METABOLIC ALTERATIONS ASSOCIATED WITH MATERNAL UNDERNUTRITION DURING THE FIRST HALF OF GESTATION LEAD TO A DIABETOGENIC STATE IN THE RAT

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Running title: Early undernutrition favors diabetes in the mother

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ABSTRACT

Background: Although recent studies have investigated the effect of maternal nutrition on metabolic programming of the offspring, the question whether a nutritional insult during early gestation favours an altered metabolic state of the mother that persist during the remainder period of pregnancy, when foetal growth is maximal, remains to be answered.

Methods: To address this issue, we analysed the effect of 40% food restriction during the first 12 days of gestation on glucose tolerance, as well as on liver and adipose tissue metabolism, in Sprague-Dawley pregnant rats.

Results: We found that undernutrition at early gestation blocks pregnancy-associated accumulation of fat, leading to a net breakdown of lipids that may account for an increased delivery of fatty acids and glycerol to the liver. Together with altered expression of hepatic enzymes, this creates a catabolic state, characterised by decreased lipogenesis and increased β-oxidation that contributes to the ketonemia of underfed mothers. Furthermore, we observed that undernutrition during early pregnancy impairs insulin sensitivity at this stage and, importantly, exacerbates insulin resistance at late gestation, contributing to a diabetogenic state.

Conclusion: Undernutrition during the first half of pregnancy not only alters liver and adipose tissue metabolism, but also exacerbates the maternal insulin resistance at late gestation, which may increase their risk of gestational diabetes.

General significance: Together, these findings highlight the persistent impact of maternal nutrition during early gestation on the metabolism of the mother during late pregnancy.

Keywords: Adipose tissue, Foetal programming, Gestational Diabetes, Insulin resistance, Liver.

Abbreviations: AUC, area under the curve; CPT1, carnitine palmitoil acyl transferase; HOMA-IR, homeostasis model assessment of insulin resistance; ISI, insulin sensitivity index; LPL, lipoprotein lipase; LXR, liver X receptor, NEFA, non-esterified fatty acids; OGTT, oral glucose tolerance test; PEPCK, phosphoenolpyruvate carboxykinase; PPAR, peroxisome proliferator activated receptor; RXR, retinoid X receptor; QUICKI, quantitative insulin sensitivity check index; SREBP-1, sterol regulatory element binding protein 1.

INTRODUCTION

Nutrition during early stages of life plays a pivotal role in foetal development and may promote permanent changes in the physiology and metabolism of the offspring [23]. It is well established that impaired pre- and postnatal growth, when followed by accelerated weight gain, potentially predispose the offspring to develop type 2 diabetes, obesity and cardiovascular disease in the adult life [24]. A deficient maternal food intake or altered metabolism, may have important consequences on foetal maturation and postnatal development. In fact, maternal malnutrition in rats affects placental transport of nutrients, and subsequently the availability of substrates for the foetus [11, 13]. Furthermore, maternal nutrient restriction during early-to-mid-gestation significantly affects placental growth and morphology in the sheep, causing weight reduction of the foetal component of the placentome [4].

Various studies have reported that undernutrition during pregnancy led to intrauterine growth retardation and long-term effects on glucose tolerance in the offspring [18, 36]. Accordingly, in a previous study of our group, undernutrition during early pregnancy in the rat decreased both the number of alive pups and their body weight [17]. Furthermore, while growth retardation disappeared during lactation, both male and female pups from underfed mothers developed glucose intolerance at 16 weeks of age [17].

Although several studies have investigated the effect of maternal malnutrition on glucose metabolism of the offspring rat [18, 36], to our knowledge little attention has been paid to the effects of nutrient restriction on maternal body composition and maternal metabolism during pregnancy. In normal pregnancy, the early phase of gestation is characterized by increased fat accumulation [13, 19] due to the enhanced insulinaemia and insulin sensitivity of the mother [29]. However, late pregnancy is characterized by insulin resistance, low lipoprotein lipase (LPL) activity in adipose tissue, and increased lipolytic activity that leads to the breakdown of fat depots [21]. Under fasting conditions during late pregnancy, glycerol is efficiently employed for gluconeogenesis by the mother, and glucose is transferred to the foetus [14]. Fatty acids can be oxidized for energy production or, alternatively, be used for ketogenesis. Ketone bodies can be

used by maternal tissues or transferred to the foetus and employed as oxidative fuels [14] or for lipid synthesis in the brain [26].

Impaired maternal fat accumulation during early pregnancy, like in hypothyroidism [1], diabetes [6] or undernutrition during early pregnancy [18], affects the disposal of energetic fuels at late pregnancy and causes intrauterine growth retardation. These antecedents point to the critical role of metabolic adaptation of the mother in early pregnancy for determining pregnancy/neonatal outcomes, although foetal growth during this phase is minimal.

We hypothesized that a nutritional insult during just the first half of gestation could favour an altered metabolic state of the mother, and that this alteration may impair foetal development, predisposing the offspring to develop diseases in the adult life [24]. To address this issue, we used an experimental rat model to determine the effect of maternal undernutrition during the first half of gestation on maternal liver and adipose tissue metabolism and maternal glucose/insulin relationships later in pregnancy.

2. MATERIALS AND METHODS

2.1 Animals and sample collection

Female Sprague-Dawley rats were housed at 22-24º C with 12 h light cycles, from 08:00 to 20:00 h, and free access to water and chow diet (Panlab, Barcelona, Spain). Animals were mated when weighing between 180-210 g. After conception (day 0 of pregnancy), one group of animals was fed *ad libitum* all the time (control), and the other was allowed to eat 60% of the amount of food consumed by the controls until day 12 of gestation (underfed). The underfed group received, between day 12 and day 20 of gestation, the same amount of food as the control animals to avoid compensatory hyperphagia.

Animals were decapitated under CO_2 anaesthesia in non-fasting conditions. Lumbar adipose pads and liver were dissected, snap-frozen in liquid nitrogen, and stored at -80ºC. Blood was collected from neck wounds in EDTA-tubes and plasma was kept at -20 ºC. All the animals were maintained in accordance with European Union Laboratory Animal Care Rules (86/609/ECC directive) and the protocols approved by the Animal Research Committee of CEU-San Pablo University.

2.2 Plasma analysis

Commercial enzymatic tests were used to determine in plasma samples: glucose (GOD-PAP, Roche Diagnostics, Spain), triacylglycerides (LPL/GPO-Trinder Roche Diagnostics, Spain), glycerol (GPO-Trinder, Sigma Diagnostic, Spain), cholesterol (Menarini, Italy), non-esterified fatty acids (NEFA) (ACS-ACOD, Wako Chemicals GmbH, Germany) and ketone bodies (Wako Chemicals GmbH, Germany). Plasma insulin was determined with a specific EIA kit for rats (Mercodia, Denmark). Corticosterone, leptin and adiponectin were determined in plasma samples by X-Map Technology (Bioplex 100X, Spain) using the "rat stress hormone" RSH69K and "rat adipocyte" RADPCYT-82 panels (Millipore, Spain).

2.3 Oral glucose tolerance test and surrogate insulin sensitivity indices

Subsets of animals from each experimental group were subjected to an oral glucose tolerance test. A solution of glucose (2g/kg) was administered to the animals by oral gavage to overnight-fasted rats. Blood samples from the tail vein were collected immediately before, and 5, 10, 15, 20, 30, 45 and 60 min after glucose administration. Plasma glucose and insulin were analysed. The areas under the curve for glucose and insulin, the insulin sensitivity index (ISI_{OGTT}), the HOMA-IR and the QUICKI index were calculated as previously [3].

2.4 Lipid content and lipoprotein lipase activity in tissues

Lipid extraction and purification from liver were performed in chloroform-methanol [9], and the different lipids fractions were resolved by thin layer chromatography and quantified using an image analyser [31]. Lipoprotein lipase (LPL) activity in adipose tissue was analysed [30].

2.5 mRNA expression of proteins in liver

RNA was isolated with the Rneasy Mini kit (Qiagen, CA, USA). cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, USA) and was used for real-time qPCR using the SYBR green RT-PCR method (Bio-Rad, USA) and a CFX96 Real Time System (Bio-Rad, USA). GADPH and β-actin were used as reference standards. The primer sequences used are shown in *Table 1.*

2.6 Statistical analysis

Results are expressed as mean ± SEM of 6 animals per group. When data were not normally distributed, log-transformed values were used for statistical analysis. Statistical comparisons between two groups were made using the Student´s t-test; comparisons between three or more groups were based on the analysis of the variance (ANOVA) with 1 or 2 ways, followed by Student-Newman Keuls as *post hoc test*, using the GraphPad Prism program (version 5).

3. RESULTS

3.1 Effect of undernutrition during early pregnancy on body weight.

As shown in *Fig1a*, maternal body weight progressively increased during gestation in control rats, whereas undernutrition abolished any weight increase during the first 12 days of pregnancy. After day 12, underfed rats were allowed to eat the same amount of food than the controls to avoid compensatory hyperfagia [28]: 30.0 ± 1.5 g/day in controls and 29.2 ± 0.30 g/day in underfed pregnant rat. Nonetheless, underfed mothers did not catch up the body weight of age-matched controls. As shown in *Fig.1b,* body weight free of conceptus progressively increased during pregnancy in controls, whereas underfed mothers didn't change body weight along the 12 days of pregnancy and latterly increased but never reached the weight attained by controls.

Although, from day 12 onwards, underfed mothers were pair-fed to controls, at day 20 of gestation their total body weight and the body weigh free of conceptus were still significantly lower than in controls. Besides, at day 20 of gestation, the number of foetuses per litter was significantly lower in the underfed mothers (11.43 ± 0.55) than in controls (13.06 ± 0.36, $p < 0.05$).

3.2 Effect of undernutrition during early pregnancy on adipose tissue fat accumulation.

In control mothers, the weight of lumbar (*Fig.2a*) and mesenteric (*Fig.2b*) adipose tissues increased during pregnancy, whereas in underfed mothers both fat depots diminished during the first 12 days of pregnancy and completely recovered by day 20 of gestation. Lumbar adipose tissue lipoprotein lipase (LPL) activity (*Fig.2c*) increased in controls during the first 12 days of pregnancy and returned to basal levels (day 0) at the end of gestation (day 20). However, LPL

activity in underfed mothers remained low throughout gestation. Liver weight increased during pregnancy in controls (*Fig.2d*), whereas liver weight decreased during the first 12 days of pregnancy in underfed mothers to be partially recovered at day 20.

3.3 Effect of undernutrition during early pregnancy on hepatic lipid content.

At day 12 of gestation, total hepatic lipid content was significantly lower in underfed than control mothers whereas no differences were observed at day 20 (*Table 2)*. Analysis of lipid fractions revealed that both phospholipids and triacylglycerides were significantly lower in underfed mothers than in control mothers at day 12 of gestation and these differences disappeared at day 20 of gestation.

3.4 Effect of undernutrition during early pregnancy on plasma metabolites and hormones.

Undernutrition during early pregnancy alters plasma parameters and the normal metabolic adaptations to pregnancy (*Fig.3)*. At day 12 of gestation, underfed mothers had significantly lower circulating levels of insulin, triacylglycerides and cholesterol than control mothers, whereas these differences disappeared at day 20 of gestation. NEFA and ketone bodies (*Fig.3e-3g*) were significantly higher at day 12 in the underfed mothers, and this increase was further exacerbated at day 20. Furthermore, at day 20 of gestation, circulating glycerol was also significantly higher in the underfed mothers (*Fig.3f*). Whereas circulating leptin increased at mid-gestation in the control group, undernutrition completely abolished this increase. Although from day 12 onwards all animals were pair-fed, leptin levels remained significantly lower in the underfed mothers (*Fig.3h*). Finally, corticosterone levels were not affected by undernutrition in the early pregnancy, but were significantly higher at late pregnancy in the underfed rats than in controls (*Fig.3i*).

3.5 Effect of undernutrition during early pregnancy on glucose tolerance.

We also evaluate whether glucose tolerance was affected by undernutrition and did not find any difference between the two groups in the areas under the curve of glucose (AUC) and insulin at

day 12 of pregnancy (*Fig.4*). At late pregnancy, the AUC for glucose and insulin were significantly higher in the underfed mothers, indicating deteriorated glucose tolerance.

Furthermore, as shown in fig. 5 the significantly higher HOMA-IR, as well as the lower QUICKI and ISI indexes in the underfed pregnant animals at 12 and 20 days of gestation further support that underfeeding during the first part of pregnancy may be associated with an insulin resistant state, that develops to a greater extent, than the insulin resistant condition normally present at late pregnancy.

3.6 Effects of undernutrition during early pregnancy on hepatic mRNA expression of key enzymes involved in lipid and glucose metabolism.

We next investigate the expression of key enzymes involved in lipid and glucose metabolism. At day 12 of gestation the expression of phosphoenolpyruvate carboxykinase 1 (PEPCK-1) and glucose 6-phosphatase were significantly higher and tend to remain higher at day 20 of gestation in underfed mothers (*Fig.6a)*. At day 20, the expression of fructose 1,6-bisphosphatase was also higher in the group of underfed mothers.

Although there was a tendency in underfed mothers to express less pyruvate dehydrogenase α1 (*Fig.6c*), these differences were neither statistically significant at day 12 nor 20 of gestation (two way anova, $F(2, 18)= 2.740$, $P= 0.0914$; $F(1, 18)= 0.7028$, $P= 0.4128$, for the effect of pregnancy and undernutrition, respectively).

We also measured the hepatic expression of key enzymes of lipogenesis (*Fig.6b)* Fatty acid synthase expression was neither modified by pregnancy nor by undernutrition (two way anova, $F(2, 18)= 1.891$, $P= 0.1797$; $F(1, 18)= 0.5507$, $P= 0.4676$, respectively). However, mRNA levels of acetyl-CoA carboxylase-α were lower in underfed rats at day 12 of pregnancy, but no differences were observed at day 20.

We also determined the expression of carnitine palmitoyltransferase 1 (CPT1) and acyl-CoA synthethase, both involved in fatty acid β-oxidation (*Fig.6d*). The expression of acyl-CoA synthethase was not modified neither by pregnancy nor by undernutrition (two way anova, F(2, 18)= 2.933, *P*= 0.0789; F(1, 18)= 0.0278, *P*= 0.8689, respectively). The expression of CPT-1, however, was affected both by pregnancy and undernutrition (F(2, 17)= 22.72, *P*< 0.0001; F(1, 17)=9.299, *P*= 0.0072, respectively). Expression of CPT1 was significantly increased in control pregnants rats and further increased in underfed mothers both at day 12 and 20 of gestation.

The expression of PPAR-α and RXR (*Fig.6f)* increased during gestation and undernutrition produced a significant increase both at day 12 and 20 of gestation in hepatic expression of PPAR-α (two way anova, F(2, 18)= 82.19, *P*<0.0001; F(1, 18)= 42.19, *P*< 0.0001, for pregnancy and undernutrition, respectively) and RXR (two way anova, F(1, 18)= 25.66, *P*<0.0001; F(2, 18)= 6.58, *P*=0.0072, for pregnancy and undernutrition, respectively). Although LXR expression was not affected by pregnancy in control mothers, the expression LXR tends to be higher in the underfed mothers.

We next analysed the expression factors involved in the transcriptional regulation of key enzymes in lipogenesis and gluconeogenesis. Although the expression of SREBP-1 *(Fig.6f)* was not modified during gestation in control mothers, early maternal malnutrition produced a significant decrease in hepatic SREBP-1 expression, both at day 12 and 20 of gestation (two way anova, F(2, 17)= 0.3978, *P*= 0.6779; F(1, 17)= 18.830, *P*= 0.0004, for pregnancy and undernutrition, respectively).

4. DISCUSSION

During the past years, an increasing number of studies has addressed the effect of maternal malnutrition during pregnancy and/or lactation on the offspring [7]. However, to our knowledge, this is the first study addressing the effects of maternal malnutrition on maternal metabolism and insulin responsiveness. We hypothesized that undernutrition at early pregnancy may alter maternal metabolic adaptations, entailing negative consequences for glucose/insulin homeostasis at late pregnancy when foetal growth is maximal. To address this issue, we analysed the effect of a 40% restriction of food intake during the first 12 days of gestation on glucose tolerance and on liver and white adipose tissue metabolism in the mother. During early pregnancy, the increase in maternal body weight is mainly due to a progressive accumulation of maternal fat, increased adipose tissue lipogenesis and glycerolgenesis, caused by enhanced insulin responsiveness [29]. Here, we show that undernutrition during the first 12 days of gestation blocks the pregnancy-associated maternal increment of body weight, and also reduces maternal fat depots in both lumbar and mesenteric adipose tissue. Although after the restriction period fat accumulation in lumbar and mesenteric adipose tissue was recovered, in the underfed mothers this compensatory fat accretion during the second half of gestation was not enough to recuperate the body weight found in control mothers. Furthermore, that altered lipid accumulation affects not only the white fat but also the liver. During the period of restriction, maternal malnutrition was associated with a decrease of both weight and hepatic lipid content, in particular of triacylglycerides and phospholipids. Although this altered pattern of lipid accumulation disappeared when normal feeding conditions were re-established, liver weight remained significantly smaller in underfed mothers during the second half of gestation. The observed decrease in hepatic lipid content in underfed mothers at day 12 but not at day 20 of gestation could be explained by either a reduced hepatic uptake of triacylglycerides from chylomicrons remnants or decreased lipogenesis. In fact, plasma non-fasting triacylglycerides were significantly lower in underfed mothers at day 12 of pregnancy but not at day 20. Secondly, undernutrition during the first 12 days of gestation may favour a catabolic condition in liver, characterized by a decrease in lipogenesis and an increase in β-oxidation, which is supported by the ketonemia and also by changes in the expression of key enzymes of hepatic lipid metabolism.

We found that, at day 12 of gestation, underfed mothers had a higher expression of CPT-1A than control mothers, presumably related to increased β-oxidation of fatty acids, but also a lower expression of acetyl-CoA carboxylase, the limiting enzyme of lipogenesis. Thus, undernutrition during early pregnancy favours a net catabolic condition in maternal liver, characterized by diminished synthesis and enhanced oxidation of fatty acids.

This catabolic state of the liver, induced by the undernutrition of the mother during the first 12 days of pregnancy, is maintained until the end of gestation despite access to the same amount of food than controls from day 12 onwards. In fact, in control rats, pregnancy was accompanied by an increase in the CPT-1A expression at late pregnancy that may reflect on increased fatty acid β-oxidation, which may facilitate a reduction in maternal glucose utilization increasing its availability to the growing foetus. In this scenario, and despite normalization of acetyl-CoA carboxylase expression at day 20, CPT-1A expression in underfed mothers increased even more than in controls during late gestation, pointing to increased fatty acid β-oxidation and ketogenesis in the liver as a consequence of undernutrition during the first 12 days of pregnancy. This interpretation fits with the exaggerated increase in circulating ketone bodies seen at late pregnancy in these animals even in non-fasting conditions.

Since SREBP-1c is a major mediator of insulin action on hepatic gene expression and a key regulator of hepatic glucose/lipid metabolism [8], we analysed whether an altered expression of this factor may account for the metabolic alterations observed in the underfed mother. The expression of SREBP in both 12 and 20-day pregnant rats was significantly decreased by undernutrition. Different factors could account for this effect. It is known that SREBP-1c is induced by insulin in isolated hepatocytes [10], decreased in streptozotozin diabetic rats, and normalized in diabetic animals after insulin treatment [34]. Furthermore, fasting and glucagon, have also previously been reported to decrease the expression of SREBP-1c through cAMP signalling [10]. Unlike other isoforms of this transcription factor, such as SREBP-2, nuclear localization of SREBP-1c is not increased in the case of low cholesterol availability [8]. Altered glucose/insulin homeostasis, thus, may favour hepatic expression of SREBP, which may account for diminished expression of acetyl-CoA carboxylase and, consequently, for decreased synthesis of fatty acids. In

fact, in SREBP-1 knockout mice, ablation of this transcription factor severely impaired the hepatic expression of genes involved in fatty acid synthesis, such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase [33]. We therefore propose that decreased expression of SREBP-1, even during late gestation, in rats that were underfed during early pregnancy may be related to their enhanced insulin resistant condition.

We also analysed whether the expression of PPARα, LXR and RXR, may contribute to the metabolic alterations observed in the underfed mother. The expression of LXR was not affected either by pregnancy nor undernutrition. However, the expression of PPARα and RXR increased during the catabolic stage of pregnancy in the control group, being higher in the 20-day control mothers than in virgin animals. PPARα activation is responsible of the induction of several genes during starvation. Among others PPARα induce the expression of fatty acid transporters, fatty acid activation genes, and key enzymes of mitochondrial fatty acid oxidation, and knocking down PPARα completely block this induction [20]. According to this, undernutrition during the first half of gestation have shown to increase the expression of these transcription factors in the liver of underfed mothers. In fact, the expression of PPARα and RXR were higher in both 12 and 20 days underfed pregnant rats when compared to control mothers. It is well known that the expression of liver CPT-1A gene is induced via PPARα by fasting, high fat diets and PPARα ligands [35]. Thus, PPARα activate the transference of long chain fatty acids across the mitochondrial membranes and mitochondrial oxidation by activating the expression of CPT-1A. Besides, in the fasting state PPARα also stimulates the synthesis of ketone bodies, that can be used as energetic substrates by extrahepatic tissues [20] and also by the foetus during late pregnancy. Taken together, these results may also contribute to explain the exacerbated catabolic state, the increased β-oxidation of fatty acids, the higher expression of CPT-1A and the hyperketonemia that were observed in the underfed mothers.

In the early stage of normal pregnancy, increased maternal insulin sensitivity, hyperphagia and hyperinsulinemia, sets the scene for the maternal increment of body fat through increased lipid synthesis [16, 25, 29], enhanced LPL activity [15] and decreased lipolytic activity of adipose tissue

[29]. These metabolic adaptations may be impaired by nutritional alterations during early pregnancy. The present study provides evidence that undernutrition during early pregnancy blocks the physiologic induction of LPL activity in maternal adipose tissue and, in parallel, favours an increase of lipolysis as suggested by the elevation in plasma NEFA. This net breakdown of fat depots may account for an increased delivery of NEFA to the liver, and, as a consequence of enhanced β-oxidation, contribute to the ketonemia observed in underfed mothers. Glycerol, the other product of lipolysis, may be used for hepatic gluconeogenesis in order to maintain glucose availability to the foetus. This hypothesis is supported by the upregulated expression of two key enzymes of hepatic gluconeogenesis, PEPCK-1 and glucose 6-phosphatase in the liver of underfed mothers at day 12 of gestation. This increment of glucose synthesis during the period of food restriction may be explained by increased glucagon levels, and by the reduced expression of the transcription factor SREBP-1c. In fact, in mice lacking the SREBP-1c isoform [22], mRNA levels of PEPCK were 3-fold higher under post-absorptive conditions than in control mice. Moreover, it has been proposed that PPARα may have a stimulatory effect on glucose synthesis by a direct induction of glucose 6-phosphatase [37], supporting the glucocorticoid action on gluconeogenesis [27] and also modulating hepatic insulin action [5].

Although from day 12 of pregnancy onwards, all pregnant rats were pair-fed, the observed lipolytic condition of adipose tissue was further exacerbated at late pregnancy in underfed rats. At day 20 of gestation underfed mothers exhibited even higher levels of circulating NEFA and glycerol than control mothers. In the third trimester of a normal pregnancy, plasma ketone body levels remain low in a non-fasting state [12]. Ketonemia, however, greatly increases under fasting conditions [32] or in diabetes [2] as a consequence of enhanced adipose tissue lipolysis, delivery of NEFA to the liver and enhanced ketogenesis. Our results point to a strongly enhanced synthesis of ketone bodies at day 20 of gestation in the early-pregnancy underfed mothers, suggesting that the nutritional intervention in early pregnancy may favour a diabetogenic condition in the pregnant mother.

This notion is further supported by results we obtained from the OGTT and the indexes of insulin resistance or sensitivity. The increased HOMA-IR and decreased QUICKI and ISI-OGTT suggest

an insulin-resistant state at day 12 of gestation in underfed mothers that was not normalized during late pregnancy, even when the mother was not submitted to food restriction. In fact, insulin resistance and lipid turnover were further exacerbated as shown by an increment of the glucose/insulin ratio, NEFA, glycerol, ketone bodies, corticosterone, HOMA-IR, and the decrease in QUICKI and ISI-OGTT. These results together with the lower number of foetuses in the underfed mothers than in controls provide evidence for altered glucose homeostasis in underfed pregnant rats that may alter foetal development. In fact, a previous study from our group, showed that at delivery, both the number of alive newborns and their body weight were significantly lower in the early pregnancy underfed mothers than in the control mothers [18]. These results suggest that despite of the small foetal growth that takes place during the first half of pregnancy, an impairment of the mother to accumulate fat depots during this specific phase clearly damages the normal intrauterine development.

The results of present study show, to our knowledge for the first time, that undernutrition during the first half of pregnancy not only impairs insulin sensitivity at this stage of pregnancy, driving some of the maternal metabolic adaptations, but also exacerbates the insulin resistant condition at the end of gestation. Undernutrition during early pregnancy, thus, favours a diabetogenic state that may eventually lead to gestational diabetes, and might also account for glucose intolerance of the pups from these mothers when adults, highlighting the relevance of maternal metabolism at early gestation.

AUTHORS CONTRIBUTION

M.L. performed experiments, analysed data, and contributed to edition of the manuscript; J.S., performed experiments, analysed data, and contributed to edition of the manuscript; MG.S. performed experiments; E.H. designed the study, contributed to the supervision of data interpretation; MP.R. designed the study, performed data analysis, coordinated data interpretation and wrote the manuscript. All authors reviewed and accepted the manuscript.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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FIGURE LEGENDS

Figure 1. a) Body weight increase in controls (○) and underfed rats during gestation (●). b) Change of maternal body weight free of conceptus. Different capital letters show differences in control rats at different days of gestation, different lowercase letters show differences in underfed rats at different days of gestation. Differences between control and underfed pregnant rats are shown by: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Figure 2. Adipose tissue weights and LPL activity in lumbar adipose tissue in non-pregnant (day 0), control pregnant rats and pregnant rats underfed at days 0, 12 and 20 of gestation: Lumbar (a) and mesenteric adipose tissue weight (b); LPL activity in lumbar adipose tissue (c); liver weight (d). Different capital letters show differences regarding the effect of gestation (non-pregnant *vs* control pregnant); different lowercase letters show differences between non-pregnant and underfed pregnant rats. Differences between control and underfed pregnant rats are shown by: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Figure 3. Plasma biochemical parameters in control (○) and underfed pregnant rats (●). Glucose (a); Insulin (b); Triacylglycerides (c); Cholesterol (d); NEFA (e); Glycerol (f); Ketone bodies (g); Leptin (h); Corticosterone (i). Different capital letters show differences regarding the effect of gestation in control rats; different lowercase letters show differences in underfed pregnant rats regarding the effect of gestation. Differences between control and underfed pregnant rats are shown by: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Figure 4. Oral glucose tolerance test in non-pregnant (discontinuous line), control pregnant rats (gray line) and underfed pregnant rats during the first half of gestation (black line). Non-pregnant rats (a), 12-days pregnant rats (b) and 20-days pregnant rats (c). Areas under the curves for glucose (AUG) and insulin (AUI) during the oral glucose tolerance test are shown as insets. Differences in the AUG and AUI between control and underfed pregnant rats are shown by: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Figure 5. Indexes of insulin sensitivity of non-pregnant (day 0), control (○) and pregnant rats underfed during the first half of gestation (●) at different days of gestation. a) HOMA-IR, an insulin resistance index; b) QUICKI and c) ISI-OGTT are insulin sensitivity indexes. Different capital letters show differences regarding the effect of gestation (non-pregnant *vs* control pregnant); different lowercase letters show differences between non-pregnant and underfed pregnant rats. Differences between control and underfed pregnant rats are shown by: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Figure 6. Hepatic expression of enzymes (mRNA) in non-pregnant rats (0d) (□), and control (C) (■) and underfed rats (U) (■) at 12 and 20 days of gestation. Data represent fold expression relative to a non-pregnant rat. **a**) Enzymes of gluconeogenesis: PEPCK: Phosphoenolpyruvate carboxykinase 1; Glucose 6-phosphatase; Fructose 1,6-bisphosphatase 1. **b)** Enzymes of lipogenesis: Fatty acid synthase; Acetyl-CoA carboxylase α. **c)** Pyruvate dehydrogenase α1. **d)** Enzymes of β-oxidation: Acyl-CoA synthetase; CPT1. **e)** Transcription factors: PPARα, RXR and LXR. **f)** Transcription factors: SREBP-1. Different capital letters show differences in controls regarding the effect of gestation different lowercase letters show differences in underfed rats regarding the effect of gestation. Differences between control and underfed pregnant rats are shown by: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

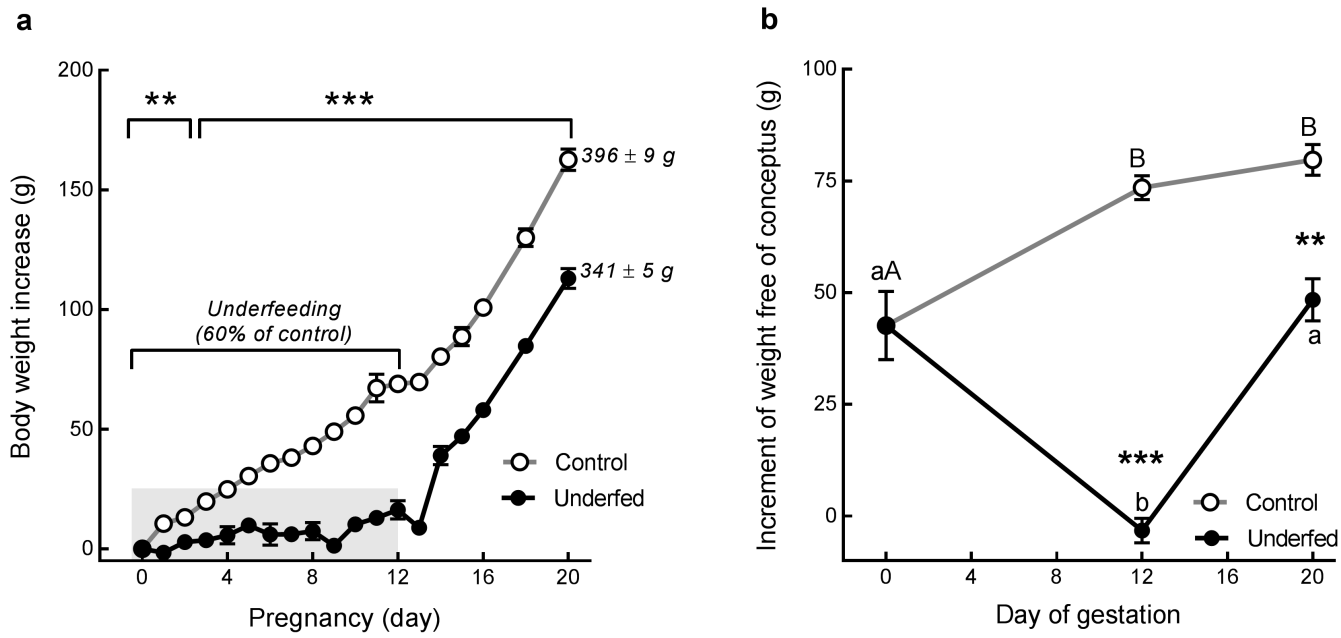
Fatty acid synthase	Forward-	CTATTGTGGACGGAGGTATC
	Reverse-	TGCTGTAGCCCAGAAGAG
Phosphoenolpyruvate carboxykinase	Forward-	CTACAACTTCGGCAAGTACC
	Reverse-	TTCCTTAGAGATTCCGAACA
Fructose 1,6-bisphosphatase	Forward-	TCACCTGCCTGCACCTTTAG
	Reverse-	GTCACATTGGTTGAGCCAGC
Glucose-6-phosphatase	Forward-	CGTGGAAAGAAAAAGTCAAC
	Reverse-	GTAAAATCCAAGTGCGAAAC
Sterol regulatory element binding protein-1c	Forward-	GACGACGGAGCCATGGATT
	Reverse-	GGGAAGTCACTGTCTTGGTTGTT
Ppar-α	Forward-	CATGGTGGACACAGAGAGCC
	Reverse-	CTGGAAGCTGGAGAGAGGGT
Rxr-α	Forward-	ACATCTGCGCTATCTGTGGG
	Reverse-	TGTCGATCAGGCAGTCCTTG
Lxr	Forward-	TGCCCACTTTACTGAGCTGG
	Reverse-	GTTACCTCCGCGATGTCTCC
Glyceraldehyde 3-phosphate dehydrogenase	Forward-	TGACTCTACCCACGGCAAG
	Reverse-	GCCAGTAGACTCCACGACA
β-actin	Forward-	GGCACCACCATGTACCCA
	Reverse-	ACACAGAGTACTTGCGCTCA

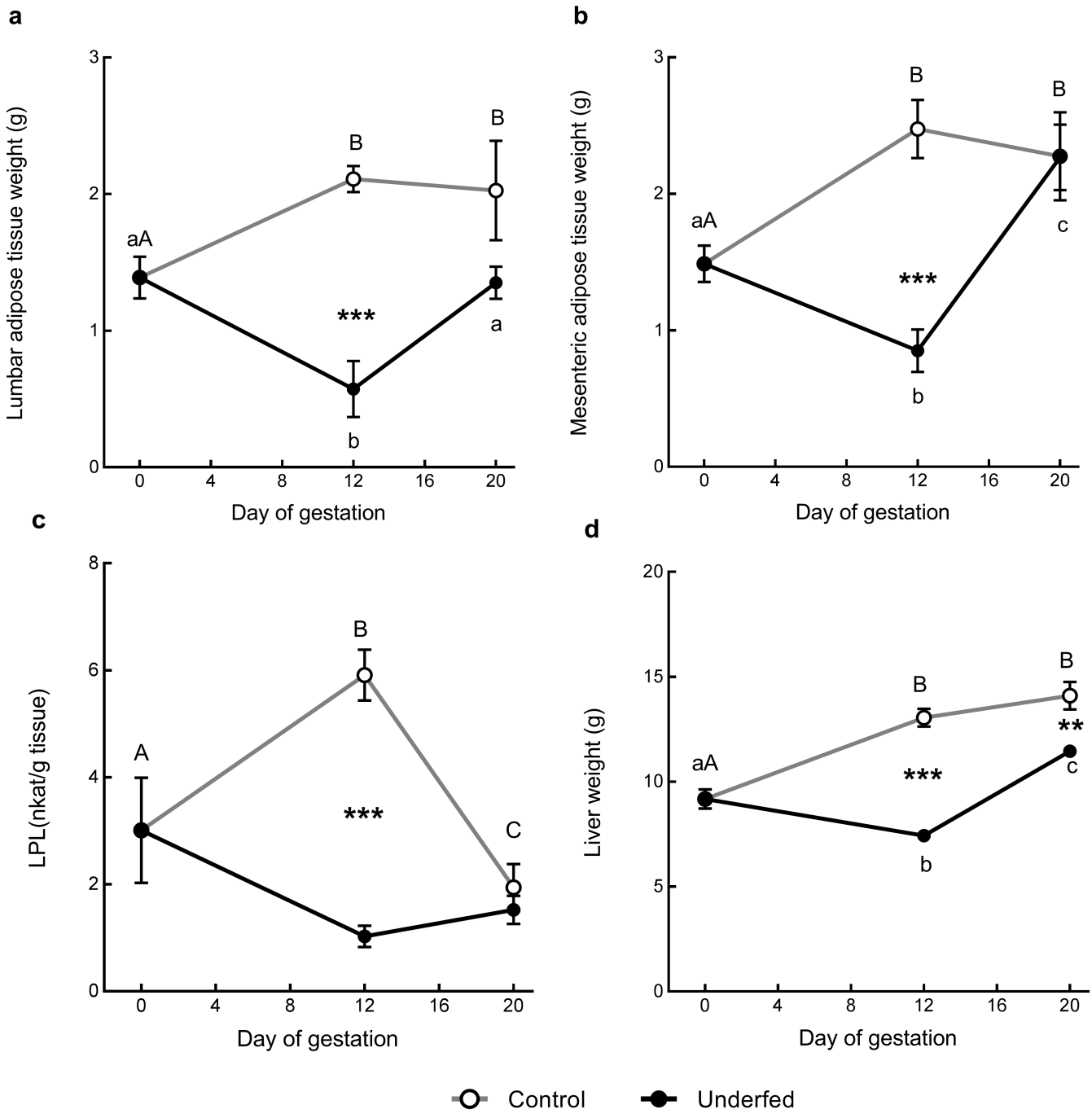
Table 1. Primer sequences used for real-time PCR

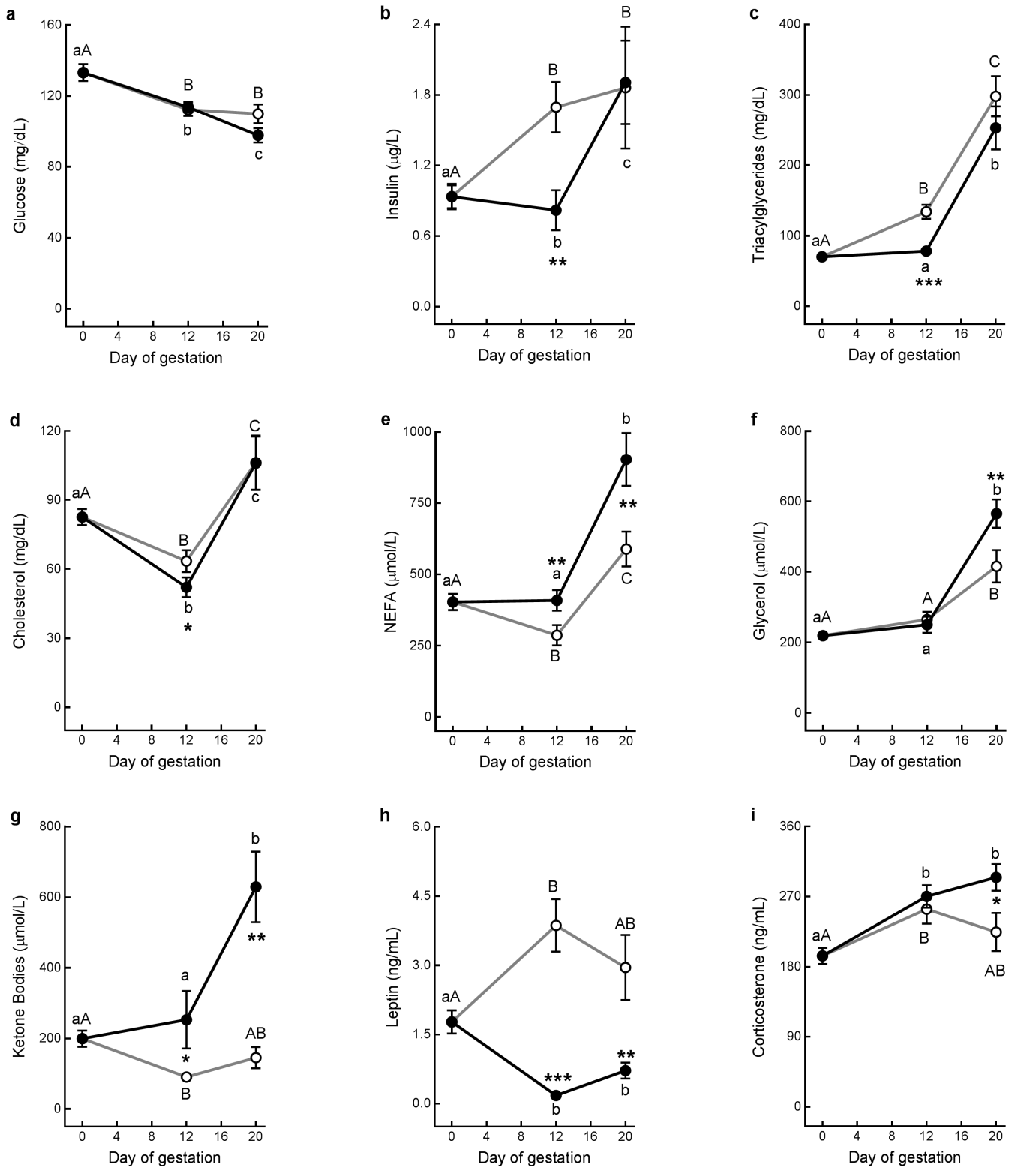
Table 2. Hepatic lipid content in non-pregnant, control pregnant rats and pregnant rats underfed during the first half of gestation.

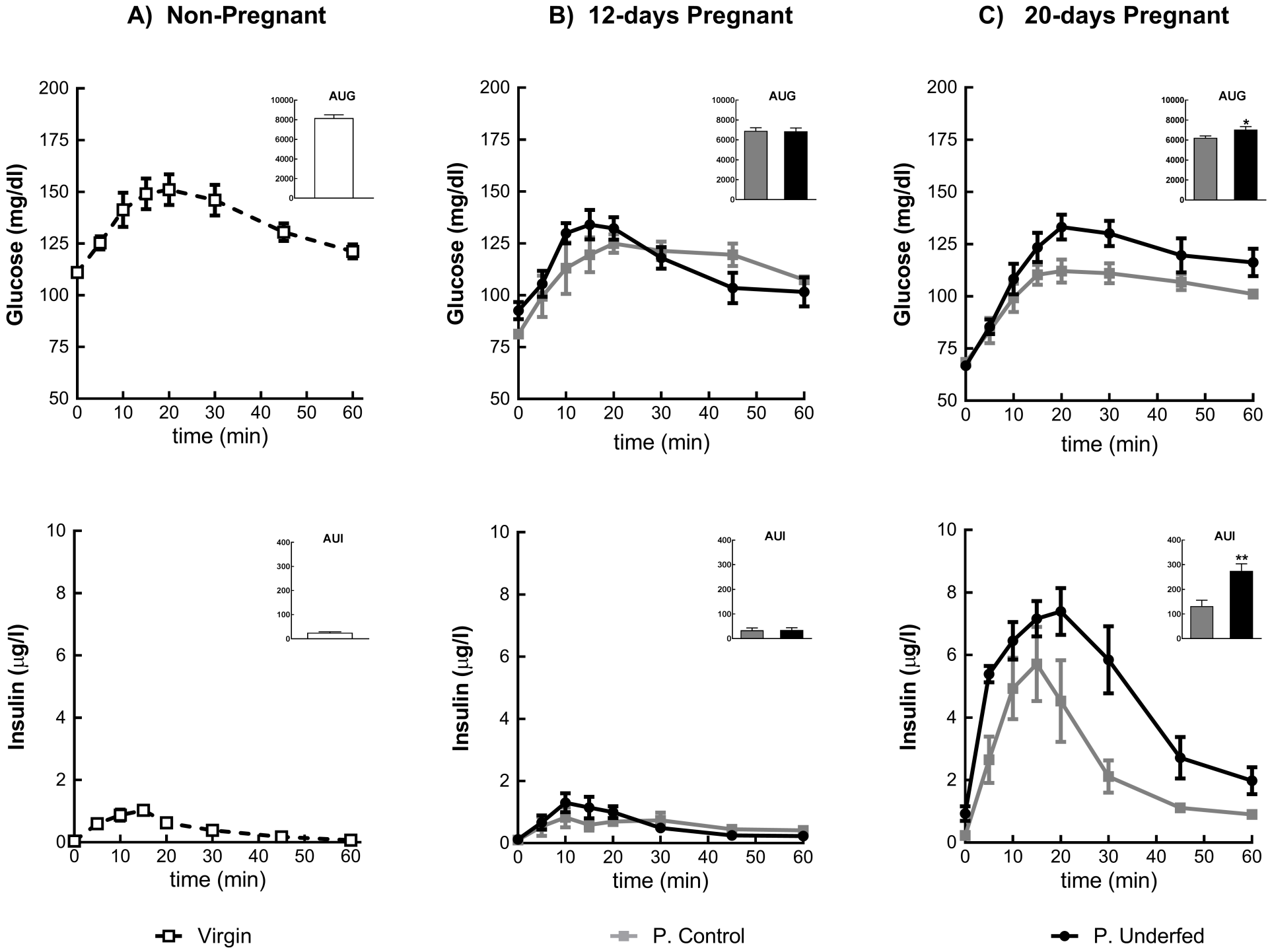
		Day of gestation		
		0	**12**	**20**
Total lipids	Control	417.2 ± 30.3 $^{A\ a}$	560.7 ± 28.3 ^{B}	575.3 ± 84.2 ^{B}
(mg/organ)	Underfed		290.3 ± 13.1 ^{b} ***	576.4 ± 23.8 ^{c}
Phospholipids	Control	283.0 ± 15.4 ^{ab}	315.7 ± 16.1	363.1 ± 43.8
(mg/organ)	Underfed		222.7 ± 29.5 ^{a} *	288.3 ± 25.9 ^{b}
Triacylglycerides	Control	45.5 ± 6.3 $^{A\ a}$	25.0 ± 4.1 ^{B}	20.9 ± 3.1 ^{B}
(mg/organ)	Underfed		9.7 ± 2.2 ^{b} *	34.2 ± 6.3 ^{a}
Cholesterol	Control	16.6 ± 1.1 $^{A\ ab}$	19.0 ± 2.1 ^{AB}	21.9 ± 1.2 ^{B}
(mg/organ)	Underfed		13.3 ± 1.4 ^{a}	21.3 ± 2.9 ^{b}
Cholesteryl esters	Control	2.04 ± 0.40	1.91 ± 0.21	1.89 ± 0.18
(mg/organ)	Underfed		1.31 ± 0.17	2.07 ± 0.41

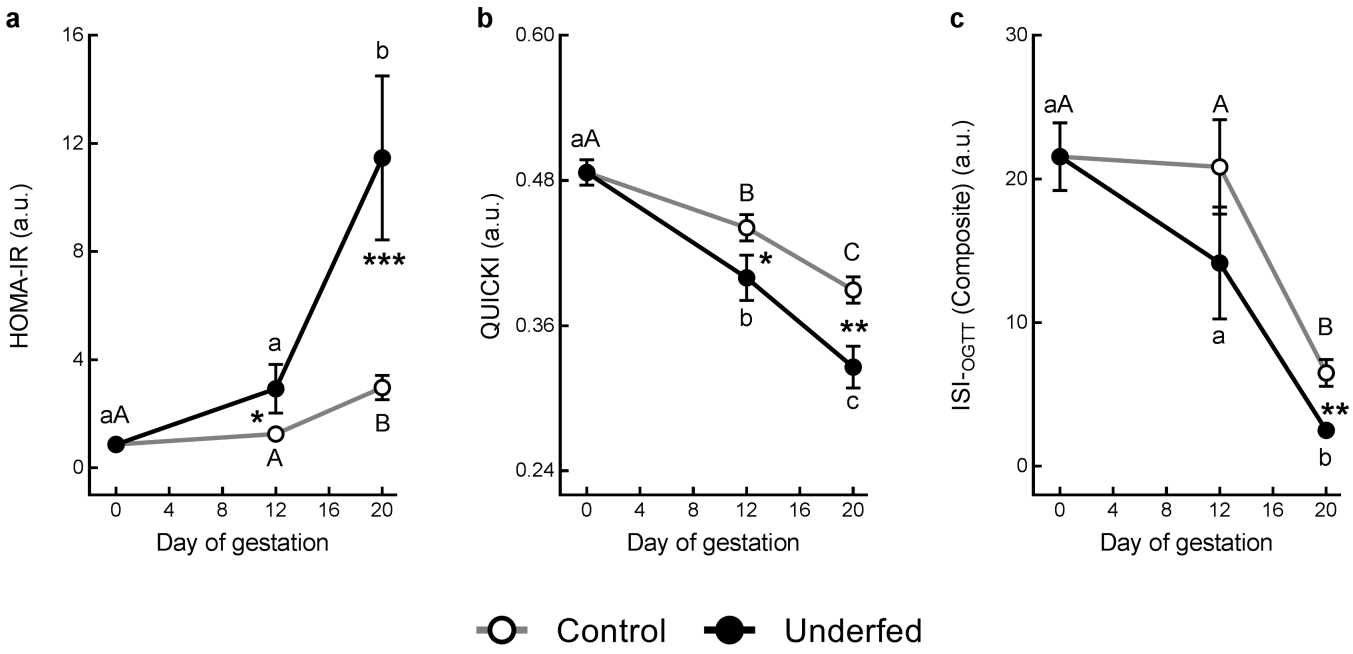
Different capital letters show differences in controls regarding the effect of gestation; different lowercase letters show differences in underfed rats regarding the effect of gestation. Differences between control and underfed pregnant rats are shown by: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

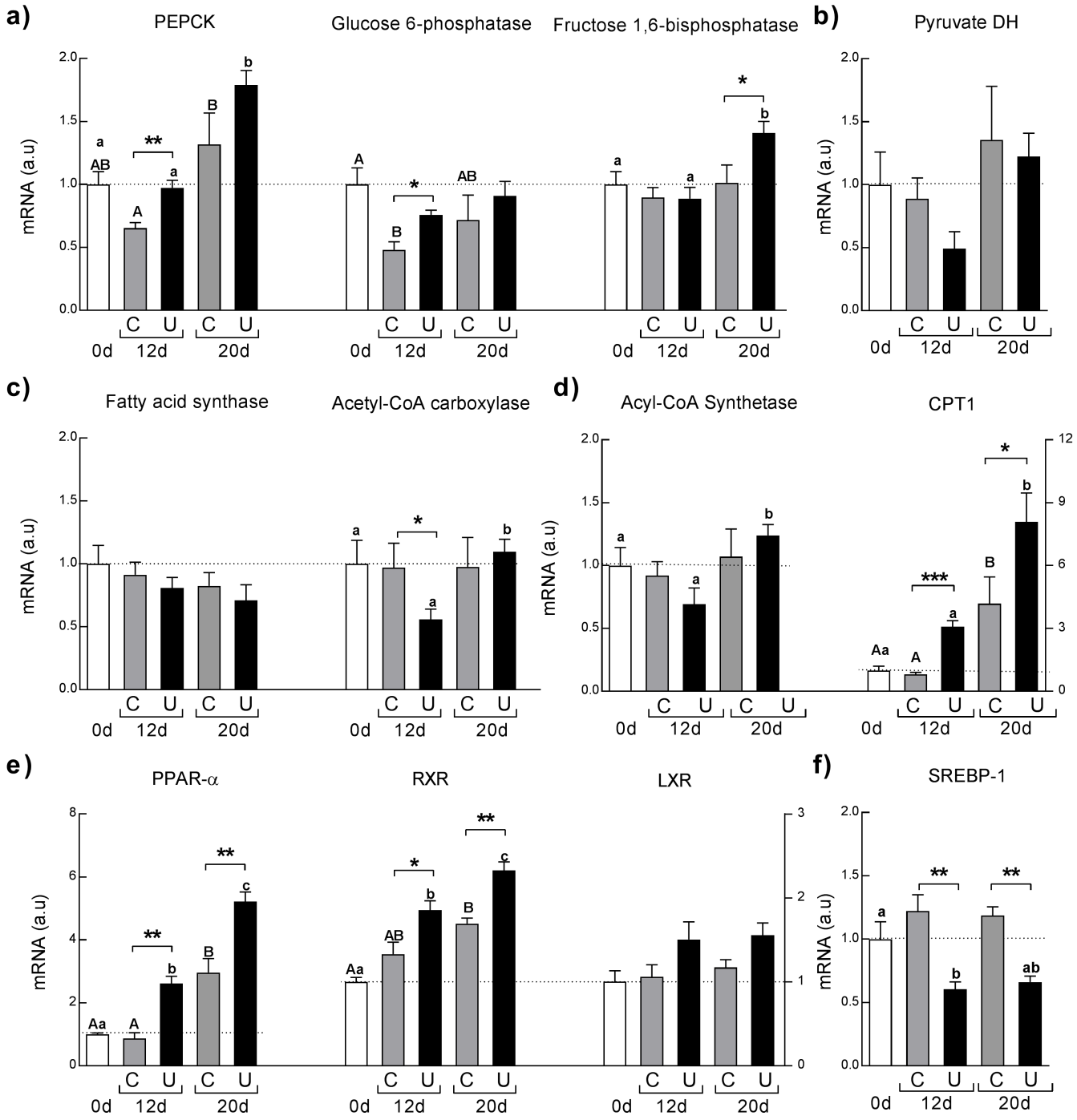

a
Body weight increase (g)
200
150
100
50
0
**
***
396 ± 9 g
341 ± 5 g
Underfeeding
(60% of control)
Control
Underfed
0
4
8
12
16
20
Pregnancy (day)
b
Increment of weight free of conceptus (g)
100
75
50
25
0
aA
B
B
**
a
***
b
Control
Underfed
0
4
8
12
16
20
Day of gestation


a
Lumbar adipose tissue weight (g)
3
2
1
0
aA
B
B
***
b
a
0
4
8
12
16
20
Day of gestation
b
Mesenteric adipose tissue weight (g)
aA
B
B
***
b
c
Day of gestation
c
LPL(nkat/g tissue)
8
6
4
2
0
A
B
C
***
Day of gestation
d
Liver weight (g)
20
15
10
5
0
aA
B
B
***
**
b
c
Day of gestation
Control
Underfed


a
Glucose (mg/dL)
Day of gestation
b
Insulin (µg/L)
Day of gestation
c
Triacylglycerides (mg/dL)
Day of gestation
d
Cholesterol (mg/dL)
Day of gestation
e
NEFA (µmol/L)
Day of gestation
f
Glycerol (µmol/L)
Day of gestation
g
Ketone Bodies (µmol/L)
Day of gestation
h
Leptin (ng/mL)
Day of gestation
i
Corticosterone (ng/mL)
Day of gestation
Control
Underfed


A) Non-Pregnant
B) 12-days Pregnant
C) 20-days Pregnant
Glucose (mg/dl)
Insulin (µg/l)
time (min)
AUG
AUI
*
**
Virgin
P. Control
P. Underfed


a
HOMA-IR (a.u.)
16
12
8
4
0
aA
a
b
A
B
*
***
0
4
8
12
16
20
Day of gestation
b
QUICKI (a.u.)
0.60
0.48
0.36
0.24
aA
B
C
b
c
*
**
0
4
8
12
16
20
Day of gestation
c
ISI-OGTT (Composite) (a.u.)
30
20
10
0
aA
A
a
B
b
**
0
4
8
12
16
20
Day of gestation
Control
Underfed


a)
PEPCK
Glucose 6-phosphatase
Fructose 1,6-bisphosphatase
b)
Pyruvate DH
c)
Fatty acid synthase
Acetyl-CoA carboxylase
d)
Acyl-CoA Synthetase
CPT1
e)
PPAR-α
RXR
LXR
f)
SREBP-1
mRNA (a.u)
C U C U
0d 12d 20d