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Fructose consumption during pregnancy and lactation induces fatty liver and glucose intolerance in rats

Mi Zou^{2,1}, Emily J. Arentson², Dorothy Teegarden², Stephanie L. Koser³, Laurie Onyskow³, and Shawn S. Donkin^{2,3}

²Interdepartmental Nutrition Program, Purdue University, IN

³Department of Animal Sciences, Purdue University, IN

Abstract

Nutritional insults during pregnancy and lactation are health risks for mother and offspring. Both fructose and low protein diets are linked to hepatic steatosis and insulin resistance in non-pregnant animals. We hypothesized that dietary fructose or low protein intake during pregnancy may exacerbate the already compromised glucose homeostasis to induce gestational diabetes and fatty liver. Therefore, we investigated and compared the effects of low protein or fructose intake on hepatic steatosis and insulin resistance in unmated controls and pregnant and lactating rats. Sprague-Dawley rats were fed either a control (CT), a 63% fructose (FR) or an 8% protein (LP) diet. Glucose tolerance test at day 17 of the study revealed greater ($P < 0.05$) blood glucose at 10 (75.6 vs. 64.0 ± 4.8 mg/dl) and 20 (72.4 vs. 58.6 ± 4.0 mg/dl) min after glucose dose and greater area under the curve (4302.3 vs. 3763.4 ± 263.6 mg·dl⁻¹·min⁻¹) for FR-fed dams compared with CT-fed dams. The rats were euthanized at 21 days postpartum. Both the FR- and LP-fed dams had enlarged ($P < 0.05$) livers ($9.3, 7.1$ vs. 4.8 ± 0.2 % body weight) and elevated ($P < 0.05$) liver triacylglycerol ($216.0, 130.0$ vs. 19.9 ± 12.6 mg/g liver weight) compared with CT-fed dams. FR induced fatty liver and glucose intolerance in pregnant and lactating rats, but not unmated control rats. The data demonstrate a unique physiological status response to diet resulting in the development of gestational diabetes coupled with hepatic steatosis in FR-fed dams, which is more severe than a LP diet.

Keywords

rat; fructose; low protein; fatty liver; pregnancy

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Corresponding author: Shawn S. Donkin, Department of Animal Sciences, Lilly Hall of Life Science, 915 W. State Street, Purdue University, West Lafayette, IN 47907-2054, Tel. 765-494-4847; Fax, 765-494-9346; sdonkin@purdue.edu.

¹Current Address: Berry Baker Laboratory of Head and Neck Cancer, Vanderbilt University Medical Center, Nashville, TN

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1. Introduction

Pregnancy represents a physiological state hallmarked by transient, compromised insulin sensitivity and glucose homeostasis. Shifts in the maternal hormonal milieu and metabolism are designed to ensure adequate nutrition for the growing fetus [1]. Adverse dietary manipulations in laboratory animals are shown to further perturb maternal insulin-sensitive processes, mimicking gestational diabetes [2]. This result is concerning in light of epidemiological evidence and controlled laboratory studies [3–6] that convincingly demonstrate a link between adverse dietary conditions during pregnancy that disrupt the maternal metabolic milieu and adult metabolic abnormalities in offspring, including hyperglycemia, insulin resistance, and dyslipidemia. To better understand the physiological underpinnings responsible for fetal programming, it is imperative to better characterize the unique maternal metabolic adaptations to diet during gestation in contrast to unmated animals.

A low protein diet fed from conception until weaning to pregnant and lactating rats is one of the most studied experimental models with regard to *in utero* programming of offspring [5]. However, the effects of dietary protein restriction on maternal glucose metabolism and health have not been fully described. Available data indicate that feeding low protein diets to pregnant rats leads to oral glucose intolerance and elevated insulin to glucose ratio during a glucose challenge, [2] indicative of gestational diabetes. The impairments in maternal metabolism in response to a low protein diet consumed during pregnancy and lactation persist even after the rats are returned to a balanced diet [7], suggesting that low protein diets consumed during pregnancy and lactation present a health risk for the mother as well as offspring.

Fructose consumption among adults in the United States has risen dramatically to approximately 80g/day [8] and is reported to account for between 10 [9] and 20% [10] of the daily calorie intake. Excessive fructose consumption is linked to numerous adverse metabolic outcomes including insulin resistance [11], increased adiposity, hypertriacylglycerolemia, hyperleptinemia [12], hyperglycemia, hyperinsulinemia, impaired glucose tolerance [13] and hepatic steatosis [14 in laboratory animals and human subjects [15]. Some data exist that show feeding high amounts of fructose to pregnant rats induces enlarged liver, elevated hepatic FAS and PEPCK-C mRNA abundance, exacerbated pregnancy-induced hypertriacylglycerolemia as well as significantly elevated fasting glucose during mid-pregnancy [16]. However, relatively little is known regarding the maternal or offspring response to fructose consumption during pregnancy that persists through the lactation period; likewise comparison between physiological states, non-pregnant and pregnant, is lacking.

Hepatic steatosis, characterized by triacylglycerol infiltration of liver [17] affects approximately 31% of the adults in the US, with more than half the patients being women [18]. Hepatic steatosis resulting from non-alcoholic fatty liver disease (NAFLD) is linked to an imbalance among the uptake, synthesis, export, and oxidation of fatty acids [19]. Development of fatty liver has been postulated as one of the maternal conditions that engages maladaptive responses in offspring during development in response to inadequate

maternal folate intake [20]. Women with gestational diabetes are at greater risk for subsequent type 2 diabetes and postpartum fatty liver [21]. Liver lipid accumulation also plays a key role in the development of insulin resistance and the metabolic syndrome [22]. Although the relationship between insulin resistance and fatty liver is not fully developed, the expression of many insulin-responsive genes is altered during fatty liver which may affect hepatic insulin sensitivity and metabolic control [14, 18].

Progressive insulin resistance occurs during pregnancy. Fructose is shown to cause the development of insulin resistance in non-pregnant individuals. We hypothesized that dietary fructose intake during pregnancy and lactation may exacerbate the already compromised glucose homeostasis to induce gestational diabetes. Protein-restricted diets lead to hepatic lipid infiltration in non-pregnant adult rats [23]. Pregnancy and especially lactation are associated with an increase in lipid mobilization. We further hypothesized restricting protein consumption during pregnancy and lactation would create metabolic conditions to induce fatty liver. Hepatic steatosis and insulin resistance are closely associated. Therefore we hypothesized that both low protein diets and fructose during pregnancy would lead to gestational diabetes. Therefore, the objectives of this study were to determine the effects of protein restriction and high fructose feeding on mated and pregnant and lactating rats on glucose tolerance, hepatic steatosis and expression of key genes for glucose and lipid metabolism in liver.

2. Methods and materials

2.1 Animals and diets

Female Sprague Dawley rats were received from Harlan (Indianapolis, IN) within 3 days after confirmed mating and housed individually in standard polycarbonate rat cages (Ancare, Bellmore, NY) containing wood shavings at a constant temperature (25°C), 40 to 50% relative humidity, and a 12-h light 12-h dark cycle. Mated rats were given free access to water and ad libitum access to one of the diet (Table 1). Unmated age and weight matched female Sprague-Dawley rats (n=24) were received at the same time as the mated animals and housed in wire bottom cages with free access to water. Unmated control rats were assigned to control diet (CT), a diet containing 63% fructose (FR) or a diet containing 8% of protein (LP) for 6 weeks (n=8/group). Mated dams were fed either a control diet (MatNuCT, n=9), a diet containing 63% fructose (MatNuFR, n=6) or a diet containing 8% of protein (MatNuLP, n=9) during both the pregnancy and nursing phase. Originally 11 dams were assigned to each of the maternal diet groups; however, as the study progressed it became apparent that several of the animals in each group were not successfully mated and therefore not included in analysis (MatNuCT n=2, MatNuFR n=5, MatNuLP n=2). Unmated controls and pregnant rats were housed together in the same room for the six week study period. Rats were weighed weekly. Food intake was measured 3 times weekly and was calculated by the difference in food offered and food remaining. Cumulative food intake was calculated as the food consumed over the six week study period. All procedures involving animals were carried out with prior approval of the Purdue University Animal Care and Use Committee.

2.2 Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed following 14 d of diet treatment, and after 3 h of food deprivation. The oral glucose challenge was conducted between 7am and 10am in the morning. Glucose (200g/L in water) was introduced into the stomach of the rats via oral gavage at a final dose of 2 g/kg body weight. Blood samples were obtained from a tail tip puncture at 10, 20 and 30 min post glucose dose [24] for analysis of glucose concentrations using a handheld glucometer (One Touch, Johnson & Johnson, Langhorne, PA). Glucose responses during the OGTT were evaluated by the estimation of the total area under the curve (AUC) using the trapezoidal method [25]. Data are only included up until 30 minutes post-gavage because pregnant animals had already returned to baseline.

2.3 Offspring handling

Within 24 hours of birth, the pups were individually weighed, commingled within maternal diet group, normalized to 10 pups per litter and cross-fostered to dams within maternal diet group to minimize the effect of dam. At 21 days of age offspring were removed from the dams, individually weighed, and nose-anus length was determined.

2.4 Tissue and blood collection and handling

Dams and unmated controls were killed by decapitation under a CO₂ overdose when pups were weaned. Blood was collected into glass tubes, allowed to clot on ice for 15 min, and centrifuged at 2000 × g. Serum was stored at -20 °C pending analysis for glucose, non-esterified fatty acids (NEFA), triacylglycerols and insulin. Liver was excised, weighed and subdivided for mRNA analysis, metabolite analysis and histological examination. The remaining liver was stored in scintillation vials and kept frozen immediately after removal until analyses.

2.5 Serum analysis

Immediately following collection blood was allowed to clot on ice prior to centrifugation to obtain serum. The serum was removed and stored frozen in microcentrifuge tubes until analyses for glucose, NEFA, triacylglycerol and insulin concentrations. Serum glucose was quantified using the glucose oxidase method [34] and reagents supplied as Autokit Glucose (439-90901, Wako Diagnostics, Mountain View, CA). Serum NEFA was quantified using coenzyme A acylation method [27] and reagents supplied as HR Series NRFA-HR(2) (Wako Diagnostics, Mountain View, CA). Serum triacylglycerol was quantified using triacylglycerolhydrolyzation [28] and reagents supplied as Serum Triglyceride Determination Kit (TR0100, Sigma-Aldrich, St Louis, MO). Serum insulin concentration was quantified using immunoblotting method and reagents supplied as Rat Insulin EIA Kit, Ultrasensitive (80-INSRTU-E10, Alpco Diagnostics, Salem, NH). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as fasting serum glucose times fasting serum insulin divided by 2430 [29]. Fasting serum glucose was in the unit of mg/dl and fasting serum insulin was in the unit of μ IU/ml [29].

2.6 Liver histology

Liver tissues were fixed in 10% formalin phosphate (SF100-4, Fisher Scientific, Fairlawn, NJ) at the time of collection and embedded in paraffin. Sections of 5- μ m thickness were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to determine morphologic changes. The photographs were taken at 400 $\times$ magnification.

2.7 Liver RNA isolation, cleanup and reverse transcription

A 0.2 g sample of each liver was immersed in TRIzol Reagent (15596-018, Invitrogen, Carlsbad, CA) upon collection and frozen immediately in liquid nitrogen and stored at -80°C pending RNA extraction and analysis. Total liver RNA was extracted using TRIzol Reagent (15596-018, Invitrogen, Carlsbad, CA). Samples (50 μ g) were further purified using RNeasy Mini Kit (74104) and genomic DNA was eliminated with the addition of RNase-Free DNase Set (79254, Qiagen, Germantown, MD). Purified RNA was quantified with NanoDrop 1000 (Thermo Scientific, Wilmington, DE) and 2 μ g total RNA was reverse transcribed using Omniscript RT Kit (205113, Qiagen, Germantown, MD) with 2 μ l of 10 μ M Oligo-dT (79237, Qiagen, Germantown, MD) and 0.4 μ l of 10 μ M Random Decamer (0702002, Ambion, Austin, TX) per reaction.

2.8 Real-time PCR

Carnitine palmitoyltransferase-1 (CPT-1a), fatty acid synthase (FAS), pyruvate carboxylate (PC), phosphoenol pyruvate carboxykinase (PEPCK-C), PPAR γ coactivator-1 α (PGC-1 α) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were determined in liver samples using real-time PCR (MXP 3005, Stratagene Technologies, La Jolla, CA) and the primer sets listed in Table 2. Expression of GAPDH was not affected by treatment and was used to normalize expression of other mRNA transcripts. All reactions used the following protocol: 12.5 μ l 2 $\times$ master mix (Brilliant SYBR Green QPCR Master Mix, 600548, Stratagene Technologies, La Jolla, CA), 100 μ M forward primers, 100 μ M reverse primers, 0.375 μ l 1:500 diluted reference dye, 2–5 μ l cDNA and the total volume adjusted with nuclease-free H $_2$ O to 25 μ l. The reactions were initialized at 95 $^{\circ}\text{C}$ for 10 min, denatured at 95 $^{\circ}\text{C}$ for 30 s, annealed at 55 $^{\circ}\text{C}$ for 1 min and elongated at 72 $^{\circ}\text{C}$ for 30 s for 40 cycles. Dissociation curve was achieved by melting the DNA at 95 $^{\circ}\text{C}$ for 1 min, incubating the DNA at 55 $^{\circ}\text{C}$ for 30 s and followed by a ramp up to 95 $^{\circ}\text{C}$ for 30 s. Relative mRNA abundance was obtained using $2^{-\text{Ct}}$ method [30], with a pool of all cDNA samples as calibrator. All values were arbitrated to the unmated control as 1.00.

2.9 Liver triacylglycerol content

Liver triacylglycerol content was determined as previously described [31] with the following modifications, liver triacylglycerol was adjusted using ^{14}C -labeled palmitate ($76.7 \pm 10.9\%$) as the sample internal standard rather than ^{14}C -labeled oleic acid to adjust for triacylglycerol recovery. Mean triacylglycerol recovery for all samples was $76.7 \pm 10.9\%$.

2.10 Statistical analyses

Data were tested for normality by PROC CAPABILITY procedure of SAS software (version 9.1; SAS Institute Inc, Cary, NC). Heteroscedacity of data (Kolmogorov-Smirnov test,

$P < 0.05$) was corrected with natural log transformation. Normally distributed data and transformed data were analyzed using PROC GLM procedure of SAS software. Means were compared using Tukey's Honestly Significant Difference (HSD) test. Normally distributed data are presented as least-square means and pooled standard errors. Transformed data were back transformed and presented as least-square means with the corresponding confidence intervals. Means were considered different when $P < 0.05$ and trends were identified when $0.05 < P < 0.10$. Power calculations, based on the effect of fructose on blood glucose and HOMA scores in a pilot study, indicated that 8 animals per treatment was sufficient for a power of the test of 0.80 when $\alpha = 0.05$.

3. Results

Main effect of physiological status

Body weight of the unmated and mated rats were 201.8 and 214.1 $\pm$ 1.7 g respectively upon receipt and did not differ between groups at the initiation of the experiment. Cumulative food intake (338.3 vs. 320.7 $\pm$ 5.3 g; pregnant vs. unmated) and body weight gain (82.0 vs. 22.9 $\pm$ 1.5 g; pregnant vs. unmated) were significantly greater ($P < 0.05$, Table 3) in the pregnant compared with the unmated rats for the first 3 week interval. During the subsequent lactation phase cumulative food intake remained higher ($P < 0.05$, Table 3) in the mated animals compared with unmated controls (840.7 vs. 337.6 $\pm$ 12.75 g; lactating vs. unmated), although body weight accretion was no longer significantly different (5.14 vs. 9.92 $\pm$ 1.74 g; lactating vs. unmated).

Two weeks into the study baseline glucose was lower ($P < 0.05$, Table 4) for pregnant rats compared with unmated controls (53.6 vs. 64.3 $\pm$ 1.4 mg/dL; pregnant vs. unmated). In response to an oral glucose challenge, pregnant rats showed a lesser glucose response ($P < 0.05$, Table 4) at each time point resulting in a lower AUC ($P < 0.05$, Table 4) compared with the unmated controls (3965.0 vs. 4782.0 $\pm$ 152.2 mg·dL⁻¹·min⁻¹; pregnant vs. unmated).

At the conclusion of the experiment following six weeks on the diets, the lactating dams had greater ($P < 0.05$, Table 5) serum glucose (166.9 vs. 117.7 $\pm$ 20.6 mg/dL; lactating vs. unmated), insulin (0.92 vs. 0.15 $\pm$ 0.23 ng/dL; lactating vs. unmated) and triacylglycerols (62.4 vs. 37.5 $\pm$ 6.5 mg/dL; lactating vs. unmated) concentrations compared with unmated females. Likewise HOMA-IR was elevated ($P < 0.05$, Table 5) in lactating rats (1.47 vs. 0.14 $\pm$ 0.50; lactating vs. unmated). Lactating rats had larger ($P < 0.05$, Table 5) livers relative to body weight (7.1 vs. 2.7 $\pm$ 0.1 % body weight; lactating vs. unmated) and increased liver triacylglycerol content (122.0 vs. 10.7 $\pm$ 7.3 g/mg liver weight; lactating vs. unmated) compared with unmated controls, regardless of the diet. Serum NEFAs were not different between lactating dams and unmated controls. The transcript abundance of PEPCK-C (0.09 vs. 0.65; lactating vs. unmated), PC (0.09 vs. 0.95; lactating vs. unmated), CPT-1 α (0.05 vs. 0.72; lactating vs. unmated) and PGC-1 α (0.62 vs. 2.27; lactating vs. unmated) was decreased ($P < 0.05$, Table 6) in liver of lactating rats compared with unmated controls, whereas FAS mRNA (33.3 vs. 2.77; lactating vs. unmated) was elevated ($P < 0.05$, Table 6) in liver of lactating dams.

Main effect of diet

Regardless of physiological status, during the first three weeks of the study rats fed the control and the low protein diets had similar intakes but rats fed the fructose diet had reduced ($P<0.05$, Table 3) intake (344.1, 304.4, and 340.0 ± 6.5 g for Control, Fructose and Low Protein, respectively). Rats fed the control and low protein diets had similar body weight gain during the first 3 weeks of the experiment, but rats fed the fructose diet had decreased ($P<0.05$, Table 3) body weight gain (55.1, 48.0, and 54.2 ± 1.9 g for Control, Fructose and Low Protein, respectively). At the conclusion of the second half of the study, rats fed the control diet had consumed more ($P<0.05$, Table 3) than the fructose-fed and low protein-fed animals (718, 608 and 501 ± 15.5 g for Control, Fructose and Low Protein, respectively). Low protein-fed rats lost weight during the second half of the study, whereas lactating dams fed the control and fructose diets gained similar amounts of weight (14.9, 11.9 and -4.26 ± 2.26 g for Control, Fructose and Low Protein, respectively).

Fructose feeding during pregnancy had no effect on offspring birth weight whereas protein restriction during pregnancy resulted in a 34% decrease in birth weight (6.7, 6.7 and 5.8 ± 0.2 g for MatNuCT, MatNuFR, and MatNuLP respectively, Table 3). Offspring from MatNuFR and MatNuLP dams had weaning weights that were 87% and 66%, ($P<0.05$) respectively, of the weight of the MatNuCT offspring (57.7, 48.5, and 28.8 ± 1.2 g for MatNuCT, MatNuFR and MatNuLP, respectively, Table 3). The same trend was mirrored in offspring BMI at weaning, indicating increased body thinness ($P<0.05$) of the offspring of dams fed fructose or protein restricted (5.51, 4.77 and $3.61\pm .11$ kg/m² for MatNuCT, MatNuFR and MatNuL Prespectively; Table 3).

Fructose induced glucose intolerance after 14 d of feeding regardless of physiological status, but feeding a low protein diet had no effect (Table 4). The AUC for fructose-fed animals was $4751.8\text{ mg}\cdot\text{dL}^{-1}\cdot\text{min}^{-1}$ compared with 4208.7 and $4159.9\pm 1864.4\text{ mg}\cdot\text{dL}^{-1}\cdot\text{min}^{-1}$ for control-and low protein-fed animals, respectively. Rats fed fructose had greater ($P=0.05$) blood glucose concentrations after 10 min (83 vs. 71 ± 3.4 mg/dl; Fructose vs. Control) after glucose dosing compared controls. At the conclusion of the study, fructose significantly feeding lowered HOMA-IR (Table 5, $P<0.05$) compared with control; low protein feeding had an intermediate effect (1.59, 0.29 and 0.53 ± 0.70 for Control, Fructose and Low Protein, respectively). There was no effect of diet on serum concentrations of glucose, insulin, NEFA, triacylglycerols or hepatic transcripts.

Interaction of physiological status and diet

There was an interaction of physiological status and diet during the lactation phase on both food intake and body weight change. For unmated controls there were no differences in food intake among the diet groups (Table 3). Among the lactating rats there was a reduction in intake for animals fed the fructose diet relative to the control diet and a further reduction for those fed the low protein diet (Table 3). Similarly, body weight change in the unmated control, low protein and fructose-fed rats and the lactating control and fructose-fed did not differ; however, the low protein lactating animals lost weight while fructose and control lactating animals gained weight. Therefore the physiological state $\times$ diet effect on food

consumption and body weight change seems the result of a specific effect of the low protein diet to reduce food intake and weight gain during lactation (Table 3).

There was an interaction of physiological status and diet during the pregnancy phase on fasting blood glucose (Table 4). Fructose feeding to unmated rats for three weeks induced elevated fasting glucose compared with CT and LP rats, but failed to do so in pregnant animals (Table 4). At the end of the lactation phase there was an interaction of physiological status and diet for fasting glucose. The effect of fructose feeding on fasting glucose was abolished in the unmated animals at the end of six weeks (Table 5). However, serum glucose was reduced with low protein feeding compared with the control diet for the lactating rats (Table 5, $P<0.05$). Consequently HOMA-IR was numerically reduced in low protein-fed rats and significantly reduced with fructose feeding in lactating rats (Table 5).

Liver lipid did not differ between lactating and unmated rats fed the control diet (Table 5). However, liver weight and liver triacylglycerol content were elevated for rats fed either the high fructose or the low protein diet during pregnancy and the ensuing lactation. Feeding fructose during pregnancy and lactation led to liver lipid content that was 10.8 times greater than values from lactating rats fed the control diet. The low protein diet fed group had liver lipid concentrations that were 6.5 times greater than controls (Table 5). Histological examination of liver slices prepared for each group of animals revealed lipid deposition in periportal hepatocytes that extends towards the central venous regions of the liver acinus (Figure 1).

There was a significant physiological status $\times$ diet interaction effect ($P<0.05$) for hepatic transcripts PC, PGC-1 α and CPT-1 α expression and a tendency for an effect ($P=0.05$) on FAS expression (Table 6). Low protein unmated controls had elevated PC compared with the CT and FR animals while all of the lactating animals had suppressed PC expression. Fructose unmated animals had suppressed CPT-1 α abundance compared with CT and LP animals while lactating animals all had lower CPT-1 α abundance. PGC-1 α abundance was higher in the FR and LP than the CT animals; conversely hepatic PGC-1 α abundance was lower in MatNuFR and MatNuLP animals compared with MatNuCT. FR and LP animals had increased FAS abundance compared with the CT animals. Lactating dams fed fructose had slightly reduced FAS compared with the MatNuCT and MatNuLP.

4. Discussion

In this present study we found that fructose feeding to pregnant and lactating elicited glucose intolerance and fatty liver in pregnant and lactating rats, but failed to do so in unmated female controls, which was in support of our hypotheses. In further agreement with our hypotheses, low protein feeding also induced fatty liver in lactating animals; however contrary to our hypothesis low protein feeding did not result in glucose intolerance in lactating animals. These data highlight the additional metabolic burden of fructose feeding during pregnancy and lactation exerts a deleterious effect in concert with the already compromised insulin sensitivity of the dam. Added sugar represents 14% of energy intake in diets consumed by pregnant women [32] and sweetened beverage consumption has been linked to greater risks for gestational diabetes(GDM) [33]. Many juices and soft drinks

contain a high proportion of fructose [34] which has been linked to insulin resistance in non-pregnant subjects [35] and therefore fructose consumption during pregnancy may be a concern for the health of the mother and developing offspring. The fructose diet used in this present study was designed with fructose as the sole carbohydrate source so that any physiological effects could be attributed to fructose. Furthermore, the high level of fructose fed was intended to magnify any potential effects of fructose feeding during pregnancy and lactation.

Incidence rate of GDM has doubled during the past decade [36] and current estimated prevalence ranges are between 4 to 14% of all human pregnancies [37]. GDM is a major risk factor for subsequent onset of type 2 diabetes in the mother [38]. Furthermore, children who are exposed to an intrauterine environment concomitant with pregnancy diabetes are at increased risk of developing metabolic syndrome including type 2 diabetes [39]. Gestational diabetes was induced within 2 weeks of fructose feeding for our pregnant rats as determined by glucose AUC and glucose concentrations at 10 and 20 minutes after a glucose challenge. These data corroborate previous findings indicating hyperglycemia and greater maternal glucose concentrations during 2 hour glucose tolerance test in the dams fed 50% fructose compared with 50% sucrose [16]. Similar to the present study a replacement of sucrose with fructose increased maternal liver weight [16] although a reduction in feed intake with fructose feeding was not observed previously. The present data extends the previous observations to demonstrate that the effects of fructose on glucose metabolism are unique to pregnancy and lactation. The effect of fructose consumption during pregnancy has not been evaluated in human subjects despite evidence from epidemiological studies and variable-controlled studies linking dietary fructose intake with impaired glucose tolerance and insulin resistance in non-pregnant individuals [15]. The present data support the effect of fructose consumed during pregnancy to precipitate manifestation of gestational diabetes. Gestational diabetes increases the risk for future type 2 diabetes [38]. Thus, fructose consumption during pregnancy may indicate long-term health risk in mothers by inducing gestational diabetes.

Non-alcoholic fatty liver disease (NAFLD) has emerged as a prevalent health disorder [18, 39]. Triacylglycerol accumulation in the liver results when hepatic fatty acid uptake and endogenous lipid synthesis exceed the capacity for fatty acid oxidation and export as triacylglycerol rich very low-density lipoprotein (VLDL) particles [19]. Higher incidence of fatty liver is found in non-pregnant humans and laboratory animals with dietary protein deficiency [40] or high fructose consumption [41, 42]. Severe fatty infiltration has been demonstrated with low protein intake during lactation [43]. The data reported here extend those observations to indicate that the impact of low protein feeding is more severe in the lactating state compared with non-pregnant animals. Furthermore the data indicate that although both low protein and high fructose both induce liver lipid infiltration the severity of infiltration with fructose is significantly greater. The effects of the fructose diet cannot be attributed to a reduction in protein intake as dietary protein consumption dams receiving the fructose diet was 79% of the control dams compared with 29% of control values for low protein-fed dams.

Pregnancy and lactation are hallmarked by an increase in energy mobilization. In the present study we found lactation induced fatty liver that was further exacerbated by fructose or low

protein feeding. Although the lactating dams did not display increased NEFAs, serum TAGs and hepatic FAS were elevated while CPT-1a abundance was suppressed indicating perturbed lipid homeostasis. The low abundance of CPT-1a mRNA in lactating dams is consistent with elevated concentrations of insulin and mirrors the differences in CPT-1a in liver observed in cows between the non-lactating and lactating state [44]. The reduction in CPT-1a with fructose in the unmated controls is consistent with the effects of fructose to reduce lipid oxidation. Furthermore, insulin resistance in liver is linked to an increase in hepatic TAG production and VLDL output [45]. Low protein feeding during pregnancy and lactation has been previously reported to induce fatty liver in rats [42], similar to what we found. In previous reports, rats fed 60% fructose through only gestation had livers that were 26% heavier on the day of parturition than pregnant rats fed a diet containing 60% glucose [46]. The nature of the increase in liver weight was not determined in those studies. Fructose feeding through lactation in the current study caused a 10.8-fold increase in liver lipid. Because fructose was fed during pregnancy and lactation but liver lipid was only profiled at the end of lactation, we are uncertain of the timing of the onset of fatty liver. Based on previous literature we would expect that fatty liver was initiated during gestation in response to fructose feeding. The extremely elevated concentrations of liver lipid in the present study, compared with other data when fructose is fed during gestation, suggest that the effects of fructose on liver lipid are amplified during lactation.

It has been known that maternal protein restriction leads to metabolic disarrangements, such as insulin resistance, hypertension and dyslipidemia, in adult offspring [47]. Several protein restriction models result in reduced birth weight, a crude indicator of developmental compromise [48], which was also found in this study. As previously discussed, protein deficiency in the dams resulted in triacylglycerol accumulation in the liver. Dams fed the fructose diet had more severe liver lipid infiltration than did low protein-fed dams. It appears that fatty liver results from protein-restriction and may be a contributing factor for offspring. If fatty liver is a common predisposing factor for adverse metabolic programming on offspring then fructose feeding may present a more severe challenge.

Offspring of MatNuFR dams had lower weaning weights compared with MatNuCT offspring despite similar birth weights. The underlying reasons for these differences are not apparent. Although food intake was reduced for dams fed fructose there were no differences in body weight. Energy needs for milk production are met through dietary energy and body tissue mobilization, therefore a lack of difference in body weight change for fructose-fed rats and reduced weaning weight of offspring suggests reduced milk production. Milk production and composition was not determined in response to fructose feeding in the present experiment however lactating multiparous sows fed fructose had reduced milk production and reduced milk fat percentage [49]. Therefore it is reasonable to assume that impaired growth in the suckling phase for offspring from fructose-fed rats is the result of a reduction in quantity of nutrients available from milk to support growth. Food intake was also reduced for the low protein-fed dams and resulted in a loss of body weight in dams and reduced weaning weight of pups. The reduction in food intake for the low protein maternal group is similar to the effect of low protein-fed throughout pregnancy and lactation or low protein feeding initiated at parturition [50] where the effects of protein restriction during lactation act to limit growth to approximately 60% of the growth achieved from cross

fostering pups onto control dams during the suckling phase of growth [50]. The implications of restricted protein intake during gestation and lactation on growth and metabolic parameters of the offspring are well characterized and leads to predisposition to the metabolic syndrome [47] however the effects of maternal fructose consumption are less well-described.

We determined the effects of low protein and fructose feeding during pregnancy and lactation on key insulin-responsive transcripts in liver involved in glucose metabolism. All transcripts were sensitive to physiological status. Expression of gluconeogenic transcripts PEPCK-C, and PC as well as the metabolic regulator PGC-1 α were reduced in lactating rats. Expression of PEPCK-C in liver is reduced in response to insulin *in vivo* and *in vitro* [51, 52]. Under normal conditions pregnancy is associated with increased maternal insulin resistance whereas lactation is characterized by increased maternal insulin sensitivity in some tissues such as a muscle and a reduction in insulin sensitivity in adipose tissue [53]. Differences in food intake and weight change during lactation obscured our ability to determine the interactions effect of diet $\times$ physiological state. In particular the reduction in food intake during lactation in rats fed the MatNuFR diet resulted in significant weight loss during the 21 d lactation.

Fructose-fed pregnant rats in our study provide a fetal programming model of gestational diabetes. Consumption of added sugar by pregnant women is 14% of energy intake [32] only slightly less than the 16% of total energy for all Americans aged >2 years [8]. Although direct measures of fructose intake during pregnancy are not available in the literature there is no reason to suspect fructose consumption patterns differ for pregnant women compared with the general population. Data presented here suggest that the fructose consumption during pregnancy and lactation has a significant impact on gestational diabetes and hepatic steatosis. Additional information is necessary to determine the impact of lower amounts of dietary fructose on these parameters and potential risk for pregnant women.

Early life events that culminate in abnormal birth weight are widely accepted to be the origin that programs adverse long-term metabolic consequences in later life [54]. In our study, maternal fructose consumption did not appear to have changed offspring birth weight. However, these pups might have been influenced by two factors – maternal growth and gestational diabetes, impacting offspring birth weight in opposite directions. On one hand, gestational diabetes is a risk factor for increased birth weight and subsequently, metabolic syndrome in childhood and adolescence [55, 56]. On the other hand, lower maternal food intake and blunted body weight gain with fructose feeding prevents the offspring from adequate growth. As a result, the birth weight remained similar between offspring from dams fed the control (MatNuCT) and the dams fed the fructose (MatNuFR) diet. However, weaning weight differed with maternal diet. Maternal fructose feeding during lactation impaired offspring growth compared with the control offspring, observed in the offspring born to low protein-fed dams, which has long been considered a consequence of the maternal diet and predicts higher risk for the metabolic syndrome in later life of the offspring. Similarly using a model that does not restrict early postnatal growth is needed in order to ascribe causal relationship to maternal fructose consumption and metabolic diseases in offspring.

Fructose consumption during pregnancy and lactation induces glucose intolerance and fatty liver in the dams. Therefore, fructose intake during pregnancy and lactation not only leads to adverse responses in the dams, but also provides a fetal environment that may lead to topogramming that influences the health risk in offspring.

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Abbreviations

CT	control
FR	fructose
LP	protein diet
NALFD	non-alcoholic fatty liver disease
OGTT	oral glucose tolerance test
AUC	area under the curve
NEFA	non-esterified fatty acids
HOMA-IR	homeostasis model assessment of insulin resistance
CPT-1a	carnitine palmitoyltransferase-1
FAS	fatty acid synthase
PC	pyruvate carboxylate
PEPCK-C	phosphoenol pyruvate carboxykinase
PGC-1α	PPAR γ coactivator-1 α
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HSD	Honestly Significant Difference
VLDL	very low-density lipoprotein
TAG	triacylglycerol

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Figure 1.

Representative H&E staining of rat liver after 6 weeks of dietary treatment. Unmated female Sprague-Dawley rats (a, c, e) and 3-day-pregnant Sprague-Dawley rats (b, d, f) were fed either a control (a, b), a fructose (c, d) or a low protein (e, f) diet for 6 weeks. Liver samples were collected at the end of the diet treatment and stained with H&E. Representative Liver sample from rat dams fed the fructose diet was infiltrated with lipid droplets (d) compared with the liver sample from dams fed the control diet (b). Low protein feeding to the dams (f) also resulted in lipid droplet accumulation in the liver compared with the control diet, but the consequence was not as pronounced as fructose feeding. The central vein (CV) and portal vein (PV) and indicated where visible and the lipid droplets indicated within panels d and f, magnification for all panels is 100 $\times$.

Table 1

Ingredient and nutrient composition of diets used to treat rats during pregnancy and lactation and unmated controls.

Item	Control ³	Fructose ⁴	Low Protein ⁵
Casein, g/kg	200	200	90
L-cystine, g/kg	3	3	1.35
Corn Starch, g/kg	497.5	0	585.74
Maltodextrin, g/kg	132	0	155.41
Fructose, g/kg	0	629.5	0
Soybean Oil, g/kg	70	70	70
Cellulose, g/kg	50	50	50
Mineral ¹ , g/kg	35	35	35
Vitamin ² , g/kg	10	10	10
Choline, g/kg	2.5	2.5	2.5
Total Protein, g/kg	17.7	17.7	8.0
Total Carbohydrate, g/kg	59.1	64.7	69.2
Total Fat, g/kg	7.2	7.2	7.1
Total Energy, kcal/g	3.66	3.89	3.67

¹Mineral Mix: AIN-93G-MX (TD. 94046) (calcium carbonate, 35.70%; potassium phosphate, 19.60%; potassium citrate, 7.08%; sodium chloride, 7.40%; potassium sulfate, 4.66%; magnesium oxide, 2.43%; ferric citrate, 0.61%; zinc carbonate, 0.17%; manganous carbonate, 0.06%; cupric carbonate, 0.03%; potassium iodate, 0.001%; sodium selenate, 0.001%; ammonium paramolybdate, 0.0008%; sodium meta-silicate, 0.15%; chromium potassium sulfate, 0.03%; lithium chloride, 0.002%; boric acid, 0.008%; sodium fluoride, 0.006%; nickel carbonate hydroxide, 0.003%; ammonium meta-vanadate, 0.0007%; sucrose, 22.07%).

²Vitamin Mix: AIN-93-VX (TD. 94047) (niacin, 0.30%; calcium pantothenate, 0.16%; pyridoxine HCl, 0.07%; thiamin HCl, 0.06%; riboflavin, 0.06%; folic acid, 0.02%; biotin, 0.002%; Vitamin B₁₂ (0.1% in mannitol), 0.25%; Vitamin E, DL- α tocopheryl acetate (500 IU/g), 1.5%; Vitamin A Palmitate (500,000 IU/g), 0.08%; Vitamin D₃, cholecalciferol (500,000 IU/g), 0.02%; Vitamin K₁, phylloquinone, 0.008%; sucrose, 97.5%).

³Diet used for CT and MatNuCT treatments

⁴Diet used for FR and MatNuFR treatments

⁵Diet used for LP and MatNuLP treatments

Table 2

Real-Time PCR primers used to quantify CPT-1a, FAS, PEPCK-C, PC and PGC-1a RNA in liver from dams fed control, fructose or low protein diets during pregnancy and lactation and livers from unmated controls. All transcripts were normalized to the abundance of GAPDH.

Transcripts	Primer Direction	Primer Sequence
CPT-1a	F	TTGGGAAGCACTTGAGACAAGCCA
	R	TGTGCCCAATATTCCTGGAGCCAA
FAS	F	ACGTGACATTTTCATCAGGCCACCA
	R	TGTCTTTCCAGAGCAGCTTGCCTT
PEPCK-C	F	TCCGAACGCCATTAAGACCATCCA
	R	AGGTACTTGCCGAAGTTGTAGCCA
PC	F	AGAGTGCGCACACACGATCTCAAA
	R	ATGCCATTCTCTTTGGCCACCTCA
PGC-1a	F	ACCCACAGGATCAGAACAAACCCT
	R	ACTGCGGTTGTGTATGGGACTTCT
GAPDH	F	TCATGACCACAGTCCATGCCATCA
	R	TCATACTTGGCAGGTTTCTCCAGG

Table 3

Effect of treatment of fructose and low protein diets on food intake, body weight change for unmated controls and pregnant and lactating rats, as well as effect of maternal diet on offspring birth weight, weaning weight and BMI¹.

Physiological status	Unmated Controls				Pregnant / Lactating				p ²	
	Diet treatment	CT	FR	LP	MatNuCT	MatNuFR	MatNuLP	SE	Pir ³	PhxD
Prepartum, weeks 1 – 3										
Food intake, g		337 ^a	294 ^b	330 ^{ab}	351 ^a	314 ^{ab}	349 ^a	9	<0.05	<0.05
BW change ³ , g		23.8 ^b	19.0 ^b	25.9 ^b	86.4 ^a	77.0 ^a	82.5 ^a	2.7	<0.05	<0.05
Postpartum, weeks 4 – 6										
Food intake, g		426 ^d	336 ^d	370 ^d	1009 ^a	800 ^b	632 ^c	15	<0.05	<0.05
BW change, g		11.2 ^a	10.2 ^a	8.4 ^a	18.7 ^a	13.6 ^a	–16.9 ^b	3.0	0.06	<0.05
Birthweight ⁴ , g		--	--	--	6.7 ^a	6.7 ^a	5.8 ^b	0.23	--	<0.05
Weaned weight ⁵ , g		--	--	--	57.7 ^a	48.5 ^b	28.8 ^c	0.82	--	<0.05
BMI ⁶ , kg/m ²		--	--	--	5.51 ^a	4.77 ^b	3.61 ^c	0.08	--	<0.05

¹Least-square means and standard errors

²P value associated main effects

³Physiological status

⁴Body weight change

⁵Offspring birthweight

⁶Offspring weaning weight

⁷BMI of offspring at weaning

^{a, b, c, d}Means with different superscripts in the same row differ (P < 0.05)

Effects of fructose and low protein diets on glucose tolerance in unmated controls and pregnant rats following two weeks of experimental diet feeding¹

Table 4

Physiological status	Unmated Controls				Pregnant			p ²				
	Diet	Treatment	CT	FR	LP	MatNuCT	MatNuFR	MatNuLP	SE	Ph ³	Diet	Ph×D
Baseline, mg/dL			58.6 ^{bc}	71.0 ^a	63.3 ^b	55.8 ^{bc}	50.9 ^c	54.2 ^{bc}	1.7	<0.05	0.34	<0.05
10 min, mg/dL			79.0 ^{ab}	91.9 ^a	82.6 ^{ab}	64.0 ^b	75.6 ^{ab}	68.8 ^b	4.8	<0.05	0.05	0.97
20 min, mg/dL			82.9 ^a	84.7 ^a	81.2 ^a	58.6 ^b	72.4 ^{ab}	58.6 ^b	4.0	<0.05	0.06	0.27
30 min, mg/dL			82.3 ^a	78.8 ^a	81.0 ^a	56.7 ^b	56.6 ^b	57.7 ^b	3.0	<0.05	0.82	0.86
AUC, mg.dL ⁻¹ .min ⁻¹			4654 ^a	5201 ^a	4490 ^{ab}	3763 ^b	4302 ^{ab}	3829 ^b	263	<0.05	0.07	0.87

¹Least-square means and standard errors

²P value associated main effects

³Physiological status

*a, b, c, d*Means with different superscripts in the same row differ (P < 0.05)

Table 5

Effects of fructose and low protein diets on serum glucose, insulin, HOMA-IR, serum NEFAs, serum TAGs and liver lipid in unmated controls and lactating rats following six weeks of experimental diet feeding¹.

Physiological status	Unmated Controls				Lactating			p ²	
Diet Treatment	CT	FR	LP	MatNuCT	MatNuFR	MatNuLP	SE	Ph ³	Ph×D
Serum Glucose, mg/dL	115 ^c	127 ^c	110 ^c	180 ^a	169 ^{ab}	151 ^b	4	<0.05	<0.05
Serum Insulin, ng/mL	0.13 ^{ab}	0.10 ^b	0.15 ^{ab}	1.66 ^a	0.25 ^{ab}	0.70 ^{ab}	0.26	<0.05	0.11
HOMA-IR	0.15 ^a	0.13 ^a	0.17 ^a	2.99 ^a	0.41 ^a	1.01 ^a	0.46	<0.05	<0.05
Serum NEFA, mmol/mL	0.83 ^a	0.73 ^a	0.82 ^a	0.83 ^a	0.85 ^a	0.75 ^a	0.05	0.71	0.59
Serum Triglyceride, mg/dL	27.8 ^b	39.2 ^{ab}	45.4 ^{ab}	79.0 ^a	47.1 ^{ab}	61.2 ^{ab}	11.3	<0.05	0.61
Liver / Body Weight %	2.6 ^d	2.9 ^d	2.6 ^d	4.8 ^c	9.3 ^a	7.1 ^b	0.2	<0.05	<0.05
Liver TAG, mg/g liver weight	13.9 ^c	2.3 ^c	15.9 ^c	19.9 ^c	216.0 ^a	130.0 ^b	12.6	<0.05	<0.05

¹Least-square means and standard errors

²P value associated main effects

³Physiological status

a, b, c, d Means with different superscripts in the same row differ (P < 0.05)

Table 6

Effects of fructose and low protein diets on liver mRNA in unmated controls and lactating rats¹.

Physiological Status	Unmated Controls			Lactating			CI	P ²		
	Diet Treatment	CT	FR	LP	MatNuCT	MatNuFR	MatNuLP	Ph ³	Diet	Ph×D
PEPCK-C		1.00 ^a	0.37 ^{ab}	0.73 ^a	0.14 ^{ab}	0.22 ^{ab}	0.024 ^b	<0.05	0.27	0.12
PC		1.00 ^{ab}	0.18 ^{ab}	4.80 ^a	0.20 ^{ab}	0.10 ^b	0.03 ^b	<0.05	0.31	<0.05
CPT-1a		1.00 ^a	0.25 ^{ab}	1.46 ^a	0.09 ^{bc}	0.07 ^{bc}	0.02 ^c	<0.05	0.30	<0.05
FAS		1.00 ^c	5.90 ^{abc}	3.60 ^{bc}	47.1 ^a	14.9 ^{ab}	52.5 ^a	<0.05	0.51	0.05
PGC-1α		1.00 ^{abc}	4.13 ^a	2.81 ^{ab}	1.28 ^{abc}	0.47 ^{bc}	0.39 ^c	<0.05	0.79	<0.05

¹Least-square means and confidence intervals

²P value associated main effects

³Physiological status

a, b, c, dMeans with different superscripts in the same row differ (P < 0.05)