Manuscript ID: JoVE61179
Title: Acclimation prior to an intraperitoneal insulin tolerance test mitigates stress-induced hyperglycemia in conscious mice.

Response to Reviewers

Please note that changes within the manuscript are shown in red font.

Reviewer #1:

1. The blood sugar in control mice during fasting seems to be a bit high? Due to stress?

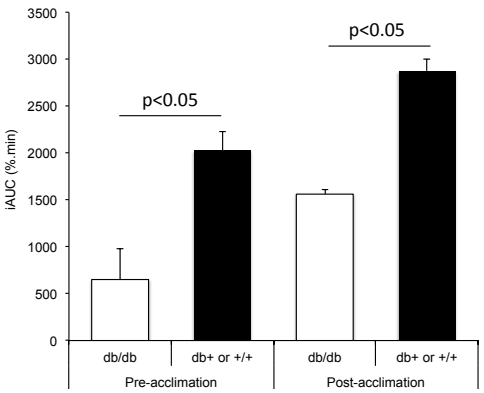
Our glucose data suggest fasted control mice experience some stress during handling prior to the insulin tolerance test (ITT). However, at the t=0 timepoint of the ITT, the blood glucose levels are lower than pre-ITT levels at -60 min (150±6 vs. 175±8 mg/dL; n=4, p=0.02), suggesting a reduced stress response following acclimation. These data are now highlighted in the results section of the manuscript. Also, in other studies from our lab where control mice of the same genotype are chronically catheterized and blood is sampled without handling (i.e. via the arterial catheter) in the conscious, unstressed state, we have found that blood glucose after a 6-hour fast is comparable to what is seen at t=0 in the present study (156±8 mg/dL, n=6). This suggests that if fasted control mice experience some stress, it is likely minimal after they have been acclimated to handling. While it is possible stress is not completely eliminated following acclimation, the relatively short duration of fasting (i.e. 3.5 hours) may be another reason fasting glucose levels are not lower than observed.

2. Statistics analysis results should indicate in the figure 3.

We have now performed statistics to determine whether the fall in glucose levels following insulin administration is significantly different between acclimated and non-acclimated mice. To do this, we compared the slopes from 0 to 15 minutes, since this 15-minute period most closely estimates insulin sensitivity, whereas counter-regulatory responses to hypoglycemia can occur beyond this timepoint. We found that the rate of glucose disappearance in the first 15 minutes is significantly different (p=0.002) between control mice that are acclimated and those that are not. In db/db mice, this is not the case, likely because (i) baseline glucose levels (at t=0) were highly variable amongst the four non-acclimated mice, and (ii) glucose levels had already decreased between t=-150 and -60 minutes in mice that underwent acclimation. The latter resulted in overall lower glucose levels during the ITT in acclimated versus non-acclimated db/db mice (p<0.05, 2-way ANOVA). Statistics have been described in the results section and added to the figure legends.

3. Insulin 2U/kg seems to be too strong for db/db because a possible "insulin resistance" only appeared within the first 15 min. If authors can demonstrate that this protocol provide a clear difference of insulin sensitivity between control and db/db mice, it will be very attractive for readers in the field of diabetes research.

Our study was not intended to compare glucose responses between control and db/db mice, but rather to demonstrate that acclimation of mice to handling can mitigate stress-induced hyperglycemia within a group of mice. That said, we have now performed statistical analysis to show that the glucose response to insulin (represented as inverse area under the curve, iAUC) is significantly different between control and db/db mice, regardless of whether they have undergone acclimation. We feel this is not surprising given that there is a large difference in insulin sensitivity between control and db/db mice. These data are shown below. Note that raw glucose data were first expressed as % of baseline, in order to adjust for the differing baseline glucose levels between control and db/db mice.



4. What time during a day is recommended for the protocol?
We recommend food withdrawal in the morning after the dark cycle has ended. Fasting time of day and duration should be consistent across groups of mice. For this protocol, food was removed between 0700 and 0800 hours, which is 1-2 hours after the light cycle begins. This information has been added to the protocol in the manuscript.

5. Can legends of each colored lines used in each Figures be added?
We have now added a legend to each figure to denote the different groups.

6. Strain of control mice in the demonstration?
The control mice were db/+ or +/+ littermates from Jackson Labs, where they are not genotyped to differentiate the two. We have now added this information to the Results section and figures.

Reviewer #2:
1. Interesting thought that corn cob bedding may be a carb source in mice. Is there a reference or any data to support this? If no, please soften the statement to conjecture.
The following reference has been added to support the statement regarding use of corncob bedding: Zahorsky-Reeves J, Castellani LW. Housing Mice on Corncob Bedding versus Hardwood Chip May Confound Research Models. Am Assoc Lab Anim Sci (10 October 2010). AALAS Scientific Session.
Also, data from our lab show that when compared to mice fasted overnight with CareFresh bedding, mice fasted overnight with corncob bedding have elevated glucose levels (CareFresh 163±12 vs. corncob 228±14 mg/dL; n=3, p=0.004). We have not added these data to the manuscript as they were derived from another cohort of C57BL/6 mice.

2. Section 2.2 it sounds like the tail is snipped while the mouse is suspended in the air. Is the mouse placed on a wire rack or other grippy surface? Please describe.
Mice are not suspended when the tail is snipped. They are placed on a flat tabletop surface. This information has been added to the protocol in the manuscript.

3. Line 234 in the discussion hyperglycemia should not be in quotes, it is real, just not due to the injected substance.
We agree and have now removed the quotation marks.

4. Line 241 in the discussion a failure of glucose levels to fall over the entire period may be more related to insulin dose or insulin resistance than stress, recommend soften that statement.
We have now edited the sentence to say: “Finally, an additional 10 studies failed to observe a decrease in glucose levels following insulin administration in db/db mice over the course of the ITT, though this may have been related to the dose of insulin used or a state of marked insulin resistance in the mice”.

5. Line 258 do you mean elevated above baseline?
Yes, we have now modified this statement for clarity.

6. Line 304 it's not really valid to say that this method more accurately reflects insulin sensitivity since gold standard wasn't measured. Perhaps change to more accurately reflects insulin action?
We agree and have now made the suggested change to the text.

Reviewer #3:
Figure 3 - Can the authors provide statistics to support the conclusion that the fall in glucose level between procedures are significant?
We have now performed statistics to determine whether the fall in glucose levels following insulin administration is significantly different between acclimated and non-acclimated mice. To do this, we compared the slopes from 0 to 15 minutes, since this 15-minute period most closely estimates insulin sensitivity, whereas counter-regulatory responses to hypoglycemia can occur beyond this timepoint. We found that the rate of

glucose disappearance in the first 15 minutes is significantly different ($p=0.002$) between control mice that are acclimated and those that are not. In db/db mice, this is not the case, likely because (i) baseline glucose levels (at t=0) were highly variable amongst the four non-acclimated mice, and (ii) glucose levels had already decreased between t=-150 and -60 minutes in mice that underwent acclimation. The latter resulted in overall lower glucose levels during the ITT in acclimated versus non-acclimated db/db mice ($p<0.05$, 2-way ANOVA). Statistics have been described in the results section and added to the figure legends.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have now proofread our manuscript to ensure there are no spelling or grammar issues.