Maternal intermittent fasting during pregnancy induces fetal growth restriction and downregulated placental system A amino acid transport in the rat

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Abstract

During Ramadan, many pregnant Muslim women fast between dawn and sunset. Although the impacts of prolonged maternal intermittent fasting (IF) on fetal growth and placental function are under-researched, reported effects include reduced placental weight and birth weight. In this study, pregnant Wistar rats were used to model repeated cycles of IF on fetal development and placental function and to examine sex-specific effects. In the IF group, food was withdrawn daily from 17:00 to 09:00 over 21 days of gestation, while the control group received food *ad libitum*. Both groups had free water access. IF dams consumed less food, had significantly reduced weight compared to controls, with reduced plasma glucose and amino acids. Both fetal sexes were significantly lighter in the IF group, with reduced fetal plasma amino acids. Placental weights and morphology were unchanged. The profile of placental metabolites was altered in the IF group with sex-specific responses evident. Transplacental flux of ^{14}C-methylaminoisobutyric acid (^{14}C-MeAIB), a system A amino acid transporter substrate, was significantly reduced in both fetal sexes in the IF group. Sodium-dependent ^{14}C-MeAIB uptake into isolated placental plasma membrane vesicles was unchanged. The gene expression of system A transporter *Slc38a1, Slc38a2* and *Slc38a4* was upregulated in IF male placentas only. No changes were observed in placental SNAT1 and SNAT2 protein expression. Maternal IF results in detrimental impacts on maternal physiology and fetal development, with changes in the placental and fetal metabolite profiles. Reduced placental system A transporter activity may be responsible for fetal growth restriction in both sexes.

Abbreviations

IF	Intermittent fasting
JZ	Junctional zone
K_{mf}	Unidirectional maternofetal clearance
LZ	Labyrinth zone
SNAT	Sodium-dependent neutral amino acid transporter

Introduction

Fasting during the month of Ramadan is one of the five pillars of Islam. During this period, it is a religious requirement that healthy adult Muslims abstain from consuming food and drink from dawn to sunset for the duration of this holy month. From sunset to dawn, there are no restrictions on food and fluid intake [1]. Although this religious mandate excludes pregnant women who are allowed to postpone their Ramadan fast until after delivery, many still elect to partake in the fast with their families for spiritual and cultural reasons [2, 3], as well as other motivational considerations [4, 5]. It has been estimated that 50-90 % of pregnant Muslim women fast for at least part of the month of Ramadan [2, 4, 5], with some choosing to extend their fast over the whole month [4, 5]. By observing this fasting practice, pregnant women experience repeated cycles of intermittent fasting (IF). As the Islamic calendar follows the lunar cycle, fasting may last between 8-18 h a day depending on the season of the year in which Ramadan falls as well as geographical location [6]. Ramadan fasting is therefore associated with an alteration in the timing of food consumption as well as a change commonly in the quantity and quality of foods consumed [3, 7]. Hence fasting pregnant women potentially expose their developing babies *in utero* to an altered nutrient and metabolic environment [6, 8].

The impacts of sustained maternal IF on fetal development and the health of adult offspring are not understood fully and observations associated with Ramadan fasting are conflicting [9]. Differences in reported pregnancy outcomes may be related to the gestational timing when fasting occurred [9, 10]: women are reported to participate in Ramadan fasting across each trimester of pregnancy [3, 5, 10]. Studies on the impact of Ramadan fasting on maternal and fetal health have, however, reported detrimental impacts for the mother and fetus, as well as effects on placental development. Adverse maternal outcomes reported include hyperemesis

gravidarum, dizziness, fatigue and nausea [3, 11]. Fasting pregnant women have higher cortisol concentrations [12], hypoglycaemia [1, 8] and diminished weight gain [13]. With regard to fetal health, studies have shown reduced fetal breathing movements [14], altered heart rate accelerations [15], and reductions in amniotic fluid index [16], fetal anthropometric measurements [16] and birthweight [12]. However, in a recent systematic review and meta-analysis, we did not find sufficient evidence to suggest that Ramadan fasting had an adverse effect on birthweight [9]. Other impacts on perinatal outcomes were not reported generally, so firm conclusions about the broader potential risks of fasting could not be made [9].

Notwithstanding this, exposure *in utero* to Ramadan fasting has been associated with lower cognitive test scores, learning difficulties and poorer academic performance in children and fewer employment hours worked in adulthood [3, 10, 17]. There is also an increased propensity to long-term health issues such as coronary heart problems and type 2 diabetes [2]. Impacts on placental growth and development have also been reported with placental weight and placental weight:birthweight ratio significantly reduced for both sexes in mothers who had fasted during Ramadan in the second and third trimesters [18].

The potential global impacts of Ramadan fasting whilst pregnant are considerable, with well in excess of 0.5 billion babies in each generation likely to be exposed *in utero* to a repeated cyclical pattern of maternal IF and ensuing associated maternal metabolic perturbations [9]. Hence, it has been estimated that more than 1 billion living Muslim adults could have been exposed to maternal fasting during Ramadan during their *in utero* development [5, 10]. It is now well established that forms of maternal nutrient restriction or a sub-optimal maternal diet during pregnancy can have detrimental effects on fetal growth trajectory with enduring impacts on physiological function in the later life of the offspring [19]. Such developmental programming

phenomena have been recapitulated in several animal models in which restriction of protein or caloric intake during pregnancy has resulted in fetal growth restriction (FGR) with alterations in placental development, morphology and function [20, 21]. Offspring from these models are predisposed to diseases in adulthood such as hypertension, diabetes and impaired cognitive function [20, 21]. Further, sub-optimal maternal nutrition and restricted fetal growth are known to be associated with altered expression and activity of placental transporters [22, 23], supporting the concept that the placenta acts as a nutrient sensor to modulate nutrient transfer to the fetus and regulate fetal growth [23].

Normal fetal growth is supported by the collective activity of several placental amino acid transporters [24]. The activity of system A, a sodium-dependent co-transporter that transports both essential and non-essential amino acids [24], is known to be crucial for optimal fetal growth [25-27]. Three isoforms of system A, sodium-dependent neutral amino acid transporter (SNAT) 1, 2 and 4, are expressed in the placenta of humans and rodents encoded by *Slc38a1*, *Slc38a2* and *Slc38a4* gene family subtypes respectively [28, 29]. Inhibition of placental system A activity during rat pregnancy leads to diminished fetal weight [30], arguing for its integral involvement in supporting normal fetal growth.

It is particularly complex to replicate human maternal Ramadan fasting behaviour fully in an animal model, bearing in mind differences related to the duration of the fast, the variable adherence of women to the fasting period, the gestational timing of the fast and maternal food choices when the fast is broken. Furthermore, the molecular impact of regular and repeated episodes of maternal IF on fetal development across gestation, and the effects on placental function remain poorly defined. The investigative approach taken here was to simulate the repeated cycles of food abstinence, analogous to those experienced during Ramadan fasting, in

pregnant dams as a prolonged fasting model. This allowed us to investigate the broader impacts of maternal IF on maternal metabolism, placental function and fetal development, thereby identifying mechanistic pathways that are modulated by a regular, repeated pattern of maternal IF. We hypothesized that placental nutrient transport function and fetal growth would be compromised by repeated cycles of maternal IF in a rat model, designed to mimic the repeated cyclical nature of Ramadan fasting in humans. Arising phenomena can then be related to subsequent offspring impacts and traits in later life, broadening our understanding of how maternal IF might contribute to the pathogenesis of later disease in the offspring within the context of a developmental programming framework.

Therefore, the aims of the study were to assess the effects of repeated cycles of maternal IF during pregnancy on maternal physiology, fetal growth and maternal and fetal metabolic profiles, as a prelude to investigating later enduring effects in the offspring. Changes in placental morphology were examined, as well as placental metabolism and placental system A amino acid transport function. As a number of studies have shown that placental responsiveness may differ between sexes [31], and that developmental diseases exhibit sex-specific differences [32], the sex-dependent specificity of fetal responses to maternal IF was also examined.

Methods

Ethical Approval

All experiments involving animals were conducted under the authority of a project licence (PPL 40/3646) issued in accordance with the UK Animals (Scientific Procedures) Act 1986. Local ethical approval was granted by the University of Manchester Animal Welfare and Ethical Review Body. All animal work was conducted at the University of Manchester.

Animals

Virgin female Wistar rats (Charles River, UK) weighing 250-275 g were mated with a Wistar male; gestational day 1 of pregnancy (GD1) was defined as the day that a vaginal plug was found (term is GD23). Females were housed singly and maintained under a constant 12 h:12 h dark/light cycle (19:00 dark cycle/07:00 light cycle) at 21-23 °C. Animals were allocated randomly to one of two dietary groups. To mimic the pattern of repeated fasting-feeding cycles adopted by pregnant women who fast over Ramadan, the maternal IF group (IF, N = 36) was subjected to food restriction for 16 h overnight, predominantly over the active dark cycle phase, with food (standard rat chow diet, (BK001 (E) SDS Rodent Breeder and Grower, LBS Biotech, Redhill, UK) removed at 17:00 and returned at 09:00 daily, from GD1 onwards until the end of the experiment on GD21. The control group (C, N = 36) was fed standard rat chow *ad libitum* throughout pregnancy. Both groups had free access to water at all times. Food and water intakes and the dam's body weight were recorded daily. Dams were excluded from the study when a vaginal plug was absent preventing the accurate determination of the start of pregnancy (N=6), or when the IF regimen resulted in a lack of pregnancy weight gain with greater than 15 % maternal weight loss (N=3).

Collection of blood and placenta

At GD21 between 09:00 and 10:00, the pregnant dams were anaesthetised with isoflurane by inhalation (4% in oxygen at 2 L/min), and maternal blood was collected by cardiac puncture. Dams were killed by cervical dislocation after which placentas and fetuses were harvested rapidly. Individual fetal blood samples were collected from an axillary vessel incision using heparinised capillary tubes. Following centrifugation, maternal and fetal plasma were stored at -80 °C until further analysis. Placentas were stored in RNAlater (Sigma Aldrich, UK) for gene expression analysis, snap-frozen for biochemical analysis including the metabolomic assays and stored at -80 °C, or fixed in 10% neutral buffered formalin for histological analyses. In some instances, the junctional (JZ) and labyrinth zones (LZ) of the placenta were dissected separately and each frozen rapidly. Placentas and fetuses were selected randomly for subsequent analysis, dependent on fetal sex, and analyses were not performed in a blinded manner with respect to dietary intervention.

Fetal anthropometric measurements

In a set of dams (N = 5 control and N = 7 IF litters), fetal anthropometric measurements of crown-rump length, abdominal circumference and head circumference were performed as described previously [33].

Fetal sex determination

For fetal sex determination, genomic DNA was isolated from fetal tail tips using a DNeasy kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. PCR was performed using primer sequences specific for rat *Sry* gene encoding the sex-determining region Y protein (sequences 5'→3'; forward primer: CATCGAAGGGTTAAAGTGCCA; reverse primer:

ATAGTGTGTAGGTTGTTGTCC), as described previously [34]. The presence or lack of a *Sry* gene amplification product, as visualised by agarose gel electrophoresis, was designated as male (M) or female (F) respectively.

Plasma glucose and hormone analyses

Glucose concentration in maternal and fetal plasma was determined using a commercial kit (GAHK-20; Sigma Aldrich, UK). Insulin and glucagon concentrations in maternal and fetal plasma were measured by the Ultrasensitive Rat Insulin and Rat Glucagon ELISA kits respectively (10-1251-01 and 10-1271-01 respectively, Mercodia, Uppsala, Sweden). All assays were performed according to the manufacturer's instructions.

Plasma amino acid concentration

The concentration of amino acids in maternal and fetal plasma was measured by reversed-phase high pressure liquid chromatography (HPLC). Maternal (50 µL) or fetal plasma (10 µL) was deproteinised with 10 % trichloroacetic acid, and amino acid concentrations measured using an Agilent 1100 HPLC (Agilent Technologies, Stockport, UK) fitted with a Gemini 50 x 4.6 mm column (Phenomenex, Macclesfield, UK) and an Agilent fluorescence detector, as described previously [35].

Placental morphology

Sections (5 µm) of formalin-fixed, paraffin-embedded placentas were stained with haematoxylin and eosin and scanned by the Bio-imaging Facility (University of Manchester) and the images processed by Panoramic Viewer software (3DHISTECH Ltd, Budapest, Hungary). JZ and LZ areas

were quantified using ImageJ software analysis from two representative placental sections (6 placentas per sex from 3 litters per dietary group).

Tissue glycogen content

Glycogen content of the placental JZ and fetal liver was measured using a glycogen assay kit (MAK016; Sigma Aldrich, UK), according to the manufacturer's instructions.

Placental metabolomics

Placentas from control and IF dams at GD21 (n = 6 placentas per fetal sex from N = 6 litters per dietary group) were harvested and a placental-quarter was flash-frozen immediately and stored at -80 °C prior to metabolomic analysis at the University of Birmingham.

Untargeted UHPLC-MS study

Extraction of placental tissue

All solvents and chemicals applied were of HPLC analytical grade (J.T. Baker, UK). Placental tissues (n = 24) were weighed and subsequently extracted in a randomised order in Precellys ceramic bead soft tissue homogenisation tubes (Bertin Technologies, Stretton Scientific UK). An extraction solution of 2.5 : 1 : 1 of methanol : chloroform : water cooled to -20 °C was applied to perform a monophasic extraction, with the volume applied normalised to tissue weight (9.2 μL per mg tissue). Homogenisation was performed in a Precellys 24 homogeniser (Bertin Technologies, Stretton Scientific UK) with the following homogenisation cycle: 6400 rpm for 15 s, 0 rpm for 15 s and 6400 rpm for 15 s. The samples were then centrifuged at 10 000 *xg* for 5 min at 3 °C to pellet the insoluble material. Samples (450 μL) were transferred to 2 mL

microcentrifuge tubes and dried under a nitrogen gas stream (room temperature) prior to storage at -80 °C until analysis. An additional homogenisation tube, without placental tissue, was employed to generate a blank control sample. Quality control (QC) samples were prepared by pooling all remaining extraction supernatants followed by transfer of 450 μL aliquots to 2 mL microcentrifuge tubes followed by drying under a nitrogen gas stream (room temperature) prior to storage at -80 °C until analysis.

UHPLC-MS analysis

All solvents and chemicals applied were of HPLC analytical grade (J.T. Baker, UK). UHPLC-MS analysis of tissue extracts and QC samples was performed applying a Dionex U3000 coupled to an electrospray LTQ-FT-MS Ultra mass spectrometer (Thermo Scientific Ltd, UK). Samples were reconstituted in 50 μL methanol : water (50 : 50), mixed and centrifuged at 10 000 *xg* for 15 min and transferred to vials with 200 μL fixed inserts (Thermo-Fisher Ltd, UK). All samples were stored in the autosampler at 5 °C and analysed separately in negative and positive electrospray ionisation (ESI) modes within 48 h of reconstitution. UHPLC separations were performed applying a Hypersil Gold C_{18} reversed phase column (100 x 2.1 mm, 1.9 μm) at a flow rate of 400 $\mu L.min^{-1}$, temperature of 40 °C and with two solvents: solvent A (HPLC grade water + 0.1 % formic acid) and solvent B (HPLC grade methanol + 0.1 % formic acid). A gradient elution was performed as follows: hold 100 % A 0–1.5 min, 100 % A–100 % B 1.5–6 min curve 3, hold 100 % B 6–12 min, 100 % B–100% A 12–13 min curve 3, hold 100% A 13–15 min. Injection volume was 5 μL. UHPLC eluent was introduced directly in to the electrospray LTQ-FT Ultra mass spectrometer with source conditions as follows: spray voltage -4.5 kV (ESI-) and +5 kV (ESI+), sheath gas 30 arbitrary units, aux gas 15 arbitrary units, capillary voltage 35 V, tube lens voltage

-100 V (ESI-) and +90 V (ESI+), capillary temperature 280 °C, ESI heater temperature 300 °C. Data were acquired in the FT mass spectrometer in the *m/z* range 100-1000 at a mass resolution of 50 000 (FWHM defined at *m/z* 400), with a scan speed of 0.4 s and an AGC setting of 1×10^6. Analysis order was composed of 10 QC sample injections for system conditioning followed by a QC sample injection every 6th injection with two QC sample injections at the end of the analytical run. Tissue extracts were analysed in a random order. Blank samples were analysed as injection 6 and as the last injection of the analysis batch.

Data processing and statistical analysis

UHPLC-MS raw data profiles were first converted into a NetCDF format within the Xcalibur software's File Converter program. Each NetCDF based three-dimensional data matrix (intensity x *m/z* x time – one per sample) was converted (or deconvolved) into a vector of peak responses, where a peak response is defined as the sum of intensities over a window of specified mass and time range (e.g. *m/z* = 102.1 and time = 130 s). In this experiment the deconvolution was performed using the freely available XCMS software [36]. Data were exported from XCMS as a .csv file for further data analysis. The quality of data was assessed applying QC data with all metabolite features with a relative standard deviation (RSD) of > 20% for QC samples being removed from the dataset prior to data analysis [36]. The data for each sample were normalised (as a percentage) to the total peak area for all metabolites in the sample. Metabolite annotation was performed applying the PUTMEDID_LCMS workflow [37] at level 2 or 3 (putatively annotated compounds) according to MSI reporting standards [38]. The processed data were analysed in R applying the unsupervised multivariate principal components analysis (PCA) and

the univariate non-parametric Wilcoxon–Mann–Whitney U test and Kruskal-Wallis test. No correction for false discovery rate was applied.

Placental system A amino acid transporter activity and expression

System A gene expression

Slc38a1, Slc38a2 and *Slc38a4* gene expression was measured by real-time quantitative PCR. Total RNA was extracted from placentas (n = 1 of each sex from N = 8-10 litters per group) according to the manufacturer's instructions (RNeasy mini kit, Qiagen, UK). Total RNA (2 µg) was reverse-transcribed to cDNA using Quantitect Reverse Transcription kit (Qiagen, UK). Real-time qPCR was performed over 35 cycles using gene-specific primers (Quantitect Primer Assay, Qiagen, UK; Supplementary Table 1) with QuantiFast SYBR Green PCR Master Mix (Qiagen, UK). Gene expression was normalised to *Ywhaz* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide) as a reference gene; *Ywhaz* mRNA expression was stable across dietary groups and fetal sex (*data not shown*).

System A protein expression

Rat placental membrane vesicles, prepared from the maternal-facing plasma membrane of syncytiotrophoblast layer II were isolated from placentas at GD21 using one cycle of Mg^{2+} precipitation, as described previously [39, 40]. Vesicle membrane purity was measured as enrichment of alkaline phosphatase activity in the vesicle suspension relative to that in placental homogenate [39, 40]. Protein expression of SNAT1 and SNAT2 in rat placental membrane vesicles was determined by immunoblotting. For SNAT1, samples were heated at 60 °C for 5 min in reducing buffer (4 % SDS, 0.004 % bromophenol blue, 20 % glycerol, 10 % β-mercaptoethanol in 0.125 M Tris-HCl pH 6.8); for SNAT2, samples were added to a non-boil reducing buffer (8 M

urea, 5 % SDS, 455 mM DTT, 0.4 % bromophenol blue in 50 mM Tris-HCl, pH 6.9). Placental vesicles (20 μg protein) were electrophoresed using 4 – 20 % Mini-PROTEAN TGX precast protein gels (Bio-Rad, UK) and proteins transferred to Immobilon-FL PVDF membrane (Merck Millipore). Blots were blocked using Odyssey Blocking Buffer (LiCOR Biotechnology, UK) for 1 h and then probed with either rabbit affinity-purified anti-SNAT1 (1.5 μg/mL) or anti-SNAT2 (1.58 μg/mL) antibodies [41] diluted in Odyssey Blocking Buffer with 0.2 % Tween 20 overnight at 4 °C, followed by a further 2 h antibody incubation at room temperature. A blocking peptide was included as negative control. Following washes with PBS/0.1 % Tween, blots were incubated with 1:20,000 anti-rabbit secondary antibody (IRDye® 800CW, green, Li-COR Biotechnology, UK). Anti-mouse β-actin antibody (1:2000; A2228, Sigma Aldrich, UK) was used to confirm protein integrity. Immunoreactivity was detected (Li-COR Odyssey) and quantification performed with ImageStudio Lite software (Li-COR Biotechnology, UK).

System A activity in isolated rat placental vesicles

Placentas were placed in tissue preserving medium (Bridge to Life Ltd, USA) overnight at 4 °C whilst fetal sextyping was performed. Placentas of male (M) and female (F) fetuses were pooled from each litter (2-12 placentas/litter) and placental vesicles isolated (control N = 13, IF N = 15), prepared as described previously [39, 40]. Alkaline phosphatase enrichment (mean ± SEM) was not different between control (M, 9.7 ± 0.6; F, 9.3 ± 0.4) and IF (M, 9.9 ± 0.6; F, 8.4 ± 0.3) groups. System A activity in isolated rat placental vesicles was measured as the Na^+-dependent uptake of ^{14}C-methylaminoisobutyric acid (MeAIB), a non-metabolisable specific substrate for the system A amino acid transporter, as described previously [40].

Unidirectional maternofetal clearance of ^{14}C-MeAIB

Unidirectional maternofetal clearance of ^{14}C-MeAIB ($^{MeAIB}K_{mf}$) was measured at GD21 in pregnant rats (control N = 6, IF = 5), and all procedures were conducted under Inactin anaesthesia (sodium thiobutabarbital 100 mg/kg body weight i.p., T133, Sigma Aldrich). A bolus dose of ^{14}C-MeAIB (0.37MBq (10 µCi) ^{14}C-MeAIB in 0.9 % saline) was injected into the maternal jugular vein at time zero. Repeated maternal blood samples were taken at regular intervals until 5 min post-injection, when the dam was killed by cervical dislocation. Fetuses were solubilised in 10 mL 3 % KOH overnight at 55 °C, followed by neutralisation with 0.54 M HCl. Maternal plasma and solubilised fetal lysates were counted for ^{14}C-MeAIB (Packard 2000CA). $^{MeAIB}K_{mf}$ was calculated, normalised per g placenta, as described previously [33].

Statistics

Data are presented either as box (with median) and whisker plots (whiskers represent 5th and 95th centiles) or as litter mean ± SEM as appropriate, with N = number of dams/litters and n = number of individual fetuses. The Shapiro-Wilk test was used to evaluate the normal distribution of the data. Data were analysed by two-way ANOVA (with repeated measures where appropriate) and Tukey tests or Kruskal-Wallis and Dunn's multiple comparison tests, or unpaired t-tests. Litter size was analysed by Fisher's exact test. Fetal weight frequency distribution curves were compared by non-linear regression (Gaussian distribution), with the 5th percentile weight calculated as described previously [42]. Data were analysed using SPSS (version 22.0, IBM SPSS Statistics, IBM United Kingdom Ltd, Hampshire, UK) or GraphPad Prism (version 7.0, GraphPad Software, Inc, La Jolla, CA, USA). For placental metabolomics data, analysis was performed using R with an unsupervised multivariate principal components analysis followed by univariate one-way ANOVA with Benjami-Hochberg's correction for multiple

comparisons. Pathway analysis was performed in MetaboAnalyst v3. For all analyses, $P < 0.05$ was considered statistically significant.

Results

Maternal food intake and weight gain

Dams in the IF group consumed 30 ± 1 % less food at all stages of pregnancy compared with control dams, despite having free access to food daily over a 8 h period (from 09:00 to 17:00) ($P < 0.001$, Figure 1A). Pregnant dams of both control and IF groups exhibited an incremental weight gain as pregnancy progressed ($P < 0.001$, Figure 1C), sustained by a daily food intake that remained relatively stable throughout gestation when normalised to maternal body weight (Figure 1A). Daily water consumption was comparable between diet groups (Figure 1B).

Although pregnant dams in both groups gained weight steadily throughout pregnancy, daily weight gain in IF dams was significantly lower compared to control dams from GD8 onwards ($P < 0.001$, Figure 1C). All dams were weighed routinely at 17:00 before food was removed from the IF group. However on GD21, the day of harvest, dams were weighed at 09:00 prior to sacrifice, accounting for the apparent fall of 19 ± 1 g in IF dam body weight at GD21 following the overnight fast (Figure 1C). This also implies that there was some recovery of the dams' overnight weight loss achieved by the opportunity to feed again following the re-introduction of food in the morning to the IF group.

Normalisation of maternal organ weights to 100 g dam body weight (after subtraction of uterine content weight) showed that variable effects were associated with maternal IF. Maternal liver weight in IF dams was significantly reduced ($P < 0.01$), while kidney weight was significantly increased, compared with the control dams ($P < 0.05$); heart weight was not affected (Supplementary Table 2).

Glucose concentration and regulatory hormones in maternal and fetal plasma

Maternal IF had differing effects on circulating glucose and associated regulatory hormone concentrations in dams and fetuses. Plasma glucose concentration was reduced by 33 ± 3 % in IF dams compared with controls ($P < 0.001$, Table 1). This was associated with a significant reduction in plasma glucagon concentration ($P < 0.01$, Table 1); insulin concentration was unaffected in IF dams. In contrast, plasma glucose and glucagon concentrations did not differ between IF and control fetuses, whereas the plasma insulin concentration was significantly lower in IF fetuses ($P < 0.05$, Table 1).

Fetal glycogen content

To investigate whether the maintained fetal glucose concentration in the IF group was underpinned by catabolism of fetal glycogen as a potential source of mobilisable glucose, the glycogen content of the placental JZ, which harbours clusters of glycogen cells, and fetal liver was measured. Placental glycogen content was unaltered in IF fetuses compared with controls (Table 2). However, fetal liver glycogen content was reduced significantly in both sexes of IF fetuses compared with control fetuses ($P_{Male} < 0.01$, $P_{Female} < 0.05$, Table 2).

Fetal and placental weights

There were no differences in litter size or fetal sex ratio between the IF and control groups (Table 3). Neither was the number of fetal resorptions (as % of total number of implantations) different between groups (2.50 ± 1.06 and 1.26 ± 0.43 % for IF and control respectively). However, the mean fetal weight per litter was significantly reduced by 15 ± 1 % in the IF group compared with the controls ($P < 0.001$, Figure 2A). Male fetuses were heavier than females in

the control group only ($P < 0.05$, Figure 2A). Plotting fetal weight frequency distribution curves revealed that 61 – 67 % fetuses in the IF group fell below the 5th centile of the control population (Figure 2B, C), taken as a clinical indicator of FGR [46]. In contrast, placental weight (as a proxy of placental growth) was unaffected by exposure to maternal IF (Table 3). The fetal:placental weight ratio (as an index of placental transport efficiency [43]), was significantly reduced in both sexes of the IF group ($P_{Male} < 0.01$, $P_{Female} < 0.05$, Table 3), suggesting an adverse effect of maternal IF on placental transport function.

The growth restriction in IF fetuses was accompanied in both sexes by a significant reduction in crown-rump length ($P < 0.01$) and head circumference ($P < 0.05$), while abdominal circumference was unaltered (Table 3). Fetal organ weights relative to body weight did not differ between groups (Table 3). However, the fetal brain:liver weight ratio was significantly increased in both sexes of IF fetuses compared with controls ($P < 0.01$, Table 3).

Sex-related differences in placental morphology

The gross morphology of the placenta was not affected by exposure to maternal IF (Figure 3A), with organisation of the placental cellular layers appearing normal, with clear discrimination of the JZ and LZ. There were no differences observed in relative area of the JZ and LZ for either sex between the two dietary groups (Figure 3B, C). However, sex-dependent differences were apparent independent of diet; placentas of female fetuses of both dietary groups had a proportionately thicker JZ, whilst male fetuses had a thicker LZ (Figure 3A-C). Hence females of both dietary groups had a significantly higher relative ratio of JZ:placental total area compared to their respective male counterparts, whereas the converse was apparent as regards the relative ratio of LZ:placental total area (Figure 3B, C).

Maternal and fetal plasma amino acid concentrations

The amino acid concentrations in maternal and fetal plasma on GD21 of pregnancy are shown in Table 4. Irrespective of dietary regimen, amino acid concentrations in fetal plasma were significantly higher compared with maternal plasma ($P < 0.05$), with a fetal:maternal concentration ratio of variable magnitude and greater than a value of 1 across all amino acids, reflective of amino acid concentrative capacity within the fetal compartment (Supplementary Figure 1). In the case of IF animals, the fetal:maternal amino acid concentration ratio was significantly increased for several amino acids including neutral aromatic (tyrosine, phenylalanine and tryptophan), neutral non-aromatic (asparagine, alanine, glutamine, serine and threonine) and cationic (lysine and arginine) amino acids ($P < 0.05$, Supplementary Figure 1). This increased fetal:maternal concentration ratio was driven in part by significant reductions in maternal tyrosine, asparagine, alanine, serine, threonine and arginine concentrations ($P < 0.05$, Table 4). Other amino acids that demonstrated a significantly reduced concentration in IF maternal plasma (histidine, methionine and valine, Table 4) were accompanied by a significant reduction in fetal plasma concentration ($P < 0.05$, Table 4), resulting in an unchanged fetal:maternal concentration ratio (Supplementary Figure 1). Interestingly, there were two amino acids, isoleucine and aspartate, that demonstrated a significant reduction in IF fetal plasma, yet were unaltered in maternal plasma (Table 4). A subset of amino acids (glutamine, glycine, leucine, tryptophan, phenylalanine, lysine and glutamate) revealed no significant changes in either maternal or fetal plasma concentration in response to maternal IF compared to control (Table 4). Therefore, there were differential effects of maternal IF on amino acid concentrations within both maternal and fetal plasma, with divergent trends across the same subclasses of amino acids.

A number of sex-dependent differences were also observed in fetal amino acid concentrations; in control fetuses lysine, arginine and tyrosine were significantly lower in females compared with males ($P < 0.05$, Table 4) and in IF fetuses, the aromatic amino acids phenylalanine, tryptophan and tyrosine were significantly lower in females compared with males ($P < 0.05$, Table 4). Maternal IF diet was also associated with sex-specific changes in fetal amino acid concentration; threonine, arginine and valine were reduced significantly in IF male fetuses compared to control males ($P < 0.05$, Table 4), while serine was reduced significantly in IF female fetuses compared to control females ($P < 0.05$, Table 4).

Placental system A amino acid transport activity

The selective reduction in fetal plasma amino acid concentrations of the IF group, together with the particularly marked reduction in fetal alanine concentration (of 26 % in male and 23 % in female fetuses, respectively), led us to postulate that the IF dietary regimen caused a reduction in placental amino acid transporter activity and compromised placental nutrient transport capacity, leading to FGR. As alanine is a preferred amino acid substrate for the system A amino acid transporter [24], we hypothesized that placental system A activity would be reduced by the IF dietary regimen. To investigate this hypothesis *in vivo*, the unidirectional maternofetal clearance of ^{14}C-MeAIB ($^{MeAIB}K_{mf}$), a non-metabolisable specific substrate for system A [33], was measured across the placenta. As shown in Figure 4A, there was a significant reduction in $^{MeAIB}K_{mf}$ across the placentas of both IF male and female fetuses by 37 ± 9 % ($P < 0.05$) and 41 ± 8 % ($P < 0.01$) respectively, compared with control litters. Measurement of system activity A *in vitro*, as the Na^+-dependent uptake of ^{14}C-MeAIB into isolated placental membrane vesicles from

both groups, revealed a linear rate of intravesicular ^{14}C-MeAIB accumulation over the time course (15-60 s, Figure 4C). However there were no significant differences in the rate of ^{14}C-MeAIB uptake between dietary groups (Figure 4C), and at 60 s the amount of accumulated tracer was comparable between groups (Figure 4B).

Placental expression of *Slc38a1*, *Slc38a2* and *Slc38a4* genes and SNAT protein expression

Based on our observation that transplacental system A activity was reduced when dams were exposed to a repeated pattern of maternal IF (Figure 4A), we examined the placental expression of genes that encode the SNAT1, 2 and 4 protein isoforms which mediate system A activity. Expression of *Slc38a1*, *Slc38a2* and *Slc38a4* was significantly increased in IF male placentas only compared to control, with no differences between groups in female fetuses, highlighting a sex-specific induction in response to IF diet in male fetuses only ($P < 0.05$, Figure 5A-C).

To assess whether the expression and functional changes observed in the IF group were accompanied by altered SNAT protein expression, we examined SNAT1 and SNAT2 expression in rat placental vesicles, applying the reasoning that these SNAT isoforms were most likely to be implicated functionally as SNAT1 and SNAT2 have shared functional transport characteristics and a much higher affinity for MeAIB as a substrate relative to SNAT4 [44]. For SNAT1, an intense immunoreactive species at 52 kDa was observed in all samples, whereas for SNAT2, two distinct bands at approximately 60 and 150 kDa were visualized (Supplementary Figure 2A, C). Quantitation of immunoreactive species intensity revealed that expression of SNAT1 (52 kDa) and SNAT2 (both 60 and 150 kDa species) were similar between the control and IF groups for both sexes ($P > 0.05$, Figure 6A-C). All immunoreactive signals were abolished in the absence of primary antibody (*data not shown*) or following pre-absorption of antibody with excess blocking

peptide (*data not shown*). Confirmation of protein integrity in all samples was achieved by visualization of a single immunoreactive signal for β-actin, which was stably expressed across groups (Supplementary Figure 2B), allowing it to be used to normalise SNAT protein expression (Figure 6).

Placental metabolomics

Bearing in mind the differences observed for glucose and amino acid concentrations in both the maternal and fetal compartments in response to a maternal IF diet (Tables 1 and 4), we postulated that this dietary regimen would be associated with an altered placental concentration of other metabolite classes not investigated so far. Table 5 shows that 22 metabolites, of different metabolite classes, were significantly altered in the placentas of both sexes of the IF group compared to controls, with the majority (16 metabolites) demonstrating an increased placental concentration, the most striking being ophthalmic acid. However, in contrast, five metabolites related to fatty acid and lipid metabolism were significantly lower in both fetal sexes of the IF group compared to control. There was a significant increase in aromatic amino acids such as tryptophan, tyrosine and phenylalanine in the IF male placentas compared to IF females (Table 6); these amino acids were also significantly higher in the plasma of IF males as compared to IF females (Table 4). In addition, the metabolite class of trisaccharides (which are catabolized to free hexose sugars) was also elevated in IF male placentas (Table 6). Collectively, the placental metabolites altered in association with the IF dietary regimen, as listed in Tables 5 and 6, suggest that divergent metabolic pathways are affected in the placenta, with evidence of amino acid, fatty acid, phospholipid, steroid and nucleotide metabolism being particularly perturbed.

DISCUSSION

The primary aim of this study was to recapitulate in an animal model aspects of maternal IF that occur during Ramadan fasting, in order to elucidate the impact of repeated dietary 'fasting-feeding' cycles during pregnancy on placental function and fetal development. The model was not designed to mimic Ramadan fasting in humans fully, in so much as the pregnant rats were deprived of food for a period of 16 h each day throughout 21 days of gestation and had free access to water. This contrasts with religious practice during Ramadan, where daily repeated fasting lasts for one lunar month and involves abstaining from both food and water intake during daylight hours.

In this rat model, in order to mimic food abstinence adopted by humans during Ramadan fasting over daylight active hours, food was withdrawn during the rat's active phase over the dark cycle. As already outlined, the duration of daily Ramadan fasting undertaken by humans can be variable within the range of 8-18 h dependent on geographical location and the season in which Ramadan falls. Our approach in this study was to examine a fasting interval that approached the maximal time of fasting in humans, to understand more fully the impact that daily prolonged maternal fasting may have. We elected not to simultaneously withhold water, as is the practice in humans during Ramadan fasting, as we sought to identify the effects of intermittent food abstinence alone without any confounding effects of dehydration. We acknowledge, however, that water deprivation may have further impacts upon fetal developmental and placental function.

As already mentioned, women may partake in the Ramadan fast at any stage of pregnancy. However because little is known about the impact of IF on the fetus, we opted to fast rats

throughout pregnancy in order to gain a more detailed understanding of the consequences of fasting across all phases of fetal growth and development. Nonetheless, we acknowledge that the dietary challenge imposed on the rats in this study is greater than that experienced by a pregnant woman fasting during Ramadan. We further recognise that we were unable to model the changes in both the quantity and quality of foods consumed after the daily fast in broken in humans, not least because the type of food eaten varies between different communities.

Notwithstanding these limitations, the study allowed us to evaluate the impact of maternal food restriction alone during pregnancy on maternal physiology, fetal development, placental development and function. It also enabled us to explore how fetal sex might influence phenomena that associate with an altered maternal dietary intake and a changed *in utero* nutrient environment.

The dietary regimen of maternal IF imposed was tolerated well by the pregnant rat dams, with no observed impact on either fetal loss or litter size, in agreement with others using a comparable Ramadan-type fasting regimen in pregnant rats [45]. As expected, maternal food intake and maternal gestational weight gain were reduced significantly following the regular withdrawal of food overnight, as observed previously [45]. Although we did not measure lean body mass, it is interesting to note that there was no consistent pattern of change in the relative weight of different maternal organs in the IF dams. These observations are in broad agreement with previous studies where pregnant rats were exposed to food-restriction for 19 h per day [46]. Dams subjected to IF consumed ~30 % less food than controls. The failure to compensate when food was made freely available in the morning was accounted for by the dams' inclination to sleep following their morning feeding session and their observed relative inactivity thereafter. Hence overall food consumption was insufficient to sustain normal weight gain during

pregnancy, in common with other food-restricted models in pregnant rats [45, 47]. Although caloric deficiencies and diminished maternal weight gain in pregnant women undertaking Ramadan fasting have been reported previously [13], others emphasize that caloric intake may in fact increase during Ramadan due to the consumption of calorie-dense foods when the fast is broken [48].

Fetal weights of male rat fetuses were heavier than females in the control group at GD21, in agreement with others [49], and consistent with findings in human newborns [18]. However, in the IF group this sex-divergence in fetal weight was not apparent suggesting that normal fetal growth trajectory patterns had been disrupted. Indeed, fetal weight of both sexes in the IF group was reduced significantly at GD21 compared to the control group, with a substantial proportion of fetuses exhibiting FGR. Additional evidence of altered fetal growth dynamics is provided by the increase in the brain:liver weight ratio of IF fetuses. This is a marker of fetal 'brain-sparing' and an index of intrauterine growth restriction, reflective of a redirection of nutrients to vital organs such as the brain at the expense of other organs [50]. Whilst this action serves to try to protect the brain, it is associated with impaired neurocognitive function later in development [51]. This is of interest in light of the lower cognitive test scores, learning difficulties and poorer academic performance reported in children exposed *in utero* to Ramadan fasting [3, 17].

The reduced maternal glucose concentration observed in IF dams accords with previous studies [45], yet a normal glucose concentration was maintained in the fetuses of the IF group. As control dams were not fasted prior to collecting blood samples we cannot rule out the possibility that the difference in plasma glucose concentration is due, in part, to prior food intake. However, it is established that fasting regimens of a similar pattern to that adopted in

this study [45] or as short as 24 h [52] in pregnant rat dams result in significantly reduced maternal glucose concentrations, so our data are in agreement with these previous reports. The observation that fetal liver glycogen content was reduced, whereas placental glycogen content was preserved in IF fetuses, is consistent with this being achieved primarily through catabolism of fetal liver glycogen, rather than a change in the contribution from placental glycogen stores. During normal rat pregnancy, fetal plasma glucose concentration is lower, and insulin concentration higher, than in the maternal circulation [52], as replicated here. This may reflect a high fetal glucose demand, with the higher fetal insulin concentration promoting glucose utilisation by fetal insulin-sensitive organs. It is therefore possible that the fetal hypoinsulinaemia observed in the IF group might also contribute to the fetal growth impairment.

Along with maternal undernutrition, placental insufficiency can contribute to impaired fetal growth and accompanying FGR [33]. Morphological differences in the placental exchange barrier, reduced placental blood flow arising from placental maldevelopment with impaired vascular development, together with dysregulation of key placental nutrient transporters, may all contribute to drive different patterns of fetal growth [53]. Maternal IF did not affect placental weight, and consistent with this, placental junctional and the labyrinth areas (as a percentage of placental total area) of IF fetuses were unaltered compared to controls, suggesting a lack of overt morphological change. Interestingly, male fetuses had a relatively larger labyrinth area compared to females, consistent with a greater exchange area to support fetal growth and compatible with a heavier fetal weight in male fetuses. However, in humans, the placental weight of babies *in utero* during Ramadan fasting is significantly reduced in both sexes [9], but only when maternal fasting occurred during the second and third trimesters of pregnancy [18].

By definition, the genesis of a FGR phenotype in the IF group must reflect a reduced net placental nutrient flux. Therefore the FGR phenotype seen is most likely to be attributable to dysregulated placental function. Indeed, the collective data are consistent with maternal IF eliciting effects both on placental nutrient transport and metabolic function. The fetal:placental weight ratio, a proxy of placental transport efficiency [43], was reduced significantly in both fetal sexes of the IF group. Additionally, the concentration of several fetal plasma amino acids (as a reflection of placental transport capacity) were altered, including histidine, isoleucine, methionine, threonine, valine, alanine, serine, arginine and aspartate; sometimes independent of a changed maternal concentration (e.g. isoleucine, aspartate). Conversely, fetal plasma asparagine was unaltered in the face of a profound reduction in maternal plasma concentration. However, on the whole, a significant reduction in both essential and non-essential amino acids in pregnant IF dams was accompanied by reductions in fetal plasma (albeit with some sex-specific differences). Similar effects have been observed previously in other rodent fasting models of shorter duration [52]. Nonetheless, fetal amino acid concentrations were maintained at higher concentrations than their mother's, suggesting that fetal concentrative capacity was sufficient to maintain a positive fetal:maternal concentration ratio for all amino acids in both dietary groups.

During Ramadan fasting pregnant women show a significant reduction in fasting blood alanine [54], in agreement with the profound reduction in maternal (~54 %) and fetal (~25 %) alanine concentration observed in the IF group. Others have also shown that fasting in rodents causes a striking reduction in the concentration of alanine, a major gluconeogenic substrate in both maternal and fetal compartments [52], which could reflect increased gluconeogenesis, or alternatively, a greater flux of pyruvate into acetyl-CoA for fatty acid synthesis and/or ATP

synthesis via the TCA cycle, which would be required for the brain 'sparing' process as lipids are a major component of the brain. Alanine is a high affinity substrate for system A amino acid transporter subtypes [24] and its activity is crucial for fetal growth; impairment of placental system A activity leads to FGR in humans and in rodent models [22, 25, 27, 30]. Our observation that the maternofetal clearance of ^{14}C-MeAIB across the placentas of IF fetuses *in vivo* was reduced significantly in both sexes compared with that of control fetuses, is in keeping with the postulate that diminished placental system A activity is associated with FGR. Surprisingly, in contrast, when system A-mediated uptake of ^{14}C-MeAIB into isolated plasma membrane vesicles was measured *in vitro*, no differences were observed between dietary groups. This contrasts with other rodent models displaying a FGR phenotype, where these two experimental approaches to measure placental system A activity show good correspondence [33]. A possible explanation for the discordance observed in placental system A activity between these two experimental approaches is that the reduction in maternofetal clearance of ^{14}C-MeAIB arises downstream of transport across the maternal-facing plasma membrane of syncytiotrophoblast layer II from which the plasma membrane vesicles are derived, across another plasma membrane within the syncytial layers of the labyrinth villi. Alternatively, regulatory factors or prevailing driving forces *in vivo* may modulate transplacental amino acid flux mechanisms.

As pregnancy advances, fetal growth is supported by increased placental system A transporter expression and activity [55, 56]. As in human placenta [28], rat placenta expresses all three genes, *Slc38a1*, *Slc38a2* and *Slc38a4*, encoding the SNAT1, 2 and 4 isoforms of system A [29], also confirmed here. Interestingly, maternal IF elicited significant increases in *Slc38a1*, *Slc38a2* and *Slc38a4* gene expression solely in the placentas of male fetuses compared to controls; no changes were observed in IF females. Likewise, in other dietary models, placental *Slc38a2* and

Slc38a4 expression was also only upregulated in the placentas of male fetuses [57]. This may be an adaptive response elicited by male fetuses to a compromised maternal amino acid environment to try to stimulate fetal growth investment [31]. However, this up-regulation in mRNA in IF male placentas was not accompanied by increased SNAT1 and SNAT2 expression at the protein level at GD21. Nor was it effective in overcoming the reduction in SNAT-mediated transplacental system A activity observed in IF fetuses at this gestational stage. Divergent trends between SNAT gene and protein expression and system A transporter activity have been reported previously [58]. However, some dietary models show an allied reduction in SNAT protein expression and placental system A transport activity [59], highlighting complexity in the regulation of SNAT-mediated placental system A activity.

The demonstration of higher placental aromatic amino acid concentrations (phenylalanine, tyrosine and tryptophan) in IF males compared with IF females further suggests sexual dimorphism in placental transport or fetal metabolism. The increased placental ophthalmic acid concentration of IF fetuses suggests increased oxidative stress and altered glutathione metabolism [60], and is synthesized from gamma-glutamyl-gamma-aminobutyrate which is also increased in placentas of IF fetuses. N-(4-guanidinobutyl)-4-hydroxycinnamide and gamma-glutamyl-gamma-aminobutyrate are both involved in arginine and proline metabolism and this is of interest in relation to the reduced plasma arginine concentrations observed in male fetuses.

In summary, although it is difficult to model all aspects of Ramadan fasting during pregnancy precisely, this study has demonstrated that a principal feature of Ramadan fasting, namely repeated cycles of maternal IF, has a negative impact on maternal physiology, as reflected by a

reduction in food intake and reduced weight gain during pregnancy when compared to the control group. Maternal IF resulted in reduced maternal glucose, glucagon and amino acid concentrations. In contrast, IF fetuses were able to maintain normal glucose and glucagon concentrations while insulin concentrations were reduced. The normal fetal glucose concentration was coupled with a reduction in fetal liver glycogen content. Fetuses exposed *in utero* to IF exhibited growth restriction but the fetal brain seem to be 'spared', as evidenced by an increased fetal brain:liver weight ratio. Fetuses exposed to IF suffered from reductions in certain essential and non-essential amino acids in parallel with their mothers. Interestingly, sex-specific differences emerged in both groups, with females having a lower concentration of certain amino acids compared to males. Likewise, aromatic amino acids and trisaccharides were lower in the placentas of IF female fetuses compared to IF male fetuses. Sub-optimal maternal nutrition induced by IF was associated with a reduction in transplacental ^{14}C-MeAIB flux measured *in vivo*. This alteration in system A activity was not observed in the maternal-facing plasma membrane of syncytiotrophoblast layer II *in vitro*, suggesting that placental efflux may be rate-limiting. However, regulation of placental transport *in vivo* by circulating factors may also exert an influence. Placental weight near-term was unaltered by IF and this was associated with unchanged junctional and labyrinth area ratios, although sex-specific differences appeared in both dietary groups, with males having higher labyrinth and lower junctional relative ratios compared with females.

These data have provided evidence that maternal IF has physiological effects within the maternal, placental and fetal compartments. This raises the possibility that pregnant women who observe the practice of Ramadan fasting may experience adverse effects on maternal

physiology, affecting placental function and impacting on fetal development, with the potential for possible longer term effects on the child later in life.

Clinical perspectives

- Many pregnant women of Muslim faith fast between dawn and sunset during the month of Ramadan, undertaking the regular cycle of fasting then feeding over many days, although the impacts on maternal and fetal physiology remain poorly defined.
- In a rat model which mimics this regular, repeated cyclical pattern of intermittent fasting during pregnancy, maternal glucose and amino acid concentrations were reduced, placental metabolites were altered and fetuses were growth restricted with reduced amino acid concentrations accompanied by reduced placental amino acid transport.
- Maternal intermittent fasting resulted in detrimental impacts on maternal physiology, placental function and fetal growth, which could compromise fetal developmental trajectory with the potential for enduring effects on the offspring's physiology later in life and an increased predisposition to disease**.**

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Data Availability

Data are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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FIGURE LEGENDS

Figure 1. Maternal food intake, water intake and gestational weight gain.

(A) Maternal food intake, **(B)** water intake and **(C)** weight gain in pregnant rats throughout gestation. Food was removed from IF rats for 16 h per day between 17:00 and 09:00 from GD1 until GD21 (N = 30, open squares); water was available *ad libitum*. Control rats (N = 30, closed circles) had free access to food and water at all times. Data are presented as mean ± SEM, except when SEM falls within the size of the symbol. Statistical comparisons were by two-way ANOVA with repeated measures and Tukey's test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ IF vs control.

Figure 2. Fetal weight and weight frequency distribution curves of control and IF fetuses at GD21.

(A) Body weight was determined in male and female control (N = 36 open boxes) and IF (N = 36 grey boxes) fetuses at GD21, with the number of fetuses (n) as given in (B). Statistical comparisons were by two-way ANOVA and Tukey's test. *** $P < 0.001$ IF vs control; $^{\#}$ $P < 0.05$ male vs female. **(B)** Fetal weight frequency distribution curves were constructed for control male (n = 269, black line), and IF male (n = 255, grey line) and **(C)** control female (n = 280, black dashed line), and IF female (n = 240, grey dashed line) offspring. The 5^{th} centile of control fetal weight for each sex is shown as a dotted vertical line. Statistical analysis was by non-linear regression (Gaussian distribution).

Figure 3. Placental morphology and thickness of the labyrinth and junctional zones in control and IF placentas at GD21. (A) Representative images for histological comparison of control and IF placental morphology. Contoured dashed line in **(A)** shows demarcation between JZ and LZ. D,

decidua. Scale bar = 500 µm. **(B, C)** The thickness of the JZ (red dashed line in **(A)**) and LZ (black dashed line in **(A)**) was measured in placentas of male and female control (n = 5 from N = 3 litters, open bars) and IF (n = 5 from N = 3 litters, grey bars) fetuses. **(B)** LZ and **(C)** JZ layer areas are presented as % of total placental area. Statistical comparisons were by two-way ANOVA and Tukey's test. ** $P < 0.05$, *** $P < 0.01$ female vs male.

Figure 4. Placental system A transporter activity in control and IF fetuses at GD21.

(A) Unidirectional maternofetal clearance of ^{14}C-MeAIB ($^{MeAIB}K_{mf}$) measured *in vivo* across the placentas of control (N = 6, open boxes; male n = 48 and female n = 47) and IF (N = 5, grey boxes; male n = 28 and female n = 27) fetuses at GD21. **(B)** Na^+-dependent ^{14}C-MeAIB uptake at 60 s into placental vesicles of control (N = 13, open boxes) and IF (N = 15, grey boxes) groups isolated from placentas of male (control n = 92, IF n = 111) and female (control n = 111, IF n = 104) fetuses. **(C)** Linearity of Na^+-dependent ^{14}C-MeAIB uptake into placental vesicles over 15-60 s of control male (N=5, open circles), control female (N=5, open square), IF male (N=7, grey circle) and IF female (N=7, grey square) fetuses (linear regression $r^2 \geq 0.98$, $P < 0.05$). Statistical comparisons were by two-way ANOVA and Tukey's test (Figure 4A, B) or by two-way ANOVA with repeated measures (Figure 4C). * $P < 0.05$, ** $P < 0.01$ IF vs control.

Figure 5. Relative mRNA expression of *Slc38a1*, *Slc38a2* and *Slc38a4* in control and IF rat placenta at GD 21.

(A) *Slc38a1,* **(B)** *Slc38a2* and **(C)** *Slc38a4* gene expression in the placentas of male and female (n = 1 per sex from individual litters) of control (N = 8, open boxes) and IF (N =10, grey boxes) fetuses at GD21. Statistical comparisons were by two-way ANOVA and Tukey's test. * $P < 0.05$, ** $P < 0.01$ IF vs control.

Figure 6. Expression of SNAT1, SNAT2 in control and IF rat placental vesicles at GD 21. Expression of **(A)** SNAT 1 and **(B)** SNAT2 (60kDa) and **(C)** SNAT2 (150kDa), normalised to β-actin, in placental vesicles from male and female control (N = 8, open boxes) and IF (N = 8, grey boxes) fetuses. Statistical comparisons were by two-way ANOVA. No significant differences were identified.

TABLES

Table 1. Maternal and fetal plasma glucose and hormone concentrations at GD21.

	Control	**IF**
	Maternal (N = 9-14)	
Glucose (mmol/L)	7.5 ± 0.3	5.0 ± 0.2***
Glucagon (pmol/L)	10.8 ± 2.4	2.2 ± 0.7**
Insulin (μg/L)	0.4 ± 0.1	0.3 ± 0.1
	Fetal (N = 5-6)	
Glucose (mmol/L)	1.9 ± 0.2	1.5 ± 0.2
Glucagon (pmol/L)	3.2 ± 0.4	3.9 ± 0.6
Insulin (μg/L)	1.7 ± 0.2	1.0 ± 0.1*

Data are shown as mean ± SEM. There were no differences between fetal sexes in either dietary group, thus the data were pooled (n = 11-12 per group). Statistical comparisons were by unpaired t-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ IF vs control.

Table 2. Placental and fetal liver glycogen contents at GD21.

	Control (N = 5-6)		**IF** (N = 5-6)	
	Male	Female	Male	Female
Placental glycogen	2.3 ± 1.4	2.2 ± 0.2	1.9 ± 0.3	2.2 ± 0.2
Fetal liver glycogen	46 ± 4	46 ± 6	24 ± 3**	27 ± 3*

Tissue glycogen content data (μg/mg wet weight) represent average values per litter (for placenta n = 6, for liver n = 10 per sex) and are shown as mean ± SEM. Statistical comparisons were by two-way ANOVA and Tukey's test. * $P < 0.05$, ** $P < 0.01$ IF vs control.

Table 3. Placental and fetal organ weights, and fetal anthropometric measurements at GD21.

	Control		IF	
	Male	Female	Male	Female
Litter size	8 ± 1	8 ± 1	7 ± 1	7 ± 1
Placental weight (g)	0.46 ± 0.01	0.46 ± 0.02	0.43 ± 0.01	0.42 ± 0.01
Fetal:placental weight ratio	8.3 ± 0.2	8.1 ± 0.2	7.5 ± 0.1**	7.4 ± 0.2*
	Fetal anthropometric measurements (cm)			
Crown:rump length	4.33 ± 0.01	4.27 ± 0.09	3.99 ± 0.07**	3.97 ± 0.05**
Abdominal circumference	3.64 ± 0.05	3.62 ± 0.07	3.44 ± 0.07	3.37 ± 0.07
Head circumference	3.89 ± 0.07	3.79 ± 0.06	3.59 ± 0.08*	3.51 ± 0.06*
	Relative fetal organ weights (mg/g body weight)			
Brain	44.9 ± 0.8	45.8 ± 1.3	45.6 ± 1.1	45.3 ± 1.8
Kidney	7.3 ± 0.4	7.3 ± 0.4	6.6 ± 0.3	6.5 ± 0.3
Liver	71.0 ± 2.4	69.1 ± 2.7	61.9 ± 5.1	60.2 ± 4.6
Heart	4.9 ± 0.4	4.9 ± 0.5	4.6 ± 0.2	4.7 ± 0.3
Brain/liver weight ratio	0.56 ± 0.01	0.56 ± 0.01	0.68 ± 0.03**	0.69 ± 0.02**

Data are shown as mean ± SEM. Values represent the average per litter; N is the number of litters. For litter size, N=35; for placental weight and fetal:placental weight ratio N = 33 (control male n = 241, control female n = 251, IF male n = 233, IF female n = 218); for anthropometric measurements N = 5-7 (control male n = 27, control female n = 46, IF male n = 50, IF female n = 51); for relative organ weights N = 10-11 (control male n = 92, control female n = 82, IF male n = 60, IF female n = 72). Statistical comparisons were by two-way ANOVA and Tukey's or Fisher's exact test (litter size). * $P < 0.05$, ** $P < 0.01$ IF vs control.

Table 4. Maternal and fetal plasma amino acid concentrations at GD21.

	Dam		Fetal Male		Fetal Female	
	C (N = 6)	**IF (N = 6)**	**C (N = 6)**	**IF (N = 7)**	**C (N = 6)**	**IF (N = 7)**
Essential Amino Acids						
His	49 ± 3	30 ± 2***	173 ± 18	69 ± 9***	137 ± 13	61 ± 8***
Ile	59 ± 5	47 ± 3	162 ± 8	123 ± 8**	136 ± 5	100 ± 3**
Leu	72 ± 7	64 ± 3	277 ± 14	232 ± 16	237 ± 11	192 ± 8
Lys	709 ± 100	588 ± 66	1695 ± 79	1839 ± 138	1345 ± 51$^{\#}$	1471 ± 73
Met	29 ± 2	22 ± 1*	99 ± 4	80 ± 3**	82 ± 2	69 ± 2*
Phe	39 ± 1	36 ± 1	212 ± 9	241 ± 9	181 ± 12	200 ± 5$^{\#}$
Thr	228 ± 21	148 ± 15**	327 ± 18	255 ± 19*	282 ± 16	218 ± 10
Trp	38 ± 3	35 ± 5	97 ± 6	111 ± 4	79 ± 2	86 ± 7$^{\#}$
Val	99 ± 9	75 ± 5*	328 ± 21	264 ± 14*	272 ± 14	220 ± 6
Non-Essential Amino Acids						
Ala	607 ± 38	277 ± 22***	1003 ± 66	740 ± 48**	889 ± 50	682 ± 18*
Arg	101 ± 10	62 ± 3**	215 ± 10	154 ± 10**	166 ± 12$^{\#}$	131 ± 8
Asn	80 ± 4	42 ± 2***	144 ± 6	113 ± 6	122 ± 7	95 ± 2
Asp	7 ± 0.7	6 ± 0.8	42 ± 4	32 ± 3	44 ± 2	29 ± 3*
Glu	55 ± 5	53 ± 4	262 ± 24	245 ± 25	262 ± 32	244 ± 31
Gln	529 ± 36	437 ± 22	793 ± 19	791 ± 30	716 ± 27	736 ± 24
Gly	122 ± 6	126 ± 8	226 ± 9	241 ± 10	200 ± 18	227 ± 8
Ser	223 ± 17	151 ± 13**	350 ± 10	300 ± 19	321 ± 13	254 ± 9*
Tyr	28 ± 1	23 ± 0.9*	157 ± 4	166 ± 4	132 ± 6$^{\#}$	147 ± 4$^{\#}$

Data for amino acid concentration (μM) are shown as mean ± SEM. Values represent data from individual dams or fetuses from different litters; N is the number of dams or litters. Statistical comparisons were by unpaired t-test or Mann-Whitney test for maternal samples and by two-way ANOVA and Tukey's test or Kruskal-Wallis test and Dunn's test for fetal samples. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ IF vs control; $^{\#}$ $P < 0.05$ female vs male.

TABLE 5. Metabolites with significant changes in IF placentas of both sexes compared to controls at GD 21.

Metabolite	C M	IF M	C F	IF F	q value
Ophthalmic acid	Not Detected	0.010	Not Detected	0.006	6.3E-15
Octahydroindole-2-Carboxylic Acid	Not Detected	0.015	Not Detected	0.010	9.0E-13
N-(4-Guanidinobutyl)-4-hydroxycinnamide	0.001	0.021	Not Detected	0.016	5.4E-11
1-(beta-D ribofuranosyl)nicotinamide	0.002	0.021	0.003	0.017	5.8E-07
1-(5-Phosphoribosyl)imidazole-4-acetate and/or 5-Hydroxymethyluridine-2'-Deoxy-5'-Monophosphate and/or 5-Methyluridine 5'-Monophosphate	Not Detected	0.011	0.003	0.006	1.4E-05
2-Hydroxybutyric acid 3-Hydroxybutyric acid and/or 4-Hydroxybutanoic acid	0.001	0.005	0.001	0.003	6.2E-05
(6R)-6-(L-erythro-1,2-Dihydroxypropyl)-7,8-dihydro-6H-pterin	0.001	0.011	0.001	0.006	0.0008
3-Sulfocatechol and/or 4-Sulfocatechol	0.009	0.005	0.015	0.003	0.001
Pseudouridine 5'-phosphate and/or UMP	0.002	0.004	0.001	0.002	0.001
gamma-Glutamyl-gamma-aminobutyrate	0.001	0.007	0.001	0.006	0.002
3,3-Dimethylglutaric acid and/or 3-Methyladipic acid and/or 6-Carboxyhexanoate	0.005	0.011	0.005	0.008	0.003
LysoPS(20:4)	0.001	Not Detected	0.002	0.003	0.005
PC(O-18:1)) and/or PC(P-18:0)	0.018	0.009	0.014	0.012	0.007
MG (12:0)	0.002	0.007	0.003	0.004	0.009
Oxo-nonanoic acid and/or hydroxy-nonenoic acid	0.001	0.013	0.002	0.006	0.009
Acetylcarnitine	0.006	0.012	0.008	0.012	0.011
N6,N6-Dimethyladenosine	0.006	0.012	0.008	0.012	0.012
Desmosine and/or Isodesmosine	0.001	0.003	0.001	0.001	0.021
DG(43:6) and/or DG(41:3)	0.049	Not Detected	0.061	0.053	0.023
S-(11-hydroxy-9-deoxy-delta12-PGD2)-glutathione and/or S-(9-hydroxy-PGA1)-glutathione	0.001	0.004	0.001	0.002	0.026
(22alpha)-hydroxy-cholestanol and/or 3alpha,7alpha-dihydroxy-5beta-cholestane and/or 5beta-cholestane-3alpha,12alpha-diol and/or 5-beta-cholestane-3alpha,26-diol and/or 5-beta-cholestane-3-alpha,7-alpha-diol and/or; 6alpha-hydroxycholestanol	0.002	0.001	0.003	0.002	0.043
PE(36:3) and/or PE(38:6)	0.030	0.014	0.024	0.020	0.044

Values shown are median. q value = P value control versus IF with black and red values indicating a significant increase and reduction respectively in those metabolites in IF placentas compared to control (one-way ANOVA followed by Tukey post hoc). C F, control female; C M, control male; DG, diglyceride; IF F, IF female; IF M, IF male; LysoPS, lysophosphatidylserine; PC, glycerophosphatidylcholine; PE, glycerophosphatidylethanolamine ; UMP, uridine monophosphate.

n = 6 placentas for each fetal sex from N = 6 litters per dietary group.

TABLE 6. Metabolite differences between sexes of IF placentas at GD 21.

Metabolite	IF F	IF M	q value
Phenylalanine	0.027	0.062	0.044
Tryptophan	0.007	0.020	0.015
Tyrosine	0.007	0.010	0.039
Trisaccharide (e.g maltotriose)	0.002	0.004	0.033

Values are shown as median. q value = *P* value, male versus female (one-way ANOVA followed by Tukey post hoc). IF F, IF female; IF M, IF male.
n = 6 placentas for each fetal sex from N = 6 litters per dietary group.

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FIGURES
FIGURE 1

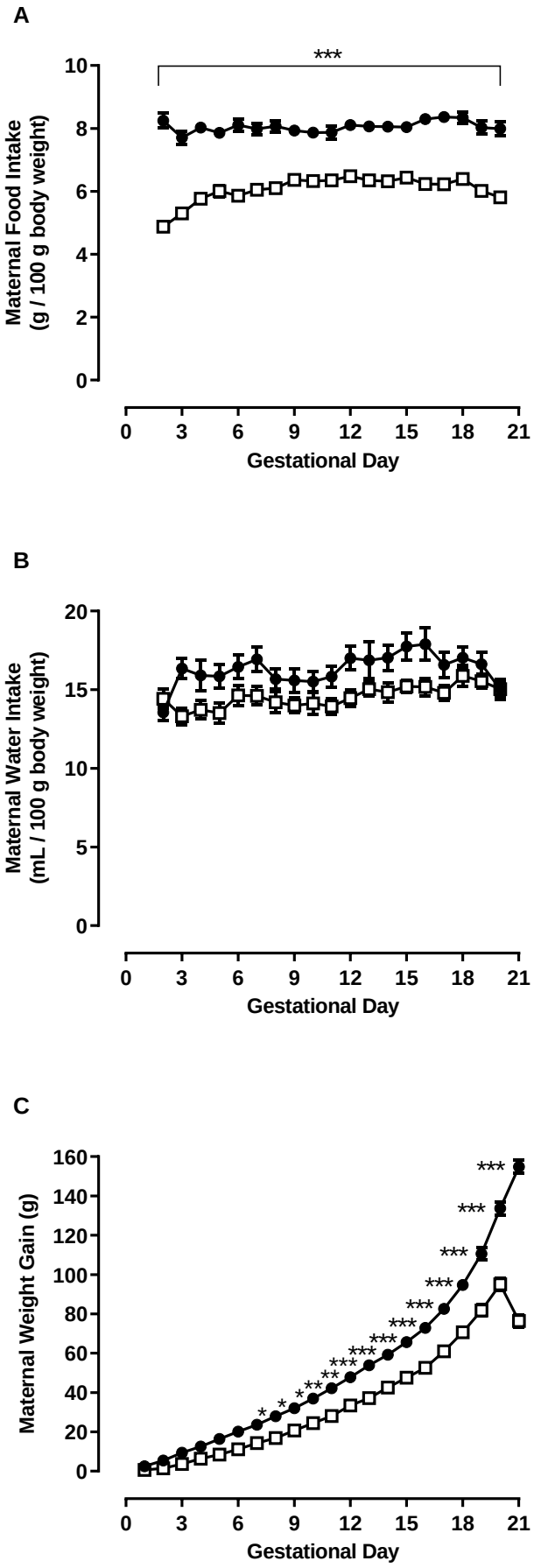


FIGURE 2

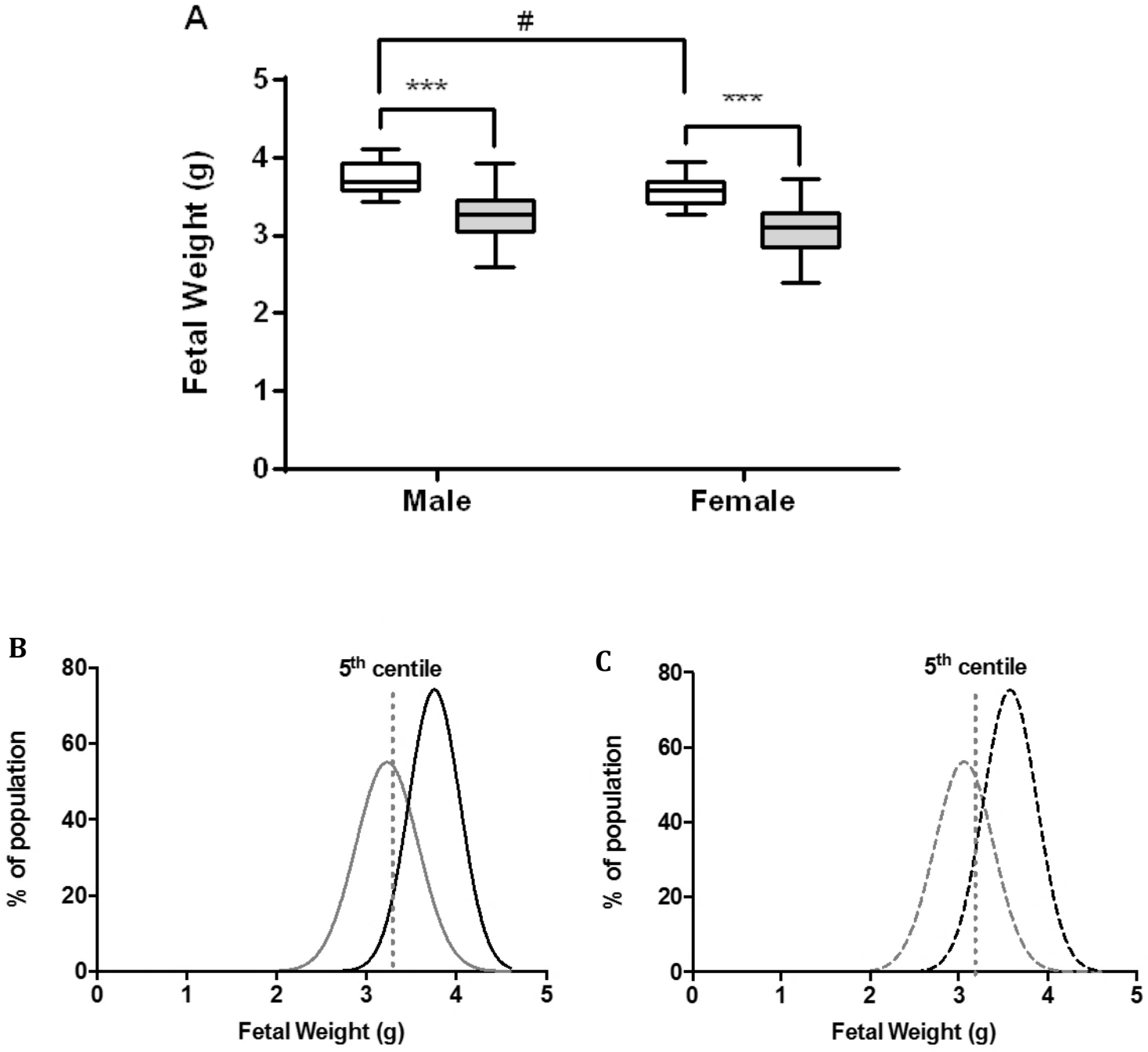
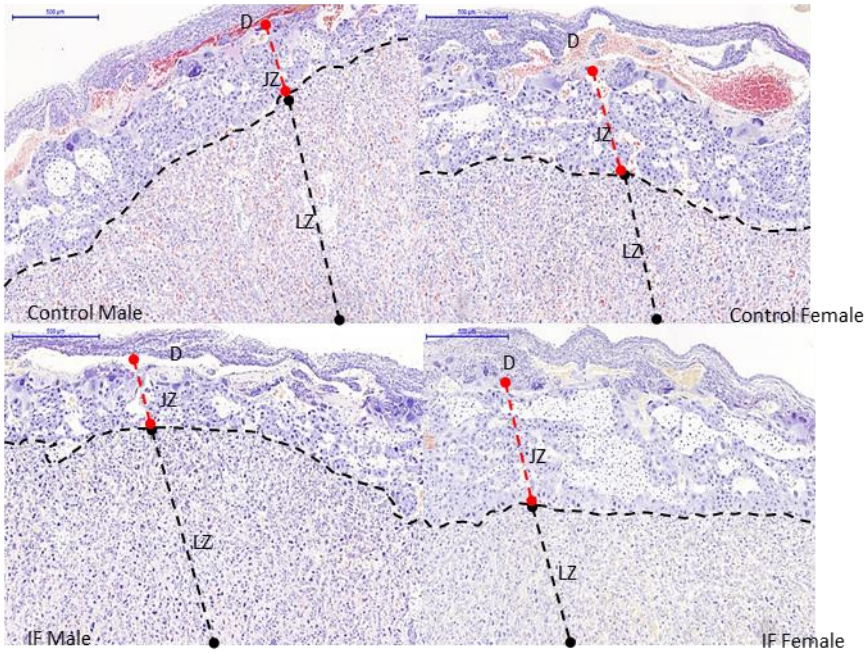
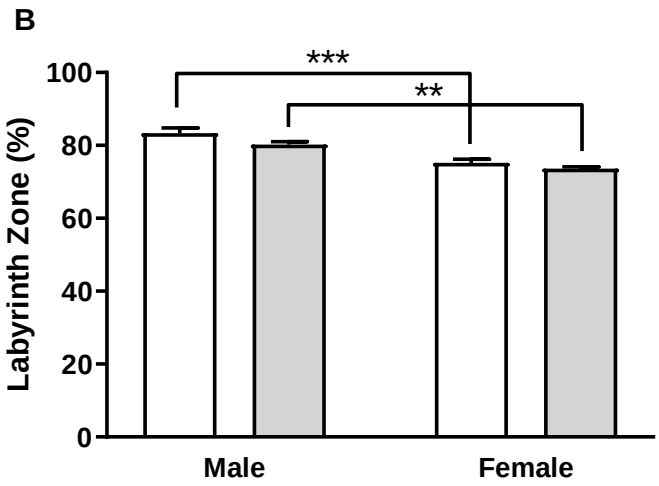

A
#
***
***
Fetal Weight (g)
0
1
2
3
4
5
Male
Female
B
5th centile
% of population
0
20
40
60
80
0
1
2
3
4
5
Fetal Weight (g)
C
5th centile
% of population
0
20
40
60
80
0
1
2
3
4
5
Fetal Weight (g)

FIGURE 3

A


D
JZ
LZ
Control Male
Control Female
IF Male
IF Female

B


***
**
Labyrinth Zone (%)
100
80
60
40
20
0
Male
Female

C

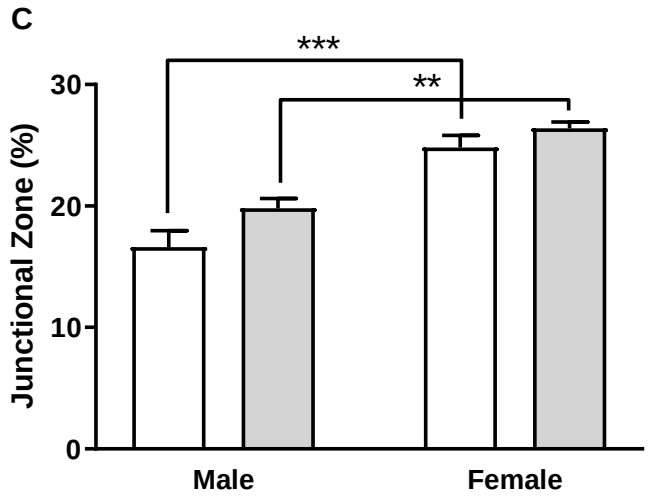
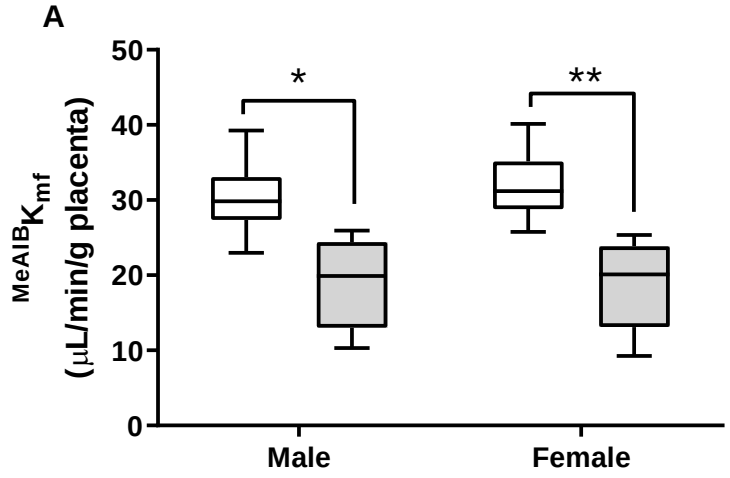
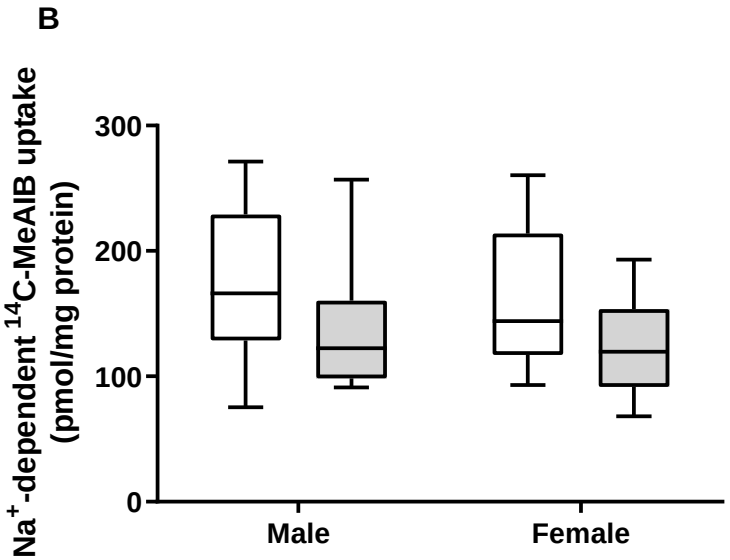

***
**
Junctional Zone (%)
30
20
10
0
Male
Female

FIGURE 4


A
50
40
30
20
10
0
*
**
$^{MeAIB}K_{mf}$
(μL/min/g placenta)
Male
Female


B
300
200
100
0
Na^+-dependent ^{14}C-MeAIB uptake
(pmol/mg protein)
Male
Female

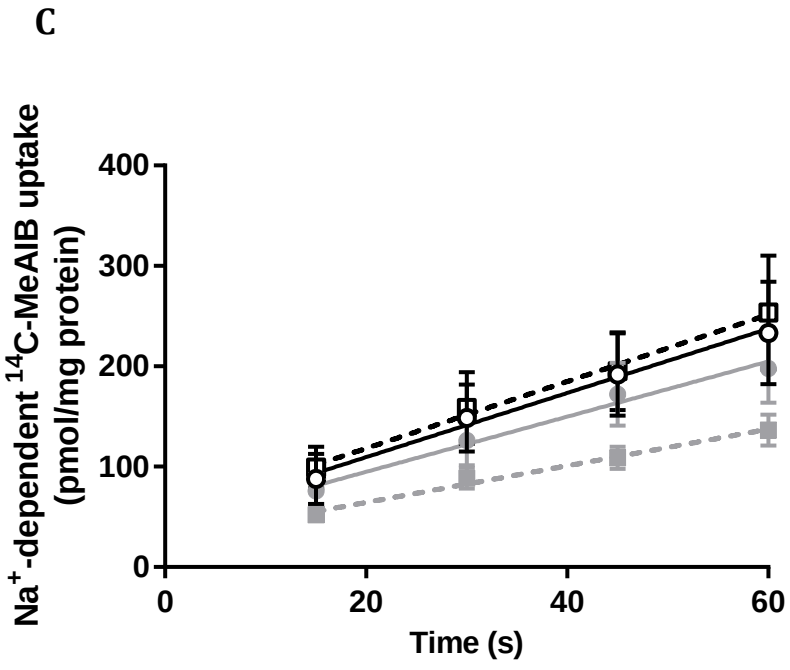

C
400
300
200
100
0
Na^+-dependent ^{14}C-MeAIB uptake
(pmol/mg protein)
0
20
40
60
Time (s)

FIGURE 5

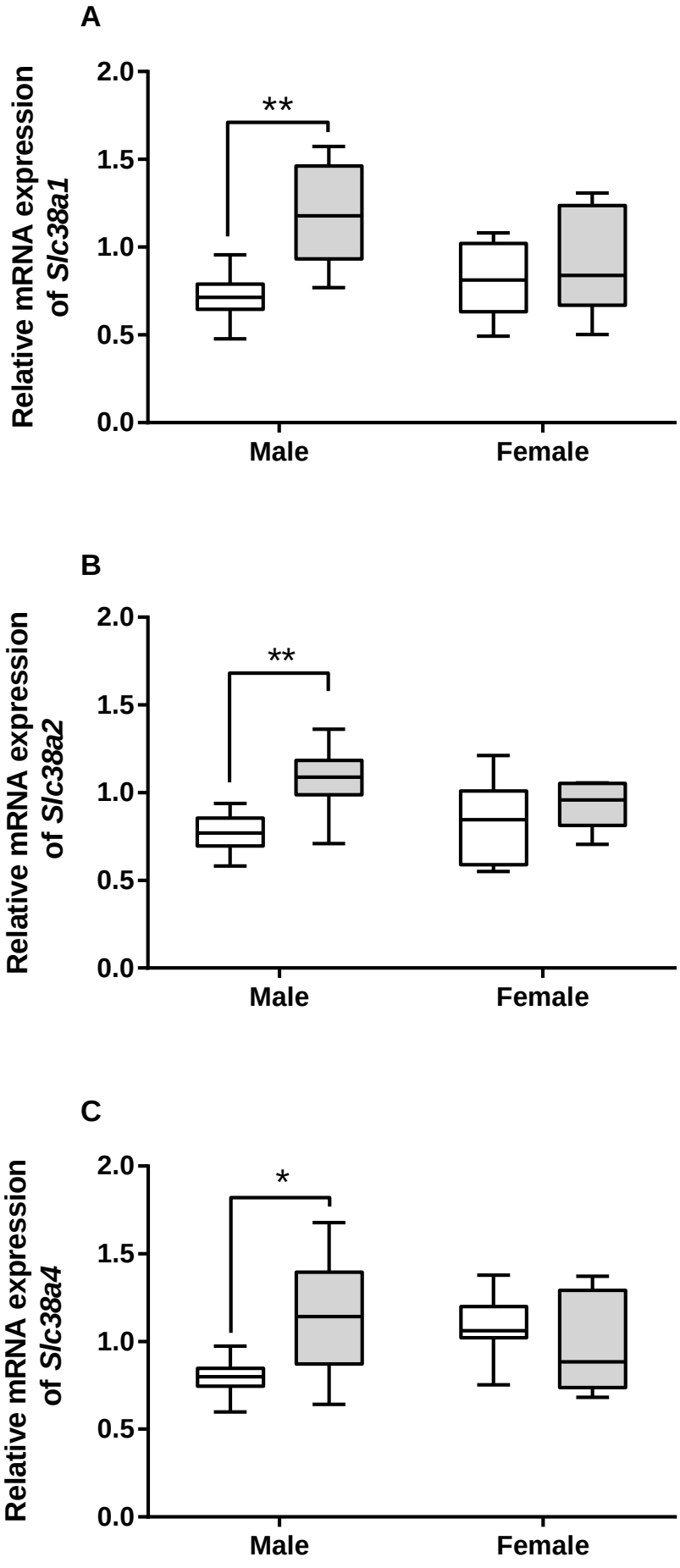
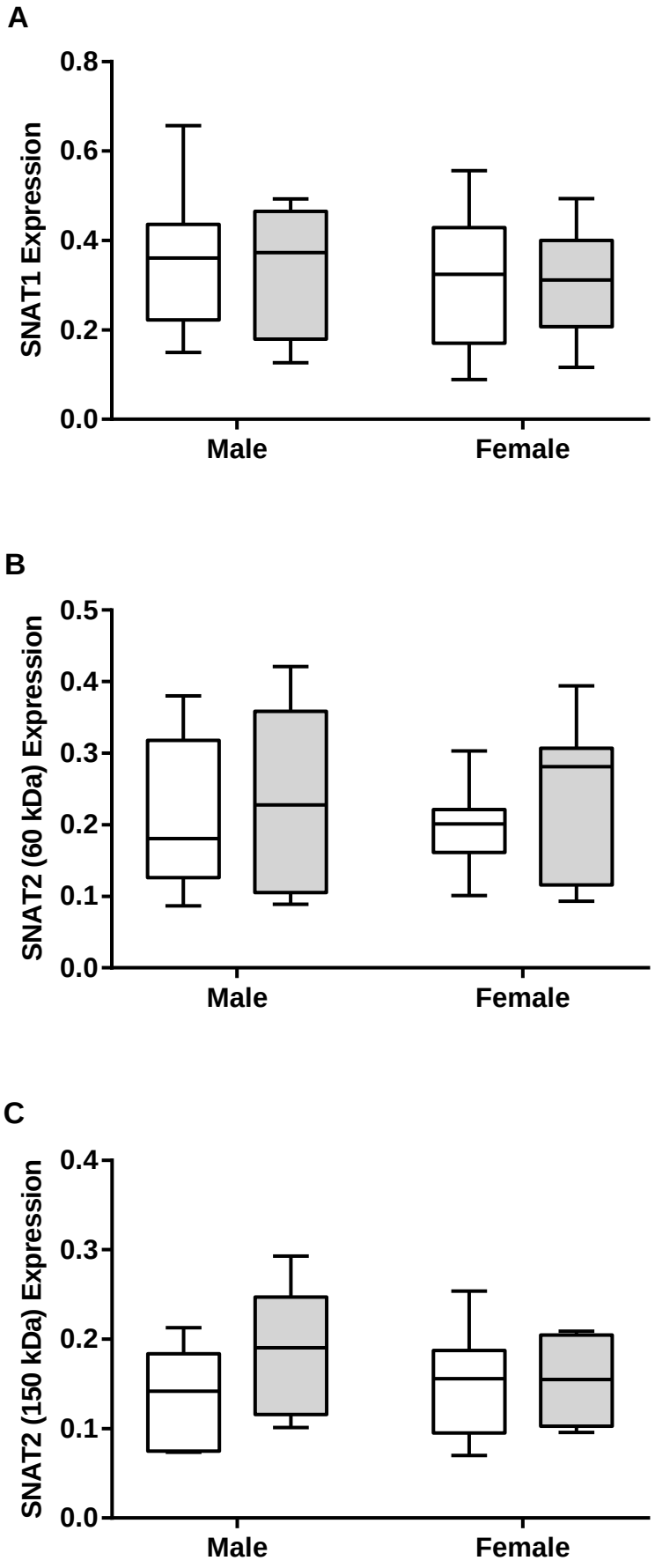

A
Relative mRNA expression of Slc38a1
2.0
1.5
1.0
0.5
0.0
**
Male
Female
B
Relative mRNA expression of Slc38a2
2.0
1.5
1.0
0.5
0.0
**
Male
Female
C
Relative mRNA expression of Slc38a4
2.0
1.5
1.0
0.5
0.0
*
Male
Female

FIGURE 6


A
SNAT1 Expression
0.8
0.6
0.4
0.2
0.0
Male
Female
B
SNAT2 (60 kDa) Expression
0.5
0.4
0.3
0.2
0.1
0.0
Male
Female
C
SNAT2 (150 kDa) Expression
0.4
0.3
0.2
0.1
0.0
Male
Female

SUPPLEMENTARY TABLES

Supplementary Table 1. Primers for real-time qPCR.

Gene	Quantitect Primer Assay	Genbank Accession No.
Slc38a1	QT00187586	NM_138832
Slc38a2	QT00186116	NM_181090
Slc38a4	QT00187943	NM_130748
Ywhaz	QT02382184	NM_013011

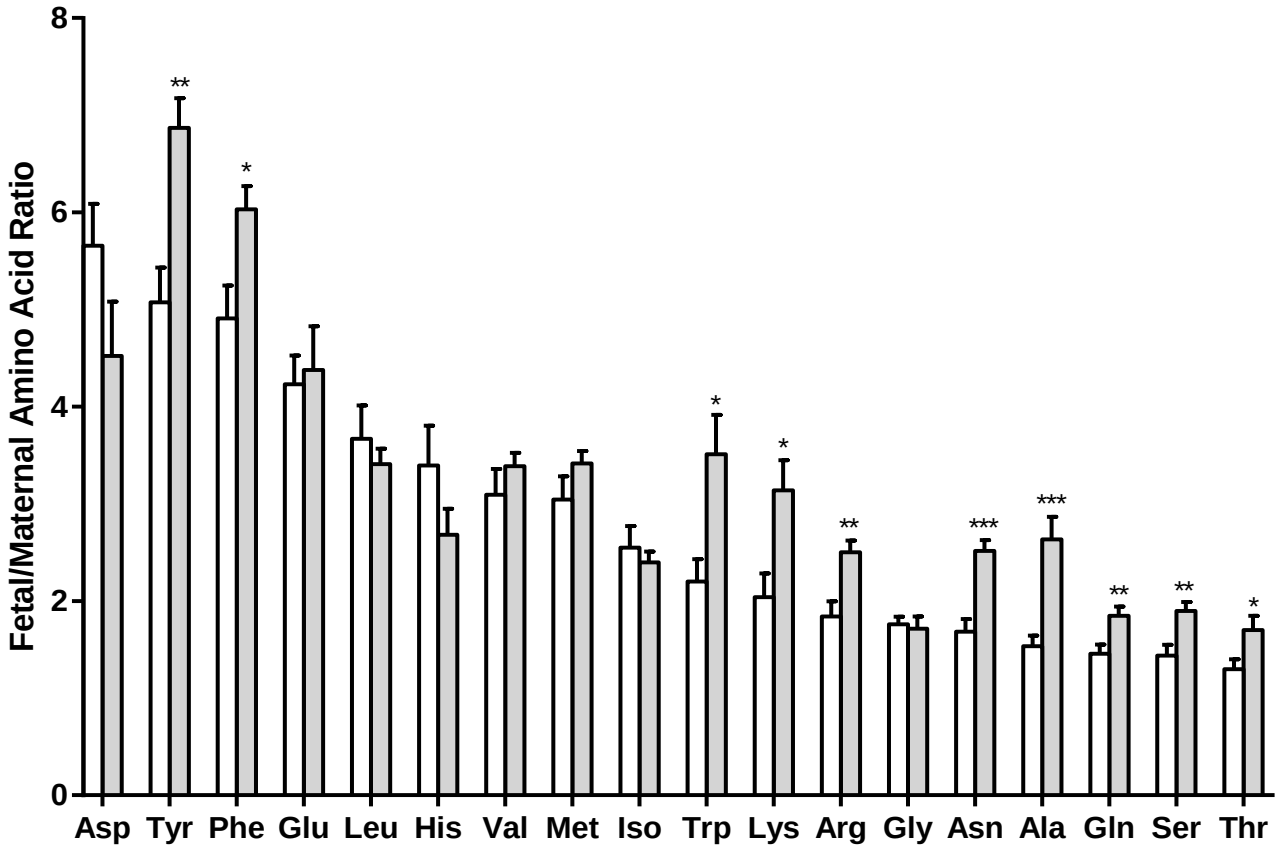
Supplementary Table 2. Maternal organ weights at GD21.

Organ	**Control** (N = 7)	**IF** (N = 7)
	Relative maternal organ weight (g/100 g body weight)	
Heart	0.28 ± 0.01	0.27 ± 0.02
Liver	4.7 ± 0.1	4.0 ± 0.1**
Kidney	0.5 ± 0.0	0.6 ± 0.02*

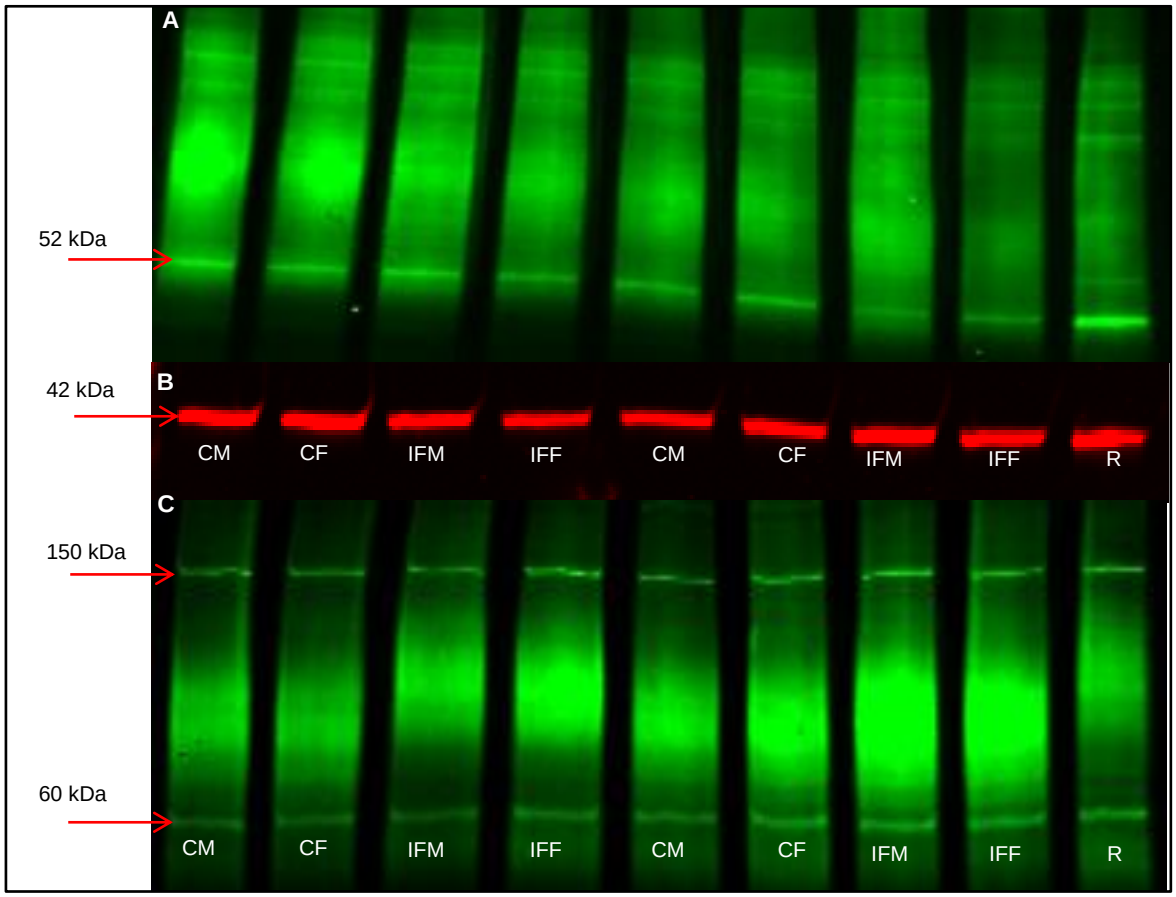
Data are shown as mean ± SEM; N is the number of dams. Statistical comparisons were by unpaired t-test. * $P < 0.05$, ** $P < 0.01$ IF vs control.

SUPPLEMENTARY FIGURES

SUPPLEMENTARY FIGURE 1



SUPPLEMENTARY FIGURE 2



SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Fetal:maternal plasma amino acid concentration ratios in control and IF dams at GD21.

As there were no significant differences between fetal sexes of either dietary group, data are shown as the litter average for control (N = 6, open bars) and IF (N = 6 grey bars) rats for clarity. Statistical comparisons for each amino acid between control and IF groups were by unpaired t-test or Mann-Whitney test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ IF vs control group.

Supplementary Figure 2. Western blot of SNAT1 and SNAT 2 expression in control and IF placental vesicles at GD21.

(A) The SNAT1 antibody detected a band at the predicted size of 52 kDa (A, arrow). **(B)** β-actin antibody generated a single immunoreactive band at 42 kDa in all samples (B, arrow). **(C)** SNAT2 showed two distinct bands at 60 and 150 kDa respectively (C, arrows). Abbreviations: CF control female; CM control male; IFF IF female; IFM IF male; R reference sample of pooled placentas from control dams at GD21. Image is representative of 4 blots.