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Analysis of LH receptor in canine ovarian follicles throughout the estrous cycle

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The aim of this study was to determine the mRNA LHR and LHR protein expression pattern in the canine ovarian follicles at different stage of development throughout the estrous cycle. Dog ovaries were obtained from 1-6y bitches at proestrus/estrus, anestrus and diestrus stages following ovariohysterectomy. Follicular cells were mechanically recovered from follicles distributed into four types (preantral, small antral, medium antral and large antral). Total RNA extraction was performed and the evaluation of gene expression levels was achieved by relative quantification q-PCR analysis. Intrafollicular amounts of LHR were assessed by western blot method. All results were evaluated by ANOVA. The expression levels of mRNA LHR in follicular cells were observed in every stage of development, however this gene expression varied over the estrous cycle. LHR transcripts increased ($P < 0.05$) from preantral to antral stage. There were not differences in LHR gene expression among follicles at preantral stages; however, at antral stages the lowest ($P < 0.05$) LHR mRNA expression was found at anestrus and the highest ($P < 0.05$) at proestrus/estrus. The LHR protein was also detected in dog follicles in all reproductive phases with patterns varying with stage of follicular development over the reproductive cycle. The antibody against human LHR revealed two bands at ~90 and ~67 kDa, probably representing the matured protein and its precursor respectively. Both bands LHR appeared already at preantral follicles increasing ($P < 0.05$) with growth. A high proportion of LHR was presented as immature forms in all follicles stages during different phases of the estrous cycle. In conclusion, the gene and protein of LHR are differentially expressed in dog follicles over the estrous cycle, increasing with growth and the precursor protein is the most predominant LHR form present in canine follicles.

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1. Introduction

Luteinizing hormone (LH) plays a major role in reproductive processes. This pituitary gonadotropin regulates follicle/oocyte maturation, ovulation, as well as optimal fertilization and corpus luteum (CL) development [1]. In the ovary, granulosa, theca, and luteal cells are capable of secreting steroid hormones, such as estrogens, testosterone and progesterone in response to LH [2,3]. The type and amount of hormone released vary according to the reproductive-physiological status of the follicle and CL [4,5].

This gonadotropin exerts its effects through binding to its receptor (LHR), which is one of the seven transmembrane domain G-protein-coupled receptor (GPCR) superfamily [6]. It has been demonstrated in many species that at the ovarian tissue LHR is mainly expressed in theca cells, but it is also present in granulosa cells of preovulatory follicles [7,8]. The interaction LH-LHR activates adenylate cyclase, phospholipase C and ion channels, which in turn control cellular cyclic AMP, inositol phosphates, Ca^{2+} and other secondary messengers [9,10]. Through this pathway, the final differentiation of the granulosa cells and the enzymes responsible for androgen production in the theca cells are enhanced [11,12]. However, alternative second messenger pathways, which may not include stimulation of cAMP levels also exists [13,14]; but, none seems to be as effective as cAMP at mediating the induction of steroids synthesis [15].

In several species the LHR expression levels increase with follicle growth in response to follicle stimulating hormone (FSH), estradiol and paracrine factors, reaching maximum levels prior to ovulation [16]. At this time, granulosa cells also express sufficient receptors for LH. Therefore, the activity of LH increases, supporting the

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growth and maturation of larger ovarian follicles and the oocyte as a result of specific LH granulosa cell receptors [17,18]. In dogs, the estrous cycle is longer than that of other mammals, with a non-seasonal anestrus of about 3–12 months [3]. The LH pulses appear to occur in all stages of the estrous cycle, including anestrus [4]. In fact, termination of the anestrus involves selection of LH-sensitive follicles, and this final follicles selection is caused by an increased frequency of high-amplitude LH pulses at the end of anestrus [19], since the responsiveness to gonadotropins increase circulating basal LH concentrations [20]. This period of increased LH pulsatility has been reported as an important determinant in the start of a new follicular phase [3,21].

It has even been suggested that changes in LH secretion may be more important than changes in FSH secretion in the initiation of a follicular phase leading to ovulation [21]. However, the endocrine mechanisms by which a cohort of follicles is recruited in anestrus for further development in proestrus is unclear, as it is not known when this sensitivity to LH begins in those follicles. Expression of both LHR gene and LHR protein may be involved in the process of recruitment and selection of anestrous follicles, but unlike the evidences in rodents and other mammals, the canine LHR expression pattern is poorly understood.

Luteinization process begins in preovulatory follicles prior to ovulation in the bitch [22]; therefore, canine follicular cells may increase receptors for LH before the end of proestrus. An early acquisition of LH receptors on granulosa cells has been reported in dogs around the period of LH surge, by measuring the binding of radioactive labeled porcine gonadotrophins by autoradiography [23]. However, there are not available data regarding the precise follicular stage at which LHR gene and LHR protein start to become expressed. Therefore, the objective of this study was to analyze the messenger LHR gene expression and LHR protein on canine follicles cells from preantral to preovulatory follicles throughout the estrous cycle.

2. Material and methods

All animals used in the present study were treated according the protocols approved by the Chilean Bioethics Committee of the National Foundation for Scientific and Technological Research (FONDECYT).

2.1. Ovaries and follicles collection

Canine ovaries were collected from clinically healthy bitches of different breeds aged from 1 to 6 years at anestrus ($n = 64$), proestrus/estrus ($n = 42$) and diestrus ($n = 43$) stages after neutering. Immediately after surgery, the stage of estrous cycle was assessed according the ovarian structures (type of growing follicles and CL) [24] and also by measurements of progesterone obtained from blood samples on the day of surgery, according previous reports [25]. Blood samples (2 mL) were collected without anticoagulant, centrifuged at 3000 rpm for 10 min (Eppendorf Centrifuge 5415 D, Hamburg, Germany) and the plasma was stored at −20 °C. Plasma progesterone concentration was assessed by enzyme-linked immunosorbent assay (ELISA) [26], (PHomo Microplate Reader®, Autobio Labtec Instruments, Zhenghaidong, China) using a progesterone canine kit (Prog ELISA Kit, MyBioSource®, San Diego, CA, USA) [25]. Sensitivity of the assay was 0.33 ng/mL. The mean intra- and inter assay precision was 7.2% and 8%, respectively. Dilution curves of samples were proved parallel to the standard curve.

Only the ovaries with no visual abnormalities were used for experiments. The ovaries were washed in phosphate buffer solution (PBS) (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM; KH_2PO_4; pH 7.4). The growing follicles were observed by stereomicroscope (Leider MZ-730-J6 American Scientific, Portland, OR, USA), the preantral and antral follicles were classified as: a) preantral corresponding to follicles which contain oocytes surrounded by granulosa cells and an intact basal membrane without an antral cavity within the granulosa, and, antral follicles at different sizes of: b) Small antral ~0.2–0.39 mm; c) medium antral ~0.4–5.9 mm and d) large antral or preovulatory ~6–10 mm [25]. Selected follicles were trimmed off the surrounding connective tissue and follicular cells from each follicle were retrieved manually using 1 mL-gauge needle (Nipro Corporation, Miami, FL USA) after puncture for release of intra-follicular contents. The method we used for the isolation of the granulosa and theca cells was used in previous studies [24,25].

The follicular cells were pooled in each essay, classified according the estrous cycle phase (anestrus, proestrus/estrus and diestrus) and follicle type (preantral, small antral, medium antral or large antral). The diameter of each individual follicle was measured using a graticule in the eyepiece of the stereomicroscope before releasing the follicular cells, excluding the oocyte. Follicles with signs of degeneration, such as debris in their isolated cells or non healthy-looking oocyte, were excluded for the experiments.

After isolation, follicles for further q-PCR analysis were transferred to RNAlater® (Ambion™Invitrogen™ Eugene, OR, USA) and subsequently stored at −20 °C until total RNA isolation. For Western Blot analysis, samples were put in PBS medium and then stored at −80 °C. For each bitch, the number and classification of estrous phase was recorded prior to pooling.

2.2. RNA extraction, reverse transcription, and quantitative real-time PCR

Evaluation of gene expression levels was achieved by relative quantification RT-PCR analysis. Follicular samples were thawed at room temperature (22 °C) for about 5 min and then homogenized in each vial. Total RNA was extracted from follicles cells by affinity columns using the Gene JET™ RNA Purification Kit (Thermo Scientific, Eugene, OR, USA), according to the manufacturer's instructions. Due to the fragility of the RNA, the extraction was performed in a laminar flow chamber under ribonuclease-free conditions using refrigerated racks (0–5 °C). After extraction, RNA was dissolved in RNase-free media and the concentration of the total RNA was determined by fluorometric measurements in a Qubit® 2.0 Fluorometer (Invitrogen™ Eugene, OR, USA), using the quantification kit Qubit® RNA Assay (Molecular Probes® Invitrogen™).

RNA samples were stored at −80 °C until use. Reverse transcription (RT) was performed using the enzyme conjugate SuperScript II™ First-Strand Synthesis System (Invitrogen™, Eugene, OR, USA) after a DNAse treatment. The complementary DNA (cDNA) concentration was also determined by fluorometry with the Kit ssDNA Qubit® Assay (Molecular Probes®, Invitrogen™, Eugene, OR, USA). cDNA samples were stored at −20 °C.

PCR reactions were performed using the Maxima SYBR Green/ROX qPCR Master mix Kit (Thermo Fisher Scientific ™, Waltham, MA, USA), according to the manufacturer's instructions. Amplification was performed using the Two Steps real-time Eco ™ PCR system (Ilumina®, San Diego, CA, USA). All samples were run in duplicates using 10 ng of complementary DNA (cDNA) in an 18-mL total reaction volume.

Control samples without reverse transcriptase and without the template, were included in each plate. The following thermal profile was used: 1 cycle at 95 °C for 10 min for initial denaturation and 40 cycles at 95 °C for 15 s (denaturation), and 60 °C for 60 s (annealing) and 72 °C for 30 s (extension).

All samples were run in duplicates; the β-actin RNA and Histone

2A RNA were chosen as normalization control genes at 1000 y 200 nm, respectively.

Canine-specific primers for LHR were used for reverse transcription (Promega, Catalys AG; Wallisellen, Switzerland) [27] (Table 1). Results were obtained by the comparative Ct method, using DCt (the value obtained by subtracting the Ct value of LHR from the Ct value of β-actin and H2A mRNA and of an individual sample).

2.3. Western blotting

For Western Blot analysis, preantral and antral follicles previously pooled according follicle sizes and reproductive status, were then put into 1 mL of homogenization RIPA buffer (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 2 mM EDTA, 1% NP-40) (Pierce®, Thermo Fisher Scientific ™, Waltham, MA, USA) plus protease inhibitor cocktail (Thermo Fisher Scientific ™, Waltham, MA, USA). After 5 min of incubation on ice, samples were sonicated for 30 s with 50% pulse in a Branson 5450D Gigital Sonifier®, (Branson Ultrasonics, Danbury, CT, USA). Then, the samples were centrifuged at 14,000 × g for 15 min at room temperature using the Hettich Universal 320R Centrifuge (Lab Technology, Beverly, MA, USA). The supernatant was collected and the total protein concentration was estimated in the Qubit® Fluorometer (Invitrogen™, Eugene, OR, USA), using the quantification kit Qubit® Protein Assay (Molecular Probes® Invitrogen™, Eugene, OR, USA).

Aliquots were boiled for 5 min and separated by SDS-PAGE under reducing conditions according Laemmli, 1970 [28]. Equal amounts (30 μg) of protein were applied per lane in a Mini Protean Tetra (Bio-Rad, Hercules, CA, USA) and the proteins were separated at 100 V. The proteins were transferred onto Immobilon®-P PVDF membrane (Merk Millipore, Billerica, MA, USA) and incubated (1:100 dilution) with the Goat Polyclonal Anti-LHR antibody SC-26341 (Santa Cruz Biotechnology Inc. Dallas, TX, USA). After washings, the samples were incubated with the Rabbit Anti-Goat IgG antibody conjugated with alkaline phosphatase (Santa Cruz Biotechnology, Inc. Dallas, TX, USA) (1:500 dilution). Protein bands were visualized with NBT/BCIP substrate (Nitro blue tetrazolium; Bromo-chloroindolyl phosphate-p-toluidine salt) (Santa Cruz Biotechnology, Inc. Dallas, TX, USA). Molecular weights of separated proteins were estimated by comparison with positions of a molecular mass standard run in parallel. A negative control was carried out by incubating only with the second antibody. Densitometry of band intensities in Western Blotting were evaluated using the Gel Documentation System Biosens SC-645 (Biotop, Shanghai Bio-Tech Co, Shanghai, China), where a volume value expresses the intensity for each band.

2.4. Statistical analysis

Preantral follicles and antral follicles were collected from 149 bitches. Data from 128 ovaries at anestrus and 84 at proestrus/estrus and 86 at diestrus for both qPCR and Western Blot analysis, were carried out by a two-way ANOVA (follicle type, stage of estrous cycle) followed by a Duncan's test to compare differences in mRNA and protein levels of LHR among follicle types and reproductive phase of the estrous cycle. Data that were not normally distributed were transformed logarithmically.

The statistical model included the effects type of follicle, stage of estrous cycle and their interactions. The possible association between the levels of mRNA LHR and LHR throughout follicular development was evaluated by the Spearman correlation coefficient. For all evaluations, differences were considered statistically significant when $P < 0.05$.

3. Results

The preantral and antral follicles were classified according to size (stage of development) and reproductive phase of the donor. The total number of each type of follicles used in this study throughout the estrus cycle is shown in Table 2.

The plasma progesterone concentration obtained from bitches at different reproductive status was 0.01–0.15 ng/mL in anestrus phase: 0.40–15.00 ng/mL in proestrus/estrus and 19.02 ng/mL to 38.02 ng/mL in diestrus. All of these ranges were in accordance to those previously reported in the different phases of the canine estrous cycle [26].

3.1. Expression of mRNA in the follicles

Quantitative real-time PCR analysis revealed mRNA encoding for LHR in all evaluated follicles throughout the reproductive cycle (Fig. 1). The mRNA LHR expression abundance of preantral follicles was different ($P < 0.05$) when comparing with different type of antral follicles at proestrus/estrus and diestrus stages, whereas follicles obtained at anestrus did not show differences between preantral with small antral follicles, however the mRNA levels increased significantly from preantral to medium antral stage. There were no differences among dog preantral follicles comparing the phases of the estrous cycle.

Significant differences in receptor mRNA expression abundance between large follicles at proestrus/estrus versus other sizes were noticed and also LHR mRNA expression between small versus medium size antral follicles at diestrus was different ($p < 0.05$).

3.2. Western blotting results

In the current experiments, expression of LHR was detected in dog follicles with patterns varying with stage of follicular development over the reproductive cycle.

The SC-26341polyclonal antibody used in the present study, raised against the human LHRH receptor, was previously proven to have cross reactivity in dogs [26]. This antibody revealed two bands at ~90 and ~ 67 kDa, probably representing the matured protein and its precursor, respectively. In the literature, the 85–95 kDa band corresponds to the mature LHR present at the cell surface, and the 65–75 kDa band is the precursor of the mature receptor [10,14].

Table 1
Sequences and PCR characteristics of LHR and references genes used in this study

Gene	Sequence 5′-3′	Accession number	Amplicon	Tm °C	Efficence
ACTB	ATTGTCATGGACTCTGGGGATG TCCTTGATGTCACGCACGAT	AF021873.2	191 bp	56,7	1,99
H2A	AGTACCTGACGGCCGAGAT AGGGCAAATCAATCCAGAGA	XM_545419.4	245 bp	59,6	1,97
LHR	CATCGTGGGAAATGTGACTG GCACTACACCCACTCCCTGT	AF389885.1	204 bp	60	2,01

Table 2
Number of follicles in different stages of development (sizes) obtained over the estrous cycle

	Stages of Follicular Development			
	Preantral	Small Antral	Medium Antral	Large Antral
Reproductive Cycle	q-PCR-WB	q-PCR-WB	q-PCR-WB	q-PCR-WB
Anestrus	297–112	177–107	93–15	–
Proestrus/Estrus	163–49	124–195	45–71	15–21
Diestrus	137–94	56–176	66–55	–
Total	597–255	357–478	204–141	15–21

Abbreviation: qPCR, quantitative real-time polymerase chain reaction; WB, western blot analysis.

Densitometric analysis of each immunoreactive band showed that LHR protein level changed among the different development stages and phases of reproductive cycle. These bands appeared to be specific for LH, as the secondary antibody alone did not produce cross-reactivity (data not shown).

During proestrus/estrus (Fig. 2 A), follicular cells expressed mostly the precursor form, increasing significantly with an increase in follicular diameter. The mature form showed a lower intensity band which increased ($P < 0.05$) from preantral to preovulatory stage (large follicles). At diestrus (Fig. 2B) the relative abundance of these bands was different between the precursors and mature forms, with a higher expression of precursor form in all follicle stages. Increasing ($P < 0.05$) immunoreactivity from preantral to medium antral stage was detected. The mature form also exhibited LHR variation among follicle sizes ($P < 0.05$). At anestrus (Fig. 2C) both bands were expressed with increasing ($P < 0.05$) levels from preantral to antral follicle stages.

The presence of LHR of each follicular cell group was also compared within the same size group at the different phases of estrous cycle. As shown in Fig. 3, preantral follicular stage and small size follicles did not exhibit differences ($P < 0.05$) in 90 Kd (Fig. 3A) and 67 kd (Fig. 3B) bands intensity, whereas the mature form showed higher ($P < 0.05$) levels in medium antral follicles at anestrus compared to proestrus and diestrus in both protein forms (Fig. 3A and B).

Messenger RNA expression and LHR, with either precursor or matur form, was positively correlated ($P < 0.05$) in all phases of the estrous cycle (Table 3).

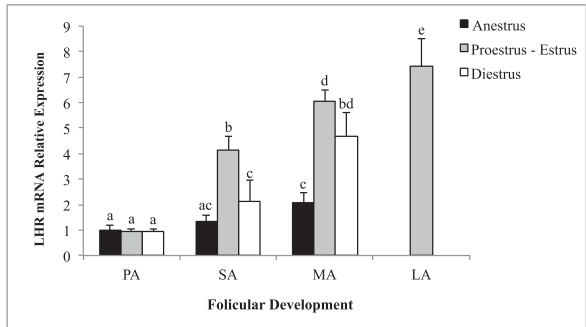


Fig. 1. Comparison of the relative expression of luteinizing hormone receptor (LHR) mRNA among follicles in different stage of development and estrous cycle. The LHR mRNA levels are expressed relative to β-actin RNA and Histone 2A RNA as control genes. (PA) Pre-antral, (SM) small antral, (MA) medium antral and (LA) large antral follicles. Different letters above bars indicate significant differences ($P < 0.05$).

4. Discussion

The pattern of LH endocrine profile during estrous cycle has been well characterized in canine [4,19–21]; however, the dynamic expression of mRNA LHR/LHR during the entire reproductive cycle had not been described in this species. In the present study we examined the presence of mRNA LHR and LHR by q-PCR and western blots respectively in dog follicles and we determined for the first time the stage of follicular development at which LHR protein and its messenger RNA are produced. It was found that both were expressed during the whole follicular growth including pre-antral stage, and in all phases of the estrous cycle. This demonstrates that the LHR expression is not restricted to the large preovulatory or antral stages follicles but is expressed even before antrum formation. Previous studies in canine follicles using autoradiography found that specific binding of LH was not detected in follicles smaller than 350 μm in diameter [23]. The difference with our study may be due to the technical methods used for this purpose. By western blotting, the whole protein of the LHR was quantified from total cellular extracts. In binding experiments, possibly only one form of the LHR present at the cell surface was quantified. Some receptor forms internalized or present in the endosome might not be accessible for binding [10].

The LHR proteins in rodent granulosa cells are not found in the preantral and early antral follicles stages [29], but are induced after FSH exposure in antral and preovulatory follicles [12]. However, in human follicles LHR appears on granulosa and theca layers at the pre-antral stage [30,31]. Although our study did not differentiate the cell type (theca or granulosa cells) where the receptor is produced, we found that preantral follicles were able to express the messenger RNA LHR and its encode protein. The differences with other studies can arise from a species-specific pattern and/or different technical approaches. The early expression pattern found herein raises the question about the function of LH at the beginning of follicular growth in dogs, considering that LH action seems to become essential for follicular development during the antral stage [19]. The finding of LH receptors during the onset of follicular development, however, suggests that LH could have functions at that time.

The expression of mRNA LH and LHR protein increased during follicular development similar to previous results in canine LH receptor [23] that described levels of granulosa LH binding sites, increased with follicular diameter. The same pattern of expression was previously observed in human [17,32] and other mammals as bovine [33] porcine [34] and murine [35] follicles.

The expression pattern of mRNA LHR levels and the level of LH receptor underwent dynamic changes during the ovarian cycle. These cyclic variations were mostly in agreement with the major

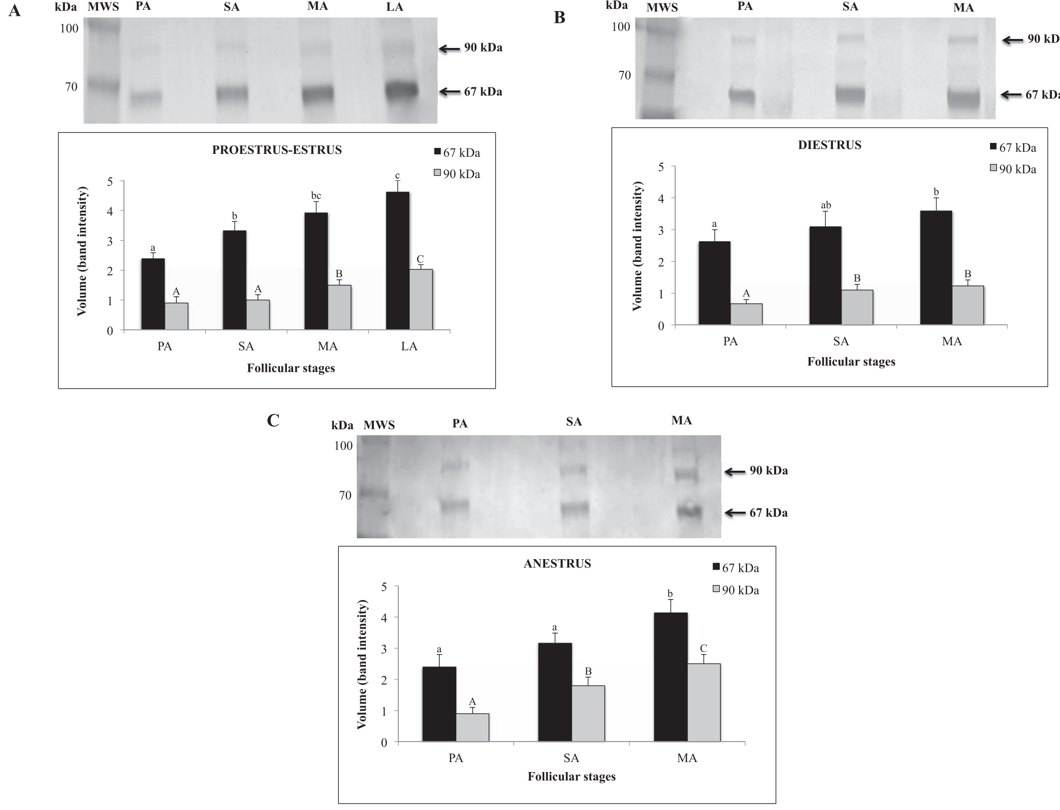


Fig. 2. Western blot analysis of luteinizing hormone receptor (LHR) using the polyclonal Anti-LHR antibody SC-26341. In all follicular stages and estrus phases, western blot analysis revealed two distinct bands. The first one with molecular mass around 67 kDa representing the LHR precursor protein and another band of approximately 90 kDa corresponding to the mature LHR. Bands were analyzed by densitometry using the Gel Documentation System Biosens SC-645. The volume value in the graph represents the intensity of each band, the black and grey bars represent the precursor and the mature protein, respectively. (PA) Pre-antral, (SM) small antral, (MA) medium antral and (LA) large antral follicles. Different letters above the bars indicate significant differences ($P < 0.05$). ($^{A-C}$) 90 kDa bands, ($^{a-c}$) 67 kDa bands.

changes of plasmatic concentration of LH found in bitches during estrous cycle [4,36]. The variation observed over the estrous cycle supports the idea that the sensitivity and response to LH can be different across the estrous cycle. The most notable increase in mRNA LHR and the increase in the encode protein LHR was exhibited in proestrus/estrus. The developing follicles increase LHR mainly during these follicular phases to allow them to respond to the LH surge, which occurs in canines at the end of proestrus and beginning of estrus [5,18]. In most mammals the LH increase permits the final stages of follicular maturation to the preovulatory stage [37]. The rise in estradiol at proestrus that initiates the surge of LH possibly influenced the high expression levels of mRNA LHR and LHR observed in this study during this period. The evidences have shown that the cyclic expression of gonadotrophin receptors in granulosa or theca cells of growing follicles is developmentally and hormonally regulated, reflecting the changes in receptor levels [36]. It is known that estrogen increases the number of LH receptors of many species [38]. The increase of transcript and protein LHR expression observed with follicle growth during proestrus/estrus, might also result from the up-regulated LHR levels in response to FSH and other paracrine factors [16].

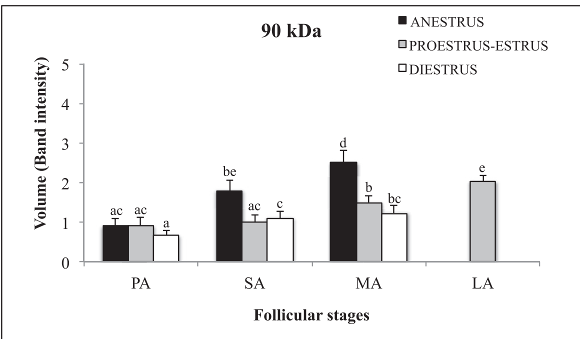
The Western blot analysis revealed the presence of two bands at ~67 and ~90 kDa. The size of these bands were within the range previously described for this protein, since post-translational changes in glycosylation and phosphorylation result in several LHR variants migrating between ~93 and 44 kDa [13,39]. It has been shown that the cell surface LHR is an 83–93 kDa protein synthesized from a 68–73 kDa intracellular precursor [40,41]. Accordingly, the 90 kDa band found herein might correspond to the mature protein, whereas the 67-kDa band to its precursor.

A high proportion of LHR was presented as immature forms in dog follicular cells. It has been shown that the murine LH receptor is also expressed predominantly in the precursor immature form, suggesting that the receptor is not efficiently processed into mature cell surface form [16]. Ovarian follicular cells from other mammals such as porcine [42], equine [43] and ovine [44] also express mainly the 60–70 kDa form. By contrast, the human LHR is expressed predominantly as the mature 90 kDa form [45].

All follicle types exhibited these two bands with different intensity depending on its developing stage and reproductive phase, demonstrating a clear influence of the reproductive status on LH receptor expression. However, inefficient maturation of the canine LHR appears to be an inherent property of this receptor in canines, as an abundance of immature receptor was observed during all reproductive phases.

During the course of anestrus, the immunoblots bands of LHR showed increasing density from preantral to medium antral follicle in both immature and mature forms. This rise in LHR could be in accordance with the increasing levels of LH reported in bitches at the end of anestrus [3]. In addition, during anestrus there is enhanced expression of the genes encoding for the estrogen receptor [46], which has an influence in up regulates LHR expression [38]. The precise role of the increase of LH secretion is not clear, but it has been suggested that the rise in LH pulses at the end of anestrus might be one factor leading to termination of anestrus in bitches [47]. In fact, the administration of exogenous LH can terminate anestrus by inducing proestrus [48]. Studies using bovine models suggest that expression of LHR mRNA and LHR in granulosa

A



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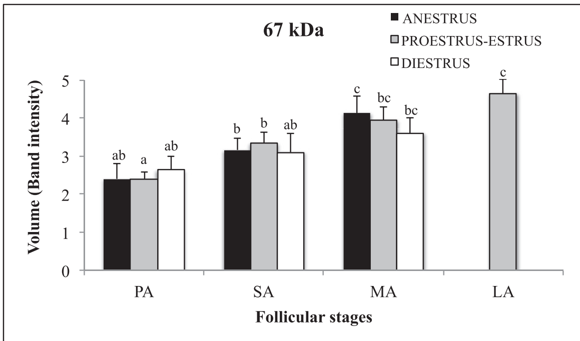

Fig. 3. Western blot result of luteinizing hormone receptor (LHR) expression, comparing the intensity of the mature protein bands (A) or precursor bands (B), among follicles in different stage of development and estrus cycle. (PA) Pre-antral, (SM) small antral, (MA) medium antral and (LA) large antral follicles. Different letters above the bars indicate significant differences (P < 0.05).

cells is required for follicles growth and selection of the dominant follicle [49]. It appears then that the major event associated with the selection of follicles is the shift in follicle dependence from FSH to LH. Therefore, possibly the follicles responding to LH at the end of anestrus will be those selected to advance to proestrus and estrus.

In contrast to the other phases of the reproductive cycle, the difference between the mature form and its precursor was less noticeable in anestrus because the mature form appeared to be expressed at higher levels as compared with the other phases of the estrous cycle.

At proestrus/estrus, LH receptor content in follicular cells expressed mostly the precursor form that increased with an increase in follicular diameter, whereas the mature form of the protein was less expressed, showing also an increase from preantral to preovulatory stage. The different processing of the LH receptor al proestrus compared to the LH receptor at anestrus might be due to the relatively short duration of the proestrus phase requiring rapid changes in the LH receptor expression level by post-transcriptional

Table 3
Spearman correlation coefficients between the LHR mRNA relative expression level and both 90 kD and 67 kD western blot bands, in the different phases of the estrous cycle.

Reproductive Phases	90 kDa	67 kDa	mRNA LHR
Anestrus	0.99	0.98	*
Proestrus/Estrus	0.89	0.99	*
Diestrus	0.82	0.98	*

*P < 0.05.

modification because LH pulsatility increase rapidly during proestrus to the preovulatory LH surge, reaching the highest concentration at the beginning of estrus [19]. Interestingly, the increasing levels of mRNA LHR, during proestrus/estrus suggest a high transcription rate, almost at the same time of translation. At anestrus, the increase of LHR seems to be less abrupt. During this period in the bitch the basal LH is low with sporadic pulses until the mid-end of anestrus [47]; therefore, this phase could allow major further processing of the precursor form to mature form of LHR than the preovulatory period.

At diestrus the LHR was expressed mainly as the precursor form. In this phase of the reproductive cycle mRNA LHR expression increased more noticeable than the protein, suggesting a major transcriptional activity in those follicular cells.

In conclusion, the present study provides information on LHR expression in canines, finding that mRNA LHR and the LHR protein appear already in preantral follicles and increased during follicles developed over the estrous cycle. The precursor form is the most predominant LHR form present in canine follicular cells.

Acknowledgments

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