ENDOCRINE CORRELATES OF FECUNDITY IN THE EWE

by

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This thesis is dedicated to my mother and father.

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Declaration

I hereby declare that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text. No part of this thesis has been submitted to any other University for any degree or diploma. I consent to this thesis being made available for photocopying and loan, if accepted for the award of the degree.

M.M. Ralph

PREFACE

Aspects of the work herein have been published as follows:

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ABBREVIATIONS

A	Androstenedione (4-androsten-3, 17-dione)
B(+)	Booroola Merino ewes (homozygous or heterozygous)
B(-)	Control Merino ewes
BSA	Bovine serum albumin
CL.	Corpus luteum
CMO	Carboxymethyl oxime
CV.	Coefficient of variation
[ ]	Concentration
cyclic AMP	cyclic 3',5'-adenosine monophosphate
E_2	Oestradiol, oestradiol-17β (1,3,5(10)-estratrien-3, 17β-diol)
FSH	Follicle stimulating hormone
GnRH	Gonadotrophin-releasing hormone
h	hour
hCG	human chorionic gonadotrophin
LH	Luteinizing hormone
min.	minute
n	number
n.s.	not significant
P	Progesterone (4-pregnen-3, 20-dione)
PG	Prostaglandin
PMSG	Pregnant mares' serum gonadotrophin
s.d.	standard deviation
s.e.m.	standard error of the mean
T	Testosterone (4-androsten-17β-ol-3-one)

SUMMARY

This thesis is concerned with a study of the role of pituitary and ovarian hormones in the control of fecundity in the ewe.

1. The increased post-ovulatory FSH secretion on day 1 of the oestrous cycle which occurs in high fecundity ewes has been correlated with the number of antral follicles present in the ovary at the subsequent oestrus. The importance of the second FSH peak in the determination of ovulation rate was, therefore, investigated. No increase in the ovulation rate of Merino ewes was evident following the administration of exogenous FSH(5-10 mg) as one or two doses on day 1 of the oestrous cycle. Twice-daily injections of FSH (3 or 12 mg), on days 1-3 of the oestrous cycle, also failed to increase the subsequent ovulation rate of ewes. These data do not support the view that the size of the second FSH peak influences ovulation rate during the following 2 oestrous cycles.

2. A detailed study of the follicular and pituitary endocrine characteristics of the high fecundity Booroola Merino was, therefore, undertaken. Follicles $\geqslant$ 3 mm diameter were isolated from the ovaries of control and Booroola ewes during the follicular phase of an induced oestrous cycle. There were approximately twice the number of preovulatory follicles, identified by high antral fluid concentrations of oestradiol ($\geqslant$ 1000 nM), in the ovaries of Booroola ewes. The preovulatory follicle of the Booroola Merino was smaller, with approximately one-third the thecal mass and one-half the number of granulosa cells of preovulatory control follicles. Despite their reduced follicular mass, Booroola follicles secreted oestradiol, androstenedione and testosterone at the same rate as the larger control follicle in response to LH in perifusion. Thus, the full steroidogenic capacity of the Booroola follicle is achieved at a much smaller follicle size. *In vivo* and *in vitro* results suggested that each preovulatory Booroola

follicle secreted oestradiol at the same rate as control follicles which contained twice the number of granulosa cells. The aromatase activity per granulosa cell, therefore, appeared to be enhanced in Booroola follicles.

3. Determination of the in vitro aromatase activity of granulosa cells, isolated from the preovulatory follicles after perifusion, failed to reveal the source of the enhanced oestrogen synthetic capacity of the Booroola follicle. No significant difference was found between Booroola and control cells in the amount of oestradiol produced during 48 h of culture, when supplied with androstenedione as substrate. FSH induction of aromatase activity was not evident in cells isolated from either genotype.

4. The hypothalamic-pituitary axis of several breeds of prolific sheep exhibits a decreased sensitivity to the feedback effects of oestradiol. In order to determine whether this characteristic occurred in the Booroola, all ewes were implanted with oestradiol at ovariectomy during the follicular phase of the oestrous cycle. The time interval to the ensuing gonadotrophin surge and the rate of increase of LH levels following the surge were similar in control and Booroola ewes. It was, therefore, concluded that the feedback responses to oestradiol did not differ in Booroola ewes.

5. Analysis of gonadotrophin concentrations in samples taken 2 hourly throughout the periovulatory phase of an induced oestrous cycle revealed no differences between control and Booroola ewes. The pulsatile secretion of LH, measured in samples taken every 15 min for 6 h, did not differ between genotypes during either the luteal or follicular phase of the oestrous cycle. The significance of decreased FSH pulse frequency during the follicular phase of Booroola ewes, the only characteristic of FSH secretion which differed between genotypes, is questioned.

6. The possibility that decreased ovarian androgen concentrations revealed by this study might contribute to the high fecundity of Booroola ewes by increasing the number of developing follicles and/or reducing the incidence of atresia was also investigated. To determine whether ovulation rate could be decreased by high intraovarian androgen levels, testosterone was implanted in the ovaries of Merino ewes. The ovulation rate of testosterone-treated ewes did not, however, differ from that of control ewes following superovulation with PMSG (1000 i.u.) 2 weeks or 4 months after implantation.

7. It was, therefore, concluded that the enhanced capacity of the smaller Booroola follicle to synthesize oestrogen, the hormone essential for follicular development, oocyte maturation, exhibition of oestrus and the potentiation of the preovulatory gonadotrophin discharge, is one of the major factors contributing to the high fecundity of the Booroola Merino.

CHAPTER I

INTRODUCTION

The annual reproduction rate of a species is determined by the number of ova shed per cycle (the ovulation rate), the frequency of the ovarian cycle and the duration of successful pregnancies. The ovulation of fertilizable ova during each reproductive cycle is the culmination of the highly integrated processes of follicular growth and oocyte maturation in response to cyclic pituitary gonadotrophin secretion. Although it is well known that follicular development is dependent on a complex interplay of hormones from the hypothalamus, pituitary and ovary, the endocrine mechanisms which ultimately determine the number of ovarian follicles which successfully mature to ovulation have not been elucidated. This thesis is concerned with an investigation of the role of pituitary and ovarian hormones and their interrelationship in the control of ovulation rate in the ewe.

Most breeds of sheep characteristically ovulate only one or occasionally 2 ova per oestrous cycle. However, a range of physiological and environmental factors are known to influence ovulation rate. By studying the associated endocrine changes, progress towards understanding the underlying determinants of fecundity may be achieved. In particular, examination of the endocrine characteristics of the several breeds of sheep with exceptional fecundity may reveal the physiological basis of the observed differences. Of these breeds, only the Booroola Merino, developed by CSIRO in Australia, demonstrates the presence of a single major gene or closely linked group of genes affecting prolificacy (Piper and Bindon, 1982b).

An understanding of the mechanisms that control fecundity in the ewe would facilitate improvement schemes within existing sheep breeds as

well as the development of improved techniques for the artificial enhancement of fertility in sheep and other species. Rapid genetic improvement of breeding stock through embryo transfer requires a reliable method for the production of high numbers of viable embryos, not currently available. As many aspects of follicular development have been shown to be similar in all species, further knowledge of the control of ovulation might be applied to ovarian dysfunction in the human.

The number of oocytes present in the ovary of the ewe is thought to be at a maximum soon after birth. (Trounson et al., 1974 ; Worthington and Kennedy, 1976). These oocytes, arrested in meiotic prophase, are surrounded by a single layer of flattened epithelial cells, and form a pool of small, non-growing follicles. Once stimulated, the primordial follicle grows through a series of stages until it ultimately develops into a preovulatory follicle or, the fate of more than 99%, undergoes degeneration by atresia at some intermediate stage. Follicular growth is a continuous process throughout the life of the animal. Follicles commence development irrespective of the reproductive state of the animal or stage of oestrous cycle and take approximately 6 months to reach preovulatory size (Cahill and Mauléon, 1980). For ease of discussion this process is divided into three phases:

(1) initiation of growth and slow growth phase;

(2) antrum formation and rapid growth phase; and

(3) preovulatory growth and maturation.

This introduction describes each of these phases separately with reference to the determinants of ovulation rate which might be operating within that phase. Data is drawn from other species when information is unavailable for sheep. The Finnish Landrace (Finn), Romanov, D'Man and Booroola Merino breeds of sheep have all been shown to exhibit mean

ovulation rates of approximately 3, with some individuals shedding up to 11 ova in one cycle (Donald and Read, 1967 ; Piper et al., 1976 ; Bindon et al., 1979 ; Lahlou-Kassi and Marie, 1981). Comparison of follicular and endocrine characteristics of these ewes may elucidate the underlying causes of their high fecundity. Evidence relating to the control of ovulation rate is also drawn from studies in which fecundity has been influenced by environmental or exogenous means. For the purposes of this review prolificacy and fecundity are used interchangeably to describe both ovulation rate and litter-size.

A. Initiation of growth and slow growth phase

1. Initiation of growth

Although Mauléon (1969) has stated that oogonial mitoses in the sheep cease prior to birth, the number of primordial follicles in lambs has been shown to increase from soon after birth to several months of age (Trounson et al., 1974 ; Worthington and Kennedy, 1976). This pool of primordial follicles has been shown to be morphologically heterogeneous in rodents (Lintern-Moore and Moore, 1979) and in sheep (Cahill and Mauléon, 1981). In the ewe, the majority (70 - 80%) of these follicles are dormant, being characterized by their small size, the lack of a relationship between oocyte size or nuclear diameter and follicle size and by being frequently arranged in 'nest' formation (Cahill and Mauléon, 1981). At a follicular diameter of approximately 0.03 mm follicle diameter, the number of granulosa cells and oocyte diameter begin to increase significantly with increases in oocyte nuclear diameter (Cahill and Mauléon, 1981). The authors suggest that it is from this transitory category that follicles enter the growth phase. The rate of change of follicular parameters with subsequent increases in oocyte

nuclear diameter decreases at a follicle diameter of approximately 0.06 mm when there are 3-4 layers of granulosa cells. At this size, follicles are considered to have entered the growth phase.

In sheep, follicular growth commences prenatally as numerous growing follicles (preantral and antral) are present in the ovaries of lambs at birth (Kennedy et al., 1974). Initial recruitment of follicles appears to be controlled by intraovarian mechanisms. In rodents, the size of the primordial follicle pool influences the number of follicles beginning to grow (Krarup et al., 1969). As the size of this pool decreases with the advancing age of the animal (Jones and Krohn, 1961), so does the number of follicles beginning to grow and the speed with which follicles grow (Pedersen, 1969). Reduction of this pool by radiation or chemicals decreases the number of growing follicles (Peters, 1979). After the first weeks of life, the number of growing follicles in the ovaries of lambs and mice decreases to a level which remains relatively constant in the mature animal (Kennedy et al., 1974 ; Peters, 1979). This suggests that a factor restraining growth initiation becomes active at this early stage. Such a factor appears to be present in large, degenerating follicles which do not occur in the first weeks of life, since the injection of antral fluid from these follicles into mice reduces early growth initiation (Peters, 1979).

In rodents, initiation of follicular growth is independent of gonadotrophins as it is unaffected by blocking the action of endogenous gonadotrophin with antiserum (Eshkol et al., 1970), hypophysectomy (Smith, 1930), or by injection of exogenous gonadotrophins (Peters et al., 1973). However, 70 days after hypophysectomy in the ewe, fewer follicles with 3 or more layers of granulosa cells were found in the ovaries. Thus, recruitment from the transitory to the growth phase may be facilitated by gonadotrophins (Dufour et al., 1979).

2. Slow growth phase

Once growth is initiated, the follicle enters a slow phase of growth during which the oocyte increases in size, granulosa cells divide slowly and theca cells differentiate to surround the developing follicle. The first thecal development is apparent when only one layer of granulosa cells are present in the developing follicle and by early preantral stages, the thecal layer is well defined (Peters, 1979). In the ewe, granulosa cell number increases from approximately 200 cells (3 layers) to 5000 cells approximately 130 days later at the start of antrum formation (Cahill and Mauléon, 1980). The ovaries of adult ewes, therefore, contain a large pool of primordial and small follicles with $\leqslant$ 2 layers of granulosa cells (12,000 - 86,000) with fewer larger follicles (100 - 400) throughout the growth phase (Cahill et al., 1979a).

Deprivation of endogenous gonadotrophins by injection of antiserum in neonatal rats resulted in abnormal granulosa and thecal development demonstrating the dependence of this stage of development on gonadotrophic support (Eshkol et al., 1970). In these animals, follicle-stimulating hormone (FSH) alone stimulated follicular growth indicated by granulosa cell proliferation and formation of the basement membrane (Eshkol and Lunenfeld, 1972). These findings are supported by autoradiographic studies in the rat ovary which have shown that receptors for FSH appear very early in development, occurring in follicles containing only one or two layers of granulosa cells, and are confined to granulosa cells (Zeleznik et al., 1974 ; Richards and Midgely, 1976). Thecal cells of preantral follicles contain low levels of luteinizing hormone (LH) receptors (Midgley, 1973 ; Zeleznik et al., 1974) and both theca and granulosa cells contain adenylate cyclase systems which are responsive to the respective gonadotrophin (Hunzicker-

Dunn and Birnbaumer, 1976). Although aromatase activity, which can be induced *in vitro* or *in vivo* by FSH (Erickson and Hsueh, 1978a), is present in the granulosa cells of these follicles, they only have a limited capacity to produce oestradiol (E_2) *in vitro* (Richards, 1980). In spite of this, oestrogens stimulate granulosa cell proliferation in hypophysectomized rats as well as increased ovarian incorporation of FSH (Goldenberg *et al.*, 1972), suggesting that it is only when large, preantral follicles gain the ability to produce significant amounts of oestrogen that they can progress to the rapid growth phase (Richards and Midgely, 1976).

Very little atresia is evident in preantral follicles (Cahill *et al.*, 1979a ; Turnbull *et al.*, 1977a).

3. Factors influencing ovulation rate

(a) Number of primordial and small follicles

The influence of the size of the primordial follicle population on the number of follicles recruited to grow and thus, on the number of follicles progressing towards ovulation has been demonstrated. However, Finnish Landrace cross ewe lambs (Land, 1970) were found to have fewer oocytes (11-15 x 10^4) in their ovaries at birth than the low ovulation rate Blackface or Welsh ewe lambs (20 and 16 x 10^4, respectively). In Peppin Merinos which had been selected for multiple births, a similar relationship was found at, or 1 week after birth, but this difference was not apparent at 5 months of age (Trounson *et al.*, 1974). This result was not supported by a similar study in which ovaries of Booroola Merino lambs were also included. No significant difference was found in primordial or small follicle numbers either within strains selected for multiple births or between strains selected for ovulation rate (Tassell

et al., 1982). However, negative correlations between total oocyte population at birth and subsequent ovulation rate have also been seen in rodents (Jones and Krohn, 1961 ; Land et al., 1974).

This difference in the population of small follicles also persists in the adult ewe. Romanov ewes, with a mean ovulation rate of 3.1, had approximately half the number of follicles with $\leqslant 2$ granulosa layers than ewes of the Ile-de-France breed with a mean ovulation rate of 1.4 (30,501 vs 56,236 respectively ; Cahill et al., 1979a). Adult Booroola and control Merino ewes also exhibit this negative relationship between small follicle number and ovulation rate at 2 and 8 years of age (Cahill et al., 1982).

However, once follicles are recruited into the slow growth phase, there is a reversal in the relationship between ovulation rate and follicle populations in the ovary. There are approximately twice the number of preantral follicles with 3 or more layers of granulosa cells in the ovaries of adult Booroola and Romanov ewes compared to control Merino and Ile-de-France ewes, respectively (Cahill et al., 1979a; Cahill et al., 1982). Therefore, as the high ovulation rate ewe has fewer primordial but more growing, preantral follicles, recruitment into the growth phase must be higher in these ewes. As so little is known of the factors controlling the initiation of follicular growth, the reasons for this increased recruitment are unknown.

It has also been shown that more follicles enter the preantral phase during anoestrus in sheep, but the role, if any, that this increased recruitment plays in seasonal variation in ovulation rate is unknown (Cahill and Mauléon, 1980).

(b) Follicular growth rate

No significant difference was found in the mitotic index of granulosa cells of preantral follicles between breeds of differing ovulation rates or stage of oestrous cycle (Cahill and Mauléon, 1980). Parameters of growth of small follicles such as oocyte diameter and number of granulosa cells were similar in breeds of differing fecundity in relation to follicular size (Cahill and Mauléon, 1980) but follicle size was larger in ewes of low ovulation rate (Cahill and Mauléon, 1981).

(c) Gonadotrophins

The compensation in ovulation rate which occurs in the remaining ovary after unilateral ovariectomy has often been studied as a model to elucidate the factors controlling ovulation rate. Seventy days after unilateral ovariectomy in the ewe, an increased number of follicles of all sizes including preantral was found, which was not evident at 4 days (Dufour et al., 1979). During the period 5.5 - 12 h after unilateral ovariectomy in the ewe there is an increase in FSH, and to a lesser extent LH (Findlay and Cumming, 1977). However, it is unlikely that the follicular compensation seen in increased follicular growth after 70 days is a result of this transient rise. Little difference was found between entire lambs and those unilaterally ovariectomized at birth in plasma LH or FSH levels or pituitary LH content and concentration measured 5 months later (Trounson et al., 1974). However, pituitary FSH content and concentration was higher in hemicastrates. Therefore, long-term changes in follicular growth which occur in hemicastrate ewes may be due to changes in FSH production not detectable by conventional assays of peripheral plasma or to, as yet undefined, intraovarian mechanisms.

(d) Nutrition

As it takes approximately 6 months for a follicle to grow to preovulatory size, it would take this length of time for an effect of nutrition on preantral follicles to alter ovulation rate (Cahill and Mauléon, 1980). Severe undernutrition imposed on ewes for a period of 7 months has been shown to decrease ovulation rate 4-5 months later, after all ewes had returned to similar live-weights (Fletcher, 1974).

B. Antrum formation and rapid growth phase

1. Follicular growth

At a follicular diameter of 0.2 -0.4 mm, antrum formation begins, oocyte growth slows and the follicle enters a rapid growth phase (Turnbull et al., 1977a ; Cahill and Mauléon, 1980). The mitotic index of granulosa and theca cells increases rapidly until it reaches a maximum at 0.7 - 0.8 mm diameter. This rate is maintained until the diameter reaches about 2 mm and then decreases slowly to a low level in preovulatory follicles. Antral volume also increases rapidly up to a diameter of approximately 0.8 mm when the increase slows until the antrum occupies >90% of the volume of the preovulatory follicle. During this rapid growth phase a follicle grows from a diameter of approximately 0.3 mm containing about 5000 granulosa cells to preovulatory size (>2 mm) with about 3 million cells in 34-45 days (Turnbull et al, 1977a ; Cahill and Mauléon, 1980). Three to four follicles a day enter this growth phase (Turnbull et al., 1977a), resulting in 5-24 antral follicles $\geqslant$ 1 mm in diameter being present in the ovary on any day of the oestrous cycle (McNatty, 1982). The proportion of the total number of follicles in the ovary that exhibit signs of atresia increases from almost none prior to antrum formation to

3% at a diameter of 0.5 mm, 27% at 0.9 mm, to approximately two-thirds of all follicles at 1-2 mm (Brand and de Jong, 1973 ; Turnbull et al., 1977a ; Cahill et al., 1979a). Thus, the number of follicles at any stage of growth is, from this point on, also dependent on the rate of follicular loss due to atresia.

2. Endocrine control of follicular growth

The increase in growth during the rapid growth phase requires the presence of pituitary hormones as well as an interaction between granulosa and theca cells in the production of oestradiol.

(a) Gonadotrophins

Seventy days after hypophysectomy, the ovaries of ewes contained 33% more atretic small antral follicles (0.36 mm - 1.12 mm diameter) than 4 days after hypophysectomy (Dufour et al., 1979). In hypophysectomized rodents, ovarian follicles never develop beyond a few cell layers, steroid secretion does not occur and atresia is evident in all medium and large-sized follicles (Schwartz, 1974 ; Peters, 1979). Simultaneous injection of gonadotrophin prevents atresia and maintains follicular growth (Welschen, 1973).

FSH, as has already been shown, stimulates growth in follicles of all sizes (Mauléon and Mariana, 1977). It is also required for antrum formation (Evans et al., 1939 ; Lostroh and Johnson, 1966) and has been shown to stimulate 3β-hydroxysteroid dehydrogenase activity in granulosa cells of larger, preantral follicles of immature rats (Zeleznik et al., 1974). For follicular growth to continue beyond the early stages, LH is required as well as FSH (Eshkol and Lunenfeld, 1972 ; Welschen, 1973), through the role it plays in oestradiol production (Lostroh and Johnson, 1966).

(b) Oestradiol

The capacity of oestradiol to stimulate granulosa cell proliferation in preantral follicles has already been demonstrated. Location of oestradiol receptors in granulosa cells has confirmed these cells as the site of action (Richards, 1975). Oestradiol also prevents atresia in follicles of hypophysectomized rats (Harman et al., 1975). By increasing the number of gap junctions between granulosa cells, oestradiol facilitates the movement of cyclic AMP between cells which may allow activation of the adenylate cyclase system to become generalized throughout the follicle (Merck et al., 1972). Therefore, the ability to synthesize E_2 is an important step in the maturation of preantral follicles to antral follicles.

As both preantral and small antral follicles contain FSH receptors and an active aromatase system induced by FSH (Zeleznik et al., 1977) it has been suggested that their limited capability to produce oestradiol is due to the low LH receptor content of their thecal cells. Early in this phase thecal LH receptor content increases (Uilenbroek and Richards, 1979), and, in response to low levels of LH, theca cells synthesize androgen. Either directly or via transformation to E_2, androgen acts synergistically with cAMP generated in granulosa cells by FSH to induce in the granulosa cell:

(1) increased aromatase activity (Richards, 1980) ;

(2) LH receptors (Zeleznik et al., 1974 ; Richards and Williams, 1976 ; Erickson et al., 1979) ; and

(3) receptors for prolactin (PRL) (Midgely, 1973 ; Richards and Williams, 1976 ; Wang et al., 1979).

Thecal cells have the steroidogenic capacity to produce the androgens androstenedione (A) and testosterone (T), but only a limited capacity to

aromatize these androgens to E_2 (Makris and Ryan, 1975 ; Armstrong et al., 1979). In contrast, granulosa cells are unable to synthesize large amounts of androgen when compared with theca (sheep ; Seamark et al., 1974 ; Moor, 1977), as they lack the $C_{17,20}$-lyase enzymes necessary to produce androgens from C_{21} precursors (Short, 1962 ; Fortune and Armstrong, 1977). Interaction of FSH with granulosa cell receptors induces the aromatase enzymes which convert androgens synthesized in the granulosa cells or which have diffused from the thecal layer (Erickson and Hsueh, 1978a ; McNatty, 1982).

The synergistic action of E_2 and FSH on granulosa cell proliferation occurs without increasing the number of FSH binding sites per cell, thus, the increased FSH binding which occurs during this growth phase is a result of the increasing numbers of granulosa cells per follicle (Richards, 1980). The second action of this synergism is to increase the number of LH receptors on granulosa cells as the follicle enlarges at this stage (Zeleznik et al., 1974 ; Richards et al., 1976). At the end of this rapid growth phase the follicle has enlarged to approximately 2 mm with maximum increases in the thecal and granulosal layer, formed an antrum filled with fluid and begun to acquire receptors for LH and PRL on granulosa cells, thus enabling it to respond to the preovulatory gonadotrophin surge. At this stage of development (1-2 mm diameter) the antral fluid concentrations of oestradiol are similar to or lower than that of androgen (androstenedione and testosterone) in healthy or atretic follicles (McNatty et al., 1982).

3. Factors influencing ovulation rate

(a) Gonadotrophins

The total dependence of preantral follicles on LH and FSH for growth and maturation has been clearly demonstrated but whether

gonadotrophin concentrations are solely facilitative or can influence the number or rate of growth of follicles is less clear. Changes or differences in gonadotrophin concentration relating to ovulation rate may only occur transiently at a vital stage of development during this period of growth, extending over 2.5 oestrous cycles in the sheep, thus making it difficult to correlate with subsequent ovulation rate.

Pituitary weight and content of FSH and LH (measured by bioassay) were similar in Finn cross and low fecundity Merino cross ewes 4 h and 36 h after the beginning of oestrus, but pituitary LH content was lower in Finn ewes on day 10 of the oestrous cycle (Land et al., 1972a). This finding was interpreted as reflecting higher LH release in these ewes which was supported by the finding that Romanov ewes had higher plasma LH concentrations 8 days prior to oestrus (Land et al., 1973). Cahill et al. (1981) also reported higher mean LH concentrations on days 9-11 of the oestrous cycle in Romanov ewes, but as these levels were not correlated with any follicular characteristics of the high fecundity breed, their significance is not known. No significant breed differences were found in mid-luteal LH levels of 4 breeds of ewe of differing fecundity, including Romanov ewes, in a similar study by Bindon et al. (1979). No variation in the total amounts and maximum concentration of LH released during the preovulatory LH discharge have been found which correlate with ovulation rate either within a breed or between breeds (Thimonier and Pelletier, 1971 ; Land et al., 1973 ; Bindon et al., 1978 ; Bindon et al., 1979 ; Quirke et al., 1979 ; Cahill et al., 1981).

Studies of peripheral FSH concentrations in breeds of sheep of varying ovulation rate are few and show little correlation with ovulation rate. When plasma FSH concentrations were compared in four breeds of differing fecundity, no difference was found between breeds in the luteal phase of the cycle (Bindon et al., 1979). The mean FSH

concentration in samples taken hourly during prostaglandin-induced luteolysis was similar in the highest (Romanov) and lowest (Ile-de-France) fecundity breeds and Ile-de-France ewes had the highest mean FSH level in samples taken 2-hourly during oestrus and the following 24 h. However, in a similar study, alignment of two-hourly peripheral plasma FSH determinations in relation to the preovulatory LH discharge showed that the area of the second FSH peak occurring on days 1-2 was greater in Romanov ewes (Cahill et al., 1981). When the data from all ewes was pooled, the area of this peak was significantly correlated with the number of antral follicles present in the ovary at the following oestrus.

In ewes of the same breed maintained on different nutritional régimes, ovulation rate increased with body weight but there was no correlation with plasma FSH on any day of the cycle or with the metabolic clearance rate of FSH on days 10-12 of the cycle (Findlay and Cumming, 1976). As gonadotrophin-releasing hormone (GnRH) infusion resulted in the release of similar quantities of FSH, there appeared to be no difference in pituitary stores.

(b) Number and growth rate of follicles

Even though ovaries removed during the luteal phase of the oestrous cycle from Merino ewes with differing numbers of ovulations had the same total numbers of follicles, the ovaries from ewes with twin ovulations contained fewer early antral follicles (0.3 - 0.42 mm diameter), ie. those just entering the rapid growth phase, and more non-atretic large follicles (diameter > 2.4 mm) than ovaries of non or single-ovulatory ewes (Turnbull et al., 1978). The greater number of follicles entering the rapid growth phase in twin-ovulatory ewes (5.2 vs 4.5 and 3.7 in single and non-ovulatory ewes, respectively) would account for the

decreased number of early antral follicles in these ewes. As the follices from twin-ovulatory ewes took less time to grow through the early stages of rapid growth (0.5 - 0.95 mm diameter) and a correspondingly longer time to grow from 1 mm to preovulatory size, an increase in the number of follicles in intermediate classes did not occur and the overall mean time for follicles to complete the rapid growth phase was similar in all ewes. As the incidence of atresia in follicles > 1 mm diameter was the same in all ewes, the number of follicles present in the ovaries that were capable of being induced to ovulate (> 2.4 mm diameter) was influenced by differences in follicle development occurring 17-21 days (time to grow from 0.4 - 4 mm diameter) earlier. Similar patterns of growth rates have been found in the high fecundity Finn and Booroola Merino breeds (Turnbull et al., 1977b). Thus, for the same total number of follicles in all size classes, the multiple ovulator has more large antral follicles which could respond to the LH surge. The recent finding that the Booroola Merino has more antral follicles than control Merino ewes would further emphasise this relationship (Cahill et al., 1982).

Not all high fecundity breeds of sheep appear to follow this pattern. Romanov ewes had 1.5 - 2 times more antral follicles throughout the entire growth phase than Ile-de-France ewes (ovulation rate 1.4, Cahill et al., 1979a). Within Ile-de-France ewes, a similar trend with ovulation rate was obvious. No differences were found between the two breeds in the growth rate of follicles of any size but for each animal there was a highly significant, linear, negative correlation between the mean mitotic index of the granulosa cells and the number of follicles in each size class (Cahill and Mauléon, 1980). The authors suggest, therefore, that the number of follicles at any stage of growth is limited and that the growth rate of follicles is adjusted to maintain

this limit. Thus, the growth rate of all follicles would be controlled by the largest follicles, as well as by intraovarian follicle dynamics.

(c) Incidence of atresia

The number of growing follicles in this class is also determined by the incidence of atresia. However, no differences in the rate of atresia that correlate with ovulation rate have been found either within a breed (Turnbull et al., 1977b) or between breeds (Cahill et al., 1979a).

(d) Season

Ovulation rate in sheep has frequently been reported to vary with time of year, being highest at the beginning of the breeding season in late summer and autumn and decreasing throughout winter to its lowest level just before anoestrus (Radford, 1959 ; Dun et al., 1960 ; Turnbull et al., 1978). Several reports, however, have failed to show any seasonal variation in the incidence of multiple ovulation (Fletcher and Geytenbeek, 1970 ; Fletcher, 1971 ; Fletcher, 1981).

The numbers of small preantral (< 0.1 mm diameter) and small antral (< 0.4 mm diameter) follicles in the ovaries of sheep are highest during anoestrus, and decline during the subsequent breeding season (Turnbull et al., 1978 ; Cahill and Mauléon, 1980). There are, however, more antral follicles in the ovaries during the breeding season in comparison to anoestrus, even though follicular growth rates are similar at both times of the year (Cahill and Mauléon, 1980). This suggests that a re-stocking of the preantral follicle population occurs during anoestrus and that ovulation rate is highest when this population is at its maximum. This finding may also demonstrate the necessity of the anoestrous period in ewes.

C. Preovulatory growth and maturation

1. Follicular growth

(a) Recruitment of the preovulatory follicle

The final stages of preovulatory growth and maturation of the follicle(s) destined to ovulate are initiated at luteal regression in the ewe. One to three large antral follicles can be found in the ovaries of sheep throughout the luteal phase, but the majority undergo atresia unless luteal regression occurs at a certain stage of development (Smeaton and Robertson, 1971 ; Brand and de Jong, 1973 ; Behrer et al., 1977 ; Turnbull et al., 1977a ; Cahill et al., 1979a ; McNatty et al., 1981c). Oestradiol is secreted by these follicles into ovarian venous blood (Baird et al., 1976b ; McNatty et al., 1981c), resulting in peaks of oestradiol secretion occurring in peripheral plasma on days 2-6, 8-11 and 14-17 of the oestrous cycle (Cox et al., 1971 ; Holst et al., 1972 ; Hauger et al., 1977 ; Herriman et al., 1979). The maintenance of FSH secretion in the sheep at this time probably accounts for this continuous follicular activity (Baird et al., 1975). FSH secretion appears to fluctuate in two waves during the luteal phase (Miller et al., 1981 ; Bister and Paquay, 1983), which may correlate with the reported waves of follicular growth (Smeaton and Robertson, 1971 ; Brand and de Jong, 1973). The development of antral follicles results in increased E_2 and, possibly, inhibin secretion so depressing FSH secretion via negative feed-back to the hypothalamic-pituitary axis (Knobil, 1974). As both tonic LH secretion and LH pulse frequency are inhibited by progesterone secreted by the corpus luteum, an ovulatory LH surge does not occur (Baird and Scaramuzzi, 1976a ; Hauger et al., 1977 ; Karsch et al., 1977). In the absence of increased LH levels, the

follicles undergo atresia, allowing FSH levels to rise. This continuous follicular activity means that a pool of antral follicles is always present in the sheep ovary which can be induced to ovulate if the influence of the corpus luteum is removed.

Unilateral ovariectomy as late as day 14 of the oestrous cycle does not affect the ovulation rate at the subsequent oestrus, suggesting that the follicle destined to ovulate develops later than this (Land, 1973 ; Findlay and Cumming, 1977). This finding is confirmed in studies in which only those follicles marked on day 14, at, or just prior to oestrus ovulated and developed into corpora lutea (Smeaton and Robertson, 1971 ; Bherer et al., 1977). Luteal regression, whether it is spontaneous or induced by chemical or surgical means, results in ovulation approximately 72 h later (Mallampati and Casida, 1970 ; Acritopoulu et al., 1978). Thus, complete maturation of the preovulatory follicle must be possible in this time. The size class from which follicles are selected is, however, undecided.

Using indian ink to mark follicles, Smeaton and Robertson (1971) showed that the largest follicle present in the ovary on days 13-15 of the cycle rarely ovulated at the following oestrus, whereas a similar study reported that the largest follicle present on day 14 ovulated in approximately 80% of ewes (Bherer et al., 1977). McNatty et al (1981c and 1982) found that the preovulatory follicle was recruited from a pool of small (1-3 mm diameter) follicles with high antral fluid E_2 ($\geqslant$ 367nM) and FSH concentrations after the onset of prostaglandin-induced luteolysis. However, preovulatory follicle recruitment may not be restricted solely to this size class. The time to ovulation was delayed by 24 h in ewes in which all follicles $\geqslant$ 2 mm diameter were removed by electrocautery prior to prostaglandin injection, but was unaffected when

only those follicles 2-4 mm diameter were removed (Tsonis et al., 1982). This suggests that follicles > 4 mm diameter may also be recruited.

Thus, it appears that the final selection of the preovulatory follicle occurs from quite a wide range of follicle size after the onset of luteal regression, although the actual mechanism of selection is unclear. It is clear, however, that a capacity for further oestradiol synthesis is essential for continued growth of the follicle, the potentiation of the LH surge, oestrous expression and the development of LH receptors on granulosa cells which allows luteinization and consequent progesterone production. It can be seen that the determination of ovulation rate could be a passive process controlled by the number of follicles reaching a specific stage of development at luteal regression enabling them to be selected for preovulatory growth. Factors occurring at any stage of follicular growth during the previous six months could influence this number. In the late luteal phase of the oestrous cycle the number of follicles containing LH receptors in theca and granulosa cells (England et al., 1981b) or with intrafollicular E_2 concentrations $\geqslant$ 367 nM (McNatty, 1982) is equal to the ovulation rate at the subsequent oestrus suggesting that the number of follicles capable of recruitment is determined prior to luteal regression. If, as suggested previously, the selection criteria are more flexible than this, controls may also be applied during regression.

(b) Preovulatory hormonal events

To follow preovulatory follicular growth, an understanding of the hormonal events which precede ovulation is necessary. The release of prostaglandin (PG) $F_{2\alpha}$ from the uterus primed with progesterone during the luteal phase becomes sufficient by day 13 of the cycle, when LH secretion is low, to cause luteolysis of the corpus luteum, thus

resulting in a decline in progesterone (P) secretion (McCracken et al., 1972 ; Baird et al., 1976a ; Baird and McNeilly, 1981). By studying the periovulatory period after administration of exogenous $PGF_{2\alpha}$, the sequence of hormonal events following this decline in progesterone secretion has been elucidated and shown to be similar to the normal sequence of events at luteolysis in the ewe (Chamley et al., 1972 ; Baird et al., 1976a). As progesterone concentration falls basal LH secretion rises and the frequency of LH pulses increases from one pulse every 3 h in the luteal phase to 1-2 pulses per hour by 24 h after the decline in progesterone secretion (Baird and Scaramuzzi, 1976a ; Hauger et al., 1977 ; Baird, 1978 ; McNatty et al., 1981a). This increase in LH secretion stimulates increased secretion of E_2, A and T into the ovarian vein by the dominant preovulatory follicle(s) (Baird et al., 1981). E_2 levels continue to rise until peak concentrations are reached prior to the LH surge, but A and T concentrations plateau 12 h after luteolysis, maintaining this level for approximately 48 h. The increased secretion of E_2 by the follicle limits LH pulse amplitude by its negative feedback action on the pituitary, and probably also increases LH pulse frequency (Goodman and Karsch, 1980 ; Baird and McNeilly, 1981) as well as suppressing FSH secretion by the pituitary (Salamonsen et al., 1973 ; Pant et al., 1977 ; Baird et al., 1981).

The proportion of antral follicles $\geqslant$ 1 mm diameter which are healthy, defined as containing $\geqslant$ 50% of the maximum number of granulosa cells expected for that diameter, decreases from 50% prior to luteolysis to less than 20% by 24 h after induced luteolysis (McNatty et al., 1982). The decline in peripheral FSH levels may be partly responsible for this increased rate of atresia as injection of pregnant mares' serum gonadotrophin (PMSG) at this time prevents the decrease (Turnbull et al., 1977a ; McNatty et al., 1982), the dominant follicle presumably

maintaining growth with the high intrafollicular concentrations of FSH present (McNatty et al., 1981c). Increased LH-stimulated thecal androgen production by follicles with insufficient aromatase activity to convert androgen to oestrogen may also contribute to increased atresia, as the administration of androgens has been shown to increase follicular atresia in preantral and antral follicles (Hillier and Ross, 1979 ; Zeleznik et al., 1979).

Peripheral E_2 and LH levels continue to rise while FSH levels continue to fall slowly until approximately 54 h after prostaglandin injection. At this time, E_2, T and A secretion by the preovulatory follicle(s) rises to peak concentrations, the rise in E_2 secretion then stimulating the preovulatory surge of LH and FSH six hours later (Goding et al., 1973 ; Baird et al., 1981). Either an increase in, or maintenance of the frequency of GnRH secretion (Webb et al., 1981) as well as increased oestrogen-induced sensitivity of the pituitary to GnRH (Reeves et al., 1971) both contribute to the positive feedback effect of E_2 in inducing the LH surge. The production of E_2 and androgens by the preovulatory follicle is initially stimulated by the LH surge and then declines rapidly (Chamley et al., 1972 ; Baird and Scaramuzzi, 1976a) oestrogen inhibition occurring before that of androgens, suggesting inhibition of aromatase by androgens (Baird et al., 1981). Ovulation occurs approximately 24 h after the LH/FSH peak (Cumming et al., 1971) when steroid secretion is at the lowest level in the oestrous cycle (Baird et al., 1981).

By 24 h after the LH surge there are more atretic follicles in the ovary than on any other day of the oestrous cycle (Turnbull et al., 1977a). When human chorionic gonadotrophin (hCG) was administered 48 h after PMSG in sheep, most of the follicles 1.25 - 3.5 mm diameter became atretic, an effect which may be mediated by androgens produced in

response to the LH surge, acting locally (Louvet et al., 1975). This decrease in the number of antral follicles present in the ovary is thought to result in decreased ovarian inhibin production (Welschen, 1980) which then allows FSH, but not LH, to rise again to similar levels as the preovulatory surge 24 h later (L'Hermite et al., 1972 ; Salamonsen et al., 1973 ; Pant et al., 1977 ; Bolt et al., 1981 ; Baird et al., 1981 ; Goodman et al., 1981 ; Miller et al., 1981 ; Bister and Paquay, 1983). The secondary FSH rise appears to be independent of GnRH control as the administration of sodium pentobarbitone (Dobson and Ward, 1977) or antisera to GnRH (Narayana and Dobson, 1979) decreases the first but not the second peak of FSH. The development of the corpus luteum following ovulation results in increased peripheral levels of progesterone, rising from approximately 0.6 nM on day 1 to 3 nM on day 4 and 10 nM on day 8 on the luteal phase of the oestrous cycle (Thorburn et al., 1969 ; Hauger et al., 1977 ; Herriman et al., 1979).

(c) Growth and maturation of the ovulatory follicle

During the period from luteal regression to the onset of the LH surge, the selected preovulatory follicle continues to enlarge in size by granulosa cell division and accumulation of antral fluid. It takes approximately 70 h to grow from 2 mm diameter to the maximum size reached before the preovulatory surge (Turnbull et al., 1977a), growing to 4 mm diameter by 6 h after the induction of luteolysis, 5 mm at 10 h and 8-10 mm at ovulation approximately 60 h later (McNatty et al., 1982). At oestrus, only the largest follicle detected in the ovary 12 h previously has increased in weight, the weight of smaller follicles remaining constant or declining (Murdoch et al., 1981). The weight of follicular tissue of the preovulatory follicle reaches a maximum at oestrus, no further increase occurring during the LH/FSH surge. The

volume of follicular fluid, however, continues to increase after the onset of the surge, reaching a maximum volume 8 h later (Murdoch et al., 1981).

The rate of blood flow to the preovulatory follicle has increased by the time of the first detected increase in the LH surge (Murdoch et al., 1983). This flow rate is maintained for approximately 12 h, then declines to levels less than those prior to the LH surge at follicular rupture 8 h later. The increased follicular blood flow is not confined to the ovulatory follicle, occurring in follicles of all sizes (Bruce and Moor, 1975) and is probably due to the increased LH concentrations at this time (McCracken et al., 1969), mediated via histamine release (Wurtman, 1964), perhaps augmented by later PGE_2 release (Murdoch et al., 1983).

The number of FSH receptors per granulosa cell does not vary with follicular diameter (Carson et al., 1979) so increases in FSH binding to whole follicles only occurs in the period prior to the LH surge when mitotic division of granulosa cells still continues. The binding of LH (hCG) to granulosa cells, however, increases with follicle diameter (Carson et al., 1979). Only the largest, oestrogen-secreting follicle(s) in the ovary during the preovulatory period exhibits LH binding to granulosa cells (England et al., 1981b). This follicle has a greater number of thecal LH receptors than non-ovulatory follicles and LH receptor number increases in both granulosa and theca cells of this follicle alone, during the ascending limb of the preovulatory gonadotrophin surge (Webb and England, 1982a). Following peak LH secretion, a fall in LH receptor capacity of both tissues occurs, a change which corresponds to the decrease in antral fluid E_2 concentration of these follicles and their in vitro E_2 production from

levels occurring prior to the LH surge (Moor, 1973 ; Seamark et al., 1974 ; Webb and England, 1982b).

LH concentration in antral fluid does not reflect the rapid changes which occur in peripheral blood prior to and during the preovulatory LH rise. Intrafollicular levels only rise after plasma LH levels have been elevated for several hours (McNatty et al., 1981b). Antral fluid concentrations of LH were elevated in small (< 5 mm) and large (⩾ 5 mm diameter) follicles isolated from sheep on the day of the LH surge, whereas prolactin concentrations in large follicles were lowest at this time (McNatty et al., 1981c).

E_2 concentrations in the dominant follicle increase after luteal regression (McNatty et al., 1981c), reaching a peak in theca, granulosa and antral fluid before the first elevation of LH at the onset of the preovulatory surge (Murdoch and Dunn, 1982). Levels then decline until minimal 8 h after commencement of the LH surge. Only those follicles with LH receptors in both granulosa and theca show increased E_2 concentrations in antral fluid prior to the LH peak (England et al., 1981b). Testosterone in follicular tissues and fluid follows a similar pattern to E_2 but a significant decrease in levels does not occur until 4 h after the onset of the LH surge (Murdoch and Dunn, 1982). The progesterone concentration in antral fluid of follicles ⩾ 5 mm diameter does not rise until 16 h after the onset of the LH surge, this high level being maintained until follicular rupture (Murdoch and Dunn, 1982).

The decline in E_2 production of the ovulatory follicle at the beginning of the preovulatory surge is thought to be initially due to LH inhibition of aromatase activity as it occurs before substrate concentrations decrease (Rado et al., 1970 ; Moor, 1974 ; Baird et al., 1981 ; Murdoch and Dunn, 1982 ; Webb and England, 1982b). The continued

secretion of androgens which have been shown to inhibit aromatization in human granulosa cells *in vitro* (Hillier *et al.*, 1980) may augment this effect. The decrease in LH receptor number which occurs after peak LH levels would account for steroid secretion becoming minimal after the LH surge (Baird *et al.*, 1981 ; Webb and England, 1982b).

The high preovulatory LH levels also act on granulosa cells of the ovulatory follicle, which now has increased LH receptor numbers, to initiate luteinization and progesterone production (Moor, 1974 ; Seamark *et al.*, 1974).

The stimulation of follicular steroidogenesis by gonadotrophins is mediated by cyclic AMP (Lindner *et al.*, 1974). Addition of LH to follicles in culture increases tissue cAMP levels within 5 min, maximum levels being reached at 60-90 mins (McIntosh and Moor, 1973 ; Weiss *et al.*, 1976). While theca cells of both large (4-6 mm diameter) and small (1-3 mm) follicles from sheep responded to LH in culture with cAMP production, only granulosa cells of large follicles responded to LH stimulation, reflecting the acquisition of functional receptors during maturation (Weiss *et al.*, 1978). FSH stimulated cAMP production in granulosa cells of both small and large follicles. However, cAMP content of the wall (granulosa and theca) of the preovulatory follicle does not increase above pre-surge levels until 4 h after the beginning of the surge (Murdoch *et al.*, 1981).

Cyclic AMP also appears to mediate LH stimulation of prostaglandin synthesis which is required for follicular rupture. The content of prostaglandins E_2 and $F_{2\alpha}$ in the wall (granulosa and theca) of the preovulatory follicle alone increase approximately 8 h after the commencement of the LH rise. This level of $PGF_{2\alpha}$ is maintained for a further 12 h but PGE_2 levels have decreased 8-12 h later (Murdoch *et al.*, 1981). Concentrations of these prostaglandins in antral fluid or

ovarian venous plasma remain unchanged throughout this time. LH stimulation of prostacyclin production *in vitro* by granulosa cells from mature porcine follicles has also been reported (Veldhuis *et al.*, 1982). The role of these prostaglandins in follicular rupture has not been defined. One or more of the following effects have been implicated in their mechanism of action:

(1) initial vasodilation and increased capillary permeability in the wall of the preovulatory follicle (Murdoch *et al.*, 1981) ;

(2) decreased collagen synthesis (Espey, 1974) ;

(3) increased smooth muscle activity (Walles *et al.*, 1974) ;

(4) release of lysosomal enzymes (Kaley and Weiner, 1975).

(d) Oocyte maturation

Throughout follicular development the oocyte has been maintained at the dictyate stage of meiotic prophase surrounded by a densely packed mass of granulosa cells forming the cumulus oophorous. Meiotic arrest is maintained in the oocyte by the surrounding follicle cells through limitation of nutrient supply or by secretion of a meiotic inhibitor (Moor *et al.*, 1982). Candidates for this inhibitor are:

(1) steroids (Rice and McGaughey, 1981) ;

(2) cyclic AMP (Dekel and Beers, 1978) ; and

(3) one or several peptides of molecular weight < 2000 daltons (oocyte maturation inhibitor), secreted by granulosa cells (Channing *et al.*, 1982).

Throughout oocyte growth, the close contact maintained between follicle cells and oocyte facilitates the transmission of lipid precursors, energy sources and perhaps, amino acids to the oocyte via permeable gap junctions (Moor *et al.*, 1982). The preovulatory gonadotrophin surge appears to trigger the final stages of oocyte maturation including the

resumption of meiosis, cytoplasmic and membrane maturation, and mucification of the cumulus oophorous (Dzuik, 1965). At this time the oxygen consumption of cumulus cells decreases while that of the oocyte increases (Dekel et al., 1976 ; Magnusson et al., 1977).

Although resumption of meiosis does not occur in follicle-enclosed oocytes in vitro unless gonadotrophins are included in the culture medium (Moor and Trounson, 1977), their role appears to be an indirect one. Oocytes removed from preovulatory follicles resume meiosis spontaneously in culture (see Thibault, 1977) and no LH or FSH binding to the oocyte has been demonstrated (Amsterdam et al., 1975). However, gonadotrophins act directly on cumulus cells increasing their progesterone production, enhancing the synthesis of glycoproteins and promoting their dispersal and embedding in a viscous matrix which reduces the number of gap junctions (Hillensjo et al., 1976 ; Thibault, 1977).

While nuclear maturation appears to be hormone- and follicle-independent, the oocyte does not acquire the capacity for successful fertilization and full developmental competence unless enclosed in the follicle for at least 6-8 h (Thibault, 1977 ; Moor et al., 1982). Sheep oocytes cultured outside the follicle showed abnormalities at fertilization or blastocyst stages, despite an apparently normal developmental sequence in the nucleus and surrounding cells (Moor and Trounson, 1977). Inclusion of FSH, LH, PRL and E_2 in the culture medium of follicle-enclosed oocytes resulted in full maturation and subsequent embryonic development in more than 50% of oocytes (Moor et al., 1982). Cytoplasmic maturation, particularly the acquisition of the capacity to change the sperm head into the male pronucleus, appears to be influenced by steroid concentrations. The developmental abnormalities occuring during fertilization and blastocyst formation in sheep embryos were

particularly affected by imbalances in the steroid profile of antral fluid,especially during the first 8 h of maturation (Moor et al., 1980).

Thus, both the early and final stages of oocyte maturation seem to be controlled by the follicle surrounding it. The cells of the follicle inhibit nuclear maturation while development of the follicle progresses, providing precursors necessary for growth. The signal (as yet unknown) of the preovulatory gonadotrophin surge is then transmitted via these cells to the oocyte. When maturation of the oocyte resumes, the follicle cells provide the correct steroidogenic environment for induction of proteins required for complete maturation.

2. Factors influencing ovulation rate

As reviewed in previous sections the number of antral follicles present in the ovary on any day of the oestrous cycle is dependent on the number of growing follicles, their rate of growth and the incidence of atresia. Therefore, the number of follicles available to be recruited for ovulation at luteal regression could solely be a passive process controlled by factors effectual at any time during the six months prior to ovulation. However, extrinsic factors such as the level of nutrition or the administration of gonadotrophins just prior to or at luteal regression have been shown to alter ovulation rate. As the number of antral follicles $\geqslant$ 1 mm diameter (5-24) present in the sheep ovary (McNatty, 1981c) at any time is usually in excess of the ovulation rate, controls imposed during luteal regression may influence the number of follicles recruited for preovulatory growth.

(a) Gonadotrophins

The ability of exogenous gonadotrophins given late in the luteal phase or at luteal regression to increase ovulation rate in ewes is well

known (see Bindlon and Piper, 1982a, for review) although the mechanism of action is not clear. Breeds of sheep in which only one or two follicles normally ovulate each cycle will produce up to 10 ovulations after injection of PMSG or FSH. The ovarian stimulation caused by PMSG has been suggested to arise from a reduction in the number of follicles undergoing atresia at this time and/or an increase in the rate of entry of follicles into the rapid growth phase (Turnbull et al., 1977a ; Dott et al., 1979 ; Cahill, 1982). McNatty et al., (1982) found no difference on PMSG treatment at luteal regression in the total number of antral follicles $\geqslant$ 1 mm diameter but that the number of healthy follicles was greater 10 h after PMSG than in control ovaries. Thus, PMSG reduced atresia in the large ($\geqslant$ 5 mm diameter) follicle population and temporarily prevented small antral follicles from undergoing atresia.

If increases in ovulation rate can result from administration of gonadotrophin during luteal regression, then breeds of sheep which naturally have a high ovulation rate may have increased levels of circulating gonadotrophins at this time. Several studies have already been described in Section B.3a, in which no difference was found in high fecundity ewes. A more detailed study of plasma hormone levels during the follicular phase has also revealed no differences in FSH levels between breeds of different ovulation rates (the Finn and the Suffolk) in the 14 h after removal of progesterone implants (Webb and England, 1982a). The Finn ewes (ovulation rate 2.7) had peripheral LH levels lower than Suffolk ewes (ovulation rate 1.3). FSH concentrations in plasma samples (10 taken at 20 minute intervals) on the three days prior to ovulation revealed no consistent differences between Booroola and control Merino ewes and LH pulse frequency was similar in both groups (Bindon et al., 1982a). No decrease in the rate of atresia of large, antral follicles (> 1.1 mm diameter), a characteristic of PMSG-

treated ewes, has been found in high fecundity ewes (Cahill et al., 1979a).

The failure to demonstrate differences in gonadotrophin secretion either within or between breeds of differing prolificacy may be due to gonadotrophin levels being permissive to ovulation rather than determinants of ovulation rate or to the failure of current methodology. Brown (1978) has shown that above the threshold requirement of the ovary, that is, the level of hormone below which follicular development does not occur, a change of 10% in FSH levels can alter ovulation rate. Present radioimmunoassays would have difficulty detecting such a change, particularly if the biological activity of secreted gonadotrophin was not accurately reflected by immunoreactivity.

Pituitary secretion rates and the resulting peripheral concentrations may not be the only factor controlling the amount of gonadotrophin reaching the preovulatory follicle(s). The Booroola Merino has been shown to have a much higher capillary blood flow than the control Merino which would result in the delivery of more gonadotrophin to each follicle despite similar peripheral hormone concentrations (Brown et al., 1981).

(b) Ovarian sensitivity to gonadotrophins

With the difficulty experienced measuring small differences in plasma levels of hormone, it is of value to determine whether ovulation rate increases originate at the level of the ovary or the hypothalamic-pituitary axis. In this regard, total receptor populations in the follicles of ewes of differing fecundity could be useful, but no studies have been reported.

An enhanced ovulatory response to exogenous gonadotrophin has provided evidence for increased ovarian sensitivity in prolific strains

(e.g. mice ; McLaren, 1962 ; Bindon and Pennycuik, 1974 : sheep ; Bindon et al., 1971 ; Bindon and Piper, 1982a and 1982b). However, high fecundity strains have been shown to have a greater number of large growing follicles present in their ovaries. Thus, more follicles of an appropriate size range (≃ 1 mm in the sheep) are available to be stimulated by PMSG (Mauléon, 1969 ; Cahill et al., 1979a). This does not mean that increased ovarian sensitivity to gonadotrophin does not operate as well in fecund strains. Booroola and control Merino ewes have a similar time interval from the onset of oestrus to the start of the preovulatory LH discharge (Bindon et al., 1978), but the Booroola ovulates earlier than controls (Bindon and Piper, 1979), suggesting the preovulatory follicles are more sensitive to gonadotrophin in the Booroola.

(c) Sensitivity to negative and positive feedback

Romanov and Finn breeds of sheep exhibit a common difference in preovulatory hormone profiles when compared with low fecundity breeds. In these ewes, the time interval between oestrus and the onset of the LH surge, as well as the duration of oestrus are longer than ewes of low ovulation rate (Land et al., 1973 ; Wheaton et al., 1977 ; Bindon et al., 1979 ; Quirke et al., 1979 ; Cahill et al., 1981). Peripheral gonadotrophin levels in ovariectomized ewes injected with oestradiol initially decrease through the negative feedback action on the pituitary, followed by the positive feedback reaction of an LH surge. Finn ewes injected with 50 µg oestradiol benzoate during the breeding season showed less initial reduction in plasma LH levels, a longer time interval to the onset of the surge and a lower maximum concentration of LH reached during the surge than similarly injected Blackface ewes (Land et al., 1976). From these findings Land postulated that the

hypothalamic-pituitary axis of high fecundity breeds may be less sensitive to the positive and negative feedback effects of oestrogen. Thus, the time interval for preovulatory follicular development, before cessation of E_2 secretion by the LH discharge, is longer in these ewes. Also, higher levels of E_2 would be tolerated before the suppression of FSH secretion in the follicular phase. Further evidence for this theory comes from a study in which Finn and Blackface ewes were injected with E_2 on days 3-14 of the oestrous cycle (Land, 1976). The proportion of Blackface ewes which ovulated at the subsequent oestrus was less than uninjected ewes, but the proportion of Finn ewes that ovulated was unaffected by E_2 treatment, demonstrating the decreased sensitivity of the Finn breed to exogenous oestrogen

A reduction in the sensitivity of the hypothalamic-pituitary axis might be expected to result in increased circulating levels of E_2 and/or gonadotrophins. Gonadotrophin concentrations have already been shown to be similar in breeds of differing fecundity and published data on oestradiol secretion is conflicting. The positive association between duration of oestrus and the amount of oestradiol administered to progesterone-treated, ovariectomized ewes was considered to suggest that the longer duration of oestrus in prolific ewes arose from their increased oestrogen secretion (Land et al., 1972b). The ovarian venous secretion rate and ovarian venous concentration of E_2 has been reported to be higher in Finn or Finn cross ewes during the periovulatory period (Baird and Scaramuzzi, 1976 ; Wheeler et al., 1977 ; Scaramuzzi and Land, 1978). However, this difference is often not reflected in peripheral hormone concentrations (Bindon et al., 1975 ; Scaramuzzi and Land, 1976 ; Bindon et al., 1979). Cahill et al (1981), however, found that the amount of E_2 secreted in the 24 h prior to the LH discharge was

greater and that peripheral E_2 concentrations at the onset of the LH surge were higher in Romanov than in Ile-de-France ewes. These levels were correlated with the number of large antral and preovulatory follicles. Despite increased E_2 levels, the time from luteolysis to the onset of the LH surge was longer in Romanov ewes. LH and FSH levels during the preovulatory period in the two breeds were similar, thus supporting Land's hypothesis. In contrast, the Booroola Merino does not differ from the control Merino in the timing of the LH discharge (Bindon et al., 1978) or duration of oestrus (Bindon et al., 1982b). Peripheral LH concentrations also decreased similarly in ovariectomized control and Booroola ewes, implanted with oestradiol just prior to anoestrus (Cummins et al., 1982).

The sensitivity of the hypothalamic-pituitary axis to the negative feedback effect of E_2 may also underly the differences in breeding season exhibited by ewes of differing fecundity. The Finn (Wheeler and Land, 1977), Romanov (Thimonier and Mauléon, 1969) and Booroola Merino (Bindon and Piper, 1976) have a shorter anoestrous period than low fecundity ewes in the same environment. In the ewe, seasonal anoestrus appears to result from increased responsiveness of the hypothalamus to the negative feedback effect of E_2 (Legan and Karsch, 1979). Highly prolific breeds in which a decreased sensitivity to E_2 has been demonstrated (the Finn and Romanov), appear to be more resistant to this change. No evidence of decreased sensitivity to E_2 has been demonstrated in the Booroola Merino which also has a long breeding season. However, ovariectomized ewes of this strain show increased pituitary sensitivity to low doses of GnRH, both during the breeding season and anoestrus, than control Merinos (O'Shea et al., 1981), which may allow ovulation during the normal anoestrous period. However, no such difference was found between ovariectomized Finnish Landrace, Scottish Blackface and

Tasmanian Merino ewes injected with GnRH at three different times of the year (Land et al., 1979). This suggests once again, that the underlying mechanism may vary between breeds.

(d) Inhibin

The failure of oestradiol and progesterone to fully suppress FSH secretion in the ovariectomized ewe has led to the hypothesis that another ovarian hormone also controls tonic FSH secretion during the oestrous cycle (Goodman et al., 1981). Evidence from rats, pigs and ewes suggests that a protein (inhibin) of > 10,000 Daltons, present in follicular fluid and secreted by granulosa cells, is able to inhibit pituitary FSH, but not LH, secretion in vitro and in vivo and may play a major role in the regulation of FSH secretion (see Channing et al., 1982 and Grady et al., 1982 for review). Inhibin is secreted in vitro by granulosa cells from porcine follicles of all sizes with cells from larger follicles having double the activity of cells from small and medium-sized follicles. Antral fluid from large follicles of the cow and pig, however, contains less inhibin than small follicles, perhaps due to higher clearance from these follicles (Channing et al., 1982). In ewes, the inhibin content of large follicles ($\geqslant$ 3.5 mm diameter) is higher than that of small follicles (Tsonis et al., 1983). A preliminary report found that the inhibin content of pooled antral fluid from follicles judged to be morphologically atretic was less than healthy follicles (Scott et al., 1980b). Subsequently, it has been found that although inhibin content and concentration in individual follicles does not differ with morphological assessments of atresia, both were correlated with the aromatase activity of granulosa cells and antral fluid oestradiol concentrations in large ($\geqslant$ 3.5 mm diameter), but not small follicles (Tsonis et al., 1983).

Inhibin is secreted into the ovarian vein of rats, varying inversely with plasma FSH concentrations during the oestrous cycle, which suggests that its secretion may be controlled by gonadotrophins (De Paolo et al., 1979). A significant increase in serum inhibin concentration, and a concomitant decrease in FSH, occurs 6-64 h after PMSG injection in immature female rats (Lee and Findlay, 1982). The authors suggest that the increased inhibin secretion arises from the increased granulosa cell numbers of non-atretic follicles resulting from PMSG treatment. In vitro, inhibin production by porcine granulosa cells of large follicles was stimulated by dihydrotestosterone, LH and FSH (Channing et al., 1982). However, inhibin secretion by bovine granulosa cells was unaffected by LH and FSH, stimulated by dihydrotestosterone, testosterone and androstenedione and suppressed by progesterone (Henderson and Franchimont, 1981).

As inhibin appears to be involved in the regulation of FSH secretion, it might be expected to play a role in folliculogenesis. In rodents, the post-ovulatory FSH peak at oestrus and the rise in FSH which occurs on unilateral ovariectomy, control the number of antral follicles which ovulate at the following oestrus (rat : Richards, 1980 ; hamster : Greenwald and Siegel, 1982). As these surges can be suppressed by follicular fluid containing inhibin-like activity, but not steroids, this inhibin-mediated control of FSH appears to regulate the number of maturing follicles (Welschen et al., 1978 ; Welschen et al., 1980). Whether a similar mechanism exists in the ewe is unknown. Administration of bovine or ovine follicular fluid to ewes during natural or induced luteolysis delays oestrus and decreases ovulation rate (Miller et al., 1979 ; O'Shea et al., 1980 ; Cummins et al., 1980), by suppressing FSH levels (Findlay et al., 1981) so decreasing the growth rate of the largest follicles (Miller et al., 1979). Immunization of ewes with a

partially purified inhibin preparation of bovine follicular fluid increased the ovulation rate of ewes temporarily, without affecting oestrous cycle length or oestrous expression (O'Shea et al., 1982). Studies of the Booroola Merino suggest a role of inhibin in determining the high ovulation rate of this strain. The ovaries of Booroola ewes contain only one-third the inhibin of ovaries of control Merino ewes (Cummins et al., 1983). However, Booroola ewes are more sensitive to the negative feedback effects of inhibin as demonstrated by the ability of ovine follicular fluid to suppress plasma FSH in ovariectomized ewes (Cummins et al., 1983) and to decrease ovulation rate when injected during luteolysis (Cummins et al., 1980).

(e) Immunization against steroids

Active or passive immunization of ewes during the breeding season against the steroids oestradiol-17β, oestrone, androstenedione or testosterone results in increased ovulation rates (Cox et al., 1982 ; Land et al., 1982). High titres achieved during active immunization result in anoestrus but moderate titres (1:500 to 1:3000) at joining of ewes give rise to an increased twinning rate without the variability and the frequent exceptionally high ovulation rates seen with gonadotrophin treatment (Cox et al., 1982). As passive treatment is effective when given on the day of mating, irrespective of stage of oestrous cycle, immunization only effects follicles in the latter stages of growth and maturation, persisting in animals returning to service in the following cycle (Land et al., 1982).

The ovarian stimulation as evidenced by increased ovarian weight, greater numbers of large active follicles and increased ovarian vein secretion of steroids is thought to be due to elevated basal LH levels and pulse frequency in immunized ewes (Martensz et al., 1976 ;

Scaramuzzi et al., 1977 ; Martensz and Scaramuzzi, 1979 ; Martensz et al., 1979 ; Scaramuzzi et al., 1980b). The role of FSH is less clear with decreased levels in androstenedione-immunized, similar levels in testosterone-immunized (Martensz and Scaramuzzi, 1979) and increased levels in oestrogen-immunized ewes (Scaramuzzi et al., 1980b). While the increased ovulation rate in oestrogen-immunized ewes appears to be due to a reduction in negative feedback action resulting in increased gonadotrophin secretion to stimulate follicular development, the underlying mechanism in androgen-immunized ewes is less clear. Antibodies to oestradiol were also formed in testosterone-immunized ewes, therefore a similar reduction in negative feedback may occur in these ewes. However, no binding of oestradiol was found in the plasma of androstenedione-immunized ewes and preovulatory oestradiol secretion was increased which has led to the postulate that androstenedione normally modulates the negative feedback control of LH in the ewe during the breeding season (Martensz and Scaramuzzi, 1979), as well as during anoestrus (Martensz et al., 1976). The failure to demonstrate androstenedione binding in the pituitary or hypothalamus of the ewe (Clarke et al., 1982) and the failure of androstenedione to alter basal LH secretion or LH pulse frequency in ovariectomized ewes (Martin and Scaramuzzi, 1980) suggests that androstenedione exerts this effect after conversion to oestrogens.

The second mechanism proposed to explain the increased ovulation rate in androgen-immunized ewes is that the removal of androgens from antral fluid by antibodies reduces the rate of atresia in large, antral follicles (Scaramuzzi, 1979). This might explain the persistence of large, cystic follicles (> 5 mm diameter) in testosterone-immunized ewes (Scaramuzzi et al., 1981a). However, in this study, no decrease was observed in the incidence of atresia in follicles 0.3 - 5 mm diameter.

(f) Nutrition

For many years, the short-term nutritional status and/or bodyweight of ewes has been known to effect ovulation rate but much of the reported data is conflicting in defining the basis of the response. The practice of 'flushing' ewes, that is, providing a short-term increase in the level of nutrition prior to mating, results in increases of 10-20% in lambing percentages (Coop, 1966). This response comprises both an increased ovulation rate and an increase in the number of ewes lambing (Knight et al., 1975). The increased ovulation rate achieved by flushing depends on both the pre-mating nutritional level and the body weight at mating (Allen and Lamming, 1961 ; Coop, 1966 ; Gunn and Doney, 1979 ; Gunn et al., 1979), and, as they are related, it is often difficult to distinguish between these causes in many studies. However, there appears to be strong correlation of ovulation rate with liveweight, each kilogram extra in liveweight resulting in 1-3% increase in ovulation rate (Edey, 1968 ; Fletcher, 1971 ; Cumming, 1977 ; Morley et al., 1978). Certainly, below a certain body weight, ewes may not ovulate at all (Fletcher, 1981) and oestrous expression is depressed (Allen and Lamming, 1961).

Only ewes in poor or moderate body condition respond to flushing with an increase in ovulation rate, and the rate achieved is similar to that of ewes maintained in good body condition (Allen and Lamming, 1961). The effect of flushing on ovulation rate can be achieved, however, without an increase in body weight. Ewes fed a supplement of lupins during the 6-8 week period of mating, or for two weeks prior to and after the beginning of mating, had an ovulation rate 13-20% higher than non-supplemented ewes, without any concurrent change in bodyweight (Knight et al., 1975). Lindsay (1976) demonstrated that this response occurred after only six days of lupin feeding with no associated

bodyweight change, and disappears soon after the cessation of feeding, suggesting that it is a transient effect on the final stages of antral follicle development. Up to approximately 4 weeks (2 oestrous cycles) a significant correlation of ovulation rate with the time of additional feeding has been found, even though bodyweight change was minimal during the second oestrous cycle (Allen and Lamming, 1961). No further increase in ovulation rate was noted up to twelve weeks. This relationship does not always appear to hold as the increase in ovulation rate recorded after 17 days of differential feeding of ewes at three different times of the year, was also achieved without any associated change in bodyweight, but did not change after a further 17 days feeding (Fletcher, 1981). The feeding of high protein supplements such as lupins appears to increase ovulation rate only at low levels of energy intake (Fletcher, 1981). Above a certain threshold requirement for protein, the level of which is dependent on the type of protein and its rate of ruminal degradation, energy, either together with protein or alone, becomes the limiting factor in ovulation rate (Fletcher, 1981 ; Smith et al., 1981).

The mechanism of the nutritional effect on ovulation is unclear. The ovaries of ewes which responded to 18 days supplementary feeding with an increased ovulation rate had the same number of follicles 1-2 mm diameter, but more follicles 2-3 mm diameter at the onset of and 18 and 48 h after the following oestrus, than ewes not given additional feed (Haresign, 1981). The number of follicles > 3 mm diameter remained the same at oestrus and 18 h later in flushed ewes, but decreased at 18 h in non-supplemented ewes, suggesting an increased incidence of atresia at the lower level of feeding. Following ovulation (48 h after oestrus), more follicles > 3 mm diameter were found in the ovaries of supplemented ewes, which is consistent with the higher number of 2-3 mm diameter

follicles in these ewes. Thus, more rapid growth or higher recruitment into the rapid growth phase may occur in supplemented ewes. That nutrition can affect earlier stages of follicle growth or recruitment was demonstrated by Allen and Lamming (1961). Ewes fed a submaintenance diet for 3 months resulting in 27% loss of liveweight had fewer follices < 2 mm diameter in their ovaries.

It is unclear whether changes in gonadotrophin levels accompany changes in ovulation rate at different nutritional levels. The finding that ewes maintained on differing levels of nutrition for 6-7 weeks had similar peripheral FSH concentrations, FSH metabolic clearance rates and FSH response to GnRH (Findlay and Cumming, 1976) has already been mentioned (B. 3.a). Significant pituitary stores of gonadotrophin were present in ewes fed a submaintenance diet for 3 months (Allen and Lamming, 1961). Nutritional level does not appear to affect total pituitary LH content at oestrus, or the magnitude of the preovulatory LH surge (Lishman et al., 1974 ; Haresign, 1981). The increased ovulation rate of ewes fed a dietary supplement of lupins during the previous 1 or 2 oestrous cycles was, however, associated with increased FSH plasma concentrations 4-5 days before oestrus (Brien et al., 1976 ; Davis et al., 1981 ; Knight et al., 1981). This difference was not maintained in the days immediately preceding oestrus. In contrast, no relationship was found between the patterns of release and concentrations of FSH or LH in the luteal or follicular phase of the oestrous cycle and supplementation with lupins or ovulation rate in a study by Radford et al., (1980). The finding that plasma FSH and E_2 levels are both increased 4 days prior to oestrus in ewes fed lupins, but not in ewes of high live-weights, despite increased ovulation rates of both groups implies the mechanisms operating in each instance may differ (Knight

et al., 1981). A decreased sensitivity to the negative feedback effects of oestrogen appears to occur in lupin-fed ewes.

Ovarian sensitivity to gonadotrophins is also unaffected by nutritional status as ewes fed submaintenance diets responded similarly to flushed or maintained ewes when given PMSG late in the luteal phase (Allen and Lamming, 1961 ; Lamond, 1963).

D. Summary

Ovulation rate appears, therefore, to be determined by the number of follicles that have developed sufficient maturity at a specific stage of the oestrous cycle to respond to the preovulatory gonadotrophin surge. This development incorporates structural growth of the follicle, acquisition of hormonal receptors and maturation of the oocyte. In most breeds of sheep, the number of preovulatory follicles is precisely controlled so that only one, or occasionally two, ovulate at each oestrus. It is not clear, however, at which stage of follicular development this control of ovulation rate is imposed. Examination of environmental, genetic and extrinsic variations in ovulation rate has implicated the number of growing follicles, rate of follicular growth, incidence of atresia and the recruitment of preovulatory follicles in the determination of ovulation rate.

Although gonadotrophins are clearly essential for folliculogenesis, a definitive role in the control of fecundity has not emerged. This may be explained by the failure of current methodology to accurately distinguish small changes in biologically active material or by sampling régimes which were infrequent or at inappropriate stages of follicular development. Thus, while FSH increases correlated with ovulation rate have been observed during the late luteal phase with increased nutrition and unilateral ovariectomy and in the post-ovulatory peak in high

fecundity ewes, no consistent pattern has been demonstrated. LH secretion is similar in ewes with genetic differences in ovulation rate but increased in ewes with high ovulation rates resulting from immunization against steroids.

A complex interrelationship between ovarian hormones and the hypothalamic-pituitary axis controls gonadotrophin secretion. Yet, little is known of either the individual follicular or total ovarian response to gonadotrophins in prolific ewes. Until the secretion rates of steroids and inhibin are defined in ewes with high ovulation rates, the role of feedback sensitivity of the pituitary to these hormones in the determination of ovulation rate can not be ascertained.

The development of the potential for ovulation within each follicle requires interactions between granulosa and thecal cells and gonadotrophins. However, comparative studies of receptor populations or the relative response of whole follicles or their separate compartments to gonadotrophins have not been reported. This thesis examines the interrelationship between follicular development and gonadotrophins in the determination of ovulation rate in the ewe.

CHAPTER II

GENERAL MATERIALS AND METHODS

A. Laparoscopy procedure

All laparoscopic examinations required for these studies were carried out by Mr. Peter Geytenbeek. At each examination the number of corpora lutea on both ovaries was recorded. Ewes were fasted for 15-22 h before laparoscopy. Each ewe was restrained in a horizontal, dorsal position in a laparoscopy cradle (Lamond and Urquhart, 1961). The ventral abdominal area was shorn closely and scrubbed with chlorhexidine cetrimide (Delta West Pty. Ltd., Adelaide, Australia). At each of two sites approximately 5 cm each side of the midline, 10 cm cranial to the mammary glands, 2.5 ml 2% xylocaine (Astra Pharmaceuticals, Sydney, Australia) was injected subcutaneously. The ewe was covered in a sheet of plastic soaked in chlorhexidine solution and raised to an angled position, the head resting close to the floor. A 7 mm trocar and cannula were inserted into the abdomen at one site and the abdomen insufflated with medical air through a value on the cannula. The trocar was removed from the cannula and replaced by a 30°C, 6.5 mm laparoscope (Storz ; Tuttlingen, W. Germany) attached to a light source via a fibre optic cable. Atraumatic manipulating forceps were inserted similarly at the other site and the ovaries of the ewe examined. On completion, all instruments were removed, the incisions sprayed with antibiotic (Neotracin ; Ethnor Pty. Ltd., Sydney, Australia) and the ewe removed from the table and returned to the flock. When not in use, all surgical equipment was kept in chlorhexidine solution.

B. Steroid analysis

1. Reagents

(a) General

Common reagents and solvents were obtained from either Ajax Chemicals (Sydney, Australia) or B.D.H. Chemicals (Melbourne, Australia). All solvents used for extraction of steroids or column chromatography were redistilled. Bovine serum albumin (Fraction V) was purchased from Sigma (St. Louis, MO, U.S.A.)

Normal human immunoglobulin (160 mg/ml) was purchased from CSL (Melbourne, Australia), polyethylene glycol 6000 from BDH Chemicals and 2,5 diphenyloxazole (P.P.O.) from Koch-Light (Bucks, England).

(b) Chromatography

Lipidex 5000 (Packard Instrument Co., Ill. U.S.A.) was utilized for chromatography of steroids. The Lipidex slurry in methanol was transferred to a Buchner funnel and washed with copious volumes of hexane. The gel was then mixed with at least 5 volumes of hexane, sonicated 30 min to remove air and equilibrated overnight. This slurry was packed into siliconised glass columns (3.5 mm i.d.), with reservoir attached, to a height of 80 mm. The columns were stored in hexane and reused frequently, being washed with ethyl acetate (5 ml) and reequilibrated in hexane (10 ml) between each use.

(c) Standards

Steroids used as standards were obtained from Steraloids (Wilton, N.H., U.S.A.). 1 mM stock solutions in ethanol were prepared for all steroids except progesterone, and further diluted to 2 µM with ethanol.

These solutions were stored at 4°C and used to prepare a working dilution in ethanol (10 nM), immediately prior to use. Stock solutions of progesterone in ethanol (4 mM and 10 μM) were similarly maintained but working dilutions (0.2 μM and 10 nM) were prepared by dilution into assay buffer.

(d) Tracers

All tritiated steroids were purchased from New England Nuclear (Boston, Mass. U.S.A.). [1,2,6,7-^{3}H] progesterone, androstenedione and testosterone and [2,4,6,7-^{3}H] oestrone of specific activities 80-115 mCi/mmol were used as tracers in the appropriate assay, 10,000 cpm (≃100 pmol) being added to each assay tube. Oestradiol-17β-6-(O-carboxymethyl) oxime (CMO)-^{125}I-iodohistamine, prepared according to Nars and Hunter (1973), was used as tracer, each assay tube containing 10-30,000 cpm. Tritiated steroids, including [2,4,6,7-^{3}H] oestradiol-17β, were used for estimation of recovery in extracted samples.

(e) Antisera

Antisera were raised in goats to oestradiol-17β-6-CMO conjugated to bovine gamma globulin (bGG; Fraction II, Calbiochem, San Diego, Calif., U.S.A.), progesterone-11α-hemisuccinate-bGG, and testosterone-15β-(3-thiopropionic acid) - BSA. The oestradiol and progesterone haptens were purchased from Steraloids. The synthesis of the testosterone hapten (Rao and Moore, 1976) and conjugation of haptens to the appropriate protein (Bauminger et al., 1974) were carried out in our laboratory. Androstenedione and oestrone were assayed using antisera, kindly donated by Dr. R.I. Cox (C.S.I.R.O., Prospect, N.S.W.), which were raised in sheep immunized with androstenedione-3-CMO-BSA and oestrone-6-CMO-BSA,

respectively. Titres and cross-reactivities of these antisera, calculated as defined by Abraham (1969), are given in Table 1.

Table 1 Titres and per cent cross-reactivity of various steroids with antisera for androstenedione (A), oestradiol-17 $\beta(E_2)$, oestrone (E_1), progesterone (P) and testosterone (T).

Steroids	Antisera (Final dilution in assay tube)				
	A (1:75,000)	E_2 (1:100,000)	E_1 (1:40,000)	P (1:10,000)	T (1:10,000)
Androstenedione	100	0.02	<0.01	0.07	0.3
Cortisol	0.02	<0.01	<0.01	<0.05	-
Dehydroepiandro-sterone	3.5	0.04	<0.01	-	<0.02
5α-dihydro-testosterone	0.5	0.14	-	-	11.9
17α-hydroxy-progesterone	0.3	<0.01	<0.01	1.2	-
Oestradiol-17β	<0.02	100	0.33	<0.03	<0.02
Oestriol	-	0.9	0.04	-	-
Oestrone	0.1	14	100	-	<0.02
Progesterone	0.5	0.6	<0.01	100	0.24
Testosterone	0.9	0.14	<0.01	<0.05	100

(f) Steroid-free plasma

Steroid-free ovine plasma for inclusion in standard curves and as blank samples was prepared by charcoal treatment. Plasma was incubated with 10% (w/v) distilled water-washed charcoal (Ajax Chemicals, Sydney, Australia) for 1-2 h at 37°C. Charcoal was then removed by

centrifugation (4°C) at 4000 g for 30 min followed by 27,000 g for 20 min. The steroid-free plasma was stored at -20°C until required.

2. General radioimmunoassay procedure

A common radioimmunoassay procedure, developed by the author, was used to measure steroids in samples of plasma or culture medium after extraction and/or chromatography, if necessary. The diluent buffer for antisera and tracers was 0.1 M sodium phosphate, pH 7.0 containing 0.9% sodium chloride, 0.1% sodium azide, and 0.1% BSA, the final assay volume being 0.2-0.4 ml. Equilibrium was achieved after 10 mins incubation at 37°C and 30 mins at 4°C for the progesterone, oestrone and androstenedione assays but 2 h incubation at 37°C followed by overnight at 4°C was required for testosterone and oestradiol assays. Precipitation of bound steroid was achieved by adding 0.05 ml (1-2 mg) human immunoglobulin and 0.8 ml 27% (w/v) polyethylene glycol 6000 at equilibrium (4°C). The supernatant was aspirated after centrifuging at 4000 g, for 15 min at 4°C. For the iodinated tracer, the precipitate was counted directly in a Nuclear Enterprises 1600 gamma counter. The precipitate was mixed with toluene scintillant containing 5 g PPO/l, 10% (v/v) ethanol when tritiated tracers were used before radioactivity was determined in a Nuclear Chicago Isocap 300 scintillation counter. To prevent adsorption of progesterone and androstenedione, incubations were carried out in glass tubes (50 x 10 mm ; HLS Scientific, Adelaide, Australia) whereas oestradiol, oestrone and testosterone were assayed in polypropylene tubes (51 x 8 mm ; Bunzl Pty. Ltd., Adelaide, Australia). All extractions were carried out in freshly-washed 13 x 100 mm glass tubes (Maple Leaf, Canada).

Assay data were calculated by computer as described by McIntosh and McIntosh (1980). The sensitivity, defined as the dose corresponding to

the bound count which was significantly ($p < 0.05$) lower than that of the zero dose, was calculated by this program for individual assays, the highest value obtained for the assays within each experiment being taken as the minimum detectable concentration. Values were only accepted if they were within the range of steroid for which the coefficient of variation was $\leqslant 20\%$ and, if duplicates differed by $\leqslant \pm 10\%$ of their mean. Steroid-free plasma or medium as well as solvent blanks were included in every assay, the values of which were always below the limit of sensitivity of the assay.

3. Specific sample procedures

(a) Progesterone in peripheral plasma

Duplicate 0.2 ml aliquots of plasma and standard amounts of progesterone (0.1-5 pmoles) equilibrated in 0.2 ml steroid-free plasma were extracted with hexane after the addition of ethanol (15% v/v). The plasma was frozen at -25°C and the solvent decanted into assay tubes. The solvent was evaporated to dryness at 37°C with air, and the residue assayed. The minimum level able to be detected in plasma was 0.25 nM. The intra- and inter-assay coefficients of variation for all assays required for this study were 9.1% and 12.8%, respectively, at 5.7 nM.

(b) Ovarian vein plasma

One to two ml of hexane:ethyl acetate 2:3 (v/v) was used to extract duplicate samples (0.1-0.5 ml) of ovarian vein plasma and standard amounts of oestradiol (0.1-8 pmoles) equilibrated in similar volumes of steroid-free plasma. The residue of the solvent fraction was assayed after being decanted from the frozen plasma and evaporated to dryness at 37°C with air. The minimum detectable concentration was 0.1 nM. Only two

assays were required for these samples for which the intra-assay coefficients of variation were 4.2% at 1.7 nM and 11.3% at 4.5 nM.

Progesterone was determined in ovarian vein plasma as described for peripheral plasma. Aliquots of 0.02 or 0.2 ml plasma were assayed. The intra- and inter-assay coefficients of variation for the three assays of 0.02 ml were 6.3% and 7.3%, respectively, at 35 nM.

Androstenedione and testosterone were determined on the same plasma sample after extraction and chromatography. Trace amounts of each [^{3}H]-labelled steroid (1000 cpm, ≃10 pmol) were equilibrated in 1-2 ml plasma prior to extraction of steroids with hexane:ethyl acetate 2:3 (v/v). The extract was decanted from the frozen plasma, evaporated to dryness at 37°C with air, and the residue dissolved in hexane:chloroform 9:1 (0.2 ml). The sample was transferred to a column of Lipidex 5000 in 0.7 ml solvent and the eluate discarded. Androstenedione was then eluted in 1.5 ml of the same solvent. After discarding 1 ml hexane:chloroform 4:1, testosterone was eluted in 2 ml of the same solvent. These fractions were evaporated to dryness and redissolved in assay buffer, aliquots of which were taken to estimate recovery by liquid scintillation counting and for radioimmunoassay. The steroid concentration of these aliquots was determined by comparison with standard amounts of androstenedione or testosterone (0.1-2 pmol) in ethanol, dispensed directly into assay tubes and evaporated to dryness prior to assay. Duplicate 0.1 aliquots of the total androstenedione fraction (1 ml buffer) were assayed, resulting in a minimum detectable level of 0.1 nM. Sample concentration was corrected for recovery which averaged 79.5±0.9% for 65 individual estimates. Testosterone was analyzed in single aliquots of 0.12 ml of the total 0.2 ml buffer volume. Mean recovery was 71.1±1.1% (n=57) and the minimum concentration able to be detected 0.05 - 0.1 nM. The intra- and inter-assay coefficients of variation for the three assays required

for these samples were 9.3% and 17.6% respectively, at 6.2 nM for androstenedione and 5.3% and 11.5% at 1.5 nM for testosterone.

(c) Antral fluid

Antral fluid was diluted in medium C on collection and, for measurement of steroids, 1-50 µl aliquots were assayed in duplicate, occasionally singly, without extraction. The sensitivity of the assays were 0.02 pmol for oestradiol, 0.1 pmol for testosterone and, 0.5 pmol for androstenedione and progesterone, but the limit of analysis was often the volume of antral fluid able to be recovered. For all steroid assays of antral fluid the intra-assay coefficient of variation was <8% and the inter-assay coefficient of variation for the 2-5 assays required was < 15%.

(d) Perifusion medium

Twenty representative samples of perifusion medium were assayed for each steroid either directly or after solvent-extraction, depending on sample size, and after extraction and chromatography to separate possible cross-reacting steroids. There were no significant differences between the values obtained with or without chromatography. Therefore, all subsequent analyses were measured without chromatography and unextracted, if sample size permitted. When extraction of samples was required, standard amounts of the respective steroid were also extracted from similar volumes of blank medium.

Oestradiol in perifusion medium from follicles with antral fluid E_2 concentrations $\geqslant$ 1000 nM and androstenedione in the medium from all follicles was determined in duplicate 0.01-0.2 ml aliquots which were assayed without extraction. Standard amounts of each steroid in ethanol were dispensed directly into assay tubes and evaporated to dryness prior

to the addition of a volume of blank medium equal to that of the samples. When > 20 µl of medium was analysed, the final concentration of polyethylene glycol 6000 was decreased from 20.5% to 15%. The minimum detectable concentration of androstenedione was 0.3 nM. Medium obtained from the perifusion of follicles with antral fluid oestradiol concentrations < 1000 nM was assayed for oestradiol in duplicate 0.5 ml aliquots after extraction with hexane:ethyl acetate 2:3, resulting in a limit of detection of 0.05 nM.

The limited amount of medium available (approximately 2.5 ml) meant that testosterone and progesterone were each assayed singly in 0.2-1 ml of medium. Progesterone was extracted with hexane (2.5 ml) after the addition of 25% (v/v) ethanol to the medium and hexane:ethyl acetate 2:3 was used to extract testosterone. The minimum detectable concentration of these steroids in medium was 0.1 nM.

For these samples, the intra- and inter-assay coefficients of variation were < 10% and <15%, respectively. All 19 samples from the perifusion of one follicle were assayed in a single assay for each steroid.

(e) Granulosa cell culture medium

Oestradiol in the incubation medium from the culture of granulosa cells was measured by radioimmunoassay after chromatographic separation. Tritiated oestradiol (1000 cpm, ≈10 pmol) was equilibrated on 0.4-0.8 ml medium at 4°C overnight. Steroids were then extracted into hexane:ethyl acetate 2:3 (2.5 ml). The medium was frozen at -25°C, the solvent decanted, evaporated to dryness at 37°C with air and the residue dissolved in hexane:ethyl acetate 9:1 (v/v). The solvent (0.7 ml) was transferred to a column of Lipidex 5000 equilibrated in hexane. Androstenedione and progesterone were eluted in a further 2.5 ml

hexane : ethyl acetate 9:1, which was discarded. Oestrone was eluted in 2.5 ml hexane:ethyl acetate 4:1, after discarding 1.5 ml. Oestradiol was then eluted with 1.5 ml ethyl acetate which was evaporated at 37°C with air, and the residue dissolved in 0.2 ml assay buffer. An aliquot (0.05 ml) of this fraction was taken for liquid scintillation counting to determine recovery in each sample assayed. An aliquot (0.1 ml) of the remaining solution was assayed with standard amounts of ethanolic oestradiol standard, evaporated and redissolved in 0.1 ml assay buffer. Each sample concentration was corrected for recovery which averaged 66.1±0.4% for 541 samples. The limit of detection of oestradiol in incubation medium was 0.1 nM. Intra- and inter-assay coefficients of variation were 12% and 17%, respectively, at 1.2 nM, for ten assays.

Oestrone was measured in the chromatographic eluate of 70 samples. The minimum concentration able to be detected was 0.02 nM with a mean recovery of 70.0±1.2%.

(f) Implant release rates in vitro

(i) Oestradiol

The plasma or saline (0.1-0.2 ml) in which the implants were incubated was extracted with hexane:ethyl acetate 2:3 (1 ml). After freezing, the solvent was decanted and evaporated to dryness at 37°C with air. The residue was dissolved in assay buffer (0.2-1.5 ml) and duplicate 0.05 ml aliquots assayed with a non-extracted standard curve. Concentrations were corrected for extraction recovery (63.4±0.4%), determined by the concurrent extraction of tritiated steroid from 16 plasma samples. The concentration of oestradiol in ovine plasma incubated without implants was < 0.05 nM, the minimum detectable dose.

All samples were assayed in a single assay with an intra-assay coefficient of variaton of 5.9% at 4.8 nM.

(ii) Testosterone

The saline or plasma (0.1 ml) from incubation of implants was extracted with hexane:ethyl acetate 2:3 (1 ml). The solvent was decanted, evaporated to dryness at 37°C with air, and the residue dissolved in 1 ml assay buffer. Duplicate aliquots (2 µl) were assayed with a non-extracted standard curve. Sample concentrations were corrected for recovery which was similar for saline and plasma (86.0±0.6%), determined by the extraction of tritiated steroid from 10 samples. The result obtained for pooled human plasma or saline incubated without implants containing testosterone, or with empty implants was < 0.3 nM, the limit of detection. The within-assay coefficient of variation for the single assay required was 9.8% at 2.98 nM.

C. Gonadotrophin assays

The concentration of LH and FSH were measured in duplicate aliquots (0.1-0.2 ml) of plasma by radioimmunoassay as described by Kenneway et al. (1984). Highly purified ovine LH, provided by Dr. L.E. Reichert and bovine FSH, provided by Dr. K.W. Cheng, were iodinated by the lactoperoxidase method (Miyachi et al., 1972). Pre-precipitated goat anti-rabbit gamma-globulin (Midgely and Hepburn, 1980) was used to separate anti-body bound hormones.

The LH antiserum (UWA-3B), generously provided by Dr. C.M. Oldham was raised in rabbits and exhibited cross-reactions of < 1% with ovine prolactin (NIH-P-S12), FSH (NIH-FSH-S12) and growth hormone (NIH-GH-S11) and < 10% with thyrotropin (NIH-TSH-S8). The sensitivity of the assay was < 0.3 ng/ml using NIH-LH-S19 (biological potency 1.01 NIH-LH-S1

units per mg) as standard. The intra- and inter-assay coefficients of variation were < 10% and < 15%, respectively, in the range 0.3-20 ng/ml.

The rabbit antibovine FSH serum, kindly supplied by Dr. K.W. Cheng exhibited low cross-reactions (< 1%) with NIH-LH-S19, NIH-P-S12, NIH-GH-S11 and NIH-TSH-S8. The sensitivity of the assay was < 2 ng/ml when NIH-FSH-S15 (biological potency 20 NIH-FSH-S1 units per mg) was used as standard. Intra- and inter-assay coefficients of variation were < 10% and < 15%, respectively, throughout the range 2-300 ng/ml.

D. Culture

1. Media

A: Transport Medium

Dulbecco's phosphate-buffered saline (Flow laboratories, Stanmore, N.S.W., Australia) containing 0.5% (v/v) solution K (CSL, Melbourne, Australia).

B: Dissection Medium

Medium A containing 20% (v/v) calf serum (Flow).

C: Perifusion Medium

Medium 199 (modified) with Earle's salts and L-glutamine, without sodium bicarbonate (Flow). The medium was reconstituted immediately before use and sodium bicarbonate (2.2 g/l), kanamycin sulphate (10 mg/l ; Boehringer Mannheim, Adelaide, South Australia) and BSA (20 g/l) added, prior to adjusting the pH to 7.4.

D: Granulosa cell culture medium (McNatty et al., 1979)

Foetal calf serum (25 mls ; Flow), 200 mM glutamine (0.34 ml ; Flow) and antibiotic-antimycotic solution (1 ml ; GIBCO, N.Y., U.S.A.) were added aseptically to each 100 ml of sterile medium 199 (modified) with Hanks' salts and 20 mM Hepes without L-glutamine and sodium bicarbonate (Flow).

2. Gonadotrophin preparations

LH and FSH were kindly supplied by the National Institute of Arthritis, Metabolism and Digestive diseases. From the information supplied by N.I.A.M.D.D., the ovine LH preparation, NIH-LH-S19 used in perifusion studies, had a potency 1.01 NIH-LH-S1 units/mg and contained less than 0.05 NIH-FSH-S1 units/mg. The potency of ovine FSH (NIH-FSH-S12) used in granulosa cell culture was 1.25 NIH-FSH-S1 units/mg. The LH contamination of this preparation was less than 0.01 NIH-LH-S1 units/mg.

E. Statistical analysis

Non-parametric statistics were used whenever possible to avoid assumptions of normality and homoscedasticity in the data. Many data sets were obviously skewed or too small to enable confirmation of normality. However, to enable comparison with other reports in the literature, all data are quoted as sample mean ± standard error of the mean (s.e.m.), as well as the median, if applicable. The Mann-Whitney U test was used to determine the significance of differences between two independent groups. For > 2 groups, Kruskal-Wallis one-way analysis of variance was used, followed by an appropriate critical range test (Colquhoun, 1971) when a significant difference was found. Significant differences between 2 related samples were determined by the Wilcoxon signed-rank test. Fisher's exact test was used to test significance of

differences if measurements gave rise to proportions. The significance of correlations within the data was most commonly determined non-parametrically ; r, the Spearman rank correlation coefficient, being the measure of association between groups. Only significant correlations with r values $\geqslant 0.5$ were considered. When testing for differences between associations, the t-test was used to determine the significance of differences between the slope and intercept of parametric correlations (Pearson's). All tests were done by computer.

CHAPTER III

THE ROLE OF FSH SECRETION ON DAY 1 IN DETERMINATION OF OVULATION RATE

A. Introduction

That FSH is essential for the growth and maturation of follicles is well known, but few correlations of the secretion of FSH with ovulation rate have been demonstrated in the ewe. A peak of FSH, occurring approximately 24 h after the preovulatory LH surge, was larger (193 ng/ml/hr) in Romanov ewes of ovulation rate 3.2 than Ile-de-France ewes (57 ng/ml/hr) of ovulation rate 1.5 (Cahill et al., 1981), and when data from all ewes was pooled there was a significant correlation between the amount of FSH released in this peak and the number of antral follicles present in the ovaries 17 days later. This post-ovulatory peak of FSH is not suppressed by oestradiol and/or progesterone in the ewe (Goodman et al., 1981) which may indicate a separately regulated mechanism whereby FSH controls the number of follicles available for recruitment in ewes of high fecundity.

Of the two similar peaks of FSH occurring in the periovulatory period of the hamster oestrous cycle, only the second is required to recruit the large preantral follicles which will ovulate 4 days later (Greenwald and Siegel, 1982). In the rat, there is only one peak of FSH which occurs concurrently with the preovulatory LH surge, but is maintained throughout oestrus when LH has returned to basal levels (Gay et al., 1970). When the secondary phase of FSH secretion is suppressed by the injection of bovine follicular fluid, the normal recruitment of antral follicles which occurs at this time is prevented (Welschen et al., 1980). The FSH rise occurring at oestrus in the rat is not coincident with LH secretion and cannot be reduced by administration of

oestradiol, progesterone or testosterone (Welschen et al., 1980). However, it can be suppressed by steroid-free follicular fluid, containing inhibin-like activity. This, together with the finding that there is a negative correlation between FSH levels and the number of large follicles present in the ovaries, suggests that the loss of follicles at ovulation results in diminished negative feedback by inhibin so causing the second phase of FSH release (Welschen et al., 1980).

Thus, the involvement of the post-ovulatory FSH peak in the recruitment or growth of follicles to ovulate at the subsequent oestrus is clear in species with 4 day cycles. However, it remains to be determined whether this relationship exists in the 17 day oestrous cycle of the ewe, as suggested by Cahill et al. (1981). In a preliminary report, administration of PMSG on day 2 of the cycle, to enhance the post-ovulatory FSH peak, was shown to increase the proportion of ewes with twin ovulations (Cahill et al., 1979b). The aim of the present experiments was to confirm and extend this observation utilizing an exogenous source of purified FSH given at the beginning of the oestrous cycle in the ewe.

The secondary rise of FSH in the ewe lasts 13.5 - 28 h with peak values occurring approximately 24 h after the preovulatory surge (Pant et al., 1977 ; Baird et al., 1981). As it was desirable to increase the size of this peak without extending it into the luteal phase, porcine pituitary FSH was chosen as the exogenous gonadotrophin since its biological half-life in sheep has been estimated to be 2-6 h (Laster, 1972 ; Akbar et al., 1974). The longer half-life (approximately 21 h) of PMSG (McIntosh et al., 1975) would have meant that stimulation continued further into the luteal phase.

Two experimental protocols were followed. Firstly, FSH treatment was confined to day 1 of the oestrous cycle, either as a single injection or, in one group, two injections 6 h apart. In the second experiment, FSH was administered twice daily over the first three days of the cycle. Two dose rates of FSH were tested ; one equivalent to a superovulatory dose (10-12 mg) if given during the late luteal phase, and a lower rate (3-5 mg), as exceedingly high ovulation rates were not desirable. The occurrence of the second oestrus after treatment and the ovulation rate at this oestrus was also studied to determine if a prolonged effect of FSH administration was apparent.

B. Materials and Methods

1. Animals

Merino ewes (Bungaree strain), 6-7 years of age, were maintained outdoors at Mortlock Experiment Station, near Clare, South Australia on a subterranean clover and annual grass pasture, supplemented with barley during the summer months as necessary. The experiments were carried out from late January to April when ewes were exhibiting regular oestrous cycles, detected by running the ewes with raddled, vasectomized rams and observing raddle marks at frequent intervals.

2. FSH

The FSH for injection (FSH-P) was purchased from Burns-Biotec (Omaha, Nebraska, U.S.A.) and stored at 4°C in the lyophilized form. Immediately prior to use, each vial, equivalent to 50mg Armour standard, was reconstituted in 10 ml sterile, isotonic saline (David Bull Lab., Mulgrave, Victoria, Australia) and aliquots of the solution drawn into sterile, plastic syringes which were kept on ice until injection.

Animals in experiment 1. received 1 ml (5 mg) or 2 ml (10 mg) of the stock 5 mg/ml solution. In experiment 2., sterile isotonic saline was used to prepare a further dilution of 1.25 mg/ml. Ewes at the high dose rate received 0.4 ml (2 mg) of the original stock solution at each injection, whereas ewes at the low dose rate received 0.4 ml (0.5 mg) of the diluted stock.

3. Blood sampling

All blood samples were taken by jugular venepuncture into heparinized, evacuated containers (Vacutainer ; Becton-Dickinson, Rutherford, N.J., U.S.A.). Samples were kept on ice until centrifuged to separate plasma, which was stored frozen prior to assay.

Progesterone was measured in these samples by radioimmunoassay (see II.3.a).

C. Experiment 1

1. Experimental design

Fifty nine ewes, on days 4-13 of the oestrous cycle, were injected i.m. with 100µg of a synthetic prostaglandin, cloprostenol (Imperial Chemical Industries, Melbourne, Australia) to induce luteolysis (Trounson et al., 1976). In a previous experiment, a similar dose of cloprostenol given to 65 ewes resulted in 6% of ewes being marked in oestrus during the following 24 h, 35% by 48 h, 51% by 72 h and the remainder by 96 h. Thus, 72 h after prostaglandin injection was designated day 0 of the induced oestrous cycle. Forty-two ewes exhibited oestrus and were randomly allocated to 4 treatment groups. All ewes were injected subcutaneously between 0900 and 1100 hrs on the morning of day 1 ; group C being reinjected at 1600-1700 hrs on the same day. Group A

(16 ewes) received saline alone (control), group B (10 ewes) 5 mg FSH-P, group C (10 ewes) 5 mg FSH-P, and group D (6 ewes) 10 mg FSH-P. After treatment the ewes were returned to pasture with raddled, vasectomized rams and raddle markings were recorded daily. On day 12 of the induced oestrous cycle all ewes were injected with 100µg cloprostenol, i.m. The incidence of oestrus was noted daily and ovulation rate, assessed by the number of corpora lutea present in the ovaries of each ewe, determined by laparoscopy 9 days after prostaglandin injection (See II.A)

2. Results

Two ewes (1 from group B and 1 from group C) exhibited shortened luteal phases being marked in oestrus 3 and 7 days after treatment and so were removed from their respective groups. Only 10 of the 16 group A ewes and 7 of the remaining 9 group B ewes exhibited oestrus after the second prostaglandin injection, probably due to only 2 rams being present in the flock. However, as all ewes had ovulated at laparoscopy, the data from ewes not detected in oestrus were included in the results. The ovaries of one ewe in group C could not be visualized at laparoscopy as they were obscured by adhesions.

No significant difference in ovulation rate between the 4 treatment groups was detected. The mean ovulation rate of control ewes was 1.63±0.13 ($n = 16$) ; ewes receiving 5 mg FSH-P, 1.78±0.15 ($n = 9$) ; ewes receiving two injections of 5 mg FSH-P, 1.88±0.23 ($n = 8$) ; and ewes receiving 10 mg FSH-P, 2.0±0.26 ($n = 6$). The proportion of ewes detected in oestrus was higher in the FSH-treated groups (21/23) than in the control group (10/16 ; $p < 0.05$).

D. Experiment 2

1. Experimental design

As previously, luteal regression was induced in sixty ewes, on days 4-13 of the oestrous cycle, by i.m. injection of 100µg cloprostenol. A blood sample was taken from each ewe at the time of injection to confirm luteal phase progesterone levels. On the morning of day 1, 96 h after prostaglandin, a blood sample was taken from each ewe, raddle marks recorded and the ewes randomly allocated to three treatment groups, each containing 20 ewes. Only 38 of the 60 ewes had exhibited oestrus, probably due to only 2 rams being present in the flock. All ewes were initially included in treatment groups until confirmation of luteolysis could be assessed by plasma progesterone concentration, an equal number of unmarked ewes being allocated to each group. For three days, beginning on day 1, all ewes were injected subcutaneously at 1000-1100 hrs and 1600-1700 hrs. Control ewes (group A) received injections of saline ; ewes in group B received 0.5 mg FSH-P at each time or 3 mg total ; ewes in group C received 2 mg FSH-P twice daily or 12 mg total. A blood sample was taken on day 3 to confirm that each ewe had ovulated and was in the early luteal phase.

An increased number of rams (5) were continuously present in the flock ; oestrus was recorded daily during treatment and every 2-3 days for the next 10 days. The length of the two subsequent oestrous cycles was recorded for each ewe and laparoscopy carried out 1-8 days after each oestrus to determine the number of corpora lutea present in the ovaries. A blood sample was taken from each ewe at laparoscopy.

2. Results

Fifty-six of the sixty ewes had plasma progesterone concentrations > 1.5 nM at the time of prostaglandin injection confirming that they were in the mid- or late luteal stage of the cycle. The mean progesterone concentration of these ewes decreased from 4.24±0.23 nM prior to PG to < 0.75 nM on day 1, demonstrating induced luteolysis, 46 ewes having levels below the sensitivity of the assay (0.25 nM) at this time. In the remaining 4 ewes luteolysis had commenced prior to prostaglandin injection as the mean progesterone concentration for these ewes was 2.71±0.28nM on day 1, rising to 6.63±0.95nM on day 3. The results of these ewes (2 from group C, 1 from each of group A and B) were excluded from the data as FSH administration did not occur on days 1-3 of the cycle. The data from a further 3 ewes (1 in group A, 2 in group C) was excluded as ovulation did not occur prior to treatment as judged by peripheral progesterone concentrations < 0.25 nM on day 3 compared to a mean concentration of 1.54±0.09 nM (n = 50) for all other ewes. One ewe (group C) was removed from the experiment due to illness and one control ewe exhibited a shortened luteal phase following treatment, being remated on day 5, and was subsequently excluded.

All remaining ewes were marked in oestrus 14 to 22 days after the beginning of FSH treatment. At laparoscopy (cycle 2), corpora lutea were present in the ovaries of all but one ewe (group A). Plasma progesterone concentrations at this were > 1.4 nM in all ewes. As the one ewe with no detectable corpus luteum had a plasma progesterone level of 5.5 nM, the laparoscopy result was assumed to be in error and excluded from the results. The ovulation rate of ewes (Table 2) did not differ between treatments. The mean time to oestrus was similar for the three treatment groups being 16.2±0.4 days for group A, 15.6±0.2 days for group B, 16.7±0.4 days for group C.

Table 2 Distribution of zero, single and multiple ovulations and mean ovulation rates of ewes ovulating in the two successive cycles following treatment with saline (A), 3 mg (B) or 12 mg (C) FSH on days 1-3 of the first oestrous cycle.

Oestrous cycle	FSH treatment	No. of ewes	No. Corpora lutea per ewe				
			0	1	2	3	Mean ± s.e.m.
2	A	16	0	2	13	1	1.94 ± 0.11
	B	19	0	2	16	1	1.95 ± 0.09
	C	15	0	4	9	2	1.87 ± 0.17
3	A	17	0	2	14	1	1.94 ± 0.10[a]
	B	19	1	1	15	2	2.06 ± 0.10[a]
	C	14†	1	6	7	0	1.54 ± 0.14[b]

† The ovulation rate of one ewe was not recorded.
Values with different superscipts (a,b) differ significantly ($p < 0.05$).

Thirty-three to thirty-six days after the beginning of FSH treatment, a second oestrus was recorded in all but three ewes. At the laparoscopy following this oestrus (cycle 3), anovulation in two of these ewes (one each from group B and C) was confirmed by the absence of corpora lutea and peripheral progesterone concentrations < 0.25 nM. There was a significant difference ($p < 0.05$) between treatments in the ovulation rate when expressed either as per ewe ovulating or per total number of ewes in the group (Table 2). The group (C) receiving the highest dose of FSH (12 mg) in the previous cycle had a lower ovulation rate than ewes receiving the lower dose (3 mg) or no FSH. The mean length of the second oestrous cycle was similar in all groups (A, 16.0±0.4 days ; B, 16.6±0.2 days ; C, 16.7±0.3 days). For group B, cycle 2 was longer than cycle 1 ($p < 0.01$).

E. Discussion

A dose of 5 or 10 mg of exogenous FSH, given on day 1 of the oestrous cycle did not increase the ovulation rate of treated ewes at the following oestrus above that of control ewes. Ovulation rate was also unaffected when the time of dosage of 3 or 12 mg FSH was extended to twice daily during the first three days of the oestrous cycle. Both the incidence and duration of oestrus in ovariectomized ewes have been demonstrated to increase with the dose of oestrogen used to induce oestrus (Fletcher and Lindsay, 1971 ; Scaramuzzi et al., 1971 ;Land et al., 1972). The finding that a greater proportion of FSH-treated ewes were marked in oestrus in experiment 1. suggests that increased follicular development resulting in higher oestrogen secretion may have occurred in these ewes. With a limited number of rams, expression of oestrus for a longer period would increase the likelihood of ewes being marked. The failure to detect a significant increase in the number of multiple ovulations in these ewes (control, 10/16 ; FSH-treated, 18/23 ; p=0.2) may be due to insufficient numbers in each group.

That exogenous gonadotrophin administered at the beginning of the oestrous cycle does result in follicular development has been demonstrated recently. Cran (1983) showed that PMSG (1200 i.u.) given on day 2 or 3 results in the formation of follicular cysts by day 8 or 9, some of which persist for up to 14 days after injection. In the majority of these cysts, the granulosa layer degenerates and luteinization of the theca interna results in progesterone secretion. However, approximately 50% of those follicles which develop after PMSG injection on days 5-7 of the oestrous cycle are capable of producing sufficient oestrogen to elicit an LH surge and ovulate after luteal regression 6-10 days later (Cran, 1983). If similar follicular development occurred in this experiment it did not result in an increased ovulation rate 14-16 days

later, suggesting either that the class of follicles destined to ovulate was unaffected by the FSH given on days 1-3 or that the number of follicles recruited for ovulation is determined prior to or after this time.

The results of this study differ from those reported by Cahill et al. (1979b), which may be explained by the difference in the biological half-life of PMSG (21 h) and FSH (2-6 h). If residual PMSG remained in the circulation for 3-4 days after the injection given on day 2 by Cahill et al. (1979b), the follicles stimulated at that time may have gone on to ovulate at the next oestrus. However, this seems unlikely as Cran (1983) found that the follicles stimulated by PMSG on days 2 or 3 had not ovulated 12-14 days later. A recent study has also failed to demonstrate an effect of the post-ovulatory FSH peak on ovulation rate. When the second FSH peak of control or Booroola Merino ewes was decreased 50-70% by the injection of ovine follicular fluid 8-35 h after oestrus, the subsequent ovulation rate and cycle length were unaffected (Cummins, 1983, cited by Bindon, 1984). Three-hourly plasma sampling throughout the periovulatory period has now confirmed that the area of the second FSH peak increases with age and ovulation rate in Booroola Merino ewes and that FSH concentrations at this time are higher in the fecund Booroola than control Merinos (Bindon, 1984 ; Bindon et al., 1984). In D'man ewes (ovulation rate 3.1), a similar sampling protocol demonstrated that the maximum value of the preovulatory surge as well as the amount of FSH released during the second peak were both greater than those of Timahdite ewes with ovulation rate 1.1 (Lahlou-Kassi et al., 1984).

Increased FSH secretion at this time may be the result, rather than the cause, of differences existing in high fecundity ewes. Evidence from studies on unilateral ovariectomy and suppression of the FSH rise at

oestrus in the rat suggests that a feedback loop exists between FSH secretion and the antral follicles it recruits. Inhibin, in concentrations proportional to the number of antral follicles in the ovary, may regulate the amount of FSH secreted by the pituitary which, in turn, determines the number of antral follicles (Welschen et al., 1978 ; Welschen et al., 1980). Ovulation and the wave of follicular atresia which occurs after the preovulatory gonadotrophin surge (Turnbull et al., 1977a) probably results in decreased inhibin secretion and the consequent rise in FSH. The growth of follicles recruited by this surge would lead to increased inhibin secretion which then once again suppresses FSH secretion to baseline levels. The ovaries of the Booroola Merino contain one-third the inhibin of ovaries of control Merinos during the mid-luteal phase of the cycle (Cummins et al., 1983). Thus, inhibin levels in Booroola ewes may increase more slowly with follicular development, allowing FSH concentrations to rise higher and/or remain elevated for longer in the high fecundity ewe. Increased sensitivity of Booroola ewes to inhibin may, however, negate this effect (Cummins et al., 1983).

Any follicular development which may have occurred on FSH treatment appears to have been asynchronous with the following two gonadotrophin surges. The lower ovulation rate of the high dosage group following the second oestrus after treatment suggests that steroid and/or inhibin secretion from the follicles produced by FSH treatment may have resulted in subsequent depression of FSH secretion, so inhibiting growth of the class of follicles recruited for ovulation at this oestrus. Alternatively, this size class might have been depleted by earlier follicular development.

Thus, although increased post-ovulatory FSH secretion has been found in several prolific sheep breeds, the failure of the present study

and that of Bindon (1984) to demonstrate a change in ovulation rate when the size of this peak was altered suggests that FSH secretion at this time is not the major factor controlling ovulation rate in the ewe. A detailed investigation of the follicular and enodocrine characteristics which might influence ovulation rate was, therefore, undertaken in the high fecundity Booroola Merino.

CHAPTER IV

ENDOCRINE PARAMETERS OF THE PREOVULATORY FOLLICLE OF THE BOOROOLA MERINO: 1. OVARIAN CHARACTERISTICS

A. Introduction

Comprehensive studies of the endocrinology of follicles of high fecundity sheep have not been reported. Detailed analysis of the follicle populations and the growth rates of the different size classes, as measured by the mitotic index of granulosa cells, within these populations are available in breeds of differing fecundity (Turnbull et al., 1977b ; Cahill et al., 1979a ; Cahill and Mauléon, 1980 ; Cahill and Mauléon, 1981 ; Cahill et al., 1982). Reports on steroid secretion are conflicting and limited to the measurement of oestradiol in ovarian vein or peripheral plasma (Baird and Scaramuzzi, 1976a ; Wheeler et al., 1977 ; Scaramuzzi and Land, 1978 ; Bindon et al., 1979 ; Cahill et al., 1981), and do not clarify the interrelationship between follicular development and gonadotrophin secretion. It is clear that there is a paucity of information about the basal or gonadotrophin - stimulated steroidogenic potential of the follicles of high fecundity ewes as would be demonstrated by ovarian vein secretion rates, antral fluid concentrations or secretion by individual follicles in vitro. Thus, a detailed comparison of the follicles of the Booroola Merino and the control Merino of low fecundity was undertaken.

The Booroola Merino has one of the highest natural ovulation rates of all sheep breeds. Mean ovulation rates of 2.5-4 have been reported, with a range of 1-11 ovulations occurring in individual animals (Bindon et al., 1971 ; Bindon et al., 1978 ; Piper and Bindon, 1979 ; Piper and Bindon, 1982a). The origins and development of the Booroola Merino flock maintained by CSIRO at Armidale have been described by Turner (1978 and

1980). The foundation stock of this flock, obtained from the Seears brothers of "Booroola" (Cooma, New South Wales), had been selected on their dam's or their own litter size records when mated to rams, unselected for litter size, continuously introduced from outside. Further selection was continued at CSIRO, initially based on the presence of multiple births in the dam's, or in the ewe's lambing record (Turner, 1978). In later years, selection was based on the dam's lifetime reproduction index calculated from deviations of average lambing performance at the ages of record as well as the heratibility of repeated records (Piper and Bindon, 1982a). Reexamination of litter size records of the foundation ewes and their offspring has shown that the high fecundity of the Booroola Merino is due to the action of a single major gene or closely linked group of genes (Piper and Bindon, 1982a), which is additive for ovulation rate (Piper and Bindon, 1979) and completely dominant for litter size (Piper and Bindon, 1982b). This finding has since been confirmed in other Booroola flocks (Davis et al., 1982). In this respect, the Booroola Merino appears to differ from other high fecundity breeds as there are no other reports of a similar genetic basis for increased litter size.

The physiological and endocrine characteristics that are known of the Booroola Merino have been described in Chapter I. To summarise briefly, the Booroola attains sexual maturity earlier than control Merinos (Bindon and Piper, 1982b) and exhibits oestrous cycles for more of the year when mature (Bindon and Piper, 1976), which might be explained by enhanced pituitary sensitivity to low doses of GnRH (O'Shea et al., 1981). Unlike other high fecundity breeds the duration of oestrus (Bindon and Piper, 1982b) and time interval between the onset of oestrus and the start of the preovulatory LH surge (Bindon et al., 1978) are similar in control and Booroola Merinos, but the Booroola ovulates

significantly earlier than controls after the onset of oestrus (Bindon and Piper, 1979). When ovariectomized ewes were implanted with oestradiol just prior to anoestrus, LH levels in Booroola and control ewes declined similarly, confirming the lack of difference of response of genotypes to the feedback actions of oestradiol (Cummins et al., 1982). No strain differences are apparent in the maximum LH concentration reached during the preovulatory rise, the duration of the LH discharge or the total LH released (Bindon et al., 1979). Few studies of FSH concentrations have been reported. The significance of higher FSH levels in Booroola ewe lambs at 30 days of age when compared to control lambs (Findlay and Bindon, 1976) is questionable as, at birth, the ovaries of Booroola lambs contain very few antral follicles (Tassell et al., 1982) which may result in decreased negative feedback control. In adult ewes, repeated sampling during the periovulatory period or mid-luteal phase has not demonstrated any consistent differences between the genotypes (Scaramuzzi et al., cited in Bindon and Piper, 1982b ; Bindon et al., 1982a). This finding is surprising considering that the inhibin content of Booroola ovaries is one third that of control Merinos but may be accounted for by the increased sensitivity of plasma FSH levels in the Booroola to the negative feedback action of inhibin (Cummins et al., 1983).

In common with the Romanov breed, the Booroola ovary has been found to contain fewer primordial and small follicles (< 3 layers of granulosa cells) and more larger follicles than ovaries of control ewes (Cahill et al., 1982). This finding, however, differs to that of Turnbull et al. (1977b) who found that Booroola ovaries contained the same number of antral follicles as the ovaries of Merino ewes with single ovulations. These authors found that differing growth patterns within the follicle population in ewes of different strains resulted in more large

follicles, capable of ovulation, being present in the ovaries of the high fecundity breed. Yet another study found that Booroola ovaries contained approximately half the number of growing follicles as control ovaries but that the incidence of atresia was reduced in these Booroola follicles (Scaramuzzi and Turnbull, 1980 ; cited by Bindon and Piper, 1982b). The greater ovarian response of Booroola ewes to exogenous PMSG (Bindon and Piper, 1982a and b) confirms the presence of increased numbers of large follicles in Booroola ovaries, as well as the possibility of increased ovarian sensitivity to gonadotrophins.

The steroid secretion rate of ovaries *in vivo* or *in vitro* or of individual follicles *in vitro* have not been examined in the Booroola Merino. The greater number of developing follicles of the Booroola does not appear to result in increased peripheral oestradiol concentrations in these ewes (Scaramuzzi *et al* ; cited by Bindon and Piper, 1982b), though the stage of oestrous cycle when measurements were carried out is not clear. Comparison of progesterone levels in the plasma of control and Booroola Merino ewes during the luteal phase has produced equivocal results. Bindon *et al*. (1981) and Bindon and Piper (1982b) found that, at ovulation rates of 1 and 2, plasma progesterone was higher in Booroola than control ewes. Within Booroola ewes, progesterone concentration did not increase with the number of corpora lutea present. This result was confirmed by the finding that the total volume of luteal tissue per Booroola ewe remained constant as ovulation rate increased (Scaramuzzi *et al*., 1981b), but these authors found no difference in progesterone concentration between Booroola and control ewes.

Thus, it is clear that little is known of the relative steroidogenic capacity and response to gonadotrophins of individual ovarian follicles of each genotype, an interrelationship which is vital in the development of ovulatory follicles. The experiments described in

Chapters IV to VI were designed to investigate these endocrine characteristics of preovulatory follicles of Booroola and control Merinos. A comparison of the sensitivity of ewes to the negative and positive feedback effects of oestradiol was carried out (Chapter VII), as well as a detailed study of peripheral gonadotrophin levels throughout the periovulatory period (Chapter VIII). This study was planned in conjunction with Professor D.T. Baird, Professor D.T. Armstrong and Dr. R.F. Seamark. Due to the intensity of the program Mr. K. Porter and Professor Baird assisted with the surgery, Mrs. R. Jeffrey with follicle dissection, Mr. F. Amato with the perifusion of follicles, and the staff of the Cytogenetics laboratory of the Queen Elizabeth Hospital with the counting of granulosa cells. This assistance is gratefully acknowledged.

B. Materials and Methods

1. Animals

The animals were kindly provided by Dr. B.M. Bindon from a flock maintained by CSIRO at Armidale, New South Wales. The ewes were drawn from a flock which was originally created by crossing Booroola rams and control Merino ewes and the progeny then mated inter se to create F_2 and F_3 generations. Twenty ewes, aged 1.5-2.5 years, of the F_3 generation where chosen for the experiment. Based on ovulation rates $\geqslant 3$, assessed at repeated laparoscopy, ten ewes were classified as carrying the Booroola gene (B(+) ; either homozygous or heterozygous) and ten ewes with ovulation rates of one or two as controls (B(-)).

In February, these ewes were transported to Adelaide where they were maintained on pasture in the presence of raddled, vasectomized rams. Oestrous observations were made daily. Three days prior to

ovariectomy, ewes were transferred to pens in the animal house at the Queen Elizabeth Hospital, where the lighting régime was similar to the outside environment.

2. Experimental design

In the mid-luteal phase of the oestrous cycle (day 7-12), luteolysis was induced in each ewe by injection i.m. of cloprostenol at 21.00. 36 ± 1 h later, between 09.00 and 10.00, laparotomy was performed through a mid-line ventral incision. Anaesthesia was induced with 500-650 mg thiopentone sodium (May and Baker, West Footscray, Victoria) and maintained with 2% halothane in oxygen in closed circuit. Blood samples were obtained by jugular venepuncture for progesterone determination 48 h, 24 h and immediately prior to ovariectomy. The ovaries and uterus were exteriorized and the presence of all visible follicles and corpora lutea recorded. A blood sample was aspirated from each ovarian vein above the junction with the middle uterine vein with a syringe attached to a 23 gauge needle. All samples were kept on ice until centrifugation to remove red blood cells within 3 h. Collection of ovarian venous blood was completed within 5 min and the ovaries were then removed, placed in transport medium (medium A ; see II.D.1) on ice, and transferred immediately to the laboratory where they were weighed.

The incision was then closed with 1 Dexon sutures (Davis and Geck, American Cyanamide Co., N.Y., U.S.A.) and sprayed with antibiotic (Neotracin, Ethnor, Sydney, Australia). A cannula (17G, Viggo, Helsingborg, Sweden) was inserted in the jugular vein and the ewe returned to a pen to recover. Two to three ewes were ovariectomized on each occasion.

3. Treatment of follicles and corpora lutea

Follicles $\geqslant$ 3 mm diameter and the regressed corpora lutea were transferred to dissection medium (medium B ; see II.D.1) and carefully dissected free of stroma using watchmaker's forceps (No. 4), under a binocular microscope at approximately 5x magnification.

The diameter of each follicle was determined from the mean of two measurements perpendicular to each other, made with a calibrated eyepiece graticule in a binocular microscope at a magnification of 1:10. Follicles were then classified as to morphological stage of atresia according to Moor et al. (1978) and Carson et al. (1981). In this classification Stage 1 follicles are non-atretic with degeneration of the granulosa layer, thecal capillaries and oocyte-cumulus complex increasing through stages II to V, in which follicles are terminally atretic. Each corpus luteum was weighed and then fixed in Bouin's solution for subsequent histological examination.

The follicles were then individually perifused for 3 h (see Chapter V). At the end of this time, the follicle was removed from the perifusion chamber, rinsed in perifusion medium (II.D.1) and slit open in a sterile petri dish. A measured aliquot (5-50 µl) of antral fluid was diluted in medium C (200 µl) and frozen until analysis. The follicle wall was transferred to a sterile petri dish (50 x 13 mm, Disposable Products, Adelaide, South Australia) containing 0.5 ml medium 199 and the granulosa cells scraped from the thecal shell, using a blunt, glass hook. The separated cells were transferred with a sterile, siliconized Pasteur pipette to a sterile centrifuge tube (12 x 75 mm, Medos, South Melbourne, Victoria). Both thecal shell and petri dish were rinsed thoroughly with an additional 0.5-1 ml of medium to ensure removal of all granulosa cells and all washings transferred to the tube. The remaining thecal shell was blotted dry and weighed.

Dispersal of the granulosa cells was carried out by passing them several times through a 25 gauge needle attached to a disposable 1 ml syringe (Terumo, Melbourne, Australia). Residual antral fluid and dispersal medium were removed by centrifugation (500 g, 10 min) at 4°C and the cells resuspended in 0.2 ml medium D (II.D.1). Aliquots (0.01 ml) of this suspension were diluted with differing volumes (0.05-0.2 ml) of trypan blue (0.1% (v/v) in 0.9% saline). The total number and percentage viable cells was quantitated in a haemocytometer at two different dilutions, 200-300 cells being counted at each dilution. From the number of cells per aliquot, the total number of granulosa cells per follicle was calculated.

4. Hormone assays

The steroid concentrations in ovarian venous plasma were measured by radioimmunoassay after solvent extraction and, if necessary, chromatography (II.3.b).

Steroids in diluted antral fluid were measured without extraction (II.3.c). Inhibin activity in antral fluid was measured by an *in vitro* pituitary cell culture system using FSH cell content as the end point (Scott *et al.*, 1980a), in the laboratory of Dr. J.K. Findlay, whose assistance is gratefully acknowledged.

C. Results

At the start of the experiment the mean weight of control and Booroola ewes was similar (B(-), 29.5 ± 1.1 kg ; B(+), 28.1 ± 0.7 kg).

Injection of a synthetic prostaglandin, cloprostenol, induced luteolysis, the plasma concentration of progesterone falling to below 1.4 nM in all ewes by 36-38 h after administration (Table 3). There was no significant difference in progesterone concentration between control

and Booroola ewes in the luteal phase prior to prostaglandin (-12 h) or during luteolysis.

Table 3 Concentration of progesterone (nM) in jugular venous plasma in control and Booroola Merino ewes, prior to and after cloprostenol injection (mean ± s.e.m. ; n = 10 ewes/group).

Time (h)± cloprostenol	Control	Booroola	Significance of difference
-12	6.2 ± 0.6	7.0 ± 0.9	n.s.
+12	2.6 ± 0.2	2.6 ± 0.3	n.s.
+36-38	0.3 ± 0.1	0.6 ± 0.2	n.s.

1. Ovarian characteristics

(a) Ovarian weight

Total ovarian weight was similar in both groups of ewes (Table 4).

Table 4 Total ovarian weight and weight of ovaries with or without corpora lutea in control and Booroola Merino ewes (mean ± s.e.m., n = 10 ewes/group).

	Control	Booroola	Significance of difference
Total ovarian weight (g)	2.07 ± 0.21	1.72 ± 0.12	n.s.
Ovaries with corpora lutea			
Number	11	16	
Weight (g)	1.16 ± 0.12	0.88 ± 0.05	n.s.
Ovaries without corpora lutea			
Number	9	4	
Weight (g)	0.89 ± 0.10	0.78 ± 0.14	n.s.

Within each strain, there was no significant difference in the weight of ovaries either with or without corpora lutea.

(b) Corpora lutea (CL)

There were more than twice the number of corpora lutea in B(+) than B(-) ewes, corresponding to the ovulation rates previously recorded by laparoscopy (Table 5). The mean weight of individual CL was significantly smaller in the B(+) ewes, resulting in the same total mass of luteal tissue per ewe in both groups. Mean peripheral progesterone concentrations in the luteal phase preceding oopherectomy had reflected this, being the same for each group (Table 3).

Table 5 Number and weight of corpora lutea in control and Booroola ewes (mean ± s.e.m. ; n = 10 ewes/group).

	Control	Booroola	Significance of difference
CL/ewe at laparoscopy†	1.1 ± 0.1	3.1 ± 0.2	$p < 0.0001$
No. CL at laparotomy	11	25	-
CL/ewe	1.1 ± 0.1	2.5 ± 0.3	$p < 0.002$
Weight CL (g)	0.29 ± 0.02	0.12 ± 0.01	$p < 0.0001$
Total Weight CL/ewe (g)	0.31 ± 0.01	0.30 ± 0.04	n.s.

† 11 observations on 10 control ewes ; 15 observations on 10 Booroola ewes

Within Booroola ewes, the number of CL per ewe was correlated with the total weight of luteal tissue per ewe ($r = 0.80$, $p < 0.005$), but not

with luteal peripheral progesterone concentration preceding luteolysis ($p > 0.1$) or the weight of individual CL ($p > 0.1$).

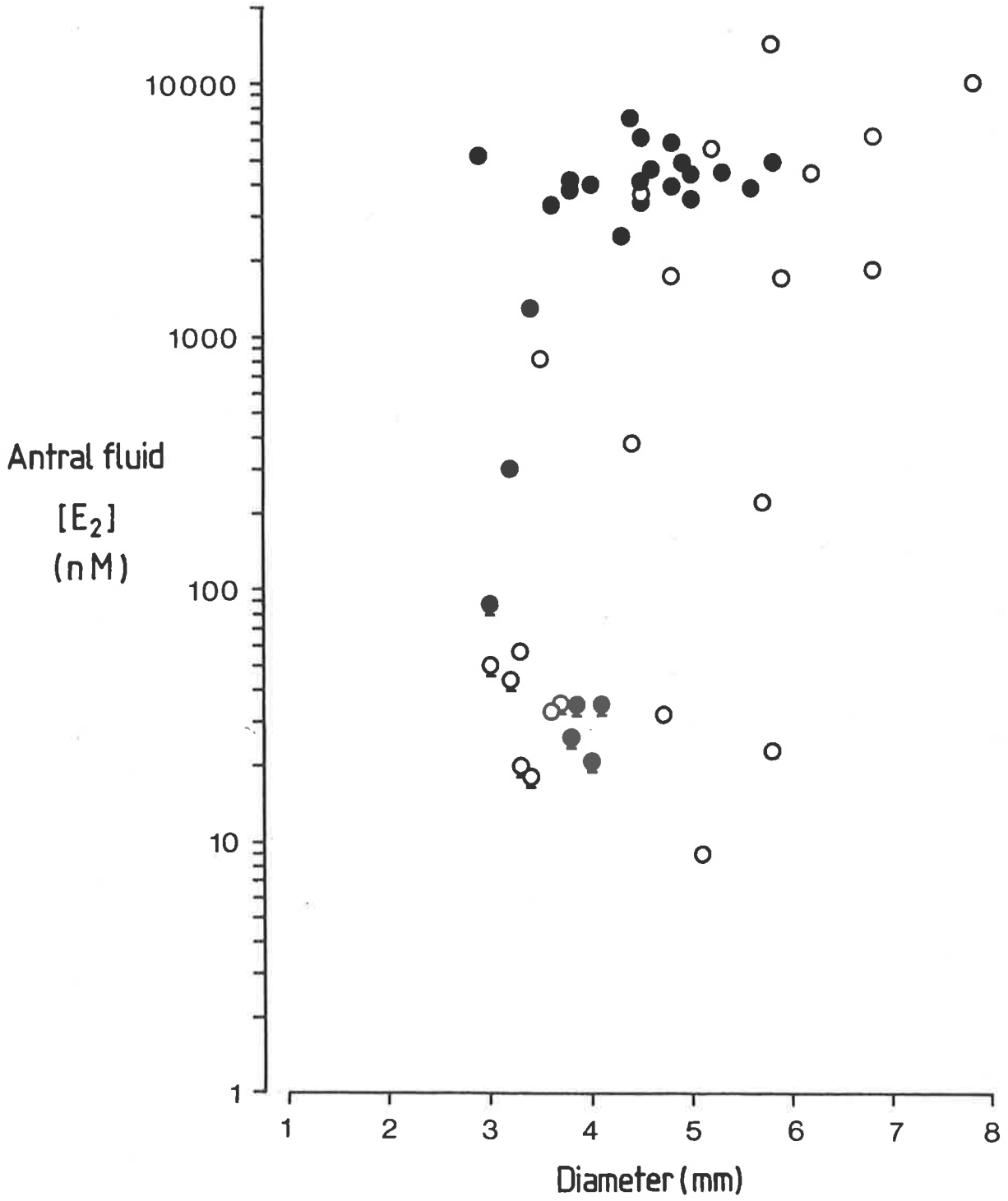
2. Follicular characteristics

(a) Number and size of follicles

In 4 of the 20 ewes (2 from each group) one follicle was ruptured during dissection. As the data from these ewes is incomplete, they have been omitted from the follicular studies. Antral fluid was recovered to allow classification of one Booroola follicle which broke while being photographed, but measurement of diameter, thecal weight or granulosa cell number was not possible.

There was no significant difference in the total number or size of follicles $\geqslant$ 3 mm in diameter isolated from the ovaries of B(-) and B(+) ewes (Table 6). The concentration of oestradiol in antral fluid of these follicles ranged from 9 to 10,458 nM. 92% of all follicles had E_2 concentrations either > 1000 nM or < 100 nM (Figure 1). High concentrations of oestradiol in antral fluid have been shown to be characteristic of preovulatory follicles (McNatty et al., 1981c). As the number of follicles with antral fluid E_2 concentration $\geqslant$ 1000 nM in this study corresponded closely to the previous ovulation rate as indicated by the number of CL (Table 6), these 'oestrogenic' follicles were, presumably, those that would go on to ovulate. Coincident with the emergence of the dominant, oestrogen-secreting follicle(s) at luteolysis, there is an increase in the incidence of atresia (50-80%) in all other follicles $\geqslant$ 1 mm diameter (McNatty et al., 1982). Therefore, most of the follicles with low antral fluid E_2 concentrations were probably undergoing atresia, though some of the small follicles may have been healthy growing follicles passing through an androgen-dominant

Figure 1 Concentration of oestradiol in antral fluid of individual follicles isolated from the ovaries of control (○) and Booroola Merino (●) ewes with respect to follicle diameter (n = 8 ewes/group). Values below the assay sensitivity are plotted at the limit of detection (●○).


10000
1000
100
10
1
Antral fluid
[E2]
(nM)
1
2
3
4
5
6
7
8
Diameter (mm)

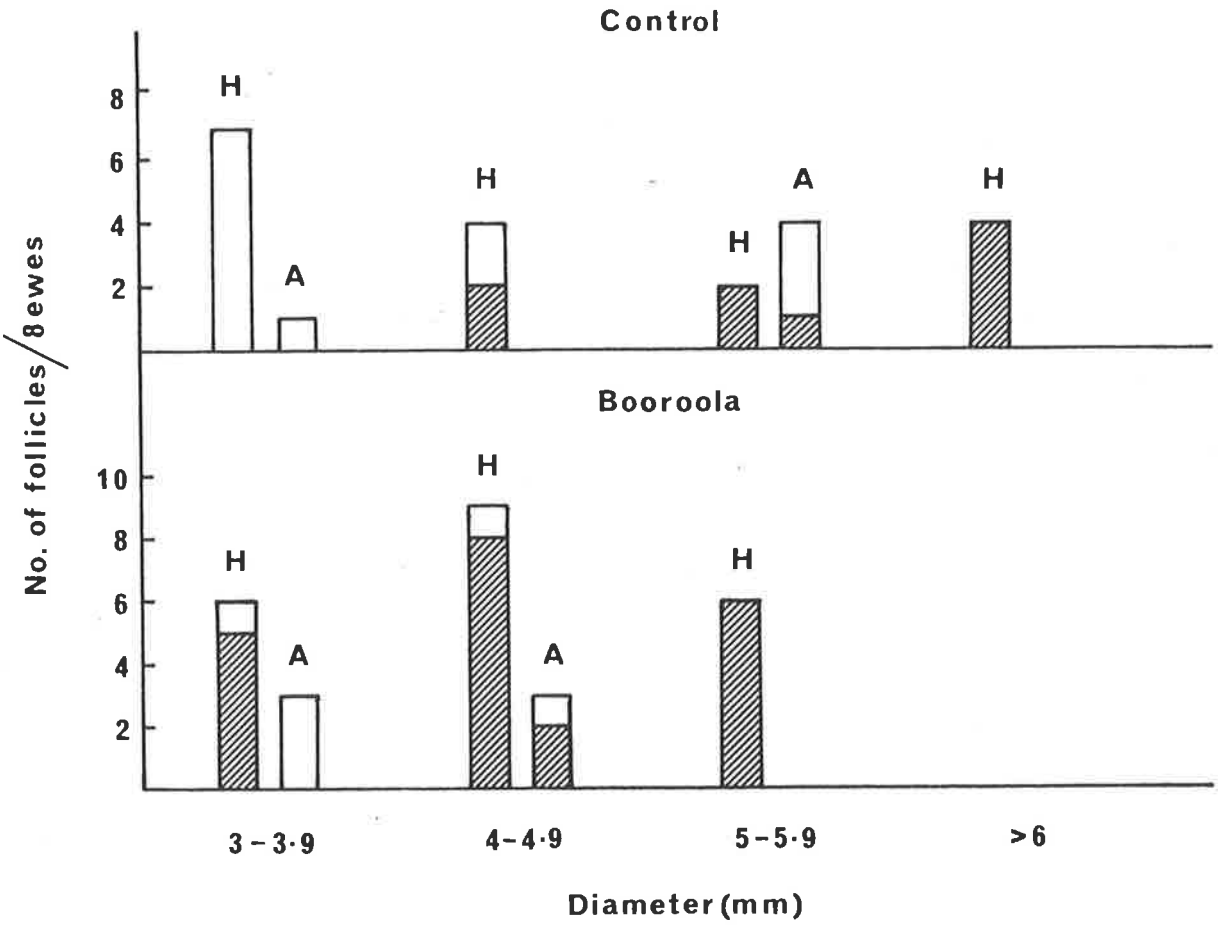
phase prior to acquiring the capacity to produce oestradiol (Carson et al., 1981 ; McNatty, 1982). Thus, follicles were classified according to antral fluid E_2 concentrations of $\geqslant$ 1000 nM (preovulatory or 'oestrogenic') or < 1000 nM (atretic or small, healthy follicles).

Table 6 Number and size of follicles and corpora lutea isolated from the ovaries of control and Booroola Merino ewes (mean ±s.e.m. ; n = 8 ewes/group).

	Control	Booroola	Significance of difference
No. of follicles $\geqslant$ 3 mm diameter	22	27	-
No. of follicles $\geqslant$ 3mm diameter/ewe	2.8 ± 0.7	3.4 ± 0.3	n.s.
Diameter of follicles $\geqslant$ 3mm (mm)	4.8 ± 0.3	4.3 ± 0.2	n.s.
No. 'oestrogenic' follicles/Total no. of follicles > 3 mm	$^{9}/_{22}$	$^{21}/_{27}$	p < 0.01
No. 'oestrogenic' follicles/ewe	1.1 ± 0.1	2.6 ± 0.3	p < 0.001
No. CL/ewe	1.1 ± 0.1	2.5 ± 0.4	p < 0.02
Diameter of 'oestrogenic' follicles (mm)	6.0 ± 0.4	4.5 ± 0.2	p < 0.005
Diameter of 'non-oestrogenic' follicles (mm)	4.1 ± 0.3	3.6 ± 0.2	n.s.

The distribution of all follicles and 'oestrogenic' follicles with respect to size and morphological stage of atresia is shown in Figure 2. No follicles were assessed as terminally atretic (Stage V) and only one follicle was considered to be Stage IV. For comparison between strains,

Figure 2 The distribution of follicles $\geqslant$ 3 mm diameter isolated from the ovaries of 8 control and 8 Booroola Merino ewes, with respect to follicle diameter and stage of atresia. The number of 'oestrogenic' follicles in each classification is represented by the solid portion of each column.


Control
H
A
H
H
A
H
8
6
4
2
Booroola
H
A
H
A
H
10
8
6
4
2
No. of follicles/8 ewes
3 – 3·9
4 – 4·9
5 – 5·9
>6
Diameter (mm)

follicles have been grouped as essentially non-atretic (Stages I & II) and atretic (Stages III & IV), distinguished by the appearance of distinct perforations in the granulosa layer. Of the 17 control follicles $\geqslant$ 3mm diameter classified as healthy, 8 contained E_2 concentrations $\geqslant$ 1000 nM whereas 19 of the 21 class I or II follicles in the Booroola were 'oestrogenic'. Conversely, 1 of the 9 control and 2 of the 21 Booroola follicles with antral fluid E_2 concentrations > 1000 nM were misclassified morphologically. Therefore, the criteria utilized for the morphological assessment of atresia appeared to be equally suitable for both strains of ewe. However, it was not possible to determine whether those 'non-oestrogenic' follicles classified as healthy (B(-), 9 ; B(+), 2) were actually in the very early stages of atresia or were small, growing follicles. The number of 'non-oestrogenic' follicles morphologically classified as atretic was identical in each strain(4).

There were significantly more 'oestrogenic' follicles per Booroola ewe than per control ewe (Table 6). The mean diameter of these preovulatory follicles in Booroola ewes was smaller than those of control ewes (Table 6). In Booroola ewes there were no follicles of diameter > 6 mm, while the diameter of 4 of the 9 preovulatory follicles in controls ranged from 6.2 - 7.5 mm. None of the eight follicles $\leqslant$ 3.9 mm diameter in controls contained E_2 concentrations $\geqslant$ 1000 nM whereas 5 of the 9 Booroola follicles of this size were preovulatory ($p < 0.05$). The 'oestrogenic' follicles of both genotypes were larger than 'non-oestrogenic' follicles (B(-), $p < 0.002$; B(+), $p < 0.05$), but the diameter of 'non-oestrogenic' follicles (Table 6) did not differ significantly between strain of ewe. In contrast to B(-) ewes, the majority of B(+) ewes had at least one preovulatory follicle on each ovary (B(-), 1/8 ; B(+), 6/8 ; $p < 0.05$).

(b) Thecal weight

The theca of 'oestrogenic' B(-) follicles was heavier ($p < 0.005$) than that of similar B(+) follicles (Table 7).

Table 7 Thecal weight and granulosa cell number of follicles isolated from the ovaries of control and Booroola Merino ewes (mean ± s.e.m. (no. of follicles) ; n = 8 ewes/group).

	Control	Booroola	Significance of difference
'Oestrogenic' follicles			
Thecal weight (mg)	8.6 ± 1.7 (9)	3.3 ± 0.3 (16)	$p < 0.005$
No. granulosa cells ($\times 10^6$)	1.36 ± 0.19 (9)	0.68 ± 0.05 (20)	$p < 0.001$
'Non-oestrogenic' follicles			
Thecal weight (mg)	3.8 ± 0.7 (13)	2.2 ± 0.4 (5)	n.s.
No. granulosa cells ($\times 10^6$)	0.67 ± 0.08 (13)	0.45 ± 0.06 (6)	n.s.

An unavoidable delay prior to weighing resulted in the dessication of tissue from 2 Booroola ewes. The weights from these 5 follicles (4 'oestrogenic', 1 'non-oestrogenic') have been omitted from the data. When 'oestrogenic' follicles of similar size (4-6 mm diameter) were compared, thecal weight did not differ between genotype (Table 8). 'Non-oestrogenic' follicles of the two strains of sheep did not differ in the thecal weight. In control ewes, but not Booroola ewes, the thecal weight of 'non-oestrogenic' follicles was less ($p < 0.05$) than that of 'oestrogenic' follicles. The total thecal weight of all 'oestrogenic' follicles per ewe did not differ between strains (B(-), 9.64 ± 2.13 mg, n = 8 ewes ; B(+), 9.68 ± 1.17 mg, n = 5 ewes).

Table 8 Diameter, thecal weight and granulosa cell number of 'oestrogenic' follicles, 4-6 mm diameter, isolated from the ovaries of control and Booroola Merino ewes (mean ±s.e.m. (median)).

	Control (n=5)	Booroola (n=15)	Significance of difference
Diameter (mm)	5.24 ±0.27(5.20)	4.80 ±0.13(4.78)	n.s.
Thecal weight (mg)	5.43 ±1.40(5.90)	3.16 ±0.33(3.17)	n.s.
No. granulosa cells ($x10^6$)	1.14 ±0.22(1.07)	0.71 ±0.06(0.67)	$p < 0.05$

The increase in thecal weight which occurred with diameter in 'oestrogenic' follicles (Figure 3) differed between genotypes, the slope of the regression lines being significantly different ($p < 0.05$). The relationship between thecal weight (y, mgs) and diameter (x, mm) in each group of ewes can be expressed by the following equations:

B(-) $y = -13.5 \pm 7.1 + (3.7 \pm 1.18)\ x$; $r = 0.76$, $p < 0.01$, $n = 9$

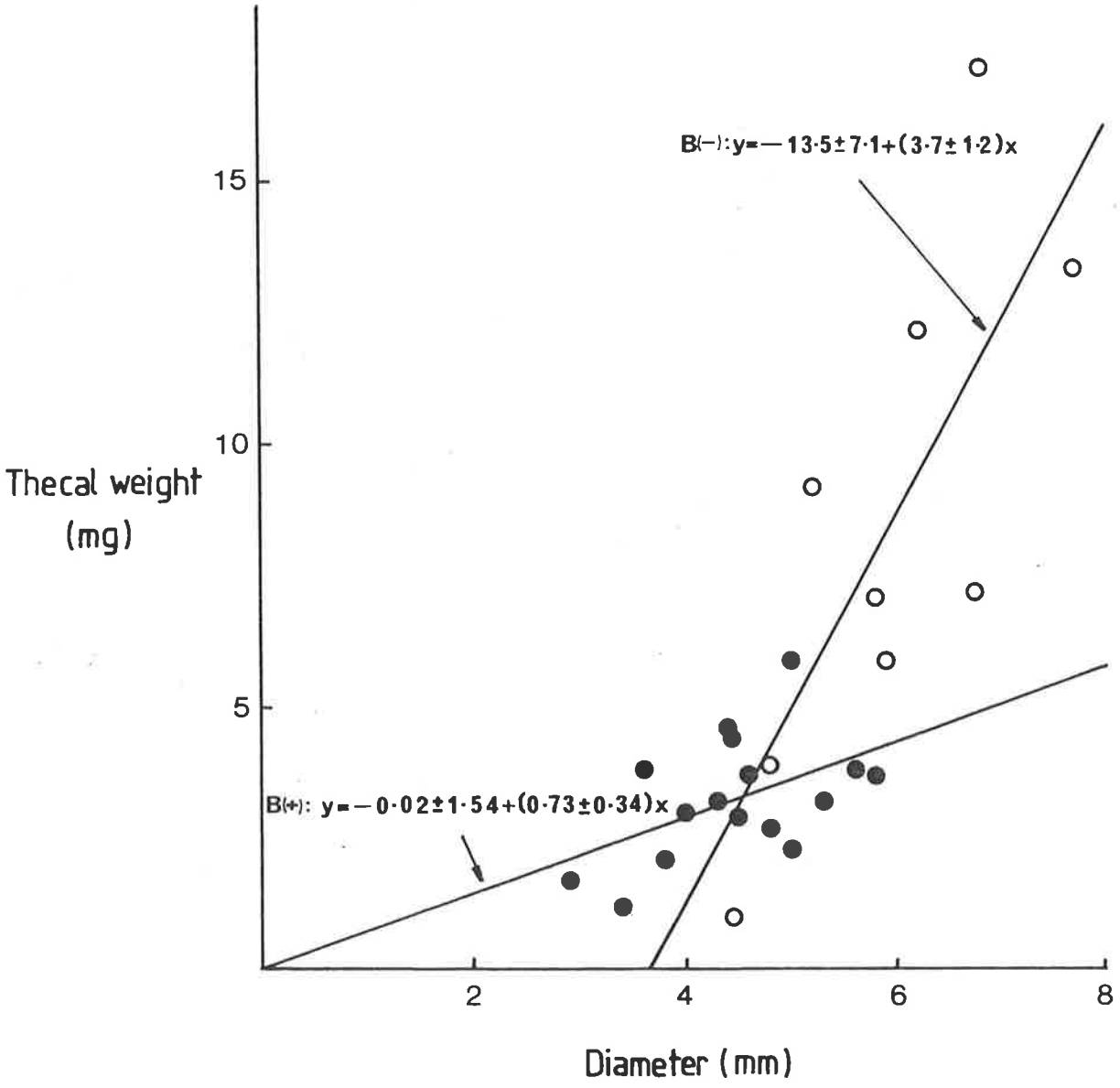
B(+) $y = -0.015 \pm 1.54 \pm (0.73 \pm 0.34)\ x$; $r = 0.50$, $p < 0.05$, $n = 16$

The intercepts of these equations did not differ from each other or from zero.

(c) Granulosa cell number

'Oestrogenic' follicles of both strains of ewe contained significantly more granulosa cells (Table 7) than those follicles with lower oestradiol concentrations (B(-), $p < 0.005$; B(+), $p < 0.05$). Viability ranged from 4-28% and did not differ between 'oestrogenic' and 'non-oestrogenic' follicles. However, the 'oestrogenic' B(-) follicle contained twice the number of granulosa cells as this B(+) follicle. When follicles of a similar size (4-6 mm diameter) were compared the number of granulosa cells was still greater in control follicles (Table 8) The granulosa cell number of 'non-oestrogenic' follicles did not

Figure 3 The relationship between thecal weight and follicle diameter found in 'oestrogenic' follicles isolated from the ovaries of control (O) and Booroola Merino (●) ewes. The calculated regression lines are superimposed on the data.


B(−): y = −13·5 ± 7·1 + (3·7 ± 1·2)x
B(+): y = −0·02 ± 1·54 + (0·73 ± 0·34)x
Thecal weight (mg)
15
10
5
2
4
6
8
Diameter (mm)

differ between strains. The mean total number of granulosa cells contained in all 'oestrogenic' follicles per ewe was 1.84 ± 0.23 for Booroola ewes (n = 7), not significantly different from that of control ewes (1.53 ± 0.27, n = 8), which had one half of the number of preovulatory follicles.

There was no correlation between the number of granulosa cells per 'oestrogenic' follicle and diameter in control ewes and only a weak correlation in Booroola ewes ($r = 0.4$, $p < 0.05$). Thecal weight and granulosa cell number per 'oestrogenic' follicle were not correlated.

3. Hormonal characteristics

(a) Ovarian venous plasma

The presence of a greater number of 'oestrogenic' follicles in the ovaries of B(+) ewes was also reflected by the ovarian vein secretion of oestradiol in these ewes. Only 5 control ewes had E_2 concentrations of > 1.85 nM (range, 1.85 - 7.9 nM) in a single ovarian vein per ewe. All eight Booroola ewes had at least one ovarian vein with E_2 concentration > 1.44 nM (range, 1.44 - 13.2 nM), 2 ewes having E_2 concentrations higher than this in both ovarian veins. The ovarian venous E_2 concentration in the remaining veins was 0.27 ± 0.06 nM in control ewes and 0.61 ± 0.14 nM in Booroola ewes.

As more than 90% of ovarian vein E_2 derives from the largest non-atretic follicle(s) present in the ovary (Bjersing et al., 1972 ; Moor, 1973 ; Baird and Scaramuzzi, 1976b ; McNatty et al., 1981b and 1981c), the concentrations of steroids in ovarian venous plasma were analysed with respect to the presence in the ovary of at least one preovulatory follicle (Table 9). Although atretic and small antral follicles, stroma and corpora lutea secrete androgen, the majority of these steroids in

ovarian venous plasma also arise from the dominant follicle (Moor et al., 1975 ; Baird and Scaramuzzi, 1976b ; Moor et al., 1978 ; Scaramuzzi et al., 1980a ; McNatty et al., 1981b and 1981c ; Scaramuzzi et al., 1981a). There were no significant differences between control and Booroola ewes in the concentration of any of the steroids measured in ovarian venous plasma draining ovaries containing at least one 'oestrogenic' follicle. The mean concentration of androstenedione was not significantly different to (B(-)), or higher (B(+), $p < 0.05$) than the mean concentration of oestradiol, which exceeded that of testosterone (B(-), $p < 0.01$; B(+), $p < 0.002$).

Table 9 Concentration of steroids (nM) in ovarian venous plasma of control and Booroola Merino ewes (mean ± s.e.m. (median) ; n = 8 ewes/group).

	Control	Booroola	Significance of difference
Ovaries containing ⩾ 1 'oestrogenic' follicle			
No. of ovaries	9	14	
Oestradiol	2.78 ± 0.98(1.85)[a]	3.89 ± 0.91(3.25)	n.s.
Androstenedione	8.80 ± 5.50(4.47)	5.98 ± 1.25(5.42)	n.s.
Testosterone	0.52 ± 0.24(0.30)	0.34 ± 0.10(0.19)	n.s.
Progesterone	69.8 ± 50.1 (12.2)	30.7 ± 4.6 (28.0)	n.s.
Ovaries without 'oestrogenic' follicles			
No. of ovaries	7	2	
Oestradiol	0.15 ± 0.03(0.13)[b]	0.37, < 0.1	-
Androstenedione	2.29 ± 1.24(0.98)	2.11, 0.90	-
Testosterone	0.27 ± 0.10(0.16)	0.50, 0.21	-
Progesterone	23.9 ± 14.7 (2.75)	66.7, 79.6	-

Within control group, values with different superscripts differ significantly ($p < 0.001$).

In the B(-) group, oestradiol was the only mean steroid concentration that was significantly higher in plasma draining the ovaries containing the preovulatory follicle(s) than in plasma from ovaries with no 'oestrogenic' follicles. There were too few ovaries without preovulatory follicles in B(+) ewes to perform a similar comparison. In all ewes, the ovarian venous concentration draining ovaries containing one or more preovulatory follicles was $\geqslant$ 0.41 nM whereas that draining ovaries devoid of 'oestrogenic' follicles was $\leqslant$ 0.37 nM. While the concentration of progesterone in ovarian venous plasma did not vary with the presence of preovulatory follicles, in control ewes it was higher in veins draining ovaries containing at least one corpora lutea than those from ovaries without corpora lutea (87.0 ± 48.5 nM, n = 9 vs 1.74 ± 0.27 nM, n = 7 ; $p < 0.01$), but not in Booroola ewes (39.5 ± 6.0 nM, n = 13 vs 21.0 ± 9.4 nM, n = 3).

The mean concentrations of steroids in plasma draining ovaries in which there was only a single 'oestrogenic' follicle were similar in Booroola and control ewes (Table 10). The mean diameter, thecal weight and granulosa cell number of the Booroola follicles secreting into these veins were less than those of control follicles. However, follicles from both strains of ewe contained similar levels of oestradiol (Table 10).

As ovarian blood flow was not measured in this experiment, the ovarian secretion rate of steroids is not known. In an attempt to estimate the overall secretion of steroids in each ewe, the total concentration of steroid in plasma of both ovarian veins was calculated (Table 11). The total oestradiol concentration of Booroola ewes was twice that of control ewes, a difference which approached significance ($p = 0.06$)

Table 10 Concentration of steroids in ovarian venous plasma draining ovaries containing a single 'oestrogenic' follicle and the antral fluid oestradiol concentrations, diameter, thecal weight and granulosa cell number of the follicles in those ovaries (mean ±s.e.m. (median)).

	Control	Booroola	Significance of difference
Ovarian venous concentration (nM)			
No. of ovaries	9	9	
Oestradiol	2.78 ± 0.98(1.85)	2.79 ± 0.78(2.05)	n.s.
Androstenedione	8.80 ± 5.50(4.47)	4.36 ± 0.76(5.29)	n.s.
Testosterone	0.52 ± 0.24(0.30)	0.20 ± 0.04(0.18)	n.s.
Progesterone	69.8 ± 50.1 (12.2)	26.0 ± 4.4 (31.8)	n.s.
Follicular data			
No. of follicles	9	6-9	
Antral fluid [E_2] (nM)	5054 ± 1113(4554)	3971 ± 419 (3927)	n.s.
Diameter (mm)	6.0 ± 0.4 (5.9)	4.0 ± 0.3 (3.9)	p <0.005
Thecal weight (mg)	8.6 ± 1.7 (7.2)	2.4 ± 0.4 (2.4)	p <0.005
No. of granulosa cells (x 10^6)	1.36 ± 0.19 (1.1)	0.68 ± 0.09(0.66)	p <0.001

Table 11 Total concentration of steroids in right and left ovarian venous plasma of control and Booroola Merino ewes (mean ±s.e.m. (median) ; n = 8 ewes/group).

Steroid Concentration (nM)	Control	Booroola	Significance of difference
Oestradiol	3.26 ± 1.06(2.36)	6.90 ± 1.21(6.70)	p = 0.06
Androstenedione	11.9 ± 7.2 (5.47)	10.8 ± 2.2 (8.14)	n.s.
Testosterone	0.82 ± 0.34(0.55)	0.68 ± 0.21(0.32)	n.s.
Progesterone	99.4 ± 55.5 (32.9)	72.0 ± 12.5 (58.8)	n.s.

The significant correlations found between the number of follicles present in the ovary and the ovarian venous concentrations draining that ovary, as well as the correlations between steroids, are presented in Table 12.

Table 12 Correlations (r, Spearman correlation coefficient) of ovarian venous steroid concentrations and follicle numbers in veins draining ovaries containing $\geqslant$ 1 'oestrogenic' follicle (control, n = 9 ; Booroola, n = 14).

	Control		Booroola	
Correlation	r	Significance	r	Significance
$[E_2]$ v [A]	0.75	$p < 0.05$	0.77	$p < 0.002$
$[E_2]$ v [T]	0.83	$p < 0.005$	0.61	$p < 0.05$
[A] v [T]	0.98	$p < 0.002$	0.81	$p < 0.002$
$[E_2]$ v No. of 'oestrogenic' follicles in ovary	0.87	$p < 0.002$	0.69	$p < 0.005$
[T] v No. of follicles $\geqslant$ 3 mm diameter in ovary	-	-	0.61	$p < 0.01$

(b) Antral fluid

(i) Steroids

The concentrations of steroids measured in antral fluid are shown in Table 13. Concentrations below the limit of detection are included in the data at a value equal to the assay sensitivity. However, as the volume of antral fluid recovered was frequently the limiting factor, particularly in smaller follicles, this detection limit is quite

Table 13 Antral fluid concentrations of steroids (nM) and inhibin (kU/ml) of follicles isolated from the ovaries of control and Booroola Merino ewes (n=8 ewes/group).

Hormone	Control		Booroola		Significance of difference
	No. of follicles	Mean ±s.e.m. (Median)	No. of follicles	Mean ±s.e.m. (Median)	
'Oestrogenic' follicles					
Oestradiol	9	5054 ±1113 (4554)	21	4257 ±269 (4039)	n.s.
Androstenedione	9	193 ± 32 (221)	21	266 ± 40 (180)	n.s.
Testosterone	9	257 ± 45 (280)	21	339 ±106 (147)	n.s.
Progesterone	9	39.7 ± 9.2 (30.6)	21	35.9 ±3.0 (37.4)	n.s.
Inhibin	8	13.6 ± 2.9 (8.0)[a]	18	7.7 ±1.1 (6.2)	p<0.05
'Non-oestrogenic' follicles					
Oestradiol	13	134 ± 64 (35)	6	83.3 ±43.6 (35)†	n.s.
Androstenedione	13	235 ± 37 (179)	6	241 ± 34 (213)	n.s.
Testosterone	13	826 ± 141 (604)	6	689 ±221 (430)	n.s.
Progesterone	13	45.6 ± 6.3 (41)	6	63.7 ±17.7 (41.2)††	n.s.
Inhibin	5	2.60± 1.26(1.30)[b]	-	-	-

Within control group, values of inhibin with different superscripts differ significantly (p<0.02).

† Only one value was detectable.
†† Only two values were detectable.

variable. There were no significant differences between control and Booroola ewes in the concentration of any steroid measured in either 'oestrogenic' or 'non-oestrogenic' follicles. Oestradiol was the predominant steroid in preovulatory follicles (E_2 > A, T or P : B(-), p < 0.01 ; B(+), p < 0.001), the molar ratio of steroids being E_2:A:T:P, 100:5:5:1. While concentrations of the two androgens were similar in preovulatory follicles, the testosterone concentration in 'non-oestrogenic' follicles exceeded that of androstenedione, oestradiol and progesterone (B(-), p < 0.001 ; B(+), p < 0.05), the molar ratio of steroids being E_2:A:T:P, 1:5:15:1. The data from all ewes (B(+) and B(-)) were pooled to test for differences between the two follicle types (Table 14).

Table 14 Concentrations of steroids in antral fluid of 'oestrogenic' (n = 30) and 'non-oestrogenic' (n = 19) follicles of control and Booroola ewes.

Hormone Concentration (nM)	'Oestrogenic' follicles	'Non-oestrogenic' follicles	Significance of difference
	Mean ± s.e.m. (median)	Mean ± s.e.m. (median)	
Oestradiol	4496±377(4042)	118± 46(35)	p < 0.0001
Androstenedione	244± 30(181)	237± 27(197)	n.s.
Testosterone	314± 75(186)	783±117(522)	p < 0.0001
Progesterone	37.1±3.4(36.4)	51.3±7.1(41.1)	n.s.

As expected, E_2 concentrations were higher in preovulatory follicles. Although A and P concentrations were similar in all follicles T concentrations were higher in 'non-oestrogenic' follicles.

Utilizing the relationship, $V = 0.52D^{2.7}$, between antral fluid volume (V, µl) and follicular diameter (D, mm) derived by Carson et al. (1981), the content of each steroid per follicle was calculated for those classes of follicle with sufficient detectable concentrations (Table 15). For preovulatory follicles, the mean content of E_2, A and P per follicle were similar in control and Booroola ewes. However, the mean P, T and total androgen (A + T) content per Booroola follicle was less than that of each control follicle (P, $p < 0.05$; T, $p < 0.02$; (A + T), $p < 0.05$). 'Non-oestrogenic' control and Booroola follicles contained similar quantities of each steroid. The larger diameter of 'oestrogenic' follicles resulted in an higher E_2 content ($p < 0.002$, B(-) follicles only) and A content ('oestrogenic', 9.73 ± 1.62, n = 29 ; 'non-oestrogenic', 5.58 ± 1.08, n = 9 ; $p < 0.05$, pooled data from B(+) and B(-) follicles) in these follicles when compared to 'non-oestrogenic' follicles. However, in both strains of ewe, the content of T or (A + T) were similar in 'oestrogenic' and 'non-oestrogenic' follicles.

To ascertain whether B(-) and B(+) preovulatory follicles of the same size had similar steroidogenic capacities, those follicles of a similar diameter (4-6 mm) were compared, as previously (see Table 8). The antral fluid steroid concentrations of these follicles (Table 16) and therefore, content per follicle did not differ between strain of ewe.

Significant correlations of antral fluid and follicular characteristics are presented in Table 17.

Table 15 Antral fluid contents of steroids (pmol/follicle) and inhibin (kU/follicle) of follicles isolated from the ovaries of control and Booroola Merino ewes (n=8 ewes/group).

Hormone	Control		Booroola		Significance of difference
	No. of follicles	Mean ±s.e.m. (Median)	No. of follicles	Mean ±s.e.m. (Median)	
'Oestrogenic' follicles					
Oestradiol	9	388 ± 131 (245)	20	138 ± 16 (141)	n.s.
Androstenedione	9	14.5 ± 4.3 (10.8)	20	7.57 ±1.15 (5.1)	n.s.
Testosterone	9	16.6 ± 3.2 (19.5)	20	7.93 ±1.48 (5.8)	$p<0.02$
Progesterone	9	3.12± 1.18(1.42)	20	1.13 ±0.18 (0.91)	$p<0.05$
A + T	9	31.1 ± 7.0 (31.3)	20	15.5 ±2.1 (10.8)	$p<0.05$
Inhibin	8	0.91± 0.16 (0.74)[a]	17	0.27±0.04 (0.26)	$p<0.002$
'Non-oestrogenic' follicles					
Oestradiol	13	3.26± 1.39 (0.62)	-	-	-
Androstenedione	13	6.34± 1.54 (3.57)	6	3.92± 0.32 (3.86)	n.s.
Testosterone	13	18.4 ± 3.7 (13.5)	6	10.2 ± 2.1 (8.80)	n.s.
A + T	13	24.7 ± 5.0 (16.2)	6	14.1 ± 2.3 (12.5)	n.s.
Inhibin	5	0.11± 0.05 (0.07)[b]	-	-	-

Within control group, values of inhibin with different superscripts differ significantly ($p<0.02$).

Table 16 Concentration of steroids (nM) and inhibin (kU/ml) in the antral fluid of 'oestrogenic' follicles of similar size (4-6 mm diameter) isolated from the ovaries of control and Booroola Merino ewes (n=8 ewes/group).

Hormone	Control		Booroola		Significance of difference
	No. of follicles	Mean ±s.e.m. (Median)	No. of follicles	Mean ±s.e.m. (Median)	
Oestradiol	5	4615 ±1618 (3634)	15	4520 ± 298 (4392)	n.s.
Androstenedione	5	184 ± 29 (216)	15	278 ± 52 (179)	n.s.
Testosterone	5	321 ± 53 (326)	15	224 ± 37 (138)	n.s.
Progesterone	5	36.8 ±11.3 (30.6)	15	35.6 ± 3.9 (36.0)	n.s.
Inhibin	4	18.2 ± 4.6 (18.7)	15	7.74±1.26 (5.90)	$p<0.05$

Within control group, values of inhibin with different superscripts differ significantly ($p<0.02$).

Table 17 Correlations of antral fluid concentrations and antral fluid content and follicular characteristics of follicles isolated from control and Booroola Merino ewes.

Type of follicle (n)	Correlation	r	Significance of correlation
Control - 'oestrogenic' (9)	[E_2] v [P]	0.85	$p < 0.005$
	[A] v [T]	0.64	$p < 0.05$
	A content v T content	0.82	$p < 0.005$
Booroola - 'oestrogenic' (20)	[A] v [T]	0.85	$p < 0.002$
	A content v T content	0.81	$p < 0.002$
	[A] v Granulosa cell number/follicle	-0.73	$p < 0.002$
	[T] v diameter	-0.75	$p < 0.002$
Control - 'non-oestrogenic' (13)	A content v T content	0.83	$p < 0.002$
Booroola - 'non-oestrogenic' (6)	[A] v [T]	0.82	$p < 0.05$

(ii) Inhibin

Sufficient antral fluid for inhibin measurement was only recovered from 8 control and 18 Booroola 'oestrogenic' follicles and 6 'non-oestrogenic' control follicles. The antral fluid inhibin concentration of control preovulatory follicles was twice that of similar Booroola follicles (Table 13). These smaller Booroola follicles therefore contained approximately one-third as much inhibin as those from control ewes (Table 15). When control and Booroola 'oestrogenic' follicles of the same size (4-6 mm diameter) were compared, antral fluid inhibin concentration was significantly less in Booroola follicles (Table 16).

The content and concentration of inhibin in 'non-oestrogenic' control follicles was less (content, $p < 0.01$; concentration, $p < 0.02$) than that in 'oestrogenic' follicles of the same ewes (Table 13 and 15).

When all B(-) follicles $\geqslant$ 3 mm were considered, 'oestrogenic' and 'non-oestrogenic' combined, antral fluid oestradiol concentration was correlated significantly with inhibin concentration and content (inhibin concentration ; $r = 0.7$, $p < 0.005$, $n = 13$; inhibin content ; $r = 0.80$, $p < 0.002$, $n = 13$). Inhibin concentration was not correlated with follicular diameter in preovulatory follicles, but a weak correlation existed when all control follicles $\geqslant$ 3 mm were considered ($r = 0.59$, $p < 0.05$). The inhibin content and concentration in all control follicles also increased with granulosa cell number (inhibin concentration, $r = 0.64$, $p < 0.05$, inhibin content, $r = 0.61$, $p < 0.05$).

D. Discussion

The ovulation rate of the Booroola Merino ewes used in this study corresponds with previous reports of Booroola ewes of a similar age (Piper and Bindon, 1982a ; Bindon *et al.*, 1984). Full expression of the Booroola gene does not occur until ewes reach maturity at approximately 3 years of age, but a clear difference was already evident between control and Booroola ewes.

Despite the increased numbers of CL per Booroola ewe, luteal peripheral progesterone concentrations were similar to control ewes, reflecting similar amounts of total luteal tissue, as found by Scaramuzzi *et al.* (1981b). Recently, Kelly *et al.* (1983/1984) reported that Booroola ewes had less total luteal tissue and that this difference was more pronounced at ovulation rates > 3, whether ovulation occurred naturally or after administration of low doses of PMSG (250 - 750 iu). They also found that luteal progesterone concentrations were higher in

control than Booroola ewes. However, Bindon et al. (1981) have previously shown that progesterone concentration increases with ovulation rate in PMSG-treated Merino ewes, but not in Booroola Merino ewes, and these two effects may have been confounded in the study of Kelly et al. (1983/1984). This study also found that progesterone secretion did not increase concurrently with ovulation rate in Booroola ewes as there were no significant regressions between the number of CL/ewe and either luteal peripheral progesterone concentration ($p > 0.1$) or ovarian vein progesterone concentration ($p > 0.05$). However, total luteal tissue weight per Booroola ewe did increase with ovulation rate.

Although similar numbers of large follicles ($\geqslant 3$ mm diameter) were isolated from Booroola and control ewes, the number of follicles with high antral fluid oestradiol concentrations, characteristic of preovulatory follicles, differed markedly between strains. In accordance with their higher ovulation rate, approximately twice the number of 'oestrogenic' follicles were isolated from the ovaries of Booroola ewes. Accounting for approximately 90% of the total steroid content, oestradiol was the predominant steroid in these follicles. The antral fluid concentrations of all steroids measured in preovulatory follicles were similar to those previously reported in follicles during the follicular phase of the oestrous cycle (McNatty et al., 1981c and 1982 ; McNatty, 1982 ; Webb and England, 1982b) and higher than those from follicles isolated during the luteal phase (Moor et al., 1978) or indeterminate stage (Carson et al., 1981) of the oestrous cycle. The significant negative correlation of testosterone concentration with diameter in 'oestrogenic' follicles is consistent with the precursor-product role of testosterone in follicular oestradiol production (Moor, 1977) by FSH-stimulated aromatase activity in granulosa cells.

The most striking characteristic of preovulatory Booroola follicles was their smaller size, each follicle having only one-third the weight of thecal tissue and one-half the number of granulosa cells as control follicles. The number of granulosa cells recovered from 'oestrogenic' follicles was less than that estimated to be present by Cahill and Mauléon (1980) or recovered by McNatty et al. (1982). Control Merino follicles contained 22.5% (range 12.2-35.6%) and Booroola Merino follicles 15.1% (range 8.0-24.8%) of the maximum number of recoverable granulosa cells for diameter predicted by McNatty's formula, derived from follicles isolated from Romney ewes, which may reflect differences between breeds of sheep. Deane et al. (1966) have shown that the majority of the volume of the corpus luteum is derived from granulosa cells of the preovulatory follicle, and the weight of the corpora lutea found in these control and Booroola ewes supports the relativity of granulosa cell counts between strains in this study. The mean weight of corpora lutea from Booroola ovaries was 45% of those from control ovaries. 'Oestrogenic' Booroola follicles yielded 50% of the number of granulosa cells found in similar control follicles.

The Booroola may, therefore, differ from other high ovulation rate breeds of sheep, as no difference was found in the preovulatory follicle size or in the number of granulosa cells per follicle size between the Romanov with an ovulation rate of 3.0 and the Ile-de-France with an ovulation rate 1.6 (Cahill et al., 1979 ; Cahill and Mauléon, 1980). A similar reduction in granulosa cell number in Booroola follicles has previously been noted (Scaramuzzi and Turnbull, cited by Bindon and Piper, 1982b).

In the Booroola, it is apparent that the steroidogenic environment essential for ovulation and oocyte maturation is generated within a follicle with reduced granulosa and thecal mass. The high levels of

oestradiol in antral fluid, together with FSH, stimulate an increase in the mitotic rate, aromatase activity and the formation of LH receptors in granulosa cells. Oocyte maturation requires not only high levels of oestradiol, particularly during early phases, to allow normal blastocyst formation (Moor and Trounson, 1977), but also the correct steroid ratio during development. Alterations to the steroid profile during maturation results in developmental abnormalities in the oocyte, particularly at fertilization (Moor et al., 1980), probably by altering the pattern of proteins synthesized by the oocyte (Moor et al., 1982). When compared to control follicles, the smaller volume of the Booroola follicle resulted in the maintenance of antral fluid steroid concentrations despite a reduced total steroid content ($p < 0.05$), consistent with their lesser follicular mass. However, when follicles of the same size were compared, steroidogenic capacity, as indicated by antral fluid steroid concentrations, did not differ between genotypes.

Therefore, similar levels of oestradiol were achieved by the Booroola follicle with only 60% of the number of granulosa cells of control follicles. The weight of the theca, the source of androgen substrate for oestrogen synthesis, (Moor, 1977) did not vary with strain of ewe in follicles of the same size. This suggests either that enhanced aromatase activity is present in the granulosa cells of Booroola follicles or that an alternate source of aromatase activity exists in the Booroola follicle. Armstrong et al. (1981) have demonstrated that the thecal tissue of large preovulatory follicles (> 6 mm diameter) isolated from Merino-cross ewes produced approximately the same quantity of oestradiol in vitro as the granulosa cells obtained from the same follicles. The relative contributions of oestrogen derived from thecal or granulosal sources to ovarian venous or antral fluid oestrogen concentrations in vivo is unknown. Thus, the possibility exists that an

enhanced capacity for thecal oestrogen synthesis exists in the Booroola follicle or, is attained at a smaller preovulatory follicle size.

The negative correlation between antral fluid androstenedione concentration and granulosa cell number in 'oestrogenic' Booroola follicles suggests that both thecal mass (and thus, androgen substrate) and aromatase activity may be rate-limiting in granulosal oestrogen production in Booroolas as no such relationship was evident in control follicles.

'Oestrogenic' follicles of all ewes were steroidogenically more active, containing more granulosa cells and, in control ewes, a greater thecal mass than follicles with low antral fluid oestradiol concentrations. In contrast to preovulatory follicles, androgens (androstenedione and testosterone) accounted for 97% of the steroid content of 'non-oestrogenic' follicles, a characteristic feature of both atretic (Moor et al., 1978 ; Carson et al., 1981) and small, healthy follicles (Carson et al., 1981 ; McNatty, 1982). More 'non-oestrogenic' follicles were isolated from control ewes, but the number of these morphologically classified as atretic, did not differ between strains. The loss of aromatase activity is one of the earliest changes in follicular function at the onset of atresia (Moor et al., 1978), occurring prior to detectable structural changes (Carson et al., 1981). Therefore, as some of the 'non-oestrogenic' follicles morphologically assessed as healthy might have been in the earliest stages of atresia, it was not possible to determine whether the incidence of atresia differed between control and Booroola ewes.

That preovulatory follicles are the prime source of oestradiol entering the ovarian vein was confirmed in both control and Booroola ewes by the significant correlation existing between the number of 'oestrogenic' follicles present in an ovary and the E_2 concentration in

the ovarian vein draining that ovary, thus supporting previous findings (Bjersing et al., 1972 ; Baird et al., 1975 ; Baird and Scaramuzzi, 1976b ; England et al., 1981b ; McNatty et al., 1981c). The levels of steroids measured in ovarian vein plasma were similar to those previously reported (Bjersing et al., 1972 ; McNatty et al., 1981c ; Murdoch and Dunn, 1982). In contrast to the ratio of steroids found in antral fluid of 'oestrogenic' follicles, mean androstenedione concentration was approximately twice that of oestradiol and twenty times that of testosterone in veins draining ovaries containing 'oestrogenic' follicles. The concentration of either androgen did not vary with the presence of preovulatory follicles. Small healthy and atretic, as well as large atretic follicles secrete androgens (Moor et al., 1978) and though Moor et al. (1975) found that ovarian stromal tissue was steroidogenically inactive in vitro, Baird and Scaramuzzi (1976b) concluded that the stroma and corpus luteum secreted some androstenedione in vivo. In this study, the lack of a relationship between the number of follicles $\geqslant$ 3 mm and androstenedione concentrations in ovarian venous plasma also suggests that the larger follicles present in the ovary are not the only source of this steroid. Testosterone levels in ovarian venous plasma were, however, correlated with the number of follicles, irrespective of their oestrogenic status. The difference in androstenedione : testosterone ratios found in antral fluid and ovarian vein plasma is consistent with the view that the majority of androstenedione is secreted unchanged by the follicle, whereas testosterone is aromatized to E_2 within the follicle (Moor et al., 1978). As ovarian vein progesterone concentration did not vary with the presence of preovulatory follicles, but with the presence of the regressing corpora lutea, these must have been the source of this steroid in the ovarian vein.

Oestradiol production is not only essential for preovulatory follicular maturation but also the regulation of gonadotrophin secretion by the pituitary. The expression of oestrus (Radford, 1967), increased LH pulse frequency (Karsch et al., 1983), sensitization of the pituitary (Reeves et al., 1971 ; Nett et al., 1984) and induction of the preovulatory gonadotrophin surge (Goding et al., 1973) are dependent on increasing levels of oestradiol during the follicular phase. The mean concentration of oestradiol in ovarian venous plasma draining ovaries containing at least one preovulatory follicle were similar in control and Booroola ewes, even though there were 1.5 ±0.2 follicles per Booroola ovary compared to only one follicle in each of these control ovaries. However, comparison of the ovarian vein concentrations in veins draining a single 'oestrogenic' follicle suggests that the oestradiol secretion rate of each Booroola follicle is equivalent to that of control follicles in vivo. The antral fluid E_2 concentrations of the B(-) and and B(+) follicles secreting into these veins were also similar, despite the reduced follicular mass of B(+) follicles, thus demonstrating, once again, the enhanced oestrogen biosynthetic capacity of the Booroola follicle. As Booroola ewes were more likely to have preovulatory follicles in both ovaries, the secretion rate and, therefore, peripheral oestradiol levels might be expected to be higher in these ewes. Also, as control and Booroola ewes had the same total thecal mass and granulosa cell number per ewe, the increased E_2 production per unit follicular mass of the Booroola follicle might be expected to result in increased total E_2 synthesis in these ewes. This difference, however, was not reflected when the total ovarian vein E_2 concentration per ewe was calculated. Measurement of ovarian steroid secretion rates, incorporating blood flow measurements would be required to clarify this point.

In a recent report, no difference in peripheral oestradiol levels was noted between control and Booroola Merino ewes during frequent sampling on days 2, 9 or 16 of the oestrous cycle (Scaramuzzi and Radford, 1983). No evidence was found in the present experiment to support the finding by Scaramuzzi and Radford that peripheral androstenedione concentrations were elevated in Booroola ewes during the preovulatory period. The ovarian vein concentration of either androstenedione or testosterone did not vary with genotype. With reports of similar or higher FSH and LH concentrations in Booroola ewes (Bindon et al., 1982 ; Scaramuzzi and Radford, 1983 ; Bindon et al., 1984), a difference in the sensitivity of the hypothalamic-pituitary axis to the negative feedback effects of oestradiol would be necessary if peripheral oestradiol levels were elevated in Booroola ewes. However, no such difference has been reported in Booroola ewes (Bindon et al., 1978 ; Cummins et al., 1982).

Ovarian inhibin, as well as oestradiol, may contribute to the control of FSH secretion in the ewe (Goodman et al., 1981 ; Cummins et al., 1983). As might be expected from the decreased numbers of granulosa cells, the source of ovarian inhibin (Erickson and Hsueh, 1978b ; Sato et al., 1978 ; Henderson and Franchimont, 1981 and 1983), the concentration and content of inhibin in the antral fluid of Booroola preovulatory follicles was less than that of control follicles. The higher concentrations of inhibin found in preovulatory follicles as well as the correlation of inhibin content and concentration in follicles with antral fluid oestradiol concentrations confirms the view that inhibin is produced by large, non-atretic follicles (Tsonis et al., 1983 ; Henderson et al., 1983). While individual B(+) follicles contained only one-third the inhibin of B(-) follicles, the total amount of inhibin contained in all the 'oestrogenic' follicles of each ewe was

similar in both genotypes, due to the greater number of follicles per Booroola ewe. During the luteal phase, the inhibin content of Booroola ovaries has been found to be approximately one-third that of ovaries from control ewes (Cummins et al., 1983). Such a difference may not occur during the follicular phase or, the release rate of inhibin from follicles may be increased at this stage of the oestrous cycle, concomitant with the increased blood flow to all follicles (Bruce and Moor, 1975 ; Murdoch et al., 1983). It has been proposed that differences in ovarian inhibin secretion might be responsible for the higher FSH levels reported during luteolysis in Booroola ewes and, therefore, the higher ovulation rate of Booroola ewes (Bindon, 1984 ; Bindon et al., 1984). As preovulatory follicle selection had probably occurred by the time of ovariectomy, differences in inhibin secretion may no longer be apparent. Resolution of this point awaits correlation of temporal changes between ovarian inhibin and pituitary FSH secretion.

CHAPTER V

ENDOCRINE PARAMETERS OF THE PREOVULATORY FOLLICLE OF THE BOOROOLA MERINO : 2. STEROID PRODUCTION BY FOLLICLES IN PERIFUSION

A. Introduction

Preovulatory follicular growth and steroid production are dependent on the actions of both LH and FSH (see Richards, 1980 for review). LH binds to specific receptors on the plasma membrane of thecal cells and stimulates the synthesis of androgen which, under the influence of FSH, is converted to oestrogen by the aromatase enzyme system within the granulosa cells. Oestrogen and FSH act synergistically to promote granulosa cell proliferation and thus, follicular growth, as well as the development of granulosa cell LH receptors. The increased oestrogen secretion by the preovulatory follicle(s) induces oestrus in the ewe (Radford, 1967) and the ovulatory gonadotrophin surge (Goding et al., 1973). To respond to gonadotrophins a follicle requires not only plasma membrane receptors but an active adenylate cyclase system and the enzymes of the steroidogenic pathways. Measurement of the steroidogenic response of follicles to gonadotrophins in vitro incorporates all these components of hormone responsiveness.

The technique of perifusion enables the study of dynamic changes in the steroid production of individual follicles, deprived of endogenous influences, in response to the controlled addition of substances. A continuous flow of medium minimizes the accumulation of metabolites, thus more closely resembling conditions in vivo than does static culture. Direct serial measurement of steroid secretion is possible without disturbance to the follicle which is apparent during continual ovarian vein sampling. The rate of steroid secretion by follicles isolated from control and Booroola Merino ewes was, therefore, compared

in perifusion. The response of each follicle to a preovulatory pulse of LH was measured.

B. Materials and Methods

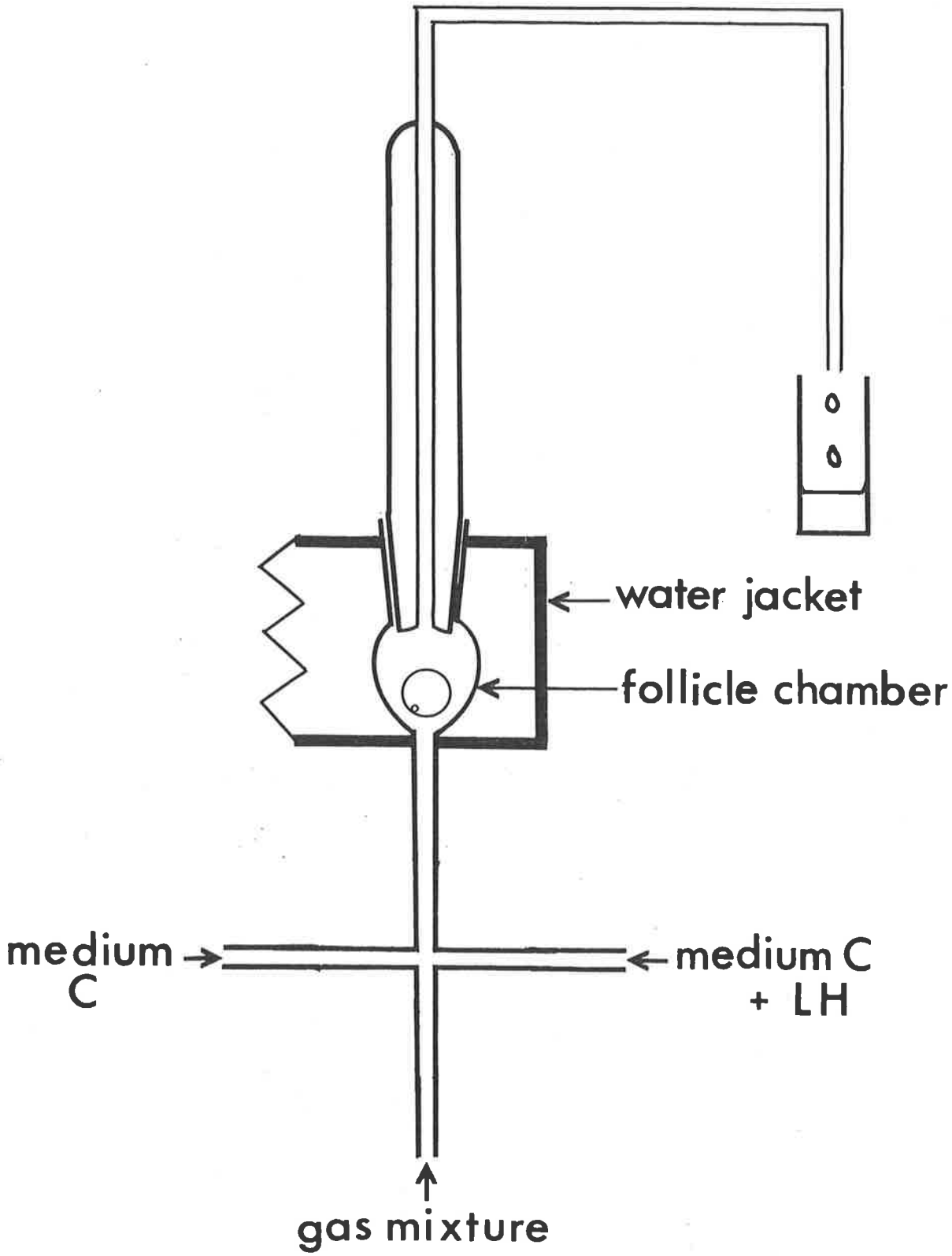
1. Perifusion System

All follicles $\geqslant$ 3mm diameter were dissected free of adhering ovarian stroma, measured and classified as to stage of atresia (see IV.B), and transferred to 1-2 ml perifusion medium (medium C, see II.D.1) in a sterile petri dish until perifusion (1-2h).

Each follicle was then placed in an individual glass chamber filled with medium C in the perifusion apparatus (Figure 4). Each chamber of the apparatus consisted of the top portion of a glass test tube (Quickfit, Corning, Stone, England), 1 ml or 1.5 ml volume, shaped into a bulb with a glass extension and stoppered with an air-stream inlet tube (small, 10/19 ; large 14/23 ; Quickfit, Corning, Stone, England). The apparatus comprised a total of 15 chambers supported in a perspex bath through which warm water (37-38°C) constantly circulated. Medium C, warmed to 37-38°C and saturated with 45% O_2 : 5% CO_2 : 50% N_2, was delivered at a constant rate of 0.25 ml/min to each chamber by a peristaltic pump (Technicon Instr. Co., N.Y., U.S.A.), a flow rate similar to the capillary flow to follicles *in vivo* (Bruce and Moor, 1976). The inclusion of 2-4% BSA in the medium is essential for maximal steroid release (Seamark, Keast and Amato, unpublished results) and minimal tissue oedema (Janson *et al.*, 1978; Bjersing *et al.*, 1981). The medium and gas entered the base of each chamber through a teflon connector, the bubbles of gas resulting in continual rotation of the follicle. The effluent leaving the top of the chamber was collected every 10 min in test-tubes using a fraction collector (Paton Industries,

Figure 4 Schematic drawing of perifusion system.

PERIFUSION SYSTEM



South Australia). The weight of each timed sample was determined to reduce errors due to fluctuations in the rate of flow between individual lines. In order to reduce steroid adsorption, all connecting tubing was made of rigid teflon (Becton-Dickinson, N.J., U.S.A.), except for the tubing in the pump, which was silicone rubber. The pH of the medium was checked intermittently during each perifusion and found to remain between 7.35 and 7.5.

Medium C containing LH was introduced into the chamber by switching to a separate pump.

The apparatus was sterilized between each experiment by flushing with 70% alcohol, water and medium C.

2. Preparation of LH

NIH-LH-S19 was dissolved in Dulbecco's phosphate-buffered saline (Flow laboratories, Stanmore, Australia) containing 0.1% BSA (Fraction V; Sigma, M.O., U.S.A.) at a concentration of 20 µg/ml and stored frozen. Aliquots of this solution were thawed and diluted in medium C immediately prior to use to yield a concentration of 5 ng/ml.

3. Hormone assays

Oestradiol and androstenedione were assayed in duplicate 0.01-0.2 ml aliquots of the perifusate without extraction. Testosterone and progesterone were measured singly after extraction of 0.2-1 ml of the perifusate (see II.B.3(d)).

LH was assayed in 0.1-0.2 ml of perifusate from a representative sample of experiments, each of the small (1.0 ml) chambers being represented twice and the large (1.5 ml) chambers at least once in the measurements (II.C.).

4. Experimental design

2-13 chambers were used for the perifusion of all follicles $\geqslant$ 3mm diameter isolated from 2-3 ewes on each occasion, the 9 small chambers being used for the majority of follicles. The 6 large chambers were used for follicles > 6 mm diameter, or when > 9 follicles were perifused at any one time. After a 60 min stabilization period to determine basal secretion rate, the follicles were exposed to a pulse of LH (5 ng/ml) lasting 30 min. Perifusion with LH-free medium then continued for a further 90 min. Samples of perifusate were collected every 10 min throughout the entire 3 h. The secretion rate of steroids is expressed as pmol/follicle/min for each 10 min collection period.

C. Results

The results of all follicles with known antral fluid steroid concentrations collected from all 20 ewes have been included in this study. Any values below the detectable limits of the assays were included in the data at a value equal to 0.9 times the assay sensitivity.

In perifusion there was an initial release of steroids from the follicles and then the establishment of a steady state production rate. Exposure to LH resulted in a pulse of increased steroid secretion from most follicles, the responses varying markedly between individual follicles. The steroid secretion rates of eight 'oestrogenic' follicles are shown in Figures 5 and 6, depicting the range of response. The rates of steroid secretion by follicles perifused with LH-free medium, remains constant or declines over 3 h (Amato, F and Seamark, R.F., unpublished results).

Figure 5 Steroid secretion (E_2, A, T and P) by individual B(-) follicles during 3 h of perifusion. The secretion rate (pmol/min) is plotted for each 10 min sample. Each follicle was exposed to LH during the time interval represented by the solid bars. Values plotted below the axis were non-detectable. Note that the T scale of follicle 6 C1L differs from all other follicles.

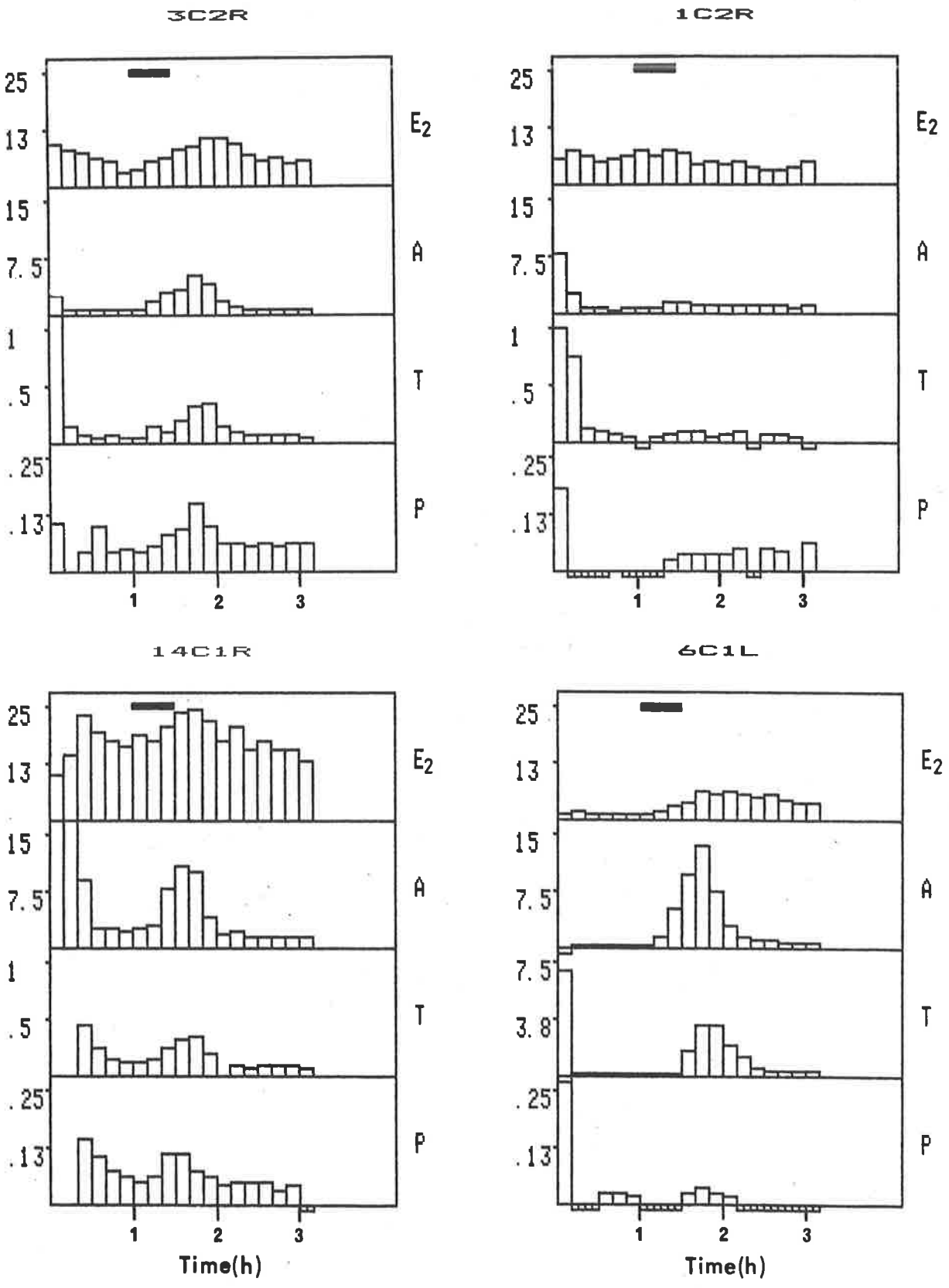
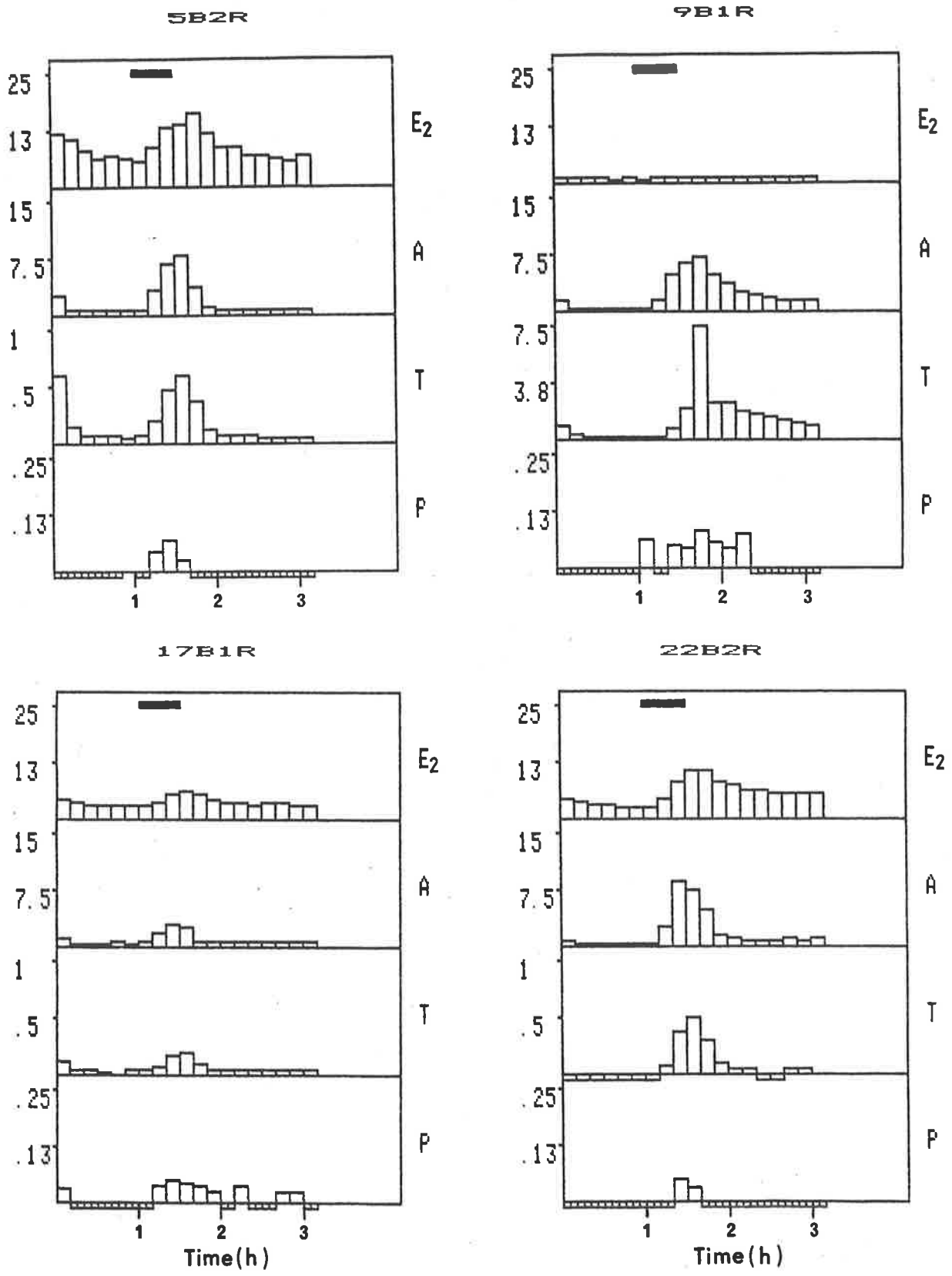

3C2R
1C2R
14C1R
6C1L
25
13
15
7.5
1
.5
.25
.13
3.8
E2
A
T
P
1
2
3
Time(h)

Figure 6 Steroid secretion (E_2, A, T and P) by individual B(+) follicles during 3 h of perifusion. The secretion rate (pmol/min) is plotted for each 10 min sample. Each follicle was exposed to LH during the time interval represented by the solid bars. Values plotted below the axis were non-detectable. Note that the T scale of follicle 9 B1R differs from all other follicles.


5B2R
9B1R
17B1R
22B2R
25
13
15
7.5
1
.5
.25
.13
7.5
3.8
E_2
A
T
P
1
2
3
Time(h)

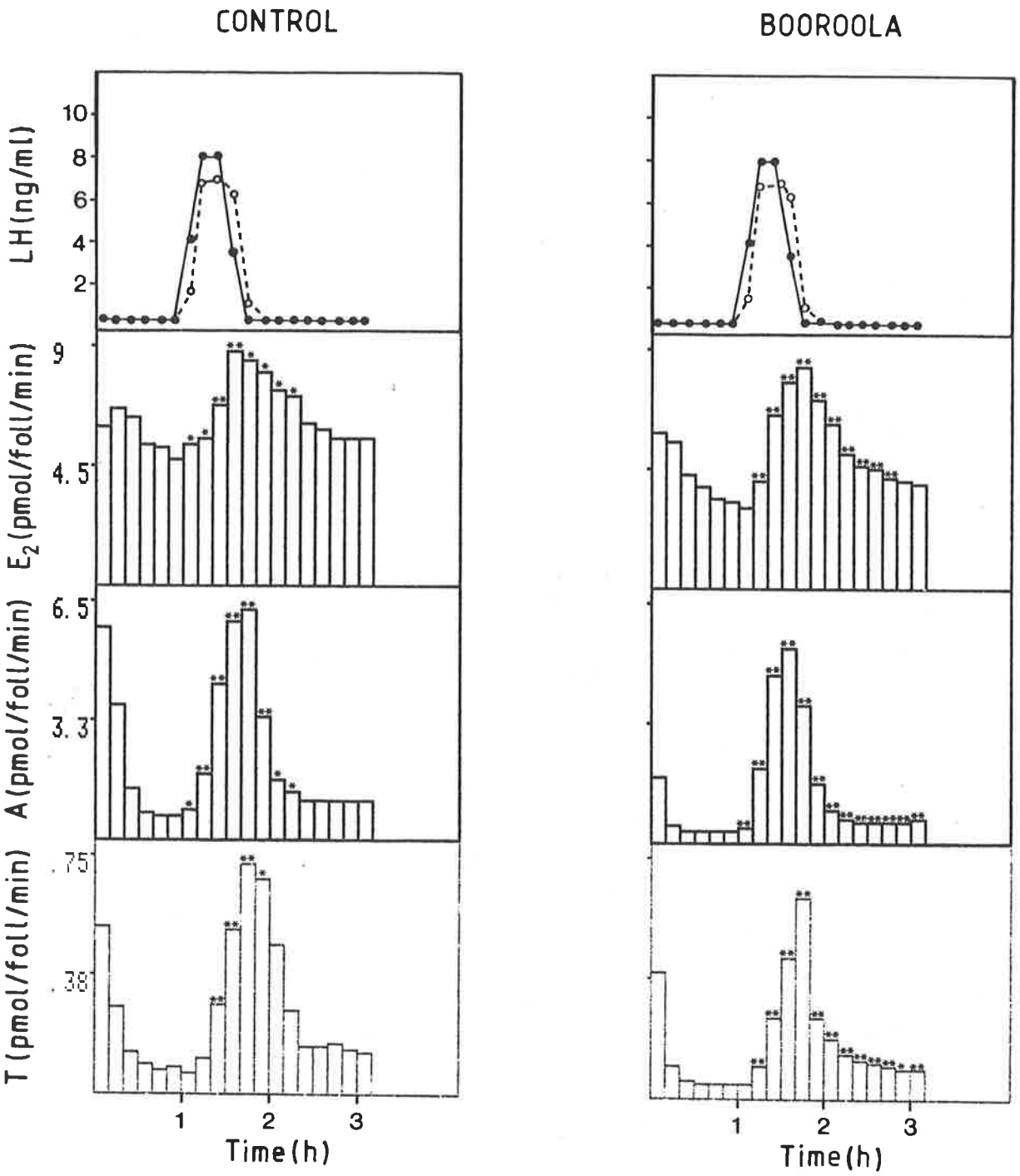
1. 'Oestrogenic' follicles (antral fluid $E_2 \geqslant 1000$ nM)

The basal production (defined as the secretion rate during the 10 min period prior to LH introduction) of oestradiol by 'oestrogenic' follicles in perifusion was approximately 9 times that of androstenedione and 60 times that of testosterone (Figure 7). Although progesterone secretion became transiently elevated after LH, basal production was frequently below the assay sensitivity (0.02-0.04 pmol/min) thus precluding any further analysis of the data. After LH, maximum E_2 secretion was 2-3 times higher than basal secretion, this rise exceeding 5 assay standard deviations (range, 5.3-56 s.d.) in 28 of the 30 follicles. In the 2 follicles not showing this response the secretion rate after LH increased by 1.2 and 2.0 times the assay s.d. The mean secretion rate of A and T after LH was approximately 10 and 20 times higher, respectively, than basal secretion. These increases exceeded 10 assay s.d. (range, 13-1600 s.d.) in all follicles.

The steroid production by 'oestrogenic' control and Booroola follicles in perifusion is shown in Table 18. The total amount of steroid released by each follicle in the pulse of secretion during and after LH exposure was calculated from the area under the curve above the basal secretion rate. The mean secretion rate of the final three ten minute periods of perifusion of each follicle was used to determine the basal secretion rate after LH.

Both before and after LH administration, the basal secretion rate of E_2 was similar in B(-) and B(+) follicles, but the secretion of A and T was lower in B(+) follicles. During the first ten minute period of LH infusion, there was a significant increase in the secretion rate of E_2 and A by B(-) follicles and of A by B(+) follicles above pre-LH levels (B(-):E_2, $p < 0.05$; A, $p < 0.05$; B(+):A, $p < 0.005$). E_2 and T secretion by B(+) follicles rose significantly by the second ten minute

Figure 7 Steroid secretion (E_2, A and T) by 'oestrogenic' B(-) (n=9) and B(+) (n=21) follicles during 3 h of perifusion. Values are the mean rate for each 10 min sample. The concentration of NIH-LH-S19 in the medium of small (●) and large (○) perifusion chambers is shown in the upper graph.
* $p < 0.05$, ** $p < 0.01$ indicate steroid secretion significantly higher than basal production prior to LH.


CONTROL
BOOROOLA
LH(ng/ml)
10
8
6
4
2
E2(pmol/foll/min)
9
4.5
A(pmol/foll/min)
6.5
3.3
T(pmol/foll/min)
.75
.38
1
2
3
Time(h)
1
2
3
Time(h)

sample (E_2, $p < 0.005$; T, $p < 0.001$). T secretion of B(-) follicles increased ($p < 0.01$) during the final ten minute period of LH administration.

There was no significant difference between B(-) and B(+) follicles in the maximum secretion rate of any steroid or the total amount of steroid released per follicle in response to LH (Table 18).

Table 18 Steroid secretion in perifusion by 'oestrogenic' follicles isolated from the ovaries of control and Booroola Merino ewes (mean ±s.e.m. (median)).

Steroid	Control (n=9)	Booroola (n=21)	Significance of difference
Basal secretion prior to LH (pmole/follicle/min)			
E_2	4.7 ±1.6 (3.0)	3.2 ±0.4 (2.8)	n.s.
A	0.64±0.19(0.40)	0.31±0.03(0.31)	$p < 0.05$
T	0.08±0.01(0.07)	0.04±0.003(0.03)	$p < 0.05$
Maximum secretion after LH (pmole/follicle/min)			
E_2	9.7 ±2.4 (7.0)	8.6 ±0.8 (7.2)	n.s.
A	6.7 ±1.5 (4.9)	5.5 ±0.5 (5.3)	n.s.
T	0.76±0.34(0.35)	0.70±0.34(0.31)	n.s.
Total steroid released per follicle (area units)			
E_2	27.2 ±6.6 (36.5)	27.3 ±2.8 (26.2)	n.s.
A	22.1 ±4.5 (22.9)	17.9 ±1.7 (16.6)	n.s.
T	3.2 ±1.6 (1.2)	2.1 ±1.2 (0.8)	n.s.
E_2+A+T	52.0±11.0(62.5)	47.1±3.8 (49.7)	n.s.
Basal secretion after LH (pmol/follicle/min)			
E_2	5.5 ±1.3 (4.2)	4.0 ±0.4 (3.4)	n.s.
A	1.03±0.26(0.74)	0.57±0.06(0.51)	$p < 0.05$
T	0.14±0.04(0.07)	0.10±0.05(0.04)	$p < 0.05$

The time from the beginning of LH infusion to the peak secretion of the measured steroids was similar in B(-) and B(+) follicles (A, 41.7 ± 1.0 min : T, 44.1 ± 1.36 min ; E_2, 48.3 ± 1.9 min) with the period of maximum A secretion occurring before that of T ($p < 0.05$) which, in

turn, preceeded the period of maximum E_2 secretion ($p < 0.05$). It was, however, found that the basal secretion rate of B(+) follicles remained elevated (E_2, $p < 0.05$; A, $p < 0.005$; T, $p < 0.05$) beyond the time when the secretion rate of B(-) follicles had returned to pre-LH levels (Table 18).

The thecal weight and granulosa cell number per follicle for perifused 'oestrogenic' follicles were quoted in Chapter IV (Table 7).

All significant correlations found between steroid production in perifusion and follicular parameters are shown in Tables 19 and 20.

2. 'Non-oestrogenic' follicles (antral fluid $E_2 < 1000$ nM)

Oestrogen secretion by these follicles was <0.2 pmol/follicle/min.

In 'oestrogenic' follicles the ratio of the basal secretion rates of A : T was 7 : 1 whereas in 'non-oestrogenic' follicles this was reduced to a ratio of 3 : 1 due to the increased secretion of testosterone (Figure 8). In contrast to 'oestrogenic' follicles, the secretion rate of androgens by 'non-oestrogenic' follicles did not differ between genotypes prior to LH (Table 21). Following exposure to LH, the mean secretion rate of B(-) follicles increased to maximal levels approximately 8 times greater than basal compared to a 4 fold increase of B(+) follicles. For all follicles, this rise in secretion rate was > 25 assay s.d. above basal secretion (range, 25-420 s.d.) for androstenedione and > 4 assay s.d. (range, 4-350 s.d.) for testosterone. A secretion increased above pre-LH levels during the first ten-minute period of LH infusion (B(-), $p < 0.001$; B(+), $p < 0.05$) but, in contrast to 'oestrogenic' follicles, increased T secretion ($P < 0.05$) was already evident in the first ten minute period of LH infusion.

Table 19 Correlations (r=Spearman correlation coefficient) between steroid production in perifusion and follicular parameters of 'oestrogenic' B(-) follicles ($n \leqslant 9$).

	E_2			A			T		
	Basal	Maximum	Total	Basal	Maximum	Total	Basal	Maximum	Total
Perifusion production									
Basal E_2 (pmol/min)	-	-	-	-	-	-	-	-	-
Maximum E_2 (pmol/min)	0.78**	-	-	-	-	-	-	-	-
Total E_2	-	-	-	-	-	-	-	-	-
Basal A (pmol/min)	0.85**	0.90**	-	-	-	-	-	-	-
Maximum A (pmol/min)	-	-	-	-	-	-	-	0.67*	0.67*
Total A	-	-	-	-	-	-	-	0.82**	0.79*
Basal T (pmol/min)	-	-	-	-	-	-	-	-	-
Maximum T (pmol/min)	-	-	-	-	-	-	-	-	-
Total T	-	-	-	-	-	-	-	0.98**	-
Antral fluid concentration									
E_2(nM)	0.59*	0.77**	0.58*	0.67*	-	-	-	-	-
A(nM)	-	-	-	-	0.64*	0.71*	-	0.63*	0.69*
T(nM)	-0.69*	-	-	-	-	0.67*	-	0.85**	0.81**
P(nM)	-	0.69*	0.60*	-	-	-	-	-	-
Inhibin (kU/ml)	-	-	-	-	-	-	-0.68*	-	-
Antral fluid content									
E_2(pmol)	0.62*	0.83*	0.60*	0.70*	-	-	-	-	-
A(pmol)	-	-	-	-	0.73*	0.80**	-	-	0.69*
T(pmol)	-0.69*	-	-	-	-	0.67*	-	0.85**	0.93**
P(pmol)	-	0.70*	-	-	-	-	-	-	-
Inhibin (kU)	-	-	-	-	-	-	-0.63*	-	-
Follicular characteristics									
Diameter	0.73*	0.84**	-	0.78**	-	-	-	-	-
No. granulosa cells	-	-	-	-	-	-	-	-	-
Thecal weight (mg)	0.60*	0.68*	-	0.78**	-	-	-	-	-

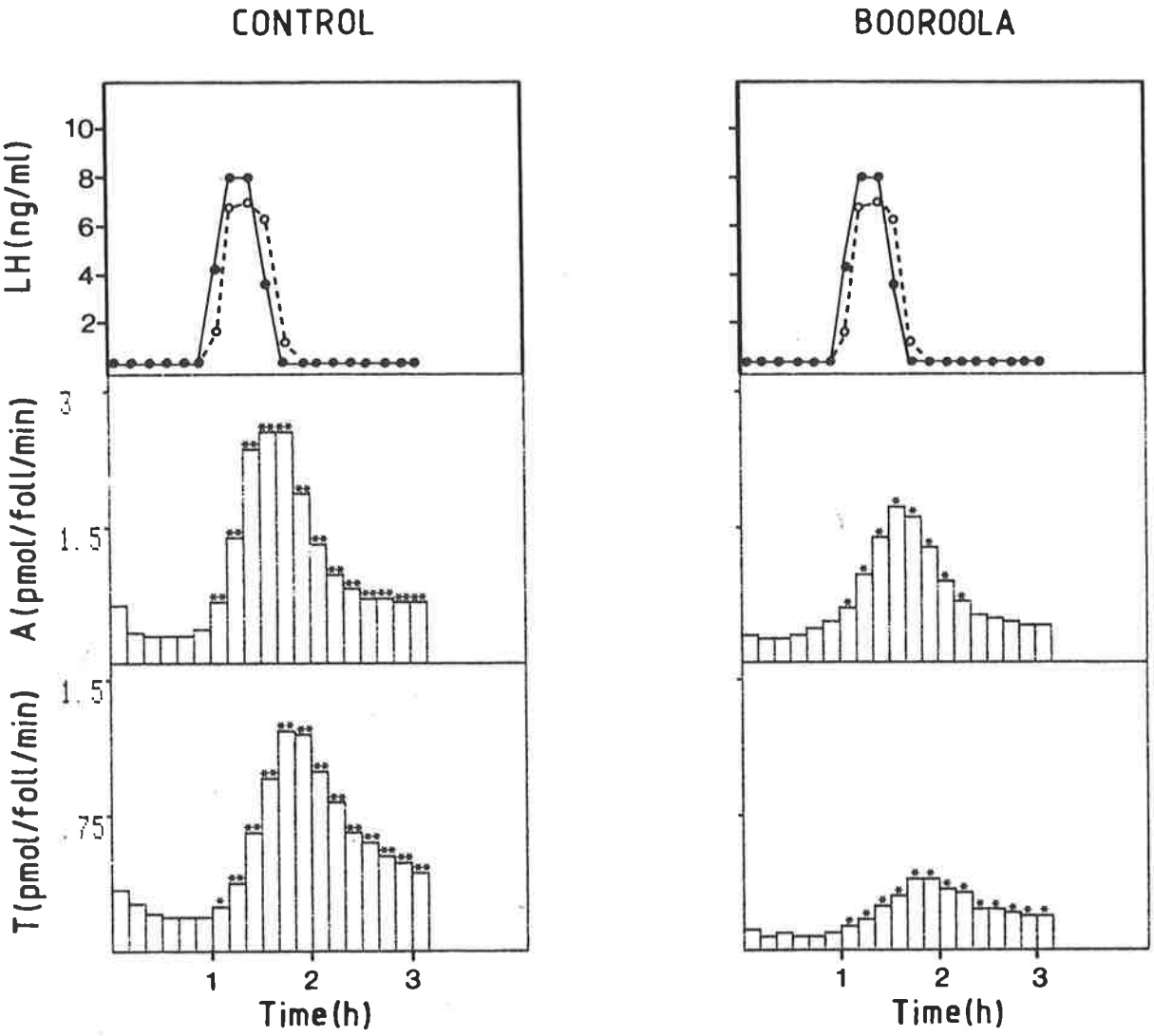
* $p < 0.05$ ** $p < 0.01$

Table 20 Correlations (r=Spearman correlation coefficient) between steroid production in perifusion and follicular parameters of 'oestrogenic' B(+) follicles ($n \leqslant 21$).

	E_2			A			T		
	Basal	Maximum	Total	Basal	Maximum	Total	Basal	Maximum	Total
Perifusion production									
Basal E_2 (pmol/min)	-	-	-	-	-	-	-	-	-
Maximum E_2 (pmol/min)	0.79**	-	-	-	-	-	-	-	-
Total E_2	-	-	-	-	-	-	-	-	-
Basal A (pmol/min)	-	-	-	-	-	-	-	-	-
Maximum A (pmol/min)	-	0.5*	0.5*	-	-	-	-	0.79**	0.88**
Total A	-	-	-	-	-	-	-	0.81**	0.94**
Basal T (pmol/min)	-	-	-	-	-	-	-	-	0.51*
Maximum T (pmol/min)	-	-	-	-	-	-	-	-	-
Total T	-	-	-	-	-	-	-	0.91**	-
Antral fluid concentration									
E_2(nM)	-	-	0.64**	-	-	-	-	-	-
A(nM)	-	-	-	-0.53**	-	-	-0.72**	-	-
T(nM)	-	-	-	-0.64**	-	-	-0.52*	-	-
P(nM)	-	-	0.55*	-	-	-	-	-	-
Inhibin (kU/ml)	-	-	-	-	-	-	-	-	-
Antral fluid content									
E_2(pmol)	-	0.58**	0.62**	-	-	-	-	-	-
A(pmol)	-	-	-	-0.52**	-	-	-0.53*	-	-
T(pmol)	-	-	-	-0.63**	-	-	-	-	-
P(pmol)	0.55*	0.61*	0.56*	-	-	-	-	-	-
Inhibin (kU)	-	-	-	-	-	-	-	-	-
Follicular characteristics									
Diameter	0.67**	0.77**	-	-	-	-	-	-	-
No. granulosa cells	-	-	-	-	-	-	-	-	-
Thecal weight (mg)	-	-	-	-	-	-	-	-	-

* $p < 0.05$ ** $p < 0.01$

Figure 8 Steroid secretion (A and T) by 'non-oestrogenic' B(-) (n=21) and B(+) (n=7) follicles during 3 h of perifusion. Values are the mean rate for each 10 min sample. The concentration of NIH-LH-S19 in the medium of small (●) and large (○) perifusion chambers is shown in the upper graph.
* $p < 0.05$, ** $p < 0.01$ indicate steroid secretion significantly higher than basal production prior to LH.


CONTROL
BOOROOLA
LH(ng/ml)
10
8
6
4
2
A(pmol/foll/min)
3
1.5
T(pmol/foll/min)
1.5
.75
1
2
3
Time(h)
1
2
3
Time(h)

Although the maximum A secretion rate of B(+) follicles was similar to B(-) follicles the total amount of steroid released per follicle in response to LH was less (Table 21).

Table 21 Steroid secretion in perifusion by 'non-oestrogenic' follicles isolated from the ovaries of control and Booroola Merino ewes (mean ±s.e.m. (median)).

Steroid	Control (n=21)	Booroola (n=7)	Significance of difference (B vs C)
Basal secretion prior to LH (pmole/follicle/min)			
A	0.35±0.06(0.26)	0.44±0.06(0.52)	n.s.
T	0.17±0.03(0.14)*	0.09±0.02(0.09)**	n.s.
Maximum secretion after LH (pmole/follicle/min)			
A	2.92±0.44(2.12)*	1.75±0.18(1.77)***	n.s.
T	1.31±0.25(0.73)*	0.42±0.10(0.37)	$p < 0.05$
Total steroid released per follicle (area units)			
A	12.5 ±2.03(10.3)	5.78±0.84(5.46)***	$p < 0.05$
T	6.83±1.66(3.82)	2.02±0.46(2.20)	$p < 0.05$
E_2+A+T	18.7 ±3.4 (13.8)*	7.8 ±1.2 (6.8)**	$p < 0.05$
Basal secretion after LH (pmol/follicle/min)			
A	0.67±0.10(0.49)	0.40±0.03(0.37)*	n.s.
T	0.47±0.09(0.38)**	0.18±0.04(0.17)*	$p < 0.05$

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Significantly different to 'oestrogenic' follicles of the same genotype (see Table 18).

The maximal and total production of testosterone of B(+) follicles in response to LH were also less than that of B(-) follicles ($p < 0.05$). The time from the introduction of LH to maximal steroid production was similar for both B(+) and B(-) follicles (A, 44.6 ± 1.4 min ; T, 58.2 ± 2.5 min ; A < T, $p < 0.001$).

In contrast to 'oestrogenic' follicles, the mean basal secretion rate of B(+) follicles after LH exposure was similar (A) or less than

(T, $p < 0.05$) that of B(-) follicles. After exposure to LH, the basal secretion rate of A and T by B(-) follicles remained above pre-LH levels (A, $p < 0.005$; T, $p < 0.05$). Only basal T secretion by B(+) follicles remained higher ($p < 0.05$) than pre-LH secretion.

Thecal weight per follicle did not differ between genotype in perifused 'non-oestrogenic' follicles (B(-), 4.0±0.59 mg, n=21 ; B(+), 2.33±0.37, n=6).

The significant correlations found between steroid production by 'non-oestrogenic' follicles in perifusion and other follicular parameters are given in Tables 22 and 23.

The steroid production in perifusion of 'non-oestrogenic' follicles was compared to that of 'oestrogenic' follicles within the same genotype (Tables 18 and 21). Decreased maximal secretion was the only characteristic of A secretion by B(-) 'non-oestrogenic' follicles which differed from 'oestrogenic' follicles. The maximal, total and basal secretion of A after LH by 'non-oestrogenic' B(+) follicles were all less than for 'oestrogenic' follicles. The basal T secretion rates of 'non-oestrogenic' follicles (B(-) and B(+)) were higher than 'oestrogenic' follicles, both before and after LH. Maximum T secretion and total T output after LH were similar in 'non-oestrogenic' and 'oestrogenic' B(+) follicles, as was the total T output by B(-) follicles, despite an increased maximal secretion by 'non-oestrogenic' follicles.

Although the initial rise in testosterone secretion produced by LH administration occurred earlier in 'non-oestrogenic' follicles, the time interval to reach maximal A and T secretion was longer in these follicles (A, $p < 0.05$; T, $p < 0.001$).

Table 22 Correlations (r, Spearman correlation coefficient) between steroid production in perifusion and follicular parameters of 'non-oestrogenic' B(-) follicles ($n \leqslant 21$).

	A			T		
	Basal	Max.	Total	Basal	Max.	Total
Perifusion production						
Basal A (pmol/min)	-	0.55**	-	-	-	-
Maximum A (pmol/min)	-	-	-	0.73**	0.70**	0.68**
Total A	-	0.96**	-	0.75**	0.77**	0.79**
Basal T (pmol/min)	-	0.73**	0.75**	-	-	-
Maximum T (pmol/min)	-	0.70**	0.77**	-	-	-
Total T	-	0.68**	0.79**	-	0.92**	-
Antral fluid concentration						
A(nM)	0.56**	0.54**	-	-	-	-
T(nM)	-	0.62**	0.72**	0.68**	0.77**	0.79**
Antral fluid content						
A(pmol)	0.79**	-	-	-	-	-
T(pmol)	0.70**	0.71**	0.68**	0.59**	-	-
Follicular characteristics						
Diameter (mm)	0.82**	-	-	-	-	-
No. granulosa cells	-	-	-	-	0.55**	-
Thecal weight (mg)	-	-	-	-	-	-

** $p < 0.01$

Table 23 Correlations (r, Spearman correlation coefficient) between steroid production in perifusion and follicular parameters of 'non-oestrogenic' B(+) follicles ($n \leqslant 7$).

	A			T		
	Basal	Max.	Total	Basal	Max.	Total
Perifusion production						
Basal A (pmol/min)	-	-	-	-	-	-
Maximum A (pmol/min)	-	-	-	-	-	-
Total A	-	0.86**	-	-	0.75*	0.71*
Basal T (pmol/min)	-	-	-	-	-	-
Maximum T (pmol/min)	-	-	0.75*	-	-	0.89**
Total T	-	-	0.71*	-	-	-
Antral fluid concentration						
A(nM)	-	-	-	0.76*	0.89**	0.71*
T(nM)	-	-	0.75**	0.74*	0.89**	0.75*
Antral fluid content						
A(pmol)	-	-	-	0.79*	-	0.75*
T(pmol)	-	0.86**	0.89**	-	0.75*	-
Follicular characteristics						
Diameter (mm)	0.71*	-	-	-	-	-
No. granulosa cells	-	-	-	-	-	-
Thecal weight (mg)	-	0.82*	0.98**	-	-	-

* $p < 0.05$, ** $p < 0.01$

D. Discussion

The perifusion technique described has the advantage of allowing controlled studies of dynamic changes in steroidogenesis by individual follicles, which is difficult to achieve *in vivo*. Previous estimates of oestrogen production by preovulatory ovine follicles *in vivo* have been obtained from the venous outflow of ovaries transplanted to the neck (Baird and Scaramuzzi, 1976a ; Baird *et al.*, 1976a) or *in situ* (McNatty *et al.*, 1981b, and c). Oestrogen secretion by perifused follicles was of the same magnitude as these estimates. The *in vivo* secretion rates of steroids in this experiment were calculated from the median concentrations in veins draining a single 'oestrogenic' follicle (Table 9, Chapter IV), assuming a preovulatory ovarian blood flow of 1-3 ml/min (Mattner and Thorburn, 1969 ; Bruce and Moor, 1976 ; Janson *et al*, 1983) and an haematocrit value of 30%. *In vivo*, 'oestrogenic' follicles were found to produce 2-5 pmol/min, a secretion rate comparable to that found *in vitro*.

Differences were, however, apparent in the estimates of androgen production by the two methods. In perifusion, the secretion rates of androstenedione and testosterone were less than the calculated production rates *in vivo* (A, 4-12 pmol/min ; T, 0.2-0.5 pmol/min). This finding supports the view that androgens secreted into the ovarian vein derive not only from preovulatory follicles but also from ovarian stroma and corpus luteum (Baird and Scaramuzzi, 1976b).

Comparison of the steroidogenic capacity of Booroola and non-Booroola follicles in perifusion revealed several interesting differences. Preovulatory Booroola follicles appeared to have achieved their full oestrogenic potential at a smaller size as throughout perifusion Booroola follicles produced more oestradiol per granulosa cell than did control follicles. In all follicles, oestrogen production

increased with follicle diameter, the basal secretion rate prior to LH indicating the capacity for increased E_2 output in response to LH. The enhanced oestrogen production per unit tissue mass *in vitro* explains how the Booroola follicle maintains antral fluid concentrations and ovarian venous secretion of E_2 at levels similar to the larger control follicle. However, it was only in control ewes that the antral fluid steroid concentration or content of follicles reflected their secretory capacity in perifusion.

Androgen production rates in perifusion also showed differences between genotypes. As might be expected from the reduced thecal weight of Booroola 'oestrogenic' follicles, the secretion of androgens before LH stimulation was less than control follicles. The negative correlation of this release rate with androgens in antral fluid suggests that thecal steroids are more readily released into the follicular blood supply than into the antrum. On exposure to LH, the androgen production of Booroola and control follicles was similar, despite marked differences in tissue weight, demonstrating an enhanced response to gonadotrophin in these follicles. The strong correlations between LH-stimulated androstenedione and testosterone secretion rates supports the view that these steroids exist in dynamic equilibrium in the ovine follicle (Seamark *et al.*, 1974). As correlations of oestrogen secretion with androgen substrate and thecal weight were only apparent in control follicles, the steroidogenic potential of the theca does not appear to be limiting in oestrogen biosynthesis of the Booroola follicle.

The basis of these apparent differences in steroidogenic capacity requires further study. Spearow (1984) has shown that one of the major genetic controls of ovarian responsiveness to gonadotrophins in mice is linked to a single gene and is probably mediated by changes in cAMP production. He also noted genetic differences in LH receptor number and

affinity between strains. Studies of thecal LH receptor populations and the dynamics of cAMP production by Booroola follicles are necessary to differentiate the site of enhanced responsiveness. In this regard, it is of interest to note that, following stimulation with LH, steroid secretion by Booroola follicles returned to baseline more slowly than that of control follicles. This finding suggests some difference in the control mechanism of steroid response, possibly in the decay of cAMP (phosphodiesterase?).

Whether the apparent differences in oestrogen secretion reflect quantitative or qualitative differences in enzyme activity or induced influences such as receptor content or cAMP release remains to be investigated. More detailed studies of the FSH-induction of aromatase activity in Booroola granulosa cells are reported in Chapter VI. Although high doses of LH inhibit oestrogen production by ovine follicles (Moor, 1974), it is not known whether lower doses might stimulate aromatase activity of granulosa cells, as has been demonstrated in the rat (Erickson et al., 1979 ; Wang et al., 1981). If so, the enhanced response of the Booroola follicle to LH might extend to the aromatase system. However, as thecal cells may contribute significantly to oestrogen synthesis in large ovine preovulatory follicles (Armstrong et al., 1981), the possibility that an increased proportion of oestrogen derives from this source in the Booroola follicle cannot be discounted.

There are few assessments of steroid production by 'non-oestrogenic' follicles. Although 'non-oestrogenic' follicles responded to LH stimulation with increased steroidogenesis, the total steroid output per follicle was less than 'oestrogenic' follicles, as would be expected for small growing or atretic follicles (Moor et al., 1978). The low aromatase activity of these follicles resulted in testosterone

secretion initially rising more rapidly. The longer time to maximal androgen secretion in 'non-oestrogenic' follicles suggests that, in ovine preovulatory follicles, high oestradiol concentrations inhibit the synthesis of androgens as has been found in the rat (Leung et al., 1978 ; Evans et al., 1981), thus regulating their own production via an intraovarian short-loop feedback mechanism.

In contrast to the production rates of 'oestrogenic' follicles 'non-oestrogenic' Booroola follicles, of similar thecal mass to control follicles, secreted less androgen in perifusion. This result provides further evidence that the enhanced steroidogenic capacity of the Booroola follicle only occurs during preovulatory development. As the steroidogenic response per unit mass of bovine theca did not vary with follicle size but decreased with atresia (McNatty et al., 1984 ; Henderson et al., 1984), a higher incidence of atresia might have been present in Booroola follicles. A correlation was demonstrated between LH-stimulated androstenedione production and thecal weight in Booroola 'non-oestrogenic' follicles, but not in 'oestrogenic' follicles. This suggests that atresia has decreased the steroidogenic capacity of thecal cells. However, the finding that the steroid output of all 'non-oestrogenic' follicles significantly increased in response to LH, albeit by lesser amounts than 'oestrogenic' follicles, contrasts with the results of McNatty et al. (1984) in bovine theca. In perifusion, theca isolated from bovine follicles did not respond to LH with increased androgen production.

CHAPTER VI

ENDOCRINE PARAMETERS OF THE PREOVULATORY FOLLICLE OF THE BOOROOLA MERINO : 3. *IN VITRO* AROMATASE ACTIVITY OF GRANULOSA CELLS

A. Introduction

There is little information available on oestrogen production in the Booroola Merino. The failure of peripheral oestradiol concentrations to increase with the more numerous follicles of Booroola ewes (Scaramuzzi *et al.*, cited by Bindon and Piper, 1982b) and the report of decreased numbers of granulosa cells per follicle (Scaramuzzi and Turnbull, cited by Bindon and Piper, 1982b) suggest that the capacity for oestrogen synthesis might be reduced in the Booroola follicle.

It is accepted that the granulosa cells of preovulatory follicles are the prime source of oestrogen during the follicular phase of the ovine oestrous cycle (Moor, 1977). Although thecal preparations obtained from large, preovulatory follicles secrete significant amounts of E_2 *in vitro*, the extent of thecal contribution to oestrogen production *in vivo* is unknown (Armstrong *et al.*, 1981). Granulosa cells are unable to synthesize large amounts of androgen when compared with theca (eg. Seamark *et al.*, 1974) as they lack the $C_{17,20}$-lyase enzymes necessary to produce androgens from C_{21} precursors (Short, 1962 ; Fortune and Armstrong, 1977). However, granulosa cells contain aromatase activity and thus, can readily convert androgens to oestrogens (Moor, 1977), under the influence of FSH (McNatty, 1982). These androgens are supplied by the LH-stimulated thecal layer, although it is unclear whether testosterone or androstenedione is the major precursor for oestrogen production (Rado *et al.*, 1970 ; Moor, 1977 ; Scaramuzzi *et al.*, 1980a). Thus, in culture, granulosa cells must be supplied with substrate to measure their capacity to aromatise androgen to oestradiol, the

predominant oestrogen secreted by ovine follicles *in vivo* (Moore *et al.*, 1969).

The induction of aromatase activity by FSH has been demonstrated both *in vivo* and *in vitro* in the rat (Erickson and Hsueh, 1978a) and in ovine follicles < 5 mm diameter *in vitro* (McNatty, 1982). In this study the aromatase activity of granulosa cells isolated from control and Booroola preovulatory follicles was determined, both with and without FSH stimulation.

B. Materials and Methods

1. Granulosa cell culture

Following determination of the total granulosa cell number per follicle (Chapter IV), all cells from each follicle were further diluted with incubation medium (medium D, see II.D.1), resulting in a suspension of 1-12 x 10^4 cells per 0.1 ml aliquot. Each aliquot was added to a single well (17 x 16 mm) of a sterile plastic Linbro multiwell tissue culture plate (Flow Laboratories, Stanmore, N.S.W., Australia), containing 0.9 ml of incubation medium. The cells were cultured at 37°C for 48 h in humidified air, either without substrate or with 0.35 µM or 3.5 µM androstenedione, each level of substrate in the presence or absence of 0.2 µg/ml NIH-FSH-S12 (McNatty, 1982). These six treatment groups were cultured in triplicate for every follicle. After 48 h, the medium was aspirated from the cells and stored frozen until assayed for oestradiol.

2. Preparation of hormones

NIH-FSH-S12 was dissolved in sterile Dulbecco's phosphate-buffered saline containing 0.1% BSA at a concentration of 1 mg/ml and stored

frozen. Immediately prior to use, an aliquot of this solution was thawed and diluted in medium D to yield a concentration of 0.2 µg/ml.

Androstenedione was dissolved in absolute alcohol at a concentration of 1 mg/ml. Dilutions of this stock were made with medium D immediately before use. The alcohol content of the medium was 0.04% at 0.35 µM A and 0.4% at 3.5 µM A.

3. Steroid Radioimmunoassay

Oestradiol and oestrone in the incubation medium were measured by radioimmunoassay after chromatographic separation of the solvent-extracted steroid fraction (see II.3.e). In 70 samples, the levels of oestrone were < 5 pmol/10^6 cells/48 h, not varying with A concentration and, therefore, assaying this fraction was discontinued.

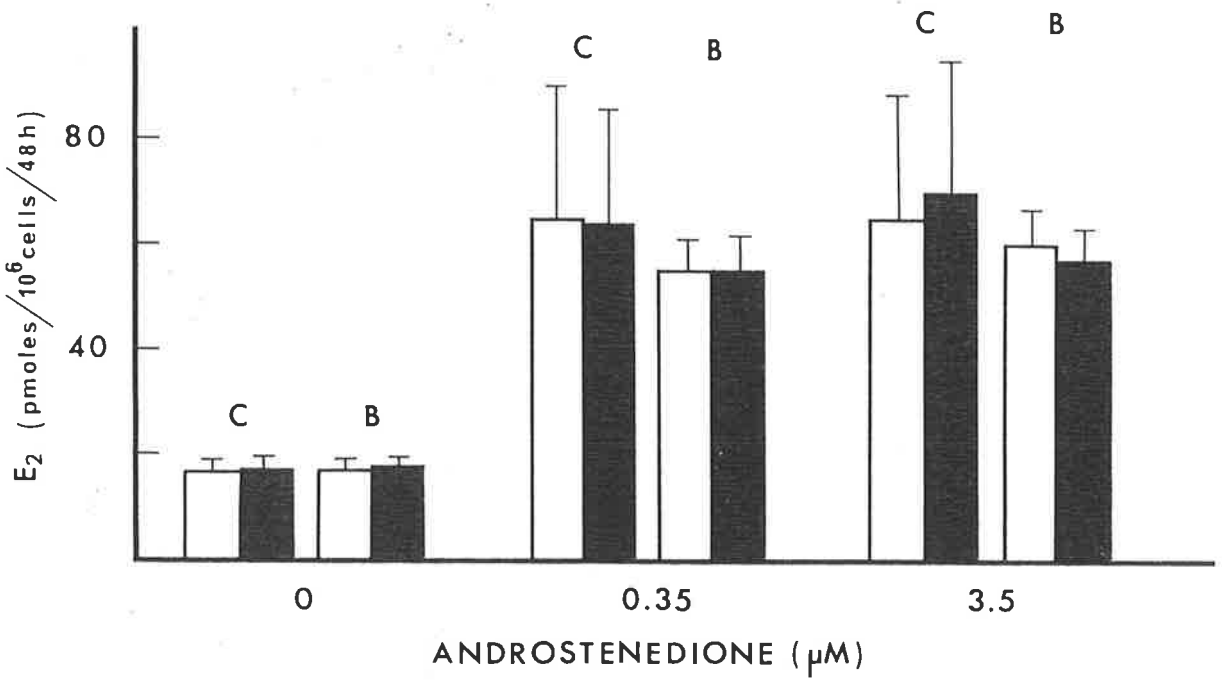
The production of oestradiol per 10^6 cells during 48 h of incubation was used as the index of aromatase activity. The mean value of steroid results from the triplicate cultures for each treatment group per follicle was used for all statistical evaluations.

C. Results

No oestradiol (< 10 pmol/10^6 cells/48 h) was detected in culture medium incubated without cells or with cells isolated from follicles with antral fluid oestradiol concentrations < 1000 nM. All 'oestrogenic' follicles, except the follicle with the lowest antral fluid oestradiol concentration of 1308 nM, produced oestradiol in culture. The results from this one follicle are included in the data at a value equal to the sensitivity of the assay (2.6 pmol/10^6 cells/48 h).

Granulosa cells cultured in medium containing no substrate, with or without FSH, produced low, but detectable levels of oestradiol (Figure 9). The addition of 0.35 µM androstenedione to the medium increased the

Figure 9 In vitro aromatase activity of granulosa cells isolated from 'oestrogenic' control (C, n = 9) and Booroola (B, n = 21) follicles. Values represent mean + s.e.m. Granulosa cells from each follicle were cultured at three levels of substrate, with (solid histograms) or without (open histograms) NIH-FSH-S12 (0.2 µg/ml).


C
B
C
B
C
B
80
40
E_2 (pmoles/10^6cells/48h)
0
0.35
3.5
ANDROSTENEDIONE (μM)

oestradiol production of cells from all follicles (B(-), $p < 0.01$; B(+), $p < 0.0005$). Increasing androstenedione concentration ten-fold to 3.5 µM, increased oestradiol production by granulosa cells from B(+) follicles significantly ($p < 0.001$), but did not alter the production by control follicles.

The inclusion of NIH-FSH-S12 (0.2 µg/ml) did not, at any substrate concentration, significantly stimulate *in vitro* aromatase activity above non-FSH levels, in either B(-) or B(+) cells.

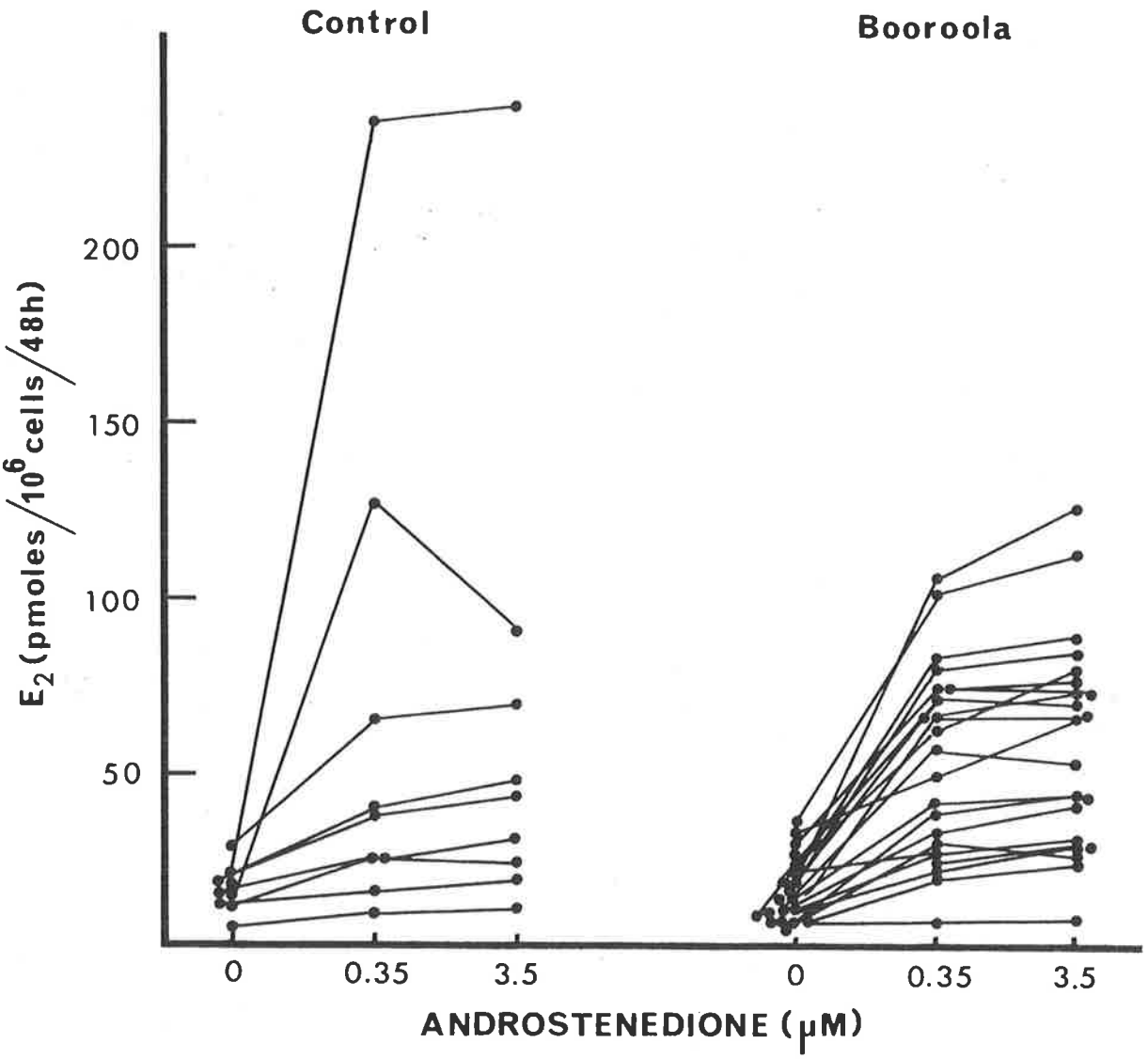
There was no significant difference in the *in vitro* aromatase activity of control and Booroola granulosa cells in any treatment group. A wide range in the aromatase activity of cells isolated from different follicles was found, particularly in control ewes (Figure 10).

The only correlation of maximal oestradiol production by granulosa cells in culture was that with androstenedione concentration in Booroola follicles ($r = 0.67$, $p < 0.005$).

D. Discussion

The *in vitro* aromatase activity of granulosa cells isolated from 'oestrogenic' follicles was similar to that found by McNatty (1982). As shown previously (e.g. Moor, 1977), provision of substrate (0.35 µM androstenedione) increased the *in vitro* oestradiol production by granulosa cells approximately three-fold over that without substrate. In Booroola, but not control cells, a further rise in oestradiol production occurred when substrate concentration was increased, indicating that aromatase might be limiting in control cells. However, the *in vitro* aromatase activity of cells did not differ between genotypes at any level of substrate. Thus, no conclusions can be drawn from this result about how preovulatory Booroola follicles secrete oestradiol *in vivo* - as demonstrated by antral fluid and ovarian venous

Figure 10 Individual changes in oestradiol production of granulosa cells isolated from control and Booroola follicles cultured in medium containing no substrate, 0.35 µM androstenedione or 3.5 µM androstenedione. Values are the mean of triplicate cultures.


Control
Booroola
200
150
100
50
E_2 (pmoles/10^6cells/48h)
0
0.35
3.5
0
0.35
3.5
ANDROSTENEDIONE (μM)

concentrations - and *in vitro* - in perifusion - at a rate comparable to the control follicle, when it contains one-half the number of granulosa cells. Measurement of extant aromatase activity at shorter incubation times with a range of substrate concentrations, as well as determination of thecal oestrogen production may resolve this question.

The total number of granulosa cells contained in 'oestrogenic' follicles per ewe was also similar for both genotypes (Chapter IV). Follicular oestradiol production and peripheral concentrations would, therefore, also be expected to be the same, provided granulosa cells are the major source of oestradiol during the follicular phase of the oestrous cycle.

FSH has been shown to stimulate *in vitro* aromatase activity in granulosa cells isolated from ovine follicles < 5 mm diameter (McNatty, 1982). In the present study, this effect was not evident. Maximal FSH stimulation of these preovulatory follicles may have occurred *in vivo*, so that further FSH stimulation of aromatase *in vitro* was not possible. The correlation between oestradiol production in culture and androstenedione concentration in antral fluid supports the view that androgens not only act as substrates for aromatase activity but can also increase the FSH-induced activity of the enzyme directly and, after conversion to oestradiol (Daniel and Armstrong, 1980).

The low aromatase activity of granulosa cells isolated from follicles with antral fluid E_2 concentrations < 1000 nM and decreased numbers of granulosa cells which were morphologically assessed as Stage III or IV, confirms their atretic state. Follicles deficient in granulosa cells have been shown to contain low levels (< 100 ng/ml, or 367 nM) of oestradiol (McNatty *et al.*, 1982) and the granulosa cells isolated from these follicles are unable to produce > 3 ng (11 pmol)/10^6 cells/48 h *in vitro* (McNatty, 1982). At approximately 3-4 mm, healthy,

growing follicles emerge from an androgen-dominant phase as aromatase activity and thus, intrafollicular oestrogen levels being to increase with preovulatory follicle enlargement (Carson et al., 1981 ; McNatty, 1982). Therefore, it is still not possible to determine whether the 3-4 mm follicles (7 B(-) ; 1 B(+)) with low antral fluid E_2 concentrations which were morphologically classified as healthy and exhibited little aromatase activity were in the early stages of atresia or growth. Follicles in this category but > 4 mm diameter (2 B(-) ; 1 B(+)) were most likely to be atretic.

CHAPTER VII

THE RESPONSE OF GONADOTROPHIN LEVELS IN OVARIECTOMIZED CONTROL AND BOOROOLA MERINO EWES TO THE FEEDBACK EFFECTS OF OESTRADIOL

A. Introduction

The hypothalamic-pituitary axis of Finn and Romanov ewes exhibits a decreased sensitivity to the feedback effects of oestrogen (Land et al., 1976 ; Cahill et al., 1981). However, it is not resolved whether this characteristic is a cause or an effect of increased fecundity. Land et al. (1976) have proposed that the tolerance of higher E_2 levels in high fecundity ewes would allow increased follicular recruitment before gonadotrophin levels become limiting. Increased gonadotrophin concentrations during preovulatory follicular development have not, however, been found in these ewes. With similar levels of gonadotrophins being reported in ewes of widely varying ovulation rate, it is difficult to see how the sensitivity of gonadotrophin secretion to oestrogen can determine ovulation rate.

Bindon et al. (1978) concluded that Booroola Merino ewes do not exhibit a reduced sensitivity to the positive feedback effect of oestradiol since the time interval from the onset of oestrus to the preovulatory LH discharge was similar to that of control ewes. Their conclusion was based on the assumption that less oestradiol is secreted by each preovulatory Booroola follicle. The finding that LH levels declined similarly in long term ovariectomized control and Booroola Merino ewes implanted with oestradiol, just prior to and during anoestrus, suggests that no difference exists between the two genotypes in the negative feedback effects of oestradiol (Cummins et al., 1982). As there is evidence that the hypothalamic-pituitary axis becomes less sensitive to the inhibitory effects of steroids with time after

ovariectomy (Karsch et al., 1979), subtle differences operating during the normal oestrous cycle might not be reflected in these ewes. Thus, the changes in gonadotrophin levels were compared in control and Booroola Merino ewes implanted with oestradiol at ovariectomy during the breeding season.

B. Materials and Methods

1. Experimental design

Ovariectomy was performed 36 ±1 h after injection of a synthetic prostalandin, cloprostenol during the mid-luteal phase of the oestrous cycle (Chapter IV). At the time of ovariectomy all 20 ewes were given subcutaneous implants containing oestradiol (Steraloids, Wilton, N.H, U.S.A.). Ewes were maintained indoors, subject to lighting similar to the external environment, and fed a diet of lucerne hay, in the absence of rams. Blood was sampled through jugular cannulae every 4 h, except at night when samples were taken 8 hourly, for 56 h and then daily at 3,4,5,6 and 7 days post-ovariectomy. Plasma was immediately separated by centrifugation and stored at -20°C until assayed for LH and FSH, together with those samples taken -48, -24 h prior to and at ovariectomy.

2. Oestradiol implants

The silastic implants were constructed as described by Martin et al. (1983). The rate of release of oestradiol from the implants was measured in vitro. To ensure constant secretion of steroid, 9 implants were preincubated for 8 days in 20 ml 0.14 M saline, changed daily and maintained at 37°C in a shaking water bath. The implants were then transferred to 20 ml pooled ewe plasma containing 0.1% sodium azide, and

samples (0.5 ml) taken at 30 min, 1 h, 2 h, 4 h and 6 h during incubation in a shaking water bath at 37°C. The oestradiol content of these samples was measured by radioimmunoassay after extraction (see II.B.3f(i)), and used to calculate the release rate per implant. For the 9 implants, the mean release rate of oestradiol was 10.2±1.6 nmol/day *in vitro*. Prior to implantation each implant was soaked overnight in 0.14 M saline and then sterilized in 70% ethanol.

3. Gonadotrophin assays

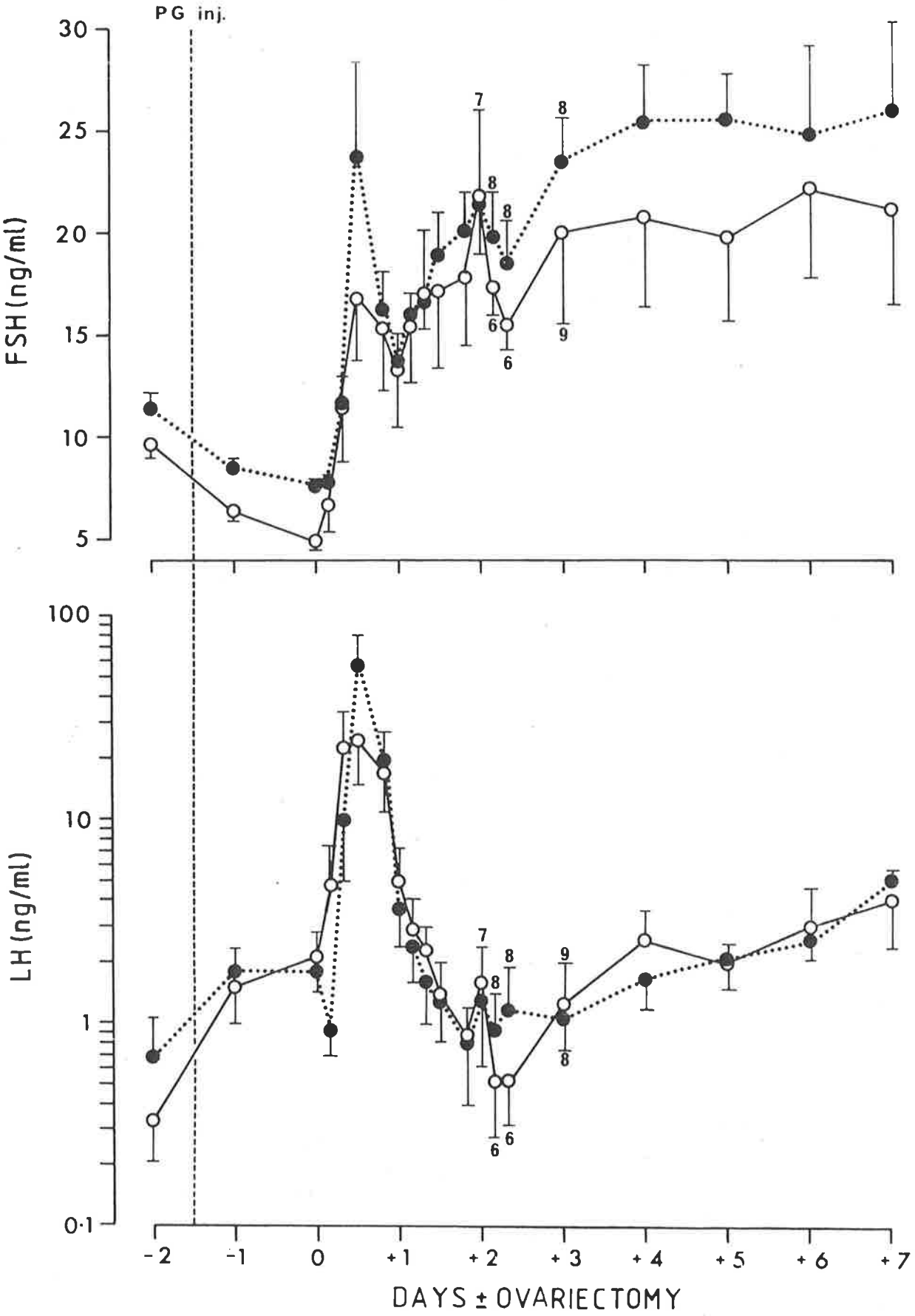
The assistance of Dr. Rex Scaramuzzi (CSIRO Division of Animal Production, Prospect, N.S.W.) in assaying LH in the samples of this experiment is gratefully acknowledged. A specific double-antibody radioimmunoassay (Martensz *et al.*, 1976) was used with ovine NIH-LH-S14 as the reference standard and an ovine LH preparation, provided by Dr. L.E. Reichert Jr, for iodination.

FSH was measured in duplicate aliquots of all samples with a radioimmunoassay as described by Bremner *et al.* (1980) in the laboratory of Dr. J.K. Findlay, whose assistance is gratefully acknowledged. The sensitivity of the assay, calculated according to Burger *et al.* (1972), was 2.28±0.11 ng/tube using NIH-FSH-S13 as the standard hormone preparation. The initial 5 samples from each ewe were assayed at a volume of 300 µl ; the remainder of the samples from each ewe in one assay at 100 µl. The within-assay CV was < 16% for all samples. The between assay CVs were 13.8% at 14.9 ng/ml, 5.0% at 31.0 ng/ml and 6.9% at 92.1 ng/ml for 4 assays.

C. Results

Plasma concentrations of LH and FSH in the luteal phase prior to prostaglandin injection and during luteolysis before ovariectomy were similar in control and Booroola ewes (Figure 11). By 12 h after

Figure 11 Concentrations of LH and FSH in peripheral plasma of control (○) and Booroola (●) Merino ewes. Ewes were implanted with oestradiol at ovariectomy, performed 36 ±1 h after injection of prostaglandin (indicated by the vertical broken line) during the mid-luteal phase of the oestrous cycle. Each point represents the mean ±s.e.m. of observations on 10 ewes, unless n is indicated for that point.


PG inj.
FSH (ng/ml)
30
25
20
15
10
5
LH (ng/ml)
100
10
1
0·1
-2
-1
0
+1
+2
+3
+4
+5
+6
+7
DAYS ± OVARIECTOMY

prostaglandin injection the mean LH concentration in all samples had risen significantly ($p < 0.001$) above luteal phase levels. While progesterone levels had shown a further decrease at ovariectomy, approximately 37 h after prostaglandin (see IV.C), mean LH concentrations remained constant. In contrast, by 12 h after prostaglandin, the mean plasma FSH concentrations of all ewes were less than luteal phase levels ($p < 0.001$), and had decreased further ($p < 0.001$) by ovariectomy.

The onset of the LH surge was defined as an LH concentration > 10 ng/ml followed by increasing LH levels. An LH discharge was considered to have occurred when concentrations exceeded 15 ng/ml. Two of the ten control ewes and one of the ten Booroola ewes did not exhibit an LH discharge after ovariectomy. There was no difference between the remaining control and Booroola ewes in the time from prostaglandin injection (or ovariectomy) either to the onset of the LH discharge or to the maximum concentration of LH reached during the discharge (Table 24). Maximum plasma concentrations of LH and FSH were similar in both genotypes (Table 25). As night-time sampling was less regular it was not possible to measure the area of the gonadotrophin discharges.

Table 24 Interval (h) from prostaglandin administration to the LH discharge of control and Booroola Merino ewes which were ovariectomized and implanted with oestradiol 36±1 h after prostaglandin injection during the mid-luteal phase of the oestrous cycle (mean ±s.e.m.).

Interval	Control (n = 8)	Booroola (n = 9)	Significance of Difference
To onset (>10 ng/ml)	49.5±2.4	49.9±1.5	n.s.
To maximum concentration	52.0±2.0	51.7±1.3	n.s.

Table 25 Maximum LH and FSH concentrations (ng/ml) in plasma during the discharge following ovariectomy and oestradiol implantation 36±1 h after prostaglandin injection during the mid-luteal phase of oestrous cycle of control and Booroola Merino ewes (mean ±s.e.m. (median)).

Hormone	Control (n = 8)	Booroola (n = 9)	Significance of Difference
LH	59.9±9.6(48.4)	74.3±24.3(56.5)	n.s.
FSH	21.3±3.2(22.1)	26.9± 4.6(20.4)	n.s.

Following the preovulatory discharge, peripheral FSH and, after an initial plateau, LH levels continued to rise. (Figure 11). Thus, the release rate of oestradiol by the implants was sufficient to induce a preovulatory discharge, but did not result in total suppression of gonadotrophin secretion. Linear regression analysis conducted on the median LH concentrations in samples taken 56 h and 3-7 days post-ovariectomy revealed no difference between control and Booroola ewes in the rate of increase of LH.

D. Discussion

The results of this experiment confirm the view that no difference exists between control and Booroola Merino ewes in the sensitivity of the hypothalamic-pituitary axis to the feedback effects of oestradiol. When control and Booroola ewes were implanted with similar levels of oestradiol, the positive feedback response did not differ between genotypes, as demonstrated by a similar time interval to the oestrogen-induced gonadotrophin discharge. As the rate of oestradiol release by the implants did not fully suppress gonadotrophin secretion, a gradual rise in LH levels followed the discharge. The similarity of this rate of increase in all ewes demonstrated that no difference existed between

genotypes in the negative feedback response of LH secretion to oestradiol.

The previous report of Cummins et al. (1982) demonstrated a far greater suppression of LH levels in oestradiol-implanted ovariectomized ewes. During anoestrus, the hypothalamic-pituitary axis is thought to become more sensitive to the negative feedback effects of oestradiol (Legan and Karsch, 1979) hence, a greater response would be expected at this time. Another reason for the disparity is likely to be the difference in the level of oestradiol used in each experiment. The implants used by Cummins et al. released 25-37 nmol/day in vitro (Karsch et al., 1973), while those used in this study released approximately 10 nmol/day. Despite these differences, both studies found that control and Booroola Merino ewes responded similarly to the negative feedback effect of oestradiol. Thus, it would seem that a difference in the sensitivity of the hypothalamic-pituitary axis does not contribute to the high ovulation rate of the Booroola Merino.

CHAPTER VIII

PERIPHERAL GONADOTROPHIN LEVELS DURING THE PERIOVULATORY PERIOD OF CONTROL AND BOOROOLA MERINO EWES

A. Introduction

It is not known whether increased gonadotrophin levels contribute to the high fecundity of the Booroola ewe. Frequent sampling throughout the periovulatory period in Romanov (Bindon et al., 1979) and Finn ewes (Webb and England, 1982a) revealed no differences in gonadotrophin concentrations which could be correlated with ovulation rate. The role of increased post-ovulatory FSH secretion which has been found in Romanov ewes (Cahill et al., 1981) was questioned in Chapter III. However, it is well known that administration of exogenous PMSG or FSH during the late luteal or follicular phase of the oestrous cycle induces superovulation (Bindon and Piper, 1982a ; Wright et al., 1981). This finding suggests that increased levels of circulatory gonadotrophin might occur in high fecundity ewes. To explore the significance of peripheral FSH levels, plasma gondadotrophin concentrations of control and Booroola Merino ewes were studied in samples taken 2 hourly just before and during the periovulatory phase of the oestrous cycle.

The essential role of LH in the final stages of preovulatory follicular development has been discussed in Chapter I. LH pulse frequency increases from approximately 1 pulse per 3 h during the luteal phase to > 1 per hour during the late follicular phase (Baird and McNeilly, 1981). In steroid-immunized ewes with increased ovulation rates, LH pulse frequency is enhanced (Scaramuzzi, 1979). The characteristics of pulsatile gonadotrophin secretion of both genotypes were, therefore, determined during the luteal and follicular phases of the oestrous cycle.

B. Materials and Methods

1. Animals

The animals for this experiment were kindly provided by the South Australian Department of Agriculture. The flock was initially created by crossing Booroola rams with South Australian Merino ewes (Ashrose). The female progeny of this cross were then mated to South Australian Merino rams (Ashrose). From the resulting progeny, 10 ewes, aged 2 years, were chosen for the experiment. On the basis of litter size records $\geqslant 3$ in the dam and ovulation rates $\geqslant 3$, assessed by repeated laparoscopy, in both the dam and the ewe herself, four ewes were classified as carrying the Booroola gene (B(+) ; either homozygous or heterozygous). 6 control ewes (B(-)) with ovulation rates of 1 or 2 in their own and their dam's records were chosen as controls.

These ewes were maintained on pasture at Mortlock Experiment Station, near Clare, South Australia during the breeding season (March to May), in the presence of raddled, vasectomized rams. All ewes were recorded in oestrus following the injection of 100 µg cloprostenol i.m. on days 5-14 of the oestrous cycle. Six days after prostaglandin injection the ewes were transferred to pens in the animal house at the Queen Elizabeth Hospital, where they were maintained on lucerne chaff in lighting similar to the external environment.

2. Experimental design

8 days after prostaglandin injection one jugular vein in each ewe was cannulated with either a commercial catheter (Angiocath, The Deseret Co., Utah, U.S.A.) or with sterile, pre-washed silastic tubing (0.76 mm i.d., 1.65 mm o.d. ; Dow-Corning Corp, U.S.A.) inserted 20 cm into the vein via a 13G stainless steel needle, which was then removed. Each

cannula was extended with silastic tubing attached to a 3-way stopcock (Top Surgical Mfg. Co., Tokyo, Japan) fastened to the dorsal surface of the head of the ewe. The cannulae were filled with sterile, heparinized saline (50 i.u./ml) containing 20 µg/ml antibiotic (amoxycillin sodium ; CSL, Melbourne, Australia), and the animals returned to pens.

Beginning on day 9±1 of the induced oestrous cycle, 5 ml samples of jugular venous blood were collected every 2 hours for 8 h prior to and 96 h after a second i.m. injection of 100 µg cloprostenol. Samples were collected more frequently (every 15 min) for 2 periods of 6 h during the luteal and follicular phases of the cycle i.e. -6 to 0 h and 24-30 h after injection of cloprostenol.

The heparinized blood samples were centrifuged immediately and the aspirated plasma stored at -20°C until analysis. A blood sample was taken 13 days after prostaglandin injection to ascertain, by progesterone concentration, if ovulation had occurred in all ewes.

3. Hormone assays

LH and FSH were measured in duplicate aliquots of each sample by radioimmunoassay as described in II.C. All samples from one ewe were included in a single assay.

Plasma progesterone concentrations were determined by radioimmunoassay as previously described (II.B.3(a)) only in the samples taken 2 hourly from -8 to +24 h and at 36, 48, 60, 72, 84 and 96 h after prostaglandin injection.

4. Analysis of hormone profiles

The values of LH and FSH for each ewe were initially alligned to the injection of prostaglandin and then to the peak concentration of LH during the preovulatory discharge. As the mean time to the LH peak was

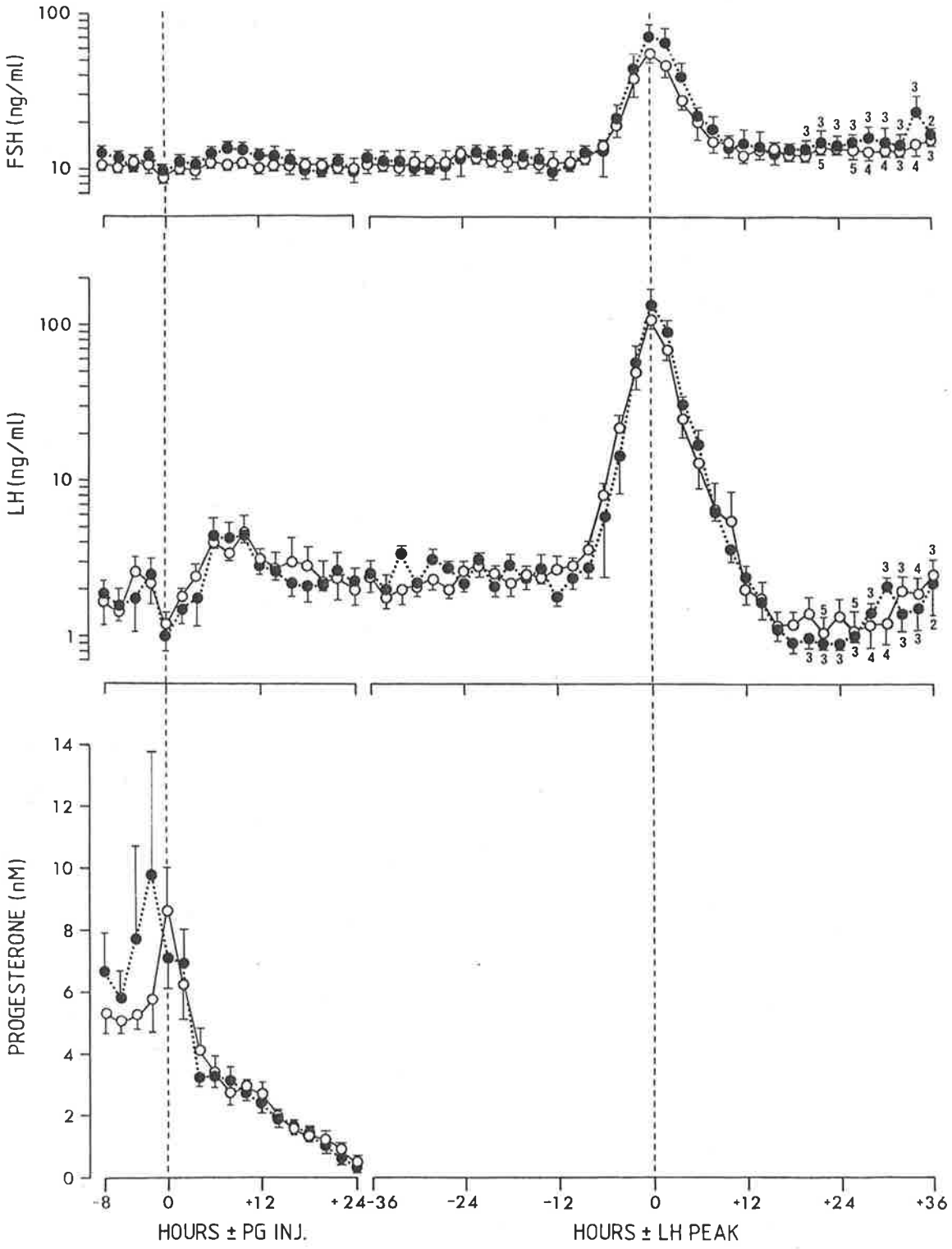
60 h (range, 46-76 h), the mean results illustrated in Figure 12 accurately represent the time-scale, in spite of the break in the ordinate.

Luteal phase comparisons between control and Booroola ewes were carried out on the mean progesterone concentration of the 2 hourly samples and the mean LH and FSH concentrations of all 15 min samples taken during the 6 h prior to cloprostenol injection.

For the periovulatory period after cloprostenol injection, the hormonal characteristics considered were: (1) the area under the LH, FSH and progesterone curves in the 24 h after cloprostenol injection; (2) the area under the LH and FSH curves corresponding to the time periods -36 to 6 h and -6 to +6 h of the preovulatory discharge ; (3) the mean LH and FSH concentrations +6 to +36 h after the LH surge (missing values due to the allignment to the LH peak made comparable area calculation not possible) ; (4) the maximum LH and FSH concentration reached during the preovulatory discharge. The time intervals considered were those between cloprostenol injection and the onset (defined as > 10 ng/ml) and maximum concentration of the LH surge.

Pulse frequency and amplitude during the 2 periods of intensive sampling were determined by computer, utilizing the program 'PULSAR' as described by Merriam and Wachter (1982). This program first calculates base line secretion using a weighted moving-average procedure, which removes long-term trends. Pulses are identified by both the number of elevated points and their distance above the baseline. Using the assay s.d. calculated from all replicates in the profile, a pulse was defined as having occurred if 1 point was elevated by 3.8 s.d., 2 consecutive points by 2.6 s.d., 3 points by 1.9 s.d., 4 points by 1.5 s.d. and 5 points by 1.2 s.d. The mean LH and FSH level of all samples in each profile, as well as the basal levels of each hormone over which the

Figure 12 Concentration of progesterone, LH and FSH in jugular venous plasma of control (O) and Booroola (●) Merino ewes during the periovulatory period. The results have been grouped around the injection of synthetic prostaglandin (PG) and the peak LH value. Each point represents the mean ± s.e.m. of observations on 6 control and 4 Booroola ewes, unless n is stated for that point.


FSH (ng/ml)
100
10
LH (ng/ml)
100
10
1
PROGESTERONE (nM)
14
12
10
8
6
4
2
0
-8
0
+12
+24
-36
-24
-12
0
+12
+24
+36
HOURS ± PG INJ.
HOURS ± LH PEAK

pulses had been superimposed, estimated as the mean of the 5 lowest samples, were compared between control and Booroola ewes.

C. Results

There were no significant differences between control and Booroola ewes in any of the parameters of gonadotrophin secretion measured during the luteal phase (Table 26). The individual gonadotrophin profiles of each ewe during the period of frequent sampling are shown in Figures 13 and 14. The mean progesterone concentration of both genotypes was similar (B(-), 5.38±0.53 nM ; B(+), 6.91±1.80 nM).

During the 24 h after prostaglandin injection, the area under the curves of LH, FSH and progesterone secretion were similar in all ewes (Figure 12, Table 27). By 2 h after cloprostenol, progesterone concentrations in all ewes had decreased ($p < 0.05$), and both LH and FSH levels had risen (LH, $p < 0.01$; FSH, $p < 0.01$). Plasma progesterone levels continued to decrease to $\leqslant$ 0.5 nM in all ewes by 36 h after prostaglandin, a level which was maintained for the next 60 h. LH concentrations remained above luteal phase levels throughout luteolysis, while FSH, after being elevated for 10 h, returned to luteal phase levels by 24 h after prostaglandin.

The pulsatile secretion of LH during the follicular phase of the oestrous cycle did not differ between control and Booroola ewes (Figures 15 and 16 ; Table 26). The only characteristic of FSH secretion which differed between genotype was that of pulse frequency which was decreased in Booroola ewes($p < 0.05$). In control ewes, but not Booroola ewes, the frequency of both LH and FSH pulses had increased from luteal phase levels (LH, $p < 0.05$; FSH, $p < 0.05$). Pulse amplitude and gonadotrophin levels did not differ significantly with the phase of the cycle within each group of ewes, but when the data from all ewes was

Table 26 Characteristics of the pulsatile secretion of LH and FSH of control (n=6) and Booroola (n=4) Merino ewes in the luteal and follicular phase (+24 h after cloprostenol injection) of the oestrous cycle. Values are mean ±s.e.m. (median) except for pulse frequency where median and range are quoted.

	Control		Booroola	
	LH	FSH	LH	FSH
Luteal phase				
Pulses/6 h	3(2-4)	0.5(0-2)	2(1-4)	0.5(0-2)
Pulse amplitude (ng/ml)	2.42 ±0.23 (2.27)	3.0 ±0.39 (2.94)	2.41 ±0.67 (1.42)	5.16, 3.01
Mean level (ng/ml)	1.69 ±0.19 (1.61)	10.3 ±0.8 (9.89)	1.54 ±0.22 (1.42)	10.9 ±0.6 (10.4)
Mean basal level (ng/ml)	0.92 ±0.18 (0.81)	8.97±0.74 (8.20)	0.90 ±0.22 (0.83)	9.51±0.60(8.95)
Follicular phase				
Pulses/6 h	5(3-6)	2(1-4)	5(1-8)	1(0-1)*
Pulse amplitude (ng/ml)	1.80 ±0.33 (1.87)	3.57±0.62 (3.43)	1.30 ±0.19 (1.22)	4.0 ±1.26 (3.54)
Mean level (ng/ml)	2.35 ±0.36 (2.18)	10.6 ±1.09 (11.5)	2.21 ±0.38 (2.11)	11.1 ±1.1 (9.66)
Mean basal level (ng/ml)	1.55 ±0.24 (1.41)	8.71±0.89 (9.34)	1.60 ±0.38 (1.63)	9.29±1.08 (8.04)

* Significantly different to control ewes, $p<0.05$

Figure 13 Concentration (ng/ml) of LH and FSH in jugular venous plasma of control Merino ewes during the luteal phase of the oestrous cycle. Samples were taken every 15 min for 6 h. Pulses identified by computer program are indicated by asterisks.

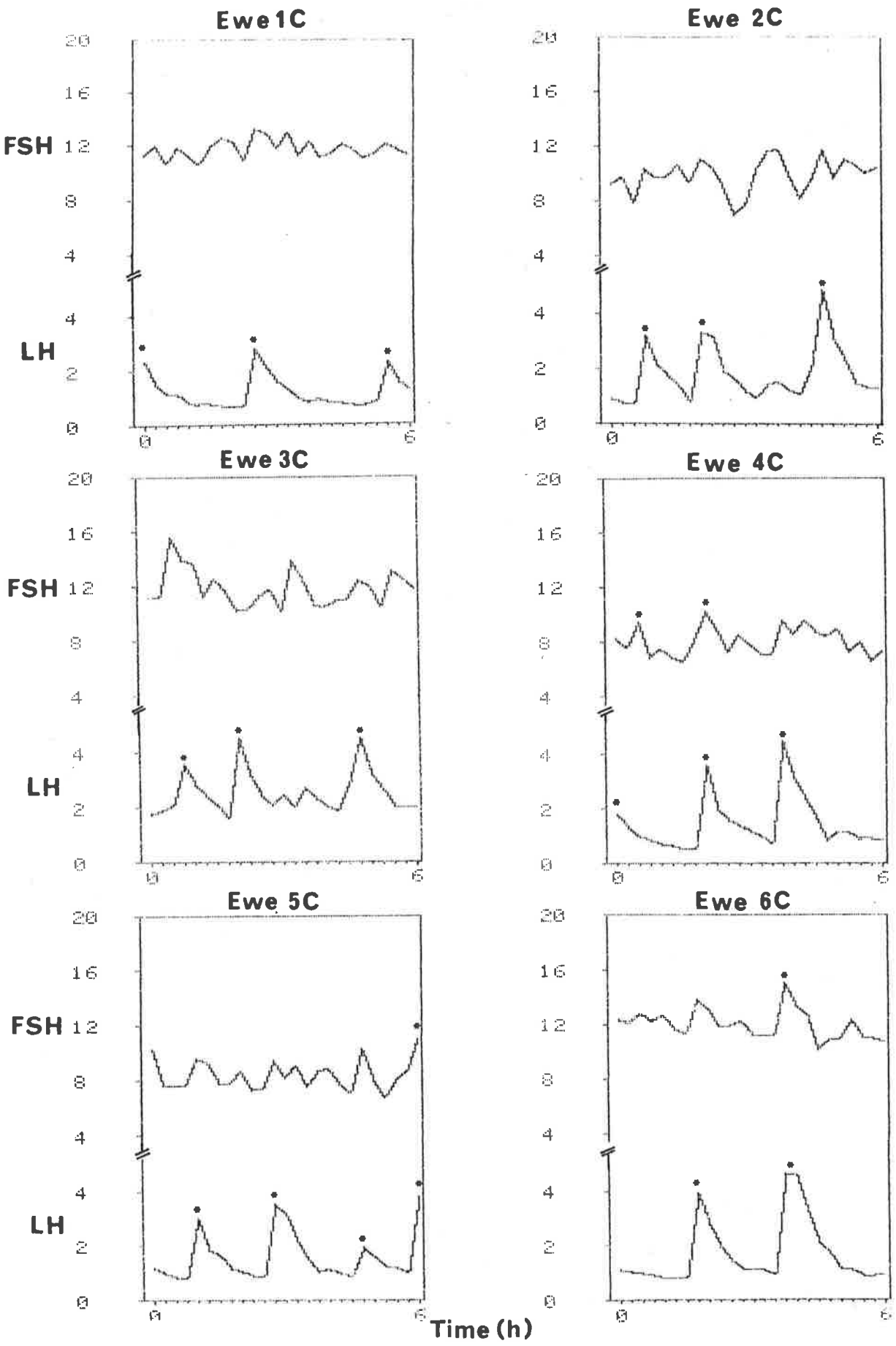

CONTROL-LUTEAL
Ewe 1C
Ewe 2C
Ewe 3C
Ewe 4C
Ewe 5C
Ewe 6C
FSH
LH
20
16
12
8
4
4
2
0
0
6
Time (h)

Figure 14 Concentration (ng/ml) of LH and FSH in jugular venous plasma of Booroola Merino ewes during the luteal phase of the oestrous cycle. Samples were taken every 15 min for 6 h. Pulses identified by computer program are indicated by asterisks.

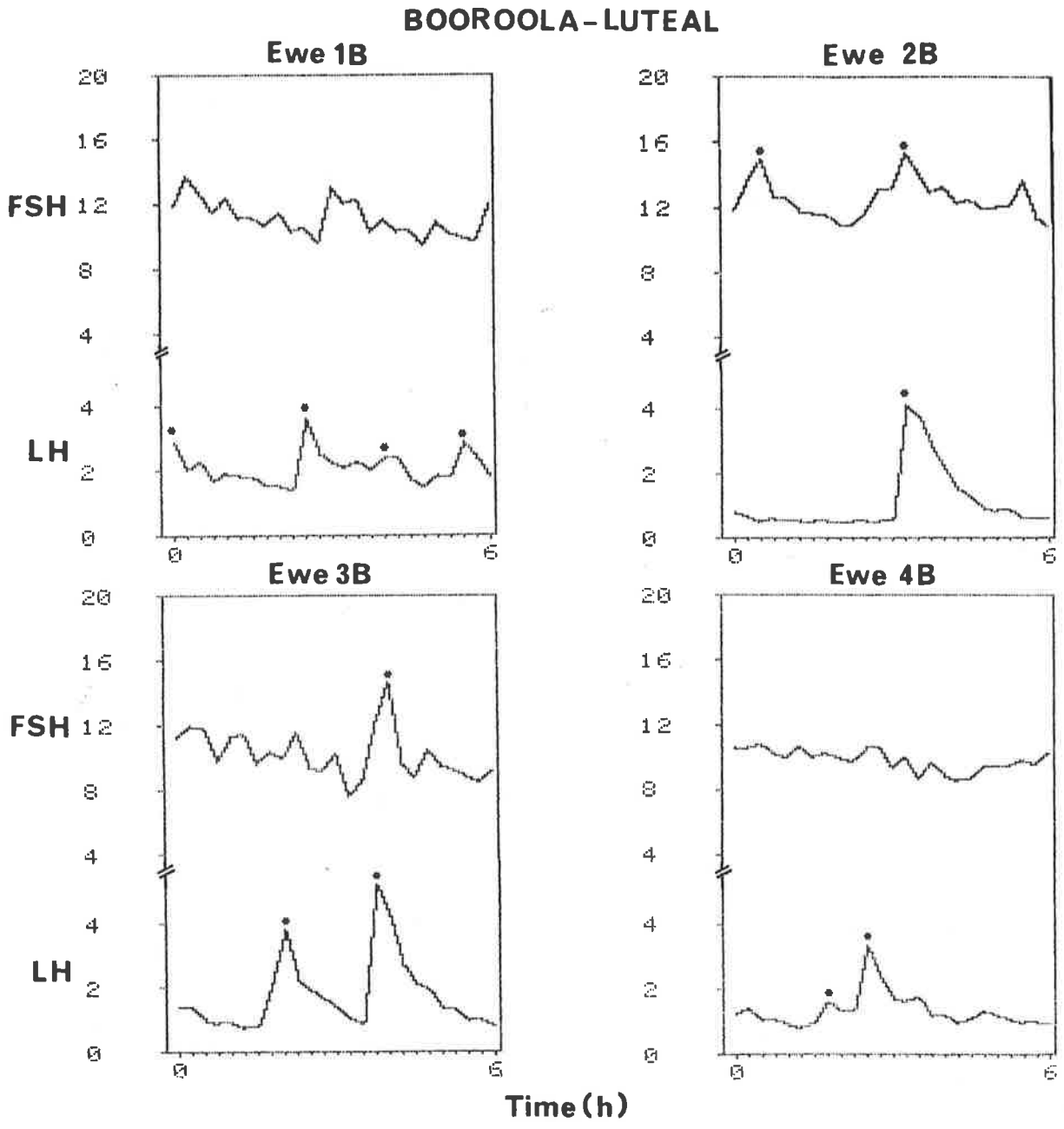

BOOROOLA - LUTEAL
Ewe 1B
Ewe 2B
Ewe 3B
Ewe 4B
FSH
LH
20
16
12
8
4
4
2
0
0
6
Time (h)

Figure 15 Concentration (ng/ml) of LH and FSH in jugular venous plasma of control Merino ewes during the follicular phase of the oestrous cycle. Samples were taken every 15 min for 6 h. Pulses identified by computer program are indicated by asterisks.

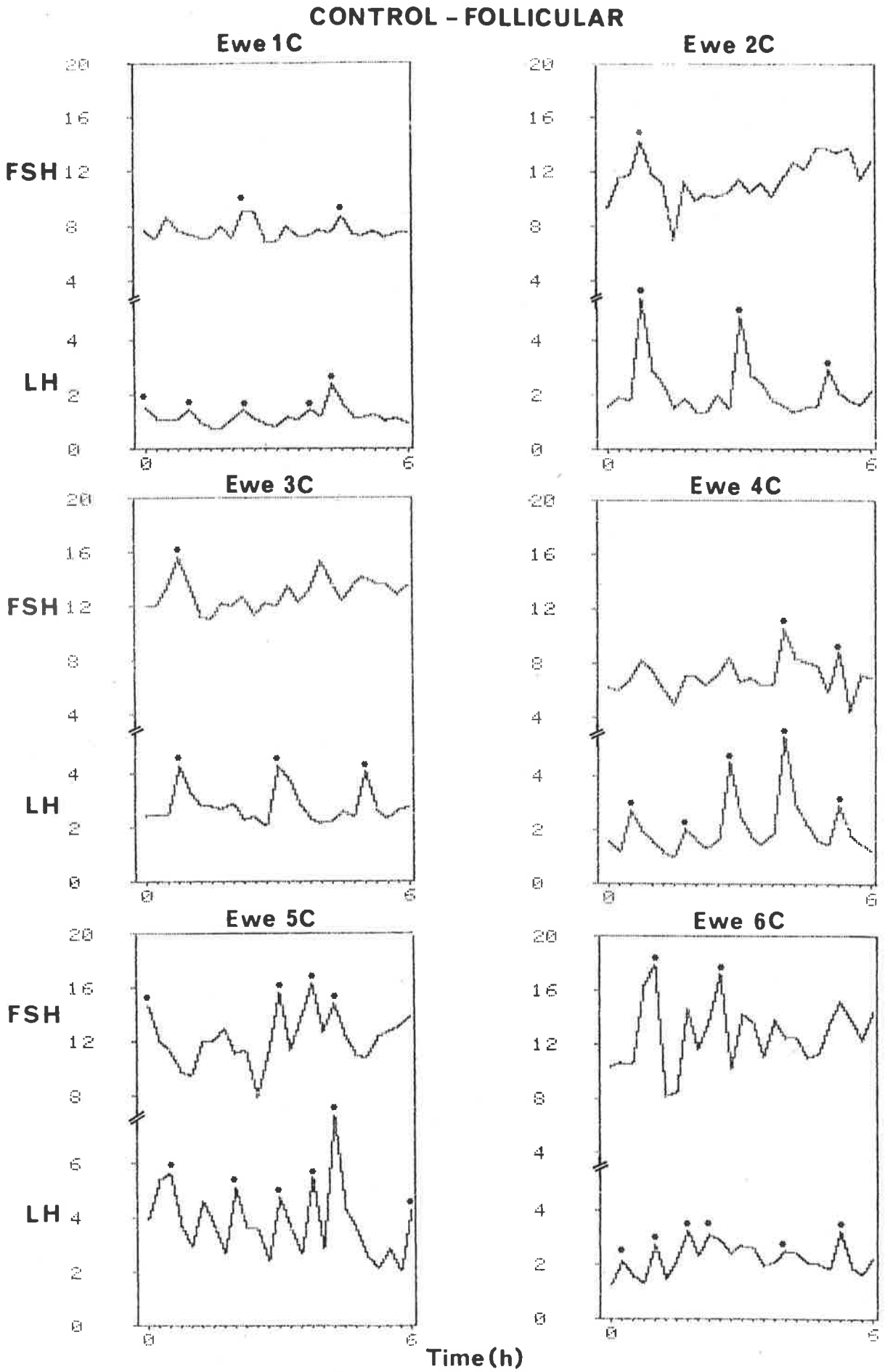

CONTROL - FOLLICULAR
Ewe 1C
Ewe 2C
Ewe 3C
Ewe 4C
Ewe 5C
Ewe 6C
FSH
LH
Time (h)

Figure 16 Concentration (ng/ml) of LH and FSH in jugular venous plasma of Booroola Merino ewes during the follicular phase of the oestrous cycle. Samples were taken every 15 min for 6 h. Pulses identified by computer program are indicated by asterisks.

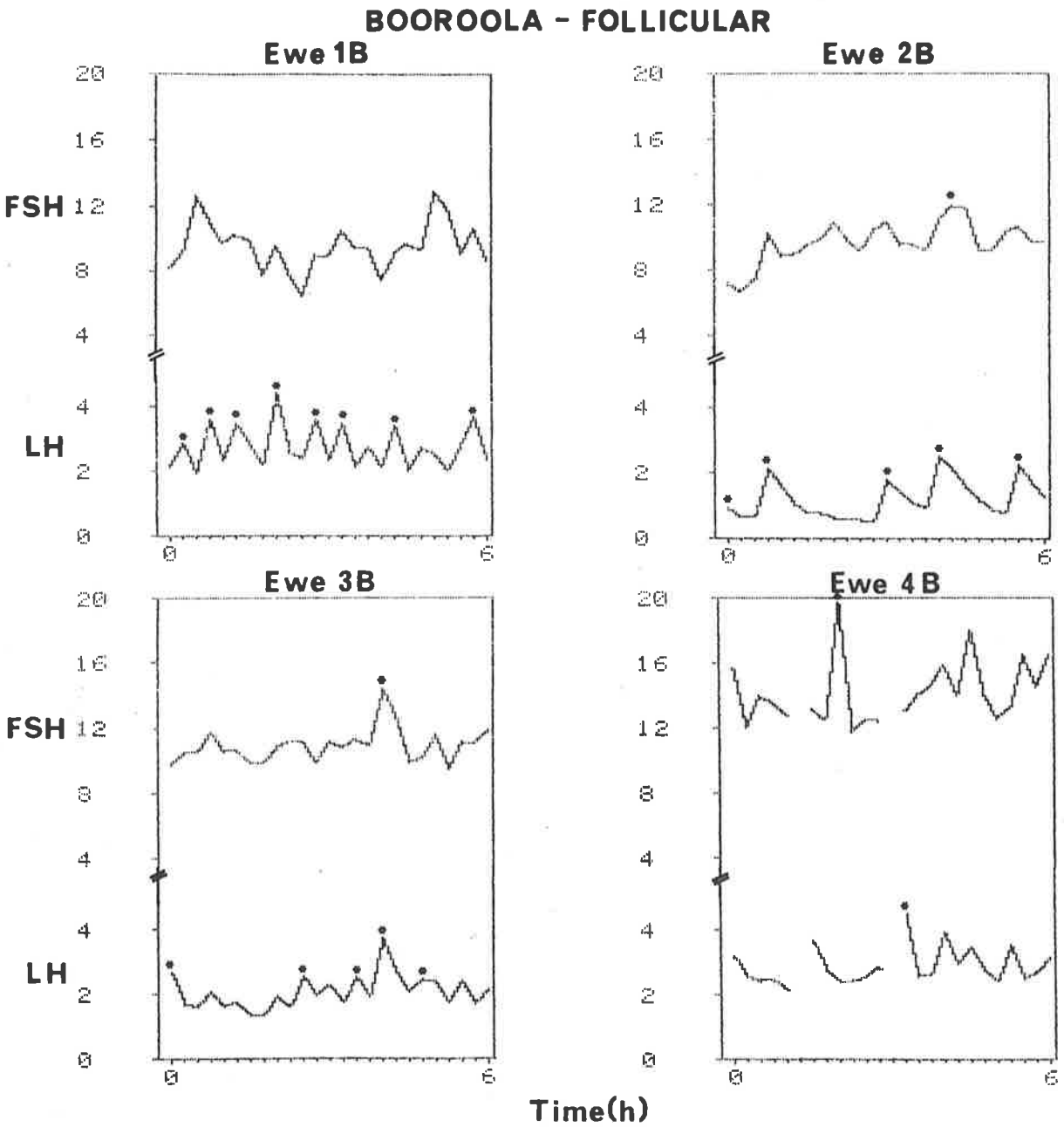

BOOROOLA - FOLLICULAR
Ewe 1B
Ewe 2B
Ewe 3B
Ewe 4B
FSH
LH
20
16
12
8
4
4
2
0
0
6
Time(h)

Table 27 Comparison of progesterone, LH and FSH profiles (area units) of control and Booroola Merino ewes during the 24 h after injection of 100 µg cloprostenol (mean ± s.e.m. (median)).

Hormone	Control (n = 6)	Booroola (n = 4)	Significance of difference
Progesterone	33.6±3.9(31.0)	31.9±4.0(29.8)	n.s.
LH	34.1±5.3(28.0)	32.9±5.1(35.6)	n.s.
FSH	125±8(119)	135±13(122)	n.s.

pooled for those parameters which were not significantly different, an increase in LH frequency, ($p < 0.05$), a decrease in LH amplitude ($p < 0.05$) and an increase in LH levels ($p < 0.05$), both mean and basal, was evident during the follicular phase.

During the 30 h prior to the preovulatory discharge LH and FSH levels remained unchanged. There were no breed differences in the amount of gonadotrophin released before, during or after the preovulatory discharge (Figure 12 ; Table 28). The maximum concentration of LH or FSH reached during the discharge was similar in all ewes (LH ; B(-), 109±12 ng/ml ; B(+) 135±22 ng/ml : FSH ; B(-), 54.7±5.7 ng/ml ; B(+), 71.0±13.7 ng/ml). Neither the interval from prostaglandin injection to the onset of the preovulatory discharge, nor to the maximum concentration of the discharge, differed between genotypes (Table 29).

All ewes appeared to ovulate, as indicated by progesterone concentrations $\geqslant$ 2.8 nM on day 13 after cloprostenol injection.

Table 28 Comparison of LH and FSH profiles of control (n=6) and Booroola (n=4) Merino ewes in the periovulatory period after induction of luteolysis by injection of 100 µg cloprostenol (mean ±s.e.m. (median)).

Time period (h relative to LH peak)	Measure	Control		Booroola	
		LH	FSH	LH	FSH
-36 to -6	Area	67.8 ±6.8 (69)	310 ±26 (326)	70.5 ±7.1 (72)	311 ±40 (283)
-6 to +6	Area	603 ±59 (563)	464 ±63 (414)	718 ±100 (788)	582 ±119 (540)
+6 to +36	Mean concentration (ng/ml)	2.56 ±0.98(1.63)	13.6 ±1.2(14.3)	1.95±0.16(1.98)	14.5 ±1.9 (13.6)

Table 29 Interval from cloprostenol injection (PG) to the onset and maximum concentration of the preovulatory LH surge in control and Booroola Merino ewes (mean ±s.e.m. (median)).

Interval from PG (h)	Control (n = 6)	Booroola (n = 4)	Significance of difference
To onset of surge (>10 ng/ml)	57.3 ±2.8 (55)	54.0 ±6.9 (47)	n.s.
To maximum concentration	61.7 ±2.9 (59)	60.0 ±3.0 (58)	n.s.

D. Discussion

The changes in LH levels observed in both genotypes during the periovulatory period and the concentrations measured were similar to those previously described (see Baird and McNeilly, 1981). Pulsatile gonadotrophin secretion was characteristic of the stage of oestrous cycle, although no decrease in FSH pulse amplitude, as previously described (Baird et al., 1981), was evident during the follicular phase in this study. A wide range of FSH values have been reported during the ovine oestrous cycle, some lower (Cahill et al., 1981 ; Miller et al., 1981) and some higher (L'Hermite et al., 1972 ; Salamonsen et al., 1973 ; McNeilly et al., 1976 ; Pant et al., 1977 ; Baird and McNeilly, 1981 ; Goodman et al., 1981 ; Bister and Paquay, 1983 ; Bolt, 1983 ; Bindon et al., 1984 ; Lahlou-Kassi et al., 1984) than those found in the present study. It is generally agreed, however, that FSH levels decline preceding the preovulatory discharge and that a post-ovulatory peak of FSH alone occurs. In the FSH profiles of ewes in this study neither of these changes was observed. Robertson et al. (1984) have recently reported differences between FSH radioimmunoassays in the detection of immunoreactive material, which was inactive in radioreceptor assay, resulting in over estimation at low pituitary FSH concentrations.

Similar nonspecific interference in this assay may have obscured fluctuations at low FSH concentrations. Also, in previous reports these characteristics of FSH secretion have not been consistently observed (McNeilly et al., 1976 ; Goodman et al., 1981).

In this study, there was no evidence of increased FSH secretion during the luteal or periovulatory phase which followed prostaglandin-induced luteolysis which might account for the increased ovulation rate of Booroola ewes. Breed differences in FSH secretion were also not evident prior to ovariectomy in the experiment reported in Chapter VII with increased numbers of ewes and utilizing a different radioimmunoassay (Bremner et al., 1980). This finding confirms the less extensive report of Bindon et al. (1982a), but more recent evidence from these workers suggests that increased FSH secretion might occur in the Booroola Merino. Booroola ewes (heterozygous or homozygous) had higher FSH concentrations in the 24 h preceding the preovulatory discharge and released more FSH during the post-ovulatory peak of a normal oestrous cycle than control ewes (Bindon et al., 1984 ; Bindon, 1984). In the same study, adult ewes with higher ovulation rates also had a larger second FSH peak than young ewes. Elevated FSH concentrations were found in plasma samples and 3 h urine collections taken from Booroola ewes on day 15 of the cycle following induction of ovulation during anoestrus by progesterone treatment (Bindon et al., 1984 ; Bindon, 1984). The finding that the pituitary glands of Booroola ewes killed on day 3 of the cycle contained more immunoactive or bioactive FSH (Robertson et al., 1984) has provided further evidence for increased FSH secretion. Evidence of increased FSH release during the late follicular phase, preovulatory discharge and post-ovulatory peak has also recently been reported in another prolific breed, the D'Man (Lahlou-Kassi et al., 1984).

Both the assay procedures used and genotypic variation in animals studied may account for the disparity between reports of FSH differences in Booroola and control Merino ewes. Robertson et al. (1984) have proposed that the failure of radioimmunoassays to accurately reflect low concentrations of biologically-active hormone (as distinguished by radioreceptor assay) might explain why differences in plasma FSH levels have not been detected between genotypes. As the effect of the Boorola gene on ovulation rate is additive (Piper and Bindon, 1982b) a similar effect might be apparent in FSH levels, if FSH is the major determinant of ovulation rate. A higher proportion of homozygous Booroola ewes may, therefore, have been included in the experimental animals of Bindon (1984). It is also possible that FSH concentrations following prostaglandin-induced luteolysis do not accurately reflect those during the normal oestrous cycle.

If, however, enhanced FSH secretion does occur during the late follicular phase of the oestrous cycle of prolific ewes, it may account for the increased follicular recruitment which occurs in these ewes. Recently, it has been shown that the final selection of ovulatory follicles occurs as late as 30-54 h after prostaglandin-induced luteolysis in the ewe (Driancourt and Cahill, 1984). PMSG, injected just prior to or at luteolysis, increases the ovulation rate of ewes by reducing atresia (Dott et al., 1979 ; McNatty et al., 1982), increasing the growth rate (Turnbull et al., 1977a) and stimulating oestrogen secretion (Moor et al., 1973 ; McNatty et al., 1982) of antral follicles. Although the ovaries of control and Booroola ewes were shown to contain the same number of morphologically atretic follicles 36±1 h after prostaglandin (Chapter IV), a greater proportion of the large antral follicles contained high levels of oestrogen, a characteristic of PMSG-treated animals (McNatty et al., 1982). The failure to ascribe a

role to the increased post-ovulatory FSH secretion in the determination of ovulation rate has been discussed in Chapter III.

The significance of the reduced FSH pulse frequency of Booroola ewes during the follicular phase is difficult to assess, particularly without a concurrent decrease in FSH levels. While the peak secretion of LH during each pulse is approximately 3 times higher than basal secretion, peak FSH secretion is only 1.3 times that of basal levels (Table 26). Thus, recognition of each secretory episode is more difficult. Although pulsatile FSH secretion was reported by Baird et al. (1981) during both the follicular and luteal phases of the oestrous cycle, Bister and Paquay (1983) failed to demonstrate episodic FSH secretion in anoestrous ewes. Increased sampling frequency will be required to accurately define pulses in Booroola ewes during the follicular phase of the oestrous cycle.

In agreement with the majority of previous reports in high fecundity breeds of sheep, no differences in LH secretion were found between control and Booroola ewes. A recent study which measured LH pulse frequency on days 2, 9 and 16 of the oestrous cycle also revealed no differences between strains (Scaramuzzi and Radford, 1983). The prolific D'Man breed actually has lower basal concentrations of LH extending from the late follicular phase to the first 7 days of the oestrous cycle and releases less LH during the preovulatory discharge than low fecundity Timahdite ewes (Lahlou-Kassi et al., 1984). When follicular phase LH levels are increased by injection or infusion of LH or GnRH, ovulation rate of ewes is unaffected (McNatty et al., 1981a ; McLeod and Haresign, 1984). The finding that exogenous LH does not increase ovulation rate of ewes above that obtained with FSH alone (Wright et al., 1981) confirms the view that LH secretion does not influence ovulation rate. The increased LH pulse frequency of steroid-

immunized ewes may, therefore, be a result of immunization which is unrelated to the increased ovulation rate of these ewes.

The similarity of timing of the preovulatory LH surge in both strains of ewe provides further evidence that there is little difference in the feedback response to oestradiol in Booroola ewes. Thus, as ovariectomized Booroola ewes have similar or lower gonadotrophin levels than ovariectomized control ewes, (Cummins et al., 1983), increased FSH levels in entire Booroola ewes may result from decreased negative feedback control of another ovarian factor. From the finding of the decreased inhibin content of Booroola ovaries (Cummins et al., 1983), Bindon (1984) has proposed that this factor is inhibin. Further studies demonstrating decreased ovarian secretion of inhibin and elucidating the role of increased sensitivity of FSH to the negative feedback effects of inhibin in Booroola ewes are necessary to confirm this view.

CHAPTER IX

THE ROLE OF INTRAOVARIAN ANDROGEN IN THE DETERMINATION OF OVULATION RATE IN THE EWE

A. Introduction

The previous chapters have shown that the smaller Booroola follicle possesses an enhanced capacity to synthesize oestrogen, the hormone essential for follicular development and oocyte maturation. Differences in peripheral gonadotrophin concentrations which could contribute to the increased ovulation rate of Booroola ewes were not demonstrated. However, a difference in ovarian androgen concentration may have been revealed between genotypes which could influence follicular recruitment.

Androgens produced locally within the ovary in response to gonadotrophic stimulation appear to play a role in follicular development beside that as substrates for aromatization. The administration of androgens or hCG to hypophysectomized immature female rats has been shown to promote follicular atresia (Payne and Runsen, 1958 ; Hillier and Ross, 1979), and inhibit the mitogenic action of oestrogen (Louvet et al., 1975) in preantral follicles. In antral follicles, androgens appear to induce atresia by antagonizing the biological effects of FSH in vivo (Zeleznik et al., 1979), thus interfering in the production and/or action of oestradiol in the follicle (Bagnell et al., 1982). Specific granulosa cell androgen receptors have been reported (Schreiber et al., 1976). A decrease in the number of antral follicles in the ovaries of rats occurs by 26 h after testosterone is implanted inside the bursa ovarica, but not when implanted subcutaneously (Welschen et al., 1980). Reduced rates of atresia have been implicated in the increased ovulation rates of androgen immunized ewes (Scaramuzzi, 1979). Thus, there is evidence that

intraovarian androgen concentrations influence the number of follicles available for preovulatory recruitment.

Although the concentrations of androstenedione and testosterone were similar in the antral fluid of 'oestrogenic' control and Booroola follicles, the total content of these two androgens within the Booroola follicle was less (Chapter IV, Table 15). As might be expected from their lower thecal weight, the basal secretion rate of androgens by 'oestrogenic' Booroola follicles in perifusion was lower than control follicles (Chapter V, Table 18). 'Non-oestrogenic' Booroola follicles released less androgen in response to LH in perifusion than control follicles of a similar size (Chapter V, Table 21). It is not known whether these differences arise from decreased androgen production or increased aromatization by the Booroola follicle, but, together they suggest that decreased intraovarian androgen levels may contribute to the increased ovulation rate of the Booroola Merino. Further confirmation of this view provided by the relative rates of atresia in control and Booroola ewes could not be obtained as it was not possible to use microscopic assessment of atresia to distinguish between early atretic and small growing follicles.

Thus, the possibility that increased ovarian concentrations of testosterone might decrease the number of follicles available for recruitment for ovulation in control Merino ewes remains. To investigate this possibility an experiment was carried out in which silicone capsules containing testosterone were implanted either in the ovaries or subcutaneously, to enable discrimination between a local or a central steroid effect. Blood samples were taken frequently during the periovulatory period following implantation and on 3 days during the ensuing luteal phase to determine whether gonadotrophin levels were affected by testosterone administration. As androgen administration has

been shown to affect both antral and preantral follicular development, the ovulation rate of ewes was determined 24 days and 5 months after implantation. On each occasion superovulation was induced in all ewes by PMSG administration, prior to induction of luteolysis with cloprostenol, to maximise differences between treatments.

B. Materials and Methods

1. Animals

During the breeding season (June), oestrus was synchronized in 44 mature Merino ewes (Bungaree strain) maintained at Mortlock Experiment Station, near Clare, South Australia, by the insertion of progesterone sponges (Repromap, Upjohn, Kalamazoo, U.S.A.) for a period of 14 days. 30 of those ewes detected in oestrus within 4 days of sponge removal were weighed and randomly allocated to three treatment groups of 10 ewes each. Except for a period of one week, these ewes were maintained outdoors on natural grass and subterranean clover pasture. During the week of frequent blood sampling following implant insertion, ewes were housed indoors under natural lighting and fed a maintenance diet of lucerne hay, barley grain and water.

2. Testosterone implants

The maximum ovarian secretion rate of testosterone was estimated to be approximately 7 nmol/day from the secretion rate of follicles in perifusion and the *in vivo* experiments of Baird *et al.* (1981). Utilizing the data of Kincl and Rudel (1971) an implant was designed to deliver approximately 10 times this amount of testosterone per day. The silastic implants were prepared by sealing one end of Silastic medical grade tubing (1.98 mm i.d., 3.18 mm o.d.) with a 2 mm plug of Silastic medical

grade adhesive type A (Dow Corning, East Hawthorn, Vic., Australia) which was allowed to harden for approximately 24 h in a water-saturated atmosphere. Testosterone (Steraloids, Wilton, N.H., U.S.A.) was packed into the tubing to a length of 4 mm (measured under a binocular microscope) and the open end sealed. Each implant was rinsed thoroughly in 70% ethanol and then incubated in 20 ml sterile saline in a shaking water bath at 37°C. The saline was changed daily and the testosterone concentration of the samples from 6 implants measured by radioimmunoassay (II.3.f(ii)). After 7 days incubation, the daily release rate of testosterone was found to have stabilized. Each implant was then incubated in 20 ml pooled human plasma containing 0.1% sodium azide in a shaking water bath at 37°C, the in vitro release rate being estimated in a single plasma sample taken at 24 h. The mean release rate of all implants was 100±2 nmol/day (n = 52).

Three days before use, the implants were sterilized and then stored in sterile saline until implantation.

3. Experimental design

On day 9 of the oestrous cycle following progesterone removal, a laparotomy was performed by mid-ventral incision on each ewe. To ensure minimal disturbance of gonadotrophin secretion (Clarke and Doughton, 1983), anaesthesia was induced with 20 ml Alphathesin (alphaxalone, 9 mg/ml ; alphadolone acetate, 3 mg/ml ; Glaxo, Boronia, Australia). Only 4 ewes required a further 10 ml Alphathesin to maintain anaesthesia until completion of laparotomy. The reproductive tract was exteriorized and either a blank (groups A and C) or testosterone implant (group B) was inserted into a hole, well removed from large follicles or corpora lutea, made in the stroma of each ovary with fine haemostats. The implant was secured with a single stitch of 3/0 surgical catgut (Ethnor

Pty. Ltd., Sydney, Australia), and excessive bleeding prevented with Gelfoam (Upjohn, Michigan, U.S.A.). Care was taken to handle the ovaries as little as possible and after implantation, the reproductive tract was thoroughly rinsed with sterile heparinized saline (20 IU/ml), before being returned to the peritoneal cavity. The abdominal incision was closed and sprayed with antibiotic (Neotracin ; Ethnor Pty. Ltd., Syndey, Australia). Two testosterone implants were inserted subcutaneously on the ventral thigh of each ewe in group B. Thus, group A (control) and group C ewes received two blank implants, one in each ovary. Group B ewes received one testosterone implant in each ovary and group C also received 2 subcutaneous testosterone implants.

On day 10 of the oestrous cycle, 28-36 h after laparotomy, luteolysis was induced in all ewes by the injection of 100 µg cloprostenol, i.m. Blood samples were taken from each ewe by jugular venepuncture on day 9, before laparotomy and prior to the injection of prostaglandin. From 36 to 92 h after prostaglandin, blood samples were taken 4 hrly (except at 56 h), and then 8 hrly until 108 h. During the ensuing luteal phase, single blood samples were taken 7, 9 and 13 days after prostaglandin injection. 1000 i.u. PMSG (Folligon ; Intervet (Aust.) Pty. Ltd., Knoxfield, Vic. Australia ; Batch 5682) was injected i.m. into each ewe on this last occasion and luteolysis induced by the injection of 100 µg cloprostenol 2 days later. Oestrus was recorded and the number of CL per ewe determined at laparoscopy 8 days after the second prostaglandin injection. A blood sample was taken to confirm ovulation by progesterone concentration.

Towards the end of the normal anoestrous period 4 months after implant insertion (November), the ewes were reweighed and oestrus was once again synchronized by the insertion of progesterone sponges for 14 days. During the intervening period, weekly oestrous observations had

demonstrated continuous cyclic activity in the majority (24/30) of ewes. Superovulation was induced by the injection of 1000 i.u. PMSG and 100 µg cloprostenol at 13 and 15 days, respectively, after sponge removal. Prior to slaughter at the local abbatoirs 6 days after prostaglandin injection, a blood sample was taken from all ewes and the presence of subcutaneous implants confirmed. The reproductive tracts were removed from each ewe at slaughter and the number of CL per ewe, extent of adhesions, and retention of ovarian implants determined by inspection in the laboratory.

4. Hormone analysis

LH and FSH concentrations were determined by radioimmunoassay (II.C) in the samples of plasma taken at laparotomy, at prostaglandin injection and throughout the periovulatory period and luteal phase following implantation. Samples taken > 36 h after prostaglandin injection were alligned to the peak of the preovulatory LH discharge and the hormonal characteristics considered for comparison between treatments were (1) maximum LH and FSH concentration reached during the preovulatory discharge (2) mean FSH level 8-48 h after the preovulatory discharge (the time of the post-ovulatory peak) (3) mean LH and FSH concentration of the 3 luteal phase samples. It was not possible to calculate the area under the preovulatory discharge due to the missing 56 h sample.

The plasma progesterone concentration of samples taken were measured by radioimmunoassay (II.3.(a)).

C. Results

The mean body weight of ewes did not differ significantly between treatments in either June or November (Table 30).

Table 30 The bodyweight (kg) of control ewes (group A) and ewes implanted with testosterone (group B and C) in June and November (mean ± s.e.m.(n)).

Month	Treatment group		
	A	B	C
June	57.9±1.7(10)	57.8±2.3(10)	57.8±2.0(10)
November	62.0±2.1(10)	61.5±2.6(10)	62.3±3.1(9)

1. Hormone levels during the induced oestrous cycle following implantation

Luteal phase levels of progesterone (> 3 nM) were found in the plasma of all ewes at laparotomy and all but one ewe at prostaglandin injection 28-36 h later. The gonadotrophin results from this one ewe (group C) have been omitted from the data. 36 h after prostaglandin, progesterone levels in all ewes had decreased to < 0.8 nM.

Implantation of ewes with testosterone did not alter any of the characteristics of LH secretion measured during the periovulatory period or luteal phase of the oestrous cycle (Table 31). Coincident with luteolysis, LH levels in all ewes increased above luteal phase levels ($p < 0.001$) by 36 h after prostaglandin injection. The timing of the preovulatory discharge after cloprostenol injection was similar in all treatment groups (Table 32).

There were no differences between treatments in the concentrations of FSH in the luteal phase immediately prior to and following implantation (Table 33). However, by 28-36 h after insertion of implants the mean FSH concentration of ewes with ovarian implants containing testosterone was less than pre-implantation levels ($p < 0.05$). Whereas FSH levels in control ewes had increased ($p < 0.02$) above pre-injection levels by 36 h after prostaglandin, FSH concentrations of testosterone-implanted ewes did not change with luteolysis. Ewes with testosterone

Table 31 Plasma LH concentration (ng/ml) of control ewes (group A) and ewes implanted with testosterone either in the ovaries (group B) or subcutaneously (group C). Values are mean ±s.e.m. (median).

Time of sampling	Treatment group			Significance of difference
	A (n = 10)	B (n = 10)	C (n = 9)	
Pre implantation	0.82±0.10 (0.88)	0.91±0.11 (0.76)	1.14±0.17 (1.25)	n.s.
Post implantation				
At PG	0.88±0.12 (0.82)	0.94±0.12 (0.78)	1.04±0.15 (0.98)	n.s.
PG + 36 h	1.25±0.11 (1.13)	1.18±0.17 (1.20)	1.48±0.23 (1.50)	n.s.
Maximum during preovulatory discharge	245±55 (169)	214±49 (145)	223±52 (201)	n.s.
Mean luteal phase level	0.99±0.22 (0.82)	1.03±0.16 (0.81)	1.29±0.22 (1.20)	n.s.

Table 32 Interval (h) from the injection of cloprostenol to the onset (> 10 ng/ml) or maximum concentration reached during the preovulatory LH discharge in control ewes (group A) and ewes implanted with testosterone either in the ovaries (group B) or subcutaneously (group C,). Values are mean ± s.e.m. (median)).

Interval	Treatment group			Significance of Difference
	A (n=10)	B (n=10)	C (n=9)	
To the Onset (> 10 ng/ml)	59.1±4.3(60)	59.2±4.0(62)	57.8±3.3(60)	n.s.
To the Maximum concentration	62.0±4.4(60)	61.6±4.1(62)	59.1±3.6(60)	n.s.

Table 33 Plasma FSH concentration (ng/ml) of control ewes (group A) and ewes implanted with testosterone either in the ovaries (group B) or subcutaneously (group C). Values are mean ± s.e.m. (median).

Time of sampling	Treatment group			Significance of difference
	A (n = 10)	B (n = 10)	C (n = 9)	
Pre-implantation	18.8±0.9 (19.1)	17.3±1.2 (17.7)	18.7±1.2 (18.3)	n.s.
Post-implantation				
At PG	17.5±0.9 (18.3)	14.5±1.1 (15.4)	16.9±1.1 (16.6)	n.s.
PG + 36 h	20.2±0.9 (19.9)	16.2±1.5* (14.6)	16.1±1.3* (16.4)	$p < 0.05$
Maximum during preovulatory discharge	86.4±8.6 (80.0)	57.1±6.9* (54.7)	60.8±6.2* (52.8)	$p < 0.05$
Mean level 8-48 h after preovulatory discharge	23.6±1.0 (22.7)	21.4±2.2 (18.2)	21.9±1.3 (21.0)	n.s.
Mean luteal phase level	20.2±1.2 (19.3)	17.9±1.8 (16.3)	18.7±1.0 (18.6)	n.s.

* Significantly different from controls

implanted at either site had lower FSH levels ($P < 0.05$) than control ewes 36 h after prostaglandin injection and during the preovulatory discharge (Table 33). This difference was no longer evident in the 40 h after the preovulatory surge or in the ensuing luteal phase.

As demonstrated by normal gonadotrophin surges and peripheral progesterone concentrations $\geqslant$ 3.4 nM 9 days after cloprostenol injection, all ewes ovulated.

2. Ovulation rate

(a) 14 days after implantation

All ewes were marked in oestrus 2-5 days after the second prostaglandin injection. At laparoscopy all but one ewe (group C) had plasma progesterone concentrations > 3.7 nM, the levels not differing between treatments (Table 34). However, adhesions to either 1 or both ovaries in 7 ewes (3 in group A, 3 in group B, 1 in group C) prevented full visualization of the ovaries at laparoscopy. The results from these ewes were, therefore, omitted from subsequent analyses. The ovulation rate of those ewes which ovulated following PMSG did not differ significantly between treatments (Table 35).

Table 34 Plasma progesterone concentration (nM) in the luteal phase following superovulation with PMSG 14 days or 4 months after treatment with blank implants (group A) or testosterone implants, inserted either in the ovaries (group B) or subcutaneously (group C). Values are mean ± s.e.m. (median) for the 7-10 ewes which ovulated per group.

Time after implantation	Treatment group			Significance of difference
	A	B	C	
14 days	31.7±7.7(19.6)	16.3±3.5(14.2)	16.7±4.1(11.5)	n.s.
4 months	4.02±1.2(3.2)	2.70±0.53(2.23)	1.61±0.35(1.31)	n.s.

Table 35 Distribution of zero, single and multiple ovulations and mean ovulation rates of ewes ovulating following superovulation with PMSG 14 days or 4 months after treatment with blank implants (group A, or testosterone implants inserted either into the ovaries (group B) or subcutaneously (group C).

Time after Implantation	Treatment group	No. of ewes	No. of CL/ewe															
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Mean ± s.e.m. (median)
14 days	A	7	-	-	-	3	1	-	-	-	1	1	-	-	-	-	1	6.3±1.6(4)
	B	7	-	-	2	2	2	-	1	-	-	-	-	-	-	-	-	3.4±0.5(3)
	C	9	1	2	2	3	-	-	-	-	-	1	-	-	-	-	-	3.0±0.9(2.5)
4 months	A	8	-	-	2	4	-	-	-	-	-	1	-	-	1	-	-	4.6±1.3(3)
	B	8	-	-	3	2	2	-	-	-	-	1	-	-	-	-	-	3.6±0.8(3)
	C	8	1	2	1	2	-	2	-	-	-	-	-	-	-	-	-	2.9±0.6(3)

(b) 4 months after implantation

One ewe was humanely killed prior to superovulation due to injury. All the remaining ewes exhibited oestrus in the 5 days following prostaglandin injection.

At autopsy, all subcutaneous implants were found to be present and although 2 control ewes had either 1 or 2 ovarian implants missing, the results from these ewes were included in the data. The data from another 2 control ewes from which incomplete tracts were recovered at slaughter, and one ewe (group B) which had 1 ovarian testosterone implant missing were excluded. No corpora lutea were recorded in the ovaries of 2 ewes (groups B and C), due to either the presence of massive adhesions arising from infection or to large cystic structures. Although some adhesions were present in the majority of ewes, these did not appear to be inhibitory to ovulation.

Neither the number of CL per ewe (Table 35) nor plasma progesterone concentration of ewes prior to autopsy (Table 34) differed significantly between treatments.

D. Discussion

The ovulation rate of ewes treated with exogenous gonadotrophin was not reduced by increased levels of ovarian testosterone, either immediately following or after several months of administration. The use of PMSG to maximise treatment effects may well have obliterated the expected differences. The increased ovulation rate resulting from PMSG administration during the late luteal phase is thought to be due to a reduction in the incidence of atresia of large antral follicles (Dott et al., 1979 ; McNatty et al., 1982 ; Moor et al., 1984). Increased rates of atresia resulting from the administration of testosterone may have been reduced by PMSG treatment.

It was hypothesized that androgen influenced ovulation rate at the local ovarian level rather than having a central effect. However, a similar decrease in FSH levels occurred with both ovarian and subcutaneous administration of testosterone. These findings contrast with those of Welschen et al. (1980) who found decreased numbers of antral follicles and increased FSH concentrations at oestrus in rats implanted with testosterone inside the bursa ovarica. FSH levels were also increased in rats with subcutaneous implants, although no decrease in follicle number occurred. Both sites of implantation increased peripheral testosterone levels 4 fold above non-implanted animals (Welschen et al., 1980). The authors suggest that, as steroids are unable to suppress FSH secretion at oestrus, decreased inhibin release by the reduced number of follicles in testosterone-treated rats is responsible for the elevated FSH levels.

As no direct effects of androgen per se on the control of gonadotrophin secretion in the ewe have been demonstrated (Clarke et al., 1982 ; Martin et al., 1983), the reduction of FSH levels found in PMSG-stimulated testosterone-treated ewes may have resulted from peripheral or central aromatization of androgen to oestrogen. However, the finding that LH secretion was unaffected suggests that increased inhibin secretion might have occurred in PMSG-stimulated testosterone implanted ewes. Henderson and Franchimont (1981) have proposed that androgens are involved in the regulation of ovarian inhibin production in the cow, through their ability to stimulate inhibin secretion of granulosa cells in vitro. Thus, the possibility exists that exogenous testosterone stimulated increased inhibin production by the preovulatory follicle(s), so depressing FSH secretion. This effect of testosterone appears to have been synergistic with PMSG as no decrease in FSH levels occurred in testosterone-treated ewes during the post-ovulatory or

luteal phase of the following oestrous cycle. This finding suggests that the increased inhibin secretion following PMSG treatment of immature female rats (Lee and Findlay, 1982) may be mediated by androgens.

It is not possible to conclude from these results whether increased intraovarian androgen levels during the normal oestrous cycle can decrease the ovulation rate of ewes.

CHAPTER X

GENERAL DISCUSSION

While much is known regarding the endocrine control of the growth and maturation of ovulatory follicles, the selection mechanism which determines the number of ova shed per cycle remains unclear. The endocrine system appears to be involved in this selection process as ovulation rate is unaffected by the removal of one ovary. Monotocous species with 2 functional ovaries exhibit "communication" between ovaries; a single follicle ovulating from only one ovary each cycle. Gonadotrophins are obligatory to follicular development and precise titration of FSH levels in blood by ovarian oestrogen and/or inhibin may control recruitment of the characteristic number of follicles in each cycle (Goodman and Hodgen, 1983). The set-point of this titration could be higher in fecund animals. The negative feedback action of oestradiol and/or inhibin secreted by the preovulatory follicle(s), once recruited, suppresses FSH levels, thus inhibiting the maturation of other follicles (Zeleznick, 1981). The dominant follicle is protected from its own inhibitory influence by high antral fluid oestradiol and FSH concentrations (McNatty et al., 1981c) and, enhanced vascularity (Zelenick et al., 1981).

However, unless differences in the vascularity of individual follicles exist prior to recruitment, all follicles in both ovaries are exposed to similar gonadotrophin levels. Thus, it is difficult to explain the emergence of one or more follicles from the several growing follicles present in the ovary or development of a single follicle on one ovary. No consistent differences in FSH secretion have been demonstrated with widely varying numbers of preovulatory follicles

(Chapter I). Therefore, it has been proposed that intraovarian factors are also able to influence follicle selection and recruitment (e.g. Lindner et al., 1977; Channing et al., 1982; Goodman and Hodgen, 1983). These intraovarian factors would control the selection process within each ovary, the dominant follicle then communicating with the other ovary either classically, via the hypothalamic-pituitary axis or, by secretion of an hormone which directly inhibits development of other follicles (Goodman and Hodgen, 1983).

Both steroids and proteins secreted by the follicle have been implicated in the intraovarian control of folliculogenesis. Androgens have been shown to promote atresia (Louvet et al., 1975), and to act synergistically with gonadotrophins to stimulate oestrogen and progestagen biosynthesis, as well as the FSH induction of LH receptors (see Hsueh et al., 1983). Oestradiol enhances gonadotrophin-stimulated oestrogen production (Hsueh et al., 1983), inhibits premature luteinization of granulosa cells (Channing et al., 1982) and may directly inhibit follicular development in the rat ovary (Dierschke et al., 1983). Local follicular development appears to be inhibited by progesterone secreted by the corpus luteum in the human (Goodman and Hodgen, 1983) but enhanced in the ewe (Dufour et al., 1971).

Several nonsteroidal follicular regulators have now been isolated from antral fluid. Low- and high-molecular weight proteins have been found that inhibit FSH induction of LH receptors and stimulation of progesterone secretion (Channing et al., 1982). As the follicle enlarges a luteinization stimulator appears which enhances these actions of FSH upon the granulosa cell (Channing et al., 1982). Nonsteroidal substances in human, porcine and ovine antral fluid, which are detectable in ovarian venous effluent (diZerega et al., 1982), have been reported to inhibit gonadotrophin-stimulated follicular growth (diZerega et al.,

1983a; Cahill et al., 1984), perhaps by suppressing granulosa cell aromatase activity (diZerega et al., 1983b). Other factors have been isolated from bovine antral fluid which can either stimulate or inhibit FSH or LH binding to receptors (Reichert et al., 1981). Further characterization of these proteins is required before their role, if any, in follicle selection becomes apparent. However, it does seem likely that both intra- and extraovarian factors play a role in regulating the number of follicles which are recruited for ovulation each cycle.

Evidence from studies carried out in this thesis and from recent work of other researchers suggests that both extra- and intraovarian endocrine mechanisms determine the selection of increased numbers of ovulatory follicles in the Booroola Merino. At least three characteristic appear to contribute to the high fecundity of this genotype:

(1) increased numbers of antral follicles available for recruitment (Turnbull et al., 1977b ; Cahill et al., 1982), which may result from

(2) increased pituitary secretion of FSH (Bindon, 1984 ; Bindon et al., 1984), and

(3) the maturation of the steroidogenic potential of the Booroola follicle at a smaller follicle diameter.

The physiological significance of increased postovulatory FSH concentrations is not clear, as neither increasing (Chapter III) nor decreasing (Bindon et al., 1984; Bindon, 1984) this peak affects ovulation rate. In rodents, there is a close relationship between FSH levels at this time and the number of follicles forming antra, which then ovulate at the following oestrus (Greenwald and Siegel, 1982). A similar effect would not be apparent for 2-2.5 oestrous cycles in the

ewe (Turnbull et al., 1977a; Cahill and Mauléon, 1980), but was not evident (Chapter III). It is possible that the increased numbers of antral follicles (Cahill et al., 1982) and the increased mitotic index of small antral follicles (Turnbull et al., 1977b) found in the Booroola might be related to increased post-ovulatory FSH concentrations.

Increased FSH concentrations have been reported in the Booroola Merino during the late luteal and follicular phases of the oestrous cycle (Bindon et al., 1984; Bindon, 1984), though are not consistently observed (Bindon et al., 1982a; Chapter VII and VIII). As preovulatory follicle selection occurs at this time (Driancourt and Cahill, 1984), the normal occurrence of atresia might be reduced by increased FSH levels. However, a reduced incidence of atresia has not been found in Booroola ewes (Cahill et al., 1982; Chapter IV), except in a group of aged ewes (Scaramuzzi and Radford, 1983). Further microscopic assessment of atresia in follicle populations during or after luteal regression is required to clarify this point. However, increased FSH levels during luteal regression might also act by increasing the numbers of follicles which develop sufficient maturity in time to respond to the preovulatory gonadotrophin surge.

The capacity for oestradiol synthesis is essential for preovulatory follicular maturation. The unique feature of the Booroola follicle is that it attains this capacity at a smaller size than the control follicle. Full thecal responsiveness to LH is acquired in Booroola follicles with only 1/3 the thecal mass of control follicles. Maintenance of equivalent antral fluid oestradiol concentrations and ovarian venous secretion occurs in Booroola follicles which contain only 1/2 the number of granulosa cells as control follicles. This enhanced aromatase activity was not evident in long-term cultures of granulosa cells isolated from preovulatory follicles. Measurement of extant

aromatase activity in granulosa and thecal preparations over shorter time periods may reveal the source of this heightened activity. The prolonged secretion of all steroids from the entire follicle following a single pulse of LH *in vitro* suggests that increased gonadotrophin-stimulated adenylate cyclase activity or decreased cAMP breakdown may also be involved in the enhanced steroidogenic capacity of the smaller Booroola follicle.

A remarkable similarity existed between genotypes in the total number of preovulatory thecal or granulosa cells per ewe, irrespective of the number of follicles. Thus, for the same or higher FSH concentrations, exactly the same preovulatory follicular mass develops in Booroola and control ewes. Given the role of FSH in follicular development, this finding would suggest a precise titration of FSH levels by a secretion arising from one or both of these cell types. Inhibin, secreted by granulosa cells, to specifically inhibit pituitary FSH secretion may fulfil this role. The mean inhibin content of individual preovulatory Booroola follicles was less than control follicles, as would be expected from the decreased granulosa cell number of Booroola follicles. However, the total inhibin content of preovulatory follicles per ewe, the major source of inhibin during the follicular phase (Tsonis *et al.*, 1983 ; Henderson *et al.*, 1983), did not differ between genotype when peripheral FSH levels were the same. Correlation of changes in FSH secretion with direct measurements of follicular and ovarian inhibin secretion is required to confirm this view.

Large variations in the ovarian response to gonadotrophins observed in mice have also been found to be controlled by only a small number of genes (Spearow, 1984). The mechanisms of action of these genes which have been demonstrated by this author include variation in follicle

number, LH receptor induction and affinity and, LH-stimulated cAMP and steroid production. In mice, this genetic variation in ovulation rate is normally hidden by feedback regulation. The Booroola gene, however, bypasses this regulation, providing a major opportunity to elucidate the biochemical and physiological controls of ovulation rate.

It cannot be assumed, however, that the mechanisms which control the recruitment of preovulatory follicles in the Booroola Merino also operate in other high fecundity breeds or in environmentally or exogenously induced variations in ovulation rate. The ovulation rate of Booroola and non-Booroola ewes increased similarly to nutritionally induced increases in live-weight (Montgomery et al., 1983/1984). This suggests either that different mechanisms are involved in the increase in ovulation rate associated with nutrition and the Booroola gene or that the control mechanism in Booroola ewes can be further 'reset' to higher levels, just as in control ewes. The many differences cited between the ovulatory process in Booroola and other high fecundity breeds suggests that not only does the Booroola have a unique genetic basis for its increased fecundity but also different endocrine mechanisms underlying the development of preovulatory follicles. The evidence of this thesis suggests that this difference may reside in the endocrine characteristics of these preovulatory follicles.

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