Studies of seasonality in red deer (*Cervus elaphus*): with special emphasis on the reproductive physiology of red deer hinds.

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Four trials were conducted to investigate factors controlling the seasonal onset of reproductive activity in red deer hinds.

Firstly (Chapter 4), the role of photorefractoriness to long daily photoperiods in the initiation of the seasonal reproductive activity in breeding red deer hinds was examined. Red deer hinds (n=10) were prematurely exposed to a long daily photoperiod of 15.3 h from 22 July to 8 November 1986 i.e. winter-spring (EPW), or maintained under natural photoperiods (NP). Six hinds experienced the natural changes in daily photoperiod until mid-summer but were exposed to a 15.5 h of light each day from 30 January to 30 April 1987, i.e. summer-autumn (EPS), whilst hinds in the other groups experienced naturally decreasing daily photoperiods. On 5 occasions (July 1986, January, February, March and April 1987), blood samples were collected from 4 NP and 4 EPW hinds every 20 minutes for 4 h to monitor secretion of luteinising hormone and half hourly for another 4 h following an i.v. injection of 2 μg GnRH to measure pituitary responsiveness. In January, March and April 1987EPS hinds were also intensively sampled for 4 h. Plasma progesterone concentrations and mean date of calving indicated that the onset of breeding activity was not affected by light treatment in the EPW hinds but was delayed by 3 weeks in the EPS hinds. In contrast, supplementary lighting caused a premature elevation of plasma prolactin concentrations and advanced pelage moulting in EPW hinds only. Plasma LH secretion patterns indicated that LH pulse frequency and mean LH concentrations were greater during the breeding season than during pregnancy or, seasonal or postpartum, anoestrus. The reduction in LH secretion was partially explained by a diminished pituitary responsiveness to GnRH. Daily plasma

melatonin secretion patterns indicated that the duration of the nocturnal increase in melatonin concentrations was responsive to changes in photoperiod and provided a suitable endocrine signal for measuring daylength. The results suggest that, unlike the sheep and prepubertal red deer hind, the onset of seasonal breeding activity in breeding red deer hinds is not affected by long daily photoperiods in spring but is delayed if the autumnal decrease in daily photoperiod is delayed. Therefore neither the development of photorefractoriness nor the the spring increase in daily photoperiods initiated the transition from seasonal anoestrus to reproductive activity in the breeding hind. However, long daily photoperiods may have entrained the annual cycle of pelage shedding and prolactin secretion. It is possible that the neuroendocrine pathway by which photoperiodic signals entrain the seasonal cycle of reproduction is separate from those which regulate other seasonal events in the breeding red deer hind.

Secondly (Chapter 5), in a study of seasonality of reproduction, 4 pubertal hinds were monitored for live weight and plasma LH and progesterone concentrations from December 1987 to October 1988 (i.e. 12-22 months of age). In addition the pattern of LH secretion was also studied in 4 ovariectomised pubertal hinds implanted s.c. with controlled release implants containing 12 mg oestradiol-17β between 4 March and 25 May and between 15 June and 19 September, 1988. On several occasions (15 December, 29 February, 15 March, 24 April, 14 June, 29 June, 18 September (all hinds) and 3 October (ovariectomised hinds only) blood samples were collected every 20 minutes for 4 h to monitor secretion of luteinising hormone and following an i.v. injection of 2 μg GnRH to measure pituitary responsiveness. Plasma progesterone profiles indicated that 4-6 ovarian cycles, lasting about 19 d each, occurred in each intact hind. Regular ovarian cycles commenced in late April (26 April ± 3.4 d, mean ± s.e.m.) and ceased 3 months later in July (21 July ± 7.2 d). The number of LH pulses in the intact hinds was higher in June (1-2 pulses/4 h) than in the non-breeding season (< 1 pulse/4 h) probably due to a seasonal increase in GnRH secretion. It appears that the seasonal increase in LH pulsatility and onset of reproductive activity in the entire hinds were temporally related to a reduction in the sensitivity of LH secretion to the negative feedback effects of oestradiol in ovariectomised pubertal hinds.

In the third trial (Chapter 6), 20 male and 20 female red deer calves were immunised at birth against a melatonin conjugate or injected with adjuvant only (controls). Booster injections were given on 5 occasions over the next 2 years. Stags which produced significant melatonin binding activity in response to immunisation, were heavier than the controls between 7 and 11 months of age and at 16, 20, 30 and 34 months of age. A similar but smaller effect on live weight was seen in the immunised hinds. Immunisation against melatonin did not affect the calving date of the hinds or antler development and time of casting of antlers in the stags. These results indicated that disruption of the photoperiodic signal by immunisation against melatonin may have prevented the entrainment of annual rhythms in feed intake and growth but without affecting the seasonal cycles in antler growth and ovarian activity.

Finally in a series of 3 trials (Chapter 7), anoestrous red deer hinds were induced to ovulate with the GnRH analogue, buserelin. Hinds were pretreated with intravaginal devices containing 0.6 g progesterone (CIDR-Type S) for 14 days prior to CIDR withdrawal on 4 March. In Year 1, 15 hinds were treated with 1 CIDR each and 8 hinds were injected i.m. with 4 μg buserelin (a GnRH analogue) at CIDR removal followed by 2nd injection of 10 μg 48 h later. In Year 2, 16 hinds were treated with 2 CIDRs each and 8 hinds were injected i.m. with 4, 3, 2, 2 and 10 μg buserelin at -48, -24, 0, 24 and 48 h respectively from CIDR withdrawal. In Year 3, 15 hinds were treated with 2 CIDRs and 11 hinds (Groups B and BO) injected with buserelin identical to the protocol in Year 2. At CIDR withdrawal 6 of these buserelin treated hinds (Group BO) and 4 other progesterone-primed hinds (Group O) were injected with 500 μg oestradiol benzoate. In addition, during the breeding season, 4 hinds (Group C) were treated with double CIDRs only for 14 d from 1 April. When oestrous behaviour and the pattern of plasma LH secretion were monitored in Year 3, oestrous behaviour was less noticeable and delayed in Group B hinds and peak LH levels were lower and increased later relative to Group O, BO and C hinds. Two weeks after CIDR withdrawal, 6 buserelin-treated hinds in both Year 1 and Year 2, and 3/5 B, 2/4 O, 3/6 BO and 4/4 C group hinds in Year 3 had a single corpus luteum present. Plasma progesterone concentrations were elevated for about 12 d in most hinds with a corpus luteum in Year 2 and in most B, BO and C Group hinds in Year 3. However, progesterone secretion was low in several Year 1 and all Group O hinds in Year 3 indicating that the induced corpora lutea in these hinds were functionally

subnormal. There was no evidence that any of the buserelin-induced ovulations resulted in pregnancy, probably because the induced ovulations were not accompanied by normal hormonal and behavioural patterns.

The experiments described in this thesis have contributed to our understanding of the regulation of seasonal breeding in red deer hinds. This knowledge may eventually enable reproductive activity to be effectively manipulated, thereby improving the efficiency of deer production systems.

Dedication

To my brother, Stuart Allan Harry Duckworth

(1 February 1966 - 6 May 1992)

If you are lost, and you look,

I will be there.

Waiting.

Time after time.

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Chapter 1

Introduction

Between 1861 and 1923, red deer (*Cervus elaphus*), originating from England and Scotland, were introduced into New Zealand (Logan & Harris, 1967) and became successfully established throughout both islands (Challies, 1985). From the 1930s wild populations of red deer were commercially exploited for skins and venison and in 1969 the first red deer farm was established (Coop & Lamming, 1976). Since then the deer farming industry in New Zealand has expanded rapidly so that in 1991 there were about 1 million red deer behind fences.

Red deer are seasonal breeders. Mating occurs in mid autumn (Kelly & Moore, 1977; Clutton-Brock *et al.*, 1982) and calving during early summer (Kelly & Drew, 1977; Bray & Kelly, 1979; Coop & Lamming, 1977; Asher & Adam, 1985). Seasonal breeding is a survival mechanism that ensures that offspring are born when the climatic conditions and food availabilitymost favour the survival of the offspring (Sadlier, 1969; Lincoln & Short, 1980; Karsch *et al.*, 1984). As the gestation length of most species is fixed, it is the time of conception which determines the date of parturition. Most mammals that have evolved in cool climates show changes in reproductive activity in response to changes in photoperiod (see Thibault *et al.* 1966). At a given locality the length of daylight changes in a very predictable manner and therefore provides a reliable proximate enviromental cue for precise timing of the mating season (see Lincoln & Short 1980; Bronson, 1985). In red deer, calves are born during the summer, presumably at the time most favourable for reproductive success in their native habitat. On New Zealand farms however, summer calving is too late to synchronise the spring peak in pasture production with the high feed demands of hinds during late lactation. With the recent increase in the numbers of farmed red deer there has been much interest in the artificial control of the calving season in order to align more efficiently the feed demands of the lactating hind with feed supply.

These studies aimed to provide information on the physiological mechanisms controlling seasonal breeding in red deer hinds and examine the influence of photoperiod and

melatonin on the seasonality of the species. The thesis also evaluates a method for advancing the calving date using a GnRH analogue, buserelin.

Chapter 2

Literature Review

2.1 Seasonal Breeding

Deer live in a wide range of habitats in cold, temperate and tropical climates (Whitehead, 1972) and produce their offspring at a time of year suited to the seasonal variation in their food supply (Lincoln, 1985). Cervids of temperate or arctic origin exhibit strict annual seasonality of births whereas species of equatorial origin tend to breed throughout the year (Zuckerman, 1953; Lincoln, 1985). In red deer, which originated in the temperate regions of Europe (38 ° N to 65 ° N) (Whitehead, 1972), seasonal changes in reproductive activity are characterised by a limited oestrous cyclicity in the female (Kelly & Moore, 1977; Clutton-Brock *et al.*, 1982; Lincoln, 1985) and dramatic changes in antler growth, behaviour and spermatogenesis in the stag (Lincoln, 1979; Clutton-Brock *et al.*, 1982).

2.2 Reproductive activity in red deer hinds

The breeding season of red deer hinds begins in the autumn and calves are born during the following spring (Guinness *et al.*, 1971; Kelly & Moore, 1977). Although hinds are polyoestrous, under farming conditions most (> 80%) conceive at the first mating (Asher & Adam, 1985; Fennessy *et al.*, 1990a). Mating is generally confined to a period in mid April and most calves are born in early December. The mating period occurs in the same season in New Zealand as in Europe (Marshall, 1937) and mean calving dates differ by exactly 6 months between the southern and northern hemisphere (Fletcher, 1974). Thus, when dates are adjusted by 6 months, the time of the onset of the breeding season and of calving reported in British studies closely correspond to dates of conception and parturition for red deer in New Zealand (see Table 2.1 & 2.2).

Table 2.1. Date of onset of breeding activity for red deer hinds in New Zealand and United Kingdom as determined from plasma progesterone profiles. (* indicates dates were adjusted by 6 months to the Southern Hemisphere equilavent).

Locality	Onset of breeding activity (mean ± s.e.m.)			Reference
New Zealand				
Otago	18	April ±	4.2	Jopson *et al.*, 1990
United Kingdom				
Scotland	17	April* ±	2.2	Adam *et al.*, 1985
England	26	April* ±	4.4	Curlewis *et al.*, 1988b
England	29	April* ±	6.7	Loudon *et al.*, 1989
England	23	April* ±	2.3	Adam *et al.*, 1989a

Red deer are generally monotocous with a low incidence (0.1-0.7%) of twins (Chapman, 1974; Mitchell *et al.*, 1981). Mean gestation length has been variously estimated at between 230 and 236 days (Guinness *et al.*, 1971; Lincoln & Guinness, 1973; Kelly & Moore, 1977; Clutton-Brock, 1982; Adam *et al.*, 1985; Moore & Cowie, 1986). Variation in mean gestation length is small (c.v. 0.5-3%) and variations in calving date most likely reflect differences in the time of conception. An unusual phenomenon in red deer is that some hinds have a small accessory corpus luteum present during pregnancy (Douglas, 1966; Guinness *et al.*, 1971; Kelly & Challies, 1978), the significance and function of which is uncertain (Kelly *et al.*, 1985).

Table 2.2. Calving dates for red deer hinds in New Zealand and United Kingdom. (* indicates dates were adjusted by 6 months to the Southern Hemisphere equilavent)

Locality	Calving date (mean ± s.e.m)			Reference
New Zealand				
Otago	9	December	± 1.9	Bray & Kelly, 1979
Otago	13	December	± 1.4	Bray & Kelly, 1979
Otago	16	December	± 0.9	Moore & Cowie, 1986
Otago	11	December	± 4.7	Fennessy & Fisher, 1988
Canterbury	13	December	± 8.0	Webster & Barrell, 1985
Canterbury	9	December	± 3.8	Barrell, 1985
United Kingdom				
Scotland	20	December*	± 1.4	Hamilton & Blaxter, 1980

When pregnancy is prevented, the breeding season of adult red deer hinds lasts about 5 months, ending in September/October[1] (Curlewis *et al.*, 1988b; Adam *et al.*, 1989a; Loudon *et al.*, 1989; Meikle *et al.*, 1991). The period of reproductive activity appears to be shorter in pubertal hinds than in the adults. In the absence of pregnancy, 2-year-old hinds display oestrus over a period of about 3 months ending in late July[1] (Guinness *et al.*, 1971).

Therefore in the female red deer conception is restricted to a certain time of year, the breeding season. During the anoestrous season hinds remain anovulatory and do not exhibit mating behaviour, whereas in the breeding period 6-9 ovulatory cycles recur at about 19 d intervals in non-pregnant hinds (Guinness *et al.*, 1971; Kelly & Moore, 1977; Adam *et al.*, 1985, 1989a).

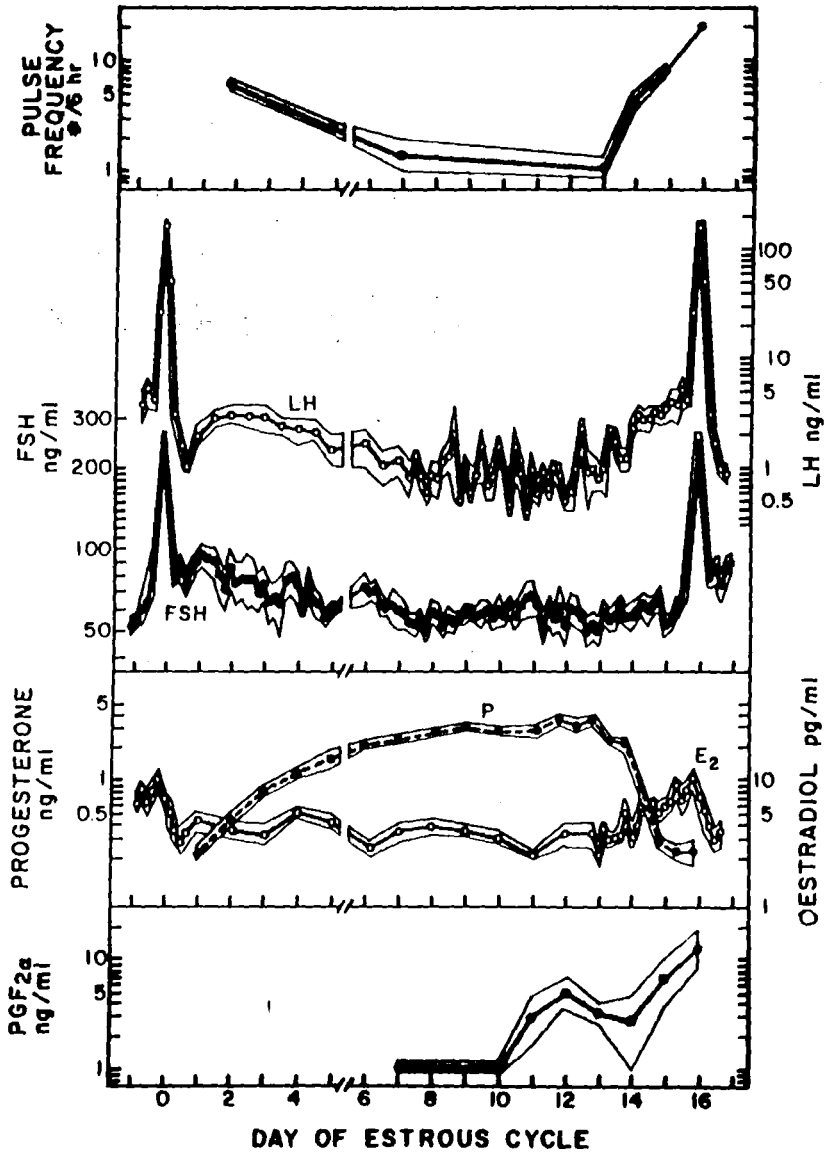
2.3 The oestrous cycle

In spontaneous ovulators the oestrous cycle results largely from the integrated action of luteinising hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary, oestradiol and progesterone from the ovary and prostaglandin ($PGF_{2\alpha}$) from the uterus. Changes in the circulating concentrations of these hormones occur in a specific sequence at predictable times. A generalised summary of the major hormonal events during the ovine oestrous cycle is presented in Figure 2.1.

Many facets of the reproductive physiology of female red deer hinds are yet to be described. The following section, which outlines the general neuroendocrine mechanisms controlling the oestrous cycle of the ruminant, is based on literature about the female sheep. The sheep is a seasonally breeding ruminant which has been extensively studied.

1/ indicates dates were adjusted by 6 months to the Southern Hemisphere equilavent

Figure 2.1. Changes in LH pulse frequency, mean peripheral concentrations of LH, FSH, progesterone (P) and oestradiol (E_2) and uterine vein concentrations $PGF_{2\alpha}$ throughout the ovine oestrous cycle. Shaded areas depict ± s.e.m. for each hormone. Values from day -1 to 5 are normalised to the first LH surge (on day 0); values from day 6 to 17are normalised to the second LH surge (on day 15). Gonadotrophin and steroid values are taken from Goodman *et al.* (1981); $PGF_{2\alpha}$ values are taken from Inskeep & Murdoch (1980); LH pulse frequencies are compiled from Foster *et al.* (1975), Baird (1978a), Karsch *et al.* (1983) and Martin & Thomas (1985). From Goodman (1988).



At the beginning of the oestrous cycle the increase in LH secretion (Hauger, 1977; Baird & Scaramuzzi, 1976a; Baird, 1978a; Baird *et al.*, 1981) stimulates the release of oestradiol from the developing follicles and the resulting sustained increase in oestradiol triggers oestrous behaviour (Robinson, 1954; Fairclough *et al.*, 1976; Karsch *et al.*, 1980) and the preovulatory LH and FSH surge (Scaramuzzi *et al.*, 1970; Beck & Reeves, 1973; Symons *et al.*, 1973; Fairclough *et al.*, 1976; Baird *et al.*, 1980). The LH surge in turn terminates oestradiol secretion (Baird *et al.*, 1981; Goodman *et al.*, 1981a), induces ovulation (Martin, 1984) and initiates luteinisation of the follicular remains (Niswender *et al.*, 1985). As the corpus luteum develops, progesterone secretion begins to rise (Niswender *et al.*, 1985), inhibiting LH secretion (Goodman & Karsch, 1980) and allowing $PGF_{2\alpha}$ concentrations to increase several days later (Karsch *et al.*, 1971). The rise in $PGF_{2\alpha}$ causes luteolysis and consequently a rapid decrease in progesterone concentrations (Scarimuzzi & Baird, 1976; Baird, 1978b). This precipitious fall in progesterone permits tonic LH secretion to increase (Goodman, 1988) and the subsequent oestradiol rise and LH surge initiate the next oestrous cycle.

Central to the control of LH and FSH secretion is the release of gonadotrophin-releasing hormone (GnRH) from the hypothalamus. Release of gonadotrophins can be both stimulated and inhibited by GnRH depending on the pattern of GnRH administration. Small pulses of GnRH increase the production of GnRH receptors in the pituitary gland, stimulating the biosynthesis of LH and priming the pituitary gland to respond more effectively to subsequent GnRH pulses (Clayton, 1989). Whereas chronic treatment with high doses of GnRH may lead to inhibition (down-regulation) of pituitary function or depletion of pituitary gonadotrophins (Nett *et al.*, 1981).

FSH, which is released continuously from the anterior pituitary gland, stimulates growth and development of the preovulatory follicles and plays a key role in the selection of follicles which will ovulate (Baird, 1983; Baird *et al.*, 1991; McNeilly *et al.*, 1991). Although the peak in FSH, coincidental to the LH surge, is dependant on GnRH release from the hypothalamus (Narayana & Dobson, 1979), secretion of FSH is also greatly influenced by the negative feedback effects of oestradiol and inhibin secreted from the ovarian follicles (see Martin *et al.*, 1988; Baird *et al.*, 1991).

Throughout the reproductive cycle LH is secreted episodically from the pituitary in response to GnRH pulses (Caraty *et al.*, 1989; Moenter *et al.*, 1990; Barrell *et al.*, 1992). This background or 'tonic' LH secretion is important for steriod synthesis and follicular development in the ovary (McCracken *et al.*, 1970; Bjersing *et al.*, 1972; Baird & Scaramuzzi, 1976b; Baird & McNeilly, 1981). In addition to the tonic LH secretion system, a separate LH surge system generates the massive preovulatory LH release in response to increasing oestradiol concentrations around oestrus. The ovarian hormones, progesterone and oestradiol, both play a major role in the determining the pattern of LH secretion. Progesterone is always a potent suppressor of LH (Goodman & Karsch, 1980; Martin *et al.*, 1983) and during the luteal phase it acts at the level of the hypothalamus to reduce the activity of the GnRH-pulse generator (Barrell *et al.*, 1992). In contrast, the actions of oestradiol on LH secretion vary markedly with stage of the oestrous cycle. During the luteal phase oestradiol acts synergistically with progesterone to maximally suppress LH secretion (Goodman *et al.*, 1981c; Martin *et al.*, 1983) whereas in the follicular phase the progressive increase in oestradiol is instrumental in initiating the LH surge. The stimulatory effects of oestradiol during the LH surge acts at both the hypothalamus and the pituitary (Karsch, 1987; Moenter *et al.*, 1990). The LH surge induced in ovariectomised ewes by oestradiol treatment is associated with a massive increase in GnRH secretion (Caraty *et al.*, 1989; Moenter *et al.*, 1990) and in addition oestradiol also increases the ability of the pituitary to release LH in response to a GnRH pulse (Reeves *et al.*, 1971; Clarke & Cummins, 1984). The amount of LH released in response to exogenous GnRH is greatest in the follicular phase of the oestrous cycle presumably due to the increase in oestradiol at this time.

At the end of the oestrous cycle luteal function is terminated by the release of $PGF_{2\alpha}$ from the uterus (Baird *et al.*, 1976c). The time of the onset of $PGF_{2\alpha}$ secretion is determined by the rise in progesterone secretion at the beginning of the luteal phase. Oestradiol from the ovarian follicles stimulates secretion of $PGF_{2\alpha}$ (Thatcher *et al.*, 1989) by increasing the number of oxytocin receptors in the endometrium of the uterus (McCracken *et al.*, 1984). Oxytocin engages in a positive feedback loop with $PGF_{2\alpha}$ and hastens the luteolytic process (Scaramuzzi *et al.*, 1977; Flint & Sheldrick, 1983; Flint *et al.*, 1986).

The limited data available suggest that the hormone secretion patterns in red deer and other cervids are similar in many respects to those of the ovine oestrous cycle.

As in the sheep, LH secretion in red deer is controlled by the pattern of GnRH release from the hypothalamus. Active immunisation against GnRH disrupts reproductive activity in red deer stags, reducing circulating testosterone and presumably LH concentrations (Lincoln *et al.*, 1982; Lincoln, 1985). In contrast, injections of exogenous GnRH stimulate LH release in red (Lincoln & Kay, 1979; Kelly *et al.*, 1982; Suttie *et al.*,1984; Manley *et al.*, 1989) and Père David's deer (Curlewis *et al.*, 1988a; McLeod *et al.*, 1991). Daily blood sampling during the oestrous cycle of red deer (Kelly *et al.*, 1985), roe deer (Schams *et al.*, 1980) and white-tailed deer (Plotka *et al.*, 1980) occasionally showed LH peaks (>10 ng/ml) on or near the day of oestrus while more intensive sampling regimes reliably record a massive surge in plasma LH around the time of mating in fallow and Père David's deer (Asher & Thompson, 1989; Asher *et al.*, 1990; Curlewis *et al.*, 1990) very similar to those which occur during the peri-oestrus period in sheep (Baird *et al.*, 1981), cattle (Walters & Schallenberger, 1984) and goats (Mori & Kano, 1984). Changes in periodic LH secretion are yet to be studied red deer hinds. Similar to sheep, LH pulse frequency is greater in the follicular phase (1 pulse/1-2 h) than in the luteal phase (1 pulse/4 h) of the oestrous cycle in Père David's hinds (Curlewis *et al.*, 1990).

Kelly, McNatty and Moore (1985) could discern no consistent pattern of progesterone concentrations in cycling red deer hinds. However in most studies the pattern of plasma progesterone secretion during the reproductive cycle of the red deer hind (Adam & Atkinson, 1984; Adam *et al.*, 1985; Webster & Barrell, 1985; Asher *et al.*, 1991; Jopson *et al.*, 1990) was similar to that described in other deer species (fallow: Asher, 1985; Asher *et al.*, 1986, white-tailed: Plotka *et al.*, 1980, roe: Schams *et al.*, 1980, Père David's: Curlewis *et al.*, 1988a) and domestic ruminants such as the sheep (Walton *et al.*, 1977) and cattle (Pope *et al.*, 1969). During the anoestrous period plasma progesterone levels are generally low (about 0.5 ng/ml) and seldom exceed 1.0 ng/ml (Adam *et al.*, 1985). Ovulatory activity is indicated by a 3-4 fold increase in plasma progesterone concentrations for several days corresponding to the luteal phase of the oestrous cycle (Adam & Atkinson, 1984; Curlewis *et al.*, 1988b; Jopson *et al.*, 1990). Unless pregnancy

is established progesterone concentrations fall to low values (<1 ng/ml) during the 3-5 day follicular phase of each cycle. Oestrous behaviour, the period when hinds are receptive to the stag, occurs while progesterone concentrations are low (Jopson *et al.*, 1990). Kelly *et al.* (1985) suggested that anomolous patterns of progesterone secretion in red deer hinds may be due to the presence of an accessory corpus luteum (Armstrong *et al.*, 1969; Kelly *et al.*, 1985). However release of progesterone from non ovarian sources has been reported in red (Meikle, 1988; Jopson *et al.*, 1990), fallow (Asher & Thompson, 1989) and white-tailed deer (Plotka *et al.*, 1983) and in sheep (Thompson & Wagner, 1974) and cattle (Watson & Munro, 1984), especially following administration of adrenocorticotrophic hormone (ACTH). It is possible that an adrenal gland source of progesterone may contribute to plasma progesterone concentrations particularly when semi-domesticated animals such as red deer are stressed (Meikle & Fisher, 1990; Jopson *et al.*, 1990).

Data on the pattern of FSH, oestradiol and prostaglandin secretion patterns of red deer are scarce. Because of the absence of assays able to measure plasma FSH concentration nothing is known of the pattern of FSH secretion in deer nor of its role in the reproductive cycle.

Although high concentrations of oestradiol were detected during the oestrous cycle of red deer hinds (Kelly *et al.*, 1985), unlike sheep (Yuthasastrakosol *et al.*, 1975), cattle (Smith *et al.*, 1974) and goats (Mori & Kano, 1984), there was no consistent increase in oestradiol at the time of oestrus. Oestradiol concentrations do tend to increase around the time of the LH surge and oestrus in fallow (Asher, 1986a) and white-tailed deer (Plotka *et al.*, 1980) but the paucity of data means that the role of oestradiol in the cervid oestrous cycle is poorly understood.

In fallow (Asher & Thompson, 1989; Asher *et al.*, 1990) and Père David's (Curlewis *et al.*, 1988a) hinds administration of prostaglandin results in a rapid decline in plasma progesterone concentrations followed by oestrous behaviour 2-3 days later. In addition, in fallow deer luteolysis is associated with increases in $PGF_{2\alpha}$ metabolites and oxytocin which can be prevented by mid-cycle hysterectomy (Asher *et al.*, 1988). These

observations imply that the mechanisms controlling of the regression of the corpus luteum in cervids may be similar to those observed in sheep.

2.4 Oestrous behaviour

Courtship behaviour, the complex sequence of behavioural interactions between the oestrous receptive hind and the stag has been described for farmed red deer (Lincoln *et al.*, 1970; Guinness *et al.*, 1971; Veltman, 1985). An accurate estimate of the duration of oestrous behaviour is difficult to determine as the female hind plays a passive role in courtship behaviour and the onset of oestrus is virtually impossible to detect in the absence of the male. The period of oestrous behaviour generally lasts for about 12-24 h but may be extended if the hind is not mated (Guinness *et al.*, 1971; Kelly & Moore, 1977).

Hormonal events controlling the oestrous cycle of spontaneously ovulating ruminants are coordinated so that the relative timing of oestrous behaviour and ovulation is favourable for fertilisation and conception. Little is known of the neuroendocrine pathway controlling oestrous behaviour in the ruminant. Under physiological conditions, a prolonged period of progesterone treatment prior to a rise in oestradiol concentrations are prerequisites for oestrous behaviour to occur in the ewe (Robinson, 1954, Robinson *et al.*, 1956; Karsch *et al.*, 1980). It is believed that the first ovulation at the start of the breeding is not accompanied by oestrous behaviour due to a lack of progesterone priming (Robinson *et al.*, 1956; Wheeler & Land, 1977; Legan *et al.*, 1985a). The limited data available for red deer suggests that a period of elevated progesterone concentrations followed by an increase in oestradiol are both required for the expression of oestrous behaviour in the hind (Meikle & Fisher, 1990). Similar to the ewe, the transition to sexual activity in red (Adam *et al.*, 1985; Asher *et al.*, 1990; Jopson *et al.*, 1990), fallow (Asher, 1985), Père David's (Curlewis *et al.*, 1988a) and white-tailed deer (Harder & Moorhead, 1980) is associated with the occurence of silent ovulations (ovulation without oestrous behaviour) and the formation of a short-lived corpus luteum. These presumably arise due to the absence of elevated plasma progesterone concentrations prior to the first ovulation.

2.5 Neuroendocrine basis of seasonal breeding.

Transitions between breeding season and anoestrus are believed to result from changes in the pattern of GnRH secretion and hence changes in the frequency of LH pulses from the pituitary. This hypothesis is supported by the observation that GnRH secretion in ewes is reduced during anoestrum (Barrell *et al.*, 1992) and that GnRH pulses are always highly correlated with LH pulses (Clarke & Cummins, 1982; Levine *et al.*, 1982; Caraty *et al.*, 1989; Barrell *et al.*, 1992). Furthermore, in intact ewes LH pulse frequency is approximately 1 per 8 h in anoestrus (Scaramuzzi & Baird, 1977; Yuthasastrakosol *et al.*, 1977; McLeod *et al.*, 1982 a,b; Legan *et al.*, 1985b) whereas during the oestrous cycle of the breeding season it ranges from 1 per 4 h in the luteal phase to more than 1 per h during the follicular phase (Baird, 1978a; Karsch *et al.*, 1983). In addition, if LH pulse frequency is increased during anoestrous to 1 per 2 h (a frequency similar to that of the early follicular phase), by pulsatile administration of LH or synthetic GnRH, successive oestrous cycles can be initiated (McNatty *et al.*, 1982, 1984).

In the sheep, most components of the ovulatory mechanism appear to function throughout anoestrus. Follicle growth and development continues during the non breeding season and preovulatory follicles are present at all stages of the reproductive cycle (Cahill & Mauleon, 1980). During anoestrus, if an appropriate LH signal is given (McNeilly & Land, 1979; McNatty *et al.*, 1982, 1984), follicles in the ovary can generate a preovulatory oestradiol rise (Goodman *et al.*, 1981b; McNeilly *et al.*, 1982) and the LH surge mechanism is able to respond appropriately to the stimulatory feedback action of oestradiol (Land *et al.*, 1976; Goodman *et al.*, 1981a; Moenter *et al.*, 1990). Following an experimentally produced LH surge ovulation, oestrous behaviour and corpus luteum formation can occur (McNatty *et al.*, 1984; Smith *et al.*, 1988). In addition, the capacity of the pituitary to release LH in response to follicular phase frequencies of GnRH pulses does not appear to limit LH pulse frequency during anoestrus (McLeod *et al.*, 1982b; Legan, 1985c). Finally the spontaneous transition into the breeding season is characterised by an increase in the frequency of LH pulses (I'Anson & Legan, 1988a) which is consistent with the view that the onset of ovarian cyclicity results from an increase in GnRH, and thereby LH, pulse frequency. Oestrous activity during anoestrum

is therefore limited by the failure of the GnRH pulse generator to cause sustained increases in tonic LH secretion (Karsch & Moenter, 1990; Barrell *et al.*, 1992).

Two mechanisms which influences LH secretion, and therefore the seasonal reproductive function of the ewe, have been described. Firstly, when LH pulse patterns were monitored in long-term ovariectomised ewes, in the absence of gonadal steroids, there was a seasonal fluctuation in the pattern of LH pulses. Slower-frequency, higher amplitude LH pulses occurred during anoestrus (Goodman *et al.*, 1982; Robinson, 1983; Montgomery *et al.*, 1985; Thomas *et al.*, 1988). This steroid-independent effect on LH pulsatility can be partially but not completely explained by a diminished ability of the pituitary to secrete LH in response to GnRH (Bittman *et al.*, 1985). The second effect of photoperiod on LH pulsatility is observed when ovariectomised ewes are treated with oestradiol implants which maintain a constant basal level of circulating oestradiol. In anoestrum oestradiol treatment greatly reduces LH pulsatility whilst high frequency pulses persist in the presence of an oestradiol implant during the breeding season (Goodman *et al.*, 1982; Martin *et al.*, 1983). The ability of oestradiol to slow LH pulsatility and dramatically reduce circulating LH concentrations in ovariectomised ewes is temporally associated with the transitions between breeding and anoestrous seasons in ewes with intact ovaries (Legan & Karsch, 1980). It is proposed that the onset of reproductive activity is controlled by a decrease in the negative feedback action of oestradiol on tonic LH secretion (Legan *et al.*, 1977; Karsch *et al.*, 1984). Analagous changes in control of tonic LH secretion have been observed in other seasonal breeders (Turek and Campbell, 1979; Goodman & Karsch, 1981), including hamsters (Seegal & Goldman, 1975; Ellis & Turek, 1979), hares (Caillol *et al.*, 1990) and rams (Olster & Foster, 1988).

Curlewis *et al.* (1991) reported that, in Père David's hinds, the pulsatility of LH secretion varies throughout the non-breeding season. LH pulse frequency in early anoestrus (1 pulse/6 h) was significantly lower than in mid to late anoestrus (1 pulse/2-3 h) and during the oestrous cycle (1 pulse/1-4 h). In Père David's hinds release of LH from the pituitary in response to GnRH is diminished during early anoestrus and this may may account for the lower LH pulse frequency (Curlewis *et al.*, 1991). The ability of Père David's hinds to ovulate following pulsed or continuous administration of GnRH is also reduced at this

time (McLeod *et al.*, 1991). Nothing is known of the changes in endogenous and GnRH-induced patterns of LH secretion in entire red deer hinds during the annual reproductive cycle. Administration of exogenous gonadotrophin (such as PMSG) or GnRH treatments reliably inducesovulations in sheep throughout anoestrus (McNatty *et al.*, 1982, 1984; Smith *et al.*, 1988) but causes only a proportion (14-61%) of seasonally anoestrous red deer hinds to ovulate (Adam, 1982, 1983; Fisher & Fennessy, 1985; Fisher *et al.*, 1986; Fennessy *et al.*, 1986; Moore & Cowie, 1986; Manley *et al.*, 1989). This suggests that insufficient GnRH release may not be the only factor limiting ovulatory activity in anoestrous red deer hinds. Firstly, it is not known whether follicles capable of generating the rise in oestradiol concentration are present during anoestrus and it is possible that FSH, or alternatively LH, secretion during anoestrus is inadequate to induce development of mature preovulatory follicles. In addition, although administration of oestradiol to ovariectomised red deer hinds induces an LH surge and oestrous behaviour during anoestrus, the sensitivity of the LH surge generator to oestradiol does vary during the annual reproductive cycle (Meikle *et al.*, 1990). Doses of oestradiol which induce an LH surge during the breeding season fail to do so during anoestrus (S. Limisirichaikul, pers. comm.). However, as little is known of the pattern of oestradiol secretion during the reproductive cycle of the hind, the physiological significance of such findings is uncertain.

Similar to the ewe, in the ovariectomised red deer hind there is a marked seasonal change in sensitivity of LH secretion to the negative feedback effects of oestradiol (Meikle *et al.*, 1991). This decrease in sensitivity to oestradiol is temporally related to the onset of breeding activity in entire hinds (Meikle *et al.*, 1991). Steroid-independent changes in LH secretion of the ovariectomised red deer hind are considerably more marked than in the ewe (Robinson *et al.*, 1985a). Diminished LH secretion in ovariectomised hinds during anoestrus is associated with a reduction in the ability of the pituitary to release LH in response to GnRH (Meikle *et al.*, 1991). It is not known if, as has been reported in Père David's deer (Curlewis *et al.*, 1991), pituitary responsiveness declines in the entire red deer hind during anoestrus. In combination, both the steroid-dependent and steroid-independent reduction in GnRH pulse generator activity, as well as the associated decrease in pituitary responsiveness, may provide the major explanation for the lack of

ovarian activity and the depth of anoestrus experienced by the red deer hind during the nonbreeding season.

2.6 Photoperiodic control of reproductive seasonality.

Seasonal breeding is the outward manifestation of an adaption to annual climate changes and ensures that offspring are born at the most favourable time of year (Clarke, 1981; Ortavant *et al.*, 1985). Although seasonal changes in temperature, rainfall and food availability are the enviromental factors which dictate the survival of young animals, the majority of mammalian species that have evolved in temperate and cold climates use seasonal changes in length of daylight as the enviromental cue for timing the onset of the breeding season (Yeates, 1949; Thibault *et al.*, 1966; Lincoln & Short, 1980; Gwinner, 1986). Unlike other climatic variables, such as temperature and rainfall, at a given locality the annual cycle of daily photoperiod is very constant and thus provides a reliable proximate cue with a good predictive value for anticipating the favourable season for birth (Lincoln & Short, 1980; Karsch *et al.*, 1984; Ortavant *et al.*, 1985).

Deer and sheep are both regarded as 'short day' breeders because ovarian cycles commence during summer/autumn as photoperiods become shorter and terminates in winter/spring as photoperiod increases (Lincoln & Short, 1980). The simplest hypothesis for photoperiodic timing of the onset of breeding activity in short day breeders is that reproductive activity is directly induced by short or decreasing photoperiods and inhibited by long or increasing photoperiods. Indeed artificially short photoperiods can induce a period of reproductive activity in sheep and deer while long days inhibit the reproductive axis (sheep: Yeates, 1949; Lincoln & Short, 1980; red deer: Webster & Barrell, 1985; Suttie & Simpson, 1985). However annual breeding cycles still persist in sheep under constant daily photoperiods (Wodzicka-Tomaszewka *et al.*, 1967, Ducker *et al.*, 1973; Howles *et al.* 1982; Kennaway *et al.*, 1983; Almieda & Lincoln, 1984) and reproductive activity commences spontaneously even if the reduction in day length after the summer solstice is not experienced (Robinson *et al.*, 1985b; Worthy *et al.*, 1985). It appears that photoperiod may not actively drive the annual cycle of reproductive activity. One possibility is that the transitions between anoestrum and sexual activity under natural

conditions result from a temporary loss of response to the prevailing photoperiod (Almeida & Lincoln, 1984; Robinson *et al.*, 1985b). This loss of response, called photorefractoriness, has also been implicated in the the cessation of the breeding season. Ewes do not need to experience lengthening photoperiods in late winter in order to terminate the breeding season at the appropriate time of year (Worthy & Haresign, 1983; Robinson & Karsch, 1984). Alternatively, more recent studies suggest that changes in the reproductive axis represent the expression of an endogenously generated circannual rhythm and photoperiod synchronises the annual reproductive cycle to the appropriate seasons of the year (Karsch *et al.*, 1989; Malpaux *et al.*, 1989; Wayne *et al.*, 1990; Woodfill *et al.*, 1991). Although some ewes maintained on equatorial photoperiods tend to breed continuously (Jackson *et al.*, 1990), most sheep maintained for extended periods in an unchanging photoperiodic environment continue to have annual cycles of breeding of about (but not exactly) one year in duration (Howles *et al.*, 1982; Karsch *et al.*, 1989). These circannual rhythms of reproductive activity gradually become desynchronised among individuals and out of synchrony with the breeding cycle of animals maintained in natural daylight. When ewes were rendered nonphotoperiodic by pinealectomy (see section 2.7) and daylength cues were restored by infusions of physiological patterns of melatonin for only short intervals of the year, the transitions between reproductive states resynchronised appropriately to these limited melatonin cues (Woodfill *et al.*, 1991). It appears that the melatonin profile associated with the increase in daily photoperiod during the spring determines the onset of reproductive activity in the following autumn whilst photic cues during autumn and summer determine the duration and termination of breeding activity (Malpaux *et al.*, 1988, 1989; Malpaux & Karsch, 1990; Wayne *et al.*, 1990; Woodfill *et al.*, 1991; O'Callaghan *et al.*, 1991).

When red deer were transported from the northern to the southern hemisphere they established reproductive cycles appropriate to the 6 month shift in photoperiod within 2 years of the transfer (Marshall, 1937; Otway, 1985). Experiments exposing red deer and other cervids to artificially manipulated photoperiods have demonstrated that light plays an important role in the timing of seasonal events, such as antler cycles in red deer (Jaczewski, 1954; Blaxter *et al.*, 1974, Pollock, 1975; Simpson *et al.*, 1984), sika deer (Goss, 1969a, 1969b, 1977; Goss & Rosen, 1973) and white-tailed deer (Budde, 1983), onset of breeding activity in female red deer (Webster & Barrell, 1985) and white-tailed

deer (Budde, 1983) and the rut in red deer stags (Blaxter *et al.*, 1974; Pollock, 1975; Simpson *et al.*, 1984) and fallow deer (Newman *et al.*, 1990). In general, exposure of deer to increasing, or long, photoperiods induces changes associated with anoestrus, whereas shorter photoperiods initiate seasonal events associated with the breeding season. However, annual antler cycles persist in sika deer maintained for a long time under constant photoperiodic conditions (Goss & Rosen, 1973). Similarly, the annual increase in plasma testosterone in male fallow deer (Newman *et al.*, 1990) and the onset of reproductive activity in pubertal red deer hinds (Loudon & Brinklow, 1990, 1992) is not dependent on animals experiencing the autumnal decrease in daily photoperiod. It appears that the timing of the spring increase in daily photoperiod may play a role in determining the onset of the breeding season in pubertal red deer (Loudon & Brinklow, 1992). As in the sheep, the transitions between reproductive states in cervids may be endogenously generated or result from the development of photorefractoriness to the ambient photoperiod.

Fletcher (1974) studied the calving patterns of red deer populations breeding in temperate regions (ranging from 25 to 57° N latitude) and found that calving occurred at similar times regardless of distance from the equator (Fletcher, 1974). Similarly, the time of peak calving for populations of white-tailed deer breeding in temperate (30-50° N) regions is also not influenced by latitude (Budde, 1983; Bronson, 1985). However in areas close to the equator (<10° S) the reproductive cycles of individual white-tailed deer become irregular and asynchronous and breeding can occur at any time of year (Bronson, 1985). The effect of equatorial photoperiods on red deer is unknown but when sika deer, natives to the temperate zone, are exposed to constant equatorial light cycles (12L:12D) long term the annual shedding of antlers is prevented (Goss & Rosen, 1973; Goss, 1977). Cervids appear to be insensitive to the amplitude of the photoperiodic cycle unless the light to dark ratio approaches 1. Goss (1977) suggested that sika become nonseasonal when the light:dark ratio is less than 1.145. Interestingly, this corresponds to the transition zone from the seasonal to the non-seasonal mode of breeding in sika deer, about 14° from the equator.

2.7 Melatonin secretion and the pineal gland

In most mammals the hormone melatonin, secreted from the pineal gland, is strongly implicated in the adaptation of the internal daily (circadian) and seasonal (circannual) rhythms to changes in the environment (see reviews: Karsch *et al.*, 1984; Lincoln, 1985; Arendt, 1986; Armstrong, 1989). Information about external lighting conditions is conveyed from retinal photoreceptors to the non-photoperiodic mammalian pineal gland (Legan & Karsch, 1983) via the retinohypothalamic tract to the suprachiasmatic nuclei (Legan & Winans, 1981) and then via the cranial cervical ganglia (Lincoln, 1979; Yellon and Clayton, 1983) to the pineal gland. In the pineal gland a cascade of complex biochemical events synthesises melatonin throughout the dark phase (see review: Sugden, 1989). A nocturnal elevation in circulating melatonin concentrations, which is abolished by the removal of the pineal gland or of the cranial cervical ganglion, is present in many mammals (sheep: Arendt *et al.*, 1980; Almeida *et al.*, 1982; horses: Sharp *et al.*, 1981; Kilmer *et al.*, 1982).

Sheep display a circadian rhythm of melatonin secretion which is acutely sensitive to light (Rollag *et al.*, 1978a; Lincoln *et al.*, 1981; Arendt & Ravault, 1988). The melatonin pattern is free-running in sheep that are blinded (Legan & Karsch, 1983) or kept in constant darkness (Rollag & Niswender, 1976; Lincoln *et al.*, 1981) and is abolished (Rollag & Niswender, 1976; Kennaway *et al.*, 1983) or suppressed in animals kept in constant light (K.P. McNatty, pers. comm.). The circadian melatonin rhythm is believed to be endogenously generated, possibly within the suprachiasmatic nuclei, and entrained to the light-dark cycle by photoperiod (see Lincoln *et al.*, 1981; Arendt, 1986). Some exceptions to the relationship between melatonin secretion and the dark-light cycle have been reported. The duration of melatonin secretion appears to be limited to approximately 16 h as melatonin concentration declines spontaneously if the dark phase exceeds this (Lincoln *et al.*, 1981; Ravault & Thimonier, 1988). Also the entrainment of the melatonin rhythm may be disrupted when animals are exposed to light cycles with non-24-h periodicity ((Lincoln *et al.*, 1981; Ravault & Thimonier, 1988; Arendt *et al.*,1988). In addition, a 1 hour light pulse which causes only a transitory suppression of melatonin concentrations when given early in the night, causes long-term suppression when given later in the night (Brinklow *et al.*, 1984; Earl *et al.*, 1985). Transient increases in melatonin concentration during the

day have been reported in long and short-day photorefractory rams (Almeida & Lincoln, 1984) although these results are controversial (Bittman, 1985; Malpaux *et al.*, 1987, 1988). Under most conditions, however it appears that the onset of the dark period entrains the onset of the melatonin peak while the onset of the light period suppresses melatonin secretion directly. These 2 processes control the initiation and termination of melatonin secretion and interact to generate a melatonin signal which closely reflects the length of the night (Rollag, 1978b; Rollag & Niswender, 1976; Lincoln *et al.*, 1985; Arendt, 1986).

Light therefore plays a dual role in the generation of melatonin signals; it entrains the endogenous rhythm to the light-dark cycle and suppresses the release of melatonin during daylight. The ability to follow changes in daylength is attained very early in life. A 24-h rhythm of melatonin concentration has been detected in foetal lambs (Yellon & Longo, 1987; Zemdegs *et al.*, 1988). The melatonin secretion pattern, which is probably maternal in origin (Zemdegs *et al.*, 1988; Yellon & Longo, 1988), can modulate the secretion of prolactin prenatally (Ebling *et al.*, 1989) and indicates that the lamb can accumulate a photoperiodic history prior to birth. Young lambs are able to generate a melatonin secretion pattern which reflects the prevailing photoperiod soon after birth (Claypool *et al.*, 1989) and are able to re-entrainthe melatonin rhythm to new photoperiods by 6 weeks of age (Wood *et al.*, 1990). Although the amplitude of the nightly melatonin rise is lower in younger animals (Claypool *et al.*, 1989), this appears to be functionally unimportant (Foster *et al.*, 1989) as neuroendocrine pathways regulating prolactin secretion (Ebling *et al.*, 1988) and steroid-independent changes in LH pulsatility (Ebling, F.J.P. cited Claypool *et al.*, 1989) are sensitive to changes photoperiod in very young lambs.

The feature of the melatonin secretion profile which is responsible for triggering photoperiodic responses has not been clearly established. Generally the duration of the melatonin rise is believed to mediate the reproductive response of the seasonal breeder to photoperiod (Arendt *et al.*, 1988; Karsch *et al.*, 1988) although a circadian rhythm in sensitivity to melatonin can not be discounted (Ravault & Thimonier, 1988). Infusions of melatonin to create long- and short-day melatonin plasma profiles in pinealectomised ewes induces the appropriate short- and long-day reproductive response, irrespective of

the prevailing photoperiod (Bittman *et al.*, 1983 a,b; Bittman & Karsch, 1984; Bittman, 1985) or the time of day melatonin was infused (Wayne *et al.*, 1988). Administration of melatonin to mimic the duration of the melatonin profile associated with a specific dark phase is as potent as treatment with the dark phase itself for the generation of a reproductive response in sheep (Nett & Niswender, 1982; Kennaway *et al.*, 1982; Arendt *et al.*, 1983; Arendt *et al.*, 1988). Furthermore continuous release melatonin devices which maintain plasma melatonin concentrations continually at or above nighttime concentrations will also effectively induce short day responses (English *et al.*, 1986; Nowak and Rodway, 1985; Poulton *et al.*, 1987b).

The reproductive response to a given photoperiod is dependent upon prior photoperiodic exposure (called photoperiodic history). A fixed melatonin pattern can usually maintain a given reproductive response only for a limited length of time. Whether the constant photoperiodic signal is eventually overridden by an endogenous reproductive rhythm or the development of photorefractoriness is yet to be determined. Direction of the photoperiodic change is also important, as the transfer to a photoperiod can either induce or suppress reproductive activity depending on whether an increase or a decrease in the duration of the nocturnal melatonin elevation is experienced (Robinson & Karsch, 1987). The mechanism transducing the melatonin signal therefore measures the duration of the nighttime melatonin rise relative to the prior melatonin signal (Karsch *et al.*, 1988).

In sheep if the the function of the pineal gland is disrupted, by pinealectomy (removal of the pineal gland) or cranial cervical ganglionectomy (removal of the cranial cervical ganglion and hence destruction of the sympathetic innervation of the pineal gland), seasonal cycles in reproductive activity persist (Lincoln 1979; Barrell & Lapwood 1979; Kennaway *et al.*, 1981, Lincoln *et al.*, 1989) but the timing of changes may be abnormal (Lincoln *et al.*, 1989; Woodfill *et al.*, 1991), especially in pubertal animals (Kennaway *et al.*, 1985; Foster *et al.*, 1986; Foster, 1988) and in the first year after surgery (Lincoln & Almeida, 1981; Karsch *et al.*, 1984).

The pattern of plasma melatonin secretion in deer is similar to that described in other species with melatonin concentrations very low or undetectable during the daytime and elevated during the scotophase (fallow deer: Asher *et al.*, 1988; red deer: Webster *et al.*,

1991). It remains to be determined whether components of the neuroendocrine pathway which control melatonin secretion in the deer are the same as in sheep. Although the role of the cranial cervical ganglia in the neuroendocrine pathway has been partially demonstrated by the alteration of the reproductive rhythm following cranial cervical ganglionectomy in young red deer stags (Lincoln & Fletcher, 1979; Lincoln, 1985), the return to a normal breeding pattern and the ability of artificial photoperiods to influence reproductive activity in subsequent years must cause the effectiveness of the surgery or the role of the SCG in red deer to be questioned. In other deer species seasonal cycles persist after removal of the pineal (Brown *et al.*, 1978; Plotka *et al.*, 1979, 1981; Snyder *et al.*, 1983), although the timing of the rut and antler cycle is perturbed, especially when young animals are pinealectomised (Brown *et al.*, 1978).

Evidence that the pineal hormone, melatonin, is important in timing the reproductive cycle in deer has been provided by numerous studies showing that exogenous melatonin administered via daily injections (red deer: Webster & Barrell, 1985), daily oral feeding or dosing (red deer: Adam & Atkinson, 1984; Nowak & Rodway, 1985; Adam *et al.*, 1986, 1989a), or constant release implants (red deer: Barrell, 1985; Fennessy & Fisher, 1987, 1990; Fisher *et al.*, 1986, 1988, 1989, 1990; Fisher & Fennessy, 1990; Asher, 1990; fallow deer: Asher *et al.*, 1988; Père David's: Milne *et al.*, 1990) alters seasonal breeding cycles in the female deer and simulates the effects of short photoperiods.

2.8 Seasonal cycles of growth and feed intake

As with seasonal cycles of reproduction, circannual cycles of feed intake and live weight in temperate species are outward manifestations of adaptions to annual climate changes and the associated changes in food supply. Red deer exhibit marked changes in live weight gain during the year (Mitchell *et al.*, 1976; Fennessy *et al.*, 1981) which reflect not only changes in feed availability but also changes in appetite (Kay, 1979; Loudon *et al.*, 1989) and body metabolism (Milne *et al.*, 1987). Voluntary food intake and growth rate are greatest during spring and summer and decrease during autumn and winter even if the feed availability is not limited (Pollock, 1975; Kay, 1979; Loudon *et al.*, 1989). During spring and summer energy excess to maintenance requirements is preferentially partitioned into the accumulation of fat depositions especially in mature stags (Drew,

1985) presumably for increased cold insulation (Price & White, 1984) and to be utilised as an energy store during autumn and winter.

The annual patterns of food intake and growth in seasonal ruminants are influenced by photoperiod. When red deer were exposed to artificial light so that the annual changes in photoperiod were compressed into a 6 month cycle, 2 cycles of food intake, growth and reproductive activity were generated in a calender year (Pollock, 1975; Simpson *et al.*, 1984; Suttie & Simpson, 1985). Exposure to long photoperiods stimulates the food intake and liveweight gain of deer (Brown *et al.*, 1979; Kay 1979; Suttie and Corson, 1991) and sheep (Forbes, 1982; Schanbacher & Crouse, 1980) and increases the efficiency of feed utilisation in sheep (Schanbacher & Crouse, 1980). These photoperiodically induced changes in feed intake and growth appear to be mediated by the hormone melatonin. Melatonin treatment which advanced the onset of the breeding season in red deer hinds also advanced the seasonal reduction in appetite (Milne *et al.*, 1990) and growth rate (Adam *et al.*, 1986; Fisher *et al.*, 1990). When young red deer hinds (Loudon & Brinklow, 1990) or male fallow deer (Newman *et al.*, 1990) were denied exposure to the autumnal decrease in daily photoperiods, appetite declined as normal during autumn. These results indicate that the autumnal reduction in feed intake and live-weight gain in deer are not actively driven by decreasing photoperiods and suggests that the development of refractoriness to long photoperiods or an endogenous circannual rhythm may play a role in the generation of the annual appetite and growth cycles.

The neurohormonal pathway by which photoperiod and melatonin influence the seasonal pattern of growth is not known. In stags loss of appetite and liveweight during autumn and winter is associated with elevated testosterone levels during the rut (Mitchell *et al.*, 1976; Drew, 1985; Wallace and Davies, 1985). However castrated stags (Milne, 1978; Kay, 1979) and entire hinds (Kay, 1979, Loudon *et al.*, 1989) also exhibit circannual rhythms of food intake and growth indicating that seasonal changes in liveweight persist in the absence of androgens. Plasma prolactin concentrations in red deer are positively correlated with food intake (Loudon *et al.*, 1989) and administration of prolactin in winter significantly increased food intake and live weight gain in young red deer stags (Suttie & Corson, 1991), reindeer (Ryg & Jacobson, 1982b) and lambs (Brinklow & Forbes, 1982).

Furthermore bromocriptine treatment which suppressed the spring increase in prolactin also delayed the spring increase in intake (Curlewis *et al.*, 1988b). However these results conflict with those of other studies where exogenous prolactin treatment elevated plasma prolactin concentrations but not feed intake in red deer hinds (Milne, McNeilly, Sibbald, Loudon & Curlewis, cited Milne *et al.*, 1990) and lambs (Eisemann *et al.*, 1984). In addition although both melatonin and bromocriptine treatments decreased prolactin concentrations in red deer hinds in midsummer the autumn decrease in feed intake was advanced in the melatonin-treated hinds only (Milne *et al.*, 1990). The increase in the thyroid hormone, tri-iodothyronine (T3) during long days photoperiods is also temporally related to a rise in feed intake (Ryg & Jacobsen, 1982a; Brown *et al.*, 1983; Loudon *et al.*, 1989) but causal relationships are less clear. Removal of the thyroid gland from red deer stags prevented the spring increase in liveweight gain (Shi & Barrell, 1992a) unless treated with exogenous T4 (Shi & Barrell, 1992b). Although T3 administration mid-winter is reported to increase live weight gain of reindeer (Ryg & Jacobsen, 1982b), the growth cycle of red deer stags treated with T3 in autumn or T4 during winter and spring was not altered (Shi, 1991). The role of prolactin and thyroid hormones in the seasonal regulation of feed intake is therefore far from clear. The annual cycle of growth and food intake is almost certainly influenced by photoperiod-sensitive growth factors such as growth hormone and insulin-like growth factor (Suttie *et al.*, 1989; Suttie & Corson, 1991) but the endocrine mechanisms whereby melatonin information alters the secretion of such factors remains unresolved.

In both sheep and deer increasing photoperiods in spring and summer appear to stimulate food intake and live weight gain whereas decreasing photoperiods in autumn and winter diminish appetite and growth. Photoperiodic changes influence appetite and live weight via a pathway involving melatonin but the physiological control mechanisms regulating seasonal growth are largely unknown.

2.9 Seasonal cycle of pelage moulting

Red deer moult twice a year following periods of hair follicle inactivity (Ryder & Kay, 1973; Ryder, 1977). The dense winter coat of grey/brown guard hairs and fine underwool is shed in spring to expose a red wool-less summer coat. The summer coat grows

throughout the summer and is gradually replaced during the following autumn by new winter pelage. Pelage shedding in the autumn is less conspicuous as the winter coat guard hairs are nearly full length before the summer coat is shed (Lincoln, 1971; Ryder & Kay, 1973). Themoultingof pelage causes any worn outer hairs to be replaced and allows for seasonally appropriate changes in coat insulation and coloration (Ryder & Kay, 1973).

The moulting cycle is sensitive to changes in photoperiod. When the frequency of the annual photoperiod changes was doubled red deer stags displayed 2 cycles of moulting within 1 calender year (Kay & Ryder, 1978) and premature exposure to short photoperiods advanced the shedding of the summer pelage in red deer hinds (Webster & Barrell; 1985) and roe deer (Lincoln & Guinness, 1972). Melatonin administered in summer to mimic the effects of short days advanced the moulting of the summer coat in white-tailed deer (Bubenik, 1983) and red deer hinds (Webster & Barrell, 1985, Fisher *et al.*, 1988; Milne *et al.*, 1990). As with seasonal cycles of reproduction and growth both prolactin and the thyroid hormones have been suggested to influence the seasonal cycle of coat shedding. The spring moult coincides with an increase in plasma T3 concentrations in red and Père David's deer (Loudon *et al.*, 1989). In red deer stags, winter pelage was not shed following removal of the thyroid gland (Shi & Barrell, 1992a), unless the stags were treated with T4 (Shi & Barrell, 1992b). Thyroidectomy ablated T4 secretion without altering the seasonal pattern of prolactin release (Shi & Barrell, 1992a). The seasonal rise and decline in plasma prolactin concentrations are associated with the spring and autumn molting of pelage respectively. Melatonin or bromocriptine treatments which inhibit prolactin secretion also stimulate the shedding of the summer pelage in red deer hinds (Webster & Barrell, 1985; Curlewis *et al.*, 1988b) while exogenous prolactin administered to young stags in winter caused early shedding of the winter coat.

Although the hormonal mechanisms controlling pelage growth are largely unknown, the effect of photoperiod on coat shedding appears to be mediated by melatonin. Thyroid hormones and/or prolactin may also be involved.

Chapter 3
General Materials and Methods

3.1 Geography

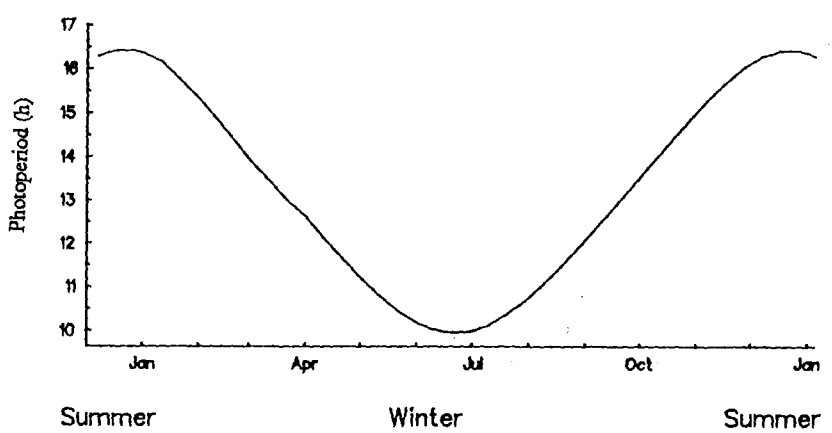
Lincoln University Deer Unit

The Lincoln University Deer Unit (formerly the Lincoln College Deer Unit) was established in 1979 and is located on the Lincoln University Research Farm (latitude 43° 39'S, longitude 172° 28'E, altitude 10 m a.s.l.), 24 km south of Christchurch in Canterbury, New Zealand. The unit consists of 27.8 ha of irrigated farmland, subdivided into 19 paddocks with 2 m high deer fences (150 mm Cyclone Tightlock). Stock, comprising of approximately 200 red deer (*Cervus elaphus*) and 100 goats (*Capra hircus*), are grazed mainly on ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) pasture and supplemented with silage during winter. Annual rainfall varies between 280 mm and 1200 mm with an average of about 600 mm. The rainfall is evenly distributed throughout the year but hot, dry northwest winds can make summer rainfall ineffective. The high stocking rate (> 18 SU/ha) is maintained by conserving grass as silage during the spring and irrigating during summer and autumn.

Photoperiodic regimen

The annual photoperiod for the nearby city of Christchurch (longitude 43° 32'S) was used to approximate the photoperiod cycle for the Lincoln University Deer Unit (longitude 43° 39'S) (Figure 3.1). Daily photoperiod was calculated from official (N.Z. Standard) sunrise and sunset times for Christchurch (N.Z. Nautical Almanac, 1987) and adjusted for civil twilight (+ 60 min.)

Figure 3.1. Annual photoperiodic cycle for the Lincoln University Deer Unit.



3.2 Livestock, facilities and field data collection

Source of red deer

Deer on the unit were derived from feral red deer hinds captured in the Rakaia River area by the New Zealand Forest Service during 1979 and 1980. Deer numbers on the unit have expanded by natural increase to over 200 head (typically about 50 stags, 100 hinds and 70 calves).

Individual identification

Mature animals were individually identified by a numbered plastic ear tag (Allflex medium, Delta Plastics Ltd, Palmerston North) and all newborn calves were tagged within 24 h of birth. In addition to the plastic ear tag, wild captured hinds had a brass tag inserted in the opposite ear.

Deer handling facilities

Enclosed handling areas and yards are located on the northeast side of the deer unit. Deer were normally mustered into the deer yards and smaller groups drafted into two darkened rooms for handling and blood sampling. Gates and passageways lead from the handling area into the deer crush and scales.

The wooden deer crush ('Carri Crush', Pattern No. 211653, Nog Construction Company Ltd, Geraldine), which was kindly donated by Cyclone Industries (now N.Z. Wiremakers Ltd), was designed so that the floor dropped down leaving the deer suspended on the bevelled walls of the cradle. Handlers had access to the deer through doors at either end or by raising plywood panels on either side. The deer was released by swinging out the entire side of the crush and allowing the deer to drop a short distance to the ground.

Live weight measurements

Deer were weighed in an enclosed laminated plywood and tube-steel box resting on clock face scales (Avery, Birmingham, England). Prior to each weighing session the scales were tared to zero and their accuracy checked with a standard weight. Deer were always weighed without fasting and live weight was recorded to the nearest 0.5 kg.

Calving data

During the calving period hind mobs were inspected each morning and evening and any newborn calves were ear tagged and their sex recorded. The dam was identified through binoculars when she approached or suckled the calf in the paddock and her identity was confirmed by observing the suckling behaviour of hinds and calves after a 2 h separation period in the yards prior to weaning.

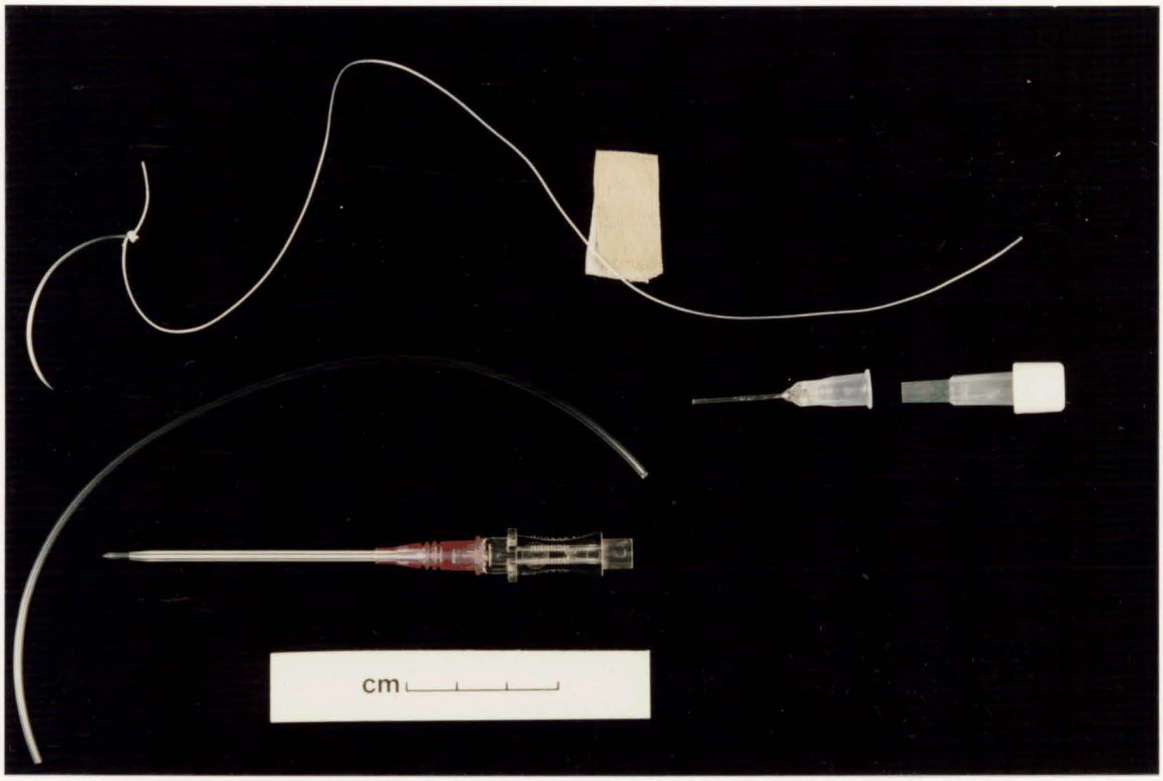
Blood sampling

Infrequent blood sampling was by jugular venepuncture. Deer were manually restrained and blood collected via a 20 G hypodermic needle (Terumo Corporation, Japan) into an evacuated glass tube containing 125 units sodium heparin (Heparin (Mucous) BP, Leo Pharmaceutical Products, Ballerup, Denmark). Blood samples were centrifuged (1200 *g* for 20 minutes) and the plasma transferred into polystyrene tubes and stored at -20 ° C until required for hormone analysis.

During intensive bleeds each hind had a cannula (Plate 1) inserted into a jugular vein for collection of blood samples. Hinds were confined in the deer crush and their heads manually restrained. Hair was clipped from the region over the right jugular vein; 10 cm below the jawline. The skin was disinfected with 70% v/v ethanol solution and locally anaesthetised with 0.5 ml of 2% xylocaine hydrochloride (Astra Pharmaceuticals Pty Ltd, NSW, Australia) injected intradermally. A 14 G x 2.5 cm introducer (Surflo I.V. catheter, Terumo Corporation, Tokyo, Japan) was inserted into the jugular vein and used to introduce 12 cm of a 15 cm length of medical grade vinyl tubing (ID 1.00 mm, OD 1.50 mm, Dural Plastics and Engineering, NSW, Australia) into the vein. The introducer was removed and the exterior end of the vinyl tubing plugged using a cut-down (20 mm) bluntened needle (22 G, Terumo Corporation, Japan) and plastic stopper. The cannula was secured by suturing a piece of tape to the skin and its patency was maintained by flushing the tubing with sterile isotonic phosphate-buffered saline solution containing 50 units/ml of sodium heparin. All hinds were injected subcutaneously with 5 ml of penicillin (Propen-S, Glaxo N.Z. Ltd, N.Z.) immediately after cannulation. Blood samples were collected from the cannula using a syringe. The first 1 ml of blood was discarded (to prevent sample dilution with heparinised saline solution) and a further 8 ml

sample was collected and expelled into a glass tube containing 125 units of heparin. The cannula was flushed with 1 ml of heparinised saline after each sample.

Plate 1. Photograph illustrating cannula and introducer (Scale 1:2).



Within an hour of collection, blood samples were centrifuged (1200 *g* for 20 minutes) and the plasma transferred into polystyrene tubes and stored at -20 °C until required for hormone analysis.

Pelage score

Pelage of hinds was scored for stage of moulting by 2 independent observers. The score was subjectively assessed with 1 representing no signs of moulting and 5 a completely moulted coat. Any signs of new pelage exposed due to physical damage to the old coat were ignored.

Laparoscopy

Hinds were mustered from pasture and fasted for 24 h. Fifteen minutes prior to laparoscopy hinds were sedated with 3.5-6.0 ml of 2% xylazine hydrochloride ('Rompun', Bayer, F.R.G.) administered intramuscularly. The sedated hinds were blindfolded and restrained in a dorsally recumbent position on a deer laparoscopy crate, raised at 30-40° to the horizontal with head down and limbs secured by straps. The

region anterior to the udder was shaved clean and the skin swabbed with a 30% chlorohexidine digluconate solution ('Savlon', ICI N.Z. Ltd, N.Z.). Local anaesthetic (1 ml 2% xylocaine, Astra Pharmaceuticals Pty Ltd, N.S.W., Australia) was injected subcutaneously 6-8 cm on either side of the mid ventral line, 12 cm anterior to the udder, and a small (0.5 cm) scalpel incision was made through the skin on each side. The abdominal wall on the left hand side was punctured with a 7 mm trochar and cannula and a manipulating probe was inserted into the other incision. The trochar was removed and the abdomen inflated with carbon dioxide gas before the laparoscope (6 mm, American Cystoscope Makers Inc., New York, U.S.A.) was introduced through the cannula. The ovaries were located and ovarian structures described. The wounds were sutured, dusted with terramycin powder (Pfizer Laboratories Ltd, N.Z.) and all hinds were injected i.m. with 5 ml of penicillin (Propen-S, Glaxo N.Z. Ltd, N.Z.) immediately after the surgery.

3.3 Hormone Assays

3.3.1 Luteinising hormone (LH) assay

LH concentration in red deer plasma was measured by a radioimmunoassay procedure similar to that described for sheep sera by Scaramuzzi *et al.* (1970) and validated for red deer sera by Kelly *et al.* (1982).

Reagents

Reagents used in the assay were ovine pituitary LH (NIADDK oLH-1-3) for radioiodination, anti-ovine LH antisera (NIADDK-anti oLH-1 (AFP-192279)), ovine LH reference preparation (NIAMMD-oLH-S20) and goat anti-rabbit gamma globulin precipitating antibody (Immuno Chemical Products, Auckland, N.Z.).

Phosphate buffer (PB) (0.5M, pH 7.4) was diluted as required for iodinations. Phosphate buffered saline solution (PBS) (0.01M (PB), 0.15M NaCl, 0.1% bovine serum albumin (BSA), 0.01% thiomersal, pH 7.4) was the diluent for all assay reagents. Spinning-down buffer consisted of 0.011 M PB, 0.1% NaEDTA, 0.28% egg albumin and 0.08% NaN_3 (pH 7.4).

Ovine LH standards (0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 and 32 ng/ml) were prepared in PBS containing 2% low-LH plasma. Low-LH plasma was collected from red deer stags treated with medroxyprogesterone acetate (MPA, Sigma Chemical Co., U.S.A.) or from pregnant red deer hinds and used if the plasma had less than 0.5 ng/ml of LH when compared with a buffer standard curve.

Radioactive LH was prepared by radioiodinating ovine LH using a modification of the chloramine T method described by Greenwood *et al.*, 1963. Ovine LH (5 μg dissolved in 25 μl 0.1M PB) was mixed with 1 mCi ^{125}I (Amersham, U.K.), followed by the addition of 20 μg chloramine T (in 10 μl 0.05M PB). After 35 seconds, sodium metabisulphate (120 μg in 50 μl 0.05M PB) was added to stop the reaction followed by 100 μl of 0.05M PB containing 2% BSA w/v. The mixture was transferred to a G75 Sephadex gel filtration column (1 cm x 30 cm) prewashed with 20 ml 0.05 M PB and 2 ml 0.05 M PB containing 2% BSA. The column was eluted with 0.05M PB to separate labelled hormone from unbound iodide. Fractions in the hormone-bound radioactive peak were stored at 4 ° C and repurified on a G75 Sephadex column (as described above) as required. Immediately prior to use, radioiodinated LH was diluted to a working concentration (20,000 cpm ^{125}I per 50 μl) with PBS containing 1% v/v rabbit serum. Labelled hormone older than 21 days was discarded.

Rabbit anti-ovine LH was diluted 1:3333 and stored at -20 ° C. A working dilution of 1:1 666 500 was used in the assay. Goat anti-rabbit gamma globulin antibody was used at a 1:50 dilution.

Radioimmunoassay procedure

Standards were added in triplicate at the beginning of each assay and in duplicate within each batch of tubes centrifuged to check for "drift" in antibody binding. Samples were assayed in duplicate and were repeated in a subsequent assay at a 1:4 dilution if values measured exceeded the working range of the assay (>16 ng/ml). Selected plasma samples were repeated in duplicate in each assay as an additional quality control measure.

Sample (100 μl) or standard (100 μl) was aliquotted with 100 μl PBS buffer into polystyrene tubes (12 x 75mm) using an automatic diluter (Dilutrend, Boehringer,

Mannhiem, F.R.G.). Ovine antiserum (100 μl) was added to all tubes, except 'non-specific binding' (NSB) tubes (which contained only 300 μl PBS so that non-antibody-bound counts in the final precipitate could be calculated) and 'total counts' (TC) tubes (which contained only radioiodinated hormone so that the total radioactivity added to each tube could be measured). All tubes were vortex mixed and incubated overnight at 10 ° C. After addition of the radioiodinated tracer (50 μl), all tubes were vortex mixed and incubated at 10 ° C for 48 h. Goat anti-rabbit gamma globulin (100 μl) was added and the tubes were vortex mixed and incubated at 4 ° C overnight. Immediately prior to centrifugation, 2 ml of spinning down buffer was added to all tubes except TC in order to dilute the amount of unbound radioactivity remaining with the antibody pellet. The antibody-bound hormone was separated from free hormone by centrifuging tubes at 3000 *g* for 20 min at 4 ° C and decanting the supernatant. The pellets were counted in a 1272 Clinigamma gamma counter (LKB, Finland) and an Apple 2E microcomputer was used to calculate unknowns from the standard curve using the spline curve fitting method described by Rawlins and Tyrjonen (1978).

Validation of the LH assay

Specificity of the antiserum to ovine LH has been determined by NIADDK who have shown that binding of the antibody was not affected by high levels of ovine pituitary hormones such as thyroid stimulating hormone (TSH), growth hormone (GH) and prolactin (cross reactivities < 1%). Ovine FSH had a cross reactivity of 5.3%. Purified pituitary hormones of cervine origin were not available.

The sensitivity of the assay (calculated as the lowest detectable LH concentration two standard deviations from zero) was 0.24 ng/ml in the LH assay. The intra- and inter-assay coefficients of variation of low, medium and high range samples, included in duplicate in each assay, were <10 % (Table 3.1).

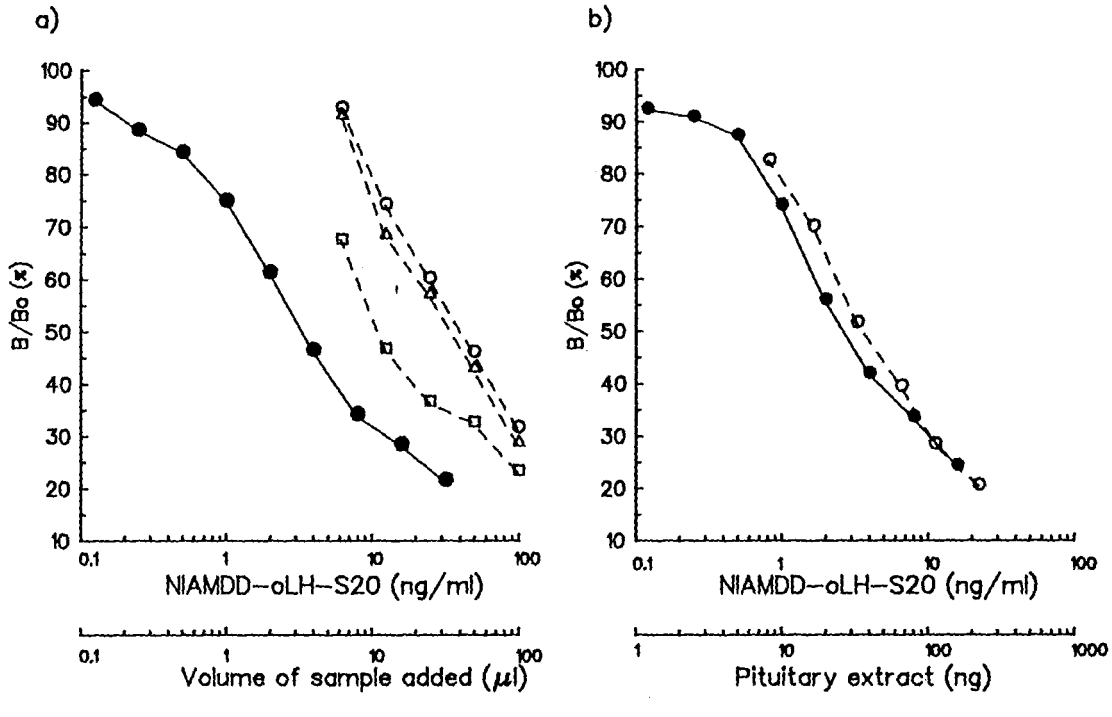
Deer plasma samples, collected when levels of immunoreactive LH were known to be elevated (from oestrus or castrated hinds), were serially diluted with PBS buffer or hypophysectomised sheep plasma (Immuno Chemical Products, Auckland, N.Z.). The resulting inhibition curves (Figure 3.2) were parallel to those of the NIAMDD-oLH-S20 standards indicating that dilution did not influence the estimation of sample LH content.

Similarly, dilutions of cervine LH pituitary extract (purified by Dr M. Lengoc, Waitaki Bioscience N.Z. Ltd) generated inhibition curves parallel to the standard curve (Figure 3.2). The assay therefore measured an LH-like immunoreactive material in deer plasma and results were expressed as ng/ml of NIAMDD-oLH-S20 for convenience.

Table 3.1 Intra- and inter-assay coefficients of variation (CV) for low, medium and high range cervine plasma samples in the LH assay.

	Mean LH (ng/ml)	Intra-assay CV (%)	Inter-assay CV (%)	n
low	1.04	6.4	9.6	25
medium	5.14	6.0	8.2	21
high	10.06	6.8	7.2	18

Figure 3.2. Binding inhibition curves for ovine LH standards (●, NIAMDD-oLH-S20) and a) representative cervine plasma samples or b) cervine pituitary extract (see text). Plasma was from an oestrous (○) or castrate (□) hind and diluted with hypophysectomised sheep plasma or from an oestrous hind and diluted with buffer (△). Cervine pituitary extract was diluted with buffer (○).



3.3.2 Melatonin assay

The radioimmunoassay for plasma melatonin (N-acetyl-5-methoxytryptamine) is based on the method of Fraser *et al.* (1983).

Reagents

Tritiated melatonin ([^{3}H] melatonin, specific activity 30.4 kCi mol/l) was obtained from New England Nuclear (USA) and sheep anti-melatonin antiserum (batch no. 704/6483) from Guildhay Antisera (Department of Biochemistry, University of Surrey, England). Melatonin for standard preparation was purchased from Sigma Chemical Co. (USA). All reagents except standards were prepared in 0.01 M tricine (Sigma Chemical Co., USA) buffer containing 9 g/l NaCl and 1 g/l gelatin (Davis Gelatine (N.Z.) Ltd, N.Z.).

Melatonin free plasma (MFP) was collected from sheep at midday when levels of melatonin were expected to be minimal. All MFP was assayed and only samples measuring less than 0.04 nmol/l were used for preparing standards. Melatonin (10 mg) was dissolved in 0.5 ml absolute ethanol and diluted to make a 10 pg/μl stock standard. Working standards were freshly prepared on the day of the assay by diluting the stock to 0.04, 0.10, 0.21, 0.43, 0.86 and 2.15 nmol/l in MFP.

Anti-melatonin serum was raised in sheep against N-acetyl-5-methoxytryptophan (Arendt & Wilkinson, 1979) and used at a working dilution of 1:4000.

Free and antibody-bound melatonin were separated using tricine buffer containing 2% w/v activated charcoal (BDH Chemicals Ltd, England) and 0.02% w/v dextran 70 (Sigma Chemical Co., U.S.A.). The scintillant solution consisted of commercial grade toluene (Shell Oil), containing 0.5 w/v 2,5-diphenyloxazole (PPO, Fluka AG, Switzerland) and 0.03% w/v ρ-bis 2-5-phenyloxazolyl benzene (POPOP, Nuclear Enterprises Ltd, Scotland).

Radioimmunoassay procedure

Standards (250 μl) were aliquotted in triplicate and samples (250 μl) in duplicate into 12 x 75 mm polystyrene tubes. Melatonin antiserum (100 μl) was added to all tubes except the NSB tubes (which contained 250 μl MFP and 100 μl buffer) and TC tubes. The tubes were vortex mixed and incubated for 30 mins at room temperature before [^{3}H]melatonin (4000 cpm in 100 μl buffer) was added. All tubes were vortex mixed and incubated at 4 ° C for 18 h. Antibody-bound melatonin was separated from the free melatonin fraction by the addition of 250 μl of activated charcoal solution and the mixture incubated at 4 ° C for 15 mins. Free melatonin was precipitated by centrifugation (1500 *g* at 4 ° C for 15 mins) and 500 μl of supernatant aliquotted into 4.5 ml vials (Omnivials, Wheaton, U.S.A.) containing 3.0 ml of scintillant solution. The vials were shaken on a rotary mixer at room temperature for 1 h, cooled at 4 ° C for 1 h and counted in a cooled automatic beta scintillation counter (LKB, Finland).

Validation of the melatonin assay

The sensitivity of the melatonin assay (defined as twice the standard deviation of maximum binding) was 0.04 nmol/l and the intra- and inter-assay coefficients of variation for samples included in duplicate in each assay ranged from 6.9 to 11.6% (Table 3.2).

Figure 3.3. Binding inhibition curves for melatonin standards (●) and a representative cervine plasma sample diluted with melatonin free plasma (○).

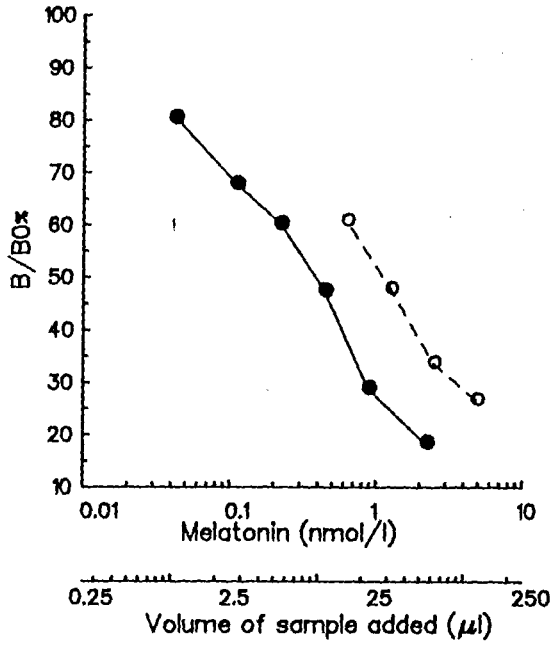


Table 3.2 Intra- and inter-assay coefficients of variation (CV) for low, medium and high range samples included in each melatonin assay.

	Mean melatonin (nmol/l)	Intra-assay CV (%)	Inter-assay CV (%)	n
low	0.29	9.4	7.6	19
medium	0.62	8.6	6.9	19
high	1.67	11.6	9.5	17

Fraser *et al.* (1983) evaluated the ability of 19 indolic analogues to compete with tritiated melatonin for antibody binding sites and reported that measurable cross reactivity was less than 0.5% for all compounds tested. When plasma samples collected from deer at night were diluted with buffer they generated inhibition curves parallel to the standard curve (Figure 3.3).

3.3.3 Enzyme-linked immunosorbent assays (ELISA)

Progesterone and prolactin concentrations in deer and sheep plasma were measured by indirect enzyme-linked immunosorbent assays using a Behring Elisa Processor M (BEPM, Behring, F.R.G.) which automatically dispenses reagents into standard 96 well microtitre plates (Falcon 3912 96-well microtitre III plate, Becton Dickenson, U.K.). The assays used common buffers and after the addition of the first antibody all steps were identical.

Buffers

Phosphate-buffered saline solution (PBS, pH 7.4) containing 0.1% w/v gelatin and 0.05% v/v Tween 20 (BDH, Chemicals Ltd, Poole, USA) was used as the assay buffer and PBS containing 0.6% Tween 20 was used for washing microtitre plates. The enzyme substrate solution was prepared immediately prior to use by adding 40 mg o-phenylene-diamine (Sigma Chemical Co., USA) and 60 µl of hydrogen peroxide (30% v/v) to 100 ml substrate buffer (consisting of 50 mmol/l Na_2HPO_4, 25 mmol/l citric acid, pH 5.0).

3.3.3.1 Progesterone Assay

Plasma progesterone levels were measured using a procedure previously described by Elder, Yeo, Lewis and Clifford (1987).

Reagents

Progesterone standards and conjugate were prepared from progesterone purchased from Sigma Chemical Co. (St Louis, USA). Progesterone-3CMO was covalently bound to bovine-thyroglobulin using the modified anhydride method of Erlanger *et al.* (1959) described by Elder *et al.* (1987). Progesterone conjugate (3.5 μl) was added to 10 ml of 6 M guanadine hydrochloride (Sigma Chemical Co., USA) to make the working conjugate solution.

A stock solution (39.9 ng/ml) of progesterone was diluted in phosphate buffer to make up a series of standards 0, 0.0.32, 0.63, 1.25, 2.5, 4.9, 9.95 and 19.9 ng/ml. The phosphate buffer used for diluting the progesterone standards and reconstituting samples consisted of 0.06 mol/l Na_2HPO_4, 0.04 mol/l NaH_2PO_4, pH 7.0 with 0.1% w/v BSA and 0.1% w/v thiomersal.

Progesterone antiserum, raised in a rabbit against progesterone-3-CMO-BSA, was obtained from Dr J.G. Lewis (Department of Clinical Biochemistry, Christchurch Hospital, N.Z.) and used at a working dilution of 1:2000. Affinity purified peroxidase labelled goat anti-rabbit IgG was purchased from Tago Immunodiagnostics Inc (USA) and used at a dilution of 1:2000.

Extraction

Plasma samples (100 μl) were vortex mixed with 4 ml of freshly redistilled hexane and frozen in an ethanol-dry ice bath. The supernatant was decanted and dried down under air at 50-60 °C. Phosphate buffer (400 μl) was added and the tubes vortex mixed to reconstitute the extract.

ELISA procedure

Microtitre plates were activated by adding 100 μl of conjugate solution to each well and incubating the plate at 4 °C overnight. Plates were washed by inverting each plate, shaking it dry and automatically rinsing each well four times with 200 μl wash buffer on the BEPM II. (All subsequent wash steps were identical to the one described here.) Assay buffer (150 μl) was added to block any remaining active protein-binding sites and to reduce the pH in each well to 7.4 and the plate was left to incubate for 1 h at room temperature. Plates were inverted and shaken dry and 50 μl of sample extract or standards were dispensed in duplicate into the appropriate wells followed by 50 μl of progesterone antibody per well. Plates were incubated at room temperature for at least 2 h, emptied and washed. Peroxidase labelled rabbit gamma globulin antibody (100 μl) was added to each well and the plate incubated for a further 2 h at room temperature. Plates were washed, dried and 100 μl of freshly prepared enzyme substrate added. Colour development was left to proceed in the dark for approximately 20 minutes and then 100 μl of 1.25M H_2SO_4 was added to stop the reaction. Absorbance was read on the BEPM at 492 nm, with a reference wavelength of 650 nm, and the progesterone concentrations of the samples determined from the dose-response curve.

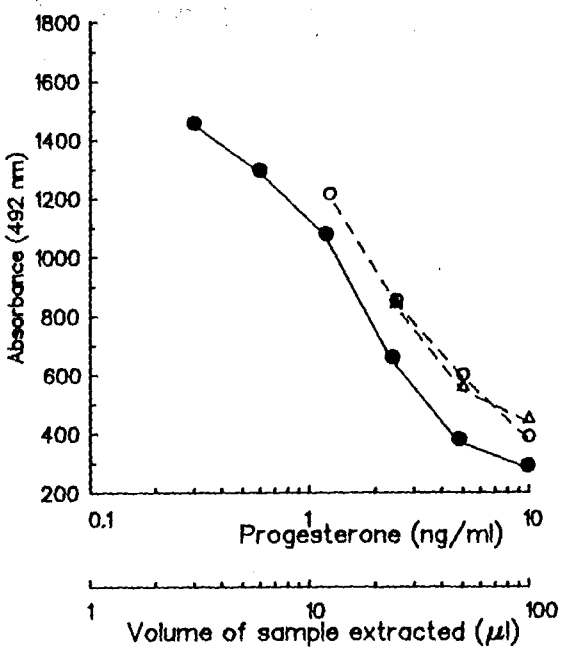
Validation of the progesterone assay

The specificity of the antibody was tested by Lewis *et al.* (1987). Cross reactivity with steriods such as cortisol, aldosterone, testosterone and 17β-oestradiol was less than 1.0%. The minimum detectable concentration of progesterone, calculated at two standard deviations from zero, was < 0.28 ng/ml. Precision of the assay, as assessed by the intra- and inter-assay CV of samples repeated in each assay, ranged from 8-16% (Table 3.3). Various volumes of a deer plasma sample, extracted and diluted with phosphate buffer, generated absorbance curves parallel to the dose response curve of the standards (Figure 3.4). This indicated that progesterone in the plasma and the standards competed for the antibody in a similar manner.

Table 3.3 Intra- and inter-assay CV for control samples in the progesterone assay.

	Mean progesterone (ng/ml)	Intra-assay CV (%)	Inter-assay CV (%)	n
low	0.68	15.9	14.2	23
medium	1.29	11.3	11.6	24
medium-high	2.85	10.9	11.4	23
high	6.19	8.3	11.5	21

Figure 3.4. Absorbance inhibition curves for progesterone standards (●) and representative cervine plasma progesterone extracts (○,△).



3.3.3.2 Prolactin Assay

Plasma prolactin levels were measured using an ELISA for ovine plasma prolactin developed and validated for ovine and cervine plasma (Lewis, Elder & Barrell, 1992).

Reagents

Prolactin standards and conjugate were prepared from ovine prolactin (NIADDK-o-PRL-16). Rabbit anti-ovine prolactin antiserum was provided by D.F.M. Van de Wiele (Research Institute for Animal Husbandry, Schoonoord, Netherlands).

Standards (0, 8, 16, 32, 64, 128 and 256 ng/ml) were prepared in horse plasma as equine prolactin did not react with the anti-ovine prolactin antiserum.

Prolactin-thyroglobulin conjugate was formed using the method of Skowsky and Fisher (1972) and 7 µl of the 10 mg/ml stock solution diluted with 10 ml of 6 M guanidine HCl to obtain the working conjugate solution.

Rabbit anti-prolactin antibody was diluted 1:50 000 with assay buffer to obtain the working concentration. Affinity purified peroxidase labelled goat anti-rabbit IgG was purchased from Tago Immunodiagnostics Inc (USA) and used at a dilution of 1:2000.

ELISA procedure

Prolactin-thyroglobulin conjugate (100 µl) was added to each well of a microtitre plate and the plate incubated overnight at 4 ° C. After washing, the plate was blocked by the addition of 150 µl assay buffer per well and incubated at 4 ° C for 1 h. The plate was shaken dry and 50 µl of standard or sample dispensed in duplicate into the appropriate wells at 4 ° C. This was immediately followed by the addition the prolactin antibody (50 µl/well) and the plate was incubated for at least 2 h at room temperature.

Subsequent steps are identical to the procedures used in the progesterone ELISA and are described in section 3.3.3.1..

Validation of the prolactin assay

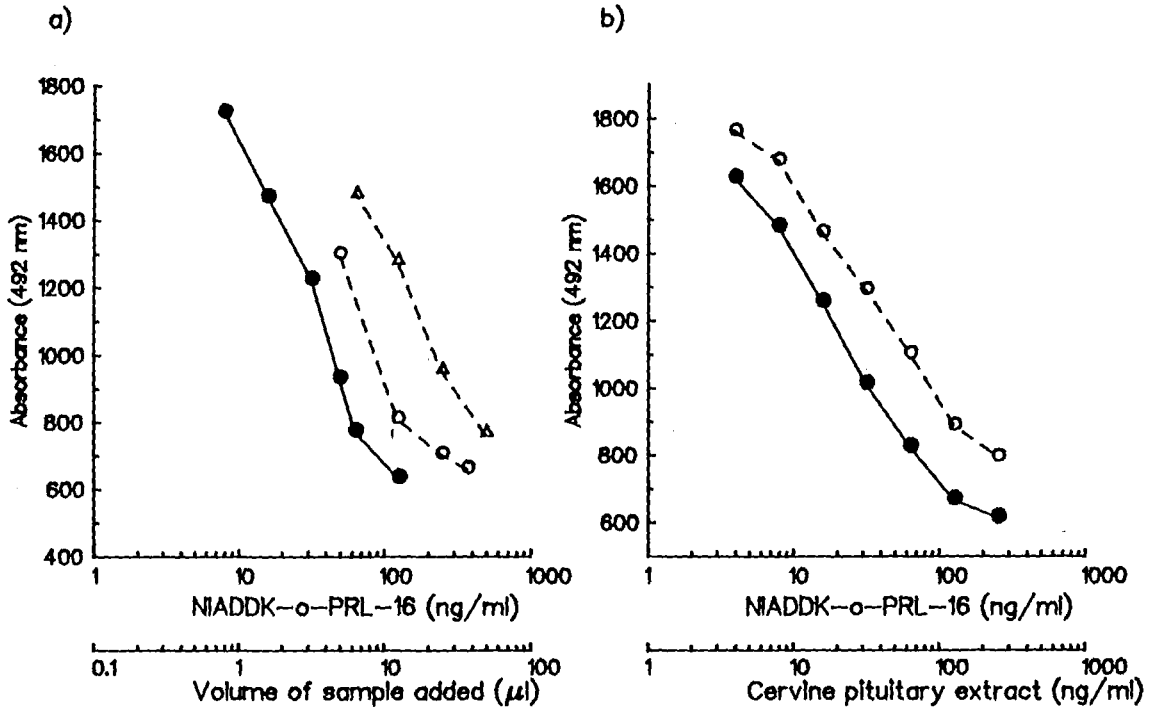
Cross reactivities with other ovine pituitary hormones were less than 1.0% for ovine GH, LH, FSH and TSH (Lewis *et al.*, 1992). No purified cervine pituitary hormones were available for testing. Cervine plasma samples and cervine prolactin pituitary extract, serially diluted with horse plasma, displayed absorbance curves parallel to those generated by ovine standards (Figure 3.5). This suggests that dilution of the sample did

not influence the estimate of prolactin concentration and that the assay measured immunoreactive cervine prolactin in the deer plasma and pituitary extract. Results were expressed as ng/ml of NIADDK-o-PRL-16 for convenience. The sensitivity of the assay was < 10 ng/ml and intra- and inter-assay CV varied from 10.7-15.1% (Table 3.4).

Table 3.4 Intra- and inter-assay CV for samples repeated in each prolactin assay.

	Mean prolactin (ng/ml)	Intra-assay CV (%)	Inter-assay CV (%)	n
low	18.8	11.3	15.1	15
medium	30.8	10.7	14.6	15
high	58.3	11.8	11.3	14

Figure 3.5. Binding inhibition curves for ovine prolactin standard (●, NIADDK-o-PRL-16) and a) representative cervine plasma samples (△○) and b) cervine pituitary prolactin extract (○) diluted with horse plasma.



3.4 Statistical Analysis

Results are expressed as mean ± standard error of the mean (s.e.m.). Initial data entry and manipulation was carried out using the Minitab statistical package (Release 7.2 VAX/VMS version 1989, Minitab Inc, USA). The SAS system (Release 6.06 1989, SAS Institute Inc, USA) was used for statistical analyses. To avoid correlations between means and variance, data were log- or rank-transformed when necessary (Ipe, 1983). Unless otherwise stated, treatment effects were detected using analysis of variance (ANOVA) in conjunction with the Duncan's least significant difference (LSD) test to separate treatment means when treatment effects (F-test) were significant. In addition, if the data set was suitable, hormone and live weight profiles were analysed using the GLM procedure repeated measures option. Time was the repeated measures factor, treatment the group factor and a profile transformation matrix was used to determine trends over time. The profile transformation matrix generates contrasts between adjacent values over time and is useful when a non-polynomial reponse to treatment is expected (SAS Institute Inc., 1989). Repeated measures analysis eliminated correlations due to multiple measurements taken over time on the same subject but was unable to cope with missing data points. Problems associated with individual data sets are described in the relevant chapter. Undetectable hormone concentrations were assigned a value equivalent to the detection limit of the assay for statistical analysis. $P < 0.05$ was considered significant.

During intensive blood sampling an LH increase was defined as a pulse if the difference between the LH peak and the previous nadir was greater than 3 times the standard deviation of the nadir. Pulse amplitude was the incremental increase between the nadir and a detected LH pulse. The mean LH concentration was the average LH concentration measured during the 4 h sampling period while basal LH was the minimum LH concentration detected during the same period. The LH response to an exogenous GnRH challenge was the incremental change in concentration between the time of GnRH administration (time 0) and the peak of LH, which always occurred in the sample collected at 10 min.

If the plasma progesterone concentration exceeded 1.0 ng/ml (upper limit of 99% confidence interval for mean plasma progesterone concentration of 62 non pregnant

anoestrous hinds) for a period of more than 5 days progesterone levels were arbitrarily deemed to be elevated.

Chapter 4

Reproductive seasonality of red deer hinds exposed to artificially extended daily photoperiods during the winter/spring or summer/autumn period

4.1 Introduction

In red deer the timing of many seasonal events, such as sexual activity in hinds (Webster & Barrell, 1985) and antler growth, rutting behaviour and testicular development in stags (Pollock, 1975; Simpson *et al.*, 1983/1984; Suttie *et al.*,1984), can be manipulated by treatment with artificial photoperiods. Photoperiod is the environmental variable utilised by most seasonally breeding animals to synchronise their fertile period so that parturition occurs at the most suitable time of the year (Karsch *et al.*, 1984).

Sheep and deer are denoted as 'short day' breeders because they are sexually active during autumn (Marshall, 1937; Kelly & Moore, 1977; Karsch *et al.*, 1984) when daily photoperiods are decreasing. Nocturnal secretion of melatonin from the pineal gland mediates the reproductive response to photoperiod (Bittman *et al.*, 1983a; Bittman & Karsch, 1984) and the onset of the breeding season may be advanced by manipulating the pattern of melatonin secretion either by reducing the length of the daily photoperiod (Bittman *et al.*, 1983a,b; Webster & Barrell, 1985) or by administering exogenous melatonin (Kennaway *et al.*, 1982; Adam & Atkinson, 1984; Lincoln *et al.*, 1984; Webster & Barrell, 1985).

Robinson *et al.* (1985b) suggested that, although the breeding cycle of the ewe may be 'driven' by exposure to short days (Bittman *et al.*, 1983b) or appropriate administration of melatonin (Kennaway *et al.*, 1982), the transition from anoestrum to sexual activity under natural conditions in autumn results from a temporary loss of response to the prevailing inhibitory photoperiod. Ewes maintained under constant photoperiodic conditions still had alternating periods of reproductive activity and quiescence (Radford, 1961; Thwaites, 1965; Ducker *et al.*, 1973) and ewes maintained on the summer solsticial photoperiod from midsummer became sexually active at the normal time (Robinson *et*

al., 1985b; Worthy *et al.*, 1985). Lack of response to the ambient daily photoperiod is known as 'photorefractoriness', a term coined by avian physiologists (Nicholls *et al.*, 1988) to describe this phenomenom. Photorefractoriness probably results from a loss of response to the melatonin secretion pattern (Karsch *et al.*, 1986).

If photorefractoriness to long daily photoperiods initiates the seasonal transition from reproductive quiescence to sexual activity in 'short day' breeders then it can be proposed that premature exposure of red deer hinds to long daily photoperiods would advance the development of photorefractoriness and therefore hasten the onset of the breeding season. This hypothesis was tested by examining the seasonal physiology of red deer hinds exposed to a long daily photoperiod from mid winter (Trial 1). This hypothesis would gain reinforcement if it could be shown that autumn breeding activity commences at the usual time in red deer hinds denied exposure to the normal autumnal decrease in photoperiod. In order to examine this point and, thus, determine the role that decreasing photoperiods during autumn played in the timing of the onset of the normal breeding season in red deer, another group of red deer hinds was exposed to long daily photoperiods from midsummer and their onset of reproductive activity was recorded (Trial 2).

Responses of hinds to these light treatments were assessed by measuring changes in secretion of the photoperiod-responsive hormones, melatonin and prolactin. The onset of oestrous cyclicity was determined using plasma progesterone profiles as an indicator of luteal activity. Calving dates were used to estimate the time of conception. Seasonal changes in reproductive activity were related to changes in the pattern of LH secretion and the ability of the pituitary to secrete LH in response to an exogenous GnRH challenge.

4.2 Materials and methods

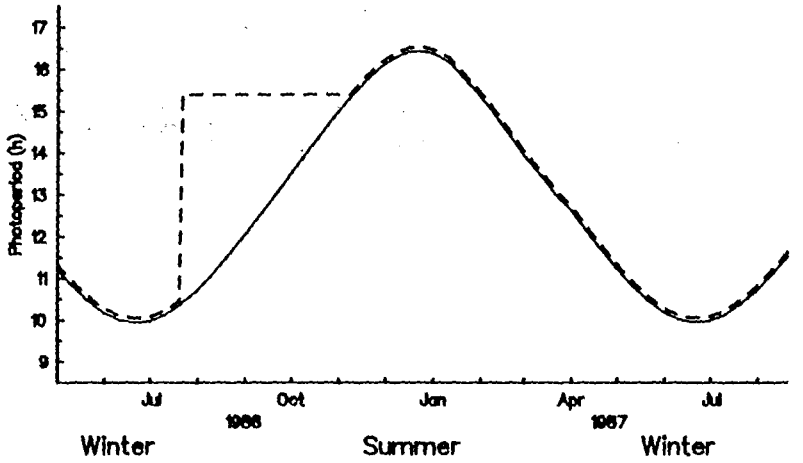
4.2.1 Trial 1

Twenty pregnant mixed-age red deer hinds (average live weight 100.4 ± 1.4 kg) were allocated to 2 treatment groups (n=10) so that the groups were approximately balanced

for age and live weight. One group was prematurely exposed to a long daily photoperiod of 15.3 h from 22 July to 7 November 1986, i.e. winter-spring (Extended Photoperiod Winter, EPW), whilst the other group was maintained under natural daily photoperiods (Natural Photoperiods, NP) (Figure 4.1).

The EPW lighting regime of a 15.3 h constant daily photoperiod was chosen so that the light treatment ceased approximately 1 month before calving, on 8 November 1986, when the natural daily photoperiod (i.e. sunrise to sunset plus 1 h civil twilight) was also 15.3 h.

Figure 4.1. Photoperiod regime for control, NP (—) and EPW (- -) hinds in Trial 1.



During the artificial light treatment period all hinds were mustered into the deer yards each day at 1700 h (NZST) and confined to an open air yard (NP hinds) or in a light proof room (12m x 5m) (EPW hinds) until release time. The room was lit by 2 pairs of fluorescent tubes (cool white/33, General Electric Company (N.Z.) Ltd, N.Z.) providing 400-800 lux (Licor Quantum Sensor Photometer, Licor) 1.3 m from the ground (at eye level of the deer). An extractor fan (Xpelair GXC6, General Electric Company (N.Z.) Ltd) was installed to improve air circulation. Plug-in programmable timers (Model KD-36, Kambrook Distributors, Australia) automatically switched off the lights and activated solenoids to open the doors and release both groups of hinds to pasture each night. This meant that all hinds were treated identically in all respects except for exposure to light. The NP hinds experienced natural changes in daily photoperiod including morning and evening twilight whereas the EPW hinds received a constant 15.3 h light/day and

morning twilight only. Release time for the EPW hinds was calculated as 14 h 50 min after sunrise.

Live weight was recorded at 1-3 week intervals during the light treatment period. During the hours when they were confined, all hinds were provided with water and hay, and from 6 August to 8 November 1986 this was supplemented with 200 g barley/hind/night. After the light treatment period ceased hinds remained on pasture and were inspected daily during the calving period. Date of birth of calves resulting from matings during the previous autumn 1986 (i.e. prior to the lighting treatment) was recorded.

Blood samples (7 ml) were collected into heparinised glass vacutainers by jugular venepuncture at approximately fortnightly intervals from June to November 1986 and 2-3 times a week from March to April 1987 for progesterone analysis. On 5 occasions (18 July 1986, 19 January 1987, 19 February 1987, 27 March 1987 and 29 April 1987) the jugular vein of the same 4 EPW hinds and 4 NP hinds was cannulated (Section 3.2) and blood samples were collected at 20 minute intervals for 4 h to establish LH secretion patterns. Immediately following each of these intensive samplings, 2 μg GnRH (1 μg/ml in PBS) was administered i.v. via the cannula and blood samples were collected 10, 30, 60, 90, 120, 150, 180, 210 and 240 minutes after this injection in order to measure the release of LH from the pituitary gland in response to an exogenous GnRH challenge. On 13 August 1986 and 27 March 1987 blood samples were collected from the same 4 hinds in each group every 1-3 h for 25 h and the plasma analysed for melatonin.

Group EPW and NP hinds were scored for pelage (see Section 2.2) on 22 October 1986, 8 April 1987 and 1 May 1987.

A mature red deer stag was introduced to the NP and EPW hinds on 1 March 1987 and removed on 30 May 1987. During November and December 1987 NP and EPW hinds was inspected daily and the date of calving for each hind recorded.

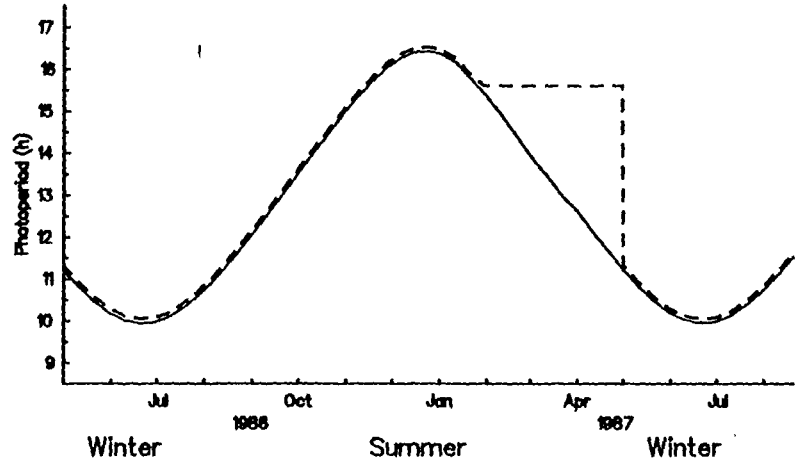
Live weight, melatonin, progesterone and LH data were log-transformed (base 10) prior to statistical analysis so that data were normally distributed and had homogeneity of variance. Prolactin data were non-normal even after this transformation so values within

animals were ranked and the rankings used for statistical analysis (Siegal, 1956; Conover & Iman, 1981). Overall treatment and time effects were determined by analysis of variance (ANOVA) with repeated measures (SAS Statistical Package, USA; see Winer, 1971) on transformed and ranked data. The profile option of the repeated measures analysis (SAS/STAT Users Guide, 1990;Cole & Grizzle, 1966) was used to detect different trends over time between treatment groups. Differences between means within treatments were established using Duncan's multiple range test and differences between treatments within times by ANOVA. Several blood samples from one control hind (19-1) were lost so data from this hind were excluded from repeated measures analysis. LH pulse frequency and pelage scores were analysed using the Friedman ranking test (Siegal, 1956; Ipe, 1987).

4.2.2 Trial 2

A group of mixed-age hinds (n=6), which had experienced the natural changes in photoperiod until mid-summer, were exposed to artificially extended photoperiods of 15.5 h of light from 30 January to 30 April 1987, i.e. summer-autumn (Extended Photoperiod Summer, EPS) (whilst hinds in Trial 1 experienced naturally decreasing daily photoperiods) (Figure 4.2). All EPS hinds had calved during November or December 1986. Four hinds were suckling calves but 2 calves had died due to misadventure prior to the start of the light treatment.

Figure 4.2. Photoperiod regime for control, NP (—) and EPS (- -) hinds in Trial 2.



The EPS lighting regime began in late January when the natural photoperiod was 15.5 h, and calves were old enough to be mustered with their dams into the yards each day without risk of mismothering. Light treatment was identical to that used in Trial 1 except hinds were released to pasture 15 h after sunrise and were provided with 500 g barley/hind during the light treatment period. As Trials 1 and 2 ran concurrently, the NP hinds described in Trial 2 were controls for both experiments. The EPS group were kept separate from NP and EPW during the light treatment periods but treatment groups were run as a single mob at all other times.

Blood samples were collected by jugular venepuncture on 26 January, 16 February, 2 March and 15 March 1987 and 2-3 times per week from mid March until mid May 1987 for progesterone and prolactin analyses. On 3 occasions (26 January 1987, 16 March 1987 and 24 April 1987) blood samples were collected at 20 min intervals for 4 h from each hind to establish LH secretion patterns. As number of animals in the trial group EPS was limited (n=6), pituitary LH responsiveness was not measured in EPS hinds in case administration of exogenous GnRH provoked the generation of an LH surge. On 24 April 1987 blood samples were also collected every 1-3 h for 25 h and plasma analysed for melatonin.

Group EPS hinds were scored for pelage change on 8 April 1987 (see Section 3.2).

A mature red deer stag was introduced to the EPS hind mob on 4 March 1987 and removed on 30 May 1987. During November and December 1987 the calving mob was inspected daily and the date of calving for each hind recorded.

4.3 Results

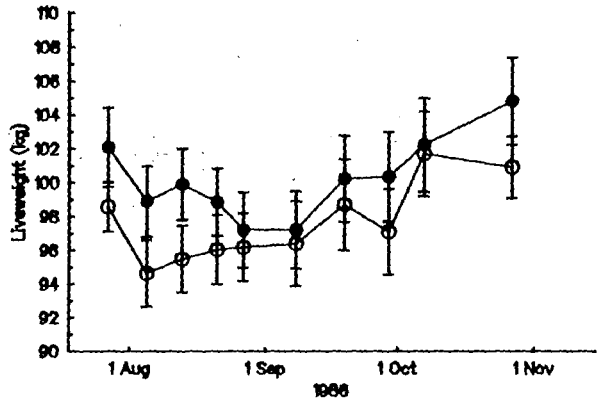
4.3.1 Trial 1

Live weight

The pattern of changes in mean live weight was similar in EPW and NP group hinds (Figure 4.3). Mean live weight in both groups of hinds decreased by about 4 kg in the

first two weeks of the experiment. Supplementary feeding began on 6 August and mean live weight was maintained at about 96 kg until early September. From September live weights gradually increased until the end of the light treatment period in November when the mean live weight of hinds was 101.9 kg (± 1.8 kg). Mean live weights on 27 February, 15 March, and 3 May 1987 were 101.4 ± 2.3, 103.7 ± 1.9 and 101.1 ± 0.9 kg, respectively.

Figure 4.3. Seasonal pattern of mean live weight of red deer hinds from 20 July to 8 November 1986. Hinds were either exposed to natural light (NP) (o, n=10) or 15.3 h photoperiods (EPW) (●, n=10) from 22 July to 8 November 1986. Vertical bars denote s.e.m.



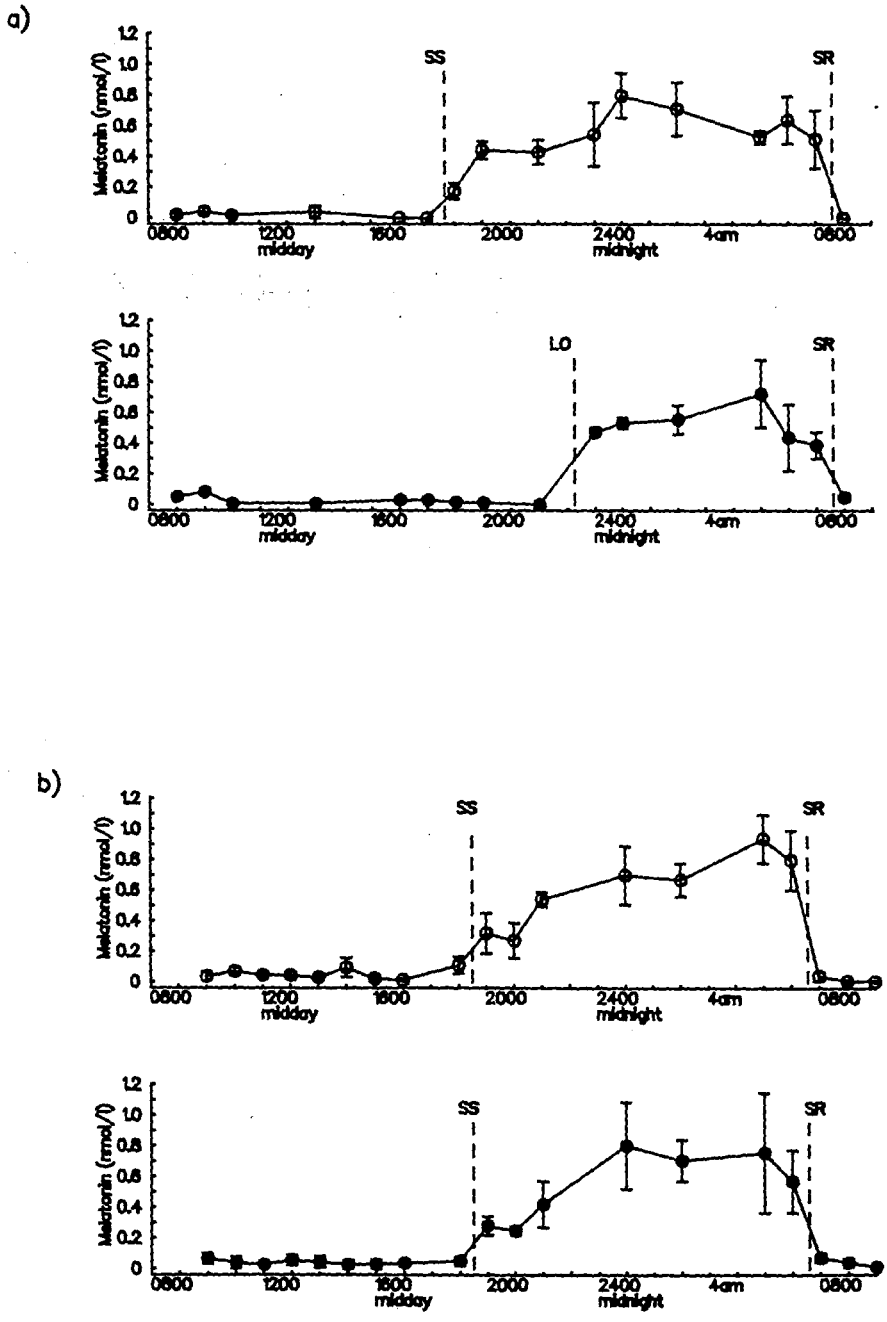
Melatonin.

Plasma melatonin concentrations on 13 August 1986 were significantly influenced by treatment ($p<0.001$) and time ($p<0.001$) (Figure 4.4 a). In the control (NP) hinds plasma melatonin concentrations were very low and often undetectable between 0800 and 1700 h but increased ($p<0.05$) rapidly after sunset and remained elevated (0.43-0.79 nmol/l) until just before sunrise the following morning. In the EPW hinds plasma melatonin concentrations were similarly very low during the hours of natural and artificial light. Melatonin levels increased ($p<0.05$) after lights out and remained elevated (0.38-0.72 nmol/l) until sunrise the next day. Mean plasma melatonin concentration was significantly higher ($p<0.01$) in NP hinds than in EPW hinds from 1800-2100 h.

On 27 March 1987 melatonin concentration was influenced by time of blood sampling only ($p<0.001$) (Figure 4.4 b). Melatonin levels in plasma were very low and often

undetectable in the NP and EPW hinds during daylight hours but increased ($p<0.01$) after sunset and remained elevated (0.25-0.87 nmol/ml) until sunrise the following morning.

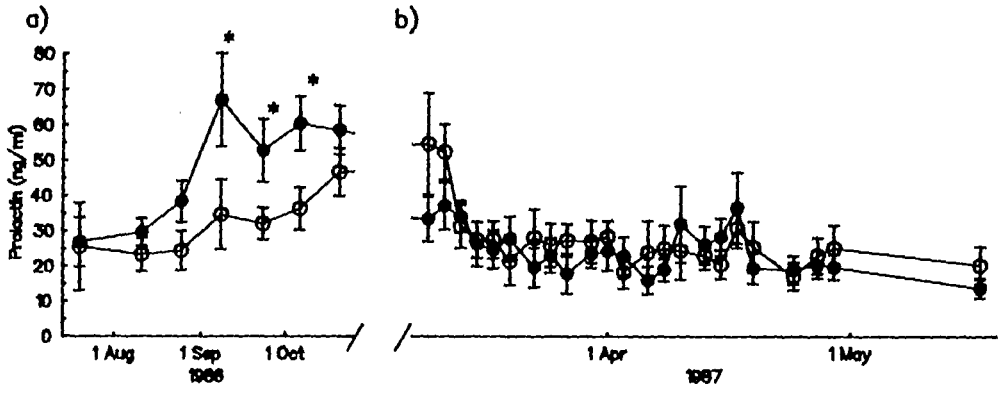
Figure 4.4 Profiles of mean plasma melatonin concentrations on a) 13 August 1986 in red deer hinds exposed to the natural photoperiod (NP) (○, n=4) or artificial light from 1630 to 2218 h (EPW) (●, n=4) and on b) 27 March 1987 in the same red deer hinds which were exposed to the natural photoperiod (NP) (n=4) or 15.3 h photoperiod (EPW) (n=4) from 22 July to 8 November 1986. Vertical bars denote S.E.M. The dashed vertical lines represent the time of sunset (SS), sunrise (SR), or lights off (LO).



Prolactin

Treatment ($p<0.001$), time ($p<0.001$) and individual animal ($p<0.001$) had significant effects on plasma prolactin concentration. Prolactin concentrations in the NP hinds were low (20-30 ng/ml) during July, August and September but gradually increased during October and November 1986. In NP hinds prolactin concentrations were highest in samples collected on 10 and 12 March 1987 (53 ± 15 and 52 ± 8 ng/ml, respectively) and levels decreased during mid March ($p<0.01$) and remained low thereafter. Plasma prolactin levels in the EPW hinds were also low during July and August but mean concentration increased in early September in the EPW hinds and was significantly higher than that of the NP hinds ($p<0.01$) on 6 September, 23 September and 8 October, 1986. Plasma prolactin levels were generally low in EPW hinds from March 1987 until the end of the trial period (Figure 4.5).

Figure 4.5. Seasonal pattern of mean plasma prolactin concentrations of red deer hinds from a) 20 July to 8 November 1986 and b) 10 March to 15 May 1987. Hinds were either exposed to natural light (NP) (○, n=10) or 15.3 h photoperiods (EPW) (●, n=10) from 22 July to 8 November 1986. Asterisks indicate significant differences between treatment group means. Vertical bars denote s.e.m.

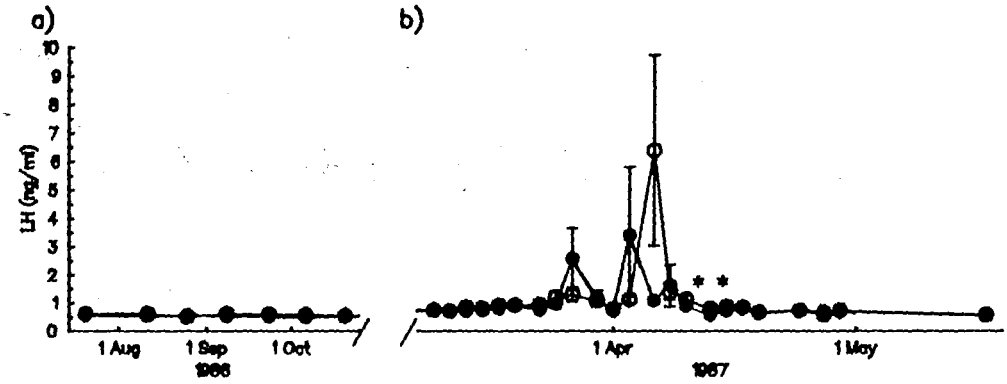


LH profile

Plasma LH concentration was influenced by treatment ($p<0.03$), time of blood sampling ($p<0.01$) and individual animal ($p<0.01$). Mean LH concentration was low (< 1 ng/ml) from July to November, 1986 and during early March 1987 (Figure 4.6). Transient increases (2.0-23.4 ng/ml) were recorded in individual hinds during late March and April. Maximum mean plasma LH concentrations in the NP and EPW hinds (5.84 ± 3.04 and

2.85 ± 1.94 ng/ml) were recorded on 6 and 3 April 1987, respectively. Mean LH levels were similar in the NP and EPW hinds except for 2 occasions (10 and 13 April, 1987) when the mean LH concentration of the NP hinds was significantly higher than that of the EPW hinds (1.12 ± 0.16 vs 0.78 ± 0.10 ng/ml and 0.81 ± 0.08 vs 0.58 ± 0.06 ng/ml respectively) (Figure 4.6).

Figure 4.6. Seasonal pattern of mean plasma LH concentrations of red deer hinds from a) 20 July to 8 November 1986 and b) 10 March to 15 May 1987. Hinds were either exposed to natural light (NP) (o, n=10) or 15.3 h photoperiods (EPW) (●, n=10) from 22 July to 8 November 1986. Asterisks indicate significant differences between treatment group means. Vertical bars denote s.e.m.



LH pulsatility and response to GnRH

The number of LH pulses and the minimum, mean and range in plasma LH concentration measured during an intensive sampling period were always similar in the NP and EPW hinds (Appendix A, Table 4.1A). These parameters did vary with time ($p<0.01$) and pooled data from NP and EPW hinds are presented in Table 4.1. During the intensive blood sampling period in July, no LH pulses were recorded and mean plasma LH concentration was very low, often below the sensitivity of the LH assay. The number of LH pulses, mean basal LH, range in LH and mean LH concentration were higher on 27 March than during sampling periods in any other month.

The increase in LH concentration in response to exogenous GnRH was not influenced by light treatment ($p>0.05$) but varied significantly with time ($p<0.01$). There was always a significant increase in plasma LH concentration after the GnRH injection but the

magnitude of the increase was significantly smaller in July 1986 and January 1987 than during February, March and April 1987 (Table 4.1).

Table 4.1. LH pulsatility and pituitary LH response to GnRH of red deer hinds in Trial I. Data from hinds exposed to natural light (NP) or 15.3 h photoperiod (EPW) between 22 July and 7 November 1986 were pooled. Means assigned different letters within rows are significantly different ($p<0.05$).

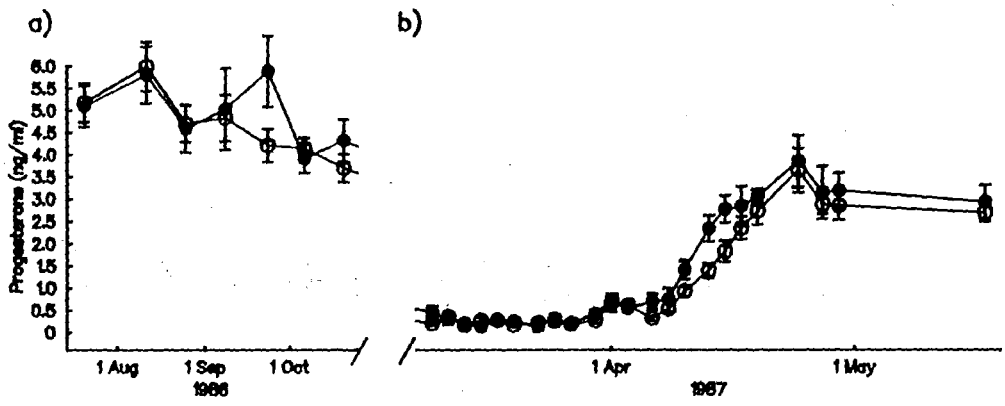
Parameter	Jul 1986	Jan 1987	Feb 1987	Mar 1987	Apr 1987
No of pulses/4 h	-	**0.38**	**0.63**	**1.25**	**0.50**
	-	(0.18)	(0.26)	(0.31)	(0.19)
		a	**a**	**b**	**a**
Basal LH	**0.27**	**0.34**	**0.35**	**0.55**	**0.32**
(ng/ml)	(0.01)	(0.03)	(0.03)	(0.07)	(0.02)
	a	**a**	**a**	**b**	**a**
Range in LH	**0.11**	**0.76**	**0.94**	**2.08**	**0.82**
concentrations	(0.01)	(0.32)	(0.27)	(0.56)	(0.48)
(ng/ml)	**a**	**b**	**b**	**c**	**b**
Mean LH	**0.32**	**0.64**	**0.60**	**1.10**	**0.46**
concentration	(0.01)	0.07)	(0.06)	(0.17)	(0.05)
(ng/ml)	**a**	**b**	**b**	**c**	**b**
LH response to	**0.48**	**0.17**	**6.17**	**4.85**	**2.98**
GnRH challenge	(0.12)	(0.08)	(0.64)	(1.06)	(0.19)
(ng/ml)	**a**	**a**	**b**	**b**	**b**

Progesterone

Plasma progesterone concentration was influenced by the time of blood sampling ($p<0.001$) but not by treatment ($p>0.05$). Plasma progesterone levels were high (about 5 ng/ml) in all hinds from June to November 1986. During March plasma progesterone concentrations were low (< 1 ng/ml) and often undetectable until levels increased in early April 1987 (Figure 4.7). The mean date of the first sustained increase in plasma progesterone levels in individual hinds (> 1 ng/ml for > 6 days) was not different for EPW and control hinds (10 April ± 0.8 d and 12 April ± 1.2 d 1987, respectively). Individual plasma progesterone profiles indicate that there was a transient increase (0.8-2.2 ng/ml) in 6/10 NP and 8/10 EPW hinds 2 to 10 d prior to the sustained increase in

plasma progesterone concentration. In 19/20 hinds progesterone levels remained elevated (about 3 ng/ml) until the end of the experimental period. In the remaining hind (5-3) plasma progesterone concentration remained elevated for only 10 d, decreased below 1 ng/ml for 3 d and then increased again in late April.

Figure 4.7. Seasonal pattern of mean plasma progesterone concentrations of red deer hinds from a) 20 July to 8 November 1986 and b) 10 March to 15 May 1987. Hinds were either exposed to natural light (NP) (○, n=10) or 15.3 h photoperiods (EPW) (●, n=10) from 22 July to 8 November 1986. Vertical bars denote s.e.m.



Elevated plasma progesterone concentrations and calving data suggest that all NP and EPW hinds were pregnant at the time of the intensive blood sampling periods in July 1986 and April 1987 and were acyclic when intensively blood sampled in January and February. In late March transient increases in plasma progesterone concentration, 2-6 days after the intensive sampling period, indicate that blood samples were collected from 4/8 animals during the transition from anoestrus to the breeding season. Plasma progesterone concentrations remained low (< 1 ng/ml) for 13-16 d after the March blood sampling period in the other 4 hinds.

Calving date

Mean calving date of the hinds resulting from matings in autumn 1986 (prior to light treatment) was similar, 22.5 November ± 2.7 d and 22.1 November ± 3.8 d for EPW and NP hinds, respectively. All hinds conceived in autumn 1987 (following light treatment). Mean calving date of the EPW hinds was 21 November 1987 (± 1.7 d), similar to that of the control hinds (23 November 1987 ± 2.2 d).

Pelage

On both occasions when it was monitored shedding of the coat was more advanced in the EPW hinds than in the control hinds ($p<0.01$). On 22 October 1986 and 8 April 1987 mean pelage scores of EPW hinds (3.7 ± 0.2 & 4.1 ± 0.2 respectively) were significantly higher than those of the control hinds (1.0 ± 0.3 & 2.5 ± 0.5, respectively).

4.3.2 Trial 2

There was no effect of calf rearing status on any parameter measured ($p>0.10$) so data for suckling and non-suckling EPS hinds were pooled.

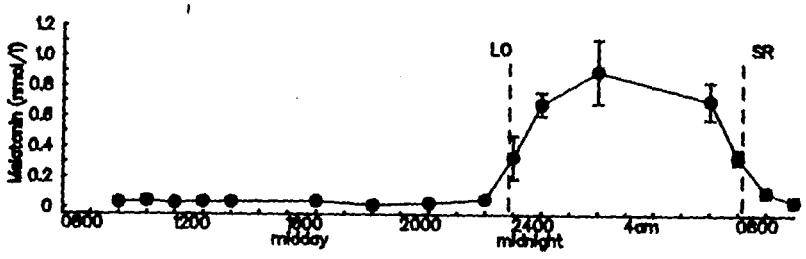
Live weight

Mean live weight of EPS hinds on 20 January, 19 February, 9 March and 10 April 1986 was 97.7 (± 2.7), 99.3 (± 2.5), 101.3 (± 2.8) and 103.5 (± 2.7) kg, respectively.

Melatonin

On 24 April 1987 mean plasma melatonin levels were low and often undetectable between 0900 and 2000 h but increased rapidly after sunset and remained elevated (0.34-0.90 nmol/l) until sunrise the following morning (Figure 4.8).

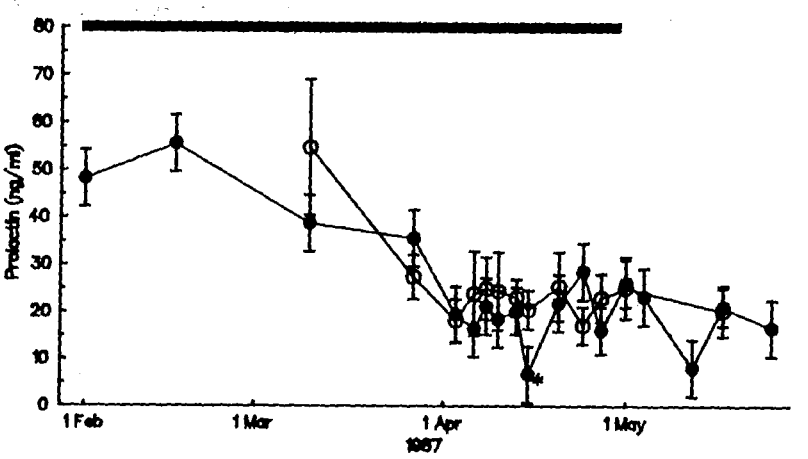
Figure 4.8 Profile of mean plasma melatonin concentrations on 24 April 1987 in red deer hinds exposed to artificial light from 1630 to 2212 h (EPS) (●, n=6). Vertical bars denote S.E.M. The dashed vertical lines represent the time of sunrise (SR), sunset (SS) or lights off (LO).



Prolactin

Plasma prolactin concentration was influenced by the time of blood sampling ($p<0.03$) and by individual animal ($p<0.001$). Mean plasma prolactin concentration was highest (28 ± 8 ng/ml) on 16 February and lowest (5 ± 3) on 25 May when values were often below the detection limit of the assay. On the 13 occasions that NP and EPS hinds were blood sampled on the same day, plasma prolactin concentrations were similar except on 15 April when prolactin concentration was significantly lower ($p<0.023$) in the EPS hinds (7 ± 1 vs 21 ± 4 ng/ml) (Figure 4.9.)

Figure 4.9. Seasonal pattern of mean plasma prolactin concentrations of red deer hinds from 10 March to 25 May 1987. Hinds were either exposed to natural light (NP) (○, n=10) or 15.5 h photoperiods (EPS) (●, n=6) from 30 January to 30 April 1987. Asterisks indicate significant differences between treatment group means. Vertical bars denote s.e.m.



LH

Plasma LH concentrations were generally low (< 1 ng/ml) throughout the trial period except on 20 April when there was a non significant increase in mean LH concentration of EPS hinds to 1.94 (± 1.42 ng/ml) (Figure 4.10). In comparison with the NP group hinds, plasma LH levels in the EPS hinds were significantly lower ($p<0.05$) on 3, 6, 8 and 13 April.

Figure 4.10. Seasonal pattern of mean plasma LH concentrations of red deer hinds from 10 March to 25 May 1987. Hinds were either exposed to natural light (NP) (o, n=10) or 15.5 h photoperiods (EPS) (●, n=6) from 30 January to 30 April 1987. Asterisks indicate significant differences between treatment group means. Vertical bars denote s.e.m.

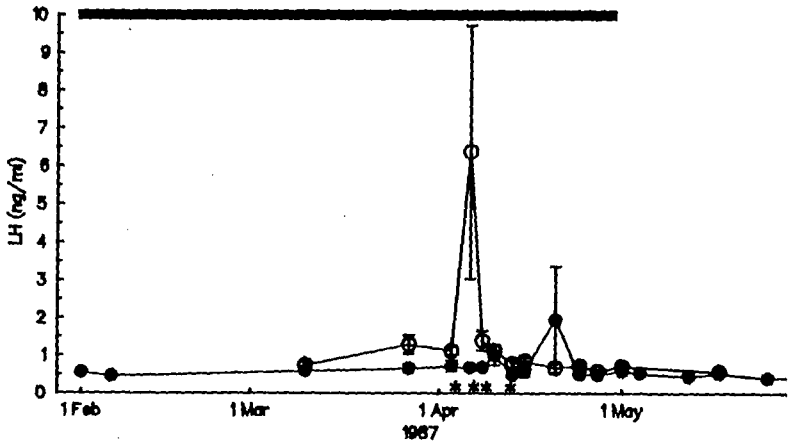


Table 4.2. LH pulsatility in hinds exposed to natural light (NP) or 15.5 h photoperiod (EPS) between 30 January and 30 April 1987. Means within parameters with different superscripts are significantly different ($p<0.05$).

Parameter		January 1987 mean	(s.e.m.)	March 1987 mean	(s.e.m.)	April 1987 mean	(s.e.m.)
No. of pulses/ 4 h period	NP	**0.50**^{a}	(0.29)	**1.50**^{b}	(0.50)	**0.50**^{a}	(0.29)
	EPS	**0.33**^{a}	(0.21)	**0.17**^{a}	(0.17)	**1.83**^{b}	(0.48)
Mean LH concentration (ng/ml)	NP	**0.64**^{a}	(0.08)	**0.80**^{b}	(0.06)	**0.42**^{a}	(0.06)
	EPS	**0.47**^{a}	(0.06)	**0.65**^{a}	(0.08)	**1.12**^{b}	(0.33)
Basal LH nadir (ng/ml)	NP	**0.37**^{a}	(0.04)	**0.46**^{b}	(0.02)	**0.31**^{a}	(0.04)
	EPS	**0.32**^{a}	(0.06)	**0.46**^{b}	(0.04)	**0.58**^{c}	(0.06)
Range in LH concentrations (ng/ml)	NP	**0.81**^{a}	(0.37)	**1.39**^{bc}	(0.31)	**0.42**^{a}	(0.18)
	EPS	**0.37**^{a}	(0.08)	**1.00**^{ab}	(0.24)	**2.23**^{c}	(0.55)

LH pulsatility

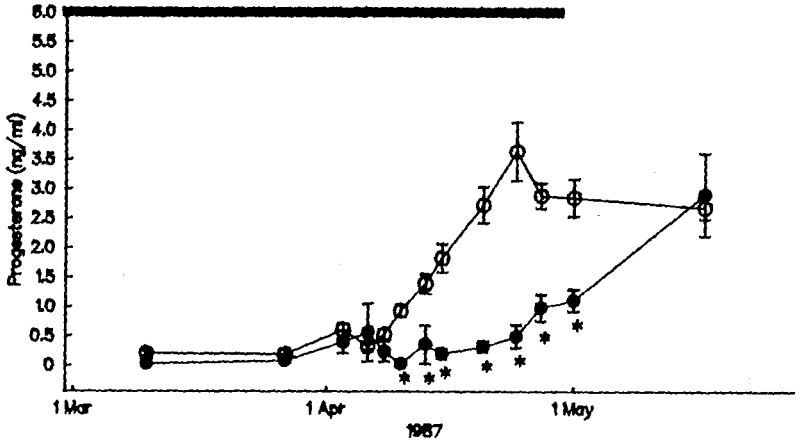
The number of LH pulses and the minimum and mean plasma LH concentration measured during the intensive blood sampling periods increased between January and April in the EPS hinds (Table 4.2). For the EPS hinds the value of all these parameters was significantly greater ($p<0.05$) during April than during January and March. When compared with LH secretion in the NP hinds in the same month of the year, LH pulsatility of the EPS hinds was similar in January, the number of pulses and mean LH levels were lower ($p<0.005$) in the EPS hinds during March and the values of all parameters were significantly greater in the EPS hinds in April ($p<0.05$).

Progesterone

Plasma progesterone concentration was significantly influenced by time ($p<0.001$) in EPS hinds. Progesterone concentrations (Figure 4.11) were generally low (< 1 ng/ml) until late April. Even then, when compared with NP hinds, mean progesterone levels were consistently lower in the EPS hinds between 8 and 27 April. The mean time of the first sustained increase in the plasma progesterone levels of EPS hinds was on 29 April ± 2.2 d, significantly later ($p<0.01$) than the increase in the NP hinds ((12 April ± 1.2 d). Individual progesterone profiles suggest that most hinds conceived at the first ovulatory cycle as plasma progesterone levels remained elevated (about 3 ng/ml) for the remainder of the trial period in 4/6 hinds. In the other 2 hinds the progesterone pattern indicates that these hinds conceived during the second ovulatory cycle.. As with the EPW and NP group hinds, a short term increase in progesterone concentration (0.7-3.2 ng/ml) was recorded in EPS hinds 5-9 days prior to the first luteal phase increase in progesterone.

Individual plasma progesterone profiles indicate that all hinds were anoestrous when intensively blood sampled in January and March. However the intensive blood sampling period in April coincided with a transitory progesterone increase (associated with the transition to breeding activity) in 2/6 hinds, with a period of low progesterone concentrations (2-4 d before a luteal phase increase in progesterone) in 2/6 hinds and with elevated plasma progesterone concentrations (the early luteal phase of the oestrous cycle) in 2/6 hinds.

Figure 4.11. Seasonal pattern of mean plasma progesterone concentrations of red deer hinds from 10 March to 25 May 1987. Hinds were either exposed to natural light (NP) (o, n=10) or 15.5 h photoperiods (EPS) (●, n=6) from 30 January to 30 April 1987. Asterisks indicate significant differences between treatment group means. Vertical bars denote s.e.m.



Calving date

In the calving period following light treatment the mean calving date of the EPS hinds was 17 December 1987 (± 3.3 d). This was significantly later ($p<0.01$) than either the calving date of the same hinds in the previous year (24 November 1986 ± 3.5 d) or of control hinds in the same year (23 November 1987 ± 2.2 d).

Pelage

On 8 April 1987, mean pelage scores were similar in the EPS and NP hinds (1.8 ± 0.4 and 2.5 ± 0.5, respectively).

4.4 Discussion

The present trial has studied the role of photoperiod in the regulation of onset of seasonal breeding in red deer hinds. Results show that the onset of seasonal breeding was not affected by premature exposure to long daily photoperiods during spring but was delayed when the autumnal decrease in daily photoperiod was delayed. This indicates that the onset of reproductive activity in red deer is initiated by stimulatory effects of short days rather than by the development of photorefractoriness to long days. This is in contrast with the case of the sheep originally put forward by Karsch and co-workers (Robinson, Wayne and Karsch, 1985b) which postulated that the normal transition from anoestrus to breeding season in the adult ewe was associated with the development of

photorefractoriness to the inhibitory ambient photoperiod. However, data from their more recent studies has caused this group to modify their conclusions as their current view is that seasonal reproductive transitions in the ewe result from an endogenously generated circannual rhythm which is entrained to a 12-month cycle by the prevailing photoperiod (Karsch *et al.*, 1989; Malpaux *et al.*, 1989; Wayne *et al.*, 1990). They have shown that it is the increase in daily photoperiod during spring which determines the subsequent onset of reproductive activity in the autumn, not the occurrence of photorefractoriness (Malpaux *et al.*, 1989, O'Callaghan *et al.*, 1991). However, this version of the working hypothesis is still at variance with the results obtained here from red deer hinds. Premature exposure of the ewe to long daily photoperiods prior to their natural breeding season advances the onset of reproductive activity but this does not appear to apply in the case of the breeding hind.

The results of this study do not exclude the likely possibility that the seasonal reproductive cycle of the hind is generated by an endogenous circannual rhythm and that photoperiodic signals entrain the breeding season to the appropriate time of year. Seasonal cycles of reproductive activity persist in the absence of photoperiodic information (following pinealectomy (Brown *et al.*, 1978; Snyder *et al.*, 1983) or cranial cervical ganglionectomy (Lincoln, 1985)) and in deer maintained on constant photoperiods (male sika deer, Goss, 1977; male fallow deer, Newman *et al.*, 1990; pubertal red deer hinds, Loudon & Brinklow, 1990, 1992). This indicates that endogenous rhythms of seasonal activity may exist in deer. That the breeding season of hinds exposed to long days after the summer solstice in the present study was not delayed indefinitely suggests, firstly, that short days are not a necessary prerequisite for the initiation of the breeding season and, secondly, that the onset of ovarian cycles may represent expression of an endogenous annual rhythm, which can be entrained by decreasing photoperiod, but will eventually be expressed anyway. However this conclusion must be tempered by the consideration that other factors may have caused the onset of the breeding season in EPS hinds. For instance, social facilitation of ovarian cyclicity by the presence of sexually active stags and hinds has been suggested by several studies (Iason & Guinness, 1985; Moore & Cowie, 1986; McComb, 1987; Fisher *et al.*, 1988; Fisher & Fennessy, 1990) and in the absence of a stimulatory short photoperiodic

signal the EPS hinds may have initiated breeding activity in response to a non-photoperiodic cue such as the sexual activity of breeding deer herds in the vicinity.

Loudon and Brinklow (1992) suggested that, in the red deer hind, exposure to lengthening photoperiods in spring synchronises the onset of the autumnal breeding season. This is similar to work reported in the ewe by Malpaux *et al.* (1989). The onset of ovarian activity can be advanced by exposing non-pregnant hinds to long photoperiod earlier than normal after the winter solstice (Loudon & Brinklow, 1992). It could be argued that premature exposure to long days during winter did not alter the onset of reproductive activity in the present study because the hinds were pregnant. The effects of pregnancy on the ability of seasonally breeding animals to transduce photoperiodic information has never been addressed. It is possible that the reproductive axis is insensitive to the melatonin signal during gestation and therefore photoperiodic models developed using findings from non-mated females (Goodman, 1988; Woodfill *et al.*, 1991) may not be applicable to breeding animals such as the red deer hinds in the present study. However, as the melatonin secretion pattern, plasma prolactin concentrations and pelage shedding cycle responded appropriately to premature long daily photoperiods in winter, it is unlikely that pregnant hinds were completely insensitive to photoperiodic signals. It is possible that more than one portion of the photoperiodic cycle may be utilised for synchronising the circannual rhythm of reproductive activity in the hind (Wayne *et al.*, 1990). Under normal conditions most adult red deer hinds conceive each year and data in the current study suggests that, in the breeding hind, the decrease in photoperiod during autumn is an important factor influencing the onset of ovarian activity.

It appears unlikely that refractoriness to the short day signals plays a role in the cessation of breeding activity in hinds, since daily treatment with melatonin commenced prior to the end of the breeding season delayed the onset of anoestrum for over a year (Adam *et al.*, 1989a). In the ewe a similar procedure only extended ovarian cyclicity for 6 weeks (Nett *et al.*, 1982). However it is possible that in red deer, unlike in the case of sheep (Robinson & Karsch, 1984), exposure to the increasing daily photoperiods during spring is necessary for the termination of breeding activity.

Adam *et al.* (1989b) suggested that once red deer hinds become reproductively quiescent there may be an obligatory period of sexual inactivity before sensitivity to an inductive photoperiod is restored. This may explain why the latency of response to short day melatonin treatment decreases as the natural breeding period approaches (Fisher *et al.*, 1988, 1990; Loudon & Brinklow, 1992). It appears that melatonin treatment will not advance ovarian activity in pubertal hinds beyond a certain limit (Fisher *et al.*, 1988; Asher *et al.*, 1990), possibly because, like the pubertal lamb (Foster *et al.*, 1985; Foster, 1988), pubertal hinds require exposure to long photoperiods before they develop the inductive response to decreasing daylength (Asher *et al.*, 1990). The response to melatonin treatment in adult hinds is also complicated by pregnancy and lactation and a confounding effect of social facilitation between treated and control hinds and stags (Fennessy & Fisher, 1988; Fisher *et al.*, 1988). Earlier treatment with melatonin does not consistently result in earlier onset of breeding activity (Adam *et al.*, 1986, 1989a). Whether there is an absolute requirement for exposure to long photoperiods or an obligatory suppression of the reproductive axis before short photoperiods will induce reproductive activity has yet to be resolved.

As in most species studied (see Arendt, 1985), the pattern of plasma melatonin concentration in red deer was characterised by elevated plasma melatonin concentrations at night and little or no evidence of melatonin secretion during the day (Figure 4.4b). Artificial light suppressed melatonin secretion so that the nightly increase in melatonin concentration was delayed until after lights were turned off in the EPW and EPS group hinds (Figures 4.4a & 4.8). The duration of melatonin elevation was directly related to the length of the dark period showing that, as in sheep (Bittman *et al.*, 1983a; Arendt *et al.*, 1981), the secretory pattern of melatonin in red deer provides a suitable endocrine signal for measuring daylength.

In the control hinds, plasma prolactin concentrations were low during winter and increased gradually during spring which is similar to the annual pattern of prolactin secretion already described in red deer (Curlewis *et al.*, 1988b; Adam *et al.*, 1989b) and sheep (Webster and Haresign, 1983; Lincoln, 1990). Elevated plasma prolactin levels in the hinds exposed to long photoperiods during winter provide evidence that, as in other species, prolactin secretion in the red deer hind is sensitive to photoperiod. Inability to

blood sample hinds regularly during late gestation and for 12 weeks post-calving meant that it was not possible to determine exactly the pattern of prolactin secretion over this period. When blood sampling resumed in March plasma prolactin concentrations tended to be lower in the EPW hinds than in the control hinds but the effect was not statistically significant. However, there was no evidence that the decrease in plasma prolactin concentration was dramatically delayed in EPS hinds maintained on long photoperiods during autumn. In sheep it appears that the annual cycle of prolactin secretion may be endogenously generated as the prolactin rhythm persists in the absence of changing photoperiodic information (Howles *et al.*, 1982; Karsch *et al.*,1989; Jackson & Jansen, 1991). In the present study the autumnal decrease in prolactin secretion did not appear to be directly 'driven' by photoperiod as prolactin concentrations declined in the hinds denied exposure to the normal autumn decrease in daily photoperiod. In addition there was a suggestion that premature exposure to long photoperiods, which advanced the spring rise in prolactin, also advanced the subsequent autumn decline in prolactin levels.

In many mammals there is a temporal relationship between the seasonal cycle of prolactin secretion and the growth of pelage. In red deer moulting of the winter coat coincides with the spring rise in plasma prolactin concentrations (present study; Curlewis *et al.*, 1988b, Loudon *et al.*, 1989) and the summer moult is associated with a decline in prolactin concentrations during autumn (Webster & Barrell, 1985; Curlewis *et al.*,1988b; Loudon *et al.*, 1989). As premature exposure to long photoperiods in spring advanced moulting of the winter and subsequent summer coats, results of the present study appear to support the theory that the annual cycle of pelage shedding is endogenously generated (Loudon & Brinklow, 1990) and is capable of being entrained by increasing daily photoperiods during spring (Loudon & Brinklow, 1990). Manipulation of prolactin secretion in red deer hinds is associated with changes in the growth and moult of pelage (Curlewis *et al.*, 1988b; Milne *et al.*, 1990). Recent work has demonstrated that prolactin administered in winter (prior to the normal spring rise in prolactin) will advance the timing of the moult from winter to summer pelage in red deer stags (Suttie & Corson, 1991). These observations provide evidence that seasonal changes in prolactin secretion may be directly involved in control of the pelage shedding cycle.

That premature exposure to long photoperiods in winter had no effect on the reproductive axis was supported by the similar calving dates, plasma progesterone profiles and LH secretion patterns in the control and EPW hinds (Figure 4.7 & Table 4.1 A). Plasma progesterone profiles indicate that luteal activity commenced in early April and that most hinds conceived at the first ovulatory cycle. This is in agreement with the estimated mean conception date for control hinds of 2 April and for EPW hinds of 4 April, calculated using a gestation length of 233 d (Kelly & Moore, 1977). In contrast maintaining hinds on long days during autumn delayed the onset of luteal activity and the subsequent calving date by 2-3 weeks.

In seasonally breeding mammals the annual cycle of reproductive activity is influenced by marked changes in LH pulse frequency (ewe, Jackson & Davis, 1979; Père David's hind, Loudon *et al.*, 1990; Curlewis *et al.*, 1991). This is believed to be regulated in females by seasonal changes in the sensitivity of LH secretion to the negative feedback effects of oestradiol (Legan *et al.*, 1977; Karsch *et al.*, 1984). In the present study, plasma LH concentration was low during pregnancy and summer. The erratic increases in LH concentration during the transition to the breeding season in late March were similar to those described in red deer (Kelly *et al.*, 1985), roe deer (Schams *et al.*, 1980) and sheep (Walton *et al.*, 1977) and highlights the variability observed when pulsatile hormones, such as LH, are infrequently sampled. Seasonal changes in the endogenous pattern of LH secretion and in the secretion of LH in response to exogenous GnRH were demonstrated during the intensive blood sampling periods.

LH secretion reflects the pattern of GnRH release from the hypothalamus as well as the ability of the pituitary to release LH in response to a GnRH pulse. Reduced ability of the pituitary to secrete LH in response to a GnRH pulse has been reported during pregnancy and the early post-partum period in several species (sheep, Jenkins *et al.*, 1977; cattle, LaVoie *et al.*, 1981; Alam & Dobson, 1987; horse, Nett *et al.*; 1987). This may explain why basal LH concentrations and number of LH pulses in the hinds were low during mid-pregnancy (July 1986) and early post-partum in January 1987. The time from parturition to restoration of pituitary responsiveness (58-89 d) was much longer in the hind than the post-partum and/or lactational period of pituitary inadequacy described in the mare (7 d, Nett *et al.*, 1987) or the cow (10-35 d, Alam & Dobson, 1987) but was similar to that of

the ewe (35-63 d, Jenkin *et al.*, 1977). Alternatively the reduced LH response in January may also reflect a seasonal reduction in pituitary responsiveness, independent of post-partum or lactational anoestrus, similar that observed in Père David's deer during mid anoestrum (Curlewis *et al.*, 1991). The extent to which melatonin, photoperiod or progesterone/gonadotrophic treatments can advance the mating date of the breeding red deer hind may therefore be limited by the time required to restore the pituitary LH response in the post-partum/lactating hind. Similar to the ewe (I'Anson & Legan, 1988a), LH pulse frequency increases in red deer hinds prior to the first ovulation in the breeding season (EPW and NP in March and EPS in April). By February pituitary responsiveness to GnRH had been restored in EPW and control hinds yet LH secretion did not peak until March (Table 4.1). This indicates that GnRH release also limited LH secretion until that time.

Estimated conception dates and individual plasma progesterone profiles suggest that during the intensive sampling periods in April EPW and NP hinds were 3-4 weeks pregnant. LH pulsatility was reduced in the pregnant hinds while pituitary responsiveness was unchanged at this time, similar to the LH relationships reported in the ewe (Chamley *et al.*, 1974; Jenkins *et al.*, 1977) and mare (Nett *et al.*, 1987) during early pregnancy. In contrast, in April most of the EPS hinds were sampled during the transition to the breeding season and differences in the LH secretion pattern between control and EPS hinds may be due to differences in the uterine and ovarian state of these hinds.

The results of this study suggest that neither the development of photorefractoriness nor the timing of the spring increase in daily photoperiods initiated the seasonal transition from anoestrum to reproductive activity in the breeding red deer hind. Instead, decreasing daily photoperiods during autumn appeared to be the major determinant of the onset of ovarian activity. Long photoperiods may be involved in entraining the annual cycle of coat shedding and the seasonal pattern of prolactin secretion and it is possible that the seasonal cycle of reproductive activity is entrained through different neuroendocrine pathways from those which regulate other seasonal events in breeding red deer hinds.

Chapter 5

Seasonal physiology of reproduction in entire and ovariectomised pubertal red deer hinds and the negative feedback effects of oestradiol

5.1 Introduction

In female deer the onset of puberty (the transition to sexual maturity) is generally regarded as the age at the first recorded oestrus, ovulation or mating. In seasonally breeding animals the timing of puberty is influenced not only by a requirement to attain sufficient body size but also the transition to sexual activity is delayed until the appropriate photoperiodic signals are received (Foster, 1988). Generally farmed yearling hinds reach the threshold live weight for puberty (65-75 kg Kelly & Moore, 1977; Blaxter & Hamilton, 1980) during their second summer and become reproductively active during the following autumn at about 16 months of age (Kelly & Moore, 1977). Little is known of the hormonal changes associated with the first breeding season of red deer hinds. In ewes and pubertal lambs, seasonal changes in the sensitivity of tonic LH secretion to the negative feedback effects of oestradiol are believed to be a major factor in the control of the onset of reproductive activity (Karsch *et al.*, 1984; Foster, 1988). During anoestrus and before puberty, low concentrations of exogenous circulating oestradiol suppress LH secretion in ovariectomised animals (Legan *et al.*, 1977; Foster & Ryan, 1979, 1981). However, prior to the onset of the breeding season the response to oestradiol feedback inhibition decreases permitting reproductive activity to begin (Karsch *et al.*, 1984; Foster *et al.*, 1986).

In this study secretion of the reproductive hormones, LH and progesterone, was monitored in red deer hinds during their first breeding season. Changes in reproductive activity were related to seasonal changes in tonic LH secretion and the release of LH from the pituitary gland in response to exogenous GnRH. In addition, seasonal changes in the negative feedback effects of oestradiol on the LH secretion pattern and pituitary sensitivity were monitored in ovariectomised, pubertal red deer hinds and related to seasonal changes in the reproductive activity of the entire hinds.

5.2 Materials and methods

In December 1987, eight 13 month old red deer hinds (average live weight 77.8 ± 0.8 kg) were allocated to one of two treatment groups so that the groups were balanced for live weight. During the experiment the hinds were grazed together outdoors on pasture and weighed every 2-4 weeks.

On 22 December 1987, one group of hinds (n=4) was ovariectomised. Hinds were removed from pasture and denied access to food and water for 24 h prior to surgery. They were immobilised with 5.5 ml 2% xylazine hydrochloride ('Rompun', Bayer, Germany) injected i.m. and restrained in deer laparoscopy crates in a dorsally recumbent position, with the head downwards at a 45° angle and forelegs and hindlegs secured by straps. Hair was removed from the area anterior to the udder, the skin thoroughly swabbed with 30% v/v savlon solution (chlorohexidine digluconate, Savlon, ICI N.Z. Ltd, Wellington, N.Z.) and 2.5 ml of local anaesthetic (2% xylocaine hydrochloride, Astra Pharmaceuticals Pty Ltd, Australia) injected subdermally along the mid ventral line. A 10 cm longitudinal incision through skin, subcutaneous tissue and peritoneum was made along the mid abdominal line, 12 cm anterior to the udder. The uterus and ovarian structures were identified and the ovaries exteriorised. The ovarian arteries and veins were ligated (Coated vicryl, Size 3.5 metric, Ethicon Inc., New Jersey, U.S.A.) and both ovaries were removed. The peritoneal and skin incisions were sutured with sheathed multifilament nylon (Braunamid, Size 6 metric, B. Braun Melsungen A.G., West Germany) and the wound was dusted with terramycin powder (Pfizer Laboratories Ltd, Auckland, N.Z.). Immediately after surgery 6 ml of antibiotic (penicillin, Propen-S, Glaxo N.Z. Ltd, N.Z.) was administered subcutaneously and the hinds were allowed to recover from sedation in the shade before being released back to pasture.

A 'Compudose 200' controlled release implant (Elanco Product Co, NSW, Australia) was cut in half transversely (Plate 2) and soaked in sterile isotonic phosphate-buffered saline solution overnight. On 4 March 1988, an halved implant, containing 12 mg oestradiol-17β, was inserted into each ovariectomised hind. Prior to implant insertion, hinds were injected i.m. with 1.5 ml of 2% xylazine hydrochloride and a small area of skin (3cm x

5cm) on the back of the neck and 8 cm to the left of the midline was shaved. The shaven region was swabbed with 30% v/v savlon solution and locally anaesthetised with 1 ml of 2% xylocaine administered intradermallly. A 7 mm cut was made through the skin with a scalpel and blunt forceps were used to create a small pocket under the skin into which the implant was inserted. The wound was closed with sheathed multifilament nylon, dusted with terramycin powder and 6 ml of Propen-S administered subcutaneously. On 25 May 1988, hinds were sedated with 1.2 ml of 2% xylazine hydrochloride i.m. and the implants were located by palpation. The area was shaved, disinfected and anaesthetised and the implant removed through a small incision made parallel to the insertion scar. On 15 June 1988, a fresh 12 mg oestradiol implant was inserted into each ovariectomised hind using the procedures described above about 2 cm below the first incision site. These implants were removed on 19 September 1988.

Blood samples (7 ml) were collected into heparinised glass vacutainer tubes by jugular venepuncture at approximately fortnightly intervals from December 1987 to March 1988 and 2-3 times a week from April to October 1988 for progesterone, prolactin and LH analysis. On 7 occasions (15 December 1987, 29 February 1988, 15 March 1988, 24 April 1988, 14 June 1988, 29 June 1988 and 18 September 1988) the entire hinds were cannulated (see section 3.2) and blood samples collected at 20 minute intervals for 4 h to establish LH secretion patterns. Immediately following this intensive blood sampling, 2 μg GnRH (1 μg/ml in sterile isotonic phosphate-buffered saline solution, LH-RH/FSH-RH (amide form), NIAMDD, Lot 26-306 AL) was administered i.v. via the jugular cannula and blood samples were collected at 10, 30, 60, 90, 120, 150, 180, 210 and 240 minutes in order to measure the response of the pituitary to an exogenous GnRH challenge. Ovariectomised hinds were blood sampled following a similar protocol on the same occasions and also on 13 October 1988. On 29 February, 15 March and 18 September 1988 blood samples were collected from all hinds at 1-3 h intervals for 24 h and the plasma analysed for melatonin concentration.

A vasectomised stag was run with the hinds from January until September 1988. On 30 March the stag was sedated and fitted with a ram harness and crayon. Thereafter hinds were inspected 2-3 times a week for crayon marks until the harness was removed on

Plate 2. Photograph illustrating an oestradiol implant (Scale 3:2).

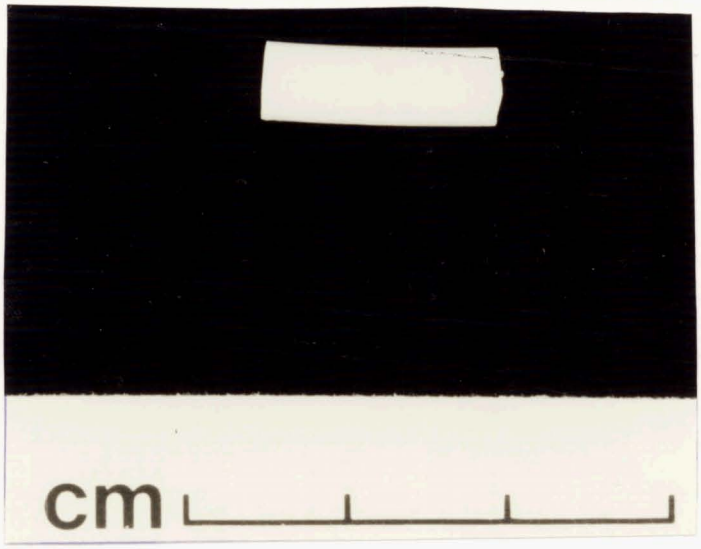


Table 5.1. Time periods (P1-P8) of ovariectomised hinds subdivided according to breeding activity in the entire hinds and oestradiol treatment and associated dates of intensive blood samplings (B1-B8).

	Period (dates)	Intensive Sampling Date	
P1	pre-ovariectomy (15 December 1987)	B1	15 December 1987
P2	anoestrum without oestradiol (11 January-29 February 1988)	B2	29 February 1988
P3	anoestrum with oestradiol (5 March-13 April 1988)	B3	15 March 1988
P4	breeding season with oestradiol (15 April-25 May 1988)	B4	24 April 1988
P5	breeding season without oestradiol (27 May-15 June 1988)	B5	14 June 1988
P6	breeding season with oestradiol (17 June-15 July 1988)	B6	29 June 1988
P7	anoestrum with oestradiol (19 July-19 September 1988)	B7	18 September 1988
P8	anoestrum without oestradiol (23 September-13 October 1988)	B8	3 October 1988

10 May. Although the crayon was scraped weekly and replaced when necessary, the tendency of the stag to wallow in mud greatly interfered with the ability of the crayon to function. Mating of one hind was observed on 4 May but as no crayon marking was detected the hind was not identified. A shoulder strap broke on 9 May and the harness was removed the following day.

During the experiment 2 hinds, both in the ovariectomised treatment group died; 1 from acute malignant catarrhal fever on 24 June 1988 and 1 from yersiniosis on 8 July 1988.

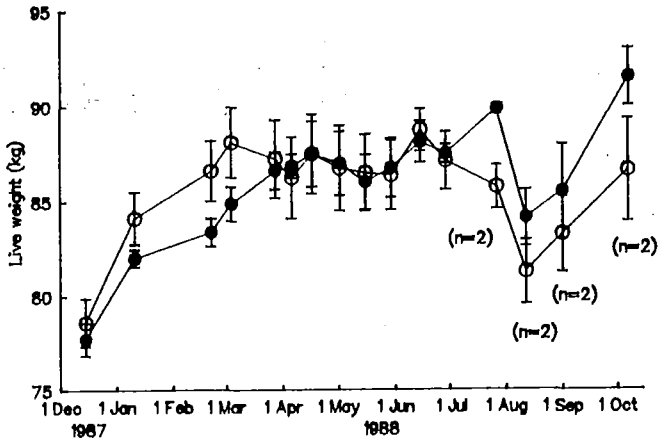
Live weight, prolactin, melatonin, progesterone and LH data were log-transformed (base 10) prior to statistical analysis. The LH profile from the weekly blood sampling of the ovariectomised hinds was subdivided into 8 time periods according to the breeding activity in the entire hinds and oestradiol treatment of the ovariectomised hinds (see Table 5.1) and the mean LH concentration of each hind for each time period was calculated. These mean concentrations and LH secretion parameters from the intensive bleeds were analysed using ANOVA followed by mean separation with Duncan's LSD when appropriate. The onset of the breeding season was defined as the date when luteal phase progesterone concentrations > 1 ng/ml were first recorded (15 April). The onset of seasonal anoestrus was defined as the date when plasma progesterone concentrations fell below 1 ng/ml following the last luteal phase of the breeding season (19 July). Alternative definitions of the onset (first sustained increase in progesterone i.e. > 1 ng/ml for more than 5 days, 26 April) or cessation (mean estimated date of the beginning of the last ovarian cycle i.e. 5 July) of breeding activity did not alter the relationships between mean LH concentrations and season. An LH increase during intensive blood sampling was defined as a pulse if the difference between the LH peak and the previous nadir (the pulse amplitude) was greater than 3 times the standard deviation of the nadir. Basal LH was the minimum plasma LH concentration and mean LH concentration was the average plasma LH concentration measured during the 4 h sampling period in each individual. The LH response to exogenous GnRH was the difference between the plasma LH concentrations immediately prior to (time 0) and 10 minutes after GnRH administration.

5.3 Results

Live weight

There was no significant effect of ovariectomy or oestradiol treatment on the live weight of hinds (Figure 5.1). Mean live weight increased from 77.8 (± 0.8) kg to 86.5 (± 1.2) kg between December 1987 and March 1988 and remained at between 84-88 kg during autumn and early winter. In early August there was a sharp decrease (6 kg) in mean live weight to about 82 kg. Live weight gradually increased in spring and at the end of the trial period, in early October 1988, hinds weighed 88.3 ± 2.0 kg.

Figure 5.1 Mean live weight (kg) of entire (●) and ovariectomised (○) red deer hinds from December 1987 to October 1988. Each point represents n=4 unless otherwise indicated. Vertical bars denote s.e.m.



LH profiles

Entire hinds

Mean plasma LH concentration in the entire hinds (Figure 5.2 a) was low (about 0.5 ng/ml) between December and late February but gradually increased in March to peak at 2.0 ± 0.5 ng/ml on 6 April. From April until the end of the trial in October, mean plasma LH levels ranged from 0.9 to 1.5 ng/ml. Throughout the trial period plasma LH concentration in individual hinds varied considerably, especially between March and August when occasional values of up to 3 ng/ml were recorded in some hinds (Figure 5.3).

Figure 5.2 Mean plasma LH concentrations of a) entire (n=4, ●) and b) ovariectomised (n=4, ○) red deer hinds. The horizontal block represents the mean breeding period of entire hinds (± s.e.m.) and the dashed lines the periods of oestradiol implantation. Ovx indicates the time of ovariectomy and the small arrows (B1-B8) the intensive blood sampling periods. Ψ indicates the death of an ovariectomised hind and after 8 July data from individual ovariectomised hinds are presented. Vertical bars denote s.e.m.

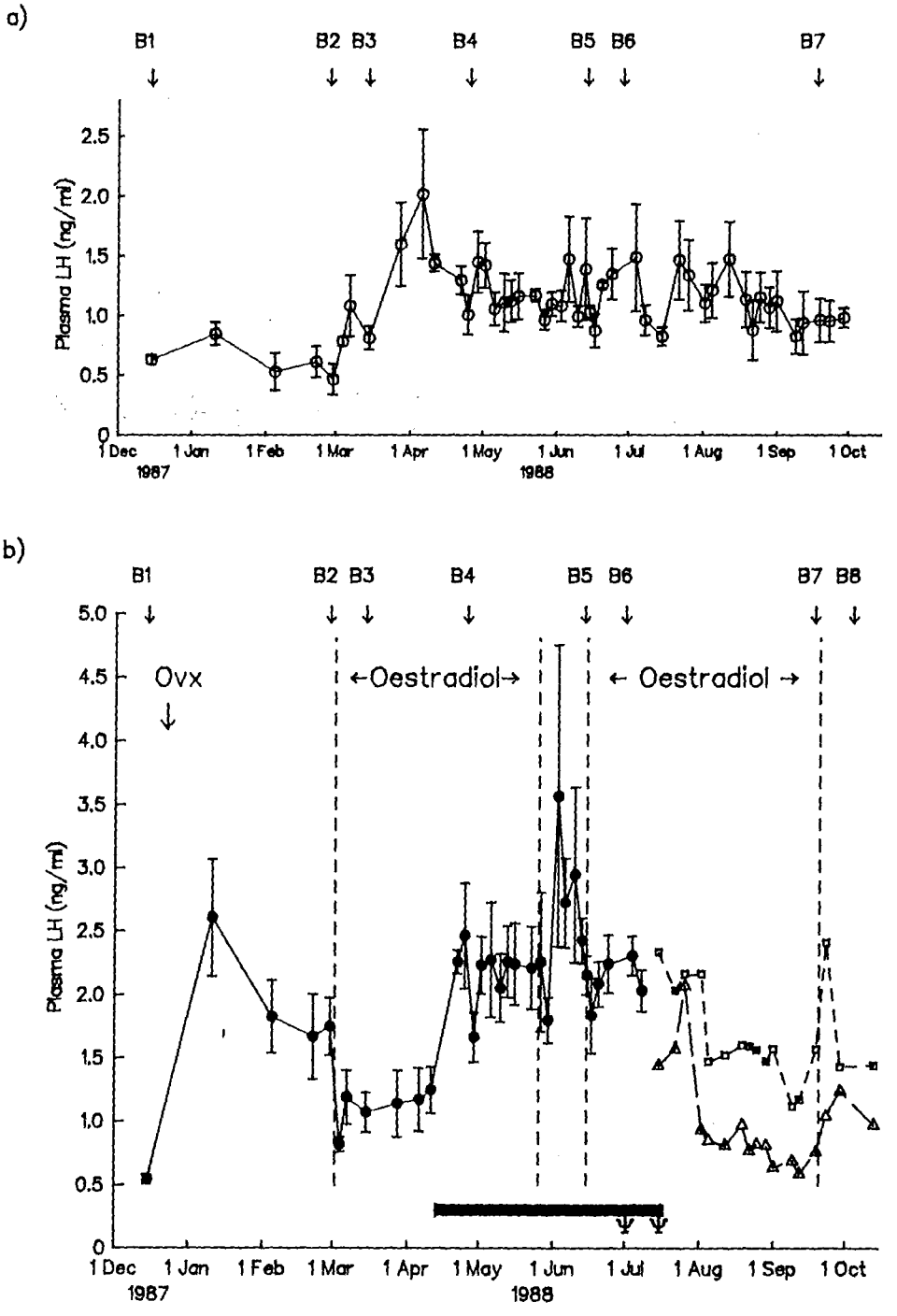


Figure 5.3. Individual plasma progesterone (●) and LH (○) profiles of entire deer hinds from December 1987 to October 1988.

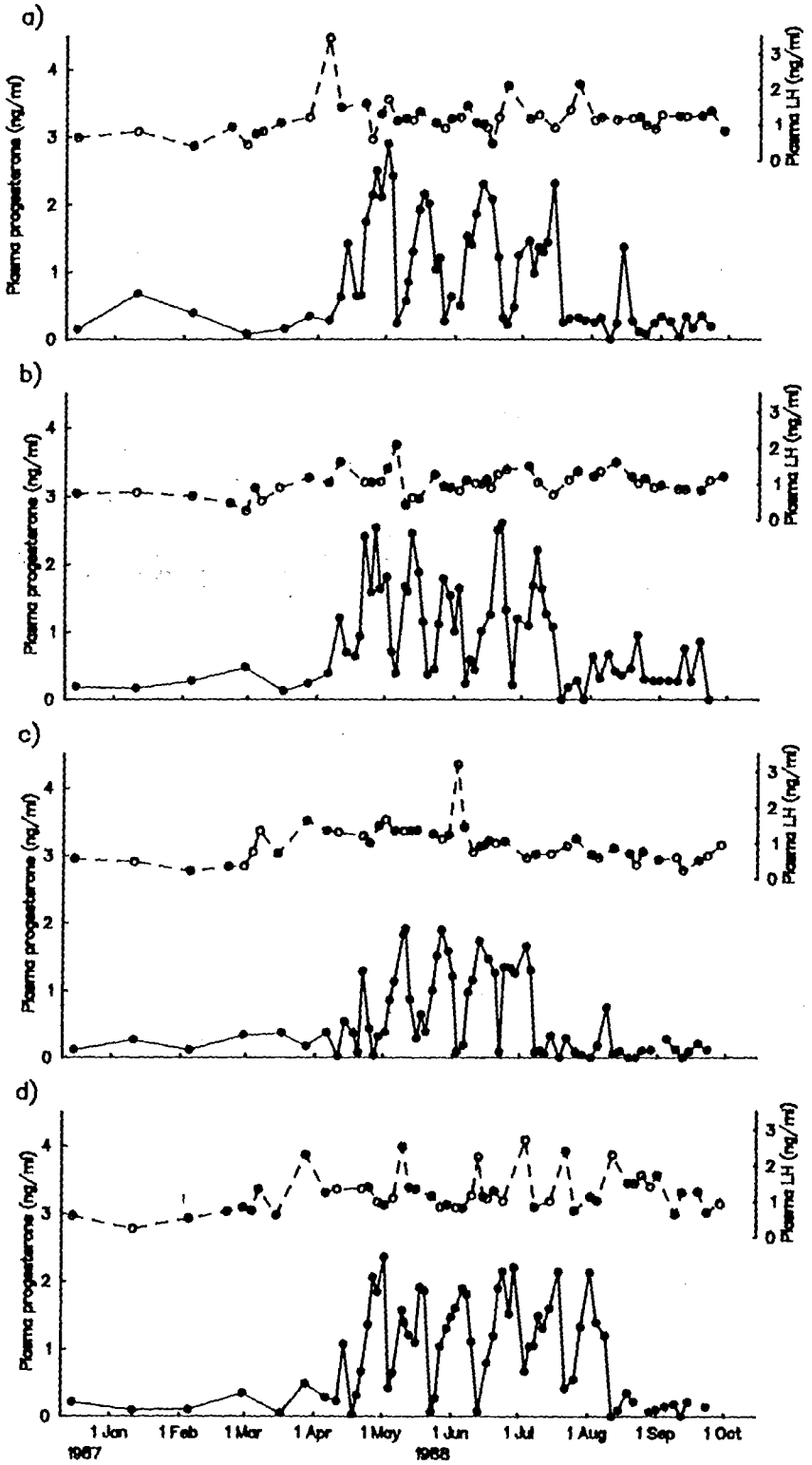


Table 5.2. Effect of oestradiol treatment on mean plasma LH concentration of ovariectomised red deer hinds in relation to oestradiol treatment and reproductive activity in intact hinds. Means assigned different letters are significantly different ($p<0.05$). Asterisks indicate data were not included in statistical analysis ($n \leq 2$).

	Period (dates)	no. of hinds n	Mean plasma LH (ng/ml) mean	s.e.m. [range]
P1)	pre-ovariectomy (15 December 1987)	4	**0.54**[a]	0.03
P2)	anoestrum without oestradiol (11 January-29 February 1988)	4	**1.96**[c]	0.18
P3)	anoestrum with oestradiol (5 March-13 April 1988)	4	**1.10**[b]	0.08
P4)	breeding season with oestradiol (15 April-25 May 1988)	4	**2.16**[c]	0.09
P5)	breeding season without oestradiol (27 May-15 June 1988)	4	**2.56**[c]	0.22
P6)	breeding season with oestradiol (17 June-15 July 1988)	3	**2.10**[c]	0.11
P7)	anoestrum with oestradiol (19 July-19 September 1988)	2	**1.17**[*]	[0.88, 1.41]
P8)	anoestrum without oestradiol (23 September-13 October 1988)	2	**1.42**[*]	[1.02, 1.92]

Ovariectomised hinds

Mean plasma LH concentrations of the ovariectomised hinds (Figure 5.2 b, Table 5.2) were low (about 0.5 ng/ml) on 20 December (P1) but increased after ovariectomy to about 2 ng/ml (P2). After the oestradiol implant was inserted in early March plasma LH concentrations decreased and remained at about 1 ng/ml until mid April (P3) when LH concentrations increased again. Mean plasma LH concentrations remained elevated at about 2 ng/ml from mid April until early June (P4) and although mean plasma LH concentrations peaked (3.5 ng/ml) in mid June after removal of the oestradiol implant, the LH secretion pattern varied between individual hinds and mean plasma LH concentration

did not change significantly after the removal (P5) or reinsertion (P6) of implants during the breeding season. In July mean plasma LH concentrations were about 2 ng/ml but tended to decrease in the surviving hinds in early August and remained at about 1 ng/ml (P7) even after the oestradiol implant was removed in mid September (P8).

LH pulsatility and response to GnRH

Mean plasma LH concentration, basal plasma LH concentration, LH pulse frequency and LH pulse amplitude during the initial 4 h of the intensive blood sampling period and LH increase in response to exogenous GnRH are presented in Tables 5.3 and 5.4 for entire and ovariectomised hinds, respectively.

Entire hinds

Mean plasma LH concentration and basal LH were significantly greater on 14 and 29 June (B5, B6) than in December (B1), February (B2) and March (B3) while values in April (B4) and September (B7) were intermediate (Table 5.3). It was not possible to define actual pulse frequency and amplitude as generally less than 2 LH pulses were detected during each intensive sampling bleed. The greatest number of LH pulses was detected on 14 June (B5) (2.0 ± 0.7 pulses/4 h); significantly more than in December (B1) or September (B7). Pulse amplitude was estimated for the intensive bleeds in April and June (B4, B5, B6) and was significantly lower on 24 April (B4) than on 29 June (B6). Mean LH and basal LH concentrations were positively correlated with pulse frequency (Spearman rank correlation coefficient $r=0.66$, $p < 0.01$ and $r=0.45$, $p < 0.01$, respectively). There was no relationship between LH pulse frequency and amplitude ($r=0.25$, $p > 0.05$).

Plasma LH concentrations always peaked 10 mins after the administration of GnRH and the magnitude of the increase did not change over time ($p>0.05$).

Table 5.3 LH secretion in young entire red deer hinds during intensive blood sampling periods and in response to GnRH challenge between December 1987 and September 1988. Values within columns with different letter superscripts are statistically different ($p<0.05$). Empty slots (-) indicate means were not estimated (< 1 pulse detected in > 2 hind).

	Date	n	Mean plasma LH (ng/ml) mean	s.e.m.	Basal LH (ng/ml) mean	s.e.m.	Pulse frequency (pulses/4 h) mean	s.e.m.	Pulse amplitude (ng/ml) mean	s.e.m.	LH response (ng/ml) mean	s.e.m.
B1)	15 December 1987	4	**0.63^{ab}**	0.04	**0.50^{ab}**	0.03	**0.25^{a}**	0.25	-	-	**2.28^{a}**	0.52
B2)	29 February 1988	4	**0.38^{a}**	0.08	**0.32^{a}**	0.04	**0.50^{ab}**	0.29	-	-	**3.97^{a}**	1.11
B3)	15 March 1988	4	**0.78^{abc}**	0.04	**0.46^{ab}**	0.04	**0.50^{ab}**	0.29	-	-	**2.60^{a}**	0.48
B4)	24 April 1988	4	**1.05^{bcd}**	0.10	**0.84^{bc}**	0.06	**1.25^{bc}**	0.48	**0.43^{a}**	0.02	**3.60^{a}**	0.58
B5)	14 June 1988	4	**1.85^{e}**	0.29	**1.22^{c}**	0.19	**2.00^{c}**	0.48	**0.82^{ab}**	0.17	**3.92^{a}**	0.41
B6)	29 June 1988	4	**1.69^{de}**	0.30	**1.30^{c}**	0.23	**1.25^{bc}**	0.25	**1.11^{b}**	0.11	**3.86^{a}**	0.55
B7)	18 September 1988	4	**1.27^{cde}**	0.30	**1.02^{bc}**	0.30	**0.25^{a}**	0.25	-	-	**3.55^{a}**	1.40

Ovariectomised hinds

In the ovariectomised hind each intensive blood sampling period (B1-B8) corresponded to one of the 8 time periods (P1-P8) described in Table 5.1. Mean LH and mean basal plasma LH concentrations recorded in the intensive blood sampling period prior to ovariectomy (B1) and when hinds were oestradiol-treated during anoestrus (B3) were significantly lower than when hinds were oestradiol-treated during the breeding season (B4, B6) and when oestradiol implants were absent, regardless of season (anoestrus (B2, B5). After the breeding season, mean LH and mean basal plasma LH concentrations in the surviving 2 hinds tended to be low regardless of oestradiol treatment (B7, B8).

No LH pulses were detected prior to ovariectomy (B1). The number of LH pulses was lower when oestradiol was present in anoestrus (B3) than when oestradiol was absent in the breeding (B2) and non-breeding season (B5), or when oestradiol was present during the breeding season (B4 & B6). Few pulses were detected after the breeding season ended and no pulses were detected in the remaining hinds after the second oestradiol implant was removed. There was no relationship between LH pulse frequency and pulse amplitude ($r=0.21$, $p > 0.05$). LH pulse amplitude tended to be lower when hinds were treated with oestradiol during anoestrum than during any other period.

Plasma LH concentrations always increased after the GnRH challenge. The magnitude of the LH response was greater on 24 April (B4) and 29 June (B6) although the increase was only significantly greater on 24 April.

Progesterone

Individual plasma progesterone profiles of the entire hinds are shown in Figure 5.3 and mean plasma progesterone concentrations of intact and ovariectomised hinds are shown in Figure 5.4.

Entire hinds

Mean plasma progesterone concentration of the entire hinds was low (about 0.5 ng/ml) until early April (Figure 5.4). In mid April (15 April ± 2.4 d) a short term increase in plasma progesterone concentration (> 1 ng/ml) was recorded in each hind and 9-14 d later (26 April ± 3.4 d) the first sustained increase (> 1 ng/ml for > 5 d) occurred. At the

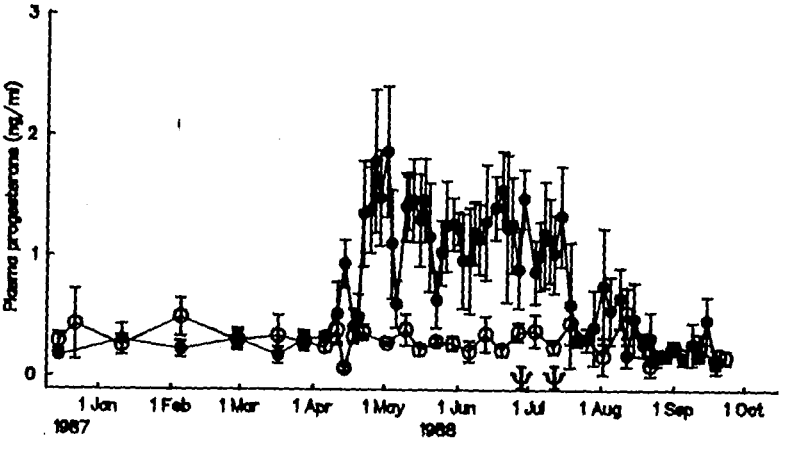
Table 5.4. LH secretion in young ovariectomised red deer hinds during intensive blood sampling periods and in response to GnRH challenge between December 1987 and September 1988. Hinds had oestradiol implants from 4 March to 25 May and from 15 June to 19 September 1988. Values within columns with different letter superscripts are statistically different ($p<0.05$). Empty slots indicate that means were not estimated as < 1 pulse was recorded in > 2 hinds. Asterisk (*) indicate data were not included in statistical analysis ($n \leq 2$).

Intensive Sampling date	n	Mean plasma LH (ng/ml) mean	s.e.m.	Basal LH (ng/ml) mean	s.e.m.	Pulse frequency (pulses/4 h) mean	s.e.m.	Pulse amplitude (ng/ml) mean	s.e.m.	LH response (ng/ml) mean	s.e.m.
B1) **pre-ovariectomy** 15 December 1987	4	**0.54^{a}**	0.03	**0.47^{a}**	0.03	-	-	-	-	**2.28^{a}**	0.53
B2) **anoestrum without oestradiol** 29 February 1988	4	**1.72^{b}**	0.27	**1.00^{abc}**	0.20	**3.25^{c}**	0.25	**1.21^{b}**	0.17	**3.13^{a}**	0.71
B3)**anoestrum with oestradiol** 15 March 1988	4	**0.80^{a}**	0.04	**0.57^{a}**	0.11	**1.25^{a}**	0.25	**0.45^{a}**	0.02	**2.71^{a}**	0.17
B4)**breeding season with oestradiol** 24 April 1988	4	**2.46^{bc}**	0.36	**1.35^{bc}**	0.12	**2.75^{bc}**	0.63	**1.11^{b}**	0.26	**6.35^{b}**	0.76
B5)**breeding season without oestradiol** 14 June 1988	4	**2.58^{c}**	0.48	**1.49^{c}**	0.21	**3.00^{c}**	0.71	**1.95^{b}**	0.72	**2.29^{b}**	0.37
B6)**breeding season with oestradiol** 29 June 1988	3	**2.17^{bc}**	0.12	**1.45^{bc}**	0.29	**2.33^{bc}**	0.58	**1.02^{ab}**	0.19	**5.53^{ab}**	1.28
B7)**anoestrum with oestradiol** 18 September 1988	2	**1.30***	0.04	**0.95***	0.05	**0.5***	0.5	-	-	**3.97***	3.35
B8)**anoestrum without oestradiol** 3 October 1988	2	**1.05***	0.07	**0.88***	0.05	**0.0***	0.0	-	-	**3.94***	1.95

beginning of each full-length luteal phase plasma progesterone concentration increased and peaked at 2.2 (± 0.1) ng/ml about 8 (± 1) d later. Progesterone concentrations subsequently decreased and remained low (< 1 ng/ml) for 4.5 ± 0.4 d before the luteal phase pattern of progesterone secretion was repeated. Individual progesterone profiles (Figure 5.3) suggest that 4-6 ovulatory cycles occurred in each hind. Excluding the short term increases in progesterone at the beginning of the breeding season, the mean ovulatory cycle length (time from the increase in progesterone concentration > 1 ng/ml until the beginning of the subsequent luteal phase) was 18.6 ± 0.9 d (n=19). The period of ovarian cyclicity (time from the beginning of the first luteal phase to the end of the last luteal phase) varied considerably from 44 to 107 d (mean 80 ± 13 d) with the last cycle ending in mid July (19 July ± 7 d).

Individual plasma progesterone profiles (Figure 5.4) indicate that in December (B1), February (B2) and March (B3) intensive blood sampling periods occurred before the onset of puberty. On 24 April (B4) 3 hinds and on 14 and 29 June (B5, B6) all 4 hinds were cycling. On 18 September (B7) all hinds were anoestrus. When hinds were cycling, individual plasma progesterone profiles indicate that the intensive blood sampling period always coincided with the luteal phase of oestrous cycle.

Figure 5.4 Mean plasma progesterone concentrations of entire (●) and ovariectomised (○) red deer hinds. Ψ indicates the death of an ovariectomised hind, otherwise each point represents n=4. Vertical bars denote s.e.m.



Ovariectomised hinds

Plasma progesterone concentrations were generally low (<0.5 ng/ml) throughout the trial period (Figure 5.4).

Melatonin

Profiles of mean plasma melatonin concentrations recorded for 25 h on 29 February, 15 March, and 18 September are shown in Figure 5.5. The pattern of melatonin secretion was similar in ovariectomised and entire hinds and melatonin concentration was influenced only by the time of blood sampling. Plasma melatonin concentrations were low and often undetectable during daylight hours but increased within 1-2 h after the onset of darkness. Mean plasma melatonin concentrations remained elevated at about 0.2-0.6 nmol/l during the dark phase and decreased to low levels around sunrise. The maximum plasma melatonin concentration (0.68 ± 0.11 nmol/l) and the timing (0100 ± 0.6 h) of the melatonin peak was not affected by treatment or date of the blood sampling ($p < 0.05$).

Prolactin

Mean plasma prolactin concentrations are shown in Figure 5.6. Plasma prolactin concentrations were similar in the entire and ovariectomised hinds but changed significantly with time. Mean plasma prolactin concentration was 60-100 ng/ml during summer and gradually decreased in late autumn. Levels were generally low during winter (about 20-40 ng/ml) but increased again in September.

Reproductive performance of other hinds.

Hinds from the same age cohort but not involved in the intensive study were run with an entire red deer stag from 1 March and most (25/28, 89%) calved (as 2-year-olds) during the following December (mean calving date, 16 December ± 3.6 d). The calving percentage of untreated mature hinds (> 2 years old) on the same property was also high (45/49, 92%) but the mean calving date (23 November ± 1.5 d) was earlier ($p<0.001$). Estimated mean conception dates of these 2-year-old and adult hinds were 27 April and 4 April, respectively.

Figure 5.5 Mean plasma melatonin concentration of entire (●) and ovariectomised (○) red deer hinds during a 25 h period on a) 29 February, b) 15 March and c) 18 September 1988. The horizontal block represents the period from sunset to sunrise. Each point represents n=4 except for the ovariectomised hinds in c) where n=2. Vertical bars denote s.e.m..

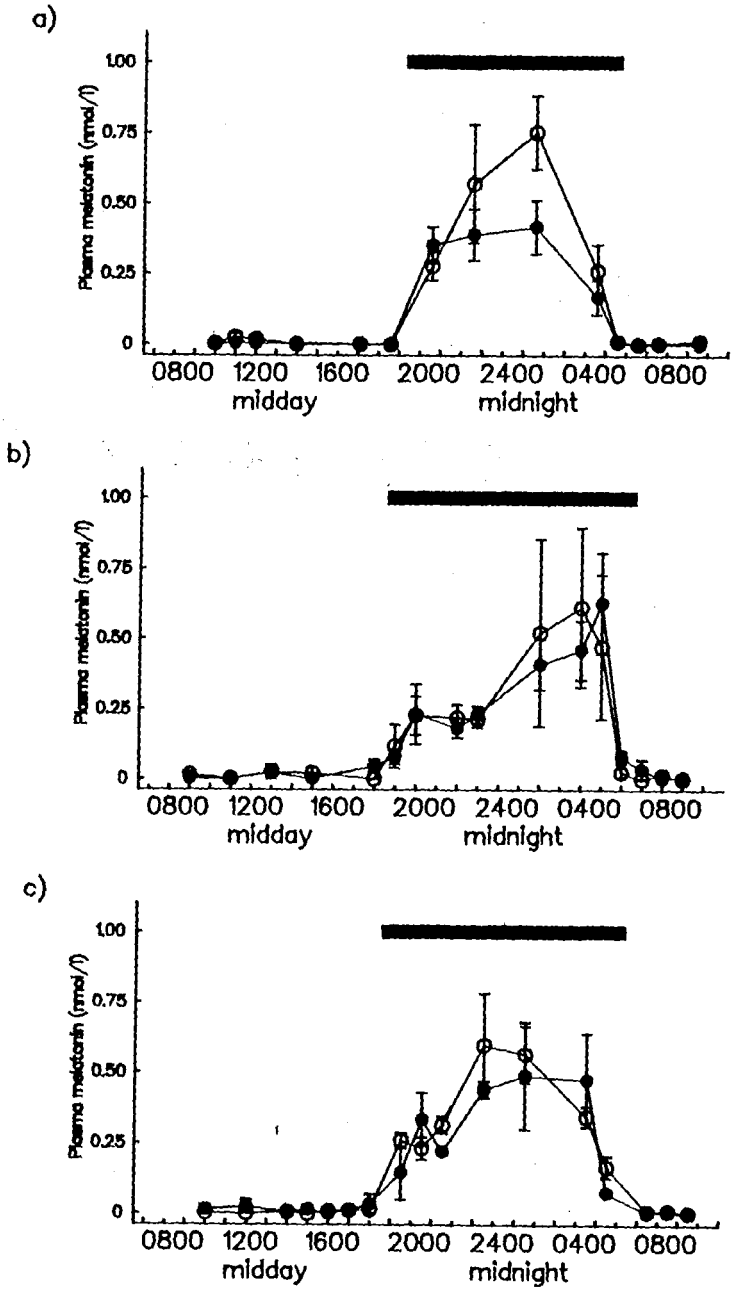
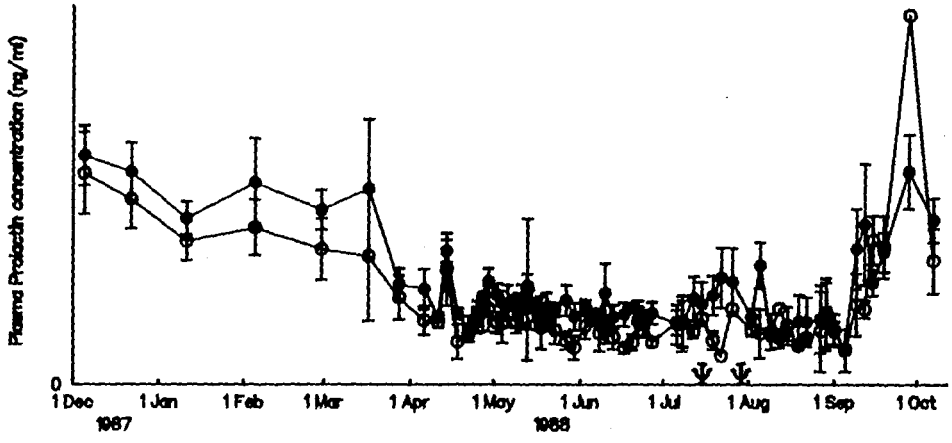


Figure 5.6 Mean plasma prolactin concentrations of entire (●) and ovariectomised (○) red deer hinds. Ψ indicates the death of an ovariectomised hind, otherwise each point represents n=4. Vertical bars denote s.e.m.



5.4 Discussion

Plasma progesterone profiles indicate that in the yearling red deer hinds ovarian cycles began in late April and ceased between mid June and August. The duration of the breeding season therefore appears to be shorter in the pubertal hind (about 3 months) than in the adult. Unmated mature hinds cycle for about 5 months with ovarian cycles beginning in April (October, Northern Hemisphere) and continuing through until September (March, Northern Hemisphere) each year (Curlewis *et al.*, 1988b; Loudon *et al.*, 1989; Adam *et al.*, 1989a; Meikle *et al.*, 1991). One factor contributing to the shorter duration of breeding period in yearling hinds would be the later onset of oestrous activity in young hinds (present study; Loudon *et al.*, 1989). This tendency was reflected in the calving data from the Lincoln Deer Unit where older hinds calved 3 weeks earlier than the mated 2-year-old hinds. A tendency for older hinds to calve earlier (McManus & Hamilton, 1991) was not evident in all studies (Guinness *et al.*, 1971, 1978) and when live weight was taken into account in one study the perceived association between late calving and young hinds disappeared (Hamilton & Blaxter, 1980). It is therefore possible that the association between calving date and age in farmed New Zealand deer (Lincoln University calving records; Bray & Kelly, 1979) is mainly a reflection of the lighter weight of young hinds. In other seasonally breeding animal, such as fallow deer (Asher,

1986) and sheep (Hafez, 1952; Dyrmundsson, 1973, 1978), the breeding season of pubertal animal also tends to be shorter than that of the adult.

In addition to the late onset of oestrous activity, the early onset of seasonal anoestrus also shortened the duration of the breeding period in the yearling hinds. Data from Guinness *et al.* (1971) showed that the mating season of captive yearling red deer hinds in Scotland was only about 3 months long. Oestrous behaviour ceased in most pubertal hinds during December (July, Southern Hemisphere) and, as in the present study, the date of the last cycle varied greatly between hinds (17 May-20 September, Southern Hemisphere). Early onset of seasonal anoestrus in young animals has also been reported in the ewe lamb (Hafez, 1952; Foster & Ryan, 1981). The shorter breeding period decreases the opportunities for seasonally breeding animals to conceive in their first season. If the onset of breeding activity is delayed, due to poor nutrition or to a late date of birth, the young female may remain anovulatory until the following breeding season. This situation is unlikely to arise in well-grown red deer yearlings on New Zealand farms but may explain the impaired reproductive performance of young red deer hinds in the wild (Clutton-Brock *et al.*, 1982).

Hinds in the present study gained weight during summer and early autumn, exceeding the threshold live weight for puberty of 65-70 kg (Kelly & Moore, 1977; Hamilton & Blaxter, 1980; Fisher & Fennessy, 1985) throughout the trial period. A large decrease in live weight (-6 kg, Figure 5.1) was experienced in late winter when feed reserves ran short and the feed requirements of other animals on the Deer Unit had highest priority. It is unlikely that this loss of live weight influenced the cessation of reproductive activity in this trial as 3 of the entire hinds had ceased to cycle before the onset of the feed shortage.

Prior to the onset of puberty plasma progesterone concentrations were low in the red deer hinds. This is in contrast with pubertal fallow does where progesterone concentrations are elevated between 10-15 months of age and decline to low levels immediately before the onset of first oestrus (Asher, 1986a). Puberty in the yearling red deer hinds observed here was characterised by a short-lived increase in progesterone followed by 4-6 full-length ovarian cycles, lasting approximately 19 days each. The progesterone profile of each cycle was typical of the progesterone pattern observed in adult hinds during the

breeding season (Adam *et al.*, 1989a; Jopson *et al.*, 1990). In lambs the first LH surge marking the pubertal transition to breeding activity is also associated with a short term rise in circulating progesterone concentrations which is followed by a second LH surge and a normal luteal-phase increase in progesterone (Foster & Ryan, 1979; Foster *et al.*, 1986). In most lambs these early ovarian cycles are 'silent' and not accompanied by oestrous behaviour (Foote *et al.*, 1970). It is purported that the progesterone rise prior to first oestrus ensures that oestrous behaviour is expressed (Foote *et al.*, 1970) and that the corpus luteum functions normally (Berardinelli *et al.*, 1980; Pearce *et al.*, 1985) and it is believed to synchronise oestrus and the LH surge so that conception occurs (McLeod & Haresign, 1984; Legan *et al.*, 1985a). A limitation of the present study was the inability to determine when oestrous behaviour was expressed. Evidence from pubertal red deer hinds in other studies suggests that although progesterone concentrations increase in some hinds before the first oestrus (Webster & Barrell, 1985) a full-length luteal phase is not necessarily a prerequisite for the expression of oestrous behaviour and conception (Asher, 1990). In this respect endocrine changes around puberty appear to be similar to those accompanying the onset of the breeding season in adult hinds (Jopson *et al.*, 1990).

Since the increase in plasma LH concentration associated with ovulation is of short duration (< 24 h, Chapter 7), the relatively long (2 to 3 d) sampling interval used in this study would not reliably detect the preovulatory LH rise. Despite this limitation, peaks in LH preceeding a luteal phase increase in plasma progesterone concentration were detected on several occasions during the breeding period. Transient peaks of LH also have been detected in plasma samples collected daily at or around the time of oestrus in red (Kelly *et al.*, 1985), roe (Schams *et al.*, 1980) and white-tailed deer (Plotka *et al.*, 1980), in female sheep (I'Anson, 1983) and in cattle before puberty (Gonzales-Padilla *et al.*, 1975). Mean plasma LH concentrations of the occasional blood samples increased at the onset of the ovarian cyclicity and the pulsatility of LH secretion differed markedly between the breeding and nonbreeding seasons. Progesterone profiles indicate that all hinds in April and June were intensively blood sampled during the luteal phase of the oestrous cycle. All other intensive blood samplings occurred whilst hinds were acyclic. As in the ewe lamb (Foster *et al.*, 1975; Huffman *et al.*, 1987), LH pulse frequency was low (< 1 pulse/4 h) in the hinds before puberty and relatively high (about 1-2 pulses/4 h) during the breeding period. The increase in LH pulsatility at the beginning of the

breeding season presumably plays a central role in the initiation of ovarian cyclicity in the pubertal hind as it does in the lactating adult red deer hind (Chapter 4) and in the pubertal and adult ewe (Foster, 1988; Goodman, 1988).

In contrast to the adult Père David's hind (Curlewis *et al.*, 1991) and the lactating red deer hind (Chapter 4), the LH response to exogenous GnRH in young red deer hinds was not reduced during the anovulatory period (Table 5.3). Thus it appears unlikely that low LH pulse frequency before puberty was due to failure of the pituitary to respond to endogenous GnRH. In fact, most of the mechanisms required for the initiation of reproductive activity appear to be functional in the prepubertal hind. Administration of exogenous gonadotrophin (e.g. PMSG) or GnRH to progesterone-primed yearling hinds has resulted in conceptions prior to the normal onset of the breeding season (Fisher *et al.*, 1986). This indicates that not only was the ovary of the young red deer hind capable of responding to gonadotrophic stimulation in an adult-like manner, but also that prepubertal hinds treated with GnRH were able to generate the LH surge and oestradiol rise required for the induction of ovulation and oestrous behaviour. In the present study, removal of the ovaries resulted in a dramatic increase in LH pulse frequency and mean circulating concentrations of LH (Table 5.3). As in the ewe lamb (Foster *et al.*, 1986), the hypothalamic-pituitary axis of the young hind was able to generate high-frequency LH pulses well before puberty but LH secretion was suppressed, probably by an input from the ovary.

Current hypotheses (Foster, 1988) suggest that concentrations of circulating gonadotrophins are low in immature lambs because the system governing LH secretion, the GnRH pulse generator, is very sensitive to the inhibitory feedback action of ovarian steroids such as oestradiol. This sensitivity to oestradiol decreases as the time of puberty approaches (Ebling *et al.*, 1990), thereby allowing LH secretion to increase and ovarian cyclicity to commence. In the present study, the presence of oestradiol suppressed LH pulse frequency in hinds before the typical age of puberty (B3) but the same oestradiol treatment was ineffective in this regard during the normal breeding period (B4 & B6). In addition, circulating LH concentrations increased in the oestradiol-treated ovariectomised hinds at the same time as ovulations were beginning in the entire females (Figure 5.2) suggesting that the decrease in sensitivity to negative feedback by oestradiol was

temporally related to the expression of puberty in the intact hinds. As in the female sheep (Foster, 1988; Ebling *et al.*, 1990), it appears that a decrease in oestradiol inhibition at puberty results in the increase in pulsatile LH secretion which occurs during sexual maturation of the red deer hind.

The present study, and recent work by Meikle *et al.* (1991) in adult hinds, suggest that not only was the sensitivity of LH secretion to inhibition by oestradiol reduced during the breeding period, but that the presence of oestradiol at that time also increased the pituitary LH response to a GnRH pulse (Table 5.4). Interestingly, this increase in the pituitary responsiveness associated with oestradiol treatment in the ovariectomised hinds did not result in an increase in mean LH concentrations (Table 5.4, Figure 5.3). In fact LH concentrations tended to be higher (markedly so in the data of Meikle *et al.*, 1991) when oestradiol was absent during the breeding season. These seemingly conflicting results may be due to oestradiol acting on **both** the pituitary and the GnRH pulse generator. In sheep, LH secretion reflects the frequency of GnRH released from the hypothalamus (Moenter *et al.*, 1990; Barrell *et al.*, 1992). The same is probably true in the the deer as administration of exogenous GnRH will induce an LH pulse (present study; Suttie *et al.*, 1984; Manley *et al.*, 1989). The increase in pituitary responsiveness in the oestradiol-treated ovariectomised hind may have been offset by a reduction in the number or size of GnRH pulses secreted by the hypothalamus. It should also be noted, that although a seasonal increase in pituitary LH responsiveness was detected in oestradiol-treated ovariectomised hinds, in the intact hinds sensitivity to GnRH was constant. However as the intensive blood samplings coincided with the luteal phase of the oestrous cycle, it is possible that, as in sheep (Jackson, 1975; Nett *et al.*, 1984; Kaynard *et al.*, 1988), oestradiol does enhance pituitary sensitivity to GnRH in the red deer hind during the follicular phase when plasma progesterone concentrations are low.

Another factor contributing to changes in reproductive activity in the hind may be the seasonal modulation of LH secretion by a pathway independent of oestradiol inhibition. In untreated ovariectomised ewes a small, but significant, reduction in LH pulse frequency during the anoestrous period was associated with an increase in pulse amplitude so that mean LH concentrations did not change (Goodman *et al.*, 1982; Montgomery *et al.*, 1985). In the red deer hinds however the steroid-independent

changes in LH secretion appear to be more dramatic. In adult ovariectomised hinds plasma LH concentrations decreased during October and remained at extremely low levels until mid December (Meikle *et al.*, 1991). This steroid-independent reduction in LH secretion during early anoestrus was associated with a significant decrease in pituitary LH responsiveness (Meikle *et al.*, 1991). Although LH secretion in untreated ovariectomised yearling hinds in the present study was similar in mid anoestrus (February) and the breeding season (July), plasma LH pulsatility and mean plasma LH concentrations tended to be lower during early anoestrus in October. It should be noted that this steroid-independent reduction in LH secretion was based on data from only 2 hinds but it may be sufficient to indicate that steroid-dependent and steroid-independent mechanisms both contribute to the reduction in LH secretion associated with the period of ovulatory inactivity in the hind.

Plasma progesterone profiles indicate that ovarian cyclicity ended in July and August in the entire hinds. In 2 hinds a transient increase in LH was recorded after the last full-length luteal phase and in one of these hinds, the LH increase was followed by a short term increase in plasma progesterone concentration. It can be argued that, because circulating LH concentrations tended to decrease in the surviving oestradiol-treated ovariectomised hinds at about the same time as ovarian cycles ceased in the entire hinds, the breeding season of intact hinds was terminated by an increase in the sensitivity of LH secretion to oestradiol negative feedback. However, because this reasoning is based on data from only 2 individuals and the exact time course of the steroid-independent changes in LH secretion were not studied in this trial, this conclusion is only tentative.

The timing of puberty in seasonally breeding animals is markedly influenced by photoperiod via the secretion of melatonin from the pineal gland during the hours of darkness. Plasma melatonin profiles in the pubertal hinds in the present study indicate that, as in the adult hind (Chapter 4), melatonin secretion was responsive to the light/dark cycle. Similarly the seasonal pattern of plasma prolactin concentrations observed during in the present trial was typical of prolactin profiles previously described in adult red deer (Chapter 4; Barrell *et al.*, 1985; Curlewis *et al.*, 1988b; Loudon *et al.*, 1989) and there was no evidence that secretion of melatonin or prolactin was influenced by ovariectomy or the presence of an oestradiol implant. Prolactin secretion, which is highly responsive

to changes in photoperiod and melatonin levels in red deer (Webster & Barrell, 1985; Adam *et al.*, 1989b), has been implicated in the control of seasonal reproductive activity. Although elevated plasma prolactin concentrations were associated with the termination of the breeding activity in red deer hinds (Curlewis *et al.*, 1988b), in the present study ovarian cyclicity ceased while prolactin levels were low and a causal relationship between high prolactin concentration and the onset of seasonal anoestrum appears unlikely.

A limitation of the present study was the inability to describe the pattern of circulating oestradiol in the ovariectomised hinds after the insertion of the oestradiol implants. It is possible to speculate that the rise in mean plasma LH concentrations in the ovariectomised hinds in mid April or alternatively the decline in LH secretion during August were due to exhaustion of the oestradiol implants (about 45 d after implantation). The manufacturers claim that the compudose implants release small amounts of oestradiol for over 200 d in cattle (Elanco product information sheet) and the 2 implants in this study were in place for 82 and 96 d, respectively. If exhaustion of the oestradiol implant was the only cause of the April rise in plasma LH concentrations then reimplantation with a fresh implant in July might be expected to suppress subsequent LH secretion. This did not happen. Insertion of the second oestradiol implant did not significantly alter the LH secretion pattern and oestradiol enhancement of LH responsiveness, evident during the breeding period when implants had been in place 14 d (2nd implant), was similar to that after 51 d (1st implant). These observations indicate that the implants were releasing oestradiol continuously during the study and that there were seasonal changes in the sensitivity of LH secretion due to oestradiol negative feedback.

The results of the present study indicate that hormonal changes associated with onset of reproductive activity in yearling red deer hinds are very similar to those recorded during the breeding season of adult red deer hinds and in pubertal and mature female sheep. Puberty in the red deer hind was associated with a reduction in the sensitivity of the GnRH pulse generator to oestradiol inhibition. The resulting increase in the frequency of LH pulses and in mean LH concentrations initiated ovulatory activity and 4-6 oestrous cycles of about 19 d duration were observed in each hind during the breeding season.

The early onset of seasonal anoestrus was associated with an increase in the sensitivity of the LH pulse generator system to oestradiol negative feedback and possibly with a steroid-independent reduction in LH concentration in ovariectomised hinds. These changes were manifested as a reduction in LH pulse frequency and the cessation of ovarian cyclicity.

Chapter 6

Effect of melatonin immunisation on the seasonal live weight, antler and reproductive cycles of red deer

6.1 Introduction

In red deer seasonal changes in breeding activity, antler development, pelage, feed intake and growth are regulated by changes in daily photoperiod (Pollock, 1975; Kay & Staines 1981; Webster & Barrell, 1985; Suttie & Simpson, 1985). In sheep and deer the length of darkness determines the duration of the nocturnal secretion of melatonin from the pineal gland (Lincoln *et al.*, 1981; Bittman *et al.*, 1983a; Kennaway *et al.*, 1983; Kennaway, 1984; Chapter 4) and thus changes in daily photoperiod entrain the timing of seasonal events via changes in the daily pattern of melatonin secretion (Arendt, 1985). When the night time rise in melatonin is abolished, by the removal of the pineal gland or disruption of its innervation (by removal of the cranial cervical ganglia) (Arendt *et al.*, 1980; Lincoln *et al.*, 1981; Bittman *et al.*, 1983b), adult sheep remain seasonal but do not respond appropriately to artificially manipulated photoperiods and in the long term they gradually lose synchrony with entire animals (Lincoln 1979; Barrell & Lapwood 1979; Lincoln *et al.*, 1989; Woodfill *et al.*, 1991). However, if pinealectomy or ganglionectomy is performed on young animals the onset of breeding activity is markedly delayed, presumably because their perception of photoperiodic information is inadequate to entrain the endogenous cycle of reproductive activity (Kennaway *et al.*, 1985; Foster *et al.*, 1986). Disruption of the daily pattern of melatonin secretion by active immunisation against melatonin has been attempted in mature rams (Lincoln & Almeida, 1981) and ewes (Arendt, 1986) and did not have any effect on reproductive activity. However, the consequences of such immunisation at an early age, possibly before the annual cycles have become established, are not known.

This study investigated the effect of active immunisation of young red deer against melatonin on live-weight gain and seasonal antler, pelage and reproductive changes.

6.2 Materials and methods

Forty red deer calves (20 of each sex) born during November and December 1985 (mean date of birth, 26 November 1985 ± 9.9 d) were randomly allocated to two treatment groups so that the groups were balanced for sex. Throughout the trial calves were grazed on ryegrass/white clover pasture. They were weaned from their dams on 5 May 1986 and separated by sex into two mobs in February 1987. Within the first two days of birth, calves were injected (subcutaneously in the frontal axilla) either with melatonin conjugate (melatonin conjugated to rabbit gamma globulin, prepared by T. Stelmasiak and S. van Mourik) plus adjuvant mixture (10 of each sex, Immunised), or with adjuvant mixture only (10 of each sex, Controls). For Immunised animals the melatonin conjugate was suspended in sterile 0.9% saline solution before being mixed with adjuvant. All calves received a booster immunisation on 20 February and at approximately 6 month intervals until February 1988 and on 4 May 1988 (Table 6.1).

Plasma samples were collected from all calves on 27 February and 23 May 1986, 17 March 1987 and 23 February 1988 for estimation of melatonin binding activity in the plasma. Duplicate 100 μl aliquots of plasma were incubated (either undiluted or diluted 1:100 in tricine buffer) at 10° C with 60 pg (2500 cpm) of tritiated melatonin ([^{3}H] melatonin, specific activity = 38.6 Ci/mmol, New England Nuclear, Boston). After 24 h bound and unbound melatonin were separated by centrifugation with dextran coated charcoal and the bound melatonin tracer was counted. Non-specific binding (NSB) of the tracer was estimated using 100 μl of sheep plasma and this varied from 1.6 to 2.9% of the total radioactivity.

Live weights were recorded for all animals on 21 February, 20 March, 5 and 28 April, 23 May, 19 August, 22 September, 6 November, 2 December, 1986, 5 March, 29 July, 1987 and 24 February, 1988. Stags only were weighed on 4 April, 27 May and 16 August 1988 while hinds were weighed on 14 March and 27 June 1988.

On 2 December 1986 the degree of moulting of the winter coat was recorded in all animals.

Table 6.1 Melatonin immunisation schedule for red deer calves.

Date	Total injection volume (ml/dose)	Melatonin conjugate * (mg/dose)	Immunisation mixture
At birth November/December 1985	2.0	1	25 mg conjugate * 22 ml saline solution 22 ml sorbitan triolate ϕ 6 ml Sontex π
20 February 1986	1.0	1	25 mg conjugate * 11 ml saline 11 ml sorbitan triolate ϕ 3 ml Sontex π
16 June 1986	0.5	1	25 mg conjugate * 12 ml saline 12 ml Freund's incomplete adjuvant ¶ 500 ug muramyl dipeptide §
5 March 1987 29 July 1987 23 February 1988 4 May 1988	2.0	1	25 mg conjugate * 25 ml saline solution 25 ml Freund's incomplete adjuvant ¶

* Immunised animals only.

ϕ polyoxythene 20 sorbitan triolate : sorbitan triolate (ratio 1:1); Sigma Chemical Co., St Louis, Missouri.

π Sontex, Marathon Marco Co., Texas.

¶ Freund's incomplete adjuvant, Gibco Laboratories, Grand Island, New York.

§ N-acetyl muramyl-L-alanyl-D-isoglutamine-6-O-steroyl, Behring Diagnostics, La Jolla, California.

Male calves were checked on 21 February, 16 June, 19 August, 22 September, 6 November, 2 December and 23 December 1986 for pedicle and antler development. The presence of a pedicle was recorded when the bony protrusion on the frontal bone of the skull was greater than 10 mm in height. The length of the pedicle and antler was measured from the skull (on the medial aspect) to the tip. In 1987 and 1988 stags were observed weekly between 15 September and 15 November and the date of antler casting recorded.

Hinds were run in a single mob with a red deer stag between March and May in 1987 and 1988 and calving dates were recorded in both years.

In March 1986 an immunised hind calf died after an accident and a control hind died from malignant catarrhal fever in January 1987. On 15 March 1988, a control hind broke a leg in an accident and died. In March 1988, 2 Control stags, 2 Immunised stags and 2 Immunised hinds were removed from the study.

Effects of treatment on live weight were examined using analysis of covariance followed by Duncan's LSD test when appropriate. Date of birth was used as the covariate. Prior to analysis live weights were transformed to their logarithms (base 10). Data were also subjected to repeated measures analysis using a profile transformation. Animals with missing data points were excluded from repeated measures analysis so that data from 31 animals (16 stags and 15 hinds) were considered.

6.3 Results

Melatonin binding activity of plasma

Plasma from 11 of the 19 Immunised calves sampled on 27 February 1986 bound significant amounts of the melatonin tracer (NSB 1.6%). Binding ranged between 5 and 26%. On 23 May 1986 plasma from 15 of the 19 Immunised calves bound between 7 and 33% of the tracer added (NSB 2.8%). On 17 March 1987 and 23 February 1988 plasma from the same 15 calves bound between 5-24% (NSB 1.8%) and 7-18% (NSB 2.1%) of tracer, respectively. In all cases a 1:100 dilution of the sample reduced binding to within the range of the NSB. Plasma from 4 of the Immunised animals (2 male and 2 female)

did not bind melatonin at any time and these animals are subsequently described as non responders. There was no detectable binding of melatonin in plasma samples from Control animals.

Live weight

Initially treatment and sex of the calf had no effect on live weight. However, by 20 March 1986 stag calves weighed significantly more than hinds (mean ± SEM; males 50.7 ± 1.1 *vs* females 48.7 ± 1.0 kg, $p<0.05$) and remained heavier throughout the trial. Also from 19 August until 6 November 1986 and on 5 March and 29 July 1987 Immunised stags were significantly heavier ($p<0.05$) than the male Controls (Fig. 6.1). The greatest between-treatment difference in mean live weight was recorded in November 1986 when Immunised and Control stags weighed 95.5 ± 1.01 and 87.4 ± 1.02 kg, respectively. Immunised hinds tended to be heavier than their female Controls (Figure 6.2) but the difference in live weight was not significant.

Figure 6.1 Adjusted mean live weight (corrected for birth date) of red deer stags immunised against melatonin (●, n=10) and non-immunised controls (○, n=10) recorded from 3 to 26 months of age. Vertical bars represent s.e.m.. Asterisks indicate significant differences between groups for log transformed data.

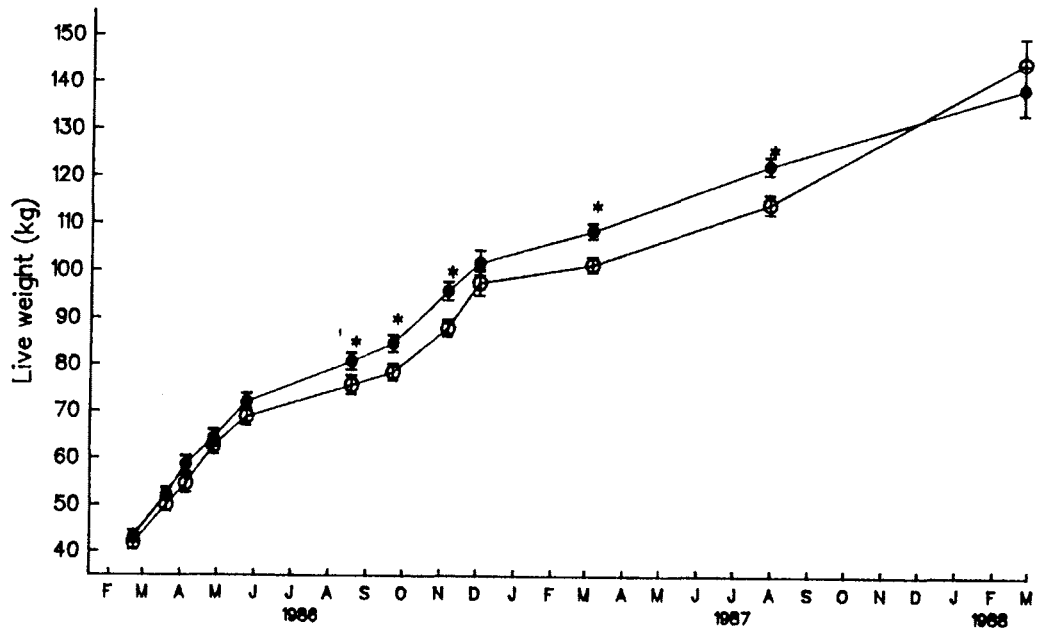


Figure 6.2 Adjusted mean live weight (corrected for birth date) of red deer hinds immunised against melatonin (●, n=9) and non-immunised controls (○, n=10) recorded from 3 to 26 months of age. Vertical bars represent SEM.

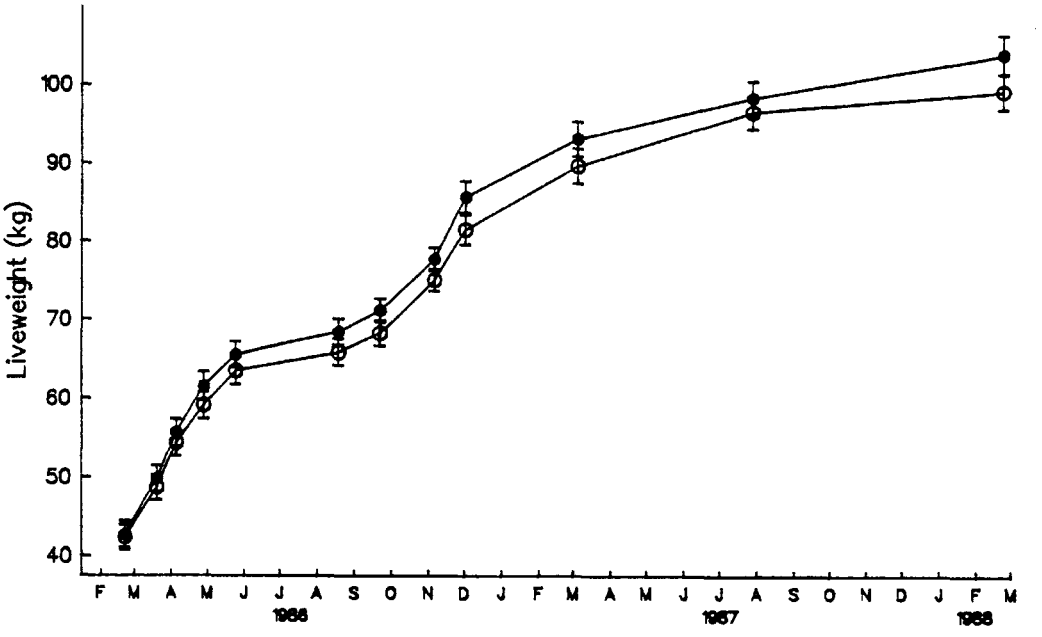


Figure 6.3 Adjusted mean live weight (corrected for birth date) of responder red deer stags immunised against melatonin (●, n=8) and non-immunised controls (○, n=8) recorded from 3 to 32 months of age. Vertical bars represent SEM. Asterisks indicate significant differences between groups for log transformed data.

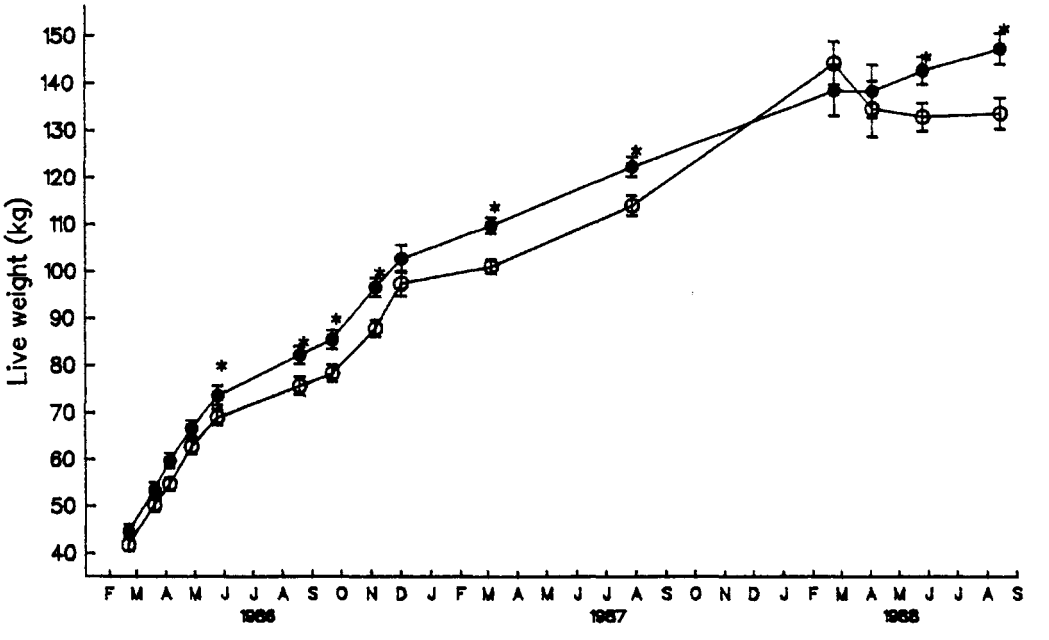
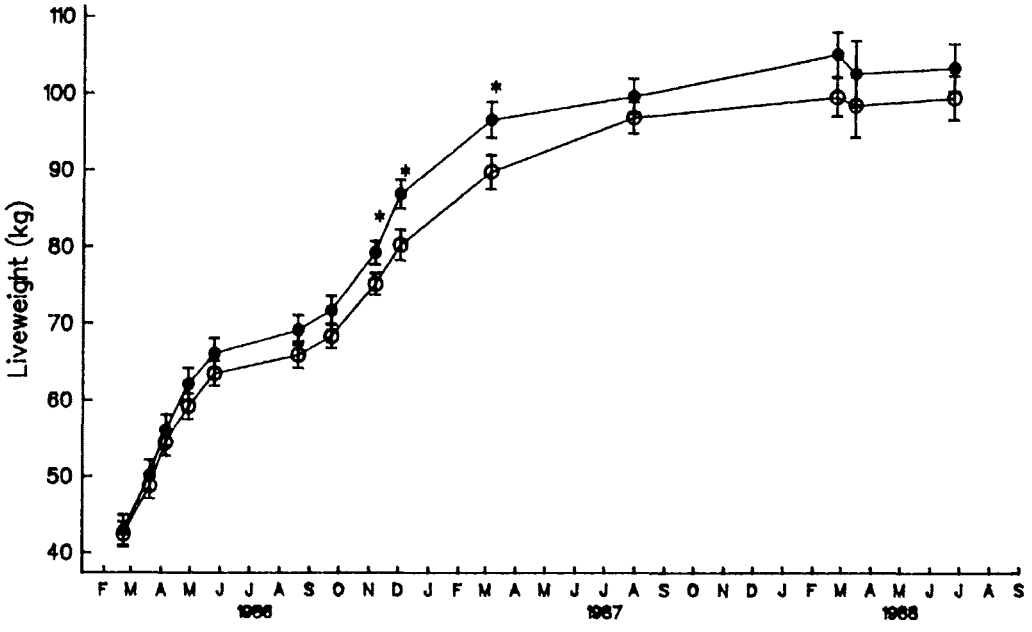


Figure 6.4 Adjusted mean live weight (corrected for birth date) of responder red deer hinds immunised against melatonin (●, n=7) and non-immunised controls (○, n=8) recorded from 3 to 31 months of age. Vertical bars represent s.e.m.. Asterisks indicate significant differences between groups for log transformed data.



Immunised animals which did not produce significant melatonin binding activity in response to immunisation (non-responders; 2 male and 2 female) had similar live weights to Controls. Consequently, elimination of non responders from the Immunised groups enhanced treatment differences so that Immunised animals with significant binding activity in plasma were heavier ($p<0.05$) than their controls at most weighing dates for stags (Figure 6.3) and on three occasions in the case of hinds (Figure 6.4).

Repeated measures analysis using profile transformation indicated that responder Immunised stags grew more rapidly than the Control stags during 3 time periods; between 20 March and 5 April 1986 ($p<0.04$), between 2 December 1986 and 5 March 1987 ($p<0.04$) and between 4 April and 16 August 1988 ($p<0.04$). The pattern of growth of Immunised and Control males was similar between April and November 1986 ($p>0.10$) and between 5 March and 29 July 1987 ($p>0.05$) but Control stags grew more rapidly between 6 November and 2 December 1986 ($p<0.04$) and between 29 July 1987 and 24 February 1988 ($p<0.03$). No differences in the pattern of live-weight gain were detected between Responder and Control hinds.

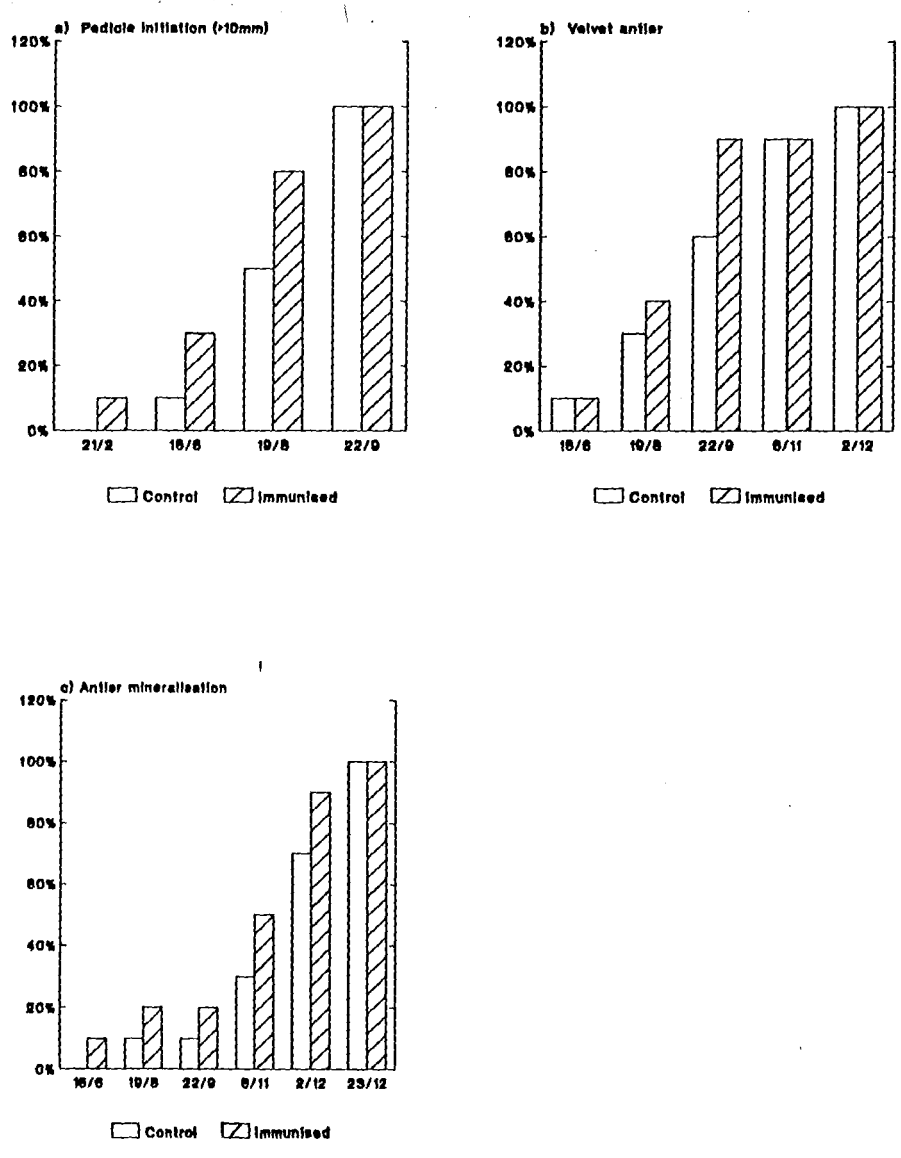
Pelage

On 2 December 1986, shedding of the winter coat was more advanced in stags than in hinds (mean pelage score ± SEM, 4.7 ± 0.2 and 4.0 ± 0.3 for stags and hinds respectively, $p<0.01$). There was no effect of treatment on pelage score.

Antler development

Pedicle and antler development varied considerably between individual stags (Figure 6.5 a-c). Pedicle development was initiated between February and August 1986 and most stags grew their first set of velvet antlers during winter and spring. These antler hardened in early summer and the velvet skin was shed during the following autumn.

Figure 6.5. Cumulative percentage of red deer stags immunised against melatonin (n=10) and non-immunised controls (n=10) to have a) developed pedicles, b) initiated velvet antlers and c) mineralised velvet antlers on observation dates during 1986.



The proportion of Immunised and Control stags that had initiated pedicle development, grown or mineralised their first antlers was similar at all measurement dates. Three stags (2 immunised and 1 control) showed precocious antler development. Their first set of antlers hardened early, were cleaned of velvet by mid August and the antlers cast between October and December. These 3 stags subsequently grew a 2nd set of antlers which they cast during the following spring at the same time as other stags. Immunisation against melatonin did not effect the date of antler casting in 1987 or 1988 (Table 6.2).

Table 6.2. Mean antler casting date in 1987 and 1988 of red deer stags immunised (Immunised) or not immunised (Control) against melatonin.

	Treatment Group			
	Immunised		Control	
Year	n	(mean date ± SEM)	n	(mean date ± SEM)
1987	10	16 October ± 2.2 d	10	14 October ± 2.8 d
1988	8	2 October ± 3.7 d	8	30 September ± 2.4 d

Calving date

All hinds conceived in 1987 although 2 of the calves (one from each treatment group) died following calving difficulties. In 1988 all surviving hinds produced live off-spring. There was no effect of treatment on the mean date of calving in 1987 or 1988 (Table 6.3).

Table 6.3. Mean calving date in 1987 and 1988 of red deer hinds immunised (Immunised) or not immunised (Control) against melatonin.

	Treatment Group			
	Immunised		Control	
Year	n	(mean date ± SEM)	n	(mean date ± SEM)
1987	9	9 December ± 2.0 d	9	7 December ± 3.4 d
1988	7	5 December ± 2.6 d	8	3 December ± 2.5 d

6.4 Discussion

In this study active immunisation of red deer calves against a melatonin conjugate altered the pattern of live-weight gain in the animals that responded to the treatment, but did not affect the cycle of reproductive activity. Live-weight gain in red deer varies with season, feed availability and the age and sex of the animal (Mitchell *et al.*, 1976; Fennessy 1981; Moore *et al.*, 1988a, 1988b). In farmed red deer growth is usually rapid during spring and summer but slows down in winter (Fennessy, 1981; Adam & Asher, 1986; Adam & Moir, 1987; Moore *et al.*, 1988a) when voluntary intake is decreased (Loudon *et al.*, 1989; Milne *et al.*, 1990), possibly in response to the shorter daily photoperiod (Pollock, 1975; Simpson *et al.*, 1984). It is of note that in the present study the effect of immunisation did not appear until the first winter. This is when the duration of melatonin secretion at night is increasing and any perturbation of the melatonin secretion profile might be expected to have its greatest effect on 'short day' photoperiod-entrained effects.

The fact that elimination of the non-responders (deer without appreciable melatonin binding activity in plasma) from the immunised group of deer has enhanced the differences in live weight between Immunised and Controls provides support for the view that the effect was due to the production of anti-melatonin antibodies in the responders. The observation that melatonin binding activity was measurable in responder deer on 23 February 1988 indicates that anti-melatonin antibodies were present in plasma for up to 8 months after a booster immunisation. In spite of this it was not possible to demonstrate the presence of high antibody titres against melatonin in plasma even at a 1:100 dilution. The present results are in contrast with those of Lincoln and Almeida (1981) where active immunisation of mature Soay rams against melatonin-bovine serum albumin conjugate resulted in high antibody titres (1:1600 to 1:32000 at 50% binding of 10 pg [^{3}H] melatonin) in all animals. Presumably the procedure used in the present trial to estimate binding activity in plasma was unable to quantify antibody titre adequately or samples were taken at inappropriate times, so it is possible that substantial antibody production did occur at some occasions during the study.

In sheep (Kennaway *et al.*, 1982; Nett & Niswender, 1982; Arendt *et al.*, 1983; Lincoln & Ebling, 1985) and deer (Bubenik & Smith, 1985; Adam & Atkinson, 1984; Webster &

Barrell, 1985; Fisher *et al.*, 1988; Milne *et al.*, 1990) administration of melatonin or provision of 'short days' advances the onset of autumnal events such as loss of appetite, decline in growth rate and testicular development or oestrous activity, shedding of antler velvet and moulting of the summer coat. If immunisation against melatonin prevented the deer from perceiving the natural autumnal decrease in photoperiod, then the associated decline in appetite and growth rate may have been delayed, which would provide the major explanation for the heavier live weight of the immunised animals recorded here. Immunisation against melatonin appears to have caused a shift in the pattern of live weight gain so that the onset of the autumn decline in growth rate was delayed causing Immunised stags to maintain higher live weights during winter and spring. The greater weight gain of Control stags during late spring in the first year suggests that the spring increase in live weight also may have been delayed in the Immunised stags and may explain why live weights of Immunised and Control stags became similar each summer (see Figure 6.3).

The differences in live weight in the present study probably reflect differences in appetite. Although there is some evidence that the efficiency with which nutrients are used for maintenance and growth changes with season (Milne *et al.*, 1987), changes in live weight in red deer can generally be explained by changes in feed intake (Suttie & Simpson, 1985; Curlewis *et al.*, 1988b; Milne *et al.*, 1990). Red deer exhibit a distinct annual cycle of appetite with voluntary feed intake peaking in late summer and being low during winter (Kay, 1979; Milne *et al.*, 1987; Curlewis *et al.*, 1988b; Milne *et al.*, 1990). There is some evidence that the annual cycle of intake is endogenously generated (Loudon & Brinklow, 1990) and entrained to the appropriate season by changes in daily photoperiod (Suttie & Simpson, 1985). Long photoperiods stimulate appetite and live-weight gain while short days decrease food intake and growth (Suttie & Simpson, 1985). Melatonin treatments, which mimic the effects of short daily photoperiods, are also associated with a decrease in appetite and reduced rate of live-weight gain (Milne *et al.*, 1990). In the absence of changing photoperiods, young red deer hinds maintained on a summer solsticial daily photoperiod from birth displayed circannual changes in voluntary feed intake similar to hinds exposed to natural photoperiods but the phase of the cycle was delayed by 4-6 weeks (Loudon & Brinklow, 1990). The effect of immunisation against melatonin may have prevented the red deer in the present study from perceiving changes

in daily photoperiod and caused a similar delay in the seasonal cycle of the voluntary food intake.

The response of red deer to immunisation against melatonin in other studies has been variable. A live-weight gain response to immunisation against melatonin was recorded in only 1 of 3 immunisation trials at Massey University (Ataja *et al.*, 1992; Barry & Wilson, 1991). The variation in results between experiments may be due to the different immunisation procedure. Calves were immunised at 4-6 months of age (Ataja *et al.*, 1992) in 2 trials and significant antibody titres against melatonin did not development until after the first winter in the trials. In the only Massey trial where fawns were immunised at birth, fawns immunised with DEAE dextran produced significant antibody titres for a short time during winter and tended to grow faster during the subsequent spring. In addition, the correct choice of adjuvants in such trials appears to be critical. In contrast to the adjuvants used in the present study (sorbitan triolate/Sontex and incomplete Freund's) and DEAE dextran, the action of complete Freund's adjuvant was associated with a depression live-weight gain prior to weaning (Ataja *et al.*, 1992).

Immunisation against melatonin did not affect the reproductive seasonality of Suffolk-cross ewes (Arendt, 1986) or Soay rams (Lincoln & Almeida, 1981) or the wool production of mature Merino sheep (Foldes *et al.*, 1991) maintained under natural photoperiods. However immuno-neutralisation of circulating melatonin increased the wool production of young Merino sheep (Foldes *et al.*, 1991). This is similar to what could be expected following surgical modifications such as pinealectomy or cranial cervical ganglionectomy. When adult white-tailed deer were pinealectomised changes in their seasonality were minor (Brown *et al.*, 1978) yet when the pineal gland was removed from young white-tailed deer, antler, testicular and pelage changes were delayed by several months (Brown *et al.*, 1978; Snyder *et al.*, 1983). Similarly, although pinealectomised or ganglionectomised sheep retained their annual rhythms (Lincoln, 1979; Kennaway *et al.*, 1981), the first seasonal breeding cycle was delayed and wool production increased in sheep modified prepubertally (Lincoln & Almeida, 1981; Kennaway, 1984; Foster *et al.*, 1988; Foldes *et al.*, 1991). Ganglionectomy of immature red deer stags also delayed the cycle of antler development during the following season although its timing returned to normal in subsequent years (Lincoln, 1985). The live-

weight patterns of animals in these studies were not reported. On balance, these results suggest that disruption of the melatonin pattern, whether by immunisation against melatonin or by surgical modification, would be expected to have little effect on the seasonality of adult animals but that it may alter the annual cycles of immature animals, possibly by interfering with entrainment of endogenous rhythms to the natural photoperiod. The results of the present study indicate that this view may hold for immature red deer immunised against melatonin, at least for live-weight patterns.

The antler growth cycle of stags in the present study was similar to that seen in previous studies over the first 24 months of life (Lincoln, 1971; Suttie *et al.*, 1984; Fennessy & Suttie, 1985). Most stags initiated pedicle development at 7-9 months of age, grew their first set of velvet antlers during late winter and spring and cast the hard antlers as 2 year olds in the following spring. Incidents of precocious puberty, as displayed by the 3 stags which cast their first antlers in the first year of life, have also been recorded in other studies (Suttie *et al.*, 1984; Meikle *et al.*, 1992). The significance of these aberrant antler cycles is not known. In the red deer stag pedicle development and the antler growth cycle are controlled by circulating concentrations of testosterone (Wislocki *et al.*, 1947; Lincoln *et al.*, 1970; Suttie *et al.*, 1984). The observation that in the present study all stags cast their hard antlers at the normal time as 2-year-olds and 3 -year-olds (Suttie *et al.*, 1984; Fennessey *et al.*, 1992) suggests that immunisation against melatonin had no effect on testicular steriodogenesis or the seasonal reproductive cycle of stags. Similarly the onset of the oestrous cyclicity, as indicated by the calving date of the female red deer, was not affected by treatment.

In this study disruption of the melatonin signal, possibly from an early age, may have prevented the entrainment of endogenous annual rhythms in feed intake and growth to the appropriate photoperiod without affecting the seasonal antler and reproductive rhythms in the same way. This apparent dichotomy is difficult to explain but it is possible to conjecture that melatonin must influence growth and reproduction by separate neurohormonal pathways or that different thresholds of sensitivity to melatonin secretion are involved.

Chapter 7
Induction of ovulation in seasonally anoestrous red deer hinds with a GnRH analogue.

This chapter describes 3 trials, carried out in successive years, to investigate the effectiveness of a GnRH analogue, buserelin (Receptal, Hoechst N.Z. Ltd), for inducing ovulation in progesterone-primed anoestrous red deer hinds.

7.1 Trial I

7.1.1 Introduction

In sheep, GnRH release evokes the tonic and the surge secretion of LH from the pituitary gland (Clarke & Cummins, 1982; Caraty *et al.*,1989; Karsch, 1987; Moenter *et al.*, 1990). During the anoestrous season the diminished pulsatility of LH (Goodman & Karsch, 1981; Karsch et al, 1984; Martin, 1984), presumably due to reduced GnRH secretion (Moenter *et al.*, 1990; Barrell *et al.*, 1992), is reflected in a lack of reproductive activity, specifically ovulation and associated oestrous behaviour. GnRH and LH appear to have roles in the reproductive seasonality of deer similar to those described for sheep. Active immunisation against GnRH disrupts reproductive activity in red deer stags, reducing circulating testosterone and presumably LH levels (Lincoln *et al.*, 1982; Lincoln, 1985). Injections of exogenous GnRH stimulate LH release (Lincoln & Kay, 1979; Kelly *et al.*, 1982; Suttie *et al.*,1984; Manley *et al.*, 1989) and may induce ovulation in anoestrous red deer hinds (Fisher *et al.*, 1986; Manley *et al.*, 1989).

Various treatments involving intravaginal progesterone followed by gonadotrophins or GnRH administration have been used in attempts to advance the breeding season of red deer hinds. Many studies have utilised pregnant mare serum gonadotrophin (PMSG) instead of GnRH to induce ovulation (Kelly *et al.*, 1982; Kelly & Moore; 1982/83; Adam, 1982, 1983; Fisher & Fennessy, 1985; Fisher *et al.*, 1986; Moore & Cowie, 1986), but fertility is generally low. Low level infusions of synthetic GnRH for 48-72 h will also induce ovulation in seasonally anoestrous red deer hinds (Fisher *et al.*, 1986)

but the expense and complexity of using a mini-osmotic pump to provide long term infusions of GnRH has precluded this method for on-farm use.

Anoestrous ewes have been induced to ovulate using a single injection of potent analogues of GnRH (Kinder *et al.*, 1976; Frandle *et al.*, 1977; Swift & Crighton, 1980; McNeilly *et al.*, 1981). These analogues were more effective than GnRH at releasing LH and FSH from the pituitary in terms of the peak plasma gonadotrophin levels and the duration of the response (Siddall & Crighton; 1977; Swift & Crighton, 1979), possibly because the analogues are catabolised more slowly than GnRH (Swift & Crighton; 1979).

Trial I attempted to advance the breeding season of progesterone-primed red deer hinds using one of the GnRH analogues, buserelin, in a simple twin-injection protocol. A small dose of buserelin was administered at the end of progesterone treatment to stimulate gonadotrophin release and therefore follicular development. This was followed 48 h later by a second and larger dose of buserelin in order to trigger an LH surge and ovulation.

7.1.2 Materials and methods

Sixteen lactating mixed-age red deer hinds (average live weight 94.5 ± 1.7 kg) were grazed with their calves as a single mob from 18 February to 2 May 1986. Hinds were allocated into 2 treatment groups so that the groups (n=8) were approximately balanced for age and live weight on 18 February 1986 and a controlled internal drug release device (CIDR, 12% progesterone w/w, AHI Plastic Moulding Co.) containing 0.6 g progesterone was inserted intravaginally into each hind. At CIDR withdrawal 14 d later (4 March), hinds in one group were injected intramuscularly with 4 μg buserelin (Receptal, Hoechst N.Z. Ltd) followed by a second injection of 10 μg 48 h later. Control hinds in the other group were not injected.

An entire red deer stag, fitted with a ram harness and crayon, was run with the hinds following CIDR withdrawal. Crayon marks were recorded daily from 4 March until 31 March and then 2 times a week until 30 April. On 18 March the ovaries of all the hinds were examined by laparoscopy and descriptions of any ovarian structures recorded. The hind mob was monitored daily during November and December and calving dates were

recorded. Blood samples were collected by jugular venepuncture (see Section 3.2) each day during March and once a week during April and the plasma analysed for progesterone.

Data from only 15 hinds were included in the analysis as the CIDR was not removed from 1 buserelin-treated hind. Differences within treatment groups were tested using the two-sample Student's t-test and between treatment effects were determined using analysis of variance (SAS Statistical Package). Progesterone concentrations were log-transformed (base 10) prior to statistical analysis. The date of conception was calculated from calving dates and an estimated gestation length of 233 d (Kelly & Moore, 1977).

7.1.3 Results

Ovarian activity.

Six buserelin-treated hinds and 1 control hind each had a single corpus luteum (C.L.) present at laparoscopy.

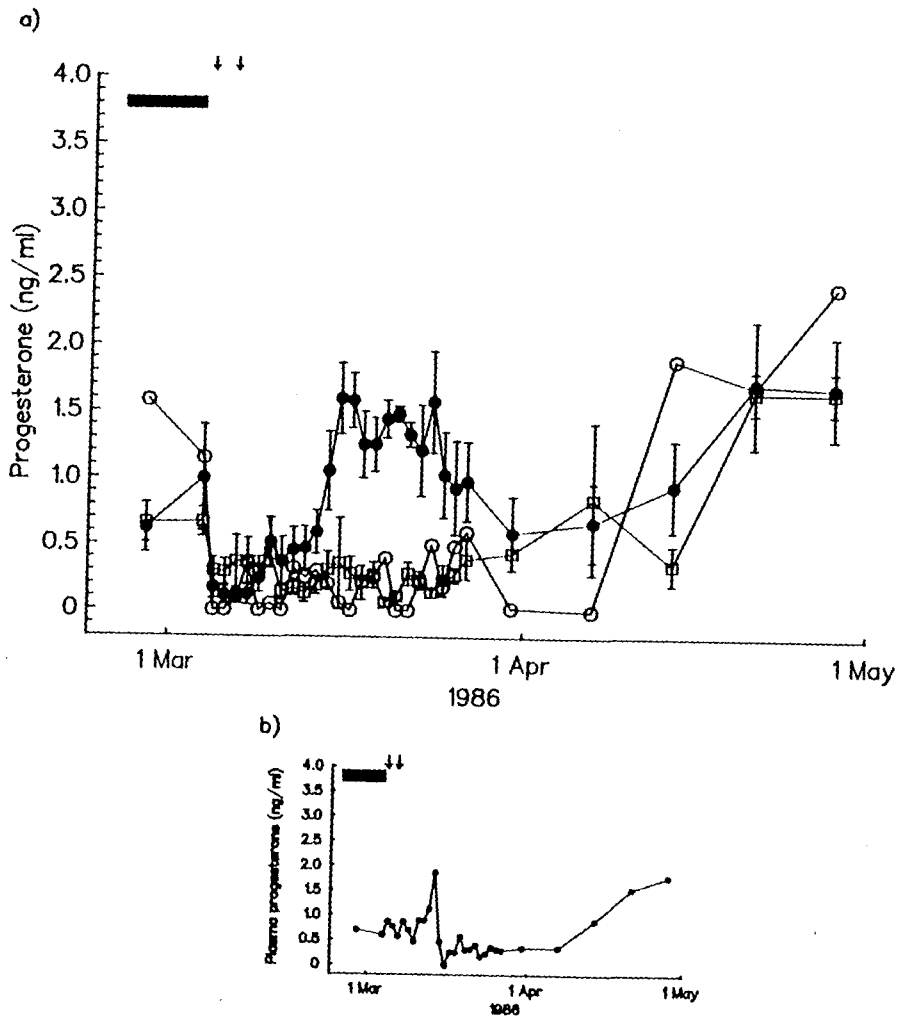
Plasma progesterone.

Mean plasma progesterone concentration at the end of the period that CIDRs were present was 0.74 ± 0.22 ng/ml and after CIDR withdrawal mean plasma progesterone level fell significantly ($p < 0.01$) to 0.24 ± 0.07 ng/ml. Plasma progesterone levels increased (> 1 ng/ml) 7-12 days (mean 10.7 ± 0.8 d) after CIDR removal in treated hinds which had a corpus luteum present at laparoscopy (Figure 7.1 a), but remained low (< 1 ng/ml) in the treated hind without a corpus luteum. Progesterone concentrations remained elevated (> 1 ng/ml) for 8-12 days (mean 9.3 ± 0.6 d) in 5 of the 6 treated hinds which had a corpus luteum but fell again after only 3 days in one treated hind with a corpus luteum(Figure 7.1 b). The mean peak plasma progesterone concentration was 2.14 ± 0.18 ng/ml during the induced cycle. Plasma progesterone levels were generally low (< 1 ng/nl) in control hinds (Figure 7.1 a) (including the hind which had a corpus luteum) until early April. Mean plasma progesterone concentrations of the treated hinds with a corpus luteum were consistently greater ($p < 0.05$) than both control and treated hinds without a corpus luteum between days 13 and 23 (17 March to 27 March) after CIDR removal.

Oestrous detection.

A total of 21 crayon markings were detected but markings which were observed to result from accidental contact with the stag during yarding (n=8) were excluded from these data, making the corrected total of 13 incidents of marking. Ten of the 15 (66%) hinds were marked and 3 hinds were marked on 2 separate occasions. Two buserelin-treated hinds were marked between 5 and 9 d after CIDR withdrawal. Only 5 of the 12 (42%) hinds which conceived before the 30 April were marked within 5 days (± 5 d) of their estimated date of conception and 2 hinds were marked at least 10 d after the estimated date of conception.

Figure 7.1. a) Mean plasma progesterone concentrations of red deer hinds following progesterone (CIDR) treatment in Trial I. Hinds either received GnRH analogue and had a corpus luteum present on the 18 March (n=6,●) or did not have a corpus luteum present (n=1,○), or did not receive GnRH analogue (n=8,□). Vertical bars denote s.e.m. b) Plasma progesterone profile of a buserelin-treated hind in Trial I with subnormal luteal function. The horizontal block represents progesterone (CIDR) treatment and arrows indicate the administration of the GnRH analogue.



Calving date.

All hinds delivered live calves at term. The mean calving date of treated hinds was 27 November ± 2.3 d, similar to that of the controls (26 November ± 2.0 d). No hinds calved on a date corresponding to conception at the induced ovulation.

7.1.4 Discussion

Single ovulations were induced in most of the red deer hinds treated with the GnRH analogue, buserelin. The presence of the corpora lutea observed in the treated hinds at laparoscopy was confirmed by the elevated plasma progesterone concentrations in these deer 7-12 days after CIDR withdrawal. Plasma progesterone levels remained elevated for about 9 days, shorter than the luteal phase recorded during the natural oestrous cycle (Adam *et al.*, 1985; Chapter 5) and maximum plasma progesterone levels during the induced cycle were generally lower than the peak levels reported elsewhere (Adam and Atkinson, 1984; Adam *et al.*, 1985; Jopson *et al.*, 1990). Also when compared with the progesterone profile in progesterone-treated hinds during the breeding season (see section 7.3) the rise in plasma progesterone levels was delayed.

In anoestrous ewes, subnormal luteal function or premature luteolysis occurred when the induced corpus luteum developed from an immature follicle (Haresign & Lamming, 1978; Coleman & Dailey, 1983; Murdoch *et al.*, 1983) or from a follicle that was not sufficiently exposed to elevated progesterone levels prior to ovulation (McLeod & Haresign, 1984; Hunter *et al.*, 1988; Southee *et al.*, 1988; Hunter, 1991). It is possible that in the present trial luteal inadequacy arose from insufficient exposure of the developing follicles to progesterone because plasma progesterone concentrations during CIDR treatment (0.74 ng/ml) were lower than those occurring during the normal luteal phase (1-4 ng/ml) (Group C hinds in Trial III, below; Adam *et al.*, 1985; Jopson *et al.*, 1990). Furthermore, the response of an anoestrous ewe to GnRH and its analogues appears to be dependent on the timing of the treatment during the non-breeding season (Shareha *et al.*, 1976; Swift & Crighton, 1980). During deep anoestrous, the release of FSH in response to a buserelin injection was minimal in ewes (Swift & Crighton, 1980). In red deer hinds nothing is known about the dynamics of FSH secretion or its role in the

control of ovulation and the oestrous cycle. However endogenous LH secretion and LH response to GnRH varies during the anoestrous period of Père David's (Curlewis *et al.*, 1991) and lactating red deer hinds (Chapter 4). If the reproductive axis was still suppressed during early March in the present study, the gonadotrophic stimulation of the developing follicles may have been inadequate, resulting in the ovulation of immature follicles and development of subnormal corpora lutea It was also possible that some structures identified as corpus luteum were actually unruptured luteinised cysts. The presence of these cysts, macroscopically identical to a corpus luteum, was associated with a late-occurring, short-duration increase in plasma progesterone levels in ewes treated with GnRH prior to the breeding season (Hunter *et al.*, 1989).

All hinds that were induced to ovulate after buserelin treatment possessed a single corpus luteum at laparoscopy. Red deer usually have a single calf (Clutton-Brock, 1982) and although multiple ovulations can be induced using progesterone/PMSG treatment they do not always result in multiple births (Kelly *et al.*, 1982). Often, response to treatment is variable with few of the treated hinds conceiving (Kelly *et al.*, 1982; Moore, 1983/1984; Fisher *et al.*, 1989; Fennessy *et al.*, 1990b). Also since multiple births are associated with premature deliveries (Adam *et al.*, 1985), increased neonatal calf losses (Adam *et al.*, 1985; Fennessy *et al.*, 1990b), reduced growth performance (Fennessy *et al.*, 1990b) and the occurrence of free-martinism among mixed-sex multiple births (Stewart-Scott *et al.*, 1989 cited Fennessy *et al.*, 1990b), the induction of multiple ovulations may be disadvantageous.

Although progesterone treatment of red deer hinds will synchronise ovulatory cycles in the breeding season (Fennessy *et al.*; 1990a; Asher *et al.*, 1991), it is seldom a sufficient stimulus to induce ovulation in lactating hinds during anoestrum (Barrell, 1985; Fisher *et al.*, 1986). Consequently the absence of corpus luteum in most of the control hinds was not unexpected. The ovarian structure that was recorded in a control hind was probably not a corpus luteum as it was small and pale in colour and did not secrete significant amounts of progesterone. It may have been a large follicle or the residue of the corpus albicans from the previous pregnancy.

No early calvings resulted from the induced ovulations. This is similar to other studies where PMSG and synthetic GnRH have been used to induce ovulation but where few hinds calved early (Kelly *et al.*, 1982; Kelly & Moore, 1982/83; Adam, 1982, 1983; Fisher & Fennessy, 1985; Fisher *et al.*, 1986; Manley *et al.*, 1989). One possible cause of low fertility following induction of ovulation in red deer is that ovulations may have been *silent* and not accompanied by oestrous behaviour. Pretreatment with progesterone is essential for ewes induced to ovulate to display oestrous behaviour (Robinson, 1954). In the present study plasma progesterone concentrations prior to CIDR removal were lower than levels normally experienced during the luteal phase of the oestrous cycle (Adam *et al.*, 1985; Fennessy *et al.*, 1989; Jopson *et al.*,1990). It was possible that the single CIDR did not elevate plasma progesterone concentrations sufficiently to ensure that courtship behaviour was expressed. Even if progesterone levels were sufficient to allow oestrous behaviour to be expressed, the hormonal pattern associated with the buserelin-induced ovulations may have been inappropriate to support pregnancy.

In this trial the attempt to use a ram crayon harness for oestrous detection was not successful as there was no relationship between the markings of the hinds and the estimated date of conception. During copulation the stag stands vertically (Veltman,1985) with no contact between the the stag's brisket and the hind, hence the crayon harness may not mark the hind. Although it was possible for crayon contact to occur during the late courtship phase when the stag adopted the non-ejaculatory, low mount posture, little weight was bourne by the hind (Veltman, 1985) and markings may have been very faint and thus undetectable. Furthermore, it was difficult to prevent the stag accidentally marking hinds during yarding for blood sample collection. Although observed incidents of nonsexual contact were excluded from the data, some of the crayon markings that were detected may also have been accidental. Consequently it was not possible to use the crayon markings to ascertain whether any of the hinds displayed oestrous behaviour after the induced ovulation or whether any were actually mated. Treated and control hinds had 100% calving and a similar range of calving dates, which indicates that the treatment regime had no subsequent detrimental effect on reproductive performance.

It was concluded that the GnRH analogue, buserelin, had potential for controlling ovulation in red deer hinds prior to the normal breeding season, but further trials were required to develop a protocol that would lead to successful establishment of pregnancy.

7.2 Trial II

7.2.1 Introduction

In Trial I single ovulations were induced in the majority of red deer hinds treated with the GnRH analogue, buserelin. The reason for the failure of treated hinds to conceive was unknown. The single CIDR may not have sufficiently elevated plasma progesterone levels to ensure that the induced ovulation was accompanied by overt oestrous behaviour and formation of a functional corpus luteum. Inadequate luteal function may also have occurred because the induced corpus luteum may have arisen from an immature follicle lacking in gonadotrophic stimulation prior to ovulation. A second study, Trial II, is described here. It utilised additional progesterone stimulation and extra doses of the GnRH analogue, buserelin, in an attempt to overcome possible limitations in Trial I.

7.2.2. Materials and methods

In 1987, 16 lactating mixed-age red deer hinds were allocated to two groups (n=8) balanced for age and live weight of the hinds. Two CIDRs (Type S), tied together, were inserted intravaginally into every hind on 19 February and removed on 4 March. From 2 March, hinds in one group were injected daily with buserelin according to the protocol in Table 7.1. The CIDRs were not removed from one control hind so her data have been excluded from trial analysis.

Following CIDR withdrawal the hinds were run with an entire red deer stag. Blood samples were collected by jugular venepuncture (See section 3.2) at 2 to 3 day intervals (on Mondays, Wednesdays and Fridays) throughout the mating period and the plasma progesterone concentration measured. On 19 March the ovaries of all hinds were examined by laparoscopy (See section 3.2) and during the following summer individual calving dates were recorded.

Table 7.1. Injection regime for GnRH-analogue (buserelin) used with red deer hinds in Trial II.

Date	Dose (i.m.) of buserelin	
2 March	4 μg	
3 March	3 μg	
4 March	2 μg	(CIDR withdrawal)
5 March	2 μg	
6 March	10 μg	

Differences within treatment groups were tested using the two-sample Student's t-test while treatment effects were determined using analysis of variance (SAS Statistical Package). Progesterone concentrations were log-transformed (base 10) prior to statistical analysis.

7.2.3 Results

Ovarian activity.

At laparoscopy, 6 of the buserelin-treated hinds had a single corpus luteum present.

Plasma progesterone.

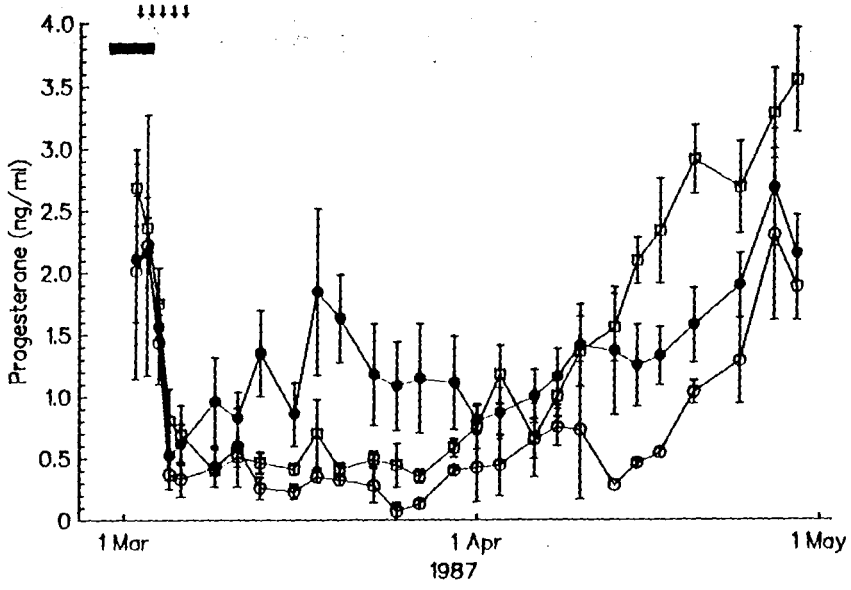
After CIDR withdrawal plasma progesterone levels fell significantly in all hinds from a mean plasma progesterone concentration of 1.65 ± 0.37 ng/ml to 0.45 ± 0.15 ng/ml. In the treated hinds with a corpus luteum present at laparoscopy, progesterone levels increased to greater than 1 ng/ml 2 to 9 d after CIDR removal (mean 7.2 ± 1.2 d). In 4 of these hinds, circulating progesterone levels remained elevated for 13 to 17 days (mean 15.0 ± 0.8 d). In the other 2 treated hinds with a corpus luteum the increase in plasma progesterone concentration was for a shorter duration (3 d). The mean peak plasma progesterone concentration was 3.02 ± 0.43 ng/ml during the induced cycle. In treated hinds without a corpus luteum and in all control hinds plasma progestesone concentrations remained low (< 1 ng/ml) until early April. Mean plasma progesterone

concentrations in treated hinds with a corpus luteum present were significantly higher ($p < 0.05$) than in control hinds from day 9 to day 26 after CIDR withdrawal (12 March to 30 March) (Figure 7.2).

Calving date.

All hinds delivered live calves at term. The mean calving date of treated hinds was 27 November ± 2.9 d and similar to that of the control hinds (23 November ± 1.1 d). No hinds calved on a date corresponding to conception at the induced ovulation.

Figure 7.2. Mean plasma progesterone concentrations of red deer hinds following progesterone (CIDR) treatment in Trial II. Hinds either received GnRH analogue and had a corpus luteum present on the 18 March (n=6,●) or did not have a corpus luteum present (n=2,○), or did not receive GnRH analogue (n=8,□). Vertical bars denote sem. The horizontal block represents progesterone (CIDR) treatment and arrows indicate administration of the GnRH analogue.



7.2.4 Discussion

In the present study, the majority of ovulations induced in red deer hinds using the GnRH analogue, buserelin, were associated with changes in plasma progesterone concentrations similar to those recorded during the luteal phase of the natural oestrous cycle (Adam *et al.*, 1985; Jopson *et al.*,1990). This suggests that, unlike the previous trial, most of the corpora lutea which developed after the induced ovulation were functional. A lack of progesterone pretreatment in anoestrous ewes is associated with the occurrence of an abnormal and short-lived corpus luteum which undergo premature luteolysis (Hunter *et*

al., 1989). In the present study plasma progesterone levels prior to CIDR removal were greater than those recorded in Trial I and it is possible that subsequent luteal function was improved because progesterone levels maintained by the double CIDR treatment were nearer those occurring during the luteal phase of the natural oestrous cycle. Alternatively, it may be that multiple low dose injections of buserelin provided extra stimulation of follicular growth, thus increasing the likelihood that the induced corpus luteum developed from fully luteinised cells.

Although the treatment regime used here produced 4 corpora lutea which were associated with normal patterns of progesterone secretion, there were also 2 corpora lutea which were considered to have subnormal luteal function. The elevation of plasma progesterone concentration in these latter cases was short-lived (< 6 d) and maximum progesterone concentrations were lower than those normally recorded during the luteal phase of oestrous cycle. It is possible that these structures, although macroscopically identical to a corpus luteum were actually luteinised cysts similar to those described by Hunter *et al.* (1989) in anoestrous ewes following treatment with GnRH.

Although progesterone secretion from most of the induced corpora lutea appeared to be normal, no hinds calved prior to the onset of the normal calving season. This indicates that no conceptions resulted from the induced ovulations. It was not known if hinds had been mated or if they had displayed courtship behaviour. Even if the hinds had displayed oestrus, it was possible that the libido of the stag was low prior to the breeding season and that he was still behaviourally incompetent to mate hinds at the time of the induced ovulation.

In summary, buserelin treatment did induce a single, functional corpus luteum in anoestrous red deer hinds but no early calvings resulted.

7.3 Trial III

7.3.1 Introduction

Trials I and II showed that although the GnRH analogue, buserelin, induced ovulation in progesterone-primed red deer hinds prior to the breeding season no calves were born as a

result of the procedure. It was not known if hinds had been mated or even if they had displayed oestrous behaviour. In the anoestrous ewe failure to establish pregnancy after buserelin treatment has been associated with; absence of oestrous behaviour (Rodway & Swift, 1985), inadequate luteal function (Swift & Crighton, 1980; McNeilly *et al.*, 1981; Rodway & Swift, 1985) or a hormonal environment inappropriate for conception (Rodway & Swift; 1985). To investigate the cause of the barrenness in red deer hinds following buserelin treatment Trial III examined oestrous behaviour, plasma LH levels and luteal function in progesterone-primed anoestrous hinds induced to ovulate with buserelin. Parameters were compared with those of; 1) progesterone-primed hinds treated with oestradiol prior to the breeding season, 2) progesterone-primed hinds treated with buserelin and oestradiol prior to the breeding season and 3) progesterone-primed hinds during the breeding season. Oestradiol benzoate was used to induce oestrous behaviour in hinds prior to the breeding season.

7.3.2 Materials and methods

In 1988 24 lactating mixed-age red deer hinds with an average live weight of 100.5 ± 2.0 kg were allocated to 4 groups (n=6) balanced for age and live weight of the hinds. On 19 February 1988, two progesterone CIDR (Type S), tied together, were inserted intravaginally into each hind and withdrawn 14 days later. From 2 March 1988, 12 hinds (Groups B and BO) were injected i.m. with 4, 3, 2, 2 and 10 μg buserelin (Receptal, Hoechst) at -48, -24, 0, 24, and 48 h respectively from CIDR withdrawal. At CIDR withdrawal on 4 March 6 of the buserelin-treated hinds (Group BO) and 6 other progesterone-primed hinds (Group O) were injected i.m. with 500 μg oestradiol benzoate (Sigma Chemical Company, USA) in 500 μl of peanut oil to induce oestrous behaviour (Robinson, 1954). During the breeding season, ovulation was synchronised in 6 hinds (Group C) by inserting 2 CIDRs into each hind on 1 April and removing the CIDRs 14 days later.

Blood samples were collected at 3 h intervals for 75 h following CIDR withdrawal for LH analysis and at 2 to 3 day intervals during the mating period for progesterone analysis. Mating behaviour was recorded by running the hinds with an entire (Groups B, O and BO) or vasectomised (Group C) red deer stag for periods of 3 h followed by a 3 h separation, repeated over the 75 h following CIDR withdrawal. During this period the

hind mob was confined to the main deer yard and grass/clover silage, hay and water provided *ad libitum*. Individual hinds were identified by numbered ear tags and by symbols painted from a spray can (Fluoro pink, Dulux (N.Z.) Ltd.) on both flanks of each hind. During the 3 h observational periods hinds were continuously observed through a 15 mm x 1.25 m gap in the wall planking of the yard and courtship activity of the mating mob was recorded. The observational area was lit at night by 2 x 200 watt floodlights. The onset of oestrus was defined as the time that a hind was first observed to be in standing heat, equivalent to the third phase of courtship described by Veltman (1985). The entire stag, initially run with Groups B, O and BO hinds, did not display rutting behaviour prior to the breeding season and was replaced after 24 h. Ovaries of all hinds were examined by laparoscopy 14 d after CIDR withdrawal (See section 3.2) and calving dates were recorded for Groups B, O and BO hinds.

Data from only 19 hinds were included in the analysis as CIDRs were not removed from 1 Group B, 2 Group O and 2 Group C hinds. As the number of hinds mated in each treatment group was small (1-3 hinds/group) and there was considerable variation in the pattern of courtship behaviour of individual hinds within groups, only the incidence and timing of mating and oestrous behaviour and the main courtship activities (those common to most hinds) are described. Progesterone and LH values were log-transformed (base 10) prior to statistical analysis using analysis of variance. An increase in LH was deemed to be significant when the LH concentration of 2 consecutive samples were greater than 2 ng/ml and the LH concentration in the first sample exceeded the mean of the previous 2 baseline values by 3 x the intra-assay C.V.. Plasma progesterone concentrations were defined as elevated when plasma progesterone concentrations exceeded 1 ng/ml for 3 consecutive samples (5-7 d) .

7.3.3 Results

Behaviour

Within 72 h of CIDR withdrawal 3/5 B and all O, BO and C hinds displayed oestrous behaviour and 1/5 B, 2/4 O, 3/6 BO and 3/4 C hinds were mated.

The mating sequence

Most hinds in oestrus (14/17) were attracted to the stag, rubbing up against him, licking his face and neck or resting their heads on his back and rump and 8/17 oestrous hinds attempted to mount him. Oestrous hinds also interacted with other hinds (10/17) (usually hinds also displaying signs of oestrus); grooming their shoulders and neck and mounting each other. Hinds displaying oestrus were exceptionally docile when blood samples were taken and stood still, wagging the tail, in response to pressure applied to the rump (11/17).

The entire stag which initially ran with the hind mob did not respond to oestrous hinds. The stag did not display rutting behaviour; he did not herd the hinds, he moved away when hinds rubbed up against him and made no attempt to groom or mount hinds in standing heat. This stag was replaced 27 h after CIDR withdrawal by two other entire red deer stags. Within 30 minutes of being introduced into the hind mob, the new stags had mated one hind each. One of these stags was removed and the study proceeded with a 5 year-old stag.

Mating behaviour displayed by the replacement stag included chasing hinds with his head down and neck extended while emitting low pitch grunts. If the hind did not move away the stag might sniff and lick the hind's head and neck or vulva (12/17) or attempt a non copulatory low mount (11/17). Prior to mating most hinds were detected to be in standing heat by handlers during the previous blood sampling (7/9 hinds). The copulatory mount was often preceded by the hind grooming (7/9 matings) or low mounting (8/9 matings) the stag and the stag grooming (9/9 matings) or low mounting (7/9 matings) the hind. The stag low mounted a hind on average 1.2 (± 0.3) times prior to mating and a hind mounted the stag about 1.1 (± 0.2) times prior to mating. After copulation oestrous behaviour ceased in most (7/9) hinds.

Oestrous behaviour and mating

In Group B oestrous behaviour was recorded in 3 hinds 32-49 h after CIDR removal and 1 hind was mated 52 h after CIDR removal (see Table 7.2).

In Group O all hinds first displayed oestrous behaviour 18-23 h following CIDR removal and 2 hinds were mated approximately 10 h later (28.5 ± 1.5 h after CIDR removal).

In Group BO behavioural oestrus was detected in all hinds 19-30 h after CIDR removal and 3 hinds were mated 27-32 h after CIDR removal.

In 3 of the control hinds oestrous behaviour was first recorded 18-24 h after CIDR withdrawal and these hinds were mated 13-18 h later (32-39 h after CIDR removal). The onset of oestrus was later (33 h after CIDR removal) in the hind which was not mated.

Table 7.2. Incidence and time of oestrus and mating. Means in columns with different superscripts are significantly different ($p <\sim 0.05$).

Treatment Group	Oestrous Behaviour		Mating	
	No of hinds detected	Onset (h from CIDR withdrawal) mean s.e.m. [Range]	No of hinds mated	Time of mating (h from CIDR withdrawal) mean s.e.m. [Range]
B	3/5	41.6^{b} 4.84 [32.5-49.0]	1/5	52.2 - [-]
O	4/4	20.4^{a} 1.38 [18.2-23.4]	2/4	28.5 1.8 [27.5-29.5]
BO	6/6	21.2^{a} 2.35 [18.7-30.2]	3/6	28.3 1.8 [27.0-32.4]
C	4/4	23.5^{a} 3.43 [18.5-33.1]	3/4	36.5 2.2 [31.5-39.0]

The onset of oestrous behaviour, when hinds were first seen to either 1) stand still when groomed or be low mounted by the stag, 2) attempt to low mount the stag or 3) to be in standing heat during blood sampling, was significantly later ($p < 0.001$) in Group B hinds than for other treatment groups (Table 7.2). Hinds displayed oestrous behaviour over a period of between 6 and 54 h. Time from onset of oestrous behaviour to mating varied

considerably between hinds (mean 9.6 ± 1.7 h, range 2.5-17.5 h) and tended to be longer in control hinds (13-17.5 h).

Plasma LH

Mean plasma LH profiles of Group B, O, BO and control hinds are shown in Figure 7.3. Mean peak LH concentrations and mean time of the major LH pulse in each treatment group are presented in Table 7.3. In the 3 days following withdrawal of progesterone treatment, two peaks of plasma LH were detected in all Group B and 1/6 Group BO hinds while a single LH peak was recorded in all Group O, Group C and 5/6 Group BO hinds. Between peaks plasma LH concentrations were generally low (0.3-0.8 ng/ml) and seldom exceeded 1 ng/ml.

Figure 7.3. Mean plasma LH concentrations of red deer hinds following progesterone (CIDR) treatment in Trial III. Hinds either received a) GnRH analogue (n=5), b) oestradiol (n=4), c) GnRH analogue and oestradiol (n=6) prior to the breeding season or d) received progesterone treatment only during the breeding season. B and O represent the time of buserelin and oestradiol administration, respectively. Vertical bars denote s.e.m.

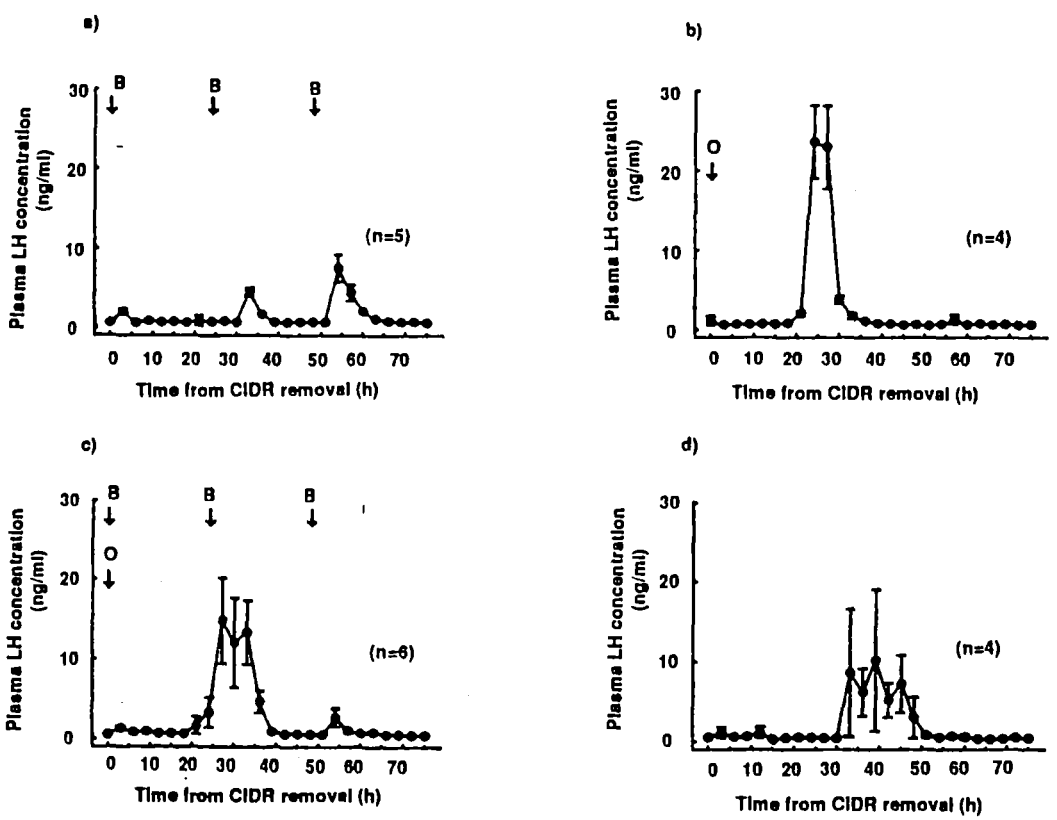


Table 7.3. Magnitude and time of maximum LH concentration. Means in columns with different superscripts are significantly different ($p < 0.05$).

Treatment Group	n	Maximum LH (ng/ml) mean s.e.m. [range]	Time of maximum LH (h from CIDR removal) mean s.e.m. [range]
B	5	8.3^{a} 1.5 [4.1-11.4]	54.6^{c} 0.6 [54-57]
O	4	33.6^{b} 3.8 [20.4-37.8]	24.5^{a} 0.8 [24-27]
BO	6	20.4^{b} 4.1 [11.7-37.4]	28.0^{a} 1.3 [24-33]
C	4	24.3^{b} 6.2 [10.8-36.8]	41.3^{b} 3.3 [33-45]

In Group B hinds two peaks in LH occurred about 33 and 54 h after CIDR removal. There was also a short term increase in plasma LH concentrations (1.6-3.2 ng/ml) 3 h after CIDR removal. The increase in LH associated with first LH peak was small (3.3-5.8 ng/ml) and LH concentrations returned to basal levels (< 1 ng/ml) 6 h later. The 2nd LH peak was slightly larger (4.1-11.4 ng/ml) and LH concentration remained elevated (> 1 ng/ml) for a longer period (9-12 h).

In Group O plasma LH concentrations began to increase 18-21 h after CIDR removal and peaked 24-27 h after CIDR removal. LH concentrations returned to basal levels (< 1 ng/ml) 12-15 h after the onset of the LH surge.

In Group BO hinds, the LH concentrations began to increase 21-27 h after CIDR removal and peaked 24-30 h after CIDR removal. There was also a small (1.1-2.1 ng/ml) short-term increase in plasma LH concentration in all hinds 3 h after CIDR removal and a third increase in LH concentrations (1.1-8.4 ng/ml) in 4 BO hinds 54 h after CIDR removal.

All control hinds had a single pronounced surge of plasma LH concentrations (10-40 fold increase over basal levels). LH concentrations increased above basal levels (> 1 ng/ml) 30-39 h after CIDR withdrawal and peaked 33-45 h after CIDR removal. LH concentrations remained elevated (> 1 ng/ml) for 12-18 h after the onset of the LH surge.

Group BO, O and C hinds had similar maximum plasma LH values which ranged from 12 to 38 ng/ml. The magnitude of the LH maxima in the Group B hinds was significantly less than the peak values recorded in the other groups ($p < 0.05$). The mean interval from the withdrawal of CIDR to maximal LH concentrations in the Group C hinds was significantly later than the time of the peak LH values in Group O and Group BO hinds but earlier than the Group B maximum ($p < 0.01$) (see Table 7.3).

Ovarian activity

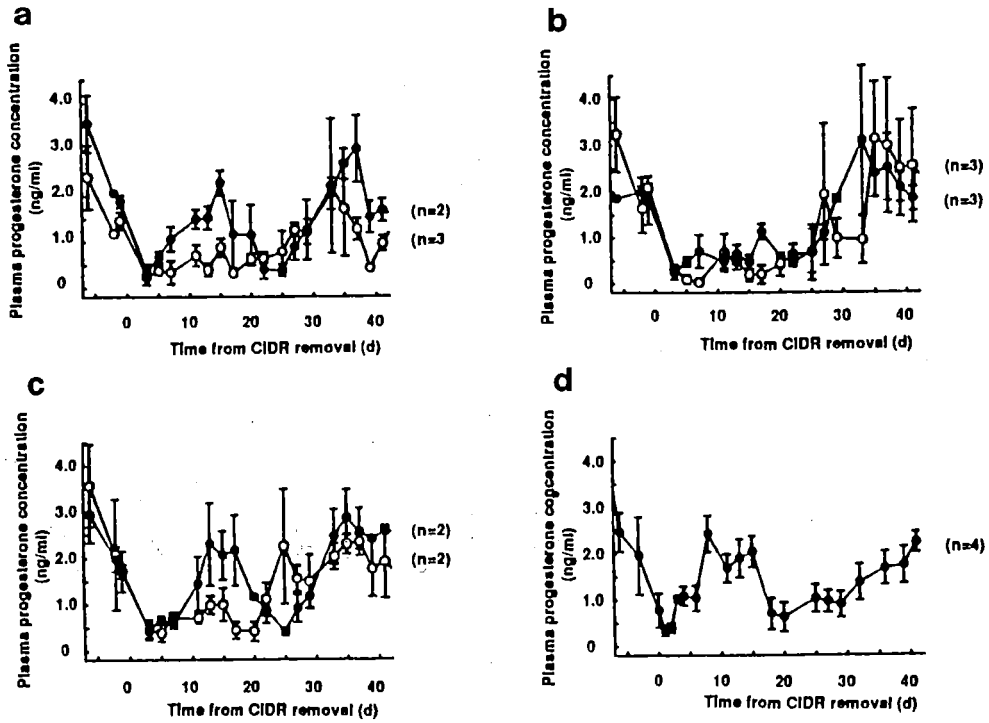
Two weeks after CIDR withdrawal 3/5 B, 2/4 O, 3/6 BO and 4/4 C group hinds had a single corpus luteum present at laparoscopy.

Plasma progesterone concentration

Mean plasma progesterone profiles are represented in Figure 7.4. The average time and duration of the plasma progesterone increase are recorded in Table 7.4. Immediately prior to CIDR withdrawal plasma progesterone concentrations averaged 2.1 ± 0.4 ng/ml and there were no significant differences between treatment groups. After CIDR removal plasma progesterone concentrations decreased (0.42 ± 0.03 ng/ml) and generally remained low (< 1 ng/ml) for 4-11 days.

In Group B and BO hinds with a corpus luteum progesterone concentrations increased and remained elevated for 11-16 days except for one Group B hind whose progesterone concentrations were elevated for only 6 days (Figure 7.5 a). Plasma progesterone concentrations in hinds without a corpus luteum and in all Group O hinds (including those with a corpus luteum) generally remained low until at least 20 d after CIDR withdrawal.

Figure 7.4. Mean plasma progesterone concentrations of red deer hinds following progesterone (CIDR) treatment in Trial III. Hinds either received a) GnRH analogue (n=5) b) oestradiol (n=4) c) GnRH analogue and oestradiol (n=6) prior to the breeding season or d) received progesterone treatment only during the breeding season and had (●) or did not have (o) a corpus luteum present at laparoscopy. Vertical bars denote s.e.m..



In Group C hinds plasma progesterone concentrations began to increase 4-6 d after CIDR removal in the 3 hinds that were mated. Plasma progesterone concentrations peaked at 1.8-3.5 ng/ml and remained elevated (> 1 ng/ml) for 12-14 days. In the control hind that was not mated (Figure 7.5 b) a transient increase in plasma progesterone concentration (2.5 ng/ml) was detected 24 h after CIDR removal and a sustained increase in plasma progesterone concentration was not recorded until d 11.

The time interval from CIDR withdrawal to an elevation in plasma progesterone concentrations was longer in Group B and Group BO hinds which ovulated than in Group C hinds.

Calving date

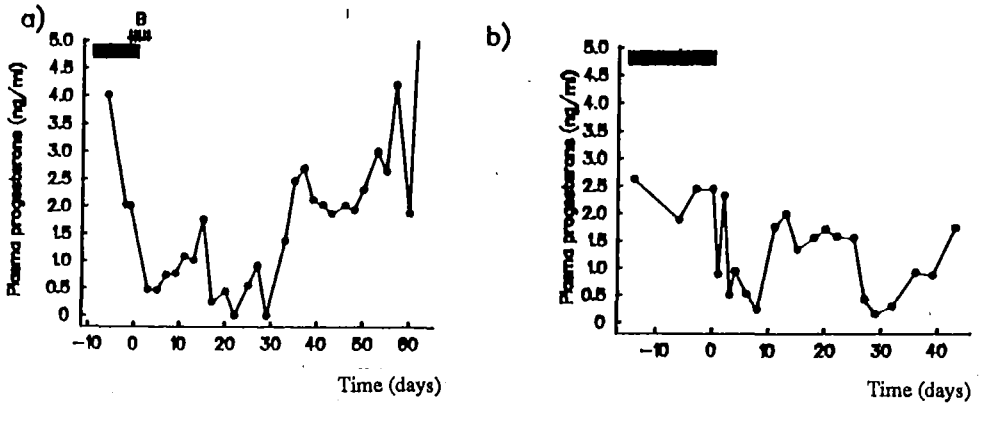
No pregnancies resulted from the induced ovulations but all hinds conceived at a subsequent mating and delivered live calves at term. The mean calving dates of Group B, O and BO hinds were 20 November ± 3 d, 17 November ± 2 d and 16 November ± 2 d respectively, with no significant difference between treatments. Calving was advanced

approximately 12 days relative to untreated hinds (1 December ± 4 d) mated in a separate mob on the research unit.

Table 7.4. Onset and duration of the increase in plasma progesterone concentrations. Means in columns with different superscripts are significantly different ($p < 0.05$).

Treatment Group			Onset of increase in plasma progesterone (d from CIDR removal) mean	s.e.m. [Range]	Duration of increase in plasma progesterone (d) mean	s.e.m. [Range]
B	With C.L.	3	9.0[b]	1.2 [7-11]	9.7 [6-13]	0.7
	Without C.L.	2	26.0[c]	1.0 [25-27]		
O	With C.L.	2	25.0[c]	- [25]		
	Without C.L.	2	27.0[c]	2.0 [25-29]		
BO	With C.L.	4	10.0[b]	0.6 [9-11]	11.5 [11-16]	0.9
	Without C.L.	2	21.0[c]	1.0 [20-22]		
C	With C.L.	4	6.3[a]	1.0 [4-11]	14 [12-16]	0.8

Figure 7.5. Plasma progesterone profiles of a) the buserelin-treated hind with a short-lived corpus luteum and b) the control hind which was not mated. The horizontal block represents progesterone (CIDR) treatment and arrows indicate administration of the GnRH analogue.



7.3.4 Discussion

Once again no early calvings resulted from the ovulations induced in progesterone-primed red deer hinds using the GnRH analogue, buserelin, prior to the breeding season. Results from this last trial did however suggest several aspects of the behavioural and hormonal sequence resulting from the treatment regime which may not have been suitable for out-of-season conception.

The inadequate rutting behaviour of one of the stags used in this trial highlights the variability in rutting behaviour of untreated red deer stags prior to the normal breeding season (Moore & Cowie, 1986; Fisher *et al.*, 1988) and may explain the failure to advance conception dates in previous trials. Advancing the onset of rutting behaviour in the stag by using melatonin treatment improved the the fertility of progesterone-PMSG induced ovulations in anoestrous hinds (Moore & Cowie, 1986; Fisher & Fennessy, 1990). In fact treating the stag alone may prove to be an effective means of advancing calving in red deer hinds (Fennessy & Fisher, 1988).

In general the range of courtship behaviour in the present trial was similar to the mating behaviour of spontaneously ovulating captive red deer hinds described in other studies (Guinness *et al.*, 1971; Veltman; 1985). Non copulatory mounting of oestrous hinds by the stag and oestrous hinds mounting the stag or other hinds were common events and oestrous behaviour, which was expressed over a period of 12-54 h, ceased in most hinds after mating. However the number of non copulatory mounts by the stag before mating was low (present study *vs* Veltman (1985), 1 *vs* 4-7 low mounts/copulation) as was the number of times the hind rode the stag prior to mating (present study *vs* Veltman (1985), 1 *vs* 4 mounts/copulation). Also the mean time from the first low mount by the stag to copulation was considerably longer than previously reported (present study *vs* Veltman (1985), 9 h *vs* 45 min). These differences may be due to variation in the reproductive competence of individual stags prior to the breeding season, effects of the exogenous hormone treatment or the artificial conditions under which courtship behaviour was observed. Confinement of the mating mob in a small yard, intermittent exposure to the stag, stress of handling and blood sampling and the presence of several hinds displaying oestrous behaviour simultaneously might perturb the normal pattern of courtship

behaviour. As 13 hinds were in oestrus over a 2 d period it is possible that the stag may have become exhausted and that increasing the stag to hind ratio would have improved the proportion of hinds mated in the study.

Unlike anoestrous ewes (Rodway & Swift, 1985), some hinds treated with buserelin in this trial did display oestrus but onset of oestrous behaviour was delayed compared with either progesterone-synchronised oestrous cycles during the breeding season or oestradiol-induced courtship behaviour during anoestrum. In the ewe (Goodman, 1988) and probably in the red deer hind (Meikle & Fisher, 1990), progesterone pretreatment followed by a rise in circulating oestradiol levels is a critical prerequisite for the expression of oestrous behaviour. This is borne out by the ability of oestradiol benzoate treatment to induce oestrous behaviour in progesterone-primed entire red deer hinds in this study. In the ewe, oestradiol is secreted from the developing follicle in response to LH released from the pituitary (Baird & Scarimuzzi, 1976b; McNatty *et al.*, 1981). The amount of LH secreted by the buserelin-treated hinds was less than in the control hinds and may not have stimulated the release of sufficient oestradiol to induce oestrous behaviour. It is possible that courtship behaviour was delayed or absent in the buserelin-treated hinds because of a late and inadequate increase in secretion of endogenous oestradiol.

In the 3 control hinds which were mated, oestrous behaviour was recorded about 24 h and mating about 36 h after CIDR withdrawal. This was earlier than the onset of oestrus reported in progesterone-treated deer by Fisher and Fennessy (1985) where no hinds displayed oestrus until at least 24 h after CIDR removal and most hinds were detected in oestrous about 48-72 h after CIDR withdrawal (Fennessy *et al.*, 1989). Variability in the interval from CIDR withdrawal to the detection of oestrus in fallow and Père David's deer has been linked to changes in the timing and method of progesterone pretreatment (Asher *et al.*, 1990; McLeod *et al.*, 1991)). In the present study hinds were treated with double CIDRs instead of the more usual single CIDR (Fisher & Fennessy, 1985) and this may have advanced the onset of oestrus in these hinds. Also different methods of oestrous detection and a different definition of oestrous behaviour were used in these studies. In the present trial direct observations of the mating mob enabled changes in activity associated with the onset of oestrous behaviour and the time of mating to be

defined. Pigment markings made on hinds by a greased stag may not be a reliable method for oestrous detection (See; Discussion of Trial I; Asher *et al.*, 1991) and as markings may reflect any stag/hind interactions, such as non-copulatory mounts, the actual time of copulation is difficult to determine.

Following progesterone treatment, plasma LH concentrations were elevated in the buserelin treated hinds 3-9 h after each buserelin injection but the maximum LH concentrations achieved were always less than those recorded in the LH surge during the breeding season. This is in contrast to studies in anoestrous ewes where a single buserelin (6-40 μg, i.v.) injection produced an immediate LH response within the physiological range of the natural preovulatory peak (Siddall & Crighton, 1977; Swift & Crighton, 1980; Rodway & Swift, 1985). The maximal LH response to buserelin recorded in this trial may have been suppressed or delayed because the analogue was administered intramuscularly (i.m.) instead of intravenously (i.v) or, alternatively, the LH peak was missed due to infrequent blood sampling. Also pituitary reserves of LH may have become exhausted or LH receptors in the pituitary down regulated by the multiple injection regime used in this trial. Interestingly, multiple injections or continuous infusion of buserelin has been previously shown to reduce release of LH in ewes (Dobson, 1985; McNeilly & Fraser, 1987; Picton *et al.*, 1990), rams (Lincoln *et al.*, 1986) and cows (Dr K.L. Macmillan, *pers comm.*) but not in red deer stags (Lincoln, 1987). Regardless of whether they were treated with buserelin or not, plasma LH concentrations increased dramatically in oestradiol-treated hinds 24-33 h after oestradiol administration, demonstrating that the LH surge system is functional in the anoestrous hind. Oestradiol elicited large increases in LH secretion after a similar time interval in entire (Goodman *et al.*, 1980) and ovariectomised ewes (Karsch, 1987) and in ovariectomised red deer hinds (Meikle & Fisher, 1990) which indicates that this is a universal response to oestradiol in progesterone-primed animals.

All control red deer hinds in this study ovulated after progesterone treatment during the breeding season. The hormone profiles associated with these ovulations presumably represent normal endocrine relationships associated with the oestrous cycle of red deer hinds. There is little doubt that the surge in plasma LH concentration recorded immediately after mating represented the 'pre-ovulatory' LH surge which occurs during

the peri-oestrous period in many domestic ruminants (sheep, Baird *et al.*, 1981; cattle, Walters & Schallenberger, 1984; goats, Mori & Kano, 1984). Daily blood sampling during the oestrous cycle of red deer (Kelly *et al.*, 1985), roe deer (Schams *et al.*, 1980) and white-tailed deer (Plotka, 1980) occasionally showed LH peaks (>10 ng/ml) on or near the day of oestrus but fallow and Père David's are the only deer species in which temporal relationships between the onset of oestrus and the preovulatory LH surge have been described (Asher & Thompson, 1989; Asher *et al.*, 1990; Loudon *et al.*, 1990). Similar to progesterone-synchronised ovulations in fallow does (Asher & Thompson, 1989; Asher *et al.*, 1990), the onset of the preovulatory LH surge in red deer hinds occurred at about the same time as mating and LH concentrations peaked (at about 25 ng/ml) about 8 h later. In contrast, the LH surge in Père David's hinds, after treatment with progesterone implants and prostaglandin injections, occurred much later (about 60 h after progesterone withdrawal) and oestrous behaviour was not detected until about 9 h after the onset of the LH increase. The short interval from CIDR removal to the onset of the LH surge and the close synchrony between the LH surge and behavioural oestrous suggests that, in these respects, red deer hinds appear to be very similar to other domestic animals such as sheep (McLeod & Haresign, 1984) and the goat (BonDurant *et al.*, 1981).

The plasma progesterone profiles recorded in the control hinds were similar to those observed during the oestrous cycle in previous studies of red deer hinds (Adams & Atkinson, 1984; Adam *et al.*, 1985; Jopson *et al.*, 1990) and in fallow (Asher, 1985), white-tailed (Plotka *et al.*, 1980), roe (Schams *et al.*, 1980) and Père David's (Curlewis *et al.*, 1988a) deer. The pattern is also characteristic of the oestrous cycle in domestic ruminants such as the sheep (Walton *et al.*, 1977) and cattle (Pope *et al.*, 1969). Erratic fluctuations of plasma progesterone concentrations observed about oestrus in red deer (Kelly *et al.*, 1985) and Pere David's hinds and in fallow does (Asher & Thompson, 1989; Asher *et al.*, 1990) were also evident in the control hind which was not mated in this study. Kelly *et al.* (1985) suggested that the erratic fluctuations in progesterone observed around oestrus were due to the presence of a functional 'accessory' corpus luteum but the hinds in the present study had only one corpus luteum present at laparoscopy and it is more likely that progesterone was released from the adrenal gland in response to handling stress (Meikle, 1988; Jopson *et al.*, 1990). In female sheep and fallow deer increases in plasma progesterone concentrations during the preovulatory

period have also been associated with a delay or absence of oestrous behaviour (Karsch *et al.*, 1980; Asher & Thompson, 1989; Asher *et al.*, 1990). The one red deer which exhibited fluctuations in plasma progesterone concentrations prior to the LH surge in the present study failed to mate, possibly due to the inhibitory effects of progesterone on the expression of oestrous behaviour.

A single ovulation was induced in the majority of red deer hinds treated with buserelin and/or oestradiol prior to the breeding season or after progesterone treatment during the breeding season. Except for one hind, the elevation in plasma progesterone concentrations in buserelin and buserelin/oestradiol-treated hinds with a corpus luteum was similar to that recorded during the luteal phase in the breeding season (in this study; Adam *et al.*, 1985; Jopson *et al.*, 1990) and indicates that most of the corpora lutea observed here functioned normally. The rise in plasma progesterone concentrations was delayed in the Group B and BO hinds. In the buserelin-treated hinds this may have been because the LH levels peaked later. However the rise in LH was not delayed in the hinds treated with both buserelin and oestradiol and the late-occurring increase in plasma progesterone may be due to inappropriate hormonal environment following this treatment regimen or to the timing of the induced ovulation relative to the onset of the natural breeding season.

Lack of an elevation of plasma progesterone concentrations in the oestradiol-treated hinds indicates that their corpora lutea were nonfunctional. It is unlikely that this luteal incompetence arose from insufficient exposure of the developing follicle to progesterone because CIDR treatment elevated plasma progesterone to concentrations similar to those occurring during the normal luteal phase (1-4 ng/ml) (Group C hinds in the present study, Adam *et al.*, 1985). Stimulation of the developing follicles by endogenous gonadotrophins may have been inadequate in these hinds as oestradiol-treated hinds which also received buserelin treatment produced a normally functioning corpus luteum. Also the negative feedback effect of oestrogens on endogenous gonadotrophin secretion prior to the LH surge (Goodman *et al.*, 1980, Meikle & Fisher, 1990) may have retarded follicular development prior to ovulation. Ewe lambs treated with 2.5 mg oestradiol valerate exhibited oestrus but most did not develop a corpus luteum (Burfening & Berardinelli, 1986). In the oestradiol-treated ewes which did develop corpora lutea the

pregnancy rate was low suggesting that pharmacological doses of oestradiol may reduce fertility.

Although none of the buserelin and/or oestradiol-induced ovulations resulted in pregnancy, the breeding season of the treated hinds was advanced relative to that of untreated animals, which indicates that the procedures used here stimulated subsequent ovarian activity. Social facilitation of reproductive activity has been reported in sheep (Knight, 1983; Martin *et al.*, 1986) and red deer (Moore & Cowie, 1986; McComb, 1987; Fisher *et al.*, 1988) with the introduction of a male advancing the onset of ovulatory activity during late anoestrus. In sheep the 'ram effect' is more dramatic if the ram is exposed to oestrous ewes (Knight, 1985). In the present study exposure of hinds to a sexually active stag and/or oestrous hinds may have advanced the transition to the breeding season.

It was concluded that after priming with progesterone, ovulation and formation of a corpus luteum could be induced in seasonally anoestrous red deer hinds by the use of a GnRH analogue. However no hinds conceived at the induced ovulation, possibly because ovulation was not accompanied by the same hormonal and behavioural sequences as those exhibited by hinds in the normal breeding season or because the stag was reproductively incompetent prior to the onset of the normal breeding season.

7.4 General Summary.

This series of trials showed that the GnRH analogue, buserelin, can be used to induce single ovulations in lactating red deer hinds prior to the onset of the breeding season. However, no calvings resulted from these treatments and reasons for the low fertility of buserelin-induced ovulations are hard to find.

In Trial I the luteal phase pattern of progesterone secretion indicated that corpora lutea induced by the twin-injection buserelin protocol were functionally subnormal. Luteal function was improved in Trial II by increasing the exposure of the developing follicle to progesterone and/or gonadotrophic stimulation prior to ovulation but still no early pregnancies resulted. Evidence from Trial III suggested several factors that may have

reduced fertility. One factor was that the oestrous behaviour was delayed or absent in the buserelin-treated hinds. Secondly, even if the hinds had displayed oestrus the inadequate rutting behaviour of the stag and large numbers of hinds displaying oestrus over a short period reduced the likelihood that hinds would be successfully mated. Thirdly, maximal LH concentrations were reduced and delayed compared with hinds exhibiting oestrus during the breeding season. This indicates that even if hinds were mated the hormonal environment arising after buserelin treatment may have been inappropriate for conception and establishment of pregnancy.

Protocols of any future buserelin trials should therefore utilise seasonally advanced stags and a high stag to hind ratio to increase the likelihood that an oestrus hind would be mated by a reproductively competent male prior to the normal breeding season. It is also recommended that hinds, progesterone-pretreated with double CIDRs, be injected with 3 small (2 μg) doses of buserelin 48, 24 and 0 h before CIDR removal followed by a final large (10 μg) dose of buserelin about 36 h after CIDR removal. This protocol should provide sufficient gonadotrophic stimulation to ensure mature follicles were present at ovulation as well as advancing the onset of the LH surge and oestrous behaviour and therefore mimicking more closely the hormonal pattern exhibited by oestrous hinds during the breeding season.

Chapter 8

General Discussion

The number of red deer farmed in New Zealand increased dramatically during the 1980's and there was much interest in the manipulation of the onset of breeding activity in order to align more efficiently the feed demands of the lactating hind with feed supply. However information on the mechanisms controlling the reproductive activity of the red deer hind was lacking and the aim of these trials was to increase our understanding of the physiology of seasonal breeding in red deer.

The timing of sexual activity in most seasonally breeding mammals is influenced by photoperiod. The first study in this thesis (Chapter 4) focussedon whether, as had been suggested in the ewe (Robinson *et al.*, 1985; Worthy *et al.*, 1985), the transition from anoestrus to breeding activity in red deer was due to the development of a temporary loss of response to long inhibitory photoperiods, called photorefractoriness. Results of this study supported the view that the cycles of reproductive activity, pelage shedding and prolactin secretion in the red deer were influenced by daily photoperiod, probably via changes in the circadian pattern of melatonin secretion. Results also indicated that the onset of ovarian activity was not due to the development of photorefractoriness to long photoperiods and that the autumnal decrease in daily photoperiod played an important role in determining the timing of the breeding season in the breeding red deer hind. It is important to note that, although reproductive activity was delayed in hinds denied exposure to the autumn decrease in photoperiod, oestrous activity did eventually begin. This finding was in agreement with recent evidence which suggests that the seasonal reproductive transitions in the ewe and red deer hind result from an endogenously generated circannual rhythm (Karsch *et al.*, 1989; Loudon & Brinklow, 1990). However in contrast to the ewe (Malpaux *et al.*, 1989) and pubertal red deer hind (Loudon & Brinklow, 1992), the timing of the spring increase in photoperiod did not influence the onset of ovarian activity in these hinds. Rather, in the breeding hind the autumn decrease in photoperiod appeared to entrain the onset of breeding season to the appropriate time of year. Interestingly, premature exposure to a long daily photoperiod during winter advanced the shedding of both the winter and the subsequent summer coat in both pubertal (Loudon & Brinklow, 1992) and breeding (Chapter 4) hinds, indicating that the spring increase in photoperiod may entrain the seasonal cycle of pelage shedding. It is

possible that this apparent dichotomy arose because a different photoperiodic signal determines the onset of oestrous activity in pubertal and adult hinds or, alternatively, the neuroendocrine pathway which regulates seasonal changes in the reproductive axis was insensitive to photoperiodic signals during gestation. The ability of seasonally breeding animals to interpret changes in the melatonin signal during pregnancy has never been addressed. This factor should be considered in future experiments as results of photoperiodic studies using pubertal or non-pregnant females may not be applicable to the majority of adult red deer hinds which under farming conditions conceive each year.

Seasonal changes in the pattern of LH secretion had been previously described in red deer stags (Suttie *et al.*, 1984; Lincoln & Kay, 1979) but information on LH pulsatility in red deer hinds was lacking. The present studies therefore provided the first description of seasonal changes in secretory patterns of LH in the pubertal (Chapter 5) and breeding red deer hind (Chapter 4). Results indicated that the mean LH concentration and the frequency of LH pulses were greater during the luteal phase of the breeding season than during anoestrus which was consistent with the hypothesis that the anovulatory state in seasonally breeding animals is due to reduced LH secretion during the anoestrous period.

Little was known of the hormonal changes associated with the onset of the first breeding season of red deer hinds. A study of reproductive seasonality in pubertal red deer hinds (Chapter 5) showed that hormonal secretion patterns during puberty were similar to those recorded during the seasonal transition to oestrous activity in the adult hind but the breeding period of non-pregnant young red deer hinds was shorter than that reported for older hinds. This study also investigated whether, as in sheep, seasonal changes in the sensitivity of tonic LH secretion to the negative feedback effect of oestradiol was a factor in the neuroendocrine control of seasonal breeding in red deer. Results of this study indicated that seasonal changes in LH pulsatility and the onset of reproductive activity in these entire hinds were temporally related to changes in the sensitivity of LH secretion to the negative feedback effects of oestradiol in ovariectomised pubertal hinds. It appears that before puberty oestradiol suppresses LH concentrations but after the transition to the breeding season the ability of oestradiol to inhibit LH diminishes permitting reproductive activity to begin. Similar seasonal changes in the sensitivity of LH secretion to the negative feedback effects of oestradiol in adult red deer hinds have subsequently been

reported by Meikle *et al.* (1991). In addition it is also possible that a pathway independent of oestradiol inhibition influences seasonal changes in LH secretion in red deer hind (Meikle *et al.*, 1991). Mean plasma LH concentrations and LH pulsatility tended to be lower in the untreated ovariectomised hind during early anoestrus than in the breeding season or late anoestrus in the present study. However as this steroid-independent reduction in LH was based on data from only 2 hinds and the time course of steroid-independent changes in LH secretion were not studied, more work is required to determine the roles that steroid-dependent and steroid-independent mechanisms play in the reproductive seasonality of the pubertal red deer hind.

LH secretion reflects the pattern of GnRH release from the hypothalamus as well as the ability of the pituitary to release LH in response to a GnRH pulse. The release of LH in response to exogenous GnRH was constant throughout the trial period in the unmated pubertal hinds (Chapter 5) indicating that the differences in LH pulsatility recorded primarily reflected seasonal changes in release of GnRH from the hypothalamus. However it should be noted that in the present studies (Chapter 4 & 5) hinds were sampled during the luteal phase of the ovarian cycle and it is possible that seasonal changes in LH responsiveness were masked by elevated progesterone concentrations during the luteal phase of these entire hinds. Pituitary responsiveness to GnRH and LH pulsatility during the follicular phase of the oestrous cycle of the red deer hind were not studied in the present trials and have not been reported in the literature. Furthermore, the increase in LH secretion during the breeding season in oestradiol-treated ovariectomised hinds (Chapter 5) was associated with an increase in pituitary responsiveness and additional studies are required to determine whether a change in pituitary responsiveness contributes to the increase in LH secretion associated with ovarian cyclicity in the entire red deer hind.

In contrast to pubertal hinds (Chapter 5) the varying LH response to GnRH indicated that the pattern of LH secretion in breeding hinds (Chapter 4) was markedly influenced by the ability of the pituitary to secrete LH. It is not known whether seasonal changes in LH secretion and pituitary responsiveness, similar to those described in Père David's hinds (Loudon *et al.*, 1990; McLeod *et al.*, 1991), occur in mature red hinds. In addition it is possible that the seasonal pattern of LH release was modified by the effects of gestation

and/or lactation. These results suggest that advancement of mating date using melatonin or progesterone/gonadotrophin treatments in the breeding red deer hinds may ultimately be limited by a period of post-partum/lactational/seasonal anoestrus. All these factors require further investigation if the mechanisms controlling the onset of reproductive activity in breeding red deer hinds are to be better understood.

Recent evidence increasingly supports the hypothesis that seasonal cycles of reproductive activity, pelage moulting, prolactin secretion, voluntary food intake and live-weight gain are endogenously generated and entrained to the appropriate time of year by the daily pattern of melatonin secretion. The close temporal relationship between such seasonal cycles has led to the assumption that these rhythms were always tightly linked and perhaps regulated by a common hormonal signal. However, in a study (Chapter 6) where young red deer were immunised against melatonin, the entrainment of the seasonal cycle of live-weight gain was apparently disrupted without influencing the reproductive seasonality of the animals. The results from these studies (Chapter 4 & Chapter 6) provide support for an hypothesis that the pathway by which melatonin signals entrain the seasonal cycle of reproductive activity may differ from those controlling other seasonal events such as growth and pelage shedding. The pathway by which melatonin signals cause seasonal changes in the reproductive axis, growth and pelage remain largely unknown.

The final study (Chapter 7) used a GnRH analogue, buserelin, to induce ovulation in progesterone-treated hinds prior to the onset of the normal breeding season. The treatment regimen was only partially successful. Although a single ovulation was induced in the majority of the anoestrous hinds, no early calvings resulted from the treatments and the progesterone profile indicated that some of the induced corpora lutea were functionally subnormal. The low fertility of the buserelin-induced ovulations may have resulted because the associated hormonal sequence was inappropriate for normal oestrous behaviour or conception or because the stags were reproductively incompetent prior to the onset of the normal breeding season.

One factor which may limit progress in the understanding of the reproductive physiology of red deer hinds is the lack of a reliable hormone assay to measure FSH concentrations

in the plasma of red deer hinds. Attempts made during these studies to purify cervine FSH from the pituitaries of red deer and develop radioimmunoassay and biological assay methods for this had limited success and, consequently, nothing is known of the secretion patterns of FSH in red deer hinds. It is possible that measuring the pattern of FSH secretion may suggest the cause of the low fertility following progesterone/gonadotrophin treatment of anoestrous hinds and may provide information on factors which influence the onset of breeding activity in red deer hinds.

Although the simple cannulation technique developed during these studies permitted blood samples to be collected frequently over an extended period of time with apparently minimal disturbance to the hinds, some individuals displayed atypical hormonal secretion patterns (Chapter 7, Trial III) possibly in response to the stress of experimental procedures. The adoption of remote sampling techniques in future studies may determine if these unusual endocrine patterns were due to natural variation between hinds or whether the stress of repeated handling and sample collection influences the reproductive physiology of some individuals.

The experiments described in this thesis have contributed to our understanding of the regulation of seasonal breeding in red deer hinds and this knowledge may eventually enable reproductive activity to be effectively manipulated thereby improving the efficiency of deer production systems. The findings suggest that the autumnal decrease in daily photoperiods plays an important role in determining the onset of reproductive activity of the breeding hind and that, in contrast to the pubertal hind, artificial manipulation of photic stimuli during spring is unlikely to provide a suitable method for advancing the calving season of breeding red deer hinds. In addition, immunisation of young red deer against melatonin, which delayed the onset of the autumnal reduction in growth and caused immunised stags to attain greater live weights during the spring and autumn periods of their second year, may be applied to venison production systems where early attainment of desirable slaughter weights may improve the efficiency of production. Studies on the seasonal patterns of LH secretion indicate that seasonal change in sensitivity of the GnRH pulse generator to the negative feedback effects of oestradiol is a major factor regulating the onset of breeding activity in the red deer hind. It also appears that seasonal and/or post-partum/lactational anoestrus reduces pituitary function during

mid seasonal anoestrus. This may limit the ability of melatonin or progesterone/gonadotrophin treatments to advance the onset of calving by more than a few weeks in breeding hinds.

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Appendix A

Table 4.1A. LH pulsatility and pituitary LH response to GnRH of red deer hinds exposed to natural light (NP) or 15.3 h photoperiod (EPW) between 22 July and 7 November 1986.

Parameter		July 1986		January 1987		February 1987		March 1987		April 1987	
No of pulses/ 4 h period	NP	-	-	**0.50**	(0.29)	**0.75**	(0.48)	**1.50**	(0.50)	**0.50**	(0.29)
	EPW	-	-	**0.25**	(0.25)	**0.50**	(0.29)	**1.00**	(0.41)	**0.50**	(0.29)
Basal LH (ng/ml)	NP	**0.27**	(0.01)	**0.37**	(0.04)	**0.38**	(0.06)	**0.46**	(0.02)	**0.31**	(0.04)
	EPW	**0.26**	(0.02)	**0.30**	(0.02)	**0.32**	(0.02)	**0.64**	(0.13)	**0.32**	(0.01)
Range in LH concentrations (ng/ml)	NP	**0.11**	(0.01)	**0.81**	(0.37)	**1.36**	(0.39)	**1.39**	(0.31)	**0.42**	(0.18)
	EPW	**0.12**	(0.03)	**0.72**	(0.58)	**0.52**	(0.24)	**2.77**	(1.01)	**1.23**	(0.97)
Mean LH concentration (ng/ml)	NP	**0.32**	(0.01)	**0.64**	(0.08)	**0.69**	(0.10)	**0.80**	(0.06)	**0.42**	(0.06)
	EPW	**0.32**	(0.02)	**0.65**	(0.11)	**0.51**	(0.06)	**1.22**	(0.33)	**0.50**	(0.10)
LH response to GnRH challenge (ng/ml)	NP	**0.56**	(0.19)	**0.20**	(0.14)	**5.73**	(0.91)	**3.93**	(0.40)	**2.98**	(0.38)
	EPW	**0.40**	(0.10)	**0.14**	(0.09)	**6.62**	(0.99)	**5.82**	(1.48)	**2.98**	(0.14)

Appendix B

Some results from experiments described in this thesis have been published or presented at conferences. These are listed below.

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