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# Role of Luteinising Hormone in ovarian follicle development and maturation in the mare

**Stephanie Nicole Schauer**

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Doctor of Philosophy

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# Dedication

In memory of my uncle Charly

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# List of Abbreviations

| | |
|---|---|
| °C | degree Celsius |
| $^{125}I$ | Iodine |
| $^{3}H$ | Tritium |
| ADAMTS | a disintegrin and metalloproteinase with thrombospondin-like motifs |
| AGO | argonaute |
| Amhr | antimüllerian hormone receptor |
| ATP | adenosine triphosphate |
| BMP | bone morphogenetic protein |
| bw | body weight |
| C/EBP | CCAAT/enhancer binding protein |
| CA | corpus albicans |
| Ca2+ | calcium ion |
| cAMP | cyclic adenosine monophosphate |
| CEG | crude equine gonadotropin |
| CH | corpus haemorrhagicum |
| CL | corpus luteum |
| cm | centimetre |
| COC | cumulus-oocyte-complex |
| cpm | counts per minute |
| CREB | cAMP responsive element binding protein |
| CV | coefficient of variation |
| CYP11A1 | cytochrome P450, family 11, subfamily A, polypeptide 1 |
| CYP17A1 | cytochrome P450, family 17, subfamily A, polypeptide 1 |
| CYP19A1 | cytochrome P450, family 19, subfamily A, polypeptide 1 / aromatase |
| DARS | donkey-anti-rabbit serum |
| DGCR8 | DiGeorge syndrome critical region gene 8 |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleotide |
| DTT | dithiothreitol |
| e, h | species specification as prefix (eLH, hCG): equine, human |
| E2 | oestradiol |
| eCG | equine chorionic gonadotropin |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| FF | follicular fluid |
| FGF | fibroblast growth factor |
| FSH | follicle stimulating hormone |
| FSHR | follicle stimulating hormone receptor |
| g | g-force |

| | |
|---|---|
| G protein | GTP-binding protein |
| GC | granulosa cells |
| GDF | growth and differentiation factor |
| GDP | guanosine diphosphate |
| GH | growth hormone |
| GnRH | gonadotropin releasing hormone |
| GPCR | G protein-coupled receptor |
| GTP | guanosine triphosphate |
| HAF | haemorrhagic anovulatory follicle |
| hCG | human chorionic gonadotropin |
| HSD17B | 17-beta-hydroxysteroid dehydrogenase |
| HSD3B | 3-beta-hydroxysteroid dehydrogenase |
| i.m. | intramuscular |
| i.v. | intravenous |
| IGF | insulin-like growth factor |
| IGF1R | insulin-like growth factor 1 receptor |
| IGFBP | insulin-like growth factor binding protein |
| INHA | inhibin A |
| INRA | Institut national de la recherche agronomique |
| IP3 | inositol triphosphate |
| IU | international units |
| kg | kilogram |
| KHCO3 | potassium bicarbonate |
| LH | luteinising hormone |
| LHCGR/LHR | luteinising hormone receptor |
| LLR | leucine-rich repeat |
| mg | milligram |
| µg | microgram |
| MHz | megahertz |
| min | minutes |
| miRNA | micro ribonucleic acid |
| ml | millilitre |
| µl | microlitre |
| mm | millimetre |
| mmu | mus musculus (mouse) |
| µm | micrometre |
| mM | millimolar |
| MMP | matrix metalloproteinase |
| MRE | microRNA response element |
| mRNA | messenger ribonucleic acid |
| n | statistical sample size |

| | |
|---|---|
| nm | nanometre |
| NRS | normal rabbit serum |
| NTC | no template control |
| OD | optical density |
| P4 | progesterone |
| PA | plasminogen activator |
| PAPP-A | pregnancy-associated plasma protein-A |
| PBS | phosphate-buffered saline |
| PBSG | phosphate-buffered saline with 0.1% swine skin gelatine |
| PCOS | polycystic ovarian syndrome |
| PCR | polymerase chain reaction |
| pg | picogram |
| PGE | prostaglandin E |
| PGF | prostaglandin F |
| PGR | progesterone receptor |
| PGS2 | prostaglandin synthase 2 |
| PKA | protein kinase A |
| PLC | phospholipase C |
| pre-miRNA | precursor micro ribonucleic acid |
| pri-miRNA | primary micro ribonucleic acid |
| PTGS2 | prostaglandin G/H synthase 2 |
| qPCR | quantitative polymerase chain reaction |
| RBMS1 | ribonucleic acid binding motif single stranded interacting protein 1 |
| RIA | radioimmunoassay |
| RISC | RNA-induced silencing complex |
| RNA | ribonucleic acid |
| RNAsIn | RNase Inhibitor |
| rno | rattus norvegicus (rat) |
| rtPCR | reverse transcription polymerase chain reaction |
| SF1 | steroidogenic factor 1 |
| SMAD | homolog of Sma (C.elegans) and mad (Drosophila; mothers against decapentaplegic) |
| Srebf1 | sterol regulatory element-binding protein 1 |
| STAR | steroidogenic acute regulatory protein |
| TGF | transforming growth factor |
| TRBP | TAR RNA-binding protein |
| TSH | thyroid stimulating hormone |
| UTR | untranslated region |
| V | volt |
| VEGF | vascular endothelial growth factor |

# Declaration of Originality

I hereby declare that the work presented in this thesis and the thesis itself have been composed and originated by myself, unless otherwise specified.

Stephanie Nicole Schauer

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## Research Publications

**Schauer, S.N.,** Guillaume, D., Decourt, C., Watson, E.D., Briant, C., Donadeu, F.X. : *Effect of luteinizing hormone overstimulation on equine follicle maturation* **2012** (Theriogenology, accepted for publication on 20th of August 2012)

**Schauer, S.N.**, Briant, C., Ottogalli, M., Decourt, C., Handel, I.G., Donadeu, F.X.: *Supplementation of equine early spring transitional follicles with luteinizing hormone stimulates follicle growth but does not restore steroidogenic activity*. Theriogenology. **2011** Apr 1; 75(6):1076-84

F.X. Donadeu, C.L. Esteves, L.K. Doyle, C. A. Walker, **S.N. Schauer**, C.A. Diaz: *Phospholipase Cβ3 Mediates LH-Induced Granulosa Cell Differentiation*, Endocrinology **2011,** 152: 2857-2869

# Conference Abstracts

**Schauer, S.N.,** Bittner, L., Donadeu, F.X.: *Changes in miRNA expression induced by hCG in equine preovulatory follicles.* Annual Conference of the Society of Reproduction and Fertility (SRF), Edinburgh, UK, 2012 **(Poster)**

**Schauer, S.N.,** Briant, C., Decourt, C., Donadeu, F.X.: *Effects of elevated luteinising hormone (LH) levels on equine ovarian follicle development.* Annual Conference of the Society of Reproduction and Fertility (SRF), Brighton, UK, 2011 **(Presentation)**

**Schauer, S.N.,** Briant, C., Ottogalli, M., Donadeu, F.X.: *Administration of equine luteinizing hormone promotes the development of ovulatory follicles in early transitional mares.* Supp. Anim. Repro. Sci. 121; 24-25, 10th International Symposium on Equine Reproduction (ISER), Kentucky, US, 2010 **(Presentation)**

**Schauer, S.N.,** Briant, C., Ottogalli, M., Donadeu, F.X.: *Effects of Exogenous Luteinizing Hormone on Development and Acquisition of Ovulatory Competence by Dominant Follicles During the Equine Anovulatory Season.* 43$^{rd}$ Annual Meeting of the Society for the Study of Reproduction (SSR), Milwaukee, US, 2010 **(Poster)**

Donadeu, F.X., **Schauer, S.N.,** Briant, C., Ottogalli, M.: *Follicles and LH: a love-hate relationship?* Tierärztekongress Leipzig and 6th International Conference on Equine Reproductive Medicine ICERM, Leipzig, DE, 2010 **(Presentation)**

# Abstract

Luteinising hormone (LH) is a crucial regulator of ovarian follicle maturation, ovulation and luteinisation. Development of healthy follicles and fertile ovulation can only occur within a specific range of circulating LH concentrations, with differing upper and lower limits depending on the stage of the oestrous cycle. The objective of the three studies in this thesis was to investigate the effects of both physiological and non-physiological circulating LH levels on equine follicular maturity by examining ovulatory and steroidogenic capacity, gene expression profiles and miRNA expression in ovulatory-size follicles at various stages of the oestrous cycle and/or in response to supplementation with LH.

The aim of the first study was to investigate the hypothesis that deficient circulating LH is a primary cause for the inability of equine follicles to ovulate during the physiological anovulatory season. A LH-rich equine pituitary fraction (eLH) given twice daily to early transitional mares did not restore steroidogenic capacity of the ovulatory-size follicle or advance the onset of the natural breeding season; however, it significantly stimulated follicular growth to a level similar to that occurring during the normal oestrous cycle. The results demonstrated that a deficiency in LH is critically involved in reduced follicle growth during the anovulatory season.

The second study examined the effects of elevated circulating LH levels early during follicle development on follicle maturation and ovulatory ability in cycling mares, with the hypothesis that excessive LH would disrupt ovulation and produce haemorrhagic anovulatory follicles (HAFs). Treatment with eLH or a luteolytic dose of prostaglandin F2α (to stimulate an increase in endogenous levels of LH) did not have any effects on follicle growth or ovulation, but did impair follicular production of androstenedione and insulin-like growth factor 1 (IGF1), suggesting a deleterious effect of high LH on follicle and oocyte maturation.

The third study examined the expression of different follicular factors associated with follicle maturation as well as microRNAs (miRNAs) in ovulatory-size follicles naturally developing under different LH milieus (oestrus, dioestrus and spring

transitional period). Progesterone and IGF1 were significantly reduced in follicles developing in a low LH environment (dioestrus and transition). All four miRNAs measured, miR-378, miR-542, miR-202 and miR-21 were found at higher levels in subordinate follicles than in preovulatory follicles during oestrus. In addition miR-202 and miR-21 were significantly increased in transitional follicles relative to oestrous follicles. The results of this study indicate that follicles developing during both the spring transitional and dioestrous periods are developmentally immature and suggested potential important roles of miRNAs in follicle maturation in the horse.

In summary, although LH is a key factor promoting follicular growth, it is by itself not sufficient to restore steroidogenic activity in transitional follicles. Elevated LH levels during follicle development do not disrupt ovulation, but induce changes in follicular fluid factors related to follicle maturation and oocyte quality. Follicles developing under different LH milieus show altered miRNA expression, suggesting an important role of miRNAs in follicle maturation.

# Chapter 1

# Introduction

# Chapter 1: Introduction

## 1.1 Equine reproductive cycle

To maximise the chance of offspring survival, mares have evolved to align their breeding pattern with the seasons, so that foals are born preferably in spring or early summer, when nutrients are plentiful. Pregnancy in the mare lasts around 11 months and the active breeding season on average encompasses the months of March to October in the Northern Hemisphere, thus the horse is a poly-oestrous long-day breeder. There has long been a need for horse breeders to efficiently detect signs of impeding ovulation in the mare to allow for successful mating, but even more so, to advance the onset of the breeding season, so that mares can be bred early during the year. This is because in the thoroughbred racing community all foals born within one calendar year get the same artificial birth date (the 1st of January), and will later race against each other, with an age difference of each month having an impact on performance and therefore earnings (Langlois and Blouin 1998).

An oestrous cycle is defined as the interval between two consecutive oestrous cycles with the first day of oestrous behaviour defined as Day 0 of the cycle. Closely related to the definition of oestrous cycle is the ovulatory cycle, which is the interval between two successive ovulations. Each ovulatory cycle during the equine breeding season is around 22 days long with a luteal phase lasting around 15 days followed by an average seven day long follicular phase that comprises the period of behavioural oestrus; ovulation usually occurs around 24 hours before the end of oestrus (Ginther 1992e). At the end of the breeding season, mares cease ovulating and enter a period of transition (autumn transition) that leads to winter anoestrus (or deep anoestrus) when follicular activity is reduced to a minimum under the effects of short daylight. At the end of the deep anoestrous period and in response to increasing daylight during late winter, gonadotropin secretion and follicular growth progressively resume with the development of one or several anovulatory follicular waves during the spring transitional period followed by ovulation and the re-initiation of oestrous cycles (reviewed in Ginther 1992a; Donadeu and Watson 2007). Three types of

reproductive hormones, GnRH, gonadotropins and steroids, play a key role in regulating the equine reproductive cycle.

## 1.2 Reproductive hormones

### 1.2.1 Gonadotropin releasing hormone

Mammalian gonadotropin releasing hormone (GnRH) is a decapeptide produced by neuroendocrine cells in the hypothalamus, where neural signals are converted into endocrine signals under the effects of kisspeptin and other regulators (Colledge 2009), resulting in secretion of GnRH in a pulsatile manner to act directly on the anterior pituitary. In response to GnRH, the gonadotropins follicle stimulating hormone (FSH) and luteinising hormone (LH) are also subjected to a pulsatile release from pituitary gonadotrophs (Handler and Aurich 2005). GnRH pulsatility is highly variable during the oestrous cycle. In the build-up to the ovulatory LH surge, two to five pulses of GnRH are secreted per hour, which is three times more frequent than during the mid-luteal phase (Irvine and Alexander 1994).

Gonadotropin pulsatility has been studied in detail in the horse with the finding that FSH and LH peaks are coupled during dioestrus but not during oestrus. This is explained by the fact that low frequency GnRH pulses stimulate both FSH and LH secretion, whereas high frequency GnRH pulsatility mainly stimulates LH release (Ginther 1992c; Bernard et al. 2010). Furthermore, not only LH pulsatility increases towards ovulation, but also the bioactivity of the circulating LH (Alexander and Irvine 1982). If high levels of GnRH are experimentally infused in a constant manner to mares, down-regulation of gonadotropin release occurs even to the point of arrested ovarian function (Fitzgerald et al. 1993), however, constant low-dose GnRH infusion led to an increase in the secretion of LH (Porter et al. 1997). This shows that gonadotropin secretion in mares occurred in the absence of a pulsatile pattern of GnRH secretion, which is in contrast to other species (Gong et al. 1996; Porter et al. 1997).

## 1.2.2 Gonadotropins

### 1.2.2.1 Follicle stimulating hormone

Like LH and thyroid stimulating hormone (TSH), FSH is a glycoprotein produced in the anterior pituitary. Each pituitary glycoprotein contains an α- and a β-subunit. The α-subunit is common to the three glycoproteins, whereas the β-subunit is specific and therefore gives biologic specificity to each hormone (Ojeda 2004a). Endogenous FSH production rate for ovariectomised mares is around 3.2 mg/day and has a mean clearance rate of 0.25 ml/kg per minute (Briant et al. 2004b).

In mammals, FSH stimulates follicle growth and in the absence of FSH or its receptor (FSHR), follicular development arrests before the antral stage (Bernard et al. 2010). FSH secretion is stimulated by activins, which are produced in the pituitary, in ovarian follicles and in a variety of other tissues (DePaolo 1997). Activin is also under feedback control from the ovary; specifically, inhibins produced in response to gonadotropin stimulation of antral growing follicles antagonise activins, therefore lowering FSH secretion (Bernard et al. 2010). At the same time, FSH and LH stimulate the production of follicular oestrogen, which in turn negatively regulates FSH secretion by the pituitary (Bernard et al. 2010). Additional regulators of FSH secretion may include bone morphogenetic proteins (BMPs); however, their mechanism of action is not yet known (Bernard et al. 2010).

### 1.2.2.2 Luteinising hormone

LH is a heterodimeric glycoprotein with a specific β-subunit, which is 149 amino acids long in the horse (Bousfield et al. 1987). LH is secreted in the anterior pituitary by basophilic gonadotroph cells, which can produce LH, FSH or both (Ojeda 2004a). Multiple isoforms of LH and FSH can be secreted that have different levels of glycosylation and bioactivity (Irvine 1979; Adams et al. 1986). It has been shown that circulating LH in the mare has the lowest biopotency during luteal regression and the highest during the preovulatory and luteal phases (Adams et al. 1986), when LH is needed to induce ovulation and stimulate luteal development and function, respectively. LH secretion is strongly regulated by ovarian steroids. As in other species, oestradiol can either have a negative feedback on LH secretion during the follicular phase or be part of a positive feedback mechanism by sensitising the

pituitary to GnRH stimuli (Ojeda 2004a). This view was recently questioned by an extensive study on the effects of oestradiol on LH levels during oestrus. If physiological levels of oestradiol are injected into the mare at different time points during the follicular phase, only a negative effect on LH secretion was found (Ginther et al. 2007d); the positive rebound effect found in previous studies was potentially caused by the use of supraphysiological doses during these experiments, causing circulating oestradiol levels over four times higher than during the physiological oestradiol peak.

The preovulatory rise in circulating LH, which lasts for around one week in the horse and peaks one day after ovulation, is necessary to stimulate follicle development and maturation as well as to induce ovulation (Handler and Aurich 2005). The role of LH includes furthermore the stimulation of progesterone secretion of the corpus luteum to maintain pregnancy (Ginther 1992c). The effect of LH during the reproductive cycle include the induction of luteinising hormone receptor (LHCGR or LHR) in granulosa cells, as well as the stimulation of expression of steroidogenic acute regulatory protein (STAR) and CYP17A1 in theca cells to further promote oestradiol synthesis at deviation as shown in cows (Luo et al. 2011) and mares (Bergfelt et al. 2001).

Equine chorionic gonadotropin (eCG) is produced by endometrial cups formed by the developing placenta beginning at around day 35 of pregnancy (Ginther 1992b) and promotes the development of additional copora lutea and stimulates their progesterone production (Carr and Rehman 2004). The β-subunit of eCG shows high similarity with the β-subunit of LH as well as human chorionic gonadotropin (hCG). Therefore all three hormones effectively stimulate the LH receptor. Because of its functional similarity to LH, hCG is used commercially to induce ovulation in domestic species. LH and chorionic gonadotropins have however very different half-lives in circulation due to high levels of glycosylation of chorionic gonadotropins compared to LH (Smith et al. 1993). hCG has a half-life of more than 24 hours whereas the half-life of eCG is several days. In contrast, the half-life of LH in mares is between 2 to 5 hours (Irvine 1979). Interestingly, administration of hCG to a mare

during the periovulatory period results in an increase in circulating endogenous levels of LH (Ginther et al. 2009).

LH induces its effects on follicular and other target cells by binding to its cognate receptor on the cell membrane. The equine luteinising hormone receptor is a member of the glycoprotein receptor subfamily of the G protein-coupled receptor (GPCR) family. The equine LHR sequence contains 699 amino acids (Saint-Dizier et al. 2004). The LHR comprises extracellular, transmembrane and intracellular domains (Figure 1.1). The extracellular domain is involved in binding the hormone ligand and contains a N-terminal cysteine-rich region, nine leucine-rich repeats (LLRs) and a C-terminal cysteine-rich region. The serpentine transmembrane region contains seven segments linked by three extracellular loops and three intracellular loops. Finally, the C-terminal domain is intracellular and is primarily involved in receptor signalling (Ascoli et al. 2002). The equine LHR, though highly conserved within mammals, was found to have an additional seventh N-glycosylation site, one more than human and bovine sequences (Saint-Dizier et al. 2004).

**Figure 1.1 Schematic of the human LH receptor amino acid sequence (Huret et al. 2003). EC: extracellular domain, TM: transmembrane domain, IC: intracellular domain**

When a GPCR such as the LHR is activated by its ligand it changes its conformation to stimulate a heterotrimeric GTP-binding protein (G protein) inside the cytosol (Figure 1.2) (reviewed in Alberts et al. 2008). Heterotrimeric G proteins contain three subunits α, β and γ, of which subunit α has a guanosine diphosphate (GDP) molecule bound in its inactive state. Upon G protein activation, the α-subunit

exchanges a GDP with a guanosine triphosphate (GTP) molecule. This triggers uncoupling of the Gα-subunit from the βγ complex allowing interaction of each of Gα and Gβγ with downstream effectors. Hydrolysis of Gα-bound GTP to GDP triggers a return of the receptor into its inactivated state (Alberts et al. 2008).

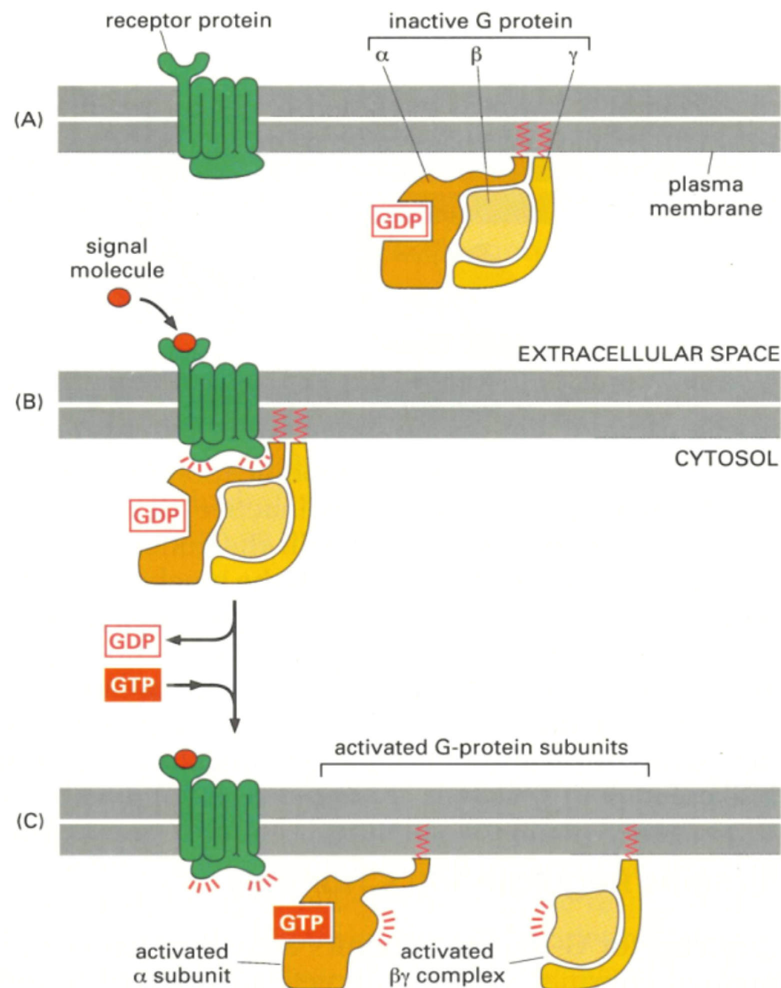


**Figure 1.2 GPCR activation of heterotrimeric G proteins. A) Localisation of the GPCR and heterotrimeric G protein within the cell. B) A ligand binds to the GPCR and triggers coupling to the G protein followed by conformational changes, which C) lead to the exchange of a GTP molecule for a GDP molecule in the Gα subunit, causing the Gα and Gβγ complexes to separate. Both complexes can now activate target proteins. (Alberts et al. 2008)**

The LHR can couple with different types of heterotrimeric G proteins (Gs, Gq, Gi) each of which can activate different downstream pathways. Upon activation by its ligand, the LHR predominantly couples with Gs leading to the activation of adenylyl cyclase (Figure 1.3) (Latronico and Segaloff 1999) and synthesis of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). cAMP then activates cyclic-AMP-dependent protein kinase (PKA) among other targets, which act on downstream proteins such as the transcription factor cAMP responsive element binding protein (CREB), to effect gene expression (Stocco et al. 2005). When expressed at high levels in the cell surface or when stimulated by high LH levels, the LHR can also couple to additional types of G proteins (Gq and Gi), which act by activating alternative pathways, predominantly the phospholipase C (PLC) pathway (Donadeu and Ascoli 2005; Donadeu et al. 2011).

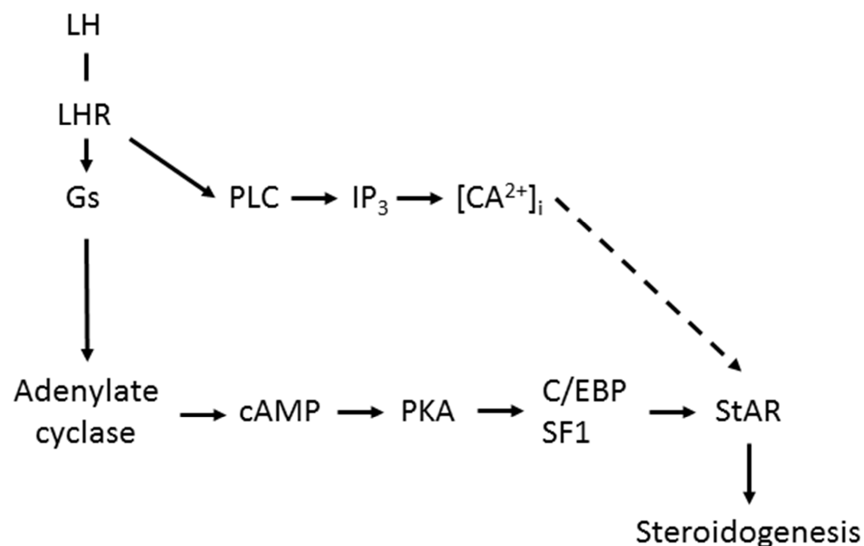


**Figure 1.3 LH stimulation of the cAMP pathway leading to steroidogenesis (adapted from Stocco et al. 2005). LH: luteinising hormone; LHR: luteinising hormone receptor; Gs: GTP-binding protein (stimulating); PLC: phospholipase C; $IP_3$: inositol triphosphate; Ca2+: calcium ion; cAMP: cyclic adenosine monophosphate, PKA: protein kinase A; C/EBP: CAAT/enhancer binding protein; SF1: steroidogenic factor 1; StAR: steroidogenic acute regulatory protein.**

### 1.2.3 Ovarian steroids

Ovarian steroids have important regulatory roles on reproductive function both through endocrine and paracrine effects. The first step in ovarian steroidogenesis (Figure 1.4) is the transport of cholesterol, a steroid precursor, into the inner mitochondrial membrane of the follicular cells by steroidogenic acute regulatory protein (STAR), which is regulated by LH. Cholesterol is subsequently converted to pregnenolone by cholesterol side-chain cleavage enzyme (CYP11A1), before undergoing transformation into progesterone, catalysed by 3β-hydroxysteroid dehydrogenase (HSD3B). Progesterone and pregnenolone can then be converted into androstenedione in the endoplasmic reticulum by the action of 17α-hydroxylase (CYP17A1). Within the ovarian follicle, androstenedione is released from theca cells and transferred to neighbouring granulosa cells, where it is directly metabolised into oestrone by aromatase (CYP19A1) or into testosterone by 17β-hydroxysteroid dehydrogenase (HSD17B) in the endoplasmic reticulum. Both oestrone and androstenedione can then be converted into oestradiol, the major oestrogen produced by the ovary, through the enzymatic activity of HSD17B and CYP19A1, respectively (Ojeda 2004a). Even though most of progesterone and androstenedione synthesis in equine follicles seems to occur in the theca cells, the granulosa cells are able to produce oestradiol all the way from progesterone (Channing 1969; Sirois et al. 1991).

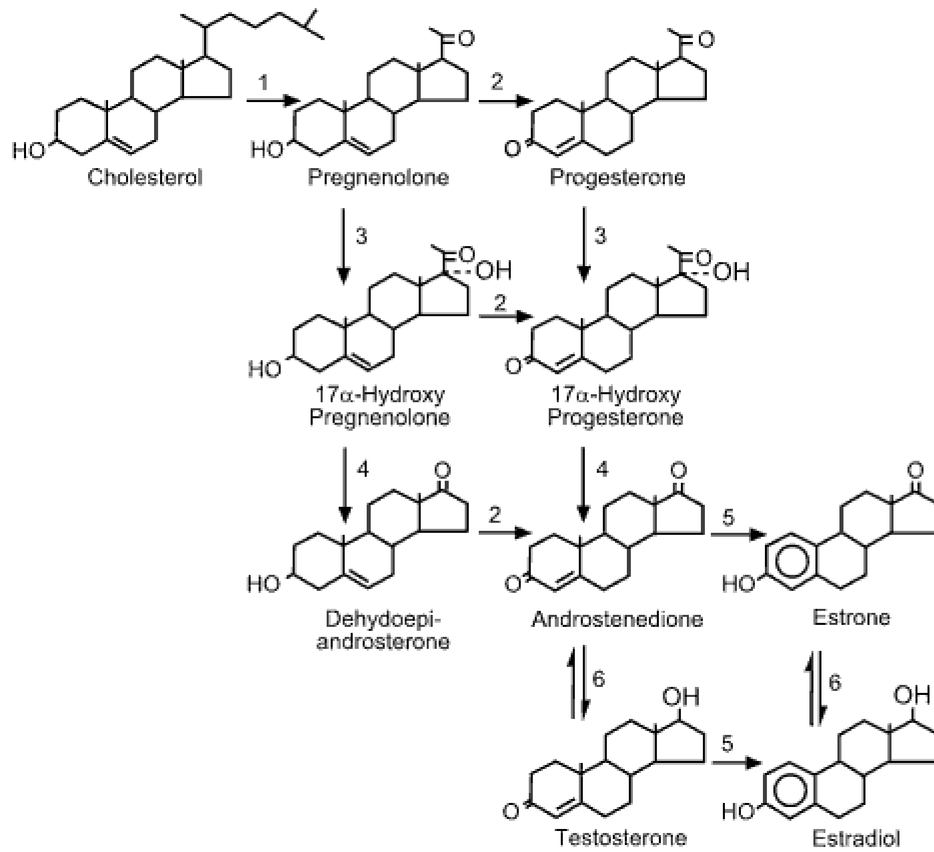


**Figure 1.4 Steroidogenic pathway in the ovarian follicle. 1) Cholesterol side-chain cleavage enzyme (CYP11A1), 2) 3β-hydroxisteroid dehydrogenase (HSD3B), 3) 17α-hydroxylase (CYP17A1), 4) 17,20-lyase, 5) aromatase (CYP19A1), 6) 17β-hydroxysteroid dehydrogenase (HSD17B) (Ojeda 2004b)**

Progesterone plays a main role inducing ovulation. When steroid synthesis was experimentally inhibited in primates (Hibbert et al. 1996) or the progesterone receptor was knocked out in mice (Robker et al. 2000a), no ovulation occurred. Additionally the maturation of the oocyte has been found to be primed by progesterone, concomitant with rising levels inside the follicle while oestradiol levels decrease between the preovulatory LH surge and ovulation (Fair and Lonergan 2012). Following from that, increased fertility was found in cows with high progesterone levels before ovulation (Wiltbank et al. 2011). Furthermore, progesterone, the main product of the corpus luteum, is the hormone preparing the

reproductive tract for pregnancy; it promotes firm closure of the cervix, reduces myometrium contractions and changes the ability of the endometrium for secretions (Handler and Aurich 2005) and is necessary for the fixation of the embryo (Ginther 1992b).

Oestradiol is mainly produced by the granulosa cells of the ovary and exerts a variety of functions. It can in an autocrine manner stimulate granulosa cell differentiation, stimulate cyclic changes of the uterus, and in an endocrine manner affect the release of gonadotropins, but furthermore has an influence on bone growth and metabolic changes (Ojeda and Griffin 2004). Additionally, oestradiol is responsible for triggering oestrous behaviour. Oestradiol is mainly under the positive regulation by FSH and LH and therefore increases up to 50-fold inside the dominant follicle during oestrus, with systemic levels reaching a peak around two days before ovulation (Handler and Aurich 2005), which possibly increases the response of the anterior pituitary to GnRH to intensify LH secretion (Karsch 1987). Further roles of oestradiol will be discussed below in relation to follicle development.

## 1.3 Folliculogenesis

### 1.3.1 Preantral follicle development

Folliculogenesis begins in the equine foetus during mid-pregnancy, when primary oocytes, arrested in the first phase of meiosis, become surrounded by a layer of somatic cells (the precursor of the granulosa cell layer) and become primordial follicles (Ginther 1992h). Initial follicle recruitment from the primordial follicle pool and up to the secondary follicle stage has been shown to be gonadotropin independent and in humans can take up to 280 days (Gougeon 1996), during which the oocyte and surrounding somatic follicular cells develop under the control of local growth factors. In a study in mares the mitotic index of follicular cells was measured and an estimated period of around 7 weeks for preantral follicles to develop from a size of around 60 µm to 1 mm was found (Driancourt and Cardon 1979). At any one time the number of recruited follicles with a size larger than 50 µm is around 50 (Driancourt and Cardon 1979), which is about a quarter the number of preantral follicles present in the ewe (Cahill et al. 1979) and half the number of preantral

follicles in the cow (Mariana et al. 1973). A primordial follicle consists of a small primary oocyte arrested in prophase I with a single layer of squamous granulosa cells and a basal lamina (Ginther 1992h). Once a primordial follicle is recruited to enter the pool of growing follicles it will sequentially develop into primary, secondary and tertiary (or antral) stages. Recruitment from the primordial follicle pool involves a change in granulosa cell morphology from squamous to cuboidal. At the same time granulosa cells begin to express FSH receptors and the enclosed oocyte increases in size by about 6-fold while becoming surrounded by a zona pellucida (Picton 2001). As development progresses into the secondary follicle stage, multiple layers of granulosa cells develop around the oocyte and a double-layer of theca cells, an outer theca externa and an inner theca interna, appears (Picton 2001). At this time the follicle acquires its own blood supply through angiogenesis mediated by vascular endothelial growth factor (VEGF) (Yang and Fortune 2007). At the end of the secondary stage, when follicles in the mare reach a size of around 0.2 to 0.4 mm, an antrum forms (Driancourt et al. 1982). This process includes first the formation of small fluid pockets before a larger continuous antrum develops and intrafollicular fluid build-up is thought to occur by osmotic pressure caused by hyaluronan and proteoglycan accumulation (Rodgers and Irving-Rodgers 2010).

## 1.3.2 Follicular waves

### 1.3.2.1 Overview

Equine antral follicles grow at an average rate of 3 mm per day (Gastal et al. 2004). Growth of antral follicles occurs in the form of periodic waves involving cohorts of follicles simultaneously responding to increases in circulating FSH. All follicles of a wave initially grow in synchrony at a similar rate until the largest follicle of the cohort reaches a size of around 23 mm (about 8 – 10 mm in cattle and humans) (Baerwald 2009). At that time, a diameter deviation between the follicles of the wave begins by which the largest follicle continues to grow in solitude as a dominant follicle whereas smaller follicles begin to regress (subordinate follicles), as part of the process called follicle deviation (Ginther et al. 2003a). Follicular waves and associated FSH surges occur periodically throughout a female’s reproductive life although the diameter attained by the largest follicle of a wave will vary during

different reproductive stages (oestrous cycles, anovulatory season, pregnancy and postpartum) and within different periods at each stage (reviewed in Ginther 1992f; Donadeu and Pedersen 2008).

Several follicular waves usually occur during the equine oestrous cycle and they have been classified as major and minor (Ginther 1993). A major wave is described by the synchronised growth of five to nine follicles, with a dominant follicle developing that grows to a size larger than 30 mm. If this follicle ovulates, the wave is termed ovulatory or primary. If the dominant follicle regresses, the wave is termed anovulatory or secondary. The development of a primary ovulatory wave is a consistent feature during an oestrous cycle and characterises the follicular phase of the cycle; in contrast, a major anovulatory wave may or may not occur during the luteal phase of the oestrous cycle. Furthermore, when follicles of a wave regress before or shortly after attaining dominance, these waves are termed minor waves (Ginther 1992e). Minor waves can occur at different stages of the oestrous cycle and are a predominant feature during other stages such as the anovulatory season (Donadeu and Ginther 2002b). Secondary major and minor waves in the mare were found in only around 25% of oestrous cycles and developed faster than primary waves, with the largest follicle growing to a smaller diameter in secondary waves than in primary waves (Ginther 1993). Furthermore, season seems to have an effect on the occurrence of major secondary or minor waves, as during the breeding season most such waves occurred during spring oestrous cycles and only one minor wave was found during autumn oestrous cycles (Ginther 1993).

### 1.3.2.2 Control of ovulatory follicular wave development

#### 1.3.2.2.1 Systemic control of follicle development

The future dominant follicle is usually the one emerging first in a wave and it retains its size advantage over smaller follicles throughout the wave so that when diameter deviation begins it can block further development of smaller follicles (Gastal et al. 2004). The selection of the dominant follicle from a wave of cohorts is needed in monovular species to avoid multiple ovulations, which could turn into twin or multiple pregnancies. Previous studies have shown that any of the follicles of a cohort is able to become dominant, if larger (future dominant) follicles are removed,

which leads to the conclusion, that instead of selection of the best follicle, there is an action of suppression on the subordinate follicles, stopping them from developing further (Ginther et al. 2002).

Before a new follicular wave emerges, the frequency of FSH pulses as well as the amplitude increases significantly (Bergfelt and Ginther 1993). When FSH was experimentally suppressed in mares, follicular wave emergence was delayed and growth stagnated (Bergfelt and Ginther 1985). During a follicular wave, FSH levels peak around the day the largest follicle reaches a size of 13 mm and subsequently decrease. This decrease is mainly caused by inhibin secreted by the growing follicles themselves (Donadeu and Ginther 2001) and follicle ablation results in an immediate increase in circulating levels of FSH (Ginther et al. 2001). In addition, when inhibin antiserum is injected into mares, their FSH levels increase, resulting in a higher number of large follicles and ovulations (Nambo et al. 1998). In addition to inhibin, follicular oestradiol also has an inhibiting effect on FSH secretion, particularly during follicle deviation (Donadeu and Ginther 2003b). Follicle deviation is triggered by the decreasing levels of FSH becoming insufficient to sustain follicle growth (Donadeu and Pedersen 2008). This results in the demise of all follicles except the largest, which at the time acquires increased sensitivity to both FSH and LH, through increased LHR expression and increased expression of growth factors, which amplify gonadotropin responses in follicular cells (Ginther et al. 2001).

At the end of the luteal phase and in association with the emergence of an ovulatory wave, circulating levels of LH begin to increase as the negative feedback of progesterone on LH secretion is lifted in response to natural luteolysis (see Figure 1.5). Levels of LH slowly begin to increase alongside the increase of FSH at the beginning of the emergence of a new wave, and LH continues to rise when FSH levels begin to drop (Gastal et al. 1997a). Before the beginning of deviation, in addition to increasing its sensitivity to FSH, the dominant follicle acquires an increased sensitivity to LH through the upregulation of LHR expression in granulosa cells (Goudet et al. 1999; Ginther et al. 2003a). The increase in LHR has been reported to occur as early as eight hours before deviation (Beg et al. 2001) and allows the dominant follicle to continue growth while subordinate follicles begin to

regress. Indeed, when LH is experimentally suppressed by administration of progesterone to mares, deviation is not disrupted but the growth of the dominant follicle is reduced or stagnates completely, depending on the level of LH suppression (Gastal et al. 1999a). A further increase of LH levels is needed to prime the dominant follicle’s responsiveness for the ovulatory LH surge (Donadeu and Watson 2007). One of the key roles of LH during follicle deviation is stimulation of oestradiol production by the dominant follicle, which further decreases FSH secretion. The dominant follicle is the main source for circulating oestradiol during a follicular wave as an ablation of the largest but not the second largest follicle reduces systemic oestradiol levels (Gastal et al. 1999b). Besides preparing the reproductive organs for coitus and fertilisation, oestradiol is responsible for oestrous behaviour in the mare (Handler and Aurich 2005). The preovulatory LH surge induces oocyte release as well as luteinisation of follicular cells involving, among many other changes (see below), a switch from oestradiol to progesterone production (Tucker et al. 1991).

Thus, while FSH is the main drive for the emergence and initial growth of a follicular wave, LH is most important beginning before deviation, and during subsequent development of the dominant follicle and ovulation.

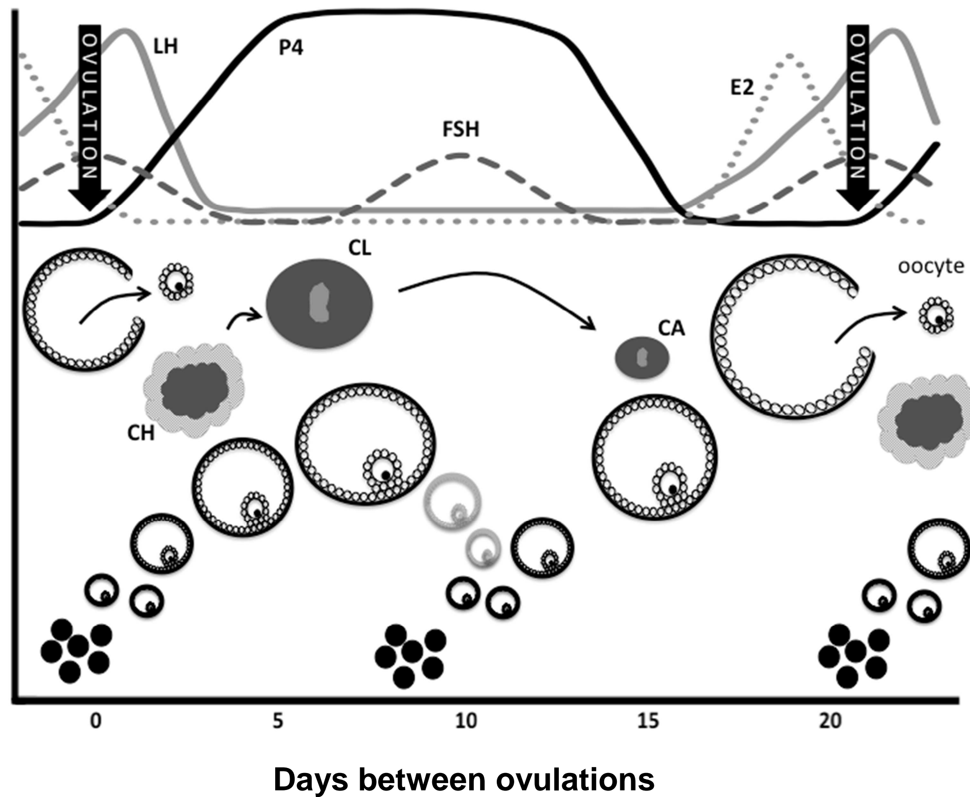


**Figure 1.5 Circulating hormone levels and follicular growth, atresia and ovulation during the equine ovulatory cycle. (Donadeu et al. 2012) LH: luteinising hormone; FSH: follicle stimulating hormone; P4: progesterone; E2: oestradiol; CH: corpus haemorrhagicum; CA: corpus albicans**

#### 1.3.2.2.2 Local control of follicle development

As the dominant follicle begins growing around six to eight days before ovulation, it produces increasingly higher levels of oestradiol (see Figure 1.6) (Ginther 1992d). Intrafollicular oestradiol levels were shown to increase shortly before deviation, suggesting a role during follicle selection (Gastal et al. 1999c). Oestradiol has been found to significantly stimulate the expression of LHR in the granulosa cells of rats (Richards et al. 1976), and this is thought to facilitate follicle selection similarly to other species (Ginther et al. 1996). Oestrogen deficiency, as occurs in large follicles of anoestrous mares, leads to anovulation and regression of the dominant follicle (Weedman et al. 1993). Furthermore, oestradiol is associated with causing oedemas, attributable to increased vascularity in the preovulatory follicle (Kerban et al. 1999).

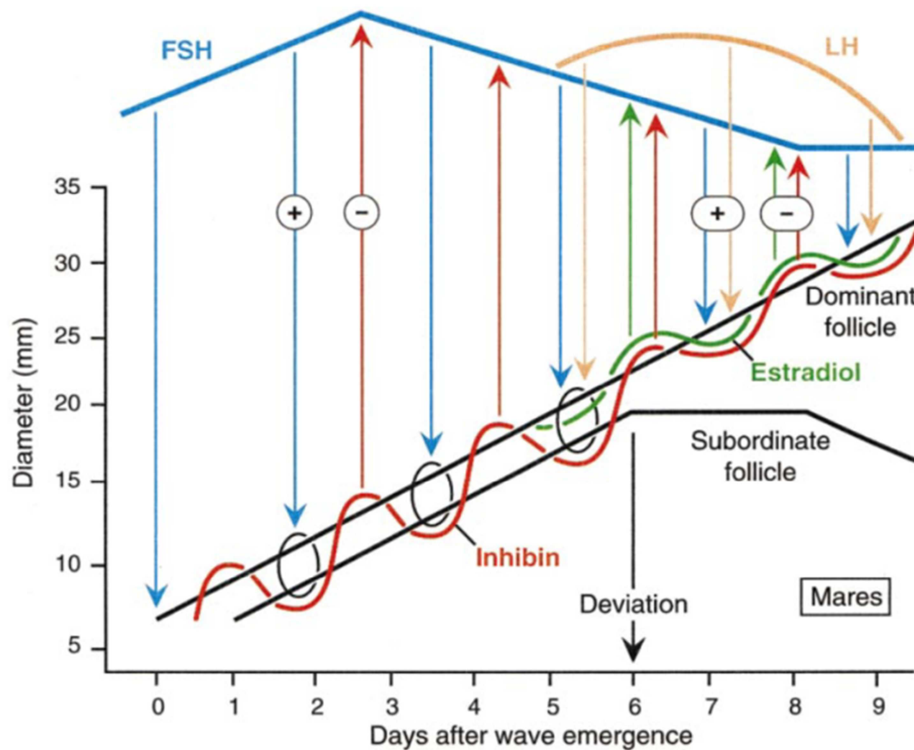


**Figure 1.6 Model for the endocrine regulation of an equine follicular wave. Growing follicles begin to secrete inhibin once they reach 13 mm. Inhibin in turn reduces circulating levels of follicle stimulating hormone (FSH). At deviation, the future dominant follicle continues growing by harnessing the increasing levels of luteinising hormone (LH), whereas the future subordinate follicle, still in need of FSH support, begins to regress. Increasing oestradiol levels secreted by the dominant follicle under stimulation by rising LH, further suppress FSH secretion. (Ginther et al. 2001)**

Insulin-like growth factor 1 (IGF1) is a key factor during deviation as demonstrated by the observations that 1) IGF1 levels begin to increase in the future dominant follicle just before deviation (Donadeu and Ginther 2002a) and 2) experimental injection of IGF1 into a future subordinate follicle resulted in that follicle becoming dominant (Doyle et al. 2008). Additionally, IGF1 receptor (IGF1R) expression increases during the growth of equine follicles (Doyle et al. 2008). IGF bioavailability in the follicle is regulated by IGF binding proteins (IGFBPs), which bind to IGF thus preventing it from interacting with its receptor and by IGFBP proteases, such as pregnancy-associated plasma protein-A (PAPP-A)(Fortune et al.

2004), which act by degrading IGFBPs, thus increasing the levels of bioactive IGFs (Beg and Ginther 2006). Injection of IGFBP3 into a future dominant follicle lead to follicular regression associated with decreased levels of IGF1, oestradiol, activin-A and inhibin-A (INHA) and increasing androstenedione (Ginther et al. 2004a), all of which are changes associated with subordinate follicles (Donadeu and Ginther 2002a). In mares, when PAPP-A was injected into the largest subordinate follicle, free IGF1 levels as well as inhibin-A, follistatin and VEGF increased, thus mimicking changes occurring in the dominant follicle during deviation (Ginther et al. 2005b). In general, intrafollicular concentrations of most IGF binding proteins, including IGFBP2, IGFBP4 and IGFBP5, but not IGFBP3, were found to increase in subordinate follicles compared to the dominant follicle (Gerard and Monget 1998; Bridges et al. 2002; Donadeu and Ginther 2002a). Taken together, the above results highlight the importance of the IGF system during follicle selection.

Activin-A, in addition to other members of the transforming growth factor β (TGFβ) superfamily, is associated with follicle growth in mammals (Knight and Glister 2006) and a potential association has been shown between activin-A (and follistatin) and oocyte competence in cows (Silva et al. 2003). Activin-A levels differentially increased in the largest follicle before deviation in mares (Donadeu and Ginther 2002a) and an injection of activin-A into the largest subordinate follicle promoted a diameter increase together with a decrease in follistatin and androstenedione levels (Ginther et al. 2005b).

Inhibins are produced by granulosa cells of small growing follicles during the follicular phase as well as the large dominant follicle in mares (Goudet et al. 1999) and have a negative effect on FSH secretion by antagonising activins (Bernard et al. 2010). Inhibin-A levels have been shown to increase during follicle growth in the mare, particularly in the dominant follicle during deviation (Donadeu and Ginther 2002a). In contrast, inhibin-B levels decreased in the dominant follicle (Donadeu and Ginther 2002a). Furthermore, inhibin is known to stimulate androgen production by the theca layer (Wrathall and Knight 1995).

The angiogenic factor, vascular endothelial growth factor (VEGF) is produced by the granulosa cells and its levels differentially increased in the future dominant follicle beginning before deviation (Ginther et al. 2004b), consistent with findings in other species (Mattioli et al. 2001). This differential increase in VEGF levels in the dominant follicle is thought to promote an increase in blood flow around the future dominant follicle one to two days before deviation, as determined using colour Doppler ultrasonography (Acosta et al. 2004b).

Additional factors like fibroblast growth factors (FGFs), which have been shown to be expressed at the mRNA level in small follicles and are capable of down-regulating the expression of CYP19A1, are currently being investigated in relation to folliculogenesis (Gasperin et al. 2012).

Taken together, follicle growth and development including deviation is a multi-factorial process, the control of which in mares is still lacking considerable knowledge. Further research into connecting these factors and investigating underlying pathways is necessary in the mare.

#### 1.3.2.3 Ovulation and luteal development

The morphology of the equine ovary is fundamentally different from most other mammals. Whereas in other species the cortex is by definition the outside functional layer surrounding the vascularised medulla at the centre, a folding event during embryonic development in the mare turns the medulla outwards to enclose the cortex containing the germ cells (Kimura et al. 2005). This results in the formation of a small area on the inside face of the kidney-shape ovary, called the ovulation fossa, which is devoid of medulla tissue and the only area where ovulation can occur (Witherspoon and Talbot 1970). The fact that ovulation can only occur through the ovulation fossa and not through the entire ovarian surface such as in other species, has long been thought to be the main reason for the difficulty of inducing superovulation in mares (McCue 1996).

Ovulation is a complex, inflammatory process involving many genes responding to the ovulatory LH surge in a multi-step cascade resulting in follicular cell

differentiation and profound tissue remodelling (Robker et al. 2000b). The genes and molecular pathways involved in ovulation have been extensively studied in rodents (reviewed by Richards et al. 2002).

In response to the LH surge, the preovulatory follicle in the mare softens and elongates towards the ovulation fossa indicating that other factors than increasing inner-follicular pressure are necessary for the expulsion of the oocyte at the apex (Ginther 1992e). Ovulation involves rupture of all layers of the follicular wall and the overlying ovarian stroma, which have to undergo integrity changes in the process. The granulosa cell layer thickens to double its size before ovulation, which presumably lessens the cell-cell attachments (Kerban et al. 1999) in response to an increase of acidic mucosubstances around cells. At the apex of the preovulatory follicle, collagenases cause the basement membrane to dissociate and the surrounding granulosa cells to undergo apoptosis, whereby they release hydrolases that add to degradation (Pierson 1993). The theca cell layer on the other hand appears to get thinner and is invaded by eosinophils (Kerban et al. 1999). Hyperaemia, oedema and haemorrhage are identifiable before ovulation around the ovulatory follicle (Kerban et al. 1999) comparable to an acute inflammatory reaction. Blood supply however is reduced around the apex, which facilitates degradation and cell death (Koenig and Probst 2005). Tissue degradation is triggered by LH-induced proteolytic enzymes like plasminogen activator (PA), plasmin and matrix metalloproteinases (MMPs) (Tsafriri and Reich 1999). The increased vascularisation at the base of the follicle and decreased vascularisation at the apex are mediated by changes in levels of VEGF, cytokines and eicosanoids amongst other factors (Tsafriri and Reich 1999). Two proteins induced by the LH surge have been shown to be essential for ovulation – progesterone receptor (PGR) and prostaglandin G/H synthase 2 (PTGS2) (Ojeda 2004a). Various studies showed that non-steroidal anti-inflammatory drugs (NSAIDs) inhibit prostaglandin synthesis and block ovulation, including in the mare (De Silva and Reeves 1985; Sogn et al. 1987; Hester et al. 2010; Cuervo-Arango and Domingo-Ortiz 2011), and that PTGS2-deficient mice are infertile (Lim et al. 1997). Prostaglandins have been found to mediate dissociation of cumulus cells leading to cumulus expansion, a necessary step during ovulation

(Ojeda 2004a). Also, PGR knock-out mice are infertile (Lydon et al. 1995). An increase in PGR expression leads to the downstream expression of a disintegrin and metalloproteinase with thrombospondin-like motifs 1 (ADAMTS1) and cathepsin L (Ojeda 2004a), both of which are involved in the ovulatory process. PGR expression increases in association with a sharp increase in progesterone production by the preovulatory follicle and if progesterone is experimentally inhibited, ovulation does not occur (Tsafriri and Reich 1999).

The development, morphology and luteolysis of the corpus luteum in the mare has been a subject of study for more than a century (reviewed in Harrison 1946; Ginther 2012). Following expulsion of the oocyte and follicular fluid during ovulation, the remaining cell layers of the follicle fold into each other and blood fills the antral space, forming a corpus haemorrhagicum (Ginther 1992e). In the horse it has been reported that the theca interna degenerates and only the granulosa cells contribute to the cell component of the future corpus luteum (Van Niekerk et al. 1975; Boerboom 2000). An increase in circulating progesterone begins immediately after ovulation and lasts for about five days, before it stabilises for the next ten days (Ginther 1992d). At the end of the luteal phase, the non-pregnant uterus secretes PGF2α, which lyses the corpus luteum leading to a drop in circulating progesterone to basal levels within two days (Ginther 1992d), to allow the development of a new ovulatory follicular wave.

## 1.4 Follicle development during periods of reduced LH secretion

As discussed above, LH is critical for normal development of ovulatory follicular waves, however, follicular growth still occurs in situations of reduced LH secretion, for example during periods of high circulating progesterone (luteal phase and pregnancy) and during short day length (anovulatory season). Physiological stages of low LH secretion therefore constitute an excellent model to study the role of LH in follicle development.

### 1.4.1 Follicle development during the anovulatory season

During the middle of the anovulatory period or deep anoestrus, follicular growth is reduced and usually only minor waves occur (i.e. dominant-size follicles do not develop) in association with seasonal suppression of LH release, which is mediated by substances such as opioids (Aurich et al. 1994). In response to increasing day length during late winter and early spring, recrudescence of the hypothalamus-pituitary axis results in a period of transition, which is characterised by renewed growth of a dominant follicle within waves, although ovulation does not occur and follicles subsequently regress (see Figure 1.7) (Donadeu and Watson 2007). At this time, levels of progesterone are basal due to the absence of a corpus luteum (Donadeu and Ginther 2002b).

Equine transitional dominant follicles have been studied with some detail. During spring transition follicular waves develop that produce follicles >21 mm in size (Donadeu and Ginther 2002b). A variable number of waves can develop during the one to three month long transition with some mares developing up to four waves with ovulatory-size follicles, which grow slower than ovulatory follicles and subsequently regress (Ginther 1990). Morphologically, compared to ovulatory follicles, dominant follicles during transition present a poorly developed theca cell layer with significantly reduced blood vessels and deficient levels of angiogenic growth factors in addition to lower proliferation activity of granulosa cells (Watson and Al-Zi'abi 2002). Follicular fluid has also been found to be deficient in oestradiol, progesterone, IGF1, INHA and VEGF during transition, and the reduced levels of oestradiol and INHA are associated with relatively high circulating FSH levels at that time (Donadeu and Ginther 2002b; Acosta et al. 2004a). Indeed, the follicular deficiencies are likely to be attributable to low stimulation by circulating LH rather than FSH, as FSH levels, in contrast to LH, are higher during transition than during the breeding season (Hines et al. 1991; Donadeu and Ginther 2003a). Circulating levels of LH and pulsatility as well as follicular fluid oestradiol levels progressively increase in association with each successive anovulatory wave developing during transition (Fitzgerald et al. 1987; Davis and Sharp 1991; Watson et al. 2002b). In addition, the first ovulatory wave is associated with a preovulatory LH surge of

lower magnitude and a dominant follicle that is steroidogenically less active but larger than subsequent ovulatory waves during the breeding season (Ginther 1990; Donadeu and Ginther 2004).

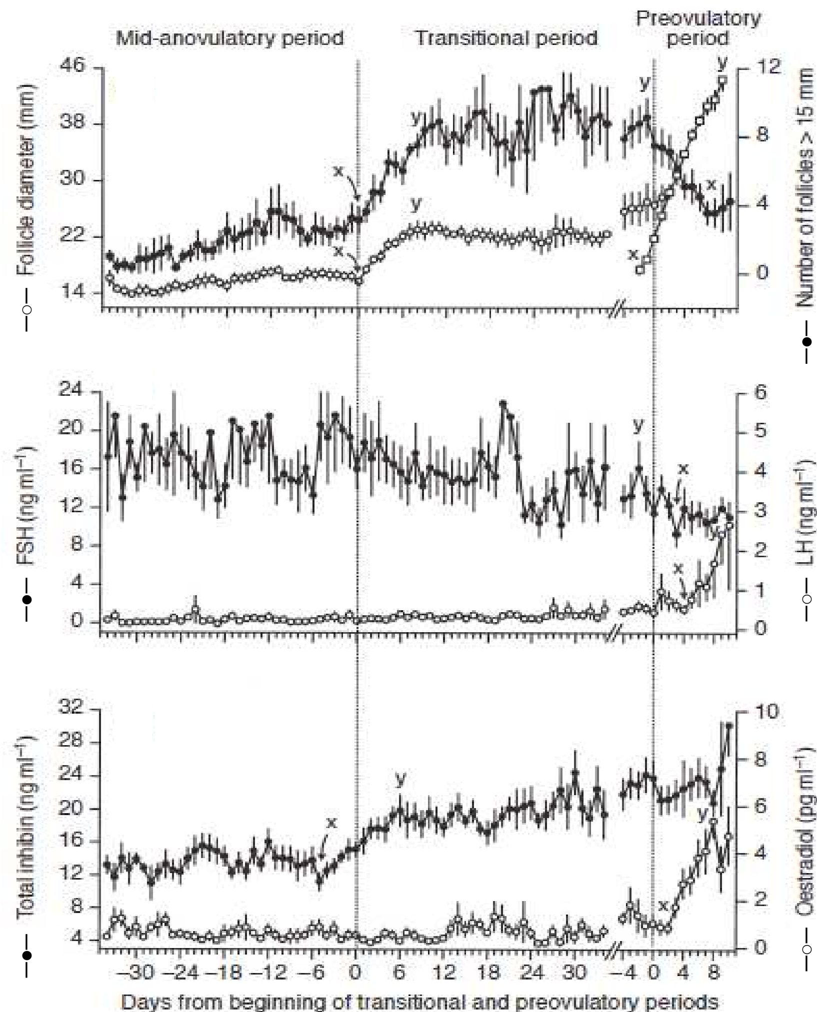


**Figure 1.7 Follicle development and associated hormone levels during deep anoestrus, spring transition and the first pre-ovulatory period of the breeding season in pony mares. (Donadeu and Ginther 2002b) x,y denotes significantly different day means within measurement ($P < 0.05$)**

**Methods to advance the onset of the breeding season**

There is great interest by the equine industry for foals to be born early during the year and therefore to advance the onset of the breeding season (see above).

Hastening the onset of the breeding season has been achieved by the use of artificial lighting regimes as early as 1947 (Burkhardt 1947; Scraba and Ginther 1985; Palmer and Guillaume 1992). Artificial light treatments bring the date of the first ovulation of the year forward but do not eliminate the development of transitional anovulatory waves, which are a source of considerable frustration to veterinarians and breeders eager to get their mares in foal.

Various therapeutic strategies have been investigated to shorten or omit the transitional period including GnRH, hCG, eFSH and progesterone. Early studies hypothesised that treatment with GnRH would increase the secretion of LH from the pituitary and restore cyclicity in transitional mares. However, responses of anoestrous, early transitional and even late transitional mares regarding LH secretion were poor (Silvia et al. 1987). Fifty per cent of mares during late transition ovulated within 30 days of treatment with GnRH or GnRH agonist (Harrison et al. 1990) and some become pregnant after ovulation induction with hCG, although another study reported reduced pregnancy rates from mares stimulated with GnRH during the natural anovulatory season (Ginther and Bergfelt 1990). In another study, mares with transitional follicles >35 mm ovulated in response to an injection of hCG and had similar fertility as normally cycling mares (McCue et al. 1992). Following a reported negative effect of dopamine on LH pulse frequency in ewes (Thiéry et al. 1995), Besognet et al. (1996) examined the effects of the dopamine antagonist, sulpiride, in transitional mares. Even though the first ovulation of the season was significantly advanced by sulpiride, circulating LH levels were not different between groups but mean FSH pulse frequency and plasma concentration was significantly elevated in sulpiride-treated mares. Some subsequent studies (Besognet et al. 1997; Daels et al. 2000; Mari et al. 2009) but not others (Donadeu and Thompson 2002) confirmed the efficacy of sulpiride on advancing the onset of the ovulatory season. The efficacy of administration of purified or recombinant equine FSH or porcine FSH to advance the breeding season in mares has also been studied. In those studies, the onset of the breeding season was significantly hastened, however, the occurrence of multiple ovulation was greatly increased (Niswender et al. 2004). Similar results were obtained when crude equine gonadotropin was used (Lapin and Ginther 1977).

Finally, consistent with the notion that anovulation during the transitional period may be attributable to deficient LH rather than FSH, repeated low dose treatment with hCG effectively advanced the onset of the breeding season in mares (Bour and Palmer 1984), however, the practicality of using such approach commercially would be questionable due to the potential antigenicity of repeated hCG administration in mares (Wilson et al. 1990). However, whether repeated injection of equine LH rather than hCG could effectively induce ovulation during the transitional period has not been examined.

In conclusion, pharmacological strategies to advance the onset of the breeding season in mares are only efficient when used during the late transitional period, when ovaries already contain large follicles, and this may require repeated administration of GnRH or gonadotropin preparations (Ginther and Bergfelt 1990; McCue et al. 1991; Turner and Irvine 1991). Such treatments may also favour the occurrence of multiple ovulations (Johnson 1987; Ginther and Bergfelt 1990), with the resulting risk for twin pregnancies. Furthermore, some of these treatments are not licensed for use with horses.

### 1.4.2 Follicle development during the luteal phase

Relatively little is known about the development of large follicles during the luteal phase in mares, when high circulating progesterone keeps circulating LH levels below a threshold of around 2 ng/ml (Ginther et al. 2006b). Major waves occur during the luteal phase in around 25% of mares (Ginther 1993), whereas cows normally have one or two major anovulatory waves during each luteal phase (Ginther et al. 1989). In some instances, such large follicles can ovulate naturally in horses, in association with moderately elevated LH levels (Vandeplassche et al. 1979). Such dioestrous ovulations may occur in around 3 – 4% of oestrous cycles (Ginther 1992e), but have been found to occur not at all in some other studies (Ginther 1993), which may reflect an effect of breed, as thoroughbred mares seem to have a higher incidence of dioestrous ovulations than pony mares (Wesson and Ginther 1981).

### 1.4.3 Follicle development during pregnancy

As during the luteal phase, elevated progesterone levels during pregnancy have a negative effect on circulating LH levels. During the first half of pregnancy follicular waves continue to occur although major waves no longer develop in some mares (Ginther and Bergfelt 1992). Follicle numbers are highest at around day 40 of pregnancy and then begin to decrease, possibly in response to decreasing LH concentrations, so that throughout the second half of pregnancy follicular growth is at its minimum (Ginther 1992h). Even during pregnancy, season has an influence on follicle numbers, with reduced numbers per ovary during winter months (Ginther and Bergfelt 1990). However, formation of supplementary corpora lutea occurs during early pregnancy from ovulation or luteinisation of follicles in response to eCG (Allen 1984). These accessory corpora lutea produce high levels of progesterone and are active until approximately day 200 of pregnancy before they regress (Ginther 1992h). During early pregnancy, LH spikes were observed in mares (Nett et al. 1975) and circulating LH levels increased transiently around day 75 of pregnancy (Aurich et al. 2001). LH levels during the second half of pregnancy decrease between day 140 and 170 of gestation following the regression of the endometrial cups and therefore decreasing levels of eCG, while levels begin to increase again from day 210 together with an increase of circulating FSH with maximal concentrations around day 280, before dropping again to basal level around one week before parturition (Nambo et al. 1996).

### 1.4.4 Follicle development during the postpartum period

Within 72 hours of parturition in mares, circulating LH levels increase in response to the removal of negative feedback from the feto-placental unit, together with an increase in oestradiol (Ginther 1992g). Because foaling usually occurs during spring, seasonal effects may also play a role in the postpartum elevation in LH levels (Ginther 1992g). When mares are lactating, LH remains at reduced levels for the first 16 weeks after parturition (Heidler et al. 2003), potentially inducing a prolonged postpartum anovulatory phase. Furthermore, poor nutritional body score at parturition significantly decreases circulating LH levels and the mechanism

responsible for anovulation during that time may be the same as during the spring transition (Hines et al. 1987).

## 1.5 Effects of overexposure to LH on follicle development

Chronically raised levels of circulating LH have been found to be associated with sub-/infertility and multiple ovarian abnormalities. Women with elevated LH levels have a lower chance to conceive and a much higher rate of miscarriages than women with lower levels (Regan et al. 1990), and pregnancy rates in in-vitro fertilisation programmes were significantly decreased in high LH level patients (Stanger and Yovich 1985). Chronically elevated LH levels present together with other hormonal disruptions like elevated testosterone and androstenedione and reduced oestradiol levels (Regan et al. 1990). In women with polycystic ovarian syndrome (PCOS) circulating LH levels are significantly higher than in healthy women and FSH levels are lower, with a complete loss of a coordinated cyclic pattern resulting in anovulation (Yen et al. 1970), often accompanied by insulin resistance, obesity, hyperandrogenism, hyperprolactinaemia, hirsutism and acne (Franks 1995). Therefore it can be challenging to establish whether excessive circulating LH levels is a cause or an effect of such syndrome.

The development of transgenic mouse models has opened the possibility to understand the pathogenesis and mechanisms of disorders associated with high LH. One study showed that female mice expressing a chimeric LH beta subunit and that hypersecreted LH developed uterine and ovarian pathologies, including cystic, haemorrhagic and anovulatory follicles (HAFs), leading to infertility (Risma et al. 1995). Further study on these mice showed the formation of ovarian cysts, early luteinisation and tumours (Risma et al. 1997). Elevated LH is also associated with accelerated ovarian follicle development, which has been shown to cause a depletion of the primordial follicle pool in mice (Flaws et al. 1997). Furthermore, rats exposed to human chorionic gonadotropin stopped ovarian cyclicity and induced the formation of ovarian follicular cysts (Poretsky et al. 1992). A model to explain how elevated LH may lead to ovarian malformations has been proposed. In transgenic

mice with elevated LH secretion, the ovary synthesises an excess of oestrogens, leading to an increase in prolactin secretion and a positive feedback on LH secretion together with an increase in LHR expression both in the ovary and the adrenal cortex, causing excess corticosterone production (Kero et al. 2000).

In domestic species very little is known about the effects of persistently elevated LH levels. Anovulation in cattle often occurs due to the formation of follicular cysts, which have been associated with continuously elevated circulating levels of LH (Hamilton et al. 1995) as well as with increased expression of LH receptor mRNA in granulosa cells (Calder et al. 2001).

Follicles that grow to preovulatory size but fail to ovulate instead filling up with blood and luteinising occur in women and mares and are termed luteinised unruptured follicles (LUFs) or haemorrhagic anovulatory follicles (HAFs). Equine haemorrhagic anovulatory follicles have been studied in some detail. In the mare these are more common later in the breeding season (Ginther et al. 2007a) and are seen more often in older animals (McCue and Squires 2002). Studies have speculated about the reasons of HAFs occurrence: overstimulation by gonadotropins, insufficient gonadotropin stimulation, insufficient oestrogen production or haemorrhage into the preovulatory follicle (Ginther and Pierson 1989; Cuervo-Arango and Newcombe 2010). In control cycles an incidence of HAFs between 3.1% (Ginther and Pierson 1989) and 11.9% (Lefranc and Allen 2003) has been reported. Ultrasound indicators of approaching ovulation (oedema, size, follicle wall) were similar for normally ovulating follicles and HAFs and only oestradiol was elevated three days before HAF formation (Ginther et al. 2006a). Most HAFs apparently contained luteinised cells (Lefranc and Allen 2003) with no differences in progesterone levels between HAF and normally ovulating mares (Ginther et al. 2006a).

A connection between HAFs and elevated LH in the mare has been suggested. An increase in the incidence of HAFs was found when crude equine gonadotropin or equine FSH was used to induce superovulation (Palmer and Hajmeli 1992; Hofferer et al. 1993; Briant et al. 2004b). Three separate studies where prostaglandin

administration was used to manipulate the equine oestrous cycle also reported an increased incidence of HAFs (up to 10-fold). In two studies, in which PGF2α was administered during the luteal phase to induce the development of a follicular wave, a high incidence of ovulatory anomalies was found with an increased double ovulation rate in some mares and HAF formation in others (Ginther et al. 2008b; Ginther and Al-Mamun 2009). In a third study a synthetic analogue of PGF2α, cloprostenol, was given to mares to shorten oestrous cycle length and a significant increase in the formation of HAFs was observed (Cuervo-Arango and Newcombe 2009). Prostaglandin F2α is secreted by the non-pregnant uterus of the mare and naturally induces luteolysis at the end of a normal luteal phase thus removing the negative feedback of luteal progesterone on gonadotropin secretion. Because of this property, PGF2α analogues have long now been used commercially to shorten the natural luteal phase and hasten ovulation. Based on the results of the above studies, it has been suggested that the an increase in circulating LH associated with induction of luteolysis with PGF2α in mares may result in exposure of follicles to excessive LH early during development thus disrupting their development, normal maturation and ovulatory capacity (Cuervo-Arango and Newcombe 2009; Ginther and Al-Mamun 2009).

## 1.6 Regulation of ovarian gene expression by microRNAs

### 1.6.1 miRNA biogenesis

Mature microRNAs (miRNAs) are a group of small (about 22 nucleotides long) non-coding ribonucleic acid molecules, which alter posttranscriptional gene expression through sequence-specific binding of target mRNA, leading to its translational repression or degradation (Du and Zamore 2005). Since the discovery of the first miRNA in 1993 (Lee et al.), great advances have been made in understanding post-transcriptional regulation in animal cells, including in relation to reproductive physiology. miRNAs have so far been shown to be involved in cell differentiation, growth and apoptosis, tissue development and angiogenesis, both in normal and pathological processes (Huang et al. 2011). miRNA sequences are highly conserved between species, highlighting their important role as master regulators of cell

differentiation processes (Berezikov 2011). Genomic miRNA sequences are found as independent genes (intergenic), intronic (mirtrons), and can be clustered within genomic regions (Berezikov 2011). In the horse, 360 mature miRNA sequences have so far been identified (miRBase, release 19.0; http://www.mirbase.org) in comparison to over 2000 different miRNA sequences for humans.

miRNA genes are transcribed into primary miRNAs (pri-miRNAs), which can be thousands of nucleotides long, and are then processed in the nucleus by the microprocessor complex including Drosha into stem-loop precursors (pre-miRNAs). Pre-miRNAs are around 70 nucleotides long and are exported into the cytoplasm by Exportin-5 (Lee et al. 2003). Once in the cytoplasm, Dicer, a member of the superfamily of RNases III, cleaves the loop off the pre-miRNAs producing a miRNA duplex, a process followed by strand selection, which results in mature miRNA and miRNA* sequences (Kim 2005). An argonaute protein (AGO1 or AGO2) incorporates the miRNA sequence into an RNA-induced silencing complex (RISC) where it induces the repression of target mRNAs, whereas the miRNA* strand is usually degraded (see Figure 1.8) (Okamura et al. 2009).

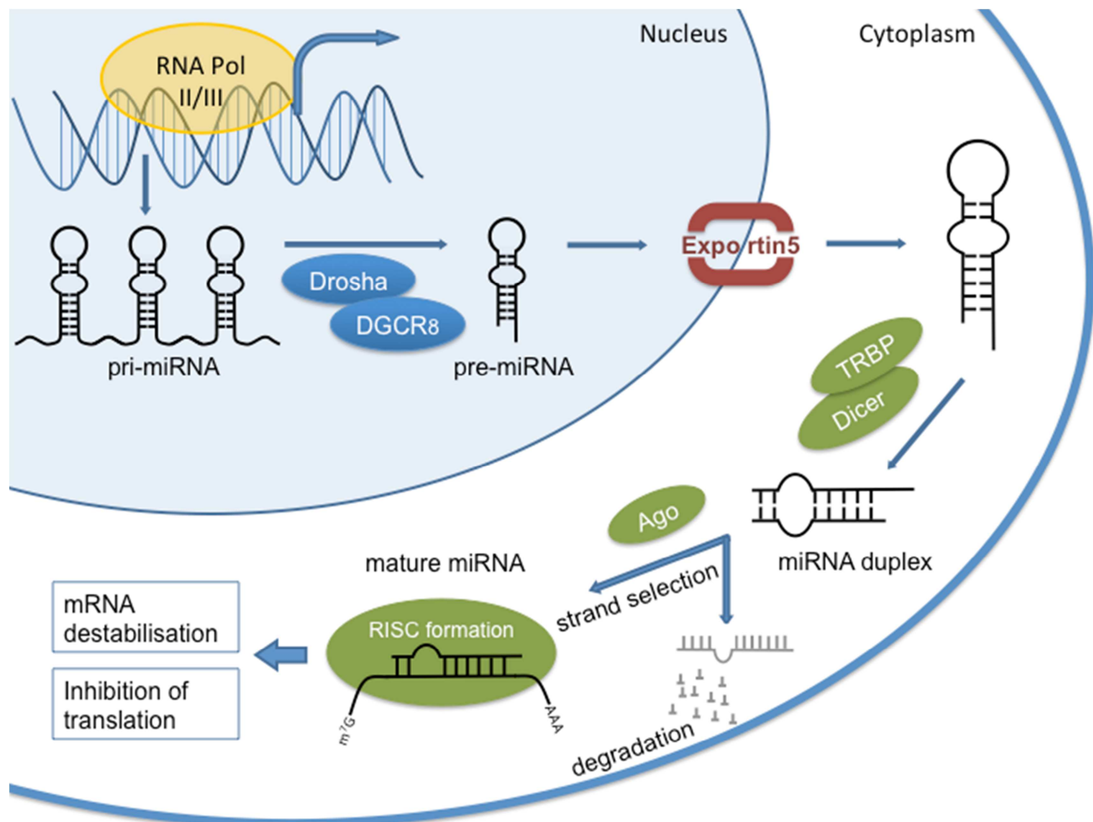


**Figure 1.8 Canonical pathway for miRNA biogenesis in eukaryotic cells. miRNA genes are mainly transcribed by RNA polymerase II to generate primary miRNAs (pri-miRNAs), followed by cleavage (cropping) by the microprocessor complex (Drosha and DGCR8). The resulting pre-miRNA is exported from the nucleus to the cytoplasm by Exportin 5, where the stem loop is removed by the RNase complex containing Dicer. Here one strand of the miRNA duplex is loaded into the RISC complex, guiding it to target mRNA, which is destabilised or its translation is inhibited, while the opposite strand is usually degraded. (Donadeu et al. 2012) DGCR8: DiGeorge syndrome critical region 8; TRBP: TAR RNA-binding protein; Ago: Argonaute; RISC: RNA-induced silencing complex.**

### 1.6.2 miRNA expression in the ovary

miRNA expression in the ovary has been investigated through RNA sequencing and array-based analyses, providing a wealth of new information. In addition, computational analyses have been used to predict thousands of mRNA targets (Thomas et al. 2010), however, some of these turned out not to be *bona fide* targets and for many biologically relevant targets computational predictions failed. In

addition, all predicted miRNA-mRNA interactions need to be experimentally validated and verified for biological relevance. Further difficulty is added by the fact that miRNA targets can be cell-context dependent (Pasquinelli 2012) and that relevant effects on target tissues may need co-expression of more than one miRNA as the effect of individual miRNAs on their targets are often modest and may be difficult to detect experimentally (Berezikov 2011).

The first approach to investigate miRNA functions was to produce mice carrying knockouts for Dicer, Ago2 or Dgcr8. Because the general Dicer1 knockout mouse turned out to be embryonically lethal, potentially because of the loss of stem cells or impaired angiogenesis (Bernstein et al. 2003; Yang et al. 2005), a mouse line was created to knock out Dicer1 under the control of anti-müllerian hormone receptor 2 promoter (Amhr2Cre/+), therefore specifically affecting the female reproductive tract (Hong et al. 2008; Nagaraja et al. 2008; Lei et al. 2010). The Amhr2Cre/+ mice showed stunted reproductive organ development and the development of cysts in the oviduct (Hong et al. 2008), leading to infertility. Other groups using the same mouse model found increased recruitment and degeneration of early follicles and changes in the expression of genes associated with steroidogenesis by and growth of granulosa cells caused by Dicer1 knockout (Lei et al. 2010). The reproductive abnormalities described above together with reduced ovulatory capacity were associated with specific changes in the levels of miRNAs and their target mRNAs (Nagaraja et al. 2008), showing that Dicer is crucial for miRNA biosynthesis in the ovary. Another strategy to study the effects of miRNA deficiency in vivo was to create a mutant mouse with a hypomorphic Dicer1 allele. These mice displayed female infertility caused by impaired angiogenesis in the corpus luteum which could be reversed by the injection of specific miRNAs putatively involved in angiogenesis (Otsuka et al. 2008). Interestingly, like Dicer1 knockouts, hypomorphic Dicer1 mice showed no changes in circulating oestrogen levels or mating behaviour (Hong et al. 2008; Nagaraja et al. 2008; Otsuka et al. 2008; Lei et al. 2010). In addition, only 28 miRNAs were downregulated in Dicer1 knock out oviducts (Nagaraja et al. 2008), providing further evidence for an additional Dicer-independent pathway as well as a

Drosha-independent pathway for miRNA maturation, which adds further layers to the complexity of miRNA expression and action in the cell (Miyoshi et al. 2010).

Numerous studies have used sequencing of whole ovaries or ovarian fractions to identify miRNA populations. Interestingly, in all those studies as few as 10 miRNAs, some of them common between studies, accounted for more than half of all ovarian miRNA sequences suggesting these miRNAs have important functions in the ovary (see Table 1.1). Some studies reported expression differences between the ovarian and follicular tissue compartments, including expression of miR-503, miR-224 and miR-383, which are expressed predominantly in granulosa cells and oocytes of mice (Lei et al. 2010; Yao et al. 2010a; Yin et al. 2012).

**Table 1.1 Ten most abundant miRNAs in whole ovaries[1] or follicular/luteal tissues[2] as reported in different studies using cloning-based[a] or next-generation sequencing[b].**

| Landgraf et al., 2007[1b] | Huang et al., 2011[1b] | Tripurani et al., 2010[1a] | Hossain et al., 2009[1a] | McBride et al., 2012[2a] | Li et al., 2011[1b] | Mishima et al., 2008[1a] | Ahn et al., 2010[1a] |
|---|---|---|---|---|---|---|---|
| human (adult) | cow (neonatal and adult combined) | cow (fetal) | cow (adult) | sheep (adult) | pig (adult) | mouse (adult) | mouse (neonatal) |
| miR-143 | miR-143* | miR-99a | let-7b | miR-21 | miR-21 | miR-125b | miR-320 |
| miR-125b | let-7f | miR-125b | let-7c | miR-125b | miR-143-3p | miR-21 | let-7i |
| let-7b | let-7a | let-7c | miR-143* | let-7b | let-7i | miR-199a-3p | let-7d |
| let-7a | let-7c | miR-125a | miR-23b | let-7a | let-7f | miR-99a | miR-298 |
| miR-16 | miR-10b | miR-26a | let-7a | miR-16b | miR-148a-3p | miR-145 | miR-199a |
| miR-126 | let-7b | let-7b | miR-24 | miR-142-3p | let-7a | miR-351 | miR-30a |
| miR-99a | miR-26a | miR-10b | miR-27a | let-7c | let-7c | miR-214-3p | miR-140 |
| miR-26a | miR-148a | let-7a | miR-652 | miR-202 | miR-24-3p | miR-143-3p | miR-322 |
| miR-29a | miR-21 | miR-145 | miR-21 | miR-199a | miR-34c | miR-93 | miR-152 |
| let-7c | miR-140 | miR-126* | miR-126 | let-7f | miR-29a-3p | miR-322 | miR-26a |

### 1.6.3 miRNAs involved in follicle and luteal development

Altered miRNA expression profiles associated with different follicle or luteal developmental stages have been described in mice (Yao et al. 2009; Lei et al. 2010; Yin et al. 2012), pigs (Xu et al. 2011; Lin et al. 2012) and sheep (McBride et al. 2012). Furthermore, experiments with cultured granulosa cells have provided insight into the regulation of miRNA expression by gonadotropins (Fiedler et al. 2008; Lei et al. 2010; Yao et al. 2010b; Yin et al. 2012) or growth factors (Yao et al. 2010a). Treatment of granulosa cells from mouse pre-antral follicles with TGFβ1 in culture showed upregulation of miR-224. This miRNA was shown to mediate the stimulatory effects of TGFβ1 on granulosa cell proliferation, Cyp19a1 expression and oestradiol production possibly by targeting Smad4 (Yao et al. 2010a). Also, miR-383, which is downregulated by TGFβ1, was shown to positively regulate Cyp19a1 expression and oestradiol production by targeting RNA binding motif, single stranded interacting protein 1 (RBMS1) (Yin et al. 2012). miR-383 itself is transcribed in response to steroidogenic factor 1 (SF1), a known gonadotropin-induced regulator of steroidogenic genes including STAR, CYP11A1 and CYP19A1 (Parker and Schimmer 1997) and miRNA-383 expression decreased following hCG administration. Another miRNA, miR-378, has been shown to directly target CYP19A1 in porcine granulosa cells. miR-378 is expressed at high levels in small follicles and at lower levels in large follicles (Xu et al. 2011). miR-378 recognised two potential miRNA response elements (MREs) in the 3' UTR of the porcine CYP19A1 gene, and when miR-378 was overexpressed or inhibited in cultured granulosa cells its effects were negatively correlated with CYP19A1 expression. Together, these data show how oestradiol production can be regulated by more than one miRNA and further investigation into the mechanisms and spatio-temporal expression patterns involved is needed. Furthermore, some miRNAs have been identified to play a role in atresia, including miR-21, miR-503 and miR-26b. Carletti et al. (2010) showed that inhibition of miR-21 induced apoptosis of granulosa cells from mouse preovulatory follicles both *in vivo* and *in vitro* followed by 2-fold reduced ovulatory rates. Additionally miR-21 has been shown to be upregulated in response to an ovulatory LH surge in mice (Fiedler et al. 2008), which suggests a role of miR-21 in ovulation and in luteinisation. Another miRNA which is mainly

expressed in the ovary (Ahn et al. 2010), miR-503, has been found to be differentially expressed during the peri-ovulatory period in mice (Lei et al. 2010) and sheep (McBride et al. 2012). Finally, expression levels of miR-26b increased during follicular atresia, potentially inducing granulosa cell death by directly targeting ataxia telangiectasia mutated (ATM), a gene involved in DNA repair (Lin et al. 2012).

Studies so far have identified miRNA populations putatively involved in follicular development and atresia, ovulation and the follicular-luteal transition (see Table 1.2). Recent progress in understanding small RNA biology and physiology has brought new and exciting perspectives about the regulation of reproductive function by miRNAs. Furthermore miRNAs can be used as potential biomarkers for disease as well as for physiological developmental processes.

**Table 1.2 Summary of reported roles of miRNAs during ovarian follicular/luteal development**

| miRNA | Site of expression | Reported function(s) | Target(s)* | Reported changes during development | Species *(reference)* |
|---|---|---|---|---|---|
| miR-224 | Granulosa | Stimulates cell proliferation, *cyp19a1*, oestradiol levels | *Smad4* | TGFβ1-induced increase in preantral follicles | Mouse *(Yao et al., 2010a)* |
| miR-383 | Granulosa, Oocyte | Stimulates *cyp19a1*, oestradiol levels | *Rbms1* | TGFβ1-induced decrease in preantral follicles, gonadotropin-induced increase in antral follicles, decrease before ovulation | Mouse (*Yin et al., 2012)* |
| miR-378 | Granulosa | Inhibits *CYP19A1*, oestradiol levels | *CYP19A1* | Decrease during antral follicle growth | Pig (*Xu et al. 2011)* |
| | CL | | *(INFGR1)* | Increase during luteal development, decrease during luteal regression | Cow *(Ma et al. 2011)* |
| miR-23a | n.d. | Pro-apoptotic in granulosa cells | *XIAP* | n.d. | Human *(Yang et al. 2012)* |
| miR-26b | Follicle | Pro-apoptotic in granulosa cells | *ATM* | Increase during follicular atresia | Pig *(Lin et al. 2012)* |
| miR-132/ miR-212 | Granulosa | n.d. | *Ctbp1* | Increase during hCG-induced ovulation | Mouse (*Fiedler et al. 2008)* |
| miR-21 | Granulosa | Anti-apoptotic (shown *in vivo*) | n.d. | Increase during hCG-induced ovulation | Mouse (*Carletti et al. 2010)* |
| miR-125b | Granulosa, theca, CL | n.d. | *(LIF)* | Decrease during luteinisation | Sheep (*McBride et al. 2012)* |
| miR-145 | Theca, CL | n.d. | *(CDKN1A)* | Decrease during luteinisation | Sheep *(McBride et al. 2012)* |
| | n.d. | Inhibits granulosa cell proliferation | *Acvr1b, Ccnd2* | n.d. | Mouse *(Yan et al. 2012)* |
| miR-199a-3p | Theca, luteal | n.d. | *(PTGS2)* | Decrease during luteinisation | Sheep *(McBride et al. 2012)* |
| miR-503 | Granulosa, oocyte | n.d | *Acvr2, Bcl2, Ccnd2, Inha, Cyp19a1, Lhcgr, Esr2, Cdkn1b* | Gonadotropin-induced decrease during follicle development | Mouse *(Lei et al. 2010)* |
| let-7b/ miR-17-5p | Luteal | Pro-angiogenic (shown *in vivo*) | *Timp1* | n.d. | Mouse (*Otsuka et al. 2008)* |

* targets were identified using luciferase reporter constructs
( ) miRNAs that were downregulated leading to increased gene expression

## 1.7 Objectives

The overall objective of this series of studies described in the following chapters was to investigate the role of LH in ovarian follicle development by examining the effects of under- and overexposure of developing follicles to LH on follicle maturation and ovulatory capacity, both at the physiological and molecular levels.

### 1.7.1 Effects of LH supplementation of transitional follicles on steroidogenic and ovulatory capacity

The aim of this study was to test the hypothesis that anovulatory transitional follicles develop under insufficient circulating LH levels and that supplementation of these follicles with LH would restore steroidogenic capacity, allowing normal ovulation.

This experiment involved treating early transitional mares twice daily with exogenous equine LH (eLH) or saline and monitoring their follicular and oestradiol responses by daily ultrasonography and blood sampling. Further, luteal function resulting from hCG-induced ovulation in these mares was studied by daily measurements of circulating progesterone.

### 1.7.2 Effects of elevated circulating LH levels on follicle maturation and ovulatory capacity

The hypothesis was tested that experimentally inducing an increase in circulating levels of LH early during a follicular wave would disrupt normal follicle maturation and ovulation.

In study one, throughout the second half of an oestrous cycle mares were administrated twice daily injections of an ovulatory dose of eLH or vehicle followed by induction of ovulation with hCG once a follicle reached >32 mm. The effects of treatment on total circulating LH levels and ovulation were recorded. Furthermore, luteal activity resulting from hCG-induced ovulations was assessed by measuring circulating progesterone levels.

In study two, the effects of twice daily eLH administration or an increase in endogenous LH following a single $PGF_{2\alpha}$ injection at mid-cycle were determined in

relation to follicular growth and levels of follicular fluid factors associated with maturation.

### 1.7.3 Comparative molecular analyses of ovulatory and anovulatory follicles

The aim of this study was to determine the levels of steroidogenic enzymes and selected miRNAs associated with reduced maturation of follicles naturally exposed to low circulating LH levels. Antral follicular fluid and granulosa cells from follicles at four different stages – oestrus (dominant and subordinate follicles), dioestrus and spring transition – were collected and compared in relation to follicular fluid factors and genes associated with steroidogenesis as well as key miRNAs putatively involved in regulating follicle maturation.

# Chapter 2

# Materials and Methods

# Chapter 2: Materials and Methods

## 2.1 Experimental animals

A total of nine mares, mainly Welsh pony cross, were used from 2009 to 2012 in the Northern Hemisphere (55° N; Edinburgh, UK). The age of these mares ranged from three to 16 years and the bodyweight from 225 kg to 530 kg. The mares were kept under natural light with ad libidum access to haylage during experimental periods, and on grass pasture between experiments, always with free access to mineral salt and water. All experimental procedures were carried out under the UK home Office Animals (Scientific Procedure) Act 1986, after approval by the Ethical Review Committee, University of Edinburgh.

To assess ovarian status and follicle development throughout experiments, mares were examined by trans-rectal ultrasonography using a 7.5 Mhz rectal transducer on a DP-6600 Vet Digital Ultrasonic Diagnostic Imaging System (BCF Technology, Livingston, UK). The diameter of follicles smaller than 10 mm was estimated by comparison with the graduation marks on the scanner screen, and larger follicles were measured twice in two perpendicular planes with electronic callipers and the average of length and width of both measurements was taken as the actual diameter of the follicle. Ovulation was defined as the time when a corpus luteum was first detected ultrasonically.

## 2.2 Equine LH preparation

A purified pituitary fraction, supplied by Dr. Christine Briant, *Unite Physiologie de la Reproduction et des Comportements*, INRA, Nouzilly, *Universite de Tours*, France, was prepared from crude equine gonadotropin (CEG 1.98) as described (Hofferer et al. 1993), and was used as source of equine LH (eLH). This fraction was previously shown to contain only residual FSH and to effectively induce ovulation when injected into oestrous mares (Hofferer et al. 1993). The preparation used in the present study was diluted in physiological saline to contain 160 µg eLH/ml, and its eFSH content was determined to be below assay sensitivity, i.e. <1.56 µg/ml.

## 2.3 Sample collection and preparation for analysis

### 2.3.1 Blood samples

Blood samples were collected daily during experiments into heparinised vacutainers via i.v. puncture. Samples were stored on ice immediately after collection until centrifugation at 1500 g for 13 minutes. The plasma was then stored at -20 °C until hormone analyses.

### 2.3.2 Follicular fluid (FF) and granulosa cell pellet (GCs)

Before experiments, all follicles were transvaginally ablated on both ovaries to remove regressing follicles and allow a new follicular wave to emerge. For this procedure mares were sedated with Sedivet® at a dose of 0.4 ml/100 kg bodyweight i.v. (equivalent to 40 µg romifidine/kg bw) and rectal straining was reduced by the administration of 5 ml/100 kg bw i.v. Buscopan® Compositum (equivalent to 0.2 mg butylscopolamine bromide and 25 mg metamizole/kg bw). After each procedure the mares received Depocillin® at a dose of 4 ml/100 kg bw i.m. (equivalent to 12 mg Procaine Benzylpenicillin/kg bw). Ablated follicles that refilled with fluid to >15 mm were re-ablated. Transvaginal ablation and follicular fluid aspiration were performed using an Aloka 500V ultrasound scanner (BCF Technology, Livingston, UK) with a 5 mHz curved array transducer with needle guide for a 17-gauge 55 cm ovum pick-up needle (Popper & sons Inc., New Hyde Park, NY, USA).

Collected follicular fluid was immediately stored on ice until centrifugation at 700 g for 10 minutes at 4 °C. The follicular fluid was carefully taken off without disturbing the granulosa cell pellet and was frozen at -20 °C until analyses. The leftover fluid including the granulosa cell pellet was pipetted into a 1.5 ml eppendorf tube and spun again for 5 minutes at 600 g. The supernatant was discarded and the remaining pellet was visually evaluated for red blood cell contamination and if necessary, treated with 1 ml of red blood cell lysis buffer (10 mM KHCO3, 150 mM NH4Cl, 0.1 mM EDTA, pH 8.0) for 1 minute before adding 10 ml physiological PBS and centrifuging again for 5 minutes at 600 g. The supernatant was discarded and the pellet was stored in 500 µl RNA-Bee (AMS Biotechnology, Abingdon, UK) at -80 °C until RNA extraction.

## 2.4 Hormone analyses

Assay sensitivity and CVs for all assays are given for each experiment separately. All RIAs and ELISAs were validated for use with equine samples in our laboratory by showing parallelism between serial sample dilutions and the provided assay standard curve. Sensitivity was in all cases calculated by subtracting two standard deviations from the mean cpm or OD at maximum percentage binding.

### 2.4.1 Solvent extraction for oestradiol in plasma

Briefly, 400 µl of plasma were pipetted in triplicates into extraction tubes (borosilicate glass PYREX, 100 x 16 mm, with screw top; Mackay and Lynn Ltd, Loanhead, UK) and topped up with 4 ml of "fresh" diethyl ether (Fisher scientific, Loughborough, UK) under a fume hood and closed tightly with PYREX screw caps (PTFE lining, Mackay and Lynn Ltd.). Tubes were then vigorously vortexed on a multitube vortexer for 15 minutes and left to settle for 5 minutes before being immersed for 1 to 2 minutes in methanol containing dry ice to freeze the aqueous layer. The solvent layer was then carefully tipped into 75 x 12 mm borosicilate glass extraction tubes (Appleton Woods Ltd., Birmingham, UK). The tubes were left overnight under a ventilated hood with gentle agitation to evaporate the ether. On the next morning the walls of the extraction tubes were flushed down with 0.8 ml of diethyl ether to recover any remaining oestradiol and were left to evaporate again for 2 hours. The dried steroid residue was then resuspended in 100 µl of PBSG (phosphate-buffered saline with 0.1% swine skin gelatine) by covering the tubes with parafilm and vigorous vortexing for 15 minutes on a multitube vortexer. Resuspended samples were immediately used for radioimmunoassay analysis. All glassware was twice washed and baked before use.

To estimate the efficiency of plasma oestradiol recovery, a trial experiment was conducted where 1500 cpm of $^{3}H$ was added to the plasma sample before the extraction procedure. The dried oestradiol was resuspended in 150 µl of PBSG of which 40 µl was removed to count $^{3}H$ cpm. Recovery efficiency of $^{3}H$ was at an average of 74% (± 7%) and this value was used for all further experiments to calculate final extraction efficiencies for oestradiol samples.

## 2.4.2 Radioimmunoassays

### 2.4.2.1 Luteinising hormone & Follicle stimulating hormone

The LH and FSH radioimmunoassays were performed by Caroline Decourt, Christine Briant and Daniel Guillaume at the *Unite Physiologie de la Reproduction et des Comportements*, INRA, Nouzilly, *Universite de Tours*, France.

### 2.4.2.2 Oestradiol

Oestradiol in plasma and follicular fluid was measured using a competitive Double Antibody radioimmunoassay kit (Siemens Healthcare Diagnostics Inc., Los Angeles, USA) following manufacturer's instructions, but the final reaction volume was halved. For plasma, the full 100 µl of the resuspended oestradiol was directly used in the assay. Follicular fluid samples were diluted 1:10,000 in PBSG for oestrous follicles, 1:1,000 for dioestrous and transitional follicles and 1:100 for subordinate follicles. For the assay 100 µl of sample was pipetted into a 75 x 12 mm borosilicate glass tube in duplicates for follicular fluid and in triplicates for extracted plasma samples. Fifty µl of Oestradiol Antiserum was added and incubated for 2 hours at room temperature. Afterwards, 50 µl of $^{125}$I Oestradiol was added to all tubes and vortexed before another incubation period for 1 hour at room temperature. Finally 500 µl of cold Precipitating Solution was added to all tubes, vortexed, incubated for 10 minutes and then centrifuged for 30 minutes at 1500 g before decanting of the supernatant. The tubes were left to stand inverted for 10 minutes before counting for 60 seconds on a gamma counter. Results were obtained from a logit-log representation of the calibration curve from the known kit standard values.

### 2.4.2.3 Progesterone

Progesterone levels in plasma and follicular fluid were measured with a Coat-a-Count radioimmunoassay kit (Siemens Healthcare Diagnostics Inc.) following manufacturer's instructions. The assay amount of plasma for each sample was halved to simulate a 1:2 dilution. Follicular fluid samples were diluted 1:10 in PBSG for dioestrous and subordinate follicles and 1:50 for transitional and oestrous follicles. Samples were run in singles as excellent repeatability (coefficient of variance (CV) below 3.3 for 16 trial samples) had been found in previous assays. For the procedure, 50 µl of plasma or 100 µl of diluted follicular fluid was added to the provided

antibody-coated polypropylene tubes and 1 ml of $^{125}$I Progesterone was added to every tube and vortexed. The incubation period was extended to overnight at 4 °C to improve sensitivity. The tubes were then decanted and left to drain for 3 minutes before being counted on a gamma counter for 60 seconds and results were calculated as indicated above.

#### 2.4.2.4 Androstenedione

Levels of androstenedione were measured using an in-house assay developed at Roslin (Corrie et al. 1981; Armstrong et al. 1998).

Standards were previously prepared in PBSG to contain 1250, 625, 313, 186, 78, 39, 19.5, 9.6, 4.8 and 2.4 pg/tube. Hundred µl of standards and samples were added in triplicates to 75 x 12 mm polypropylene assay tubes and 100 µl of rabbit antibody (dilution 1:30,000) was added followed by 100 µl of $^{125}$I Androstenedione label (diluted to 15,000 cpm/100 µl) prepared by Ms Anne Kelly at the Institute of Cardiovascular and Medical Sciences, Royal Infirmary, Glasgow. The tubes were then incubated over-night at 4 °C. Then, 100 µl of donkey-anti-rabbit serum (dilution 1:20; 500 ml of DARS in 1 ml 0.1M EDTA and 8.5 ml PBSG for 100 tubes) and 100 µl of normal rabbit serum (dilution 1:200; 50 µl NRS in 9.95 ml PBSG for 100 tubes) were added to each tube before repeated incubation of the tubes over-night at 4 °C. Finally 1 ml of cold PBSG was added to each tube immediately before centrifugation at 1500 g for 35 minutes at 7 °C, before decanting and counting on a gamma counter for 60 seconds. Results were calculated as above.

#### 2.4.2.5 Total Testosterone

Total testosterone was measured only in follicular fluid. A Coat-a-Count radioimmunoassay kit (Siemens Healthcare Diagnostics Inc.) was used following the manufacturer's instructions. A dilution of 1:20 in PBSG was used for all samples. Twenty µl of each sample were pipetted into antibody-coated polypropylene tubes in duplicate. One ml of $^{125}$I Total Testosterone was added and the tubes were vortexed. Tubes were then incubated over-night at 4 °C and decanted and counted for 60 seconds in a gamma counter the next morning. Results were calculated as described above.

#### 2.4.2.6 Cortisol

Cortisol levels were only measured in follicular fluid. A Coat-a-Count radioimmunoassay kit (Siemens Healthcare Diagnostics Inc.) was used with the provided antibody-coated polypropylene tubes. Twenty-five µl of each sample were added undiluted to assay tubes in duplicate, topped up with 1 ml of $^{125}$I Cortisol and vortexed. Tubes were then incubated over-night at 4 °C and decanted and counted for 60 seconds in a gamma counter the next morning. Results were calculated as described above.

### 2.4.3 ELISAs

#### 2.4.3.1 Oestradiol

Oestradiol levels in follicular fluid were measured using a competitive solid phase enzyme-linked immunosorbent assay (DRG Instruments GmbH, Marburg, Germany). ELISA was used for measuring oestradiol in follicular fluid but not in plasma, as a higher sensitivity method (RIA) was required to measure the low levels of oestradiol in plasma. Follicular fluid from oestrous follicles (including oestrous follicles collected from mares treated with LH and PGF2α, see Chapter 4) was diluted 1:10,000 in PBSG, fluid from transitional and dioestrous follicles was diluted 1:1,000 and fluid from subordinate follicles was diluted 1:500 before the assay procedure. For the assay, 25 µl of each standard and sample were pipetted in duplicates into antibody-coated wells. Two-hundred µl of enzyme conjugated were added and mixed before incubation for 120 minutes at room temperature. Three washing steps were performed. 100 µl of substrate solution was subsequently added to each well and incubated for 15 minutes at room temperature. The enzymatic reaction was stopped by adding 50 µl of stop solution to each well and the absorbance was read at 450 nm on a microtiter plate reader and converted into pg/ml by reading the values for the percentage binding off the standard curve.

#### 2.4.3.2 PGE2

Levels of prostaglandin E2 (PGE2) were measured using a competitive Biotrak™ enzyme immunoassay system (GE Healthcare, Little Chalfont, UK), which included an extraction step to dissociate PGE2 from soluble receptors and interfering binding proteins. Samples were used neat following protocol 8.5 for “measurement of PGE2

in plasma using novel lysis reagents” provided by the manufacturer. Follicular fluid samples were prepared for the assay by adding 300 µl of sample to 33.3 µl of buffer A, of which 50 µl were used for the assay procedure. Fifty µl of each of the standards were pipetted into wells precoated with sheep anti-mouse antibody. Fifty µl of mouse anti-PGE2 antibody and 50 µl of conjugate were added to each well and incubated for 1 hour at room temperature. Four washing steps were performed. After that, 150 µl of enzyme substrate were added to each well and the plate was incubated for 30 minutes at room temperature. The developing blue colour was immediately read at 630 nm and converted into pg/ml.

#### 2.4.3.3 IGF1

A two-step sandwich-type immunoassay was used to quantify free insulin-like growth factor 1 (IGF1) (Diagnostic Systems Laboratories Inc., Webster, TX, USA) in undiluted follicular fluid. Fifty µl of standards and samples were pipetted into pre-coated wells and 20 µl of the Free IGF1 Sample Buffer were added before incubating the plate on an orbital microplate shaker for 1 hour at room temperature. Five washing steps were performed. Then, 100 µl of the antibody-enzyme conjugate solution were pipetted into each well and the plate was incubated by shaking for 30 minutes at room temperature. Another five washing steps were performed. Finally, 100 µl of the TMB chromogen solution were added to each well, the plate was incubated in darkness for 10 minutes on an orbital shaker before 100 µl of stopping solution was added. The absorbance was read immediately on a microplate reader at 450 nm and absorbance values were converted into ng/ml.

## 2.5 Quantification of mRNA levels in granulosa cells

### 2.5.1 Total RNA extraction

Granulosa cell pellets stored at -80 °C in 500 µl RNABee (AMS Biotechnology, Abingdon, UK) were thawed on ice. First, 100 µl of Chloroform (Sigma-Aldrich, Gillingham, UK) were added and the mixture was shaken for 30 seconds then placed on ice for 5 minutes, followed by a centrifugation step at 12,000 g for 15 minutes on 4 °C. Next, 250 µl of the upper layer were carefully pipetted into a new eppendorf tube and 250 µl of Isopropanol (Sigma-Aldrich) were added and the mixture left at

room temperature for 5 to 10 minutes. The mixture was again centrifuged at 12,000 g for 10 minutes at room temperature and the supernatant was then discarded. The remaining pellet was resuspended in 500 µl of 75% Ethanol and spun at 7,500g for 5 minutes at room temperature. Again the supernatant was discarded, the pellet left to dry for 5 to 10 minutes and finally dissolved in 25 µl of RNAse free water and stored at -80 °C until further use. The RNA concentration was measured in 1.2 µl of the extracted sample using the ND-1000 spectrophotometer (NanoDrop Technologies, Willmington, DE) and checked for potential phenol and protein contamination using 260/280 and 260/230 ratios.

### 2.5.2 Reverse transcription (RT)

To produce cDNA a reverse transcription reaction was carried out, which included, in addition to the test samples, a no template control tube (NTC – consisting of PCR grade water) and a noRT tube, which contained all components of an RT reaction except for the RT enzyme. The noRT control was used to allow detection of potential genomic DNA contamination of samples. Mastermix A was prepared by mixing 1 µl (250 ng/µl) Random Primers (Promega, Madison, WI) and 1 µl dNTP mix (10mM, Invitrogen) for each tube. Each sample was diluted with water to a final amount of RNA of 100 ng and 11 µl of this was added to 2 µl of Mastermix A. This mixture was then heated to 65 °C for 5 minutes and cooled for 1 minute to 4 °C. Mastermix B was prepared by mixing 4 µl First strand buffer (x5), 1 µl DTT (0.1 M), 1 µl RNasIn (40 units/µl; Invitrogen) and 1 µl SuperScript III (Invitrogen, 200 units/µl), and this was then added to each sample tube and the final mixture was vortexed briefly and stored on ice. Tubes were subjected to a programme cycle in the Biometra TGradient Thermocycler of 25 °C for 5 minutes, 50 °C for 60 minutes, 70 °C for 15 minutes and a cool-down to 4 °C before storage of the cDNA at -20 °C.

### 2.5.3 Quantitative PCR (qPCR)

Expression for the genes listed in Table 2.1 was analysed using the SensiFAST™ SYBR Lo-ROX Kit (Bioline Reagents Ltd, London, UK) on the Mx3005P real-time PCR system (Stratagene, La Jolla, CA) following manufacturer instructions using 2

µl of sample to achieve a final reaction volume of 10 µl. A cDNA dilution of 1:20 was chosen for the house keeping gene 18S, and 1:4 was used for all other genes.

**Table 2.1 Primer pair sequences used for qPCR on granulosa cell cDNA**

| gene | | sequence (5'-3') | product size | |
|---|---|---|---|---|
| 18S | FW | GCTGGCACCAGACTTG | 209 | * |
| | RV | GGGGAATCAGGGTTCG | | |
| CYP19A1 | FW | CGCAAAGCCTTAGAGGATGA | 212 | # |
| | RV | ACCATGGCGATGTACTTTCC | | |
| CYP17A1 | FW | ATCCGGCCTGTGGCCCCTA | 226 | |
| | RV | CTGCTCCAAAGGGCAAGTAG | | |
| CYP11A1 | FW | ACAGGCGCATGGAGCAGCAG | 114 | |
| | RV | TCCCCTGGCGCTCCCCAAAT | | |
| FSHR | FW | TCTTTGGCATCAGCACCTAC | 399 | * |
| | RV | AGAAATCCCTGCGGAAGTTC | | |
| LHR | FW | CCCGGTTAAAATACCTAAGC | 229 | * |
| | RV | AGTGTCGTCCCGTTGAA | | |
| IGF1R | FW | AAGCCGAGAAGCAGGCCGAG | 265 | |
| | RV | CGGAGGTTAGAAATGACAGT | | |

* Doyle et al. 2008
# Donadeu et al. 2011

Oligonucleotide primers for CYP17A1 (NM_001082523.1), CYP11A1 (NM_001082521.1) and IGF1R (XM_001489765.2) sequence specific to equus caballus obtained from e!Ensembl (http://www.ensembl.org) were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast), which is based on the Primer3 algorithm. Primers were designed to span two exons to minimise amplification of genomic DNA in the samples. Primers were ordered from Invitrogen.

The following cycling conditions were used on a Mx3005P real-time PCR system (Stratagene): Activation for 2 minutes at 95 °C, followed by 40 cycles of denaturation for 5 seconds at 95 °C, annealing for 11 seconds at 60 °C and extension for 5 seconds at 72 °C, with fluorescence data collection. After the last cycle a

melting curve analysis was performed, which served to confirm the amplification of a single product of the expected size. All mRNA expression levels are shown normalised to 18S expression levels.

## 2.6 Quantification of miRNA levels in follicular fluid

Follicular fluid was aspirated and prepared as described above (2.3.2) and stored at -20 °C until extraction.

### 2.6.1 miRNA extraction

The extraction of miRNA from follicular fluid was performed following the Exiqon protocol for RNA purification from blood serum and plasma using a miRNeasy mini kit (Qiagen, Crawley, UK). Follicular fluid was spun at 10,000 g for 10 minutes at 4 °C to remove cell debris before 200 µl of each sample were mixed with 750 µl of QIAzol master mix, containing 800 µl QIAzol (Qiagen) and 1.25 µl 0.8 µg/µl MS2 RNA (Roche Diagnostics Ltd., Burgess Hill, UK) as carrier RNA, and vortexed for 30 seconds. A carrier RNA-only sample was prepared as control. Each sample was spiked-in with 1 µl of a 5 fmol solution of Caenorhabditis elegans (cel)-mir-39 (Qiagen), which was used to control for RNA extraction and RT efficiencies. The samples were incubated for 5 minutes at room temperature before 200 µl of chloroform were added, vortexed and incubated for 2 minutes at room temperature. After the centrifugation at 12,000 g for 15 minutes at 4 °C, the upper aqueous phase (500 µl) was transferred to a 2 ml tube and mixed with 750 µl of pure ethanol. This was transferred to an RNeasy Mini Spin Column and washed following manufacturer's instructions. Finally miRNAs were eluted with 30 µl of PCR grade water and stored at -80 °C until further use.

### 2.6.2 RT

For the reverse transcription, the miScript II RT Kit (Qiagen) was used following the manufacturer's instructions with the following modifications. To prepare the mastermix, 4 µl HiSpec buffer were combined with 2 µl of Nucleic Mix and 2 µl of Reverse Transcriptase Mix. This was then added to 12 µl of template and incubated for 60 minutes at 37 °C followed by an RT inactivation for 5 minutes at 95 °C before storage at -20 °C.

### 2.6.3 QPCR

All samples were diluted after the reverse-transcription reaction 1:20 before being used in the qPCR. A miScript SYBR Green PCR Kit (Qiagen) was used to analyse miRNA expression following the manufacturer's instructions with a total reaction volume of 10 µl. For each sample, 5 µl of SYBR Green Master Mix were combined with 1 µl Universal Primer (x10), 1 µl miRNA specific Primer Assay, 1.5 µl PCR-grade water and 1.5 µl of sample template (See Table 2.2 for list of primers used).

**Table 2.2 Qiagen Primer Assays used for miRNA qPCR (a universal reverse primer was used)**

| miRNA | sequence (5'-3') |
|---|---|
| miR-378a-3p | ACUGGACUUGGAGUCAGAAGG |
| miR-202-5p | UUCCUAUGCAUAUACUUCUUUG |
| miR-21-5p | UAGCUUAUCAGACUGAUGUUGA |
| miR-542-5p | UCGGGGAUCAUCAUGUCACGAGA |

The following conditions were used on a Mx3005P real-time PCR system (Stratagene): Activation for 15 minutes at 95 °C, followed by 40 cycles of denaturation for 15 seconds at 94 °C followed by annealing for 30 seconds at 55 °C and extension for 30 seconds at 70 °C, with fluorescence data collection. After the last cycle a melting curve analysis was performed.

All miRNA and mRNA expression was quantified using the ΔΔCt method as performed by the MXPro software by aligning sample Ct's with a double diluted standard curve of a sample pool. All follicular fluid miRNA expression data was normalised against cel-mir-39, which was used as a spike-in control prior to the RNA extraction process and rtPCR.

## 2.7 Sequencing for miR-202

First, the qPCR product for miR-202 from granulosa cell samples was run on an agarose gel to confirm the amplification of a single product. Then, the product from duplicate wells was used for sequencing.

### 2.7.1 Gel electrophoresis

A 2% agarose gel (UltraPure Agarose, Invitrogen) was prepared and the wells were flushed with 1x TAE running buffer (40 mM Tris base, 20 mM acetic acid, 1mM EDTA) once the combs were removed.

The samples taken directly from the qPCR plate were prepared by mixing 6 µl of the sample with 1 µl of 6x Blue Orange loading dye (Promega).

The gel was run on 90 V for about 60 minutes, and the bands were then made visible under ultra-violet light with a Gel LOGIC 200 Imaging system.

### 2.7.2 Cloning

To clone the qPCR product obtained using the human miR-202* primer into the sequencing plasmid, the TOPO TA Cloning Kit (Invitrogen) was used. First, two agar plates containing 50 µg/ml ampicillin per sample were prepared and left to cool down. Then cloning was performed following manufacturer's instructions. The TOPO cloning reaction was set up as follows. Two µl of fresh PCR product of each sequence of interest were mixed with 1 µl of Salt Solution, 2 µl of water and 1 µl of TOPO vector for a final volume of 6 µl. This mixture was then incubated for 15 minutes at room temperature. During this time One Shot Mach1-T1$^R$ Competent Cells were thawed on ice. Two µl of the TOPO cloning reaction were pipetted into a vial of One Shot Chemically Competent *E. coli,* gently mixed and incubated on ice for 15 minutes. Then the cells were heat-shocked for 30 seconds at 42 °C without shaking and transferred back to ice. Two hundred and fifty µl of S.O.C medium at room temperature was added and the tube incubated on a 200 rpm shaker for 1 hour at 37 °C. For each sample two different volumes of the transformed bacteria (10 and 50 µl) were spread on a pre-warmed selective agar plate and incubated over-night at 37 °C. The next morning the plates were checked for colonies and 4 colonies were picked and each added into 5 ml LB (Luria Broth) mixed with 1 µl/ml carbenicillin at room temperature. All tubes (universals) were then kept overnight in a shaking incubator at 37 °C. The next morning, the contents of all 4 universals for each miRNA were transferred to a 1.5 ml eppendorf tube and centrifuged at 1000 g for 5

minutes, the supernatant was discarded and the process repeated until all the broth was transferred and centrifuged. The final pellet was frozen at -20 °C until use.

To recover the recombinant DNA from transformed bacteria, a PureLink® Quick Plasmid DNA Miniprep Kit (Invitrogen) was used. The thawed pellet was resuspended using 250 µl of Resuspension Buffer with RNase A and homogenised, and 250 µl of Lysis Buffer was added and the mixture incubated for 5 minutes at room temperature. Next, 350 µl of Precipitation Buffer was added and the tube was vigorously shaken before centrifugation at 12,000 g for 10 minutes. The supernatant was then loaded onto a Spin Column in a 2-ml Wash Tube and centrifuged at 12,000 g for 1 minute. Flowthrough was discarded and 500 µl Wash Buffer was added to the column, incubated for 1 minute at room temperature and the tube was centrifuged at 12,000 g for 1 minute. Flowthrough was discarded and the tube was spun again at 12,000 g for 1 minute. The Spin Column was placed in a 1.5 ml eppendorf tube, 75 µl of preheated TE Buffer were added, then incubated for 1 minute at room temperature and spun at 12,000 g for 2 minutes. Finally, 1.2 µl of the eluted DNA was quantified using the ND-1000 spectrophotometer. The recovered plasmid DNA was stored at 4 °C until further use.

Since the plasmid contained an EcoR1 restriction site at each side of the insert, to confirm integration of the PCR product, 8 µl of the 600 ng/µl DNA per sample was added to a tube and mixed with 1 µl EcoR1 (Biolabs) and 1 µl NEBuffer1 (Biolabs). This mixture was incubated for one hour at 37 °C, then stored at -20 °C until run on a gel.

Samples positive for an insert were then prepared to be submitted for sequencing. In a 0.2 ml eppendorf tube, 200 ng DNA and 3.2pMol Topo kit M13 primer were added to give a total volume of 6 µl. The sequencing was performed by Genepool (University of Edinburgh).

# Chapter 3

# Effects of eLH supplementation during early spring transition on follicular development and ovulatory competence

# Chapter 3: Effects of eLH supplementation during early spring transition on follicular development and ovulatory competence

## 3.1 Introduction

The seasonal pattern of reproductive activity in the mare involves a reduction in ovarian activity during the winter months followed by a period of transition during spring when follicular growth re-initiates leading to the successive development of up to three or more waves with large dominant follicles which do not ovulate (Ginther 1990). This leads to a remarkable waste of time and resources by veterinarians and breeders who unsuccessfully try to get mares in foal early in the year. Although exposure to artificial lights is a proven method to effectively hasten the onset of the ovulatory season in mares, there has long been a need in the horse industry for reliable, shorter-term interventions that reduce the length of the spring transitional period resulting in early ovulations. Various hormonal treatments have been used to this end (reviewed in Nagy et al. 2000), including daily injection of low doses of hCG (Bour and Palmer 1984), however, these treatments have often proven to be efficient only during the latter stages of the transitional period, when follicle activity is already significant. Some success has been recently achieved by using FSH in spring transitional mares. Specifically, FSH injected twice daily early during the transitional period promoted the development of ovulation-competent follicles in many treated mares (Niswender et al. 2004; Raz et al. 2009), however, not surprisingly, many of these mares double-ovulated thus increasing the risk of multiple pregnancy.

Based on earlier reports (reviewed in Donadeu and Watson 2007) a deficiency of LH, rather than FSH, may physiologically underlie deficient follicle development during the transitional period and in that regard LH supplementation may provide a more rational approach to induce early ovulations. Scientific evidence clearly indicates that available circulating FSH is not a limiting factor for follicle development during the transitional period (Donadeu and Pedersen 2008). In contrast, the renewed growth of dominant follicles at the onset of the spring

transitional period is associated temporally with an increase in LH pulsatility (Fitzgerald et al. 1987; Donadeu and Pedersen 2008). In addition, both during the fall and spring transitional periods significantly higher LH concentrations were associated with follicular waves involving a dominant follicle than with waves producing only smaller follicles (Donadeu and Ginther 2003a; Ginther et al. 2003b). Another report showed higher circulating LH concentrations early during development of the first ovulatory follicle of the season than during the development of large anovulatory follicles during the spring transition (Acosta et al. 2004a). Developmental deficiencies of transitional follicles include an underdeveloped and poorly vascularised theca layer together with reduced follicular cell expression of steroidogenic enzymes and LH receptors as well as low IGF1 bioactivity, all of which are associated with severely reduced steroid production by these follicles and are attributable, at least partially, to low circulating LH levels (reviewed in Donadeu and Watson 2007). Taken together, these observations indicate that adequate LH levels are required during the anovulatory season in mares both for the growth of dominant follicles and for the acquisition of ovulatory competence by these follicles, a conclusion which is consistent with experimental evidence that LH is required for the growth of dominant follicles during the ovulatory season in mares (Gastal et al. 1999a; Guillaume et al. 2002; Briant et al. 2004a) and in cycling cows (Gong et al. 1996).

Based on the above evidence, the aim of this study was to directly test the hypothesis that supplementation of growing follicles with LH during early transition would stimulate development of steroidogenically active dominant follicles with the ability to respond to an ovulatory stimulus and that this approach could therefore be potentially used to efficiently hasten the onset of the ovulatory season.

## 3.2 Materials and Methods

### 3.2.1 Experimental treatments and data collection

Seven seasonally anovulatory mares of mixed breeding, mainly welsh pony cross, three to 14 years of age, with weights of 250 – 500 kg and a history of reproductive health were used as described in 2.1.

Ovarian ultrasonography (as described in 2.1) was performed twice weekly in all mares starting during mid-December to ensure that mares were in deep anoestrus before the start of the experiment, i.e., that no follicles ≥25 mm or a CL had been detected by ultrasound for at least the previous month.

Once the first follicle 25 mm in diameter was detected after deep anoestrus (starting early February) all follicles larger than 15 mm were ablated using an ultrasound-guided transvaginal aspiration system to allow a new follicular wave to grow as described in 2.3.2. Once the largest follicle of the ablation-induced wave reached 20 mm, corresponding to just before the emergence of the dominant follicle (Ginther et al. 2001), mares were randomly assigned to receive eLH or an equivalent volume of physiological saline vehicle (n = 7 mares per group). In mares assigned to receive eLH, this was injected at a concentration of either 0.4 µg/kg bw (n = 4 mares) or 0.1 µg/kg bw (n = 3 mares). Because there was no significant main effect of Dose or an interaction between Dose and Day for any follicular growth or hormone level endpoints considered ($P > 0.4$), data from the two dose groups were combined for comparative analyses with controls and are shown as such throughout the manuscript. Treatments were administered every 12 hours continuing until a follicle reached >32 mm at which time 3000 IU of hCG (Chorulon®) were injected i.v. to induce ovulation. If no follicle reached 32 mm, treatments were stopped once the largest follicle of the wave had ceased growing for at least three days. Twice daily eLH injections were performed in an attempt to simulate the natural pattern of LH secretion during late dioestrus, when the ovulatory wave begins to develop, and which involves a mean of one pulse of circulating LH every 12 hours (Fitzgerald et al. 1987; Watson et al. 1995). Daily ultrasound monitoring of animals continued until the dominant follicle reached >32 mm followed by twice daily monitoring between

the time of hCG injection and ovulation; this increased ultrasound frequency was used to precisely determine the time of ovulation after hCG and to distinguish true corpora lutea from potential haemorrhagic anovulatory follicles (Ginther et al. 2007a; Ginther et al. 2008a). The day of ovulation was assigned to the ultrasound scanning session immediately before a CL was first detected. The same mares that were used during transition were also used during the ovulatory season (June and July, n = 7). In this ovulatory control group, all follicles >15 mm were ablated 10 days after ovulation and mares were given saline injections once a follicle reached 20 mm and every 12 hours until a follicle reached a size >32 mm, when hCG was administered to induce ovulation. Ovaries were monitored and plasma samples were taken as described for experiments during early transition (see Figure 3.1).

Throughout the experimental periods during both, early transition and ovulatory season, blood samples were collected daily starting before the first eLH injection and continuing up to 13 days after ovulation or, if the dominant follicle did not ovulate, until the follicle stopped growing for at least 3 consecutive days.

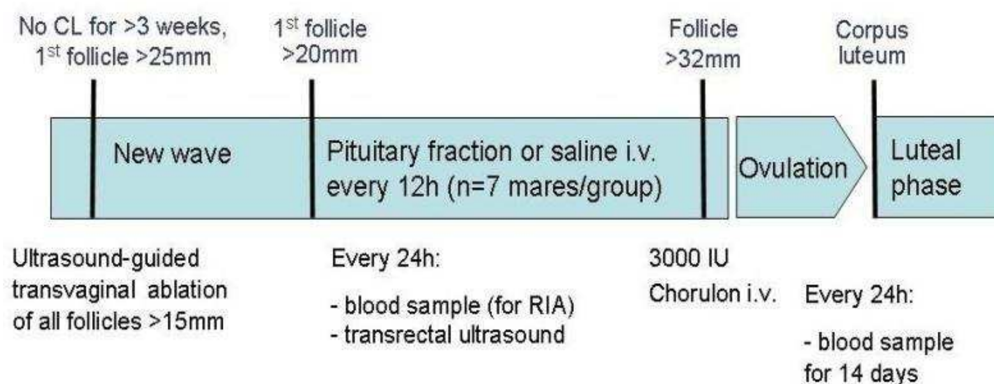


**Figure 3.1 Schematic of experimental protocol used during transition. During the ovulatory season a new wave was induced by ablation of all follicles >15 mm on day 10 after a natural ovulation.**

## 3.2.2 Hormone analyses

### 3.2.2.1 LH and FSH

Plasma concentrations of LH and FSH were measured by radioimmunoassay as described in 2.4.2.1. Intra-assay and interassay CVs and sensitivity were 14.8%, 17.5% and 0.4 ng/ml, respectively, for LH and 14.0%, 20.0% and 1.56 ng/ml, respectively, for FSH.

### 3.2.2.2 Oestradiol and progesterone

Plasma levels of oestradiol and progesterone were measured as described in 2.4.2. Intra-assay and interassay CVs and sensitivity were 17.9%, 19% and 0.1 pg/ml, respectively, for oestradiol, and 9.5%, 13.7% and 0.015 ng/ml, respectively, for progesterone.

### 3.2.2.3 Statistical analyses

Before statistical analyses, follicular and hormone data were normalised to the day the future dominant follicle reached 20 mm (Day 0; determined retrospectively). All data were tested for normality using a Kolmogorov-Smirnoff test ($P < 0.05$) and log-transformed if necessary. Data were then subjected to analysis of variance using a GLS procedure (R version 1.10.1) which accounted for the autocorrelation between samples taken over time, and the effects of Group, Day and the interaction were determined. Whenever there was a significant main effect or interaction, individual means were compared using Tukey’s test. Group means involving percentages were compared using Chi-square analyses. Statistical significance was considered at $P < 0.05$.

# 3.3 Results

## 3.3.1 Short-term circulating gonadotropin responses

Changes in circulating gonadotropin levels following injection of the two different doses of eLH are shown in Figure 3.2. Circulating LH levels increased after injection (Time, $P < 0.01$) and this increase was more pronounced in response to the 0.4 μg/kg than the 0.1 μg/kg dose (Group, $P=0.03$), so that pre-treatment circulating LH levels were restored ($P > 0.05$) by 30 min after injection of 0.1 μg LH/Kg but only by 150

min after injecting the larger dose. In contrast, FSH levels did not change after injection of any of the two eLH doses (Day, P > 0.6).

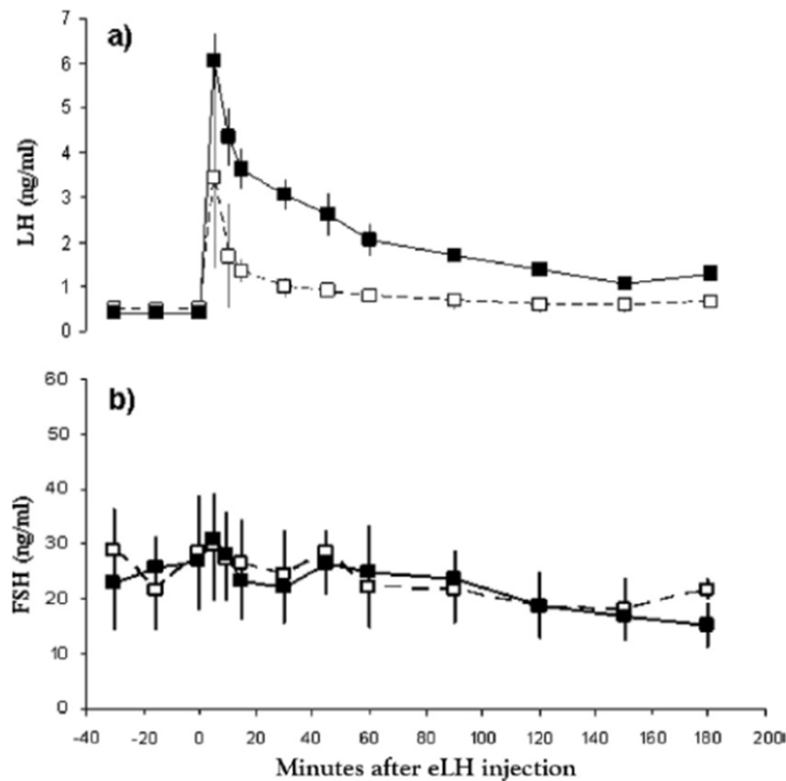


**Figure 3.2 Mean (± SEM) values for plasma concentrations of a) LH and b) FSH obtained from frequent jugular sampling after injection of eLH to deep anoestrous mares at doses of 0.4 µg/kg (solid line, closed square) or 0.1 µg/kg (dashed line, open square; n = 5 mares/group). Whereas there were no main effects of Time and Dose or an interaction for FSH (P > 0.4), there were significant effects of both Time (P < 0.01) and Dose (P = 0.03) for LH concentrations. For LH, Dose means were different within each time point between 10 and 90 min (P < 0.05). Within the 0.1 µg/kg dose, each of the means at 5, 10 and 30 min were different from the mean at 0 min (P < 0.05) and within the 0.4 µg/kg dose, each mean from 5 to 120 min was different from the mean at 0 min (P < 0.05).**

### 3.3.2 Follicular growth and ovulation

Mean follicular responses to treatments are presented in Figure 3.3. Administration of eLH to early transitional mares stimulated growth of the dominant follicle (Group x Day, $P < 0.0001$; Figure 3.3a). As a result, on Days 4 and 5 follicle diameters in that group were higher ($P < 0.05$) than in transitional controls and similar ($P > 0.1$) to ovulatory controls. However, there were no differences in diameter of the largest subordinate follicle (Figure 3.3b) or number of follicles > 10 mm (Figure 3.3c) during treatment, as indicated by the absence of main effect of Group or an interaction for each of the two endpoints ($P > 0.3$).

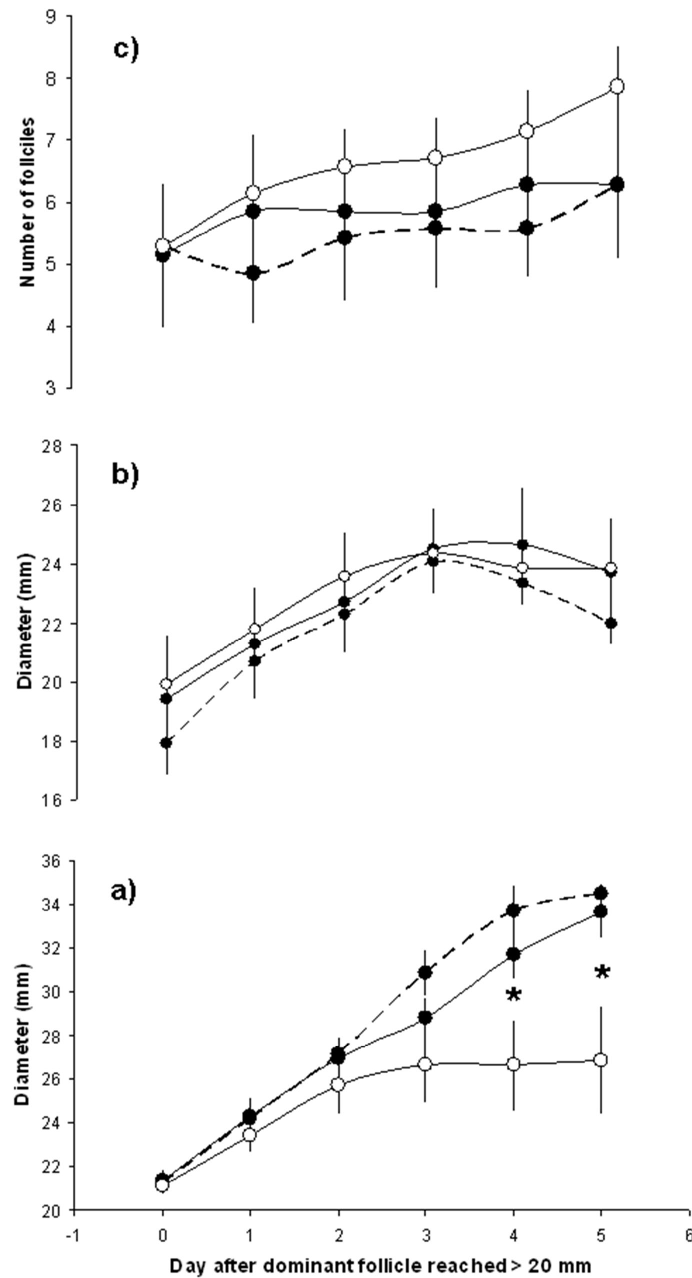


**Figure 3.3 Mean (± SEM) values for a) diameter of the dominant follicle, b) diameter of the largest subordinate follicle and c) total number of follicles >10 mm, normalised to the day the dominant follicle of the post-ablation wave reached >20 mm (Day 0; determined retrospectively) in transitional mares treated with saline (solid line, open circle) or eLH (solid line, closed circle) and in saline-treated seasonally ovulatory mares (dashed line, closed circle; n = 7 mares/group). There was an interaction between Group and Day for Diameter of the dominant follicle (P < 0.0001). An asterisk indicates a significant difference between the Transitional control group and each of Transitional LH and Ovulatory control groups within a day (P < 0.05).**

The stimulatory effect of LH on follicle growth resulted in a higher proportion of transitional mares (P < 0.05) developing a follicle >32 mm during LH treatment (6 of 7) than during treatment with saline (2 of 7; Figure 3.4). For reasons unrelated to the experiment, injection of one of the eLH-treated mares with hCG once the dominant follicle reached >32 mm was not possible. Of the remaining 5 mares, 3 ovulated in response to hCG, whereas the two transitional control mares that developed a >32 mm follicle also ovulated after hCG injection (Figure 3.4). For mares injected with hCG, there were no differences among the 3 groups in the diameter of the dominant follicle at hCG injection (P = 0.4) or the diameter at hCG-induced ovulation (P = 0.6), with overall means of 33.9 ± 0.3 mm and 37.7 ± 1.9 mm, respectively. However, as shown in Figure 3.5, the interval from hCG-induced ovulation to the next (spontaneous) ovulation was significantly longer (P = 0.02) in the two transitional groups than in ovulatory controls. In addition, the interval from beginning of treatment to onset of cyclic ovulatory activity was not different between LH- and saline-treated transitional mares (P = 0.3).

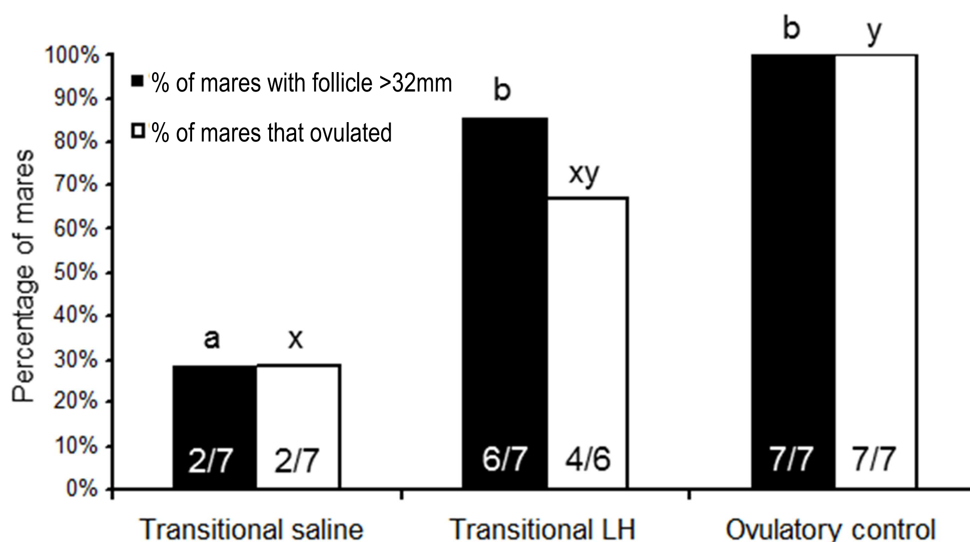


**Figure 3.4 Percentage of mares in Transitional saline, Transitional LH and Ovulatory control groups that grew a follicle larger than 32 mm (black bars) and that ovulated in response to hCG injection (white bars). a,b and x,y denote P < 0.05**

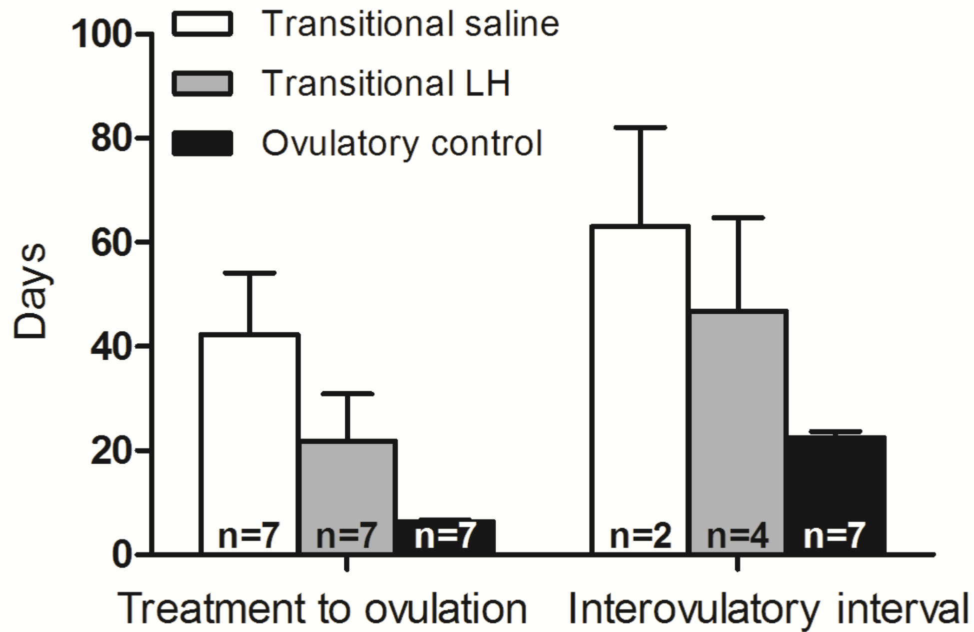


**Figure 3.5 Number of days from beginning of treatment to ovulation for Transitional saline, Transitional LH and Ovulatory control groups (left graph). Interovulatory interval from first (spontaneous or induced) ovulation to second ovulation (right graph). a,b denotes P < 0.05**

## 3.3.3 Circulating steroid levels

There was a Group by Day interaction for plasma oestradiol levels (P < 0.013; Figure 3.6). Oestradiol levels during transition did not increase in eLH- or saline-treated mares but did increase in ovulatory controls between Days 1 and 4 (P < 0.05) so that levels were higher in the ovulatory group than in each of the transitional groups between Days 3 and 5 (P < 0.05)

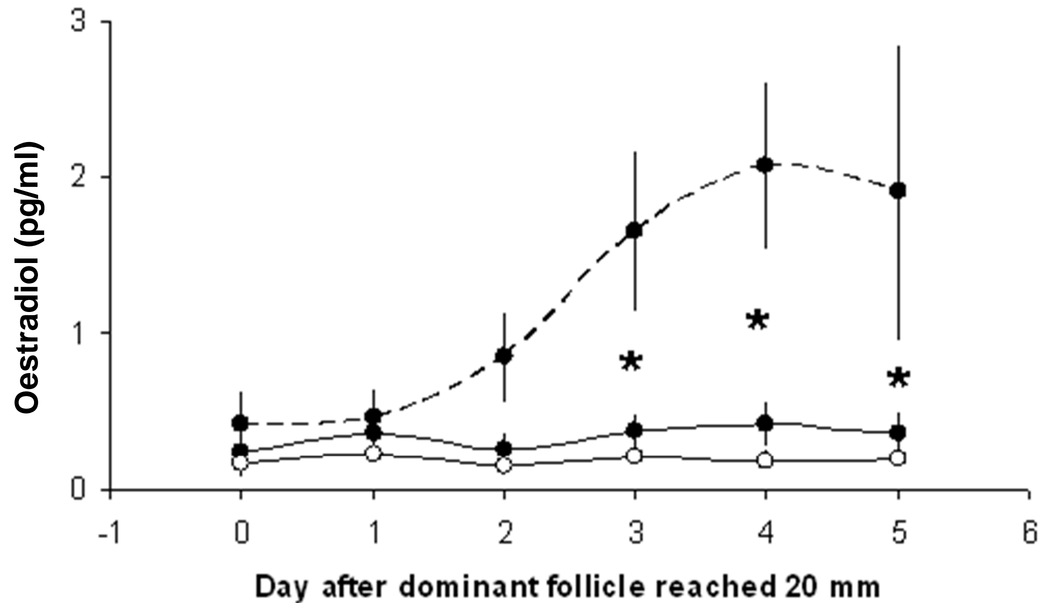


**Figure 3.6 Mean (± SEM) values for plasma oestradiol concentrations normalised to the day the dominant follicle of the post-ablation wave reached >20 mm (Day 0) in transitional mares treated with saline (solid line, open circle) or eLH (solid line, closed circle) and in saline-treated seasonally ovulatory mares (dashed line, closed circle; n = 7 mares/group). There was an interaction between Group and Day (P = 0.013). Within the ovulatory control group, there was a mean difference between Days 1 and 4 (P < 0.05). An asterisk indicates a significant difference between the Ovulatory control group and each of Transitional LH and Transitional control groups within a day (P < 0.05).**

Circulating levels of progesterone after ovulation in transitional mares that responded to hCG and in ovulatory controls are shown in Figure 3.7. Mean progesterone levels in the two transitional control mares that ovulated were similar to levels in eLH-treated mares and the two groups were therefore combined for comparative analyses with the ovulatory control group. Although overall progesterone levels increased after ovulation (Day, P < 0.00001), there was a significant effect of Group (P = 0.045) due to higher levels in ovulatory controls than in transitional mares.

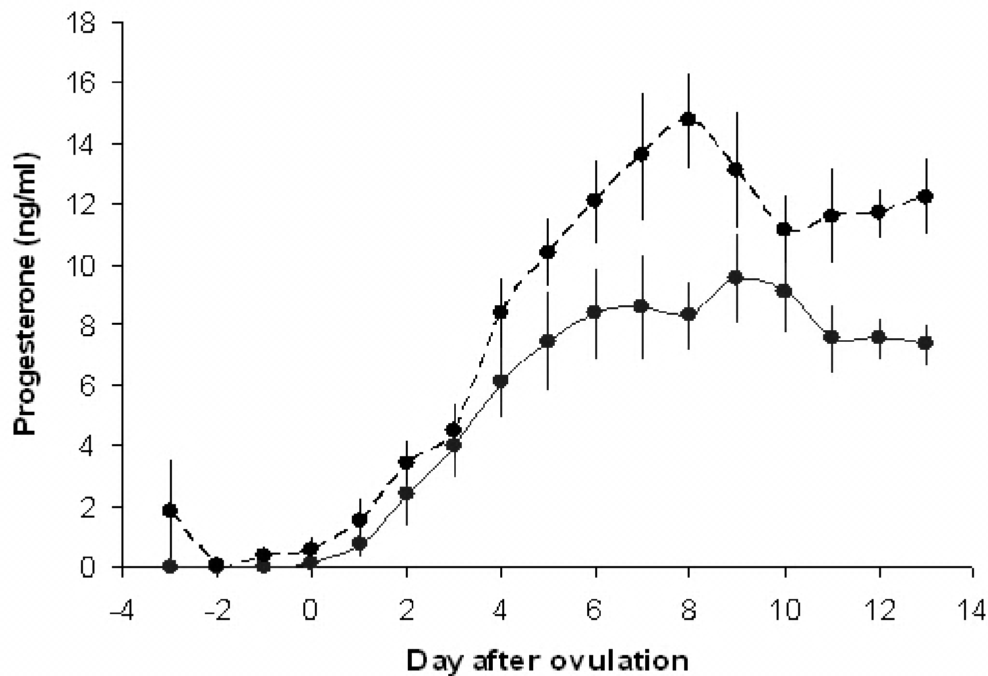


**Figure 3.7 Mean (± SEM) values for plasma progesterone concentrations normalised to the day of ovulation in transitional mares that responded to an ovulatory dose of hCG (solid line, closed circle; data from eLH- and saline treated mares combined; n = 5 mares) and in ovulatory controls (dashed line, closed circle; n = 7 mares). There were main effects of Group (P = 0.045) and Day (P < 0.00001).**

## 3.4 Discussion

While it is known that follicular responses to gonadotropins naturally change throughout follicle development and across seasons (Donadeu and Pedersen 2008), previous attempts to stimulate follicular activity and ovulation in seasonally anovulatory mares have not considered follicular wave status at the beginning of treatments. In this regard, this study used a novel approach by administering follicle-stimulating treatments to specifically target the growing dominant follicle of early transitional waves, an approach that led to 6 of 7 mares developing an ovulatory-size follicle at a mean of 5 ± 0.5 days after the start of eLH-treatments. This result demonstrated that eLH supplementation can effectively stimulate growth of the

dominant follicle of early transitional waves thus confirming previous evidence of an essential role of LH in promoting growth of dominant follicles during both the anovulatory season (Bour and Palmer 1984; Donadeu and Ginther 2003b) and the ovulatory season (Gastal et al. 1999a) in mares as well as in other species (Gong et al. 1996). The observed effects of treatment on follicle development could be attributed primarily to LH rather than to residual FSH levels in the pituitary fraction, a conclusion that is based on 1) injection of the pituitary fraction to deep anoestrous mares, in which levels of both LH and FSH in circulation are expected to be minimal (Silvia et al. 1987; Watson et al. 2002a), resulted in a clear increase in LH but no detectable changes in FSH for up to 180 min after injection and 2) an increased incidence of co-dominant follicles and double ovulation, which is a common observation during administration of recombinant eFSH to stimulate early ovulation in transitional mares (Niswender et al. 2004; Raz et al. 2010), was not observed in mares injected with eLH in this study; specifically, a co-dominant follicle (>28 mm) was detected in the experimental wave in one mare from each of the transitional control and transitional eLH groups and no mare double-ovulated in any group during the study. In this regard, as physiological upregulation of LH receptors is presumed to occur exclusively in the dominant follicle of a wave during selection (Ginther et al. 2001), it is expected that supplementation with eLH, unlike treatment with eFSH, will selectively stimulate growth of the dominant follicle.

Although eLH robustly stimulated follicle growth, the experimental hypothesis that supplementation with eLH would promote the development of steroidogenically active, hCG-responsive follicles was not supported. An effect (positive or negative) on ovulation could not be demonstrated as, although only 3 of 5 mares in the LH group ovulated in response to hCG (compared to 7 of 7 mares in the ovulatory control group), the proportion of mares that ovulated in response to hCG was not significantly different among the 3 experimental groups. This result needs to be confirmed in future studies involving a larger number of mares. In a previous report (Bour and Palmer 1984), daily low-dose hCG treatment of seasonally anovulatory mares successfully resulted in ovulation of most mares when administered in March (7 of 8) but not in February (1 of 7), although responses to hCG may have been

compounded by the formation of hCG antibodies. It should be noted that in the present study the two transitional control mares treated with hCG ovulated. Although reports have shown that most late transitional mares with an ovulatory-size follicle will ovulate in response to hCG (Bour and Palmer 1984), no other critical studies have been undertaken to determine the response of early transitional follicles to an ovulatory dose of hCG. In the present study, transitional mares took 58 ± 14 days to ovulate again after hCG-induced ovulation, averaged over eLH and saline groups, compared with a mean interovulatory interval of 22.5 ± 1.2 days in ovulatory season controls. This indicates that, consistent with previous attempts to hormonally stimulate ovulatory activity during deep anoestrus or early transition (Nagy et al. 2000), induced ovulations were followed by a return to the anovulatory state. As a result of this, mean intervals between beginning of treatment and onset of the natural ovulatory season were similar in eLH- and saline-treated transitional mares (53 ± 11.7 and 60 ± 9.5 days, respectively) and they were similar to the mean duration of the spring transitional period (54 days) reported for pony mares (Donadeu and Pedersen 2008), thus confirming that mares in this study were indeed in early transition at the time of eLH injections.

One of the most interesting observations in this study was that although LH administration robustly promoted the growth of dominant follicles during early transition, it did not restore the steroidogenic capacity of these follicles, as demonstrated by the absence of an increase in circulating oestradiol during eLH treatments. Examination of oestradiol profiles of individual mares from the two transitional groups revealed no apparent increase in oestradiol during the treatment period regardless of diameter attained by the dominant follicle or ovulation outcome. This was in contrast with a clear increase in oestradiol during growth of the dominant follicle in ovulatory controls, which was expected (Donadeu and Ginther 2004). In agreement with the present results, ovulatory follicles induced by daily low-dose hCG injection in transitional mares produced lower levels of oestradiol than spontaneously growing ovulatory follicles (Bour and Palmer 1984). Moreover, in the present study, corpora lutea derived from transitional follicles that ovulated tended to produce lower progesterone than corpora lutea from ovulatory season controls, as

indicated by differences in circulating progesterone levels between the two groups of mares. This latter result is consistent with an earlier study showing reduced circulating progesterone after ovulation in mares stimulated with GnRH during deep anoestrus relative to seasonally ovulating mares (Bergfelt and Ginther 1992).

Transitional dominant follicles are naturally steroidogenically deficient, owing to a large extent to an underdeveloped theca layer and low expression of steroidogenic enzymes in theca and granulosa, as well as low expression of LH receptors (Watson and Al-Zi'abi 2002; Doyle et al. 2008). Steroid production by such follicles gradually increases during the late transition and this seems to be attributable to an increase in circulating LH (Sharp et al. 1991). Indeed, the first ovulatory follicle of the season has been shown to have restored oestrogen-producing capacity (Donadeu and Ginther 2004). It seemed therefore plausible that LH supplementation of early transitional waves beginning just before emergence of a dominant follicle (i.e., when the largest follicle reached >20 mm) in the present study would promote follicle maturation including increased steroidogenesis. In this regard, the failure of LH to stimulate steroid synthesis despite its clear positive effects on follicle growth could be linked to the observation that restoration of full steroidogenic capacity during the natural period of spring transition occurs progressively over several follicular waves (Watson et al. 2002b), presumably in response to increasing circulating LH levels (Fitzgerald et al. 1987). Conceivably, an important effect of the increasing LH during this period would be to stimulate development of follicular theca (Watson and Al-Zi'abi 2002) so that 1) androgen production can be restored to provide adequate substrate for the synthesis of oestradiol by late transitional dominant follicles (Davis and Sharp 1991) and 2) theca-derived luteal cells can later on contribute to adequate levels of progesterone secretion by the CL. Based on this, it can be hypothesised that stimulation of steroid synthesis by dominant follicles during early transition may be more effectively achieved by LH supplementation beginning at earlier stages of development of the future dominant follicle, for example once it reaches 10 mm or earlier. This would stimulate theca development and steroidogenic activity early on so that adequate levels of androgen could be made available later on to meet the high demands of the dominant follicle for oestrogen precursor, a hypothesis that should be

tested in future studies. Additionally, other factors, such as prolactin, may be required in addition to LH for ovulation-competent follicles to fully develop in transitional mares. In that regard, prolactin secretion naturally increases in the mare in temporal association with seasonal reproductive recrudescence (Johnson and Becker 1987) and it has been shown that experimentally increasing circulating prolactin levels can hasten ovulatory activity during spring (Besognet et al. 1997; Donadeu and Thompson 2002; Mitcham et al. 2010).

## 3.5 Conclusion

In summary, supplementation of a follicular wave with exogenous eLH during the early spring transition in mares stimulated the growth of ovulatory-size follicles indicating that deficient LH levels are naturally implicated in the reduced growth of follicles during the equine anovulatory season. Supplementation with eLH, however, did not hasten the onset of the ovulatory season. In addition, LH failed to stimulate steroid production by the early transitional follicles and this was associated with reduced levels of progesterone produced by corpora lutea derived from hCG-induced ovulation of these follicles. In conclusion, although LH supplementation of early transitional waves beginning after the largest follicle reaches 20 mm promotes growth of ovulatory-size follicles, additional supplementation with LH or exposure to other trophic factors may be necessary to induce full maturation of these follicles.

# Chapter 4

# Effects of LH overstimulation on follicle development and maturation

# Chapter 4: Effects of LH overstimulation on follicle development and maturation

## 4.1 Introduction

In monovular species such as humans and horses, fluctuations in concentrations of circulating gonadotropins stimulate the development of ovarian follicular waves and ovulation of a dominant follicle at oestrus (Donadeu and Pedersen 2008). Follicular wave emergence is induced by a surge in circulating FSH levels followed by a progressive decrease in FSH as the wave continues to develop (Donadeu and Ginther 2001). In contrast, mean circulating LH levels remain relatively low throughout the development of a wave and only distinctly increase before ovulation of a mature dominant follicle (Irvine and Alexander 1994).

There are several lines of evidence indicating that a premature increase in circulating LH levels during follicular wave development may disrupt follicle maturation and potentially lead to ovulation failure. Transgenic mice expressing a chimeric LH beta subunit displayed multiple endocrine and ovarian dysfunctions including anovulation and large haemorrhagic follicular cysts (Risma et al. 1997). There is also a clear association between high LH, hyperandrogenism and the formation of anovulatory cystic follicles in women with polycystic ovarian syndrome (Ehrmann 2005). In horses, an increased incidence of ovulatory dysfunction, including premature ovulation or anovulation, and reduced fertility (Squires and McCue 2007) were attributed to the relatively high LH content of pituitary preparations used to stimulate multiple ovulations (Hofferer et al. 1993; Briant et al. 2004b). More importantly, separate studies by Ginther and co-workers (2008b; 2009) and Cuervo-Arango and Newcombe (2009) reported an association between the use of prostaglandin F2α (PGF2α) to artificially shorten the oestrous cycle in mares and a 10-fold increase in the incidence of haemorrhagic anovulatory follicles (HAFs). The authors suggested that a premature elevation of endogenous LH levels resulting from induced luteolysis may act to disrupt or hasten follicle maturation leading to anovulation (Ginther et al. 2008b). Despite the widespread use of PGF2α as a luteolytic agent for the control of oestrous cycles and the potential implications this could have in terms of overall

reproductive efficiency, the hypothesis that an increase in endogenous LH levels after administration of PGF2α may be deleterious to follicle development has not been critically assessed in farmed species.

The present study tested the hypothesis that a premature elevation in LH levels during a follicular wave induced by administration of exogenous LH or administration of a luteolytic dose of PGF2α would disrupt normal follicle maturation and that this would account, at least partially, for the recently reported effects of PGF2α-induced luteolysis on ovulation in mares (Ginther et al. 2008b; Cuervo-Arango and Newcombe 2009; Ginther and Al-Mamun 2009).

Specific follicular fluid factor levels were measured, which were known to naturally respond to the ovulatory LH surge and which are involved in oocyte maturation (IGF1), the inflammatory response associated with follicle rupture (PGE2, cortisol), oocyte maturity (cortisol) and luteinisation (progesterone, androstenedione and oestradiol) (Murphy 2000; Espey and Richards 2002; Sirois et al. 2004; Velazquez et al. 2009).

## 4.2 Materials and Methods

### 4.2.1 Experimental animals and equine LH preparation

Eight of the pony mares were kept as described in 2.1. The equine LH preparation is described in 2.2.

### 4.2.2 Experimental procedure and data collection

In a preliminary experiment the short-term circulating gonadotropin responses to a single eLH injection were analysed.

Subsequently, two different experiments were conducted sequentially to determine the effects of elevated LH levels on 1) the response of the dominant follicle to an ovulatory stimulus (Experiment 1) and 2) follicle growth and the levels of follicular fluid factors associated with pre-ovulatory maturation (Experiment 2). In the second experiment, the effects of eLH administration were compared to responses to the

high endogenous LH levels induced after administration of a luteolytic dose of PGF2α.

#### 4.2.2.1 Preliminary experiment – single eLH injection

Equine LH (eLH) was administered in the form of a highly purified pituitary fraction from crude equine gonadotropin as described in 2.2. To determine gonadotropin levels after a single eLH injection, 9 to 11 days after ovulation, mares were fitted with a jugular catheter in sterile conditions under local anaesthesia and given i.v. 1.6 μg eLH per kg body weight or vehicle (saline, n = 4 or 5 mares per group). Blood samples were collected through the same catheter into heparinised tubes at 0, 2, 5, 15, 30, 60, 180, 360, and 720 min after eLH treatment and stored as described in 2.3.

#### 4.2.2.2 Experiment 1 – Effects of eLH administration on ovulatory responses

Ten days after a natural ovulation, all ovarian follicles >10 mm were ablated in each mare by trans-vaginal ultrasound-guided follicle puncture to remove atretic follicles and induce a new follicular wave, as described in 2.3.2. Mares were randomly assigned to receive an i.v. injection of eLH (1.6 μg eLH per kg body weight) or an equivalent volume of vehicle (physiological saline) every 12 hours (n = 8 mares/group) starting one day after ablation (Day 0) and continuing until a dominant follicle reached >32 mm in diameter, at which time hCG (3000 IU; Chorulon®; i.v.) was administered to induce ovulation. A cross-over design was used whereby each mare was randomly assigned once to each of the two treatments leaving a complete oestrous cycle between treatment periods. Throughout the treatment period, follicle growth was monitored daily by transrectal ultrasound as described in 2.1. After administration of hCG, the frequency of scanning was increased to every 12 h to precisely determine the time of ovulation, which was detected by the disappearance of the previous preovulatory follicle and was confirmed by the presence of an echoic corpus luteum. The development of HAFs was established based on published ultrasound criteria (Ginther et al. 2007a). Blood samples were collected daily until 12 days post-ovulation as described in 2.3.1.

#### 4.2.2.3 Experiment 2 – Effects of administration of eLH or a luteolytic dose of PGF2α on follicular growth and follicular fluid factor levels

Following ablation of all follicles >10 mm ten days after ovulation, mares were randomly assigned to one of three groups (n = 7 mares/group). Two groups received either an i.v. injection of eLH or vehicle every 12 hours as in Experiment 1. Mares in the third group received a luteolytic dose of PGF2α (250 µg; Cloprostenol®; i.m.) on the day of ablation followed by twice daily injections of physiological saline. A cross-over design was used as in Experiment 1, so that each mare was randomly assigned once to each of vehicle, eLH and PGF2α groups and follicular and blood hormone data were collected similarly. When a dominant follicle reached a diameter of >32 mm, follicular fluid was completely aspirated by trans-vaginal puncture, centrifuged and stored as described in 2.3.2 until further analysis.

### 4.2.3 Hormone and follicular-fluid factor analysis

All samples were analysed in assays as described in Chapter 2.

Intra-assay CVs and sensitivities were 10.5% and 0.25 ng/ml for LH and 14.4% and 2.9 ng/ml for FSH.

For progesterone the intra-assay CV was 8.6% and the sensitivity was 0.01 ng/ml.

The intra-assay CV of cortisol was 6% and the sensitivity was 0.56 ng/ml.

The androstenedione assay had an intra-assay CV was 7% and the sensitivity was 2.18 pg/ml.

The IGF1 ELISA had an intra-assay CV of 9.9% and a sensitivity of 15 pg/ml.

Prostaglandin E2 (PGE2) was measured with an intra-assay CV of 7.9% and a sensitivity of 40 pg/ml.

Oestradiol levels were measured with a sensitivity of 7.4 pg/ml and an intra-assay CV of 0.7%.

### 4.2.4 Statistical Analyses

All data were tested for normality using the Anderson-Darling test ($P > 0.05$) and log-transformed if necessary. Data were then subjected to analysis of variance using a GLS procedure (R version 1.10.1), which accounted for the autocorrelation between samples taken over time, and the effects of Group, Day and the interaction were determined. Whenever there was a significant main effect or interaction, individual means were compared using Tukey's test. Unpaired t-tests were used for comparisons involving only two sample means. Statistical significance was considered at $P < 0.05$. All results are shown as Mean ± SEM.

## 4.3 Results

### 4.3.1 Short-term circulating gonadotropin responses to eLH administration

Circulating gonadotropin levels following a single i.v. injection of 1.6 μg eLH/kg body weight were determined in the preliminary experiment during the mid-oestrous cycle of the mares (Figure 4.1). Levels of LH increased more than 15-fold after injection, with a peak at 5 minutes followed by a progressive decrease during which LH concentrations remained higher ($P < 0.05$) than in vehicle-treated mares until at least six hours post injection. FSH levels, in contrast, were not different between the two groups (Group, $P > 0.6$).

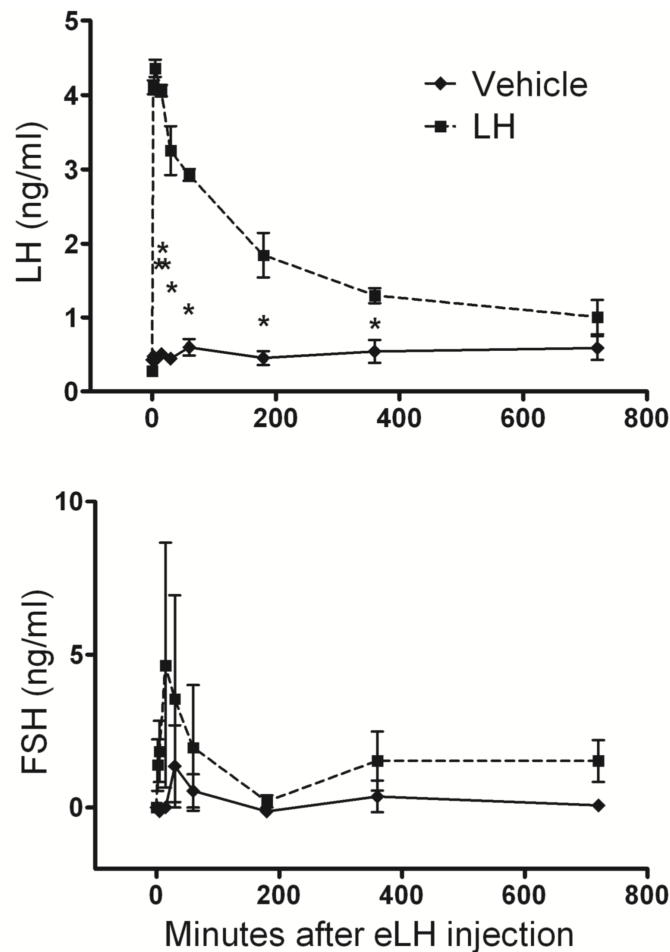


**Figure 4.1 Plasma levels of LH and FSH after a single intravenous injection of vehicle (♦, continuous line, n = 4) or eLH (■, broken line, n = 5) in mid-dioestrous mares. There was a significant interaction of Treatment by Day for LH ($P < 0.001$). An asterisk indicates a significant difference between treatment means ($P < 0.05$). To account for differences in pre-treatment levels between animals, the FSH value at 0 min was subtracted from all other values within each animal.**

## 4.3.2 Effects of eLH administration on ovulatory responses

There were no significant differences ($P > 0.1$) between groups in Experiment 1 in the growth rate of the dominant follicle (3.3 ± 0.3 mm/day and 2.9 ± 0.2 mm/day for eLH- and vehicle groups, respectively) or the day the dominant follicle ovulated after the onset of treatment (11.8 ± 1 days and 11.6 ± 0.9 days). The dominant follicle ovulated in response to an injection of hCG in seven out of eight mares in the vehicle

group and in six out of eight mares in the eLH group, and both the mean interval from hCG administration to ovulation (57 ± 9 h and 54.9 ± 9 h in eLH- and vehicle-treated groups, respectively) and the diameter of the dominant follicle at ovulation (35.8 ± 1 mm and 36.8 ± 0.9 mm) were similar ($P > 0.2$) between the two groups. No mare double-ovulated. Among the three mares that failed to ovulate in response to hCG, one mare in each of the two experimental groups developed a HAF based on the appearance of specks in the follicular fluid beginning at the time of expected ovulation, followed over the next seven days by a continued increase in follicle diameter (to a maximum of 63.0 and 53.0 mm in the eLH- and vehicle-treated mare, respectively) and the progressive organisation of follicular fluid specks into a distinct hyperechogenic network within the follicle cavity. At the same time, each of these two mares developed another preovulatory follicle which ovulated 14 and 20 days after hCG administration (eLH- and vehicle-treated mares, respectively). In the third mare (eLH group), the target follicle did not ovulate in response to hCG but ovulation occurred from a smaller follicle 4.5 days after hCG administration.

### 4.3.3 Effects of elevated LH levels on follicular growth and follicular fluid factors

In Experiment 2, plasma progesterone levels in eLH- and vehicle-treated groups remained elevated during the first few days of treatment and then decreased in association with natural luteolysis (Figure 4.2). In contrast, in the remaining group, injection of PGF2α on day 10 after ovulation effectively hastened luteolysis as indicated by an immediate decrease in progesterone levels.

Daily circulating LH levels increased in response to eLH treatment and this increase was similar to that induced by injection of a luteolytic dose of PGF2α at mid-cycle (Figure 4.2). As a result, LH levels in the eLH and PGF2α groups were 2- to 3-fold higher ($P < 0.05$) than in the vehicle group throughout the treatment period. In contrast, FSH levels decreased over the treatment period in all three groups (Day, $P = 0.0009$) and were not significantly different between groups (Figure 4.2).

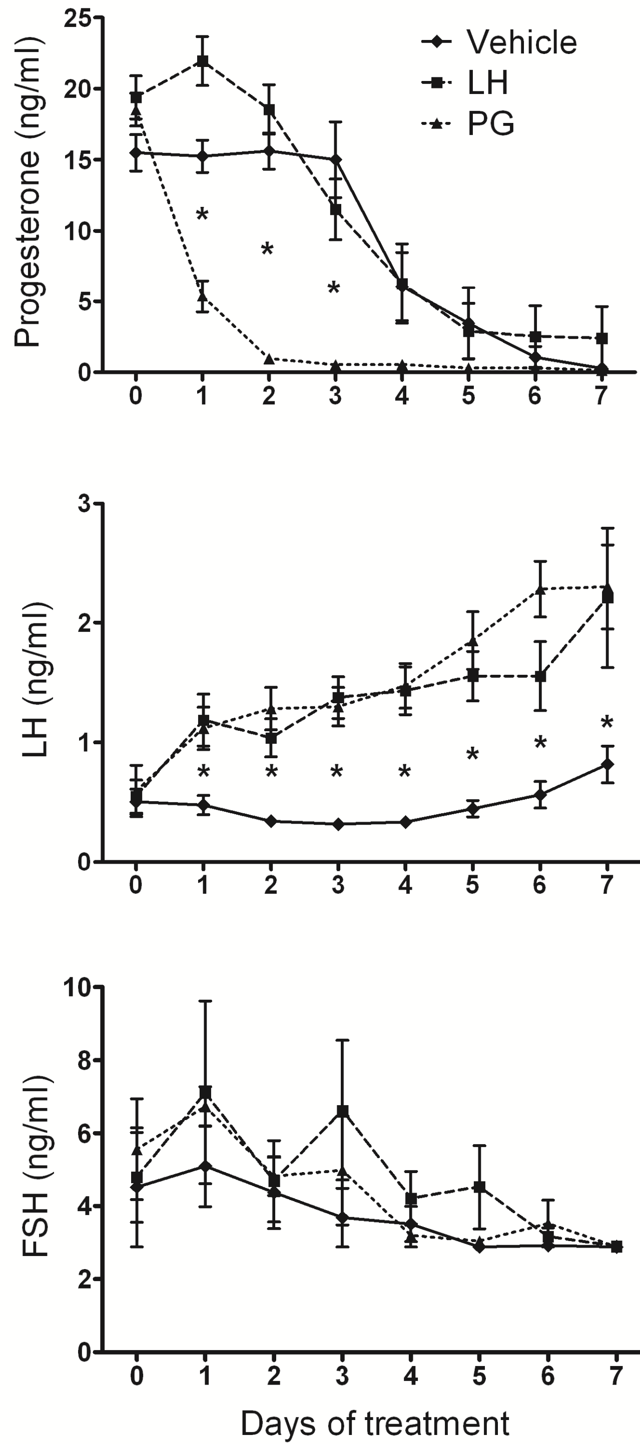


**Figure 4.2 Plasma levels of progesterone, LH and FSH in mares treated with vehicle (♦, continuous line), eLH (■, broken line) or PGF2α (▲, dotted line) (n = 7 mares/group). There was a significant Treatment by Day interaction for progesterone ($P = 0.004$) and LH ($P = 0.049$). Significant mean differences between groups within each day are indicated by asterisks ($P < 0.05$).**

Follicular responses to treatments are shown in Figure 4.3. There were no significant differences among groups (P > 0.1) in the growth of the dominant follicle or total numbers of follicles >10 mm in the ablation-induced wave. A single dominant follicle developed in all mares.

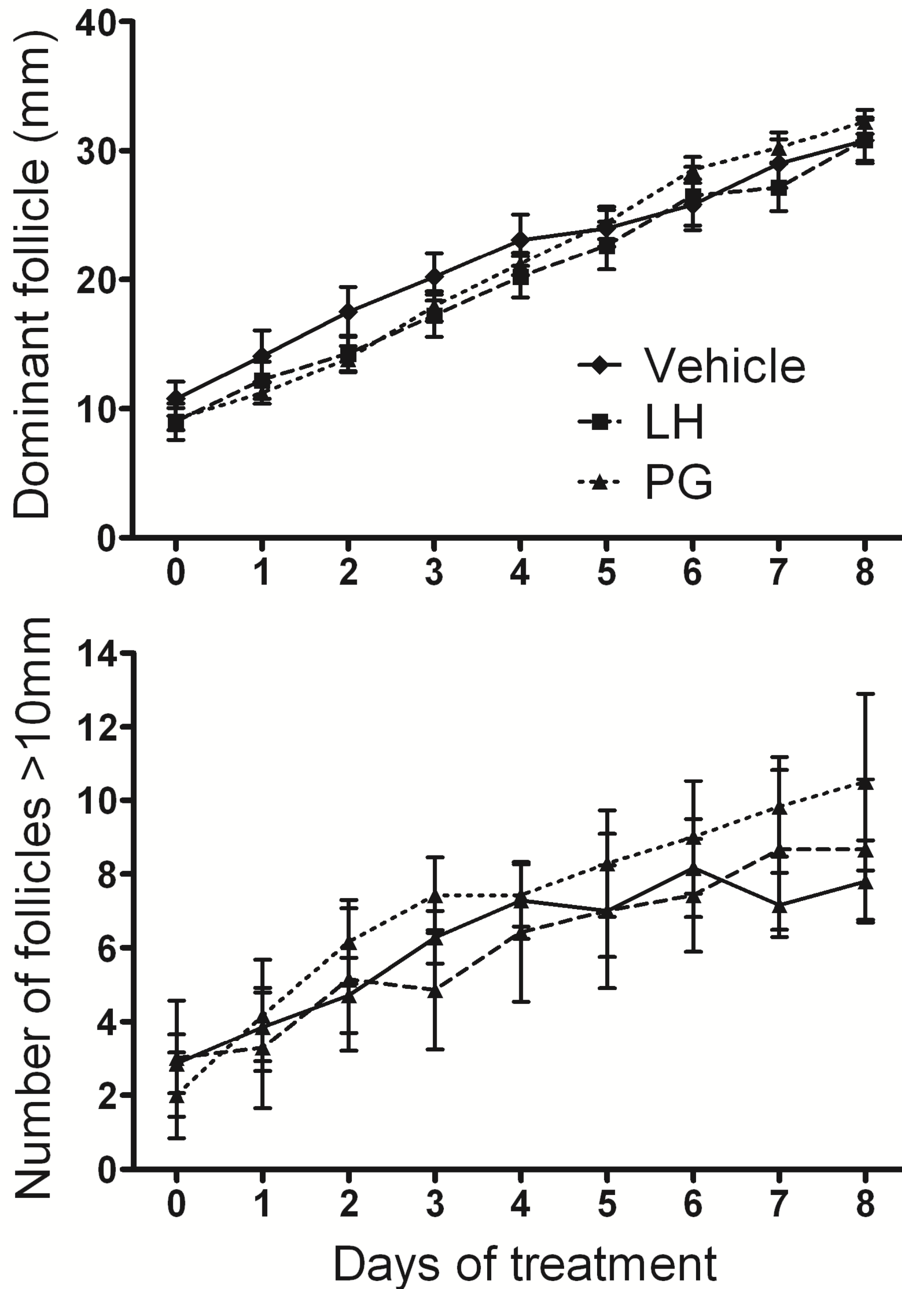


**Figure 4.3 Diameter of the dominant follicle and numbers of follicles >10 mm in mares treated with vehicle (♦, continuous line), eLH (■, broken line) or PGF2α (▲, dotted line) (n = 7 mares/group). The effect of Day was significant for each of the two endpoints (P < 0.0001).**

The dominant follicle was aspirated in all treatment groups once it reached >32 mm and the follicular-fluid levels of different factors known to be physiologically involved in LH-induced maturation were analysed (Figure 4.4). For reference, follicular fluid aspirated from the largest subordinate follicle in the vehicle group was included in the analyses. The dominant follicle aspirate from one mare in the vehicle group was grossly contaminated with blood and was discarded. In one additional mare from each of the vehicle and LH groups the dominant follicle had a diameter ≥36 mm on the day of aspiration; this was larger than expected based on the reported average daily growth of equine dominant follicles (Donadeu and Pedersen 2008) and the two samples were therefore removed from analysis. Analyses of data from the remaining mares revealed that mean levels of androstenedione in the dominant follicle were lower (about 2-fold) in the eLH and PGF2α groups combined than in the vehicle group ($P = 0.03$). However, there were no significant differences ($P > 0.1$) in the levels of progesterone and oestradiol in the dominant follicle among groups. The levels of progesterone and oestradiol were lower ($P < 0.05$) in subordinate follicles than in dominant follicles (vehicle group) whereas mean androstenedione levels tended to be higher in subordinate follicles ($P = 0.08$). Combined for eLH and PGF2α groups, levels of IGF1 in the dominant follicle were higher than in the vehicle group ($P = 0.03$) whereas the levels of PGE2 tended to be lower ($P = 0.06$). The levels of IGF1 were much lower ($P = 0.001$) in dominant follicles than in subordinate follicles of the vehicle group. Finally, mean levels of cortisol in dominant follicles were not different among groups ($P > 0.1$), but were significantly lower in subordinate follicles than in dominant follicles of the vehicle group ($P < 0.01$).

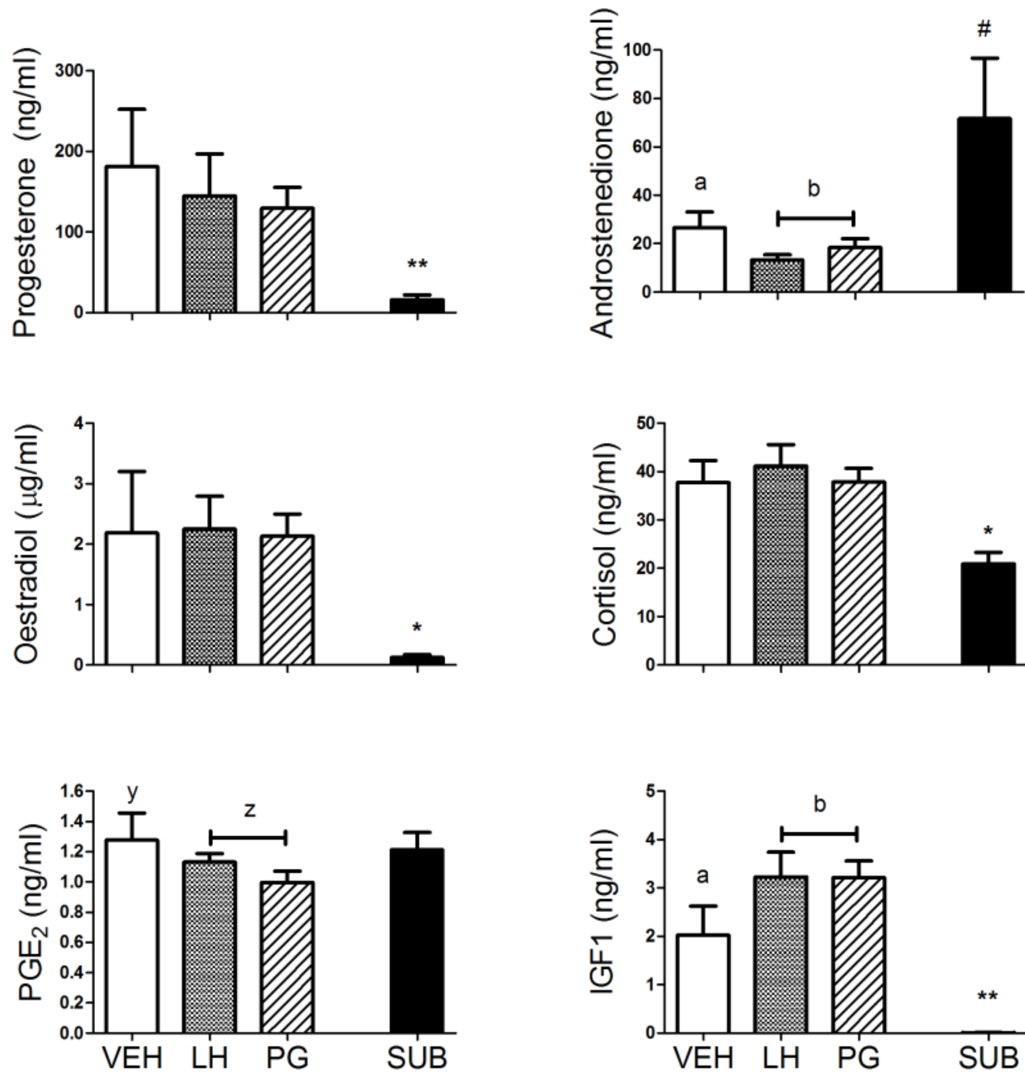


**Figure 4.4 Follicular fluid factor levels in pre-ovulatory follicles of mares treated with vehicle (VEH; n = 5), eLH (LH; n = 6) or PGF2α (PG; n = 7), and in subordinate follicles from vehicle-treated mares (SUB; n = 7). Differences in mean values between the vehicle group and the eLH and PGF2α groups combined are indicated by ab (P<0.05) or yz (P=0.06), whereas differences between dominant and subordinate follicle in the vehicle group are indicated by ** (P < 0.01), * (P < 0.05) or # (P = 0.08).**

### 4.3.4 Effects of elevated LH on circulating hormone levels during the subsequent luteal phase

The effect of over-stimulating follicles with LH on the subsequent luteal phase was assessed by measuring circulating progesterone levels from two days before to 12 days after hCG-induced ovulation in eLH- and vehicle-treated mares (Experiment 1). Overall, there were no significant differences (P = 0.6) in the post-ovulatory increase in progesterone levels between the two groups (Figure 4.5), although, when the period between 6 and 12 days after ovulation was separately analysed, mean progesterone levels tended to be lower in the eLH group than in the vehicle group (P = 0.095). Circulating LH levels peaked in both groups one day after ovulation and this was followed by a progressive decrease during the ensuing luteal phase (Day, P = 0.0004). In addition, mean LH levels during the entire period remained higher in eLH- than in vehicle-treated mares (Group, P = 0.02; Figure 4.5).

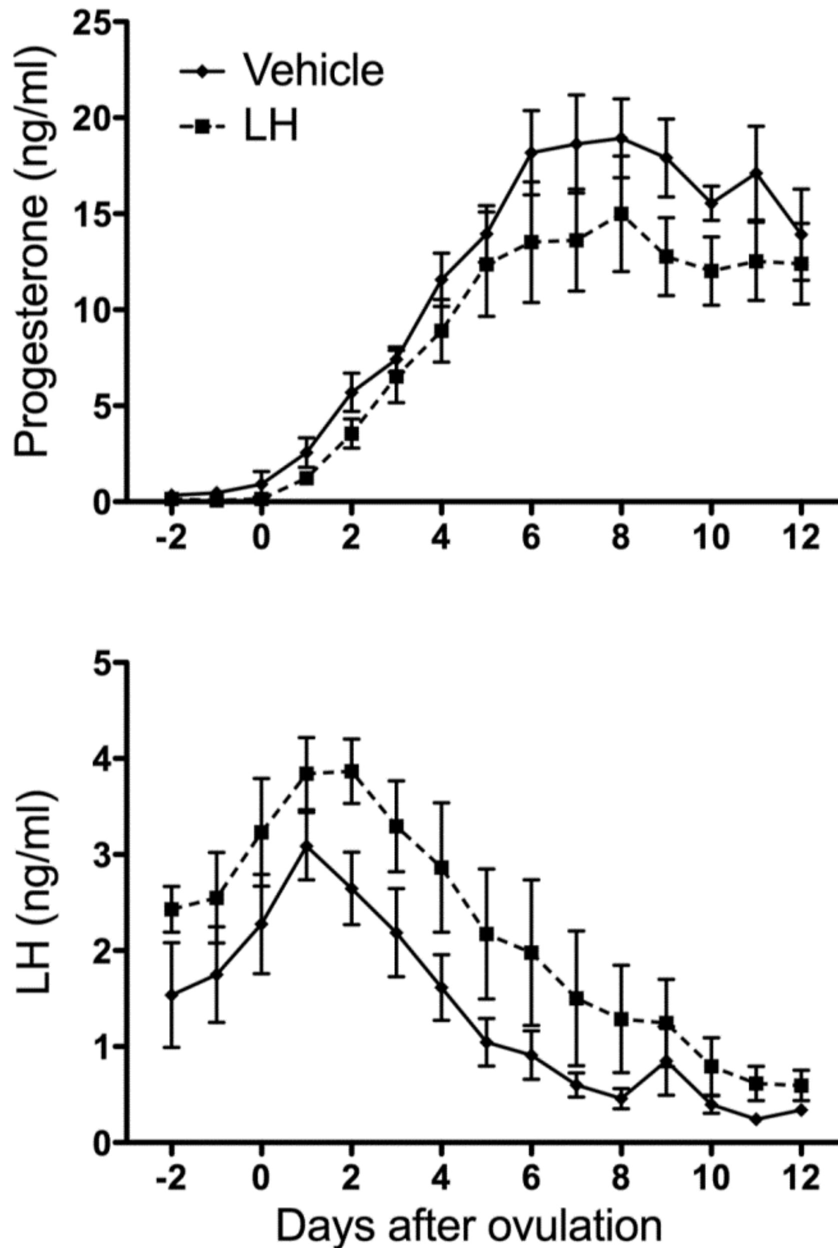


**Figure 4.5 Circulating levels of progesterone and LH from two days before to 12 days after hCG-induced ovulation following treatment with vehicle (♦, continuous line; n = 6) or eLH (■, broken line; n = 7) in Experiment 1. There was a significant effect of Day for each of Progesterone (P = 0.0001) and LH (P = 0.0004), and an effect of Group for LH (P = 0.02).**

## 4.4 Discussion

Given that PGF2α and its derivatives are routinely used for the control of oestrous cycles in horses and other farmed animals, the recent reports of a deleterious effect of induction of luteolysis with PGF2α on subsequent ovulation in mares (Ginther et al. 2008b; Cuervo-Arango and Newcombe 2009) strongly indicated the need to precisely investigate the effects of exogenous PGF2α on folliculogenesis and ovulation. In this study, a follicular ablation model in horses was used which allows close monitoring of very early follicle development stages in vivo (Gastal et al. 1997b; Donadeu and Ginther 2001; Donadeu and Ginther 2002a; Doyle et al. 2008). By using frequent administration of an enriched pituitary fraction, we investigated the effects that the exposure of growing follicles as small as 10 mm in diameter to a sustained elevation in LH would have on subsequent growth, maturation and ovulatory capacity. We then compared these to the follicle responses associated with high levels of endogenous LH after administration of a luteolytic dose of PGF2α during mid-cycle. Our results did not support the hypothesis that a premature elevation in LH levels during a follicular wave would disrupt normal follicle growth or ovulation; however, based on differences in mean follicular fluid factor levels between experimental groups, the present results did provide evidence that elevated LH levels may disrupt normal follicle maturation.

Circulating LH levels increased in response to daily administration of eLH or PGF2α injection and were 2- to 3-fold higher in each of these two groups than in vehicle-treated animals throughout the treatment period, which is consistent with reported LH values attained after administration of PGF2α ten days post-ovulation (Johnson et al. 1988; Gastal et al. 2000; Ginther et al. 2007b). These findings demonstrated the adequacy of the eLH administration regime used for investigating potential LH-mediated effects of PGF2α administration on follicle development.

The bioactivity of the eLH preparation used in the present study has been demonstrated previously by ovulation induction in mares (Hofferer et al. 1993). Twice daily injections of the same preparation at doses that were 4- to16-fold lower than the ovulation-inducing dose used in the present report readily stimulated

follicular growth in early transitional mares (Chapter 3). Those effects were attributable to the LH activity of the eLH preparation rather than to any residual FSH (Chapter 3). Consistent with that conclusion, in the present study no significant differences were found in FSH levels between eLH- and vehicle-treated mares, either in samples collected immediately following eLH administration or at daily intervals throughout the treatment period. As expected, daily FSH concentrations were also not changed by PGF2α treatment (Johnson et al. 1988; Gastal et al. 2000), but decreased for all groups progressively during the development of the induced follicular waves (Donadeu and Ginther 2001).

High levels of circulating LH induced by eLH administration in Experiment 1 did not have any apparent effects on hCG-induced ovulation and did not increase the incidence of HAFs resulting from the ablation-induced wave, which were detected in one mare from each of the eLH and vehicle groups. In retrospect, considering that in earlier studies (Cuervo-Arango and Newcombe 2009; Ginther and Al-Mamun 2009) the incidence of HAFs in mares after treatment with PGF2α was only ≤20 %, a larger number of oestrous cycles than examined in the present study may be necessary to detect effects of high LH on ovulation in mares, and therefore the results of Experiment 1 may not be considered conclusive.

We reasoned that, despite the lack of clear effects of eLH administration on ovulation, high LH levels may still induce changes in normal follicle maturation that under certain circumstances could increase the likelihood of ovulatory failure, thus explaining previous observations in larger mare populations (Ginther et al. 2008b; Cuervo-Arango and Newcombe 2009; Ginther and Al-Mamun 2009). To test this possibility, in Experiment 2 we analysed the follicular fluid of pre-ovulatory follicles that had developed in the presence of high exogenous or endogenous levels of LH (eLH and PGF2α groups, respectively). Follicular levels of selected factors were measured that are known to naturally respond to the ovulatory LH surge and which are involved in oocyte maturation (IGF1), the inflammatory response associated with follicle rupture (PGE2 and cortisol) and luteinisation (progesterone, androstenedione and oestradiol) (Murphy 2000; Espey and Richards 2002; Sirois et al. 2004; Velazquez et al. 2009). As expected (Donadeu and Ginther 2002a), the levels of

progesterone, oestradiol and IGF1 were higher in dominant follicles than in subordinate follicles whereas androstenedione levels tended to be higher in subordinate follicles. More importantly, mares in the eLH and PGF2α groups had decreased mean levels of androstenedione and increased levels of IGF1 in pre-ovulatory follicles, in temporal association with elevated circulating LH levels in those two groups. Follicular-fluid levels of androstenedione and oestradiol physiologically decreased in response to high gonadotropin stimulation during the preovulatory period (Voss and Fortune 1993; Belin et al. 2000). The reason for a decrease in androstenedione but not oestradiol in response to high LH in the present study is not known, however, a reduction in follicular production of androstenedione rather than oestradiol has been reported after administration of high levels of LH in other species (Rousey and Terranova 1985), and it is well-known in other species that lower doses of LH stimulate follicular androstenedione production whereas high doses are inhibitory (Berndtson et al. 1995; Campbell et al. 1998). In relation to IGF1, it has been shown in ruminants that follicular levels of this growth factor increase during super-ovulatory treatments and that high IGF1 levels can impair oocyte quality and reduce embryo development (Velazquez et al. 2009). Furthermore, a study in mares (Ginther et al. 2007c) found that IGF1 levels were 2-fold lower in preovulatory follicles containing a mature oocyte than in those containing an immature oocyte. In light of those findings, the results of the present study warrant critical examination of the potential effects that high IGF1 levels induced by treatment with luteolytic PGF2α may have on oocyte quality in mares and, potentially, other livestock.

Recent studies by Cuervo-Arango and collaborators (2011) and Ginther et al. (2011) showed that pharmacological inhibition of PTGS2, a prostaglandin synthesising enzyme which is upregulated by the ovulatory LH surge (Sirois et al. 2004), blocked ovulation in mares, consistent with results in other species (Peters et al. 2004). In the present study, high levels of LH in the eLH and PGF2α groups were associated with slightly lower levels of endogenous PGE2 in the pre-ovulatory follicle. We were not able to collect enough granulosa cells from our follicular aspirates to properly determine whether the expression or activity of PTGS2 was decreased in the eLH

and PGF2α groups. However, although the present finding will need confirmation in future studies, a reduced ability of preovulatory follicles exposed to high LH to synthesise prostaglandins would be consistent with the reported predisposition of mares treated with PGF2α to develop anovulatory follicles (Ginther et al. 2008b; Cuervo-Arango and Newcombe 2009).

## 4.5 Conclusion

In summary, although treatment with eLH or PGF2α did not have any apparent effects on follicle growth or ovulation, the results of this study indicate that induction of luteolysis with PGF2α in mares can alter the levels of follicular-fluid factors involved in follicular and oocyte maturation, including IGF1 and PGE2, and that these responses are mediated by high circulating LH. These LH-induced changes may in turn increase the predisposition of mares to develop HAFs (Cuervo-Arango and Newcombe 2009; Ginther and Al-Mamun 2009). Further studies in larger mare populations will be required to more definitely establish the effects of high LH levels on ovulatory outcome. However, the present results are novel and clearly warrant further investigation of the precise effects of luteolysis-inducing treatments on the fertility of farm animals.

# Chapter 5

# Comparative molecular analyses of ovulatory and anovulatory follicles

# Chapter 5: Comparative molecular analyses of ovulatory and anovulatory follicles

## 5.1 Introduction

The different stages of the equine reproductive cycle provide an excellent model to study the effects of different LH milieus on follicular growth and maturation. During the follicular phase of an oestrous cycle, follicular waves develop under circulating LH levels that are 2-fold higher than during the luteal phase (Vandeplassche et al. 1979; Ginther et al. 2005a) and 2- to 10-fold higher than during the anovulatory season (Watson et al. 2002b; Donadeu and Ginther 2003a). Furthermore, LH pulse frequency is minimal during the middle of the anovulatory season and increases through the period of transition into the ovulatory season, whereas LH pulse amplitude simultaneously decreases (Fitzgerald et al. 1987). Similarly during an oestrous cycle, LH pulsatility increases during the follicular phase in the mare leading to a preovulatory LH surge during which LH pulse frequency is highest (Alexander and Irvine 1987).

The critical requirement of LH for the normal growth of a dominant follicle has been shown in cattle (Ginther et al. 1998) and mares (Gastal et al. 2000) by experimental suppression of LH using progesterone. In addition, previous studies in mares showed that follicles growing during the transitional period contain an under-developed and poorly vascularised theca cell layer, express reduced levels of steroidogenic enzymes and LHR, and have reduced follicular fluid levels of steroids and growth factors such as IGF1 and VEGF (Watson and Al-Zi'abi 2002; Acosta et al. 2004a; Watson et al. 2004a; Donadeu 2006). Large anovulatory follicles developing during the luteal phase in mares have been less well studied with one study reporting decreased oestradiol and progesterone levels in follicular fluid compared to ovulatory follicles (Vandeplassche et al. 1979). Although large equine follicles developing during the luteal phase (Hedberg et al. 2006) and the spring transitional period (Colbern et al. 1987) can be induced to ovulate with hCG, these ovulations may be associated with low pregnancy rates (Ginther and Bergfelt 1992).

miRNAs are key post-transcriptional regulators of cell differentiation. Recently, different miRNAs have been shown to be involved in follicle maturation and ovulation. In mice, Fiedler et al. (2008) looked at the changes in miRNA expression in response to an ovulatory dose of hCG and found 13 miRNAs differentially expressed, including miR-21, which they subsequently showed to promote the survival of granulosa cells (Carletti et al. 2010). miR-378 has been reported to regulate steroidogenesis by targeting aromatase expression in porcine granulosa cells and therefore it may be involved in regulating ovulatory capacity (Xu et al. 2011). Other miRNAs, including miR-202 (Ahn et al. 2010) and miR-542 (Mishima et al. 2008) have been reported to be expressed exclusively or predominantly in the ovary, suggesting that they may have unique functions during follicle or luteal development. miR-202 has been shown to be regulated by oestradiol during embryonic gonad development in chicken (Bannister et al. 2011), whereas miR-542 expression was linked to the maturity of bovine oocytes (Tesfaye et al. 2009).

To better understand the molecular mechanisms associated with poor follicle maturation during periods of low circulating LH in the mare, this study sought to compare the levels of different follicular fluid factors as well as expression of genes associated with follicular maturity in equine preovulatory-size follicles (≥30 mm) developing during three different stages of the equine reproductive cycle, namely follicular phase, luteal phase and the spring transitional period. In addition, the expression of key miRNAs potentially involved in regulating follicle maturation was also determined.

## 5.2 Materials and Methods

### 5.2.1 Experimental animals and sample collection

Seven mainly Welsh-cross ponies were used in this experiment and kept as described in 2.1. Ovaries were scanned daily by transrectal ultrasonography as described in 2.1 to determine the diameter and status (growing or regressing) of the two largest ovarian follicles during normal oestrous cycles occurring during May to October 2009/2010 and during the spring transitional period (February and March) of 2011/2012. Follicular fluid and granulosa cell samples were collected by transvaginal

ultrasound-guided puncture as described in 2.3.2 from the largest growing (dominant) follicle (>32 mm) during the follicular phase (Oestrous follicles; 16 to 20 days after ovulation; n = 7) and the luteal phase (Dioestrous follicles; 9 to 11 days after ovulation; n = 7) of different oestrous cycles. During each follicular phase, the second largest follicle (Subordinate follicles; n = 7) was also aspirated. Seasonally anovulatory mares were identified by the absence of a detectable corpus luteum for at least four weeks beginning in January; the first follicle to grow to a size >32 mm in these mares was aspirated (Transitional follicles; n = 4).

### 5.2.2 Hormone analyses

Follicular fluid concentrations of progesterone, testosterone and cortisol were assayed using a commercial radioimmunoassay kit (Coat-a-Count, Siemens Healthcare Diagnostics, Surrey, UK) as described in 2.4.2. The intra-assay CVs were 4.3%, 1.8% and 4.2% and the sensitivities were 0.01 ng/ml, 7.0 ng/ml and 0.56 ng/ml, respectively.

Concentrations of oestradiol and IGF1 were measured using commercial ELISA kits (DRG Instruments GmbH, Marburg, Germany and Diagnostic Systems Laboratories, Inc., Webster, TX, USA, respectively), as described in 2.4.3. The intra-assay CVs were 9.8% and 9.9% and the sensitivities were 5.2 pg/ml and 15 pg/ml, respectively.

mRNA extraction from granulosa cells and miRNA extraction from follicular fluid, and RT-qPCR, were performed as described in 2.5 and 2.6. Expression levels of 18S and cel-mir-39 (spike-in control) were used for normalisation of mRNAs and miRNAs, respectively, in follicular samples.

### 5.2.3 Statistical analysis

Dixon’s outlier test was used to identify suspected outlier values from the data set ($P < 0.01$), which were subsequently removed before analyses. Data normality was tested with the Anderson-Darling test and non-normal data were analysed using non-parametric statistical tools. Oestrous, Dioestrous and Transitional follicles were therefore compared using ANOVA or Kruskal-Wallis test followed by Tukey’s or Dunn’s multiple comparison test, respectively. Subordinate follicles were compared

to Oestrous follicles with a one-tailed student's t-test. Significance was always considered at $P < 0.05$ whereas a $P < 0.1$ was taken as indicative of approaching significance. All results are shown as mean ± SEM.

## 5.3 Results

### 5.3.1 Follicle size and circulating hormone levels

Average follicle sizes at the time of sampling are shown in Figure 5.1. Oestrous, Transitional and Dioestrous follicles had mean diameters of 33.9 ± 0.5, 34.5 ± 0.6 and 32.1 ± 0.6 mm ($P = 0.06$), respectively. Subordinate follicles had a diameter of 25.1 ± 0.7 mm.

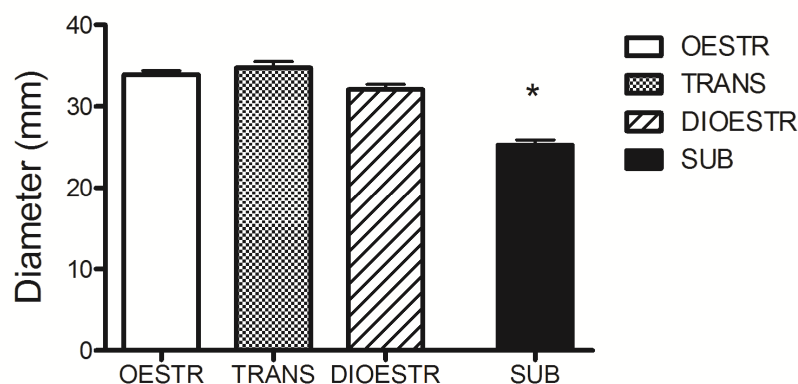


**Figure 5.1 Follicle size of Oestrous (n = 7), Transitional (n = 4), Dioestrous (n = 7) and Subordinate follicles (n = 7) at the time of sampling. Subordinate follicles were significantly smaller than Oestrous follicles (P < 0.0001).**

### 5.3.2 Follicular fluid factors

Progesterone concentrations in follicular fluid were significantly higher in Oestrous follicles than in Transitional or Dioestrous follicles ($P < 0.05$), whereas there was no difference among these three follicle groups for oestradiol, testosterone and cortisol (Figure 5.2). Subordinate follicles had significantly lower levels of progesterone, oestradiol and cortisol ($P < 0.05$) than Oestrous follicles (Figure 5.2). In addition, levels of IGF1 were lower in Transitional and Dioestrous follicles than in Oestrous

follicles (P < 0.05), and were also lower in Subordinate follicles than in Oestrous follicles (P < 0.001).

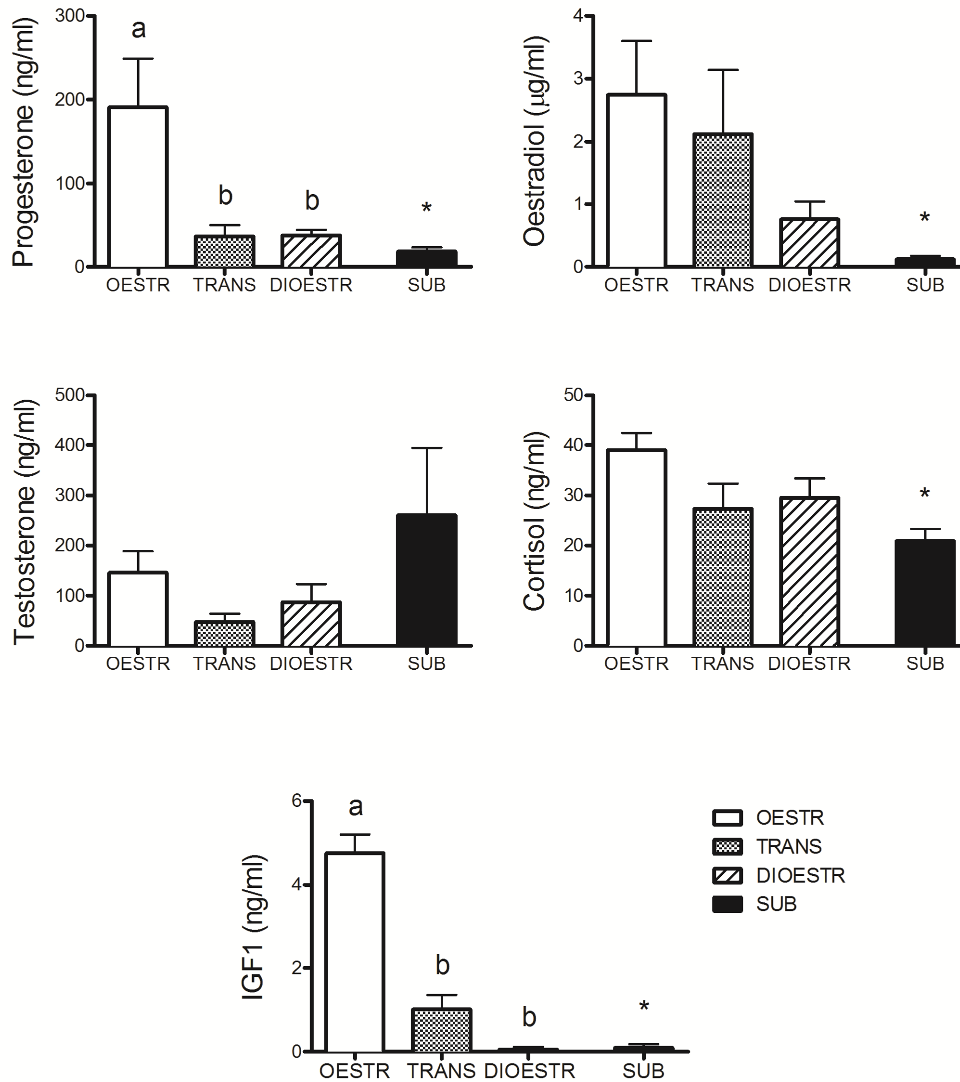


**Figure 5.2 Follicular fluid factor levels in Oestrous (n = 6-7), Transitional (n = 3-4), Dioestrous (n = 5-7) and Subordinate (n = 6-7) follicles. Overall significance for analyses involving the first three groups were P = 0.004 for progesterone and P < 0.0001 for IGF1. Differences in mean values between Oestrous, Transitional and Dioestrous follicle groups are indicated by a, b (P < 0.05), differences between Oestrous and Subordinate follicles are indicated by * (P < 0.05)**

### 5.3.3 Gene expression in granulosa cells

The levels of different genes associated with follicle maturation were measured in granulosa cells isolated from follicular fluid aspirates from each of the experimental groups (Figure 5.3). Differences in CYP11A1 involving Oestrous, Transitional and Dioestrous follicles approached significance (overall, $P = 0.07$). Although mean expression of CYP17A1 and CYP19A1 was lower in Transitional follicles and, particularly, Dioestrous follicles than in Oestrous follicles, these differences did not attain significance ($P > 0.1$). Expression levels of the three genes were however significantly lower in Subordinate follicles than in Oestrous follicles ($P < 0.04$).

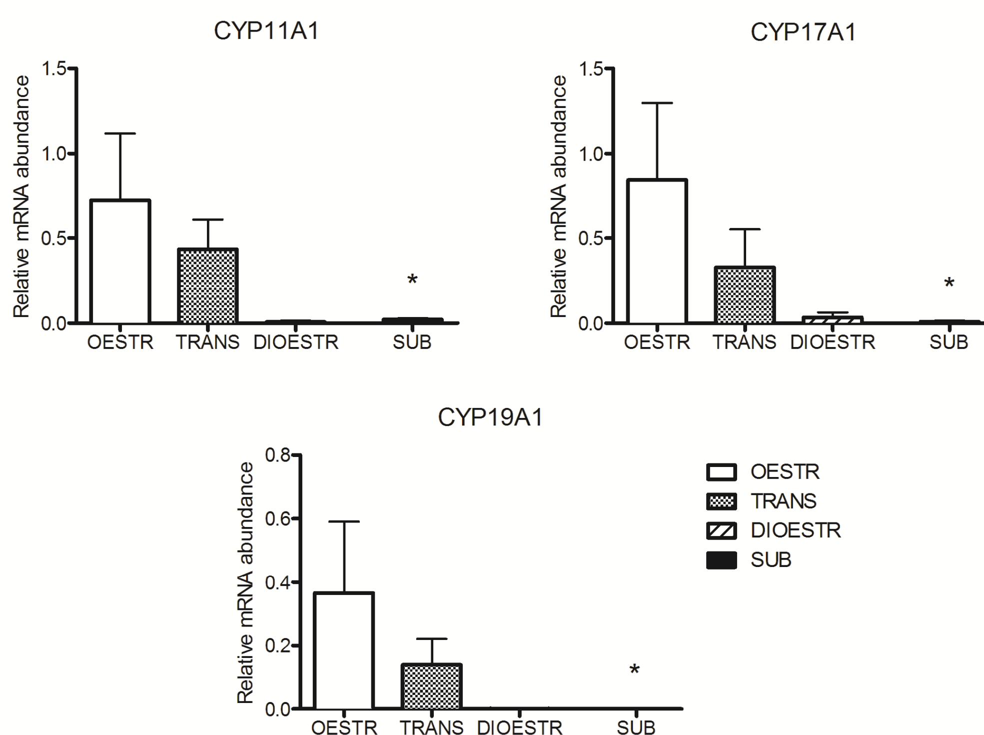


**Figure 5.3 mRNA levels of different steroidogenic enzymes normalised to 18S in Oestrous (n = 4-5), Transitional (n = 4), Dioestrous (n = 3) and Subordinate (n = 6) follicles. Differences in mean values between Oestrous and Subordinate follicles are indicated by * (P < 0.05)**

Furthermore, expression levels of each of LHR, FSHR and IGF1R (Figure 5.4) were not different among Oestrous, Transitional and Dioestrous follicles (P > 0.1), whereas Subordinate follicles showed low expression of FSHR relative to Oestrous follicles (P = 0.008) and also tended to have lower expression of IGF1R (P = 0.07).

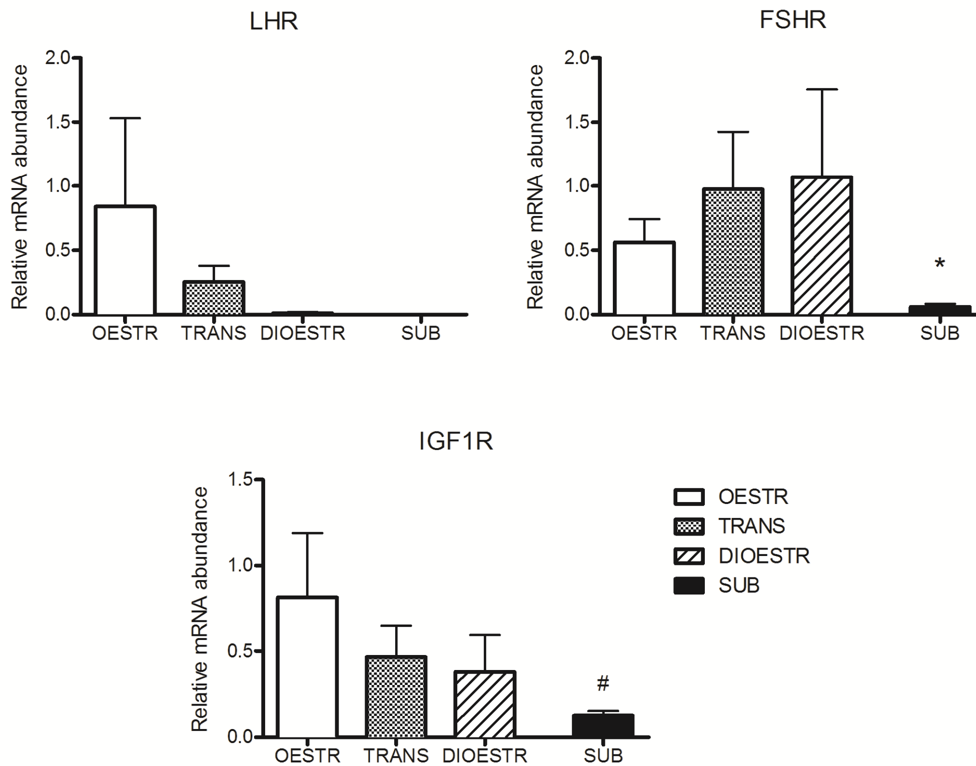


**Figure 5.4 mRNA levels of different receptors normalised to 18S in Oestrous (n = 4-5), Transitional (n = 4), Dioestrous (n = 3-4) and Subordinate (n = 5-6) follicles. Differences in mean values between oestrous and subordinate follicles are indicated by * (P < 0.05), # (P < 0.1)**

## 5.3.4 miRNA expression profiles

QPCR analyses using equine-specific primers revealed the presence of eca-miR-378, eca-miR-21 and eca-miR-542 in equine follicular fluid. An equine sequence corresponding to hsa-miR-202* has not been reported and therefore the corresponding human primer was used for qPCR; sequencing revealed that the resulting product had 100% homology to mmu-miR-202-5p (mouse) and rno-miR-

202-5p (rat), and, compared to the human and bovine sequences, had one additional 3' nucleotide ("T") (Figure 5.5).

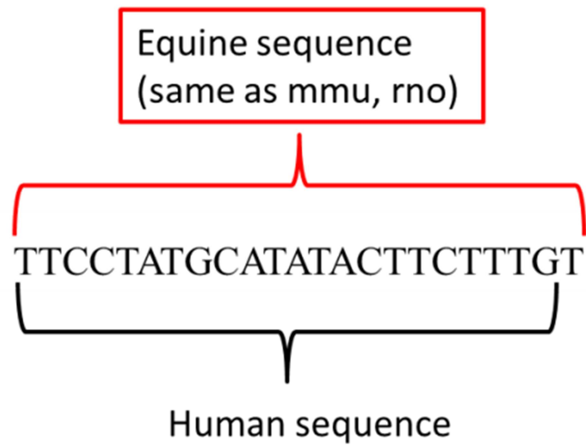


**Figure 5.5 Sequence of product obtained by qPCR of equine granulosa cells using a hsa-miR-202-5p primer.**

There were no differences (P > 0.1) in the levels of miR-378 and miR-542 among Oestrous, Transitional and Dioestrous follicles. Expression levels of miR-202 and miR-21 were significantly higher in Transitional than Oestrous follicle groups (P < 0.05) but were not significantly different (P > 0.1) between Transitional and Dioestrous follicles (Figure 5.6). Expression of miR-378, miR-202 and miR-542 was significantly higher in Subordinate follicles than in Oestrous follicles (P < 0.04), whereas miR-21 levels tended to be higher in subordinate follicles (P = 0.06; Figure 5.6).

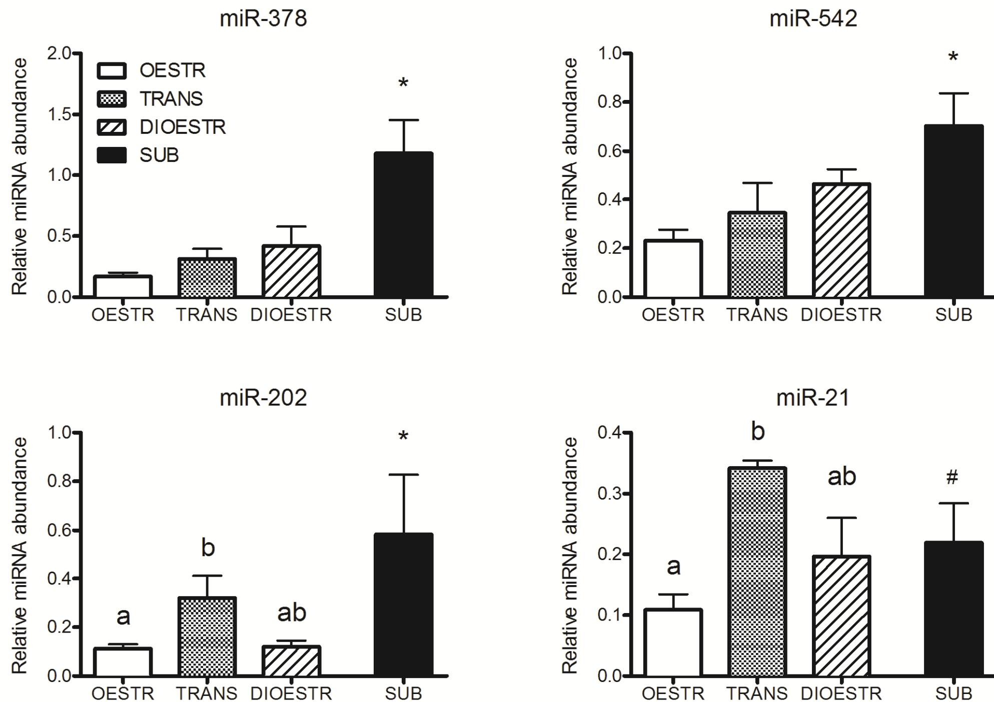


**Figure 5.6 Expression levels of miR-378, miR-542, miR-202 and miR-21 normalised to levels of spiked-in cel-miR-39 in follicular fluid of Oestrous (n = 6), Transitional (n = 4), Dioestrous (n = 4) and Subordinate follicles (n = 5-6). Analyses involving Oestrous, Transitional and Diestrous follicles revealed a significant effect of group for miR-21 (P = 0.003) and for miR-202 (P = 0.04). Specific differences in mean values between these three groups are indicated by ab (P < 0.05). Differences between Oestrous and Subordinate follicles are indicated by * (P < 0.05), # (P < 0.1)**

To determine whether, as reported in pig (Xu et al. 2011), the horse CYP19A1 3'UTR (NC_009144 REGION: 139140381..139204552) could contain a potential target for eca-miR-378, the two sequences were aligned using RNAhybrid software (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). This revealed significant pairing between the eca-miR-378 sequence and a sequence in the equine CYP19A1 3'UTR (nucleotides 1428 - 1454), which produces a 7mer match in the seed region of the miRNA (nucleotides 2 - 8) (Figure 5.7).

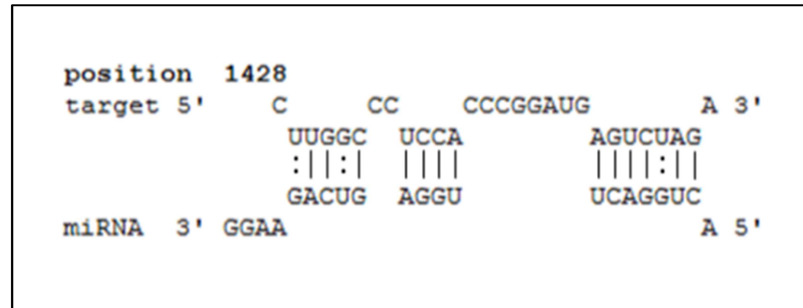


**Figure 5.7 Potential eca-miR-378 target sequence in the equine CYP19A1 3'UTR (presented with a 2-8 base seed match; allowing for GU wobbles) identified using the RNAhybrid software (Krüger and Rehmsmeier 2006)**

## 5.4 Discussion

Understanding the molecular features of anovulatory follicles is important in the horse because it could lead to the development of strategies to increase the fertility rates in this species. In this regard, this is the first study to undertake comparative analyses of dioestrous, transitional and ovulatory follicles, and the first to examine the potential involvement of miRNAs in equine follicle maturation.

The results of this study showed that mean levels of both, steroids in follicular fluid and steroidogenic enzyme mRNA in granulosa cells were lower in Transitional and Dioestrous follicles than in Oestrous follicles, although significance (or approaching significance) was only reached for progesterone and CYP11A1. These results are consistent with low levels of StAR, CYP11A1, CYP17A1 and CYP19A1 (Watson et al. 2004a) as well as progesterone and oestradiol (Davis and Sharp 1991; Watson et al. 2002b; Acosta et al. 2004a) in transitional compared to ovulatory follicles in previous experiments, which led to the conclusion that equine transitional follicles are deficient in all major steps of the steroidogenic pathway (Donadeu and Watson 2007). The present results also confirm previous limited data indicating steroidogenic deficiencies in dominant follicles developing during the luteal phase compared to the follicular phase of the oestrous cycle in mares (Vandeplassche et al. 1979; Fay and Douglas 1987). The steroidogenic deficiencies of transitional and dioestrous follicles are predictably detrimental to normal follicle maturation, as oestradiol plays a main

role in promoting both follicle maturation and the production of an LH surge, and progesterone is involved in mediating the ovulatory process (Brännström and Janson 1989; Robker et al. 2000b).

In the horse, IGF1 levels (Acosta et al. 2004a) or activity (Watson et al. 2004b) have been previously shown to be deficient in transitional follicles, although no information existed to-date on the IGF status of dominant follicles during dioestrus. Previous studies have shown that IGF1 is required for normal development of dominant ovulatory follicles in the mare (Ginther et al. 2004b). Further, in some studies high IGF1 levels in follicular fluid have been used as an indicator of good oocyte quality and positive fertilisation outcome (Artini et al. 1994; Fried et al. 2003). In light of these findings, the present results are consistent with equine transitional and dioestrous follicles being underdeveloped and potentially containing low quality oocytes.

Based on the present and previous data (Watson et al. 2004a; Doyle et al. 2008), the deficiencies observed in dominant follicles developing during the transitional and the dioestrous period may be to a significant extent attributed to reduced expression of receptors for trophic factors, namely LHR and IGF1R that are key stimulators of steroidogenesis in mature ovulatory follicles. Despite their developmental deficiencies and low LHR expression, equine transitional and dioestrous follicles can be induced to ovulate by administration of hCG (Colbern et al. 1987; Hedberg et al. 2006). However, there is some evidence that ovulations resulting from such follicles may be associated with low fertility. For example, in one study, follicle development was stimulated in seasonally anovulatory mares by treatment with GnRH; although ovulation could be induced in about 26% of those mares, early pregnancy loss after insemination was high (about 64%) and was associated with lower circulating LH and progesterone levels compared to non-treated mares (Bergfelt and Ginther 1992). Consistent with this, the first ovulation in the post-partum cow, which occurs under lower LH levels, results in short luteal cycles and subsequent reduced pregnancy rates (Copelin et al. 1987; Vasconcelos et al. 2001). These results are consistent with developmental somatic and/or germ cell deficiencies in these follicles.

Based on previous information in horses (da Silveira et al. 2011), differences in gene expression and steroidogenesis between follicles developing under different LH milieus may be accounted for at least partially by differences in posttranscriptional regulation by miRNAs. miRNAs investigated in this study have previously been reported to be specific to the ovary - miR-202 (Ahn et al. 2010) and miR-542 (Mishima et al. 2008) - or to be involved in steroidogenesis - miR-378 (Xu et al. 2011) - and survival of granulosa cells - miR-21 (Carletti et al. 2010). A recent study reported that miRNA profiles in equine follicular fluid provided, for the most part, a good indication of miRNA expression in surrounding granulosa cells and cumulus cells (da Silveira et al. 2011); therefore in this study we used follicular fluid analyses to investigate differences in miRNA levels between different types of equine follicles.

Mean miR-378 levels were higher, although not significantly, in Transitional and Dioestrous follicles than in Oestrous follicles and significantly much higher (about 6-fold) in Subordinate follicles than in Oestrous follicles. Recently, miR-378 has specifically been reported to regulate CYP19A1 expression and oestradiol production in pig granulosa cells (Xu et al. 2011). In the present study, mean expression levels of miR-378 in equine follicular fluid were clearly negatively correlated with expression levels of CYP19A1 in granulosa cells and follicular fluid oestradiol levels. As no reporter assay was performed to confirm a direct interaction between miR-378 and CYP19A1 in the horse, the present results have to be interpreted with caution. The reported interaction between miR-378 and the CYP19A1 3'UTR in the pig (Xu et al. 2011) is not conserved in human and horse. We found a potential target site for eca-miR-378 in the equine CYP19A1 3'UTR. Whether this site is a true target of equine miR-378 will need to be determined in future studies. In addition to its role in regulating aromatase expression in the pig, miR-378 has been found to promote angiogenesis (Hua et al. 2006; Lee et al. 2007), bone growth (Kahai et al. 2009) and to regulate CYP2E1 expression in the liver (Mohri et al. 2010), and therefore it may have additional actions in the ovary.

The expression pattern of miR-542, an ovarian-specific miRNA (Mishima et al. 2008) for which there is little published information, was similar to that of miR-378,

with expression levels significantly increased in Subordinate follicles compared to Oestrous follicles. miR-542 was also increased in immature compared to mature oocytes in bovine (Hailemariam 2011); taken together with the present result this suggests that miR-542 may have a role in follicle and/or oocyte maturation.

The results for miR-202, another miRNA putatively specific to the ovary (Mishima et al. 2008) showed that its expression levels were significantly higher in Subordinate follicles than in Oestrous follicles. Interestingly, Transitional follicles, but not Dioestrous follicles, also expressed significantly higher levels of miR-202 than Oestrous follicles, suggesting a distinct function of miR-202 during follicle maturation, which may not be related to the LH-deficient status common to both Transitional and Dioestrous follicles. McBride et al. (2012) reported that in sheep miR-202 was upregulated in growing follicles relative to preovulatory (early luteinising) follicles. In another study, oestradiol was shown to modulate miR-202 expression during the differentiation of the chick gonad (Bannister et al. 2011), however, a role for oestradiol in regulating levels of miR-202 in the horse was not supported by the data in the present study.

In the present study, similar to miR-202, miR-21 levels were significantly upregulated in Transitional follicles relative to Oestrous follicles, and Subordinate follicles tended to have higher miR-21 levels than Oestrous follicles. In equine follicles, miR-21 was found to be expressed in cumulus cells and in microvesicles within the follicular fluid but not in mural granulosa cells (da Silveira et al. 2011). Although not studied in relation to follicle maturation, miR-21 has been shown to have an anti-apoptotic effect in mice granulosa cells during ovulation (Carletti et al. 2010) and has also been reported to regulate sterol regulatory element-binding protein 1 (Srebf1) (Larsen et al. 2011), a gene involved in lipid biosynthesis, which is upregulated in granulosa cells in response to hCG (Richardson et al. 2005). Higher mean levels of miR-21 in regressing (Subordinate) than healthy growing (Oestrous) follicles is consistent with data in cattle (Sontakke and Donadeu, unpublished) and may indicate a role of miR-21 in follicular atresia. Based on the present results, the involvement of miR-21 in Transitional follicles will clearly need to be investigated in the future; furthermore, the substantial difference (>3-fold) detected in miR-21 levels

between Oestrous and Transitional follicles may provide a potential marker to assess follicular maturity during the transitional period in mares.

## 5.5 Conclusion

In summary, follicles developing under conditions of low circulating LH (the Transitional period and Dioestrus) showed low steroidogenic activity as well as reduced expression of different genes associated with follicle maturation in comparison with naturally ovulatory (Oestrous) follicles. Furthermore, different follicular fluid expression profiles were identified for miR-378, miR-202, miR-21 and miR-542 among different types of follicles (Oestrous, Transitional, Dioestrous and Subordinate), which are consistent with a role of these miRNAs in follicle maturation and regression. Further research will be necessary to identify precise targets and involvement of these miRNAs during equine follicle development.

# Chapter 6

# Discussion and Conclusion

# Chapter 6: Discussion and Conclusion

## 6.1 Motivation and experimental approaches

The British horse racing industry contributes a cumulative impact of more than £3 billion to the British economy, of which over £200 million are spent on breeding (British Horseracing Authority 2009). A foal born in January/February has a significantly higher annual earning rate in flat races than foals born later in the year, with a decrease in earnings for each month, and this difference only seems to even out for horses over 5 years of age (Langlois and Blouin 1998). A main goal for breeders and veterinarians is to better understand the equine reproductive system so that broodmare management can be manipulated to allow foals to be born as early as possible in the year and improve mare fertility. Other objectives include developing methods for effective suppression of oestrous behaviour in mares during the racing season. But furthermore, the horse is an excellent model for many aspects of ovarian reproduction in humans including follicle development and maturation (Baerwald 2009) and reproductive aging (Carnevale 2008), processes which are physiologically very similar between the two species. The relatively large size of equine ovarian follicles allows easy manipulation including frequent transrectal ultrasound and follicular fluid sampling and injection of test substances. In addition, as the horse is a seasonal breeder, the natural occurring anovulatory period can be used as a model to study infertility in general.

The overall aim of the studies described herein was to elucidate the effects of luteinising hormone on follicle development and maturation. Circulating LH levels were manipulated by administration of an equine LH-rich pituitary fraction or PGF2α. Established methods were used for follicular monitoring and sampling as well as quantification of gonadotropin, steroid and growth factor levels in plasma and follicular fluid as well as miRNA levels in follicular fluid of mares (Gastal et al. 1997a; Donadeu and Ginther 2002a; Doyle et al. 2008; McBride et al. 2012), that had already been validated for the use with equine samples in our laboratory.

The ovarian puncture method used in these studies has been extensively used in our laboratory (Doyle et al. 2008) and elsewhere (Hinrichs et al. 1991; Gastal et al.

1997a; Vanderwall et al. 2006) for collection of a cell-free follicular fluid fraction. In addition to follicular fluid, we also collected the granulosa cell fraction. Based on visual inspection, the collected cell pellet was smaller and often contaminated with blood, especially in the case of preovulatory-size follicles and grossly contaminated samples were discarded. For all granulosa cell samples, mRNA quality was very variable and depended not on the size of the follicle but on sample processing batch. Also, mRNA quality increased as more experience was gained in sample handling and RNA extraction procedures. These factors led to low samples numbers which together with the inherent variability associated with mRNA quantification by qPCR, reduced the power of analyses, thus limited conclusions could be drawn from gene expression measurements in cell samples. Another alternative for collecting ovarian cell populations for analyses would have been ovariectomy, however this is a terminal procedure which would not have allowed performing the whole set of pre-planned experiments in this population of mares. In this regard, the use of a cross-over design within each experiment accounted for the within-mare variability. A third option would have been collecting follicles from ovaries obtained from a horse abattoir, however, there are only two such abattoirs in the UK and that would have involved transport of the samples for hours and working with animals of unknown reproductive status.

Two of the experiments described herein investigated the effects of LH supplementation on follicle development and ovulation during a period of naturally reduced LH (anovulatory season) or during the normal oestrous cycle, when LH levels are highest. So far, studies aimed at determining the effects of stimulation of follicle development with gonadotropins in mares have involved administration of GnRH (McCue et al. 1991), hCG (Bour and Palmer 1984), or pituitary fractions, which often contained high levels of both LH and FSH (Lapin and Ginther 1977). None of these approaches can adequately test the sole effects of LH on the ovary. GnRH treatments stimulate the release of both LH and FSH from the pituitary. Furthermore, high levels of FSH in pituitary preparations administered as a substitute for LH can produce confounding results as FSH supplementation can lead to rescue of future subordinate follicles from atresia leading to multiple ovulations (Logan et

al. 2007). Finally, administration of hCG, particularly when frequent, leads to the production of antibodies, which can quickly inhibit hCG bioactivity, making this approach unsuitable to study the effects of LH in vivo (Wilson et al. 1990). In this set of studies, the high LH to FSH ratio of the purified pituitary fraction used (crude equine gonadotropin, provided by collaborators at INRA, France) was preliminarily confirmed by determining its effects on circulating LH and FSH levels when injected into anoestrous and dioestrous mares. The LH bioactivity of this pituitary fraction had previously been shown by its ability to induce ovulation when administered into cycling mares (Hofferer et al. 1993).

## 6.2 Relevant findings

### 6.2.1 Effects of reduced LH levels on follicle development

The aim of the first study in Chapter 3 was to test the hypothesis that anovulatory transitional follicles develop under insufficient circulating LH levels and that supplementation of transitional mares with LH would restore follicular steroidogenic capacity, allowing normal ovulation. Findings from previous studies in mares (Donadeu and Ginther 2002b; Donadeu and Watson 2007), cattle (Gong et al. 1996) and sheep (McNatty et al. 1981; McNeilly et al. 1982) suggested the lack of LH rather than FSH was the cause for the stunted development of follicles during periods of transition between anovulatory and ovulatory stages including those associated with season and postpartum.

The main conclusion of the study was that supplementary circulating LH is needed during the anovulatory season for follicular growth to proceed to the stage of follicle deviation and for the dominant follicle to reach preovulatory size. However, the supplementation of early spring transitional follicles with LH was not able to reinstate the follicle's full ability for steroidogenesis, leaving eLH-stimulated dominant follicles devoid of oestradiol and unable to ovulate in response to hCG administration, except in limited instances. Yet, hCG is not only an agonist to LHR but when administered to mares hCG also stimulates LH secretion by the anterior pituitary (Ginther et al. 2009). In this study, an increase in endogenous LH secretion at the time of hCG injection was not detected, and because the pars tuberalis of the

anoestrous equine pituitary has been reported to contain a reduced proportion of LH secreting gonadotrophs (Eagle and Tortonese 2000), leading to significantly lower LH storage than during the breeding season (Hart et al. 1984), this, together with low follicular expression of LHR (Chapter 5) could potentially render ovulation induction with hCG in transitional mares less effective. Some mares that developed large transitional follicles (in the eLH- or saline group) did ovulate in response to hCG, however, the capacity of the resulting corpus luteum to produce progesterone was diminished, attesting to the immaturity of these follicles and consistent with data in other species (McNeilly et al. 1981; Bergfelt and Ginther 1992).

Given the results of the study described in Chapter 3, a better understanding of the reasons for reduced steroidogenic capacity in transitional follicles was sought in a subsequent study (Chapter 5) by determining the expression of steroidogenic enzymes and growth factor receptors in (anovulatory) follicles growing during periods of naturally reduced LH levels (spring transition and luteal phase) compared to ovulatory follicles growing during a normal follicular phase. Furthermore, levels of selected miRNAs which, based on previous reports, are potentially involved in regulating follicle development, were also determined in follicular fluid. The results of that study showed that consistent with the study in Chapter 3, steroidogenic enzyme levels were reduced in anovulatory dominant follicles developing during spring transition and dioestrus which was attributable at least partially to reduced levels of IGF1 as well as lower receptor mRNA expression levels for gonadotropins and IGF1. The lack of receptors in granulosa cells of transitional follicles presumably occurs in addition to the reported deficiencies in vascularisation and LHR expression in the theca layer of these follicles (Watson and Al-Zi'abi 2002). Considered in retrospect, supplementation with eLH earlier during follicle development in the initial study (Chapter 3), e.g., when the largest follicle of a transitional wave was 10 mm rather than 20 mm, may have stimulated theca development and steroidogenesis more effectively and potentially increased the number of ovulations in eLH-supplemented mares.

The findings above are consistent with previous data in transitional follicles (Watson et al. 2004b) and provide novel data for dioestrus follicles, for which there is very

limited information on their molecular characteristics (Vandeplassche et al. 1979). In addition, the results from Chapter 5 provide evidence of a potential role of miR-202, miR-378, miR-542 and miR-21 in equine follicle development. In particular, temporal interrelationship between steroid/steroidogenic enzyme levels and miRNA levels warrant further investigation of an involvement of miR-378 and miR-542 in regulating steroidogenesis in equine follicles, as reported in pigs (Xu et al. 2011). Two miRNAs, miR-202 and mir-21, were upregulated in transitional follicles, but not in dioestrous follicles, compared to large oestrous follicles, suggesting a role of these miRNAs in seasonally anovulatory follicles. An unexpected observation in Chapter 5 was that, in contrast to the results on circulating levels of oestradiol in transitional mares in Chapter 3 and previous data in transitional follicles (Watson et al. 2002b), oestradiol levels in follicular fluid were similar in transitional and oestrous follicles. However, intrafollicular levels of other key factors associated with follicle maturation, including progesterone and IGF1, were clearly deficient in transitional follicles, in agreement with previous reports (Watson et al. 2004b). The higher-than-expected oestradiol levels in transitional follicles in the study in Chapter 5 may be explained by the fact that the research ponies underwent progressive weight gain between the time they arrived at Roslin Institute's experimental farm (December 2008), shortly before the onset of the study described in Chapter 3 and the time the study described in Chapter 5 was performed (2011). This was a result of their progressive adaptation to a new environment including housing and feeding, factors which led to an improvement of their nutritional status and body condition, which is known to have a positive effect on follicular activity, particularly during the winter period (Gentry et al. 2002). Nevertheless, it is worth reiterating again that except for oestradiol levels, transitional follicles showed similar characteristics to dioestrous follicles, and both were clearly different to oestrus follicles, consistent with the hypothesis that reduced LH levels during spring transition and dioestrus have a limiting effect on follicle maturation.

### 6.2.2 Effects of LH overexposure on follicle development

The study described in Chapter 4 in this thesis was designed to examine the effects of excessive rather than deficient circulating LH levels on follicle maturation in mares. In other studies, elevated levels of luteinising hormone during follicular development caused anovulatory polycystic ovaries in mice (Risma et al. 1995), whereas the spontaneous development of follicular cysts in cows was also associated with elevated LH secretion (Cook et al. 1991). In mares it was reported that shortening the oestrous cycle by an injection of PGF2α caused a significant increase in the incidence of HAFs (Cuervo-Arango and Newcombe 2009), leading to the suggestion that this effect may be causally related to an early rise in circulating LH levels during follicle development induced by PGF2α treatment (Ginther et al. 2008b). In the study described in this thesis, LH supplementation was shown to provide an adequate model to investigate the effects of a PGF2α-induced rise in circulating LH on follicle maturation (Ginther et al. 2007b). A relevant finding in that experiment was that elevated exogenous LH did not disrupt follicular growth or ovulation, which did not support the initial hypothesis. Similarly, short-term treatment of cows with exogenous LH to experimentally induce follicular cysts did not impair ovulation (Hampton et al. 2003). These findings may indicate that either equine follicles are relatively resistant to the potentially deleterious effects of supra-physiological LH levels during development or that longer-term exposure to high LH, as in the above cited studies in mice, are necessary to induce defects in ovulation. Additionally, eCG which has LH-like effects but with much longer half-life than LH, may more readily induce the formation of anovulatory follicles in mares (Allen 1979; De Mestre, personal communication, 2011). Furthermore in this study (Chapter 4), elevated circulating levels of LH, whether induced by exogenous LH administration or by PGF2α stimulation of endogenous LH, during the early development of future dominant follicles were associated with clear changes in follicular fluid factors, mainly a reduction of androstenedione and PGE2 and an increase of IGF1, suggesting potential detrimental effects on follicle maturation and oocyte quality (Ginther et al. 2007c; Velazquez et al. 2009), which need to be investigated in the future.

## 6.3 Conclusions

The set of studies in this thesis have confirmed the importance of LH in the regulation of the equine oestrus cycle and have provided additional information in regard to its effects on follicle development, maturation and ovulation, which can be summarised as follows:

- LH is key for promoting follicle growth in mares, and a deficiency in LH is responsible for reduced follicle growth and/or maturation during spring transition and, putatively, during the luteal phase.

- However, although effective in stimulating follicle growth, short-term supplementation with LH is, by itself, not sufficient to restore steroidogenic activity of transitional follicles indicating more complex requirements of follicles for steroidogenesis than for growth. The relative lack of responsiveness of transitional follicles to LH supplementation is presumably due to reduced gonadotropin and growth factor receptor levels in these follicles.

- Immature transitional follicles can ovulate in response to hCG although they produce a steroidogenically deficient corpus luteum.

- Follicles developing under different LH milieus exhibit differences in miRNA expression and have altered steroidogenic capacity indicating potential roles of these miRNAs in follicle maturation.

- High circulating LH levels during follicular wave development do not disrupt ovulation but induce changes in intrafollicular fluid factors (androstenedione, IGF1, PGE2) consistent with effects on follicle maturation, and may impact oocyte quality and fertility.

## 6.4 Future work

Following from the encouraging results in relation to the response of early transitional follicles to LH treatment (Chapter 3), further studies should be undertaken to better elucidate the effects of exogenous LH during anoestrus in mares as well as examine the potential of treating follicles earlier during development (beginning at a size of 10 mm rather than 20 mm) for advancing the onset of the breeding season.

On the other hand it is common practice by veterinarians and breeders to manipulate the ovulatory cycle of mares by shortening the luteal phase through an injection of PGF2α and by inducing follicular growth and/or ovulation by an injection of hCG or by administration of GnRH analogues, which can lead to sustained, elevated high secretion of gonadotropins by the pituitary. All of these approaches are used for reproductive management not only of horses but also livestock without thorough consideration of their potential long term effects on follicle maturation and oocyte quality. Therefore, future studies will need to investigate the impact of induced high circulating LH levels on oocyte maturity and quality to clarify conflicting evidence on fertility following oestrous cycle manipulations of farm animals.

The novel results on changes in miRNA levels between different follicle types in Chapter 5 will provide the basis for further studies on the posttranscriptional regulation of gene expression during follicle maturation and the search for potential target genes and physiologically relevant roles of these miRNAs in the mare. Furthermore, these results also bring exciting prospects in relation to the potential of miRNAs as biomarkers of reproductive function in mares and other species (Brase et al. 2010) and as potential therapeutic targets for reproductive disorders (Ohlsson Teague et al. 2009; Heneghan et al. 2010).

# Chapter 7

# Literature cited

# Chapter 7: Literature cited


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