

# Expression of melanocortin receptors *mRNA*, and direct effects of ACTH on steroid secretion in the bovine ovary

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## Abstract

Melanocortin receptors (MCRs) are involved in physiological responses to ACTH, as well as to  $\alpha$ -,  $\beta$ - and  $\gamma$ -melanocyte-stimulating hormone ( $\alpha$ -,  $\beta$ - and  $\gamma$ -MSH). Their expression has previously been analyzed in various bovine tissues; however, there are apparently no reports regarding their localization in the ovaries. In the present study, the expression of MCR mRNA in various bovine ovarian structures was characterized with reverse transcription polymerase chain reaction (RT-PCR). Furthermore, whether ACTH affected follicular components by affecting steroid secretion in fragments of ovarian follicular wall of medium and large antral follicles cultured in serum free medium with 1, 10, and 100 nM ACTH, was also determined. *Melanocortin receptors* mRNA was localized in the theca cells of various follicular stages, whereas only *MC3R* mRNA was weakly evident in granulosa cells. *Melanocortin receptors* 1, 2, and 3 mRNA were present in the CL, whereas in stroma, only *MC2R* mRNA was expressed. There were significant increases in estradiol and cortisol concentrations in response to ACTH in medium follicles, as well as increased concentrations of testosterone and cortisol in large follicles. These results confirmed earlier reports in other species, and demonstrated that MCRs were present in bovine ovaries. Since ACTH induced steroid secretion from the ovary *in vitro*, we inferred that melanocortin peptides could be involved in regulatory mechanisms related to ovarian functions, e.g. ovulation, steroidogenesis, and luteal function.

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**Keywords:** Ovary; POMC; ACTH; Melanocortin; Cattle

## 1. Introduction

Adrenocorticotrophic hormone (ACTH) and  $\alpha$ -,  $\beta$ - and  $\gamma$ -melanocyte-stimulating hormone ( $\alpha$ -,  $\beta$ - and  $\gamma$ -MSH) are derived from the post-translational processing of the precursor molecule proopiomelanocortin

(POMC) [1]. These POMC products are collectively called melanocortin peptides or melanocortins. The principal source of melanocortins is the pituitary gland, but the POMC gene is also expressed in a variety of other brain regions, as well as a number of peripheral tissues, in particular skin [2].

Recognition and cloning of melanocortin receptors (MCRs) has greatly improved the understanding of peptide-target cell interactions. To date, five MCRs (termed MC1R to MC5R) have been cloned. Express-

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sion of *MC2R* mRNA was reported in the ovaries of fish [3,4] and mice [5], and *MC5R* was identified in human ovaries [6]. Furthermore, expression of MCRs has been analyzed in various bovine tissues [7–15]. However, there are apparently no reports regarding expression of specific *MCR* mRNA or its localization in ovaries. It should, however, be considered that the interactions between melanocortins and reproduction have been clearly established, associating them with various processes related to metabolism, stress, immunity, and neuroendocrine signalling pathways [2,16].

Stress-associated stimulation of the hypothalamic-pituitary-adrenal/ovary axis disrupted reproductive function [17]. Poor ovarian follicular development, reduced ovulation rates, and ovarian cyst formation were reported following ACTH treatment and resulting increases in glucocorticoid concentrations [18–21]. Also, high glucocorticoid concentrations during the late follicular phase of the estrous cycle can disrupt gonadotropin release [22,23].

Although these studies indicated an effect of glucocorticoids at the cellular level within the ovary, little research has been performed to understand the direct effects of ACTH on ovarian function, although there could be receptors for this hormone within the ovary of some species. Therefore, the objective of the current study was to examine the expression of *MCR* mRNA in bovine ovaries using reverse transcription polymerase chain reaction (RT-PCR). We postulated that these receptors, differentially expressed in ovarian components, could mediate the actions of melanocortins directly affecting follicular development and ovarian function. To confirm this, we analyzed *in vitro* the direct effects of ACTH on constitutive follicular wall steroidogenesis.

## 2. Materials and methods

### 2.1. Collection and preparation of tissues

Ovaries from 66 randomly cycling cattle with apparently normal follicular activity were collected at a local abattoir, within 20 min after death. These ovaries were from mixed breeds of *Bos taurus* cows, assessed visually as non-pregnant and with no apparent abnormality in the reproductive system. Intact ovaries were washed in sterile PBS, refrigerated, and transported immediately to the laboratory.

Each batch of ovaries was placed on ice, and antral follicles were removed using dissecting scissors. Prior to dissection of the ovaries, follicle diameter was measured with calipers, and follicular fluid from each fol-

licle was aspirated and stored separately at  $-20\text{ }^{\circ}\text{C}$  pending determination of progesterone and estradiol concentrations. Follicles with an obviously atretic appearance (debris in the antrum) were discarded. Large antral follicles were obtained only from ovaries without a visible, active CL.

For RT-PCR, follicles were classified into three categories according to their calculated diameters, as described previously [24]: small ( $<5\text{ mm}$ ,  $n = 15$ ), medium ( $5\text{--}10\text{ mm}$ ,  $n = 15$ ) or large ( $>10\text{ mm}$ ,  $n = 15$ ). To avoid cross contamination and damage due to manipulation, only one follicular structure was obtained from each ovary. Follicles were hemisected in PBS, and granulosa cells (GCs) were gently scraped into tubes containing approximately 20 mL of sterile PBS. The cell suspension was centrifuged at  $400 \times g$  for 10 min, the supernatant discarded, and the GC pellets resuspended in Trizol LS reagent (Invitrogen, Carlsbad CA, USA). Follicular walls were further washed several times with PBS to remove residual GC. The surrounding stroma was also removed from follicular walls and regarded as theca tissues [25]. Samples of CL ( $n = 5$ ) were also obtained from cattle in the luteal phase. All samples were snap-frozen in liquid nitrogen and stored at  $-80\text{ }^{\circ}\text{C}$  pending total RNA extraction. The adrenal cortex, liver, spleen and spinal cord tissue and white blood cells of a non-pregnant cow, which served as both positive and negative controls (Table 1), were obtained and conserved in the same manner.

### 2.2. Total RNA extraction

Total RNA was isolated from the samples after treatment with Trizol LS reagent (Invitrogen), according to the manufacturer's instructions with slight modifications. Briefly, 50 to 100 mg of tissue was homogenized with 750  $\mu\text{L}$  of Trizol reagent (Invitrogen) and incubated for 10 min at  $25\text{ }^{\circ}\text{C}$ . Then, RNA was purified by vigorously homogenizing with chloroform and incubating for 15 min at  $4\text{ }^{\circ}\text{C}$ . After centrifugation at  $12,000 \times g$ , the aqueous phase was incubated with an equal

Table 1  
Positive (+) and negative (–) controls used to analyze mRNA expression of melanocortin receptors in bovine tissues.

	MC1R	MC2R	MC3R	MC4R	MC5R
Liver	–	–	+	–	+
Spleen	–	–	+	–	–
Spinal cord	+	+	+	+	+
White blood cells	+	+	+	+	+
Adrenal cortex	+	+	+	+	+

For review see [1,30].

volume of isopropanol for 60 min at  $-80^{\circ}\text{C}$  and centrifuged at  $12,000 \times g$  to obtain the pellet of mRNA that was then washed with 75% ethanol for 10 min at  $4^{\circ}\text{C}$ . Alcohol was replaced by DEPC-water pre-warmed at 55 to  $60^{\circ}\text{C}$ . The extracted RNA was DNase treated with deoxyribonuclease I (amplification grade; Invitrogen) to eliminate contaminating DNA, assessed for quality and quantity using a fluoroscopic method (Qubit, Invitrogen), aliquoted, and stored at  $-80^{\circ}\text{C}$  pending further use.

### 2.3. PCR primer design

#### 2.3.1. Melanocortin receptors specific primers

Bovine sequences for MC1R, MC2R, MC3R, MC4R and MC5R were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/Entrez/index.htm>). Specific primers were designed to obtain PCR amplification products between 400 and 500 bp, spanning two mRNA-splicing sites, using the PrimerSelect program in the LASERGENE software (DNASar, Madison, WI, USA). The primers were purchased from Invitrogen, and the sequences are summarized (Table 2). Oligonucleotide primers and amplification products were tested using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) software to confirm gene specificity and determine exon locations,

assuring that they were not designed from any homologous regions coding for other genes.

#### 2.3.2. Other primers

The primer sequences for cytochrome P450 aromatase (*CYP19a1*) and cytochrome P450 17 $\alpha$ hydroxylase/17,20-lyase (*CYP17a1*) were designed following the same rules as MCR primers, and validated to confirm bovine granulosa and theca cell mRNA purity (no cross-contamination) [26]. Primers for housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously [27].

### 2.4. RT-PCR

The first strand of cDNA was synthesized in a  $20 \mu\text{L}$  reaction mixture using  $1 \mu\text{g}$  of total RNA. The reaction tubes contained  $0.25 \mu\text{L}$  random hexamers ( $1 \mu\text{g}/\mu\text{L}$ ),  $0.4 \mu\text{L}$  dNTPs (25 mM),  $4 \mu\text{L}$   $5 \times$  reaction buffer,  $2 \mu\text{L}$  DTT (0.1 M),  $0.5 \mu\text{L}$  ribonuclease inhibitor (40 U/ $\mu\text{L}$ ), and  $1 \mu\text{L}$  M-MLV reverse transcriptase (200 U/ $\mu\text{L}$ ); all reactives were from Invitrogen). Reactions were incubated at  $25^{\circ}\text{C}$  for 10 min,  $37^{\circ}\text{C}$  for 50 min, and inactivated for 15 min at  $70^{\circ}\text{C}$ .

Each PCR assay was optimized with regard to annealing temperature and magnesium ( $\text{MgCl}_2$ ) concen-

Table 2  
Primer sequences, regions of the target genes and conditions used for semi-quantitative RT-PCR of bovine tissues.

Name	Sequence (5'–3')	Gene accession no.	Amplicon position	Amplicon size (bp)	Annealing temperature ( $^{\circ}\text{C}$ )	No. cycles
<i>MC1R</i>						
Forward	CAGCCTGCTCTTCATCACCTACTA	NM_174108	808–1229	422 bp	58.8	30
Reverse	AGCACTGCAGCACCTCTTG					
<i>MC2R</i>						
Forward	GGCATTACCATCGTGACCTTC	NM_174109	609–1057	449 bp	57.2	30
Reverse	TATATCCCTGTGGCTCCTAAAATC					
<i>MC3R</i>						
Forward	CGATCCCCGTGGTCAAC	FJ433881	771–1213	443 bp	57.4	30
Reverse	GCAACTGCCCTTCATACA					
<i>MC4R</i>						
Forward	TCGATCGCGGTGGACAGGTA	NM_174110	637–1129	493 bp	58.9	32
Reverse	TCCGCAGGGCATAAATCAGAGG					
<i>MC5R</i>						
Forward	CCAGCTGCGGCACGGTCTT	NM_001015542	512–942	431 bp	57.6	32
Reverse	GCTCTGAAAACAAACAATCTCCTT					
<i>CYP17</i>						
Forward	GGAGGCGACCATCAGAGAAGTGC	NM_174304	1105–1423	319 bp	60.8	35
Reverse	CAGCCGGGACATGAAGAGGAAGAG					
<i>CYP19</i>						
Forward	TAAAACAAAGCGCCAATCTCTACG	BTCYP19	8–348	341 bp	55.4	35
Reverse	GGAACCTGCAGTGGGAAATGA					
<i>GAPDH</i>						
Forward	CACCCTCAAGATTGTCTAGCA	BC102589	492–594	103 pb	52	31
Reverse	GGTCATAAGTCCCTCCACGA					

tration. Firstly, amplification of control samples was performed with annealing temperatures between 51 and 69 °C and MgCl<sub>2</sub> between 1 and 2 mM, using the gradient feature of a gradient thermal cycler Techne TC-3000G (Techne Inc, Princeton, NJ, USA). The number of PCR cycles was determined in preliminary experiments by performing 20 to 35 amplification reactions. Then, PCR was carried out in a final volume of 25 µL containing 75 ng cDNA (previously quantified by the Qubit method), 1.5 mM MgCl<sub>2</sub>, 0.5 µM forward primer, 0.5 µM reverse primer, 0.2 mM dNTP, 2.5 µL Taq buffer 10X, and 2 U Taq polymerase (5 U/µL; all reagents from Invitrogen). For every sample, cDNA (volume, 3 µL) was added to the master-mix solution. Amplification conditions included 30 to 35 cycles of denaturation at 94 °C for 45 s, annealing at 52 to 60.8 °C (Table 2) for 30 s, and extension at 72 °C for 1 min 30 s. Finally, a final extension step at 72 °C for 10 min were performed.

The PCR products were resolved by electrophoresis alongside a 1 kb DNA ladder (BioLogicos, Buenos Aires, Argentina) through a 2% agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA), and visualized and digitized under UV light to confirm the presence of a single band at the appropriate size. The mRNA levels of the house-keeping gene GAPDH were similar for all follicle samples. Only samples positive for GAPDH mRNA expression were used to investigate the mRNA expression of the other genes. In addition, only granulosa samples positive for *CYP19a1* mRNA and negative for *CYP17a1* mRNA and the theca samples positive for *CYP17a1* mRNA and negative for *CYP19a1* mRNA expression were used to detect MCR mRNA expression.

## 2.5. Image analysis

Agarose gel images were digitized using an Olympus digital camera and PCR products analyzed using the Image Pro-Plus 3.1 program (MediaCybernetic, Bethesda, MD, USA). For these assessments, *GAPDH* mRNA was selected as an internal control, since expression of *GAPDH* mRNA remained constant in all tissues studied. In comparative PCR analysis, the absolute optical density (OD) values for each PCR product were obtained by densitometry and were normalized with *GAPDH* levels, whereas relative levels of the specific mRNA were expressed in arbitrary units.

## 2.6. Nucleotide sequencing

The specificity of PCR products was checked by direct sequencing, to verify amplification of the correct sequences, at the University of Maine (Orono, ME, USA) DNA sequencing facility. The resulting sequences were verified using the MegAlign Tool, in the LASERGENE software (DNASStar).

## 2.7. Secretion of steroids by the follicular wall

The size of bovine ovaries precluded the whole-ovary culture system used successfully with rodents [28,29], but these methods can be adapted to permit follicle wall culture. In a laminar flow hood, ovaries were rinsed, and medium and large antral follicles were taken using a scalpel and placed in Krebs-Ringer bicarbonate buffer pH 7.4 (in mM: NaCl, 118; KCl, 4.7; KH<sub>2</sub>PO<sub>4</sub>, 1.2; MgSO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 25; NaHCO<sub>3</sub>, 2.5; glucose, 11.5; bovine serum albumin, 0.1%; and ascorbic acid, 100 µg/mL; all were obtained from Sigma Chemical Co., St Louis, MO, USA). The culture medium had previously been equilibrated for 2 h in a humidified incubator (Forma Scientific 3154 Water-Jacketed Incubator, Asheville, NC, USA) gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37 °C. Small follicles were not cultured because was technically impossible to obtain enough tissue samples without stromal contamination.

Each large antral follicle was sectioned into four fragments (one for basal and three for treatments, approximately 50 mg each). For each medium follicle, only one fragment was obtained for each follicle and different follicles were used for each treatment. The fragments were cultured on 24-well (1.5 mL medium/well) culture plate (Nunc plate, Applied Scientific, South San Francisco, CA, USA), and incubated in Krebs-Ringer bicarbonate buffer, pH 7.4 for 20 min. After pre-culture, tissues were placed in medium with 1, 10, or 100 nM ACTH (Laboratorios ELEA, Buenos Aires, Argentina) for 3 h at 37 °C. As a control (basal secretion), tissues were incubated in the same way without ACTH. Each set of cultures (n = 4) was performed under identical conditions. Then, the culture media was taken from each well and maintained at –20 °C until assayed.

## 2.8. Hormone assays

Estradiol, progesterone, testosterone, and cortisol secreted into the incubation medium were measured by ELISA kits (Estradiol EIA, DSL-10-4300; Progesterone EIA, DSL-10-3900; Testosterone EIA, DSL-10-4000; Cortisol DSL-1-200; Diagnostic Systems Labo-

Table 3

Mean  $\pm$  SEM concentrations of estradiol and progesterone in the follicular fluid of small, medium, and large bovine antral follicles.

Steroid (ng/mL)	Small	Medium	Large
Estradiol	97.31 $\pm$ 6.65 <sup>a</sup>	143 $\pm$ 12.48 <sup>a,b</sup>	282.41 $\pm$ 22.18 <sup>b</sup>
Progesterone	62.12 $\pm$ 9.01	66.75 $\pm$ 10.72	81.52 $\pm$ 9.48

<sup>a,b</sup> Within a row, means without a common superscript differed ( $P < 0.05$ ).

ratories, Webster, TX, USA), according to the manufacturer's instructions. The concentrations of progesterone and estradiol were also determined directly in the follicular fluid. The assay sensitivity was 7 pg/mL for estradiol, 0.13 ng/mL for progesterone, 0.04 ng/mL for testosterone, and 0.10  $\mu$ g/dL for cortisol. For incubation medium, results were normalized in relation to tissue weight and expressed as hormone concentration/mg of tissue.

### 2.9. Statistical analyses

A statistical software package (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA) was used to perform all statistical tests. The relative levels of the specific MCRs mRNA in ovarian components and the effects of different concentrations of ACTH on the secretion of steroids in culture medium were compared by ANOVA, followed by Duncan's multiple range tests. The comparison of basal secretion between medium and large antral follicles was performed using a nonpaired two-tailed Student's *t*-test. Regression analysis was used to determine the dose-response effects. For all statistical analyses,  $P < 0.05$  was considered significant. Results are expressed as mean  $\pm$  SEM.

## 3. Results

### 3.1. Follicular characterization

Concentrations of estradiol and progesterone in the follicular fluid from various follicular sizes are shown (Table 3). All follicles were categorized as estrogen-active, with an increase in large antral follicles ( $P < 0.05$ ). However, progesterone concentrations were not significantly different among follicle categories.

### 3.2. Melanocortin receptors mRNA expression

Expression of MCRs mRNA were compared among RT-PCR samples containing the same amount of GAPDH cDNA. Controls were performed in parallel using water (no cDNA) and RNA samples (without RT), and no PCR product was visible. All granulosa

samples were positive for *CYP19a1* mRNA and negative for *CYP17a1* mRNA, whereas theca samples were positive for *CYP17a1* mRNA and negative for *CYP19a1* mRNA expression. Data obtained in representative RT-PCRs for all primers in ovarian and control tissues are shown (Fig. 1 and 2).

The identity of the MCRs PCR products was confirmed by sequencing (range 97–99% homology with bovine sequences). *Melanocortin receptor 1* mRNA was weakly detected in the theca of small and medium antral follicles and higher ( $P < 0.05$ ) in CL. The theca in large antral follicles, granulosa cells and stroma showed no expression. *Melanocortin receptor 2* mRNA levels were moderate in the theca cells of small and medium follicles and CL, and low ( $P < 0.05$ ) in the theca of large antral follicles and stroma. No specific MC2R mRNA was detected in the granulosa.

The expression of MC3R mRNA was moderate in the theca cells of small antral follicles, but weak in the theca of medium and large antral follicles. The GC had a low expression, but CL a strong expression of MC3R mRNA ( $P < 0.05$ ). No MC3R mRNA was detected in stroma. *Melanocortin receptor 4* mRNA was moderately expressed in the theca cells of small and medium antral follicles, whereas MC5R mRNA was only weakly expressed in the theca cells of small and medium antral follicles, with a decrease in the theca of large antral follicles ( $P < 0.05$ ). No MC4R or MC5R mRNA was identified in the granulosa, CL, or stroma.

The comprehensive screening of MCRs mRNA in various organs and tissues was performed to check the specificity of the primers (Table 1, Fig. 1). In the spleen, only a moderate expression of MC3R was observed, whereas in the liver, a high expression of MC3R and low expression of MC5R were evident. In the spinal cord, the expression of all MCRs was weakly detected, except MC2R (strongly detected). In white blood cells, MC1R, MC2R and MC3R were moderately expressed, whereas MC4R and MC5R had a low expression. Finally, in the adrenal cortex a high expression of MC2R, a moderate expression of MC1R and MC3R, and a low expression of MC4R and MC5R were observed.

### 3.3. Effects of ACTH on the secretion of steroids by the follicular wall

After incubating for 3 h, basal levels of steroids secretion were detected in all samples, whereas secretion of estradiol, progesterone and testosterone were significantly lower in small antral follicles, and cortisol was higher in large antral follicles (Fig. 3).



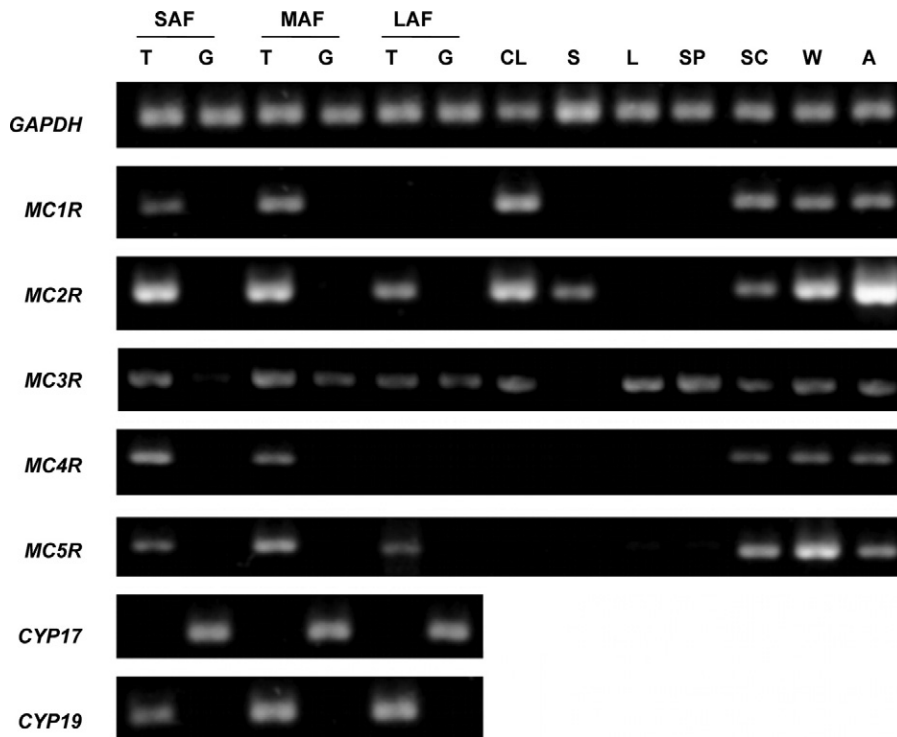


Fig. 1. Gel electrophoresis of specific RT-PCR products, showing the expression of specific primers in bovine ovarian components and control tissues.

SAF, small antral follicle; MAF, medium antral follicle; LAF, large antral follicle; G, granulosa; T, theca; CL, corpus luteum; S, stroma; L, liver; SP, spleen; SC, spinal cord; W, white blood cells; A, adrenal cortex.

In medium antral follicles, progesterone and testosterone secretion was not modified by a range of increasing doses of ACTH in the incubation medium. However there were dose-dependent increases for estradiol and cortisol, with a significant increase in response to 10 and 100 nM ACTH. In large follicles, progesterone and estradiol secretion was not modified by the presence of ACTH. A significant dose-dependent increase however was evident in testosterone and cortisol secretion. Significantly higher values of cortisol and testosterone secretion also occurred in response to 10 and 100 nM ACTH.

#### 4. Discussion

In this study, there was clear evidence that mRNA for all MCRs was expressed in bovine ovaries. The use of bovine primers and the results of controls tissues were in agreement with the expression patterns of MCRs previously described [1,30] and were also confirmed by sequencing. Based on semi-quantitative analyses, relative levels of mRNA for various MCRs changed at each stage of follicular development. There-

fore, this study was undertaken to determine the gene expression of all components in a single study, to start to establish a relationship among various subtypes of the receptors in ovarian structures. Overall, the present results confirmed earlier reports in other species, and introduced new findings, including the existence of additional receptors in various ovarian components.

Unfortunately, there are no antibodies for MCRs for immunohistochemical or western blot studies with bovine tissues. Although we only demonstrated the presence of mRNA coding for the MCRs, not the protein, our findings confirmed previously published reports describing the presence of MC2R and MC5R in ovaries of various species [1,4]. To our knowledge, there has been no investigation into the expression and localization of MC1R, MC3R, and MC4R in reproductive organs. In this sense, although ACTH bound to all MCRs, it has been hypothesized that it exerts its effects on follicular steroidogenesis via MC2R and MC5R, thereby centralizing the studies on these receptors. Nonetheless, MC1R, MC3R and MC4R might also mediate actions related to other functions in ovarian physiology.

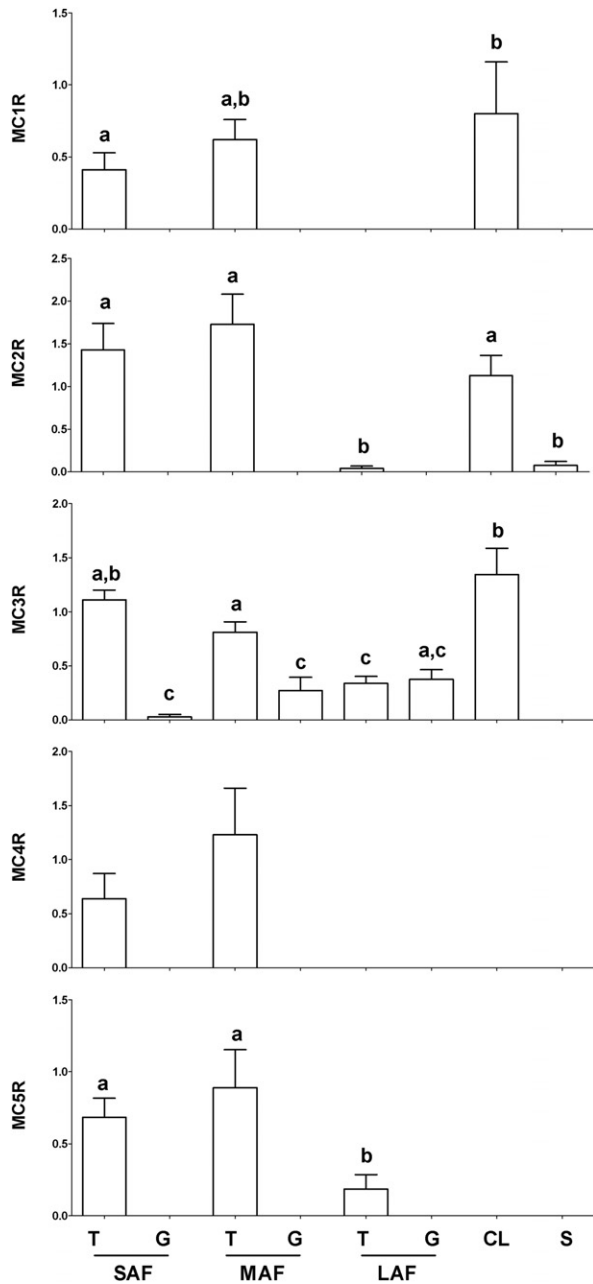


Fig. 2. Mean ( $\pm$  SEM) relative expression of melanocortin receptors (mRNA MCRs/mRNA GDPH) in bovine ovarian components and control tissues.

SAF, small antral follicle; MAF, medium antral follicle; LAF, large antral follicle; G, granulosa; T, theca; CL, corpus luteum; S, stroma; L, liver; SP, Spleen; SC, spinal cord; W, white blood cells; A, adrenal cortex.

<sup>a,b</sup>Means without a common superscript differed ( $P < 0.05$ ).

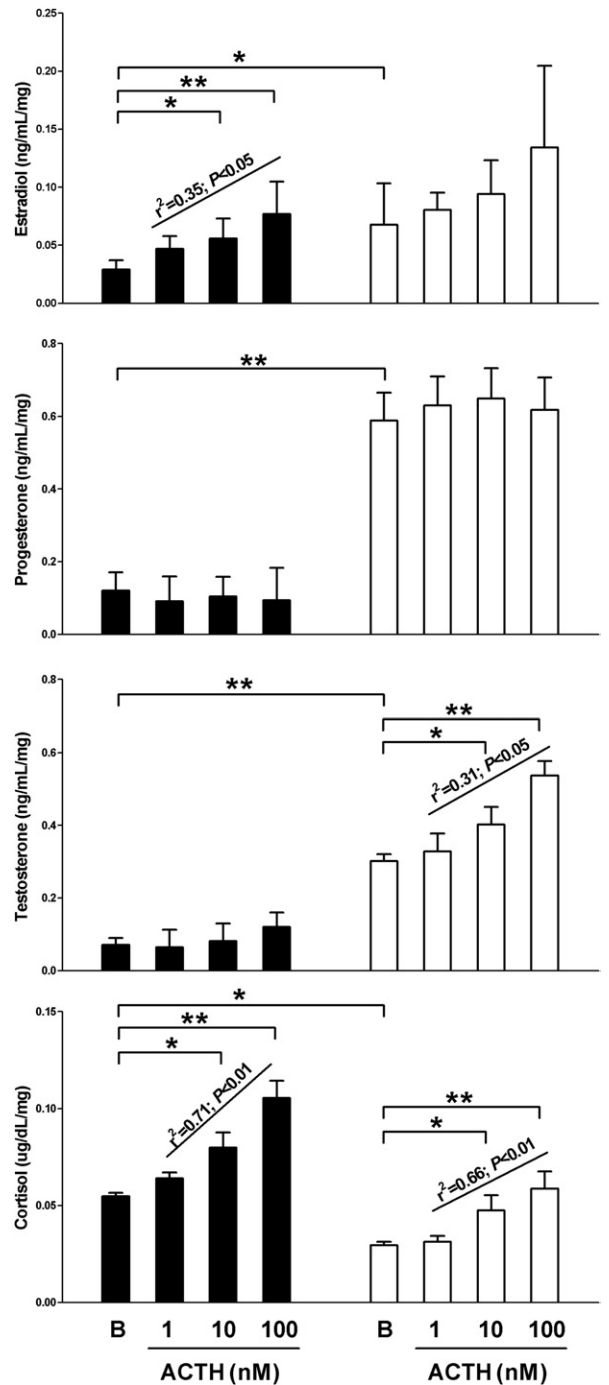


Fig. 3. Mean ( $\pm$  SEM) effects of ACTH on the secretion of steroids by the follicular wall of medium (black bar) and large (white bar) bovine antral follicles. Differences relative to basal secretion and differences in basal secretion between medium and large antral follicles are indicated: \*  $P < 0.05$  and \*\*  $P < 0.01$ .

Dose-response effects are also indicated.

Few studies have addressed the role of MCRs in mediating the effects of melanocortins on reproduction. It is well known that ACTH is acutely released from the pituitary gland in response to stressor stimulation and is the primary signal for adrenal cortisol biosynthesis. The stress-induced release of glucocorticoids is known to inhibit the immune [31], reproductive [20,32], and endocrine [33] systems. Furthermore, exogenous ACTH treatment in cattle, which increases endogenous cortisol secretion, induces follicular cysts [34,35]. An intra-ovarian effect of ACTH has been demonstrated in fish, where ACTH suppresses gonadotrophin-stimulated E2 production in ovarian follicles, and the expression of MC2R in ovaries and testes suggest a role for ACTH in modulating gonadal function throughout MCRs [4]. In agreement with this, in the present study, MC2R mRNA (the major signaling receptor for ACTH in the adrenal gland) and MC5R mRNA were expressed mainly in the theca cells of small and medium follicles, but not in the CL. Because theca cells express *CYP17a1*, a key enzyme in the steroidogenic pathway that produces the precursors of cortisol [4,36], it was expected that cortisol secretion could be stimulated by ACTH, by the presence of their receptors in theca.

In this study, basal secretion of estradiol, progesterone and testosterone were lower in medium than in large antral follicles, in agreement with previous results [37]. In contrast, basal cortisol secretion was higher in medium antral follicles than in large antral follicles. Giving ACTH to gilts during the luteal phase significantly elevated both plasma and intrafollicular cortisol concentrations [38] without disrupting gonadotrophin secretion [39]. Acosta et al [40] reported a temporal rise in follicular cortisol concentrations occurred around the preovulatory LH surge in cattle and that although concentrations of active glucocorticoids were suppressed during ovarian follicular development and maturation, they were increased during the ovulatory process triggered by an LH surge, suggesting a functional role for ACTH in the stimulation of ovarian cortisol secretion. In addition, that the follicular wall of medium antral follicles had higher levels of MCRs could be related to the stimulatory effect of ACTH on estradiol and cortisol secretion. The expression of MC2R could also be associated with the stimulatory effect of ACTH on testosterone secretion in large antral follicles, perhaps associated with a decrease in P450 aromatase activity. If we take into account the differentiation of follicular walls, the increased ACTH secretion previously reported during stress in cattle [41] could be responsible

for the increase in testosterone that characterized cystic follicles during COD in cattle.

By contrast, evidence has accumulated to support the role of the immune system in reproduction. In these processes, cytokines act through either a paracrine or autocrine mechanism and are involved in follicle rupture and remodeling, leukocyte infiltration, angiogenesis, steroid hormone production, and oocyte maturation [42,43]. In addition, many features of the ovulatory process are common in the inflammatory process, and ovulation is often likened to an acute inflammation [43,44]. In these contexts, our findings contributed to the hypothesis that attributed anti-cytokine and anti-inflammatory effects to melanocortin peptides [45]. Virtually all cells responsive to the anti-inflammatory effect of melanocortins express MC1R, the receptor with the greatest affinity for  $\alpha$ -MSH [1]. Consistent with the idea of multiple receptor involvement in the anti-inflammatory effects of melanocortins, we found the evident expression of MC1R and MC3R in the CL and some components of the follicular wall, which could indicate a close interaction between the immune and reproductive functions mediated by melanocortins. Also, although neither MC3R nor MC4R knockout mice have shown any obvious malfunction in reproduction [2], the CL was decreased in MC4R $-/-$  mice [46], supporting a role in the ovarian physiology.

To conclude, we demonstrated for the first time that MCRs mRNA were present in bovine ovaries and that ACTH affected steroid secretion in bovine ovarian follicles. We inferred that melanocortins, through their stimulatory effect on steroid secretion, could be directly involved in the regulatory mechanisms of ovarian function.

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