

—Original Article—

## Heat stress impairs gap junction communication and cumulus function of bovine oocytes

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**Abstract.** The intimate association of cumulus cells with one another and with the oocyte is important for regulating oocyte meiotic arrest and resumption. The objective of this study was to determine the effects of heat stress on cumulus cell communication and functions that may be related to accelerated oocyte meiosis during early maturation. Bovine cumulus-oocyte complexes underwent *in vitro* maturation for up to 6 h at thermoneutral control (38.5°C) or elevated (40.0, 41.0 or 42.0°C) temperatures. Gap junction communication between the cumulus cells and the oocyte was assessed using the fluorescent dye calcein after 4 h of *in vitro* maturation. Dye transfer was reduced in cumulus-oocyte complexes matured at 41.0°C or 42.0°C; transfer at 40.0°C was similar to control ( $P < 0.0001$ ). Subsequent staining of oocytes with Hoechst revealed that oocytes matured at 41.0 or 42.0°C contained chromatin at more advanced stages of condensation. Maturation of cumulus-oocyte complexes at elevated temperatures reduced levels of active 5' adenosine monophosphate activated kinase ( $P = 0.03$ ). Heat stress exposure had no effect on active extracellular-regulated kinase 1/2 in oocytes ( $P = 0.67$ ), associated cumulus cells ( $P = 0.60$ ) or intact cumulus-oocyte complexes ( $P = 0.44$ ). Heat-induced increases in progesterone production by cumulus-oocyte complexes were detected during the first 6 h of maturation ( $P = 0.001$ ). Heat-induced alterations in gap junction communication and other cumulus-cell functions likely cooperate to accelerate bovine oocyte meiotic progression.

**Key words:** Cumulus cell, Gap junction, Heat stress, Meiotic maturation, Oocyte

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Oocyte maturation is a critical developmental period for acquiring competence to progress to embryonic stages after fertilization. Exposure of the cumulus-oocyte complex to heat stress during the first half of meiotic maturation decreases rates of development to the blastocyst stage after fertilization (*in vivo* hyperthermia, [1]; *in vitro* heat stress, [2–5]). Some of the influences of heat stress on oocyte competence may in part relate to heat-induced hastening of meiotic progression.

In the bovine, heat stress during the first 12 h of *in vitro* maturation (hIVM) accelerated progression to metaphase-I and metaphase II, with more oocytes completing meiotic progression at 16 and 18 h compared with oocytes matured only at a thermoneutral control temperature [6]. Heat-induced hastening of meiotic maturation is not without consequence because subsequent efforts by Schrock *et al.* [7] demonstrated that earlier fertilization of heat-stressed bovine oocytes improved blastocyst development rates. In 2015, Hooper and others [8] demonstrated that heat-induced hastening of meiotic maturation is detectable as early as 4 hIVM. Specifically, more heat-stressed bovine oocytes underwent germinal vesicle breakdown compared to

control counterparts; this difference was more prominent by 6 hIVM. The possibility for heat stress to hasten the onset of maturation has been observed in other species. Baumgartner and Chrisman [9] noted an increase in the bicellular presentation of oocytes obtained from hyperthermic murine females (i.e., one cell presumed to be the first polar body, the other being the oocyte). Kim *et al.* [10] reported that an *in vitro* heat stress exposure accelerated germinal vesicle breakdown in murine oocytes.

The control of oocyte meiotic arrest and resumption is largely mediated by the cumulus cells. The oocyte is most susceptible to heat stress during the first 12 h of maturation [1, 2], when the surrounding cumulus cells are intimately associated with one another and the oocyte through intercellular gap junctions [11–13]. A reduction in gap junction permeability occurs around the time of oocyte resumption of meiosis in the bovine [13–15]. In mice, Norris and others [16] demonstrated LH-induced closure of gap junctions in cumulus cells reduces cGMP content in the oocyte. Gap junction communication is partly regulated by kinases, either directly through phosphorylation by extracellular signal regulated kinase 1/2 (ERK1/2) [17, 18] or indirectly through control of protein synthesis by 5' adenosine monophosphate activated kinase (AMPK; [19]). ERK1/2 and AMPK may also mediate other cumulus cell functions during oocyte maturation, including progesterone production [20, 21]. In turn, progesterone regulates levels of connexin 43, the primary cumulus-derived gap junction protein [22, 23]. Furthermore, increases in cumulus-derived progesterone during early maturation may accelerate germinal vesicle breakdown and meiotic progression in the bovine oocyte [24, 25].

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Taken together, we hypothesized that heat stress exposure during early maturation may alter cumulus cell communication and function to hasten onset of meiotic progression. To that end, we evaluated the effects of heat stress on gap junction function and chromatin configuration in the maturing bovine cumulus-oocyte complex. To further evaluate gap junction communication we also examined the effect of heat stress on cGMP content in oocytes and their surrounding cumulus cells. We also investigated the impact of heat stress on activation of ERK1/2 and AMPK as well as the production of progesterone during early maturation.

## Materials and Methods

### *Collection and in vitro maturation of cumulus-oocyte complexes*

Unless otherwise noted, reagents and chemicals were procured from MilliporeSigma (St. Louis, MO, USA). Oocytes were collected from abattoir-derived bovine ovaries as described previously [3]. Oocyte maturation medium (OMM) and HEPES-TALP were prepared as described by Rispoli *et al.* [26]. Cumulus-oocyte complexes (COCs) were placed into OMM in polystyrene tubes (Sarstedt AG and Co., Nümbrecht, Germany) and matured at 38.5°C or at an elevated temperature observed in heat-stressed cows [1, 27–31]. Care was taken to ensure that only COCs with similarly sized cumulus cell vestments were selected.

### *Study one: Gap junction communication, chromatin configuration, and cGMP content of heat-stressed cumulus-oocyte complexes*

Gap junction communication between oocytes and associated cumulus cells was assessed as described previously [13] in COCs (35 per 500  $\mu$ l phenol red-free OMM) matured at 38.5, 40.0, 41.0 or 42.0°C for 4 h. Maturation time was selected based on previous efforts in our laboratory documenting that heat-induced hastening of meiotic maturation occurs as early as 4 hIVM [8]. Elevated temperatures were selected based on rectal temperatures recorded in cows experiencing moderate (40 to 41°C; [27–30]) or severe (42°C; [1, 31]) hyperthermia. A separate subset of COCs were cultured at 38.5°C in the presence of the gap junction inhibitor carbenoxolone (Alfa Aesar, Tewksbury, MA, USA) at a concentration (243  $\mu$ M) previously shown to be effective [13]. After 4 hIVM, COCs were incubated with 5  $\mu$ M calcein acetoxymethyl (calcein-AM; Cayman Chemicals, Ann Arbor, MI, USA) in phenol red-free OMM for 15 min at 38.5°C. COCs were then washed and incubated in calcein-AM-free, phenol red-free OMM for 30 min to allow cleavage of the acetoxymethyl group and transfer of the fluorescent calcein molecule into the oocyte. Phenol red-free OMM was used to prevent non-specific cleavage of the calcein-AM molecule [13]. Oocytes were denuded of surrounding cumulus by vortexing for 5 min in HEPES-TALP. Fluorescence (excitation 450–490 nm; emission > 515 nm) was measured using a Nikon Eclipse TE300 inverted microscope and the NIS Elements BR imaging software (version 3.0; Nikon Instruments, Melville, NY, USA). This study was replicated across six different days of COC collection (n = 360 to 393 total COCs/group).

After measuring fluorescence, oocytes were fixed in 3% paraformaldehyde in Dulbecco's phosphate buffered saline (DPBS) for 15 min and then stained with 0.5  $\mu$ g/ml Hoechst 33342 (~6 h after

placement into OMM). Chromatin configuration was evaluated with fluorescence (excitation 330–380 nm; emission > 420 nm) using a Nikon Eclipse TE300 inverted microscope for three different days of COC collection (n = 74 to 135 total oocytes/group) and scored based on previously defined criteria [14]. Those with a diffuse chromatin taking up approximately 20–30% of the oocytes surface were assigned a score of one, oocytes with mostly diffuse chromatin with a few condensed foci beginning to form were assigned a score of two, oocytes with chromatin condensed into clumps were assigned a score of three, oocytes with a single mass of condensed chromatin were assigned a score of four, and oocytes at prometaphase-I, where individual chromosomes were apparent, were assigned a score of five.

To further evaluate gap junction communication we examined the effect of heat stress on cGMP content in oocytes and their surrounding cumulus cells separately. Intact COCs (55 to 60 per 500  $\mu$ l OMM) were cultured at 38.5 or 41.0°C for 4 h. Thereafter, oocytes were denuded of associated cumulus cells by vortexing for 5 min in HEPES-TALP. Disassociated cumulus cells were washed with DPBS and collected after centrifugation at 300  $\times$  g for 10 min. Denuded oocytes that were not lysed and completely free of cumulus cells were collected (50 oocytes/pool as per [32]). Cumulus cells and cumulus-free oocytes were lysed as separate pools in 40  $\mu$ l 0.1 N HCl before being stored at –80°C. Samples were acetylated and intracellular cGMP concentrations were determined in triplicate in denuded oocytes and associated cumulus cells using a commercially available cGMP ELISA kit (Cayman Chemicals) according to the manufacturer's instructions. Intra-assay coefficient of variation was 10.3%. This study was replicated using nine different COC pools across three different days of collection (n = 450 total oocytes or cumulus masses/treatment group).

### *Study two: Levels of protein kinases ERK1/2 and AMPK in heat-stressed cumulus-oocyte complexes*

To determine levels of active ERK1/2 (phosphorylated at Thr185/Tyr187), total ERK1/2, and active AMPK (phosphorylated at Thr172), COCs (55 to 60 per 3 ml OMM) were matured at 38.5, 40.0 or 41.0°C for 4 or 6 hIVM (*a priori* test of hypothesis using a 3  $\times$  2 factorial treatment arrangement). Maturation times of 4 and 6 hIVM were chosen based on previous efforts of Hooper *et al.* [8] reporting heat-induced differences in meiotic progression in heat-stressed oocytes. Omission of a maturation temperature (i.e., 42°C) was required to ensure that COCs were processed in a timely manner for assessing kinase activity after maturation for 4 or 6 h. This temperature is severe and is seldom observed in heat-stressed cows. When interactions were not significant, main effect means for maturation time and temperature were reported. Kinase levels were also examined in immature COCs when possible (i.e., after collection but before placement into OMM, 0 hIVM) as a reference starting point. In instances where a surplus of COCs were available on a given collection day (n = 5), separate pools of COCs were also matured for 24 h at 38.5°C to evaluate the effect of maturation time on activity of AMPK and ERK1/2 under thermoneutral conditions.

Active ERK1/2 and total ERK1/2 were determined in oocytes (n = 30 of the original pool of 55 to 60 total) and their associated cumulus cells using a Milliplex MAP phosphorylated/total ERK1/2 duplex kit (MilliporeSigma) as described by Chouzouris *et al.* [33].

After the indicated maturation period, oocytes were denuded of associated cumulus cells by vortexing for 5 min in HEPES-TALP. Cumulus cells were washed with DPBS, centrifuged at  $300 \times g$  for 10 min, and cell pellets were stored at  $-80^{\circ}\text{C}$ . Oocytes that were completely free of cumulus cells and were not lysed were stored at  $-80^{\circ}\text{C}$ . Oocytes and cumulus cells were lysed by incubating in lysis buffer [33] supplemented with 1 mM phenylmethylsulfonyl fluoride for 10 min on ice, sonicating for 1 min at 100% amplitude, and then a 10 min incubation on ice. Cell lysates were centrifuged at  $10,000 \times g$  for 10 min at  $4^{\circ}\text{C}$  to remove debris. Total protein concentrations in the cumulus cell lysate were determined using the BradfordRED (Expedeon, San Diego, CA, USA) or FluoroProfile (MilliporeSigma) kits against a bovine serum albumin standard curve, according to manufacturer's instructions. The proportion of total ERK1/2 that was active was determined in duplicate cumulus cell (equivalent 6.25  $\mu\text{g}$  total protein/well) and oocyte (equivalent 15 oocytes/well) lysates using the Milliplex MAP kit according to the manufacturer's instructions. Median fluorescence intensity was determined using a Luminex 200 (Luminex, Austin, TX, USA). Wells that did not reach a minimum of 35 bead counts (manufacturer's recommendation) or that had a bead coefficient of variation  $> 200\%$  [34, 35] were excluded from the analysis. Intra-assay coefficient of variation for active and total ERK1/2 were 22.0 and 8.2%, respectively, consistent with previously published studies using a magnetic bead approach [36, 37]. Effort to examine active ERK1/2 and total ERK1/2 was replicated across eleven different days of COC collection ( $n = 330$  total oocytes or cumulus masses/group). Outcomes of these efforts prompted us to conduct a follow-on study to examine the extent to which separating the oocyte from its cumulus cells by vortexing influenced the proportion of total ERK1/2 that was active in intact COCs cultured for 4 h at 38.5 or  $41.0^{\circ}\text{C}$ . This effort was replicated using five different COC pools on a single day of collection ( $n = 150$  total COCs/treatment group).

Active AMPK levels were determined in intact COCs (20 COCs out of the original pool of 55 to 60 total) using a commercial ELISA kit (Cell Signaling Technology, Danvers, MA, USA). To this end, COCs were lysed with the provided lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and total protein concentrations were determined as described above. Levels of active AMPK phosphorylated at Thr172 were determined in COC cell lysates in duplicate at  $37^{\circ}\text{C}$  as per manufacturer's instructions. Absorbance (450 nm) was measured using a Synergy H1 microplate reader (BioTek Instruments, Burlington, VT, USA). Intra-assay coefficient of variation was 4.1%. Absorbance was corrected for relative protein amount. This study was replicated across ten different days of COC collection ( $n = 200$  total COCs/treatment group).

#### Study three: Cumulus-derived progesterone production by heat-stressed cumulus-oocyte complexes

The amount of progesterone released into the conditioned maturation medium after culture of COCs (30 to 40 per 500  $\mu\text{l}$  OMM) at 38.5, 41.0 or  $42.0^{\circ}\text{C}$  for 1, 2, 3, 4, 5 or 6 hIVM. Effort to include earlier maturation times in this study allowed for assessing when levels in the conditioned medium become detectable. Priority for examining consequences of  $41^{\circ}\text{C}$  was based on previous efforts reporting heat-induced increases in progesterone during the latter part of maturation

[38, 39]. Effort to examine consequences of a more severe heat stress ( $42^{\circ}\text{C}$ ) was prioritized over examining consequences of  $40.0^{\circ}\text{C}$  to increase the likelihood of detecting heat-induced differences during times when progesterone production by the cumulus is low. A double antibody  $^{125}\text{I}$  radioimmunoassay progesterone assay (MP Biomedicals, Costa Mesa, CA, USA) was used according to the manufacturer's instructions. The assay sensitivity was 0.2 ng/ml, intra-assay coefficient of variation was 5.1%, and the inter-assay coefficient of variation was 5.9%. This study was replicated across nine different days of COC collection ( $n = 296$  total COCs/treatment group).

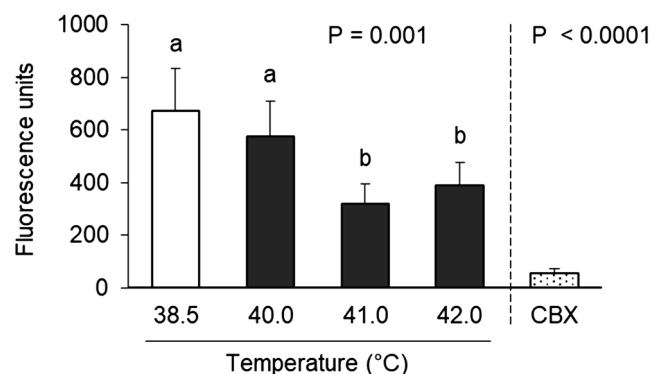
#### Statistical analyses

Data were analyzed using generalized linear mixed models (PROC GLIMMIX, SAS 9.4, SAS Institute, Cary, NC, USA) as a randomized block design, blocking on replicate (i.e., day of COC collection). For study one, the fixed effect included in statistical model was IVM temperature. The ERK1/2 data were log-transformed to achieve normality before analysis; fixed effects included in the model for this variable and AMPK included IVM temperature, IVM time and respective interaction. Relevant for study three, fixed effects in the model included IVM temperature, IVM time and respective interaction. A  $P$ -value  $< 0.05$  was considered significant. In instances where interactions were not significant ( $P > 0.05$ ), main effect means were reported. Treatment differences were determined using F-protected least significant differences and are reported as least squares means  $\pm$  standard error of the mean (SEM).

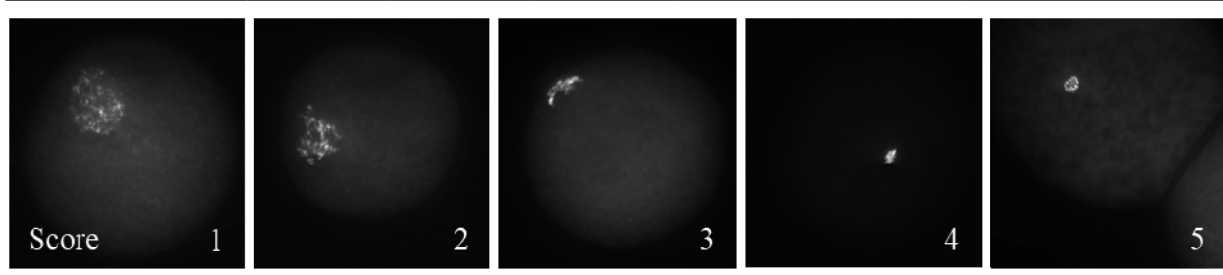
## Results

#### Study one: Gap junction communication, chromatin configuration, and cGMP content of heat-stressed cumulus-oocyte complexes

Exposure of COCs to heat stress for the first 4 hIVM reduced transfer of the calcein dye ( $P < 0.001$ ; Fig. 1). Impaired gap junction communication was noted in COCs matured at  $41.0$  or  $42.0^{\circ}\text{C}$ ,

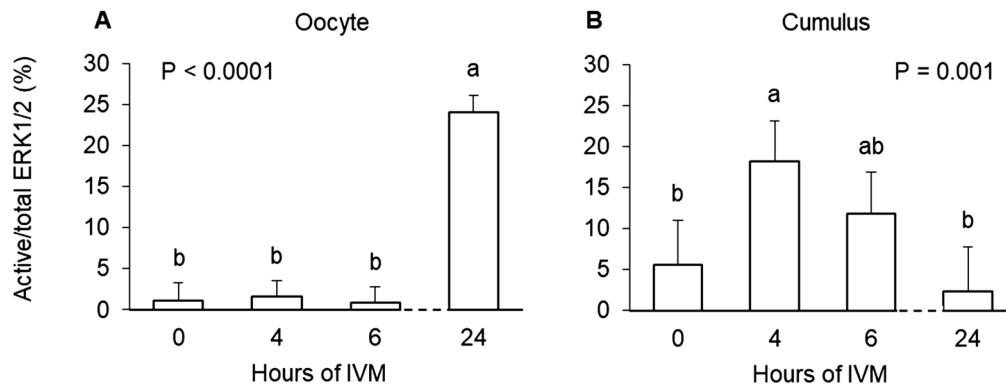


**Fig. 1.** Effect of maturation of bovine cumulus-oocyte complexes for 4 h at either 38.5, 40.0, 41.0 or  $42.0^{\circ}\text{C}$  on gap junction communication as determined by the transfer of the fluorescent dye calcein from cumulus cells into the oocyte. Groups that do not share a letter are significantly different ( $P = 0.001$ ). A separate subset of cumulus-oocyte complexes were matured at  $38.5^{\circ}\text{C}$  in the presence of the gap junction inhibitor carbenoxolone (CBX).

**Table 1.** Chromatin configuration in control and heat-stressed oocytes after assessment of gap junction function


IVM temperature (°C)	Chromatin configuration score (%) *					Mean chromatin score
	1	2	3	4	5	
38.5	8.6	38.3	43.8	9.4	0.0	2.54 ± 0.07 <sup>b</sup>
40.0	8.7	40.2	43.5	6.5	1.1	2.51 ± 0.08 <sup>b</sup>
41.0	1.5	20.7	51.9	25.9	0.0	3.02 ± 0.06 <sup>a</sup>
42.0	2.8	16.0	64.2	17.0	0.0	2.95 ± 0.07 <sup>a</sup>
CBX **	1.4	21.6	59.5	16.2	1.4	2.95 ± 0.09 <sup>a</sup>

Images depict representative images of oocytes with diffuse chromatin (score 1), diffuse chromatin with some condensed foci (score 2), clumped, mostly condensed chromatin (score 3), a single condensed chromatin mass (score 4), or at the pro-metaphase-I stage (score 5). Oocytes were fixed (~6 h after placement of cumulus-oocyte complexes into maturation media) after *in vitro* maturation (IVM) at indicated temperature, and determination of calcein transfer. \* Percentage of total oocytes evaluated at each stage. \*\* CBX: Oocytes derived from cumulus-oocyte complexes matured at 38.5°C with the gap junction inhibitor carbenoxolone. Means that do not share a letter are significantly different ( $P < 0.0001$ ).



**Fig. 2.** The proportion of total ERK1/2 that was active in bovine oocytes (panel A) and associated cumulus cells (panel B) after *in vitro* maturation (IVM) of cumulus-oocyte complexes at 38.5°C for 0, 4, 6 or 24 h. Groups that do not share a letter are significantly different.

whereas dye transfer in COCs matured at 40.0°C was similar to the thermoneutral control. Exposure to a known gap junction inhibitor, carbenoxolone, also reduced dye transfer compared to the control ( $P < 0.0001$ ; Fig. 1). Heat-induced changes in gap junction communication were coincident with differences in chromatin configuration (Table 1). Mean oocyte chromatin configuration scores were higher in COCs matured at 41.0 or 42.0°C or with carbenoxolone compared to those matured at 38.5 or 40.0°C ( $P < 0.0001$ ). In other words, oocytes exposed to temperatures  $\geq 41.0^\circ\text{C}$  had progressed further through meiosis compared to oocytes matured at 38.5 or 40.0°C.

Heat stress of COCs for the first 4 hIVM did not alter intracellular cGMP levels in denuded oocytes ( $0.527 \pm 0.04$  vs.  $0.502 \pm 0.04$  fmol/oocyte at 38.5 and 41.0°C, respectively;  $P = 0.68$ ) or their associated

cumulus cells ( $1.663 \pm 0.445$  vs.  $1.578 \pm 0.920$  fmol/cumulus at 38.5 and 41.0°C, respectively;  $P = 0.43$ ).

#### Study two: Levels of protein kinases ERK1/2 and AMPK in heat-stressed cumulus-oocyte complexes

The proportion of total ERK1/2 that was active was highest in oocytes matured at 38.5°C for 24 h ( $P < 0.0001$ ) compared to those matured for 6 h or less (Fig. 2A). Culture of intact COCs at an elevated temperature of 40 or 41°C for 4 to 6 h did not impact levels of active ERK1/2 in the oocyte ( $P = 0.67$ ; Table 2).

When evaluating the cumulus enveloping immature vs. matured oocytes (i.e., 0 vs. 24 hIVM), no differences were evident in the proportion of total ERK1/2 that was active ( $P = 0.02$ ; Fig. 2B).

**Table 2.** Effects of *in vitro* maturation time (hIVM), temperature and hIVM × temperature interaction on the activation of extracellular-regulated kinase 1/2 (ERK1/2) in oocyte and associated cumulus cells

	Active/total ERK1/2 (%)	
	Oocytes	Cumulus cells
hIVM <sup>1</sup>		
4	0.75 ± 0.51	10.63 ± 3.39 <sup>a</sup>
6	0.58 ± 0.52	8.15 ± 3.38 <sup>b</sup>
Temperature (°C) <sup>2</sup>		
38.5	0.67 ± 0.52	10.23 ± 3.42
40.0	0.76 ± 0.52	8.74 ± 3.47
41.0	0.57 ± 0.52	9.21 ± 3.44
hIVM × Temperature		
4 / 38.5°C	0.91 ± 0.54	12.82 ± 3.57
4 / 40.0°C	0.72 ± 0.53	8.53 ± 3.61
4 / 41.0°C	0.63 ± 0.52	10.55 ± 3.61
6 / 38.5°C	0.43 ± 0.54	7.64 ± 3.57
6 / 40.0°C	0.81 ± 0.53	8.95 ± 3.65
6 / 41.0°C	0.50 ± 0.58	7.87 ± 3.58
hIVM P value	0.92	<b>0.046</b>
Temperature P value	0.67	0.60
hIVM × Temperature P value	0.07	0.18

<sup>1</sup>Main effect of time (4 or 6 h) averaged across temperatures. <sup>2</sup>Main effect of temperature (38.5, 40.0 or 41.0°C) averaged across maturation times.

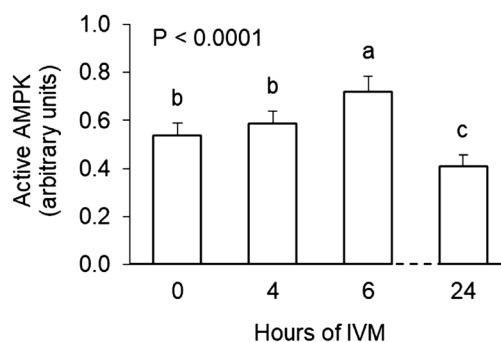
<sup>a-b</sup>Means with different superscripts within a column differ ( $P < 0.05$ ).

Interestingly, the proportion of total ERK1/2 that was active was lower at 6 vs. 4 h ( $P = 0.046$ ; Table 2). Relevant for examining the main effect of temperature, culture of intact COCs for 4 to 6 h at an elevated temperature of 40 or 41°C did not impact levels of active ERK1/2 in the surrounding cumulus ( $P = 0.60$ ; Table 2). Lack of an effect heat stress to impact active ERK1/2 was also noted when levels were examined in intact COCs at 4 hIVM ( $23.46 \pm 1.43$  vs.  $25.20\% \pm 1.43$  at 38.5 and 41.0°C, respectively;  $P = 0.44$ ).

Active AMPK levels in intact COCs were lower after 24 h of maturation at 38.5°C compared to 0 hIVM ( $P < 0.0001$ ; Fig. 3). However, during the early part of maturation, active AMPK was higher at 6 vs. 4 hIVM ( $P < 0.02$ ; Figs. 3 and 4A). Culture of intact COCs at an elevated temperature during the first 6 h reduced levels of active AMPK ( $P = 0.03$ ; Fig. 4B). Maturation at 40.0°C decreased active AMPK levels ( $P = 0.008$ ); exposure to 41.0°C resulted in levels that were intermediate to those obtained for COCs matured at 38.5 or 40.0°C ( $P = 0.08$ ).

### Study three: Cumulus-derived progesterone production by heat-stressed cumulus-oocyte complexes

Progesterone levels in the conditioned maturation medium increased progressively during the first 6 hIVM ( $P < 0.0001$ ; Fig. 5A). Exposure of COCs to an elevated temperature of 41 or 42°C increased progesterone production ( $P = 0.001$ ; Fig. 5B). Heat-induced increases in progesterone did not differ depending on time of maturation (hIVM × Temperature interaction,  $P = 0.80$ ).

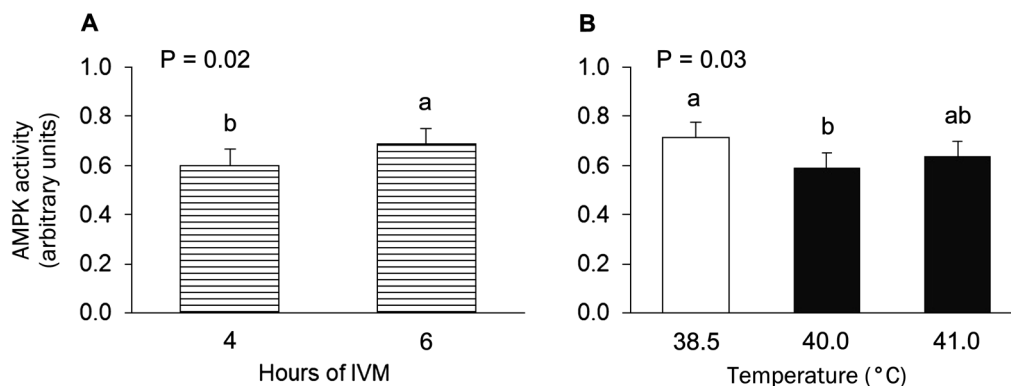
**Fig. 3.** Active AMPK in bovine cumulus-oocyte complexes after *in vitro* maturation (IVM) at 38.5°C for 0, 4, 6 or 24 h Groups that do not share a letter are significantly different ( $P < 0.0001$ ).

## Discussion

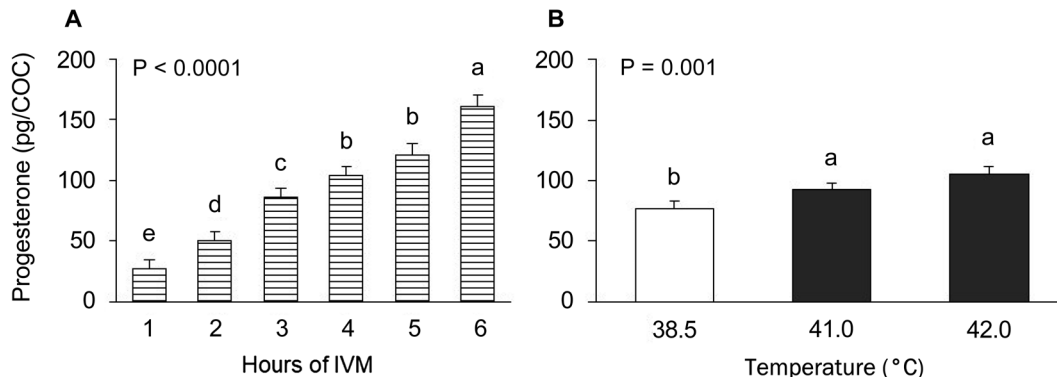
The findings presented herein identify novel cumulus cell components/pathways affected by heat stress exposure that may explain, in part, heat-induced hastening of the onset of meiotic progression reported in study one and previously by others. To this end, accelerated condensation of chromatin was coincident with heat-induced reductions in gap junction communication. Heat stress exposure during this early time period also reduced AMPK activity in the intact COC and heightened progesterone release into the maturation medium.

Intercellular communication through gap junctions is a key pathway by which the cumulus cells regulate oocyte meiotic arrest and resumption [13, 14]. In the current study, exposure to an elevated temperature during early maturation impaired gap junction communication, which was coincident with accelerated oocyte chromatin condensation. Heat-induced changes in chromatin status are likely the result of heat-induced changes in cumulus cell communication because treatment of COCs with the gap junction inhibitor carbenoxolone markedly reduced gap junction transfer (~90%) and also stimulated condensation of maternal chromatin. This is consistent with prior studies on bovine oocytes that demonstrated gap junction connectivity is highly correlated to chromatin status; efforts to uncouple gap junction channels with heptanol stimulated germinal vesicle breakdown [14, 15]. Although heat stress exposure for 4 h reduced gap junction transfer of calcein by approximately 50%, cGMP content was not impacted in oocytes or surrounding cumulus cells. In retrospect this finding is not surprising because Xi *et al.* [40] reported that treating bovine COCs with a different gap junction inhibitor (octanol) did not alter cGMP content in the oocyte or cumulus. This same study showed that the bovine oocyte may have an intrinsic ability to produce cGMP suggesting that, unlike what occurs in the mouse [16], transfer of cGMP from the cumulus may not be entirely required to maintain meiotic arrest.

Permeability of gap junctions in the COC is primarily regulated by ERK1/2-dependent phosphorylation in the mouse [18]. However, species-specific differences likely exist because despite having observed heat-induced reductions in gap junction transfer by approximately 50%, a corresponding change in active ERK1/2 in bovine cumulus cells was not detected in our second study. Heat



**Fig. 4.** Main effects of maturation time (4 or 6 h of *in vitro* maturation (IVM) averaged across temperatures; panel A) and maturation temperature (38.5, 40.0 or 41.0°C averaged across maturation times; panel B) on active AMPK levels in bovine cumulus-oocyte complexes. Groups that do not share a letter are significantly different.



**Fig. 5.** Main effects of maturation time (1 to 6 h of *in vitro* maturation (IVM) averaged across temperatures; panel A) and maturation temperature (38.5, 41.0 or 42.0°C averaged across maturation times; panel B) on progesterone released into conditioned medium from bovine cumulus-oocyte complexes. Groups that do not share a letter are significantly different.

stress exposure and carbenoxolone addition in our first study elicited similar oocyte phenotypes (i.e., reduced gap junction communication and earlier changes in chromatin) suggesting heat stress may be perturbing gap junction transfer and/or meiotic progression in a similar manner. The mechanism of action for glycyrrhetic acid derivatives, such as carbenoxolone, appears to be through the activation of protein kinases (e.g., ERK1/2, CamKII and/or PKA) which in turn may phosphorylate connexin subunits of gap junctions to decrease communication (reviewed by [41]). In other species and cell types, hyperthermia induces phosphorylation of connexin 43, however the protein kinase responsible for mediating heat stress effects differ depending on the cellular context [42–44]. Heat stress may be affecting gap junction communication through any number of kinases in the COC because connexins have multiple phosphorylation sites responding to numerous different kinases, including protein kinase A, protein kinase B, protein kinase C, and EGFR tyrosine kinase to name a few (reviewed by [45]).

Lack of an effect of heat stress exposure on ERK1/2 activation at the temperatures tested was initially surprising. Mild heat shock

exposure has been shown to activate ERK1/2 in other cell types [46] and premature activation of MAPK activity in bovine oocytes had been shown to accelerate germinal vesicle breakdown and reduce the time required to reach metaphase I [47]. Our initial efforts examining levels in the oocyte and their surrounding cumulus after denuding by vortex resulted in different values than those for an equivalent number of intact COCs (proportion of active ERK1/2 ranged from 23 to 25% in intact COCs, 8 to 13% in cumulus cells, and 0.4 to 0.9% in denuded oocytes). Thus, additional effort was taken to examine consequences of heat stress exposure in intact COCs. Regardless of whether COCs were intact or not, heat stress exposure in the manner applied in our studies had no detectable effect on ERK1/2.

Related to the intact COC, a heat stress exposure of 40°C during the first part of maturation in study two reduced levels of AMPK in COCs by 17%, whereas 41°C exposure reduced levels by 11%. Because heat stress mediates AMPK inhibition in other cell types [48] and a decline in AMPK activity has been associated with accelerated meiotic progression in bovine oocytes [21] it was tempting to speculate that heat-induced changes described herein may be contributing to

chromatin changes (study one and previously reported [8]). However, heat-induced hastening of chromatin changes described herein were only noted when maturation temperatures were  $\geq 41^\circ\text{C}$ . Furthermore, heat-induced reductions in AMPK were most prominent after maturation of COCs at  $40^\circ\text{C}$ ; effects after  $41^\circ\text{C}$  exposure were subtle.

Heat-induced increases in progesterone production during maturation have been described previously by our laboratory [38, 39]. Efforts of study three extend these findings and document an effect of heat stress exposure to increase progesterone production during the early period of maturation. Although consequences are not yet understood, bovine oocytes express genomic and non-genomic progesterone receptors [49]. Others have shown that supplementation of culture medium with progesterone stimulates germinal vesicle breakdown [25] and meiotic progression [24]. While species differences do exist, progesterone supplementation accelerated meiosis in porcine oocytes [50] and other non-mammalian species (reviewed in [51]). Therefore, we cannot preclude the possible role of progesterone in the heat-induced hastening of chromatin changes in the bovine oocyte after resumption of meiosis. In support of this, Shimada and Terada [22] reported that suppression of progesterone production in porcine COCs using aminoglutethimide was sufficient to almost entirely prevent germinal vesicle breakdown; inhibitory effects on germinal vesicle breakdown were overcome by additional progesterone.

Heightened release of progesterone into the medium by heat-stressed COCs is likely due to enhanced synthesis. Previous efforts of our laboratory noted elevated expression levels of CYP11A1 and HSD3B1 [38]. Heat-induced increases in progesterone production by the COC were likely not related to changes in ERK1/2 despite the reported role of ERK1/2 in progesterone synthesis [20]. Regardless of the underlying mechanism, elevated progesterone production by heat-stressed COCs document that cumulus cells are “luteinizing” earlier, which may be influencing dissociation of the gap junction connections [22]. Preliminary results from our laboratory indicate that carbenoxolone treatment effective for reducing gap junction communication by  $> 90\%$ , increased progesterone production by COCs ( $N = 2$  replicates;  $40.2$  vs.  $84.8$  and  $93.3$  vs.  $176.5$  pg progesterone/COC for control and carbenoxolone, respectively). This is suggestive that heat-induced alterations in the interconnected cumulus may precede or contribute to changes in progesterone production. Increased progesterone production is associated with reduced AMPK activity in bovine COCs [21] and both components are influential on meiotic resumption. Nonetheless, our findings highlight the progress of our studies towards identifying putative components problematic in the heat-stressed oocyte during the early maturation.

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