We thank the reviewers and editors for their thoughtful consideration of this work. These insights have been invaluable as we have revised our manuscript and we truly appreciate your help in this process. The major changes we have made in this revised manuscript are to clarify the sample size of our study (an n of 11-17/group for most experiments, including a replication cohort), consider more thoughtfully the lack of many phenotypes for a fairly robust dietary restriction, and to include caveats to our provisional conclusion that sex-specific islet function under HFD conditions underlies the major phenotype observed, and more careful consideration in framing the phenotype as it could be a result of male being sensitive to the exposure or females being resilient. We now provide additional new data both in the main manuscript and in the supplementary figures, a better description of our methods and rationale for studying this dietary exposure in the given model system, and an updated discussion. Specific points addressed are noted below.

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?

**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 2, Lines 4-5. Revised text below:**

**“TRE currently is thought to improve metabolism, even in some cases without weight loss.”**

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 69-71:**

**“Another recent work demonstrated that up to 23.7% of a human pregnant and recently post-partum cohort said they would be willing to try TRE during pregnancy (1). 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 87-99**

**“At the time of this manuscript, two studies of TRF during pregnancy in rodents exist. The first emphasized fetal health and was completed in the context of preventing complications from 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 (2) and placental oxidative stress markers (3) 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, evaluated 12 hour access in light and dark cycles to a chow diet during pregnancy and followed male and female resultant offspring until 150 days of age (4). Adult female offspring of dams fed in the dark cycle with TRF were found to be glucose intolerant *in vivo*, and reduced glucose stimulated glucose secretion *in vitro* in both male and female offspring islets. altered glucose metabolism in adult offspring of TRF fed dams (4). However, this study compared 12 hour 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 33-36**

**“Body composition was similar between groups in both sexes from weaning to adulthood, with minor increases in food intake in eTRF females and slightly 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 It contains 45% of energy from fat, 20% from protein, and 35% from carbohydrate compared to our (For reference, our chow is 5% of Calories from fat, 24% from protein, 71% from carbohydrates). 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. We also have worked to change the language of the manuscript to focus less on sex-specific phenotype, as the reviewer pointed out that we are not sure if it is resilience in females or responsiveness in males. HFHS feeding is expected to cause some metabolic and adiposity changes in adult mice, most notably an increase in fat mass. We see that both groups in each sex increase fat mass and food intake, but in a comparable way.**

**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 36-38 we now state:**

**“Further Studies should assess the susceptibility of males, and apparent resilience of females, to gestational eTRF related changes in islet physiology and HFHS diet in adulthood.”**

**In the conclusion, we added the additional language about chow feeding to delineate the distinct effect between the two diets on page 20 line 420-421:**

**“Offspring who are exposed to eTRF of NCD *in utero* have similar body composition, glucose tolerance, and insulin tolerance in early adulthood 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 41-51:**

**“All mammals have cell-autonomous clocks that coordinate the rhythm of metabolism. The molecular 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) (5). The nuclear hormone receptors ROR(α, β, and γ) and REV-ERB (α and β) activate or repress expression of BMAL1 respectively (6, 7). 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 (6). Factors capable of manipulating, or entraining, this system are called zeitgebers. One such potent zeitgeber is food intake (8).”**

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, and were more specific about the examples of outcomes from Ramadan-related literature on page 4 line 58- page 5 line 78 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 (9). There are critical periods of development in the lifespan where changes to dietary behaviors can impact current and future health 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 (10). Another 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 (1). However, there is currently no information on the long-term implications of this dietary strategy for progeny. The most available literature examines fasting during the month of Ramadan while pregnant. Review of these studies found that children born to those who fasted during pregnancy have similar birth weights and rates of pre-term birth as those who did not fast (11). In a recent review, Ramadan exposure *in utero* was associated with smaller body size and stature in later periods of life (12). 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. 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 74-78 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 (12). 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 126-129**

**“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.

**We used ZT to refer to zeitgeber time in this manuscript, so the first use of ZT is described on page 7 lines 119-120**

**“All animals were maintained on a, 12-hour light/dark (12 dark (ZT12, 6pm):12 light (ZT0, 6am); ZT = zeitgeber time) cycle in a temperature and humidity-controlled room.”**

1. 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 randomized to negate possible anticipatory stress in the second procedure?

**This was an intraperitoneal GTT as noted in the revised methods section on page 8, line 152-153:**

**“Baseline intraperitoneal insulin (ITT) and glucose tolerance tests(GTT) were assessed at young adulthood towards the end of the NCD diet period (PND 60-70, in that order).”**

**We completed the tests in the same order for both cohorts, first ITT, then GTT as noted in the manuscript on page 8, line 151-152. 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 et al. evaluated the effect of pregnancy on food intake timing and ambulatory activity in mice (13). They compared age-matched pregnant and non-pregnant female mice of the same strain used in the current study (C57BL/6J). 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 195-199:**

**“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). This period represents the active phase of both pregnant and non-pregnant mice (13)”**

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 measured the effect of this intervention on the dams. Those data are intended for a separate publication after a series of replication experiments focusing on maternal physiology and fertility. We have found in these previous cohorts that the 6h window is sufficient for similar daily calorie 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). We have included these data in the revised manuscript in the Supplementary Figure reproduced below and described on page 11 lines 201-203:**

**“We find no evidence of maternal eTRF causing significantly lower daily food intake during pregnancy nor are there changes in body weight (Supplementary Figure 1A&B).”**



**Supplemental Figure 1: Maternal Food Intake and Body Weight during Gestation**

**A)** Maternal food intake from one week before pregnancy until delivery B) Maternal body weight from one week before pregnancy until delivery. Dams in analysis n 8= eTRF, 9=AL.

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 describing 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 is very likely to have defects in insulin secretion. Those two data points work in concert with the data on GSIS to implicate functional differences in islet physiology. Importantly, we obtained this data for only a validation subset of mice. This preliminary islet validation study was only performed after two cohorts agreed on impaired glucose tolerance but normal insulin responsiveness. We agree that standing alone, the insulin secretion data is less robust, but stronger 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 263-266 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 substantially softened the language to reflect the non-significant finding regarding insulin secretion. Our interpretation is that the fold change in response to glucose shows no difference because the baseline values for eTRF offspring were considerably lower than they were for the AL offspring, which in and of itself reflects a difference in islet physiology. Our aim was to fulsomely present these data in several ways, and leave the conclusion to the reader. To clarify the results in 3K we include the table of showing all values below (two were below the limit of detection for the assay, those observations being omitted from our 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 for Insulin ELISA during in vivo GSIS in adult offspring of AL and eTRF dams.  eTRF = gestational exposure to early Time-Restricted Feeding  AL = gestational exposure to ad libitum feeding | | | | |
| Sample size 4 eTRF male, 4 eTRF females, 5 AL males, 7 AL females  NA\* indicates sample was below the limit of detection and was omitted from our analysis | | | | |

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 distinct cohorts of mice treated similarly. Phenotypes were robustly replicable across both cohorts. We have clarified this in the manuscript. The study included much larger sample sizes than suggested for the body composition, food intake, GTT and ITT data (eTRF males = 11, eTRF females = 19, AL males = 16, eTRF females = 17). We view that this is a reasonable number of animals for those outcomes and were in line with our pre-study power analyses. 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 only decided to complete this provisional experiment after the replication of the GTT and ITT results from the first two cohorts on page 7, line 123-126.:**

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

**These data inspired us to characterize *in vivo* glucose stimulated insulin secretion further in the second cohort by a more exploratory analysis of insulin secretion. We agree that these insulin secretion results, standing alone are somewhat less 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 a more detailed technical analysis of islet biology. The physiological characterization of these sex and diet-dependent differences will including islet morphology, studies on isolated islets, a comprehensive survey of incretins and islet communication networks and single-cell RNA sequencing. We are eager to perform those studies, but are feel those are beyond what we consider reportage of an interesting, robust and relevant phenotype in this manuscript, that of sex specific glucose intolerance in the offspring.**

**We also explain the lower n for in vivo GSIS, page 14 Line 282-284 as such:**

**“After noticing a consistent trend in both cohorts of eTRF males developing glucose intolerance after HFHS diet exposure, we sought to explore 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 perspective is that this is the first rigorous study of eTRF on long term metabolic health. Using a relatively large number of animals across multiple cohorts we believe that the lack of metabolic abnormalities in general, aside from HFD-induced sex-specific glucose intolerance is an important advance and to our minds, somewhat surprising. 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 hope to characterize what changes, what doesn’t and what counter adaptations occur in future studies, but these will take several years to complete. What we are comfortable sharing at this stage is that in the animals that underwent GSIS, we also conducted an *in vitro* GSIS (see figure Response Figure 1). However, the results had high levels of inter-replicate variability and will require multiple cohorts to be repeated. 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. This is the primary novel difference we are reporting here. As such we agree that the *in vivo* GSIS reported in this manuscript 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 page 19 lines 398-403**

**“we were not powered to conclusively establish lower insulin secretion in male eTRF offspring in adulthood, and have not yet evaluated islet size or beta cell mass to determine the mechanisms driving the worsening of glucose tolerance in adulthood in male mice or the resilience of female mice. We hope that future studies will describe these effects in larger samples and with higher resolution so that more in-depth conclusions can be drawn.”**

***In Vitro* GSIS Methods (previously described in (14)) :**

Response Figure 1: High Inter-replicate variability during *in vitro* GSIS in adult male offspring



**A)** Fold change of insulin secretion in AL vs eTRF islets collected from adult male offspring, n=4 per group, 3 replicates per measurement **B)** Maximal insulin secretion divided by well protein **C)** Insulin secretion in response to 22mM glucose divided by well **D)** Insulin secretion in response to 2mM glucose divided by well protein **E)** Maximal (KCl) divided by low glucose (2mM) insulin secretion.

**Four eTRF and 4 AL male offspring from cohort 2 were fasted overnight for primary mouse islet collection. Animals were euthanized by inhaled anesthesia overdose and cervical dislocation. Immediately after sacrifice, the abdomen of the mouse was washed thoroughly with 70% ethanol and a midline incision was made to expose the peritoneal cavity. The common bile duct was perfused with 1mg/mL collagenase in HBSS via handheld syringe and pancreas tissue was dissected, placed into falcon tube, and allowed to digest at room temperature for 15 minutes. Pancreata were then transferred to cold HBSS and centrifuged for 2-minutes and the pellet washed 3 times in HBSS and FBS. The interphase layer was collected by use of a 70-micrometer cell strainer, which was rinsed thoroughly with cold HBSS. Islets were then handpicked with a pipette and placed into 5micromolar glucose medium and recovered overnight before the experiment was conducted the next day.   
The next day, 10 islets were transferred to a 24-well plate for each condition. All *in vitro* GSIS measurements were completed in triplicate for each animal. Islets were incubated in 2 micromolar glucose for one hour at room temperature. This process was also completed using high glucose (22mM) and KCl (30mM) to assess response to low, high, and maximal insulin secretion in the collected islets. Insulin concentration was assessed via ELISA**

**Furthermore, since submitting our work, another group has recapitulated 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 15 line 300-page 16 lines 320:**

**“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. We find minimal effects associated with eTRF during gestation while male and female offspring are consuming a chow diet through early adulthood. However, after prolonged HFHS diet feeding, there are deleterious effects on glucose tolerance only in adult male progeny. Although inconclusive, we suspect from GSIS testing, there may be differences in insulin secretion for eTRF males compared to their AL counterparts. A recent study of gestational 12-hour TRF of chow diet in rats also found evidence of glucose intolerance and insulin sensitivity in the offspring of TRF dams, which is similar to the phenotype we note in male eTRF offspring after prolonged HFHS feeding (4). However, there are some differences compared to the current study. Most notably, they found impaired glucose stimulated insulin secretion in both male and female offspring who had not been exposed to high fat diet. These glycemic effects *in vivo* were apparent in female offspring, but were present in both male and female offspring *in vitro.* Furthermore, this group found further impairments in eTRF offspring *in vivo* when timed feeding was during the light cycle. The modest reduction of insulin at baseline during GSIS in eTRF offspring may contribute to the modest insulin sensitivity seen after HFHS feeding in the current study, and insulin sensitivity in vivo was evident in females in Prates and colleagues** (4)**. There were reductions in insulin secretion in response to high glucose in male and female dark-cycle fed islets after gestational TRF, suggesting this may be a contributing mechanism for metabolic disruption in our model of gestational TRF.”**

**Further, on page 18 lines 364-371 we now clarify:**

**“In contrast the previous study and some other models of nutrient restriction in pregnancy, we did not observe major differences until a HFHS diet challenge in adulthood, which may suggest that gestational eTRF may be relatively safe to practice in the context of a healthful diet or absent a second challenge. However, it also suggests that in the context of unhealthy diet patterns, adult offspring may be ill-equipped to adapt to high-calorie food environments, leading to metabolic dysfunction. These studies differ both in the age of onset and duration of food restriction that are required to initiate glucose intolerance in offspring of TRF dams which also may explain these differences.”**

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 123-126:**

**“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 and the response to point 13 from reviewer #1 above). This suggests the intervention does not induce overall caloric restriction during pregnancy in our model. This important point has now been described in the revised manuscript.**

# 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 (which for nocturnal animals is not when feeding normally occurs). As it pertains to disrupted feeding in pregnancy, there is now evidence, albeit limited, that pregnant women may adopt this practice (1, 15), referenced page 5 lines 79-82. We also find that the idea of doing this in a pre-existing metabolically unhealthy mouse model is interesting, but is an entirely different study and would be harder to interpret in the absence of these data. As we describe in this manuscript many phenotypes including virtually all metabolic measures in female offspring are not different in spite of this relatively aggressive feeding restriction *in utero* throughout pregnancya 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 duration of time spent eating during pregnancy can range from 3.25 to 15.75 hours on the average weekday. Six hour feeding periods have been used in previous TRF studies in both humans** (16–20) **and animals (21, 22) and is a good balance between overly restrictive 4h windows and only modestly restrictive 12h regimens. The additional rigor of maintaining circadian feeding windows also was critical do our design and strengthens our data herein. 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 seems 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 rodent IUGR literature, there are often metabolic phenotypes that only arise in adulthood, after a dietary challenge, or even in sex-specific manors. Upon further detailed review, some, but not all of these rodent nutrient restriction models cause a reduction in birth weight or body weight in early life, something we did not observe here. This is distinct from the human definition of IUGR which requires reduced birth weight. We edited our manuscript such that our conclusions are milder and to clarify that our model does not find any differences in offspring weight. We also use nutrient restriction in pregnancy in lieu of IUGR to better describe the phenotype and avoid conflating the term with low birth weight. Our data however is consistent with the other papers evaluating gestational TRF in a rodent model. On page 16 lines 334-page 17 lines 341 of the revised manuscript we note:**

**“The phenotype in male offspring from time-restricted feeding bears resemblance to animal models of mild intrauterine nutrient restriction, where glucose intolerance in resultant offspring can be a common phenotype. First described by Barker and colleagues, offspring who were deprived of nutrition *in utero* were more likely to develop chronic, nutrition-related disease in adulthood (23). Since that time, multiple animal models for gestational nutrient restriction were developed; maternal overnutrition during pregnancy, maternal caloric restriction, maternal protein restriction, and surgically induced placental insufficiency through late gestation uterine artery ligation.”**

**Page 17 lines 348-350**

**“Of note, these studies routinely find reductions in body weight as early as immediately postnatal**(24–32)**, which is inconsistent with the current study where we see no statistical reductions in body weight on either NCD or HFHS.”**

Minor:

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

composition in early life”

**Updated on page 10 line 195** :

**“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 32-68% increase in weekly caloric intake. This overnutrition challenge was done as animals on NCD alone had very modest phenotypes (see point 1 in response to reviewer 2), 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.**

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