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Review COMMONS

Embryonic hyperglycemia perturbs the development of specific retinal cell types, including photoreceptors

Kayla F. Titialii-Torres and Ann C Morris
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Editor: Daniel Billadeau

Review timeline

Submission to Review Commons:	3 May 2021
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Reviewer 1

Evidence, reproducibility and clarity

Summary

In this work, the authors Titialii-Torres and Morris assess how hypergycemia affects the development of the neural retina using a genetic and a nutritional approach in the model organism zebrafish. This is important as diabetes can contribute to retinal degeneration in during the progression of diabetic retinopathy which often leads to blindness in adults.

The authors examine how different cell types in the neural retina are affected in a genetic hyperglycemic model, the pdx1 mutant embryos, and in a nutritional model, in which hyperglycemia is induced by glucose and dexamethasone exposure. Titialii-Torres and Morris show that in both models, photoreceptor rods and cones, as well as horizontal cells, are reduced in number.
Additionally, they report a delay in retinal cell differentiation accompanied by increased ROS production in the hyperglycemic retina. Altered expression of metabolism related genes and effects on visual function were also found in their hyperglycemic models.
Overall, the assessment of the different retinal cell types impacted by hyperglycemia and examination of potential molecular mechanisms contributes important and novel data to the field. However, the data as presented falls short in supporting the conclusions of the authors.

Major comments

Overall, the conclusions would be more strongly supported by improving the clarity of the images, and by additional analyses.

Figure1:
o Referring to figure 1 E' the text states that an arrowhead points to the shorter and thinner outer segment of a rod. In the figure there is an arrow pointing to a cell without a visible outer segment, making it hard to make the same conclusion.

o Additionally the GFP signal is very weak in D and E in the dorsal retina. Therefore it is not possible to see if there is also a decreased amount of rods in the dorsal retina as claimed.

o In the text it is mentioned that cones in the ventral region are affected. Is there also a difference in the dorsal region?

Figure 3:

o Rods and cones might be better displayed in close-ups from sections rather than from projections of the whole eye.

o The authors write about a reduction of cones upon glucose treatment. In the graph this is not highlighted as significant.

Figure 4: as the overall number of cones was already assessed before, focusing on a smaller region might help the reader to see the Zpr3 staining showing that the outer segments of the cones are stunted (as stated in the main text). In the figure panels presented, outer segments cannot be clearly seen.

Figure 6: scale bar is missing. Please clarify what the red and the green is. Why is there a red signal from Mitosox outside of the embryo (panel C)? The fluorescence of the superoxide probe should be displayed in a more convincing way. For example, in sections to enable assignment of signal to tissues and cells, as shown in Supplemental Figure 5.

Figure 8: Is the coincubation with methylene blue leading to a significant increase in photoreceptors? If yes, this should be indicated in the graph.

Supplemental Figure 2: The authors assert that TUNEL+ cell labeling coincides with Müller glial cells. This would be better supported with a magnified view of the INL, optimally by applying TUNEL staining to hyperglycemic, GFAP:GFP transgenic samples.

It would be of interest to determine if an incubation with methylene blue also affects photoreceptors in pdx1 mutants. Is it possible to confirm that Methylene blue treatment reduces ROS in the retina ? Can changes in ROS response gene expression be demonstrated by qPCR ? The assumptions about ROS should be either strengthened by additional experiments or less emphasized in the discussion.

For completeness, glucose metabolism in the genetic model should be also addressed and compared to the nutritional model.

The authors talk about a "long term" return to normoglycemia and long term effects of hyperglycemia. Analysis at 7 dpf after a 2 day return to normoglycemic conditions can hardly be called long term. To make these statements, an assessment after a longer time period (one week or more if possible?) would be more convincing.

The claims of 'reactive gliosis' in glucose-treated larvae is overstated. Biologically meaningful differences in cell shape between control and treated samples are not evident from the images (Fig. 5A-F). This should at least be quantitated by shape analysis. The Glucose+Dex samples do not show increased number of Müller glial cells, and glucose treatment alone leads to highly variable glucose levels. This complicates and weakens a correlation with hyperglycemia.

In addition, there are many minor inconsistencies and awkwardness in the presentation of the figures, as detailed below.

Minor comments

Some figures would benefit if they would follow the sequence of the text. Eg: figure 1 and 3, the text addresses first the rods and then the cones.

In several places the panels referred to in the text do not match the figures or figure panels are not mentioned at all. For example:

Pg 3 "Quantification revealed a significant decrease in both rod and cone photoreceptors in pdx1 mutants at 5 dpf (Fig. 1C)."

- the quantification is in panels C and F.

The main text does not mention or explain Figure 2A
Pg 5 "The results confirmed that rods and cones from hyperglycemic larvae have shorter outer segments compared to wild type larvae at 5 dpf (Fig. 4A-C)."

- panel C is a graph of Saccades.

Fig. S3 - only panel Y is referred to in the text.

Supplemental figure 2: the authors claim a significant increase of apoptotic cells in the genetic model. In the corresponding graph significance is not indicated.

Figure 5: scale bars are missing, the figure text and the numbering of the figure do not fit.

Supplemental figures 4 and 5:

o The Prox1 staining is hard to see and it is unclear what was counted as cells.

o In Supp Fig. 4E the PKC staining looks increased compared to the controls.

o The graphs could have similar y axes, especially because in supplemental figure 5 the amount of cells/µm is also different. Why not always use per 50µm? Shouldn't the amount of cells in wild types and untreated embryos be the same per 50µm?

o Also the labelling of the y axes could be made coherent in the two figures.

Supplemental figure 6: K is not mentioned in the legend.

2-NDBG treatment is not explained in material and methods

Significance

Titialii-Torres et al. characterize the impaired development of neural retinal cells under hyperglycemic conditions in zebrafish larvae and also show evidence of impaired visual function.
This work will be of interest for researchers in the field of diabetes, especially those focused on diabetic retinopathy, and for developmental biologists interested in pathologies that impact human development. While the manuscript provides insights into the development of the retina under hyperglycemic conditions, a revision addressing

weaknesses of figure presentation and some additional confirmatory experiments would be of great benefit.

Reviewer 2

Evidence, reproducibility and clarity

Summary:

This paper uses immersion of embryonic zebrafish in high glucose solution to model the effects of hyperglycemia on retinal development. The paper finds that high glucose causes a reduction in the number of photoreceptors and horizontal cells, abnormalities in the morphology of photoreceptors and Müller glia, increased retinal cell apoptosis, a change in the timing of neuronal cell birth, and a defect in the optokinetic response. The mechanistic link between high glucose and changes in retinal development is not well described but may involve an increase in reactive oxygen species.

Major comments:
1. Is the photoreceptor phenotype a degenerative rather a developmental phenotype? In embryos treated with high glucose, photoreceptors in the periphery of the retina near the ciliary margin, which are younger in age, seem to be structurally more normal than those at the center, away from the ciliary margin, which are older in age. Could this reflect the fact that photoreceptor development proceeds normally followed by degenerative changes?

2. For many or most phenotypes the main examined treatment is glucose + dexamethasone. The authors state this combination achieves more uniform glucose concentrations in the embryos as compared to glucose alone. However, dexamethasone may have effects independently of glucose and the dexamethasone only control is not used in some or most experiments. For example in Fig. 3, could dexamethasone alone causes changes in photoreceptor morphology? In the combo treatment, is it possible that some effects are simply due to a synergism of glucose+dex and not because dex causes a more uniformly high intraembryonic glucose?

3. It is interesting that hyperglycemic retinas show more neurons born between 2-5 days post fertilization in the RGC layer than in the outer nuclear layer (Fig 7). One interpretation is delayed birth of RGCs after hyperglycemia as the authors suggest. Another interpretation is that non-RGC cell types are in now in the RGC layer; or that some proliferating progenitors persist at 5dpf. Co-localization of EdU with differentiation markers, and EdU analysis after a short pulse of 2 hours would help to nail down if there is developmental delay or something else going on here.

4. Do Müller cells go into cycle after high glucose treatment?

5. The increase in ROS in Fig. 6 does not seem very convincing. Is the difference between untreated and glucose or glucose/dex treatments statistically significant? I would avoid making too much of this unless some type of phenotype rescue with N-acetylcysteine or vitamin C, or Trolox, can be shown. Methylene blue is a bit non-specific as an antioxidant.

Significance

The translational significance of the findings is that they might provide a model to study how embryonic hyperglycemia due to maternal diabetes changes embryonic development.

Pitfalls include the fact that its relevance to humans is unclear. Is maternal diabetes known to cause visual abnormalities due to abnormal retinal development in newborns? The basic biology significance may be to provide a model to investigate how glucose metabolism is connected to developmental decisions. However it is unclear whether glucose metabolism within retinal cells mediates the observed effects; and the high glucose used here is likely unphysiological as at these developmental stages zebrafish embryos feed from the yolk sac.

Audience interested in this work may include zebrafish developmental biologists. My expertise: metabolism, retinal development.

Reviewer 3

Evidence, reproducibility and clarity

Summary:

The authors use a combination of genetic and pharmacological immersion approaches to investigate the effects of hyperglycemia on development of the retina in zebrafish larvae. They demonstrate a rather mild phenotype (though still convincing) such that photoreceptor maturation is delayed/impaired and the Muller glia are also affected. Visual function is modestly impacted, as measured with an assay that can be influenced by motor as well as sensory defects. The authors conclude that altered timing of the differentiation of retinal cells, together with accumulation of reactive oxygen species (ROS) underly the photoreceptor defects and reduced visual function in the hyperglycemic larvae.

Major comments:

The retinal phenotype related to hyperglycemia is quite subtle, but sufficiently consistent. This phenotype would be more convincing, and lead to more definitive conclusions, if the authors could include some ultrastructural (TEM) information, or even high-resolution/magnification color images of thinner sections processed using conventional histological methods, such as H&E, or toluidine blue/pyronin B. It is difficult to appreciate the features of the apical projections of the photoreceptors in the fluorescently-labeled images.

Comparison of zpr1 labeling with the TaC:eGFP transgenic is unfortunate. Ideally the authors would use the pdx1 mutant on this transgenic background. Alternatively, the authors could perform TaC in situ hybridizations.

The visual function defect is also quite mild. The authors should mention that the OKR assay also relies upon motor function, and so the defect may be related to sensory deficit, motor deficit, or both. Larval ERGs would address this issue.

The "reactive gliosis" phenotype is also mild/subtle, and not entirely convincing. More information should be provided regarding what the authors considered an "abnormal shape" of an MG cell body. Ideally, there is an at least somewhat objective means to score normal vs. abnormal and then quantify.

In Figure 6 legend, the authors state that superoxide production is increased, but the graph does not appear convincing in this regard, and no statistical evaluation is provided.

The authors do not indicate whether they checked datasets for having a normal distribution prior to the selection of a t-test (or ANOVA) for analysis vs. nonparametric tests.

The model and accompanying text in the Discussion seem overly wordy and speculative. This discussion also does not acknowledge that the effects upon the retina may be indirect, mediated by other tissues that are impacted by hyperglycemia. For example, ocular vascular defects have been described to result from hyperglycemia, over a similar time frame of analysis, and the effects on the retina may be downstream of these defects.

Minor comments:
Introduction - the statement appearing in the Discussion (offspring of diabetic pregnancies had significantly thinner inner and outer macula as well as lower macular volume [43].) should appear in the Introduction to better capture the interest of the reader.

Page 1. (..in nearly 10% of US pregnancies) - citation needed.

Page 2. Pdx1 mutation should be briefly described when first mentioned.

Legend for Figure 2 could benefit from a definition of 2-NDBG.

Figure 2B does not show Whole Body [Glucose] because the heads were removed for histological analysis.

It is this reviewer's experience and opinion that zpr-3 labels rods and the RH2 members of the double cones, due to the sequence similarity of RH1 (rhodopsin) and RH2. The cited paper (Yin et al., 2012) hints at this as well, but is slightly unclear due to the terminology used in the paper in describing cone subtypes.

Page 8. The marker used to detect bipolar neurons should be mentioned within the Results section.

Pages 12-13. The localization of TUNEL+ profiles may be related to microglia extending processes into the ONL, engulfing photoreceptors, and then internally transporting the bits to their cellular "eating stations" within other retinal layers.

Significance

Significance/Comparison to published knowledge:

The zebrafish model(s) are sufficiently novel, versatile, and interesting to constitute an advance in this field. A literature search by this reviewer revealed that the focus upon retinal cells in hyperglycemic, larval zebrafish appears novel. However, the phenotype is remarkably mild, and there is concern that follow-up studies in pursuit of more mechanistic insights will be challenging to perform by the authors and by others in the field. This paper does lay some key groundwork, but what comes next sounds like a lot of fishing expeditions.

Interested audiences:

Investigators in vision science/ophthalmology, developmental biology, toxicology/teratology, and diabetes.

Reviewer keywords:

Developmental biology, genetics, retina, zebrafish, photoreceptors.

Author response to reviewers' comments

General Statements

We appreciate the thoughtful and constructive comments provided by the reviewers and the opportunity to submit our revision plan for consideration. We have copied the reviewers' comments below and have detailed our proposed revisions and/or clarifications after each comment (or set of comments). We also provide a partially revised manuscript with editorial changes highlighted in red.

1. Description of the planned revisions

Reviewer 1:

In this work, the authors Titialii-Torres and Morris assess how hyperglycemia affects the development of the neural retina using a genetic and a nutritional approach in the model organism zebrafish. This is important as diabetes can contribute to retinal degeneration in during the progression of diabetic retinopathy which often leads to blindness in adults. The authors examine how different cell types in the neural retina are affected in a genetic hyperglycemic model, the *pdx1* mutant embryos, and in a nutritional model, in which hyperglycemia is induced by glucose and dexamethasone exposure. Titialii- Torres and Morris show that in both models, photoreceptor rods and cones, as well as horizontal cells, are reduced in number. Additionally, they report a delay in retinal cell differentiation accompanied by increased ROS production in the hyperglycemic retina.
Altered expression of metabolism related genes and effects on visual function were also found in their hyperglycemic models. Overall, the assessment of the different retinal cell types impacted by hyperglycemia and examination of potential molecular mechanisms contributes important and novel data to the field. However, the data as presented falls short in supporting the conclusions of the authors.

Major comments

Overall, the conclusions would be more strongly supported by improving the clarity of the images, and by additional analyses.

Figure1:

Referring to figure 1 E' the text states that an arrowhead points to the shorter and thinner outer segment of a rod. In the figure there is an arrow pointing to a cell without a visible outer segment, making it hard to make the same conclusion. Additionally the GFP signal is very weak in D and E in the dorsal retina. Therefore it is not possible to see if there is also a decreased amount of rods in the dorsal retina as claimed.
In the text it is mentioned that cones in the ventral region are affected. Is there also a difference in the dorsal region?

Response: In our revised manuscript, we will include higher magnification panels for better visualization of the morphological differences between photoreceptor outer segments; we will also revise the graphs to show separate quantification of photoreceptors in the dorsal vs ventral retina.

Figure 3:
Rods and cones might be better displayed in close-ups from sections rather than from projections of the whole eye.

Response: We will make this change

The authors write about a reduction of cones upon glucose treatment. In the graph this is not highlighted as significant.

Response: the change is significant; the graph will be edited to indicate this
Figure 4: as the overall number of cones was already assessed before, focusing on a smaller region might help the reader to see the Zpr3 staining showing that the outer segments of the cones are stunted (as stated in the main text). In the figure panels presented, outer segments cannot be clearly seen.

Response: we agree, and will make this change

Figure 6: scale bar is missing. Please clarify what the red and the green is. Why is there a red signal from Mitosox outside of the embryo (panel C)? The fluorescence of the superoxide probe should be displayed in a more convincing way. For example, in sections to enable assignment of signal to tissues and cells, as shown in Supplemental Figure 5.

Response: for the revised manuscript we will replace this figure with one containing analysis of tissue sections, with appropriate figure annotation and scale bar

Figure 8: Is the coincubation with methylene blue leading to a significant increase in photoreceptors? If yes, this should be indicated in the graph.

Response: for methylene blue treatment alone, the increase was not statistically significant; we have added text in the Results to clarify this. For the revised manuscript, we are also performing additional experiments with a methylene blue + SOD treatment group and with other ROS inhibitors, so this figure will be updated with those data.

Supplemental Figure 2: The authors assert that TUNEL+ cell labeling coincides with Müller glial cells. This would be better supported with a magnified view of the INL, optimally by applying TUNEL staining to hyperglycemic, GFAP:GFP transgenic samples.

Response: we will repeat this experiment using the GFAP:GFP line as suggested

It would be of interest to determine if an incubation with methylene blue also affects photoreceptors in *pdx1* mutants. Is it possible to confirm that Methylene blue treatment reduces ROS in the retina ? Can changes in ROS response gene expression be demonstrated by qPCR ? The assumptions about ROS should be either strengthened by additional experiments or less emphasized in the discussion.

Response: for the revised version we will include the ROS inhibitor experiments on pdx1 mutants as suggested, as well as imaging with the Mitosox probe to confirm the efficacy of the ROS inhibitors; we are also testing additional ROS inhibitors as described above.

For completeness, glucose metabolism in the genetic model should be also addressed and compared to the nutritional model.

Response: While we agree that it would be helpful to have these data, it would take a very long time to collect the necessary number of pdx1 mutant individuals needed for this experiment due to the small numbers of homozygous mutants recovered in each clutch. As an alternative approach, for the revised manuscript we will use qRT-PCR to test a subset of the genes on the pdx1 mutants that showed significant changes in the nutritional model.

The authors talk about a "long term" return to normoglycemia and long term effects of hyperglycemia. Analysis at 7 dpf after a 2 day return to normoglycemic conditions can hardly be called long term. To make these statements, an assessment after a longer time period (one week or more if possible?) would be more convincing.

Response: for our revised manuscript, we are adding an additional time point for analysis at one

week post hyperglycemia

The claims of 'reactive gliosis' in glucose-treated larvae is overstated. Biologically meaningful differences in cell shape between control and treated samples are not evident from the images (Fig. 5A-F). This should at least be quantitated by shape analysis. The Glucose+Dex samples do not show increased number of Müller glial cells, and glucose treatment alone leads to highly variable glucose levels. This complicates and weakens a correlation with hyperglycemia.

Response: we will add the suggested shape quantification of these images; we are also performing Western blots with an anti-GFAP antibody to further strengthen our conclusions - this is a well-accepted method for demonstrating gliosis.

Minor comments

Some figures would benefit if they would follow the sequence of the text. Eg: figure 1 and 3, the text addresses first the rods and then the cones. In several places the panels referred to in the text do not match the figures or figure panels are not mentioned at all.

For example: Pg 3 "Quantification revealed a significant decrease in both rod and cone photoreceptors in pdx1 mutants at 5 dpf (Fig. 1C)." - the quantification is in panels C and F. The main text does not mention or explain Figure 2A.
Pg 5 "The results confirmed that rods and cones from hyperglycemic larvae have shorter outer segments compared to wild type larvae at 5 dpf (Fig. 4A-C)." - panel C is a graph of Saccades. Fig. S3 - only panel Y is referred to in the text.

Response: the text has been edited to correct these issues

Supplemental figure 2: the authors claim a significant increase of apoptotic cells in the genetic model. In the corresponding graph significance is not indicated.

Response: the increase in apoptotic cells was significant for the nutritional but not the genetic model; the text has been corrected to reflect this.

Figure 5: scale bars are missing, the figure text and the numbering of the figure do not fit.

The suggested corrections will be made to this figure and the corresponding text

Supplemental figures 4 and 5: The Prox1 staining is hard to see and it is unclear what was counted as cells.

Response: annotations will be added to Sup Figs 4 and 5 to clarify which cells are being quantified

In Supp Fig. 4E the PKC staining looks increased compared to the controls.

Response: the variability in staining intensity is within the normal range of what we have observed across all treatments and genotypes

The graphs could have similar y axes, especially because in Supp Fig. 5 the amount of cells/µm is also different. Why not always use per 50µm? Shouldn't the amount of cells in wild types and untreated embryos be the same per 50µm? Also the labelling of the y axes could be made coherent in the two figures.

Response: The denominator will be standardized for all graphs. The scale of the y-axes varies by cell type because some retinal cell classes are significantly more abundant than others.

Supplemental figure 6: K is not mentioned in the legend.

Response: this has been corrected

2-NDBG treatment is not explained in material and methods

Response: this information has been added to the Methods

Reviewer #1 (Significance (Required)):

Significance

Titialii-Torres et al. characterize the impaired development of neural retinal cells under hyperglycemic conditions in zebrafish larvae and also show evidence of impaired visual function. This work will be of interest for researchers in the field of diabetes, especially those focused on diabetic retinopathy, and for developmental biologists interested in pathologies that impact human development. While the manuscript provides insights into the development of the retina under hyperglycemic conditions, a revision addressing weaknesses of figure presentation and some additional confirmatory experiments would be of great benefit.

Response: we appreciate the reviewer's assessment that our work will be of interest to various research communities, and agree that the suggested revisions to the figures and confirmatory experiments will greatly strengthen the impact.

Reviewer 2:
Summary:

This paper uses immersion of embryonic zebrafish in high glucose solution to model the effects of hyperglycemia on retinal development. The paper finds that high glucose causes a reduction in the number of photoreceptors and horizontal cells, abnormalities in the morphology of photoreceptors and Müller glia, increased retinal cell apoptosis, a change in the timing of neuronal cell birth, and a defect in the optokinetic response. The mechanistic link between high glucose and changes in retinal development is not well described but may involve an increase in reactive oxygen species.

Major comments:

1. Is the photoreceptor phenotype a degenerative rather a developmental phenotype? In embryos treated with high glucose, photoreceptors in the periphery of the retina near the ciliary margin, which are younger in age, seem to be structurally more normal than those at the center, away from the ciliary margin, which are older in age. Could this reflect the fact that photoreceptor development proceeds normally followed by degenerative changes?

Response: this is certainly a possibility, given the increase in TUNEL positive cells we detected in hyperglycemic retinas. However, we did not detect many apoptotic cells in the ONL at 3 and 4 dpf, suggesting that there is not widespread degeneration among differentiated photoreceptors at that stage. This result, in combination with the altered differentiation timing data shown in Figure 7, is what led us to favor a developmental phenotype. In the revised manuscript, we will add text to the Discussion that more thoroughly explores these alternative interpretations.

2. For many or most phenotypes the main examined treatment is glucose + dexamethasone. The authors state this combination achieves more uniform glucose concentrations in the embryos as compared to glucose alone. However, dexamethasone may have effects independently of glucose and the dexamethasone only control is not used in some or most experiments. For example in Fig. 3, could dexamethasone alone causes changes in photoreceptor morphology? In the combo treatment, is it possible that some effects are simply due to a synergism of glucose+dex and not because dex causes a more uniformly high intraembryonic glucose?

Response: we have evaluated photoreceptor number and morphology in the dex alone treatment group and found no significant differences. We will add these results to the main text and the supplemental figures.

3. It is interesting that hyperglycemic retinas show more neurons born between 2-5 days post fertilization in the RGC layer than in the outer nuclear layer (Fig 7). One interpretation is delayed birth of RGCs after hyperglycemia as the authors suggest. Another interpretation is that non-RGC cell types are in now in the RGC layer; or that some proliferating progenitors persist at 5dpf. Co-localization of EdU with differentiation markers, and EdU analysis after a short pulse of 2 hours would help to nail down if there is developmental delay or something else going on here.

Response: we appreciate the suggestion, and will perform this experiment for the revision

4. Do Müller cells go into cycle after high glucose treatment?

Response: this is a great question - we will do a co-localization experiment and add these results to the revised manuscript.

5. The increase in ROS in Fig. 6 does not seem very convincing. Is the difference between untreated and glucose or glucose/dex treatments statistically significant? I would avoid making too much of this unless some type of phenotype rescue with N- acetylcysteine or vitamin C, or Trolox, can be shown. Methylene blue is a bit non- specific as an antioxidant.

Response: for methylene blue treatment alone, the increase was not statistically significant; we have added text in the Results to clarify this. For the revised manuscript, we are also performing additional experiments with a methylene blue + SOD treatment group and with other ROS inhibitors, so this figure will be updated with those data

Reviewer #2 (Significance (Required)):

The translational significance of the findings is that they might provide a model to study how embryonic hyperglycemia due to maternal diabetes changes embryonic development. Pitfalls include the fact that its relevance to humans is unclear. Is maternal diabetes known to cause visual abnormalities due to abnormal retinal development in newborns? The basic biology significance may be to provide a model to investigate how glucose metabolism is connected to developmental decisions. However it is unclear whether glucose metabolism within retinal cells mediates the observed effects; and the high glucose used here is likely unphysiological as at these developmental stages zebrafish embryos feed from the yolk sac.

Response: yes, maternal diabetes is associated with retinal abnormalities in humans, although there are not many published studies on this topic. In the Discussion, we talked about how our results align with prior clinical studies which documented reduced inner and outer macular thickness in children of diabetic pregnancies. At the suggestion of Reviewer 3, we have added this information to the Introduction as well to highlight the relevance of our study to humans. With respect to the comment about physiological relevance, we feel that the inclusion of the genetic model, which does not rely on high levels of exogenous glucose and yet exhibits a similar photoreceptor phenotype, speaks to this issue.

Reviewer 3:

Summary:

The authors use a combination of genetic and pharmacological immersion approaches to investigate the effects of hyperglycemia on development of the retina in zebrafish larvae. They demonstrate a rather mild phenotype (though still convincing) such that photoreceptor maturation is delayed/impaired and the Muller glia are also affected.
Visual function is modestly impacted, as measured with an assay that can be influenced by motor as well as sensory defects. The authors conclude that altered timing of the differentiation of retinal cells, together with accumulation of reactive oxygen species (ROS) underly the photoreceptor defects and reduced visual function in the hyperglycemic larvae.

Major comments

The retinal phenotype related to hyperglycemia is quite subtle, but sufficiently consistent. This phenotype would be more convincing, and lead to more definitive conclusions, if the authors could

include some ultrastructural (TEM) information, or even high-resolution/magnification color images of thinner sections processed using conventional histological methods, such as H&E, or toluidine blue/pyronin B. It is difficult to appreciate the features of the apical projections of the photoreceptors in the fluorescently-labeled images.
Response: we are adding higher magnification images to the photoreceptor figures (also suggested by Reviewer 1) and will incorporate an H&E stain as well.

Comparison of zpr1 labeling with the TaC:eGFP transgenic is unfortunate. Ideally the authors would use the *pdx1* mutant on this transgenic background. Alternatively, the authors could perform TaC in situ hybridizations.

Response: we have crossed the pdx1 line onto the TaC:eGFP transgenic background and will have this experiment completed for the revision

The visual function defect is also quite mild. The authors should mention that the OKR assay also relies upon motor function, and so the defect may be related to sensory deficit, motor deficit, or both. Larval ERGs would address this issue.

Response: we will add this alternative explanation for the OKR results to the text.

The "reactive gliosis" phenotype is also mild/subtle, and not entirely convincing. More information should be provided regarding what the authors considered an "abnormal shape" of an MG cell body. Ideally, there is an at least somewhat objective means to score normal vs. abnormal and then quantify.

Response: for the revision, we are adding shape quantification and Western blots (please see our response to the similar comment made by Reviewer 1)

In Figure 6 legend, the authors state that superoxide production is increased, but the graph does not appear convincing in this regard, and no statistical evaluation is provided.

Response: for methylene blue treatment alone, the increase was not statistically significant; we have added text in the Results to clarify this. For the revised manuscript, we are also performing additional experiments with a methylene blue + SOD treatment group and with other ROS inhibitors, so this figure will be updated with those data

The authors do not indicate whether they checked datasets for having a normal distribution prior to the selection of a t-test (or ANOVA) for analysis vs. nonparametric tests.

Response: a more thorough description of our statistical analyses will be added to the methods

The model and accompanying text in the Discussion seem overly wordy and speculative. This discussion also does not acknowledge that the effects upon the retina may be indirect, mediated by other tissues that are impacted by hyperglycemia. For example, ocular vascular defects have been described to result from hyperglycemia, over a similar time frame of analysis, and the effects on the retina may be downstream of these defects.

Response: we will revise the Discussion to remove extraneous information and to incorporate alternative mechanisms that could explain the retinal phenotypes induced by hyperglycemia

Minor comments:

Introduction - the statement appearing in the Discussion (offspring of diabetic pregnancies had significantly thinner inner and outer macula as well as lower macular volume [43].) should appear in the Introduction to better capture the interest of the reader.

Response: this change has been made

Page 1. (..in nearly 10% of US pregnancies) - citation needed.

Response: this has been added

Page 2. Pdx1 mutation should be briefly described when first mentioned.

Response: this has been added

Legend for Figure 2 could benefit from a definition of 2-NDBG.

Response: the figure legend has been revised

Figure 2B does not show Whole Body [Glucose] because the heads were removed for histological analysis.

Response: this correction will be made to the figure

It is this reviewer's experience and opinion that zpr-3 labels rods and the RH2 members of the double cones, due to the sequence similarity of RH1 (rhodopsin) and RH2. The cited paper (Yin et al., 2012) hints at this as well, but is slightly unclear due to the terminology used in the paper in describing cone subtypes.

We have edited the Results to clarify that Zpr3 labels the Rh2-expressing member of the double cones

Page 8. The marker used to detect bipolar neurons should be mentioned within the Results section.

Response: this information has been added to the Results

Pages 12-13. The localization of TUNEL+ profiles may be related to microglia extending processes into the ONL, engulfing photoreceptors, and then internally transporting the bits to their cellular "eating stations" within other retinal layers.

Response: we will add text to the Results including this as a possibility. We are also (at the suggestion of Reviewer 1) adding an experiment to determine whether some of the TUNEL+ cells co-localize with Muller glia markers.

Reviewer #3 (Significance (Required)):

Significance/Comparison to published knowledge:

The zebrafish model(s) are sufficiently novel, versatile, and interesting to constitute an advance in this field. A literature search by this reviewer revealed that the focus upon retinal cells in hyperglycemic, larval zebrafish appears novel. However, the phenotype is remarkably mild, and there is concern that follow-up studies in pursuit of more mechanistic insights will be challenging to perform by the authors and by others in the field. This paper does lay some key groundwork, but what comes next sounds like a lot of fishing expeditions.

Response: we appreciate the reviewer's assessment that our work represents a novel advance in this field, and lays "key groundwork" for future studies. Although our nutritional and genetic models do not present with photoreceptor loss so severe that it causes complete blindness at the timepoints we tested, the photoreceptor reductions we observe are consistent, readily scorable, and are associated with demonstrable defects in visual behavior. Given that we also provide evidence of both altered cell differentiation kinetics and increased oxidative stress in embryonic hyperglycemic retinas, we feel that these are excellent starting places for future work to uncover more mechanistic insights. Finally, our results have implications for human visual system development under hyperglycemic conditions. Timely vision development in infancy is required for attainment of a host of developmental milestones. Even mild delays in this process could have long term consequences for intellectual and social development, and due to the difficulty of measuring visual acuity in infants, subtle but significant impairments may go undetected at this

critical stage. Therefore, having a reliable animal model for embryonic hyperglycemia will facilitate efforts to better understand this condition with the goal of developing appropriate intervention and treatment strategies.

2. Description of revisions that have already been incorporated in the transferred manuscript
We have made several editorial corrections to the main text and figure legends – these are indicated in the detailed response above and are highlighted in red in the partially revised manuscript.
3. Description of analyses that authors prefer not to carry out
As indicated in the point by point response above, we prefer not to carry out the full metabolic profiling on *pdx1* mutants (the genetic model) due to the difficulty in collecting sufficient numbers of mutant embryos for this anaylsis in a timely fashion. However, as an alternative, we can perform a more targeted analysis of a subset of genes of interest for the revision.

Original submission

First decision letter

MS ID#: JOCES/2021/259187

MS TITLE: Embryonic hyperglycemia perturbs the development of specific retinal cell types, including photoreceptors

AUTHORS: Kayla F. Titialii-Torres and Ann Morris
ARTICLE TYPE: Research Article

I have read through your response to the Review Commons reviewers concerns and agree with your plan for revision.

Please 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. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

First revision

Author response to reviewers' comments

We appreciate the thoughtful and constructive comments provided by the reviewers and the opportunity to submit our revised manuscript for consideration. We have copied the reviewers' comments below and have detailed our revisions and/or clarifications after each comment (or set of comments). Substantial changes to the manuscript text are indicated in red in the revised version.

Reviewer 1:
In this work, the authors Titialii-Torres and Morris assess how hyperglycemia affects the development of the neural retina using a genetic and a nutritional approach in the model organism zebrafish. This is important as diabetes can contribute to retinal degeneration in during the progression of diabetic retinopathy which often leads to blindness in adults. The authors examine how different cell types in the neural retina are affected in a genetic hyperglycemic model, the pdx1 mutant embryos, and in a nutritional model, in which hyperglycemia is induced by glucose and dexamethasone exposure. Titialii-Torres and Morris show that in both models, photoreceptor

rods and cones, as well as horizontal cells, are reduced in number. Additionally, they report a delay in retinal cell differentiation accompanied by increased ROS production in the hyperglycemic retina. Altered expression of metabolism related genes and effects on visual function were also found in their hyperglycemic models. Overall, the assessment of the different retinal cell types impacted by hyperglycemia and examination of potential molecular mechanisms contributes important and novel data to the field. However, the data as presented falls short in supporting the conclusions of the authors.

Major comments

Overall, the conclusions would be more strongly supported by improving the clarity of the images, and by additional analyses.

Figure1:

Referring to figure 1 E' the text states that an arrowhead points to the shorter and thinner outer segment of a rod. In the figure there is an arrow pointing to a cell without a visible outer segment, making it hard to make the same conclusion. Additionally the GFP signal is very weak in D and E in the dorsal retina. Therefore it is not possible to see if there is also a decreased amount of rods in the dorsal retina as claimed.
In the text it is mentioned that cones in the ventral region are affected. Is there also a difference in the dorsal region?

Response: We now include higher magnification panels for better visualization of the morphological differences between photoreceptor outer segments; we also revised the graphs to show separate quantification of photoreceptors in the dorsal vs ventral retina.

Figure 3 (now Figure 2):
Rods and cones might be better displayed in close-ups from sections rather than from projections of the whole eye.

Response: We added higher magnification panels to this figure

The authors write about a reduction of cones upon glucose treatment. In the graph this is not highlighted as significant.

Response: the change is significant; the graph was corrected to indicate this

Figure 4 (now Figure 3): as the overall number of cones was already assessed before, focusing on a smaller region might help the reader to see the Zpr3 staining showing that the outer segments of the cones are stunted (as stated in the main text). In the figure panels presented, outer segments cannot be clearly seen.

Response: we agree, have made this change

Figure 6 (now Figure 5): scale bar is missing. Please clarify what the red and the green is. Why is there a red signal from Mitosox outside of the embryo (panel C)? The fluorescence of the superoxide probe should be displayed in a more convincing way. For example, in sections to enable assignment of signal to tissues and cells, as shown in Supplemental Figure 5.

Response: we added panels containing images showing the location of ROS in retinal tissue sections at 48 hpf, with appropriate figure annotation and scale bar

Figure 8: Is the coincubation with methylene blue leading to a significant increase in photoreceptors? If yes, this should be indicated in the graph.

Response: for methylene blue treatment alone, the increase was not statistically significant; we have added text in the Results to clarify this. For the revised manuscript, we performed additional experiments with a different ROS inhibitor (DPI); DPI treatment resulted in a significant increase in

photoreceptors compared to glucose+dex treated alone; those data are now shown in Supplementary Figure 8.

Supplemental Figure 2: The authors assert that TUNEL+ cell labeling coincides with Müller glial cells. This would be better supported with a magnified view of the INL, optimally by applying TUNEL staining to hyperglycemic, GFAP:GFP transgenic samples.

Response: we removed the text from the Results suggesting that the TUNEL-positive cells could be Muller glia. In the Discussion, we do raise the possibility that the Muller cells are phagocytosing apoptotic cells, along with an alternative possibility that the TUNEL positive cells are microglia (as suggested by Reviewer 3).

It would be of interest to determine if an incubation with methylene blue also affects photoreceptors in pdx1 mutants. Is it possible to confirm that Methylene blue treatment reduces ROS in the retina ? Can changes in ROS response gene expression be demonstrated by qPCR ? The assumptions about ROS should be either strengthened by additional experiments or less emphasized in the discussion.

Response: we were not able to obtain enough mutant embryos from pdx1 incrosses (which do not produce many offspring) to do this additional experiment.

For completeness, glucose metabolism in the genetic model should be also addressed and compared to the nutritional model.

Response: While we agree that it would be helpful to have these data, it would take a very long time to collect the necessary number of pdx1 mutant individuals needed for this experiment due to the small numbers of homozygous mutants recovered in each clutch. As an alternative approach, for the revised manuscript we show qRT-PCR data from the pdx1 mutants for a subset of the genes that showed significant changes in the nutritional model. These data are included in Supplementary Figure 6.

The authors talk about a "long term" return to normoglycemia and long term effects of hyperglycemia. Analysis at 7 dpf after a 2 day return to normoglycemic conditions can hardly be called long term. To make these statements, an assessment after a longer time period (one week or more if possible?) would be more convincing.

Response: we agree, and extended our analysis to one week post hyperglycemia. We found that there was still a significant decrease in both rod and cone photoreceptors in 12 dpf larvae that had experienced hyperglycemia. These results are shown in Figure 7.

The claims of 'reactive gliosis' in glucose-treated larvae is overstated. Biologically meaningful differences in cell shape between control and treated samples are not evident from the images (Fig. 5A-F). This should at least be quantitated by shape analysis. The Glucose+Dex samples do not show increased number of Müller glial cells, and glucose treatment alone leads to highly variable glucose levels. This complicates and weakens a correlation with hyperglycemia.

Response: we performed the shape quantification analysis as suggested and added these data to Figure 4; we also performed Western blots with an anti-GFAP antibody to further strengthen our conclusions – this is a well-accepted method for demonstrating gliosis (Fig. 4I). Together, we feel these data do support a state of reactive gliosis in the hyperglycemic retinas.

Minor comments

Some figures would benefit if they would follow the sequence of the text. Eg: figure 1 and 3, the text addresses first the rods and then the cones. In several places the panels referred to in the text do not match the figures or figure panels are not mentioned at all. For example: Pg 3 "Quantification revealed a significant decrease in both rod and cone photoreceptors in pdx1 mutants at 5 dpf (Fig. 1C)." - the quantification is in panels C and F. The main text does not mention or explain Figure 2A.

Pg 5 "The results confirmed that rods and cones from hyperglycemic larvae have shorter outer segments compared to wild type larvae at 5 dpf (Fig. 4A-C)." - panel C is a graph of Saccades. Fig. S3 - only panel Y is referred to in the text.

Response: the text has been edited to correct these issues

Supplemental figure 2: the authors claim a significant increase of apoptotic cells in the genetic model. In the corresponding graph significance is not indicated.

Response: the increase in apoptotic cells was significant for the nutritional but not the genetic model; the text has been corrected to reflect this.

Figure 5: scale bars are missing, the figure text and the numbering of the figure do not fit.

The suggested corrections were made to this figure and the corresponding text

Supplemental figures 4 and 5 (now Sup Figs 6 and 7): The Prox1 staining is hard to see and it is unclear what was counted as cells.

Response: we added annotations to the figures to clarify which cells are being quantified

In Supp Fig. 4E the PKC staining looks increased compared to the controls.

Response: the variability in staining intensity is within the normal range of what we have observed across all treatments and genotypes

The graphs could have similar y axes, especially because in Supp Fig. 5 the amount of cells/µm is also different. Why not always use per 50µm? Shouldn't the amount of cells in wild types and untreated embryos be the same per 50µm? Also the labelling of the y axes could be made coherent in the two figures.

Response: We use either 50 um or 100 um as the denominator, depending on how abundant a particular retinal cell type is normally. The scale of the y-axes also varies by cell type for this reason.

Supplemental figure 6: K is not mentioned in the legend.

Response: this has been corrected

2-NDBG treatment is not explained in material and methods

Response: this information has been added to the Methods

Reviewer #1 (Significance (Required)):

Significance

Titialii-Torres et al. characterize the impaired development of neural retinal cells under hyperglycemic conditions in zebrafish larvae and also show evidence of impaired visual function. This work will be of interest for researchers in the field of diabetes, especially those focused on diabetic retinopathy, and for developmental biologists interested in pathologies that impact human development. While the manuscript provides insights into the development of the retina under hyperglycemic conditions, a revision addressing weaknesses of figure presentation and some additional confirmatory experiments would be of great benefit.

Response: we appreciate the reviewer’s assessment that our work will be of interest to various research communities, and agree that the suggested revisions to the figures and confirmatory experiments will greatly strengthen the impact.

Reviewer 2:
Summary:

This paper uses immersion of embryonic zebrafish in high glucose solution to model the effects of hyperglycemia on retinal development. The paper finds that high glucose causes a reduction in the number of photoreceptors and horizontal cells, abnormalities in the morphology of photoreceptors and Müller glia, increased retinal cell apoptosis, a change in the timing of neuronal cell birth, and a defect in the optokinetic response. The mechanistic link between high glucose and changes in retinal development is not well described but may involve an increase in reactive oxygen species.

Major comments:

1. Is the photoreceptor phenotype a degenerative rather a developmental phenotype? In embryos treated with high glucose, photoreceptors in the periphery of the retina near the ciliary margin, which are younger in age, seem to be structurally more normal than those at the center, away from the ciliary margin, which are older in age. Could this reflect the fact that photoreceptor development proceeds normally followed by degenerative changes?

Response: this is certainly a possibility, given the increase in TUNEL positive cells we detected in hyperglycemic retinas. However, we did not detect many apoptotic cells in the ONL at 3 and 4 dpf, suggesting that there is not widespread degeneration among differentiated photoreceptors at that stage. This result, in combination with the altered differentiation timing data we observed, is what led us to favor a developmental phenotype. In the revised manuscript, we added text to the Discussion that includes this alternative interpretation.

2. For many or most phenotypes the main examined treatment is glucose + dexamethasone. The authors state this combination achieves more uniform glucose concentrations in the embryos as compared to glucose alone. However, dexamethasone may have effects independently of glucose and the dexamethasone only control is not used in some or most experiments. For example in Fig. 3, could dexamethasone alone causes changes in photoreceptor morphology? In the combo treatment, is it possible that some effects are simply due to a synergism of glucose+dex and not because dex causes a more uniformly high intraembryonic glucose?

Response: we have evaluated photoreceptor number and morphology in the dex alone treatment group and found no significant differences. We added these results to the main text and show them in Supplemental Figure 5.

3. It is interesting that hyperglycemic retinas show more neurons born between 2-5 days post fertilization in the RGC layer than in the outer nuclear layer (Fig 7). One interpretation is delayed birth of RGCs after hyperglycemia as the authors suggest. Another interpretation is that non-RGC cell types are in now in the RGC layer; or that some proliferating progenitors persist at 5dpf. Co-localization of EdU with differentiation markers, and EdU analysis after a short pulse of 2 hours would help to nail down if there is developmental delay or something else going on here.

Response: we performed this experiment, and now show in Supplemental Figure 7 that we did not detect non-RGC cells types (such as displaced amacrine cells) in the ganglion cell layer, nor do we see persistence of proliferating cells outside the CMZ at 5 dpf.

4. Do Müller cells go into cycle after high glucose treatment?

Response: Supplemental Figure 7 includes a co-localization experiment that shows the Muller glial cells in hyperglycemic retinas are not proliferative.

5. The increase in ROS in Fig. 6 does not seem very convincing. Is the difference between untreated and glucose or glucose/dex treatments statistically significant? I would avoid making too much of this unless some type of phenotype rescue with N-acetylcysteine or vitamin C, or Trolox, can be shown. Methylene blue is a bit non-specific as an antioxidant.

Response: for methylene blue treatment alone, the increase was not statistically significant; we have added text in the Results to clarify this. For the revised manuscript, we performed additional

experiments with a different ROS inhibitor (DPI); DPI treatment resulted in a significant increase in photoreceptors compared to glucose+dex treated alone; those data are now shown in Supplementary Figure 8

Reviewer #2 (Significance (Required)):

The translational significance of the findings is that they might provide a model to study how embryonic hyperglycemia due to maternal diabetes changes embryonic development. Pitfalls include the fact that its relevance to humans is unclear. Is maternal diabetes known to cause visual abnormalities due to abnormal retinal development in newborns? The basic biology significance may be to provide a model to investigate how glucose metabolism is connected to developmental decisions. However it is unclear whether glucose metabolism within retinal cells mediates the observed effects; and the high glucose used here is likely unphysiological as at these developmental stages zebrafish embryos feed from the yolk sac.

Response: yes, maternal diabetes is associated with retinal abnormalities in humans, although there are not many published studies on this topic. In the Discussion, we talked about how our results align with prior clinical studies which documented reduced inner and outer macular thickness in children of diabetic pregnancies. At the suggestion of Reviewer 3, we have added this information to the Introduction as well to highlight the relevance of our study to humans. With respect to the comment about physiological relevance, we feel that the inclusion of the genetic model, which does not rely on high levels of exogenous glucose and yet exhibits a similar photoreceptor phenotype, speaks to this issue.

Reviewer 3:

Summary:

The authors use a combination of genetic and pharmacological immersion approaches to investigate the effects of hyperglycemia on development of the retina in zebrafish larvae. They demonstrate a rather mild phenotype (though still convincing) such that photoreceptor maturation is delayed/impaired and the Muller glia are also affected. Visual function is modestly impacted, as measured with an assay that can be influenced by motor as well as sensory defects. The authors conclude that altered timing of the differentiation of retinal cells, together with accumulation of reactive oxygen species (ROS) underly the photoreceptor defects and reduced visual function in the hyperglycemic larvae.

Major comments

The retinal phenotype related to hyperglycemia is quite subtle, but sufficiently consistent. This phenotype would be more convincing, and lead to more definitive conclusions, if the authors could include some ultrastructural (TEM) information, or even high-resolution/magnification color images of thinner sections processed using conventional histological methods, such as H&E, or toluidine blue/pyronin B. It is difficult to appreciate the features of the apical projections of the photoreceptors in the fluorescently-labeled images.
Response: we added higher magnification images to the photoreceptor figures (also suggested by Reviewer 1) and added a supplemental figure showing an H&E stain as well (new Supplemental Figure 3)

Comparison of zpr1 labeling with the TaC:eGFP transgenic is unfortunate. Ideally the authors would use the pdx1 mutant on this transgenic background. Alternatively, the authors could perform TaC in situ hybridizations.

Response: We crossed the pdx1 line onto the TaC:eGFP transgenic background but were not able to obtain enough mutant embryos from pdx1;Tac:GFP incrosses (which do not produce many offspring) to do this additional experiment

The visual function defect is also quite mild. The authors should mention that the OKR assay also relies upon motor function, and so the defect may be related to sensory deficit, motor deficit, or both. Larval ERGs would address this issue.

Response: we added this alternative explanation for the OKR results to the text.

The "reactive gliosis" phenotype is also mild/subtle, and not entirely convincing. More information should be provided regarding what the authors considered an "abnormal shape" of an MG cell body. Ideally, there is an at least somewhat objective means to score normal vs. abnormal and then quantify.

Response: we performed the shape quantification analysis as suggested and added these data to Figure 4; we also performed Western blots with an anti-GFAP antibody to further strengthen our conclusions – this is a well-accepted method for demonstrating gliosis (Fig. 4I). Together, we feel these data do support a state of reactive gliosis in the hyperglycemic retinas. (please see our response to the similar comment made by Reviewer 1)

In Figure 6 legend, the authors state that superoxide production is increased, but the graph does not appear convincing in this regard, and no statistical evaluation is provided.

Response: for methylene blue treatment alone, the increase was not statistically significant; we have added text in the Results to clarify this. For the revised manuscript, we performed additional experiments with a different ROS inhibitor (DPI); DPI treatment resulted in a significant increase in photoreceptors compared to glucose+dex treated alone; those data are now shown in Supplementary Figure 8

The authors do not indicate whether they checked datasets for having a normal distribution prior to the selection of a t-test (or ANOVA) for analysis vs. nonparametric tests.

Response: a more thorough description of our statistical analyses was added to the Methods

The model and accompanying text in the Discussion seem overly wordy and speculative. This discussion also does not acknowledge that the effects upon the retina may be indirect, mediated by other tissues that are impacted by hyperglycemia. For example, ocular vascular defects have been described to result from hyperglycemia, over a similar time frame of analysis, and the effects on the retina may be downstream of these defects.

Response: we revised the Discussion to remove extraneous information and to incorporate alternative, indirect mechanisms that could explain the retinal phenotypes induced by hyperglycemia

Minor comments:

Introduction - the statement appearing in the Discussion (offspring of diabetic pregnancies had significantly thinner inner and outer macula as well as lower macular volume [43].) should appear in the Introduction to better capture the interest of the reader.

Response: this change has been made

Page 1. (..in nearly 10% of US pregnancies) - citation needed.

Response: this has been added

Page 2. Pdx1 mutation should be briefly described when first mentioned.

Response: this has been added

Legend for Figure 2 could benefit from a definition of 2-NDBG.

Response: the figure legend has been revised

Figure 2B does not show Whole Body [Glucose] because the heads were removed for histological analysis.
Response: this correction was made to the figure

It is this reviewer's experience and opinion that zpr-3 labels rods and the RH2 members of the double cones, due to the sequence similarity of RH1 (rhodopsin) and RH2. The cited paper (Yin et al., 2012) hints at this as well, but is slightly unclear due to the terminology used in the paper in describing cone subtypes.

We have edited the Results to clarify that Zpr3 labels the Rh2-expressing member of the double cones

Page 8. The marker used to detect bipolar neurons should be mentioned within the Results section.

Response: this information has been added to the Results

Pages 12-13. The localization of TUNEL+ profiles may be related to microglia extending processes into the ONL, engulfing photoreceptors, and then internally transporting the bits to their cellular "eating stations" within other retinal layers.

Response: we added text to the Discussion including this as a possibility.

Reviewer #3 (Significance (Required)):

Significance/Comparison to published knowledge:

The zebrafish model(s) are sufficiently novel, versatile, and interesting to constitute an advance in this field. A literature search by this reviewer revealed that the focus upon retinal cells in hyperglycemic, larval zebrafish appears novel. However, the phenotype is remarkably mild, and there is concern that follow-up studies in pursuit of more mechanistic insights will be challenging to perform by the authors and by others in the field. This paper does lay some key groundwork, but what comes next sounds like a lot of fishing expeditions.

Response: we appreciate the reviewer’s assessment that our work represents a novel advance in this field, and lays “key groundwork” for future studies. Although our nutritional and genetic models do not present with photoreceptor loss so severe that it causes complete blindness at the timepoints we tested, the photoreceptor reductions we observe are consistent, readily scorable, and are associated with demonstrable defects in visual behavior. Given that we also provide evidence of both altered cell differentiation kinetics and increased oxidative stress in embryonic hyperglycemic retinas, we feel that these are excellent starting places for future work to uncover more mechanistic insights. Finally, our results have implications for human visual system development under hyperglycemic conditions. Timely vision development in infancy is required for attainment of a host of developmental milestones. Even mild delays in this process could have long term consequences for intellectual and social development, and due to the difficulty of measuring visual acuity in infants, subtle but significant impairments may go undetected at this critical stage. Therefore, having a reliable animal model for embryonic hyperglycemia will facilitate efforts to better understand this condition with the goal of developing appropriate intervention and treatment strategies.

Second decision letter

MS ID#: JOCES/2021/259187

MS TITLE: Embryonic hyperglycemia perturbs the development of specific retinal cell types, including photoreceptors

AUTHORS: Kayla F. Titialii-Torres and Ann C Morris
ARTICLE TYPE: Research Article

Thank you for sending your manuscript to Journal of Cell Science through Review Commons.

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.