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

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Running title: eTRF and offspring glucose intolerance

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Word Count: 5296

Answers to study importance questions:

What is already known about this subject?
- TRE is a dietary strategy that can impact metabolic health in adults and is currently thought to improve metabolism, even in some cases without weight loss.
- Recent work demonstrates that up to 23.7% of a pregnant sample would consider attempting TRE during gestation, yet there is no study that evaluates the long-term implications of this diet on the resultant children.

- What are the new findings in your manuscript?
- We find that the effects of this gestational exposure to TRE in a mouse model are minimal and not deleterious while offspring consume a healthful diet until young adulthood.
- After offspring are switched to a high fat, high sucrose diet, we see glucose intolerance in adult males whose mothers were assigned to TRE without body weight changes or food intake changes. Females are do not experience this glucose intolerance.

- How might your results change the direction of research or the focus of clinical practice?
- These findings highlight the critical missing populations where TRE might affect long-term health, mothers and their children. It demonstrates the need to evaluate this dietary practice for further safety and efficacy information, especially since trials in humans cannot be completed until we have a deeper understanding of the safety implications in animal models.

Abstract

The timing of food intake is a novel dietary component that impacts health. Time-restricted feeding (TRF), a form of intermittent fasting, manipulates food timing. The timing of eating may be an important factor to consider during critical periods, like pregnancy. Nutrition during pregnancy, too, can have lasting impact on offspring health. The timing of food intake has not been thoroughly investigated in models of pregnancy, despite evidence that interest in the practice exists. Therefore, using a mouse model, we tested body composition and glycemic health of gestational early TRF (eTRF) in male and female offspring from weaning to adulthood on a chow diet and after a high fat, high sucrose (HFHS) diet challenge. 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 while on a chow diet. However, after 10 weeks of HFHS, male eTRF offspring developed glucose intolerance. Further studies should assess the susceptibility of males, and apparent resilience of females, to gestational eTRF and assess mechanisms underlying these changes in adult males.

38

Introduction

40   Behaviors that impact circadian rhythms, such as sleep, light exposure, and shift work

41 have long been associated with human health. The circadian rhythm follows a 24-hour cycle

42 which is governed at the cellular level by a transcription factor system (2–4).This highly

43 coordinated system can be entrained according to external cues. The system imparts a rhythm to

44 many physiological systems, including metabolism (5). Recently, food intake has been found to

45 impact the oscillations of circadian rhythm (6).

46   Recent evidence demonstrates the timing of food intake in reference to circadian rhythms

47 can impact propensity for health or disease (7). Time-restricted feeding/Eating (TRF/E), a

48 method of intermittent fasting, is thought to align caloric intake with naturally occurring

49 circadian rhythms of metabolism (8). Timing of food intake is capable of influencing metabolic

50 systems for either poor health from chronodisruption, or good health with either diurnal or

51 nocturnal feeding, depending on the species (9).

52   To our knowledge, no estimate of the prevalence of TRE in humans exists. However,

53 according to one sample, up to ten percent of people surveyed that stated they followed a diet in

54 the year 2020 had attempted "intermittent fasting," making it the most prevalent dietary

55 intervention in that sample (10). There are critical periods of development in the lifespan where

56 changes to dietary behaviors can impact current and future health status. One such critical period

57 is pregnancy. During pregnancy, habitual timing of food intake may be altered for many reasons:

58 religious practice, food insecurity, disordered eating behaviors, nausea and vomiting of

59 pregnancy/morning sickness, changes in taste/food preferences, or intentional timing of eating

60 for weight maintenance. Very little research has evaluated the timing of eating during pregnancy

61 and its impact on offspring health. One cross-sectional analysis found that extending the

62   overnight fast during pregnancy was associated with lower blood glucose levels at mid gestation

63   (11). Another recent work demonstrated that up to 23.7% of a human pregnant and recently post-

64   partum cohort said they would be willing to try TRE during pregnancy (12). However, there is

65   currently no information on the long-term implications of this dietary strategy for progeny. The

66   most available literature examines fasting during the month of Ramadan while pregnant. Review

67   of these studies found that children born to those who fasted during pregnancy have similar birth

68   weights and rates of pre-term birth as those who did not fast (13). In a recent review, Ramadan

69   exposure *in utero* was associated with smaller body size and stature in later periods of life (14).

70   However, these studies are limited, and Ramadan fasting is an imperfect model for TRF, as food

71   intake is not only limited in duration but also not permitted during the normal active phase for

72   humans.

73   There is much interest in the TRE diet and interruptions in food intake are known to

74   occur during pregnancy; however, research about the effects of intentional fasting during

75   pregnancy is limited to the observance of Ramadan, a cross-sectional study about attitudes

76   toward the practice (12), and one case report of fasting to improve gestational diabetes (15).

77   Detailed modeling of TRF in pregnancy is warranted, as TRE is currently thought to exist in

78   human populations (12, 15) yet, long-term effects are unknown.

79   Other groups have demonstrated that circadian rhythm and entrainment with external

80   cues, like phase shifts in lighting, during gestation can affect perinatal health outcomes in rodent

81   models. In fact, chronic use of photoperiod shifts during gestation and early postnatal life in rats

82   can result in altered oscillations of hormones and behaviors in dams, to impact gestational age

83   and birth weight, and to cause endocrine abnormalities, elevations in mean glucose, and glucose

84   intolerance in adult male offspring (16). Others have found worsened glycemic health in both

85   male and female adult offspring with chronodisruption despite no differences in body weight or

86   litter size (17). This is important, because it demonstrates that external cues, impact health

87   outcomes during pregnancy. Light cues are the most powerful zeitgebers, but other external cues

88   like the timing of food intake have not been investigated in pregnant populations.

89   Previous studies of maternal diet during pregnancy have focused on dietary restriction or

90   macronutrient excess in pregnancy, with little-to-no attention directed toward temporality of food

91   intake. At the time of this manuscript, two studies of TRF during pregnancy in rodents exist. The

92   first emphasized fetal health and was completed in the context of preventing complications from

93   a high fat, high sucrose diet (HFHS) during gestation in a rat model. Upadhyay and colleagues

94   found that 9-hour TRF improved fetal lung development (18) and placental oxidative stress

95   markers (19) at embryonic day (E)18.5 compared to *ad libitum* fed dams. This approach did not

96   evaluate the long-term, postnatal effects of TRF and the independent effects of TRF are

97   complicated by the use of a high fat, high sucrose diet. The second, also in rats, evaluated 12

98   hour access in light and dark cycles to a chow diet during pregnancy and followed male and

99   female resultant offspring until 150 days of age (20). Adult female offspring of dams fed in the

100 dark cycle with TRF were found to be glucose intolerant *in vivo*, and reduced glucose stimulated

101 insulin secretion *in vitro* in both male and female offspring islets. altered glucose metabolism in

102 adult offspring of TRF fed dams (20). However, this study only assessed offspring body

103 composition at birth and once during adulthood. It also did not evaluate glycemic health until late

104 adulthood, leaving the developmental trajectory of gestational-eTRF exposed offspring

105 unexamined.

106   The effects of TRF in non-pregnant human populations are inconsistent. Similarly,

107 insulin sensitization results in some (21, 27–30), but not all trials of TRF (22, 26). The duration

108 and timinig of feeding windows for TRF employed in human can vary. Lengths of feeding

109 windows can vary between 4 (21) and 12 hours(23), the feeding window can be early(27–29, 31)

110 vs late (24, 26, 28) in the day, control of caloric intake isocaloric (27) vs unrestrained caloric

111 intake (22, 26, 32), and inpatient observation (27) or outpatient adherence monitoring (22, 26).

112 As such, the biological effects of this eating strategy are not clear, even in non-pregnant humans.

113　　　Results from rodent models of TRF are more consistent than human trials. These have

114 found TRF of a HFHS reduces body weight compared to ad libitum feeding (33–38), can

115 improve Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) (35, 38, 39), and

116 may limit complications like insulin resistance (36, 37) from HFHS feeding.

117　　　Taking together the likelihood that food intake can be time-disrupted in pregnancy and

118 the evidence of TRF being a potent method to improve body composition and glycemic health in

119 adult mice, we sought to evaluate the impact of TRF of normal laboratory chow (6-hour, early

120 dark-cycle) before and during pregnancy on resulting offspring body composition and glycemic

121 health through adulthood.

Methods

123 *Animal care and use*
124 Male and Female C57BL/6J mice were obtained from Jackson Laboratory (RRID

125 IMSR_JAX:000664). All animals were maintained on a, 12-hour light/dark (12 dark (ZT12,

126 6pm):12 light (ZT0, 6am); ZT = zeitgeber time) cycle in a temperature (70° C) and humidity (40-

127 60%)-controlled room. After one week of acclimatization, females were single-housed for the

128 remainder of the experiment and males were socially housed until mating. Dams and sires were

129 randomized to either early time-restricted feeding (eTRF) or *ad libitum* (AL) feeding(dams n 8=

130 eTRF, 9=AL). This study was completed in two independent cohorts of animals. The phenotypes

131   noted in offspring were highly consistent Between cohorts and we found no statistical effect

132   modification by cohort (data not shown). Therefore, data shown is the combined total from

133   cohorts one and two and statistical tests do not include effects of cohort in the model. Dams and

134   sires fed AL had 24-hour access to a chow diet (NCD, Picolab Laboratory Rodent diet, 5L0D;

135   5% of Calories from fat, 24% from protein, 71% from carbohydrates). Dams and sires fed eTRF

136   had 6 hours of NCD food access during the early dark cycle (ZT 14-ZT 20). Water was provided

137   *ad libitum* throughout the study to both experimental groups. After one week of either AL or

138   eTRF feeding (beginning age 120 days), age-matched sires were introduced into cages for

139   breeding. Males were kept in the cage until a copulatory plug was detected. Daily, dams were

140   transferred to a clean cage at ZT20, allowing for a cage free of food for eTRF animals and

141   similar levels of handling between experimental groups. After birth, all dams switched to AL and

142   were maintained on this diet until weaning at postnatal day (PND) 21.5. Therefore, any

143   phenotype in the offspring is attributable to modifications to the pre-gestational/gestational diet.

144   All experimental protocols were reviewed and approved by The University of Michigan

145   Institutional Animal Care and Use Committee.

146

147   *Offspring growth and food intake monitoring*
148   Pups born were weighed and counted within 24 hours of birth. Litters were reduced to 4 pups (2

149   male, 2 female, when possible) at PND 3.5 to standardize milk supply between litters. At PND

150   21.5, offspring were weighed and body composition was assessed using EchoMRI 2100

151   (EchoMRI) before being weaned by sex and maternal feeding regimen and housed 4-5 per cage

152   (eTRF males = 11, eTRF females = 19, AL males = 16, AL females =17). Offspring were given

153   AL access to NCD until PND 70. Food intake and body composition were assessed weekly.

154   Food intake is represented as an average per animal per day. To correct to food spillage, during

155   weekly food measurements, cages were examined for excessive levels of pellet shredding or food

156   loss from the hopper. Cages meeting these criteria for spillage were excluded from statistical

157   from analysis for that week. After PND 70, all animals began AL 45% High Fat, High Sucrose

158   Diet (HFHS; Research Diets D12451; 45% Fat/ 20% Protein/ 35% Carbohydrate). Weekly body

159   composition and food intake measurement continued during HFHS feeding. Feeding efficiency

160   was calculated for the two periods of diet (NCD and HFD). Fat and lean mass measurements

161   collected via EchoMRI at the beginning of the period were subtracted from the final fat and lean

162   mass measurements for that feeding period. This represented the total gain in fat and lean mass

163   during this diet period. These values were then multiplied by 9 and 4 respectively (Atwater

164   factors for fat and carbohydrate/proteins). The product was then divided by total kcals consumed

165   during the feeding period. The result is expressed as a percentage, where larger numbers

166   represent greater efficiency in turning consumed kcals into bodily tissues (eTRF males = 9,

167   eTRF females = 16, AL males = 14, AL females =14).

168

169   *Insulin Tolerance and Glucose Tolerance Testing*
170   Baseline intraperitoneal insulin (ITT; eTRF males = 9, eTRF females = 17, AL males = 18, AL

171   females =19) and glucose tolerance tests (GTT; eTRF males = 4, eTRF females = 4, AL males =

172   7, AL females =6) were assessed at young adulthood towards the end of the NCD diet period

173   (PND 60-70, in that order). Animals were transferred into a cage with no food during the early

174   light cycle (ZT 2), with water freely available. After 6 hours, fasting blood glucose was assessed

175   using a tail clip and a handheld glucometer (OneTouch Ultra). Shortly thereafter, an

176   intraperitoneal injection of insulin was administered (Humulin, u-100; 0.75U/kg lean mass).

177   Blood glucose was assessed by glucometer every 15 minutes for 2 hours. One week later,

178   glucose tolerance was assessed in a similar way (D-Glucose,1.5g/kg lean mass). Insulin and

179 glucose tolerance were then re-assessed after HFHS feeding (PND 140-160; eTRF males = 9,

180 eTRF females = 18, AL males = 18, AL females =18) (insulin dose 2.5U/kg lean mass, glucose

181 dose 1.0g/kg lean mass). Area under curve was calculated for each animal by taking the sum of

182 glucose at each time point, and then was averaged by sex and maternal feeding regimen. Rates of

183 drop for ITT were calculated by limiting the dataset to the initial period after insulin

184 administration (<60 minutes), taking the log of the glucose values and generating a slope for

185 each animal. After each animal's rate of drop was calculated, values were averaged by sex and

186 treatment.

187 *Glucose Stimulated-Insulin Secretion testing in vivo*

188 As an exploratory analysis, one week after GTT and ITT, animals underwent intraperitoneal

189 glucose stimulated insulin-secretion (GSIS) testing (PND 160-170; eTRF males = 4, eTRF

190 females = 4, AL males = 5, AL females =8). At ZT2, animals were placed in a clean cage

191 without food and with unrestricted access to water. After a 6-hour fast, animals were lightly

192 anesthetized with isoflurane via drop jar and a baseline blood sample was collected via retro-

193 orbital bleed with a heparinized capillary. Following baseline blood collection, an intraperitoneal

194 injection of D-glucose (1.0g/kg lean mass) was given. After 15 minutes, animals were lightly

195 anesthetized in the same manner and another blood sample was collected. Blood samples were

196 allowed to clot on wet ice (~20 minutes), then were spun down in a cold centrifuge (4° C,

197 Eppendorf microcentrifuge, model 5415R) for 20 minutes at 2000 g. Serum was pipetted off and

198 stored at -80 °C until analysis. Serum insulin was assessed via a commercially available ELISA

199 kit (ALPCO 80-INSMSU-E10). Serum insulin was assessed in 5uL samples and read via

200 colorimetric assay.

201   *Statistical analysis*
202   All measures with p-values <0.05 were considered statistically significant. Data are presented as

203   mean ± standard error throughout. All statistical analyses were performed using R version 4.0.2

204   (40). To minimize potential bias,the analysis plan was chosen prior to the start of experiments,

205   and unchanged upon data analysis. Repeated measures, such as body composition, cumulative

206   food intake, and responses to GTT or ITT were assessed via mixed linear effects modeling with

207   random effects of mouse ID and dam and fixed effects of maternal dietary treatment, age, and

208   sex using lme4 version 1.1-26 (41). Body composition and food intake were measured repeatedly

209   in two separate conditions: during NCD feeding, and after being switched to HFHS. Analyses

210   were tested for significant interactions between sex and maternal dietary treatment. Models were

211   assessed using a two-way ANOVA for sex and maternal dietary treatment, with an interaction

212   between the two. If a significant interaction was observed, data were sex-stratified and pairwise

213   comparison was repeated, reporting the effect size and p-value for the interaction. Otherwise, sex

214   was used as a covariate in a non-interacting model. Observations were tested for normality by

215   Shapiro-Wilk test and equivalence of variance by Levene's test. Pairwise measures that were

216   normal and of equal variance utilized Student's *t*-tests. Measures that were not normally

217   distributed used non-parametric Mann-Whitney tests.

Results

218

219   *Gestational eTRF increases food intake, but not body weight in early life*
220   To model gestational early time restricted feeding (eTRF), we used a normal chow diet (NCD)

221   and assigned female mice to either unrestricted (*ad libitum,* AL) or 6 hours of restricted food

222   availability between ZT14-20 (eTRF) (**Figure 1A**). This period represents the active phase of

223   both pregnant and non-pregnant mice (42). This approach limits potential sleep disruptions and is

224   more translationally relevant to human dietary restriction. This treatment started a week before

225   mating in both dams and sires and continued through delivery (**Figure 1B**). We find no evidence

226   of maternal eTRF causing significantly lower daily food intake during pregnancy nor are there

227   changes in body weight (Supplementary Figure 1A&B). Litters were normalized to equal sizes

228   on postnatal day 3 to reduce variability and effects of lactation.

229

230   The pups resulting from this experiment were weighed and their body composition was

231   assessed weekly, then analyzed using linear mixed effect modeling. We found significant and

232   expected effects of age and sex (older mice weigh more than younger mice and male pups weigh

233   more than females), but no effect modification of maternal eTRF on body weight (**Figure 2A,**

234   p_{diet}=0.47), lean mass (**Figure 2C,** p_{diet}=0.45), or fat mass (**Figure 2B**, p_{diet}=0.47). There was no

235   interaction between sex and maternal feeding regimen in cumulative food intake ($p_{diet*sex}$=0.38).

236   However, cumulative food intake in the NCD period is 22% higher in eTRF females than AL

237   females and 10% higher in eTRF males than AL males (**Figure 2D**, $p_{diet\,=}$ 0.016). Assessing the

238   efficiency by which food is converted into stored mass resulted in a 12% reduced feeding

239   efficiency in eTRF female offspring (p_{sex}<0.00001) which is not present in males

240   (**Supplementary Figure 2A**).

241   *Gestational eTRF modestly improves glucose tolerance in young adult males*
242   To assess glucose homeostasis in the offspring, we conducted ITTs and GTTs between PND 60

243   and 70. Male offspring averaged 15mg/dL higher blood glucose during insulin tolerance testing

244   compared to females (p_{sex}=0.0018), but no effect of maternal dietary restriction was evident

245   through linear mixed effect modeling (**Figure 2E**, p_{diet}=0.73). Summarizing the ITT by

246   calculating the area under the curve (AUC) demonstrated there was no diet:sex interaction

247   ($p_{diet:sex}$=0.069), but an effect of maternal restriction where eTRF offspring had lower AUC

248   compared to AL offspring, 8.5% and 2.2% lower in females and males respectively (p_{diet}=0.013),

249 and a significant effect of sex (p_{sex}<0.0001). As expected, males had a higher AUC than females

250 (**Figure 2F**). The initial response to insulin (the rate of glucose decline over the first 60 minutes,

251 not pictured) was not significant for sex (p_{sex}=0.10) or treatment (p_{diet}=0.83). These data suggest

252 that gestational eTRF slightly improves the response to insulin challenge in adult mice, and that

253 this is not driven by reduced fat mass.

254 Glucose tolerance was similar in young adulthood between groups in both males and females

255 (**Figure 2G**). We found no significant effect of diet (p_{diet}=0.53) on the rise in blood glucose

256 during GTT, but there was an effect of sex (p_{sex}=0.0093) on glucose tolerance, again with

257 expected higher glucose levels in male mice. The summarized AUC for the GTT (**Figure 2H**)

258 shows a significant interaction between sex and maternal dietary treatment ($p_{sex:diet}$=0.00082).

259 eTRF males had an 8.2% lower AUC than their AL counterparts (p_{diet}<0.0001) while this was

260 absent in females (p_{diet}=0.99). Fasting blood glucose, assessed before ITT and GTT, was 10.4%

261 higher in males than in females (p_{sex}=0.0054; **Figure 2I**), but did not differ significantly by

262 maternal dietary treatment (p_{diet}=0.18). Taken together these data suggest that gestational eTRF

263 has very a mild effect on adult offspring, despite the narrow feeding window. Offspring whose

264 mothers were fed eTRF had slightly improved responses to insulin and glucose challenge but no

265 differences in body weight or in fat mass.

266 *HFHS feeding in adult offspring exposed to eTRF during gestation generates sex-*
267 *specific glucose intolerance*

268   Given that adult offspring were minimally affected by gestational eTRF exposure, we

269 administered a high fat, high sucrose (HFHS) overnutrition challenge; *ad libitum* access to 45%

270 of energy from fat and 17% of energy from sucrose after PND 70. Food intake and body

271 composition measurements continued weekly. The average weekly food intake increased by

272 67.6% in AL offspring and by 31.8% in eTRF offspring after switching to HFHS, both of which

273   exceed energy needs for adult mice (43). Similar to the findings on chow, with HFHS, there were

274   no major differences between eTRF and AL offspring in body weight (**Figure 3A**, p_{diet}=0.99), fat

275   mass (**Figure 3B,** p_{diet}=0.65), or lean mass (**Figure 3C,** p_{diet}=0.47). Therefore, offspring of eTRF

276   and AL experienced similar changes in body composition in response to overnutrition.

277   Cumulative HFHS consumption was comparable between females and males (p_{sex}=0.72), and

278   maternal restriction groups (**Figure 3D**, p_{diet}=0.72). Feeding efficiency, a ratio comparing food

279   intake to stored fat and lean mass, was greater in males than in females, which is consistent with

280   the NCD period (**Supplemental Figure 2B,** p_{sex} = 0.00023). However, unlike the NCD period,

281   efficiency was indistinguishable between eTRF and AL offspring (p_{diet}=0.93).

282   We repeated an ITT and GTT after 10 weeks of HFHS feeding. During the ITT, there

283   was a significant interaction between sex and diet using mixed linear effect modeling (**Figure**

284   **3E**, $p_{sex:diet}$=0.03). Female eTRF had a similar response to insulin, with less than a 1 mg/dL

285   difference from their AL counterparts (p_{diet}=0.85), but male eTRF offspring tended to be more

286   insulin sensitive with 25mg/dL lower glucose compared to AL males (p_{diet}=0.17). It could also

287   be true that females were more resilient to changes from HFHS. These findings were confirmed

288   by calculating the AUC where eTRF females showed no difference in AUC compared to AL

289   females (**Figure 3F**, p_{diet}=0.20) while eTRF males had 20.4% lower AUC than AL males

290   (p_{diet}<0.0001). The initial rate of glucose decline (not pictured) was greater in females compared

291   to males (p_{sex}=0.029) but there were no differences between eTRF and AL offspring (p_{diet}=0.23).

292   The trend toward insulin sensitivity from the ITT was not explained by fasting blood glucose, as

293   females had 23% lower fasting blood glucose than males (p_{sex}<0.0001) but were similar between

294   eTRF and AL offspring within the same sex (**Figure 3I,** p_{diet}=0.83). Glucose tolerance tests in

295   **Figure 3G**, also showed significant effect of interaction ($p_{sex:diet}$=0.011), although now in the

296 opposite direction. During GTT, eTRF males trended toward glucose intolerance with an average

297 of 53mg/dL higher glucose than AL males during the course of the experiment (p_{diet}=0.14). This

298 was not observed in female eTRF offspring, which had similar blood glucose during the GTT

299 compared to AL females (p_{diet}=0.61). The GTT AUC showed interaction between effects of sex

300 and treatment (**Figure 3H,** ($p_{sex:diet}$<0.0001)). AUC was 5% lower in eTRF females (p_{diet}=0.07)

301 but was 13.5% higher in eTRF male offspring compared to AL (p_{diet}<0.0001). Taken together,

302 these tests suggest eTRF results in males who experience glucose intolerance and insulin

303 sensitivity whereas females are more resilient to glycemic changes after gestational eTRF. Given

304 that we cannot explain glucose intolerance in males via reduced insulin sensitivity, we next

305 evaluated insulin secretion.

306   After noticing eTRF males developed glucose intolerance after HFHS diet exposure in

307 both cohorts, we sought to explore cohort 2 more closely for insulin secretion defects, via an *in*

308 *vivo* glucose stimulated insulin secretion (GSIS) assay (**Figure 3J**). Females had lower levels of

309 insulin than males (p_{sex}<0.0001). There was a non-significant trend toward lower insulin levels in

310 eTRF compared to AL offspring of both sexes (p_{diet}=0.071). Females had similar increases in

311 insulin in response to glucose injection, 139% in AL versus 137% eTRF. Male AL offspring had

312 a 48% increase in insulin whereas this was just an 18% increase for eTRF males. There was no

313 interaction between sex and maternal restriction ($p_{sex:diet}$=0.064). Females have 94% greater fold-

314 change insulin secretion in response to glucose challenge than male offspring (p_{sex}=0.0027) but

315 there was no impact of maternal restriction on fold change secretion (p=0.85, **Figure 3K**). Male

316 and female offspring of eTRF dams had lower baseline insulin values compared to AL dams,

317 which we believe resulted in the similarity of fold change insulin secretion between maternal

318 restriction groups. This study was not conclusive as it had a lower sample size and failed to reach

319 statistical significance, but could indicate that insulin secretion is modestly impaired in male

320 eTRF offspring after HFHS challenge in males.

321

Discussion

323   This study is the second to describe the long-term effects of gestational eTRF on

324 offspring health and the first to describe their response to a high fat, high sucrose diet challenge.

325 We find minimal effect of eTRF during gestation while male and female offspring are

326 consuming a chow diet through early adulthood. However, after prolonged HFHS diet feeding

327 and advanced age, glucose intolerance develops in adult male progeny. Taken together, results

328 from insulin and glucose tolerance testing, and exploratory GSIS after HFHS feeding suggest

329 modest reduction in insulin secretion between eTRF and AL males. Although, the latter was

330 exploratory and did not reach statistical significance. The other study of gestational (12-hour)

331 TRF of chow diet in rats also found evidence of glucose intolerance and insulin sensitivity in the

332 offspring of TRF dams (20). However, two studies were not completely consistent. Most

333 notably, they found impaired GSIS in both male and female without exposure to HFHS. The

334 modest reduction of insulin at baseline during GSIS in eTRF offspring may contribute to the

335 modest insulin sensitivity seen after HFHS feeding in the current study, and this is consistent

336 with others noting modest improvements in insulin sensitivity in females (20). There were

337 reductions in insulin secretion in response to high glucose in male and female dark-cycle fed

338 islets after gestational TRF, suggesting this may be a contributing mechanism for metabolic

339 disruption in our model of gestational TRF.

340   Other studies that focus on lighting manipulations during gestation highlight similar

341 effects among adult offspring. Perinatal exposure to chronodisruption in rats and mice also

342 resulted in mild phenotypes of glycemic dysmetabolism (16, 17, 44, 45). This is similar to the

343 current study, as this effect is present without reductions in birth weight or litter size (17, 44).

344 Taken together, these data imply that the chronological timing of multiple zeitgebers can impact

345 perinatal health outcomes.

346   Comparing the current study with other studies utilizing HFHS diets and TRF

347 demonstrates some consistencies in glycemic outcomes. Fasting insulin can be lowered (35–38,

348 46), similar to our findings, and resulting HOMA-IR can be improved with TRF (37, 46, 47).

349 Our finding that fasting blood glucose is unchanged in eTRF mice is consistent with other groups

350 examining TRF with HFHS (35, 46, 47). Some differences in the current studies are not reflected

351 in the literature, such as elevated food intake while on NCD in female offspring exposed to eTRF

352 *in utero* and was not seen in the other longitudinal analysis of offspring health following

353 gestational TRF (20). Studies of adult mice pairing TRF and HFHS report reduced food intake in

354 TRF groups (39, 48) or equivalent caloric intake when matched by diet (36–38, 49). This could

355 indicate a compensatory response in the female offspring resulting from eTRF *in utero*.

356 Interestingly, this did not result in differing body weight or composition, suggesting that this

357 increased food intake is matched by decreased caloric extraction or increased energy expenditure

358 in these mice.

359   The phenotype in male offspring from time-restricted feeding bears resemblance to

360 animal models of adverse intrauterine development, where glucose intolerance in resultant

361 offspring can be a common phenotype. First described by Barker and colleagues, offspring who

362 were deprived of nutrition *in utero* were more likely to develop chronic, nutrition-related disease

363 in adulthood (50). Undernutrition (51–53), overnutrition (54, 55), placental insufficiency (56,

364 57), and chronodisruption (16, 17, 44, 58) during pregnancy have all been reported to induce

365 offspring glucose intolerance . The extent to which male-predominate phenotypes and female

366 resilience to changes are difficult to deduce as many groups either study male offspring

367 exclusively (53, 59) or analyze males and females together (52, 60).

368   Although we did not evaluate insulin conclusively in the current study, glucose

369 intolerance in adverse intrauterine development models has been found to co-occur with insulin-

370 related abnormalities in the offspring, including lower insulin content in the pancreas (52), lower

371 basal circulating insulin levels (60), impaired insulin secretion (53, 61), reduced beta cell mass

372 (62). However, in the present study we find modest improvement in male insulin sensitivity in

373 adulthood in male offspring exposed to gestational eTRF. This finding is similar to the previous

374 study where females exposed to gestational TRF had greater rates of glucose disappearance

375 during insulin tolerance testing (20). We believe that the insulin sensitivity during high fat, high

376 sucrose diet feeding in eTRF males could be attributed to having lower basal levels of insulin

377 compared to AL males in our model. This could result in peripheral tissues being more sensitive

378 to insulin action despite an apparent insulin secretion impairment at the level of the pancreas.

379 However, without formal experimental evaluation of islet form and function or insulin peripheral

380 insulin signaling, we cannot conclude this is the mechanism for these phenotypic differences in

381 eTRF offspring.

382   In contrast to the previous study and some models of adverse intrauterine environment in

383 pregnancy, we did not observe major metabolic differences between restricted and unrestricted

384 offspring until a HFHS diet challenge occurred in adulthood. Our findings bears similarity to the

385 phenotype of adult offspring born to dams that experienced chronodisruption during pregnancy

386 metabolic consequences only manifested after 12 months of age (17, 44). This could suggest that

387 gestational eTRF may be relatively safe to practice in the context of a healthful diet or absent a

388 second challenge. However, it also suggests that in the context of unhealthy diet patterns, adult

389 offspring may be ill-equipped to adapt to high-calorie food environments, leading to metabolic

390 dysfunction. These studies differ both in the age of onset and duration of food restriction that are

391 required to initiate glucose intolerance in offspring of TRF dams which also may explain these

392 differences. Modeling of this dietary strategy remains incomplete, so translation to human

393 clinical populations is not possible at this time. The similarity of the present study to those using

394 diverse gestational stressors suggests that restriction of the total time pregnant dams is a novel

395 dietary component that can have lasting impact on the spent eating in metabolic health of

396 offspring and recommends further research on this novel component in the diet as a modulator of

397 maternal and child metabolic health outcomes.

398   Although we have not investigated offspring pancreatic tissues, we hypothesize that

399 alterations in the development of the pancreas may underlie the susceptibility of males for

400 glucose intolerance and modest insulin sensitivity in eTRF offspring after HFHS feeding. This is

401 confirmed by one study of early post-natal exposure to TRF, which found that adolescent males

402 who were fed TRF the first 4 weeks after weaning developed smaller islets of Langerhans and

403 higher blood glucose compared to those fed AL (49). Therefore, future studies of gestational or

404 developmental eTRF should examine islet size, pancreatic beta cell mass, and insulin secretion to

405 investigate the mechanism of offspring glucose intolerance more conclusively.

406   This study and the conclusions to be made from it have some limitations. First, the model

407 of gestational eTRF may have resulted in differences in maternal behaviors that were not noted

408 by the study team, and therefore could play a part in the effects seen in the offspring. Second, we

409 assessed the effect of a dietary insult in young adulthood by switching all animals to HFHS. As

410 such, disentangling the effect of HFHS diet from that of aging and gestational eTRF is not

411 possible in this model. It is also worth noting that several metabolic diseases are highly linked to

412 age, and while our study ended at approximately six months of age, mice can live much longer

413 under laboratory conditions typically 26-30 months. As metabolic, physical, cognitive, and other

414 phenotypes that do not appear until towards the end of the mouse's lifespan were not detectable,

415 and we look forward to future studies on geriatric mice treated *in utero* with eTRF. Finally,

416 although we see a robust effect on glucose intolerance, we were not powered to conclusively

417 establish lower insulin secretion in male eTRF offspring in adulthood and have not yet evaluated

418 islet size or beta cell mass to determine the mechanisms driving the worsening of glucose

419 tolerance in adulthood in male mice or the resilience of female mice. Furthermore, while dams

420 were manipulated simultaneously, we cannot rule out that our eTRF treatment induced other

421 confounding differences that we have not accounted for, including potential maternal stress or

422 chronodisruption. Our model used healthy, non-obese dams; therefore, we cannot extend the

423 effects of the current study to the context of metabolic syndrome, diabetes, or obesity during

424 pregnancy. Future work should prioritize assessing the pancreas and islets in larger samples and

425 with higher resolution so that more in-depth conclusions can be drawn.

426   There are many strengths to this study. Among them are the use of a preclinical model

427 which facilitates consistency when compared to existing literature and allows for careful control

428 of diet, genetics, and environment throughout gestation, which would be impossible at this point

429 in a human trial. Further strengths include the long follow up period for a gestational exposure,

430 controlling for the effect of litter size, repeated measurement of body composition, and food

431 intake measurements over the life course in the resultant offspring. Finally, the inclusion of both

432 male and female offspring in the study, as many metabolic assessments of TRF either focus

433 exclusively on the effects of the regimen in males (37, 38) or female mice (35, 36) is a strength.

Conclusion

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. Gestational eTRF resulted in male impairments in glucose tolerance in adulthood only after chronic HFHS feeding, whereas females appeared resilient to and did not develop differences. 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.

Acknowledgements

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602 **Figure Legends**

603 **Figure 1: Experimental Protocol and Timing**
604 **A)** Food availability and timing for dams during pregnancy. Food access began at ZT13 for early
605 Time-Restricted Feeding dams (eTRF, light gray, n=8) and continued until ZT129, total of 6
606 hours. Food was available 24 hours a day for ad libitum dams (AL, dark gray, n=9). **B)** Offspring
607 experimental protocol. After birth, all dams had AL access to laboratory chow (NCD). Litters
608 were reduced to 4 (2 males, 2 females when possible) on post-natal day (PND) 3. Offspring were
609 weaned by maternal feeding regimen at PND 21 and maintained on AL NCD for 70 days.
610 Weekly body composition and food intake measurements were taken throughout the experiment.
611 At 70 days of age, insulin tolerance tests (ITT; eTRF males = 9, eTRF females = 17, AL males =
612 18, AL females = 19) and glucose tolerance tests (GTT; eTRF males = 4, eTRF females = 4, AL
613 males = 7, AL females = 6) were conducted before switching all animals to a 45% high fat, high
614 sucrose diet (HFHS) with sucrose. Animals were on HFHS for 10 weeks before repeating ITT
615 and GTT (eTRF males = 9, eTRF females = 18, AL males = 18, AL females = 18), and an in
616 vivo glucose stimulated insulin secretion test (GSIS; eTRF males = 4, eTRF females = 4, AL
617 males = 5, AL females = 8). Animals were euthanized after these tests. Abbreviations: zeitgeber
618 time (ZT), ZT0 = lights on, ZT12 = lights off.
619
620 **Figure 2: Early Life Body Composition, Food Intake, and Glycemic Homeostasis**
621 **A)** Body weight in grams from PND21-PND70 in males and females, averaged by age, maternal
622 feeding regimen, and sex. **B)** Fat mass in grams from PND21-PND70 in males and females,
623 averaged by age, maternal feeding regimen, and sex. **C)** Lean mass in grams from PND21-
624 PND70 in males and females, averaged by age, maternal feeding regimen, and sex. **D)** Food
625 intake in kcals per mouse per day, averaged by week, maternal feeding regimen, and sex. *p-
626 value <0.05 for diet. **E)** Insulin tolerance test (ITT) ~PND 70, averaged by maternal feeding
627 regimen, sex, and time in minutes. **F)** Area under the curve (AUC) for ITT, averaged by maternal
628 feeding regimen, and sex. * indicates p-value <0.05 for effect of diet in males. **G)** Glucose
629 tolerance test (GTT) ~PNG 70, averaged by maternal feeding regimen, sex, and time in minutes.
630 **H)** AUC for GTT, averaged by maternal feeding regimen, and sex. * indicates p-value <0.05 for
631 effect of diet in males. **I)** Fasting blood glucose (FBG) PND 70, averaged by maternal feeding
632 regimen and sex. Animals included in body composition measurements, FBG, ITT, and GTT,
633 n=11 eTRF males, 16 AL males, 19 eTRF females, 17 AL females. Number of cages in food
634 intake analysis n=4 eTRF males, 5 AL males, 4 eTRF females, 5 AL females.
635
636 **Figure 3: Body Composition, Food Intake, and Glycemic Response to High Fat, High**
637 **Sucrose Diet Feeding in Adulthood**
638 **A)** Body weight in grams from PND 70-175 in males and females, averaged by age, maternal
639 feeding regimen, and sex. **B)** Fat mass in grams from PND 70-175 in males and females,
640 averaged by age, maternal feeding regimen, and sex. **C)** Lean mass in grams from PND 70-175
641 in males and females, averaged by age, maternal feeding regimen, and sex. **D)** High fat, high
642 sucrose diet (HFHS) intake in kcals per mouse per day averaged by week, maternal feeding
643 regimen, and sex. **E)** Insulin tolerance test (ITT) after 10 week of HFHS, averaged by age,
644 maternal feeding regimen, sex, and time in minutes. **F)** Area under the curve (AUC) for insulin
645 tolerance test, averaged by maternal feeding regimen, and sex. * indicates, p-value <0.05 for diet
646 in males. **G)** Glucose tolerance test (GTT) after 10 weeks of HFHS, averaged by maternal

647   feeding regimen, sex and time in minutes. **H)** Area under the curve (AUC) for GTT after 10
648   weeks of HFHS, averaged by maternal feeding regimen and sex. * indicates p-value <0.05 for
649   effect of diet in males. **I)** Fasting blood glucose (FBG) after 10 weeks HFHS, averaged by
650   maternal feeding regimen, and sex. **J)** Glucose stimulated insulin secretion (GSIS), averaged by
651   maternal feeding regimen, sex, and time. **K)** Fold change of insulin secretion during GSIS,
652   averaged by maternal feeding regimen and sex. * indicates p-value <0.05 for effect of sex.
653   Animals included in body composition, FBG, ITT, GTT, and GSIS: n=11 eTRF males, 16 AL
654   males, 19 eTRF females, 17 AL females. Cages in food intake analysis: n=4 eTRF males, 5 AL
655   males, 4 eTRF females, 5 AL females.
656
657   **Supplemental Figure 1: Maternal Food Intake and Body Weight during Gestation**
658   **A)** Maternal food intake from one week before pregnancy until delivery B) Maternal body
659   weight from one week before pregnancy until delivery. Dams in analysis n 8= eTRF, 9=AL.
660
661   **Supplemental Figure 2**: **Feeding Efficiency Throughout Adulthood**
662   **A)** Feeding efficiency (%) in males and females, calculated based on food intake and body
663   composition changes during the NCD period (before PND 70). (p_{sex}<0.001, p_{diet}=0.002). **B)**
664   Feeding efficiency in males and females during the HFHS period (after PND 70). (p_{sex} =
665   0.00023, p_{diet} = 0.093).
666
667
668

Figure 2

Figure 2:

Figure 3: