CSIRO PUBLISHING

Reproduction, Fertility and Development, 2015, **27**, 957–968
http://dx.doi.org/10.1071/RD14450

Maternal age and *in vitro* culture affect mitochondrial number and function in equine oocytes and embryos

W. Karin Hendriks[A,F], *Silvia Colleoni*[C], *Cesare Galli*[C,D], *Damien B. B. P. Paris*[A,G], *Ben Colenbrander*[A], *Bernard A. J. Roelen*[A,B] *and Tom A. E. Stout*[A,B,E,H]

[A]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 114, 3584 CM Utrecht, The Netherlands.
[B]Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM Utrecht, The Netherlands.
[C]Avantea, Laboratorio di Tecnologie della Riproduzione, Via Porcellasco 7f, 26100 Cremona, Italy.
[D]Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia, Italy.
[E]Section of Reproduction, Faculty of Veterinary Science, University of Pretoria, Private Bag XO4, Onderstepoort, 0110, South Africa.
[F]Present address: Hendriks EQ Repro Consultancy, Zutphensestraatweg 32, 6955 AH Ellecom, The Netherlands.
[G]Present address: College of Public Health, Medical and Veterinary Sciences, James Cook University, Solander Drive, Townsville, Qld 4814, Australia.
[H]Corresponding author. Email: t.a.e.stout@uu.nl

Abstract. Advanced maternal age and *in vitro* embryo production (IVP) predispose to pregnancy loss in horses. We investigated whether mare age and IVP were associated with alterations in mitochondrial (mt) DNA copy number or function that could compromise oocyte and embryo development. Effects of mare age (<12 vs ≥12 years) on mtDNA copy number, ATP content and expression of genes involved in mitochondrial replication (mitochondrial transcription factor (*TFAM*), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single-stranded DNA-binding protein (*SSB*)), energy production (ATP synthase-coupling factor 6, mitochondrial-like (*ATP-synth_F6*)) and oxygen free radical scavenging (glutathione peroxidase 3 (*GPX3*)) were investigated in oocytes before and after *in vitro* maturation (IVM), and in early embryos. Expression of *TFAM*, *mtPOLB* and *ATP-synth-F6* declined after IVM ($P < 0.05$). However, maternal age did not affect oocyte ATP content or expression of genes involved in mitochondrial replication or function. Day 7 embryos from mares ≥12 years had fewer mtDNA copies ($P = 0.01$) and lower mtDNA : total DNA ratios ($P < 0.01$) than embryos from younger mares, indicating an effect not simply due to lower cell number. Day 8 IVP embryos had similar mtDNA copy numbers to Day 7 *in vivo* embryos, but higher *mtPOLB* ($P = 0.013$) and a tendency to reduced *GPX3* expression ($P = 0.09$). The lower mtDNA number in embryos from older mares may compromise development, but could be an effect rather than cause of developmental retardation. The general down-regulation of genes involved in mitochondrial replication and function after IVM may compromise resulting embryos.

Additional keywords: ATP, gene expression, mitochondrial quantity, mitochondrial replication.

Received 18 November 2014, accepted 6 March 2015, published online 17 April 2015

Introduction

Mares selected for breeding based on their performance in sports other than flat-racing (e.g. show jumping, dressage, polo) are often of advanced age (8–16 years old) at the onset of their breeding career. Unfortunately, fertility also begins to decline from around 12–14 years of age, as indicated by lower pregnancy rates on Day 15 after ovulation and an increased incidence of pregnancy loss, particularly in the Day 15–42 period (Morel *et al.* 2005; Allen *et al.* 2007). Embryo transfer (ET) is attractive because it enables sport and breeding to be combined and it can also help overcome one of the age-related contributors to reduced fertility, namely chronic endometrial

fibrotic degeneration (Carnevale and Ginther 1992). However, endometrial degeneration is probably not the primary contributor to the age-related decline in mare fertility. Embryos flushed from the oviducts of mares ≥20 years of age have been reported to undergo delayed cleavage and to contain fewer cells than those from younger mares (Carnevale and Ginther 1995), analogous to the situation in older compared with younger women (Baird *et al.* 2005). Combined with studies demonstrating that embryos from mares >18 years show lower survival rates than embryos from mares <6 years after transfer to young recipients (Ball *et al.* 1989), this indicates that the loss of developmental potential is intrinsic to the ovulated oocyte or arises during very early (intra-oviductal) embryo development. Further studies have clearly demonstrated that the principal contributor to the age-related decline in mare fertility is reduced oocyte quality, because oocytes from mares ≥15 years often exhibit morphological abnormalities associated with functional deficiencies that, in turn, contribute to a decrease in developmental competence (Carnevale and Ginther 1992; Carnevale 2008; Altermatt *et al.* 2012).

Another way of producing offspring from mares with impaired fertility, or without interrupting their sporting career, is *in vitro* embryo production (IVP). Despite the relatively low per oocyte success rates (18%–36% of recovered oocytes develop into a transferable blastocyst; Hinrichs *et al.* 2005; Galli *et al.* 2007), during the past 5 years IVP has become a commercially viable means of breeding horses. The increasing popularity of IVP despite the relatively high costs is explained by the number of transferable blastocysts per ovum pick-up (OPU) session (0.6–0.8; Galli *et al.* 2014), which compares favourably with embryo flushing in the field. Moreover, OPU–IVP is applicable to mares incapable of producing embryos in the normal manner (e.g. due to severe damage to the uterus or oviducts or because they repeatedly fail to ovulate normally; Galli *et al.* 2007) and/or when stallion semen availability or fertility is limiting (Lazzari *et al.* 2002).

In women, the principal symptom of the age-related reduction in oocyte quality is an increased risk of numerical chromosome abnormalities (aneuploidy) as a result of aberrant chromosome segregation (Franasiak *et al.* 2014). One of the proposed underlying predispositions to aneuploidy and compromised developmental competence of oocytes and embryos from older women is mitochondrial dysfunction. Compromised mitochondrial function appears to stem from defective mitochondrial replication (leading to a fall in mitochondrial numbers), an increase in the incidence of mitochondrial (mt) DNA aberrations, such as point mutations and deletions, and increased production and release of reactive oxygen species (ROS); Takeuchi *et al.* 2005; Carnevale 2008; Fragouli *et al.* 2011). The number of mitochondria in an oocyte can be estimated by analysis of mtDNA copy number because oocytes contain only one to two mtDNA copies per mitochondrion (Pikó and Matsumoto 1976; Pikó and Taylor 1987; Jansen 2000; Santos *et al.* 2006*b*; Jiao *et al.* 2007; Chiaratti and Meirelles 2010). For successful early embryonic development, a species-specific minimum number of mitochondria appears to be required; for example, mouse oocytes are thought to require at least 50 000–200 000 mtDNA copies (Cao *et al.* 2007; Cree *et al.* 2008; Wai *et al.* 2008, 2010), whereas bovine, porcine and human oocytes have been reported to require between 100 000 and 700 000 mtDNA copies if they are to retain developmental competence (Steuerwald *et al.* 2000; Reynier *et al.* 2001; May-Panloup *et al.* 2005*a*; Almeida-Santos *et al.* 2006*b*; Spikings *et al.* 2007; Zeng *et al.* 2007). However, during early embryonic development, mtDNA replication is transiently arrested (St. John *et al.* 2010) and therefore the mtDNA copy number either remains constant (e.g. mouse and rat; Thundathil *et al.* 2005; Kameyama *et al.* 2007; Wai *et al.* 2010) or decreases over time (e.g. cow and pig; May-Panloup *et al.* 2005*b*; Spikings *et al.* 2007). Mitochondrial replication is not reinitiated until the blastocyst stage in the cow and pig (May-Panloup *et al.* 2005*b*; Spikings *et al.* 2007) or until gastrulation in the mouse (Facucho-Oliveira *et al.* 2007). This temporary arrest in mitochondrial replication, despite a near exponential increase in the number of cells, underlines why the number of functional mitochondria present in a mature oocyte is of critical importance for subsequent developmental competence (May-Panloup *et al.* 2007).

The combination of advancing maternal age and IVP has been reported to negatively influence mtDNA copy number in oocytes and embryos. For example, maturation of oocytes from young (21–89 months) bovine donors resulted in a mean increase in mitochondrial copy number and ATP content, whereas maturation of oocytes from 'aged' (>89 months) bovine donors led to a decrease in both parameters (Iwata *et al.* 2011). Similarly *in vitro* maturation (IVM) of oocytes retrieved from older mares (≥12 years) led to a significant decrease in mitochondrial copy number that was not seen in younger mares (<12 years; Rambags *et al.* 2014).

Advancing maternal age and IVP have both been reported to induce alterations in mitochondrial function that can result in mitochondrial damage (Wilding *et al.* 2001; Wang *et al.* 2009). This manifests as a decrease in mtDNA copy number, a reduction in mitochondrial gene expression and an increased production of ROS within embryonic cells (Giritharan *et al.* 2007, 2010). Changes in mtDNA quantity can also result in alterations in the expression of genes involved in various aspects of the mitochondrial replication process, such as mitochondrial transcription factor (*TFAM*; Facucho-Oliveira *et al.* 2007), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single-stranded DNA-binding protein (*SSB*; St. John *et al.* 2010). Expression of these genes is normally upregulated when mtDNA replication is reactivated (Pikó and Taylor 1987; May-Panloup *et al.* 2005*b*; Thundathil *et al.* 2005; Spikings *et al.* 2007). Glutathione peroxidase (GPX) and ATP synthase (ATP synthase-coupling factor 6, mitochondrial-like (ATP-synth_F6)) are enzymes involved in balancing the generation and decomposition of ROS (Ufer and Wang 2011) to help prevent ROS levels reaching a point likely to induce oxidative damage. Increased expression of *ATP-synth_F6* has been described in oocytes collected from women >35 years, and in embryonic cells during IVP (Wang *et al.* 2009).

The aim of the present study was to determine whether advancing maternal age and IVP induce changes at the level of mitochondrial number and function in horse oocytes that may underlie reduced developmental competence. To this end we examined the effects of maternal age and IVP on mtDNA copy

number and the expression of genes involved in mitochondrial replication (*TFAM*, *mtPOLB*, *SSB*). Mitochondrial function was evaluated by measuring total oocyte ATP content and the expression of genes involved in energy production (*ATP-synth_F6*) and oxygen free radical scavenging (*GPX3*).

Materials and methods

Collection of cumulus–oocyte complexes

Ovaries were collected from 101 mares immediately after they had been killed and were divided into two groups based on donor mare age, namely younger (<12 years) or older (≥12 years), which was determined on the basis of the date of birth recorded in the mare's passport. The ovaries were transported to the laboratory at 30°C in a thermos flask and processed within 4 h postmortem. Cumulus–oocyte complexes (COCs) were collected as described previously (Tharasanit *et al.* 2006). Only oocytes with a complete multilayered cumulus investment (Hinrichs *et al.* 1993) were selected and maintained in HEPES-buffered M199 (GIBCO BRL Life Technologies; Bleiswijk, The Netherlands) supplemented with 0.014% (w/v) bovine serum albumin (BSA; Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands). For each mare age group, the COCs recovered were divided randomly into two groups. In the first group (not-IVM), oocytes were immediately denuded by vortexing for 4 min in calcium- and magnesium-free Earle's balanced salt solution (EBSS; GIBCO BRL Life Technologies) containing 0.25% (v/v) trypsin-EDTA (GIBCO BRL Life Technologies); removal of all cumulus cells was confirmed by microscopic examination. The denuded oocytes were washed twice in phosphate-buffered saline (PBS; Sigma-Aldrich Chemicals) containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich Chemicals) and placed individually in 10 µL lysis buffer (RLT + β-mercaptoethanol; Qiagen, Venlo, The Netherlands) in a 0.5-mL Eppendorf tube, snap-frozen and stored at −80°C until further processing (ATP measurement or DNA and RNA extraction). For the second group (IVM), COCs were incubated in groups of four to five for 30 h in 500 µL maturation medium consisting of M199 (GIBCO BRL Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; GIBCO BRL Life Technologies), 0.01 units mL^{-1} porcine FSH and 0.01 units mL^{-1} LH (Sigma-Aldrich Chemicals) in four-well plates (Nunc, Roskilde, Denmark) at 38.7°C in a humidified atmosphere of 5% CO_2 in air. After incubation, only the fully expanded COCs were transferred to EBSS containing 0.25% (v/v) trypsin-EDTA (GIBCO BRL Life Technologies) and denuded by vortexing for 4 min. The completely denuded oocytes were further processed as for Group 1 and stored at −80°C until ATP quantification or DNA and RNA extraction.

Embryo collection

Ten Dutch warmblood mares (age 4–22 years) were used to recover embryos on Day 7 after ovulation. All animal procedures were approved by Utrecht University's Institutional Animal Care and Use Committee (DEC 2007.III.02.036). During oestrus, the ovaries and uteri of the mares were examined daily by transrectal palpation and ultrasonography using a MyLab Five ultrasound machine (Esaote Pie Medical, Maastricht, The Netherlands) equipped with a 7.5-MHz linear array transducer. Once the dominant follicle exceeded 35 mm in diameter, ovulation was induced with intravenous injection of 1500 IU hCG (Chorulon; Intervet, Boxmeer, The Netherlands) and the mare was inseminated with >500 million motile spermatozoa from a single fertile stallion. Embryos were collected by non-surgical uterine lavage using three flushes with 1 L prewarmed (37°C) lactated Ringer's solution (LRS; Baxter, Lessines, Belgium) supplemented with 0.5% FCS (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Eight embryos each were recovered from younger (<12 years; average age 6.87 years, range 4–11 years) and older (>16 years; average age 19.25 years, range 17–22 years) mares. Embryos were washed 10 times with LRS to remove maternal cells and residual FCS before assessment under a dissecting microscope (SZ60; Olympus, Zoeterwoude, The Netherlands). Embryos were classified according to developmental stage (morula, early blastocyst or expanded blastocyst) and quality (1–4, good to degenerate), as described previously (Tremoleda *et al.* 2003), and their diameter was measured using a calibrated eyepiece micrometer. The embryos were then transferred with 10 µL LRS into 0.5-mL Eppendorf tubes, snap frozen in liquid nitrogen and stored at −80°C until DNA and RNA extraction.

Eight *in vitro* embryos were produced as described by Galli *et al.* (2001), with minor modifications. Briefly, COCs recovered from the ovaries of mares of mixed breed and unknown age that had been killed were subjected to IVM by incubating for 22–24 h in Dulbecco's modified Eagle's medium (DMEM)–F12 containing 10% serum replacement (SR; GIBCO BRL Life Technologies), ITS supplement (1.0 mg mL^{-1} insulin, 0.55 mg mL^{-1} transferrin, 0.5 µg mL^{-1} sodium selenite; Sigma-Aldrich Chemicals), 1 mM sodium pyruvate, 50 ng mL^{-1} epidermal growth factor (Sigma-Aldrich Chemicals), and 0.1 IU LH and FSH (Pergovet, Serono, Italy). Next, oocytes were denuded and returned to maturation medium for a further 2–4 h (i.e. up to 26 h maximum IVM). Thirty-two oocytes with an extruded first polar body were then fertilised by intracytoplasmic sperm injection (ICSI) with spermatozoa selected by Redigrad (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. Sperm injection was performed using a Piezo-driven unit (PrimeTech, Tsuchiura-shi, Japan) fixed on a micromanipulator equipped with a 37°C heated stage. Oocytes were held with the polar body oriented at the 6- or 12-o'clock position using a holding pipette with an inner diameter of 50 µm and outer diameter of 150 µm and injected with a blunt-ended needle with an inner diameter of 5 µm. Only motile spermatozoa were selected for ICSI and immobilised by two or three Piezo pulses before injection. The resulting presumptive zygotes were cultured in synthetic oviductal fluid (SOF) supplemented with minimum essential medium (MEM) essential and non-essential amino acids, glutamine and BSA and examined for cleavage on Day 2 after injection; those showing development to the 2-cell stage or more (26/32; 81.25%) were cultured *in vitro* for an additional 6 days, with the culture medium refreshed on Days 4 and 6 (Galli *et al.* 2007). On Day 8 of development, eight of the 12 resulting embryos (12/32; 37.5%) were washed twice in PBS containing 0.1% polyvinyl alcohol (PVA; Sigma-Aldrich Chemicals) and transferred individually to DNase–RNase-free tubes

in RLT buffer (Qiagen, Milan, Italy), snap-frozen in liquid nitrogen and stored at −80°C.

RNA and gDNA extraction and cDNA synthesis

Total RNA and genomic (g) DNA were extracted from groups of pooled oocytes (nine per group) and from individual embryos in 350 or 600 μL RLT buffer, respectively, using an AllPrep DNA/RNA/Protein Mini Kit and subjected to on-column DNase I digestion using an RNase-Free DNase Set (both from Qiagen), as described previously (Paris *et al.* 2011). DNA was eluted in 50 μL elution buffer (Qiagen). RNA was eluted in 35 μL RNase-free water (Qiagen) and then transcribed into cDNA. For conventional polymerase chain reaction (PCR), the total reaction volume was 25 μL, containing 1 μL cDNA, 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Promega, Madison, WI, USA), 0.5 μM forward primer, 0.5 μM reverse primer and 0.625 U HotStarTaq DNA polymerase (all Qiagen, except dNTPs). The PCR cycle conditions consisted of 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at the primer-specific annealing temperature (Table 1) and 1 min at 72°C, with a final extension for 10 min at 72°C. Products were visualised on 1% agarose gels. If suitable for PCR amplification and free of gDNA contamination, both +RT (with RT) and –RT (without RT) cDNA samples were diluted 1 : 9 and frozen at −20°C until further quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis.

DNA quantification

Total cell number was estimated by quantifying total gDNA with a Quant-iT PicoGreen dsDNA assay kit (Molecular Probes, Invitrogen, Eugene, OR, USA). A standard curve ranging from 25 ng mL^{-1} to 2.5 pg mL^{-1} was created via a 1 : 9 dilution series. Briefly, 50 μL Pico Green reagent was mixed with 9.95 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and 40 μL was added to 20 μL sample (standard curve or embryo extract) per well in a FLUOTRAC 600 96-well microplate (Greiner Bio-One, Frickenhausen, Germany). During preparation and plate setup, the materials were protected from light. Fluorescence was measured using a multimode detector (Beckman Coulter DTX 880; Beckman, Woerden, The Netherlands) and the Fluorescein Top Read software program for Anthos Multimode detectors (Anthos Microsystem, Krefeld, Germany).

qRT-PCR

Primers for the *TFAM*, *mtPOLB*, *SSB*, *ATP-synth_F6* and *GPX3* genes were optimised and tested for specificity using an iQ5 RT-qPCR Detection System with iQ5 Optical System Software v2.0 (Table 1; BioRad, Hercules, CA, USA); equine kidney served as the positive control tissue.

One hundred and sixty-five oocytes were recovered from younger mares (<12 years) and 170 were recovered from older mares (≥12 years). Seventy-seven oocytes from younger mares and 92 oocytes from older mares were analysed directly after harvesting from follicles, whereas 88 from younger mares and 78 from older mares were subjected to IVM before analysis. The same oocytes and embryos were used to study mtDNA copy number and to examine the relative expression of genes associated with mitochondrial replication or function.

Quantitative real-time PCR was performed separately for oocytes and embryos, but using the same equipment, settings

Table 1. Primers used for gene amplification in quantitative reverse transcription–polymerase chain reaction
T_A, annealing temperature; F, forward; R, reverse

Symbol	Gene name	GenBank Accession no.	Primer sequence (5′–3′)	Product size (bp)[C]	T_A (°C)
PGK1	Phoshoglycerate kinase 1	XM_001502668	F: 5′-CAAGAAGTATGCTGAGGCTG-3′ R: 5′-AGGACTTTACCTTCCAGGAG-3′	260	57.0
SRP14	Signal recognition particle 14kDa	XM_001503583	F: 5′-ACTCACCCTCAAGTATGTCC-3′ R: 5′-CCAGTATGTCCATCTTGACG-3′	100	55.0
RPL4	60S ribosomal protein L4-like	XM_001497094	F: 5′-CATCCCTGGAATTACTCTGC-3′ R: 5′-CGGCTAAGGTCTGTATTGAG-3′	203	61.5
TFAM	Transcription factor A, mitochondrial	NM_001034016	F: 5′-GGCAGGTATACAAGGAAGAG-3′ R: 5′-GTTATAAGCTGAGCGAGGTC-3′	170	56.5
mtPOLB	DNA polymerase subunit gamma-2, mitochondrial	NM_015810	F: 5′-CCGAGTAAGGAACAGCTAGT-3′ R: 5′-ACTCCAATCTGAGCAAGACC-3′	155	57.5
SSB	Mitochondrial single stranded DNA binding protein	XM_003364867	F: 5′-CATGAGACAGGTGGAAGGAA-3′ R: 5′-GATATGCCACATCTCTGAGG-3′	167	57.1
ATP-synt_F6	ATP synthase-coupling factor 6, mitochondrial-like	XM_001499999	F: 5′-ACTCACCCTCAAGTATGTCC-3′ R: 5′-CCAGTATGTCCATCTTGACG-3′	148	59.5
GPX3	Glutathione peroxidase 3 (plasma)	XM_001917631	F: 5′-ACTCACCCTCAAGTATGTCC-3′ R: 5′-CCAGTATGTCCATCTTGACG-3′	289	58.9
mtDNA	Mitochondrial DNA	NC_001640.1	F: 5′-CATGATGAAACTTCGGCTCC-3′ R: 5′-TGAGTGACGGATGAGAAGGCAG-3′	118	67.7

and software described by Paris *et al.* (2011). The total reaction volume was 25 µL per well, and included 1× iQ5 SYBR Green Supermix (BioRad), 0.5 µM forward primer and 0.5 µM reverse primer (Ocimum Biosolutions, IJsselstein, The Netherlands). Each well included 10 µL standard or sample, and the following were included in the final plates: (1) standard curve: 100 fg–6.4 ag fivefold dilution series ($n = 7$); (2) 1 : 9 diluted sample cDNA (pooled oocytes $n = 24$; embryos $n = 24$); (3) 1 : 9 diluted –RT sample (pooled oocytes $n = 24$; embryos $n = 24$); and (4) DNase and RNase-free water (Invitrogen) as a no-template control.

All samples except the –RT samples were run as duplicates. Both frozen–validated and freshly prepared standards were included on each plate. Because of space limitations, –RT samples for the embryos were run on a separate plate in a subsequent run on the same day but using identical standards. The PCR cycle conditions consisted of 4.5 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at optimal annealing temperature (T_A; Table 1) and 30 s at 72°C, during which fluorescence was acquired, followed by a melting curve protocol consisting of 1 min at 95°C, 1 min at optimal T_A, then 10 s at optimal T_A increasing to 95°C by 0.5° per cycle during which a second round of fluorescence was acquired. Baseline and threshold (~100 relative fluorescence units (RFU)) were manually adjusted and samples with non-uniform or failed amplification, primer dimers or with amplified products in the corresponding –RT sample were excluded from further analysis for all genes (only 12 samples in total from all the reactions for gene expression and mtDNA quantification). Raw gene expression data from non-matured pooled oocytes and embryos obtained from younger mares was set at 1, and the results of the other groups were expressed relative to these values. Relative gene expression was calculated using iQ5 Optical Software v2.0 (BioRad, Hercules, CA, USA). The relative expression of all genes of interest was then normalised using three stable reference genes (phosphoglycerate kinase 1 (*PGK1*), ribosomal protein L4 (*RPL4*) and signal recognition particle 14kDa (*SRP14*)) previously identified to be suitable for early equine embryos (Paris *et al.* 2011).

For mtDNA quantification, DNA was used from pooled groups of oocytes and individual embryos. The same protocol was used as for cDNA quantification, and all samples were amplified on one plate using the same plate setup as above. The PCR product length for mtDNA is 118 bp, which is equivalent to 1.21×10^{-4} fg and represents 1 mtDNA copy. Therefore, mtDNA copy number was calculated by dividing the starting quantity (i.e. absolute quantity in fg measured during the amplification process) by 1.21×10^{-4}.

Analysis of ATP quantity

The ATP content was determined using an ATP-dependent luciferin–luciferase bioluminescence assay (ATP Bioluminescent Somatic Cell Assay Kit; Sigma-Aldrich) as described by Van Blerkom *et al.* (1995). After removal of any adherent cumulus cells, zona-intact oocytes were frozen at −80°C in 200 µL ultrapure water. They were subsequently thawed immediately before the automated addition of the luciferin–luciferase mixture. A standard curve containing 11 ATP concentrations ranging from 10 fmol to 10 pmol was generated for each analysis. Oocytes from the different experimental groups were analysed simultaneously on a single 96-well plate (all samples on one plate). Measurements were repeated five times within 10 min and the mean values of these five measurements were used for statistical analysis.

Statistical analysis

Data were analysed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). For gene expression, the effects of maternal age, IVM (oocytes) and IVP (embryos) were analysed using one-way ANOVA, followed by a post-hoc Bonferroni test to determine the source of any differences. Data for mtDNA (mtDNA copy number and mtDNA : DNA ratio) and for ATP quantity were $\log_{10}$ transformed before analysis to achieve equivalence of variance. After transformation, data were analysed using univariate analysis. Differences were considered significant at two-tailed $P < 0.05$.

Results

mtDNA copy number

The eight Day 7 embryos recovered from younger and older mares were all Grade 1–2 and ranged in developmental stage from the morula to the expanded blastocyst stage, and in size from 150 to 800 µm in diameter (younger mares: two early blastocysts, six expanded blastocysts; older mares, two morulae, three early blastocysts, three expanded blastocysts). The eight Day 8 IVP embryos were all early blastocysts. The gDNA quantity (an indicator of cell number) did not differ significantly among embryos retrieved from younger mares, older mares or those produced *in vitro* (Fig. 1). In contrast, there was considerable variation in mtDNA copy number among groups. Indeed, both mtDNA copy number per embryo and the ratio of mtDNA : total DNA (i.e. corrected for approximate cell number) were significantly ($P < 0.05$) lower in embryos recovered from older (>16 years) mares ($0.26 \times 10^6 \pm 0.09 \times 10^6$ and

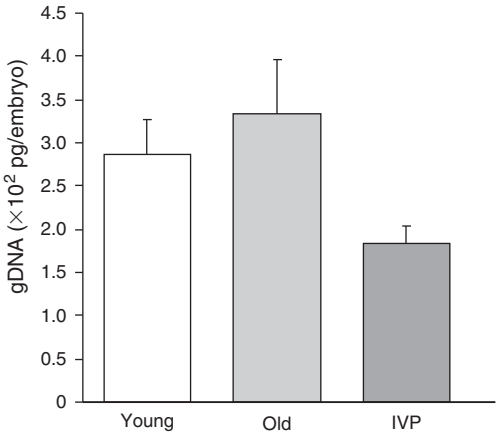

Fig. 1. Total DNA quantity (pg per embryo) in Day 7 *in vivo* equine embryos recovered from younger mares (<12 years) and older mares (>16 years), as well as in *in vitro*-produced (IVP) embryos recovered from mares of unknown ages. Values are the mean ± s.e.m. No significant differences were observed between the different groups. gDNA, genomic DNA.

$1.42 \times 10^{-4} \pm 0.45 \times 10^{-4}$, respectively) than in those from younger mares ($32.91 \times 10^{6} \pm 21.54 \times 10^{6}$ and $2.84 \times 10^{-2} \pm 1.89 \times 10^{-2}$, respectively) or produced *in vitro* ($7.64 \times 10^{6} \pm 3.13 \times 10^{6}$ and $0.95 \times 10^{-2} \pm 0.51 \times 10^{-2}$, respectively; Fig. 2).

Expression of genes involved in mitochondrial replication or function

All –RT cDNA samples were free of gDNA contamination. The PCR products for each primer pair were of the expected size when examined by agarose gel electrophoresis (Fig. 3) and DNA sequencing confirmed that the products were specific to the target genes (data not shown). Evaluation of the stability of gene expression for *PGK1*, *RPL4* and *SRP14* in the pooled oocyte groups, using geNorm v3.5 (Biogazelle NV, Zwijnaarde, Belgium), confirmed that they were suitable for use as reference genes (gene expression stability measure (M) values of 0.94, 1.40 and 0.96, respectively).

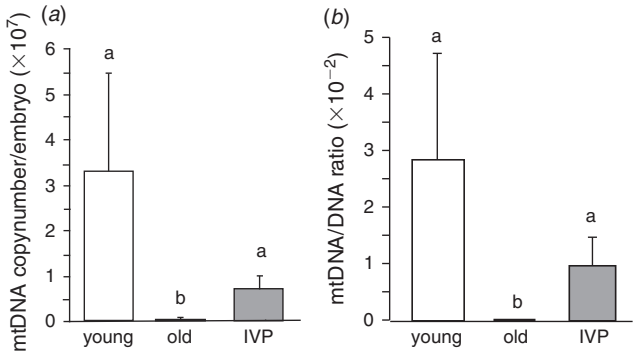

Fig. 2. (*a*) Mitochondrial (mt) DNA copy number per embryo and (*b*) mtDNA quantity in relation to total DNA quantity (mtDNA : DNA ratio) in Day 7 *in vivo* equine embryos recovered from younger mares (<12 years) and older mares (>16 years), as well as in *in vitro*-produced (IVP) embryos recovered from mares of unknown ages. Values are the mean ± s.e.m. Columns with different letters differ significantly ($P < 0.05$).

Most oocytes or embryos contained detectable quantities of mRNA for the genes of interest. The notable exception was *GPX3*, for which expression was below the detection limit in 30 of the 48 pooled oocyte samples. However, there was no group effect or bias in that 6–8 of the 12 samples were below the detection limit in all four groups examined. For *mtPOLB*, mRNA expression was undetectable in one IVP embryo and one embryo from an older mare, such that there was also no group or treatment bias. For the purposes of statistical analysis, samples with gene expression values under the detection limit were assigned a value of 0.

Normalised relative gene expression for *TFAM*, *mtPOLB* and *ATP-synth_F6* was lower in oocytes after IVM than in oocytes that had not been cultured ($P < 0.05$; Fig. 4*b*), although the differences were not statistically significant for *TFAM* and *ATP-synth_F6* in young mare oocytes. Relative expression of the five target genes in embryos was unaffected by maternal age. However, IVP embryos showed higher expression of *mtPOLB* than *in vivo* embryos ($P = 0.013$), whereas their expression of *GPX3* tended ($P = 0.09$) to be lower than in *in vivo* embryos from young mares (Fig. 5*e*).

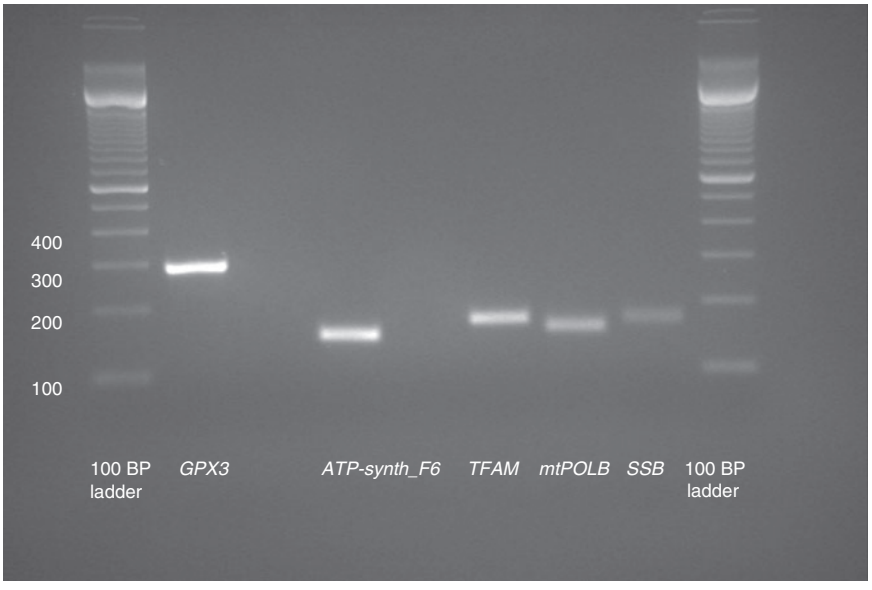

Fig. 3. Amplified products of glutathione peroxidase 3 (*GPX3*), ATP synthase-coupling factor 6, mitochondrial-like (*ATP-synth_F6*), mitochondrial transcription factor (*TFAM*), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single-stranded DNA-binding protein (*SSB*) separated on a 2% agarose gel in parallel with a 100-bp DNA ladder and visualised by ethidium bromide staining. Products were of the expected size (*GPX3*, 289 bp; *ATP-synth_F6*, 148 bp; *TFAM*, 170 bp; *mtPOLB*, 155 bp; *SSB*, 167 bp).

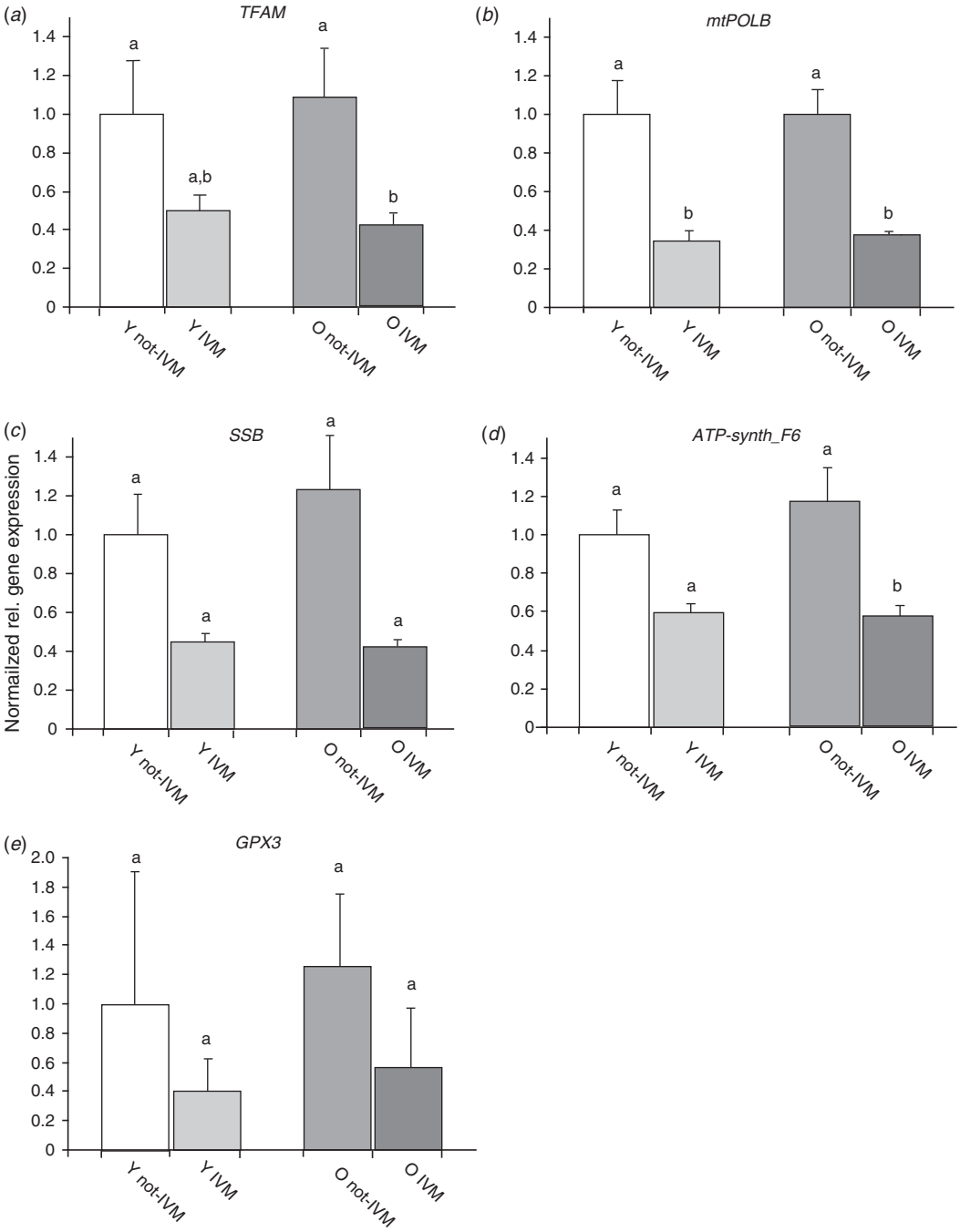

Fig. 4. Normalised relative gene expression (mean ± s.e.m.) for genes involved in mitochondrial replication, namely mitochondrial transcription factor (*TFAM*), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single-stranded DNA-binding protein (*SSB*), and function, namely glutathione peroxidase 3 (*GPX3*) and ATP synthase-coupling factor 6, mitochondrial-like (*ATP-synth_F6*), in pooled equine oocytes obtained from younger mares (<12 years) and older mares (≥12 years) that were either matured *in vitro* (IVM) or not matured (not-IVM). The expression of most genes was detectable in all oocyte pools in each treatment group with the exception of *GPX3*, for which only a few oocyte pools per group yielded a product. Columns with different letters differ significantly ($P < 0.05$).

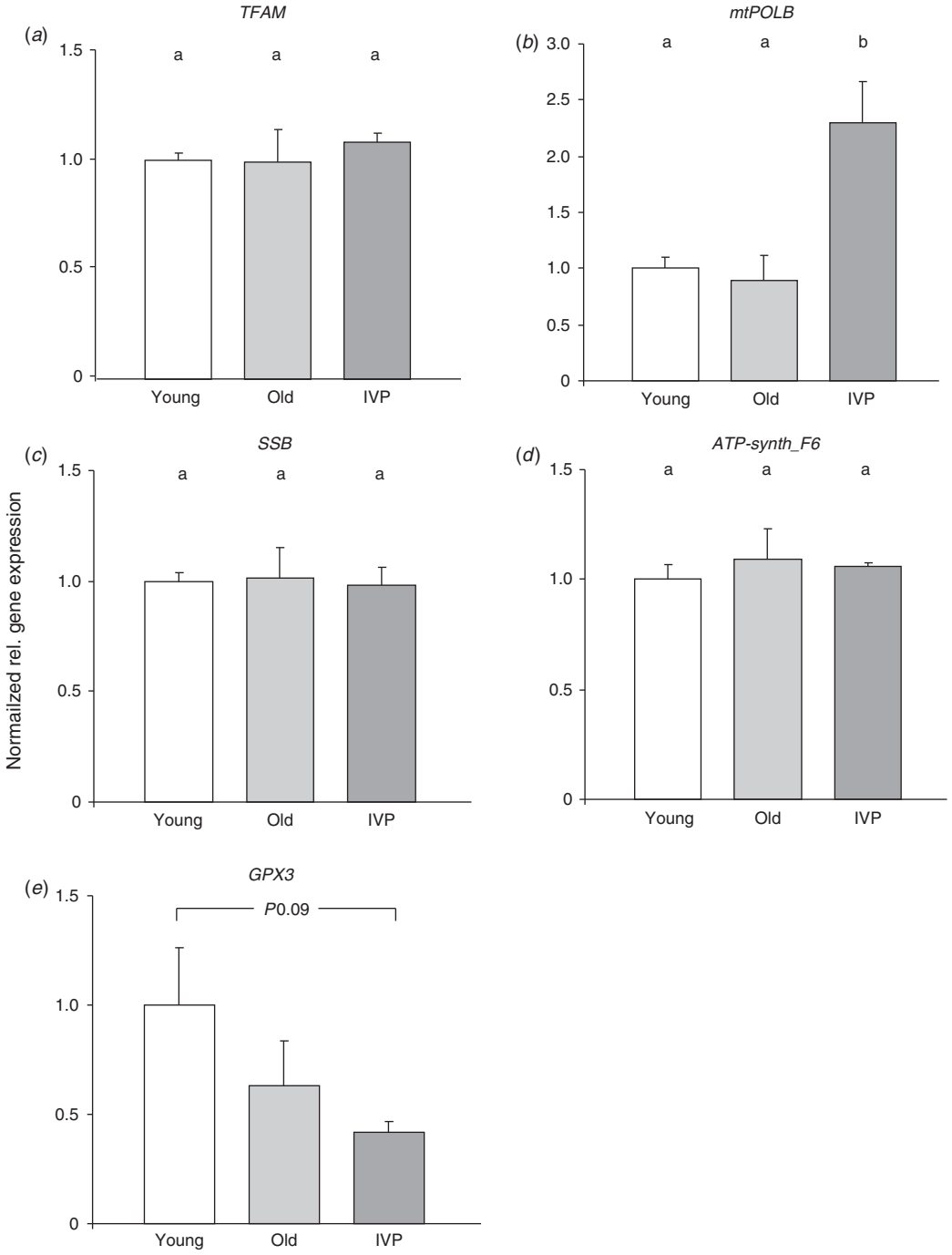

Fig. 5. Normalised relative gene expression (mean ± s.e.m.) for genes involved in mitochondrial replication, namely mitochondrial transcription factor (*TFAM*), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single-stranded DNA-binding protein (*SSB*), and function, namely glutathione peroxidase 3 (*GPX3*) and ATP synthase-coupling factor 6, mitochondrial-like (*ATP-synth_F6*), in day 7 equine embryos obtained from younger mares (<12 years) and older mares (>16 years) and in day 8 *in vitro*-produced (IVP) embryos from mares of unknown ages. Columns with different letters differ significantly ($P < 0.05$).

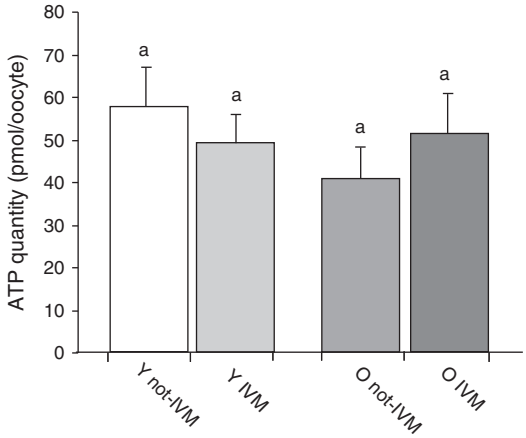

Fig. 6. ATP quantity (pmol per oocyte) in individual equine oocytes obtained from younger mares (<12 years) and older mares (≥12 years) that were either matured *in vitro* (IVM) or not matured (not-IVM). Values are the mean ± s.e.m. No significant differences were observed between the different groups.

ATP quantity

The effect of maternal age on metabolic capacity of oocytes during IVM was evaluated by measuring ATP quantity in oocytes before and after IVM. The mean ATP quantity before IVM was 40.5 ± 7.3 pmol per oocyte in oocytes from older mares and 57.6 ± 9.1 pmol per oocyte in those from younger mares. ATP quantities did not change significantly after oocyte maturation and did not differ significantly between the two mare age groups (Fig. 6).

Discussion

Equine oocytes contain an average of more than one million mtDNA copies (Rambags *et al.* 2014) and, presumably, a similar number of mitochondria. During early development, the mitochondria in a blastomere are distributed sequentially over the newly formed daughter cells, leading to falling numbers of mitochondria per cell until replication is restarted (i.e. at the time of gastrulation in the mouse (Facucho-Oliveira *et al.* 2007) and at blastocyst formation in cattle and pigs (May-Panloup *et al.* 2005*b*; Spikings *et al.* 2007)).

In the present study, embryos collected from older mares on Day 7 after ovulation contained significantly fewer mtDNA copies than those from younger mares. The mtDNA : gDNA ratio in older mares was also lower, indicating that there were also fewer mitochondria per cell. IVP embryos showed an intermediate mtDNA copy number that could reflect, at least in part, their generation from oocytes recovered from mares of unknown, but presumably mixed, age. The reduced mtDNA copy number per embryo (and cell) in aged mares could arise in one of at least three different ways: (1) a lower number of mitochondria at the onset of development, as suggested by a reduced mtDNA content in oocytes from mares ≥12 years after IVM (Rambags *et al.* 2014); (2) a delay in the onset of mitochondrial replication or a reduced replication rate; or (3) a higher rate of mitochondrial deterioration induced, for example, by oxidative stress. In this respect, various genes involved in mitochondrial replication and mtDNA maintenance, namely *TFAM*, *mtPOLB* and *SSB* (Facucho-Oliveira *et al.* 2007; St. John *et al.* 2010), were not differently expressed between embryos from younger versus older mares in the present study. The relative gene expression of *GPX3* (a key factor in neutralising the effects of ROS; Eichenlaub-Ritter *et al.* 2011) was also not significantly affected by maternal age.

The relative mRNA abundance of *TFAM* and *mtPOLB*, both of which are enzymes involved in mitochondrial replication, decreased after IVM of equine oocytes. A similar general trend was observed for all target genes examined and may explain, in part, why mtDNA copy numbers have been reported not to increase during IVM of equine oocytes (Rambags *et al.* 2014). A decrease in relative gene expression for *TFAM* and *mtPOLB* after oocyte maturation has previously been reported for porcine and murine oocytes (Spikings *et al.* 2007; Mahrous *et al.* 2012) and related to the arrest in mitochondrial replication that is observed during early embryonic development. However, the absolute reduction in mtDNA copy number in oocytes of mares ≥12 years after IVM observed by Rambags *et al.* (2014) was thought to be a factor of mitochondrial deterioration, as indicated by electron microscopic evidence of mitochondrial swelling and disintegration.

Expression of genes associated with mitochondrial replication or function differed little between embryos of differing origins (older mares, younger mares, IVP). The only verifiable difference was an increase in *mtPOLB* expression in IVP compared with *in vivo* embryos. *mtPOLB* is primarily involved in mtDNA maintenance, by mediating replication, recombination and repair (St. John *et al.* 2010). This increase may therefore indicate a response to a suboptimal environment (*in vitro* culture) aimed at maintaining sufficient mtDNA and mitochondria to safeguard embryonic development. Under suboptimal conditions, ROS production is likely to be higher, resulting in an increased risk of mtDNA damage; an increase in *mtPOLB* expression could therefore be a reaction to mitigate mtDNA damage (Takahashi 2012; Giritharan *et al.* 2007).

Expression of *GPX3* is associated with the protection of mitochondria against their own potentially toxic by-products, namely ROS, which *GPX3* can neutralise (Guérin *et al.* 2001; Eichenlaub-Ritter *et al.* 2011). Reduced *GPX3* expression can result from elevated production of ROS and is associated with an increased risk of damage to mitochondrial and nuclear DNA, which can lead to developmental retardation and arrest (Takahashi 2012). Although there were no significant differences in *GPX3* expression in oocytes recovered from younger versus older mares, and no significant effect of *in vitro* oocyte maturation on *GPX3* expression, relative gene expression for *GPX3* was approximately halved after IVM. It is therefore possible that there was a fall in ROS-scavenging capacity within oocytes during IVM that failed to reach statistical significance because of the low number of oocyte pools examined. Alternatively, the apparent decrease in gene expression may be a more general phenomenon, given that the abundance of all other target mRNAs roughly halved during IVM. It is also possible that ROS-scavenging activity is not a function of the oocyte alone, because an effect of IVM on *GPX3* expression has been recorded in the surrounding cumulus cells (Luciano *et al.* 2006).

Future studies examining ROS-scavenging activity during oocyte maturation should therefore encompass both the oocyte and its cumulus investment. The trend to reduced *GPX3* gene expression in IVP equine embryos may have similar implications for the prevention of ROS accumulation, and could therefore contribute to the elevated levels of apoptosis and DNA damage seen in equine IVP compared with *in vivo* embryos (Tremoleda *et al.* 2003; Pomar *et al.* 2005).

Relative expression of *ATP-synth_F6* mRNA was reduced in oocytes from older mares after IVM. However, this was not reflected by a reduction in the ATP content of aged mare oocytes following IVM. *ATP-synth_F6* is involved in ATP production via the electron transport system; however, oocyte maturation, both *in vivo* and *in vitro*, takes place in a hypoxic environment. This may promote ATP production via glycolysis rather than via oxidative phosphorylation and the electron transport system (Leese *et al.* 2008; Ramalho-Santos *et al.* 2009). During blastocyst formation, embryos are thought to become more dependent on oxygen consumption for the production of energy from mitochondria (Houghton and Leese 2004) and reduced *ATP-synth_F6* expression at this stage may therefore be detrimental. In the present study, we did not find any evidence of a difference in *ATP-synth_F6* expression between Day 7 embryos produced *in vitro* or recovered from mares <12 or >16 years.

In conclusion, Day 7 equine embryos recovered from older mares have lower mitochondrial DNA copy numbers than Day 7 embryos recovered from younger mares; this seems to be independent of any change in the rate of development because the mtDNA : gDNA ratio (i.e. corrected for cell number) is also reduced. This reduction in the number of mtDNA copies per cell may push the cells in some developing embryos below the threshold number required for normal development and thereby contribute to compromised cell survival and, as a result, reduced embryo developmental competence. This rationale has been used previously to propose treatments to improve the developmental capacity of oocytes from aged females undergoing fertilisation by ICSI, namely using the transfer of cytoplasm containing 'high-quality mitochondria' from a young oocyte (for a review, see Chappel 2013).

Two genes were found to be differentially expressed in IVP compared with *in vivo* embryos, namely *mtPOLGB* and *GPX3*. This suggests that some of the detrimental effects of present *in vitro* culture procedures on developmental competence (as evidenced by higher rates of early pregnancy loss than after fertilisation *in vivo*; Carnevale *et al.* 2005; Alexopoulos *et al.* 2008; Giritharan *et al.* 2010) may be mediated via effects on mitochondrial replication, repair or ability to resist the effects of ROS. In addition, IVM led to a generalised reduction in the expression of genes involved in mitochondrial replication and function, irrespective of maternal age. However, because these comparisons have not yet been performed on oocytes matured *in vivo*, it is not possible to determine whether this is an effect of IVM or inherent to the oocyte maturation process.

Acknowledgements

The authors thank L. van Tol for advice on oocyte recovery and *in vitro* maturation, C. van de Lest for help in establishing the ATP and DNA quantification assays, E. de Vries for assisting with the luminescence measurement, M. Vos-Loohuis for assistance with primer sequencing and J. van den Broek for assistance with statistical analysis.

References

Alexopoulos, N. I., Maddox-Hyttel, P., Tveden-Nyborg, P., D'Cruz, N. T., Tecirlioglu, T. R., Cooney, M. A., Schauser, K., Holland, M. K., and French, A. J. (2008). Developmental disparity between *in vitro*-produced and somatic cell nuclear transfer bovine Days 14 and 21 embryos: implications for embryonic loss. *Reproduction* **136**, 433–445. doi:10.1530/REP-07-0392

Allen, W. R., Brown, L., Wright, M., and Wilsher, S. (2007). Reproductive efficiency of Flatrace and National Hunt thoroughbred mares and stallions in England. *Equine Vet. J.* **39**, 438–445. doi:10.2746/042516407X1737581

Altermatt, J. L., Marolf, A. J., Wrigley, R. H., and Carnevale, E. M. (2012). Effects of FSH and LH on ovarian and follicular blood flow, follicular growth and oocyte developmental competence in young and old mares. *Anim. Reprod. Sci.* **133**, 191–197. doi:10.1016/J.ANIREPROSCI.2012.06.021

Baird, D. T., Collins, J., Egozcue, J., Evers, L. H., Gianaroli, L., Leridon, H., Sunde, A., Templeton, A., Van Steirteghem, A., Cohen, J., Crosignani, P. G., Devroey, P., Diedrich, K., Fauser, B. C., Fraser, L., Glasier, A., Liebaers, I., Mautone, G., Penney, G., and Tarlatzis, B., ESHRE Capri Workshop Group (2005). Fertility and ageing. *Hum. Reprod. Update* **11**, 261–276. doi:10.1093/HUMUPD/DMI006

Ball, B. A., Little, T. V., Weber, J. A., and Woods, G. L. (1989). Survival of Day-4 embryos from young, normal mares and aged, subfertile mares after transfer to normal recipient mares. *J. Reprod. Fertil.* **85**, 187–194. doi:10.1530/JRF.0.0850187

Cao, L., Shitara, H., Horii, T., Nagao, Y., Imai, H., Abe, K., Hara, T., Hayashi, J., and Yonekawa, H. (2007). The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells. *Nat. Genet.* **39**, 386–390. doi:10.1038/NG1970

Carnevale, E. M. (2008). The mare model for follicular maturation and reproductive aging in the woman. *Theriogenology* **69**, 23–30. doi:10.1016/J.THERIOGENOLOGY.2007.09.011

Carnevale, E. M., and Ginther, O. J. (1992). Relationships of age to uterine function and reproductive efficiency in mares. *Theriogenology* **37**, 1101–1115. doi:10.1016/0093-691X(92)90108-4

Carnevale, E. M., and Ginther, O. J. (1995). Defective oocytes as a cause of subfertility in old mares. *Biol. Reprod.* **1**, 209–214.

Carnevale, E. M., Coutinho da Silva, M. A., Panzani, D., Stokes, J. E., and Squires, E. L. (2005). Factors affecting the success of oocyte transfer in a clinical program for subfertile mares. *Theriogenology* **64**, 519–527. doi:10.1016/J.THERIOGENOLOGY.2005.05.008

Chappel, S. (2013). The role of mitochondria from mature oocyte to viable blastocyst. *Obstet. Gynecol. Int.* **2013**, 183024. doi:10.1155/2013/183024

Chiaratti, M. R., and Meirelles, F. V. (2010). Mitochondrial DNA copy number, a marker of viability for oocytes. *Biol. Reprod.* **83**, 1–2. doi:10.1095/BIOLREPROD.110.084269

Cree, L. M., Samuels, D. C., de Sousa Lopes, S. C., Rajasimha, H. K., Wonnapinij, P., Mann, J. R., Dahl, H. H., and Chinnery, P. F. (2008). A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. *Nat. Genet.* **40**, 249–254. doi:10.1038/NG.2007.63

Eichenlaub-Ritter, U., Wieczorek, M., Luke, S., and Seidel, T. (2011). Age related changes in mitochondrial function and new approaches to study redox regulation in mammalian oocytes in response to age or maturation conditions. *Mitochondrion* **11**, 783–796. doi:10.1016/J.MITO.2010.08.011

Facucho-Oliveira, J. M., Alderson, J., Spikings, E. C., Egginton, S., and St John, J. C. (2007). Mitochondrial DNA replication during differentiation

of murine embryonic stem cells. *J. Cell Sci.* **120**, 4025–4034. doi:10.1242/JCS.016972

Fragouli, E., Wells, D., and Delhanty, J. D. (2011). Chromosome abnormalities in the human oocyte. *Cytogenet. Genome Res.* **133**, 107–118. doi:10.1159/000323801

Franasiak, J. M., Forman, E. J., Hong, K. H., Werner, M. D., Upham, K. M., Treff, N. R., and Scott, R. T., Jr. (2014). The nature of aneuploidy with increasing age of the female partner: a review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. *Fertil. Steril.* **101**, 656–663. doi:10.1016/J.FERTNSTERT.2013.11.004

Galli, C., Crotti, G., Notari, C., Turini, P., Duchi, R., and Lazzari, G. (2001). Embryo production by ovum pick up from live donors. *Theriogenology* **55**, 1341–1357. doi:10.1016/S0093-691X(01)00486-1

Galli, C., Colleoni, S., Duchi, R., Lagutina, I., and Lazzari, G. (2007). Developmental competence of equine oocytes and embryos obtained by *in vitro* procedures ranging from *in vitro* maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Anim. Reprod. Sci.* **98**, 39–55. doi:10.1016/J.ANIREPROSCI.2006.10.011

Galli, C., Duchi, R., Colleoni, S., Lagutina, I., and Lazzari, G. (2014). Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. *Theriogenology* **81**, 138–151. doi:10.1016/J.THERIOGENOLOGY.2013.09.008

Giritharan, G., Talbi, S., Donjacour, A., Di Sebastiano, F., Dobson, A. T., and Rinaudo, P. F. (2007). Effect of *in vitro* fertilization on gene expression and development of mouse preimplantation embryos. *Reproduction* **134**, 63–72. doi:10.1530/REP-06-0247

Giritharan, G., Li, M. W., De Sebastiano, F., Esteban, F. J., Horcajadas, J. A., Lloyd, K. C., Donjacour, A., Maltepe, E., and Rinaudo, P. F. (2010). Effect of ICSI on gene expression and development of mouse preimplantation embryos. *Hum. Reprod.* **25**, 3012–3024. doi:10.1093/HUMREP/DEQ266

Guérin, P., El Mouatassim, S., and Ménézo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum. Reprod. Update* **7**, 175–189. doi:10.1093/HUMUPD/7.2.175

Hinrichs, K., Schmidt, A. L., Friedman, P. P., Selgrath, J. P., and Martin, M. G. (1993). *In vitro* maturation of horse oocytes: characterization of chromatin configuration using fluorescence microscopy. *Biol. Reprod.* **48**, 363–370. doi:10.1095/BIOLREPROD48.2.363

Hinrichs, K., Choi, Y. H., Love, L. B., Varner, D. D., Love, C. C., and Walckenaer, B. E. (2005). Chromatin configuration within the germinal vesicle of horse oocytes: changes post mortem and relationship to meiotic and developmental competence. *Biol. Reprod.* **72**, 1142–1150. doi:10.1095/BIOLREPROD.104.036012

Houghton, F. D., and Leese, H. J. (2004). Metabolism and developmental competence of the preimplantation embryo. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **115**(Suppl. 1), S92–S96. doi:10.1016/J.EJOGRB.2004.01.019

Iwata, H., Goto, H., Tanaka, H., Sakaguchi, Y., Kimura, K., Kuwayama, T., and Monji, Y. (2011). Effect of maternal age on mitochondrial DNA copy number, ATP content and IVF outcome of bovine oocytes. *Reprod. Fertil. Dev.* **23**, 424–432. doi:10.1071/RD10133

Jansen, R. P. (2000). Origin and persistence of the mitochondrial genome. *Hum. Reprod.* **15**(Suppl. 2), 1–10. doi:10.1093/HUMREP/15.SUPPL_2.1

Jiao, F., Yan, J. B., Yang, X. Y., Li, H., Wang, Q., Huang, S. Z., Zeng, F., and Zeng, Y. T. (2007). Effect of oocyte mitochondrial DNA haplotype on bovine somatic cell nuclear transfer efficiency. *Mol. Reprod. Dev.* **74**, 1278–1286. doi:10.1002/MRD.20698

Kameyama, Y., Filion, F., Yoo, J. G., and Smith, L. C. (2007). Characterization of mitochondrial replication and transcription control during rat early development *in vivo* and *in vitro*. *Reproduction* **133**, 423–432. doi:10.1530/REP-06-0263

Lazzari, G., Crotti, G., Turini, P., Duchi, P., Mari, G., Zavaglia, G., Barbacini, S., and Galli, G. (2002). Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of *in vitro* matured oocytes with frozen–thawed spermatozoa from semen of different fertilities. *Theriogenology* **58**, 709–712. doi:10.1016/S0093-691X(02)00777-X

Leese, H. J., Baumann, C. G., Brison, D. R., McEvoy, T. G., and Sturmey, R. G. (2008). Metabolism of the viable mammalian embryo: quietness revisited. *Mol. Hum. Reprod.* **14**, 667–672. doi:10.1093/MOLEHR/GAN065

Luciano, A. M., Goudet, G., Perazzoli, F., Lahuec, C., and Gérard, N. (2006). Glutathione content and glutathione peroxidase expression in *in vivo* and *in vitro* matured equine oocytes. *Mol. Reprod. Dev.* **73**, 658–666. doi:10.1002/MRD.20469

Mahrous, E., Yang, Q., and Clarke, H. J. (2012). Regulation of mitochondrial DNA accumulation during oocyte growth and meiotic maturation in the mouse. *Reproduction* **144**, 177–185. doi:10.1530/REP-12-0113

May-Panloup, P., Chretien, M. F., Jacques, C., Vasseur, C., Maltiery, Y., and Reynier, P. (2005*a*). Low oocyte mitochondrial DNA content in ovarian insufficiency. *Hum. Reprod.* **20**, 593–597. doi:10.1093/HUMREP/DEH667

May-Panloup, P., Vignon, X., Chrétien, M. F., Heyman, Y., Tamassia, M., Malthièry, Y., and Reynier, P. (2005*b*). Increase of mitochondrial DNA content and transcripts in early embryogenesis associated with upregulation of mtTFA and NRF1 transcription factors. *Reprod. Biol. Endocrinol.* **3**, 65. doi:10.1186/1477-7827-3-65

May-Panloup, P., Chretien, M. F., Malthiery, Y., and Reynier, P. (2007). Mitochondrial DNA in the oocyte and the developing embryo. *Curr. Top. Dev. Biol.* **77**, 51–83. doi:10.1016/S0070-2153(06)77003-X

Morel, M. C., Newcombe, J. R., and Swindlehurst, J. C. (2005). The effect of age on multiple ovulation rates, multiple pregnancy rates and embryonic vesicle diameter in the mare. *Theriogenology* **63**, 2482–2493. doi:10.1016/J.THERIOGENOLOGY.2004.09.058

Paris, D. B., Kuijk, E. W., Roelen, B. A., and Stout, T. A. (2011). Establishing reference genes for use in real-time quantitative PCR analysis of early equine embryos. *Reprod. Fertil. Dev.* **23**, 353–363. doi:10.1071/RD10039

Pikó, L., and Matsumoto, L. (1976). Number of mitochondria and some properties of mitochondrial DNA in the mouse egg. *Dev. Biol.* **49**, 1–10. doi:10.1016/0012-1606(76)90253-0

Pikó, L., and Taylor, K. D. (1987). Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Dev. Biol.* **123**, 364–374. doi:10.1016/0012-1606(87)90395-2

Pomar, F. J., Teerds, K. J., Kidson, A., Colenbrander, B., Tharasanit, T., Aguilar, B., and Roelen, B. A. (2005). Differences in the incidence of apoptosis between *in vivo* and *in vitro* produced blastocysts of farm animal species: a comparative study. *Theriogenology* **63**, 2254–2268. doi:10.1016/J.THERIOGENOLOGY.2004.10.015

Ramalho-Santos, J., Varum, S., Amaral, S., Mota, P. C., Sousa, A. P., and Amaral, A. (2009). Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells. *Hum. Reprod. Update* **15**, 553–572. doi:10.1093/HUMUPD/DMP016

Rambags, B. P. B., van Boxtel, D. C. J., Tharasanit, T., Lenstra, J. A., Colenbrander, B., and Stout, T. A. E. (2014). Advancing maternal age predisposes to mitochondrial damage and loss during maturation of equine oocytes *in vitro*. *Theriogenology* **81**, 959–965. doi:10.1016/J.THERIOGENOLOGY.2014.01.020

Reynier, P., Chretien, M. F., Savagner, F., Larcher, G., Rohmer, V., Barriere, P., and Malthiery, Y. (2001). Mitochondrial DNA content affects the fertilizability of human oocytes. *Mol. Hum. Reprod.* **7**, 425–429. doi:10.1093/MOLEHR/7.5.425

Santos, J. H., Meyer, J. N., Mandavilli, B. S., and Van Houten, B. (2006*a*). Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells. *Methods Mol. Biol.* **314**, 183–199. doi:10.1385/1-59259-973-7:183

Santos, T. A., El Shourbagy, S., and St. John, J. C. (2006*b*). Mitochondrial content reflects oocytes variability and fertilization outcome. *Fertil. Steril.* **85**, 584–591. doi:10.1016/J.FERTNSTERT.2005.09.017

Spikings, E. C., Alderson, J., and St John, J. C. (2007). Regulated mitochondrial DNA replication during oocyte maturation is essential for successful porcine embryonic development. *Biol. Reprod.* **76**, 327–335. doi:10.1095/BIOLREPROD.106.054536

St. John, J. C., Facucho-Oliveira, J., Jiang, Y., Kelly, R., and Salah, R. (2010). Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells. *Hum. Reprod. Update* **16**, 488–509. doi:10.1093/HUMUPD/DMQ002

Steuerwald, N., Barrit, J. A., Adler, R., Malter, H., Schimmel, T., Cohen, J., and Brenner, C. A. (2000). Quantification of mtDNA in single oocytes, polar bodies and subcellular components by real-time rapid cycle fluorescence monitored PCR. *Zygote* **8**, 209–215. doi:10.1017/S0967199400001003

Takahashi, M. (2012). Oxidative stress and redox regulation of mammalian embryos. *J. Reprod. Dev.* **58**, 1–9. doi:10.1262/JRD.11-138N

Takeuchi, T., Neri, Q. V., Katagiri, Y., Rosenwaks, Z., and Palermo, G. D. (2005). Effect of treating induced mitochondrial damage on embryonic development and epigenesist. *Biol. Reprod.* **72**, 584–592. doi:10.1095/BIOLREPROD.104.032391

Tharasanit, T., Colleoni, S., Lazzari, G., Colenbrander, B., Galli, C., and Stout, T. A. (2006). Effect of cumulus morphology and maturation stage on the cryopreservability of equine oocytes. *Reproduction* **132**, 759–769. doi:10.1530/REP.1.01156

Thundathil, J., Filion, F., and Smith, L. C. (2005). Molecular control of mitochondrial function in preimplantation mouse embryos. *Mol. Reprod. Dev.* **71**, 405–413. doi:10.1002/MRD.20260

Tremoleda, J. L., Stout, T. A., Lagutina, I., Lazzari, G., Bevers, M. M., Colenbrander, B., and Galli, C. (2003). Effects of *in vitro* production on horse embryo morphology, cytoskeletal characteristics, and blastocyst capsule formation. *Biol. Reprod.* **69**, 1895–1906. doi:10.1095/BIOLREPROD.103.018515

Ufer, C., and Wang, C. C. (2011). The roles of glutathione peroxidases during embryonic development. *Front. Mol. Neurosci.* **4**, 12.

Van Blerkom, J., Davis, P. W., and Lee, J. (1995). ATP content of human oocytes and developmental potential and outcome after *in-vitro* fertilization and embryo transfer. *Hum. Reprod.* **10**, 415–424.

Wai, T., Teoli, D., and Shoubridge, E. A. (2008). The mitochondrial DNA genetic bottleneck results from replication of a subpopulation of genomes. *Nat. Genet.* **40**, 1484–1488. doi:10.1038/NG.258

Wai, T., Asangla, A., Zhang, X., Cyr, D., Dufort, D., and Shoubridge, E. A. (2010). The role of mitochondrial DNA copy number in mammalian fertility. *Biol. Reprod.* **83**, 52–62. doi:10.1095/BIOLREPROD.109.080887

Wang, L. Y., Wang, D. H., Zou, X. Y., and Xu, C. M. (2009). Mitochondrial functions on oocytes and preimplantation embryos. *J. Zhejiang Univ. Sci. B* **10**, 483–492. doi:10.1631/JZUS.B0820379

Wilding, M., Dale, B., Marino, M., di Matteo, L., Alviggi, C., Pisaturo, M. L., Lombardi, L., and De Placido, G. (2001). Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. *Hum. Reprod.* **16**, 909–917. doi:10.1093/HUMREP/16.5.909

Zeng, H. T., Ren, Z., Yeung, W. S., Shu, Y. M., Xu, Y. W., Zhuang, G. L., and Liang, X. Y. (2007). Low mitochondrial DNA and ATP contents contribute to the absence of birefringent spindle imaged with PolScope in *in vitro* human oocytes. *Hum. Reprod.* **22**, 1681–1686. doi:10.1093/HUMREP/DEM070