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

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1 **Abstract**

2 Our understanding of the effects of temperature on granulosa cell (GC) physiology is

3 primarily limited to *in vitro* studies conducted under atmospheric (~20% O_2) conditions. In

4 the current series of factorial experiments we identify important effects of O_2 level (i.e. 5% vs

5 20% O_2) on GC viability and steroidogenesis, and go onto report effects of standard (37.5°C)

6 vs high (40.0°C) temperatures under more physiologically representative (i.e. 5%) O_2 levels

7 in the presence of different levels of melatonin (0, 20, 200 and 2000 pg/mL); a potent free-

8 radical scavenger and abundant molecule within the ovarian follicle. Cells aspirated from

9 antral (4 to 6 mm) follicles were cultured in fibronectin-coated wells using serum-free M199

10 for up to 144 h. At 37.5°C viable cell number was enhanced and luteinization reduced under

11 5 vs 20% O_2. Oxygen level interacted (P<0.001) with time in culture to affect aromatase

12 activity and cell estradiol (E_2) production (pg/mL/10^5 cells). These decreased between 48 and

13 96 h for both O_2 levels but increased again by 144 h for cells cultured under 5% but not 20%

14 O_2. Progesterone (P_4) concentration (ng/mL/10^5 cells) was greater (P<0.001) under 20 vs 5%

15 O_2 at 96 and 144 h. Cell number increased (P<0.01) with time in culture under 5% O_2

16 irrespective of temperature. However, higher doses of melatonin increased viable cell number

17 at 40.0°C but reduced viable cell number at 37.5°C (P=0.004). Melatonin also reduced

18 (P<0.001) ROS generation at both O_2 levels across all concentrations. E_2 increased with time

19 in culture at both temperatures under 5% O_2, however P_4 declined between 96 to 144 h at

20 40.0 but not 37.5°C. Furthermore, melatonin interacted (P<0.001) with temperature in a dose

21 dependent manner to increase P_4 at 37.5°C but to reduce P_4 at 40.0°C. Transcript expression

22 for *HSD3B1* paralleled temporal changes in P_4 production, and those for *HBA* were greater at

23 5% than 20% O_2, suggesting that hemoglobin synthesis is responsive to changes in O_2 level.

24 In conclusion, 5% O_2 enhances GC proliferation and reduces luteinization. Elevated

25 temperatures under 5% O_2 reduce GC proliferation and P_4 production. Melatonin reduces

26 ROS generation irrespective of O_2 level and temperature, but interacts with temperature in a

27 dose dependent manner to influence GC proliferation and luteinization.

28

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

62 processes. Culturing under low O_2 may provide a more physiologically relevant system to

63 investigate the effects of this potent antioxidant in helping to attenuate thermal stress on

64 somatic cells within the ovarian follicle. The current series of experiments, therefore, sought

65 initially to characterise the effects of O_2 level (i.e. 5% vs 20% O_2) on GC viability and

66 steroidogenesis and then to investigate the effects of standard (37.5°C) vs high (40.0°C)

67 temperatures under low (i.e. 5%) O_2 levels in the presence of different concentrations of

68 melatonin (0, 20, 200 and 2000 pg/mL).

69 **2. Materials and methods**

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

71 *2.1. Granulosa cell culture*

72 Antral follicles (4 to 6 mm) were aspirated from abattoir derived ovaries using a 21 G needle

73 and GCs prepared for serum-free culture. These GCs were therefore likely to represent a

74 population of largely luminal GCs and cumulus cells. Compared to mural GCs (scraped from

75 dissected follicles of comparable size) these cells are more estrogenic [12] and mitotically

76 active [23]. Viable cells (determined by trypan blue exclusion [24] were re-suspended in 1 ml

77 of pre-warmed M199 culture medium supplemented with (Penicillin (50 IU/ml),

78 Streptomycin (50 μg/ml), bovine serum albumin free fatty acid (BSA; 1 mg/ml), testosterone

79 (100 ng/ml), FSH (1 ng/ml; Cat. No. F2293), insulin (10 ng/ml), transferrin 2.5 (μg/ml),

80 sodium selenite (4 ng/ml) and L-glutamine (365 μg/ml)) prior to plating in fibronectin coated

81 wells (Nunclon Delta, Thermo Fisher, Denmark) at seeding densities depicted in Table S1.

82 Fibronectin facilitates the attachment and proliferation of GCs [25], whilst low insulin (10

83 ng/mL) in serum-free media allows cells to form aggregates, proliferate and maintain a

84 primary GC phenotype [26]; hence their responsiveness to trophic hormones [27].

85

86 *2.2. Experimental designs*

87 *2.2.1. Experiment 1.A. Effect of atmospheric vs physiological O_2 level on cell number,*

88 *steroidogenesis and aromatase activity*

89 This was a 2 x 3 factorial experiment with two O_2 levels (~5 vs 20%; using two humidified

90 incubators (Model Innova CO-14, New Brunswick Scientific, Edison, NJ, USA) at 37.5°C)

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

95 *2.2.2. Experiment 1.B. Effect of melatonin on cell number, steroidogenesis and gene*
96 *expression under atmospheric vs physiological O_2 levels*

97 This was a 4 x 2 x 3 factorial experiment with four levels of melatonin (0, 20, 200, and 2000
98 pg/ ml), two O_2 levels (~5 vs 20%; using two humidified incubators at 37.5°C) and three
99 culture endpoints (48, 96 and 144 h from seeding), replicated four times using a 12-well plate
100 format (Table S1). Melatonin levels for this and subsequent experiments were selected on the
101 basis of concentrations reported previously in ovarian follicular fluids [16-20], and from a
102 small pilot study where we determined melatonin concentrations by ELISA
103 (MyBioSource.com; San Diego, CA, USA; Bovine kit - MBS743340) in follicular fluids
104 from 15 heifers slaughtered at a local abattoir (Figure S1). Simple and geometric means for
105 ovarian follicular-fluid melatonin in that study were 1,600 (95% CI = 173 - 3036) and 320
106 pg/mL respectively. Media were changed and cells harvested as described for Experiment
107 1A.

108 *2.2.3. Experiment 1.C. Effect of O_2 level and melatonin on ROS*

109 This experiment adopted the factorial arrangement described for Experiment 1B but using a
110 96-well format (Table S1). Media were changed as described for Experiment 1A. Generation
111 of ROS was assessed at 48, 96 and 144 h of culture (described later).

112 *2.2.4. Experiment 2.A. Effect of temperature and melatonin on cell number, steroidogenesis*
113 *and gene expression under physiological O_2 levels*

114 This experiment adopted a factorial arrangement similar to Experiment 1B but treatments
115 (37.5 vs 40.0 °C; using two humidified incubators at 5% O_2 with four melatonin doses (0, 20,
116 200 and 2000 pg/ml)) commenced after 48 h of culture (Table S2). Incubator temperature
117 was monitored using two thermometers (temperature loggers, EL-USB-1, Lascar Electronics,
118 Salisbury, UK) in addition to that built into the incubator. Media were changed and cells
119 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 ($\sim$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.

148   *2.5. Transcript expression (Experiments 1B and 2A)*

149   Methodologies reported were those used previously in our laboratory [28]. Briefly, total RNA

150   was extracted from cultured GCs using RNeasy Mini Kit (Qiagen Ltd., West Sussex, UK)

151   and treated with DNase (Promega, Southampton Science Park, Southampton, UK) to remove

152   genomic DNA contamination. DNase-treated RNA was then transcribed into complementary

153   DNA (cDNA) using Omniscript cDNA synthesis kit (Qiagen Ltd) in a 20 µl volume

154   according to manufacturer's instruction. Quantitative Real time PCR (qPCR) was performed

155   using a Roche LightCycler 480 (Roche Diagnostics Ltd, Penzberg, Germany) with gene-

156   specific primers and TaqMan probes (Eurofins Genomics, Ebersberg, Munich, Germany) that

157   were labelled with the 6-carboxyfluorescein (FAM) and tetramethylrhodamine (TAMRA) at

158   5' and 3' ends respectively (Table S3). PCR was performed in 20 µl volume containing 10 µl

159   of 2x Probe Master mix, 0.3 µM each primer, 0.2 µM Taqman probe and 1 µl cDNA. Before

160   quantification, standard curves using each primer/probe set for a particular gene were

161   generated and only those which gave an efficiency of 1.8 to 2.0 used. To ensure no genomic

162   DNA contamination, –RT for genes were performed. A negative control (without cDNA) was

163   also included in each qPCR run. Four biological replicates were conducted per experiment

164   and cDNA from each sample was run in duplicate for each gene.

165   Several housekeeping genes (*RPL19, RPLP0, B2M and TBP)* were tested (Table S3) and it

166   was found that *TBP* was the most stable housekeeping gene when analysed by NormFinder

167   and RefFinder. Hence, all target genes in this study were normalized to *TBP*. Relative

168   quantification was calculated using the formula of [33].

169   2.6. *Measurement of ROS (Experiments 1C and 2B)*

170   Reactive oxygen species generated from cultured GCs were measured by Nitroblue

171   tetrazolium (NBT; N6876, Sigma-Aldrich) as previously described [34-36] but with some

172   modifications. Briefly, media were removed and 50 µl of 1 mg/ml NBT added to each well

173   and incubated for 2 min. Reactions were terminated by adding 100 µl of 1 M HCl. Solutions

174   in each well were then removed and wells washed three times with PBS. Then 150 µl of

175   dimethyl sulfoxide (DMSO) was added to each well to solubilise formazan produced inside

176   the cells. Finally, 10 µl of 1 M NaOH was added to each well and shaken for 20 min. Colour

177   production was measured at 630 nm using a plate reader (Thermo Fisher, Loughborough,

7

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

179 assay.

2.7. Statistical analyses

181 Results were analysed by ANOVA using GenStat (GenStat, 17th ed.). For Experiment 1A

182 terms fitted to this 2 x 3 factorial model were O_2 (physiological vs atmospheric) and time in

183 culture (48, 96 and 144 h). A third interactive term, melatonin (0, 20, 200 and 2000 pg/ml),

184 was included for Experiments 1B and 1C. Oxygen level, melatonin and culture duration

185 were considered as fixed effects and blocked by culture date, incubator and plate. For

186 Experiments 2A and 2B, terms fitted to these 2 x 4 x 2 factorial models were temperature

187 (37.5°C *vs* 40.0°C), melatonin (0, 20, 200 and 2000 pg/ml) and time in culture (96 and 144

188 h). For transcript expression in Experiment 1B and 2A, terms fitted to these 2 x 2 x 2 factorial

189 models were environmental treatment (1B, physiological vs atmospheric O_2; 2A, 37.5°C vs

190 40.0°C), melatonin (0 and 2000 pg/ml) and culture duration (96 and 144 h). These models

191 were blocked by culture date, incubator and plate. Again, temperature, melatonin and culture

192 duration were considered as fixed effects. Estradiol (pg/ml) and P_4 (ng/ml) production were

193 expressed per 10^5 cells. Natural log transformations of these data were used to correct for

194 heteroscedasticity of the residuals. The data are shown as natural logs of the means with a

195 SED.

3. Results

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

steroidogenesis

200 Granulosa cell number in 6-well plates declined between initial plating and 48 h of culture

201 (1.5 to 1.0 x 10^6 cells/well; SED = 0.049; P<0.001) but subsequently recovered (P<0.001)

202 with time so that, by the end of culture, cell density was similar to that initially seeded. Mean

203 cell number averaged across all time points was greater under low than high O_2 level (1.19 vs

204 1.03 x 10^6 cells/well; SED = 0.027; P = 0.004).

205 There was a culture time by O_2 levels interaction (P = 0.014) which indicated that the

206 increase in P_4 production between 48 h and 96/144 h was greater under high than low O_2

207 level (Fig 1A). Granulosa-cell E_2 production declined between 48 and 96 h of culture (Fig

208   1B). However, there was a culture time by O_2 level interaction (P<0.001) which indicated

209   that, in contrast to 20% O_2 where E_2 production didn't change, E_2 production increased

210   between 96 and 144 h for cells cultured under 5% O_2. Consequently, at both 96 and 144 h,

211   the E_2: P_4 ratio was greater (P<0.001) for cells cultured under 5% than under 20% O_2. The

212   temporal pattern of E_2 production depicted in Fig 1B was confirmed by determining

213   aromatase activity (Fig. 2). Testosterone conversion to E_2 in the presence of NADPH

214   indicated a decline (P = 0.014) in aromatase activity at 96 and 144 h for cells cultured under

215   20% O_2 but not for cells cultured under 5% O_2

216   *3.2. Experiment 1 B. Effect of melatonin on GCs cultured under 5% or 20% O_2*

217   Confirming observations from Experiment 1A, cell numbers using a 12-well format declined

218   between 48 and 96 h of culture but increased again by 144 h (647,250 vs 595,391 vs 658,906

219   cells/ml; SED = 15,000; P<0.001). At 20 pg/mL, melatonin increased (P<0.001) cell number

220   relative to non-treated cells for both O_2 levels, but higher doses melatonin did not alter cell

221   number relative to non-treated cells (621,479, 673,938, 603,604 and 590,308 cells/well for 0,

222   20, 200 and 2000 pg/mL respectively; SED = 16,258).

223   Again confirming results from Experiment 1A, P_4 production increased (P<0.001) between

224   48 h and 96/144 h, and was lower (P = 0.024) under 5% than 20% O_2 (data not shown).

225   Similarly, E_2 production matched that of Experiment 1A, declining (P<0.001) by 96 h and

226   remaining low by 144 h under 20% O_2, but increasing again (P<0.001) by 144 h under 5%

227   O2 (data not shown). In contrast to cell number, melatonin had no effect on P_4 and, at 20

228   ng/ml only, marginally reduced (P = 0.023) E_2 production relative to untreated cells (log E_2 =

229   7.09 vs 7.24 pg/ml/10^5 cells; SED = 0.063). Transcript expression for selected genes involved

230   in steroidogenesis, apoptosis and O_2 metabolism varied with time in culture and, for *HSD3B1,*

231   *SOD1* and *HBA* there were O_2 level by culture time interactions (Table 1). However, there

232   was no effect of melatonin on transcript expression. Consistent with measured concentrations

233   of P_4 in spent culture media, *HSD3B1* mRNA expression increased (P = 0.003) with time for

234   cells cultured under 20%. However, this was not the case under 5% O_2. *HBA* mRNA

235   expression was greater (P = 0.015) under 5% than 20% O_2, but decreased (P = 0.006) with

236   time under these conditions. In contrast, transcripts for the antioxidant enzymes *SOD1* and *2*

237   were broadly similar for both O_2 treatments, and *ASMT* expression was unaffected by

238   treatment.

239 *3.3 Experiment 1C. Effect of O_2 level and melatonin on ROS*

240 Using a 96-well format ROS generation determined by Nitroblue tetrazolium assay did not

241 differ between 5% and 20% O_2 culture treatments. However, ROS generation increased

242 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

243 48, 96 and 144 h respectively; SED = 0.011; P<0.001). The inclusion of melatonin to culture

244 media under both O_2 levels reduced (P<0.001) ROS generation irrespective of dose (0.31,

245 0.27, 0.25 and 0.26 OD units/10^5 cells for 0, 20, 200 and 2000 pg/ml respectively; SED =

246 0.012).

247 *3.4. Experiment 2A. Effect of temperature on cell proliferation and steroidogenesis at 5% O_2*

248 *in the presence or absence of melatonin*

249 Working with a 12-well plate format, viable cell number by 48 h of culture in basal media

250 and under standard temperature (i.e. 37.5°C) decreased from 6.00 to 4.54 x 10^5 cells. At this

251 time plates were randomly allocated to standard or high temperature (40.0°C) incubators and

252 melatonin treatments introduced. Cell number subsequently increased (P<0.001) with time in

253 culture to 7.05 x 10^5 cells by 144 h, and this was independent of temperature. However, there

254 was an interaction (P = 0.004) between temperature and melatonin treatment which indicated

255 that the inclusion of melatonin increased viable-cell number at 40.0°C but reduced viable-cell

256 number at 37.5°C, particularly at the higher doses (Fig. 3). Cell number was greater at 37.5°C

257 than 40.0°C when melatonin was not included in the media.

258 Consistent with Experiments 1A and B, P_4 production increased between 48 and 96 h for

259 cells cultured at both 37.5°C and 40.0°C. In contrast to 37.5°C, however, P_4 production

260 declined (P<0.001) between 96 and 144 h culture at 40.0°C (Fig. 4A). The pattern of E_2

261 production during the 144 h culture period (Fig. 4B) was similar to that observed in

262 Experiments 1A and B at 5% O_2 for both 37.5°C and 40.0°C. There was an interaction (P =

263 0.007) between melatonin dose and temperature on P_4 production (Fig. 5). Whereas the

264 higher concentrations of melatonin (i.e. ≥ 200 ng/ml) increased P_4 production at 37.5°C they

265 reduced P_4 production at 40.0°C. The two lower doses of melatonin (i.e. 20 and 200 pg/ml)

266 reduced E_2 by GCs whereas the highest dose (2000 pg/ml) had no effect (data no shown).

267 Consequently, the E_2:P_4 ratio increased (P<0.001) between 96 (1.47:1) and 144 h (2.51:1) of

268 culture, and the overall effect of melatonin was similar to that observed for E_2; that is the two

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

270 effect.

271 In contrast to the effects of O_2 level (Table 1), temperature generally had little effect on

272 transcript expression in cultured GCs (Table 2). Importantly, however, there was a

273 temperature x time of culture interaction (P = 0.009) for *BAX* mRNA expression. Consistent

274 with the results of Experiment 1B (Table 1), there was a decline in *BAX* mRNA expression

275 with time at 37.5°C, but this did not occur at 40.0°C (Table 2). Transcript expression for

276 *ASMT* was greater (P = 0.019) at 40.0°C than at 37.5°C. There was no significant effect of

277 melatonin on transcript expression.

278 *3.5. Experiment 2B. Effect of temperature and melatonin on ROS production at 5% O_2*

279 Production of ROS by bovine GCs was not affected by temperature. The presence of

280 melatonin at all three concentrations reduced (P<0.001) ROS production by cultured GCs

281 (0.31, 0.26, 0.25 and 0.27 OD units/10^5 cells for 0, 20, 200 and 2000 pg/ml respectively; SED

282 = 0.01) at both temperatures.

283

4. Discussion

285 The most significant novel findings to emerge from this study were, firstly, the relatively

286 small overall effect that elevated temperature (40.0 vs 37.5°C) had on GC physiology when

287 these cells were cultured under low (5%) O_2 as opposed to atmospheric (20%) O_2 and,

288 secondly, the interaction between melatonin dose and temperature on viable cell number and

289 P_4 production at low O_2 levels. Extended culture of GCs at 40.0°C led to a decline in P_4

290 production, a response which was exacerbated with the inclusion of high-dose ($\geq$ 200 pg/mL)

291 melatonin (Fig. 5). In contrast, at 37.5°C P_4 production remained high at 144 h and the

292 inclusion of high-dose melatonin appeared to contribute to this increase. The corresponding

293 changes in viable cell number with increasing dose of melatonin for low and high

294 temperatures (Fig. 3) suggest that high doses of melatonin interacted with temperature to

295 differentially influence the extent of GC luteinisation at low O_2 levels.

296 In contrast to temperature, O_2 level had a more marked effect on GC physiology. Unlike GCs

297 cultured under atmospheric (20%) O_2 levels, GCs cultured under low O_2 retained their

298 primary GC phenotype to a greater extent, being more proliferative and estrogenic (Fig. 1).

299   These observations are consistent with those of [12] for GCs from small-medium sized

300   follicles cultured in 5% O_2, and indicate that studies investigating environmental effects on

301   cultured primary GCs are best carried out under low O_2 levels which better represent the

302   ovarian follicle [14,15].

303   *4.1. Responses to O_2 level*

304   Cell proliferation, and hence mean cell number, from 48 h of culture in the current study was

305   greater at 5% than 20% O_2, an observation consistent with that of Shiratsuki et al. [13]. The

306   increase in aromatase activity and E_2 production between 96 and 144 h for GCs cultured

307   under 5% O_2 in our study (Fig. 1B and 2) is also in general agreement with observations of

308   Roberts and Echternkamp [12]. Collectively, these results suggest that under 5% O_2 from

309   around 96 h of culture a population of proliferating and steroidogenic cells exits which, in

310   contrast to cells cultured under 20% O_2, better represent luminal GCs observed in medium-

311   sized growing antral follicles. The decline in aromatase activity and E_2 production under 20%

312   O_2 may be due to our use of fibronectin-coated plates. Plates pre-coated with attachment

313   factors such as serum have been found to reduce E_2 production by GCs cultured under 20%

314   O_2 [27]. In our study, the assumption is that the steady decline in aromatase activity (pg

315   E_2/mg protein) up to 144 h under 20% O_2 was due to a parallel decline in enzyme. However,

316   we were not able to confirm the mechanism of this decline. It was not possible to establish

317   differential transcript expression for *CYP19A1* which, for GCs in our system, was close to the

318   detection limit of the method and so the data are not presented. Transcript levels for *HSD3B1*

319   (which catalyzes the conversion of pregnenolone to P_4) were greater at 20% than at 5% O_2,

320   and increased with time in culture at 20% O_2, consistent with increased production of P_4 by

321   these cells (Fig. 1A).

322   Of the transcripts measured (Table S3) the only other to be affected by O_2 level was *HBA*

323   (Hemoglobin alpha) (Table 1); transcript expression for Hemoglobin beta (*HBB*) was barely

324   detectable and unresponsive to culture conditions. In fact transcript expression for *HBA* at 48

325   h culture was similar to that for freshly aspirated GCs (data not presented), but under low O_2

326   declined with time during culture. Transcripts for *HBA* and *HBB* have previously been

327   reported in mouse and human granulosa and cumulus cells [37], and transcripts for *HBA* were

328   recently reported in bovine GCs in a micro-array study that assessed the effects of plating

329   density on gene expression [38]. Working with aspirated GCs from small to medium (<

330   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

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

379 The most striking and novel observation in our study was the differential effect of the two
380 higher doses of melatonin (i.e. 200 and 2000 pg/mL) on viable cell number and P_4 production
381 at 37.5 and 40.0°C (Fig. 3 and Fig. 5). The levels were within the range used previously in
382 culture studies with bovine cumulus and GCs (i.e. pg/mL to µg/mL levels [22, 45-47]) and,
383 for the most part, comparable to levels reported in human, porcine and bovine follicular fluid
384 (i.e. 10 to 300 pg/mL [16-20]). The higher doses of melatonin employed in the current study
385 could therefore be considered to be towards the upper end of physiological. The stimulatory
386 effect of increasing doses of melatonin on P_4 at 37.5°C is consistent with previous reports for
387 human and bovine GCs cultured at 37°C but in the presence of serum and under atmospheric
388 O_2 levels [22, 48]. The concomitant reduction in GC numbers in the current study further
389 suggests that the higher doses of melatonin induced GCs to luteinise under these conditions.
390 This is in stark contrast to GCs cultured at 40.0°C where the effects of higher doses of
391 melatonin were to increase cell proliferation and reduced P_4 production. Although melatonin
392 has previously been found to increase sheep GC numbers at high (43°C) temperatures (in the
393 presence of 10% FCS and under atmospheric O_2 [49]), a suppressive effect on P_4 production
394 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

6. References

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558

559 **Figure captions**

560 **Fig. 1**. Effect of atmospheric (20%) or physiological (5%) O_2 level on granulosa-cell P_4 (A)

561 and E_2 (B) production after 48, 96 and 144 h of culture in Experiment 1.A. A 2 x 3 factorial

562 arrangement replicated 5 times. Data were $\log_e$ transformed prior to analysis. Superscripts

563 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.

21

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

591

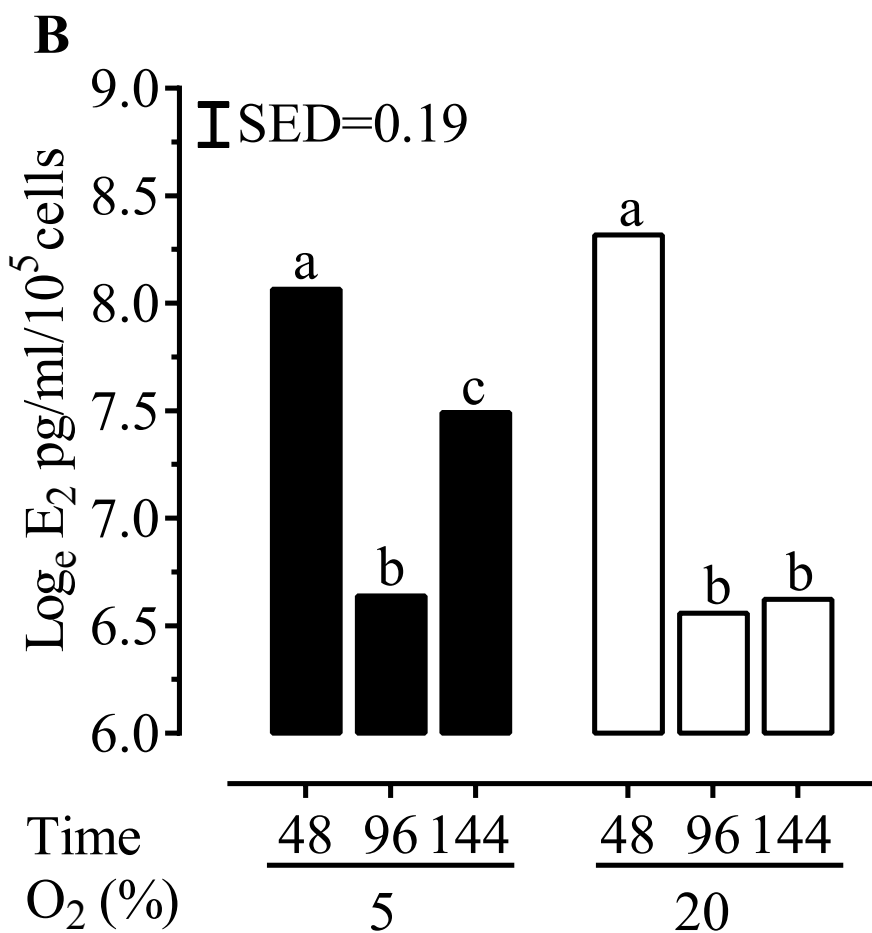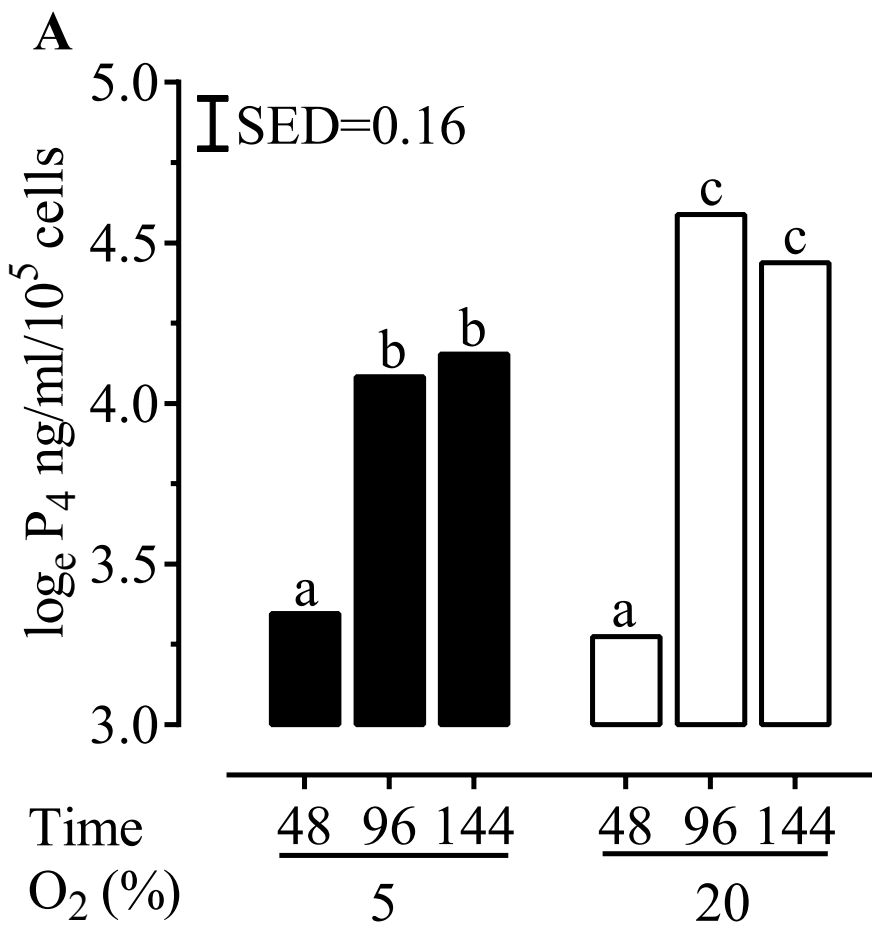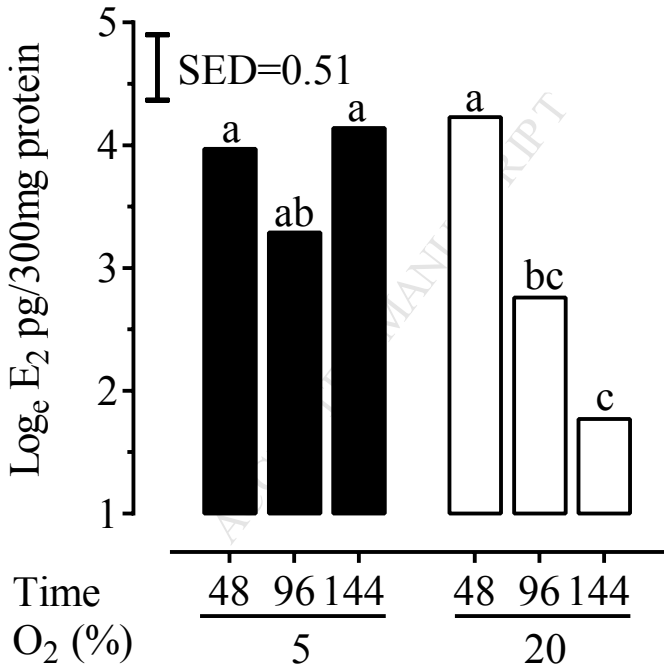| Oxygen (O$_2$)		5%			20%			Significance (P)		
Culture time (h)	48	96	144	48	96	144	SED	O$_2$	h	O$_2$ x h
Steroidogenesis										
HSD3B1	11.02[a]	11.47[a]	11.47[a]	11.25[a]	12.06[b]	13.17[c]	0.31	0.028	<0.001	0.003
Apoptosis										
BAX	11.99[ab]	11.67[ab]	11.37[a]	12.12[b]	11.27[a]	11.43[a]	0.37	-	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

22

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

595

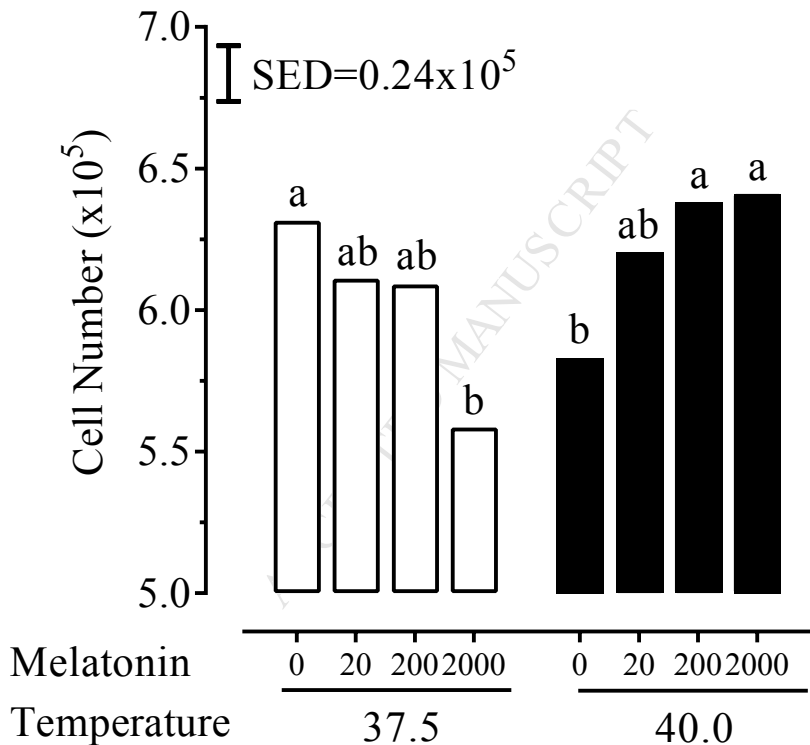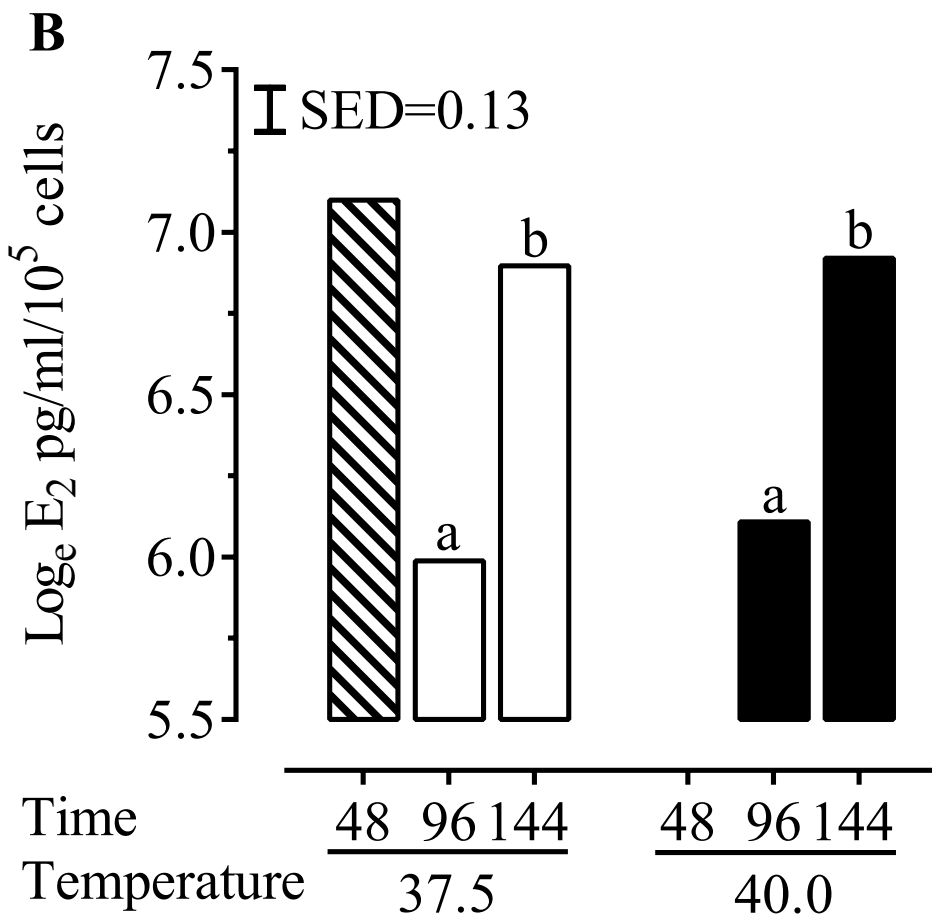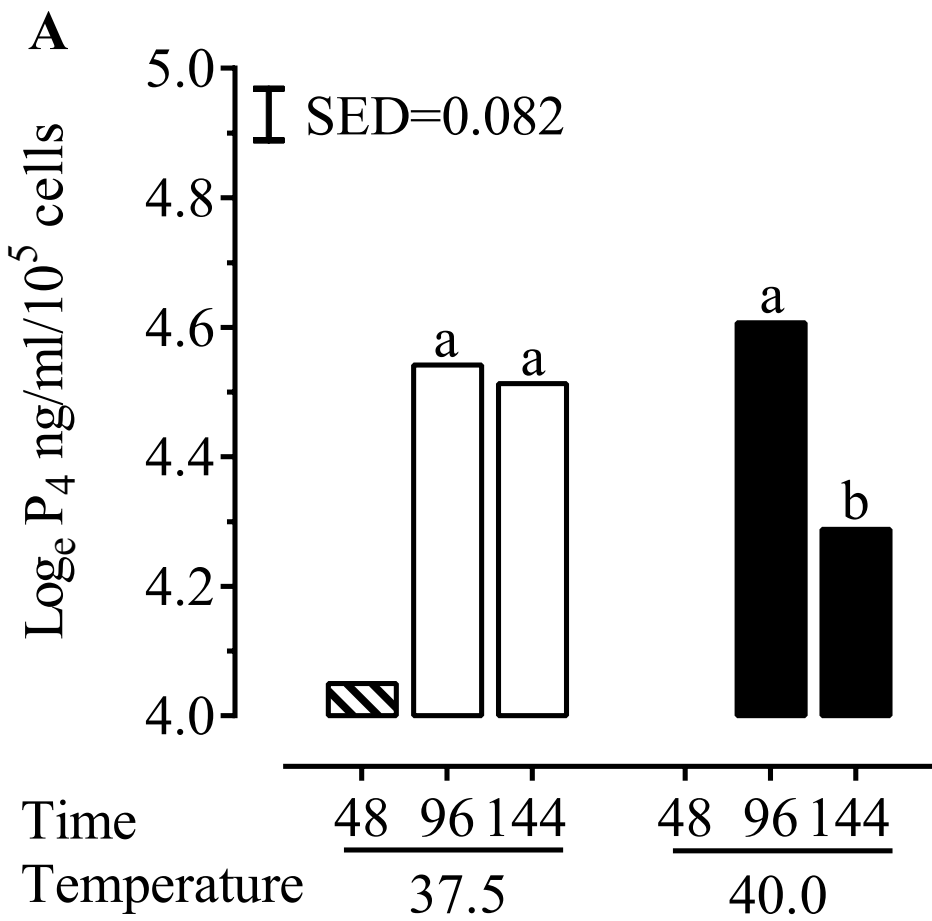| Temperature (°C)	37.5		40.0°C			Significance (P)		
Culture time (h)	96	144	96	144	SED	°C	h	°C x h
Steroidogenesis								
HSD3B1	13.32	13.42	14.03	13.42	0.31	-	-	-
Apoptosis								
BAX	12.56 [a]	12.19 [b]	12.49 [a]	12.66 [a]	0.12	0.068	-	0.009
P53	12.58	12.39	12.54	12.86	0.36	-	-	-
HSPA1A	13.9	13.72	14.38	14.2	0.40	-	-	-
O₂ 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	-	-

596

23

A

log$_e$ P$_4$ ng/ml/10^5 cells

SED=0.16

Time: 48 96 144 | 48 96 144
O$_2$ (%): 5 | 20

B

Log$_e$ E$_2$ pg/ml/10^5 cells

SED=0.19

Time: 48 96 144 | 48 96 144
O$_2$ (%): 5 | 20

SED=0.51

Log$_e$ E$_2$ pg/300mg protein

Time
O$_2$ (%)

48 96 144   48 96 144
5   20

A

Log$_e$ P$_4$ ng/ml/10^5 cells

SED=0.082

Time: 48 96 144   48 96 144
Temperature: 37.5   40.0

B

Log$_e$ E$_2$ pg/ml/10^5 cells

SED=0.13

Time: 48 96 144   48 96 144
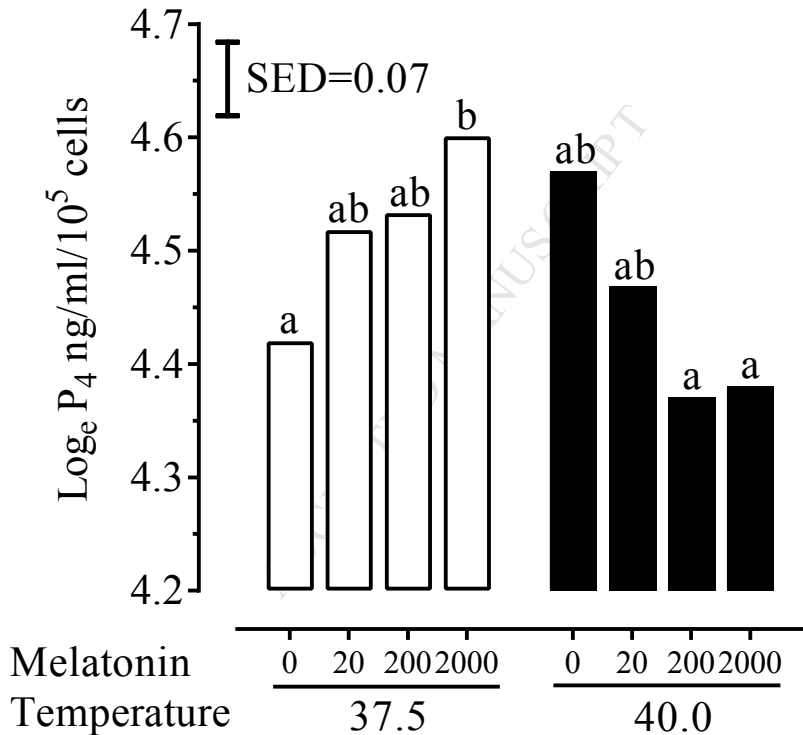Temperature: 37.5   40.0

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