Sex-dependent Effects of Developmental Hypoxia on Cardiac Mitochondria from Adult Murine Offspring

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Sex-dependent Effects of Developmental Hypoxia on Cardiac Mitochondria from Adult Murine Offspring

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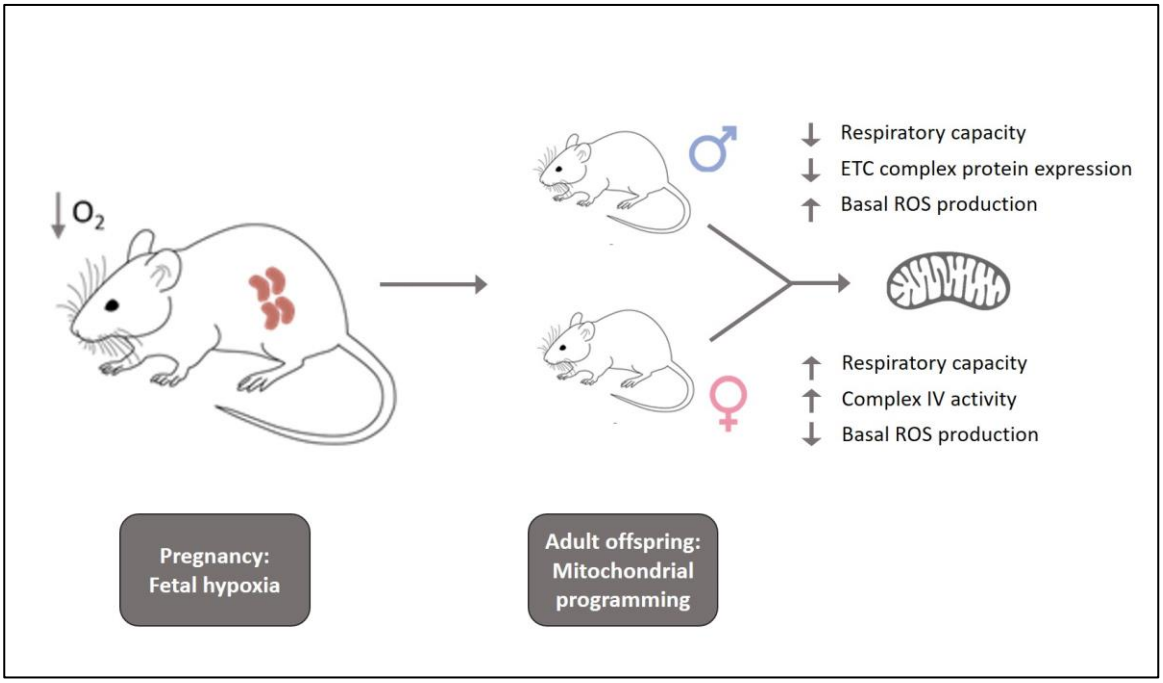
Abstract

Insufficient oxygen supply (hypoxia) during fetal and embryonic development can lead to latent phenotypical changes in the adult cardiovascular system, including altered cardiac function and increased susceptibility to ischemia reperfusion injury. While the cellular mechanisms underlying this phenomenon are largely unknown, several studies have pointed towards metabolic disturbances in the heart of offspring from hypoxic pregnancies. To this end, we investigated mitochondrial function in the offspring of a mouse model of prenatal hypoxia. Pregnant C57 mice were subjected to either normoxia (21%) or hypoxia (14%) during gestational days 6-18. Offspring were reared in normoxia for up to 8 months and mitochondrial biology was assessed with electron microscopy (ultrastructure), spectrophotometry (enzymatic activity of electron transport chain complexes), microrespirometry (oxidative phosphorylation and H_20_2 production) and Western Blot (protein expression). Our data showed that male adult offspring from hypoxic pregnancies possessed mitochondria with increased H_20_2 production and lower respiratory capacity that was associated with reduced protein expression of complex I, II and IV. In contrast, females from hypoxic pregnancies had a higher respiratory capacity and lower H_20_2 production that was associated with increased enzymatic activity of complex IV. From these results, we speculate that early exposure to hypoxia has long term, sex-dependent effects on cardiac metabolic function, which may have implications for cardiovascular health and disease in adulthood.

Graphical Abstract



Keywords

Heart

Mitochondria

Developmental programming

Fetal hypoxia

Reactive oxygen species

Abbreviations

ADP - adenosine diphosphate

CS - citrate synthase

ETC – electron transport chain

ET_{CI+CII} - electron transfer capacity with complex I and II substrates

ET_{CII} - electron transfer capacity with complex II substrates

FCCP - carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone

GD – gestational day

H_20_2 – hydrogen peroxide

HRP - horseradish peroxidase

I/R – ischemia reperfusion

$LEAK_{CI}$ - Leak respiratory state with complex I substrates

MPG - malate pyruvate and glutamate

O^{-}_2 - superoxide

OXPHOS – oxidative phosphorylation

$OXPHOS_{CI}$ - oxidative phosphorylation with complex I substrates

$OXPHOS_{CI+CII}$ - oxidative phosphorylation with complex I and II substrates

PKCε - protein kinase C epsilon

RCR - respiratory control ratio

ROS – reactive oxygen species

ROX - residual non-mitochondrial oxygen consumption

SOD - superoxide dismutase

TMPD - TM/AS, N,N,N,N-tetramethyl-p-phenylenediamin

1.0. Introduction

Heart disease remains the leading cause of death worldwide [1]. Despite significant advances in treatment options, prevention strategies are the most cost-effective way to reduce the socioeconomic burden of these diseases. Such strategies have traditionally targeted behavioral and lifestyle risk factors, such as smoking and obesity. However, the seminal work of Barker [2] demonstrated that adverse events during pregnancy can predispose offspring to heart disease in adulthood. This phenomenon, termed developmental programming, provides a window of opportunity to prevent the development of heart disease in early life. Nevertheless, before effective treatments can be designed, it is crucial to understand the mechanisms leading to cardiac dysfunction in offspring from high-risk pregnancies.

Insufficient oxygen supply to an embryo or foetus, termed developmental hypoxia, occurs in a wide range of high-risk pregnancies, including preeclampsia, placental insufficiency, placental infection, maternal anaemia, gestational diabetes and high altitude pregnancy [3,4]. Animal models have shown the foetus initially responds to hypoxia by preferentially distributing blood flow to vital organs, such as the heart and brain [4-6]. While this strategy is protective in the short term, a sustained redistribution of blood flow is associated with asymmetric fetal growth restriction, increased peripheral resistance and cardiac abnormalities, including hypertrophy [4]. In adulthood, the hearts of offspring from hypoxic pregnancies continue to express abnormal phenotypes, including diastolic dysfunction, sustained increases in myocardial contractility and enhanced responsiveness to β-adrenoreceptor stimulation [7-9]. Additionally, prenatal hypoxia appears to sensitise the adult heart to ischaemia and reperfusion (I/R) injury [10-12]. Interestingly, females from hypoxic pregnancies appear to be partially protected

from cardiac dysfunction, suggesting that the effects of developmental hypoxia are gender-specific [11,13,14]. In aggregate, these studies suggest developmental hypoxia programmes a dysfunctional cardiac phenotype in offspring that cannot be reversed by normalising oxygen availability after birth.

Given that most of these experiments have been conducted in isolated hearts, the cardiac dysfunction in offspring from hypoxic pregnancies cannot be explained by autonomic influences, altered peripheral resistance (cardiac afterload) or circulating catecholamines. Therefore, the intrinsic properties of the myocardial cells have been altered. While multiple cellular mechanisms may account for cardiac dysfunction, recent evidence suggests intrauterine stress can alter offspring cardiac metabolism, particularly at the level of the mitochondria. For example, nutritional stress during development leads to a range of cardiac mitochondrial abnormalities in fetal and adult offspring, including structural disorganization, impaired mitophagy, reduced oxygen consumption, decreased proton leak and altered fission/fusion dynamics [15-17]. Similarly, exposure to excess glucocorticoids during development can programme cardiac mitochondrial dysfunction in adult offspring, leading to increased sensitivity to ischemia/reperfusion injury, higher levels of H_20_2 production and a reduced capacity to produce ATP [18]. Collectively, these studies suggest mitochondrial remodeling represents a major mechanism underlying the developmental programming of heart disease.

To our knowledge, only one group has investigated the long-term effects of developmental hypoxia on offspring mitochondrial function. Thompson and colleagues has shown pregnant guinea pigs exposed to 10.5% atmospheric oxygen between gestational days (GD) 28-65 (term 65-72 days) led to sex-specific alterations in

mitochondrial enzymatic complex activities, mitochondrial DNA content, protein expression and respiration [19-22]. However, the moderate level of hypoxia used in these studies (10.5%) caused a significant reduction in maternal weight, which is indicative of nutritional stress. It is therefore difficult to conclude whether mitochondrial function is being altered by developmental hypoxia or nutritional stress, or a combination of the two. To this end, we have undertaken a comprehensive investigation into the effects of developmental hypoxia on adult offspring mitochondrial morphology, respiratory capacity, reactive oxygen species production, enzymatic activity and protein expression. Our results suggest developmental hypoxia has long-term, sex-dependent effects on cardiac mitochondrial function; in particular, males from hypoxic pregnancies have a lower mitochondrial respiratory capacity and generate more H_20_2 under basal conditions, and females from hypoxic pregnancies have greater respiratory scope and produce less H_20_2 under basal conditions, compared to their normoxic counterparts. We speculate that these phenotypes have long-term implications for metabolic health and the susceptibility to heart disease.

2.0. Methods

2.1. Animal model

There are several strategies to model chronic fetal hypoxia, but most (e.g. placental embolisation, reduced uterine blood flow) cause impaired placental perfusion, thereby decreasing the delivery of nutrients as well as oxygen to the foetus [23,24]. In these instances, it is difficult to separate the effects of fetal nutrient restriction versus fetal hypoxia in programming cardiovascular dysfunction in the offspring. To this end, we have utilised a rodent model of prenatal hypoxia developed by the Giussani laboratory

that does not affect maternal food intake, thereby allowing the effects of developmental hypoxia to be studied in isolation [8,25-27].

2.2. Oxygen incubation protocols

All procedures comply with The UK Animals (Scientific Procedures) Act 1986 and EU directive 2010/63. The ARRIVE guidelines were followed for reporting the use of animals in scientific experiments. Local ethical approval was granted by The University of Manchester Animal Welfare Ethical and Review Board. Pregnant C57BL/J6 mice (aged 12 weeks, 24.1 ± 6.0 g), were bred, mated and maintained at The University of Manchester (UK). Mice were housed in standard Individually Ventilated Cages (IVC) with normal oxygen levels and a 12:12 light cycle with ad libitum food and water. The pregnant mice were randomly assigned to two groups; normoxia (N, 21% O_2) or hypoxia (H, 14% O_2). For hypoxic incubations, mice were transferred to an environmental chamber (Coy O_2 In Vivo Glove Box, Coy Laboratory Products, Grass Lake, MI) at gestational day (GD) 6 where they were subjected to 14% oxygen. Levels of humidity (60%), CO_2 (<1%) and temperature (22^{o}C) were controlled throughout chamber incubation. Maternal food intake and water intake was monitored at regular intervals throughout the procedure, and maternal body weight was measured before and after chamber incubation. Mice were removed from the chamber at GD 18 and allowed to litter in normoxia (GD 21 ±1 day). Litters were culled down to six (3 males and 3 females) to assure standardized maternal care and feeding. Sexing was done by visual determination of the presence or absence of dark pigmentation on the perineum [28,29]. Offspring were weaned from the mother at 3 weeks of age, and group housed in normal conditions. Experiments were performed on mouse offspring aged between 25-32 weeks.

2.3. Electron microscopy

We used electron microscopy to assess left ventricular mitochondrial morphology in adult mice aged between 30-32 weeks. 5 images were analyzed from each animal (n = 2 males and 2 females from normoxic pregnancies, and n = 5 males and 5 females from hypoxic pregnancies). In brief, hearts were removed from the animal, the atria were discarded, and the ventricles were cut longitudinally to separate the left and right chambers. A section of the left ventricular free wall was isolated and 2mm horizontal slices were taken from the mid myocardial layer; care was taken to isolate the same area from each animal. The slices were immediately fixed by immersion with 4% formaldehyde and 2.5% glutaraldehyde in 0.1M HEPES. The tissue was then removed from the fixation solution and prepared according to the Elisman protocol [30]. Briefly, the tissue was stained with heavy metals, dehydrated (stepwise), infiltrated with Taab 812 Hard Resin (stepwise), embedded in silicon wells and finally polymerized at 60°C for 24 hours [31]. A single block was randomly selected from a bag and muscle orientation was determined in semithin sections. Ultrathin sections (up to $1mm^2$, Reichert Ultracut ultramicrotome) were cut longitudinally in relation to the muscle fibres. Samples were then attached to 200 mesh copper EM grids and imaged at x890 magnification with a FEI Tecnai 12 Biotwin microscope at 80kV acceleration voltage with a Gatan Orius SC1000 CCD camera. A grid was randomly selected, and squares were given numerical values. A random number generator was used to select 5 grid squares per animal and the images were analyzed using the free hand tool in Image J (version 1.52k, National Institute of Health, MD). Mitochondria were traced and total mitochondrial area was expressed relative to the total area of the cell (students were blinded to the experimental sample).

2.4. Mitochondrial function

Mitochondrial function was investigated in male (n = 7 normoxic and 9 hypoxic) and female (n = 7 normoxic and 8 hypoxic) offspring aged between 26-31 weeks.

2.4.1. Mitochondrial oxygen consumption and H_20_2 production

Mitochondrial respiration was assessed by high-resolution respirometry using an Oroboros Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) coupled to a fluorescent LED2-Module, allowing simultaneous measurement of O_2 consumption and H_20_2 production, respectively. Mice were killed by cervical dislocation and the heart was immediately excised. ~50mg of fresh left ventricular tissue was homogenized (IKA Ultra-turrax T25) in mitochondrial respiration media (MiR05; containing: EGTA 0.5mM, $MgCl_2$ 3mM, K-MES 60mM, KH_2PO_4 10mM, HEPES 20mM, sucrose 110mM and 1% BSA). 0.16 ± 0.04 mg of homogenised tissue was injected into each of the two chambers of the Oxygraph-2K for measurement of mitochondrial respiration and H_2O_2 production. The rest of the homogenate was frozen at -80^{o}C for analysis of enzymatic function (see section 2.4.2). To measure H_20_2 production, 10µM Amplex® UltraRed and 1U/ml horseradish peroxidase (HRP) were added to each chamber. Amplex® UltraRed oxidizes in the presence of H_2O_2 and forms resorufin, using HRP as a catalyst. Amplex® UltraRed was excited at 563nm and emission was read at 587nm. 5U/ml superoxide dismutase (SOD) was also added to the chambers to convert any extramitochondrial superoxide (O^{-}_2) to H_2O_2.

Substrate inhibitor titration protocols (SUIT protocols) were designed according to Pesta et al [32] (see Fig. 1). Firstly, pyruvate (5mM), malate (2 mM) and glutamate (10mM) were added to achieve LEAK respiratory state with complex I (CI) substrates in the absence of

adenylates ($LEAK_{CI}$). When oxygen consumption was stable, saturating ADP (5mM) was injected to activate oxidative phosphorylation with CI substrates ($OXPHOS_{CI}$). Succinate (10mM) was then added to assess the additive effects of complex II (CII) substrates on oxidative phosphorylation ($OXPHOS_{CI+CII}$).

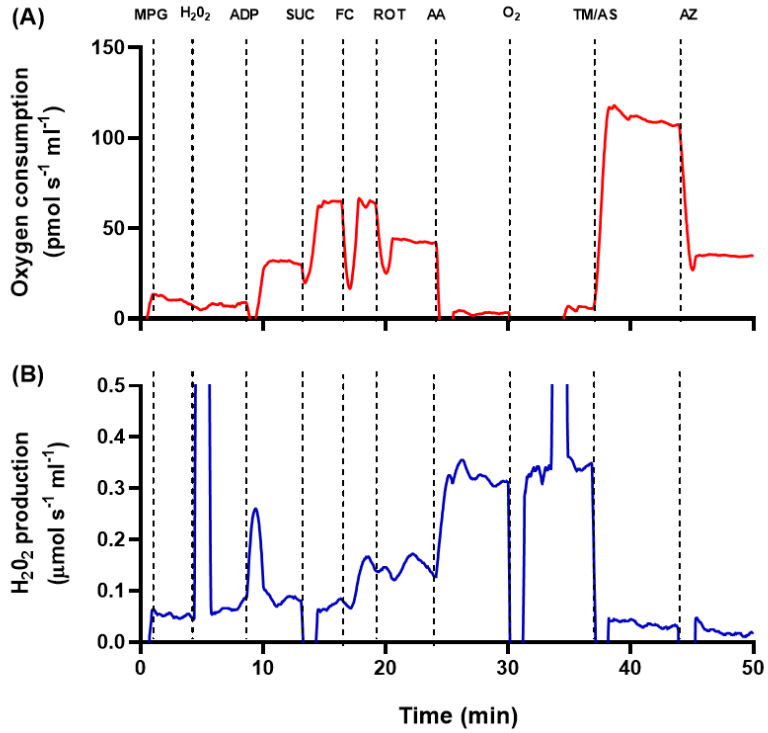


Figure 1: Original trace of simultaneous measurement of mitochondrial oxygen consumption and H_20_2 production in mouse ventricular homogenate. *Data is from a male offspring from a hypoxic pregnancy. Ventricular homogenate (0.1 mg ml^{-1}) was added to the chamber and a range of substrates and inhibitors were injected to investigate the electron transport chain (see methods section for details). Abbreviations; MPG, malate pyruvate and glutamate; ADP, adenosine diphosphate; SUC, succinate; FC, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP); ROT, rotenone; AA, antimycin-A; O_2,*

the chamber was opened for reoxygenation; TM/AS, N,N,N,N-tetramethyl-p-phenylenediamine (TMPD) and ascorbate; AZ, azide.

To uncouple mitochondria and assess maximum electron transfer capacity with CI+CII substrates (ET_{CI+CII}), carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) was titrated in steps to a final concentration of 0.1-0.3µM. Next, the CI inhibitor rotenone (0.5µM) was added to assess ET_{CI+CII} with CII substrates only. To block the ET-pathway and assess residual non-mitochondrial oxygen consumption (ROX), the complex III (CIII) inhibitor, antimycin A, was added (2.5 µM). To assess complex IV (CIV) activity in isolation, the electron donor N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD, 0.5 mM) was added in combination with ascorbate (2mM) to avoid autooxidation of TMPD. Lastly, the CIV inhibitor sodium azide (50mM) was added to assess background non-mitochondrial oxygen consumption from the addition of TMPD.

A separate protocol was used to assess the impact of CIV inhibition on H_20_2 production in control adult mice (n = 4). Ventricular homogenates were incubated with substrates to achieve a steady-state $OXPHOS_{CI+CII}$ (pyruvate, malate, glutamate, succinate and ADP) whilst measuring H_20_2 (HRP, amplex red and SOD). Next, sodium azide was titrated in 0.25mM steps from 0 to 1.75 mM to selectively inhibit CIV.

All O_2 consumption and H_20_2 production data were normalized to a marker of mitochondrial density, citrate synthase (CS) activity. When analyzing the effect of sodium azide on H_20_2 production, H_20_2 was expressed as a percentage of the amount of O_2 consumed (H_20_2/O_2). To estimate mitochondrial efficiency of ATP production, the OXPHOS-coupling efficiency ratio was calculated as 1 – ($LEAK_{CI}$ / $OXPHOS_{CI}$), and the respiratory control ratio (RCR) was calculated as $OXPHOS_{CI}$ / $LEAK_{CI}$) [33].

2.4.2. *Spectrophotometric analysis of enzymatic activities and antioxidant capacity*

Spectrophotometric assays were undertaken on ventricular homogenates from male (n = 5 normoxic and 5 hypoxic) and female (n = 6 normoxic and 6 hypoxic) frozen tissue samples from (aged between 25-29 weeks). Homogenates were assayed for protein content, citrate synthase activity and enzymatic activity of electron transport chain complexes. For all assays, absorbance was measured with a BioTek Synergy HTX multimode reader (BioTek, Swindon, UK). For enzymatic assays, values are expressed as maximum enzymatic activity per min divided by protein content. Protein content of ventricular homogenates were assessed using the Quick start Bradford dye reagent kit (Bio-Rad laboratories, Watford, UK); absorption was read at 550nm at 25°C.

Protocols for measuring CI, CII and CIV enzyme activities, as well as citrate synthase activity, were designed according to Spinazzi et al. [34]. In brief, approximately 20-50mg of frozen ventricular tissue was homogenized using a FastPrep-24™ 5G instrument (MP Biomedicals, Santa Ana, CA) in solution containing 20mM TRIZMA-base, 40mM KCl, 2mM EGTA and 250mM Sucrose (pH 7.4). The tissue was homogenized in two cycles of 30 seconds with a 180-second pause in between each trial at 4°C. Samples were then spun at 600g for 10 minutes at 4°C and the supernatant was stored in -80°C until the day of the assay. Maximal enzymatic activity rate (V_{max}) was assessed over a ten-minute period with a BioTek Synergy HTX multimode reader (BioTek, Swindon, UK) at 37°C. The buffer components for each individual assay are given in Supplementary Table S1.

2.5. Mitochondrial protein expression of electron transfer chain complexes

Protein expression of complexes in the electron transport chain was measured with Western Blot in males from normoxic (n = 5) and hypoxic (n = 5) pregnancies, and females from normoxic (n = 5) and hypoxic (n = 5) pregnancies, aged between 26-32 weeks. The protocols have been described previously in detail [35-37]. Following cardiac excision, a ~0.5 cm^3 region of the left ventricular free wall was removed, snap frozen and stored in liquid nitrogen until analysis. Whole homogenates (~50 mg starting material) were prepared in RIPA buffer with protease and phosphatase inhibitors (0.1mg/ml phenylmethanesulphonylfuroide, 100 mmol/l sodium orhtovanadate, 1 mg/ml aprotonin, 1 mg/ml leupeptin) and protein content was determined (DC Protein Assay, BioRad, UK). Proteins were separated by PAGE and transferred to nitrocellulose membranes. 10µg of protein was used for each sample, and membranes were blocked with 5% milk in TBS-T and incubated with the primary antibody cocktail (Abcam-110413, Cambridge, UK: 1:1000 concentration) and the secondary antibody IRDye® 800CW IgG_{2a}-Specific (Licor, UK: 1:20000 concentration). Membranes were visualized by chemiluminescence (Syngene, UK) or IR-Dye labeled secondary antibodies (Licor, UK). As the 'classical' housekeeping proteins can prove problematic with experimental treatments we opted for a total protein stain to normalize for sample loading, as suggested by Li et. Al., [38], and an internal control to normalize between gels. Total protein transferred to the membrane was determined by REVERT total protein stain (Licor, UK). Blots were repeated in triplicate on separate occasions and data was averaged.

The antibody generated five separate bands, one for each protein corresponding to the five complexes of the ETC pathway (Fig. 1 supplementary material). The value for each separate protein in each sample was divided by the total value for the total protein as well as the internal control.

344

345 *2.6. Calculations and statistics*

346 Maternal BW, FI, WI are expressed as scatter plots (including all measured points) and
347 statistically compared by fitting linear regression curves to compare slopes. Offspring
348 BW is presented as scatter plots expressing means ± SEM and compared with mixed-
349 effect analysis using maternal oxygen level as the random effect and the Tukey's
350 correction for multiple comparisons test. For CIV inhibition, a linear regression curve was
351 fitted to the data to assess if the slope was non-zero. For mitochondrial density where
352 multiple observations (n) have been obtained from the same animal (N), linear mixed
353 modeling (SPSS Statistics. IBM, USA) was performed thus accounting for the nested
354 (clustered) design of the experiment e.g. multiple observations from the same heart (with
355 treatment (hypoxic/normoxic), sex and animal as co-factors). All remaining data were
356 statistically analyzed using a 2-way analysis of covariance (ANCOVA), with sex and
357 intrauterine oxygen levels as independent variables, and age as a covariate (n-values
358 and p-values are given in the figure legends). For mitochondrial H_20_2 production, data
359 were log-transformed to obtain a normal distribution prior to performing the ANCOVA.

360

361 **3.0. Results**

362 *3.1. Maternal and offspring biometry*

363 There were no differences in maternal body weight, water intake or food intake between
364 normoxic and hypoxic dams (Fig. 2A-C), confirming that any programmed effects in the
365 offspring are most likely due to hypoxia alone, rather than differences in maternal food
366 consumption. There were no differences in offspring body weight between hypoxic and
367 normoxic groups at any of the ages tested (Fig. 2D-E).

368

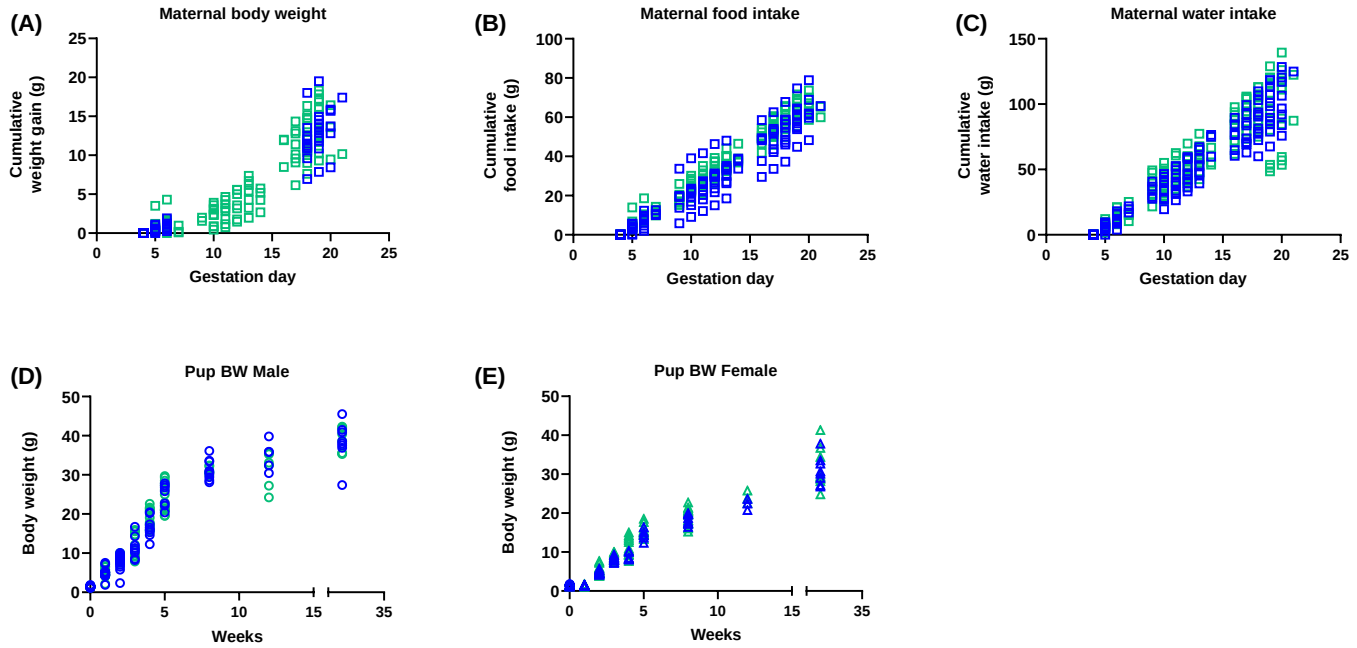


Figure 2: Effects of developmental hypoxia on maternal and fetal biometry. *Maternal body weight (A), food (B) and water intake (C) were monitored in normoxic (green squares, n = 16) and hypoxic dams (blue squares, n = 11). Due to space restrictions within the environmental chamber, the body weight of pregnant dams could not be measured during the hypoxic incubation period (gestational days 6-18). Offspring body weight was also monitored in males (D, circles) from normoxic and hypoxic pregnancies (n = 24 offspring from 9 pregnancies and 23 offspring from 8 pregnancies, respectively) and females (E, triangles) from normoxic and hypoxic pregnancies (n = 20 offspring from 8 pregnancies and 20 offspring from 7 pregnancies, respectively) from birth to 15 weeks. Data are presented as scatter points for measurements of an individual animal. Statistics were run using a mixed-model effects with Tukey's correction for multiple comparisons.*

388 *3.2. Offspring mitochondrial oxygen consumption*

389 Mitochondrial homogenate preparations were of good quality, as attested by high
390 respiratory control ratios (9.2 +/- 0.3) and OXPHOS-coupling efficiency ratios (0.89 +/-
391 0.006) with complex I substrates (malate, pyruvate and glutamate). Mitochondrial
392 oxygen consumption and H_20_2 production responded to substrates and inhibitors in the
393 expected manner [39] (Fig. 1), but FCCP had modest effects on mitochondrial respiration
394 with only 9.3% of preparations responding positively. In the other preparations, even
395 very low concentrations of FCCP (<0.1 μM) had no effect on oxygen consumption or
396 caused a small inhibition (see Fig. 1A), suggesting that mouse cardiac homogenates are
397 already operating at or near their theoretical maximum rate of oxygen consumption.
398
399 In male mice, developmental hypoxia reduced mitochondrial oxygen consumption in the
400 $Leak_{CI}$ and $OXPHOS_{CI}$ states, as well as flux through CIV alone (Fig. 3, circular
401 symbols). In contrast, developmental hypoxia increased mitochondrial oxygen
402 consumption in female mice in all states, except for $LEAK_{CI}$ (Fig. 3, triangular symbols).
403 Within the normoxic group, male mitochondrial oxygen consumption was higher than
404 females in all respiratory states (Fig. 3, green symbols), but there were no sex-
405 dependent differences in the hypoxic group (Fig. 3, blue symbols). There was no effect
406 of treatment or gender on the OXPHOS-coupling efficiency ratio (Fig. 3F) or the RCR
407 (data not shown).
408

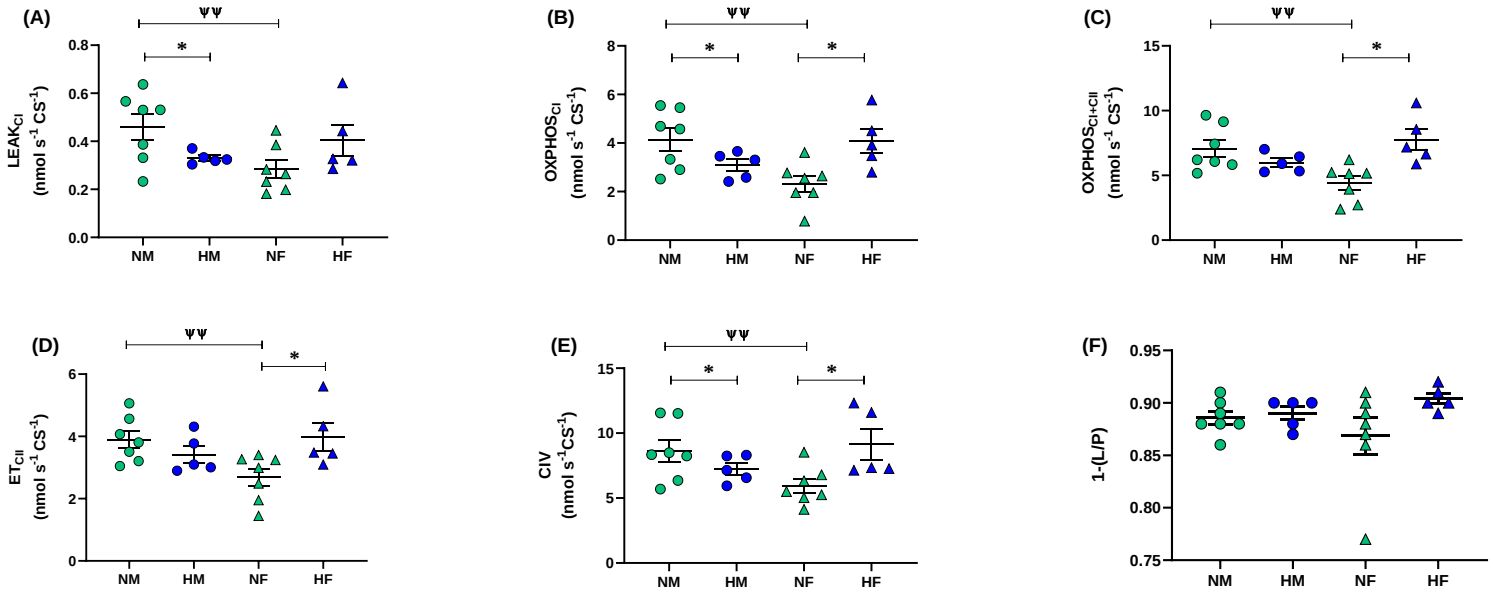


Figure 3: Effect of developmental hypoxia on mitochondrial oxygen consumption. *Mitochondrial oxygen consumption was measured in males from normoxic (green circles, n = 6-7) and hypoxic (blue circles, n = 8-9) pregnancies, and females from normoxic (green triangles, n = 7) and hypoxic (blue triangles, n = 8) pregnancies. Each panel represents a different respiratory state; (A) Leak respiration with substrates for complex I (LEAK$_{CI}$), (B) oxidative phosphorylation with substrates for complex I (OXPHOS$_{CI}$), (C) oxidative phosphorylation with substrates for Complex I and II (OXPHOS$_{CI+CII}$), (D) electron transfer capacity with substrates for Complex II (ET$_{CII}$), (E) electron donation to Complex IV (CIV) and (F) OXPHOS-coupling efficiency ratios (1-(L/P)). Data are mean ± SEM. Asterix denotes a difference between mice from normoxic and hypoxic pregnancies, and Ψ denotes a difference between male and female mice (Two-way ANCOVA, one symbol = $p < 0.05$, two symbols = $p<0.01$).*

3.3. Offspring mitochondrial H_2O_2 production

In male mice, developmental hypoxia increased H_2O_2 production when it was measured in the $LEAK_{CI}$ state and when CIII was inhibited with antimycin A (Fig. 4A and F, circular symbols). In contrast, females from hypoxic pregnancies produced less H_2O_2 compared to their normoxic counterparts in the $OXPHOS_{CI+CII}$ state, and in the ET states (Fig. 4, triangular symbols). With regard to sex-dependent differences, normoxic male H_2O_2 production was higher than normoxic females in all respiratory states (Fig. 4, green symbols), but there were no differences in H_2O_2 production between hypoxic males and hypoxic females (Fig. 4, blue symbols).

Having discovered differences in mitochondrial oxygen consumption and H_2O_2 production between treatment groups, we next sought to determine the underlying mechanisms. In principle, changes in CIV oxygen consumption (Fig. 3E) may account for differences in basal H_2O_2 production between experimental groups. To explore this possibility, we performed experiments in control adult mice where we partially inhibited CIV with sodium azide and simultaneously measured H_2O_2 production (Fig. 5). We found that a dose-dependent inhibition of CIV caused a stepwise increase in H_2O_2 production.

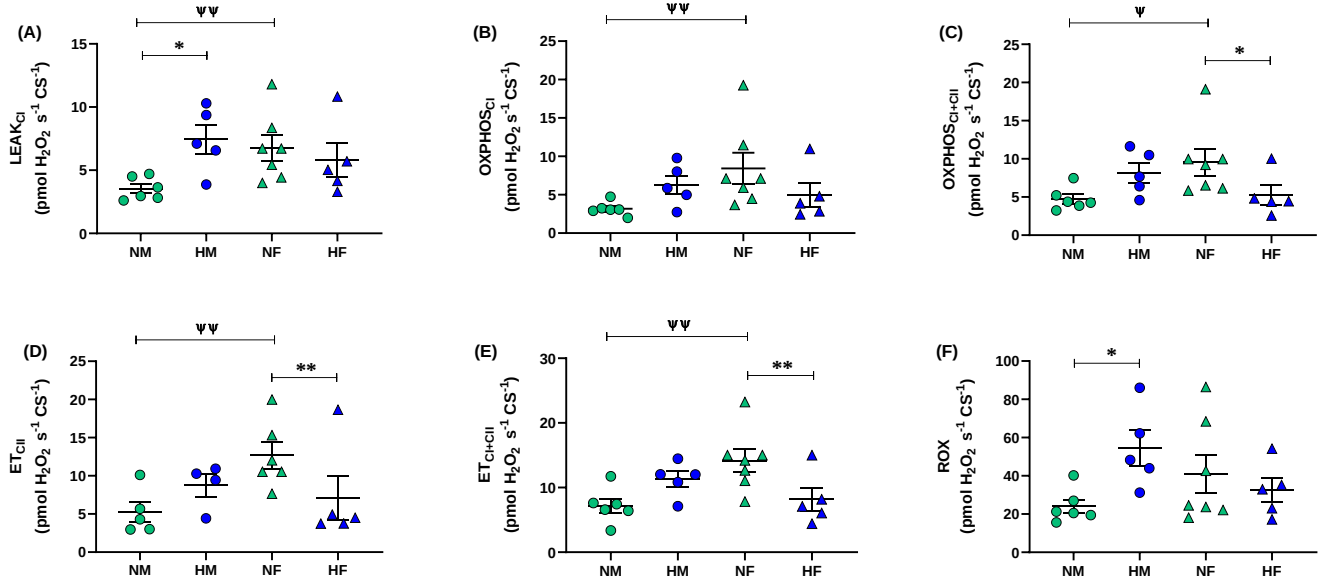


Figure 4: Effects of developmental hypoxia on mitochondrial H_20_2 production. *H_20_2 production was measured in males from normoxic (green circles, n = 5) and hypoxic (blue circles, n = 5) pregnancies, and females from normoxic (green triangles, n = 5) and hypoxic (blue triangles, n = 6) pregnancies. Each panel represents a different respiratory state; (A) Leak respiration with substrates for complex I ($LEAK_{CI}$), (B) oxidative phosphorylation with substrates for complex I ($OXPHOS_{CI}$), (C) oxidative phosphorylation with substrates for complex I and II ($OXPHOS_{CI+CII}$), (D) electron transfer capacity with substrates for complex II (ET_{CII}), (E) electron transfer capacity with substrates for complex I+II (ET_{CI+CII}), and (F) residual oxygen consumption (ROX) in the presence of antimycin A. Data are mean ± SEM. Asterix denotes a statistically significant difference between mice from normoxic and hypoxic pregnancies, and Ψ denotes a difference between male and female mice (Two-way ANCOVA, one symbol = $p < 0.05$, two symbols = $p<0.01$).*

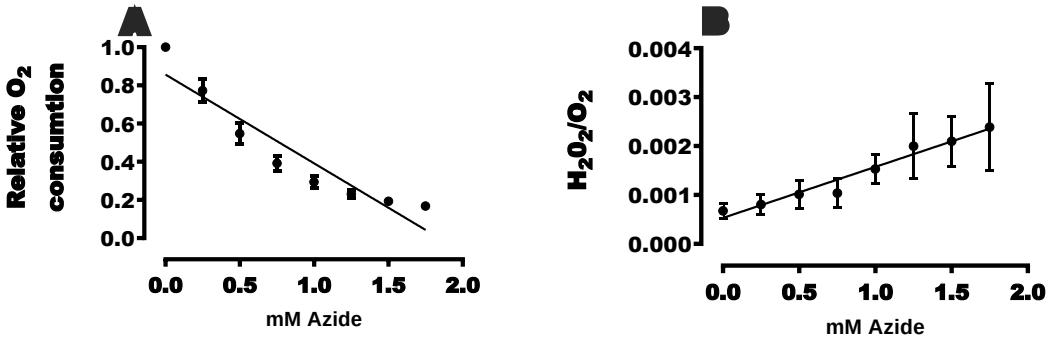


Figure 5: Relationship between complex IV activity and H_20_2 production. *Oxygen consumption (A) was measured simultaneously with H_20_2 production (B) during dose-dependent inhibition of complex IV with sodium azide. Data are presented as mean ± SEM, n=4 adult females from normoxic pregnancies in both panels. A linear regression curve was fitted to the data and was found to be significantly different from zero.*

3.4. Offspring mitochondrial morphology

Differences in mitochondrial oxygen consumption and H_20_2 production between experimental groups may also be explained by variable mitochondrial densities. Therefore, we investigated mitochondrial morphology with electron microscopy and measured the enzymatic activity of a common marker for mitochondrial content, citrate synthase. Developmental hypoxia had no effect on mitochondrial density or citrate synthase activity in any of the treatment groups (Fig. 6) and there were no differences between genders.

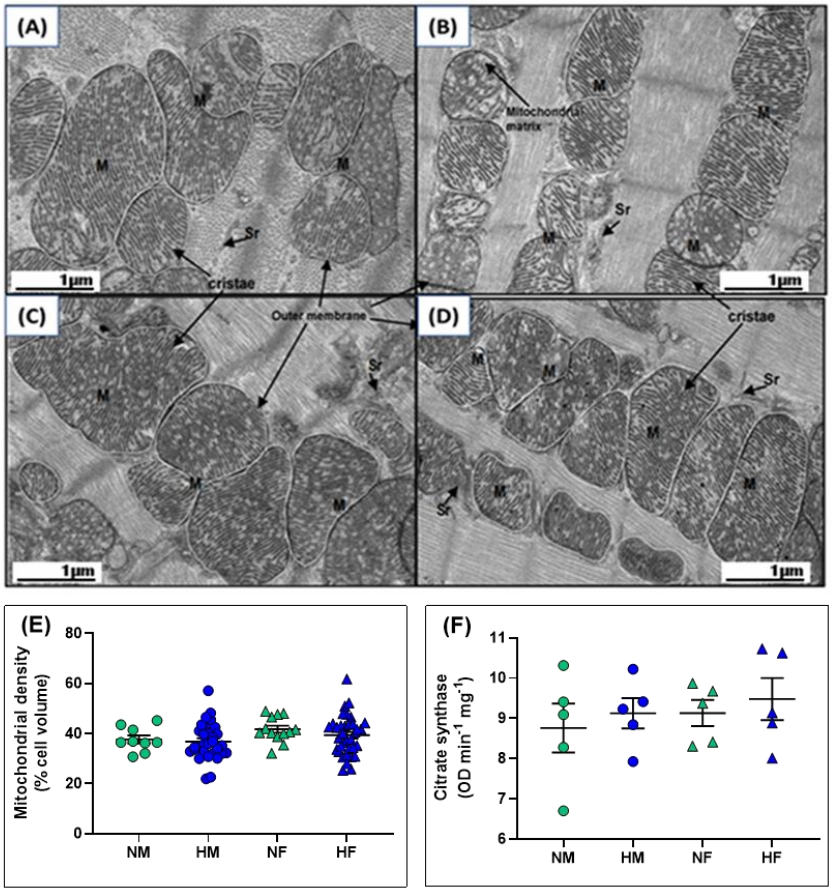


Figure 6: Effects of developmental hypoxia on mitochondrial density and citrate synthase activity. *Transmission electron microscope images of left ventricular mitochondria in male offspring from normoxic (A, N = 2) and hypoxic (B, N = 5) pregnancies, and female offspring from normoxic (C, N = 2) and hypoxic (D, N = 5) pregnancies. Labels in image represent; M: mitochondria; Sr: sarcoplasmic reticulum; Scale bar: 1 uM. Panel E-F show mean data ± SEM for mitochondrial density (n = 5 images per mouse) and citrate synthase activity (n = 5 offspring per group), respectively. For mitochondrial density, linear mixed modeling was performed to account for the nested (clustered) design of the experiment e.g. multiple observations from the same animal. For citrate synthase activity, a 2-way ANCOVA was performed. No statistical differences were found between any of the experimental groups.*

3.5. Offspring mitochondrial enzymatic activity

Next, we investigated the possibility that differences in mitochondrial oxygen consumption and H_20_2 production were underlined by variable enzymatic activities of complexes in the electron transport chain. We found that male offspring from hypoxic pregnancies had higher CI enzymatic activity (Fig. 7A), compared to their normoxic counterparts, and females from hypoxic pregnancies had lower CII activity (Fig. 7B) and higher CIV activity (Fig. 7C), compared to their normoxic counterparts.

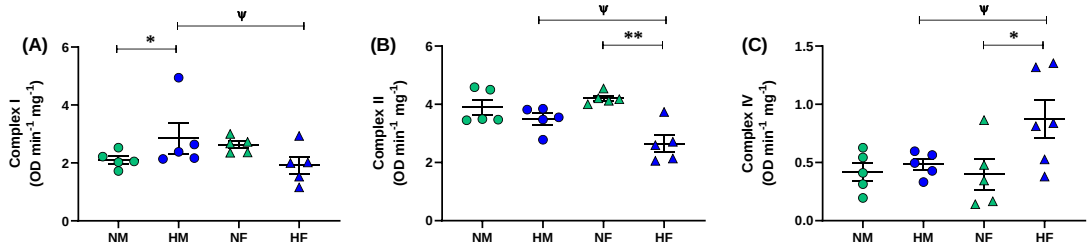


Figure 7: Effects of developmental hypoxia on the activity of key mitochondrial enzymes. *Enzymatic activity of complex I (A), complex II (B) and complex IV (C) were measured in males from normoxic (green circles, n = 5) and hypoxic (blue circles, n = 5) pregnancies, and females from normoxic (green triangles, n = 5) and hypoxic (blue triangles, n = 6) pregnancies. Data are presented as mean ± SEM. Asterix signifies a statistically significant difference between mice from normoxic and hypoxic pregnancies (Two-way ANCOVA, one symbol = p < 0.05, two symbols = p<0.01).*

With regards to gender-specific effects, there were no differences in enzymatic activity between males and females in offspring from normoxic pregnancies, but females from hypoxic pregnancies had lower CI and CII activity, and higher CIV activity, compared to their male counterparts (Fig. 7A-C).

3.6. Offspring mitochondrial complex I-V protein expression

Lastly, we investigated the possibility that differences we observed in mitochondrial function could be explained by differential protein expression of electron transport chain complexes. CI, CII and CIV protein expression were lower in males from hypoxic pregnancies, compared to their normoxic counterparts (Fig. 8A, B and D), but there were no differences in the female group. Within the normoxic group, males had higher CI, CII and CIV protein expression compared to females (Fig. 8A, B and D), but there were no sex-specific effects within the hypoxic group.

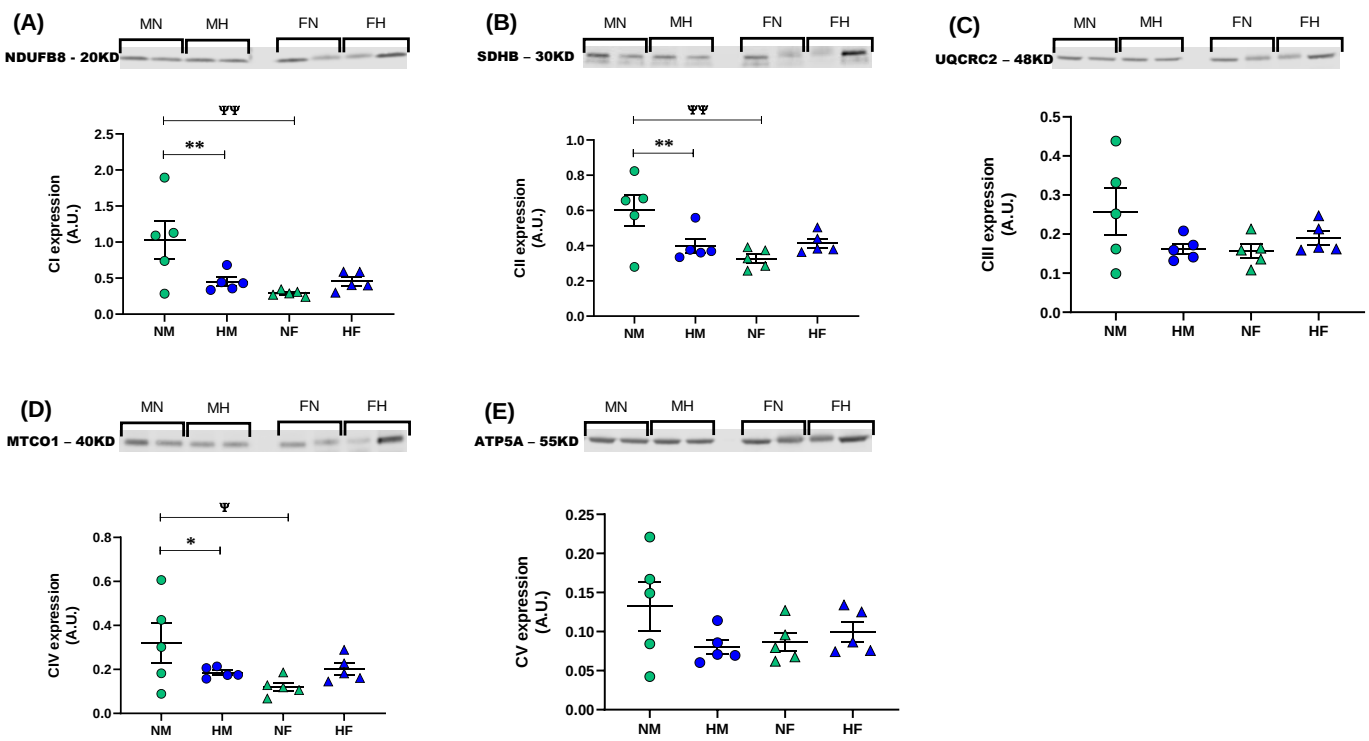


Figure 8: Effect of developmental hypoxia on protein expression of respiratory chain Complexes. *Protein expression was measured with Western Blot in males from normoxic (green circles, n = 5) and hypoxic (blue circles, n = 5) pregnancies, and females from normoxic (green triangles, n = 5) and hypoxic (blue triangles, n = 6) pregnancies. Each panel represents a different complex in the respiratory chain; complex I (A), II (B), III (C), IV (D) and V (E). Data are mean ± SEM. Asterix denotes a difference between mice from normoxic and hypoxic pregnancies, and Ψ denotes a statistically significant difference between male and female mice (Two-way ANCOVA, one symbol = $p < 0.05$, two symbols = $p<0.01$). The full Blot is given in supplementary Figure S1.*

4.0. Discussion

This is the first comprehensive study to address the effects of developmental hypoxia on mitochondrial function in adult offspring. We show that male adult offspring from hypoxic pregnancies possess mitochondria with a reduced respiratory capacity, increased H_20_2 production and a lower protein expression of CI, CII and CIV. In contrast, females from hypoxic pregnancies had a higher respiratory capacity, greater CIV enzymatic activity, and reduced H_20_2 production, despite lower enzymatic activity of CII. From these results, we speculate that early exposure to hypoxia has long term sex-dependent effects on metabolic function, which may have implications for susceptibility to cardiac disease in adulthood.

4.2. Effects of developmental hypoxia on male offspring mitochondrial function

The first major and novel finding from this study is that male offspring from hypoxic pregnancies had greater levels of basal H_20_2 production, compared to their normoxic counterparts. Previous studies have shown developmental hypoxia can cause oxidative stress in fetal [22] and perinatal [40] mammals, but to our knowledge, this is the first study to show developmental hypoxia can programme ROS levels in adulthood. It is well-known that the over-production of mitochondrial ROS plays a major role in cardiac pathologies, particularly I/R injury [41]. Interestingly, several studies have shown developmental hypoxia programmes cardiac sensitivity to I/R injury in adult male offspring [7,11,12], partly due to the downregulation of protein kinase C epsilon (PKCε) via DNA methylation [13]. In addition to differential expression of PKCε, it is tempting to speculate that elevated basal H_20_2 production sets a functional deficit in the hearts of males from hypoxic pregnancies, which may predispose them to postischemic oxidative stress.

623 Our data provides some information on the possible mechanism underlying the elevated
624 basal H_20_2 production in males from hypoxic pregnancies. H_20_2 production in hypoxic
625 males was higher than their normoxic counterparts in all respiratory states (although it
626 only reached statistical significance in the $LEAK_{CI}$ state), and the difference was also
627 apparent in the presence of rotenone and antimycin A, which block the CI_Q and $CIII_{Qi}$
628 ROS sites, respectively [42]. In the presence of these inhibitors, ROS production
629 commonly occurs at the $CIII_{Qo}$ site, which is a major source of oxidative stress in the
630 postischemic heart [43]. Nevertheless, pharmacological inhibition of the $CIII_{Qo}$ site (e.g.
631 with myxothiazol or stigmatellin) would be necessary to confirm its involvement because
632 other sites proximal to CIII can contribute to ROS production under these conditions [42].
633 Furthermore, other factors may explain our results, including differential antioxidant
634 profiles. Clearly, further research is necessary to confirm the site or source of elevated
635 H_20_2 production in males from hypoxic pregnancies.
636
637 In addition to H_20_2 production, developmental hypoxia significantly reduced mitochondrial
638 oxygen consumption in $LEAK_{CI}$ and $OXPHOS_{CI}$ states, as well as flux through CIV alone.
639 The reduction in mitochondrial respiration was associated with a decrease in CI, CII and
640 CIV protein expression, suggesting that these complexes are downregulated by
641 developmental hypoxia, leading to lower respiratory capacity. These results are in
642 agreement with data from Thompson's group [19,20] that showed developmental hypoxia
643 reduces mitochondrial respiration rate and enzymatic activities of CI and CIV in adult
644 male guinea pigs from hypoxic pregnancies. A reduction in mitochondrial respiratory
645 capacity compromises ATP production, especially under situations of increased
646 metabolic demand, such as exercise or disease. Therefore, in addition to elevated basal

H_20_2 production, limitations in mitochondrial electron transport may contribute to cardiac dysfunction and sensitivity to I/R in males from hypoxic pregnancies.

The mitochondrial phenotype in males from hypoxic pregnancies may be explained by several factors. It is possible that developmental hypoxia directly altered fetal mitochondrial function and the phenotype persisted into adulthood. Indeed, several studies have shown intrauterine stress alters embryonic and fetal mitochondria, leading to the overproduction of ROS and a self-reinforcing cycle of mitochondrial dysfunction that persists into adulthood, and may even be inherited [44,45]. In support of this idea, a previous study found evidence of oxidative stress in male fetal guinea pig hearts exposed to 10.5% oxygen, and this was associated with a reduction in the enzymatic activity of CIV [22]. Alternatively, developmental hypoxia may have altered fetal mitochondria indirectly due to its effects on cardiac structure. The foetus initially responds to hypoxia by preferentially distributing blood flow to the heart and brain, which eventually leads to increased peripheral resistance and cardiac remodeling [4-6]. This remodeling is apparent at the cellular level [46-48], which may require mitochondrial alterations. Lastly, mitochondrial programming by intrauterine stress could also be achieved via stable epigenetic alterations to the nuclear genome [49]. Indeed, recent studies have shown developmental hypoxia alters global DNA methylation patterns in rats leading to a reprogramming of the cardiac transcriptome, with a major focus on mitochondrial genes [50]. Therefore, the observed differences in male mitochondrial protein expression in the present study may have epigenetic origins. Integrative longitudinal studies that monitor the effects of developmental hypoxia on mitochondrial function across the life course would help to discern between these possibilities.

4.2. Effects of developmental hypoxia on female offspring mitochondrial function

673 The second major and novel finding from this study is that adult female offspring from
674 hypoxic pregnancies possess mitochondria with a higher respiratory capacity, increased
675 mitochondrial efficiency and lower levels of basal H_20_2 production. These results
676 suggest developmental hypoxia programmes a mitochondrial phenotype in females that
677 can generate ATP at a higher capacity and efficiency, while limiting ROS production. To
678 our knowledge, this is the first study which has shown developmental hypoxia can
679 programme a seemingly advantageous metabolic phenotype. From this perspective, it is
680 interesting to note that several studies have shown female hearts are less affected by
681 developmental hypoxia than males [20,51,52], and they also recover better from I/R injury
682 [7,11,12]. In theory, a greater mitochondrial respiratory capacity and lower H_20_2 production
683 could help to sustain ATP production and limit oxidative stress during oxygen and/or
684 nutrient deprivation. Therefore, we propose that mitochondrial adaptations programmed
685 by developmental hypoxia may protect the female heart from subsequent I/R stress in
686 adulthood.
687
688 The increased respiratory capacity in females from hypoxic pregnancies was present
689 under all respiratory states (except for Leak respiration), and with substrate
690 combinations for CI and CII, as well as direct electron transfer to CIV with TMPD.
691 Therefore, one mechanistic explanation is that developmental hypoxia increased
692 mitochondrial density in female mice. However, analysis of cardiomyocyte EM images
693 found no significant differences in mitochondrial content between females from normoxic
694 or hypoxic pregnancies. While we acknowledge that our EM analysis has limitations and
695 a more robust approach should be taken to confirm these morphological findings (i.e.
696 using stereology or 3D reconstruction), we also found no differences in female citrate
697 synthase activity, which is an excellent marker of mitochondrial content[53]. Taken
698 together, it seems unlikely that the differences in respiratory capacity between females

699 from normoxic and hypoxic pregnancies is related to mitochondrial content. An
700 alternative explanation is that developmental hypoxia increases CIV activity in female
701 mice. In support of this explanation, enzymatic activity of CIV was elevated in females
702 from hypoxic pregnancies, compared to their normoxic counterparts, and this was
703 associated with a trend towards higher CIV protein expression. In addition to increasing
704 respiratory capacity, enhanced complex IV activity is also known to increase
705 mitochondrial oxygen affinity which is beneficial for maintaining ATP production under
706 conditions of low oxygen availability, such as I/R injury [54,55]. Therefore, it would be
707 interesting to investigate the effects of developmental hypoxia on female mitochondrial
708 oxygen kinetics.
709
710 With regard to the mechanism underlying the reduced basal H_20_2 production, enzymatic
711 activity and protein expression of the main ROS producing complexes (CI and CIII) were
712 not altered by developmental hypoxia in female mice. However, theoretically, the
713 increase in CIV activity in females from hypoxic pregnancies could decrease the
714 reduction of redox centers in CI or CIII, thereby reducing electron leak and ROS
715 generation from these complexes [56]. To explore this possibility, we performed
716 experiments in control adult mice where we partially inhibited CIV with sodium azide and
717 simultaneously measured H_20_2 production. We found that a dose-dependent inhibition of
718 CIV caused a stepwise increase in H_20_2 production. This relationship has been
719 demonstrated previously in isolated cardiomyocytes from embryonic chick
720 cardiomyocytes where reduced CIV leads to enhanced ROS production [57]. Therefore,
721 greater CIV activity in females from hypoxic pregnancies may contribute to the observed
722 reduction in H_20_2 production in this experimental group. Nevertheless, there are other
723 mechanistic explanations to explore, including differences in antioxidant capacity.
724

The mechanisms underlying mitochondrial programming in female mice are likely to be similar to males (discussed above), but the resultant phenotype is obviously very different. These results are strongly aligned to numerous studies that have demonstrated sex-dependent differences in the susceptibility of offspring to fetal stress (reviewed in[45,58,59]). Identifying the underlying cause of these differences is an active area of research, but recent work suggests several fundamental differences between males and females may influence programming susceptibility, including; pattern of development (genetic, transcriptional and morphological), growth rate, sex hormones, placental plasticity, the regulation of epigenetic processes, metabolic hormones, the rate of ageing and lifespan[45,58]. With regard to the cardiovascular system, several studies have shown oestrogen plays a protective role in the programming of hypertension, while testosterone is detrimental[59]. Given that oestrogen is also known to modulate cardiac mitochondrial biogenesis, oxidative phosphorylation and ROS production[60], this hormone may have played a role in orchestrating the gender-specific responses in the present study. In this respect, it would be interesting to repeat the experiment in ovariectomized and castrated mice. There is also strong evidence that sex-dependent differences in placental plasticity play a major role in cardiovascular programming [61]. Indeed, numerous studies have shown developmental hypoxia leads to placental remodeling in rodents [62-65], with female placentas adapting much better than males [66]. Given the essential role that the placenta plays in the provision of fetal nutrients, sex-dependent placental remodeling may have important consequences for cardiac function and metabolism. Lastly, recent work has shown that epigenetic and transcriptomic signatures associated with developmental hypoxia are sex-dependent [67], and predominantly mitochondrial [50]. Therefore, the gender-specific mitochondrial protein expression that we observed may represent persistent epigenetic marks.

4.3. Effects of gender on mitochondrial structure and function in offspring from normoxic pregnancies

Previous work has demonstrated substantial differences between males and females in mitochondrial structure and function, but the results are highly tissue-specific. In most tissues, including liver, brain, adipose and skeletal muscle, females exhibit a higher respiratory capacity than males, which is often associated with greater mitochondrial content [68]. In contrast, two studies found no differences in cardiac mitochondrial respiratory capacity between male and female rats, despite a reduced mitochondrial content in females [69,70]. Furthermore, a third study found mitochondrial respiration with glutamate and malate was higher in males compared to females [71]. In line with this latter study, we found male normoxic mice had a greater respiratory capacity than their female counterparts, and this difference was present in all respiratory states and substrate combinations, as well as CIV flux with TMPD. We go further to show the increase in male respiratory capacity was associated with higher male CI, CII and CIV protein expression, providing a mechanistic explanation for these sex-dependent differences.

In addition to respiratory capacity, previous work has shown sex-dependent differences in cardiac ROS production. In general, female cardiomyocytes generate less ROS than males at rest and after pathological stimuli, and they are also less prone to age-dependent oxidative stress [69,72,73]. Some studies suggest the reduced ROS production in females is due to a superior antioxidant capacity [73], while others point towards reduced electron leak from CI and CIII [69]. In contrast to these studies, normoxic males in our study had lower basal H_20_2 production than their female counterparts in all respiratory states and substrate combinations. We cannot explain the discrepancy between these studies, but factors such as species, strain and age may play a role in determining sex-dependent differences.

5.0. Conclusions and future directions

In conclusion, our study has shown developmental hypoxia has long-term, sex-specific implications for metabolism. We speculate that these differences may have implications for disease susceptibility. For example, increased basal H_20_2 production and lower respiratory capacity in males from hypoxic pregnancies may predispose mitochondria to cardiac dysfunction and I/R injury, a condition that is largely driven by oxidative stress [41]. In contrast, the greater respiratory capacity and lower H_20_2 production in females from hypoxic pregnancies may help to sustain ATP production and limit oxidative stress under conditions of oxygen deprivation. These sex-specific differences in response to developmental hypoxia may help to explain why females from hypoxic pregnancies are less susceptible to I/R injury, compared to their male counterparts [11,12]. While purely speculative at this stage, the present study provides the foundation to test this hypothesis and design future gender-specific metabolic therapies to prevent cardiac dysfunction in offspring from hypoxic pregnancies.

6.0 Acknowledgements

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Supplementary Tables and Figures

Table S1: Conditions for spectrophotometric assays of respiratory chain enzymes and citrate synthase activities in ventricular homogenates

	CI	CII	CIV	CS
λ (nm)	600	600	550	412
Buffer	KP, 50mM	KP, 25 mM	KP, 50mM	TRIS, 100mM
pH	7.50	7.50	7.5	8.0
Substrates/electron acceptors	NADH 100 µM; DCPIP 80 µM; Ub-1 60 µM	Succinate 20 mM; DCPIP 80 µM; DUB 50 µM	Cyt c H_2; 60 µM	DTNB 100 µM; Ac CoA 300 µM; Oxaloacetate 0.5mM
Other reagent(s)	BSA 3 mg ml^{-1}; KCN 300 µM	BSA 1 mg ml^{-1}; KCN 300 µM	KCN 300 µM	Triton X-100 0.1%;
Specific inhibitor	Rotenone 10 µM	Malonate 10 mM	KCN 300 µM	-

Abbreviations: CI, complex I; CII, complex *II; CIV,* complex *IV; CS, citrate synthase; λ, selected wavelength for the assay; ε, extinction coefficient; Ac CoA, acetyl coenzyme A; BSA, fatty acid–free bovine serum albumin; Cyt c H_2, reduced cytochrome c; DCPIP, 2,6-dichlorophenolindophenol; DUB, decylubiquinol; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); KCN, potassium cyanide; KP, potassium phosphate buffer; Tris, Tris buffer; Ub1, biquinone1*

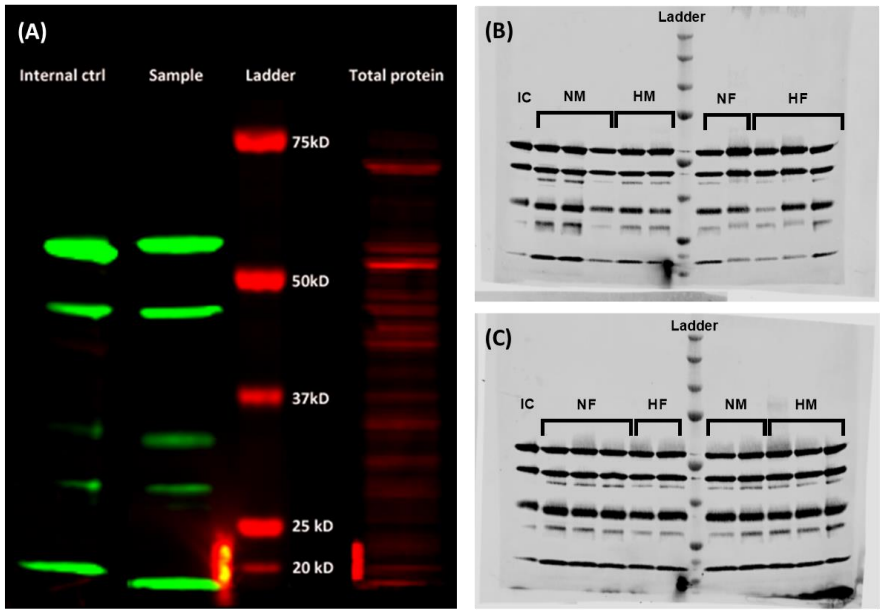

Figure S1: Representative images of Western Blots for one replicate. (A) Each gel was loaded and imaged at two wavelengths; the samples and an internal control (historical left ventricular mouse sample that was used for all blots) were loaded with the antibody and imaged at 800nm (green bands: top to bottom; complex V, complex III, complex IV, complex II, and complex I). In addition, a ladder and the total protein stain, REVERT, was imaged on the same Blot at 700nm (red bands). (B-C) Original Blots for one replicate showing sample protein expression of Complexes V, III, IV, II and I (top to bottom) in an internal control (IC), normoxic female (NF, n = 5), hypoxic female (HF, n = 5), normoxic male (NM, n = 5) and hypoxic male (HM, n = 5). Averages for three replicates are given in Figure 8 of the main manuscript.

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