We thank the reviewers…

Comments to the Author

Reviewer 1:  
This study investigates the impact of maternal time restricted feeding on metabolic outcomes in male and female offspring. This is an important question, however, there are a number of issues that need to be addressed:  
Major  
Minor

1. Page 2, line 7: Should be “TRE is currently thought to….”. In addition, check through the whole document to ensure consistency with the high fat diet abbreviation – is it high fat high sugar or a high fat diet?

**To be clearer, we changed all mentions of HFD to HFHS (high fat high sugar) diet in the manuscript and figures.**

**The word choice was updated as requested. Page 5, Lines101-102. Revised text below:**

**“Detailed modeling of TRF in pregnancy is warranted, as TRE is currently thought to exist in human populations (Ali & Kunugi, 2020; Flanagan et al., 2022) yet, long-term effects are unknown.”**

1. Page 2, line 8: Should be “Recent work demonstrated up to 23.7% of a pregnant cohort…….. However, there is no information on the long-term implications of this diet on the progeny”

**We agree, so updated page 5, Line 87-89:**

**“Recent work demonstrated that up to 23.7% of a pregnant and recently post-partum cohort said they were willing to try TRE during pregnancy (Flanagan et al., 2022). However, there is currently no information on the long-term implications of this dietary strategy for progeny.”**

1. Page 2, lines 14-17: Make it clear these studies are in mice.

**We included species in the description of those TRF studies during pregnancy and whether they were done on mice or humans. In the interim there was also was a second manuscript that was published since we submitted our work, which we thought was relevant so the text was updated. On page 6 line 123-131**

**“To date, two studies of TRF during pregnancy in animals exist. The first emphasized fetal health and was completed in the context of preventing complications from overnutrition (a high fat, high sucrose diet, HFHS) during gestation in a rat model. Upadhyay and colleagues found that 9-hour TRF improved fetal lung development (Upadhyay et al., 2020) and placental oxidative stress markers (Upadhyay et al., 2019) at embryonic day (E)18.5 compared to ad libitum fed dams. This approach did not evaluate the long-term, postnatal effects of TRF and the independent effects of TRF are complicated by the use of a high fat, high sucrose diet. The second, also in rats, found altered glucose metabolism in adult offspring of TRF fed dams** (Prates et al., 2022)**. However, this study compared 12 hr feeding to ad libitum feeding in pregnancy, leaving more restrictive windows unexamined.”**

1. In the answer to study importance the authors state “We see glucose intolerance in adult males fed on a HFD” but in the abstract the authors state “…and improved glucose tolerance in males” (Page 3, lines 24-25).

**Thank you for pointing this out. This section describes the modest effect of gestational TRF in males before being exposed to HFHS feeding. To clarify, the language has been modified on page 3 line 27-30**

**“Body composition was similar between groups in both sexes from weaning to adulthood, with minor increases in food intake in eTRF females and improved glucose tolerance in males. After 10 weeks of high fat, high sucrose diet, male eTRF offspring developed glucose intolerance.”**

1. On page 2 the authors state a high fat diet was used whereas in the abstract the authors state a high fat high sucrose diet was used. There needs to be consistency.  
   The final sentence of the abstract doesn’t make sense. Why would you look at the male pancreas to elucidate the mechanisms that protect females. The same can be said for the conclusion in the main paper.

**The obesogenic diet used in our study is best described as a high-fat, high-sucrose diet. We describe it this way to be consistent with other studies in the lab comparing it to ketogenic diets, which are just high in fat. We have changed the language to be consistent throughout the manuscript and the figures.**

**The last line was meant to describe 2 future directions, one for male pancreatic tissue and another to understand the protective factors for females. The reviewer also brings up an excellent point, whether we are looking at a sensitivity in males or a protection in females. We have incorporated this insight throughout, but to this specific point the language has been changed to more clearly define them as separate goals. On page 3 Line 31-33 we now state:**

**“Further studies in both sexes are needed to determine the effect gestational eTRF has on the insulin secretion and glycemic health in males and to understand why females are protected.”**

**In the conclusion, we added the additional language about chow feeding to delineate the distinct effect between the two diets on page 18 line 436-438:**

**“Offspring who are exposed to eTRF of NCD *in utero* have similar body composition, glucose tolerance, and insulin tolerance in early adulthood with normal chow feeding in both males and females.”**

# introduction

1. The first paragraph should probably provide a brief description of the circadian system and its involvement in the alignment of metabolic processes. This should be followed by the role of the timing of food intake as a zeitgeber.

**A paragraph describing the internal clock system and food as a zeitgeber is now included on page 4 Line 50-60:**

**“All mammals have cell-autonomous clocks that coordinate the 24-hour rhythm of metabolism. The clock consists of the CLOCK:BMAL1 heterodimer that binds to regulatory elements in DNA (E boxes), among them are its own repressors cryptochrome (1 & 2) and period (1-3) (Lee et al., 2001). The nuclear hormone receptors ROR(α, β, and γ) and REV-ERB (α and β) activate or repress expression of BMAL1 respectively (Panda, 2016; Takahashi, 2017). This highly coordinated transcription factor system entrains circadian rhythm in the central clock, the suprachiasmatic nucleus (SCN) of the brain, according to external cues. Peripheral tissues also possess internal clocks that can be entrained. This system is imparts a rhythm of metabolism, programming predominance of melatonin during the night hours and cortisol/corticosterone during early waking hours (Panda, 2016). Factors capable of manipulating, or entraining, this system are called zeitgebers (ZT). One such potent ZT is food intake(Pickel & Sung, 2020).”**

1. The second paragraph of the introduction needs more structure and the last sentence is meaningless without examples.

**We appreciate this suggestion and have updated the language to transition more naturally between topics in this paragraph on page 4 line 67-page 5 line 86 to now read:**

**“To our knowledge, no estimate of the prevalence of TRE in humans exists. However, according to one sample, up to ten percent of people surveyed that stated they followed a diet in the year 2020 had attempted “intermittent fasting,” making it the most prevalent dietary intervention in that sample (International Food Information Council, 2020). There are critical periods of development in the lifespan where changes to dietary behaviors can impact health current and future status. One such critical period is pregnancy. During pregnancy, habitual timing of food intake may be altered for many reasons: religious practice, food insecurity, disordered eating behaviors, nausea and vomiting of pregnancy/morning sickness, changes in taste/food preferences, or intentional timing of eating for weight maintenance. Very little research has evaluated the timing of eating during pregnancy and its impact on offspring health. One cross-sectional analysis found that extending the overnight fast during pregnancy was associated with lower blood glucose levels at mid gestation(Loy et al., 2017).”**

1. Perhaps with the Ramadan example the authors should also point out that the food consumption is out of phase with normal feeding behaviour.

**We have now included clarification about the phase-shifted eating of Ramadan on page 5 Line 92-96 of the revised manuscript:**

**“In a recent review, Ramadan exposure in utero was associated with smaller body size and stature in later periods of life (Oosterwijk et al., 2021). However, these studies are limited and Ramadan fasting is an imperfect model for TRF, as food intake is not only limited in duration but also not permitted during the normal active phase for humans.”**

1. The authors need to be more specific/precise throughout the manuscript, such as naming the animals used in the studies e.g. page 5, line 22 “ad libitum fed what dams”

**All dams were fed a normal chow diet in this study. This is indicated in the revised methods section on page 7 lines 161-164**

**“Dams fed AL had 24-hour access to a chow diet (NCD, Picolab Laboratory Rodent diet, 5L0D; 5% of Calories from fat, 24% from protein, 71% from carbohydrates). Dams fed eTRF had 6 hours of NCD food access during the early dark cycle (ZT 14-ZT 20).”**

Methods:

1. Zeitgeber should be abbreviated to ZT the first time it is used in the introduction.

**Added on page 4 lines 63-64:**

**“Factors capable of manipulating, or entraining, this system are called zeitgebers (ZT).”**

1. Page XX, Line XX, Is the GTT an intraperitoneal GTT or an oral glucose tolerance test. Please go through the methods and ensure that all drug administrations are reported. Should the order of the ITT and GTT have been randomised to negate possible anticipatory stress in the second procedure?

**This was an intraperitoneal GTT as noted in the revised methods section on page XX, line YY:**  
  
  
**We completed the tests in the same order for both cohorts, first ITT, then GTT as noted in the manuscript on page XX, line YY. The randomization is an interesting issue, but if there is anticipatory stress we wanted to normalize this as well, so we were intentional about all mice being exposed to measures in the same consistent order.**

Results:

1. In the first paragraph of the results section the authors state the eTRF is 50% of their active nocturnal window. This is for non-pregnant mice, is this true for pregnant mice. In addition, this statement should be referenced. In addition, is this early TRF in pregnant mice? Do we know what meal patterns are in pregnant mice?

**This is an interesting point we hadn’t previously considered. Ladyman, Carter, and Grattam (Ladyman et al., 2018) evaluated the effect of pregnancy on food intake and ambulatory activity in mice. This study compared age-matched pregnant and non-pregnant female mice of the same strain used in the current study (C57/BL6J). Meal events and duration of meals increased in pregnant dams, but percent of food intake consumed during the light and the dark cycle remained similar between pregnant and non-pregnant females. However, they did not report food intake hourly. To clarify the context of the experiment, we eliminated the language about the active window in the manuscript. On page 10 line 231-233:**

**“To model gestational early time restricted feeding (eTRF), we used a normal chow diet (NCD) and assigned female mice to either unrestricted (*ad libitum,* AL) or 6 hours of restricted food availability between ZT14-20 (eTRF) (Figure 1A). ”**

1. It is important to understand the energy intake in the dams. Did eTRF eat less than the ad libitum or did they compensate when food was available and consume the same energy as the ad libitum fed dams?

**We have carefully measured the effect of this intervention on the dams. It is intended for a separate publication after a series of replication experiments focusing on maternal physiology and fertility. These are currently underway, but are the subject of a separate manuscript . We have found in these previous cohorts that the 6h window is sufficient for similar daily kcal intake between AL and eTRF dams and that body weights remain similar to AL dams before, during, and after pregnancy (see Figure 1 of this response).**



1. In the results section if something is not significant then just state there is no difference. For example, Page 11, line 52-52 should be “…where there was no difference in the AUC between eTRF and AL female mice but ~20% lower AUC for eTRF meals compared to AL male offspring…”. Please change the results section accordingly.

**We respectfully disagree that noting near significant differences adds no value, so we have chosen to describe statistical analyses for some key near-significant differences though with more care about the interpretation of these results. For example, it is our view that an animal with significantly impaired intraperitoneal glucose intolerance, but unimpaired insulin sensitivity (via an ITT) is highly likely to have defects in insulin secretion. We show this data for only a subset of mice, as we only performed this experiment for the second cohort. Therefore, we agree that insulin secretion data is less robust standing alone, but quite strong in the context of the GTT and ITT. We have clarified that point in the revised manuscript. In terms of which comparisons, we have are now more clear about the comparisons. On page 13 line 293-294 we now state:**

**“These findings were confirmed by calculating the AUC where eTRF females no difference in AUC compared to AL females (Figure 3F, pdiet=0.20) while eTRF males had 20.4% lower AUC than AL males (pdiet<0.0001)”**

Discussion.

1. The insulin secretion studies are inconclusive and not significant and therefore you can’t make bold statements in the conclusion that the impaired glucose tolerance in HFD conditions is due to impaired insulin secretion. In addition, it is not clear where you obtained Figure 3K from because it doesn’t seem to reflect the results in Fig 3J.

**We have softened the language to reflect the inconclusive nature of this study. We did complete. We believe the fold change figures shows no difference because the baseline values for eTRF offspring were considerably lower than they were for the AL offspring. We included the table of showing all values below (2 were below the limit of detection for the assay, this resulted in those observations being omitted from the analysis).**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Table 1: Insulin Values, *In Vivo* GSIS** | | | | |
| **ID** | **time (mins)** | **maternal diet** | **sex** | **Insulin (ng/mL)\*** |
| 453 | 0 | eTRF | male | 2.98 |
| 456 | 0 | AL | male | 5.12 |
| 464 | 0 | eTRF | male | 7.39 |
| 465 | 0 | eTRF | male | 2.49 |
| 454 | 0 | eTRF | female | 0.49 |
| 455 | 0 | eTRF | female | 1.48 |
| 466 | 0 | eTRF | female | 0.49 |
| 467 | 0 | eTRF | female | 0.14 |
| 453 | 15 | eTRF | male | 4.00 |
| 464 | 15 | eTRF | male | 5.47 |
| 465 | 15 | eTRF | male | 4.89 |
| 452 | 15 | eTRF | male | 2.61 |
| 454 | 15 | eTRF | female | 1.51 |
| 455 | 15 | eTRF | female | 2.94 |
| 466 | 15 | eTRF | female | 1.16 |
| 467 | 15 | eTRF | female | 0.55 |
| 460 | 0 | AL | male | 9.68 |
| 461 | 0 | AL | male | 4.97 |
| 468 | 0 | AL | male | 3.60 |
| 469 | 0 | AL | male | 4.03 |
| 458 | 0 | AL | female | 0.49 |
| 459 | 0 | AL | female | 0.61 |
| 462 | 0 | AL | female | 0.49 |
| 463 | 0 | AL | female | 0.82 |
| 470 | 0 | AL | female | NA |
| 471 | 0 | AL | female | 0.14 |
| 473 | 0 | AL | female | 3.33 |
| 474 | 0 | AL | female | 1.64 |
| 460 | 15 | AL | male | 14.81 |
| 461 | 15 | AL | male | 4.34 |
| 468 | 15 | AL | male | 7.42 |
| 456 | 15 | AL | male | 6.95 |
| 458 | 15 | AL | female | 1.08 |
| 459 | 15 | AL | female | 3.21 |
| 462 | 15 | AL | female | NA |
| 463 | 15 | AL | female | 1.54 |
| 471 | 15 | AL | female | 1.00 |
| 473 | 15 | AL | female | 6.97 |
| 474 | 15 | AL | female | 1.61 |
| \*values measured in 5uL of serum via a commercially available ELISA kit (ALPCO 80-INSMSU-E10) | | | | |
| Sample size 4 eTRF male, 4 eTRF females, 5 AL males, 7 AL females | | | | |

1. The huge issue with this paper is the low n numbers (n=4 eTRF males and n=4 eTRF females and only n=5 for the AL groups). This is simply not enough and has led to inconclusive findings.

**We apologize for this lack of clarity. As noted in the revised manuscript, this study utilized two entirely distinct cohorts of mice treated similarly. Phenotypes were robustly replicable across both cohorts. We have clarified this in the manuscript. The study included many animals for the body composition, food intake, (eTRF males = 11, eTRF females = 19, AL males = 16, eTRF females = 17). We view that this is a reasonable number of animals for almost every outcome of the study. The only experiment that had lower n was the *in vivo* glucose stimulated insulin secretion in figures 3J and 3K. As we note in the revised manuscript, we had predicted the phenotype through the analysis of the GTT and ITT in the first two cohorts and wanted to characterize it further in the second cohort by a somewhat more exploratory analysis of insulin secretion. We agree that these insulin secretion results, standing alone are somewhat lest robust than the other experiments we report, but want to be clear that the majority of the data is n>11/group and replicable across cohorts. Repeating the insulin secretion studies would take >8 months and in our view add little to this initial report. We look forward to further characterization of this novel sex-specific developmental phenotype, including mechanistic studies noted by reviewer 2 in a future manuscript.**

**Page 7 Line 171, “Results are shown for 2 cohorts of animals that have been combined.”**

**Explaining lower n for in vivo GSIS, page XX Line 317**

**“After noticing a consistent trend in both cohorts of eTRF males developing glucose intolerance after HFHS diet exposure, we sought to assess cohort 2 more closely for insulin secretion defects, via an *in vivo* glucose stimulated insulin secretion (GSIS) assay (Figure 3J).”**

# Reviewer 2

In this manuscript, Mulcahy et al explore the consequence of an early time-restricted

feeding regimen (eTRF) during gestation on the offspring’s glucose homeostasis.

Overall, their results show that no differences were observed when the male and female

offspring were later fed a chow diet, whereas glucose intolerance and increased insulin

sensitivity were observed in the male offspring only when fed a high-fat high-sucrose

(HFHS) diet.

The question of the effect of maternal feeding on metabolic risk of the offspring in later

life addressed in this manuscript is deemed highly relevant. Indeed, studies in the

context of dam malnutrition or metabolic dysfunctions management during gestation

have highlighted a spectrum of deleterious long-term consequences in the offspring’s,

which are important to consider when estimating the risks/benefits ratio of the

therapeutic intervention (e.g. metformin/insulin treatment during pregnancy with

gestational diabetes) and can inform diet recommendation for the offspring. Additionally,

this study investigating the effect of TRF during pregnancy is novel since only one study

has looked into the effect of TRF during gestation in a different nutritional context and

did not look at long-term consequences in the offspring.

Unfortunately, the study falls short in providing convincing results of the effect of

gestational eTRF. Overall, although the results are interesting, it is the reviewer’s opinion

that the conclusions are mostly overstated and that further major experimental evidence

are required to illuminate the mechanisms by which glucose intolerance in the males

offspring might be happening and confirm the overall reproducibility of the results. In

addition, it is unclear why so much of the emphasis of the manuscript is made on the

potential sex- and diet-specific deleterious effect when other data seems to support the

safety of the intervention opening a route to testing TRF in the context of maternal

obesity/gestational diabetes which seems like a more translationally relevant question.

Specific comments are as follow:

1. Male’s offspring fed HFHS “developed glucose intolerance” (title), “with impaired insulin

secretion” (abstract l28-30, results p13 l6-8, discussion)

As pointed out in the discussion of the manuscript, the fact that the males showed

glucose intolerance in a GTT associated with insulin-sensitivity and a non significant

trend towards lower insulin secretion in a GSIS assay supports the idea that they might

have a defect in insulin secretion. However, this claim, that is the only one conveyed in

the title and discussed extensively in almost 2 pages of the discussion, remains mostly

speculative and needs to be substantiated by additional experiments such as:

- Conclusive GSIS assay: the defect in insulin secretion is entirely based on a

non-significant trend. These measures are highly variable and a trend is not

dimmed sufficient to support the major conclusion of this manuscript.

Additionally, Fig 3K shows a significant difference between males eTRF and

AL with significantly higher fold chance in insulin response which contradicts

the interpretation of the results. Please clarify .

- Insulin secretion in response to other substrate (e.g. arginine TT)

- Islet size and pancreatic beta cell mass quantification

The results from these experiments would also shed light on the mechanisms behind the

sexually dimorphic response observed in which the female’s offspring are not affected by

gestational eTRF.

**These are all excellent suggestions, and we appreciate how these studies could further inform what is a novel and un-reported phenotype. Our view is that this is the first rigorous study of eTRF on long term metabolic health. Using a high number of animals across multiple cohorts we strongly believe that the lack of metabolic abnormalities in general, aside from HFD-induced sex-specific glucose intolerance is an important advance. We agree that the mechanism of susceptibility in males (or resilience in females) warrants further study at the physiological, molecular and epigenetic level, but believe those are beyond the scope of the first report of this phenotype. We look forward to these future studies which will be explicitly designed to evaluate islet biology across the lifespan, but these will take several years to complete. What we can share is that in the animals that underwent GSIS, we also conducted an *in vitro* GSIS(See figure below). However, the results had high levels of inter-replicate variability. As noted above in the response to reviewer 1, comment 16 we only identified the potential insulin secretion differences after confirmation of impaired glucose tolerance in the context of unimpaired insulin sensitivity in HFD-fed males after the second cohort of mice. As such the GSIS which had a smaller number of animals is less robust, and the reviewers are correct that we should be more cautious of these interpretations. The language in the revised manuscript has been altered to be less definitive toward an islet specific defect, for example XXX. Furthermore, since submitting our work, another group has recaptiulated our phenotype in rats using a chow-fed TRF during gestation model. They conducted further pancreatic studies, including the in vitro GSIS, and found what we speculate to be consistent with their work. We altered the language to be less definite and included much more discussion of the other paper in the discussion on page 14 line 323-334:**

**“This study is the second to describe the long-term effects of gestational eTRF on offspring health and the first to describe their response to a high fat, high sucrose diet challenge in adulthood. We find minimal effects associated with eTRF during gestation while male and female offspring are consuming a chow diet. However, after prolonged HFHS diet feeding, there are significant deleterious effects of gestational eTRF on glucose tolerance only in adult male progeny. Although inconclusive, we proposed based on provisional GSIS data, that differences in insulin secretion may exist in eTRF males compared to their AL counterparts. Whether this is due to sensitivity of males or resilience in females is not clear. A recent study of gestational TRF of chow diet in rats also found evidence of glucose intolerance and insulin sensitivity in the offspring (Prates et al., 2022). There were however some key distinctions. First, these effects *in vivo* were found only in female offspring instead of in males as in the current study. Furthermore, islets collected from adult male offspring of TRF fed dams had impaired glucose-stimulated insulin (Prates et al., 2022). The similarities to the current study demonstrate that changes in islet programming may be a likely mechanism for metabolic disruption from gestational TRF.”**

**Further, on page 16 lines 385-394 we now now clarify:**

**“Given that negative effects are not seen until a HFHS diet challenge occurs in adulthood for the offspring, this suggests that gestational eTRF may be relatively safe to practice in the context of a healthful diet. However, it also demonstrates that in the context of unhealthy diet patterns, adult offspring may be ill-equipped to adapt to their food environments, leading to metabolic dysfunction. Furthermore, the age of onset and stressors that are required to initiate glucose intolerance in offspring of TRF dams are not clearly defined in this study, and translation to human clinical populations would be difficult at this stage. The similarity of the present study to those using diverse gestational stressors suggests that restriction of the total time spent eating in dams is a novel dietary component that may have lasting impact on the metabolic health of offspring.”**



In Vitro GSIS Results

1. Of importance, these additional experiments will also test whether the described results

are reproducible across at least 2 different animal cohorts, which is dimmed critical to

support the results of the study at this point.

**As both reviewers noted this critique, we agree that we were unclear in the design description. This study was completed in two entirely independent cohorts of animals, and the lack of differences in the chow phase and the male-specific glucose intolerance was present in both cohorts of mice. The methods section has been updated to reflect that this was a multiple cohort study on page 7 line 161163:**

**“This study was completed in two independent cohorts of animals. The phenotypes noted in offspring were highly consistent between cohorts and including cohort as a covariate in analyses did not materially alter the results. Therefore, data shown is the combined total from cohorts one and two and statistical tests do not include effects of cohort in the model.”**

1. The manuscript would benefit from a characterization of the effect of eTRF on the dam

during gestation. A lack of evidence on how TRF affects the dam during pregnancy

makes it difficult to ascertain whether the effects on the offspring are a result of caloric

restriction, time restricted feeding, or a host of other side-effects that may have occurred

from the intervention.

**We have tested two independent cohorts to this dietary intervention. We are still evaluating the effect on the dams, but our last 2 cohorts have shown that food intake is comparable and weight gain over pregnancy is similar between eTRF and AL dams (Figure 1 of this response). This suggests the intervention does not induce caloric restriction during pregnancy in our model. For some data, please see comments to reviewer 1, item #13.**

# Rationale & design:

1. A very strong point is made about eTRF during gestation as a model of feeding

disruption observed during pregnancy (abstract l12-19, introduction p4 l50-53, p5 l52-

540). There are several reasons why the reviewer respectfully disagrees with this

statement, amongst which the idea that adhering to a rigorous short daily feeding

interval can represent disrupted gestational eating behavior characterized by changes in

food preferences and tolerability. In addition, in most cases, whether in rodents or

human studies, TRF/TRE has been studied in the context of obesity and metabolic

disease. The effect of early and short 6h eTRF of normal chow in female rodents itself is

unknown to the best of the reviewer’s knowledge.

**The literature is inconsistent in the total time spend fasting vs eating for TRF. It often ranges from 4-12 hours of eating in both human and animal studies. Although we agree that 6 hour is on the more restrictive side, it is still within the range seen in the literature. When we designed this study we felt that this was a reasonable starting point. Another key advantage of our approach is that unlike some studies, our restriction is during their normal feeding cycle not during the daytime. As it pertains to disrupted feeding in pregnancy, there is now evidence, albeit limited, that pregnant women appear to adopt this practice (Ali & Kunugi, 2020; Flanagan et al., 2022). We also find that the idea of doing this in a pre-existing obese mouse model is interesting, but is an entirely different study. We decided against doing so because we wanted to understand what the independent effects of TRF were outside of the context of metabolic disease and weight loss as a first step. As we describe in this manuscript many phenotypes including virtually all metabolic measures in female offspring are not different in spite of a relatively aggressive feeding restriction *in utero* a finding as surprising to us as it is to the reviewers.**

Thus, in the reviewer’s opinion, there

is a missed opportunity to study the effect on the dams as well as the impact of the

intervention in the context of diet-induced obesity and/or metabolic disease in the dams

since, in the reviewers opinion, the idea that (1) healthy pregnant women would

deliberately restrict their eating window to 6 hours daily for the duration of the pregnancy

– a very restrictive intervention - or that (2) this model can recapitulate some aspects of

eating disruption associated with pregnancy is farfetched.

**As referenced in the previous comment, we agree that although the 6h time restriction is relatively narrow (reviewer 1, comment 13), and there is evidence that TRF happens in pregnant women. As of yet, there are no known rigorous studies that look at this in human beings. Furthermore, identifying and following offspring of fasted pregnant people over decades while controlling for diet, genetics and environment would be difficult to impossible. In order to begin to study this in humans, an understanding of the basic safety of this practice must be ascertained. This is why we chose to study this model in mice, to begin to assess the safety of this intervention. We agree that 6 hours is not the norm in terms of restriction. However, but the range of 4-12 hours is normal in the literature and our 6h intervention fits within that range. Again, a separate observational study that we are conducting will follow time restricted feeding in pregnant people. While this study is in its initial phases, we find that restriction is XXXX. Six hour feeding periods have been used in previous TRF studies in both humans (Cienfuegos et al., 2020; Hutchison et al., 2019; Jamshed et al., 2019; Ravussin et al., 2019; Sutton et al., 2018) and animals (Boucsein et al., 2019; García-Gaytán et al., 2020). Again, we would like to note that we had the extra rigor of a high number of animals, longitudinal evaluation, and restriction only during the dark cycle. We feel that this indicates that this first of its kind report will be important as this field continues to develop.**

# Conclusion:

1. The conclusion is essentially entirely focused on the effect of gestational eTRF being

similar to intrauterine growth restriction (IUGR). Again, we respectfully disagree as the

evidence provided are too weak to make such a strong comparison. A quick review of

the literature cited sieems to suggest that IUGR usually leads to low birth weight and

differences in fat content that is not observed here and that usually glucose and insulin

intolerance go hand by hand.

**In the literature, there are often phenotypes that only arise in adulthood and do not have lowered birth weight. In mild models of restriction there is no effect on birthweight. However, given that the metabolic disruption is only evident after additional stressors (HFHS diet), we have edited the language to make the conclusions much more mild. On page 18 lines 436-438 of the revised manuscript we note:**

**“Offspring who are exposed to eTRF of NCD *in utero* have similar body composition, glucose tolerance, and insulin tolerance in early adulthood with normal chow feeding in both males and females. Gestational eTRF led to sex-specific impairments in male glucose tolerance in adulthood after chronic HFHS feeding. This occurs without increase in body weight, fat mass, or food intake compared to age matched AL males. More research is warranted to understand the mechanisms that underlie this novel phenotype.”**

Minor:

1. Result section 1 title: “Gestational eTRF increases food intake, but not body

composition in early life”

**Updated on page 10 line 230** :

**“Gestational eTRF increases food intake, but not body weight in early life.”**

1. Result section 3: what is meant by “overnutrition challenge” ?

**By overnutrition challenge we mean diets that exceed recommended levels of energy. We use this term to refer to our HFHS diet. As we now note in the revised manuscript this diet caused a XXX% increase in caloric intake. This was done as animals on NCD alone had modest phenotypes, but we were only able to elicit glucose intolerance in males via this challenge.**

1. Statistics: sounds very elaborate and cool but n per group still very low.

**We appreciate the recognition of the care we have put into the design and statistical approaches into this study. As we noted above to this point from reviewer 1 (comment 16) the sample size for the vast majority of experiments is >11 per group. Upon review, we see that this was not clear in the previous version, so we have clarified this in the revised manuscript. As noted above, the only exception to this is the *In vivo* GSIS, which we have now described as a provisional confirmation.**

**References**

Ali, A. M., & Kunugi, H. (2020). Intermittent Fasting, Dietary Modifications, and Exercise for the Control of Gestational Diabetes and Maternal Mood Dysregulation: A Review and a Case Report. *International Journal of Environmental Research and Public Health*, *17*(24), 9379. https://doi.org/10.3390/ijerph17249379

Boucsein, A., Rizwan, M. Z., & Tups, A. (2019). Hypothalamic leptin sensitivity and health benefits of time-restricted feeding are dependent on the time of day in male mice. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, *33*(11), 12175–12187. https://doi.org/10.1096/fj.201901004R

Cienfuegos, S., Gabel, K., Kalam, F., Ezpeleta, M., Wiseman, E., Pavlou, V., Lin, S., Oliveira, M. L., & Varady, K. A. (2020). Effects of 4- and 6-h Time-Restricted Feeding on Weight and Cardiometabolic Health: A Randomized Controlled Trial in Adults with Obesity. *Cell Metabolism*, *32*(3), 366-378.e3. https://doi.org/10.1016/j.cmet.2020.06.018

Flanagan, E. W., Kebbe, M., Sparks, J. R., & Redman, L. M. (2022). Assessment of Eating Behaviors and Perceptions of Time-Restricted Eating During Pregnancy. *The Journal of Nutrition*, *152*(2), 475–483. https://doi.org/10.1093/jn/nxab397

García-Gaytán, A. C., Miranda-Anaya, M., Turrubiate, I., López-De Portugal, L., Bocanegra-Botello, G. N., López-Islas, A., Díaz-Muñoz, M., & Méndez, I. (2020). Synchronization of the circadian clock by time-restricted feeding with progressive increasing calorie intake. Resemblances and differences regarding a sustained hypocaloric restriction. *Scientific Reports*, *10*. https://doi.org/10.1038/s41598-020-66538-0

Hutchison, A. T., Regmi, P., Manoogian, E. N. C., Fleischer, J. G., Wittert, G. A., Panda, S., & Heilbronn, L. K. (2019). Time-Restricted Feeding Improves Glucose Tolerance in Men at Risk for Type 2 Diabetes: A Randomized Crossover Trial. *Obesity*, *27*(5), 724–732. https://doi.org/10.1002/oby.22449

International Food Information Council. (2020). *2020 Food & Health Survey*. https://foodinsight.org/2020-food-and-health-survey/

Jamshed, H., Beyl, R. A., Della Manna, D. L., Yang, E. S., Ravussin, E., & Peterson, C. M. (2019). Early Time-Restricted Feeding Improves 24-Hour Glucose Levels and Affects Markers of the Circadian Clock, Aging, and Autophagy in Humans. *Nutrients*, *11*(6), 1234. https://doi.org/10.3390/nu11061234

Ladyman, S. R., Carter, K. M., & Grattan, D. R. (2018). Energy homeostasis and running wheel activity during pregnancy in the mouse. *Physiology & Behavior*, *194*, 83–94. https://doi.org/10.1016/j.physbeh.2018.05.002

Lee, C., Etchegaray, J.-P., Cagampang, F. R. A., Loudon, A. S. I., & Reppert, S. M. (2001). Posttranslational Mechanisms Regulate the Mammalian Circadian Clock. *Cell*, *107*(7), 855–867. https://doi.org/10.1016/S0092-8674(01)00610-9

Loy, S. L., Chan, J. K. Y., Wee, P. H., Colega, M. T., Cheung, Y. B., Godfrey, K. M., Kwek, K., Saw, S. M., Chong, Y.-S., Natarajan, P., Müller-Riemenschneider, F., Lek, N., Chong, M. F.-F., & Yap, F. (2017). Maternal Circadian Eating Time and Frequency Are Associated with Blood Glucose Concentrations during Pregnancy. *The Journal of Nutrition*, *147*(1), 70–77. https://doi.org/10.3945/jn.116.239392

Oosterwijk, V. N. L., Molenaar, J. M., van Bilsen, L. A., & Kiefte-de Jong, J. C. (2021). Ramadan Fasting during Pregnancy and Health Outcomes in Offspring: A Systematic Review. *Nutrients*, *13*(10), 3450. https://doi.org/10.3390/nu13103450

Panda, S. (2016). Circadian physiology of metabolism. *Science (New York, N.Y.)*, *354*(6315), 1008–1015. https://doi.org/10.1126/science.aah4967

Pickel, L., & Sung, H.-K. (2020). Feeding Rhythms and the Circadian Regulation of Metabolism. *Frontiers in Nutrition*, *7*. https://www.frontiersin.org/article/10.3389/fnut.2020.00039

Prates, K. V., Pavanello, A., Gongora, A. B., Moreira, V. M., de Moraes, A. M. P., Rigo, K. P., Vieira, E., & Mathias, P. C. de F. (2022). Time-restricted feeding during embryonic development leads to metabolic dysfunction in adult rat offspring. *Nutrition*, 111776. https://doi.org/10.1016/j.nut.2022.111776

Ravussin, E., Beyl, R. A., Poggiogalle, E., Hsia, D. S., & Peterson, C. M. (2019). Early Time-Restricted Feeding Reduces Appetite and Increases Fat Oxidation But Does Not Affect Energy Expenditure in Humans. *Obesity*, *27*(8), 1244–1254. https://doi.org/10.1002/oby.22518

Sutton, E. F., Beyl, R., Early, K. S., Cefalu, W. T., Ravussin, E., & Peterson, C. M. (2018). Early Time-Restricted Feeding Improves Insulin Sensitivity, Blood Pressure, and Oxidative Stress Even without Weight Loss in Men with Prediabetes. *Cell Metabolism*, *27*(6), 1212-1221.e3. https://doi.org/10.1016/j.cmet.2018.04.010

Takahashi, J. S. (2017). Transcriptional architecture of the mammalian circadian clock. *Nature Reviews Genetics*, *18*(3), 164–179. https://doi.org/10.1038/nrg.2016.150

Upadhyay, A., Anjum, B., Godbole, N. M., Rajak, S., Shukla, P., Tiwari, S., Sinha, R. A., & Godbole, M. M. (2019). Time-restricted feeding reduces high-fat diet associated placental inflammation and limits adverse effects on fetal organ development. *Biochemical and Biophysical Research Communications*, *514*(2), 415–421. https://doi.org/10.1016/j.bbrc.2019.04.154

Upadhyay, A., Sinha, R. A., Kumar, A., & Godbole, M. M. (2020). Time-restricted feeding ameliorates maternal high-fat diet-induced fetal lung injury. *Experimental and Molecular Pathology*, *114*, 104413. https://doi.org/10.1016/j.yexmp.2020.104413