BIOLOGY OF REPRODUCTION (2016) **94**(3):66, 1–10
Published online before print 3 February 2016.
DOI 10.1095/biolreprod.115.133017

The Infertility of Repeat-Breeder Cows During Summer Is Associated with Decreased Mitochondrial DNA and Increased Expression of Mitochondrial and Apoptotic Genes in Oocytes[1]

Roberta Machado Ferreira,[2,3,4,5] Marcos Roberto Chiaratti,[3,6,7] Carolina Habermann Macabelli,[6,7] Carlos Alberto Rodrigues,[8] Márcio Leão Ferraz,[9] Yeda Fumie Watanabe,[10] Lawrence Charles Smith,[11] Flávio Vieira Meirelles,[5,6] and Pietro Sampaio Baruselli[4]

[4]*Departamento de Reprodução Animal, Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, São Paulo, São Paulo, Brazil*
[5]*Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, Pirassununga, São Paulo, Brazil*
[6]*Departamento de Ciências Básicas, Faculdade de Zootecnia e Engenharia de Alimentos da Universidade de São Paulo, Pirassununga, São Paulo, Brazil*
[7]*Departamento de Genética e Evolução, Centro de Ciências Biológicas e da Saúde, Universidade Federal de São Carlos, São Carlos, São Paulo, Brazil*
[8]*Clínica Veterinária Samvet de São Carlos Ltda., São Carlos, São Paulo, Brazil*
[9]*Vida Reprodutiva Consultoria, Cravinhos, São Paulo, Brazil*
[10]*Vitrogen-WTAvet, Cravinhos, São Paulo, Brazil*
[11]*Centre de Recherche em Reproduction Animale, Faculté de Médecine Vétérinarie, Université de Montréal, St. Hyacinthe, Quebec, Canada*

ABSTRACT

Oocyte quality is known to be a major cause of infertility in repeat-breeder (RB) and heat-stressed dairy cows. However, the mechanisms by which RB oocytes become less capable of supporting embryo development remain largely unknown. Thus, the aim of this study was to investigate whether the decreased oocyte competence of RB cows (RBs) during summer is associated with an altered gene expression profile and a decrease in mitochondrial DNA (mtDNA) copy number. Therefore, oocytes collected from heifers, non-RBs in peak lactation (PLs), and RBs were used to evaluate mtDNA amounts as well as the expression levels of genes associated with the mitochondria (*MT-CO1*, *NRF1*, *POLG*, *POLG2*, *PPARGC1A*, and *TFAM*), apoptosis (*BAX*, *BCL2*, and *ITM2B*), and oocyte maturation (*BMP15*, *FGF8*, *FGF10*, *FGF16*, *FGF17*, and *GDF9*). The oocytes retrieved from RBs during winter contained over eight times more mtDNA than those retrieved from RBs during summer. They also contained significantly less mtDNA than oocytes retrieved from heifers and PLs during summer. Moreover, the expression of mitochondria- (*NRF1*, *POLG*, *POLG2*, *PPARGC1A*, and *TFAM*) and apoptosis-related (*BAX* and *ITM2B*) genes, as well as of *GDF9*, in RB oocytes collected during summer was significantly greater than that in oocytes collected from heifers and PLs during the same season. In oocytes from heifers and PLs, the expression levels of these genes were lower in those collected during summer compared with winter, but this difference was not observed in oocytes collected from RBs. Altogether, these data provide evidence of altered gene expression and reduced mtDNA copy number in the oocytes collected from RBs during summer. This indicates a loss of fertility in RBs during summer, which might be caused by a possible mitochondrial dysfunction associated with a greater chance of oocytes to undergo apoptosis.

apoptosis, dairy cow, heat stress, mitochondria, oocyte quality, repeat breeding, reproductive efficiency

INTRODUCTION

Repeat breeding is a major problem in the dairy industry, with an incidence ranging from 9% to 24% of lactating animals [1–4]. Repeat-breeder (RB) cows are described as subfertile animals without any anatomical or infectious abnormalities that do not become pregnant until after the third or later breeding attempt, or that remain infertile after numerous services [2, 4, 5]. Although a multitude of factors may be involved in repeat breeding [4, 6, 7], several studies have suggested that oocyte quality plays a major role in its occurrence [8–15]. For instance, we have recently reported that oocytes from RB cows (RBs) exhibit a low capacity to develop into viable blastocysts in vitro [12]. Båge et al. [11] have also reported that oocytes from RB heifers are of poor quality, as judged by their morphology and cytoplasmic maturation profiles. These findings are in agreement with other reports that repeat breeding is characterized by low fertilization rates [8] and early embryonic loss [6, 9, 10], but that RBs experience similar rates of gestational loss compared to non-RBs [15]. Moreover, the transfer of embryos from non-RBs into RB surrogates has been shown to result in conception rates that are comparable to

[1]Supported by the São Paulo Research Foundation grants 2009/00938-3, 2010/13384-3, 2010/09561-7, 2011/14207-0, and 2012/07510-1, and the Coordination for the Improvement of Higher Level Personnel.
[2]Correspondence: Roberta Machado Ferreira, Avenida Duque de Caxias Norte, 225, Jardim Elite, CEP. 13635-000, Pirassununga, SP, Brazil. E-mail: robertinhavet@yahoo.com.br
[3]These authors contributed equally to this manuscript and are considered co-first authors.

Received: 30 June 2015.
First decision: 25 August 2015.
Accepted: 29 January 2016.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

those for non-RB surrogates, indicating that the lower conception rates of RBs are determined by the quality of the embryo and not by the uterine environment [15]. In summary, despite the significant economic losses caused by repeat breeding [2], little is known about the underlying molecular mechanisms by which oocyte competence is compromised in RBs.

Heat stress is another important factor that causes large reductions in fertility in lactating dairy cows [16, 17]. The extent of this problem is increasing, because intense genetic selection for high milk production is associated with decreased thermoregulatory competence [16, 17]. Follicular oocyte development represents one of the most critical periods of the reproductive cycle that is affected by heat stress, which alters patterns of follicular development [18], steroid production [19, 20], and gene expression [21, 22]. As a consequence, oocytes harvested from Holstein cows exposed to heat stress show reduced competence in developing into blastocysts in vitro [12, 16, 22–25]. Moreover, the effect of heat stress on oocyte competence may persist for long periods, even after the heat stress has ended [19, 26]. The effect of heat stress on fertility is even greater in RBs, resulting in very low blastocyst development and conception rates [12, 15]. This observation suggests that the reduced oocyte quality observed in RBs is exacerbated by heat stress, further highlighting the need for studies of the mechanisms by which oocyte competence is compromised in RBs.

The microenvironment of the preovulatory follicle is so critical for the developmental competence of oocytes that any perturbation during this period may affect subsequent development [27–29]. Transcription and regulation of mRNA stability are among the key events that occur during this period; these events play critical roles in establishing the molecular program for early embryogenesis [28–30]. There is evidence that exposure of the ovarian pool of oocytes to environmental stress disturbs oocyte/embryo gene expression, which, in turn, impairs development [22, 25]. The extensive mitochondrial replication that results in hundreds of thousands of mitochondria in fully grown oocytes is another important event that affects preovulatory follicles [31, 32]. The number of mitochondrial DNA (mtDNA) copies per oocyte has been associated with oocyte competence in mice [33], pigs [34, 35], humans [36–38], and cattle [39–42]. Therefore, any insult that disturbs mitochondrial replication during folliculogenesis might impair oocyte competence by rendering the organelle dysfunctional. Because oocyte quality represents an important factor in repeat breeding [8–15], evaluating gene expression patterns and mtDNA copy numbers in oocytes from RBs might increase the understanding of the molecular mechanisms that are involved in this syndrome.

The aim of this study was to address the hypothesis that the decreased oocyte competence that is observed in RB Holstein cows during summer is associated with altered gene expression profiles and decreased mtDNA copy numbers in oocytes. Hence, oocytes were collected from heifers, non-RBs in peak lactation (PL), and RBs [12]. Mitochondrial DNA copy number and the expression levels of genes associated with the mitochondria (*MT-CO1*, *NRF1*, *POLG*, *POLG2*, *PPARGC1A*, and *TFAM*), apoptosis (*BAX*, *BCL2*, and *ITM2B*), and oocyte maturation (*BMP15*, *FGF8*, *FGF10*, *FGF16*, *FGF17*, and *GDF9*) were then evaluated in these oocytes.

MATERIALS AND METHODS

All of the chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise stated.

Ethics Statement

This study was approved by the Bioethics Commission of the Faculdade de Medicina Veterinária e Zootecnia of the Universidade de São Paulo (protocol number 1571/2008), which complies with ethical principles for animal research. The cattle were provided by local commercial farms, as described below, with the consent of their owners. We acknowledge the farms Santa Rita–Agrindus S/A (Descalvado, SP, Brazil) and São Jorge (São Pedro, SP, Brazil) for supplying the animals and for the management required to conduct this study.

Experimental Design and Sample Collection

All analyses were conducted as described in a previous study [12]. In brief, Holstein (*Bos taurus*) cattle of three different categories were analyzed: heifers, PL, and RBs. The heifers were on average 16.8 ± 0.3 mo old, were cycling, and had never been inseminated. The RB group was composed of normal-cycling, lactating cows that had been inseminated several times (ranging from 4 to 13 services) without becoming pregnant and that had no anatomical or infectious abnormalities. The PL group consisted of normal-cycling cows, averaging 110.4 ± 3.8 days in milk, which had been inseminated less than three times (non-RBs). More information regarding the characteristics of the animals is provided in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org). Follicular wave emergence was synchronized using a standard protocol [12] before the females were subjected to ovum pick up (OPU). Four OPU sessions, two during winter (heifers, n = 34; PL, n = 32; and RB, n = 31) and two during summer (heifers, n = 36; PL, n = 37; and RB, n = 36), were conducted for each of the three categories of animals. However, each animal was subjected to only one OPU session. Retrospective analysis was conducted to choose the coolest and warmest periods of the year at the farms where the experiments were conducted [12]. Cumulus-oocyte complexes (COCs) that were recovered during the OPU sessions were morphologically classified as viable or unviable based on the cytoplasmic characteristics of the oocyte and on the number of cumulus cell layers [12]. Approximately 10% of the viable COCs were used for molecular analyses, whereas the remaining 90% were used for in vitro embryo production; the results of the in vitro embryo production experiments have been published previously [12]. The oocytes that were used for molecular analyses were mechanically separated from cumulus cells by vortexing (3 min at maximum speed) and were then thoroughly washed in PBS with 0.1% polyvinyl-pyrrolidone (PVP) three times to completely remove the cumulus cells. Every oocyte was closely checked for the presence of somatic cells under a stereomicroscope. Moreover, in a pilot experiment, denuded oocytes were tested for the absence of contaminating cumulus cells to validate our protocol [43]. The oocytes were stored individually at −80°C in 0.2 ml polystyrene PCR tubes with 1 μl PBS with 0.1% PVP and 1 U/μl RNase inhibitor (RNase OUT; Invitrogen, Carlsbad, CA).

Quantification of mtDNA and mRNA

Isolation of genomic DNA and total RNA. Both genomic DNA and total RNA were extracted from each individual oocyte using TRIzol reagent (Invitrogen), as previously described [39, 43]. The extracted RNA was directly dissolved in 10 μl DNase I solution (Invitrogen) plus 1 U/μl RNase OUT for DNA degradation, as suggested by the manufacturer. The concentration of RNA was not measured or normalized before reverse transcription. The total content of RNA from an oocyte was immediately reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The cDNA was then stored at −20°C until use. The extracted DNA was dissolved in 20 μl of 8 mM sodium hydroxide as suggested by the manufacturer [39]. To adjust the pH, 1.72 μl of 0.1 M Hepes was added, and then 3.3 μl of ultrapure H_2O was added to a final volume of 25 μl. The samples were immediately used for quantitative PCR to avoid DNA degradation [39].

Relative quantification of mtDNA. The relative quantification of mtDNA was performed as described previously [39]. In brief, fragments of mtDNA and of the external standard were added to samples that were amplified by real-time PCR before TRIzol extraction. The amount of mtDNA in each sample was calculated relative to the external standard using the standard curve method [44].

Quantification of mRNA. The expression of 15 genes was evaluated in single oocytes that were collected from animals in each of the different categories during winter and summer (Supplemental Table S2). These genes were selected due to their roles in important events that occur during folliculogenesis and early development. The chosen genes were grouped into three categories: genes related to mitochondria (*MT-CO1*, *NRF1*, *POLG*, *POLG2*, *PPARGC1A*, and *TFAM*), genes related to apoptosis (*BAX*, *BCL2*, and *ITM2B*), and genes related to oocyte maturation (*BMP15*, *FGF8*, *FGF10*,

FGF16, *FGF17*, and *GDF9*). We analyzed these target genes without normalization to internal controls, because we used single oocytes and because the expression of candidate reference genes was highly variable during heat stress [22, 43]. The stability of 10 candidate reference genes (*ACTH*, *GAPDH*, *GUSB*, *HIST1H2AG*, *HPRT1*, *PPIA*, *RPL15*, *SDHA*, *TBP*, and *YWHAZ*) was previously studied in oocytes collected from heifers, PL, and RBs during winter and summer, but an optimal number of reference genes could not be determined using the geNorm application, demonstrating their high degree of variability [43]. According to this result, nine reference genes would be needed to calculate a normalization factor for bovine oocytes exposed to seasonal variation, which would be impractical and expensive to apply to several experimental conditions. Thus, the normalization factor was calculated using the geometric average of the three housekeeping genes (*RPL15*, *PPIA*, and *GUSB*) that were considered to be the most stable [43], as previously suggested [45]. Normalization of the target genes using these criteria is presented as supplementary material (Supplemental Figs. S1–S3). Gene-specific mRNA transcripts were quantified by real-time RT-PCR using TaqMan assays, as described previously [43]. Briefly, primers and TaqMan probes were designed based on sequences available in GenBank (Supplemental Table S2) using Primer Express software v. 3.1 (Applied Biosystems). Whenever possible, primers or probes were designed to anneal to exon-exon junctions, avoiding genomic DNA amplification. The primers and probes were purchased from Applied Biosystems or Sigma-Aldrich, as indicated in Supplemental Table S2. Before performing real-time RT-PCR, cDNA was preamplified using a TaqMan PreAmp Master Mix Kit (Applied Biosystems), as previously described [43]. The linearity of amplification of each of the transcripts was determined as suggested by the manufacturer [43]. Real-time RT-PCR was performed in 15-μl reactions containing 1× TaqMan assay (consisting of 900 nM of primers and 250 nM of probe), 1× TaqMan Gene Expression Master Mix, and 2 μl of template. For each sample, preamplified cDNAs were used as a template after they were diluted by 100-fold (*GDF9*, *BMP15*, *FGF8*, *FGF10*, *FGF16*, and *FGF17*), 25-fold (*PPARGC1A*, *NRF1*, and *MT-CO1*), or 10-fold (*BCL2*, *BAX*, *ITM2B*, *POLG*, *POLG2*, and *TFAM*). All gene-specific cDNAs that were amplified for a particular sample were always run in duplicate in the same real-time RT-PCR plate using an ABI PRISM SDS 7500 HT Real-Time PCR System (Applied Biosystems). The following cycling conditions were applied for amplification: initial denaturation at 95°C for 15 min, followed by 40 cycles consisting of 95°C for 15 sec and 60°C for 1 min. Probe fluorescence was read at the end of each extension step (60°C). Standard curves were generated for each gene-specific cDNA using four five-fold serial dilutions of sample pools to evaluate amplification efficiency [43]. Because all of the assays (except for *BCL2* and *FGF17*) showed high amplification efficiency (roughly 100%), the target transcript amounts of each sample were linearized according to Livak and Schmittgen [44] using the $2^{-\Delta\Delta Ct}$ (normalized values) or 2^{-Ct} (nonnormalized values) method, as described previously [43]. The linearized average values of duplicate samples were used to evaluate the expression levels of specific genes with respect to season and animal category. The PCR products of each amplification assay were run on 2% agarose gels to assess the specificity of the amplified fragment.

Statistical Analysis

The real-time RT-PCR data were tested for the normality of residuals and for the homogeneity of variance. ANOVA was then performed using the GLIMMIX procedure of SAS v. 9.3 (SAS/STAT; SAS Institute Inc., Cary, NC) for a log-normal distribution [43]. The explanatory variables of animal category, season, and the interaction between animal category and season were considered for inclusion in the models. For each gene transcript, the expression level is presented following normalization to the mean level of the same transcript found in oocytes collected from heifers during winter on a natural log (Ln) scale (because the log-normal distribution was considered). Due to the Ln scale, a reduction in gene expression in a specific experimental group may result in a negative value when the expression level is expressed relative to the value obtained for oocytes collected from heifers during winter. Therefore, to avoid generating negative values, the mean used for data normalization was divided by e^5 [43], meaning that all data were compared against 5, which is the relative mean expression level found for oocytes retrieved from heifers during winter. The data are expressed in relation to oocytes collected from heifers during winter, because this is the category and season in which the physiological (lactation) and environmental (heat stress) influences on fertility are smallest. Significance was considered at $P < 0.05$. Values are presented as the mean ± SEM.

RESULTS

Low Oocyte Competence of RBs During Summer Heat Stress Is Related to Decreased mtDNA Copy Number in Oocytes

Because mtDNA copy number has been reported as a possible marker of oocyte viability, we sought to determine whether the low competence of oocytes retrieved from RBs during summer was associated with decreased mtDNA amounts. Immature oocytes that were collected via OPU from heifers, PL, and RBs [12] were used to measure mtDNA amounts. In agreement with our hypothesis, we found that oocytes retrieved from RBs during summer contained less mtDNA compared to oocytes from heifers ($P = 0.01$) or PLs ($P = 0.0008$) during the same season (Fig. 1). Furthermore, the mtDNA content was reduced by over eight-fold ($P = 0.0004$) in oocytes from RBs during summer in comparison with oocytes retrieved from the animals of the same category during winter (Fig. 1). Interestingly, the mtDNA content was negatively correlated ($r = 0.24$; $P = 0.04$) with the respiration rate at the time that the animals were subjected to OPU. However, although the amount of mtDNA was positively correlated with the total number of oocytes ($r = 0.24$; $P = 0.04$) and number of viable oocytes ($r = 0.25$; $P = 0.03$), no association between the mtDNA amount and developmental rate was found (Supplemental Table S3), corroborating our previous findings [39]. In summary, these data provide evidence of a reduced mtDNA copy number in oocytes from RBs during summer.

Increased Expression of Genes Associated with Mitochondrial Function Suggests a Compensatory Response to the Low Amounts of mtDNA in Oocytes Retrieved from RBs During Summer

To more deeply investigate the relationship between mtDNA copy number and oocyte competence in RBs, we evaluated the expression of genes associated with mitochondrial function (*MT-CO1*, *NRF1*, *POLG*, *POLG2*, *PPARGC1A*, and *TFAM*). As previously explained, the expression of the target genes was analyzed without normalization to reference gene expression (Fig. 2) to provide more precise information regarding the influence of heat stress and animal category on gene expression. An increase of *NRF1* ($P < 0.006$), *POLG* ($P < 0.003$), *POLG2* ($P < 0.002$), *PPARGC1A* ($P < 0.004$), and *TFAM* ($P < 0.0006$) expression was found in RB oocytes collected during summer compared to oocytes collected from heifers and PLs during the same season (Fig. 2). In addition, the expression of these genes during summer was decreased compared with that during winter in oocytes from heifers ($P < 0.001$) and PLs ($P < 0.01$), but not in oocytes from RBs (Fig. 2). In contrast, the expression of *MT-CO1* in heifers ($P = 0.04$) and RB ($P = 0.0008$) oocytes was greater than that in oocytes collected from PLs, regardless of season (Fig. 2). These results provide evidence of a potential compensatory response of mitochondria-related genes to the lower levels of mtDNA found in RB oocytes collected during summer. This compensatory effect is further supported by a negative correlation (Supplemental Table S3) between the mtDNA amount and expression of *MT-CO1* ($r = -0.26$; $P = 0.02$), *NRF1* ($r = -0.44$; $P < 0.0001$), *POLG* ($r = -0.51$; $P < 0.0001$), *POLG2* ($r = -0.47$; $P < 0.0001$), *PPARGC1A* ($r = -0.44$; $P < 0.0001$), and *TFAM* ($r = -0.45$; $P < 0.0001$). Moreover, positive correlations between the blastocyst rate and the expression of *NRF1* ($r = 0.29$; $P = 0.01$) and *POLG2* ($r = 0.23$; $P = 0.04$)

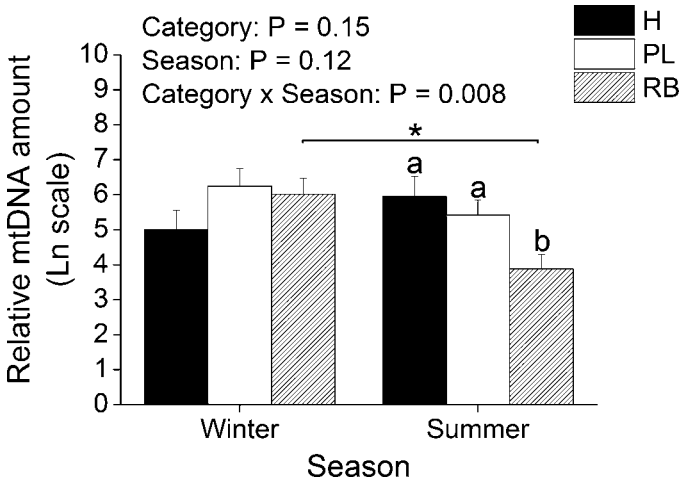

FIG. 1. Mitochondrial DNA content in Holstein cattle oocytes. Oocytes were retrieved from heifers (H; n = 17 and 15 oocytes, respectively), high-producing cows in PL (n = 16 and 18 oocytes, respectively), and RBs (n = 17 and 17 oocytes, respectively) during winter (W) and summer (S). The amounts of mtDNA are expressed relative to the amount detected in oocytes collected from heifers during winter. *P* values for animal category, season, and animal category × season are denoted in the insets above each graphic. The different letters over the bars denote significant differences between the categories within a season ($P < 0.05$). *Difference between seasons within a category ($P < 0.05$).

were identified, indicating that the expression of these genes was associated with oocyte competence.

Increased ITM2B and BAX Expression Provides Evidence of Apoptosis of Oocytes Collected from RBs During Summer

To further investigate whether the poor oocyte competence that was identified in oocytes collected from RBs during summer [12] was associated with altered expression of apoptotic genes, we evaluated the expression of *BAX*, *BCL2*, and *ITM2B*, as well as the *BAX*/*BCL2* ratio. Briefly, *ITM2B* has been described as a proapoptotic regulator that possesses the same BH3 domain that is present in BCL-2 gene family members [46–51]. When the expression levels of *BAX* and *ITM2B* were evaluated, both were increased in RB oocytes retrieved during summer compared with heifer ($P < 0.05$) and PL ($P < 0.01$; Supplemental Table S3) oocytes collected during the same season. Furthermore, the expression of *BAX* and *ITM2B* during summer was decreased compared with that during winter in heifer ($P < 0.05$) and PL ($P < 0.05$) oocytes, but not in RB oocytes. As for the mitochondrial genes, the expression patterns of these apoptotic genes in RB oocytes differed from those in oocytes collected from heifers and PLs; these differences might be related to differences in oocyte quality among the tested categories. Accurate analysis of *BCL2* expression could not be performed, due to the absence of detectable levels of *BCL2* transcripts in many oocytes retrieved during both summer (heifers = 14/15, PLs = 6/18, and RBs = 8/17 [failed/total samples]) and winter (heifers = 12/17, PLs = 11/16, and RBs = 8/17). As a result, the effects of season and animal category on the expression level of *BCL2* and *BAX*/*BCL2* had to be evaluated separately (Fig. 3), but no effects were found. In summary, the increased levels of *BAX* and *ITM2B* in the oocytes collected from the RBs during summer provide evidence of apoptosis. The increased levels of these transcripts might be a consequence of the lower amounts of mtDNA found in the RB oocytes that were retrieved during summer, as the expression of both *BAX* ($r = -0.39$; $P = 0.0006$) and *ITM2B* ($r = -0.46$; $P < 0.0001$) were negatively correlated with the mtDNA amount (Supplemental Table S3). A positive correlation (Supplemental Table S3) of *BAX* ($P < 0.0001$), *BCL2* ($P < 0.001$), and *ITM2B* ($P < 0.0001$) expression with *MT-CO1* ($r = 0.82$, 0.47, and 0.83, respectively), *NRF1* ($r = 0.76$, 0.71, and 0.84), *POLG* ($r = 0.88$, 0.66, and 0.96), *POLG2* ($r = 0.85$, 0.61, and 0.93), *PPARGC1A* ($r = 0.86$, 0.64, and 0.94), and *TFAM* ($r = 0.71$, 0.49, and 0.79) expression suggests that these genes were coregulated.

Altered Expression of GDF9 and FGF16 Suggests that These Genes Play Roles in the Low Developmental Competence of Oocytes Retrieved from RBs During Summer

Finally, we evaluated the expression of *GDF9*, *BMP15*, *FGF8*, *FGF10*, *FGF16*, and *FGF17* (Fig. 4) to investigate whether the poor oocyte competence identified in oocytes collected from RBs during summer [12] was associated with altered expression of genes related to oocyte maturation. These genes encode local factors that are secreted by the oocyte and that provide essential control over gamete maturation. The amplification of *FGF17* failed in all samples, leading to its exclusion from further analysis. The expression of *BMP15* ($P = 0.006$) and *FGF10* ($P = 0.004$) was lower in oocytes collected during summer compared with those collected during winter, regardless of the animal category. Furthermore, the expression of *FGF16* (Fig. 4) in oocytes collected from RBs during winter was decreased compared with that in oocytes collected from heifers ($P = 0.01$) and PLs ($P = 0.03$) during the same season. In heifer ($P < 0.0001$) and PLs ($P = 0.0003$), *FGF16* expression was also lower in oocytes collected during summer compared with those collected during winter. However, the expression of *GDF9* in oocytes collected from RBs during summer was greater than that in oocytes collected from heifers ($P = 0.04$) and PLs ($P = 0.01$) during the same season (Fig. 4). In heifers ($P < 0.0001$) and PLs ($P < 0.0001$), *GDF9* expression was also lower in oocytes collected during summer compared with those collected during winter. Altogether, these data provide evidence that *GDF9* and *FGF16* were differentially expressed in RB oocytes during summer and winter, suggesting that these genes may play roles in the low developmental competence of RB oocytes. This hypothesis is further supported by the finding that the expression of *GDF9* ($P < 0.05$) and *FGF16* ($P < 0.05$) were positively correlated (Supplemental Table S3) with the total number of oocytes ($r = 0.27$ and 0.24, respectively), the number of viable oocytes ($r = 0.27$ and 0.23), and the blastocyst rate ($r = 0.24$ and 0.21). Overall, the expression of individual genes was positively correlated with the expression of the other genes that were evaluated, whereas the expression of most of the evaluated genes was negatively correlated with the content of mtDNA in oocytes (Supplemental Table S3).

DISCUSSION

The results presented here strongly support the hypothesis that altered gene expression and reduced mtDNA copy number in oocytes are linked to decreased competence of oocytes collected from RBs during summer. The importance of mtDNA copy number in fertility has been extensively studied, because mtDNA replication is reported to be downregulated during the preimplantation period [52–58]. Previous studies have provided strong evidence of a link between mtDNA copy number and fertility in several species [34, 36–38], including cattle [40–42]. Indeed, studies have indicated that mitochondria play an important role in development [32, 59, 60]; however, it remains

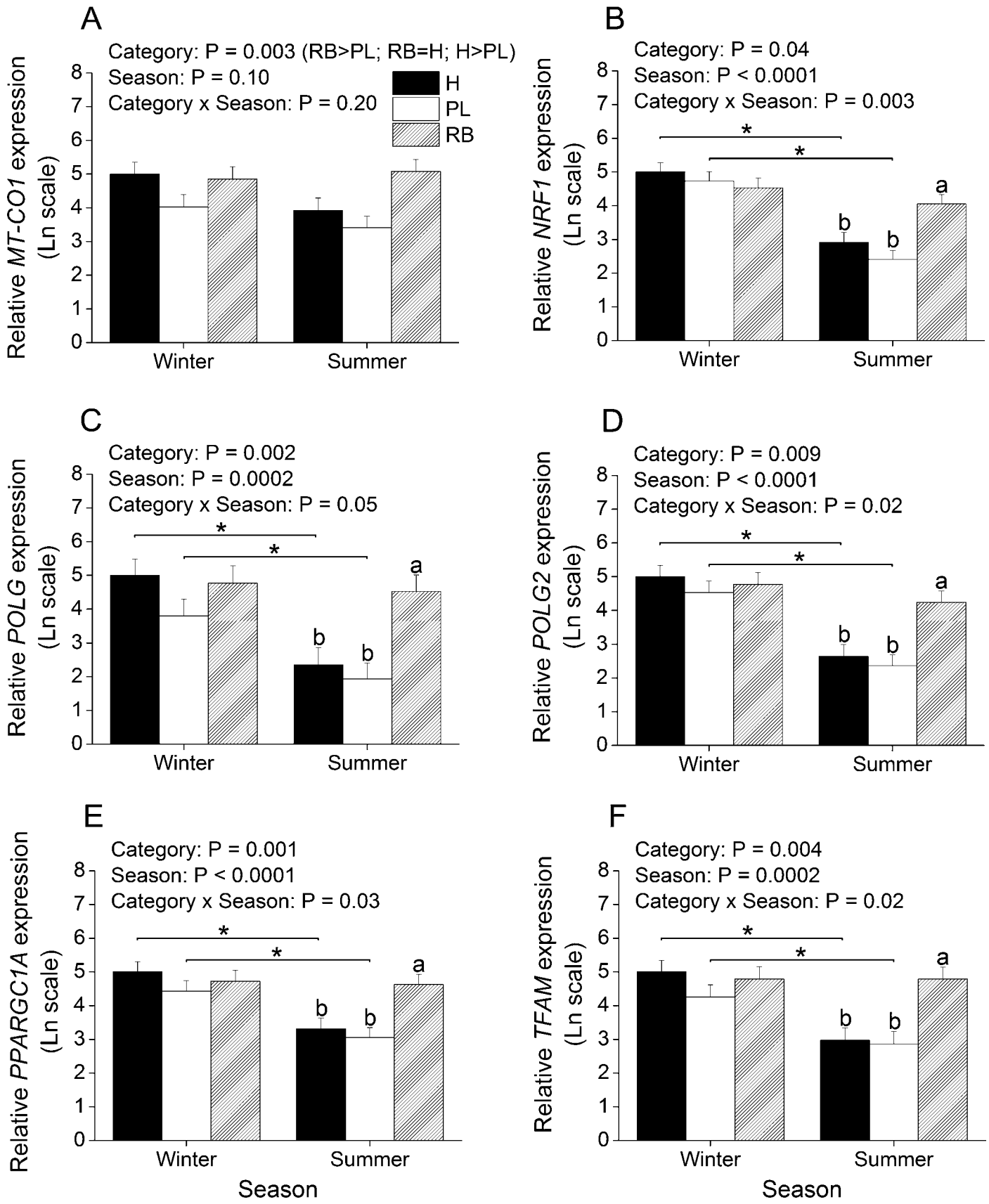

FIG. 2. Expression of mitochondria-related genes without normalization to reference gene expression. Oocytes were retrieved from heifers (H; n = 17 and 15 oocytes, respectively), high-producing cows in PL (n = 16 and 18 oocytes, respectively), and RBs (n = 17 and 17 oocytes, respectively) during winter (W) and summer (S). The amounts of *MT-CO1* (**A**), *NRF1* (**B**), *POLG* (**C**), *POLG2* (**D**), *PPARGC1A* (**E**), and *TFAM* (**F**) transcripts are expressed relative to the values obtained for oocytes that were collected from heifers during winter. *P* values for animal category, season, and animal category × season are denoted in the insets above each graphic. The different letters over the bars denote significant differences between categories within a season ($P < 0.05$). *Difference between seasons within a category ($P < 0.05$).

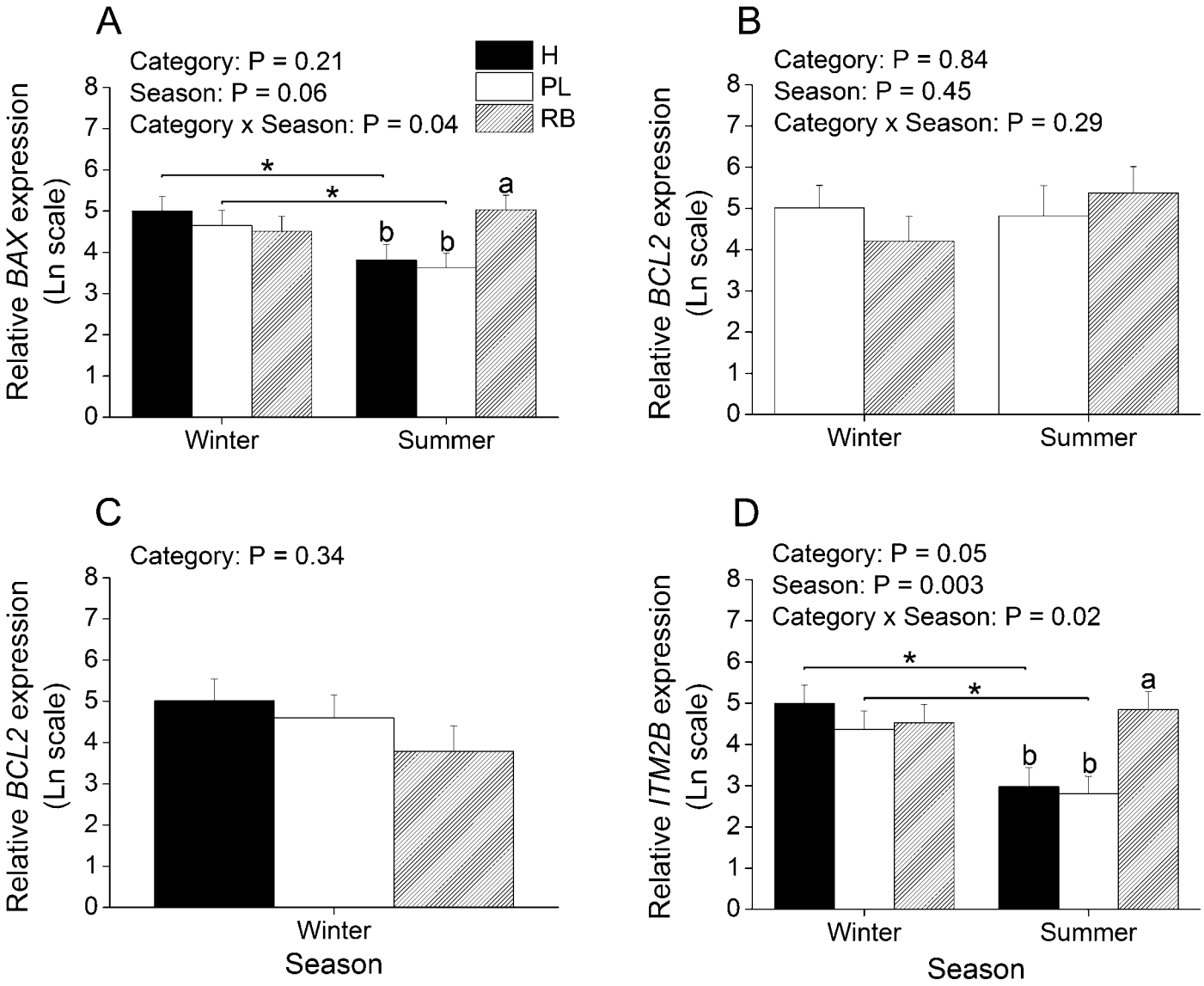

FIG. 3. Expression of apoptotic genes without normalization to reference gene expression. Oocytes were retrieved from heifers (H; n = 17 and 15 oocytes, respectively), high-producing cows in PL (n = 16 and 18 oocytes, respectively), and RBs (n = 17 and 17 oocytes, respectively) during winter (W) and summer (S). The amounts of *BAX* (**A**), *BCL2* (**B**), *BCL2* (**C**), and *ITM2B* (**D**) transcripts are expressed relative to the values obtained for oocytes collected from heifers during winter. *P* values for animal category, season, and animal category × season are denoted in the insets above each graphic. The different letters over the bars denote significant differences between categories within a season ($P < 0.05$). *Difference between seasons within a category ($P < 0.05$).

unclear whether the number of mtDNA copies that are present in cow oocytes is related to their competence to develop to term [61]. This relationship is clearer in some species, such as mice and humans [33, 61]. Moreover, recent reports in humans found an inverse correlation between mtDNA amount in embryos and their developmental potential [62, 63]. Although these findings are contradictory compared with those of previous reports, it seems that the differences were determined by the moment (e.g., oocyte vs. embryo) mtDNA amount was measured [33, 61–63]. Thus, it is believed that, regardless of the species, a minimum number of mtDNA copies in the oocyte is necessary to support development after fertilization [61]. If the number of mtDNA copies in oocytes is below this threshold, the embryo seems to have to activate mtDNA replication during preimplantation [39], explaining why less mtDNA in embryos is better [62, 63]. Herein, the amount of mtDNA in RB oocytes was decreased by over eight-fold during summer compared with winter. Furthermore, blastocyst rate in RBs dropped substantially from winter to summer (22.5 ± 5.4% vs. 7.9 ± 4.3%), whereas the percentage of TUNEL-positive cells in blastocysts increased by more than two-fold during summer (2.2 ± 0.2% vs. 4.9 ± 0.7%) [12]. Therefore, these findings provide further evidence of a link between mtDNA and oocyte viability in cattle, which may be associated with the lower fertility of RBs during summer.

In addition to the above findings, RB oocytes also had an increased amount of nuclear-encoded transcripts related to the transcription and replication of mtDNA (e.g., *NRF1*, *POLG*, *POLG2*, *PPARG*, and *TFAM*) [64]. Expression of these genes was not altered in comparison with RB oocytes during winter, but it was altered in relation to oocytes collected from heifers and PLs during summer. Since the oocytes collected from heifers and PLs presented better developmental rates [12], the altered expression pattern may also be associated with the

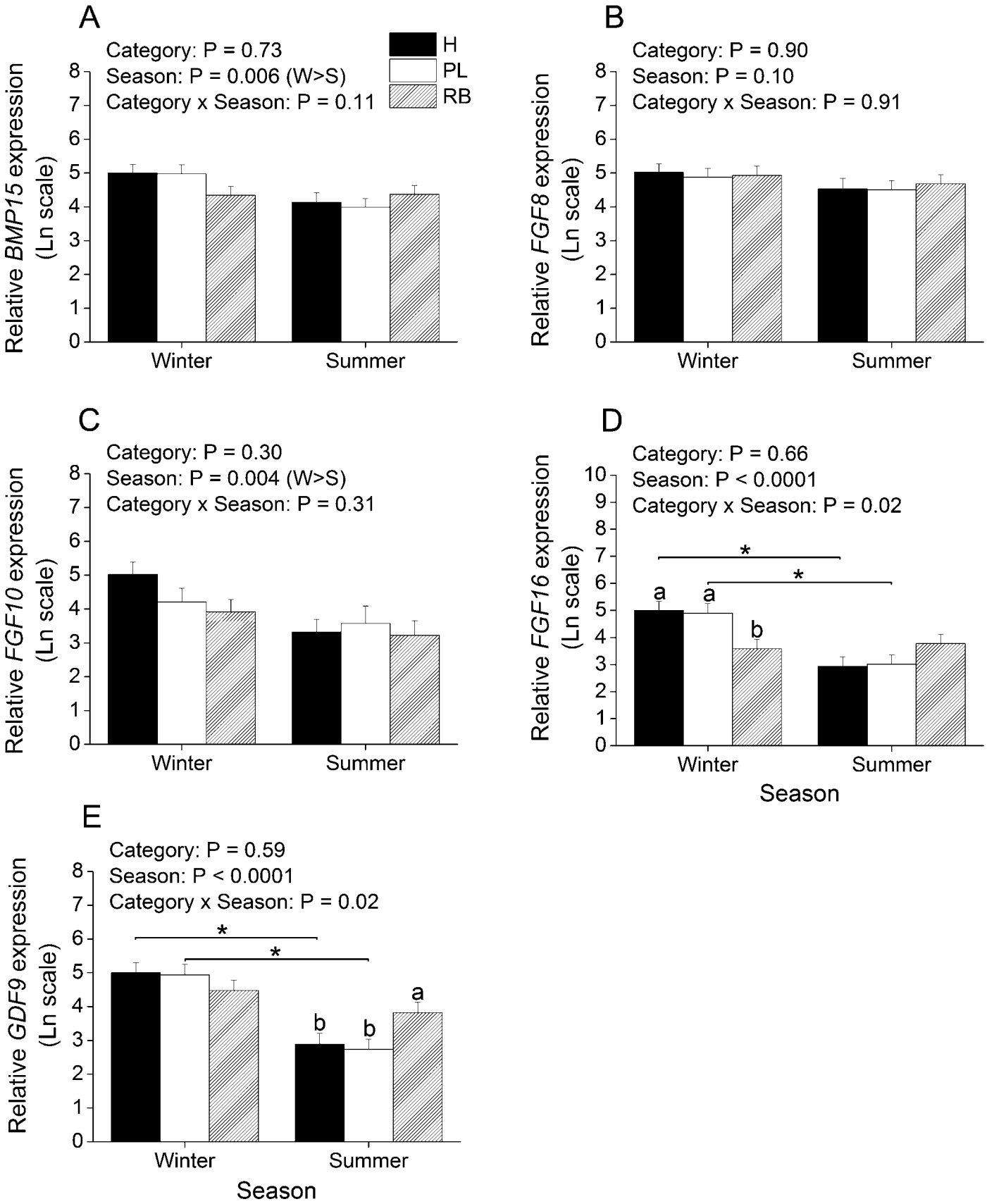

FIG. 4. Expression of maturation genes without normalization to reference gene expression. Oocytes were retrieved from heifers (H; n = 17 and 15 oocytes, respectively), high-producing cows in PL (n = 16 and 18 oocytes, respectively), and RBs (n = 17 and 17 oocytes, respectively) during winter (W) and summer (S). The amounts of *BMP15* (**A**), *FGF8* (**B**), *FGF10* (**C**), *FGF16* (**D**), and *GDF9* (**E**) transcripts are expressed relative to the values obtained for oocytes collected from heifers during winter. *P* values for animal category, season, and animal category × season are denoted in the insets above each graphic. The different letters over the bars denote significant differences between categories within a season ($P < 0.05$). *Difference between seasons within a category ($P < 0.05$).

poorer developmental competence of RB oocytes. In humans, increased expression of mitochondrial-targeted genes encoded by the nuclear DNA (nDNA) is a hallmark of mitochondrial dysfunction seen in patients with mitochondrial diseases [65]. In the presence of mtDNA mutations or depletion, the nDNA upregulates genes related to the mitochondrion in an attempt to rescue its function [65]. This may be the case here, as the lower content of mtDNA in RB oocytes might have triggered a nuclear response toward a mitochondrial rescue. This hypothesis is supported by the finding that the mtDNA content and expression of these genes was inversely correlated. Although such nuclear response may not have been sufficient to restore the mtDNA content in the oocyte, it might have supported an increase in the content of mtDNA in the developing embryo, as described in humans [62, 63]. Furthermore, expression of *MT-CO1*, an mtDNA-encoded transcript, did not differ among heifer, PL, and RB oocytes within summer, suggesting an attempt to rescue mitochondrial function. Altogether, these results demonstrate that gene expression in RB oocytes was altered during summer, providing evidence of a possible mitochondrial dysfunction in the oocytes of poorer developmental competence. This is in agreement with the result that oocytes that developed with better rates expressed greater levels of *NRF1* and *POLG2*. Moreover, upregulation of apoptotic genes (*BAX* and *ITM2B*) in RB oocytes, further confirms their greater sensitivity to summer. As blastocysts produced using RB oocytes presented higher rates of TUNEL-positive cells [12], increased expression of *BAX* and *ITM2B* might indicate that these oocytes were already more prone to undergo apoptosis.

An overall downregulation of gene expression during summer was also noticed in the oocytes from heifers and PLs compared with winter. This is demonstrated by the lower expression of genes associated with mitochondria (*NRF1*, *POLG*, *POLG2*, *PPARGC1A*, and *TFAM*), apoptosis (*BAX* and *ITM2B*) and oocyte maturation (*FGF16* and *GDF9*). In addition, we showed previously that these oocytes downregulated *HSP90AA1* and several candidate housekeeping genes (*ACTB*, *GAPDH*, *GUSB*, *HIST1H2AG*, *PPIA*, and *RPL15*) during summer [43]. These results provide further evidence of the profound effect of summer on oocyte development on expression of several genes (*HSP90AA1*, *HSPA1AB*, *NRF1*, *POLG2*, *GDF9*, *BMP15*, and *FGF16*), as indicated by the positive correlation between transcript abundance and development outcome into blastocysts. As previously suggested, this may be a consequence of cumulative heat stress on mRNA synthesis and storage during oocyte growth [22], perhaps as far back as the secondary follicle stage [26]. Herein, cows were exposed to heat stress during the entire summer period, and oocytes were collected only at the end of the season. The accumulation of maternal mRNA during the growth phase is known to provide oocytes with developmental competence [66], and perturbations during follicular development have been shown to be capable of reducing oocyte quality [22]. For instance, exposing oocytes at the germinal vesicle stage to heat stress impaired maternal mRNA storage and/or the mechanism of transcription renewal, which, in turn, affected gene expression (e.g., *MOS*, *GDF9*, *POU5F1*, and *GAPDH*) in the developing embryo [22]. These previous reports may explain the lower transcript levels found during summer for several genes in the present study and the poorer development of oocytes from heifers and PLs during summer [12]. Moreover, as follicle development lasts ~60 days in cattle, such effect on the oocyte may remain for long periods beyond the end of heat stress [26]. In summary, these data support a remarkable effect of summer on oocyte gene expression, which may impact on oocyte developmental competence and fertility of dairy cows.

In conclusion, herein we provide evidence that RB oocytes collected during summer present a possible mitochondrial dysfunction (illustrated by reduced mtDNA content and increased expression of mitochondrial genes) and are more prone to undergo apoptosis, which might be associated with their poorer developmental potential. In addition, an overall downregulation of gene expression was seen in the oocytes from heifers and PLs during summer, which corroborates the poorer developmental rates during heat stress. These findings are relevant to the management of Holstein cows in dairy farms, as the association of summer heat stress and repeat breeding causes significant economic losses through their impact on cow fertility.

ACKNOWLEDGMENT

We acknowledge hormone donations from Ourofino Saúde Animal (Cravinhos, SP, Brazil), the cooperation and assistance of the WTA Tecnologia Aplicada LTDA (Cravinhos, SP, Brazil), and Vida Reprodutiva Consultoria (Cravinhos, SP, Brazil) companies, the farms, Santa Rita–Agrindus S/A (Descalvado, SP, Brazil) and São Jorge (São Pedro, SP, Brazil), for supplying the animals and management necessary to conduct this study, and Professor José Buratini, Jr. for critical suggestion of the genes related to oocyte maturation with which we worked.

REFERENCES

1. Bulman DC, Lamming GE. Milk progesterone levels in relation to conception, repeat breeding and factors influencing acyclicity in dairy cows. J Reprod Fertil 1978; 54:447–458.
2. Bartlett PC, Kirk JH, Mather EC. Repeated insemination in Michigan Holstein-Friesian cattle: incidence, descriptive epidemiology and estimated economic impact. Theriogenology 1986; 26:309–322.
3. Waldmann A, Reksen O, Landsverk K, Kommisrud E, Dahl E, Refsdal AO, Ropstad E. Progesterone concentrations in milk fat at first insemination—effects on non-return and repeat-breeding. Anim Reprod Sci 2001; 65:33–41.
4. Yusuf M, Nakao T, Ranasinghe RBK, Gautam G, Long ST, Yoshida C, Koike K, Hayashi A. Reproductive performance of repeat breeders in dairy herds. Theriogenology 2010; 73:1220–1229.
5. Zemjanis R. 'Repeating breeding' or conception failure in cattle. In: Morrow DA (ed.), Current Therapy in Theriogenology. Philadelphia: WB Saunders; 1980:205–213.
6. Gustafsson H, Larsson K. Embryonic mortality in heifers after artificial insemination and embryo transfer: differences between virgin and repeat breeder heifers. Res Vet Sci 1985; 39:271–274.
7. Gustafsson H, Emanuelson U. Characterisation of the repeat breeding syndrome. Acta Vet Scand 2002; 43:115–125.
8. Graden AP, Olds D, Mochow CR, Mutter LR. Causes of fertilization failure in repeat breeding cattle. J Dairy Sci 1968; 51:778–781.
9. Linares T. A morphological study of the blastocysts collected from repeat breeder heifers. Theriogenology 1981; 15:128.
10. Linares T. Embryonic development in repeat breeder and virgin heifers seven days after insemination. Anim Reprod Sci 1982; 4:189–198.
11. Båge R, Petyim S, Larsson B, Hallap T, Bergqvist A-S, Gustafsson H, Rodríguez-Martínez H. Oocyte competence in repeat-breeder heifers: effects of an optimized ovum pick-up schedule on expression of oestrus, follicular development and fertility. Reprod Fertil Dev 2003; 15:115–123.
12. Ferreira RM, Ayres H, Chiaratti MR, Ferraz ML, Araújo AB, Rodrigues CA, Watanabe YF, Vireque AA, Joaquim DC, Smith LC, Meirelles FV, Baruselli PS. The low fertility of repeat-breeder cows during summer heat stress is related to a low oocyte competence to develop into blastocysts. J Dairy Sci 2011; 94:2383–2392.
13. Kurykin J, Waldmann A, Tiirats T, Kaart T, Jaakma Ü. Morphological quality of oocytes and blood plasma metabolites in repeat breeding and early lactation dairy cows. Reprod Domest Anim 2011; 46:253–260.
14. Puglisi R, Cambuli C, Capoferri R, Giannino L, Lukaj A, Duchi R, Lazzari G, Galli C, Feligini M, Galli A, Bongioni G. Differential gene expression in cumulus oocyte complexes collected by ovum pick up from repeat breeder and normally fertile Holstein Friesian heifers. Anim Reprod Sci 2013; 141:26–33.
15. Baruselli PS, Ferreira RM, Sales JNS, Gimenes LU, Sá Filho MF, Martins

CM, Rodrigues CA, Bó GA. Timed embryo transfer programs for management of donor and recipient cattle. Theriogenology 2011; 76: 1583–1593.
16. Hansen PJ. Exploitation of genetic and physiological determinants of embryonic resistance to elevated temperature to improve embryonic survival in dairy cattle during heat stress. Theriogenology 2007; 68(suppl 1):S242–S249.
17. Roth Z. Heat stress, the follicle, and its enclosed oocyte: mechanisms and potential strategies to improve fertility in dairy cows. Reprod Domest Anim 2008; 43(suppl 2):238–244.
18. Roth Z, Meidan R, Braw-Tal R, Wolfenson D. Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. J Reprod Fertil 2000; 120: 83–90.
19. Roth Z, Meidan R, Shaham-Albalancy A, Braw-Tal R, Wolfenson D. Delayed effect of heat stress on steroid production in medium-sized and preovulatory bovine follicles. Reproduction 2001; 121:745–751.
20. Bridges PJ, Brusie MA, Fortune JE. Elevated temperature (heat stress) in vitro reduces androstenedione and estradiol and increases progesterone secretion by follicular cells from bovine dominant follicles. Domest Anim Endocrinol 2005; 29:508–522.
21. Argov N, Moallem U, Sklan D. Summer heat stress alters the mRNA expression of selective-uptake and endocytotic receptors in bovine ovarian cells. Theriogenology 2005; 64:1475–1489.
22. Gendelman M, Roth Z. Seasonal effect on germinal vesicle-stage bovine oocytes is further expressed by alterations in transcript levels in the developing embryos associated with reduced developmental competence. Biol Reprod 2012; 86:1–9.
23. Al-Katanani YM, Paula-Lopes FF, Hansen PJ. Effect of season and exposure to heat stress on oocyte competence in Holstein cows. J Dairy Sci 2002; 85:390–396.
24. Rocha A, Randel RD, Broussard JR, Lim JM, Blair RM, Roussel JD, Godke RA, Hansel W. High environmental temperature and humidity decrease oocyte quality in *Bos taurus* but not in *Bos indicus* cows. Theriogenology 1998; 49:657–665.
25. Gendelman M, Roth Z. In vivo vs. in vitro models for studying the effects of elevated temperature on the GV-stage oocyte, subsequent developmental competence and gene expression. Anim Reprod Sci 2012; 134: 125–134.
26. Torres-Júnior JR de S, Pires M de FA, de Sá WF, Ferreira A de M, Viana JHM, Camargo LS, Ramos AA , Folhadella IM, Polisseni J, de Freitas C, Clemente CA, de Sá Filho MF, et al. Effect of maternal heat-stress on follicular growth and oocyte competence in *Bos indicus* cattle. Theriogenology 2008; 69:155–166.
27. Moor RM, Dai Y, Lee C, Fulka J. Oocyte maturation and embryonic failure. Hum Reprod Update 1998; 4:223–236.
28. Fair T. Mammalian oocyte development: checkpoints for competence. Reprod Fertil Dev 2010; 22:13–20.
29. Sirard M. Factors affecting oocyte and embryo transcriptomes. Reprod Domest Anim 2012; 47(suppl 4):148–155.
30. Sirard M-A, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. Theriogenology 2006; 65:126–136.
31. Shoubridge E a, Wai T. Mitochondrial DNA and the mammalian oocyte. Curr Top Dev Biol 2007; 77:87–111.
32. Dumollard R, Duchen M, Carroll J. The role of mitochondrial function in the oocyte and embryo. Curr Top Dev Biol 2007; 77:21–49.
33. Wai T, Ao A, Zhang X, Cyr D, Dufort D, Shoubridge EA. The role of mitochondrial DNA copy number in mammalian fertility. Biol Reprod 2010; 83:52–62.
34. El Shourbagy SH, Spikings EC, Freitas M, St. John JC. Mitochondria directly influence fertilisation outcome in the pig. Reproduction 2006; 131:233–245.
35. Sato D, Itami N, Tasaki H, Takeo S, Kuwayama T, Iwata H. Relationship between mitochondrial DNA copy number and SIRT1 expression in porcine oocytes. PLoS One 2014; 9:e94488.
36. Reynier P, May-Panloup P, Chrétien MF, Morgan CJ, Jean M, Savagner F, Barrière P, Malthièry Y. Mitochondrial DNA content affects the fertilizability of human oocytes. Mol Hum Reprod 2001; 7:425–429.
37. May-Panloup P, Chrétien MF, Jacques C, Vasseur C, Malthièry Y, Reynier P. Low oocyte mitochondrial DNA content in ovarian insufficiency. Hum Reprod 2005; 20:593–597.
38. Santos TA, El Shourbagy S, St. John JC. Mitochondrial content reflects oocyte variability and fertilization outcome. Fertil Steril 2006; 85: 584–591.
39. Chiaratti MR, Bressan FF, Ferreira CR, Caetano AR, Smith LC, Vercesi AE, Meirelles FV. Embryo mitochondrial DNA depletion is reversed during early embryogenesis in cattle. Biol Reprod 2010; 82:76–85.
40. Tamassia M, Nuttinck F, May-Panloup P, Reynier P, Heyman Y, Charpigny G, Stojkovic M, Hiendleder S, Renard J-P, Chastant-Maillard S. In vitro embryo production efficiency in cattle and its association with oocyte adenosine triphosphate content, quantity of mitochondrial DNA, and mitochondrial DNA haplogroup. Biol Reprod 2004; 71:697–704.
41. Hua S, Zhang Y, Li X-C, Ma L-B, Cao J-W, Dai J-P, Li R. Effects of granulosa cell mitochondria transfer on the early development of bovine embryos in vitro. Cloning Stem Cells 2007; 9:237–246.
42. Iwata H, Goto H, Tanaka H, Sakaguchi Y, Kimura K, Kuwayama T, Monji Y. Effect of maternal age on mitochondrial DNA copy number, ATP content and IVF outcome of bovine oocytes. Reprod Fertil Dev 2011; 23:424–432.
43. Macabelli CH, Ferreira RM, Gimenes LU, De Carvalho NAT, Soares JG, Ayres H, Ferraz ML, Watanabe YF, Watanabe OY, Sangalli JR, Smith LC, Baruselli PS, et al. Reference gene selection for gene expression analysis of oocytes collected from dairy cattle and buffaloes during winter and summer. PLoS One 2014; 9:e93287.
44. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−delta delta C(T)) method. Methods 2001; 25:402–408.
45. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3:RESEARCH0034.
46. Pittois K, Deleersnijder W, Merregaert J. cDNA sequence analysis, chromosomal assignment and expression pattern of the gene coding for integral membrane protein 2B. Gene 1998; 217:141–149.
47. Deleersnijder W, Hong G, Cortvrindt R, Poirier C, Tylzanowski P, Pittois K, Van Marck E, Merregaert J. Isolation of markers for chondro-osteogenic differentiation using cDNA library subtraction. J Biol Chem 1996; 271:19475–19482.
48. Fleischer A, Ayllón V, Dumoutier L, Renauld J-C, Rebollo A. Proapoptotic activity of ITM2B(s), a BH3-only protein induced upon IL-2-deprivation which interacts with Bcl-2. Oncogene 2002; 21: 3181–3189.
49. Fleischer A, Rebollo A. Induction of p53-independent apoptosis by the BH3-only protein ITM2Bs. FEBS Lett 2004; 557:283–287.
50. Van den Plas D, Merregaert J. In vitro studies on Itm2a reveal its involvement in early stages of the chondrogenic differentiation pathway. Biol Cell 2004; 96:463–470.
51. Choi DK, Yoo KW, Hong SK, Rhee M, Sakaki Y, Kim CH. Isolation and expression of Napor/CUG-BP2 in embryo development. Biochem Biophys Res Commun 2003; 305:448–454.
52. Pikó L, Taylor KD. Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. Dev Biol 1987; 123:364–374.
53. Cao L, Shitara H, Horii T, Nagao Y, Imai H, Abe K, Hara T, Hayashi J-I, Yonekawa H. The mitochondrial bottleneck occurs without reduction of mtDNA content in female mouse germ cells. Nat Genet 2007; 39: 386–390.
54. Thundathil J, Filion F, Smith LC. Molecular control of mitochondrial function in preimplantation mouse embryos. Mol Reprod Dev 2005; 71: 405–413.
55. Cree LM, Samuels DC, de Sousa Lopes SC, Rajasimha HK, Wonnapinij P, Mann JR, Dahl H-HM, Chinnery PF. A reduction of mitochondrial DNA molecules during embryogenesis explains the rapid segregation of genotypes. Nat Genet 2008; 40:249–254.
56. Cree LM, Hammond ER, Shelling AN, Berg MC, Peek JC, Green MP. Maternal age and ovarian stimulation independently affect oocyte mtDNA copy number and cumulus cell gene expression in bovine clones. Hum Reprod 2015; 30:1410–1420.
57. Iwata H, Goto H, Tanaka H, Sakaguchi Y, Kimura K, Kuwayama T, Monji Y. Effect of maternal age on mitochondrial DNA copy number, ATP content and IVF outcome of bovine oocytes. Reprod Fertil Dev 2011; 23:424–432.
58. Takeo S, Goto H, Kuwayama T, Monji Y. Effect of maternal age on the ratio of cleavage and mitochondrial DNA copy number in early developmental stage bovine embryos. J Reprod Dev 2013; 59:174–179.
59. Machatkova M, Jeseta M, Hulinska P, Knitlova D, Nemcova L, Kanka J. Characteristics of bovine oocytes with different meiotic competence in terms of their mitochondrial status and expression of nuclear-encoded factors. Reprod Domest Anim 2012; 47:806–814.
60. Van Blerkom J. Mitochondrial function in the human oocyte and embryo and their role in developmental competence. Mitochondrion 2011; 11: 797–813.
61. Chiaratti MR, Meirelles FV. Mitochondrial DNA copy number, a marker of viability for oocytes. Biol Reprod 2010; 83:1–2.

62. Fragouli E, Spath K, Alfarawati S, Kaper F, Craig A, Michel CE, Kokocinsk IF, Cohen J, Munne S, Wells D. Altered levels of mitochondrial DNA are associated with female age, aneuploidy, and provide an independent measure of embryonic implantation potential. PLoS One 2015; 11:e1005241.
63. Diez-Juan A, Rubio C, Marin C, Martinez S, Al-Asmar N, Riboldi M, Díaz-Gimeno P, Valbuena D, Simón C. Mitochondrial DNA content as a viability score in human euploid embryos: less is better. Fertil Steril 2015; 104:534–541.
64. Falkenberg M, Larsson N-G, Gustafsson CMDNA. Replication and transcription in mammalian mitochondria. Annu Rev Biochem 2007; 76: 679–699.
65. Schon E a, DiMauro S, Hirano M. Human mitochondrial DNA: roles of inherited and somatic mutations. Nat Rev Genet 2012; 13:878–890.
66. Trounson A, Anderiesz C, Jones G. Maturation of human oocytes in vitro and their developmental competence. Reproduction 2001; 121:51–75.