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## Effect of reproductive ageing on pregnant mouse uterus and cervix.

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**Table of Contents category:** Reproductive physiology

### Key points summary

- Older pregnant women have greater risk of operative delivery, still birth and post-term induction.
- This suggests that maternal age can influence the timing of birth and processes of parturition.

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- We have found that increasing maternal age in C57BL/6J mice is associated with prolongation of gestation and length of labour.
- Older pregnant mice also had delayed progesterone withdrawal and impaired myometrial function.
- Uterine ageing and labour dysfunction should be further investigated in older primigravid women.

### **Abstract**

Advanced maternal age ( $\geq 35$  years) is associated with increased rates of operative delivery, stillbirth, and post-term labour induction. The physiological causes remain uncertain, although impaired myometrial function has been implicated. To investigate the hypothesis that maternal age directly influences successful parturition, we assessed the timing of birth and fetal outcome in pregnant C57BL/6J mice at 3 months (young), 5 (intermediate) months vs. 8 months (older) of age using infra-red video recording. Serum progesterone profiles, myometrium and cervix function, and mitochondrial electron transport chain complex enzymatic activities were also examined. Older pregnant mice had longer mean gestation and labour duration ( $P < 0.001$ ), as well as reduced litter size ( $P < 0.01$ ) vs. 3 month old mice. Older mice did not exhibit the same decline in serum progesterone concentrations as younger mice. Cervical tissues from older mice were more distensible than younger mice ( $P < 0.05$ ). Oxytocin receptor and connexin-43 mRNA expression were reduced in myometrium from 8 month vs. 3 month old mice ( $P < 0.05$ ,  $P < 0.01$  respectively) in tandem with more frequent, but shorter duration spontaneous myometrial contractions ( $P < 0.05$ ) and an attenuated contractile response to oxytocin. Myometrial mitochondrial copy number was reduced in older mice, but there were no age-induced changes to the enzymatic activities of the mitochondrial electron transport chain complexes. In conclusion, 8 month old mice provide a useful model of reproductive ageing. This study has identified potential causes of labour dysfunction amenable to investigation in older primigravid women.

### **Abbreviations**

B2M,  $\beta_2$  microglobulin; CAP, contractile associated protein; Cx43, connexin-43; ELISA, enzyme-linked immunosorbent assay; ETC, electron transport chain; GAPDH, glyceraldehyde

3-phosphate dehydrogenase; MIT, mean integral tension; MMP2, matrix metalloproteinase-2; MtDNA, mitochondrial DNA; Mt/N, mitochondrial/nuclear genome ratio; OT, oxytocin; OTR, oxytocin receptor; PBS, phosphate-buffered saline; PGE2, prostaglandin E2; PTGS2, prostaglandin-endoperoxide synthase 2; PSS, physiological saline solution; qPCR, quantitative reverse transcription polymerase chain reaction; ROS, reactive oxygen species.

## Introduction

Over recent decades, the average age of primigravid mothers in developed countries has progressively increased, with women (including multiparous women) over the age of 35 comprising a significant proportion of the pregnant population (Office for National Statistics for England and Wales, 2013; Matthews & Hamilton, 2014). Such trends are accompanied by a simultaneous rise in the incidence of pregnancy complications such as post-term induction, failure to progress in labour, and postpartum haemorrhage (Ecker *et al.*, 2001; Roos *et al.*, 2010; Yogev *et al.*, 2010). Advanced maternal age is also associated with an increase in Caesarean section and instrumental delivery rates (Smith *et al.*, 2008; Ludford *et al.*, 2012; Karabulut *et al.*, 2013; Herstad *et al.*, 2015).

There is limited understanding of the links between maternal age and the processes of timing of parturition. Spontaneous contraction of isolated human pregnant and non-pregnant myometrium *ex vivo* is reported to decline with advancing maternal age, with myometrium from pregnant women exhibiting increased multiphasic contractions (Smith *et al.*, 2008; Arrowsmith *et al.*, 2012). Others have suggested that myometrial tissue responds less effectively to uterotonic agents such as oxytocin or prostaglandins with increasing maternal age (Greenberg *et al.*, 2007; Arrowsmith *et al.*, 2012), supported by the observation that women of older age are more likely to require oxytocin-augmentation for induction of labour (Adashek *et al.*, 1993; Main *et al.*, 2000).

Age may also influence cervical ripening. A retrospective, cohort study revealed that maternal age above 30 years was an independent and significant predictor of cervical ripening failure in response to prostaglandin E2 (PGE2) (Melamed *et al.*, 2010), suggesting abnormal age-related timing of cervical ripening or impaired PGE2 responsiveness. Maternal age greater than 35 years has also been implicated as an independent risk factor of 'failure to pro-

gress in labour', during the first stage of labour, normally associated with cervical dilatation and regular myometrial contractions (Sheiner *et al.*, 2002).

Studies in rodents may give further insight into mechanism and improve upon experimental control and tissue availability compared to the clinical setting, but have seldom been undertaken in appropriately aged and nulliparous rodents. Holinka *et al.*, (1978) suggested that multiparous mice at the extreme limit of reproductive viability (11-12 months) have extended gestations compared to younger multiparous mice (3-7 months), possibly due to changes in the maternal progesterone status (Holinka *et al.*, 1978). However, the impact of maternal age in nulliparous mice at an age more relevant to older pregnant women, i.e. at the initial stages of reproductive ageing (~ 8 months) was not studied. In another report, spontaneous myometrial contractility *in vitro* was reduced in tissues from older 24 week old Wistar rats compared to 8 week old animals, but this was unrelated to expression of myometrium contraction associated proteins, or plasma lipid or pregnancy-related hormone profiles. There was also no evidence of a delay in timing of delivery (Elmes *et al.*, 2015) suggesting their model does not reflect adequately the situation in older pregnant women. A possible reason for this is that a rat age of 24 weeks of age is more representative of an 18-20 year adult rather than a 35 year old (Sengupta, 2013).

The aim of this study, therefore, was to develop a more appropriate mouse model of reproductive ageing to address parallels with older (~ 35 years) nulliparous pregnant women and explore the impact on gestational age at parturition as well as uterine and cervical function. We hypothesised that the timing/initiation of labour would be prolonged in older dams as a consequence of a delay in parturition signals affecting the physiological ripening of the cervix and the activation of myometrium. We also explored the notion that ability of myometrium to contract at term would deteriorate in older mothers as a result of age-related mitochondrial dysfunction.

## **Materials and Methods**

### **Ethical Approval**

Institutional Animal Welfare Committee approval was not required as no regulated procedure were used for this study. No animal was anaesthetised or underwent any surgical procedure.

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All animals were treated and killed in accordance with the Animals (Scientific Procedures) Act 1986 guidelines and experimental details conform to the animal ethics checklist (Grundy, 2015).

### **Animals**

C57BL/6J mice (21.5-30.5 g; 3-8 months of age, Charles River Laboratories, UK) were maintained under controlled conditions (25°C, 12-hour light/dark cycle), and received water and standard chow diet *ad libitum*. Females (all ages) were mated with C57BL/6J males (3-4 months of age, Charles River Laboratories, UK). Conception was determined by the presence of a vaginal plug (day 0 of gestation). A total of 510 mice were used for this study. This number comprised non-pregnant 3 month old (n=60), 5 month old (n=50), 8 month old mice (n=60), late pregnant 3 month old (n=90), 5 month old (n=40), 8 month old mice (n=110), full term 3 month old (n=8), and 8 month old mice (n=8) as well as 84 pups born to the full term mice.

### **Development of the mouse model of advanced maternal age**

Three age groups of female mice were selected for experimentation; all were capable of reproduction (3, 5 and 8 months of age). This was informed by previous studies of the oestrous cycle in ageing C57BL/6J mice (Nelson *et al.*, 1982, Felicio *et al.*, 1984) which reported that peak cycling occurred at 3 to 5 months of age, but that by 9 months normal cycling was disrupted and average cycle lengths became progressively longer, indicative of reproductive ageing. Virgin C57BL/6J mice at 8 months of age were used to model human nulliparous advanced maternal age. The 3 month old mice acted as controls, assuming this to be a period of maximal reproductive potential. The 5 month old mice represented an intermediate age group for some experiments. Where appropriate, experimental techniques were repeated with age matched non-pregnant female mice.

### **Tissue collection**

Mice were killed by cervical dislocation and exsanguination, in accordance with Schedule 1 of The Animals (Scientific Procedures) Act 1986. To control for changes to uterine smooth muscle during the oestrous cycle, all non-pregnant tissues were collected in the oestrous stage of the cycle, confirmed by the presence of large cornified epithelial cells with very few or no visible nuclei in vaginal fluid collected by daily vaginal smearing. To control for changes to

uterine smooth muscle during the oestrous cycle, all non-pregnant tissues were collected in the oestrous stage of the cycle, confirmed by the presence of large cornified epithelial cells with very few or no visible nuclei in vaginal fluid collected by daily vaginal smearing.

Myometrium and cervixes from time mated late pregnant mice were collected on day 18 of gestation and blood samples for progesterone analyses taken between day 15 and 19 of gestation. Blood samples were immediately collected by cardiac puncture. Following centrifugation, (10 minutes, 13000 x g), serum was aspirated and stored at -80°C until analysis.

Uterine horns and cervixes were dissected and placentas and fetuses removed. Fetuses were killed by destruction of the brain and decapitation, in accordance with Schedule 1. Mouse uterine horns were cut longitudinally down the midline, and the endometrium gently removed with a cotton bud. Small, full thickness myometrium strips were dissected longitudinally with fibre structure and mounted vertically in the organ bath.

Uterine and cervical tissues were either immediately snap frozen in liquid nitrogen for DNA extraction and protein isolation, placed into RNAlater (Ambion, Warrington, UK) according to manufacturer's recommendations for RNA extraction and stored at -80°C, or transferred to ice-cold phosphate-buffered saline (PBS; Sigma-Aldrich, UK) for isometric tension recording. Cervical biopsies for histological analysis were fixed by overnight immersion in 10% (v/v) formal saline solution (formaldehyde solution 10% v/v in 0.9% NaCl solution; Fisher Scientific, UK), and then embedded in paraffin. Non-pregnant uterine and cervical tissues were similarly collected and processed.

### **Serum progesterone assay**

Serum progesterone concentrations from primiparous mice through late gestation (n=5-8) were determined using a progesterone ELISA kit (Enzo Life Sciences, UK).

### **RNA isolation and cDNA synthesis**

Myometrium (30 mg) was homogenised (TissueLyser; Qiagen, UK), and total RNA extracted from the lysate (RNeasy mini kit; Qiagen, UK) according to the manufacturer's recommendations. RNA sample quality and concentrations were verified by gel electrophoresis and spectrophotometric analysis using a NanoDrop ND-1000 spectrophotometer (Labtech, International Ltd, UK). RNA (500 ng) was used to synthesise cDNA using 0.25 µg random hexanu-

cleotide primers (Promega, Southampton, UK) and 200 IU Superscript III (Invitrogen, Paisley, UK).

### **DNA isolation**

Total genomic DNA was extracted from 25 mg of myometrial tissue. Tissue was homogenised in 160  $\mu$ l of PBS (Sigma-Aldrich, UK). DNA was extracted from the lysate using the ReliaPrep gDNA Purification kit (Promega, UK) according to manufacturer's recommendations. DNA was quantified as ng/ $\mu$ l and DNA integrity checked (NanoDrop ND-1000 spectrophotometer). Dilution was standardised to 50 ng/ $\mu$ l in a volume of 100  $\mu$ l using RNase/DNase free water (Qiagen, UK), and samples sonicated for 10 minutes (Pulsatron 55; Kerry Ultrasonics Ltd., UK) to shear the DNA prior to Mt/N ratio determination by quantitative real-time polymerase chain reaction (qPCR).

### **Quantitative real-time polymerase chain reaction**

qPCR was performed in accordance with Bustin et al., (2008). SYBR green chemistry (2x QuantiFAST SYBR green; Qiagen, UK) on a RotorGene 6000 (Qiagen, UK) using primers listed in Table 1. Forward and reverse primers were optimised to be used at 300 nM working concentrations, in a total reaction volume of 10  $\mu$ l. A pre-PCR cycle was run for 5 minute at 95°C followed by 42 cycles of 95°C for 10 s denaturation step, followed by 60°C for 30 s combined annealing/extension step. Test samples (volume of 2  $\mu$ l cDNA) were run in duplicate in parallel with cDNA standards of known gene copy number abundance ( $10^8$ - $10^1$  copies). Melt-curve analysis was performed to confirm the presence of one single product and non-template controls were run to assess genomic DNA contamination. Quantification cycle (also referred to as cycle threshold) values were used for analysis and abundance data was obtained for test samples by the generation of a standard curve based on the quantified cDNA. Quantification data for the genes of interest were expressed relative to the most stable reference genes from a panel of 3 [glyceraldehyde 3-phosphate dehydrogenase (GAPDH),  $\beta$ -actin and  $\beta_2$  microglobulin (B2M)], using GeNorm software (Vandesompele et al, 2002).

### **Infra-red video camera recording of mouse parturition**

Primiparous late pregnant (day 17 of gestation onwards, 3 and 8 months of age, n=8 for each) females were singly housed. Females were monitored continuously, using infrared video



cameras (600TVL Eyeball Dome; Qvis, USA) from the appearance of the first pup until the completion of parturition. Minimal nesting bedding was provided (Enviro-dri; Shepherd Speciality Papers, USA), to prevent the camera view being obscured. Mice were maintained under controlled conditions (25°C, 12-hour light/dark cycle: 0700-1900 h), with access to standard chow and water *ad libitum*. Cameras were connected to a Zeus Lite HDMI LX 4 Channel Full D1 Networked CCTV Recorder DVR 1TB Hard Drive (Qvis, USA), which recorded continuously with tracked time and date. Litter size, live pup weights and any pup mortality were recorded. Mice that failed to delivery after 21 days were culled and uteri dissected to confirm pregnancy loss. The precise time of birth was determined from the video recording by the appearance and complete delivery of the first pup, and gestation length calculated (days). Total parturition duration (hours) was determined as the time between the complete delivery of the first pup to the complete delivery of the final pup.

### ***Ex vivo* isometric tension measurements of mouse myometrium**

Myometrial muscle strips (approximately 5 x 2 x 2 mm) were dissected from mouse uterine horns from non-pregnant females in oestrous or late pregnant dams (gestation day 18). Strips were mounted in a 10 ml organ bath chamber (Panlab 8 Chamber Organ Bath System; ADInstruments, UK) and maintained in oxygenated physiological salt solution (PSS, pH 7.4; mM: NaCl 119, KCl 4.7, MgSO<sub>4</sub> 1.17, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.18, EDTA 0.025, glucose 6 and CaCl<sub>2</sub> 2.5; 37°C, 95% air-5% CO<sub>2</sub>; BOC Gases, UK). A resting tension of 29 mN (x2 slack length) was applied before an equilibration period (45 minutes) to establish spontaneous contractions. After 10 minutes of baseline measurement, spontaneous contractile activity (minimum 1 hour), and response to the contractile agonist oxytocin were evaluated (10<sup>-12</sup>-10<sup>-7</sup> M oxytocin concentration response curve; Syntocinon; Alliance Pharmaceuticals, UK). Contractile capacity of each myometrial tissue strip in response to a depolarising K<sup>+</sup> solution (PSS with 60 mM KCl substituted for NaCl) was measured following the recording of spontaneous contractility.

All data were recorded and analysed using LabChart 6 software (ADInstruments Limited, UK). Contractile periods were assessed using mean integral tension (MIT; the sum of the contraction integrals divided by the duration of the period assessed, and corrected for myometrial active tissue wet weight, expressed as mN.s/g). Contraction force was calculated as the mean amplitude of contractions over the assessment period. Contraction frequency was expressed as contractions/second, and the duration of a contraction as the time (seconds) be-

tween start and end. As oxytocin dose response curves were not sigmoidal,  $EC_{50}$  and  $E_{max}$  values could not be calculated; the slope of the linear response across the concentration range was therefore analysed.

### ***Ex vivo* tensile strength measurements of mouse cervix**

Cervical tensile strength was measured following an adaptation of a published method (Read *et al.*, 2007). Intact cervixes from non-pregnant in oestrous or late pregnant (day 18) females were dissected and all vaginal tissue removed (n=6-9). The preparation was mounted in a 10 ml organ bath chamber in oxygenated PSS (37°C). Cervixes were initially held at a slack length (at which a further 0.25 mm stretch led to an increase in passive tension), and equilibrated for 15 minutes. The inner diameter (resting diameter of cervical os) was measured as 0 mm stretch and the os was then isometrically stretched incrementally (1 mm every two minutes) until tension plateaued or, infrequently, when the tissue snapped/tore. The force required to distend the cervix and the tension exerted by the stretched tissue were analysed, force-strain curves generated and the slope of the linear component measured as a gauge of tissue elasticity /stiffness.

### **Histological studies in mouse cervix**

Whole intact cervical samples from non-pregnant females in oestrous or late pregnant (day 18) were fixed overnight (10% (v/v) formaldehyde solution in 0.9% NaCl solution; Fisher Scientific, UK), followed by automated processing (TP1020 tissue processor; Leica Biosystems, UK), and wax embedding (EG1150 heated paraffin embedding module; Leica Biosystems, UK). Paraffin wax sections (5-10  $\mu$ m longitudinally through cervical samples) were mounted on SuperFrost Plus slides (VWR International Ltd, UK). Masson's Trichrome Kit (Accustain® Trichrome stains; Sigma-Aldrich, UK) was used to identify structures and detect collagen (blue stain), including a positive control section (Trichrome TISSUE-TROL, Sigma Aldrich, UK). Images (x10 magnification of original) were taken using AZ100 multizoom microscope. Collagen content (area of blue stain) in each cervical section was calculated (% of total section area, binary units; NIS-Elements Version 4.0 software, Nikon Instruments Europe, UK). Identities of the sections were unknown to the observer.

MMP2 (matrix metalloproteinase-2) expression was localised by immunostaining (ImmunoCruz rabbit LSAB Staining System; Santa Cruz Biotechnology, Germany). Cervical sections were deparaffinised, immersed in 10 mM Tris-EDTA buffer (10 mM Tris Base, 1

mM EDTA, 0.05% Tween 20, pH 8.0), and heated at highest power for 10 minutes in a microwave (antigen retrieval). Sections were incubated with MMP2 primary antibody (1:500; Anti-MMP2 ab37150; Abcam, UK) overnight at room temperature in a humidified chamber, and counterstained with hematoxylin (Gill's formulation number 2 Hematoxylin; Sigma-Aldrich, UK). Images were taken at x10 magnification of original (AZ100 multizoom microscope and NIS-Elements version 4.0 software; Nikon Instruments Europe, UK). Brown staining was quantified using ImageScope (Aperio Technologies Ltd, UK). The percentage of positive labelled cells per x10 magnification field was determined using the 'positive pixel count' function. Results were expressed as 'positivity', accounting for the number of positive pixels and intensity of staining. Identities of the sections were unknown to the observer.

### **Mitochondrial DNA copy number in mouse myometrium**

Genomic DNA was extracted from mouse myometrium from non-pregnant females in oestrous and late pregnant (day 18) mice as described; n=8. Real Time qPCR was performed for the primers listed in Table 2 (Malik *et al.*, 2016). Data were expressed as Mt/N ratio: the qPCR derived copy number for the mouse mitochondrial genome relative to the mouse B2M copy number (Malik *et al.*, 2011; Malik *et al.*, 2016).

### **Mitochondrial electron transport chain enzymatic activities in mouse myometrium**

Myometrial samples (non-pregnant in oestrous and late pregnant gestation day 18, 50 mg, n=8) were homogenised (4°C in 1:9 (w/v) 100 mM potassium phosphate ( $K_2HPO_4 \cdot 3 \cdot H_2O$ ) buffer; pH 7.4. and centrifuged ( $1,500 \times g$ , 5 min, 4°C). Supernatants were stored at -80°C until required. Enzymatic activities of mitochondrial respiratory chain complexes: NADH-ubiquinone oxydo-reductase (complex I), succinate-ubiquinone oxydo-reductase (complex II), ubiquinol cytochrome c reductase (complex III) and citrate synthase were assayed using a spectrophotometer (Uvikon 941; Kontron Instruments, UK) according to previously published methods (Mourmoura *et al.*, 2011).

Protein content in homogenates was determined using the bicinchoninic acid (BCA) method with a commercially available kit (Thermo Scientific, Rockford, IL). The activities of the electron transport chain complexes were expressed in units/mg of homogenate protein content. To evaluate myometrial mitochondrial density, citrate synthase activity was adjusted for protein content.

## Statistical analysis

Student's *t*-test was used to assess data between two groups. One-way analysis of variance (ANOVA) followed by all pairwise multiple comparison (Tukey's test) when comparing three or more groups (GraphPad Prism version 5.0; GraphPad Software, USA); with comparisons presented are between pregnant and non-pregnant animals at the same age and between animals at different ages with the same pregnant status.

Data were expressed as mean  $\pm$  standard error of the mean (SEM) and  $P < 0.05$  was considered significant; 'n' refers to the number of animals or tissues used per test group. Linear regression analyses were carried out using Stata version 11.2 (StataCorp, College Station, USA),  $P < 0.05$  was considered significant. Pup weight was calculated as an average per litter. For assessment of myometrium spontaneous contractile activity, when there was more than one tissue strip per animal, the average value across all related strips was used. For assessment of oxytocin augmented concentration-responses, the rate of change in mean integral tension (slope) in response to increasing concentrations of oxytocin was calculated for each myometrium tissue strip and the results compared generalised least squares regression clustering on animal and with robust standard errors clustered by animal.

Power calculations were carried out based on published data. Animal numbers were calculated to give a minimum of 80% power to detect a minimum of 20% difference between groups at the  $P < 0.05$  level.

## Results

### Parturition in mice of advancing reproductive age

Eight mice per group were monitored in pregnancy and at delivery. Of the 8 month old mice, only 6 delivered at term; 25% failed to maintain pregnancy (confirmed by uterine dissection post day 21). All mice, regardless of age group, were confirmed pregnant on gestation day 15, but 63% of the older mice had some degree of intrauterine fetal loss and/or fetal resorption. All 3 month old mice delivered at term, with no fetal loss. Older 8 month old mice demonstrated increased gestational length ( $20.1 \pm 0.2$  days,  $n=6$ , *versus*  $19.1 \pm 0.1$  days for 3 month mice,  $n=8$ ,  $P < 0.001$ ) (Figure 1A). 8 month old pregnant mice also had longer labour duration ( $3.7 \pm 0.3$  hours,  $n=6$  *versus*  $1.0 \pm 0.2$  for 3 month old mice,  $n=8$ ,  $P < 0.001$ ) (Figure

1B) and reduced litter size ( $4.8 \pm 0.9$  pups,  $n=6$  litters, *versus*  $7.5 \pm 0.2$  pups for 3 month old mice;  $n=8$  litters,  $P < 0.01$ ; Figure 1C). Reproductive age had no effect on average pup weight per litter; 8 month old mice ( $1.59 \pm 0.07$  g, 29 pups from  $n=6$  litters, *versus*  $1.54 \pm 0.04$  g, 60 pups from  $n=8$  litters).

Linear regression analyses determined whether the prolongation of gestation and duration of labour in older mice could be explained not only by an influence of maternal age but also mechanistically because of the reduction in litter size. The impact of older age on gestation length was 1.03 days (95% CI: 0.59 to 1.47;  $P < 0.001$ ), which remained significant after adjustment for litter size. The effect of maternal age on gestation length was 0.64 days (95% CI: 0.12 to 1.16;  $P < 0.05$ ). Litter size had no significant influence on gestation length for both 3 and 8 month old mice (3 months slope:  $0.04 \pm 0.19$ ,  $r^2$ : 0.007,  $P = 0.848$ ; 8 months slope:  $-0.16 \pm 0.08$ ,  $r^2$ : 0.523,  $P = 0.104$ ; Figure 1D). The impact of maternal age on labour duration was 2.67 hours (95% CI: 1.93 to 3.41;  $P < 0.001$ ); after adjustment for litter size, the effect of maternal age on labour duration was 1.77 hours (95% CI: 1.14 to 2.39;  $P < 0.001$ ).

### **Progesterone measurements in late gestation**

The serum progesterone profile differed between 3 and 8 month old mice; in 3 month old mice the progesterone concentration fell between gestation day 15 to day 18 ( $109.5 \pm 22.55$  ng/ml *versus*  $30.5 \pm 6.59$ ;  $P < 0.01$ ; Figure 2) whereas this decline was not observed in older mice between gestation day 15 to day 19 ( $80.9 \pm 12.13$  to  $58.4 \pm 11.57$  ng/ml; Figure 2).

### **Effect of reproductive ageing on cervical distensibility**

Maximal cervical distension (increase in cervical os diameter) was similar in non-pregnant mice aged 3 and 5 months, but at 8 months was increased 1.7 fold compared to both 3 month old ( $10.6 \pm 0.2$  mm,  $n=8$ , *versus*  $6.2 \pm 0.2$  mm,  $n=6$ ,  $P < 0.001$ ), and 5 month old non-pregnant mice ( $6.3 \pm 0.2$  mm,  $n=8$ ,  $P < 0.001$ ; Figures 3A and 3B). In late pregnancy, cervixes from 8 month old mice were more distensible compared to the 3 month old mice ( $19.3 \pm 0.4$  mm,  $n=6$ , *versus*  $16.0 \pm 0.5$  mm,  $n=9$ ,  $P < 0.001$ ), and those of the 5 month old mice more distensible than 3 month animals ( $18.0 \pm 0.3$  mm,  $n=6$ ,  $P < 0.01$ ; Figures 3A and 3B).

Maximum distension was greater in mice of all ages in late pregnancy compared to non-pregnant mice; implying that cervical softening had occurred in all groups by day 18 of gesta-

tion. The maximal distension for late pregnant cervical tissues compared to maternal age matched non-pregnant animals was 2.59, 2.88 and 1.82 fold higher for 3, 5 and 8 month old mice respectively (n=6-9,  $P < 0.0001$  for all; Figures 3A and 3B).

Cervical stiffness was determined as the slope of cervical distension stress-strain curves shown in Figure 3A. There was no difference in cervical stiffness between non-pregnant 3 and 5 month old mice ( $7.1 \pm 0.2$  g/mm, n=6;  $6.6 \pm 0.2$  g/mm, n=8 respectively) but stiffness at both ages was approximately twice that at 8 months ( $3.4 \pm 0.2$  g/mm, n=8,  $P < 0.001$ ),  $P < 0.001$  for both comparisons (Figure 3C). Slopes of the cervical distension stress-strain curves for all ages of late pregnant mice were similar and all were lower than age matched non-pregnant animals by a factor of 0.32, 0.27 and 0.49 for 3, 5 and 8 month old mice respectively (n=6-9,  $P < 0.0001$  for all; Figure 3C).

#### **Determination of collagen in cervical tissues**

Masson's trichrome blue staining (expressed as a percentage of the total cervical area) was similar between the three age groups in the non-pregnant and late pregnant cervical tissues. The transition from non-pregnant to late pregnancy in all age groups was associated with reduced cervical collagen content; a 28 % decline in 3 month old mice compared to age matched non-pregnant animals ( $63.0 \pm 4.2$  to  $34.6 \pm 3.5$  %,  $P < 0.001$ ), a 29 % reduction in 5 month old mice ( $64.1 \pm 3.4$  to  $34.9 \pm 1.7$  %,  $P < 0.001$ ), and a 32 % reduction for 8 month old mice ( $69.8 \pm 2.4$  to  $37.8 \pm 2.1$  %,  $P < 0.001$ ); n=6 for all groups (Figure 4C). This was evidenced by weaker and sparser blue staining as shown in Figures 4A and 4B.

#### **Expression of MMP2 in cervical tissues**

MMP2 staining was localised in the columnar and squamous epithelium as well as the stroma in both non-pregnant and late pregnant cervical tissues for all three ages. MMP2 protein expression (mean  $\pm$  SEM arbitrary units of positivity), was not significantly different between the three age groups in non-pregnant or late pregnant cervical tissues. MMP2 expression was  $0.086 \pm 0.025$ ,  $0.074 \pm 0.011$  and  $0.082 \pm 0.010$  arbitrary units in cervical tissues from non-pregnant 3, 5 and 8 month old mice respectively. Expression was  $0.040 \pm 0.015$ ,  $0.055 \pm 0.007$  and  $0.058 \pm 0.015$  arbitrary units in cervical tissues from late pregnant 3, 5 and 8 month old mice respectively (Figure 5, representative staining).

#### **Contractile-associated protein gene expression analysis in late pregnant myometrium**

PTGS2 (prostaglandin endoperoxide synthase 2) mRNA expression was similar in non-pregnant and late pregnant myometrium (n=8 per group) and there were no age related differences in expression in myometrium from both groups. Cx43 (connexin 43) mRNA expression was reduced in late pregnant myometrium from 8 month ( $4.18 \pm 0.03$  log[Cx43 copy number]) compared with 3 month old mice ( $4.40 \pm 0.03$ ;  $P < 0.01$ , n=8 for both groups, Figure 6A). There were no other age-related differences in expression across groups. Cx43 expression was low in non-pregnant myometrium from all age groups, compared with late pregnancy, which was associated with increased expression in mice of all ages (fold change 6.16, 9.12 and 8.17 for 3, 5 and 8 month old mice respectively compared to non-pregnant tissues;  $P < 0.001$ , n = 8 for all groups; Figure 6A).

Myometrial expression of OXTR (oxytocin receptor) was reduced in 8 month late pregnant mice ( $2.67 \pm 0.19$  log[OXTR copy number]) compared to younger mice (3 month;  $2.11 \pm 0.12$ ,  $P < 0.05$ , n=8 for both groups). There were no other age-induced differences in OXTR expression levels across groups. Late pregnancy was associated with increased OXTR mRNA expression in all ages of mice, compared with non-pregnant myometrium; OXTR copy number increased 2.1 fold in 3 month old ( $P < 0.001$ , n=8 for both groups), 2.4 fold in 5 month old ( $P < 0.001$ , n=8 for both groups), and 1.8 fold in 8 month old ( $P < 0.01$ , n = 8 for both groups) late pregnant myometrium (Figure 6B).

### **Spontaneous contractile activity of non-pregnant and late pregnant myometrium**

MIT values for spontaneous myometrial contractions was similar between all age groups in both non-pregnant and late pregnant cohorts (n=8 mice per group, average of 4 tissue strips per animal) (Figure 7A). Spontaneous contractile activity was significantly higher in all late pregnant mice compared to non-pregnant mice of all age groups ( $P < 0.001$ ,  $P < 0.01$ ,  $P < 0.05$ , n=8 mice per group, average of 4 strips per animal).

The contractile response (mean peak amplitude) induced by 40 mM  $K^+$  PSS), was similar in myometrium from all non-pregnant age groups. In the late pregnant mouse, there numerical age-associated reduction in the contraction amplitude was not significant ( $P = 0.073$ , Figure 7B). High  $K^+$ -induced contraction amplitudes were significantly increased in myometrium from late pregnant mice compared to tissues from non-pregnant mice in all three age categories;  $P < 0.01$ ,  $P < 0.05$ , n=8 mice for each group, average of 4 tissue strips per animal (Figure 7B).

The frequency and duration of spontaneous contractions in myometrium from non-pregnant mice was similar regardless of age. In contrast, spontaneous contractions were more frequent (1.7 times) in myometrium from late pregnant 8 month old mice (0.05 Hz, n=8 mice, average of 4 tissue strips per animal) compared to 3 month old mice (0.03 Hz,  $P < 0.05$ , n=8 mice, average of 4 tissue strips per animal) (Figures 7C, D and E).

Individual contraction duration in tissues from late pregnant mice aged 8 months was reduced by 36 % and 41 % compared with 5 month old and 3 month old myometrium respectively ( $P < 0.01$  for both n = 8 mice for all groups, average of 4 tissue strips per animal). No statistical difference was identified between mean contraction duration of myometrium from 3 *versus* 5 month old mice (Figure 7F).

### **Effect of oxytocin on spontaneous contractions of non-pregnant and late pregnant myometrium**

In non-pregnant mice, oxytocin ( $10^{-12}$ - $10^{-7}$  M) did not augment contractile activity of spontaneously contracting myometrium *in vitro*. All statistical comparisons across all age groups were also non-significant (n=8 mice per group, average of 4 tissue strips per animal).

Oxytocin ( $10^{-12}$ - $10^{-7}$  M) augmented contractile activity (MIT) compared to baseline spontaneous contractile activity in late pregnant myometrium from mice in all age groups (Figure 8). Compared to 3 month old mice (slope 27.95, 95% CI: 23.41 to 32.49), the slope of the dose response was lower in 5 month (mean difference: -7.29, 95% CI: -12.31 to -2.28,  $P < 0.01$ ) and 8 month old mice (mean difference: -7.63, 95% CI: -13.92 to -1.35,  $P < 0.05$ ).

### **Mitochondrial DNA (MtDNA) content in myometrium**

MtDNA copy number (Mt/N ratio) was 26% lower in myometrium from non-pregnant 8 month ( $23.56 \pm 1.59$  copy number) compared to non-pregnant 3 month old mice ( $31.75 \pm 2.07$ ;  $P < 0.05$ ) (Figure 9). There was an age related decline in MtDNA copy number in late pregnant myometrium from 3 months ( $48.63 \pm 1.67$ ) to 5 months ( $38.61 \pm 1.60$ ; -25%,  $P < 0.01$ ) from 3 months to 8 months ( $28.80 \pm 1.72$ ; -41%,  $P < 0.001$ ) and from 5 months to 8 months (-21%)  $P < 0.01$ ; Figure 9). MtDNA copy number was higher in late pregnant than non-pregnant myometrium from 3 month (53%,  $P < 0.001$ ) and 5 month old mice (34%,  $P < 0.01$ ; Figure 9).

### **Mitochondrial electron transport chain enzyme activities in myometrium**



Figure 10A demonstrates that citrate synthase activity in myometrial tissue protein was not significantly affected by age in neither non-pregnant (3 months *versus* 8 months,  $P = 0.896$ ,  $n=8$  for both groups); nor late pregnant mice (3 months *versus* 8 months,  $P = 0.952$ ,  $n=8$  for both groups). Citrate synthase enzyme activity was numerically similar in late pregnant mouse myometrium compared to the non-pregnant state for both age sets; the differences were not significant ( $n=8$  for all groups; Figure 10A).

In tissues from the same groups of animals, complex I (NADH dehydrogenase) activity in myometrial protein was not modified by age in either non-pregnant (3 month old *versus* 8 month old,  $P = 0.465$ ) nor late pregnant mice (3 month old *versus* 8 month old,  $p = 0.075$ ) (Figure 10B). Myometrial tissue NADH dehydrogenase activity was not significantly altered by pregnancy in either age group compared to the non-pregnant state (Figure 10B).

Similarly, complex II (succinate dehydrogenase) activity was not significantly altered by age in both non-pregnant (3 months *versus* 8 months,  $P = 0.205$ ) and late pregnant mice (3 months *versus* 8 months,  $P = 0.654$ ) (Figure 10C). However, succinate dehydrogenase activity was significantly increased by pregnancy in myometrium from both 3 and 8 month old mice ( $p < 0.001$ ,  $P < 0.01$  respectively) (Figure 10C). Succinate dehydrogenase activity was significantly higher in myometrium from pregnant 3 month old (64%) and 8 month old (43%) mice compared to age-matched non-pregnant controls ( $P < 0.001$  and  $0.01$  respectively).

Complex III (ubiquinol cytochrome c reductase) activity was also not significantly altered by age in non-pregnant (3 months *versus* 8 months,  $P = 0.299$ ) and late pregnant mice (3 months *versus* 8 months,  $P = 0.399$ ) (Figure 10D). For both age groups, the enzyme activity in the myometrium was numerically higher in myometrial tissues from pregnant mice compared to the non-pregnant state, but these did not reach significance (Figure 10D).

## Discussion

This study confirmed the validity of using 8 month old pregnant C57BL/6J mice, a common transgenic mouse strain, as a model of reproductive ageing in relation to parturition by demonstrating that maternal age was associated not only with prolonged gestation, but also greater duration of labour. Mechanistically, this was associated with an absence of progesterone withdrawal at term in older mice and disrupted uterine priming activation, but the process of cervical softening appeared to be unaffected. There was also a significant reduction in

MtDNA copy number in myometrium from older mice which could contribute to impaired contractile activity via a reduction in overall ATP synthesis or altered intracellular  $\text{Ca}^{2+}$  regulation

### **The impact of mouse maternal age on gestation length and labour duration**

Accurate evaluation of timing of birth demonstrated that the older the age of mice at conception, the more likely they are to have prolonged pregnancies and longer labours compared to younger mice. This supports previous data that report a marked increase in gestation length seen with advancing maternal age across various species including mice and humans (Asdell, 1929; Terrill & Hazel, 1947; Brakel *et al.*, 1952; Soderwall *et al.*, 1960; Moore, 1963; Holinka *et al.*, 1978; Silk *et al.*, 1993; Jolly *et al.*, 2000; Roos *et al.*, 2010). The only previous study of aged C57BL/6J mice used proven breeders, rather than virgin mice, up to the age of 11 to 12 months and reported a gestation increase of 2.2 days (Holinka *et al.*, 1978). Our data shows that the length of gestation in primiparous C57BL/6J mice is prolonged from 8 months, an age that is more relevant to the human situation.

Labour duration was also significantly lengthened in older mice; previous animal studies have not examined this in any detail, so as far as can be ascertained. Our data agrees, however, with observations on reproductive ageing in humans where length of labour and risk of prolonged labour increases with advancing maternal age (Greenberg *et al.*, 2007). There are a number of possible explanations for the maternal age-related prolonged gestation length in the mouse model, but the most likely is the observed absence of an effective progesterone withdrawal, which is essential for the onset of parturition in rodents (Weiss, 2000). Young animals showed a reduction in progesterone (72 %) the day before delivery; in contrast older animals maintained progesterone concentrations the day before delivery (only 28 % reduction). Of interest is that despite the lack of progesterone withdrawal the older mice still went into labour. This brings into question the role of progesterone withdrawal in labour onset, although we cannot rule out that progesterone withdrawal occurs later and more quickly in older mothers and was not detected by our daily measurements up to 19 days. The apparent lack of progesterone withdrawal could also represent a disruption in the luteolysis pathway that inhibits/delays progesterone decline in older mice. This was not assessed in our study, but it has been reported that a significant decline in murine corpus luteum function during pregnancy normally only occurs at 10 to 11 months of age in mice (Harman & Talbert, 1970). One study of the effect of age on maternal progesterone concentrations in term parturient rats

aged 8 weeks and 24 weeks showed no difference, but this model was not one of advanced reproductive age (Elmes *et al.*, 2015).

We also identified a negative relationship between reproductive age at conception and litter size at term. Older mice delivered smaller litters and had a greater incidence of stillbirth, with a small number of older mice failing to deliver by 22 days of gestation, most likely due to pup resorption. A reduction in litter size in older animals has been reported previously in various species (Soderwall *et al.*, 1960; Finn, 1963; Holinka *et al.*, 1978, 1979; Lopes *et al.*, 2009; Monclus *et al.*, 2011; Kong *et al.*, 2012). Increased rates of resorption of conceptuses, intrauterine fetal deaths as well as stillbirth rate have also been reported in ageing rodents (Talbert, 1971; Holinka *et al.*, 1979; Niggeschulze & Kast, 1994; Akiyama *et al.*, 2006; Lopes *et al.*, 2009; Kong *et al.*, 2012).

The reduction in litter size in older mothers in this study supports data from humans that indicate that miscarriage increases with maternal age (Nybo Andersen *et al.*, 2000; Nagaoka *et al.*, 2011; Khalil *et al.*, 2013; Qiao *et al.*, 2014). Stillbirth and fetal loss are also common risk factors for mothers of advanced maternal age (Haavaldsen *et al.*, 2010). Possible causes for fetal loss in older animals in this study could be a series of reproductive senescence changes such as oocyte depletion and poor oocyte quality in aged ovaries resulting in the development of fewer and/or non-viable fetuses (Liu *et al.*, 2013). Decreased litter size can also be related to an increase in the number of defective blastocysts (as a consequence of abnormal embryonic development patterns) available for implantation. (Day *et al.*, 1991) or failure of the aged uterus to maintain embryonic development (Talbert, 1971; Holinka *et al.*, 1979; Kong *et al.*, 2012). Furthermore, as 8 month old mice tended to be slightly heavier, there may have been some influence of increased adiposity, but this was not controlled for in this study. Unlike, the study by Holinka *et al.*, (1978) on older animals, we did not find a significant relationship between litter size and gestation length. However, as this was not the primary focus of our study the numbers were small; it is possible that this relationship would become more apparent if more animals had been studied.

### **The impact of mouse maternal age on cervical function**

Having demonstrated that older mice have prolonged gestation and labour in parallel with altered progesterone withdrawal, we determined how this impacted on reproductive tissues and mechanisms underpinning the observed age-related phenotypic changes. Firstly, we hypothesised that delayed cervical softening (which occurs prior to ripening) in older mice could influence gestation length. Pregnant cervixes were generally more distensible than those of non-pregnant mice, data which complements the existing body of literature in rodents (Harkness & Harkness, 1959; Read *et al.*, 2007; Word *et al.*, 2007; Vargis *et al.*, 2012). Cervixes from older pregnant mice still displayed greater distensibility than the younger pregnant mice, but intriguingly this did not result in shorter labours. It suggests perhaps, that the acute cervical ripening phase may have more functional importance for labour progress than cervical softening. We attempted to examine the contribution of collagen and MMP2 expression (Stygar *et al.*, 2002; van Engelen *et al.*, 2008; Choi *et al.*, 2009; Akins *et al.*, 2011) to these changes in cervix biomechanics and softening using immunolocalisation techniques. There were no apparent age related changes in collagen and MMP2 in non-pregnant mouse tissues. This did not mirror the generalised age-associated changes reported for other collagen rich tissues and organs such as skin and bone (Wang *et al.*, 2002; Quan *et al.*, 2010).

In pregnant compared to non-pregnant animals, there was a reduction in cervical tissue collagen content, which could directly account for the pregnancy related change in cervical distension. There were no significant differences in collagen and MMP2 expression in cervixes on day 18 of gestation between younger and older animals. However, the period for cervical softening is long (day 12-18) so differences may not be that apparent when labour is delayed. Indeed, the role of MMPs has been attributed more to break down of extracellular matrix during the ripening rather than the softening phase of parturition (Read *et al.*, 2007). As ripening usually occurs within 24 hours of parturition (Read *et al.*, 2007) this may be a potential explanation as to why no significant differences between MMP2 expression in non-pregnant and late pregnant cervical tissues were detected. Cervical ripening also requires a drop in progesterone levels (Mahendroo *et al.*, 1999; Mahendroo, 2012) and since circulating progesterone remains relatively stable at the end of gestation in older 8 month old mice, this may be an indication that the onset of cervical ripening was also perturbed.

Taken together, it is difficult to conclude that age-induced changes in collagen and MMP2 cervical properties and cervical distension have a major impact on the progress of labour in this model. To definitively confirm this, further quantitative methods for comparing soluble

and interstitial collagen content should be performed, along with Western blot and zymography for MMP2. Also the ~ 24 hour delay in parturition seen in 8 month old mice needs to be taken into account by examining cervixes on day 19 for this group.

### **The impact of mouse maternal age on uterine function**

We also ascertained there was an inherent decrease in myometrial contractile function *in vitro* which could explain the prolongation of pregnancy and labour in older mice. These data provide additional insight to previous literature where a negative correlation between contractile activity and increasing maternal age has been reported for women (Smith *et al.*, 2008) and in rats where mature parturient females demonstrate reduced spontaneous myometrial contractile activity compared to adolescent rats (Elmes *et al.*, 2015).

At the molecular level, we detected small changes in Cx43 and OXTR expression in the older group of mice in parallel with the alterations in contractile frequency and duration in myometrium. This suppression may be due to the lack of progesterone withdrawal on day 18 in the older mice, or reduced fetal occupancy (reduced litter size) limiting the influence of uterine stretch on the induction of these CAPs (Ou *et al.*, 1997; Ou *et al.*, 1998; Lyall *et al.*, 2002; Terzidou *et al.*, 2005). This contrasts with Elmes *et al.* (2015) who reported that maternal age had no influence on Cx43 protein expression in pregnant rat myometrium, but as discussed previously this study used 24 week old rats which represents full maturity rather than the point of reproductive decline as used in our model (Elmes *et al.*, 2015). The upregulation of Cx43 and OXTR in late pregnancy compared to the non-pregnant state was similar to other studies in rodents (Ou *et al.*, 1997; Ou *et al.*, 1998).

The functional impact of Cx43 and OXTR changes are difficult to directly elucidate as expression was still relatively high in older animals. However, assuming that the majority of mRNA signal comes from smooth muscle rather than embedded blood vessels, this small decrease in mRNA could result in a reduction in gap junction number and oxytocin receptor in myometrium and in part explain the reduction in oxytocin-augmented contractility in tissues from older mice. These data are consistent with published findings that report reduced contractile responses to oxytocin in myometrium from pregnant women aged 40 and above (Arrowsmith *et al.*, 2012).

In the present study, there was no specific age-induced decrease in spontaneous contractile activity of non-pregnant mice and oxytocin did not augment contractile activity, most likely

because OXTR mRNA expression was low. In contrast, Arrowsmith and colleagues have reported a striking reduction in spontaneous contractile force in non-pregnant human myometrium from older women (post 30 years old) when compared to myometrium from non-pregnant 25-29 year olds (Arrowsmith *et al.*, 2012).

Returning to molecular events, despite evidence to support the role of PTGS2 in human and mouse pregnancy (Reese *et al.*, 1999; Slater *et al.*, 1999; Gross *et al.*, 2000; Tsuboi *et al.*, 2000; Tsuboi *et al.*, 2003), expression of PTGS2 was not induced in term myometrium or regulated by age. This might be because we assessed myometrium on the day before delivery for the younger mice and two days before the 8 month old group. Tsuboi *et al.*, (2000) reported an absence of PTGS2 mRNA upregulation in the myometrium of wild-type mice on the day before parturition, but strong signals for PTGS2 mRNA on the day of parturition. Since myometrial tissues were taken on day 18 of gestation, it would be beneficial to repeat this study using myometrial tissues collected on day 19 of gestation in older primiparous mice as well as on the day of parturition for both young and older mice.

### **The impact of mouse maternal age on uterine mitochondrial number and function**

The unique set of experiments we undertook to assess mitochondrial number and activity in myometrium from primiparous mice in early stages of reproductive ageing showed a clear reduction in the Mt/N DNA copy number seen with age in myometrial tissues from pregnant and non-pregnant animals. Interestingly, there was a pregnancy-associated enhancement in the Mt/N in younger mice, but this was suppressed in older mice. In contrast, mean activities of mitochondrial electron transport chain enzymes NADH dehydrogenase and ubiquinol cytochrome c reductase (complexes I and III), as well as citrate synthase activity in mouse myometrium were not influenced by age or pregnancy state. Succinate dehydrogenase (complex II) activity was also unaffected by age, but enhanced in pregnancy.

These data differ from the bulk of published data for cardiac and skeletal muscles and to some extent smooth muscle, where the process of ageing has often been associated with an alteration in the enzymatic activities of all or some of the mitochondrial electron transport chain complexes (Yen *et al.*, 1989; Cooper *et al.*, 1992; Torii *et al.*, 1992; Sugiyama *et al.*, 1993; Takasawa *et al.*, 1993; Ojaimi *et al.*, 1999; Kwong & Sohal, 2000; Lin *et al.*, 2000; Short *et al.*, 2005; Figueiredo *et al.*, 2008; Muller *et al.*, 2010; Padrao *et al.*, 2012). Some

contradictory data do exist, with alterations in mitochondrial respiratory complex activities not always apparent across all tissues in aged animals (Kwong and Sohal, 2000).

The reasons for minimal age-related changes in the present study may relate to the age of mice used as the majority of published studies have reported alterations in mitochondrial electron transport chain complex enzyme activities in muscles from animals far older than 8 months of age.

Irrespective of age, complex II was found to be significantly increased in pregnant myometrium. This is novel finding could be explained, in part, by increased cell number and/or hypertrophy in pregnant uterus (Douglas *et al.*, 1988; Shynlova *et al.*, 2006). However, we would also expect to see an increase in activities of all other electron transport complexes, which was not the case. Increased complex II activity is often associated with increased rates of ROS production by reverse electron flux from complex II to complex I (Quinlan *et al.*, 2012; Dedkova *et al.*, 2013). Increased ROS production leads to eventual reduction of oxidative capacities and ATP synthesis rate, which may be why activities of complex I and III were not increased during pregnancy. To definitively determine mitochondrial function in terms of oxidative capacity and ATP production, NADH availability (NADH/NAD<sup>+</sup> ratio) in the mitochondrial matrix should be measured.

The impact of pregnancy on one complex, cytochrome c-oxidase/complex IV activity, myometrium has been studied by Geyer & Riebschläger (1974) and they showed an increase in pregnant human and rat myometrium compared to non-pregnant myometrium (ages of human subjects were not reported; pregnant rats were 40 days of age).

The generalised age-related reduction in Mt/N DNA copy number in non-pregnant and pregnant myometrium at 8 month of age suggests that this reduction precedes any change in mitochondrial complex activity. Reduced mitochondrial copy number has been previously reported as a consequence of ageing in rodent and human skeletal muscle (Barazzoni *et al.*, 2000; Short *et al.*, 2005; Peterson *et al.*, 2012). According to the mitochondrial theory of ageing, a decline in MtDNA is a direct result of mitochondrial ROS accumulation which in turn causes progressive damage to MtDNA and other mitochondrial constituents. The limited increase in mitochondrial copy number in pregnant older animals compared to non-pregnant animals is likely to have physiological consequences in parturition and could help explain the reduction in contractile potential in myometrium from older mice. A reduction in Mt/N could reflect a

concurrent reduction in capacity to generate sufficient cellular energy (ATP) to drive uterine contractions but since several copies of MtDNA can be present in one mitochondria (Wiesner *et al.*, 1992) ATP production in mitochondria from older animals would have to be measured to confirm this.

The role of mitochondria and ATP provision in the control of uterine contractions has previously been studied in mouse non-pregnant myometrium (Gravina *et al.*, 2010). Pharmacological inhibition of mitochondrial complex I and disruption of the mitochondrial membrane potential caused a reduction in the force of myometrial contractions *in vitro*. The authors hypothesised that these reductions were not linked to ATP generation but suggested that mitochondrial  $\text{Ca}^{2+}$  handling has an independent role in determining uterine contractility (Chalmers & McCarron, 2008; Gravina *et al.*, 2010; Gravina *et al.*, 2011). This could be an alternative explanation; given the reduction in MtDNA copy number, for our observations that myometrium from older pregnant mice has altered contractility *in vitro*.

### Summary

This study confirms that both gestation and parturition are prolonged in a primiparous mouse model of maternal ageing, as well as exhibiting deterioration in reproductive capacity reflected by a decline in litter size. The mouse model mimics characteristics associated with ageing in humans including delayed onset of parturition, prolonged duration of labour as well as greater risk of fetal loss. It is a suitable model to further assess the mechanisms responsible for the age-induced changes demonstrated in this study.

Our study demonstrates that maternal age influences expression of CAPs and alterations in spontaneous and oxytocin augmented myometrial contractions in older mice. These data suggest that myometrium from older mice on day 18 of gestation may not be fully “activated” for parturition mediated either by the limited progesterone withdrawal in the older mice or reduced uterine stretch due to litter size. Reduced Mt/N copy number in myometrial tissues from older pregnant mice implies myometrium in older mice have fewer mitochondria which will also potentially impact on myometrial contractile function in these animals.

Taken together, the data from this study highlight the physiological and cellular changes that occur with reproductive ageing. The reproductive tissue that is predominantly affected by ageing is the myometrium. This work highlights the need for additional research in this nas-



cent area in both animal models and human tissues in order to develop a more detailed understanding of the mechanistic impact of maternal ageing on labour. This is necessary to inform management of labour in older women, a burgeoning clinical problem.

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## Additional Information

### Competing interests

The authors have no conflicts of interest to declare

### Author contributions

RP, RMT, JDM, EM, LD and LP designed the experiments. RP performed most experiments at King's College London, U.K. Immunohistochemistry was carried out by RP and JDM at St George's University of London, U.K. Experiments to measure mitochondrial electron transport chain enzymatic activities were performed by RP and EM at Université Joseph Fourier, Grenoble, France. RP, EM and PTS analysed and interpreted data. RP, RMT, JDM, EM, LD, PTS and LP wrote the manuscript and critically revised it before submission.

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## TABLES

Table 1. Primer sequences used in qPCR

Gene	Primer Sequences
<b>mouse GAPDH</b> (glyceraldehyde-3-phosphate dehydrogenase) (accession number: NM_001001303)	<b>Forward primer:</b> 5'-TTGATGGCAACAATCTCCAC-3' <b>Reverse primer:</b> 5'-CGTCCCGTAGACAAAATGGT-3'
<b>mouse B2M</b> (beta-2 microglobulin) (accession number: NM_009735)	<b>Forward primer:</b> 5'-TTCAGTATGTTCCGGCTTCCC-3' <b>Reverse primer:</b> 5'-TGGTGCTTGCTCTCACTGACC-3'
<b>mouse <math>\beta</math>-actin</b> (accession number: NM_007393)	<b>Forward primer:</b> 5'-ATGGAGGGGAATACAGCCC-3' <b>Reverse primer:</b> 5'-TTCTTTGCAGCTCCTTCGTT-3'

**mouse OTR**

(oxytocin receptor)

(accession number: NM\_001081147.1)

**mouse Cx43**

(connexin 43)

(accession number: X61576.1)

**mouse PTGS2**

(Prostaglandin-endoperoxide synthase 2)

(accession number: NM\_011198.3)

**Forward primer:**

5'-GTGCAGATGTGGAGCGTCT-3'

**Reverse primer:**

5'-GTTGAGGCTGGCCAAGAG-3'

**Forward primer:**

5'-GTGCCGGCTTCACTTTCA-3'

**Reverse primer:**

5'-GGAGTAGGCTTGGACCTTGTC-3'

**Forward primer:**

5'-GGGAGTCTGGAACATTGTGAA-3'

**Reverse primer:**

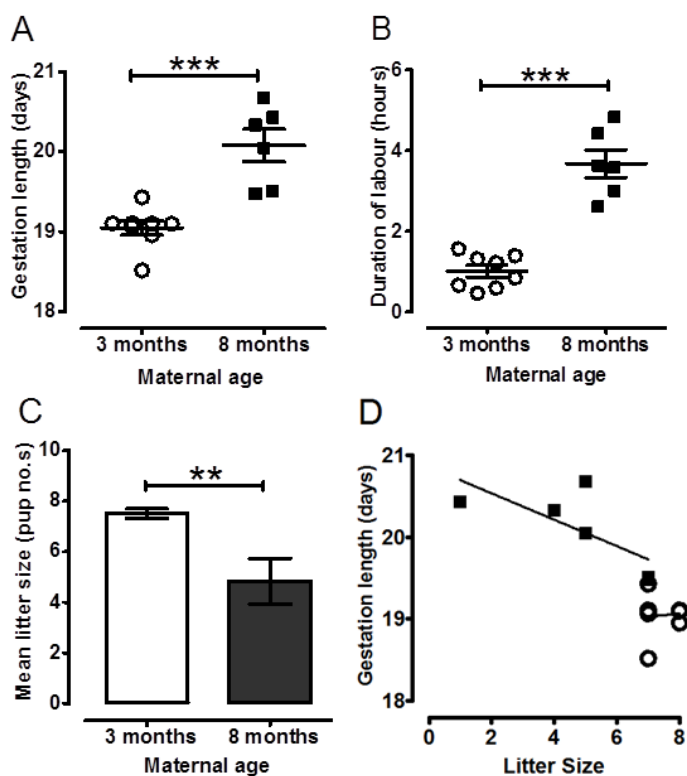
5'-GTGCACATTGTAAGTAGGTGGACT-3'

**Table 2. Primer sequences used to determine mitochondrial copy number**

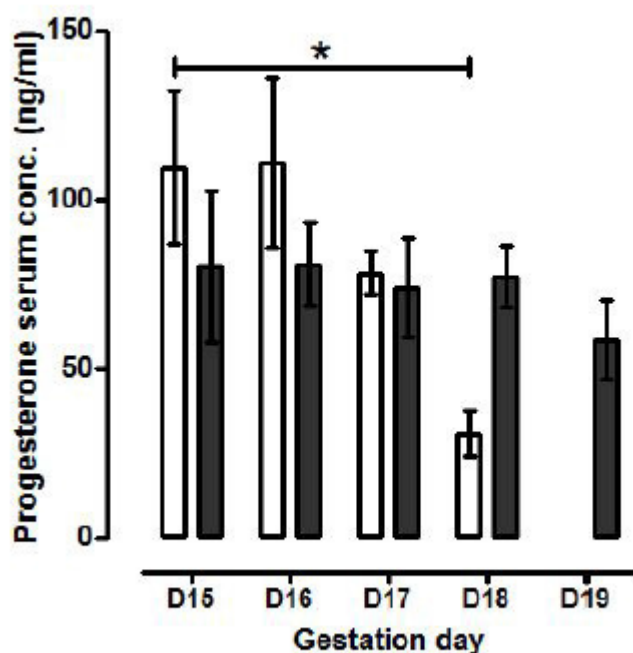
Gene	Primer Sequences
<b>mouse mitochondrion complete genome</b> (accession number: NC_005089.1)	<b>Forward primer:</b> 5'-CTAGAAACCCCGAAACCAA-3' <b>Reverse primer:</b> 5'-CCAGCTATCACCAAGCTCGT-3'
<b>mouse B2M</b> (accession number: NC_000068.8)	<b>Forward primer:</b> 5'-ATGGGAAGCCGAACATACTG-3' <b>Reverse primer:</b> 5'-CAGTCTCAGTGGGGTGAAT-3'

**FIGURE LEGENDS****Figure 1. Influence of maternal age on gestation length, labour duration and litter size.**

(A) Scatter graph presenting individual gestation lengths in 3 month old (n=8, open circles) and 8 month old (n=6, closed squares) pregnant mice. Gestation was longer in 8 month old mice, \*\*\*  $P < 0.001$ . (B) Scatter graph presenting individual labour durations in 3 month old (n=8, open circles) and 8 month old (n=6, closed squares) pregnant mice. Parturition was longer in 8 month old mice, \*\*\*  $P < 0.001$ . Horizontal bars indicate means, and error bars indicate  $\pm$  SEM in both (A) and (B). (C) Bar graph illustrating litter size for 3 month old (n=8, open bars) and 8 month old (n=6, closed dark grey bars) pregnant mice. Litter size was reduced in 8 month old mice, \*\*  $P < 0.01$ . Data expressed as mean  $\pm$  SEM. Data were analysed using Student's *t*-test for all (A), (B) and (C). (D) Scatter plot and linear regression lines illustrating the relationship between gestation length and litter size in 3 month old (n=8, open circles) and 8 month old (n=6, closed squares) pregnant mice. There was no significant influence of litter size on gestation length (3 months slope:  $0.04 \pm 0.19$ ,  $r^2$ : 0.007,  $P = 0.848$ ; 8 months slope:  $-0.16 \pm 0.08$ ,  $r^2$ : 0.523,  $P = 0.104$ ).

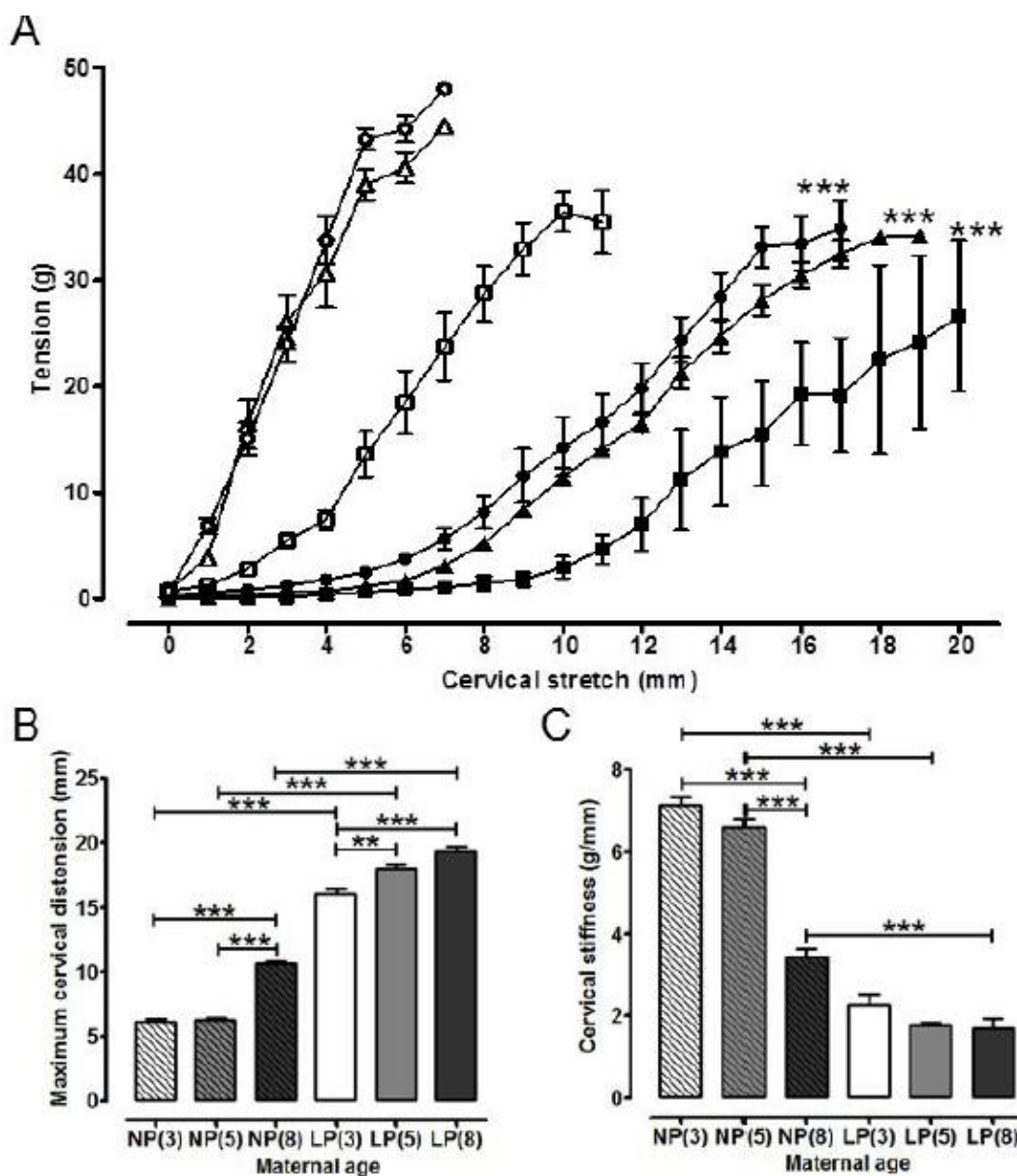


**Figure 2. Effect of maternal age on serum progesterone concentration.** Bar graph presenting serum progesterone concentrations through gestation days 15 to 19 (D15 – D19) for 3 month old ( $n=8$  for each day, open bars) and 8 month old ( $n=5-8$  for each day, closed dark grey bars) pregnant mice. 3 month old mice had a significant reduction in serum progesterone concentration from day 15 of gestation to day 18,  $* P < 0.05$ ; however serum progesterone concentrations were similar throughout gestation (D15-19) for older mice. Data were analysed using ANOVA followed by all pairwise multiple comparison Tukey's test and are presented as mean  $\pm$  SEM.



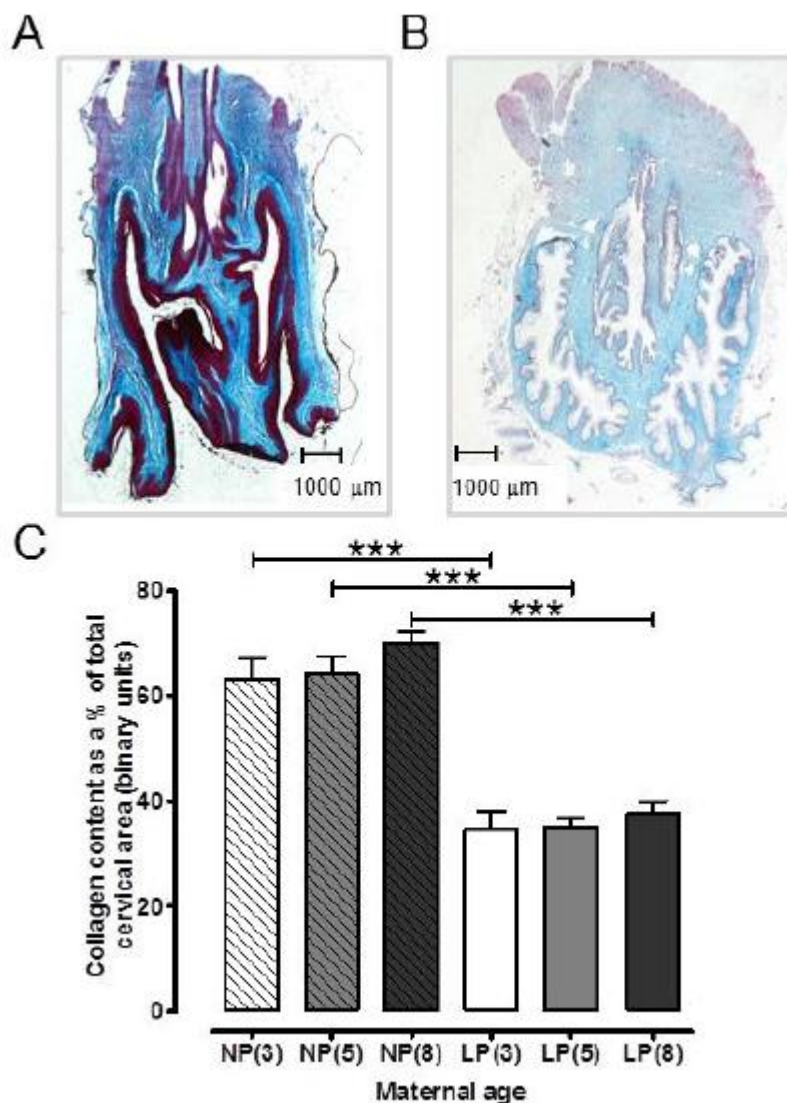
**Figure 3. The impact of maternal age on cervical distension and stiffness in non-pregnant and late pregnant tissues.** (A) Tension generated by the cervix is plotted as a function of incremental stretch of the cervical os diameter as stress-strain curves. Tissues obtained from non-pregnant 3 month (n=6, open circles), 5 month (n=8, open triangles), and 8 month (n=8, open squares) old mice, as well as late pregnant 3 month (n=9, closed circles), 5 month (n=6, closed triangles), and 8 month (n=6, closed squares) old mice. Cervical stretch (distension) means were different between non-pregnant compared to late pregnant mice of all three age groups (\*\*\*)  $P < 0.001$ . Cervices obtained from non-pregnant 3 month [NP(3), n=6, open bar with diagonal lines], 5 month [NP(5), n=8, closed grey bar with diagonal lines], and 8 month [NP(8), n=8, closed dark grey bar with diagonal lines] old mice, as well as late pregnant 3 month [LP(3), n=9, open bar], 5 month [LP(5), n=6, closed grey bar], and 8 month [LP(8), n=6, closed dark grey bar] old mice for (B) and (C). (B) Transition from non-pregnant to pregnant state, significantly increased maximal cervical distension in all three age groups, \*\*\*  $P < 0.001$ . Advance in age from 3 to 8 months, and 5 to 8 months in non-pregnant mice, and from 3 to 5 to 8 months in late pregnant mice also significantly increased maximal cervical distension, \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ . (C) Bar graph presenting cervical stiffness as determined by the slope of cervical distension stress-strain curves. Transition from non-pregnant to pregnant state, significantly reduced cervical stiffness in 3, 5 and 8

month old mice, \*\*\*  $P < 0.001$ . Advance in age from 3 to 8 months and 5 to 8 months in non-pregnant mice also significantly reduced cervical stiffness \*\*\*  $P < 0.001$ , however advance in age did not change cervical stiffness in tissues from late pregnant mice. Data were analysed using ANOVA followed by all pairwise multiple comparison Tukey's test and are presented as mean  $\pm$  SEM for (A), (B) and (C).



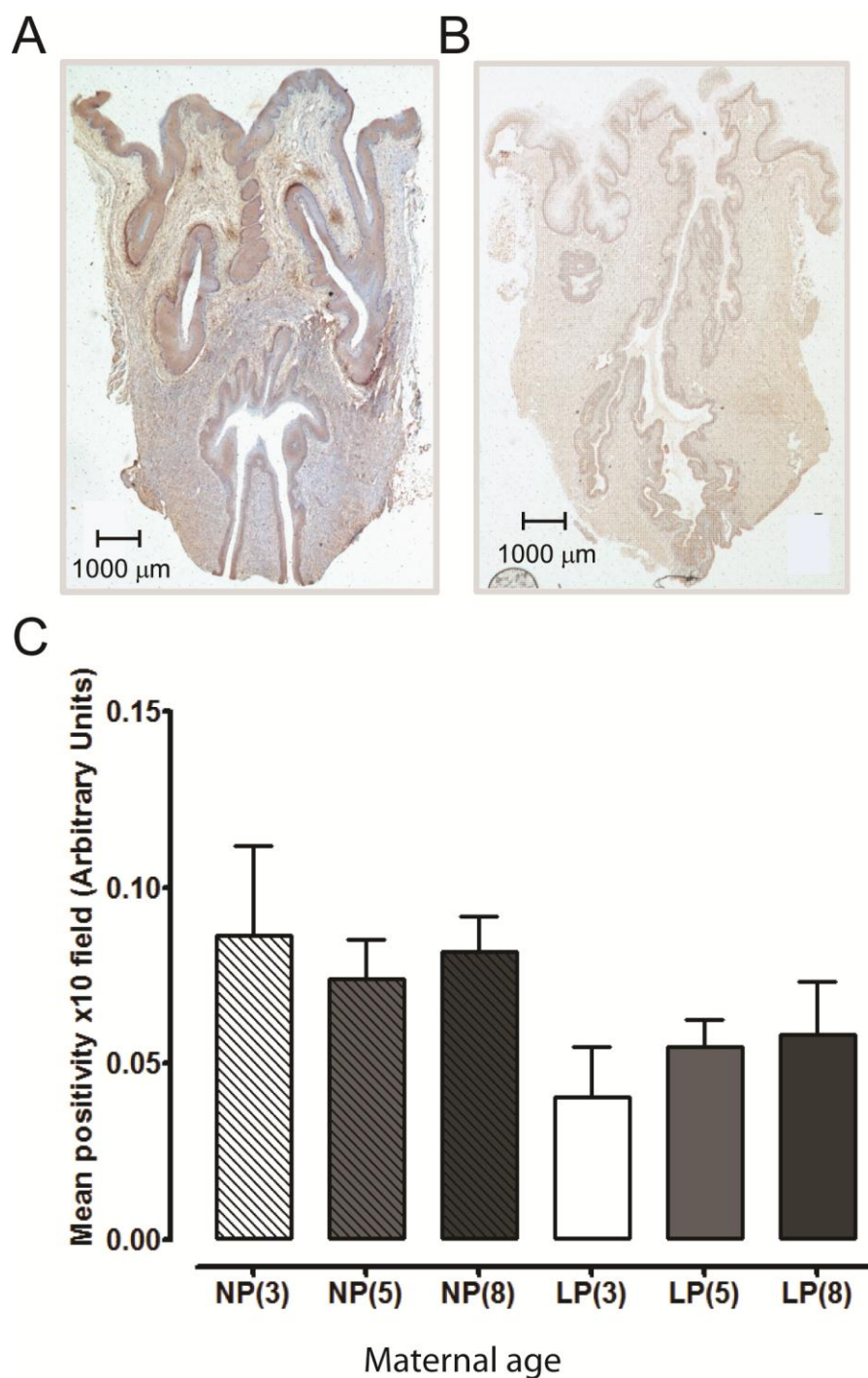
**Figure 4. Collagen content is lower in cervical tissues from late pregnant mice compared to non-pregnant mice.** Representative images of Masson's trichrome staining of collagen in cervical tissues from 8 month old non-pregnant (A) and late pregnant (B) mice. Collagen staining is noticeably weaker in the late pregnant cervix. Original magnification  $\times 10$ . (C) Bar graph presenting percentage of collagen content in cervical tissues obtained from non-pregnant 3 month [NP(3),  $n=6$ , open bar with diagonal lines], 5 month [NP(5),  $n=6$ , closed grey bar with diagonal lines] and 8 month [NP(8),  $n=6$ , closed dark grey bar with diagonal lines] old mice, as well as late pregnant 3 month [LP(3),  $n=6$ , open bar], 5 month [LP(5),  $n=6$ , closed grey bar], and 8 month [LP(8),  $n=6$ , closed dark grey bar] old mice. Transition from non-pregnant to pregnant state, signifi-

cantly reduced cervical collagen content in 3, 5 and 8 month old mice, \*\*\*  $P < 0.001$ . Data were analysed using ANOVA followed by all pairwise multiple comparison Tukey's test and are presented as mean  $\pm$  SEM.



**Figure 5. Expression of MMP2 is not significantly altered by the effect of age in cervical tissues from both non-pregnant and late pregnant mice**

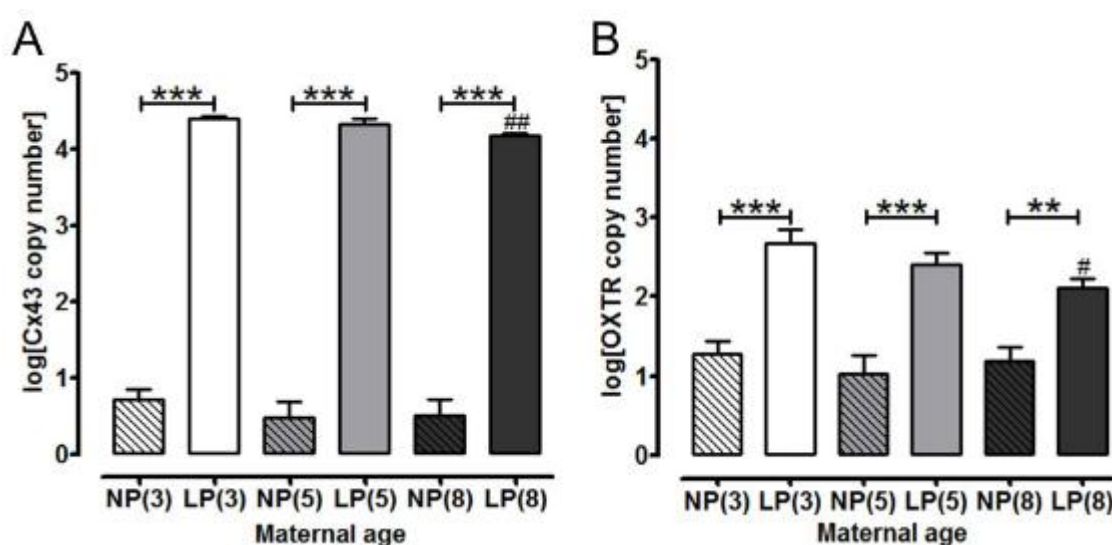
Representative images of immunohistochemical staining of MMP2 in cervical tissues from 8 month old non-pregnant (A) and late pregnant (B) mice. (C) Bar graph presenting expression of MMP2 in cervical tissues obtained from non-pregnant 3 month [NP(3), n=6, open bar with diagonal lines], 5 month [NP(5), n=6, closed grey bar with diagonal lines] and 8 month [NP(8), n=6, closed dark grey bar with diagonal lines] old mice, as well as late pregnant 3 month [LP(3), n=6, open bar], 5 month [LP(5), n=6, closed grey bar], and 8 month [LP(8), n=6, closed dark grey bar] old mice. Comparisons across all groups did not reach significance. Data were analysed using ANOVA followed by all pairwise multiple comparison Tukey's test and are presented as mean  $\pm$  SEM arbitrary positivity score.



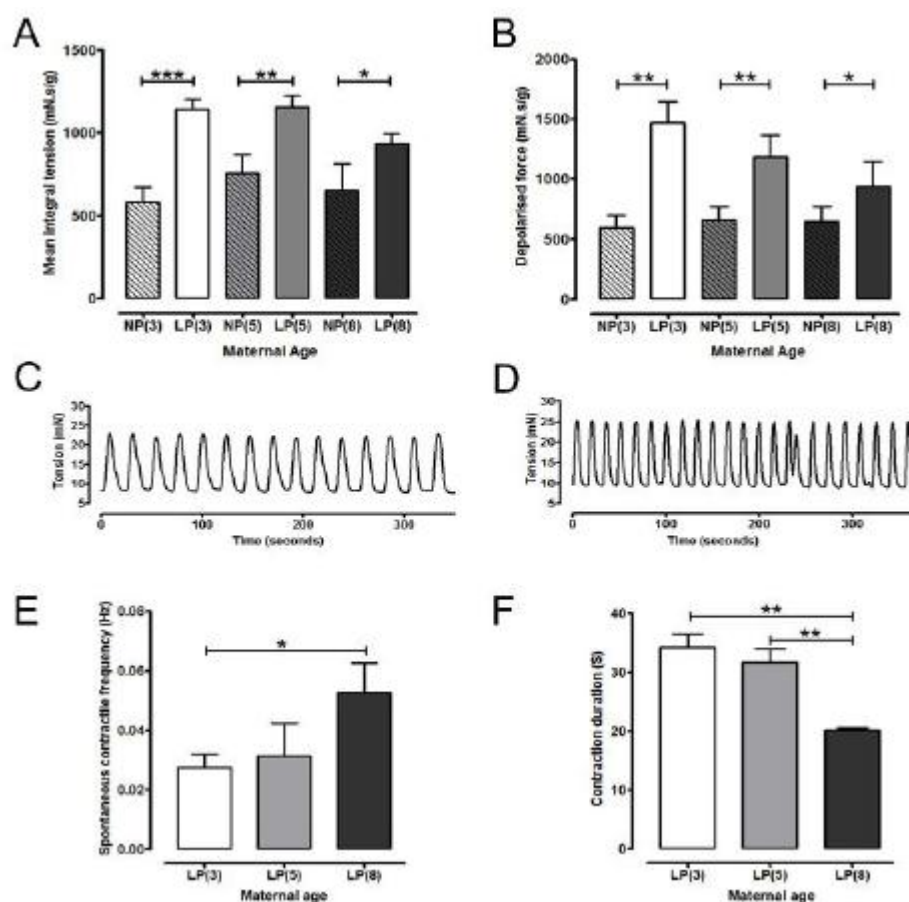
**Figure 6. Effect of maternal age on mRNA expression of connexin-43 and oxytocin receptor in myometrium from non-pregnant and late pregnant mice.** Myometrium was obtained from non-pregnant 3 month old [NP(3), n=8, open bars with diagonal lines] late pregnant 3 month old [LP(3), n=8, open bars], non-pregnant 5 month old [NP(5), n=8, closed grey bars with diagonal lines], late pregnant 5 month old [LP(5), n=8, closed grey bars] non-pregnant 8 month old [NP(8), n=8, closed dark grey bars with diagonal lines] and late pregnant 8 month old [LP(8), n=8, closed dark grey bars] mice for both (A) and (B). (A) Express-



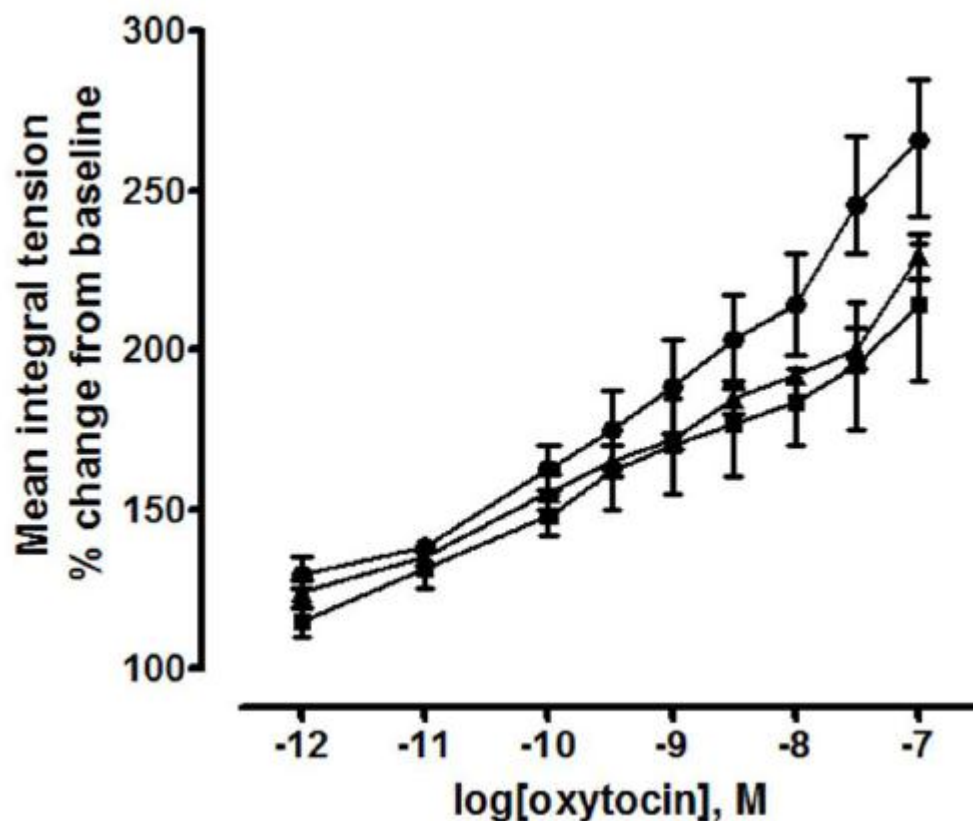
sion of connexin-43 was increased in late pregnant myometrium of all age groups compared to non-pregnant, \*\*\*  $P < 0.001$ , and lower in late pregnant myometrium from 8 month old mice compared to late pregnant 3 month old, <sup>#</sup>  $P < 0.01$ . **(B)** Expression of oxytocin receptor was significantly increased in late pregnant myometrium of all age groups compared to non-pregnant; \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , and lower in late pregnant myometrium from 8 month old mice compared to late pregnant 3 month old, <sup>#</sup>  $P < 0.05$ . Data were analysed using ANOVA followed by all pairwise multiple comparison Tukey's test and are expressed as  $\log(\text{mean of copy number}) \pm \text{SEM}$  normalised to reference genes B2M and GAPDH.



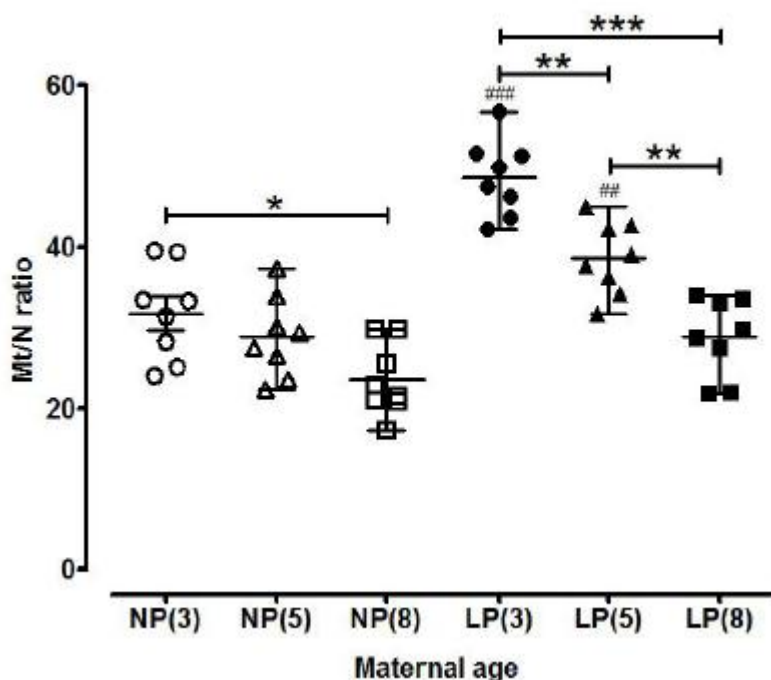
**Figure 7. Effect of maternal age on spontaneous contractile activity in myometrium from non-pregnant and late pregnant mice.** Myometrium was obtained from non-pregnant 3 month old [NP(3), n=32 strips from n=8, open bars with diagonal lines] late pregnant 3 month old [LP(3), n=32 strips from n=8, open bars], non-pregnant 5 month old [NP(5), n=32 strips from n=8, closed grey bar with diagonal lines], late pregnant 5 month old [LP(5), n=32 strips from n=8, closed grey bar] non-pregnant 8 month old [NP(8), n=32 strips from n=8, closed dark grey bar with diagonal lines] and late pregnant 8 month old [LP(8), n=32 strips from n=8, closed dark grey bar] mice for (A), (B), (E) and (F). (A) Mean integral tension was significantly higher in late pregnant myometrium compared to non-pregnant across all age groups, \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$ . (B) Force amplitude was significantly higher in late pregnant myometrium compared to non-pregnant across all age groups, \*\*  $P < 0.01$ , \*  $P < 0.05$ . (C) Representative trace showing spontaneous myometrial contractions from late pregnant 3 month old mouse. (D) Representative trace showing spontaneous myometrial contractions from late pregnant 8 month old mouse. (E) Spontaneous myometrial contraction frequency was significantly greater in late pregnant 8 month vs. late pregnant 3 month old mice, \*  $P < 0.05$ . (F) Myometrial spontaneous contraction duration was shorter in late pregnant 8 month vs. late pregnant 5 month and 3 month old mice, \*\*  $P < 0.01$ . Data were analysed using ANOVA followed by all pairwise multiple comparison Tukey's test and are presented as mean  $\pm$  SEM for (A), (B), (E) and (F), all significant comparisons ( $P < 0.05$ ) comparisons are indicated.



**Figure 8. Effect of oxytocin ( $10^{-12}$ - $10^{-7}$  M) on the contractile activity in myometrial tissues taken from late pregnant mice at different ages.** Line graph illustrating the effect of oxytocin ( $10^{-12}$ - $10^{-7}$  M) on contractile activity in myometrium from late pregnant 3 month old [LP(3), n=32 strips from n=8, closed circles], late pregnant 5 month old [LP(5), n=32 strips from n=8, closed triangles], and late pregnant 8 month old [LP(8), n=32 strips from n=8, closed squares] mice. Data expressed as mean integral tension expressed as a percentage increase of the baseline spontaneous activity  $\pm$  SEM. Application of oxytocin at concentrations  $10^{-12}$ - $10^{-7}$  M were able to augment mean integral tension in myometrium from mice of all ages; linear regression analysis.



**Figure 9. MtDNA copy number ratio (Mt/N) declines by the influence of maternal age in non-pregnant and late pregnant mouse myometrium.** Scatter graph depicting Mt/N in myometrium from non-pregnant 3 month old [NP(3), n=8, open circles], non-pregnant 5 month old [NP(5), n=8, open triangles] and non-pregnant 8 month old [NP(8), n=8, open squares], late pregnant 3 month old [LP(3), n=8, closed circles], late pregnant 5 month old [LP(5), n=8, closed triangles] and late pregnant 8 month old [LP(8), n=8, closed squares] mice. Mt/N was significantly reduced by the influence of age between NP(3) and NP(8) myometrium, \*  $P < 0.05$ , LP(3) and LP(8) myometrium, \*\*\*  $P < 0.001$ , LP(3) and LP(5) myometrium, \*\*  $P < 0.01$ , and between LP(5) and LP(8) myometrium, \*\*  $P < 0.01$ . Mt/N was increased by the influence of pregnancy between NP(3) and LP(3) myometrium, ###  $P < 0.001$ , and NP(5) and LP(5) myometrium, ##  $P < 0.01$ . Data were analysed using ANOVA followed by all pairwise multiple comparison Tukey's test and are presented as mean  $\pm$  SEM.



**Figure 10. Effect of maternal age on citrate synthase, complex I, complex II and complex III enzymatic activities in myometrium from non-pregnant and late pregnant mice.** Myometrium was obtained from non-pregnant 3 month old [NP(3), n=8, open bar with diagonal lines], non-pregnant 8 month old [NP(8), n=8, closed dark grey bar with diagonal lines], late pregnant 3 month old [LP(3), n=8, open bar], and late pregnant 8 month old [LP(8), n=8, closed dark grey bar] mice for (A), (B), (C) and (D). (A) There were no differences in citrate synthase enzyme activity. (B) There were no differences in complex I/NADH dehydrogenase enzyme activity. (C) Transition from non-pregnant to pregnant state increased complex II/succinate dehydrogenase activity in myometrium from both 3 and 8 month old mice, \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ . (D) There were no differences in complex III/ubiquinol cytochrome c reductase enzyme activity. Data were analysed using ANOVA followed by all pairwise multiple comparison Tukey's test and are presented as mean  $\pm$  SEM.

