**Title: Gestational Early-Time Restricted Feeding Results in Altered Glucose Homeostasis in Adult Male Mice**

**Abstract   
Introduction**

Recent research has begun to correlate not only food quality and volume with health, but also with timing of intake in reference to circadian rhythms. 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.  **Methods**

*Animal care and use*

Virgin female C57/bl6J mice were obtained from Jackson Laboratory. All animals were maintained on a 12-hour (12 dark:12 light) 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). After one week of dietary treatment (beginning age 120 days), age-matched males were introduced into cages for breeding. Males were kept in the female cage until copulatory plug appeared. The day a copulatory plug appeared was designated gestational day 0.5 (E 0.5). Measures of maternal health during pregnancy will be described separately. After birth (post-natal day, PND 0.5), offspring were weighed multiple times (PND 3.5, 7.5, 14.15) before reaching weaning age (PND 21.5). Litters were culled to 4 pups (2 males, 2 females, when possible) at PND 3.5. At weaning, offspring were weaned by sex and maternal feeding regimen and given normal chow diet (NCD) AL until 70 days of age. All experimental protocols were reviewed and approved by The University of Michigan Institutional Animal Care and Use Committee.

*Maternal dietary treatment*

Dams were randomized to either early time-restricted feeding (eTRF) or *ad libitum* (AL) feeding during gestation (8= eTRF, 9=AL). Dams fed AL had 24 hour access to NCD (Lab diet, 5L0D) and water. Dams fed eTRF had 6 hours of food access over the early portion of the dark cycle (zeitgeber time , ZT 13-ZT 19) for the whole of pregnancy. Each day, all animals were transferred to a clean cage at ZT20, allowing for similar levels of handling of all experimental animals. After birth, all dams were switched to AL feeding of NCD and maintained on this diet until PND21.5. This meant that any phenotype in the offspring could be attributable to the gestational diet exclusively.

*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. Pups were weighed again at PND 7.5, 14.5, and 21.5. At PND 21.5, offspring were weighed, and body composition was assessed using 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 (research diets D12451, 4.73kcal/gram).

*Insulin Tolerance and Glucose Tolerance Testing*

Baseline glucose and insulin tolerance were assessed at young adulthood during NCD diet period (~ postnatal day 70). Animals were transferred into a cage with no food during the early light cycle, 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 body weight). 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,1.5u/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 1.0u/kg lean mass).

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### *Sacrifice and tissue collection:*

Offspring were sacrificed after the high fat diet glucose tolerance test (**Figure 1B**). Animals were fasted for 16 hours with *ad libitum* access to water. Animals were lightly anesthetized by isofluorane inhalation. Fasting blood glucose was determined by glucometer and a fasted blood sample was collected by retro-orbital bleed and immediately put on ice to clot. Once clot formed, whole blood was spun down in a cold centrifuge (4 degrees C) for 20 minutes at 5000 RCF. Serum was pipetted off and placed in the -80 degrees C freezer until analysis. After blood collection, animals were euthanized by isofluorane overdose and cervical dislocation. Animal body weight was measured immediately after euthanasia on an electronic scale to the nearest 0.1 gram. Liver, inguinal white adipose tissue (iWAT), gonadal white adipose tissue (gWAT), and quadriceps femoris muscle were dissected from the right side of each mouse and snap frozen in liquid nitrogen and stored at -80C until later use.

*Statistical analysis*

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. The models were separated by offspring diet (NCD or HFD) and analyses were tested for interaction of sex and diet. If interaction was present, sex-stratified models were then used. Static measures, such as fasting blood glucose and area under the curves, 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 linearity 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. All measures whose p-values <0.05 were considered statistically significant.

**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 (p=0.45), lean mass (p=0.47), or fat mass (p=0.48) **Figures 2A-2C**. Cumulative food intake over the first 70 days of life demonstrated significant effect of age (p<0.0001) and of gestational feeding regimen (p=0.0024), where by 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. Fasting blood glucose, assessed before ITT and GTT was 10.4% higher in males than in females (p=0.0045) but did not differ by maternal dietary treatment (p=0.1873). Insulin tolerance testing showed a significant effect of time, and sex where males glucose was ~15 mg/dL higher than females at each time point after insulin administration, but no effect of maternal dietary treatment was evident (p=0.72, **Figure 2F**). Area under the curve (**Figure 2G**) during the ITT 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). This suggest that gestational eTRF impacts insulin sensitivity in adult mice. Glucose tolerance was similar in young adulthood between groups in both males and females (**Figure 2H**). In mixed linear effect modeling, there was no significant effect of diet (p=0.58), or sex (p=0.28) on glucose tolerance. However, AUC for the GTT (**Figure 2I**) had a significant interaction for sex and maternal dietary treatment (p=0.0093) where eTRF males had lower AUC than their AL counterparts. 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 generates glycemic deficits 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 3F**, 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 3G**, p=0.20) and eTRF males had 20.4% lower AUC during the insulin tolerance test than AL males (p<0.0001). This 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 3E,** p=0.83). Glucose tolerance testing (**Figure 3H**), also showed significant time\* sex interaction (p=0.011), so sex-stratified models were employed. During glucose tolerance testing males trended toward glucose intolerance (p=0.14), which was absent in females (p=0.61). AUC (**Figure 3I**) 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.

*Insulin secretion in eTRF males is blerg*

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 4A**). Utilizing 2-way ANOVA, 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=0.25).

* HOMA-IR
* Include sacrifice data?
* Check stats with Dave for GSIS

**Discussion**

* **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).

**Conclusion**

References

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

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