PITUITARY AND OVARIAN FUNCTION IN FEMALE CATTLE TREATED WITH DESLORELIN, AN AGONIST OF GONADOTROPHIN-RELEASING HORMONE

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Declaration

I declare that this thesis is my own work and that it has not been submitted in any form for another award at any other university or institution of tertiary education. All experimental results, discussion and conclusions drawn are the work of the author.

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Publications arising from this thesis

Pitcher, DJ, Aspden, WJ, Scott, PT, Rodgers, RJ, D'Occhio, MJ. (1997). Pituitary desensitisation, increased progesterone secretion and changes in corpus luteum weight and steroidogenic enzyme content in heifers treated with the GnRH agonist, deslorelin. *Proceedings of the 28th Annual Conference of the Australian Society for Reproductive Biology,* 29 September – 1 October, 1997, Abstract 28.

Acknowledgments

I would like to thank my supervisor, Professor Michael J. D'Occhio (Animal Sciences and Production Group, CQU), for his advice, support and direction throughout my Masters degree. I would also like to thank his co-supervisors, Dr Paul Scott (CQU) and Dr Ray Rodgers (Flinder's Medical Centre).

I would especially like to thank my co-workers in the Animal Sciences and Production Group: Mr Tim Whyte, Ms Lisa Maclellan and Dr William Aspden for their technical support in field experiments and their friendship and support throughout my Masters. Thanks also to the staff at *Belmont* National Cattle Breeding Station for their expert assistance with field experiments and management of heifers. I thank Dr Paul McKosker for performing the ovariectomies. I thank Mr and Mrs Fausto and Simona Cremonesi for their technical support in field experiments, particularly with ultrasound experiments. Thanks to Dr Tim Trigg and Mr J Walsh (Peptech Animal Health Pty Ltd) for providing the deslorelin implants. I thank Dr Douglas M. Stocco for his assistance with the Western Analyses of StAR protein. Thanks also to the members of Dr Ray Rodgers' lab for passing on their extensive experience in Western Analyses. Funding for this thesis was provided, in part, by a Meat Research Corporation grant and a CQU post-graduate research award.

I would like to take this opportunity to thank my parents, John and Janice Pitcher, and my brother, Darren, for their unconditional support, encouragement and faith throughout my studies. I could never have done this without them.

Dedication

This thesis is dedicated to my grandmother

Grace Margaret Pitcher

2 December 1909 - 4 February 1997

Abstract

Female cattle (heifers) treated with deslorelin, an agonist of gonadotrophin-releasing hormone (GnRH), typically show a biphasic gonadotrophin response to treatment. During the acute phase (0 to 24 h), plasma concentrations of luteinising hormone (LH) are elevated. The acute phase is followed by GnRH receptor downregulation in the anterior pituitary gland, and the pituitary becomes desensitised to natural sequence GnRH. Heifers treated chronically with deslorelin therefore do not have pulsatile secretion of LH. However, heifers treated chronically with deslorelin, commencing early in the oestrous cycle (Day 3), typically had elevated plasma concentrations of progesterone during the luteal phase compared with contemporary untreated heifers.

It is unlikely that increased plasma progesterone in heifers treated with deslorelin, commencing early in the oestrous cycle, is due to ovulation in response to the acute rise in plasma LH, and subsequent formation of an accessory corpus luteum. The increase in plasma progesterone in these treated heifers might be due, therefore, to changes in patterns of LH secretion, which are accompanied by changes in the size and/or function of the corpus luteum. Heifers treated with deslorelin commencing later in the oestrous cycle may be induced to ovulate and develop an accessory corpus luteum.

The aim in Experiments 1 and 2 of this thesis was to characterise LH secretion, and determine changes in size and steroidogenic activity of the corpus luteum, in heifers treated chronically with deslorelin, commencing on Day 3 of the oestrous cycle. Steroidogenic activity was ascertained by measuring tissue contents of progesterone, steroidogenic acute regulatory (StAR) protein and the key steroidogenic enzymes,

cytochrome P450scc (P450scc) and 3β-hydroxysteroid dehydrogenase, Δ^5-Δ^4 isomerase (3β-HSD). The recently described StAR protein facilitates the transport of cholesterol to the inner mitochondrial membrane of steroidogenic cells, where P450scc is localised. Transport of cholesterol to the inner mitochondrial membrane is considered the rate-limiting step in steroidogenesis in all steroidogenic tissues.

A further aim in the thesis was to determine the relationship between stage of the oestrous cycle (i.e. stage of the ovarian follicular wave) and the ability of deslorelin to induce ovulation during the acute LH response phase.

In Experiment 1, stage of the oestrous cycle was synchronised in randomly cycling Brahman heifers, using a standard progestagen treatment. On Day 3 of the ensuing oestrous cycle, heifers were assigned to: Control (n=9), no treatment; Deslorelin (n=9), implanted subcutaneously with GnRH agonist (deslorelin) bioimplants. Heifers treated with deslorelin received approximately 25 μg deslorelin/kg live weight/24 h).

Blood samples for LH and progesterone analyses were taken immediately before implanting (Day 0) and 6 hours later, and on Days 1, 3, 5, 8, 9, 10. On Day 10 of treatment (Day 13 of the oestrous cycle), all heifers were ovariectomised and the corpus luteum dissected from the ovary and weighed. Immediately after ovariectomy, all heifers received an injection of natural sequence GnRH (50 μg i.m.) and blood samples were taken at 0 and 30 min to determine pituitary LH release. Progesterone content of the corpus luteum was determined by radioimmunoassay. Contents of StAR protein, P450scc, and 3β-HSD were determined using Western Blot analyses.

Experiment 2 was similar in design to Experiment 1, except that heifers treated with deslorelin received approximately 50 μg deslorelin/kg live weight/24 h. Also, in Experiment 2, heifers were bled on Days 1, 3, 5, 7, 9, 10.

The trends in the treatment responses were similar for Experiments 1 and 2 and pooled data are presented below.

Treatment with deslorelin caused an acute increase in the plasma concentration of LH and, at 6h after implanting, treated heifers had greater ($P<0.01$) plasma LH (1.4 $\pm$ 0.1 ng/ml) than control heifers (0.6 $\pm$ 0.1 ng/ml). Plasma LH in implanted heifers had declined by Day 1; however, mean LH from Day 1 to 10 of treatment was greater ($P<0.01$) for implanted heifers (0.8 $\pm$ 0.1 ng/ml) than control heifers (0.5 $\pm$ 0.1 ng/ml). On Day 10, control heifers had an increase ($P<0.01$) in plasma LH after injection of GnRH (ΔLH 1.73 $\pm$ 0.23 ng/ml), but this did not occur in heifers implanted with deslorelin (ΔLH 0.11 $\pm$ 0.08 ng/ml).

Plasma progesterone concentrations increased from Day 0 to Day 10 for both control heifers and heifers treated with deslorelin. On Day 10, plasma progesterone was greater ($P<0.01$) for heifers implanted with deslorelin (18.9 $\pm$ 3.5 ng/ml) than control heifers (9.1 $\pm$ 1.3 ng/ml).

Corpus luteum weight was greater ($P<0.05$) for implanted (4.2 $\pm$ 0.4 g) than control (3.1 $\pm$ 0.2 g) heifers. The amount of progesterone per corpus luteum was also greater ($P<0.01$) for treated heifers (164 $\pm$ 20 μg per corpus luteum) than control heifers (88 $\pm$ 13 μg per corpus luteum).

The amount of StAR protein per total corpus luteum was greater ($P<0.05$) for heifers treated with deslorelin (2.2 $\pm$ 0.3 arbitrary units) than control heifers (1.2 $\pm$ 0.2 arbitrary units). The relative content of StAR protein per unit weight of corpus luteum was greater in treated heifers (1.8 $\pm$ 0.3 arbitrary units/g) compared with control heifers (1.5 $\pm$ 0.1 arbitrary units/g), but this difference was not significant.

The relative content of P450scc per total corpus luteum was greater ($P<0.01$) in treated heifers (2.2 ± 0.2 arbitrary units) compared with control heifers (1.4 ± 0.2 arbitrary units). The relative content of P450scc per unit weight corpus luteum was also greater ($P<0.05$) in treated heifers (1.9 ± 0.2 arbitrary units/g) compared with control heifers (1.2 ± 0.1 arbitrary units/g).

Relative content of 3β-HSD per unit weight of corpus luteum tissue tended to be greater (Experiment 1: $P=0.09$; Experiment 2: $P=0.72$) in heifers treated with deslorelin (1.57 ± 0.15 arbitrary units/g) than in control heifers (1.49 ± 0.15 arbitrary units/g) but this was not significant. Relative content of 3β-HSD per total corpus luteum likewise tended to be greater ($P=0.20$) in heifers treated with deslorelin (1.91 ± 0.25 arbitrary units) than in control heifers (1.51 ± 0.28 arbitrary units), but this also was not significant.

In Experiment 3, stage of the oestrous cycle was synchronised in Brahman heifers, which were in random stages of the oestrous cycle, using a standard progestagen treatment. Heifers were then assigned to: Control (n=4), no treatment; or implanted with deslorelin (50 μg/kg live weight/24 h) on different days of the oestrous cycle: D2 (n=4) implanted on Day 2; D4 (n=4) implanted on Day 4; D6 (n=4) implanted on Day 6; or D8 (n=4), implanted on Day 8 of the oestrous cycle. Ovarian follicle and corpus luteum status were monitored using ultrasonography for approximately 40 days.

Heifers treated with deslorelin commencing on Day 2 of the oestrous cycle had follicles with a maximum diameter of 4.7 ± 0.3 mm at the time of treatment and did not ovulate or develop an accessory corpus luteum. The diameter of the largest follicle on Day 4 of the oestrous cycle was 6.5 ± 0.7 mm and two of four heifers ovulated and

developed an accessory corpus luteum. The diameters of the two follicles that ovulated were 5.0 and 6.0 mm, while those that did not ovulate were 6.0 and 7.0 mm. On Day 6 of the oestrous cycle, each of the heifers had a 10 mm follicle that ovulated and developed an accessory corpus luteum. Heifers treated with deslorelin on Day 8 of the oestrous cycle had a relatively large follicle (8.63 ± 0.94 mm), but no heifers in this group ovulated.

In 4 of 6 heifers that ovulated and developed an accessory corpus luteum, the spontaneous corpus luteum persisted for 11 to 19 days longer than the spontaneous corpus luteum in control heifers, even after the accessory corpus luteum had regressed. The induced corpus luteum may have increased $PGF_{2\alpha}$ receptors, or increased numbers of large luteal cells in the corpus luteum. Also, the presence of two corpora lutea might have produced sufficient oxytocin to cause downregulation of endometrial oxytocin receptors.

The acute increase in plasma concentration of LH subsequent to implantation of heifers with deslorelin was consistent with previous findings. The rise in plasma LH was sufficient to induce ovulation of follicles of relatively small diameter (5 to 6 mm). This observation indicated that LH receptors sufficient for a response to a preovulatory LH surge are expressed in 6-7 mm follicles in Brahman (*Bos indicus*) heifers.

Basal concentrations of plasma LH were consistently greater in heifers receiving deslorelin compared with untreated heifers. Increased basal LH in heifers treated with deslorelin was associated with greater concentrations of plasma progesterone. Because heifers treated with deslorelin would not have pulsatile secretion of LH, it could be concluded that basal secretion of LH is an important determinant of corpus luteum function and progesterone secretion in cattle. Heifers treated with deslorelin also had a

larger corpus luteum and increased corpus luteum contents of StAR protein and P450scc. The latter findings are the first demonstration of concomitant increases in progesterone secretion, size of the corpus luteum and steroidogenic activity of luteal cells in a female mammal treated with GnRH agonist.

Progesterone is required for the establishment and maintenance of pregnancy in cattle, and it is possible that treatment with a GnRH agonist may stimulate increased progesterone secretion sufficient to enhance conceptions to artificial insemination and embryo transfer in cattle.

Chapter 1: Introduction and Literature Review

1.0 Introduction

Gonadotrophin releasing hormone (GnRH) has a critical role in reproductive processes. Released from the basal hypothalamus, GnRH stimulates the gonadotrophe cells of the anterior pituitary gland to secrete luteinising hormone (LH) and follicle-stimulating hormone (FSH), which are required for normal ovarian function including follicular growth, maturation and ovulation.

Agonists of GnRH have been developed with a greater resistance to enzymatic degradation and an increased affinity for the GnRH receptor (Karten and Rivier, 1986). The GnRH agonists induce a biphasic response from the anterior pituitary gland. The acute phase, which in cattle lasts approximately 24 hours, consists of an increase in plasma concentrations of LH. This is followed by the chronic phase in which pulsatile secretion of LH is suppressed (Rettmer *et al.*, 1992b; Gong *et al.*, 1995, 1996).

In most mammals studied, basal plasma concentrations of LH are suppressed during the chronic phase of treatment with a GnRH agonist, whereas in heifers basal LH can be increased during the chronic phase of treatment (D'Occhio *et al.*, 1996; Gong *et al.*, 1995; Evans and Rawlings, 1994; Maclellan *et al.*, 1997). Increased basal concentrations of plasma LH during the chronic phase of GnRH agonist treatment have been associated with increased secretion of progesterone by the corpus luteum in heifers (Milvae *et al.*, 1984; Macmillan *et al.*, 1985; Bergfeld *et al.*, 1996; Gong *et al.*, 1996; Rajamahendran *et al.*, 1996, 1999, Ambrose *et al.*, 1999). Increased plasma progesterone in heifers treated with GnRH agonist may have resulted from an induced ovulation and formation of an accessory corpus luteum, or from increased steroidogenic

activity of the existing corpus luteum.

The small and large luteal cells of the corpus luteum are responsible for the production of progesterone. Cholesterol is converted to pregnenolone by cytochrome P450 side chain cleavage enzyme (P450scc), which is localised to the inner mitochondrial membrane. Transport of cholesterol from the cytoplasm to the inner mitochondrial membrane is the rate-limiting step in steroidogenesis, and is mediated by steroidogenic acute regulatory (StAR) protein. Pregnenolone is then converted to progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). Because StAR protein mediates the rate-limiting step in progesterone biosynthesis, increased progesterone secretion by the corpus luteum may be associated with increased amounts of StAR protein in the corpus luteum.

One objective in this thesis was to characterise corpus luteum function in heifers treated chronically with a GnRH agonist, commencing early in the oestrous cycle, to determine the mechanisms associated with increased progesterone secretion. Observations included corpus luteum size and amounts of StAR protein, P450scc enzyme (protein) and 3β-HSD (protein).

In cattle, each oestrous cycles consists of two or three waves of follicular growth. In an oestrous cycle which has three waves of follicular growth, the first and second dominant follicles usually develop in the presence of a corpus luteum and do not ovulate. However, the first and second wave dominant follicles can be induced to ovulate by injecting prostaglandin to induce luteolysis of the corpus luteum, or by gonadotrophin injection (e.g. LH, hCG).

A second objective in this thesis was to determine the relationships between the LH response during the acute phase of treatment with a GnRH agonist, size of the

largest follicle at initiation of treatment, ovulation, and plasma concentrations of progesterone during the subsequent luteal phase. Accordingly, treatment with a GnRH agonist was initiated at different stages during the first follicular wave of the oestrous cycle.

1.1 Overview of the Hypothalamic-Pituitary-Ovarian Axis

Endocrine regulation of reproductive function in female cattle (heifers) occurs through the hypothalamic-pituitary-ovarian axis. The hypothalamus, situated at the base of the brain, secretes GnRH which stimulates the anterior pituitary gland to release the gonadotrophins, LH and FSH. The pituitary gland is formed by the fusion of two tissues from different embryological origins (Berne and Levy, 1990). The neurohypophysis (posterior pituitary gland) develops as a downward outpocketing of neuroectoderm from brain tissue; this differentiates into the neurons of the hypothalamus. The lowest part of this downward-growing neural stalk forms the bulk of the neurohypophysis, while the upper part expands to form the median eminence. The adenohypophysis (anterior pituitary gland) develops from an upward outpocketing of ectoderm from the floor of the oral cavity; the adenohypophysis becomes separated from the mouth by the sphenoid bone of the skull (Berne and Levy).

The median eminence is vascularised by the superior hypophyseal artery (and to a lesser extent, the inferior hypophyseal artery) (Berne and Levy, 1994). Hypothalamic neurons are the source of releasing and inhibiting neurohormones that regulate adenohypophysis function. The capillary plexus of the median eminence forms a set of portal veins that descend into the adenohypophysis. The portal veins give rise to a second fenestrated capillary plexus, which serves two roles. Hypothalamic releasing and inhibiting hormones carried from the median eminence exit the second plexus and

regulate secretion from respective cells of the adenohypophysis (Berne and Levy, 1994). The trophic hormones of these cells enter the same capillary plexus and are delivered, via the general circulation, to target organs.

1.2 GnRH and GnRH agonist structure

GnRH, also termed luteinising hormone-releasing hormone (LHRH), is a decapeptide neurohormone that has a critical role in reproductive function (Figure 1.1; Matsuo *et al.*, 1971; Burgus *et al.*, 1972)). GnRH has a short half-life in circulation (2-5 minutes, Candas *et al.*, 1990) and is metabolised and cleared rapidly after endogenous release for the hypothalamus, or after exogenous injection. Both the NH_2- and COOH-terminal domains of mammalian GnRH are involved in receptor binding, while the NH_2-terminal domain is important for receptor activation (Millar *et al.*, 1987; Karten and Rivier, 1986). It is considered that $pGlu^1$, His^2 and Trp^3 may be involved in receptor activation (Figure 1.1). The Arg^8 is associated with high affinity binding of GnRH to its receptor (Figure 1.1).

GnRH agonists have been developed that have greater affinity for the GnRH receptor and increased resistance to proteolytic degradation (Karten and Rivier, 1986). Replacement of the Gly^{10} amine terminus with alkyl amides produced a nonapeptide alkyl amide agonist that induced release of LH and ovulation with greater potency than GnRH (Fujino *et al.*, 1972). Also in the latter study, it was concluded that the terminal Gly^{10} amide was not essential for GnRH activity, and that the total chain length of the peptide was important for receptor binding. Natural sequence GnRH is proteolytically cleaved at Gly^6. Monahan *et al.* (1973) demonstrated that replacement of the Gly^6 residue with D-alanine produced a peptide with potency approximately 350-450% greater than that of GnRH. The corresponding L-$alanine^6$ derivative had only 4% of the

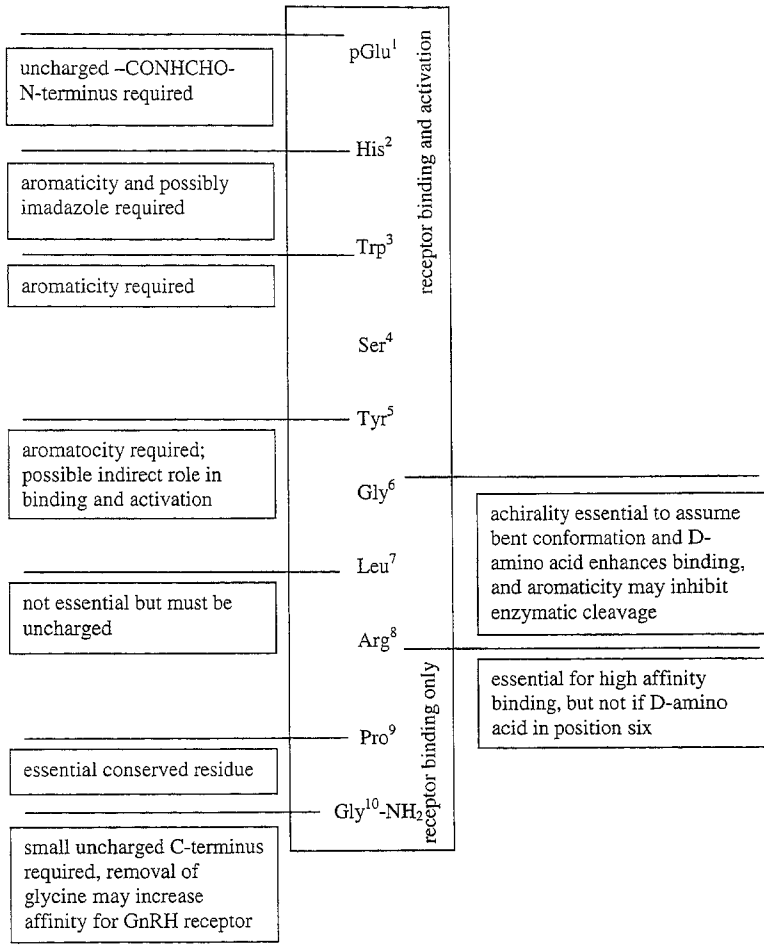


Figure 1.1: Schematic of GnRH, summarising functional properties of individual amino acid residues (adapted from Karten and Rivier, 1986; Sealfon *et al.*, 1997).

potency of GnRH. It was concluded that the increased biological potency of GnRH agonists with D-amino acid substitutions at position 6 resulted from the conformational stabilising effect of D-amino acids, which aids in resistance to proteolytic cleavage (Karten and Rivier, 1986).

1.2.1 Deslorelin

Deslorelin [D-Trp^6-Pro^9-Net-GnRH], the GnRH agonist used exclusively in this study has a D-tryptophan replacing Gly^6 (the site of enzymatic cleavage), and Gly^{10} has been removed and replaced with ethylamide (Net), enhancing the affinity of deslorelin for the GnRH receptor. The NEt residue protects the peptide from postproline-cleaving enzyme activity (Karten and Rivier, 1986). Hence, deslorelin has a greater resistance to enzymatic cleavage and a higher affinity for the GnRH receptor than natural sequence GnRH (approximately 144 times the *in vitro* potency: Karten and Rivier, 1986). The half-life of deslorelin in humans is increased 10-fold compared with natural sequence GnRH (Karten and Rivier, 1986).

1.2.2 GnRH receptor and binding

GnRH binds exclusively to gonadotrophe cells of the anterior pituitary gland (Hazum and Conn, 1988). The action of GnRH is mediated by its specific receptor, which is localised to the plasma membrane of gonadotrophe cells (Figure 1.2). The receptor is a member of the family of 7-transmembrane G-protein coupled receptors (Sealfon *et al.*, 1997). The bovine GnRH receptor cDNA contains 1,326 nucleotides, which code for a protein containing 328 amino acids (Kakar *et al.*, 1993). The bovine GnRH receptor contains cysteine residues in the first and second intracellular loops, which are reported to be essential for stabilising binding of GnRH to the receptor (Dohlman *et al.*, 1991). The bovine GnRH receptor has 91% homology with the human

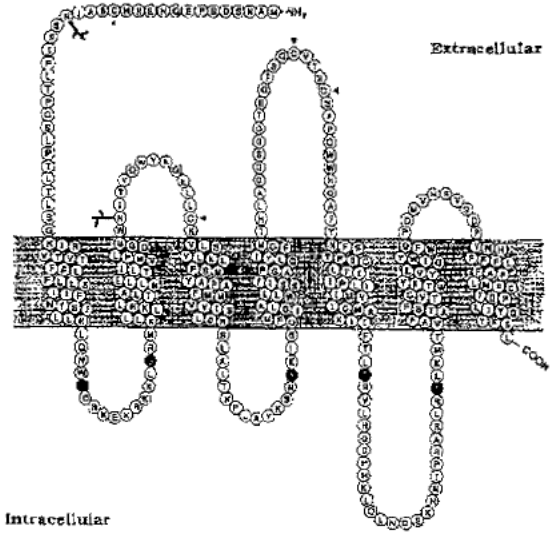


Figure 1-2 Schematic representation of the GnRH receptor, a member of the seven-transmembrane G-protein coupled receptor family. Glycosylation sites are shown (Y). (adapted from: Kakar *et al.*, 1992).

GnRH receptor and 86% homology with the rat and mouse GnRH receptor (Kakar *et al.*, 1992).

1.2.3 GnRH receptor downregulation

Following binding of GnRH to its receptor, GnRH receptor complexes dimerise and form clusters that are subsequently internalised through coated pits and vesicles (Hazum and Conn, 1988). Proteolytic degradation follows, leading to reduced receptor number, while secretory granules containing LH and FSH fuse with the plasma membrane of gonadotrophe cells and release their contents (Hazum and Conn, 1988; Kaiser *et al.*, 1997). Downregulation is a reduction in the number of GnRH receptors on the surface of gonadotrophe cells, which occurs as a result of internalisation of the receptor-GnRH complex (Conn and Hazum, 1981; Nett *et al.*, 1981). After LH and FSH release, upregulation of GnRH receptors occurs (Hazum and Conn, 1988).

After binding to the GnRH receptor, GnRH agonists cause microaggregation and internalisation of the receptor, which results in insensitivity of the gonadotrophe cell to further GnRH stimulation (Conn and Hazum, 1981). Receptor replenishment does not occur during continued exposure to GnRH agonist (Huckle et al., 1988; Hawes *et al.*, 1992).

1.2.4 GnRH signal transduction

Intra- and extra-cellular Ca^{2+} is necessary but not sufficient to mediate GnRH action (Naor, 1986; Chang *et al.*, 1988; Gilman, 1987). When GnRH binds to its receptor, it stimulates phosphoinositide turnover, Ca^{2+} mobilisation, protein kinase C (PKC) activation, arachadonic acid release and activation of membrane-bound adenylate cyclase. Adenylate cyclase catalyses the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), a second messenger (Stryer, 1995). Cyclic

AMP activates serine/threonine specific protein kinases (Naor, 1990).

Mammalian GnRH receptors are the only G-protein coupled receptors that lack the entire intracellular C-terminal domain. The cytoplasmic C-terminal of 7-transmembrane receptors is a region that is important for desensitisation of the receptor (Hausdorf *et al.*, 1990). Since this region is missing in the GnRH receptor, it follows that desensitisation of the GnRH receptor is regulated through different mechanisms, perhaps through interference with second messenger pathways.

1.2.5 GnRH-like proteins

GnRH-like proteins have a direct anti-gonadotrophic effect on luteal and granulosa cells in the rat, probably mediated by high-affinity GnRH receptor sites in the ovaries (Hsueh and Jones, 1981). GnRH-like proteins have been isolated from human, rat, ovine and bovine ovaries and are known to have anti-gonadotrophic effects in rats (Aten *et al.*, 1986, 1987a,b).

In the bovine, GnRH-like proteins were localised to granulosa cells, and to a lesser extent to luteal cells (Ireland *et al.*, 1988). The GnRH-like proteins were present in large amounts in follicles; however the concentration of GnRH-like proteins decreased markedly during luteinisation of the follicle (Ireland *et al.*, 1988). Concentrations of luteal GnRH-like proteins were greater in developing corpora lutea compared with mature or regressed corpora lutea (Ireland *et al.*, 1988). Because GnRH-like proteins have an anti-LH effect, greater concentrations of plasma LH in the late-luteal phase may be associated with small amounts of GnRH-like proteins (Ireland *et al.*, 1988). In cattle, GnRH receptors could not be detected in the hypothalamus, testes, pancreas, spleen, hippocampus, liver, adrenals, kidney, myometrium, corpus luteum and ovarian follicle (Brown and Reeves, 1983; Kakar *et al.*, 1993). While GnRH agonists

may not function directly at the ovaries, they may regulate amounts of GnRH-like proteins in the ovaries and in turn regulate LH secretion.

1.2.6 GnRH secretion

During the luteal phase of the oestrous cycle in the sheep, GnRH is released in discrete pulses, at relatively low frequency, because progesterone inhibits GnRH secretion at luteal phase concentrations (Clark *et al.*, 1987). During the early follicular phase, GnRH secretion increases as progesterone declines (Clark *et al.*, 1987). Karsch *et al.*, (1992) monitored GnRH secretion throughout the oestrous cycle in ewes and found that GnRH pulse frequency increased from approximately 1 pulse per 4 hours in the luteal phase, to approximately 1 pulse per 45 minutes in the follicular phase (Figure 1.3). Karsch *et al.* (1992) demonstrated that the GnRH surge was initiated by the preovulatory increase in 17β-oestradiol (oestradiol) secreted by the growing dominant follicle. It is the rising frequency and amplitude of pulsatile secretion of GnRH that induces the preovulatory LH surge. Because pulsatile LH release has been shown to occur as a result of pulsatile GnRH release in ewes (Karsch *et al.*, 1987; Clarke and Cummins, 1987), it has been inferred that pulsatile GnRH release causes pulsatile LH release in heifers. In young bulls, each pulse of GnRH stimulated the release of a pulse of LH, which in turn stimulated a pulse of testosterone (Rodriguez and Wise, 1989).

In a recent study, pulsatile secretion of GnRH into the third-ventricle of the brain was demonstrated in ovariectomised mature cows (Gazal *et al.*, 1998). This was the first direct evaluation of the hypothalamic GnRH pulse generator in mature cows. Pulses of GnRH were closely related to pulses of LH in the peripheral circulation in ewes and heifers (Skinner *et al.*, 1997; Gazal *et al.*, 1998).

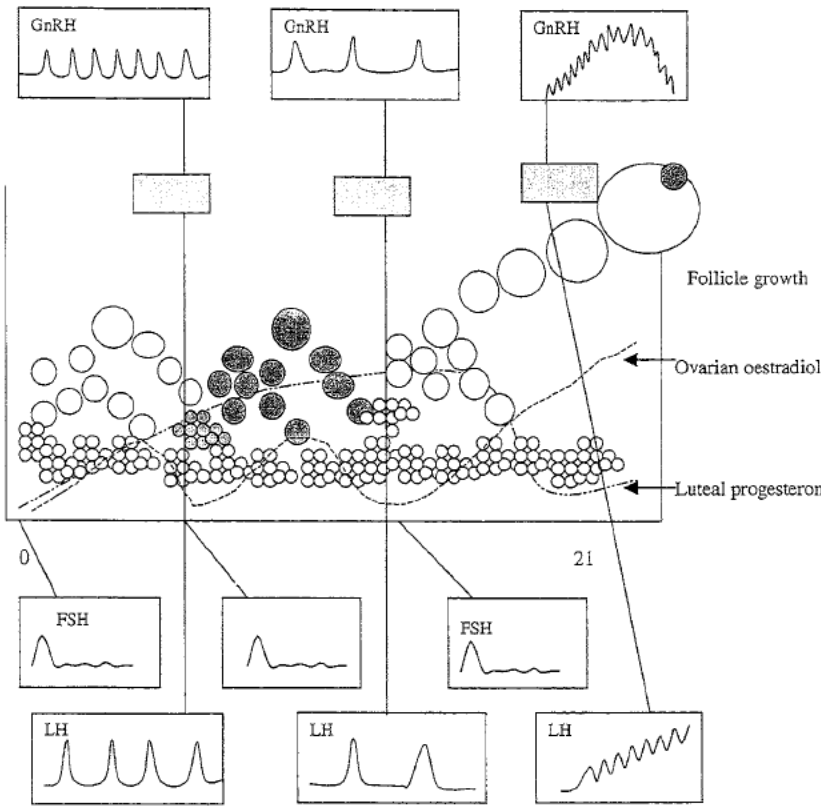


Figure 1.3 Schematic representation of major events during the bovine oestrous cycle. In each follicular wave, a 'cohort' of follicles is selected from a pool of small antral follicles, to grow in response to a transient increase in FSH. One follicle becomes dominant, secreting increased amounts of oestradiol. If this wave of growth occurs in the luteal phase (when progesterone is high), then the dominant follicle undergoes atresia and a new cohort of follicles is selected. If the dominant follicle is present in the follicular phase when progesterone is low, the dominant follicle will ovulate in response to the preovulatory LH surge, which occurs in response to a surge in GnRH.

1.3 Structures of LH and FSH

Luteinising hormone (LH) and follicle-stimulating hormone (FSH) are glycoproteins, each with a molecular weight of approximately 30kDa (Segaloff and Ascoli, 1992). Both LH and FSH consist of two covalently-linked peptide chains: the α- and β-chains. Both the α- and β-chains are required for activity (Baenziger *et al.*, 1988). The α-chains of LH and FSH are identical, whereas the β-chains are specific for each hormone, thus determining the respective biological specificity. Both LH and FSH are synthesised by the gonadotrophe cells of the anterior pituitary gland (Peters, 1985a). In cattle, the half-lives of LH and FSH are around 35 and 300 minutes, respectively (Schams and Karg, 1969; Laster, 1972).

1.3.1 Receptors for LH and FSH

Both LH and FSH receptors are members of a unique subclass of 7-transmembrane–spanning receptors that possess a large extracellular hormone-binding region and are coupled to adenylyl cyclase and phospholipase-C (Segaloff and Ascoli, 1992; Richards, 1994).

The LH receptor is composed of a single polypeptide with a molecular weight of 93kDa (Ascoli and Segaloff, 1989). The extracellular domain of the LH receptor is entirely responsible for high affinity binding of LH (Segaloff and Ascoli, 1992). This is in contrast to other 7-transmembrane G-protein coupled receptors, which have relatively small amino-terminal extracellular domains and in which the ligands interact directly with the amino acids within the transmembrane helices (Segaloff and Ascoli, 1992). Binding of LH to its receptor results in coupling of the inner receptor domain to a G-protein (Herrlich *et al.*, 1996). This causes activation of adenylate cyclase and subsequent production of cAMP and activation of inositol phosphate/calcium pathways

which in turn leads to the stimulation of steroidogenesis (Dufau, 1988; Milvae *et al.*, 1996).

Messenger RNA encoding LH receptor is first detectable in theca interna cells of small antral follicles shortly after antrum formation, and increases linearly with size of the follicle in heifers; in granulosa cells of *Bos taurus* genotype heifers, LH receptor mRNA was only detected in growing follicles larger than 9mm in diameter (Xu *et al.*, 1995a). Both FSH and oestradiol are required to induce expression of LH receptors in granulosa cells (Richards, 1980; Segaloff *et al.*, 1990).

As with the LH receptor, the extracellular domain is important for FSH binding (Simoni *et al.*, 1997). Binding of FSH to its receptor causes coupling of the receptor to its G-protein and subsequent activation of the PKA pathway (Simoni *et al.*, 1997). Continued exposure to FSH leads to uncoupling, downregulation and desensitisation of the FSH receptor (Simoni *et al.*, 1997). The FSH receptor has been localised exclusively to the granulosa cells (Tisdall *et al.*, 1995; Zheng *et al.*, 1996). In *Bos taurus* genotype heifers, FSH receptor mRNA was localised to the granulosa cells of small, growing follicles (i.e. shortly after recruitment from the primordial pool) (Xu *et al.*, 1995a).

1.3.2 LH secretion

Patterns of LH secretion fluctuate throughout the oestrous cycle in heifers (Cupp et al., 1995) (Figure 1.3). Rahe *et al.* (1980) demonstrated that approximately one pulse of LH was released per hour in the early luteal phase, compared with one pulse every three hours in the mid-luteal phase (Figure 1.3) (Walters *et al.*, 1984). The LH pulse frequency is decreased and LH pulse amplitude is increased in the mid-luteal phase (Day 10-11) compared with the early luteal phase (Day 3) (Rahe *et al*, 1980) (Figure 1.3). Peters (1985b) also reported that LH pulse frequency is low (2-3 pulses /

8hr) during the mid-luteal phase. During the late-luteal phase, LH pulse amplitude is low, with increasing frequency, culminating in the preovulatory LH surge (Rahe *et al.*, 1980). Cupp and co-workers (1995) reported fluctuations in the amplitude of LH pulses in female cattle between Days 16 and 19, due to declining progesterone. Cupp et al. (1995) also demonstrated fluctuations in LH pulse amplitude between Days 7 and 12. This suggests that between Days 7 and 12, LH pulse amplitude is regulated by the development and/or regression of dominant ovarian follicles (Cupp et al., 1995).

1.3.2.1 Effects of ovarian steroids on LH secretion

In the early luteal phase, rising plasma concentrations of progesterone secreted by the developing corpus luteum exert negative feedback on the hypothalamus to decrease GnRH, and hence LH, pulse frequency (Clark *et al.*, 1987). As plasma concentrations of oestradiol increase with the growth of the first wave dominant follicle, oestradiol, in concert with progesterone, exerts negative feedback on the hypothalamo-pituitary axis, decreasing LH pulse amplitude and frequency (McNatty *et al.*, 1984; Price and Webb, 1988). Price and Webb (1988) demonstrated that progesterone alone could not suppress LH pulse amplitude but did suppress LH pulse frequency. Progesterone also enhanced the suppressive action of oestradiol on LH pulse amplitude while oestradiol alone had no effect on LH pulse frequency but suppressed LH pulse amplitude (Price and Webb, 1988).

In the late luteal phase, when the corpus luteum regresses and plasma concentrations of progesterone decline, the negative feedback effect of progesterone on the hypothalamus is substantially reduced and LH pulse frequency increases, stimulating follicle maturation and increased synthesis of oestradiol. The positive feedback effect of oestradiol on the hypothalamo-pituitary axis (McNatty *et al.*, 1984; Prado *et al.*, 1990;

Roche *et al.*, 1992) and the withdrawal of the negative feedback effect of progesterone on the hypothalamus combine to increase GnRH and LH secretion to the extent that the preovulatory LH surge occurs, inducing ovulation (Figure 1.3).

1.3.3 FSH secretion

The preovulatory surge of GnRH elicits corresponding surges in plasma concentrations of LH and FSH. In heifers, this is followed 0.5 to 1.2 days after oestrus by a transient increase in plasma FSH in cattle (Cooke *et al.*, 1997). The secondary transient increase in plasma FSH precedes the emergence of the first follicular wave of the oestrous cycle (Figure 1.3).

Turzillo and Fortune (1990) showed that suppression of the secondary increase in plasma FSH in heifers delayed the emergence of the first follicular wave and growth of the first-wave dominant follicle, but it had no effect on LH, progesterone or oestrous cycle length. This indicated that the secondary transient increase in FSH induces the emergence of a new follicular wave subsequent to the preovulatory LH/FSH surge. Kastelic *et al.* (1990a) demonstrated that suppressing FSH by treating heifers with follicular fluid caused suppression of dominant and subordinate follicle growth. Adams *et al.* (1992) subsequently confirmed that an increase in plasma concentration of FSH precedes the emergence of a new follicular wave by 1 to 2 days, and that FSH decreases just after selection of the dominant follicle in heifers. Sunderland *et al.* (1994) also confirmed the occurrence of a transient increase in plasma concentrations of FSH, which precedes the recruitment of a new follicular wave.

1.3.3.1 Effects of oestradiol, inhibin, activin and follistatin on FSH secretion

In heifers, inhibin (consisting of two dissimilar subunits, α and β) is produced in the granulosa cells (Knight *et al.*, 1990). Inhibin finctions at the anterior pituitary

gland to suppress FSH synthesis (de Jong and Sharp, 1976; Kaneko *et al.*, 1993). Taya *et al.* (1991) reported an inverse relationship between inhibin and FSH in the follicular and early luteal phases in cows. Immunoneutralisation of endogenous inhibin during the early luteal phase (Kaneko *et al.*, 1997) and during the mid-luteal phase (Kaneko *et al.*, 1993) resulted in increased plasma concentrations of FSH and oestradiol in cows, followed by a faster follicular growth rate. A negative correlation also exists between plasma concentrations of oestradiol and FSH in the follicular phase of the bovine oestrous cycle (Sunderland *et al.*, 1994). It is thought that oestradiol acts synergistically with inhibin to suppress FSH production (Kaneko *et al.*, 1995).

Activin (consisting of two inhibin β subunits) is also produced by the granulosa cells in heifers and has FSH-stimulating activity (Wrathall and Knight, 1995). Follistatin, a specific binding protein for activin, is produced by the granulosa cells and suppresses FSH secretion (Ueno *et al.*, 1987). The effects of follistatin are mediated primarily through its ability to neutralise activin (Nakamura et al., 1990). Messenger RNA expression of follistatin increases as follicle size increases. (Shukovski *et al.*, 1992).

1.4 Follicle morphology

Erickson (1966) estimated that approximately 130,000 primordial follicles are present in the bovine ovary at the time of birth. McNatty *et al.* (1998) recently described a classification system for small bovine follicles:

Type 1. *primordial follicle* – oocyte surrounded by one layer of 'flattened granulosa cells;

Type 1a. *transitory follicle* – oocyte surrounded by one layer of flattened and cuboidal granulosa cells (Van Wezel and Rodgers, 1996);

Type 2 *primary follicle* – oocyte surrounded by one or two layers of cuboidal granulosa cells;

Type 3 *small preantral follicle* – oocyte surrounded by two or three layers of granulosa cells;

Type 4 *large preantral follicle* – oocyte surrounded by four, five or six layers of granulosa cells;

Type 5 *small antral follicle* – oocyte surrounded by more than five layers of granulosa cells.

Messenger RNA for FSH receptor is expressed in bovine granulosa cells at the Type 2-3 stage of development (Xu *et al.*, 1995a). At the Type 3-4 stage, granulosa cells are FSH-responsive *in vitro* (McNatty *et al.*, 1992; Boland *et al.*, 1993). In the cow, theca interna cells develop in Type 4 follicles (Lundy *et al.*, 1998). Messenger RNA for LH in theca interna cells is expressed in Type 4-5 preantral follicles (Xu *et al.*, 1995a).

1.4.1 Follicular dynamics during the bovine oestrous cycle

The length of the bovine oestrous cycle varies from 17-24 days. The life span of the corpus luteum dictates the length of the cycle and therefore the number of follicular waves in the cycle. A follicular wave is characterised by periods of recruitment, selection, dominance and regression of follicles (Savio *et al.*, 1988; Sirois and Fortune, 1988; Ginther *et al.*, 1989; Lucy *et al.*, 1992). Generally, oestrous cycles in cattle consist of two or three follicular waves. However, cycles with one and four waves of follicular growth also occur (Savio *et al.*, 1988; Sirois and Fortune, 1988; Murphy *et al.*, 1990; Rhodes *et al.*, 1994; 1995a; Figueiredo *et al.*, 1996).

In short oestrous cycles (8-12 days), the first wave dominant follicle is the

ovulatory follicle (Savio *et al.*, 1990a). In contrast, if the corpus luteum has a long lifespan, a fourth wave of follicular growth can occur, and the fourth wave dominant follicle is the ovulatory follicle. In a two wave cycle, the emergence of the first and second follicular waves occurs on Days 2 and 11 (Savio *et al.*, 1988; Sirois and Fortune, 1988; Pierson and Ginther, 1988; Ginther *et al.*, 1989; Knopf *et al.*, 1989; Taylor and Rajamahendran, 1991). In a three wave cycle, follicular waves emerge on Days 2, 9 and 16 (Savio *et al.*, 1988; Sirois and Fortune, 1988, Bergelft *et al.*, 1991; Taylor and Rajamahendran, 1991b).

Within each follicular wave, synchronous recruitment of a group or 5-7 antral (Type 5) follicles 5 mm in diameter occurs in response to a transient increase in plasma FSH (Bigelow and Fortune, 1998; D'Occhio et al 1999). One follicle is selected as the dominant follicle of the wave. Because the first follicular wave generally occurs during the luteal phase when progesterone is inhibiting LH pulse frequency, the first-wave dominant follicle generally undergoes atresia (Rahe *et al.*, 1980; Cupp *et al.*, 1990; Lucy *et al.*, 1990). The first wave dominant follicle can ovulate and release an oocyte with normal fertility if the corpus luteum is induced to regress (Savio *et al.*, 1990a; Lavoir and Fortune, 1990), or the follicle is stimulated to ovulate with GnRH (Pratt *et al.*, 1982; McDougall *et al.*, 1995), LH (D'Occhio *et al.* 1997) or hCG (Schmitt *et al*, 1996a,b,c,d; Rajamahendran and Sianangama, 1992; Fricke *et al.*, 1993; Sianangama and Rajamahendran, 1995).

In a 2-wave oestrous cycle, the second dominant follicle ovulates, and in a 3-wave cycle, the third dominant follicle ovulates. The dominant follicle is defined morphologically as the largest follicle present on the ovary, and functionally as a follicle that has the ability to ovulate, inhibit growth of subordinate follicles, and suppress FSH

secretion (Fortune, 1993). It has been suggested that oestradiol and inhibin, produced by the dominant follicle, may regulate LH and FSH secretion, depriving subordinate follicles of gonadotrophic stimulation (Kastelic *et al.*, 1990a; Goulding, 1991). However, the dominant follicle is also gonadotrophin-dependent, so it is possible that oestradiol and inhibin act directly on subordinate follicles to inhibit growth.

1.5 Corpus luteum structure and function

The preovulatory LH surge induces a series of events which results in the re-organisation of follicular cells into the corpus luteum, a transient endocrine gland (Niswender *et al.*, 1994). Formation of the corpus luteum is achieved by a process called luteinisation, which involves a series of morphological and biochemical changes in the cells of the theca interna and membrana granulosa cells of the preovulatory follicle (Gore-Langton and Armstrong, 1988).

In response to the preovulatory LH surge, the basement membrane between the theca interna and the membrana granulosa breaks down, blood vessels invade the antral cavity of the follicle, and an extensive vascular network begins to develop (Niswender *et al.*, 1994). There is significant hypertrophy (increase in size) and hyperplasia (increase in number) of the thecal cells, which begin to migrate into the antral cavity and become dispersed among the luteinising granulosa cells (O'Shea *et al.*, 1980).

Luteal growth is dependent upon growth of new blood vessels (angiogenesis; Reynolds *et al.*, 1992; Zheng *et al.*, 1993) as the corpus luteum is a highly vascularised tissue. In the early luteal phase, there is rapid growth and proliferation of endothelial cells and invasion of capillaries (Zheng *et al.*, 1993). In the mid-luteal phase, a dense microvascular network of capillaries develops (Zheng *et al.*, 1993). In the late luteal phase, the capillaries begin to regress, there are increases in the amount of connective

tissue and large microvesicles, and there is regression and loss of luteal parenchyma (Zheng *et al.*, 1993).

The cells of the corpus luteum are derived from the theca and granulosa cells which are thought to respectively give rise to small luteal cells and large luteal cells, the two distinct morphological types of steroidogenic luteal cells in the corpus luteum (Alila and Hansel, 1984; Farin *et al.*, 1986; O'Shea *et al.*, 1990; Meidan *et al.*, 1990; Jablonka-Shariff *et al.*, 1993; Girsh *et al.*, 1995). Small and large luteal cells constitute ~70% of luteal weight (O'Shea *et al.*, 1989; Parry *et al.*, 1980; Wiltbank *et al.*, 1994).

During the process of luteinisation, large luteal cells accumulate agranular (smooth) endoplasmic reticulum, their mitochondria become rounded with tubulovesicular cristae and glycogen-containing granules accumulate (Niswender *et al.*, 1994). These are characteristics of steroidogenic cells. The Golgi complex is quite extensive in large luteal cells, and there is an abundance of electron-dense, membrane-bound secretory granules in their cytoplasm (Niswender and Nett, 1988). Both oxytocin and relaxin have been isolated as components of these granules in bovine large luteal cells (Wathes *et al.*, 1983; Fields *et al.*, 1980). Oxytocin is involved in luteal regression (Section 4.2).

Small luteal cells are morphologically and functionally distinct from large luteal cells. The nucleus of small luteal cells is irregular in shape and there are a moderate number of mitochondria of variable size (Niswender and Nett, 1988). Both tubular and lamelliform cristae have been observed, as have large amounts of smooth endoplasmic reticulum and lipid droplets (Niswender and Nett, 1988). The Golgi complex is less pronounced in small luteal cells compared with large luteal cells and there are no secretory granules (Niswender and Nett, 1988).

The preovulatory follicle weighs ~200 mg, and during the process of luteinisation, it grows in 11 days to a corpus luteum that weighs ~4 g in cattle (Fields and Fields, 1996). Bovine large luteal cells tend to proliferate only during the early stages of luteal development, after which there is little or no mitotic activity in these cells (Zheng *et al.*, 1994). It is considered that a proportion of small luteal cells develop into large luteal cells (Alila and Hansel, 1984; Smith *et al.*, 1994).

The primary function of the corpus luteum is to secrete the steroid hormone, progesterone, although it also secretes oxytocin and relaxin (Baird, 1984; Pough *et al.*, 1989). Luteinising hormone promotes progesterone production by the corpus luteum (Niswender *et al.*, 1994). The mechanism for LH stimulation of progesterone secretion (Section 3.1.1) by the small luteal cells (see Section 1.5.1) involves the formation of cAMP and subsequent activation of protein kinase A (PKA) (Niswender *et al.*, 1994). This stimulates cholesterol esterase activity, and may also enhance transport of cholesterol to the inner mitochondrial membrane for steroid biosynthesis (Niswender *et al.*, 1994).

Luteinising hormone stimulates progesterone synthesis both *in vivo* and *in vitro* (Niswender and Nett, 1988) by binding to its receptor as described in Section 1.3.1. Small luteal cells are six times more responsive to LH compared with large luteal cells (Niswender *et al.*, 1994). Large luteal cells, however, secrete over 80% of the progesterone secreted by the corpus luteum during the midluteal phase of the oestrous cycle (Niswender *et al.*, 1985).

1.5.1 Luteal regression

Luteal regression involves the process of apoptosis, a type of 'programmed' cell death in which distinct populations of cells are eliminated by activation of

endonucleases within the cells (Arends *et al.*, 1990; Sawyer *et al.*, 1990; Compton and Cidlowski, 1992). There are two types of luteal regression: functional luteal regression (decreased progesterone synthesis) and structural luteal regression (decreased cell numbers) (Juengel *et al.*, 1993). In the latter study, initiation of apoptosis did not occur until after functional luteal regression had begun. Sawyer *et al.* (1990) proposed that a reduction in blood flow initiated apoptosis. In ewes, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) caused a rapid decrease in blood flow to the ovary (Nett *et al.*, 1976).

In heifers, premature luteal regression occurred when LH activity was removed by immunoneutralisation (Snook *et al.*, 1967). Similar regression of the corpus luteum occurred in ewes treated with a GnRH antagonist (Baird, 1992). Peters *et al.* (1994) treated heifers with a GnRH antagonist on Days 2-7 of the oestrous cycle, which resulted in diminished luteal function. Heifers treated with GnRH antagonist on Days 7-12 also had reduced progesterone secretion (Peters *et al.*, 1994). However, treatment of heifers with a GnRH antagonist on Days 12-17 of the oestrous cycle did not influence luteal function (Peters *et al.*, 1994). This would indicate that pulsatile release of LH may not be required for luteal function after Day 12 of the oestrous cycle.

$PGF_{2\alpha}$ is the luteolysin in the cow and the ewe. $PGF_{2\alpha}$ is released from the uterus and binds to receptors on large luteal cells (Powell *et al.*, 1976) to cause luteal regression. Rueda *et al.* (1997) proposed that $PGF_{2\alpha}$ stimulated uncontrolled production of reactive oxygen species, leading to functional and structural luteolysis. In some cell types, reactive oxygen species, such as free radicals, have been shown to stimulate apoptosis (Buttke and Sandstrom, 1994).

Kindahl (1976) showed that $PGF_{2\alpha}$ was released on approximately Day 17 of the oestrous cycle in cattle. It was considered that oxytocin, produced by the large luteal

cells in the corpus luteum, binds to specific endometrial receptors to cause $PGF_{2\alpha}$ release (Kindahl (1976). Bazer *et al.* (1994) demonstrated that in sheep, the concentration of endometrial oxytocin receptors was low until approximately Day 17. Continuous infusion of oxytocin during the mid- to late-luteal phase extended the lifespan of the corpus luteum and inhibited $PGF_{2\alpha}$ secretion in heifers, possibly through down-regulation of endometrial oxytocin receptors (Lutz *et al.*, 1991). Wathes and Lamming (1995) demonstrated in ewes, that while oestradiol was not necessary for oxytocin receptor expression, it could cause upregulation of oxytocin receptors when oestradiol concentrations and oxytocin receptor numbers were high.

In sheep, $PGF_{2\alpha}$ secretion is dependent on oxytocin release from the corpus luteum. During the mid-luteal phase in ewes, progesterone inhibited synthesis of endometrial oxytocin receptors (McCracken *et al.*, 1984). As progesterone concentrations decreased towards the end of the luteal phase, and oestradiol concentrations increased, endometrial oxytocin receptors were synthesised and the uterus became responsive to oxytocin, secreting $PGF_{2\alpha}$ (McCracken *et al.*, 1984; Zollers *et al.*, 1993). In order for the uterus to become responsive to oxytocin, progesterone concentrations must decrease.

Recently, it was demonstrated that this might not occur in heifers. In fact, luteal regression can occur in the absence of oxytocin release in cattle both *in vitro* (Blair *et al.*, 1997) and *in vivo* (Hansel and Blair, 1996). Kotwica *et al.* (1998) suggested that, in cattle during luteolysis, $PGF_{2\alpha}$ either decreases plasma progesterone, allowing oxytocin receptors to increase, or that $PGF_{2\alpha}$ directly stimulates oxytocin receptor synthesis.

1.5.2 Pregnancy

In heifers, progesterone inhibits the development of the luteolytic signal; low plasma concentrations of progesterone resulted in the release of larger amounts of PGF2α (Mann and Lamming, 1995). This may help to explain why cows with relatively low plasma concentrations of progesterone are prone to lowered conception rates and increased embryo mortality (Folman *et al.*, 1973; Erb *et al.*, 1976; Rosenberg *et al.*, 1977; Favero *et al.*, 1993; Cavalieri *et al.*, 1998).

After conception, the bovine conceptus secretes trophoblast protein-1, a complex glycoprotein also known as interferon-τ, from the trophoblastic vesicle, from about Day 12 or 13 until about Day 19, when the synthesis rate is markedly decreased (Bartol *et al.*, 1985; Godkin *et al.*, 1988; Roberts *et al.*, 1992). Maternal plasma progesterone concentrations in the bovine have been positively correlated with concentrations of interferon-τ produced by the conceptus (Kerbler *et al.*, 1997), suggesting that relatively greater maternal plasma progesterone concentrations may provide a uterine environment more supportive for the developing conceptus.

A number of studies have shown that progesterone supplementation may enhance conception rate in heifers (Robinson *et al.*, 1989; Walton *et al.*, 1990). Robinson and co-workers (1989) administered progesterone to dairy cows between Days 5 and 12 after insemination, which resulted in significantly increased pregnancy rates. The use of human chorionic gonadotrophin (hCG) on Day 5 after mating to induce ovulation of the first wave dominant follicle (Figure 1.3) and subsequent formation of an accessory corpus luteum, was associated with increased plasma progesterone concentrations and enhanced pregnancy rates (Price and Webb, 1989; Walton *et al.*, 1990; Rajamahendran and Sianangama, 1992). Gonadotrophin-releasing hormone and

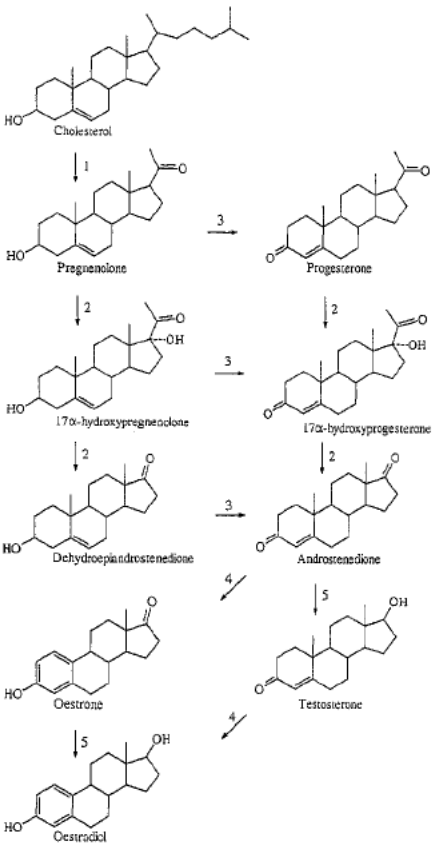
its agonists can be used in a similar manner to induce formation of an accessory corpus luteum and increase progesterone secretion (Table 1.1; Milvae *et al.*, 1984; Bishop and Wetteman, 1993; Gong *et al.*, 1995; Rajamahendran *et al.*, 1996). Morgan and Lean (1993) reported increased conception rates when GnRH or GnRH agonist was injected at the time of insemination. Generally, the use of a GnRH agonist at the time of artificial insemination or at 10-12 days following insemination, results in increased fertility (Coleman *et al.*, 1988; Lajili *et al.*, 1991; Troxel *et al.*, 1993; Macmillan and Burke, 1996; Ambrose *et al.*, 1999).

1.6 Steroidogenesis

The process of steroidogenesis is summarised in Figure 1.4. All steroid hormones are synthesised from cholesterol. Removal of the side chain on cholesterol produces pregnenolone, which is then converted to progesterone by 3β-hydroxysteroid dehydrogenase, Δ^5,Δ^4-isomerase (3β-HSD). This enzyme is localised in the theca and granulosa cells of the preovulatory follicle and in the corpus luteum (Baird, 1994). Cleavage of the side chain between C-20 and C-22 of pregnenolone produces a series of C_{21}-steroids, known as the pregnane series. Further cleavage of the C-17, C-20 side chain produces the C_{19}-steroids, termed the androstane series. Finally, removal of the C-10 methyl group forms the C_{18}-steroids, known as the oestrane series (Gore- Langton and Armstrong, 1988). The steroid hormones are classified according to their chemical structure and their biological actions. The three major classes of ovarian steroids are the progestins, androgens and oestrogens, which belong to the pregnane, androstane and oestrane series, respectively.

1.6.1 The role of cholesterol

Cholesterol is synthesised from acetate within steroidogenic cells, or it is obtained from



1. C_{27} P450scc, adrenodoxin, cholesterol 22-hydroxylase, cholesterol 20α-hydroxylase, $C_{20,22}$-lyase
2. 3β-HSD
3. C_{21} P450scc, 17α-hydroxylase, $C_{17,20}$-lyase, NADPH, O_2
4. 17α-hydroxylase, NADPH, O_2
5. C17,20-lyase, NADPH, O_2
6. P450 aromatase, NADPH, O_2
7. 17β-HSD, NADH

Figure 1.4 The steroidogenic pathway in cattle.

high- or low-density lipoproteins (HDL and LDL, respectively) sequestered from the bloodstream. Uptake of LDL occurs through receptor-mediated endocytosis, whereas HDL uptake involves binding to specific membrane binding sites and shuttle of cholesterol into the cell (Niswender *et al.*, 1994). Cholesterol is directly utilised for steroid biosynthesis, or it is incorporated into cholesterol esters by acyl Coenzyme A cholesterol acyltransferase and stored as lipid droplets within steroidogenic cells (Niswender *et al.*, 1994).

1.6.2 Steroidogenic acute regulatory (StAR) protein

The first reaction in the progesterone biosynthesis pathway occurs in the mitochondria. Stocco (1997) developed a model for the transport of cholesterol to the inner mitochondrial membrane, involving a mitochondrial phosphoprotein called steroidogenic acute regulatory (StAR) protein.

It was proposed that in response to stimulation by LH, StAR protein is synthesised in the cytosol as a 37 kDa protein that is rapidly imported across the mitochondrial membrane, via its N-terminal leader sequence, at 'contact sites' which form between the inner and outer mitochondrial membranes (King *et al.*, 1995). The 37 kDA protein is the active form of StAR and has a short half-life (3-5 minutes; Clark and Stocco, 1996).

Mitochondrial uptake of StAR protein is associated with the processing of StAR to yield four 30 kDa phosphoproteins whose functions remain unknown (Stocco and Sodeman, 1991; King *et al.*, 1995). The 30 kDa forms (half life 3-5 minutes; Clark and Stocco, 1996) remain associated with the inner mitochondrial membrane; however, they are believed to be no longer active in cholesterol transfer (Pescador *et al.*, 1996). Synthesis of the 30 kDa proteins relies on a cAMP-dependent PKA pathway (Stocco,

1997). The exact mechanism of LH stimulation of StAR synthesis is not understood, but is thought to involve cAMP (Stocco, 1997). It is considered that, during the processing of the 37 kDa protein, cholesterol is transferred from the outer to the inner mitochondrial membrane through the contact sites and after processing, the membranes separate such that no further cholesterol transport can occur without additional synthesis and processing of StAR protein (Stocco, 1997).

Pescador *et al.* (1996) demonstrated the presence of StAR mRNA and StAR protein in bovine theca cells, granulosa cells, corpus luteum, and placenta. They showed that, in cattle, both StAR mRNA and StAR protein in lesser concentrations during luteal development, elevated in functional corpora lutea, and absent in regressed corpora lutea.

1.6.3 Cytochrome P450 side chain cleavage enzyme and 3β-hydroxysteroid dehydrogenase

Cholesterol, once it has entered the mitochondria, is converted to pregnenolone by the cytochrome P450scc enzyme; P450scc is part of a three-enzyme complex known as the cholesterol side chain cleavage complex that also includes adrenodoxin and adrenodoxin reductase (Simpson and Boyd, 1966, 1967; Churchill and Kimura, 1979; Tuls *et al.*, 1979). The amount and activity of P450scc enzyme are not affected by LH stimulation (Lambeth *et al.*, 1982; Tuckey and Stevenson, 1984; Tuckey, 1992).

In cattle, mRNAs for the steroidogenic enzymes, including P450scc and 3β-HSD, increase during luteal development during the mid-luteal phase (Rodgers *et al.*, 1987; Couet *et al.*, 1990). Messenger RNA for P450scc is localised to the theca interna of all growing follicles, and is not detectable in granulosa cells until follicles reach approximately 4 mm in diameter (Xu *et al.*, 1995b). Messenger RNAs for P450scc and 3β-HSD decline during luteal regression (Tian *et al.*, 1994; Rogers *et al.*, 1995).

Tian *et al.* (1994) demonstrated that immunohistochemically detectable amounts of 3β-HSD remained present during late luteal regression, even after the message for 3β-HSD had declined. Rodgers *et al.* (1995) observed that tissue concentrations of P450scc and 3β-HSD did not decline within 24 hours of $PGF_{2\alpha}$-induced luteolysis. This indicates that a "pool" of 3β-HSD may exist in the corpus luteum, such that rate of 3β-HSD gene expression may be maximal throughout the luteal phase of the oestrous cycle. As a result, regulation of 3β-HSD secretion may not be critical for increased progesterone secretion.

The half-life of P450scc in adrenocortical cells of cattle is 40 hours (Boggaram *et al.*, 1984) and that of 3β-HSD has not been measured. It is likely that the enzymes do not decline at the same rate as the messenger RNAs; hence, amounts of P450scc or 3β-HSD are probably not related to the decline in plasma concentrations of progesterone associated with luteolysis, or increased progesterone secretion during luteal development.

It can be concluded that the rate-limiting step in progesterone biosynthesis is the StAR protein-mediated step. Increased plasma concentrations of progesterone are likely a result of increased StAR protein synthesis. As speculated in Section 1.6.2, increased rate of StAR protein synthesis may induce increase P450scc and 3β-HSD complex synthesis.

1.6.4 Steroid synthesis by follicles

1.6.4.1 Theca cells

In the ovarian follicle, cholesterol is converted to pregnenolone by the theca cells Figures 1.4, 1.5). Cytochrome P450scc enzyme was localised to the theca interna from

preantral follicles, while it did not appear in granulosa cells until the follicle reached approximately 4 mm in diameter (Xu *et al.*, 1995b). Gong *et al.* (1996) demonstrated that follicles become FSH-dependent at 4 mm in diameter. This suggests that expression of P450scc gene in granulosa cells may require FSH stimulation. In the theca interna, cholesterol is transported across the mitochondrial membrane in response to LH stimulation (Section 1.6.2) and is converted to pregnenolone. In turn, pregnenolone is converted to dehydroepiandrostenedione by the cytochrome P450 17α-hydroxylyasea/C-17,20 lyase enzyme (Figures 1.4, 1.5), which has been localised to the theca interna and was first observed about the time of antrum formation (Xu *et al.*, 1995b). Dehydroepiandrostenedione is converted to androstenedione which traverses the follicular basement membrane to be utilised in oestrogen biosynthesis in the granulosa cells (Figures 1.4, 1.5) (Xu *et al.*, 1995b).

1.6.4.2 Granulosa cells

Androstenedione from the theca interna is converted directly to oestradiol by the cytochrome P450 aromatase enzyme (Figures 1-4, 1-5), which was localised to the granulosa cells of follicles ≥ 4 mm in diameter (Voss and Fortune, 1993; Xu *et al.*, 1995b). Activity of the aromatase enzyme is increased by FSH in granulosa cells *in vitro*, indicating that the production of oestradiol may be FSH-stimulated (Gore-Langton and Armstrong, 1988).

1.6.5 Progesterone synthesis by the corpus luteum

Receptors for LH are localised to the small luteal cells (Fitz *et al.*, 1982) coupled with an adenylate cyclase system (Hoyer and Niswender, 1985). In contrast, large luteal cells possess the majority of oestradiol (Glass *et al.*, 1985) and $PGF_{2\alpha}$ receptors (Fitz *et al.*, 1982). Both small and large luteal cells have expression of the P450scc gene (Rodgers *et*

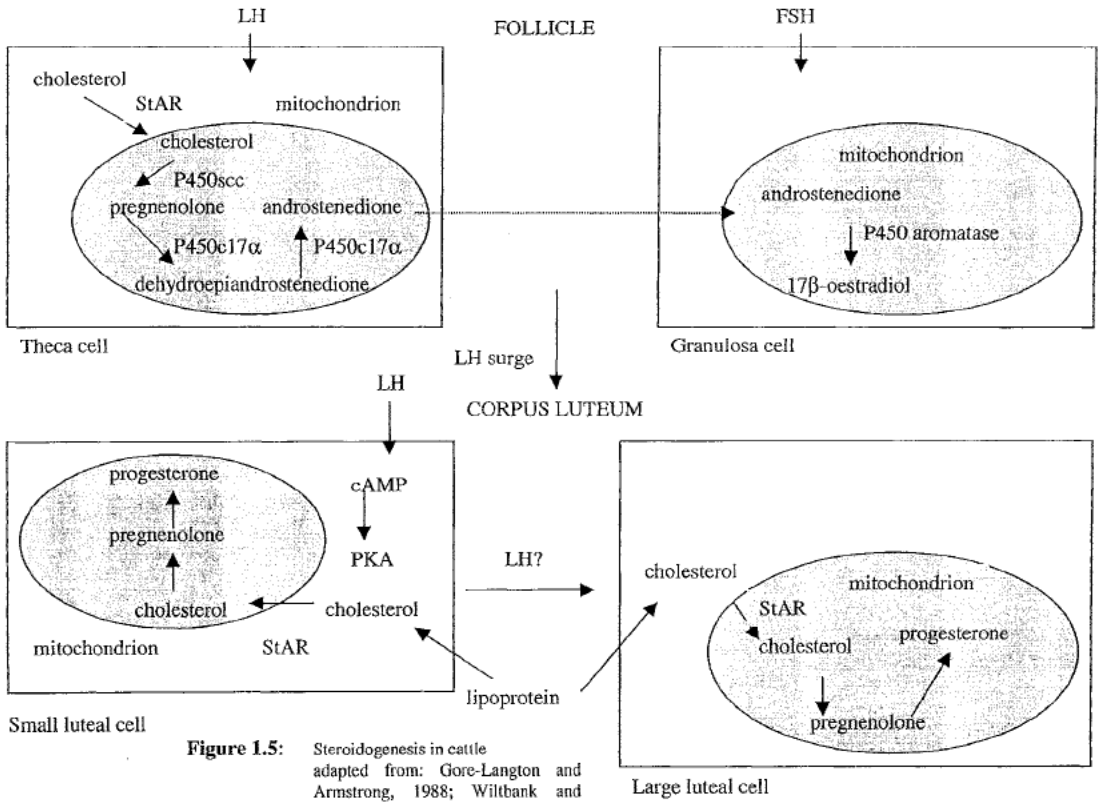


Figure 1.5: Steroidogenesis in cattle adapted from: Gore-Langton and Armstrong, 1988; Wiltbank and Niswender, 1992; Xu et al., 1995.

al., 1986). Despite possessing fewer LH receptors and therefore being less responsive to LH stimulation, large luteal cells produce a greater amount of progesterone compared with small luteal cells (Koos and Hansel, 1981).

1.7 Pituitary and ovarian responses to treatment with GnRH agonists in heifers

1.7.1 Biphasic response of the anterior pituitary to GnRH agonist

As discussed above, GnRH agonists have a longer half-life than natural sequence GnRH, and a greater affinity for the GnRH receptor (Section 1.2). Implantation or continuous infusion of a GnRH agonist results in a biphasic response from the anterior pituitary gland (Table 1.1). The acute phase (0-24h) consists of an increase in LH secretion by the anterior pituitary gland. During the chronic phase that follows, the pituitary is desensitised to natural sequence GnRH and pulsatile secretion of LH is abolished. As a result, the preovulatory LH surge does not occur (Rettmer *et al.*, 1992b; Chenault *et al.*, 1990; Gong *et al.*, 1995; Bergfeld *et al.*, 1996; Gong *et al.*, 1996). In heifers treated with GnRH agonist, non-pulsatile LH secretion has been associated with elevated basal concentrations of LH (Evans and Rawlings, 1994; Gong *et al.*, 1995; D'Occhio *et al.*, 1996) and elevated plasma concentrations of progesterone (D'Occhio *et al.*, 1996; Rajamahendran, 1996).

1.7.2 Acute phase response to GnRH agonist

It has been shown that the increase in plasma concentrations of LH, during the acute phase of treatment with a GnRH agonist, is sufficient to induce ovulation and/or luteinisation of ovulatory follicles present at the initiation of GnRH agonist treatment (Table 1.1; Macmillan and Thatcher, 1991; Wolfenson *et al.*, 1994; Gong *et al.*, 1995, 1996; Schmitt *et al.*, 1996a,b,c,d, Ambrose *et al.*, 1999). An induced ovulation and subsequent formation of an accessory corpus luteum results in increased plasma

Table 1.1 Effects of treatment with an agonist of GnRH on Day 0 to 4, Day 5 to 8, Day 9 to 16, or at random stages of the oestrous cycle in heifers

Agonist	Dose	Day of Administration	Effects	Reference
Day 0 – 4				
Buserelin	8μg	Day 0; Day 6: those that had not exibited oestrus received PGF AI 12hrs after oestrus	no effect on P4 or conception rates; d0-6: less cows exhibited oestrous; d6-10: greater synchrony and precision of oestrous.	Twagiramungu *et al.*, 1992 70:1904.
Fertirelin	100μg	6 or 12 hrs after oestrus	acute LH release; 6hr group had greater LH release; no effect on P4.	Coleman *et al.*, 1988
Fertirelin	50 or 100μg	AI 12hrs after oestrus, agonist at AI	conception rates tended to be greater : 100μg > 50μg > controls	Coleman *et al.*, 1988
Buserelin or Deslorelin	8μg 75μg 150μg 700μg 2100μg	Day 4	maximum acute LH response greater in 700 and 2100 deslorelin than buserelin; basal LH greater in 700 than 2100; induced CL in all groups; cycle length and CL lifespan extended in 2100; all groups: P4 greater than controls, extremely so in 700 and 2100; number of follicles >5mm reduced in 2100 group from Days 15-21.	Rajamahendran *et al.*, 1996
Day 5 –8				
Buserelin	8μg	Day 7: PGF Day 8: buserelin TAI 15hrs after buserelin OR AI at oestrous	acute LH release; ovulation of dominant follicle; pregnancy rates greater in AI at oestrus group.	Schmitt *et al.*, 1996 (74:1084)

Agonist	Dose	Day of Administration	Effects	Reference
Buserelin	5 or 10µg	Day 5: twice daily for 21 days	no dose-response effect; acute LH/FSH release after first injection, then LH returned to basal; FSH peak before first follicular wave then peaked again before second wave and remained at peak until end of treatment; induced ovulation followed by increased P4; induced and endogenous CLs regressed at the same time as controls CLs	Gong *et al.*, 1995
Buserelin	osmotic mini-pump releasing 2.5µg/hr	Day 5; replaced 28 days later	acute LH/FSH release, then non-pulsatile LH; FSH lowered from Day 31 to end of treatment; follicles grew to 7-9mm with FSH, without LH; follicles arrested at 4mm without FSH/LH.	Gong *et al.*, 1996.
Deslorelin		Day 7 for 28 or 56 days	increased LH; return to oestrous 3 weeks after implant removal	D'Occhio *et al.*, 1996
Day 9 – 16				
Buserelin	5µg	Day 7-16: PGF; buserelin 0.25, 24 or 72hrs after PGF OR Mid-luteal: buserelin, then PGF OR Day 7: buserelin; Day 8, 12 or 16: ovary with CL removed	buserelin increased the post-PGF return to oestrous, no time effect; decreased P4, but delayed structural luteolysis; post-op interval to oestrous not effected conclusion: buserelin temporarly inhibited luteolysis.	Macmillan *et al.*, 1985 8:213
GnRH	250µg	day after largest follicle reaches 10mm, implant for 3weeks	acute LH release; ovulation of second or third wave DF.	McDougall *et al.*, 1995

Agonist	Dose	Day of Administration	Effects	Reference
Buserelin	10 or 20μg	one injection on Day 8-16	dose-response acute LH/FSH release.	Chenault *et al.*, 1990
GnRH	100, 200, 500μg	one injection Day 8-16	acute LH release.	Chenault *et al.*, 1990
Buserelin		Day 9-12: inject 4 times daily	increased P4 until end of treatment period	Milvae *et al.*, 1984
Buserelin	8μg	Day 12: buserelin Day 19: PGF	induced ovulation; increased oestradiol; decreased number of small follicles, indicating dominance	Wolfenson *et al.*, 1994
Buserelin	10μg	12-14 Days after AI (AI at oestrus)	enhanced conception rates in females that had previously received PGF; increased conception rate to subsequent AI increased.	Lajili *et al.*, 1991
Random stages				
Deslorelin	50μg/kg/day	Implant for 28 days, beginning at random stages of the oestrous cycle	acute LH release; pituitary desenstised for 12 days after treatment; increased rate of follicular growth; increased oestradiol; increased size of dominant follicles.	Bergfeld *et al.*, 1996
GnRH	2μg/4hr or 2μg/hr	Pulsatile, beginning at random stages of the oestrous cycle	dose-response acute LH release; increased P4 for treatment period.	Bishop and Wettemann, 1993
Deslorelin	2 or 4 implants	Random stages	cycles ceased	D'Occhio *et al.*, 1996
	1 or 2 implants	Random stages	cycles ceased	

concentrations of progesterone during the ensuing luteal phase (Table 1.1).

1.7.2.1 Increased activity of the endogenous corpus luteum

Initiation of treatment with a GnRH agonist early in the oestrous cycle (Days 2 to 5) resulted in increased plasma concentrations of progesterone in the subsequent luteal phase (Gong *et al.*, 1995; Rajamahendran *et al.*, 1999; Ambrose *et al.*, 1999; Table 1.1). Early in the oestrous cycle, there are no preovulatory follicles present in the ovary. Hence, an accessory corpus luteum would not be formed in response to treatment with a GnRH agonist. Increased plasma concentrations of progesterone as a result of GnRH agonist treatment commencing early in the oestrous cycle is likely due to increased steroidogenesis by the endogenous corpus luteum.

Increased progesterone synthesis and secretion by the endogenous corpus luteum during treatment with a GnRH agonist may involve increased synthesis of StAR protein, the mediator of the rate-limiting step in steroidogenesis. Since treatment with a GnRH agonist commencing early in the oestrous cycle results in tonically elevated plasma concentrations of LH (Bergfeld *et al.*, 1996; Maclellan *et al.*, 1997, Pitcher *et al.*, 1997), it would follow that LH-induced StAR synthesis may be increased by GnRH agonist treatment. Other key steroidogenic enzymes in progesterone biosynthesis, P450scc enzyme and 3β-HSD, may also be increased. However, from previous studies, it is thought that regulation of amounts of P450scc enzyme and 3β-HSD may not be critical in steroidogenesis (Section 1.6.3).

1.7.2.2 Accessory corpus luteum

The dominant follicle of each follicular wave can be induced to ovulate by injecting or implanting a GnRH agonist (Macmillan and Thatcher, 1991). A follicle which has entered its regression phase will not respond to the acute increase in LH

induced by a GnRH agonist; hence, the timing for treatment with a GnRH agonist is critical for inducing ovulation of the dominant follicle. Treatment of heifers with a GnRH agonist, commencing on Day 5 to 8 of the oestrous cycle, induced ovulation of the first wave dominant follicle (Gong *et al.*, 1995, 1996; Schmitt *et al.*, 1996a,b,c,d; Table 1.1). Treatment with buserelin on Day 12 of the oestrous cycle induced ovulation of the second wave dominant follicle (Macmillan and Thatcher, 1991; Rettmer *et al.*, 1992a,b; Wolfenson *et al.*, 1994).

Schmitt *et al.* (1996b) treated heifers with GnRH agonist on Day 5 of the oestrous cycle to induce ovulation of the first wave dominant follicle and formation of an accessory corpus luteum. In those heifers treated with GnRH agonist, progesterone concentrations were greater in the ensuing luteal phase (Schmitt *et al.*, 1996b). There are data to support the concept that increased plasma concentrations of progesterone at the time of insemination may be beneficial to conception and pregnancy rates (Section 1.5.3).

Rajamahendran *et al.* (1999) implanted cows with deslorelin on Day 5 of the oestrous cycle, inducing ovulation of the first wave dominant follicle and subsequent formation of an accessory corpus luteum that, on Day 15 of the oestrous cycle, was the same diameter as the spontaneous corpus luteum. Basal plasma concentrations of LH and plasma concentrations of progesterone were increased between Days 12 and 21 of the oestrous cycle (Rajamahendran *et al.*, 1999). Cows treated with large doses of deslorelin (700 and 2100 μg) had greater agonist-induced LH release and greater plasma concentrations of progesterone (Rajamahendran *et al.*, 1999).

Macmillan and Thatcher (1991) demonstrated that treatment of cows with buserelin (GnRH agonist) on Day 11, 12 or 13 of the oestrous cycle resulted in an

increase in the number of medium follicles, which may have been a result of recruitment of small follicles or luteinisation of large follicles. Treatment of heifers with buserelin on Day 12 of the oestrous cycle induced ovulation of the second wave dominant follicle (Wolfenson *et al.*, 1994). When the same heifers were injected with buserelin again on Day 19 of the oestrous cycle, progesterone declined and oestradiol increased as normal (Wolfenson et al., 1994). However, the preovulatory oestradiol peak was higher in the treated group compared with the controls (Wolfenson *et al.*, 1994). In the same study, the sizes of the preovulatory and second-largest follicles were larger in the buserelin group and the numbers of small- and medium-sized follicles were smaller (Wolfenson *et al.*, 1994). It could be suggested from these results that the preovulatory follicle in buserelin-treated heifers is functionally and morphologically more dominant, as indicated by the greater plasma concentrations of oestradiol and the smaller numbers of subordinate follicles.

1.7.3 Chronic phase response to GnRH agonist

Non-pulsatile secretion of LH during the chronic phase of treatment with a GnRH agonist was associated with suppression of follicular growth beyond 9 mm in diameter in *Bos taurus* genotype beef heifers (Gong *et al.*, 1996) and non-lactating dairy cows (Rajamahendran *et al.*, 1988). Messenger RNA for LH receptors is undetectable in the granulosa cells of follicles until they reach approximately 9 mm in diameter in *Bos taurus* genotype beef heifers (Xu *et al.*, 1995a). Beyond a diameter of 9 mm, follicles are dependent on LH for maturation and dominance.

Gong *et al.* (1996) demonstrated that if GnRH agonist treatment was longer-term, FSH secretion became suppressed. As a result, follicular growth was arrested at 4mm in diameter. As discussed in Section 3.0, Type 3-4 follicles require FSH for

growth and development. FSH is also essential for the recruitment of new follicular waves, so suppressed FSH secretion arrests the emergence of follicular waves (Adams *et al.*, 1992; Gong *et al.*, 1996). From these studies, it is apparent that FSH is required for development of follicles from a diameter of 4 mm to 9 mm, and that further development beyond 9 mm requires pulsatile secretion of LH.

1.8 Aims and significance of thesis

In females of most mammals studied, treatment with a GnRH agonist is associated with decreased progesterone secretion (Balmaceda *et al.*, 1984; McRae *et al.*, 1985; Zorn *et al.*, 1986; McNeilly *et al.*, 1987; Fraser *et al.*, 1989, 1990; Lacoste *et al*, 1989; Picton *et al.*, 1991; Lunn *et al.*, 1992; Filicori *et al.*, 1993). In female cattle, however, plasma concentrations of progesterone are increased by treatment with a GnRH agonist. There would appear to be fundamental differences, therefore, in the pituitary response and hence the response of the corpus luteum to GnRH agonists among different species. An understanding of the mechanisms associated with the pituitary and luteal responses in cattle would provide important information on the hypothalamic-pituitary-ovarian axis in cattle, and could also indicate fundamental differences between species in the response to treatment with GnRH agonist. Examination of changes in the amounts of StAR protein, P450scc enzyme and 3β-HSD as a result of treatment with a GnRH agonist may also provide insight into the mechanisms associated with regulation of progesterone biosynthesis.

If GnRH agonist treatment is commenced early in the oestrous cycle (Day 0–4), the endogenous corpus luteum secretes increased progesterone. Increased progesterone synthesis by the endogenous corpus luteum may be a result of increased amounts of steroidogenic enzymes or StAR protein in the corpus luteum.

StAR protein regulates the rate-limiting step in steroidogenesis which is the transport of cholesterol to the inner mitochondrial membrane in response to trophic stimulation. Since the response in most mammals treated with GnRH agonist is a decrease in plasma progesterone concentrations, an understanding of the roles that LH and StAR protein have in causing increased steroidogenic activity of the corpus luteum in cattle may provide fundamental information about the regulation of the steroidogenic pathway. Tonically elevated basal secretion of LH, as observed in the chronic phase of GnRH agonist treatment, may be at least partly responsible for increased progesterone secretion.

Cytochrome P450scc enzyme which mediates the first reaction in the steroidogenic pathway, and 3β-HSD which catalyses the reaction that produces progesterone, may also be increased as a result of treatment with a GnRH agonist.

Objective One: The first objective in this thesis was to characterise corpus luteum function in heifers treated chronically with deslorelin to determine the mechanisms associated with increased progesterone secretion. This was done by establishing whether increased progesterone secretion is associated with changes in size of the corpus luteum, and/or amounts of StAR protein, P450scc enzyme or 3β-HSD.

Objective Two: The second objective in this thesis was to examine the relationships between follicle size (day of the oestrous cycle) at the initiation of treatment with GnRH agonist and ovulation in heifers, and whether the corpus luteum which results from the induced ovulation has a longer life span than the endogenous corpus luteum. Treatment with deslorelin was initiated on different days during the first follicular wave of the oestrous cycle (Days 2, 4, 6, and 8).

The information obtained in this thesis will provide important insight on the

mechanism(s) associated with increased progesterone secretion induced by GnRH agonist treatment in heifers. Amounts of StAR protein, P450scc enzyme and 3β-HSD in GnRH-agonist treated heifers have not been reported in the literature, and this thesis will provide new information on the role(s) that StAR protein and key steroidogenic enzymes have in progesterone biosynthesis in heifers.

Chapter 2: Materials and Methods

2.0 Oestrous detection

Rump-mounted oestrous detectors (Bulling Beacon®) were glued to the sacral region of each heifer ten days before the beginning of each experiment. This was accompanied by twice daily observations for behavioural oestrus.

2.1 Ultrasonography

Ovarian function was monitored by transrectal ultrasonography using an Aloka 210 scanner equipped with a 7.5 MHz linear array transducer (Veterinary Medical and Surgical Supplies, Newcastle, NSW, Australia). The size (> 2 mm) and numbers of follicles and corpora lutea were recorded.

2.2 GnRH agonist bioimplants

The GnRH agonist, deslorelin (D-Trp^{6}-Pro^{9}-des-Gly^{10}-GnRH ethylamide) (Karten and Rivier, 1986) was formulated into bioimplants containing either 6 or 12 mg of deslorelin (Peptech Animal Health Pty Ltd, North Rhyde, NSW, Australia). Previous studies have shown that *in vitro*, the implants initially released 50-100 µg deslorelin/24 h for 2-3 days, after which the release rate stabilized at approximately 20 µg/24 h (J. Walsh, unpublished results). The implants were placed subcutaneously in the ear using a conventional implanting device.

2.3 Blood Collection

Blood samples were collected using coccygeal venepuncture in all experiments. Blood was collected into evacuated tubes containing lithium heparin (Vacutainer®, Becton-Dickinson, Rutherford, New Jersey, USA) and immediately placed on ice.

Blood samples were stored on ice until centrifuged at 800xg, for 20 min at 7°C on a Beckman J-6M Induction Drive Centrifuge. The plasmas were stored at -20°C.

2.4 Hormone radioimmunoassays

Plasma concentrations of LH were measured by double-antibody radioimmunoassay (RIA) (D'Occhio *et al.*, 1986). Plasma concentrations of progesterone were determined using an extraction single antibody RIA (D'Occhio *et al.*, 1988). Assay sensitivities were 0.2 ng LH/ml and 0.2 ng progesterone/ml. Duplicate samples were performed, resulting in intra- and inter-assay coefficients of variation of <10%.

2.4.1 Phosphate buffers

PBS (phosphate-buffered saline)

1.47 g Na2HPO4 (anhydrous)

0.37 g EDTA

1.00 g NaN3

8.18 g NaCl

gel-PBS

2 g of gelatin per 1L of PBS

Phosphate buffers were prepared in advance and stored at 4°C until used.

2.4.2 Iodination of bovine LH

Iodination of bovine-LH was performed using a modification of the chloramine-T method described by D'Occhio *et al.* (1986). Iodine-125 (0.5µl, 0.5mCi, specific activty 37 MBq/mCi; NEZ-033A, Dupont, NEN Research Products, Boston,

MA) was added to 5 µg bovine-LH (USDA-bLH-1-I) in 25 µl of 0.01M PBS, followed by the addition of 5 µl chloramine-T (1 mg/ml) in 0.01M PBS (pH 7.5). After 90 seconds, 50 µl of sodium metabisulphite (2 mg/ml 0.01M PBS) was added to stop the reaction. After 60 seconds, 200 µl of potassium iodide (10 mg/ml of 0.01M PBS) was added. The radioiodinated and free hormone were separated on a BIOGEL P-60 column (Bio-Rad Laboratories, Richmond, California, USA). Fractions (0.5 ml) were collected and aliquots (10 µl) of each fraction were counted for 2 min using a gamma counter (LKB Wallac 1277 Gammamaster, Pharmacia, Wallac OY, Finland). The two fractions with the highest concentration of iodinated bLH were kept and diluted in 0.01M gel-PBS to obtain approximately 20,000 cpm/100 µl.

2.4.3 LH assay protocol

In each LH assay, three samples of low, medium and high LH concentration were used, in duplicate, as quality controls. Purified bLH (USDA-bLH-B-5) was serially diluted in gel-PBS to concentrations ranging from 100 to 0.19 ng/ml for a standard curve. Standards (100 µl) were pipetted in triplicate, into glass tubes with 300 µl gel-PBS. Quality controls and plasma samples (400 µl) were pipetted in duplicate. Antiserum to bLH (400 µl; USDA-309-684 P, 1:300,000) was added to all tubes, with the exception of the non-specific binding tubes, which received 400 µl of rabbit IgG (25 mg/L in gel-PBS). Iodinated bLH (100 µl, 20,000 cpm) was added to each tube and the tubes were shaken for two min. Tubes were incubated overnight at room temperature. Sheep-anti-rabbit gamma globulin (100 µl) was added to each tube and tubes were incubated overnight at room temperature, followed by an overnight incubation at 4°C. All tubes received PBS (1800 µl, pH 7.5, 4°C) and the tubes were centrifuged at 1000xg

for 30 min at 4°C (Beckman J-6M induction drive centrifuge). The supernatant was decanted and pellets counted for 2 min using a gamma counter (LKB Wallac 1277 Gammamaster, Pharmacia, Wallac Oy, Finland). Plasma concentrations of LH were then calculated using Prince Henry's Hospital Institute of Medical Research Radioimmunoassay System (Monash Medical Centre, Melbourne, Australia).

2.4.4 Progesterone assay protocol

In each assay, standards of low, medium and high concentrations of progesterone were used as quality controls. A standard curve was obtained by serial dilution of stock progesterone in the range 10 to 0.07 ng/ml. Standards (100 µl) were pippeted in triplicate into appropriately labeled tubes. Quality controls (100 µl) and samples (100 µl) were pippeted in duplicate. To each tube, 1 ml of heptane was added and samples were extracted for 10 min. After extraction, tubes were allowed to stand for 10 min. Tubes were placed in a 70% ethanol bath containing liquid nitrogen to freeze the aqueous layer. The supernatant was poured off into appropriately labeled tubes and the heptane evaporated under a stream of filtered air in a water bath at 45°C. When dry, 200 µl of tritiated progesterone was added (10,000 cpm/100 µl in gel-PBS; 1,2,6,7-^{3}H-progesterone; 80-100Ci/mmol TRK413; Amersham Australia Pty Ltd, North Ryde, NSW, Australia). Progesterone antiserum was then added to each tube (200 µl; sheep-anti-progesterone-11α-hemisuccinate-BSA conjugate, Dr RI Cox, Hormone Assay Development Group, Blacktown, NSW, Australia). The antiserum was used at a 1:15.000 dilution to ensure 45% reference binding. All tubes were shaken by hand and incubated for at least 16 h at 4°C. Dextran-coated charcoal (200µl; 62.5g dextran T70 and 625 mg charcoal Norit A in 100 ml PBS) was added to each tube and mixed. Tubes

were then incubated for 10 min at 4°C, following which tubes were centrifuged 1000xg for 20 min at 4°C in a Beckman J-6M Induction Drive centrifuge. The supernatant was poured off into appropriately labeled tubes and scintillation fluid (Optiphase HiSafe 2) was added to the supernatant. Each tube was counted on a β-counter (Wallac 1410 Liquid Scintillation Counter, Pharmacia, Wallac Oy, Finland) for 2 min. Progesterone concentrations were calculated using Prince Henry's Hospital Institute of Medical Research Radioimmunoassay System (Monash Medical Center, Melbourne, Australia).

2.4.5 Pituitary responses to GnRH

The LH response of the anterior pituitary gland to natural sequence GnRH was determined by administering 50 μg natural sequence GnRH i.m. and taking blood samples at 0 and 30 min. Peak LH concentrations were previously found to occur within 30 min of GnRH administration (Bergfeld *et al.*, 1996). The change in LH (ΔLH) following injection was calculated by subtracting concentrations at Time 0 min from respective concentrations at Time 30 min post-injection.

2.5 Corpus luteum processing

Frozen corpus luteum tissue samples were homogenised in 5 volumes of homogenisation buffer (1.0% cholate, 0.1% sodium dodecyl sulphate (SDS) in PBS, pH 7.4), using a Polytron PT 1200 (setting 4.5) (Kinematica, Switzerland). Homogenates were centrifuged on a Beckman Microfuge® for 30 min at 4°C and aliquots of the supernatants were stored at –70°C.

2.5.1 Protein assay

Concentration of protein in supernatants from corpus luteum tissue homogenates were measured using a Bradford Assay (Bradford, 1976). A standard

curve using BSA (Sigma Chemical Company, Castle Hill, NSW) was prepared and tissue homogenate supernatants were diluted and pipetted in triplicate into separate microtitre plate wells containing 40 µl Dye Reagent (Bio-Rad Laboratories Pty Ltd, North Ryde, Australia). Each plate was shaken and incubated at room temperature for 10 min. The absorbance at 595 nm was measured using a Bio-Rad 3550 Microplate Reader (Bio-Rad Laboratories Pty Ltd, North Ryde, Australia). Protein concentrations were calculated using Microtitre Plate Software (Bio-Rad Laboratories Pty Ltd, North Ryde, Australia).

2.5.2 Western blot analyses of P450scc and 3β-HSD

Aliquots (30 µg protein as determined by Bradford assay) of corpus luteum homogenates were heated at 100°C for 10 min in sample buffer (25mM Tris, pH 6.8; 1% w/v SDS; 0.2M β-mercaptoethanol; 1mM EDTA; 4% v/v glycerol, 0.005% w/v bromophenol blue). Proteins were then size-separated by polyacrylamide/sodium dodecyl sulphate gel electrophoresis (PAGE) (10% separating gel, 5% concentrating gel) on a Protean II xi Cell (Bio-Rad Laboratories, North Ryde, Australia). Protein was Western blotted onto "Hybond™-C extra" nitrocellulose membrane (Hybon C Extra, Amersham, Buckinghamshire, UK) in transfer buffer (20mM Tris, 150mM glycine, 20% methanol, pH 8.3) using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories Pty Ltd, North Ryde, Australia).

Antibodies to P450scc and 3β-HSD were used to determine relative contents of these enzymes in the corpus luteum homogenates. The P450scc primary antibody was purified rabbit anti-bovine cytochrome P450SCC (1:1000 dilution) (Rodgers *et al.*,

1986). The 3β-HSD primary antibody was purified rabbit anti-human 3β-HSD (Lorence *et al.*, 1990).

Non-specific binding sites on the nitrocellulose membranes were blocked by incubating them at 37°C for 60 min in Buffer A (10mM Tris, 0.15M NaCl, 5% BSA, 0.2% Non-Idet P40 non-ionic detergent, pH 7.4). The membranes were incubated in appropriate primary antibody in Buffer A for 150 min at room temperature, then washed 3 x 15 min in buffer B (10mM tris, 0.15M NaCL, 0.2% non-ionic detergent, 0.25% sodium deoxycholic acid, 0.1% SDS, PH 7.4) and 1 x 10 min in buffer C (10mM Tris, 0.15M NaCl, pH 7.4). The membranes were then incubated in ^{125}I-goat-anti-rabbit IgG second antibody (R4880, Sigma Immunochemicals, Castle Hill, NSW, Australia) in Buffer A for 45 min at room temperature, then washed 4 x 10 min in buffer B, 1 x 10 min in buffer C.

A strip of the nitrocellulose membrane containing the protein molecular weight marker (161-0317, BioRad Laboratories, North Ryde, NSW, Australia) was cut off and stained in amido black stain. The membranes were dried at room temperature and placed in autoradiography cassettes with KODAK Biomax-MS film and intensifying screen at –70°C. Film was developed using Kodak GBX developer and fixative (Eastman Kodak Company, Rochester, NY). Protein bands were quantified using the KODAK Digital Science 1D Scientific Imaging System (Eastman Kodak Company, Rochester, NY).

2.5.3 Western blot analysis of StAR protein

Aliquots (25 µl) of corpus luteum homogenates were separated by PAGE and Western blotted onto polyvinyl difluoride membrane (BioRad) in transfer buffer (20mM Tris, 150 mM glycine, 10% methanol, 0.01% SDS, pH 7.4).

Non-specific binding sites on the membranes were blocked by incubating the membranes at room temperature for at least 60 min in blocking buffer (4% milk solution and 0.25% Tween 20 in PBS, pH 7.4). The membranes were incubated in primary antibody (rabbit-anti-mouse StAR protein; Clark et al., 1994) in 2% milk (skim milk powder) solution (pH 7.4) for 60 min at room temperature, washed 3 x 5 min in wash buffer (0.25% Tween in PBS, pH 7.4) and incubated in second antibody (donkey-anti-rabbit) in 2% milk solution (pH 7.4) for 30 min at room temperature. Membranes were then washed 2 x 30 min in wash buffer and StAR protein bands detected by Chemiluminescence (NEN Research Products).

The membranes were dried at room temperature and placed in autoradiograpghy cassettes with KODAK Biomax-MS film and intensifying screen at –70°C. Film was developed using Kodak GBX developer and fixative (Eastman Kodak Company, Rochester, NY). Bands were analysed by densitometry using the KODAK Digital Science 1D Scientific Imaging System (Eastman Kodak Company, Rochester, NY).

2.6 Statistical Analyses

Data for LH and progesterone at single time points were examined by analysis of variance (ANOVA) procedures using the General Linear Models (GLM) procedure of SAS/STAT (SAS, 1990). Data analyses over time were accomplished by repeated measures analysis using SAS/STAT procedure MIXED (SAS, 1992). The model was y=treatment, time, treatment x time, with the repeated measure being animal. Group

means of two treatment groups were compared using a Student's T-test (SAS/STAT). In cases where there were more than two treatment groups, the CONTRAST statements of SAS/STAT procedures MIXED and GLM were used to compare group means. Where necessary, data were $\log_{10}$ transformed for homogeneity of variance.

Liveweights and results for corpus luteum size and StAR protein, P450scc, 3β-HSD and progesterone content are means ± SEM.

Chapter 3: Pituitary and corpus luteum responses in heifers treated with deslorelin

3.0 Introduction

Heifers treated chronically with a GnRH agonist, commencing early in the oestrous cycle, had significantly elevated plasma concentrations of progesterone (D'Occhio *et al.*, 1996b; Rajamahendran *et al.*, 1996). In these studies, treatment with a GnRH agonist was commenced early in the oestrous cycle when there would not have been a preovulatory follicle present on the ovaries. Hence, elevated progesterone as a result of GnRH agonist treatment was not due to the formation of an accessory corpus luteum (Section 1.7.2). Increased plasma concentrations of progesterone may have been due, therefore, to increased size of the spontaneous corpus luteum and/or increased steroidogenic capacity of the corpus luteum.

Steroidogenic acute regulatory (StAR) protein, which mediates transport of cholesterol into the inner mitochondrial membrane, has been demonstrated in the corpus luteum of cattle (Pescador *et al.*, 1996). Both StAR protein mRNA and StAR protein are present in small amounts during luteal development, elevated in functional corpora lutea, and absent in regressed corpora lutea (Pescador *et al.*, 1996). Because StAR protein mediates the rate-limiting step in steroidogenesis (Section 1.6.2), it could be assumed that increased concentrations of plasma progesterone, as a result of treatment with a GnRH agonist, might be associated with increased amounts of StAR protein and other key steroidogenic enzymes in the corpus luteum.

The aim in the experiments described in this chapter was to examine the basis for increased secretion of progesterone in heifers treated with a GnRH agonist

(deslorelin) commencing early in the oestrous cycle. Observations included plasma concentrations of LH and progesterone throughout the treatment period, size of the corpus luteum after treatment, and corpus luteum content of progesterone, StAR protein, P450 side chain cleavage enzyme and 3β-hydroxysteroid dehydrogenase (Sections 1.6.2 and 1.6.3).

3.1 Materials and Methods

3.1.1 Experimental Design

3.1.1.1 Experiment One

The oestrous cycles of nulliparous Brahman (Zebu, *Bos indicus*) heifers (3-yr-old, 452 ± 9kg) were synchronized using the Crestar® oestrous synchrony treatment for 11 days (Intervet, Australia). Visual observations for signs of oestrous were carried out every 12 hours for 3 days. On Day 3 of the ensuing oestrous cycle (Day 0=oestrus), heifers were assigned to: Control (n=16), no treatment; Deslorelin (n=16), implanted with one deslorelin bioimplant containing 6mg deslorelin (Section 2.2). Blood samples for LH and progesterone RIA were taken on days 0, 1, 3, 5, 8, 9 and 10 of treatment. On Day 0 of treatment (Day 3 of cycle), blood samples were taken 6 h after implanting to determine the acute release of LH in response to agonist treatment. On Day 10 of treatment (Day 13 of oestrous cycle), all heifers were ovariectomised and the corpus luteum weighed and dissected. Corpus luteum samples were frozen in liquid nitrogen and then stored at –80°C until required for measurement of progesterone content by RIA (Section 2.4.4) and for Western Blot analyses for amounts of StAR protein (Section 2.5.3), P450scc and 3β-HSD (Section 2.5.2). Immediately after ovariectomy, all heifers received an injection of natural sequence GnRH and blood samples for LH RIA (Section 2.4.3) were collected at 0 min and 30 min to test for capacity of the pituitary to release

LH (i.e. desensitisation in heifers treated with GnRH agonist) (Section 2.4.5).

3.1.1.2 Experiment Two

The oestrous cycles of nulliparous Brahman heifers (3-yr-old, 363 ± 7.6 kg) were synchronized using CIDR® treatment for 10 days (InterAg, New Zealand). The day before CIDR removal, all heifers were injected with prostaglandin $F_{2\alpha}$ (Lutalyse, Upjohn, NSW, Australia). Visual observations for signs of oestrous were carried out every 12 hours for 3 days. On Day 3 of the ensuing cycle (Day 0=oestrus) heifers were assigned to Control (n=16), no treatment; Deslorelin (n=16), implanted with 3 deslorelin bioimplants, each containing 6 mg deslorelin (Section 2.2). Blood samples for LH and progesterone RIA were taken on days 0, 1, 3, 5, 7, 9 and 10 of treatment. On Day 0 of treatment (Day 3 of cycle), blood samples were taken 6 h after implanting to determine the acute release of LH in response to GnRH agonist treatment. On Day 10 of treatment (Day 13 of the oestrous cycle), all heifers were ovariectomised and the corpus luteum weighed and dissected. Corpus luteum samples were frozen in liquid nitrogen and stored at –80°C until required for measurement of progesterone content by RIA (Section 2.4.4) and for Western Blot analyses for levels of StAR protein (Section 2.5.3), P450scc and 3β-HSD (Section 2.5.2). Immediately following ovariectomy, all heifers received an injection of natural sequence GnRH and blood samples for LH RIA (Section 2.4.3) were collected at 0 min and 30 min to test for LH-releasing capacity of the anterior pituitary gland (i.e. desensitisation in heifers treated with GnRH agonist) (Section 2.4.5).

3.1.2 Statistical analyses

Data were analysed statistically as described in Section 2.6.

3.2 Results

Data from Experiments 1 and 2 were pooled in all cases where there was no

experiment difference. In cases where there was an experiment difference, results are shown separately.

3.2.1 Concentrations of plasma LH and progesterone during treatment with deslorelin

The LH profiles in Experiments 1 and 2 are shown in Figure 3.1. There was an experiment difference ($P<0.05$) in plasma concentrations of LH for heifers treated with deslorelin; however, a similar difference also occurred in control heifers, suggesting that there may have been seasonal differences in absolute plasma concentrations of LH; inter-assay variation may also have accounted, in part, for the experiment difference. The general 'features' of the LH responses were the same for both experiments.

Treatment with deslorelin caused an acute increase in plasma concentrations of LH and, at 6 h after implanting, treated heifers had greater ($P<0.01$) plasma LH (1.4 ± 0.01 ng/ml, pooled data for Experiments 1 and 2) than control heifers (0.6 ± 0.01 ng/ml, pooled data). Plasma LH in treated heifers had declined by Day 1; however, mean LH from Day 1 to Day 10 of treatment was greater ($P<0.01$) for implanted heifers (0.8 ± 0.1 ng/ml, pooled data) than control heifers (0.5 ± 0.1 ng/ml, pooled data) (Figure 3.1). On Day 10, control heifers had an increase ($P<0.01$) in plasma LH after injection of natural sequence GnRH, but this did not occur in heifers implanted with deslorelin (Table 3.1).

Plasma progesterone increased from Day 0 to Day 10 of treatment for both control heifers and heifers treated with deslorelin. In Experiment 1, plasma progesterone on Day 10 of treatment was greater ($P<0.01$) for heifers implanted with deslorelin (10.5 ± 2.4 ng/ml) than control heifers (5.4 ± 0.4 ng/ml) (Figure 3.2). Similarly in Experiment 2, plasma progesterone on Day 10 of treatment was greater ($P<0.01$) for heifers implanted with deslorelin (29.7 ± 5.2 ng/ml) than control heifers

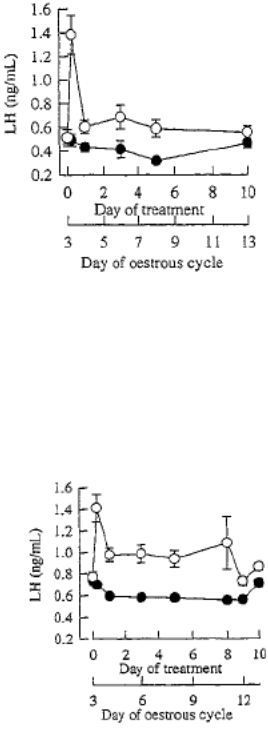


Figure 3.1 Plasma concentrations of LH (means±SEM) throughout the treatment period in control heifers (closed circles) and heifers treated with deslorelin (open circles). Heifers treated with deslorelin received either 6 mg (top panel) or 18 mg (lower panel) of deslorelin.

Table 3.1 Pooled data for Experiments 1 and 2: Changes in plasma LH (means ± SEM) in control heifers and heifers treated with deslorelin after injection of natural sequence GnRH (50 μg, i.m.)

	Relative to LH injection	
	0 min	30 min
Control	$0.61\pm0.04^{a,y}$	$2.33\pm0.25^{b,y}$
Deslorelin	$0.73\pm0.05^{a,y}$	$0.84\pm0.10^{a,z}$

^{a,b}means within rows without a common superscript differ ($P<0.05$);
^{y,z}means within columns without a common superscript differ ($P<0.01$)

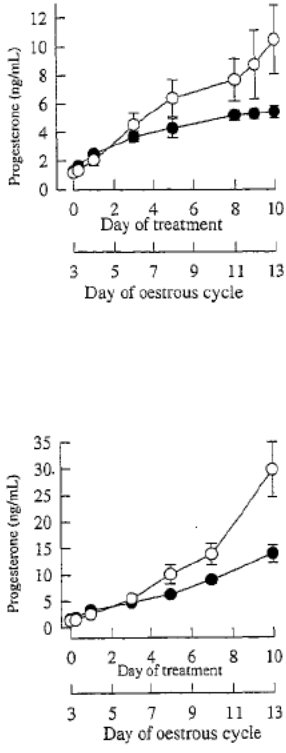


Figure 3.2 Plasma concentrations of progesterone (means ± SEM) in control heifers (closed circles) and heifers implanted with 6 mg deslorelin (open circles; top panel) or 18 mg deslorelin (open circles, lower panel)

(13.9 ± 1.7 ng/ml) (Figure 3.2). Progesterone concentrations were greater (P<0.05) in treated heifers in Experiment 1 compared with treated heifers in Experiment 2.

3.2.2 Corpus luteum size, progesterone, StAR protein, P450scc and 3β-HSD

There were no experiment differences for corpus luteum size, progesterone, StAR protein or P450scc. There was, however an experiment difference for 3β-HSD per unit weight of corpus luteum.

In Experiment 1, the weight of the corpus luteum was greater (P<0.05) in heifers treated with deslorelin than in control heifers (Figure 3.3). In Experiment 2, the weight of the corpus luteum tended to be greater (P = 0.19) in heifers treated with deslorelin When the data were pooled (Figure 3.3), heifers treated with deslorelin had a heavier (P<0.01) corpus luteum than control heifers.

Progesterone content per unit weight of corpus luteum was greater (P<0.05) in deslorelin-treated heifers compared with control heifers in Experiment 1, but not (P=0.53) in Experiment 2 (Figure 3.4). Progesterone content per total corpus luteum was not significantly different between treated and control heifers in Experiment 1 (P=0.12) and Experiment 2 (P=0.78). However, when the data were pooled, progesterone content per corpus luteum was greater (P<0.01) for treated heifers than control heifers (Figure 3.4).

The relative content of StAR protein per total corpus luteum was greater (P<0.05) in treated heifers (2.2 ± 0.3 relative arbitrary units, pooled data) compared with control heifers (1.2 ± 0.2 relative arbitrary units, pooled data) (Figure 3.5, Figure 3.6). The relative content of StAR protein per unit weight of corpus luteum tended (P=0.11) to be greater in heifers implanted with deslorelin compared with control heifers.

The amount of P450scc per total corpus luteum was greater (P<0.01) in heifers

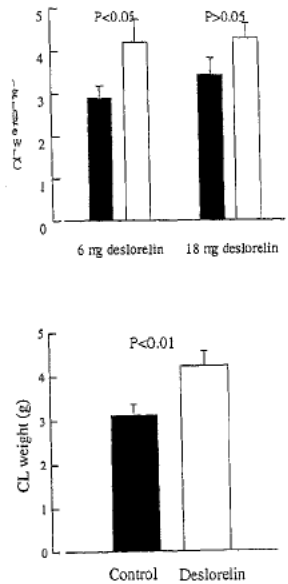


Figure 3.3 Upper panel: Weight of corpus luteum (means±SEM) in control heifers (solid bar, upper panel) and heifers treated with 6 mg deslorelin (left, open bar) or 18 mg deslorelin (right, open bar) of deslorelin. Lower panel: Data are pooled for Experiments 1 and 2.

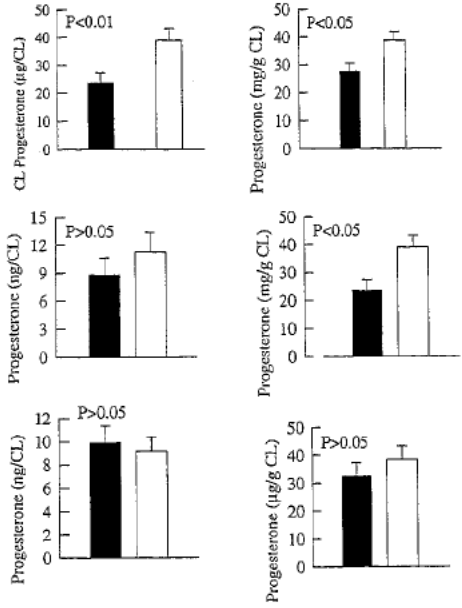


Figure 3.4 Amount of progesterone (means±SEM) per corpus luteum (left) and per unit weight of corpus luteum (right) in control heifers (solid bars) and heifers treated with deslorelin (open bars). Top panel: pooled data; Middle panel: Experiment 1; Lower panel: Experiment 2.

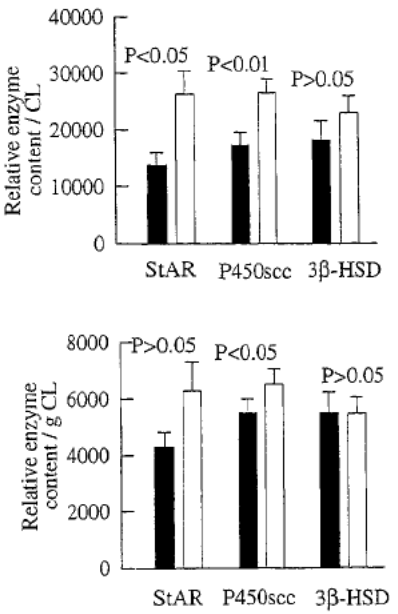


Figure 3.5 Relative contents (means±SEM) of StAR protein (left), P450scc enzyme (middle), and 3β-HSD (right) in control heifers (filled bars) and heifers treated with deslorelin (open bars). Contents are represented as arbitrary units per corpus luteum (top panel) and arbitrary units per unit weight of corpus luteum (lower panel). Data are pooled for Experiments 1 and 2.

treated with deslorelin (2.2 ± 0.2 relative arbitrary units, pooled data) compared with control heifers (1.4 ± 0.2 relative arbitrary units, pooled data) (Figure 3.5). P450scc content per unit weight of corpus luteum was also greater ($P<0.05$) in heifers treated with deslorelin (1.9 ± 0.2 relative arbitrary units, pooled data) compared with control heifers (1.2 ± 0.1 relative arbitrary units, pooled data) (Figure 3.5).

Content of 3β-HSD per total corpus luteum did not differ ($P=0.20$) between implanted and control heifers. There was an experiment difference ($P<0.05$) for the amount of 3β-HSD per unit weight of corpus luteum, treatment was not significant (Experiment 1: $P=0.09$; Experiment 2: $P=0.72$).

3.3 Discussion

The acute increase in plasma LH after implanting heifers with deslorelin was consistent with previous results in heifers (Bergfeld *et al.*, 1996). In the present study, plasma LH decreased within 24 h, but remained tonically elevated compared with control heifers. Heifers in the present experiments probably had non-pulsatile LH secretion since the anterior pituitary was desensitised to natural sequence GnRH.

Tonically elevated basal concentrations of LH in heifers treated with GnRH agonist were associated with elevated plasma concentrations of progesterone relative to control heifers, throughout the treatment period in Experiments 1 and 2. This suggested that basal secretion of LH may be an important factor in the stimulation of progesterone secretion by the corpus luteum. Conversely, since LH secretion was likely non-pulsatile in Experiments 1 and 2, pulsatile LH may not be obligatory of progesterone secretion. The mechanisms by which basal secretion of LH is maintained in agonist-treated heifers, which have a desensitised anterior pituitary gland, are not understood. Maintenance of

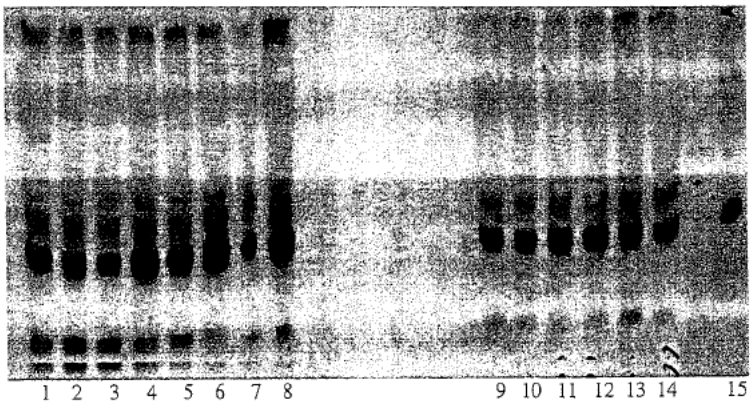


Figure 3.6: Representative autoradiographed Western blot for StAR protein. Lanes 1-8 are Control heifers; lanes 9–14 are heifers treated with deslorelin; lane 15 is a StAR standard.

LH secretion may involve constitutive release of LH that is independent of agonist stimulation or endogenous GnRH, or it may involve agonist stimulation of second messenger pathway systems.

Peters *et al.* (1994) demonstrated in heifers treated with a GnRH antagonist, that in the absence of LH from days 12 – 17 of the oestrous cycle, the corpus luteum continued to function normally; however, the corpus luteum required pulsatile LH support from Days 2 – 12 of the oestrous cycle. They concluded that in the absence of pulsatile LH secretion, basal concentrations of LH probably contributed to the development of an 'inferior' corpus luteum. In the present experiments, tonically elevated basal concentrations of LH were more than sufficient to maintain a fully functional corpus luteum in heifers treated with deslorelin, with plasma progesterone concentrations greater than in control heifers.

In the present experiment, amounts of StAR protein were increased in the luteal cells of heifers treated with GnRH agonist compared with control heifers. In the Stocco and Clark (1996) model for StAR protein production and function, StAR protein is synthesized in response to trophic hormone stimulation. Without further StAR protein synthesis, cholesterol transport to the mitochondria ceases. It has been demonstrated in sheep (Juengel *et al.*, 1995) that hypophysectomy results in decreased StAR protein mRNA amounts in the corpus luteum and decreased serum progesterone concentrations. The effect was reversible upon treatment with LH or growth hormone. Thus, since LH initiates StAR protein synthesis, it could be assumed that tonically elevated concentrations of LH might result in constant production of StAR protein, which may lead to constant transport of cholesterol to the mitochondria and therefore increased progesterone secretion. It may be possible that the increased amounts of cholesterol

being transported to the inner mitochondrial membrane may stimulate the synthesis of P450scc and 3β-HSD enzyme 'complexes' within the mitochondrial membranes which in turn aids in increased progesterone production.

As previously discussed (Section 1.6.3), 3β-HSD mRNA was maximal by Day 3 of the oestrous cycle and remained relatively constant through to Day 12 (Niswender et al., 1994). In the same study, maximum P450scc mRNA was not reached until mid-luteal phase, indicating that there is a maximal "pool" of 3β-HSD present by Day 3 of the oestrous cycle (Niswender *et al.*, 1994). In the present study, amounts of 3β-HSD were not significantly different between control heifers and those treated with deslorelin. As discussed in Section 1.6.3, the half-life of P450scc in bovine adrenocortical cells is 40 h (Boggaram *et al.*, 1984) and that of 3β-HSD has not been measured. It is likely that the enzymes do not decline at the same rate as the messenger RNAs; hence P450scc or 3β-HSD levels probably do not regulate the fall in plasma concentrations of progesterone associated with luteolysis, or increased progesterone secretion during luteal development. It is concluded, therefore, that LH-induced increases in StAR protein was likely directly responsible for increased progesterone secretion in heifers treated with deslorelin in the present study.

Chapter 4: Relationships between stage of the first follicular wave of the oestrous cycle and ovulatory response to treatment with deslorelin.

4.0 Introduction

When treatment with a GnRH agonist is initiated on different days of the oestrous cycle in cattle, different follicular responses occur (Table 1.1; Macmillan and Thatcher, 1991; Gong et al., 1995; Schmitt et al., 1996a,b,c). Treatment with GnRH agonist on Day 5 (Gong *et al.*, 1995; Schmitt *et al.*, 1996a; Rajamahendran *et al.*, 1999) or Day 12 (Macmillan and Thatcher, 1991; Wolfenson *et al.*, 1994) of the oestrous cycle induced ovulation of the respective dominant follicle of the first or second follicular waves. Ovulation of the dominant follicle was associated with the formation of an accessory corpus luteum and relatively greater plasma concentrations of progesterone during the luteal phase (Macmillan and Thatcher, 1991).

If GnRH agonist treatment commences when a preovulatory follicle is not present, ovulation does not occur. Treatment with GnRH agonist 6 to 12 h after oestrous resulted in acute LH release, with no effect on progesterone secretion (Coleman et al., 1988). Milvae et al. (1984) administered GnRH agonist injections 4 times daily from Days 9 to 12 of the oestrous cycle, leading to increased progesterone secretion. If GnRH agonist treatment was commenced in the mid-luteal phase, plasma concentrations of progesterone were decreased but structural luteolysis was delayed (Macmillan et al., 1985b). Treatment with GnRH agonist did not effect the time when luteolysis of either the induced or spontaneous corpora lutea occurred (Gong *et al.*, 1995; Rajamahendran et al., 1999).

The differing responses observed when GnRH agonist treatment is initiated at

different stages of the oestrous cycle may be related to the amplitude and/or duration of the acute LH response. At threshold concentrations, oestradiol provides positive feedback on LH release from the anterior pituitary gland. Hence, the acute LH response may differ depending on the amount of ovarian oestrogenic activity.

The aims in the present experiment were to determine: (i) the relationships between size of the largest follicle present in the ovary, the acute LH response to treatment with GnRH agonist, and ovulation in heifers treated with GnRH agonist at different times during the first follicular wave of the oestrous cycle; and (ii) plasma concentrations of progesterone during the subsequent luteal phase and life span of the spontaneous and any induced corpora lutea.

4.1 Materials and Methods

4.1.1 Experimental design

Stage of the oestrous cycle was synchronised in Brahman heifers at random stages of the oestrous cycle, by using a CIDR® (Interag, Australia) device for 10 days, with an injection of 2mg oestradiol benzoate (Ciderol, 100μg, Genetics Australia Bacchus Marsh, Victoria, Australia) given on the day of CIDR® implantation. The day before CIDR® removal, heifers received an i.m. injection of $PGF_{2\alpha}$ (500 μg).

Heifers were assigned to a Control Group (n=4) that received no treatment, or treatment groups that were implanted with deslorelin (50 μg/kg live weight/24 h) (Section 2.2) on different days of the oestrous cycle: D2 (n=4) implanted with deslorelin on Day 2; D4 (n=4) implanted with deslorelin on Day 4; D6 (n=4) implanted with deslorelin on Day 6; or D8 (n=4), implanted with deslorelin on Day 8 of the oestrous cycle. Ovarian follicle and corpus luteum status were monitored using ultrasonography for 39 days. Blood samples were collected every second day throughout the experiment

to determine changes in plasma concentrations of LH and progesterone.

4.2 Results

Treatment with deslorelin induced an acute increase in plasma concentrations of LH (Figure 4.1). The amplitude of the acute rise in plasma LH differed among the treatment groups (Table 4.1). The acute increase in LH was greater ($P<0.05$) for the D6 heifers compared with the D2, D4 and D8 heifers (Table 4.1). There was no significant differences observed between the amplitude of the acute rise in plasma LH of the D2 and D4 heifers or the D4 and the D8 heifers (Table 4.1).

The oestrous cycles of 4 of 4 Control heifers consisted of three waves of follicular growth. The formation of a corpus luteum in all Control heifers was first noticeable by ultrasound on Day 2 of the oestrous cycle, and the corpus luteum showed signs of regression by Day 20. On Day 2 of the cycle, each Control heifer had a small corpus haemorragicum. The corpus luteum from the first oestrous cycle in Control heifers regressed by Day 20 of treatment in 4/4 heifers.

Heifers treated with deslorelin commencing on Day 2 of the oestrous cycle had follicles with a maximum diameter of 4.7 ± 0.3 mm at the time of treatment, and did not ovulate or develop an accessory corpus luteum. The spontaneous corpus luteum in the D2 heifers had regressed by Day 20 of treatment in 2 of 4 heifers (#523, #593). In the remaining two heifers, the corpus luteum persisted until Day 29 (#530) and Day 39 (#583) of treatment (Figure 4.2).

The diameter of the largest follicle on Day 4 of the oestrous cycle was 6.5 ± 0.7 mm and 2 of 4 heifers ovulated (#566, #574) and developed an accessory corpus luteum. The diameters of the follicles that ovulated were 5 and 6 mm, respectively. In both heifers the accessory corpus luteum had regressed by Day 22 of the treatment period.

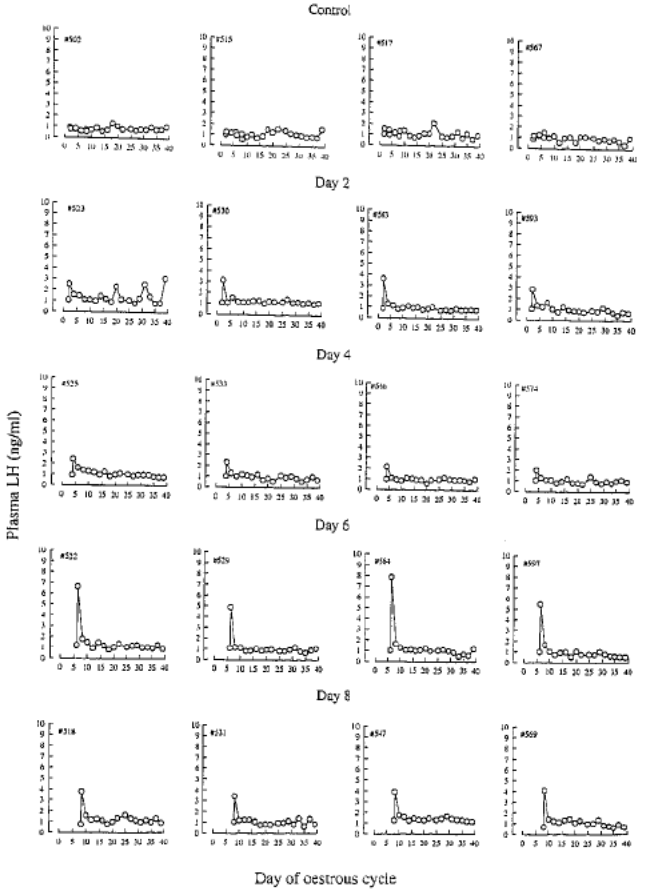


Figure 4.1: Plasma concentrations of LH throughout the treatment period. Control heifers (top panel); Day 2 - heifers implanted on Day 2 had an acute increase in plasma LH and 0/4 ovulations; Day 4 - heifers implanted on Day 4 had an acute increase in plasma LH and 2/4 ovulations; Day 6 - heifers implanted on Day 6 had the largest ($P<0.05$) acute increase in plasma LH and 4/4 ovulations; Day 8 - heifers implanted on Day 8 had an acute rise in LH and 0/4 ovulations.

Table 4.1 Acute increase in plasma concentrations of LH, size of the largest follicle and occurrence of ovulation after GnRH agonist treatment commencing on Day 2 (D2), 4 (D4), 6 (D6), or 8 (D8) of the oestrous cycle in heifers.

Treatment group	Increase in plasma LH ΔLH (ng/ml)	Size of largest follicle (mm)	Ovulation
Control	0.35 ± 0.75^{a}	-	0/4
D2	$2.03\pm0.28^{b,c}$	4.7 ± 0.3^{a}	0/4
D4	1.20 ± 0.11^{b}	6.5 ± 0.7^{b}	2/4
D6	5.14 ± 0.61^{d}	10.0 ± 0.0^{c}	4/4
D8	2.87 ± 0.22^{c}	$8.6\pm0.9^{b,c}$	0/4

^{a,b,c,d} means within columns with different superscripts are significantly different ($P<0.05$)

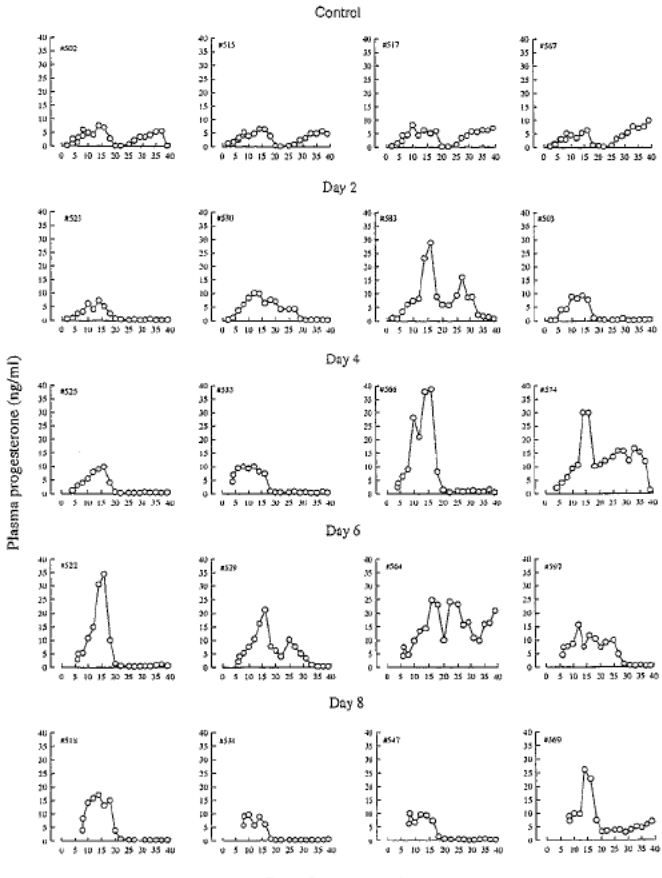


Figure 4.2: Plasma concentrations of progesterone throughout the treatment period. Control heifers (top panel) - 3 follicular waves in 4/4 heifers; Day 2 - heifers implanted on Day 2 had 0/4 ovulations and two persistent CLs (#530, #583); Day 4 - heifers implanted on Day 4 had 2/4 ovulations and one persistent CL (#574); Day 6 - heifers implanted on Day 6 had 4/4 ovulations and three persistent CLs (#529, #564, #597); Day 8 - heifers implanted on Day 8 had 0/4 ovulations and one persistent CL (#569).

The spontaneous corpus luteum in one of these heifers (#566) had regressed by Day 22 of the treatment period, whilst the spontaneous corpus luteum in the remaining heifer (#574) was still present (10 mm) and functional (plasma progesterone > 2 ng/ml) at the end of the treatment period (Day 39). In the two heifers that did not ovulate (#525, #533), the sizes of the largest follicles present at the start of treatment were 6.0 and 7.0 mm respectively, and the spontaneous corpus luteum had regressed by Day 18-20 of the treatment period.

On Day 6 of the oestrous cycle, each heifer had a 10 mm follicle that ovulated and developed into an accessory corpus luteum. In heifer #522, both the spontaneous and accessory corpora lutea had regressed by Day 22 of the treatment period (Figure 4.2). For heifer #597, both the spontaneous and accessory corpora lutea were present until Day 31 of treatment. The accessory corpus luteum in heifer #529 had regressed by Day 22 of the treatment period, while the spontaneous corpus luteum persisted until Day 33. In heifer #564, both the spontaneous and accessory corpora lutea were persistent (10 mm) and the plasma progesterone concentration was 20 ng/ml at the end of the treatment period (Day 39).

Heifers treated with deslorelin on Day 8 of the oestrous cycle had a relatively large follicle (8.6 ± 0.9 mm), but no heifer in this group ovulated. In three of the D8 heifers (#518, #531, #547), the spontaneous corpus luteum had regressed by Day 18, 20 and 22 of the treatment period, respectively (Figure 4.2). In one of the D8 heifers (#569), the spontaneous corpus luteum was persistent with a small diameter (5 mm) and elevated progesterone (7 ng/ml) at the end of treatment (Day 39).

4.3 Discussion

The acute increase in plasma concentrations of LH subsequent to implantation of

heifers with deslorelin was consistent with previous findings (Bergfeld *et al.*, 1996; Rajamahendran *et al.*, 1996). The amplitude of the acute increase in plasma LH differed depending on the day of the oestrous cycle on which deslorelin treatment commenced. The acute increase in plasma LH was relatively small in the D2 heifers compared with the other treatment groups. It is possible that, following the preovulatory LH surge, the stores of LH in the anterior pituitary gland may have been depleted, and so replenishment of the anterior pituitary reserves may not have been complete by Day 2 of the oestrous cycle. The largest acute increase in LH occurred on Day 6 of the oestrous cycle, with the second largest rise occurring on Day 8. Hence, in the present experiment, reserves of LH may have been replenished by Day 6 of the oestrous cycle.

As described in Section 1.3.2.1, plasma concentrations of oestradiol increase to a threshold level at which oestradiol begins to provide a positive feedback stimulus on LH release (McNatty *et al.*, 1999). The D6 dominant follicles were probably functionally and morphologically dominant (i.e. had greater oestrogenic activity) compared with the D8 dominant follicles, which had probably lost their functional dominance supported by the decrease in diameter. As a result, the acute release of LH from the anterior pituitary was greater in heifers treated on Day 6 compared with Day 8. The largest follicles present in the D8 heifers at the initiation of treatment had begun to regress (8.6 ± 0.9 mm on Day 8, compared with 10.0 ± 0.0 mm on Day 6, $P>0.05$) and had likely lost their oestrogenic activity.

All of the D6 heifers ovulated in response to deslorelin treatment and none of the D8 heifers did. The increase in plasma LH in response to treatment with deslorelin was sufficient to induce ovulation of follicles of relatively small diameter (5 to 6 mm). This observation indicated that LH receptors sufficient for a response to a preovulatory LH

surge are present in 5 to 6 mm follicles in Zebu (*Bos indicus*) beef heifers compared with 9 mm in *Bos taurus* crossbreed beef heifers (Xu et al., 1995). Rhodes *et al.* (1995a) reported that maximal size of the first dominant follicle (~ 10 mm) and ovulatory follicle were smaller in *Bos indicus* heifers than those in *Bos taurus* heifers.

In those heifers that were induced to ovulate and develop an accessory corpus luteum, progesterone concentrations were markedly elevated. In Chapter 3, the chronic phase of GnRH agonist treatment was associated with tonically elevated basal LH secretion, which could contribute to increased luteotrophic support of the induced and existing corpora lutea, and hence greater plasma progesterone.

In 4 of 6 treated heifers that developed an accessory corpus luteum, the spontaneous corpus luteum persisted for 11-19 days longer than the spontaneous corpus luteum in control heifers, even after the accessory corpus luteum had regressed. In 3 of 10 treated heifers that did not develop an accessory corpus luteum, the spontaneous corpus luteum persisted for 9-19 days longer than the spontaneous corpus luteum in control heifers. The persistence of the corpus luteum as determined by ultrasonography was associated with elevated progesterone, indicating a functional corpus luteum. The corpus luteum produces oxytocin, which binds to specific uterine receptors to cause the release of prostaglandin $F_{2\alpha}$ (Section 1.5.1). However, constant infusion of heifers with oxytocin during the mid- to late-luteal phase extended the life span of the corpus luteum and inhibited prostaglandin $F_{2\alpha}$ secretion, possibly through down-regulation of endometrial oxytocin receptors (Garverick *et al.*, 1992). In the present study, with the presence of two corpora lutea, the amount of oxytocin may have been sufficient to cause downregulation of oxytocin receptors in the uterus. Additionally, luteotrophic support provided by tonically elevated basal concentrations of LH might have aided the

extension of the life span of the corpus luteum. Injections of LH extended luteal life span in cattle (Donaldson and Hansel, 1965; Schomberg *et al.*, 1967), while administration of anti-bovine LH induced luteolysis of the corpus luteum (Snook *et al.*, 1969).

In the present study, the spontaneous corpus luteum persisted after the accessory corpus luteum had regressed, suggesting that the spontaneous corpus luteum resisted the uterine luteolytic signal. The induced corpus luteum may have increased $PGF_{2\alpha}$ receptors, or increased numbers of large luteal cells in the induced corpus luteum. These possibilities warrant further study.

Treatment with deslorelin on Day 4 to 6 of the oestrous cycle in *Zebu (Bos indicus)* heifers can cause ovulation of the first wave dominant follicle and subsequent formation of an accessory corpus luteum, associated with increased plasma concentrations of progesterone. As discussed in Chapter 3, treatment with GnRH agonist induced tonically elevated plasma concentrations of LH, which were associated with increased size of the corpus luteum, and increased amounts of StAR protein and P450scc enzyme. The increased luteotrophic support for StAR protein synthesis, combined with the larger amount of luteal tissue, likely contributed to the elevated plasma concentrations of progesterone.

Chapter 5: Conclusions and Future Directions

The first aim in this thesis was to gain a better understanding of the pituitary and ovarian basis for increased plasma concentrations of progesterone in heifers treated chronically with GnRH agonist, commencing early in the oestrous cycle. Of particular interest was the effect of treatment with GnRH agonist on content in the corpus luteum of the newly discovered steroidogenic acute regulatory (StAR) protein. The StAR protein controls the transport of cholesterol from the outer to inner mitochondrial membrane, which is considered to be the rate-limiting step in steroid biosynthesis (Stocco, 1997).

The acute response of the anterior pituitary gland to GnRH agonist treatment consists of a significant increase in concentrations of plasma LH. The chronic response of the anterior pituitary gland to treatment with a GnRH agonist in cattle consists of non-pulsatile secretion of LH; however, concentrations of plasma LH are tonically elevated for at least 10 days after initiation of treatment. Longer-term treatment with GnRH agonist was associated with non-pulsatile secretion of LH, but not necessarily elevated basal concentrations of LH (Gong *et al.*, 1996).

In the present study, when heifers were treated with GnRH agonist from Day 2 to Day 13 of the oestrous cycle, the pituitary became desensitised to exogenous natural sequence GnRH and plasma concentrations of LH were tonically elevated compared with control heifers. Additionally, plasma concentrations of progesterone were increased compared with control heifers. Increased plasma concentrations of progesterone were associated with increased size of the corpus luteum and increased amounts of StAR protein and cytochrome P450scc enzyme. While amounts of 3β-HSD

were not significantly increased compared with control heifers in the present study, they tended to be greater in deslorelin-treated heifers compared with control heifers.

Increased LH may have provided increased trophic stimulation of StAR protein synthesis (Stocco, 1997). Subsequently, it is possible that the increased amounts of cholesterol being transported to the inner mitochondrial membrane may have stimulated the synthesis of P450scc enzyme and, in turn increased progesterone synthesis.

This thesis has demonstrated for the first time greater relative amounts of StAR protein and P450scc enzyme in the corpus luteum of heifers treated with GnRH agonist. This was associated with increased content of progesterone in the corpus luteum and elevated plasma concentrations of progesterone.

Since plasma concentrations of progesterone are increased in the presumed absence of pulsatile secretion of LH in heifers treated with GnRH agonist, it is possible that basal LH secretion may be a key factor in controlling progesterone synthesis. Additionally, pulsatile secretion of LH may not be an absolute requirement for luteal growth and progesterone synthesis.

The second aim in this thesis was to establish the relationships between the acute increase in plasma LH and associated ovarian follicular response, when treatment with GnRH agonist was initiated on different days during the first follicular wave of the oestrous cycle. When treatment was commenced on Day 4 or Day 6 of the oestrous cycle, the first wave dominant follicle was induced to ovulate, resulting in the formation of an accessory corpus luteum, and increased plasma concentrations of progesterone. In heifers that developed an accessory CL, this CL regressed at the normal time of the oestrous cycle, whilst in 4 of 6 of the heifers the spontaneous CL persisted for up to 19 days after the induced corpus luteum had regressed. Luteal regression occurs when

oxytocin is secreted by the corpus luteum to stimulate $PGF_{2\alpha}$ release from the uterus. $PGF_{2\alpha}$ subsequently binds to large luteal cells, causing luteolysis. It would be expected that both the spontaneous and induced corpora lutea would respond to prostaglandin and regress simultaneously. However, the presence of two corpora lutea may provide increased oxytocin sufficient to cause downregulation of endometrial oxytocin receptors, thereby reducing secretion of $PGF_{2\alpha}$. The preferential receptiveness of the induced corpus luteum to luteolysis, compared with the spontaneous corpus luteum, may have been the result of increased $PGF_{2\alpha}$ receptor expression or increased numbers of large luteal cells in the induced corpus luteum. However, this requires further study.

Treatment with GnRH agonist commencing on Day 6 of the oestrous cycle resulted in ovulation of the largest growing follicle in a proportion of heifers. By Day 6 of the oestrous cycle, when the dominant follicle had reached 10 mm, ovulation occurred in all heifers in response to the acute rise in plasma LH. In the present study, treatment with GnRH agonist commencing on Day 4 resulted in an acute rise in plasma LH which induced ovulation of follicles as small as 5 to 6 mm in diameter, indicating that in Zebu (*Bos indicus*) genotypes, sufficient LH receptors are present on follicles of this size, compared with 9 mm which was reported for *Bos taurus* genotypes (Xu *et al.*, 1995a).

The amplitude of the acute rise in LH was greater on Day 6 of the oestrous cycle compared with Days 2, 4 and 8. The suggestion has been made in this thesis that this was due to positive feedback from oestradiol secreted from the dominant follicle on Day 6. Also on Day 6, the dominant follicles were of greater diameter that on Days 2, 4 and 6. A relatively large increase in plasma LH combined with a large growing dominant follicle probably combined to result in ovulation, and formation of an

accessory CL in 4/4 heifers on Day 6.

The use of GnRH agonists to increase plasma concentrations of progesterone has potential for application in artificial insemination and embryo transfer programs. Increased maternal plasma concentrations of progesterone at the time of conception or embryo transfer may aid in embryo survival by providing an optimal uterine environment (Section 1.5.3). A practical, reliable and relatively non-invasive method for increasing maternal plasma concentrations of progesterone would be worthwhile. The GnRH agonist bioimplant (deslorelin) used in the present thesis provides a new practical approach to examining relationships between maternal plasma concentrations of progesterone and conception rates. In the present thesis, implanting heifers with deslorelin on Days 4 to 8 of the oestrous cycle increased the life span of the endogenous corpus luteum in a proportion of heifers, which may provide an advantage in the establishment of pregnancy.

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