1 **Title:** Placental mitochondrial function, nutrient transporters, metabolic signalling and steroid metabolism relate

2 to fetal size and sex in mice.

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4 **Running title:** Placental alterations relate to fetal size and sex

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21 **Abbreviations:** AMPK, 5' AMP-activated protein kinase; AKT, protein kinase B; C, complex; CI_L, complex I

22 respiration in LEAK state; CI_P, OXPHOS capacity via complex I; CII_P, OXPHOS capacity via complex II;

23 CLPP, caseinolytic mitochondrial matrix peptidase proteolytic subunit (CLPP); CS, citrate synthase; DRP1,

24 dynamin related protein; ET, Electron Transfer; ETS, electron transfer system; FAO, fatty acid oxidation; FC,

25 fetal capillaries; FCCP, trifluoromethoxy carbonyl-cyanide phenylhydrazone; F:P, fetal weight to placenta

26 weight ratio; HSP60/70, chaperonin; LEAK respiration, non-phosphorylating state of intrinsic uncoupling;

27 LGA, large for gestational age; Lz labyrinth zone; OPA1, mitochondrial dynamin like GTPase, OXPHOS,

28 oxidative phosphorylation; MAPK, mitogen activated kinase; MBS, maternal blood spaces; MFN1/2, mitofusin

29 1/2 ; ns, not-significant; PGC1A, peroxisome proliferator-activated receptor gamma coactivator 1-α; PPARγ,

30   peroxisome proliferator activated receptor-γ, SGA, small for gestational age; TB, trophoblast; TBS, tris-

31   buffered saline; TMPD, N, N, N', N'-tetramethyl-p-phenylenediamine; TID1, tumorous imaginal disc

32   1/mitochondrial DnaJ co-chaperone protein; 1-P/E, ETS excess capacity.

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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].

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90   In this study in mice, we employed an integrative approach to evaluate placenta morphology, mitochondrial

91   OXPHOS capacity and mitochondrial regulator expression (electron transport system (ETS) complexes and

92   biogenesis and fission-fusion regulators), in relation to growth of the lightest and heaviest female and male

93   fetuses within the litter. We also examined the activity of signalling pathways governing placental growth and

94   metabolism, as well as the expression of nutrient transporter and steroid hormone handling genes to further

95   understand how placental phenotype is modulated by fetal weight and sex within the litter. Analyses were

96   conducted on the labyrinth zone (Lz) of the mouse placenta as it is primarily responsible for controlling the

97   transport of nutrients, oxygen and hormones from mother to fetus. Importantly, since the mouse is a polytocous

98   species, normal variation of fetal weight is expected within the litter, even under a normal, healthy gestational

99   environment.

100

Methods

Animals

103   All experiments were performed under the U.K. Animals (Scientific Procedures) Act 1986 after ethical approval

104   by the University of Cambridge. A total of 12 C57BL/6J virgin female mice were housed in the University of

105   Cambridge Animal Facility using a 12/12 dark/light system and received *ad libitum* water and chow food

106   (Rodent No. 3 breeding chow; Special Diet Services, Witham) during the study. At 4 months age, females were

107   mated overnight and the day a copulatory plug was found was designated as gestational day (GD) 1. On GD18,

108   pregnant dams were killed by cervical dislocation, uteri were recovered, and fetuses and placentas cleaned from

109   fetal membranes. All fetuses and placentas from the litter were weighed, and the Lz subsequently micro-

110   dissected. Lz samples from 7 litters were cut in half, after microdissection of placental layers, one Lz half was

111   placed into ice-cold biopsy preservation medium (0.21 M Mannitol, 0.07 M Sucrose, 30% DMSO in H_2O and

112   pH7.5) and frozen at -80°C until respirometry analysis. The remaining half of the Lz was snap frozen and stored

113   at -80°C for molecular analyses (RT-qPCR and Western Blot). Placental Lz samples from the additional 5 litters

114   were placed into 4% paraformaldehyde, histologically processed, and used for structural analysis. Fetal brain

115   and liver were dissected and weighed from each fetus. Fetal tails were kept for sex determination by detection

116   of the *Sry* gene using the Taq Ready PCR system (Sigma), specific primers (*Sry*: FPrimer: 5'-

117   GTGGGTTCCTGTCCCACTGC-3', RPrimer: 5'-GGCCATGTCAAGCGCCCCAT-3' and PCR autosomal

118   gene control: FPrimer: 5'-TGGTTGGCATTTTATCCCTAGAAC-3', RPrimer: 5'-

119   GCAACATGGCAACTGGAAACA-3') and agarose gel electrophoresis.

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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 ditriothreitol, 6.56 mM MgCl2, 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 $MgCl_2x6H_2O$, 20 mM taurine, 10 mM KH_2PO_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 ($CI+\acute{I}I_P$). 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- $CI+\acute{I}I_P$/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.

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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.

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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].

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Placental immunohistochemistry analysis

183 Morphology of the Lz was assessed by double-labelling placental sections with cytokeratin and lectin antibodies

184 to identify trophoblast and fetal capillaries, respectively. Details about the staining protocol have been described

185 in detail elsewhere [27]. Stained sections were then scanned using a NanoZoomer 2.0-RS Digital Pathology

186 System (NDP Scan Hamamatsu, Japan) and stereological analysis of the Lz was performed as described

187 previously [27].

188

Statistical analysis

190 Statistical analyses were performed using GraphPad Prism version 8 (GraphPad, La Jolla, CA, USA). The

191 lightest and heaviest fetuses for each sex were identified and statistical analyses were performed using paired *t*-

192 test. Similarly, comparisons of the lightest females versus lightest males and heaviest females versus heaviest

193 males were performed using paired *t*-test. Relationships between data were undertaken using Pearson's

194 correlation coefficient (r). Values are expressed as individual data points and/or mean ± SEM and p values <

195 0.05 were considered statistically significant. The number of samples per group for each analysis is shown in

196 each figure and described in the legends of figures and footnotes of tables.

197

Results

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

200 Considering all fetuses together, conceptus biometric data were different between females and males within the

201 litter at gestational day 18 (term on day 20; Figure S1). In particular, fetus, placenta and Lz weights were lower

202 in females when compared with male fetuses (p=0.03, p<0.001 and p=0.02, respectively). However, there were

203 no differences in fetal brain and liver weights (relative to body weight), or placental efficiency (p=0.06),

204 calculated as the ratio of fetal weight to placental weight between females and males (Figure S1).

205

206 Placental weight was not significantly correlated with fetal weight when analysing all conceptuses within the

207 litter collectively (n=71; r=0.17; p=0.15). Similarly, when data was separated into females and males, again no

208 correlation was detected (Figure 1A, females: n=35; r=0.24; p=0.15, males: n=36; r=-0.07 p=0.64). However,

209 when data were segregated to only assess the heaviest and lightest fetuses per sex within the litter, a positive

210 correlation between placental and fetal weight was found for the lightest females (Figure 1B, r=0.74; p=0.005),

211 but not for the lightest males (Figure 1C, r=0.32; p=0.31) or the heaviest fetuses of either sex. These data

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212 suggest that the placenta may be supporting growth of the female and male fetuses in different ways within the

213 litter.

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

216 To understand why there may be a sex-related difference in the relationship between placental weight and fetal

217 weight, the lightest and heaviest fetuses from each litter were selected and conceptus biometry were compared

218 for each sex separately (Figure 2). As expected, fetal weight was lower for the lightest compared to the heaviest

219 for each fetal sex in the litter (Figure 2A, females; p=0.002; males; p<0.0001), and the mean weight difference

220 between them were similar for females and males (14.1% and 13.6% less than heaviest, respectively). Fetal

221 brain and liver weights as a proportion of body weight did not vary, which suggests that the lightest fetuses are

222 symmetrically smaller when compared to the heaviest fetuses (Figure 2B-D). Moreover, placental weight, Lz

223 weight and placenta and Lz efficiency did not vary between the lightest and the heaviest fetuses within the litter,

224 regardless of fetal sex (Figure 2E-H)

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226 Stereological analysis of the placental Lz zone revealed that there were no differences in trophoblast and fetal

227 capillary volumes (Figure 2I-K). However, there was less maternal blood spaces in the placental Lz of the

228 lightest females, compared to the heaviest females, and this difference was not found for the males (Figure 2M).

229 Similarly, maternal blood space surface area was lower in the lightest, compared to heaviest female fetuses, an

230 effect not observed for the male fetuses (Figure 2N). The surface area of the fetal capillaries (Figure 2L) and

231 barrier thickness (Figure 2O) of the Lz did not vary between the lightest and the heaviest fetuses, for either

232 females or males.

233

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

236 To investigate whether sex-dependent structural changes in the Lz zone between the lightest and heaviest

237 fetuses may be related to mitochondrial functional alterations, high resolution respirometry was performed

238 (Figure 3A). Oxygen flux rate analysis revealed that in LEAK state, mitochondrial CI related oxygen

239 consumption was ~60% greater for the placental Lz of lightest compared to the heaviest females, but no effect

240 was seen for the males (Figure 3B, p=0.003). Whilst CI oxygen flux under OXPHOS state was not different

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241 between the lightest and heaviest fetuses of either sex (Figure 3C), after adding succinate, Lz CI+CII oxygen

242 consumption rate was ~44% greater in the lightest compared to the heaviest females within the litter (p=0.01); a

243 difference that was not observed for the males (Figure 3D). Fatty acid oxidation (FAO), total ETS capacity and

244 CIV associated oxygen consumption by the placental Lz were not different between lightest and heaviest fetuses

245 for either fetal sex (Figure 3E-G). When oxygen consumption rates for CI in LEAK state and CI+II in OXPHOS

246 state were corrected to total ETS oxygen flux to provide a qualitative indication of changes in mitochondrial

247 function per mitochondrial unit, these values were also increased in only the lightest compared to heaviest

248 females (not different for the lightest compared to heaviest males) (Figure 3H, p=0.02; 3I and 3J, p=0.03). In

249 addition, calculation of 1-P/E indicated that ETS excess capacity was lower in the lightest females compared to

250 the heaviest (p=0.03), again a difference not identified for the males (Figure 3K).

251

252 **Expression of mitochondrial ETS components, dynamic genes, and regulatory proteins in the placental**

253 **Lz for the lightest *versus* the heaviest fetuses of each sex**

254 To gain further information on the sex-dependent differences in placental mitochondrial respiratory capacity,

255 western blotting and qPCR was performed to determine the expression ETS complex proteins (CI-V),

256 biogenesis, fusion and fission genes, and additional mitochondrial regulatory proteins in the placental Lz of the

257 lightest and heaviest fetuses for both sexes (Figure 4). These analyses revealed that CI abundance was lower in

258 the lightest compared to heaviest females (Figure 4A), meanwhile CIII and CV were lower only in the lightest

259 compared to the heaviest males (Figure 4B). In addition, the expression of mitochondria biogenesis gene, *Nrf1*

260 and mitochondrial fission genes, *Drp1* and *Fis1,* was lower in the Lz of the lightest females, when comparing

261 with the heaviest females (Figure 4C). Whereas the expression of *Tfam,* a mitochondria biogenesis transcription

262 factor gene, was greater in the Lz of the lightest males versus the heaviest males (Figure 4D). Mitochondrial

263 content, informed by citrate synthase abundance, did not vary in the Lz between the lightest and heaviest fetuses

264 within the litter, regardless of fetal sex (Figure 4E). In addition, abundance of proteins involved in

265 mitochondrial biogenesis (PGC-1α), fusion (MNF2 and OPA1), heat shock (HSP60, HSP70) and chaperone

266 (TID1) proteins did not differ in the Lz between the lightest and heaviest fetuses within the litter, in either sex

267 (Figures 4E). However, CLPP a key protease involved in mitochondrial protein clearance and a marker of the

268 mitochondrial unfolded protein response (UPRmt), was lower in the lightest females compared with the heaviest

269 females; an effect not seen for males (Figure 4E).

270

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

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302 (abundance of phosphorylated normalized to total AMPKα, Figures 6B and D). While the total abundance of

303 AKT did not vary between the lightest and the heaviest fetuses, activated AKT (phosphorylated to total) was

304 ~32% lower in the Lz zone supporting the lightest compared to the heaviest males (p=0.032), but no difference

305 was found for the females (Figures 6B and D). The abundance and activation of p44/42 MAPK and p38 MAPK

306 was not different between the lightest and the heaviest fetuses, irrespective of fetal sex (Figure 6A and D).

307 Interestingly, PPARγ an important transcription factor involved mitochondrial metabolism and lipid synthesis,

308 was greater in the lightest female compared to the heaviest female, whereas PPARγ was lower in the lightest

309 males when compared to the heaviest males from the litter (Figure 6E, p=0.04 and p < 0.05, respectively).

310

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

312 To gain further insight into the sex-dependent intra-litter differences in placental phenotype, retrospective

313 comparisons between the lightest females and lightest males and heaviest females and heaviest males within the

314 litter were performed (Supplementary Table 1). These data showed that the heaviest males were ~5% heavier

315 than the heaviest female fetuses within the litter (p <0.05). The placenta of the heaviest males in the litter was

316 also greater by ~13% when compared to the heaviest females (p=0.04), with a tendency for this to also vary

317 with sex for the lightest female littermates (p=0.054). The placental expression of glucose (*Slc2a1*: -37%,

318 p=0.003) and lipid (*Fatp1*: +17%, tendency p=0.07) transporter genes were also differentially expressed

319 between the lightest (but not heaviest) male and female fetuses of the litter. Placental respirometry rates

320 associated with CI_{Oxphos} (+39%, tendency p=0.05) and with $CI+CII_{Oxphos}$/Total ETS (+30%, tendency p=0.08)

321 together with biogenesis (*Nrf1*: +37%, p=0.03; *Tfam*: +25%, tendency p=0.06) and dynamic (*Opa1*: +13%,

322 tendency p=0.06, *Mfn1*: +11%, p=0.05) genes were all greater in the lightest males compared to the lightest

323 females. Meanwhile, biogenesis genes *Pparγ* (-38%, p=0.03) and *Tfam* (-19%, tendency p=0.07) were

324 decreased in the heaviest males compared to females. Finally, the expression of the steroidogenic gene *Cyp11a1*

325 was lower (-38%, p=0.02) in the lightest males compared to females and in heaviest fetuses, gene expression of

326 *Cyp17a1* (-41%, p=0.01) were decreased on males compared to females. There was also no effect of fetal sex in

327 the placental Lz morphology of the lightest and heaviest fetuses.

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329 **Discussion**

330 The present study in mice shows that placental mitochondrial functional capacity varies in relation to natural

331 differences in the weight of females and male fetuses within the litter. The placenta Lz of both the lightest

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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 [12]. 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 [3]. 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 [3]. Similarly, calcium transfer across lightest placenta was higher than the heaviest placentas within the litter, resulting in similar calcium accretion levels in the fetus [6]. Variations in placental structure and transport within the litter were related to an increase in placental efficiency in both of these previous studies [3,6]. 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

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363   litter. These data indicate that there may be differences in the way in which the placenta may be supporting

364   growth of the female and male fetuses within the litter.

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366   While there was no difference in placental fetal capillaries, trophoblast volume or barrier thickness between the

367   lightest and heaviest of either fetal sex, maternal blood space volume and surface area of the placenta was lower

368   in the lightest compared to the heaviest female fetuses. These differences in placental structure suggest mal-

369   perfusion of the placenta of the lightest females and would be expected to decrease the delivery of nutrient and

370   oxygen to fetus and could explain the weight discrepancy when comparing to the heaviest females in the litter.

371   Previous studies in pigs have shown that compared to placentas supporting fetuses weighing closest to the litter

372   mean, placentas supplying the lightest fetuses within the litter have impaired angiogenesis [28]. Moreover, there

373   are differences in the extent of endothelial cell branching morphogenesis with conditioned media from the

374   placenta of females compared to males [28]. Work in rats has suggested that an angiogenic imbalance may

375   underlie regional differences in uteroplacental vascularization and fetoplacental development within the litter [29].

376   Our study was not designed to assess the contribution of uterine position to the sex-related differences in

377   placental morphology of the lightest *versus* heaviest fetuses in the litter, which could be a focus of future work.

378

379   **Placental mitochondrial function**

380   Previous work has shown that in human pregnancies associated with FGR, although mRNA expression of ETS

381   complexes (II, III and IV) is lower, there is a higher mitochondrial DNA (mtDNA) content and higher oxygen

382   consumption related with mitochondrial bioenergetics in the placenta [30]. Similarly, FGR induced by maternal

383   caloric restriction in rats is associated with augmented mitochondrial biogenesis, as evidenced by the increased

384   expression of PGC-1α, NRF1 and TFAM, as well as elevated complex I and IV dependent respiration in the

385   placenta [20]. These data suggest that a common response of the placenta to try and match the genetically

386   determined demands of the fetus for growth during gestation involves the modulation of placental mitochondrial

387   respiratory capacity. The current study supports this notion and shows that the functional characteristics of

388   placental mitochondria also adapts with natural variations of fetal growth in normal pregnancy. In females,

389   biogenesis promoter *Nrf1*, fission regulators *Drp1* and *Fis1*, and mitochondrial complex I were lower, yet

390   complex I LEAK and complex I+II OXPHOS were greater in the placenta supporting the lightest compared to

391   the heaviest female. Whereas in males, biogenesis gene *Tfam* was greater, yet mitochondrial complexes III and

392   V were lower and LEAK and OXPHOS capacity were not different in the placenta supporting the lightest

393   compared to the heaviest male. Therefore, for both sexes, the placenta of the lightest fetuses appears to increase

14

394   mitochondrial respiratory efficiency (as there was reduced mitochondrial complexes yet unaltered/increased

395   respiration), although the underlying mechanisms and extent to which this may occur is different for females

396   and males. Indeed, our results suggest that mitochondria in the placenta sustaining the lightest female fetuses in

397   the litter are more responsive and susceptible to adaptative mechanisms, as they exhibited increased

398   mitochondrial respiration rates. This enhanced adaptive response may have been beneficial in providing the

399   energy to sustain the expression of glucose transporters for the lightest fetuses. Previous work has demonstrated

400   there is reduced abundance of all mitochondrial complexes and lower OXPHOS respiration rates in placental

401   trophoblast from obese women who deliver high birth weight babies [31]. Moreover, mitochondrial complex

402   activity is also decreased in placentas from women with pre-pregnancy obesity or pre-gestational diabetes who

403   have LGA babies [32]. Thus, the natural variation in intra-litter placental mitochondrial function in the current

404   study is likely the outcome of adaptive responses in operation for both the lightest and heaviest fetuses.

405

406   In the lightest female, but not lightest males, there was lower CLPP protein abundance when compared to the

407   heaviest female fetuses of the litter. CLPP is also decreased along with mitochondrial complex abundance in the

408   placenta of preeclamptic women delivering FGR babies [33]. However, no other members associated with the

409   UPRmt pathway that was analysed varied in the placenta between the lightest and heaviest fetuses (HSP60,

410   HSP70 and TID1), which may be expected given that our study was performed on mouse litters from normal,

411   healthy pregnancies. The biological relevance of the sex-specific difference in placental CLPP level between the

412   lightest and heaviest fetuses is currently unknown. However, differences in placental CLPP may be particularly

413   relevant for the outcome of female and male fetuses if the gestation is challenged, such as by a hypoxic or

414   nutritional stimulus.

415

416   **Placental sex steroid handling**

417   The placental expression of key steroid synthetic enzymes was differentially expressed between the lightest and

418   heaviest females only. The greater *Cyp11a1* while lower *Cyp17a1* in the lightest compared to the heaviest

419   female fetus, would be expected to enhance the synthesis of the steroid hormone precursor pregnenolone, but

420   also limit the synthesis of sex steroids. CYP11A1 and CYP17A1 are both cytochrome P450 monooxygenases

421   located in the mitochondrial membrane that use oxygen for steroidogenesis, and changes in their expression

422   may have relevance for understanding the greater rate of oxygen consumption in LEAK state for the placenta of

423   the lightest *versus* heaviest females. Other work has shown that CYP11A1 is upregulated in the placenta of

424   women with preeclampsia and overexpression of CYP11A1 in human trophoblast cells reduces proliferation

15

425 and induces apoptosis [34,35]. In addition, *in vitro* studies using cell lines have implicated an important role of

426 CYP17A1 in estrogen production by the placenta [36]. Thus, further work would value from quantifying steroid

427 hormone levels in the placenta. The expression of the androgen receptor was greater in the placenta of the

428 lightest compared to the heaviest males; a difference not seen in females. These data suggest enhanced

429 sensitivity of the lightest male placenta to androgens, namely testosterone, which can be produced by the fetal

430 testes from approximately day 12-13 of mouse [37]. Interestingly, in rats elevated testosterone levels disrupts the

431 number and structure of mitochondria in the placenta and decreases fetal weight [38,39]. Additionally, DHT (5α-

432 reduced metabolite of testosterone) and insulin treatment of rats induces mitochondrial damage and an

433 imbalance between oxidative and antioxidative stress responses in the placenta in association with defects in

434 uterine function and FGR [40]. Thus, differences in steroid production and signalling are likely involved in the

435 mechanisms underlying alterations in placental morphology and mitochondrial functional capacity supporting

436 the lightest fetus of each sex.

437

Placental signalling pathways

439 The mechanisms underlying the differences in intra-litter placental mitochondrial function for each fetal sex are

440 unknown. However, intra-litter differences in placental morphology and mitochondrial functional capacity

441 likely stem from variations in the abundance of AMPK, AKT and PPARγ between the lightest and heaviest

442 female and male fetuses [15]. Increased levels of AMPK in the placenta were seen for both the lightest females

443 and lightest males compared to their heaviest counterparts and may reflect a beneficial adaptation occurring

444 during normal late gestation in mice. AMPK is activated by an increase in the AMP to ATP ratio and hence, is

445 reflective of a decline in energy status. In turn, AMPK activates metabolic enzymes that allow cells to switch on

446 catabolic pathways that generate ATP, including glycolysis and fatty acid β-oxidation [41]. We did not observe

447 differences in placental fatty acid oxidation between the lightest and heaviest of each fetal sex within the litter.

448 However, it would be beneficial to assess glycolysis and glycolytic enzyme expression in the placenta to assess

449 whether there are intra-litter differences for females or males. In addition to its role in energy sensing, AMPK

450 regulates placental trophoblast differentiation, proliferation and nutrient transport [42]. Moreover, AMPK in the

451 placenta has been linked to maternal vascular responses and changes in placental morphology and fetal growth

452 in hypoxic pregnancy [8,43]. Interestingly, compared to their heavier counterparts, the magnitude of increase in

453 placental AMPK was greater for the lightest females *versus* for the lightest males (increased by 67% and 41%,

454 respectively). Whether this may relate to the observation of altered placental morphology in only the lightest

455 females requires further study.

16

456

457   Activation of AKT (phosphorylated AKT) was lower in the Lz of the lightest compared to heaviest male fetuses

458   only. The AKT-mTOR signalling pathway plays a crucial role in the regulation of placental transport function

459   and it has been shown to be up-regulated in pregnancies from obese women delivering LGA babies [44] and

460   down-regulated in placentas from SGA/FGR babies [45]. Moreover, placental trophoblast specific loss of

461   phosphoinositol-3 kinase (PI3K) signalling, which is upstream of AKT leads to FGR in mice [46]. In line with the

462   reduced AKT activation, only the lightest males presented lower glucose transporter (*Slc2a1*, encodes GLUT1)

463   expression in their placenta, when compared to the heaviest males. Insulin is a major fetal growth factor that

464   signals via AKT to mediate its metabolic effects. Reduced activation of AKT in the placenta of the lightest

465   males may therefore reflect reduced fetal insulin signalling to the placenta [47]. These data also reinforce the idea

466   that the placentas of females and males may execute their own molecular mechanisms to best support fetal

467   growth and development. In humans, placental GLUT1 is down-regulated in preeclampsia, and this might play

468   a role on the development of FGR [48]. Conversely, women with insulin-dependent diabetes who have an

469   increased incidence of LGA show increased GLUT1 expression and a higher mediated uptake of D-glucose by

470   the placenta [49]. Since glucose is the most important energetic substrate for fetal wellbeing and growth, lower

471   expression of placental *Slc2a1* in the lightest relative to the heavier male fetus may explain their fetal growth

472   discrepancy.

473

474   Sexually dimorphic differences in the placenta phenotype may also relate to changes in PPARγ abundance;

475   PPARγ was greater in the lightest females, but lower in the lightest males compared to their respective

476   counterparts. In humans, PPARγ expression was found to be reduced in the placenta of SGA fetuses and to

477   associate positively with fetal and placental weights within this subpopulation [50]. In addition, it has been

478   reported that PPARγ modulates the expression of amino acid transporters LAT1 and LAT2 (encoded by *Slc7a5*

479   and *Slc3a2,* respectively) and plays a key role in the control of fetal growth [51]. PPARγ is also important for the

480   regulation of lipid uptake and metabolism and regulates mitochondrial function via multiple routes [52]. While in

481   our study no differences were detected in the placental expression of amino acid and fatty acid transporters

482   between lightest and heaviest fetuses, changes in PPARγ could in part mediate the differential changes in

483   mitochondrial respiratory capacity and regulatory factor expression. PPARγ is a transcription factor that can be

484   modulated by numerous signals, including hormonal/growth factor signalling pathways, inflammatory/stress

485   signalling pathways and cellular metabolite levels [53]. Hence, the sex-related alterations in PPARγ abundance in

486   the placenta of the lightest females and the lightest males likely reflects the very different metabolic and

487   hormonal environment of the fetuses relative to their heaviest counterparts within the litter.

17

488

Placental phenotype and fetal sex comparisons

490 Previous work has shown there are changes in placental Lz morphology, function, mitochondrial respiratory

491 capacity and mitochondrial-related regulators that help to support the growth demands of the fetus during

492 normal late mouse pregnancy [23]. In the current study the heaviest males and their placentas were heavier than

493 the heaviest females within the litter, although no differences were found in placental morphology,

494 mitochondrial functional capacity, or transport/hormone genes between them. In contrast, the lightest males and

495 their placentas did not differ in weight when compared to the lightest females (tendency for placental weight to

496 be greater in the lightest males *versus* females), yet they varied in the placental expression of nutrient

497 transporters, steroidogenesis genes, mitochondrial respiratory capacity and mitochondrial-related gene

498 expression. These data suggest that male and female fetuses differentially execute a placental response

499 depending on their ability to reach (lightest fetuses) or supersede (heaviest fetuses) their genetic growth

500 potential. Future work would benefit from assessing the timing of changes occurring in the placenta relative to

501 the pattern of fetal growth for the males and females within the litter. This will help to identify whether fetal

502 weight discrepancies within the litter are the cause or consequence of placental adaptations that started during

503 early mouse pregnancy.

504

Summary

506 In summary, our data show that the placental transport Lz adopts different strategies morphologically,

507 functionally, and at the mitochondrial level to support growth of the lightest and the heaviest fetuses within the

508 litter. These adaptations are likely mediated via metabolic (e.g. lipids, energy status) and endocrine cues

509 (insulin, sex steroids) within the fetus that trigger signalling pathways (e.g. AMPK, PPARγ, AKT) in the

510 placenta, initiating pleiotropic effects. From a clinical perspective, our data may have relevance for

511 understanding the pathways leading to placental insufficiency and fetuses not reaching (FGR/SGA) or

512 exceeding their genetically determined growth potential (LGA). They may also have significance in

513 understanding the discordance in weight and perinatal outcomes between babies of multiple gestations in

514 women. Moreover, since the spectrum of pregnancy outcomes and the factors causally involved are likely to be

515 many, determining how placental phenotype interacts with the weight of female or male fetuses within normal

516 mouse litter may be useful to the design of sex-specific therapeutic agents to improve pregnancy outcomes in

517 humans. This is highly relevant given the profound impacts of fetal growth and pregnancy complications on

518 immediate and life-long health of the child.

519

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527

Author's contribution

529 ESP, JLT and ANSP designed the study. ESP, JLT and DPC performed the experiments and analyzed and

530 graphed the data. ESP and ANSP wrote the paper. All authors contributed to data interpretation and performed

531 final editing checks and approve the final manuscript.

532

Conflicts of interest/Competing interests

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

535

References

537 1.   Napso T, Yong HEJ, Lopez-Tello J, Sferruzzi-Perri AN. The role of placental hormones in mediating

538   maternal adaptations to support pregnancy and lactation. *Front Physiol*. 2018;9(AUG):1-39.

539   doi:10.3389/fphys.2018.01091

540 2.   Coan PM, Vaughan OR, Sekita Y, et al. Adaptations in placental phenotype support fetal growth during

541   undernutrition of pregnant mice. 2010;3:527-538. doi:10.1113/jphysiol.2009.181214

542 3.   Coan PM, Angiolini E, Sandovici I, Burton GJ, Constˆ M, Fowden AL. Adaptations in placental nutrient

543   transfer capacity to meet fetal growth demands depend on placental size in mice. 2008;18:4567-4576.

544   doi:10.1113/jphysiol.2008.156133

545 4.   Constância M, Angiolini E, Sandovici I, et al. Adaptation of nutrient supply to fetal demand in the mouse

546   involves interaction between the Igf2 gene and placental transporter systems. 2005;102(52):19219-

547   19224.

548 5.   Dilworth MR, Kusinski LC, Cowley E, et al. Placental-speci fi c Igf2 knockout mice exhibit

549   hypocalcemia and adaptive changes in placental calcium transport. 2010. doi:10.1073/pnas.0911710107

550 6.   Hayward CE, Renshall LJ, Sibley CP, Greenwood SL, Dilworth MR. Adaptations in Maternofetal

551   Calcium Transport in Relation to Placental Size and Fetal Sex in Mice. 2017;8(December):1-10.

552   doi:10.3389/fphys.2017.01050

553 7.   Sferruzzi-Perri AN, Vaughan OR, Haro M, et al. An obesogenic diet during mouse pregnancy modifies

554   maternal nutrient partitioning and the fetal growth trajectory. *FASEB J*. 2013;27(10):3928-3937.

555   doi:10.1096/fj.13-234823

556 8.   Higgins JS, Vaughan OR, Fernandez de Liger E, Fowden AL, Sferruzzi-Perri AN. Placental phenotype

557   and resource allocation to fetal growth are modified by the timing and degree of hypoxia during mouse

558   pregnancy. *J Physiol*. 2016;594(5):1341-1356. doi:10.1113/JP271057

559 9.   Mayhew TM, Manwani R, Ohadike C, Wijesekara J, Baker PN. The Placenta in Pre-eclampsia and

560   Intrauterine Growth Restriction: Studies on Exchange Surface Areas, Diffusion Distances and Villous

561   Membrane Diffusive Conductances. *Placenta*. 2007;28(2-3):233-238. doi:10.1016/j.placenta.2006.02.011

562 10.   Jansson N, Pettersson J, Haafiz A, et al. Down-regulation of placental transport of amino acids precedes

563   the development of intrauterine growth restriction in rats fed a low protein diet. *J Physiol*. 2006;576(Pt

564   3):935-946. doi:10.1113/jphysiol.2006.116509

565 11.   McIntyre KR, Vincent KMM, Hayward CE, et al. Human placental uptake of glutamine and glutamate is

566   reduced in fetal growth restriction. *Sci Rep*. 2020;10(1):1-12. doi:10.1038/s41598-020-72930-7

567 12.   Burton GJ, Jauniaux E. Expert Reviews Pathophysiology of placental-derived fetal growth restriction.

568   *Am J Obstet Gynecol*. 2018;218(2):S745-S761. doi:10.1016/j.ajog.2017.11.577

569 13.   Chatuphonprasert W, Jarukamjorn K, Ellinger I. Physiology and pathophysiology of steroid biosynthesis,

570   transport and metabolism in the human placenta. *Front Pharmacol*. 2018;9(SEP):1-29.

571   doi:10.3389/fphar.2018.01027

572 14.   Lu M, Sferruzzi-perri AN. Placental mitochondrial function in response to gestational exposures.

573   *Placenta*. 2021;104(October 2020):124-137. doi:10.1016/j.placenta.2020.11.012

574 15.   Mishra P, Chan DC. Metabolic regulation of mitochondrial dynamics. 2016:1-9.

575   doi:10.1083/jcb.201511036

576   16.   Jones ML, Mark PJ, Lewis JL, Mori TA, Keelan JA, Waddell BJ. Antioxidant Defenses in the Rat

577   Placenta in Late Gestation : Increased Labyrinthine Expression of Superoxide Dismutases , Glutathione

578   Peroxidase 3 , and Uncoupling Protein 2 1. 2010;260(April):254-260. doi:10.1095/biolreprod.110.083907

579   17.   Stark MJ, Hodyl NA, Butler M, Clifton VL. Localisation and characterisation of uncoupling protein-2 (

580   UCP2) in the human preterm placenta. *Placenta*. 2012;33(12):1020-1025.

581   doi:10.1016/j.placenta.2012.09.010

582   18.   Holland O, Nitert MD, Gallo LA, et al. Review: Placental mitochondrial function and structure in

583   gestational disorders. *Placenta*. 2017. doi:10.1016/j.placenta.2016.12.012

584   19.   Gnanalingham MG, Williams P, Wilson V, et al. Nutritional manipulation between early to mid-

585   gestation: Effects on uncoupling protein-2, glucocorticoid sensitivity, IGF-1 receptor and cell

586   proliferation but not apoptosis in the ovine placenta. *Reproduction*. 2007;134(4):615-623.

587   doi:10.1530/REP-06-0369

588   20.   Mayeur S, Lancel S, Theys N, et al. Maternal calorie restriction modulates placental mitochondrial

589   biogenesis and bioenergetic efficiency : putative involvement in fetoplacental growth defects in rats.

590   2013:14-23. doi:10.1152/ajpendo.00332.2012

591   21.   Rebelato HJ, Esquisatto MAM, Moraes C, Amaral MEC, Catisti R. Gestational protein restriction

592   induces alterations in placental morphology and mitochondrial function in rats during late pregnancy. *J

593   Mol Histol*. 2013;44(6):629-637. doi:10.1007/s10735-013-9522-7

594   22.   Borengasser SJ, Faske J, Kang P, Blackburn ML, Badger TM, Shankar K. In utero exposure to

595   prepregnancy maternal obesity and postweaning high-fat diet impair regulators of mitochondrial

596   dynamics in rat placenta and offspring. *Physiol Genomics*. 2014;46(23):841-850.

597   doi:10.1152/physiolgenomics.00059.2014

598   23.   Sferruzzi-Perri AN, Higgins JS, Vaughan OR, Murray AJ, Fowden AL. Placental mitochondria adapt

599   developmentally and in response to hypoxia to support fetal growth. *Proc Natl Acad Sci U S A*.

600   2019;116(5):1621-1626. doi:10.1073/pnas.1816056116

601   24.   Rosenfeld CS. Sex-specific placental responses in fetal development. *Endocrinology*.

602   2015;156(10):3422-3434. doi:10.1210/en.2015-1227

25. Barke TL, Money KM, Du L, et al. Sex modifies placental gene expression in response to metabolic and inflammatory stress. *Placenta*. 2019;78(615):1-9. doi:10.1016/j.placenta.2019.02.008

26. Romero-Calvo I, Ocón B, Martínez-Moya P, et al. Reversible Ponceau staining as a loading control alternative to actin in Western blots. *Anal Biochem*. 2010;401(2):318-320. doi:10.1016/j.ab.2010.02.036

27. De Clercq K, Lopez-Tello J, Vriens J, Sferruzzi-Perri AN. Double-label immunohistochemistry to assess labyrinth structure of the mouse placenta with stereology. *Placenta*. 2020;94(February):44-47. doi:10.1016/j.placenta.2020.03.014

28. Stenhouse C, Hogg CO, Ashworth CJ. Associations between fetal size, sex and placental angiogenesis in the pig. *Biol Reprod*. 2019;100(1):239-252. doi:10.1093/biolre/ioy184

29. Dias-Junior CA, Chen J, Cui N, et al. Angiogenic imbalance and diminished matrix metalloproteinase-2 and -9 underlie regional decreases in uteroplacental vascularization and feto-placental growth in hypertensive pregnancy. *Biochem Pharmacol*. 2017;146(3):101-116. doi:10.1016/j.bcp.2017.09.005

30. Mandò C, De Palma C, Stampalija T, et al. Placental mitochondrial content and function in intrauterine growth restriction and preeclampsia. *Am J Physiol - Endocrinol Metab*. 2014;306(4):404-413. doi:10.1152/ajpendo.00426.2013

31. Mele J, Muralimanoharan S, Maloyan A, Myatt L. Impaired mitochondrial function in human placenta with increased maternal adiposity. *Am J Physiol - Endocrinol Metab*. 2014;307(5):E419-E425. doi:10.1152/ajpendo.00025.2014

32. Hastie R, Lappas M. The effect of pre-existing maternal obesity and diabetes on placental mitochondrial content and electron transport chain activity. *Placenta*. 2014;35(9):673-683. doi:10.1016/j.placenta.2014.06.368

33. Yung HW, Colleoni F, Dommett E, et al. Noncanonical mitochondrial unfolded protein response impairs placental oxidative phosphorylation in early-onset preeclampsia. *Proc Natl Acad Sci U S A*. 2019;116(36):18109-18118. doi:10.1073/pnas.1907548116

34. Enquobahrie DA, Meller M, Rice K, Psaty BM, Siscovick DS, Williams MA. Differential placental gene expression in preeclampsia. *Am J Obstet Gynecol*. 2008;199(5):566.e1-11. doi:10.1016/j.ajog.2008.04.020

35. He G, Xu W, Chen Y, Liu X, Xi M. Abnormal Apoptosis of Trophoblastic Cells Is Related to the Up-

631   Regulation of CYP11A Gene in Placenta of Preeclampsia Patients. *PLoS One*. 2013;8(3).

632   doi:10.1371/journal.pone.0059609

633   36. Escobar JC, Patel SS, Beshay VE, Suzuki T, Carr BR. The human placenta expresses CYP17 and

634   generates androgens de novo. *J Clin Endocrinol Metab*. 2011;96(5):1385-1392. doi:10.1210/jc.2010-

635   2504

636   37. Büdefeld T, Jezek D, Rozman D, Majdic G. Initiation of steroidogenesis precedes expression of

637   cholesterologenic enzymes in the fetal mouse testes. *J Vet Med Ser C Anat Histol Embryol*.

638   2009;38(6):461-466. doi:10.1111/j.1439-0264.2009.00975.x

639   38. Yao S, Lopez-Tello J, Sferruzzi-Perri AN. Developmental programming of the female reproductive

640   system - a review. *Biol Reprod*. 2021;104(4):745-770. doi:10.1093/biolre/ioaa232

641   39. Mishra JS, Blesson CS, Kumar S. Testosterone decreases placental mitochondrial content and cellular

642   bioenergetics. *Biology (Basel)*. 2020;9(7):1-14. doi:10.3390/biology9070176

643   40. Hu M, Zhang Y, Guo X, et al. Hyperandrogenism and insulin resistance induce gravid uterine defects in

644   association with mitochondrial dysfunction and aberrant reactive oxygen species production. *Am J*

645   *Physiol - Endocrinol Metab*. 2019;316(5):E794-E809. doi:10.1152/ajpendo.00359.2018

646   41. Aye ILMH, Aiken CE, Charnock-Jones DS, Smith GCS. Placental energy metabolism in health and

647   disease—significance of development and implications for preeclampsia. *Am J Obstet Gynecol*. 2020.

648   doi:10.1016/j.ajog.2020.11.005

649   42. Carey EAK, Albers RE, Doliboa SR, et al. AMPK Knockdown in Placental Trophoblast Cells Results in

650   Altered Morphology and Function. 2014;23(23):2921-2930. doi:10.1089/scd.2014.0092

651   43. Skeffington KL, Higgins JS, Mahmoud AD, et al. Hypoxia , AMPK activation and uterine artery

652   vasoreactivity. 2016;5:1357-1369. doi:10.1113/JP270995

653   44. Welsh P, Doolin O, McConnachie A, et al. Circulating 25OHD, dietary vitamin D, PTH, and calcium

654   associations with incident cardiovascular disease and mortality: The MIDSPAN family study. *J Clin*

655   *Endocrinol Metab*. 2012;97(12):4578-4587. doi:10.1210/jc.2012-2272

656   45. Yung HW, Calabrese S, Hynx D, et al. Evidence of placental translation inhibition and endoplasmic

657   reticulum stress in the etiology of human intrauterine growth restriction. *Am J Pathol*. 2008;173(2):451-

658   462. doi:10.2353/ajpath.2008.071193

659 46. López-Tello J, Pérez-García V, Khaira J, et al. Fetal and trophoblast PI3K p110α have distinct roles in
660   regulating resource supply to the growing fetus in mice. *Elife*. 2019;8:1-25. doi:10.7554/eLife.45282.001

661 47. Hiden U, Maier A, Bilban M, et al. Insulin control of placental gene expression shifts from mother to
662   foetus over the course of pregnancy. *Diabetologia*. 2006;49(1):123-131. doi:10.1007/s00125-005-0054-x

663 48. Lüscher BP, Marini C, Joerger-Messerli MS, et al. Placental glucose transporter (GLUT)-1 is down-
664   regulated in preeclampsia. *Placenta*. 2017;55:94-99. doi:10.1016/j.placenta.2017.04.023

665 49. Jansson T, Wennergren M, Powell TL. Placental glucose transport and GLUT 1 expression in insulin-
666   dependent diabetes. *Am J Obstet Gynecol*. 1999;180(1 I):163-168. doi:10.1016/S0002-9378(99)70169-9

667 50. Díaz M, Bassols J, López-Bermejo A, Gómez-Roig MD, De Zegher F, Ibáñez L. Placental expression of
668   peroxisome proliferator-activated receptor γ (PPARγ): Relation to placental and fetal growth. *J Clin
669   Endocrinol Metab*. 2012;97(8):1468-1472. doi:10.1210/jc.2012-1064

670 51. Chen Z, He P, Ding X, Huang Y, Gu H, Ni X. PPAR γ stimulates expression of L-type amino acid and
671   taurine transporters in human placentas : the evidence of PPAR γ regulating fetal growth. *Nat Publ Gr*.
672   2015;(December 2014):1-13. doi:10.1038/srep12650

673 52. Mello T, Materozzi M, Galli A. PPARs and mitochondrial metabolism: From NAFLD to HCC. *PPAR
674   Res*. 2016;2016. doi:10.1155/2016/7403230

675 53. Lee YK, Park JE, Lee M, Hardwick JP. Hepatic lipid homeostasis by peroxisome proliferator-activated
676   receptor gamma 2. *Liver Res*. 2018;2(4):209-215. doi:10.1016/j.livres.2018.12.001

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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) $CI+II_P$: $CI+CII_{Oxphos}$, (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,

25

709   MNF2, OPA1, HSP60, HSP70, TID1 and CLPP in the placental labyrinth zone supporting the lightest and

710   heaviest fetuses of each sex within the litter on gestational day 18. Images from each antibody and

711   representative Ponceau staining are included. For western blot results sample size is 5 lightest and 5 heaviest

712   fetuses per sex from n = 5 litters. For qPCR results sample size is 7 lightest and 7 heaviest fetuses per sex from

713   n = 7 litters. Data are displayed as individual data points with mean ± S.E.M. Data analysed for each sex

714   separately by paired t test; ∗p < 0.05, ∗∗p <0.01, ∗*∗p <0.001.

715

716   **Figure 5.** Relative mRNA expression of amino acid (A), glucose (B), lipid (C) transporters and steroid hormone

717   metabolism and signalling related genes (D) in the placental labyrinth zone supporting the lightest and heaviest

718   fetuses of each fetal sex within the litter on gestational day 18. Data are from 7 lightest and 7 heaviest fetuses

719   per sex from n = 7 litters. Data displayed as individual data points with mean ± S.E.M. Data analysed for each

720   sex separately by paired t test; ∗p < 0.05.

721

722   **Figure 6.** Protein abundance of key growth and metabolic signalling proteins in the placental labyrinth zone

723   supporting the lightest and heaviest fetuses of each sex within the litter on gestational day 18. (A and C) Total

724   AKT, AMPKα, MAPK 44/42 and P38 MAPK protein levels, and (B and D) AKT, AMPKα, MAPK 44/42 and

725   P38 MAPK phosphorylation levels as a ratio to total protein in the heaviest *versus* the lightest fetuses for

726   females (A and B) and males (C and D). Total protein abundance for PPARγ (E). Representative images from

727   each antibody and Ponceau staining are included. Data are 5 lightest and 5 heaviest fetuses per sex from n=5

728   litters. Data are displayed as individual data points with mean ± S.E.M. Data analysed for each sex separately

729   by paired t test; *p < 0.05

730

731   **Figure 7.** Summary figure representing the alterations in placental phenotype of the lightest *versus* heaviest

732   fetuses of each sex in normal wildtype pregnant mice at gestational day 18.

733

734   **Figure S1.** Conceptus biometrical data from female and male fetuses within the same litter at gestational day

735   18. (A) Fetal weight, (B) Brain and (C) Liver weights as a proportion of fetal weight, (D) Placental weight, (E)

736   Labyrinth zone (Lz) weight and (F) Placental efficiency (F:P ratio; determined as the ratio of fetal weight to

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737   placental weight). Data are from n = 12 litters were averaged and displayed as individual data points with mean

738   ± S.E.M. Data analysed by paired t test; $*p < 0.05$, $***p < 0.001$.

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743 **Table 1. Primers used for qPCR analysis.**

744

Symbol	GenBank ID	Primer sequences (5'=>3') Fw/Rv	Amplicon length (bp)
Nutrient transporters genes			
Slc2a1	NM_011400.3	Fw: GCTTATGGGCTTCTCCAAACT Rv: GGTGACACCTCTCCCACATAC	123
Slc2a3	NM_011401.4	Fw: GA TCGGCTCTTTCCAGTTTG Rv: CAATCATGCCACCAACAGAG	176
Fatp1	NM_011977.4	Fw: GGCTCCTGGAGCAGGAACA Rv: ACGGAAGTCCCAGAAACCAA	65
Fatp3	NM_011988.3	Fw: GAGAACTTGCCACCGTATGC Rv: GGCCCCTATATCTTGGTCCA	162
Fatp4	NM_011989.5	Fw: GATTCTCCCTGTTGCTCCTGT Rv: CCATTGAAGCAAACAGCAGG	174
Fatp6	NM_001081072.1	Fw: AACCAAGTGGTGACATCTCTGC Rv: TCCATAAAGTAAAGCGGGTCAG	158
Slc38a2	NM_001355633.1	Fw: TAATCTGAGCAATGCGATTGTGG Rv: AGATGGACGGAGTATAGCGAAAA	129
Slc38a1	NM_001166456.1	Fw: CCTTCACAAGTACCAGAGCAC Rv: GGCCAGCTCAAATAACGATGAT	127
Slc38a4	NM_001358060	Fw: GCGGGGACAGTATTCAGGAC Rv: GGAACTTCTGACTTTCGGCAT	102
Slc7a5	NM_011404.3	Fw: CTGCTGACACCTGTGCCATC Rv: GGCTTCTTGAATCGGAGCC	161
Slc7a8	NM_016972.2	Fw: CCAGTGTGTTGGCCATGATC Rv: TGCAACCGTTACCCCATAGAA	161
Mitochondrial genes			
Pgc-1	NM_008904.2	Fw: GCAGTCGCAACATGCTCAAG Rv: GGGAACCCTTGGGGTCATTT	83
Nrf1	NM_001164226.1	Fw: AGAAACGGAAACGGCCTCAT Rv: CATCCAACGTGGCTCTGAGT	96
Nrf2	NM_010902.4	Fw: ATGGAGCAAGTTTGGCAGGA	96

28

		Rv: GCTGGGAACAGCGGTAGTAT	
Tfam	NM_009360.4	Fw: TCCACAGAACAGCTACCCAA	84
		Rv: CCACAGGGCTGCAATTTTCC	
Pparγ	NM_001113418.1	Fw: TGCAGCCTCAGCCAAGTTGAA	77
		Rv: TTCCCGAACTTGACCAGCCA	
Opa1	NM_001199177.1	Fw: TGGGCTGCAGAGGATGGT	60
		Rv: CCTGATGTCACGGTGTTGATG	
Mfn1	NM_024200.4	Fw: TTGCCACAAGCTGTGTTCGG	148
		Rv: TCTAGGGACCTGAAAGATGGGC	
Mfn2	NM_001285920.1	Fw: AGAGGCAGTTTGAGGAGTGC	103
		Rv: ATGATGAGACGAACGGCCTC	
Drp1	NM_152816.3	Fw: ATGCCAGCAAGTCCACAGAA	86
		Rv: TGTTCTCGGGCAGACAGTTT	
Fis1	NM_025562.3	Fw: CAAAGAGGAACAGCGGGACT	95
		Rv: ACAGCCCTCGCACATACTTT	
Steroid metabolism and signaling			
Star	NM_011485.5	Fw: TCGCTACGTTCAAGCTGTGT	184
		Rv: GCTTCCAGTTGAGAACCAAGC	
Cyp11a1	NM_001346787.1	Fw: GCCCCCGGAGAGCTTG	193
		Rv: TCCCATGCTGAGCCAGA	
Cyp17a1	NM_007809.3	Fw: TGGAGGCCACTATCCGAGAA	119
		Rv: CACATGTGTGTCCTTCGGGA	
Hsd11b1	NM_008288.2	Fw: GAGGAAGGTCTCCAGAAGGTA	143
		Rv: ATGTCCAGTCCGCCCAT	
Hsd11b2	NM_008289.2	Fw: GGCTGGATCGCGTTGTC	132
		Rv: CGTGAAGCCCATGGCAT	
Esr2	NM_207707.1	Fw: CCTCGTTCTGGACAGGTCCTC	70
		Rv: CCTTGGGACAGCACTCTTCG	
Ar	NM_013476.4	Fw: GGATTCTGTGCAGCCTATTGC	90
		Rv: TCAGGAAAGTCCACGCTCAC	

745

746   Annealing temperature was 60 °C for all genes. Androgen receptor, *Ar*; Cytochrome P450 Family 11 Subfamily

747   A Member 1, *Cyp11a1*; Cytochrome P450 Family 17 Subfamily A Member 1, *Cyp17a1*; Dynamin 1-like, *Drp1*;

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748   Estrogen receptor 2 (beta), *Esr2*; Solute Carrier Family 27 (Fatty Acid Transporter), Member 1, *Fatp1*; Solute

749   Carrier Family 27 (Fatty Acid Transporter), Member 3, *Fatp3*; Solute Carrier Family 27 (Fatty Acid

750   Transporter), Member 4, *Fatp4*; Solute Carrier Family 27 (Fatty Acid Transporter), Member 6, *Fatp6*;

751   Mitochondrial fission factor, *Fis1*; Hydroxysteroid 11-Beta Dehydrogenase 1, *Hsd11b1*; Hydroxysteroid 11-

752   Beta Dehydrogenase 1, *Hsd11b2*; Mitofusin 1, *Mfn1*; Mitofusin 2, *Mfn2*; Nuclear respiratory factor 1, *Nrf1*;

753   Nuclear respiratory factor 2, *Nrf2*; Mitochondrial dynamin like GTPase, *Opa1*; Peroxisome proliferator-

754   activated receptor gamma coactivator 1-alpha, *Pgc-1α*; Peroxisome proliferator activated receptor alpha *Pparγ*;

755   Solute Carrier Family 2 (Facilitated Glucose Transporter), Member 1, *Slc2a1*; Solute Carrier Family 2

756   (Facilitated Glucose Transporter), Member 3, *Slc2a3*; Solute carrier family 38, member 2, *Slc38a2*; Solute

757   carrier family 38, member 1, *Slc38a1*; Solute carrier family 38, member 4, *Slc38a4*; Solute Carrier Family 7

758   (Amino Acid Transporter Light Chain, L System), Member 5, *Slc7a5*; Solute carrier family 7 (cationic amino

759   acid transporter, y+ system), member 8, *Slc7a8*; Steroidogenic Acute Regulatory Protein, *Star*; Transcription

760   factor A, mitochondria, *Tfam*.

761

762 **Table 2. List of primary antibodies used in this study.**

763

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
Phospo-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

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766 **Supplementary Table 1. Comparisons between fetal sexes on lightest and heaviest fetuses.**

	Lightest			Heaviest		
	Female	**Male**	**p-value**	**Female**	**Male**	**p-value**
Conceptus biometry						
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 ± 1.82	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						
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
Transporter gene expression						
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

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						
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
1-P/E	0.14 ± 0.02	0.18 ± 0.03	NS	0.21 ± 0.03	0.13 ± 0.03	NS
ETS complex proteins			ND			ND
Mitochondria-related gene expression						

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
Steroid handling genes						
Star	1.05 ± 0.09	1.22 ± 0.20	NS	1.12 ± 0.16	1.38 ± 0.20	NS
Cyp11a1	1.03 ± 0.09	0.78 ± 0.08	p < 0.05	1.13 ± 0.22	1.15 ± 0.15	NS
Cyp17a1	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.
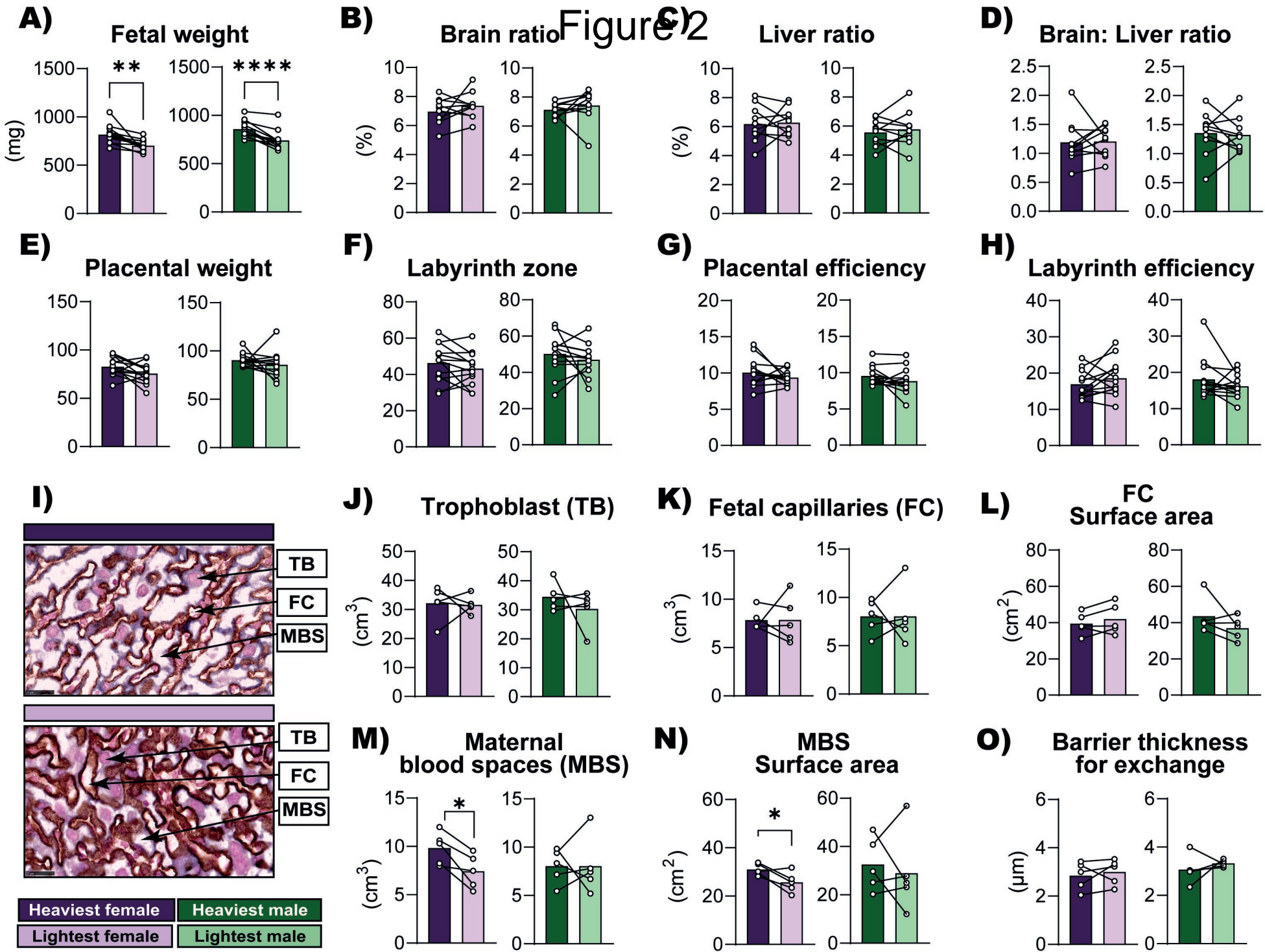
Figure 1

A) Litter-Sex

Females
r= 0.24
p= ns

Males
r= -0.07
p= ns

Placenta weight (mg) vs Fetus weight (mg)

B) Females

Heaviest female
r=-0.30
p=ns

Lightest female
r=0.74
p= **✱✱**

Placenta weight (mg) vs Fetal weight (mg)

C) Males

Heaviest male
r=-0.10
p=ns

Lightest male
r=-0.32
p=ns

Placenta weight (mg) vs Fetal weight (mg)

Figure 2

A) Fetal weight

B) Brain ratio

C) Liver ratio

D) Brain: Liver ratio

E) Placental weight

F) Labyrinth zone

G) Placental efficiency

H) Labyrinth efficiency

I)

J) Trophoblast (TB)

K) Fetal capillaries (FC)

L) FC Surface area

M) Maternal blood spaces (MBS)

N) MBS Surface area

O) Barrier thickness for exchange

Heaviest female   Heaviest male
Lightest female   Lightest male

Figure 3

Figure 4

Figure 5

A) Amino Acid Transporters

B) Glucose Transporters

C) Lipid Transporters

D) Steroid metabolism and signalling

Legend: Heaviest female, Lightest female, Heaviest male, Lightest male

Figure 6

Figure 7

Mother
= Environment
= Maternal signals
= Nutrients

= Genotype to their sibling / = Genetic growth potential

Lightest Female

↔ Placental weight ↔
↔ Labyrinth weight ↔
Labyrinth structure
↓ MBS volume & surface area ↔
↔ FC ↔
↔ TBS ↔
↔ BT ↔

Lightest Male

Placental labyrinth zone

Placental/fetal sex signals

Lightest females:
↑ PPARγ protein levels

Signalling Pathways
AMPK PPARγ AKT

Lightest males:
↓ AKT phosphorilation
↓ PPARγ protein levels

Lightest females & males: total AMPK

Mitochondria dynamics

Fission
Fusion
Biogenesis

Mitochondria energetics

Krebs cycle
CI
CII
CIII
CIV
CV

Gene expression

Mitochondria genes
Fis1 Drp1 Nrf1 Tfam
Slc2a1 Cyp17a1 Cyp11a1 Ar
Glucose and steroid related genes

Lightest females:
↓ Fission (*Drp1* and *Fis1*)

Lightest females:
↑ CI$_L$ and CI+CII$_P$ oxygen flux
↓ CI protein levels
↓ CLPP protein levels

Lightest males:
= respiratory rates
↓ CIII and CV protein levels

Lightest females:
↓ *Cyp17a1* gene expression
↑ *Cyp11a1* gene expression
↓ *Nrf1* gene expression

Lightest males:
↓ *Slc2a1* gene expression
↑ *Tfam* gene expression
↑ *Ar* gene expression

Female fetuses | **Male fetuses**
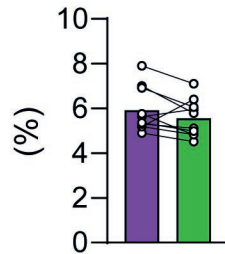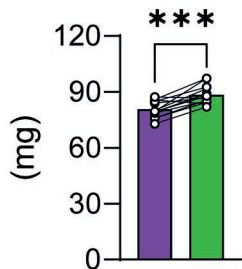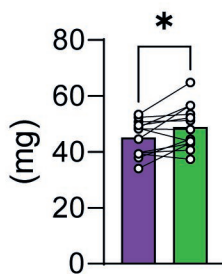
A) Fetal weight

B) Brain ratio

C) Liver ratio

D) Placental weight

E) Lz weight

F) F:P ratio