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?

We changed all mentions of HFD to HFHS diet in the manuscript.

The word choice was updated as requested. Page 5, Lines101-102 “Detailed modeling of TRF in pregnancy is warranted, as TRE is currently thought to exist in human populations (8, 11) 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”

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 manuscript describing TRF studies during pregnancy. There also was a second manuscript that was published since we submitted our work, so the text was updated.  
  
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 (6) and placental oxidative stress markers (7) 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 (1). 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.   
  
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 diet used in the latter part of 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 language has been changed to more clearly define them as separate goals.

Page 3 Line 31-33 “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. 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 was included. 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) (1). The nuclear hormone receptors ROR(α, β, and γ) and REV-ERB (α and β) activate or repress expression of BMAL1 respectively (2, 3). 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 (2). Factors capable of manipulating, or entraining, this system are called zeitgebers (ZT). One such potent ZT is food intake(4).”

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

We updataed the language to transition more naturally between topics in this paragraph.

Page 4 line 67-page 5 line 86 “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 (6). 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(7).”

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

This is an excellent point. We included clarification about the phase-shifted eating of Ramadan. Page 5 Line 92-96  
  
“In a recent review, Ramadan exposure in utero was associated with smaller body size and stature in later periods of life (10). 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. We indicated this in the methods section. 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.

Page 4 lines 63-64   
“Factors capable of manipulating, or entraining, this system are called zeitgebers (ZT).”

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 randomised to negate possible anticipatory stress in the second procedure?

We intentionally completed the tests in the same order for both cohorts, first ITT, then GTT. This is a standard in our laboratory, and we find we have reproductible results with this method.   
  
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?

Ladyman , Carter, and Grattam (PMID29738792) 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 taken in 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.   
  
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 3rd cohort (which is currently underway). We have found in 2 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 figures below).



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.  
   The language was changed. Page 13 line 293-294  
     
   “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 now differences because the baseline values for eTRF offspring were considerably lower than they were for the AL offspring. We included the table of values below (2 were below the limit of detection for the assay).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Wells | ID | time | diet | sex | full.conc |
| A1 | 464 | 15 | eTRF | male | 5.47209 |
| A2 | 459 | 15 | AL | female | 3.21024 |
| A3 | 474 | 15 | AL | female | 1.605816 |
| A4 | 474 | 0 | AL | female | 1.636854 |
| A5 | 464 | 0 | eTRF | male | 7.39251 |
| A6 | 463 | 0 | AL | female | 0.820296 |
| B1 | 463 | 15 | AL | female | 1.542576 |
| B2 | 467 | 15 | eTRF | female | 0.55356 |
| B3 | 471 | 15 | AL | female | 0.99894 |
| B4 | 453 | 0 | eTRF | male | 2.982939 |
| B5 | 456 | 0 | AL | male | 5.11809 |
| B6 | 455 | 0 | eTRF | female | 1.477662 |
| C1 | 460 | 15 | AL | male | 14.80584 |
| C2 | 461 | 15 | AL | male | 4.34211 |
| C4 | 471 | 0 | AL | female | 0.1364961 |
| C6 | 465 | 0 | eTRF | male | 2.491551 |
| D1 | 456 | 15 | AL | male | 6.95367 |
| D2 | 454 | 15 | eTRF | female | 1.510338 |
| D3 | 453 | 15 | eTRF | male | 3.99936 |
| D4 | 459 | 0 | AL | female | 0.612618 |
| D5 | 460 | 0 | AL | male | 9.67845 |
| D6 | 467 | 0 | eTRF | female | 0.1364961 |
| E1 | 458 | 15 | AL | female | 1.08102 |
| E2 | 455 | 15 | eTRF | female | 2.940441 |
| E4 | 458 | 0 | AL | female | 0.49023 |
| E5 | 454 | 0 | eTRF | female | 0.49023 |
| E6 | 466 | 0 | eTRF | female | 0.49023 |
| F1 | 466 | 15 | eTRF | female | 1.159353 |
| F2 | 465 | 15 | eTRF | male | 4.88883 |
| F3 | 468 | 15 | AL | male | 7.41642 |
| F4 | 462 | 0 | AL | female | 0.49023 |
| F6 | 452 | 0 | eTRF | male | 1.477662 |
| G2 | 452 | 15 | eTRF | male | 2.608287 |
| G3 | 462 | 15 | AL | female | NA |
| G5 | 468 | 0 | AL | male | 3.59961 |
| G6 | 469 | 0 | AL | male | 4.03446 |
| H1 | 468 | 15 | AL | male | 7.1508 |
| H2 | 473 | 15 | AL | female | 6.96612 |
| H3 | 461 | 0 | AL | male | 4.96599 |
| H4 | 470 | 0 | AL | female | NA |
| H5 | 473 | 0 | AL | female | 3.33003 |

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.

This study utilized 2 cohorts of mice (1 cohort in 2019, another in 2020). We found glucose intolerance in males to be consistent between the two 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). The only study that had lower n was the *in vivo* GSIS, as we had noted the phenotype through a GTT in the first cohort and wanted to characterize it further in the second cohort.

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

# 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.

In the animals that underwent GSIS, we also conducted an *in vitro* GSIS. However, the results had high levels of inter-replicate variability. Because of this, we did not feel that the data were reliable and shouldn’t be included in the manuscript. No further cohorts of offspring are planned at this time, as it will take >6 months to generate a third cohort and run them through the full experimental protocol. We also do not have the expertise to conduct the studies mentioned above. The language in the manuscript has been altered to be less definitive toward an islet specific defect. 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.

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 suspect from GSIS testing, differences in insulin secretion for eTRF males compared to their AL counterparts. A recent study of gestational TRF of chow diet in rats also found evidence of glucose intolerance and insulin sensitivity in the offspring (14). However, these effects *in vivo* were apparent 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 (14). The similarities to the current study demonstrate that changes in islets may be a likely mechanism for metabolic disruption from gestational TRF.”

Page 16 lines 385-394 “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 consistent, so translation to human clinical populations is not possible at this time. 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 can have lasting impact on the metabolic health of offspring.”

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.

This study was completed in 2 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 repeated experiment.   
page 7 line 171, “Results are shown for 2 cohorts of animals that have been combined”

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 completed 2 cohorts of study to understand the maternal effects of this dietary intervention. We are still ascertaining the effect on 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. This suggests the intervention does not induce caloric restriction during pregnancy in our model. For some data, please see comments to reviewer 1. This data is intended for a separate publication. See comments for 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 isn’t consistent 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 it comes to disrupted feeding in pregnancy, there is now evidence, albeit limited, that pregnant women have adopted this practice (Ali & Kunugi, 2020; Flanagan et al., 2022). We also considered evaluating this eating modality with a diet-induced obesity model. We decided against doing so for 2 reasons: 1 – we were concerned about having too few litters to draw conclusions from using a DIO model, and 2 – we wanted to understand what the independent effects of TRF were outside of the context of metabolic disease and weight loss. Since beginning this review, a similar work has been published (Prates et al., 2022) that also utilizes a chow model of TRF in pregnant rodents (rats) and also finds insulin secretory differences in the resultant offspring, although stronger responses are seen in females in this work.

1. 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 know that although the 6h time restriction is very narrow(reviewer 1, comment 13), 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. 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. 6 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).

# 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 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.

Page 18 lines 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. 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”

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” ?

Overnutrition challenge is a common term for dietetic analysis in diets that exceed recommended levels of energy, fat, and refined carbohydrates. We use this term to refer to our HFHS diet.

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

The n for the vast majority of experiments is >12 per group(see reviewer 1 comment 16). The exception to this is the *In vivo* GSIS. However as stated previously, we are not able to repeat this study due to time and resource constraints.