Poor maternal nutrition and accelerated postnatal growth induces an accelerated aging phenotype and oxidative stress in skeletal muscle of male rats

Jane L. Tarry-Adkins¹,*†, Denise S. Fernandez-Twinn¹, Jian Hua Chen¹, Iain P. Hargreaves², Viruna Neergheen², Catherine E. Aiken¹ and Susan E. Ozanne¹

ABSTRACT

Developmental programming, which occurs as a consequence of suboptimal in utero and early environments, can be associated with metabolic dysfunction in later life, including an increased incidence of cardiovascular disease and type 2 diabetes, and predisposition of older men to sarcopenia. However, the molecular mechanisms underpinning these associations are poorly understood. Many conditions associated with developmental programming are also known to be associated with the aging process. We therefore utilized our well-established rat model of low birth weight and accelerated postnatal catch-up growth (termed ‘recuperated’) in this study to establish the effects of suboptimal maternal nutrition on age-associated factors in skeletal muscle. We demonstrated accelerated telomere shortening (a robust marker of cellular aging) as evidenced by a reduced frequency of long telomeres (48.5-8.6 kb) and an increased frequency of short telomeres (4.2-1.3 kb) in vastus lateralis muscle from aged recuperated offspring compared to controls. This was associated with increased protein expression of the DNA-damage-repair marker 8-oxoguanine-glycosylase (OGG1) in recuperated offspring. Recuperated animals also demonstrated an oxidative stress phenotype, with increased citrate synthase activity, increased electron-transport-complex activities of complex I, complex II-III and complex IV (all markers of functional mitochondria), and increased xanthine oxidase (XO), p67phox and nuclear-factor kappa-light-chain-enhancer of activated B-cells (NF-κB). Recuperated offspring also demonstrated increased antioxidant defense capacity, with increased protein expression of manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD), catalase and heme oxygenase-1 (HO1), all of which are known targets of NF-κB and can be upregulated as a consequence of oxidative stress. Recuperated offspring also had a pro-inflammatory phenotype, as evidenced by increased tumor necrosis factor-α (TNFα) and interleukin-1β (IL1β) protein levels. Taken together, we demonstrate, for the first time to our knowledge, an accelerated aging phenotype in skeletal muscle in the context of developmental programming. These findings may pave the way for suitable interventions in at-risk populations.

KEY WORDS: Skeletal muscle, Oxidative stress, Mitochondria, Developmental programming

INTRODUCTION

For over 25 years, it has been known that a suboptimal in utero environment is strongly associated with increased risk of development of age-associated disease in later life, including cardiovascular disease (CVD) (Barker et al., 1989) and type 2 diabetes (T2D) (Barker et al., 1993). These findings have been robustly confirmed in both humans and animals (Tarry-Adkins and Ozanne, 2014; Zambrano et al., 2016), and these studies support the ‘thrifty phenotype hypothesis’ (Hales and Barker, 1992), which states that, under conditions of suboptimal nutrition, the fetus permanently alters its organ structure, metabolism and function to ensure immediate survival of the organism. Although beneficial in continued conditions of poor postnatal nutrition, such ‘developmental programming’ is known to be detrimental in postnatal conditions of adequate or over-nutrition, both of which can cause accelerated postnatal growth.

The development of skeletal muscle is especially vulnerable to nutritional deficiency compared to other tissues, owing to muscle mass being lost at the expense of brain-sparing in utero (Desai et al., 1996). Indeed, maternal nutrient restriction, a widely used model of developmental programming, is known to reduce offspring birth weight due to reductions in fetal circulating amino acids (Jansson et al., 2006; Pantham et al., 2015). This is highlighted in studies of ovine fetuses (Zhu et al., 2004) and offspring (Zhu et al., 2006) exposed to a suboptimal in utero environment, which demonstrate low birth weight as well as dysregulation of muscle development, including changes in the number and composition of myofibers. Numbers of myofibers and neuromuscular junctions are also altered in a rat model of maternal protein restriction (Comfortin et al., 2016) and this effect is long-lasting, into old age (Comfortin et al., 2015). There is also evidence that a suboptimal early environment (nutrient restriction) in the mouse can impact on muscle metabolism/function and molecular changes, including decreased mitochondrial content (Beauchamp et al., 2015) and reduced expression of mitochondrial genes, especially those involved in oxidative phosphorylation (Mortensen et al., 2010). Decreased muscle fiber score has also been observed in vastus lateralis muscle of low-birth-weight elderly men (Patel et al., 2012).

Muscle mass is known to decline with age, which can contribute to age-associated muscular dysfunction; however, the rate of decline shows great inter-individual variation (Sayer et al., 2010). With age, skeletal muscle can accumulate oxidative stress,
which can cause issues such as a reduction in force generation and muscle atrophy. Muscle atrophy contributes to progressive weakness and an increased risk of mobility impairment, falls and physical frailty in very advanced age (Cruz-Jentoft et al., 2010). Among the most frequently implicated mechanisms of aging muscle atrophy is mitochondrial dysfunction, which leads to increased reactive oxygen species (ROS) generation (Marzetti et al., 2013).

Oxidative stress accumulation occurs when cellular ROS overwhelm the endogenous antioxidant defense capacity and thus redox homeostasis is not maintained (Ray et al., 2012). This excess ROS generation can cause macromolecular damage to proteins, lipids and DNA (Valko et al., 2007). Telomeres (hexamer repeats of DNA: [TTAGGG]n), which are found at the ends of chromosomes, are particularly susceptible to ROS damage because of their guanine-rich sequences (Oikawa and Kawanishi, 1999). In normal somatic cells, telomeres shorten with every cellular division. This makes telomere length measurement a robust marker of aging in many species, including humans and rodents, and this has been shown to be associated with longevity (Haussman et al., 2003; Heidinger et al., 2012). It is known that suboptimal in utero nutrition can lead to accelerated aging in a number of tissues (Tarry-Adkins and Ozanne, 2014). The high metabolic activity of skeletal muscle renders it particularly susceptible to oxidative stress; however, accelerated aging in skeletal muscle as a consequence of developmental programming has never been explored.

This study therefore aimed to investigate the effects of a poor maternal diet followed by accelerated postnatal growth upon skeletal muscle (vastus lateralis) of aging male rat offspring, focusing specifically upon telomere length, and indices of oxidative stress, antioxidant defense capacity and inflammation.

RESULTS
In all cases, the reported data are expressed as mean±s.e.m.

Anthropometrical data
Re recuperated offspring were significantly (P<0.001; 6.3±0.3 g) smaller compared to controls (7.4±0.2 g) on day 3, and remained significantly (P<0.001) smaller at day 7 (13.4±0.6 vs 16.8±0.8 g). By 14 days of age, the recuperated offspring had undergone rapid postnatal catch-up growth and so were similar in weight to control offspring (33.7±0.7 g vs 34.1±1.7 g), and this was maintained at weaning (52.2±0.9 g vs 50.7±1.2 g) and at 12 months of age (920±29 g vs 956±25 g). These values reflect average male pup weight in the litter.

Poor maternal nutrition and accelerated postnatal growth lead to increased skeletal-muscle oxidative stress
(a) NF-κB:
There was a significantly increased in recuperated offspring compared to controls (Fig. 2A). There was, however, no effect of maternal diet (control 442±60 vs recuperated 377±60 copy number) upon NF-κB gene expression. This suggests that the mechanism underlying the NF-κB dysregulated expression involves post-transcriptional regulation.

(b) NADPH oxidase 2 (NOX2), xanthine oxidase (XO) and cytochrome c:
XO protein expression was significantly (P<0.05) increased in recuperated offspring compared to controls and there was a trend towards an increase in P67phox (P<0.1) (Fig. 2B); however cytochrome c protein expression was similar between groups (Fig. 2B). mRNA levels of the NOX2 protein-complex components Gp91phox (P<0.05) and P22phox (P<0.05) were significantly increased in recuperated animals compared to controls (Fig. 2C). Expression levels of P67phox and Nox4 were unchanged between groups (Fig. 2C). Gene expression of Xo was also unchanged between groups (control 444±54, recuperated 344±40 copy number).

(c) Mitochondrial indices of ROS:
Levels of citrate synthase (CS), a marker of functional mitochondria, were decreased (P<0.05) in recuperated offspring compared to controls (Fig. 2D). Increased levels of complex I (P<0.001), linked complex II-III (P<0.01) and complex IV (P<0.001) activities were observed in recuperated offspring compared to controls (Fig. 2E). The increased complex II-III activity was not associated with any differences in coenzyme Q9 (CoQ9) (control 9675±660 vs recuperated 8410±695 pmol/mg protein). mRNA levels of Cytochrome c oxidase 1 (Cox1), a subunit of complex IV, was increased (P<0.05) in recuperated offspring compared to controls (Fig. 2F).

(d) Direct indices of Reactive Oxygen Species (ROS):
Markers of lipid peroxidation (4-Hydroxynonenal) and protein tyrosination (3-nitrotyrosine) were undetectable in skeletal muscle.

Poor maternal nutrition and accelerated postnatal growth altered skeletal-muscle antioxidant defense capacity
Protein expression of the antioxidant enzymes manganese superoxide dismutase (MnSOD) (P<0.05), copper-zinc superoxide dismutase (CuZnSOD) (P<0.05) and heme oxygenase-1 (HO1) (P<0.05) were increased in recuperated offspring compared to controls (Fig. 3A). Protein expression of peroxiredoxin-1 (PRDX1), peroxiredoxin-3 (PRDX3) and glutathione reductase (GR) were unaffected by maternal diet (Table 1). mRNA expression of MnSOD, CuZnSOD, extracellular superoxide dismutase (ECSD), catalase and Hmox1 were unaffected by maternal diet (Fig. 3B).

NF-κB1 correlates with antioxidant defense and oxidant capacity
Positive correlations were observed between NF-κB1 protein expression and XO (XO=0.05; r2=0.3596), MnSOD (P=0.0432; r2=0.3805), CuZnSOD (P=0.0181; r2=0.4801), catalase (P=0.05; r2=0.3585), HO1 (P=0.048; r2=0.3590) and IL1β (P=0.029; r2=0.4277). The correlation between Gp91phox and NF-κB1 was non-significant (P=0.1619; r2=0.2051) (Fig. 4A-F).
Poor maternal nutrition and accelerated postnatal growth lead to altered markers of inflammation

TNFα (P=0.05) and IL1β (P<0.001) protein levels were increased in recuperated offspring compared to controls; however, IL6 protein expression was similar between groups (Fig. 5A). No effect of maternal diet was observed upon Tnfα or Il6 mRNA levels; however, there was a trend toward increased Tgfβ1 mRNA expression levels (P=0.11) in recuperated offspring compared to controls (Fig. 5B).

DISCUSSION

The aging process is associated with a decline in muscle ‘fitness’, with distinct muscle mass decline and loss of muscle strength occurring from 40 years of age in humans (Keller., 2013). This is becoming of increasing concern in an aging population. The environment to which an individual is exposed in utero and in early life has also been shown to have an effect on skeletal muscle in old age; skeletal muscle from elderly monozygotic twins, in which the lower-birth-weight twin developed type 2 diabetes (TD2) in later life, had perturbations in glycogen metabolism and insulin resistance, which are not apparent in young monozygotic twins (Poulsen et al., 2007). An accelerated postnatal growth trajectory in low-birth-weight children is also known to reduce physical fitness in pre-pubescent children (van Deutekom et al., 2015). We have shown previously using an animal model that low birth weight followed by accelerated postnatal growth leads to a reduction in lifespan (Hales et al., 1996). Low birth weight alone without postnatal catch-up growth did not impact on lifespan (Hales et al., 1996). It is unknown whether low birth weight and accelerated early postnatal growth impacts on skeletal muscle aging. We addressed this knowledge gap using a well-established rat model of maternal protein restriction, which generates low-birth-weight offspring and accelerated postnatal growth (recuperated), by cross-fostering to control-fed mothers.

Accelerated telomere shortening, a robust marker of cellular aging, was observed in aged skeletal muscle from recuperated rats compared to control animals, suggesting that low birth weight and rapid postnatal growth causes accelerated skeletal-muscle aging. This was not associated with premature cellular senescence; two markers of cellular senescence, p53 and p21, were unaltered at the mRNA level; however, changes at the protein level cannot be disregarded. Ku70 and Ku80, two of the major telomere-length maintenance proteins, which are also instrumental in non-homologous end-joining (NHEJ) DNA repair (a mechanism that repairs double-stranded DNA breaks), were unchanged at the mRNA level. However, we cannot discount the possibility that changes may occur at the protein level: there is a lack of antibodies to these molecules that work well in muscle. OGG1 the major

**Fig. 1. Telomere length and markers of DNA damage.** The effect of in utero protein restriction and accelerated postnatal growth upon (A) telomere length (the percentage of telomeres at each length is shown), (B) mRNA expression of telomere-length maintenance proteins (Ku70 and Ku80) and (C) protein expression of DNA-damage-repair protein (OGG1) in vastus lateralis skeletal muscle of 12-month-old male rats (shown as a percentage of the total amount in control rats). Results are expressed as means±s.e.m. **P<0.01 (control versus recuperated). Statistics were calculated using a Student’s t-test (two-tailed) and a linear regression model was used to analyze the telomere length data, which included effects of maternal diet (P<0.05), category of telomere length (P<0.001). C, control; R, recuperated. n=6 per group for telomere-length analysis and protein expression; n=8 per group for mRNA expression.
enzyme involved in the excision of 8-oxo-7,8-dihydroguanine (8-oxodG) DNA base lesions via the base excision repair (BER) mechanism, which is key in repairing oxidative base damage specifically in telomeres, was increased at the protein level in recuperated offspring. It is worthwhile to note that we previously reported an increase in OGG1 protein expression in hearts from recuperated rats (Tarry-Adkins et al., 2012).

The increase in skeletal-muscle OGG1 is particularly noteworthy given that recuperated offspring demonstrated a strong oxidative stress phenotype with increased XO protein expression, elevated components of the NADPH oxidase-2 (Gp91phox, P22phox, and cytochrome c) mRNA expression of Gp91phox_P22phox_P67phox, and Xo, (D) ETC complex activity, (E) citrate synthase (CS) activity and (F) Cox I mRNA expression in vastus lateralis skeletal muscle in 12-month-old male rats. Results in A and B are shown as a percentage of the total amounts in control rats. Results are expressed as mean±s.e.m. *p<0.05 and **p<0.01, ***P<0.001 (control versus recuperated). C, control; CS, citrate synthase; R, recuperated. n=6 per group for protein expression; n=8 per group for mRNA expression analysis; n=10 per group for ETC complex activity analysis.

The observed upregulation of OGG1 is a compensatory mechanism to attempt to repair the oxidative base damage.

Skeletal muscle is one of the most aerobically and metabolically active tissues in the body, and is therefore extremely mitochondrially rich, and a major source of oxidative stress. Therefore, we investigated muscle mitochondria as a potential source of ROS in vastus lateralis muscle of the recuperated offspring. Evidence of mitochondrial dysfunction was observed in the muscle of recuperated offspring by a reduction in CS activity (a marker for functional intact mitochondria), and increased complex I, linked complex II-III and complex IV electron transport chain (ETC) activity. It has been shown that, in states of ‘mitochondrial hyperactivity’ (in which ETC activities are upregulated), the ETC
mitochondria might have to compensate for fewer mitochondria by increased ETC activity suggests that the recuperated muscle evidence of a reduced number of functional mitochondria and complex IV of the ETC (Heilbronn et al., 2007), was also upregulated group for protein analysis and hypothesis testing where appropriate. C, control; R, recuperated.

Table 1. Effect of maternal diet upon antioxidant defense capacity

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control (%)</th>
<th>Recuperated (%)</th>
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</thead>
<tbody>
<tr>
<td>GR</td>
<td>100±6</td>
<td>95±4</td>
</tr>
<tr>
<td>PRDX1</td>
<td>100±9</td>
<td>112±15</td>
</tr>
<tr>
<td>PRDX3</td>
<td>100±10</td>
<td>134±21</td>
</tr>
</tbody>
</table>

Control values are set at 100% and other values are relative to the control value. Results are expressed as mean±s.e.m. and n=6 per group. GR, glutathione reductase.

In conclusion, we have shown evidence for accelerated aging as a consequence of suboptimal nutrition and provide a molecular basis through which this can occur. This includes accelerated telomere shortening and increased DNA damage, which was associated with a strong oxidative stress phenotype, a compensatory increased antioxidant defense capacity and inflammation – all of which may be regulated by NF-κB signaling. These findings provide an explanation of why some individuals are at greater risk of developing age-associated muscular dysfunction than others. Given that oxidative stress is a major phenotype in this model, this study provides a strong rationale for a targeted postnatal antioxidant intervention as a potentially safe and cost-effective therapy in at-risk individuals.

MATERIALS AND METHODS

Animal experimentation
All procedures involving animals were conducted under the British Animals (Scientific Procedures) Act (1986) and underwent ethical review by the...
Fig. 4. Correlations of NF-κB protein expression with markers of oxidative stress, antioxidant enzymes and markers of inflammation. The effect of in utero protein restriction and accelerated postnatal growth upon correlations of protein expression of NF-κB versus (A) Gp91^phox^, (B) XO, (C) MnSOD, (D) CuZnSOD, (E) catalase, (F) HO1 and (G) IL1β in vastus lateralis skeletal muscle of 12-month-old male rats. P-values are shown in the graphs (NF-κB versus antioxidants). Statistics were calculated using a Student’s t-test (two-tailed). Results are expressed as mean±s.e.m. n=6 per group. IDV, integrated density value.
University of Cambridge Animal Welfare and Ethical Review Board. Stock animals were purchased from Charles River. Dams were produced from in-house breeding from stock animals, and each was paired with a different stock male for mating. Pregnant Wistar rats (rattus norvegicus) were maintained at room temperature in specific pathogen-free (SPF) housing to diets and water was provided ad libitum.

The day of birth was recorded as day 1 of postnatal life. Pups born to LP dams were maintained on a 20% protein diet (control) or, an isocaloric low protein diet (LP) (8%) diet, as previously described (Snoeck et al., 1990). Access to diets and water was provided ad libitum. All animals used in this study were SPF-housed individually at 22°C on a controlled 12:12-h light-dark cycle. Diets were purchased from Arie Blok (Woerden, The Netherlands).

Methods

Pups born to LP dams were cross-fostered to control-fed mothers on postnatal day 3, in order to create a recuperated litter. Each recuperated litter was standardized to four male pups at random to maximize their plane of nutrition. The control group was the offspring of mothers fed the 20% protein diet (control) or, an isocaloric low protein diet (LP) (8%) diet, as previously described (Snoeck et al., 1990). Access to diets and water was provided ad libitum. All animals used in this study were SPF-housed individually at 22°C on a controlled 12:12-h light-dark cycle. Diets were purchased from Arie Blok (Woerden, The Netherlands).

The day of birth was recorded as day 1 of postnatal life. Pups born to LP diet-fed dams were cross-fostered to control-fed mothers on postnatal day 3, in order to create a recuperated litter. Each recuperated litter was standardized to four male pups at random to maximize their plane of nutrition. The control group was the offspring of mothers fed the 20% protein diet and suckled by 20% protein-fed dams. Each control litter was culled to eight pups as a standard. Animals in this group were suckled by their own dams. To minimize stress to the animals when cross-fostered, pups were transferred with some of their own bedding. Body weights were recorded at postnatal days 3, 7, 14 and 21, and at 12 months. For time points up until 21 days of age, these reflect average male pup weight in the litter. At 21 days, two males per litter were weaned in their home-cage onto standard laboratory chow fed ad libitum (Special Diet Services) and were maintained on this diet until 12 months of age. All animals were killed by CO2 asphyxiation at approximately 10 am. At post-mortem, vastus lateralis tissue was removed, weighed and snap-frozen in liquid nitrogen and then stored at −80°C until analysis. Ten litters per group were used in this study; this was based on power calculations. In all cases, n refers to the number of litters (with one animal used from each litter).

Reagents

All general reagents for western blotting were purchased from Sigma (Poole, UK), except for the antibodies, which are detailed in the Protein analysis section. All general reagents for gene expression analysis were purchased from Applied Biosystems (Warrington, UK) and all general reagents for CoQ9 and ETC activities were sourced from Sigma (Poole, UK).

Protein analysis

Protein was extracted from samples of vastus lateralis muscle tissue and assayed as described previously (Tarry-Adkins et al., 2016). Protein (20 µg) was loaded onto 10%, 12% or 15% polyacrylamide gels, dependent upon the molecular weight of the protein to be measured. The samples were electrophoresed and transferred to polyvinylidene fluoride membranes (Tarry-Adkins et al., 2016), and detected using the following dilutions of primary antibody: OGG1 (Novus Biologicals, Leiden, UK; cat. no.: NB100-106, 1:500), XO (Santa-Cruz, Cambridgeshire, UK; cat. no.: SC-20991, 1:200), GPx1 (ProteinTech, Cambridge, UK; cat. no.: 19013-1-AP, 1:1000), P67phox (ProteinTech, Cambridge, UK; cat. no.: 15551-1-AP, 1:1000), cytochrome c (Abcam, Cambridge, UK; cat. no.: Ab90529, 1:2000), MnSOD (Upstate, Loughborough, UK; cat. no.: 06-984, lot 26654, 1:1000), CuZnSOD (ProteinTech, Cambridge, UK; cat. no.: 10269-1-AP, 1:1000), TNFa (Cell Signaling Technology, Danvers, MA, USA; cat. no.: 11948S). NF-kB (cat. no.: Ab89600), catalase (cat. no.: Ab1877-10), GR (cat. no.: Ab16801), PRDX1 (cat. no.: Ab15571), PRDX3 (cat. no.: 6751), TGFβ1 (cat. no.: Ab6672), and HO1 (cat. no.: Ab972) were all diluted 1:1000 and purchased from Abcam (Cambridge, UK). All antibodies used anti-rabbit IgG secondary antibodies from Cell Signaling Technology (Danvers, MA, USA) at a dilution of 1:2000.

Gene expression

RNA was extracted using an RNeasy Plus mini kit (Qiagen, Manchester, UK) following the manufacturer’s instructions. A DNase digestion step was performed in order to ensure no genomic DNA contamination. RNA (1 µg) was used to synthesize cDNA using oligo-dT primers and M-MLV reverse transcriptase (Promega, Southampton, UK). Gene expression was determined using custom-designed primers (Sigma, Poole, UK) and SYBR Green reagents (Applied Biosystems, Warrington, UK). Primer sequences are presented in Table 2. Quantification of gene expression was performed using a Step One Plus RT-PCR machine (Applied Biosystems, Warrington, UK). Equal efficiency of the reverse transcription of RNA from all groups was confirmed through quantification of expression of the housekeeping gene Ppia. Expression of Ppia did not differ between groups (effect of maternal diet P=0.99; control 37±6, recuperated 39±6 average copy number). Sample sizes were n=8 per group.

Mitochondrial ETC complex activities and CoQ measurement

Activities of complex I (NADH: ubiquinone reductase; EC 1.6.5.3), complex II-III (succinate: cytochrome c reductase; EC 1.3.5.1+EC 1.10.2.2) and complex IV (cytochrome oxidase; EC 1.9.3.1) as well as citrate synthase (CS; EC1.1.1.32) were assayed as described previously (Tarry-Adkins et al., 2016). Vastus lateralis CoQ9 was quantified by reverse phase high-performance liquid chromatography (HPLC) with UV detection at 275 nm as described previously (Tarry-Adkins et al., 2016).

4-hydroxynonenal (4-HNE) and 3-nitrotyrosine (3-NT) analysis

Protein nitrotyrosination was assayed using a 3-Nitrotyrosine ELISA kit (MitoSciences, Cambridge, UK), according to the manufacturer’s instructions. 3-NT (a marker of lipid peroxidation) was analyzed using an Oxiselect HNE Adduct ELISA kit (Cambridge Biosciences), according to the manufacturer’s instructions.
Table 2. Primer sequences and PCR product sizes

<table>
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<tr>
<th>Primer</th>
<th>Sequence (5'-3' forward)</th>
<th>Sequence (5'-3' reverse)</th>
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Statistical analysis

Maternal-diet effects were compared between groups using Student’s t-test for single hypotheses. In order to correct for multiple hypothesis testing where relevant, P-values were transformed to take account of the false discovery rates using the p.adjust function in R stats package. A linear regression model was used to analyze the telomere length data, which included effects of maternal diet, category of telomere length and an interaction term between these. Data are represented as mean±SEM. All statistical analyses were performed using either Statistica 7 software (Statsoft Inc., Bracknell, UK) or R version 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria). Where P-values or adjusted P-values are reported, an alpha level <0.05 was considered statistically significant. Data was checked for normal distribution. In all cases, n refers to the number of litters (with one animal used from each litter).

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