Accepted Manuscript

Physiological responses of cultured bovine granulosa cells to elevated temperatures under low and high oxygen in the presence of different concentrations of melatonin

Bayar K. Zeebaree, Wing Y. Kwong, George E. Mann, Carlos G. Gutierrez, Kevin D. Sinclair

PII: S0093-691X(17)30442-9

DOI: 10.1016/j.theriogenology.2017.09.014

Reference: THE 14261

To appear in: *Theriogenology*

Received Date: 24 May 2017

Revised Date: 8 September 2017

Accepted Date: 9 September 2017

Please cite this article as: Zeebaree BK, Kwong WY, Mann GE, Gutierrez CG, Sinclair KD, Physiological responses of cultured bovine granulosa cells to elevated temperatures under low and high oxygen in the presence of different concentrations of melatonin, *Theriogenology* (2017), doi: 10.1016/j.theriogenology.2017.09.014.

Physiological responses of cultured bovine granulosa cells to elevated temperatures under low and high oxygen in the presence of different concentrations of melatonin

Bayar K Zeebaree[1], Wing Y Kwong[1], George E Mann[1],
Carlos G Gutierrez[1,2] and Kevin D Sinclair[1*]

[1]University of Nottingham, School of Biosciences, Sutton Bonington, Leicestershire, LE12 5RD, UK

[2]Universidad Nacional Autonoma de Mexico, Facultad de Medicina Veterinaria, Mexico City 04510, Mexico

*Correspondence: kevin.sinclair@nottingham.ac.uk

Abstract

Our understanding of the effects of temperature on granulosa cell (GC) physiology is primarily limited to *in vitro* studies conducted under atmospheric (~20% O_2) conditions. In the current series of factorial experiments we identify important effects of O_2 level (i.e. 5% vs 20% O_2) on GC viability and steroidogenesis, and go onto report effects of standard (37.5°C) vs high (40.0°C) temperatures under more physiologically representative (i.e. 5%) O_2 levels in the presence of different levels of melatonin (0, 20, 200 and 2000 pg/mL); a potent free-radical scavenger and abundant molecule within the ovarian follicle. Cells aspirated from antral (4 to 6 mm) follicles were cultured in fibronectin-coated wells using serum-free M199 for up to 144 h. At 37.5°C viable cell number was enhanced and luteinization reduced under 5 vs 20% O_2. Oxygen level interacted ($P<0.001$) with time in culture to affect aromatase activity and cell estradiol (E_2) production (pg/mL/10^5 cells). These decreased between 48 and 96 h for both O_2 levels but increased again by 144 h for cells cultured under 5% but not 20% O_2. Progesterone (P_4) concentration (ng/mL/10^5 cells) was greater ($P<0.001$) under 20 vs 5% O_2 at 96 and 144 h. Cell number increased ($P<0.01$) with time in culture under 5% O_2 irrespective of temperature. However, higher doses of melatonin increased viable cell number at 40.0°C but reduced viable cell number at 37.5°C ($P=0.004$). Melatonin also reduced ($P<0.001$) ROS generation at both O_2 levels across all concentrations. E_2 increased with time in culture at both temperatures under 5% O_2, however P_4 declined between 96 to 144 h at 40.0 but not 37.5°C. Furthermore, melatonin interacted ($P<0.001$) with temperature in a dose dependent manner to increase P_4 at 37.5°C but to reduce P_4 at 40.0°C. Transcript expression for *HSD3B1* paralleled temporal changes in P_4 production, and those for *HBA* were greater at 5% than 20% O_2, suggesting that hemoglobin synthesis is responsive to changes in O_2 level. In conclusion, 5% O_2 enhances GC proliferation and reduces luteinization. Elevated temperatures under 5% O_2 reduce GC proliferation and P_4 production. Melatonin reduces ROS generation irrespective of O_2 level and temperature, but interacts with temperature in a dose dependent manner to influence GC proliferation and luteinization.

Keywords: Granulosa cells, Oxygen level, Melatonin, Heat stress, hemoglobin

1. Introduction

Thermal stress can have a detrimental effect on ovarian function and endometrial receptivity in the cow leading to reduced expression of estrus, impaired post-fertilisation development of oocytes and implantation failure [1-3]. It is particularly problematic in the metabolically challenged high-yielding dairy cow that struggles to dissipate heat under moderate to high (typically ≥25°C) ambient temperatures [4,5]. Reduced blood flow to the ovary in such animals can contribute to observed delays in emergence of dominant/pre-ovulatory follicles [6] which in turn can indirectly compromise oocyte quality. However, the effects of heat stress are also believed to directly and negatively affect both pre- and early-antral stages of follicle development, and the pool of germinal vesicle-stage oocytes contained therein [7].

In order to gain a better understanding of the mechanisms underlying the effects of thermal stress on follicular development several studies have undertaken short-term *in vitro* culture of granulosa and/or thecal cells simulating normal or high core-body temperatures. They confirmed reductions in cell viability associated with upregulation of apoptotic pathways and reduced steroidogenic capacity [1,8,9]. However, whilst providing valuable insights into underlying mechanisms, these and other related studies [10], invariably cultured cells under atmospheric (~20%) O_2 levels often in the presence of serum. To the best of our knowledge there are no *in vitro* culture studies that have assessed the effects of thermal stress on somatic cells of the ovary under more physiological O_2 levels (~5% O_2), although one recent study considered temperature and atmospheric environment in the context of ROS generation during bovine oocyte maturation [11]. This issue is important because it is believed that both bovine and porcine GCs cultured under low O_2 are more prolific, glycolytic and estrogenic than GCs cultured in 5% CO_2 in air [12,13]. A low O_2 culture environment probably better recapitulates intra-follicular atmospheric conditions as fractional O_2 concentrations in follicular fluid range between 2 and 9% [14,15].

Variable concentrations (10 to > 400 pg/mL) of the indole amine melatonin have been reported in follicular fluid of different species including the cow [16-20]. Melatonin is believed to exert protective effects on ovarian cells during thermal stress due to its capacity to act as a potent antioxidant [21]. However, as with the studies described earlier, *in vitro* culture experiments with melatonin have to date invariably been conducted under atmospheric O_2 conditions [17, 22] so that the described modes of action and benefits of melatonin may be specific to these situations and less representative of intra-follicular

processes. Culturing under low O_2 may provide a more physiologically relevant system to investigate the effects of this potent antioxidant in helping to attenuate thermal stress on somatic cells within the ovarian follicle. The current series of experiments, therefore, sought initially to characterise the effects of O_2 level (i.e. 5% vs 20% O_2) on GC viability and steroidogenesis and then to investigate the effects of standard (37.5°C) vs high (40.0°C) temperatures under low (i.e. 5%) O_2 levels in the presence of different concentrations of melatonin (0, 20, 200 and 2000 pg/mL).

2. Materials and methods

All reagents were obtained from Sigma–Aldrich unless otherwise stated.

2.1. Granulosa cell culture

Antral follicles (4 to 6 mm) were aspirated from abattoir derived ovaries using a 21 G needle and GCs prepared for serum-free culture. These GCs were therefore likely to represent a population of largely luminal GCs and cumulus cells. Compared to mural GCs (scraped from dissected follicles of comparable size) these cells are more estrogenic [12] and mitotically active [23]. Viable cells (determined by trypan blue exclusion [24] were re-suspended in 1 ml of pre-warmed M199 culture medium supplemented with (Penicillin (50 IU/ml), Streptomycin (50 μg/ml), bovine serum albumin free fatty acid (BSA; 1 mg/ml), testosterone (100 ng/ml), FSH (1 ng/ml; Cat. No. F2293), insulin (10 ng/ml), transferrin 2.5 (μg/ml), sodium selenite (4 ng/ml) and L-glutamine (365 μg/ml)) prior to plating in fibronectin coated wells (Nunclon Delta, Thermo Fisher, Denmark) at seeding densities depicted in Table S1. Fibronectin facilitates the attachment and proliferation of GCs [25], whilst low insulin (10 ng/mL) in serum-free media allows cells to form aggregates, proliferate and maintain a primary GC phenotype [26]; hence their responsiveness to trophic hormones [27].

2.2. Experimental designs

2.2.1. Experiment 1.A. Effect of atmospheric vs physiological O_2 level on cell number, steroidogenesis and aromatase activity

This was a 2 x 3 factorial experiment with two O_2 levels (~5 vs 20%; using two humidified incubators (Model Innova CO-14, New Brunswick Scientific, Edison, NJ, USA) at 37.5°C)

and three culture endpoints (48, 96 and 144 h from seeding)), replicated five times using a 6-well plate format (Table S1). 80% of media was replaced every 48 h during culture. Upon harvesting, spent media and cell pellets were snap frozen in liquid N and stored at -80°C until analysis.

2.2.2. Experiment 1.B. Effect of melatonin on cell number, steroidogenesis and gene expression under atmospheric vs physiological O_2 levels

This was a 4 x 2 x 3 factorial experiment with four levels of melatonin (0, 20, 200, and 2000 pg/ ml), two O_2 levels (~5 vs 20%; using two humidified incubators at 37.5°C) and three culture endpoints (48, 96 and 144 h from seeding), replicated four times using a 12-well plate format (Table S1). Melatonin levels for this and subsequent experiments were selected on the basis of concentrations reported previously in ovarian follicular fluids [16-20], and from a small pilot study where we determined melatonin concentrations by ELISA (MyBioSource.com; San Diego, CA, USA; Bovine kit - MBS743340) in follicular fluids from 15 heifers slaughtered at a local abattoir (Figure S1). Simple and geometric means for ovarian follicular-fluid melatonin in that study were 1,600 (95% CI = 173 - 3036) and 320 pg/mL respectively. Media were changed and cells harvested as described for Experiment 1A.

2.2.3. Experiment 1.C. Effect of O_2 level and melatonin on ROS

This experiment adopted the factorial arrangement described for Experiment 1B but using a 96-well format (Table S1). Media were changed as described for Experiment 1A. Generation of ROS was assessed at 48, 96 and 144 h of culture (described later).

2.2.4. Experiment 2.A. Effect of temperature and melatonin on cell number, steroidogenesis and gene expression under physiological O_2 levels

This experiment adopted a factorial arrangement similar to Experiment 1B but treatments (37.5 vs 40.0 °C; using two humidified incubators at 5% O_2 with four melatonin doses (0, 20, 200 and 2000 pg/ml)) commenced after 48 h of culture (Table S2). Incubator temperature was monitored using two thermometers (temperature loggers, EL-USB-1, Lascar Electronics, Salisbury, UK) in addition to that built into the incubator. Media were changed and cells harvested as described for Experiment 1A.

120 *2.2.5. Experiment 2.B. Effect of temperature and melatonin on ROS production under*
121 *physiological O_2 levels*

122 This experiment also adopted the factorial arrangement described for Experiment 1B but
123 using a 96-well format (Table S2) and with treatments described for Experiment 2A. Media
124 were changed as described for Experiment 1A. Generation of ROS was assessed at 96 and
125 144 h of culture (described later).

126 *2.3. Hormone analyses*

127 Progesterone and E_2 production by GCs after 48, 96 and 144 h of culture in Experiment 1A,
128 1B and 2A was assessed by ELISA using commercial kits provided by Ridgeway Research
129 Ltd, Gloucestershire, UK (P_4 product code RIDGE-P), and DRG GmbH, Marburg, Germany,
130 (E_2; product code EIA-2693)) as described previously [28,29]. Spent media were initially
131 diluted (P_4, 1:100; E_2 1:20) and analysed in duplicate. Inter- and intra-assay CV for P_4 were
132 11.1% and 5.1% respectively, and corresponding values for E_2 were 8.6% and 6.8%.

133 *2.4. Aromatase activity (Experiment 1A)*

134 Granulosa cells (~1 x 10^6 cells/tube) were homogenized in 200 µl of aromatase buffer
135 solution PH 7.4 (20 mM TES, 10 mM EDTA, 150 mM KCL, protease inhibitor) on ice using
136 tissue and cell homogenizer (Fast Prep-24, model 6004-500, Strasbourg, France) for 30s.
137 Homogenates of two wells per plate were pooled. The protein was then extracted by
138 centrifugation at 1000g for 5 min at 4°C, quantified (BCA method [30] and samples stored at
139 – 80°C until assayed for aromatase activity as described by Satoh et al. [31] and Tinwell et al.
140 [32] with slight modifications. Briefly, duplicate aliquots of 60 µg cell protein were incubated
141 for 25 min at 37°C with testosterone (100 nM) and NADPH (10 mM) (Santa Cruz, sc
142 202725; Cofactor, Cytochrome P450 reductase) in aromatase working buffer (final reaction
143 volume of 200 µl/tube at pH 7.5). Enzyme activity was then terminated by heating the tube at
144 100°C for 5 min. In addition, background E_2 (i.e. intracellular E_2) was estimated for each
145 sample after heat inactivation prior to enzyme reaction. Following centrifugation,
146 supernatants were stored at -80°C until E_2 assay by ELISA. The experiment was replicated 5
147 times.

2.5. Transcript expression (Experiments 1B and 2A)

Methodologies reported were those used previously in our laboratory [28]. Briefly, total RNA was extracted from cultured GCs using RNeasy Mini Kit (Qiagen Ltd., West Sussex, UK) and treated with DNase (Promega, Southampton Science Park, Southampton, UK) to remove genomic DNA contamination. DNase-treated RNA was then transcribed into complementary DNA (cDNA) using Omniscript cDNA synthesis kit (Qiagen Ltd) in a 20 µl volume according to manufacturer's instruction. Quantitative Real time PCR (qPCR) was performed using a Roche LightCycler 480 (Roche Diagnostics Ltd, Penzberg, Germany) with gene-specific primers and TaqMan probes (Eurofins Genomics, Ebersberg, Munich, Germany) that were labelled with the 6-carboxyfluorescein (FAM) and tetramethylrhodamine (TAMRA) at 5' and 3' ends respectively (Table S3). PCR was performed in 20 µl volume containing 10 µl of 2x Probe Master mix, 0.3 µM each primer, 0.2 µM Taqman probe and 1 µl cDNA. Before quantification, standard curves using each primer/probe set for a particular gene were generated and only those which gave an efficiency of 1.8 to 2.0 used. To ensure no genomic DNA contamination, –RT for genes were performed. A negative control (without cDNA) was also included in each qPCR run. Four biological replicates were conducted per experiment and cDNA from each sample was run in duplicate for each gene.

Several housekeeping genes (*RPL19, RPLP0, B2M and TBP)* were tested (Table S3) and it was found that *TBP* was the most stable housekeeping gene when analysed by NormFinder and RefFinder. Hence, all target genes in this study were normalized to *TBP*. Relative quantification was calculated using the formula of [33].

2.6. *Measurement of ROS (Experiments 1C and 2B)*

Reactive oxygen species generated from cultured GCs were measured by Nitroblue tetrazolium (NBT; N6876, Sigma-Aldrich) as previously described [34-36] but with some modifications. Briefly, media were removed and 50 µl of 1 mg/ml NBT added to each well and incubated for 2 min. Reactions were terminated by adding 100 µl of 1 M HCl. Solutions in each well were then removed and wells washed three times with PBS. Then 150 µl of dimethyl sulfoxide (DMSO) was added to each well to solubilise formazan produced inside the cells. Finally, 10 µl of 1 M NaOH was added to each well and shaken for 20 min. Colour production was measured at 630 nm using a plate reader (Thermo Fisher, Loughborough,

UK). Optical density (OD) was adjusted to viable cell number as assessed by crystal violet assay.

2.7. Statistical analyses

Results were analysed by ANOVA using GenStat (GenStat, 17th ed.). For Experiment 1A terms fitted to this 2 x 3 factorial model were O_2 (physiological vs atmospheric) and time in culture (48, 96 and 144 h). A third interactive term, melatonin (0, 20, 200 and 2000 pg/ml), was included for Experiments 1B and 1C). Oxygen level, melatonin and culture duration were considered as fixed effects and blocked by culture date, incubator and plate. For Experiments 2A and 2B, terms fitted to these 2 x 4 x 2 factorial models were temperature (37.5°C *vs* 40.0°C), melatonin (0, 20, 200 and 2000 pg/ml) and time in culture (96 and 144 h). For transcript expression in Experiment 1B and 2A, terms fitted to these 2 x 2 x 2 factorial models were environmental treatment (1B, physiological vs atmospheric O_2; 2A, 37.5°C vs 40.0°C), melatonin (0 and 2000 pg/ml) and culture duration (96 and 144 h). These models were blocked by culture date, incubator and plate. Again, temperature, melatonin and culture duration were considered as fixed effects. Estradiol (pg/ml) and P_4 (ng/ml) production were expressed per 10^5 cells. Natural log transformations of these data were used to correct for heteroscedasticity of the residuals. The data are shown as natural logs of the means with a SED.

3. Results

3.1. Experiment 1A. Atmospheric vs physiological O_2 levels on cell number and steroidogenesis

Granulosa cell number in 6-well plates declined between initial plating and 48 h of culture (1.5 to 1.0 x 10^6 cells/well; SED = 0.049; P<0.001) but subsequently recovered (P<0.001) with time so that, by the end of culture, cell density was similar to that initially seeded. Mean cell number averaged across all time points was greater under low than high O_2 level (1.19 vs 1.03 x 10^6 cells/well; SED = 0.027; P = 0.004).

There was a culture time by O_2 levels interaction (P = 0.014) which indicated that the increase in P_4 production between 48 h and 96/144 h was greater under high than low O_2 level (Fig 1A). Granulosa-cell E_2 production declined between 48 and 96 h of culture (Fig

1B). However, there was a culture time by O_2 level interaction (P<0.001) which indicated that, in contrast to 20% O_2 where E_2 production didn't change, E_2 production increased between 96 and 144 h for cells cultured under 5% O_2. Consequently, at both 96 and 144 h, the E_2: P_4 ratio was greater (P<0.001) for cells cultured under 5% than under 20% O_2. The temporal pattern of E_2 production depicted in Fig 1B was confirmed by determining aromatase activity (Fig. 2). Testosterone conversion to E_2 in the presence of NADPH indicated a decline (P = 0.014) in aromatase activity at 96 and 144 h for cells cultured under 20% O_2 but not for cells cultured under 5% O_2

3.2. Experiment 1 B. Effect of melatonin on GCs cultured under 5% or 20% O_2

Confirming observations from Experiment 1A, cell numbers using a 12-well format declined between 48 and 96 h of culture but increased again by 144 h (647,250 vs 595,391 vs 658,906 cells/ml; SED = 15,000; P<0.001). At 20 pg/mL, melatonin increased (P<0.001) cell number relative to non-treated cells for both O_2 levels, but higher doses melatonin did not alter cell number relative to non-treated cells (621,479, 673,938, 603,604 and 590,308 cells/well for 0, 20, 200 and 2000 pg/mL respectively; SED = 16,258).

Again confirming results from Experiment 1A, P_4 production increased (P<0.001) between 48 h and 96/144 h, and was lower (P = 0.024) under 5% than 20% O_2 (data not shown). Similarly, E_2 production matched that of Experiment 1A, declining (P<0.001) by 96 h and remaining low by 144 h under 20% O_2, but increasing again (P<0.001) by 144 h under 5% O2 (data not shown). In contrast to cell number, melatonin had no effect on P_4 and, at 20 ng/ml only, marginally reduced (P = 0.023) E_2 production relative to untreated cells (log E_2 = 7.09 vs 7.24 pg/ml/10^5 cells; SED = 0.063). Transcript expression for selected genes involved in steroidogenesis, apoptosis and O_2 metabolism varied with time in culture and, for *HSD3B1, SOD1* and *HBA* there were O_2 level by culture time interactions (Table 1). However, there was no effect of melatonin on transcript expression. Consistent with measured concentrations of P_4 in spent culture media, *HSD3B1* mRNA expression increased (P = 0.003) with time for cells cultured under 20%. However, this was not the case under 5% O_2. *HBA* mRNA expression was greater (P = 0.015) under 5% than 20% O_2, but decreased (P = 0.006) with time under these conditions. In contrast, transcripts for the antioxidant enzymes *SOD1* and *2* were broadly similar for both O_2 treatments, and *ASMT* expression was unaffected by treatment.

3.3 Experiment 1C. Effect of O_2 level and melatonin on ROS

Using a 96-well format ROS generation determined by Nitroblue tetrazolium assay did not differ between 5% and 20% O_2 culture treatments. However, ROS generation increased between 48 and 96 h and then declined to 144 h (0.26 vs 0.32 vs 0.28 OD units/10^5 cells for 48, 96 and 144 h respectively; SED = 0.011; P<0.001). The inclusion of melatonin to culture media under both O_2 levels reduced (P<0.001) ROS generation irrespective of dose (0.31, 0.27, 0.25 and 0.26 OD units/10^5 cells for 0, 20, 200 and 2000 pg/ml respectively; SED = 0.012).

3.4. Experiment 2A. Effect of temperature on cell proliferation and steroidogenesis at 5% O_2 in the presence or absence of melatonin

Working with a 12-well plate format, viable cell number by 48 h of culture in basal media and under standard temperature (i.e. 37.5°C) decreased from 6.00 to 4.54 x 10^5 cells. At this time plates were randomly allocated to standard or high temperature (40.0°C) incubators and melatonin treatments introduced. Cell number subsequently increased (P<0.001) with time in culture to 7.05 x 10^5 cells by 144 h, and this was independent of temperature. However, there was an interaction (P = 0.004) between temperature and melatonin treatment which indicated that the inclusion of melatonin increased viable-cell number at 40.0°C but reduced viable-cell number at 37.5°C, particularly at the higher doses (Fig. 3). Cell number was greater at 37.5°C than 40.0°C when melatonin was not included in the media.

Consistent with Experiments 1A and B, P_4 production increased between 48 and 96 h for cells cultured at both 37.5°C and 40.0°C. In contrast to 37.5°C, however, P_4 production declined (P<0.001) between 96 and 144 h culture at 40.0°C (Fig. 4A). The pattern of E_2 production during the 144 h culture period (Fig. 4B) was similar to that observed in Experiments 1A and B at 5% O_2 for both 37.5°C and 40.0°C. There was an interaction (P = 0.007) between melatonin dose and temperature on P_4 production (Fig. 5). Whereas the higher concentrations of melatonin (i.e. ≥ 200 ng/ml) increased P_4 production at 37.5°C they reduced P_4 production at 40.0°C. The two lower doses of melatonin (i.e. 20 and 200 pg/ml) reduced E_2 by GCs whereas the highest dose (2000 pg/ml) had no effect (data no shown). Consequently, the E_2:P_4 ratio increased (P<0.001) between 96 (1.47:1) and 144 h (2.51:1) of culture, and the overall effect of melatonin was similar to that observed for E_2; that is the two

lower doses of melatonin decreased this ratio (P<0.001) whereas the highest dose had no effect.

In contrast to the effects of O_2 level (Table 1), temperature generally had little effect on transcript expression in cultured GCs (Table 2). Importantly, however, there was a temperature x time of culture interaction (P = 0.009) for *BAX* mRNA expression. Consistent with the results of Experiment 1B (Table 1), there was a decline in *BAX* mRNA expression with time at 37.5°C, but this did not occur at 40.0°C (Table 2). Transcript expression for *ASMT* was greater (P = 0.019) at 40.0°C than at 37.5°C. There was no significant effect of melatonin on transcript expression.

3.5. Experiment 2B. Effect of temperature and melatonin on ROS production at 5% O_2

Production of ROS by bovine GCs was not affected by temperature. The presence of melatonin at all three concentrations reduced (P<0.001) ROS production by cultured GCs (0.31, 0.26, 0.25 and 0.27 OD units/10^5 cells for 0, 20, 200 and 2000 pg/ml respectively; SED = 0.01) at both temperatures.

4. Discussion

The most significant novel findings to emerge from this study were, firstly, the relatively small overall effect that elevated temperature (40.0 vs 37.5°C) had on GC physiology when these cells were cultured under low (5%) O_2 as opposed to atmospheric (20%) O_2 and, secondly, the interaction between melatonin dose and temperature on viable cell number and P_4 production at low O_2 levels. Extended culture of GCs at 40.0°C led to a decline in P_4 production, a response which was exacerbated with the inclusion of high-dose (≥ 200 pg/mL) melatonin (Fig. 5). In contrast, at 37.5°C P_4 production remained high at 144 h and the inclusion of high-dose melatonin appeared to contribute to this increase. The corresponding changes in viable cell number with increasing dose of melatonin for low and high temperatures (Fig. 3) suggest that high doses of melatonin interacted with temperature to differentially influence the extent of GC luteinisation at low O_2 levels.

In contrast to temperature, O_2 level had a more marked effect on GC physiology. Unlike GCs cultured under atmospheric (20%) O_2 levels, GCs cultured under low O_2 retained their primary GC phenotype to a greater extent, being more proliferative and estrogenic (Fig. 1).

These observations are consistent with those of [12] for GCs from small-medium sized follicles cultured in 5% O_2, and indicate that studies investigating environmental effects on cultured primary GCs are best carried out under low O_2 levels which better represent the ovarian follicle [14,15].

4.1. Responses to O_2 level

Cell proliferation, and hence mean cell number, from 48 h of culture in the current study was greater at 5% than 20% O_2, an observation consistent with that of Shiratsuki et al. [13]. The increase in aromatase activity and E_2 production between 96 and 144 h for GCs cultured under 5% O_2 in our study (Fig. 1B and 2) is also in general agreement with observations of Roberts and Echternkamp [12]. Collectively, these results suggest that under 5% O_2 from around 96 h of culture a population of proliferating and steroidogenic cells exits which, in contrast to cells cultured under 20% O_2, better represent luminal GCs observed in medium-sized growing antral follicles. The decline in aromatase activity and E_2 production under 20% O_2 may be due to our use of fibronectin-coated plates. Plates pre-coated with attachment factors such as serum have been found to reduce E_2 production by GCs cultured under 20% O_2 [27]. In our study, the assumption is that the steady decline in aromatase activity (pg E_2/mg protein) up to 144 h under 20% O_2 was due to a parallel decline in enzyme. However, we were not able to confirm the mechanism of this decline. It was not possible to establish differential transcript expression for *CYP19A1* which, for GCs in our system, was close to the detection limit of the method and so the data are not presented. Transcript levels for *HSD3B1* (which catalyzes the conversion of pregnenolone to P_4) were greater at 20% than at 5% O_2, and increased with time in culture at 20% O_2, consistent with increased production of P_4 by these cells (Fig. 1A).

Of the transcripts measured (Table S3) the only other to be affected by O_2 level was *HBA* (Hemoglobin alpha) (Table 1); transcript expression for Hemoglobin beta (*HBB*) was barely detectable and unresponsive to culture conditions. In fact transcript expression for *HBA* at 48 h culture was similar to that for freshly aspirated GCs (data not presented), but under low O_2 declined with time during culture. Transcripts for *HBA* and *HBB* have previously been reported in mouse and human granulosa and cumulus cells [37], and transcripts for *HBA* were recently reported in bovine GCs in a micro-array study that assessed the effects of plating density on gene expression [38]. Working with aspirated GCs from small to medium (< 6mm) antral follicles, this latter study adopted a culture system similar to ours (i.e. serum free

media with 10 ng/mL insulin), but under 20% O_2. Of the 906 transcripts upregulated by increased plating density, those for *HBA* were the most affected, which the authors suggested was due to increased hypoxic conditions. This would certainly be consistent with current theories for the role of hemoglobin within the ovarian follicle [39] and with observations from the present study where *HBA* mRNA expression was 1.6 fold greater on average at 5% than 20% O_2. Brown et al. [37] found hemoglobin transcript expression to be regulated by gonadotrophins (hCG) in the mouse and proposed a model that linked increasing *HBA* mRNA levels to events leading to follicular maturation and luteinization. In keeping with this model our findings that *HBA* mRNA levels decrease with time under 5% O_2 are consistent with a population of proliferating and estrogenic GCs.

4.2. Responses to temperature

Studies assessing the effects of elevated temperature on cultured bovine, porcine and murine GCs have invariably been conducted in 5% CO_2 in air for variable periods of time often in the presence of high levels of gonadotrophins and/or growth factors and serum [9,10,40,41]; that is under conditions that favour or promote luteinization. For bovine and murine GCs cultured in this way elevated temperatures were found to increase BAX/BCL-2 and Caspase-3 mediated apoptosis and to reduce steroidogenesis [9,41]. Results from the current study where GCs were cultured under 5% O_2 in serum free media were less dramatic. Elevated temperature did reduce viable cell number in the absence of melatonin but had little effect on apoptotic gene expression (Table 2). Progesterone production declined with time in culture for GCs cultured at 40.0°C but not 37.5°C (Fig. 4A). The inhibitory effect of elevated temperature on P_4 production occurred in the absence of differences in transcript expression for *HSD3B1* (Table 2). Instead this may have been due to reduced expression of steroidogenic acute regulatory protein (*STAR*) and cytochrome P450 (*CYP11A1*) as observed by Li et al. [9] in bovine GCs, but not determined in the current study. The absence of an effect of elevated temperature on E_2 production (Fig. 4B) contrasts with the observations of Li et al. [9] who also reported a decline in *CYP19A1*. Insufficient details of the culture system employed by these authors negates a more direct comparison. However, it is clear that aromatase activity in our study was not impeded by elevated temperature. It may be that the provision of a readily available substrate for aromatization (i.e.100 ng/mL testosterone) under 5% O_2 in our study helped alleviate the effects of elevated temperature on E_2 production.

4.3. Responses to melatonin

Elevated (atmospheric) levels of O_2 [42] and temperature [7] each can disturb the intricate balance between the generation of ROS and antioxidant defence mechanisms leading to increased oxidative stress during either embryo or cell culture. However, neither O_2 level nor temperature affected ROS generation in the current series of experiments, but the inclusion of melatonin at all three levels significantly reduced ROS production by GCs. The antioxidant and anti-apoptotic properties of melatonin are well recognized [21], and variable levels of this indole amine are found in ovarian follicular fluid [16-18]. It is believed that follicle levels are derived from both systemic and local sources within the ovary [43, 44], and the current study confirmed the presence of transcripts for *ASMT* (Acetlyserotonin O-methyltransferase) and *AANAT* (Aralkylamine N-acetyltransferase) involved in melatonin synthesis in bovine GCs. However, transcripts for *AANAT* were extremely low and barely detectable, and those for *ASMT* were unaffected by O_2 level and only marginally increased at 40°C. These responses may have been influenced by the level of tryptophan (10 µg/mL) in our basal medium (M199, Sigma-Aldrich). Kim et al. [45] observed that adding an additional 50 µg/mL of tryptophan to M199 (Gibco) during human GC culture lead to an 8- to 60-fold increase in expression of these two transcripts.

The most striking and novel observation in our study was the differential effect of the two higher doses of melatonin (i.e. 200 and 2000 pg/mL) on viable cell number and P_4 production at 37.5 and 40.0°C (Fig. 3 and Fig. 5). The levels were within the range used previously in culture studies with bovine cumulus and GCs (i.e. pg/mL to µg/mL levels [22, 45-47]) and, for the most part, comparable to levels reported in human, porcine and bovine follicular fluid (i.e. 10 to 300 pg/mL [16-20]). The higher doses of melatonin employed in the current study could therefore be considered to be towards the upper end of physiological. The stimulatory effect of increasing doses of melatonin on P_4 at 37.5°C is consistent with previous reports for human and bovine GCs cultured at 37°C but in the presence of serum and under atmospheric O_2 levels [22, 48]. The concomitant reduction in GC numbers in the current study further suggests that the higher doses of melatonin induced GCs to luteinise under these conditions. This is in stark contrast to GCs cultured at 40.0°C where the effects of higher doses of melatonin were to increase cell proliferation and reduced P_4 production. Although melatonin has previously been found to increase sheep GC numbers at high (43°C) temperatures (in the presence of 10% FCS and under atmospheric O_2 [49]), a suppressive effect on P_4 production has not previously be reported.

4.4. Conclusions and perspective

The results demonstrate that culturing GCs under low O_2 more accurately reflects the follicular environment resulting in the expression of a more physiological phenotype than is seen under atmospheric O_2 concentrations. This more physiological approach revealed a lesser impact of elevated temperature on GC function than has previously been reported. However, it should be noted that granulosa cells were cultured in the absence of theca cells and in the presence of high levels of androgen, so we cannot rule out a potential theca-cell mediated impact of temperature on granulosa cell function. Nevertheless, the results do strongly support the need to consider O_2 concentration more carefully when investigating the impact of heat stress on ovarian function. As anticipated, the potent antioxidant melatonin consistently reduced ROS. However, the effects of melatonin on GC function were dependant on O_2 concentration, once again emphasising the importance of considering culture conditions when designing these experiments.

5. Acknowledgments

This work was supported by the University of Nottingham. BK Zeebaree received financial support from the Kurdistan regional government of Iraq. CG received financial support from DGAPA, UNAM, and CONACyT towards a sabbatical visit to the University of Nottingham.

6. References

[1] Wolfenson D, Roth Z, Meidan R. Impaired reproduction in heat-stressed cattle: basic and applied aspects. Ani Reprod Sci 2000; 60: 535-547.

[2] Hansen PJ. To be or not to be - Determinants of embryonic survival following heat shock. Theriogenol 2007; 68 Suppl 1: S40-S48.

[3] De Rensis F, Garcia-Ispierto I, López-Gatius F. Seasonal heat stress: Clinical implications and hormone treatments for the fertility of dairy cows. Theriogenol 2015; 84: 659-666.

[4] Sartori R, Rosa GJM, Wiltbank MC. Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci 2002; 85: 2813-2822.

[5] Dash S, Chakravarty AK, Singh A, Upadhyay A, Singh M, Yousuf S. Effect of heat stress on reproductive performances of dairy cattle and buffaloes: a review. Vet World 2016; 9: 235-244.

[6] Honig H, Ofer L, Kaim M, Jacobi S, Shinder D, Gershon E. The effect of cooling management on blood flow to the dominant follicle and estrous cycle length at heat stress. Theriogenol 2016; 86: 626-634.

[7] Roth Z. Physiology and endocrinology symposium: Cellular and molecular mechanisms of heat stress related to bovine ovarian function. J Anim Sci 2015; 93: 2034-2044.

[8] Wolfenson D, Lew BJ, Thatcher WW, Graber Y, Meidan R. Seasonal and acute heat stress effects on steroid production by dominant follicles in cows. Ani Reprod Sci 1997; 47: 9-19.

[9] Li L, Wu J, Luo M, Sun Y, Wang G. The effect of heat stress on gene expression, synthesis of steroids, and apoptosis in bovine granulosa cells. Cell Stress and Chaperones 2016, 1-9.

[10] Sirotkin AV, Kacaniova M. The effect of high temperature on swine ovarian function in vitro. Veterinarni Medicina 2010; 55: 377-382.

[11] Ortega MS, Rocha-Frigoni NAS, Mingoti GZ, Roth Z, Hansen PJ. Modification of embryonic resistance to heat shock in cattle by melatonin and genetic variation in HSPA1L. J Dairy Sci 2016.

[12] Roberts A, Echternkamp S. In vitro production of estradiol by bovine granulosa cells: evaluation of culture condition, stage of follicular development, and location of cells within follicles. Biol Reprod 1994; 51: 273-282.

[13] Shiratsuki S, Hara T, Munakata Y, Shirasuna K, Kuwayama T, Iwata H. Low oxygen level increases proliferation and metabolic changes in bovine granulosa cells. Mol Cell Endocr 2016; 437: 75-85.

[14] Van Blerkom J, Antczak M, Schrader R. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Human Reprod 1997; 12: 1047-1055.

[15] De Castro E Paula LA, Andrzejewski J, Julian D, Spicer LJ, Hansen PJ. Oxygen and steroid concentrations in preovulatory follicles of lactating dairy cows exposed to acute heat stress. Theriogenol 2008; 69: 805-813.

[16] Shi JM, Tian XZ, Zhou GB, Wang L, Gao C, Zhu SE, Zeng SM, Tian JH, Liu GS. Melatonin exists in porcine follicular fluid and improves in vitro maturation and parthenogenetic development of porcine oocytes. J Pineal Res 2009; 47: 318-323.

[17] Taketani T, Tamura H, Takasaki A, Lee L, Kizuka F, Tamura I, Taniguchi K, Maekawa R, Asada H, Shimamura K, Reiter RJ, Sugino N. Protective role of melatonin in progesterone production by human luteal cells. J Pineal Res 2011; 51: 207-213.

[18] Tian X, Wang F, He C, Zhang L, Tan D, Reiter RJ, Xu J, Ji P, Liu G. Beneficial effects of melatonin on bovine oocytes maturation: a mechanistic approach. J Pineal Res 2014; 57: 239-47.

[19] He CJ, Ma T, Shi JM, Zhang ZZ, Wang J, Zhu KF, Li Y, Yang MH, Song YK, Liu GS. Melatonin and its receptor MT1 are involved in the downstream reaction to luteinizing hormone and participate in the regulation of luteinization in different species. J Pineal Res 2016: 61: 279-290.

[20] Tong J, Sheng S, Sun Y, Li H, Li WP, Zhang C, Chen ZJ. Melatonin levels in follicular fluid as markers for IVF outcomes and predicting ovarian reserve. Reprod. 2017; 153: 443-451.

[21] Cruz MH, Leal CL, Cruz JF, Tan DX, Reiter RJ. Essential actions of melatonin in protecting the ovary from oxidative damage. Theriogenol 2014, 82, 925-932.

[22] Wang SJ, Liu WJ, Wu CJ, Ma FH, Ahmad S, Liu BR, Han L, Jiang XP, Zhang SJ, Yang LG. Melatonin suppresses apoptosis and stimulates progesterone production by bovine granulosa cells via its receptors (MT1 and MT2). Theriogenol 2012; 78: 1517-1526.

[23] Duda M, Gasińska A, Gregoraszczuk EL. Flow cytometric cell cycle analysis of two subpopulations of porcine granulosa cells. Exp Clin Endocrinol Diabetes 1999; 107: 203-207.

[24] Tennant JR. Evaluation of the trypan blue technique for determination of cell viability. Transplantation 1964; 2: 685-694.

[25] Saumande J. Culture of bovine granulosa cells in a chemically defined serum-free medium: the effect of insulin and fibronectin on the response to FSH. J Steroid Biochem Mol Biol. 1991; 38:189-96.

[26] Gutierrez CG, Glazyrin AL, Robertson GW, Campbell BK, Gong JG, Bramley TA, Webb R. Ultra-structural characteristics of bovine granulosa cells associated with maintenance of oestradiol production in vitro. Mol Cell Endocr 1997a; 134: 51-58.

[27] Gutierrez CG, Campbell BK, Webb R. Development of a long- term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics. Biol Reprod 1997b; 56: 608-616.

[28] Hughes J, Kwong WY, Li D, Salter AM, Lea RG, Sinclair KD. Effects of omega-3 and-6 polyunsaturated fatty acids on ovine follicular cell steroidogenesis, embryo development and molecular markers of fatty acid metabolism. Reprod 2011; 141: 105-118.

[29] Kia HD, Safdar AHA. Effects of calcium salts of fatty acids (csfa) with different profiles (ω3 and ω6) during the flushing period on reproductive performance of Afshari ewes. Small Rum Res 2015; 126: 1-8.

[30] Catteau-Jonard S, Jamin SP, Leclerc A, Gonzales J, Dewailly D, Di Clemente N. Anti-Mullerian Hormone, Its Receptor, FSH Receptor, and Androgen Receptor Genes Are Overexpressed by Granulosa Cells from Stimulated Follicles in Women with Polycystic Ovary Syndrome. J Clin Endocr Metab 2008; 93: 4456-4461.

[31] Satoh K, Nonaka R, Ishikawa F, Ogata A, Nagai F. In vitro screening assay for detecting aromatase activity using rat ovarian microsomes and estrone ELISA. Biol Pharm Bulletin 2008; 31: 357-362.

[32] Tinwell H, Rascle JB, Colombel S, Al Khansa I, Freyberger A, Bars R. A novel method for measuring aromatase activity in tissue samples by determining estradiol concentrations. J App Toxicol 2011; 31: 446-454.

[33] Hughes S, Poh, TY, Bumstead N, Kaiser, P. Re-evaluation of the chicken MIP family of chemokines and their receptors suggests that CCL5 is the prototypic MIP family chemokine, and that different species have developed different repertoires of both the CC chemokines and their receptors. Devel Comp Immunol 2007; 31: 72-86.

[34] Sharma RK, Reynolds N, Rakhit M, Agarwal A. Methods for detection Of ROS in the female reproductive system. In: Agarwal A, Aziz N, Rizk B editors. Studies on Women's Health, Springer; 2013.

[35] Amarasekara DS, Wijerathna S, Fernando C, Udagama PV. Cost-effective diagnosis of male oxidative stress using the nitroblue tetrazolium test: useful application for the developing world. Andrologia 2014; 46: 73-79.

[36] Huang, Y-C, Li R-Y. Preparation and characterization of Antioxidant nanoparticles composed of chitosan and fucoidan for antibiotics delivery. Marine Drugs 2014; 12, 4379-4398.

[37] Brown HM, Anastasi MR, Frank LA, Kind KL, Richani D, Robker RL, Russell DL, Gilchrist RB, Thompson JG. Hemoglobin: a gas transport molecule that is hormonally regulated in the ovarian follicle in mice and humans. Biol Reprod 2015; 92:26.

[38] Baufeld A, Koczan D, Vanselow J. Induction of altered gene expression profiles in cultured bovine granulosa cells at high cell density. Reprod Biol Endocr 2017; 15: article number 3.

[39] Thompson JG, Brown HM, Kind KL, Russell DL. The ovarian antral follicle: living on the edge of hypoxia or not? Biol Reprod 2015; 92: 153.

[40] Wolfenson D, Sonego H, Bloch A, Shaham-Albalancy A, Kaim M, Folman Y, Meidan R. Seasonal differences in progesterone production by luteinized bovine thecal and granulosa cells. Dom Anim Endocr 2002; 22: 81-90.

[41] Luo M, Li L, Xiao C, Sun Y, Wang G-L. Heat stress impairs mice granulosa cell function by diminishing steroids production and inducing apoptosis. Mol Cell Bioch 2016; 412: 81-90.

[42] Takahashi M. Oxidative Stress and Redox Regulation on In Vitro Development of Mammalian Embryos. J Reprod Devel 2012; 58: 1-9.

[43] Itoh MT, Ishizuka B, Kuribayashi Y, Amemiya A, Sumi Y. Melatonin, its precursors, and synthesizing enzyme activities in the human ovary. Mol Human Reprod 1999; 5: 402-408.

[44] Kim MK, Park EA, Kim HJ, Choi WY, Cho JH, Lee WS, Cha KY, Kim YS, Lee DR, Yoon TK. Does supplementation of in- vitro culture medium with melatonin improve IVF outcome in PCOS? Reprod Biomed Online 2013; 26: 22-29.

[45] El-Raey M, Geshi M, Somfai T, Kaneda M, Hirako M, Abdel-Ghaffar AE, Sosa GA, El-Roos ME, Nagai T. Evidence of melatonin synthesis in the cumulus oocyte complexes and its role in enhancing oocyte maturation in vitro in cattle. Mol Reprod Devel 2011; 78: 250-62.

[46] Takada L, Junior AM, Mingoti GZ, Balieiro JC, Cipolla-Neto J, Coelho LA. Effect of melatonin on DNA damage of bovine cumulus cells during in vitro maturation (IVM) and on in vitro embryo development. Res Vet Sci 2012; 92: 124-7.

[47] Wang F, Tian X, Zhang L, Gao C, He C, Fu Y, Ji P, Li Y, Li N, Liu G. Beneficial effects of melatonin on in vitro bovine embryonic development are mediated by melatonin receptor 1. J Pineal Res 2014; 56: 333-342.

[48] Webley GE, Luck MR. Melatonin directly stimulates the secretion of progesterone by human and bovine granulosa cells in vitro. J Reprod Fert 1986; 78: 711-7.

[49] Fu Y, He C-J, Ji P-Y, Zhuo Z-Y, Tian X-Z, Wang F, Tan D-X, Liu G-S. Effects of melatonin on the proliferation and apoptosis of sheep granulosa cells under thermal stress. Int J Mol Sci 2014; 15: 21090-21104.

Figure captions

Fig. 1. Effect of atmospheric (20%) or physiological (5%) O_2 level on granulosa-cell P_4 (A) and E_2 (B) production after 48, 96 and 144 h of culture in Experiment 1.A. A 2 x 3 factorial arrangement replicated 5 times. Data were $\log_e$ transformed prior to analysis. Superscripts highlight differences ($P<0.05$) between groups.

564

565 **Fig. 2.** Effect of atmospheric (20%) or physiological (5%) O_2 level on aromatase activity in
566 granulosa cells after 48, 96 and 144 h of culture in Experiment 1A. A 2 x 3 factorial
567 arrangement replicated 5 times. Data were $\log_e$ transformed prior to analysis. Superscripts
568 highlight differences (P<0.05) between groups.

569

570 **Fig. 3.** Effect of melatonin (pg/ml) and incubation temperature from 48 h culture on
571 granulosa-cell number averaged across 96 and 144 h of culture at 5% O_2 in Experiment 2.A.
572 A 4 (melatonin) x 2 (temperature) x 2 (time points) factorial arrangement replicated 4 times.
573 Data were $\log_e$ transformed prior to analysis. Superscripts highlight differences (P<0.05)
574 between groups.

575

576 **Fig. 4.** Effects of incubation temperature on granulosa-cell P_4 (A) and E_2 (B) production in
577 vitro after 96 and 144 h of culture at 5% O_2 in Experiment 2.A. A 4 (melatonin) x 2
578 (temperature) x 2 (time points) factorial arrangement replicated 4 times. Cells were incubated
579 for 48 h at 37.5°C and thereafter exposed to 37.5°C or 40.0°C. Data were $\log_e$ transformed
580 prior to analysis. Superscripts highlight differences (P<0.05) between groups. Hatched bars
581 represent P_4 and E_2 production after 48 h culture at 37.5°C in 5% O_2 prior to commencement
582 of treatments.

583

584 **Fig. 5.** Effect of incubation temperature and melatonin dose (pg/ml) on granulosa-cell P_4
585 production in vitro in Experiment 2.A. A 4 (melatonin) x 2 (temperature) x 2 (time points)
586 factorial arrangement replicated 4 times. Cells were incubated for 48 h at 37.5°C in 5% O_2
587 and then exposed to 37.5°C or 40.0°C in the presence or absence of melatonin. Data were
588 $\log_e$ transformed prior to analysis. Superscripts highlight differences (P<0.05) between
589 group.

590 **Table 1.** Normalized transcript expression in GCs cultured under physiological (5%) and atmospheric (20%) oxygen levels.

591

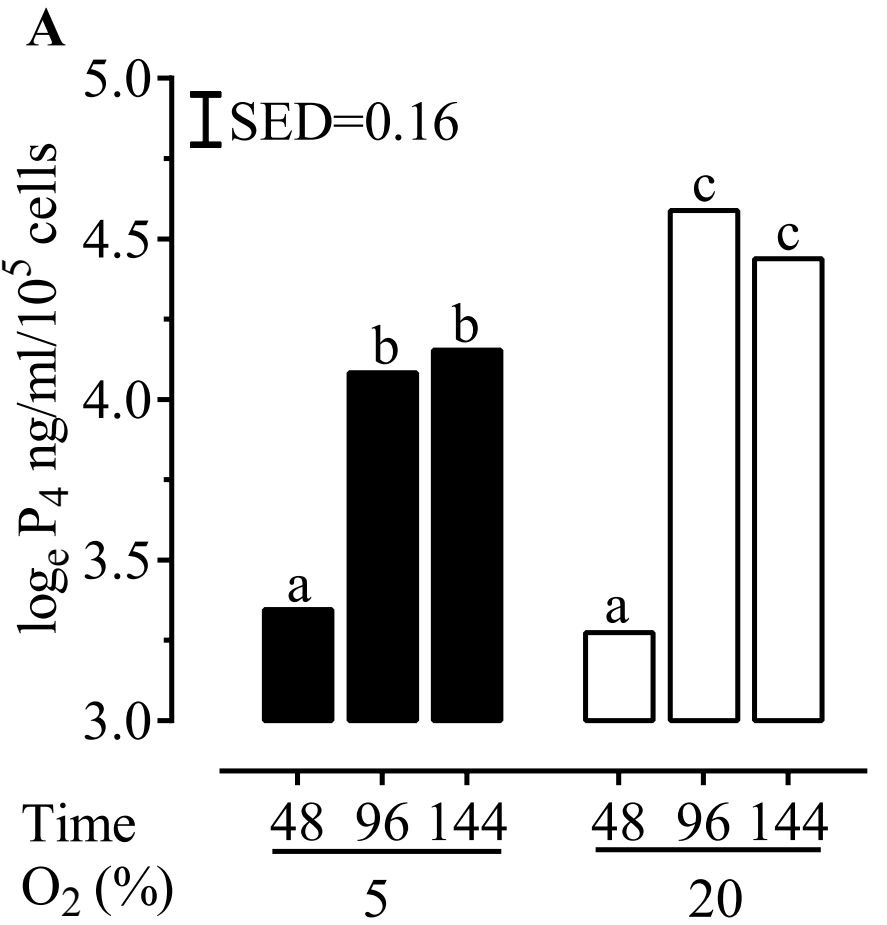
Oxygen (O_2)	5%			20%				Significance (P)		
Culture time (h)	48	96	144	48	96	144	SED	O_2	h	O_2 x h
Steroidogenesis										
HSD3B1	11.02 [a]	11.47 [a]	11.47 [a]	11.25 [a]	12.06 [b]	13.17 [c]	0.31	0.028	<0.001	0.003
Apoptosis										
BAX	11.99 [ab]	11.67 [ab]	11.37 [a]	12.12 [b]	11.27 [a]	11.43 [a]	0.37	-	0.015	-
P53	11.39	11.38	11.40	11.37	11.41	11.45	0.034	-	-	-
HSPA1A	13.13 [a]	11.94 [b]	11.92 [b]	12.52 [ab]	11.69 [b]	12.08 [ab]	0.53	-	0.018	-
O_2 metabolism										
SOD1	11.31	11.93	11.49	11.89	11.30	11.41	0.34	-	-	0.045
SOD2	14.55 [a]	14.12 [ab]	13.80 [b]	14.59 [a]	13.49 [b]	13.23 [b]	0.29	-	<0.001	-
HBA	15.43 [a]	11.62 [b]	10.51 [b]	8.65 [c]	7.82 [c]	7.51 [c]	1.11	0.015	<0.001	0.006
Melatonin synthesis										
ASMT	9.11	9.41	9.27	9.4	9.24	9.33	0.35	-	-	-

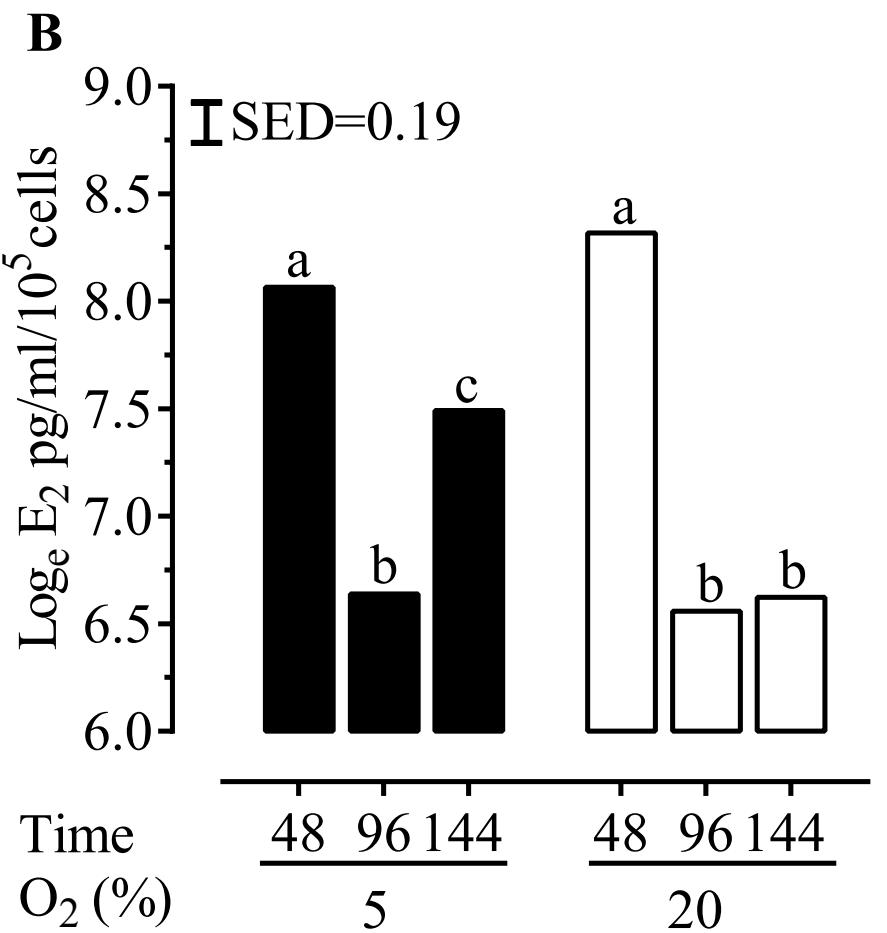
592

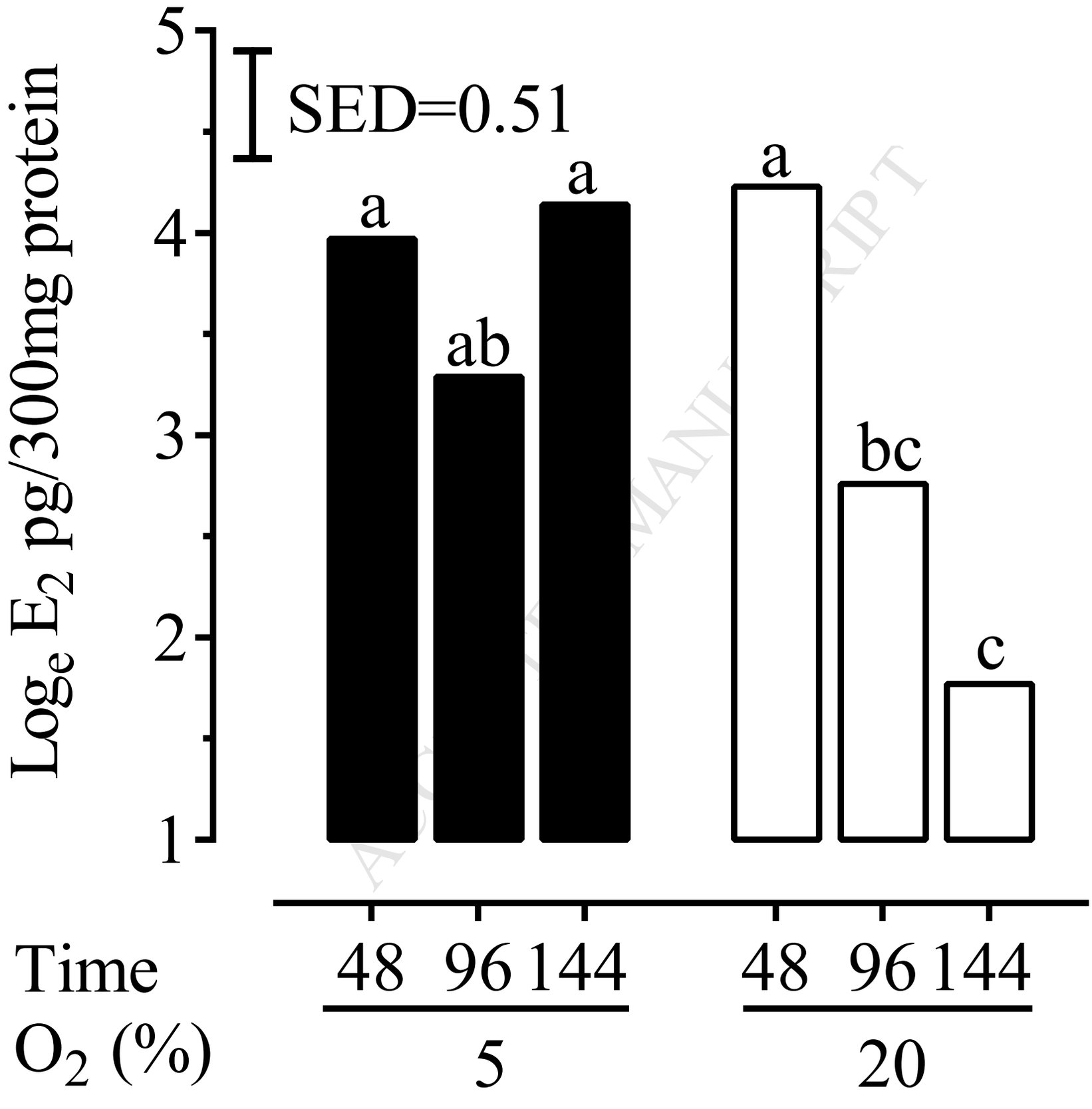
593

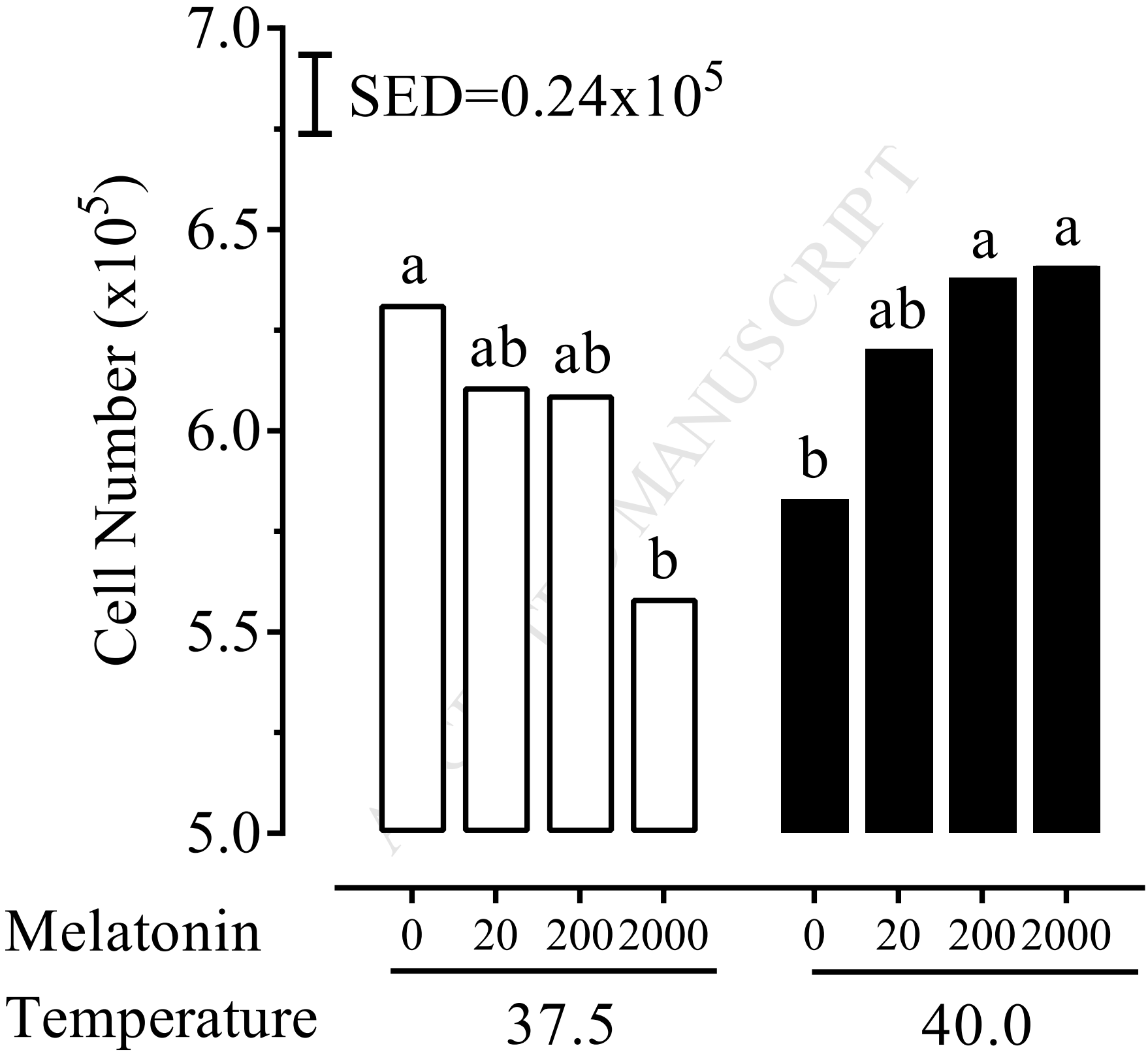
Table 2 Normalized transcript expression in GCs cultured under normal (37.5°C) and high (40.0°C) temperatures.

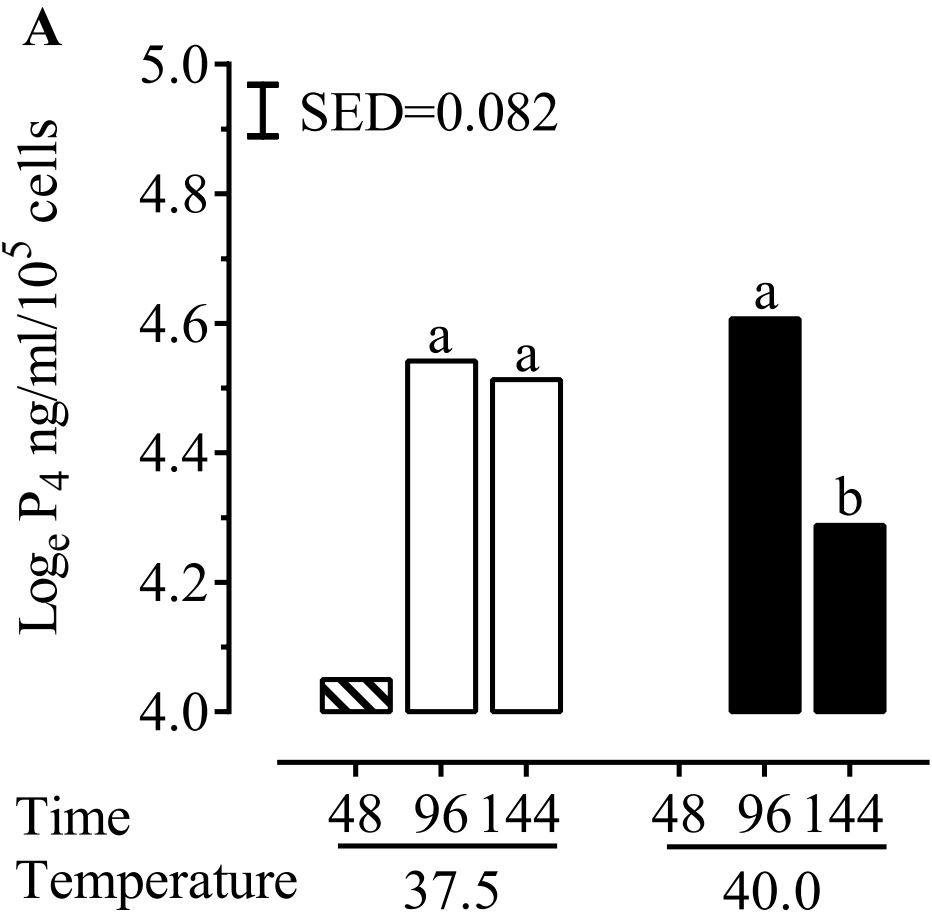
Temperature (°C)	37.5		40.0°C			Significance (P)		
Culture time (h)	96	144	96	144	SED	°C	h	°C x h
Steroidogenesis								
HSD3B1	13.32	13.42	14.03	13.42	0.31	-	-	-
Apoptosis								
BAX	12.56 [a]	12.19 [b]	12.49 [a]	12.66 [a]	0.12	0.068	-	0.009
P53	12.58	12.39	12.54	12.86	0.36	-	-	-
HSPA1A	13.9	13.72	14.38	14.2	0.40	-	-	-
O_2 metabolism								
SOD1	12.69	12.6	13.09	12.41	0.31	-	-	-
SOD2	14.02	13.96	14.21	14.24	0.18	-	-	-
HBA	14.08	13.54	15.25	14.6	1.04	-	-	-
Melatonin synthesis								
ASMT	10.38	10.03	10.94	10.98	0.49	0.019	-	-

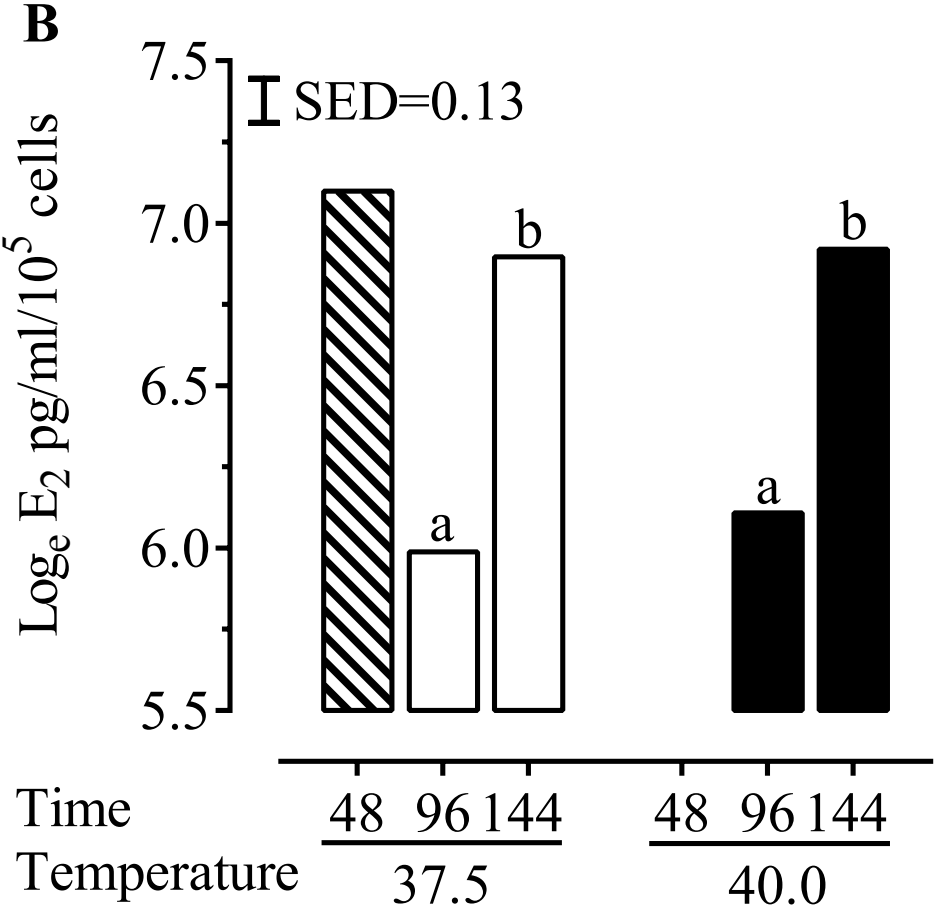

A
5.0
4.5
4.0
3.5
3.0
SED=0.16
log$_e$ P$_4$ ng/ml/10^5 cells
a
b
b
a
c
c
Time
48 96 144
48 96 144
O$_2$ (%)
5
20

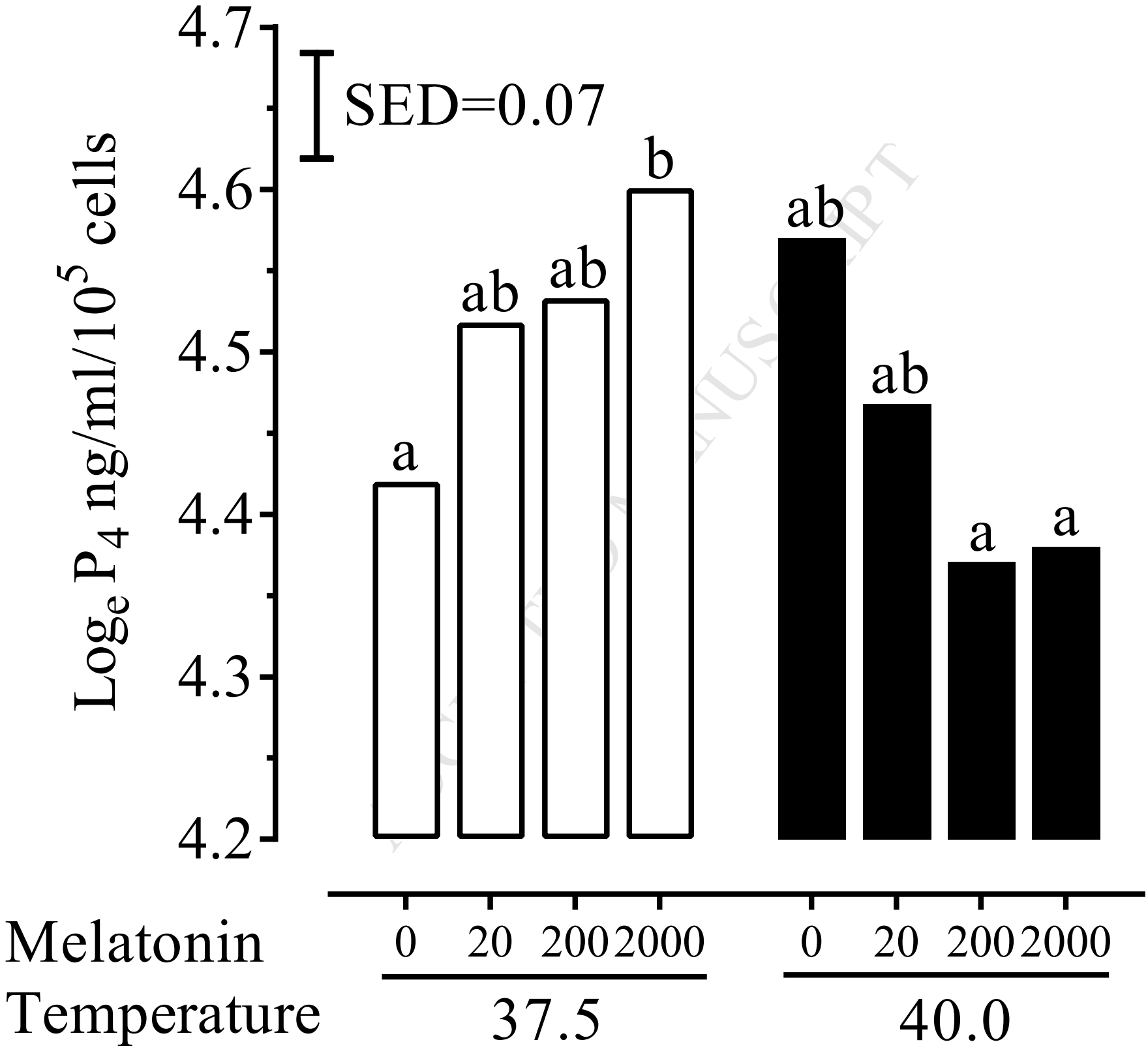

B
9.0
8.5
8.0
7.5
7.0
6.5
6.0
SED=0.19
Log$_e$ E$_2$ pg/ml/10^{5}cells
a
b
c
a
b
b
Time
48 96 144
48 96 144
O$_2$ (%)
5
20






A
5.0
4.8
4.6
4.4
4.2
4.0
SED=0.082
Loge P4 ng/ml/10^5 cells
a
a
a
b
Time
48 96 144
48 96 144
Temperature
37.5
40.0


B
7.5
7.0
6.5
6.0
5.5
SED=0.13
Loge E2 pg/ml/10^5 cells
a
b
a
b
Time
48 96 144
48 96 144
Temperature
37.5
40.0



Highlights

- Granulosa cells cultured under 5% than 20% O_2 better retain primary phenotype
- Culturing granulosa cells under 5% than 20% O_2 lessens impact of heat stress
- Melatonin interacts with temperature to affect cell number and progesterone at 5% O_2