

**OVARIAN FUNCTION IN THE GILT  
AND LACTATING SOW**

by

**Shona Agnes GRANT, B.Sc. (Edinburgh)**

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University of Nottingham  
School of Agriculture  
Sutton Bonington  
Loughborough, Leics., LE12 5RD

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ABSTRACT

Maturation changes during the follicular phase of the porcine oestrous cycle were investigated in spontaneously cycling gilts. The results revealed considerable variation in the morphological and biochemical development of dominant follicles in the early follicular phase. This asynchrony was emphasised by the marked differences in follicular fluid steroid concentrations and LH binding to granulosa cells and theca tissue between follicles of identical size within the same ovary. The nature of this variation may have great significance on our present understanding of asynchronies in embryo development.

On the basis of the variables assessed in this study, those follicles destined to ovulate or to become atretic were only readily distinguishable on Day 20 of the oestrous cycle, suggesting that the selection of the preovulatory population continues over a 4 day period from about Day 16 to Day 20 of the cycle and, furthermore, that the selection process involves some initial maturation even in those unselected follicles that ultimately join the atretic pool.

Leading on from this study, the control of the initiation of follicular development in lactating primiparous sows was examined by assessing the relationships between ovarian activity and circulating hormone levels following manipulation of the suckling stimulus.

In the first experiment, piglet suckling behaviour and follicular development were investigated following a reduction in litter size to five piglets (split-weaning), seven days before weaning at 21 days post-partum. Overall, morphological and biochemical follicular development were significantly advanced ( $p < 0.001$ ) in the treatment

sows. The significantly ( $p < 0.001$ ) greater concentration of follicular fluid oestradiol in follicles recovered from the split-weaned sows suggested that the supply of androgen substrate was probably limiting production of this steroid in the control animals. It was proposed that the latter was due to inadequate gonadotrophin stimulation.

The comparative ethological study revealed that the remaining split-weaned piglets 'multiple-suckled' quarters vacated by their heavier litter mates. Despite a constant suckling frequency, suckling intensity was reduced in the split-weaned litters as evidenced by a regression in mammary tissue growth in the dams.

In a subsequent experiment, further manipulation of the suckling stimulus was achieved by physically restricting the number of mammary quarters available to the piglets. Canvas sheeting strapped around the anterior quarters of one group of sows (cover) limited the five piglets remaining after split-weaning to suckle only the posterior quarters. An investigation of circulating hormone levels, 12 hours before and 48 hours after the start of treatment, revealed that, compared to the split-weaned and control sows, LH levels were significantly ( $p < 0.001$ ) elevated in the cover sows with a concomitant significant ( $p < 0.001$ ) depression in circulating prolactin. Although split-weaning induced a similar endocrine response in several sows, this effect was short-lived and overall, levels of both hormones were similar in the split-weaned group to those recorded in the control sows. The concentration of oxytocin released at suckling was unaffected by litter size or the intensity of mammary stimulation.

Ovarian activity at weaning was significantly enhanced in the cover ( $p < 0.001$ ) and split-weaned ( $p < 0.001$ ) groups. Hence, it was concluded that the critical factor influencing hormone levels and follicular development in the lactating sow was the neural intensity of the suckling stimulus.

The cumulative data of the lactation experiments suggest that in practice, serious consideration should be given to the use of litter size reduction as a means of effectively improving the performance of the primiparous sow and her litter during lactation.

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To Mum and Dad

## INTRODUCTION

The first part of the report deals with the general situation of the country and the position of the industry. It is followed by a detailed description of the various types of products and the methods of their production. The third part of the report is devoted to the study of the market for these products and the factors which influence their demand. The fourth part of the report is devoted to the study of the various types of costs and the methods of their calculation. The fifth part of the report is devoted to the study of the various types of taxes and the methods of their payment. The sixth part of the report is devoted to the study of the various types of insurance and the methods of their payment. The seventh part of the report is devoted to the study of the various types of financing and the methods of their payment. The eighth part of the report is devoted to the study of the various types of distribution and the methods of their payment. 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The accumulated knowledge of agricultural science acquired within the last century has revolutionised the efficiency of pig farming. A recent Pig Improvement Services Newsletter (MLC, 1988) indicates that the majority of producers surveyed now wean between 19 and 25 days post-partum, compared to an 8 week lactation which was traditional. Hence, assuming an average litter size of 10.5, each sow should theoretically produce approximately 22-24 pigs per year.

However, in practice this target is rarely achieved due to a prolonged period after the sow is weaned until she is successfully served again. This reduction in sow productivity is estimated at 2-4 pigs/year or in financial terms, approximately £50/sow/year.

It is obviously economically desirable to reduce the amount of time breeding stock remain unproductive. Although follicular development, oestrus and ovulation may be stimulated in the anoestrus sow by the administration of various hormonal regimens, the induced physiological response is characterised by enormous variability between treatments and individual animals. Alternatively, post-weaning sow performance may be significantly improved by altering the nursing pattern of the sow and her litter during lactation.

In order that these approaches to increase sow productivity may be optimized, it is essential to have a greater understanding of the fundamental endocrine and ovarian events controlling folliculogenesis in the pig. It is hoped that the results of the present studies might provide a sound basis of information upon which to design a suitable treatment for the induction of oestrus and ovulation in the sow, with a view to establishing pregnancy concurrent with lactation and thus shortening the interval between successive parturitions.

# CHAPTER 1

## LITERATURE REVIEW

## I ENDOCRINOLOGY OF REPRODUCTION

### a) Regulation of Gonadotrophin Secretion

In the context of reproduction, most importance attaches to the integration of the nervous and endocrine systems, achieved in the brain at the level of the hypothalamus and in the anterior and posterior components of the pituitary gland (Schally, Arimura and Kastin, 1973; Sawyer, 1978).

The idea that the anterior pituitary trophic hormones might be controlled by humoral mediators from the hypothalamus was first proposed by Hinsey and Markee (1933). However, it was not until the potential of a vascular route between the two structures was exposed by the histological studies of Wislocki and King (1936) that this theory gained acceptance. In the 1950's, a neuro-vascular concept was firmly established when Harris showed that pituitary transplants regained function only if placed under the median eminence where they could be reached by regenerating portal vessels (Harris, 1955, 1972). This view was also supported by Nikitovitch-Winer and Everett (1958).

Thus, the synthetic and secretory activities of the cells of the adenohypophysis are largely controlled by small peptide molecules, of molecular weight 1200-1500 or less, formulated in the hypothalamus (reviewed by McCann and Porter, 1969). These chemical mediators or "releasing hormones" are produced by specialised cells or "nuclei" in the hypothalamus and released from nerve endings into a network of capillary loops over the median eminence. From this point, they are transported humorally via the hypothalamo-hypophysial portal system

to the anterior pituitary, where their specific action controls the synthesis and release of at least six hormones (Schally et al., 1968; McCann and Porter, 1969; Schally, Arimura and Kastin, 1973), although only three, luteinising hormone (LH), follicle stimulating hormone (FSH) and prolactin (PRL) have a direct critical influence on the reproductive cycle.

b) Role of Neurohormones in the Regulation of Gonadotrophin

Secretion

The presence of an LH-releasing hormone (LH-RH) and FSH releasing hormone (FSH-RH) in the hypothalamic extracts of rats and domestic animals was first demonstrated in the early 1960's (McCann, Taleisnik and Friedman, 1960; Campbell et al., 1961; Courrier et al., 1961; Schally and Bowers, 1964a,b; Igarashi and McCann, 1964; Mittler and Meites, 1964; Kuroshima et al., 1965). Later, the gonadotrophin-releasing fraction was purified and in 1971 the decapeptide structure of both porcine and ovine LH-RH were determined (Matsuo et al., 1971b; Amoss, Rivier and Guillemin, 1972) and the molecule synthesised (Geiger et al., 1971; Matsuo et al., 1971a; Burgus et al., 1972).

Although the synthetic LH-RH molecule has been shown to release both LH and FSH, this does not itself prove that this molecule is the main releasing agent of both hormones in normal physiological situations and opinion is still divided as to whether or not this is the case. Active immunisation against a conjugate of synthetic LH-RH caused a substantial decrease in serum pituitary LH and FSH in ovariectomised rats (Fraser et al., 1975) and sheep (Foster, Webb and

Crighton, 1977). However, chromatographic studies on rat hypothalamic extract indicated the presence of another Gn-RH, although this second factor was closely related to the original peptide (Fawcett, Beezley and Wheaton, 1975). The latter finding is further supported by *in vitro* studies investigating the patterns of LH and FSH release evoked by synthetic Gn-RH or hypothalamic extracts of female rats from isolated pituitary tissues in a continuous perfusion system (Yu, Namiki and Gorbman, 1978). The results suggested that the hypothalamic extract contained an entity other than the decapeptide which was capable of releasing both LH and FSH and this component caused a differential release of LH and FSH under particular circumstances.

c) Role of Neurotransmitters in the Regulation of Gonadotrophin Secretion

The regulation of LH-RH release may involve neurotransmitters produced in and around the medial basal hypothalamus. Ample evidence supports the hypothesis that neuronal production of serotonin, dopamine (DA) and norepinephrine (NE) are involved in regulating the secretion of gonadotrophin as well as other hypophysial trophic hormones (McCann and Moss, 1975). Morphological findings in experimental animals indicate that these biogenic amines in the brain, particularly in the hypothalamus, affect peptidergic neurons which contain the releasing and/or inhibiting factors. Both DA and NE are found to be associated with LH-RH neurons in the rat and to influence the release of LH-RH.

Several other neurotransmitters influence reproductive functions: acetylcholine (Hökfelt et al., 1978), amino acids (Hökfelt et al., 1978), amines (Rivier and Vale, 1978), prostaglandins (Ojeda, Jameson and McCann, 1977), and the protein hormones (Fuxe et al., 1977). However, knowledge of the activity of most of them is still fragmentary or, in the case of prostaglandins, controversial (reviewed by Edwards, 1980).

#### d) Feedback Control at the Hypothalamic and Pituitary Levels

##### i) Negative Feedback

The most convincing evidence for the control of tonic gonadotrophin secretion by the feedback action of gonadal steroids has been obtained from bilateral gonadectomy and hemi-castration experiments performed in the rat (Piacsek and Meites, 1966; Knobil, 1974; Yen et al., 1972; Gay and Seth, 1972), primate (Seyler and Reichlin, 1973; Carmel, Araki and Ferin, 1976) and sow (Brinkley et al., 1964; Short et al., 1968), respectively. The treated animals all exhibited a rapid rise in gonadotrophin levels elicited by the hypophysiotrophic effect of enhanced GnRH secretion in the absence of gonadal feedback. Although exogenous steroid replacement of ovariectomised rats decreased LH levels, neither the administration of oestradiol alone or a combination treatment with progesterone inhibited the acute rise of serum FSH levels (Blake, Norman and Sawyer, 1972; Kalra et al., 1973; Ramirez and Sawyer, 1974; Chappel and Barraclough, 1977; Bronson and Channing, 1978). This differential secretion of LH and FSH has been attributed to an additional gonadal peptide which acts selectively on FSH secretion (see this Chapter, Section II(d)).

In contrast to the negative feedback action of oestradiol on LH release, the maintenance of physiological or pharmacological serum levels of progesterone was totally ineffective in suppressing serum gonadotrophin levels in the absence of other ovarian steroids (Yamaji et al., 1972; Karsch et al., 1973). However Karsch and his colleagues (Karsch et al., 1973) reported that progesterone can synergise with oestradiol to inhibit tonic secretion of LH and FSH in the ovariectomised monkey. Nonetheless, the physiological relevance of the latter remains speculative because an inhibitory action of the steroid on the tonic secretion of gonadotrophins is not demonstrable in the intact Rhesus monkey (Dierschke et al., 1973; Resko et al., 1974).

The precise feedback role of oestradiol during the ovine oestrous cycle also awaits clarification. Although Legan and her associates reported that physiological serum levels of the hormone effectively inhibit LH secretion during anoestrus (Legan, Karsch and Foster, 1977), a similar situation has not been observed during the breeding season (Karsch et al., 1978). Hence, it was proposed that progesterone operates as an important negative feedback hormone in the ewe (Baird and Scaramuzzi, 1976; Hauger, Karsch and Foster, 1977; Karsch et al., 1977), although, following ovariectomy, the inhibitory effect was observed to decrease with time (Karsch et al., 1978). Thus, an overall consensus would appear to favour the presence of additional ovarian hormones as an essential prerequisite for progesterone negative feedback (Scaramuzzi and Martensz, 1975; Foster and Karsch, 1976; Martensz et al., 1976; Karsch et al., 1977).

An important feature of the negative feedback action of oestradiol is its site(s) of action and effects at both the pituitary and the hypothalamic level have been proposed. Direct feedback at the level of the pituitary was initially suggested following the observation of a) pituitary cytological changes (Bogdanove, 1963; Gersten and Baker, 1970; Kingsley and Bogdanove, 1973), b) gonadotrophin inhibition (Ramirez, Abrams and McCann, 1964; Chowers and McCann, 1965) or c) changes in sensitivity to hypothalamic releasing factors (Spies et al., 1969; Debeljuk, Arimura and Schally, 1972) after implantation of crystalline steroid. *In vitro* studies which demonstrated a reduction in GnRH induced LH release from rat pituitary cells following the inclusion of oestradiol in the culture medium also support the latter findings. Conversely, in subsequent culture experiments oestradiol had no effect on GnRH induced LH release from bovine pituitary cells (Beck and Convey, 1977; Padmanabhan and Convey, 1978; Convey et al., 1981). Indeed, the situation is further complicated by the well documented observation that intra-hypothalamic steroid implantation is more effective in inhibiting gonadotrophins than any of the other afore-mentioned techniques (Ramirez, Abrams and McCann, 1964; Davidson, 1969). Kato and Villet (1967) further postulated the presence of specific hypothalamic oestradiol binding sites similar to those found in the uterus and vagina.

## ii) Positive Feedback

A consideration of the hormonal inter-relationships of the oestrous cycle (see next section) suggests the existence of an

oestrogen trigger for the preovulatory gonadotrophin surge, and hence a positive oestrogen feedback mechanism. Evidence in support of this concept was initially obtained from experiments in the rhesus monkey and anoestrous sheep, in which exogenous administration of oestradiol induced premature increases in LH (Goding *et al.*, 1969; Radford, Wheatley and Wallace, 1969; Beck and Reeves, 1973) and FSH secretion (Jonas *et al.*, 1973; Pant and Ward, 1974; Pant, Dobson and Ward, 1978) at levels comparable to those of the natural cycle. Further identical results have now been obtained using similar techniques in the ovariectomised ewe (Goding *et al.*, 1970; Pelletier and Signoret, 1969; Scaramuzzi *et al.*, 1971), pigs (Foxcroft and Elsaesser, 1977; Edwards and Foxcroft, 1983), cattle (Beck and Convey, 1977) and rats (Ramirez and Sawyer, 1965).

While progesterone has been shown to have an inhibitory effect on this sensitising action of oestradiol (Labrie *et al.*, 1978), an enhanced response of the pituitary to LH-RH during the mid-luteal phase in women (Yen *et al.*, 1972) and the pig (Van de Weil *et al.*, 1978) has implicated the possibility of additive and synergistic actions of progestin at this time. However, progesterone on its own has little effect in either the rat (Goodman, 1978), sheep (Hauger, Karsh and Foster, 1977) or human (Van Look *et al.*, 1977).

Although inhibitory oestradiol feedback receptors have been located within the median-eminence and pituitary, the situation is less clear in the case of stimulatory feedback. Selective brain lesioning experiments have suggested several potential loci within the hypothalamus such as the median eminence (Kannwischer, Wagner and

Critchlow, 1967; Motta, Piva and Martini, 1973), the anterior hypothalamus (Bogdanove, 1963; Smith and Davidson, 1968), the posterior tuberal region and mamillary body (Bogdanove, 1963) and the preoptic area (Barracough and Gorski, 1961). However, no specific site can be favoured as a discrete localisation for positive feedback as fundamental species differences exist with regard to the precise mechanism which governs the surge mode of LH secretion (reviewed by Karsh et al., 1978). Indeed, the issue is further complicated by the discovery that steroid receptor neurons are not restricted to the hypothalamus (Pfaff, 1968; Anderson and Greenwald, 1969; Stumpf, 1971) thus implicating the potential involvement of additional brain regions (Gorski, 1974).

Initial evidence for a pituitary site of oestradiol positive feedback action came from investigations using multiple injections of synthetic GnRH, a technique previously demonstrated to induce a much greater release of LH in anoestrous ewes than a single injection of the same total dose (Crichton et al., 1975). In general, pituitary responsiveness to the releasing hormone increased during the late follicular period, although in rats it was found to be dependent on the nature and sequence of steroid hormones to which the hypothalamo-hypophysial axis had previously been exposed (Yen et al., 1972; Cooper, Fawcett and McCann, 1973; Legan and Karsh, 1975; Labrie et al., 1978). Further related studies in humans resulted in an hypothesis which proposed the involvement of two pools of gonadotrophins; the first being immediately releasable and the second requiring continued stimulation (Bremner and Paulsen, 1974). Since

the individual content of these pools can be seen to vary during the menstrual cycle, Diebel and his colleagues (Diebel, Yamamoto and Bogdanove, 1973) suggested that gonadal steroids may affect the type of LH in the pituitary gland. Hence, in synchrony with the rising circulating oestradiol concentrations during the early to mid-follicular phase, the size of the second pool is preferentially augmented. Similarly, there is an increase in the 'releasable' pool during the late follicular phase.

Although the precise mechanism for this phenomenon is unknown, the biphasic patterns of pituitary LH release observed after constant infusion or repeated injections of GnRH in several species (rats: Aiyer, Chiappa and Fink, 1974; Edwardson and Gilbert, 1976; Blake, 1978; Pickering and Fink, 1979; Evans et al., 1983; sheep: Crighton and Foster, 1976, 1977; women: Bremner and Paulsen, 1974; Yen et al., 1975; Wang et al., 1976; Hoff et al., 1977) would indicate an oestrogen-dependent self-priming effect of GnRH. In fact, some workers have speculated that the size of the pool of readily releasable LH may be equated to the number of GnRH receptors and that oestradiol acts to increase GnRH receptor numbers (Spona, 1974). Undoubtedly, additional conclusive evidence is required in this field.

#### e) Endocrinology of the Oestrous Cycle

Female eutherian mammals achieve sexual maturity with the establishment of regular cyclic ovarian activity; the menstrual cycle of primates and the oestrous cycle of non-primates. These breeding

cycles are characterised by a repeated series of ovarian changes, particularly in the secretion of steroid hormones which influence the histological state of the reproductive tract. The key stage of the oestrous cycle can be recognised as the time when the animal is receptive to the male and allows mating to take place. This period of receptivity is referred to as heat or oestrus, which in laboratory animals and pigs is associated with a series of behavioural lordosis responses involving immobility, raising the hind quarters or arching the back; there may also be mounting or riding of other females. Species like the pig also show conspicuous alterations in their external genitalia, notably vulva oedema and an increased liquefaction and flow of mucus from the cervix.

The literature concerning endocrinological changes during the mammalian sexual cycle is exhaustive and the following section has therefore been restricted to literature connected with the large farm species with particular reference to the pig.

Oestrus, which occurs at a precise interval characteristic of each species is generally taken as a reference point for the description of the oestrous cycle and designated day 0. Following ovulation, and the development of a corpus luteum or corpora lutea, there is an increase in blood progesterone which achieves a peak concentration on days 10-12 in the ewe and cow (Hunter, 1980) and on days 13-14 in the sow (Edqvist and Lamm, 1971; Hunter, 1980).

This is in contrast to a basal preovulatory level which is generally less than 1.0 ng/ml (Stabenfeldt *et al.*, 1969a,b,c). This period of the cycle is the recognised luteal phase and in sheep and

cattle the maintenance of the corpora lutea requires hypophysial support (Denamur, Martinet and Short, 1973; Hansel, Concannon and Lukaszewska, 1973). Whether a similar situation exists in the pig has been a subject of considerable contention, with much of the early evidence (Nalbandov, 1970; Hansel, Concannon and Lukaszewska, 1973) suggesting that once the signal for luteinisation had occurred the corpora lutea were essentially autonomous. By contrast, several authors have reported a distinct pattern of high amplitude low frequency LH episodes during the porcine luteal phase (Rayford, Brinkley and Young, 1971; Watson and Leask, 1975; Parvizi *et al.*, 1976; Foxcroft, 1978; Van de Weil *et al.*, 1981). In addition, subsequent studies in the miniature pig and an *in vitro* perfusion study (Watson and Leask, 1975) have indicated an association between LH and elevations in peripheral progesterone. Hence, a specific role for LH at this time still awaits clarification.

In the presence of the low basal levels of LH which exist in the latter half of the luteal phase, the corpora lutea become increasingly sensitive to the effects of  $\text{PGF}_2\alpha$ , a luteolytic compound which is produced in the endometrium (Pharris and Wyngarden, 1969). Thus, by days 14-15 and days 16-17 of the oestrous cycle in the sheep and cow respectively, sufficient  $\text{PGF}_2\alpha$  reaches the ovary via a counter current mechanism between the utero-ovarian vein and the ovarian artery (McCracken, 1971) to cause a decline in the secretion of progesterone. Although  $\text{PGF}_2\alpha$  has also been postulated to be a major signal for luteolysis in the pig, the *in vitro* studies of Watson and Walker (1977) have suggested that some factor, other than

or acting in concert with  $\text{PGF}_2\alpha$  may be required for luteolysis. Indeed Wathes and Swann (1982) have recently proposed a release of luteal oxytocin to be this 'missing link'.

After the initiation of luteal regression, there is a striking increase in the secretion of oestradiol from the pre-ovulatory follicles. The factor responsible for this is thought to be a rise in the basal secretion of LH which occurs in response to the decline in the concentration of circulating progesterone (Baird and Scaramuzzi, 1976). Associated with this rise in LH concentration is a marked increase in the frequency of LH pulses previously observed in the luteal phase (Baird, 1978). Thus, in the ewe, by 24 hours after the decline of progesterone secretion, the pulse frequency has doubled to one pulse every 75 minutes (Baird and McNeilly, 1981) and consequently, by the onset of oestrus, the pulsatile discharges of LH are occurring so rapidly that it becomes difficult to distinguish individual pulses from the rising basal LH concentration.

Plasma oestradiol levels normally reach a peak on day 20 of the porcine oestrous cycle (Shearer *et al.*, 1972; Van de Weil *et al.*, 1981) and this period of elevated oestrogens exerts a positive feedback on the hypothalamus and pituitary, stimulating a surge of gonadotrophins from the anterior pituitary. The evidence to support this was obtained by Spies and Niswender (1972) who found that administration of oestradiol blocking agents would prevent the spontaneous LH surge. In addition, exogenous oestrogen has been shown to elicit a release of gonadotrophins in pigs (Foxcroft and Elsaesser, 1977; Edwards and Foxcroft, 1983), monkeys (Yamaji *et al.*,

1971), cattle (Beck and Convey, 1977), sheep (Goding et al., 1969) and rats (Ramirez and Sawyer, 1965). The characteristics of this abrupt rise and fall in LH, termed the pre-ovulatory LH surge, were initially determined in the pig by an ovarian cholesterol depletion assay (Liptrap and Raeside, 1966) then subsequently by radioimmunoassay (Rayford, Brinkley and Young, 1971; Henricks, Guthrie and Handlin, 1972; Parvizi et al., 1976; Vandalem et al., 1979; Van de Weil et al., 1981). These authors reported that the duration of the surge, which was approximately 12-16 hours, was coincident with the onset of oestrus and achieved peak values of 5-10 ng/ml plasma.

In contrast to the pre-ovulatory LH surge, the FSH response to oestradiol positive feedback is variable. Van de Weil and his colleagues (1981) have suggested that this divergent secretion might be explained solely on the basis of the withdrawal of the inhibitory effects of oestrogen or some other ovarian factor. Nevertheless, there is conclusive evidence for a direct oestrogen-induced surge of FSH release coincident with the LH surge, though of reduced magnitude (Elsaesser and Foxcroft, 1978).

In the pig, the pre-ovulatory surge of gonadotrophins initiates a sequence of ovarian events which culminates in ovulation 36-40 hours after the onset of behavioural oestrus. At this point the LH levels are consistently low, in contrast to an additional increase in the release of FSH during days 2-3 of the oestrous cycle (Wilfinger, 1974). The biological significance of this surge remains speculative and, at least in short cycle species (Schwartz, 1979) and prolific breeds of sheep (Cahill, Mariana and Mauléon, 1979), it has been

associated with the recruitment of the crop of follicles destined to ovulate at the subsequent oestrus. There have also been consistent reports of markedly elevated levels of prolactin at this time (Wilfinger, 1974; Van Landeghem and Van de Weil, 1977; Van de Weil et al., 1981). However, the significance of this remains inconclusive, with one group of workers suggesting an inhibitory effect of prolactin on progesterone secretion (Rolland, Gunsalus and Hammond, 1976) while others favour a possible role for the hormone in the stimulation of follicle growth and steroidogenesis (Van de Weil et al., 1981).

Thus, in summary, the oestrous cycle consists of an alternation between a relatively prolonged luteal phase during which progesterone secretion from the corpus luteum is the principal ovarian event, and a compact follicular phase when oestrogen from one or more follicles predominates.

## II THE OVARIAN FOLLICLE

### a) Follicle Development

#### i) Differentiation of Ovarian Tissues

The early development of the female gonad is characterised by the migration of a mitotically active cohort of germ cells from the region of the embryonic hind-gut. Prior to birth, rapid mitotic activity ceases and the resultant primary oocytes remain arrested in meiotic prophase until they either degenerate or are released by ovulation. The actual process of follicular development commences in the central cortex of the ovary where the oocytes are in intimate

contact with a network of cords and tubules, the intra-ovarian rete (Byskov, 1975). A comprehensive study over the past decade concerning the exact role of the latter relationship in folliculogenesis has revealed that the cells of the rete, which travel by amoeboid motion (Byskov, 1975) become attached to the surface of the oocytes and differentiate into granulosa cells. The resultant structures known as a primordial follicles are located in large numbers in the adult ovary and it is this definitive population of follicles which is gradually depleted as follicles enter the growth phase and develop toward ovulation or degenerate by atresia (Ingram, 1962).

The age at which follicular growth commences is species dependent. However, once initiated, the process is continuous throughout life and occurs daily irrespective of the physiological status of the animal. However, the number of follicles beginning to grow each day does vary and is primarily related to the number of follicles in the non-growing pool (Jones and Krohn, 1961). The signal which stimulates one resting follicle to grow while its neighbour remains quiescent is a mystery. In the light of the results of hypophysectomy experiments with rats (Smith, 1930) and hamsters (Moore and Greenwald, 1974), and the observation that only a few oocytes respond to this stimulus, it would appear that extra-ovarian factors may not be necessary for initial steps in the process of follicular development. However, folliculogenesis cannot proceed beyond these early stages in the complete absence of the pituitary (Dufour, Cahill and Mauléon, 1979) and it is now well established

that the gonadotrophic hormones and the ovarian steroid hormones are required for continuous development (reviewed by Richards, 1979; 1980).

The end result of granulosa cell proliferation is the formation of a multilayered, stratified, epithelial-like structure, the stratum granulosum, which surrounds the oocyte. At this stage of development, locally produced androgen and oestrogen stimulate FSH secretion which in turn induces the granulosa cells to secrete a variety of products, including proteins and mucopolysaccharides that accumulate in a cavity termed the antrum (Lindner et al., 1977) which forms in the centre of the follicle. This follicular fluid is an essential component of follicle development since it provides a means by which the avascular granulosa cells can be exposed to an environment different from that of serum and neighbouring follicles.

During the final processes of follicular growth, the cells of the stroma which surrounds the follicle become organised into a series of concentric compressed layers, the theca folliculi. Although, this layer is discernible even before the appearance of an antrum, it is only after the development of the latter that the cellular components of the theca differentiate into two distinct layers (Brambell, 1956): a richly vascularised theca interna containing epithelial-like cells (Priedkalns et al., 1968) and a theca externa characterised by the presence of spindle-shaped cells, similar in appearance to the cells in the surrounding stroma (Van Blerkom and Motta, 1979). As the cells of the theca interna enlarge, assuming a polyhedral appearance, ultra-structural features characteristic of steroidogenic cells may be observed (Christensen and Gillim, 1969).

ii) Patterns of Follicular Growth in the Oestrous Cycle

Many workers have attempted to establish the actual patterns of follicular development; research with sheep (Smeaton and Robertson, 1971) and cattle (Rajakoski, 1960; Hancock, 1962; Choudary, Gier and Marion, 1968; Dufour et al., 1972; Ireland, Coulson and Murphree, 1979; Matton et al., 1981) has indicated that it is not only those follicles which are destined to ovulate that develop, but that accessory follicular development coupled with extensive atresia occurs throughout the cycle.

Histological observations of bovine and porcine follicles led Rajakoski (1960), Thibault (1977) and Wrathall (1980) to propose the existence of two waves of follicular growth during the oestrous cycle: an initial minor wave emerging from the pre-antral stage at about the time of oestrus and a second wave which commences in the early to mid-cycle (Thibault, 1977; Wrathall, 1980). These workers concluded that the follicles of the first wave, which rarely exceed 3-4 mm in diameter (Wrathall, 1980) are normally destined for atresia, although they may be induced to ovulate by an injection of gonadotrophins (Hunter, 1964; 1966). It is therefore apparent that the ovulatory population of follicles are determined from the second wave of growth which occurs after luteal regression.

Contrary to this wave hypothesis, Choudary, Gier and Marion (1968), Donaldson and Hansel (1968) and Matton et al. (1981) considered that follicular growth and atresia are continuous processes which act to maintain a proliferating pool of follicles. In the case of the pig; Foxcroft and Hunter (1985) suggest that it is

only follicles achieving a size greater than 5 mm during the follicular phase which are subject to the action of gonadotrophins. Follicles attaining this optimum size at other stages of the cycle become atretic due to inadequate hormonal stimulation. The observation that the number of follicles was greater in breeds with high ovulation rates (Kirkpatrick et al., 1967; Clark et al., 1973) lends support to this theory.

In either case, the largest follicles present in the ovary 2-3 days prior to ovulation seem destined to ovulate (Dufour et al., 1972; Thibault, 1977; Matton et al., 1981). Thus as the cycle advances there is an increase in the rate of replacement of large follicles, an enhancement in the growth rate of medium-sized follicles (Matton et al., 1981) and a block to the replacement of smaller follicles into the proliferating pool (Robinson and Nalbandov, 1951; Anderson, 1980; Clark et al., 1982). The existence of an intra-ovarian inhibitory action of the largest follicle upon the smaller developing population, especially in monovular species, has also been postulated (Bherer, Dufour and Matton, 1976; diZerega and Hodgen, 1981; Ireland, Coulson and Murphree, 1979; Matton et al., 1981).

Many of the experiments to determine the patterns of follicular dynamics involve the use of markers, specifically Indian ink (Dufour et al., 1972; Matton et al., 1981). It is questionable whether or not this technique disrupts the integrity of the follicle and, therefore, influences the validity of the aforementioned results.

## b) Cellular Sites of Follicular Steroidogenesis

### i) Cellular Interactions in Follicular Steroidogenesis

As the isolated follicle is a multi-component system, the question of the relative contribution of the ovarian somatic cells to follicular steroidogenesis has been an area of intense interest.

The classical micro-dissection/autotransplantation experiments of Falck (1959) first demonstrated that oestrogen formation by the rat follicle depended upon a synergistic action between the theca interna and granulosa cell layer. This interaction was embodied in a "two-cell" type theory which regarded the theca interna as the major site of follicular oestrogen formation, using progesterone produced by luteinised granulosa cells as a substrate (Short, 1962). Later, Ryan, Petro and Kaizer (1968) showed that theca and granulosa cells from FSH/LH stimulated human follicles could each synthesise oestrogen from exogenous labelled acetate *in vitro*. However, the yield of labelled hormone was more than additive when the two cell types were incubated together, providing the first direct evidence of coordinated cellular interaction in the formation of follicular oestrogen.

Present concepts are based on a better understanding of the cellular and biochemical basis of gonadotrophin action and on the application of sensitive radio-immunoassays to determine the steroidogenic potential of isolated follicular cell types *in vitro*. Hence, a "two-cell type, two gonadotrophin" theory has been proposed (Armstrong, Goff and Dorrington, 1978) in which LH-stimulated androgen biosynthesis initially occurs in the vascularised theca

interna; this androgen then diffuses across the lamina basalis and serves as a substrate for an active androgen aromatase enzyme system present in the avascular granulosa layer. Indeed, the ability of cellular incubations (equine: Channing, 1969; porcine: Bjersing and Carstensen, 1967; Tsang, Moon and Armstrong, 1978, 1982; rabbit: Erickson and Ryan, 1975; rat: Dorrington, Moon and Armstrong, 1975) to synthesise significant amounts of oestradiol-17 $\beta$  supports this theory. Additional supportive *in vivo* evidence was provided by Baird (1977), who showed that in ewes bearing an ovarian autotransplant to the carotid-jugular circulation, the infusion of a high titre anti-serum against testosterone inhibited LH-induced oestradiol production. Presumably, the aromatase substrate was intercepted by antibodies as it crossed the intercellular space and basement membrane.

## ii) Androgen Synthesis

The theca interna is regulated by LH and is the major site of androgen formation. Granulosa cells show little *de nova* synthesis of C19 steroid: they either lack the requisite C17-20 lyase enzymes or the enzyme system is repressed (Younglai and Short, 1970; Lacroix, Eechaute and Leusen, 1974; Fortune and Armstrong, 1978; Fowler et al., 1978; Hamberger, Hillensjo and Ahren, 1978; Tsang, Moon and Armstrong, 1978, 1982; Johnson and Hoversland, 1983). However, an increase in androgen production *in vitro* by isolated thecal tissue in the presence of exogenous pregnenolone or 17-hydroxyprogesterone (Makris and Ryan, 1980; Fortune, 1981; Lischinsky and Armstrong, 1983) supports a metabolic role for the C21 precursors formed in the granulosa layer.

Consequently, the granulosa layer is literally drenched in androgen throughout most of the preovulatory follicle's antral phase of development, and, depending upon its intra-follicular metabolic profile, the steroid may effect acute control of steroidogenesis through direct interaction with steroid transforming enzymes. At physiological concentrations, testosterone and  $5\alpha$ -dihydrotestosterone have been shown to inhibit 3-ene- $3\beta$ -hydroxysteroid dehydrogenase/isomerase activity in FSH-treated porcine granulosa cell cultures (Lischinsky, Evans and Armstrong, 1983; Tan and Armstrong, 1984). Other data suggest a possible modulatory action of androgens on granulosa cell  $17\beta$  hydroxysteroid hydrogenase and/or 4-ene- $5\alpha$ -reductase activity (Moon and Leung, 1983). Moreover, it has also been reported that androgen augments FSH-induced stimulation of aromatase activity (Katz, Leung and Armstrong, 1979). It is likely that these effects represent specific actions of androgens, since an androgen receptor has been identified in rat (Schreiber, Reid and Ross, 1976), sheep (Campo, Carson and Findlay, 1984) and human (Milwidsky et al., 1980) granulosa cell cytosol.

### iii) Oestrogen Synthesis

The highly responsive granulosa cell androgen aromatase system is induced by FSH binding to membrane associated cell receptors on the cell surface (Nimrod, Erickson and Ryan, 1976; Gore-Langton and Dorrington, 1981 - see previous section). *In vitro* measurement of aromatase activity in cell suspensions prepared from maturing follicles has shown that the activity appears to be directly correlated to the stage of follicular maturity and general health

status of the follicle (Haney and Schomberg, 1981). A potential alternative route of androgen metabolism is via 4-ene-5 $\alpha$ -reductase, an enzyme system present in granulosa and theca cells from the adult hamster (Makris and Ryan, 1980) and human ovary (McNatty et al., 1979a,b; Moon and Duleba, 1982). Its activity is reportedly highest at puberty (Karakawa et al., 1976; Inaba, Imori and Matsumoto, 1978; Eckstein, 1983) and progressively declines as the first ovulation approaches (Suzuki, Kawakari and Tamaoki, 1978; Eckstein and Ravid, 1979; Toorop et al., 1984). However, its regulation and contribution, if any, to the control of follicular oestrogen biosynthesis in the adult ovary remains to be established.

Although androstenedione and testosterone are present in follicular fluid at concentrations sufficient to drive the aromatisation reaction maximally in isolated granulosa cell suspensions (Hillier, Reichert and Van Hall, 1981), some of the steroid may be unavailable for cellular metabolism due to its association with steroid binding protein in the follicular fluid (Edwards, 1974; McNatty, 1978; Martin et al., 1981). Nonetheless, this would be unlikely to affect cells in the mural granulosa layer which are in close association with the thecal vasculature (Reynolds, 1973) and hence, a readily available source of androgens. Indeed the discovery in the rat that mural granulosa cells are the only site of cytochemically detectable cytochrome P-450 in the preovulatory follicle emphasises the active steroidogenic potential of these cells (Zöller and Weisz, 1978).

The preferential aromatisation of particular androgens has yet to be proven and this topic remains an area of intense controversy. Results obtained from sheep (Moor, 1977; Scaramuzzi et al., 1980) and rat (Oakey and Stitch, 1967) granulosa preparations *in vitro* suggest that these cells are more able to convert testosterone than androstenedione to oestrogen. In contrast Evans et al., (1981) proposed androstenedione to be the major androgen precursor in porcine granulosa cells, since it was found that androstenedione production by thecal tissue from preovulatory follicles exceeded testosterone production in the ratio of 4:1. Subsequent *in vitro* investigations by Mahajan and Samuels (1974) and McNatty et al. (1984) provided further contradictory evidence when both groups postulated that the two androgens were equipotent as aromatase substrates in equine and bovine ovarian tissue respectively.

In addition to the granulosa cells, the theca interna cannot be totally excluded from follicular oestrogen formation. Thecal tissue dissected from FSH/LH stimulated human follicles possesses a full complement of enzymes required for oestrogen biosynthesis from exogenous acetate *in vitro* (Ryan, Petro and Kaiser, 1968) and the theca of large and small antral follicles recovered at all stages of the human menstrual cycle produced limited but significant amounts of oestrogen when cultured *in vitro* (McNatty et al., 1975; Batta, Wentz and Channing, 1980). In the porcine situation, the thecal cells also produce significant quantities of oestradiol (Evans et al., 1981; Haney and Schomberg, 1981; Stoklosowa, Gregoraszczyk and Channing, 1982) although the granulosa cells are still the major source of this

steroid. In fact, the primary site of follicular oestrogen formation may be a species specific variable (Ryan, 1979), as granulosectomy of the preovulatory follicle of a Rhesus monkey had no marked effect on the concentration of oestrogen in the venous effluent from the 'active' ovary, whereas the concentrations were very low after the complete removal of the follicle (Channing and Coudert, 1976). Conversely, bovine studies (Bjersing and Carstensen, 1967; Baird, 1977) have provided apparently conclusive evidence in support of the 'two-cell type-two gonadotrophin' theory and it was experiments with isolated rodent follicular tissue (Makris and Ryan, 1977; Armstrong, Goff and Dorrington, 1978) which prompted the initial synergistic concept. Undoubtedly, these conflicting results may be partially attributed to the problems encountered in obtaining thecal preparations which are free of granulosa cell contamination.

#### iv) Progesterone Synthesis

The preovulatory follicle is also capable of synthesising progesterone. The comparative synthesising ability of both granulosa and thecal compartments to secrete progesterone *in vivo* has been widely studied by a number of workers in various species. Current results for the pig and laboratory rodents suggest that it is the granulosa cells that are primarily responsible for progesterone production (Makris and Ryan 1975; Evans et al., 1981; Stoklozowa, Gregoraszczyk and Channing, 1982). Conversely, from studies on bovine follicles, Fortune and Hansel (1979) reported that the theca interna and granulosa were equipotent in the secretion of progesterone.

Evidence from *in vitro* research has implicated androgens in the regulation of progesterone biosynthesis. The addition of testosterone, androstenedione or dihydrotestosterone to porcine (Schomberg, Stouffer and Tyrey, 1976), rat (Nimrod and Lindner, 1976; Lucky *et al.*, 1977) or bovine (Shemesh and Ailenberg, 1977) granulosa cells in culture was observed to markedly stimulate progestin accumulation. Similarly, during a four day period (day 15-19) of the porcine oestrous cycle, the administration of the anti-androgen, flutamide, by means of an intra-ovarian implant *in vivo* resulted in a significant 50% reduction in progesterone secretion (Schomberg *et al.*, 1978). Since this compound exerts its effect by inhibiting the association of the androgen with cytoplasmic receptors in the target cells, and thereby preventing nuclear translocation of the active androgen-receptor complexes, it may be concluded that the androgenic stimulation of progesterone synthesis is a receptor-mediated event.

In rats, manipulation of progesterone synthesis could only be demonstrated in intact but not hypophysectomised animals (Leung, Goff and Armstrong, 1979) suggesting the essential involvement of some pituitary factor. FSH was subsequently identified as this factor and although the gonadotrophin may act alone to stimulate progesterone production by granulosa cells (Leung, Goff and Armstrong, 1979), it also acts synergistically with androgens to further increase their effect (Armstrong and Dorrington, 1976; Dorrington and Armstrong, 1979; Goff, Leung and Armstrong, 1979; Leung, Goff and Armstrong, 1979).

Evidence that these aforementioned stimulatory actions are a direct action of androgens and do not result from the aromatisation of androgen to oestrogen was initially presented by Lucky *et al.* (1977). They demonstrated that oestrogen had no effect on progesterone production by rat granulosa cells. Nonetheless, antagonistic actions of oestrogens and androgens on progesterone biosynthesis have been observed in cultures of preantral granulosa cells from immature hypophysectomised oestrogen-primed rats and in cultures of porcine granulosa cells from small (1-2 mm) (Thanki and Channing, 1976), medium (3-5 mm) (Schomberg, Stouffer and Tyrey, 1976) and large follicles (8-10 mm) (Haney and Schomberg, 1978). In the former *in vitro* system an intriguing pattern of interaction between oestrogen and androgens was revealed which depended upon their relative concentrations. High oestrogen (diethylstilbestrol,  $10^{-5}M$ ) suppressed, whereas low concentrations ( $10^{-9}M$ ) enhanced the stimulatory effect of low concentrations of testosterone ( $10^{-9}M$ ) on progesterone production. The response to high concentrations of testosterone ( $10^{-7}M$ ) was not affected by either high or low concentrations of oestrogen (Hillier, Knazek and Ross, 1977). To explain this phenomenon, the authors postulated the existence of an interaction between the two classes of steroid at the androgen receptor level.

Additional *in vivo* evidence has further indicated an inhibitory action of oestrogen on progesterone biosynthesis. Treatment of immature hypophysectomised rats with oestradiol resulted in an impaired ovarian response to LH in the production of progesterone *in*

*vivo* (Leung et al., 1978). This inhibitory action was not observed in intact rats and was antagonised by the administration of purified FSH in hypophysectomised rats *in vivo* (Leung, Henderson and Armstrong, 1979).

Several non-steroidal regulators of reproduction have been identified in follicular fluid (reviewed by Channing et al., 1982) and one of these isolated in porcine follicular fluid stimulates granulosa cell progesterone secretion (Ledwitz-Rigby et al., 1977; Ledwitz-Rigby and Rigby, 1981). There is also evidence for an effect of certain metabolic hormones on follicular cell activity. Adashi and co-workers (1985) demonstrated a synergistic action between FSH and somatomedin-C on progesterone accumulation by granulosa cells *in vitro*. These workers showed no significant effect of insulin which is contradictory to the findings of Ciancio and La Barbera (1984). This suggests that the stimulatory effect of insulin on progesterone and c'AMP production may act by increasing the transport and metabolism of glucose and amino acids in the cultured cells.

### c) Gonadotrophin Interaction with the Ovary

#### i) Gonadotrophin receptors

The investigations of inter-cellular biochemical relationships have been complemented by studies demonstrating the intraovarian/intrafollicular distribution of gonadotrophin receptors, mainly utilising radioisotopic methods (Rajaniemi, Hirshfield and Midgley, 1974; Hseuh, Dufau and Catt, 1976).

Initial *in vivo* binding studies employing radio-iodinated LH or hCG demonstrated labelled hormone in the interstitial cells of the testis (de Krester, Catt and Paulsen, 1971) and the luteinised cells of the ovary (Lunenfeld and Eshkol, 1967; Espeland, Naftolin and Paulsen, 1968). However, a more detailed characterisation of the binding sites was eventually achieved using homogenate preparations, tissue slices and cell suspensions (reviewed by Moyle, 1980). It was finally established that FSH binding is restricted to granulosa cells (Ireland and Richards, 1978; Carson et al., 1979; Louvet and Vaitukaitis, 1976) whereas LH binding is present on theca, interstitial and luteal cells and in mural granulosa cells of large preovulatory follicles (Channing and Kammerman, 1973; 1974).

Since follicles within the ovary at any given time are equally exposed to fluctuations in gonadotrophin, it seems reasonable to postulate that changes in cell receptor content might determine the follicular response to specific hormones. Early porcine studies demonstrated that the granulosa cells of preovulatory follicles have 10-500 fold more LH/hCG receptors than those obtained from adjacent 1-2 mm follicles (Channing and Kammerman, 1973; 1974; Kammerman and Ross, 1975; Lee, 1976; Stouffer, Tyrey and Schomberg, 1976). Histological examination of the cultured tissue showed that during maturation there was no increase in cell size *per se*, which suggested that to increase the binding ability of the cell, either new receptors were synthesised or the receptors present in the cell altered their binding affinity for the gonadotrophin. However, in support of the former concept, subsequent scatchard plot analysis

indicated no change in the apparent binding affinity of the receptor for the hormone (Stouffer, Tyrey and Schomberg, 1976; Zeleznik, Midgley and Reichert, 1974; Kammerman and Ross, 1975; Channing and Tsafiriri, 1977).

The pattern of FSH binding during follicular maturation contrasts markedly to that of LH (Nakano *et al.*, 1977) with small follicles of only one or two discernible cell layers exhibiting an enhanced ability to bind radio-iodinated FSH when compared to medium or large follicles. This infers that the presence of FSH receptors on the initial cohort of follicles is a prime requirement for subsequent unimpaired growth and maturation. Indeed, Eshkol and Lunfield (1972) employing an anti-serum against rat FSH, intimated that in animals devoid of FSH only exogenously administered FSH was capable of stimulating granulosa cell proliferation.

#### ii) Relationship between Receptors and Adenylyl Cyclase

There is considerable evidence indicating that the effect of gonadotrophin binding is mediated through activation of an adenylyl cyclase system (Marsh and Savard, 1966; Marsh *et al.*, 1966; Channing and Seymour, 1970; Erickson and Ryan, 1975; Mills, 1975). The concurrent involvement of 3',5'-AMP was first postulated by Haynes (1958) who found that the steroidogenic response of bovine adrenal tissue to Adrenocorticotrophic hormone was associated with elevated levels of c'AMP. Subsequent *in vitro* gonadal studies (cows: Marsh and Savard, 1964, 1966; women: LeMaire, Askari and Savard, 1971; rat: Hermier and Jutisz, 1969; rabbit: Dorrington and Kilpatrick, 1967) have shown that, although the magnitude of the response was species

specific, in each case maximum stimulation by 3',5'-AMP produced a comparable response to that produced by saturating concentrations of LH (or hCG), both in terms of total mass and the relative proportions of steroid produced. Thus, it was concluded that 3',5'-AMP was the intra-cellular mediator of gonadotrophin action on steroid synthesis in the ovary, even though negligible levels of the phosphate were detected in the follicular fluid of small porcine follicles, known to possess a highly responsive FSH-adenylyl cyclase system - an anomaly which has yet to be satisfactorily explained.

The rising levels of oestradiol associated with follicular maturation causes a proliferation of granulosa cells (Goldenberg, Vaitukaitus and Ross, 1972) and hence, gonadotrophin receptor numbers. The increase in FSH receptors enables the cells to respond, via the adenylyl cyclase system to the elevated levels of circulating FSH. In the rat and sheep, this results in an additional increase in both FSH and LH receptors (Zelevnik, Midgley and Reichert, 1974; Weiss et al., 1978) and a stimulation of aromatase activity (Armstrong and Papkoff, 1976; Erickson and Hseuh, 1978; Moon et al., 1978). The entire process has been termed 'coordinated receptor regulation' (Richards, 1979).

Hormonal activation of adenylyl cyclase is nonetheless tissue specific and largely dependent upon the receptor coupling mechanism. In most systems, coupling is rapidly reversible and in consequence, the state of cellular activity is under the moment to moment control of the circulating hormone concentration. Hence, the exposure of the adenylyl cyclase system in mature follicles to the preovulatory LH

surge as well as to exogenous gonadotrophin, results, within approximately the time-span necessary for ovulation to occur, in a gonadotrophin-induced desensitisation of the system to further gonadotrophin stimulation. In fact, LH not only acts in preovulatory follicles but also rapidly reduces the responsiveness of all follicles to FSH, a process of heterodesensitisation (Ross and Gilman, 1977; Richards, 1979; Jonassen and Richards, 1980). Numerous theories exist to explain this phenomenon (reviewed by Ryan *et al.*, 1977), and it seems that at least in some cases, desensitisation is a transcriptionally mediated event that occurs coincident with a loss of hormone specific receptors. However, the significant difference in apparent  $K_m$  for stimulation and that for desensitisation of the adenylyl cyclase system observed in porcine follicle membrane particles (Hunzicker-Dunn, Bockaert and Birnbaumer, 1978) suggests that the follicular refractoriness extends beyond a simple loss of receptors. Additional causative factors include conformational changes in the receptor, uncoupling of the receptor-catalytic unit complex or inactivation of the catalytic unit.

Finally, the functional physiological significance of desensitisation to gonadotrophins during follicle growth awaits clarification. The increase in 3',5'-AMP induced by the gonadotrophic surge, directly or indirectly triggers many events in the follicle, e.g. morphological changes of the granulosa cells, increased steroidogenesis, an increased rate of glycolysis, increased production of prostaglandins and a resumption of oocyte meiosis (Channing *et al.*, 1978). A sustained increase of 3',5'-AMP levels

seems, however, to be able to block oocyte maturation (Hillensjo et al., 1978), to prevent the normal preovulatory rise of prostaglandins (Challis, Erickson and Ryan, 1974) and to prevent ovulation (LeMaire et al., 1973). Thus, a very delicate balance between the rate of hormone synthesis and the rate of 3',5'-AMP production must exist in the preovulatory follicle, and the phenomenon of adenylate cyclase desensitisation probably plays a key role.

#### d) Bioregulators of Reproduction

The study of non-steroidal intra-gonadal regulators was postulated over half a century ago by Mottram and Cramer (1923) and McCullagh (1932), who independently demonstrated that an aqueous substance from the seminiferous tubules was a major inhibitor of pituitary FSH secretion. Despite the appearance of a number of reports providing circumstantial evidence for the existence of such a factor, the 'inhibin' hypothesis was only revived in the past 15-20 years. Direct evidence is now readily available for the occurrence of inhibin-like activities in extracts or fluids related to reproductive tissues in several species; human seminal plasma, ram rete testis fluid (Baker et al., 1976; Setchell, Davies and Main, 1977; Franchimont et al., 1978, 1979; Lee et al., 1979), ram testicular lymph (Baker et al., 1978), Sertoli cell culture medium (Steinberger and Steinberger, 1976; Lagacé et al., 1979; De Jong et al., 1978) and follicular fluid (Marder, Channing and Schwartz, 1977; Welschen et al., 1977; Lorenzen, Channing and Schwartz, 1978; Chari et al., 1979). The active material has been a difficult molecule to

isolate and purify and there has been considerable confusion about its physico-chemical characteristics (see de Jong and Robertson, 1985; Findlay, 1986). However, recent reports from cow (Robertson et al., 1985, 1986; Fukuda et al., 1986), sheep (Leversha et al., 1986) and pig (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985) follicular fluid studies have indicated that inhibin has an apparent molecular weight ranging from 31,000 to 100,000. At present two differing forms of bovine (Fukuda et al., 1986; Robertson et al., 1986), ovine (Leversha et al., 1986) and porcine (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985) inhibin have been purified. Indeed, the availability of pure bovine inhibin has enabled the development of a radioimmunoassay applicable to cattle and humans (McLachlan et al., 1986).

Convincing evidence of the action of inhibin on FSH synthesis was provided by the experiments of Chowdhury, Steinberger and Steinberger (1978). These authors studied the incorporation of  $^3\text{H}$ -Leucine in FSH and LH produced by organ cultures of rat anterior pituitaries suspended in a medium previously used for 2-3 days to culture Sertoli cells. The Sertoli cell factor present in the medium selectively reduced the incorporation of labelled hormone into immunoprecipitable FSH while decreasing its incorporation into LH. Further studies in the female rat have shown that injection of charcoal-treated porcine follicular fluid, a presumed source of inhibin, can completely inhibit the post-castrational rise in FSH (Marder, Channing and Schwartz, 1977) without altering the normal pattern of LH secretion (Schwartz and Channing, 1977). Indeed, the secondary

surge in FSH appears to coincide with a marked nadir in inhibin-like activity in rat ovarian vein serum (DePaolo et al., 1979). Recent *in vitro* studies have also demonstrated that addition of pure inhibin at low and high doses has a suppressive effect on FSH and LH release by pituitary cell cultures (Farnworth et al., 1986). Similarly in sheep, large doses of ovine follicular fluid given to ovariectomised ewes caused a significant reduction in plasma FSH and LH concentration (Clarke et al., 1986). These results were attributed to a reduction in LH pulse amplitude and confirmed by experiments using ovariectomised ewes with hypothalamo-pituitary disconnection to be mediated through a direct action of inhibin on the pituitary (Clarke et al., 1986). Whether inhibin also affects the hypothalamus by inhibiting LH-RH synthesis, transport or secretion is unknown at this time, however this possibility has been suggested by both *in vivo* (Lugaro et al., 1974) and *in vitro* experiments (Demoulin et al. 1979). Nonetheless the suppressive effect is reversible, since removal of an inhibin preparation and continuation of the incubation results in the re-establishment of FSH at control levels (Shander et al., 1980).

The discovery of inhibin activity in follicular fluid (De Jong and Sharpe, 1976; Welschen et al., 1977; Schwartz and Channing, 1977; Marder, Channing and Schwartz, 1977; Franchimont et al., 1979) raised the possibility that it was produced by granulosa cells and this was confirmed by Erickson and Hseuh (1978). However, although it appears that granulosa cells harvested from all sizes of follicles can synthesise inhibin (Channing, 1979), data from the literature are

contradictory concerning the actual physiological levels produced (Welschen et al., 1977; Lorenzen, Channing and Schwartz, 1978). Welschen and his colleagues (1977) found maximum concentrations in medium and large (11-20 mm) bovine follicles. In contrast, an inverse relationship between follicular size and inhibin activity has been reported in the pig (Lorenzen, Channing and Schwartz, 1978; Anderson and de Paolo, 1981), cow (Franchimont et al., 1981) and human (Channing et al., 1981), even though the data presented by Anderson and de Paolo (1981) confusingly indicated that granulosa cells acquire a greater ability to secrete inhibin as follicular maturation progresses. The authors suggest various hypotheses to explain these differences such as destruction due to protease activity, rapid removal secondary to a better vascularisation or the possibility that inhibin may be secreted as a pro-hormone regulated by enzymatic degradation (Strickland and Beers, 1976).

In addition to inhibin, the existence of several other intra-ovarian factors or 'cybernins' has been postulated (Ledwitz-Rigby and Rigby, 1981). An oocyte maturation inhibitor (OMI) produced by the granulosa, which is antagonised by LH has been described by Tsafriri and Channing (1975). OMI does not seem to be species specific since bovine follicular fluid inhibits maturation of hamster oocytes (Gwatkin and Andersen, 1976), pig follicular fluid inhibits rat (Tsafriri et al., 1977) and mouse oocytes (Channing and Pomerantz, 1981) and human follicular fluid inhibits pig oocytes (Hillensjo et al., 1978). It has been suggested that this factor acts to maintain a functional association between the oocyte and cumulus cells

(Channing et al., 1982), arresting the oocyte in the dictyate stage prior to ovulation.

Another factor, apparently a polypeptide, has been identified in a 500-1000 molecular weight fraction from bovine (Darga and Reichert, 1978) and human (Chari et al., 1979) follicular fluid. It inhibits the binding of FSH to granulosa cells *in vitro*, possibly via an interaction with the receptor itself or with a receptor related membrane domain (Darga and Reichert, 1978). However, the existence of a similar factor in human serum has questioned the follicular origin of this so-called FSH binding inhibitor (FSH-BI: Reichert, Sanzo and Darga, 1979; reviewed by Reichert, Sanzo and Dias, 1981). Channing and her colleagues postulated that OMI and FSH-BI may act on granulosa cells through a common mechanism (Channing et al., 1982), but the active principles have yet to be isolated in a pure form so their physiological role in the control of follicular function has not been assessed.

Ying and Guillemin (1979) have reported on the extraction of a polypeptide fraction (molecular weight <3500) from rat ovaries treated with PMSG and from spent rat granulosa cell culture medium. This material designated 'gonadocrinin' was shown to be hypophysiotrophic *in vivo* and *in vitro*, and this stimulatory effect was inhibited by a competitive LH releasing hormone analogue-antagonist. The strong parallelism between gonadocrinin and synthetic luteinising hormone releasing factor implies that gonadocrinin may bind to a similar site at the pituitary level. From the discovery that mature follicles contain high levels of gonadocrinin activity, the authors

suggest that the protein may mediate dominance of more advanced follicles by inhibiting the growth of adjacent, less developed ones.

Ledwitz-Rigby and her colleagues have presented evidence for two distinct but opposing regulatory actions of follicular fluids, a 'luteinisation inhibitor' (LI) and a 'maturation stimulator' (LS). The existence of the former was suggested following a demonstration that follicular fluid taken from immature (1-2 mm) porcine follicles reduced the accumulation of 3',5'-AMP in response to LH and decreased the secretion of progesterone when added to granulosa cell cultures obtained from large (8-10 mm) follicles (Ledwitz-Rigby et al., 1977). The morphological transformation of these cells, normally seen when luteinisation occurs was also blocked by the addition of either pure or charcoal-treated follicular fluid (Ledwitz-Rigby and Rigby, 1981) - similar effects of follicular fluid have been reported in several species (cow: Shemesh, 1979; rat: Amsterdam et al., 1979; horse: Younglai, 1972; human: Kraiem, Druker and Lunenfeld, 1978; Channing et al., 1980). However, LI activity is greatest in the fluid from atretic follicles (Shemesh, 1979; Channing et al., 1980) or fluid obtained from small, immature follicles (Ledwitz-Rigby et al., 1977; Ledwitz-Rigby and Rigby, 1981). Perhaps the loss of this factor constitutes a part of the normal follicular maturation.

In sharp contrast to the latter inhibitory influences, fluid collected from large preovulatory follicles has been shown to be quite permissive of *in vitro* luteinisation of granulosa cells from mature follicles and additionally potentiates the effects of LH and FSH from immature follicles. Younglai (1972) reported stimulation of

both progesterone and oestrogen synthesis in equine granulosa cell cultures when fluid from large preovulatory follicles was added to the medium.

In conclusion, if both LI and LS activity are present to some extent in follicles at various stages of maturation, the changing ratio of LS:LI (or lack of change) within individual follicles may modulate their response to circulating gonadotrophins. Consequently, as an ovulatory follicle matures, the interaction of these two factors determines not only a gonadotrophic response, but the overall rate of local steroid production. Follicles lacking adequate LS support may be doomed to atresia, a concept favoured by Channing and her colleagues (1980).

diZerega and Kling and their colleagues (diZerega et al., 1982, 1983; Kling et al., 1984) reported the presence of a heat and trypsin labile substance secreted by human granulosa cells which suppresses the follicular response to gonadotrophins and inhibits aromatase activity of rat and porcine granulosa cells in culture. Although they proposed that this substance is an important inter- and/or intraovarian regulator of folliculogenesis, their results were based upon material derived from clomiphene and hCG stimulated ovarian cycles and therefore no conclusion can be drawn regarding production of this protein by normally developing granulosa cells.

Finally, the most recent potential intragonadal regulator of follicular function was discovered by Aten, Williams and Behrman, (1986) in rat, human, sheep and cow ovaries. This factor, which is absent from follicular fluid, inhibits binding of radioactive GnRH to

rat ovarian membranes. It is however not GnRH, since it is heat sensitive, its activity elutes differently than GnRH during HPLC and it does not possess GnRH immuno-reactivity. Nonetheless, whether this ovarian factor has anti-gonadal properties in humans and domestic species awaits clarification.

e) Follicular Atresia

The vast majority of germ cells in the mammalian ovary do not mature to be expressed at ovulation, but are eliminated by a degenerative process known as atresia. This phenomenon occurs at all stages of life, including periods of anovulation, during pubertal development, pregnancy and lactation (Brambell, 1956; Hage, Groen-Klevant and Welschen, 1978), throughout the ovarian cycle (Greenwald, 1961; Turnbull, Braden and Mattner, 1977) and during oestrus in reflex ovulators (Hill and White, 1933). Although, in most cycling mammals atresia occurs primarily in small antral follicles (Byskov, 1979; Ryan, 1981), in a number of species including rats and sheep, even large follicles of preovulatory size become atretic (Hirshfield and Midgley, 1978; Carson et al., 1979).

Degenerative cellular changes are the first morphologically recognisable signs of atresia. In the granulosa layer, cessation of mitosis is followed by nuclear karyolysis which results in the breakdown of the entire follicular epithelium (Ingram, 1962). These degenerated cells then detach from the follicular wall and float into the antrum, whereupon the follicular fluid may become gelatinous and cloudy due to an accumulation of cellular debris. Such structural

alterations are associated with enhanced hydrolytic enzyme activity which actively degrades the cellular fragments (Lobel, Rosenbaum and Deane, 1961; Deane, Lobel and Romney, 1962; Dhanasekharan, Sheela-Rani and Moudgal, 1983). In contrast, although some of the theca cells disintegrate, most of them frequently undergo hypertrophy, accumulate lipid droplets and *in vitro* secrete considerable quantities of steroid hormones (Moor et al., 1978). In some species, thecal cells become arranged in irregular short columns radiating outward from the margin of the contracting antrum and separated by fibroblasts forming radial bands between the columns (human: Himelstein-Braw et al., 1976; monkey: Koering, 1969).

Most atretic follicles also exhibit meiotic-like changes such as germinal vesicle breakdown, chromosomes at metaphase or even expulsion of polar bodies (Austin, 1961; Himelstein-Braw et al., 1976; Braw and Tsafiriri, 1980; Oakberg, 1979). These maturation-like changes are accompanied by the disruption of oocyte-cumulus connections (Chiquoine, 1960; Thibault, Gérard and Ménézo, 1976) except in the sheep where connection is maintained even at advanced stages of atresia (Hay et al., 1979). As atresia progresses the follicle eventually collapses, possibly due to dislocation and shortening of the smooth muscle-like cells on the theca externa (Motta and Familiari, 1981).

Although the intra-follicular biochemical events "responsible" for atresia have yet to be satisfactorily clarified, the involvement of endocrine mechanisms was soon recognised. Thus, it was found that hypophysectomy resulted in atresia of antral follicles (Ingram, 1953;

Jones and Krohn, 1959) and that administration of oestrogen or gonadotrophins to hypophysectomised animals reduced the rate of atresia (Pencharz, 1940; Williams, 1944; 1966; Ingram, 1959; Jones and Krohn, 1961; Goldenberg, Vaitukaitis and Ross, 1972; Harman, Louvet and Ross, 1975). Employment of these techniques has readily established the steroidogenic ability of atretic follicles and explanted rat and hamster follicles exhibit a progressive decline in both androgen and oestrogen production *in vitro* (Uilenbroek, Wouterson and Van der Schoot, 1980; Braw and Tsafriri, 1980; Terranova, 1981) although progesterone synthesis is either unchanged (Uilenbroek, Wouterson and Van der Schoot, 1980) or even enhanced (Uilenbroek, Wouterson and Van der Schoot, 1980; Braw and Tsafriri, 1980; Terranova, 1981). These results have also been repeated *in vivo* (Bill and Greenwald, 1981; Terranova and Ascanio, 1982).

Inhibition of androgen biosynthesis, however, is not a universal early atretic change. Atretic follicles from sheep (Moor et al., 1978), pigs (Meinecke, Meinecke-Tillman and Gips, 1982) and primates (Mori et al., 1982) secrete increasing quantities of androgen and very small amounts of oestradiol indicating a possible interference with aromatising enzymes. As a consequence of the latter, several authors have proposed that the resultant enhanced androgen levels promote the atretic process both directly and by further inhibition of residual aromatase following conversion to 5 $\alpha$ -dihydrotestosterone (DHT: McNatty, 1980; Chan and Tan, 1986). This premise is supported by McNatty and his co-workers who reported the preferential conversion of androstenedione to DHT in granulosa cells of small, atretic human follicles (McNatty et al., 1979b).

Nonetheless, thecal androgen production increases principally in developing preovulatory follicles, whereas most follicles appear to undergo atresia at an earlier stage of growth. Therefore, additional conditions are required to explain failure of these follicles to sustain their development. Radiolabelled gonadotrophin binding studies by Carson *et al.* (1979) and Shaha and Greenwald (1982) clearly demonstrated a depressed ability of follicles to specifically bind radio-iodinated gonadotrophins as atresia advances. It has been hypothesised that this progressive inhibition may be partially attributed to increasing quantities of chondroitin sulphate (CS), a follicular fluid glycosaminoglycan (Bellin and Ax, 1984): normally CS intercalates with surface membranes and binds divalent cations and other macromolecules; however, at the abnormally high concentrations observed in atretic follicles, this substance inhibits gonadotrophic binding and prevents LH stimulation of adenylate cyclase (Bellin and Ax, 1984).

Whether such changes are a consequence of atresia *per se* or merely a manifestation of the entire process remains a controversial issue but undoubtedly serum concentrations of gonadotrophins appear to be particularly important (Talbert, Meyer and McShan, 1951; Greenwald, 1978; Braw, Bar-Ami and Tsafiriri, 1981) and in the absence of an appropriate sustained increment of LH, FSH or both, follicles are unable to grow and thus degenerate. Inappropriate hormonal stimuli may also alter follicular function e.g. basement membrane permeability allowing the entrance of previously excluded serum factors. If this leads to activation of a complement system

(Farookhi, 1981) then it is possible that granulosa cell destruction can occur. Furthermore, since complement activation releases a macrophage chemotactic factor (Müller-Eberhard, 1975), atresia may be considered as a localised inflammatory response (Espey, 1980).

In conclusion, the high steroidogenic activity of atretic follicles lends support to the notion that atresia is not a completely degenerative process and that atretic follicles may play an essential role in ovarian physiology (Nicosia, Evangelista and Batta, 1975). How long these cells remain functional is not known but in most species they eventually dedifferentiate and disappear (Mossman and Duke, 1973, cited by Byskov, 1978).

### III REPRODUCTIVE PHYSIOLOGY OF THE LACTATING AND WEANED SOW

#### a) Neuro-endocrine Control of Lactation

##### i) Oxytocin and the Suckling Stimulus

In most mammalian species, successful lactation depends upon the smooth operation of two related and interdependent processes, milk secretion and milk ejection. However, for milk to become available to suckling offspring another mechanism, the milk ejection reflex must operate.

The involvement of oxytocin in the phenomenon of milk ejection was first postulated by Ely and Peterson in 1941. Since then the role of the hormone in this neuro-endocrine reflex has been well established in a number of species using bioassays of plasma withdrawn during suckling or milking (Folley and Knaggs, 1966; Bisset,

Clark and Haldar, 1970; Cleverley and Folley, 1970) or by cannulating a mammary gland and measuring the increase in milk ejection pressure (Wakerley and Lincoln, 1971). Briefly, the nipple and skin covering the mammary gland have a rich supply of nerve endings responding to touch and pressure (Findlay, 1966) and during suckling afferent stimuli are relayed via the spinal chord to the hypothalamic supra-optic and paraventricular nuclei where activation of oxytocin neurones (Lincoln and Wakerley, 1974; 1975) results in a discharge of the hormone from the posterior pituitary terminals. Oxytocin is then transported in the blood stream to the mammary gland where, via an oxytocin receptor mediated depolarisation of the cell membrane (Lincoln and Paisley, 1982), it causes the contraction of specialised myoepithelial cells surrounding the mammary alveoli (Richardson, 1944; Linzell, 1955) and the ejection of the stored presynthesised milk. Although this basic pattern is common to most mammals, the relative importance of oxytocin in the efficient transfer of milk varies from species to species and depends upon the architecture of the mammary glands. Hence, in certain domesticated ruminants large cisterns allow removal of a sizeable fraction of milk by passive withdrawal and indeed, research has revealed a very poor relationship between milk yield and blood oxytocin levels in these species (Folley and Knaggs, 1966; Cleverley and Folley, 1970). By contrast, animals whose milk stores are mainly "alveolar" e.g. the sow, require an active milk ejection response. Thus it is only possible to hand-milk a sow after an intravenous injection of oxytocin (Braude and Mitchell, 1952).

The process of lactation has been studied in some detail in the pig (Forsling et al., 1979; Ellendorff, Forsling and Poulain, 1982) and the rat (Lincoln, Hill and Wakerley, 1973). The young are normally suckled at regular intervals over a 24 hour period and characteristic patterns of behaviour are repeated by both mother and young on each occasion (Barber, Braude and Mitchell, 1955; Gill and Thompson, 1956; Whittemore and Fraser, 1974; Fraser, 1973, 1975a,b; Forsling et al., 1979). Of these much attention has been focussed in the sow on the pattern of vocalisation demonstrated at each suckling and in particular to the change from a slow to a fast rhythmic grunt at the time of milk ejection (Whittemore and Fraser, 1974). However, the mechanisms responsible for the acceleration remain unknown. They may be common with those allowing the activation of oxytocin neurones, or alternatively, the massive electrical discharge of oxytocin neurones could facilitate grunting, since oxytocinergic fibres have been described in the dorsal and dorsomedial vagal nucleus (Swanson and Sawchenko, 1980) which "inter alia" innervates the larynx.

Many researchers have attempted to detect changes in intramammary pressure (IMP) during nursing. In the rat, Wakerley and Lincoln (1971) reported that abrupt rises in IMP, each lasting about 15 seconds recur at regular intervals of 3-10 minutes throughout each nursing. They concluded that these IMP recordings reflected a pulsatile pattern of oxytocin release, although insensitive radio-immunoassay and a limitation of blood sample volume has prevented a detailed analysis of the actual hormone profiles. A similar pattern

of results has been obtained in the human (Cobo et al., 1967) and more recent studies in the cow and pig have also revealed a 'spurt-like' release of the hormone in both these species (Bruhn et al., 1981).

The mechanism which brings about repeated milk ejection is poorly understood. Undoubtedly, the intermittent nature of milk ejection in the pig is partially due to behavioural factors, since the piglets do not suckle continuously. However a refractory period lasting much longer than the actual period of suckling is essential if a subsequent successful suckling is to ensue. Indeed, if the young suckle earlier than usual, a phenomenon of "incomplete suckling" occurs in which the piglets display all the normal behavioural characteristics but there is no release of oxytocin and hence no "milk let down" (Ellendorff, Forsling and Poulain, 1982). Experiments which have demonstrated mammary gland responses to either electrical stimulation or oxytocin injections shortly after a natural milk ejection (Ellendorff, Forsling and Poulain, 1982) suggest that the inhibitory mechanism seems to be located centrally within the afferent arc of the milk ejection reflex as Cross (1955) originally proposed for the rabbit.

#### ii) Prolactin and the Suckling Stimulus

Prior to the onset of lactation in the sow extremely high levels of prolactin (PRL) have been detected on the day before farrowing (Bevers, Willemsse and Kruip, 1978; Van Landeghem and Van de Weil, 1978; Dusza and Krzymowska, 1981). This increase is an essential prerequisite to a normal lactation. After parturition the hormone

levels gradually decrease to the nevertheless elevated concentrations associated with lactation (Bevers, Willemsse and Kruip, 1978; Van Landeghem and Van de Weil, 1978; Benjaminsen, 1981; Stevenson, Cox and Britt, 1981; Kirkwood et al., 1984). Although a number of exteroceptive stimuli play an important role in maintaining the elevation of the circulating prolactin (Jakubowski and Terkel, 1985), undoubtedly the frequent application of the suckling/milking stimulus is one of the most crucial, as has been demonstrated in a number of species.

The concept is further supported by the observation of a gradual fall in prolactin levels toward the end of lactation when the young are less dependent upon their mother and consequently suckle less often (Mulloy and Malven, 1979).

The identity of the neural substance(s) which mediate the suckling-induced PRL secretion has become a central issue of PRL research. PRL secretion is generally thought to be under the influence of a PRL inhibiting factor (PIF). The existence of such a substance in the hypothalamus which was released into the hypothalamo-hypophysial portal veins was first suggested by Everett (1954, 1956) to explain his observation that transplantation of the pituitary gland away from the median eminence favoured prolactin secretion. Subsequently, it was shown that an injection of crude hypothalamic extracts could block the suckling-induced release of prolactin from the pituitary gland (Grosvenor, 1965; Grosvenor, McCann and Nallar, 1965). However, the most convincing evidence for the existence of PIF was provided in a series of elegant studies by

Kamberi, Mical and Porter, (1971a,b). They demonstrated that crude hypothalamic extract administered through a microcannula inserted into a pituitary portal vessel of a living rat depressed the release of prolactin. Moreover, the decrease was directly related to the amount of hypothalamic extract administered.

Following the discovery of dopamine receptors on pituitary membranes (Brown, Seeman and Lee, 1976), in particular those membranes of lactotrophes (Goldsmith, Cronin and Weiner, 1979) and the location of dopaminergic secretory granules adjacent to portal vessels leading to the pituitary gland (MacLeod and Lehmeyer, 1974), much attention was concentrated on the role of the catecholeamines in the control of PRL secretion. By employing specific catecholeamine inhibitors, researchers have now conclusively proven that hypothalamic dopamine is a direct inhibitor of PRL release and could be the major PIF. In harmony with this proposal are reports that suckling is followed by an increase in hypothalamic dopamine synthesis, and that inhibition of catecholeamine synthesis results in an enhanced suckling-induced release of prolactin (Voogt and Carr, 1974). However, there is growing scepticism that dopamine alone can entirely account for the prolactin inhibiting activity found in median eminence extracts because the concentration of the catecholeamine measured in hypophysial stalk plasma will significantly, but not completely suppress spontaneous PRL release (reviewed by Leong, Frawley and Neill, 1983). Several additional compounds have been implicated in this process, in particular gamma aminobutyric acid (GABA) (Schally et al., 1977; Enjalbert et al., 1979) which coexists

with dopamine in the neurones of the arcuate nucleus and receptors of which are found on pituitary membranes. However, the moderate maximal effect of this protein on PRL release *in vitro*, as well as the observation that GABA antagonists do not markedly reduce the PRL inhibiting activity of hypothalamic extracts (Enjalbert et al., 1979) suggest that this compound does not play a major physiological role in the regulation of prolactin secretion. Finally, the gonadotrophin-releasing hormone associated peptide, GAP has recently been shown to inhibit the release of PRL (Nikolics et al., 1985). It possesses marked FSH and LH releasing properties which led to the suggestion that the release of GAP with Gn-RH will promote the secretion of the gonadotrophins while simultaneously inhibiting PRL secretion.

Besides an inhibitory mechanism of control for prolactin secretion, the concept of a supporting stimulatory mechanism has enjoyed wide acceptance. Unlike the prolactin inhibitory mechanism, the clear requirement for a prolactin stimulatory mechanism has been difficult to demonstrate unambiguously. The concept extends mostly from numerous reports of PRL releasing activity in purified fractions of hypothalamic extracts (Arimura and Schally, 1977) and it has now been claimed that thyrotrophin releasing hormone (TRH) may itself be the PRL releasing factor (PRF) (Bowers et al., 1973; Hill-Samli and MacLoed, 1974). Nonetheless, although TRH releases PRL in man, sheep, cow, goat (Tindall, 1974) and the rat (Mueller et al., 1973), there is considerable doubt as to whether TRH is a physiological, as opposed to merely a pharmacological releasing agent for PRL (Chen and

Meites, 1975). Furthermore, in a long term study in which sheep were actively immunized against TRH, there was little or no evidence of any change in PRL secretion (Fraser and McNeilly, 1980). Numerous other substances have been proposed as PRFs (reviewed by Leong, Frawley and Neil, 1983) in particular vasoactive intestinal polypeptide, serotonin, the opiates and oestradiol. Undoubtedly they all exhibit PRF activity but as yet their physiologic role in the normal regulation of PRL release remains to be determined.

b) Lactational Anoestrus

i) Ovarian Changes

It is commonly observed that a number of sows exhibit oestrus behaviour approximately 2 days after farrowing, and a few animals may even come into heat prior to parturition (Warnick, Casida and Grummer, 1950; Burger, 1952; Baker et al., 1953; Self and Grummer, 1958). However, although the proportion of sows displaying this post-partum oestrus varies very widely according to different authors, there is universal agreement that, regardless of whether the sows are suckled or have been weaned, ovulation never occurs. Hence, the whole period of lactation is generally characterised by a state of relative follicular quiescence. Palmer, Teague and Venzke (1965a,b) have shown that follicle size and number decrease significantly in the first week of lactation; thereafter a steady increase occurs which culminates in a marked follicular development following weaning (Lauderdale et al., 1965; Palmer, Teague and Venzke, 1965a,b; Crighton, 1966; Crighton and Lamming, 1969; Edwards, 1980; Shaw, 1984; Britt et al., 1985).

ii) Gonadotrophin Secretion

The factors controlling this lactational anoestrus are poorly understood. The important inhibitory effect of the suckling stimulus has been emphasised experimentally.

In rat studies the protraction of lactation through regular litter replacement (Nicoll and Meites, 1959) resulted in a sustained arrest of oestrous cyclicity and the establishment of prolonged diestrus (Bruce, 1961; Jakubowski and Terkel, 1980). The presence of a suckling infant profoundly inhibits ovarian activity in the higher primates, including man (Yamaji et al., 1971; Williams, Johnson and Hodgen, 1979; Baird et al., 1979; Plant et al., 1980). However, the effect of suckling on the length of the post-partum period in sheep is controversial. Fletcher (1973) and Moss et al. (1980) found no difference in time from birth to first post-partum ovulation between suckling and non-suckling ewes. By contrast, Kann and Martinet (1975) reported that denervation of the udder was associated with a more rapid return to oestrus.

Since suckling also promotes lactation, it has been postulated that it may inhibit ovulation by depressing the neural stimulus to gonadotrophin synthesis. This was confirmed by experiments in which sows were ovariectomised during lactation (Crighton and Laming 1969). The sows failed to show the significant increase in the synthesis and release of LH which is normally expressed following similar treatment in regular cycling animals (Parlow, Anderson and Melampy, 1964; Rayford, Brinkley and Young, 1971). Studies in cows have shown that frequent suckling will continue to suppress

concentrations of LH in the peripheral circulation (Short et al., 1972; Randel, Short and Bellows, 1976) and Kunavongkrit (1984) has recently reported that LH was higher in sows nursing small (2-4 pigs) litters compared to sows nursing normal (7-12 pigs) litters.

From these and earlier observations (Zawadowsky, Eskin and Ovsjannikov, 1935; Casida et al., 1943) it may be inferred that the lack of ovarian activity following parturition is a direct consequence of inadequate gonadotrophic stimulation. Much research, primarily in cattle and sheep was focussed upon various components of the hypothalamic-hypophysial axis in an effort to discover the prime cause of this inhibition (reviewed by Nett, 1987). Moss and Carruthers and their co-workers (Carruthers et al., 1980; Moss et al., 1980) were unable to show any significant change in the hypothalamic GnRH content in the ewe and cow respectively. Neither was it possible to demonstrate a depressed sensitivity of the pituitary gland to GnRH during the early post-partum period using both *in vivo* (Cermak et al., 1983; Moss et al., 1985) and *in vitro* techniques (Moss et al., 1980). However, the LH content of the anterior pituitary gland was found to be dramatically reduced (Nalbandov and Casida, 1940; Chamley, Jonas and Parr, 1976; Jenkin, Heap and Symons, 1977). Current data in the pig on whether basal LH secretion during the final stages of lactation differs from that seen throughout the oestrous cycle is extremely contradictory. Whilst Booman and van de Weil (1980) observed a suppression in basal LH, other groups (Parvizi et al., 1976; Edwards, 1980; Shaw, 1984) have shown no significant change. To further complicate the issue,

Stevenson and Britt (1981) reported a decrease in LH secretion early in lactation with a progressive increase toward late lactation.

Recently, much speculation has centred on the endogenous opioid peptides as factors which might be involved in the suckling induced inhibition of LH secretion. Administration of naloxone, an opioid antagonist, will stimulate LH secretion in suckling rats (Sirinathsinghji and Martini, 1984), ewes (Gregg et al., 1986), cows (Gregg et al., 1985; Whisnant et al., 1986), women (Ishizuka et al., 1984) and sows (Barb et al., 1986; Mattioli et al., 1986; Armstrong, Kraeling and Britt, 1988). Furthermore, the treatment also suppressed serum prolactin levels in rats (Miki et al., 1981), ewes (Gregg et al., 1986) and sows (Barb et al., 1986; Mattioli et al., 1986; Armstrong, Kraeling and Britt, 1988).

The earliest data available on pituitary FSH indicate that levels of the hormone are high with little evidence of change throughout lactation (Lauderdale et al., 1965; Melampy et al., 1966; Crighton and Lamming, 1969). Aherne et al. (1976) have reported FSH levels in the plasma of lactating sows at 3 weeks post-partum which were similar to those observed by Rayford et al. (1974) in cyclic sows and gilts. Stevenson, Cox and Britt (1981) compared the concentration of gonadotrophins in intact and ovariectomised sows. As only FSH levels increased after ovariectomy they proposed that FSH secretion is controlled by a non-steroidal ovarian factor, probably inhibin (see section II(d)) and that the *de novo* synthesis of the hormone is totally unaffected. In support of the latter, several authors (Aherne et al., 1976; Edwards, 1980; Stevenson, Cox and Britt, 1981;

Duggan, Bryant and Cunningham, 1982) have described an increase in FSH levels as lactation progresses. Shaw (1984) reported that the aromatase enzyme system (see section II(b)iii) was active in follicles removed from lactating sows and that the degree of activity was positively correlated with peripheral FSH levels. Thus, the levels of FSH observed during lactation are sufficient to induce aromatase activity in granulosa cells (Shaw, 1984; Foxcroft et al., 1987).

### iii) Steroid Hormones

The pituitary gland is not only dependent upon hypothalamic stimulation but is most susceptible to the feed-back control of the ovarian steroids (see section I(d)i) and ii)). As a consequence of an experiment in ovariectomised ewes which demonstrated that progesterone reduced the frequency of pulsatile LH release and oestradiol inhibited the amplitude of the LH pulses (Goodman and Karsh, 1980), it was proposed that the high levels of circulating oestradiol and progesterone observed during late gestation could exert an inhibitory effect which would directly suppress the occurrence of oestrus and ovulation post-partum. However, from a number of studies in the sow which have detailed circulating steroid levels during lactation, it is apparent that, aside from an initial elevation in plasma (Sasser et al., 1973; Ash et al., 1973; Robertson and King, 1974; Ash and Heap, 1975) and urinary (Raeside, 1963) oestrogens, the concentration of both hormones decreases rapidly around the time of parturition (Ash and Heap, 1975; Baldwin and Stabenfeldt, 1975; Parvizi et al., 1976; Stevenson, Cox and Britt, 1981). Thus, it was originally

concluded that a block on pituitary gonadotrophin secretion induced by ovarian steroids would not appear to account for the anovulatory condition of the lactating sow. Nevertheless, current work in ovariectomised ewes does suggest that steroids may have an anti-gonadotrophic effect in sheep (Tamanini, Crowder and Nett, 1986). The results of this experiment showed that each steroid alone or in combination produced a dramatic reduction in the pituitary concentration of LH. A similar decrease in the mRNAs for the subunits of pituitary LH has been observed during gestation in the ewe (Wise et al., 1985). In agreement with previous studies (Goodman and Karsch 1980; Tamanini, Crowder and Nett, 1986), the authors concluded that there did not appear to be a direct progesterone mediated effect on the secretion or synthesis of LH, but if the anterior pituitary gland was under the influence of oestradiol, the GnRH-induced release of LH was dramatically diminished. Whether the inhibitory effect of oestradiol is mediated primarily at the hypothalamus (Karsch et al., 1978) or directly on the gonadotroph (Wise, Sawyer and Nett, 1986) is yet to be conclusively determined.

#### iv) Prolactin

The association of hyperprolactinaemia with various infertility syndromes in man (Edwards, 1980) and the general observation that a reciprocal relationship exists between prolactin and LH levels during suckling (Lu et al., 1976; Smith, 1978; Isherwood and Cross, 1980; Sirinathsingji and Martini, 1984) has lent support to the view that lactational infertility, particularly in primates is the consequence of a physiologic suckling induced hyperprolactinaemia (Rolland et

al., 1975; Maneckjee, Sinath and Moudgal, 1976; McNeilly, 1979). Indeed, PRL has been considered an anti-gonadal, anti-gonadotrophic agent in several species (McNatty, 1974; Short, 1984) and high levels of the hormone have been demonstrated to suppress oestrogen sensitisation of the pituitary to GnRH in sheep (Kann, Martinet and Schirar, 1978) and pigs (Elsaesser and Parvizi, 1980). Additional supporting evidence has been obtained from *in vitro* experiments in which increasing amounts of prolactin were observed to inhibit progesterone secretion in human and porcine granulosa cells (McNatty, 1974; Veldhuis and Hammond, 1980; Veldhuis, Klase and Hammond, 1981) and from whole mouse follicles (McNatty, Neal and Baker, 1976). Furthermore, McNeilly et al. (1982) postulated that high levels of PRL inhibit follicular steroidogenesis not only by interfering with aromatase but also by reducing the production by the theca of the androgen precursors necessary for oestrogen production.

Nonetheless, these observations are open to question because in spite of maintained hyperprolactinaemia, normal ovarian activity can, and does occur, post-partum in dairy cows (Carruthers and Hafs, 1980), dairy goats (Hart, 1975) and in marmosets (McNeilly et al., 1981). Controversial evidence has also arisen from experiments in which no change in LH levels are observed even after the concentration of circulating PRL had been artificially reduced by the dopamine receptor agonist bromocryptine (Evans et al., 1980; Smith, 1981; van der Schoot et al., 1982; Sirinathsinghji and Martini, 1984). Moreover, ovulation with normal luteal function can be induced in hyperprolactinaemic states by appropriate gonadotrophin

treatment in sheep (LH: McNeilly, O'Connell and Baird, 1980; PMSG: Rhind et al., 1980), rhesus monkeys (LHRH: Knobil et al., 1980) and women (Kemmann et al., 1977). Indeed, studies in artificially hyperprolactinaemic ovariectomised ewes (Kann, Martinet and Schirar, 1978) and hyperprolactinaemic amenorrhagic women (Thorner et al., 1974) have demonstrated a pituitary response to GnRH independent of PRL levels.

From studies in man (Short, 1984), cattle (Carruthers et al., 1980) and rats (Minaguchi and Meites, 1967), a more attractive hypothesis has arisen to explain the inhibitory effects of the neuro-endocrine events frequently observed during lactation. Neural inputs from the nipples stimulate the release of the opiate  $\beta$ -endorphin which is thought to suppress the discharge of hypothalamic gonadotrophin-releasing hormone. As a consequence of this decrease, LH-RH "self-priming" is reduced, which decreases the releasable pool of LH in the pituitary and the frequency and magnitude of episodic LH release. The same afferent inputs from the nipple also inhibit the secretion of dopamine, the PIF, enabling the secretion of PRL to rise immediately (Minaguchi and Meites, 1967). Hence, the level of PRL is a useful index of the degree of hypothalamic inhibition caused by suckling, but it may be quite unrelated to the cause of the ovulatory stimulus (Short, 1984).

#### v) Oxytocin

The recent establishment of oxytocin as an important ovarian hormone (reviewed by Wathes, 1984) concomitant with its essential role during lactation has led researchers to speculate that this

hormone may be critically involved in lactational anoestrus and ovulation. In the pig the actions of suckling in depressing follicular development has been analysed to determine whether it is due to the mere presence of the piglets, to the action of the oxytocin, or to the actual removal of the milk (Peters, First and Casida, 1969). Casida (1971) reported that mamillectomised sows receiving injections of oxytocin had the least follicular development if the litter was still present, and pituitary FSH was reduced in the sows regardless of oxytocin treatment. Hence, although actual milk removal may not be necessary for depressing follicular growth, the presence of the piglets plus the action of oxytocin may be of importance.

By contrast, there is evidence in sheep to suggest that oxytocin is not involved in post-partum anoestrus. Fletcher (1973) compared the intervals from parturition to oestrus in control Merino ewes, ewes with restricted suckling, non-suckling ewes and non-suckling ewes administered with oxytocin in a manner designed to mimic suckling induced releases of the hormone. The latter group had the smallest weaning-oestrus interval and it was concluded that oxytocin did not suppress fertility and, if anything, in the absence of suckling or other lactationally associated stimuli, appeared to decrease the time taken to return to oestrus. Finally, recent attempts to maintain post-weaning anoestrus in sows through oxytocin administration (Ellendorff, 1984; Ellendorff *et al.*, 1985) were only partially successful, providing further conflicting and inconclusive results.

c) Endocrinology of the Weaning to Oestrus Interval

The removal of the entire litter from the sow after a period of lactation normally results in overt oestrus and ovulation within 4-5 days. Initial investigations relating the response of gonadotrophins to weaning produced conflicting results, with several groups unable to demonstrate any elevation in LH secretion (Aherne et al., 1976; Parvizi et al., 1976; Dyck, Palmer and Simaraks, 1979; Stevenson and Britt, 1981) while others obtained direct evidence of a significant post-weaning increase in pituitary LH levels (Lauderdale et al., 1965; Melampy et al., 1966; Crighton and Lamming, 1969; Van de Weil et al., 1979; Edwards, 1980; Cox and Britt, 1981). This anomaly is undoubtedly related to the experimental design employed by respective research laboratories. The frequent sampling studies of Edwards and Foxcroft (1983) and Shaw (1984) revealed an increase in the concentration of serum LH within 9-12 hours after weaning with a concomitant rise in the frequency of episodic LH pulses over the next 2-3 days (Edwards and Foxcroft, 1983; Shaw and Foxcroft, 1985). Furthermore, the latter is also associated with elevated hypothalamic GnRH content (Cox and Britt, 1981, 1982) within 60 hours of weaning, which increases the potential for LH synthesis by the pituitary in preparation for sustained LH secretion at the post-weaning oestrus. The magnitude of this post-weaning preovulatory LH surge is directly related to the length of the preceding lactation with early weaning (10-21 days) depressing the surge (Edwards and Foxcroft, 1983; Kirkwood et al., 1984a,b). This supports previous evidence for a gradual recovery in the responsiveness of the oestrogen positive feedback mechanism (Elsaesser and Parvizi, 1980).

In contrast there is only a transient rise in the post-weaning FSH levels (Aherne et al., 1976; Edwards and Foxcroft, 1983; Shaw and Foxcroft, 1985) and there is no distinct episodic pattern of secretion. In fact, Edwards and Foxcroft (1983) observed no increase in FSH secretion in several sows that returned to oestrus normally. However, the establishment of a significant and positive correlation between plasma FSH levels and granulosa cell aromatase activity by Shaw (1984), further emphasises a role for the hormone in the control of follicular development during lactation.

Plasma oestradiol levels begin to rise 2-6 days after weaning and attain peak levels on the day prior to, or on the day of oestrus (Ash and Heap, 1975; Aherne et al., 1976; van de Weil et al., 1979; Edwards and Foxcroft, 1983). Oestradiol profiles between weaning and oestrus have been observed to be dependent upon lactation length in some animals (Kirkwood et al., 1984a,b) but not in others (Edwards and Foxcroft, 1983). Indeed, Varley, Atkinson and Ross (1981) reported that very early weaned sows exhibited a prolonged oestradiol surge at oestrus.

#### d) Manipulation of Lactational Anoestrus

##### i) Hormone Treatment

The use of exogenous hormone treatment to shorten and/or synchronise the post-weaning interval has been an active area of research. Pregnant mare's serum gonadotrophin (PMSG), alone or in combination with human chorionic gonadotrophin (hCG) has been used extensively to induce follicular development and ovulation during

lactation (Schilling and Cerne, 1972; Dyck, Palmer and Simaraks, 1979; King, Williams and Barker, 1982). In early experiments, oestrus was induced in 96% (Cole and Hughes, 1946) and in 86% (Heitman and Cole, 1956) of sows given a single injection of 1000 to 2000 iu of PMSG 40 days or greater post-partum. Stimulation earlier in lactation led to a less consistent and lower response. Later, Longenecker and Day (1968) showed that an injection of 1200 iu PMSG on the day of weaning, after variable lactation periods of 4-8 weeks caused a significant reduction in the average interval from weaning to the onset of oestrus. Conception rate in the treated animals was very high, but although PMSG increased ovulation rate and the number of living embryos in the sows examined between 21-40 days of gestation, litter size at the time of farrowing was not significantly increased. The possibility that genetic and management factors may influence the response of sows to stimulation by PMSG early in lactation was proposed by Crighton (1970). He obtained only a small response in sows injected with PMSG alone on the 21st day of lactation, but when a system of partial weaning was adopted before the injection of PMSG a high proportion of sows came on heat. In general, an enhanced ovarian response to PMSG is observed as the interval from parturition to injection increases, however no consistently good results have been obtained (Heitman and Cole, 1956; Epstein and Kadmon, 1969; Crighton, 1970a,b; Martinat-Botte, 1975).

The use of hCG alone in order to induce post-weaning ovulation in sows not previously stimulated has not been very satisfactory. Injection of hCG 2-3 days after weaning following an 8 week lactation

period will induce ovulation quite consistently in all animals from 40-42 hours later (Buttle and Hancock, 1967). Under these circumstances, however, the occurrence of oestrus in a large proportion of sows is inhibited, and if such animals are inseminated, the conception rate is much reduced (Radford, 1965). There is some evidence that the transport of embryos in the oviducts of sows induced to ovulate at this time is disturbed (Hancock and Buttle, 1968), although this is inconsistent with results previously obtained from gilts (Polge, Day and Groves, 1968).

There have been fewer attempts to stimulate ovulation following very short periods of lactation. In a similar experiment to that of Lauderdale et al. (1965) in which sows were weaned for 5 days before slaughter at 6, 11 or 16 days post-partum, Kirkpatrick et al. (1965) examined the effects of injecting pituitary extracts. When pituitary FSH was given daily for 5 days a marked stimulation of follicular development was observed in all animals. The ovulatory response was greatest when the gonadotrophins were given immediately after parturition. Peters et al. (1969) adopted a similar regimen but inseminated the animals immediately after removing the litters; no fertilisation was obtained, the ovulatory response was poor, a high proportion of the follicles were cystic and transport of the eggs in the reproductive tract abnormal.

Cox and Britt (1982b) were the first to demonstrate that a fertile oestrus could be induced in multi-parous lactating sows by pulsatile administration of GnRH. Three out of six sows given GnRH every 2 hr and six out of six sows given GnRH hourly exhibited

oestrus at 96 and 91 hours respectively after the onset of the GnRH pulses. The crucial factor in stimulating pituitary responsiveness to GnRH appears to be the timing and concentration of the injected hormone (Bevers *et al.*, 1981; Stevenson, Cox and Britt, 1981; Rojanasthien *et al.*, 1987; Rojanasthien *et al.*, 1988). The experimental regimen employed by Clark, Komkov and Tribble (1986) was not sufficient to overcome the inhibitory effects of lactation and suckling.

Induction of oestrus after weaning has also been attempted by means of steroid hormone preparations. Although treatment with oestradiol benzoate induced an early and synchronous oestrus (Edwards and Foxcroft, 1983; Kirkwood *et al.*, 1984a,b), the technique has not proven successful because of an associated reduction in ovulation rate. Meredith (1979) and Wrathall (1975) reported field problems of prolonged anoestrus following the use of hCG and oestradiol benzoate. Similarly androgen-oestrogen (Jöchle and Schilling, 1965) or oestrogen-progesterone (Dyck, 1976) have been marginally effective and variable in inducing a post-weaning oestrus. Dyck (1976) concluded that the steroids either stimulated or inhibited gonadotrophin secretion from the pituitary depending upon the reproductive stage of the sow at the time of treatment. Weaning to oestrus intervals are reported to have been shortened using diethylstilbestrol (Rigor, Villareal and Illagen, 1968) but conception rates and litter sizes were again reduced.

Finally, vitamin E is included in some hormone preparations for the treatment of anoestrus in pigs but there is little scientific

basis for its use. Kupferschmied (1966) has shown that a combination of vitamin E and hCG can be successfully used to treat anoestrus but the efficacy was not significantly different from that of hCG alone. By contrast, Sporri and Odermatt (1957) obtained a pregnancy rate of only 1% when vitamin E was used on its own for the treatment of anoestrus. Hence, although a number of vitamins (Brooks, Smith and Irwin, 1977) are sometimes used for the treatment of post-weaning infertility, there is little evidence that they are beneficiary or that a deficiency of them causes anoestrus.

#### ii) Management and Environmental Factors

Various regimens for altering the nursing pattern of the sow and her litter and hence reducing the intensity of the suckling stimulus have been investigated by a number of workers as possible means of inducing oestrus and ovulation during lactation. Partial weaning experiments in which the piglets were removed for various time periods/day beginning the second or third week of lactation have induced lactational oestrus in a high percentage of sows in most studies (Smith, 1961; Walker and England 1978; Thompson, Hanford and Jensen, 1981; Stevenson and Davis, 1984) although not in all (Crighton, 1970a; Cole, Brooks and Kay, 1972; Kirkwood, Smith and Lapwood, 1983; Henderson and Hughes, 1984). The technique of split-weaning or abruptly reducing the litter size (RLS) prior to final weaning has also increased the percentage of sows exhibiting oestrus 5-10 days after weaning (Stevenson and Britt, 1981; Cox et al., 1983; Stevenson and Davis, 1984). Stevenson and Britt (1981) reported that when the litter size was altered (3,8 or 13-14 piglets) during the

last 5 days of lactation, the onset of cyclic activity was significantly hastened (1.1, 4.1 and 4.0 days respectively). In a comparable study, 44% of "treated" sows were detected in oestrus on the day of weaning following a reduction in the number of nursing piglets to 2-4 per sow a week prior to final weaning (Stevenson and Davis, 1984). The results of the latter study, in agreement with Kunavongkrit (1984), also indicated that lactation lengths of two weeks in association with litter reduction, separation or total weaning of the whole litter did not extend weaning to oestrus intervals beyond those observed for sows with longer (4-5 week) lactations. This is in contrast to most studies in which increased duration of lactation is associated with decreased weaning to oestrus intervals or decreased weaning to conception intervals (Self and Grummer, 1958; Aumaitre and Rettagliati, 1972; Svajgr et al., 1974; Cole, Varley and Hughes, 1975; Hays et al., 1978).

Other factors also influence sow reproductive activity during and after lactation. Exposure of lactating sows to a synthetic boar pheromone (5-androst-16ene-3one) or to mature boars has reduced the weaning to oestrus interval (Hillyer, 1976; Petchey and English, 1980). In British management systems, grouping sows together with their pigs in the presence or absence of boars has successfully induced lactational oestrus and fertile matings (Petchey and Jolly, 1979; Rowlinson and Bryant, 1981; 1982a,b) even when the suckling behaviour of the pigs remained unchanged after grouping (Rowlinson, 1977). In an earlier experiment, 80% of sows showed oestrus during lactation when they were grouped together in the presence of boars

and fed *ad libitum* (Rowlinson and Bryant, 1976). Partial boar contact (visual, auditory or olfactory) or no boar contact induced oestrus in only 42% and 4% of the sows respectively. However, similar treatment in American studies under different conditions of management and with different breeding stock had no effect (Guthrie, Pursel and Frobish, 1978). Gadd (1969) and Petherick, Rowlinson and Bryant (1977) found that grouping of sows and litters stimulated lactational oestrus when the boar was not present.

It is apparent from several studies that the reproductive performance of the sow is also directly affected by the level of protein and energy intakes during lactation. An intake of approximately 50 MJ/day is required for satisfactory follicular development after weaning. Animals which fail to meet this requirement have greater weight loss and ultimately a prolonged weaning to oestrus interval. Nutrition trials which have examined the effect of a post-weaning supplementation of the diet have provided contradictory and inconclusive results. Some authors support such a practice (Brooks and Cole, 1972; Cox *et al.*, 1983) while others reported no beneficial effect whatsoever (Dyck, 1972; Brooks *et al.*, 1975; Fahmy and Dufour, 1976; Tribble and Orr, 1982).

Finally, there is clear evidence that season affects reproductive performance in the pig (Mauget, 1982). There is an increase in weaning to oestrus interval when litters are weaned during summer or early autumn, and this delay is greater in primiparous compared to multiparous sows (Aumaitre *et al.*, 1976; Benjaminsen and Karlberg, 1981; Britt, Szarek and Levis, 1983). Armstrong, Britt and Cox.

(1984) demonstrated distinct differences between seasons in post-weaning endocrine changes and feed intake. Sows weaned in September had decreased levels of hypothalamic gonadotrophic releasing hormone, anterior pituitary gonadotrophic content and appetite compared to sows weaned in March.

## CHAPTER 2

### EXPERIMENT 1

#### FOLLICULAR DEVELOPMENT IN THE CYCLIC GILT

## I INTRODUCTION

Although the gilt has been extensively used to study porcine folliculogenesis, ovarian material has frequently been obtained from either PMSG or hCG treated prepubertal gilts (Ainsworth et al., 1980; Evans et al., 1981) or collected at abattoirs from animals of unknown physiological status. In the former case, it is not possible to assume that an injection of such exogenous hormones induces completely normal follicular development and in the latter, previous nutritional or management factors may have enhanced or diminished the reproductive development of the animals prior to slaughter. Moreover, ovarian material obtained from hormonally stimulated animals is often pooled in terms of follicular size (Evans et al., 1981; Stoklosowa, Gregoraszczyk and Channing, 1982; Tsang et al., 1985) thus preventing a comparison of data between morphologically similar follicles. Consequently, follicles collected from these sources may not provide accurate information on the progressive morphological and biochemical changes which occur during the follicular phase.

In addition, attention is generally focussed upon the factors affecting the development of the selected ovulatory population and hence, the ultimate ovulate rate. However, such studies have been criticised for assuming that these follicles represented this chosen population.

The aim of this experiment was to investigate the maturational changes which occur within and between developing follicles during the follicular phase of a normal porcine oestrous cycle, and, in particular, to record any characteristics specific to the selected

ovulatory population which would facilitate identification of these follicles in ovarian material from any source.

## II MATERIALS AND METHODS

### a) Animals

Twenty prepubertal Landrace x (Landrace x Large White) gilts were obtained from the School of Agriculture's main herd. Animals were group-penned according to their size and age to facilitate easier management and fed approximately 2.3 kg of a standard sow ration/day. Water was available *ad libitum*. To stimulate the onset of puberty and to detect oestrus, a vasectomised boar was introduced into each pen twice daily for a minimum period of one hour. Once gilts were known to be postpubertal and exhibiting regular oestrous cycles, the date of the last oestrus for each animal was utilised to predict the stage of oestrous cycle and five gilts were then slaughtered locally on predicted days 16 (d16), 18 (d18), 20 (d20) and 21 (d21) of the cycle, respectively. Slaughter of animals on day 22/day 1 of the cycle was discontinued early in the study due to an inability to harvest the ovarian tissue (see Section IV a(i)).

### b) Collection and Isolation of Follicular Tissues

Immediately after slaughter, the ovaries were collected into phosphate buffered saline (PBS) and transferred within 5 mins to the laboratory. Gross morphological features were recorded. For each pair of ovaries, all follicles  $> 2$  mm were dissected free of surrounding connective tissue using fine iris scissors and pooled

into ice-cold PBS. The diameter of each follicle was recorded from the mean of two measurements taken at 90° to one another and the degree of follicular vascularisation assessed. Follicular fluid was aspirated using 25, 100 or 250  $\mu$ l fixed-needle Hamilton syringes and added to 1 ml of Hanks buffer (Flow Laboratories, UK). The Hanks buffer and follicular fluid were then centrifuged at 160 x g for 10 mins to pellet any granulosa cells. The supernatant was decanted and stored at -20°C for subsequent oestradiol, testosterone and progesterone radio-immunoassay (see Section II(e)).

Each follicle was bisected and the granulosa cells removed by gently scraping the inner surface of each half with a heat bent pasteur pipette into 3 mls PBS containing 4 mM Ethylenediamine-tetracetic acid (EDTA; PBS-EDTA) to prevent cellular clumping. The remaining theca tissue was agitated in a further 2 mls PBS-EDTA to remove any adhering granulosa cells. These cell suspensions were pooled and added to the granulosa cell pellet obtained from centrifugation of the follicular fluid. The cells were collected by centrifugation at 160 x g for 10 mins at 4°C and resuspended in 0.5mls Modified Eagles Basal Medium (Flow Laboratories, UK) containing 0.1% Bovine Serum Albumin (Sigma Chemical Company, UK) (Med. BSA). Cell counts for each follicle were determined using a haemocytometer and a light microscope under low magnification. The follicles were then arranged in an order of descending diameter and cell suspensions were either a) incubated *in vitro* to assess aromatase activity (see Section II(d)) or b) frozen at -196°C in 1 ml polypropylene cryotubes (Nunc: Gibco Europe Ltd., Uxbridge,

Middlesex, UK) containing 0.5 ml PBS and 20% glycerol for subsequent determination of  $^{125}\text{I}$ -hCG binding capacity (see Section II(c)). The theca tissue was individually wrapped in foil and frozen at  $-196^\circ\text{C}$ . It was subsequently used for either a)  $^{125}\text{I}$ -hCG binding studies or b) homogenised to determine the testosterone content of the tissue (see Section II(e)ii).

c)  $^{125}\text{I}$ -labelled hCG ( $^{125}\text{I}$ -hCG) Binding Analysis

$^{125}\text{I}$ -hCG binding to granulosa cells (BG) and theca tissue (BT) was carried out according to the method of Foxcroft et al. (1987) with no modifications.

d) In Vitro Incubations for Aromatase Estimation (AR)

Replicate 125  $\mu\text{l}$  aliquots of granulosa cell suspensions were pipetted into polystyrene tubes (12 x 75 mm) and incubated in the presence (100 ng/ml) or absence of testosterone in a total volume of 1 ml Med BSA. Cell suspensions were incubated at  $37^\circ\text{C}$  in a shaking water bath and incubations were stopped after 2 hrs by transferring the tubes to a freezer at  $-20^\circ\text{C}$ . For subsequent oestradiol determination, the contents of each tube were thawed and centrifuged at  $160 \times g$  for 10 mins and the supernatant decanted for assay.

The concentration of the testosterone substrate and the optimum length of the incubation period were previously established by Foxcroft et al. (1987) and estimated aromatase activity was expressed as ng  $\text{E}_2/2\text{h}/\text{follicle}$ .

e) Radioimmunoassay of Steroids in Follicular Fluids, Incubation Medium and Theca Tissue

i) Oestradiol-17 $\beta$  (E<sub>2</sub>)

Measurement of the concentration of E<sub>2</sub> in follicular fluid (FFE) and incubation medium was carried out according to the method of Foxcroft et al. (1984) but without extraction. The direct follicular fluid assay was validated by comparing the values recorded from the measurement of a range of samples and standards with and without prior extraction. The correlation obtained from this comparison is shown in Fig. 2.1. The correlation coefficient of E<sub>2</sub> measured after extraction and E<sub>2</sub> measured without extraction was 0.9925 ( $p < 0.001$ ). These results indicated that the measurement of E<sub>2</sub> in follicular fluid without prior extraction was acceptable and samples were assayed at either 1, 2.5, 5, 10, 20, 100 or 200  $\mu$ l. Samples less than 100  $\mu$ l were made up to a final volume of 100  $\mu$ l with Hanks buffer. A pool of follicular fluid diluted in Hanks buffer (potency = 340 pg/ml) was used as a quality control and routinely assayed at four volumes (10, 20, 50 or 100  $\mu$ l) to measure intra- and inter-assay coefficients of variation (C.V.) and to determine parallelism. At 100  $\mu$ l, the intra- and inter-assay C.V. were 2.4% and 9.3% respectively and the mean limit of assay sensitivity was 2.5 pg/100  $\mu$ l.

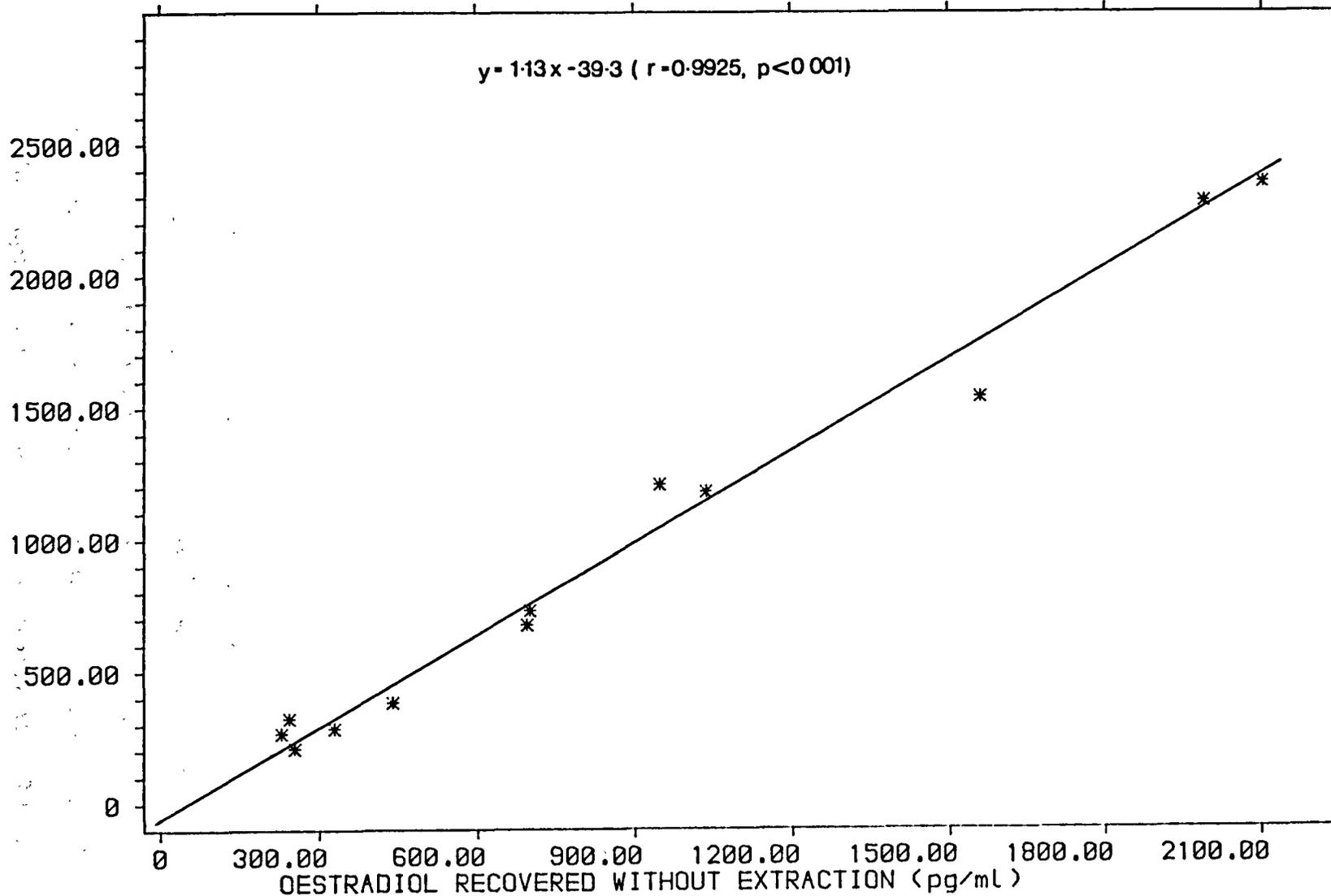
The direct measurement of E<sub>2</sub> in incubation medium was validated by Foxcroft et al. (1987). For this experiment, media was assayed at either 25, 50, 100 or 200  $\mu$ l. Samples less than 100  $\mu$ l being made up to a final volume of 100  $\mu$ l with Modified Eagles Basal Medium. A

**Figure 2.1**      A comparison of oestradiol measurements assayed in porcine follicular fluid with and without extraction with diethyl ether.

OESTRADIOL RECOVERED WITH AND WITHOUT EXTRACTION (pg/ml)

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A  
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T  
I  
O  
N

pg/ml



control pool of medium (potency = 400 pg/ml) was routinely assayed at 50, 100 and 200  $\mu$ l to determine parallelism and intra- and inter-assay C.V. which were 3.6 and 11.7%, respectively. The mean sensitivity of these assays was 6.3 pg/100  $\mu$ l.

ii) Testosterone

The assay used for the measurement of the concentration of testosterone in a) follicular fluid (FFT) and b) theca tissue (THT) was as described by Purvis, Illius and Haynes (1974) with minor modifications.

Follicular fluid samples were assayed at either 1, 5, 10, 20, 50, 100 or 200  $\mu$ l. Samples less than 100  $\mu$ l were made up to a final volume of 100  $\mu$ l with Hanks buffer. The samples were extracted with 1 ml diethyl ether ('Pronalys AR') by shaking for 20 mins on a multi-purpose horizontal shaker. Mean extraction efficiency was 94%, and the mean sensitivity of this series of assays was 5.2 pg/100  $\mu$ l. A pooled sample of follicular fluid in Hanks buffer (potency = 0.55 ng/ml) was routinely assayed at 20, 50 and 100  $\mu$ l which confirmed parallelism and to measure intra- and inter-assay C.V. At 100  $\mu$ l, the intra- and inter-assay C.V. were 4.4% and 6.0 % respectively.

Theca tissue samples were quartered and homogenised in 2 mls double distilled ethanol using a polytron homogeniser. The probe was washed in a further 2 ml of ethanol to make up a final volume of 4 mls ethanol extract which was then centrifuged at 160 x g at 4°C for 30 mins. The supernatant was decanted and stored at -20°C for subsequent testosterone assay. Aliquots of 100 or 200  $\mu$ l ethanol

extract were evaporated to dryness at 37°C and reconstituted in assay buffer (PBS). The volume of buffer added to the tubes was adjusted to between 50 and 200  $\mu$ l to obtain a value of B/Bo in the assay of between 30 and 80%. After adding the buffer, the tubes were shaken on a multi-vortex for 10 mins and left to reconstitute in a 37°C water bath for a further 30 mins. The tubes were then revortexed and allowed to cool prior to normal assay procedure. A spiked quality control pool of double distilled alcohol (1.6 ng/ml) was routinely dried down, reconstituted and assayed at 50, 100 and 200  $\mu$ l to determine parallelism and the intra- and inter-assay C.V. At 100  $\mu$ l, the intra-assay C.V. was 12.7% and the inter-assay C.V. 13.8%. The mean limit of assay sensitivity was 14.1 pg/100  $\mu$ l.

### iii) Progesterone

Measurement of the concentration of progesterone in follicular fluid (FFP) was determined using a modification of the radioimmunoassay method as described by Southee (1986).

Follicular fluid samples were measured without extraction and the direct assay was validated by comparing results obtained from a range of samples and standards which were either extracted or measured directly after heating at 70°C for 30 mins. The correlation obtained from this comparison is shown in Fig. 2.2. The correlation coefficient of progesterone measured after extraction and progesterone measured directly after heat denaturation was 0.9966 ( $p < 0.001$ ). This result was acceptable and samples were subsequently heat denatured then assayed directly in volumes between 1 and 100  $\mu$ l. Samples less than 100  $\mu$ l were made up to a final

volume of 100  $\mu$ l in Hanks buffer for assay. A quality control pool of follicular fluid (potency  $\approx$  25 ng/ml) was routinely assayed at 50 and 100  $\mu$ l to determine intra- and inter-assay C.V. which were 6.4% and 4.4% at 100 $\mu$ l. The limit of assay sensitivity was 13.7 pg/ 100  $\mu$ l.

### III STATISTICAL ANALYSIS

#### a) Calculation of Radioimmunoassay Data

The counting data were evaluated using a statistical program initially developed by Rodbard and Lewald (1970).

#### b) Statistical Analysis

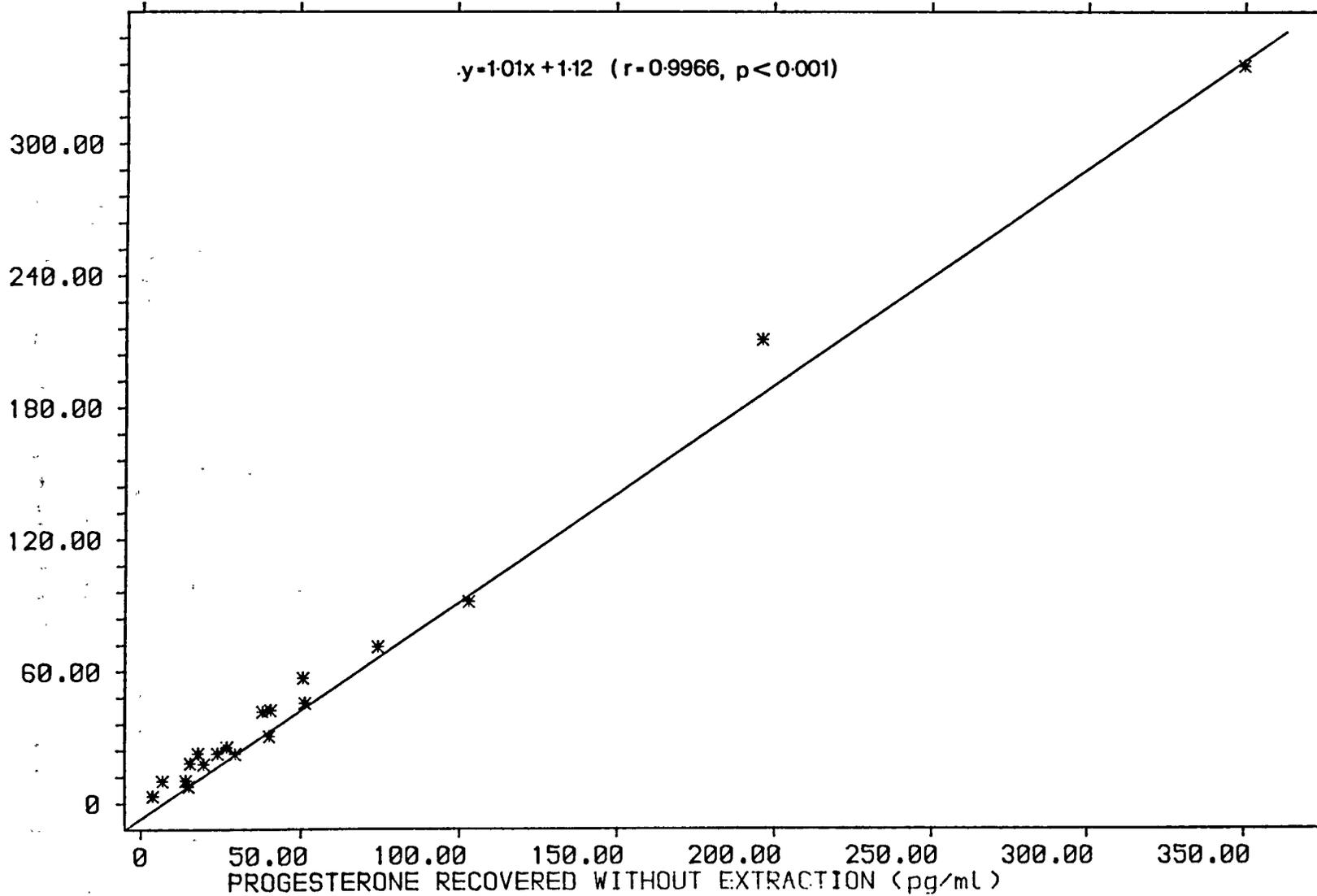
The results of all the measured variables are represented as geometric means for each day. For results represented in histogram form, where appropriate the standard error of the mean (sem) is illustrated as scaled bars on each histogram block.

An initial analysis to determine linear and quadratic trends within the data showed that the majority of variation could be accounted for by a linear fit. Hence, to determine significant relationships between variables within days, the relevant data were subjected to multiple linear correlation and regression analysis. However, since the granulosa cells were used to assess either  $^{125}$ I-hCG binding (see Section II(c)) or aromatase activity (see Section II(d)) and the theca tissue used to determine  $^{125}$ I-hCG binding (see Section II(c)) or testosterone content (see Section II(e)ii), it was statistically impossible to regress/correlate BG and

**Figure 2.2** A comparison of progesterone measurements assayed in porcine follicular fluid with and without extraction with diethyl ether

PROGESTERONE RECOVERED WITH AND WITHOUT EXTRACTION (pg/ml)

PROGESTERONE RECOVERED BY EXTRACTION  
pg/ml



BT against AR or THT and vice versa. For clarity, individual data points have been omitted from the figures which depict regression lines of best fit between variables for each day. The equation of each line and the standard error of the slope are shown.

Significant differences of individual variables between days were analysed by one way analysis of variance (ANOVA) using a Genstat computer program on the Nottingham University ICL 2900 mainframe computer. Once analysis of variance had demonstrated a significant effect, individual means were compared by the students 't' test.

The data in Table 2.2 are presented for visual appraisal only. It was not possible to statistically analyse this data because of the large variation in the number of follicles/category/day.

#### IV RESULTS

##### a) Morphological Data

##### i) Follicle Number, Diameter (FD) and Volume (FV)

Overall, dissected follicles ( $n = 511$ ) ranged from 2.0 to 10.5 mm in diameter with a progressive increase in size from d16 to d21. Within days there was a greater distribution of size in d18 (2.0 mm  $\rightarrow$  8.2 mm) and d20 animals (2.5 mm  $\rightarrow$  9.00 mm) (Fig. 2.3). An inverse relationship ( $p < 0.001$ ) existed between groups for the mean FD (d16, 4.0 mm ( $n = 180$ ); d18, 4.9 mm ( $n = 164$ ); d20, 5.5 mm ( $n = 110$ ); d21, 8.8 mm ( $n = 57$ )) and the mean total number of follicles recovered/animal/group (Fig. 2.3: d16, 36; d18, 33; d20, 22; d21, 11).

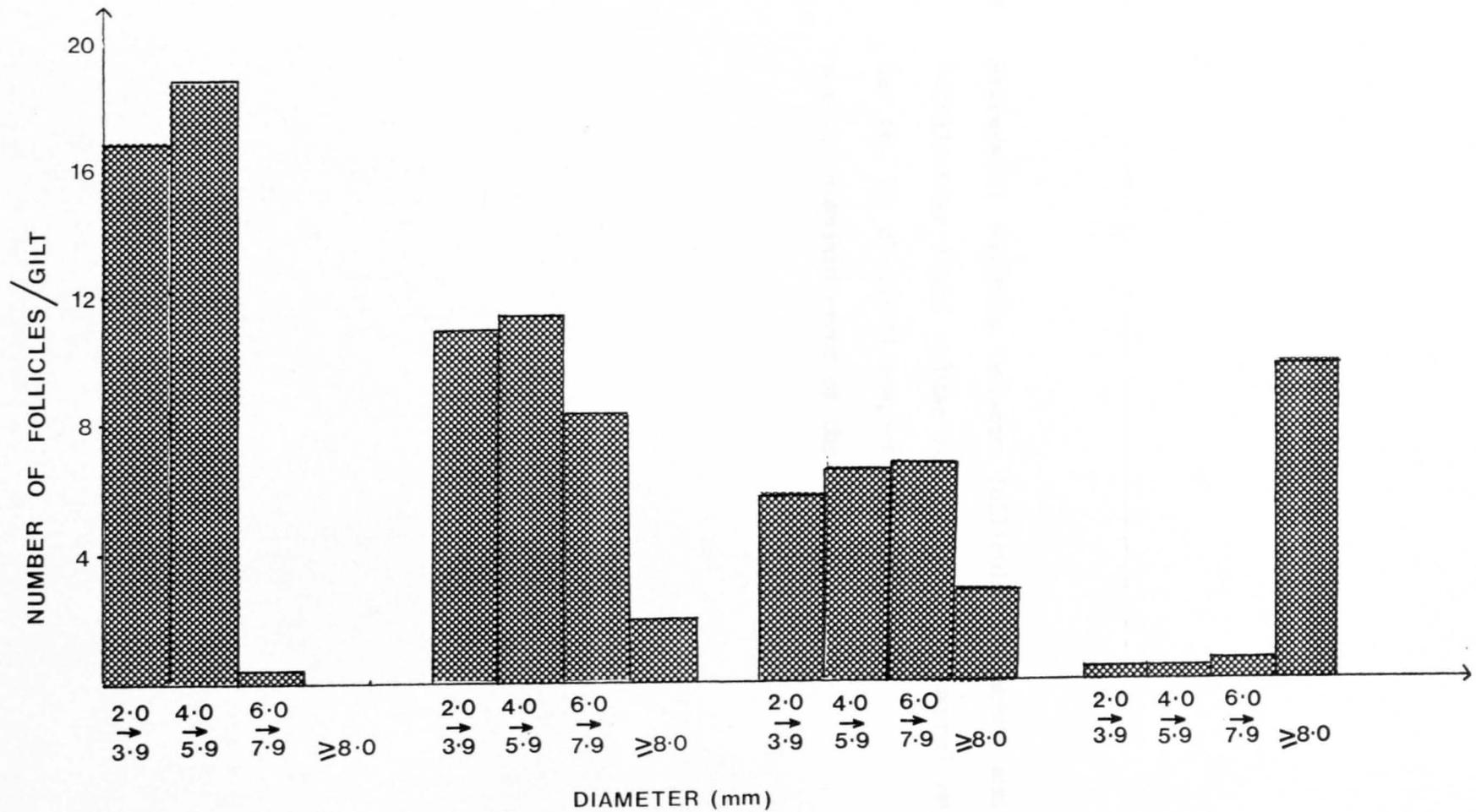
A highly significant ( $p < 0.001$ ) positive relationship existed between FD and FV for all days (for regression analysis see Fig. 2.4) and for the combined data from all gilts ( $r = 0.98$ ,  $df = 440$ ,  $p < 0.001$ ; see Fig. 2.5:  $FD = 1.397541 \times \sqrt[3]{v} + 0.069$  where  $v =$  volume of follicular fluid). The follicular tissue volume (TV; see Fig. 2.5) i.e. the difference between the theoretical volume (assuming the volume of a sphere,  $V = \frac{4}{3}\pi r^3$ ) and the actual recorded volume of fluid, in follicles of 4, 6 and 8 mm diameter was 0.42, 0.53 and 0.75 mm<sup>3</sup> respectively. This volume increased dramatically on Day 21 to 1.66 mm<sup>3</sup> in follicles of 10 mm diameter.

The follicular fluid from the animals ( $n = 3$ ) initially slaughtered on d22 was extremely viscous and difficult to remove from the intact follicle. A histological examination of these follicles revealed a considerable infolding of both the granulosa and theca layers with a concomitant decrease in follicular fluid volume (Plate 1). As a consequence of this, slaughter on d22 was discontinued and animals were subsequently slaughtered on the first day of oestrus, d21.

#### ii) Number of Granulosa Cells (NG)

Mean NG/follicle  $\times 10^4$  was 121.4, 145.7, 148.2 and 377.6 for d16, d18, d20 and d21, respectively (Table 2.1). Correlation analysis of NG with FD established a highly significant positive relationship overall (Fig. 2.6:  $r = 0.68$ ,  $d.f. = 465$ ,  $p < 0.001$ ) and in follicles from d16 ( $r = 0.71$ ,  $d.f. = 159$ ,  $p < 0.001$ ), d18 ( $r = 0.61$ ,  $d.f. = 125$ ,  $p < 0.001$ ) and d20 ( $r = 0.45$ ,  $d.f. = 44$ ,  $p < 0.001$ ) however, within follicles on d21, a less significant

**Figure 2.3** Histogram showing the range of follicular diameters and the mean number of follicles recovered/gilt on day 16, 18, 20 and 21 respectively.



DAY 16

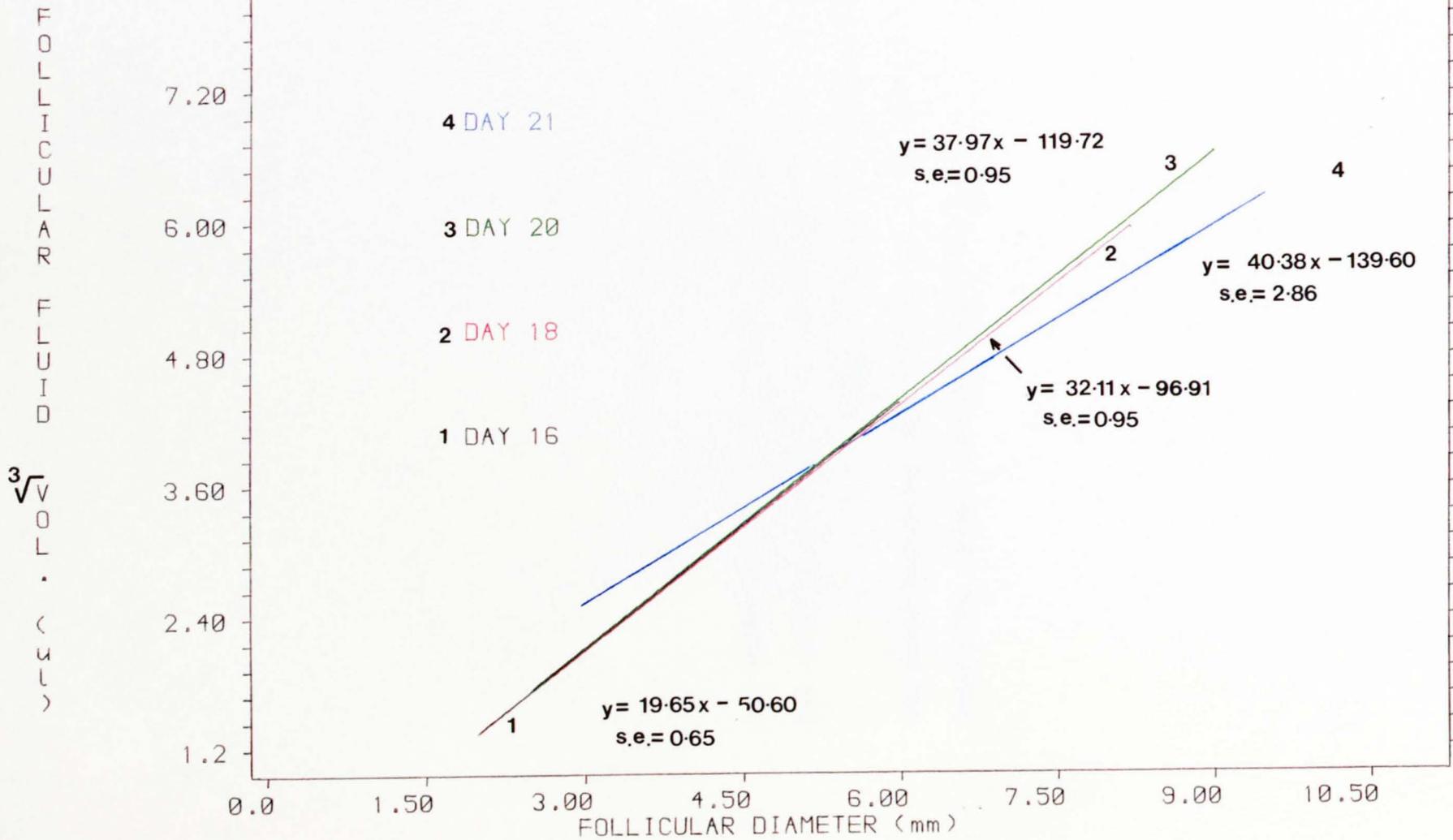
DAY 18

DAY 20

DAY 21

**Figure 2.4** Regression analysis between follicular diameter and  $\sqrt[3]{\text{follicular fluid volume}}$  for follicles recovered on day 16, 18, 20 and 21 respectively.  
s.e. ; standard error of the slope.

EXPT1:REGRESSION ANALYSIS. DIAM. (mm)  $\sqrt[3]{\text{VOL}}$  ( $\mu\text{L}$ )

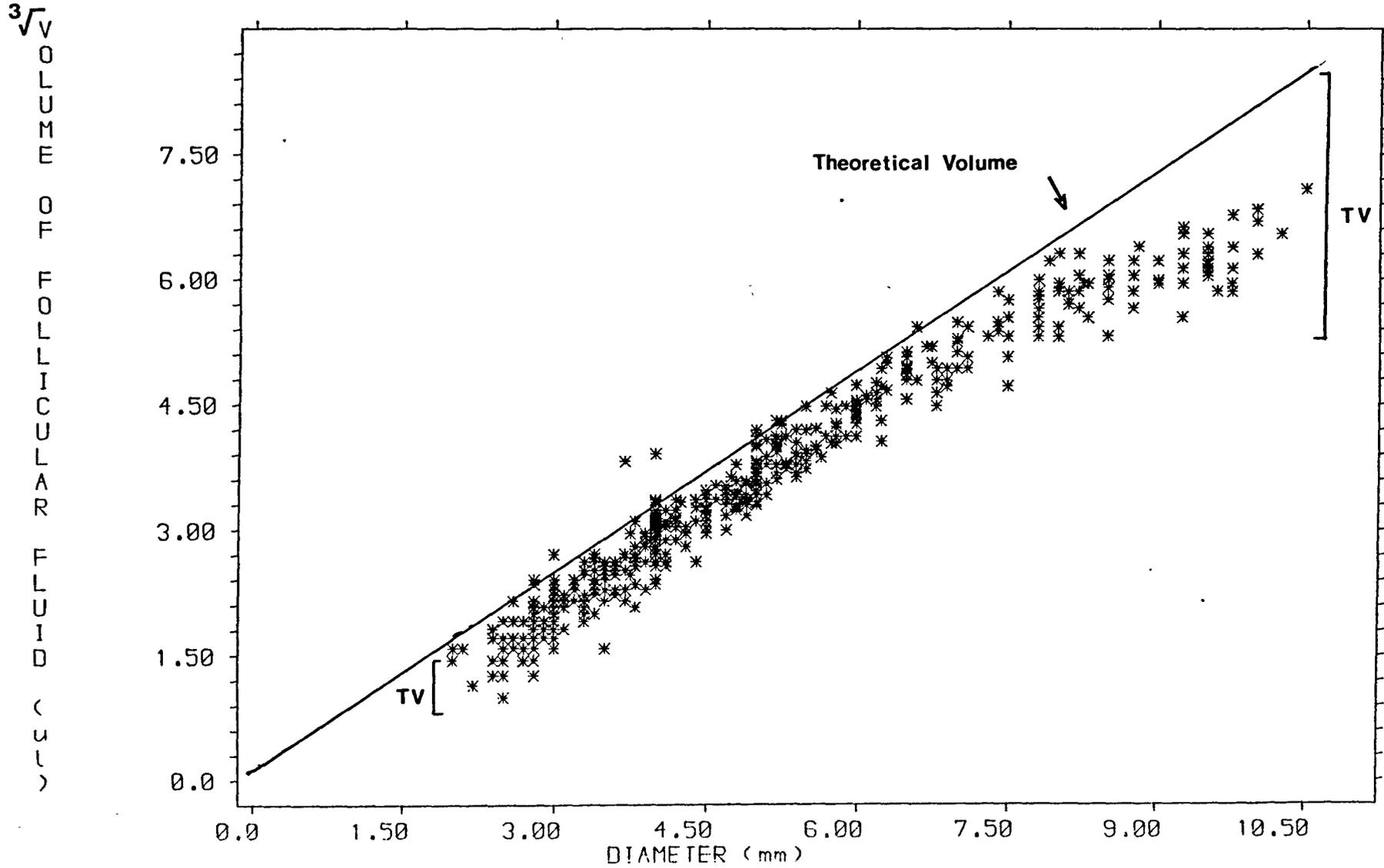


**Figure 2.5** The overall relationship between total follicular volume and the volume of aspirated follicular fluid for the combined data from all days.

Each follicle was assumed to be spheroid in shape and hence the theoretical volume was calculated using the formula  $V = \frac{4}{3}\pi r^3$ .

T.V. ; estimated tissue volume.

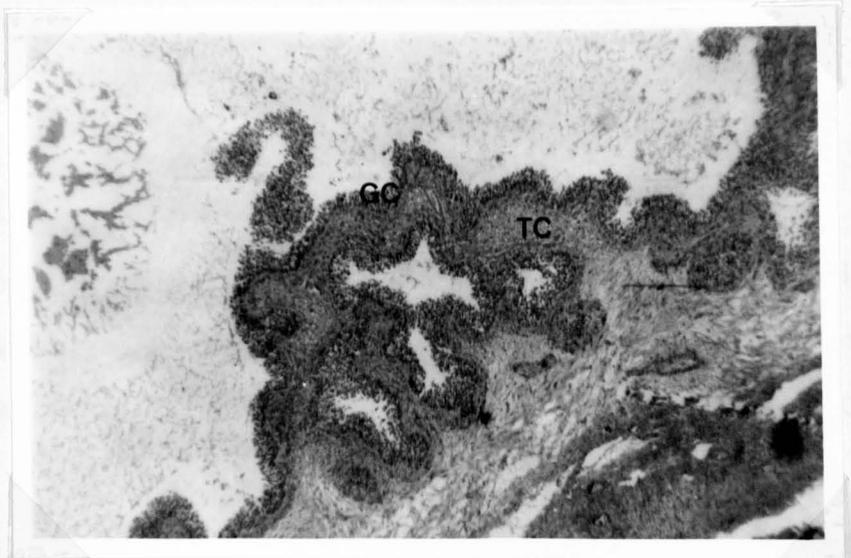
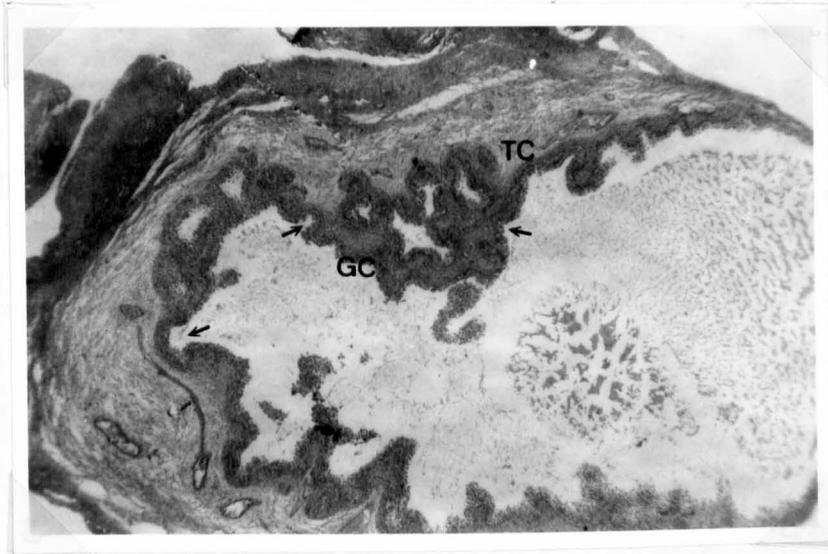
DIAMETER (mm)  $\sqrt[3]{}$  VOLUME OF FOLLICULAR FLUID ( $\mu$ l)



**Plate 1** Histological section through follicles recovered from a gilt slaughtered on Day 22. The arrows are pointing to the considerable infolding and thickening of the granulosa and theca layers.

GC ; Granulosa cell layer

TC ; Theca layer.



( $r = 0.37$ , d.f. = 44,  $p < 0.05$ ) relationship was evident. The small follicles (2.0-3.5 mm) of d20 and d21 contained many fragmented cells.

b) Biochemical Data

i) Aromatase Activity (AR)

The mean AR (ng  $E_2$ /2h/follicle) for each day is shown in Table 2.1. Maximum activity occurred on d21 (4.65) and minimum activity on d16 (1.13). Subdivision of the follicles into four diameter classes (Table 2.2) showed that the largest follicles/day possessed the greatest enzyme activity. As expected AR was significantly correlated to FFE for all groups (d16,  $r = 0.61$ , d.f. = 66,  $p < 0.001$ ; d18,  $r = 0.44$ , d.f. = 60,  $p < 0.001$ ; d20,  $r = 0.68$ , d.f. = 41,  $p < 0.001$ ; d21,  $r = 0.39$ , d.f. = 24,  $p < 0.05$ : for regression analysis see Fig. 2.7) and to FD and FV ( $p < 0.001$ ) for d18 ( $r = 0.66$ , d.f. = 60;  $r = 0.63$ , d.f. = 60) and d20 ( $r = 0.71$ , d.f. = 41;  $r = 0.74$ , d.f. = 41). AR was correlated to FFT for d20 only ( $r = 0.64$ , d.f. = 41,  $p < 0.001$ ).

Analysis of variance between mean AR/day showed a highly significant difference between all days except for d16 and d20 (d16 v d18, d16 v d21, d18 v d20 and d20 v d21;  $p < 0.001$ : d18 v d21,  $p < 0.05$ ).

ii)  $^{125}I$ -hCG binding to a) Granulosa Cells (BG) and b) Theca Tissue (BT)

The mean BG (cpm/follicle  $\times 10^3$ )/day is shown in Table 2.1. Maximum binding of 82.9 was observed on d20 and a minimum of 21.2 on

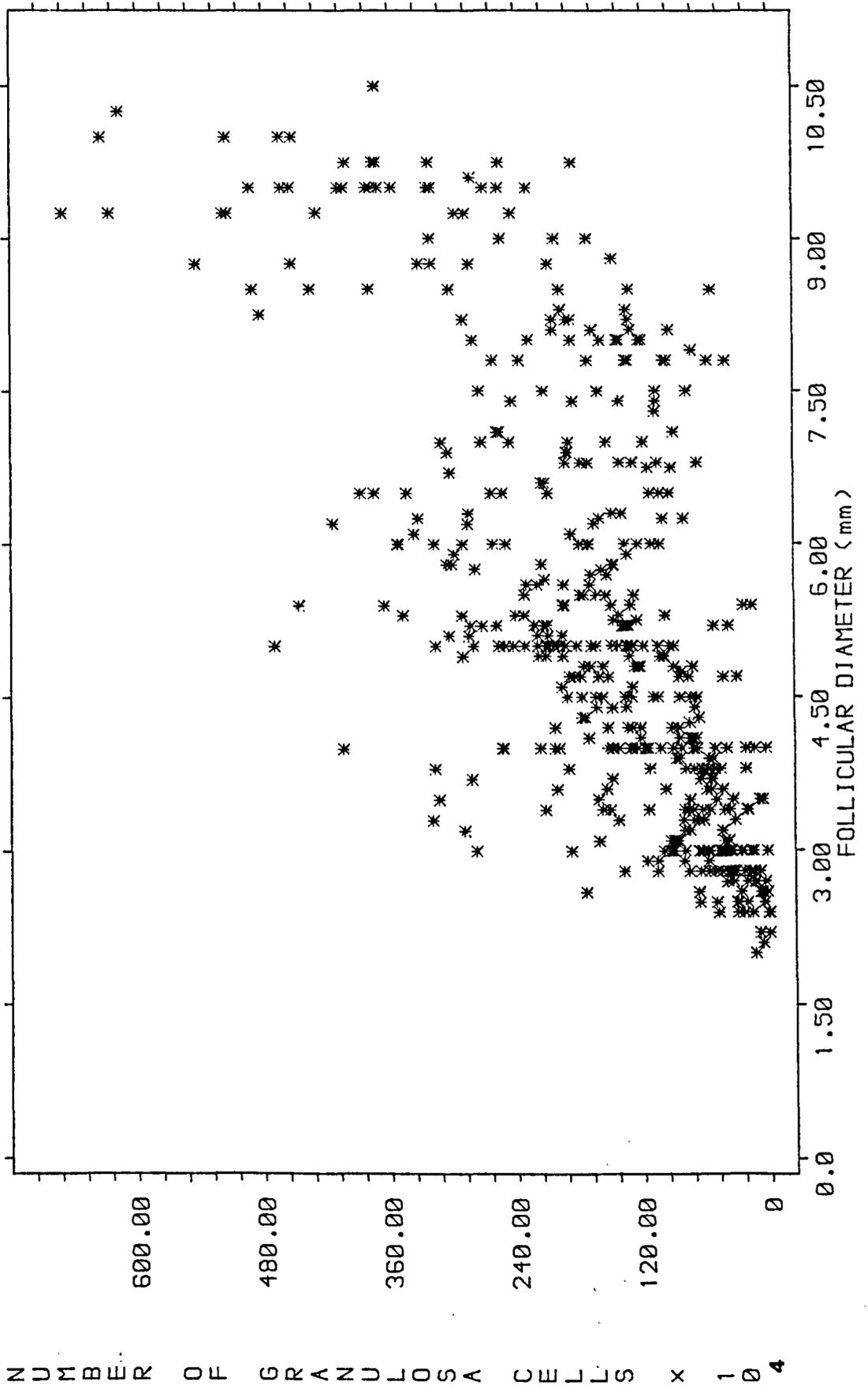
Table 2.1 Mean follicular diameter (mm), number of granulosa cells ( $\times 10^4$ ),  $^{125}\text{I}$ -hCG binding to granulosa and theca cells ( $\text{cpm} \times 10^3$ ), follicular fluid oestradiol, testosterone and progesterone concentrations (ng/ml), concentration of testosterone in thecal tissue (ng/foll) and *in vitro* aromatase enzyme activity ( $\text{ngE}_2/2\text{h/foll}$ ) recorded for day 16, 18, 20 and 21 respectively.

Variables measured	Day 16 ( $\underline{n}=180$ )	Day 18 ( $\underline{n}=164$ )	Day 20 ( $\underline{n}=110$ )	Day 21 ( $\underline{n}=57$ )	SED
Diam. (mm)	3.99	4.91	5.50	8.80	0.47
No. of granulosa cells/follicles ( $\times 10^4$ )	121.4	145.7	148.2	377.6	44.2
granulosa cell aromatase activity (ng oestradiol/2h/follicle)	1.13	3.70	1.73	4.65	2.04
$^{125}\text{I}$ -labelled hCG binding to granulosa cells (cpm bound $\times 10^{-3}$ /follicle)	21.2	52.1	82.9	59.7	23.5
$^{125}\text{I}$ -labelled hCG binding to theca tissue (cpm bound $\times 10^{-3}$ /follicle)	7.7	24.2	38.8	53.0	14.3
oestradiol in follicular fluid (ng/ml)	22.0	110.6	235.5	102.6	89.8
testosterone in follicular fluid (ng/ml)	22.3	55.8	75.7	34.3	31.4
progesterone in follicular fluid (ng/ml)	102.1	83.4	76.2	497.2	175.8
testosterone content of theca tissue (ng/follicle)	0.06	0.69	1.76	2.12	1.36

S.E.D. = standard error of difference for comparing between means

**Figure 2.6** The relationship between follicular diameter and granulosa cell numbers for the combined data from all days

EXPT 1: FOLL. DIAM(mm) v NUMBER OF GRANULOSA CELLS x 10<sup>4</sup>



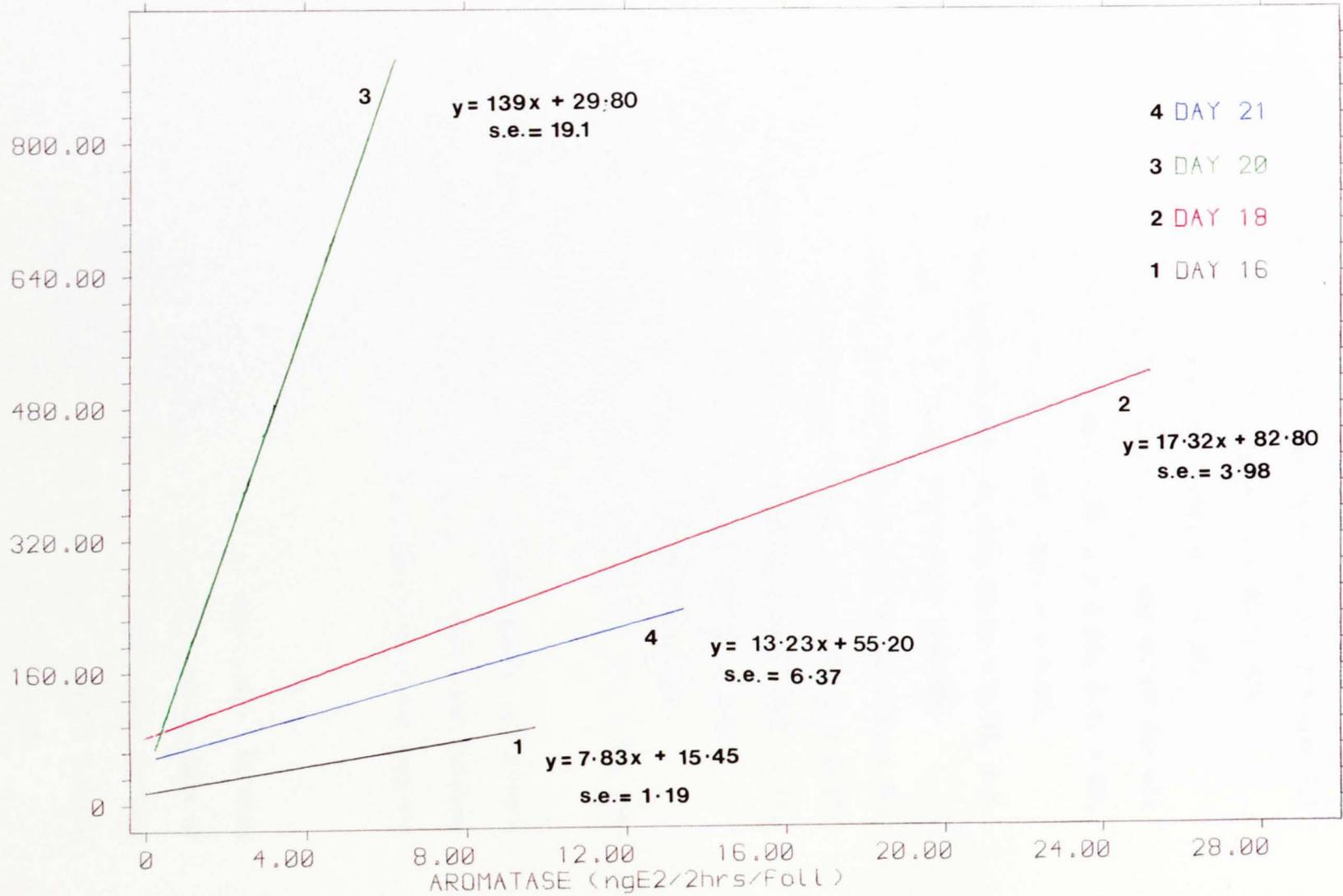
**Table 2.2** Mean aromatase activity in granulosa cells (ng oestradiol/2h/follicle),  $^{125}\text{I}$ -labelled hCG binding to granulosa and theca tissue (cpm bound  $\times 10^3$ /follicle) and follicular fluid concentrations (ng/ml) of oestradiol, testosterone and progesterone of follicular populations recovered from day 16, 18, 20 and 21 respectively following subdivision into four restricted diameter classes.

Day of Cycle	FOLLICULAR DIAMETER (mm)			
	2.0-3.9	4.0-5.9	6.0-7.9	$\geq 8.0$
16	n=84	n=94	n=2	
aromatase activity	0.3	1.6	7.3	
granulosa binding	7.4	30.4	131.5	
theca binding	4.7	10.2	13.1	
oestradiol	14.5	27.5	81.8	
testosterone	30.8	15.7	25.7	
progesterone	118.7	89.4	110.8	
18	n=55	n=57	n=42	n=10
aromatase activity	0.2	1.3	6.2	10.7
granulosa binding	9.2	28.8	119.3	144.0
theca binding	6.7	16.1	45.2	95.9
oestradiol	6.7	33.4	243.4	514.3
testosterone	22.0	42.1	94.9	127.8
progesterone	69.2	44.2	124.9	169.0
20	n=29	n=33	n=34	n=14
aromatase activity	0.1	1.1	2.6	3.8
granulosa binding	10.9	26.9	163.2	157.7
theca binding	9.3	24.0	56.3	95.3
oestradiol	5.2	64.9	372.2	782.8
testosterone	19.0	37.4	98.5	213.1
progesterone	67.3	34.6	82.1	180.1
21	n=2	n=2	n=2	n=49
aromatase activity	-	-	0.2	5.1
granulosa binding	6.6	13.9	57.4	63.5
theca binding	5.7	4.3	35.8	58.3
oestradiol	3.0	2.4	38.2	116.7
testosterone	12.3	8.3	21.8	38.4
progesterone	73.6	63.2	92.0	501.5

**Figure 2.7** Regression analysis between *in vitro* aromatase enzyme activity (ng E<sub>2</sub>/2h/foll) and the concentration of follicular fluid oestradiol (ng/ml) for days 16, 18, 20 and 21 respectively).  
s.e. ; standard error of the slope.

EXPT1:REGRESSION ANALYSIS. AR (ngE2/2hrs/Foll) v FF E2(ng/ml)

FOLLICULAR FLUID E2 (ng/ml)



d16. Similar binding of 52.1 and 59.7 was recorded for d18 and d21 respectively. BG was significantly correlated with NG on d16 ( $r = 0.49$ , d.f. = 68,  $p < 0.001$ ), d18 ( $r = 0.58$ , d.f. = 57,  $p < 0.001$ ) and d21 ( $r = 0.58$ , d.f. = 21,  $p < 0.01$ ) and to BT for all days (d16,  $r = 0.39$ , d.f. = 68,  $p < 0.001$ ; d18,  $r = 0.63$ , d.f. = 57,  $p < 0.001$ ; d20,  $r = 0.62$ , d.f. = 45,  $p < 0.001$ ; d21,  $r = 0.62$ , d.f. = 21,  $p < 0.01$ ). BG was also correlated with FD ( $r = 0.70$ , d.f. = 68,  $p < 0.001$ ), FV ( $r = 0.80$ , d.f. = 68,  $p < 0.001$ ) and FFE ( $r = 0.48$ , d.f. = 68,  $p < 0.001$ ) for d16 and FD ( $r = 0.78$ , d.f. = 57;  $r = 0.77$ , d.f. = 45,  $p < 0.001$ ), FV ( $r = 0.79$ , d.f. = 57;  $r = 0.77$ , d.f. = 45,  $p < 0.001$ ), FFE ( $r = 0.66$ , d.f. = 57;  $r = 0.61$ , d.f. = 45,  $p < 0.001$ : for regression analysis see Fig. 2.8), FFT ( $r = 0.29$ , d.f. = 57,  $p < 0.05$ ;  $r = 0.63$ , d.f. = 45,  $p < 0.001$ ) and FFP ( $r = 0.57$ , d.f. = 57,  $p < 0.001$ ;  $r = 0.32$ , d.f. = 45,  $p < 0.05$ ) for d18 and d20 respectively.

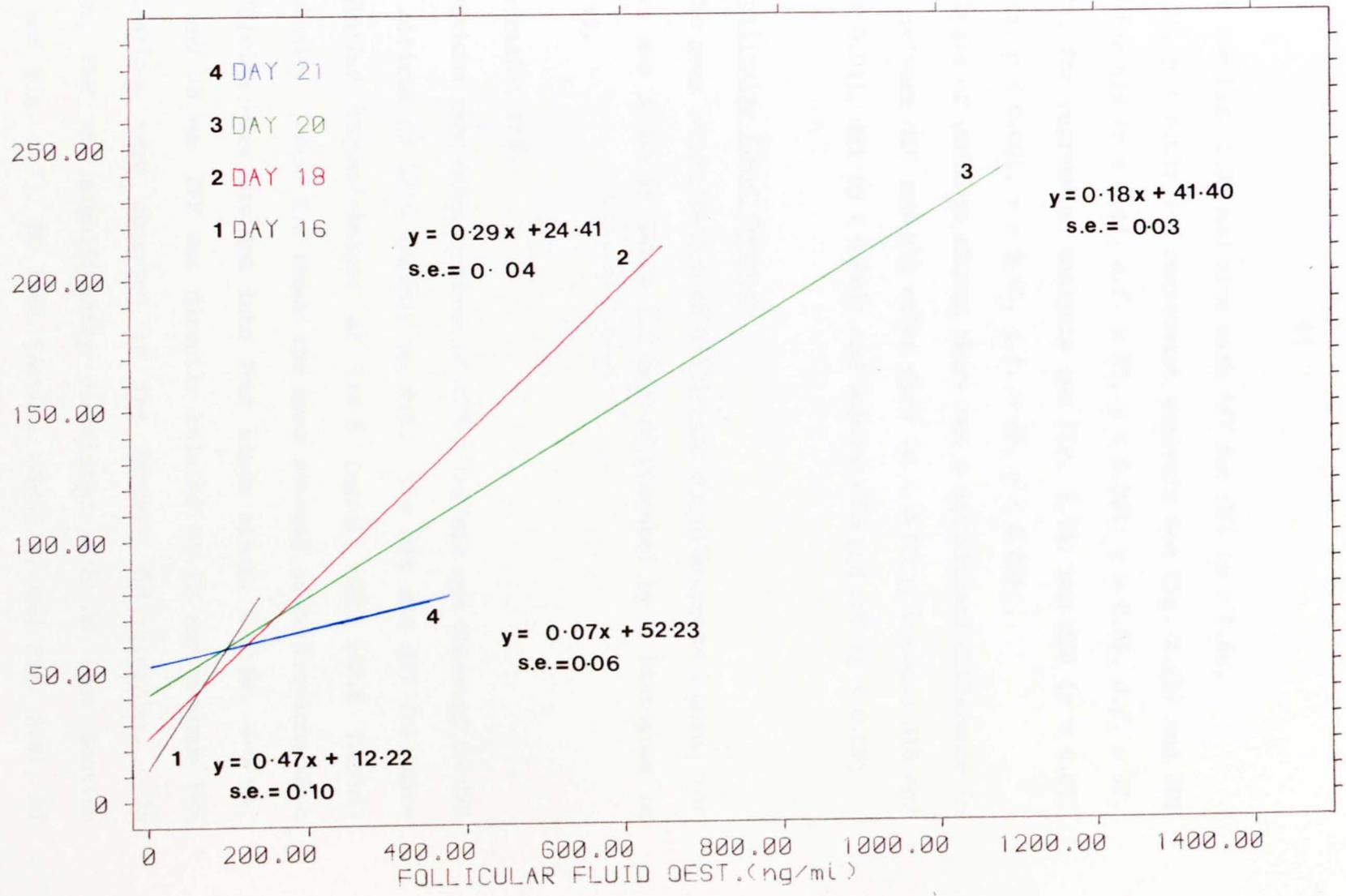
Analysis of variance showed there was a significant difference between mean BG for d16 and all other days ( $p < 0.001$ ) and between d18 and d20 ( $p < 0.001$ ). There was no significant difference between d20 and d21.

b) The mean BT (cpm  $\times 10^3$ /follicle) is shown in Table 2.1. Binding increased progressively from a minimum of 7.7 on d16 to a maximum of 53.0 on d21. BT was significantly correlated with BG (see Section IVb(ii)a) and FFE for all groups (d16,  $r = 0.28$ , d.f. = 68,  $p < 0.05$ ; d18,  $r = 0.75$ , d.f. = 57,  $p < 0.001$ ; d20,  $r = 0.83$ , d.f. = 45,  $p < 0.001$ ; d21,  $r = 0.53$ , d.f. = 21,  $p < 0.05$ : for regression

Figure 2.8      Regression analysis between follicular fluid oestradiol concentration (ng/ml) and  $^{125}\text{I}$ -hCG binding to granulosa cells (cpm  $\times 10^3$ ) for day 16, 18, 20 and 21.  
s.e. ; standard error of the slope.

GRANULOSA BINDING (CPM X 10<sup>-3</sup> / FOLL)

EXPT1:REGRESSION ANALYSIS. FF E2(ng/ml) v GRAN.BIND(cpm x 10<sup>-3</sup>/FOLL)



analysis see Fig. 2.9) and also with FFT for d21 ( $r = 0.48$ , d.f. = 21,  $p < 0.05$ : for regression analysis see Fig. 2.10) and FFT and FFP for d18 ( $r = 0.47$ , d.f. = 57,  $p < 0.001$ ;  $r = 0.37$ , d.f. = 57,  $p < 0.01$ : for regression analysis see Fig. 2.14) and d20 ( $r = 0.69$ , d.f. = 48,  $p < 0.001$ ;  $r = 0.49$ , d.f. = 45,  $p < 0.001$ ).

Analysis of variance showed there was a significant difference in mean BT between d16 and all other days ( $p < 0.001$ ), between d18 and d20 ( $p < 0.01$ ), d21 ( $p < 0.001$ ) and between d20 and d21 ( $p < 0.05$ ).

### iii) Follicular Fluid Steroids

The mean concentration of follicular fluid steroids (ng/ml) for all days are shown in Table 2.1 and represented by a histogram in Fig. 2.11.

#### a) Oestradiol $17\beta$

A maximum mean concentration of 235.5 (ng/ml) was observed on d20 with a minimum of 22.0 (ng/ml) on d16. The d18 and d21 follicles have similar concentrations of 110.6 (ng/ml) and 102.6 (ng/ml) respectively. Table 2.2 shows the mean steroid concentrations when the follicles were divided into four class sizes; 2-4 mm, 4-6 mm, 6-8 mm and >8 mm. FFE was directly related to FD and maximum FFE concentrations were observed in the largest follicles/day. In addition, FFE was significantly correlated with AR (see Section IVb(i) and Fig. 2.7), BG (see Section IVb(ii)a) and Fig. 2.8), BT (see Section IVb(ii)b) and Fig. 2.9), FFT ( $r = 0.56$ , d.f. = 159,  $p < 0.001$ ; for regression analysis see Fig. 2.12) and THT for d16 ( $r = 0.41$ , d.f. = 66,  $p < 0.001$ ) and also with FFP (for regression

**Figure 2.9** Regression analysis between follicular fluid oestradiol concentrations (ng/ml) and  $^{125}\text{I}$ -hCG binding to theca cells (cpm  $\times 10^3$ ) for day 16, 18, 20 and 21 respectively.

s.e. ; standard error of the slope.

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EXPT1:REGRESSION ANALYSIS. FF E2 (ng/ml) v BT (cpm x 10<sup>-3</sup>/FOLL)

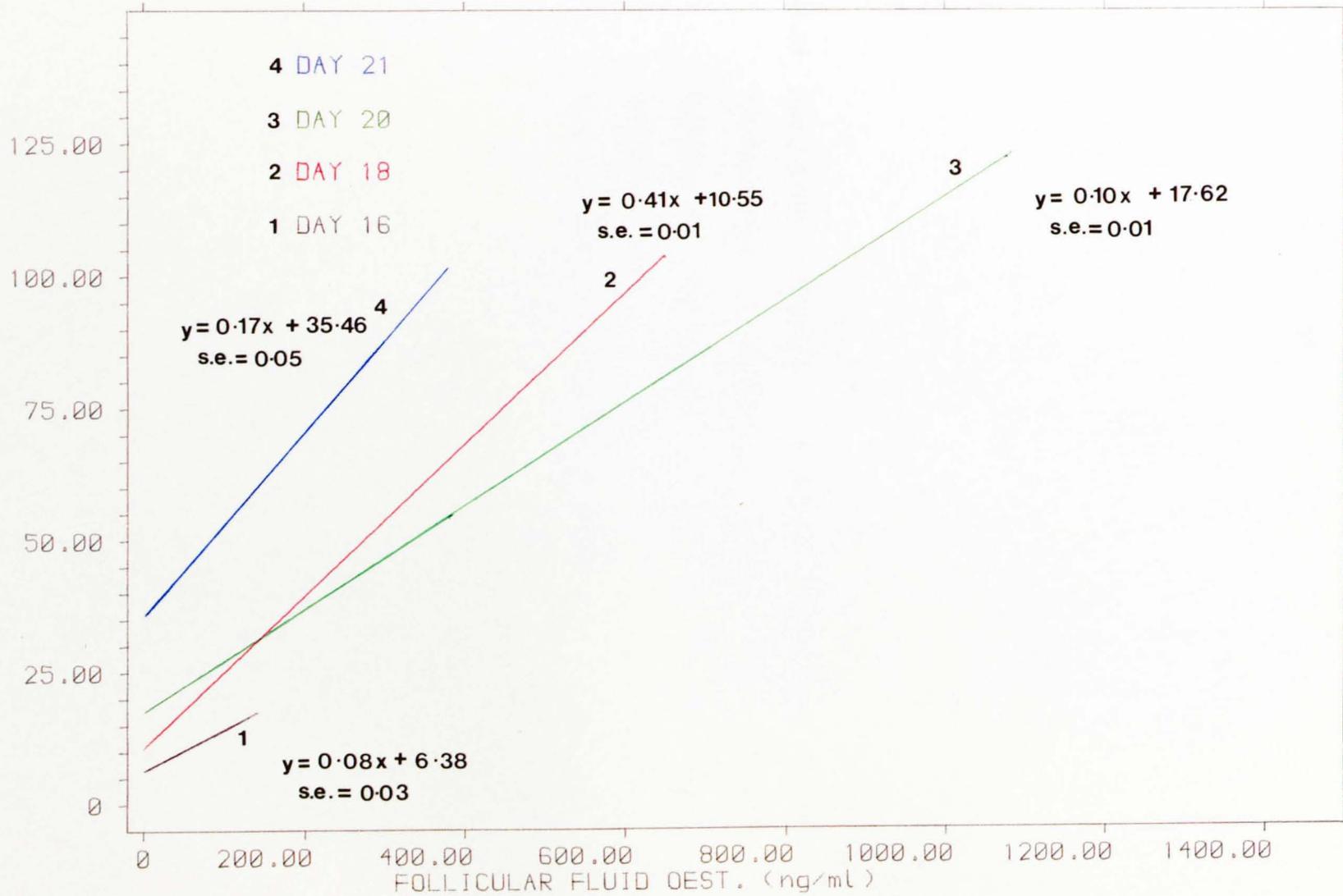
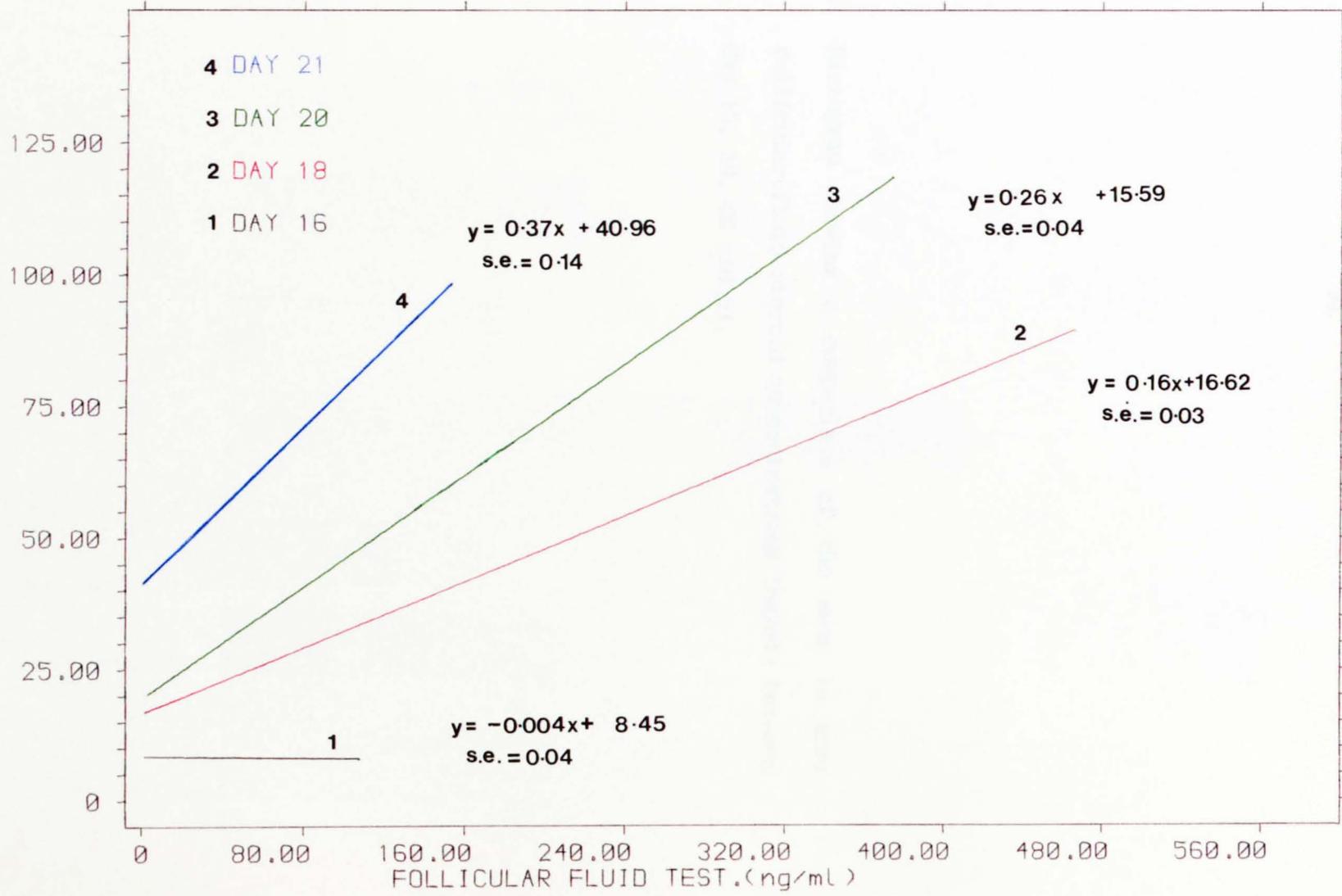


Figure 2.10 Regression analysis between follicular fluid testosterone concentrations (ng/ml) and  $^{125}\text{I}$ -hCG binding to theca cells on day 16, 18, 20 and 21 respectively.

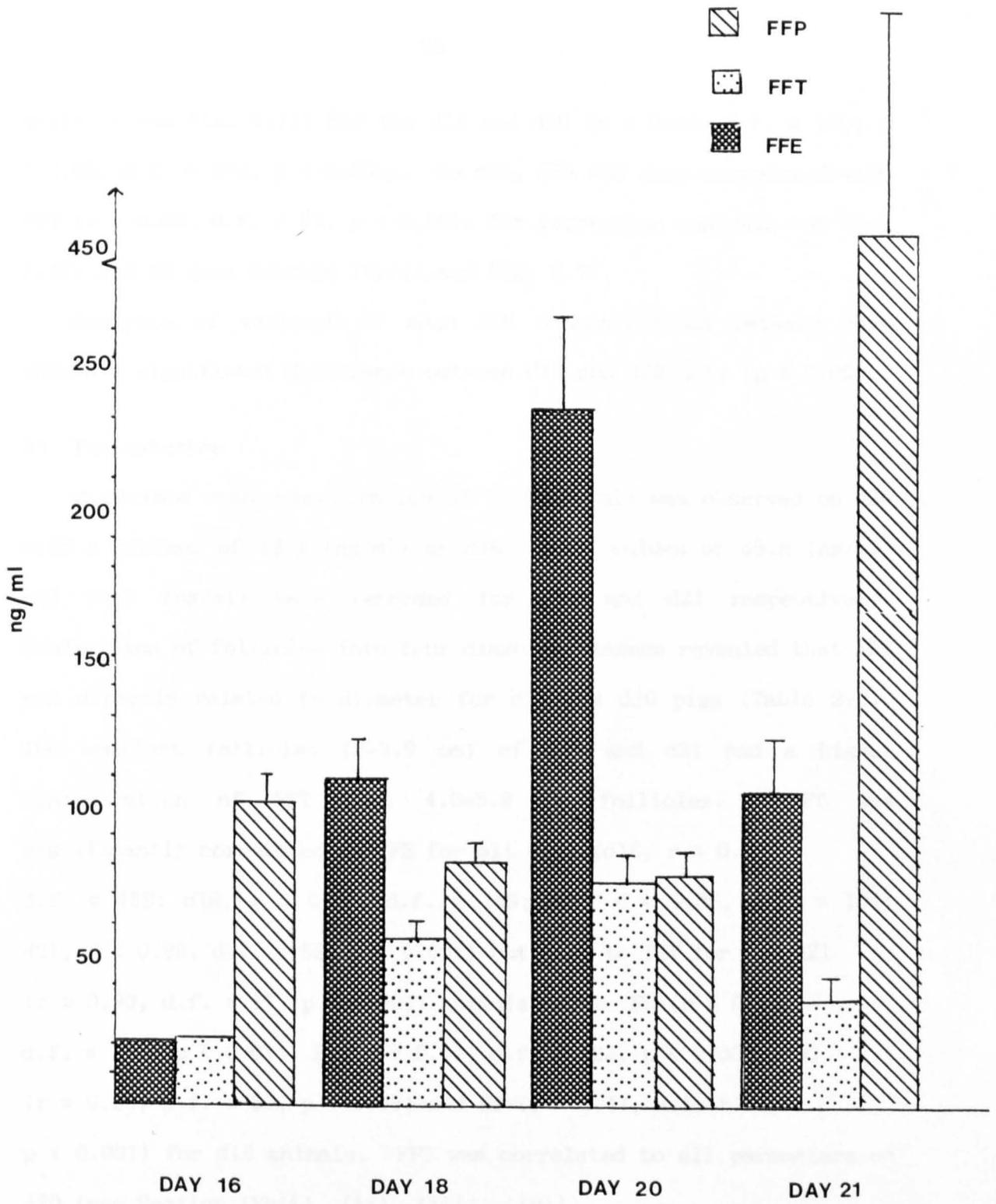
s.e. ; standard error of the slope.

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EXPT1:REGRESSION ANALYSIS. FF T(ng/ml) v BT(cpm x 10<sup>3</sup>/FOLL)



**Figure 2.11** Histogram showing a comparison of the mean ( $\pm$  sem) follicular fluid steroid concentrations (ng/ml) between day 16, 18, 20 and 21.



$p < 0.001$  for all values. FFT was correlated to all parameters ( $r = 0.70$ ,  $r = 0.65$ ,  $r = 0.65$ ,  $r = 0.65$  respectively).

Analysis of variance of mean FFT between days revealed a significant difference between day 16 and 20 ( $p < 0.05$ ) and 20 and 21 ( $p < 0.01$ ) respectively.

analysis see Fig. 2.11) for the d18 and d20 ( $r = 0.49$ , d.f. = 150;  $r = 0.68$ , d.f. = 104,  $p < 0.001$ ). On d21, FFE was only correlated with FFT ( $r = 0.88$ , d.f. = 52,  $p < 0.001$ : for regression analysis see Fig. 2.12) and AR (see Section IVb(i) and Fig. 2.7).

Analysis of variance of mean FFE concentrations between days showed a significant difference between d16 and d20 only ( $p < 0.05$ ).

#### b) Testosterone

A maximum mean concentration of 75.7 (ng/ml) was observed on d20 with a minimum of 22.3 (ng/ml) on d16. Mean values of 55.8 (ng/ml) and 34.3 (ng/ml) were recorded for d18 and d21 respectively. Subdivision of follicles into four diameter classes revealed that FFT was directly related to diameter for d18 and d20 pigs (Table 2.2). The smallest follicles (2-3.9 mm) of d16 and d21 had a higher concentration of FFT than 4.0-5.9 mm follicles. FFT was significantly correlated to FFE for all days (d16,  $r = 0.56$ , d.f. = 159; d18,  $r = 0.62$ , d.f. = 149; d20,  $r = 0.86$ , d.f. = 104; d21,  $r = 0.88$ , d.f. = 52:  $p < 0.001$ ) but also to THT for the d21 ( $r = 0.93$ , d.f. = 27,  $p < 0.001$ ) animals and to FD ( $r = 0.40$ , d.f. = 122,  $p < 0.01$ ), FV ( $r = 0.41$ , d.f. = 122,  $p < 0.001$ ), BG ( $r = 0.29$ , d.f. = 57,  $p < 0.05$ ) and BT ( $r = 0.47$ , d.f. = 57,  $p < 0.001$ ) for d18 animals. FFT was correlated to all parameters on d20 (see Section IVb(i), (ii), (iii), (iv)).

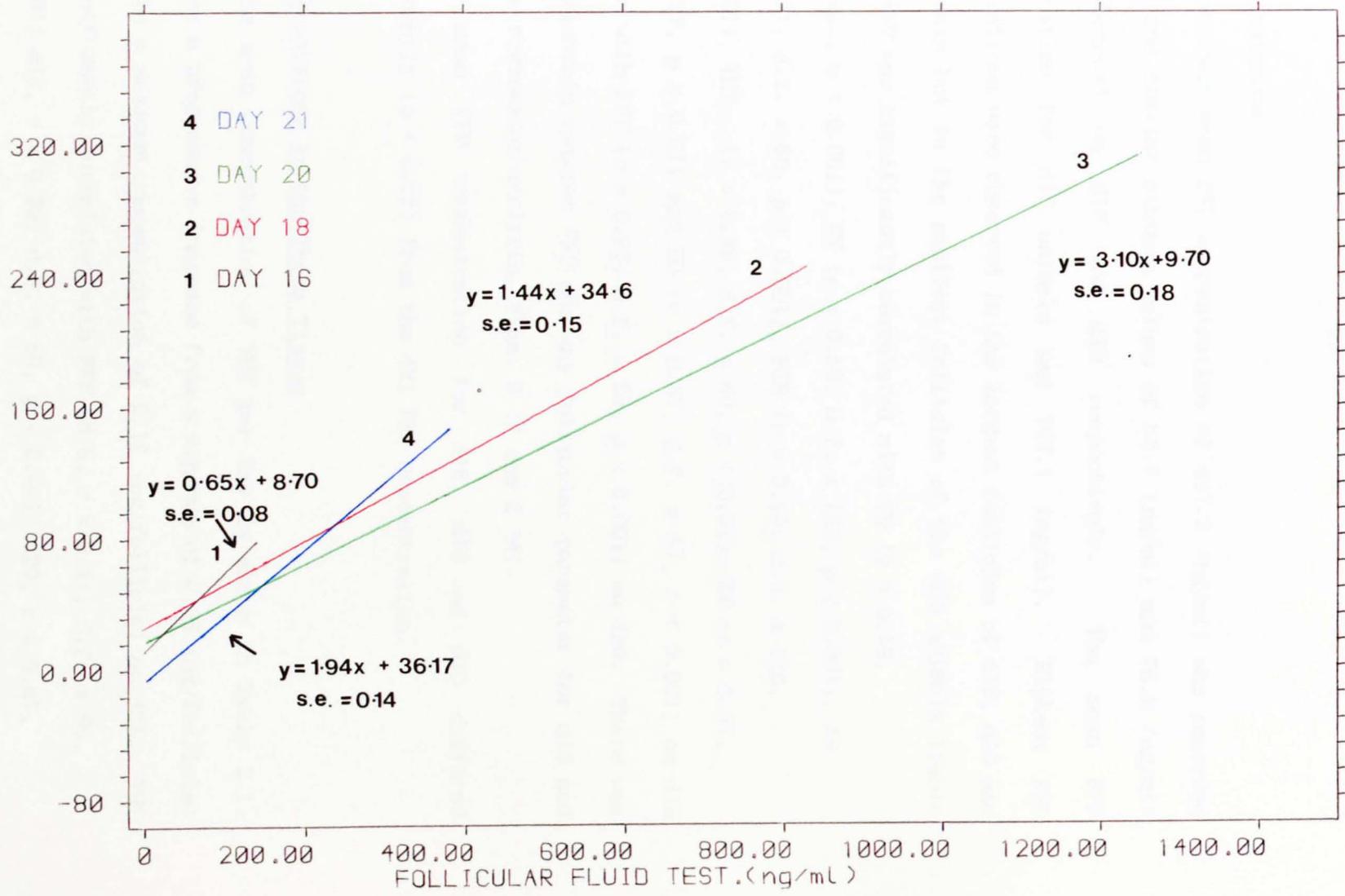
Analysis of variance of mean FFT between days revealed a significant difference between d16 and d18 ( $p < 0.05$ ) and d20 ( $p < 0.01$ ) respectively.

Figure 2.12 Regression analysis between follicular fluid testosterone (ng/ml) and follicular fluid oestradiol (ng/ml) concentrations on day 16, 18, 20 and 21 respectively.

s.e. ; standard error of the slope.

EXPT1:REGRESSION ANALYSIS. FF T(ng/ml) v FF E2(ng/ml)

FOLLICULAR FLUID TEST.(ng/ml)



### c) Progesterone

A maximum mean FFP concentration of 497.2 (ng/ml) was recorded for d21 and similar minimum values of 83.4 (ng/ml) and 76.2 (ng/ml) were observed on d18 and d20 respectively. The mean FFP concentration for d16 animals was 102.1 (ng/ml). Highest FFP concentrations were observed in the largest follicles of d18, d20 and d21 animals but in the smallest follicles of the d16 animals (Table 2.2). FFP was significantly correlated with FD ( $r = 0.49$ , d.f. = 122,  $p < 0.001$ ), FV ( $r = 0.50$ , d.f. = 122,  $p < 0.001$ ), AR ( $r = 0.71$ , d.f. = 60,  $p < 0.001$ ), FFE ( $r = 0.49$ , d.f. = 150,  $p < 0.001$ ), THT ( $r = 0.36$ , d.f. = 60,  $p < 0.01$ ), BT ( $r = 0.37$ , d.f. = 57,  $p < 0.01$ ) and BG ( $r = 0.57$ , d.f. = 57,  $p < 0.001$ ) on d18 and also with FFT ( $r = 0.61$ , d.f. = 98,  $p < 0.001$ ) on d20. There was no relationship between FFP and any follicular parameter for d16 and d21 (see regression analysis, Figs. 2.13 and 2.14).

The mean FFP concentration for d16, d18 and d20 differed significantly ( $p < 0.05$ ) from the d21 FFP concentration.

### iv) Testosterone in the Theca Tissue

The mean concentration of THT per day is shown in Table 2.1. There was a progressive increase from a minimum of 0.06 (ng/follicle) on d16 to a maximum concentration of 2.12 (ng/follicle) on d21. THT was significantly correlated with FFE (d16,  $r = 0.41$ , d.f. = 66,  $p < 0.001$ ; d18,  $r = 0.86$ , d.f. = 60,  $p < 0.001$ ; d20,  $r = 0.87$ , d.f. = 47,  $p < 0.001$ ; d21,  $r = 0.93$ , d.f. = 27,  $p < 0.001$ ) for all days and with FFT on d18 ( $r = 0.41$ , d.f. = 60,  $p < 0.001$ ), d20

Figure 2.13 Regression analysis between follicular fluid testosterone (ng/ml) and follicular fluid progesterone (ng/ml) concentrations on day 16, 18, 20 and 21 respectively.

s.e. ; standard error of the slope.

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EXPT1:REGRESSION ANALYSIS. FF T(ng/ml) v FF P(ng/ml)

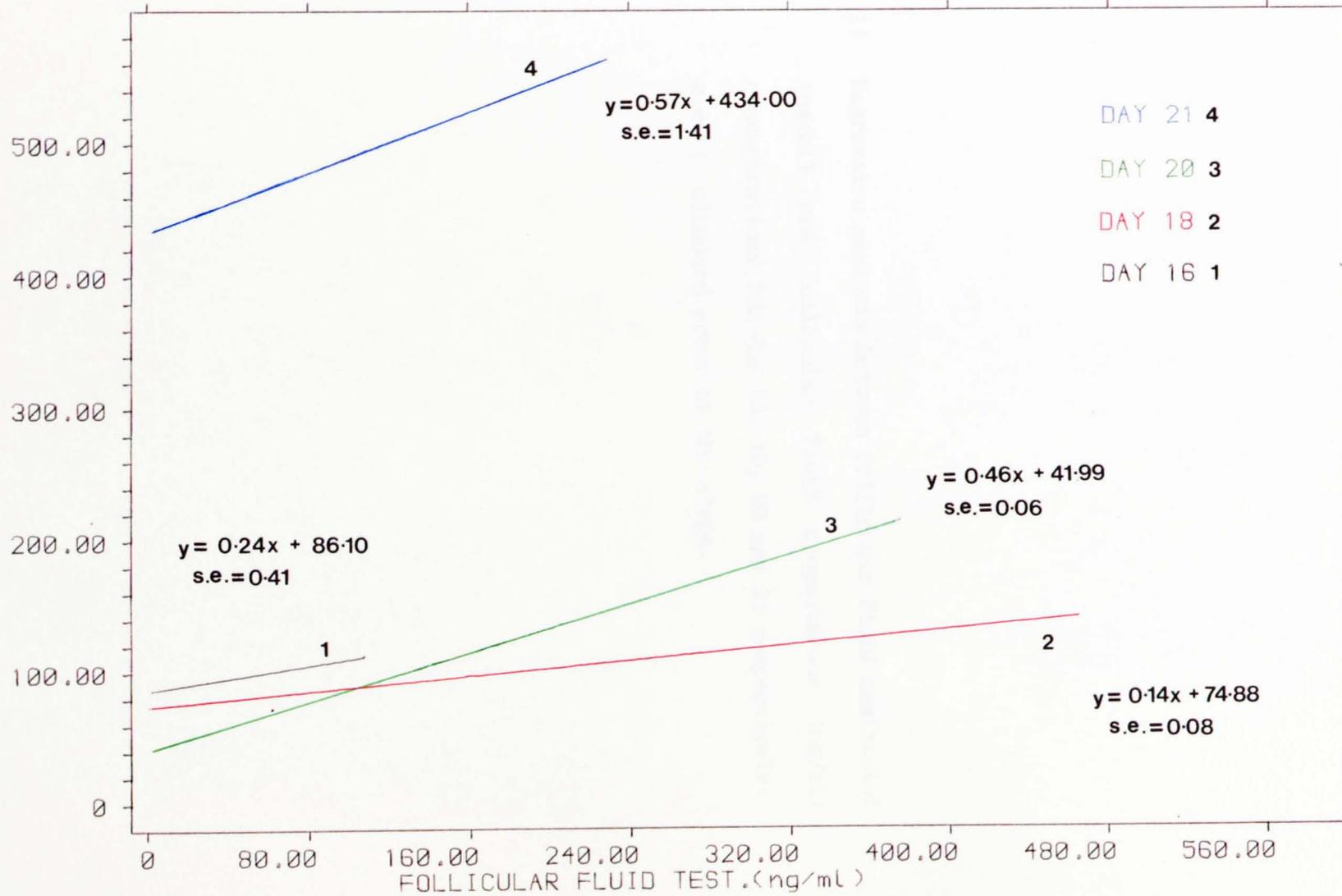
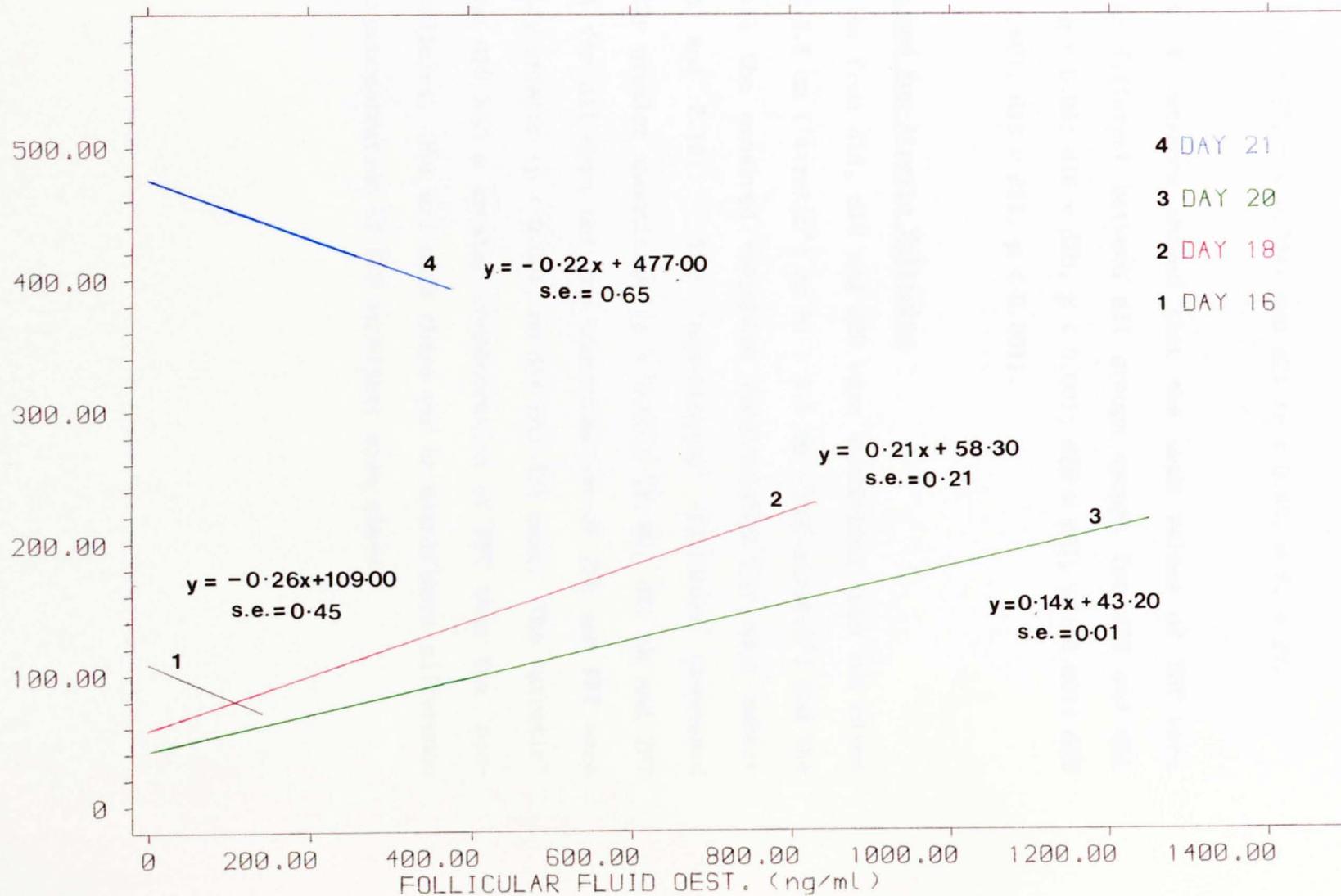


Figure 2.14 Regression analysis between follicular fluid oestradiol (ng/ml) and follicular fluid progesterone (ng/ml) concentrations for day 16, 18, 20 and 21 respectively. s.e. ; standard error of the slope.

EXPT1:REGRESSION ANALYSIS. FF E2 (ng/ml) v FF P (ng/ml)

FOLLICULAR FLUID PROG. (ng/ml)



( $r = 0.82$ , d.f. = 47,  $p < 0.001$ ) and d21 ( $r = 0.93$ , d.f. = 27,  $p < 0.001$ ).

Analysis of variance showed that the mean values of THT were significantly different between all groups except for d20 and d21 (d16 v d18,  $p < 0.05$ ; d16 v d20,  $p < 0.001$ ; d20 v d21,  $p < 0.001$ ; d18 v d20,  $p < 0.001$ ; d18 v d21,  $p < 0.001$ ).

c) Atretic and Non-Atretic Follicles

Follicles from d16, d18 and d20 were subdivided into two class sizes; a)  $\leq 3.5$  mm ('atretic') or b)  $> 3.5$  mm ('non-atretic') and the means of all the measured variables recalculated for each subset (Fig. 2.15 and 2.16). The 'non-atretic' follicles possessed significantly greater quantities ( $p < 0.001$ ) of BG, BT, AR and THT (Fig. 2.15) for all days but the concentration of FFE and FFT were significantly greater ( $p < 0.001$ ) on d18 and d20 only. The 'atretic' follicles on d16 had a greater concentration of FFT than the 'non-atretic' follicles. For all days there was no significant difference between the concentration of FFP in either size class.

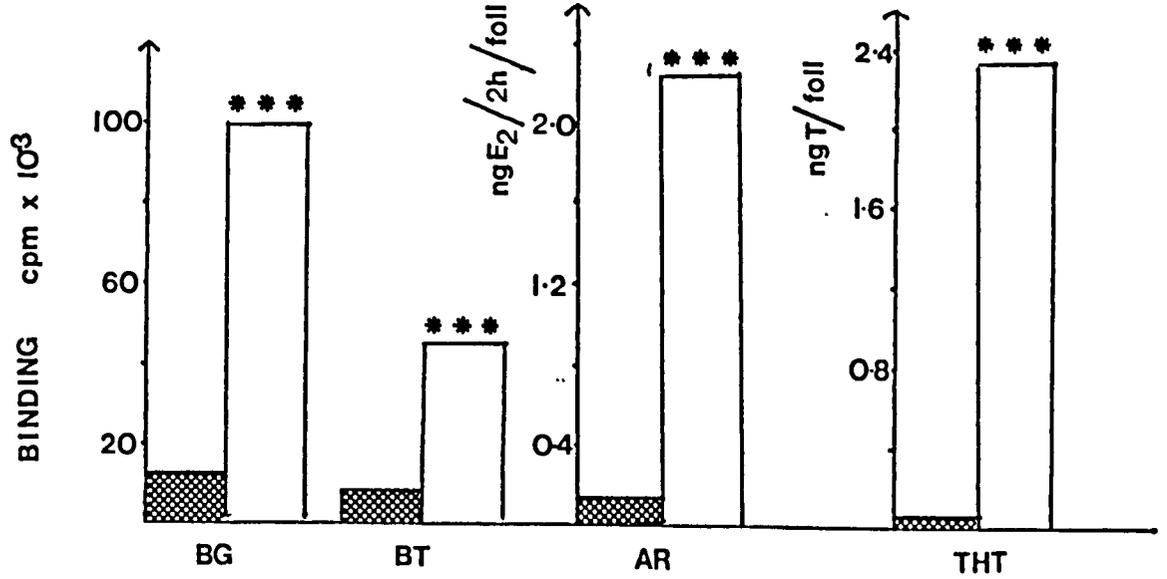
Figure 2.15 Histogram showing a comparison between atretic (hatched) and non-atretic follicles of the levels of  $^{125}\text{I}$ -hCG binding to granulosa and theca cells ( $\text{cpm} \times 10^3$ ), *in vitro* aromatase enzyme activity ( $\text{ngE}_2/2\text{h}/\text{foll}$ ) and testosterone concentration in the theca tissue ( $\text{ng}/\text{foll}$ ).

Values of non-atretic follicles significantly greater than values of atretic follicles as indicated by

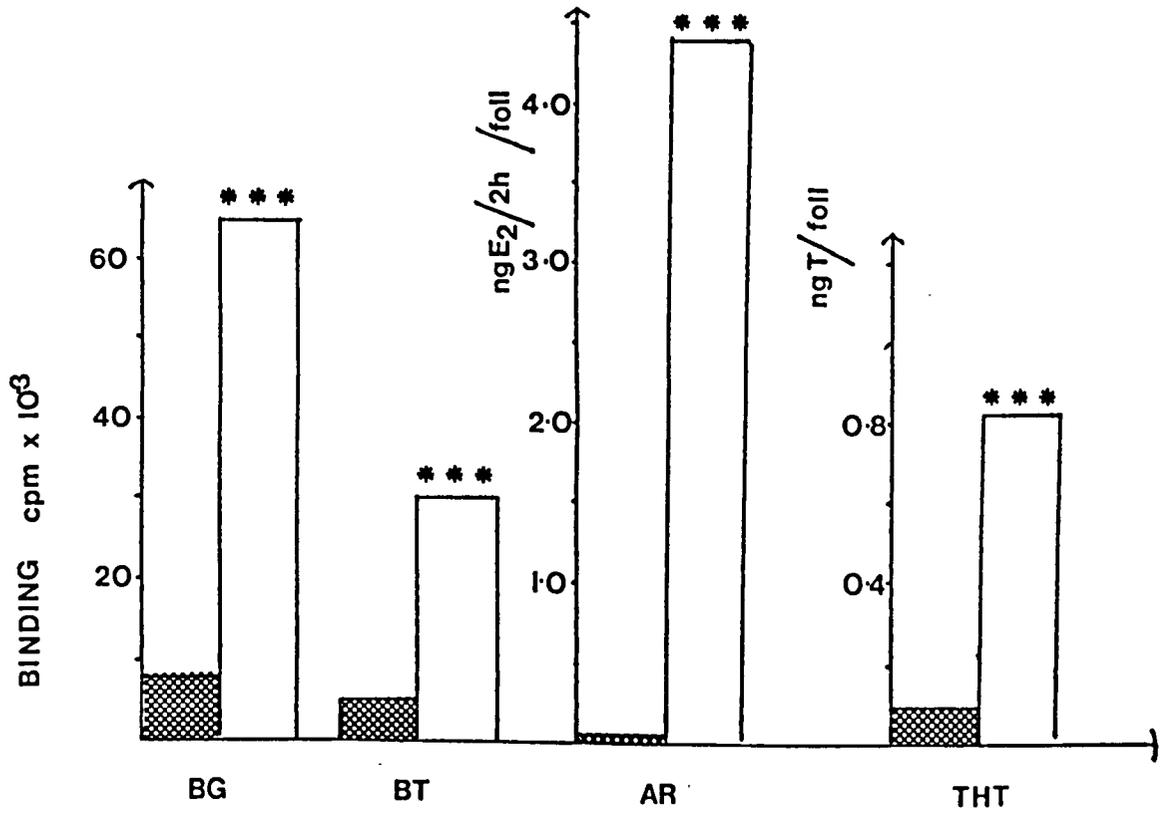
\*\*\*  $p < 0.001$ .

N.S. ; no significant difference between values.

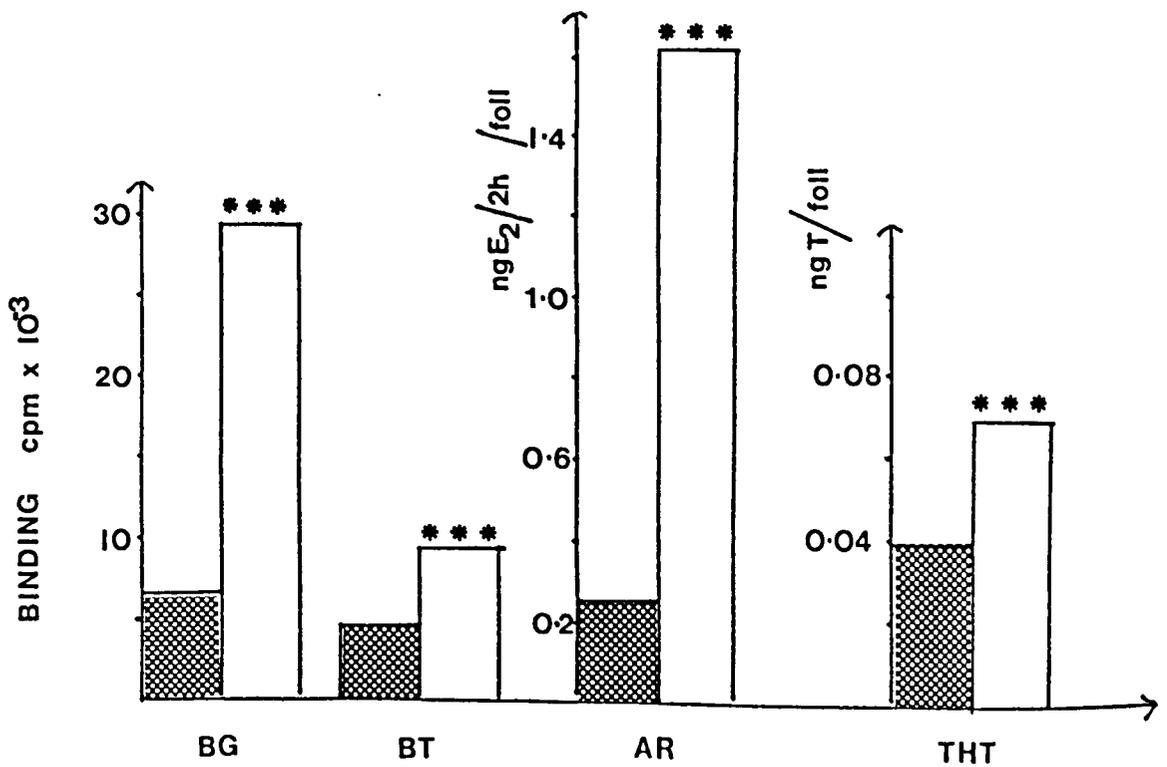
DAY 20



DAY 18



DAY 16

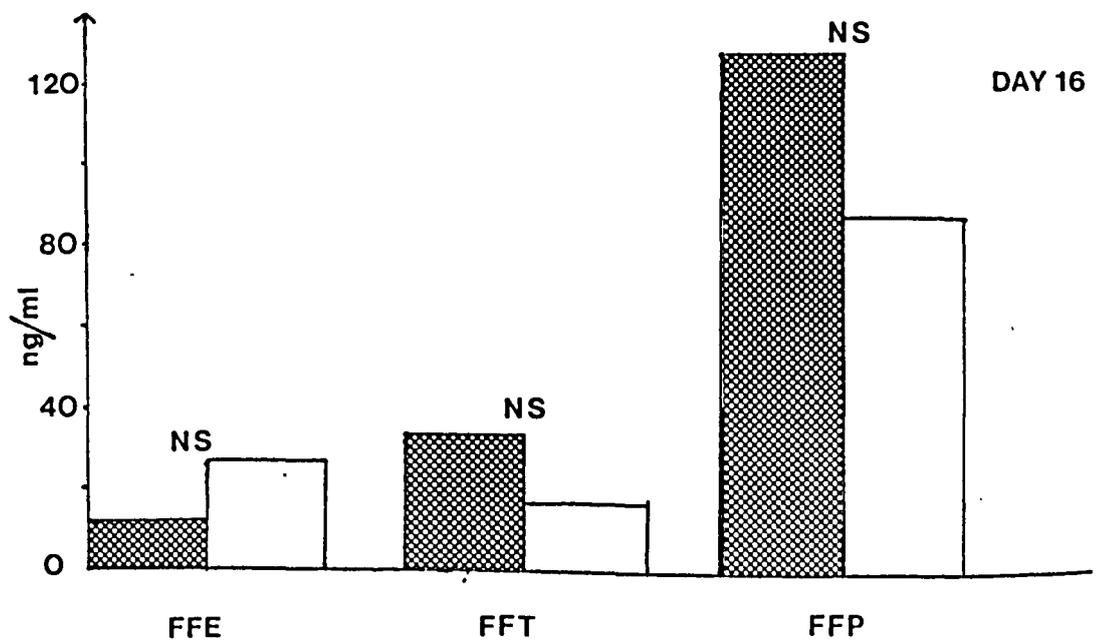
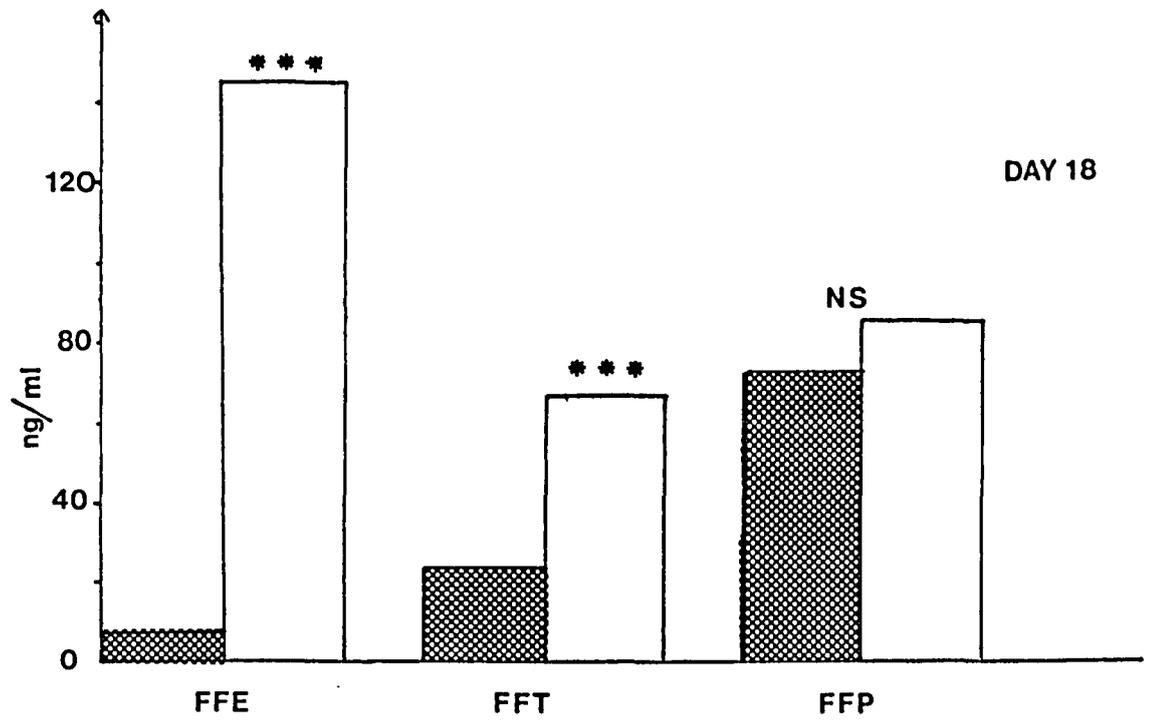
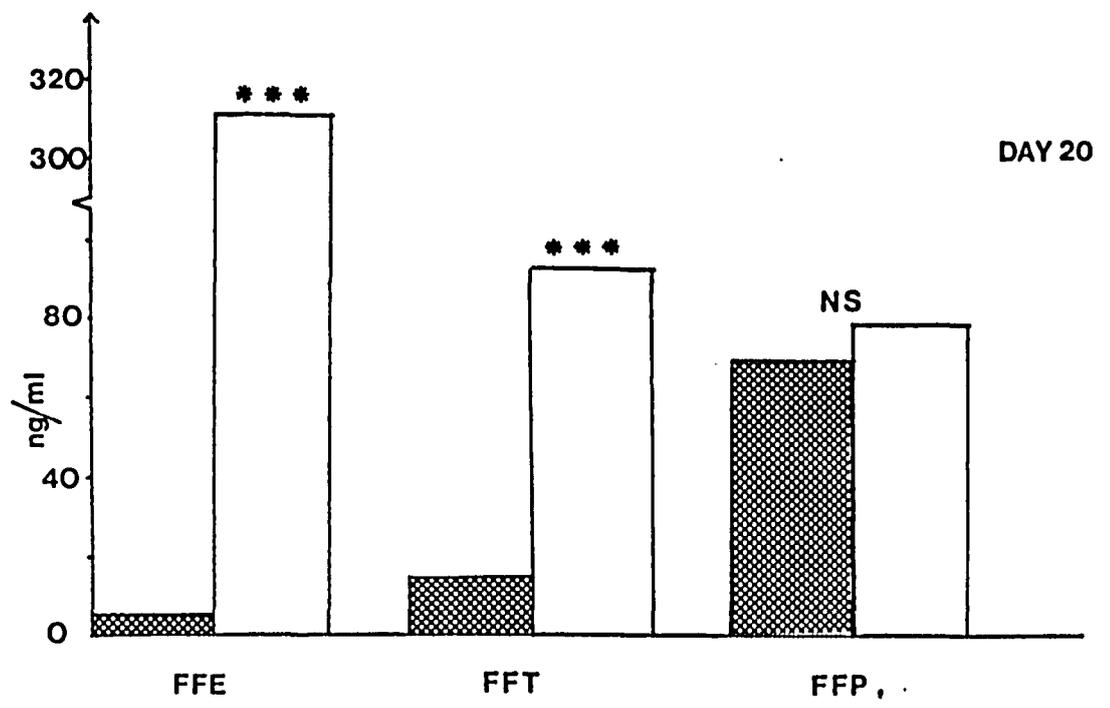


**Figure 2.16** Histogram showing a comparison of the concentrations of follicular fluid oestradiol, testosterone and progesterone (ng/ml) between atretic (hatched) and non-atretic follicles.

Values of non-atretic follicles significantly greater than values of atretic follicles as indicated by

\*\*\*  $p < 0.001$

N.S ; no significant difference between values.



## V DISCUSSION

### a) Morphological Development

The large number of dissectable follicles present in the d16 animals is consistent with previous observations in the cyclic gilt (Robinson and Nalbandov, 1951; Kirkpatrick et al., 1967; Clark et al., 1973) and confirms that during the luteal phase of the porcine oestrous cycle, a proliferating pool of follicles 1-6 mm in diameter is maintained by a continuous process of development and subsequent atresia. At the onset of the follicular phase (d16), recruitment occurs from this proliferating pool which establishes a group of selected follicles destined to ovulate. In the ewe, ink-labelling experiments have shown that this recruitment coincides with luteolysis and only follicles > 2 mm in diameter are able to be selected (Draincourt and Cahill, 1984). Unfortunately, this technique has met with limited success in the cyclic gilt but experiments involving exogenous gonadotrophins (Phillippo, 1968; Hunter, 1972; Hunter, Cook and Baker, 1976), electrocautery of follicles (Clark et al., 1979) and unilateral ovariectomy (Coleman and Dailey, 1979; Clark et al., 1982) indicate that recruitment occurs between days 14-16 of the oestrous cycle. The progressive decrease in follicle number/animal from d16 and d21 suggests that during the follicular phase there is a physiological block which prevents the replacement of atretic follicles into the proliferating pool. Consequently, by d21 only the ovulatory population remained. Aside from their size and biochemical characteristics which will be discussed later, these follicles protruded from the surface of the ovary and possessed extremely well-vascularised theca tissue.

The highly significant linear relationship between follicular diameter and volume recorded in this study agrees with previous observations in the weaned sow (Shaw, 1984) and the ewe (Carson *et al.*, 1981; England, Dahmer and Webb, 1981). In addition, it lends support to the proposal of England, Dahmer and Webb (1981) that each of these variables is interchangeable as an estimate of gross morphological development. Thus, the diameter of follicles deeply embedded within the stroma of an ovary may be satisfactorily estimated by antral fluid measurements alone without dissection. As shown in Fig. 2.4, the difference between the follicular volume and the theoretical total volume of the follicle represents follicular tissue volume. In the follicular phase (d21), a dramatic increase in tissue volume occurred concomitant with a slight decrease in the correlation between follicular diameter and volume of follicular fluid. An explanation for the latter was provided by the initial slaughter of some animals on the second day of oestrus, about 12-16 hours before ovulation. Only a small volume of viscous follicular fluid could be removed from the 10-11 mm follicles dissected from the ovaries of these 'd22' animals and subsequent histological examination revealed considerable infolding of the granulosa and theca layers. This phenomenon was obviously responsible for the increase in tissue volume and has previously been described in the preovulatory follicles of carnivores (Brambell, 1956). Although, the elegant studies of Corner (1919) recorded extensive cytological changes in preovulatory porcine follicles, he detailed infolding of the follicular walls only in the ruptured Graafian follicle.

However, recently Bagnell et al. (1987) observed an increase and loosening of the theca and granulosa cell layers in PMSG treated gilts about 6 hours before the expected time of ovulation. Nevertheless, neither group describe changes in the viscosity of follicular fluid.

Although, the mean number of granulosa cells increased with follicular development and a positive correlation was demonstrated between number of granulosa cells and follicular diameter for the combined data of all days, the significance of this relationship decreased as the follicles matured and attained preovulatory size. Figure 2.6 clearly shows an excellent correlation within the small follicles of all groups but once a diameter of approximately 6 mm had been achieved, a plateau effect occurred after which a haphazard explosion in cell number ensued with little relationship to follicular diameter. This pattern agrees with previous recorded results for the sheep and human (McNatty, 1982) but to date a suitable explanation has not been provided for such an effect. It is conceivable that the development of the more advanced follicles is temporarily arrested while the remaining potentially ovulatory population achieves a comparable state of maturity. Within and between animals, there was great variability in the number of granulosa cells for follicles of identical diameter. This however did not appear to be related to atresia because such follicles were observed to be equally active oestrogenically; this situation is analogous to that described in the weaned sow (Shaw, 1984). Hence, the number of granulosa cells is a poor indicator of health/atresia in pig follicles.

By d20 and d21 a subset of 14-16 large follicles was readily distinguishable from the remaining follicular population (Appendices 3 and 4). Within this subset, there was a difference in follicular diameter of approximately 1.5 mm between the smallest and largest follicles. Such a range in size within the presumed ovulatory population was observed in all pigs in the d20 and d21 groups (Appendices 3 and 4). This suggests that all follicles will not be at an identical stage of development/maturity at the assumed time of ovulation, 40-48 hours after the LH surge. This suggestion is supported by the recent experiment by Wilde and his colleagues (1987); using natural and hCG induced ovulatory gilts they reported that although the majority ( $73 \pm 2\%$ ) of follicles ovulated simultaneously, there was a latent period of four hours before the entire process was completed. Furthermore their data also demonstrated that a protracted ovulation interval was associated with morphological disparity among littermate embryos. Hence, the asynchronous development of early embryos (Anderson, 1978; Pope *et al.*, 1986) and the subsequent occurrence of embryonic mortality may be a consequence of the initial range of follicular development within the selected ovulatory population.

#### b) Biochemical Development

The positive significant correlations between follicular fluid oestradiol and aromatase activity and follicular fluid oestradiol and follicular fluid testosterone recorded for each of the days studied are consistent with the 'two-cell' theory of steroidogenesis (see

Chapter I, Section IIb(ii)) originally proposed by Falck (1959) for the rat and verified in the initial porcine *in vitro* experiments by Haney and Schomberg (1981) and Evans et al. (1981). In fact, in the present study aromatase activity in granulosa tissue *in vitro* was demonstrable in all the follicles studied and the wide range in the level of activity recorded appeared to be directly related to follicular diameter, and hence maturity, for d16, d18 and d20. However, between d18 and d20 the mean aromatase activity actually declined. This unexpected decrease happened in the face of rising follicular fluid oestradiol levels and suggests that the level of granulosa aromatase activity is not the limiting factor in the control of oestradiol synthesis at this time. The precipitous decline in follicular fluid oestradiol which occurred on d21 in the presence of maximal aromatase activity further supports this theory. Consequently, these data imply that the availability of androgen substrate from the theca is of critical importance in maintaining the synthesis of oestradiol. The significant correlation between the testosterone content of the theca tissue and follicular fluid oestradiol throughout the entire follicular phase strongly supports this concept.

Similar to aromatase activity, there was a wide variation in follicular fluid steroid concentrations both within and between animals (Appendices 1-4). On d16 there was only a weak relationship between follicular fluid oestradiol levels and follicular diameter but this relationship strengthened as the follicles matured and by d20 there was a highly significant correlation between these two

variables. At this stage of development, a sub-section of follicles with very high levels of follicular fluid oestradiol was readily distinguishable from the remaining follicular population (Appendix 3). As predicted by the correlation between follicular fluid oestradiol and follicular diameter, the 14-16 follicles within this oestrogenically active subset were the largest of the dissected follicles for each d20 animal.

In accordance with the earlier observations of Channing and Kammerman (1973, 1974) these highly oestrogenic follicles also had many more granulosa and theca LH receptors than the adjacent smaller follicles (Appendices 1-4). Undoubtedly, the interaction of endogenous LH with these specific binding sites increased synthesis and accumulation of oestradiol and this was consistent with a highly significant correlation between granulosa and theca binding and follicular fluid oestradiol. In addition it has been reported in the rat (Hillier, 1985) that the rising levels of follicular fluid oestradiol associated with follicular maturation cause a proliferation in the number of granulosa cells and hence, gonadotrophin receptor numbers.

The correlation between hCG binding to theca tissue and follicular fluid testosterone for d18, d20 and d21 suggests that LH stimulates androgen synthesis, thereby providing the essential precursors for the aromatase system (see Chapter I, Section IIb(ii)). Although Shaw and Foxcroft (1985) reported no relationship between any of these variables in the weaned sow, the follicles in their study were relatively small and at an equivalent stage of development

to those dissected from the present d16 animals, in which no correlation existed either. These results would seem to confirm that LH has only a limited influence on the synthesis of follicular fluid testosterone in small immature follicles.

The strong correlation between follicular fluid testosterone and testosterone in the theca tissue and follicular fluid testosterone and hCG binding to the theca tissue supports previous observations that the theca layer, regulated by LH is the major follicular site of  $C_{19}$  steroid synthesis (rat: Fortune and Armstrong, 1977; hamster: Makris and Ryan, 1975, 1980; cow: McNatty *et al.*, 1984a; sheep: Moor, 1977 and pig: Evans *et al.*, 1981, Tsang *et al.*, 1985). The detection of  $^{125}I$ -hCG binding to thecal tissue in most follicles studied indicates that specific membrane associated LH receptors are present on the thecal layer at all stages of antral follicular development. However, the level of binding to the theca tissue recorded for the d16 animals was obviously insufficient to stimulate maximal thecal androgen biosynthesis. Consequently, the concentration of testosterone in the thecal tissue was very low in these animals and as expected there was no correlation between any of the aforementioned variables. Although, on d21 the peak concentration of testosterone in the theca tissue coincided with maximal aromatase activity, the concomitant fall in follicular fluid testosterone levels in these animals suggests two possibilities. Thecal androgen may no longer be required as an oestrogen precursor in the mature preovulatory follicle and hence the steroid simply accumulates within the theca tissue; alternatively the preovulatory LH surge may block

the aromatase pathway by inducing androgen metabolism via the  $5\alpha$  reduction pathway, with  $5\alpha$ -reduced androgens acting as potential competitive inhibitors of extant aromatase activity and unable to serve as oestrogen precursors (Hillier, 1985). Either explanation requires experimental confirmation.

The notable change in the follicular fluid steroids of the d21 animals (Fig. 2.11; Appendix 4) was undoubtedly a consequence of the LH surge. In accordance with other species (sheep: Moor, 1974; Webb and England, 1982; cow: Staigmiller *et al.*, 1982; rat: Goff and Henderson, 1979; Hillensjo, Dekel and Ahrén, 1976; Lieberman *et al.*, 1975; hamster: Saidapur and Greenwald, 1978) there was a transient increase in progesterone synthesis and a dramatic fall in both androgen and oestradiol- $17\beta$  production. This effect of LH is well established and is understood to be mediated through c'AMP (Channing and Tsafiriri, 1977 - see Chapter I, Section IIc(ii)). Unfortunately, the mechanism of this inhibitory action of LH, just prior to ovulation, is still obscure. It has been suggested that LH may stimulate production of a protein which inhibits the enzymes involved in the cleavage of the 17-sidechain of progesterone ( $17\alpha$  hydroxylase and/or  $17:20$  lyase) thereby resulting in a simultaneous inhibition of both follicular fluid testosterone and oestradiol synthesis (Lieberman *et al.*, 1975). Katz and Armstrong (1976) and Henderson and Moon (1979) proposed that LH actually inhibits oestradiol  $17\beta$  production by reducing aromatase activity. However, the d21 group possessed the highest mean aromatase activity of all the animals studied which infers that, at least in the pig, the latter theory does not apply.

The physiological significance of this changing steroidal environment on d21 is uncertain. It is possible that it may influence the mechanism of follicle rupture. Rondell (1974) has shown that progesterone can increase the distensibility of strips of sow ovarian follicles that are incubated in solutions containing the steroid but studies using intra-follicular injections of progesterone actually inhibited ovulation (Swanson and Lipner, 1977). However, others favour a role for the steroid in inducing the resumption of meiosis by the oocyte (Zuckerman and Baker, 1977; Gerard et al., 1979; Ainsworth et al., 1980).

Thus, similar to the morphological data, the 14-16 follicles within the selected ovulatory population were readily distinguishable on d20 by distinct biochemical characteristics, notably a high follicular fluid oestradiol:testosterone ratio, elevated granulosa aromatase activity and increased LH binding to granulosa and theca cells. However, as with morphological development, there was also a considerable range of biochemical development among follicles within the same ovary. Follicles of identical size and with equal numbers of granulosa cells showed great dissimilarity in follicular fluid steroid concentrations and in LH binding (Appendix 2). Indeed, this diversity was apparent at all stages of follicular development, both between days and within animals, a phenomenon which has previously been described in the weaned sow (Shaw and Foxcroft, 1985). Thus, the pooling of follicles from within the same ovary on the basis of size, in addition to the pooling of similar sized follicles from different animals before *in vitro* study, is likely to confound the interpretation of the data obtained.

In addition to the selected ovulatory population, some of the characteristics of atretic 'unselected' follicles were also apparent. Although LH binding to theca and granulosa cells, granulosa cell aromatase activity and testosterone in the theca tissue were detectable in the majority of the 'atretic' follicles and especially in the d16 animals, the non-atretic' follicles always possessed significantly greater amounts ( $p < 0.001$ ) of all these parameters (Fig. 2.15). Similarly, the concentration of follicular fluid oestradiol and testosterone was significantly greater in the 'non-atretic' follicles on d18 and d20, whereas on d16 there was no significant difference between the two classes and, in fact, the 'atretic' follicles had a greater concentration of follicular fluid testosterone. For all days there was no significant difference between the concentration of follicular fluid progesterone in either class which implies that this follicular fluid steroid is definitely not a good indicator of follicular atresia.

Overall, these results suggest that the less mature follicles do continue to develop morphologically and to a limited extent steroidogenically beyond d16. However, as they possess few LH receptors, there is insufficient gonadotrophin support to stimulate further development. Consequently, by d18, there was a notable decline in aromatase and follicular fluid oestradiol synthesis, a fact which may explain the enhanced androgen levels in some of these potentially atretic follicles. Indeed, several authors have proposed that testosterone promotes the atretic process both directly and by further inhibition of residual aromatase activity following

conversion to 5 $\alpha$ -dihydrotestosterone (McNatty et al., 1979b; McNatty, 1980; Chan and Tan, 1986). Nonetheless, the limited steroidogenic activity of these small follicles lends support to the notion that atresia is not a completely degenerative process and therefore, these follicles may play an essential role in ovarian physiology.

Finally, although the overall pattern of changing follicular fluid steroids observed in this study compares favourably with the results obtained from previous experiments in PMSG/hCG primed gilts (Ainsworth et al., 1980; Meinecke, Gips and Meinecke-Tillmann, 1987) there were several notable differences. While Ainsworth and his colleagues (1980) reported that follicular fluid androgen concentrations remained relatively constant throughout preovulatory follicular development at 20-40 ng/ml, a distinct rise and fall of follicular fluid testosterone was observed in the naturally cycling animals (Fig. 2.11) of this study. In addition, the maximal concentration of follicular fluid oestradiol was notably higher in PMSG/hCG primed gilts ( $389 \pm 74$  ng/ml, 72 hrs post PMSG;  $264 \pm 47$  ng/ml, 76 hrs post PMSG, 4hrs post hCG: Ainsworth et al., 1980) compared to the cyclic animals ( $235 \pm 30.2$  ng/ml, d20;  $105 \pm 30.2$  ng/ml, d21); also in contrast to the cycling gilts, follicular fluid progesterone was the predominant steroid at 72 hrs post PMSG (Ainsworth et al., 1980; Meinecke, Gips and Meinecke-Tillmann, 1987). These elevated progesterone concentrations suggest that the PMSG preparation could have a high LH activity which would stimulate progesterone secretion by the granulosa cells and this has been demonstrated in experiments using isolated granulosa cells of PMSG

pre-treated gilts (Tsang *et al.*, 1985). Hence, although the synchronised PMSG/hCG treated pig represents a well established experimental model, the observed discrepancies between spontaneous and synchronised animals should not be overlooked.

## VI CONCLUSION

It is evident from this experiment that preovulatory ovarian follicular development in a polyovulatory species such as the pig consists of a very complex array of events.

The considerable body of information gathered from each of the days studied in this experiment is summarised in Fig. 2.17 which depicts a model for the sequential maturation or atresia of pig follicles during the follicular phase. It was obvious from the range of morphological and biochemical development on d16 that not all destined ovulatory follicles are at the same stage of maturity at the time of recruitment. This asynchrony was highlighted by the marked differences in steroid synthesising capacity of follicles of identical size within the same ovary. A further consequence of the asynchrony in the rate at which follicles become oestrogenically active, is the period of time in which follicles are in a 'mature' state prior to the onset of the preovulatory LH surge. Presumably the high oestrogenic activity of the dominant follicles will provide the trigger for the preovulatory LH surge mechanism and therefore set the timing of the surge and ultimately of ovulation. From a consideration of the reported timing between oestrogen stimulation and the LH surge in gilts and weaned sows (Foxcroft *et al.*, 1984;

**Figure 2.17** A model for the sequential maturation or atresia of pig follicles. Sizes (mm) represent initial diameter of follicles within the proliferating pool (PP) before selection (S). Probably subsequent development or atresia of each size class in the follicular phase (FP) is then shown. The final stages represent initial follicular responses to the preovulatory LH surge.

The outer zone of each follicle represents theca and  $\text{ThR}_{\text{LH}}$  (solid sector) the relative amount of theca LH binding. The middle zone represents granulosa cells and  $\text{GrR}_{\text{LH}}$  (solid sector) the relative amount of LH binding to granulosa cells. The inner zone represents granulosa tissue and follicular fluid:  $\text{R}_{\text{FSH}}$  (clear areas), presence of, but not relative differences in, granulosa FSH binding; AR (stippled area), relative differences in demonstrable aromatase activity *in vitro*; FFE (dark stippled area), relative concentrations of follicular fluid oestradiol-17 $\beta$ ; FFT (clear area) relative concentrations of follicular fluid testosterone; FFP (cross-hatching), relative concentrations of follicular fluid progesterone. Arrows and  $\text{E}_2$  signify origin and relative amounts of oestradiol-17 $\beta$  secretion from follicles and possible intraovarian as well as peripheral effects of  $\text{E}_2$ .



Edwards and Foxcroft, 1983) of approximately 55 hours, the activation of the surge mechanism would be expected on d18 of the cycle at a time when selection of ovulatory follicles is in progress. Thus, the interval between the final selection of the least mature ovulatory follicles and the dramatic maturational changes induced by the LH surge must be very limited. This could affect the later development of the oocyte and embryo (Pope *et al.*, 1986; Wilde *et al.*, 1987) and also be causally related to the different populations of functional corpora lutea reported in the pig by Rao and Edgerton (1984).

On the basis of the characteristics in this study, those follicles destined to ovulate were only readily distinguishable on d20 (Appendix 3) suggesting that the selection of the preovulatory population continues over a four day period from about d16 to d20 of the cycle. Aside from being the largest of the dissected follicles, the notable characteristics of the selected ovulatory population included a high ratio of follicular fluid oestrogen to testosterone, high hCG binding to the theca and granulosa cells, high granulosa cell aromatase activity *in vitro* and the presence of significant correlations between the majority of these variables. However, approximately 25-30 follicles of the d16 animals and 20-25 follicles of the d18 animals also possessed these qualities further emphasising that the selection process involves some initial maturation even in those unselected follicles which ultimately join the atretic pool. The onset of atresia could be identified by a low ratio of follicular fluid oestrogen to testosterone, low hCG binding to the theca and granulosa cells, negligible aromatase activity *in vitro* and a

distinct lack of variable inter-relationships. Nevertheless, the limited steroid synthesising capacity of these small follicles may well exert a regulatory role in the final development of the ovulatory population (Fig. 2.17).

## CHAPTER 3

### GENERAL MATERIAL AND METHODS

The following text is extremely faint and largely illegible. It appears to be the beginning of a section describing the general material and methods used in the study. The text is too light to transcribe accurately, but it seems to follow a standard scientific format for a methods section, likely starting with a description of the materials and the experimental procedures.

## I INTRODUCTION

The materials and methods described in this section are common to Experiment 2 and Experiment 3. Procedures specific to the individual experiments are detailed in Chapter 4 Section II and Chapter 5 Section II.

## II ANIMALS

All animals were primiparous gilts Large White x (Large White x Landrace) from the School of Agriculture's main herd. Every month from May 1986 to January 1987, oestrus was synchronised in groups of ten gilts by feeding 20 mg/day of the orally active progestagen, altrenogest or (Regumate, Roussel, Uclaf) for 10 days. Within 3-6 days of withdrawing this treatment, all animals exhibited a standing oestrus and were artificially inseminated with double or triple doses of pooled Large White boar semen (MLC Semen Delivery Service, Selby, Yorkshire) at approximately 12 hr intervals.

Pregnant gilts were group penned during gestation and then transferred to standard farrowing crates one week before parturition where they remained throughout the 21 day lactation. Artificial lighting within the farrowing house was regulated to give a photoperiod regimen of 8 hours daylight and 16 hours darkness. Litter size was adjusted to between 9 to 11 piglets by cross-fostering between litters or alternatively acquiring neonates from the University's commercial piggery.

During gestation gilts were fed approximately 2.3 kg/day of a standard sow ration which was increased to 1.0 kg/sow plus 0.5 kg/piglet or to 5.5 kg, whichever was the lesser during lactation. Water was available ad libitum and no creep ration was fed to the piglets.

Any animals showing signs of stress during parturition were sedated with an intra-muscular injection of 'Stresnil' (4% solution of azaperon or 4'fluoro-4[4-2-pyridyl-1-piperazinyl] butyrophenone, Janssen, Pharmaceutica, Beerse, Belgium) at a dose of 2.2 mls/50 kg.

Piglets with milk scour were orally treated with 2 mls of Neftin Piglet Medicator (Smith Kline Animal Health Ltd., Stevenage, Herts.) twice daily. Piglets showing signs of swollen joints were given an intra-muscular injection of Lincocin (UpJohn Limited, Crawley, Sussex).

### III SLAUGHTER AND *IN VITRO* PROCEDURES

Sows were slaughtered at the School of Agriculture abattoir by electrical stunning and exsanguination. The mammary tissue and the overlying epidermis was dissected from the carcass and the weight and original position of the individual glands ('quarters') recorded. The ovaries were recovered within 5 minutes of slaughter, transferred to the *in vitro* laboratory and their gross morphology recorded. The 50 largest follicles/sow were dissected free of extraneous tissue. Details of dissection procedures, collection of follicular fluid, granulosa cell recovery, estimation of aromatase activity and assessment of  $^{125}\text{I}$ -hCG binding to granulosa cells were as described in Chapter 2 Section II(b), (c) and (d) of this thesis.

#### IV RADIOIMMUNOASSAY OF OESTRADIOL-17 $\beta$ IN FOLLICULAR FLUID AND INCUBATION MEDIA

Measurement of oestradiol-17 $\beta$  in follicular fluid and incubation media was carried out according to the methods detailed in Chapter 2 Section II(e)(i) with the following minor modifications.

##### a) Follicular Fluid

A pool of follicular fluid diluted in Hanks buffer (potency: 400 pg/ml) was used as a quality control and routinely assayed at 50, 100 or 200  $\mu$ l to measure the inter- and intra-assay C.V. and to confirm parallelism. At 100  $\mu$ l, the inter- and intra-assay C.V. were 7.9% and 11.9% respectively. The limit of assay sensitivity was 6.8 pg/ 100  $\mu$ l.

##### b) Incubation Media

For these experiments, media was assayed at 50, 100 or 200  $\mu$ l. A control pool of medium (potency = 400 pg/ml) was routinely assayed at similar volumes to confirm parallelism and inter- and intra-assay C.V. which were 9.7% and 3.1% respectively. The sensitivity of these assays (n = 50) was 7.6 pg/100  $\mu$ l.

#### V STATISTICAL ANALYSIS

##### a) Calculation of Radioimmunoassay Data

The assay data were interpolated using a statistical program developed by Rodbard and Lewald (1970).

b) General Results

For clarity, the results of all the measured ovarian and piglet related data are represented as means, both for individual sows and for the combined data of each group of sows. An initial analysis to determine linear and quadratic trends within the data revealed that the majority of variation was accounted for by a linear fit. Hence, to determine significant relationships between ovarian and production parameters within groups, the relevant data were subjected to multiple linear and correlation analyses. However, as in Experiment I (Chapter 2), since the granulosa cells from particular follicles were used to assess either  $^{125}\text{I}$ -hCG binding (see Section II(c)) or aromatase activity (see Section II(d)), it was not possible to correlate BG with AR. Similar to Experiment 1, individual data points have been omitted from the figures which depict regression lines of best fit between follicular variables for each group of sows. The equation of each line and the standard error of the slope are shown.

Significant differences between individual parameters between groups of sows were analysed by one way analysis of variance (ANOVA). Once analysis of variance had demonstrated a significant effect, individual means were compared by the students 't' test.

Comparisons of suckling behaviour between the piglets from the control and treatment litters was achieved using the chi-squared distribution test.

c) Statistical Analysis of Ovarian Parameters

Evidence from Experiment 1 (Chapter 2) suggests that it is the largest follicles within a follicular population which are ultimately destined to ovulate. Consequently, to compare differences between potentially ovulatory follicles from the control and treatment sows analysis was restricted to the largest twenty follicles dissected from each sow. In addition, although follicular fluid samples were assayed completely at random., the detectable levels of oestradiol in the majority of samples were extremely low. Consequently, to avoid the possibility that differing sensitivities of individual assays would negatively bias any one sow or group of animals, only follicles with  $\geq 1.0$  ng  $E_2$ /ml follicular fluid were included in statistical analysis with other ovarian parameters.

## CHAPTER 4

### EXPERIMENT 2

PIGLET SUCKLING BEHAVIOUR AND OVARIAN FOLLICULAR DEVELOPMENT  
ASSOCIATED WITH SPLIT-WEANING IN LACTATING PRIMIPAROUS SOWS

## I INTRODUCTION

The domestic sow generally remains anoestrous during the first 3-4 weeks of lactation. Weaning of the litter typically leads to a rapid increase in the size of antral follicles associated with oestrus and ovulation within 3 to 10 days. Initially, it was assumed that the entire period of lactation was characterised by a state of follicular quiescence; however detailed studies of the endocrinology and ovarian morphology of the lactating and weaned sow (see Chapter I, Section III b & d) have provided convincing evidence for folliculogenesis during lactation. Indeed, a survey of the literature quoted earlier in this thesis (see Chapter 1, Section III d(ii)) has shown that various regimens for altering the nursing pattern of the sow and her litter have provided a possible means of inducing oestrus and ovulation during lactation.

The enhanced ovarian development observed in these early studies was presumed to be directly related to a reduction in the intensity of the piglet suckling stimulus. However, this general assumption has never been accurately confirmed nor have the hormonal or biochemical changes associated with enhanced follicular development been adequately described.

Hence, the aims of this experiment were to initiate follicular development during lactation by manipulating litter size in an attempt to 1) study the factors associated with follicular maturation in treated compared to control sows, and 2) to closely study the suckling behaviour and growth performance of each litter and to relate these observations to any subsequent ovarian response.

## II EXPERIMENTAL PROCEDURE

### a) Animals

From the day of parturition to day seven of lactation to determine whether suckling frequency was influenced by photoperiod, the suckling frequency of each litter was recorded during three 12 hours daytime periods and compared to three 12 hours night-time periods. Between days seven to fourteen of lactation the teat order of each litter was recorded. This was achieved by closely observing the litter during several suckling bouts and numbering each piglet on the back with indelible marker pen in ascending numerical order from the sow's head to her tail. The position and the number of mammary quarters preferentially suckled by each piglet was then recorded. Initially, the suckling bouts of several sows were filmed using a video camera in an attempt to record the exact time piglets spent at a particular teat. However, this technique was abandoned as it was not possible to accurately observe those piglets suckling the lower layer of teats beneath their siblings.

On the fourteenth day of lactation, the piglets from all the litters were weighed and the sows allocated to one of two treatment groups, a) Control (n = 8) or b) Split-weaned (n = 8). The heaviest piglets were removed from the split-weaned animals to leave each sow suckling the five lightest piglets for the remaining seven days of lactation. The control sows continued to suckle their entire litter.

Between day fourteen and weaning on day twenty-one of lactation the new teat order, the general suckling behaviour and the suckling frequency of the split-weaned group were recorded. At weaning, the remaining piglets were reweighed and the sows slaughtered.

### III RESULTS

#### a) Ethological Study

##### i) Development of a Fixed Teat Position

Initial organisation at the mammary quarters appeared muddled but within 7 days of parturition all litters had developed a stable teat order. This meant that at every suckling session, piglets consistently returned to their 'chosen' teat(s) (Plate 2). Each piglet strongly defended these teats from their siblings and fighting between litter-mates was common if one piglet deliberately attempted to suckle a different teat.

##### ii) General Suckling Behaviour

Suckling bouts were initiated both by the piglets and the sow. The feeding call of the sow was generally given in response to the piglets which had already started to squeal and pester her. Periods of nursing lasted approximately two minutes and were divided into five distinct phases:-

- a) Jostling for position at the udder
- b) Nosing the udder with vigorous up and down movements of the head
- c) A "quiet" phase during which the piglets suck on the teats with slow mouth movements
- d) Rapid suckling with ears cocked backwards
- e) A brief return to sucking with slow mouth movements and nosing of the udder.

**Plate 2** Photograph showing the stable teat order of the litter of Sow 5 (Control) during one suckling session. Each piglet is avidly suckling its own chosen teat.



Normal suckling recurred approximately every 45 minutes and was synchronised between all sows. If nursing began soon after the previous milk ejection, although piglet behaviour appeared superficially normal, milk let down did not occur.

iii) Suckling Behaviour in Split-Weaned Litters

During the two to three suckling periods immediately following the removal of their siblings, the remaining piglets continued to suckle only their chosen teat. However, within 24 hours of split-weaning 88% of the piglets were suckling an average of 2 teats each; 35% of these piglets suckled their original chosen teat plus an additional teat within close proximity, while 60% had moved to completely 'new' mammary quarters vacated by their heavier littermates (see Figures 4.1, 4.2 and 4.3). A significantly lower proportion of piglets (25%,  $\chi^2 = 25.69$ ,  $p < 0.001$ ) were observed to multiple suckle in the control litters.

iv) Sow Vocalisation

Sows vocalised continuously when nursing their piglets regardless of litter size. Grunt rate increased from approximately 1 grunt/second to a crescendo of 2-3 grunts/second during suckling and milk ejection occurred approximately 20 seconds after this increase in grunt rate.

v) Suckling Frequency

The mean ( $\pm$  sem) suckling frequencies recorded during the 12 hours day and night-time periods are shown in Table 4.1. Although the piglets suckled less frequently at night, there was no

Figures 4.1, 4.2, 4.3 The teat order of six litters before and after split-weaning.

FIGURE 4.1

SOW 4

← HEAD →			
TEAT ORDER PRE SPLIT-WEANING	FIVE HEAVIEST PIGLETS REMOVED	TEAT ORDER POST SPLIT-WEANING	
1 1		3 2	PECTORAL TEATS
3 2	3 2	3 2	
4 4		5 9	
5 6	5	5 9	
- 7		- 9	
8 9	8 9	8 8	POSTERIOR TEATS
11 10		8 -	
← TAIL →			

SOW 21

← HEAD →			
TEAT ORDER PRE SPLIT-WEANING	FIVE HEAVIEST PIGLETS REMOVED	TEAT ORDER POST SPLIT-WEANING	
2 1	2	2 2	PECTORAL TEATS
3 4	3	3 3	
5 3	5 3	5 5	
6 6	6 6	6 6/5	
7 7		6 9	
8 8		9/6 9	POSTERIOR TEATS
9 9	9 9	9 9	
← TAIL →			

FIGURE 4.2

SOW 11

← HEAD →			
1 2	2	2 4	PECTORAL TEATS
4 3	4 3	2 4	
5 -	-	3 -	
6 -	-	3 -	
8 7		9 10	
10 9	10 9	9 10	POSTERIOR TEATS
← TAIL →			
TEAT ORDER PRE SPLIT-WEANING	FIVE HEAVIEST PIGLETS REMOVED	TEAT ORDER POST SPLIT-WEANING	

SOW 27

← HEAD →			
2 1	2 1	2 2	PECTORAL TEATS
3 4	3 4	1 1	
6 5		4 1	
7 7		4 4	
8/9 8	8 8	3 4	
- 9		- 3	POSTERIOR TEATS
10 10		8 -	
← TAIL →			
TEAT ORDER PRE SPLIT-WEANING	FIVE HEAVIEST PIGLETS REMOVED	TEAT ORDER POST SPLIT-WEANING	

**FIGURE 4.3**

**SOW 33**

← HEAD →			
1 2	2	2 2	PECTORAL TEATS
3 4		5 7	
7 5	7 5	7 -	
5 -	5	7 -	
6 -		8 -	
8 -	8	8 -	
9 -		- -	POSTERIOR TEATS
10 -		10 -	
← TAIL →			
TEAT ORDER PRE SPLIT-WEANING	FIVE HEAVIEST PIGLETS REMOVED	TEAT ORDER POST SPLIT-WEANING	

**SOW 34**

← HEAD →			
1 2	1	1	PECTORAL TEATS
4 6	4	3 3	
3 7	3	- -	
5 -		4 4	
8 8		10 9	
9 10	9 10	9	POSTERIOR TEATS
- -	- -	10	
← TAIL →			
TEAT ORDER PRE SPLIT-WEANING	FIVE HEAVIEST PIGLETS REMOVED	TEAT ORDER POST SPLIT-WEANING	

Table 4.1 Suckling frequencies recorded over 12 hr day and night-time periods for control and split-weaned sows (before and after split weaning)

TREATMENT GROUP	SOW NUMBER	SUCKLING FREQUENCY IN THREE 12 HR DAYTIME PERIODS			SUCKLING FREQUENCY IN TWO 12 HR NIGHT-TIME PERIODS	
		1	2	3	1	2
CONTROL	NT	18	19	22	18	18
	5	21	18	22	19	17
	7	22	19	22	18	18
	16	21	20	23	17	20
OVERALL MEAN		20.58 ± 0.5			18.13 ± 0.35	

TREATMENT GROUP	SOW NUMBER	SUCKLING FREQUENCY IN THREE 12 HR DAYTIME PERIODS			SUCKLING FREQUENCY IN TWO 12 HR NIGHT-TIME PERIODS		SUCKLING FREQUENCY IN FOUR 12 HR DAYTIME PERIODS AFTER SPLIT-WEANING			
		1	2	3	1	2	1	2	3	4
SPLIT-WEANED	1	22	21	20	18	17	20	17	17	18
	4	21	20	22	18	18	19	19	19	22
	11	22	21	22	20	20	18	22	19	23
	18	20	20	23	17	20	21	20	18	18
OVERALL MEAN		21.17 ± 0.02			18.5 ± 0.46		19.62 ± 0.37			

significant difference between day and nighttime periods. There was also no significant effect of split-weaning on suckling frequency.

b) Ovarian Study

During the course of her second and third week of lactation, Sow 7 from the control group became thin and agalactic and her piglets failed to develop as well as the other litters. Examination of her ovaries at slaughter revealed negligible follicular development. Hence to avoid the likelihood of biasing the data from the control animals, the results from this sow have been excluded from the overall ovarian analysis.

i) Morphological Data

Dissected follicles ( $n = 336$ , mean number of follicles recovered/sow =  $48.00 \pm 1.21$ ) from the control animals ranged from 0.90 to 5.05 mm with a mean ( $\pm$  sem) diameter (FD) of  $3.45 \pm 0.04$ . Follicles recovered from the split-weaned sows ( $n = 361$ , mean number of follicles recovered/sow =  $45.13 \pm 2.43$ ) were significantly larger ( $p < 0.001$ ) with a range of 2.20 to 6.10 mm and a mean ( $\pm$  sem) FD of  $3.90 \pm 0.04$ .

As described earlier follicle populations were also subdivided to include only the largest 20 follicles/sow. These follicles ranged from 2.95 to 5.05 mm with a mean FD of  $3.92 \pm 0.04$  and 3.50 to 6.10 mm with a significantly larger ( $p < 0.001$ ) mean FD of  $4.51 \pm 0.04$  mm for the control and split-weaned sows, respectively.

Subdivision of both sets of follicular data into three diameter size classes, 2.00 - 3.99 mm, 4.00 - 5.99 mm and  $>6.00$  mm confirmed

that a greater percentage of large follicles were recovered from the split-weaned animals (Fig. 4.4).

ii) Biochemical Data

a) Aromatase Activity (AR)

The mean AR (pg  $E_2$ /2h/follicle) of the largest 20 follicles dissected from individual sows and the overall mean ( $\pm$  sem) AR for the control and split-weaned sows are presented in Tables 4.2 and 4.3. Although mean AR, calculated from all the follicular data was significantly greater ( $p < 0.001$ ) in split-weaned animals (1025.0 v 774.0) analysis of variance revealed no significant difference between the two groups for mean AR calculated from the largest twenty follicles/sow/group. This was due to the large variation in response between individual sows within the two groups especially among smaller follicles.

There was a highly significant correlation between AR and FFE ( $r = 0.54$ , d.f. = 36,  $p < 0.001$ ) for the split-weaned animals (for regression analysis see Fig. 4.5). Subdivision of these follicles into 3 diameter size classes: 2.00-3.99 mm, 4.00-5.99 mm and  $>6.00$  mm revealed that the largest follicles possessed the greatest AR activity (Table 4.4). However, a significant relationship between AR and FD was only established in the control animals ( $r = 0.40$ , d.f. = 53,  $p < 0.01$ ; for regression analysis see Fig. 4.6).

**Figure 4.4** Histograms showing the size distribution of a) all dissected follicles and b) the greatest 20 follicles within restricted size classes (2.0 - 3.99 mm, 4.0 - 5.99 mm,  $\geq$  6.0 mm diameter) for control and split-weaned animals respectively.



Control



Split-Weaned

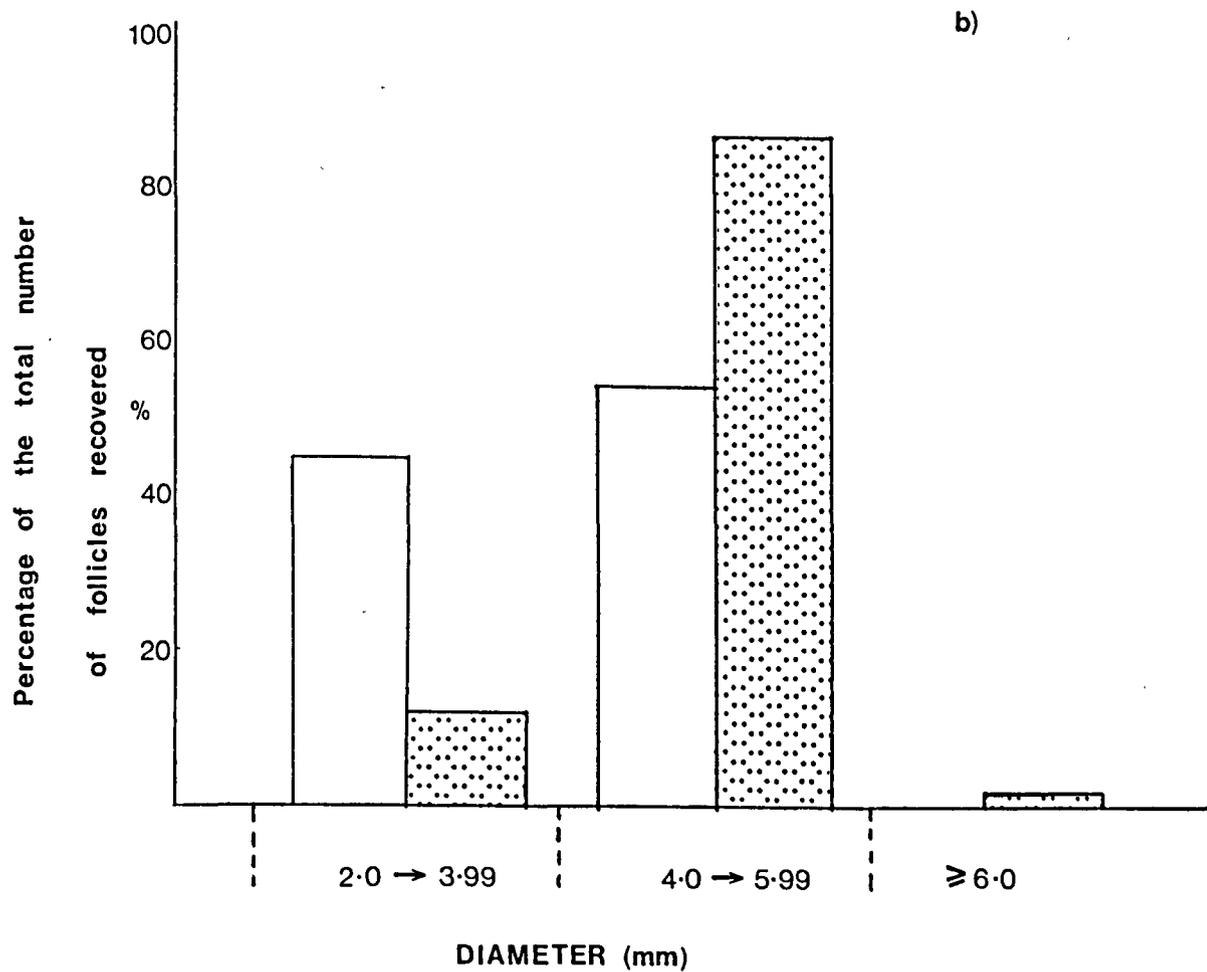
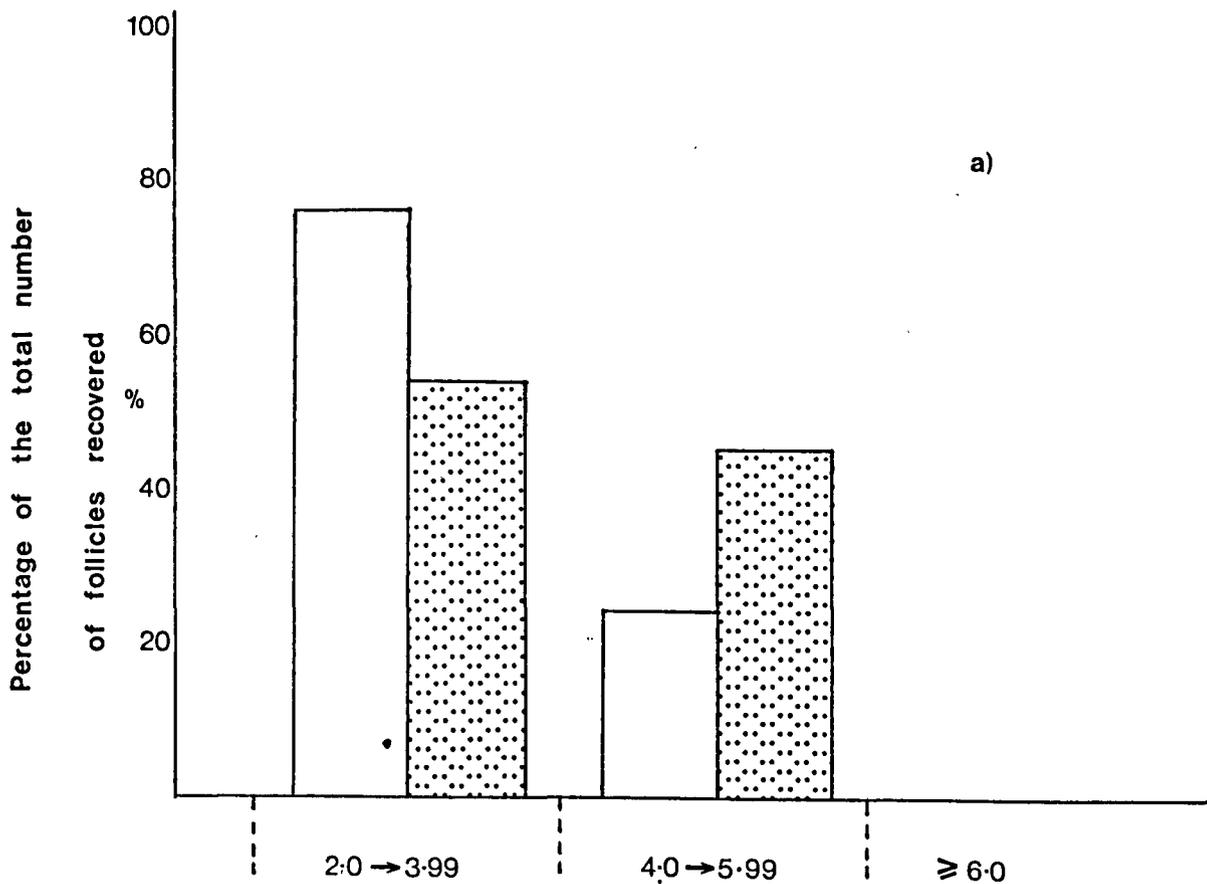


Table 4.2 Mean aromatase enzyme activity (pg E<sub>2</sub>/2h/foll), <sup>125</sup>I-hCG binding to granulosa cells and follicular fluid oestradiol concentrations (ng/ml) for individual control sows. The overall mean (± sem) of each variable is also shown.

CONTROL SOW NUMBER	MEAN AROMATASE (pg E <sub>2</sub> /2h/follicle)	MEAN <sup>125</sup> I-hCG GRAN. BINDING (cpm x 10 <sup>3</sup> /follicle)	MEAN FFE (ng/ml)	MEAN FFE (ng/ml) [for follicles ≥ 1.0 ng E <sub>2</sub> /ml]
5	381.0	2.13	0.69	1.30
16	1407.0	13.39	1.74	1.79
NT	474.0	1.63	2.63	2.63
26	1264.0	6.73	0.44	-
28	397.0	3.14	*	*
29	1040.0	12.67	0.42	-
30	1749.0	7.01	0.57	1.29
7	Removed from analysis			
OVERALL MEANS	959.0 ± 208.0	6.67 ± 1.82	1.08 ± 0.34	1.75 ± 0.31

136

\* Samples mislaid prior to assay.

Table 4.3 Mean aromatase enzyme activity (pg E<sub>2</sub>/2h/foll), <sup>125</sup>I-hCG binding to granulosa cells and follicular fluid oestradiol concentrations (ng/ml) for individual split-weaned sows. The overall mean (± sem) of each variable is also shown.

SPLIT-WEANED SOW NUMBER	MEAN AROMATASE (pg E <sub>2</sub> /2h/follicle)	MEAN <sup>125</sup> I-hCG GRAN. BINDING (cpm x 10 <sup>3</sup> /follicle)	MEAN FFE (ng/ml)	MEAN FFE (ng/ml) [for follicles ≥ 1.0 ng E <sub>2</sub> /ml]
1	1310.0	15.23	4.89	6.61
4	2090.0	16.85	4.14	5.34
11	693.0	8.45	3.02	4.06
18	971.0	19.10	0.72	1.86
21	444.0	10.73	0.52	1.31
34	437.0	10.03	1.49	2.65
27	662.0	10.51	0.39	-
33	1073.0	25.36	6.94	8.54
OVERALL MEAN	960.0 ± 194.0	14.53 ± 2.03	2.76 ± 0.85	4.34 ± 1.0

Table 4.4 Mean values of aromatase (pg E<sub>2</sub>/2h/foll), follicular fluid oestradiol concentration (ng/ml) and <sup>125</sup>I-hCG binding to granulosa tissue (cpm x 10<sup>3</sup>) following subdivision of follicular populations into three size categories

		FOLLICULAR DIAMETER (mm)		
		2.0-3.99	4.0-5.99 mm	>6.0 mm
CONTROL	AR	603.0 (n = 254)	1300.0 (n = 80)	- (n = 0)
	FFE*	1.68	0.77	-
	BG	2.89	10.35	-
SPLIT- WEANED	AR	945.0 (n = 188)	1111.0 (n = 159)	984.0 (n = 2)
	FFE*	1.70	2.86	3.75
	BG	4.51	14.54	-

\* Overall mean levels calculated from the top 20 follicles/sow/group.

Figure 4.5      Regression analysis between aromatase enzyme activity (pg E<sub>2</sub>/2h/foll) and the concentration of follicular fluid oestradiol (ng/ml: follicles with  $\geq 1.0$  ng E<sub>2</sub>/ml).  
s.e. ; standard error of the slope.

F  
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EXPT3:REGRESSION ANALYSIS AROM (pgE2/2Hrs/Follicle) v FF E2 (ng/ml)

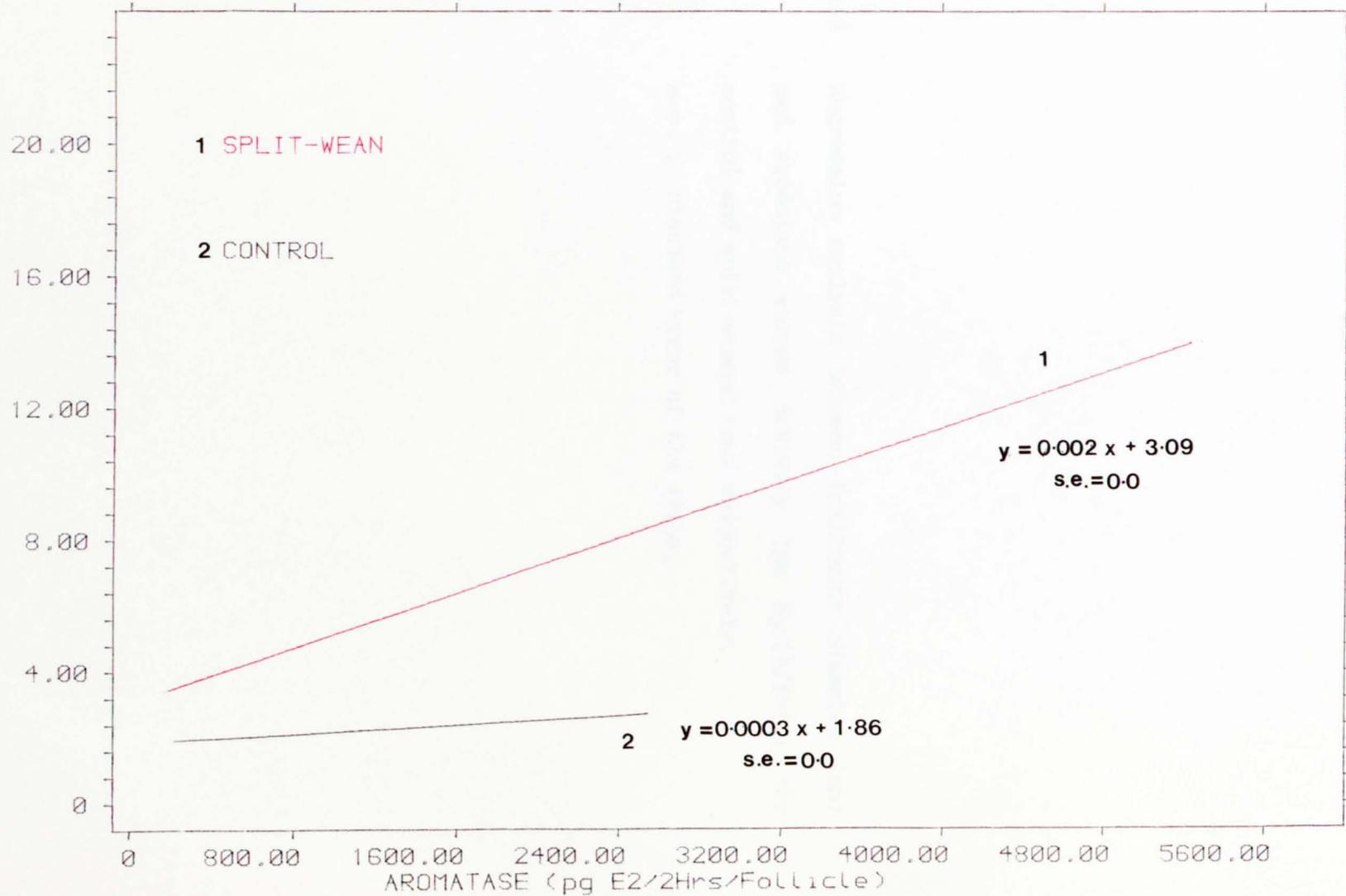
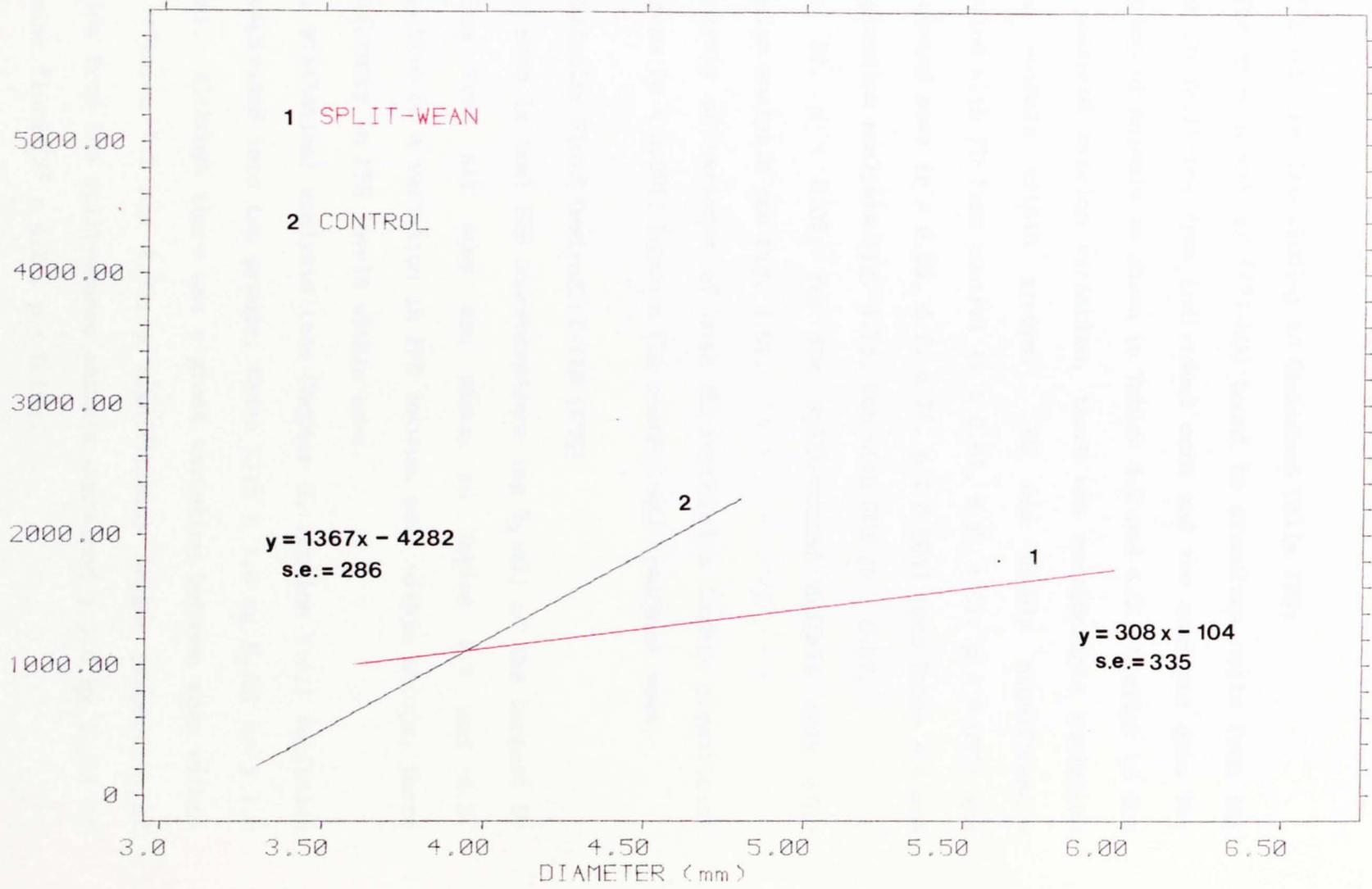


Figure 4.6 Regression analysis between follicular diameter (mm) and aromatase enzyme activity (pg E<sub>2</sub>/2h/foll) for control and split-weaned sows respectively.

s.e. ; standard error of the slope.

EXPT3:REGRESSION ANALYSIS DIAMETER (mm) v AROMATASE(pg/2Hrs/FOLL)

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(  
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F  
O  
L  
L  
I  
C  
L  
E  
)



b)  $^{125}\text{I}$ -labelled hCG binding to Granulosa Cells (BG)

The mean amount of  $^{125}\text{I}$ -hCG bound to granulosa cells from the largest 20 follicles from individual sows and the combined data for each group of animals is shown in Tables 4.2 and 4.3. Similar to the other measured ovarian variables, there was considerable variation between animals within groups. BG was highly significantly correlated with FD from control ( $r = 0.61$ , d.f. = 61,  $p < 0.001$ ) and split-weaned sows ( $r = 0.66$ , d.f. = 74,  $p < 0.001$ ) (see Table 4.4 and for regression analysis Fig. 4.7), but with FFE ( $r = 0.37$ , d.f. = 29,  $p < 0.05$ ) for the split-weaned animals only (for regression analysis see Fig. 4.8).

Analysis of variance of mean BG revealed a highly significant difference ( $p < 0.001$ ) between the control and treatment sows.

c) Follicular Fluid Oestradiol- $17\beta$  (FFE)

The mean ( $\pm$  sem) FFE concentrations (ng  $\text{E}_2$ /ml) of the largest 20 follicles from all sows are shown in Tables 4.2 and 4.3. Irrespective of a variation in FFE between sows within groups, there was uniformity in FFE levels within sows.

For statistical analysis (see Chapter 3, Section V(c)) follicles were subdivided into two groups; those with  $< 1.0$  ng  $\text{E}_2$ /ml or  $\geq 1.0$  ng  $\text{E}_2$ /ml. Although there was a great variation between sows within groups (Tables 4.2 and 4.3), a significantly larger proportion of follicles from the split-weaned animals contained  $\geq 1.0$  ng  $\text{E}_2$ /ml of follicular fluid ( $\chi^2 = 4.22$ ,  $p < 0.05$ ).

FFE was significantly correlated with AR (see Section IIIb(ii)a) and BG (see Section IIIb(ii)b) for the split-weaned animals only. There was no significant relationship between FFE and FD for either group of animals.

Figure 4.7      Regression analysis between follicular diameter (mm) and  $^{125}\text{I}$ -hCG binding ( $\text{cpm} \times 10^3$ ) to granulosa cells for the control and split-weaned sows respectively.

s.e. ; standard error of the slope.

GRANULOSA  
BINDING  
(CPM X  
10<sup>-3</sup> /  
POLL)

EXPT3:REGRESSION ANALYSIS DIAMETER(mm) v GRAN.BIND.(cpm x 10<sup>-3</sup>/POLL)

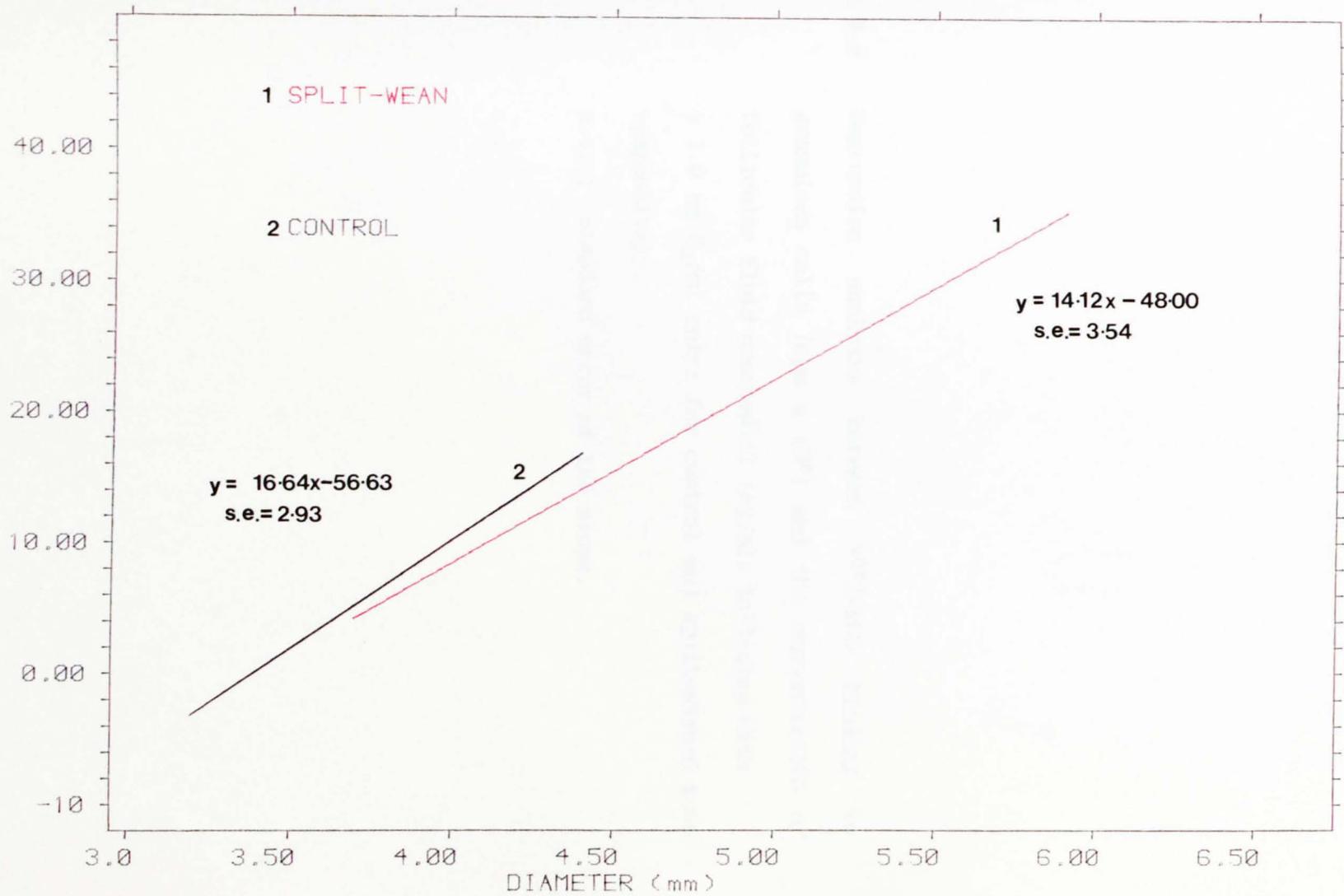
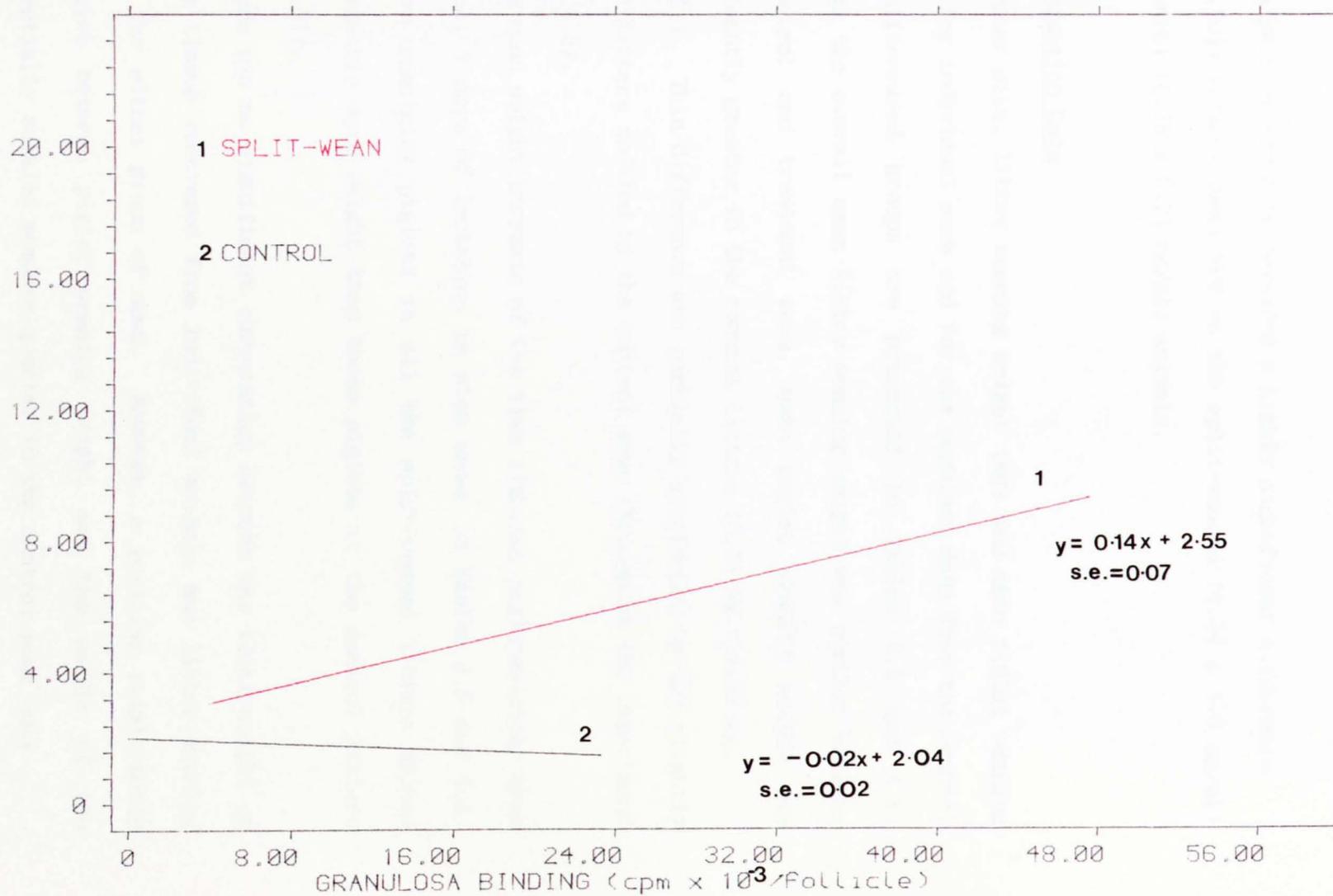


Figure 4.8 Regression analysis between  $^{125}\text{I}$ -hCG binding to granulosa cells ( $\text{cpm} \times 10^3$ ) and the concentration of follicular fluid oestradiol ( $\text{ng/ml}$ : follicles with  $\geq 1.0 \text{ ng E}_2/\text{ml}$  only) for control and split-weaned sows respectively.

s.e. ; standard error of the slope.

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EXPT3:REGRESSION ANALYSIS GRAN.BIND( $\text{cpm} \times 10^3/\text{folL}$ ) v FF E2 (ng/ml)



Analysis of variance revealed a highly significant difference ( $p < 0.001$ ) between mean FFE in the split-weaned ( $4.34 \pm 1.0$  ng/ml) and control ( $1.75 \pm 0.31$  ng/ml) animals.

c) Production Data

Litter size, litter weaning weight (kg) and mean piglet weaning weight for individual sows and for the combined data from the control and split-weaned groups are presented in Tables 4.5 and 4.6. Although the overall mean litter weaning weight was similar between the control and treatment sows, mean piglet weaning weight was significantly greater in the control litters (6.11 kg v 5.60 kg,  $p < 0.01$ ). This difference was partially attributed to the slightly smaller litters suckled by the control sows throughout the experiment (Table 4.5).

The mean weight increase of the five lightest piglets/litter over the final 7 days of lactation is also shown in Tables 4.5 and 4.6. The five remaining piglets in all the split-weaned litters gained significantly more weight than those piglets in the control litters ( $p < 0.01$ ).

There was no significant correlation between the total weight of mammary tissue recovered from individual animals and litter weaning weight for either group of sows. However, a positive relationship did exist between piglet weaning weight and the weight of the preferentially suckled mammary quarter in the control sows only ( $r = 0.44$ , d.f. = 74,  $p < 0.001$ ). It was also apparent that in 75% of the split-weaned litters and 57% of the control litters, the

Table 4.5 Litter size, litter weaning weight (kg), mean piglet weaning weight (kg) and the weight gain (kg) of the 5 lightest piglets over the final week of lactation for individual control sows. Overall means ( $\pm$  sem) are also shown.

CONTROL SOW NUMBERS	LITTER SIZE	LITTER WEANING WEIGHT	MEAN PIGLET WEANING WEIGHT (kg)	WEIGHT GAIN OF 5 LIGHTEST PIGLETS OVER FINAL 7 DAYS OF LACTATION
[7	9	38.5	4.3]*	
NT	10	67.3	6.7	NR
16	9	54.0	6.0	NR
5	10	58.7	5.9	NR
26	8	49.0	6.1	+8.5
30	9	52.8	5.9	+8.2
29	8	49.3	6.2	+8.2
28	9	53.9	6.0	+6.9
	9.00 $\pm$ 0.27	55.00 $\pm$ 0.38	5.89 $\pm$ 0.26	+7.95 $\pm$ 0.25

\* Sow 7 removed from overall analysis because of agalactia.

NR ; Values not recorded.

Table 4.6 Litter size, litter weaning weight (kg), mean piglet weaning weight (kg) and the weight gain (kg) of the 5 lightest piglets over the final week of lactation for individual split-weaned sows. Overall means ( $\pm$  sem) are also shown.

SPLIT-WEANED SOW NUMBER	LITTER SIZE	LITTER WEANING WEIGHT	MEAN PIGLET WEANING WEIGHT (kg)	WEIGHT GAIN OF 5 LIGHTEST PIGLETS OVER FINAL 7 DAYS OF LACTATION
1	10	57.0	5.7	+10.5
4	11	59.8	5.4	+12.5
11	10	54.6	5.5	+11.3
18	10	56.9	5.7	+9.0
21	9	52.8	5.9	+8.6
34	10	50.1	5.0	+9.6
27	11	63.5	5.8	+11.2
33	10	58.4	5.8	+11.3
OVERALL MEAN VALUES	10.13 $\pm$ 0.23	56.64 $\pm$ 1.48	5.60 $\pm$ 0.10	10.5 $\pm$ 0.47

heaviest piglets at weaning had been suckling the pectoral teats (see Figures 4.9 and 4.10).

The total weight of mammary tissue from the control sows <sup>(7.40kg)</sup> was significantly greater ( $p < 0.05$ ) than that recovered from the split-weaned animals (6.50kg).

**Figures 4.9 and 4.10** Schematic diagrams depicting the relationship between final piglet weaning weight (kg) and the position of the quarter(s) preferentially suckled by each piglet for all litters from the control and split-weaned sows respectively. As the majority of piglets after split-weaning were observed to 'multiple' suckle, only the position of the preferred quarter is represented.

B represents a 'blind teat' which produced no milk

\* an asterix highlights the heaviest piglet in each litter.

Fig. 4.9

CONTROL SOW NUMBERS	PECTORAL		PAIRS OF MAMMARY QUARTERS			POSTERIOR	
	1	2	3	4	5	6	7
NT	7.3	4.8	6.8	6.0	7.7*	6.8	6.8
	7.1	B	7.2	6.0	7.7	6.8	B
16	7.0*	6.1	B	B	5.2	6.9	B
	6.3	5.8	6.5	B	5.1	5.1	6.9
5	6.8	B	5.0	6.2	5.9	5.6	5.0
	6.9*	6.4	5.8	6.2	5.9	5.6	5.0
26	6.2	5.9	5.1	B	B	B	6.6
	7.5*	5.9	7.2	B	B	B	6.6
30	5.9	5.9	5.9	5.6	8.0*	6.4	6.1
	5.0	6.1	6.1	B	B	B	B
29	6.3	6.8	6.8	B	6.2	6.8	4.0
	7.1*	6.6	B	B	B	5.0	4.0
28	B	5.7	B	6.8	7.0	5.1	5.2
	5.0	B	6.0	B	7.4*	5.7	5.2

B = blind teat

Fig. 4.10

SPLIT-WEANED SOW NUMBERS	PECTORAL		PAIRS OF MAMMARY QUARTERS			POSTERIOR	
	1	2	3	4	5	6	7
1	7.2			4.6	5.9	7.1	
	7.3*				B	B	
4		6.9*		6.2	B	6.2	
	6.8					6.5	
11		5.9	B	B		6.2	
	6.4*		5.8			6.4*	
18	6.1	6.1					
	6.9*			6.3	5.9		
21		7.0*	6.4	6.2			6.3
	6.4						
27	7.7*		6.8	5.4		5.9	6.4
						B	
33	7.2*	7.6		B	B	B	B
		6.4			7.0		5.4
34				5.0			6.1
	5.8*	5.5				4.6	

B = blind teat

#### IV DISCUSSION

##### a) Ethological Study

The ability of piglets to consistently suckle at one particular teat was strikingly apparent in this experiment and has been extensively detailed in many early studies (Donald, 1937b; Barber, Braude and Mitchell, 1955; Gill and Thompson, 1956; McBride, 1963). McBride (1963) broadly suggested that sight, smell and recognition of neighbours helped piglets locate their chosen teat, but it was the elegant studies of Jeppesen (1982b) which conclusively showed that piglets distinguish a teat by means of individual olfactory chemical cues. She suggested that marking the preferred teat area may have been a form of territorial behaviour in which piglets were "warned" away from "owned" teats during the formative stages of the teat order. Kittens (Ewer, 1960) and rat pups (Hofer, Shair and Singh, 1976; Singh and Hofer, 1978) are also thought to recognise their own nipples by smell but for these animals, the odour is said to act as a release of sucking behaviour.

The time taken to form a stable teat order in this study is in agreement with previous observations by Donald (1937b) and McBride (1963). Indeed teat selection appeared to develop initially on the front and rear teats with more "errors" being made by piglets on the middle teats than by those at either extremity. Most reports of observations on teat order and competition among piglets agree that the anterior teats are the most popular (Donald, 1937a; Gill and Thompson, 1956; Hartsock, Graves and Baumgardt, 1977). The most common theory to explain this observation is that the pectoral teats

usually produce the most milk and are therefore more attractive to piglets (Donald, 1937b). In support of the latter, Gill and Thompson (1956) measured milk-intake by weighing piglets before and after feeds, and found that 15.3% more milk was produced by the front four teats than by the rear four. Conversely, some researchers have indicated that a particular gland is not consistently a large or small producer (Hartman, Ludwick and Wilson, 1962; Hartsock, Graves and Baumgardt, 1977). Although an accurate measurement of milk intake was not performed in this experiment, data from the control and split-weaned litters (Figures 4.9 and 4.10) indicate that those piglets suckling the anterior teats were amongst the heaviest at weaning. Furthermore the vacation of several teats at split-weaning enabled the remaining piglets to positively or negatively discriminate against the milk producing ability of these available quarters. In all but one (Fig. 4.3: Sow 34) of the split-weaned litters studied, vacated pectoral teats were quickly adopted by another piglet. Nevertheless, assuming that these larger piglets are generally more vigorous than their smaller siblings, it is likely that they empty their chosen gland more completely when suckling and thus stimulate greater milk production. Therefore if milk production does vary from teat to teat, it may largely be due to the ability of certain piglets to stimulate their glands efficiently as opposed to the metabolic secretory ability of the mammary tissue *per se*.

In the majority of the observed nursings, the sow gave a characteristic pattern of vocalisation consisting of an initial period of rhythmic grunting followed by a period of more rapid

grunting and then a decline toward the completion of nursing. At the same time the piglets displayed five distinct phases of suckling behaviour (see Section IIIa(ii)). These results and those of many previous authors (Fraser, 1973; Whittemore and Fraser, 1974) suggest that the pattern of vocalisation of the sow bears a reliable relationship to the suckling behaviour of the piglets and to the flow of milk. The phase of rapid sucking was only observed if there had been a period of rapid grunting and milk ejection occurred only in nursings which involved these two behaviour features. Thus, although some nursings appeared superficially normal, the sows grunt rate did not show an increase and milk "let down" did not occur. Such "unsuccessful" nursings have previously been described by Barber, Braude and Mitchell (1955), Folley and Knaggs (1966) and Whittemore and Fraser (1974). Nevertheless, throughout these observations there was a relatively low proportion of unsuccessful or incomplete nursings. Such failures were particularly common if nursing began soon after the previous milk ejection. Furthermore, the sows normally delayed a substantial time before attempting to nurse again. Other factors which caused nursing to fail were human disturbance, disorderly behaviour of the piglets during suckling and discomfort to the sow.

Suckling frequency was maintained at a constant rate after split-weaning and was comparable with that for control sows (Table 4.1). Although the exact duration of each suckling period was not monitored, suckling bouts were synchronised between all sows and consequently it was possible to observe that sows suckled for

approximately the same time. Nevertheless it was observed that the five piglets remaining after the removal of their litter-mates suckled two, three and even four teats. This phenomenon of "multiple suckling" has been described previously (McBride 1963; Wyeth and McBride, 1964; Shaw, 1984). In such cases, the piglets did not give equal attention to each teat, suckling one in preference to the other(s). Since it was impossible for the piglets to suckle several teats simultaneously, it is likely that, despite a constant suckling frequency, the suckling intensity of the split-weaned animals was reduced compared to the control animals. This suggestion is supported by the significant difference between the mammary tissue weights of the control and split-weaned sows. It is likely that the control litters stimulated greater mammary growth and milk synthesis throughout lactation.

#### b) Ovarian Data

The large number of follicles dissected from both groups of animals in this experiment is consistent with previous observations in the lactating sow (Palmer, Teague and Venzke, 1965a,b; Kunavongkrit, Einarsson and Settergren, 1982). These studies have reported that during early lactation, follicular development is characterised by a large population of small-sized follicles and a small population of medium-sized follicles. However as lactation progresses there is a gradual shift in number of follicles into medium or large sized categories and the proportion of atretic follicles declines. Although Kunavongkrit and his colleagues

(Kunavongkrit, Einarsson and Settergren, 1982) reported that after an 8 week lactation not more than 2% of follicles grew to a diameter > 4 mm, in the present study 24% of all follicles dissected from the control sows and 44.5% of those recovered from the split-weaned animals were within a range of 4.00 to 5.99 mm.

Since the follicles dissected from the split-weaned animals were significantly larger than those from the control animals, yet all sows were known to be at an identical stage of lactation it seems likely that the process of split-weaning had directly or indirectly influenced the factors controlling ovarian activity during lactation. Indeed previous reports where the split-weaning (Stevenson and Britt, 1981; Cox *et al.*, 1983; Stevenson and Davis, 1984) or partial weaning (Smith, 1961; Walker and England, 1978; Thompson, Hanford and Jensen, 1981; Stevenson and Davis, 1984) of litters has resulted in either earlier post-weaning returns to oestrus or an increased proportion of sows in oestrus at various pre- and post-weaning intervals, lend support to this theory. Unfortunately, the endocrine/ovarian events associated with this technique of manipulating litter size remain largely speculative. Undoubtedly the reduction in the intensity of the suckling stimulus is of critical importance. The detailed observations of the behaviour of the split-weaned litters concomitant with the disparity between total mammary tissue weight for the control and split-weaned sows, confirms the general assumption of early workers that split-weaning does decrease suckling intensity and the growth of mammary tissue. However the multiple suckling behaviour of the five remaining piglets indicates that the reduction

in mammary stimulation may not be as great as originally thought. Nevertheless the general enhancement of both the morphological and biochemical development of the ovary in the split-weaned sows suggests that the stimulus had dropped below the threshold level required to maintain a state of relative ovarian quiescence.

In harmony with Shaw (1984) this investigation demonstrated the presence of aromatase activity in granulosa tissue *in vitro* in the follicles of all the sows studied. However, similar to Experiment 1 in this thesis (see Chapter 2) there was a wide variation in the level of extant enzyme activity between animals within groups and as before, this range appeared to be directly related to follicular diameter and hence to follicular maturity. The significant relationship between aromatase activity and the concentration of follicular fluid oestradiol in the split-weaned sows supports the previous *in vitro* results for the preovulatory follicular study, and further confirms the "two cell" theory of follicular steroidogenesis (Falck, 1959; Haney and Schomberg, 1981; Evans *et al.*, 1981). However, the concentrations of oestradiol in the follicular fluid of follicles from both the control and split-weaned sows were 400 to 500 fold lower than those previously recorded for pre-ovulatory porcine follicles (Eiler and Nalbandov, 1977; Ainsworth *et al.*, 1980) and Experiment 1 of this thesis. This large difference emphasises the relative steroidogenic immaturity of even the largest follicles in this study and confirms earlier follicular studies in the lactating sow (Shaw, 1984).

Although the level of aromatase activity was similar between control and treatment sows, the significantly higher concentration of oestradiol in the follicular fluid of the treatment sows implies that it is the level of theca androgen substrate which is the factor limiting oestradiol synthesis in the control sows. Since it has been demonstrated that testosterone production is induced by LH stimulation (see reviews by Richards 1979; Leung and Armstrong, 1980) this would suggest that insufficient LH stimulation may be responsible for the inadequate supply of androgen precursor in these animals. This pattern of events was certainly reflected in the follicular data and is consistent with the significantly lower levels of  $^{125}\text{I}$ -hCG binding to the granulosa cells, and the lack of any significant correlation between follicular fluid oestradiol and  $^{125}\text{I}$ -labelled hCG binding for the control animals. By contrast the highly significant correlation between these two variables in the split-weaned sows suggests a functional relationship between LH binding and the activation of oestrogen synthesis in the marginally larger but more biochemically mature follicles recovered from these animals.

Current evidence from *in vivo* endocrine studies on the lactating and weaned sow substantiates this *in vitro* data. Experiments in pigs (Crighton and Lamming, 1969; Kunavongkrit, 1984) and cows (Short *et al.*, 1972; Randel, Short and Bellows, 1976) have shown that frequent suckling will continue to suppress concentrations of LH in the peripheral circulation. Furthermore, within 8-12 hours post weaning, the concentration of serum LH dramatically rises (Cox and Britt,

1982a, Edwards and Foxcroft 1983; Shaw and Foxcroft, 1985) with a concomitant increase in the frequency of episodic pulses of LH over the next 2-3 days (Cox and Britt, 1982c; Shaw and Foxcroft, 1985). Information concerning the endocrine profiles of sows nursing a reduced number of piglets is unfortunately limited and contradictory. While Shaw (1984) reported that plasma LH levels and the LH pulse frequency and amplitude were unaffected by reducing litter size, Kunavongkrit (1984) recorded a higher serum LH in sows nursing small (2-4 pigs) compared to normal (7-12 pigs) litters. However, in the latter study, the sows suckled a small litter throughout lactation and consequently on the evidence of the behaviour studies in this experiment, it seems unlikely that the suckling-induced suppression of LH secretion in Kunavongkrit's sows would be as intense as in the sows in Shaw's (1984) study.

In some of the split-weaned sows (Sows 18 and 27), although aromatase activity and granulosa binding were high relative to the other split-weaned and control animals, follicular fluid oestradiol was still low. This would indicate that an additional block may be operating at the level of the ovary. The general observation that a reciprocal relationship exists between prolactin and LH levels during suckling (Shaw and Foxcroft, 1985) and the association of hyperprolactinaemia with various infertility syndromes in man (see Edwards, 1980) has encouraged the view that lactational infertility is in part the consequence of a physiological suckling-induced hyperprolactinaemia. Indeed, there is some evidence to suggest that high levels of the hormone may have a direct inhibitory effect at an

ovarian (McNeilly et al., 1982; McNeilly and Baird, 1983) and pituitary level (Kann et al., 1977; Elsaesser and Parvizi, 1980). However, although prolactin can inhibit the induction by FSH of the rat aromatase system (Dorrington and Gore-Langton, 1982), data from this experiment would indicate that aromatase activity is evident in porcine granulosa cells when incubated *in vitro* after exposure to presumably elevated prolactin concentrations (Bevers et al., 1981; Stevenson, Cox and Britt, 1981; Kirkwood et al., 1984a; Shaw and Foxcroft, 1985) *in vivo*. Nevertheless, it is plausible that the removal of the follicles from the *in vivo* environment may have been sufficient to negate any inhibitory prolactin-mediated effects. To further confuse the issue, McNatty and his colleagues (McNatty, Sawers and McNeilly, 1974) have reported that granulosa cells require an optimal concentration of prolactin to express differential function. In fact Walters (Walters et al., 1982) has shown that although serum prolactin concentrations did not differ, pooled samples of follicular fluid from non-suckled cows had greater prolactin than suckled cows and the follicular LH receptors were more numerous in the non-suckled animals.

Hence, the close coupling of hyperprolactinaemia to the suckling stimulus makes it difficult to ascertain if either factor is responsible for the depression of LH secretion during lactation. Since a detailed endocrine study was not performed in this experiment, it is not possible to compare the endocrine status of individual sows between and within groups, nor to favour any one theory. Nevertheless, since previous experiments have shown no

obvious correlation between the level of serum prolactin in the sow and the number of piglets nursing (Bever, Willems and Kruip, 1978; Shaw, 1984) it seems unlikely that the level of serum prolactin was influenced by split-weaning and therefore the observed ovarian differences of individual sows between and within groups are probably independent of this hormone.

There is a paucity of information concerning whether the pulsatile release of oxytocin during reflex milk ejection (Ellendorff, Forsling and Poulain, 1982) affects follicular development during and after lactation in sows. Peters and his colleagues (1968, 1969a) reported that regular administration of oxytocin to sows whose litters were weaned on Day 6 of lactation did not affect pituitary levels of LH or FSH, or follicular development, compared with values for non-suckling controls. Further experiments in intact or mammillectomised sows, in the presence or absence of piglets, showed that oxytocin depressed follicular fluid weight only when the piglets were present. More recently, Ellendorff (1984) presented evidence that administration of intravenous pulses of oxytocin delayed oestrus for at least 3 weeks post-weaning, but he failed to report whether oxytocin blocked the increase in LH after weaning or whether sows ovulated without expressing oestrus. Although, it is recognised that the release of oxytocin in the sow is a direct consequence of mammary gland stimulation by the piglets, it has yet to be established if a positive relationship exists between the number of piglets nursing and the levels of circulating oxytocin or if a threshold level of stimulation is necessary to maintain

pulsatile oxytocin release during lactation. Hence, it is not possible to speculate whether split-weaning reduces the circulating levels of oxytocin at the time of nursing and whether this in turn would directly or indirectly affect follicular development. The results of Peter's (1968, 1969a) earlier studies certainly suggest that the presence of the piglets plus the action of oxytocin is of some significance.

c) Production Data

The majority of investigations which have attempted to induce ovulation in the lactating sow through the manipulation of litter size and suckling intensity (see Chapter 1, Section IIIId(ii)) have failed to record the performance of the piglets either during or subsequent to the experiment. However a few trials (Walker and England, 1977; Thompson and Jensen, 1979; Thompson et al., 1980; Stevenson and Britt, 1981) have reported weight gain in limited nursed piglets to be lower before weaning (Walker and England, 1977; Thompson and Jensen, 1979; Thompson et al., 1980) but similar (Thompson et al., 1980; Stevenson and Britt, 1981) or greater (Thompson and Jensen, 1979) than control litters after weaning. Indeed, the production data obtained from this study revealed few disadvantages to the piglets reared in the split-weaned litters. The significantly enhanced weight gain of the five lightest piglets in the treatment litters indicates that the multiple suckling behaviour of these piglets concomitant with the removal of any competition from

their heavier siblings had enabled them to suckle more milk and hence grow faster than equivalent animals in the control litters. Unfortunately, comparisons of performance were principally between the 5 lightest piglets of each group of sows and in hindsight it would have been interesting to compare the liveweight gains of the control piglets over the final week of lactation to the gains/losses of the 5 early-weaned piglets from the split-weaned litters which were fed milk substitute for 7 days in a thermoregulated "early-weaning" van.

In agreement with Newton et al. (1987), litter weaning weight was similar between control and treatment sows. Nevertheless, since the control sows had nursed slightly smaller litters, mean piglet weaning weight was significantly greater in these litters.

The correlation between piglet weaning weight and the weight of the preferentially suckled mammary quarter in only the control sows suggests that because the multiple suckling split-weaned piglets divided their attention unequally among two/three teats, they did not stimulate any one teat to the same extent as the control piglets. The significantly heavier total weight of mammary tissue recovered from the control sows further supports this suggestion. Hence, although suckling several teats allowed the split-weaned piglets access to greater quantities of milk, the reduced synthesis by the mammary tissue in the split-weaned sows suggests that the amount of piglet stimulation/teat was not as great as in the control sows.

## V CONCLUSION

The results of this experiment have shown that a certain degree of variable follicular development is detectable in the ovaries of the lactating sow irrespective of the number of piglets nursing. However, compared to previous studies on preovulatory porcine follicles (Eiler and Nalbandov, 1977; Ainsworth et al., 1980; Experiment 1), the follicles recovered from all sows were very immature. Nevertheless, the significantly advanced morphological and biochemical ovarian activity observed in the split-weaned sows emphasised a treatment effect.

In association with these ovarian changes, the comparative study of piglet suckling behaviour, concomitant with the observed regression in mammary tissue growth in the split-weaned sows, revealed that despite a constant suckling frequency, suckling intensity was undoubtedly reduced in the split-weaned litters. Since suckling promotes lactation, it has been postulated that it may inhibit ovulation by depressing the neural stimulus to gonadotrophin synthesis (Crighton and Lamming, 1969; Short et al., 1972; Randel, Short and Bellows, 1976; Kunavongkrit, 1984). Hence the removal of the five heaviest piglets from the split-weaned sows may have directly/indirectly enabled a partial "escape" of the hypothalamo-hypophysial axis from the inhibitory effects of lactation. As no detailed endocrine study was performed in this study, it is not possible to speculate if split-weaning also affects the circulating levels of prolactin and oxytocin during lactation. Whether these hormones have a direct inhibitory effect upon follicular development requires additional confirmation.

Since the levels of aromatase activity did not differ between treatments, it seems likely that the hormonal activation of this enzyme is independent of the neuroendocrine events related to suckling. However, the significantly greater concentrations of follicular fluid oestradiol in follicles recovered from the split-weaned animals implies that the supply of androgen substrate is probably the main factor limiting oestradiol production in the control sows. Previous workers have reported that the inability of thecal tissue to synthesise testosterone is due to an inadequate level of circulating LH. The low level of  $^{125}\text{I}$ -hCG binding to the granulosa cells of the control animals strongly supports this suggestion.

Although this experiment supplied some interesting ovarian data, the applied aspects to which these findings appertain must not be forgotten. The use of partial weaning to reduce the interval from parturition to conception in the lactating sow has proved unsatisfactory, partly because it is a labour intensive technique, but principally due to the conflicting results obtained from various trials (Smith, 1961; Walker and England, 1978; Thompson, Hanford and Jensen, 1981; Stevenson and Davis, 1984; Kirkwood, Smith and Lapwood, 1983). However, there are several ways in which split-weaning could be used in contemporary management systems. Weaning of all large piglets at 2 weeks and redistribution of the remaining small piglets among several sows would allow some piglets to be weaned early and some to be weaned 5 to 7 days later. Small piglets allowed to nurse for 5 to 7 extra days would, as in this study, receive more milk

because of less competition and this might improve their survival and post-weaning performance. Sows rearing small litters would probably be ready to be rebred at or near weaning which would neatly coincide with the normal onset of oestrus in the sows whose piglets were weaned 5 to 7 days previously.

## **CHAPTER 5**

### **EXPERIMENT 3**

**PIGLET SUCKLING BEHAVIOUR, HORMONAL CHANGES AND OVARIAN FOLLICULAR  
DEVELOPMENT IN LACTATING PRIMIPAROUS SOWS FOLLOWING SPLIT-WEANING  
AND MANIPULATION OF THE INTENSITY OF THE SUCKLING STIMULUS**

## I INTRODUCTION

The data from Experiment 2 (see Chapter 4) in association with the results of previous studies (Stevenson and Britt, 1981; Cox et al., 1983b; Stevenson and Davis, 1984) further confirm that the technique of split-weaning litters during lactation is an effective method of promoting follicular development. However, the ethological study of all the litters in the previous Experiment revealed that although split-weaning reduced the suckling intensity, the efficient 'multiple-suckling' behaviour of the remaining piglets was such that the difference in suckling intensity between control and split-weaned groups was not as great as originally anticipated. Consequently, to ensure that a reduction in the neural intensity of the suckling stimulus was achieved in this experiment, the number of mammary quarters available to the piglets was artificially restricted in one group of sows after split-weaning.

In addition to an analysis of ovarian activity at weaning/slaughter, detailed comparisons of LH, prolactin and oxytocin secretion were made between treated and control sows to investigate whether endocrine changes and ultimately follicular development may be initiated during lactation by manipulating the neural intensity of the suckling stimulus. A further study of piglet suckling behaviour was also performed in this Experiment.

## II MATERIALS AND METHODS

Thirty primiparous gilts were allocated to this experiment which took place from November 1986 to April 1987.

### a) Ethological Study

Between days seven to fourteen of lactation, the teat order of each litter and the number of mammary quarters preferentially suckled by each piglet was recorded as described previously (see Chapter 4, Section II). On the fourteenth day of lactation at 09.00h, the piglets from all the litters were weighed and the sows randomly allocated to one of three treatments, a) Control (n=9), b) Split-Weaned (n=10) and c) Cover (n=11). As in Experiment 2 (see Chapter 4) the heaviest piglets were removed from the split-weaned sows to leave each sow suckling only the five lightest piglets for the remaining seven days of lactation. The 'covered' animals were also split-weaned on day 14, but the number of mammary quarters available to be suckled by the five remaining piglets was reduced by strapping a piece of strong tent canvas around the sow in order to cover the 4/5 pairs of pectoral and anterior teats to leave only the three posterior pairs of teats exposed to the piglets (Plate 3). Cover sows were selected on the basis that this would offer at least five active mammary quarters to the remaining piglets. Control sows suckled an entire litter throughout the 21 day lactation.

Between day 14 and weaning on day 21 of lactation, the new teat order and the general suckling behaviour of piglets on the split-weaned and covered group sows were recorded. At weaning, all the piglets were re-weighed and the sows slaughtered.

**Plate 3** Showing Sow 104 with the canvas sheeting strapped around her anterior quarters. Only three posterior pairs of teats are left exposed to the piglets.



**b) Endocrinological Study**

Within each treatment group, a subset of eight sows received an indwelling cannula (see this chapter, section II(c)) on approximately the tenth day of lactation. 2.5 ml blood samples for subsequent LH and prolactin analysis were withdrawn at 15 minute intervals for 12 hours before and for 48 hours after split-weaning/covering. Control animals were sampled for 60 hours over the same stage of lactation.

Additional 10 ml samples for oxytocin analysis were taken at 15 second intervals from all sows during suckling periods. Samples were collected during three suckling periods/sow in the initial 12 hour pre-treatment bleed and this regimen was repeated 48 hours after split-weaning/covering and on the day before final weaning/slaughter. The presence/absence and the time of 'milk let down' was recorded during the withdrawal of these samples.

**c) Surgical Procedures**

Sows were initially sedated with an intra-muscular injection of 'Stresnil' (4% solution of azaperon or 4'fluoro-4[4-(2-pyridyl)-1-piperazinyl]-butyrophenone, Janssen, Pharmaceutica, Beerse, Belgium) at a dose of 2.2 mls/50 kg and then anaesthetized after 15 minutes with 'Hypnodil' (5% solution of metomidate, Janssen, Pharmaceutica, Belgium) administered intravenously, via an ear vein, at a dose of 3.3 ml/50 kg. The right ventral area of the neck was scrubbed with an antiseptic soap solution (Savlon - ICI Ltd., Macclesfield, Cheshire) and was then shaved free of hair. The whole area was swabbed with a skin antiseptic (Chlorhexidine; 0.5% in 70% spirit)

before an incision, approximately 200 mm in length, was made along the midline of a triangle formed by the point of the jawbone, the shoulder and the sternum. The fat and muscle layers in this region of the neck were then separated by blunt dissection to expose the external jugular vein which was dissected free of surrounding fascia. The anterior end of the exposed section of vein was ligatured and a silicone rubber cannula (Esco (Rubber) Ltd., Teddington, UK; internal diameter 1 mm, total diameter 4 mm) flushed with sterile heparinised saline, was inserted into the vein through a small incision until its tip was located anterior to the heart in the superior vena cava. To prevent the backflow of blood, a second ligature was tied around both vein and catheter, just below the point of insertion. In addition, to stop the cannula being forced or pulled out of the vein, a disc of silastic sheeting had been glued onto the cannula prior to surgery such that when the distal end of the cannula was exteriorised behind the ear using a stainless steel trochar, the disc was pulled firmly against the muscle block dorsal to the jugular vein, thus anchoring the cannula securely in position. A topical antibiotic spray was used before sealing the wound with continuous and purse string sutures. Finally, a thin layer of antiseptic film (Op-site, Smith and Nephew, Ltd., Welwyn Garden City, Herts.) sealed the wound from infection. The free end of the cannula was fitted with a 15 gauge adaptor (Becton and Dickenson Ltd., New Jersey, U.S.A.) and sealed with an obturator (Vygon, France). The cannula was wrapped with surgical tape and further protected from the piglets by two rectangular pieces of canvas which were velcroed together and

loosely sutured in position over the cannula. When blood sampling was in progress access to the cannula was easily achieved by separating the two pieces of canvas.

Sows received an intramuscular broad spectrum antibiotic (Depocillin, Mycoform Ltd., Braintree, Essex, UK) at a dose of 2 ml/50 kg for three days after surgery. In addition, the site of catheter exteriorization was treated with an antibiotic cream (Streptopen, Glaxo Laboratories) to prevent infection. The sows were returned to their litters as soon as they recovered sufficient coordination to obviate any danger of crushing their young; usually within two or three hours of initial removal to surgery. Blood sampling did not begin in any cannulated sow until at least two days after surgery was performed.

#### d) Blood Sampling Procedures

Blood samples were collected into heparinized polystyrene tubes (11 x 64 mm: LIP (Equipment and Services) Ltd., Shipley, W. Yorkshire or 16 x 95 mm: Starstedt, Leicester, UK) and immediately centrifuged at 1500 x g for 20 minutes at room temperature. Plasma was decanted into polystyrene storage tubes and stored on ice until frozen at -20°C.

Between blood samples cannulae were flushed with sterile heparinised saline (10 IU Heparin/ml) which also contained 0.5 mg/ml Ampicillin.

e) Radioimmunoassay of Hormones in Plasmai) Luteinising Hormone (LH)

Plasma LH concentrations were quantified by a recently validated homologous double-antibody radioimmunoassay using a locally produced goat antiserum (GRF-G81/1) raised against a purified porcine LH preparation SDG2-65 (0.96-1.18 x NIH-oLH S19) kindly provided by Dr. S.D. Glenn. The purified LH preparation, 'IVO' (0.77 x NIH-oLH SI), kindly provided by Dr. D.F.M. Van de Wiel, was used as reference standard and for radioiodination as previously described by Shaw and Foxcroft (1985). For the assay, 200  $\mu$ l plasma or standard (0.011 to 0.50 ng IVO pLH per tube) and 200  $\mu$ l goat antiporcine LH antiserum (at an initial dilution of 1:60,000 in PBS containing 1:600 normal goat serum (PBS/NGS)) were dispensed at least in duplicate into plastic disposable assay tubes using a Micromedic automatic dispenser, vortexed and incubated at 5°C for 24h. On the second day 100  $\mu$ l  $^{125}$ I-labelled pLH were added in assay diluent to give approximately 10,000 cpm per tube; tubes were again vortexed and incubated at 5°C for 24h. On the final day of the assay 200  $\mu$ l of a 1:40 dilution of donkey antigoat gamma globulin (AGGG raised locally) in a 10% solution of polyethylene glycol (PEG, Polyethylene glycol 6000, BDH Ltd., Poole) were added to all tubes (AGGG/PEG solution prepared and incubated for 24h at 5°C before addition to assay) and free and bound  $^{125}$ I-labelled LH were separated by centrifugation at 2000g for 30 min at 5°C after incubating with AGGG/PEG for at least 8h at 5°C. The dilution of first antibody used bound on average 18% of 5 total counts added in the absence of unlabelled hormone. Three

volumes of pooled plasma were included in all assays as internal controls; these showed parallelism to the standard curve and based on the potency estimates of these central plasmas between and within assays, the mean intra- and inter-coefficients of variance for an initial series of 22 assays were 9.4 and 18.0%, respectively. Cross reactivities of the antiserum to pFSH (NIH-FSH P2) and to pPRL (pPRL KK; 30 iu/mg) at 50% of total binding were 0.88 and 0.17%, respectively. The overall sensitivity of the assay was 0.02 ng per tube (approximately 87 to 90% binding) over the assays reported here; samples below assay sensitivity were therefore assigned a potency of 0.1 ng/ml. Accuracy, estimated as the recovery of LH standards from pig plasma ranged from 96 to 104%. Possible effects of inter-assay variability on the assessment of treatment effects were negated by including samples from sows in each of the three treatments in each assay.

ii) Prolactin (PRL)

Samples were assayed according to the method of Shaw and Foxcroft (1985) with no modifications. The intra and inter-assay coefficients of variance were 3.5 and 7.3% respectively and the mean assay sensitivity was 8.4 ng/ml.

iii) Oxytocin

Plasma oxytocin was quantified by the assay of Sheldrick et al. (1980) and Sheldrick and Flint (1981) with modifications. Oxytocin was extracted from plasma using Sep-Pak C<sub>18</sub> cartridges (Millipore, Harrow). Cartridges were washed with 10 ml 80% acetonitrile (Fison

Scientific Analysis, Loughborough) in 0.1% trifluoroacetic acid (Rathburn Chemicals Ltd., Walkerburn; sequencer grade) (AN/TFA), followed by 20 ml distilled water. Up to 6 ml plasma was then slowly applied to the cartridge. To standardize these procedures 10 cartridges were connected in parallel on the incoming end to reservoirs consisting of glass syringe barrels, and on the outflow side to a single vacuum flask evacuated by means of a water aspirator. Cartridges were then eluted with 3 ml AN/TFA into solvent resistant tubes and the eluate dried down in a water bath at 45°C to a volume of 0.5 ml under a stream of nitrogen. After adding 1 ml distilled water, samples were frozen at -20°C and then freeze dried. For assay, extracts were redissolved in 400 µl veronal assay buffer over a 1 to 2h period. Any material undissolved after this time was packed by centrifugation prior to dispensing 50 µl of the extract at least in duplicate into assay tubes. Extraction efficiency was determined for each set of extraction by pre-incubating 4 or 6 ml plasma with <sup>125</sup>I-labelled oxytocin providing approximately 30,000 cpm. Hot recovery tubes were counted after drying under nitrogen (i.e. without freeze drying and reconstituting the extract). Hot recoveries ranged from 71 to 93% (mean 84.6% over 25 sets of extractions), depending on first-time or second-time use of cartridges, and corrections were made for recovery in the final calculation of plasma potency. Routine extractions also included extracts of charcoal stripped 'blank' plasma and aliquots of a pool of blank plasma spiked with a known amount of oxytocin.

For the assay 50  $\mu$ l sample extract or standard (1.25 to 100 pg/tube), 100  $\mu$ l antiserum (GTI37 at an initial dilution of 1:20,000) and 100  $\mu$ l  $^{125}\text{I}$ -labelled oxytocin (approx. 7,000 cpm/tube) and 100  $\mu$ l assay buffer were added, vortexed and then incubated overnight at 4°C. Bound and free  $^{125}\text{I}$ -oxytocin was separated by the addition of 200  $\mu$ l of 30% PEG, mixing on a multivortex for 1 min and then centrifugation at 2,000g at 4°C for 30 min. Accuracy of the assay was confirmed by recovering unlabelled oxytocin from plasma; the addition of 100, 200 and 400 pg/4 ml plasma resulted in an estimated potency of 103 $\pm$ 12, 205 $\pm$ 3 and 405 $\pm$ 1 pg oxytocin per 4 ml sample (mean recovery 102%). Charcoal stripped 'blank' plasma gave an estimated potency of 3.75 pg/ml and the overall sensitivity of the assay taken as 85% of total binding on the standard curve across all assays was 3.0 pg/ml. For the purpose of analysis of the data therefore, samples were assigned a potency of 3.0 pg/ml if below assay sensitivity. The inter- and intra-assay coefficients of variance based on a blank plasma pool spiked with 100 pg oxytocin and run in all assays at least in duplicate were 12.3 and 6.0%.

### III STATISTICAL ANALYSIS

#### a) Calculation of Radioimmunoassay Data

See Chapter 3, Section V(a).

#### b) Analysis of Ovarian Data

See Chapter 3, Section V(c).

c) Interpretation of Endocrine Secretion Data

i) Characterisation of LH Pulses

A pulse was defined as any increase in the concentration of LH that:-

1. exceeded the 95% confidence limits for the baseline and peak samples
2. was completed within two sampling intervals
3. was followed by a decline in concentration that exceeded the 95% confidence limits, had at least two sample points between the peak value and succeeding trough or baseline and occurred at a rate no greater than the known half life of the hormone.

ii) Pulse Frequency

The number of pulses (as defined above) per unit time.

iii) Calculation of Maximum and Minimum LH and PRL Levels

The 60 hour frequent sampling period was divided into five 12 hour time blocks prior to computerized analysis of maximum and minimum levels following the method of Shaw and Foxcroft (1985). The highest and lowest points of the first eight values of any one block were extracted and a one sample (or 15 minute) shift was then made along the time axis before further extraction of the highest and lowest values of the next set of eight samples. This 'sliding window' analysis was repeated until a total of 40 values were obtained from which the maximum and minimum LH or PRL levels were calculated. The mean maximum LH value derived for any one 12 hour period was indicative of the mean pulse height and pulse frequency

during that time, whilst the mean minimum value for any one time block formed an estimate of mean basal levels during that 12 hour period.

iv) Statistical Analysis of LH and PRL

To determine whether significant differences existed in plasma hormone concentrations between groups of sows, the relevant data were subjected to split-plot analysis of variance appropriate for repeated measurements in individual animals (Gill and Hafs, 1971). Regression correlations were used to determine relationships between hormone levels and ovarian measurements.

v) Oxytocin Data

Statistical analysis of the oxytocin data was confounded by the variability in the number of samples collected/animal/suckling session and the time of peak oxytocin in plasma in relation to milk letdown. Therefore to standardise the results, the data have been plotted with respect to the peak concentration of oxytocin released at each suckling session/sow (Time 0; Fig. 5.10). A considerable proportion of samples assayed had oxytocin concentrations below assay sensitivity; any sample below assay sensitivity was given an estimated potency of 3 pg/ml. Hence, beneath each mean value plotted on Fig. 5.15 the x/y indicates x number of samples measured above assay sensitivity and y below (and given a potency of 3 pg/ml); and  $x + y$  indicates the number of samples assayed from the total number of oxytocin bleeds analysed on that day (n). Each set of oxytocin data within days and treatment groups contains data from each sow in

that group; in cases where n exceeds the number of sows studied, samples from other 'oxytocin' bleeds on that day were analysed. Because of technical limitations and the expense of these assays not all oxytocin samples were assayed; priority was given to samples from covered sows on the last day of lactation as the comparison between the pattern of oxytocin release at this time and oxytocin release in the pretreatment bleeds represented the extremes of suckling intensity.

#### IV RESULTS

##### a) Ethological Study

##### i) Development of a Fixed Teat Position

The initial behaviour of the piglets in this study was identical to that observed in Experiment 2 (see Chapter 4, Section III(a)).

##### ii) General Suckling Behaviour (see Chapter 4, Section III(a)).

##### iii) Suckling Behaviour in Split-Weaned Litters

Initial suckling behaviour in the split-weaned litters was comparable to that observed in Experiment 2 (see Chapter 4, Section III(a)). Within 24 hours of split-weaning 67.5% of the piglets were suckling an average of 2 teats each, compared to only 15% before split-weaning. Of these piglets, 4.1% suckled their original chosen teat plus another within close proximity, while 22.2% moved to completely new teats vacated by their heavier litter-mates. A significantly lower proportion of piglets (29.1%;  $\chi^2 = 16.06$ ,  $p < 0.001$ ) were observed to multiple suckle in the control litters.

#### iv) Suckling Behaviour in Covered Litters

Piglets which originally suckled a preferred teat in the posterior region of the sow were generally unaffected by the canvas sheet which covered the anterior quarters. Of the ten litters studied, 42% of piglets suckled posterior quarters. Once the canvas cover was in position 76.2% of those piglets continued to suckle their original teat while 23.8% moved to quarters vacated by their siblings.

For piglets which originally suckled a pectoral quarter, the canvas cover rendered their chosen teat inaccessible (see Plate 3). These piglets were notably distressed by the unavailability of their preferred teat and for several suckling bouts after covering they ignored the available vacated posterior quarters and moved around the sow squealing loudly. However, within 36 hours, these piglets started to suckle one of the available posterior teats. While this new 'teat order' was being established, fighting between the 'pectoral' and 'posterior' piglets was common as the latter strongly defended their original chosen teat.

#### v) Suckling Frequency

Similar to Experiment 2 (Chapter 4, Section III(a)), suckling was synchronised between all sows irrespective of treatment group. Consequently it was possible to visually assess that no group suckled more or less often than another.

b) Ovarian Study

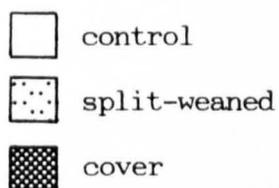
At slaughter the ovaries from sow 46 (Cover Group) were abnormally small and contained no dissectable follicles. The analysis of the ovarian data does not therefore include results from this animal.

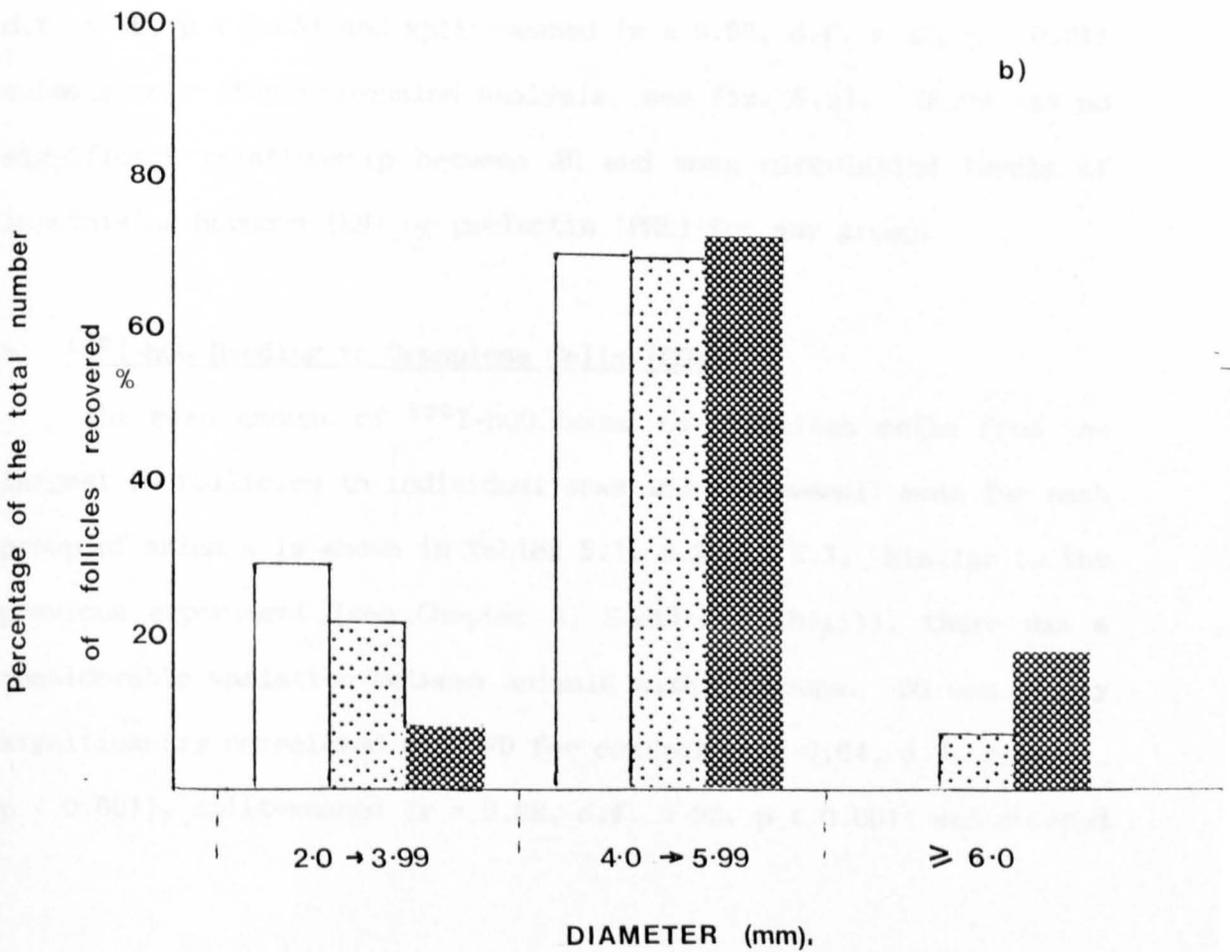
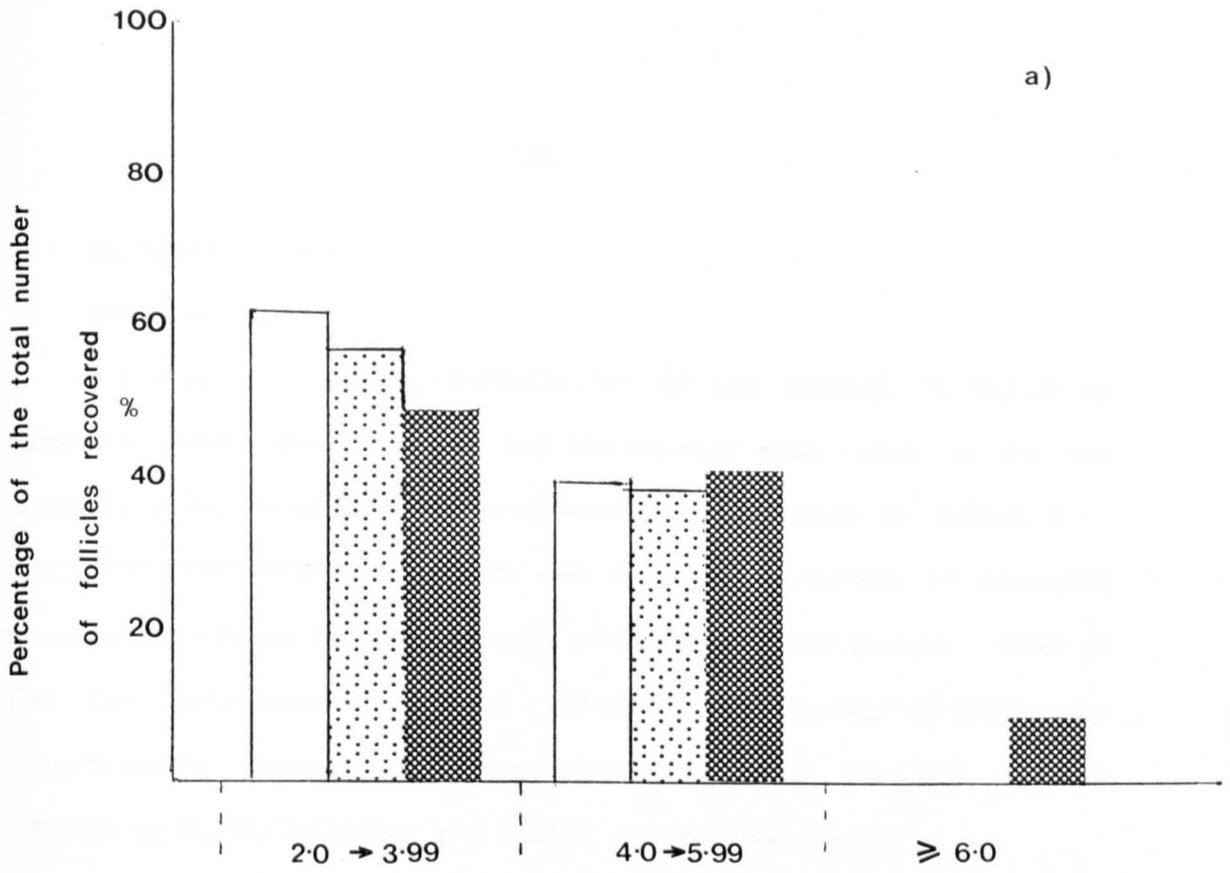
i) Morphological Data

The size distribution of follicles dissected from the control (n = 404, mean number of follicles recovered/sow =  $44.89 \pm 2.37$ ), split-weaned (n = 413, mean number of follicles recovered/sow =  $41.3 \pm 3.54$ ) and covered (n = 341, mean number of follicles recovered/sow =  $34.1 \pm 5.27$ ) sows is shown in Figure 5.1a. The mean diameter ( $\pm$ sem) of follicles recovered from the covered sows ( $4.31 \pm 0.08$ ) was significantly larger ( $p < 0.001$ ) than those dissected from the split-weaned ( $4.00 \pm 0.06$ ) and control sows ( $3.74 \pm 0.04$ ).

When larger numbers of dissected follicles were available, further analysis was restricted to the largest 20 follicles/pig. These follicles ranged from 3.06 to 5.64 mm (n=180), 3.06 to 10.43 mm (n=196) and 3.64 to 9.30 mm (n=177) in the control, split-weaned and cover groups respectively (see Fig. 5.1b). For these follicular populations, there were significant ( $p < 0.001$ ) differences between the mean FD ( $\pm$ sem) of both the covered ( $5.23 \pm 0.10$ ) and split-weaned ( $4.75 \pm 0.10$ ) sows and the control ( $4.21 \pm 0.04$ ) animals, and between the covered group and split-weaned animals ( $p < 0.001$ ).

**Figure 5.1** Histograms showing the size distribution of a) all dissected follicles and b) the largest 20 follicles within restricted size classes (2.0-3.99 mm, 4.0-5.99 mm,  $\geq 6.0$  mm diameter) for control, split-weaned and cover animals respectively.





ii) Biochemical Data

## a) Aromatase (AR)

The mean AR (pg E<sub>2</sub>/2h/follicle) of the largest 20 follicles dissected from individual sows and the overall mean ( $\pm$ sem) AR for the control, split-weaned and covered sows are presented in Tables 5.1, 5.2, 5.3, respectively. There was a large variation in measured aromatase activity between animals within the three groups. Mean AR for the split-weaned animals (2413 $\pm$ 807 pg E<sub>2</sub>/2h/follicle) was significantly greater than the values recorded for the control (804 $\pm$ 52 pg E<sub>2</sub>/2h/follicle;  $p < 0.001$ ) and covered groups (1838 $\pm$ 302 pg E<sub>2</sub>/2h/follicle;  $p < 0.05$ ). Mean AR for the covered sows was also significantly greater ( $p < 0.001$ ) than the control group.

AR was significantly correlated to FFE for the control ( $r = 0.37$ , d.f. = 26,  $p < 0.05$ ) and split-weaned ( $r = 0.50$ , d.f. = 33,  $p < 0.01$ ) animals only (for regression analysis, see Fig. 5.2). There was no significant relationship between AR and mean circulating levels of luteinising hormone (LH) or prolactin (PRL) for any group.

b) <sup>125</sup>I-hCG Binding to Granulosa Cells (BG)

The mean amount of <sup>125</sup>I-hCG bound to granulosa cells from the largest 20 follicles in individual sows and the overall mean for each group of animals is shown in Tables 5.1, 5.2 and 5.3. Similar to the previous experiment (see Chapter 4, Section IIIb(ii)), there was a considerable variation between animals within groups. BG was highly significantly correlated with FD for control ( $r = 0.64$ , d.f. = 78,  $p < 0.001$ ), split-weaned ( $r = 0.88$ , d.f. = 95,  $p < 0.001$ ) and covered

Table 5.1 Showing mean aromatase activity,  $^{125}\text{I}$ -hCG binding to granulosa cells, and the concentration of follicular fluid oestradiol in follicles from individual control sows. The overall mean ( $\pm$ sem) for each variable is also shown.

CONTROL SOW NUMBERS	MEAN AROMATASE (pg $\text{E}_2$ /2h/follicle)	MEAN $^{125}\text{I}$ -hCG GRANULOSA BINDING (cpm $\times 10^3$ /follicle)	MEAN FFE (ng/ml)	MEAN FFE (for follicles with $>1$ ng $\text{E}_2$ /ml)
47	514	3.06	1.12	1.31
51	727	14.56	1.86	2.49
52	783	2.89	0.43	-
65	821	14.51	0.82	1.09
76	469	6.30	0.35	-
90	617	14.65	0.29	-
97	1788	20.21	1.17	2.14
109	703	2.80	0.86	1.10
111	811	4.52	1.05	1.35
	804 $\pm$ 52	9.28 $\pm$ 0.65	0.88 $\pm$ 0.06	1.58 $\pm$ 0.24

Table 5.2 Showing mean aromatase activity,  $^{125}\text{I}$ -hCG binding to granulosa cells, and the concentration of follicular fluid oestradiol in follicles from individual split-weaned sows. The overall mean ( $\pm$ sem) for each variable is also shown.

SPLIT-WEANED SOW NUMBERS	MEAN AROMATASE (pg $\text{E}_2$ /2h/follicle)	MEAN $^{125}\text{I}$ -hCG GRANULOSA BINDING (cpm x $10^3$ /follicle)	MEAN FFE (ng/ml)	MEAN FFE (for follicles with $>1$ ng $\text{E}_2$ /ml)
38	2080	14.19	0.54	1.39
50	7844	107.20	2.44	5.82
53	6235	23.33	5.51	7.22
57	655	11.46	0.95	1.34
64	665	7.09	0.56	-
75	2165	14.04	1.97	4.11
77	478	2.46	1.73	5.57
98	975	9.71	1.07	2.29
102	859	7.28	1.22	2.14
103	2180	14.55	3.20	5.13
	2414 $\pm$ 808	21.13 $\pm$ 9.73	1.92 $\pm$ 0.48	3.89 $\pm$ 0.72

Table 5.3 Showing mean aromatase activity,  $^{125}\text{I}$ -hCG binding to granulosa cells, and the concentration of follicular fluid oestradiol in follicles from individual cover sows. The overall mean ( $\pm$ sem) for each variable is also shown.

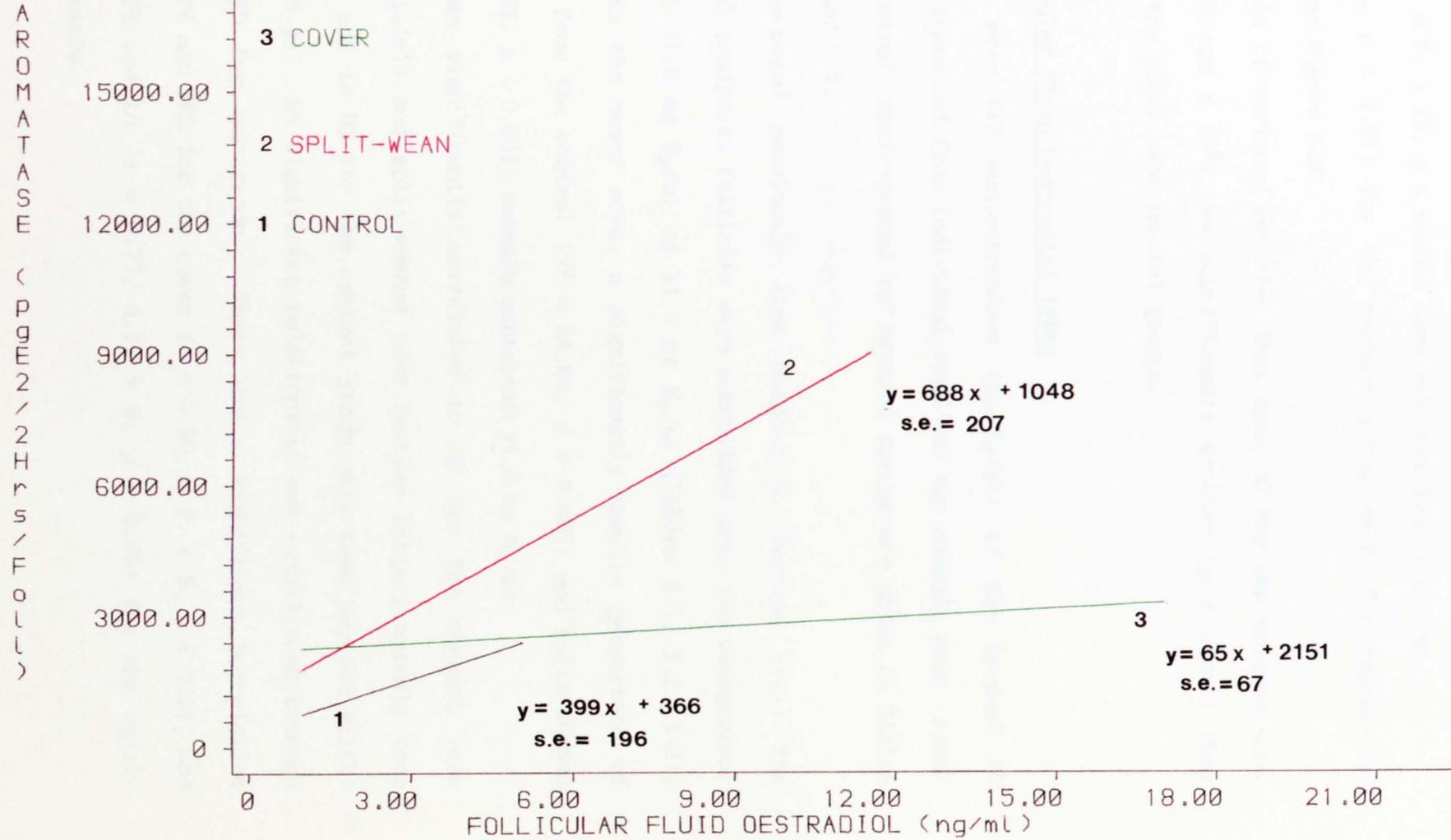
COVER SOW NUMBERS	MEAN AROMATASE (pg $\text{E}_2$ /2h/follicle)	MEAN $^{125}\text{I}$ -hCG GRANULOSA BINDING (cpm x $10^3$ /follicle)	MEAN FFE (ng/ml)	MEAN FFE (for follicles with $>1$ ng $\text{E}_2$ /ml)
42	1827	16.96	1.21	2.62
43	842	17.15	2.15	3.41
66	2080	17.70	2.12	4.02
68	2913	84.60	6.03	6.79
70	3167	28.30	3.54	4.86
71	2602	87.31	1.33	1.51
73	483	13.31	3.29	3.98
94	2418	7.26	4.54	5.23
101	1459	18.50	6.14	13.78
104	588	43.80	1.55	3.04
	1838 $\pm$ 306	33.49 $\pm$ 9.28	3.19 $\pm$ 0.59	4.92 $\pm$ 1.09

\* Sow 46 - no follicular growth

**Figure 5.2** Regression analysis between aromatase enzyme activity (pg E<sub>2</sub>/2h/follicle) and follicular fluid oestradiol (ng/ml) for the control, split-weaned and cover groups respectively.

s.e. ; standard error of the slope.

EXPT4:REGRESSION ANALYSIS:FFE2 (ng/ml) v AROM.(pgE2/2Hrs/Foll)



( $r = 0.85$ , d.f. = 79,  $p < 0.001$ ) sows but with FFE ( $r = 0.46$ , d.f. = 22,  $p < 0.05$ ) for the control group only (for regression analysis see Figure 5.3).

Analysis of variance revealed that mean BG for the covered sows ( $33.49 \pm 9.28$  cpm  $\times 10^3$ ) was significantly greater ( $p < 0.001$ ) than either of the other experimental groups.

### c) Follicular Fluid Oestradiol (FFE)

The mean FFE concentrations (ng  $E_2$ /ml) of the largest 20 follicles dissected from individual sows and the overall mean ( $\pm$ sem) for the control, split-weaned and covered groups are shown in Tables 5.1, 5.2 and 5.3.

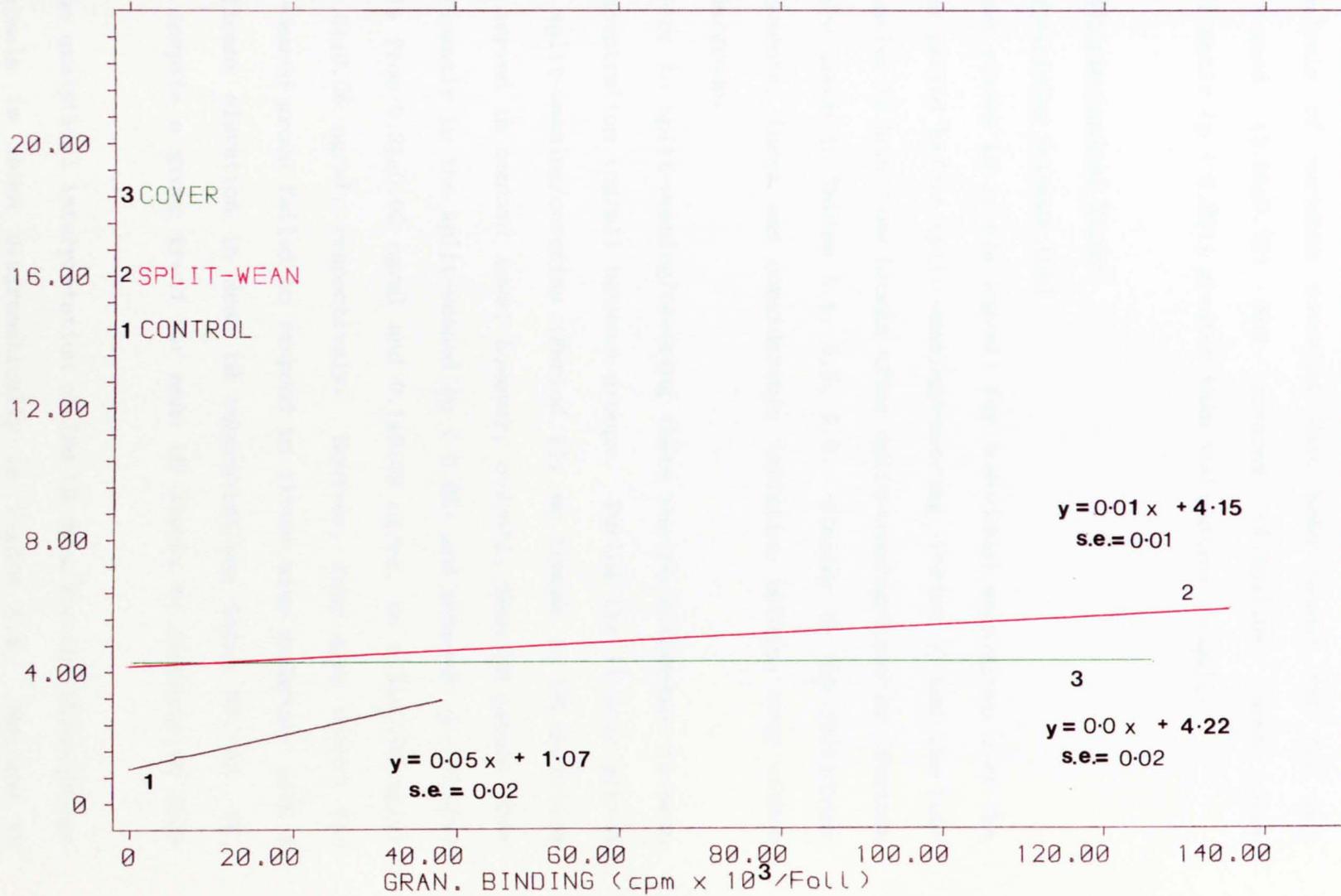
As discussed previously (see Chapter 3, Section V(c)) for statistical analysis, follicles were subdivided into two categories; those with  $< 1.0$  ng  $E_2$ /ml or  $\geq 1.0$  ng  $E_2$ /ml (Tables 5.1, 5.2, 5.3). Compared to the cover sows, a significantly smaller proportion of follicles from the control ( $\chi^2 = 34.60$ ,  $p < 0.001$ ) and split-weaned ( $\chi^2 = 22.99$ ,  $p < 0.001$ ) animals contained  $\geq 1.0$  ng  $E_2$ /ml.

FFE was significantly correlated to AR for the control (see Section Bii(a)) and split-weaned (see Section Bii(a)) animals (see Fig. 5.2) and to BG for the control group only (see Section Bii(b) and Fig. 5.3). No significant relationship was established between FFE and FD for any group. There was a significant correlation between FFE and PRL for the cover ( $r = 0.80$ , d.f. = 5,  $p < 0.05$ ) and between FFE and LH ( $r = 0.77$ , d.f. = 6,  $p < 0.05$ ) for the split-weaned animals.

Figure 5.3 Regression analysis between  $^{125}\text{I}$ -hCG binding to granulosa cells ( $\text{cpm} \times 10^3$ ) and follicular fluid oestradiol ( $\text{ng/ml}$ ) for the control, split-weaned and cover groups respectively.  
s.e. ; standard error of the slope.

EXPT4:REGRESSION ANALYSIS:GRAN.BIND.(cpm x 10<sup>3</sup>/Foll) v FFE2 (ng/ml)

F  
O  
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Analysis of variance revealed that mean ( $\pm$ sem) FFE for the split-weaned ( $3.89\pm 0.72$ ) and covered ( $4.92\pm 1.09$ ) sows were significantly ( $p < 0.001$ ) greater than the control animals.

c) Endocrinological Study

i) Luteinising Hormone (LH)

Mean plasma LH levels (ng/ml) for individual sows/group over the 12 hour period before split-weaning/covering (Period 1) and the four consecutive 12 hour time blocks after split-weaning/covering (Periods 2-5) are shown in Tables 5.4, 5.5, 5.6. Similar to the follicular measurements, there was considerable variation between sows within periods/group.

Prior to split-weaning/covering there was no difference in mean LH concentration (ng/ml) between groups. During the 12 hour period after split-weaning/covering (Period 2), no change in LH secretion was observed in control sows; however, overall, mean LH levels rose significantly in the split-weaned ( $p < 0.05$ ) and covered ( $p < 0.001$ ) animals from  $0.21\pm 0.02$  ng/ml and  $0.1\pm 0.02$  ng/ml, to  $0.31\pm 0.05$  ng/ml and  $0.51\pm 0.06$  ng/ml, respectively. However, four sows within the split-weaned group failed to respond to litter size reduction with a significant elevation in mean LH concentrations (sows 50, 38, 98, 103), despite a group trend for mean LH levels to increase at this time.

The analytical interpretation of the LH data for the three groups of animals is shown diagrammatically in Figure 5.4. Maximum LH concentrations for the cover sows were significantly greater than

Table 5.4 Mean plasma LH concentrations during five consecutive 12 hour time blocks in the control SOWS.

CONTROL SOWS	LH Concentrations (ng/ml)				
	Periods	1	2	3	4
47	0.34	0.30	0.15	0.12	0.22
51	0.14	0.15	0.12	0.16	0.16
52	0.14	0.13	0.14	0.14	0.10
76	0.12	0.21	0.20	0.10	0.11
90	0.20	0.16	0.22	0.14	0.17
97	0.36	0.42	0.25	0.34	0.30
109	0.23	0.12	0.15	0.12	0.11
111	0.47	0.35	0.39	0.23	0.41
MEAN ( $\pm$ sem)	0.25 $\pm$ 0.04	0.23 $\pm$ 0.04	0.20 $\pm$ 0.03	0.17 $\pm$ 0.03	0.20 $\pm$ 0.04

Table 5.5 Mean plasma LH concentrations for the 12 hour period before split-weaning (Period 1) and the four consecutive 12 hour periods after split-weaning. The overall mean LH ( $\pm$ sem) of each period is also shown.

SPLIT-WEANED SOWS	LH Concentrations (ng/ml)				
	Periods	1	2	3	4
53	0.25	0.59	0.43	0.36	0.43
50	0.21	0.24	0.26	0.31	0.29
38	0.25	0.14	0.20	0.35	0.15
77	0.22	0.45	0.33	0.19	0.23
75	0.11	0.33	0.21	0.27	0.22
102	0.19	0.30	0.22	0.30	0.22
98	0.17	0.20	0.18	0.20	0.14
103	0.24	0.26	0.22	0.15	0.20
MEAN ( $\pm$ sem)	0.21 $\pm$ 0.02	0.31 $\pm$ 0.05	0.26 $\pm$ 0.03	0.27 $\pm$ 0.03	0.24 $\pm$ 0.03

Table 5.6 Mean plasma LH concentrations for the 12 hour period before the attachment of the canvas cover (Period 1) and the four consecutive 12 hour periods subsequent to this treatment. The overall mean LH ( $\pm$ sem) of each period is also shown.

COVER SOWS	LH Concentrations (ng/ml)				
	Periods	1	2	3	4
43	0.17	0.34	0.49	0.31	0.27
42	0.28	0.39	0.24	0.24	0.27
46	0.12	0.27	0.37	0.30	0.24
70	0.10	0.72	0.41	0.46	0.41
73	0.28	0.55	0.79	0.60	0.66
71	0.26	0.56	0.45	0.18	0.16
101	0.23	0.57	0.40	0.35	0.36
94	0.14	0.40	0.30	0.22	0.47
104	0.11	0.81	0.90	0.49	0.45
MEAN ( $\pm$ sem)	0.19 $\pm$ 0.02	0.51 $\pm$ 0.06	0.48 $\pm$ 0.03	0.35 $\pm$ 0.03	0.37 $\pm$ 0.05

**Figure 5.4** Changes in maximum, minimum and mean LH concentrations for Control, Split-Weaned and Cover sows during the 12 hour period before (Period 1) and the four consecutive 12 hour periods after (Periods 2-5) split-weaning/covering.

Maximum values for Cover group significantly different from Control, as indicated by superscripts

a  $p < 0.001$ ; b  $p < 0.01$ ; and Split-weaned c  $p < 0.001$ ;

d  $p < 0.01$ . Minimum values for Cover group significantly different from Control, as indicated by

superscripts e  $p < 0.001$ ; f  $p < 0.05$ ; and Split-weaned

g  $p < 0.001$ .

Mean values for Cover group significantly different from Control, as indicated by superscripts h  $p < 0.001$ ;

j  $p < 0.01$ ; and Split-weaned, k  $p < 0.001$ ; l  $p < 0.05$ .

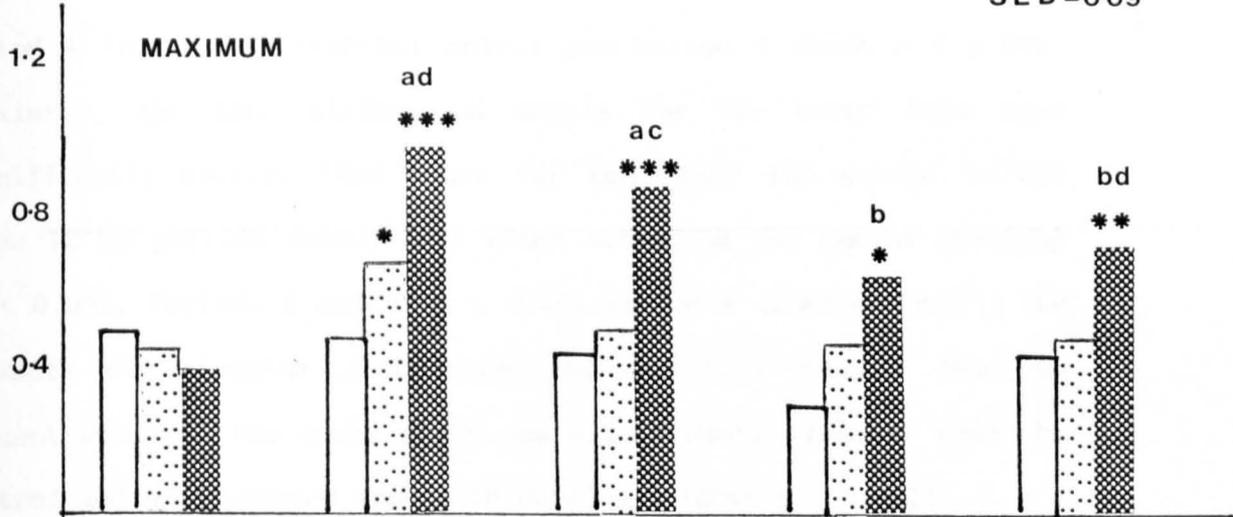
Within groups, Periods 2-5 significantly different from Period 1 as indicated by \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ,

\*  $p < 0.05$ .

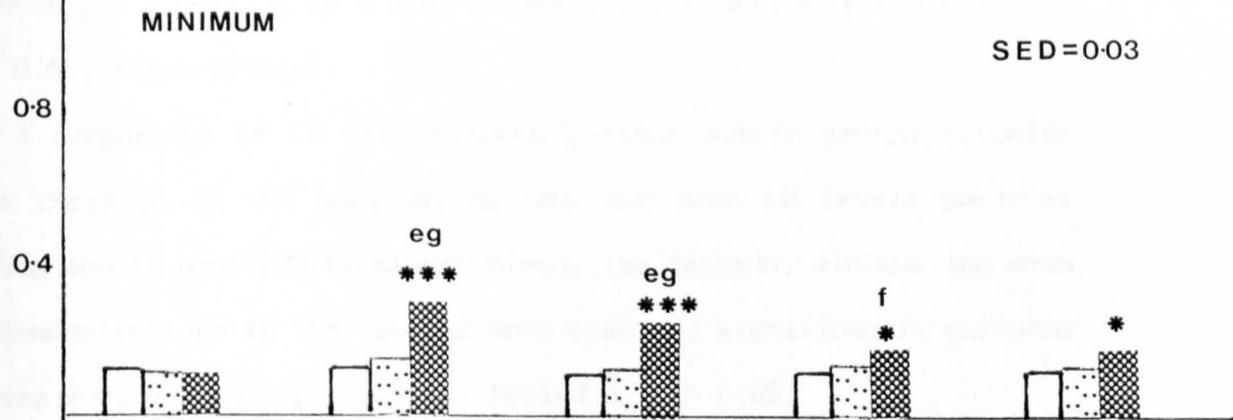
S.E.D.; standard errors of differences of means.

Control      Split-Weaned      Cover

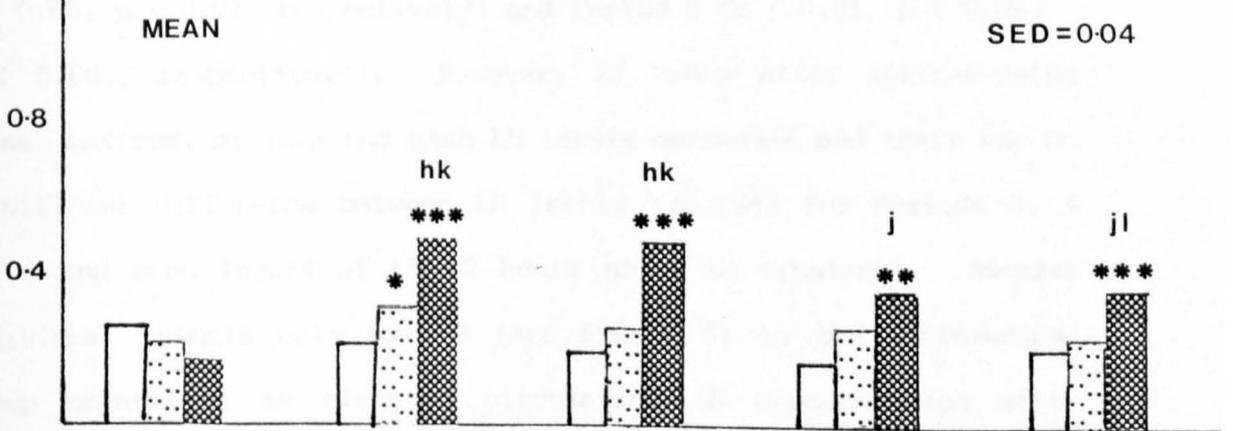
SED=0.09



SED=0.03



SED=0.04



LH (ng equiv. IVO pLH/ml)

1      2      3      4      5

PERIODS

those for both the control and split-weaned sows during Period 2 ( $p < 0.001$ ;  $p < 0.01$ , respectively), Period 3 (both  $p < 0.001$ ), Period 4 ( $p < 0.001$  (control only)) and Period 5 (both  $p < 0.01$ ). Similarly, the mean minimum LH levels for the cover sows were significantly greater than those for the other two groups in the three 12 hr periods immediately after attaching the canvas sheeting ( $p < 0.001$ , Periods 2 and 3;  $p < 0.05$ , Period 4 (control only)) and reflects the elevated LH baseline seen in this group. Mean LH concentration in the cover group was significantly greater than the control and split-weaned groups in Periods 2 (both  $p < 0.001$ ), 3 (both  $p < 0.001$ ), 4 ( $p < 0.01$  (control only)) and 5 ( $p < 0.01$ ;  $p < 0.05$ , respectively).

A comparison of LH data between periods within groups revealed that compared to the maximum, minimum and mean LH levels recorded during the 12 hour pre-treatment bleed, the maximum, minimum and mean LH concentrations in the covered sows remained significantly elevated during Period 3 (all  $p < 0.001$ ), Period 4 ( $p < 0.05$ ,  $p < 0.05$ ,  $p < 0.01$ , respectively) and Period 5 ( $p < 0.01$ ,  $p < 0.05$ ,  $p < 0.001$ , respectively). However, 24 hours after split-weaning alone, maximum, minimum and mean LH levels decreased and there was no significant difference between LH levels recorded for Periods 3, 4 and 5 and mean levels of LH 12 hours prior to treatment. Amongst individual animals only sow 53 (see Fig. 5.5) in the split-weaned group maintained an elevated circulating LH concentration after split-weaning. Throughout the 60 hour bleed, maximum, minimum and mean LH levels did not differ significantly between any period for the control sows.

Analysis of variance between groups established a treatment effect. The overall mean LH concentration in the cover sows ( $0.38 \pm 0.06$  ng/ml) was significantly greater than both the control ( $0.21 \pm 0.01$  ng/ml;  $p < 0.01$ ) and split-weaned ( $0.26 \pm 0.02$  ng/ml;  $p < 0.05$ ) animals. There was no significant difference between the split-weaned and control animals.

Representative examples of each of the three groups are shown in Figures 5.5, 5.6 and 5.7. These figures emphasise the difference in LH pulse frequency (pulses/12 hours) between the three groups before and after treatment. Mean pulse frequency during Period 1 was  $4 \pm 0.9$ ,  $3 \pm 0.6$  and  $2.3 \pm 0.7$  for the control, split-weaned and cover groups respectively. During Period 2 there was an increase in pulse frequency in the split-weaned ( $4.6 \pm 0.7$ ) and cover groups ( $8.5 \pm 1.1$ ) but no change was observed in the control animals ( $3.6 \pm 0.9$ ). A high frequency of LH pulses was maintained in the covered animals during Period 3 ( $8.8 \pm 1.1$ ), Period 4 ( $7.0 \pm 0.6$ ) and Period 5 ( $7.0 \pm 1.1$ ). However, a gradual decrease to pre-treatment levels was observed in the split-weaned group ( $5.3 \pm 0.9$ ;  $4.0 \pm 0.9$ ;  $3.6 \pm 0.9$ ) although some pigs (Sow 75, Fig. 5.6) maintained an enhanced LH pulse frequency after split-weaning. Figures 5.5, 5.6 and 5.7 also illustrate the variability in response between sows within groups.

## ii) Prolactin (PRL)

Tables 5.7, 5.8 and 5.9 show the mean PRL levels (ng/ml) for individual sows/group during the 12 hour period immediately before split-weaning/covering and for each of the four 12 hour time blocks following the start of treatment. Similar to circulating LH

Table 5.7 Mean prolactin concentrations in five consecutive 12 hour periods during lactation in the control sows.

CONTROL SOWS	PRL Concentrations (ng/ml)				
	Periods	1	2	3	4
47	13.2	14.8	18.5	19.2	17.9
51	8.2	9.5	15.4	15.9	15.6
52	25.9	32.5	31.5	37.9	32.7
76	18.4	20.1	19.2	20.2	15.7
90	17.3	16.8	14.4	7.2	5.1
97	24.5	22.7	21.7	25.9	23.8
109	15.0	10.2	11.4	16.3	16.5
MEAN ( $\pm$ sem)	17.50 $\pm$ 2.35	18.09 $\pm$ 3.01	18.87 $\pm$ 2.47	20.37 $\pm$ 3.62	18.19 $\pm$ 3.20

Table 5.8 Mean prolactin concentrations in the 12 hour period before split-weaning (Period 1) and the four consecutive 12 hour periods after split-weaning. The overall mean PRL ( $\pm$ sem) of each period is also shown.

SPLIT-WEANED SOWS	PRL Concentrations (ng/ml)				
	Periods	1	2	3	4
53	42.0	19.8	18.2	28.6	44.1
50	12.2	12.5	11.4	16.2	14.7
38	6.2	7.8	8.9	8.2	12.3
77	16.3	15.1	18.2	20.8	16.9
75	13.5	14.8	12.4	16.0	13.4
102	23.0	21.4	14.9	25.4	20.1
98	21.9	23.8	16.7	23.9	24.1
103	20.8	23.4	22.1	21.3	17.9
MEAN ( $\pm$ sem)	19.49 $\pm$ 3.79	17.33 $\pm$ 2.01	15.35 $\pm$ 1.52	20.05 $\pm$ 2.28	20.44 $\pm$ 3.64

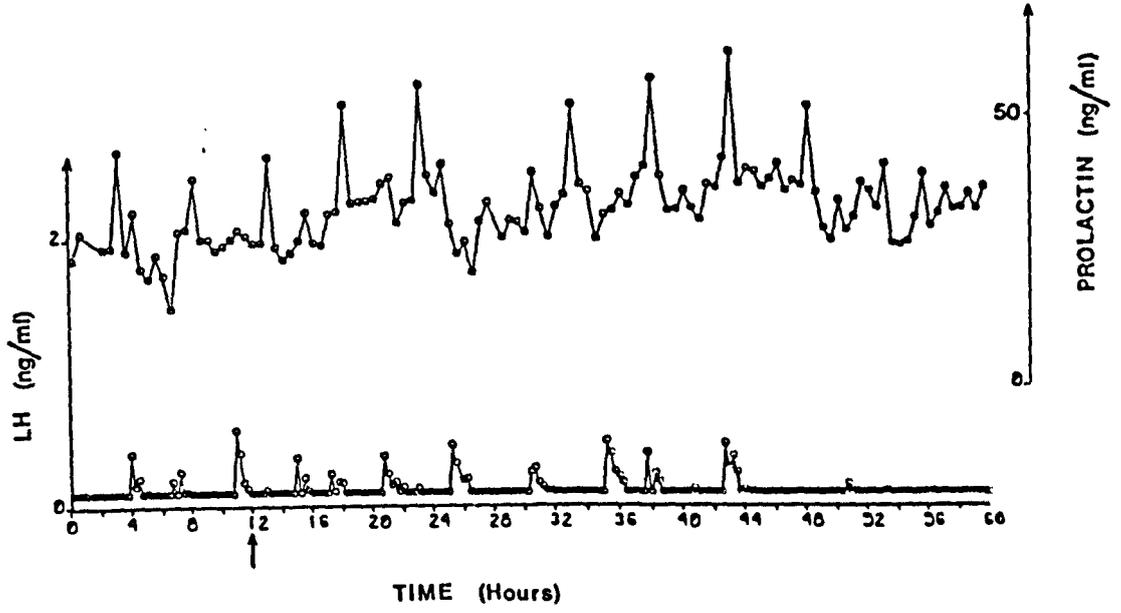
Table 5.9 Mean prolactin concentrations for the 12 hour period before the attachment of the canvas cover (Period 1) and the four consecutive 12 hour periods subsequent to this treatment. The overall mean PRL ( $\pm$ sem) of each period is also shown.

COVER SOWS	PRL Concentrations (ng/ml)				
	Periods	1	2	3	4
43	15.9	10.5	6.7	7.8	6.7
42	9.8	10.1	9.3	10.0	9.9
46	22.8	16.8	9.2	14.3	12.9
70	28.3	15.5	20.1	7.3	13.3
73	34.1	14.7	5.1	7.7	9.2
101	26.1	26.2	16.9	21.4	23.2
94	24.3	11.5	5.3	8.9	8.8
104	30.4	14.9	4.9	14.5	10.9
MEAN ( $\pm$ sem)	23.96 $\pm$ 2.79	15.03 $\pm$ 1.82	9.69 $\pm$ 2.04	11.49 $\pm$ 1.74	11.86 $\pm$ 1.79

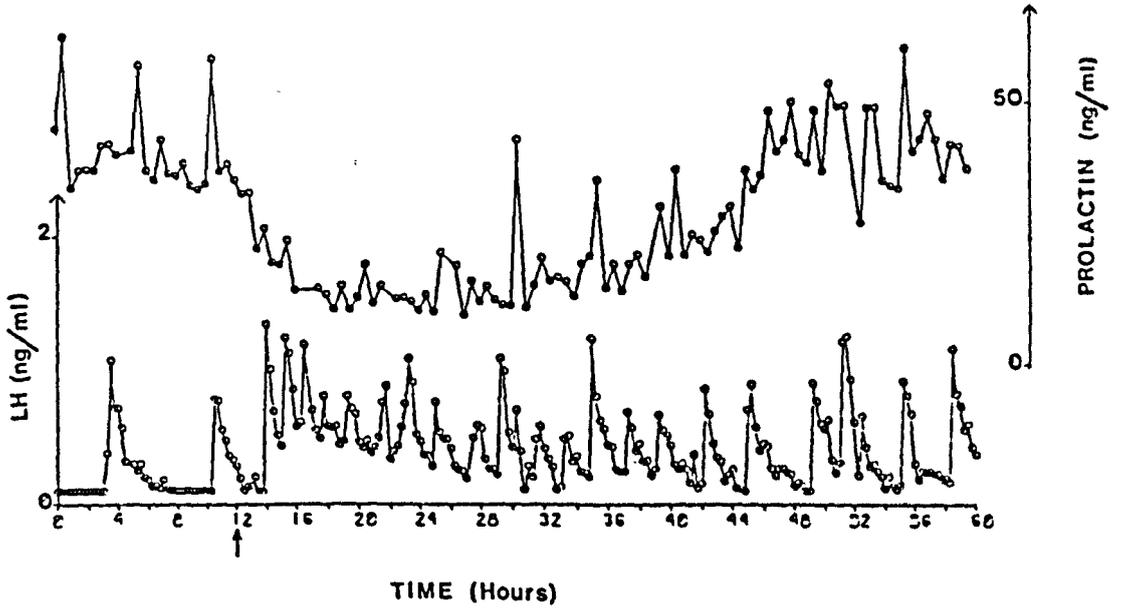
Figure 5.5 Plasma LH and PRL changes in Sow 52 (Control), Sow 53 (Split-Weaned) and Sow 73 (Cover) during 12 hours before split-weaning/covering and for 48 hours subsequent to the start of treatment.

↑ commencement of treatment.

Sow 52 Control



Sow 53. Split-Weaned



Sow 73 Cover

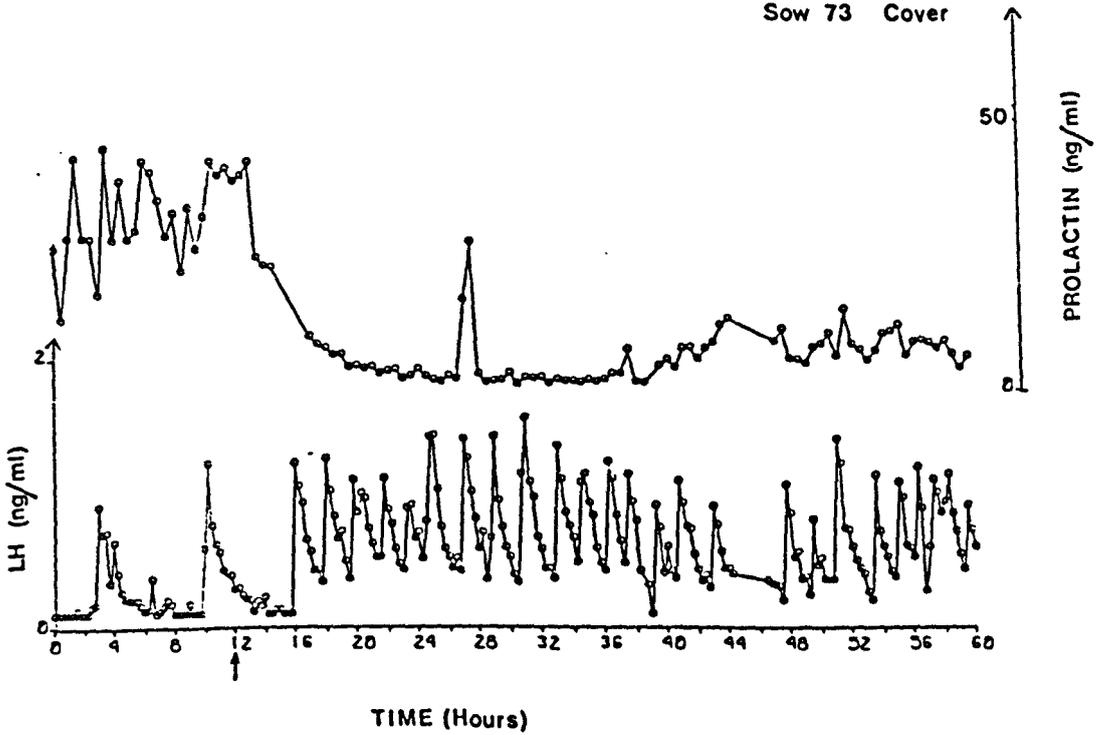
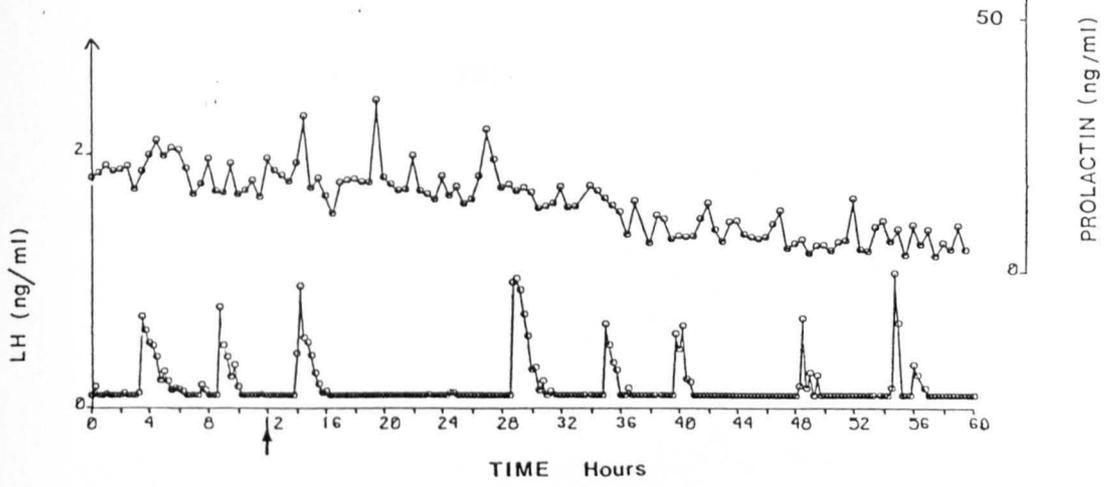


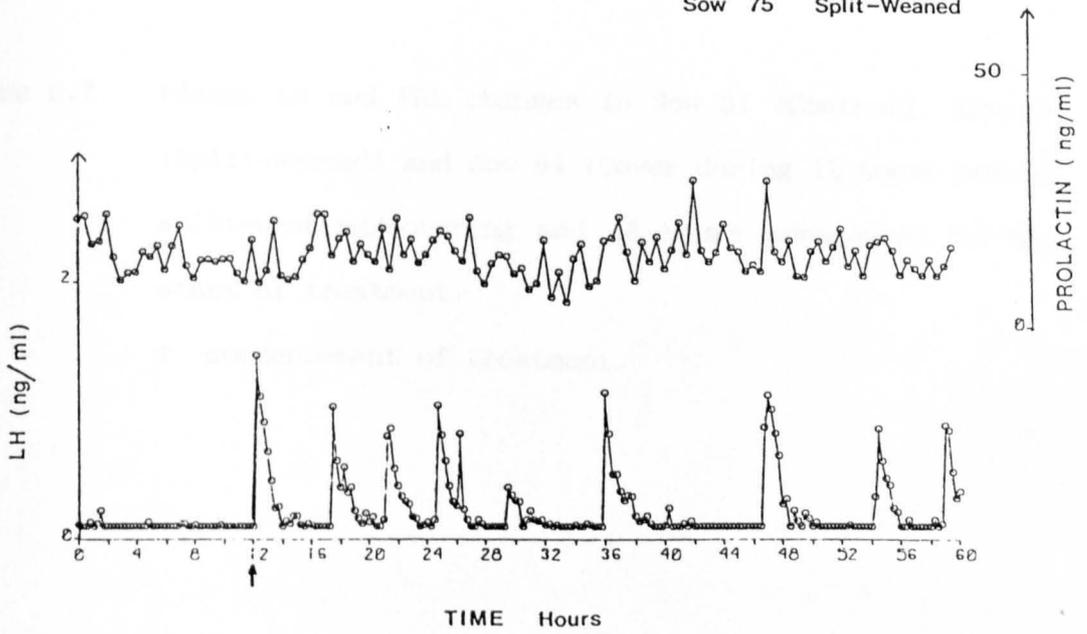
Figure 5.6 Plasma LH and PRL changes in Sow 90 (Control), Sow 75 (Split-Weaned) and Sow 46 (Cover) during 12 hours before split-weaning/covering and 48 hours subsequent to the start of treatment.

↑ commencement of treatment.

Sow 90 Control



Sow 75 Split-Weaned



Sow 46 Cover

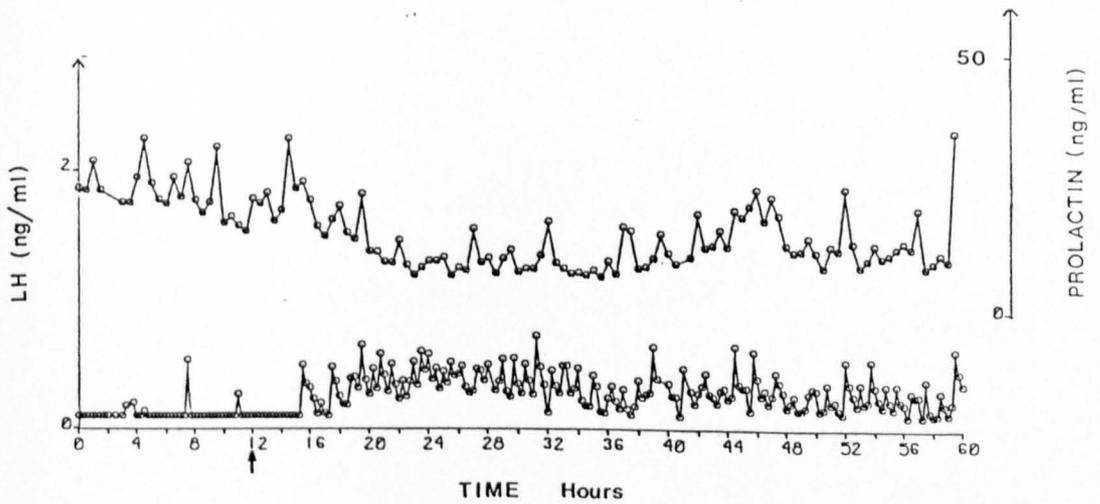
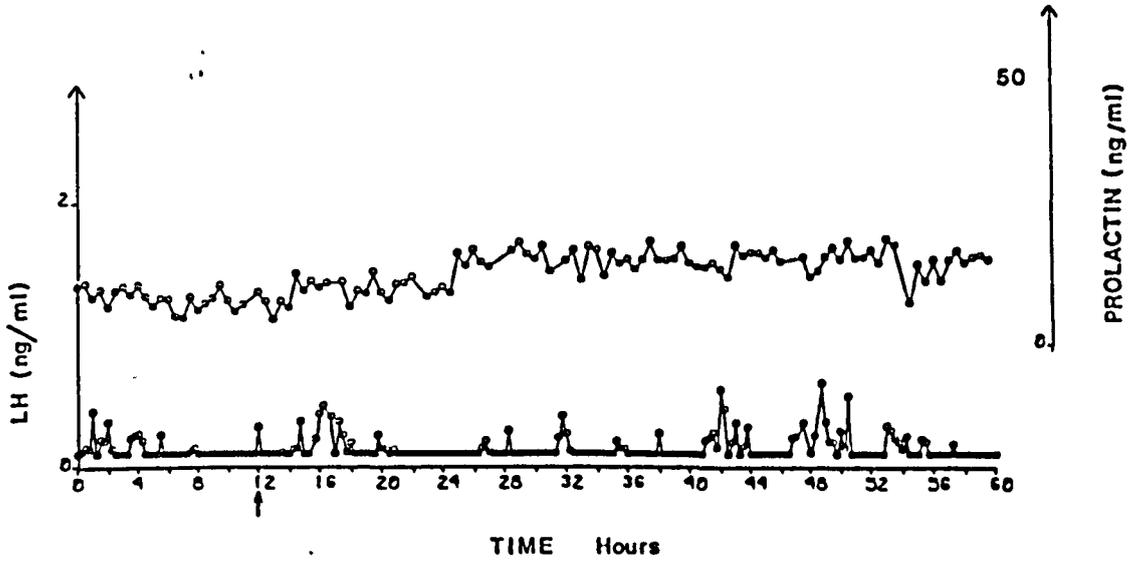


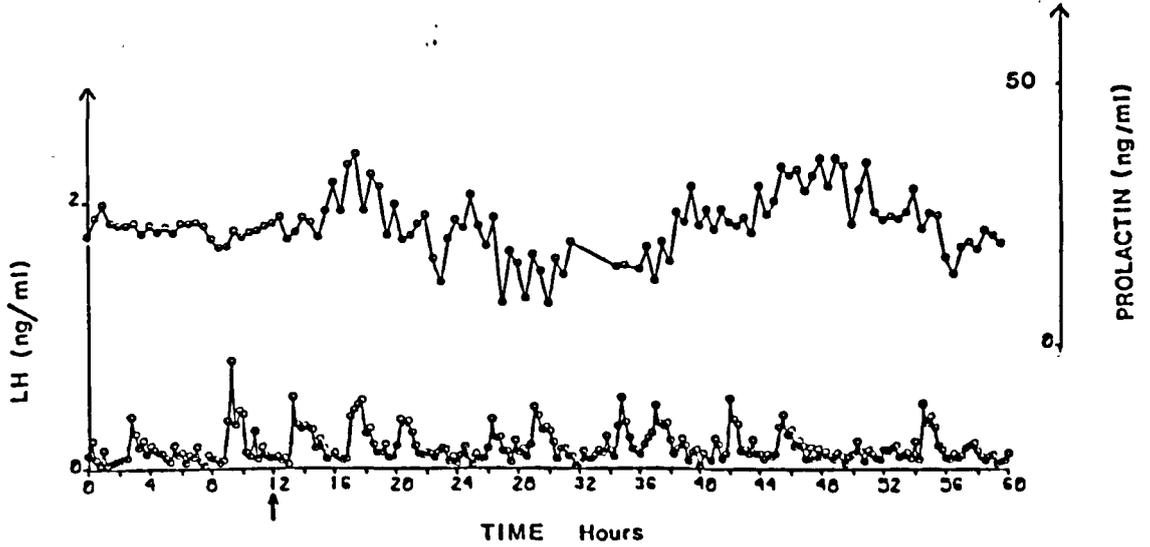
Figure 5.7 Plasma LH and PRL changes in Sow 51 (Control), Sow 98 (Split-Weaned) and Sow 94 (Cover during 12 hours before split-weaning/covering and 48 hours subsequent to the start of treatment.

↑ commencement of treatment.

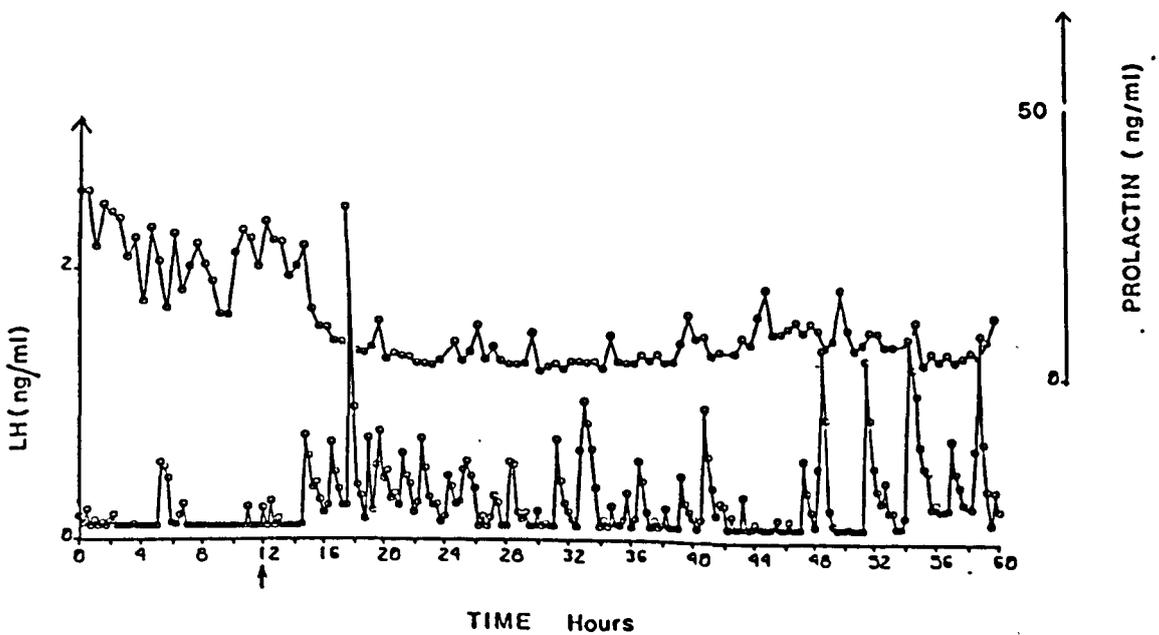
Sow 51 Control



Sow 98 Split-Weaned



Sow 94 Cover.



concentrations there was a considerable variation in PRL concentration between sows within groups.

The maximum, minimum and mean PRL concentrations recorded/period/group are presented in Figure 5.8. There was no significant difference in maximum PRL concentrations between groups of sows within time blocks. However, minimum and mean PRL levels were significantly depressed in the cover animals compared to the control group during Periods 3 and 4 (all  $p < 0.05$ ) and compared to the split-weaned group during Period 4 ( $p < 0.05$  (Mean PRL only)) and Period 5 (both  $p < 0.05$ ). There was no significant difference between any period for the control and split-weaned groups.

Analysis of variance within groups revealed that mean PRL concentrations did not differ significantly between periods for the control and split-weaned animals respectively. However, compared to the concentration of PRL circulating in the initial 12 hour pre-bleed (Period 1), mean PRL levels were significantly depressed in Period 2 ( $p < 0.01$ ) and Periods 3, 4 and 5 ( $p < 0.001$ ) in the covered animals. Only sows 42 and 101 did not conform to this trend (see Table 5.9).

A significant reciprocal correlation ( $r = -0.40$ , d.f. = 38,  $p < 0.001$ ) existed between prolactin and LH concentrations in the cover sows only (for regression analysis see Fig. 5.9).

### iii) Oxytocin

The mean concentration of oxytocin (pg/ml) detected during suckling sessions on the days before and after split-weaning/covering, and on the day before final weaning/slaughter are illustrated in Fig. 5.10 for the control, split-weaned and cover sows

**Figure 5.8** Changes in maximum, minimum and mean PRL concentrations for Control, Split-Weaned and Cover sows during the 12 hour period before (Period 1) and the four consecutive 12 hour periods after (Periods 2-5) split-weaning/covering.

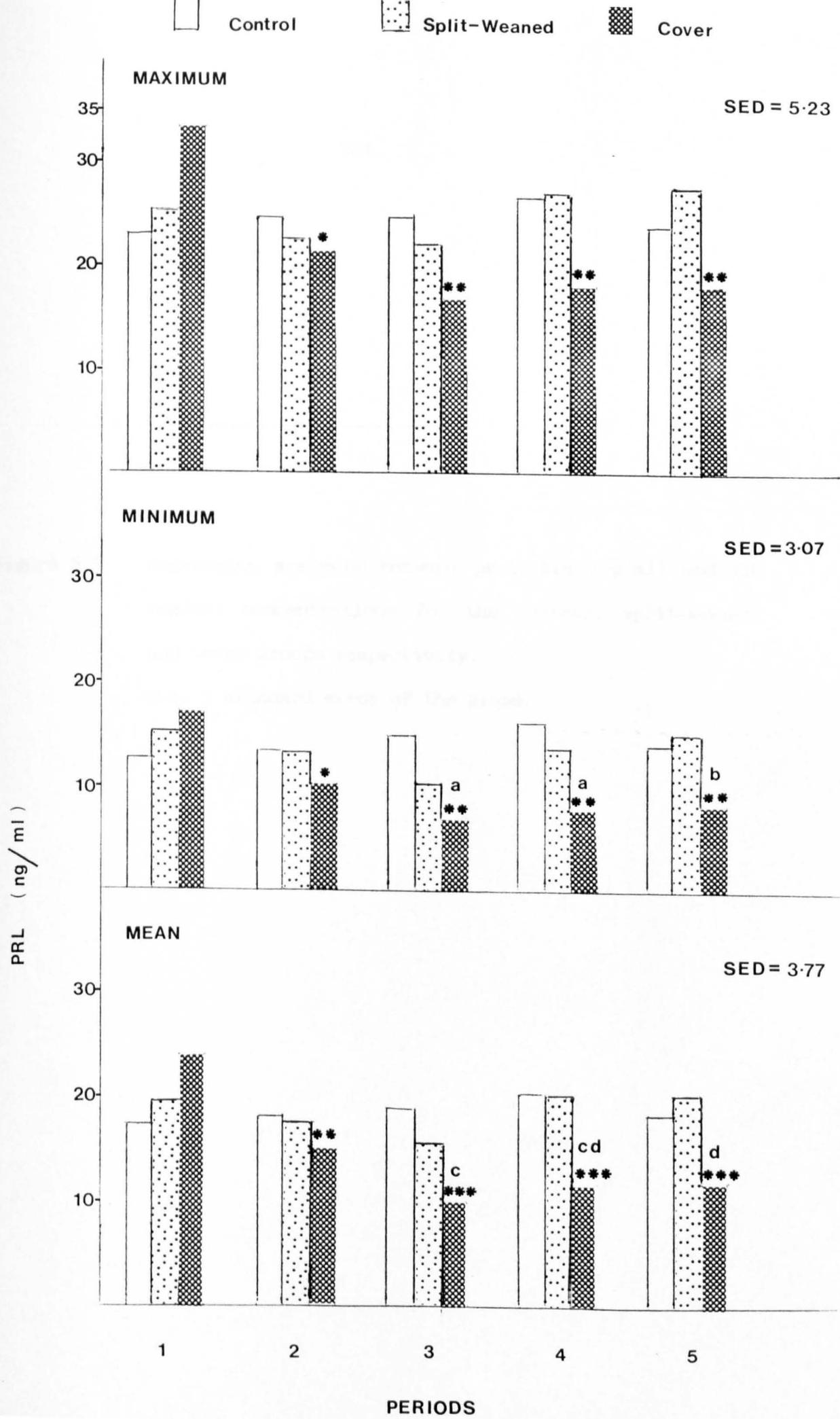
There was no significant difference in maximum PRL concentrations between groups of sows within time blocks.

Minimum values for Cover group significantly different from Control as indicated by superscripts <sup>a</sup>  $p < 0.05$ ; and Split-Weaned <sup>b</sup>  $p < 0.05$ .

Mean values for Cover group significantly different from Control as indicated by superscripts <sup>c</sup>  $p < 0.05$ ; and Split-Weaned <sup>d</sup>  $p < 0.05$ .

Within groups, Periods 2-5 significantly different from Period 1 as indicated by \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ .

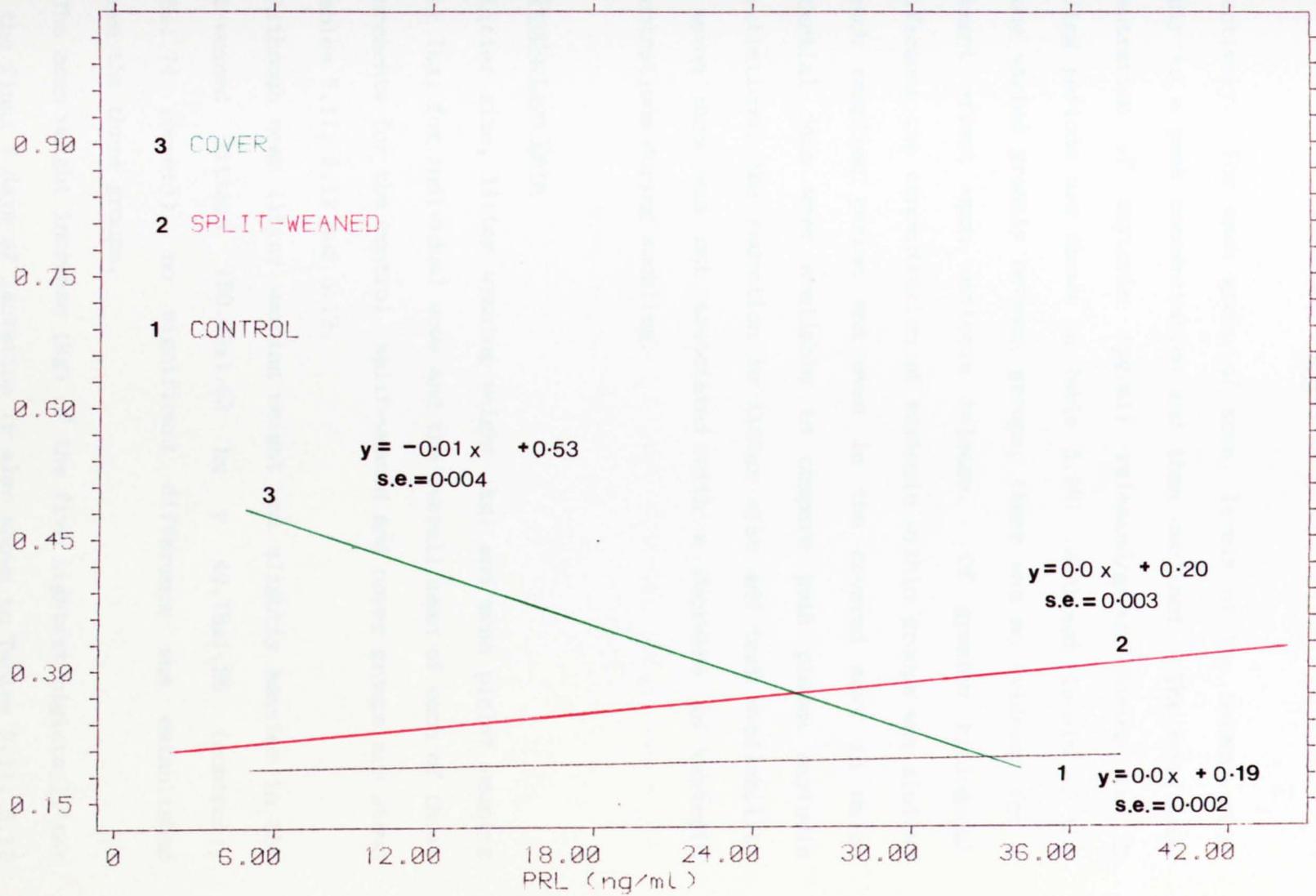
S.E.D. ; standard errors of differences of means.



**Figure 5.9** Regression analysis between prolactin (ng/ml) and LH (ng/ml) concentrations for the control, split-weaned and cover groups respectively.  
s.e. ; standard error of the slope.

EXPT 3: REGRESSION ANALYSIS. PRL (ng/ml) v LH (ng/ml)

L  
H  
(  
n  
g  
/  
m  
l  
)



respectively. For each group of sows, levels of the hormone rose rapidly to a peak concentration and then declined. The mean peak concentration of oxytocin (pg/ml) released/group during the 3 sampling periods are shown in Table 5.10. Although levels of the hormone varied greatly between groups, there was no evidence for a treatment effect upon oxytocin release. Of greater biological significance the concentration of oxytocin within groups was similar at each sampling period and even in the covered sows in which substantial data were available to compare peak plasma oxytocin concentrations, the reduction in litter size and teat availability for seven days was not associated with a decrease in oxytocin concentrations during suckling.

d) Production Data

Litter size, litter weaning weight (kg) and mean piglet weaning weight (kg) for individual sows and the overall mean of each of these measurements for the control, split-weaned and cover groups are shown in Tables 5.11, 5.12 and 5.13.

Although mean litter weaning weight was slightly heavier in the split-weaned litters ( $50.65 \pm 1.63$  kg v  $48.18 \pm 1.28$  (control);  $48.85 \pm 1.74$  (cover)), no significant difference was established between the three groups.

The mean weight increase (kg) of the five lightest piglets/litter over the final 7 days of lactation is also shown in Tables 5.11, 5.12 and 5.13. The piglets in the split-weaned litters gained significantly more weight than equivalent piglets in the control

Figure 5.10 The mean concentration of oxytocin (pg/ml) detected during suckling sessions on the days before and after split-weaning/covering and on the day before final weaning/slaughter for the control, split-weaned and cover sows respectively.

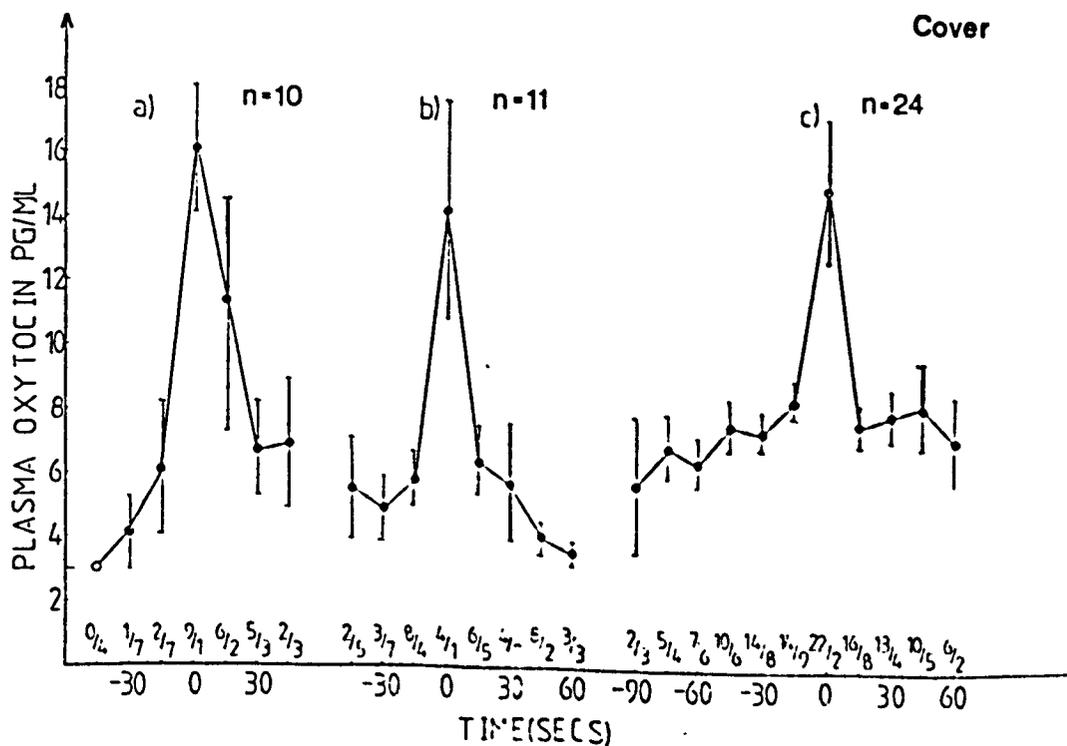
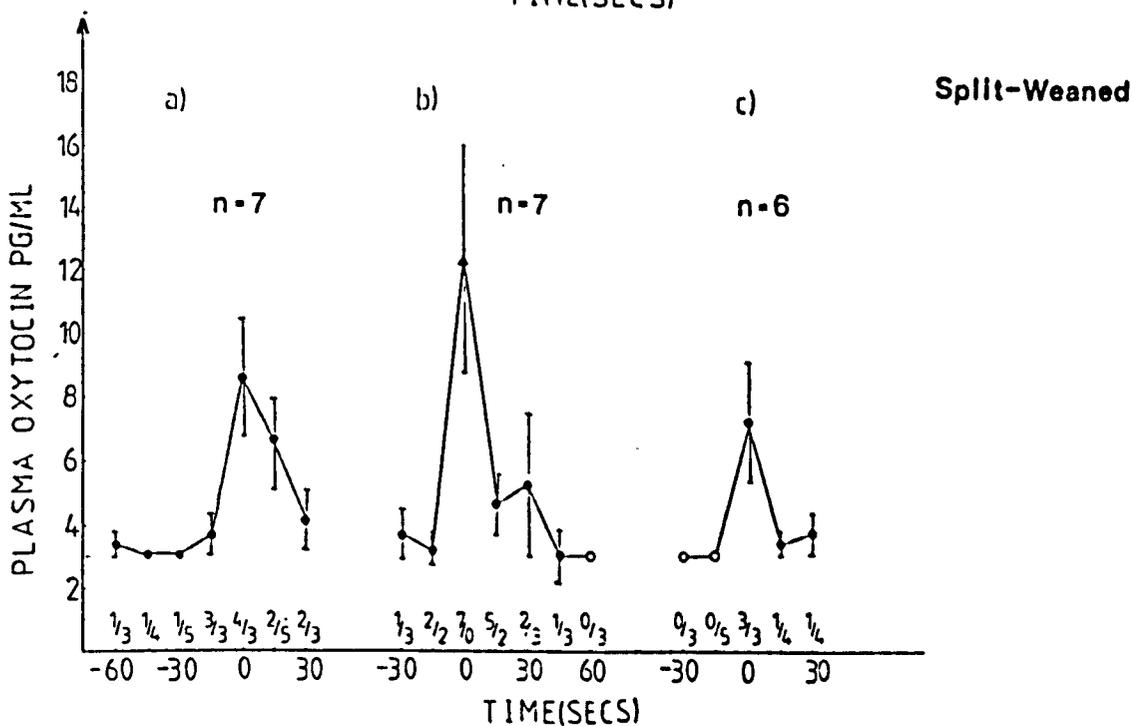
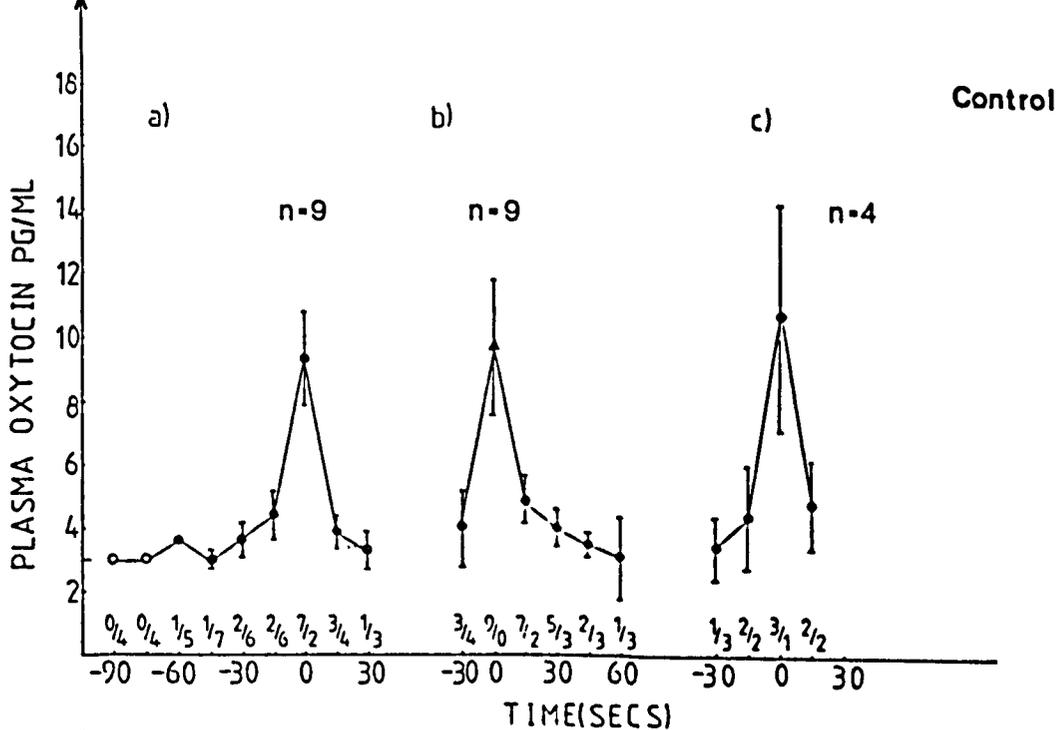


Table 5.10 The mean concentration of oxytocin (pg/ml) detected during 3 sampling periods on the days before and after split-weaning/covering and on the day before final weaning/slaughter for the control, split-weaned and cover sows respectively.

	Pre-Bleed (pg/ml)	48 Hours After Split-Weaning/Covering (pg/ml)	24 Hours Before Weaning/Slaughter (pg/ml)
CONTROL	9.3 ± 1.4	9.7 ± 2.1	10.7 ± 3.5
SPLIT-WEAN	8.6 ± 1.8	12.2 ± 3.7	7.2 ± 1.9
COVER	15.9 ± 4.5	14.0 ± 3.4	15.1 ± 4.9

**Table 5.11** Litter size, litter weaning weight (kg), mean piglet weaning weight (kg) and the weight gain (kg) of the five lightest piglets over the final week of lactation for individual control sows. Overall means ( $\pm$  sem) are also shown.

CONTROL SOW NUMBER	INITIAL LITTER SIZE	LITTER WEANING WEIGHT (kg)	MEAN PIGLET WEANING WEIGHT (kg)	WEIGHT GAIN OF 5 LIGHTEST PIGLETS OVER FINAL 7 DAYS OF LACTATION (kg)
47	10	54.3	5.4	+ 7.6
51	9	44.6	5.0	+ 4.9
52	8	45.1	5.6	+ 8.9
65	8	48.3	6.0	+ 7.0
76	9	41.6	4.6	+ 7.1
90	10	50.0	5.0	+ 6.7
97	9	51.0	5.7	+ 6.5
109	8	48.6	6.1	+ 7.3
111	8	50.1	6.3	+ 7.9
<hr/>				
$8.78 \pm 0.28$ $48.18 \pm 1.28$ $5.52 \pm 0.19$ $+ 7.10 \pm 0.36$				

**Table 5.12** Litter size, litter weaning weight (kg), mean piglet weaning weight (kg) and the weight gain (kg) of the five lightest piglets over the final week of lactation for individual split-weaned sows. Overall means ( $\pm$  sem) are also shown.

SPLIT-WEANED SOW NUMBER	INITIAL LITTER SIZE	LITTER WEANING WEIGHT (kg)	MEAN PIGLET WEANING WEIGHT (kg)	WEIGHT GAIN OF 5 LIGHTEST PIGLETS OVER FINAL 7 DAYS OF LACTATION (kg)
38	12	52.3	4.4	+ 6.6
50	11	51.5	4.7	+ 8.7
53	10	57.4	5.7	+10.0
57	10	53.4	5.3	+11.5
64	10	52.0	5.2	+ 8.7
75	10	49.5	5.0	+ 4.1
77	10	54.2	5.4	+11.4
98	10	52.8	5.3	+ 9.9
102	9	40.9	4.5	+ 7.2
103	9	42.5	5.0	+10.7
<hr/>				
	10.10 $\pm$ 0.28	50.65 $\pm$ 1.63	5.05 $\pm$ 0.13	+ 8.88 $\pm$ 0.74

Table 5.13 Litter size, litter weaning weight (kg), mean piglet weaning weight (kg), and the weight gain (kg) of the five lightest piglets over the final week of lactation for individual cover sows. Overall means ( $\pm$  sem) are also shown.

COVERED SOW NUMBERS	INITIAL LITTER SIZE	LITTER WEANING WEIGHT (kg)	MEAN PIGLET WEANING WEIGHT (kg)	WEIGHT GAIN OF 5 LIGHTEST PIGLETS OVER FINAL 7 DAYS OF LACTATION (kg)
42	10	54.2	5.4	+ 9.2
43	11	49.2	4.5	+ 5.4
46	10	47.3	4.7	+ 7.2
66	9	36.9	3.7	+ 4.6
68	10	57.0	5.7	+ 4.6
70	10	51.5	5.2	+ 7.5
71	10	53.1	5.3	+ 6.7
73	10	42.0	4.2	+ 3.7
94	11	47.4	4.3	+ 4.6
101	10	52.4	5.2	+ 6.4
104	9	46.4	5.2	+ 6.1
<hr/>				
	10.00 $\pm$ 0.19	48.85 $\pm$ 1.74	4.85 $\pm$ 0.19	+ 6.0 $\pm$ 0.49

( $8.88 \pm 0.74$  kg v  $7.10 \pm 0.36$ ;  $p < 0.05$ ) and covered ( $8.88 \pm 0.74$  v  $6.0 \pm 0.49$ ,  $p < 0.01$ ) litters. There was no significant difference between the control and covered litters.

Mean piglet weaning weight (kg) in the control litters was significantly greater than that recorded in the cover litters ( $5.52 \pm 0.19$  v  $4.85 \pm 0.19$ ,  $p < 0.05$ ). There was no significant difference between mean piglet weaning weight in the split-weaned litters and any other group.

The total weight of mammary tissue dissected from the control ( $6.69 \pm 0.36$  kg) and split-weaned ( $6.20 \pm 0.18$  kg) sows was significantly greater ( $p < 0.001$ ) than that recovered from the covered ( $4.91 \pm 0.12$ ) animals. A significant correlation was established between total mammary tissue weight and litter weaning weight ( $r = 0.73$ , d.f. = 7,  $p < 0.05$ ) in the split-weaned group only.

A significant correlation was also shown between piglet weaning weight and the weight of the preferentially suckled mammary quarter in the split-weaned ( $r = 0.28$ , d.f. = 48,  $p < 0.05$ ) and covered ( $r = 0.28$ , d.f. = 77,  $p < 0.05$ ) litters. The heaviest piglets at weaning were observed to preferentially suckle a pectoral teat in six (66.7%) of the split-weaned litters studied (see Fig. 5.12) but in only two (22.2%) of the control litters (see Fig. 5.11).

Figures 5.11 and 5.12 Schematic diagrams depicting the relationship between final piglet weaning weight (kg) and the position of the quarter(s) preferentially suckled by each piglet for all litters from the control and split-weaned sows respectively. As the majority of piglets after split-weaning were observed to 'multiple' suckle, only the position of the preferred quarter is represented.

B represents a 'blind' teat which produced no milk.

\*An asterix highlights the heaviest piglet in each litter.

Fig. 5.10

CONTROL SOWS	PECTORAL			POSTERIOR				
	1	2	3	4	5	6	7	8
65				6.8	B	5.5	6.3	
	5.0	5.7	5.8	6.8	7.3*	B	6.3	5.9
47	4.3	5.4	5.4	5.2	-	4.8	6.7	B
	4.7	5.0	-	5.8	5.2	6.9*	5.5	B
51	4.1	5.3	5.3/4.3	5.6	5.2	5.9	5.8	
	3.8	4.3	4.6	4.6	B	5.9*	5.8	
52	5.6	6.1	6.5	B	B	B	B	
	-	4.9	6.0	5.9	5.9/5.9	5.9	B	4.2
90	5.0	4.9	5.2	2.9	B	4.2	4.6	
	5.0	6.1	6.2	6.9*	B	4.0	4.6	
76	5.2	5.1	5.1	-	6.2	7.0	B	6.3
	4.7	7.4*	7.0	B	7.0	B	6.3	-
111	6.6	B	B	B	B	4.8	3.7	-
	6.6	6.5	7.2	6.7	6.7	7.8*	6.8	B
109	7.0	7.4*	B	5.3	6.3	5.5	5.2	
	7.0	6.3	B	B	B	5.6	5.6	
97	6.2	6.4	4.9	B	5.6	5.1	-	
	4.8	6.0	6.5*	4.9	5.6	5.5	5.1	

Fig. 5.11

SPLIT-WEANED	PECTORAL		PAIRS OF MAMMARY QUARTERS					POSTERIOR
	1	2	3	4	5	6	7	8
38	-	-	-	-	-	-	-	3.4
		5.1*	4.9	5.1*	-	-	4.3	-
64	6.2	4.9	3.9	B	B	B	B	-
	-	B	6.4*	-	-	-	B	5.1
57	-	-	-	5.7	5.4	B	B	B
	6.9*	6.3	5.0	-	-	-	B	B
50	5.3	-	-	5.5*	5.3	B		
	-	-	5.0	-	4.1	-	-	-
77	6.4	-	-	-	5.6	-		
	6.4*	5.9	5.7	-	-	-	-	
75	3.9	-		5.7*	-	-		
	-	-	5.1		4.5	5.2		
103	-	4.9	5.2	-	B	-	4.5	
	5.9*	5.7	B	B	B	B		
98	5.1	-	-	5.5	-	-	-	
	5.9*	5.5	-	4.7	-	-	-	
102	-	-	5.6	-	-	-		
	4.3	5.6*	-	3.1	-	-	4.2	

## V. DISCUSSION

### a) Ethological Study

During the initial two weeks of lactation, the suckling behaviour of the piglets in this study was comparable to that observed in the previous experiment. In all litters a stable teat order was established within 3 days of parturition and prior to split-weaning/covering the majority of piglets consistently suckled at one particular teat which they strongly defended from their siblings.

The critical importance of these individual teats to each piglet has been well documented (Donald, 1937b; Barber, Braude and Mitchell, 1955; Gill and Thompson, 1956; McBride, 1963; Jeppesen, 1982a,b) and was emphasised in this experiment by the distressed behaviour of some of the piglets in the 'cover' litters immediately after the attachment of the canvas sheeting had rendered their preferred teats inaccessible. Indeed during the initial suckling bouts immediately after the number of available 'quarters' had been restricted, these piglets consistently returned to the position of their original quarter ignoring the available vacated posterior teats and consequently missing a feed. Although such piglets did eventually 'adopt' a new posterior teat they were obviously reluctant to do so. Since piglets recognise teats by individual olfactory chemical cues (Jeppesen, 1982b) perhaps the smell of the previous occupier was sufficient to discourage an immediate occupation. This is consistent with Jeppeson's (1982b) theory that marking the preferred teat area is a form of territorial behaviour to warn piglets away from 'owned' teats during the formative stages of establishing the teat order.

The suckling behaviour of the split-weaned piglets was identical to that observed in Experiment 2 (see Chapter 4). Within a day of removing their heavier littermates the majority of the remaining piglets suckled at least one additional teat within close proximity to their primary teat. Although these piglets divided their attention unequally between teats, contrary to the findings of the previous investigation, there was no significant difference between the weight of mammary tissue recovered from the control and split-weaned sows. Since suckling bouts were known to be synchronised between all sows and it was previously established that split-weaning does not influence suckling frequency (see Chapter 4), it would seem that the multiple suckling behaviour of the split-weaned piglets in this study was sufficient to maintain the level of mammary growth originally stimulated by the entire litter.

Conversely, the rapid regression of the mammary glands covered by the canvas sheeting significantly reduced the total weight of mammary tissue recovered at slaughter from the cover sows. Although the remaining piglets suckled vigorously from the available posterior quarters it was apparent that partial stimulation of the sow's udder concomitant with the visual, auditory and olfactory presence of the piglets was not sufficient to maintain the growth and secretory ability of each 'quarter'. Hence, the level of stimulation received at individual teats appears to govern the ultimate growth and development and perhaps metabolic synthesising ability of that particular gland. This in turn would support the proposal of Hartman and his colleagues (Hartman, Ludwick and Wilson, 1962) that

variability in milk production among glands is largely due to the ability of vigorous piglets to stimulate their glands more efficiently.

b) Ovarian Data

The large number of follicles dissected from the majority of sows in this study confirms the results of the previous experiment and supports the theory that limited follicular development occurs during lactation (Palmer, Teague and Venzke, 1965a; Kunavongkrit, Einarsson and Settergren, 1982; Shaw, 1984). Since follicles harvested from the split-weaned and cover sows were significantly larger than those recovered from the control group, it is apparent that both treatments had directly/indirectly affected the factors regulating folliculogenesis during lactation. Moreover, as the follicles dissected from the covered sows were, in turn, larger than those recovered from the split-weaned sows, it is obvious that restricting the number of mammary quarters available to be suckled by the piglets induced a more dramatic effect upon follicular development than simply split-weaning the litters.

In accord with Experiment 2 (see Chapter 4) this investigation demonstrated aromatase activity in the granulosa cells of all the sows studied. However, as observed in the preceding chapter, there was a wide variation in the level of extant enzyme activity between animals within groups and this variability appeared to be directly related to follicular diameter and hence maturity. Although levels of aromatase activity were comparable between experiments for the

control sows, the overall mean enzyme activity in the split-weaned sows of this investigation was almost double that recorded previously. This large discrepancy may be attributed to sows 50 and 53 in which extant enzyme activity was 3-16 fold the levels recorded in other animals. The removal of the data from these two sows abolished this difference and reduced the overall mean enzyme activity in this group of sows to 1257 pg  $E_2$ /2h/follicle.

Although the morphological development of the follicles in the cover sows was significantly more advanced than in either the split-weaned or control groups, when compared to preovulatory follicles from other studies (Eiler and Nalbandov, 1977; Ainsworth *et al.*, 1980; Experiment 1 - see Chapter 2), all follicles in this experiment were morphologically and steroidogenically immature. This is consistent with earlier follicular studies in the lactating sow (Shaw 1984) and the results of Experiment 2 (see Chapter 4). Nevertheless, the elevated levels of aromatase activity, follicular fluid oestradiol and granulosa binding observed in the follicles of the cover sows indicates that the physical restriction of the number of mammary quarters available to be suckled by the piglets had directly/indirectly promoted folliculogenesis in these animals. However, contrary to the results of Experiment 1 of this thesis (see Chapter 2) the "enhanced" development of these follicles was not associated with a concomitant increase in the significance of interrelationships between the measured follicular variables. Furthermore, several significant correlations previously established in the follicular data of the last experiment could not be reproduced

in the control and split-weaned sows of this study. Since laboratory techniques were identical in each investigation, these inconsistencies between results are undoubtedly a consequence of the large variation observed not only between sows within groups but also between experiments.

The strong relationship between follicular diameter and  $^{125}\text{I}$ -hCG binding to granulosa cells evident in all groups of sows in both Experiments (2 and 3), further confirms many earlier receptor binding studies (Channing and Kammerman, 1973, 1974; Kammerman and Ross, 1975; Lee, 1976; Stouffer, Tyrey and Schomberg, 1976; Nakano *et al.*, 1977; Daguet, 1979; Shaw, 1984) that the capacity of granulosa cells to bind hCG is directly related to follicular maturity. It has been well documented that ovarian steroids increase the responsiveness of the ovary to gonadotrophins (Pencharz, 1940; Williams, 1940; Simpson *et al.*, 1941); however, despite the significantly higher levels of follicular fluid oestradiol and granulosa binding in the split-weaned and cover sows, an association between these two variables was only observed in the control sows. This anomaly may be attributed to the ambiguous follicular data of several sows from the two treatment groups, in which either high concentrations of follicular fluid oestradiol were associated with low  $^{125}\text{I}$ -hCG binding to the granulosa and negligible aromatase activity (Sows 43, 73 and 77) or else the converse was true (Sows 38, 71).

Although the contradictory nature of these *in vitro* follicular studies confuses the interpretation of the results, the significant differences in ovarian activity observed between the control,

split-weaned and cover sows undoubtedly indicate that follicular development in the lactating sow is intimately concerned with the proper sequential development of follicular gonadotrophin receptors. Factors which may be involved in the endocrine or neural mediation of gonadotrophic stimulation of folliculogenesis are discussed in the next section.

c) Endocrine Study

The low frequency episodic release of LH observed in all sows during the initial 12 hour bleed (Period I) is inconsistent with the proposal of Booman and van de Weil (1980) that LH secretion is absent during lactation in sows and confirms previous observations by several authors (Parvizi *et al.*, 1976; Stevenson, Cox and Britt, 1981; Edwards, 1980; Edwards and Foxcroft, 1983a; Shaw, 1984; Shaw and Foxcroft, 1985; Foxcroft *et al.*, 1987) that basal LH secretion in the pig is not totally suppressed during lactation. However, the great variation between sows in both the pattern of LH release and the mean concentration of plasma LH recorded during Period I, suggests that either individual animals were under different levels of suppression at the hypothalamic level or that the sensitivity of the hypothalamic-hypophysial axis to similar inhibitory signals results in differences in LH secretion in individual sows.

Although split-weaning significantly enhanced mean plasma LH concentration and LH pulse frequency in four out of the eight sows studied, this effect was short-lived and by the end of Period 5, 60 hours after the start of the bleed, LH concentrations in the

split-weaned animals had decreased to levels comparable to those recorded in the control animals. A sustained increase in LH secretion was observed in Sow 53 only. These data support the results of Shaw (1984) who observed that mean LH concentration, pulse frequency and amplitude on the day of weaning were unaffected by a reduction in litter size 5 days earlier. By comparison, mean plasma LH levels were significantly increased in all the cover sows immediately after the canvas sheeting had been fitted around the anterior mammary quarters and, moreover, this increase was maintained throughout the duration of the bleed. Since all sows in the experiment were known to be at an identical stage of lactation and the two treatment groups both nursed only five piglets/sow, the critical factor directly or indirectly affecting differences in LH synthesis/release was not the finite size of the nursing litter, but the number of quarters available to be suckled by the piglets and hence the cumulative intensity of the sensory input received by each sow across the entire udder. Although this theory disagrees with earlier rat studies (Rothchild, 1960) which demonstrated that the degree of gonadotrophin suppression during lactation was proportional to litter size, other experiments in sheep (Kann and Martinet, 1975) and tammar wallabies (Renfree, 1979) have shown that denervation of the mammary gland has no immediate effect on lactation but abolishes all inhibitory effects on reproduction. Thus, at least in these species the sensory nerve endings in the nipple itself appear to indirectly mediate the inhibitory effects of suckling upon serum LH levels.

The high levels of prolactin recorded during the 12 hour pre-bleed in this study support many previous findings in the lactating sow (Van Landeghem and Van de Weil, 1978; Bevers *et al.*, 1978; Stevenson, Cox and Britt, 1981; Benjaminsen, 1981; Kirkwood *et al.*, 1984a,b; Edwards and Foxcroft, 1983; Shaw and Foxcroft, 1985). Although the removal of the heaviest piglets from the split-weaned sows resulted in a slight decrease in the mean concentration of prolactin, levels of the hormone rapidly returned to the original concentration recorded prior to split-weaning. Since the latter was not significantly different from the values recorded for the control animals, these results confirm the proposal of Bevers and his colleagues (1978) that there is no obvious correlation between the level of prolactin in the blood and the number of piglets nursing. However, the prolonged and significant depression in prolactin levels observed in the "cover" sows after the canvas sheeting had restricted the number of mammary quarters available to be suckled by the remaining piglets strongly implies that similar to circulating levels of LH, the concentration of prolactin in the lactating sow is directly influenced by the intensity of the sensory input received by each sow across the entire udder. Consequently, although the split-weaned and cover sows were nursing litters of identical size, since only five or six quarters were available to be stimulated in the 'cover' animals the intensity of neural input received by each of these sows obviously dropped below the threshold required to suppress episodic LH release and sustain elevated prolactin levels. Hence a rise in mean LH levels and a concomitant decrease in prolactin was observed in this group. Conversely, since the endocrine profiles of the split-weaned sows (except Sow 53) were comparable to those of the

control animals, it may be inferred that the multiple suckling split-weaned litters stimulated their udders almost as efficiently as an entire litter. Indeed, the total weight of mammary tissue recovered from the control sows was only marginally greater than that from the split-weaned sows.

It is generally accepted that a major factor controlling the lack of sustained follicular development in the lactating sow is the inadequate secretion of LH (Parvizi *et al.*, 1976; Van de Weil *et al.*, 1979; Edwards and Foxcroft, 1983; Shaw and Foxcroft, 1985). Certainly in this study the relative ovarian inactivity of the control sows was associated with infrequent episodic release of LH while follicles were obviously developing in the cover sows in response to increasing episodic and baseline LH levels. However, many authors have speculated that lactational anoestrus is a consequence of a suckled induced hyperprolactinaemia (see Chapter 1, Section IIIb(iv)). Certainly, evidence that prolactin can be a potent inhibitor of ovarian function has been obtained in several species (McNeilly *et al.*, 1982). Moreover, specific *in vivo* (Tsai-morris *et al.*, 1983) and *in vitro* (Advis, Weiner and Ojeda, 1981; Dorrington and Gore-Langton, 1981, 1982; Magoffin and Erickson, 1982) experiments in rats have shown that prolactin can inhibit FSH induction of aromatase activity and LH-stimulated androgen synthesis in preovulatory follicles. Enhanced follicular development and an associated inverse relationship between plasma prolactin and episodic LH release observed in the cover sows appear to substantiate those findings; in contrast, however, the establishment of a significant

positive correlation between follicular fluid oestradiol and prolactin in these animals implicates a stimulatory role for the hormone. Furthermore, since aromatase activity was evident in follicles from all sows when incubated *in vitro* after exposure to elevated prolactin concentrations *in vivo*, there is little evidence from this study that prolactin interferes with the biochemical development of follicles in the lactating sow.

Nevertheless, there are strong indications for a negative relationship between plasma levels of prolactin and LH within animals. Evidence from the literature suggests that LH suppression is caused by an inhibition of LHRH release (Cox and Britt, 1982a,b) which indicates a blockade at the hypothalamic level or higher. Since dopaminergic pathways have been shown to be involved in the control of the release of LH (Weiner and Ganong, 1978), a possible mechanism for LH suppression by prolactin is related to the activation by the hormone of the dopaminergic terminals in the external layer of the median eminence (Hökfelt and Fuxe, 1972). However, many workers have observed no change in LH levels even after the concentration of prolactin had been artificially reduced by the dopamine receptor agonist, bromocryptine (Evans *et al.*, 1980; Smith, 1981; van der Schoot *et al.*, 1982). The conclusions of these former studies, concomitant with the endocrine profiles of the sows in this investigation suggest that high levels of circulating prolactin can only partially account for the suppression of LH during lactation. It is therefore not possible to distinguish inhibitory effects on LH caused by prolactin *per se*, from those caused by direct neural stimuli due to suckling.

The pattern and concentration of oxytocin release recorded in this investigation confirm and extend earlier reports (Forsling et al., 1979; Bruhn et al., 1981; Ellendorff, Forsling and Poulain, 1982). The relative importance of the mere presence of the piglets, the actual removal of the milk and the action of oxytocin released before each "milk let down" as mediators of the action of suckling in depressing follicular development in the lactating sow has been examined previously (Peters, First and Casida, 1969). Since the amplitude of the suckling-induced oxytocin discharge is smaller in the later stages of lactation (Forsling et al., 1979) and thus inversely related to the activity of the hypothalamic-pituitary-ovarian axis in lactation, Ellendorff and his colleagues (1985) hypothesised that oxytocin itself could be responsible for lactational anoestrus. To date their own studies to substantiate this proposal have been inconclusive. The results of this Experiment do not support a direct role for oxytocin in the regulation of LH secretion and ovarian function in lactation. The similar profiles of oxytocin release recorded for each group of sows contrast with the established significant differences in LH secretion and ovarian activity. Furthermore, the absence of a treatment effect upon the levels of oxytocin detected at various suckling sessions in the split-weaned and cover sows, respectively, suggests that there is no correlation between the neural intensity of the suckling stimulus and the magnitude of the suckling induced oxytocin release. These data indicate that in the sow there is a relatively low threshold for the suckling-induced neural stimulation of the oxytocic reflex which is

exceeded by the suckling activity of litters of five piglets. Once this threshold is exceeded the pulse of oxytocin released induces adequate milk letdown in all active mammary glands.

It is apparent from the endocrine and ovarian data for each group of sows that a striking parallelism exists between suckling related events and the depth of the lactational anoestrus. Since previous studies have shown that the pituitary gland and the ovary of the lactating sow remain responsive to gonadotrophin releasing hormone (GnRH) (Bever *et al.*, 1981; Stevenson, Cox and Britt, 1981) and exogenous gonadotrophin (Guthrie, Pursel and Frobish, 1978) respectively, it appears that central pathways inhibitory to the release of GnRH must be activated as part of the events leading to milk ejection. Recently, much speculation has centred on the endogenous opioid peptides as factors which might be involved in the suckling induced inhibition of LH secretion. Evidence for this supposition is that administration of naloxone, an opioid antagonist, will stimulate secretion of LH in lactating rats (Sirinathsinghji and Martini, 1984), cows (Gregg *et al.*, 1985, 1986; Whisnant *et al.*, 1986) and ewes (Gregg *et al.*, 1985, 1986). Similar experiments in the lactating sow (Barb *et al.*, 1986; Mattioli *et al.*, 1986; Armstrong, Kraeling and Britt, 1988) produced comparable results and naloxone administration also depressed prolactin levels. These results suggest that the high prolactin levels and low episodic LH release associated with lactation are at least in part, a consequence of the same factor acting through an opioid-mediated mechanism. This theory is consistent with results of experiments in which bromocryptine

suppressed prolactin in lactating sows without influencing LH values (Seren et al., 1984; Mattioli and Seren, 1985) in that suppression of prolactin was not mediated via the opioidergic pathway. Hence, if the level of hypothalamic inhibition is directly related to the circulating concentration of endogenous opioid peptides, then the ovarian and endocrine results of this study suggest that the release of these peptides is directly influenced by the intensity of the neural input received by each sow across her entire udder. Therefore, by physically restricting mammary (neural) stimulation in the cover sows, it is feasible that endogenous opioids were directly reduced in these animals enabling a partial escape of the hypothalamo-pituitary axis from the suppressive effects of lactation. Thus, prolactin levels fell and there was a concomitant increase in baseline LH levels and ultimately follicular development.

Paradoxically experiments in rats (reviewed by Van Wimersma Greidanus and ten Haaf, 1985) suggest that endogenous opioid peptides have an inhibitory role in the regulation of oxytocin release. This does not appear to be the case in the sow since the pulsatile release of oxytocin was similar for each group of sows after exposure to presumably differing levels of endogenous opioid peptides.

#### d) Production Data

Although mean litter weaning weight was similar between all three groups of sows, the five lightest piglets in the split-weaned litters gained significantly more weight over the final seven days of lactation than equivalent piglets in the control and cover groups.

This is consistent with the results of Experiment 2 (see Chapter 4) and further confirms that the multiple suckling behaviour of these piglets, concomitant with the removal of any competition from their heavier siblings, enabled them to suckle greater quantities of milk and consequently grow faster than comparable piglets in the other groups. Nevertheless, as observed in the previous chapter, the disparity between the total weight of mammary tissue recovered from the control and split-weaned sows indicates that the split-weaned piglets divided their attention unequally among two/three teats and hence, they did not stimulate any one teat to the same extent as the control piglets.

The five remaining piglets in the cover sows were amongst the lightest at weaning. However the significant correlation between the weaning weight of each piglet and the weight of the preferentially suckled quarter suggests that these piglets were maximising the synthetic and secretory capacity of the posterior quarters. Certainly Gill and Thompson (1956) have proposed that the posterior teats produce less milk though more recent research (Hartman, Ludwick and Wilson, 1962; Hartsock, Graves and Baumgardt, 1977) does not favour any gland to be a consistently large or small producer.

In summary, the production data were favourable for all three groups of sows. Undoubtedly, the lightest piglets in the split-weaned litters benefitted from the removal of any competition from their heavier siblings while the reduced metabolic secretory ability of the posterior glands may have limited the growth potential of the five remaining piglets in the cover sows.

## VI CONCLUSION

In conclusion the present findings suggest that the critical factor influencing the concentration of serum prolactin, LH and ultimately follicular development in the lactating sow is undoubtedly the number of mammary quarters suckled by the piglets and hence the neural intensity of the suckling stimulus. This is consistent with the data of several previous studies (Crighton and Lamming, 1969; Peters et al., 1969; Edwards and Foxcroft, 1983; Shaw, 1984) and confirms the results of Experiment 2 (see Chapter 4).

Although this investigation demonstrated the presence of aromatase activity in the majority of follicles from each sow, the oestradiol levels in the follicular fluid of these follicles were low compared with follicular fluid steroid concentrations in preovulatory follicles (Eiler and Nalbandov, 1977; Ainsworth et al., 1980; Experiment 1 of this thesis). Since the diameter of several follicles recovered from the cover sows was equivalent to those follicles harvested during the late follicular phase in the cyclic gilt study (see Chapter 2), there is strong evidence that oestrogen biosynthesis in the lactating sow is limited by a deficit in aromatizable androgen precursor. The low levels of  $^{125}\text{I}$ -hCG binding in a high proportion of dissected follicles suggests that the latter was probably a consequence of inadequate LH stimulation.

The significant differences in ovarian activity observed between the control, split-weaned and cover sows are corroborated by the variation in mean hormone levels detected in peripheral plasma. Since the attachment of the canvas sheeting to the cover sows induced

an immediate elevation in episodic LH release and a decline in PRL levels, it is apparent that the concentration of both these hormones is greatly influenced by the intensity of the suckling stimulus. However, whether the suckling stimulus suppresses gonadotrophin secretion by acting directly via neural pathways impinging on the hypothalamic neurones controlling the secretion of anterior pituitary hormones or indirectly via elevated prolactin, dopamine or opiate levels, remains to be elucidated. Certainly current theories on the suckling induced inhibition of LH secretion favour a role for the endogenous opioid peptides in the lactating sow (Barb et al., 1986; Mattioli et al., 1986; Armstrong, Kraeling and Britt, 1988).

Overall, mean hormone levels were similar between the control and split-weaned groups. The enhanced LH levels observed in half the split-weaned sows in response to the removal of the five heaviest piglets was short-lived in all animals except Sow 53. Nevertheless, as morphological and biochemical follicular development were significantly greater in the split-weaned group perhaps this brief rise in LH concentration was sufficient to advance follicular growth beyond that observed in the control sows.

The endocrine data confirmed the observations of the ethological studies in this and the previous experiment. As LH levels at the time of weaning were suppressed to a similar extent in the control and split-weaned sows, the suckling intensity of the multiple suckling split-weaned litters was obviously equivalent to an entire litter. The temporary rise in LH release detected in a few split-weaned sows immediately after the removal of the five heaviest

piglets may be attributed to a brief depression in mammary stimulation caused by the remaining siblings suckling only their chosen teat unaware of nearby vacated 'quarters'.

Irrespective of strong indications for a negative relationship between plasma levels of prolactin and LH/ovarian activity within animals, in agreement with Benjaminsen (1981) and Van de Weil *et al.* (1985) there is no conclusive evidence in this experiment that high levels of prolactin exert an inhibitory effect upon follicular development in the lactating sow. This is consistent with studies in several species (see Chapter 1, Section IIIb(iv)) in which normal ovarian activity was recorded post-partum despite a maintained state of hyperprolactinaemia. Furthermore, aromatase activity was demonstrated in all follicles *in vitro* after exposure to elevated prolactin concentration *in vivo*. However, it is feasible that disruption of the follicular environment may have altered the biochemical properties of the cells during incubation and that the continued presence of hormones/factors, such as prolactin may be required for any inhibitory/stimulatory effects to be manifest. Nevertheless, the biochemical and morphological development of the follicles from the split-weaned sows were significantly enhanced despite the fact that prolactin levels were similar to the controls. It would, therefore, appear that in the lactating sow, prolactin does not directly inhibit aromatase activity.

As prolactin levels were depressed in the cover sows in response to a decrease in the intensity of the suckling stimulus, it is apparent that levels of the hormone are closely related to the neural

input received by each sow across the entire udder. Hence, Shaw (1984) observed no effect upon serum prolactin in sows following a reduction in litter size because, similar to the split-weaned sows of this study, the multiple suckling activity of the remaining piglets maintained the neural intensity of the suckling stimulus above an unknown threshold level required to sustain a hyperprolactinaemic state. Consequently, from the data of this experiment, it is not possible to distinguish the direct inhibitory effects of prolactin on LH from those mediated by neural stimuli due to suckling.

The absence of a treatment effect upon the levels of oxytocin measured during suckling sessions suggests that the release of this hormone is quantitatively independent of varying levels of mammary stimulation. This theory supports the results of Parvizi and her colleagues (1976) who reported that only one piglet is required to sustain a normal lactation. It is thus likely that in the pig as in the rat (Lincoln *et al.*, 1973) the sudden activation of oxytocin cells takes place only after the summation of afferent impulses trigger the opening of a gate mechanism.

The production data of this study revealed few disadvantages to the piglets reared in either the split-weaned or cover sows. Although the technique of restricting mammary stimulation by covering induced a significant ovarian response, it is unfortunately not a method practically suited to intensive farming systems. Nonetheless the results of the split-weaned litters were extremely favourable, not only to the sow but also to the smaller piglets of each litter. Hence the potential of split-weaning for improving the post-weaning performance of the sow and her litter should not be overlooked.

## CHAPTER 6

### GENERAL CONCLUSION

The pig is an excellent animal in which to study the control of folliculogenesis in a polytocous species, and particularly to examine the inter-relationships between follicles of the same animal. Although previous studies (Ainsworth et al., 1980; Evans et al., 1981; Meinecke, Gips and Meinecke-Tillman, 1987) have established many of the characteristics of porcine folliculogenesis, the majority of authors have failed to describe the development of follicles within the same ovary. The results of Experiment I of this thesis emphasise the considerable range of morphological and biochemical follicular development which exists between follicles within the selected ovarian hierarchy of individual animals. Since follicles of identical size showed great dissimilarity in follicular fluid steroid concentrations and LH binding, this asynchrony in both morphological and steroidogenic development may have great bearing on our present understanding of asynchronies in embryo development. Furthermore, the results suggest that an inhibitory effect of dominant follicles on the maturation of smaller follicles in the hierarchy, proposed for other species (McNatty et al., 1982; Baird, 1983; Ireland and Roche, 1983), is not applicable to the pig.

On the basis of the characteristics assessed in Experiment 1 a process of continuous recruitment into the ovulatory population seems likely to occur during the follicular phase of the pig. A similar mechanism has recently been proposed in at least one prolific breed of sheep (Draincourt, Cahill and Bindon, 1985).

An investigation into the ovarian/endocrine changes associated with various regimens for altering the nursing pattern of the sow and

her litter (Experiments 2 and 3) confirmed previous observations (Palmer, Teague and Venzke, 1965a,b; Kunavongkrit, Einarsson and Settergren, 1982) that a certain degree of follicular development is detectable in the ovaries of the lactating sow irrespective of the number of piglets nursing. However, compared to preovulatory porcine follicles (Eiler and Nalbandov, 1977; Ainsworth et al., 1980; Meinecke, Gips and Meinecke-Tillman, 1987; Experiment 1 of this thesis), the follicles recovered from all sows were steroidogenically immature. Nevertheless, the significantly advanced morphological and biochemical ovarian activity observed in the split-weaned and cover sows emphasised that the neural intensity of the suckling stimulus may be a critical factor, directly/indirectly influencing folliculogenesis during lactation.

Extant aromatase enzyme activity was demonstrated in the follicles of all the sows studied. Since literature data suggest that the aromatase enzyme system is activated by FSH (Dorrington, Moon and Armstrong, 1975; Erickson and Hseuh, 1978) and studies in the lactating sow (Shaw, 1984) have shown that the degree of aromatase activity is positively correlated with peripheral FSH concentrations, it is likely that sufficient levels of the gonadotrophin were circulating in all sows irrespective of treatment. Indeed, Aherne et al. (1976) have reported levels of FSH in the plasma of lactating sows at three weeks post-partum which were similar to those observed by Rayford et al. (1974) in cyclic animals. However, as FSH levels increase significantly after weaning (Edwards and Foxcroft, 1983; Cox and Britt, 1982a; Shaw and Foxcroft, 1985), undoubtedly lactation

does reduce FSH output, even though this may be a marginal effect. Unfortunately, the time constraints on this thesis did not enable any samples to be assayed for FSH. Thus, whether the disparity in extant enzyme activity between groups was a direct consequence of significantly different plasma FSH concentrations requires further experimental investigation.

The observation of episodic LH release during lactation is consistent with previous data from this (Edwards and Foxcroft, 1983; Shaw and Foxcroft, 1985; Foxcroft et al., 1987) and other (Parvizi et al., 1976; Stevenson, Cox and Britt, 1981) laboratories; the inability of several authors to detect similar endocrine profiles (Booman and van de Weil, 1980; Kirkwood et al., 1984a) is related to the inadequate sampling frequencies used. The notable variability in the secretion of LH between sows during the pre-treatment bleed emphasises the differing degrees of LH suppression during lactation, even when parity and lactation length have been standardised. A consideration of the overall LH data for the treatment sows provides some support for the concept (Shaw and Foxcroft, 1985) that a marked increase in episodic LH frequency and in LH baseline after weaning is associated with, and may be the stimulus for, an enhanced development of follicles. However, other factors may influence follicular development since the ovarian response of individual sows to raised LH levels was unique to each animal with no single ovarian variable being consistently stimulated in all sows.

The possible inhibitory effects of prolactin and/or oxytocin on gonadotrophin secretion or ovarian activity in the lactating sow has

been, and remains, an area of intense controversy. However, in most species the evidence for a direct effect of either hormone is unproven. Certainly the results of Experiment 3 do not support a role for these lactogenic hormones in the regulation of LH secretion and ovarian function.

The comparative study of piglet suckling behaviour throughout both experiments revealed that despite a constant suckling frequency, suckling intensity was reduced in the treatment sows. This information in association with the significant differences in ovarian and endocrine data recorded between groups of sows suggests that the critical factor influencing the concentration of serum prolactin, LH and ultimately follicular development in the lactating sow is undoubtedly the number of mammary quarters suckled by the piglets and hence the neural intensity of the suckling stimulus. However, whether the suckling stimulus suppresses gonadotrophin secretion by acting directly via neural pathways impinging on the hypothalamic neurones controlling the secretion of anterior pituitary hormones or indirectly via elevated prolactin, dopamine or opiate levels remains to be elucidated. Certainly current theories on the suckling induced inhibition of LH secretion favour a role for the endogenous opioid peptides in the lactating sow (Barb *et al.*, 1986,; Mattioli *et al.*, 1986; Armstrong, Kraeling and Britt, 1988).

At this point it is pertinent to discuss the applied aspects to which these findings appertain. Although the technique of restricting mammary stimulation by covering induced the greatest ovarian response, it is not a technique practically suited to

commercial farming systems. Nevertheless, the results of the split-weaned group were extremely favourable, not only to the sow but also to the smaller piglets of each litter. In addition this method is less labour intensive than the partial-weaning system and would, therefore, be more economically viable. Hence, the potential of split-weaning for improving the performance of the sow and her litter should not be overlooked.

Finally, it is concluded that the cyclic gilt and lactating sow present excellent models in which to study the initiation of ovarian follicular development and also the complex phenomenon of lactational anoestrus. The Experiments in this thesis have considerably extended current literature data on these topics in the hope that a better understanding of the mechanisms which are involved in these responses may enable the techniques to be applied to systems of potentially practical significance. To comprehend these events more fully will ultimately lead to a greater ability to overcome the restriction placed on sow productivity by the otherwise anoestrus state of lactation.



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## APPENDICES

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## Appendix 1 PIG M7 DAY 16

FN	FD	NG	AR	BG	BT	FFE	FFT	FFP	THT
1	6.0	291	7.3			91.6	27.8	165.0	0.60
2	6.0	250		131.5	13.1	72.0	23.6	56.5	
3	5.8	301		84.7	18.6	36.1	17.4	65.5	
4	5.8	306	9.7			85.8	37.6	76.5	0.57
5	5.8	149	4.2			26.8	7.5	52.6	0.57
6	5.7	169		71.5	42.0	49.4	16.5	50.1	
7	5.6	195	4.3			90.6	13.8	62.8	0.61
8	5.3	233	1.9			92.0	14.2	55.2	0.65
9	5.3	241		86.3	11.4	44.6	12.7	60.1	
10	5.2	140		100.0	12.6	61.8	31.9	75.4	
11	5.2	136	8.1			55.8	29.3	119.0	0.53
12	5.1	210	8.6			51.1	20.4	67.5	0.62
13	5.1	285		70.0	22.9	45.6	10.7	52.5	
14	5.0	243	4.5			95.1	69.9	104.0	0.37
15	5.0	120		43.2	17.7	62.8	19.8	49.8	
16	5.0	251		34.0	7.8	141.0	80.6	54.0	
17	4.5	137	2.7			106.6	79.6	47.1	0.38
18	4.4	149		21.4	12.3	60.6	26.4	27.0	
19	4.0	118	0.7			84.9	46.1	52.9	0.20
20	3.2	81		7.3	7.7	46.0	40.0	65.8	

## Appendix 2 PIG M9 DAY 18

FN	FD	NG	AR	BG	BT	FFE	FFT	FFP	THT
1	6.5	375	2.2			259.0	196.0	139.0	0.3
2	6.5			160.6	102.3	241.0	93.6	21.8	
3	6.3	285	2.0			188.0	49.8	18.7	0.4
4	6.2			88.1	68.5	141.0	48.3	28.6	
5	6.2	414	2.0			161.0	59.3	38.7	0.7
6	6.1	337		114.1	44.9	99.2	23.4	18.7	
7	6.0	352		124.3	64.6	308.0	174.0	207.0	
8	6.0	262		200.1	28.2	261.0	109.6	14.6	
9	6.0	353	2.0			182.0	42.8	25.5	0.4
10	6.0	137	2.3			249.0	254.0	16.3	0.3
11	6.0	318	1.3			181.0	151.0	16.8	0.8
12	5.8	216		148.4	46.1	140.0	150.1	12.5	
13	5.8	148	3.5			178.0	213.8	30.9	0.1
14	5.6	220		83.5	58.8	53.7	26.1	5.0	
15	5.4	366		43.0	53.9	18.1	16.6	<7.0	
16	5.4	477	0.1			4.7	13.5	8.5	0.1
17	5.3	292	0.2			4.5	17.0	8.5	0.1
18	4.9	291	0.0			2.0	12.3	<4.6	0.1
19	5.0	257		16.9	26.0	<1.82	5.66	<7.5	
20	4.1	172		6.5	24.0	1.53	12.8	9.2	

## Appendix 3 FIG M1 DAY 20

FN	FD	NG	AR	BG	BT	FFE	FFT	FFP	THT
1	9.0	172		221.4	106.7	968.0	279.0	270.0	
2	9.0	203	3.2			1245.0	182.0	173.0	13.8
3	8.8	148	4.4			1251.0	253.0	331.0	
4	8.5	54	4.2			1029.0	329.0	179.0	14.0
5	8.5	132		56.9	102.9	1082.0	188.0	122.0	
6	8.3	135	2.5			1152.0	295.0	214.0	12.7
7	8.3	197		234.3	104.0	538.0	207.0	223.0	
8	8.2	205		148.8	78.9	618.0	173.0	93.6	
9	8.1	168		122.8	89.7	648.0	375.0	199.0	
10	8.1	131	5.4			738.0	317.0	246.0	10.6
11	8.1	205	5.0			626.0	197.0	137.0	10.8
12	7.8	134		132.9	79.5	1038.0	169.0	85.8	
13	7.8	41	4.6			874.0	301.0	172.0	4.8
14	7.1	258		43.5	23.7	53.5	25.9	15.9	
15	3.9	89	0.0			14.4	125.0	69.8	0.1
16	3.7	150		27.5	6.1	<1.72	3.49	47.2	
17	3.0	60		12.8	10.1	<6.63	<8.04	54.3	
18	3.0	95	0.1			<2.73	14.5	118.0	0.1
19	2.9	117	0.3			<3.43		64.7	0.0

## Appendix 4    PIG   M12   DAY 21

FN	FD	NG	AR	BG	BT	FFE	FFT	FFP	THT
1	10.5	373	1.7			16.1	2.9	121.0	0.9
2	9.75	401		18.5	25.1	22.3	1.6	65.9	
3	9.75	372	1.5	21.9	25.1	26.4	5.1	101.0	0.4
4	9.5	408	1.8			25.1	4.2	141.0	0.4
5	9.5	381		21.9	25.1	22.2	4.6	92.0	
6	9.5	454		97.2	25.2	30.8	4.8	90.9	
7	9.5	357	2.1			29.4	4.0	150.0	0.3
8	9.25	288		47.6	33.6	20.8	3.3	72.6	
9	8.75	284	3.8			23.3	4.1	155.0	0.4
10	7.0	311		57.4	35.8	30.1	15.7	143.0	
11	6.25	161	0.3	13.9	4.3	46.2	27.8	41.0	0.2
12	5.5					1.5	6.2	86.0	
13	4.0					3.4	10.4	40.3	0.1
14	3.0			6.6	5.7	2.7	6.1	60.6	