**Title: Gestational Early-Time Restricted Feeding Results in Sex-Specific Glucose Intolerance in Adult Male Mice**

**Abstract   
Introduction**

Recent research has begun uncover that not only food quality and volume can impact health, but also that timing of intake in reference to circadian rhythms can play a part in health and disease. Robust rodent studies demonstrate that the timing of food intake is a strong zeitgeber (ZT) capable of programming for either poor health with models of chrono-disruption, or good health with models of dark-cycle timed feeding. The goal of time-restricted feeding (TRF), a method of intermittent fasting, is to align eating and caloric beverage intake with naturally occurring circadian rhythms in order to optimize health.

Pregnancy is a critical period when diet impacts not only maternal health, but also offspring lifetime risk for chronic disease. Previous studies have focused on dietary restriction or macronutrient excess in pregnancy, with very little focus on temporality of food intake. Only 1 group to date has evaluated the paradigm of time-restricted feeding during gestation. These investigations were focused on fetal health and completed in the context of preventing overnutrition from a high fat diet. They found TRF was protective for fetal lung development and  **Methods**

*Animal care and use*

Virgin female C57BL/6J mice were obtained from Jackson Laboratory (RRID IMSR\_JAX:000664). All animals were maintained on a 12-hour (12 dark (ZT12):12 light (ZT0); ZT = zeitgeber time) dark cycle in a temperature and humidity controlled room. After one week of acclimatization, they were single housed and dietary treatment began (either eTRF or AL feeding). Dams were randomized to either early time-restricted feeding (eTRF) or *ad libitum* (AL) feeding during gestation (n 8= eTRF, 9=AL). Dams fed AL had 24 hour access to a chow laboratory diet (NCD, Picolab Laboratory Rodent diet, 5L0D; 5% Fat / 24% Protein/ 71% Carbohydrate). Dams fed eTRF had 6 hours of NCD food access during the early dark cycle (zeitgeber time , ZT 14-ZT 20. Water was provided *ad libitum* throughout the study. After one week of either AL or eTRF feeding (beginning age 120 days), age-matched males were introduced into cages for breeding. Males were kept in the female cage until copulatory plug appeared. Each day, dams were transferred to a clean cage at ZT20, allowing for a clean cage free of food for eTRF animals and similar levels of handling between experimental groups. After birth, all dams were switched to AL unrestricted feeding of NCD and maintained on this diet until PND 21.5. This meant that any phenotype in the offspring could be attributable to the gestational diet exclusively. All experimental protocols were reviewed and approved by The University of Michigan Institutional Animal Care and Use Committee.

*Offspring growth and food intake monitoring*

Pups born to either eTRF or AL dams were weighed and counted within 24 hours of birth. Litters were reduced to 4 pups (2 male, 2 female when possible) at PND 3.5 to standardize milk supply among litters. At PND 21.5, offspring were weighed, and body composition was assessed using EchoMRI 2100 (EchoMRI) before being weaned by sex and maternal feeding regimen and housed 4-5 per cage (eTRF males = 11, eTRF females = 19, AL males = 16, AL females =17). Offspring were given AL access to NCD until PND 70. Food intake and body composition were assessed weekly throughout the course of the experiment. Food intake is represented as a per animal per day average. After PND 70, all animals were switched to 45% High Fat Diet (HFD; Research Diets D12451; 45% Fat/ 20% Protein/ 35% Carbohydrate).

*Insulin Tolerance and Glucose Tolerance Testing*

Baseline glucose and insulin tolerance were assessed at young adulthood towards the end of the NCD diet period (PND 60-70). Animals were transferred into a cage with no food during the early light cycle (ZT 2), with water freely available. After 6 hours, fasting blood glucose was assessed using tail clip and a handheld glucometer (OneTouch Ultra). Shortly thereafter, an intraperitoneal injection of insulin was administered (Humulin, u-100; 0.75U/kg lean mass). Blood glucose was assessed by glucometer every 15 minutes for 2 hours. One week later, glucose tolerance was assessed in a similar way (D-Glucose,1.5g/kg lean mass). Insulin and glucose tolerance were then re-assessed after high fat diet feeding (PND 140-160) (insulin dose 2.5U/kg lean mass, glucose dose 1.0g/kg lean mass). Area under curve was calculated by taking the sum of glucose at each time point for each animal, and then was averaged by sex and maternal feeding regimen. Rates of drop for ITT were calculated by limiting the dataset to the initial period after insulin administration (<60 minutes), taking the log of the glucose values and generating a slope for each animal. After each animal’s rate of drop was calculated, values were averaged by sex and treatment.

*Glucose Stimulated-Insulin Secretion testing in vivo*

One week after GTT and ITT, animals underwent GSIS (PND 160-170). At ZT2, animals were placed in a clean cage without food and with unrestricted access to water. After a 6 hour fast, animals were lightly anaesthetized with isofluorane via drop jar and a baseline blood sample was collected via retro-orbital bleed with heparinized capillary tube. Following baseline blood collection, an intra-peritoneal injection of D-glucose (1.0g/kg lean mass) was given. After 15 minutes had elapsed from injection, animals were again lightly anaesthetized in the same manner and another blood sample was collected via retro-orbital bleed. Blood samples were allowed to clot on wet ice (~20 minutes), then were spun down in a cold centrifuge (4 degrees C, MODEL # HERE) for 20 minutes at 5000 RPM. Serum was pipetted off and stored at -80 degrees C until analysis. Serum insulin was assessed via commercially available ELISA kit (AIPCO 80-INSMSU-E10) Insulin was assessed in 5uL of serum and read via colorimetric assay.

*Statistical analysis*

All measures whose p-values <0.05 were considered statistically significant. Data are presented as mean +/- standard error throughout. All statistical analyses were performed using R version 4.0.2 (R Core Team, 2021). Repeated measures, such as body composition, cumulative food intake, and responses to GTT or ITT were assessed via mixed linear effects modeling with random effects of mouse ID and dam and fixed effects of maternal dietary treatment, age, and sex using lme4 version 1.1-26. Body composition and food intake were measured separately in 2 phases; one during NCD feeding, and another after being switched to HFD. Analyses were tested for significant interactions between sex and maternal dietary treatment. If a significant interaction was observed, sex-stratified models were then used and the p-value for the interaction was reported. Models were assessed using a two-way ANOVA with for sex and maternal dietary treatment, with interaction. Those with interaction present were then assessed separately by sex; observations were tested for normality by Shapiro-wilk test and equivalence of variance by Levene’s test. Measures that were normal and of equal variance utilized Student’s t-tests. Measures that were not normal used non-parametric Mann-Whitney tests.

**Results**

*Gestational eTRF affects food intake, but not body composition in early life*

Body composition analysis using mixed linear effect modeling found consistent effect of age and sex, but no effect of maternal feeding regimen on body weight (**Figure 2A,** p=0.47), lean mass (**Figure 2C,** p=0.45), or fat mass (**Figure 2B**, p=0.471). Cumulative food intake over the first 70 days of life demonstrated significant effect of age (p<0.0001) and of gestational feeding regimen (p=0.00068), whereby PND 70, female eTRF pups consumed 17.8% more kilocalories than AL females, and males eTRF animals consumed 9.43% more kilocalories than AL males (**Figure 2D**).

*Gestational eTRF* *improves insulin tolerance in young adult males*

To assess glycemic health effects from gestational eTRF, we conducted insulin tolerance (ITT) and glucose tolerance (GTT) tests between PND 60 and 70. Insulin tolerance testing showed a significant effect of time, and sex (p=0.0018) where male blood glucose was ~15 mg/dL higher than females at each time point after insulin administration, but no effect of maternal dietary treatment was evident (**Figure 2E**, p=0.73). Area under the curve during the ITT had a significant effect of maternal dietary treatment (p=0.013) and of sex (p<0.0001, **Figure 2F**). AUC was 8.5% lower in eTRF in females than in AL females, and 2.2% lower in eTRF males than AL males (p=0.0054). The initial response for insulin sensitivity was not different by sex (p=0.10) or treatment (p=0.83). This suggest that gestational eTRF impacts the response to insulin challenge in adult mice, but that it does not differ in the initial period following insulin administration. Glucose tolerance was similar in young adulthood between groups in both males and females (**Figure 2G**). In mixed linear effect modeling, there was no significant effect of diet (p=0.53), but there was an effect of sex (p=0.0093) on glucose tolerance. However, AUC for the GTT (**Figure 2H**) had a significant interaction for sex and maternal dietary treatment (p=0.00082) where eTRF males had a lower 8.2% AUC than their AL counterparts (p<0.0001), this effect was absent in females (p=0.99). Fasting blood glucose, assessed before ITT and GTT, was 10.4% higher in males than in females (p=0.0054) but did not differ by maternal dietary treatment (p=0.18). This suggests there were early effects of gestational eTRF present in male offspring that were not explained by body composition, which was comparable between groups, or food intake, which was higher in eTRF animals. Given that adult offspring had minimal effects as a result of gestational eTRF exposure, we administered an overnutrition challenge; 45% of energy from fat. After 70 days, all animals were switched to AL access to 45% HFD while weekly food intake and monitoring of body composition continued.

*Adult HFD Feeding in gestational eTRF exposed offspring generates glucose intolerance in a sex-specific manner*

After beginning high fat diet feeding, there were no distinct changes between eTRF and AL offspring in body weight (**Figure 3A**, p=0.99), fat mass (**Figure 3B,** p=0.65),or lean mass (**Figure 3C,** p=0.47). Therefore, offspring of eTRF and AL experienced a similar transition in body composition to overnutrition challenge. There was an initial increase in food consumption with the switch to HFD, but this normalized over the course of 12 weeks and was not different between experimental groups. Females and males consumed similar amount of HFD (p=0.088), but there was a significant effect of maternal dietary treatment where AL consumed 4.5% less HFD over the course of the feeding period compared to AL (**Figure 3D**, p=0.00068)

Assessments of glucose homeostasis following HFD treatment uncovered a phenotype only present in male offspring. During ITT, there was a significant sex:treatment interaction in the mixed linear effect modeling (**Figure 3E**, p=0.03), which made sex-stratified analysis necessary. Female eTRF were similar in insulin tolerance to their AL counterparts (p=0.85), but male eTRF offspring tended to be more insulin sensitive than AL males (p=0.17). This was confirmed with the AUC for the ITT where females had similar AUC (**Figure 3F**, p=0.20) and eTRF males had 20.4% lower AUC during the insulin tolerance test than AL males (p<0.0001). The initial period of blood glucose drop was significant for sex, where females had greater rates of drop than AL males (p=0.029) but there was no effect of maternal feeding regimen (p=0.23). The trend toward insulin sensitivity was not explained by fasting blood glucose, as females had was 23% lower FBG than males (p<0.0001) but were similar between maternal dietary treatment groups (**Figure 3I,** p=0.83). Glucose tolerance testing (**Figure 3G**), also showed significant treatment and sex interaction (p=0.011). During glucose tolerance testing, males trended toward glucose intolerance (p=0.14), which was absent in females (p=0.61). GTT AUC (**Figure 3H**) was similar between groups in females (p=0.07) but was 13.5% higher in eTRF males compared to AL males ( p<0.0001). Taken together, these tests suggest male-specific glucose intolerance and insulin sensitivity. To further understand the male-specific phenotype of insulin sensitivity and glucose intolerance, we sought to assess for insulin secretion defects by conducting an *in vivo* glucose stimulated insulin secretion (GSIS) assay (**Figure 3J**).There was no effect of maternal diet (p=0.071) on insulin secretion, but a significant effect of sex was present, where females had lower insulin secretion than males in both eTRF and AL groups(p<0.0001), but no diet/sex interaction was present (p=0.064).  **Discussion**

This study is the first to study the long-term effects of gestational eTRF on offspring, and to assess their response to an overnutrition challenge. We see that deleterious effects of this diet are most notable in male adults who have been fed HFD.

In human beings, studies of fasting in pregnancy have been exclusively in the context of the month of Ramadan, and the focus is largely on infant health and maternal glycemia.

While prevalence of time-restricted eating is currently unknown in the global population, research and lay materials detailing the diet are increasing, suggesting an increasing interest in this feeding paradigm. Birthing parents may have periods of time with limited food intake for many reasons; among them, food insecurity, disordered eating behavior\s, nausea and vomiting of pregnancy/morning sickness, or intentional timing of eating. Because research is limited in this area, modeling TRF in pregnancy is warranted, as it likely exists in human populations and effects are unknown.

It is also possible that this feeding paradigm might have affected maternal behaviors, which could have an effect on offspring as well.

* **Parallel to humans**
* **IUGR**
* **Reference other mouse papers not in pregnancy**
* **Can project a bit (islet development )-> islet hyperplasia**
* **Ramadan fasting in pregnant women**
* **Morning sickness and maternal behaviors**
* **Will be common because** 
  + **FI, sick, undernutrition, Ramadan, choice – almost impossible to study in humans.**

To our knowledge, this is the first report of gestational time-restricted feeding and its effects on offspring through adulthood. Although this effect fails to reach statistical significance, there is a trend toward having higher blood glucose in the initial response to glucose administration and lower blood glucose in the initial response to insulin administration. One other group has studied gestational eTRF, but their models utilize 60% HFD and follow offspring into late fetal term (E)(Upadhyay et al., 2019, 2020).

Alth

Strengths:

* Long follow up with repeated body composition and food intake data
* Males and females were evaluated throughout the life course and the statistical methods employed were very good.

Limitations:

* Lack of understanding of the mechanism that drives these changes.
* We are not able to conclude that gestational eTRF resulted in differences in maternal behaviors that weren’t part of the study.

**Conclusion**

References

R Core Team. (2021). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/

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