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## Responsiveness of bovine cumulus-oocyte-complexes (COC) to porcine and recombinant human FSH, and the effect of COC quality on gonadotropin receptor and Cx43 marker gene mRNAs during maturation in vitro

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### Abstract

Substantially less development to the blastocyst stage occurs in vitro than in vivo and this may be due to deficiencies in oocyte competence. Although a large proportion of bovine oocytes undergo spontaneous nuclear maturation, less is known about requirements for proper cytoplasmic maturation. Commonly, supraphysiological concentrations of FSH and LH are added to maturation media to improve cumulus expansion, fertilization and embryonic development. Therefore, various concentrations of porcine FSH (pFSH) and recombinant human FSH (rhFSH) were investigated for their effect on bovine cumulus expansion in vitro. Expression of FSHr, LHR and Cx43 mRNAs was determined in cumulus-oocyte complexes to determine whether they would be useful markers of oocyte competence. In serum-free media, only 1000 ng/ml pFSH induced marked cumulus expansion, but the effect of 100 ng/ml pFSH was amplified in the presence of 10% serum. In contrast, cumulus expansion occurred with 1 ng/ml rhFSH in the absence of serum. FSHr mRNA was highest at 0–6 h of maturation, then abundance decreased. Similarly, Cx43 mRNA expression was highest from 0–6 h but decreased by 24 h of maturation. However, the relative abundance of LHR mRNA did not change from 6–24 h of maturation. Decreased levels of FSHr, LHR and Cx43 mRNAs were detected in COCs of poorer quality. In conclusion, expansion of bovine cumulus occurred at low doses of rhFSH in serum-free media. In summary, FSHr, LHR and Cx43 mRNA abundance reflects COC quality and FSHr and Cx43 mRNA expression changes during in vitro maturation; these genes may be useful markers of oocyte developmental competence.

### Introduction

Generally, cumulus-oocyte complexes (COCs) are collected from 2–8 mm antral follicles from unstimulated bovine ovaries collected from an abattoir for in vitro

fertilization procedures. These follicles are several days from reaching pre-ovulatory size and may not have received sufficient exposure to hormones and growth factors in vivo to have the resulting accumulation of

maternal mRNAs to develop well in vitro. Recent evidence has shown that maturation condition (oocytes matured in vivo or in vitro) has a significant influence on the numbers of embryos developing to the blastocyst stage [1]. This suggests improvements in maturation media and protocols still could be made that would improve oocyte competence and developmental rate.

Although at least 80% of bovine oocytes collected from antral follicles undergo spontaneous nuclear maturation in culture [2], gonadotropins are often added to maturation media to induce cytoplasmic maturation, cumulus expansion and to improve embryonic development. Follicle stimulating hormone (FSH) induces expansion of mouse cumulus oocyte complexes in vitro [3] and improves bovine fertilization and cleavage rate [4]. Luteinizing hormone (LH) has beneficial effects on bovine oocyte maturation [5]. In addition, it has been reported that serum is required for hormonally induced cumulus expansion of COCs, although the percentage may be as low as 0.01–5% [3]. In most cases, supraphysiological hormone concentrations are added to in vitro maturation (IVM) media and it is not clear if these high concentrations are strictly required. Porcine FSH is usually added to IVM media at 0.5–1 µg/ml [2], but up to 10 µg/ml has been used, while reported bovine pre-ovulatory surge FSH concentrations average about 125 ng/ml [6]. Ovine LH is usually added to IVM media at 5 µg/ml [2], but the bovine pre-ovulatory LH surge averages about 200 ng/ml [6]. Recently, recombinant gonadotropins have become commercially available; these are very pure sources of hormone that can allow the individual roles of FSH and LH to be investigated without the hormone cross-contamination of pituitary, serum or urinary preparations.

For gonadotropins to act in vitro, FSH and LH receptor (FSHr and LHR) proteins and mRNAs must be expressed by the COC. Although FSHr mRNA and radiolabelled FSH binding have been detected in cumulus cells [7,8]; the presence of LHR protein and mRNA in cumulus is more controversial. LHR mRNA was not detected in cumulus cells by in situ hybridization [9] and was only detectable by RT-PCR in bovine follicular wall, and not in granulosa or cumulus cells [8]. However, radiolabelled LH and hCG binding is detectable in mouse and bovine COCs [7], and LHR protein was detected by immunocytochemistry in cumulus from mouse pre-ovulatory follicles [10]. Thus, the controversy about whether LHR mRNA and protein is expressed in cumulus-oocyte complexes could arise from the sensitivities of the assays used, the type of follicles from which the COCs are isolated, the hormonal environment and timing of the examination after isolation and culture of COCs.

The connexins are a gene family encoding transmembrane proteins that form gap junctions. Ions, second messengers and small molecules can be transmitted through gap junctions, which are important for cell-to-cell communication and coordinate responses [11]. Connexin-43 (Cx43) is a gap junction protein that is involved in follicular growth. The amount of Cx43 protein increases in granulosa of the bovine follicle from the pre-antral to antral stage [12]. Cx43 mRNA and protein is present in the bovine COC during in vitro maturation [13,14]. Although much of the protein may become inactivated during oocyte maturation [15], some gap junctions remain functional, as fluorescent dye transfer continues between corona radiata and oocyte following cumulus expansion [14,16]. Bovine oocyte maturation may rely on functional gap junctions, as gap junction blockade [14,17] or reduction of Cx43 mRNA and protein by an antisense approach [17] is associated with an inhibition of oocyte maturation. Thus, Cx43 may be an important mediator of positive oocyte maturation signals from cumulus to oocyte during maturation in vitro.

Therefore, the purpose of these experiments was to investigate whether supraphysiological concentrations of porcine and recombinant human FSH are required for bovine cumulus expansion and to determine whether serum was necessary for cumulus expansion. Because of their important roles in cell signaling and follicular growth, FSHr, LHR and Cx43 mRNAs may be important markers that could be used to predict oocyte competence in vitro. The relative abundance of FSHr, LHR and Cx43 mRNAs was examined in bovine COCs of differing qualities at 0, 6, 12, 18 and 24 h timepoints during in vitro maturation in a maturation media used in many bovine IVF laboratories.

## Materials and Methods

### **Bovine ovary collection and COC isolation**

Bovine COCs were isolated from abattoir ovaries provided by Department of Biomedical Sciences (University of Guelph, Guelph, ON) by follicular aspiration using an 18 gauge needle connected to a vacuum system. Serum-free TCM-199 (Gibco BRL, Burlington, ON), buffered with 10 mM HEPES and 26 mM bicarbonate, and containing 50 IU/ml heparin (Leo Pharma, Ajax, ON) was used for washing COCs. Pools of 50–60 COCs were matured in vitro in 0.5 ml TCM-199 containing 26 mM bicarbonate and 2.5 mM pyruvate in 4-well plates (NUNC, Denmark) at 38.5°C in a 5% CO<sub>2</sub> in a humidified air atmosphere.

### **Effect of pFSH and rhFSH on bovine cumulus expansion**

In many bovine IVM protocols, FSH is used to induce expansion of bovine cumulus and maturation of the oocyte prior to fertilization. Doses used are often supraphysiological. Therefore, a preliminary experiment was designed to examine the expansion of bovine COCs matured in

TCM-199 media with porcine FSH (pFSH, Folltropin-V®, Vetrepharm, Ottawa, ON, Canada) in doses ranging from 0–1000 ng/ml in the presence or absence of 10% serum (newborn calf serum, Gibco BRL). The positive control media (FLES) contained 1000 ng/ml FSH (porcine FSH, Follitropin-V, Vetrepharm), 5000 ng/ml LH (porcine LH, Lutropin-V, Vetrepharm) and 1000 ng/ml estradiol (estradiol-17β, Sigma-Aldrich, Oakville, ON, Canada) and 10% serum (newborn calf serum, Gibco BRL). As only minimal expansion of cumulus occurs with doses of pFSH under 1000 ng/ml in the absence of serum, a larger scale experiment was designed to compare cumulus expansion in: a) 100 ng/ml pFSH; b) 100 ng/ml pFSH plus 10% serum; c) 1000 ng/ml pFSH; or d) positive control, FLES. COCs were evaluated for cumulus expansion at 21–22 h of maturation as: I, little to no expansion; II, moderate expansion of the outer layers of the cumulus; and III, full expansion [18]. A preliminary experiment with recombinant human FSH (rhFSH) (Organon Canada, Scarborough, ON; 10 000 IU ≈ 1 mg) demonstrated that 1 ng/ml but not 0.1 ng/ml rhFSH caused substantial cumulus expansion in serum-free media. Therefore, a larger experiment was designed to compare cumulus expansion in serum-free media at 0, 1, 100 and 1000 ng/ml of rhFSH compared to pFSH. COCs evaluated as category II or III after 21–22 h of maturation were considered expanded.

**RNA isolation, reverse transcription, marker gene quantification**

For RNA studies, COCs were divided into three quality grades and cultured as described above: quality one COCs had even oocyte granulation and multiple layers of cumulus cells; quality grade two COCs had uneven granulation of the oocyte or only 1–2 layers of cumulus cells; while quality grade three COCs had a small oocyte, uneven oocyte granulation, cumulus already expanded, incomplete or absent [18]. Aliquots of COCs (50) of each quality grade were examined after 0 h or after 6, 12, 18 or 24 h of maturation in vitro in TCM-199 containing 26 mM bicarbonate and 2.5 mM pyruvate, and 1000 ng/ml pFSH, 5000 ng/ml pLH, 1000 ng/ml estradiol and 10% serum as in the positive control media above. COCs were placed into 0.5 ml microcentrifuge tubes and excess supernatant was removed after brief centrifugation. Samples were then frozen in liquid nitrogen before being stored in a -70°C freezer. The experiment was replicated four times.

Total RNA was extracted from 50 COCs with 12–24 µg E. coli rRNA (Roche Molecular Biochemicals, Laval, QC) as a carrier, by using a standard phenol/chloroform extraction method [19]. The RNA samples were quantified by measuring 260/280 nm OD absorbance using spectrophotometry. For analysis of marker gene expression, RNA samples representing 40 COC equivalents had 0.1 pg rabbit globin mRNA (Gibco BRL) added per COC equivalent as an exogenous internal standard and were reverse transcribed (RT) with oligo (dT)<sub>12–18</sub> primer (Gibco BRL) and SuperScript (SuperScript™ II RNase H-Reverse Transcriptase, Gibco BRL), as described previously [18,20]. RNA from positive control bovine tissues (liver for β-actin, ovary for FSHr, corpus luteum for LHr, brain for Cx43) was extracted using a standard laboratory phenol/chloroform technique [21] and 1–2.5 µg RNA was reverse transcribed as above. PCRs were carried out with 10 × PE Gold buffer (Perkin-Elmer, Canada Ltd., Mississauga, ON) with a final concentration of 1.25–2 mM MgCl<sub>2</sub>, 1 µM primers (2 µM for actin, 0.1 µM for Cx43), 200 µM dNTPs (Gibco-BRL) and 1 unit AmpliTaq Gold DNA Polymerase (Perkin-Elmer). Two microlitres of cDNA (2 COC equivalents) was added to each 50 µl PCR reaction; for positive control tissues, 1 µl of cDNA was added per tube. β-actin was examined in each sample after 37 cycles of amplification as a positive control to ensure that RNA extraction was efficient; as β-actin mRNA is known to vary during oocyte maturation, this mRNA was not quantified. A semi-quantitative method was used for marker gene mRNA analysis, in general, thirty-seven PCR cycles were used for FSHr and LHr, 39 cycles for Cx43 and 30 cycles for the α-globin standard. The basic program included a soak at 95°C for 10 min, followed by a cycle program of 95°C for 1 min, gene specific annealing temperature for 30 sec and 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR reactions were done in Perkin-Elmer GeneAmp 2400 thermocyclers (PE Applied Biosystems, Mississauga, ON). Twenty microlitres of product was resolved on a 2% agarose gel containing ethidium bromide and a Gene-Ruler 100 base pair (bp) DNA ladder (MBI Fermentas Inc., Flamborough, ON). Photographs were taken with a UV camera system (Amersham Pharmacia Biotech, Baie d'Urfé, QC). Image analysis and quantification was done with the ImageMaster VDS program (Amersham Pharmacia Biotech). Bands were quantified by comparing the ratio of band intensity to the α-globin standard [18,20].

**Table 1: Primers for FSH and LH receptors and Connexin-43.**

	5' (5'-3')	3' (5'-3')	Primer binding temp. (°C)	Product size (bp)
FSHr	CAACCTGCTATACATCGACC	GAGCAAGTCACATCAACCAC	53	723 537
LHr	AGAGTGAAGTGGCTGG	CAACACGGCAATGAGAGTAG	53	533
Cx43	TGGCTGCTCCTACCAACG/CGC	AGGTCATCAGGCCGAGGT/CCTG	58	334

**Table 2: The effect of varying concentrations of pFSH with and without serum, and other hormones on cumulus expansion.**

Hormone (ng/ml)	Percentage of Expanded COCs (%) <sup>1</sup>
100 pFSH	9.1 ± 3.3 <sup>a</sup>
100 pFSH + 10% serum	55.3 ± 11.1 <sup>ab</sup>
1000 pFSH	72.6 ± 10.8 <sup>b</sup>
1000 pFSH, 5000 pLH, 1000 estradiol and 10% serum (FLES)	90.3 ± 2.5 <sup>b</sup>

<sup>1</sup>COCs that were evaluated as Category II and III at 21–22 h of maturation were considered expanded. Data are Means ± SEM. <sup>a,b</sup> denotes differences between treatments, P < 0.05. Data are from eight replicates with 750–800 COCs per treatment.

**Table 3: Effect of recombinant human FSH (rhFSH) and porcine FSH (pFSH) on cumulus expansion.**

Hormone (ng/ml)	Percentage of Expanded COCs (%) <sup>1</sup>
0	3.12 ± 0.80 <sup>a</sup>
1 rhFSH	87.26 ± 2.39 <sup>b,mx</sup>
100 rhFSH	92.12 ± 1.44 <sup>b,mx</sup>
1000 rhFSH	78.65 ± 9.90 <sup>b,mx</sup>
1 pFSH	2.54 ± 0.72 <sup>a,ly</sup>
100 pFSH	5.48 ± 1.08 <sup>a,ly</sup>
1000 pFSH	76.40 ± 4.69 <sup>b,my</sup>

<sup>1</sup>COCs that were evaluated as Category II and III at 21–22 h of maturation were considered expanded. Data are Means ± SEM. <sup>a,b</sup> denotes differences between the hormone and dose groups versus the negative control (0) in a one-way ANOVA, P < 0.05. Two-way ranked ANOVA was used to test the effect of source of hormone, dose and interaction. <sup>l,m</sup> within hormone, values with different superscripts are different, P < 0.05. <sup>x,y</sup> within dose, values with different superscripts are different, P < 0.05. Data are from eight replicates with approximately 550 COCs per treatment.

The  $\beta$ -actin primers were as reported previously [20]. These primers amplify a 243 bp product at an annealing temperature of 56°C and, because they are intron spanning, were used to detect possible genomic contamination (408 bp product). The  $\alpha$ -globin primers were as reported previously [20], and amplified a product of 257 bp at 55°C. The FSHr primers (Table 1) were identical to those used in an earlier study [8], and amplified exons 4–10 to produce products of 723 bp and 537 bp at 53°C. LHR primers (Table 1) were designed by comparison of bovine sequence (Genbank accession U20504), and amplified a portion of exon 11, resulting in a single product of 533 bp when amplified at 53°C. Cx43 primers (Table 1) were a gift from Dr. G. Kidder, designed to work with porcine and bovine Cx43 (data unpublished) and amplified a 334 bp product at 58°C. All primers were made by Gibco BRL. The identity of FSHr, LHR and Cx43 products was confirmed by dye-deoxysequencing (Robarts Research Institute, London, ON); our sequences were 98–99% identical to reported bovine FSHr and LHR sequences. The Cx43 product from bovine COCs was 98% identical to the published bovine sequence (J05535, Genbank); the only discrepancies were in the region of the primers.

Throughout these studies, there were difficulties in consistently obtaining RT-PCR products from timepoint 0

COCs. Inhibition of transcription was often seen even for the rabbit globin RNA that was added just prior to reverse transcription. COCs for timepoint 0 were recovered directly from wash dishes containing 50 IU/ml heparin, while COCs at other timepoints were recovered from maturation medium that is free of heparin. Heparin has been reported recently to inhibit RT-PCR [22], and to interfere with both the RT and PCR reactions [23]. Heparin is not adequately removed by phenol-chloroform extraction [22]. Thus, residual heparin contamination may affect the efficiency of RT-PCR of COCs. Whether sequence, primer, or type of Taq polymerase affects the ability to amplify certain cDNAs is not known. Because it was determined that washing time 0 COCs in heparin-free media affected the abundance of actin and FSHr mRNAs, the abundance of all marker genes was re-examined at 0 h and 6 h of maturation in the three COC quality grades after washing twice in heparin-free wash medium. In some cases, the number of cycles was adjusted to provide greater resolution of signal.

#### Statistical Analyses

The Sigma Stat program (Version 2, 1992–1997, SPSS Inc.) was used for analysis of cumulus expansion. For these studies, category II and III COCs were considered expanded. For both experiments, data were not normal and

could not be resolved by transformation. Therefore, Kruskal-Wallis analysis of variance on ranks tests were performed. In the pFSH experiment, Tukey's mean separation test was used to detect differences due to treatment. For the rhFSH and pFSH experiment, the data were compared two ways. A one-way ANOVA was done to compare all treatments to the negative control (0). Dunnett's mean separation test was used to detect differences. A two-way ANOVA on ranks was used to compare the data in a  $2 \times 3$  factorial comparing the effects of source of hormone, dose and interaction. Tukey's test was used for mean separation.

For the gene expression data, multiple ANOVA was performed using the Practical Statistics program (Canadian Academic Technology Inc., West Flamborough, ON), with main effects of quality grade and time of maturation. Where there were significant main effects or interactions, Duncan's mean separation procedures were performed to detect differences.

## Results

### **Cumulus expansion with pFSH and rhFSH**

The first experiment examined expansion of bovine COCs matured with pFSH in the presence or absence of serum (Table 2). The positive control media contained 1000 ng/ml FSH, 5000 ng/ml LH and 1000 ng/ml estradiol-17 $\beta$ . Overall, there was a significant effect of maturation media, ( $P < 0.001$ ). Percentage cumulus expansion (category II and III at 21–22 h of maturation) was lower in the 100 ng/ml pFSH treatment than all other treatments, including the 100 ng/ml pFSH with 10% serum treatment ( $P < 0.05$ ). Expansion in 100 ng/ml pFSH and 10% serum was lower than in the 1000 ng/ml and positive control groups but the difference was not significant ( $P > 0.05$ ). Percentage expansion in 1000 ng/ml pFSH and positive control groups were similar. There were no differences in cleavage rates or development to the blastocyst stage (data not shown).

A preliminary experiment (data not shown) suggested that rhFSH induced cumulus expansion at 1 ng/ml. Therefore, cumulus expansion was compared in COCs matured at doses of 1, 100 and 1000 ng/ml rhFSH or pFSH relative to the negative control (0), COCs evaluated as category II or III at 21–22 h of maturation were considered expanded. After testing each treatment against the negative control by one-way analysis, there was a significant effect due to treatment, ( $P < 0.001$ ). All doses of rhFSH tested (1, 100 and 1000 ng/ml) induced significantly higher expansion of bovine COCs than 0 ng/ml, whereas pFSH only induced significant expansion at 1000 ng/ml (all  $P < 0.05$ , Table 3). When data were compared by two-way analysis on ranks, source of FSH, dose, and source by dose interaction were all significant (all  $P < 0.002$ ). Within rhFSH,

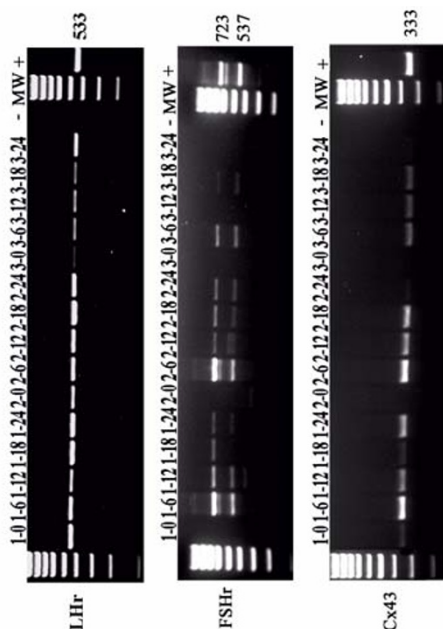
percent expansion among doses was not different, whereas within pFSH, 1000 ng/ml showed greater cumulus expansion than either 1 ng/ml or 100 ng/ml pFSH ( $P < 0.05$ ). Within each dose, rhFSH resulted in greater cumulus expansion than pFSH.

### **Marker gene expression from 0–24 h of maturation in medium containing FSH, LH, estradiol and serum**

Representative pictures of PCR-amplified products of three quality grades of oocytes at 0, 6, 12, 18 and 24 h are shown in Figure 1. Similar to observations reported previously [8], two isoforms of the FSHr mRNA were detected; a full-length isoform of 723 bp and a shorter 537 bp isoform that lacks exon 9, with the identity of both confirmed by sequencing. In Figure 2A, the abundance of the full-length FSHr mRNA in bovine COCs is shown from 6–24 h. For this isoform, there was an interaction between time of maturation and COC quality ( $P < 0.02$ ), such that abundance was higher in quality grade 1 and 2 COCs at 6 h of maturation than in quality grade 3 COCs and all other timepoints (all  $P < 0.01$ ). Because of earlier difficulties with heparin contamination of time 0 COCs, abundance of both isoforms of FSHr, LHr and Cx43 mRNAs was re-examined in fresh pools of COCs at 0 and 6 h of maturation after washing in heparin-free medium. For the full-length FSHr isoform, mRNA abundance was similar at 0 and 6 h, there was only an overall effect due to COC quality ( $P < 0.002$ , Figure 2B), abundance was higher in quality grade 1 and 2 COCs than in quality grade 3 COCs (both  $P < 0.01$ ).

In Figure 3A, abundance of the shorter FSHr mRNA isoform is shown. The abundance of this mRNA was affected only by maturation time ( $P < 0.003$ ). The highest abundance of the shorter FSHr mRNA was observed at 6 h of maturation ( $P < 0.01$  versus timepoints 18 h and 24 h). Figure 3B shows abundance of the shorter isoform from 0 to 6 h in COCs washed in heparin-free medium. There was an effect of time ( $P < 0.001$ ); the shorter FSHr isoform mRNA was higher at 0 h than 6 h of maturation. There was a tendency ( $P < 0.053$ ) towards an effect of COC quality.

LHr mRNA abundance was affected by quality grade ( $P < 0.01$ , Figure 4A) and time of maturation ( $P < 0.04$ ), but there was no interaction. Abundance of LHr mRNA was significantly higher in quality grade 1 COCs than in quality grade 3 COCs, ( $P < 0.01$ ), whereas the abundance in quality grade 2 COCs was intermediate. When data for grades were pooled, the effect of time only approached significance ( $P < 0.06$ ). Abundance of LHr mRNA tended to be higher at 24 h than from 6 to 12 h. When LHr mRNA abundance was compared in COCs at 0 and 6 h timepoints after washing in heparin-free medium, there were no differences in abundance due to time or grade (Figure 4B).



**Figure 1**  
 Representative RT-PCR figures with gene on the left, PCR product size in bp on right. The leftmost lane is the 100-bp DNA marker lane (M) followed by oocyte quality grade 1, hour 0; quality grade 1, hour 6, etc. The negative lane (-ve) is the PCR blank which contains all components except cDNA. A second marker lane and positive tissue control (+ve) lane follow.

Overall, Cx43 mRNA abundance was affected by quality grade ( $P < 0.01$ , Figure 5A) and time of maturation ( $P < 0.01$ ), but there was no interaction. Cx43 mRNA abundance was significantly higher in quality grade 1 and 2 COCs than in quality grade 3 COCs, ( $P < 0.01$  and  $P < 0.05$ , respectively). Abundance was significantly higher at 6 h than 18 and 24 h (both  $P < 0.01$ ), whereas the 12 h value was intermediate and not different from any other timepoint. When Cx43 mRNA abundance was compared in COCs at 0 and 6 h timepoints after washing in heparin-free medium, there was no effect of grade or maturation time on abundance of Cx43 mRNA (Figure 5B).

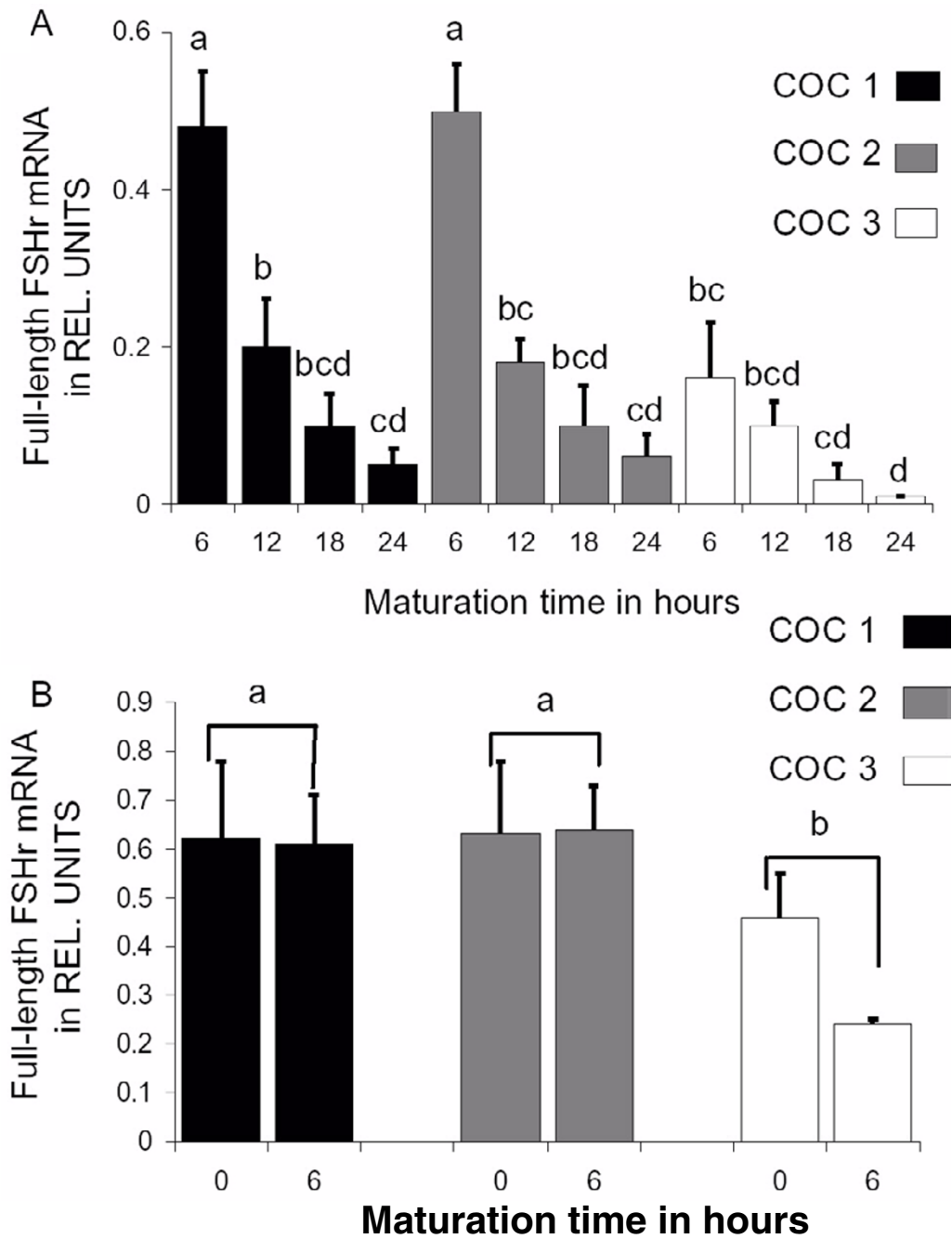
**Discussion**

In the present experiment, bovine cumulus expansion was obtained with 1000 ng/ml pFSH in serum-free TCM-199

media. Although not significant, expansion in TCM with 100 ng/ml pFSH and 10% FCS tended to be greater than in 100 ng/ml pFSH alone. Earlier studies suggested that FSH-dependent cumulus expansion occurred only when serum was present in the culture media [3] and the effects of FSH and serum were additive over FSH or serum alone [24]. However, older studies may have been done with sub-optimal culture media. The presence of glutamine and glucose or glucosamine in culture media (as precursors for hyaluronic acid synthesis) enhances FSH-induced cumulus expansion [24]. TCM-199, commonly used for bovine IVF, contains both glutamine and glucose. Therefore, in the absence of serum, adequate concentrations of glucose and glutamine may be necessary for FSH to induce cumulus expansion in vitro.

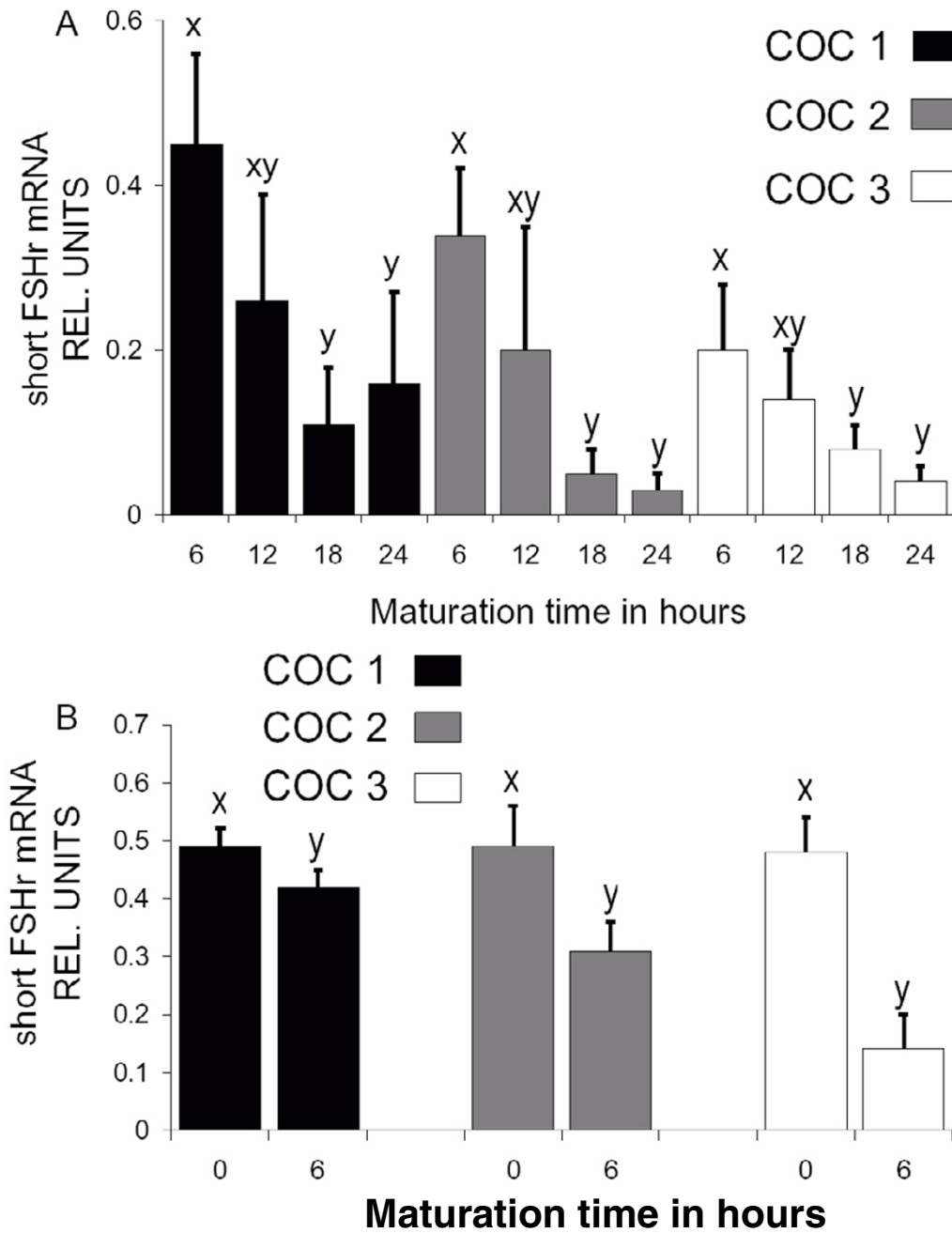
Cumulus expansion was induced with only 1 ng/ml rhFSH in serum-free media, which is in contrast to the high concentration pFSH required for cumulus expansion. These results agree with recent studies reporting low rhFSH concentrations (0.01–1 IU/ml (1–100 ng/ml)) are effective for in vitro maturation in several species [4,25,26]. It is not clear why bovine cumulus expands at lower doses of rhFSH compared to pFSH. Recombinant human FSH has been shown to be more effective than urinary human FSH for superovulation of women [27], and it was suggested that its more basic isoforms and lesser degradation (4% vs. 40%) may be responsible for its increased effectiveness. Both rat and human FSH are reported to bind well to the human FSHr but ovine FSH binds poorly [28]; whereas the rat FSHr was reported to generate cAMP and testosterone more efficiently with recombinant human FSH than with recombinant rat FSH [29]. However, only one published study has compared the binding efficiency of FSH of various species with the bovine FSHr. In that study, rhFSH bound better to calf testis than rat FSH [30]. It is possible that porcine FSH does not efficiently bind the bovine FSHr, which could explain why high concentrations are required to initiate bovine cumulus expansion. Nonetheless, the majority of bovine superovulation protocols utilize pFSH for ovarian stimulation. While cumulus expansion is not necessary to achieve nuclear maturation, expansion of the cumulus may be an outward sign of favorable cytoplasmic maturation. In vivo, cumulus expansion is necessary to achieve ovulation and fertilization. However, during bovine IVF, when several thousand sperm are added for each egg, there may be sufficient sperm to fertilize oocytes with unexpanded cumulus, resulting in unaffected developmental rates.

Multiple FSHr transcript sizes are detected by Northern analysis in human testis [31], rat granulosa [32] and bovine ovary [33]. Multiple transcripts may be a result of alternative splicing and the use of different polyadenylation sites. Primers used to amplify FSHr from bovine cu-



**Figure 2**

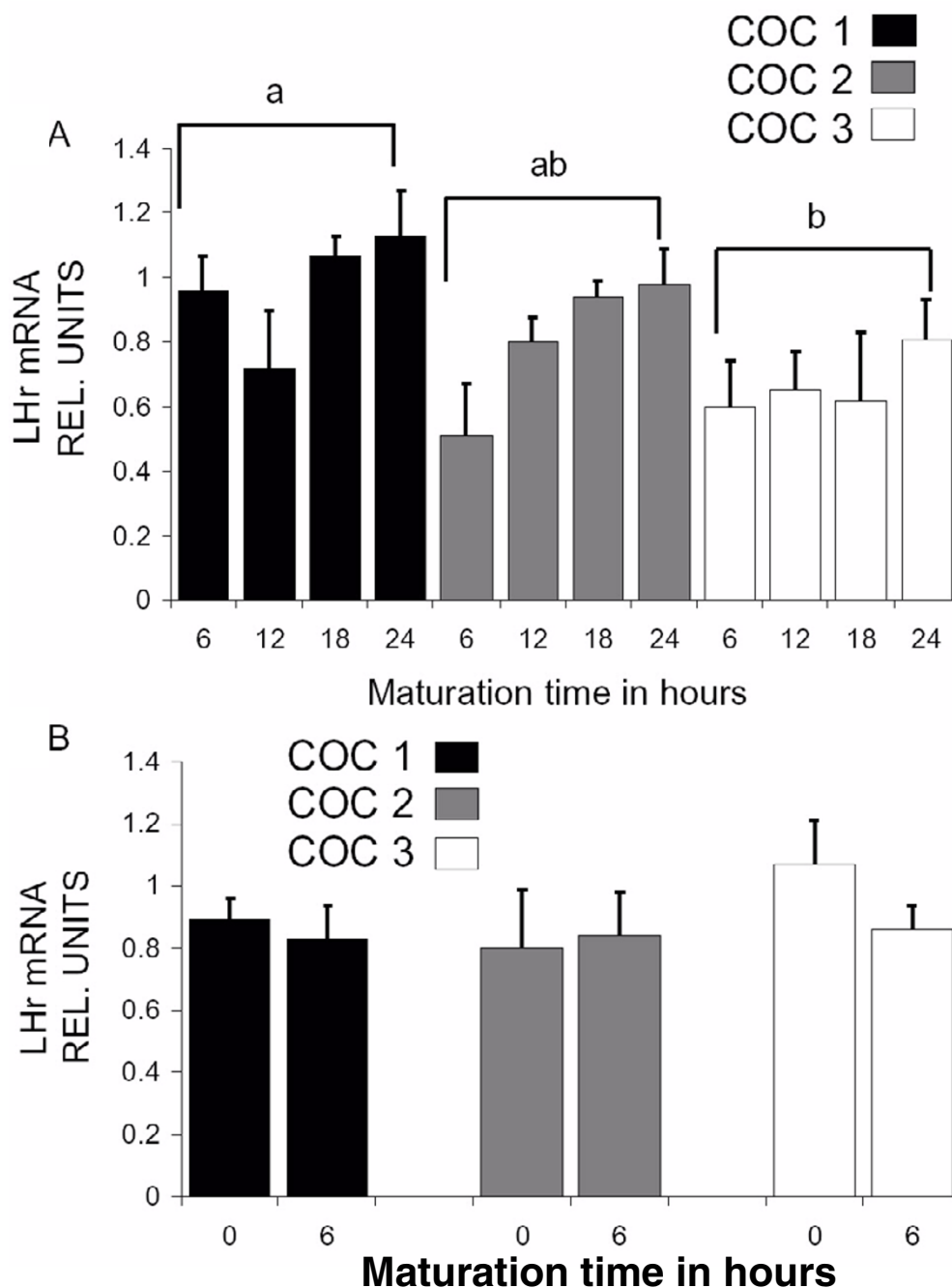
Figure 2A. Expression of full-length FSHr mRNA in three COC quality grades at 6, 12, 18 and 24 h of maturation. Mean  $\pm$  standard error (n = 4 replicates). There was a significant interaction between quality and time (P < 0.02). <sup>abcd</sup> Bars with no superscripts in common are different at P < 0.05. Figure 2B. Expression of full-length FSHr mRNA in three quality grades of COCs washed in heparin-free media at 0 h and 6 h of maturation. Mean  $\pm$  standard error (n = 4 replicates). <sup>ab</sup> COC qualities with no superscripts in common are different at P < 0.05.



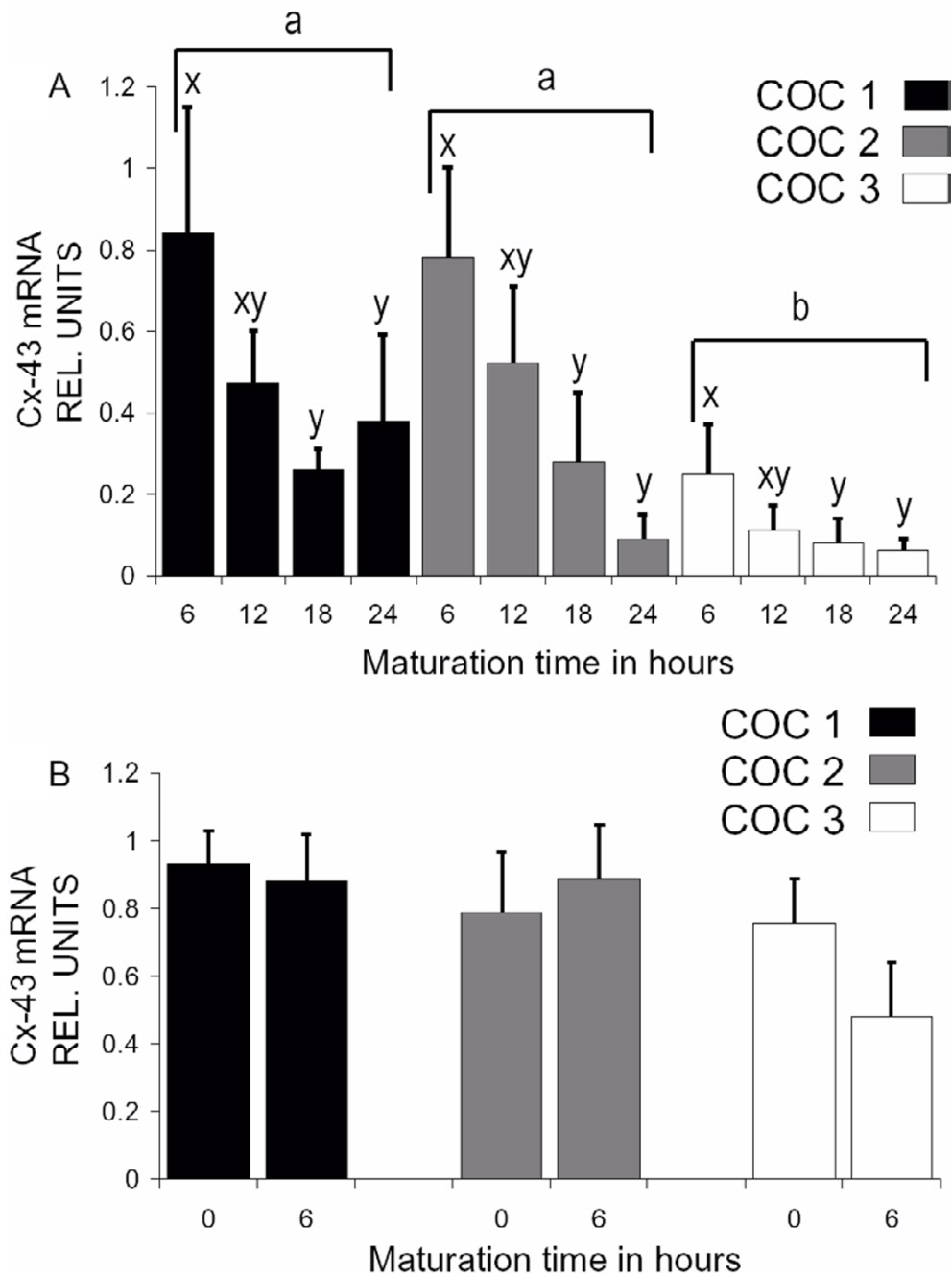
**Figure 3**

Figure 3A. Expression of exon-9 deleted FSHr mRNA in three COC quality grades at 6, 12, 18 and 24 h of maturation. Mean  $\pm$  standard error (n = 4 replicates). <sup>xy</sup> Time-points with no superscripts in common are different at P < 0.05. Figure 3B. Expression of exon-9 deleted FSHr mRNA in three quality grades of COCs washed in heparin-free media at 0 h and 6 h of maturation. Mean  $\pm$  standard error (n = 4 replicates). <sup>xy</sup> Time-points with no superscripts in common are different at P < 0.05.





**Figure 4**  
 Figure 4A. Expression of LHR mRNA in three COC quality grades at 6, 12, 18 and 24 h of maturation. Mean  $\pm$  standard error (n = 4 replicates). <sup>ab</sup>COC quality grades with no superscripts in common are different, at least P < 0.05. Figure 4B. Expression of LHR mRNA in three quality grades of COCs washed in heparin-free media at 0 h and 6 h of maturation. Mean  $\pm$  standard error (n = 4 replicates).



**Figure 5**

Figure 5A. Expression of Cx43 mRNA in three COC quality grades at 6, 12, 18 and 24 h of maturation. Mean  $\pm$  standard error (n = 4 replicates). <sup>ab</sup> COC quality grades with no superscripts in common are different, at least P < 0.05. <sup>xy</sup> Time-points with no superscripts in common are different, at least P < 0.05. Figure 5B. Expression of Cx43 mRNA in three quality grades of COCs washed in heparin-free media at 0 and 6 h of maturation. Mean  $\pm$  standard error (n = 4 replicates).

mulus-oocyte complexes were identical to those used by van Tol et al., [8] and produced the same two main product sizes, although others were detectable. A common mammalian FSHr splice isoform is missing exon 9 and this isoform was detected here and in other bovine studies [8,34].

The abundance of both FSHr mRNA isoforms decreased in COCs after 6 h of maturation in IVM medium containing gonadotropins, estradiol and serum. This is consistent with the downregulation of FSHr mRNA and FSH binding in rat granulosa after a pre-ovulatory gonadotropin stimulus [32], decreased FSH binding after culture of mouse COCs in media containing serum [7], and decreased FSHr mRNA in bovine granulosa after culture with FSH [33]. Abundance of high FSHr mRNA isoform, in particular, was higher in better quality COCs. The role of the exon 9-deleted FSHr isoform in FSH binding and signaling is unclear; other isoforms missing exon 2, 5 or 6 either do not bind FSH, or do not interfere with activities of the full-length receptor in cotransfection studies [35,36].

Several LHR transcript sizes have been detected in mammals, which may result from different transcription start sites, alternative splicing and different polyadenylation signals. Although LHR mRNA was recently detected by RT-PCR only in the wall of bovine follicles, and not in granulosa or cumulus [8]; there were two mismatches in the primers derived from porcine sequence that may have affected binding and amplification efficiency of those primer sets. In the current studies, LHR primers consistently amplified a product of 533 bp from bovine cumulus and corpus luteum. These LHR primers were located in exon 11 and thus would detect only mRNAs encoding isoforms with a complete transmembrane domain, and not other truncated isoforms reported in ruminants [37,38]. However, these primers would not distinguish the F isoform from the full-length mRNA. The F isoform, which differs from the full-length isoform in the absence of exon 10 (27aa), is the only LHR mRNA expressed in monkey testis, yet binds hCG and generates second messengers similarly to the full-length human LH receptor [39]. In bovine corpora lutea, the full-length isoform LHR mRNA is expressed at approximately a 2–3:1 ratio to the F isoform [37,38], and expression of all isoform mRNAs appears to be regulated coordinately in bovine corpora lutea throughout the estrous cycle [38]. Because  $\beta$ -actin and other primer sets amplify across intron-exon borders and yet larger product sizes were not detected, and because expression of LHR mRNA tended to increase during the culture period, the detection of LHR mRNA in these experiments was not likely a result of genomic DNA contamination. Thus, the design of primers and probes, as well as which isoforms are expressed by a tissue, may also

lead to the discrepancies in the ability to detect the LHR mRNA in various studies.

There was an effect of COC quality grade on abundance of LHR mRNA, as better quality COCs had greater LHR mRNA levels. There was a trend towards increased LHR mRNA abundance from 6 h to 24 h coincident with advancing maturation. The increase in LHR mRNA expression in cultured bovine COCs is corroborated by an increase in LH binding after culture of mouse COCs with FSH [7]. Advancing nuclear maturation of oocytes may allow increased LHR mRNA expression in cumulus, as, although germinal vesicle stage mouse oocytes suppress granulosa LHR mRNA expression, mature oocytes are much less effective [40]. It is likely that the increased abundance of LHR mRNA during maturation reflects increased luteinization of the cumulus cells.

Abundance of Cx43 mRNA was significantly lower in poorer quality COCs compared to better quality COCs. Differential expression of the Cx43 mRNA among varying COC classes indicates that this gene product may be a useful marker of oocyte competence. Reduced Cx43 mRNA could be inherent to the lower quality of these COCs, as when Cx43 mRNA is decreased in antisense experiments; Cx43 protein decreased, cumulus-oocyte dye transfer was lower and oocyte maturation rate was reduced [17]. Cx43 mRNA abundance also decreased in COCs from 6 h to 18–24 h of maturation. This agrees with decreased Cx43 protein observed in the outer cumulus layers of COCs when matured for 12 h [41].

Marker genes that predict developmental competence could be used in the design of more appropriate maturation and culture media and selection of better oocytes for culture and embryos for transfer. This study indicates that levels of mRNAs encoding FSHr, LHR and Cx43 mRNAs are dependent on time of maturation and oocyte quality. These data may indicate differences in gonadotropin signaling among varying COC qualities that could reflect variation in their developmental competence.

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