# Hormonal And Follicular Responses To Steroid Treatment Administered During Emergence Of The First Follicle Wave In Dairy Heifers

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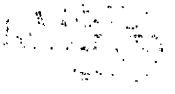

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## DECLARATION

**The work presented in this thesis is original and has been carried out solely by the author, except where collaboration with others has been acknowledged.**

**ALI KHANE**

To my mother Fatma, to my father Benaissa, to my brother Mohammed and all my family.

**July 2001**

# ACRONYMS

| | |
|---|---|
| FSH: | Follicle-stimulating hormone |
| LH: | Luteinizing hormone |
| DF: | Dominant follicle |
| CL: | Corpus luteum |
| GnRH: | Gonadotropin releasing hormone |
| PGF2α: | Prostaglandin F2α |
| AP: | Anterior pituitary |
| E2: | Oestradiol |
| BP: | Binding protein |
| bFF: | Bovine follicular fluid |
| β(TGFβ): | Transforming growth factorsβ |
| mRNA: | Messenger r |
| NWE: | New wave emergence |
| SF: | Subordinate follicle |
| EL: | Early luteal phase |
| ML: | Mid luteal phase |
| FP: | Follicular phase |
| RIA: | Radioimmunoassay |
| PRID: | Progesterone releasing intravaginal device |
| CIDR: | Controlled intravaginal device release |
| EB: | Oestradiol benzoate |
| ODB: | Oestradiol benzoate |
| EV: | Oestradiol valerate |

| | |
|---|---|
| ICAR: | International congress of animal reproduction |
| AI: | Artificial insemination |
| $P_4$: | Progesterone |
| EV+N: | Oestradiol valerate + Norgestomet |
| N: | Crestar ear implant |
| STD: | Standard |
| QC: | Quality control |
| NSB: | Non specific binding |
| TB: | Total binding |
| NRS: | Normal ram serum |
| AR: | Anti-rabbit |
| TC: | Total count |
| PBS: | Phosphate buffer solution |
| I125: | Iodine 125 |
| OXV: | Ovariectomized |
| EIA: | Buffer |
| Em: | Emergence |
| LF: | Largest follicle |
| Max: | Maximum |
| s.e.m. | Standard error to the mean |
| vs | Versus |
| MHz | Megahertz |
| mRNA | Messenger ribonucleic acid |
| oFSH | Ovine follicle stimulating hormone |
| P | Significance level |

| | |
|---|---|
| $P_4$ | Plasma progesterone |
| NaOH | sodium hydroxide |
| AI | Artificial insemination |
| cpm | Count per minute |
| ELISA | Enzyme-linked immunosorbent assay |

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# PUBLICATIONS

1) Mihm M, Khane A, Hansen H, Broadwith HM and Robertson L. The international congress on animal reproduction Stockholm, 2-6 July 2000. Abstracts volume 1, 1:43.

**" Differences in the steroid-induced suppression of growing wave foiiicies affect the timing of the next wave in dairy heifers".**

2) Mihm M, Khane A, Bleach E and Night P. The international workshop on inhibin, activins and follistatins, Melbourne, Australia, October 26-28, 2000.

**" Health and atresia in the first wave follicle is reflected in changing serum dimeric inhibin concentrations in dairy heifers".**

# Chapter 1:

# Review of Literature

# I) Introduction

Synchronising the timing of oestrus and ovulation in cattle involves the manipulation of ovarian activity such that the time of ovulation can be predicted accurately for successful pre-planned breeding (Odde, 1990; Macmillan and Burke, 1996). Synchronisation treatments aim to induce consistently and predictably a new transient Follicle Stimulating Hormone (FSH) rise intimately associated with emergence of a new wave of antral follicles approximately 3 mm in diameter, from which a single new dominant follicle (DF) is selected to continue to grow in all treated animals. If DF selection is combined with luteolysis and treatment withdrawal, an excellent synchrony of oestrous onset is predicted in all treated animals, allowing single fixed-time artificial insemination and resulting in high fertility (Roche *et al.,* 1998).

Before a transient FSH rise and emergence of a new wave can occur, growth of all existing follicles needs to be synchronously terminated irrespective of the stage of their development at start of treatment (Roche *et al.,* 1998). As the fate of growing wave and selected dominant follicles depends mainly on circulating concentrations of the two gonadotrophins FSH and luteinizing hormone (LH) (Adams *et al.*, 1992a; Savio *et al.,* 1993, Stock and Fortune, 1993), any successful manipulation of ovarian activity must modify FSH and LH concentrations in order to influence wave or dominant follicle growth.

The aims of this literature review are to highlight:

a) The dependence of antral follicle growth beyond >3mm in diameter on gonadotrophin stimulation in cattle, and;

b) The use of steroids to manipulate gonadotrophin secretion and thus modify antral follicle growth as part of existing programmes to synchronise oestrus and ovulation in cattle.

Hence, this literature review will lead to a deeper understanding of the regulation of follicle growth and provide the basis of future studies to improve the success of steroid combination synchronisation treatments.

## II) Overview of the oestrous cycle and follicle wave growth

The oestrous cycle in the bovine species varies in length from 18 to 24 days in both cows and heifers (Arthur *et al.,* 1989). The cycle consists of four different periods (Fig 1), viz. *oestrus* lasting 6 to 30 hours, followed by ovulation about 12-15 hours after the end of oestrus; the main ovarian hormone produced being oestradiol which rises before the onset of behavioural oestrus with peak values at the onset of oestrus and which stimulates the surge of LH from the anterior pituitary which is necessary for follicular maturation and ovulation. *Metoestrus* the phase succeeding oestrus where the granulosa and theca cells of the ovulated follicle give rise to lutein cells thus forming the corpus luteum which secretes progesterone; this period of corpus luteum (CL) formation is assumed to last four days. *Dioestrus,* the period when the CL is fully functional, 13- 15 days in length; and during which time no LH surge and thus oestrus can occur. *Proestrus*, when plasma oestradiol is increasing and progesterone is basal after regression of the CL (Glencross *et al.,* 1973). Cattle may also experience *Anoestrous* periods during which dominant follicles grow but do not ovulate and CLs are not present (Ginther *et al.,* 1989a; Evans *et al.,* 1994; Stagg *et al.,* 1995).

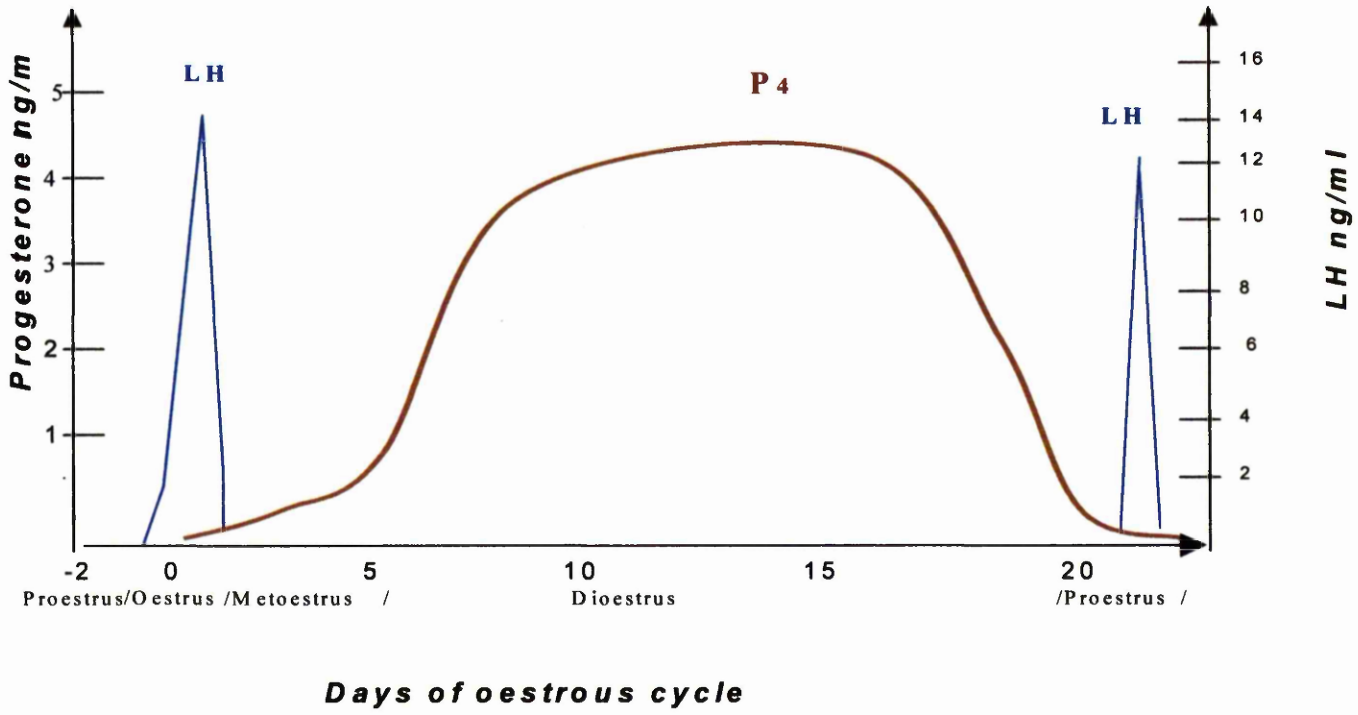



**Fig1.1 Changes in progesterone and LH concentrations during the oestrous cycle of the cow.**

## 1) Follicle growth

Follicular growth is a dynamic process beginning in the foetal ovary (Ireland, 1987; Fortune, 1994; Ginther *et al.,* 1996). Initiation of follicle growth involves the passage of primordial follicles from a quiescent growth-arrested state into a growth-committed state characterised by changes in shape of granulosa cells from squamous to cuboidal, proliferation of these cells, and enlargement of the oocyte (Hirshfield, 1991). Once follicles have entered the growth pool, they continue to grow until they become atretic (>99%) or ovulate (<1%). Thus, the process of atresia involves far more follicles than does the process of dominance, prerequisite for ovulation. Atresia means the degeneration of ovarian follicle cells and oocytes and occurs during pre-natal and post-natal development via apoptosis (Jolly *et al.,* 1994). In cattle up to $2.7x10^6$ oocytes are present in the foetal ovary up to day 110 of gestation, but this number is reduced to approximately 160,000 by parturition through the process of atresia (Erickson, 1966). By puberty, this number is further reduced, and in post pubertal cyclic animals the process of atresia continues with the exception of one or possibly two ovulatory follicles per cycle. Thus, only 200-300 follicles and oocytes, much less than 1% of the original germ cell endowment, survive atresia during foetal, post-natal or adult development. Atresia can occur during all stages of follicle development, though it is most commonly detectable in early antral follicles in a variety of species (Kaipia and Hsueh, 1997).

Follicles are classified according to their size and number of layers of granulosa cells (Rajakoski, 1960). Primordial follicles contain one layer of

squamous granulosa cells and are < 40μm in diameter. Once follicles leave their resting quiescent state and start growing (recruitment), they are termed Primary follicles which contain 1-2 layers of cuboidal granulosa cells. Subsequently, Secondary and Pre-antral follicles contain 2-6 layers of granulosa cells at the inside of the basement membrane and the theca interna layer develops on the outside. Oocytes initiate meiosis but then arrest in the diplotene phase of prophase 1; in primary follicles their growth is initiated when at least 40 granulosa cells are present (Braw-Tal and Yossefi, 1997). The acquisition of an antrum, a fluid filled cavity surrounded by granulosa cells occurs when follicles are between 200 and 500 μm in diameter and when > 6 layers of granulosa cells are present (Lussier *et al.,* 1987).

Little is currently known about progression of follicles from the primordial to the preantral stage, but it seems to be independent of gonadotrophins and involve oocyte or granulosa cell derived growth factors. Proliferating granulosa and theca cells develop FSH and LH receptors from primary or secondary stage respectively (Xu *et al.,* 1995). Thus, preantral follicles are responsive to, but not dependent on, the key endocrine regulators, FSH and LH. Various local growth factors modulate this stage of follicular development which suggests that mitogenic growth factors are active in stimulating cell division, and follicle and oocyte growth, whereas cell-differentiating growth factors are less active (Wandji *et al.,* 1996). The specific roles and nature of this growth factor cascade remain to be identified in farm animals.

Antral follicles are present in laboratory rodents, sheep and cattle in the absence of circulating gonadotrophins and in the presence of basal FSH and

absence of LH (Dufour *et al.,* 1979; Prendiville *et al.,* 1995; Gong *et al.,* 1996). Thus, antrum acquisition in sheep and cattle does not appear to be a FSH dependent event, yet antral follicles are responsive to FSH. However, on reaching 3 to 4 mm in size antral follicles are dependent on increased FSH for their continued growth (Gong *et al.,* 1996; Crowe *et al.,* 2001), but the exact mechanisms and stages at which they switch from just FSH responsiveness to FSH dependency are not known. Growth from 3 mm in cattle, as seen during emergence of a follicle wave of up to 24 follicles (Ginther *et al.,* 1996) is dependent on transient FSH rises from baseline. Ovarian transrectal ultrasound scanning determined that from this group of emerging follicles, one follicle is selected to become the dominant follicle (DF), while all other follicles belonging to the emerging wave undergo atresia (Fig 2). The DF that develops in the presence of an active corpus luteum (CL) will undergo atresia due to the absence of a frequent LH pulse pattern as a result of high progesterone concentrations. Therefore, only the dominant non-atretic follicle that is present when the CL regresses will become the preovulatory follicle and has the opportunity to ovulate releasing the egg.

Two (Ginther *et al.,* 1989b) or three (Savio *et al.,* 1988) waves of DF development occur based on the presence of a single large highly oestrogenic follicle on the ovary on two or three separate occasions during the cycle, on days 5, 13-15 of the cycle, and in 3 wave cycles at the end of the cycle during the pre-ovulatory period (Day 0 = oestrus).

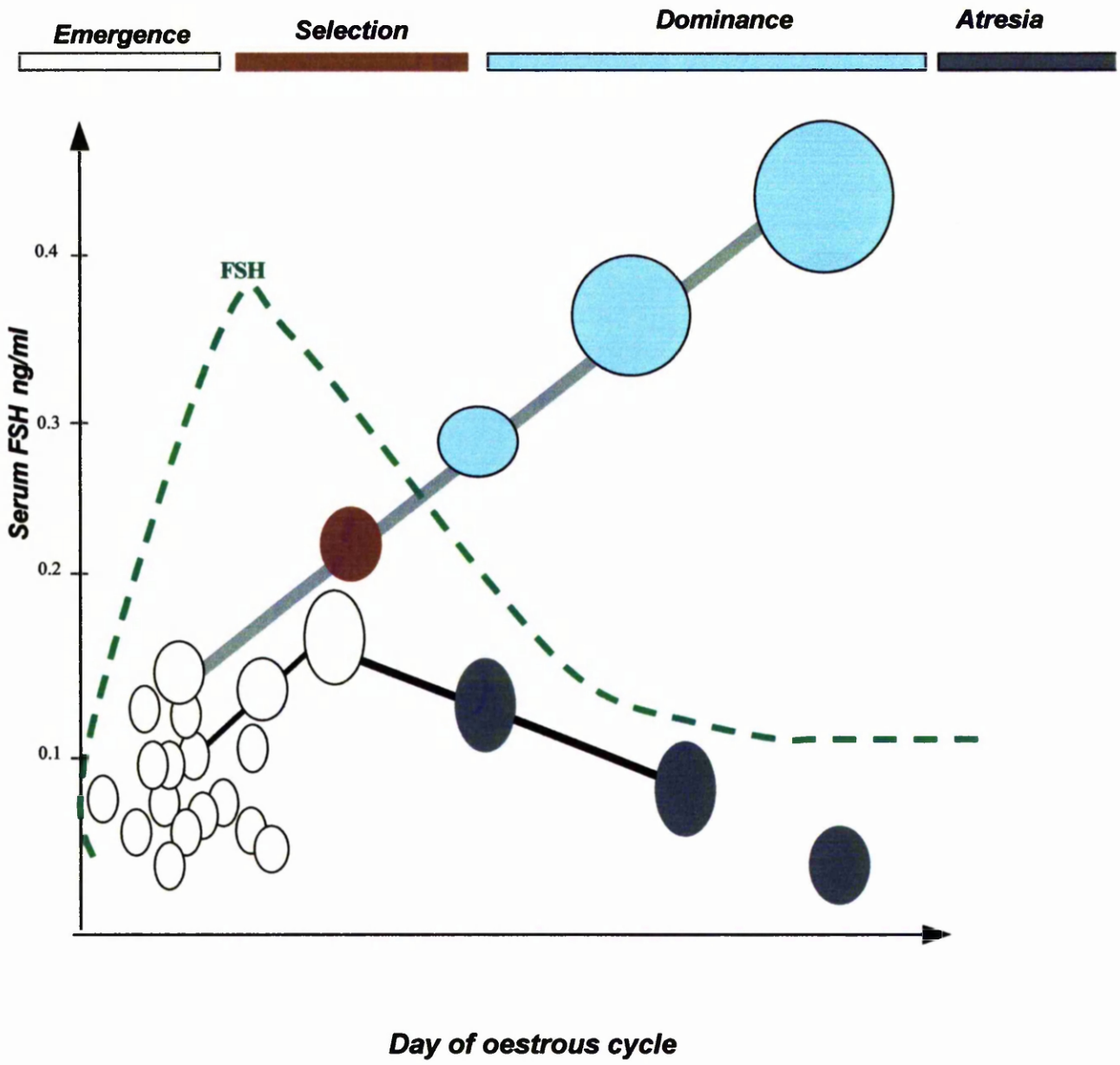



**Fig 1.2 FSH concentrations during the three phases of follicle wave development during the oestrous cycle of the cow; during emergence a group of 3-5 mm follicles are detected growing by ultrasound followed by selection phase during which one single follicle is selected to be dominant and all other cohort follicles undergo atresia; during its dominance phase no other follicles will grow.**

Antral follicle wave growth in cattle has been defined to occur in three phases namely emergence, selection and dominance (Ireland and Roche, 1987). Each wave begins with a group of follicles (cohort) being stimulated to grow simultaneously and their emergence is detected by ultrasound scanning at approximately 3 mm in diameter. Following the emergence of the new cohort, the number of follicles that continue to grow and remain oestrogen-active is sequentially reduced to that of the specific ovulatory quota of one in cattle. This process encompasses the selection period, and the selected follicle is now the DF; it continues to grow while the other follicles cease growth and start to regress in size. During its dominance period, no other follicle cohort emerges. Dominant follicle selection in cattle can be defined by the following criteria a) morphologically by daily ultrasound measurements of follicles and b) hormonally by estimating the intrafollicular oestradiol-progesterone or androgen ratio (oestrogen-active: ratio > 1; oestrogen inactive: ratio < 1; Ireland and Roche, 1983; Sunderland *et al.,* 1994). Although initially, neither size nor oestrogen activity of follicles after wave emergence are good predictors of future dominance (Ginther *et al.,* 1997a; Mihm *et al.,* 2000), over time size and/or oestrogen activity become a good predictor or indicator of dominance (Sunderland *et al.,* 1994). However, all follicles in a wave can become the DF, as random destruction of follicles in the 5-mm category does not alter the timing of DF selection (Gibbons *et al.,* 1997). The controlling factors that determine which follicle of the emerged cohort will become dominant are currently the subject of intense study and the sequence of events leading to DF selection remains to be determined. Loss of dominance and DF atresia is indicated by emergence of the next follicle wave induced by transient FSH (Adams *et al.,* 1992a; Sunderland *et al.,* 1994).

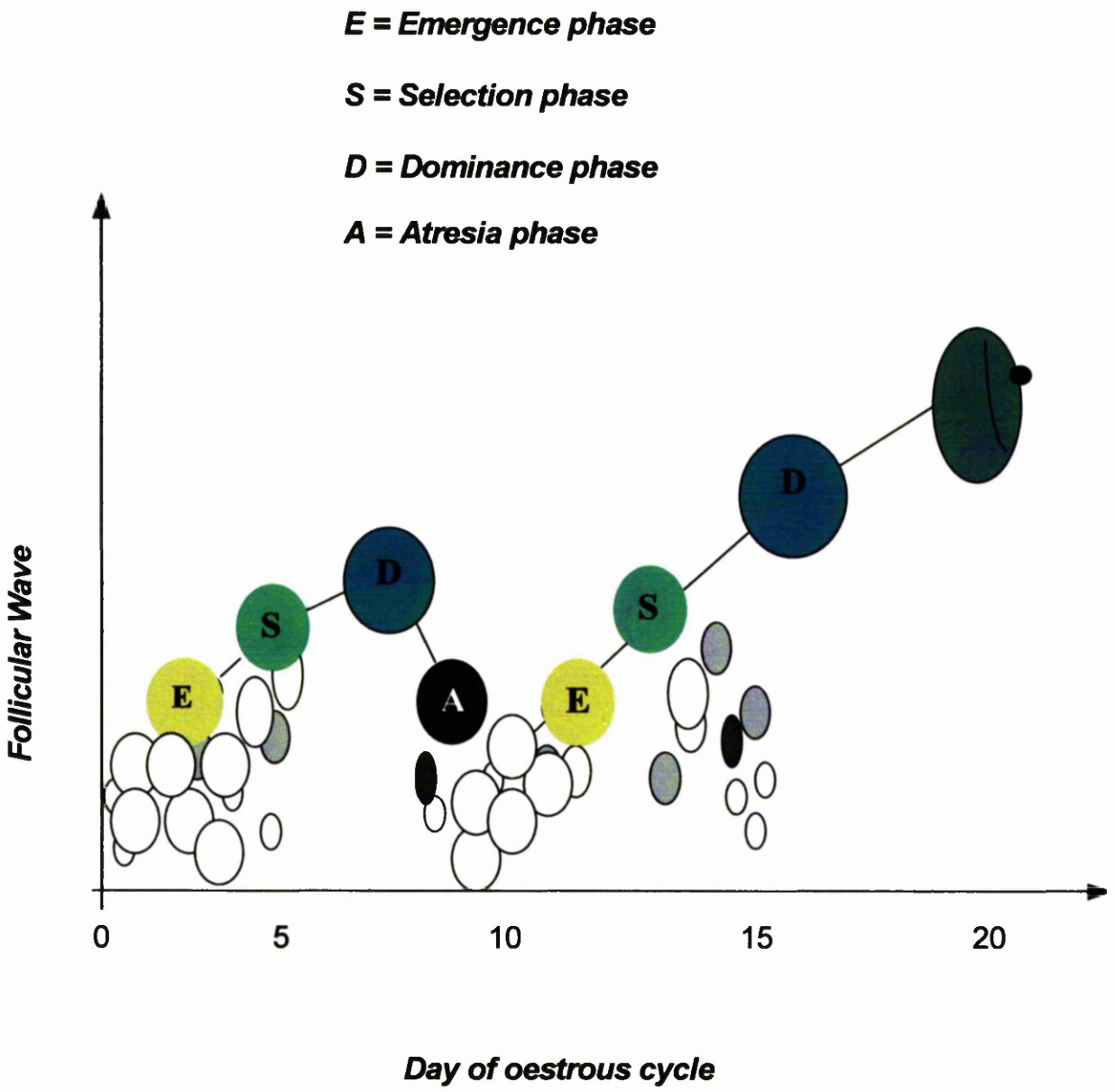



**Fig 1.3 Oestrous cycle with two follicle waves in cattle. The first dominant follicle is anovulatory, the second wave in the cycle selects the ovulatory dominant follicle.**

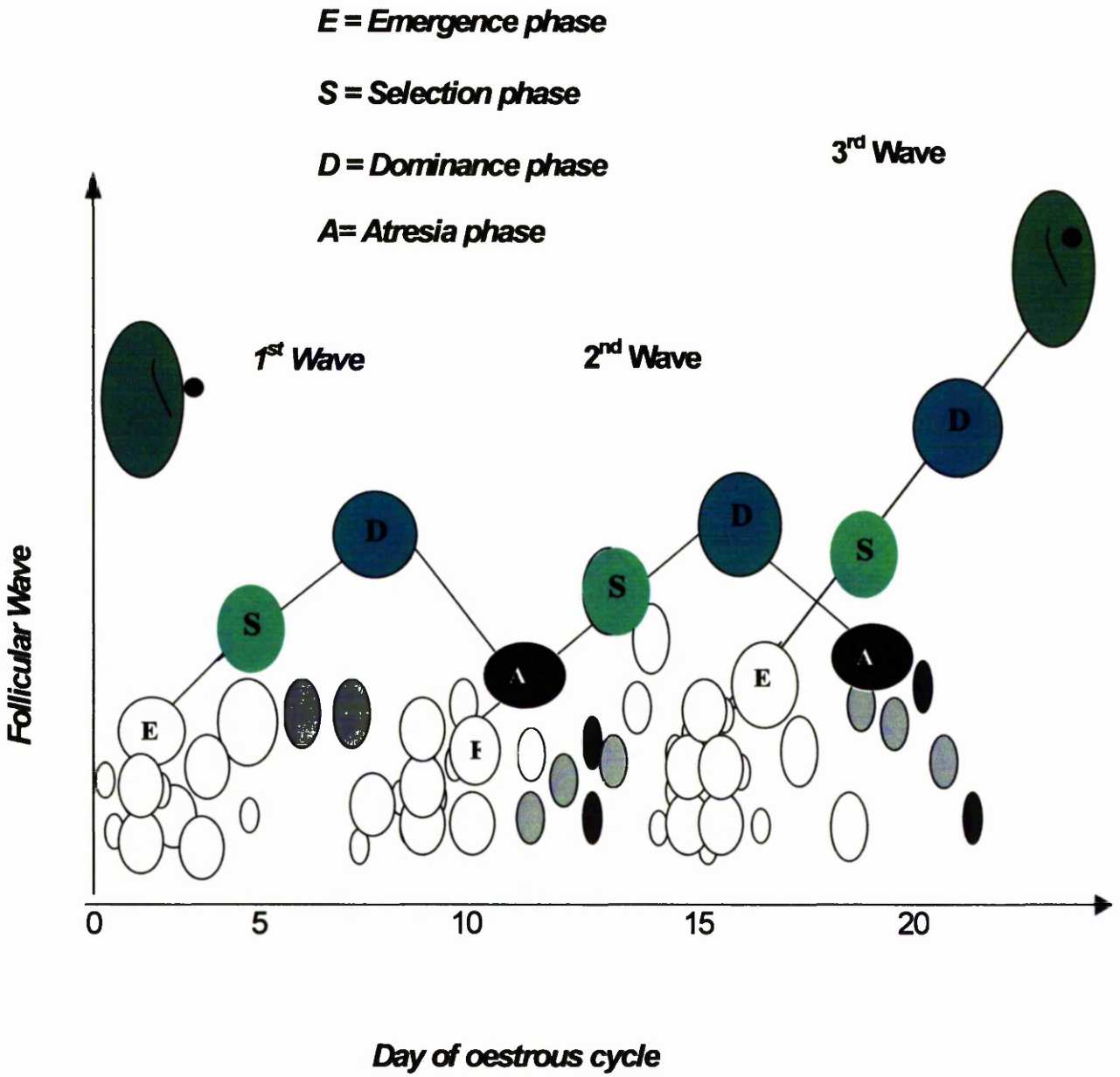



**Fig 1.4 Oestrous cycle with three follicle waves in cattle. The first and second wave have an anovulatory dominant follicle whereas the third wave selects the ovulatory dominant follicle.**

## 2) Hormonal Changes

### a) Follicle-Stimulating-Hormone

Follicle-Stimulating hormone is the key endocrine hormone responsible for stimulation of antral follicle growth in farm animals in a wave-like manner, but relatively little is known about the differential regulation of its synthesis and its dependency on ovarian status (Roche *et al.,* 1998). Gonadotropin-releasing hormone (GnRH) is a key regulator of FSH; exogenous GnRH induces FSH release, and GnRH immunization or GnRH agonist/antagonist treatments suppress transient FSH rises and follicle turnover in cattle (Prendiville *et al.,* 1995; Gong *et al.,* 1996). Transient FSH rises are detectable every 7 to 10 days during the cycle, in early pregnancy, and in postpartum anoestrous beef suckler cows independent of LH pulse frequency (Roche *et al.,* 1998). The structure of FSH is heterogeneous due to post-translational modification of the carbohydrate moiety, which leads to different isoforms in the pituitary gland and circulation. Follicle-Stimulating Hormone isoforms differ in their receptor binding affinity, metabolic clearance rate, and bioactivity (Cooke *et al.,* 1996). The proportion of different isoforms varies during different reproductive states, and it is regulated by different factors such as GnRH, oestrogen, and androgens (Cooke *et al.,* 1996). Androgens stimulate the synthesis and release of more acidic forms, which are less potent but have long half-lives. Basic or less acidic FSH isoforms are stimulated by GnRH and oestradiol, and these forms are more bioactive *in vitro* but have a shorter half-life than more acidic forms (Cooke *et al.,* 1996). The existence of FSH isoforms in circulation raises the possibility that changes in proportion rather than amount of FSH in blood could be a potent way to regulate

follicle wave dynamics. However Cooke *et al.,* (1997a) reports that the profile of FSH isoforms does not change before or at the maximum of the first or the second FSH rise of the oestrous cycle, or after declining FSH in heifers. A shift to less acidic forms was only noted before and during the gonadotrophin surge. Thus, non-ovulatory follicle waves in cattle do not seem to be regulated by shifts in proportion of FSH isoforms.

Following the early demonstration of a peri-ovulatory FSH rise in cattle (Dobson, 1978; Roche and Ireland, 1981), associated with emergence of the first new follicle wave of the cycle, it has been conclusively shown that each emergence of a cohort is linked to the transient FSH rise (Turzillo and Fortune, 1990; Adams *et al.,* 1992a) and that sequential FSH rises associated with new follicle waves occur during the oestrous cycle (Adams *et al.,* 1992a; Sunderland *et al.,* 1994), in the postpartum period (Crowe *et al.,* 1998; Stagg *et al.,* 1998), and before puberty in cattle (Evans *et al.,*1994). Thus, morphological selection of the DF in a single ovulatory species occurs in association with the decline in FSH. Prevention of this decline by administration of physiological amounts of FSH delays selection of a DF in cattle (Adams *et al.,* 1993; Mihm *et al.,* 1997). Although physiological regulation of the selection process is not well understood, the FSH decline does alter key intrafollicular growth factors involved in selection. Because FSH is a key survival factor preventing granulosa cell death via apoptosis in follicles, the putative future DF has to gain the capacity to survive the FSH decline and still continue to grow and produce oestradiol, while unselected follicles undergo apoptosis (Austin *et al.,* 2001).

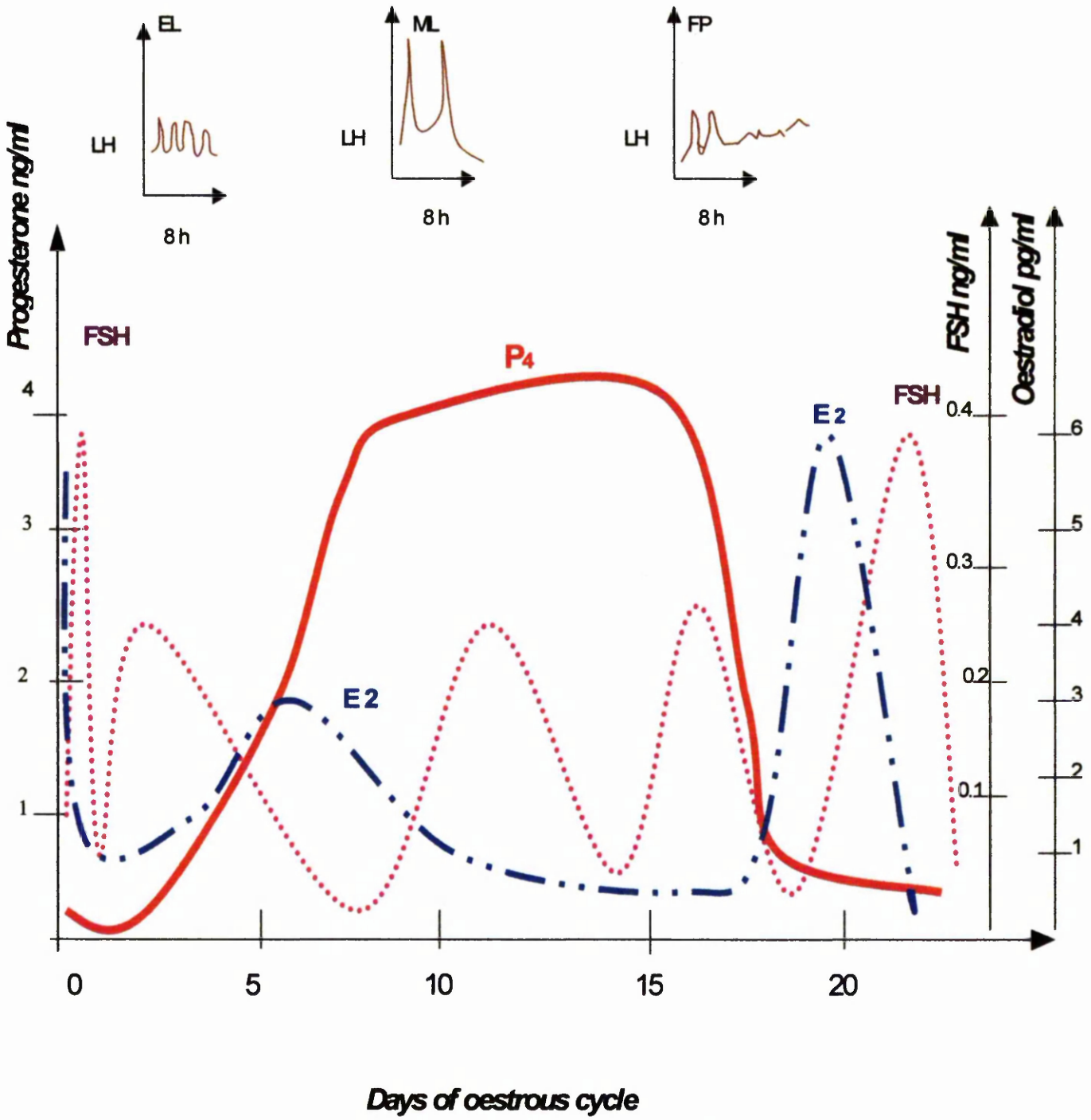



**Fig 1.5 Changes in FSH and the LH pulse profile in the early luteal (EL) and mid luteal (ML) and follicular (FP) phase with changes of progesterone and oestradiol concentrations during the oestrus cycle in cattle**

**b) Oestradiol**

During final maturation of the pre-ovulatory DF, the oestradiol concentration increases gradually during the 3 days preceding oestrus (Sunderland *et al.,* 1994; Cooke *et al.,* 1997a) and show a sharp peak about 4 h before the onset of oestrus (Shemesh *et al.,* 1972). By the time the first signs of oestrous behaviour are detected, the oestrogen level has already begun to decline, and it reaches its nadir 12 hours later (Shemesh *et al.,* 1972). Minimal oestradiol levels are maintained at the time of ovulation (20 to 32 hours after onset of oestrus). The pre-ovulatory elevation in oestradiol stimulates a further increase in GnRH pulse frequency, and a prolonged surge in GnRH (Moenter *et al.,* 1991) which induces the gonadotrophin surge, luteinization and ovulation. Subsequently, a minor oestradiol rise is observed consistently on Days 4-6 of the cycle coincident with selection of the first DF, and a more sustained increase on Days 10 to 13, with a peak on Day 11 was reported in one study, possibly coincident with selection of the second DF of the cycle (Shemesh *et al.,* 1972). Rises in oestradiol were observed 1-2 times during the luteal phase in addition to the pre-ovulatory rise following CL regression and thus Ireland and Roche (1987) hypothesized that these were the times when an oestrogen active DF was selected and growing during the oestrous cycle.

**c) Progesterone**

Once ovulation has taken place 24 – 30 hours after onset of oestrus (Austin *et al.,* 2001) the corpus luteum (CL) is formed and will be responsible for

the secretion of progesterone during the luteal phase. The CL is the major source of progesterone during the cycle, with serum concentrations of progesterone positively correlated with the amount of luteal tissue as assessed ultrasonographically (Kastelic *et al.,* 1990, Battocchio *et al.,* 1999). Progesterone concentrations in peripheral blood increase slowly, and reach 1 ng/ml approximately 3-4 days into the oestrous cycle, which is 2-3 days after ovulation. Maximum concentrations are observed by Days 9-12 and are maintained until luteolysis from Day 17 (Glencross *et al.,* 1973; Cupp *et al.,* 1995).

**d) Luteinizing hormone**

Mean concentrations of LH are low during the luteal phase of the oestrous cycle due to the strong negative feedback effect of progesterone on GnRH, resulting in decreased GnRH and hence LH pulse frequency (Goodman and Karsch, 1980). Following luteolysis, caused by oxytocin-induced release of prostaglandin F2$\alpha$ on Days 16-18 of the oestrous cycle, progesterone concentrations decline to their lowest level during the follicular phase, and thus the pulse frequency and the mean concentration of LH both increase (Rahe *et al.,* 1980). Once regression of the CL occurs, the dominant follicle present (under the influence of increasingly frequent but lower amplitude LH pulses) begins its pre-ovulatory growth phase, resulting in increased oestradiol production. This increased oestradiol results in the GnRH and therefore the LH and FSH surge, which is responsible for ovulation. Thus, changing concentrations of LH and FSH during the cycle determine growth of follicles, and the increased frequency of LH pulses during the follicular phase is responsible for stimulating the pre-ovulatory

steroidogenic changes resulting in ovulation. Subtle decreases in LH pulse frequency also occur between day of dominance of the first wave DF of the oestrous cycle Day 5 and initiation of atresia on Day 8-11 (Mihm *et al.,* 1995; Evans *et al.,* 1997), and a transient increase in mean LH has been associated with the time of deviation in growth rates between the dominant follicle and its largest subordinate follicle (Ginther *et al.,* 1999; Ginther *et al.,* 2001).

Luteinizing hormone plays an important role in determining oestrogen activity and final fate of the DF. It stimulates androgen precursor production in theca cells, which is necessary for oestradiol synthesis and continued health of the DF. The pattern of LH release is controlled by GnRH output and hence is pulsatile in character. The LH pulse pattern changes during the oestrous cycle, with frequent low amplitude pulses during Days 1 to 4 of the cycle as progesterone concentrations increase (Rahe *et al.,* 1980). There are also small but significant changes in LH pulse frequency during the luteal phase while high progesterone concentrations persist (Cupp *et al.,* 1995). Relatively minor changes in LH can determine whether a DF undergoes atresia (low LH pulse frequency) or ovulates (1 LH pulse/hour). The period of dominance is also affected by LH pulse frequency. Subluteal concentrations of progesterone that arise from use of intravaginal progesterone devices after 4 days, or ear implants of synthetic progestagen in the absence of the animal's own CL, result in an increase in LH pulse frequency (1 pulse per 1 to 2 h), prolongation of the period of dominance and formation of persistent DF (Savio *et al.,* 1993; Stock and Fortune, 1993). In contrast, atresia of the persistent DF can be caused most reliably by an acute decrease in LH pulse frequency using exogenous progesterone (Savio *et*

*al.,* 1993; Rajamahendran and Manikkam, 1994) or oestrogen (Yelich *et al.,* 1997).

In postpartum anoestrus and before puberty, infrequent LH pulses occur every 3 to 6 hours, which results in loss of oestrogen activity and dominance of the first DF resulting in a subsequent FSH rise within 1 to 2 days (Roche *et al.,* 1998). Therefore, lack of LH pulses during the luteal phase, during the early postpartum period, pre-puberty or during pregnancy does not inhibit antral follicle development, nor dominant follicle selection; however, the lack of LH leads to inadequate oestradiol production from dominant follicles, lack of gonadotrophin surges and thus lack of oestrus and ovulation (Stagg *et al.,* 1998).

## III) Regulation of antral follicle growth in cattle

### 1) Regulation of LH and FSH

#### a) Regulation via GnRH

It is the hypothalamus that is responsible for the control of release of gonadotrophins from the anterior pituitary by the action of a specific releasing substance gonadotrophin releasing hormone (GnRH); GnRH is secreted from hypothalamic neurones and carried from the median eminence of the hypothalamus by the hypothalamic-hypophyseal portal blood system. Gonadotrophin releasing hormone was isolated 30 years ago (Amoss *et al.,* 1971), and the molecular structure was determined as being a decapeptide and subsequently synthesised (Matsuo *et al.,* 1971; Geiger *et al.,* 1971). GnRH binds to specific receptors on the gonadotrophs, but the GnRH effect is transient due to gonadotroph cells becoming insensitive to GnRH if it is continually present caused by a down regulation of GnRH receptors. The main forces that affect the Hypothalamo - pituitary axis are external such as suckling and nutrition, or internal such as concentrations of progesterone and/or oestradiol.

The anterior pituitary gonadotrophin cells release LH and FSH causing ovarian steroid production, which in turns has either a negative or positive feedback on the hypothalamus or anterior pituitary gland influencing GnRH or LH surge or pulsatile release and FSH secretion. Both FSH and LH are hetero-dimeric glycoprotein hormones with a common $\alpha$-subunit but a specific $\beta$-subunit (Chappel *et al.,* 1983; Brown and McNeilly, 1999). As its name suggests FSH is involved in stimulating follicle growth in the female and is also involved in

spermatogenesis in the male. Luteinizing hormone (LH) is involved in final maturation of dominant/ovulatory follicles, a surge of LH triggers ovulation and LH is required for normal corpus luteum function

The GnRH pattern determines the specific pulsatile pattern of LH release (Clarke and Cummins, 1982; Moenter *et al.,* 1991; 1992). GnRH tightly regulates LH in most physiological situations. Except during the gonadotrophin surge, each pulse of GnRH in portal blood results in an LH pulse in peripheral circulation in ewes (Moenter *et al.,* 1992; Caraty *et al.,* 1995). However, such a close relationship has not been seen during FSH secretion, as pulses of FSH are detected in portal blood which are independent of GnRH pulses (Padmanabhan *et al.,* 1997). Thus, LH and FSH are produced in the same cell under the influence of a single releasing hormone GnRH, but are differentially regulated and thus divergent throughout the oestrous cycle (Sunderland *et al.,* 1994; Cooke *et al.,* 1997a) and postpartum period of cows (Stagg *et al.,* 1998). Thus, GnRH mediates control of LH through exocytosis of stored secretory granules containing LH (Currie and McNeilly, 1995). In contrast, FSH, once synthesised is released, implying that control of FSH is at the level of hormone synthesis (Farnworth, 1995; Brown and McNeilly, 1999). The FSH regulation must, therefore, involve an endocrine component active directly on the gonadotrophs, as well as locally controlled feedback systems in the form of growth factors including activin, inhibin, and follistatin within the pituitary gland.

**b)-Regulation via steroids**

During the luteal phase a surge of GnRH and gonadotrophin is prevented by high concentrations of progesterone secreted from the CL (Kasavubu *et al.,* 1992). Progesterone has also been shown to suppress GnRH pulse frequency and thus LH pulse frequency (Goodman and Karsch, 1980; Karsch *et al.,* 1987; Kasavubu *et al.,* 1992). After luteolysis a marked reduction in progesterone is seen, the negative feedback by progesterone on the hypothalamus is removed and GnRH pulses are released at higher amplitudes and frequencies than during the luteal phase. This causes particularly LH to be released at higher levels, thus promoting follicular development and the production of oestradiol during the follicular phase. Oestradiol in turns exerts a positive feedback on the neurones of the hypothalamus surge centre and induces a prolonged GnRH surge and thus gonadotrophin surge in 12 hours (Moenter *et al.,* 1990; Evans *et al.,* 1995).

The suppressive effects of progesterone on LH have been well documented in ovariectomised (Price and Webb 1988) and cyclic heifers (Ireland and Roche 1982a, Kinder *et al.,* 1996) in which LH pulse frequency plus amplitude differ depending on the level of progesterone during the luteal phase (Rahe *et al.,* 1980; Cupp *et al.,* 1995). Coadministration of oestradiol and progesterone enhances the suppression of LH pulses compared with progesterone alone in ovariectomised (Price and Webb, 1988; Burke *et al.,* 1996) and cyclic cattle (Rajamahendran and Manikkam, 1994). Treatment with 0.75 mg oestradiol benzoate and a progesterone device in early metoestrus suppressed transiently FSH and completely obliterated LH pulses in beef heifers (Austin, 2000). Oestradiol appears to preferentially reduce LH pulse amplitude while

progesterone inhibits LH pulse frequency (Goodman and Karsch, 1980).

Oestradiol from the DF is a likely endocrine candidate involved in regulation of FSH secretion. Oestradiol has a direct negative feedback on FSH as exogenous oestradiol suppresses FSH within 18 to 24 h in cattle (Price and Webb, 1988; Bolt *et al.,* 1990; O'Rourke *et al.,* 2000). It is thought that transiently rising oestradiol concentrations associated with dominance periods during the oestrous cycle could result in the rise and decline of FSH (Ginther *et al.,* 2000a). However, follicle waves result in undetectable peripheral oestradiol rises a) in the early postpartum period of nutritionally compromised beef suckler cows, which fail to ovulate for over 100 days, and b) in the mid-luteal phase of cyclic cattle in most studies (Ireland and Roche, 1987), yet these cows have recurrent FSH rises similar to animals, in which significant oestradiol is produced after first wave emergence and before onset of atresia of the first DF (Stagg *et al.,* 1998). In cyclic heifers FSH declines on days 2 to 3 of the cycle and this decline begins before oestradiol rises noticeably in circulation; in addition the next FSH increase does not occur immediately following the initial abrupt decline in oestradiol noted between days 5 and 8 of the cycle (Cooke *et al.,* 1997a). Thus, factors other than oestradiol are involved in the negative feedback regulation of FSH secretion. Steroid-free bovine follicular fluid suppresses FSH, but the precise roles of oestradiol or follicular fluid peptides in regulation of FSH remain to be elucidated.

**c) Regulation via members of the inhibin family**

Inhibin was first isolated from ovarian follicular fluid as a granulosa cell product, and was characterised as a disulphide-linked dimeric glycoprotein

(consisting of one α and one β subunit; Knight, 1996) capable of selectively suppressing the synthesis and secretion of FSH by pituitary gonadotrophs (de Jong and Sharpe, 1976; Findlay and Clarke, 1987; Ying, 1988; Sugino *et al.*, 1992). Two forms of inhibin (A and B) are expressed in the ovaries of most species examined; the mature, fully processed forms of inhibin A and B (*Mr*~ 32,000) share a common α subunit (Mr~ 18 - 20,000) but have one of two β subunits (*Mr*~13 – 15,000) termed βA or βB, respectively (Knight, 1996). So far, one form (inhibin A containing the βA subunit) has been isolated from bovine and ovine ovaries (Knight, 1996).

During the course of purifying inhibin from follicular fluid, two other molecules with modulatory effects on pituitary FSH release were discovered. These were termed activin and follistatin. Activin was characterised as a (*Mr*~ 25,000) disulphide-linked dimer of two inhibin β subunits which stimulates pituitary FSH release and FSHβ mRNA accumulation, thereby opposing the action of inhibin (Ling *et al.,* 1986, Vale *et al.,* 1986). Two forms of activin were initially isolated from pig follicular fluid, now referred to as activin A (βAβAdimer) and activin AB (βAβB dimer). Both inhibin and activin belong to the TGFβ superfamily. Follistatin was characterised as a monomeric, cysteine-rich polypeptide, which, although structurally unrelated to inhibin, suppresses pituitary FSH release in a similar manner to inhibin but with less potency (Ueno *et al.,* 1987; Robertson *et al.,* 1987). A single gene encodes the molecule and the existence of several size variants of follistatin (*Mr* 32-39,000) reflects alternate splicing of gene transcripts to yield carboxy-terminal truncated forms, as well as differential glycosylation (Sugino *et al.,* 1997). Follistatin, although structurally unrelated to the TGFβ

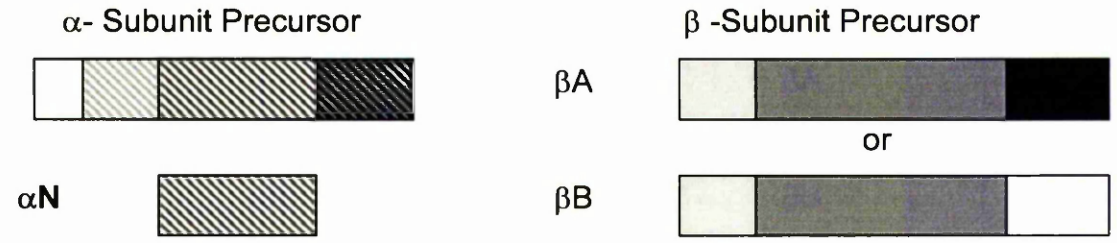
family, neutralises activin bioactivity by acting as a specific high-affinity binding protein.

Inhibins, activins, and follistatins are expressed in granulosa cells, are locally regulated and function as intragonadal autocrine or paracrine regulators of follicle cell differentiation and steroidogenesis. In addition they subserve local regulatory roles in numerous extragonadal tissues, including brain, adrenal gland, bone marrow and placenta but perhaps most notably the anterior pituitary where they can be of systemic or local origin (Knight, 1991).

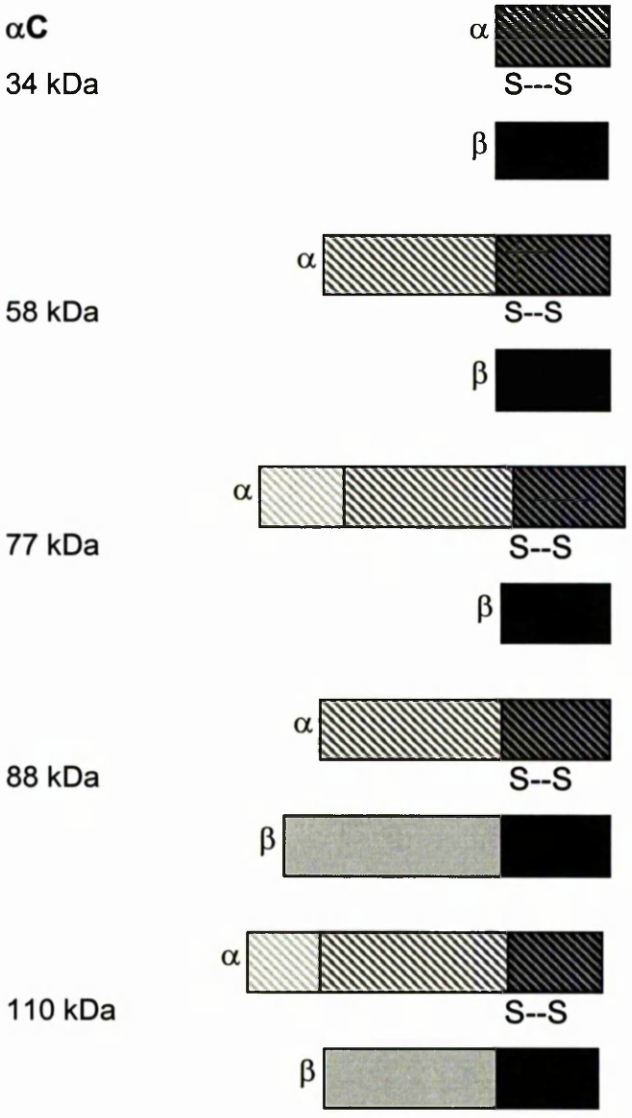
Inhibin dimers produced in the ovary suppress FSH secretion during culture of pituitary cells (Robertson *et al.,* 1987; Good *et al.,* 1995) and immunoneutralization of inhibin by passive immunization of cows during the growth phase of the first dominant follicle causes FSH to increase within 8 hours of treatment (Kaneko *et al.,* 1997) indicating an in vivo role of inhibins in suppression of FSH during the first wave.

In several species including cattle, the smallest and most predominant form of biologically active inhibin identified has an *Mr* of approximately 31,000–32,000 (Fig 4), (Miyamoto *et al.,* 1985; Robertson *et al.,* 1985). Since the $\alpha$ and $\beta$ subunits are generated by proteolytic cleavage of two independently synthesised precursor molecules (Forage *et al.,* 1986; Mason *et al.,* 1986), it is, perhaps, not surprising that inhibin forms of higher *Mr* have also been identified in ovarian follicular fluid from various species (Robertson *et al.,* 1985; Miyamoto *et al.,* 1986).

Inhibin precursors and monomeric forms in bovine follicular fluid




**(B) Dimeric inhibins forms in bovine follicular fluid**




>160 kDa forms of inhibin in follicular fluid may represent aggregation of Inhibin α and βsubunits, presence of novel proteins containing α and β subunits, or the complexing of smaller forms to larger inhibin binders, such as α2-macroglobulins.

**Fig.1.6 Structure of inhibins in bovine follicular fluid**

Several *in vivo* studies, involving intact rats (Ying *et al.,* 1987), ovariectomized ewes (Findlay *et al.,* 1987) and ovariectomized heifers (Beard *et al.,* 1990) have confirmed that highly purified inhibin preparations do indeed suppress plasma FSH concentrations with little or no effect on plasma LH although there is some *in vitro* and *in vivo* evidence that inhibin increases LH release (Muttukrishna and Knight, 1990; McKeown *et al.,* 1997). Exposure of cultured sheep pituitary cells to highly purified bovine inhibin actually enhances GnRH–induced LH release without affecting basal LH release; basal and GnRH-induced FSH release were suppressed by inhibin in a manner similar to that observed in rats (Muttukrishna and Knight, 1990).

An injection of bFF or highly purified bovine inhibin decreased plasma concentrations of FSH in ovariectomized (Beard *et al.,* 1990) and cyclic heifers (Quirk and Fortune, 1986), indicating that inhibin has the ability to suppress FSH secretion in cows *in vivo* suppressing emergence of the next wave when administered to cattle (Turzillo and Fortune, 1990; Adams *et al.,* 1992a). Also, bovine follicular fluid has the ability to suppress FSH during the dominance period of the first wave DF (Adams *et al.,* 1992a; Turzillo and Fortune, 1993; Ginther *et al.,* 1999; Bergfelt *et al.,* 2000). However, due to the possibility that other FSH-suppressing and stimulating factors are present in bovine follicular fluid, such data are not conclusive in terms of effects of inhibin. Immunoneutralization of endogenous inhibin during the luteal phase of the bovine oestrous cycle results in a marked and selective increase in plasma concentration of FSH, thus inhibin must be an important factor in the regulation of FSH secretion during the luteal phase of the cow's oestrous cycle (Kaneko *et al.,* 1993).

**2) Follicle wave development and dependence on changes in FSH and LH**

Several waves of development of dominant follicles occur during a bovine oestrous cycle. Each wave has three phases: emergence, selection and dominance followed by atresia or ovulation of the DF (Ireland and Roche, 1987). Two waves of antral follicular development were proposed initially for the bovine oestrous cycle in dairy cows, and each wave resulted in a follicle of pre-ovulatory diameter (Rajakoski, 1960). It is now well established that two or three waves of follicular development occur during the majority of bovine cycles (Pierson and Ginther 1988; Savio *et al.,* 1988; Sirois and Fortune, 1988; Knopf *et al.,* 1989; Ginther *et al.,* 1989b). However, it must be pointed out that small proportions of cycles exhibit just one or alternatively four waves per cycle (Pierson and Ginther, 1988; Sirois and Fortune, 1988).

From the simultaneously emerging cohort and over 2-3 days, the cohort follicles differentiate into one large follicle that continues to grow (the selected dominant follicle) and the remaining wave or subordinate follicles, which are characterised by a reduced growth rate and stasis with subsequent regression within 2-4 days after wave emergence (Savio *et al.,* 1988; Sirois and Fortune, 1988; Ginther *et al.,* 1989b; Knopf *et al.,* 1989).

In earlier studies it was thought that the dissociation into dominant and subordinate follicles was a gradual event beginning at emergence. Later studies (Ginther *et al.,* 1997b) demonstrated that dissociation in terms of growth rates and size was often an abrupt event and this time-point was considered to be the start of follicle deviation. Regression of the dominant follicle consistently occurs following emergence of a new follicle wave during the luteal phase of the cycle

(Ginther *et al.,* 1989b). Generally the first dominant follicle of the oestrous cycle is detectable as one of a cohort of 3-mm follicles that are present the day after ovulation. The future dominant follicle appears to emerge at 3 mm a mean of 6 h (Ginther *et al.,* 1997a) or at 4 mm a mean of 7h (Kulick *et al.,* 1999) earlier than the future largest subordinate follicle. However maintaining individual identity of up to 24 follicles in a wave is considered to be one of the limitations of ovarian ultrasound scanning and thus a growth advantage from emergence without detectable size differences up to the time of deviation may be very difficult to prove.

The DF is selected during the following 2 to 4 days and becomes dominant between Days 4 and 5 of the cycle (Ireland and Roche, 1987; Sunderland *et al.,* 1994). Deviation is characterised by enhanced growth of the largest follicle to become the dominant follicle and reduction or cessation of growth by the remaining follicles to become subordinate follicles (Ginther *et al.,* 2000a; 2000b). The mean diameter of the two largest follicles in cattle at the beginning of deviation were 8.5 and 7.7 mm with deviation beginning a mean of 2.5 days after emergence of the largest follicle at 4 mm (Ginther *et al.,* 1997b; 1999; Kulick *et al.,* 1999). The follicles grow in parallel initially, so that the largest follicle maintains about a 0.5-mm diameter advantage (non-significant!) until deviation (Ginther *et al.,* 1997b; Kulick *et al.,* 1999). However, the differences in mean diameter of the largest and the second largest follicle only become significant from follicle deviation.

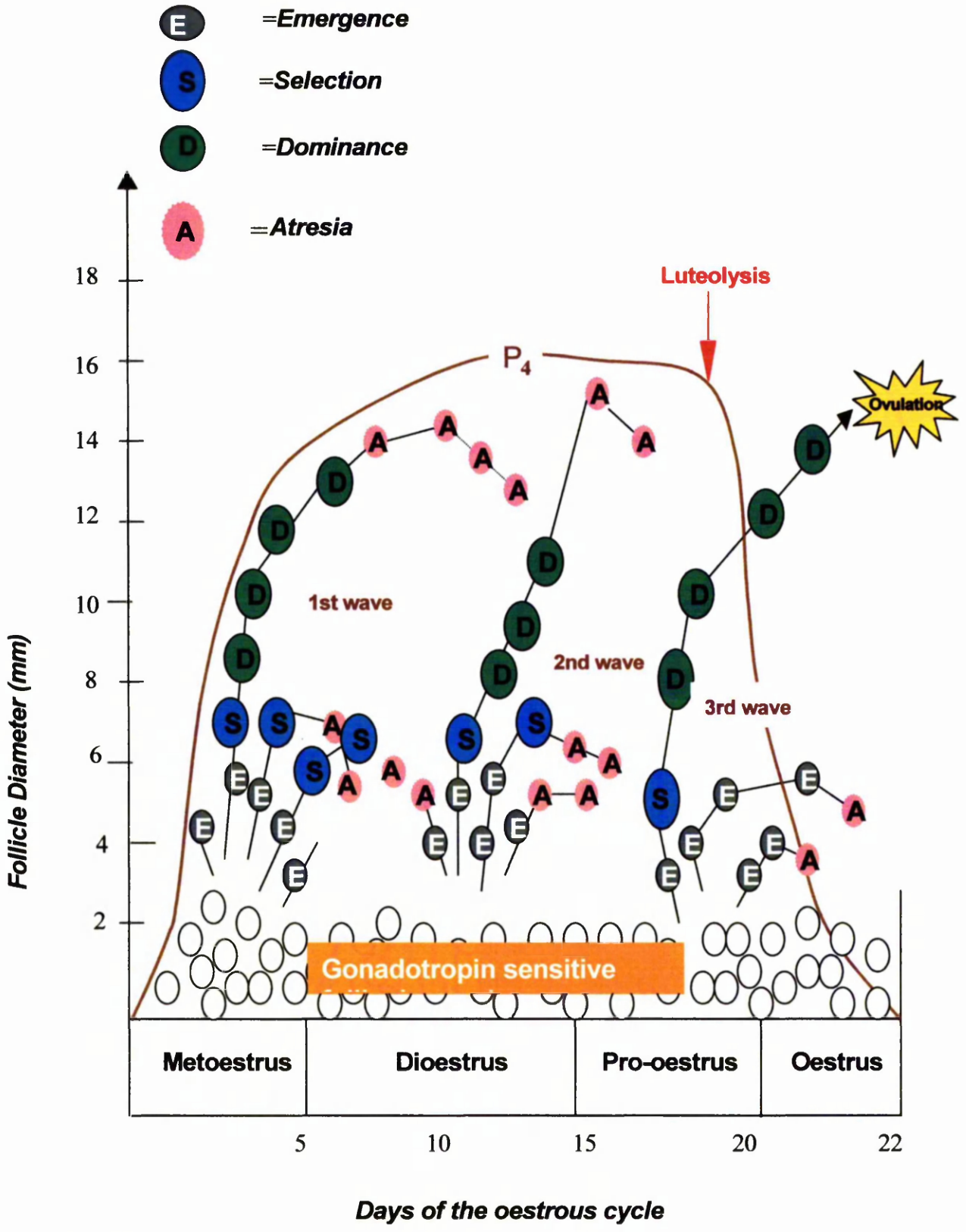



**Fig 1.7 Follicle dynamics in cattle with emerging follicles measuring 3-5 mm to reach 16 mm after dominance. The third dominant follicle is the ovulatory follicle in the three-wave cycle in the cow.**

During the dominant phase after deviation, the subordinate follicles undergo atresia (Ireland and Roche, 1983; Adams *et al.,* 1992a; Sunderland *et al.,* 1994), while the DF continues to grow, secretes oestradiol and is able to undergo final differentiation and ovulate. In the first wave the dominant follicle reaches its maximum diameter of 14.6 ± 0.4 mm on day 7 (the second largest follicle's maximum diameter was 6.3 ± 0.3 mm in Pierson and Ginther (1988), or reaches 13-16 mm between days 6 and 8 of the cycle (Roche and Boland, 1991) followed by a period of relative stability between days 6 and 10. Finally, the first DF decreases in size and is no longer identifiable by day 15. The end of each dominance phase is preceded by loss of FSH and luteinizing hormone (LH) receptors and therefore the oestrogen producing capacity of the dominant follicle (Ireland and Roche, 1983), and occurs during low or reduced LH pulse frequencies (Savio *et al.,* 1993). Loss of dominance is indicated by emergence of a new follicle wave between Days 10-14 preceded by a transient rise in FSH (Adams *et al.,* 1992a; Sunderland *et al.,* 1994).

Approximately 75% of well-fed beef heifers have three waves of development of the DF during the oestrous cycle (Fortune, 1994); the mean length of cycles with 3 dominant follicles was 21.3 ± 1.5 days. The first wave is post-ovulatory and the first dominant follicle was identified on average by Day 4 (range 2-5 days; Sirois and Fortune, 1988), reached its maximum size by Days 6-8 and was detectable on average until Day 15 (range 10-20 days). The maximal size reached by the dominant follicle in the first, second and third wave was 12.3 ± 0.2, 10.2 ± 0.5, and 12.8 ± 0.3mm, respectively (Sirois and Fortune, 1988) and 15.5, 15.9 and 18.8 mm respectively (Savio *et al.,* 1988). The dominant follicle of the second wave is present on average between Days 12 and 19 (range 10-22

days) and reaches its maximum size on Day 16 (range 13-18 days) with a significantly smaller size and slower growth rate compared with the first or third wave DF (Sirois and Fortune, 1988).

The growth rate and duration of detection were not different between the 1st and the 2nd dominant follicle but duration of growth, maximum size and day of maximum diameter were significantly different (Savio *et al.,* 1988). Oestrous cycles with two follicular waves are somewhat shorter than three wave cycles (21 versus 23 days) (Pierson and Ginther, 1988; Sirois and Fortune, 1988; Ginther *et al.,* 1989b). The ovulatory dominant follicle in animals with three follicle waves emerges around Day 16, is significantly larger than the first and the second dominant follicle (Savio *et al.,* 1988) and ovulates 7 days later.

Follicle waves occur before puberty, during the oestrous cycle, in *postpartum* anoestrus and during early pregnancy in cattle (Ginther *et al.,* 1989a, 1989b; Evans *et al.,* 1994; Stagg *et al.,* 1995). The emergence of a follicular wave in cattle is stimulated by FSH rise. The rise reaches a maximum peak by the time the follicles attain 4mm in diameter (Ginther *et al.,* 1997b; Austin *et al.,* 2001). As without transient FSH rises follicular growth stagnates at the 5mm size categories (Prendiville *et al.,* 1995; Gong *et al.,* 1996), in the following follicular gonadotrophin dependencies are outlined.

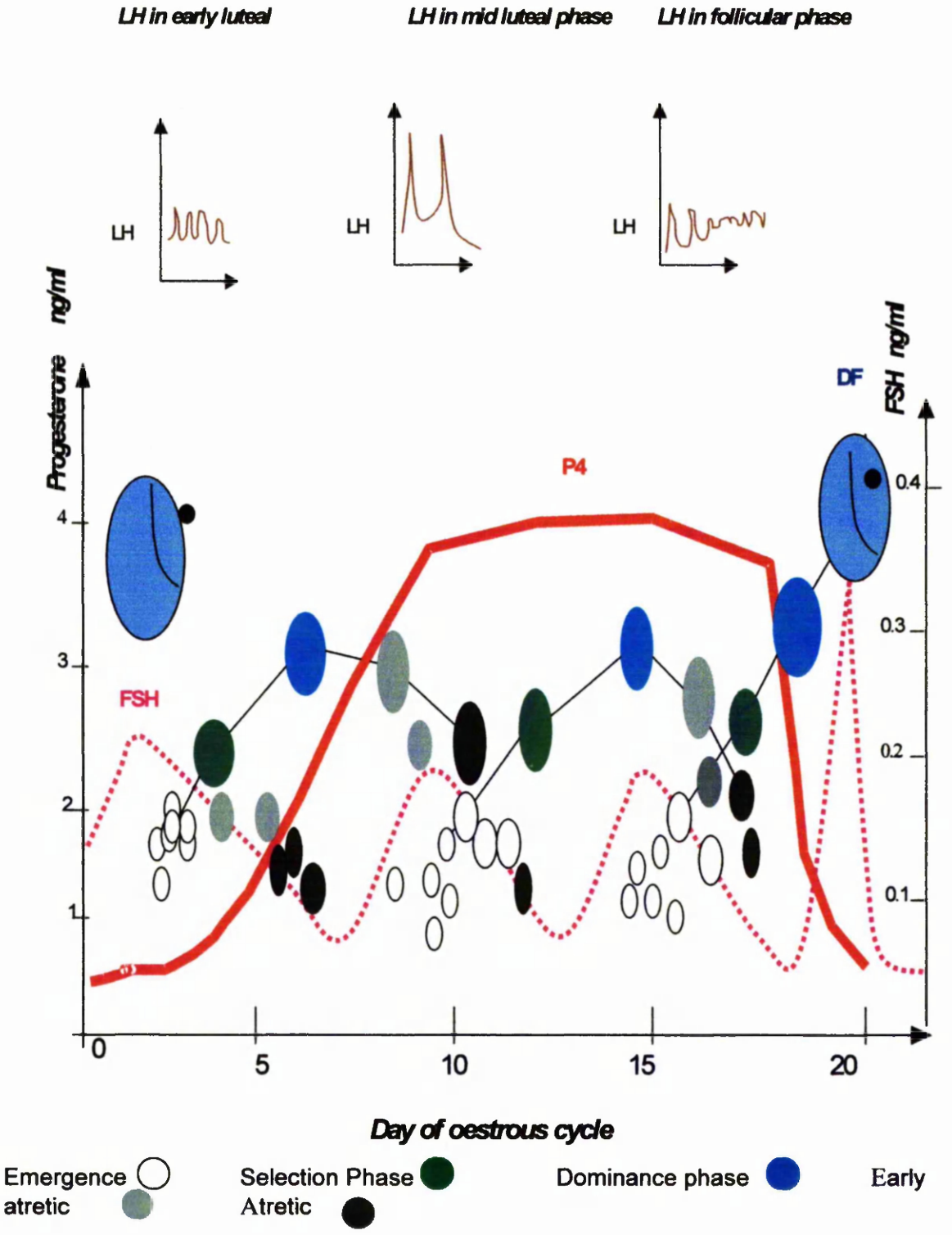



**Fig 1.8 Turnover of dominant follicles in three wave oestrous cycles in the cow: Recurrent transient rises of FSH and progesterone secretions.**

There is a clear association between transient rises in FSH secretion and the emergence of follicular waves (Adams *et al.*, 1992a). It has recently been demonstrated in cattle, that early stages of follicle development (up to 4 mm) are not dependent on gonadotrophins, but that development from 4 to 9 mm requires a rise in FSH, and further development beyond 9 mm requires LH pulses (Prendiville *et al.,* 1995; Gong *et al.,* 1996; Crowe *et al.,* 2001). These observations are clearly important for the precise regulation of follicular development. Suppression of the periovulatory FSH rise using bovine follicular fluid blocks new wave emergence (Turzillo and Fortune, 1990). The concurrent administration of bFF and FSH results in wave emergence, indicating that FSH triggers each follicular wave (Bergfelt *et al.,* 1994a). This is supported by experiments in which heifers were immunized against GnRH, which suppresses recurrent FSH increases, abolishes LH pulses (Prendiville *et al.,* 1996), and blocks follicular growth at the 4 to 5 mm stage (Prendiville *et al.,* 1996).

Declining FSH after a transient rise coincides with selection of a dominant follicle and atresia of the remaining cohort follicles (subordinates) in cattle. This declining and low FSH at the time of follicle deviation is still important for the DF, yet already too low for its subordinate follicles which are no longer FSH responsive (Ginther *et al.,* 1999; Bergfelt *et al.,* 2000). A functional coupling between follicle growth and declining FSH exists following emergence, and the DF appears responsible for FSH suppression at the time of follicle deviation (Ginther *et al.,* 2000a; 2000b). In the first 33 hours of the FSH decline, differential alterations in FSH- dependent growth factors are seen within the cohort of pre-selection follicles, simultaneously inducing growth and enhanced oestradiol-producing capacity of the surviving follicles and already committing some follicles

to their fate of subordinate follicles undergoing atresia (Mihm *et al.,* 1997; Austin *et al.,* 2001).

In cattle, all of the growing cohort follicles ≥ 5 mm contribute to the decline in FSH concentrations, thus precipitating their own atretic fate, as FSH is still needed by growing follicles (Adams *et al.,* 1993; Mihm *et al.,* 1997; Ginther *et al.,* 2000a). The main follicle-produced FSH suppressants appear to be oestradiol and inhibin. During the selection process, the largest follicle also develops the ability to utilise the reduced concentrations of FSH for its continued growth in addition to enhancing its FSH-suppressing ability. It was demonstrated that in heifers that had the largest follicle ablated, oestradiol concentrations were lower than in controls heifers by hour 4 and FSH concentrations increased between hours 4 and 12 after the ablation (Ginther *et al.,* 1999; 2000a). Thus, these results support the hypothesis that the largest follicle releases increased oestradiol into the blood at the beginning of follicular deviation, and that the released oestradiol is involved in the continuing depression of FSH concentrations below the requirement of the smaller follicles (Ginther *et al.,* 2000b).

During a prolonged dominance phase, administration of progesterone or synthetic progestagen reduces LH pulse frequency and dominant follicle atresia occurs with an associated decrease in intrafollicular oestradiol, increases in lower molecular weight insulin-like growth and binding proteins (IGFBPs), which are indicators of reduced follicle health, and granulosa cell apoptosis (Savio *et al.,* 1993; Manikkam and Rajmahendran, 1997). Subtle decreases in LH pulse frequency also occur between the first day of dominance of the first wave DF of the oestrous cycle (Day 5), loss of oestrogen activity preceding atresia on Day 8 and loss of dominance and atresia on Day 11 based on new wave emergence

(Mihm *et al.,* 1995; Evans *et al.,* 1997). In postpartum anoestrus and before puberty, infrequent LH pulses occur every 3 to 6 hours, which result in small DF, rapid loss of oestrogen activity and dominance resulting in a new transient FSH rise within 1 to 2 days after selection (Evans *et al.,* 1994; Stagg *et al.,* 1995; 1998).

Recurrent periods of turnover of dominant follicles are seen in pre-pubertal, cyclic, early pregnant or immunised heifers with persistent corpora lutea independent of presence or absence of progesterone but in a common environment characterised by low LH pulse frequency. Thus, it seems that relatively minor changes in LH can determine whether a DF undergoes atresia (one LH pulse every 3-6 hours) or stays healthy, grows and attains the ability to ovulate (1-2 LH pulses/hour), demonstrating the acute LH-dependence of selected dominant follicles. Exogenous LH pulses administered during the luteal phase (Taft *et al.,* 1996) or early postpartum (Duffy *et al.,* 2000) maintain dominant follicles that would otherwise undergo atresia. It is again worth emphasising that the dominant follicle that develops in the presence of an active corpus luteum (first DF in cycles with two and second DF in cycles with three dominant follicles) will also undergo atresia due to the absence of an LH pulsatile pattern conducive to high oestradiol synthesis and the presence of progesterone blocking the GnRH and LH surge. The non-atretic dominant follicle, which is present, or developing at the time of regression of the corpus luteum will become the ovulatory follicle due to the increased follicular LH pulse frequency which is sufficient to stimulate final maturation, pro-oestrus oestradiol and LH surges, and ovulation.

### 3) Intra-follicular health markers during follicle wave development

As DF selection is dependent on the FSH decline following its transient rise, it is likely that the biochemical factors that are involved in the selection process are FSH-dependent, although it is not known whether changes in those factors are initiated before or after wave emergence. Alterations in amounts of several key FSH–dependent growth factors and health markers in follicles progressing from wave emergence to dominance have been characterised in detail during the first follicle wave in cattle (Ireland and Roche, 1983; Badinga *et al.,* 1992; Mihm *et al.,* 1997; Austin *et al.,* 2001). These studies show that; a) intrafollicular concentrations of oestradiol, inhibins, insulin-like growth factor I (IGF-I) and insulin-like growth factor binding proteins (IGFBP) were dramatically altered between Days 3 and 5 of the heifers' oestrous cycle, during a period of declining nadir FSH concentrations and coinciding with selection of the dominant follicle and atresia of subordinate follicles (Mihm *et al.,* 1997), and b) that exogenous FSH administered in physiological amounts on Day 2 and 3 of the oestrus cycle delayed selection of the DF1 and atresia of subordinate follicles and blocked most of the aforementioned alterations in intrafollicular hormones and growth factors (Mihm *et al.,* 1997). Thus, biochemical mechanisms employed by the future DF to continue its growth and oestradiol production despite declining FSH concentrations can be identified.

Follicular factors likely are responsible for the depressed FSH concentrations during the declining portion of the FSH surge that stimulated wave emergence (Ginther *et al.,* 1999; 2000a). The identity of such factors has not been demonstrated, but oestradiol and inhibin are candidates (Ginther *et al.,*

1996). Inhibins and intrafollicular factors such as the IGF family of proteins have a role in controlling follicular development, through either regulation of systemic gonadotrophins or local intraovarian modulation of the effects of the gonadotrophins especially FSH (Roche *et al.,* 1998).

As mentioned previously systemic oestradiol concentrations increase two or three times during the oestrous cycle in cattle and most of the oestradiol in circulation is produced by a single ovary (Ireland *et al.,* 1984); periodic increases correspond to the time of DF development (Ireland and Roche, 1987). Follicular development from emergence to dominance is accompanied by increased follicular oestradiol production (Sunderland *et al.,* 1994), and an increase in the expression of mRNA for aromatase in granulosa cells of growing antral follicles (Bao *et al.,* 1997).

It was shown that intrafollicular concentrations of oestradiol were dramatically altered between Days 3 and 5 of the heifer's oestrous cycle when FSH concentrations were still declining and the DF1 was selected (Sunderland *et al.,* 1994; Mihm *et al.,* 1997). Follicular oestradiol concentrations of all follicles were relatively low at 5 hours after the peak in FSH concentrations. In addition there were no differences in oestradiol concentrations between follicles from 2.5 to 6.0 mm (Austin *et al.,* 2001). Differentiation into growing follicles with increased intrafollicular oestradiol and those with maintained or reduced follicular fluid oestradiol concentrations were seen at 33 hours after the FSH peak. At 84 hours after the FSH peak, the DF1 was the follicle in cohort with highest concentrations of oestradiol in follicular fluid (Austin *et al.,* 2001). Thus, the attainment of dominance by a single follicle is characterised by its largest size, highest intrafollicular oestradiol concentrations, continued growth despite low systemic

FSH concentrations, and its capacity to suppress growth of other follicles i.e. functional dominance (Ginther *et al.,* 1996).

Follicular theca cells and in DF also granulosa cells produce progesterone, and increased progesterone in follicular fluid (FF) may reflect onset of atresia or luteinization (Singh *et al.,* 1998). However, follicular fluid progesterone concentrations are similar in the DF and the subordinate follicles and progesterone in the DF does not necessarily change following loss of dominance, while intrafollicular oestradiol is reduced markedly (Badinga *et al.,* 1992; Sunderland *et al.,* 1994). However in late atresia, when follicles are decreasing in size, progesterone concentrations in follicular fluid increase (Singh *et al.,* 1998).

### 3.1) Changes in inhibins, IGFs and IGFBP during the first follicle wave

The intrafollicular concentrations of inhibins were also altered between Days 3 and 5 of the heifer oestrous cycle in follicles becoming dominant versus those undergoing subordinate atresia (Mihm *et al.,* 1997). At 5 hours after the peak in FSH concentrations, all follicular size classes had similar amounts of each inhibin form (Austin *et al.,* 2001). At 33 hours after the peak in FSH, an increase in the higher molecular weight inhibins (> 34 kDa) was seen which was significant in the largest two follicles, and the largest follicle already had higher amounts of the 6 precursor inhibin forms than the smallest two follicles examined. These differences were maintained without a further increase in intrafollicular inhibins, and at 84 hours after the FSH peak, the two largest follicles (DF1 and largest subordinate) had highest amounts of higher molecular weight inhibins (Austin *et al.,* 2001). In addition, subordinate status was associated with an increase in the

34 kDa inhibin dimer on Day 5 similar to what is seen in atretic follicles (Sunderland *et al.,* 1996; Mihm *et al.,* 1997).

An endocrine role of inhibin in the regulation of growth and atresia of follicles has been suggested since intrafollicular concentrations of total α-inhibin, as determined by RIA, increase during growth of oestrogen-active dominant follicles when FSH declines, but decrease during regression of oestrogen-inactive old dominant follicles (Martin *et al.,* 1991; Guilbault *et al.,* 1993) when FSH rises again. These studies suggest that oestradiol and inhibin may act to suppress FSH secretion and thus block recruitment and growth of other follicles during the growing phase of a dominant follicle (Martin *et al.,* 1991). However, inhibin may be the more important factor in the regulation of FSH secretion during the luteal phase of cows (Kaneko *et al.,* 1993) and in ewes only inhibin reduces FSH concentration to levels seen before ovariectomy (Mann *et al.,* 1992).

Only one study has so far investigated the relationship between systemic inhibin-A, FSH, oestradiol and follicle growth in cattle (Bleach *et al.,* 2001). It was demonstrated in cattle that mean plasma inhibin-A (~ 50 pg/ml before luteolysis) rises steadily during the induced follicular phase to a peak (~125 pg/ml) coincident with the preovulatory oestradiol, LH and FSH surge and after ovulation inhibin-A fell sharply to a nadir (~55 pg/ml) coincident with the secondary FSH rise (Bleach *et al.,* 2001). Similarly, both inhibin-A and oestradiol fall to basal values after the pre-ovulatory gonadotrophin surge in the ewe (Knight *et al.,* 1998). During the next 3 days in the heifer cycle (early luteal phase), inhibin-A increased to approximately 90 pg/ml in association with growth of the new wave and DF, and plasma oestradiol also rose twofold during this period, whereas FSH fell by approximately 50% (Bleach *et al.,* 2001). Thus, the growth of the first- wave (non-

ovulatory) DF from 5 to 10 mm over approximately 3 days was associated with a rise in inhibin-A and oestradiol and a fall in FSH. Thereafter, both inhibin-A and oestradiol declined despite the fact that this DF remained >10mm for a further 7 days. The above decline in inhibin and oestradiol presumably reflecting loss of functional dominance of the first wave DF was associated with a marked increase in FSH; again, growth of the ovulatory DF was associated with a 3-fold rise in inhibin-A and a 5-fold rise in oestradiol. Thus, Bleach *et al., (*2001) conclude in accordance with Ginther *et al.,* (1996) that a reduction in concentrations in both follicular secretions, oestradiol and inhibin-A, contributes to the generation of the post ovulatory FSH rise required to initiate the FSH- dependent growth of new first wave cohort follicles.

A second, increasingly important family of proteins belonging to the IGF system has been studied in relation to follicle wave growth. Members of the IGF system studied in detail in bovine follicles are the two peptides IGF-I and II, the IGF-type 1 receptor and four different and specific IGF binding proteins (IGFBPs), which regulate IGF activity by binding their ligand with high affinity thus preventing IGF receptor interaction (Spicer and Echternkamp, 1995). Follicle proliferation and steroidogenesis in response to gonadotrophins are thought to be enhanced by IGF-I (Spicer *et al.,* 1993), but no close relationship has been found between stage of follicle development and intrafollicular total IGF-I concentrations possibly due to the presence of IGFBPs (Stewart *et al.,* 1996; Mihm *et al.,* 1997). In fact, follicle oestrogen activity is negatively correlated with intrafollicular amounts of the IGFBPs < 40 kDa (that is IGFBP2, 4 and 5) with increased oestrogen activity of follicles being accompanied by decreased total IGF-BP and lower amounts of low molecular weight forms of IGF-BP (Echternkamp *et al.,* 1994; Stewart *et al.,* 1996;

de la Sota *et al.,* 1996). Increases in the lower MW IGFBP2, 4 and 5 have been seen in atretic bovine follicles (Mazerbourg *et al.,* 2000). Gong *et al.* (1991) proposed an important positive in vivo role for IGF-I in antral follicular growth showing that growth hormone treatment of heifers increased systemic total IGF-I and numbers of small follicles without affecting LH and FSH concentrations

During declining and nadir serum FSH concentrations (between Days 3 and 5 of the bovine oestrous cycle), the increased growth and enhanced oestradiol secretion of the DF are associated with increased intrafollicular concentrations of IGF-I, but markedly reduced total IGF-I binding activity. Specifically, the IGF binding proteins 2, 4 and 5 (produced by follicular cells) were maintained low during DF selection while subordinate fate was linked to an increase in IGFBP4 and 5 (Mihm *et al.,* 1997; Austin *et al.,* 2001). Thus, the selected DF has a marked net increase in overall intrafollicular IGF-I bioactivity. Also, the newly selected DF has increased IGF-BP-4-specific proteolytic activity (Chandrasekher *et al.,* 1996). Enhanced proteolytic activity results in the degradation of IGF-BP-2, 4, 5 and thus more bioavailable IGFs (Besnard *et al.,* 1997). However the mechanisms responsible for differential synthesis and processing of IGF-BP in only one follicle of a cohort of oestrogen-active follicles prior to dominance is currently unknown. One study showed recently that the ability to maintain low amounts of intrafollicular IGFBP-4 may be linked to DF selection (Mihm *et al.,* 2000). Such an ability was also seen in successful cohort follicles during the FSH decline and linked with enhancement of oestradiol synthesis (Austin *et al.,* 2001). The cessation of growth and diminished oestradiol production of subordinate follicles are dynamic events, which occur coincident with the continued growth and enhanced oestradiol production of the DF (Mihm *et*

*al.,* 1997). Loss of oestradiol production in subordinate and increased % of apoptotic granulosa cells is associated with increased amounts of all lower molecular weight forms of IGFBP, and thus reduced IGF bioavailability forms (de la Sota *et al.,* 1996; Mihm *et al.,* 1997; Austin *et al.,* 2001). Similarly, the DF shows an increase in the lower molecular weight of IGFBP and % of apoptotic granulosa cells during induced loss of dominance (Manikkam and Rajamahendran, 1997).

# IV) Manipulation of Follicle Waves Using Progestagen-Oestradiol Combination Treatments

## 1) Synchronisation

Good synchronisation of oestrus and ovulation in cattle will help us improve reproductive efficiency by allowing planned breeding with subsequent good fertility. Improved reproductive efficiency is due to reduced postpartum intervals, increased submission rates and thus reduced and pre-planned breeding and calving seasons, and due to the increased use of AI allowing disease control and an accelerated increase in genetic merit within herds and disease control (Macmillan and Burke, 1996)

For oestrous synchronisation to be considered successful, > 95 % of treated animals are expected to display oestrus within a defined 12-hour period, and fertility should at least be the same as after natural mating or insemination following natural oestrus (depending on age and production type of animals > 45-55 % calving rates). As already mentioned earlier, it is essential to manipulate follicle wave development to ensure a healthy DF at end of treatment, which will ovulate predictably following luteolysis and treatment withdrawal. Very strict criteria apply to the ovulatory DF, as duration of dominance at oestrus affects subsequent fertility with dominance periods of > 8 days drastically decreasing pregnancy rates; duration of dominance of pre-ovulatory DF at oestrus also affects the spread in oestrous onset between animals (Austin *et al.*, 1999). Least variability in oestrus onset was achieved following 2-4 day dominance periods,

and this was also combined with highest fertility (Austin *et al.,* 1999). Thus, it is no longer sufficient to cause synchronous luteolysis using PGF2α as stage of follicle wave development determines the interval from PGF2α to oestrus; follicle waves will have to be managed such that luteolysis and selection of a new DF occur at the same time. It is also no longer sufficient to ensure that all animals have a DF present at the time of luteolysis and treatment withdrawal; the treatment required for this will cause subluteal progesterone levels in blood, lead to some animals developing persistent follicles with much reduced fertility, and at the end of treatment DF with differing durations of dominance will exist which will increase variation in time to oestrous onset. In the following, the induction of synchronous atresia in follicles present at time of treatment to ensure synchronous and predictable growth of a new follicle wave and a new DF will be considered.

**2) Follicle Wave Management**

As discussed above, it is essential for successful synchronisation treatments to induce a new follicle wave synchronously in all treated animals, such that synchronous luteolysis can be initiated just when a new DF is selected. As wave emergence is linked with transient FSH rises (Adams *et al.,* 1992a; Sunderland *et al.,* 1994), and transient FSH rises can only occur following loss of dominance of a DF or removal of the inhibitory effects of the growing healthy cohort or dominant follicles, it is necessary to cause the demise of all existing follicles independent of their stage of differentiation.

Removal of a DF via electrocautery (Ko *et al.,* 1991; Adams *et al.,* 1992a) will advance the next wave causing its emergence within 2 - 2.5 days, and ultrasound- guided ablation of DF or all follicles from 5 mm will immediately lead to FSH rises and emergence of a new follicle wave 1–1.5 days later (Bergfelt *et al.,* 1994b; Bodensteiner *et al.,* 1996). Induction of ovulation of dominant follicles using exogenous GnRH agonist treatment (Twagiramungu *et al.,* 1995; Ryan *et al.,* 1998; Mihm *et al.,* 1998), thus removing the inhibitory effects of healthy DF, will cause an FSH rise within 1.5 days followed by the predictable emergence of a new wave and selection of a new DF in 4 - 5 days. Thus, it has been shown that removal of the inhibitory effects of follicles on FSH and other follicle growth will lead to a predictable transient FSH rise coupled with the emergence of a new follicle wave. However, such physical means, although considered to be very successful in synchronising follicle wave growth, are not practical at farm level. In addition, the success of any GnRH treatment in causing synchronous new wave emergence will depend on the presence of a healthy DF at the time of treatment which is able to respond to the induced gonadotrophin surge (Roche *et al.,* 1998). There is, therefore, a need to hormonally cause acute atresia of all growing follicles present at time of treatment, using treatments which are acceptable to the farmer regarding cost and labour intensity but also to the public in terms of animal welfare and subsequent product quality.

### 3) Causing Atresia of wave follicles before DF selection

The one characteristic of cohort follicles is their absolute dependence on raised FSH concentrations from baseline. Treatment with bovine follicular fluid from ovulation will prevent the secondary transient FSH rise and thus delay the emergence of the first post ovulatory follicle wave (Turzillo and Fortune, 1990). Conversely, maintenance of raised FSH will delay selection of a DF by maintaining growth of most of the wave follicles and preventing subordinate atresia (Adams *et al.,* 1993; Mihm *et al.,* 1997). Thus, removal of FSH stimulus from growing wave follicles before DF selection will cause follicle atresia; this happens naturally in all but one follicle following emergence of a follicle wave, and also occurs following a premature decline in FSH caused by treatment with oestradiol benzoate from the day of ovulation (Adams *et al.,* 1992a; Sunderland *et al.,* 1994; Cooke *et al.,* 1997b).

Oestradiol has been used extensively to suppress FSH and also LH in the presence of progesterone. It has been shown to have a transient suppressive effect on circulating FSH concentrations (Bolt *et al.,* 1990; O'Rourke *et al.,* 2000), and thus its inclusion into hormonal treatments to manipulate follicle wave development is considered essential to 1)- cause premature suppression of the transient FSH rise and thus terminate a newly emerging follicle wave (Cooke *et al.,* 1997b; Austin, 2000), 2)-delay the next transient FSH rise if administered during the dominance period by approximately 2 days thus delaying the next wave emergence (Bo *et al.,* 1993; 1995; O'Rourke *et al.,* 1998). Pharmacologically high doses of oestradiol given in combination with progestagen during the growth phase of the first follicle wave (1 day after ovulation in the absence of endogenous

progesterone) will cause an initial FSH decline, a small FSH surge one day later, cessation of follicle growth in 0.5 – 1.3 days followed by rising FSH to reach maximum concentrations 3-5 days after treatment; new wave emergence occurs 1 day after maximum FSH and is advanced by 2 days versus controls (Bo *et al.,* 1993; 1994). However, no new wave emergence or variation in intervals to new wave emergence ranging from 2-7 days from oestradiol treatment have been reported by other studies (Duffy *et al.,* 1997; O'Rourke *et al.,* 1998). Thus, variation in intervals from treatment to new wave emergence appears to exist and is so far unexplained, yet may compromise success of steroid synchronisation treatments essential for fixed-time AI. Why the next transient FSH rise is protracted reaching its maximum only 3-5 days after oestradiol treatment compared to the fast natural transient FSH rises is so far unexplained. Rapidly declining but still relatively high circulating oestradiol concentration following a single administration of oestradiol ester, or persisting and still partially functional follicles may both be responsible for such a protracted FSH rise.

Different oestradiol esters have different half-lives and thus persistence in circulation. The use of 5mg oestradiol valerate is clearly pharmacological both in relation to magnitude >100 pg/ml and duration (5 to 7 days) of the estradiol rise (Bo *et al.,* 1993; Duffy *et al.,* 1997); similarly, 5 mg of oestradiol benzoate (ODB) raises serum concentrations to the pharmacological range, but duration of elevation is reduced to 3 to 4 days (O'Rourke *et al.,* 1998). Single injections of ODB in the range of 0.5 to 1.0 mg elevate oestradiol concentrations in blood 2 to 3 times above follicular phase concentrations for 36 to 60 hours. However, intravaginal administration of high doses (5 or 10 mg) of ODB as a dissolvable capsule inserted into the groove within the CIDR or the inside of the coil of the

PRID only elevates serum concentrations to maximal (2-4 pg/ml), i.e. early luteal phase levels and concentrations above 1 pg/ml may only be achieved for approximately 2-3 days. These varying concentrations of oestradiol cause a dose dependent decrease in FSH followed by a rise in FSH, despite the fact oestradiol concentrations are elevated to 5 to 50 pg/ml (O'Rourke *et al.,* 1998). Therefore, oestradiol has only a transitory suppressive effect on FSH concentrations in cattle, which may be insufficient to block follicle growth at different stages of the follicle wave.

The i.m. injection of 5 mg oestradiol 17-β dissolved in ethanol and administered in sesame oil rapidly leads to extremely high maximum concentrations of 650 pg/ml followed by a much more rapid elimination from circulation, and high concentrations only persist for 42 hours before basal levels are reached (Bo *et al.,* 1994).

Wave follicles after emergence are also responsive to changes in the LH pulse environment. When progesterone is administered early during emergence of the first follicle wave (just after ovulation) suppressing LH pulse frequency, first follicle wave growth is suppressed, leading to reduced maximum diameter of the DF and largest SF, and a tendency for more 3-wave cycles possibly due to advanced DF atresia and second wave emergence (Adams *et al.,* 1992b; Taylor *et al.,* 1994; Burke *et al.,* 1994; Austin, 2000). Although selection of the first DF is not suppressed, attainment of maximum size and duration of dominance appear to be affected beyond treatment duration, indicating that follicles were responsive to the LH pulse environment after their emergence but before DF selection. This is not surprising as follicular oestradiol production depends on LH stimulated thecal androgen precursor synthesis and follicle growth is linked with oestradiol

synthesis in successful wave follicles (Austin *et al.,* 2001). In addition, follicles > 8 mm acquire LH receptors on their granulosa cells, which is an important step of differentiation for the selected DF. More detailed studies investigating the effects of suppression of LH via progesterone before or after follicle deviation did indicate that the freshly selected DF was compromised in terms of intrafollicular oestradiol and free IGF-1 but only after follicle deviation occurred and not before (Ginther *et al.,* 2001).

So, can treatments that suppress FSH consistently cause atresia of growing wave follicles? While treatment with bovine follicular fluid (bFF) to suppress FSH alone will delay wave emergence if administered from ovulation (Turzillo and Fortune, 1990; Adams *et al.,* 1992a), the effect on growing wave follicles before DF selection but already experiencing the FSH decline has not been studied so far. Exogenous oestradiol treatments reaching up to twice follicular phase levels in circulation and given in the absence of progesterone, i.e. very early after ovulation (at the peak of the transient FSH rise approximately 30-36 hours after onset of heat), will reduce the duration of the transient FSH rise but may also cause a mini gonadotrophin surge and an increase in the LH pulse amplitude (Bo *et al.,* 1993; Cooke *et al.,* 1997b, Austin, 2000). However, no significant differences in wave growth and intrafollicular health parameters are seen 1.5 days after a single injection of 0.75 mg ODB compared with untreated controls (Austin, 2000). When 1mg ODB injections were continued on a daily basis, however, the first cohort regressed before selecting a DF (Cooke *et al.,* 1997b). It is worth noting that endogenous progesterone was rising in this study and the combined actions of exogenous oestradiol and endogenous progesterone may have reduced LH pulse frequency and amplitude and thus effects on the

wave were probably due to modification of both FSH and LH concentrations in serum.

Indeed, the combination of oestradiol benzoate with a progesterone treatment given just after ovulation will abolish LH pulses most effectively, and delays ultrasound-detectable emergence and selection of the first wave DF, and reduces the maximum size of the DF (Austin, 2000). Such an effect on the first follicle wave may again be due the premature reduction in FSH concentrations combined with the reduction in LH pulse frequency and amplitude.

Treatment with a combination of oestradiol and progestagen given 1 day after ovulation or on the day of emergence of the first follicle wave will modify follicle wave growth depending on the dose of oestradiol and its preparation used. In beef heifers, pharmacologically high doses of oestradiol (5mg of oestradiol-17β or ODB) will lead to a reduction of the maximum size achieved to 6.4 - 8 mm, with cohort follicles becoming static 0.5–1.3 days after oestradiol treatment, will prevent selection of a DF and cause regression of the first wave and new wave emergence in 5 - 6 days (oestradiol-17β) (Bo *et al.,* 1994; 1995) or 8 days from oestradiol treatment (ODB) (O'Rourke *et al.,* 1998). Lowering the dose of ODB to twice follicular phase levels also affected selection of the first DF in most animals and caused emergence of the next wave in approximately 5 days from ODB treatment (O'Rourke *et al.,* 1998). Although Bo *et al.,* (1994; 1995) report that variation in time interval to new wave emergence is small due to the use of oestradiol -17β with its short half life (within 42 hours oestradiol concentrations are reduced to baseline levels), such consistent time intervals have not been seen using oestradiol preparations licensed for use in cattle (oestradiol benzoate or oestradiol valerate). Thus, variation in interval from oestradiol treatment to new

wave emergence in beef cattle is quite marked after ODB, and merits investigation into possible causes. Data are also not available in relation to dairy breeds, which may respond differently due to genetic, nutritional or growth characteristics. In this context it may be interesting to speculate that rising FSH concentrations after their initial suppression following oestradiol administration and despite high circulating oestradiol concentrations (Bo *et al.,* 1994; Cooke *et al.,* 1997b; O'Rourke *et al.,* 2000), may variably support wave follicles with sufficient FSH receptors left to respond which may in turn variably suppress new follicles preventing an immediate and synchronous new wave emergence. Thus the atretogenic effect of steroid combination treatments on growing wave follicles will have to be determined in the future.

**4) Causing atresia of dominant follicles**

Following its selection the DF is dependent on LH pulses (Savio *et al.,* 1993) and each LH pulse is followed by pulsatile release of oestradiol from the ovary with the dominant follicle (Walters *et al.,* 1984). Luteal phase frequencies of 1 LH pulse per 3-4 hours or less (Rahe *et al.,* 1980) will cause loss of oestradiol synthesising capacity, stasis and finally regression of the DF, followed by a transient FSH rise and emergence of a new wave. This occurs during the luteal phase of the cycle and is the fate of DF selected post-partum (Stagg *et al.,* 1998), pre-puberty (Evans *et al.,* 1994), during pregnancy (Ginther *et al.,* 1989a) or nutritional anestrous (Rhodes *et al.,* 1995; Stagg *et al.,* 1998) where very low LH pulse frequencies predominate. Conversely, an increase in LH pulse frequency,

as occurs naturally in the follicular phase following luteolysis (Rahe *et al.,* 1980) or after treatment with LH pulses during the luteal phase (Taft *et al.,* 1996), will lead to enhanced oestradiol production and continued growth of the DF. If an adequate LH pulse environment persist, DF will respond with long term growth and oestradiol production for up to 12 days and longer DF persistence (Stock and Fortune, 1993; Mihm *et al.,* 1994; 1999).

During the cycle and pregnancy it is progesterone secretion from the CL that determines LH pulse frequency and thus DF lifespan via negative feedback on GnRH pulsatile release from the hypothalamus (Goodman and Karsch, 1980). Thus, exogenous progestagen treatments routinely used to suppress the gonadotrophin surge and oestrus for a specific time period (Taylor *et al.,* 1993) also suppress LH pulse frequency with varying potencies, and thus affect DF development during treatment. Exogenous progesterone administered intravaginally in the form of a PRID (Sanofi Animal Health) or CIDR device (CIDR-B® InterAg, Hamilton, New Zealand) elevate serum progesterone concentrations in ovariectomized animals above luteal phase levels (Macmillan *et al.,* 1991; Van Cleeff *et al.,* 1992) for approximately 4 days. Subsequently, serum progesterone concentrations fall to subluteal concentrations of 1-2 ng/ml, which will cause an increase in the LH pulse frequency to 1 LH pulse every 1-2 hours (Sirois and Fortune, 1990; Stock and Fortune, 1993). In cyclic animals, administration of intravaginal devices during the luteal phase will elevate progesterone concentration to twice-luteal phase concentrations (Burke *et al.,* 1996). However, if a device is inserted towards the end of the luteal phase, concentrations of progesterone will fall to subluteal levels following luteolysis and after ca. 4-5 days of insertion time. This again is predicted to result in high LH pulse frequencies,

which will support any healthy DF sometimes for very long periods until device removal. Synthetic progestagen treatments, such as norgestomet 3mg or 6mg silastic subcutaneous ear implants or oral preparations such as megoestrol acetate will prevent the gonadotrophin surge for the duration of treatment (Taylor *et al.,* 1993) but do not mimic the CL in terms of negative feedback on LH (Kinder *et al.,* 1996). These synthetic progestagen treatments, therefore, lead to higher LH pulse frequencies in the absence of the animal's own CL which may lead to persistence of the DF present at the time of luteolysis (Rajamahendran and Taylor, 1991; Savio *et al.,* 1993; Mihm *et al.,* 1999).

Ovulation of persistent DF has been linked with reduced fertility (Stock and Fortune, 1993; Savio *et al.,* 1993; Mihm *et al.,* 1994: Cooperative regional research project NE-161, 1996). Specifically if treatment duration leads to an extended duration of dominance of the DF beyond 8 days, severe reductions in pregnancy rates are seen (Mihm *et al.,* 1994; Austin *et al.,* 1999) linked with changes in follicular fluid parameters of health, and premature resumption of meiosis in oocytes before the gonadotrophin surge (Revah and Butler, 1996; Bigelow and Fortune, 1998; Mihm *et al.,* 1999). Oviductal secretions at and after ovulation are altered (Binelli *et al.,* 1999), and early embryonic development appears to be compromised following ovulation of persistent DF and fertilisation (Ahmad *et al.,* 1995). However, the prolonged and high pre-ovulatory oestradiol rise and subsequent luteal function do not appear to be the main causes of reduced fertility as pregnancy rates following embryo transfer in recipients which ovulated persistent DF were similar to those in control recipients (Wehrman *et al.,* 1997), and fertility was not reduced if a new DF was allowed to ovulate following

long-term persistence and prolonged oestradiol secretion from the preovulatory DF (Fike *et al.,* 1997).

Thus it is essential to cause atresia of DF present at the time of treatment initiation in order to avoid DF persistence as a consequence of subluteal progesterone concentrations or the presence of synthetic progestagen after luteolysis. Abolishing LH pulses using GnRH antagonist treatment (Manikkam *et al.,* 1995) or a sudden reduction in LH pulse frequency using norgestomet will cause atresia of a persistent (maintained by high LH pulse frequency) DF (Savio *et al.,* 1993), followed by emergence of a new wave 5 days later. Progesterone induced atresia of a persistent DF maintained by one norgestomet implant following exogenous luteolysis is accompanied by characteristic changes in intrafollicular steroid concentrations, lower molecular weight IGF-Binding proteins, and granulosa cell apoptosis, with first biochemical changes occurring 5 days after treatment (Manikkam and Rajamahendran, 1997). When exogenous oestradiol, testosterone and progesterone were evaluated in their ability to cause persistent DF atresia, progesterone and oestradiol 17β led to DF atresia most consistently and emergence of a new wave occurred 5 days after treatment (Rajamahendran and Manikkam 1994). As mentioned previously, the addition of oestradiol will enhance the negative effect of progesterone and suppress LH pulse amplitude as well as frequency (Goodman and Karsch, 1980; Bolt *et al.,* 1990). Thus, a more powerful negative feedback is exerted on LH pulsatile release abolishing LH pulses more completely than progesterone alone as was seen immediately after ovulation (Austin, 2000) and in the mid-luteal phase (Burke *et al.,* 1996). Combination treatments using progestagen and oestradiol may thus

initially abolish LH pulsatility, which will lead to more consistent DF atresia than any of the steroids used on their own.

When oestradiol-progestagen combination treatments were administered to the healthy DF, its further growth and maximum size were suppressed and the next wave emerged 4-5 days (Bo *et al.,* 1994; 1995) or 7 days after oestradiol treatment (O'Rourke *et al.,* 1998); the difference may be due to the oestradiol preparations (oestradiol- 17β versus ODB) and the progestagen used (synthetic norgestomet versus progesterone). No effect on the current DF, but differences in the interval from oestradiol treatment to new wave emergence were again seen between the oestradiol-17β + norgestomet combination and ODB + progesterone combination when treatments were administered during dominance just before next (second) wave emergence: oestradiol-17β plus norgestomet delayed second wave growth by 2 days with new wave emergence occurring 4.7 days after oestradiol treatment (Bo *et al.,* 1995), while ODB plus progesterone delayed next (second) wave emergence until 7 days after oestradiol treatment (O'Rourke *et al.,* 1998). These differences warrant further research into the differential effects of oestradiol and progestagen preparations on LH and FSH and thus on DF atresia.

Thus, it appears that once a DF is selected, it is doubtful whether it can be made atretic very acutely and predictably, as the interval from treatment to emergence of a new wave may vary between 4 and 7 days (Bo *et al.,* 1995; O'Rourke *et al.,* 1998). The persistent DF (possibly a model for the pre-ovulatory DF) can reliably be induced to become atretic using progestagen or a combination of progestagen and oestradiol suppressing LH pulse frequency within 6 hours (Kinder *et al.,* 1996), however, it will take approximately 5 days from treatment for a new follicle wave to emerge, and approximately 8 days for a new DF to become

selected (Manikkam and Rajamahendran, 1997). In fact, induction of a persistent follicle first, then causing its synchronous atresia thereby inducing synchronous and predictable new wave emergence may be an easier goal to achieve than acute atresia of follicles in different stages of their development. However, this necessitates long-term treatments. For example, administration of oestradiol-17β or progesterone during the later stages of a synthetic progestagen treatment was aimed to regress persistent DF and ensure ovulation of a healthy freshly selected DF. Such a treatment was found to increase pregnancy rates (Anderson and Day, 1994; Yelich *et al.,* 1997; Cavalieri *et al.,* 1998) or leave pregnancy rates unchanged compared with animals only treated with the synthetic progestagen (Fike *et al.,* 1999). It is not clear, whether the lack of very high pregnancy rates seen in these studies expected after ovulation of DF with very short duration of dominance is due to inadequate atresia of the persistent DF or some other factors influencing fertility particular to each study.

One reason for combining oestradiol with a progestagen pre-treatment is to prevent oestradiol from inducing a GnRH and thus a gonadotrophin surge in a low endogenous progesterone environment, for example after luteolysis. Small gonadotrophin surges were seen just after ovulation in the early luteal phase (Bo *et al.,* 1993; Cooke *et al.,* 1997b). This would cause ovulation of a DF present at the time and a quicker new wave emergence than expected. It would also consistently necessitate the induction of luteolysis 7 days later. The effects of a surge on growing cohort follicles before DF selection are unclear, but preliminary data appear to indicate that cohort growth will be unaffected (Ryan *et al.,* 1998; Mihm *et al.,* 1998).

## V) Conclusions

What is the success of current progesterone and oestradiol combination treatments in terms of induction of synchronous atresia of follicles at random stages of their development? At this stage oestradiol and progestagen combination treatments have been used randomly (Bo *et al.,* 1996; Yaakub *et al.,* 1998) and at different stages of development of the first follicle wave in order to explain the variation that occurs in the interval from oestradiol treatment to new wave emergence (Bo *et al.,* 1993; O'Rourke *et al.,* 1998). The effects of treatment during the second wave or in the mid-luteal phase have not been monitored as closely. One research group concludes that the use of 5mg oestradiol-17β combined with a norgestomet treatment given one day prior to oestradiol will cause predictable atresia of existing cohort or dominant follicles and induce new wave emergence in 4 days independent of stage of follicle wave development (Bo *et al.,* 1994; 1995; 1996). However, such consistent timing and low variation in new wave emergence was not reported elsewhere using oestradiol preparations licensed for use in cattle combined with progesterone (O'Rourke *et al.,* 1998; Yaakub *et al.,* 1998). In these studies the interval from treatment to new wave emergence is on average 5-8 days depending on stage of development of the first wave, longer if treated before emergence or DF selection, shorter if treated at the time of DF selection, and with quite large variation between animals. Thus it is clear that current steroid combination treatments are unable to cause consistent and acute atresia especially of emerging wave follicles via suppression of FSH and LH, and the underlying reasons merit further research.

In addition, it is unknown whether atresia is indeed induced in growing wave follicles following steroid combination treatments: if atresia is complete, it may be possible that prolonged high oestradiol concentrations or secretions from follicles undergoing atresia may prevent immediate new wave emergence. It is also possible that atresia is incomplete in growing wave follicles following some steroid combination treatments, and follicles may be maintained and continue to grow for a certain period of time suppressing the transient FSH rise and/or immediate new wave emergence.

Therefore, as no detailed information of the effects of oestradiol-progestagen combination treatments on follicle dynamics and circulating hormone concentrations are available in dairy heifers or cows, our study aims to address some of the questions raised above in order to explain why steroid manipulation of follicle wave growth appears to be very difficult and does not result in synchronous new wave emergence in all treated animals. In particular, we are trying to determine in dairy heifers whether:

- Changes in the FSH profile occur following steroid treatment during emergence of the first follicle wave which could be responsible for lack of synchrony and prolonged intervals to new wave emergence.

- The fate of follicles belonging to the first postovulatory wave (such as: (i) no further growth; (ii) continued growth until stasis< 8mm; (iii) continued growth with selection of DF but shortened dominance period; (iv) unaffected wave growth, DF selection and dominance period) depends on the dose of ODB given with norgestomet at the time of emergence of the first follicle wave.

- Wave or dominant follicles present after treatment with ODB and norgestomet given at the time of emergence of the first follicle wave, influence the next FSH rise (rate of rise, maximum) and thus the timing of emergence of the next wave and the next DF.

- Inhibin-A concentration in serum can be used as a marker of follicle health, as has been shown for the higher molecular weight forms of inhibin in follicular fluid, and thus can be used to evaluate the atretogenic effect of ODB + norgestomet given at the time of emergence of the first follicle wave.

# Chapter 2:

# Hormonal and follicular responses to steroid treatment administered during emergence of the first follicle wave in dairy heifers

## 1) Abstract

Current oestrous synchronisation programmes in cattle include a combination of oestradiol and progestagen to cause atresia of follicles present on ovaries at the time of treatment, followed by a transient FSH rise and emergence of a new follicle wave. In order to determine why the timing of new wave emergence is relatively unpredictable and intervals from treatment are prolonged when steroids are given during emergence of a follicle wave, the aims of our study were to: 1)- compare hormonal and follicular events following dominant follicle (DF) aspiration (predicted to cause an immediate transient FSH rise and new wave emergence in all animals) with those seen after treatment with two different doses of oestradiol benzoate (ODB) combined with a synthetic progestagen during emergence of the first follicle wave of the dairy heifer oestrous cycle. 2)- determine the suppressive effect of the largest follicles present 3 days after steroid treatment on FSH and follicle emergence, and 3)- evaluate systemic inhibin-A as a marker for health or atresia of follicles present on ovaries in control and steroid manipulated oestrous cycles.

Holstein Friesian heifers (year 1: 14 heifers, 2-3 cycles each; year 2: 4 heifers 2 cycles each, total of 42 experimental oestrous cycles) were given prostaglandin $F2_{\alpha}$ in the presence of a corpus luteum to cause luteolysis and oestrus (=Day 0). On Day 2, heifers were left untreated (control cycles; n =15) or treated with 0.75mg (n =9 cycles) or 5mg (n= 18 cycles) ODB (Oestradiol Benzoate 5mg, Intervet UK Ltd, UK) in sterile corn oil i.m. and a subcutaneous ear implant containing 3mg norgestomet (P; Crestar, Intervet UK Ltd, UK) left in place for a 10 day period. Transvaginal ultrasound-guided aspiration of the largest follicles present on Day 5 was carried out in 5 control cycles, 5 0.75mg

ODB+P treated cycles and 10 5mg ODB+P treated cycles. Progression of the first and second follicle wave was monitored daily using transrectal ultrasound scanning, and changes in systemic FSH, oestradiol and inhibin-A were established using validated RIA and ELISA from blood samples collected every 12-24 hours.

Aspiration of the freshly selected first DF resulted in serum oestradiol and inhibin-A declining ($p<0.05$) within 24 hours, while FSH increased ($p<0.05$) to reach maximum concentrations 2 days after aspiration, which coincided with the time of emergence of the next wave. Selection of the next DF occurred 5 days after DF aspiration. In contrast, the intervals from steroid treatment to the next FSH maximum, emergence and selection of the next DF were longer ($p<0.05$) than following DF aspiration. FSH declined ($p<0.05$) initially, then began to rise on the second day after steroid treatment to reach a maximum 3 days earlier ($p<0.05$) than in control cycles, associated with a 2-2.5 day earlier emergence of the next wave. This wave selected the next DF in almost all cases in 5mg ODB+P treated cycles 1.7 day earlier ($p=0.06$) than in controls, while in 0.75 mg ODB+P treated cycles the new DF was selected from this or another wave at the same time ($p>0.05$) as in control cycles. Concentrations of inhibin-A declined ($p<0.05$) from the day of steroid treatment to reach nadir concentrations 2.5 (0.75mg) and 3.4 (5mg) days earlier ($p<0.05$) than in control cycles. Inhibin-A concentration increased again ($p<0.05$) associated with growth of the next wave and selection of the next DF similar to control cycles. Aspiration of the largest one (0.75mg ODB) or 1-4 (5mg ODB) follicles present 3 days after steroid treatment did not advance ($p >0.05$) the timing of the next FSH rise and next wave emergence further, and did not shorten ($p>0.05$) the intervals from steroid treatment to selection of the

next DF.

In summary, treatment with a combination of ODB and P was less efficient in advancing the transient FSH rise, next wave emergence and next DF than DF aspiration, possibly due to differing FSH profiles as well as the continuation of growth of follicles from the first wave especially when treated with 0.75 mg ODB+P. We, therefore, conclude that the lack of an immediate and short transient FSH rise and the inability of currently available steroid treatments to cause immediate and permanent follicle atresia are responsible for the variable and prolonged intervals from treatment to a new DF. We also conclude that elevated inhibin-A concentrations in serum are an excellent marker of growth of healthy wave or dominant follicles.

## 2) Introduction

The ovulation of a newly selected dominant follicle (DF) is crucial for optimum fertility and oestrous synchrony in cattle (Mihm *et al.,* 1994; Austin *et al.,* 1999). Any successful oestrous synchronisation programmes, therefore, will have to manipulate follicle wave growth such that the interval from treatment to selection of a new DF is consistent and predictable in all treated animals. Consistent intervals of 4 days to emergence of a new wave following the use of oestradiol 17β one day after synthetic progestagen treatment reported previously (Bo *et al.,* 1995) were not repeated when using a combination of oestradiol benzoate and progesterone or synthetic progestagen; in contrast, variable intervals to emergence of a new wave and selection of a new DF were seen and considered to be dependent on the stage of follicle wave development treatment was initiated (Duffy *et al.,* 1997; O'Rourke *et al.,* 1998; Roche *et al.,* 1998).

Such differences in the interval from treatment to new wave emergence may be due to oestrogen ester or progestagen used, doses administered, timing of administration in relation to follicle wave development and type of animals synchronised. Specifically, steroid combination treatments administered during growth of the first follicle wave of the oestrous cycle appear to cause prolonged intervals of 6 to more than 8 days to selection of a new DF (Bo *et al.,* 1995; O'Rourke *et al.,* 1998) with absence of new wave emergence reported in some cases (Duffy *et al.,* 1997). This is in marked contrast to the predictable and short intervals to new wave emergence and DF selection following acute removal of DF or all follicles present from 5mm via ovulation using GnRH (Mihm *et al.,* 1998;

Ryan *et al.,* 1998), electrocauterization (Ko *et al.,* 1991; Adams *et al.,* 1992a) or transvaginal ultrasound-guided ablation (Bergfelt *et al.,* 1994; Bodensteiner *et al.,* 1996; Boni *et al.,* 1997). Clearly, currently available steroid combination treatments do not cause such acute atresia and predictable new wave emergence especially when administered during follicle wave growth before DF selection, yet the atretogenic effects of steroid treatments on growing wave follicles are so far undetermined.

Several possibilities why steroid treatments do not immediately result in synchronous follicle atresia and/or new wave emergence in all treated animals, exist and need to be explored in order to improve success of current steroid synchronisation treatments. It is possible that the suppressive effect of exogenous oestradiol and progestagen on both gonadotrophins LH and FSH is not sufficient to cause immediate atresia of growing wave follicles comparable to follicle ablation, resulting in continued growth and function, i.e. suppression of FSH and other follicle growth. Conversely, steroid treatments may suppress gonadotrophin concentrations to such an extent that next wave emergence is inhibited due to a suppressed or modified transient FSH rise and the absence or severe reductions in LH pulses (Turzillo and Fortune, 1990; Gong *et al.,* 1996). Oestradiol has been shown to only have a transient suppressive effect on FSH concentrations (O'Rourke *et al.,* 2000), and FSH rises are seen within 2 days following treatment of cyclic heifers with oestradiol 17β and progestagen (Bo *et al.,* 1994). High circulating oestradiol concentrations, however, may modify any subsequent FSH rise such that the rate of increase is reduced, which may affect growth of a new FSH-dependent cohort. A comparison between the presumed

rapid FSH rise following DF aspiration (Ginther *et al.,* 1999) and the FSH rise following steroid combination treatments has not yet been reported.

Although ultrasound monitoring of individual follicle growth has proven essential to estimate morphological dominance and subordinate atresia (Sunderland *et al.,* 1994), gross morphological characteristics are insufficient to determine follicle function as shown by loss of oestrogen activity in the first dominant follicle on day 8 of the cycle despite its morphological dominance (Badinga *et al.,* 1992). Systemic markers reflecting health or atresia of the current ovarian follicle population (wave or dominant follicles) have not yet been investigated, although systemic oestradiol concentrations appear to rise or decline in the presence or absence of oestrogenic DF (Ireland and Roche, 1987). However, several intrafollicular peptide factors, such as the insulin-like growth factors, inhibins and their respective binding proteins have been characterised in detail during growth of the first follicle wave (Sunderland *et al.,* 1996; Mihm *et al.,* 1997), and an increase in intrafollicular amounts of dimeric inhibin precursors > 34 kDa has been found to be associated with wave emergence and its continued growth (Austin *et al.,* 2001). So far, only one study exists of systemic dimeric inhibin-A concentrations during the peri-ovulatory period in cattle, and appears to confirm that DF selection and growth are associated with high or rising systemic inhibin-A concentrations (Bleach *et al.,* 2001). The usefulness of inhibin-A as a systemic marker for presence of healthy or atretic follicles especially following steroid treatments remains to be determined. Therefore, this study addresses the issue of lack of predictability and consistency in intervals to new wave emergence and DF selection following a steroid combination treatment given at emergence of

the first follicle wave of the oestrous cycle in dairy heifers: specifically this study aims to i) compare DF aspiration followed by an acute and predictable FSH rise and new wave emergence with the effect of a commercially available steroid combination treatment, i.e. two doses of oestradiol benzoate combined with the synthetic progestagen norgestomet, on circulating FSH and follicle wave growth; ii) evaluate the use of systemic inhibin-A as a marker for presence of healthy or atretic wave or dominant follicles; and iii) estimate indirectly whether the steroid treatments cause complete atresia of the first follicle wave by determining the effects of aspirating the largest follicles present after steroid treatment on FSH, inhibin-A and growth of the second follicle wave. Growth and atresia of the first and growth of the second follicle wave were determined morphologically by ultrasound, as well as functionally using changes in serum FSH, oestradiol and inhibin-A concentrations.

## 3) Materials and Methods

### a) Animals and Treatments

Holstein Friesian heifers (year 1: 14 heifers, 2-3 cycles each; year 2: 4 heifers 2 cycles each, total of 42 experimental oestrous cycles) were housed in straw pens, fed a growth diet of *ad libitum* hay and twice daily concentrates to achieve a mean growth rate of 0.6 ±0.04 kg/day (year 1) and 0.8 ± 0.14 kg/day (year 2), and had *ad libitum* access to water. Heifers were between 16-22 months of age, and in year 1 and 2 heifers weighed on average 303.6 ± 4.2 kg and 285 ± 8 kg, respectively, before the study commenced. Heifers were given one or two i.m. injections of 15 mg Luprostiol, a prostaglandin $F2_{\alpha}$ analogue (PG; Prosolvin, Intervet UK Ltd., Cambridge, U.K.), in the presence of a corpus luteum to cause luteolysis. From the day of PG, heifers were checked three-four times daily for onset of oestrus (= Day 0). On Day 2 of the cycle, heifers were either left untreated (n= 15 control cycles) or received a subcutaneous ear implant of 3 mg of the synthetic progestagen norgestomet (P; Crestar, Intervet UK Ltd., U.K.) plus oestradiol benzoate (ODB; Oestradiol Benzoate, Intervet UK Ltd., U.K) diluted in sterile corn oil and given as a 3 ml i.m. injection; the dose given was either 0.75 mg (0.75ODB+P; n=9 cycles) or 5 mg ODB (5ODB+P; n=18 cycles). Implants were withdrawn after 10 days (Day 12 of cycle), and control and steroid-treated heifers were allowed to complete their oestrous cycle before re-allocation to another experimental oestrous cycle (see below).

During five of the control and 0.75ODB+P cycles, and during 10 of 5ODB+P cycles, heifers underwent ultrasound-guided transvaginal aspiration of the largest follicle (control and 0.75ODB+P) or the largest 1-4 follicles (5ODB+P) present on Day 5 of the cycle, using a scanner fitted with a 6 MHz, 31 cm long, human transvaginal transducer (model 601v TOSHIBA), a 15g 30 cm introducer with a central stylet to penetrate the vaginal wall, and 18 or 20g 45 cm needles with an echogenic tip (Casmed, Cheam, Surrey, UK) connected to a suction unit with a foot-pedal activated pump (Karri-vac 2, Rocket, London, UK; suction adjusted to 25 ml of water per minute) (Scott *et al.,* 1994). Prior to the aspiration procedure, each heifer received a 4 ml epidural injection of 20 mg/ml Lignocaine Hydrochloride BP (Lignavet Injection, C-Vet Veterinary Products, UK), 10 ml i.v. 30 μg/ml clenbuterol hydrochloride (Planipart, Boehringer Ingelheim, UK) to facilitate ovarian manipulations and 0.75 ml of 2% Xylazine solution i.m. (Rompun, Bayer plc, UK) as a sedative.

In year 1, the 14 heifers underwent 34 experimental oestrous cycles over a period of 7.5 weeks: the first experimental cycle was either a control (n=10) or 0.75ODB+P cycle without follicle aspiration (n=4), while during their second experimental cycle heifers either received 0.75mg ODB+P and the largest follicle was aspirated on Day 5 (n=5), or 5mg ODB+P with (n=4) or without (n=5) aspiration of the largest follicles on Day 5. Subsequently, six heifers were re-allocated on their second oestrus and underwent a third experimental cycle untreated (n=5 control cycles) or treated with 5mg ODB+P (n=1) with aspiration of the largest follicle on Day 5.

As there was concern that the status of the first follicle wave at the time of treatment with 5mg ODB+P determined subsequent success of the ultrasound-guided follicle aspiration, another 4 heifers and 8 experimental oestrous cycles were allocated to the 5mg ODB+P treatment in year 2 over a 5.5 week period, with (n= 5) and without (n=3) transvaginal ultrasound-guided follicle aspiration of the largest follicles on Day 5.

**b) Ovarian ultrasound scanning and blood sampling**

Starting from Day 0, growth and regression of individual follicles greater than 2 mm were monitored daily by ultrasonography using a real-time, B-mode, linear array ultrasound scanner with a 7.5 MHz rectal transducer (Toshiba Capasee, model SSA-220-A; Toshiba Medical System, Manor Royal, Crawley, UK). Ultrasound scanning continued until the first day of dominance of the second DF of the cycle (approximately Day 13). The day of emergence of a new follicle wave was defined retrospectively as the day when the first member of the new wave reached a size of 4 mm in diameter. The first day of dominance of any dominant follicle was defined by the following two morphological criteria: i) the DF had achieved a minimum diameter of 8 mm, and ii) the difference in size between the DF and the next largest (subordinate) follicle was at least 1 mm and this size difference was increased the next day.

Heifers were blood sampled every 12 hours from Day 2 (all steroid-treated cycles and control cycles without DF aspiration) or Day 5 (control cycles with DF aspiration) until the first day of dominance of the second DF of the cycle. Blood

samples were collected via jugular venepuncture (10ml into evacuated glass containers) to establish circulatory concentrations of FSH, oestradiol and Inhibin-A. Each blood sample was maintained at room temperature for 1 hour after collection, at 4° C overnight, then centrifuged at 700g for 20 min and the serum was stored at -20° C until determination of hormone concentrations.

**c) Hormone analysis and data representation**

Serum FSH concentrations were quantified using a heterologous assay previously validated (Crowe *et al.,* 1997), with the NIDDK-anti-oFSH antibody (AFP-C 5288113), ovine tracer and a bovine FSH standard preparation (USDA I2 bFSH). The following modification was introduced as reported by Amridis *et al.,* (1999): the precipitating antibody used (400μl/tube) was a donkey anti-rabbit antibody (SAPU S022, batch 8164B) at a dilution of 1:20, with normal rabbit serum (SAPU, batch 7989B) at a dilution of 1:200. The sensitivity of this assay was 0.08 ng $ml^{-1}$ and the mean intra- (n=2-6) and interassay (n=7) coefficients of variation for 2 serum samples containing 0.1 and 0.6 ng/ml FSH were 14.7 and 21.4 % (low) and 10 and 29.7 % (high).

Concentrations of oestradiol in serum were estimated using a validated RIA (Prendiville *et al.,* 1995). The sensitivity of this assay was 0.4 pg $ml^{-1}$, and the mean intra- (n=2-8) and interassay (n= 7-8) coefficients of variation for 2 serum samples containing 1.1 and 4.4 pg/ml FSH were 15.6 and 24 % (low) and 12.4 and 11.3 % (high).

Concentrations of inhibin-A in serum were estimated using a validated ELISA (Bleach *et al.,* 2001). The sensitivity of this assay was 15 pg $ml^{-1}$, and the mean intra- (n = 2-7) and inter-assay (n = 15) coefficients of variation for two serum pools containing 41.8 and 108.8 pg/ml Inhibin-A were 21.4 and 18.3 % (low) and 21.5 and 13.7 % (high). Standards and test samples were mixed with 50 μl of SDS solution (6% [w/v]) and heated for 10 min at 90 °C. After cooling, 100 μl of ELISA buffer (10%)[w/v], BSA, 5% [v/v] Triton X-100, 2% [v/v] normal mouse serum, 0.1 M Tris- HCL buffer [pH 7.5]) and 50 μl of distilled water containing 10% (v/v) hydrogen peroxide (Sigma UK ltd., Poole, Dorset, UK) were added, tubes were incubated and 100 μl aliquots transferred to microplates coated with the monoclonal antibody against the inhibin βA subunit. On the second day ELISA buffer containing 1 μg/ml of biotinylated α subunit-specific mAb (PPG 14/6) was added followed by ELISA buffer containing extravidin-alkaline phosphate conjugate (1:20 000; Sigma). Finally, bound alkaline phosphatase was quantified using a commercially available ELISA amplification kit (Immuno Select ELISA Amplification System; Gibco BRL, Uxbridge, Middelsex, Uk) according to the supplier's instructions. Data were processed by immunoassay curve-fitting software (Riacalc; Pharmacia, Milton Keynes, Bucks, UK).

**d) Statistical analysis and data representation**

Serum hormone concentrations were compared between control cycles and cycles in which heifers were treated with two doses of ODB combined with progestagen using repeated measures twoway analysis of variance, with time and treatment as the two main effects. If a significant time or treatment effect or time x

treatment interaction were seen, one way analysis of variance was used to determine whether differences in means between days in one treatment or between treatments on specific days were significant.

Comparisons between control cycles and cycles treated with the two steroid combinations of mean maximum or nadir concentrations, the mean day specific concentrations were reached, and mean ultrasound parameters of growth of the first and second follicle wave were carried out using one way analysis of variance followed by Fisher's test for individual comparisons when 3 or 4 treatment groups or student's t test when only two treatment groups were compared. Data underwent logarithmic transformation when the Bartlett's test for equal variances showed that variances differed significantly; however, data are presented in absolute values for clarity. A non-parametric test (Kruskall-Wallis) was carried out if logarithmic transformation did not restore equality to variances. Chi-square analysis was used to determine differences in the ratio of new waves resulting in new DF out of all new waves in 5 mg ODB+P treated cycles.

No differences were seen in any of the parameters analysed between 5mg ODB+P treated cycles in year 2 in which follicles were or were not aspirated on Day 5 compared with equivalent treatment cycles in year 1, and thus data from the two years for aspirated or non-aspirated 5mg ODB+P treated cycles were pooled. For analysis of serum oestradiol concentrations following 0.75 or 5mg ODB both aspirated and non-aspirated treatment cycles were pooled, as follicle aspiration did not affect any of the parameters relating to serum oestradiol concentrations.

In one control aspiration cycle, the first DF filled up again and continued to grow following ultrasound-guided follicular fluid aspiration, despite an echogenic area within the cavity indicating successful penetration of the needle and the formation of an intrafollicular blood-clot; emergence of the second wave occurred later than in all other control aspiration cycles indicating that the aspirated DF had re-attained its dominance. As this demonstrates normal variation within this model, data relating to this cycle were included in the analysis. Similarly, the first DF formed after treatment with 0.75mg ODB+P was successfully aspirated, yet filled up again, continued to grow, and resumed dominance, indicated by absence of emergence of a second wave. This DF, however, also ovulated following a short luteal phase, similar to what occurred in one 0.75mg ODB+P treated cycle without follicle aspiration; here, the selected DF also remained dominant and ovulated after a short luteal phase with no detectable emergence of a second wave. Again, as these scenarios demonstrate normal variation within the aspiration models used, hormone and ultrasound data relating to the first follicle wave were included in the analysis.

## 4) Results

*1) Serum oestradiol concentrations following treatment with two doses of ODB+P at emergence of the first follicle wave*

Treatment with ODB on Day 2 increased ($p<0.05$) maximum serum oestradiol concentrations 5.3 fold (0.75mg) or 32 fold (5mg) compared with control cycles; maximum concentrations were achieved 2 days earlier than in controls, and the subsequent decline ($p<0.05$) to concentrations below 2 pg/ml took 3 (0.75mg) or 6 days (5mg) (Table 2.1). Thus, oestradiol concentrations in 5mg ODB treated cycles were higher ($p<0.05$) than control concentrations on every day until Day 10 of the cycle; in 0.75mg ODB treated cycles oestradiol concentrations were higher ($p<0.05$) than control concentrations on Days 3 to 5, and were lower ($p<0.05$) than in 5mg ODB treated cycles on Days 3 to 8 (Figure 2.1). On the day of emergence of the next follicle wave, oestradiol concentrations were still 10-fold and 2.8-fold higher ($p<0.05$) in 5mg ODB treated cycles compared with control or 0.75mg ODB treated cycles, respectively (Table 2.1).

Figure 2.1 Serum oestradiol concentrations (mean±sem) in control cycles (n=10), and in all cycles in which dairy heifers were treated with 0.75mg (n=6-9) or 5mg ODB+P (n=11-18) on Day 2 of their oestrous cycle.

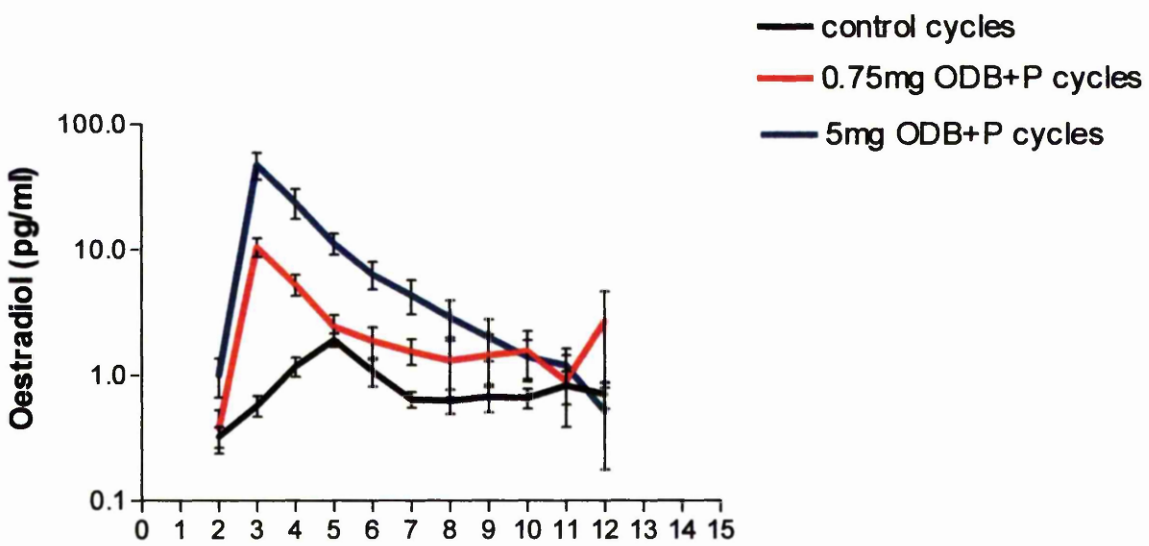


Table 2.1 Parameters (mean±sem) relating to serum oestradiol ($E_2$) in control cycles, and in all cycles in which dairy heifers were treated with 0.75mg or 5mg ODB + P on Day 2 of their oestrous cycle.

| | | **Treatment cycles** | |
|---|---|---|---|
| | **Control** | **0.75mg ODB+P** | **5mg ODB+P** |
| | n=10 | n=9 | n=18 |
| $E_2$ on Day 2 (pg/ml) | 0.3±0.1[a] | 0.5±0.1[ab] | 0.9±0.2[b] |
| Maximum $E_2$ (pg/ml) | 2.1±0.3[a] | 10.9±1.0[b] | 66.5±10.5[c] |
| Day of cycle of max. $E_2$ | 5.0±0.2[a] | 3.0±0.0[b] | 3.1±0.1[b] |
| Day of cycle $E_2$ declines to < 2pg/ml | 6.0±0.2[a] | 5.9±0.2[a] | 9.4±0.5[b] |
| $E_2$ on day of emergence of the 2nd wave (pg/ml) | 0.5±0.1[a] | 1.4±0.4[a] | 5.1±1.0[b] |

[abc]Means with different superscripts within rows are statistically different ($p<0.05$).

*2) The effects of aspiration of the freshly selected first dominant follicle on Day 5 or treatment with two doses of ODB + P at emergence of the first follicle wave on Day 2 on FSH and growth of the first and second follicle wave*

In all control aspiration cycles the largest follicle on Day 5 was aspirated which was also the freshly selected first DF, thus preventing first-wave DFs from reaching their normal maximum diameter (Table 2.2). However, during one cycle the aspirated follicle re-gained its follicular fluid and function within 2 days, and no further follicle growth was seen until emergence of the second wave on Day 9. Immediately following DF aspiration serum oestradiol concentrations declined ($p<0.05$) and remained low, while FSH concentrations increased ($p<0.05$) to reach a maximum 2 days after aspiration which was 2 days earlier than in non-aspirated control cycles ($p<0.05$), and associated with earlier emergence of the second follicle wave ($p<0.05$) (Figure 2.2a,b; Table 2.2). Subsequently, FSH concentrations declined rapidly and the first day of dominance of the second DF was advanced ($p<0.05$) by 2.4 days compared to control cycles (Figure 2.2a; Table 2.2).

Following treatment with ODB + P FSH declined by 40-50% ($p<0.05$) to reach nadir concentrations within one day which was 2 days earlier ($p<0.05$) than in control cycles; concentrations subsequently increased to reach the maximum of the next FSH rise 3 days earlier ($p<0.05$) than in control cycles (Table 2.2; Figure 2.3a). Thus, FSH concentrations were higher ($p<0.05$) in 5mg ODB+P treated cycles on Days 4-6 of the cycle compared with controls (Figure 2.3a).

Treatment with 0.75mg ODB+P at emergence of the first wave did not influence selection of the first DF in 8/9 cycles, which reached a similar ($p>0.05$) maximum size to the one reached in control cycles (Table 2.2). However, treatment with 5mg ODB+P caused suppression of first wave growth, and led to reduced ($p<0.05$) maximum size attained 1-2 days after treatment compared with control and 0.75ODB+P cycles (Table 2.2). Associated with the earlier second rise in FSH, a new wave emerged 2 days earlier ($p<0.05$) in ODB+P cycles than in control cycles. In 5mg ODB+P cycles, this new wave led to selection of a new DF in 6/8 cycles, and the first day of dominance of this new DF tended to be earlier ($p=0.06$) than in control cycles. However, in cycles treated with 0.75mg ODB this new wave did not lead to selection of a new DF in 2/3 cycles, which on average emerged later, at the same time ($p>0.05$) as in control cycles (Table 2.2).

Following treatment with ODB+P FSH increased more slowly to reach a later maximum ($p<0.05$) than following ablation of the DF (Figure 2.4a, Table 2.2), while maximum FSH concentrations were similar ($p>0.05$) between treatments. Consequently, the time from ablation or steroid treatment to emergence of the new DF was 3 or 4 days longer ($p<0.05$) in 5mg or 0.75mg ODB+P cycles, respectively, compared with DF ablation cycles (Table 2.2).

Figure 2.2 Serum concentrations (mean±sem) of a) FSH, b) oestradiol and c) inhibin-A in control cycles (n=10 for FSH and oestradiol; n=6 for inhibin-A) and in control cycles, in which the dominant follicle (DF) was aspirated on Day 5 (n=4-5).

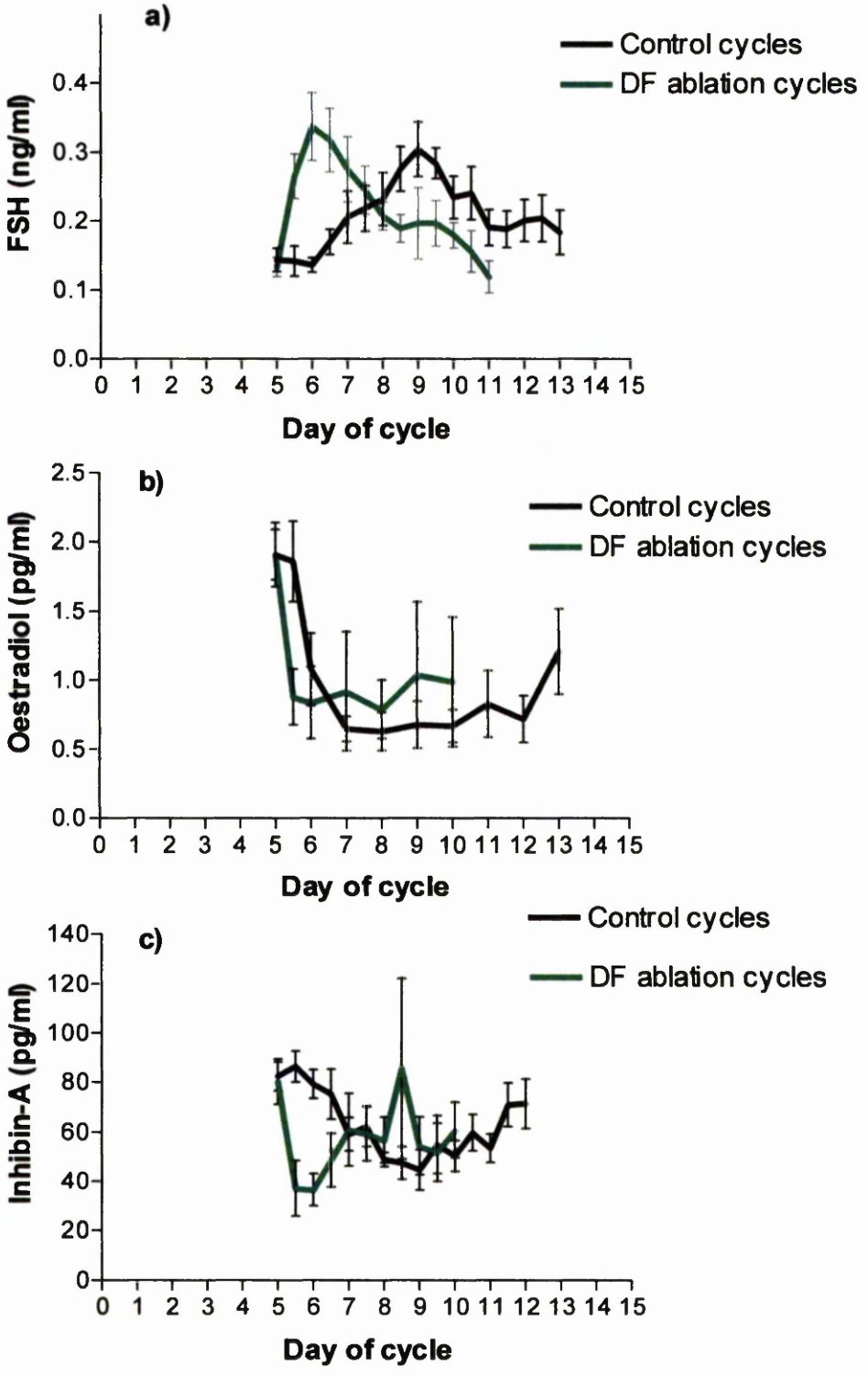


Figure 2.3 Serum concentrations (mean±sem) of a) FSH and b) inhibin-A in control cycles (n=10) and in cycles in which dairy heifers were treated with 0.75mg (n=3-4) or 5mg ODB + P (n=3-8) on Day 2 of their oestrous cycle.

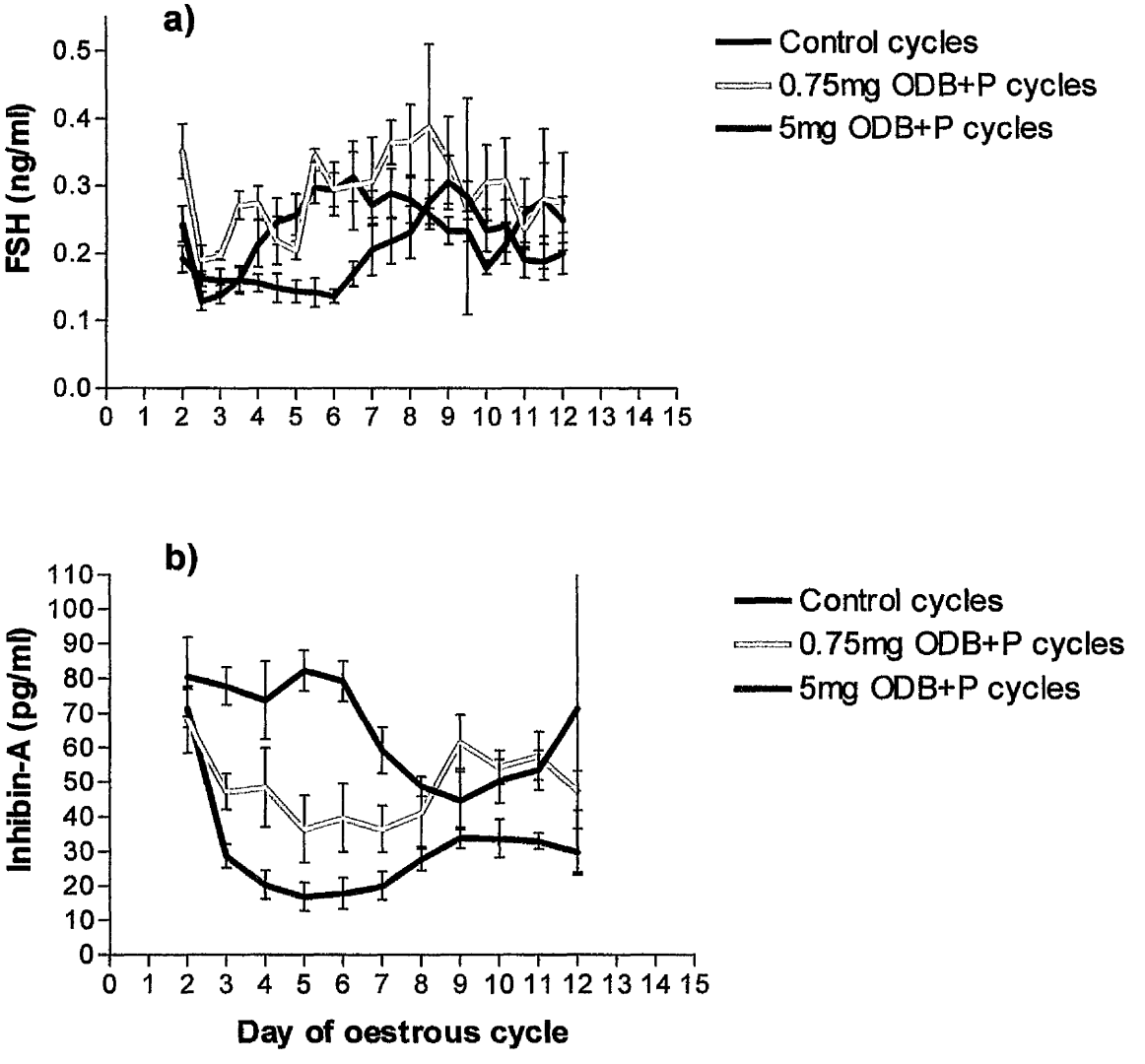


Figure 2.4 Serum concentrations (mean±sem) of a) FSH and b) inhibin-A in control cycles, in which the dominant follicle (DF) was aspirated on Day 5 (n=3-5), and in cycles in which dairy heifers were treated with 0.75mg (n=3-4) or 5mg ODB + P (n=3-8) on Day 2 of their oestrous cycle.

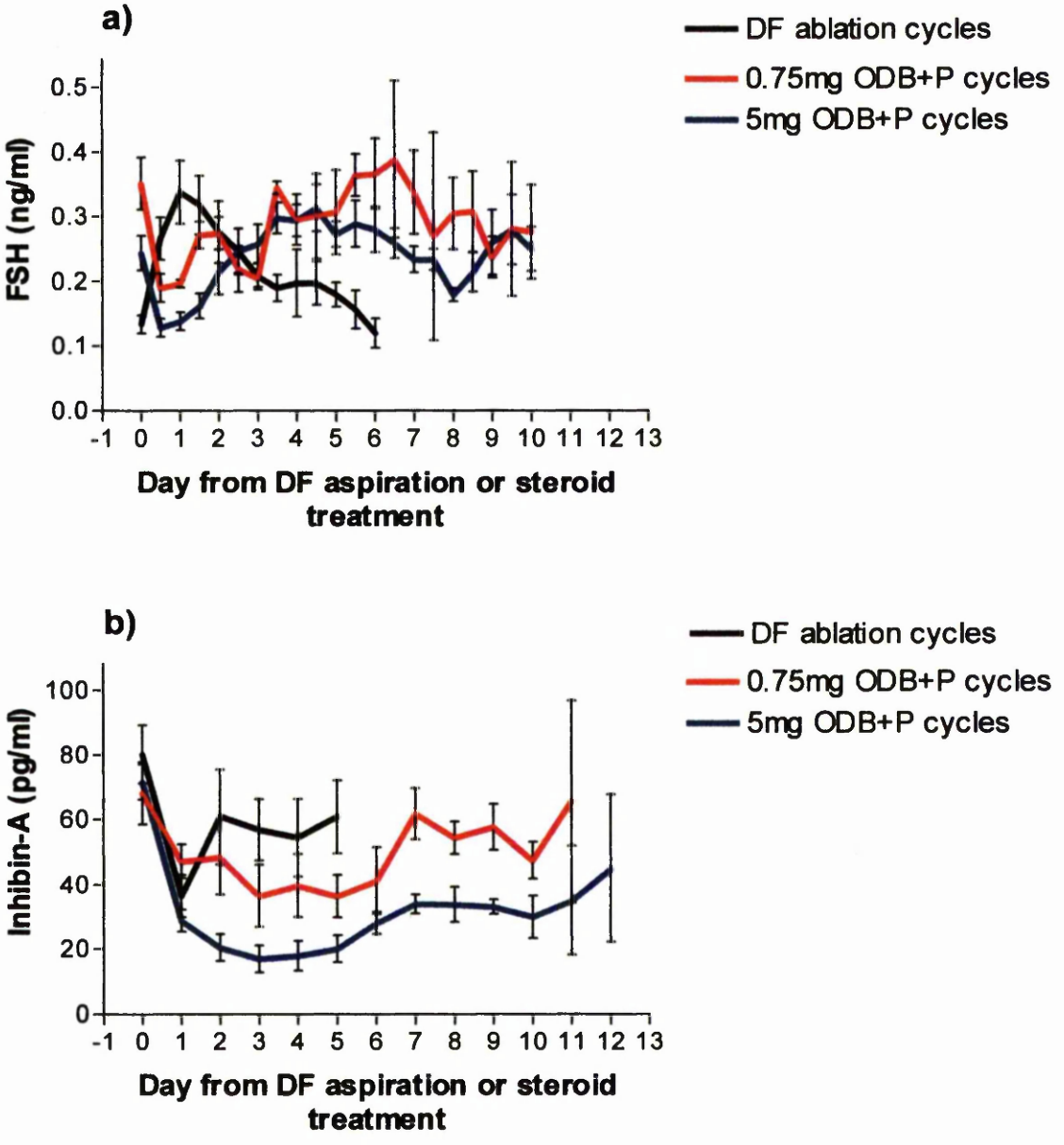


Table 2.2 Parameters (mean±sem) relating to serum FSH and growth of the first and second follicle wave in control cycles, in control cycles in which the first dominant follicle (DF) was aspirated on Day 5, and in cycles in which dairy heifers were treated with 0.75mg or 5mg ODB plus Progestagen on Day 2 of their oestrous cycle.

| | **Control cycles** | | **Treatment cycles** | |
|---|---|---|---|---|
| | **No aspiration** n=10 | **Aspiration of first DF** n=5 | **0.75mg ODB+P** n=3-4[$] | **5mg ODB+P** n=8 |
| *Serum FSH concentrations* | | | | |
| Nadir FSH (ng/ml) | 0.10±0.01[a] | -* | 0.18±0.01[b] | 0.12±0.01[a] |
| Day of cycle of nadir FSH | 4.4±0.6[a] | -* | 2.6±0.1[b] | 2.7±0.1[b] |
| Maximum FSH (ng/ml) | 0.40±0.04[a] | 0.38±0.03[a] | 0.41±0.03[a] | 0.35±0.03[a] |
| Day of cycle of max. FSH | 9.1±0.4[a] | 6.9±0.6[b] | 6.1±0.4[b] | 6.3±0.2[b] |
| Interval from nadir to next maximum (days) | 4.7±0.6[a] | -* | 3.5±0.5[a] | 3.6±0.2[a] |
| Interval from DF aspiration or steroid treatment to max. FSH (days) | - | 1.9±0.6[a] | 4.1±0.4[b] | 4.3±0.2[b] |
| *First follicle wave* | | | | |
| Maximum size of largest follicle (mm) | 13.3±0.6[a] | 10.8±0.8[b]** | 11.4±1.4[ab] | 6.3±0.3[c] |
| Day of cycle of max. size | 8.4±0.4[a] | 5.8±0.8[b]** | 8.8±1.7[a] | 3.3±0.3[c] |
| *Second follicle wave* | | | | |
| Day of cycle of 2nd wave emergence (Em) | 9.3±0.4[a] | 6.8±0.6[b] | 7.3±1.8[b] | 6.8±0.3[b] |
| Day of cycle of Em of 2nd DF | 9.6±0.3[a] | 7.2±0.6[b] | 9.7±1.7[a] | 7.0±0.3[b] |
| Interval from DF aspiration or steroid treatment to Em of 2nd DF (days) | - | 2.2±0.6[a] | 7.7±1.7[b] | 5.0±0.3[c] |
| First day of dominance of 2nd DF (day of cycle) | 12.2±0.5[a] | 9.8±0.8[b] | 11.0±2.0[ab] | 10.5±0.7[ab]*** |
| Size of 2nd DF on first day of dominance (mm) | 9.0±0.2[a] | 8.9±0.3[a] | 9.1±0.3[a] | 8.6±0.2[a] |
| Interval from DF aspiration or steroid treatment to first day of dominance of 2nd DF (days) | - | 4.8±0.8[a] | 9.0±2.0[b] | 8.5±0.7[b] |

[abc]Means with different superscripts within rows are statistically different ($p<0.05$).

[$]In one of the 0.75mg ODB+P treated cycles no new wave emergence occurred and the old DF continued to grow and ovulated following a short luteal phase, thus, only data relating to the first wave are available.

*In control cycles in which the first DF was aspirated, blood samples were only collected from Day 5, it is, therefore, not possible to determine nadir FSH concentrations.

**Size of the first DF on Day 5 (n=4) or Day 9 (n=1; follicular fluid aspiration did not terminate follicle function).

***Means in the 5mg ODB+P treatment cycles tended to differ from control cycles ($p=0.06$).

*3) Systemic inhibin-A and the presence of growing wave or dominant follicles*

In control cycles, serum inhibin-A concentrations were already high on Day 2 with a maximum of 98.0±7.1 pg/ml associated with the first wave on Day 3.5 of the cycle. Concentrations were maintained high up to Day 6 of the cycle, followed by a decline ($p<0.05$) to reach nadir concentrations of 34.8±5.4 pg/ml on Day 9.0±0.6. Subsequently, inhibin-A concentrations increased ($p<0.05$) to a second maximum of 78.1±10.5 pg/ml on Day 12 at the end of the study when the second DF was selected (Figure 2.3b).

Following aspiration of the first DF on Day 5, inhibin-A concentrations fell precipitously ($p<0.05$) on Day 6 to reach 46.4±7.9% of the previous' day values, which was reduced ($p<0.05$) compared with control cycles in which 95.8±4.7% of the previous' day values were maintained. Thus, nadir inhibin-A concentrations were reached 2 days earlier ($p<0.05$) following aspiration of the first DF on Day 6.8±0.6, followed by an increase to reach a second maximum of 74.9±4.4 pg/ml again 2.3 days earlier ($p=0.05$) than in control cycles (Figure 2.2b), associated with earlier growth of the second wave and selection of the second DF.

Following treatment with ODB+P, maximum inhibin-A concentrations associated with the first follicle wave were reduced ($p<0.05$) to 69.2±9.3 pg/ml (0.75mg) and 71.5±5.5 pg/ml (5mg) compared with control cycles, and this maximum was reached 1.5 days earlier ($p<0.05$) in 5mg ODB+P treated cycles compared with control cycles (Figure 2.3b). In fact, concentrations of inhibin-A declined ($p<0.05$) from the day of steroid treatment to reach nadir concentrations

of 21.3±5.8 (0.75mg) and 11.1±2.2 pg/ml (5mg; $p<0.05$) 2.5 (0.75mg) and 3.4 (5mg) days earlier ($p<0.05$) than in control cycles (Figure 2.3b). Subsequently, inhibin-A concentrations increased ($p<0.05$) associated with growth of the second wave to reach a second maximum of 73.5±7.3 (0.75mg) and 48.6±5.5 pg/ml (5mg; $p<0.05$) on Days 12.5±1.0 (0.75mg) and 10.4±1.1 (5mg) of the cycle (Figure 2.3b).

*4) The effects of aspiration of the largest follicles present on Day 5 three days after treatment with two doses of ODB + P at emergence of the first follicle wave*

In five of nine 0.75mg ODB+P treated cycles the largest follicle (LF) present on Day 5 was aspirated similar to control DF aspiration cycles. In all five cycles a new wave emerged following LF ablation on Day 5; the new DF was selected from this new wave in 3 cycles, while in one cycle another wave emerged causing selection of the new DF. In the remaining cycle, the aspirated follicle re-gained its follicular fluid content and its function, continued to grow and re-asserted its dominance following emergence of the new wave; this wave regressed and no other follicle growth was seen for the remainder of the (shortened) cycle. As mentioned above, in three of the four 0.75 mg ODB+P treated cycles without follicle aspiration a new wave emerged, but this new wave only led to selection of the new DF in one case.

Overall, the diameter of the largest follicle present on Day 6 of the cycle was reduced ($p<0.05$) following LF aspiration on Day 5, but LF aspiration did not change ($p>0.05$) the day of cycle when the largest follicle of the first wave reached its maximum diameter following treatment with 0.75 mg ODB+P (Table 2.3).

Largest follicle aspiration on Day 5 did not advance ($p>0.05$) the timing of the second FSH rise and second wave emergence any further, and it did not shorten ($p>0.05$) the interval from steroid treatment to selection of the second DF following treatment with 0.75 mg ODB+P (Figure 2.5a; Table 2.3). There were also no differences seen ($p>0.05$) in the serum oestradiol profile following ODB treatment (Figure 2.5b).

Figure 2.5 Serum concentrations (mean±sem) of a) FSH and b) oestradiol in cycles in which heifers were treated with 0.75mg ODB + P on Day 2 without any further treatment (n=2-4), and in which the largest follicle (LF) present on Day 5 was aspirated (n=2-5).

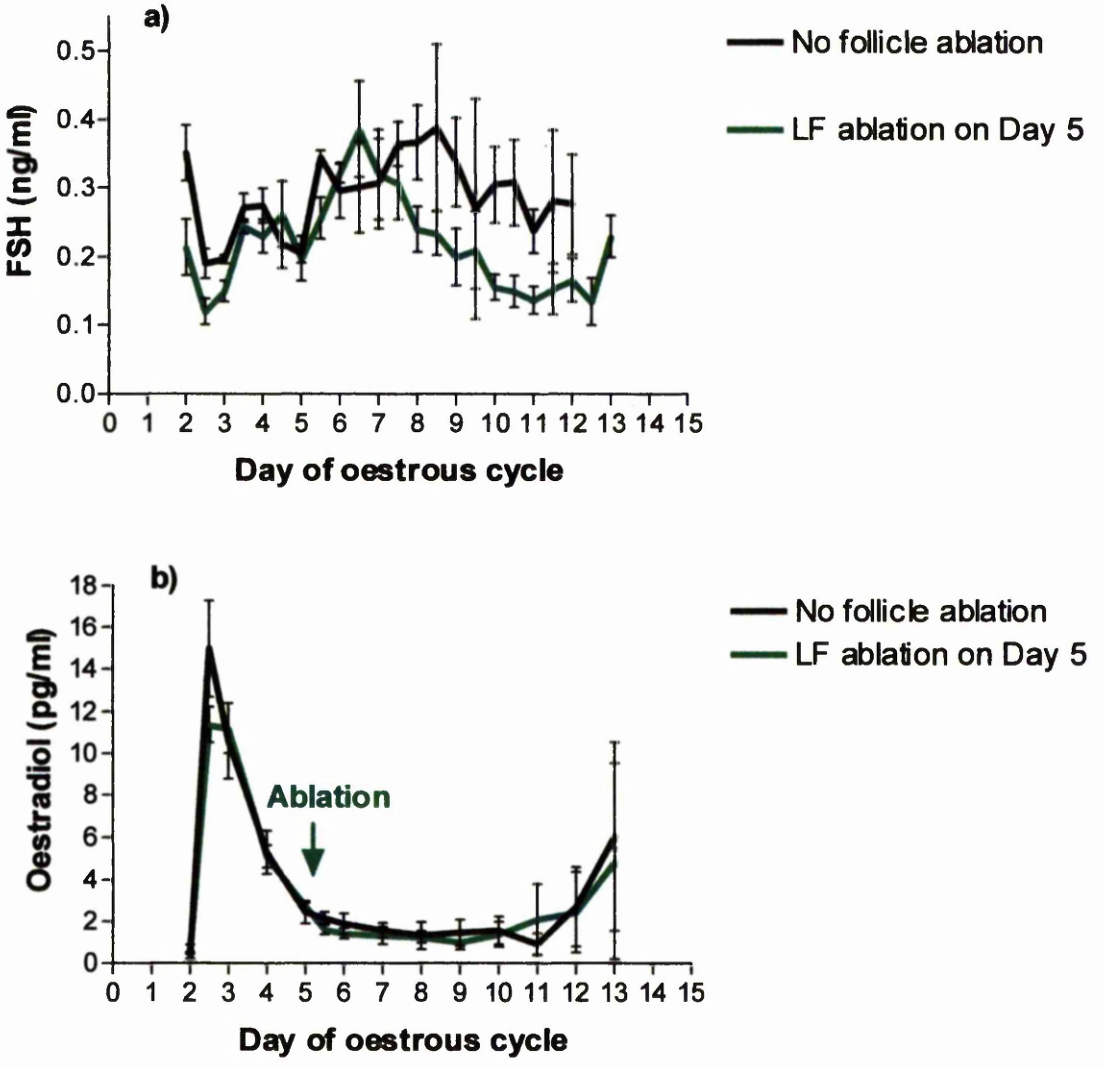


Table 2.3 Parameters (mean±sem) relating to serum FSH and growth of the first and second follicle wave in cycles in which dairy heifers were treated with 0.75mg ODB plus P on Day 2 of their oestrous cycle with or without aspiration of the largest follicle (LF) present on Day 5.

| | **0.75mg ODB+P cycles** | |
|---|---|---|
| | **No aspiration** n=3-4* | **LF aspiration Day 5** n=4-5* |
| *Serum FSH concentrations* | | |
| Nadir FSH (ng/ml) | 0.18±0.01[a] | 0.12±0.02[b] |
| Day of cycle of nadir FSH | 2.6±0.1[a] | 2.5±0.0[a] |
| Maximum FSH (ng/ml) | 0.41±0.03[a] | 0.44±0.05[a] |
| Day of cycle of max. FSH | 6.1±0.4[a] | 6.1±0.4[a] |
| Interval from nadir to next maximum (days) | 3.5±0.5[a] | 3.6±0.4[a] |
| Interval from steroid treatment to max. FSH (days) | 4.1±0.4[b] | 4.1±0.4[a] |
| *First follicle wave* | | |
| Size of largest follicle on Day 5 (mm) | 9.6±0.3[a] | 9.1±0.7[a] |
| Size of largest follicle on Day 6 (mm) | 9.6±0.3[a] | 6.3±0.8[b] |
| Maximum size of largest follicle (mm) | 11.4±1.4[a] | 10.6±1.5[a] |
| Day of cycle of max. size | 8.8±1.7[a] | 6.6±1.6[a] |
| *Second follicle wave** | | |
| Day of cycle of 2nd wave emergence (Em) | 7.3±1.8[a] | 7.0±0.0[a] |
| Day of cycle of Em of 2nd DF | 9.7±1.7[a] | 7.5±0.5[a] |
| Interval from steroid treatment to Em of 2nd DF (days) | 7.7±1.7[a] | 5.5±0.5[a] |
| First day of dominance of 2nd DF (day of cycle) | 11.0±2.0[a] | 10.8±1.1[a] |
| Size of 2nd DF on first day of dominance (mm) | 9.1±0.3[a] | 9.3±0.4[a] |
| Interval from steroid treatment to first day of dominance of 2nd DF (days) | 9.0±2.0[a] | 8.8 ±1.1[a] |

[ab]Means with different superscripts within rows are statistically different ($p<0.05$).

*In one of the 0.75mg ODB+P treated cycles without LF aspiration, no new wave emergence occurred; in one of the 0.75mg ODB+P treated cycles with LF aspiration no new DF was selected (follicle aspiration did not terminate follicle function). In both cases the old DF continued to grow and ovulated following a short luteal phase; thus, only data relating to the first wave are available.

Following treatment with 5mg ODB+P the largest follicle was aspirated in 6 of 18 cycles, while the 2-4 largest follicles were aspirated in 4 of 18 cycles. Thus, on average 1.8±0.4 follicles were ablated with a mean size of 6.4±0.3 mm and a largest size of 6.8±0.2 mm in diameter. There were no differences seen ($p>0.05$) in the follicle status on Day 2 or on Day 6 of the cycle between 5mg ODB+P treated cycles in which follicles were or were not aspirated on Day 5 (Table 2.4). However, on Day 5 of the cycle, follicle aspiration was carried out in cycles in which less follicles < 5mm and more follicles >6 mm were seen ($p<0.05$), and the largest follicle was > 1mm larger ($p<0.05$) in diameter compared with cycles in which no aspiration was carried out (Table 2.4). In addition, maximum size of any follicle of the first wave was reached 1 day later ($p<0.05$) and was larger ($p=0.05$) in 5mg ODB+P treated cycles in which follicle aspiration was carried out on Day 5 compared without follicle aspiration (Table 2.4).

Although a new follicle wave emerged in all 5mg ODB+P treated cycles approximately 5 days after steroid treatment, this new wave gave rise to the new DF in only 3/10 cycles in which follicles had grown larger for longer before aspiration on Day 5 compared to 6/8 cycles without follicle aspiration ($p<0.05$). The new DF was finally selected from another wave, which emerged subsequently in the majority of 5mg ODB+P cycles with follicle aspiration. This is reflected in a 2-day delay in the timing of the second FSH maximum ($p<0.05$) and a 1.6-day delay in the day of emergence of the second DF ($p<0.05$) in 5mg ODB+P cycles in which follicles had grown larger for longer before aspiration on Day 5 compared to cycles without aspiration (Figure 2.6a; Table 2.5). However, no further differences ($p>0.05$) were seen in the rise and decline of serum FSH

and oestradiol concentrations, and in the decline and rise of inhibin-A concentrations in 5mg ODB+P treated cycles in which follicles had grown larger for longer before aspiration on Day 5 compared to cycles without follicle aspiration (Figure 2.6).

Figure 2.6 Serum concentrations (mean±sem) of a) FSH, b) oestradiol and c) inhibin-A in cycles in which heifers were treated with 5mg ODB + P on Day 2 without any further treatment (n=2-8), and in which the largest follicle(s) present on Day 5 (average size 6.4 mm) were aspirated (n=2-18).

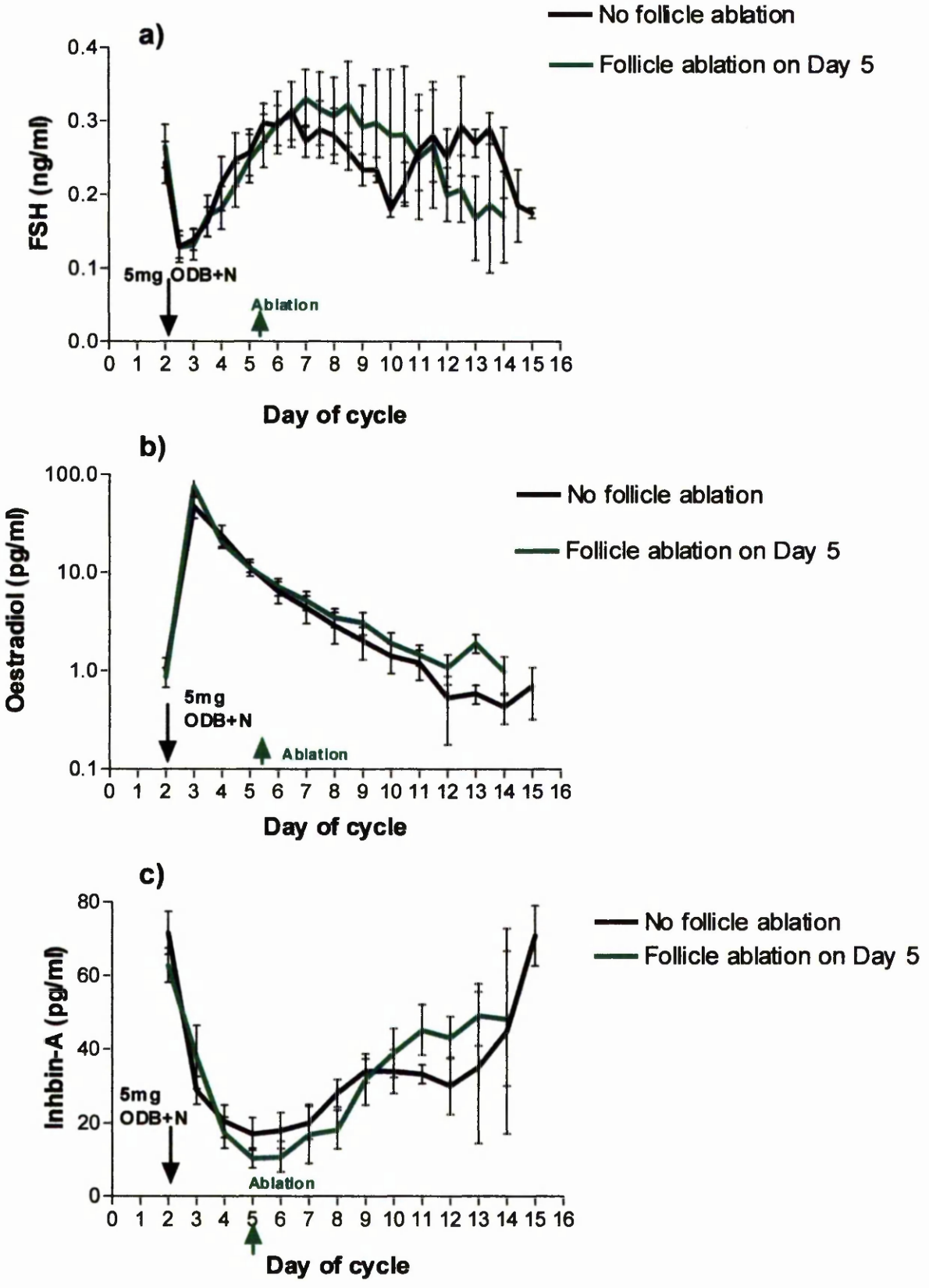


Table 2.4 Parameters (mean±sem) relating to growth of the first follicle wave in cycles in which dairy heifers were treated with 5mg ODB + P on Day 2 of their oestrous cycle with or without aspiration of the largest follicle(s) present on Day 5.

| | **5mg ODB+P cycles** | |
|---|---|---|
| | **No aspiration** n=8 | **Follicle aspiration Day 5** n=10 |
| *First follicle wave* | | |
| Follicle numbers on Day 2: | | |
| < 5mm | 4.1±0.6[a] | 4.5±0.8[a] |
| 5-6mm | 1.0±0.6[a] | 1.9±0.6[a] |
| > 6mm | 0.4±0.2[a] | 0.7±0.5[a] |
| Follicle numbers on Day 5: | | |
| < 5mm | 5.1±0.6[a] | 2.8±0.6[b] |
| 5-6mm | 1.4±0.7[a] | 1.6±0.3[a] |
| > 6mm | 0.4±0.3[a] | 1.5±0.5[b] |
| Follicle numbers on Day 6: | | |
| < 5mm | 3.9±0.9[a] | 5.1±0.8[a] |
| 5-6mm | 1.0±0.6[a] | 0.7±0.3[a] |
| > 6mm | 0.6±0.3[a] | 0.4±0.3[a] |
| Size of largest follicle on: | | |
| Day 2 (mm) | 5.4±0.3[a] | 5.6±0.3[a] |
| Day 5 (mm) | 5.3±0.4[a] | 6.8±0.2[b] |
| Day 6 (mm) | 5.5±0.3[a] | 5.4±0.4[a] |
| Maximum size of largest follicle (mm) | 6.3±0.3[a] | 7.0±0.2[b] |
| Day of cycle of maximum size | 3.3±0.3[a] | 4.3±0.2[b] |

[ab]Means with different superscripts within rows are statistically different ($p \leq 0.06$).

Table 2.5 Parameters (mean±sem) relating to serum FSH and growth of the second follicle wave in cycles in which dairy heifers were treated with 5mg ODB plus progestagen on Day 2 of their oestrous cycle with or without aspiration of the largest follicle(s) present on Day 5.

| | **5mg ODB+P cycles** | |
|---|---|---|
| | **No aspiration** n=8 | **Follicle aspiration Day 5** n=10 |
| *Serum FSH concentrations* | | |
| Nadir FSH (ng/ml) | 0.12±0.01[a] | 0.11±0.02[a] |
| Day of cycle of nadir FSH | 2.7±0.1[a] | 2.5±0.0[a] |
| Maximum FSH (ng/ml) | 0.35±0.03[a] | 0.43±0.08[a] |
| Day of cycle of max. FSH | 6.3±0.3[a] | 8.2±0.5[b] |
| Interval from nadir to next maximum (days) | 3.6±0.2[a] | 5.3±0.4[b] |
| Interval from steroid treatment to max. FSH (days) | 4.3±0.2[a] | 6.2±0.4[b] |
| *Second follicle wave* | | |
| Day of cycle of 2nd wave emergence (Em) | 6.8±0.3[a] | 6.6±0.3[a] |
| Day of cycle of Em of 2nd DF | 7.0±0.3[a] | 8.6±0.5[b] |
| Interval from steroid treatment to Em of 2nd DF (days) | 5.0±0.3[a] | 6.6±0.5[b] |
| First day of dominance of 2nd DF (day of cycle) | 10.5±0.7[a] | 12.1±1.5[a] |
| Size of 2nd DF on first day of dominance (mm) | 8.6±0.2[a] | 8.8±0.2[a] |
| Interval from steroid treatment to first day of dominance of 2nd DF (days) | 8.5±0.7[a] | 10.1±0.5[a] |

[ab]Means with different superscripts within rows are statistically different ($p<0.05$).

## 5) Discussion

Steroid combination treatments administered during emergence of the first follicle wave in heifers have been shown to cause follicle atresia and advance the time of next wave emergence (Bo *et al.,* 1994; 1995; O'Rourke *et al.,* 1998). Intervals from treatment to the next wave and selection of the next DF may depend on the dose and type of oestradiol used, and can be variable and prolonged (Bo *et al.,* 1994; 1995; Duffy *et al.,* 1997; O'Rourke *et al.,* 1998). Yet, unpredictable time intervals to selection of the next DF will compromise the success of oestrous synchronisation treatments in terms of synchrony of oestrous onset and subsequent fertility. The first main aim of our study was to compare hormonal and follicular events following DF aspiration with those seen after treatment with a low (2 x follicular phase levels) or high (pharmacological) dose of ODB combined with progestagen given at the time of first wave emergence. Predictably (Bodensteiner *et al.,* 1996; Amiridis *et al.,* 1999; Ginther *et al.,* 1999), aspiration of the freshly selected first DF generally resulted in a transient FSH rise and emergence of the next wave 2 days later, followed by selection of the next DF 5 days after aspiration. This timing of events was similar to other studies where the DF was removed by either electrocautery (Ko *et al.,* 1991; Adams *et al.,* 1992a) or ovulation using exogenous GnRH (Mihm *et al.,* 1998; Ryan *et al.,* 1998). In contrast, the intervals from steroid treatment to the next FSH maximum and new wave emergence were more than twice as long as following aspiration, with FSH declining initially, then only beginning to rise on the second day after treatment; the interval to selection of the next DF was also 4 days longer than following DF aspiration. Thus, the longer intervals from steroid treatment to new

DF selection are associated with a changed FSH profile, which in turn influences emergence of the next wave. Interestingly, follicle wave growth linked to the next FSH maximum did not lead to selection of the next DF in 12/27 (44%) of cycles following steroid treatment, further delaying intervals to selection of the next DF, although the next FSH maximum and wave emergence were advanced compared to control cycles. It is possible that first wave DFs selected despite the low dose of ODB+P may have experienced a partial or transitory loss in function causing an FSH rise stimulating follicle emergence initially, but then recovered some of their inhibitory effects on FSH and/or FSH-dependent follicles. There is some evidence regarding the direct inhibitory effects of healthy DF on other follicle growth despite raised FSH (Adams *et al.,* 1992a; Bungartz and Niemann, 1994; Mihm *et al.,* 1995). However, in cycles treated with the high dose of ODB+P where follicles continued to grow for 2 days after treatment, wave growth was transitory and unsuccessful (without DF selection) in the majority of cycles despite rising FSH and absence of follicles > 5 mm. Thus, it is proposed that factors other than FSH or healthy wave or dominant follicles control the timing of new wave emergence after steroid treatment, and these may be related to secretions from wave follicles ≥ 7mm undergoing atresia. Lack of immediate new wave emergence in response to rising FSH may also be related to an inadequate population of small FSH-dependent follicles present at the time. However, follicle waves consistently emerge following follicle ablations carried out at a random stage of the cycle or twice weekly for several months (Bergfelt *et al.,* 1994; Boni *et al.,* 1997), thus lack of follicles ready to emerge does not appear to be the reason for lack of ultrasound-detectable emergence.

The initial drop in FSH was seen following both doses of ODB+P and was most pronounced following treatment with the high ODB dose; this is in accordance with the known FSH suppressive effect of oestradiol which is dose dependent (Price and Webb, 1988; Bolt *et al.,* 1990; O'Rourke *et al.,* 2000). However, FSH rises immediately following the initial decline despite pharmacologically high serum oestradiol concentrations, similar to what was previously seen in cyclic or ovariectomized heifers (Bo *et al.,* 1993; 1994; O'Rourke *et al.,* 2000), and induces growth of the next follicle wave at oestradiol concentrations normally seen during the follicular phase of the cycle (Bo *et al.,* 1993; Sunderland *et al.,* 1994). The abrupt decline in FSH and the subsequent rise following the high dose of ODB+P are associated with follicle stasis at 6 or 7 mm in diameter and regression thereby preventing DF selection. This was followed by an advanced next FSH maximum and next wave emergence 5 days later and, if the wave follicles became static within 2 days of treatment, selection of the next DF also tended to be advanced compared with control cycles. In contrast, the low dose of ODB+P did not prevent DF selection and did not affect the timing of emergence of the next DF compared with control cycles, but it did cause a temporarily changed FSH profile associated with the advanced emergence of a new wave 5 days later. However, this wave only led to selection of the next DF in one of three cycles, while in the other two cycles follicles regressed again and new follicles emerged coincident with next wave emergence in control cycles.

By aspirating the largest follicle present on Day 5, three days after treatment with the low dose of ODB +P, we tried to determine whether this follicle

still had any residual suppressive effects on FSH or other follicles. No significant effects of largest follicle aspiration were seen following treatment with the low dose of ODB+P in contrast to control cycles, which indicates that the first wave was affected by treatment even if the largest follicle was dominant morphologically. In a previous study using the low dose of ODB and an intravaginal progesterone device the first wave showed temporary loss of function, as a delay in wave growth and reduced intrafollicular oestradiol concentrations in the largest follicles were seen (Austin, 2000). However, this largest follicle may still have been functionally dominant at times, as in 3 of 4 aspiration cycles selection of the next DF occurred from the advanced next follicle wave similar to control aspiration cycles and different to 0.75ODB+P treated cycles without aspiration.

Aspiration of the largest follicle(s) present 3 days after treatment with 5mg ODB+P did not advance the next wave or the next DF, thus, these follicles were not actively suppressing any other follicle growth. However, due to the limitation of the technique, only follicles larger than 5mm were aspirated in general, and this led to a difference in cycles treated with the high dose of ODB + P. Only cycles in which more first wave follicles grew beyond 6 mm underwent the aspiration technique, and such divergent follicle growth between Days 2 and 5 appeared to delay the next transient FSH rise and emergence of the next wave independent of aspiration (follicle status on Day 6 was similar in all cycles treated with the high dose of ODB+P). Thus, treatment with a pharmacologically high dose of ODB in combination with progestagen at the time of wave emergence may lead to two distinct responses:

1) the steroid treatment will cause immediate suppression of wave growth from 1 day after treatment at 6mm; the next wave consistently emerges 5 days after steroid treatment which is advanced compared to control cycles and similar to the intervals reported in other studies using 5 mg E-17β (Bo *et al.,* 1994; 1995).

2) the first follicle wave grows for 2 more days to a larger size of 7 mm despite the high dose of ODB +P, which is associated with a delay in the next FSH maximum and emergence of the next DF despite advanced FSH rise and follicle emergence. This response has not been reported previously, and may indeed be specific to this study, but could possibly account for the variation seen in intervals to the next new DF following the use of high ODB+P combination treatments (Duffy *et al.,* 1997; O'Rourke *et al.,* 1998). It is possible that some wave follicles continue to grow despite pharmacologically high oestradiol and acutely suppressed FSH because they are no longer as acutely FSH-dependent as others. Such growth may subsequently determine success of the next follicle wave (in terms of DF selection), and may reflect differences in the local or systemic environment not measured in our study, which needs to be explored in the future to avoid divergent responses to steroid combination treatments.

Overall, the effect of ODB+P on the growing first follicle wave appears to be dose dependent in our study and others (O'Rourke *et al.,* 1998) and may also depend on the genotype of heifers and the progestagen used in combination. In our study in postpubertal dairy heifers treated during emergence of the first wave, only the high dose of ODB+P consistently prevented DF selection and tended to advance the next DF when follicles became static within 2 days of treatment. Others saw a similar effect in beef heifers with a similarly high dose of oestradiol-

17β on its own or in combination with the same synthetic progestagen (Bo *et al.,* 1993; 1994; 1995), or with the low dose of ODB in combination with an intravaginal progesterone releasing device (O'Rourke *et al.,* 1998). In the latter study, the use of the high dose of ODB plus a progesterone releasing device also disrupted first follicle wave growth similar to our study, but did not advance the next wave. Follicles in our study were generally more suppressed at 6-7 mm than other studies report following steroid treatment at or after wave emergence. For example, Bo *et al.,* (1993) and (1995) reports cessation of the wave 1.3 and 0.6 days after treatment at a maximum size of 8 or 9.6 mm. Such divergent effects on first wave growth may also be due to the timing of treatment in relation to wave emergence and the oestradiol profile in circulation.

The relative contributions of the two gonadotrophins FSH and LH in follicle wave growth were determined in studies where GnRH actions were reduced or abolished using active GnRH immunization (Crowe *et al.,* 2001) or continuous GnRH agonist exposure (Gong *et al.,* 1996), and FSH and LH were supplemented exogenously. Thus, it emerged, that transient FSH rises are essential for wave follicle growth, while LH pulses are needed for follicular oestradiol production and growth beyond 9 mm in diameter. We assume that the severe drop in FSH caused by pharmacologically high doses of oestradiol in our combination treatment is responsible for causing stasis and regression of the first follicle wave, as these follicles are considered FSH-dependent. This is in agreement with a recent study which concluded that the suppressive effects of oestradiol on the first follicle wave were exerted indirectly rather than directly at the ovarian level (Bo *et al.,* 2000). However, oestradiol only has a transient suppressive effect on FSH,

as FSH began to rise again in this study and others within 2 days of treatment (Cooke *et al.,* 1997b; O'Rourke *et al.,* 2000). Yet, follicles were unable to respond to this FSH rise in our study and regressed in all cycles treated with the high dose of ODB+P, indicating an atretogenic effect of transiently reduced FSH concentrations.

In addition, both oestradiol and progesterone in combination also exert a potent negative feedback on GnRH and thus LH pulse frequency and amplitude (Goodman and Karsch, 1980). Whether severely reduced LH pulses, occurring within 36 hours of treatment with the low dose of ODB and progesterone (Austin, 2000), are also involved in wave atresia, is so far undetermined. However, reduced LH pulse frequencies may contribute to subtle losses in follicle function and decreased diameters of subsequently selected DF when experienced during wave emergence (Adams *et al.,* 1992b; Burke *et al.,* 1994; Austin, 2000) and cause DF atresia when experienced during a prolonged dominance period (Savio *et al.,* 1993; Taylor *et al.,* 1994; Manikkam and Rajamahendran, 1997). Although the prolonged high oestradiol concentrations following the high dose of ODB are thought to also exert prolonged inhibitory effects on LH pulsatility in combination with exogenous P and progesterone secretion from the corpus luteum, new waves are still able to emerge, thus demonstrating the relative insensitivity of emerging follicles to the LH pulse environment.

Oestradiol concentrations in serum were still relatively high when the next transient FSH rise occurred and new follicles emerged, thus oestradiol as such does not have a direct inhibitory effect on growing cohort follicles. However, high oestradiol concentrations may have been responsible for the protracted rise in

FSH seen following steroid treatment either directly at the level of the pituitary or via reducing GnRH pulsatile release from the hypothalamus (Bolt *et al.,* 1990). Whether different FSH profiles influence wave emergence or its subsequent growth is so far undetermined. Such a protracted FSH rise, however, may contribute to the delay seen in emergence of the next follicle wave or its lack of progression in 44% of steroid treated cycles when compared with the rapid events seen following DF ablation. Oestradiol preparations with a very short half life, for example oestradiol-17β (Bo *et al.,* 1994, 1995), cause systemic oestradiol concentrations to return to baseline in 42 hours, and thus may allow a more physiological transient FSH rise beneficial to rapid cohort growth and selection of a DF. This may be the reason for a shorter and more predictable interval to emergence of the next wave reported following treatment with oestradiol-17 β than following the use of oestradiol benzoate or valerate (Bo *et al.,* 1995; Duffy *et al.,* 1997; O'Rourke *et al.,* 1998).

Our second main aim was to evaluate systemic inhibin-A as a marker for health or atresia of follicles present on both ovaries. Inhibins in follicular fluid have proven to be very accurate physiological indicators of follicle health or atresia in several studies. Higher molecular weight forms of inhibins are increased in oestrogen - active versus - inactive follicles (Ireland *et al.,* 1994), in freshly selected DF versus their subordinate follicles and versus the old DF following its loss of dominance (Sunderland *et al.,* 1996; Mihm *et al.,* 1997). In addition, intrafollicular amounts of higher molecular weight inhibins (> 34 kDa) increase by 33 hours after the FSH peak in the largest cohort follicles and continue to be maintained high in the successful follicles up to DF selection (Mihm

*et al.,* 1997; Austin *et al.,* 2001). Thus, we hypothesized that the synthesis and secretion into circulation of high molecular weight inhibins are a good marker for follicle survival, assuming that ovarian sources are mostly responsible for circulating concentrations of inhibins and changes in circulation reflect changes in ovarian/follicular secretions. However, measurements of dimeric inhibin in circulation have proven difficult in the past in cattle, and assays for the higher molecular weight inhibins do not exist at the moment. Only very recently, an inhibin-A assay previously published for sheep plasma (Knight *et al.,* 1998) has been modified and validated for bovine plasma and shown to detect all dimeric forms of inhibin-A (Bleach *et al.,* 2001).

Increases in inhibin-A were seen to be associated with growth of the pre-ovulatory follicle during the follicular phase, followed by a decline and a subsequent smaller increase during growth of the first follicle wave of the cycle (Bleach *et al.,* 2001). Results from our study extend this information and show for the first time:

Firstly, the healthy first wave DF secretes inhibin-A and is, in fact, responsible for circulating inhibin-A during its dominance period, as DF aspiration on Day 5 caused an abrupt fall in inhibin-A within 12 hours coincident with a drop in oestradiol, and followed by a transient rise in FSH reaching its maximum 2 days later. This differs from control cycles, where serum oestradiol also declines quite abruptly between Days 5 and 6, while inhibin-A only declines slowly reaching nadir concentrations on Day 9, the day when the second transient FSH rise reaches its maximum. Thus, we conclude, that secretions from the DF and in

particular inhibin-A more so than oestradiol, dynamically regulate FSH concentrations during the dominance period.

Secondly, increasing and high concentrations of inhibin-A are associated with growth of a follicle wave, and no further increases are seen following DF selection. This was the case in our study in all 28 control or treated cycles studied where serum inhibin-A was determined every 12 or 24 hours, and is in agreement with findings of Bleach *et al.,* (2001) in relation to the postovulatory follicle wave. In control cycles from our study, maximum concentrations of inhibin-A were achieved on Day 3.5 after wave emergence but before DF selection, and this is reflected by the changes seen in intrafollicular amounts of inhibins reported for the first follicle wave; healthy growing wave follicles showed increased intrafollicular amounts of the higher molecular weight inhibins within 33 hours of the FSH maximum and amounts did not change any further following DF selection (Mihm *et al.,* 1997; Austin *et al.,* 2001). Maximum inhibin-A concentrations in circulation precede the FSH nadir by 1 day in control cycles, while intrafollicular and systemic oestradiol concentrations reach their maximum after the FSH nadir with completed selection of the DF. As all dimeric molecular weight forms of inhibin have been shown to suppress FSH secretion from pituitary cells (Good *et al.,* 1995), we would propose that inhibin-A secretions from the growing follicle wave are the main endocrine regulator of the FSH decline essential for DF selection, while both oestradiol and inhibin-A secreted from the DF are involved in maintaining FSH low during the dominance period thus preventing any other follicle growth.

And finally, as increases in inhibin-A after wave emergence are probably the result of stimulation of inhibin synthesis from FSH-dependent wave follicles by the transient FSH rise, the abrupt decline in inhibin-A following steroid treatment at first wave emergence must reflect the abrupt withdrawal of FSH support caused by oestradiol. Such a decline in inhibin-A was previously only seen following ovulation (Bleach *et al.,* 2001) or in our study following DF aspiration, and appears to indicate a loss in function of wave follicles similar to the first DF when it loses dominance in control cycles. This is mirrored by the reductions in higher molecular weight inhibins seen in follicular fluid during DF or subordinate atresia (Sunderland *et al.,* 1996; Mihm *et al.,* 1997). Interestingly, the decline in inhibin-A is not as pronounced in cycles treated with the low dose of ODB+P where the follicle wave still progresses to DF selection. However, as inhibin-A concentrations were still reduced compared to control cycles, the function of this wave must have been compromised to a certain extent as discussed above. Also interesting is the fact that inhibin-A concentrations declined equally abruptly and severely in all cycles treated with the high dose of ODB+P, even when follicles grew for 2 more days to reach a larger maximum diameter following steroid treatment. Thus, declining inhibin-A may be related to reduced FSH response in wave follicles, but not necessarily reflect all functions related to follicle growth and atresia. Overall, however, high inhibin-A concentrations in circulation reflect the presence of healthy growing wave or dominant follicles and declining or nadir concentrations are associated with loss of follicle function, generally followed by follicle atresia. Thus, we conclude, that systemic inhibin-A is an excellent marker of the presence of healthy follicles on ovaries, although it may not differentiate between wave or dominant follicles in the absence of ovarian ultrasound

scanning. In agreement with Bleach *et al.,* (2001), we also conclude that follicular inhibin-A very likely plays an important role in the regulation of FSH, possibly more than oestradiol, as in all control cycles of our study the time of the inhibin nadir and the time of each FSH maximum preceding new follicle emergence were identical. Thus, declining or low inhibin-A concentrations due to loss of function of follicles present allow the subsequent rise in FSH, the rate of which may be controlled by other follicular or endocrine (oestradiol) factors.

Our study evaluated the follicular and hormonal responses of 2 steroid combination treatments administered at emergence of the first follicle wave in dairy heifers. Overall, the most consistent response was seen in cycles treated with a pharmacologically high dose of ODB + P, where wave follicles became arrested in their growth within 2 days of treatment at a maximum size of 6mm. However, the time interval from treatment to the next FSH rise, wave emergence and new DF was 3-5.5 days longer in steroid treated cycles than following removal of the freshly selected DF. This delay is attributed to a different FSH profile following steroid treatment characterised by an initial decline and a subsequent protracted rise, and inadequate follicular atresia following steroid treatment: first wave follicles continued to grow between Days 2 and 5 to a DF (low ODB dose) or to 7 mm (high ODB dose) despite steroid treatment, which may have affected success of the next wave that emerged in response to rising FSH. Serum inhibin-A rose and declined coincidentally with the presence of healthy or atretic wave or dominant follicles, and thus can be used to determine the success of any steroid treatments aimed to cause predictable and acute follicle atresia. We conclude, that further studies need to be carried out regarding

the efficiency of steroid combination treatments to cause follicle atresia and thus consistently and predictably synchronise follicle wave emergence and DF selection in cattle, a prerequisite of high oestrous synchrony coupled with optimum fertility.

# Chapter 3:

# General Discussion

The success of oestrous cycle synchronisation programmes in cattle depends on the precise manipulation of ovarian follicle wave growth in such a way that a newly selected dominant follicle is present at the time of luteolysis and treatment withdrawal. This will ensure that the interval from treatment withdrawal to oestrus and ovulation is consistent and predictable in all treated animals allowing single fixed-time insemination with subsequent high fertility. However, steroid combination treatments administered during different stages of follicle wave development have so far yielded unsatisfying results with varying intervals of 2-7 days from treatment to growth of a new follicle wave selecting a new dominant follicle. Our study addressed the effects of administering oestradiol benzoate and a synthetic progestagen during growth of the first follicle wave of the cycle in dairy heifers, as this stage of wave development has been shown to respond variably to different steroid combinations and doses of oestradiol benzoate in beef heifers; in addition, no comparable studies investigating the response of wave follicles in terms of continued wave growth or follicle atresia have been carried out in dairy heifers.

In our study we compared follicular and hormonal changes occurring following ablation of the freshly selected first dominant follicle with those following two doses of oestradiol benzoate administered in conjunction with progestagen at emergence of the first follicle wave. Treatment with oestradiol benzoate and progestagen was less efficient in advancing the transient FSH rise, next wave emergence and selection of the next dominant follicle than the dominant follicle aspiration technique. In addition, an oestradiol benzoate dose effect previously reported in some beef heifers became very evident in our dairy heifers, where the

low dose did not abolish dominant follicle selection, although it appeared to compromise dominant follicle function causing an initial drop in inhibin-A, a rise in FSH, and emergence of a small wave which then regressed again. Unfortunately, the number of heifers allocated to this treatment was low and due to premature luteolysis occurring in one animal, only responses in three animals could be evaluated. Aspiration of the dominant follicle selected following treatment with the low dose of oestradiol benzoate and progestagen did not cause differences in the timing of subsequent wave growth as it did in control animals, again indicating some compromise of function of this selected dominant follicle. In this study we were unable to collect uncontaminated follicular fluid from the dominant follicles selected under exogenous steroid treatment, and in the future perfection of the aspiration technique will allow us to investigate any changes which may occur in intrafollicular parameters of health (such as oestradiol, inhibins and insulin-like growth factor binding proteins) and the oocyte following steroid treatment. Thus, we will be able to estimate the atretogenic effect of a steroid treatment using the low dose of oestradiol benzoate; at the moment, it does not appear sufficient in dairy heifers to cause immediate and predictable atresia of wave follicles.

The high dose of oestradiol benzoate combined with progestagen consistently prevented dominant follicle selection, and advanced the subsequent FSH rise and wave emergence similar to previous reports using oestradiol-17β preparations but not oestradiol benzoate in beef heifers. If follicles became static within 2 days of treatment selection of the next dominant follicle was also advanced compared with control animals; however, the interval from treatment to presence of e newly selected dominant follicle was still > 8 days, which is very long in the current climate of short synchronisation treatments and necessitates a

minimum treatment duration of 8-9 days before withdrawal. A second response to administration of the high dose of oestradiol benzoate was seen, which has not been reported previously; follicles continued to grow for 2 days after treatment reaching a larger maximum size and this was associated with a delay in the next FSH maximum and selection of the next dominant follicle. This was also the group of animals treated with the high dose of oestradiol benzoate and progestagen in which follicles from 5 mm were successfully aspirated. It was very clear that aspiration 3 days after steroid treatment did not advance new wave emergence, and this allowed us to conclude that medium follicles present at the time could not inhibit small follicles belonging to the next wave. However, it is possible that the follicles which grew for 2 more days despite treatment with the high dose of oestradiol benzoate combined with progestagen had an inhibitory effect while they were growing and this may have affected the success of subsequent wave growth (the early emerging follicle wave did not select a new dominant follicle but regressed). In the future, aspiration of growing wave follicles 1, 2 and 3 days following treatment with the high dose of oestradiol benzoate combined with progestagen should elucidate whether follicles are inhibitory during their growth phase thus affecting success of the subsequent wave. It is, of course, possible that the two different responses seen following treatment with the high dose of oestradiol benzoate were due to differences in other hormones not measured in our study, such as progesterone and its effects on LH, and that these same differences also subsequently affected new wave growth. We have to conclude, however, that treatment with the high dose of oestradiol benzoate and progestagen may lead to two different responses in terms of causing atresia of wave follicles present at the time of treatment which will again affect the

predictability and consistency of the timing to new wave and dominant follicle selection.

Finally, our study used inhibin-A measurements in serum for the first time to determine ovarian status, i.e. presence of healthy or atretic follicles. Serum inhibin-A increased and declined coincident with the presence of healthy or atretic wave or dominant follicles, and thus, we conclude that elevated inhibin-A concentrations in serum are an excellent marker of growth of healthy wave or dominant follicle. Whether low inhibin-A concentrations are an equally good marker of the presence of atretic follicles will need to be determined in the future, as no differences in the inhibin-A decline were seen in cycles treated with the high dose of oestradiol benzoate and progestagen where follicles continued to grow for 1 or 2 more days achieving different maximum sizes. Thus, secretion of inhibin-A may indicate that wave follicles are able to respond to FSH, and thus is reduced when FSH declines, but our inhibin-A measurements may have been too insensitive to detect differences between the two follicular responses to the high dose of oestradiol benzoate.

In summary, treatment of dairy heifers with a combination of oestradiol benzoate and progestagen at the time of emergence of the first follicle wave of the cycle does not cause a predictable new transient FSH rise, wave emergence and dominant follicle selection in all animals even following the use of the high dose of oestradiol benzoate. Further experiments need to be completed to improve the efficacy of steroid combination treatments in relation to their ability to cause follicle atresia, a necessary prerequisite to consistently and predictably synchronise follicle wave emergence and dominant follicle selection in cattle.

# Chapter 4:

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