Chapter 1 Literature Review

Ovarian cysts occur in 6-19% of dairy cattle (Kesler & Garverick, 1982) and can affect production to varying degrees of severity, including an extended calving to conception interval that can result in economic loss to the farmer, a decreased pregnancy rate at first insemination, and an increased number of inseminations per conception (Peter, 2004; Shrestha, *et al.,* 2004). Furthermore, they may also result in a reduction in the lifetime milking record of the animal and an increased number of involuntary culls (Chavatte, *et al.*, 1992). Some cows develop an ovarian cyst that is spontaneously resolved before it would ordinarily be noticed at the routine pre-breeding examination 8 weeks post-partum, while others develop a large non-steroidogenic cyst but may otherwise be cycling normally. However, importantly, some cows develop large steroidogenic ovarian cysts that suppress normal ovarian function by maintaining high concentrations of either oestradiol or progesterone. This will adversely affect the oestrous cycle, preventing luteolysis and a new wave of pre-ovulatory follicle growth. Dairy production is a very intense industry and animals that repeatedly fail to be observed in oestrus and/or conceiving normally are inevitably culled as the relationship between the cost of keeping the cow and its profitability becomes unfavourable (Mäntysaari *et al.,* 1993). This is reportedly costing the farmer approximately 10% of their annual income (Dijkhuizen *et al.,* 1985).

1.1 Ovarian Function

Production and ovulation of oocytes as well as synthesis and secretion of gonadal hormones (oestradiol and progesterone) are three of the main functions of the ovary, all of which are interrelated. Both processes are aided by positive and negative feedback of various hypothalamic and pituitary hormones in a cycle that typically lasts 21 days (range: 17-24d) in cattle (Ahmad *et al.*, 1997).

The ovary contains 1) a central medulla that houses blood and lymphatic vessels and nerves; 2) the ovarian cortex which is covered by a superficial simple squamous to cuboidal epithelium thought to give rise to germ cells and also which contains ovarian follicles (in most species); 3) the tunica albuginea comprised of a layer of irregular connective tissue; and 4) the surface epithelium, through which ovulation occurs (Figure 1.1).

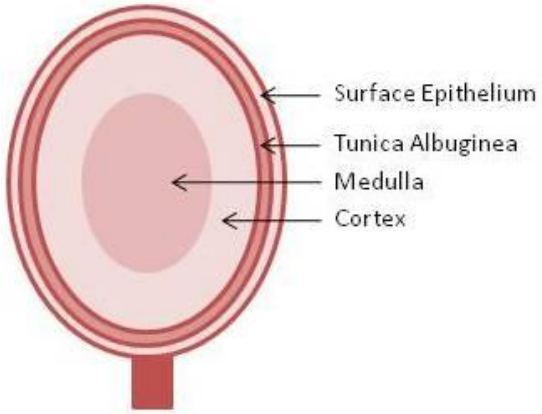

Figure 1.1: Layers of the ovary (minus follicular structures)

1.2 Control of ovarian dynamics

Ovarian dynamics are regulated both locally by hormones, steroids, cytokines and growth factors secreted from the gonads, and remotely by the hypothalamus and pituitary hormones binding to receptors in target tissues (Table 1.1).

Table 1.1: A summary of key ligands and the location of their receptors.

Ligand	Secretory Gland	Location of Receptors	Reference
Gonadotropin releasing hormone	Hypothalamus	Surface of pituitary gonadotrope cells	Millar, 2005
Luteinising hormone	Anterior Pituitary	Theca cells Granulosa cells	Dufau *et al.*, 1998 Xu *et al.*, 1995
Follicle stimulating hormone	Anterior Pituitary	Granulosa cells	Wunsch *et al.*, 2007
Oestradiol	Granulosa Cells	Granulosa and theca cells, corpus luteum Hypothalamus Anterior pituitary gland Endometrium	Berisha *et al.*, 2002 Lane *et al.*, 2009
Progesterone	Corpus Luteum	Granulosa and theca cells, corpus luteum, most cells of the ovary Hypothalamus Endometrium	Berisha *et al.*, 2002 D'Haeseleer *et al.*, 2007 Lane *et al.*, 2009
Prostaglandin $F_{2\alpha}$	Uterus	Luteal cells of the corpus luteum	Anderson *et al.*, 2001
Oxytocin	Corpus Luteum Posterior Pituitary	Granulosa cells Endometrium	Uenoyama & Okuda, 1997

Release of gonadotropin releasing hormone (GnRH) from the hypothalamus is controlled by two separate regions made up of clusters of nerve bodies called hypothalamic nuclei. The tonic centre is responsible for basal release of GnRH and can be likened to the action of a dripping tap, while the surge centre of the hypothalamus is responsible for a surge in luteinising hormone (LH) that occurs just once during a complete oestrous cycle in the cow. Both centres also secrete small basal LH pulses throughout the oestrous cycle.

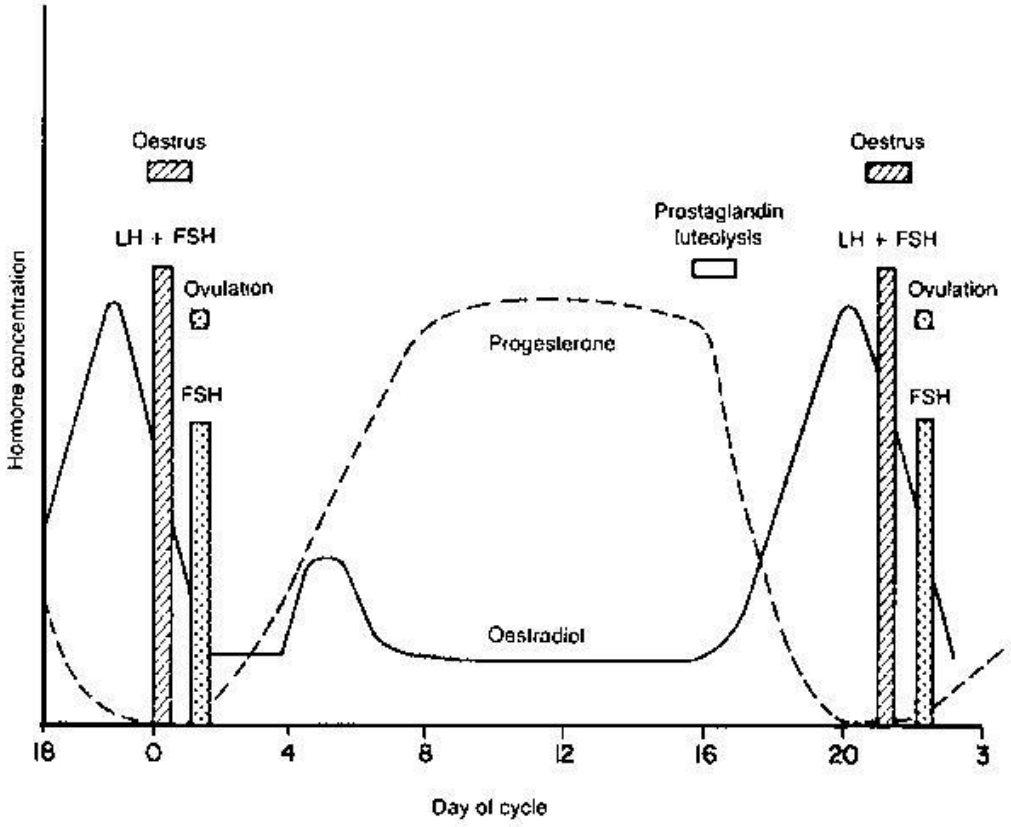

Figure 1.2: Steroidogenic profiles during the bovine oestrous cycle, Peters & Lamming (1983).

Pulsatile release of GnRH from the tonic centre of the hypothalamus travels to the anterior pituitary, via the hypophyseal portal of blood vessels, and stimulates release of LH and follicle stimulating hormone (FSH). At the

ovarian level, FSH stimulates further antral follicle development and targets granulosa cells to increase oestradiol synthesis. When progesterone secretion from the regressing corpus luteum (CL) is low and oestradiol secretion from the dominant follicle is high, oestradiol positively feedbacks on the surge centre of the hypothalamus, stimulating further release of GnRH and thus, LH and FSH. Ovulation is controlled by the aforementioned LH surge in the underlying endocrine combination of low progesterone and high oestradiol concentration. Post ovulation, LH secreted at basal levels promotes development of the CL by acting on the luteinising steroidogenic cells of the theca interna, and progesterone secretion will ultimately suppress further FSH secretion (negative feedback), thus inhibiting pre-ovulatory follicle development. At the end of the luteal phase, the CL regresses and subsequently, GnRH pulse frequency begins to increase in the early follicular phase due to low secretion of progesterone (Figure 1.2, Figure 1.3)

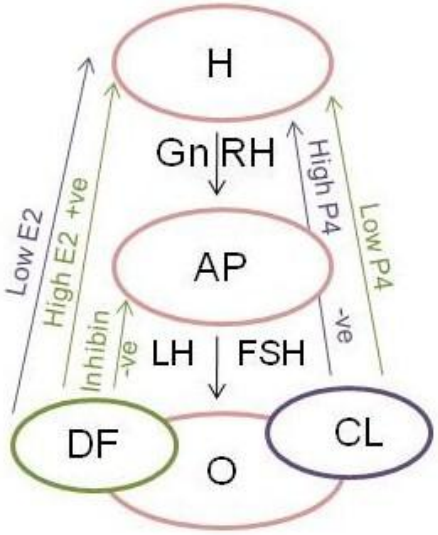

Figure 1.3: A schematic representation of positive and negative feedback when there is a dominant follicle (DF) or a corpus luteum (CL) as the dominant structure on the ovary. Removal of the progesterone (P4) block as well as high oestradiol (E2) during follicular dominance results in increased gonadotropin releasing hormone (GnRH), and increased luteinising hormone (LH) and follicle stimulating hormone (FSH) (positive feedback). Eventually inhibin is secreted from the granulosa cells of the dominant follicle which inhibits further FSH secretion (negative feedback) and prevents development of subordinate follicles. When a CL is the dominant structure on the ovary, high progesterone and low oestradiol inhibit release of GnRH, and subsequently LH and FSH, (negative feedback) resulting in suppression of follicle development. H=hypothalamus; AP= anterior pituitary; O=ovary.

1.3 Folliculogenesis

1.3.1 Follicle classification

For the purpose of this review, ovarian follicles will be classified using a system developed by Braw-Tal & Yossefi, (1997). Classification criteria is not limited to follicle diameter or responsiveness to circulating steroids, but also includes the number of cell layers as well as definition between each cell type. Follicle classes are outlined in Figure 1.4 and Table 1.2.

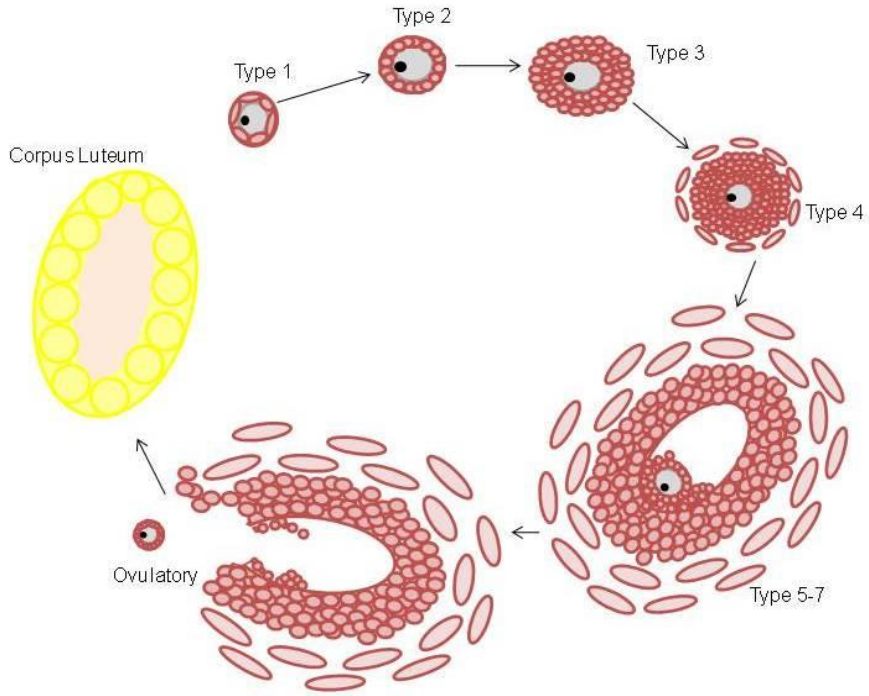

Figure 1.4: A schematic representation of follicle development from primordial (type 1) follicles through to post ovulation and corpus luteum formation, based on the follicle classification system of Braw-Tal & Yossefi (1997).

Table 1.2: Classification and characterisation of bovine follicles (Braw-Tal & Yossefi., 1997) with the addition of 2 further categories.

GC = granulosa cell. [1] range in μm; [2]Oocyte diameter in μm (mean ± SEM).

Follicle	Layers of GC	Number of GC (range)	Follicle diameter[1]	Oocyte diameter[2]	Presence of a zona pellucida	Clearly defined theca interna
Primordial (type 1)	1	<10 flattened	<40	29.75 ±0.30	-	-
Primary (type 2)	1-1.5	10-40 cuboidal	40-80	31.12 ±0.42	-	-
Small preantral (type 3)	2-3	41-100	81-130	49.48 ±2.43	-	-
Large preantral (type 4)	4-6	101-250	131-250	68.61 ±2.78	+	+
Small antral (type 5)	>6	>250	250-500	92.90 ±4.50	++	++
N.B: type 5 follicles extended to include follicles up to 5mm in diameter for the purposes of this review						
Large antral (type 6)	Large antral follicles greater than 5mm but less than 10mm will be included as type 6 follicles					
Pre-ovulatory (type 7)	Pre-ovulatory follicles 10-20mm in diameter will be included as type 7 follicles					

Despite the extensive criteria used to make up this classification system, two additional categories have been included to facilitate discussion of the various endocrinological changes associated with the advancing stages of follicle development (Figure 1.4). This division of follicle growth is useful for the

purpose of this review as ultimately ovarian cyst formation may occur during the latter stages of follicular growth.

1.3.2 Overall pattern of growth

Oogenesis and folliculogenesis begin *in utero*, with differentiation of primordial germ cells into primary oocytes by day 60 of gestation in the bovine. By day 90, the first follicular cells have developed, and at midterm, following mitosis of cells from the primordial follicles, some vesicular follicles can be seen in sections of fetal ovarian tissue (Marion & Gier, 1971). During fetal life oogenesis is arrested during the first meiotic prophase, resulting in a tetraploid primary oocyte. In adult ovaries oogenesis will resume, post LH surge, with the first, then second, meiotic division following which, degradation of accompanying polar bodies results in a haploid ovum (Dilugi *et al.,* 2008). By birth, the ovaries will maintain a pool of follicles that will develop and ovulate, or degenerate during the lifetime of the animal (Hirshfield, 1991).

In the adult ovary, antral follicular development can be divided into 3 steps: recruitment, selection, and dominance. Recruitment is the process by which a cohort of antral follicles begins to grow and produce oestradiol. The point, at which some follicles are selected to continue to develop with one becoming dominant, is known as ‘selection’ or ‘deviation’. Dominance in poly-oestrous species such as the cow is usually characterised by the maturation of only one antral follicle to a pre-ovulatory size while all others enter atresia (Pierson & Ginther, 1988), however multiple ovulations and split

embryos can result in twins. Deviation can be defined to have occurred when there is the greatest difference in size between the two largest follicles but once the second largest follicle has reached its maximum size (Ginther *et al.*, 1996). Antrum volume of a large antral bovine follicle can occupy up to 95% of total follicle volume (Rodgers *et al.*, 2001).

Understanding follicular waves is important as it provides a basis for the development of protocols of oestrous cycle synchronisation and superovulation, through a combination of intra-vaginal progesterone releasing devices and prostaglandin, as well as FSH and GnRH agonists respectively (Savio *et al.*, 1991; Ginther *et al.*, 1996). These procedures have practical applications in agriculture and human medicine; in an agricultural environment, the synchronisation of oestrous cycles of numerous animals allows for better animal management, while in human medicine and research, superovulation is a useful tool in oocyte studies as well as IVF procedures.

1.3.3 Pre-antral follicle growth and regulation

Primordial follicles are situated below the tunica albuginea, they are quiescent follicles ellipsoidal in shape measuring <40μm in diameter (Braw-Tal & Yossefi, 1997), showing little or no biological activity. They contain an immature oocyte arrested before the first meiotic division and are surrounded by flat squamous granulosa cells and a basal lamina (van Wezel & Rodgers, 1996; Rodgers & Irving-Rodgers, 2010). Histological examination of primordial follicles from bovine ovaries showed each primordial follicle contained 24 granulosa cells (van Wezel & Rodgers, 1996). Formation of

primordial follicles occurs during fetal life in the cow and ceases after birth (Yang & Fortune, 2008, Scaramuzzi *et al.,* 2011). Activation of follicles, i.e. the transition from the inactive primordial follicle to the primary follicle, can be defined as the time when the oocyte begins to enlarge and the granulosa cells begin to proliferate (van Wezel & Rodgers, 1996). However, the mechanisms by which this occurs are still unclear (Scaramuzzi *et al.,* 1993; Findlay *et al.,* 1996; Armstrong *et al.,* 2000). Primary follicles consist of the primary oocyte surrounded by a single layer of cuboidal granulosa cells and measure approximately 40-80μm in diameter (Braw-Tal & Yossefi, 1997). Despite recent research into the mechanisms that drive the transformation from flattened to cuboidal granulosa cell shapes, it is still unclear as to what signal initiates this process. However, it has been shown that cuboidal granulosa cells divide at an increased velocity in comparison to flattened granulosa cells, reaching maximum packing density on the basal lamina and resulting in the formation of a second layer (Da Silva-Buttkus *et al.,* 2008). A small pre-antral follicle (type 3) is characterised by 2-3 layers of granulosa cells measuring approximately 81-130μm in diameter with no antrum. A large pre-antral follicle (type 4) can be identified by 4-6 layers of granulosa cells, the presence of a zona pellucida, clearly defined theca interna and measures 130-250μm in diameter (Braw-Tal & Yossefi, 1997). In other literature, small pre-antral and large pre-antral follicles may otherwise be referred to as secondary follicles.

Modulation of granulosa cell proliferation and basal lamina synthesis at this stage is likely to be controlled by a series of locally produced factors due to the location of primordial follicles in an avascular region of the ovary (van Wezel & Rodgers, 1996) (Table 1.3) (Figure 1.5).

Table 1.3: A summary of the role played by key gonadotropins, hormones and growth factors during pre-antral follicle development.

Gonadotropin/ Hormone/Factor	Function	Reference
Luteinising hormone	Promotes thecal cell androgen production, as well as, growth factor production	Orisaka *et al.*, 2009
Follicle stimulating hormone	Induces aromatase expression resulting in increased conversion of thecal androgen to granulosal oestradiol	Orisaka *et al.*, 2009
Testosterone	Proliferation of granulosa cells	Yang & Fortune, 2006
Growth differentiation factor-9	Proliferation of granulosa layers	Dong *et al.*, 1996 Hayashi *et al.*, 1999 Orisaka *et al.*, 2009
KIT ligand	Maintains meiotic arrest Increased organisation of thecal layer Induction of primordial follicle development Stimulates expression of KGF & HGF Regulates theca cell function, growth and androgen production	Godin *et al.*,1991 Ismail *et al.*, 1996 Parrott and Skinner 1997a Parrott & Skinner 1999 Gougeon *et al.*, 2010 Guglielmo *et al.*, 2010
Basic fibroblast growth factor (bFGF)	Regulates granulosa cell mitosis, steroidogenesis and differentiation	Neufeld *et al.*, 1987 Nilsson & Skinner 2001
Keratinocyte growth factor (KGF) Hepatocyte growth factor (HGF)	Increased granulosa and theca cell proliferation	Parrott & Skinner 1997c, 1998
Insulin-like growth factor-1	Not essential for pre-antral follicle growth	Monget *et al.*, 2002; Silva *et al.*, 2009

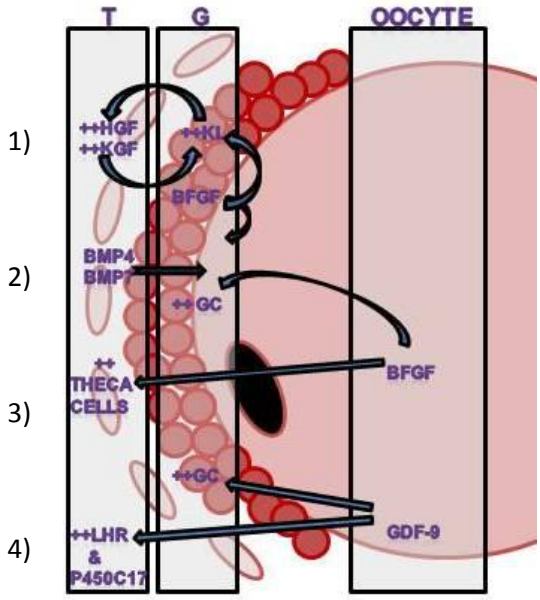

Figure 1.5: A summary of the interactions between various growth factors regulating pre-antral follicle growth; 1) KIT ligand (KL), produced by granulosa cells, is important in regulating organisation of thecal cells, forming a positive feedback loop with keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF), to regulate theca cell function and up regulate androgen production (Parrott *et al.*, 1997b). 2) Bone morphogenetic protein (BMP) -4 and -7 increase follicle stimulating hormone binding and synthesis, enhance luteinising hormone action and androgen production and increase granulosa cell proliferation (Knight & Glister, 2006). 3) Basic fibroblast growth factor (bFGF) regulate mitosis, steroidogenesis and differentiation of granulosa cells and have both autocrine and paracrine effects to increase granulosa cells proliferation (Nilsson *et al.*, 2001; Nilsson & Skinner, 2001). 4) Growth differentiation factor (GDF) -9 regulates development of granulosa layer and promotes production of CYP17A1, LH receptors (LHr) and KL (Dong *et al.*, 1996; Orisaka *et al.*, 2009).

While levels of growth factors are high, levels of aromatase activity in bovine granulosa cells are low (Parrott & Skinner, 1997c; 1998), the authors

hypothesise that these growth factors promote cellular proliferation at the expense of steroidogenic capacity.

During pre-antral follicle growth, LH receptors (LHr) are localised exclusively on theca cells whilst FSH receptors (FSHr) are unique to granulosa cells (Braw-Tal & Roth, 2005; Orisaka *et al.*, 2009). Evidence for the stimulatory role for FSH during pre-antral follicle growth is reported by Gutierrez *et al.,* (2000), who demonstrated that the addition of FSH to culture media increased the probability (from 0.19 to 0.55) of pre-antral follicles reaching antral size *in vitro.* Differentiation of the ovarian cortex, but not medullary, stromal cells into theca cells and the acquisition of LH responsiveness are stimulated by the presence of granulosa cells (Orisaka *et al.,* 2006b). Co-culturing granulosa cells with ovarian cortex cells also resulted in an increase in LHr abundance as well as LH-induced cAMP and androstenedione production (Orisaka *et al.,* 2006b), demonstrating that early follicle development is also controlled by presence of granulosa cells.

Testosterone plays a key role in the development of primary to secondary follicles. For example, during culture studies, the development of primary to secondary follicles was observed when cultured with testosterone but not when treated with oestradiol (Yang & Fortune, 2006). Furthermore, addition of an androgen receptor antagonist negated the effects of testosterone (Yang & Fortune, 2006), indicating that testosterone, rather than oestradiol, is important during early folliculogenesis and that its effects are mediated through androgen receptors. Moreover, androgen receptors have been localised in small pre-antral follicles of rodents (Tetsuka *et al.,* 1995). Steroidogenic acute regulatory protein (StAR) is a transport protein

responsible for transporting cholesterol to the inner mitochondrial membrane where it is converted to pregnenolone by CYP11A1 (Rodgers *et al.,* 1986). mRNA for *CYP17A1*, a key enzyme in steroidogenesis, has been reported in the theca interna, but not granulosa, of large type 4 pre-antral follicles (Braw-Tal & Roth, 2005), but despite the presence of CYP17A1 there is a distinct lack of StAR in theca cells at this stage of development.

It is worth noting that caution should be taken when drawing comparisons between rodents and cattle due to more progressive development of folliculogenesis observed in the rodent ovary. This is particularly important in relation to the acquisition of the theca cell layer occurring earlier in the rodent ovary when compared to other species (Knight & Glister, 2006).

The above evidence collated demonstrates how hormones control pre-antral follicle growth through indirect, growth factor mediated pathways (Orisaka *et al.,* 2006). Despite their importance, at this stage levels of KIT ligand (KL), hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) actually peak during antral follicle development, indicating an on-going role for these growth factors during both pre-antral and antral follicle development (Parrott *et al.,* 1997a,b; 1998) Pre-antral follicles seem to have the initial capacity to synthesise progesterone and following subsequent development of theca cells, are then enabled to begin the synthesis and secretion of androstenedione and testosterone. As androstenedione is the substrate precursor for oestradiol production, it is assumed that the ability to synthesise and secrete oestradiol occurs later in the antral stage of follicular growth.

1.3.4 Antral follicle growth

Both the theca and granulosa layers of antral follicles will undergo morphological differentiation; the theca into a well vascularised, steroidogenic theca interna, and a less well vascularised theca externa; and the granulosa into stratum granulosa, cumulus oophorus and corona radiata (Motta *et al.,* 1991). Follicles form a large antrum in order to allow for lateral expansion of the granulosa cells, which otherwise would expand medially into an avascular environment (Rodgers *et al.,* 2001). The theca interna provides the granulosa cells with androstenedione which is metabolised to oestradiol in the granulosa cells by CYP19A1, otherwise known as the 'two cell - two gonadotropin hypothesis' (Figure 1.6) (McNatty *et al.,* 1992; Rodgers *et al.,* 2001). Furthermore, there is evidence that communication between the granulosa and theca layers results in "reciprocal modulation" of proliferation and function of both layers. For example, co-culture systems have shown how not only cell morphology, but also cell density and steroidogenic capacity of both granulosa and theca cells are affected by the presence of theca and granulosa cells respectively (Kotsuji *et al.,* 1990). Responsiveness to gonadotropins can be defined as the point in time when the follicle begins to express the appropriate receptors as well as intracellular signal transduction systems (Findlay *et al.,* 1996).

Small antral follicles (type 5) measure 250-500μm in diameter (Braw-Tal & Yossefi, 1997), extended to 5mm for the purpose of this review. Large antral follicles measuring 5.1–9.9mm in diameter will be included as type 6 follicles in order to discuss the endocrinological changes observed at deviation,

(in other literature, type 5 and type 6 follicles may otherwise be referred to as early or late tertiary follicles respectively).

Table 1.4: A summary of the role played by key gonadotropins, hormones and growth factors during type 5 follicle development. **NB:** IGF-II is the predominant IGF ligand in ruminants.

A4 = androstenedione; P4 = progesterone; LHr = luteinising hormone receptor.

Gonadotropin/ Hormone/Factor	Function	Reference
Follicle stimulating hormone	Follicular recruitment ↑ *CYP19A1* mRNA expression ↑ P4 in granulosa cells	Ginther *et al.*, 1998 Webb *et al.*, 1999 Orisaka *et al.*, 2006a,b
Insulin-like growth factor-I	Proliferation of granulosa and theca cells	Perks *et al.*, 1999
Insulin-like growth factor-II	Proliferation of granulosa and theca cells	Armstrong *et al.*, 2000
Growth differentiation factor-9	↑ Theca cell proliferation ↓ P4 and A4 ↓ Expression of LHr and *CYP11A1* mRNA	Spicer *et al.*, 2008

Type 5 follicles can be described as gonadotropin independent as they do not require the gonadotropins LH or FSH to develop ≤4mm in diameter (Gong *et al.*, 1996). However, growth of follicles up to 9mm is dependent on FSH; before follicles then become dependent on LH for further growth (Gong *et al.*, 1996; Webb *et al.*, 1999). During all these phases locally produced cytokines and growth factors will have influenced follicular development (Orisaka *et al.*, 2009). FSH stimulates production of inhibin from the granulosa cells and

inhibin has a negative feedback effect on the anterior pituitary to inhibit further FSH secretion (Ginther *et al.,* 2001a, Figure 1.3, page 6). In sheep, FSHr have been identified in follicles with as few as two layers of granulosa cells (Tisdall *et al.,* 1995). In cattle, suppression of FSH, during follicle growth when the largest follicle is 6mm in diameter, resulted in restricted size of the dominant follicle and inhibition of subordinate follicle growth (Ginther *et al.,* 2000b). Collectively, this evidence clearly demonstrates that FSH is still needed for further growth up to and even after deviation, but in a diminished capacity.

The IGF family consists of two ligands; insulin-like growth factor I (IGF I) and IGF-II, two receptors, type 1 and type 2, and a family of IGF-binding proteins (IGFBP) (Adashi, 1998). IGF-I is secreted by the liver under the control of growth hormone (GH) which is secreted from the anterior pituitary gland (Laron, 2001). During folliculogenesis, IGF-II is produced by the theca cells of the ovary where it has autocrine (theca) and paracrine (granulosa) effects, encouraging cell proliferation (Voutilainen & Miller, 1987). It is believed that presence of IGFBP-2, -4 and -5 in gonadotropin independent follicles prevent binding of IGFs to their receptors, thus preventing initiation of the AKT signalling pathway, a stimulator of cell growth and proliferation (Webb *et al.,* 1999; Orisaka *et al.,* 2006a).

IGF-II from theca cells is the predominant locally produced IGF ligand in ruminants, from the time of antrum formation and throughout dominance (Armstrong *et al.,* 2000). At same time mRNA for the type 1 IGF receptor has been identified in both granulosa and theca cells (GC>TC) of antral follicles (Armstrong *et al.,* 2000). In cattle, treatment with GH has been shown to increase the circulating levels of IGF-I in serum leading to a subsequent

increase in the number of healthy follicles 2-5mm in diameter (Gong *et al.*, 1991) providing clear evidence for the stimulatory role of IGF during antral follicle development (Silva *et al.*, 2009). However, in IGF-I knock-out mice, administration of exogenous IGF-I ligand failed to induce growth of previously arrested follicles (Monget *et al.*, 2002).

The addition of insulin into media containing LH during the culture of large antral bovine follicles resulted in increased theca cell proliferation, as well as an increase in progesterone and androstenedione. This demonstrated that insulin directly influences antral follicle development and steroidogenesis. Interestingly, this was mediated through increased receptors for LH but not IGF-I (Stewart *et al.*, 1995).

In early antral bovine follicles, growth differentiation factor (GDF) -9 increases theca cell numbers but conversely decreases both progesterone, androstenedione as well as the abundance of LHr and *CYP11A1* mRNA *in vitro* (Spicer *et al.*, 2008). These results, as well as those of Wu *et al.*, (2004) suggest that the effects of GDF-9 are dependent on stage of follicle growth and also infer that GDF-9 plays an important role in both cell proliferation and prevention of premature cell differentiation of the theca interna during early folliculogenesis (Spicer *et al.*, 2008).

There are two types of inhibin, inhibin A and inhibin B: Inhibin A reaches a peak near or after the beginning of presumptive deviation whereas inhibin B peaks twice, once mid follicular phase and again at ovulation (Mondal *et al.*, 2008). Granulosa cells are the predominant source of inhibin in healthy antral follicles >5mm (Henderson *et al.*, 1984). A coincidental rise in inhibin during

each follicular wave has been reported in cows (Kaneko *et al.,* 1995). Over the course of an oestrous cycle, inhibin levels continue to elevate post deviation, while FSH levels begin to decline (Kaneko *et al.,* 1995). Importantly, administration of an antiserum to inhibin in cattle resulted in an increase in circulating FSH levels. Furthermore, injection of follicular fluid containing inhibin into cattle decreased the diameter of the dominant follicle (Kastelic *et al.,* 1990; Medan *et al.,* 2006). As follicle diameter increases, inhibin concentrations have been reported to remain constant (Ginther *et al.,* 2001b). Evidence to the contrary suggests a decrease in circulating inhibin levels with increasing follicle diameter in porcine and bovine ovaries (Lorenzen *et al.,* 1978; Henderson & Franchimont, 1981), while Mihm *et al.,* (2000) reported similar levels of inhibin in the three largest follicles prior to deviation. Despite these results, Ginther *et al.,* (1996) concluded that data from experiments investigating differing concentrations of inhibin can be difficult to interpret as many authors fail to differentiate between inhibin A and inhibin B.

Only 5% of follicles >5mm in diameter possess the capability of synthesising oestradiol despite earlier production of its androgen precursor (McNatty *et al.,* 1992). From a cohort of antral follicles ≥2mm in diameter (n=60), nearly all demonstrated the ability to secrete androstenedione (McNatty *et al.,* 1984). However, levels of oestradiol in recruited follicles are low (Fortune *et al.,* 2001), this is most likely due to a lack of CYP19A1 activity despite high levels of *CYP19A1* mRNA expression in follicles 4mm in diameter during the recruitment process (McNatty *et al.,* 1984; Ginther *et al.,* 1996; Armstrong *et al.,* 2000). FSH stimulates the production of androstenedione from theca cells, however increased LH secretion is

responsible for CYP19A1 mediated oestradiol production in developing antral follicles (McNatty *et al.,* 1992; Ginther *et al.,* 2001b). Expression of *CYP19A1* mRNA is first identified in granulosa cells of small antral follicles (1-5mm) but concentrations are eight fold greater in granulosa cells of large antral follicles (8-22mm) (Spicer & Aad, 2007).

Culturing granulosa cells in the presence, but not absence, of theca cells led to an increase in oestrogen production (Orisaka *et al.,* 2006a). This supports the 'two cell - two gonadotropin' hypothesis (Fortune *et al.,* 1988) (Figure 1.6) that granulosa cells need theca cells and *vice versa,* to proliferate and facilitate steroidogenesis. A summary of factors regulating type 5 and type 6 follicle growth can be found in Tables 1.4 and 1.5 respectively.

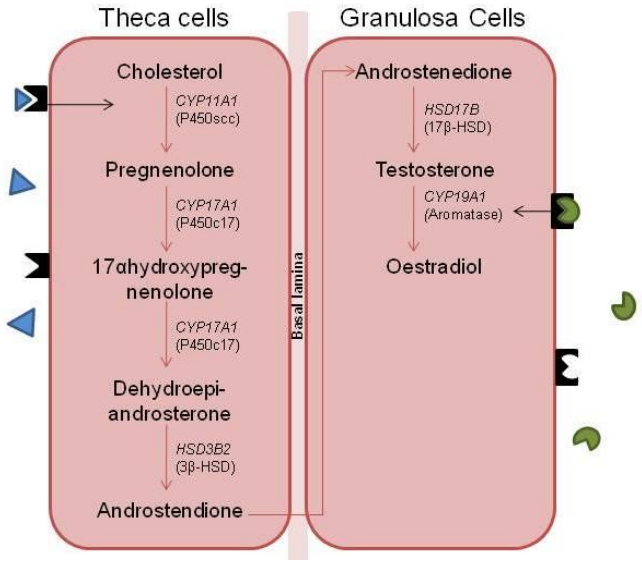

Figure 1.6: A summary of steroid hormone synthesis in the granulosa and thecal layers, otherwise known as the 'two cell - two gonadotropin' hypothesis. Theca cells can synthesise androgens but cannot convert them to oestradiol, and granulosa cells can convert androgens to oestradiol but they cannot synthesise androgens (Fortune *et al.*, 1988).

LH (blue) and its receptor; FSH (green) and its receptor.

Table 1.5: A summary of the role played by key gonadotropins, hormones and growth factors during type 6 follicle development.

FSH = follicle stimulating hormone.

Gonadotropin/ Hormone/Factor	Function	Reference
Follicle stimulating hormone	↑Androstenedione production	McNatty *et al.,* 1992
Luteinising hormone	↑ Aromatase mediated oestradiol production	Ginther *et al.,* 2001b
Oestradiol	Suppresses FSH secretion	Bo *et al.,* 1993
Steroid acute regulatory protein	Increased steroidogenesis	Bao *et al.,* 1998
Insulin-like growth factor-I	Support follicle growth ↑ Granulosa and theca cell proliferation Stimulates progesterone secretion from follicles >5mm in diameter ↑ Sensitivity of small antral follicles to gonadotropins	Monniaux & Pisselet, 1992 Beg *et al.,* 2001
Inhibin	Suppresses FSH production	Kaneko *et al.,* 1995
Insulin	↑ Theca cell proliferation ↑ Progesterone and androstenedione	Stewart *et al.,* 1995
Transforming growth factor β-1	↓ Oestradiol secretion from follicles >5mm	Ouellette *et al.,* 2005
Basic fibroblast growth factor	↑ Vascularisation of theca interna	Neufeld *et al.,* 1987
Growth differentiation factor -9	Supports follicle growth	Orisaka *et al.,* 2009

1.3.5 Recruitment of follicles

During the progesterone dominated luteal phase, follicles develop in waves, one approximately every 10 days, characterised by synchronous development of 3-6 small antral follicles >5mm in diameter. Studies have demonstrated two (Ginther *et al.,* 1989) or three (Savio *et al.,* 1988) follicular waves in cattle. Each follicular wave is preceded by a surge in FSH (Adams *et al.,* 1992; Adams and Pierson 1995; Webb *et al.,* 1999), with levels of FSH beginning to increase from 8 hours before and continuing until 8 hours after the emergence of the follicle wave; FSH is primarily responsible for follicular recruitment (Ginther *et al.,* 1998; Webb *et al.,* 1999). The number of follicular waves can be affected by dietary intake, parity and lactational status (Ginther *et al.,* 1996). Levels of FSH reach a peak when the future dominant follicle reaches 4mm in diameter and begin to decline to basal levels when all follicles in that wave reach 6mm in diameter (Adams *et al.,* 1992). Smaller, subordinate follicles are still dependent on FSH for further development and hence become deprived of their primary gonadotropin and enter atresia.

1.3.6 Selection for the dominant follicle

The selected follicle has been dependent on FSH for growth until this stage when the primary gonadotropin becomes LH. A shift in dependency to LH results in increased production of oestradiol, thus suppressing further secretion of FSH, and also may increase the amount of intracellular cAMP in the selected follicles, protecting the dominant follicle from declining concentrations of FSH (Ginther *et al.,* 1996). The protein inhibin is secreted

from the granulosa cells in response to FSH, conversely inhibin is known to down-regulate FSH synthesis and suppress FSH secretion (Martin *et al.,* 1991).

The mean day of deviation is determined to be approximately three days after the onset of the follicular wave when the two largest follicles are approximately 8-9mm in diameter (Ginther *et al.,* 1996; Ouellette *et al.,* 2005). Webb *et al.,* (1999) hypothesised that mechanisms involved in the selection of dominant follicles was linked to timing of LHr and *HSD3B2* mRNA expression in granulosa cells. HSD3B2 is another key enzyme in steroidogenesis, specifically in the production of progesterone, androstenedione and testosterone. Other authors believe that the maturation of only one follicle into dominance may be as a result of the granulosa cells' ability to respond to increasing levels of LH and FSH to increase production of cAMP and hence aid the development of the CYP19A1 enzyme complex (Hseuh *et al.,* 1994; Jolly *et al.,* 1994) (Figure 1.7).

LH pulsatility increases on completion of, and up to 32 hours after wave emergence, after which it plateaus (Ginther *et al.,* 1998). LHr have been identified in theca cells of large antral follicles 2-4 days after wave emergence and 8 hours before the onset of deviation in follicles predicted to become dominant (Ginther *et al.,* 1996; 2001a). An increase in the *LHr* mRNA expression in granulosa cells is noticeable just prior to deviation when the 2 largest follicles are between 8-8.4mm in diameter (Beg *et al.,* 2001), and thus it is possible that this acquisition of LHr plays a role in establishment of dominance. After wave emergence, LH concentrations increase 24-32 hours before presumptive deviation, remaining elevated up to 48 hours after deviation (Kulick *et al.,* 1999; Ginther *et al.,* 1998; Bergfelt *et al.,* 2000;

Ginther *et al.*, 2001b). Post deviation the increase in LHr may cause an up-regulation in aromatisation as LH secretion increases (Fortune *et al.*, 2001; Ginther *et al.*, 2001b). Furthermore, levels of FSH begin to decrease approximately 16-32 hours before deviation and remain low up until 24 hours after deviation (Ginther *et al.*, 1998). Levels of oestradiol have been shown to increase at the beginning of deviation (Ginther *et al.*, 2000a). Namely, low levels of oestradiol are detected when follicles are 5-7mm in diameter while mean concentrations of oestradiol are elevated when the largest follicle is 8-9mm, approximately two days after deviation (Martin *et al.*, 1991; Beg *et al.*, 2001; Ginther *et al.*, 2001b). Furthermore, responsiveness to LH in granulosa cells, identified by cAMP production, has been found in oestrogen active follicles >8mm in diameter (Jolly *et al.*, 1994). Expression of LHr at this stage allows the follicle to respond to low levels of LH present during the transition of gonadotropin dependencies. Evidence of the absolute need for LH at deviation was demonstrated by experimentally decreasing levels of LH at the beginning of presumptive deviation. Decreasing LH at deviation resulted in decreased concentrations of oestradiol, androstenedione and IGF-I in the follicular fluid of the two largest follicles (Gong *et al.*, 1995; Ginther *et al.*, 2001a; Ginther *et al.*, 2001b).

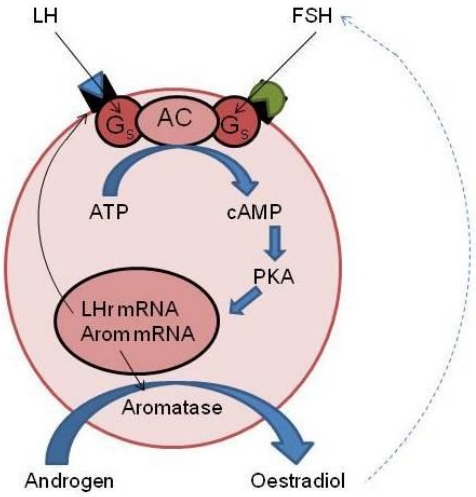

Figure 1.7: Luteinising hormone (LH) (blue) and follicle stimulating hormone (FSH) (green) interactions through their receptors with a granulosa cell post-deviation and their effect in up-regulating LH receptors (LHr) to aid development of the CYP19A1 enzyme complex (adapted from Ginther *et al.*, 1996).

G_s: stimulatory G protein, AC: adenylate cyclase, PKA: protein kinase A.

Levels of free IGF-I increase in the largest follicle during deviation (Beg *et al.*, 2001; Ginther *et al.*, 2001b) and decrease in the second largest follicle (Beg *et al.*, 2001), suggesting a role for IGF-I during deviation. Furthermore, GH deficiency in cattle has resulted in an 8-fold decrease in concentrations of IGF-I accompanied by a 2- to 4- fold decrease in the number of follicles >5mm in diameter (Chase *et al.*, 1998). This strongly indicated that IGF-I plays a major modulatory role in the development of antral follicles. Furthermore, in cows, levels of IGFBP-2 in the second largest (subordinate) follicles increase during the time of deviation, implicating an involvement of IGF-I during the transition from gonadotropin independency to dependency by increasing the sensitivity of small antral follicles to gonadotropins. Overall, this evidence suggests that

IGF-I is important during and after deviation to support healthy growth and function of dominant follicle and that decreasing concentrations of IGF-I may contribute to the cessation of follicle growth.

1.3.7 Follicular dominance

Follicle growth from early vesicular follicle up to pre-ovulatory size in the non-pregnant female cow is hypothesised to occur over 40 days (Marion & Gier, 1971). Final diameter of the dominant follicle in any wave, ovulatory or not, can be influenced by stage of, and steroidogenic control of, the oestrous cycle (Ginther *et al.,* 1996).

LH stimulated oestradiol production, as well as pregnancy associated plasma protein-A (PAPP-A), increase as follicle diameter increases and the follicle achieves dominance. PAPP-A is a gene that encodes for the metalloproteinase that cleaves IGF binding proteins, thus increasing the bioavailability of IGF-I which can then synergise with FSH to further increase oestradiol synthesis (Fortune *et al.,* 2004). Receptors for IGF-I have been identified in the theca cells of dominant follicles (Stewart *et al.,* 1996) and LH binding capacity has been shown to increase 6-10 fold in pre-ovulatory structures (McNatty *et al.,* 1986). Experimental deprivation of LH facilitated a decrease in diameter of the dominant follicle resulting in a dominant follicle similar to those observed naturally from non-ovulatory waves (Ginther *et al.,* 2001b).

Increased levels of oestradiol and IGF-I stimulated by LH within the dominant follicle result in better preparation to survive the low concentrations

of FSH caused by the aforementioned negative feedback of oestradiol and inhibin on the hypothalamus and the anterior pituitary. Ginther *et al.,* (1996) reported an extended lifespan of the dominant follicle in response to increased LH pulse frequency, demonstrating the need for LH at this stage of development. However, during this study, the authors made no differentiation between the 2 largest follicles. mRNA expression for *LHr, CYP17AI* and *CYP19A1* were higher in the granulosa and theca cells of dominant follicles than in recruited follicles (Fortune *et al.,* 2004). Interestingly, levels of *CYP11A* have been measured to be higher in the granulosa cells but not theca cells of dominant follicles (Fortune *et al.,* 2004), this was unexpected as one would expect to observe higher levels in theca cells along with StAR for the conversion of cholesterol to pregnenolone. A summary of steroid hormone synthesis in the thecal and granulosa layers of antral follicles can be found in Figure 1.6 (page 22).

Inhibin concentrations in the follicular fluid of the dominant follicle are higher than subordinate follicles (Scheyner *et al.,* 2000), providing further evidence for the requirement for inhibin in suppressing further growth of subordinate follicles, and in allowing maturation of the pre-ovulatory follicle. However, an earlier study measured no significant increase in inhibin concentrations in dominant follicles of the first or third follicular wave (Kaneko *et al.,* 1995).

Treatment with supra-basal concentrations of insulin in the late follicular phase resulted in diminished oestradiol concentrations for 8-12 hours that led to a subsequently delayed LH surge (up to 15 hours) suggesting that insulin could influence LH pulsatility and the pre-ovulatory LH surge (Scherzer *et al.,*

2009). IGFBP-4 has been detected in the follicular fluid of pre-ovulatory follicles from cattle (Mazerbourg *et al.,* 2001); divergence in levels of IGFBP-4 begins at time of deviation, when levels are higher in the second and third largest follicles compared to the DF. An increase in mRNA expression of IGFBP-4 has been observed to occur concurrently with an increase in LH concentrations in the DF (Armstrong *et al.,* 1998). However, evidence to the contrary reports that granulosa cells from dominant follicles do not express mRNA for IGFBP-4, and instead the authors attribute changes in the dominant follicle to the action of IGFBP proteases (Beg *et al.,* 2001; Yuan *et al.,* 1998; Ginther *et al.,* 2001a, b).

Unsurprisingly levels of StAR protein increase in dominant follicles owing to its crucial role during steroidogenesis, indicating an essential role during follicular function and dominance (Fortune *et al.,* 2001).

Table 1.6: A summary of the role played by key gonadotropins, hormones and growth factors during type 7 follicle development.

IGF-1 = insulin like growth factor-I; FSH = follicle stimulating hormone.

Gonadotropin/ Hormone/Factor	Function	Reference
Luteinising hormone	Supports follicle growth and oestradiol production	Ginther *et al.*, 2001b
Follicle stimulating hormone	Declining concentrations of FSH enable increased oestradiol production and morphological differentiation of the dominant follicle	Mihm *et al.*, 2002
Pregnancy associated plasma protein-A	↑ Bioavailability of IGF-1	Fortune *et al.*, 2004
Insulin-like growth factor-1	Synergises with FSH to ↑ oestradiol production	Fortune *et al.*, 2004
Inhibin	Suppresses FSH	Kaneko *et al.*, 1997
Insulin	Suppresses oestradiol	Scherzer *et al.*, 2009

1.4 Endocrine and ovarian events prior to ovulation

Bovine follicles gain ovulatory capacity from when they reach approximately 10mm in diameter (Sartori *et al.*, 2001). It is thought that this acquisition may be dependent on an increased expression of LHr on granulosa cells of the dominant follicle at the time of deviation (Sartori *et al.*, 2001). Prior to ovulation, the cumulus oophorus surrounding the ovum undergo cumulus expansion, initiated by FSH (Ralph *et al.*, 1995). This expansion results in a concomitant increase in antrum volume, swelling follicle diameter up to approximately 20mm (Fukui & Sakuma, 1980). Swelling is characterised by

the formation of a pronounced bulge, known as a 'blister' at the surface of the follicle. Cumulus oophorus granulosa cells also begin to secrete a hyaluronic acid rich 'cocktail' that gathers around the ovum to form a matrix that will accompany the ovum post-ovulation and is essential for fertilisation (Tanghe *et al.,* 2003). An LH induced signal transduction cascade initiates the secretion of proteolytic enzymes that will degrade the follicular tissue at the site of the blister, forming the stigma through which the cumulus oocyte complex (COC) will leave the follicle (Smith *et al.,* 1994). Regression of the CL resulting from the previous ovulation removes the negative feedback effects of progesterone on the hypothalamus. Low progesterone combined with high oestradiol concentrations from the dominant follicle positively feeds back on the hypothalamus to stimulate the release of GnRH pulses from the tonic centre of the hypothalamus, resulting in release of LH and FSH from the anterior pituitary. This positive feedback effect continues, culminating in an LH surge during which the pituitary releases up to 80% of its LH content (McNeilly, 2002).

1.5 Luteinisation and formation of a corpus luteum

Luteinisation is the process by which a recently ovulated follicle transforms into a steroidogenic cluster of cells known as the corpus luteum, through vascularisation, follicular cell hypertrophy and lipid accumulation (Henderson & Moon, 1979). Post ovulation, as the follicle wall collapses a corpus haemorrhagicum will form, characterised by an influx of blood from vessels broken at ovulation (Sangha *et al.,* 2002). During luteinisation, there is a

dramatic decrease in the ability of granulosa cells to aromatise androgens into oestrogens (Henderson & Moon, 1979). Furthermore, LH directly inhibits granulosa cell proliferation and instead stimulates production of progesterone and inhibin within luteal cells (Tsonis *et al.*, 1987; Stocco *et al.*, 2007). In pre-ovulatory follicles, granulosa cell output of progesterone is minimal due to the inability of the progesterone precursor, cholesterol, derived from plasma in the form of low density lipoproteins (LDLs), to cross the basal lamina (see Grummer & Carrol, 1988 for review). During luteinisation, breach of the basal lamina and increased vascularisation of the newly formed CL allows LDLs to reach the luteinised granulosa cells, facilitating production of progesterone. Progesterone is the principal hormone secreted from the CL and its function is to inhibit further GnRH and therefore LH release in order to prevent ovulation of smaller follicles and to support a potential pregnancy.

If the cow fails to recognise pregnancy (via the interferon tau signal from the developing embryo) by approximately day 17 post ovulation, luteolysis occurs (Ginther *et al.*, 2007). The mechanisms of bovine luteolysis are extensively reviewed in Milvae *et al.*, (1996), the key points are summarised as follows: Oxytocin is produced from the CL to little consequence during early CL development as oxytocin receptors do not form in the endometrium until 12-15 days of CL growth. Oxytocin binds with its receptors in the endometrium to stimulate secretion of $PGF_{2\alpha}$ which in turn further stimulates oxytocin production from luteal cells in a positive cascade system. $PGF_{2\alpha}$ levels are low early in the oestrous cycle but increase and continue to remain high until progesterone levels have declined and are at basal levels once again (Kindahl *et al.*, 1981; Sakamoto *et al.*, 1995).

Increased $PGF_{2\alpha}$ results in a decrease in blood flow to the CL (Acosta *et al.*, 2002), as well as inhibiting progesterone production, removing the inhibitory action of progesterone on GnRH secretion from the anterior pituitary, thus allowing a new wave of follicle growth

1.6 Follicular atresia

Follicular atresia is the fate of 99% of all follicles and is a hormonal and growth factor influenced process (Hseuh *et al.*, 1994; Rolaki *et al.*, 2005; Manabe *et al.*, 2008) involving the degeneration of all follicular components, eventually to be replaced with interstitial tissue (Westergaard, 1991). Atretic follicles are characterised by: (1) shortening and rounding of theca cells, (2) presence of several pyknotic granulosa cells (cells showing condensation of the chromatin in the nucleus) and (3) formation of irregular bulges, or blebs, from the cell membrane that are phagocytosed by macrophages (Marion *et al.*, 1968; van Wezel *et al.*, 1999). McNatty *et al.*, (1984) further classified atretic follicles as those characterised by an absence or lower activity of CYP19A1 in granulosa cells, which always preceded any reduction in the thecal steroidogenic response to LH. Currently, gonadotropins, epidermal growth factor (EGF), transforming growth factor alpha (TGF_{α}), basic fibroblast growth factor (bFGF), IGF-I, interleukin 1 (IL-1) as well as oestrogens have been identified as anti-atretic factors (Hseuh *et al.*, 1994; Kaipia & Hseuh, 1997), whereas androgens, GnRH, the cytokine interleukin-6 (IL-6), and tumour necrosis factor alpha (TNF_{α}) have been identified as atretic factors in the bovine and the rodent (Hseuh *et al.*, 1994; Kaipia & Hseuh, 1997; Evans *et al.*, 2004). This demonstrates that atresia is a process controlled by a balance of

hormones, cytokines and growth factors. Atresia can happen at any stage of follicular development and mechanisms of degeneration can vary depending on follicular stage. For example, during the pre-antral follicle stage, atresia is initiated by oocyte degeneration (Rajakoski, 1960), whereas, in antral follicles, apoptosis is first evident in granulosa cells (Rodgers & Irving-Rodgers, 2010). Atresia is more apparent as follicle size increases but the two most common stages of atresia are; 1) during the pre-antral to antral transition and 2) during selection for dominance. Atresia of oocytes also occurs in fetal ovaries that fail to attract granulosa cells and become involved in folliculogenesis (Edson *et al.*, 2009).

1.6.1 Atresia in large antral follicles

Large antral follicles (>5mm in diameter) undergo antral or apical atresia whereby granulosa cells in the layers closest to the antrum slough off and begin to degenerate whilst cells in layers closest to the basal lamina remain intact, numerous pyknotic nuclei are observed in the apical layers of granulosa cells during atresia of large antral follicles (Jolly *et al.*, 1994; Irving-Rodgers *et al.*, 2001). In follicles >6mm in diameter, both oestradiol and IGF-I levels were greater in healthy versus atretic follicles (Grimes *et al.*, 1987; Westergaard 1991; Porter *et al.*, 2000). The proliferative effects of oestradiol and IGF-I were also examined by Quirk *et al.*, (2004) who concluded that the increased levels of oestradiol and IGF-I observed in dominant follicles appeared to increase granulosa cell survival by preventing apoptosis. Levels of testosterone drop by 50% in atretic follicles (Grimes *et al.*, 1987), whereas

progesterone concentrations significantly increase in atretic follicles compared to non-atretic follicles. This is most likely due to the continued steroidogenic effect of different cell layers at varying stages of atresia (Grimes *et al.,* 1987). Levels of androstendione appear to remain constant in both healthy and atretic follicles >6mm in diameter (Westergaard, 1991). After initiation of atresia concentrations of steroid output from theca and granulosa cells could continue to increase and then decline at a later date due to the sequential decomposition of the follicular components (Rodgers & Irving-Rodgers, 2010).

Interestingly, despite StAR being absent in the granulosa cells of healthy follicles, it has been hypothesised that expression of StAR in the granulosa cells of atretic follicles may play a mediatory role in follicle atresia (Bao *et al.,* 1998; Braw-Tal & Roth, 2005). As atresia progresses StAR concentrations then decline, which was also accompanied by a decrease in the expression of both *LHr* and *CYP17A1* (Braw-Tal & Roth, 2005). Collectively, this results in the decreasing steroidogenic capacity of the follicle.

In pre-ovulatory follicles, it appears that the LH surge plays an important role in protecting granulosa cells from apoptosis. Approximately 12 hours after the LH surge granulosa cells undergo differentiation (i.e. exit from the cell cycle) programmed by TGF_{α}, and after this point they are resistant to apoptosis (Quirk *et al.,* 2004). Further evidence, provided by Porter *et al.,* (2001), clearly demonstrated that granulosa cells isolated both before and 14 hours after a GnRH induced LH surge, showed differing susceptibility to apoptosis likely induced by Fas ligand (Zhang *et al.,* 1998). The increase in progesterone concentration observed during antral atresia is more noticeable during atresia of pre-ovulatory follicles (Westergaard, 1991; Jolly *et al.,* 1994).

Furthermore, increasing concentrations of oestradiol in growing dominant follicles are responsible for enhanced granulosa cell expression of mRNAs for *CYP19A1*, *LHr*, *oestradiol receptor*, and *Mcl-1* (the gene that encodes for Mcl-1, a protein that inhibits cellular apoptosis in its longer form, but induces apoptosis in its shorter form), when compared to smaller subordinate follicles from the same follicular wave in cattle (Evans *et al.*, 2004).

Table 1.7: Summary of gonadotropins, hormones and growth factors associated with atresia of pre-antral, antral and pre-ovulatory follicles *vs.* healthy follicles of the same size.

StAR = steroidogenic acute regulatory protein; LH(r) = luteinising hormone (receptor); FSH = follicle stimulating hormone; IGF(BP) = insulin like growth factor (binding protein); MMP = matrix metalloproteinases; EGF = epidermal growth factor; TGF = transforming growth factor; bFGF = bovine fibroblast; IL = interleukin; TNF = tumour necrosis factor.

Pre-antral-Early Antral	Large Antral	Pre-ovulatory
↓oestradiol Irving Rodgers *et al.*, 2003b	↓ oestradiol Grimes *et al.*, 1987; Westergaard 1991; Porter *et al.*, 2000	↓oestradiol Evans *et al.*, 2004
↑ progesterone Irving Rodgers *et al.*, 2003b	↑ progesterone Grimes *et al.*, 1987	
↓ androstenedione testosterone Irving Rodgers *et al.*, 2003b	↓ testosterone Grimes *et al.*, 1987	
↑CYP11A1 ↑HSD3B2 Irving Rodgers *et al.*, 2003b	↓ CYP17A1 ↓ StAR Bao *et al.*, 1998; Braw-Tal & Roth, 2005	
	↓ LHr ↓ FSH Braw-Tal & Roth, 2005 Grimes *et al.*, 1987	↓ LH Zhang *et al.*, 1998
↑ IGFBP -2, -4, -5 ↑ Fas ligand ↑ MMP-2 & -9 Porter *et al.*, 2000; Yahia Khandoker *et al.*, 2001		↓ IGF-I ↓ Insulin ↑ EGF, TGFα, bFGF Mediatory effects of IL-1 on gonadotropins Tilly *et al.*, 1992; Chun *et al.*, 1995. ↑ TNF_{α}

The evidence presented suggests that there are clearly different mechanisms involved in the atresia of follicles, specifically of small antral and large antral follicles where atresia progresses either inwards from the antrum or outwards from the membrana granulosa. Furthermore, follicular atresia appears to be controlled by a number of hormones and growth factors (Table 1.7), but their actions cannot clearly be categorised as it is apparent that the stage at which the follicle enters atresia can have a dramatic effect on how the cells of that follicle responds to these circulating hormones and growth factors.

1.7 Bovine ovarian cysts

Typically, an ovarian cyst is defined as a follicle-like structure, 25mm, or more, in diameter that persists on the ovary for 10 days or more in the absence of a CL (Kesler & Garverick, 1982). Three classic types of cysts have been identified; 1) large thin walled anovulatory follicular cysts, characterised by high oestradiol secretion and prolonged signs of oestrus (nymphomania); 2) thick walled anovulatory luteal cysts characterised by prolonged and elevated progesterone secretion and lack of oestrous behaviour; 3) follicular cysts that luteinise. Furthermore, persistent CL have also been observed in dairy cattle, these form post ovulation with characteristics of a normal CL, if not slightly larger, they may or may not contain a vacuole of varying size. Functionality of the oestrous cycle may not be affected, however persistent CL are capable of secreting progesterone to the magnitude of maintaining a pseudo pregnancy (McEntee, 1958, Roberts, 1971). **NB:** In practice, differentiation between a persistent CL and luteal cyst at diagnosis can be incredibly difficult.

1.7.1 Cyst diagnosis

Diagnosis of cysts has developed over the last 50 years; traditionally rectal palpation of follicles was the only way to monitor follicular structures. Developments in technology have led to methods with increasing, yet varying degrees of accuracy. Transrectal ultrasonography can be utilised to monitor follicle growth and development on a frequent basis (Pierson *et al.*, 1988; Fricke *et al.*, 2002). Withdrawal and analysis of blood or milk samples for the hormones oestradiol and progesterone can also be utilised for diagnosis (Douthwaite & Dobson, 2000), although there is disagreement by many authors over what the target values should be in either diagnosis (Nakao, *et al.*, 1983; Farin, 1990; Ribadu *et al.*, 1994; Douthwaite & Dobson, 2000; Mueller, 2007). The most convenient method of diagnosis is transrectal palpation as this is quick and requires no equipment, but the best diagnosis is likely to come from a combination of both transrectal ultrasonography (Farin *et al.*, 1990) and progesterone analysis (Mueller, 2007). Currently, veterinarians in practice diagnose cysts during fertility checks when cows are not seen in oestrus. Cysts can also be detected during pregnancy diagnosis (via transrectal ultrasonography). This can provide a better differentiation between follicular and luteal cysts and also facilitates increased accuracy when estimating the size of a follicular structure.

1.7.2 Treatment of ovarian cysts

There are 3 tailored approaches currently used in practice for treating ovarian cysts (Mueller, 2007).

Oestrogenic follicular cysts (i.e., those present in the absence of a CL) can be treated with GnRH, if successful, luteinisation of the cystic structure should occur (Kesler *et al.,* 1981), and a CL may be detectable on the affected or unaffected ovary within 8 days of treatment (Jeffs *et al.,* 2011). To decrease time to oestrus a combination treatment with $PGF_{2\alpha}$ 9-14 days later, can be used to induce luteolysis of this CL (Dinsmore *et al.,* 1990), theoretically followed by a return to oestrus. Treatment of follicular cysts with a progesterone releasing intra-vaginal device (IVD) has been demonstrated to be effective at inducing atresia of oestrogenic follicular cysts (Todoroki *et al.,* 2001).

Non-steroidogenic follicular cysts (i.e. those co-existing with smaller follicular structures) can also be treated with a progesterone releasing IVD. How progesterone works to resolve cysts has not been fully established. Nevertheless it is most likely that the progesterone negatively feeds back on the anterior pituitary to down-regulate LH/FSH production, terminating cyst growth and initiating a new wave of follicle development. If treatment is successful, inserting an IVD should effectively imitate the luteal phase of the oestrous cycle, on removal of the IVD, progesterone levels should decline rapidly, resulting in an LH surge and ovulation within 14 days of treatment (Johnson & Ulberg, 1967; Zulu *et al.,* 2003).

Luteal cysts can be treated with $PGF_{2\alpha}$ to induce luteolysis and allow for a new wave of follicle growth. It is important that the pregnancy status of

the animal is determined prior to treatment as $PGF_{2\alpha}$ can induce abortion in pregnant cows. Successful return to oestrus can be observed within 3-4 days of treatment (Tebble *et al.,* 2001; Jeffs *et al.,* 2011).

Furthermore, for undifferentiated cyst types GnRH can be effective in inducing luteinisation (Nakao *et al.,* 1979) of cysts and/or follicles by stimulation of LH secretion, with cows successfully inseminated within 18-23 days from treatment (Youngquist, 1986). Some veterinarians choose to utilise a combination of treatments (as will be observed later) that they believe will effectively resolve cystic structures. Alternatively, some practitioners may choose to provide no treatment for ovarian cysts as, during the first 60 days postpartum, there is 60-65% rate of self-recovery (Vanholder *et al.,* 2006).

Treatment costs vary dependent on chosen treatment and number of treatments required to resolve the cyst, however, individual cost of one single cyst treatment range from £3-£10 on top of either a standard fee for a routine veterinary visit (£15) or an individual charge per bovine examination (£20-£30) (Burnell M, personal communication, all charges excluding VAT). Efficacy of hormonal treatments for ovarian cysts are varied. For example, Nanda *et al.,* (1988) demonstrated a number of recurrent ovarian cysts after treatment with both a prostaglandin analogue (for luteal cysts) and a GnRH analogue (for follicular cysts). Furthermore, a number of cows for each treatment required a further treatment for a new or unresponsive cyst. Recovery, defined as absence of a cyst but presence of a CL with or without an observed oestrus, 10 days (luteal cyst) or 15 days (follicular cyst) after treatment, took in excess of 30 days (Nanda *et al.,* 1988). Conversely,

treatment with a GnRH analogue has also been shown to be 90-93% effective against undifferentiated cyst types (Hooijer *et al.,* 1999).

As well as the cost of the initial treatment, increased calving to conception intervals, recurrence of cysts, further treatments as well as the cost of additional inseminations to achieve conception should also be factored into the cost of managing ovarian cysts.

1.7.3 Aetiology of ovarian cysts

Despite a broad range of research (Hamilton *et al.,* 1995; Silvia *et al.,* 2002; Evans, 2003; Peter, 2004; Vanholder *et al.,* 2006) investigating the biological mechanisms prior to cyst formation, the exact pathogenesis of this 'disease' remains unknown. "It is generally accepted that, disruption of the hypothalamo-pituitary-ovarian axis by endogenous and/or exogenous factors, causes cyst formation" (Vanholder *et al.,* 2006). Studying cyst formation is challenging due to the invasive nature of monitoring required, which in itself may induce the formation of an ovarian cyst. It is unlikely that an accurate picture of cyst formation can be acquired when cattle often need to be restrained to allow transrectal ultrasonography or withdrawal of blood for hormone detection. Furthermore, as this problem is particularly troublesome for the dairy industry, most UK dairy cows are both genetically and environmentally stressed for the majority of their working life so finding a suitable 'control' group with which to compare them is also challenging. Intensive genetic selection for milk yield may have provided a predisposing factor for ovarian cysts (Erb *et al.,* 1985; Fleischer *et al.,* 2001) and increased

corticosteroid production may result in compromised functionality of granulosa and theca layers as well as their intra-follicular functions (Liptrap & McNally, 1976). Physical signs of cysts seen by the farmer include anoestrus, irregular oestrous intervals, nymphomania, relaxation of the broad pelvic ligament, and development of masculine traits (in late lactation) (Vanholder *et al.,* 2006).

Although the exact aetiology is still unknown, significant differences in LH concentrations and pulse frequency have been identified in cows with ovarian cysts (Vanholder *et al.,* 2006 for a full review) (Figure 1.8). Altered feedback of oestradiol on the hypothalamus may result in an aberrant GnRH/LH surge, if this aberrant LH surge were to happen too early during dominant follicle maturation it is unlikely to result in an ovulation and instead leave the hypothalamus unresponsive to further oestradiol feedback (Cook *et al.,* 1991). Conversely, supra-basal concentrations of progesterone may block the LH surge, inhibiting ovulation and resulting in the formation of a large, persistent, an-ovulatory follicle with increased oestradiol output (Silvia *et al.,* 2002; Robinson *et al.,* 2006) (Figure 1.8).

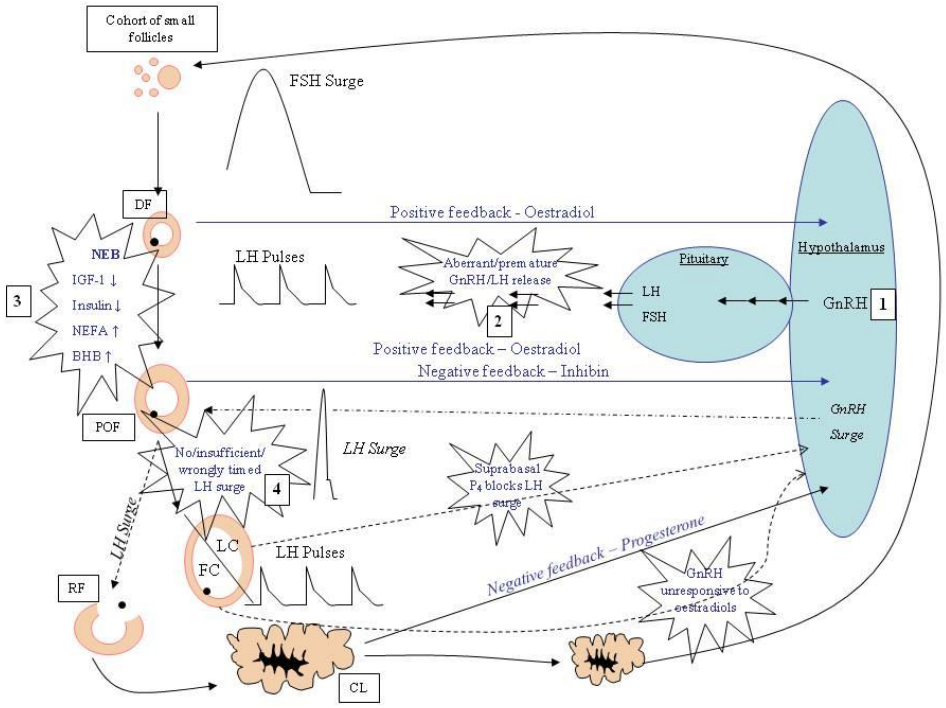

Figure 1.8: A summary of expected follicle growth (solid lines) and interference points and their possible effects (dashed lines) leading to the disruption of normal follicle development: 1) as oestradiol from granulosa cells of a dominant follicle fail to reach threshold concentrations, there is no positive feedback on the hypothalamus and therefore no further release of gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH) or luteinising hormone (LH). 2) Prolonged release of oestradiol or progesterone (P4) from a follicular (FC) or luteal cyst (LC) (respectively) can result in continuous release of LH or aberrant suppression of GnRH/FSH. 3) During a period of negative energy balance (NEB) there are reduced concentrations of insulin-like growth factor-I (IGF-I) and insulin as well as increased non-esterified fatty acids (NEFAs) and β-hydroxy butyrate (BHB), this can result in development of substandard oocytes inside anovulatory follicles. 4) Failings in the positive feedback on the hypothalamus can result in either; an LH surge during a period of no follicular dominance or no LH surge at all › no ovulation. Adapted from Vanholder *et al.,* 2006.

CL = corpus luteum; DF = dominant follicle; POF = pre-ovulatory follicle; RF = ruptured follicle.

1.7.4 Transition period, and reproductive pathologies associated with the post-partum period in lactating dairy cows

The three weeks immediately prior to, and immediately following parturition are known as the 'transition period'. The transition period is "critically important for health, production and profitability of dairy cows" (Drackley, 1999), and most health disorders, infectious (metritis), reproductive (ovarian cysts, retained fetal membranes) and metabolic (ketosis), are likely to happen at this time (Curtis *et al.,* 1985; Drackley *et al.,* 1999). Other common conditions observed during the transition period are displaced abomasum and milk fever (Drackley *et al.,* 1999).

1.7.5 Stress induced ovarian cysts

"The association between stress and ovarian cyst formation has not clearly been eludicated" (Ribadu *et al.,* 2000), this is most likely due to the multifactorial nature of cyst formation. It is known, however, that increased adrenocorticotropic hormone (ACTH) during the follicular phase of the oestrous cycle results in suppression of baseline LH (Thun *et al.,* 1998) as well as a decreased number of LH pulses (Ribadu *et al.,* 2000). At this time, the dominant follicle is likely to have developed ovulatory capacity so inhibition of LH pulse frequency is likely to render it anovulatory and cause it to persist as a large, steroidogenic cystic structure (Figure 1.8).

1.7.5.1 Metabolic stress

During the transition period there is an increase in requirement for fetal growth followed by lactogenesis. At the beginning of the transition period, dry matter intake (DMI) drops dramatically by up to 30% for reasons that are still unclear (Grummer, 1995). As the demands for production on the body total greater than the energy the cow is able to obtain from its DMI. The cow is subsequently in a state of negative energy balance (NEB) and mobilisation of body fat reserves for lipolysis is necessary, which is manifested as a drop in body condition. Lipolysis results in increased levels of non-esterified fatty acids (NEFAs), and any excess NEFAs are taken up by hepatic cells and are esterified, not oxidised as would be under normal physiological conditions, into triglycerides (Herdt *et al.*, 1988). Accumulation of triglycerides in the liver, peaking 7-13 days postpartum (Wathes *et al.*, 2007b) can result in hepatic lipidosis, leading to decreased liver function. Esterification and accumulation of these newly mobilised fatty acids prevents them from being used efficiently in either maintenance or milk production (Wensing *et al.*, 1997), and persistently elevated hepatic triglycerides have been associated with a longer calving to conception interval (CCI) (Butler, 2000). Length and severity of NEB can depend on any of the following factors; genetic merit, pre-calving body condition score (BCS), DMI before the transition period, and overall diet (Wathes *et al.*, 2007b).

Postpartum initiation of a new follicle wave is inevitable, regardless of NEB (Beam & Butler, 1999). However, follicles beginning their development during a time of NEB, 10-20 days postpartum, are significantly less likely to develop normally (Wensing *et al.*, 1997) and ovulation of an inferior oocyte is

a credible possibility (Leroy *et al.*, 2008). In a comparative study, it was demonstrated that, although there is no significant difference in the number of oocytes collected from cows with and without hepatic lipidosis, more oocytes collected from cows of normal energy balance were successfully fertilised and cultured than those collected from cows suffering with a NEB (15.9% v 5.5%) (Wensing *et al.*, 1997). Furthermore, oocytes cultured in media containing ≥2mmol/l NEFA were less likely to reach metaphase II following induction by LH, i.e resumption of meiosis previously arrested at the diplotene stage of meiosis I during oogenesis (Wensing *et al.*, 1997). It is believed that NEB delays ovulation through inhibition of LH pulse frequency in association with low levels of glucose, insulin and IGF-I. These diminish the capacity of the dominant follicle to synthesise oestrogen (Butler, 2000). Furthermore, cows with an increased LH pulse frequency have been demonstrated to have a shorter CCI (Beam & Butler, 1999). The decreased LH pulse frequency could explain why follicular development during a period of NEB (as discussed earlier) could result in incompetent oocytes. Cows suffering NEB and a drop in BCS have a longer CCI most likely due to a number of irregular cycles caused by this alteration in blood chemistry and metabolites observed during the transition period (Beam & Butler, 1999; Wathes *et al.*, 2007a, b). Furthermore, Patton *et al.*, (2007) demonstrated that cows in a state of normal energy balance that had a greater DMI during the first 1-4 weeks of lactation were more likely to successfully conceive. Force feeding and substitution of fat into the diets of postpartum NEB cows decreased, but did not eliminate lipolysis or elevated NEFA levels (Grummer, 1995).

1.7.5.2 Heat stress

The 'thermo-neutral' zone of lactating dairy cows, i.e. the temperature range at which they feel comfortable, is between 5˚C and 25˚C (Roenfeldt, 1998). Thermal stress can be defined as a period of time when the cow is experiencing difficulty maintaining normo-thermia, and applies more to hyper- rather than hypo-thermia. Effects include compromised steroidogenic capacity of the granulosa and theca cells (Table 1.8) and inappropriate secretion of gonadotropins from the anterior pituitary, resulting in suppression of follicular dominance (Wolfenson *et al.*, 1995), and impairment of oocyte quality and subsequent embryo development (Wolfenson *et al.*, 2000) (Figure 1.9)

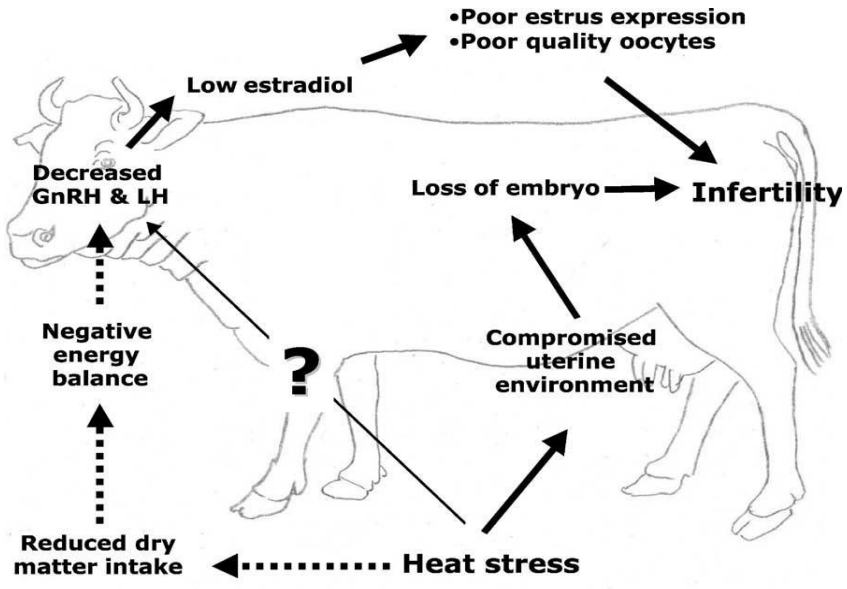

Figure 1.9: A summary of the effects of heat stress in the lactating dairy cow. Heat stress may; 1) indirectly affect ovarian function by decreasing dry matter intake, thereby inducing a state of negative energy balance and decreased gonadotropin releasing hormone (GnRH) and luteinising hormone (LH) secretion; 2) directly affect hypothalamic secretion of GnRH and LH by some, yet unknown, mechanism. Furthermore, if pregnant, heat stress can compromise the uterine environment and cause the loss of the embryo (De Rensis *et al.*, 2003).

Reduced fertility during the summer can sometimes be attributed to the effects of heat stress (>25˚C) with fertility dropping from 40-60% during the winter to 10-20% in the summer (Cavestany *et al.,* 1985). Cows experiencing experimental heat stress exhibited a greater cohort of follicles during the second follicle wave as a result of decreased inhibin and subsequent elevated FSH concentrations (Roth *et al.,* 2000). Furthermore, experimentally heat stressing cows during the winter results in a reversal of expected oestradiol and androstenedione concentrations, similar to those observed naturally during the summer (Wolfenson *et al.,* 1997). This suggested that the steroidogenic function of the theca, more so than the granulosa layer, is compromised during a period of heat stress. Inadequate supply of androstenedione to the granulosa cells will result in decreased oestradiol production, and subsequently an inability of the largest follicle to establish dominance and induce regression of the subordinate follicles. Examining hormone levels as a marker of the effects of heat stress has been a contentious topic with many differing results reported. However, evidence to date does support that heat stress has multifactorial effects on reproductive function.

Table 1.8: A summary of fluctuating concentrations of steroid hormones and gonadotropins, during follicular dominance (or lack of) throughout the year (based on data from Wolfenson *et al.,* 1997; 2000). Comparison of hormone concentrations in the summer, autumn and winter provides evidence that there may be a delayed effect of heat stress even after the summer months have ended (Roth *et al.,* 2001).

E2 = oestradiol; A4 = androstenedione.

	SUMMER	AUTUMN	WINTER
Follicular fluid	↓ E2 ↑ A4	↓ E2 ↓ A4	↑ E2, Intermediate A4
Theca cells	↓ A4	↓ A4	↑ A4
Granulosa cells	↓E2 ↓Inhibin	↑ E2	↑ E2 ↑ Inhibin
Anterior Pituitary	↓ LH ↑ FSH		↑LH ↓FSH

1.7.5.3 Genetic stress

Heritability of ovarian cysts was first suggested in 1949 (Garm, 1949). Examination of 2246 calvings involving 923 cows over 20 years highlighted an increase in the incidence of ovarian cysts up to 55% in multiparous Holstein-Friesian cows (Cole *et al.,* 1986). Of the affected cows, 30 were randomly selected for pedigree analysis, and results found that 24/30 shared common ancestory through the dam, sire or both (Cole *et al.,* 1986). These findings provide evidence that expression of this condition could be a direct result of genetics. Furthermore, culling bulls whose daughters are proven to be susceptible to developing cysts, reduces the incidence of cysts by 50% (Coleman, 2010). Predisposition to ovarian cysts has also been identified as breed specific and particularly high among Holstein Friesians, Jersey, Guernsey and Ayrshire cows (Coleman, 2010); this is unsurprising due to the

intense selection for increased milk yield traits that is not paralleled within breeding of beef breeds. Cows bred for beef are also less likely to experience extensive periods of NEB as, despite experiencing a similar demand for fetal growth, they are not expected to achieve the same magnitude of lactogenesis post-partum as dairy breeds. To the contrary, Dohoo *et al.*, (1984) reported heritability of cysts to be low. There are a number of reasons why this could be so, cohort studies often consist of a number of cows across varying age ranges and production categories. In this particular incidence, ovarian cysts were not the primary focus of the study but were observed alongside a number of other reproductive problems. High yielding dairy cows have been shown to be more susceptible to ovarian cysts when compared with a control population; this is most likely related to the NEB they will suffer at calving (Nanda *et al.*, 1989; Uribe *et al.*, 1994).

In conclusion, it is clear that the incidence of ovarian cysts is dependent on variable factors, however, it is important to remember that, despite the multifactorial nature of the condition it is ultimately a disruption in the normal endocrinological balance that results in this condition (Figure 1.8, page 45).

1.8 Hypotheses

The aim of this thesis was to develop a deeper understanding of the disruptions to the hypothalamo-pituitary-ovarian (HPO) axis that could lead to the development of aberrant follicles and ovarian cysts. Furthermore, this thesis aims to demonstrate the current failings in cyst diagnosis and consequences of the administration of inappropriate treatment.

Hypothesis 1: Milk progesterone is a valuable tool in assessing the accuracy of diagnosis as well as monitoring the efficacy of chosen treatments.

Hypothesis 2: There are predisposing metabolite and associated factors in the transition period that increase the likelihood for cows to develop an ovarian cyst.

Hypothesis 3: Prolonged exposure to a GnRH agonist leads to an LH surge but fails to induce ovulation, symptomatic of ovarian cysts.