I

EVIDENCE FOR THE FORMATION

OF

SUPPLEMENTARY CORPORA LUTEA

IN

THE PREGNANT

AND

PSEUDOPREGNANT RAT

BY

DEBORAH A GARSIDE

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'The laboratory rat has been the most intensively researched of all species for at least 40 years and we had begun to think we had a complete understanding of the rat reproductive endocrinology. That this has been proved wrong, should keep those who think they have conclusively solved a problem alert and open-minded.'

Nalbandov - 1976

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ABSTRACT

In a series of experiments investigating a possible post-coitum contraceptive, it was observed that the numbers of corpora lutea (CL) of pregnant Sprague-Dawley (CD) rats were greater than in cyclic, unmated control females ($p<0.001$).

The ontogeny of these supplementary CL (SCL) was established by the recording the mean number of CL in pregnant Sprague-Dawley rats on days 1 to 9 post-coitum (day 1 being the first day post-coitum) using the gross morphological dissection of the ovary.

Compared to cyclic controls, an initial significant increase in mean CL numbers was recorded on day 1 post-coitum ($p<0.05$) with a further significant increase between days 5 and 7 ($p<0.001$). This phenomena was neither Sprague-Dawley strain, nor pregnancy specific, as a similar increase was also recorded for Wistar rats between days 4 and 7 post-coitum and indentical increases in mean CL numbers were recorded in pseudopregnant rats of both strains.

Histological studies of ovarian tissue confirmed the presence of newly formed CL on day 5 and 6 post-coitum and an absence of entrapped ova; follicular development was also present in early pregnancy with Graafian follicles evident on day 4 post-coitum.

Plasma progesterone determinations revealed no increase in circulating progesterone as a result of SCL formation in either pregnant or pseudopregnant rats.

Supplementary ovulations may be initiated by the preimplantation surge of oestrogen, on day 4 post-coitum, as treatment with the anti-oestrogen, Tamoxifen, inhibits their formation. Investigations into the presence of an appropriately-timed preovulatory surge of gonadotrophins in the early stages of pregnancy however, proved inconclusive.

Supplementary ovulation occurs therefore between day 4 and 7 post-coitum in both pregnant and pseudopregnant Wistar and Sprague-Dawley rats, probably initiated by a consistent physiological event. This phenomena therefore obviously has an impact on the estimation of embryo mortality studies in this species.

CHAPTER 1

INTRODUCTION

In recent years, considerable research in reproduction of the female rat has lead to an established 'model of reproduction' for this species. While undertaking a series of experiments in reproductive physiology for the World Health Organisation (WHO) a phenomenum of rat reproduction not included in this model appeared to present itself, namely the formation of 'supplementary' corpora lutea (CL) in pregnancy.

In 1978, the WHO began an investigation into the possible contraceptive properties of a selection of plant materials with the aim of finding a 'safe' post-coital contraceptive. Centres were established in London, Chicago, Hong Kong, Recife, Sri Lanka and Souel, with a proportion of plants assigned to each centre. The Central Steering Committee (Geneva) drew up a standard '*in vivo*' bioassay protocol, for use by all centres, in order that results could be standardised.

From the London centre, the dried plant material was sent to Leeds University Chemistry Department, where a series of extractions were performed. The resultant crude plant extracts were then tested at the City of London Polytechnic, Department of Biology, using the standard bioassay in rats and hamsters. In both species, the extract was given by oral gavage for the first two weeks of pregnancy. The animals were then sacrificed and the total number of CL embryos/decidualisation sites noted.

This bioassay therefore, covered the possible action of the plant extract on tubal transport of the fertilised ovum, implantation and early pregnancy. If an extract prevented pregnancy, then further research would be undertaken to establish at which stage pregnancy was interrupted and the mechanism involved.

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treated rats became pregnant compared to the untreated controls. The bioassay, therefore, possessed the potential to show a post-coital contraceptive effect.

While undertaking the counts of CL and corresponding embryo/ decidualisation sites, it was noted that the CL counts of the pregnant rats at Day 12 (D12) of gestation (D1 being the day of locating sperm in the vaginal smear) were in excess of those of the unmated controls; as well as observing the expected difference between CL and embryo counts due to embryonic mortality.

During the preliminary development of the bioassay, a high percentage of mated females were not pregnant at D12 of gestation, due probably to poor mating facilities and the lack of a stable environment in the animal house. Stable environmental conditions were obtained, the bioassays re-run, and the difference between CL counts of pregnant and unmated female controls persisted. Routine statistics on the first 3 bioassays revealed this difference to be significant ($p < 0.001$) while accummulation of further data confirmed this significant difference. In contrast, there was no such significant difference in CL numbers between the pregnant and unmated hamster controls.

It was therefore decided to investigate further the ontogeny and possible physiological role of these 'supplementary CL' (SCL) of pregnancy.

The following Chapter reviews the literature related to the mechanisms involved in ovulation, formation of CL and the responsiveness of the ovary during early pregnancy, as these are all factors to be considered in the formation of SCL.

CHAPTER 1:1

THE ENDOCRINE CONTROL OF REPRODUCTION

1 Introduction to Reproduction in the Rat

The rat is a spontaneous ovulator, and as such follows a constant reproductive pattern at the end of which ovulation occurs. This sequence of events is called the oestrous cycle and is repeated every 4 or 5 days depending on the strain. The oestrous cycle can be separated into 4 sequential periods known as dioestrus , pro-oestrus , oestrus, and metoestrus , each of which is characterised by endocrine and physiological events occuring in chronological order (van Tienhoven, 1968), the resultant inter-action of which is to initiate ovulation at late oestrus. Ovulation depends on the involvement of the higher brain centres, hypothalamus, pituitary and ovary which, with their respective hormones, interrelate to bring about ovulation. An indication of the inter-relationships between the components of the endocrine axis controlling ovulation is shown in Figure 1 and will be discussed in the following sections.

2 The Hypothalamic - Pituitary Unit

In Figure 1, it is noticeable that many of the hormones involved in ovulation converge on the hypothalamus, which is closely linked to the pituitary. It may be correctly assumed therefore, that the hypothalamic - pituitary unit plays an essential part in the regulation of the endocrine control of the oestrous cycle (Schally et al, 1973).

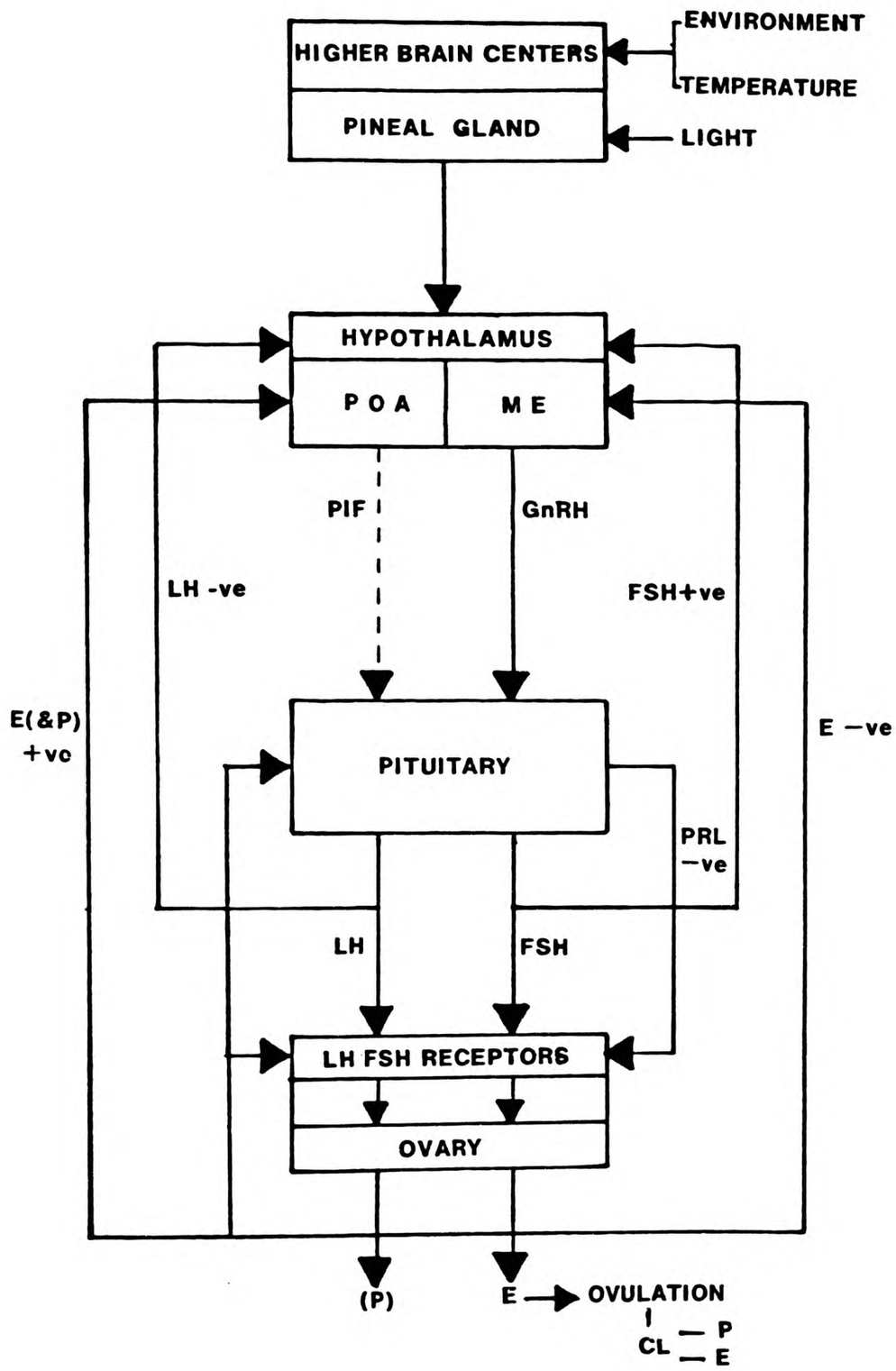
The hypothalamus synthesises and releases hormones, called releasing and inhibiting hormones, which influence the release of luteinising hormone (LH), follicle stimulating hormone (FSH) and prolactin (PRL) which in turn affect the cyclic changes in the ovaries. In detail however, the above is more complex and has been comprehensively reviewed by Everett (1969).

Legend to Figure 1

E	Oestrogen
FSH	Follicle Stimulating Hormone
GnRH	Gonadotrophin Releasing Hormone
HBC	Higher Brain Centres
LH	Luteinising Hormone
ME	Median Eminence
P	Progesterone
PIF	Prolactin Inhibiting Factor
POA	Preoptic Area
PRL	Prolactin

MODEL FOR THE ENDOCRINE CONTROL OF OVULATION

FIGURE 1



McCann et al, (1960) first reported the existence of luteinising hormone releasing hormone (LHRH) in the rat hypothalamus. It was thought that there were two different releasing hormones (RH) for LH and FSH. More recent research however, has shown that LHRH and FSHRH are identical and should be renamed gonadotrophin RH (GnRH), as both LH and FSH are released in response to the stimulus of a common RH (White, 1970). GnRH is a polypeptide of small molecular weight whose synthesis and release is controlled in part by extra-hypothalamic areas of the forebrain. Experiments by Kaves and Halez (1970) suggest that stimulation of the pre-anterior hypothalamus affects the hypophysiotrophic area to produce the cyclic release of GnRH. More recently, Kawakami and Ando (1980) have indicated by means of electrolytic lesions that the diagonal band of broca (DBB) supracommisural area is necessary for acute ovulatory gonadotrophin (GT) release. This area of research is still incomplete.

The target organ of GnRH is the anterior pituitary, (Nalbandov, 1976; Loughlin et al, 1981; Baldwin et al, 1983) and to which GnRH is transported by means of the hypophyseal portal system (Ramirez and Sawyer, 1966). There appear to be periods during the oestrous cycle when the pituitary is more responsive to GnRH and these are temporarily related to the occurance of ovulation (Ramirez and Sawyer, 1966; Savoy-Moore et al, 1980, 1981). Fink and co-workers (1975) have shown a massive increase in the response of the anterior pituitary to GnRH occuring before and during the preovulatory surge of LH. Without this increase in pituitary responsiveness between 13.30h dioestrus and 17.30h pro-oestrus , the preovulatory LH surge may not occur in the rat (Blake and Kelch, 1981). This priming effect of GnRH (Blake, 1976c), has been postulated to be responsible for ensuring that the peaks of responsiveness and GnRH coincide (Sarkar et al, 1976).

The anterior-pituitary response to GnRH is influenced also by oestrogen (Arimura et al, 1971; Libertun et al, 1974; Vilchez-Martinez et al, 1974). Libertun and co-workers (1974) administered exogenous oestrogen followed by GnRH to ovariectomised rats, whose

plasma levels of LH were subsequently enhanced. The priming effect of oestrogen on GnRH is greatest in the afternoon of pro-oestrus , producing an increase in pituitary sensitivity to GnRH during the period of the preovulatory LH surge. There appeared to be a biphasic response of oestrogen on the sensitivity of the pituitary to GnRH and recently, there is evidence that GnRH may increase the oestrogen receptor content of the pituitary, thus enhancing the oestrogen effect (Singh and Muldoon, 1982).

Although oestrogen plays an important role in determining the magnitude of the priming effect of GnRH, Aiyer et al, (1974) indicated in their research that neither oestrogen secreted by the ovary or the adrenals mediated this effect on the preovulatory LH surge, as the profile and magnitude of the LH response to GnRH of adrenalised or ovariectomised rats at pro-oestrus were the same as the controls. The ovulatory threshold of LH may be related therefore to the total amount of hormone reaching the ovary rather than the concentration present in the plasma. Legan and Karsh (1975) suggested that an ovarian hormone was required for an increase in the pituitary response to GnRH but that it was not necessarily oestrogen. However, it is generally accepted that the role oestrogen plays in increasing the pituitary sensitivity to GnRH during the period of the preovulatory LH surge is important in determining the time and magnitude of the surge (Gordon and Reichlin, 1974; Martin et al, 1974). Progesterone has been discounted as a cause of pituitary sensitisation to GnRH at pro-oestrus , as it inhibits GnRH (Gordon and Reichlin, 1974; Libertun et al, 1974). More recently however, Legace and co-workers (1980) concluded that progesterone exerts a positive feedback action on GnRH induced LH and FSH release by direct action at the pituitary level, although this effect is accelerated with oestrogen priming.

The interaction of the ovarian steroids oestrogen and progesterone and LH and FSH at the level of the hypothalamic-pituitary unit is in the form of positive and negative feedbacks, which may be further classified into short or long loops; the former consisting of the

positive and negative feedback of LH and FSH themselves on the hypothalamus and the latter, the positive and negative feedback of the steroid hormones on the preoptic area (POA) and median eminence (ME) of the hypothalamus and also on the anterior pituitary.

(a) Short Loop

These are operative mainly in the infantile stage (Ojeda and Ramirez, 1969) but are present in the adult rat. Implants of LH and FSH in the hypothalmus produced a significant depression of pituitary GT content (David et al, 1966). FSH and LH may modify the activity of the controlling system via changes in the metabolism of the hypothalamus.

There is evidence that the pituitary exercises an internal negative feedback effect on the hypothalamus, as in the absence of the pituitary, GnRH increases (Corben et al, 1970).

(b) Long Loop

The positive and negative feedbacks of oestrogen operate to control the release of GT into the circulation. The POA is responsive to the positive feedback; when oestrogen exceeds a critical plasma threshold, the negative feedback of the steroid is interrupted and the preovulatory surge of LH is released.

The role of the pituitary in ovulation is reviewed by Van Rees (1972).

3 The Ovary

Having discussed the control of the trophic hormones from the hypothalamus and pituitary, the response of the ovary, the target organ as shown in Figure 1, will be reviewed.

The ovarian steroid hormones concerned with ovulation are oestrogen and progesterone. It has been established that oestrogen of follicular origin is important in controlling the onset of ovulation (Schwartz, 1964; Trent-Williams and Lipner, 1980).

The site and formation of steroid hormones in the rat ovary was first described by Falck (1959), who, by isolating cell types, concluded that both granulosa and theca cells were required for oestrogen production. Later research confirmed that granulosa cells played a key role in the process of hormone production in the follicle (Bjersing and Corstenson, 1964; Regan and Short, 1965; Bjersing 1967). Recent views are that the theca is the source of follicular androgen (a precursor steroid in oestrogen production) synthesis, but an interaction between the theca and granulosa is required for production of oestrogen (Moor, 1977). It appears that both cell types act synergistically in order to produce the concentration of oestrogen necessary for triggering the preovulatory LH surge (Bjersing, 1978). The low levels of LH and FSH released from the pituitary (Schwartz and M^{C}Cormack, 1972) stimulate steroid production from the ovaries. Recent findings show that FSH stimulates the granulosa cells of the rat to aromatise androgen (Dorrington et al, 1975; Moor et al, 1975). FSH receptors have been demonstrated in granulosa cells (Zeleisnik et al, 1974; Richards et al, 1976). Theca cells on the other hand possess LH, not FSH, receptors (Monroe and Midgley, 1969; Rajaniemi and Vanha-Pertulla, 1972; Channing and Kammerman, 1974; Amsterdam et al, 1976). It has been postulated that LH stimulates the production of androgens by theca interna cells and FSH affects the aromatisation of these androgens in the granulosa (Armstrong and Papkoff, 1976). Richards and co-workers (1976) showed that FSH together with a local positive feedback of oestrogen, results in a rapid increase in the number of FSH receptors of the granulosa and later a positive increase in LH receptors in the granulosa of developing follicles. This influence of the GT's on their respective receptors has been confirmed by others (Rao and Saxena, 1973; Channing and Tsaf riri, 1977; Lindner et al, 1977).

The resultant production of oestrogen from the follicles results in a rapid increase in plasma oestrogen between 12.00h and 14.00h on pro-oestrus which subsequently serves to activate the neural trigger for the preovulatory release of LH (Kalra, 1975; Apfelbaum, 1983). Thus the positive feedback of oestrogen becomes operational, though this involves oestrogen from the adrenal also (Cambell et al, 1977).

The ovarian oestrogen influence on the response of the rat anterior pituitary to release LH was shown by early researchers such as Bradbury (1947) and Everett (1947). When exogenous oestrogen was administered to entire immature female rats, there followed within 96 hours an increase in plasma GT concentration with a corresponding decrease in pituitary GT content. If the rats were spayed prior to the injection of oestrogen no decrease in pituitary GT potency was seen. Further research by Everett (1948) and Brown-Grant (1969) substantiated the role of oestrogen in the release of LH and subsequent ovulation. Everett showed that the administration of oestrogen at dioestr in 5 day cyclic rats induced ovulation one day early, on pro-oestr , while Brown-Grant demonstrated the same effect in 4 day cyclic rats by injections of oestrogen on metoestrus . Oestrogen antagonists given to rats at pro-oestrus inhibited the preovulatory peaks of LH and FSH (Ferland et al, 1978). However, if an injection of human choriongonadotrophin (HCG) was given at the time of the expected LH peak, ovulation occured, thus the ovaries were still responsive to the GT stimulus (Ferin et al, 1969). Oestrogen, it was concluded, acted at the hypothalamic level to produce the LH release.

It was noted during the experiments to elucidate the role that oestrogen played in stimulating LH production, that there were in fact two effects of the steroid on GT release and oestrogen could also depress LH production from the pituitary (Gans and van Rees, 1962; Callentine et al, 1966; Barraclough and Haller, 1970; Higuchi and Kawakami, 1982), by inhibiting its responsiveness to GnRH (Negro-Vilar et al, 1973). Oestrogen administered in sufficiently large doses, produces a total blockade of pituitary GT secretion

(Nalbandov, 1976). This is the negative feedback of oestrogen previously mentioned and indicated on Figure 1.

Progesterone also has a well established role in controlling the release of GT, though it is not as pronounced as that of oestrogen (Legacé et al, 1980). In 1969, Hashimoto and Wiest found that ovarian vein progesterone peaked prior to ovulation, in the late afternoon of pro-oestrus and suggested that the maturing follicles were the source of the progesterone at this time of the oestrous cycle. This was later confirmed (Szoltys, 1981) and taken further to show that FSH increased progestrone secretion of the granulosa cells in vitro while LH alone had no effect (Armstrong and Dorrington, 1976). The stimulatory effect of progesterone on GT secretion may play a role in the periovulatory period by facilitating the positive feedback of oestrogen and GnRH on GT secretion.

Progesterone is involved also in the rupture of follicles at ovulation, which will be discussed in Chapter 1:3, and acts synergistically with oestrogen in the induction of oestrous behaviour (Boling and Blandau, 1939; Barfield and Lisk, 1970).

It was thought initially that ovarian steroids were secreted into the ovarian blood supply by simple diffusion. However, Gemmel and co-workers (1974) experimenting with ovine CL, (a large source of progesterone) concluded that progesterone, at least, is stored in the Golgi apparatus and discharged from the cell in granules, requiring calcium for actual secretion (Higuchi et al, 1976). There is speculation as to whether steroids of follicular origin are released in the same way.

CHAPTER 1:2

FOLLICULAR GROWTH

AND

DEVELOPMENT

1 Control of Follicular Growth

If ovulation is to occur, it is necessary not only for the hormones which have been discussed to have fulfilled their role, but also for there to be follicles capable of ovulating present in the ovary, to respond to the ovulatory stimulus. The way in which this is attained by the ovary will be the subject of this section.

The pituitary GT's mentioned previously are responsible for the promotion of growth and differentiation of the ovarian follicles, thereby providing mature follicles for ovulation.

The rat ovary contains a pool of germ cells which become surrounded by a layer of granulosa cells, a basement membrane and a theca layer. The growth of follicles in the rat is initiated by an unknown event, which once started, continues until the follicle ovulates or becomes atretic (Richards, 1978; Uilenbrock et al, 1980). The relative stages of growth include small follicles, large preantral follicles and antral preovulatory follicles (Pederson and Peters, 1968). Most of the small follicles grow to become large preantral follicles and then undergo atresia; only a few follicles actually enter the final stages of growth and appear to be selected by the GT surge in the preceding oestrous cycle (Schwartz, 1974).

It would seem that follicles are unequally responsive to GT and the differentiation of follicular cells may determine which follicles become preovulatory follicles. As the granulosa cells of most follicles possess FSH receptors (Richards et al, 1976), while only that of the large preovulatory follicles also contain LH receptors (Zeleisnik et al, 1974), it may be that changes in receptor content may determine the follicular response to GT. Oestrogen increases the responsiveness of rat follicles to GT (Williams, 1945) and ovarian oestrogen may therefore affect the rate of follicular growth by influencing the concentration of receptors for FSH and LH. Oestrogen can also act on granulosa cells to increase its own receptor content (Richards, 1975).

FSH appears to be the hormone responsible for the follicle to aquire the mechanisms necessary to ovulate in response to LH (Goldenberg et al, 1972; Schwartz et al, 1972; Richards, 1978). Oestrogen enhances the responsiveness of the granulosa cells to FSH by affecting the FSH stimulation of its own receptor (Ireland and Richards, 1978). FSH in turn induces an increase in granulosa cell adenosine 3'-5' - cyclic monophosphate (cAMP) (Channing and Seymour, 1970; Nilson et al, 1974) and progesterone production (Richards, 1978).

Although it is established that LH induces luteinisation and ovulation of large preovulatory follicles, its role in the early stages of ovarian follicular development is not well known. It has been indicated that LH is involved in early follicular cell differentiation (Lostroh and Johnson, 1966), but does not appear to have any further involvement until luteinisation.

It is FSH which increases the granulosa cell content of LH receptors in order to enable the follicle to ovulate (Ireland and Richards, 1978). Recent research shows that the precise roles of LH and FSH in regulating theca - granulosa cell differentiation during follicular development are still unclear. It is a topic concerning many researchers, whose results from different laboratories in varying conditions provide conclusions which are confusing when trying to standardise them.

Concerning which follicles are selected to actually become preovulatory follicles and ovulate, it has been suggested that large follicles produce a substance which causes negative selection of smaller follicles (Peters et al, 1973). Welschen and co-workers (1980) postulated that an 'inhibin-like' compound from the granulosa cells of preovulatory follicles called Follicostatin (Erikson and Hsueh, 1978) limits their own number, thereby regulating the number of follicles maturing. There has been little evidence to confirm the above but recent research appears to support this theory (Schwartz and Channing, 1977; Grady et al, 1981; Channing et al, 1981).

2 Follicular Development During the Oestrous Cycle

Follicular growth and development continues throughout the oestrous cycle in order to provide a number of follicles capable of ovulating by late oestrus . At oestrus , day 1 of the cycle, the group of follicles destined to ovulate at the end of that cycle are between 352-517μm diameter (Peppler and Greenwald, 1970). These follicles grow approximately 60 μm per day until the morning of oestrus and ovulation, when they are 650+ μm (Mandl and Zuckerman, 1952).

At the beginning of Chapter 1, it was stated that a rat can have an oestrous cycle duration of 4 or 5 days, and is usually strain dependent. This programming of a 4 or 5 day cycle depends on the timing of the regression of the CL and rise in oestrogen levels, called the luteal-follicular shift. A single injection of LH on the morning of metoestrus converts a 4 day cyclic rat to a 5 day cycle (Buffler and Roser, 1974), due to a sustained secretion rate of ovarian progesterone (Nequin et al, 1975). Genetics, is therefore another factor involved in the control of reproduction.

As previously mentioned, FSH and LH influence the growth of follicles, their increase in plasma concentration during the oestrous cycle occuring on the afternoon of pro-oestrus (Daane and Parlow, 1971). FSH is involved in the conversion of preantral to antral follicles by metoestrus (Schwartz et al, 1973), as an injection of anti-FSH delays the formation of antral follicles (Welschen and Dullant, 1976). Follicles therefore fluctuate in size and number in phase with the oestrous cycle (Mandl and Zuckerman, 1952).

CHAPTER 1:3

OVULATION AND THE CORPORA LUTEA

1 Hormones Initiating Ovulation

In Figure 1, assuming that the organs and their respective hormones have functioned as is necessary for the initiation of ovulation and mature preovulatory follicles are present in the ovary, then the mechanism by which the horomones interelate to bring about ovulation can now be discussed.

The role of the preovulatory surges of ovarian progesterone and oestrogen in the production of LH has been indicated in Chapter 1.1. The release of these preovulatory surges is accurately timed in the rat (Uilenbrock et al, 1976). The oestrogen surge occurs between 05.00h and 20.00h at pro-oestrus (Yoshinaga et al, 1968), while progesterone reaches its maximum plasma concentration on the afternoon of metoestrus. There is also a gradual increase in the level of adrenal progesterone detectable from 09.30h pro-oestrus, prior to the preovulatory oestrogen surge which appears to be required to prime the POA to the later ovulatory feedback of oestrogen (Hoffman and Schwartz, 1965; Gallo and Zarrow, 1970; Feder et al, 1971). Progesterone may exert its effect on the POA by way of enhancement of oestrogen retention by either (i) increasing the hypothalamic oestrogen receptors, or (ii) causing an increase in receptors due to a progesterone mediated block of a later step in the oestrogen-receptor-genome interaction (Reiter and Lisk, 1976). Progesterone has also been shown to have an effect on the timing but not the concentration of the resultant preovulatory surge of LH (Nequin et al, 1975; Schuling, 1980), however, progesterone alone cannot induce LH release (Caligaris et al, 1971b; Swerdloff et al, 1972; Mann and Barraclough, 1973). The following positive feedback of oestrogen initiates the preovulatory surge of GT, via GnRH from the hypothalamus (France and Pincus, 1964; Weik and Davidson 1970; Nalbandov, 1976).

However, as early as the 1940's, it was noted that there was a critical period for hypothalamic stimulation by the ovarian steroids in the rat (Everett et al, 1949; Everett and Sawyer, 1950;

Everett, 1952). Everett (1956) indicated that this critical period occured between 14.00h and 16.00h on pro-oestrus, although for any individual rat the duration of stimulation need only be for any 30 minutes within this period. It was later confirmed that this was a critical period for the initiation of the release of the 'ovulatory GT complex' from the pituitary (Kelly et al, 1963; Feder et al, 1971; Gay and Sheth, 1972). Further research by Blake (1974) using various ovulatory blocking agents prior to the onset of the 'critical period' indicated that the neurohormonal signal stimulates the adenohypophysis to release GT during approximately a 3 hour 'activation period' at pro-oestrus. To inhibit this signal, the blocking agents had to be given during a 7 hour 'potential activation period'. Thus there are 3 stages implicated in the stimulation of LH in the rat. This may account for the fact that the duration of hypothalamic activiation is more important for ovulation, than the frequency of stimulation as appears in some results (Everett, 1965; Dyer et al, 1978).

The resultant preovulatory surge of LH is also accurately timed in relation to the ovulatory surge of oestrogen, occuring between 15.00h and 19.00h on pro-oestrus, 8 to 10h after the oestrogen surge (Yoshanaga et al, 1968; Rodger and Schwartz, 1973; Ramirez and McCann, 1974; Blake, 1976). Blake (1976) detailed the characteristics of the preovulatory LH peak in the rat, which confirmed earlier results (Anderson and McShan, 1966; Monroe et al, 1968; Daane and Parlow, 1971). At 15.00h on pro-oestrus, plasma LH concentration was initially 200 ng/ml reaching a peak at 18.30h of 1700 ng/ml. Over the next 110 minutes, LH fluctuated around this peak declining by approximately 19.00h, though this decline was interrupted by one or more rapid increases in LH.

FSH is released also in response to the positive feedback of oestrogen at the same time as LH (Daane and Parlow, 1971; Rodgers and Schwartz, 1973). It is not fully understood whether the role of FSH in ovulation is facilitatory or obligatory, while the presence of LH is necessary. Kelly et al, (1963) showed that if an anti-LH serum

was injected into rats 12 hours prior to ovulation, then ovulation was inhibited. However, injections of either LH or FSH directly into a follicle will cause it to ovulate 10 hours later (James and Nalbandov, 1972), providing concentrations greater than 100 ng/ml of FSH are used. If less than 100 ng/ml of FSH is injected, the follicles do not luteimise, as they will with low doses of LH. If the two hormones are injected together, then smaller concentrations of both are required for ovulation (Nalbandov, 1976), and it may be speculated that FSH and LH are synergistic and ovulation is brought about by a complex of the two hormones. This would seem more likely from the results than the claims that FSH can act as an ovulatory hormone alone (Mahesh and Goldman, 1970). In all experiments whereby FSH has caused ovulation when administered alone, excessively large doses were used *in vitro* and it is questionable whether the physiological concentration of approximately 100 ng/ml (Uilenbrock *et al*, 1976) would initiate ovulation *in vivo*. Also to be considered, is the possible LH contamination of the preparations of FSH used, as the experiments were undertaken during the early period of obtaining pure preparations.

The actual mechanism by which FSH and LH bring about ovulation is poorly understood, but will be discussed in the following section. The ovary possesses receptors for both LH and FSH, as described earlier (Hermier *et al*, 1970), though only the large preovulatory follicles possess LH receptors (Zeleiznik *et al*, 1974; Rajaniemi and Vanha-Pertulla, 1974; Richards *et al*, 1976), which favours the theory that LH is the predominant hormone concerned with actual ovulation. There is an increase in GT receptor sites as the follicle enlarges (Kamerman and Rees, 1975), which may account for why usually only the preovulatory follicles respond by ovulating, although exogenous GT can induce ovulation in rats at all stages of the cycle other than oestrus, especially at pro-oestrus (Welschen and Rutte, 1971).

It must be noted that prolactin (PRL) is able to influence ovulation and has been postulated to be involved in the inhibition of the latter by reducing the synthesis of oestrogen by the follicle,

thereby preventing the positive feedback of oestrogen (Hamada et al, 1980; Cheung, 1983). PRL can also increase the number of LH receptors of preovulatory follicles (Holt et al, 1976; Grinwich et al, 1976).

LH it seems, is the main ovulating hormone in the rat, which combined with the facilitatory effect of FSH and PRL, initiates ovulation.

2 The Mechanism of Ovulation

Several theories have been put forward over the years as to how the ovulatory hormones actually precipitate follicle rupture, of which many of the early theories can be discounted as ovulation is not an explosive event and cannot be induced by fimbrial massage or abdominal pressure. More recently, experiments indicate that the initiation of follicle rupture is a complex process involving changes in the local blood flow and biochemistry of the follicle.

Concerning blood flow, Nalbandov (1976) postulated that the mature follicles ovulated partly due to a loss of vascularisation and hence a lack of GT support. Observations supported the possibility that, as the largest follicle approaches its ovulatory size the amount of blood flowing in its vascular system becomes proportionally less than that flowing through a smaller follicle. Hence the amount of GT available is less and the follicle has reached a stage where the GT concentration is inadequate to maintain active growth. However, other research has demonstrated, by injection of latex into the ovarian artery, that the majority of the ovarian vasculature is distributed to the large preovulatory follicles (Esprey, 1978). It is possible that, although the larger follicles possess a greater blood supply, the actual volume of blood available to them decreases, thereby supporting Nalbandov. If that is the case however, it would seem logical to assume that there would be a decrease in LH concentration to the follicle, which would not be advantageous for ovulation. There is evidence that LH can drastically reduce blood flow to follicles, which would indicate that a decrease in blood flow

is as a result of the preovulatory LH surge, rather than the decrease reducing LH to the follicle. It is more likely that LH initiates biochemical changes within the follicle when administered in concentrations of that of the LH surge. A detailed summary of how LH converts a follicle that cannot ovulate into one that can is reviewed by Nalbandov (1961).

The biochemical changes within the follicle and their effect on ovulation have been researched in greater detail than has the effect of follicular blood flow. It would appear that these biochemical changes are triggered when the follicular tissue receives the preovulatory surge of GT via the receptors, LH being the essential hormone (Schwartz, 1974; Schwartz and Ely, 1974). The role of FSH in these changes is unclear, its major function being the regulation of the number of follicles maturing (Goldenberg et al, 1972; Welschen et al, 1980). This discussion will therefore confine itself mainly to the action of LH, progesterone and prostaglandin.

The formation of an LH-receptor complex results in the activation of a membrane localised adenyl-cyclase system, which in turn increases the synthesis of cAMP (Mason and Marsh, 1975; Channing et al, 1977). This increase of intracellular cAMP induces a metabolic change in steroidogenesis, thereby increasing oestrogen and progestin production (Hilliard, 1974; Moor et al, 1975). This increase is temporary and declines before ovulation (Esprey, 1978) and may therefore be postulated to be involved with follicle rupture. Progesterone has been suggested to be important in the mechanism of follicle rupture, as intrafollicular injections of progesterone antisera inhibits ovulation in the rabbit (Swanson and Lipner, 1977). Randell (1970) suggests that progesterone acts on secretion and/or activation of an ovulatory enzyme in the interstitial compartments, which affects the collagen framework of the follicle, resulting in an increase in distensibility. These physical changes in the structural elements of the follicle wall, initiated by progesterone, increases the distensibility such that the force of continuous follicular pressure causes the follicle to swell and rupture. However,

Randell's recorded change in distensibility due to progesterone is slight compared to that which normally occurs at the time of ovulation. Thus the role of progesterone in follicle rupture is not definite.

It has been shown that ovarian prostaglandins (PG) increase significantly in response to GT (Armstrong et al, 1974; Bowring et al, 1975) and more recently to GnRH (Clark, 1982; Ekholm et al, 1982). Also, the administration of exogenous prostaglandin E_2 (PGE_2) can induce ovulation in rats (Tsaf riri et al, 1972). There is not sufficient evidence yet to assume that PG's alone can induce follicle rupture, but their action is important in the mechanism of ovulation.

There is also possibly a role for proteolytic enzymes in ovulation, as follicle rupture is dependant on the synthesis and breakdown of collagen in its cell wall (Esprey and Lipner, 1976), thus vitamins are also involved.

The actual mechanism of follicle rupture, therefore, is still uncertain and is an area for further research. Whatever the means, the connective tissue in the follicle wall is degraded and begins to disassociate under the stress of intrafollicular pressure. On rupturing, the corona radiata containing the ovum, is dislodged and expelled from the follicle, the site of rupture being marked by a stigma.

It is pertinent at this stage to mention that external factors such as day length, temperature and stress are able to influence the endocrine factors involved in ovulation, via the higher brain centres. Day length is the most prominent, controlling the time of release of the hormones involved, such as the preovulatory LH surge. Continuous light abolishes the oestrous cycle leading to persistant oestrus without ovulation while continuous dark induces anoestrus (Critchlow, 1963).

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3 The Formation and Function of the Corpora Lutea

The previous sections have dealt with the events involved with and leading to ovulation. Once ovulated, the follicle forms a structure, the CL, which is important in the rat for both the establishment and maintenance of pregnancy and as such requires discussion.

The antral follicle, having ovulated in response to the preovulatory surge of GT releases its ovum into the fimbria of the fallopian tube. The thecal and granulosa cells luteinise and form the CL. In the rat, ovulation occurs 10 to 12 hours after the preovulatory surge of LH (Everett et al, 1949).

The CL has been investigated from as early as 1899 when Gustav Bonn suggested that it was a gland of internal secretion necessary for implantation and early development of the embryo. This was later confirmed in rabbits by Fraenkel (1910). In 1966, the early knowledge of CL function was summarised by Parkes and Deansley, associating its presence with the suppression of ovulation.

Immediately after ovulation, the post-ovulatory follicular cavity is filled with lymph and blood from ruptured thecal vessels (Nalbandov, 1976). The thecal blood vessels then invade the developing CL initiating the gradual hypertrophy and hyperplasia of the granulosa cells, resulting in the eventual obliteration of the follicular cavity (Perry, 1971). The CL volume increases rapidly between 12 and 48 hours post ovulation (Boling, 1942).

The main function of the CL is to secrete progesterone, whose primary role is to condition the uterus to accept and maintain a developing embryo (Perry, 1971). This progesterone secretion has been shown to originate in the 'luteinised' granulosa cells (Short, 1964) while the thecal cells secrete oestrogen (Falk, 1959). In the rat, the CL is already producing small amounts of progesterone one day after ovulation (Eto et al, 1962). Hunzicker-Dunn and Birmbaumer (1976) have suggested that adenylate cyclase maybe related to progesterone

production by the newly formed CL, as post ovulatory progesterone exhibits a similar pattern to that of the responsiveness of adenylate cyclase to LH. 20α-hydroxy-progesterone (20α-OH-P)is a major progestin secreted by the ovary immediately after ovulation at an approximately 9 times greater concentration than progesterone (Hashimoto et al, 1968). However, 20α-OH-P has little progestational activity and in cycling rats, progesterone is reduced to 20α-OH-P by 20α-hydroxysteroid dehydrogenase. The CL of the oestrous cycle, therefore, does not secrete sufficient progesterone to condition the uterus to implantation (Yoshinaga, 1978). If mating does not occur, the CL is then induced to degenerate after 2 days (Austin and Short, 1972) by a uterine luteolytic substance identified as prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$) in the rat (Stacy and Gemmell, 1978; Thomas et al, 1978).

As the luteal phase in the rat oestrous cycle is so short, should fertilisation occur, the fertilised ovum would arrive on D5 post-ovulation in an unprepared uterus, with little progesterone to support it (Nalbandov, 1976). The rat has, therefore, developed a mechanism to overcome this, allowing pregnancy to take place. On mating, nerve endings in the vagina and cervix are stimulated by the males penile spines (Zarrow and Clarke, 1968), resulting in a neuro-hormonal reflex which stimulates PRL release from the adenohypophysis (Kollar, 1953; Carner and Taleisnik, 1970; Nallbandov, 1976). PRL is luteotrophic (Sammelwitz et al, 1961; Perry, 1971) maintaining cholesterol ester synthetase activity and hence progesterone production by the CL until D7 post-coitum (Morishige and Rothchild, 1974). This period of support of the CL is known as pseudopregnancy and will continue for 12 days in the rat, even if the mating is unfertile (de Greef et al, 1977). Psuedopregnancy is not an ovulatory cycle with the dioestrus phase extended; the CL is active and secretes progesterone which in turn plays a role in the regulation and secretation of LH during pseudopregnancy (Koiter et al, 1979).

PRL in the rat is responsible for transforming the CL of the oestrous cycle to that of pregnancy/pseudopregnancy (Smith, 1980). Dey and

between PRL and the maintenance of the luteal adenyl-cyclase response to LH and suggested that the surge of PRL early on D3 post-coitum is critical for survival of the CL and secretion of progesterone (Astwood, 1941). Dubreuil (1962) classified the CL of pregnancy into two types; progestational, prior to implantation and gestational, after implantation.

In pregnancy, it is probable that the blastocyst initiates via the release of hormones, a uterine neural reflex (Dickman and Dey, 1974) causing the release of PRL from the pituitary and thus maintaining pregnancy until D7 post-coitum (Morishige and Rothchild, 1974). However, Moudgal and co-workers (1974) established that specific LH antisera administered between D1 and D12 post-coitum lead to spontaneous abortion. They postulated that LH activates the esterase involved in the conversion of free cholesterol to progesterone. LH is required from the pituitary until D12 of pregnancy only, as a placental luteotrophic complex maintains pregnancy after this time (Astwood and Greep, 1938; Linkie and Niswender, 1973). An increase in plasma PRL is noticable also on the afternoon of pro-oestrus (Blake,1974).

In the rat it would therefore appear that both PRL and LH are luteotrophic in the initial days post-coitum, as PRL alone cannot support pregnancy in the absence of LH (Madwha and Moudgal, 1970). Richards and Williams (1976) have shown that in the antral follicles of the rat, both LH and PRL receptors increase during the differentiation of granulosa cells into luteal cells. This appeared to facilitate progesterone secretion (Smith et al, 1975). When LH and PRL are administered to rat lutein cells in vitro, progesterone secretion was maintained and 20α-OH-P was kept at a low level, unlike the situation existing in the CL of the oestrous cycle which is influenced by LH only (Wu et al, 1976). PRL suppresses 20α-hydroxysteroid dehydrogenase activity and daily surges of PRL are detectable in pregnancy and pseudopregnancy (Freeman et al, 1974).

LH may also play a role in programming the lifespan of the CL in pseudopregnant rats, during days 1 to 3 post-coitum (Takeshashi, 1978; Cochrane, 1983).

Intraluteal oestrogen has been shown to participate in the regulation of luteal function during pregnancy by acting locally to maintain luteal progesterone synthesis and exogenous oestrogen treatment in the pseudopregnant rat can prolong the CL for up to 6 to 8 weeks (Bogdanove, 1966).

During pregnancy, progesterone secretion by the CL is greater than that of the CL of the oestrous cycle and the maximum output is between D12 and D14 post-coitum, in the rat (Hashimoto et al, 1968; Madwha and Maudgal, 1970). Levels of 260 ng/ml plasma on D14 have been measured by Grota and Eik-nes (1967), declining to 10 ng/ml by D22 of pregnancy. This increase during mid-pregnancy was postulated to be due to an increase in ovarian blood flow but experiments by Bruce and co-workers (1980) have indicated that progesterone secretion is not determined by the rate of ovarian blood flow. The CL is the major source of progesterone in the rat, as the placenta does not contribute unlike in some mammals (Yoshinaga, 1978), it is therefore essential throughout pregnancy and abortion occurs if ovariectomy is performed as late as 5 days prior to parturition (D21-22 post-coitum) (Nalbandov, 1976).

This section would not be complete without reference to PG, especially $PGF_{2\alpha}$, which initiates luteal regression in the rat (Pharriss and Wyngarden, 1969). Pharriss and co-workers (1970) postulated that $PGF_{2\alpha}$, exerted its effect on the CL by venoconstriction, as injections of high doses of $PGF_{2\alpha}$, caused acute reduction in ovarian venous blood flow. This may not be the case, as $PGF_{2\alpha}$, was administered in non-physiological concentrations, in fact other researchers have reported no decrease in ovarian blood flow due to exogenous $PGF_{2\alpha}$, (Behrman et al, 1971; Labhsetwar, 1974). Thomas

et al (1978) postulated that the rapid regression of luteal cells by the action of PG is due to a block of the LH dependant production of cAMP, resulting in a decrease in progesterone secretion.

It is possible, though, that PG's, especially $PGF_{2\alpha}$, exert profound effects on the ovary and evidence indicates that they bring about regression of the CL at the end of pseudopregnancy/pregnancy in the rat. PG's originate from the uterus and a comprehensive review of the effects of PG's and the uterus on CL function and maintenance is edited by Anderson et al (1969).

4 Evidence for Coitally-Induced Ovulation

Earlier, it was stated that the rat is a spontaneous ovulator and the preceeding sections have therefore looked at reproduction of the rat in this light. It is perhaps important though to note that ovulation in the rat has been shown to occur in response to copulation (Aron et al, 1966; Zarrow and Clarke, 1968; Rodgers, 1971). The rat therefore, although normally considered as a spontaneous ovulator may produce coitally-induced ovulations, if mating occurs between 17.00h and 18.30h on pro-oestrus (Rodger, 1971).

This induction of ovulation is in response to the neuro-hormonal reflex triggered by copulation, resulting in the release of LH and pelvic neurotomy abolishes this response (Harrington et al, 1966; Zarrow and Clark,1968).

The effectiveness of the copulatory stimulus to induce LH release diminishes over the evening of pro-oestrus, due probably to a failure of GnRH release or a decrease in pituitary sensitivity to GnRH (Tyler and Gorski, 1980). Tyler and Gorski also extend the time given by Rodger (1971) in which copulation stimulus has to occur, to 21.00 hours on pro-oestrus, concluding that there is a narrow temporal window existing for copulation induced ovulation.

Investigation into the pathway involved in the copulation-induced ovulation reflex appears to involve the posterior hypothalamus and the preoptic region, where the stimulus may influence the cyclic ovulatory centre (Kalra and Sawyer, 1970). This corresponds to earlier experiments by Sawyer and co-workers (1949), which indicated that the hormonal component of the stimulus traverses the pituitary stalk via the hypothalamic portal system to initiate ovulation. The copulation stimulus may exert its effect, therefore, at the level of the hypothalamus (Harrington et al, 1966).

The surge of LH released in response to the coital stimulus appears to occur shortly after copulation, as there is a smaller time lapse between copulation and LH release than between the positive feedback of oestrogen and LH release at oestrus . LH can be released 30 to 60 minutes after copulation (Tyler and Gorski, 1980) compared to approximately 8 hours after the preovulatory oestrogen surge (Johnson and Everitt, 1972). This may be explained by the fact that the coital stimulus is neuro-hormonal, thus the neural impulse from the cervix will reach the hypothalamus in a short time, releasing the hormonal component, LH.

The CL produced by copulation-induced ovulation would appear to be functionally equivalent to those produced by spontaneous ovulation and the resultant ova capable of fertilisation. Experiments by Everett (1967) in which rats whose spontaneous ovulations had been blocked, but allowed to ovulate in response to coitus, produced a 25 per cent pregnancy rate.

This information though has implications when classifying a mammal as either a spontaneous or induced ovulator (such as a rabbit, (Heape, 1905)),as was the common practice. It is possible that a mammal can be predominantly one or the other, but capable of showing characteristics of the alternative in certain circumstances. Certainly, this appears true of the rat.

CHAPTER 1:4

OVARIAN FOLLICULAR ACTIVITY

DURING

PREGNANCY

1 Follicular Development and Steroidogenesis During Pregnancy

The follicular development of the ovary during the oestrous cycle has been reviewed and it is considered pertinent to extend this to follicular activity during pregnancy, as the latter was of concern in the following experiments.

It is commonly accepted that, with the exception of the CL, the ovary is inactive during pregnancy and pseudopregnancy. There is no cyclic follicular activity during pregnancy and follicles larger than 600 µm in diameter do not appear until D22 in the rat (Greenwald, 1966). This inhibition of follicular growth and ovulation is usually attributed to the increased secretion of oestrogen and progesterone from the CL acting to suppress the pituitary hormones responsible for ovarian stimulation (Amoroso and Finn, 1962). Throughout pregnancy, plasma LH and FSH levels are less than dioestrus values (Greenwald, 1978). Greenwald (1966) could not initiate ovulation by HCG until D20 of gestation in Holtzman rats, but Wistar rats could be induced to ovulate, although not consistently (Brown-Grant,1969). Strain difference may be responsible for this discrepancy, but there is evidence in the rat that the inhibition of ovarian activity during pregnancy is incomplete.

Swezy and Evans (1930) suggested that there was a 4 to 5 day cycle of ovagenesis occuring during the rat oestrous cycle which was not interrupted by pregnancy. Mature follicles were present on days 5, 10, 14 and 18 post-coitum and a few even resulted in the formation of CL; these were one third the size of those of pregnancy and although they did not posess retained ova, no ova could be located either in the Fallopian tube. They also noticed the presence of luteinised follicles. At D5 post-coitum the presence of, on average, three mature follicles of 500-600 µm in diameter have been recorded per rat (Greenwald, 1966). In the pseudopregnant rat, it was observed that ovulatory growth had occured by D12 post-coitum (Welschen et al, 1964). Brown-Grant (1943) agreed with Swezy and Evans (1930), suggesting that ovarian follicles develop to a stage responsive to

exogenous GT at the same rate during early pregnancy as in the normal oestrous cycle.

The follicles secrete oestrogen during early pregnancy (Kraicer, 1969) and if all follicles are destroyed at this time, the CL continue to secrete progesterone but little or no oestrogen is detectable (MacDonald et al, 1969). This output of oestrogen increases on D4 post-coitum between 14.00h and 16.00h (D1 being the day of locating a copulatory plug) (Yoshinaga et al, 1968; Shaikh, 1971) and is involved in the initiation of implantation on D5 post-coitum (Psychosos, 1963; Deansley, 1966). In ovariectomised rats, implantation occured providing progesterone was administered daily with an injection of oestrogen on D4 post-coitum. If oestrogen was not given at all or it was administered on another day other than on D4 post-coitum, implantation did not occur or was delayed (Shelesnyak et al, 1963; Perry, 1971). It would appear that FSH and LH are required to maintain this oestrogen surge as the ovary fails to secrete oestrogen after auto-transplantation of the pituitary (Perry, 1971). This oestrogen surge occurs at the same time in the pseudopregnant rat (Shelesnyak et al, 1963) and is in the region of 1.10 ng oestrogen/ml (Yoshinaga et al, 1968).

2 Evidence for Ovulation During Pregnancy/Pseudopregnancy

It is pertinent in this thesis to investigate whether it is possible to induce the formation of CL during pregnancy/pseudopregnancy and from the previous section, it is possible that the ovary may be receptive. If ovulation is to occur post-coitum then, (i) there must be follicles competent to ovulate present in the ovary and, (ii) the pituitary has to be responsive to the positive feedback of oestrogen and GnRH and to possess sufficient concentrations of LH and FSH.

In the first case, it has already been indicated that there is controversy over this issue. Early researchers concluded that follicular development did continue in early pregnancy (Swezy and

Evans, 1930; Brown-Grant, 1943) while later experiments contradicted these findings (Amoroso and Finn, 1962; Greenwald, 1966).

Brown-Grant (1943) in his experiments to confirm that follicular development is retained, at least in early pregnancy, was able to obtain ovulation when administering exogenous oestrogen on D4 post-coitum in Wistar rats. There appeared, though, to be a strain difference as to whether and when ovulation occured in response to exogenous oestrogen. This response was confirmed in pseudopregnant rats by Everett and Nichols (1968) who administered exogenous oestrogen at 12.30h on D4 post-coitum, obtaining ovulation by D6 post-coitum. They determined also that the exogenous oestrogen initiated an LH surge at hours corresponding with the 'critical period' for spontaneous release of LH at pro-oestrus. Later experiments by Everett (1977) also showed that ovulation could be induced by exogenous oestrogen in pregnant and pseudopregnant rats, but that there was a strain difference affecting this response.

As already stated, exogenous oestrogen given on D4 post-coitum was able to elicit a surge of LH (Everett, 1977). It can be concluded, therefore, that the pituitary is responsive to the positive feedback of oestrogen and GnRH at this time. There is evidence also that the pituitary concentrations of LH during the early stages of pregnancy do not remain static. Zeiner (1952) detected fluctuations in the pituitary GT content throughout pregnancy in the rat, which were most prominent during the first two weeks. These fluctuations were rhythmic in nature as regards FSH and LH and may be evidence that the rhythm of GT fluctuations seen during the oestrous cycle, are maintained in early pregnancy. This is confirmed by Everett (1977), who detected concentrations of pituitary LH on days 6 to 9 post-coitum, comparable to that of pro-oestrus.

There has been little research on this subject in recent years and this data may therefore be questionable, owing to the techniques used at that time. It does, however, correspond with Everett's (1977) later results when inducing ovulation with exogenous oestrogen.

It is possible that a further physiological source of LH is involved in the induction of ovulation during pregnancy. Bambra and Gombe (1978) showed the presence of an 'LH/FSH like' hormone produced by the chorionic cells of the rat placenta, which could be involved in the induction of ovulation during pregnancy, in a manner similar to pregnant mare's serum (PMSG) in the mare (Amoroso, 1955). Everett, however detected his LH surge on late D4 post-coitum, when the rat placenta has not been established, thus Bambra and Combes' 'GT-like' hormone could not contribute to an LH surge at this time. It is possible that 'GT-like' hormones which are possibly released by the embryo to indicate pregnancy to the maternal reproductive system (Moor, 1968) may be included in Everett's LH surge on D4 post-coitum.

It is therefore feasible that the reproductive axis shown in Figure 1 is still operational, at least during early pregnancy in the rat.

CHAPTER 1:5

THE OVUM

1 Initial Ovum Development after Ovulation

In order to complete this review, the early development of the ovum after ovulation should be mentioned as a knowledge of this subject will be valuable when considering the functional viability of the SCL, compared to the normal CL of pregnancy/pseudopregnancy.

The fertilised ovum is a single cell and has to be transformed into a multi-cellular body in order to produce the complex arrangement of cells which is the foetus. This transformation takes place at the beginning of development and is attained by a series of cell divisions following in rapid succession, called cleavage (Pincus, 1936; Balinsky, 1981).

The cleavage of the fertilised ovum is initiated by the division of the nucleus, followed by the division of the cytoplasm, resulting in the formation of two cells or blastomeres; subsequent divisions produce 4, 8, 16 and 32 cell embryos eventually leading to the morula stage of development.

Initially, blastomeric cleavage tends to occur simultaneously but this synchronisation is soon lost and the blastomeres then divide in an independant, asynchronous manner (Balinsky, 1981).

In the rat, the ova are in various stages of maturation when ovulation occurs (Nicholas, 1942). This is reflected in the variability in cleavage rates and 2, 3 or 4 cell ova can be found in the Fallopian tubes 48 hours after copulation. Generally, the cleavage rates for rat ova can be related to the post-coitum interval as follows (Nichols, 1942):

(i) cleavage to 2-cell - late D1
(ii) cleavage to 4-cell - late D2
(iii) cleavage to 8-cell - late D3
(iv) cleavage to 16-cell - late D4
(v) Entry into the uterus and implantation - late D4 to D5

However, during the period that the ova are held in the Fallopian tube by the utero-tubal junction, some ova show little or no development or definite degenerative changes resulting in fragmentation (Long and Evans, 1922). On entry into the uterus on late D4, the zona begins to degenerate, due probably to a uterine factor (Orsini and McLaren, 1967).

There is little information existing on the fate of the unfertilised ovum after ovulation, the only recently published research appearing to be that by McLaren and Orsini (1968) in the mouse. Their experiments indicated a difference in the degeneration rate between unfertilised ovum from cyclic mice and those of pseudopregnancy; the former degenerating more quickly than the latter. In mice at D4 post-ovulation, no ova at the 1-cell stage were located whereas 39 per cent of ova recovered at this stage in pseudopregnant mice were at the 1-cell stage. Unfertilised ova of pseudopregnant mice also retain their zona for a longer period of time compared to those of cyclic mice, although the vitellus does break up. This could account for the absence of 1-cell stage ova on D4 in cyclic mice, as if the zona is lost then the vitelline fragments may escape detection. These observations are in agreement with earlier work by Charlton (1917).

Initial ovum development after fertilisation, therefore proceeds in set stages, even if the time taken to reach each stage is not constant between ova, while unfertilised ova appear to follow no set pattern.

MATERIALS

AND

METHODS

CHAPTER 2

GENERAL METHODS

1 Animals

500 female Wistar (Nottingham University) and Sprague-Dawley (CD) Charles River (Margate, Kent) rats of 200 to 300g body weight, were kept in pairs on sawdust in plastic bottomed cages, with access to food and water ad-libitum and in an ambient temperature of 22°C. The Sprague-Dawley (CD) strain was used in the London experiments. These were fed on Dixons FF(G)M diet (see Appendix 1) and kept on a dark:light cycle of 9:15 hours (light period = 0700h to 2200h). The Nottingham experiments used the Wistar strain, with access to a diet prepared locally (see also Appendix 1) and on a 10:14 hours dark:light cycle (light period = 0700h to 2100h).

2 Investigation of Cyclicty

The regular cyclicity of females was established by vaginal smears using a clean pasteur pipette adapted to fit the vagina without stimulating the cervix and filled with a small amount of physiological saline: this was ejected into, and aspirated from, the vagina and then expelled onto a microscope slide for observation under a light microscope. The cell types recovered from the vagina were assessed and the stage of the cycle established by the following criteria:-

Stage of Cycle	Cell Morphology		
	Leucocytes	Cornified Epithelia	Epithelial Cells
Proestrus	±	+	+
Oestrus	-	+++	-
Metoestrus	+	++	++
Dioestrus	+	-	++

3 Mating Procedures

Females showing regular cyclicity were mated with proven intact or vasectomised males, depending on the experimental protocol. Females were placed overnight (16.30h to 10.00h) in wire bottomed cages with a male and the trays under the cages were examined for vaginal plugs, as an indication of mating. Initially, where vaginal plugs were located, a vaginal smear was taken and examined under a light microscope for the presence of sperm. If more than 10 sperm were present in the smear, the female was considered to be positively mated. In later experiments, vaginal plugs were considered to be satisfactory evidence of a positive mating, as evidenced by the pregnancy rate achieved.

After mating, females were returned to plastic bottomed cages and allocated to an experimental regime. The day of finding a plug and/or sperm was designated Day 1 (D1) post-coitum. Unless specified, controls were unmated females in which vaginal smears were used to establish the day of the oestrous cycle.

4 Sacrifice and Autopsy

All females were sacrificed using chloroform and cervical dislocation. At autopsy, for pregnant females, the total number of decidual swellings was noted for both uterine horns and was taken to represent the total number of embryos *in utero* at the time of implantation. The total number of CL was also recorded for each ovary. A preliminary count was estabished by superficial observation of the ovary and was substantiated by extruding each CL from the ovary using a dissecting microscope and microforceps. After the initial evidence for SCL had been established, each subsequent count of CL number was undertaken "blind", so as to remove technical bias. The counts were therefore conducted without the knowledge of the day of gestation at which the rat was sacrificed; counts were also done independantly by two people and recounts were carried out using a third person if necessary to reconcile differences.

In selective experiments, ovarian tissue was stored in 10% Formal saline for histological sectioning, in which case the number of CL was established by superficial observation only.

5 Radioimmunoassays

a Progesterone

Plasma samples were assayed for progesterone as described by Haresign, Foster, Haynes and Lamming (1975). Samples were assayed in duplicate using 20µl of a 1 in 10 dilution of plasma and were extracted by shaking at room temperature for 20 minutes, after the addition of 2ml of petroleum ether (Mallenchrodt Chemical Works). Samples were then frozen at -20°C for an hour and the solvent decanted and placed in a water bath at 37°C to evaporate the solvent. 0.1ml of assay buffer, 0.1ml antiserum (GSP 711/12 at 1:6000 dilution) and 0.1ml 3H-Progesterone (Amersham International Ltd) was added, vortexed and left at 4°C overnight. Charcoal dextran suspension (0.5ml) was added and incubated at 4°C, then centrifuged for 10 minutes at 2000 rpm, also at 4°C. 2ml of scintillant (Fisoflour 3, Fisons Scientific Apparatus) was added to the decanted supernatent and the scintillation vials incubated in a water bath for 30 minutes at 70°C, prior to counting.

Two ether blanks, measured in each assay, gave values of less than 0.8ng progesterone per ml and the recovery of progesterone was 95%. Standard plasma samples were assayed as for the unknown samples and were used to determine the intra-assay coefficient of variance, which was less than 8%. The inter-assay coefficient of variance was less than 10%. All results were expressed as ng of progesterone/ml of plasma.

b Follicle Stimulating Hormone (FSH)

Plasma and pituitary samples were assayed using a rat FSH kit supplied by the 'Rat Pituitary Hormone Distribution Program, NIAMDDNIH',as described by Sasamoto, Harada and Taya (1979). All plasma samples were assayed in duplicate using 100 and 200ul aliquots. Buffer was added to the plasma samples to give a final volume of 400µl. Radio-iodonated NIAMDD-r-FSH-1-5 (100µl) was added with 100µl of first antibody, to give a final tube dilution of first antibody of 1:10.000. All samples were incubated at room temperature for 24 hours prior to the addition of second antibody. For standards, lyophilised NIAMDD-r-FSH-RP-1 was solubilised in 1% bovine serum albumin (BSA) in phosphate-buffered 0.9% saline (PBS) at a concentration of 100µg/ml.

The supernatent fraction of the pituitary homogenate was diluted five-fold with 0.5% BSA in 0.001M PBS, pH 7.4(0.5%BSA) and assayed in duplicate in 50, 100 and 200µl aliquots, with a final tube dilution of first antibody of 1:10.000.

Radioimmunoassay results were expressed as ng NIAMDD-r-FSH-RP-1/ml plasma or ng NIAMDD-r-FSH-RP-1/pituitary gland. The intra-assay coefficient of variance was less than 6% and the inter-assay coefficient of variance was less than 10%. The lower limit of sensitivity was 10ng/tube (50ng/ml plasma using 200µl aliquots of plasma).

c Luteimising Hormone (LH)

Plasma and pituitary LH samples were assayed using a rat LH-RP-1 kit supplied by the 'Rat Pituitary Hormone Distribution Program, NIAMDDNIH' as also described by Sasamoto, Harada and Taya (1979). Plasma samples were assayed in duplicate in 100 to 200ul aliquots. Buffer was added to give a final tube volume of 400µl. 100µl of NIAMDDK-r-LH-1-5 and 100µl of first

antibody was added to give a final tube dilution of first antibody of 1:40000. Tubes were then incubated at room temperature for 24 hours, prior to the addition of second antibody. For standards, the vial of NIADDK-r-LH-RP-2 provided, was dissolved in 1ml of distilled water (10µg/ml in 1% BSA).

The supernatent fraction of the pituitary homogenate was prepared and assayed as described previously for FSH analysis and radioimmunoassay results were expressed as ng NIADDK-r-LH-RP-2/ml plasma or ng NIADDK-r-LH-RP-2/pituitary gland.

The intra-assay coefficient of variance was less than 5% and the inter-assay coefficient of variance was less than 15%. The lower limit of sensitivity was 0.1ng/tube (0.5ng/ml plasma using a 200µl aliquot).

6 Histology

Ovaries were stored in 10% Formal saline for at least one month and then processed through a series of changes of alcohol dilutions and xylenes and subsequently vacuum embedded in wax blocks. Serial sections were cut at 10 or 7µm, using a microtone and floated out on a hot water bath prior to mounting and staining with haematoxylin and eosin. Cover slips were placed over the sections when dry, sealing with Histomount.

7 Blood Sampling

All blood samples were taken at sacrifice by cardiac puncture. Four ml samples were usually obtained, centrifuged at 5000 rpm for 10 minutes and the resultant plasma decanted and stored at -20oC until assayed.

8 Statistics

Students' 't' test was used to determine any significant difference between two groups of data. Analysis of variance and analysis of variance for overall regression were employed for a more detailed analysis of large groups of data. A Cusum plot (Mulhalland, 1980) was used to investigate separate trends in the data.

CHAPTER 3

THE AETIOLOGY OF SUPPLEMENTARY CORPORA LUTEA

Introduction

The accumulation of data from the World Health Organisation (WHO) experiments indicated that pregnant Sprague-Dawley rats exhibited a greater number of CL than did non-pregnant controls ($p<0.001$). Subsequent experiments were therefore designed to determine the aetiology of these extra, or supplementary CL (SCL).

It was considered pertinent to determine the stage of gestation at which SCL were formed and whether they represented ovulations or lutenised follicles. Hence, after the time of SCL formation had been determined, the recovery of ova was attempted as a means of establishing whether or not ovulation had occured.

Furthermore, the study was expanded to determine whether the formation of SCL was strain specific and to investigate if SCL might enhance secretion of progesterone.

Experiment 1

Corpora Lutea Observations from the WHO Experiments

Experimental Procedure

The initial evidence that the number of CL increased during pregnancy in the rat was obtained from the WHO experiments, in which female virgin Sprague-Dawley rats were mated with proven intact males and subsequently sacrificed on D12 of gestation. The CL were observed and counted as stated in the general methods. The number of embryo/decidualisation sites were counted also after making a longitudinal antimesometrial incision in the uterine wall, thereby exposing the endometrium. The number of CL and embryo/decidual swellings were compared to those of non-pregnant cyclic females sacrificed in the luteal phase. This was also repeated for the hamsters used in the WHO protocols.

Results and Conclusions

The mean number of CL and decidual swellings for Sprague-Dawley rats are shown in Table 1.

Table 1 - Mean numbers of CL in non-pregnant and pregnant rats on D12 of gestation

	Mean No of CL ± SEM	Mean No of decidual Swellings ± SEM	n
Pregnant Females	17.75 ± 0.32	12.88 ± 0.32	146
Non Pregnant Females	10.65 ± 0.74	-	34

Students' 't' test showed a statistical difference ($p<0.001$) between non-pregnant cyclic control and pregnant rat CL numbers at D12 of gestation. These extra CL were termed supplementary CL (SCL). The mean number of CL for non-pregnant control and pregnant hamsters on D10 of gestation showed no similar significant difference; although a similar trend was apparent. (The mean number of CL for 30 non-pregnant and pregnant hamsters being 9.90 ± 0.50 and 12.40 ± 0.58 respectively.) However, while the mean number of decidualisation sites for pregnant rats was less than that of the CL number at D12 of gestation, that of the hamsters on D10 of gestation was similar.

It may therefore be that the apparent increase in hamster CL number between the non-pregnant and pregnant females may be due to coitally induced ovulations only (Zarrow & Clarke, 1968; Rodgers, 1971).

While this thesis is concerned ony with the formation of SCL in female rats post-coitum, it may be of interest to expand these studies to include the hamster.

Experiment 2

The Timing of SCL Formation

Experiment Procedure

In order to establish the aetiology of the SCL, 97 virgin Sprague-Dawley females were mated with intact males and randomly allocated to groups which were sacrificed on days 1-9 post-coitum and the number of CL and, where applicable, decidualisation sites were counted. Control females were sacrificed in the luteal phase and the number of CL per rat determined.

Data was also collated for the mean number of CL on consecutive days post-coitum, incorporating all Sprague-Dawley rats studied.

Results and Conclusions

The mean number of CL for the 97 Sprague-Dawley rats sacrificed on days 1-9 post-coitum, are shown in Table 2 and Text Figure 2.

Table 2 - The mean number of CL and decidual swellings for Sprague-Dawley rats sacrificed on days 1-9 post-coitum

Day of Gestation	Mean Number of CL ± SEM	Mean Number of Decidual Swellings ± SEM	Total Number of Rats
1	15.30 ± 0.47		10
2	14.40 ± 0.85		10
3	14.27 ± 0.82		11
4	15.80 ± 0.89		10
5	14.00 ± 1.13		11
6	15.92 ± 0.93		12
7	17.36 ± 0.78	13.21 ± 0.76	14
8	18.27 ± 0.57	15.73 ± 0.85	11
9	19.57 ± 1.04	14.14 ± 1.72	10
Control	9.94 ± 0.79		10

Results from a comprehensive study of the mean number of corpora lutea on consecutive days post-coitum for all Sprague-Dawley rats studied are shown in Table 3 and Text Figure 3.

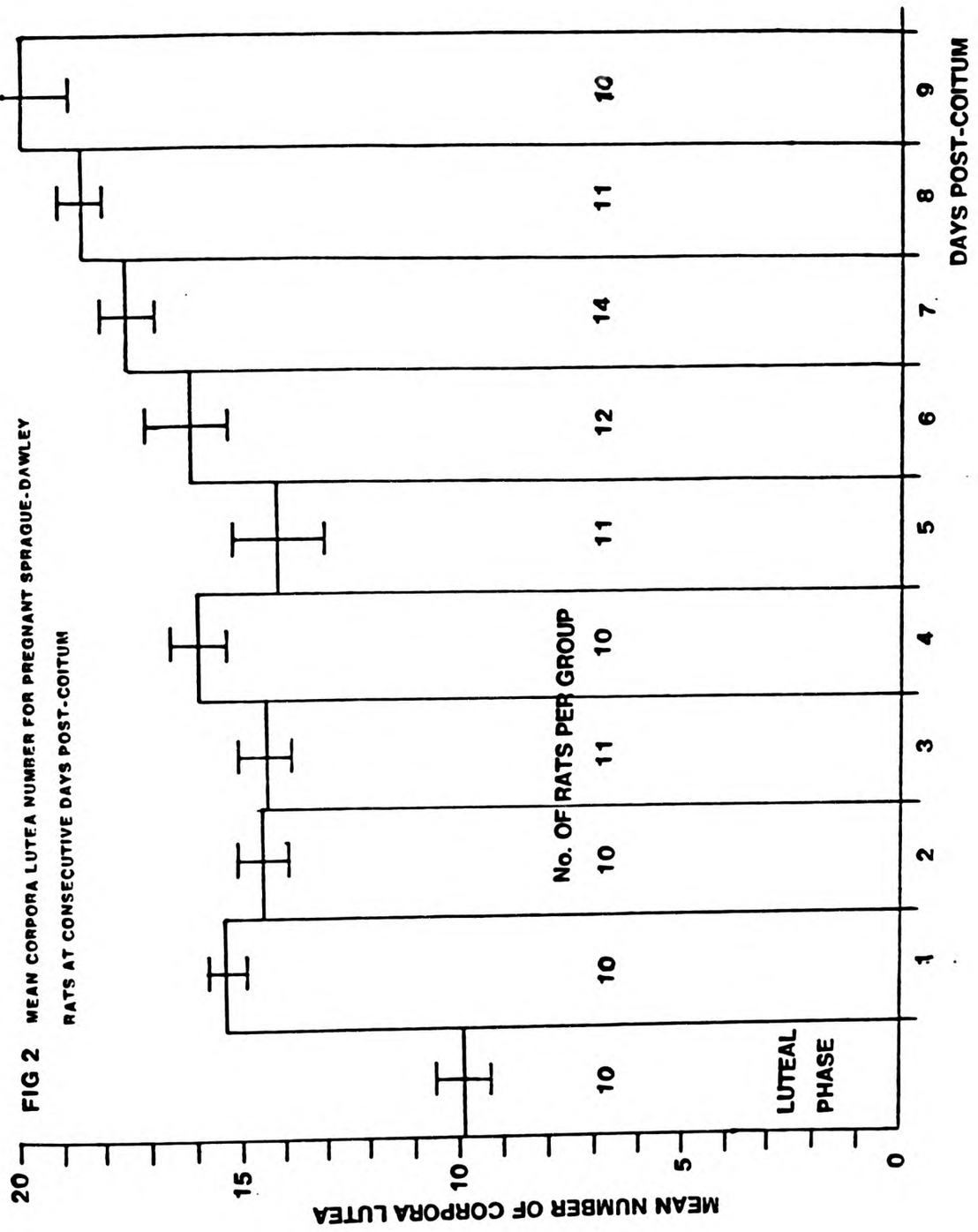


FIG 2 MEAN CORPORA LUTEA NUMBER FOR PREGNANT SPRAGUE-DAWLEY RATS AT CONSECUTIVE DAYS POST-COITUM

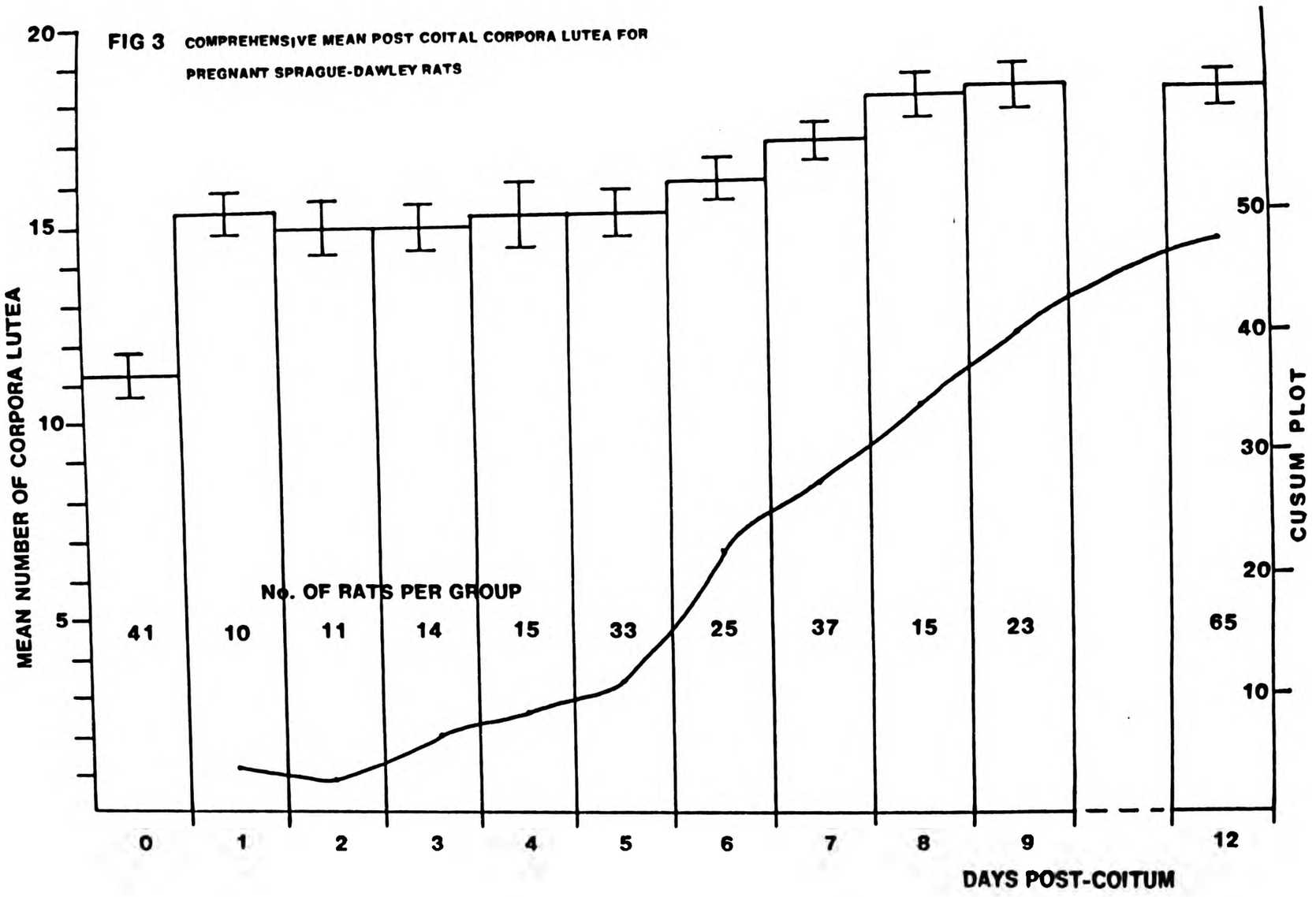
Table 3 - A comprehensive study of the mean number of CL on consecutive days post-coitum for all Sprague-Dawley rats studied

Day of Gestation	Mean Number of CL ± SEM	Total Number of Rats
1	15.3 ± 0.47	10
2	14.9 ± 0.64	11
3	14.9 ± 0.51	14
4	15.2 ± 0.66	15
5	15.2 ± 0.45	33
6	15.9 ± 0.50	25
7	16.8 ± 0.38	37
8	17.9 ± 0.53	15
9	18.2 ± 0.52	23
12	18.0 ± 0.43	65
Control	11.3 ± 0.61	41

Using Student's 't' test, a significant difference ($p<0.001$) was initially recorded between the mean number of cyclic controls and mated females on D1 post-coitum.

A Cusum plot (Mulhalland, 1980) of the data also revealed that a further sustained increase in CL numbers occured at D6, as indicated in Text Figure 2. The data could therefore be divided for further analysis on this basis and a Student's 't' test showed a significant difference ($p<0.001$), between the mean number of CL on D1 - 5 and D6 - 12 post-coitum.

FIG 3 COMPREHENSIVE MEAN POST COITAL CORPORA LUTEA FOR PREGNANT SPRAGUE-DAWLEY RATS



Analysis of variance of the CL data for all animals studied also revealed a highly significant effect of time post-partum ($p<0.001$) and regression analysis of the data from D5 - 12 post-coitum established a positive significant linear relationship between CL numbers and the stage of pregnancy ($b=0.04$, $r=0.98$, $F_{1,96}=13.92$).

Experiment 3

Strain Variations in SCL Formation

Experimental Procedure

In order to establish whether the formation of SCL was strain specific and a phenomena peculiar to the Sprague-Dawley rats used in earlier experiments, experiment 2 was repeated at the University of Nottingham using Wistar rats bred locally.

Ninety-two virgin female Wistar rats were mated with proven intact males and sacrificed at different stages of gestation.

Results and Conclusions

The distribution of CL number with respect to the stage of gestation is shown in Table 4 and Text Figure 4.

Table 4 - The total number of CL for pregnant Wistar rats on days 1-9 of gestation

Day of Gestation	Mean Number of CL ± SEM	Mean Number of Decidual Swellings ± SEM	Total Number of Rats
1	11.7 ± 0.9		10
2	14.0 ± 0.8		11
3	12.6 ± 0.7		10
4	12.7 ± 1.2		10
5	15.3 ± 0.8		10
6	15.1 ± 0.7	11.9 ± 0.5	10
7	16.5 ± 0.9	12.4 ± 0.5	11
8	16.6 ± 1.0	12.7 ± 0.5	10
9	16.6 ± 1.0	12.7 ± 0.5	10
Control	12.7 ± 1.2		10

Student's 't' test did not reveal a significant difference between the mean CL number of cyclic controls and the mated females on D1 post-coitum.

A Cusum plot (Mulhalland, 1980) of the data also revealed that a further sustained increase in CL numbers occured at D4, as indicated in Text Figure 4. The data could therefore be divided for analysis on this basis and a Student's 't' test showed a significant difference ($p<0.001$) between the mean number of CL on D1-4 and 5-9 post-coitum.

Analysis of variance of the CL data for all animals studied also revealed a highly significant effect of time post-coitum ($p<0.001$) and regression analysis of the data from D4 to 9 post-coitum established a positive significant linear relationship between CL numbers and the stage of pregnancy ($b=0.02$, $r=0.88$, $F_{2,28}=4.27$).

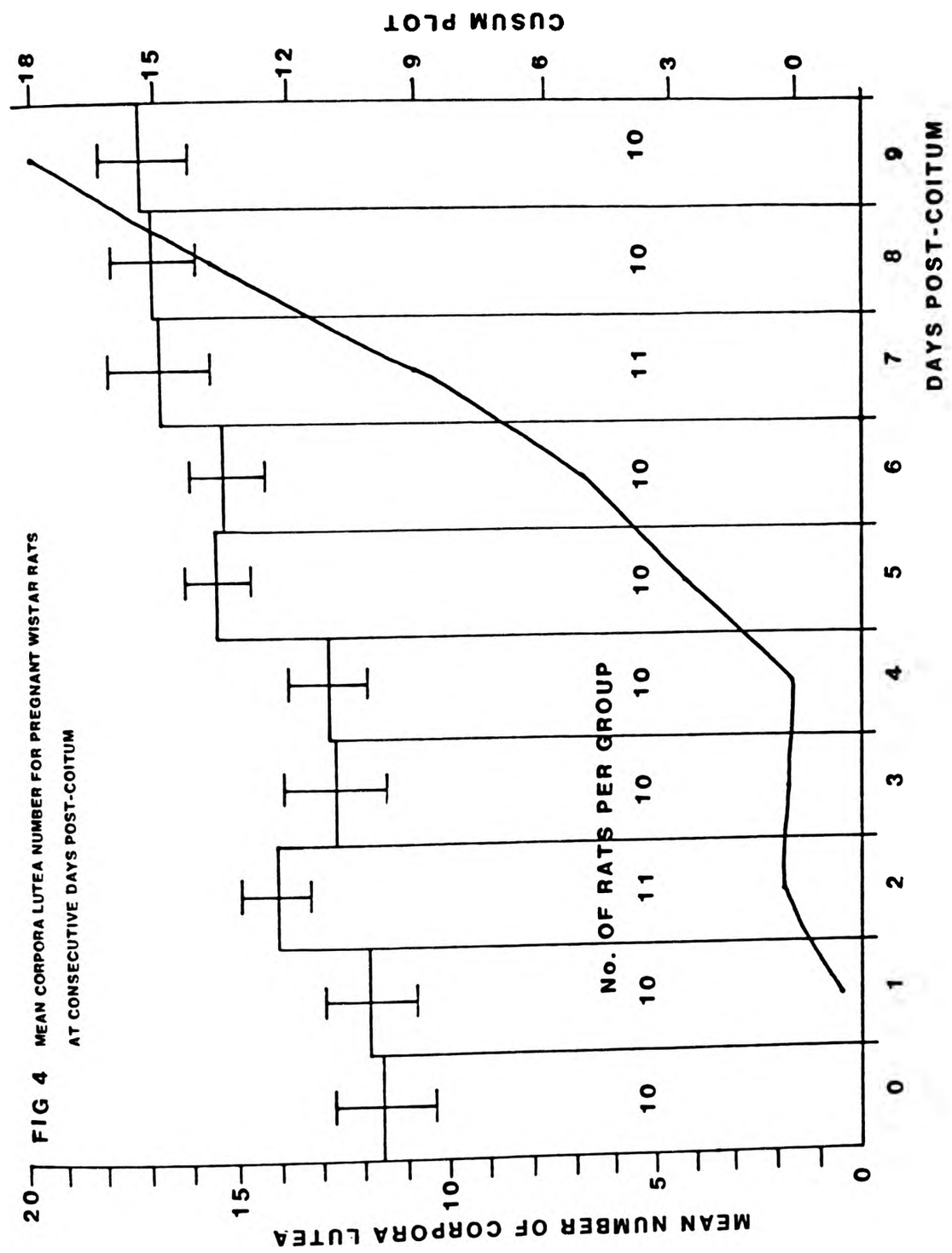


FIG 4 MEAN CORPORA LUTEA NUMBER FOR PREGNANT WISTAR RATS AT CONSECUTIVE DAYS POST-COITUM

Experiment 4

Plasma Progesterone Concentrations in Early Gestation

Experimental Procedure

In order to establish whether the formation of SCL was associated with an increase in plasma progesterone concentrations during pregnancy, progesterone levels were measured in plasma samples obtained at sacrifice on days 1-9 post-coitum, from the rats used in experiments 2 and 3.

Results and Conclusions

The level of plasma progesterone for both pregnant Sprague-Dawley and Wistar rats are shown in Tables 5 and 6 and Text Figures 5 and 6 respectively.

Table 5 - Mean plasma progesterone levels for pregnant Sprague-Dawley rats on days 1-9 of gestation

Day of Gestation	Mean Plasma Progesterone ± SEM (ng+ml)	Total Number of Rats
1	10.05 ± 2.92	4
2	16.02 ± 1.33	5
3	33.85 ± 11.07	3
4	62.10 ± 3.27	3
5	57.37 ± 6.99	4
6	67.40 ± 8.53	3
7	67.34 ± 16.05	4
8	74.00 ± 5.59	5
9	63.11 ± 3.94	6
Control	16.22 ± 7.46	9

FIG 5 MEAN PLASMA PROGESTERONE LEVELS AT CONSECUTIVE DAYS POST-COITUM FOR PREGNANT SPRAGUE-DAWLEY RATS

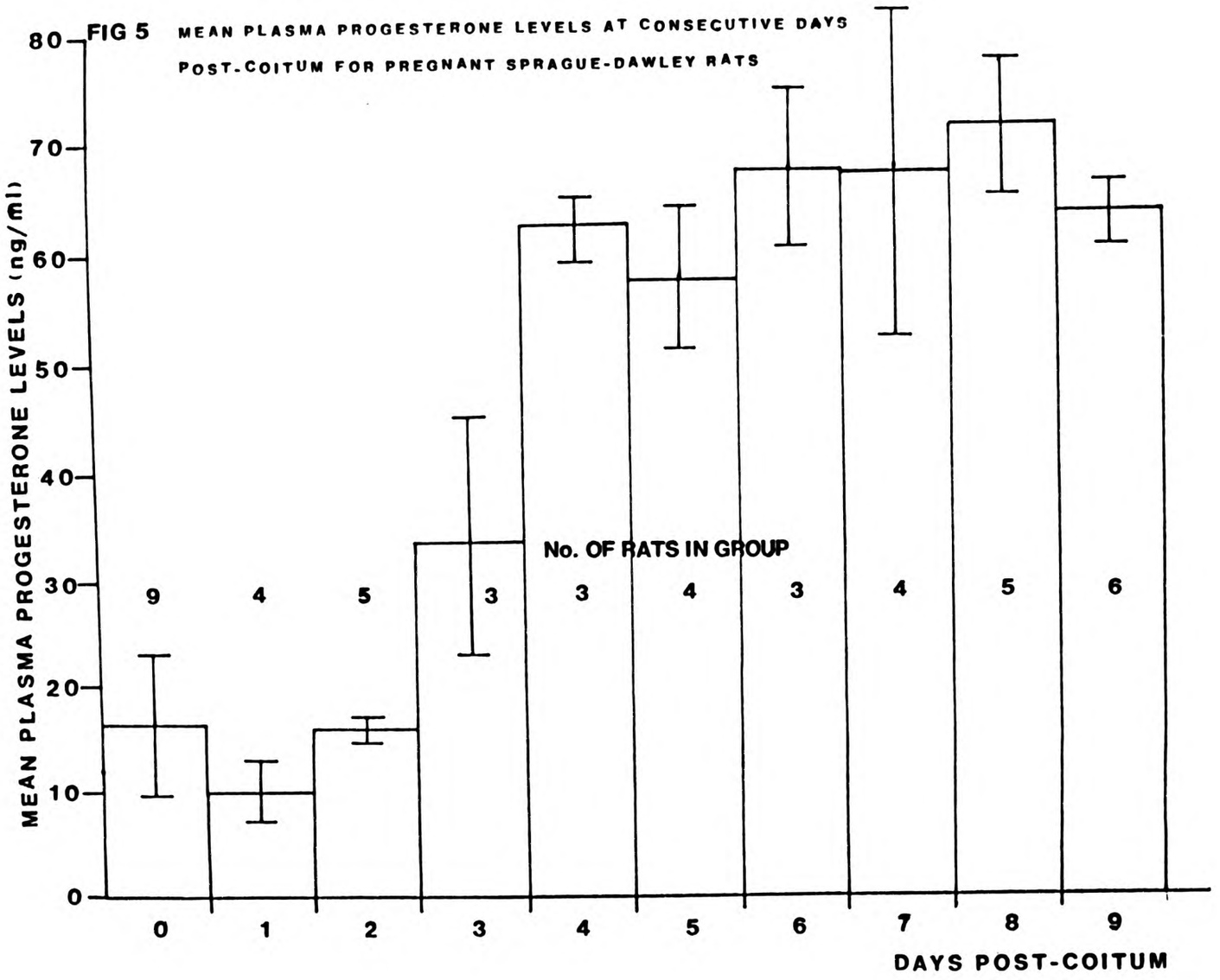
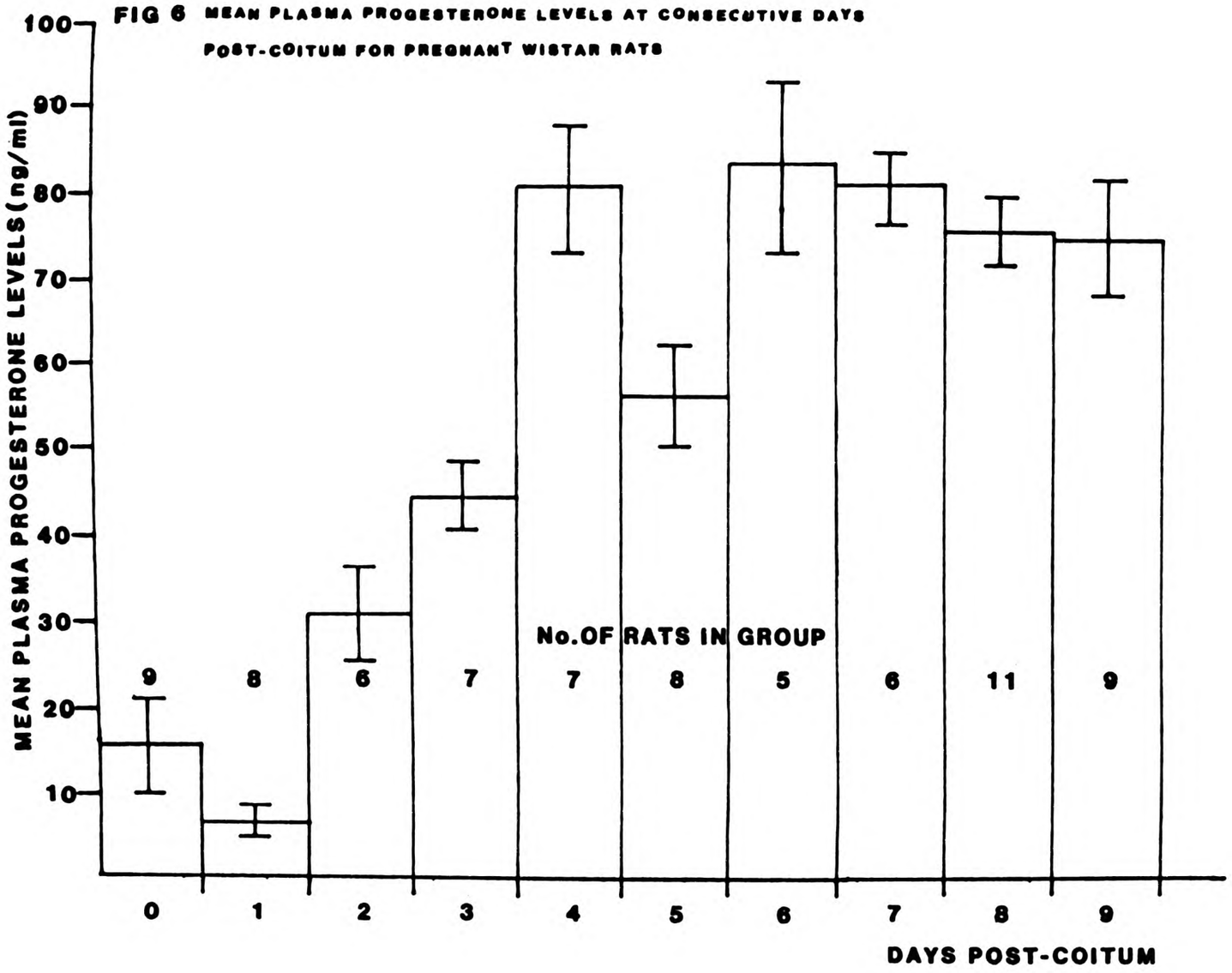


Table 6 - Mean plasma progesterone levels for pregnant Wistar rats on days 1-9 of gestation

Day of Gestation	Mean Plasma Progesterone ± SEM (ng+ml)	Total Number of Rats
1	7.63 ± 1.19	8
2	31.67 ± 5.72	6
3	43.86 ± 3.98	7
4	81.71 ± 7.81	7
5	56.25 ± 7.05	8
6	83.00 ± 10.28	5
7	81.33 ± 4.06	6
8	74.55 ± 4.41	11
9	73.56 ± 4.77	9
Control	16.22 ± 7.46	9

The data for both pregnant Wistar and Sprague-Dawley rats show that plasma progesterone levels rose sharply to a level which then plateaued over the period of days 4 to 9 of gestation. However, both profiles did exhibit a transient decrease in concentrations on D5 of gestation. Plasma progesterone levels may have been expected to show a further increase on D5-7 of gestation, with the formation of SCL. However, these progesterone levels are consistent with the observations of Moudgal (1973), who noted that the concentration of progesterone during pregnancy tends to plateau after reaching a ceiling level, regardless of the number of CL present. It is unclear from published progesterone profiles, whether a similar transient decrease also occurs on D5 of gestation.

FIG 6 MEAN PLASMA PROGESTERONE LEVELS AT CONSECUTIVE DAYS POST-COITUM FOR PREGNANT WISTAR RATS



Experiment 5

The Occurence of Ovulation in Association with SCL Formation

Experimental Procedure

To investigate whether the formation of SCL was associated with ovulation and the release of ova or whether SCL might result from the luteinisation of unovulated follicles, 12 female Sprague-Dawley and 12 Wistar rats were mated and sacrificed on days 5, 6, 7 and 8 of gestation. At autopsy, the Fallopian tubes were removed, placed in a watch glass containing isotonic saline, cut into very small pieces and then left at $20^{o}C$ for at least two hours, the presence of ova was then determined using a binocular dissecting microscope and if no ova were located, then the pieces of Fallopian tube were further maserated by teasing apart with microforceps. Any ova observed were transferred to a cavity slide for microscopic examination.

However, to initially establish the technique involved, 6 female Wistar rats were mated with proven males and subsequently sacrificed on days 1, 2, 3 and 4 of gestation. Fallopian tubes were investigated, as before, for the presence of ova. The stage of development of the ova located was noted.

Also in order to establish the age of any ova recovered from SCL formation, 3 female Wistar rats were sacrificed at metoestrus , dioestrus, and pro-oestrus respectively. At autopsy Fallopian tubes were examined for the presence of ova as previously described. Additionally, the Fallopian tubes of two further cyclic females were ligated under anaesthetic at pro-oestrus . These females were then mated with a vasectomised male at their second subsequent oestrus , to block further ovulation. Two consecutive oestrous cycles were then monitored by smearing, before mating, to ensure that operational trauma had not interfered with the cycle regularity. This would then enable the recovery of unfertilised ova which were older than 5 days. At autopsy, the Fallopian tubes were similarly investigated for ova.

Results and Conclusions

Using the technique described, it was possible to recover ova from pregnant Wistar rats, however the recovery rate was poor, as indicated in Table 7 below.

Table 7 - The stage of development of ova recovered from pregnant Wistar rats on days 1, 2, 3 and 4 of gestation

Rat	Day of Gestation	Total No of CL	Ova Recovered	Description of Ova
1	1	10	1	At the 1 cell stage. Zona pellucida (zp) intact.
2	1	8	4	All at the 1 cell stage. Zp intact. Sperm was present in the Fallopian tubes.
3	2	15	7	1(1 cell), 3(2 cell). Zp was intact and 3 empty zp's were present.
4	3	12	2	1 (1 cell), 1 (4 cell). Zp was intact.
5	3	13	6	1 (1 cell), 2 (2 cell), 1 (3 cell), 2 (4 cell). Zp was intact.
6	4	13	7	All at the Morulla stage and located in the uterus.

The stages of development of the fertilised ova recovered confirm the current published data.

Ova were recovered from Sprague-Dawley rats sacrificed around the time of SCL formation, on days 6 and 7 of gestation, however, this was not more than 4 from any one female. These ova were all in a degenerative state and although the zona pellucida was intact the vitellus had degenerated and no cell divisions were visible. No ova were recovered from Sprague-Dawley rats sacrificed on D5 and D8 of gestation.

However, this recovery of ova around the time of SCL formation may not be conclusive evidence that these were released as a result of ovulations at the time of SCL formation. It is possible that they may represent residual unfertilised ova from a previous cycle which had become entrapped in the Fallopian tube.

The recovery of unfertilised ova from pseudopregnant ligated Wistar rats also gave a poor recovery rate. It was possible to recover 5 and 2 unfertilised ova from the two rats sacrificed at metoestrus and dioestrus respectively, all of which possessed an intact vitelline membrane and zona pellucida. No ova were recovered from the female sacrificed at pro-oestrus due possibly to the former having passed into the uterus. However, only 12 ova were recovered from the two pseudopregnant ligated rats, sacrificed on D10 and D8 post-coitum. These ova would be aged 2, 5, 8, 10, 13 or 16 days, as a result of the number of the oestrous cycles the female was allowed between ligation and mating. Ten of the ova recovered were comparable to the ova recovered from the cyclic females previously described, hence it was presumed that they were either 2 or 5 days old. The other two ova recovered were at a degenerative stage but retained an intact zona pellucida.

It would seem therefore that degeneration of an unfertilised ovum does not occur within the first 5 days post ovulation. However, between 5 and 8 days after ovulation the unfertilised ovum degenerates, although the zona pellucida remains intact.

DISCUSSION

Female rats of both the Sprague-Dawley and Wistar strain are capable of SCL formation during early pregnancy. The formation of SCL during pregnancy appears to occur at a specific time, between days 5 and 7 post-coitum, and would indicate that SCL formation is a consistent physiological event.

The plasma progesterone concentrations for both Sprague-Dawley and Wistar pregnant rats exhibited similar profiles. A transient decrease in concentration was seen in both on D5 post-coitum, the reason for which is unknown. It may be that the increase in progesterone again on D6 may be attributed to the formation of SCL between D5 and D7 post-coitum and that plateau concentrations would otherwise remain similar to those shown on D5.

It is difficult to extrapalate this theory to data on progesterone levels from other researchers, as they are probably including, also, the progesterone output from SCL. A further experiment, eliminating the formation of SCL would confirm this hypothesis.

As the overall ovarian progesterone secretion may be limited by the amount of LH available, it is possible that any increase in progesterone secretion would only be temporary. The overall progesterone profile follows the usual pattern for pregnancy, reaching a peak by D6 and plateauing thereafter. The limiting factor of LH hormone available should inhibit any significant increase in progesterone once the plateau has been reached, thus an increase in CL number need not necessarily result in an overall increase in progesterone secretion by the ovary.

The recovery of ova from Sprague-Dawley females is not considered to be evidence for the release of ova at the time of SCL formation. These ova were in a degenerative state and from the data on post-ovulatory development, they should still have been similar to an unfertilised ovum, if they had recently been released. Degeneration, also is not normally

obvious until approximately 5 days post-ovulation. It would have been unlikely that ova released as a result of SCL formation would have been fertilised, therefore only recent unfertilised ova, similar to those found at dioestrus , would have been conclusive evidence of SCL formation. The levels of ova recovery were also generally below methodological expectations anyway, so this experiment was not a satisfactory method of establishing the occurence of ovulation in association with SCL formation.

The significant difference in CL numbers of the cyclic controls and those of D1 of gestation seen in the Sprague-Dawley rats, is probably as a result of coitally induced ovulations (Zarrow & Clarke, 1968; Rodgers, 1971). The absence of these in the Wistar rat, may be due to the mating procedure; coitally induced ovulations occur only if mated between 17.00 and 18.30 hours on pro-oestrus , (Rodgers, 1971) or perhaps is a strain difference.

CHAPTER 4

WHETHER SCL ARE PREGNANCY SPECIFIC

CHAPTER 4

WHETHER SCL ARE PREGNANCY SPECIFIC

Introduction

Having established the formation of SCL in the pregnant rat, it was of considerable interest to determine whether this phenomena was in fact pregnancy specific.

Gonadotrophin—like hormones are present in the rat placenta (Bambra and Combe, 1978) and the embryo and/or placenta may be associated with the stimulus for the formation of SCL. In order to verify this, data from pseudopregnant rats was obtained.

Experiment 8

The Formation of SCL During Pseudopregnancy

Experimental Procedure

To establish whether SCL were formed at a similar time in pseudopregnant as in pregnant rats, additional females of both Sprague-Dawley and Wistar strain were mated with vasectomised males and were sacrificed on days 1-14 post-coitum. The procedures were as described in Experiment 2.

Results and Conclusions

The number of CL recorded on consecutive days post-coitum for both Sprague-Dawley and Wistar rats, is shown in Table 8 and Text Figures 7 and 8 respectively.

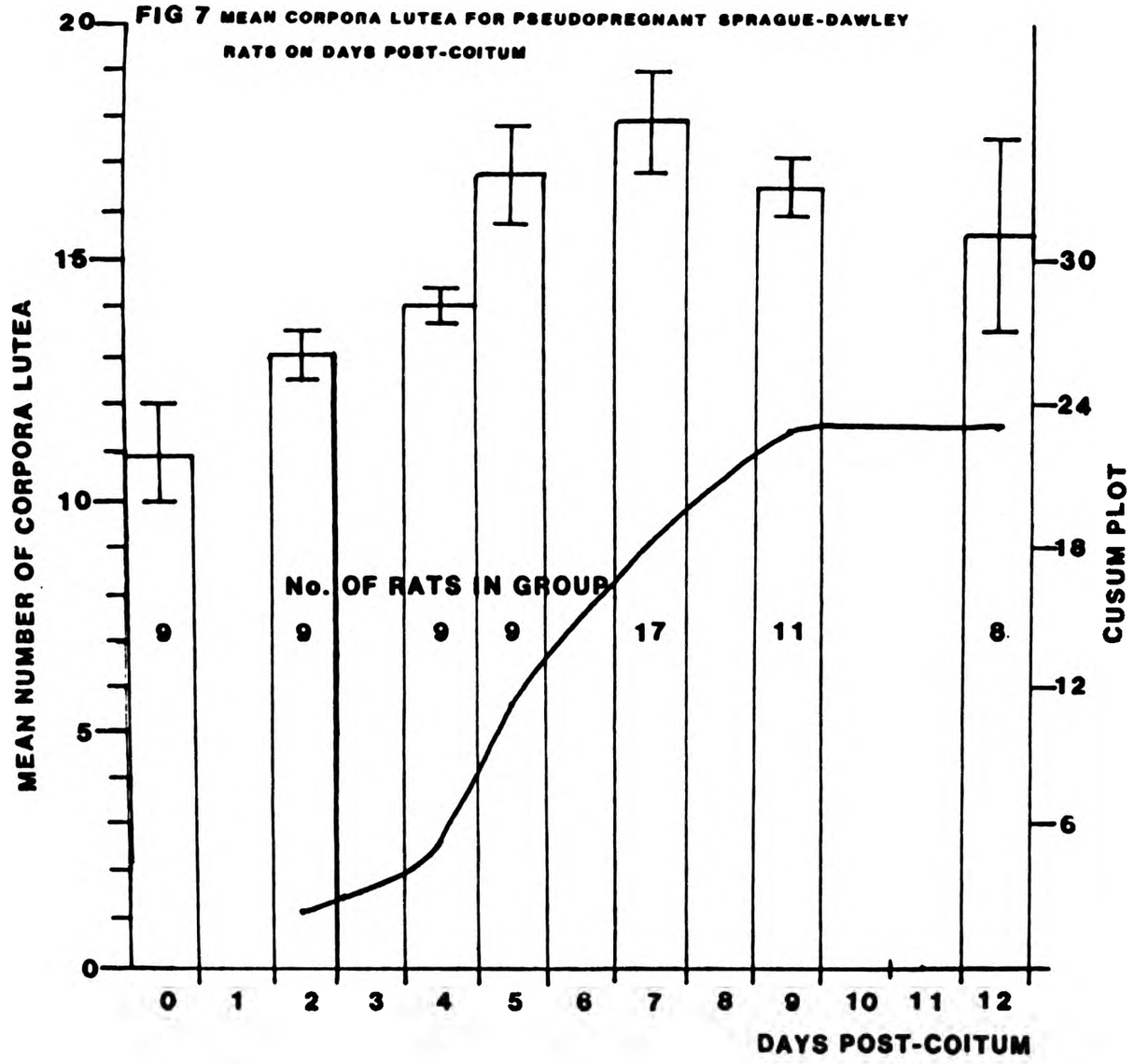
Table 8 - The mean number of CL for pesudopregnant Sprague-Dawley and Wistar rats, post-coitum

Sprague-Dawley Females			Wistar Females		
Day post-coitum	Mean Number of CL ± SEM	Total Number of Rats	Day post-coitum	Mean Number of CL ± SEM	Total Number coitum
2	13.10 ± 0.54	9	2	13.10 ± 0.43	14
4	14.00 ± 0.37	9	3	12.25 ± 0.75	12
5	16.70 ± 1.09	9	4	12.70 ± 0.78	17
7	17.90 ± 0.93	9	7	15.30 ± 0.82	18
9	16.50 ± 0.69	17	9	13.80 ± 0.70	21
12	10.89 ± 1.39	11	11	13.30 ± 1.12	10
Control	10.89 ± 1.39	9	14	12.10 ± 0.79	9
			Control	12.30 ± 0.86	15

Consistent with the corpora lutea data from the pregnant rats, Student's 't' test revealed a significant difference ($p<0.05$) between the mean CL number of cyclic controls and the mated females on D1 post-coitum, for Sprague-Dawley rats, but not for the Wistar strain.

A Cusum plot (Mulhalland, 1980) also revealed that a further sustained increase in CL numbers occured at D5 for Sprague-Dawley rats and after D4 for Wistar rats, as indicated in Text Figures 7 and 8 respectively. The data could therefore be divided for analysis on this basis and a Student's 't' test showed a significant difference ($p<0.05$) between the mean number of CL on D2-4 and 7-12 post-coitum for Sprague-Dawley rats. A similar significant difference ($p<0.05$) between the mean number of CL on D2-4 and 7-11 post-coitum was also revealed for Wistar rats.

FIG 7 MEAN CORPORA LUTEA FOR PSEUDOPREGNANT SPRAGUE-DAWLEY RATS ON DAYS POST-COITUM



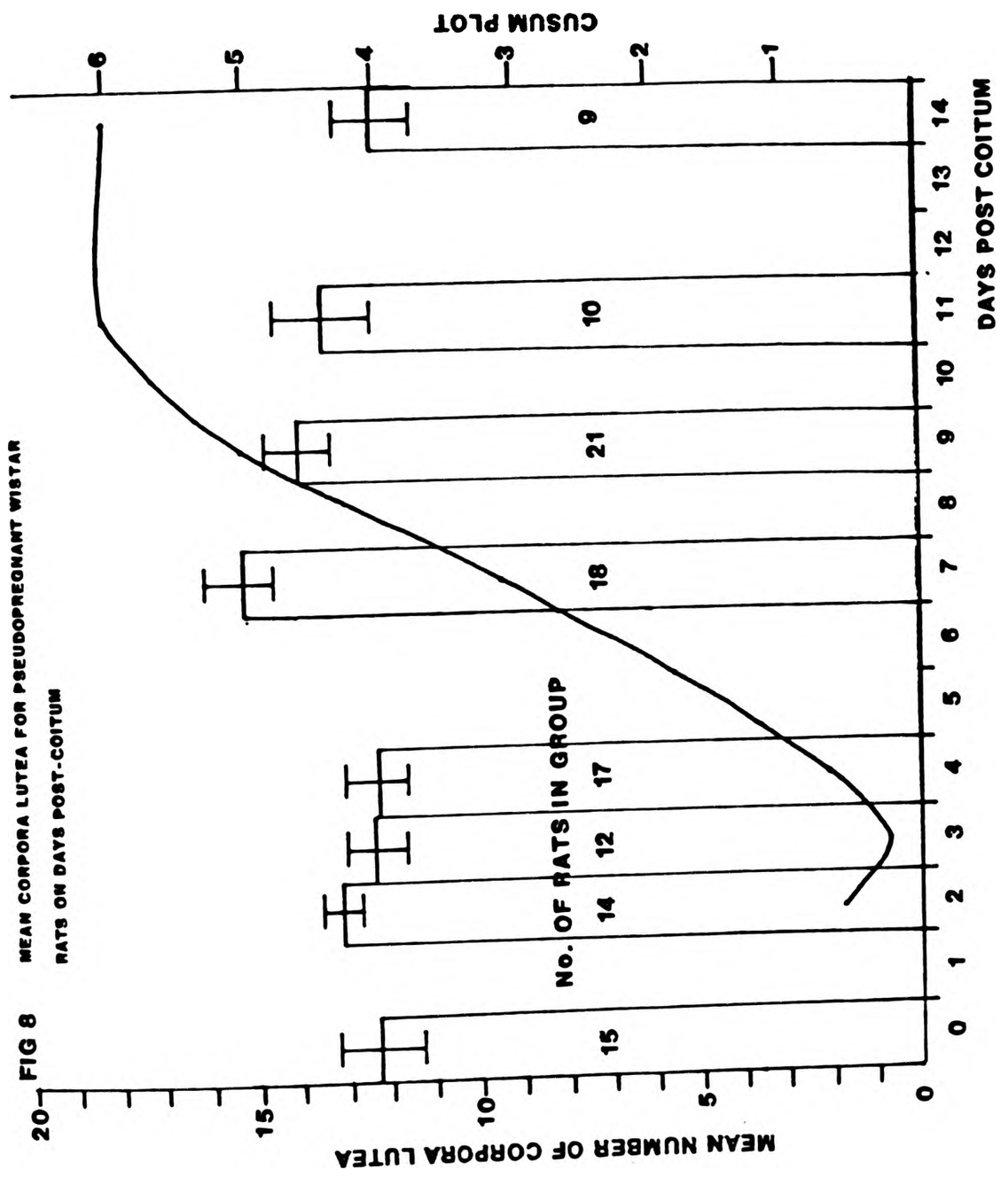


FIG 8 MEAN CORPORA LUTEA FOR PSEUDOPREGNANT WISTAR RATS ON DAYS POST-COITUM

Analysis of variance of CL data for all animals studied for both Sprague-Dawley and Wistar rats, also revealed a highly siginificant effect of time post-coitum ($p<0.001$) and regression analysis of the data from D4 post-coitum for Wistar rats and D4 post-coitum for Sprague-Dawley rats, established a positive significant linear relationship between CL numbers and the stage of pseudopregnancy (b=0.03, r=0.90, $F_{2,32}=5.49$ for Sprague-Dawley rats and b=0.03, r=1.00 $F_{1,33}=9.60$ for Wistar rats).

Experiment 9

Progesterone Levels During Pseudopregnancy in the rat

Experimental Procedure

The plasma progesterone profile during pseudopregnancy was established to determine whether the formation of SCL influenced progesterone output, by obtaining blood samples from the females used in Experiment 8. The methods used were as described in the general methods for blood sampling and radioimmunossay procedures.

Results and Conclusions

The plasma progesterone levels obtained for pseudopregnant Sprague-Dawley and Wistar rats are shown in Table 9 and Text Figures 9 and 10 respectively.

Table 9 - Mean post-coital plasma progesterone levels for pseudopregnant Sprague-Dawley and Wistar rats

Sprague-Dawley Females			Wistar Females		
Day post-coitum	Mean Plasma Progesterone (ng+ml) ± SEM	Total Number of Rats	Day post-coitum	Mean Plasma Progesterone (ng+ml) ± SEM	Total Number of Rats
2	18.44 ± 1.86	10	2	25.50 ± 2.53	4
4	52.90 ± 5.70	11	3	52.63 ± 5.97	12
8	58.41 ± 5.97	10	4	62.20 ± 13.07	6
12	29.95 ± 2.00	2	7	70.57 ± 7.11	8
Control	16.22 ± 7.46	9	8	79.00 ± 14.84	3
			9	62.00 ± 8.15	11
			11	39.10 ± 4.70	10
			14	8.50 ± 1.90	9
			Control	16.22 ± 7.46	9

The plasma progesterone profiles for the pseudopregnant rat appear to follow the same pattern as for the pregnant rat, in both strains. There was no significant increase in plasma progesterone levels after the formation of SCL and it was unclear from the pseudopregnant data whether a transient decline in progesterone concentration occurs on D5 post-coitum, as was evident in the pregnant rats. However, the data obtained is consistent with that of other research workers (Smith et al, 1975). The decline in plasma progesterone levels after D8 post-coitum is consistent with the regression of the CL at the end of pseudopregnancy; the concentrations being equivalent to that of cyclic females by D14 post-coitum.

It is not possible to postulate from the pseudopregnant progesterone profiles, whether SCL formation increases the total progesterone secretion significantly.

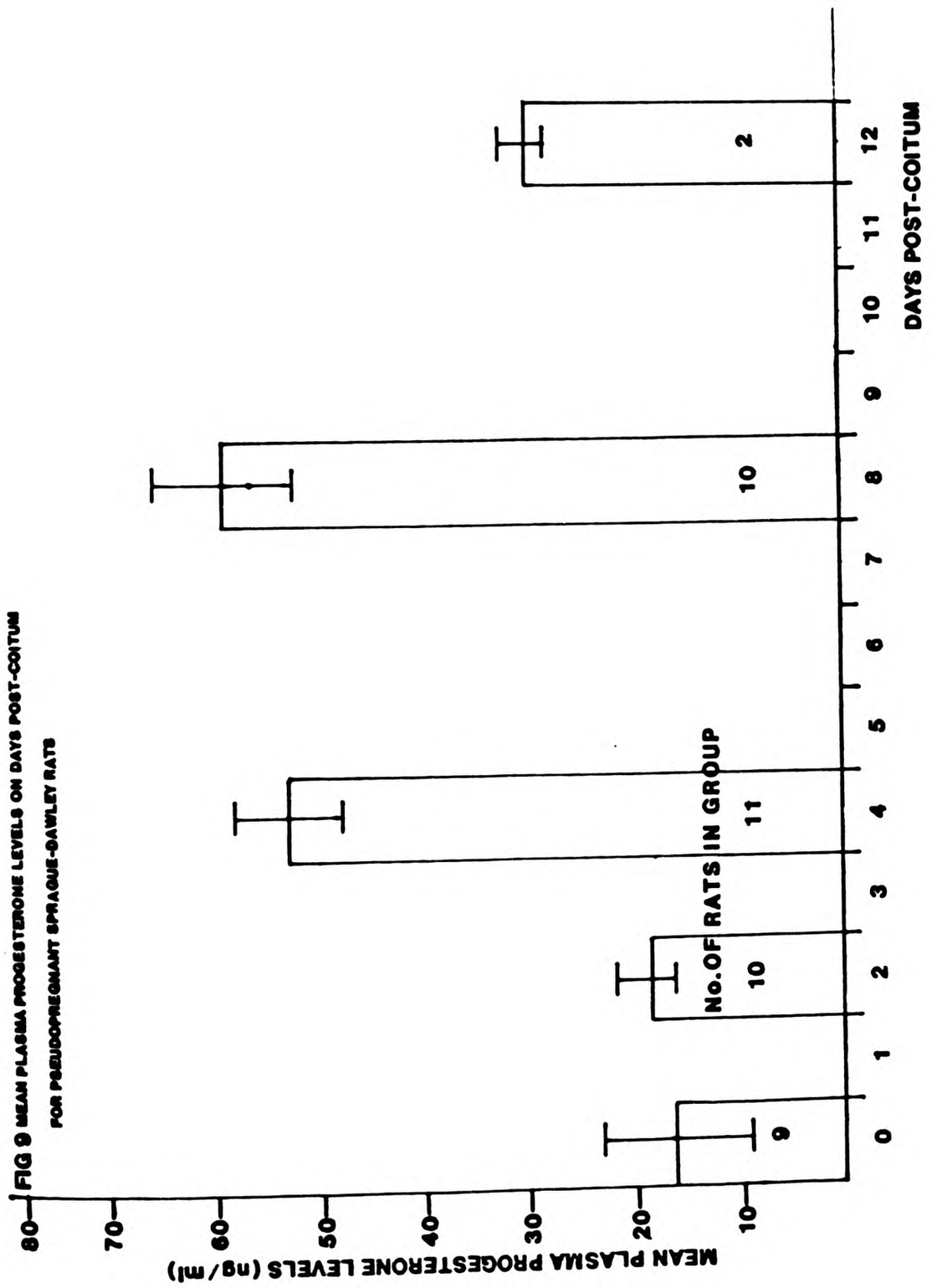


FIG 9 MEAN PLASMA PROGESTERONE LEVELS ON DAYS POST-COITUM FOR PSEUDOPREGNANT SPRAGUE-DAWLEY RATS

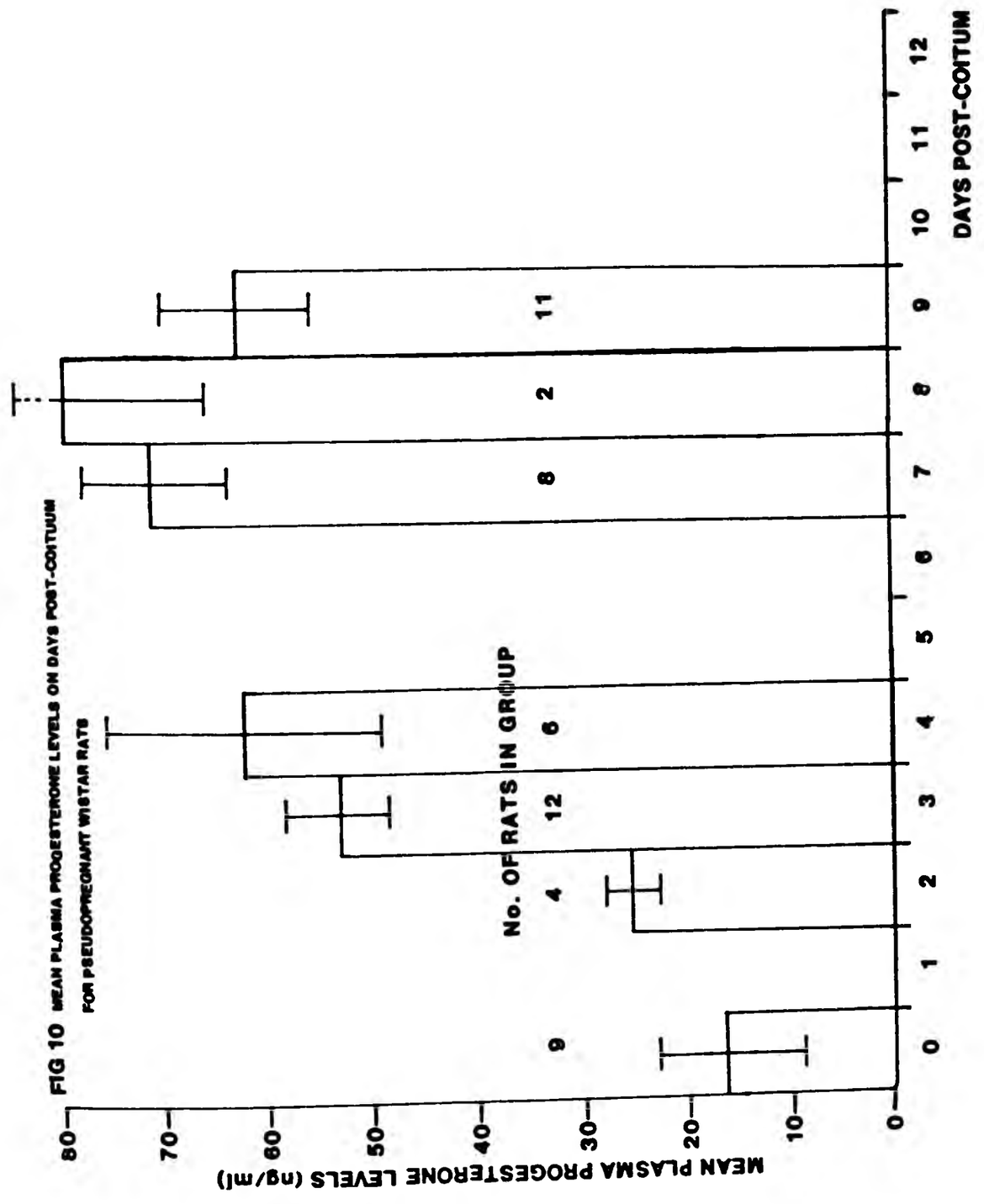


FIG 10 MEAN PLASMA PROGESTERONE LEVELS ON DAYS POST-COITUM FOR PSEUDOPREGNANT WISTAR RATS

DISCUSSION

The formation of SCL is not pregnancy specific as there was a significant increase in CL number by D7 post-coitum in both Sprague-Dawley and Wistar pseudopregnant rats, corresponding to the increase shown in pregnant females. The decrease in CL number after D7 post-coitum may be attributed to the regression of CL, as pseudopregnancy continues for 12 to 14 days only (Long and Evans, 1922). This is confirmed by the D14 post-coitum counts being similar to that of the controls.

Progesterone levels over this period do not show an apparent increase after the formation of SCL and are comparable to those obtained from pregnant Sprague-Dawley and Wistar rats, for the first few days of pseudo-pregnancy. The progesterone levels decrease around D8 post-coitum which is consistent with the CL data.

There was no evidence of an increase in ovarian progesterone secretion as a result of SCL formation, which would be expected to be obvious by D7 post-coitum, as full progesterone production usually occurs approximately 2 days after CL formation. Any apparent increases were not significant due to large standard errors.

It is therefore probable that the placenta does not play a role in the initiation of SCL formation, in Sprague-Dawley and Wistar rats. Therefore further investigation is required to elucidate the 'trigger' for SCL formation.

CHAPTER 5

THE ENDOCRINE CONTROL OF SCL FORMATION

Introduction

The previous two chapters have established the presence of SCL. This chapter will therefore be concerned with obtaining an insight into the possible endocrine control of SCL formation.

As SCL appear to be formed by D5 and D6 post-coitum and the preimplantation surge of oestrogen occurs in the afternoon of D4 post-coitum (Shaikh, 1971), it may be postulated that this is the initiator of SCL formation. The data from SCL initiation in pseudopregnant rats, showed that neither the placenta nor embryo appear to be involved in their production.

Experiments were therefore designed to determine the consequences of eliminating the preimplantation surge of oestrogen and to establish whether LH and FSH were released in response to the preimplantation surge, as postulated by Everett, (1977).

Experiment 10

The Preimplantation Surge of Oestrogen and SCL Formation

Experimental Procedure

It was considered that the preimplantation surge of oestrogen may initiate the formation of SCL. In order to verify this hypothesis, the effect of the elimination of this surge of oestrogen on SCL formation was studied by mating 29 female Sprague-Dawley rats with proven males. Fifteen females were given 0.1mg/kg of Tamoxifen (ICI Ltd) suspended in aracis oil, by oral gavage, at 11.00 hours on D4 post-coitum (Bloxham et al, 1975). Fourteen mated control females were given the vehicle only by the same regime. All rats were sacrificed on D8 post-coitum and the number of CL and decidual swellings recorded.

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Results and Conclusions

The number of CL recorded for the Tamoxifen treated rats is compared to that of the controls in the following table.

Table 10 - Total number of CL and decidual swellings per rat on D8 post-coitum in Sprague-Dawley females receiving Tamoxifen on D4 post-coitum

Tamoxifen Treated Females		Control Females	
Total Number of CL	Total Number of Decidual Swellings	Total Number of CL	Total Number of Decidual Swellings
13, 16, 15, 16, 16, 13, 16, 14, 14, 12, 16, 19, 15, 15, 15	None	17, 21, 18, 20, 19, 17, 14, 20, 19, 16, 16, 16, 21	15, 15, 16, 17, 14, 16, 15, 4, 17, 18, 16, 13, 14, 16
Mean ± SEM 15.0 ± 0.57 n 15	0 15	Mean ± SEM 18.0 ± 0.44 14	Mean ± SEM 14.7 ± 0.89 14

The preimplantation surge of oestrogen occuring on D4 post-coitum appears to influence the formation of SCL in the pregnant rat, as a Students' 't' test showed a significant difference ($p<0.001$) between the number of CL in the Tamoxifen treated rats and the control females.

The preimplantation surge of oestrogen is essential for implantation to take place in the rat (Deansley, 1966; Nalbandov, 1976) and as there was no evidence of decidual swellings in any of the rats dosed with Tamoxifen, it would imply that Tamoxifen interfered with the preimplantation surge of oestrogen.

Tamoxifen is an anti-oestrogen (Bloxham et al, 1975) and the data of this experiment would appear to confirm this as Tamoxifen was administered prior to the preimplantation surge. However, it is unclear as to how Tamoxifen exerts its anti-oestrogenic effect, whether it inhibits at an ovarian or hypothalamic/pituitary level.

Accepting that Tamoxifen has eliminated the preimplantation surge of oestrogen, then it would appear from this experiment that it is likely that the formation of SCL may be initiated by this release of oestrogen.

Experiment 11

The Involvement of an LH/FSH Surge

Experimental Procedure

The previous experiment indicated that the preimplantation surge of oestrogen influenced the formation of SCL and it was therefore considered important to establish whether this release of oestrogen initiated a corresponding LH/FSH surge, as occurs in the oestrous cycle.

The preovulatory oestrogen surge in the cyclic rat occurs 5 hours after the onset of light at pro-oestrus , followed by the LH/FSH surge 7 hours later (Nequin et al, 1975). As the preimplantation oestrogen surge occurs between 14.00h and 16.00h on D4 post-coitum (Yoshinaga et al, 1968), any resultant LH/FSH surge would be expected to occur also 7 to 8 hours later. Thus the preovulatory LH/FSH surge occurs at night (Blake, 1976) as would also, the postulated LH/FSH surge triggered by the preimplantation oestrogen surge of oestrogen. For convenience therefore, the light:dark cycle was adjusted so that the dark period occured during normal working hours, with the preovulatory LH/FSH surge therefore at its peak at 10.30 hours and the postulated LH/FSH surge occuring at 15.00 hours.

All rats were acclimatised to the adjusted light regime (light period = 22.00h to 12.00h) for 2 weeks, during which time the presence of regular oestrous cycles was monitored by vaginal smears.

Seventeen Wistar females were then mated and sacrificed between 15.00h and 15.30h (the expected time of preimplantation LH/FSH surge) on D4, 5 and 6 post-coitum. In order to compare any LH/FSH surge occuring at these times with that of the preovulatory LH/FSH surge, 6 cyclic females were sacrificed prior to the onset of the preovulatory surge, at 08.30 hours proestrus , and 7 during the period of maximum LH/FSH secretion at 10.45 hours pro-oestrus At sacrifice a blood sample was taken by cardiac puncture from all females, for plasma LH/FSH assay. The pituitary gland was removed within 5 minutes, immediately homogenised for 30 seconds in 5.0ml of cold phosphate buffer saline at $4^{o}C$ and the homogenate centrifuged for 10 minutes at 3000 rpm. The aqueous fraction was stored at $-20^{o}C$ until assayed for pituitary LH/FSH.

Results and Conclusions

The levels of plasma and pituitary LH and FSH recorded for both cyclic and pregnant Wistar rats are shown in Tables 11 and 12.

Table 11 - Mean plasma LH and FSH levels for pro-oestrus cyclic rats and for pregnant rats after the preimplantation surge of oestrogen

Day of Cycle or post-coitum	Mean Plasma LH ± SEM (ng/eqv (NIADDK-r-LH-RP-2/ml plasma))	Mean Plasma FSH ± SEM (ng/eqv (NIAMDD-r-FSH-RP-2/ml plasma))
Proestrous 08.30	1.05 ± 2.37	231.5 ± 30.7
10.30	18.08 ± 7.07	346.9 ± 55.9
Post-coitum N4	1.01 ± 0.28	271.2 ± 22.7
N5	2.06 ± 1.16	253.0 ± 17.8
N6	0.82 ± 1.13	166.3 ± 9.0

Table 12 - Mean pituitary LH and FSH levels for pro-oestrus cyclic rats and for pregnant rats after the preimplantation surge of oestrogen

Day of Cycle or post-coitum	Mean Pituitary LH ± SEM (ng/eqv) (NIADDK-r-LH-RP-2/pituitary)	Mean Pituitary FSH ± SEM (ng/eqv) (NIAMDD-r-FSH-RP-2/pituitary)
Proestrous 08.30	63.0 ± 8.45	4229 ± 233.5
10.30	60.0 ± 10.20	2904 ± 355.0
Post-coitum N4	69.0 ± 5.0	5808 ± 999.0
N5	131.5 ± 20.50	6360 ± 755.0
N6	93.5 ± 6.65	6260 ± 535.0

Students' 't' test showed a significant difference ($p<0.10$) between plasma FSH levels at 08.30 and 10.30 proestrus . There was no significant difference between N4 and N5, but there was between N4 and N6 post-coitum ($p<0.001$). LH was significantly elevated during the preovulatory surge ($p<0.10$) although there was no significant difference in plasma levels on nights 4, 5 or 6 post-coitum. Thus there was a difference in the plasma levels of FSH and LH before and during the preovulatory surge and while there was no significant differences in plasma GT levels between N4, 5 and 6 post-coitum, plasma FSH was slightly higher for N4 post-coitum, compared to N5 and 6.

A Students' 't' test gave a significant difference in pituitary LH levels between N4 and 5 post-coitum ($p<0.025$), while there was no difference before or during the preovulatory LH surge. There was a significant difference between the FSH levels before and during the preovulatory surge at proestrus , however, there were no differences between the levels on N4,5 or post-coitum. Therefore, there was a difference in the pituitary FSH levels before and during the preovulatory surge as expected, but this was not reflected in the LH levels. However, LH levels were significantly

lower on N4 post-coitum compared to N5 and although the FSH levels at this time showed no significant differences, the level on N4 post-coitum was slightly depressed.

The pitiutary FSH levels are consistent with the FSH plasma levels while this is not necessarily the case for the LH plasma and pituitary levels.

DISCUSSION

The significant difference in the increased number of CL in the pregnant Sprague-Dawley rats not receiving the anti-oestrogen, Tamoxifen, may be indicative of the preimplantation surge of oestrogen of D4 post-coitum, playing a role in the initiation of SCL. However, it would be desirable to undertake further experiments to confirm this, as it is not certain at what level Tamoxifen acts on the reproductive axis. It was therefore decided to investigate the presence of an LH/FSH surge following the preimplantation peak, to substantiate the Tamoxifen data.

The plasma gonodotrophin data in the pro-oestrus females confirmed the presence of the preovulatory FSH/LH surge in the cyclic rats acclimatised to an adjusted new light regime. The values obtained are consistent with those seen in the literature (Blake, 1976).

Pituitary LH/FSH levels did not absolutely confirm these results, although there was a significant decrease in pituitary FSH at the time of the plasma LH/FSH surge, as might be expected. However, there is some discrepancy in the literature as to whether there is a decrease in pituitary concentrations corresponding to the plasma LH/FSH surge. Sasomoto et al, (1979) observed no significant fall in pituitary GT's around the time of the preovulatory LH surge, while Blake (1976) did. It is therefore unclear as to the significance of the pituitary LH/FSH levels, at this time.

Evidence for an LH/FSH peak following the preimplantation surge is not convincing as there was no significant increase in plasma GT levels on night 4 (N4) post-coitum; the expected time of any GT surge following the preimplantation oestrogen peak. Although plasma FSH levels on N4 were significantly elevated compared to N6, they were not significantly different to levels observed at 08.30 hours pro-oestrus., before the preovulatory LH/FSH surge ($p<0.05$) and were lower than levels seen at the time of the LH/FSH surge.

The plasma LH levels on N4, 5 and 6 were of the order of those recorded prior to the preovulatory LH/FSH surge. It therefore appears that plasma GT concentrations did not attain comparable values to those of the preovulatory LH/FSH surge, during the period studied.

The pituitary GT levels on N4 were lower than those of N5 and 6 and may be indicative of a general release of GT's at this time, although this is not confirmed by the plasma data. There therefore seems to be a decreased synthesis or an earlier 'missed' GT surge.

The pituitary and plasma FSH data, although not highly significant, together with the pituitary LH and Tamoxifen data, could be indicative of the preimplantation surge of oestrogen being capable of illiciting an LH/FSH peak, after an equivalent time period to that of the oestrous cycle. This would be in keeping with the results obtained by Everett (1977); when administering exogenous oestrogen on D4 post-coitum to pseudopregnant rats, an LH surge was subsequently recorded resulting in ovulation and CL formation.

The discrepancies in the GT data may be accounted for by the fact that only one blood sample was taken during the proposed period of the preimplantation oestrogen surge induced GT release. Also, as the LH/FSH surge prior to ovulation in the oestrous cycle is episodic, around a plateau level, it would be pertinent to assume that the same pattern of release occurs if a GT surge is illicited during pregnancy; peak levels may not have, therefore, been measured. It is also an assumption that the GT surge during pregnancy would be of the same duration as that of the oestrous cycle and the peak period may have been missed using the experimental regime in experiment 11. It would be beneficial to repeat this experiment, employing a more detailed sampling regime.

CHAPTER 6

THE HISTOLOGY OF SCL

Introduction

The investigation of the histology of SCL would be more conclusive than the previous experiment involving ova recovery. Thus, in order to substantiate evidence for the formation of SCL, it was considered pertinent to undertake a detailed histological study of the ovaries of rats sacrificed during the first trimester of pregnancy.

This study would confirm the appearance of newly-formed luteal tissue around D5 post-coitum and whether or not the SCL were derived from luteinised follicles or from ovulatory follicles. Observations on the distribution of follicle sizes in the first trimester of pregnancy would also add further data to that already obtained on SCL formation.

Experiment 12

An Investigation of the Histology of SCL

Experimental Procedure

Four female Wistar rats were sacrificed between 13.00h and 14.00h on each day of the cycle (ie pro-oestrus , oestrus , metoestrus, and dioestrus) and acted as controls.

Thirty-three female Wistar rats were mated with proven intact males as stated previously and then designated to two experimental groups as follows:-

Group 1: 15 females sacrificed on D3
Group 2: 15 females sacrificed on D7.

At autopsy, the ovaries were removed and the number of CL recorded by observing the gross structure of the ovary under a low power dissecting

microscope. Ovaries were then placed in 10% Formal saline for future histological sectioning and were later processed as described in the general methods.

Serial sections of each ovary were observed as described in Chapter 2 under low power using a light microscope and the size of all CL was recorded, as well as the numbers of corpora albicantia and of follicles greater than 650µm diameter. Any CL not represented on more than eight sections (ie the diameter was less than 0.8 mm) was re-evaluated and if it was confirmed to be active luteal tissue, then it was included in the CL number. Otherwise it was discounted and classified as a regressing CL.

The ovaries of 9 Sprague-Dawley females sacrificed on D2, 4, 5, 6, 7 and 9 of gestation respectively, were similarly processed and the number of CL present recorded.

Results and Conclusions

The numbers of CL and follicles of greater than 650µm diameter recorded from the Wistar females is shown in Table 13.

Table 13 - Mean number of CL and follicles greater than 650um determined from histological examination of ovarian tissue from cyclic and pregnant Wistar rats

Day of Cycle or Post-coitum	Mean Number of CL ± SEM	Mean Number of follicles > 650µm ± SEM	Total Number of Rats
Proestrus	10.5 ± 1.19	2.8 ± 1.3	4
Oestrus	13.5 ± 1.04	1.0 ± 0.41	4
Metoestrus	13.5 ± 2.5	3.0 ± 2.9	2
Dioestrus	10.5 ± 0.29	0.8 ± 0.48	4
D3 p-c	11.7 ± 0.54	0.6 ± 0.36	15
D7 p-c	15.2 ± 0.6	1.93 ± 0.25	15

A Students' 't' test comparing the number of CL from Wistar rats sacrificed on D3 and D7 post-coitum, showed a significant difference of $p<0.001$; and the means were comparable with those obtained by macroscopic counting. A few ovaries from Sprague-Dawley rats were also investigated histologically in order to confirm previous counts of CL, and were found to compare favourably with the CL numbers recorded in experiment 1 (ie a combined mean of 14.2 for D2 and 4(n=4) and a combined mean of 16.2 for D5, 6 and 7(n=5)).

Recently-formed CL were noted in ovaries of Sprague-Dawley rats sacrificed on D5 and 6, similar to those present in metoestrus cyclic rats and those sacrificed on D2 post-coitum. The granulosa of these structures was becoming luteinised and blood clots were seen retained in the centre of some. These structures are shown in Figure 11, plates c, d, e and f, and Figure 12, plate a. No entrapped ova were recorded in any of the CL studied from rats sacrificed post-coitum, which would indicate that all these CL were formed from ovulatory follicles.

The follicles recorded greater than 650µm were present throughout the oestrous cycle and on all days post-coitum that were investigated. However, it was only on D7, pro-oestrous and metoestrous that more than 1 large follicle was recorded for Wistar rats. From the Sprague-Dawley rats studied, an average of 3 to 4 follicles larger than 650µm were recorded on D4 and 5 post-coitum (n=5), while only 1 on D6 (n=2). It would appear therefore, that follicular growth continues during early pregnancy in both Wistar and Sprague-Dawley rats, and that large ovulatory follicles are present around the time of SCL formation (Figure 11, plates a and b).

The CL counts based on histological sections therefore, compared favourably with previous results determined from the dissection of fresh ovarian tissue at sacrifice. There was also a significant difference in CL number before and after the proposed timing of SCL formation for Wistar rats, which was associated with evidence from continued follicular development during the period of early pregnancy studied.

FIGURE 11

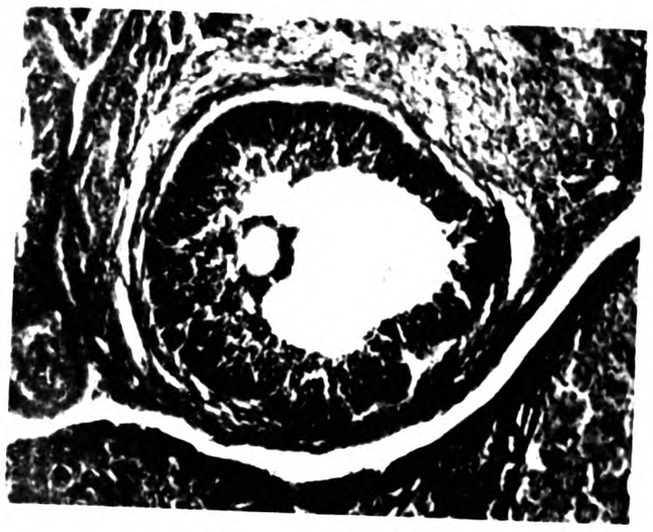
Histological Sections of Ovarian Tissue

Legend to Figure 11

Plate	
a	Graafian follicle present at Day 4 post-coitum (x 125)
b	Graafian follicle present at Day 5 post-coitum (x 160)
c	Recently formed CL present at Day 5 post-coitum (x 125)
d	Recently formed CL present at Day 7 post-coitum (x 160)
e	Recently formed CL present at Day 5 post-coitum (x 160)
f	Recently formed CL present at Day 6 post-coitum (x 160)

FIGURE II

a



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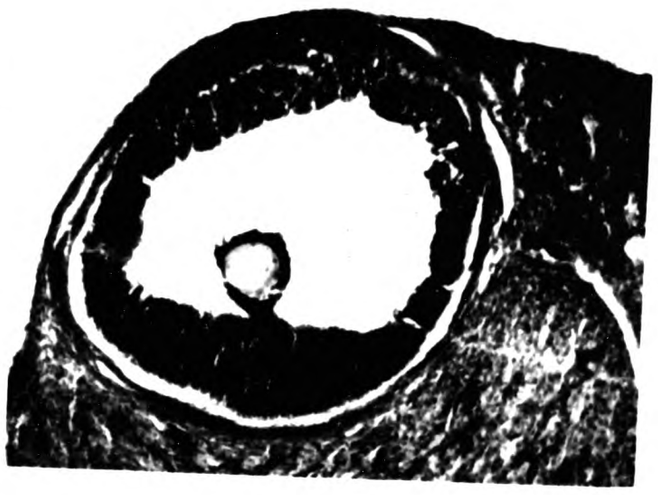

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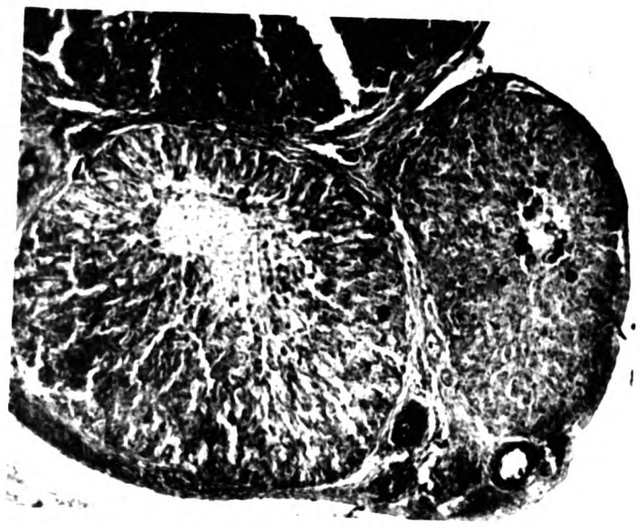

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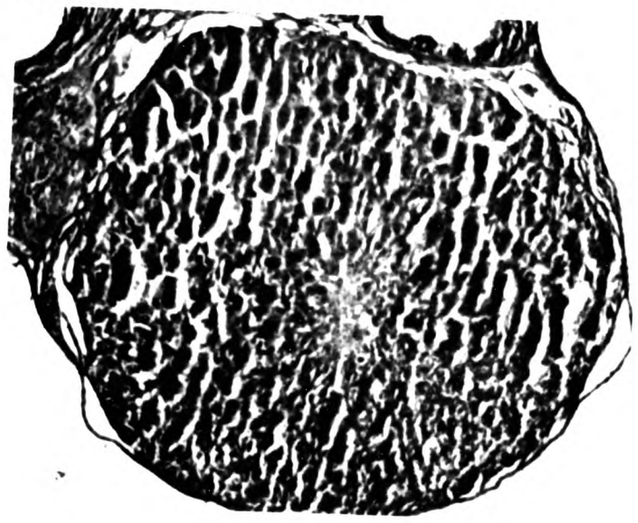

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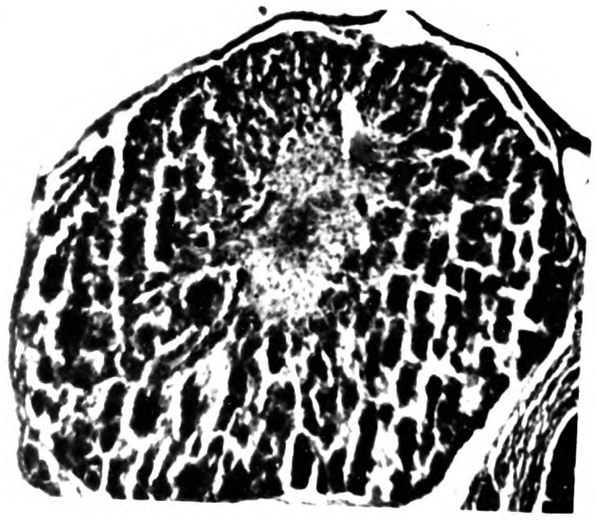

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FIGURE II

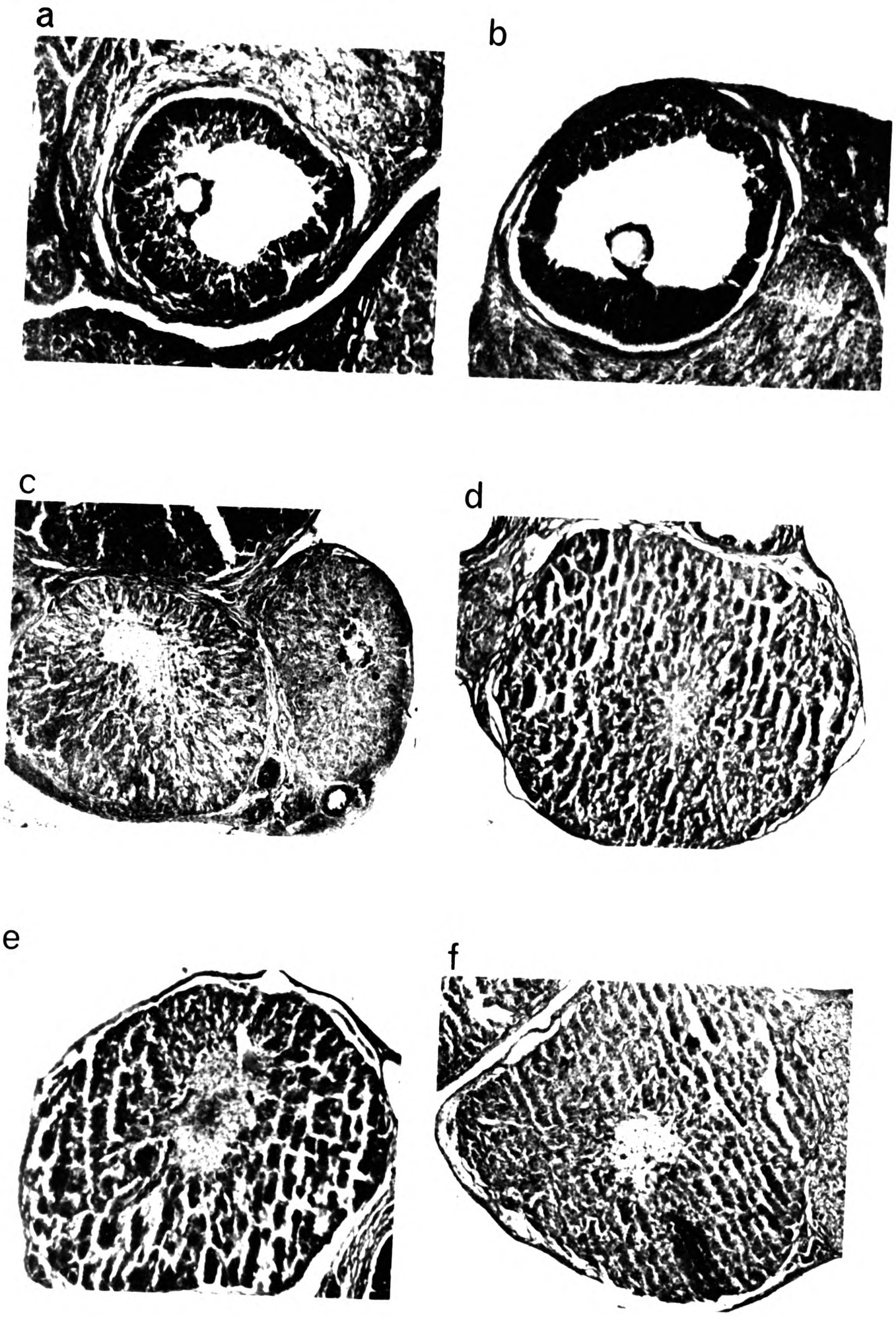


FIGURE 12

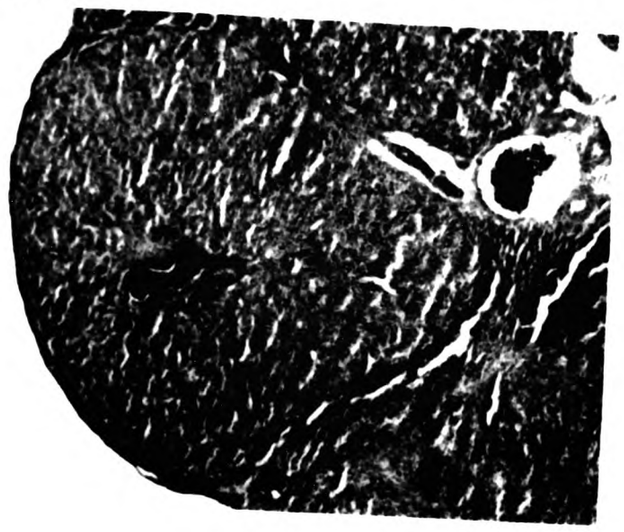
Histological Sections of Ovarian Tissue

Legend to Figure 12

Plate	
a	Recently formed CL present at Day 7 post-coitum (x 160)
b	Established CL present at Day 7 post-coitum (x 160)
c	Established CL present at Day 5 post-coitum (x 160)
d	Copora albicantia (x 125)
e	Whole ovary at D3 post-coitum, showing recently formed CL and Graafian follicles (x 15)
f	Whole ovary of cyclic control rat. (x 15)

FIGURE 12

a



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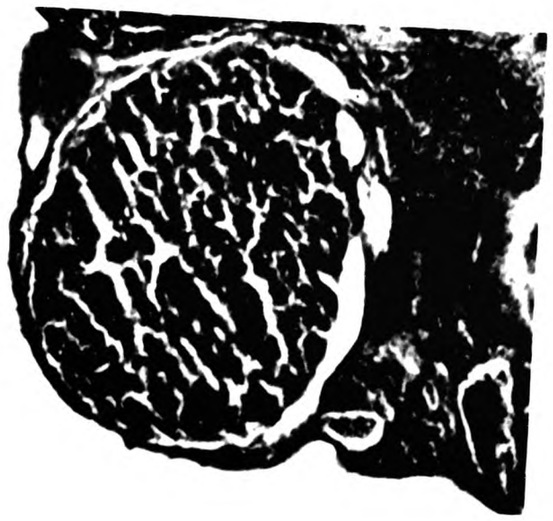

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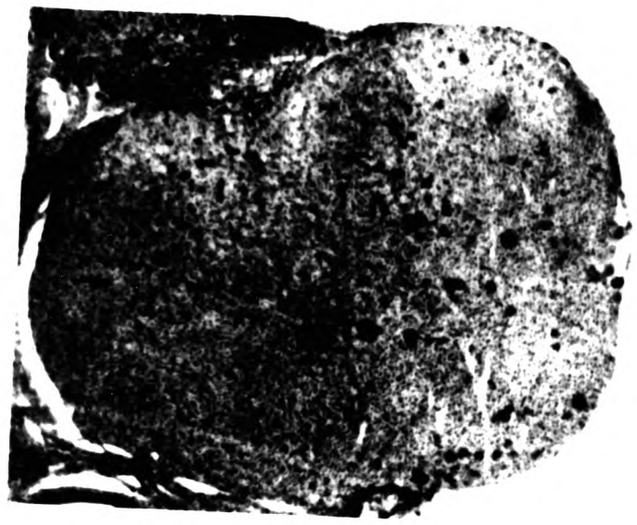

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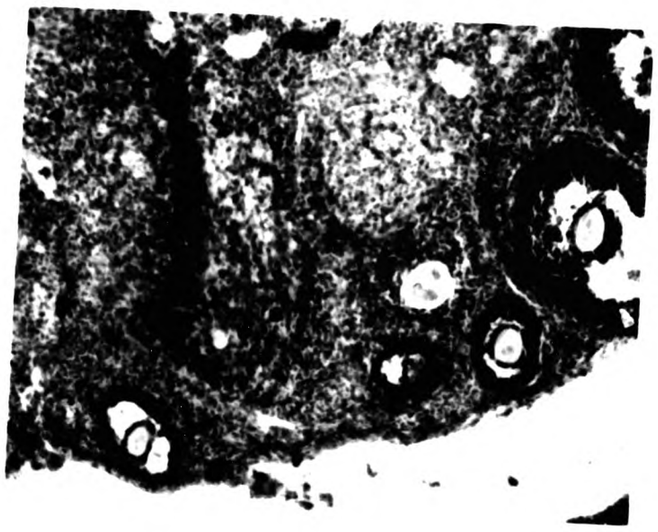

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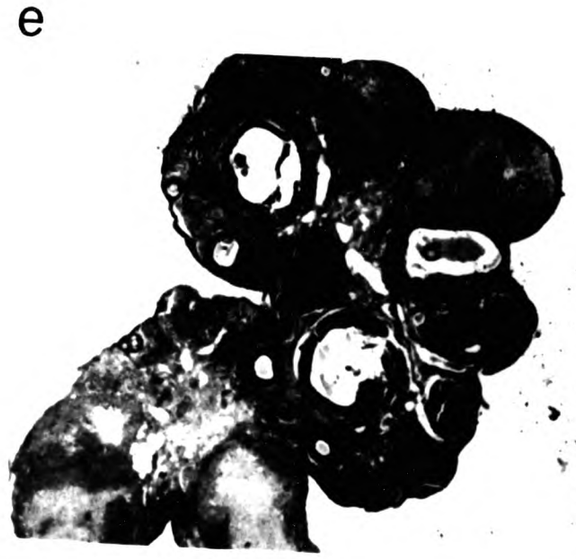

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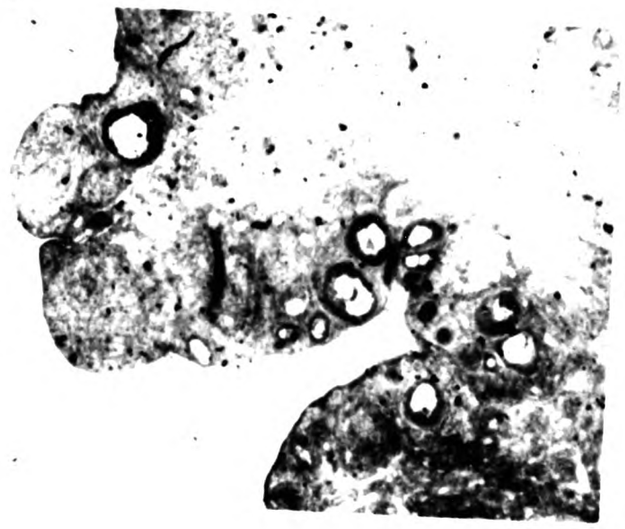

FIGURE 12

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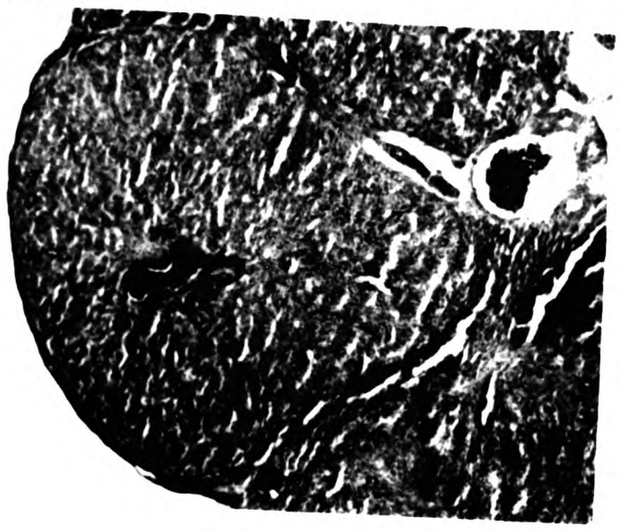

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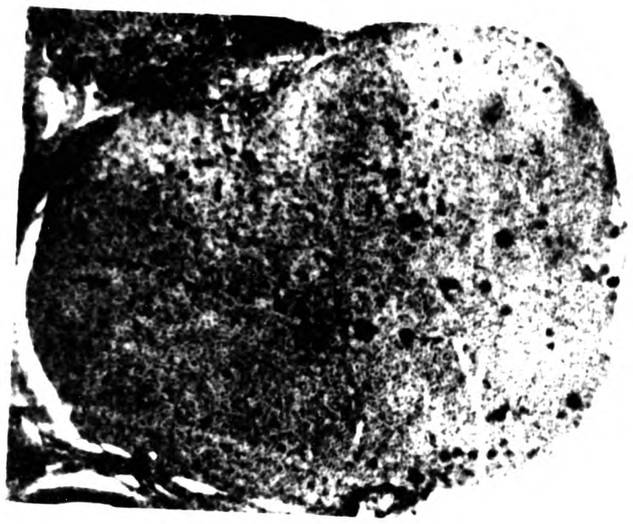

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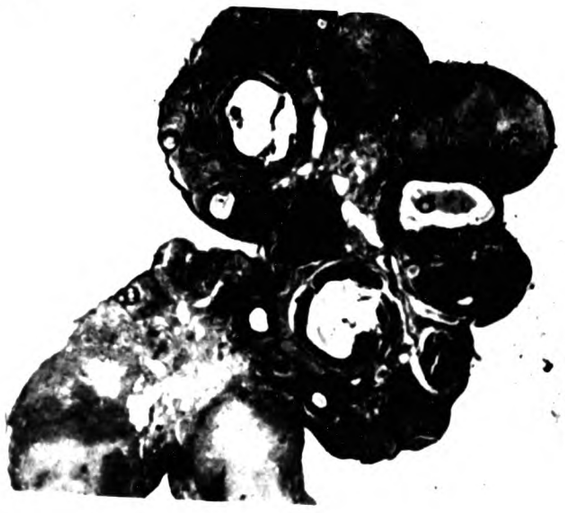

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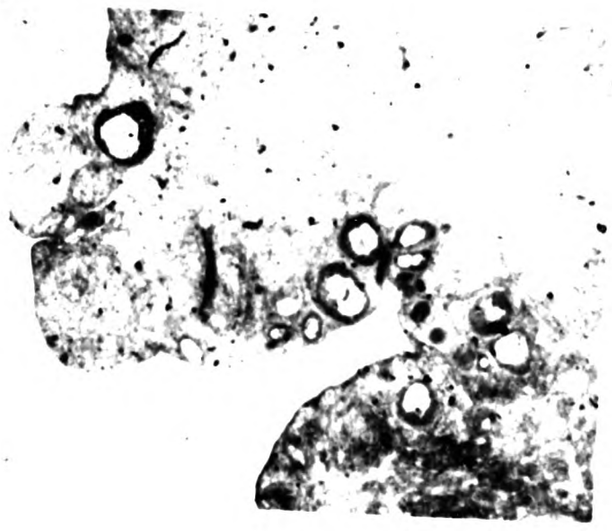

DISCUSSION

These histological studies were a more precise method of establishing the presence of SCL and follicular development during pregnancy, than the earlier ova recovery experiments. It was not certain that the ova recovered from Sprague-Dawley rats on D7 post-coitum were not tubal 'locked' ova from coitally induced ovulations. It was apparent from the data accumulated on the degenerative stages of unfertilised ova from control cyclic females, that the ova recovered at D7 post-coitum were at a more advanced degenerative stage than would be expected, even though the zona pellucida was intact. Thus, these histological experiments provided further information on follicular development and SCL formation.

The histological investigaton of ovarian tissue from Wistar rats was concentrated on D3 and 7 post-coitum, due to the time factor, as a representative of before and after SCL formation. However, although not statistically viable, ovaries from nine Sprague-Dawley rats sacrificed on D2, 4, 5, 6 and 7 post-coitum were investigated also, and provided values comparable to those of Experiment 1, substantiating the accuracy of the counts made by dissection of fresh ovarian tissue at sacrifice.

Initially, difficulty was found in differentiating between active CL and regressing corpora albicantia, in ovaries studied from D3 and metoestrus females: however, after careful comparison of the two structures, they were differentiated as shown in Figure 12, plates b, c and d. This was not a problem when examining females sacrificed on D7 post-coitum as the corpora albicantia had undergone considerable regression and could not confound counts of active CL.

The results show that follicular development does continue during the early period of gestation, confirming the data of Swezy and Evans (1930) and Brown-Grant (1943). Follicular development appears to follow a pattern of growth, repeated every 2 to 3 days. Large Graafian follicles (follicles large than 650μm) are certainly present at times during the first trimester and could ovulate if the necessary trigger were provided.

As these were found to be present on day 4 and 5 post-coitum they may possibly be the precursors of SCL formation, and average numbers of Graafian follicles located on D4 and 5 post-coitum were consistent with the numbers of SCL recorded after D7 post-coitum. The presence of Graafian follicles on D4 and 5 post-coitum is also consistent with the findings of Everett (1977).

The number of Graafian follicles located on D3 post-coitum for Wistar rats were fewer than recorded for Sprague-Dawley rats on D4 and 5 and it would perhaps be expected to locate more than an average of 0.6 Graafian follicles at this time, as SCL formation occurs around D5 post-coitum. However, at D3 post-coitum, several follicles were present which were only just smaller than the criteria of 650μm and may well have developed into Graafian follicles by D4 or 5 post-coitum. A more comprehensive study would confirm this. These small follicles were not present in the ovaries studied from the two Sprague-Dawley rats sacrificed on D6 post-coitum, each of which possessed only one Graafian follicle.

It would appear therefore, that follicular development continues during the period of early gestation studied, and that ovulatory Graafian follicles are present around the time of SCL formation. These follicles then appear not to be present immediately after SCL formation.

CHAPTER 7

GENERAL DISCUSSION

The data presented in the preceding chapters confirm the initial observations from the earlier World Health Organisation experiments, that is, the apparent formation of additional CL during early pregnancy in the rat. These extra CL, termed supplementary corpora lutea, appear between D4 and 7 post-coitum in both pregnant and pseudopregnant rats; it may therefore be concluded that the hypothalamus - hypophysial - ovarian axis is functional during the first trimester and is associated with both follicular development and ovulation.

This observation is not in agreement with some established theories that there is no cyclic follicular activity during pregnancy (Amoroso and Finn, 1962; Greenwald, 1966). The inhibition of follicular growth and ovulation is usually attributed to the increased secretion of ovarian oestrogens and luteal progesterone, acting to suppress the pituitary hormones reponsible for ovarian stimulation. This theory of ovarian/endocrine inactivity during pregnancy is relatively recent and such theories are in contrast to conclusions drawn from studies by prominent researchers in the early thirties, which suggested that there was a four day cycle of ovagenesis occuring during the rat oestrous cycle which was not interrupted by pregnancy (Swezy and Evans, 1930; Brown-Grant, 1943); the formation of CL during pregnancy had also been noted (Swezy and Evans, 1930).

It was not until investigation into follicular activity and the viability of the pituitary during pregnancy was undertaken (Zeiner, 1952; Welschen et al, 1961; Everett and Nicholls, 1968; Everett, 1977), that evidence was accumulated to confirm the earlier research of Swezy and Evans (1930). These later research workers concluded that follicular development did continue during early pregnancy and that the endocrine control of ovulation was not completely static at this time. Indeed, ovulation was obtained during the first trimester, after administration of exogenous oestrogen (Everett and Nicholls, 1968; Everett, 1977). The evidence of SCL formation presented in this theses is therefore consistent with the findings of these earlier research workers.

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It was initially postulated that SCL may have been triggered by a GT-like substance released from the rat placenta (Bambra and Combe, 1978).

However, compared to the situation in the mare (Rowlands, 1948; Amoroso, 1955), at the time of SCL formation in the rat, the placenta would probably be secreting hormones in insufficient concentrations to trigger ovulation. The possibility of the blastocyst initiating a release of GT from the pituitary, comparable to the uterine-neural reflex triggering the PRL release, is invalid as SCL are present in the pseudopregnant female and SCL are therefore not pregnancy specific. Thus, it would appear that the trigger for SCL production is maternal in origin, and an endocrine basis for such an ovulatory stimulus must be considered.

It was therefore postulated that the preimplantation oestrogen surge may be the trigger for SCL formation, as the timing between these events was comparable to that between the preovulatory oestrogen surge and ovulation in the oestrous cycle. The results presented in the previous chapters are not however entirely consistent with this hypothesis. The initial experiment, whereby the elimination of the preimplantation surge prevented SCL formation suggests strongly that this increase in oestrogen levels was infact the trigger. However, if this is the case, then a GT surge should be elicited as a result of the oestrogen release, in order that ovulation can take place. The timing between the preimplantation oestrogen surge and SCL formation is comparable with that of the oestrous cycle, and may indicate therefore that a GT surge is initiated. The data however, from the experiment undertaken to confirm this hypothesis is not conclusive of GT's being released, after a comparable time lapse to that of the oestrous cycle.

The results indicate that there may be a release of FSH from the pituitary around N4 post-coitum, although the plasma FSH concentrations were not significantly different to levels observed during the pro-oestrous period. The decrease in pituitary levels on N4 post-coitum was not reflected in the plasma levels and thus these results are not conclusive of GT release being initiated by the preimplantation surge, at least on a time scale similar to that of the oestrous cycle. However, it may be that the single measurements taken were not sufficient to adequately monitor a surge of GT release from the pituitary. LH and FSH are released episodically (Nalbandov, 1976; Knobil, 1974) and hence the more frequent measurement of

hormone levels at the expected timing of the GT surge would confirm whether release occurs in response to the preimplantation oestrogen peak. It would also be useful to monitor GT levels in females before and after the expected LH/FSH surge, in order to achieve a better comparison with hormone concentrations during the GT surge itself.

Although the endocrine data presented in this thesis does not conclusively confirm that the preimplantation oestrogen surge results in the release of LH/FSH from the pituitary, the release of oestrogen on D4 post-coitum still seems to be a logical trigger for ovulation during the first stages of pregnancy in the rat. This is supported by results presented by Everett (1977), whereby an LH surge was induced by the administration of exogenous oestrogen on D4 post-coitum in pregnant rats. There is also evidence that fluctuations in pituitary GT content occur during the first two weeks of pregnancy in the rat (Zeiner, 1952; Everett, 1977); these are comparable at times to pituitary LH/FSH concentrations at pro-oestrus .

It is possible that the ovarian oestrogen itself may be a controlling factor with regard to SCL formation as the internal feedback of oestrogen on the ovary influences ovarian LH/FSH receptors. Hence, it may be that the preimplantation surge of oestrogen increases the number of ovarian LH/FSH receptors and smaller amounts of LH/FSH are able to initiate ovulation. This could explain the small increases in GT concentration measured in the present studies in females in which ovulation nevertheless occured. As the preimplantation surge of oestrogen appears to be the only consistent maternal trigger which could initiate ovulation during pregnancy, this may explain why SCL are not formed at other times during pregnancy when continued follicular development may be present. However, further investigations with respect to the endocrine control of SCL formation would be recommended.

The initial experiments investigating the recovery of ova to confirm the presence of newly ovulated follicles were unsuccessful, mainly due to problems in developing a suitable technique which could guarantee 100 per cent collection of one or two ova per rat. It was also not certain, that the hormone environment existing during early pregnancy was conducive to

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the normal stages in ovum transport post-ovulation. Progesterone in elevated concentrations is accepted to cause tubal 'locking' (Na lbandov, 1976) and it may be that any ova released during a period of increased progesterone concentrations, would perhaps not be received by the fimbria, or would be 'locked' in the antral section of the Fallopian tube. Thus, flushing of the Fallopian tubes on D5 to 7 post-coitum, would not result in collecting ova from SCL formation.

The histological study of CL was a more conclusive method in confirming the presence of SCL. The inital knowledge gained when studying the control ovaries was invaluable when applied to those of pregnancy. This work confirmed the structures of active CL, corpora albicantia and recently formed CL, which was essential to positively identifying SCL. The finding of newly formed CL on D5 and 6 post-coitum was consistent with the earlier information as to the timing of SCL formation. The granulosa structure of these SCL located on D5 and 6 was consistent with those of recently formed CL of the oestrous cycle, and appears to confirm also that SCL are formed from true ovulatory follicles, rather than by the luteimisation of unovulated Graafian follicles. Hence it would be expected to recover ova from the formation of SCL and as this was not possible, the hypothesis that their transport is retarded due to the hostile hormone environment may be feasible. These results are consistent with those of Swezy and Evans (1930), who investigated the CL formed from the natural ovulation of mature follicles during pregnancy in the rat, and found no retained ova, nor were ova located in the Fallopian tubes.

It was considered essential throughout this research that all CL counts were recorded by at least two independant people and where possible, without the knowledge of the day of pregnancy/oestrous cycle. This criteria was strictly enforced after the day of SCL formation had been verified.

Follicular development was evident on the days studied, appearing to be cyclic in nature, which is consistent with data recorded by other researchers (Swezy and Evans, 1930, Welschen et al, 1961). Mature follicles of ovulatory size (Graafian follicles) were present at the time

of SCL formation and the average numbers recorded were consistent with the mean number of extra CL (SCL). It would have been more conclusive if this study had been expanded to include D1 to 9 post-coitum, thereby establishing a definite pattern of follicular growth. It would be pertinent to mention here that follicular activity/development must be present until day 4 post-coitum in the rat, in order to produce the preimplantation surge of oestrogen, and therefore the evidence presented here relating to follicular development is not exceptional.

Having established the presence of SCL during early pregnancy in the rat, their apparent function is not known. Morphological evidence suggests that SCL are functional and could secrete progesterone, although whether they increase the overall ovarian progesterone release is unclear. The plasma progesterone profiles for both strains of rat, pregnant and pseudo-pregnant, are consistent with those of other workers (Smith et al, 1975).

The same pattern was followed for both Sprague-Dawley and Wistar rats, including an identical transient decrease in progesterone concentrations on D5 post-coitum. To verify whether this transient decrease and subsequent increase on D6 post-coitum was due to SCL formation, hormone levels should be monitored at more regular intervals around SCL formation and progesterone concentrations measured during early pregnancy, after initially inhibiting the formation of SCL. Presumably, progesterone profiles determined by other researchers include any progesterone contribution from SCL.

Although, from the data presented, the SCL appear to have no significant function in the release of progesterone they may in fact, play a role in ensuring that plasma progesterone remains elevated at a critical time of gestation, notably D7 post-coitum, when prolactin support is withdrawn (Morishige and Rothchild, 1974). They are possibly important also in ensuring that pregnancy reaches term, since the CL are the only source of progesterone during pregnancy in the rat (Yoshinaga, 1978). It must be considered however, that SCL may be produced by accident rather than by design, due to the rather excessive concentrations of oestrogen produced to initiate implantation. However, the preimplantation surge of oestrogen

is of a concentration far greater than is required to initiate implantation (Dickman, 1980). If this is not just a case of an exuberant release of a hormone, then to what purpose is the excess hormone put? Is it possible that the preimplantation surge is released in such a concentration to overcome the limiting threshold of the pituitary and thereby precipitate the release of GT, or further, to increase the number of LH/FSH ovarian receptors, as previously mentioned.

The formation of SCL do not appear to be specific to the Sprague-Dawley females, the strain of rat used in the WHO experiments when this phenomena was noted. The presence of SCL has also been confirmed, from the data presented in this thesis, in the Wistar strain. There is evidence of their formation in the wild Norway rat (Perry, 1971).

Whether this phenomena is universal to the rat species remains to be proved. There is evidence that there may be a strain difference regarding the ability to ovulate during pregnancy. Brown-Grant (1943) was able to induce ovulation after administration of exogenous oestrogen on D4 post-coitum in pregnant Wistar rats, but not in some other strains. This response was confirmed in pseudopregnant females by Everett (1977).

It is likely that the apparent strain difference regarding CL formation post-coitum is as a result of experimental design, if the trigger is the preimplantation surge of oestrogen on D4 post-coitum. It is universally accepted that all strains of rat require a preimplantation surge of oestrogen to initiate implantation (Deansley, 1966; Na Ibandov, 1976). It would be unlikely, therefore, that some strains would not produce SCL, providing follicular growth during early pregnancy occurs at the same rate in all strains. The other variable would be the pattern of GT production by the pituitary. If either of these were out of sequence with the preimplantation oestrogen surge, SCL formation would not occur.

Further research is required to conclusively elucidate the mechanism of SCL formation and also to confirm whether their function is to contribute to progesterone output during the early stages of pregnancy.

The former would perhaps be better resolved initially by repeating experiment 11, in order that a more comprehensive evaluation of the plasma and pituitary GT levels around SCL formation could be established. More than one sample should be taken around the expected timing of an LH surge, so that a profile of the surge and its precise timing could be elucidated. The values presented in the results section of experiment 11 were designed to be taken during the peak of the LH/FSH surge. This may not however have been the case and the values presented may be representative of the inclining or declining values of the surge. Also, it would be more informative to establish the overall progesterone profile for the whole of this period, incorporating the expected LH/FSH surge; that is from after the preimplantation surge of oestrogen to D7 post-coitum, when LH/FSH concentrations should have declined to basal levels. In order to establish whether SCL contribute significantly to the overall progesterone output, at this critical stage of pregnancy (ie when PRL support is withdrawn), experiments designed to remove the SCL would be valuable.

It may be concluded, therefore, that ovulation occurs between D4 and 7 post-coitum in the pregnant and pseudopregnant Wistar and Sprague-Dawley rat. The mechanism for the trigger of this phenomena is not certain, but it would appear to be maternal in origin and possibly linked to the preimplantation surge of oestrogen. The physiological function of these extra CL formed during the first trimester is not known, but may be postulated to support pregnancy during the period of PRL withdrawal.

Finally, the presence of SCL will have a profound effect on embryo mortality studies and it is pertinent to discover the specificity of this phenomena to particular strains of rat, as their presence has a bearing on some universal research techniques used today. Their formation during early pregnancy obviously presents a problem in the estimation of embryo mortality for this species, since mortality is measured by comparing the number of CL observed to the number of embryos. Not only do extra CL appear on D1 post-coitum as a result of coitus (from which ova are available for fertilisation) (Zarrow and Clarke, 1968; Rodgers, 1971), but SCL are formed between D5 and7 post-coitum. Thus, past and present embryo mortality rates, determined routinely in toxicology studies, may be

misleading. This generally accepted technique would obviously require standardising in the light of SCL formation, or the employment of strains which perhaps do not produce SCL during pregnancy.

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APPENDIX 1

Animal Dietary Components

1 Wistar Rats

Nottingham University Rat Diet

Ingredient	% Inclusion
Ground wheat	46.00
Ground oats	24.45
Dried skim milk	2.50
Dried yeast	2.50
Fish Meal	15.00
Tallow fat	5.00
Salt	0.50
Cod liver oil	0.50
Vitamin supplement	2.50
Inert binding agent	1.05
	100.00

Crude fibre -8g
Vitamin A -3.21%

2 Sprague-Dawley (CD) Rats

Dixons FFG(M) Diet

Ingredient
Wheat
Barley
Maize
Soya bean meal (extracted)
Fish meal
Molasses
Salt
Vitamin supplement

Mineral supplement

The actual quantities of the above diet are not supplied by Dixons.

APPENDIX 2

1 Total number of CL for individual pregnant Sprague-Dawley rats sacrificed on Days 1-9 post-coitum

Day of Gestation	Total Number of CL for Individual Rats
1	15, 13, 18, 16, 17, 15, 14, 16, 14, 15
2	19, 15, 15, 12, 15, 16, 15, 16, 11, 10
3	17, 17, 15, 16, 16, 10, 9, 13, 15, 16, 13
4	15, 17, 15, 17, 19, 11, 21, 15, 14, 14
5	10, 14, 11, 12, 15, 24, 14, 11, 15, 14, 14
6	19, 9, 18, 15, 19, 13, 19, 20, 14, 16, 15
7	22, 19, 18, 18, 22, 14, 14, 17, 16, 21, 15, 13, 18, 16
8	18, 19, 22, 18, 19, 15, 19, 17, 20, 18, 16
9	18, 23, 22, 22, 19, 17, 16
Cyclic Control	3, 13, 8, 6, 11, 12, 13, 15, 11, 8

2 Total number of CL for individual pregnant Wistar rats sacrificed on Days 1-9 post-coitum

Day of Gestation	Total Number of CL for Individual Rats
1	12, 14, 15, 10, 16, 13, 8, 12, 7, 10
2	12, 17, 16, 17, 15, 11, 17, 13, 10, 14, 12
3	13, 12, 13, 18, 10, 13, 12, 10, 12, 13
4	15, 11, 13, 13, 11, 10, 22, 9, 13, 10
5	12, 16, 16, 16, 20, 14, 13, 16, 17, 13
6	15, 14, 14, 16, 17, 17, 18, 16, 12, 12
7	16, 16, 17, 19, 19, 20, 20, 15, 11, 14, 15
8	20, 17, 15, 15, 19, 17, 14, 22, 16, 11
9	15, 15, 16, 20, 18, 19, 12, 18, 22, 13
Cyclic Control	5, 13, 12, 9, 16, 11, 13, 13, 17, 18

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