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GLUCOCORTICOID MATURATION OF MITOCHONDRIAL RESPIRATORY CAPACITY IN SKELETAL MUSCLE BEFORE BIRTH

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ABSTRACT

In adults, glucocorticoids act to match the supply and demand for energy during physiological challenges, partly through actions on tissue mitochondrial oxidative phosphorylation (OXPHOS) capacity. However, little is known about the role of the natural prepartum rise in fetal glucocorticoid concentrations in preparing tissues for the increased postnatal energy demands. This study examined the effect of manipulating cortisol concentrations in fetal sheep during late gestation on mitochondrial OXPHOS capacity of two skeletal muscles with different postnatal locomotive functions. Mitochondrial content, biogenesis markers, respiratory rates and expression of proteins and genes involved in the electron transfer system (ETS) and OXPHOS efficiency were measured in the *biceps femoris* (BF) and *superficial digital flexor* (SDF) of fetuses either infused with cortisol before the prepartum rise or adrenalectomised to prevent this increment. Cortisol infusion increased mitochondrial content, biogenesis markers, substrate-specific respiration rates and abundance of ETS Complex I and adenine nucleotide translocator (ANT1) in a muscle-specific manner that was more pronounced in the SDF than BF. Adrenalectomy reduced mitochondrial content and expression of *PGC1α* and *ANT1* in both muscles, and ETS Complex IV abundance in the SDF near term. Uncoupling protein gene expression was unaffected by cortisol manipulations in both muscles. Gene expression of the myosin heavy chain isoform, *MHCIIx,* was increased by cortisol infusion and reduced by adrenalectomy in the BF alone. These findings show that cortisol has a muscle-specific role in prepartum maturation of mitochondrial OXPHOS capacity with important implications for the health of neonates born pre-term or after intrauterine glucocorticoid overexposure.

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INTRODUCTION

In adults, glucocorticoids are stress hormones with metabolic actions on a wide range of tissues that maintain functions critical to survival in adverse environmental conditions and during normal physiological challenges to homeostasis like exercise and pregnancy (Picard *et al.,* 2018, Casuro *et al.* 2020, Bartho *et al.* 2020). Many of these functions require energy in the form of ATP, which is produced mainly by oxidative phosphorylation (OXPHOS) in the mitochondria (Nunnari & Suomalainen 2012, Rodriguez-Caro *et al.,* 2020). Mitochondria, therefore, have a key role in the response to both internal and external environmental cues, and are known to be regulated by glucocorticoids in adulthood (Lee *et al.* 2013, Lapp *et al.* 2019).

Mitochondria are dynamic organelles that respond to changes in energy demand by biogenesis, fusion/fission and by alterations in the abundance of the electron transfer system (ETS) complexes and other proteins regulating ATP production (Goffat & Wiesner 2003, Liang & Ward, 2006, Chandhol *et al.* 2018). Utilising a range of metabolic substrates, ATP is produced by ATP synthase using the proton gradient across the inner mitochondrial membrane generated by redox reactions at ETS complexes with oxygen as the final electron acceptor. The efficiency of mitochondrial OXPHOS also depends on uncoupling proteins (UCPs) that dissipate the proton gradient when activated, and on transporters that shuttle adenine nucleotides across the mitochondrial membranes (Kimura & Rasmussen 1977, Nunnari & Suomalainen 2012). Glucocorticoids have been shown to influence many of these regulatory processes in mitochondria of several adult tissues, including skeletal muscle (Djouadi *et al.* 1994, Rachamim *et al.* 1995, Weber *et al.,* 2002, Du *et al.* 2009).

Glucocorticoids can also act as stress signals in the fetus but, during normal conditions in late gestation, their primary role is as a signal of impending delivery (Reynolds 2013, Fowden & Forhead 2015). In most mammals studied to date, fetal glucocorticoid concentrations rise naturally towards term and switch fetal tissues from growth to differentiation in preparation for birth (Fowden *et al.* 1998). This prepartum glucocorticoid surge also activates many processes that have little or no function *in utero* but which are essential for neonatal survival such as breathing, thermogenesis, glucogenesis and locomotion (Fowden *et al.,* 2016). All these new functions require extra energy but relatively little is known about the effects of glucocorticoids on mitochondrial function in fetal tissues during late gestation, particularly in species that are mobile from birth.

In several species, mitochondrial function is known to rise between fetal and neonatal life in several different tissues (Prieur *et al.* 1998, Lehman *et al.* 2000, Nakai *et al.* 2002, Minai *et al.* 2008, Rog-Zielinska *et al.* 2015, Davies *et al* 2020). Administration of potent synthetic glucocorticoids during rodent pregnancy has also been shown to affect the abundance of mitochondrial proteins in fetal tissues near term (Nakai *et al.* 1998, Prieur *et al.* 1998, Rog-Zielinska *et al.* 2015). In addition, a recent study in fetal sheep has demonstrated that the natural prepartum cortisol surge closely parallels the increase in mitochondrial OXPHOS capacity of skeletal muscle towards term (Davies *et al.* 2020). However, whether these changes are the direct consequence of the fetal cortisol increment remains unknown. This study, therefore, examined the hypothesis that cortisol causes maturation of mitochondrial OXPHOS capacity in skeletal muscle towards term.

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METHODS

Animals

A total of 24 time-mated pregnant ewes and 6 newborn twin lambs were used in this study. Of the pregnant ewes, twelve carried single fetuses while the remainder were twin-bearing. Pregnant ewes were group housed before surgery and single housed within sight and sound of other sheep after surgery. They had free access to hay and water at all times except for 12-18h before surgery when food was withheld. All animal procedures were carried out under the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body.

Surgical procedures

Between 114-119 days of gestational age (dGA), surgery was carried out on 6 twin-bearing and 12 single-bearing ewes under isofluorane anaesthesia (1.5-2% in 5:1 O_2:N_2O mixture) with positive pressure ventilation. In twin-bearing ewes, one fetus was adrenalectomised (AX) and its twin was sham-operated as a control (Barnes *et al.*, 1978). In the single-bearing ewes, catheters were inserted into the maternal dorsal aorta and the fetal dorsal aorta and caudal vena cava, via the femoral vessels, and exteriorised through the maternal flank (Fowden & Silver, 1995). The ewes were monitored throughout surgery using a capnograph and pulse oximeter. At surgery, the ewes were given antibiotics (oxytetracycline, 20mg/kg i.m., Allamycin, Norbrook Laboratories, Newry, UK and penicillin, Depocillin, Intervet international, Milton Keynes, UK, 15mg/kg i.m. to mother and intra-amniotically or i.v. to fetus) and analgesia (1mg/kg carprofen, s.c. Rimadyl, Zoetis, London UK). Penicillin treatment to the ewe continued for 2 days post-operatively.

126

127 **Experimental procedures**

128 All catheterised animals were sampled daily to maintain catheter patency and to collect blood
129 samples to measure blood gases and concentrations of metabolites and hormones. Following
130 post-operative recovery for at least 5 days, the catheterised fetuses were assigned randomly
131 to receive a 5-day intravenous infusion of either saline (0.9% NaCl, 3ml/day, n=6, control, 3
132 male M: 3 Female F) or cortisol (2-3mg/kg/day Solu-Cortef; Pharmacia, n=6, 4M:2F). At the
133 end of infusion (128-131dGA), the ewes and fetuses were killed by administration of a lethal
134 dose of anaesthetic (200mg/kg sodium pentobarbitone, iv, Pentoject, Animalcare Ltd, Youk,
135 UK) and tissues collected from the fetus. Similarly, at 141-145dGA, the ewes with AX (4M:2F)
136 and sham-operated fetuses (2M:4F) were euthanized with an overdose of anaesthetic as
137 above and the fetuses delivered in a random order. A blood sample was taken from the
138 umbilical artery of each fetus before administration of a lethal dose of sodium pentobarbitone
139 (200mg/kg) and tissue collection. At delivery, the two female AX fetuses had small adrenal
140 remnants (80mg and 180mg) so neither was used for any subsequent analyses.

141

142 Umbilical arterial blood and skeletal muscle were also collected from twin fetuses of 6
143 unoperated ewes at 102-105dGA as described above. Tissue from only one fetus of each pair
144 (2M:4F) was randomly selected for further study. In addition at 1-2 days of postnatal age,
145 one lamb from 6 unoperated pairs of twins (3M:3F) was euthanized for tissue collection using
146 sodium pentobarbitone (200mg/kg) after collection of a blood sample from the jugular vein.
147 All blood samples were collected into heparin-coated tubes and, after centrifugation, the
148 plasma was stored at -20°C for future hormone analysis. Immediately following euthanasia,
149 the fetal and newborn lambs were weighed and measured.

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Two hindlimb skeletal muscles with different postnatal functions in locomotion, the *biceps femoris* (BF) and *superficial digital flexor* (SDF), were immediately collected and weighed. The BF is a large powerful, multifunctional muscle producing mechanical power by shortening while the SDF is a small flexor muscle generating force predominately by isometric contraction (Fourie, 1962; Biewener, 1998). The BF controls locomotive gait through extension and abduction of the hindlimb whereas the SDF controls foot placement important for allowing the neonate to stand (Fourie 1962, Walker & Luff 1995). Both muscles are of mixed fibre type with a combination of slow-twitch Type I and fast-twitch Type II fibres (Davies, 2018). In late gestation, the SDF has proportionally more Type I fibres than the BF, although both muscles still contain undifferentiated fibres at birth (Davies 2018, Davies *et al*. 2020).

Samples of these muscles were snap-frozen in liquid nitrogen before being stored at -80°C until required. Additionally, in the fetuses at 129dGA and 144dGA, a small sample (≈100-200mg) from the centre of each muscle was collected into ice cold biopsy preservation solution (BIOPS; pH 7.1 solution containing 2.77mM CaK_2EGTA, 7.23mM K_2EGTA, 20mM imidazole, 20 mM taurine, 50 mM MES, 0.5 mM dithiothreitol, 6.56 mM $MgCl_2.H_2O$, 5.77mM Na_2ATP and 15 mM phosphocreatine; Pesta & Gnaiger 2012) before dissection for respirometry.

Respirometry

Respirometry measurements were made on the skeletal muscle samples from the AX, sham operated, cortisol- and saline-infused groups of fetuses using the protocol described

previously for this tissue (Pesta and Gnaiger, 2012, Kuznetsov *et al.* 2008). Briefly, 2-3mg pieces of tissue were dissected in BIOPS, bundles of 6-8 fibres were teased apart before incubating with saponin for 20 minutes to permeabilise the plasma membrane (100µg saponin/ml BIOPS). Samples were transferred into an isotonic respiration medium maintained at 37°C (MiR05; pH7.1 solution containing 20mM HEPES, 0.5mM EGTA, 3 mM $MgCl_2.6H_2O$, , 10 mM KH_2PO_4, 20mM taurine, 110mM sucrose, 60mM K-lactobionate and 1g/l BSA; Pesta & Gnaiger 2012, Gnaiger *et al.* 2000) in order to measure oxygen (O_2) consumption using Clark-type oxygen electrodes (Strathkelvin Instruments, Glasgow, UK). Substrates were added into the chambers at saturating concentrations according to three protocols as previously described (Davies *et al.* 2020). Malate (2mM), glutamate (10mM), ADP (10mM) and succinate (10mM) were added in sequence to give a measure of maximal ADP-coupled oxygen consumption when electron entry to both complex I and II of the ETS is saturated. The second protocol involved the addition of malate (2mM), pyruvate (5mM) and ADP (10mM) was used to obtain a measure of oxidative capacity for pyruvate (Py), a derivative of glucose. And thirdly, malate (2mM), palmitoyl-carnitine (PC, 40µM) and ADP (10mM) were added to provide a measure of fatty acid oxidation capacity. In all protocols, leak state was measured in the presence of substrates before the addition of ADP, and the experiment concluded with the addition of cytochrome *c* (10µM) to check outer mitochondrial membrane integrity. Results were excluded if there was a ≥15% increase in O_2 consumption following cytochrome *c* addition. Additionally, data were excluded if the rate of O_2 uptake over the baseline period before substrates were added, exceeded 0.001µmol O_2/min as this suggests insufficient plasma membrane permeabilisation (Kuznetsov *et al.,* 2008). Following respirometry, muscle fibres were extracted from chambers and dried for 48 hours before being weighed, and results are presented as O_2 consumption/mg dry weight.

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199 **Biochemical analyses**

200 ***Hormone assays***

201 Plasma cortisol concentrations were measured using a human ELISA (RE52061, Tecan,
202 Männedorf, Switzerland), previously validated for sheep plasma (Vaughan *et al.* 2016). Intra-
203 and inter-assay coefficients of variation for the cortisol assay were 3% and 5% respectively
204 and the limit of detection was 5.2ng/ml. Because cortisol increases fetal T_3 concentrations
205 towards term and thyroid hormones are known to affect O_2 consumption by fetal tissues
206 (Fowden & Silver, 1995; Forhead *et al.* 2006; Davies *et al.* 2020; 2021), total plasma T_3 and T_4
207 were also measured using radioimmunoassays (Kit numbers, 06B254215 and 06B 254011,
208 respectively, MP Biomedical, Eschwege, Germany), previously validated for sheep plasma
209 (Fowden & Silver, 1995). Intra- and inter-assay variations were less than 2% and 8% for T_3 and
210 3% and 5% for T_4. The limit of detection was 0.14ng/ml for T_3 and 11.3ng/ml for T_4.

211

212 ***Biochemical composition***

213 Water content was calculated as a percentage by weighing, freeze-drying overnight and then
214 re-weighing samples of frozen muscles. Following extraction from homogenised frozen tissue,
215 protein content was measured using a bicinchoninic acid assay and expressed as mg protein
216 per gram tissue (wet weight) or as mg protein per mg dry weight calculated using the
217 percentage water content of the muscle.

218

219 ***Citrate synthase activity***

220 Activity of citrate synthase (CS), an enzyme of the tricarboxylic acid cycle, is a putative marker
221 of muscle mitochondrial content (Larsen *et al.* 2012) and was measured

spectrophotometrically in the skeletal muscles. Ten to thirty micrograms of homogenised protein was added to the assay buffer (pH8) containing 0.1mM 5,5'-Dithio-bis(2-nitrobenzoic acid), DTNB, 1mM oxaloacetate and 0.3mM acetyl-CoA. CS activity was determined as the maximal rate of absorbance change at 412nm over 3 minutes (a measure of the rate of 5-thio-2-nitrobenzoic acid production). CS activity is expressed per mg protein.

Western blotting

Frozen muscle samples (55mg±10%) were homogenised, total protein extracted and diluted to 2.5µg/µl in 8% SDS solution. Protein was electrophoresed on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane and stained with Ponceau-S to allow for normalisation of protein loading. Membranes were incubated either with primary antibodies to ETS complexes I-IV and ATP synthase (OXPHOS antibody cocktail; 458099; Life Technologies; 1:1000), followed by an HRP-linked anti-mouse secondary antibody (NIF82; GE Healthcare; 1:5000) or to ANT1 (Abcam; Cambridge, UK, ab1002032, 1:1000), followed by HRP-linked donkey anti-rabbit IgG (GE healthcare; NA934V, 1:5000). Enhanced chemiluminescence was used to visualise protein bands and quantified using ImageJ (http://rsb.info.nih.gov/ij/).

qRT-PCR

Frozen skeletal muscle samples were powdered and RNA extracted using TRIzol (Thermo Fisher) and chloroform, and the aqueous phase used in the RNeasy Plus kit (Qiagen, Hilden, Germany). RNA concentration was measured using a Nanodrop ND-1000 spectrophotometer, diluted to 50ng/µl and used for cDNA synthesis (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using a MESA BLUE

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Mastermix (Eurogentec, Liège, Belgium) following the manufacturers recommended protocol (5 minutes at 95°C followed by 40 amplification cycles of 15 seconds at 95°C and 1 minute at 60°C). The genes assayed, their encoded protein and function together with the primer sequences used are given in Table 1. Results were analysed using $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak, 2008) and expressed relative to the geometric mean of S15 and 18S housekeeper genes and set relative to one experimental sample in the relevant control group. All samples were run in triplicate and housekeeper gene expression did not differ significantly between groups.

Statistical analyses

Data are presented as mean ± standard error of the mean (SEM) and GraphPad Prism Version 6.05 (www.graphpad.com) was used for analyses. A one-way ANOVA was used to assess the developmental changes in CS and plasma cortisol concentration data followed by a Tukey's multiple comparison *post hoc* test. A *t*-test or non-parametric Mann-Whitney test, as appropriate, was used to compare the data between sham-operated and AX and between cortisol- and saline-infused fetuses. Where appropriate, a t-test of the significant of a single mean was used to assess the mean difference between the AX and sham-operated twin pairs. Pearson's correlation coefficient was used to assess correlations between variables and log-transformed hormone concentrations. Partial correlation analysis was applied to determine the relationship between two variables controlling for a third. $P \leq 0.05$ was considered significant throughout.

RESULTS

Hormone concentrations, morphometry and body composition

In line with previous findings (Barnes *et al.* 1978, Fowden *et al.* 1998), cortisol concentrations increased in control animals towards term and on into the immediate neonatal period (Figure 1A). Relative to saline-infused fetuses at 129dGA, cortisol infusion significantly increased the cortisol concentration to values similar to those seen in the older sham-operated controls at 144dGA (Figure 1A). In contrast, AX prevented the normal prepartum rise in fetal cortisol concentrations; the mean value in AX fetuses was significantly lower than the concentrations in sham-operated controls at 144dGA and similar to control values at the earlier gestational ages (Figure 1A). Fetal plasma T_3 concentrations were significantly higher in cortisol- than saline-infused fetuses but were not significantly affected by AX, although values had a tendency to be lower in the AX than sham-operated fetuses (P=0.057, Table 2). There were no changes in fetal plasma T_4 concentrations with cortisol infusion or AX (Table 2).

Neither cortisol-infusion nor AX had a significant effect on fetal morphometric measurements or muscle weights compared with their respective controls (Table 2). Water content was significantly lower in the BF of cortisol- than saline-infused fetuses at 129dGA and significantly higher in both muscles of AX compared to sham-operated fetuses at 144dGA (Table 2). Cortisol infusion had no effect on protein content of either muscle, whereas AX reduced the protein content of the BF alone when expressed per gram wet weight but not per gram dry weight (Table 2). Cortisol infusion has no effect on the fetal blood gas status or concentrations of glucose and lactate during the infusion period before tissue collection (data not shown).

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Muscle mitochondrial content

In control fetuses, CS activity increased towards term with a further increase after birth in both muscles (Figure 1B & C), consistent with previous findings in the BF (Davies *et al.* 2020). Cortisol infusion had no effect on CS activity in the BF but significantly increased activity in the SDF relative to saline-infused control values (Figure 1B & C). In contrast, CS activity was significantly less in AX than sham-operated fetuses near term in the BF but not in the SDF when comparing group means (Figure 1B & C). However, a paired comparison between the AX fetus and its sham-operated twin showed CS activity was significantly less in the AX twin than its sibling for both the BF (-0.090 ± 0.006µmol/min/mg protein, n=4, $P<0.01$) and SDF (-0.040 ± 0.008µmol/min/mg protein, n=4, $P<0.05$, t-test for significance of single mean, both muscles).

When data from all the groups were combined irrespective of age or treatment, there were significant positive correlations between CS activity and the concentrations of both cortisol and T_3 in each muscle (Table 3). As the cortisol and T_3 concentrations were also correlated ($r=0.720$, n=33, $P<0.001$), partial correlation analyses were used to determine the relative importance of the two hormones when the confounding effect of the other was taken into account. This showed that both hormones were significant influences on CS activity with plasma T_3 the more significant factor statistically in both muscles (Table 3).

Muscle mitochondrial biogenesis and membrane dynamics

Consistent with the changes in mitochondrial density, manipulating fetal cortisol concentrations had muscle specific effects on gene expression of *PGC1α* and *MFN2*.

315 Expression of *PGC1α* was significantly higher in the SDF of cortisol- than saline-infused
316 fetuses, but not in the BF, and was reduced significantly by AX in both muscles near term
317 (Figure 2A & B). Expression of *MFN2* in the BF was unaffected by varying cortisol
318 concentrations (Figure 2C). In contrast in the SDF, *MFN2* expression was upregulated by
319 cortisol infusion and down-regulated by AX relative to their respective controls (Figure 2D).
320 In both muscles, varying cortisol concentrations had no significant effect on *DRP1* expression
321 (Figure 2E & F).

322

323 **Muscle oxygen consumption**

324 The ADP-coupled rates of O_2 consumption by the two muscles are shown in Figure 3 for the
325 three different respiratory protocols. In the BF, cortisol infusion had no effect on maximal
326 OXPHOS or PC-supported oxidative capacity but significantly increased Py-supported O_2
327 consumption relative to saline-infused values (Figure 3A-C). In contrast, in the SDF, cortisol
328 infusion significantly increased maximal OXPHOS and PC-supported oxidative capacity
329 together with a tendency for higher rates of Py-supported respiration compared to saline-
330 infused values (P=0.064, Figure 3D-F). In both muscles, AX had no significant effect on
331 respiratory rates using any of the substrates, although there was a tendency for lower BF
332 rates of Py-supported respiration after AX (P=0.093, Figure 3B).

333

334 When the respiratory data were combined for all fetuses irrespective of treatment or
335 gestational age for each substrate separately, there were significant positive correlations
336 between the BF rate of Py-linked respiration and the concentrations of both cortisol and T_3,

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337 although partial correlation showed no significant correlations with either hormone alone
338 (Table 3). There were no significant correlations between the BF respiratory rates with the
339 other substrates and either hormone concentration (Table 3). In the SDF, cortisol
340 concentrations were positively correlated with PC-linked respiration and maximal OXPHOS
341 but not Py-linked respiration while T_3 levels were positively correlated to all three respiratory
342 rates (Table 3). Partial correlation of the SDF data showed no effect of either cortisol or T_3
343 alone on PC-linked respiration but a statistically dominant effect of T_3 on maximal OXPHOS
344 (Table 3).

345

346 In the BF, leak state respiration, a measure of O_2 consumption for processes other than ATP
347 production, was unaffected by manipulating fetal cortisol concentrations, irrespective of
348 substrate (data not shown). In the SDF, leak state respiration with PC was significantly higher
349 in cortisol- (1.30 ± 0.21 nmolO_2/min/mg dry wt, n = 5) than saline-infused fetuses (0.69 ± 0.17
350 nmolO_2/min/mg dry wt, n = 6, $P<0.05$) but not with the other substrates (data not shown).
351 Adrenalectomy had no significant effect on the SDF leak state respiration using any of the
352 substrates (data not shown). There were no significant correlations between any of leak state
353 respiratory rates and the concentrations of either hormone ($P>0.05$, all cases).

354

355 **ETS and other mitochondrial OXPHOS regulatory proteins**

356 In the BF, cortisol infusion significantly increased protein abundance of ETS Complex I but had
357 no effect on any of the other complexes or ATP synthase (Figure 4A). Complexes I-IV and ATP
358 synthase were also unaffected by cortisol infusion in the SDF (Figure 4B). In contrast, AX had
359 no significant effect on protein abundance of Complexes I-IV or ATP synthase in the BF but

reduced Complex IV abundance alone in the SDF relative to sham-operated values (Figure 4C & D). Gene expression for the uncoupling proteins, UCP2 and UCP3, was unaffected by treatment in both muscles (Figure 5A-D). In the SDF, cortisol infusion significantly increased both gene expression and protein abundance of ANT1 whereas, in the BF, it had no significant effect on either ANT1 measure, although there was a tendency for higher protein abundance relative to saline-infused values (P=0.095, respectively, Figure 5E-H). Adrenalectomy reduced gene and protein ANT1 levels significantly in both muscles (Figure 5E-H).

Muscle expression of myosin heavy chain (*MHC*) isoforms

The effects of manipulating the fetal cortisol concentration on fibre composition of the two muscles was assessed by quantifying *MHC* isoform expression for the Type 1 slow-twitch, oxidative fibres with abundant mitochondria (*MHCI*) and the Type II fast-twitch fibres that have fewer mitochondria and are either oxidative/glycolytic, *MHCIIa*, or predominantly glycolytic, *MHCIIx* (Yates *et al.,* 2016). In both muscles, cortisol infusion had no significant effect on expression of the *MHCI* or *MHCIIa* isoforms (Figure 6A-D). In contrast, *MHCIIx* expression in the BF was increased by cortisol infusion and decreased by AX relative to their respective controls (Figure 6E). No change in *MHCIIx* expression was seen in the SDF with either treatment (Figure 6F).

DISCUSSION

The results show that variations in fetal cortisol concentrations within the physiological range affect mitochondrial OXPHOS capacity in ovine skeletal muscles near term. The effects were

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muscle-specific and were associated with changes in mitochondrial content, biogenesis markers and abundance of specific ETS complexes and ANT1. They were accompanied by substrate-specific alterations in respiratory function. In addition, there were muscle-specific changes in *MHC* isoform expression in response to altering fetal cortisol concentrations. The cortisol-dependent changes in mitochondrial function are summarised in Table 4 for the two muscles. Collectively, they indicate that the normal prepartum rise in fetal cortisol concentrations has a key role in maturing mitochondrial capacity in preparation for the increased energy demands of skeletal muscle postnatally.

In the current study, mitochondrial content was reduced in both muscles when the normal prepartum cortisol surge was prevented by fetal AX. In rats, suppressing fetal corticosterone concentrations close to term by maternal AX and metopirone treatment reduces mitochondrial content of the fetal kidney but not the liver or heart (Prieur *et al.* 1998). Short-term maternal administration of a potent synthetic glucocorticoid, dexamethasone, near term restored the normal renal mitochondrial density in these glucocorticoid-deficient rat pups and also increased the volume density of mitochondria in Type II pneumocytes of normal fetal rabbits (Snyder *et al.* 1997, Prieur *et al.* 1998). In the current study, raising cortisol level to prepartum values by cortisol infusion before the normal surge increased mitochondrial content, specifically in the SDF. In a recent study, longer-term treatment of pregnant ewes with cortisol for the last 25 days of pregnancy reduced mitochondrial DNA content of the fetal BF and heart at term (Joseph *et al.* 2020). Similarly, maternal corticosterone treatment of rats at mid-pregnancy decreased placental mitochondrial density (Bartho *et al.* 2019). In the current study, muscle mitochondrial content increased between 104dGA and 129dGA in the

absence of any cortisol increment. This coincides with a major period of muscle fibre differentiation and suggests that factors other than circulating cortisol, such as growth factors and receptor abundances, may be involved in mitochondrial development earlier in gestation (Florini *et al.* 1991, Walker & Luff 1995, Bloise *et al.* 2018). Collectively, these findings suggest that glucocorticoids are required for normal mitochondrial biogenesis near term in specific fetal tissues but that, earlier in gestation, their actions may depend not only on the tissue and its stage of development but also on the duration, timing, route and type of glucocorticoid exposure.

The changes in muscle mitochondrial density seen in response to varying fetal cortisol levels in the current study tracked closely with expression of the key regulator of mitochondrial biogenesis, *PGC1α* (Table 4). Alterations in *PGC1α* expression were more pronounced in the SDF than BF and were accompanied by parallel changes in SDF expression of *MFN2*, a gene essential for normal membrane dynamics and OXPHOS function that is regulated by *PGC1α* (Liang & Ward 2006). Previous studies on rodents have shown that *PGC1α* expression is glucocorticoid sensitive and increases towards term in fetal heart and adipose tissue (Rog-Zielinska *et al.* 2015, Chen *et al.* 2020). Deletion of *PGC1α* expression in fetal mice also impairs mitochondrial OXPHOS function, and the metabolic response to glucocorticoids in developing cardiomyocytes (Rog-Zielinska *et al.* 2015). Conversely, over-expression of *PGC1α* promotes mitochondrial biogenesis and O_2 consumption in neonatal cardiomyocytes *in vitro* (Lehman *et al.* 2000). However, no prepartum upregulation of *PGC1α* expression was seen in fetal ovine BF, despite a concomitant increase in mitochondrial density towards term (Davies *et al.* 2020).

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Previous rodent studies have shown increases in mitochondrial respiration and/or expression of Complex IV and ATP synthase in heart, liver and brain of fetal and neonatal pups in response to dexamethasone treatment (Prier *et al.* 1998, Lehman *et al.* 2000, Nakai *et al.* 2002, Rog-Zielinska *et al.* 2015). In the present study, raising cortisol levels within the physiological range increased mitochondrial OXPHOS capacity in both fetal skeletal muscles but in a substrate-specific manner. In the BF, cortisol stimulated respiration with pyruvate by about 30% but not with the other substrates. This occurred without any significant change in mitochondrial content but was accompanied by a similar percentage increase in Complex I abundance, consistent with pyruvate being an electron donor to this complex via NADH (Kuznetsov *et al.* 2008). There was, however, no accompanying increase in maximal OXPHOS capacity, supported by saturating concentrations of substrates for Complex I and Complex II, which may be due to limitations at the Q-junction for electron entry to Complex III, which did not increase in abundance. Cortisol-induced upregulation of Py-linked respiration in the BF was also accompanied by greater *MCHIIx* expression consistent with the increased BF abundance of *MCHIIx* glycolytic fibres seen previously towards term (Davies *et al.* 2020). Collectively, the current findings in the BF may suggest that the mitochondrial content of its oxidative fibres increases in response to cortisol infusion. In contrast, in the SDF, cortisol infusion resulted in significant rises in PC-linked and maximal OXPHOS capacity together with a tendency for higher rates of Py-supported respiration (Table 4). These respiratory changes occurred without alteration in *MHC* expression but concomitantly with increased mitochondrial biogenesis and content. However, preventing the prepartum fetal cortisol surge by AX had no significant effect on mitochondrial respiration in either muscle irrespective of substrate, despite decreased expression of *MHCIIX* in the BF and lower

452 mitochondrial density and *PGC1α* expression in both muscles. Thus, cortisol appears to act
453 on mitochondrial OXPHOS *in utero* through different muscle-specific mechanisms, which may
454 also depend on gestational age.

455

456 The discrepancy between the effects of cortisol on respiratory rates at 129dGA and 144dGA
457 may reflect, in part, differences in the duration of cortisol exposure between the single
458 infused and twin sham-operated fetuses as activation of the fetal hypothalamic-pituitary-
459 adrenal axis and the rise in fetal cortisol concentrations occurs more rapidly and closer to
460 term in twin than single sheep fetuses (Edwards & McMillen 2002). Since cortisol activates
461 the deiodinases converting T_4 to T_3 (Forhead *et al.* 2006), the current findings that fetal T_3
462 concentrations were increased by 5 days of cortisol infusion but did not differ significantly
463 between sham-operated and AX fetuses later in gestation would be consistent with a shorter
464 period of cortisol exposure in the sham-operated twin fetuses. Thyroid hormones are known
465 to affect mitochondrial function in adult tissues and their fetal deficiency has recently been
466 shown to impair mitochondrial OXPHOS capacity of the fetal ovine BF and brain (Bloise *et al.*
467 2018, Lombardi *et al.* 2015, Davies *et al.* 2020, 2021). Indeed, the current findings suggest
468 that both cortisol and T_3 are important factors in regulating mitochondrial content and
469 OXPHOS capacity of skeletal muscle during late gestation. The prepartum maturational
470 effects of cortisol on mitochondrial function in skeletal muscle may, therefore, be mediated,
471 in part, by T_3 as occurs with other metabolic processes essential for neonatal survival (Forhead
472 & Fowden, 2014).

473

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The current findings in AX fetuses indicate that the prepartum cortisol increment increases mitochondrial content in both muscles. However, earlier in gestation when muscle fibres were still differentiating, the effects of cortisol are more complex and appear to be muscle and possibly fibre-type specific. In the SDF, cortisol infusion increased mitochondrial biogenesis, content and maximal OXPHOS, but with no apparent increase in ETS complex abundance. In the BF, cortisol infusion had no effect on mitochondrial content or maximal OXPHOS, but specifically increased Complex I capacity and altered the relative contribution of the different muscle fibres to the mitochondrial pool. In both muscles, there were no changes in ATP synthase or UCPs with experimental manipulation of the fetal cortisol concentration that would explain the changes in OXPHOS functional capacity, although UCP expression may not reflect activity. This contrasts with the known effects of cortisol in upregulating UCP abundance in fetal ovine adipose tissue near term (Mostyn *et al.* 2004, Ghanalingham *et al.* 2008). In general, ANT1 levels were increased by cortisol infusion and reduced by AX in both muscles in the current study. As well as functioning as a mitochondrial ADP-ATP exchanger, ANT1 can induce mild mitochondrial uncoupling in adult tissues, particularly in response to fatty acids (Kimura & Rasmussen 1977, Brand *et al.* 2005, Sparks *et al.* 2016). This is consistent with the current finding of greater ANT1 abundance concurrently with increased SDF rates of both PC-linked leak and OXPHOS respiration in cortisol infused fetuses. In adult rat liver, dexamethasone has been shown to increase ANT1 content and simultaneously enhance both mitochondrial uncoupling and OXPHOS capacity (Arvier *et al.* 2007). The prepartum rise in cortisol may, therefore, act to stimulate mitochondrial biogenesis and, thus, the capacity for neonatal ATP production while minimizing the potential for oxidative stress, in part through dissipating the proton gradient. Other factors may then

497 activate the increase in mitochondrial OXPHOS after birth when the ATP demand rises with
498 the new metabolic activities (Fowden & Forhead 2015).

499

500 In summary, the current findings show that cortisol is an important regulator of mitochondrial
501 OXPHOS capacity in ovine skeletal muscle during late gestation. Its effects were muscle-
502 specific and involved changes in mitochondrial biogenesis and respiratory function. Indeed,
503 these prenatal cortisol-induced adaptations may explain, in part, the adult mitochondrial
504 dysfunction observed after adverse conditions during pregnancy that raise fetal
505 glucocorticoid concentrations (Reynolds 2013, Khamoui *et al*. 2018, Chen *et al.* 2020,
506 Gyllenhammer *et al.* 2020). While glucocorticoids are known to affect adult mitochondrial
507 function through both the nuclear and mitochondrial genomes (Lapp *et al.* 2019), further
508 studies are needed to determine the specific molecular mechanisms by which cortisol induces
509 mitochondrial maturation in skeletal muscle fibres. Greater knowledge of these
510 developmental processes will be beneficial for metabolic health of infants under- or over-
511 exposed to glucocorticoids prenatally due to stress, prematurity or maternal treatment with
512 synthetic glucocorticoids for threatened pre-term delivery or other clinical conditions.

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686 **FIGURES LEGENDS**

687

688 **Figure 1**: Individual and mean (±SEM) values of (A) fetal cortisol concentration and the activity
689 of citrate synthase (CS) in (B) the *biceps femoris* (BF) and (C) *superficial digital flexor* (SDF)
690 muscles of unoperated newborn lambs (n=6) and fetal sheep delivered either unoperated at
691 104 days of gestational age (dGA, n=6), at 129dGA after infusion with saline (S-I, n=6) or
692 cortisol (C-I, n=6) for 5 days before delivery at 129dGA or at 144dGA after adrenalectomy (AX,
693 n=4) or sham operation (Sham, for cortisol n = 6, for CS n=5 BF, n=6 SDF) at 114-119dGA.
694 Mean (±SEM) values for control animals (104dGA, S-I, sham-operated and newborn animals)
695 are shown with white columns while those for animals with cortisol concentrations that were
696 manipulated experimentally (C-I and AX) are shown with grey columns. Control columns with
697 different letters as superscripts are significantly different from each other (One-way ANOVA,
698 $P<0.05$). An asterisk indicates a significant difference from the respective control group (*
699 $P<0.05$, ** $P<0.01$, t-test or Mann-Whitney Rank sum test).

700

701 **Figure 2:** Mean (±SEM) relative gene expression of *PGC1α* (panels A &B), *MFN2* (panels C &
702 D) and *DRP1* (panels E & F) in the *biceps femoris* (BF, panels A, C & E) and *superficial digital*
703 *flexor* (*SDF*, panels B, D & F) muscles of fetal sheep either at 129 days of gestational age (dGA)
704 after 5 days of infusion of saline (S-I, n=5 BF, n=6 SDF) or cortisol (C-I, n=6, both muscles) or
705 at 144dGA after adrenalectomy (AX, n=4, both muscles) or sham operation (Sham, n=6, both
706 muscles) at 114-119dGA. An asterisk indicates a significant difference from the respective
707 control group (* $P<0.05$ t-test or Mann-Whitney Rank sum test).

708

Figure 3: Mean (±SEM) maximal (panels A & D), pyruvate supported (Py, panels B & E) and palmitoyl-carnitine supported (PC, panels C & F) rates of oxygen consumption by the *biceps femoris* (BF, panels A-C) and *superficial digital flexor* (SDF, panels D-F) muscles of fetal sheep either at 129 days of gestational age (dGA) after 5 days of infusion of saline (S-I, n=6, both muscles) or cortisol (C-I, n=4-6 BF, n=5-6 SDF) or at 144dGA after adrenalectomy (AX, n=3-4, both muscles) or sham operation (Sham, n= 6 BF, n=5-6 SDF) at 114-119dGA. An asterisk indicates a significant difference from the respective control group (* P<0.05, ** P<0.01, t-test or Mann-Whitney Rank sum test). A hash tag indicates a trend towards a significant difference from the respective control group (# P<0.10, t-test or Mann-Whitney Rank sum test)

Figure 4: Mean (±SEM) relative protein abundance of the electron transfer system complexes (CI-IV) and ATP synthase (CV) in the *biceps femoris* (*BF*, panels A & C) and *superficial digital flexor* (*SDF*, panels B & D) muscles in fetal sheep either at 129 days of gestational age (dGA) after 5 days of infusion of saline (white columns, n=5 BF, n=6 SDF) or cortisol (grey columns, n=6, both muscles) in panels A & B or at 144dGA after adrenalectomy (AX, grey columns, n=4, both muscles) or sham operation (white columns, n=6, both muscles) at 114-119dGA in panels C & D. An asterisk indicates a significant difference from the respective control group (* P<0.05, ** P<0.01, t-test or Mann-Whitney Rank sum test).

Figure 5: Mean (±SEM) relative gene expression of *UCP2* (panels A &B), *UCP3* (panels C & D), and *ANT1* (panels E & F) and of ANT1 protein abundance (panels G & H) in the *biceps femoris* (*BF*, panels A, C, E & G) and *superficial digital flexor* (*SDF*, panels B, D, F & H) muscles of fetal

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732 sheep either at 129 days of gestational age (dGA) after 5 days of infusion of saline (S-I, n=5
733 *BF,* n=6 *SDF*) or cortisol (C-I, n=6, both muscles) or at 144dGA after adrenalectomy (AX, n=4,
734 both muscles) or sham operation (Sham, n=6, both muscles) at 114-119dGA. An asterisk
735 indicates a significant difference from the respective control group (* P<0.05, ** P<0.01, t-
736 test or Mann-Whitney Rank sum test). A hash tag indicates a trend towards a significant
737 different from the respective control group (# P<0.10, t-test or Mann-Whitney Rank sum test).

738

739 **Figure 6:** Mean (±SEM) relative gene expression of *MHCI* (panels A &D), *MHCIIa* (panels D &
740 E), and *MCHIIx* (panels C & F) in the *biceps femoris* (BF, panels A-C) and (C) *superficial digital*
741 *flexor* (SDF, panels D-F) muscles of fetal sheep either at 129 days of gestational age (dGA)
742 after 5 days of infusion of saline (S-I, n=5 *BF*, n=6 *SDF*) or cortisol (C-I, n=6, both muscles) or
743 at 144dGA after adrenalectomy (AX, n=4, both muscles) or sham operation (Sham, n=6, both
744 muscles) at 114-119dGA. An asterisk indicates a significant difference from the respective
745 control group (* P<0.05 t-test or Mann-Whitney Rank sum test).

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DECLARATIONS OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

The study was designed by KLD, ORV, AJM and ALF. The *in vivo* experimental work on the animals was carried out by KLD, EJC, DJS, AJF and ALF. The *in vitro* tissue analyses were carried out by KLD, DJS, EJC and AJM. The manuscript was written by KLD and ALF. All the other authors commented on the text.

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Figure 1

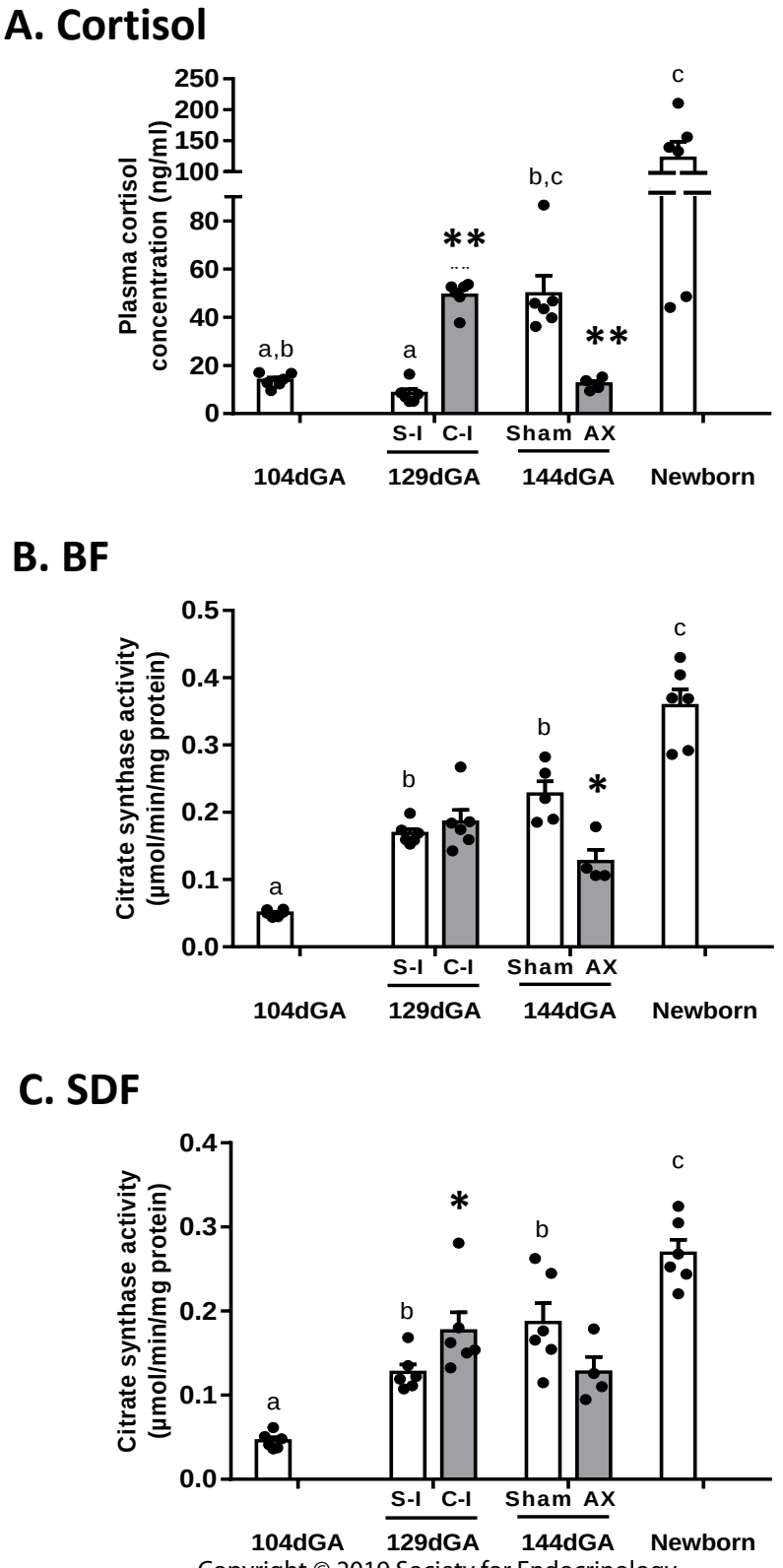

Figure 2

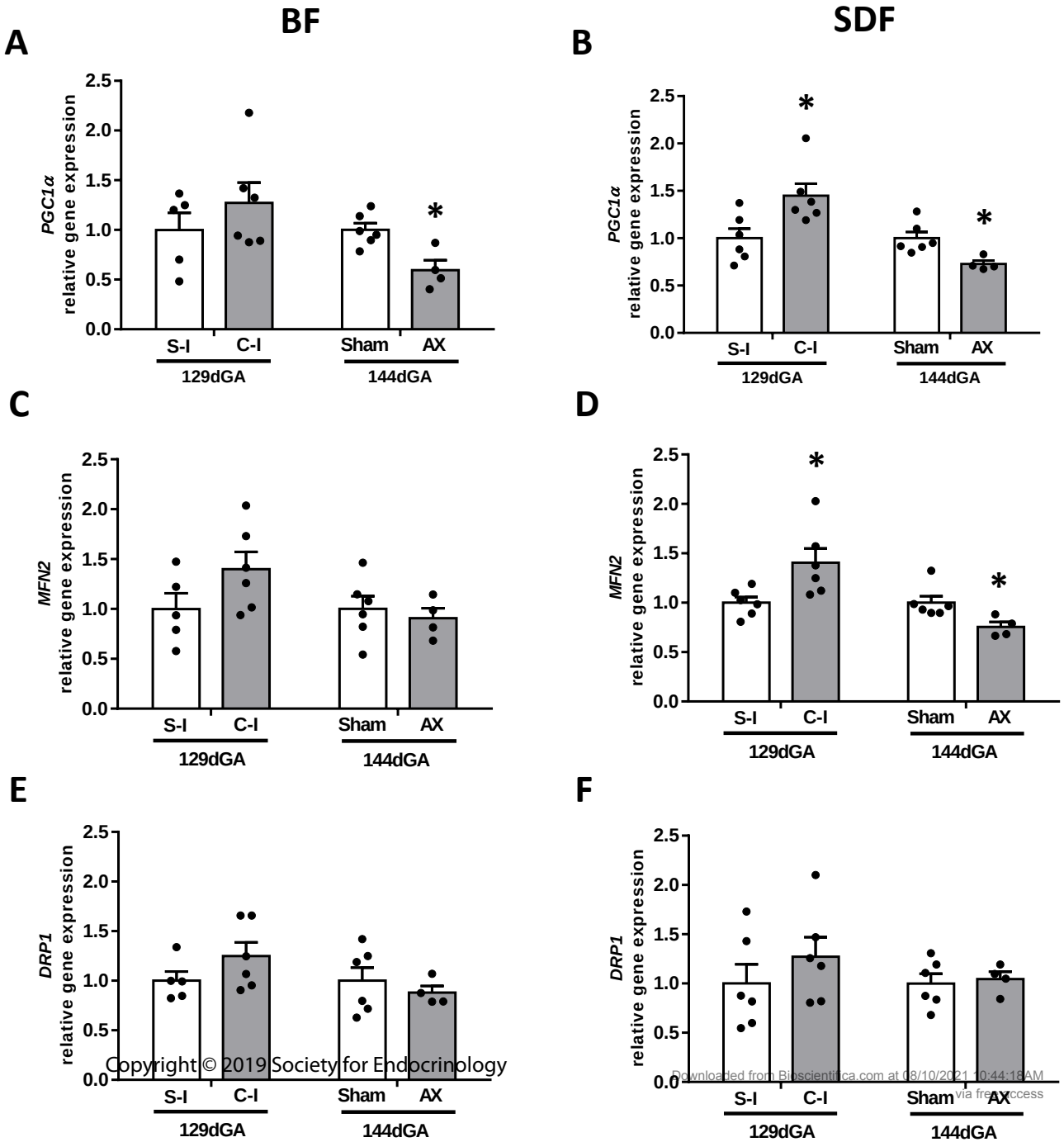

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Figure 3

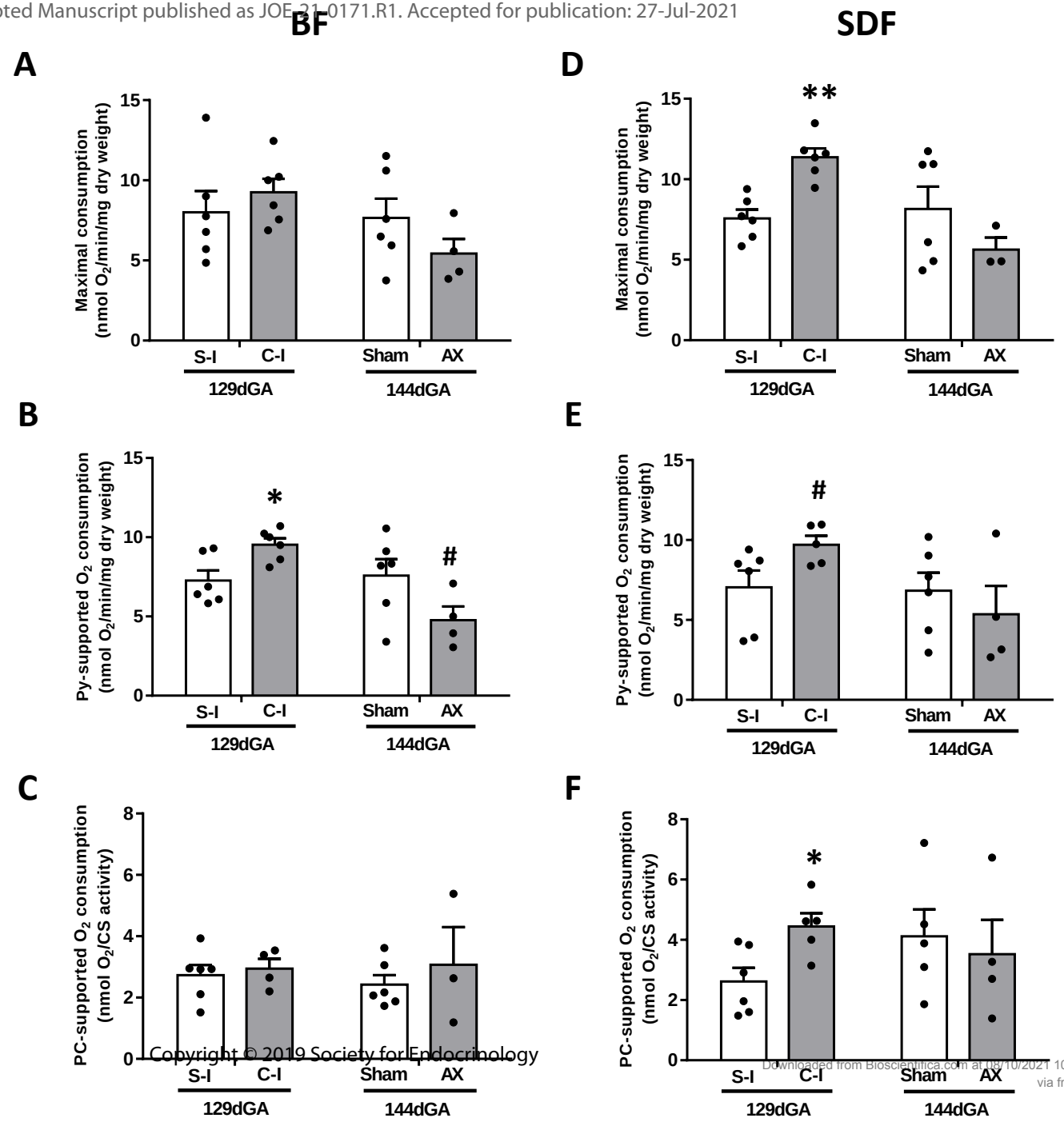

Figure 4

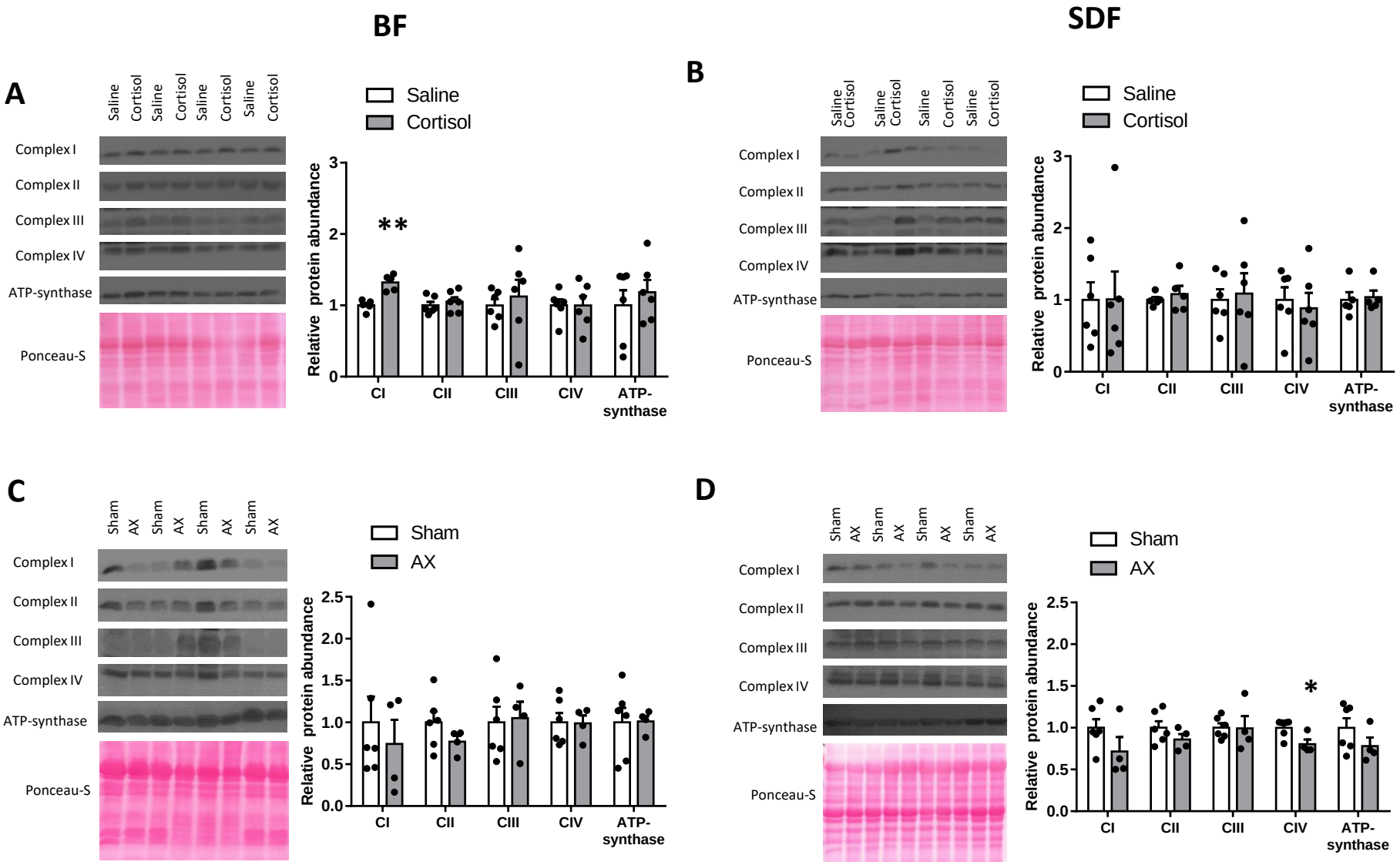

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Figure 5

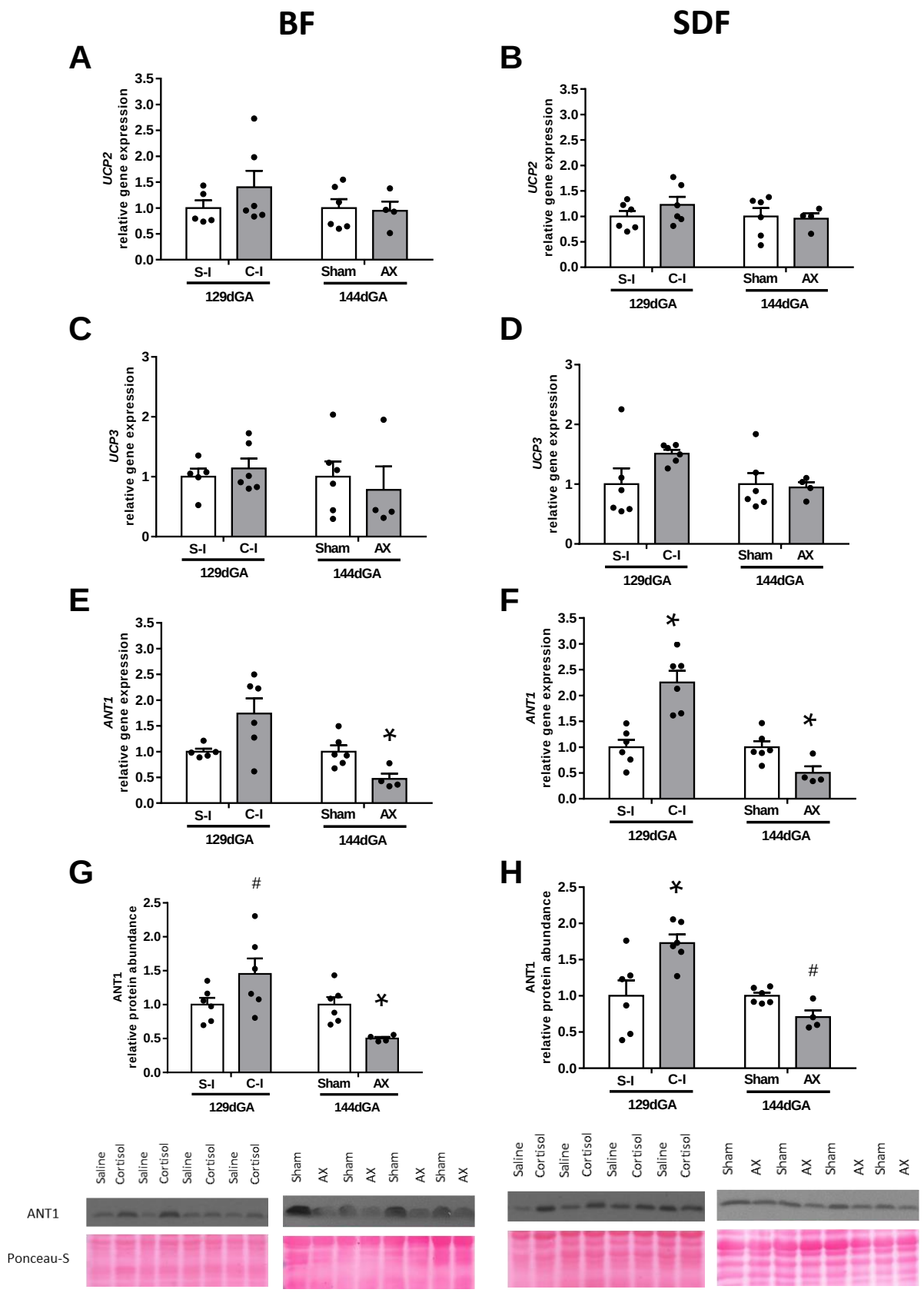

Figure 6

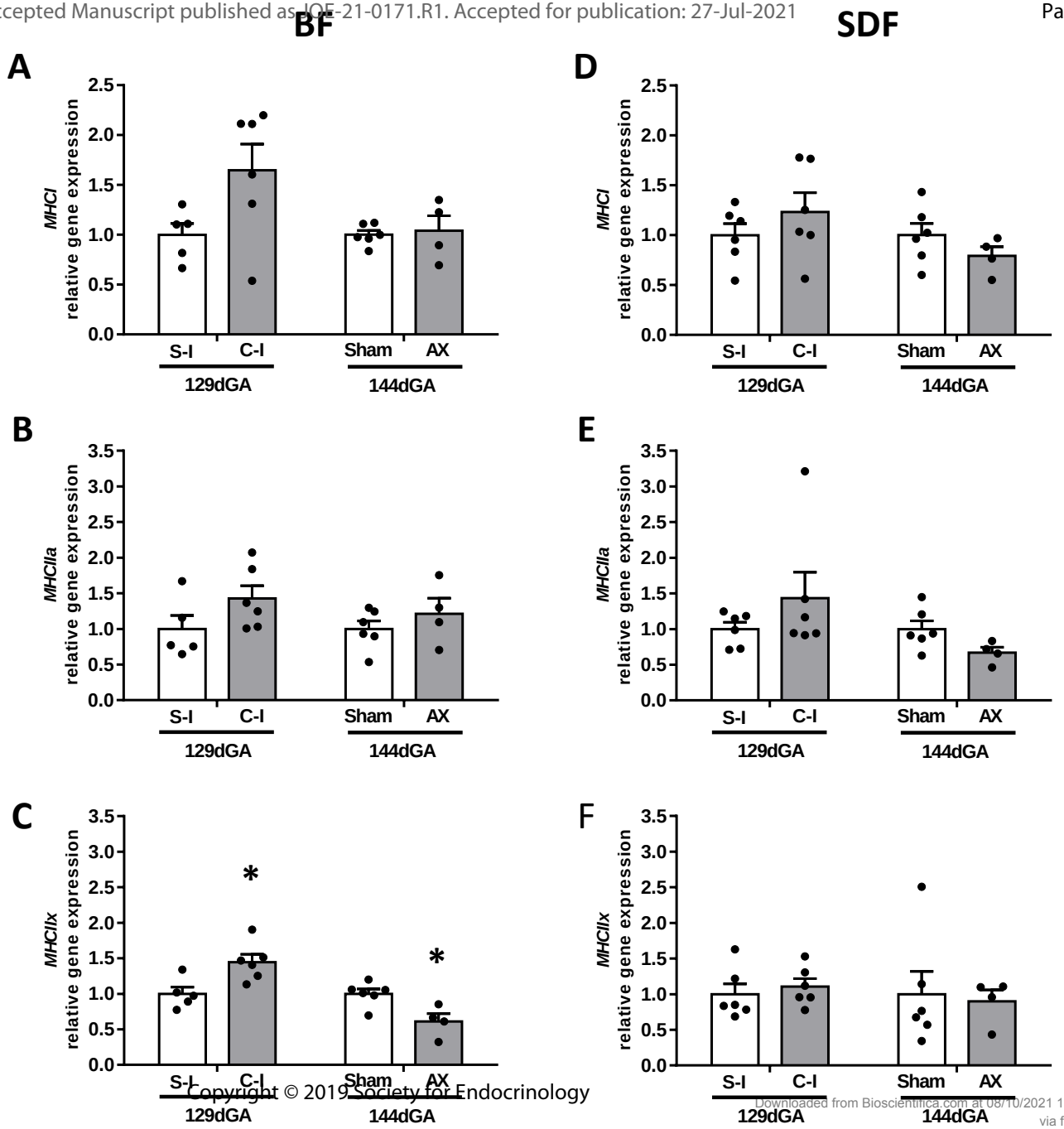

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Table 1: Forward and reverse primer sequences used for SYBR qRT-PCR.

Target Gene, Encoded Protein and Function	Primer Sequences	Reference
Ribosomal protein S15 (RPS15)	F: ATCATTCTGCCCGAGATGGTG R: TGCTTCACGGGCTTGTAGGTG	Yates et al., 2016
18S rRNA	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG	Byrne et al., 2010
Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PPARGC1A) PGC1α protein Regulator of Mitochondrial biogenesis	F: GAGATGTGACCACCGAGAATGAG R: GCTGTTGACAAATGCTCTTCGC R: CACCGCCGAATAATTCACTT	Myers et al., 2008
Mitofusin 2 (MFN2) MFN2 protein Regulator of mitochondrial membrane fusion	F: CATCAGCTATACTGGCTCCAACT R: AATGAGCAAAAGTCCCAGACA	Davies et al., 2020
Dyamin-related protein1 (DRP1) DRP1 protein Regulator of mitochondrial membrane fission	F: ATGCCAGCAAGTCCACAGAA R: TGTTCTCGGGCAGACAGTTT	Reddy et al., 2016
Uncoupling protein 2 (UCP2) UCP2 protein Mitochondrial uncoupling	F: AAGGCCCACCTAATGACAGA R: CCCAGGGCAGAGTTCATGT	Davies et al., 2020
Uncoupling protein 3 (UCP3) UCP3 protein Mitochondrial uncoupling	F: GAAAGGAATTCTGCCCAACA R: TCCAAAGGCAGAGACGAAGT	Kelly et al., 2011
SLC25A4 Adenine nucleotide translocase 1 (ANT1) protein Transport of ADP and ATP across mitochondrial membranes. Mild mitochondrial uncoupling	F: TGGTGTCCTACCCCTTTGAC R: CAGGCGCCTTTGAAGAAAGC	Kelly et al., 2011
Myosin heavy chain 7 (MHY7) MHCI protein Muscle contraction	F: GAGATGGCCGCGTTTGGGGAG R: GGCTCGTGCAGGAAGGTCAGC	Yates et al., 2016
MHY2 MHCIIa protein Muscle contraction	F: ACCGAAGGAGGGGCGACTCTG R: GGCTCGTGCAGGTGGGTCATC	Yates et al., 2016
MHY1 MHCIIx protein Muscle contraction	F: AAAGCGACCGTGCAGAGCAGG R: GGCTCGTGCAGGTGGGTCATC	Yates et al., 2016

Table 2. Hormone concentrations, morphometry and biochemical composition.

	129dGA		144dGA	
	Saline-infused	**Cortisol-infused**	**Sham**	**AX**
THYROID HORMONE CONCENTRATIONS				
Plasma T_3 (ng/ml)	0.43±0.03	0.70±0.14 *	0.51±0.09	0.23±0.03 #
Plasma T_4 (ng/ml)	122.0±9.5	131.6±4.0	98.0±19.0	101.3±13.1
MORPHOMETRY				
Body weight (kg)	3.1±0.1	3.0±0.1	3.63±0.27	3.91±0.29
Crown-rump length (cm)	43.3±0.6	43.8±0.5	48.1±0.9	48.8±1.1
Abdominal girth (cm)	33.4±0.6	33.2±1.0	35.3±1.2	37.4±2.5
BF Weight (g)	13.07±0.61	12.30±0.87	15.2±1.5	15.2±1.2
BF:BW (g/kg)	4.28±0.09	4.14±0.14	4.1±0.1	3.9±0.3
SDF weight (g)	2.06±0.17	1.97±0.09	2.5±0.3	3.5±0.6
SDF:BW (g/kg)	0.67±0.03	0.67±0.03	0.7±0.05	0.9±0.2
MUSCLE BIOCHEMICAL COMPOSITION				
BF Water content (%)	82.1±0.2	80.5±0.2**	79.1±0.3	80.8±0.2*
BF Protein content				
(mg/g wet wt)	44.0±1.2	47.5±2.6	52.5±2.9	40.8±4.3*
(mg/mg dry wt)	0.25±0.01	0.24±0.02	0.25±0.01	0.21±0.02
SDF Water content (%)	80.9±0.3	79.8±0.5	79.5±0.2	81.2±0.2*
SDF Protein content				
(mg/g wet wt)	51.1±2.1	50.9±2.3	30.4±0.8	28.9±0.5
(mg/mg dry wt)	0.27±0.01	0.25±0.01	0.15±0.003	0.15±0.002

Mean (±SEM) values of T_3 and T_4 concentrations, morphometric measurements and muscle biochemical composition of the biceps femoris (BF) and superficial digital flexor (SDF) muscles of sheep fetuses delivered either at 129 days of gestational age (dGA) after a 5 day infusion of cortisol (n=6) or saline (n=6) for 5 days or at 144dGA after adrenalectomy (n=4, AX) or sham operation (Sham, n=6) at 114-119 dGA. Asterisk: Significantly different from the value in the control fetuses at the same gestational age * P<0.05, **P<0.01, # P=0.057 (t-test or non-parametric Mann-Whitney test as appropriate)

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Table 3: Correlation and partial correlation analyses between hormone concentrations and citrate synthase activity and mitochondrial oxidative phosphorylation (OXPHOS) rates of the fetal *biceps femoris* (BF) and *superficial digital flexor* (SDF)muscles.

Muscle	Hormone	Citrate synthase	Py-linked OXPHIOS	PC-linked OXPHOS	Maximal OXPHOS
Correlations					
BF	Log_{10} Cortisol	**r=0.735** P<0.01 n=33	**r=0.482** P<0.05 n=22	r=-0.050 P>0.05 n=19	R=0.263 P>0.05 n=22
	Log_{10} T_3	**r=0.803** P<0.001 n=33	**r=0.460** P<0.05 n=22	r=0.272 P>0.05 n=19	R=0.304 P>0.05 n=22
SDF	Log_{10} Cortisol	**r=0.701** P<0.001 n=32	r=0.203 P>0.05 n=21	**r=0.507** P<0.05 n=19	**r=0.421** P<0.05 n=21
	Log_{10} T_3	**r=0.801** P<0.001 n=32	**r=0.428** P<0.05 n=21	**r=0.446** P=0.050 n=19	**r=0.566** P<0.01 n=21
Partial correlations					
BF	Log_{10} Cortisol	**r=0.387** P<0.05	r=0.350 P>0.05	Not required	Not required
	Log_{10} T_3	**r=0.582** P<0.01 n=33	r=0.310 P>0.05 n=21	Not required	Not required
SDF	Log_{10} Cortisol	**r=0.424** P<0.05	Not required	r=0.390 P>0.05	r=0.215 P>0.05
	Log_{10} T_3	**r=0.506** P<0.01 n=32	Not required	r=0.279 P>0.05 n=19	**r=0.478** P<0.01 n=21

For each muscle, data were combined from the cortisol infused and adrenalectomised and their respective control groups of fetuses. Significant correlations and partial correlations are shown in bold ($P \leq 0.05$)

Table 4: Summary of the changes induced in the *biceps femoris* and *superficial digital flexor* muscles either by pre-term cortisol infusion for 5 days to mimic the normal prepartum increase in cortisol concentration or by adrenalectomy (AX) to prevent this increment near term relative to their respective control groups.

	Biceps femoris		***Superficial digital flexor***	
	Cortisol-infused	AX	Cortisol-Infused	AX
Mitochondrial density and biogenesis				
Citrate synthase	No Δ	↓	↑	↓*
PGC1α	No Δ	↓	↑	↓
MFN2	No Δ	No Δ	↑	↓
DRP1	No Δ	No Δ	No Δ	No Δ
Mitochondrial ADP-coupled respiration				
Pyruvate (Py)	↑	Tendency ↓	Tendency ↑	No Δ
Palmitoyl-carnitine (PC)	No Δ	No Δ	↑	No Δ
Total	No Δ	No Δ	↑	No Δ
Mitochondrial OXPHOS efficiency				
ETS complexes & ATP synthase	↑ CI	No Δ	No Δ	↓ CIV
UCP2 & *UCP3*	No Δ	No Δ	No Δ	No Δ
ANT1 gene	No Δ	↓	↑	↓
ANT1 protein	Tendency ↑	↓	↑	↓
Muscle fibre composition				
MHCI	No Δ	No Δ	No Δ	No Δ
MHCIIa	No Δ	No Δ	No Δ	No Δ
MHCIIx	↑	↓	No Δ	No Δ

No Δ = No significant change. CI = ETS Complex I, CIV = ETS Complex IV

↓=Significant decrease (P<0.05) or tendency for decrease (P<0.10). * significant decrease by paired t-test to its sham-operated twin (P<0.05).

↑=Significant increase (P<0.05) or tendency for increase (P<0.10).