Title: Placental mitochondrial function, nutrient transporters, metabolic signalling and steroid metabolism relate to fetal size and sex in mice.

Running title: Placental alterations relate to fetal size and sex

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Abbreviations: AMPK, 5' AMP-activated protein kinase; AKT, protein kinase B; C, complex; CI L, complex I respiration in LEAK state; CI P, OXPHOS capacity via complex I; CI P, OXPHOS capacity via complex II; CLPP, caseinolytic mitochondrial matrix peptidase proteolytic subunit (CLPP); CS, citrate synthase; DRP1, dynamin related protein; ET, Electron Transfer; ETS, electron transfer system; FAO, fatty acid oxidation; FC, fetal capillaries; FCCP, trifluoromethoxy carbonyl-cyanide phenylhydrazone; F:P, fetal weight to placenta weight ratio; HSP60/70, chaperonin; LEAK respiration, non-phosphorylating state of intrinsic uncoupling; LGA, large for gestational age; Lz labyrinth zone; OPA1, mitochondrial dynamin like GTPase, OXPHOS, oxidative phosphorylation; MAPK, mitogen activated kinase; MBS, maternal blood spaces; MFN1/2, mitofusin 1/2; ns, not-significant; PGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-α; PPARγ,
peroxisome proliferator activated receptor-γ, SGA, small for gestational age; TB, trophoblast; TBS, tris-buffered saline; TMPD, N, N, N’, N’-tetramethyl-p-phenylenediamine; TID1, tumorous imaginal disc 1/mitochondrial DnaJ co-chaperone protein; 1-P/E, ETS excess capacity.
Abstract

Fetal growth depends on placental function, which requires energy supplied by mitochondria. Here we investigated whether mitochondrial function in the placenta relates to growth of the lightest and heaviest fetuses of each sex within the litter of mice. Placentas from the lightest and heaviest fetuses were taken to evaluate placenta morphology (stereology), mitochondrial energetics (high-resolution respirometry), and mitochondrial regulators, nutrient transporters, hormone handling and signalling pathways (qPCR and western blotting). We found that mitochondrial complex I and II oxygen consumption rate was greater for placentas supporting the lightest female fetuses, although placental complex I abundance of the lightest females and complexes III and V of the lightest males were decreased compared to their heaviest counterparts. Expression of mitochondrial biogenesis (Nrf1) and fission (Drp1 and Fis1) genes was lower in the placenta from the lightest females, whilst biogenesis-related gene Tfam was greater in the placenta of the lightest male fetuses. Additionally, placental morphology and steroidogenic gene (Cyp17a1 and Cyp11a1) expression were aberrant for the lightest females, but glucose transporter (Glut1) expression lower in only the lightest males versus their heaviest counterparts. Differences in intra-litter placental phenotype were related to sex-dependent changes in the expression of hormone responsive (androgen receptor) and metabolic signalling pathways (AMPK, AKT, PPARγ). Thus, in normal mouse pregnancy, placental structure, function and mitochondrial phenotype are differentially responsive to growth of the female and the male fetus. This study may inform the design of sex-specific therapies for placental insufficiency and fetal growth abnormalities with life-long benefits for the offspring.

Keywords Placenta, fetus, sex, mitochondria, transport, hormones
Introduction

A successful pregnancy strongly depends on balancing resource allocation between the genetically determined fetal drive for growth and the mother who needs resources to support the pregnancy state. As the functional interface between mother and fetus, the placenta plays a key role in balancing fetal and maternal resource needs. Amongst its plethora of functions, the placenta executes the metabolism and secretion of hormones that have physiological effects on the mother and fetus, and transfers nutrients and oxygen from the mother to the fetus \(^1\). Thus it is perhaps unsurprising that fetal weight is related to placental development, the uteroplacental blood supply of nutrients and oxygen, and the capacity of the placenta to transport substrates to the fetus \(^2\)–\(^8\).

Moreover, failure of the placenta to grow and function properly is associated with a divergence of the fetus away from their genetic growth potential and can lead to small for gestational age (SGA), fetal growth restriction (FGR) or large for gestational age (LGA) \(^9\)–\(^11\). SGA, FGR and LGA not only increase the risk of perinatal morbidity and mortality, but also have long-term consequences for offspring health \(^12\). Thus, it is important to understand the placental mechanisms regulating fetal growth outcomes.

To enable normal placental grow and function, the placenta depends on energy supplied by mitochondria. Mitochondria are the primary source of ATP, which is produced by oxidative phosphorylation (OXPHOS) using substrates derived from β-oxidation and the tricarboxylic acid cycle. ATP is used by the placenta to fuel growth and placental endocrine and transport functions. Mitochondria are also the place within the cell where steroidogenesis occurs; they contain several key proteins and enzymes such as STAR and CYP11A1, which are required for glucocorticoid and sex-steroid synthesis \(^13\). Mitochondria are also involved in cell signalling, homeostasis and survival via production of reactive oxygen species (ROS) and other molecules like nitric oxide. They are also dynamic organelles that can replicate (biogenesis), divide (fission) and combine (fusion) in response to metabolic, growth and stress signals \(^14\),\(^15\). During pregnancy, there are temporal changes in placental mitochondrial respiratory capacity and mitochondrial-related proteins in several species \(^14\),\(^16\)–\(^19\). Increasing evidence also suggests that placental mitochondrial function (mitochondrial OXPHOS, abundance, biogenesis, fission-fusion and efficiency) alters in line with defects in fetal growth and placental development in response to experimental reductions maternal nutrient and oxygen availability \(^20\)–\(^23\). However, little is known about the relationship between placental mitochondrial capacity, placental morphological development and natural deviations in fetal growth in normal, uncompromised pregnancies. Even less is known about whether this relationship may vary for female and male fetuses, which is highly relevant given that sex is emerging as an important contributor to changes in placental, fetal and offspring health outcomes \(^24\),\(^25\).
In this study in mice, we employed an integrative approach to evaluate placenta morphology, mitochondrial OXPHOS capacity and mitochondrial regulator expression (electron transport system (ETS) complexes and biogenesis and fission-fusion regulators), in relation to growth of the lightest and heaviest female and male fetuses within the litter. We also examined the activity of signalling pathways governing placental growth and metabolism, as well as the expression of nutrient transporter and steroid hormone handling genes to further understand how placental phenotype is modulated by fetal weight and sex within the litter. Analyses were conducted on the labyrinth zone (Lz) of the mouse placenta as it is primarily responsible for controlling the transport of nutrients, oxygen and hormones from mother to fetus. Importantly, since the mouse is a polytocous species, normal variation of fetal weight is expected within the litter, even under a normal, healthy gestational environment.

Methods

Animals

All experiments were performed under the U.K. Animals (Scientific Procedures) Act 1986 after ethical approval by the University of Cambridge. A total of 12 C57BL/6J virgin female mice were housed in the University of Cambridge Animal Facility using a 12/12 dark/light system and received ad libitum water and chow food (Rodent No. 3 breeding chow; Special Diet Services, Witham) during the study. At 4 months age, females were mated overnight and the day a copulatory plug was found was designated as gestational day (GD) 1. On GD18, pregnant dams were killed by cervical dislocation, uteri were recovered, and fetuses and placentas cleaned from fetal membranes. All fetuses and placentas from the litter were weighed, and the Lz subsequently micro-dissected. Lz samples from 7 litters were cut in half, after microdissection of placental layers, one Lz half was placed into ice-cold biopsy preservation medium (0.21 M Mannitol, 0.07 M Sucrose, 30% DMSO in H2O and pH7.5) and frozen at -80°C until respirometry analysis. The remaining half of the Lz was snap frozen and stored at -80°C for molecular analyses (RT-qPCR and Western Blot). Placental Lz samples from the additional 5 litters were placed into 4% paraformaldehyde, histologically processed, and used for structural analysis. Fetal brain and liver were dissected and weighed from each fetus. Fetal tails were kept for sex determination by detection of the Sry gene using the Taq Ready PCR system (Sigma), specific primers (Sry: FPrimer: 5’-GTGGGTTCCTGTCCACTGC-3’, RPrimer: 5’-GGCCATGTCAAGCGCCCCAT-3’ and PCR autosomal gene control: FPrimer: 5’-TGGTTGGCATTITTATCCCTAGAAC-3’, RPrimer: 5’-GCAACATGGCAACTGGAAACA-3’) and agarose gel electrophoresis.
Placental Lz respirometry

High resolution respirometry (Oxygraph 2k respirometer; Oroboros Instruments, Innsbruck, Austria) was used to assess the capacity for respiratory substrate use and ETS function. Cryopreserved Lz samples (10-15 mg) were gently thawed in ice-cold sucrose solution (0.25 M sucrose, 0.01 M TRIS-HCl, pH 7.5). Samples were then permeabilized in respiratory medium BIOPS (10 mM CaEGTA buffer, 0.1 µM free Ca$^{2+}$, 1 mM free Mg$^{2+}$, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM dithiothreitol, 6.56 mM MgCl$_2$, 5.77 mM ATP and 15 mM phosphocreatine, pH 7.1) containing saponin (5 mg in 1 ml, Sigma-Aldrich, UK) for 20 minutes on ice. Samples were then washed by three 5 minutes washes in respiratory medium MiR05 (0.5 mM EGTA, 3 mM MgCl$_2$$\cdot$6H$_2$O, 20 mM taurine, 10 mM KH$_2$PO$_4$, 20 mM Hepes, 1 mg/mL of BSA, 60 mM K-lactobionate, 110 mM sucrose, pH 7.1) on ice to remove all endogenous substrates and contaminants. Oxygen concentration (µM) and flux per tissue mass (pmol O$_2$/s/mg) were recorded in real-time using calibrated oxygen sensors and Datlab software (Oroboros Instruments, Austria). Respiratory rates were corrected for instrumental background by DatLab, considering oxygen consumption of the oxygen sensor and oxygen diffusion out of or into the oxygraph chamber measured under experimental conditions in miR05 medium without any tissue present.

A substrate-inhibitor titration protocol was performed under the presence of octanoylcarnitine and using approximately 10-15 mg of permeabilized Lz tissue placed into each oxygraph chamber. Briefly, complex I substrate malate (2mM) was added first to determine LEAK respiration (uncoupled from ATP synthesis; complex I LEAK or CI$_{Leak}$). Next, ADP (5 mM), pyruvate (20mM) and glutamate (10 mM) were added to obtain complex I oxygen flux under OXPHOS state (CI$_{P}$). Then, succinate (10 mM) was added to provoke complex I and II dependent oxidative phosphorylation (CI+II$_P$). Fatty acid oxidation (FAO) was calculated immediately after ADP addition (i.e. octanoylcarnitine, plus malate and ADP). Trifluoromethoxy carbonyl-cyanide phenylhydrazone (FCCP, two doses of 0.5 µM each) was added to obtain total ETS capacity (uncoupled state). To activate complex IV (CIV) dependent respiration, the first three complexes of the ETS were inhibited by adding rotenone (inhibits complex-I; 0.5 µM), malonic acid and myxothiazol (inhibits complex-II; 5 mM and 0.5 µM, respectively) and antimycin A (inhibits complex-III; 2.5 µM). Sodium ascorbate (2 mM) and N, N, N’, N’-tetramethyl-p-phenylenediamine (TMPD, 0.5 mM) were then added to stimulate complex IV supported respiration, which was then inhibited by adding sodium azide (200 mM). ETS excess capacity was calculated using the formula: 1 - CI+II$_P$/total ETS (1-P/E). Cytochrome c (10 µM) was added to check mitochondrial membrane integrity and data were excluded if respiration increased by >30%. All substrates used were at their saturating concentrations to assess maximal mitochondrial respiratory capacity.
**Placental Lz RT-qPCR analysis**

Placental Lz RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, UK) and the quantity of RNA obtained was determined using a NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Auburn, AL). A total of 2 μg per sample was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) according to the manufacturer’s instructions. Three dilutions of each cDNA sample (1:10, 1:20 and 1:100) were run as a triplicate along with non-template controls in the 7500 Fast Real-Time PCR thermocycler System (Applied Biosystems, UK) for gene expression quantification using gene specific primer pairs (Table 1) and SYBR Green master-mix (Applied Biosystems, UK). The standard thermal cycling protocol was conducted as follows: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 95 seconds and 60 °C for 1 min. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method and genes of interest were normalized to the mean expression of 3 housekeeping genes (Hprt, Ywhaz and Ubc), which were stable in the placental Lz between the lightest and heaviest fetuses of each sex.

**Placental Lz western blot analysis**

Protein extraction was performed on frozen Lz tissue homogenized using commercial RIPA lysis buffer (Thermo Scientific, US) supplemented with Mini EDTA-free protease inhibitor cocktail mix (Roche, CH). The protein concentration was determined using the Bicinchoninic Acid protein assay (Thermo Scientific, US). Lysates (3μg/μl in 1xSDS) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.2 μm nitrocellulose membranes (Bio-Rad Laboratories, US) using a semi-dry technique (Semi-dry Blotter, Invitrogen). Protein transfer was confirmed by Ponceau red staining of the membrane. The membrane was washed with tris-buffered saline tween (TBST) and blocked with 5% milk or fetal bovine serum (used for phosphorylated proteins) in TBST on a shaker, for 60 minutes at room temperature. Membranes were then incubated overnight at 4°C with primary antibody (Table 2). The day after, membranes were washed and incubated with rabbit or mouse secondary antibodies tagged to horseradish peroxidase (NA934 or NA931; 1:10000) diluted in TBST containing 2.5% milk for 60 minutes. Protein bands were visualised using Scientific SuperSignal West Femto enhanced chemiluminescence (ECL) substrate (Thermo Scientific, US). The captured images were downloaded, and the signal intensity of protein bands quantified using ImageJ software. Images from Ponceau staining were used for normalization of protein abundance 26.

**Placental immunohistochemistry analysis**
Morphology of the Lz was assessed by double-labelling placental sections with cytokeratin and lectin antibodies to identify trophoblast and fetal capillaries, respectively. Details about the staining protocol have been described in detail elsewhere. Stained sections were then scanned using a NanoZoomer 2.0-RS Digital Pathology System (NDP Scan Hamamatsu, Japan) and stereological analysis of the Lz was performed as described previously.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism version 8 (GraphPad, La Jolla, CA, USA). The lightest and heaviest fetuses for each sex were identified and statistical analyses were performed using paired t-test. Similarly, comparisons of the lightest females versus lightest males and heaviest females versus heaviest males were performed using paired t-test. Relationships between data were undertaken using Pearson’s correlation coefficient (r). Values are expressed as individual data points and/or mean ± SEM and p values < 0.05 were considered statistically significant. The number of samples per group for each analysis is shown in each figure and described in the legends of figures and footnotes of tables.

**Results**

**Conceptus biometry for all female versus all males within the litter**

Considering all fetuses together, conceptus biometric data were different between females and males within the litter at gestational day 18 (term on day 20; Figure S1). In particular, fetus, placenta and Lz weights were lower in females when compared with male fetuses (p=0.03, p<0.001 and p=0.02, respectively). However, there were no differences in fetal brain and liver weights (relative to body weight), or placental efficiency (p=0.06), calculated as the ratio of fetal weight to placental weight between females and males (Figure S1).

Placental weight was not significantly correlated with fetal weight when analysing all conceptuses within the litter collectively (n=71; r=0.17; p=0.15). Similarly, when data was separated into females and males, again no correlation was detected (Figure 1A, females: n=35; r=0.24; p=0.15, males: n=36; r=−0.07 p=0.64). However, when data were segregated to only assess the heaviest and lightest fetuses per sex within the litter, a positive correlation between placental and fetal weight was found for the lightest females (Figure 1B, r=0.74; p=0.005), but not for the lightest males (Figure 1C, r=0.32; p=0.31) or the heaviest fetuses of either sex. These data
suggest that the placenta may be supporting growth of the female and male fetuses in different ways within the litter.

**Conceptus biometry and placental Lz morphology for the lightest versus the heaviest fetuses of each sex**

To understand why there may be a sex-related difference in the relationship between placental weight and fetal weight, the lightest and heaviest fetuses from each litter were selected and conceptus biometry were compared for each sex separately (Figure 2). As expected, fetal weight was lower for the lightest compared to the heaviest for each fetal sex in the litter (Figure 2A, females; p=0.002; males; p<0.0001), and the mean weight difference between them were similar for females and males (14.1% and 13.6% less than heaviest, respectively). Fetal brain and liver weights as a proportion of body weight did not vary, which suggests that the lightest fetuses are symmetrically smaller when compared to the heaviest fetuses (Figure 2B-D). Moreover, placental weight, Lz weight and placenta and Lz efficiency did not vary between the lightest and the heaviest fetuses within the litter, regardless of fetal sex (Figure 2E-H).

Stereological analysis of the placental Lz zone revealed that there were no differences in trophoblast and fetal capillary volumes (Figure 2I-K). However, there was less maternal blood spaces in the placental Lz of the lightest females, compared to the heaviest females, and this difference was not found for the males (Figure 2M). Similarly, maternal blood space surface area was lower in the lightest, compared to heaviest female fetuses, an effect not observed for the male fetuses (Figure 2N). The surface area of the fetal capillaries (Figure 2L) and barrier thickness (Figure 2O) of the Lz did not vary between the lightest and the heaviest fetuses, for either females or males.

**Mitochondria respiratory capacity of the placental Lz for the lightest versus the heaviest fetuses of each sex**

To investigate whether sex-dependent structural changes in the Lz zone between the lightest and heaviest fetuses may be related to mitochondrial functional alterations, high resolution respirometry was performed (Figure 3A). Oxygen flux rate analysis revealed that in LEAK state, mitochondrial CI related oxygen consumption was ~60% greater for the placental Lz of lightest compared to the heaviest females, but no effect was seen for the males (Figure 3B, p=0.003). Whilst CI oxygen flux under OXPHOS state was not different
between the lightest and heaviest fetuses of either sex (Figure 3C), after adding succinate, Lz CI+CI\text{I} oxygen consumption rate was ~44\% greater in the lightest compared to the heaviest females within the litter (p=0.01); a difference that was not observed for the males (Figure 3D). Fatty acid oxidation (FAO), total ETS capacity and CIV associated oxygen consumption by the placental Lz were not different between lightest and heaviest fetuses for either fetal sex (Figure 3E-G). When oxygen consumption rates for CI in LEAK state and CI+CI\text{I} in OXPHOS state were corrected to total ETS oxygen flux to provide a qualitative indication of changes in mitochondrial function per mitochondrial unit, these values were also increased in only the lightest compared to heaviest females (not different for the lightest compared to heaviest males) (Figure 3H, p=0.02; 3I and 3J, p=0.03). In addition, calculation of 1-P/E indicated that ETS excess capacity was lower in the lightest females compared to the heaviest (p=0.03), again a difference not identified for the males (Figure 3K).

**Expression of mitochondrial ETS components, dynamic genes, and regulatory proteins in the placental Lz for the lightest versus the heaviest fetuses of each sex**

To gain further information on the sex-dependent differences in placental mitochondrial respiratory capacity, western blotting and qPCR was performed to determine the expression ETS complex proteins (CI-V), biogenesis, fusion and fission genes, and additional mitochondrial regulatory proteins in the placental Lz of the lightest and heaviest fetuses for both sexes (Figure 4). These analyses revealed that CI abundance was lower in the lightest compared to heaviest females (Figure 4A), meanwhile CIII and CV were lower only in the lightest compared to the heaviest males (Figure 4B). In addition, the expression of mitochondria biogenesis gene, *Nrf1* and mitochondrial fission genes, *Drp1* and *Fis1*, was lower in the Lz of the lightest females, when comparing with the heaviest females (Figure 4C). Whereas the expression of *Tfam*, a mitochondria biogenesis transcription factor gene, was greater in the Lz of the lightest males versus the heaviest males (Figure 4D). Mitochondrial content, informed by citrate synthase abundance, did not vary in the Lz between the lightest and heaviest fetuses within the litter, regardless of fetal sex (Figure 4E). In addition, abundance of proteins involved in mitochondrial biogenesis (PGC-1\text{α}), fusion (MNF2 and OPA1), heat shock (HSP60, HSP70) and chaperone (TID1) proteins did not differ in the Lz between the lightest and heaviest fetuses within the litter, in either sex (Figures 4E). However, CLPP a key protease involved in mitochondrial protein clearance and a marker of the mitochondrial unfolded protein response (UPRmt), was lower in the lightest females compared with the heaviest females; an effect not seen for males (Figure 4E).
Expression of transport genes and steroid metabolism and signalling genes in the placental Lz for the lightest versus the heaviest fetuses of each sex

Since the energy provided by mitochondria helps to fuel placenta transport and endocrine function, we evaluated whether sex-related variations found on mitochondria functional capacity (respiratory function, gene and protein regulators) are associated with the expression of nutrient transporter and steroidogenic genes between the lightest and heaviest of each sex within the litter. In particular, the mRNA expression of key transporters for glucose (Slc2a1 and Slc2a3), amino acid (Slc38a1, Slc38a2, Slc38a4, Slc7a5 and Slc3a2) and lipids (Fatp1, Fatp3, Fatp4, Fatp6 and Cd36) were quantified in the placental Lz zone by RT-qPCR (Figure 5).

We also evaluated the expression of genes involved in steroid hormone production (Star, Cyp11a1 and Cy17a1), glucocorticoid metabolism (11bhsd1 and 11bhsd2) and steroid hormone signalling (Esr2 and Ar) in the placental Lz using qPCR. These analyses showed that the expression of Slc2a1 mRNA was ~20% lower for the lightest compared to the heaviest males (Figure 5B, p = 0.021), however this difference was not observed for the lightest versus the heaviest females. In addition, no differences were found between the lightest and the heaviest fetuses within the litter for any of the other nutrient transporter genes quantified in either fetal sex (Figure 5A-C). The expression of Cyp11a1 was ~63% greater (p=0.038), while Cyp17a1 ~20% lower (p=0.035) in the lightest compared to the heaviest female fetus, with no differences in these steroidogenic genes detected in the males (Figure 5D). Whereas the expression of the androgen receptor (Ar) a steroid-hormone activated transcription factor was ~91% greater (p=0.046) in the lightest compared to the heaviest males only (Figure 5D). The mRNA expression of the estrogen receptor beta (Esr2), the 11β-hydroxysteroid dehydrogenase, isozymes 1 and 2 (Hsd11b1 and Hsd11b2) and the steroidogenic acute regulatory protein (Star) in the Lz were not different between the lightest compared to the heaviest fetuses in either sex.

Abundance of key growth and metabolic proteins in the placental Lz for the lightest versus the heaviest fetuses of each sex

To inform on the sex-dependent differences in placental morphology and mitochondrial function between lightest and heaviest fetuses, the abundance of key growth and metabolic signalling proteins, namely AKT (protein kinase B), AMPKα (5’-AMP-activated protein kinase catalytic subunit alpha-1), p44/42 MAP kinase (ERK1/ERK2), p38 MAPK (mitogen-activated protein kinase) and PPARγ (Peroxisome proliferator-activated receptor gamma) were evaluated by western blotting (Figure 6). The abundance of total AMPKα was greater in the lightest compared to the heaviest fetuses for both females and males (67% and 41%, Figures 6A and C, p=0.005 and p=0.01, respectively), however this was not related to a significant change in activation of AMPKα.
abundance of phosphorylated normalized to total AMPKα, Figures 6B and D). While the total abundance of AKT did not vary between the lightest and the heaviest fetuses, activated AKT (phosphorylated to total) was ~32% lower in the Lz zone supporting the lightest compared to the heaviest males (p=0.032), but no difference was found for the females (Figures 6B and D). The abundance and activation of p44/42 MAPK and p38 MAPK was not different between the lightest and the heaviest fetuses, irrespective of fetal sex (Figure 6A and D).

Interestingly, PPARγ an important transcription factor involved mitochondrial metabolism and lipid synthesis, was greater in the lightest female compared to the heaviest female, whereas PPARγ was lower in the lightest males when compared to the heaviest males from the litter (Figure 6E, p=0.04 and p < 0.05, respectively).

Comparisons of the effect of sex on feto-placental growth of the lightest and heaviest fetuses

To gain further insight into the sex-dependent intra-litter differences in placental phenotype, retrospective comparisons between the lightest females and lightest males and heaviest females and heaviest males within the litter were performed (Supplementary Table 1). These data showed that the heaviest males were ~5% heavier than the heaviest female fetuses within the litter (p <0.05). The placenta of the heaviest males in the litter was also greater by ~13% when compared to the heaviest females (p=0.04), with a tendency for this to also vary with sex for the lightest female littermates (p=0.054). The placental expression of glucose (Slc2a1: -37%, p=0.003) and lipid (Fatp1: +17%, tendency p=0.07) transporter genes were also differentially expressed between the lightest (but not heaviest) male and female fetuses of the litter. Placental respirometry rates associated with CI_{Oxphos} (+39%, tendency p=0.05) and with CI+CI_{Oxphos}/Total ETS (+30%, tendency p=0.08) together with biogenesis (Nrf1: +37%, p=0.03; Tfam: +25%, tendency p=0.06) and dynamic (Opal: +13%, tendency p=0.06, Mfn1: +11%, p=0.05) genes were all greater in the lightest males compared to the lightest females. Meanwhile, biogenesis genes Pparγ (-38%, p=0.03) and Tfam (-19%, tendency p=0.07) were decreased in the heaviest males compared to females. Finally, the expression of the steroidogenic gene Cyp11a1 was lower (-38%, p=0.02) in the lightest males compared to females and in heaviest fetuses, gene expression of Cyp17a1 (-41%, p=0.01) were decreased on males compared to females. There was also no effect of fetal sex in the placental Lz morphology of the lightest and heaviest fetuses.

Discussion

The present study in mice shows that placental mitochondrial functional capacity varies in relation to natural differences in the weight of females and male fetuses within the litter. The placenta Lz of both the lightest
female and male fetuses showed altered abundances of ETS complexes and mitochondrial biogenesis genes between the lightest and heaviest fetuses, although the specific nature of these changes depended on fetal sex. Moreover, the morphology, respiratory capacity, mitochondrial fission, and misfolded protein regulators of the placental Lz differed between the lightest and heaviest females, but not males. The level of nutrient (glucose) transporters varied between the lightest and heaviest males, but not females, whereas the ability to produce steroid hormones differed only between the lightest and heaviest females within the litter. There were also sex-dependent changes in the expression of hormone responsive, growth and metabolic signalling pathways in the placental Lz between the lightest and heaviest fetuses. However, despite these sex-related variations, the average weight differences between the lightest and heaviest fetuses were similar for both sexes. Together, these data suggest that in normal mouse pregnancy, placental structure, function and mitochondrial phenotype appears to respond differentially to the genetically determined growth demands of the female and the male fetus (Figure 7).

Fetal and placenta growth

In our study, placental efficiency, and other fetal biometry parameters (fractional liver and brain weights) were not different between lightest and heaviest fetuses of each sex. This is relevant since placental efficiency indicates the capacity of the placenta to support fetal growth and alterations in this measure, as well as the symmetry of fetal growth enhance the risk for chronic diseases in later life through developmental programming. Similarities in body proportionality between lightest and heaviest fetuses likely relates to the adaptative properties and efficiency of their placentas in these normal, healthy pregnancies. Interestingly, previous work exploring the implications of natural intra-litter variability of placental weight, rather than fetal weight in mice, has found morphological differences between the lightest and the heaviest placentas, which included a greater Lz volume and an increased surface area for exchange. Functional adaptations were also found, with a greater rate of amino acid transfer and enhanced expression of sodium-dependent neutral amino acid transporter-2 (Slc38a2) by the lightest versus the heaviest placentas. Similarly, calcium transfer across lightest placenta was higher than the heaviest placentas within the litter, resulting in similar calcium accretion levels in the fetus. Variations in placental structure and transport within the litter were related to an increase in placental efficiency in both of these previous studies. However, in our study the placentas sustaining the lightest or the heaviest fetus were not necessarily the lightest or the heaviest placenta within the litter. In addition, a key strength of our study is that the lightest and heaviest fetuses of each sex were analysed. Segregating the data by sex identified that there was a positive correlation between placental and fetal weight for only in the lightest females in the
litter. These data indicate that there may be differences in the way in which the placenta may be supporting growth of the female and male fetuses within the litter.

While there was no difference in placental fetal capillaries, trophoblast volume or barrier thickness between the lightest and heaviest of either fetal sex, maternal blood space volume and surface area of the placenta was lower in the lightest compared to the heaviest female fetuses. These differences in placental structure suggest mal-perfusion of the placenta of the lightest females and would be expected to decrease the delivery of nutrient and oxygen to fetus and could explain the weight discrepancy when comparing to the heaviest females in the litter. Previous studies in pigs have shown that compared to placentas supporting fetuses weighing closest to the litter mean, placentas supplying the lightest fetuses within the litter have impaired angiogenesis. Moreover, there are differences in the extent of endothelial cell branching morphogenesis with conditioned media from the placenta of females compared to males. Work in rats has suggested that an angiogenic imbalance may underlie regional differences in uteroplacental vascularization and fetoplacental development within the litter. Our study was not designed to assess the contribution of uterine position to the sex-related differences in placental morphology of the lightest versus heaviest fetuses in the litter, which could be a focus of future work.

**Placental mitochondrial function**

Previous work has shown that in human pregnancies associated with FGR, although mRNA expression of ETS complexes (II, III and IV) is lower, there is a higher mitochondrial DNA (mtDNA) content and higher oxygen consumption related with mitochondrial bioenergetics in the placenta. Similarly, FGR induced by maternal caloric restriction in rats is associated with augmented mitochondrial biogenesis, as evidenced by the increased expression of PGC-1α, NRF1 and TFAM, as well as elevated complex I and IV dependent respiration in the placenta. These data suggest that a common response of the placenta to try and match the genetically determined demands of the fetus for growth during gestation involves the modulation of placental mitochondrial respiratory capacity. The current study supports this notion and shows that the functional characteristics of placental mitochondria also adapts with natural variations of fetal growth in normal pregnancy. In females, biogenesis promoter Nrf1, fission regulators Drp1 and Fis1, and mitochondrial complex I were lower, yet complex I LEAK and complex I+II OXPHOS were greater in the placenta supporting the lightest compared to the heaviest female. Whereas in males, biogenesis gene Tfam was greater, yet mitochondrial complexes III and V were lower and LEAK and OXPHOS capacity were not different in the placenta supporting the lightest compared to the heaviest male. Therefore, for both sexes, the placenta of the lightest fetuses appears to increase
mitochondrial respiratory efficiency (as there was reduced mitochondrial complexes yet unaltered/increased respiration), although the underlying mechanisms and extent to which this may occur is different for females and males. Indeed, our results suggest that mitochondria in the placenta sustaining the lightest female fetuses in the litter are more responsive and susceptible to adaptive mechanisms, as they exhibited increased mitochondrial respiration rates. This enhanced adaptive response may have been beneficial in providing the energy to sustain the expression of glucose transporters for the lightest fetuses. Previous work has demonstrated there is reduced abundance of all mitochondrial complexes and lower OXPHOS respiration rates in placental trophoblast from obese women who deliver high birth weight babies \( ^{31} \). Moreover, mitochondrial complex activity is also decreased in placentas from women with pre-pregnancy obesity or pre-gestational diabetes who have LGA babies \( ^{32} \). Thus, the natural variation in intra-litter placental mitochondrial function in the current study is likely the outcome of adaptive responses in operation for both the lightest and heaviest fetuses.

In the lightest female, but not lightest males, there was lower CLPP protein abundance when compared to the heaviest female fetuses of the litter. CLPP is also decreased along with mitochondrial complex abundance in the placenta of preeclamptic women delivering FGR babies \( ^{33} \). However, no other members associated with the UPRmt pathway that was analysed varied in the placenta between the lightest and heaviest fetuses (HSP60, HSP70 and TID1), which may be expected given that our study was performed on mouse litters from normal, healthy pregnancies. The biological relevance of the sex-specific difference in placental CLPP level between the lightest and heaviest fetuses is currently unknown. However, differences in placental CLPP may be particularly relevant for the outcome of female and male fetuses if the gestation is challenged, such as by a hypoxic or nutritional stimulus.

**Placental sex steroid handling**

The placental expression of key steroid synthetic enzymes was differentially expressed between the lightest and heaviest females only. The greater Cyp11a1 while lower Cyp17a1 in the lightest compared to the heaviest female fetus, would be expected to enhance the synthesis of the steroid hormone precursor pregnenolone, but also limit the synthesis of sex steroids. CYP11A1 and CYP17A1 are both cytochrome P450 monoxygenases located in the mitochondrial membrane that use oxygen for steroidogenesis, and changes in their expression may have relevance for understanding the greater rate of oxygen consumption in LEAK state for the placenta of the lightest *versus* heaviest females. Other work has shown that CYP11A1 is upregulated in the placenta of women with preeclampsia and overexpression of CYP11A1 in human trophoblast cells reduces proliferation.
and induces apoptosis \cite{34,35}. In addition, \textit{in vitro} studies using cell lines have implicated an important role of CYP17A1 in estrogen production by the placenta \cite{36}. Thus, further work would value from quantifying steroid hormone levels in the placenta. The expression of the androgen receptor was greater in the placenta of the lightest compared to the heaviest males; a difference not seen in females. These data suggest enhanced sensitivity of the lightest male placenta to androgens, namely testosterone, which can be produced by the fetal testes from approximately day 12-13 of mouse \cite{37}. Interestingly, in rats elevated testosterone levels disrupts the number and structure of mitochondria in the placenta and decreases fetal weight \cite{38,39}. Additionally, DHT (5α-reduced metabolite of testosterone) and insulin treatment of rats induces mitochondrial damage and an imbalance between oxidative and antioxidative stress responses in the placenta in association with defects in uterine function and FGR \cite{40}. Thus, differences in steroid production and signalling are likely involved in the mechanisms underlying alterations in placental morphology and mitochondrial functional capacity supporting the lightest fetus of each sex.

**Placental signalling pathways**

The mechanisms underlying the differences in intra-litter placental mitochondrial function for each fetal sex are unknown. However, intra-litter differences in placental morphology and mitochondrial functional capacity likely stem from variations in the abundance of AMPK, AKT and PPARγ between the lightest and heaviest female and male fetuses \cite{15}. Increased levels of AMPK in the placenta were seen for both the lightest females and lightest males compared to their heaviest counterparts and may reflect a beneficial adaptation occurring during normal late gestation in mice. AMPK is activated by an increase in the AMP to ATP ratio and hence, is reflective of a decline in energy status. In turn, AMPK activates metabolic enzymes that allow cells to switch on catabolic pathways that generate ATP, including glycolysis and fatty acid β-oxidation \cite{41}. We did not observe differences in placental fatty acid oxidation between the lightest and heaviest of each fetal sex within the litter. However, it would be beneficial to assess glycolysis and glycolytic enzyme expression in the placenta to assess whether there are intra-litter differences for females or males. In addition to its role in energy sensing, AMPK regulates placental trophoblast differentiation, proliferation and nutrient transport \cite{42}. Moreover, AMPK in the placenta has been linked to maternal vascular responses and changes in placental morphology and fetal growth in hypoxic pregnancy \cite{8,43}. Interestingly, compared to their heavier counterparts, the magnitude of increase in placental AMPK was greater for the lightest females \textit{versus} for the lightest males (increased by 67% and 41%, respectively). Whether this may relate to the observation of altered placental morphology in only the lightest females requires further study.
Activation of AKT (phosphorylated AKT) was lower in the Lz of the lightest compared to heaviest male fetuses only. The AKT-mTOR signalling pathway plays a crucial role in the regulation of placental transport function and it has been shown to be up-regulated in pregnancies from obese women delivering LGA babies \(^{44}\) and down-regulated in placentas from SGA/FGR babies \(^{45}\). Moreover, placental trophoblast specific loss of phosphoinositol-3 kinase (PI3K) signalling, which is upstream of AKT leads to FGR in mice \(^{46}\). In line with the reduced AKT activation, only the lightest males presented lower glucose transporter (\(Slc2a1\), encodes GLUT1) expression in their placenta, when compared to the heaviest males. Insulin is a major fetal growth factor that signals via AKT to mediate its metabolic effects. Reduced activation of AKT in the placenta of the lightest males may therefore reflect reduced fetal insulin signalling to the placenta \(^{47}\). These data also reinforce the idea that the placentas of females and males may execute their own molecular mechanisms to best support fetal growth and development. In humans, placental GLUT1 is down-regulated in preeclampsia, and this might play a role on the development of FGR \(^{48}\). Conversely, women with insulin-dependent diabetes who have an increased incidence of LGA show increased GLUT1 expression and a higher mediated uptake of D-glucose by the placenta \(^{49}\). Since glucose is the most important energetic substrate for fetal wellbeing and growth, lower expression of placental \(Slc2a1\) in the lightest relative to the heavier male fetus may explain their fetal growth discrepancy.

Sexually dimorphic differences in the placenta phenotype may also relate to changes in PPAR\(\gamma\) abundance; PPAR\(\gamma\) was greater in the lightest females, but lower in the lightest males compared to their respective counterparts. In humans, PPAR\(\gamma\) expression was found to be reduced in the placenta of SGA fetuses and to associate positively with fetal and placental weights within this subpopulation \(^{50}\). In addition, it has been reported that PPAR\(\gamma\) modulates the expression of amino acid transporters LAT1 and LAT2 (encoded by \(Slc7a5\) and \(Slc3a2\), respectively) and plays a key role in the control of fetal growth \(^{51}\). PPAR\(\gamma\) is also important for the regulation of lipid uptake and metabolism and regulates mitochondrial function via multiple routes \(^{52}\). While in our study no differences were detected in the placental expression of amino acid and fatty acid transporters between lightest and heaviest fetuses, changes in PPAR\(\gamma\) could in part mediate the differential changes in mitochondrial respiratory capacity and regulatory factor expression. PPAR\(\gamma\) is a transcription factor that can be modulated by numerous signals, including hormonal/growth factor signalling pathways, inflammatory/stress signalling pathways and cellular metabolite levels \(^{53}\). Hence, the sex-related alterations in PPAR\(\gamma\) abundance in the placenta of the lightest females and the lightest males likely reflects the very different metabolic and hormonal environment of the fetuses relative to their heaviest counterparts within the litter.
Placental phenotype and fetal sex comparisons

Previous work has shown there are changes in placental Lz morphology, function, mitochondrial respiratory capacity and mitochondrial-related regulators that help to support the growth demands of the fetus during normal late mouse pregnancy. In the current study the heaviest males and their placentas were heavier than the heaviest females within the litter, although no differences were found in placental morphology, mitochondrial functional capacity, or transport/hormone genes between them. In contrast, the lightest males and their placentas did not differ in weight when compared to the lightest females (tendency for placental weight to be greater in the lightest males versus females), yet they varied in the placental expression of nutrient transporters, steroidogenesis genes, mitochondrial respiratory capacity and mitochondrial-related gene expression. These data suggest that male and female fetuses differentially execute a placental response depending on their ability to reach (lightest fetuses) or supersede (heaviest fetuses) their genetic growth potential. Future work would benefit from assessing the timing of changes occurring in the placenta relative to the pattern of fetal growth for the males and females within the litter. This will help to identify whether fetal weight discrepancies within the litter are the cause or consequence of placental adaptations that started during early mouse pregnancy.

Summary

In summary, our data show that the placental transport Lz adopts different strategies morphologically, functionally, and at the mitochondrial level to support growth of the lightest and the heaviest fetuses within the litter. These adaptations are likely mediated via metabolic (e.g. lipids, energy status) and endocrine cues (insulin, sex steroids) within the fetus that trigger signalling pathways (e.g. AMPK, PPARγ, AKT) in the placenta, initiating pleiotropic effects. From a clinical perspective, our data may have relevance for understanding the pathways leading to placental insufficiency and fetuses not reaching (FGR/SGA) or exceeding their genetically determined growth potential (LGA). They may also have significance in understanding the discordance in weight and perinatal outcomes between babies of multiple gestations in women. Moreover, since the spectrum of pregnancy outcomes and the factors causally involved are likely to be many, determining how placental phenotype interacts with the weight of female or male fetuses within normal mouse litter may be useful to the design of sex-specific therapeutic agents to improve pregnancy outcomes in humans. This is highly relevant given the profound impacts of fetal growth and pregnancy complications on immediate and life-long health of the child.
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**Author’s contribution**

ESP, JLT and ANSP designed the study. ESP, JLT and DPC performed the experiments and analyzed and graphed the data. ESP and ANSP wrote the paper. All authors contributed to data interpretation and performed final editing checks and approve the final manuscript.

**Conflicts of interest/Competing interests**

The authors declare that no conflicts of interest/competing interests exist.

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**Figure legends**

**Figure 1.** Relationships between placental and fetal weights, (A) for each fetal sex when using all data obtained for each litter, (B) using data obtained for just the lightest and heaviest female fetuses in the litter, and (C) using data obtained for just the lightest and heaviest male fetuses in the litter on gestational day 18. Data were analysed using Pearson’s correlation coefficient and r values are shown in each corresponding figure (**p < 0.01). In A, data are from 35 females and 36 males from n = 12 litters. In B and C, data are from 12 lightest and 12 heaviest fetuses and respective placentas per sex from n = 12 litters. ns = not significant.

**Figure 2.** Conceptus biometry and labyrinth zone structure of the lightest versus the heaviest fetuses of each fetal sex within the litter on gestational day 18. (A) Fetal weight (B) Brain and (C) Liver weights as a proportion of fetal weight, (D) Brain weight to liver weight ratio, (E) Placenta weight, (F) Labyrinth zone weight, (G) Placental efficiency, (H) Labyrinth efficiency (determined as the ratio of fetal weight to labyrinth weight), (I) Representative images of placental labyrinth histology of female fetuses, (J) Trophoblast volume, (K) Fetal capillaries volume, (L) Fetal capillaries surface area, (M) Maternal blood spaces volume, (N) Maternal blood spaces surface area, and (O) Barrier thickness. For conceptus biometry analysis, 12 lightest and 12 heaviest fetuses and respective placentas per sex were used. For stereological analysis, 5 lightest and 5 heaviest fetuses and respective placentas per sex were used. Data are displayed as individual data points with mean ± S.E.M. Data analysed for each sex separately by paired t test; *p < 0.05 **p < 0.01 and ****p < 0.0001. Abbreviations: TB (trophoblasts); FC (Fetal capillaries); MBS (Maternal blood spaces)

**Figure 3.** Mitochondrial respiration parameters in the labyrinth zone supporting the lightest and heaviest fetuses of each sex within the litter on gestational day 18. (A) Representative experimental trace, (B) C_L: C_Leak, (C) C_P: C_Oxphos, (D) CI+II:P: CI+II_Oxphos, (E) FAO, (F) Total ETS, (G) CIV, (H) C_L/ Total ETS, (I) C_P/ Total ETS, (J) CI+II_P/ Total ETS and (K) 1-P/E. Sample size was 7 lightest and 7 heaviest fetuses per sex from n = 7 litters. Data are displayed as individual data points with mean ± S.E.M. Data analysed for each sex separately by paired t test; *p < 0.05 and **p < 0.01.

**Figure 4.** Protein abundance of electron transport chain complexes (A and B, males and females, respectively). Relative mRNA expression of key mitochondria dynamics genes associated with biogenesis, fusion, and fission processes on females (C) and males (D) and key mitochondrial regulatory proteins; (E) citrate synthase, PGC1α,
MNF2, OPA1, HSP60, HSP70, TID1 and CLPP in the placental labyrinth zone supporting the lightest and heaviest fetuses of each sex within the litter on gestational day 18. Images from each antibody and representative Ponceau staining are included. For western blot results sample size is 5 lightest and 5 heaviest fetuses per sex from n = 5 litters. For qPCR results sample size is 7 lightest and 7 heaviest fetuses per sex from n = 7 litters. Data are displayed as individual data points with mean ± S.E.M. Data analysed for each sex separately by paired t test; *p < 0.05, **p < 0.01, ***p <0.001.

Figure 5. Relative mRNA expression of amino acid (A), glucose (B), lipid (C) transporters and steroid hormone metabolism and signalling related genes (D) in the placental labyrinth zone supporting the lightest and heaviest fetuses of each fetal sex within the litter on gestational day 18. Data are from 7 lightest and 7 heaviest fetuses per sex from n = 7 litters. Data displayed as individual data points with mean ± S.E.M. Data analysed for each sex separately by paired t test; *p < 0.05.

Figure 6. Protein abundance of key growth and metabolic signalling proteins in the placental labyrinth zone supporting the lightest and heaviest fetuses of each sex within the litter on gestational day 18. (A and C) Total AKT, AMPKα, MAPK 44/42 and P38 MAPK protein levels, and (B and D) AKT, AMPKα, MAPK 44/42 and P38 MAPK phosphorylation levels as a ratio to total protein in the heaviest versus the lightest fetuses for females (A and B) and males (C and D). Total protein abundance for PPARγ (E). Representative images from each antibody and Ponceau staining are included. Data are 5 lightest and 5 heaviest fetuses per sex from n=5 litters. Data are displayed as individual data points with mean ± S.E.M. Data analysed for each sex separately by paired t test; *p < 0.05.

Figure 7. Summary figure representing the alterations in placental phenotype of the lightest versus heaviest fetuses of each sex in normal wildtype pregnant mice at gestational day 18.

Figure S1. Conceptus biometrical data from female and male fetuses within the same litter at gestational day 18. (A) Fetal weight, (B) Brain and (C) Liver weights as a proportion of fetal weight, (D) Placental weight, (E) Labyrinth zone (Lz) weight and (F) Placental efficiency (F:P ratio; determined as the ratio of fetal weight to
placental weight). Data are from \( n = 12 \) litters were averaged and displayed as individual data points with mean 
\( \pm \text{S.E.M.} \). Data analysed by paired t test; \(*p < 0.05, \ ***p < 0.001.\)
Table 1. Primers used for qPCR analysis.

| Symbol | GenBank ID | Primer sequences (5’=>3’) Fw/Rv | Amplicon length (bp) |
|--------|------------|----------------------------------|---------------------|
|        |            | for Nutrient transporters genes  |         |
| Slc2a1 | NM_011400.3| Fw: GCTTATGGGCTTCTCCAAAACG
Rv: GGTGACACCTCTCCACACATAC | 123     |
| Slc2a3 | NM_011401.4| Fw: GA TCGGCTTTTCCAGTTTG
Rv: CAATCATGCCACCAACAGAG | 176     |
| Fatp1  | NM_011977.4| Fw: GGCTCCTGAGCAGAACA
Rv: ACGGAAGTCCACAGAAAACC | 65      |
| Fatp3  | NM_011988.3| Fw: GAGAATTGCCCACGTATGC
Rv: GGCCCCATATCTGCTCCA | 162     |
| Fatp4  | NM_011989.5| Fw: GATTCCTCCTGTGGCTCTGT
Rv: CCATGGAGCAAACAGCAG | 174     |
| Fatp6  | NM_00108107| Fw: AACCAAGTGGTGACATCTGC
Rv: TCCATAAAATGCAAGGTCAG | 158     |
| Slc38a2| NM_00135563| Fw: TAATCTGAGCAATGCCAT
Rv: AGATGGACGAGTGATACGAAA | 129     |
| Slc38a1| NM_00116645| Fw: CCTCAACGCTAACAGAC
Rv: GGCCAGCTCAATAACGATGAT | 127     |
| Slc38a4| NM_00135806| Fw: GCGGGACAGTATTCAGAC
Rv: GGAATTCTCTGACTTCGGA | 102     |
| Slc7a5 | NM_011404.3| Fw: CTGCTGACACCTGTGCCATC
Rv: GGCTTCTGGAATCGGAGCC | 161     |
| Slc7a8 | NM_016972.2| Fw: CCAGTGTGGTCAGCATGATC
Rv: TGCAACCGTTACCCATAGAA | 161     |
|        |            | for Mitochondrial genes         |         |
| Pgc-1  | NM_008904.2| Fw: GCAGTCGCAACATGCTCAAG
Rv: GGGAACCCTTGGGTCATTT | 83      |
| Nrf1   | NM_00116422| Fw: AGAAACGGGAAACGGCCTCATC
Rv: CATCAGAATGCTGGATCAGT | 96      |
| Nrf2   | NM_010902.4| Fw: ATGGAGCAAGTTTGCGAGGA | 96      |
| Gene       | Accession | Fw:                               | Rv:                               | Annealing temperature |
|------------|-----------|-----------------------------------|-----------------------------------|-----------------------|
| Tfam       | NM_009360.4 | TCCACAGAAGCTACCCCAA               | CCACAGGCTGCAATTTTCC              | 84                    |
| Pparγ      | NM_0011341 8.1 | TGCAGCCTAGCAAGTTGAA               | TTCCGAACCTTGACCAGCCA             | 77                    |
| Opa1       | NM_00119917 7.1 | TGGGCTGCAGAGGATGTTG               | CCTGATGTCACGGTGTTGATG            | 60                    |
| Mfn1       | NM_024200.4 | TTGCCACAAGCTGTTCCGG               | CCTGGACCTGAAAGATGGGC             | 148                   |
| Mfn2       | NM_00128592 0.1 | AGAGGCAGTTTGAGGAGTGC              | ATGATGAGACGAACCGCCTC             | 103                   |
| Drp1       | NM_152816.3 | ATGCCAGCAAGTCCACAGAA             | TGTTTCGGGAGGAGCATTTT             | 86                    |
| Fis1       | NM_025562.3 | CAAAGAGGAACAGCGGGACT              | ACAGCCCTGACATACATT              | 95                    |
| Steroid metabolism and signaling |           |                                   |                                   |                       |
| Star       | NM_011485.5 | TCGCTACGTTCAAGCTGTG               | GCTTCAGTTGAGAACCAAGC             | 184                   |
| Cyp11a1    | NM_00134678 7.1 | GCCCCCGGAGAGCTTG                | TCCCATGCTGAGCCGA               | 193                   |
| Cyp17a1    | NM_007809.3 | TGGAGGCCACTATCCGAGAA             | CACATGTTGCTCCTGCGGA             | 119                   |
| Hsd11b1    | NM_008288.2 | GAGGAAGGTCTCCAGAAAGGTA           | ATGTCAGTGCCCAT                 | 143                   |
| Hsd11b2    | NM_008289.2 | GGCTGGATCGCGTTGTC               | CGTGAAGCCCATGTCGAT              | 132                   |
| Esr2       | NM_207707.1 | CCTCGTCTGGACAGGTCCTC             | CTTGGGACAGCACTCTTGT             | 70                    |
| Ar         | NM_013476.4 | GGATTCTGTGACGCCTATTG             | TCAGGAAAGTCCACGCTC             | 90                    |

Annealing temperature was 60 °C for all genes. Androgen receptor, Ar; Cytochrome P450 Family 11 Subfamily A Member 1, Cyp11a1; Cytochrome P450 Family 17 Subfamily A Member 1, Cyp17a1; Dynamin 1-like, Drp1;
Estrogen receptor 2 (beta), *Esr2*; Solute Carrier Family 27 (Fatty Acid Transporter), Member 1, *Fatp1*; Solute Carrier Family 27 (Fatty Acid Transporter), Member 3, *Fatp3*; Solute Carrier Family 27 (Fatty Acid Transporter), Member 4, *Fatp4*; Solute Carrier Family 27 (Fatty Acid Transporter), Member 6, *Fatp6*; Mitochondrial fission factor, *Fis1*; Hydroxysteroid 11-Beta Dehydrogenase 1, *Hsd11b1*; Hydroxysteroid 11-Beta Dehydrogenase 1, *Hsd11b2*; Mitofusin 1, *Mfn1*; Mitofusin 2, *Mfn2*; Nuclear respiratory factor 1, *Nrf1*; Nuclear respiratory factor 2, *Nrf2*; Mitochondrial dynamin like GTPase, *Opa1*; Peroxisome proliferator-activated receptor gamma coactivator 1-alpha, *Pgc-1α*; Peroxisome proliferator activated receptor alpha *Pparγ*; Solute Carrier Family 2 (Facilitated Glucose Transporter), Member 1, *Slc2a1*; Solute Carrier Family 2 (Facilitated Glucose Transporter), Member 3, *Slc2a3*; Solute carrier family 38, member 1, *Slc38a1*; Solute carrier family 38, member 2, *Slc38a2*; Solute carrier family 38, member 1, *Slc38a1*; Solute carrier family 38, member 4, *Slc38a4*; Solute Carrier Family 7 (Amino Acid Transporter Light Chain, L System), Member 5, *Slc7a5*; Solute carrier family 7 (cationic amino acid transporter, y+ system), member 8, *Slc7a8*; Steroidogenic Acute Regulatory Protein, *Star*; Transcription factor A, mitochondria, *Tfam*. 
Table 2. List of primary antibodies used in this study.

| Primary Antibody                  | Host/ Isotype | Manufacturer, catalogue number | Dilution |
|-----------------------------------|---------------|--------------------------------|----------|
| AKT                               | Rabbit        | Cell Signalling, 9272          | 1/1000   |
| Phospho-AKT (Ser473)              | Rabbit        | Cell Signalling, 9271          | 1/1000   |
| AMPK                              | Rabbit        | Cell Signalling, 5832          | 1/1000   |
| Phospho-AMPK (Thr172)             | Rabbit        | Cell Signalling, 2535          | 1/1000   |
| P44/42 MAPK (Erk1/2)              | Rabbit        | Cell Signalling, 4695          | 1/1000   |
| Phospho-MAPK-p44/42 (Erk1/2) (Thr202/Tyr204) | Rabbit        | Cell Signalling, 4370          | 1/1000   |
| Total-p38 MAPK                    | Rabbit        | Cell Signalling, 8690          | 1/1000   |
| Phos-p38 MAPK Thr180/Tyr182       | Rabbit        | Cell Signalling, 4511          | 1/1000   |
| OPA1                              | Rabbit        | Cell Signalling, 80471         | 1/1000   |
| PGC-1alpha                        | Rabbit        | Santa Cruz, sc-13067          | 1/1000   |
| OXPHOS (ETS complexes)            | Mouse         | Thermo Fisher, 45-8099         | 1/250    |
| Citrate Synthase                  | Rabbit        | Abcam, ab9660                 | 1/1000   |
| MNF-2                             | Rabbit        | Cell Signalling, 9482          | 1/1000   |
| HSP60                             | Rabbit        | Abcam, ab46798                | 1/1000   |
| HSP70                             | Rabbit        | Abcam, ab194360               | 1/1000   |
| TID1                              | Rabbit        | Genetex, GTX111077            | 1/1000   |
| CLPP                              | Rabbit        | Abcam, ab124822               | 1/1000   |
| PPAR-γ                            | Mouse         | Santa Cruz (sc-7273)          | 1/200    |
### Supplementary Table 1. Comparisons between fetal sexes on lightest and heaviest fetuses.

| Conceptus biometry | Lightest | Heaviest | p-value | Lightest | Heaviest | p-value |
|--------------------|----------|----------|---------|----------|----------|---------|
| Fetal weight       | 702.13 ± 15.91 | 740.61 ± 26.68 | NS | 817.33 ± 25.85 | 857.60 ± 22.66 | p < 0.05 |
| Placenta weight    | 75.75 ± 2.88  | 85.74 ± 3.77  | p = 0.05 | 82.62 ± 2.67 | 90.54 ± 1.95 | p < 0.05 |
| Labyrinth zone weight | 43.18 ± 2.50  | 46.97 ± 2.34  | NS | 46.29 ± 2.90  | 50.15 ± 2.97  | NS |
| Liver weight       | 43.08 ± 2.19  | 42.55 ± 2.22  | NS | 49.61 ± 3.70  | 47.03 ± 2.41  | NS |
| Brain weight       | 50.48 ± 4.82  | 55.57 ± 3.46  | NS | 55.59 ± 2.60  | 60.25 ± 1.88  | NS |
| Brain/liver ratio  | 1.20 ± 0.08   | 1.35 ± 0.12   | NS | 1.19 ± 0.12   | 1.32 ± 0.09   | NS |
| Placenta efficiency| 9.37 ± 0.26   | 8.84 ± 0.49   | NS | 10.07 ± 0.54  | 9.53 ± 0.36   | NS |
| Labyrinth zone efficiency | 16.90 ± 1.01  | 16.19 ± 0.87  | NS | 18.59 ± 1.36  | 18.05 ± 1.53  | NS |

| Labyrinth zone structure | Lightest | Heaviest | p-value | Lightest | Heaviest | p-value |
|--------------------------|----------|----------|---------|----------|----------|---------|
| Trophoblast              | 31.6 ± 1.4 | 30.3 ± 2.9 | NS | 32.1 ± 2.7 | 34.4 ± 2.2 | NS |
| Fetal capillaries        | 7.9 ± 1.1  | 8.0 ± 1.3  | NS | 7.8 ± 0.5  | 8.0 ± 0.8  | NS |
| Maternal blood spaces    | 7.4 ± 0.8  | 9.3 ± 1.6  | NS | 9.8 ± 0.8  | 11.0 ± 1.7 | NS |
| Fetal capillaries (S.A.) | 41.9 ± 3.8 | 36.9 ± 2.9 | NS | 39.4 ± 2.7 | 43.5 ± 4.5 | NS |
| Maternal blood spaces (S.A.) | 25.7 ± 1.9 | 29.0 ± 7.5 | NS | 31.0 ± 1.1 | 32.6 ± 5.0 | NS |
| Barrier Thickness        | 3.0 ± 0.2  | 3.3 ± 0.1  | NS | 2.8 ± 0.3  | 3.1 ± 0.3  | NS |
| Growth/nutrient signalling | ND       | ND        | ND     | ND       | ND        | ND     |

| Transporter gene expression | Lightest | Heaviest | p-value | Lightest | Heaviest | p-value |
|-----------------------------|----------|----------|---------|----------|----------|---------|
| Slc2a1                      | 1.21 ± 0.07 | 0.79 ± 0.07 | p < 0.05 | 1.07 ± 0.14 | 1.03 ± 0.09 | NS |
| Slc2a3                      | 1.06 ± 0.08 | 0.93 ± 0.11 | NS | 1.06 ± 0.10 | 1.09 ± 0.14 | NS |
| Gene   | FAO       | p/E       | p/E       | 1-P/E   |
|--------|-----------|-----------|-----------|---------|
| Fatp1  | 1.04 ± 0.10 | 1.22 ± 0.12 | p = 0.07 | 1.02 ± 0.08 | 0.94 ± 0.09 | NS |
| Fatp3  | 1.03 ± 0.09 | 1.03 ± 0.19 | NS       | 1.02 ± 0.08 | 1.04 ± 0.07 | NS |
| Fatp4  | 1.01 ± 0.04 | 1.27 ± 0.20 | NS       | 1.02 ± 0.07 | 0.96 ± 0.08 | NS |
| Fatp6  | 1.02 ± 0.08 | 1.21 ± 0.15 | NS       | 1.02 ± 0.08 | 0.80 ± 0.10 | NS |
| Cd36   | 1.01 ± 0.06 | 1.09 ± 0.15 | NS       | 1.01 ± 0.05 | 0.97 ± 0.07 | NS |
| Slc38a1| 1.03 ± 0.09 | 1.22 ± 0.14 | NS       | 1.08 ± 0.11 | 0.91 ± 0.12 | NS |
| Slc38a2| 1.02 ± 0.06 | 0.96 ± 0.08 | NS       | 1.09 ± 0.17 | 0.92 ± 0.08 | NS |
| Slc38a4| 1.04 ± 0.09 | 1.23 ± 0.14 | NS       | 1.04 ± 0.08 | 1.19 ± 0.13 | NS |
| Slc7a5 | 1.02 ± 0.07 | 0.81 ± 0.14 | NS       | 1.07 ± 0.14 | 1.18 ± 0.06 | NS |
| Slc3a2 | 1.04 ± 0.09 | 1.10 ± 0.19 | NS       | 1.11 ± 0.15 | 0.96 ± 0.13 | NS |

**Mitochondria respiratory function**

| Function                  | Value     | Value     | Value     | Value     | Value     | Value     |
|---------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Complex I LEAK            | 2.09 ± 0.18 | 2.24 ± 0.40 | NS       | 1.27 ± 0.12 | 1.51 ± 0.14 | NS       |
| Complex I OXPHOS          | 2.37 ± 0.30 | 3.28 ± 0.45 | p = 0.05 | 2.05 ± 0.40 | 2.34 ± 0.40 | NS       |
| Complex I + II OXPHOS     | 15.92 ± 0.84 | 16.04 ± 0.66 | NS       | 13.40 ± 0.60 | 16.93 ± 1.31 | p = 0.08 |
| FAO                       | 2.64 ± 0.31 | 2.75 ± 0.38 | NS       | 1.79 ± 0.25 | 2.23 ± 0.35 | NS       |
| Total ETS                 | 18.48 ± 1.09 | 19.53 ± 0.78 | NS       | 16.95 ± 0.49 | 19.61 ± 1.60 | NS       |
| CIV                       | 34.94 ± 4.42 | 36.07 ± 2.21 | NS       | 32.17 ± 2.55 | 36.79 ± 4.44 | NS       |
| Complex I LEAK/ETS        | 0.12 ± 0.01 | 0.11 ± 0.02 | NS       | 0.08 ± 0.01 | 0.08 ± 0.01 | NS       |
| Complex I OXPHOS/ETS      | 0.12 ± 0.01 | 0.17 ± 0.02 | p = 0.06 | 0.12 ± 0.02 | 0.12 ± 0.02 | NS       |
| Complex I + II OXPHOS/ETS | 0.87 ± 0.02 | 0.82 ± 0.03 | NS       | 0.79 ± 0.03 | 0.87 ± 0.03 | NS       |
| ETS complex proteins      | ND        | ND        | ND        | ND        | ND        | ND       |

**Mitochondria-related gene expression**
| Protein       | Mean ± S.E.M | p < 0.05 | 0.05 ± 0.10 | 0.09 ± 0.17 | NS        |
|--------------|--------------|----------|--------------|--------------|-----------|
| Pgc1α        | 1.03 ± 0.11  | 1.28 ± 0.21 | NS           | 1.09 ± 0.15  | 1.09 ± 0.17 | NS        |
| Nrf1         | 1.01 ± 0.03  | 1.38 ± 0.14 | p < 0.05     | 1.02 ± 0.06  | 0.90 ± 0.03 | NS        |
| Nrf2         | 1.02 ± 0.08  | 1.23 ± 0.22 | NS           | 1.04 ± 0.10  | 0.87 ± 0.08 | NS        |
| Tfam         | 1.02 ± 0.07  | 1.28 ± 0.08 | p = 0.06     | 1.04 ± 0.09  | 0.84 ± 0.07 | p = 0.07  |
| Pparγ        | 1.03 ± 0.09  | 1.38 ± 0.28 | NS           | 1.07 ± 0.14  | 0.66 ± 0.10 | p < 0.05  |
| Opa1         | 1.00 ± 0.03  | 1.13 ± 0.05 | p = 0.06     | 1.00 ± 0.03  | 0.97 ± 0.06 | NS        |
| Mfn2         | 1.00 ± 0.03  | 1.13 ± 0.07 | NS           | 1.03 ± 0.09  | 0.92 ± 0.07 | NS        |
| Mfn1         | 1.01 ± 0.03  | 1.12 ± 0.04 | p = 0.05     | 1.02 ± 0.07  | 0.99 ± 0.06 | NS        |
| Drp1         | 1.03 ± 0.08  | 1.30 ± 0.15 | NS           | 1.03 ± 0.09  | 0.82 ± 0.08 | NS        |
| Fis1         | 1.00 ± 0.02  | 1.11 ± 0.04 | NS           | 1.00 ± 0.03  | 0.94 ± 0.03 | NS        |
| Mitochondria-related proteins | ND          |           | ND           | ND           | ND        |

### Steroid handling genes

| Protein | Mean ± S.E.M | p < 0.05 | 0.05 ± 0.10 | 0.09 ± 0.17 | NS        |
|---------|--------------|----------|--------------|--------------|-----------|
| Star    | 1.05 ± 0.09  | 1.22 ± 0.20 | NS           | 1.12 ± 0.16  | 1.38 ± 0.20 | NS        |
| Cyp11α  | 1.03 ± 0.09  | 0.78 ± 0.08 | p < 0.05     | 1.13 ± 0.22  | 1.15 ± 0.15 | NS        |
| Cyp17α  | 1.05 ± 0.13  | 1.05 ± 0.34 | NS           | 1.06 ± 0.15  | 0.63 ± 0.07 | p < 0.05  |
| Hsd11b1 | 1.01 ± 0.05  | 0.98 ± 0.07 | NS           | 1.05 ± 0.11  | 0.99 ± 0.08 | NS        |
| Hsd11b2 | 1.05 ± 0.13  | 0.80 ± 0.18 | NS           | 1.03 ± 0.09  | 0.97 ± 0.11 | NS        |
| Esr1    | 1.04 ± 0.10  | 1.04 ± 0.14 | NS           | 1.00 ± 0.06  | 1.10 ± 0.11 | NS        |
| Ar      | 1.40 ± 0.43  | 0.88 ± 0.12 | NS           | 1.50 ± 0.46  | 0.63 ± 0.11 | NS        |

Data from qPCR and high resolution respirometry analyses are from 7 lightest and 7 heaviest fetuses per sex from n = 7 litters. Data from fetal biometrical assessments results are from 12 lightest and 12 heaviest fetuses per sex from n = 12 litters. Data from placenta stereology are from 5 lightest and 5 heaviest fetuses per sex from n = 5 litters. Data are displayed as mean ± S.E.M and analysed for the effect of sex in each fetal weight category by paired t test *p < 0.05. ND: not determined. NS: not significant. *P<0.05.
Figure 5

A) Amino Acid Transporters

B) Glucose Transporters

C) Lipid Transporters

D) Steroid metabolism and signalling
A) Fetal weight
B) Brain ratio
C) Liver ratio
D) Placental weight
E) Lz weight
F) F:P ratio