effects on the body size response to a low sugar diet was larger in males than in females (sex:diet:genotype interaction $p=0.0114$).”

[TOR] “In w^{1118} females, larvae reared on 0S were significantly larger than genotype-matched larvae raised on 1S (Fig. 4J); however, 100% of the low sugar-induced increase in body size was blocked in $Tor^{\Delta P}$/+ female larvae (Fig. 4J; genotype:diet interaction $p<0.0001$). This suggests TOR function in females contributes to their larger body size in 0S. In males, while the low sugar-induced increase in body size was 50% blocked in $Tor^{\Delta P}$/+ larvae (Fig. 4K; genotype:diet $p<0.0001$), the magnitude of genotype effects on the diet-induced increase in body size was greater in females than in males (sex:diet:genotype interaction $p=0.0303$).”

We also included new text to comment on the reproducibility and effect size of the sex:genotype:diet interactions:

[IIS] “Genetically reducing IIS function therefore had a male-biased impact on the low sugar-induced increase in body size, an effect we replicated across multiple independent experiments despite modest interexperiment variation in the magnitude of the low sugar-induced increase in body size (Fig. S2A-D).”

[TOR] “This female-biased effect was robust, as we reproduced the sex:diet:genotype interaction in *Tor*$^{\Delta P}$/+ larvae across multiple biological replicates (Fig. S9A-D).”

3) Throughout the manuscript it is a bit difficult to determine how many replicates were done and the number of animals used in each replicate. For example, in Figure 1 is each dot = one animal? For the qPCR data in Fig2 the methods say there were 3-4 biological replicates each consisting of 10 larva and each was done twice. However, when examining the raw the data in Sup table 4 it is not clear what each value in the mRNA levels table represents. For example, there are 7-8 values given for each sugar level of Nlaz and puc mRNA levels for females and 6-7 for males. What does each value represent? Is each a different biological replicate or technical replicate? Likewise, for the transcriptome data in Sup 4 Figure 4 what does each number represent in terms of replicate number and animals used?

To address these concerns, we included the number of biological replicates for each experiment in all figure legends and we now define what constitutes a biological replicate for each technique in the Methods section. Finally, we improved our description of how qPCR data is normalized and displayed:

“Each biological replicate consists of 10 larvae. Values displayed in each graph represent the fold change for a gene’s mRNA, normalized to *Act5c* and ß-*tub*, housekeeping genes that are not differentially regulated between the sexes or between 1S and 0S.”

4) The data shown in the western blots in Fig2F and S1 does not really fit with the description given in the results where it says that the phosphorylated S6k was higher in females but not in males on 0S. This does not look to be the case. In both blots, it appears that pS6k is higher in both sexes on 0S. The blots need to be done in triplicate using different biological replicates and then normalized to actin levels.

We thank the Reviewer for the suggestions on how to improve our conclusions regarding a potential role for TOR in mediating low sugar-induced changes to metabolism and body size. We used many approaches to investigate TOR regulation in males and females in 1S and 0S, and to strengthen our findings regarding a role for TOR in regulating the sugar-induced changes to body size and metabolism.

Here is a brief summary of the new TOR-related data we include in the revised manuscript:

- RNAseq data showed that mRNA levels of two known TOR-responsive genes *unk* and *cbt* were altered only in females and not in males in 0S (Fig. 4L).
- Reduced TOR function blocked the female-specific increase in whole-body protein levels in 0S (Fig. 4I).
- Lower TOR function caused a reproducible female-biased effect on the low sugar-induced increase in body size in 0S across multiple experiments (Fig. 4J,K; Fig. S9A-D).
- Levels of p-S6k showed a trend (p=0.0625) toward increased levels in both sexes in 0S compared with 1S (Fig. S10A,B).
- RNAseq data shows altered mRNA levels of anabolic processes known to be regulated by TOR.

A potential sex-biased role for TOR is supported by our new genetic data confirming a female-biased requirement for TOR function in regulating the body size and metabolic responses to a low sugar diet. Dr. Ramon Klein Geltink, an expert in TOR-mediated metabolic changes and author on our revised manuscript, further highlighted that the female-specific regulation of several cellular processes in 0S (e.g. lysosomes, biosynthesis of amino acids, pentose phosphate pathway) are consistent with known TOR-regulated processes. Indeed, Dr. Klein Geltink pointed out that TOR acts through multiple effectors beyond S6k to regulate these diverse cellular processes, suggesting more studies will be needed to monitor sex-specific regulation of TOR targets in addition to S6k.

To present all the TOR data together, and to reflect the complexity between the TOR readouts we used (*unk*/*cbt* and p-S6k), we made several changes to the manuscript:

- We refocused the first three figures of the manuscript on our discoveries regarding the male-specific regulation and function of IIS in regulating the male body size and metabolic response to a low sugar diet.
- We limited our conclusions regarding a potential female-biased role for TOR to the metabolic and body size phenotypes.
- Because TOR is known to affect cellular metabolism via multiple downstream effectors, we state that future studies will need to monitor additional TOR targets.
- We made changes to the text to suggest more knowledge is needed about TOR biology in both sexes.

Importantly, our TOR data, when taken together with our findings on the sex-biased regulation and function of IIS in regulating metabolism and body size in a low sugar context, supports our overall conclusion that the mechanisms underlying the low sugar-induced increase in body size are not fully shared between the sexes.

5) Is it known whether there are sex-specific differences in organ size relative to overall body volume or weight in larva? For example, if there is more fat, muscle or cuticle relative to volume or weight in one sex versus another then this might skew the metabolic and gene expression analysis data.

The Reviewer raises an interesting point. We gathered data for the fat, an important nutrient-storing organ, which shows no difference in organ size relative to body size between the sexes in either 1S or 0S (Fig. S6A). This suggests that there are no sex differences in the relationship between organ size and body size that account for the sex-specific effects on metabolism, which we note in the revised manuscript:

“Importantly, these sex-specific metabolic changes in protein, glycogen, and trehalose cannot be attributed to sex differences in the relationship between organ and body size, as we found no male-female difference in scaling between the fat body, an important nutrient-storing larval organ, and the whole larva in either 1S or 0S (Fig. S6A).”

6) In this study, caloric content of the food does not appear to be accounted for. What happens if caloric level is held constant while sugar concentration is varied? Might it be that the effects are due to changes in caloric content and not sugar?

We thank the Reviewer for raising this important point. In the revised manuscript we added text and a reference to our recent paper where we showed that reducing dietary calories to match the caloric content of the low sugar diet did not affect body size:

“Because removing cornmeal from 1S to match the caloric content of the reduced sugar diet did not affect body size (Millington et al., 2021a), our findings suggest that the larger body size of larvae raised in 0S can be attributed to less dietary sugar.”

Minor issues

1) At what time point where larva sexed? What was the error rate in the sorting (as determined by actual males versus females that eclosed from each group)?

We added text in the “Fly Husbandry” section of the Methods to clarify that larvae were sexed as third instars at the time of collection for gene expression or similar assays (108 hr after egg-laying), whereas pupae were sexed 0-12 hr after puparium formation (prior to head eversion):

“Larvae were raised at a density of 50 animals per 10 mL food at 25°C, and sexed by gonad size as mid-third instar larvae at the time of collection for gene expression or metabolic experiments (108 hr after egg-laying). For pupal volume experiments, animals were separated by sex between 0-12 hr after puparium formation.”

To confirm the accuracy of our sorting, we added qPCR data to the revised manuscript. Using primers directed against the male- and female-specific isoforms of sex determination genes *transformer* and *doublesex* is a sensitive and accurate way to detect the presence of unwanted material from the opposite sex. In our samples, we show that females and not males showed amplification of the female-specific *transformer* and *doublesex* splice isoforms, whereas males and not females showed amplification of the male-specific *doublesex* isoform (Fig. S1A). This suggests our sorting of larvae by sex is accurate, which we state in the text:

"Given that we verified the accuracy of our larval sorting by detecting the expected male- and female-specific isoforms of sex determination genes (Fig. S1A)"

2)At what stage where larva picked for biochemical and gene expression studies?

We added text to the "Fly Husbandry" section of the Methods to explicitly state the age of the larvae collected for biochemical and gene expression studies:

"For all metabolic, gene expression, imaging, insulin stimulation, and Western blotting experiments, larvae were collected at 108 hr after egg-laying."

Reviewer 3 Advance Summary and Potential Significance to Field:
The manuscript by Millington and colleagues investigates the effects of nutrients on larval growth in Drosophila, specifically focusing on differences between the sexes in response to a diet low in sugar. The authors start by confirming that reducing the levels of sugar in the diet results in larger body size for both males and females. Interestingly, the authors present evidence that the reduction in sugar activates the growth promoting signalling pathways, IIS and TOR, in a sex specific manner. Indeed, using genetic tools, the authors show that IIS activity is required for a low sugar diet to increase body size in males, while TOR is required for the same effect in females. Additionally, the authors identify differences in gene expression and metabolite content between males and females under low sugar conditions.

Overall, the manuscript presents a very interesting observation that substantially advances the field: despite an apparent lack of sexual dimorphism in the wild-type's growth response to dietary sugar, the underlying mechanisms ensuring growth differ between males and females. The experiments are generally well conducted, appropriately analysed, interpreted and presented. I believe the paper would be a very strong candidate for publication in Development after the authors address the comments below. I thank the authors for their acknowledgement of the First Nations.

We thank the Reviewer for their positive comments, and for their thoughtful suggestions on how to improve our manuscript.

Reviewer 3 Comments for the Author:
1) Differential regulation of IIS/TOR in males/females by the low sugar diet is an important part of the manuscript, supporting the main conclusion. However, I think that the analysis of pathway activation, as it stands in the manuscript, has important limitations that should be addressed.

Firstly, with respect to IIS activation, the authors measure the levels of Foxo targets to infer IIS activity. However, are the authors certain that the chosen genes are targets of Foxo in both sexes? More direct evidence of IIS activation could be obtained by quantifying total and phosphorylated AKT, for example. I suggest authors do this additional experiment.
With respect to TOR, the authors only quantify the levels of phosphorylated S6K (not total) and present a single blot. The increased levels of pS6K could be due to changes in the total levels of the S6K and not TORC1 activity. For this reason, I think that the authors should look at both phospho and total S6K, and present experimental repeats.

We thank the Reviewer for these suggestions. In our revised manuscript we strengthened our support for the sex-specific regulation of both IIS and TOR by gathering additional lines of evidence:

IIS

We first used a GFP-based reporter called GFP-PH, where membrane localization of the GFP-PH reporter is increased when IIS is higher (PMID 11832249). In our revised manuscript we show that the localization of GFP-PH is higher in males reared in 0S compared with males raised in 1S (Fig. 2C,D), an increase we did not observe in females.

Second, when we analyzed our newly-generated RNAseq data, we found that males cultured in 0S had a significant reduction in "FoxO signaling pathway" compared with males reared in 1S, a decrease that was not observed in females (Fig. 4D). Given that repression of FoxO is an important way that high levels of IIS promote larval growth, increased repression of FoxO targets in males reared in 0S suggests higher IIS activity.

Third, given that sugar has been shown to cause insulin resistance in larvae, we directly tested insulin sensitivity in male and female larvae reared in 0S and 1S. We found that in males reared in 1S, adding insulin to cultured male carcasses did not stimulate increased GFP-PH localization to the membrane (Fig. 2G). In males raised in 0S on the other hand, there was an increase in GFP-PH localization to the membrane (Fig. 2G). Males raised in the 1S diet are therefore less insulin-sensitive than males reared on 0S, suggesting one reason for the increased IIS activity in males raised on 0S. In females, there was a significant increase in GFP-PH localization to the membrane in larvae cultured on both 0S and 1S, suggesting that females are insulin-sensitive in both contexts (Fig. 2F).

Together, this new data supports a male-specific increase in IIS in 0S compared with 1S, and suggests that an increase in insulin sensitivity explains this sex-specific increase. We added a significant amount of text and figures to the revised manuscript to reflect the importance of this additional data.

TOR

We used many approaches to investigate TOR regulation in males and females in 1S and 0S, and to strengthen our findings regarding a role for TOR function in regulating the low sugar-induced changes to body size and metabolism.

While I provide detailed comments on measuring TOR activity in 1S and 0S below (see "Detailed comments on measuring TOR activity"), here is a brief summary of the new TOR-related data we include in the revised manuscript:

- RNAseq data showed that mRNA levels of two known TOR-responsive genes *unk* and *cbt* were altered only in females and not in males in 0S (Fig. 4L).
- Reduced TOR function blocked the female-specific increase in whole-body protein levels in 0S (Fig. 4I).
- Lower TOR function caused a reproducible female-biased effect on the low sugar-induced increase in body size in 0S across multiple experiments (Fig. 4J,K; Fig. S9A-D).
- Levels of p-S6k showed a trend ($p=0.0625$) toward increased levels in both sexes in 0S compared with 1S (Fig. S10A,B).
- RNAseq data shows altered mRNA levels of anabolic processes known to be regulated by TOR.

A potential sex-biased role for TOR is supported by our new genetic data confirming a female-biased requirement for TOR function in regulating the body size and metabolic responses to a low sugar diet. Dr. Ramon Klein Geltink, an expert in TOR-mediated metabolic changes and author on our revised manuscript, further highlighted that the female-specific regulation of several cellular processes in 0S (e.g. lysosomes, biosynthesis of amino acids, pentose phosphate pathway) are consistent with known TOR-regulated processes. Indeed, Dr. Klein Geltink pointed out that TOR acts through multiple effectors beyond S6k to regulate these diverse cellular processes, suggesting more studies will be needed to monitor sex-specific regulation of TOR targets in addition to S6k.

To present all the TOR data together, and to reflect the complexity between the TOR readouts we used (*unk*/*cbt* and p-S6k), we made several changes to the manuscript:

- We refocused the first three figures of the manuscript on our discoveries regarding the male-specific regulation and function of IIS in regulating the male body size and metabolic response to a low sugar diet.
- We limited our conclusions regarding a potential female-biased role for TOR to the metabolic and body size phenotypes.
- Because TOR is known to affect cellular metabolism via multiple downstream effectors, we state that future studies will need to monitor additional TOR targets.
- We made changes to the text to suggest more knowledge is needed about TOR biology in both sexes.

Importantly, our TOR data, when taken together with our findings on the sex-biased regulation and function of IIS in regulating metabolism and body size in a low sugar context, supports our overall conclusion that the mechanisms underlying the low sugar-induced increase in body size are not fully shared between the sexes.

Detailed comments on measuring TOR activity

4E-BP. Unfortunately, when we tested a commercial phospho-4E-BP antibody, no bands were lost in *4E-BP* null mutant larvae. Because we could not be sure which of the many bands detected by this antibody corresponded to 4E-BP, we were unable to draw any conclusions from this experiment.

TOR gene expression readouts. Our RNAseq data allowed us to monitor mRNA levels of known TOR-responsive genes *unk* and *cbt* (Guertin et al., 2006; Ingaramo et al., 2020; Tiebe et al., 2015) (Supplementary file 2). We found that *unk* and *cbt* were significantly downregulated in female, but not male, larvae reared in 0S compared with larvae raised in 1S (Fig. 4L). Because both genes are repressed by TOR, this suggests a female-specific upregulation of TOR in 0S.

Additional S6k Western blotting. To improve the S6k blot quantification, we were fortunate to receive a small aliquot of total S6k antibody. Unfortunately, we observed significant diet-induced changes to total S6k levels in 1S and 0S. Because drawing accurate conclusions about TOR activity using the ratio of phospho:total S6k requires that levels of total S6k do not vary with the experimental manipulation, we were concerned about the sugar-dependent effect on total S6k levels. In consultation with Dr. Hilla Weidberg, an expert in Western blotting, we normalized phospho-S6k levels to the total amount of protein loaded in each lane using the stain-free labeling system from BioRad (Cat# 1610185). This avoids overreliance on loading levels of one protein which may be subject to regulation by nutrients. We found that levels of p-S6k showed a trend ($p=0.0625$) toward increased levels in both sexes in 0S compared with 1S (Fig. S10A,B).

2)The dietary condition used is presented as "low sugar" but it can also be framed as high protein/sugar ratio. This is an important point to discuss.

We thank the Reviewer for this comment. We added text in the revised manuscript to ensure readers are aware that the low sugar diet and a low protein:sugar ratio have similar effects on development:

"It remains unclear whether these growth effects are mediated by dietary sugar alone or by changing the protein to carbohydrate ratio, as both factors affect body size and the length of the larval period, respectively (Kim et al., 2020; Matzkin et al., 2011; Musselman et al., 2011; Pasco and Léopold, 2012); however, our findings extend prior knowledge by demonstrating an equivalent body size response between the sexes to this dietary manipulation."

3)The authors appropriately use 2-/3-way ANOVA for the testing of effects of genotype/diet/sex on growth. I think they can extend this statistical approach to other data presented in the manuscript, where relevant.

We thank the Reviewer for this suggestion. We limited our use of 2- and 3-way ANOVAs to body size data due to smaller sample sizes in our metabolic and gene expression experiments (n=6-8).

4)Can the authors speculate on why these sex differences in how growth is promoted under low sugar exist and what effect they may have on the adult, if any?

In our revised manuscript, we added data showing that the low sugar diet improves insulin sensitivity in male larvae, leading to higher insulin pathway activity in 0S (Fig. 2G). Because insulin sensitivity was unchanged in female larvae between 0S and 1S conditions (Fig. 2F), this provides one explanation for why there is a male-female difference in the signaling pathways activated between 0S and 1S. To ensure this important point is communicated to readers, we added the following text to the revised manuscript:

“Together, these results indicate that the increase in IIS activity between 1S and 0S in males was caused by improved insulin sensitivity in a low sugar context. In females, there was no increase in IIS activity because females are insulin sensitive in both diets. This data adds to a growing body of evidence demonstrating sex differences in the nutrient-dependent regulation of IIS (Millington et al., 2021b). Significantly, our data suggests that in flies, as in mammals (Guerre-Millo et al., 1985; Mittendorfer, 2005; Macotela et al., 2009; Yki-Järvinen, 1984), males exhibit lower insulin sensitivity than females in some contexts.”

Second decision letter

MS ID#: DEVELOP/2021/200491

MS TITLE: A low sugar diet enhances Drosophila body size in males and females via sex-specific mechanisms

AUTHORS: Jason W Millington, Puja Biswas, Charlotte Chao, Yi Han Xia, Lianna W Wat, George P Brownrigg, Ziwei Sun, Paige J Basner-Collins, Ramon I Klein Geltink, and Elizabeth J Rideout
ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.