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Doctoral Thesis

DETERMINATION OF LUTEINIZING HORMONE (LH) AND FOLLICLE STIMULATING HORMONE (FSH) IN BOVINE PLASMA: DEVELOPMENT AND VALIDATION OF SPECIES-SPECIFIC MONOCLONAL ANTIBODY-BASED ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

(SSD BIO/12)

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1. INTRODUCTION

1.1 Hypothalamo-pituitary-gonadal axis and the female cattle reproductive cycle

Reproductive functions in the bovine species are governed, as in all mammalian species, by the hypothalamus-pituitary-gonadal (HPG) axis. The HPG axis is composed of three anatomically and functionally distinct structures, that interact trough a complex system of positive and negative hormonal feedback loops. In the female it is specifically named the hypothalamus-pituitary-ovarian (HPO) axis.

In 1910 a work from Crowe linked for the first time the pituitary gland function to the development of genital organs *(Crowe et al., 1910)*. Further studies have been developed till 1926 when Smith showed that the ovarian atrophy after removal of the hypophysis was reversed by pituitary implants *(Smith, 1926-A,B)* and Zondek evoked precocious sexual maturation in immature animals with external implantation of anterior pituitary glands *(Zondek, 1926)*. Thus confirming the pituitary-ovarian link hypothesis.

It was first in 1929-1930 when Zondek proposed the idea that two hormones might be required for normal ovarian function *(Zondek, 1929, 1930)*. It was after he showed that extracts from urine collected from postmenopausal women produced a predominant follicle-stimulating effect, whereas urine from pregnant women showed a strong luteinizing activity (*Göretzlehner et al., 1978*).

Fevold and a co-worker proved the latest hypothesis isolating two crude pituitary hormones with distinct actions in the ovary of the rat *(Fevold et al., 1931)*. The first, the follicle stimulating hormone (FSH), stimulated the ovarian follicular development and the luteinizing hormone (LH) which caused FSH-stimulated follicle to luteinize.

It was many years later when Guillemin suggested that a higher centre in the brain controlled the secretion of gonadotropins from pituitary gland, proposing the hypothalamus-pituitary link *(Guillemin, 1967)*. The existence of HPG axis was finally confirmed in early 1970s by Schally and

Guillemin when they elucidated the chemical structure of gonadotropin releasing hormone (GnRH) *(Schally et al., 1971; Burgus et al., 1972).*

Nowadays, it is well well-known HPO is a complex neuroendocrine system regulated through several feedback loops using different chemical messengers such as neurotransmitters, hormones and growth factors. Figure 1 shows a schematic representation of the HPO axis.

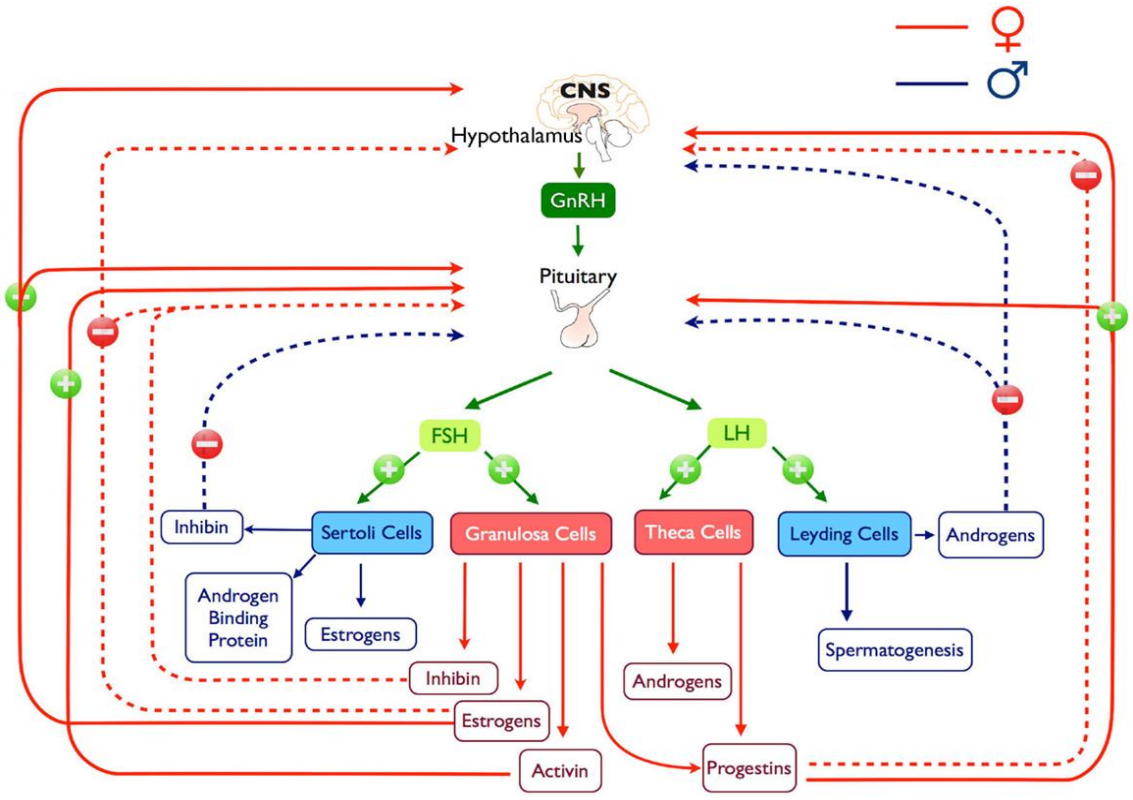

Figure 1

Hypothalamicï pituitaryï gonadal axis. The figure shows the complexity of posisitve and negative feedback system involved in the regulation of gonadotropin production and release. Loops and products are illustrated. *(Duran-Pasten et al., 2013).*

GnRH modulates the activity of anterior pituitary gonadotropes, controlling FSH and LH synthesis and release. It has been demonstrated that while both LH and FSH are released from the gonadotrope in a basal manner independent of GnRH only the release of pulses of LH is totally dependent on pulsatile GnRH input, while the release of FSH is associated only with a small amount proportion of GnRH pulse *(Kile et al., 1994; Padmanabhan et al., 1997; Clarke et al., 2002).*

The GnRH, LH, and FSH synthesis and secretion are modulated by a complex feedback mechanisms of gonadal molecules. First of all, sex-steroids, inhibins and activins. Nevertheless, over the last decade a series of new gonadal proteins have been discovered, including Mullerian inhibiting substance (MIF), epidermal growth factor (EGF), transforming growth factor (TGF), and insulinlike growth factors (IGF-I and IGF-II). It has become evident that regulation of gonadal function and gametogenesis can no longer be explained solely by the effects of the sex-steroids and the two gonadotropins.

1.2. Molecular structure of bovine GnRH

Gonadotropin-releasing hormone is the master hormone controlling the HPO axis and therefore female reproductive physiology. It belongs to a group of neuropeptides isolated as factors of hypothalamic origin *(Schneider et al., 2006)*. There are currently 23 identified naturally occurring GnRH structural variants across the vertebrate species *(Millar et al., 2004; Morgan et al., 2004)*.

The hypothalamic form of GnRH is designated GnRH-I *(Sealfon et al., 1997; Pawson et al., 2005)*. It is a decapeptide synthesized from a 92 amino acid precursor. The GnRH-I signal peptide is composed of a 23 amino acid N-terminal peptide, the biological active decapeptide, a 3 amino acidic sequence of enzymatic cleavage and a 56 amino acid peptide named GAP (GnRH-associated-peptide) at the C-terminal. The GnRH-I decapeptide with pyroglutamyl amino (N) and amidated carboxy (C) terminals has an identical amino acidic sequence in all mammalian species studied *(Dellovade et al., 1998; Pei-San, 2006)*, while GnRH-I precursor is species-specific (Figure 2). Table 1 reports the percentages of amino acid identity between the GnRH-I precursor of nine mammalian species in comparison with the bovine.

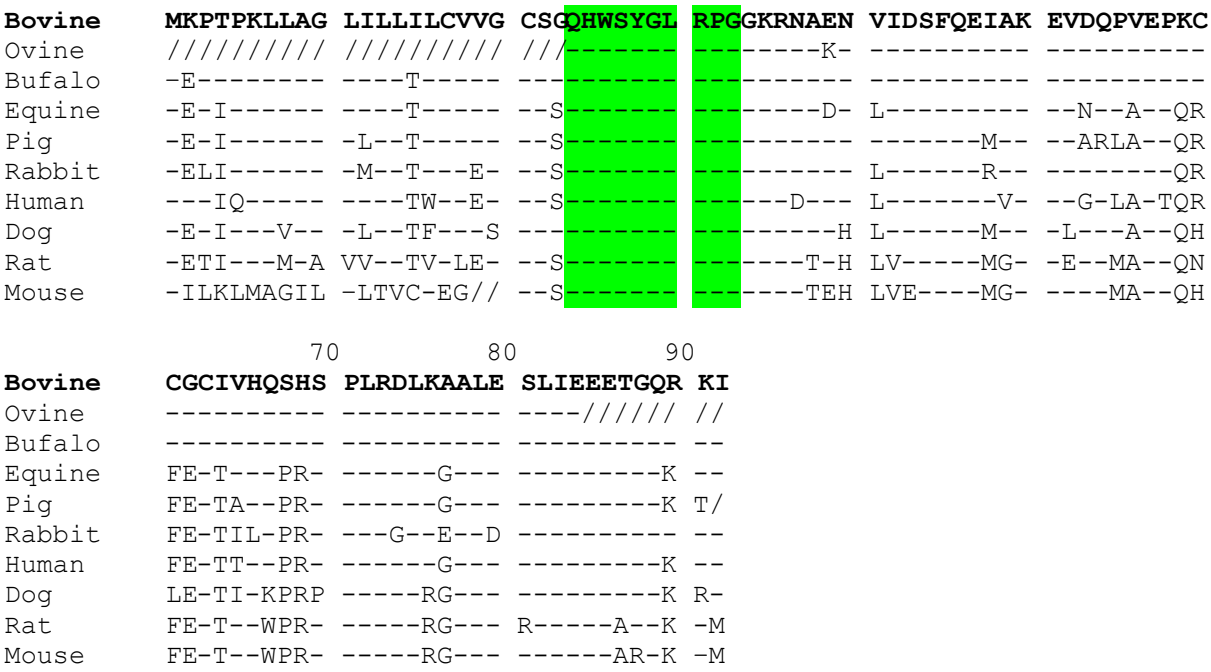

Figure 2

Amino acidic sequence of pre-GnRH-I in 9 mammalian species compared to the bovine GnRH-I precurosor. The green box highlight the ten identical amino acid of GnRH-I.

Table 1

Identity between GnRH-I sequence of 9 mammalian species in respect of bovine GnRH-I.

	Number of different Amino acids	Identity (%)
Ovine	1	99
Bufalo	2	98
Equine	17	82
Pig	21	77
Rabbit	21	77
Human	23	75
Dog	26	72
Rat	35	62
Mouse	40	57

1.3. Molecular structures of bovine FSH and LH

FSH and LH together with thyroid stimulating hormone (TSH) and chorionic gonadotropin (CG) belongs to the glycoprotein hormone family which is structurally considered the most complex hormone family in animal kingdom (*Dirnberger et al., 2001*). FSH, LH and TSH are produced and secreted by the anterior pituitary gland, while CG is produced by placental trophoblasts only in primates and equids.

These are all heterodimers consisting of a common Ŭ-subunit non-covalently bound to a β-subunit. Within a given animal species the Ŭ-subunit has an identical amino acid sequences in all four glycoprotein hormones as is encoded by the same gene; while the β-subunit arise from different genes and so is unique and determine the hormone biological and immunological activity *(Ulloa-Aguirre et al., 2001-A).*

Each subunit forms intra-chain disulfides bridges and present glycosylation sites which makes the hormone produced and secreted not as a single structure but as a mixture of glycosylation isoforms *(Szkudlinski et al., 1995).*

1.3.1 FSH and LH differently glycosylated isohormone

Carbohydrates are an important structural component of the gonadotropins, comprising nearly 20-30% of the hormone mass. Glycosylation represent the most pronounced and most complex form of protein post-translational modification (*Geyer et al., 2006*). It involves a complex biosynthetic pathways beginning in the rough endoplasmic reticulum (RER) continuing through the Golgi apparatus until the mature hormone is transported to secretory granules *(Ulloa-Aguirre et al., 2001-A)*. There are mainly two types of protein glycosylation: N-glycosylation and O-glycosylation. In pituitary gonadotropins (FSH and LH), we only find the N-glycosylation type in all mammalian species, with the exception of equine LH (see section 1.3.3.1).

Figure 3 shows a summary of the biosynthetic pathway for N-linked oligosaccharides. During the N-glycosylation the common core, from a lipid-linked precursor (dolichol) is transferred to the amide group of asparagines within an Asn-X-Ser/Thr motif, where X is any amino acid apart from proline *(Geyer et al., 2006; Ulloa-Aguirre et al., 2001-A).* This precursor is further modified by Ŭ-glucosidase I and II removing terminal glucose residues and a 2-mannosidases removing one mannose residue. A common core is so generated composed by two N-acetyl glucosamine (GlcNac) residues linked to three mannose. After dimer formation and trimming of glucose and mannose residues to a Man5GlcNac2 in RER and cis-Golgi apparatus further processing occurs in the medial and trans-Golgi involving several enzymes, such as N-acetyl galactosamine (GalNac) transferase, N-acetyl glucosamine transferase, syaliltransferase, etc..., till mature oligosaccharides is formed *(Ulloa-Aguirre et al., 2001-A).*

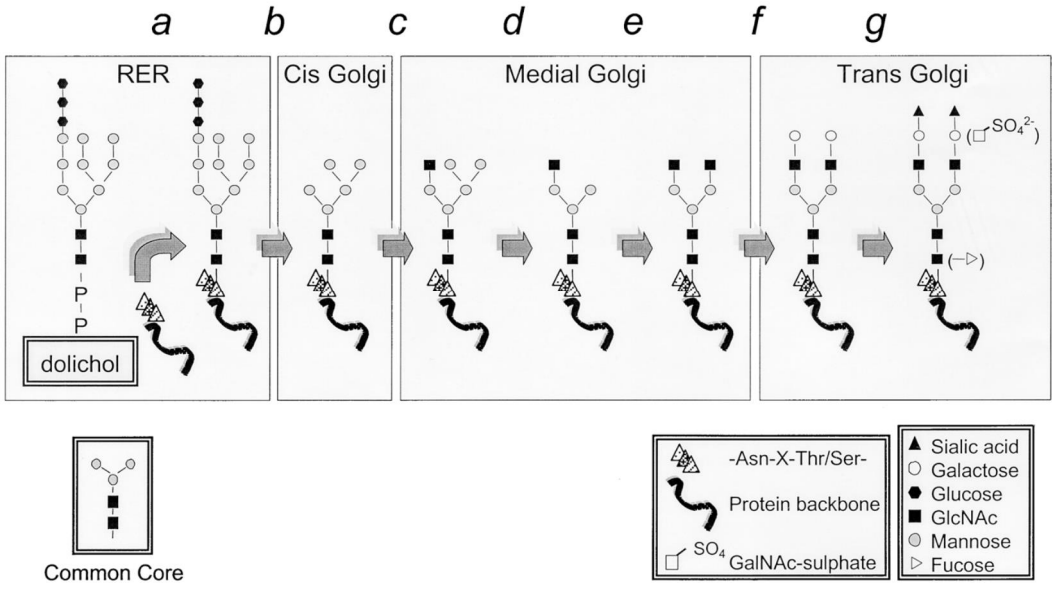

Figure 3

Summary of the biosynthetic pathway for N-linked oligosaccharides. Some key steps are shown, starting with en bloc transfer of the common core from a lipid-linked precursor (dolichol). Steps include: oligosaccharyltransferase-catalyzed transfer of dolichol-linked Glc3Man9GlcNAc2 oligosaccharide to aspargine at glycosylation siteï Asn-Xaa-Thr/Ser **(step a)**; _-glucosidase I and II remove terminal glucose residues, endoplasmic reticulum (RER) _-1, 2-mannosidase removes one mannose residue, and Golgi _-mannosidase I removes an additional three mannose residues **(step b)**; N-acetylglucosamine-transferase adds N-acetylglucosamine, forming precursor for a structure having only one completed branch **(step c)**, or leading to continued processing in which Golgi _-mannosidase II removes two mannose residues **(step d)**, and N-acetylglucosamine-transferase II adds a second N-acetylglucosamine residue **(step e)**, leading to further processing **(steps f and g)** (*Ullua-Aguirre et al., 2001-A*).

Oligosaccharides structure of glycoprotein hormones have been shown to play an essential role in many of the functional characteristic of the hormones. They determine the maintenance of protein folding, subunit assembly, conformational maturation and heterodimer secretion, as well as metabolic clearance, interaction with its cognate receptor and signal transduction. *(Fiete et al., 1991; Bousfield et al., 1996; Ulloa-Aguirre et al., 2001-B, Perrera-Marìn et al., 2007).* In fact deglycosylated glycoproteins by chemical or enzymatic procedures are rapidly cleared from the circulation and are practically inactive *in vivo* when compared to the corresponding intact variant. Oligosaccharides on the ß-subunit play a major role in determining the metabolic clearance rate of gonadotropins, while glycosilation of the Ŭ-subunit is essential to activation of the receptor/signal transducer (G-protein) system and the subsequent biological response. In fact the removal of Ŭ-subunit oligosaccharides result in an increased capacity to bind the receptor, but a significantly reduced signal transduction capability *(Ulloa-Aguirre et al., 2001-B).*

Glycoprotein hormone FSH and LH must be viewed as a heterogeneous mixture of closely related variants, so called isoforms. This polymorphism occurs due to variations in the structure of the oligosaccharides *(Baenzinger et al., 1988; Manzella et al., 1996; Ulloa-Aguirre et al., 1999, 2001a,b; Dias, 2001),* which affects the physicochemical characteristics and biological and immunological activities of the hormone.

The asparagine-linked oligosaccharides on the pituitary glycoprotein hormones LH and FSH, consist of a heterogeneous array of neutral, sulfated, sialylated, and sulfated/sialylated structures. The glycosilation differences are both qualitative and quantitative and usually involve the content of sialic acid (Sia), N-acetil-galactosamine sulphate (SO_4-4GalNAc), galactose (Gal), mannose (Man) and N-acetilglucosamine (GlcNAc). The sulfated oligosaccharides consist of mono- (S-1) and di- (S-2) sulfated structures containing the characteristic peripheral sequence SO_4-4GalNAcβ1, 4GlcNAcβl,2ManŬ. The sialylated oligosaccharides consist of a wide array of structures differing in number (1 (N-l), 2 (N-2), and 3 (N-3)) and linkage (Ŭ2,3 versus Ŭ2,6) of sialic acid moieties as well as underlying oligosaccharide structure *(Stockell et al., 1992; Green et al., 1988-A,B).* The major

difference between the LH and FSH oligosaccharides is the significantly different distributions of sulfated and sialylated oligosaccharides. Bovine LH for example only contains sulfated oligosaccharides, with 78% of monosulfated branches and 22% of disulfated branches, while bFSH contains both sialylated to sulfated oligosaccharides, with sialylated structures dominating. Sialylated oligosaccharides are also heterogeneous with respect to sialic acid linkage (Ŭ2,3 versus Ŭ2,6), and substitution (i.e.: *N*-acetyl- and *N*-glycolyl-neuraminic).

Interestingly, there are not only hormone- but also animal species-specific differences in the types and distributions of sulfated, sialylated, and sulfated/sialylated structures. The differences are both qualitative and quantitative. For example, relative distribution of sialylated, sulfated and neutral Asn-linked oligosaccharides in human-LH is: 58%, 49% and 0%, and in bovine-LH is: 0%, 67% and 33%. The relative distribution of the same sugars in human FSH is: 88%, 7% and 5% and 56%, 13% and 31% in bovine FSH *(Green et al., 1988-A,B)*. Furthermore, it has been shown that bovine FSH carries both *N*-acetyl- and *N*-glycolyl-neuraminic acids (Neu5GC and Neu5Ac) *(Borromeo et al., 2004)*, while Neu5Gc is absent in primates and chicken, due to the lack of a functional CMP-Neu5Ac-hydroxylase (Chenu et al., 2003; Varki, 2001). This adds further evidence to the role of sugars in determining FSH species-specificity.

The functional significance of this diversity of isoforms is not yet fully understood. Sialic acid seems to be the major determinant for the circulatory stability of glycoprotein hormones. This sugar content and the number of exposed terminal galactose residues is essential in determining the hormone survival in circulation. In fact the exposure of terminal galactose residues dramatically increases the rate of glycoprotein clearance from plasma through a mechanism that involves hepatocites receptors for the asialo galactose-terminal complex molecules. In contrast terminal GalNAc-4-sulfate residues or oligosaccharides bearing terminal mannose or GlcNAC accelerate the molecule clearance by specific receptors present in hepatic endothelial and Kupffer cells (*Fiete et al., 1991, Fiete et al., 1997, Dirnberger 2001*). As a consequence, heavily sialylated glycoproteins, such as FSH, have longer plasma half life and higher *in vivo* bioactivity, despite lower *in vitro* bioactivity,

(Dirnberger et al., 2001; Szkudlinski et al., 1995), than less sialylated molecules (e.g. LH) and, although the mixture of charge isoforms in serum contains most of the isoforms detected in pituitary extract (for example the spectrum of the sialylation variants of human FSH extracted from the anterior pituitary includes approximately 20 isoforms exhibiting both basic to very acid pH values), the strongly acidic isoforms predominate due to the rapid clearance of their less sialylated counterparts *(Ulloa-Aguirre et al., 2001-B)*.

Several studies performed by Bogdanove and associates showed that androgen treatment altered the B/I ratio, (B) bioactivity, (I) immunoreactivity, suggesting for the first time that changes in the endocrine milieu could modify the gonadotropin structure. Further studies detected that the molecular weight of pituitary gonadotropins differed depending on the endocrine status of the donor animal *(Bogdanove et al., 1974; Peckham et al., 1973)*. Subsequently, significant variations in the relative abundance of intrapituitary and circulating gonadotropin glycoforms have been documented in several physiologic conditions characterized by profound changes in the endocrine environment to which the pituitary is exposed, including puberty (*Phillips et al., 1997; Padmanabhan et al., 1992*), the menstrual cycle (*Padmanabhan et al., 1988; Wide et al., 1993; Anobile et al., 1998*), and senescence *(Wide, 1985; Wide, 1989; Anobile et al., 1998)*. For example, intrapituitary LH and/or FSH are more acidic/sialylated in males and in older individuals of both sexes, and circulating gonadotropins in the postmenopausal state (a physiologic estrogen- deprived condition) are more sialylated and circulate for longer periods of time than those from women at reproductive age *(Ulloa-Aguirre et al., 2001-B)*. Several studies have also detected the occurrence of significant changes in the relative distribution of the glycosylated variants of serum LH and FSH throughout the menstrual cycle, with greater proportions of less acidic forms released during midcycle than in the early-, mid-follicular, and luteal phases.

1.3.2 Bovine glycoprotein hormone common Ŭ-subunit

In Figure 4 the bovine Ŭ-subunit amino acid sequence is compared to the Ŭ-subunits from 18 mammals. Table 2 reports the pair-wise comparisons (bovine vs other species) for amino acid sequences of these animal species.

```
                  10         20         30         40         50         60
BOVINE    FPDGEFTMQG CPECKLKENK YFSKPDAPIY QCMGCCFSRA YPTPARSKKT MLVPKNITSE
BUFFALO   ---------- ---------- ---------- ---------- ---------- ----------
OVINE     ---------- ---------- ---------- ---------- ---------- ----------
PIG       ---------- ---------- ----LG---- ---------- ---------- ----------
GOAT      ------M--- ---------- ---------- ---------- ---------- ----------
DEER      ------M--- ---------- ---------- ---------- ---------- ----------
DOG       ---------- ---------- ----LG---- ---------- ---------- ----------
RABBIT    ------A--- ---------- ----LG---- ---------- ---------- ----------
TIGER     ---------- ---------- ----LG--V- ---------- ---------- ----------
CAT       ---------- ---------- ----LG---- ---------- ---------- ----------
KANGAROO  ------I--- ---------- ----LG---- ---------- ---------- ----------
RAT       L---D-II-- ---------- ----LG---- ---------- ---------- ----------
MOUSE     L---D-II-- ---------- ----LG---- ---------- ---------- ----------
WHALE     --B-Z---Z- --Z----ZB- ----LG---- Z--------- ---------- ---------Z
DONKEY    -------T-D -------K-- ----LGV--- ---------- ---------- ----------
MACACO    ---------D -----PR--- F----G---- ---------- ----V----- ---Q--V---
UISTITI   L------AEE ---------- ---RLGS--- ---------- ----L--Q-- ------V---
HORSE     -------T-D ------R--- --F-LGV--- --K------- -------R-- ----------
HUMAN     AP-////V-D ----T-Q--P F--Q-G---L ---------- ----L----- ---Q--V--E

                  70         80         90     96
BOVINE      ATCCVAKAFT KATVMGNVRV ENHTECHCST CYYHKS
BUFFALO     ---------- ---------- ---------- ------
OVINE       ---------- ---------- ---------- ------
PIG         ---------- -------A-- ---------- ------
GOAT        ---------- ----T----- ----D----- ------
DEER        ---------- ---------- ----D-H--- ------
DOG         ---------- -------AK- ---------- ------
RABBIT      ---------- -------AK- ---------- ------
TIGER       ---------- -------AK- ---------- ------
CAT         ---------- -------AK- ---------- --H--I
KANGAROO    ---------- -----D--KI ---------- ------
RAT         ---------- -------A-- ---------- ------
MOUSE       ---------- -------A-- ---------- ------
WHALE       ---------- ------BA-- Z---Z----- ------
DONKEY      ---------I RV-L---I-L ----Q-Y--- --H--I
MACACO      S------SL- RVM---S--- ---------- -----F
UISTITI     S-------Y- -------I-- ---------- --H--F
HORSE       S--------I RV-----IKL ----Q-Y--- --H--I
HUMAN       S-------SYN RV----GFK- ----A----- ------
```

Figure 4

Glycoprotein hormone Ŭ-subunit amino acid sequence of 18 mammalian species in comparison to bovine amino acid sequence. Cys residues forming the four intra-chain disulfides bridges are highlighted in yellow.

Table 2

Identity percentage between glycoprotein hormone Ŭ-subunit of 18 different mammalian species in comparison to bovine Ŭ-subunit.

Species	N°aa different	Identity percentage (%)
Buffalo	0	100
Ovine	0	100
Pig	3	97
Goat	3	97
Deer	3	97
Dog	4	96
Rabbit	5	95
Tiger	5	95
Cat	6	94
Kangaroo	6	94
Rat	7	93
Mouse	7	93
Whale	14	85
Donkey	16	83
Macaco	16	83
Uistitì	16	83
Horse	20	79
Human	25	74

In general, the glycoprotein hormone Ŭ-subunit amino acid sequence is specie-specific. Nonetheless, the Ŭ-subunit of bovine, ovine and buffalo have the same sequence. Furthermore, all the ten cysteine residues, forming four intra-chain disulfides bridges, are conserved among mammalian species, suggesting that the glycoprotein hormone Ŭ-subunit have similar tridimensional structure in different species (see section 1.3.4**)**.

In the bovine species, the glycoprotein hormone common Ŭ-subunit is encoded by a gene composed of four exons and three introns (gene ID:280749). The gene is localized on chromosome 9 and has 22 kb dimension.

The mature protein is composed of 96 amino acids, with five disulphide bridges at Cys_{11}-Cys_{35}, Cys_{14}-Cys_{64}, Cys_{32}-Cys_{86}, Cys_{36}-Cys_{88}, Cys_{61}-Cys_{91} positions and two N-glycosidic linked

oligosaccharides, at Asn[56] and Asn[82]. The calculated molecular weight (Mr) is 10791.5 and the isoelectric point (pI) 8.70. However, subunit glycosylation determines a significant difference between the Mr and pI theoretical values and experimental data *(Ulloa-Aguirre et al., 2001-A).*

1.3.3 Bovine glycoprotein hormone β-subunits

As mentioned before the ß-subunit is unique for each hormone determining itôs biological activity. In Figure 5 the β-subunit amino acid sequences of bovine LH, FSH and TSH are compared. Table 3 reports the pair-wise comparisons for amino acid sequences of these three bovine hormone β-subunits.

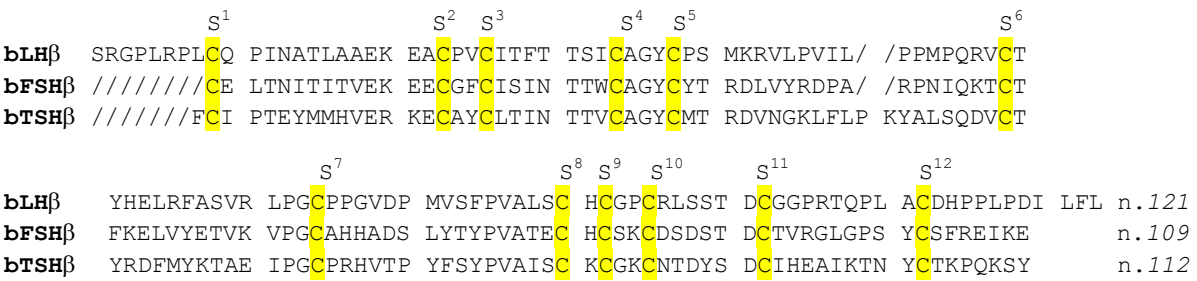

Figure 5

Amino acid sequence of the ß-subunits of bovine LH, FSH e TSH, aligned to mixmimise identity. Yellow box highlight the Cys residues forming 12 intrachain disulfides bridges (S^1-S^{12}).

Tabel 3

Number of amino acid identitity between β-subunits of bovine LH, FSH and TSH and identity percentage respect to LHβ sequence.

Hormone	Conserved aa	Sequence identity (%)
LHβ vs FSHβ vs TSHβ	25	22%
LHβ vs FSHβ	(25 +13) = 38	31%
LHβ vs TSHβ	(25 + 6) = 31	26%
FSHβ vs TSHβ	(25 + 8) = 33	30%

Despite the low sequence homology (around ~30%) all twelve cysteine residues forming six intra-chain disulfides bridges, are conserved, suggesting that ϭ-subunit tridimensional structure is very similar in the three pituitary glycoprotein hormones (see section 1.3.4).

The bFSH ϭ-subunit contains two N-linked oligosaccharides while the bLH ϭ-subunit only contains one N-linked oligosaccharide.

1.3.3.1 Bovine LH β-subunit

In the bovine species, the LH β-subunit (bLHβ) is encoded by a gene composed of three exons and two introns (ID: 280839). The gene is localized on chromosome 18 and is rather short, more or less 1,3 kb.

The gene encode for a sequence of 121 amino acids with six disulphide bridges at Cys_{9}-$Cys_{57,}$ Cys_{23}-$Cys_{72,}$ Cys_{26}-Cys_{110}, Cys_{34}-$Cys_{88,}$ Cys_{38}-Cys_{80}, Cys_{83}-Cys_{100} and one glycosilation site (N-glycosidically linked oligosaccharide) at Asn^{13}. The theoretical Mr is 12972,4 and pI 8,30. As the β-subunit is glycosylated these theoretical Mr are different from experimental values.

In Figure 6 the bLHβ amino acid sequence is compared to the bLHβ from 14 different mammal species. Table 4 reports the pair-wise comparisons (bovine vs other species) for amino acid sequences of these species.

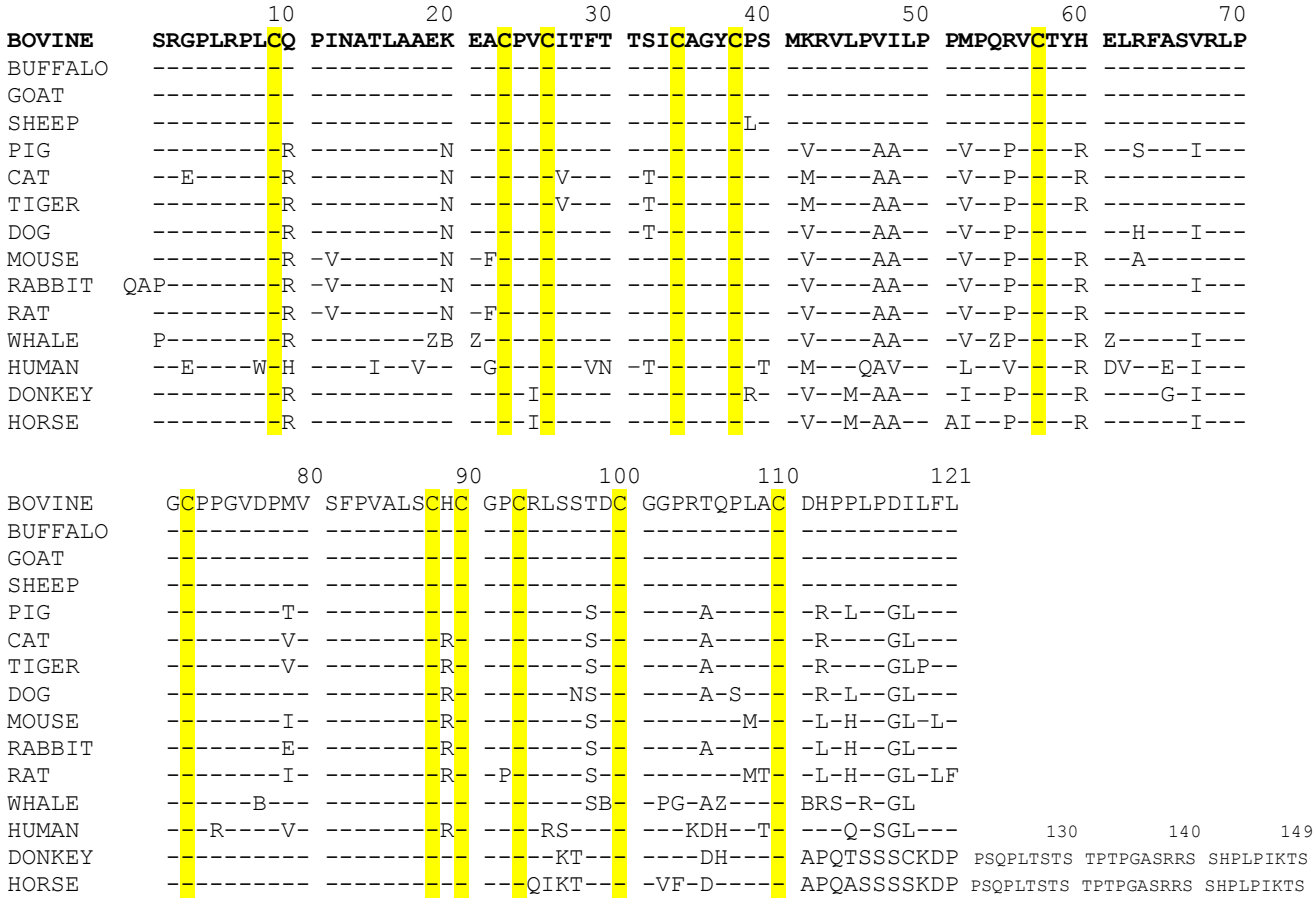

Figure 6

LH ɓ-subunit amino acid sequence of 14 mammalian species compared to bovine LH ɓ-subunit. Cys residues forming the six intrachain disulfides bridges of the ɓ-subunit are highlighted in yellow.

Table 4

Identity between LH ɓ-subunit of 14 different mammalian species in comparison to bovine LH ɓ-subunit

Specie	N°aa differing	Identity (%)
Buffalo	0	100
Goat	0	100
Ovine	1	99
Pig	17	86
Cat	18	85
Tiger	18	85
Dog	19	84
Mouse	20	83
Rabbit	21	83
Rat	22	82
Whale	27	78
Human	34	72
Donkey	55	55
Horse	57	53

The sequences of bovine, buffalo and goat bLHβ are identical and differ from sheep LHβ at one residues. The sequences of any of these LHβ differ from the other terrestrial mammalian sequences by 17-22 residues, and from that of human LHβ by 34 residues.

It is interesting to notice that equine and donkey LHβ, contain a 28 amino acid peptide at the C-terminal responsible of the low percentage of identity with the other mammalian species (total of 149 amino acid sequence vs 121 of all mammalian species). Moreover, the additional 28 amino acid peptide at C-terminal is reach of glycosylated Ser residues making the equids LH (eLH) the only LH with O-glycosidically linked oligosaccharides *(Bousfield., 2001; Galet et al., 2009)*.

This particular eLHɓ structure is very similar to the ɓ-subunit of the chorionic gonadotropin (CGβ). In fact both human CGɓ and equine CGɓ are formed of a 145-149 aminoacidic sequence and contains O-glycosidically linked oligosaccharides at their C-terminals *(Fares, 2006; Galet et al., 2009)*.

The longer ɓ-subunit sequence and the presence of O-linked oligosaccahrides are responsible of the considerably longer half life of the CG (about 36 h) compared to LH (30 min) *(Matzuk et al.,1990; Murphy et al., 1991; Allegra, 2011)*. As eLHɓ and eCGɓ are encode by the same gene *(Sherman et al., 1992)* it is reasonably to hypothesize that eLH has a longer half life compared to the other mammalian LH.

1.3.3.2 Bovine FSH β-subunit

In the bovine species, the FSH β-subunit (bFSHβ) is encoded by a gene composed of three exons and two introns (ID: 281171). The gene is localized on chromosome 15 and is more or less 4 kb.

The gene encode for a sequence of 109 amino acids with six disulphide bridges at Cys_1-Cys_{49}, Cys_{15}-Cys_{64}, Cys_{18}-Cys_{102}, Cys_{26}-$Cys8_0$, Cys_{30}-Cys_{82}, Cys_{85}-Cys_{92} and two glycosylation sites (N-glycosidically linked oligosaccharides) at Asn^5 and Asn^{22}. The theoretical molecular weight is

12972,4 and pI 8,30. As β-subunit is glycosylated theoretical molecular weight and pI are different from experimental values.

In Figure 7 the bFSHβ amino acid sequence is compared to the FSHβ sequences from 12 different mammalian species. Table 5 reports the pair-wise comparisons (bovine vs other species) for amino acid sequences of these species.

There is no identical amino acid sequence even between very related animal families (ruminants). The sequences of any of these FSHβ differ from the other from 5 (pig) to 19 (dog) residues.

Table 5
Identity percentage between FSH ϐ-subunit of 12 different mammalian species in comparison to bovine LH ϐ-subunit.

	Number of different Amino acids	**Identity (%)**
Bufalo	7	93
Goat	11	90
Ovine	9	91
Pig	5	95
Cat	9	91
Tiger	11	90
Dog	19	82
Mouse	13	88
Rabbit	12	89
Rat	15	86
Human	12	89
Equine	10	91

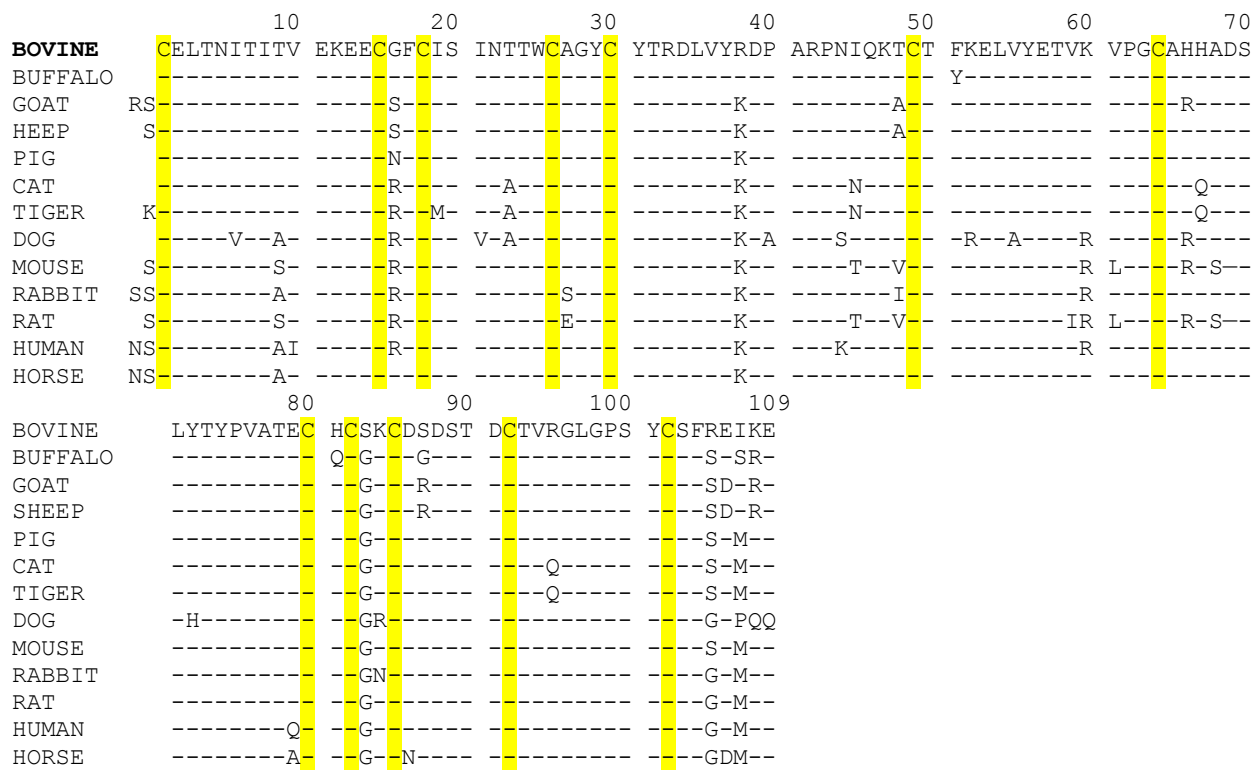

Figure 7

FSH ɓ-subunit amino acid sequence of 12 mammalian species in comparison to bovine FSH ɓ-subunit. Cys residues forming the six intrachain disulfides bridges of the ɓ-subunit are highlighetd in yellow.

1.3.4 Bovine LH and FSH Ŭβ heterodimers

The Ŭ and β subunits of the glycoprotein hormones are non-covalently bound to form the biological active Ŭβ heterodimer. The three different β-subunits may combine with the same common Ŭ-subunit (*Pierce et al., 1971*). This data together with the fact that the β-subunit amino acid sequence maintain, in all three glycoprotein hormones, the same 12 Cysteine residues (Cys), suggest that some regions within the three-dimensional structure of the various β-subunits may be very similar among the glycoprotein hormones *(Ulloa-Aguirre et al., 2001-A)*. Therefore the non-similar regions are probably those to confer the immunological and biological specificity to the hormone.

The three-dimensional structure of human CG and FSH (hCG and hFSH) have been determine through X-ray crystallography (*Fox et al., 2001)*. In Figure 8 the crystal structure of hFSH is represented. The Ŭ- and β- subunits have similar folds, consisting on a central cysteine-knot motifs from which three β-hairpins extend. The two subunit associate in a head to tail arrangement, forming an elongated, slightly curved structure similar to that of hCG.

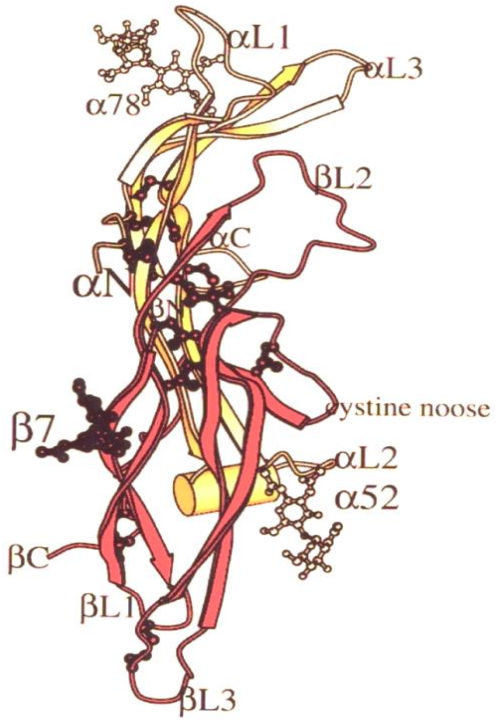

Figure 8
Overall fold of hFSH. The β-subunit is shown in red and the Ŭ-subunit in yellow. The carbohydrate and the disulfide bonds in both subunits are represented by ball-and-stick models. The N and C termini of each subunit, the N-glycosylated Asn residues and the loops are labeled.

No three-dimensional structure of gonadotropins hormones for other mammalian species has yet been determined, but alignment of amino acid sequence of both Ŭ- and ɓ- subunits (Figure 4, 6, 7) show the cysteine residues, forming the intra-chain disulfides bridges, are conserved, suggesting that the glycoprotein hormone have similar tridimensional structure in different species.

The bLH dimer has a theoretical Mr of 23745.89 and a pI of 8.60 while the bFSH as a theoretical Mr of 23072.4 and a pI of 7.47. However, since both subunits are glycosylated, experimental values significantly differ from theoretical Mr and pI.

1.4 Assays to determine LH and FSH in plasma

Various assay systems for the estimation of gonadotropin hormones in biological samples have been developed through years, and can be classified into two groups. Those that determine some response of a biological system to stimulation with LH or FSH (bioassays, both *in vivo* and *in vitro*) and those that estimate high affinity binding of gonadotropin hormones to molecules which exhibit specific properties of molecular recognition (immunoassays and receptor assays) *(Rose et al., 2000; Kalia et al., 2004).*

Independently from the type of assay, one of the major problems in gonadotropin measure is that often there are significant differences between results obtained by different methods. In fact, the concentration of gonadotropins measured in the same biological sample can significantly vary depending on the assay protocol and on the standard preparation used as reference. The reason is that each gonadotropin is intrinsically an heterogeneous mixture of different molecular variants, and assays can be selective for particular array of isoforms, as well as standard preparations can have a different isoform composition.

The use of international reference standards with a defined composition to which calibrate the FSH and LH assays is of great help in reducing this inter-assay variability. Several highly purified reference standards are now available for both human and animal gonadotropins. These standard hormone are provided by the National Institute of Diabetes and Digestive and Kidney diseases

(NIDDK) or the United States Department of Agriculture (USDA) through the National Hormones and Pituitary Program (NHPP, Baltimore, MD). However, despite the use of these international standards, discontinuity on quantification of gonadotropins in biological samples by different methods still occurs, especially using bioassays. It is thus essential that any measurement of gonadotropins include specification both of the standard with which the measured gonadotropins is compared and the assay method used for that comparison *(Rose et al., 2000)*.

1.4.1 Bioassay for gonadotropins

Gonadotropins were first identified and defined on the basis of *in vivo* endocrine activity and the assays developed for gonadotropins were based on classical endocrine principles. These early assays had two main drawbacks. First, there was no assay specific for FSH and LH, and second, quantification and hence between-laboratory comparisons were made difficult by a lack of standardization. However, with the advancement in biotechnology, various reliable *in vitro* bioassays were developed. Follicle-stimulating hormone bioassays have relied on measurement of aromatase activity in primary cultures of immature rat Sertoli cells or rat granulosa cells. Luteinizing hormone bioassays have relied on measurement of androgen production in primary cultures of rat interstitial testicular cells or mouse Leydig cells.

While sensitive and accurate, those bioassays are cumbersome and often lacking of analytical precision (high intra assay variability). The cloning of the cDNAs of FSH and LH receptors has allowed the establishment of cell lines expressing gonadotropin receptors. A permanent cell line is used avoiding primary culture of granulosa, Sertoli or Leydig cells, thus significantly improving reproducibility and simplifying laboratory procedures. Furthermore, no live animals are used. (*Christin-Maitre et al., 2000; Christin-Maitre et al., 1996)*.

Bioassay based on cloned receptors are now a valuable tool for both diagnostic and research applications, but have not so far enabled to routinely measure gonadotropins in plasma, and immunoassays (see section 1.4.2) are still used for most of the physiological and clinical studies on

serum gonadotropins *(Rose et al., 2000; Kalia et al., 2004)*. The immunoassays offer improvement in sensitivity, precision and convenience over bioassays, but cannot provide information about the biological activity of the gonadotropin measured *(Rose et al., 2000; Kalia et al., 2004*). In fact, the major advantage of bioassay over immunoassay is the ability to detect changes in the patterns and proportions of gonadotropin glycoforms which may be of biological and clinical significance.

Bioassay for the measurement of the activity of bovine pituitary gonadotropins were previously developed both by *in vivo* and *in vitro*. The bioactivity of bLH was measured *in vivo* by the ovarian ascorbic acid depletion assay *(Reichert, 1962; Courte et al., 1972; Perrera-Marin et al. 2004)*, quantitative hyperemia of Ellis *(Reichert, 1962)* and ventral prostate test in hypophysectomized rat *(Courte et al., 1972)*. *In vitro* by primary culture of dispersed mouse Leydig cells (*Dahal et al., 1993; Henricks et al, 1984; Stumpf el al., 1992*) and by the MA-l0 cell line, that is a clonal strain of mouse Leydig tumor cells that has receptors for LH and respond to LH stimulation by progesterone secretion (*Dahal et al., 1993; Baenzinger et al. 1992).*

The bioactivity of bFSH has been previously tested *in vivo* by the ovarian weight augmentation assay of Steelman and Pohely *(Reichert, 1971)*, *in vitro* by a method based on the estradiol produced from dispersed Sertoli cells *(Grimeck et al., 1979; Wu et al., 1993)*. The rat granulosa cell line FSHR-17 has been recently shown to be a reliable in vitro model for studying the FSH activity in the bovine species (*Borromeo et al., 2004*). This cell line was established by immortalisation of preovulatory rat granulosa cells by co-transfection of primary cells with SV40 DNA, Ha-ras gen and rat FSH receptor (r-FSHR) plasmid (*Keren-Tal et al., 1993*). The cells express approximately 27,000 r-FSHR per cell with a *K*d of 115 pM, and respond specifically to FSH by dose-dependent secretion of progesterone. The sensitivity of the FSHR-17 bioassay is one order of magnitude lower than in the classical primary Sertoli cell bioassay (Ritzen et al., 1982), but much better than in other bioassays based on cloned receptors (Christin-Maitre et al, 2000).

1.4.2 Immunoassay for gonadotropins

Since the development of the first radioimmunoassay (RIA) for insulin *(Yalow et al., 1959)*, the use of antibodies in endocrinological research has become widespread, and now various immunological assay methods have been developed for the measurement of gonadotropins in human as well in domestic animals *(Niswender et al., 1969; Adam et al., 1975; Matteri et al., 1987; Crowe et al., 1997; Ginther et al., 1999; Valares et al., 2007; etc)*. Immunochemical systems improved considerably the sensitivity and lowered inter-assay variation compared to bioassays, especially with the introduction of hybridoma techniques, which allowed the production of specific mAbs to the given hormone. In fact, the immunoassays using polyclonal antisera, often lack specificity and sufficient sensitivity to measure the relative low gonadotropin concentration in biological fluids (*Zou et al., 1991)*. Furthermore, conventional polyclonal antisera is hampered by Ŭ-chain identity within a given species, by ɓ-chain similarities, by cross-contamination of immunogens and/or standards with other hormones, as well as by batch to batch variations of polyclonal antisera. Monoclonal antibodies have the potential to overcome these problems *(Hojo et al., 1985)*.

Serum gonadotropins levels are often determined by radioimmunoassay (RIA), both in humans and animals (see section 1.4.2.1). However, even though RIA is a reliable and accurate method, the procedure uses radioisotopes ^{125}I as label, suffering of various disadvantages. In fact, there are safety consideration to take in account when using isotopic label, such as health hazards, restrictive regulations for radioactive waste disposal, more over there is immunological instability of the labeled substance, limited shelf-life of iodine tracers, limited licensing and expensive equipment *(Valares et al., 2007; Peclaris et al., 2003; Zou et al., 1991)*. Such constraints have necessitated the development of non-isotopic methods, such as enzyme immunoassay (EIA) (see section 1.4.2.1).

The selectivity of immunoassays is dependent upon the epitope specificity of the antibodies used in the systems. For the glycoprotein hormones, epitope specificity is dependent largely upon amino acid sequences and only to a minor extent on oligosaccharide structures (*Jeffcoate, 1993*). The

consequence is that immunoassays are largely blind to changes in the patterns and proportions of gonadotropin glycoforms.

There is considerable experimental evidence that glycosylation plays little part in the immunological properties of glycoprotein hormones. Luteinizing hormone, for example, has to be extensively deglycosylated before there is any effect on antigenic structure (*Sairam et al., 1988)*. The hormone erythropoietin (which is 40% glycosylated) can be completely deglycosylated without affecting its immunogenicity, (*Jeffocate, 1993*). For hCG also immunogenicity and epitope mapping are not affected by deglycosylation (*Schwarz et al., 1991)*.

It is possible that glycosylation could have subtle effects on conformation and thus antigenicity. Deglycosylation of TSH has been shown to have no effect on β-chain epitopes but certain Ŭ-chain epitopes were altered (*Papandreou et al., 1990)*. It should be recognized that complete removal of oligosaccharides is far from subtle and not representative of the physiological variants that occur.

The overall conclusion remains that glycosylation is immunologically unimportant. Incidentally it is worth pointing out that any subtle changes affecting antibody binding affinity are more likely to be revealed in traditional competitive RIAs than in the reagent excess two-site immunometric methods.

1.4.2.1 Immunoassay for bovine FSH and LH

Numerous immunoassays to quantify bLH and bFSH in the peripheral circulation have been described in literature. In Tables 6 and 7 are reported a schematic review of the RIAs and EIAs developed for the measurement of these two gonadotropins in the bovine specie. Tables highlight the technique, the standard and the antibody used by each author.

Table 6

Schematic review of the RIAs (A) and EIAs (B) developed for the measurement of LH in the bovine plasma. Tables highlight the technique, the standard and the antibody used by each author.

(A) RADIO-IMMUNO-ASSAYS FOR BOVINE LH	
REFERENCE	**METHOD**
Niswender et al., 1969	- Std and labeled hormone: bLH (LER-791-1) - In house antiserum anti-bLH and anti-rabbit gamma globulin
Golter et al; 1973	- Std: bLH (NIH-LH-B7) - First antibody: rabbit anti-bLH serum - Second antibody: goat anti-rabbit gamma globulin - Sensitivity 0.1 ng/ml
Adam et al., 1975	- Std: NIH-LH-S16 - Labelled hormone: ovine LH (LER-1056-C2) - Sensitivity 0.25 ng/ml
Zaied et al., 1980	- Modified from *Niswender et al., 1969.* - Standard: NIH-LH-B-8 - Label ^{125}I used instead of ^{131}I
Rawlings et al., 1984	- Modified from *Niswender et al., 1969.* - Labelled hormone: ovine LH (LER 1017-2) - First antibody in house produced - Sensitivity: 50 pg/ml
Hamernik et al., 1987	- Modified from *Niswender et al., 1969.* - Standard: NIH-LH-S-21 - Sensibility: 0.15 ng/tube
Schillo et al., 1988	- Modified from *Niswender et al., 1969.* - Std and labeled hormone: NIADDK-oLH-25 - Ovine LH antiserum (GDN-15) - Sensitivity 0.025 ng/tube
Wolfe et al., 1989	- Modified from *Adams et al., 1975.* - Standard: NIH-LH-B-7 - Labelled hormone: ovine LH (LER-1056-C2) - Rabbit antisera anti-oLH (TEA-RAOLH #35) - Sensitivity: 88 pg/ml
Chenault et al., 1990	- Modified from *Niswender et al; 1969.* - Standard: NIH-oLH-S24
Evans et al., 1992	- Modified from *Rawlings et al., 1984.* - Standard: NIDDK-bLH-4 - Sensitivity 0.1 ng/ml
Wolfe et al., 1992	- Modified from *Adams et al., 1975* and *Wolfe et al., 1989.* - Standard: NIH-LH-B7 - Labelled hormone: highly purified iodinated ovine LH (LER-1056-C2) - Antiserum anti-oLH in rabbits (JJRRaOLH #%I) - Sensitivity: 50 pg/ml
Imwalle et al., 1998	- Modified from *Hall et al., 1994.* - Labelled hormone: highly purified LH (LER-1374A), supplied by Dr. L.E. Reichart - Ovine LH antiseru AFP-192279, supplied by NHPAgency (Bethesda, MD) - Sensitivity: 0.05 ng/tube
Ginther et al., 1999	- Standard and labeled serum: USDA-bLH-B-6 - USDA-309-684 as the primary antiserum - Sensitivity 0.07 ng/ml
Honaramooz et al., 2000	- Modified from *Rawlings et al., 1984* and *Evans et al., 1992.* - Standard: NIDDK-bLH4 - Sensitivity 0.1 ng/ml
Hampton et al., 2003	- Modified from *Zaied et al., 1980* - Ovine LH antiserum TEA # 35 (J. J. Reeves, Washington State Univ., Pullman, WA).

Valares et al., 2007	- Fist antibody: Mab 0220-518b7 anti-bLH ϐ-subunit (518b7 antiLH) supplied by Dr Jan Roser - Labelled antibody: anti-ovine-LH (NIDDK, oLH-26; AFP 5551B) - Standards: oLH (NIDDK-oLH-I-4)
Atkins J.A., et al; 2008	- Modified from *Hampton et al., 2003.* - Primary antibody: antibovine LH monoclonal antibody (518B7; Matteri et al., 1987) - Sensitivity 0.06 ng/ml
Perry et al., 2008	- LH antisera (NHPP) - Sensitivity 0.125 ng/m
Palhao et al., 2009	- Modified from *Bolt & Rollins., 1983*, and *Ginther et al., 1999.* - Standard and labelled hormone: USDA-bLH-B-6 - Primary antiserum: USDA-309-684P - Sensitivity: 0.1 ng/ml
Ginther et al., 2010	- Modified from *Palhao et al., 2009* and *Hannan et al., 2010.* - Sensitivity: 0.06 ng/ml
Hannan et al., 2010	- RIA method as *Palaho et al., 2009* - Primary antibody: AFP192279 from National Hormones and Pituitary Program - Sensitivity: 0.12 ng/ml
Wankowska et al., 2010	- Double-antibody RIA for bLH - Standard: NIH-LH-B6 - Antisera: anti-rabbit-gammaglobulin - Sensitivity: 0.312 ng/ml
Giordan et al., 2012	- Modified from *Palhao et al., 2009* and *Hannan et al., 2010.* - Sensitivity: 0.12 ng/ml

(B) IMMUNOENZIMATIC ASSAYS FOR BOVINE LH	
REFERENCE	**METHOD**
Spearow et al., 1987	- different systems: - a) Three different standard: USDA-bLH-B5, bLH-LER-1072-7 supplied by Dr. Leo Reichert, oLH β-subunit supplied by Dr. Darrel Ward - Anti-oLH number 15 serum obtained from Dr. Gordon Niswender and Dr. Terry Nett. - Mouse monoclonal antibody clone 0220-518B7 anti-bLH β-subunit (518B7 anti-LH) obtained from Dr. Jan Roser - Chicken anti-oLH β-subunit Dl 1-61 (chicken anti-LH) produced by immunizing hens with oLH β-subunit.
Prakash et al., 2002	- Standard: Bovine LH (USDA-bLH-B-6). - Labeled Biotinylated Bovine LH (USDA-bLH-B-6). - Rabbit polyclonal anti-bovine LH antiserum (USDA- 309-684P) - First coating with goat IgG anti-rabbit IgG - Sensitivity 0.31 ng/ml
Schneider et al., 2002	- Standard bLH was provided from Biotrend (Cologne, Germany) - Polyclonal rabbit anti-bLH mAb - Labelled Mab 518 B7 against bLH - Sensitivity 0.03 ng/ml
Faure et al., 2005	- Standard: oLH CY1083 - Monoclonal antibody to bovine LH β-subunit 518B7 - Biotinylated monoclonal antibody to human α-subunit - Sensitivity: 0,1 ng/ml

Table 7.

Schematic review of the RIAs (A) and EIAs (B) developed for the measurement of FSH in bovine plasma. Tables highlight the technique, the standard and the antibody used by each author.

(A) RADIO-IMMUNO-ASSAYS (RIA) FOR BOVINE FSH	
REFERENCES	**METHOD**
Bolt and Rollins., 1983	- Std and labeled hormone: USDA-FSH-BP-1 - Antiserum: USDA-5-0122
Hamernik et al., 1987	- NIADDK-oFSH radioimmunoassay kit
Garverick et al., 1988	- Standard: USDA-bFSH-B-1 - Labelled hormone: NIAMMD-oFSH-I-1 - Antiserum anti-ovine FSH: NIAMMD-anti-oFSH-1 - Sheep anti-rabbit gamma globulin
Henderson et al., 1989	- FSH antiserum: NIAMDD-anti-oFSH-I (AFP-C5288113) - Standard: NIAMDD-oFSH-RP-1 - Labelled hormone: NIAMDD-oFSH-I-1 (AFP-5679C) - Sensitivity: 0.05-0.1 ng/tube
Chenault et al., 1990	- NIADDK-oFSH radioimmunoassay kit
Adams et al., 1992	- Modified from *Bolt and Rollins 1983.* - Standard: USDA-bFSH-B-1 - Labelled hormone: USDA-FSH-BP-3 - Antiserum anti-bovine FSH: USDA 5-pool - Sensitivity: 5 ng/ml
Cupp et al., 1995	- Standard and labelled hormone: oFSH (LER-1976-A2) - Rabbit antisera o-FSH (JAD-RAoLH n. 17-6 7,9) - Sensitivity 45 pg/ml
Gong et al.,1995	- Anti ovine FSH serum:NIDDK-anti-oFSH-1 - Standard: USDA-bFSH-I-2 - Labelled hormone: NIDDK-oFS-I-1 - Sensitivity: 0.11 ng/ml
Crowe et al., 1997	- anti-FSH antibodies: NIDDK anti-ovine FSH, JAD anti-ovine FSH, USDA anti-bovine FSH - Standard: USDA bFSH-B-1/I-2 - Labelled hormones: USDA-oFSH-I-2, LER 1976a ovine FSH, USDA bFSH-I-2
Hampton et al., 2003	- Modified from *Garverick et al., 1988.* - Standard: NIAMMD-oFSH-RP-1
Lane et al., 2008	- Modified from *Crowe, et al., 1997.* - Standard: USDA-bFSH-I-2 - Labelled hormone: ovine FSH AFP-4117A - Antisera: NIDDK anti-ovine FSH (AFP-C5288113) - Sensitivity: 0,01 ng/ml
Palhao et al., 2009	- Modified from *Bolt & Rollins 1983*, and *Adams et al. 1992.* - Standard and labelled hormone: USDA-bFSH-I-2 - Primary antiserum: NIDDK-anti-oFSH-1 - Sensitivity: 0.02 ng/ml

(B) IMMUNOENZIMATIC ASSAYS (EIA) FOR BOVINE FSH	
REFERENCE	**METHOD**
Prakash et al., 1999	- Standard: USDA-bFSH-1-2 - Antisera: sheep IgG and anti-bovine FSH beta (USDA-5 pool) - Sensitivity: 0,125ng/ml
Zou et al.,1991	- Standard: purified bFSH from Institute of Zoology (Chinese Acad Sci) - In house produced mAbs - Sensitivity: 1 ng/ml

Despite the relatively high number of publications reporting the measure of bFSH and bLH, the two gonadotropins in bovine plasma are currently measured only in a limited number of specialized laboratories. The first reason is that no reliable kits can be purchased on the market, and therefore all immunoassay have to be developed in house. Second, that antibodies as well as hormone standards to set up such immunoassays are almost exclusively provided in small and defined quantity by the USDA. Third, that bFSH and bLH are species-specific (both amino acid and oligosaccharides differs among species), limiting the accurate hormone detection to homologous assays.

As reported above, a major problem with the development of LH and FSH immunoassays in the bovine species is the limited availability of pure bLH and bFSH to be used as immunogen and/or reference standard. The only source of bLH and bFSH is in fact the pituitary gland, since no recombinant protein is available. Biologically active bLH has been obtained from CHO cells (*Kaetzel et al., 1985; Kaetzel et al., 1988)*, and biologically active bFSH from plant and insects *(Van De Wiel et al., 1998; Dirnberger et al., 2001)*, but the process did not reach the efficiency required for large-scale production. The use of recombinant human gonadotropins is hampered by the fact that anti-bLH and anti-bFSH antibodies bind with very low affinity to human gonadotropins *(Kofler et al., 1981; Matteri et al., 1987)*. Furthermore, because LH, TSH and FSH have similar structures and overlapping charge, their separation by methods based on acid-base separation is problematic and often the LH and FSH preparations recovered are contaminated with other hormones.

Finally, despite the many advantages of mAbs over polyclonal antibodies, very few mAbs have been produced to animal LH and FSH. To the best of our knowledge, only two reports have been published on the production and characterization of anti-bLH mAbs *(Kofler et al., 1981; Matteri et al., 1987)*, and only one of these mAbs (i.e: mAb 518B7, *Matteri et al., 1987*) resulted suitable for application in ELISA (*Matteri et al., 1987*; *Spearow et al., 1987; Schneider et al., 2002*). As well, while mAbs against hFSH are widely available, mAbs generated against bFSH are much less common *(Miller et al., 1987; Zou et al., 1991)*, and they have never been used for developing an immunoassay.

2. AIM OF THE STUDY

In cattle plasma bLH and bFSH are currently measured only in a limited number of laboratories, using non-commercial immunoassays, developed using antibodies and/or reference hormones provided from external research institutions. Furthermore despite the well-known advantages of mAbs over polyclonal antibodies and their significant contribution to the development of modern research in endocrinology in both human and veterinary medicine, very few mAbs have been produced to bovine LH and FSH.

The aim of the present study was therefore to develop and validate two novel species-specific mAb-based ELISAs for measuring bLH or bFSH in bovine plasma, based on reagents (antibody and reference standard) produced in our laboratory, thus to ensure long-term continuity in large-scale operation with this ELISAs. Specifically, the objectives were: (1) to produce a panel of anti-bLH and anti-bFSH mAbs by immunizing with standard hormones obtained from the USDA; (2) to select mAbs suitable for immuno-affinity chromatography purification of bLH and bFSH from pituitary glands; (3) to combine the mAbs and the purified gonadotropins to develop two ELISAs for bLH and bFSH detection.

3. MATERIALS AND METHODS

3.1 MAbs production and characterization

BALB/c mice were immunized with bFSH obtained from Fitzgerald Industries International (Concord, MA, USA) or with bLH from USDA (USDA-bLH-B-6, kindly provided by Dr. A.F. Parlow through the USDA Animal Hormone Program, Germoplasm and Gamete Physiology Laboratory, Beltsville Agricultural Research Center, Beltsville. MD, USDA). Reference standard of USDA-bTSH-I-1 and USDA-bFSH-I-2 were also kindly provided by Dr. A.F. Parlow.

The protocol was approved by the Animal Experimentation Ethic Committee of the State University of Milan and consisted in immunizing BALB/c mice, weighing 10-12 g. by two intramuscular injections of 50 and 25 μg of bLH or bFSH in 150 μL saline solution containing 12 μg AbISCO-100 adjuvant (ISCONOVA AB, Uppsala, Sweden) with a three-week interval. They then received a booster intramuscular injection of 25 μg of bLH or bFSH in saline-AbISCO-adjuvant four days before fusion.

Splenic lymphocytes were fused with P3X63Ag8U.11 myeloma cells using poly(ethylene glycol) 1450 (PEG solution 50%, Hybri Max, SIGMA) according to a standard procedure *(Secchi et al., 1991)*.

The so produced hybridomas were screened for their ability to bind respectively bLH and bFSH in enzyme immunoassay. For screening we used a direct ELISA, where ninety-six-well microtiter plates were coated with bLH or bFSH (0.1 μg/well) in Na2CO3ï NaHCO3 buffer 50 mM, pH 9.6, 50 ul/well; overnight at 4 °C. The plates were then washed with PBS-T, and saturated with 100 ul/well of BSA, 1% in PBS-T (1 h at 37 °C). After washing with PBS-T, hybridoma culture medium was added (50 ul/well) and the plates were incubated at 37 °C for 3 h. They were then washed again with PBS-T and incubated with 50 ul/well of rabbit anti-mouse immunoglobulins-alkaline phosphatase conjugated (Sigma-Aldrich, Milan, Italy), 1/2000 in PBS-T containing BSA 0.1%; 1.5 h at 37 °C.

The wells were washed with PBS-T and the enzymatic reaction was carried out by adding 50 ul/well of freshly prepared p-nitrophenyl phosphate (pNPP) 1 mg/ml in 10 mM diethanolamine (pH 9.8) containing 0.5 mM MgCl2. The reaction was let proceed for 1 h at 37°C and then the absorbance was measured at 405 nm using an automated microtiter plate reader (SpectraCount, Canberra Packard, Milano, Italy).

Hybridomas found to be positive against bLH were also screened for binding to bFSH (USDA-bFSH-I-2) and bTSH (USDA-bTSH-I-1), while hybridomas positive against bFSH were also screened against bLH (USDA-bLH-B-6) and bTSH (USDA-bTSH-I-1). For screening a direct ELISA was again performed. 96-well plates were coated with the hormone (0.1 μg/well) and, after incubation and washing, hybridoma culture medium was added. The binding of mAbs to the adsorbed hormone was revealed with AP-conjugated rabbit anti-mouse immunoglobulins (Sigma-Aldrich, Milan, Italy) as described above.

The antibody class of mAbs was determined by enzyme immunoassay using the mouse-hybridoma subtyping kit (Life Technology, Italy).

Positive clones, producing antibodies belonging to the IgG class, were subcloned by limiting dilution and expanded in culture to obtain antibodies. The so obtained monoclonal antibodies were precipitated from the culture supernatants with 50% ammonium sulphate solution, dialyzed against 10 mM phosphate buffer, 0.148 M NaCl, pH 7.2 (PBS) and purified by affinity chromatography on HiTrap r-Protein A columns (GE Healthcare Life Science, Italy). The dissociation constant (Kd) of each mAb to his antigen was determined by the immunoenzymatic method described by Schots *(Schots et al., 1988).*

According to their characteristics four mAbs: two anti-bLH ɓ-subunit (N6H7 and N3G8), one anti-bFSH ɓ-subunit (A3C12) and one anti-Ŭ subunit (D2H1) were selected for further studies. Samples of each (0.5 mg) were conjugated with HRP using the periodate method (*Tijssen., 1985*). MAbs N3G8 (0.5 mg) and D2H1 (0.5 mg) were conjugated with biotin *(Secchi et al., 1988)*, fractionated and stored at -80°C.

3.1 Epitope mapping

Bovine LH and bFSH antigenic epitope mapping was performed by a labeled antibody competition test (LACT) *(Aston et al., 1985*). Serial dilutions of unlabeled mAb were added to bLH or bFSH-coated plates (0.05 µg/well) to displace the mAb-HRP from the immobilized hormone. The labeled mAb was used at a dilution giving absorbance of about 1.0 in the absence of the competing mAb (B_0) under the conditions used for the assay. The percentage of competition was established on the basis of the EC_{50} calculated from each curve *(Borromeo et al., 2003)*. We took the EC_{50} obtained using the same mAb as competing and labeled mAb as 100%.

3.3 Immunoaffinity purification

HiTrap NHS-activated HP columns (1 mL; GE Healthcare Life Science, Italy) were used. According to the manufacturer's directions we coupled 10 mg of the protein A purified mAb-N6H7 and A3C12 for bLH and bFSH purification respectively. Monoclonal antibodies were chosen according to their characteristics. The derivatized columns were stored in PBS.

Bovine pituitary glands were collected just after slaughter in a local abattoir and immediately frozen, then stored at -20°C until required. The pituitaries were not selected for age, sex, reproductive status or breed of the donor. Crude bFSH and bLH extracts were obtained from bovine pituitaries following the first steps of the procedure described by Reichert *(Reichert et al., 1975).* Briefly, 100 g of pituitaries were homogenized in distilled water at 4°C and extracted for 8h at pH 5.5. After centrifugation (4500 x g, 1h) supernatant and precipitate were separated. The supernatant containing bFSH was precipitated with 1.8 M ammonium sulphate and the so obtained supernatant was precipitated a second time with 3 M ammonium sulphate. The first precipitate, containing bLH, was extracted at pH 4.0 with 0.1 M ammonium sulphate for 3h. The supernatant from centrifugation was recovered and precipitated the first time with 3 M ammonium sulphate at pH 4.0 and the second time with 3.5 M ammonium sulphate at pH 4.0. The bFSH and bLH precipitates were recovered and

dialyzed against several changes of distilled water for 24h. The precipitates were removed by centrifugation (10000 x g, 30 min), while the supernatants (bLH-1 or bFSH-1) were adjusted to 10 mM phosphate, 0.148 M NaCl, pH 7.2.

The crude hormone fractions, bLH-1 and bFSH-1, were then loaded onto the N6H7-affinity column and A3C12-affinity column respectively, at a flow-rate of 1 mL/min. The columns were then washed with 5-10 column volumes of PBS and the bound bLH or bFSH were eluted with citrate buffer 0.1 M, pH 3.0. The fractions containing the eluted bLH (N6H7-bLH) and bFSH (A3C12-bFSH) were immediately neutralized with TRIS 1M and concentrated to 0.5 ml in PBS 10 M, pH 7.2 using Amicon Ultra-4 concentrators (10000 MWCO, Millipore Corporation, MA, USA). The columns were re-equilibrated with PBS between batches.

Purified bLH and bFSH were fractionated and stored at -80°C. The protein content of each fraction was determined by the method of Lowry *(Lowry et al., 1951).*

3.4 Protein characterization

3.4.1 Biological activity

bLH-biological activity: The N6H7-bLH biological activity was determined *in vitro* on MA-10 cells (a kind gift from Dr. Mario Ascoli, University of Iowa, Iowa City, IA), a mouse Leydig tumor cell line responding specifically to LH by dose-dependent secretion of progesterone.

Cells were maintained with standard techniques *(Ascoli, 1981; Swinnen et al., 1991)* in RPMI medium with penicillin (100 U/ml), streptomycin (100 µg/ml) and 15% horse serum (HS). For bioassay, cells were cultured in 0.5 ml medium containing 15% HS for 48 h on NUNC 24-well plates (0.5×10^5 cells/well). After this, medium was removed and the cells were washed with DPBS. After medium removal and DPBS washing bovine LH at various concentrations (0.5-250 ng/ml) was added in RPMI medium without serum and cells maintained in CO_2 incubator for 4h.

Progesterone accumulated in the culture medium was determined by a competitive enzyme immunoassay, using an in-house produced anti-P4 monoclonal antibody as capture antibody and progesterone-11-HS-HRP (Fitzgerald Industries International, Concord, MA, USA) as labeled hormone. The cross-reactivity of the antibody was 5% for testosterone and 0% for 17ß-estradiol. Assay sensitivity was 0.5 ng/ml. The range of the standard curve was from 250 to 0.5 ng/ml, and intra- and interassay coefficients of variation were 9.2 % and 12.4%.

Bioactive bLH was measured in triplicate and the experiment was replicated five times. The relative potency was interpolated from the standard curve plotted using the standard reference preparation USDA-bLH-B6.

Before statistical analysis, the dose-response curves were log-dose-transformed. Parallelism of the response curves generated by the various bLH preparations was determined by comparing the slopes and the y intercept of the regression lines fitting the data points within the linear range of the curves (ie, 1-2.5 ng/ml) with a statistical method included in the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The method tests the null hypothesis that the slopes are all identical: if the P value (two-tailed) is greater than 0.05, it concludes that the lines are parallel. If slopes are indistinguishable the software calculate a second P value testing the null hypothesis that the y intercepts are equal. If also this second P value (two-tailed) is greater than 0.05, it concludes that the lines are identical. The ED_{50} of the curves was defined as the amount of bLH required to give 50% of the maximum response, under the conditions established for the assay with maximum response defined as the top of the dose-response sigmoid curve plotted with increasing concentrations of each treatment (all preparations gave rise to the same maximal stimulation) and was calculated using a 4-parameter log dose-response sigmoid curve fitting the whole range of bLH doses tested (0.5-250 ng/mL).

bFSH-biological activity: The A3C12-bFSH biological activitiy was determined in vitro on FSHR-17 cells *(Keren-Tal et al., 1993)*, a preovulatory rat granulosa cell line immortalized by co-transfection of primary cells with SV40 DNA, Ha-ras gen and rat FSH receptor (r-FSHR) plasmid.

The cells express approximately 27,000 r-FSHR per cell with a *K*d of 115 pM, and respond specifically to FSH by dose-dependent secretion of progesterone.

Cells were maintained on Petri dishes (100mm) (NUNC, Roskilde, Denmark) containing 8ml DMEM/ F12 medium (1:1) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 5% fetal calf serum (FCS). For bioassays, cells were cultured in 0.5 ml medium containing 5% FCS for 24 h on NUNC 24-well plates (0.5 x 10^5 cells/well) before hormone stimulation, which was done without changing the medium. Bioactive bFSH was measured in triplicate at four or five dose levels (from 140.0 to 8.7 ng/ml for A3C12-bFSH) and the experiment was replicated three times. The relative potencies were interpolated from the standard curve plotted using the standard reference preparations USDA-bFSH-I-2 and USDA-bFSH-B-1.

Progesterone accumulated in the culture medium after 24 h incubation with the appropriate treatment was determined by competitive enzyme immunoassay as described above for bLH bioassay. Statistical analysis was carried out as described for bLH bioassay.

3.4.2 SDS-PAGE and Western blot

SDS-PAGE was done both under reducing and non-reducing conditions in polyacrylamide gradient gel (7%-20%) overlaid with 5% stacking gel. We used a Minigel apparatus (Biometra, Göttingen, Germany) and applied a constant current of 10 mA for 15 min and successively a constant voltage (200 V) for about 1 h (*Berrini et al., 1991).*

Gels were loaded with 1 µg of N6H7-bLH, 1 µg of USDA-bLH-B-6, 1 µg of A3C12-bFSH and 1 µg of USDA-bFSH- I-2 and stained with silver nitrate.

All samples, separated in SDS-electrophoresis in reducing and non-reducing conditions were transferred by Western blotting to nitrocellulose sheets. Blotting was performed at 350 mA for 2.30 h in transfer buffer (25 mM Tris, 192 mM Gly, 20% MetOH). After blotting nitrocellulose sheet was first washed in PBS-T and saturated with BSA 1% in PBS-T for 1 h. After washing the nitrocellulose sheet was immunodetected overnight with mAbs (mAb-D2H1 and N6H7, 10 µg/ml; mAbs N3G8 and

A3C12, 2 µg/ml). Nitrocellulose sheet was washed in PBS-T and HRP-labeled anti-mouse immunoglobulins was added for 3 h. After more PBS-T washing, a chemiluminescent substrate (LiteAbot PLUS, Euroclone, Pero (MI), Italy) was added and the chemiluminescence signal was measured with Bio-Rad Chemi-Doc (Bio-Rad, Italy).

3.4.3 Isoelectric focusing

IEF was carried out in 5% polyacrylamide pre-cast gels (Servalyt precotes, pH 3ï 10, 125 x125mm, 300 mm; SERVA, Heidelberg, Germany) at 7°C using Multiphor equipment (GE Healthcare Life Science, Italy). After pre-focusing (20 min at 6 mA), N6H7-bLH, A3C12-bFSH (10 µg both) and isoelectric point standards (SERVA Liquid Mix IEF Markers 3ï 10, SERVA, Heidelberg, Germany) were loaded, and the electrophoresis proceeded for another 3.5 h at 6 mA (2000 V). Gels were stained with silver nitrate.

3.4.4 NanoLC-ESI-MS/MS and protein sequencing

N6H7-bLH: Affinity purified bLH was identified using nanoLC-ESI-MS/MS (Proteome Factory AG, Berlin, Germany). The MS system consisted of an Agilent 1100 nanoLC system (Agilent, Waldbronn, Germany), PicoTip electrospray emitter (New Objective, Woburn, MA) and an Orbitrap XL mass spectrometer (ThermoFisher, Bremen, Germany), PicoTip electrospray emitter (New Objective, Woburn, MA) and an Orbitrap XL mass spectrometer (ThermoFisher, Bremen, Germany).

Protein bands obtained after SDS-PAGE under reducing conditions were trypsin-digested in-gel (Promega, Mannheim, Germany) and applied to nanoLC-ESI-MS/MS. Reduction of the disulphide bonds was deemed essential, as digestion of the nonreduced subunits results in large tryptic cores which are of little analytical significance. We also proceeded with treatment with DTT and iodoacetamide before the gel run, to alkylate cysteines and prevent incorrect disulphide pairs from reforming, which would have complicated trypsin digestion and mass assignments. Peptides

were trapped and desalted on the enrichment column (Zorbax SB C18, 0.3 x 5 mm, Agilent) for 5 min using 2.5% acetonitrile and/or 0.5% formic acid as eluant, then separated on a Zorbax 300 SB C18, 75 mm x 150 mm column (Agilent) using an acetonitrile and/or 0.1% formic acid gradient from 5% to 35% acetonitrile within 40 min. MS/MS spectra were recorded data-dependently by the mass spectrometer according to the manufacturerôs recommendations. Proteins were identified using the Mascot search engine MS/MS ion search (Matrix Science, London, England) and nr protein database (National Center for Biotechnology Information, Bethesda). Ion charges in search parameters for ions from ESI-MS/MS data acquisition were set to ñ1+, 2+, or 3+ò according to the instrumentôs and methodôs common charge state distribution.

A3C12-bFSH: Affinity purified bFSH was identified by N-terminal sequence analysis after SDS-PAGE and blotting onto PVDF membranes, following the method described by Dunbar (*Dunbar et al., 1994)*. The analyisis was kindly done by Dr. B. Dunbar (University of Aberdeen, Scotland).

3.4.5 Amino acid analysis

N6H7-bLH and A3C12-bFSH (2 ug) were hydrolyzed in 6 M HCl under vacuum at 105°C for 24h. The amino acid analyses were done by reversed-phase LC with pre-column derivatization with AccQ-fluor reagent, as indicated by manufactures (Waters AccQ-tag Chemistry Package, Instruction Manual Waters Corp) (Water Corporation, Milford, MA).

3.5 Development and validation of the ELISA for bLH

Polystyrene microtiter plates (96 wells, flat bottom; Maxisorp, Nunc) were used. For the first coating we added 1 µg of mAb-N6H7 dissolved in 100 µL of Na_2CO_3/$NaHCO_3$ buffer 50 mM, pH 9.6. The plates were incubated overnight at 4°C. After washing three times with 10 mM phosphate buffer, 0.3 M NaCl, 0.1% Tween 20 pH 7.2 (PBS-T), the plates were saturated for 1h at 37°C with 300 µL of BSA 1% in PBS-T. After washing with PBS-T, bLH (N6H7-bLH) and samples were

added (100 µL/well). Standard and samples were diluted in PBS-T containing 0.1% BSA. The plates were incubated overnight at 4°C. The next day plates were decanted and washed five times with PBS-T before addition of 100 µL of biotinylated mAb-N3G8 diluted 1/10000 in PBS-T, BSA 0.1%. The plates were incubated for 2h at 37°C with constant agitation. After five washes the wells were filled with 100 µL of avidin-peroxidase (0.1 µg/mL in PBS-T, BSA 0.1%) and further incubated for 2h at 37°C. The plates were washed five times and the enzyme substrate solution (3 mM o-phenylenediamine in 0.1M phosphate citrate buffer pH 5.0, 0.01% H_2O_2) was added (100 µL/well). The plates were incubated in the dark for 15 min at room temperature and the reaction stopped with 1 M H_2SO_4 (100 µL/well). Absorbance was measured at 490 nm with a microtiter plate reader.

3.5.1 Analytical specificity

The slope of the curve with N6H7-bLH was compared with the slope of the curves obtained using serial dilutions of USDA-bLH-B6, bovine plasma and bovine pituitary extract. The pituitary extract was prepared as previously described *(Secchi C., 1988)*, and assayed starting at the protein concentration of 0.8 mg/ml. The experiment was repeated 3 times and parallelism was assessed within each experiment using GraphPad PRISM 5.0 software package, as described in the bLH biological activity (see section 3.3.1). The percentage of cross-reactivity was calculated on the basis of the ED50 calculated from each curve.

3.5.2 Cross-reactivity

Serial dilutions of bFSH (USDA-bFSH-I-2), bTSH (USDA-bTSH-I-1), ovine-LH (USDA-oLH-I-2, kindly provided by dr. Parlow), human chorionic gonadotropin (hCG, Vetecor 2000 U.I.) and pig, horse, dog, and rabbit pituitary extracts, prepared as described previously for bovine extract, were tested in the ELISA starting at a protein concentration of 8 mg/ml. The hormones were diluted in PBS-T, BSA (0.1%). Cross-reactivity coefficients (%) were calculated as the bLH concentration divided by the cross-reactant concentration providing the same signal in ELISA.

3.5.3 Accuracy and reproducibility

Graded doses of bLH standard were added to a bovine plasma with known bLH content. The samples were tested by ELISA and the correlation between the measured and the added concentrations was established. Intra- and interassay coefficient of variation was calculated analyzing 5 bovine plasma samples. Plasmas were assayed several times either in the same or in different plates.

3.5.4 Clinical validation

The method was biologically validated by a gonadotropin stimulation test using a synthetic analog of GnRH (buserelin acetate) (Receptal, MSD Animal Health, Milan, Italy) in 4 Holstein Friesian heifers (15-month-old). The experiment was conducted at the Milan University Centro Zootecnico Didattico Sperimentale (Experimental zootechnical teaching station) (Lodi) and met the requirements of the Italian and European Community Commission for Scientific Procedures. The protocol was approved by the Animal Experimentation Ethics Committee of the State University of Milan. Ten days before the experiment, the 4 heifers were given a single intramuscular (i.m.) injection of 150 mg of a PGF2alpha analog (d-cloprostenol, Dalmazin, Fatro, Italy) during the luteal phase.

To minimize stress the animals were surgically implanted with an indwelling catheter in the jugular vein (Nutricath, Vygon, Ecouen, France). On the day after cannulation, the animals received an intravenous (i.v.) bolus injection of the GnRH analog buserelin acetate (20 µg/5 mL saline). Blood samples were collected into heparinized tubes starting 75 min before treatment, then every 15 min up to 6 h 30 min after treatment. The plasma was centrifuged at 2500 rpm for 10 min, divided and stored at -80°C until assayed.

Basal bLH concentration was calculated as the mean (± standard deviation [SD]) of the blood values before GnRH infusion. The start, end, and duration of the secretory peak were calculated as described by Merriam *(Merriam et al., 1982)*. The magnitude of the peak was the highest hormone

level achieved during the peak. The amplitude was expressed as the fold-increase of the magnitude from baseline. The total amount of LH released during the peak was expressed as area under the curve (AUC).

3.6 Development and validation of the ELISA for bFSH

The ELISA for bFSH was carried out substantially as described for bLH, with the difference of mAbs and standard used. The first coating was performed adding 1ug/100ul/well of anti-bFSH mAb-A3C12. bFSH standard and samples were diluted as described for the bLH ELISA. The second biotinylated antibody was the anti-Ŭ-subunit mAb-D2H1, diluted 1/1000. All the other steps were as described for bLH.

3.6.1 Analytical specificity

The slope of the curve with A3C12-bFSH was compared with the slope of the curves obtained using serial dilutions of USDA-bFSH-B1, bovine plasma and bovine pituitary extract. The pituitary extract was prepared as previously described (Secchi C., 1988), and assayed starting at the protein concentration of 0.2 mg/ml. The experiment was repeated 3 times and parallelism was assessed within each experiment using GraphPad PRISM 5.0 software package, as described in (see section 3.3.1). The percentage of cross-reactivity was calculated on the basis of the ED50 calculated from each curve.

3.6.2 Cross-reactivity

Serial dilutions of bLH (USDA-bLH-B-6) and bTSH (USDA-bTSH-I-1) were tested in the ELISA. The hormones were diluted in PBS-T, BSA (0.1%). Cross-reactivity coefficients (%) were calculated as the bLH concentration divided by the cross-reactant concentration providing the same signal in ELISA.

3.6.3 Accuracy and reproducibility

Graded doses of bFSH standard were added to a bovine plasma with known bFSH content. The samples were tested by ELISA and the correlation between the measured and the added concentrations was established. Inter-assay coefficient of variation was calculated analyzing one bovine plasma sample which had a measurable hormone concentration bovine plasma samples. Plasmas were assayed several times in different plates

3.6.4 Clinical validation

The method was biologically validated using the samples obtained by the gonadotropin stimulation test described for bLH clinical validation (see paragraph 3.4.4).

Basal bFSH concentrations were calculated as the mean (± standard deviation [SD]) of the blood values before GnRH infusion. The magnitude of the peaks was the highest hormone level achieved during the peak.

4. RESULTS

4.1 Production and characterization of anti-bFSH and anti-bLH mAbs

About 300 viable hybridomas clones were obtained after immunization with the purified bLH and bFSH we obtained in small amount from external laboratories (bLH-USDA-B-6 and bFSH-Fitzgerald Industries). At a first screening, 17 of these hybridomas resulted reactive to the bLH-USDA-B-6, and 20 to the Fitzgerald Companyôs pituitary bFSH.

A second screening was performed to assess the cross-reactivity of each produced mAb toward bFSH, bLH and bTSH, sharing a common Ŭ-subunit (Figure 9).

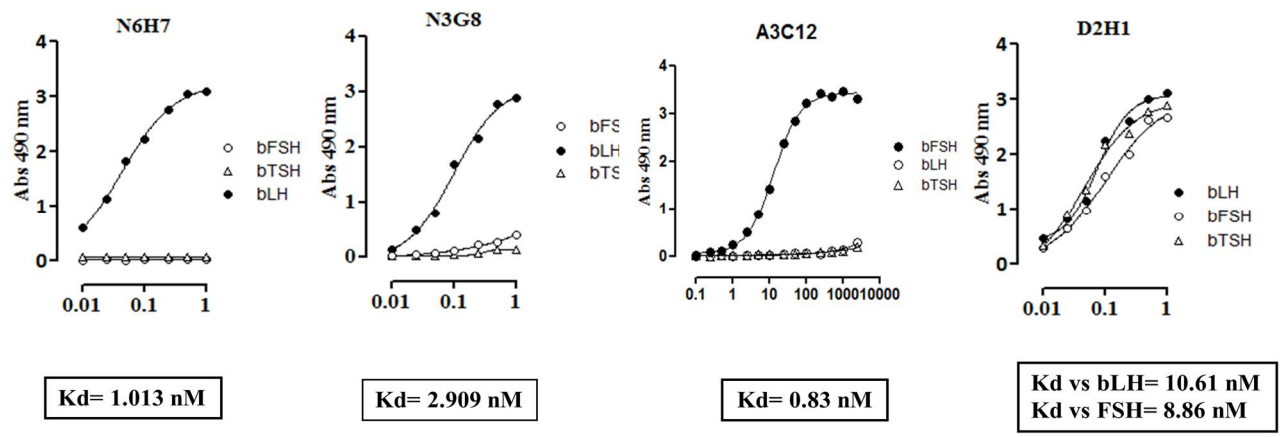

Figure 9

mAbs specificity measured by direct ELISA. Microtiter plates were coated with USDA-bLH-B-6 (ŏ), USDA-bFSH-I-2 (ʒ), or USDA-bTSH-I-1 (æ) and serial dilutions of 4 different mAbs were added. The bound mAbs were revealed with HRP-conjugated rabbit anti-mouse immunoglobulins. The means of triplicate wells in a single experiment are shown. The affinity (Kd) to the specific antigen of each mAb is reported in the box below the graph.

Two anti-bLH mAbs showed no reactivity to bFSH and bTSH (cross reactivity <0.1%), and were thus considered against the ß-subunit of bLH. This two mAbs were named anti-bLH mAb-N6H7 and anti-bLH mAb-N3G8.

Only one anti-bFSH mAb did not recognize bLH and bTSH (cross reactivity <0.1%), and was considered against the ß-subunit of bFSH. This mAb was named anti-bFSH mAb-A3C12.

These three mAbs (N6H7, N3G8 and A3C12) proved to be of the IgG_1 k subclass. They were therefore subcloned and purified. The affinity to their specific antigens was then measured. The Kd were 2.91 nM, 1.01 nM and 0.83 nM for N6H7, N3G8 and A3C12, respectively.

The remaining mAbs showed 100% cross-reactivity with bLH, bFSH and bTSH. They were therefore considered against an epitope located on the common pituitary glycoprotein hormones Ŭ-subunit. Of these Ŭ-subunit mAbs, mAb-D2H1 showed the highest reactivity and was therefore selected for further evaluation. Anti-Ŭ mAb-D2H1 proved to be of the IgG_1 k subclass with similar affinity to bLH and bFSH (Kd 10.061 nM and 8.86 nM respectively).

Table 8 shows the results of competition binding analysis of pairs of these four selected mAbs (N6H7, N3G8, A3C12, D2H1) to bLH and bFSH by the LACT test. The four mAbs recognized four non overlapping epitopes.

Table 8

Competition binding analysis of pairs of mAbs to bLH or to bFSH using the LACT assay. The assay involved the displacement of an HRP-labeled mAb (listed in the first row) from immobilized bLH or bFSH with increasing concentrations of a second unlabeled mAb (in the left-hand column). The EC50 of the self-inhibition curve was taken as 100%.

Competing mAb	HRP-mAb (inhibition %)			
	Anti-βLH N6H7	Anti-βLH N3G8	Anti-βFSH A3C12	Anti-Ŭsubunit D2H1
Anti-βLH N6H7	100.0	9.1	/	6.7
Anti-βLH N3G8	7.9	100.0	/	3.8
Anti-βFSH A3C12	/	/	100.0	-
Anti-Ŭsubunit D2H1	1	0.2	-	100.0

4.2 Purification and characterization of bLH and bFSH

The key step of the purification procedure was the mAb based immunoaffinity chromatography of bovine pituitary extract. The antibodies used to this purpose were the anti-βbLH mAb-N6H7 and the anti-βbFSH mAb-A3C12, for purification of bLH and bFSH respectively.

From 100 g of pituitary homogenate (about 50 pituitary glands), by subsequent precipitation steps we firstly recovered two crude extracts: 343.6 mg of crude bLH (bLH-1) and 337.5 mg of crude bFSH (bFSH-1).

These two pituitary extract were then loaded into the two immunoaffinity chromatography columns. For every chromatographic run we loaded about 50 mg of bLH-1 or bFSH-1.

In total, from 100 g of pituitary homogenate we recovered 1.58 mg of purified bLH (N6H7-bLH) and 400 µg of purified bFSH (A3C12-bFSH).

4.3 Characterization of purified A3C12-bFSH and N6H7-bLH

4.3.1 Biological activity

The biological activity of the purified N6H7-bLH was measured *in vitro*, using the MA-10 cell line. Figure 10 shows the linear regression analysis of progesterone production after 4 h incubation with increasing concentration of reference standard USDA-bLH-B-6 and the immunoaffinity purified N6H7-bLH.

The regression lines were calculated by fitting the points within the linear portion of the log transformed dose-response curve (1ï 25 ng/mL). There was no appreciable difference between the slopes and y intercepts of the dose-response lines (P > 0.1). Therefore we concluded that N6H7-bLH was as potent as the USDA-bLH-B-6, with specific activity 2.1 U/mg. The ED50 of both curves was 15.74 ng/ml. The specificity of progesterone production in response to bLH was demonstrated by the fact that these cells did not respond to USDA-bFSH-I-2 and USDA-bTSH-I-1 (data not shown).

The biological activity of A3C12-bFSH was measured *in vitro* with the FSHR-17 cell line (*Keren-Tal et al., 1993*). Figure 11 shows the linear regression analysis of progesterone production after 24 h incubation with increasing concentrations of reference standards USDA-bFSH-I-2 and USDA-bFSH-B-1 and the immunoaffinity purified A3C12-bFSH.

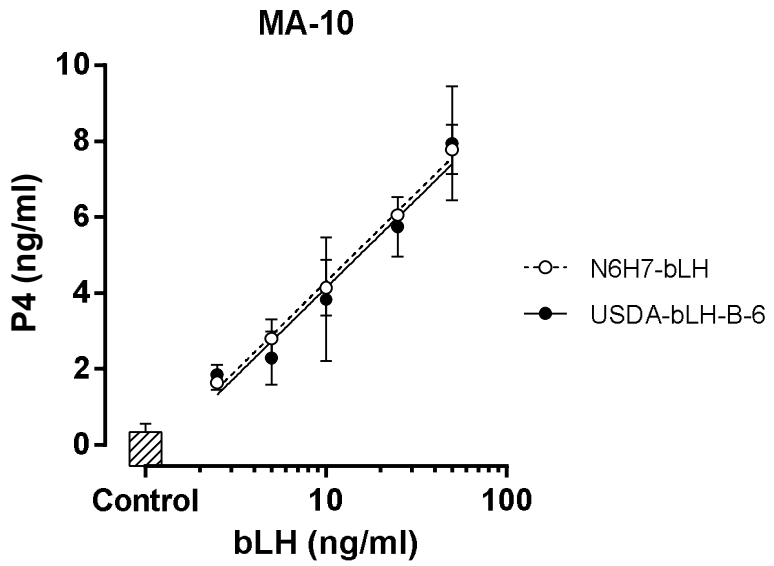

Figure 10

Linear regression analysis of the dose-related progesterone production by MA-10 cells in response to reference preparation USDA-bLH-B-6 and N6H7-bLH. Progesterone values are means ±SD of triplicate cultures from a representative experiment. The accumulation of progesterone in the absence of LH is shown by the hatched area (control).

There was no appreciable difference between the slopes of the lines after log transformation (P=0.25, F=1.54). This parallelism indicates the analytical specificity of the FSHR-17 bioassay. On comparing the EC50, we found that A3C12-bFSH was about 160 times more potent than USDA-bFSH-B-1 and 7 times less than USDA-bFSH-I-2 (A3C12-bFSH: EC50=31.3 ng/ml; USDA-bFSH-I-2: EC50=4.3 ng/ml; and USDA-bFSH-B-1: EC50=5013.4ng/ml). Considering the specific activity of the standard USDA-bFSH-I-2 (854 U/mg), the calculated specific activity of A3C12-bFSH was 117.3 U/mg. The specificity of the progesterone production in response to bFSH was demonstrated by the fact that these cells did not respond to USDA-bLH-B-6 and USDA-bTSH-I-1 (data not shown).

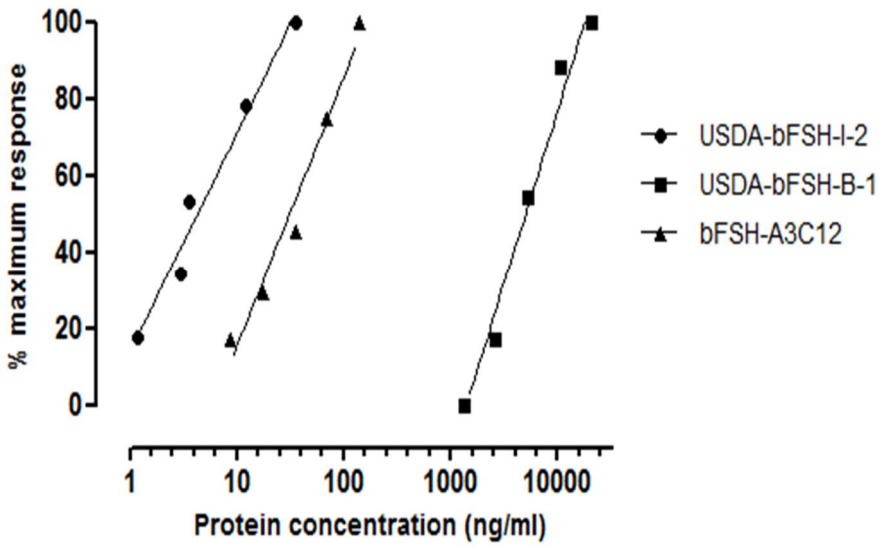

Figure 11

Parallelism in the FSHR-17 cell bioassay of the doseï response curves of USDA-bFSH-I-2 (36, 12, 4, and 1.2 ng/ml), USDA-bFSH-B-1 (21000, 10500, 5250, 2625, and 1312.5 ng/ml) and A3C12-bFSH (140, 70, 35, 17.5, and 8.7 ng/ml).

4.3.2 SDS- electrophoresis (SDS-PAGE)

In SDS-PAGE, purified N6H7-bLH (Figure 12) showed 2 major bands, both under reducing (lane 1) and non-reducing conditions (lane 3). The electrophoresis pattern exactly matched that of USDA-bLH-6 (lanes 2 and 4). The calculated molecular weights (Mr) of these bands were 20300 and 18690 in the reducing gel, and 21980 and 19500 in the non-reducing gel.

In SDS-PAGE purified A3C12-bFSH (Figure 12), showed only one band under reducing (lane 5) and two under non-reducing conditions (data not shown). The electrophoresis pattern of A3C12-bFSH exactly matched that of USDA-bFSH-I-2 (lanes 6). The single band has calculated Mr of 22358.

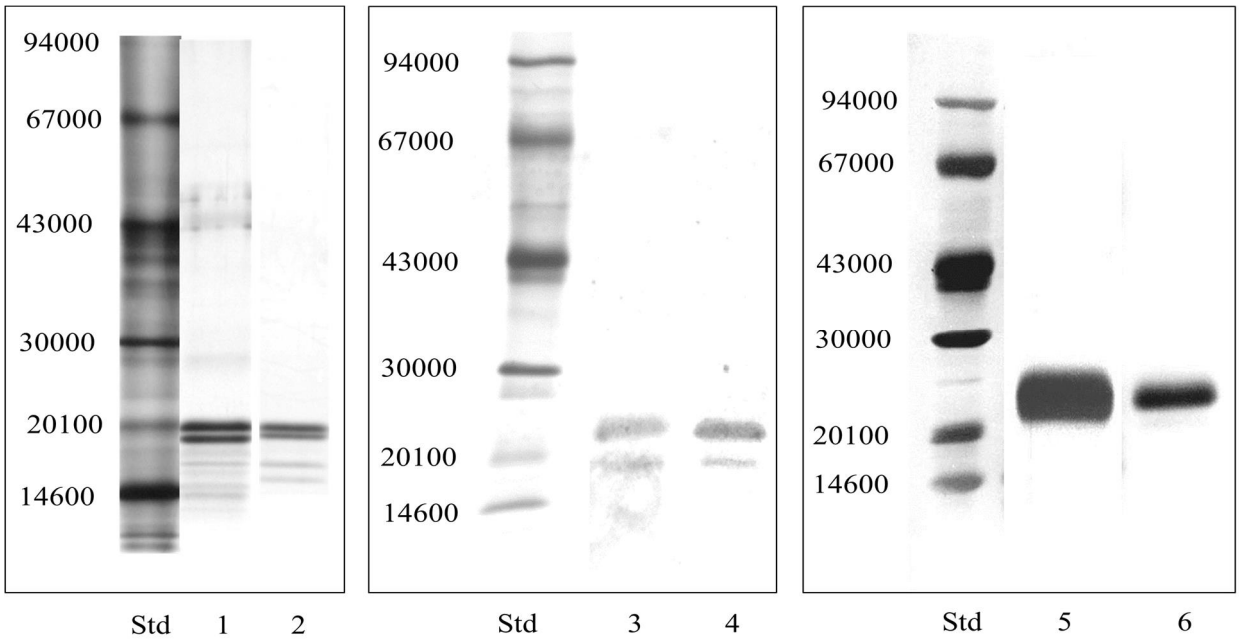

Figure 12

SDS-PAGE of purified bLH and bFSH. Hormone samples were electrophoresed on 7% to 20% gradient polyacrylamide gels under reducing or non-reducing conditions and were stained with silver nitrate. Lane 1: N6H7-bLH, after reduction; lane 2: USDA-bLH-6, after reduction; lane 3: N6H7-bLH, no reduction; lane 4: USDA-bLH-6, no reduction; lane 5: A3C12-bFSH, after reduction; lane 6: USDA-bFSH-I-2 after reduction. Std: Mr wt markers. The positions of the molecular-weight markers are indicated.

4.3.3 Western blot

Figure 13 shows the analyses in western blot with different mAbs of the purified N6H7-bLH (A and B) and A3C12-bFSH (C and D). Gel A and C were run under reducing conditions, and gel B and D under non-reducing conditions.

The anti-Ŭ subunit mAb-D2H1 recognized the bands at: Mr 20300 (lane 1, bLH after reduction), Mr 19500 (lane 4, bLH no reduction), Mr 22358 (lane 8, bFSH after reduction), Mr 20771 (lane 9, bFSH no reduction). The anti-bLHɓ mAb-N3G8 recognized the bands at: Mr 18690 (lane 3, bLH, after reduction), 21980 (lane 6, bLH no reduction).

The anti-bFSHɓ mAb-A3C12 recognized the bands: at Mr 22358 (lane 7, bFSH, after reduction), Mr 27640 (lane 11, bFSH no reduction).

Lanes 2 and 5 (bLH) were probed with a mixture of the anti-Ŭ subunit mAb-D2H1 and anti-bLHɓ mAb-N3G8. Lane 10 with a mixture of the anti-Ŭ subunit mAb-D2H1 and anti-bFSHɓ mAb-A3C12.

In western blotting, the anti-bLHβ mAb-N6H7 did not recognize any of the various bLH and bFSH bands, analyzed both under reducing and non-reducing conditions.

These data confirmed the ELISA results (see section 4.1), which indicated that mAb-D2H1 recognize an epitope on the glycoprotein common Ŭ-subunit, and mAb-N3G8 and A3C12 recognize epitopes localized on the ɓ-subunit of bLH and bFSH, respectively.

The western blot data also revealed that the Ŭ and the β subunits of bLH when analyzed under reducing or non-reducing conditions, inverted their relative positions on the gel. In the non-reducing gel the bLHɓ-subunit migrated more slowly than the bLHŬ-subunit, the reverse under reducing conditions. As for bLH also bFSH migrated in a different way under reducing and non-reducing conditions. Under reducing conditions only a single band for both Ŭ- and ɓ-subunit was evident, while in the absence of the reducing agent the two subunits migrated in two separate bands.

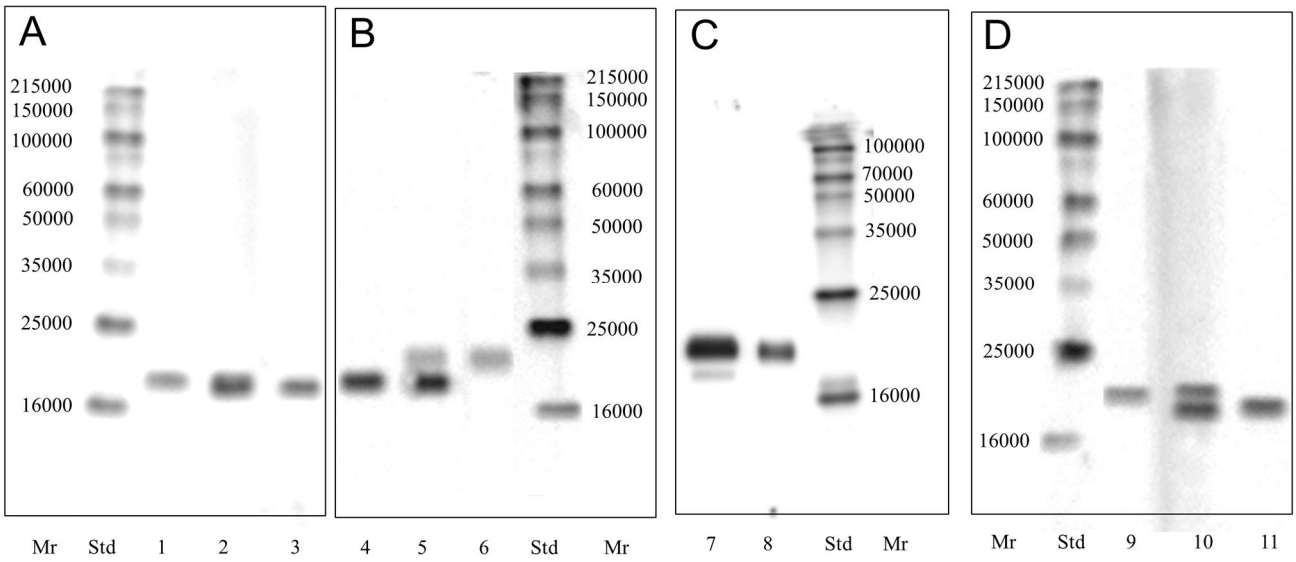

Figure 13

Western blot immunodetection of N6H7-bLH (gel A and B) and A3C12 b-FSH (gel C and D) in reducing (gel A and C) and non-reducing conditions (gel B and D). Gels were electro-transferred onto nitrocellulose, and the membrane was probed with anti-Ŭ mAb-D2H1, anti-bLHβ mAb-N6H7 and anti-bFSHβ mAb-A3C12, singly or combined. Lane 1: N6H7-bLH, after reduction, probed with anti-Ŭ mAb-D2H1; lane 2: N6H7-bLH, after reduction, probed with a mix of anti-Ŭ mAb-D2H1 and anti-bLHβ mAb-N3G8; lane 3: N6H7-bLH, after reduction, probed with anti-bLHβ mAb-N3G8; lane 4: N6H7-bLH, no reduction, probed with anti-Ŭ mAb-D2H1; lane 5: N6H7-bLH, no reduction, probed with a mix of anti-Ŭ mAb-D2H1 and anti-bLHβ mAb-N3G8; lane 6: N6H7-bLH, no reduction, probed with anti-bLHβ mAb-N3G8; lane 7; A3C12-bFSH, after reduction, probed with anti-bFSHβ mAb-A3C12; lane 8: A3C12-bFSH, after reduction, probed with anti-Ŭ mAb-D2H1; lane 9: A3C12-bFSH, no reduction, probed with anti-Ŭ mAb-D2H1; lane 10: A3C12-bFSH, no reduction, probed with a mix of anti-Ŭ mAb-D2H1 and anti-bFSHβ mAb-A3C12; lane 11: A3C12-bFSH, no reduction, probed with anti-bFSHβ mAb-A3C12. Std: Mr wt markers. The positions of the molecular-weight markers are indicated.

4.3.4 Isoelectric focusing (IEF)

Figure 14 shows the IEF analysis of N6H7-bLH (lane 1) and A3C12-bFSH (lane 2). The patterns show a large number of tight bands with pI ranging from 9.0 to 4.2 and from 8.0 to 4.5 respectively for N6H7-bLH and A3C12-bFSH, indicating extensive charge heterogeneity.

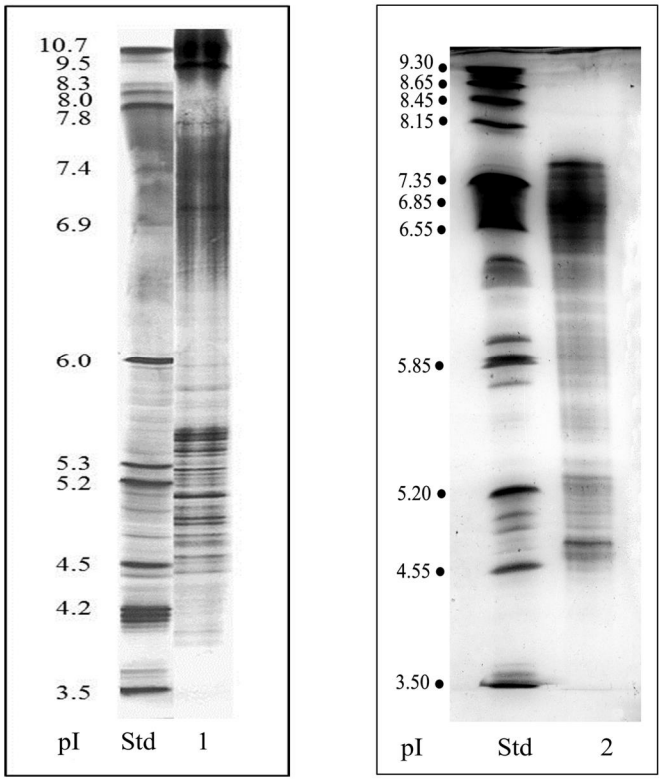

Figure 14

Analytical IEF in polyacrylamide gel stained with silver nitrate. Lane 1: N6H7-bLH; lane 2: A3C12-bFSH; Std: standard proteins; pI: Isoelectric points of standard proteins.

4.3.5 Protein identification

The two bands of N6H7-bLH obtained in the reducing SDS-PAGE were extracted and analyzed by nanoLCES MS/MS. The upper band (Mr 20300) corresponded to the Ŭ-bLH subunit, and the lower band (Mr 18690) to the ɓ-bLH subunit. Identification was unambiguous and no peptides not corresponding to a theoretical trypsin fragment of the bLH were detected.

The band of A3C12-bFSH obtained in the reducing SDS-PAGE was electro-blotted unto a PVDF membrane, cut and the protein extracted analysed by N-terminal sequencing. The first 10 amino acids yielded two sequences with an equal molar ratio. All the amino acids of the Ŭ-bFSH subunit were detected (F_{19}-P_{19}-D_{15}-G_{18}-E_{13}-F_{8}-T_{16}-M_{5}-Q_{6}-G_{7}, where the subscripts indicates the pmoles of amino acid for each sequencing cycle), while two residues were missing in the ɓ-bFSH subunit sequence (X_{0}-E_{31}-L_{20}-T_{21}-X_{0}-I_{12}-T_{16}-I_{10}-T_{6}-V_{9}, where X indicates an undetected amino acid). These gaps were expected, because the first residue in the mature ɓ-bFSH subunit is a C, and the fifth is a glycosylated N. Both Ŭ- and ɓ- bFSH sequences were obtained free of contamination by other amino acids.

4.3.6 Amino acid composition

Table 9 illustrates the amino acid composition measured for the purified N6H7-bLH and A3C12-bFSH. The numbers of each amino acid residue are in agreement with the expected bLH and bFSH composition (calculated on the Ŭand ɓ subunit complementary DNA sequences).

Table 9

Amino acid composition of purified N6H7-bLH and A3C12-bFSH. Data are Mol/mol of protein, mean (±SD) of 5 different preparations

Amino Acid	bLH Theoretical[a]	N6H7-bLH	bFSH Theoretical[a]	A3C12-bFSH
Asp	11	11.9±1.0	15	14.7±0.9
Ser	14	16.2±1.3	13	12.8±2.4
Glu	14	14.7±1.2	18	18.1±1.4
Gly	11	13.7±1.5	9	10.3±2.0
His	6	4.2±1.1	6	5.9±0.5
Arg	11	11.4±1.0	8	7.6±1.2
Thr	16	16.3±1.6	22	21.1±2.2
Ala	15	18.4±2.8	12	12.7±1.0
Pro	27	21.3±3.0	12	11.0±1.1
Cys	22	n.d.[b]	22	n.d.[b]
Tyr	7	6.9±0.6	12	10.9±0.9
Val	13	14.0±0.9	12	12.9±0.6
Met	7	5.9±0.5	4	3.6±1.1
Lys	12	14.8±1.9	16	15.1±0.6
Ile	7	6.4±0.3	8	7.4±0.6
Leu	15	14.0±0.7	7	7.6±0.3
Phe	9	6.7±0.5	8	8.1±0.8
Trp	0	n.d.[b]	1	n.d.[b]

[a] Mol/mol of protein based on the bLH- or bFSH- cDNA.
[b] n.d, not determined.

4.4 Development and validation of the ELISA for bLH

The combination of anti-bLHβ mAb-N6H7 adsorbed to the solid phase and the biotin-labeled anti-bLHβ mAb-N3G8 was used to develop a sandwich ELISA to measure bLH in bovine plasma. All other mAb combinations tested gave poorer analytical performances.

4.4.1 Analytical sensitivity

The bLH standard curve for the sandwich ELISA with mAb-N6H7 as capturing antibody and the labeled mAb-N3G8 to reveal the bound hormone is shown in Figure 15.

To check for plasma interference with the assay sensitivity, standards were diluted in either bovine plasma, which had a virtually undetectable amount of endogenous bLH (± 0.05 ng/ml), or PBS-T, BSA (0.1%). There were no significant differences between slopes and y intercepts of the 2 curves. The standard curve, plotted as T% vs log of ng/ml of bLH, was linear over the range 0.05 to 2.5 ng/ml. The regression coefficient was $r^2 = 0.99$ and the ED_{50} (x intercept when y = 50) was 0.27 ng/ml. The values plotted were the mean of 12 experiments ± SD and the coefficient of variation between the slopes was 2.88%.

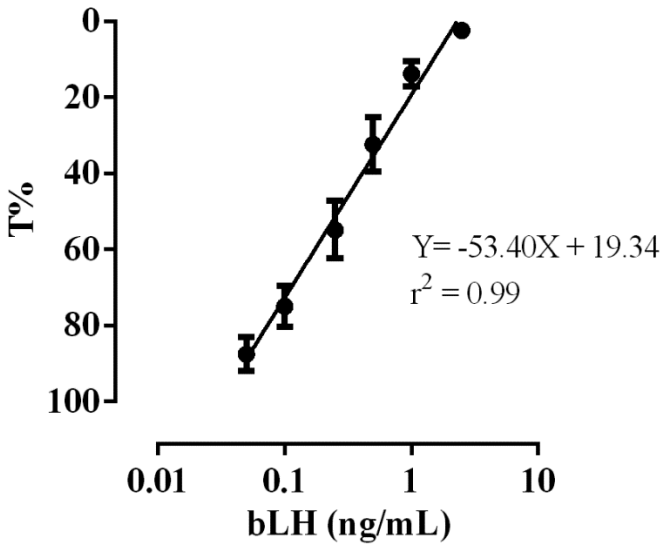

Figure 15

Standard curve for the sandwich ELISA of bLH, with mAb-N6H7 as solid phase antibody and the labeled mAb-N3G8 to reveal the bound hormone. Standards were diluted in PBS-T, BSA (0.1%). Points are plotted as T% versus log of ng/ml of the hormone. Each point is the mean of 12 experiments ±SD.

4.4.2 Analytical specificity

Dilutions of bovine pituitary extract, plasma and reference USDA-bLH-6 were compared with the dose-response curve for N6H7-bLH in this ELISA (Figure 16). There was no significant difference between the slopes, after log transformation of the dilutions ($P > 0.1$). The sandwich ELISA showed 99.95% cross-reaction with standard USDA-bLH-6.

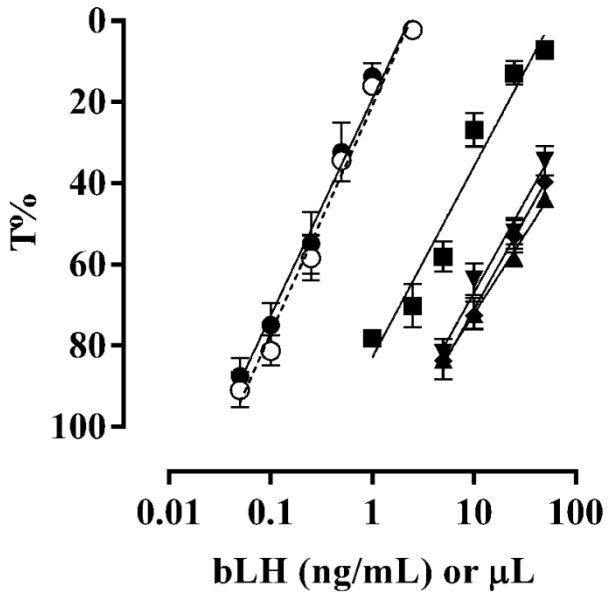

Figure 16

Regression lines obtained by serial dilution of the N6H7-bLH (ŏ), USDA-bLH-6 (ƺ), pituitary extract (y), and plasma from 3 heifers (ƶ , Ɛ , Dž. Dilutions ranged between 2.5 and 0.05 ng/ml for the two bLHs, 0.8 and 0.02 mg/ml for pituitary extract, and 1/5 to 1/50 for plasma samples. Points are plotted as T% versus log of ng/ml of hormone or ml of plasma and pituitary extract. The means of duplicate wells in a representative experiment are shown.

4.4.3 Cross-reactivity

Standard USDA-oLH-I-2 showed 99.5% cross-reactivity. Conversely, no cross-reactivity (Ò 0.1%) was observed with bFSH and bTSH (Figure 17).

No reactivity was observed with hCG, horse, dog, pig, rabbit, and mouse pituitary extracts, even when assayed at a protein concentration of 8 mg/ml, thus 400-fold higher than the lowest concentration of the bovine pituitary extract showing significant reactivity (0.02 mg/ml, Figure 16).

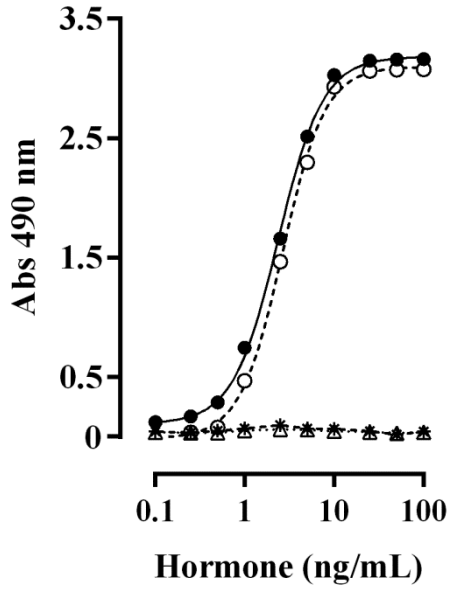

Figure 17

Cross-reactivity of the sandwich ELISA to bLH (ŏ), oLH (ƺ), bTSH (æ), and bFSH (*).

4.4.4 Accuracy

Various amounts of bLH were added to pools of bovine plasma, and the overall recovery was summarized in linear regression analysis (Figure 18). Recoveries ranged between 83.2% and 119.3%, and the slope of the regression was not different from unit (slope, 1.03 ± 0.01; y-intercept, - 0.04 ± 0.04; $r^2 = 0.99$, n = 67).

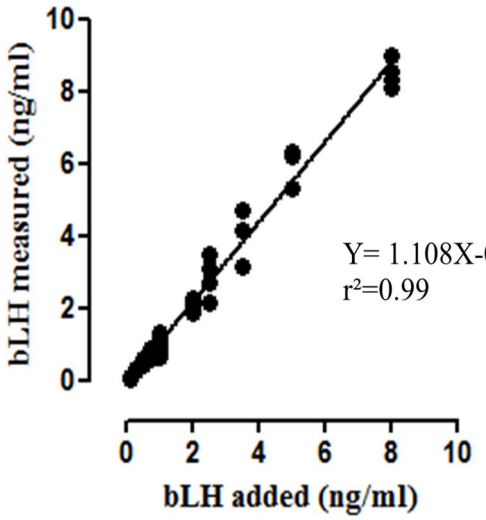

Figure 18

Linear regression with recoveries ranging between 83.2% and 119.3%.

4.4.5 Reproducibility

The intra- and inter-assay coefficients of variation were calculated by measuring the plasma LH concentration in 5 heifers (Table 10). Bovine LH concentrations ranged between 0.32 and 1.70 ng/mL. The intra-assay CV ranged between 3.41% and 9.40% and the inter-assay CV between 9.29% and 15.84%.

Table 10

Inter- and intra-assay variability of the ELISA for the detection of bLH in bovine plasma.

Animal	bLH ng/ml ± SD	Inter-assay CV (%)	bLH ng/mL ± SD (n)	Intra-assay CV (%)
1	1.67 ± 0.16 (27)	9.29	1.70 ± 0.09 (15)	5.49
2	1.54 ± 0.19 (28)	12.23	1.50 ± 0.12 (16)	8.30
3	1.50 ± 0.24 (28)	15.84	1.43 ± 0.13 (16)	9.40
4	1.44 ± 0.20 (28)	14.06	1.39 ± 0.05 (16)	3.41
5	0.35 ± 0.05 (31)	14.68	0.32 ± 0.03 (12)	8.99

Abbreviations: (n), number of determinations; SD, standard deviation

4.4.6 Clinical validation

The analytical specificity of the method was confirmed by stimulation tests for LH using GnRH (20 µg) in 4 heifers. As expected, plasma bLH rose significantly in all animals shortly after the injection (Figure 18), with some differences in the LH surge (Table 11). In general, plasma LH started to rise between 30 and 75 min after injection, reaching a peak at 120 to 150 min, and rapidly declined to pretreatment levels by 225 to 255 min. Intravenous injection of 5 ml saline did not change plasma LH levels throughout the test period.

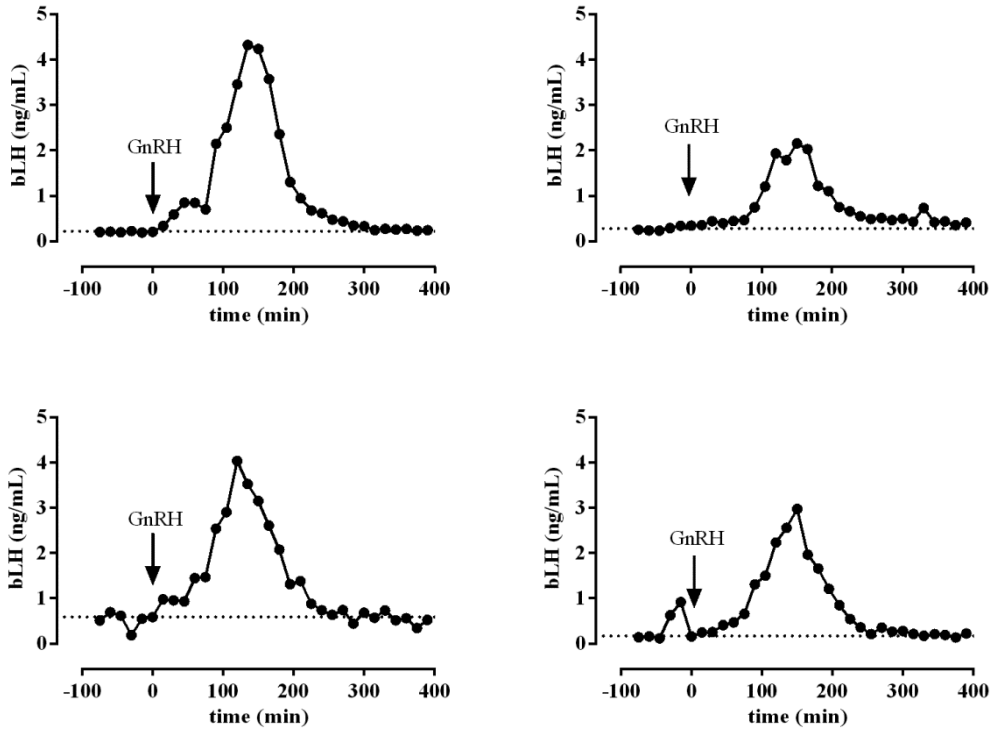

Figure 18

Plasma bLH patterns after injection of GnRH (20 μg, arrows) in 4 heifers. The horizontal line indicates the individual baseline bLH concentrations calculated as the means before GnRH.

Table 11
Individual baseline bLH concentrations and GnRH-induced peaks in 4 heifers.

Heifer No	Baseline (ng/ml)	GnRH induced bLH response						
		Start (min)	Stop (min)	Duration (h:m)	Magnitude		Amplitude (fold increase)	AUC[a] (ng/ml)
					Time (min)	Level (ng/ml)		
1	0.22 ± 0.05	30	255	3:45	135	4.32	20	29.60
2	0.28 ± 0.05	75	255	3:00	150	2.16	8	15.10
3	0.59 ± 0.07	60	255	2:45	120	4.03	7	27.33
4	0.17 ± 0.05	45	240	3:15	150	2.98	18	18.70

[a]AUC, area under the curve;

4.5 Development and validation of the ELISA for bFSH

The combination of the anti-bFSHβ mAb-A3C12 adsorbed to the solid phase and the biotin-labeled anti-Ŭ subunit mAb-D2H1 was used to develop a sandwich ELISA to measure bFSH in bovine plasma.

4.5.1 Analytical sensitivity

The bFSH standard curve for the sandwich ELISA with mAb-A3C12 as capturing antibody and the labeled mAb-D2H1 to reveal the bound hormone is shown in Figure 19. To check for plasma interference with the assay sensitivity, standards were diluted in either bovine plasma, which had a virtually undetectable amount of endogenous bFSH, or PBS-T, BSA (0.1%). There were no significant differences between slopes and y intercepts of the 2 curves. The standard curve, plotted as T% vs log of ng/ml of FSH, was linear over the range 0.25 to 10 ng/ml. The regression coefficient was r2 = 0.97 and the ED_{50} (x intercept when y = 50) was 1.65 ng/ml. The values plotted were the mean of 14 experiments ± SD and the coefficient of variation between the slopes was 3.51%.

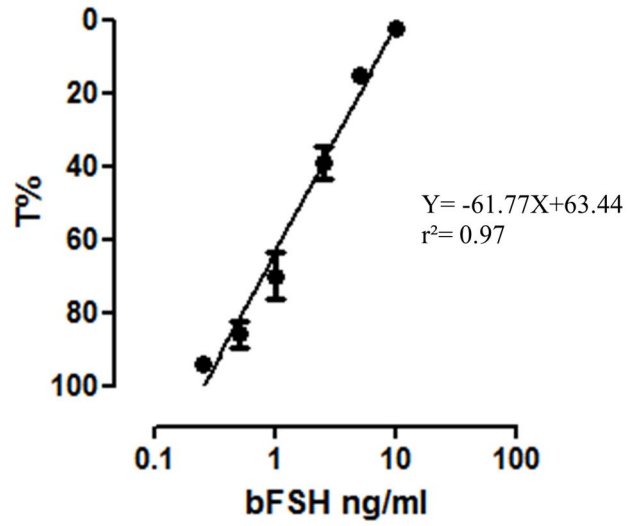

Figure 19

Standard curve for the sandwich ELISA of bFSH, with mAb-A3C12 as solid phase antibody and the labeled mAb-D2H1 to reveal the bound hormone. Standards were diluted in PBS-T, BSA (0.1%). Points are plotted as T% versus log of ng/mL of the hormone. Each point is the mean of 14 experiments ±SD.

4.5.2 Analytical specificity

Dilutions of reference standard USDA-bFSH-I-2, bovine pituitary extract and plasma were compared with the dose-response curve for purified A3C12-bFSH in ELISA (Figure 20).

There was no significant difference between the slopes, after log transformation of the dilutions ($P > 0.1$). The sandwich ELISA showed 84.7% cross-reaction with standard USDA-bFSH-I-2 .

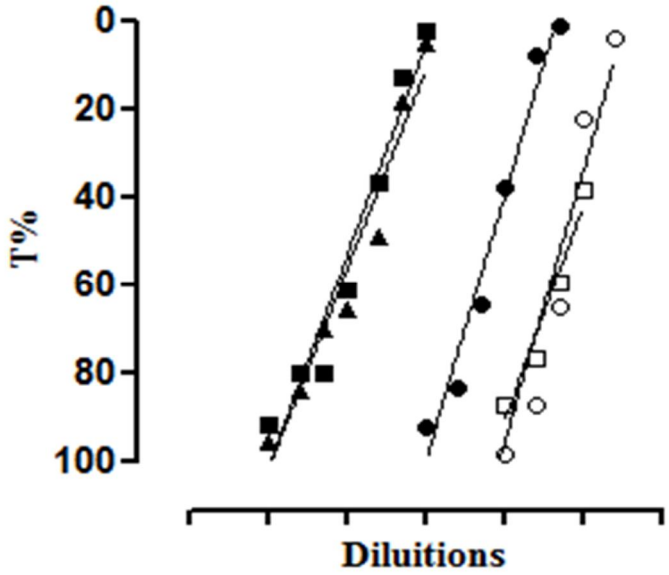

Figure 20

Regression lines obtained by serial dilution of theA3C12-bFSH (ƶ), reference standard USDA-bFSH-B-1 (ƴ), pituitary extract (ŏ), and plasma from 2 heifers (Ĭ , ƺ). Dilutions ranged between 0.25 and 10 ng/ml for A3C12 bFSH and USDAbFSH-B-1, 0.2 and 0.0002 mg/ml for pituitary extract, and 1/1 to 1/100 for plasma samples. Points are plotted as T% versus log of ng/ml of hormone or ml of plasma and pituitary extract. The means of duplicate wells in a representative experiment are shown.

4.5.3 Cross-reactivity

No cross-reactivity (Ò0.1%) was observed with bTSH and bLH (Figure 21), even though these three hormones share the same Ŭ-subunit and similar tridimensional structure.

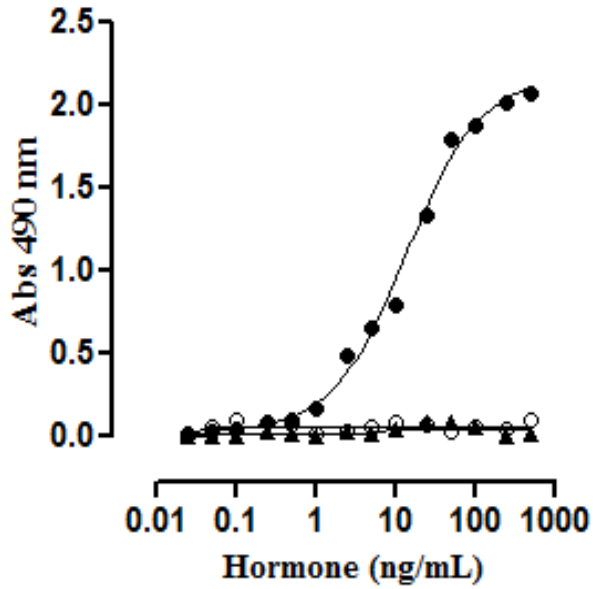

Figure 21

Cross-reactivity of the bFSH sandwich ELISA to bFSH (ŏ), bTSH (ʒ) and bLH (ƶ).

4.5.4 Accuracy

Various amounts of bFSH were added to pools of bovine plasma, and the overall recovery was summarized in linear regression analysis (Figure 22).

Recoveries ranged between 69.6% and 129.7%, with regression values: slope 0.77 ± 0.04; y-intercept, 0.39 ± 0.19; $r^2 = 0.97$; n = 14.

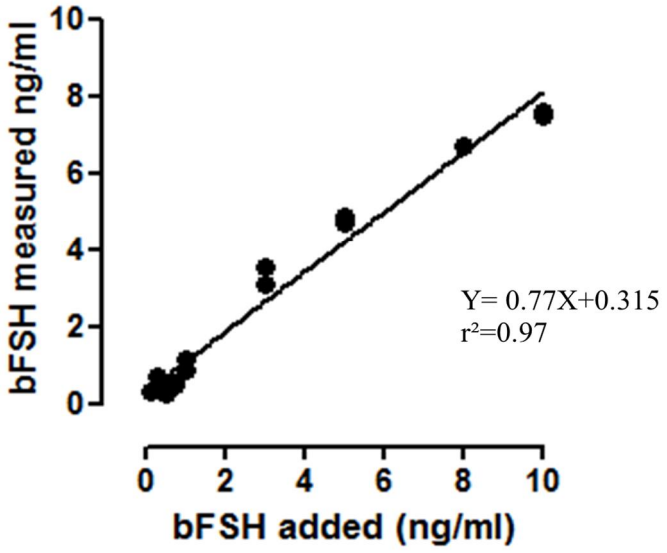

Figure 22

Linerar regression with recoveries ranging between 69.6% and 129.7%.

4.5.5 Reproducibility

The inter-assay coefficients of variation was calculated by measuring the bFSH concentration in a bovine plasma. bFSH concentration was 1.51 ng/ml ± 0.21 (SD) (n=15) with the inter-assay CV of 13.79%.

4.5.6 Clinical validation

Figure 23 shows the bFSH plasma patterns measured in four heifers, before and after GnRH stimulation. Secretion of bFSH was clearly pulsatile, and no clear secretory peak was evident after GnRH injection. Plasma hormone peaks were irregularly distributed throughout the 8h of plasma collection, were of variable length (15-60 min) and of small amplitude and magnitude.

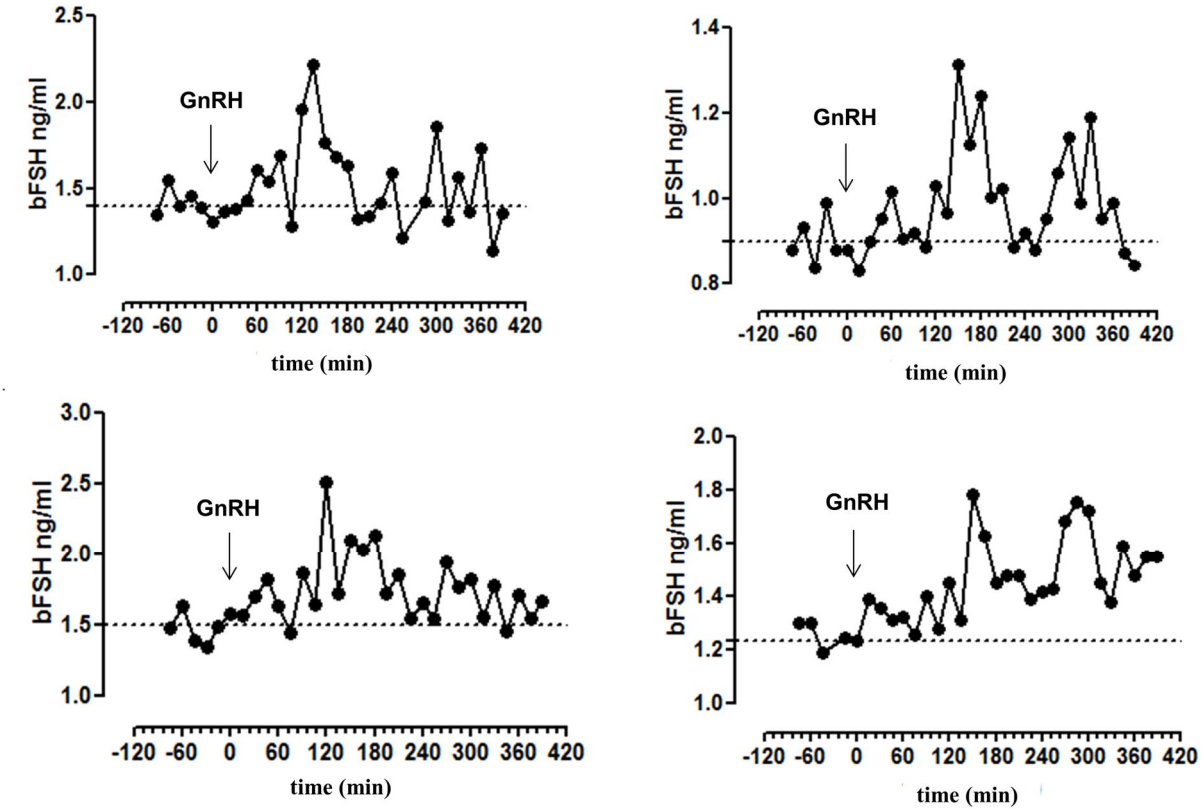

Figure 23

Plasma bFSH pattern after injection of GnRH (20 μg, arrows) in 4 heifers. The horizontal line indicates the individual baseline bFSH concentrations calculated as the means before GnRH.

5. DISCUSSION

Maximizing livestock reproductive potential is of primary importance in business farming, and itôs the reason for the increasing interest in the development as well as the application of innovative bio-techniques addressed to the improvement of *in vivo* and *in vitro* farm animal fertility. Most of the achievement in this field relies on the knowledge of the complex mechanisms involved in the control of follicular growth, oocyte maturation and early embryonic development. Pivot molecules regulating these aspects of animal reproduction are the pituitary gonadotropins. Methods able to study FSH and LH plasma patterns in the bovine species have therefore implications in improving cattle reproductive efficiency, for example by helping detection of dysfunction of the pituitary-ovarian axis, diagnosis of reproductive disorders, monitoring of antifertility programs and monitoring endocrine therapy.

However, the determination of gonadotropins in bovine plasma by immunoassay itôs not straightforward. It rely on in-house validated methods, developed by specialized laboratories with species-specific reagents which are provided in small quantity by International Institutions (e.g. USDA). In fact a bottle neck step for the development and large-scale application of bLH and bFSH immunoassays is the limited supply of pure bLH and bFSH to be used as immunogen and/or reference standard.

In the present PhD thesis we describe the production of a panel of anti-bLH and anti-bFSH mAbs and their successful application in the immunoaffinity purification of bLH and bFSH from pituitary glands. These reagents were then combined for the development of new homologous, specific, sensitive, and reproducible sandwich ELISAs for the measurement of the two gonadotropins in bovine plasma.

For the production of the mAbs against pituitary gonadotropins, we firstly requested small amounts of purified bLH and bFSH provided by international institutes. We then used these standard hormones as immunogens, to obtain a panel of species-specific anti-bLH and anti-bFSH mAbs. While monoclonal antibodies against human gonadotropins are widely available, in literature there

are only few reports on the production and characterization of mAbs to bovine FSH and LH. Specifically, two reports on the production of anti-bLH mAbs *(Matteri et al., 1987; Kofler et al., 1981)* and two on the development of anti-bFSH mAbs *(Miller et al., 1987; Zou et al., 1991).* Furthermore, to the best of our knowledge, no mAbs nor hybridoma clones to bovine gonadotropins are commercially available.

Among more than 300 hybridoma clones produced, we selected two clones secreting mAbs recognizing with high affinity two non-overlapping epitopes on the ß-subunit of the bLH molecule (mAb-N3G8 and mAb-N6H7), one clone producing mAbs to an epitope localized on the ß-subunit of the bFSH molecule (mAb-A3C12), and one clone secreting mAbs binding to the pituitary glycoprotein common Ŭ-subunit (mAb-D2H1). These epitopes were localized firstly by testing the cross-reactivity of each mAb toward bLH, bFSH and bTSH: mAbs reacting with the ß-subunit of one hormone did not cross-react with the other hormones, while mAbs binding to the Ŭ-subunit bound to the three bovine pituitary glycoprotein hormones with the same affinity. Second, by testing the ability of mAbs to recognize the specific subunits in immunoblotting. In fact, in SDS-PAGE, the subunits of bLH and bFSH migrated separately.

Immunoblotting analyses also allowed to acquire some information about the nature of the epitopes. We suggest that anti-bLHβ mAb-N3G8, anti-bFSHβ mAb-A3C12 and anti-Ŭsubunit mAb-D2H1 bind to stable epitopes, while anti-bLHβ mAb-N6H7 is conformation sensitive. In fact, mAbs N3G8, A3C12 and D2H1, but not N6H7, recognized the separate subunits even when the protein conformation was altered, as in SDS-PAGE after disulphide bridge reduction. Anti-bLHβ mAb-N6H7 might possibly recognize an epitope on the conformationally intact ß subunit or a structure that is only expressed in the holo-hormone but not in the free subunits. Further studies using isolated bLH subunits are needed to confirm this.

In general, mAbs recognizing denatured hormones, such as N3G8, A3C12 and D2H1, might have other applications besides their use for hormone subunit detection after blotting, such as immuno-histochemical investigations. Conformation sensitive antibodies such as N6H7 could be

used to study the changes in pituitary bLH during purification, to verify the appropriate folding of the hormone produced by recombinant technology, and to check artefactual changes during storage *(Borromeo et al., 2003)*.

The ability of anti-bLHβ mAb-N6H7 and of anti-bFSHβ mAb-A3C12 to bind with high affinity to the hormones was exploited in 1-step purification by immunoaffinity chromatography of substantial amount of biologically active bLH and bFSH, respectively.

Immunoaffinity-chromatography has already been used to purify bFSH either from pituitary extracts *(Miller et al., 1987)*, or produced by recombinant technology *(Dirnberger et al., 2001; Van De Wiel et al., 1998)*, while to the best of our knowledge this is the first time that it has been used for bLH purification.

The two purification procedures developed were rapid and easy, allowing the one-step recovery of about 4 mg of bFSH and 16 mg of bLH from 1 kg of pituitary gland homogenate. Nevertheless, the purification yields are somewhat poorer of those previously reported by other authors that used different purification methods. The yields of bLH recoveries are in fact lower than that of multistep extraction procedures from fresh glands described by Reichert (60 mg/kg) *(Reichert et al., 1962)*, Courte (170 mg/kg) *(Courte et al., 1972)*, and Papkoff (90 mg/kg) *(Papkoff et al., 1970)*. As well, the yields of bFSH are lower than Millerôs figures (22.2 mg/kg) *(Grimek et al., 1979; Miller et al., 1987; Wu et al., 1993)*, and similar to what was obtained by Wu (1.7, 2.5, and 1.5mg/kg for three different bFSH isoforms), and Grimek (1.3 mg/kg).

The low column capacity was not the reason of the relatively low efficiency shown by our bLH and bFSH purification methods. In fact, despite the rather low bLH and bFSH binding capacity of our immunoaffinity columns (i.e. about 230 µg, compared to a theoretical capacity of about 1 mg of bLH or bFSH, on a 1:1 molar ratio with 10 mg of mAb coupled to the column), the procedure was carried out without exceeding the column binding capacity. In fact in preliminary experiments, we loaded onto the column increasing amount of pituitary extracts (25-200 mg), and we selected the 50 mg dose because the pure bLH or bFSH eluted from the columns did not increased significantly using greater

pituitary extract amounts (> 60-70 mg). Furthermore, the bLH or bFSH eluted from a second chromatographic run in which the unbound fraction was loaded onto the column, became significant when the extract dose was higher than 50 mg. We therefore considered 50 mg of pituitary extracts as the maximum loading capacity of our columns.

We are now examining ways of improving the bLH and bFSH recoveries of our immunoaffinity chromatography procedure. However, modifying the elution buffer (e.g., lower pH in the presence of 6 M guanidine) only minimally improved recovery, and significantly reduced their biological activity. In fact, bovine gonadotropin dissociation into subunits is greatly facilitated by pH below 4 or in the presence of a denaturant such as 6 M guanidine hydrochloride *(Sairam, 1991; Gospodarowicz,1972)*. The yield was also not improved by loading the column with partially purified pituitary extract, or by coupling other anti-ɓ subunits mAb to the affinity column (data not shown).

The immune-purified bLH and bFSH both showed high biological activity. That of N6H7-bLH was equal to that of reference USDA-bLH-B-6 (2.1 U/mg), and equivalent to that of bLH from pituitary glands purified by other authors: the first purified bLH had specific activity of 1.69 U/mg *(Reichert et al., 1962)*, Papkoff purified a bLH with biological activity of 1.5 U/mg *(Papkoff et al., 1970)*, and Courte achieved 2 U/mg *(Courte et al., 1972)*.

The biological activity of the purified A3C12-bFSH was 91.8 U/mg, which is 7 times less than that of reference bFSH-USDA-I-2, but 160 times more potent than that of standard bFSH-USDA-B-1. Furthermore, this bio-potency is comparable to that reported previously for other pituitary purified bFSH: Grimek (43.0 U/mg) *(Grimek et al., 1979)* and Wu (217.0, 86.0, and 62.0 U/mg for three different bFSH isoforms) *(Wu et al., 1993)*.

In general it is difficult to compare the specific activity of bFSH and bLH purified in different laboratories. Quantification of the activity of FSH and LH mostly depends on the bioassay and standard chosen *(Christin-Maitre et al.., 1996; Kalia, 2004; Rose et al., 2000; Ulloa-Aguirre et al., 2003)* and reliable comparison is further complicated for the results of in vitro and in vivo bioassays *(Christin-Maitre et al., 1996)*.

For testing the biological activity of bFSH, we used the *in vitro* FSHR-17 cells, that allowed sensitive, reproducible and specific measurment of bFSH biological activity. The sensitivity of this bioassay is one order of magnitude lower than in the classical primary Sertoli cell bioassay *(Ritzen et al., 1982)*, but much better than that of other bioassays based on cloned receptors *(Albanese et al., 1994; Christin-Maitre et al., 1996; Dirnberger et al., 2001; Van De Wiel et al., 1998)*. Expression of the rat instead of the human FSH receptor (FSHR) on the FSHR-17 cells may be one reason accounting for the higher sensitivity of this recombinant bioassay for the bovine hormone. In fact, ruminant FSH was shown to have higher affinity to rat than to human FSHR *(Tilly et al., 1992)*.

For testing the biological activity of bLH we used the MA-10 cell line, that is a well-established assay to measure LH activity *(Zhou et al., 2013; Dahl K.D., 1993)*. Nonetheless, it has never been used before to quantify the biological activity of bLH in terms of U/mg. The bLH dose-response curves obtained with this bioassay in our experiments were very similar to those reported by others using different standard USDA-bLH preparations (i.e.: ED_{50} in the 10ï 20 ng/mL range) *(Dahl K.D., 1993; Smith et al., 1990)*. This confirms that the bLH we purified has a biological activity equivalent to the reference USDA-bLH independently of the U/mg calculated and the assay used.

In SDS-PAGE the bLH Ŭ- and ɓ-subunits migrated as two separate bands, under reducing and non-reducing conditions. This was expected, since the subunits of gonadotropins promptly dissociate after boiling in the presence of SDS *(Reichert et al., 1973; Parsons et al.,1985; Bousfield et al., 1996)*. After reduction, the ɓ-subunit showed greater electrophoretic mobility than the Ŭ-subunit (ɓ-subunit: Mr 18690; Ŭ-subunit: Mr 20300), while in absence of the reducing agent, the ɓ-subunit migrated more slowly than the Ŭ-subunit (ɓ-subunit: Mr 21980; Ŭ-subunit: Mr 19500). This anomalous behavior is consistent with previous reports, showing that under non-reducing conditions the Ŭ-subunit migrates more than the bLH ɓ-subunit (*Reichert et al., 1973)*, and the reverse is true under reducing conditions *(Reichert et al., 1973; Bousfield et al., 1996)*.

Taking into account that (1) the bLH ɓ-subunit is 25 residues longer than the Ŭ-subunit, but possesses only a single N-linked oligosaccharide (Asn^{13}) whereas the latter has 2 (Asn^{56} and Asn^{82});

and that (2) bLH is a disulphide-rich heterodimer, with a cysteine-knot motif in the central core of each subunit *(Jiang et al., 2014)*, it is reasonable to hypothesize that upon reduction, the subunits folding and the sugar chain display is so greatly altered that it overturns the SDS-PAGE migration.

In SDS-PAGE under reducing conditions the purified bFSH showed a single band, at Mr 22358, while in the absence of the reducing agent, the bFSH Ŭ- and ɓ-subunits migrated as two separate bands (Ŭ-subunit: 20771; ɓ-subunit: 27640). The slower migration of the Ŭ- compared to the bFSH ɓ-subunit in non-reducing conditions is consistent with the fact that both subunit have two N-linked oligosaccharides, but the ɓ-subunit is 13 residues longer. The finding that the two subunits after reduction had the same SDS-PAGE mobility is less evident. We can hypothesize that, as for bLH subunits, breaking the cysteine-knot core of the bFSH subunits alter the protein folding and sugar chain display so much to distort the apparent Mr.

In IEF the immunopurified bFSH and bLH revealed extensive charge heterogeneity, indicating the presence of a wide array of differently glycosylated isoforms. In fact, it is well known that sugars form the major chemical basis of heterogeneity of gonadotropins in pituitary and the bloodstream in many animal species, including bovines *(Perrera-Marin et al., 2008; Baenziger et al., 1988)*. The fact that the two gonadotropins were purified from a pool of unselected bovine pituitaries, regardless to sex, physiological status and age, might have increased the heterogeneity. In fact glycoprotein hormones are known to exist in the pituitary as a dynamic population of isoforms that exhibit changes in relative abundance reflecting the endocrine status of the individual *(Cooke et al., 1997; Padmanabhan et al., 1999; Rose et al., 2000; Ulloa-Aguirre et al., 2003)*.

The possibility exists that the immuno-purification procedure has missed some of the bLH and bFSH differently glycosylated isoforms present in the pituitary extract, because not recognized by the anti-bLH or anti-bFSH mAb bound to the solid phase. However, contemporary research points to a minor role of sugar in glycoprotein hormones antigen structure *(Fox et al., 2001; Rose et al., 2000)*, and mAbs routinely used to detect hFSH in biological samples are considered to be almost blind to variations in glycosylation *(Jeffcoate, 1993)*. Nevertheless, it is of relevance the report of mAbs that

can indeed distinguish between native and desialylated hCG *(Rose et al., 2000; Simoni et al., 1994)*. Studies in our laboratory are currently underway to investigate whether binding is affected by the bFSH/bLH *N*-linked oligosaccharides.

The amino acid sequence and composition analysis of the purified bLH and bFSH showed that the polypeptide chain of both hormones was highly homogeneous, suggesting that amino acid modifications do not contribute to isoform differences and that the oligosaccharides chains are the only source of extensive charge heterogeneity shown in IEF.

The highly purified bioactive bLH and bFSH were combined with anti-bFSH and anti-bLH mAbs to develop sensitive, specific and reproducible sandwich ELISA to measure bLH and bFSH in bovine plasma. We selected the best pair to develop sandwich ELISA among more than 300 hybridoma clones. It is important to develop a library of a sufficient number of different mAbs, to improve the likelihood of identifying combinations of antibodies capable of recognizing many forms of hormones in biological fluids. This is an existing problem for the detection of human gonadotropins *(Rose et al., 1998; Costagliola et al. 1994)*. For example in humans more than 30 different assays are now available for LH and FSH, but numerous publications have highlighted that caution should be still exercised in interpreting assay results. Variations among kits are such that coefficients of variation (CV) of 30% or more have been observed. This is claimed to be primarily caused by differences in specificity or affinity of different antibodies.

For the development of the ELISA to determine bLH in bovine plasma we selected the combination of anti-ϐbLH mAb-N6H7 adsorbed to the solid phase and the biotin labeled anti-ϐbLH mAb-N3G8. The ELISA based on these two mAbs allowed the detection of bLH concentrations as low as 0.05 ng/mL. The sensitivity is similar to or better than with other bLH ELISAs *(Abdul-Ahad et al., 1987; Spearow et al., 1987; Mutayoba et al., 1990; Schneider et al., 2002)* and radioimmunoassay *(Niswender et al.,1969; Wolfe et al., 1992; Evans et al., 1992;Schneider et al., 2002; Perry et al., 2008; Hannan et al., 2010)*, previously used successfully to detect bLH plasma patterns.

Plasma components did not interfere with the assay (parallelism and recovery tests), which means it is suitable for measuring bLH without any prior treatment of samples. Furthermore, the parallelism between serial dilutions of crude pituitary extract, plasma, and the purified bLH suggests that the latter was not only pure, as discussed previously, but also was not damaged during the purification procedure, at least with regard to its immunoreactivity.

The precision of the assay, shown by the intra- and inter-assay statistical quality control, appears to be acceptable in relation to other methods for LH determination in bovine and other species *(Kalia., 2004)*.

The bLH ELISA developed is species-specific. We tested the reactivity of LH from various species, and only ovine LH showed high cross-reactivity. The LH sequences in these two ruminants differ only in the substitution of one amino acid in the ɓ subunit (Pro^{39} vs Leu^{39}). The 100% cross-reactivity of oLH was therefore expected and confirms previous reports *(Niswender et al., 1969; Spearow et al., 1987; Valares et al., 2007)*. Further studies are needed to test the precision and accuracy of this ELISA for detection of LH in ovine plasma.

No reactivity was observed with horse, dog, pig, rabbit, and mouse pituitary extracts. This was somewhat unexpected. The bLHɓ- subunit amino acid sequence is closely homologous to that of all the other species tested (Figure 6). Furthermore, previous studies showed that anti-bLH antibodies might have broad species-specificity, reacting to LH from diverse species *(Kofler et al., 1981; Matteri et al., 1987)*. Nonetheless, there are a few amino acid sequences in the bovine LHɓ that are species-specific (eg, β_{40}-β_{60} and the C-terminal end), clearly distinguishing sheep and cattle from non-ruminant species *(Wallis, 2001)*. Furthermore, these sequences seem to be ñhot spotsò where gonadotropin epitopes cluster in antigenic domains (*Berger et al., 1996)*. The absence of cross-reactivity in our ELISA to horse, dog, pig, rabbit, and mouse LH needs to be confirmed using purified LH, but it suggests that one or both mAbs used recognize epitopes not conserved among these species LHβ subunits.

The analytical specificity of the method was confirmed by the capacity to detect plasma LH levels in heifers under conditions known to affect LH secretion *(Pawson et al., 2005; Fields et al., 2009)*. The stimulation of bLH secretion by GnRH was clearly detectable, with an induced pattern of circulating LH concentrations similar to that reported in the bovine species by many other authors *(Bolt et al., 1990; Chenault et al., 1990; Gong et al., 1995; Schneider et al., 2002; Atkins et al., 2008; Colazo et al., 2009; Rantala et al., 2009; Ginther et al., 2012)*. The bLH plasma patterns induced by i.v. injection of GnRH were similar to those previously obtained using the same route of administration *(Perrera-Marin et al., 2005),* but, unexpectedly also to those reported after i.m. GnRH treatment *(Golter et la.,1973; Chenault et al., 1990; Atkins et al., 2008; Rantala et al.,2009; Ginther et al., 2012)*. We cannot rationalize why in the bovine the GnRH can induce a bLH release with similar pattern after i.m. and i.v. administration. Nevertheless, it is well known that comparing the effects of GnRH treatment is a very challenging goal because of the fact that many variables beside route of injection affect secretory bLH response *(Chenault et al., 1990; Schneider et al., 2002; Atkins et al., 2008; Rantala et al., 2009; Colazo et al., 2009)*. In particular variation among GnRH products and doses, circulating steroid hormone milieu at the time of treatment (e.g.: estrous cycle phase), bLH assay method, number and timing of blood samples, parameters used to analyze the secretory pattern.

Among these variables, we believe that animal category and high P4 plasma concentrations at the time of GnRH treatment may also explain why we found GnRH stimulated bLH peaks with magnitude and amplitude smaller than that reported by other authors *(Perrera-Marin et al., 2005; Atkins et al., 2008; Colazo et al.,2009)*. In fact, bLH secretion was stimulated with a GnRH analog at dose that as reported by Chenault (*Chenault et al., 1990*) is the most potent in releasing LH in the bovine compared to other products. However, we treated young heifers, and the GnRH-induced LH release has been shown to be reduced in heifers compared to cows (*Lucy., 1986; Atkins et al., 2008*). Furthermore, the animals were in diestrous, and during the luteal phase of the estrous cycle, when circulating P4 concentrations are high, the number of GnRH receptors on the pituitary cells are

decreased, which significantly lower the magnitude of the GnRH-induced LH surge in ruminants (*Giordano et al., 2012; Colazo et al., 2008; Atkins et al., 2008*). In general, cows with P4 concentrations greater than 1 ng/ml had low total LH secretion and LH peak below 5 ng/ml compared with cows with lower P4 concentrations. We did not measure P4 levels in our animals, but we trust that the heifers had high plasma P4 concentration since the GnRH treatment was done 10 days after a $PGF_{2Ů}$ treatment, and the presence of the corpus luteum was verified by transvaginal ultrasonography. The fact that the animals were in the lutein phase is also consistent with the low and stable bLH concentrations measured before GnRH administration (*Giordano et al., 2012; Colazo et al., 2009; Rantala et al., 2009; Atkins et al., 2008; Perera-Marin et al., 2005*).

For the development of the ELISA system to determine bFSH in bovine plasma we selected the combination of anti-ɓbLH mAb-A3C12 adsorbed to the solid phase and the biotin labeled anti-Ů-subunit mAb-D2H1.

In literature several RIA for bFSH have been described (Table 7), but to the best of our knowledge, only Prakash (*Prakash et al. 1999*) and Zou *(Zou et al. 1991)* described the development of ELISAs for bFSH. The assay developed by Zou is, like our ELISA, a mAb based species-specific assay, but while the method has proved useful for measuring FSH concentrations in tissue culture media and extracts, it was inadequate for analyzing plasma samples, due to insufficient detection limit (i.e.: 1 ng/mL).

The sandwich ELISA we developed was specific and reproducible. No cross-reactivity was measurable with other bovine glycoprotein hormones (bLH and bTSH), and plasma components did not interfere with the assay (parallelism and recovery tests), confirming the accuracy of the assay for measuring bFSH without any prior treatment of samples. The inter-assay statistical quality control were similar to that of other methods for FSH determination *(Adams et al., 1992-A* (9%)*; Adams et al., 1992-B (15%); Cupp et al., 1995 (9.6%); Gong et al., 1995 (8.4%))*.

The ELISA resulted rather sensitive, allowing the detection of bFSH plasma levels as low as 0.25 ng/mL. This detection limit is similar to that of the ELISA described by Prakash *(Prakash et al.,*

1999) and of several RIAs, but is one order of magnitude higher than that of other RIAs for bFSH (*Palhao et al; 2009; Lane et al., 2008).* Experiments are currently underway to improve the sensitivity of our assay. In fact, while LH appears to be released in a clear pulsatile manner with large secretory burst, the release of FSH is relatively smooth and stable, with small variations respect to basal plasma levels. Therefore, more sensitive is the assay, the better it can unravel the pulse frequency dynamics of plasma FSH.

Nevertheless, the sensitivity of the bFSH assay resulted suitable to detect plasma bFSH levels in vivo, as shown by the hormone patterns measured in heifers after GnRH stimulation. Variations of bFSH levels were clearly detectable in the plasma of all the four heifers tested, with irregular peaks of variable duration and small amplitude and magnitude. This is consistent with the minor bFSH surges described in bovine during luteal phase (*Edwards et al. 2013; Ginther et al., 2014*). As well, the absence of a well-defined secretory peak after GnRH injection agree with the current view that FSH release is only associated with a small proportion of the GnRH pulses. The majority of FSH secretion occurs in fact independently of signals arising from the GnRH receptor to the release mechanisms. Thus, GnRH is crucial for the activation of FSH gene, but minimally controls the pulsatile secretion of FSH episodes of secretion (*Pawson el al., 2005; Padmanabhan et al., 1997; Clarke et al., 2002; Kile et al., 1994).*

It should be mentioned that few other studies reported a significant bFSH increase over basal levels within 15 min after GnRH administration, reaching maximum peak concentrations approximately after 1-2h from treatment *(Gong et al., 1995; Gong et al., 1996; Peters et al., 1985).* This bFSH induced secretory pattern is therefore very similar to that of bLH after GnRH administration. To measure plasma bFSH the mentioned authors used well-established RIAs, but a cross-reactivity of these RIAs with bLH could rationalize this finding. Nonetheless, as discussed above, comparing the effects of GnRH treatment is difficult, because of the many variables that affect secretory gonadotropin response (e.g.: experimental protocol, estrus cycle phase, eccé).

6. CONCLUSIONS AND FUTURE PERSPECTIVES

In the present PhD thesis we describe the production of a panel of anti-bLH and anti-bFSH mAbs and their successful application in the development of: (1) alternative methods for the purification of LH and FSH from bovine pituitary glands, and (2) sandwich ELISAs to measure these two gonadotropins in bovine plasma.

The mAb-based immunoaffinity chromatography protocols adopted allowed the one-step purification of substantial amount of biologically active bLH and bFSH. These methods, besides being simple and rapid, appear to have a major advantage over classic methods (e.g.: ion exchange): the effect of charge on the separation is minimal. Since LH, TSH and FSH isoforms have similar structures and overlapping charge, their separation by methods based acid-base separation is difficult and often lead to the selection of specific isoforms, and to the recovery of small amount of hormone devoid of most of the original heterogeneity. Conversely, the bFSH and bLH we purified by immunoaffinity chromatography consist of a complex mixture of differently glycosylated isoforms.

Our knowledge of the structure-function relationships and of the endocrine mechanisms regulating FSH and LH glycosylation is still limited. In order to assign distinct functional properties to defined structural isoforms, information on the respective structures is required. Chemical and biochemical characterization, however, are often impeded by the small amounts of sample available, thus necessitating extremely highly sensitive and efficient methods of investigation. We trust that the immunoaffinity purified bFSH and bLH are a good starting material to isolate differently glycosylated isohormones in sufficient amount for detailed functional and structural characterization

The two homologous, specific, sensitive, and reproducible sandwich ELISAs developed satisfy all the criteria required to investigate LH and FSH secretory patterns in the bovine species. Both capture and detection antibodies are produced taking advantage of hybridoma cell lines, that produce antibodies in unlimited quantities. This guarantee the almost unlimited availability of reagents for

such ELISAs and can ensure their long-term continuity in large-scale operation. These methods can therefore be useful for rapid, inexpensive and quantitative measurement of the hormone in the pursuit of knowledge concerning the reproductive physiology of the bovine species. Understanding of gonadotropin-secretory regulation is also important from the Veterinary practical point of view, for example in treatment of acyclicity and in improving the superovulatory responses for embryo transfer.

7. REFERENCES

Abdul-Ahad W.G., Gosling J.P. ñAn enzyme-linked immunosorbent assay (ELISA) for bovine LH capable of monitoring fluctuations in baseline concentrationsò, J Reprod Fertil (1987); 80:653ï 61.

Adams T.E., Kinder J.E., Chakraborty P.K. Estergreen V.L., Reeves J.J. ñEwe luteal function influenced by pulsatile administration of synthetic LHRH/FSHRHò, Endocrinol (1975); 97(6): 1460-7.

Adams G.P., Matteri R.L., Kastelic J. P., Ko J. C. H., Ginther O. J. ñAssociation between surges of follicle-stimulating hormone and the emergence of follicular waves in heifersò, J Reprod Fert (1992-A); 94: 177-88.

Adams G.P., Matteri R. L., Ginther O. J. ñEffect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifersò J Reprod Fert (1992-B); 95: 627-40.

Albanese C., Christin-Maitre S., Sluss P.M., Crowley W.F., Jameson J.L., ñDevelopment of a bioassay for FSH using a recombinant human FSHR and a cAMP responsive luciferase reporter geneò, Mol Cell Endocrinol (1994); 101: 211ï 9.

Allegra A. Andros Day Surgery. Atti della Società Italiana di Ginecologia e Ostetricia. vol. LXXXVII, Palermo 2011.

Anobile C.J., Talbot J.A., McCann S.J., Padmanabhan V., Robertson W.R. ñGlycoform composition of serum gonadotrophins through the normal menstrual cycle and in the post-menopausal stateò, Mol Hum Reprod (1998); 4 (7) : 631-9.

Ascoli M. ñCharacterization of several clonal lines of cultured leydig tumor cells: gonadotropin receptors and steroidogenic responsesò, Endocrinol (1981); 108: 88ï 95.

Aston R., Ivanyi J. ñMonoclonal antibodies to growth hormone and prolactinò, Pharmacol Ther (1985); 27: 403ï 24.

Atkins J.A., Busch D.C., Bader J.F., Keisler D.H., Patterson D.J., Lucy M.C., Smith M.F. ñGonadotropin-releasing hormone-induced ovulation and luteinizing hormone release in beef heifers: effect of day of the cycleò, J Anim Sci (2008); 86: 83ï 93.

Baenzinger J.U., Green E.D. ñPituitary glycoprotein hormone oligosaccharides: structure, synthesis and function of the asparagine-linked oligosaccharides on lutropin, follitropin and thyrotropinò, Biochim Biophys Acta (1988); 947:287ï 306.

Baenzinger U.J., Kumar S., Brodbeck R.M., Smith P.L., Beranek M.C. ñCirculatory half-life but not interaction with the lutropin/chorionic gonadotropin receptor is modulated by sulfation of bovine lutropin oligosaccharidesò Proc Natl Acad Sci U.S.A. (1992); 89: 334ï 8.

Berger P., Bidart J.M., Delves P.S., Dirnhofer S., Hoermann R., Isaacs N., Jackson A., Klonisch T., Lapthorn A., Lund T., Mann K., Roitt I., Schwarz S., Wick G. ñImmunochemical mapping of gonadotropinsò, Mol Cell Endocrinol (1996); 125: 33ï 43.

Berrini A., Borromeo V., Secchi C. ñPurification to homogeneity of bovine prolactin by high-performance ion-exchange chromatographyò, J Chromatogr (1991); 547: 457ï 61.

Bogdanove E.M., Campbell G.T., Peckham W.D. ñFSH pleomorphism in the ratð regulation by gonadal steroidsò, Endocr Res Commun (1974); 1: 87-99.

Bolt D.J., Rollins R. ñDevelopment and application of a radioimmunoassay for bovine follicle-stimulating hormoneò, J Anim Sci (1983); 56(1): 146-54.

Bolt D.J., Scott V., Kiracofe G.H. ñPlasma LH and FSH after estradiol, norgestomet and Gn-RH treatment in ovariectomized beef heifersò, Anim Reprod Sci (1990); 23: 263ï 71.

Borromeo V., Gaggioli D., Berrini A., Secchi C. ñMonoclonal antibodies as a probe for the unfolding of porcine growth hormoneò, J Immunol Methods (2003); 272: 107ï 15.

Borromeo V., Amsterdam A., Berrini A., Gaggiolia D., Dantes A., Secchi C. ñCharacterization of biologically active bovine pituitary FSH purified by immunoaffinity chromatography using a monoclonal antibodyò, Gen Com Endocrinol (2004); 139: 179ï 89.

Bousfield G.R., Butnev V.Y., Russell Gotschall R., Baker V.L., Moore W.T. ñStructural features of mammalian gonadotropinsò, Mol Cell Endocrinol (1996); 125(1-2): 3-19.

Bousfield G.R., Butnev V.Y. ñIdentification of twelve O-glycosylation sites in equine chorionic gonadotropin beta and equine luteinizing hormone ss by solid-phase Edman degradationò, Biology of Reproduction (2001); 64: 136ï 47.

Burgus R., Butcher M., Amoss M., Ling N., Monahan M., Rivier J., Fellows R., Blackwell R., Vale W., Guillemin R. ñPrimary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF) (LH-hypothalamus-LRF-gas chromatography-mass spectrometry-decapeptide-Edman-degradation)ò, Proc Natl Acad Sci U.S.A. (1972); 69: 278ï 82.

Chenault J.R., Kratzer D.D., Rzepkowski R.A., Goodwin M.C. ñLH and FSH response of Holstein heifers to fertirelin acetate, gonadorelin and buserelinò, Theriog (1990); 34: 81ï 98.

Chenu S., Gregoire A., Malykh Y., Visvikis A., Monaco L., Shaw L., Shauer R., Marc A., Goergen J.L. ñReproduction of CMP*N*- acetylneuraminic acid hydroxylase activity in engineered Chinese hamster ovary cells using an antisense-RNA strategyò Biochim Biophys Acta (2003); 1622: 133ï 44.

Christin-Maitre S., Bouchard P. ñBioassays of gonadotropins based on cloned receptorsò, Molec Cell Endocrinol (1996); 125: 151-9.

Christin-Maitre S., Vasseur C., Fauser B., Bouchard P. ñBioassays of Gonadotropinsò, Methods (2000); 21: 51-7.

Clarke I.J. ñMultifarious effects of estrogen on the pituitary gonadotrope with special emphasis on studies in the ovine speciesò, Arch Physiol Biochem (2002); 110: 62ï 73.

Colazo M.G., Kastelic J.P., Davis H., Rutledge M.D., Martinez M.F., Small J.A., Mapletoft R.J. ñEffects of plasma progesterone concentrations on LH release and ovulation in beef cattle given GnRHò, Domest Anim Endocrinol (2008); 34(1): 109-17.

Colazo M.G., Ree T.O., Emmanuel D.G., Ambrose D.J. ñPlasma luteinizing hormone concentrations in cows given repeated treatments or three different doses of gonadotropin releasing hormoneò, Theriogen (2009); 71: 984ï 92.

Costagliola S., Niccoli P., Carayon P. ñGlycoprotein hormone isomorphism and assay discrepancy: The paradigm of luteinizing hormone (LH)ò, J Endocrinol Invest (1994); 17: 291-99.

Cooke D.J., Crowe M.A., Roche J.F. ñCirculating FSH isoform patterns during recurrent increases in FSH throughout the oestrus cycle of heifersò, J Reprod Fertil (1997); 110: 339ï 45.

Courte C., Hurault M., Clary C., De la Llosa P., Jutisz M. ñPurification and physicochemical properties of bovine luteinizing hormone (LH) and comparison between bull and cow LH-preparationsò, Gen Comp Endocrinol (1972); 18(2): 284-91.

Crowe S.J.C., Cushing H., Homans J. ñExperimental hypophysectomy. Bullò, Johns Hopkins Hosp (1910); 21: 128ï 69.

Crowe M.A., Padmanabhan V., Hynes N., Sunderland S.J., Enright W.J., Beitins I.Z., Roche J.F. ñValidation of a sensitive radioimmunoassay to measure serum follicle-stimulating hormone in cattle: correlation with biological activityò, Anim Reprod Sci (1997); 48(2-4): 123-36.

Cupp A.S., Stumpf T.T., Kojima F.N., Werth L.A., Wolfe M.W., Roberson M.S., Kittok R.J., Kinder J.E. ñSecretion of gonadotropins change during the luteal phase of the bovine oestrous cycle in the absence of corresponding changes in progesterone or 17ß-oestradiolò, Animal Reprod Science 37 (1995); 109-19.

Dahl K.D., Sarkissian A. ñValidation of an improved in vitro bioassay to measure LH in diverse speciesò, J Androl (1993); 14: 124ï 9.

Dellovade T., Schwanzel-Fukuda M., Gordan J., Pfaff D. ñAspects of GnRH neurobiology conserved across vertebrate formsò, Gen Comp Endocrinol (1998); 112: 276ï 82.

Dias J.A. ñIs there any physiological role for gonadotrophin oligosaccharide heterogeneity in humans?ò, Human Reprod (2001); 16: 825ï 30.

Dirnberger D., Steinkellner H., Abdennebi L., Remy J.J., Van de Wiel D. ñSecretion of biologically active glycoforms of bovine follicle stimulating hormone in plantsò, Eur J Biochem (2001); 268: 4570ï 79.

Dunbar B., Wilson S.B. ñA buffer exchange procedure giving enhanced resolution to polyacrylamide gels prerun for protein sequencingò, Anal Biochem (1994); 216: 227ï 8.

Durán-Pastén M.L., Fiordelisio T. ñGnRH-induced Ca^{2+} signaling patterns and gonadotropin secretion in pituitary gonadotrophs. Functional adaptations to both ordinary and extraordinary physiological demandsò Front Endocrinol (2013); 4(127): 1-13

Edwards S.A., Phillips N.J., Boe-Hansen G.B., Bo G.A., Burns B.M., Dawson K., McGowan M.R. ñFollicle stimulating hormone secretion and dominant follicle growth during treatment of Bos indicus heifers with intra-vaginal progesterone releasing devices, oestradiol benzoate, equine chorionic gonadotrophin and prostaglandin F(2Ŭ)ò, Anim Reprod Sci 2013;137(3-4): 129-36.

Evans A.C., Currie W.D., Rawlings N.C. ñEffects of naloxone on circulating gonadotrophin concentrations in prepubertal heifersò, J Reprod Fertil (1992); 96: 847ï 55.

Fares F. ñThe role of O-linked and N-linked oligosaccharides on the structureï function of glycoprotein hormones: Development of agonists and antagonistsò, Bioc Biop Acta (2006); 1760: 560ï 7.

Faure M.O., Nicol L., Fabre S., Fontaine J., Mohoric N., McNeilly A., Taragnat C. ñBMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitaryò J Endocrinol (2005); 186(1): 109-21.

Fevold H.L., Hisaw F.L., Leonard S.L. ñThe gonad stimulating and the luteinizing hormones of the anterior lobe of the hypophesisò, Am J Physiol (1931); 97: 291ï 301.

Fields S.D., Perry B.L., Perry G.A. ñEffects of GnRH treatment on initiation of pulses of LH, LH release, and subsequent concentrations of progesteroneò, Domest Anim Endocrinol (2009); 37: 189ï 95.

Fiete D., Srivastava V., Hindsgaul O., Baenziger J.U. ñA hepatic reticuloendothelial cell receptor specific for SO4-4GalNAc beta 1,4GlcNAc beta 1,2Man alpha that mediates rapid clearance of lutropinò, Cell (1991); 67: 1103ï 10.

Fiete D., Beranek M.C., Baenziger J.U. ñThe macrophage/endothelial cell mannose receptor cDNA encodes a protein that binds oligosaccharides terminating with SO4-4 GalNAcbeta1,4GlcNAcbeta or Man at independent sitesò, Proc Natl Acad Sci USA. (1997); 94 (21): 11256-61.

Fox K.M., Dias J.A., Van Roey P. ñThree-dimensional structure of human follicle-stimulating hormoneò, Mol Endocrinol (2001); 15: 378ï 89.

Galet C, Guillou F., Foulon-Gauze F., Combarnous Y., Chopineau1 M. ñThe b104ï 109 sequence is essential for the secretion of correctly folded single-chain ƃŬ horse LH/CG and for its FSH activityò, J Endocrinol (2009); 203: 167ï 74.

Garverick H.A., Parfet J.R., Lee C.N., Copelin J.P., Youngquist R.S., Smith M.F. ñRelationship of pre- and post-ovulatory gonadotropin concentrations to subnormal luteal function in postpartum beef cattleò J Anim Sci (1988); 66(1): 104-11.

Geyer H., Geyer R. ñStrategies for analysis of glycoprotein glycosylationò, Biochim et Biophys Acta (2006); 1764: 1853ï 69

Ginther O.J., Bergfelt D.R., Kulick L.J., Kot K. ñSelection of the dominant follicle in cattle: establishment of follicle deviation in less than 8 hours through depression of FSH concentrationsò, Theriogen (1999); 52: 1079ï 93.

Ginther O.J., Fuenzalida M.J., Shrestha H.K., Beg M.A. ñConcomitance of luteinizing hormone and progesterone oscillation during the transition from preluteolysis to luteolysis in cattleò, Dom Anim Endocrinold (2010); 40(2): 77-86.

Ginther O.J., Beg M.A. ñStimulation of a pulse of LH and reduction in PRL concentration by a physiologic dose of GnRH before, during, and after luteolysis in heifersò, Anim Reprod Sci (2012); 133: 52ï 62.

Ginther O.J., Rakesh H.B., Bashir S.T., Hoffman M.M. ñMinor FSH surge, minor follicular wave, and resurgence of preovulatory follicle several days before ovulation in heifers ñTheriogenò (2014); 81(3): 437-45.

Giordano J.O., Fricke P.M., Guenther J.N., Lopes Jr G., Herlihy M.M., Nascimento A.B., Wiltbank M.C. ñEffect of progesterone on magnitude of luteinizing hormone surge induced by two different doses of gonadotropin-releasing hormone in lactating dairy cowsò, J Dairy Sci (2012); 95: 3781-93.

Golter T.D., Reeves J.J., OôMary C.C., Arimura A., Schally A.V. ñSerum LH levels in bulls treated with synthetic luteinizing hormone-releasing hormone-follicle stimulating hormone-releasing hormone (LH-RH/FSH-RH)ò, J Anim Sci (1973); 37: 123ï 7.

Gong J.G., Bramley T.A., Gutierrez C.G., Peters A.R., Webb R. ñEffects of chronic treatment with a gonadotrophin-releasing hormone agonist on peripheral concentrations of FSH and LH, and ovarian function in heifersò J Reprod Fertil (1995); 105: 263ï 70.

Gong J.G., Campbell B. K., Bramley T.A., Gutierrez C.G., Peters A.R., Webb R. ñSuppression in the Secretion of Follicle-Stimulating Hormone and Luteinizing Hormone, and Ovarian Follicle Development in Heifers Continuously Infused with a Gonadotropin-Releasing Hormone Agonistò, Biol Repr (1996); 55: 68-74.

Göretzlehner G., Rudolf K. ñ50 years ago--1927--the 1st demonstration of chorionic gonadotropin in the urine of pregnant women by Aschheim and Zondekò, Zentralbl Gynakol (1978); 100(10): 638-41.

Gospodarowicz D. ñSingle step purification of ovine luteinizing hormone by affinity chromatographyò, J Biol Chem (1972); 247: 6491ï 8.

Green E.D., Baenziger J.U. ñAsparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin. I. Structural elucidation of the sulfated and sialylated oligosaccharides on bovine, ovine, and human pituitary glycoprotein hormonesò, J Biol Chem (1988-A); 263(1): 25-35.

Green E.D., Baenziger J.U. ñAsparagine-linked oligosaccharides on lutropin, follitropin, and thyrotropin. II. Distribuition of sulfated and sialylated oligosaccharides on bovine, ovine, and human pituitary glycoprotein hormonesò, J Biol Chem (1988-B); 263(1): 36-44.

Grimek H.J., Gorski J., Wentworth B.C. ñPurification and characterization of bovine follicle-stimulating hormone: comparison with ovine follicle-stimulating hormoneò, Endocrinol (1979);104(1): 140-7.

Guillemin R. ñChemistry and physiology of hypothalamic releasing factors for gonadotrophinsò, Int J Fertil (1967); 12: 359ï 67.

Hamernik D.L., Kim K.E., Maurer R.A., Nett T.M. ñProgesterone does not affect the amount of mRNA for gonadotropins in the anterior pituitary gland of ovariectomized ewesò, Biol Reprod (1987); 37(5): 1225-32.

Hampton J.H., Salfen B.E., Bader J.F., Keisler D.H., Garverick H.A. ñOvarian follicular responses to high doses of pulsatile luteinizing hormone in lactating dairy cattleò, J Dairy Sci (2003); 86(6): 1963-9.

Hannan M.A., Fuenzalida M.J., Siddiqui M.A., Shamsuddin M., Beg M.A., Ginther O.J. ñDiurnal variation in LH and temporal relationships between oscillations in LH and progesterone during the luteal phase in heifersò, Theriogenol (2010); 74: 1491ï 8.

Henderson K.M., Ellen R.L., Savage L.C., McNatty K.P. òStudies of the effectiveness of gonadotrophin-releasing hormone, steroids and follicular fluid in modulating ovine gonadotrophin output in vivo and in vitroò, J Reprod Fertil (1989); 86(1): 105-17.

Henricks D.M., Poffenbarger J.I. ñMouse Leydig cell bioassay shows lack of LH in the bovine preimplantation conceptusò J Reprod Fertil (1984); 71(2): 419-26.

Hoier R., Callesen H., Greve T. ñA semiquantitative and rapid radioimmunosorbent assay of luteinizing hormone for early prediction of time of ovulation in cattleò, Theriogenol (1988); 30: 235ï 43.

Hojo H., Ryan R.J. ñMonoclonal antibodies against human follicle-stimulating hormoneò, Endocrinol (1985); 117(6): 2428-34.

Honaramooz A., Chandolia R.K., Beard A.P., Rawlings N.C. ñOpioidergic, dopaminergic and adrenergic regulation of LH secretion in prepubertal heifersò, J Reprod Fertil (2000); 119(2): 207-15.

Imwalle D.B., Patterson D.J., Schillo K.K. ñEffects of melengestrol acetate on onset of puberty, follicular growth, and patterns of luteinizing hormone secretion in beef heifersò, Biol Reprod (1998); 58(6): 1432-6.

Jeffcoate S.L. ñAnalytical and clinical significance of peptide hormone heterogeneity with particular reference to growth hormone and luteinizing hormone in serumò, Clin Endocrinol (1993); 38(2):113-21.

Jiang X., Dias J.A., He X. ñStructural biology of glycoprotein hormones and their receptors: insights to signalingò, Mol Cell Endocrinol (2014); 382: 424ï 51.

Kaetzel D.M., Browne J.K., Wondisford F., Nett T.M., Thomason A.R., Nilson J.H. ñExpression of biologically active bovine luteinizing hormone in Chinese hamster ovary cellsò, Proc Natl Acad Sci U.S.A. (1985); 82(21): 7280-3.

Kaetzel D.M., Nilson J.H. ñMethotrexate-induced amplification of the bovine lutropin genes in Chinese hamster ovary cells. Relative concentration of the alpha and beta subunits determines the extent of heterodimer assemblyò, J Biol Chem (1988); 263(13): 6344-51.

Kalia V., Jadhav A.N., Bhutani K.K. ñLuteinizing hormone estimationò, Endocr Res (2004); 30(1), 1-17.

Keren-Tal I., Dantes A., Sprengel R., Amsterdam A. ñEstablishment of steroidogenic granulosa cell lines expressing follicle stimulating hormone receptorsò, Mol Cell Endocrinol (1993); 95: R1ï R10.

Kile J.P., Nett T.M. ñDifferential secretion of follicle-stimulating hormone and luteinizing hormone from ovine pituitary cells following activation of protein kinase A, protein kinase C, or increased intracellular calciumò, Biol Reprod (1994); 50: 49ï 54.

Kofler R., Kalchschmid E., Berger P., Wick G. ñProduction and characterization of monoclonal antibodies against bovine luteinizing hormoneò, Immunobiol (1981); 160(2):196-207.

Lane E.A., Sweeney T., Ryan M., Roche J.F., Crowe M.A. ñRelationship between serum gonadotropins and pituitary immunoreactive gonadotropins and steroid receptors during the first FSH increase of the estrous cycle and following steroid treatment in heifersò, Anim Reprod Sci (2008); 112(1-2): 66-82.

Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. ñProtein measurement with the folin phenol reagentò, J Biol Chem (1951); 193: 265ï 75.

Lucy M.C., Stevenson J.S. ñGonadotropin-releasing hormone at estrus: luteinizing hormone, estradiol, and progesterone during the periestrual and postinsemination periods in dairy cattleò, Biol Reprod (1986); 35(2): 300-11.

Manzella S.M., Hooper L.V., Baenzinger J.U. ñOligosaccharides containing ß1,4-linked *N* acetylgalactosamine, a paradigm for protein-specific glycosylationò, J. Biol. Biochem (1996); 271: 12117ï 20.

Matteri R.L., Roser J.F., Baldwin D.M., Lipovetsky V., Papkoff H. ñCharacterization of a monoclonal antibody which detects luteinizing hormone from diverse mammalian speciesò, Domest Anim Endocrinol (1987); 4(3): 157-65.

Matzuk M.M., Hsueh A.J.W., Lapolt P., Tsafriri A., Keene J.L., Boime I. ñThe biological role of the carboxyl-terminal extension of human chorionic gonadotropin b-subunitò, Endocrinology (1990); 126: 376ï 83.

McCann S.M. ñHypothalamic control of FSH and LH by FSH-RF, LHRH, cytokines, leptin and nitric oxideò, Neuroimmunomod (1998); 5:193ï 202.

Merriam G.R., Wachter K.W. ñAlgorithms for the study of episodic hormone secretionò, Am J Physiol (1982); 243: E310ï 8.

Millar R.P., Lu Z.-L., Pawson A.J., Flanagan C.A., Morgan K., Maudsley, S. ñGonadotropin-releasing hormone receptors2ò, Endocrinol (2004); 25: 235ï 75.

Miller K.F., Goldsby R.A., Bolt D.J. ñImmunoaffinity chromatography of bovine FSH using monoclonal antibodiesò, J Endocrinol (1987);115(2): 283-8.

Morgan K., Millar R.P. ñEvolution of GnRH ligand precursors and GnRH receptors in protochordate and vertebrate speciesò, Gen Comp Endocrinol (2004); 139: 191ï 7.

Murphy B.D., Martinuk S.D. ñEquine chorionic gonadotropinò, Endocr Rev (1991); 12: 27ï 44.

Mutayoba B.M., Meyer H.H., Schams D., Schallenberger E. ñDevelopment of a sensitive enzyme immunoassay for LH determination in bovine plasma using the streptavidin-biotin techniqueò, Acta Endocrinol (1990); 122: 227ï 32.

Niswender G.D., Reichert Jr L.E., Midgley Jr A.R., Nalbandov A.V. ñRadioimmunoassay for bovine and ovine luteinizing hormoneò, Endocrinol (1969); 84: 1166ï 73.

Nunemaker C.S. ñGonadotropin- releasing hormone neurons generate interacting rhythms in multiple time domainsò, Endocrinol (2003); 144: 823ï 31.

Padmanabhan V., Lang L.L., Sonstein J., Kelch R.P., Beitins I.Z. ñModulation of serum follicle-stimulating hormone bioactivity and isoform distribution by estrogenic steroids in normal women and in gonadal disgenesisò, J Clin Endocrinol Metab (1988); 67: 465ï 73.

Padmanabhan V., Mieher C.D., Borondy M., lôAnson H., Wood R.I., Landerfeld T.D. Foster D.L., Beitins I.Z. ñCirculating bioactive follicle-stimulating hormone and less acidic follicle-stimulating hormone isoforms during experimental induction of puberty in the female lambò, Endocrinol (1992); 131: 213ï 20.

Padmanabhan V., McFadden K., Mauger D.T., Karsch F.J., Midgley Jr. A.R. ñNeuroendocrine control of follicle-stimulating hormone (FSH) secretion I. Direct evidence for separate episodic and basal components of FSH secretionò, Endocrinol (1997); 138: 424ï 32.

Padmanabhan V., Lee J.S., Beitins I.Z. ñFollicle-stimulating isohormones: regulation and biological significanceò, J Reprod Fertil Suppl (1999); 54: 87ï 99.

Palhao M.P., Beg M.A., Rodrigues M.T., Ginther O.J. ñFollicle and hormone dynamics in single versus double ovulating heifersò, Reproduction (2009); 138: 561ï 70.

Papandreou M.J., Sergi I., Benkirane M., Ronin C. ñCarbohydrate-dependent epitope mapping of human thyrotropinò, Molecular and Cellular Endocrinol (1990); 73: 15-26.

Papkoff H., Gan J. ñBovine interstitial cell-stimulating hormone: purification and propertiesò, Arch Biochem Biophys (1970); 136: 522ï 8.

Parsons T.F., Strickland T.W., Pierce JG. ñDisassembly and assembly of glycoprotein hormonesò, Methods Enzymol (1985); 109: 736ï 49.

Pawson A.J., McNeilly A.S. ñThe pituitary effects of GnRH2, Anim Reprod Sci (2005); 88: 75ï 94.

Peckham W.D., Yamaji T., Dierschke D.J., Knobil E. ñGonadal function and the biological and physicochemical properties of follicle-stimulating hormoneò, Endocrinology (1973); 92: 1660-6.

Peclaris G.M., Pappa A., Deligiannis K., Koutsotolis K. ñEnzyme immunoassays for the determination of ovine LH and FSHò, Reprod Domest Anim (2003); 38(5): 367-72.

Pei-San T. ñGonadotropin-releasing hormone in invertebrates: Structure, function, and evolutionò Gen Comp Endocrinol 148 (2006); 48ï 53.

Perera-Marin G., Falcon A.A., Murcia M.C., Hernandez C.J., Gonzalez P.E. ñPurificacion de cinco isoformas de la hormona luteinizante bovina (bLH). Caracterizacion fisicoquēmica, biologica e inmunologicaò, Vet Mex (2004); 35: 129ï 45.

Perera-Marin G., Murcia C., Rojas S., Hernandez-Ceron J., Gonzalez- Padilla E. ñPattern of circulating luteinizing hormone isoforms during the estrous and luteal phases in Holstein heifersò, Anim Reprod Sci (2005); 86: 53ï 69.

Perera-Marìn G., Murcia C., Gonzàlez-Padilla E. ñLuteinizing hormone (LH) isoforms in ruminants: Characterization and physiological relevanceò Anim Reprod Sci (2007); 101: 187ï 207.

Perera-Marín G[1], Gutiérrez CG, Murcia C, León H, González-Padilla E**. ñ**Progesterone and the distribution of pituitary gonadotropin isoforms in cattle**ò,** Anim Reprod Sci (2008); 104(2-4): 164-76.

Perry G.A., Perry B.L. ñEffect of preovulatory concentrations of estradiol and initiation of standing estrus on uterine pH in beef cowsò, Domest Anim Endocrinol (2008); 34: 333ï 8.

Peters A. R., Pimentel M. G., Lamming G. E. ñHormone responses to exogenous GnRH pulses in post-partum dairy cowsò, J Reprod Fert (1985): 75: 557-65.

Phillips D.J., Albertsson-Wikland K., Eriksson K., Wide L. ñChanges in the isoforms of luteinizing hormone and follicle-stimulating hormone during puberty in normal childrenò, J Clin Endocrinol Metab (1997); 82(9): 3103-6.

Pierce J.G., Bahl O.P., Cornell J.S., Swaminathan N. ñBiologically active hormones prepared by recombination of the alpha chain of human chorionic gonadotropin and the hormone-specific chain of bovine thyrotropin or of bovine luteinizing hormoneò, J Biol Chem (1971); 246(7): 2321-4.

Prakash B.S., Wallenhorst S., Metten M., Holtz W., Wuttke W. ñDevelopment of a sensitive enzymeimmunoassay (EIA) for FSH determination in bovine plasmaò, Anim Reprod Sci (1999); 55: 183ï 92.

Prakash B.S., Paul V., Anandlaxmi N. ñDevelopment and validation of a simple, sensitive, second antibody format enzyme immunoassay for LH determination in plasmaò J Immunol Methods (2002) 15; 270(2): 281-90.

Rantala M.H., Peltoniemi O.A., Katila T., Taponen J. ñEffect of GnRH dose on occurrence of short oestrous cycles and LH response in cyclic dairy heifersò, Reprod Domest Anim (2009);44:647ï 52.

Rawlilngs N.C., Jeffcoate I.A., Rieger D.L. ñ The influence of estradiol-17ß and progesterone on peripheral serum concentrations of luteinizing hormone and follicle stimulating hormone in the ovariectomized eweò, Theriogen (1984); 22(5): 473-88.

Reichert Jr L.E. ñPreparation of purified bovine luteinizing hormoneò, Endocrinol (1962); 71: 729-33.

Reichert Jr L.E., Lawson Jr G.M. ñMolecular weight relationships among the subunits of human glycoprotein hormonesò Endocrinol (1973); 92: 1034ï 42.

Reichert Jr L.E. ñElectrophoretic properties of pituitary gonadotropins as studied by electrofocusingò, Endocrinol (1971); 88(4): 1029-44.

Reichert Jr LE. ñPurification of anterior pituitary hormones (ovine, bovine, rat, rabbit)ò, Methods Enzymol (1975); 37: 360ï 80.

Ritzen E.M., Froysa B., Gustafsson B., Westerholm G., Diczfalusy E. ñImproved in vitro bioassay of follitropinò, Horm Res (1982); 16: 42ï 8.

Rose M.P. Follicle stimulating hormone International standards and reference preparation for the calibration o f immunoassay and bioassayò, Clin Chim Acta (1998); 103-17.

Rose M.P., Gaines Das R.E., Balen A.H. ñDefinition and measurement of follicle stimulating hormoneò, Endocr Rev (2000); 21(1): 5-22.

Sairam M.R., Linggen J., Bhargavi G.N. ñAlterations in antigenic structure of gonadotropins following deglycosylationò, Biosci Reports (1988); 8: 271-8.

Sairam M.R. ñFacile separation of the subunits of ovine and bovine gonadotrophins by high-performance liquid chromatography: microheterogeneity and biological activityò J Endocrinol (1991); 130: 415ï 24.

Sealfon S.C., Weinstein H., Millar R.P. ñMolecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptorò, Endocr Rev (1997); 18: 180ï 205.

Schillo K.K., Green M.A., Hayes S.H. ñEffects of adrenalectomy on photoperiod-induced changes in release of luteinizing hormone and prolactin in ovariectomized ewesò, J Reprod Fertil (1988); 83(1): 431-8.

Schally A.V., Nair R.M., Redding T.W., Arimura A. ñIsolation of the luteinizing hormone and follicle-stimulating hormone-releasing hormone from porcine hypothalamiò, J Biol Chem (1971); 246: 7230ï 6.

Sherman G.B., Wolfe M.W., Farmerie T.A., Clay C., Threadgill D.S., Sharp D.C., Nilson J.H. ñA single gene encodes the ß-subunits of equine luteinizing hormone and chorionic gonadotropinò, Molec Endocrinol (1992); 6: 951ï 9.

Schneider F., Bellmann A., Becker F., Bambang Poernomo S., Rehfeldt C., Nürnberg G., Kanitz W. ñGonadotropin release in periovulatory heifers after GnRH analogs measured by two types of immunoassaysò Exp Clin Endocrinol Diabetes (2002);110(5): 235-44.

Schneider F., Tomek W., Gru¨ndker C. ñGonadotropin-releasing hormone (GnRH) and its natural analogues: A reviewò, Theriogenol (2006); 66: 691ï 709.

Schots A., Van Der Leede B.J., De Jongh E., Egbert, E. ñA method for the determination of antibody affinity using a direct ELISAò, J Immunol Methods (1988); 109: 225.

Schwarz S., Krude H., Klieber R., Dirnhofer S., Loftesberger C., Merz W.E., Wick G. Berger P. ñNumber and topography of epitopes of hCG are shared by desialylated and deglycosylated hCGò, Molec Cell Endocrinol (1991); 80: 33-4.

Secchi C., Biondi P.A., Berrini A., Simonic T., Ronchi S. ñA biotin-avidin sandwich enzyme-linked immunosorbent assay of growth hormone in bovine plasmaò, J Immunol Methods (1988); 110: 123ï 8.

Secchi C., Berrini A., Borromeo V., Gamba D., Brocchi E. ñBinding characteristics of monoclonal antibodies raised against bovine growth hormoneò, Hybridoma (1991); 10: 499ï 505.

Simoni M., Jockenhovel F., Nieschlag E. ñPolymorphysm of human pituitary FSH: analysis of immunoreactivity and in vitro bioactivity of different molecular speciesò, J Endocrinol (1994);141: 359ï 67.

Smith P.E. ñAblation and transplantation of the hypophyses of the ratò, Anat Rec (1926-A); 32: 221.

Smith P.E. ñHastening development of female genital system by daily hymoplastic pituitary transplantsò, Proc Soc Exp Biol Med (1926-B); 24: 131ï 2.

Smith P.L., Kaetzel D., Nilson J., Baenziger J.U. ñThe sialylated oligosaccharides of recombinant bovine lutropin modulate hormone bioactivityò J Biol Chem (1990) ;265: 874ï 81.

Spearow J.L., Trost B.A. ñDevelopment of a sensitive enzyme-linked immunosorbent assay for cattle, sheep, rat, and mouse luteinizing hormoneò, Biol Reprod (1987); 37(3): 595-605.

Stockell-Hartree A., Renwick G.C. ñMolecular structures of glycoprotein hormones and functions of their carbohydrate componentsò, Biochem J (1992); 287: 665ï 79.

Stumpf T.T., Roberson M.S.,Wolfe M.W., Zalesky D.D., Cupp A.S.,Werth L.A., Kojima N., Hejl K.L., Kittok R.J., Grotjan H.E., Kinder J.E. ñA similar distribution of gonadotropin isohormones is maintained in the pituitary throughout sexual maturation in the heiferò, Biol Reprod (1992); 46: 442ï 50.

Swinnen J.V., DôSouza B., Conti M., Ascoli M. ñAttenuation of CAMP-mediated responses in MA-10 leydig tumor cells by genetic manipulation of a camp-phosphodiesteraseò, J Biol Chem (1991); 266: 14383ï 9.

Szkudlinski M.W., Thotakura N.R., Tropea J.E., Grossmann M., Weintraub B.D. ñAsparagine-linked oligosaccharide structures determine clearance and organ distribution of pituitary and recombinant thyrotropinò, Endocrinol (1995); 136: 3325ï 30.

Tijssen P. ñPreparation of enzyme-antibody or other enzymemacromolecule conjugates. In: Burdon RH, van Knippenberg PH, editorsò, Practice and theory of enzyme immunoassays Elsevier (1985); 221ï 78.

Tilly J.L., Aihara T., Nishimori K., Jia X.C., Billig H., Kowalski K.I., Perlas E.A., Hsueh A.J.W. ñExpression of recombinant human follicle-stimulating hormone receptor: species-specific ligand binding, signal transduction, and identification of multiple ovarian messenger ribonucleic acid transcriptsò, Endocrinol (1992); 131: 799ï 806.

Ulloa-Aguirre A., Timossi C., Damián-Matsumura P., Dias J.A.òRole of glycosylation in function of follicle-stimulating hormoneò, Endocrine (1999); 11(3): 205-15.

Ulloa-Aguirre A., Maldonado A., Damián-Matsumura P., Timossi C. ñEndocrine Regulation of Gonadotropin Glycosylationò, Arch Medical Res (2001-A); 32: 520ï 32.

Ulloa-Aguirre A., Timossi C., Méndez J.P. ñIs there any physiological role for gonadotrophin oligosaccharide heterogeneity in humans? I. Gondatrophins are synthesized and released in multiple molecular forms. A matter of factò, Human Reprod (2001-B); 16(4): 599-604.

Ulloa-Aguirre A., Timossi C., Barrios-de-Tomasi J., Maldonado A., Nayudu P. ñImpact of carbohydrate heterogeneity in function of FSH: studies derived from in vitro and in vivo modelsò, Biol Reprod (2003); 69: 379ï 89.

Valares J.A., Abecia J.A., Forcada F., Palacin I., Mata L., Razquin P. ñDevelopment of a simple enzyme immunoassay for the determination of ovine luteinizing hormoneò, Vet Res Commun (2007); 31: 427ï 36.

Van de Wiel D.F., van Rijn P.A., Meloen R.H., Moormann R.J. ñHigh-level expression of biologically active recombinant bovine follicle stimulating hormone in a baculovirus systemò, J Mol Endocrinol (1998); 20(1): 83-98.

Varki A. ñ*N*-Glycolylneuraminic acid deficiency in humansò, Biochimie (2001); 83: 615ï 22.

Wallis M. ñEpisodic evolution of protein hormones in mammalsò, J Mol Evol (2001); 53: 10ï 8.

WaCkowska M., Polkowska J., Misztal T., Romanowicz K. ñInfluence of ovarian hormones on endocrine activity of gonadotroph cells in the adenohypophysis of lambs during the postnatal transition to prepubertyò, Anim Reprod Sci (2010); 121(1-2): 84-93.

Wide L. ñMedian charge and charge heterogeneity of human pituitary FSH, LH and TSH. II. Relationship to sex and ageò, Acta Endocrinol (1985); 109: 190ï 7.

Wide L. ñFollicle-stimulating hormones in anterior pituitary glands from children and adults differ in relation to sex and ageò, J Endocrinol (1989);123(3): 519-29.

Wide L., Bakos O. ñMore basic forms of both human follicle-stimulating hormone and luteinizing hormone in serum at midcycle compared with the follicular or luteal phaseò, J Clin Endocrinol Metab (1993); 76: 885ï 9.

Kronenberg H.M., Memed S., Polonsky K.S., Larsen P.R. ñWilliams Textbook of Endocrinologyò, Chapter: Neuroendocrinology; Page: 132; Publisher: Elsevier Health Sciences; Edition: 11; 2007.

Wolfe M.W., Stumpf T.T., Roberson M.S., Wolfe P.L., Kittok R.J., Kinder J.E. ñEstradiol influences on pattern of gonadotropin secretion in bovine males during the period of changed responses to estradiol feedback in age-matched femalesò, Biol Reprod (1989); 41: 626ï 34.

Wolfe M.W., Roberson M.S., Stumpf T.T., Kittok R.J., Kinder J.E. ñCirculating concentrations and pattern of luteinizing hormone and folliclestimulating hormone in circulation are changed by the circulating concentration of 17 beta-estradiol in the bovine male and femaleò, J Anim Sci (1992); 70: 248ï 53.

Wu J.B., Stanton P.G., Robertson D.M., Hearn M.T. ñIsolation of FSH from bovine pituitary glandsò, J Endocrinol (1993); 137(1): 59-68.

Yalow R.S., Bersen S.A. ñAssay of plasma insulin in human subjects by immunological methodsò, Nature (1959); 184: 1648-9.

Zaied A.A., Garverick H.A., Bierschwal C.J., Elmore R.G., Youngquist R.S., Sharp A.J. ñEffect of ovarian activity and endogenous reproductive hormones on GnRH-induced ovarian cycles in postpartum dairy cowò J Anim Sci. (1980); 50(3): 508-13.

Zhou L., Beattie M.C., Lin C.Y., Liu J., Traore K., Papadopoulos V., Zirkin B.R., Chen H. ñOxidative stress and phthalate-induced downregulation of steroidogenesis in MA-10 leydig cellsò, Reprod Toxicol (2013); 42: 95ï 101.

Zondek B. ñUeber die Funktion des Ovariumsò, Z Geburtsh Gynäkol (1962); 90: 372ï 6.

Zondek B. ñWeitere Untersuchungen zur Darstellung, Biologie und Klinik des Hypophysenvorderlappen-hormons (Prolan)ò, Z Gynäkol (1929); 14: 834ï 48.

Zondek B. ñÜber die Hormone des Hypophysenvorderlappens. II. Follikelreifungshormon Prolan A-Klimakterium-Kastrationò, Klin Wochenschr (1930); 9: 393ï 6.

Zou Y.Q., Yu-Cui L., Jing-Hui Y. ñGeneration of monoclonal antibodies to bovine follicle-stimulating hormone and application in an enzyme immunoassayò, Hybridoma (1991); 10: 493ï 8.